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Induction of lens regeneration from the ventral iris of the newt, *Notophthalmus viridescens*, through BMP inhibition-driven regulation of *six3*

Matthew William Grogg
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**INDUCTION OF LENS REGENERATION FROM THE VENTRAL IRIS OF THE
NEWT, *NOTOPHTHALMUS VIRIDESCENS*, THROUGH BMP INHIBITION-
DRIVEN REGULATION OF *SIX3***

Dissertation

Submitted to

The College of Art and Sciences of the

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The Degree

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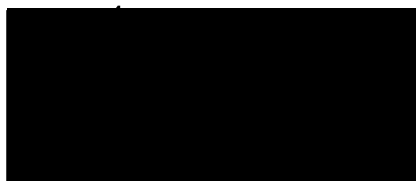
Matthew William Grogg

UNIVERSITY OF DAYTON

Dayton, Ohio

April 2006

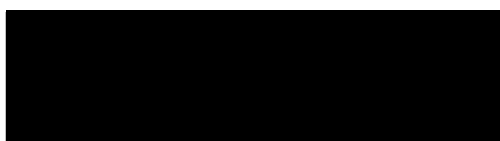
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ABSTRACT

INDUCTION OF LENS REGENERATION FROM THE VENTRAL IRIS OF THE NEWT, *NOTOPHTHALMUS VIRIDESCENS*, THROUGH BMP INHIBITION- DRIVEN REGULATION OF *SIX3*

Matthew William Grogg
University of Dayton, 2006

Advisor: Dr. Panagiotis Tsonis

The ability to regenerate body parts is one of the most amazing feats of nature that scientists have the opportunity to study. The urodeles perhaps possess the greatest regenerative abilities in the animal kingdom, with some of them able to regenerate limbs, tails, lenses, retinas, brains, hearts, and more. It has been clearly established that *in vivo* lens regeneration in urodele amphibians comes from the dorsal iris only. Some studies have shown that lens regeneration from the dorsal iris can be blocked or interrupted successfully. Several studies have shown an induction of lens regeneration from the dorsal iris with and without lens removal. While many scientists have successfully inhibited lens regeneration from the dorsal iris, the "Holy Grail" of lens regeneration studies is the one that is able to induce lens regeneration from the regeneration incompetent ventral iris. To date only one known treatment, that of the potent carcinogen MNNG (methyl-nitro-nitrosoguanidine), has elucidated such an

induction. Nevertheless, these experiments clearly show that induction is possible and remains one of the greatest challenges in the field.

We initially set out to perform two tasks, the first being to induce lens regeneration from the ventral iris in the newt. The second goal was to examine expression levels of some important developmental eye genes in the newt intact iris and regenerating iris in the attempt to underscore the mechanism of regeneration. In our attempts at inducing lens regeneration from the ventral iris, we focused on factors that play a role in lens development. The study shows that we indeed were able to induce lens regeneration from the ventral iris in the newt. This was accomplished in two separate ways. The first was by inhibiting the bone morphogenetic protein (BMP) pathway in newt ventral iris tissue either using the BMP signaling antagonist Chordin or a truncated form of bone morphogenetic protein receptor-IA (BMPRI-IA). The second was accomplished by transfecting newt ventral iris pigmented epithelial cells (PECs) with *Six3*, a homeobox-containing transcription factor, and adding retinoic acid (RA). These results demonstrate the ventral iris of the newt has been successfully induced to regenerate a lens with known molecules. We were also able to show in our study that expression levels of some important developmental eye genes are drastically different than originally thought. The expression levels of transcription factors *Six3* and *Pax6* are much higher in the intact ventral iris than the intact dorsal iris. Similarly, the levels of *Six3* and *Pax6* remain slightly higher in

regenerating ventral iris than regenerating dorsal iris, although the change in expression from intact dorsal to regenerating dorsal was higher than the change in intact ventral to regenerating ventral. This suggests that a threshold of some sort might exist that must be overcome for the initiation of lens regeneration *in vivo*. This study also demonstrates the complexity of the molecular mechanism that underlies lens regeneration. Although we were not able to delineate the exact mechanism behind lens regeneration, our study was able to pinpoint key players that are part of a molecular network.

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TABLE OF CONTENTS

ABSTRACT.....	iii
ACKNOWLEDGEMENTS.....	vi
TABLE OF CONTENTS.....	viii
LIST OF FIGURES.....	x
LIST OF TABLES.....	xi
LIST OF ABBREVIATIONS.....	xii
CHAPTERS	
I. Review of Literature	1
Anatomy of the Eye.....	1
History of Lens Regeneration.....	4
Process of Newt Lens Regeneration.....	9
Transdifferentiation and Stem Cells.....	11
Studying Lens Regeneration	14
Factors in Lens Regeneration and Development.....	16
Induction of Lens Regeneration.....	27
References.....	30

II.	BMP Inhibition-Driven Regulation of <i>Six3</i> Underlies Induction of Newt Lens Regeneration.....	39
III.	Conclusions and Future Research	70
APPENDIX.....		81
I.	Lens Regeneration in Mice: Implications in cataracts.....	82
II.	Eye on Regeneration.....	85
III.	Gene Expression and discovery during lens regeneration in mouse: regulation of EMT and lens differentiation.....	92

LIST OF FIGURES

Chapter I

Figure 1.	The Vertebrate Eye.....	3
Figure 2.	Lens Regeneration in <i>Xenopus</i> and newt.....	5
Figure 3.	Stages of newt lens regeneration.....	10
Figure 4.	Lens development and genetic pathway model in the mouse.....	18

Chapter II

Figure 1.	Lens Induction from ventral PECs.....	56-57
Figure 2.	Expression during lens regeneration and induction.....	58-59
Figure 3.	Expression of <i>six-3</i> and <i>BMPR-1A</i> during regeneration.....	60-61
Figure 4.	Expression in axolotl.....	62-63
Supplementary Figure 1.....		65-66

Chapter III

Figure 1.	Potential molecular mechanism of lens regeneration.....	77
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LIST OF TABLES

Chapter I

Summary of known factors in lens development and lens regeneration.....	27
--	----

Chapter II

Supplementary Table 1 – Induction of transdifferentiation.....	64
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LIST OF ABBREVIATIONS

RPE.....	Retinal pigmented epithelium
PECs.....	Pigmented epithelial cells
FGFs.....	Fibroblast growth factors
FGFRs.....	Fibroblast growth factor receptors
Shh.....	Sonic hedgehog
Ihh.....	Indian hedgehog
twhh.....	tiggy-winkle hedgehog
BMPs.....	Bone morphogenetic proteins
RPE.....	Retinal pigmented epithelium
MNNG.....	Methyl-nitro-nitrosoguanidine
BMPR.....	Bone morphogenetic protein receptor

Chapter I

Review of Literature

I. Anatomy of the Eye

The vertebrate eye is a camera-type eye that is more complex than the simple eyes or compound eyes of animals in the lower phyla. There are 3 main layers to the vertebrate eye: the sclera, the uvea, and the retina. The sclera is the thick, white fibrous tissue and makes up the outermost layer. It is continuous with the transparent cornea that allows light to pass through while refracting it on the lens. The extraocular muscles attach to the sclera and control eye movement.

The uvea is the middle layer and consists of the choroid, the iris, and the ciliary body. The choroid is composed of layers of blood vessels that nourish the back of the eye. The iris is the colored part of the eye that divides the back of the eye from the front. Similar to the aperture of a camera, the iris controls the levels of light inside the eye by dilating or constricting the pupil (opening) through the use of the sphincter muscle. Just behind the iris lies the ciliary body. The ciliary body has two main functions. One is to secrete the clear aqueous humor into the anterior chamber of the eye that will nourish the cornea and lens. The second function is to suspend the lens in place with the use of ciliary zonules, suspensory ligaments that connect to the lens capsule. These zonules allow the ciliary body to control accommodation and change the shape of the lens by relaxing or contracting.

The third layer of the eye is the retina. The retina is a multi-layered sensory tissue that lines the back of the eye. The two layers of the retina are the retinal pigmented epithelium (RPE) and the neural retina. The RPE functions to absorb light and prevent scattering as well as forming the blood:retina barrier and providing nutrients to the neural retina and vitreous humor. The neural retina contains five different types of neurons: the photoreceptors (rods and cones), horizontal cells, bipolar cells, amacrine cells, and ganglion cells. The photoreceptors capture light rays and convert them into electrical impulses. These impulses are sent back towards the front of the eye with the help of the horizontal, amacrine, and bipolar cells and finally travel along the ganglion cell axons, which constitute the optic nerve, to the brain where they are turned into images.

The lens of the eye plays one of the most important roles in vision. It focuses light onto the retina. The lens can undergo the process of accommodation, the ability to change shape to adjust for close or distance vision, with the help of the ciliary body. The lens has a nucleus made up of compacted primary lens fibers. Surrounding the nucleus are secondary lens fibers. The lens also has an anterior epithelial layer that is continuously dividing producing more epithelial cells, some of which will form more secondary lens fibers. Therefore, the lens is constantly growing and the nucleus is getting more and more compact over time. The lens is composed mainly of water and protein. By far the most abundant protein found in lens fibers are the crystallin proteins, which give the lens fibers their transparency.

The lens is also surrounded by a capsular bag that functions to hold the lens in place by attaching to the ciliary zonules. The lens helps separate the anterior and posterior chambers. The vitreous humor is located in the posterior chamber and is a thick, transparent substance that fills the center of the eye. The vitreous is composed mainly of water and comprises almost two-thirds of the eye's volume, giving it form and shape.

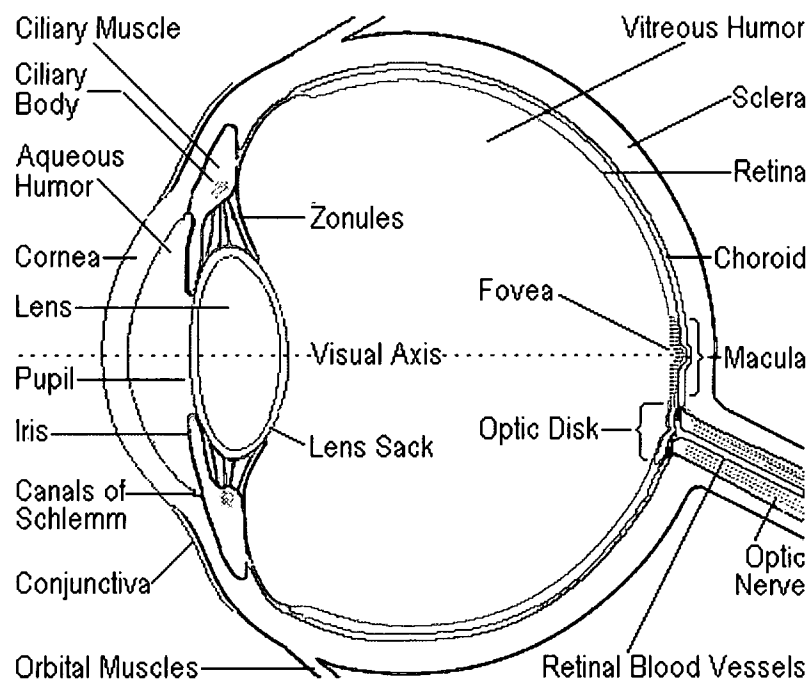


Figure 1. The Vertebrate Eye. Diagram depicting structures of the eye.

II. History of Lens Regeneration

The ability to regenerate body parts is one of the most amazing feats of nature that scientists have the opportunity to study. Ancient civilizations were even fascinated with regeneration as evidenced in mythological tales. Many organisms can regenerate appendages or organs. Even higher orders such as mammals possess some ability to regenerate. The urodeles perhaps possess the greatest regenerative abilities, with some of them able to regenerate limbs, tails, lenses, retinas, brains, jaws, and more.

Lens regeneration in the newt has been a documented phenomenon for over 100 years. Both Colluci (1891) and Wolff (1895) independently observed the process, sometimes referred to as Wolffian regeneration after Wolff's studies. Lens regeneration is seen predominantly in amphibians, but most spectacularly in some species of urodeles as this feat can occur in an adult organism. Lens regeneration in the newt occurs through a process known as transdifferentiation. Upon removal of the lens, the pigmented epithelial cells of the dorsal iris transdifferentiate and form an entire new, functional lens (Eguchi, 1988; Tsonis, 2000).

The newt is not, however, the only organism that can regenerate its lens. Anurans such as *Xenopus laevis* can also regenerate a lens through the process of transdifferentiation. Unlike the newt, which regenerates the lens through transdifferentiation of the dorsal iris pigmented epithelial cells (PECs), *Xenopus* regenerates a lens from the inner layer of the outer cornea (Freeman, 1963; Filoni et al., 1997). This process appears to be facilitated by

a factor secreted from the retina. Following removal of the lens, this factor is no longer hindered from making contact with the outer cornea (Filoni et al., 1982). Besides the type of cells that undergo transdifferentiation, another difference exists between the newt and *Xenopus* and that is the stage at which regeneration is possible. As was mentioned earlier, the newt can regenerate a lens throughout its lifespan, which is one of the reasons that scientists use this animal as a model system for studying regeneration. *Xenopus* can only regenerate during early stages of life. The capacity for regeneration is lost after metamorphosis (Freeman, 1963).

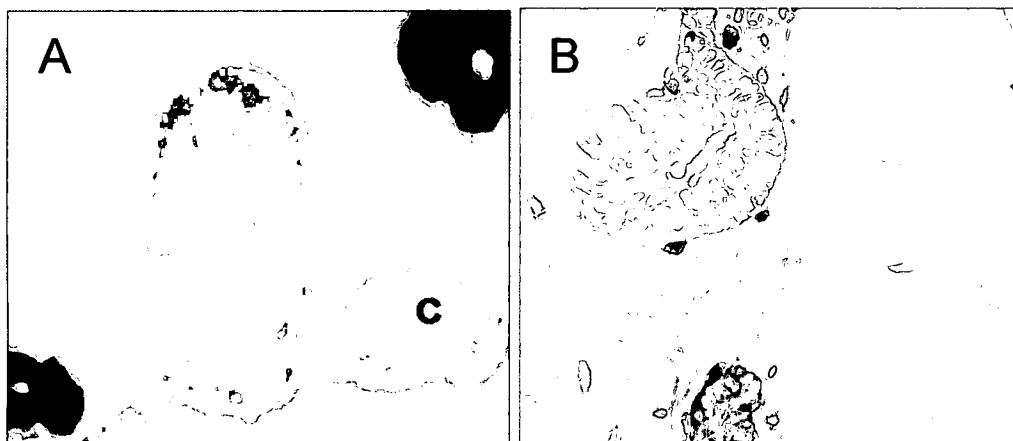


Figure 2. Lens Regeneration in *Xenopus* and Newt. A – *Xenopus* lens regeneration from cornea (c – cornea). B – Newt lens regeneration from dorsal iris (~15 days post-lentectomy).

Lens regeneration is not only restricted to amphibians but has been shown to take place in other vertebrates as well. Lens regeneration has been reported to occur from a layer of cells found at the border of the iris near the choroid in the chick embryo (Deth, 1940). These findings, however, are controversial. The controversy lies in the fact that scientists are not sure if the newly formed lens is a result of transdifferentiation of the cells near the iris or if it is merely an inductive response due to competent ectoderm being left behind after surgery (McKeehan, 1961). The lack of markers to follow the process was a major problem with these studies. Similar to salamanders, some species of adult fish can regenerate a lens through transdifferentiation of cells of the dorsal iris (Sato, 1961; Mitashov, 1966).

Other vertebrates, such as mice, rabbits, and cats, can also undergo lens regeneration to a degree. In mammals, lens regeneration studies have been largely restricted to rabbits. Unlike the other organisms examined, these mammals do not regenerate a lens through the process of transdifferentiation. Instead, regeneration occurs from lens epithelial cells remaining on the capsular bag following removal of the lens. Removal of the lens while leaving the lens capsule results in a lens regeneration event that is sometimes not complete (Stewart and Espinase, 1959; Gwon et al., 1990, 1993a,b). Without the capsular bag, regeneration in these organisms will not take place (Gwon et al., 1989, 1990; Call et al., 2004). Lens epithelial cells that remain attached to the capsule following surgery have the ability to proliferate, migrate, and differentiate and "fill in" the capsule, and in some cases create a normal lens

(Gwon et al., 1993b). Recently Call and colleagues have shown that mice can regenerate a lens in similar fashion (Call et al., 2004). This process is sometimes considered more of a repair mechanism than a true regenerative event. Lens epithelial cells are constantly proliferating, migrating, and differentiating throughout the lifetime of an organism so in this sense mammalian lens regeneration is merely a continuation of that. In contrast to mammals, newt lens regeneration is regarded as true regeneration in that a terminally differentiated cell can undergo transdifferentiation to replace the lost body part.

Some invertebrates also carry the amazing ability to regenerate parts of the eye or the complete eye itself. Snails have a very similar eye to that of vertebrates with pigment epithelium, retina, and lens in common. If the eye of the snail, located at the end of the cephalic eyestalk, is removed by amputation through the mid-eyestalk, it will regenerate the entire eye through transdifferentiation in about 2 weeks (Miller, Bever, and Borgens, 1988). Planaria, another invertebrate, can also regenerate its eye as well as the entire body. Planaria have a simple eye known as an "eye spot" that consists of two cell types; a bipolar nerve cell with photoreceptive properties and a set of pigmented cells that form a cup shape. Upon head amputation, these cells differentiate from neoblasts, totipotent cells found in the adult animal. The cells of the eye spots express Pax-6, along with a group of pigmented cells that form after amputation and mark eye spot regeneration (Callaberts et al., 1999).

As was illustrated above, lens regeneration occurs via many different mechanisms and even within transdifferentiation different tissues are utilized (cornea in the *Xenopus* and dorsal iris in the newt). With this in mind, is there a common strategy used in regeneration or does each organism employ a unique strategy to regenerate? It may be that organisms that do not normally regenerate still maintain the ability to do so if the right switches are turned on. The key to answering this lies in elucidating the mechanism in regeneration-competent animals and then applying those findings to noncompetent ones.

Through the use of *in vitro* cell culture systems, it has been demonstrated that PECs from many organisms can undergo the transdifferentiation process to form lentoids, lens-like structures. Tissues that were once thought to lack regenerative capabilities have been shown to have the potential for regeneration through these culturing systems. It has been shown that PECs from the ventral iris of the newt could undergo transdifferentiation to form a lentoid (Eguchi et al., 1974). This potential for transdifferentiation is also seen with retinal PECs of chick embryos (Eguchi and Okada, 1973) as well as in human iris and retinal PECs from adult and fetal eyes (Eguchi, 1988; Tsonis et al., 2001).

Not all salamanders possess the ability to regenerate their lens (Stone, 1967). The axolotl, a salamander with extraordinary abilities in limb and tail regeneration, lacks the ability to regenerate a lens. What allows some salamanders to possess regeneration in some tissues and not others is of major interest to researchers worldwide. The fact that the newt will

regenerate a lens *in vivo* from the dorsal iris while the ventral iris cannot regenerate a lens *in vivo* makes the newt a wonderful model for studying lens regeneration.

III. Process of Newt Lens Regeneration

Many events are necessary in order for normal lens regeneration in urodeles to proceed. Histological, cellular, and molecular events all take place during the complex process of regeneration. Soon after removal of the lens, the PECs of the dorsal iris dedifferentiate, that is, they lose cellular characteristics such as pigmentation that define their cell type. Macrophages recruited to the area help mediate this process. There is also an early initiation of cell cycle reentry, which is paramount to proliferation. The first peak of proliferation is observed at about 4 days postlentectomy. At approximately 10 days postlentectomy, the depigmented cells are visible as a vesicle, containing both an inner and an outer layer, at the tip of the dorsal iris (Eguchi, 1963; Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004) (Figure 1). The inner layer of the vesicle begins to thicken at 12–16 days postlentectomy as the cells elongate and differentiate into primary lens fiber cells (Figure 1). It is also at this time point that the second peak of proliferation is observed and the synthesis of crystallins begins. As regeneration ensues, primary lens fibers continue to form in the inner layer, while nondividing secondary fibers start to form in the external layer of the lens vesicle (days 15–19). At 18–20

days postlentectomy, crystallins are continually synthesized. A complete lens is formed with a layer of lens epithelial cells on the anterior surface and lens fibers cells on the interior of the lens 25 days after lens removal (Eguchi, 1964; Yamada, 1977).

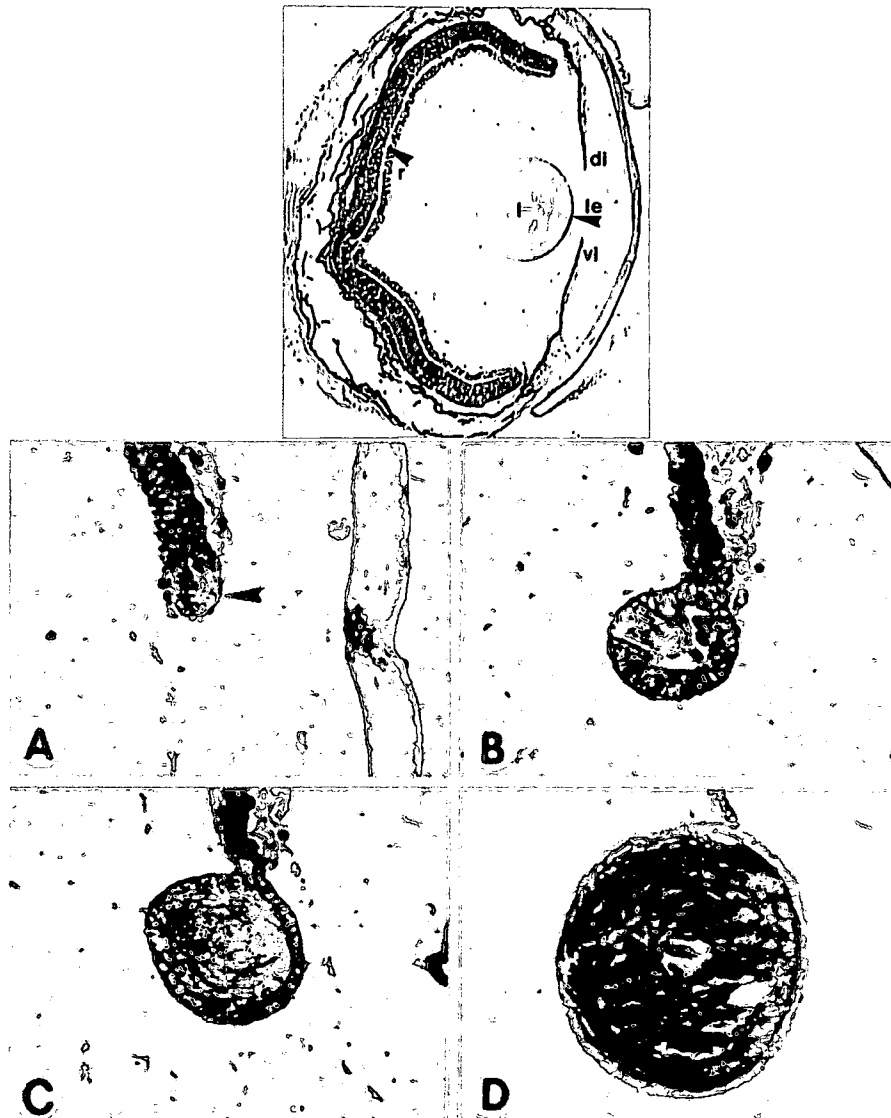


Figure 3. Stages of newt lens regeneration. Top panel – Histology of intact adult newt eye (di – dorsal iris, vi – ventral iris, le – lens epithelium, l – lens, r – retina). A-D – Regenerating lens at 10 days, 15 days, 20 days, and 25 days post-lentectomy, respectively.

IV. Transdifferentiation and Stem Cells

First coined by Selman and Kafatos (1974) as a description for the switch in cell differentiation that takes place during insect development, this term has revolutionized a way of thinking for developmental and cellular biologists. In its simplest definition, transdifferentiation is the process by which a fully differentiated cell or tissue gives rise to a totally different cell or tissue type (Eguchi, 1995). It involves the dedifferentiation of a terminally differentiated somatic cell followed by a differentiation of that cell into another fully differentiated cell or tissue (Tsonis and Del Rio-Tsonis, 2004).

The purest forms of transdifferentiation are seen in salamander limb regeneration, lens regeneration, and metaplasias (Eguchi, 1995). During limb regeneration, cells of varying types such as muscle cells can produce many different mesodermal cell types such as chondrocytes, osteocytes, and fibroblasts of connective tissue (Eguchi, 1995; Tsonis and Del Rio-Tsonis, 2004). Proof that differentiated cells were actually giving rise to completely different cell types came from Eguchi and Okada (1973) when they elegantly showed that well-differentiated retinal pigmented epithelial cells from chick embryos (9 days) changed phenotype to that of lens cells and formed lentoids. These lentoids expressed both structural and molecular characteristics of the lens.

The most recent approach to transdifferentiation is seen in stem cell research. In the past few years studies have shown that stem cells, those

reserved and used for repair, may play more of a role in regeneration than originally thought. In this case the stem cells involved are local (i.e., located in the brain and involved in nervous tissue repair) or non-local (i.e., hematopoietic and involved in repair of several tissues such as liver, nervous, or cardiac) multipotent cells which differ from the urodele repair strategy in that they do not undergo dedifferentiation. It has been hypothesized that there may also be similarities between stem cells and transdifferentiating cells (Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004). For instance, mesenchymal stem cells located in the bone marrow can differentiate to any number of cells (chondrocytes, myocytes, osteoblasts, or adipocytes) much like that of the cells of the blastema. It has also been shown that stem cells residing in the brain can become blood or muscle cells (Blau et al., 2001). It has been shown that mammalian myotubes can transdifferentiate through the generation of progenitor cells (Chen et al, 2004). Chen and colleagues screened over 50,000 discrete small molecules and found a compound, myoreversin, that reversed a terminally differentiated cell into progenitor cells, which were then able to differentiate into osteocytes or adipocytes (Chen et al, 2004; Tsonis, 2004). These findings are of great importance in the regeneration field. If these compounds can induce dedifferentiation in multiple cell types then it begs the question of there being a common signal for dedifferentiation.

One of the major issues concerning "transdifferentiation" of stem cells is that of cell fusion. It has been shown that stem cells can acquire

characteristics of other cells by fusion which might explain the observed transdifferentiation effect of non-local stem cells (Terada et al., 2002; Ying et al., 2002; Wang et al., 2003; Vassilopoulos et al., 2003). The point here being that even though the picture of stem cells that transdifferentiate is unclear, the transdifferentiation that is seen in classical regeneration remains relatively clearer. Having asked the question of whether stem cells can transdifferentiate begs the opposite question of whether non-stem cells that transdifferentiate are "stem cell like". One of the more popular theories these days in the regeneration field suggests that cells capable of regeneration such as the newt PECs in lens regeneration can actually revert back to a "younger" state that resembles developing cells or that these cells have a unique quality in which they are multipotent but do not resemble their "younger" selves. The newt PECs can transdifferentiate to neural retina cells and to lens cells and can also renew themselves (Del Rio-Tsonis and Tsonis, 2003). Thus might they be considered transdifferentiating stem cells? Sustar and Schubiger also tackled this question using *Drosophila* imaginal disc cells and found that in fact, the cells in the imaginal discs that are capable of transdetermination do not revert back to their "younger" selves, but instead convert into a unique cell type (Sustar and Schubinger, 2005). These findings will have a large impact on the mechanisms of the two strategies of regeneration, that of the urodeles and transdifferentiation and that of recruitment of stem cells.

V. Studying Lens Regeneration

The observations of Colucci and Wolff led to a new era of studies on lens regeneration. These studies, a classical series of experimental approaches attempted in order to understand exactly which cells were involved in lens regeneration, are some of the most fascinating in regenerative science. Although many questions were answered by these studies, the overall mechanism of lens regeneration remained a mystery.

The most basic experiment showed that when grafts of dorsal iris were placed into lentectomized eyes they gave rise to a lens (Wachs, 1914; Sato, 1930, 1935; Mikami, 1941). When dorsal irises were placed into the lentectomized eyes of non-regenerating salamanders they were also able to form a lens (Ikeda, 1934; Amano and Sato, 1940; Reyner, 1956). Conversely, regeneration competent dorsal irises that were implanted into the body cavity or subcutaneously in both lens regenerating and non lens regenerating animals did not form a lens (Ikeda, 1935, 1936; Stone, 1958a; Reyner, 1953, 1954). This suggested that perhaps something in the eye cavity was necessary for lens regeneration to take place. Studies went on to show that the neural retina was sufficient to rescue regeneration of the lens in the previous attempts (Stone, 1958a). It was further shown that by removing the neural retina and leaving the iris, lens regeneration could be prevented (Stone, 1958b). The likely reason is that there is a certain factor provided by the retina that plays a role in lens regeneration. Speculation on the identity of

this factor includes fibroblast growth factors (FGFs) because of their known roles in lens development and polarity (see below).

Several of the classical experiments have shown that the lens itself might provide a factor that is involved. If the lens is removed from lens regeneration competent animals and then replaced near the dorsal iris no regeneration occurs or it is minimal depending on the distance from the dorsal iris (Eguchi, 1961). The same lack of regeneration is seen from a dorsal iris explant that is placed in the anterior part of the eye and the lens is either left in or removed and then replaced (Reyer, 1961). It has also been speculated that the lens might play a mechanical hindrance role in regeneration by physically blocking lens regeneration. These classical approaches led to a greater understanding of the parameters of lens regeneration and helped pave the way to the molecular era with many questions looming, mainly that of the actual molecular mechanism.

In order to understand the molecular aspects of lens regeneration, many scientists begin their quest by examining molecules which are known to play a role in vertebrate lens development. Do the same factors that play a role in developing body parts also play a role in regeneration? In other words, does regeneration recapitulate development? It is in this mode of thinking that some of the strongest data has been generated.

VI. Factors in Lens Development and Lens Regeneration

Vertebrate lens development is brought about by a series of inductive interactions in the embryo which leads to the initiation of differentiation of the head ectoderm (Coulombre and Coulombre, 1963). Three primordial tissues (ectoderm from the neural tube, surface ectoderm, and mesoderm) are required for formation of the vertebrate eye. The lens as well as the corneal epithelium is derived from the surface ectoderm. In newt lens regeneration the PECs of the dorsal iris, which derive from neural ectoderm, will form the lens. In most vertebrates, lens development is initiated by proliferation of the ectodermal cells overlying the optic vesicle, which forms the lens placode. This region seems predisposed to become a lens as it expresses *Pax6* even before optic vesicle formation (Li et al., 1994). It has been shown in multiple vertebrates that *Pax6* is essential for lens development (Fujiwara et al., 1994; Ashery-Padan et al., 2000). The placode then invaginates along with the optic vesicle to form the lens pit and optic cup, respectively. The lens pit deepens and is eventually closed off to form a lens vesicle. The lens vesicle soon separates from the overlying ectoderm forming the lens.

Once the lens vesicle has formed, during both lens development and regeneration, a period of differentiation begins. At this point, the process of lens development and regeneration are very similar in terms of differentiation and even that of crystallin synthesis. β -crystallin and γ -crystallin proteins are the first to be detected (McDevitt and Brahma, 1982) in the lens vesicle

followed by α -crystallin (Takata et al , 1964). It is not until later stages that crystallins are detected in the lens epithelial cells (Takata et al, 1966). The similarities in crystallin gene expression between regeneration and development have also been shown via *in situ* hybridization (Mizuno et al., 2002). The crystallins are but a few of the many factors involved in both lens development and lens regeneration. The similarities between lens development and regeneration make the factors/molecules present during lens development a prime starting point in the search for key players in the molecular mechanism of lens regeneration.

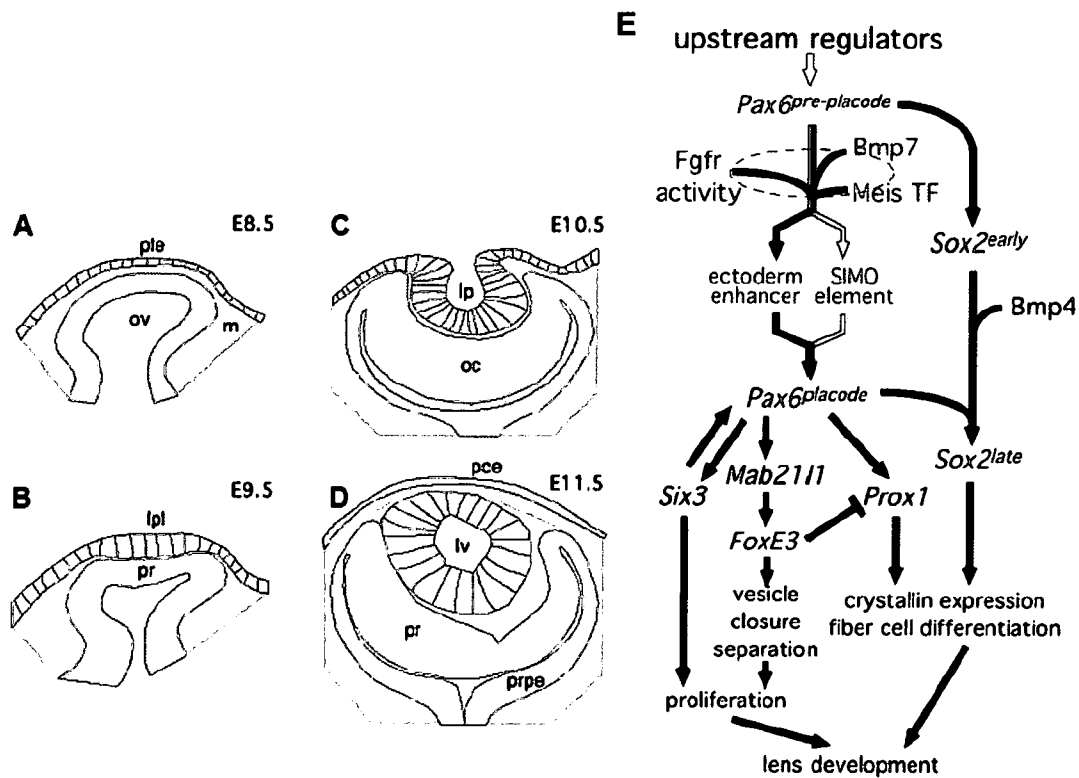


Figure 4. Lens development and genetic pathway model in the mouse. A-D – From E8.5 to E11.5 in daily intervals. The three tissue layers involved in eye development include the surface ectoderm (red) the mesenchyme (blue) and the neuroepithelium of the optic vesicle (green). ple – presumptive lens ectoderm, ov – optic vesicle, m – mesenchyme, lpl – lens placode, pr – presumptive retina, lp – lens pit, oc – optic cup, pce – presumptive corneal ectoderm, lv – lens vesicle, prpe – presumptive retinal pigmented epithelium. E – Model of genetic pathway regulating lens induction. Adapted from Lang, 2004.

Homeobox Genes

Another similarity between lens regeneration and lens development is seen in the expression pattern of two important eye genes, *Pax6* and *Prox1* (Mizuno et al., 1999). *Pax6* has long been known to be one of the most important determinants for eye formation. Mutations in *Pax6* cause aniridia in humans and the "small eye" phenotype in mice and rats (Mizuno et al., 1999). In mice it is expressed in the head ectoderm early in development and at later stages (E10.0) is expressed in the lens vesicle and optic cup. At E13.5, *Pax6* is expressed in the proliferating anterior epithelial cells, but is not detected in the lens fiber cells beyond this stage (Koroma et al., 1997). Another homeodomain protein important in lens development is *Prox1*. *Prox1* is first detected at E9.5 in the lens placode with subsequent expression in the lens vesicle, anterior epithelium, bow region, and lens fibers (Oliver et al., 1993; Tomarev et al, 1996; Glasgow and Tomarev, 1998). Null *Prox1* mice die due to its role in lymphatic vessel development. These mutant mice also show a defect in lens differentiation, which is in turn due to the absence of crystallin (Wigle et al., 1999). In fact both *Pax6* and *Prox1* regulate crystallin expression (Cvekl and Piatigorsky, 1996; Tomarev et al., 1996).

In the regenerating eye, *Pax6* is expressed soon after lentectomy in a broad region that includes both the dorsal and ventral iris. As regeneration continues, *Pax6* expression becomes restricted to the dorsal iris (Del Rio-Tsonis et al., 1995, Mizuno et al., 1999). It is now believed that *Pax6* is more involved in the later events of lens regeneration including proliferation of

PECs in the dorsal and ventral iris and differentiation of lens fibers through control of crystallin synthesis (unpublished). Eventually *Prox1* is expressed within the *Pax6* expression range. This sequential expression of *Pax6* and *Prox1* is also seen in the lens placode during lens development indicating that there may be a common genetic program to both development and regeneration (Mizuno et al, 1999). However, once dedifferentiation is apparent the expression of *Pax-6* is localized to the dorsal iris PECs and subsequently becomes restricted to the lens epithelium of the regenerating lens (Del Rio-Tsonis et al., 1995; Mizuno et al., 1999). *Prox1* expression is more restricted to the dorsal iris during regeneration and not in the regeneration incompetent ventral iris (Del Rio-Tsonis et al., 1999).

Fibroblast Growth Factors

Fibroblast growth factors and their receptors are critical for lens development. FGFs play dominant roles in controlling crystallin gene expression and regulating the spatial and temporal pattern of expression (delongh et al., 1997; Lang, 1999). In addition, they also play a role in lens fiber differentiation and maintenance. In chicks, FGF-8 expression in the distal optic vesicle leads to the expansion of the lens field (Vogel-Höpkner et al., 2000). Targeted overexpression of FGFs in transgenic mice leads to inappropriate differentiation of the lens epithelium (Robinson et al., 1995, 1998; Lovicu and Overbeek, 1998).

In regeneration, several FGFs and their receptors are expressed but only FGFR-1 is present in the dorsal iris during dedifferentiation (Del Rio-Tsonis et al., 1997; Del Rio-Tsonis et al., 1998; McDevitt et al., 1997). Further examination showed that FGFR-1 plays a role in regulating lens regeneration. This was shown by inhibiting the function of FGFR-1, which in turn led to inhibition of lens regeneration and lens fiber differentiation (Del Rio-Tsonis et al., 1998).

Several of the FGF ligands have also been found to have implications in lens regeneration. Hayashi and colleagues have shown that intraocular injection of recombinant FGF2 can trigger lens regeneration from the dorsal iris without previously removing the host lens (Hayashi et al., 2004). Injection of FGF2 also induced expression of several transcription factors such as *Pax6*, *Sox2*, and *MafB* (Hayashi et al., 2004). Intraocular injections of recombinant newt FGF1 also had effects on newt lens regeneration (Yang et al., 2005). In these experiments, the injection of FGF1 caused depigmentation and dedifferentiation of both the dorsal and ventral irises with lens like structures forming from the dorsal irises, albeit with a very thin or missing lens epithelium and abnormal lens fiber cells (Yang et al., 2005). Del Rio-Tsonis and colleagues were the first to show this second lens production after FGF4 treatment in lentectomized eyes (Del Rio-Tsonis et al., 1997). In this experiment treatment with both FGF1 and FGF4 induced interesting abnormalities in the regenerating lenses that seems to coincide with abnormalities induced during lens development in transgenic FGF mice, that

being lenses with abnormal polarity, transformation of lens epithelial cells to lens fibers, and double lens formation from the dorsal iris (Del Rio-Tsonis et al., 1997).

Retinoids

Retinoids and their receptors play major roles in both lens development and regeneration. Retinoic acid is thought to play a role in induction during lens development. Retinoic acid deficiency in mouse embryos leads to a failure to form the lens placode and optic vesicle invagination, which leads to a small eye phenotype (Bavik et al., 1996; Grindley et al., 1995). Retinoic acid receptors play a role in transcriptional control of α B and E crystallins (Gopal-Srivastava et al., 1998) during development. It also appears that retinoic acid and its other analogs regulate gene expression in lens cells and play an important role in maintaining the epithelial layer (Lovicu and Robinson, 2004). Retinoic acid has also been shown to control the fate of neural retinal cells during development (McCaffrey et al., 1993; Wagner et al., 2000). In addition to these roles in development, exogenous retinoic acid has led to the formation of ectopic lens differentiation (Manns and Fritzsch, 1991).

Retinoic acid and retinoic acid receptors not only play a role in development but they also play a crucial role in newt lens regeneration. Treatment of newts, following removal of the lens, with an antagonist to the retinoic acid receptors or with disulfiram (a chemical which inhibits the

synthesis of retinoic acid) severely retards the regenerative capability of the dorsal iris. While inhibition was the most prevalent outcome there were also a few cases of ectopic lenses being formed (Tsonis et al., 2000; Tsonis et al., 2002).

Sonic Hedgehog

While it has not been implicated directly in lens development, sonic hedgehog (Shh) plays different roles in eye development and patterning. Shh expression from the ventral forebrain regulates the spatial expression of Pax6 thereby setting up the midline and preventing cyclopia (Ekker et al., 1995; Macdonald et al., 1995). Interfering with normal Shh expression underlies its importance. Shh mutants, overexpressing Shh, and inhibiting the hedgehog pathway in several animal models results in eye defects and in some cases cyclopia (Belloni et al., 1996; Chiang et al., 1996; Huh et al., 1999; Macdonald et al., 1995; Perron et al., 2003; Roessler et al., 1996; Sasagawa et al., 2002; Stenkamp et al., 2000; Zhang and Yang et al., 2001). In zebrafish, Gli-2 mutants show a transdifferentiation of the adenohypophysis to lens (Kondoh et al., 2000). Tsonis and colleagues were the first to show expression of *Shh* and *lhh* as well as their receptors *Ptc-1* and *Ptc-2* in the lens during both development and regeneration (Tsonis et al., 2004). Interestingly, however, *Shh*, *lhh* and their receptors are absent in the intact adult lens (Tsonis et al., 2004). It has also been shown that interfering with the hedgehog pathway results in inhibition of the lens regeneration process including decreased cell

proliferation and defective fiber cell differentiation (Tsonis et al., 2004). Hedgehog signaling has also been shown as the cause of eye degeneration in blind cavefish (Yamamoto et al., 2004). Overexpression of *shh* or *twhh* can also lead to eye degeneration through hyperactivation of downstream genes, lens apoptosis and arrested growth and development of the eye in surface fish as well (Yamamoto et al., 2004).

Bone Morphogenetic Proteins

Although the expression of bone morphogenetic proteins (BMPs) and their receptors during newt lens regeneration remains largely untested, BMPs are known to play a key role in development of the mammalian lens. Both BMP4 and BMP7 are expressed in developing eye tissues with overlapping expression patterns (Dudley and Robertson, 1997; Furuta and Hogan, 1998; Wawersik et al., 1999). BMP7 is expressed in the presumptive lens ectoderm, presumptive RPE, and the dorsal optic cup (Dudley et al., 1995; Lang, 2004). The deletion of *BMP7* results in eye defects such as microphthalmia to anophthalmia (Dudley et al., 1995; Luo et al., 1995). Lens specific defects such as failure or defects in lens placode formation as well as loss of *Pax6* expression in the placode are also seen in *BMP7* deletion studies (Wawersik et al., 1999). These studies have led to the thinking that *BMP7* is directly upstream of *Pax6* lens placode expression (Dimanlig et al., 2001; Lang, 2004).

BMP4 has also been shown to be critical in mammalian lens development. Null *BMP4* mice lack lens formation (Furuta and Hogan, 1998). Lens formation can be rescued by culturing the eye primordia in the presence of recombinant BMP4 (Furuta and Hogan, 1998). Interestingly, the absence of BMP4 does not affect *Pax6* expression (Furuta and Hogan, 1998). It is thought, therefore, that BMP4 is likely to regulate *Sox2*, which works in concert with *Pax6* to regulate crystallin expression and lens fiber differentiation (Lang, 2004). BMPs have implications in almost every developmental process and are a very likely candidate for some role in lens regeneration.

Six3

A member of the six-homeodomain family, it was the expression pattern of *Six3* that first implicated it in lens development (Lang, 2004). In mice, *Six3* is first expressed in the lens placode followed by expression in the lens epithelium during differentiation stages (Oliver et al., 1995). A mildly different pattern is seen in *Medaka*, where expression is seen in the presumptive lens ectoderm but down-regulated in the lens placode prior to lens differentiation (Loosli et al., 1998). *Six3* expression in the chick is similar to the mouse in that it is seen in the presumptive lens ectoderm overlying the optic vesicle but remains through the lens placode stage to end up localized in the lens epithelium (Bovolenta et al., 1998).

Functional studies have shown the importance of *Six3* in lens induction. Misexpression of *Six3* in *Medaka* resulted in the formation of ectopic lenses (Oliver et al., 1996). It seems that *Six3* induced ectopic lenses arise from transformation of the otic vesicle (Oliver et al., 1996). As opposed to *Pax6* induced ectopic lenses, the *Six3* induced ectopic lenses appear to be produced in a non-autonomous manner (Oliver et al., 1996). It is now thought that *Six3* induces a soluble factor that changes the bias of the otic placode towards a lens fate (Oliver et al., 1996). Furthermore, *Six3* and *Pax6* are now known to be able to mutually activate each other through *Six3* binding sites in the *Pax6* ectoderm enhancer and *Pax6* binding sites in the *Six3* gene (Goudreau et al., 2002). Although elaborate *Six3* expression studies have not been conducted during newt lens regeneration, the possibility is high that *Six3* is involved making it a strong candidate.

Factors in Lens Development	Expressed During Lens Regeneration	Role in Lens Regeneration
Pax6	Yes	Unknown
Prox1	Yes	Unknown
FGF1	Yes	Dorsal Induction
FGF2	Yes	Dorsal Induction
FGF4	Yes	Dorsal Induction
FGFR1	Yes	Inhibits if Blocked
RA	Unknown	Inhibits if Blocked
RARs	Yes	Inhibits if Blocked
Shh	Yes	Inhibits if Blocked
Ihh	Yes	Unknown
Ptc1	Yes	Unknown
Ptc2	Yes	Unknown
BMP4	Unknown	Unknown
BMP7	Unknown	Unknown
BMPR-IA	Unknown	Unknown
Six3	Unknown	Unknown

Table 1. Summary of known factors in lens development and lens regeneration. Expression – factor is expressed at some point in development or regeneration. Role in lens regeneration – Factors have been shown to play some antagonistic or agonistic role in lens regeneration.

VII. Induction of Lens Regeneration

It has been clearly established that *in vivo* lens regeneration in urodele amphibians comes from the dorsal iris only. Several studies have also shown an induction of lens formation from the dorsal iris when the lens is not removed. Both FGF1 and FGF2 have been implicated in lens formation from the dorsal iris through intraocular injection (Yang et al., 2005; Hayashi et al., 2004). FGF4 has also been shown to induce double lenses from the dorsal

iris when injected after lentectomy (Del Rio-Tsonis et al., 1997). Other studies have shown that lens regeneration from the dorsal iris can be blocked or interrupted successfully (Tsonis et al., 2004; Del Rio-Tsonis et al., 1998; Tsonis et al., 2002). While many scientists have successfully inhibited lens regeneration from the dorsal iris, the "Holy Grail" of lens regeneration studies is the one that is able to induce lens regeneration from the ventral iris. To date only one known treatment, that of the potent carcinogen MNNG, has elucidated such an induction. MNNG was added to regenerating eyes and the result was ectopic lenses from the ventral iris (Eguchi and Watanabe, 1973). This was also done on cultured ventral irises *in vitro* that were treated with the carcinogen and implanted back into a lentectomized eye. Some of the implants produced a lens in this study as well (Eguchi and Watanabe, 1973). While the mechanism is still unknown, it was suggested in that study that MNNG altered the cell surface properties of the ventral PECs. However, this has not been documented. Nevertheless, these experiments clearly show that induction is possible and remains one of the greatest challenges in the field.

When looking at the possibility of inducing lens regeneration from the ventral iris in newts, it is hard to ignore the similarities of the factors involved in lens development and lens regeneration. As seen above, there are many factors to choose from that play significant roles in lens development and lens regeneration. Some of the candidates are soluble factors involved in cell signaling. Others are transcription factors with known and unknown targets.

Once the factors are chosen then the methodology needs to be decided upon. Normally one would consider looking at endogenous expression levels in intact and regenerating tissue. Also, utilizing knockout or knockdown technology is one worth considering. Finally, over-expression of certain genes or proteins is also a worthy goal. Transgenesis in the newt is still in the early stages of development. At the time when most of this work was done transgenesis in the newt or other salamanders was virtually nonexistent. It was only in the last year that the first case of successful transgenesis in newts was reported (Ueda et al., 2005). We therefore had to be creative in our methods.

Our goals consisted of two main aims. The first was to over-express our candidate genes and proteins in dorsal and ventral iris cells and tissue explants of the newt. These studies represent our functional studies. We examined the effects of over-expressing the different candidate genes during lens regeneration. The second was to conclusively study the expression levels of several of the candidate genes in newt dorsal and ventral iris in intact and regenerating tissue. These studies showed that, in fact, induction of lens regeneration from the ventral iris in newts is possible. Specifically, by blocking the BMP pathway using the inhibitor Chordin or a truncated form of the BMPRII protein lens induction from the ventral iris was possible. Further, over-expressing *Six3* and adding retinoic acid also led to induction of lens regeneration from the ventral iris in newts.

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Chapter II

BMP inhibition-driven regulation of *six-3* underlies induction of newt lens regeneration

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Abstract

Lens regeneration in adult newts is a classic example of faithfully regenerating an entire organ via transdifferentiation¹⁻⁶. After lentectomy, intriguing regulation allows the pigment epithelial cells (PECs) of the dorsal iris, but not the ventral, to dedifferentiate and then differentiate to form a new lens. This regulation might provide clues to the lack of lens regeneration in higher vertebrates. *Six-3* and *pax-6* known for their ability to induce ectopic lenses during embryogenesis^{7,8} and members of the BMP pathway, which are regulators of the dorsal/ventral axis establishment in embryos⁹ were examined for their role in induction of lens regeneration. Here we show that lens regeneration from the ventral iris is possible by inhibiting the BMP pathway or by transfecting ventral iris cells with *six-3* and concomitant treatment with retinoic acid. In intact irises *six-3* is expressed higher in the ventral iris. During regeneration, however, only levels in the dorsal iris are significantly increased. Such an increase is seen in ventral irises only when they are induced to transdifferentiate by *six-3*/RA or BMP inhibitors. Therefore, transcriptional regulation associated with competency for lens regeneration, aims to increase levels over established thresholds and not to merely render a regulatory gene as dorsal-specific. Lack of induction in the axolotl, a salamander incapable of lens regeneration seems to be associated with repression of *six-3* expression.

Methods

All methods not listed here can be found in Supplementary Information.

Cloning of newt *six-3* and *BMPR-1A* partial cDNAs. BMPR-1A cloning was performed with RNA isolated from newt forelimb blastema (~ 2 weeks post-amputation) using TRI REAGENT[®] (Molecular Research Center, INC.) according to manufacturer's instructions. Dorsal PECs were used to clone a partial cDNA for *six-3*. One microgram of RNA was used to synthesize cDNA using iScript[™] cDNA Synthesis Kit (BioRad). For PCR, a portion of the DNA was used along with *Taq* polymerase, 200 μ M dNTPs, and 800 nM primers. Primers used were as follows: *BMPR-1A* forward 5'- TGCTGYATTGCTGAYYTDGG, reverse 5'- GGRTCATTYGGCACCA; *six-3* forward 5'-CACTACCAGGAGGCCGAGAA, reverse 5'- TCCTTGAAGCAGTGCGTCTT. DNA was purified using Qiagen MinElute[™] Gel Extraction Kit. The fragment was cloned using the pGEM[®]-T Easy Vector Systems (Promega) and sequenced.

Immunostaining. Affinity purified polyclonal antibodies were made against *six-3* and *BMPR-1A* peptides. The *six-3* antibody was made in rabbit (New England Peptide, Inc.) and the *BMPR-1A* in chick (Cocalico). Newts were anesthetized and the lens was removed through a slit in the cornea. They were sacrificed at 2, 4, 8, 12, 15 and 25 days post-lentectomy. The eyeballs were enucleated and fixed in 4% formaldehyde for 4 hours, washed in 1X PBS and cryoprotected in 30% sucrose, followed by embedding in OCT (Andwin Scientific, Warner Center,

CA), freezing and sectioning at 10µm. Slides with frozen serial sections were washed several times in PBS and 1% saponin (Sigma, St. Louis, MO) and incubated in 10% goat serum/PBS. Occasionally, for reduction of pigmentation, sections were bleached in 0.1% potassium permanganate for 10 minutes, followed by immersion in 0.5% oxalic acid for 5 minutes and rinses in PBS. The samples were incubated at 4°C overnight with primary antibody (anti-newt six3 diluted 1:10 in blocking solution or anti-newt BMPR diluted 1:100 in blocking solution), followed by washes in 0.3% PBST and PBS, and incubation secondary antibody for 2 hours at 37°C: goat anti-rabbit- Alexaflor 546 (Molecular Probes, Invitrogen, Eugene, OR) for six3, and rabbit anti-chicken-FITC (Sigma, St. Louis, MO) for BMPR, diluted 1:200 in 10% goat serum in PBST. The sections were again washed with PBST and PBS, and coverslipped using Vectashield (Vector labs, Burlingame, CA). Pictures were taken using confocal microscopy.

Real-Time PCR. RNA was isolated from iris tissue and pigmented epithelial cells (PECs) using TRI REAGENT® (Molecular Research Center, INC.) according to manufacturer's instructions. The following tissues and cells were used: Intact dorsal and ventral iris, 2, 4, 8-day dorsal and ventral iris, isolated cells from dorsal and ventral iris, transfected cells with *six-3/RA*, *six-3* alone, RA alone from the dorsal and ventral iris, explants from dorsal and ventral iris, explants treated with chordin or BMPR-1A from dorsal and ventral iris, axolotl intact and 8-day dorsal and ventral iris and chordin-treated axolotl dorsal and ventral iris. The isolated RNA was used to evaluate expression of *six-3*, *BMPR-1A* and *pax-6*

(along with a suitable reference gene) via Real Time PCR and RT-PCR. RT-PCR was employed to verify that the correct fragment was amplified. Appropriate negative controls were included in all sets. 0.75 micrograms of RNA was used to synthesize cDNA using iScript™ cDNA Synthesis Kit (BioRad). All Real-Time PCRs were performed using the iCycler™ (BioRad). For each Real-Time PCR reaction run in triplicate 2 microliters of cDNA, 800 nM primers, and iQ™ SYBR® Green Supermix (BioRad) were used. Primers were designed from the cloned cDNAs for *six-3* and *BMPR-1A* and from previously published sequence for *pax-6*. Newt Primers – *rpL27*: Forward 5'- TACAACCACTTGATGCCA, reverse 5'- CAGTCTTGTATCGTTCCTCA, *pax-6*: Forward 5'- CTGGGCAGGTATTACGAG, reverse 5'- GTCTCTGATTTCAGGC, *six-3*: Forward 5'- CAAGAAGTTCCCGCTGC, reverse 5'- GGTAGGGGTCCTGTAGGTAC, *BMPR-1A*: Forward 5'- TGCTGTATTGCTGATTAGG, reverse 5'- ATAGGTATCAAAGCAGTCCA. Axolotl Primers – *RP*: Forward 5'- CATCAGATCAAGCAAGCAGTA, reverse 5'- CCAATGCAGCAGTTTAGATG, *pax-6*: Forward 5'- GAGTGCTCCGCAACCTG, reverse 5'- ATTCGTGTTCTCGCCTCC, *six-3*: same as newt, *BMPR-1A*: Forward 5'- CAG TGC TGC ATT GCT GAT, reverse: 5'- GGC TAC TTC CCA AAT AAC C. For each Real-Time PCR the basic program was as follows: Denaturation at 95°C, annealing at 50.6°C, and extension at 72°C (40 cycles). To minimize the background caused by primer-dimer formation, an extra step was added (78°C for 6 seconds) at the end of each cycle. The readings were taken during this step. Data analysis was performed using the Pfaffl method²⁴. The reference

genes were *rpL27* for the newt and *RP* for the axolotl. They both encode ribosomal proteins.

Supplementary Information

Culture of iris epithelial cells

The newt, *Notophthalmus viridescens*, was used in this study. Animals were anesthetized with 0.1% ethyl 3-aminobenzoate, methanesulfonic acid. Eyeballs were enucleated and washed twice in Ca^{2+} - Mg^{2+} free newt Hanks (110 mM NaCl, 4mM KCl, 0.4 mM KH_2PO_4 , 0.3 mM Na_2HPO_4 , 5 mM HEPES, 0.1 g/L kanamycin sulfate, pH 7.4). Eyeballs were sterilized for 3 seconds in 5% Lugol's solution in 70% ethanol and rinsed in newt Hanks (add 0.11 g CaCl_2 and 0.08 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ to 1 L Ca^{2+} - Mg^{2+} free Hanks). The iridocorneal complex was removed in newt Hank's solution and cleared of the lens and any remaining neural retina. The iridocorneal complex was transferred to a new dish of newt Hanks where the ventral and dorsal halves of the iris were separated. The isolated iris halves were separately placed in complete L-15 culture medium modified for the newt (L-15, 10 mM HEPES pH 7.4, 10% fetal bovine serum, 1% amphotericin B, 1% kanamycin sulfate) with 1000 U/ml dispase (Godoshusei Co. Ltd, Tokyo, Japan) for enzymatic dissociation and incubated for 3 hours at 27° C. Following dispase treatment, the iris pigmented epithelial cells were separated from the stroma and collected into a microcentrifuge tube. The cells were

pelleted by centrifugation at 1200 r.p.m. for 30 seconds. The cells were washed in newt Hanks and treated with newt trypsin solution (Ca^{2+} - Mg^{2+} free newt Hanks, 1 mM MgCl_2 , 0.25% trypsin, 0.01% DNaseI, pH 8.0) for 2 hours at 27° C for further dissociation. Dissociated iris cells (1×10^4 cells) were placed onto a 35-mm type I collagen-coated dish with complete L-15 and incubated at 27° C.

Transfection

LipofectAMINE PLUS™ Reagent (Gibco Life Technologies, Rockville, Maryland) was used for DNA transfection 15 days after culturing. Methods used were based on the manufacturer's protocol. The expression vectors used are as follows: mouse *six-3* cloned into CMV5 and human *pax-6* cloned into pRC/CMV. Both genes are driven by the CMV promoter. Briefly, 4 µg of plasmid DNA and 16 µl of each LipofectAMINE and PLUS reagent were used for each transfection. The DNA/lipofectAMINE solution was added to the cultured iris epithelial cells in which the complete L-15 was replaced by serum-free L-15 culture medium and incubated for 3 hours at 27° C. Following incubation the transfection medium was removed and replaced by complete L-15 culture medium with or without 10^{-8} M all-trans-retinoic acid (Acros Organics, New Jersey) added. Treatment with retinoic acid was continuous. Transfection efficiency was approximately 75-80% as determined by β -galactosidase staining (Fig. 1a). Transgene expression was present only in the transfected iris PECs and not in the untransfected cultures (Figure 1b). We also examined whether the products of the transgenes were

made. Indeed, both human pax-6 and mouse six-3 proteins were detected in the nucleus (Fig. 1c-f).

RT-PCR of partial cDNA for the transfected genes and detection of the exogenous proteins

RNA was isolated from transfected PEC cultures using TRI REAGENT[®] (Molecular Research Center, INC.) according to the manufacturer's instructions. The total RNA (approximately 2 µg) was reverse transcribed in a reaction containing random hexamers using the First-strand cDNA Synthesis Kit (Amersham). For PCR, a portion of the DNA was used along with *Taq* polymerase, 200 µM dNTPs, and 800 nM primer. Primers used were specific to each transfected gene construct. Following amplification, the samples were run on an agarose gel and analyzed for bands of the expected size (Fig. 1b). Human pax-6 protein and mouse six-3 were detected by immunostaining with specific antibodies that do not cross-react with the endogenous counterparts (Santa Cruz) (Fig. 1c-f).

Preparation of aggregates and transplantation

In order to prepare aggregates, iris epithelial cells were harvested 4 days after transfection by dispase treatment (1000 U/ml in L-15 complete medium) for 7 hours at 27° C. Cells were collected into a microcentrifuge tube, pelleted by centrifugation at 1200 r.p.m. for 30 seconds, and washed twice in L-15 complete medium. Cells for each aggregate (6000-8000 cells) were placed into a

microcentrifuge tube with L-15 complete medium, pelleted by centrifugation at 1200 r.p.m. for 30 seconds, and incubated at 27° C for 48 hours. Aggregates from dorsal and ventral iris cells were transplanted into the pupillary region of lentectomized host newts. The aggregate must be placed and remain within the pupillary region of the eye. If the aggregate is posterior to the host lens it would not transdifferentiate¹⁰. During transplantation, however, exact positioning is not always feasible and uncontrolled movement of the transplant can occur. Therefore, in order to account for all possibilities, the results in Table 1 are divided into three columns. The first column shows the total number of double lenses per host eye, while the second column depicts the number of double lenses per case where a transplant (aggregate) was found. The third column represents what we call the normalized data, which consist of only those aggregates located in the anterior region of the eye. After 20-30 days the eyes were collected and processed for histological examination.

Iris explant treatment

Newt dorsal and ventral irises were isolated using the same procedure used for culturing the PECs. The isolated iris halves were separately placed in complete L-15 culture medium. All irises (control and treated) were incubated for 48 hours at 27°C. The treatments were added daily to the media during the 48 hour incubation and were as follows: 300 ng/ml rhBMPR-1A, 3-6 µg/ml rmChordin, 30 ng/ml rhBMP-4, and 600 ng/ml rhBMP-7. All proteins were from R&D Systems. Following treatment the iris explants were implanted into lentectomized newt

eyes. The explants were positioned in the pupil region of the eye. The newts were then maintained for 30 days at which point the eyes were collected for histology.

In order to determine the role that *six-3* and *pax-6* play in the induction of transdifferentiation of the ventral iris, ventral iris cells were transfected in the presence or absence of retinoic acid (RA) with the appropriate constructs and examined for induction by utilizing an *in vitro* transfection/*in vivo* transplantation system that reproduces the conditions seen *in vivo*¹⁰⁻¹². Retinoids have been shown to affect regeneration and to determine morphogenesis and differentiation of several tissues including the eye and limb¹³⁻¹⁶. In addition, dorsal or ventral iris explants were treated with soluble BMP-4, BMP-7, chordin and a soluble competitor for BMPR-1A.

Following transfection and implantation of aggregated PECs, scores of eyes were examined (see Supplementary Information). As a rule, untransfected dorsal PEC aggregates transdifferentiate to lens while the ventral ones do not. Under the conditions outlined in Methods short term culturing of cells does not interfere with the potential for lens transdifferentiation. Dorsal aggregates produced a lens in over 83% of the cases (10/12), while the ventral ones, as expected, did not (0/11) (Fig. 1a-c). It has been shown before, through beta galactosidase staining, that the lens is indeed derived from the aggregate¹². Dorsal aggregates transfected with the constructs with RA treatment also transdifferentiated to lens (not shown). However, with ventral PECs, only one particular protocol, transfection of PECs with *six-3* in the presence of retinoic acid, led to the induction of lens transdifferentiation (Fig 1d-f). This induction occurred at a comparable rate (3/4; 75%) to that seen in the dorsal aggregates. Neither treatment with retinoic acid alone nor transfection of ventral PEC cultures

with *six-3* alone was able to induce transdifferentiation. In the BMP series we found that inhibition of the pathway by either the BMPR-1A competitor or chordin resulted in the induction of a lens from the ventral explants (3/15 and 1/8 respectively) (Figure 1g-j). The incidence of induction was low (17%), however, we regard this as highly significant in light of the failure of the untreated ventral explants to differentiate to lens (0/27; 0% induction). This is in agreement with the established role of BMPs in maintaining ventral identity during embryogenesis and the fact that inhibition of BMPs binding to receptors results in dorsalization⁹. Interestingly, treatment of the dorsal iris explants with BMP-7, and to a lesser degree BMP-4, significantly inhibited their ability to transdifferentiate to lens (1/12; 8.3% and 5/12; 41.6% respectively). Such results clearly indicate that BMPs maintain the ventral identity and inhibition of the pathway dorsalizes the ventral iris allowing transdifferentiation.

In order to further probe the mechanism of induction, we decided to undertake a detailed gene expression profiling of *six-3* and *BMPR-1A* during lens regeneration and during the experimental treatments that lead to the induction of lens regeneration from the ventral iris. *Pax-6* expression was also assessed because of its known association with *six-3*. We selected to work with samples of iris isolated 2, 4 and 8 days post lentectomy. During this time, dedifferentiation events that lead to regeneration from the dorsal iris have been initiated. Moreover, at later stages the vesicle starts expressing crystallins and differentiating to lens. Since these genes are also expressed in the differentiating lens their induction-related expression might be 'contaminated.' Several

interesting points emerged from the expression patterns, which somewhat were very surprising and call for a revision of our view of the mechanism of lens regeneration. First, both dorsal and ventral iris showed expression of all three genes. When the data were analyzed to compare between the dorsal and ventral iris we found that the three genes were expressed higher in the intact ventral iris. This pattern was maintained by day 8 but with a lesser relative fold change (Fig. 2a). When the data, however, were analyzed in a different way to compare expression in the 2, 4 and 8-day dorsal iris with the intact dorsal iris and the 2, 4 and 8-day ventral iris with the intact ventral iris, to correlate expression with the process of regeneration, an interesting pattern emerged: The levels of *six-3* were elevated in the dorsal iris only and seem comparable at this time to the ventral ones. *BMPR-1A* and *pax-6* were also slightly up-regulated (Fig. 2b-d). Up-regulation of *six-3* in the dorsal iris started at day 4 (Fig. 2c), while for *pax-6* and *BMPR-1A* at day 8 (Fig. 2d). In other words, increase of *six-3* levels seems to be important during the dedifferentiation process in the dorsal iris. Since regeneration occurs only from the dorsal iris and since the ventral iris also expresses these genes, our data suggest that gene regulation associated with the competency for lens regeneration aims to increase levels over a particular threshold and not simply rendering a regulatory gene as dorsal-specific. Such a pattern for *six-3* is clearly shown when the expression of the different time points is also presented in comparison to intact dorsal iris in one cluster (Fig. 2e).

Treatment of ventral iris cells with *six-3*/RA, which resulted in induction of transdifferentiation, showed a similar pattern of up-regulation of *six-3*, *pax-6*, and

BMPR-1A when compared to the untransfected ventral cells (Fig. 2f). Treatment of the cells with RA alone or transfection of *six-3* alone, which failed to induce irises to differentiate to lens, did not show such a pattern (not shown). Similarly, treatment of ventral iris explants with chordin, which also resulted in induction, invoked marked up-regulation of *six-3* and *pax-6*, and to a lesser degree of *BMPR-1A*, in the treated ventral irises, as compared with the increase in the untreated irises (Fig. 2g). *BMPR-1A* transcriptional regulation might not be that important for the induction. Interestingly the rate of increase (as relative fold change) in the treated ventral irises is comparable to the increase in the regenerating 8-day dorsal iris. In other words the treated ventral irises that were able to transdifferentiate to lens adopted a gene expression profile (especially for *six-3*) that is seen only in the dorsal iris during dedifferentiation and regeneration. This in turn indicates that when the ventral irises are coaxed to mimic patterns of regulatory events seen in the dorsal iris they are able to be "dorsalized" and therefore transdifferentiate into lens.

These expression patterns for *six-3* pose the following question: Are there subpopulations in the dorsal or ventral iris that might account for these differences? To answer this question we used immunostaining to assess the distribution of *six-3* expressing cells. We stained serial sections along the nasal-temporal axis that spanned the whole iris (with distinct dorsal and ventral portions) and we counted the positive cells. *Six-3* positive cells were found throughout the examined 8-day dorsal and ventral irises without apparent differences in their distribution (Fig. 3a). These results show that *six-3* up-

regulation is not attributed to expression in more cells. When the aggregates (or explants) transdifferentiated to lens nearly all cells participated, arguing against *six-3* expressing subpopulations as well (Fig. 1). Since expression of *six-3* and *BMPR-1A* in lens regeneration has not been reported before we also examined their expression throughout the process. In Fig. 3b we show expression in dorsal and ventral iris at early stages (before vesicle formation) and in Fig. 3c during later stages (in vesicle or regenerating lens). Ventral iris of the later stages is also positive (not shown). The presence of *six-3* in dorsal and ventral iris is consistent with the QPCR data, immunostaining, however, is not quantitative.

The next question is whether the "newt treatments" are unique to the newt or if they can induce lens transdifferentiation in irises from other vertebrates. To answer this question we used another salamander, the axolotl, which possesses the ability to regenerate limbs or tail, but not the lens. None of the treatments induced transdifferentiation, indicating that the treatments are most likely newt-specific. However, it might be too premature to preclude the participation of *six-3* and BMP inhibition in lens regeneration in other species. In the newt the lens is regenerated from the dorsal iris and in the premetamorphic frog from the cornea, two strategies that differ from the embryonic induction of lens development as well. This might argue against absolute conservation of the inductive mechanisms. To receive some insight that could explain the axolotl data we examined gene expression in intact irises, in irises 8 days after lentectomy and in treated irises. In contrast to what was observed in the newt, the expression profiles in the axolotl differ considerably in both intact and 8-day irises (Fig. 4a).

The intact and 8-day ventral irises do not show higher levels of expression over the dorsal ones. Moreover, *six-3* was severely down regulated in the irises 8-days after lentectomy when compared with the intact irises. *Pax-6* and *BMPR-1A* were slightly upregulated in both dorsal and ventral irises (Fig. 4b). This expression pattern is diametrically opposite to what we observed in the newt and indicates that a negative regulation of *six-3* as well as regulation in establishing thresholds might result in inability for lens regeneration in the axolotl. So, why did the treatments fail to induce lens transdifferentiation? We believe because repression of *six-3* persists even after the treatments. Indeed, treatment with chordin, which mediated *six-3* and *pax-6* up-regulation in the induced newt ventral irises failed to do so in the axolotl (compare Fig. 4c with Fig.2g). Several explanations can account for the axolotl results. First, *six-3* repression could be regulated more tightly than in the newt possibly by an inhibitor of its transcription and function. Second, the axolotl PECs do not respond to these treatments equally and they may require optimized conditions. Third, the mechanism of induction of lens regeneration in different vertebrates follows unique pathways.

It is rather interesting that *pax-6* was unable to induce transdifferentiation of the ventral iris, even though it was shown to be up-regulated in the chordin-treated ventral irises. However, in *pax-6* transfected cells *six-3* was not upregulated (not shown) and this might be the reason why *pax-6* was not able to induce transdifferentiation. Based on other results from our laboratories we now believe that *pax-6* is rather involved in later events of lens regeneration, such as the proliferation of PECs in both the dorsal and ventral iris and control of

crystallin synthesis¹⁷. The fact that RA was also necessary for the induction most likely indicates that other factor(s) regulated by RA are involved, a synergism that has been shown in other studies as well^{16, 18,19}. Interestingly, both inhibitors of the BMP pathway and the *six-3/pax-6* loop are part of a network identified during induction of eye development²⁰. The up-regulation of *six-3* and *pax-6* in chordin treated iris explants (Fig. 3f) suggests that the BMP signaling is upstream of the *six-3/pax-6* regulatory loop.

Previous work by us and others has shown that other important regulators of lens differentiation, such as *FGFs*, *Sox2*, *MafB* and members of the hedgehog pathway are expressed in both dorsal and ventral iris^{21,22}. This goes against the commonly held belief that regulatory genes involved in lens regeneration should be dorsal-specific. However, the detailed quantitative studies reported here suggest novel regulatory events involved in the induction of lens regeneration. Collectively, our data presented here demonstrate that induction of lens regeneration can be achieved in non-competent adult tissues. This is important because ectopic lens formation has never been shown in adults and opens new avenues in the field of vertebrate lens regeneration.

Figure 1. Lens induction from ventral PECs. a-f, Lens induction by transplantation of PEC aggregates examined 30 days later. Thick arrows indicate the host regenerated lens and arrowheads the PEC aggregate or the induced lens from the PEC aggregate. **a,** A control untransfected dorsal PEC aggregate, which has transdifferentiated to lens. le: lens epithelium, lf: lens fibers, di: dorsal iris, vi: ventral iris. **b,** A control untransfected ventral PEC aggregate (arrowhead), which has remained pigmented and failed to transdifferentiate to lens. **c,** Detection of crystallin synthesis in both a host lens and an induced lens with a lens fiber-specific antibody to β -crystallin²³. **d-f,** Induced lenses from ventral PEC aggregates transfected with *six-3* and treated with RA. **g-j,** Induced lenses from ventral iris explants treated with BMPR-1A inhibitor (**g-i**) and with chordin (**j**).

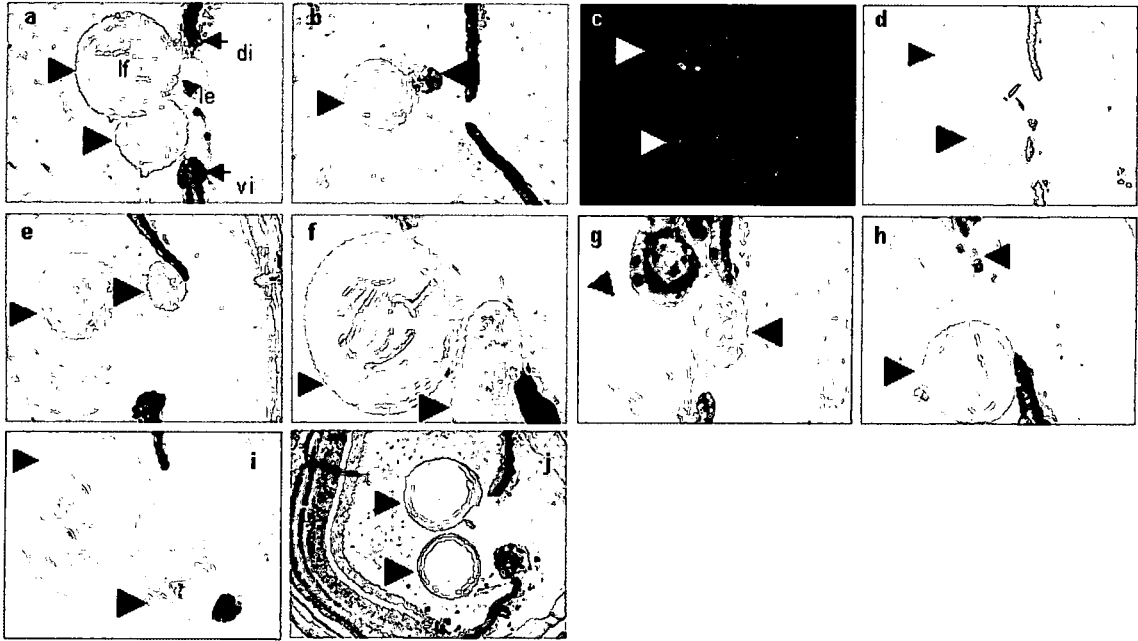


Figure 2. Expression during lens regeneration and induction. **a**, Expression of *pax-6*, *six-3* and *BMPR-1A* in the newt intact and 8-day post-lentectomy dorsal and ventral iris. Comparison between intact dorsal and ventral iris. The values of the dorsal irises have been set to 1 and those of the ventral iris are shown as relative fold changes. **b-d**, Expression of *pax-6*, *six-3* and *BMPR-1A* shown by comparing 2, 4 and 8-day irises with intact irises. **e**, Expression of *six-3* at all time points compared with its expression in the intact dorsal iris. **f**, Expression in *six-3*/RA treated PECs in relation to the untransfected cells. Note significant increase of exogenous *six-3* levels in the ventral PECs. Levels of *pax-6* and *BMPR-1A* are much lower and are represented by the numbers on the right y axis (to accommodate the graph with the very high fold increase for *six-3*). **g**, Expression in chordin treated iris explants shown as relative fold change to the untreated explants. Results are means \pm s.d.

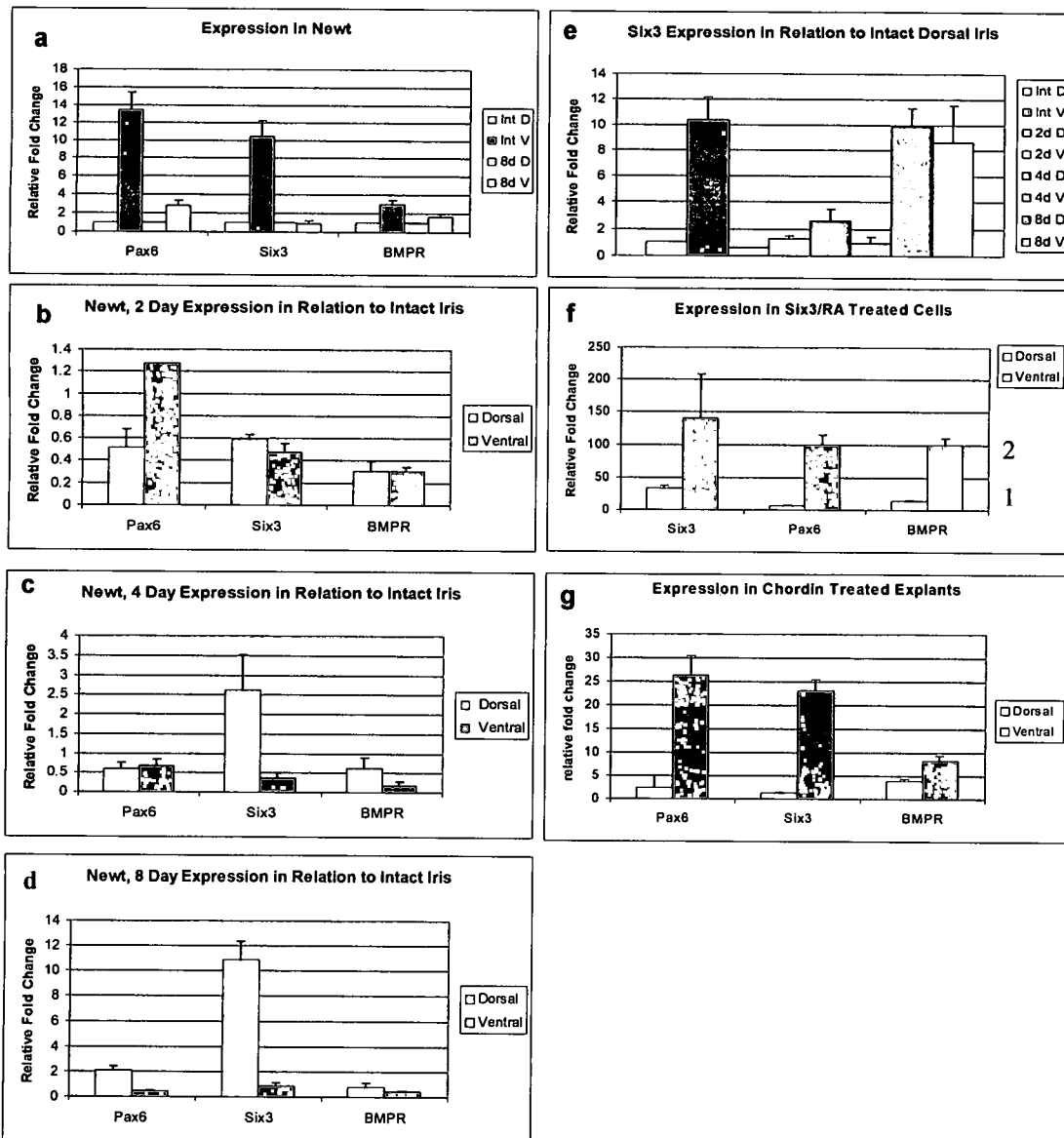
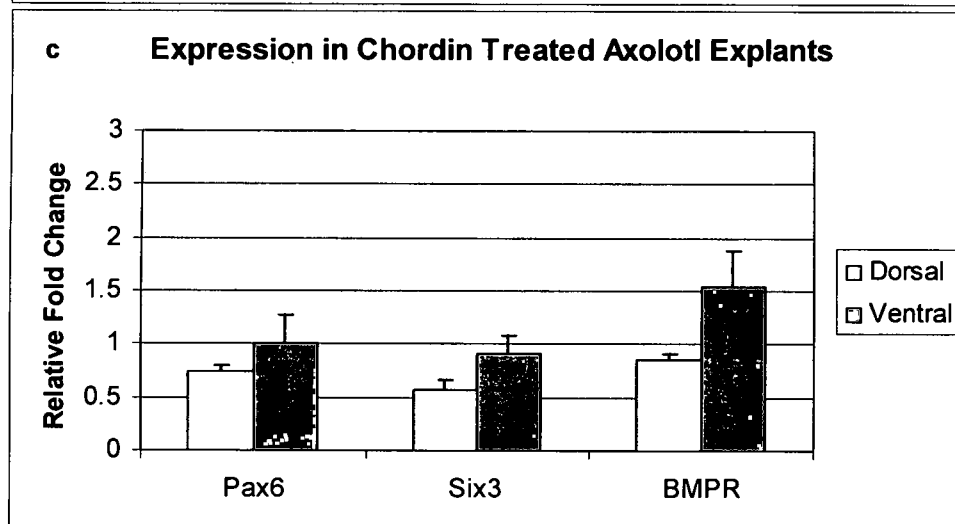
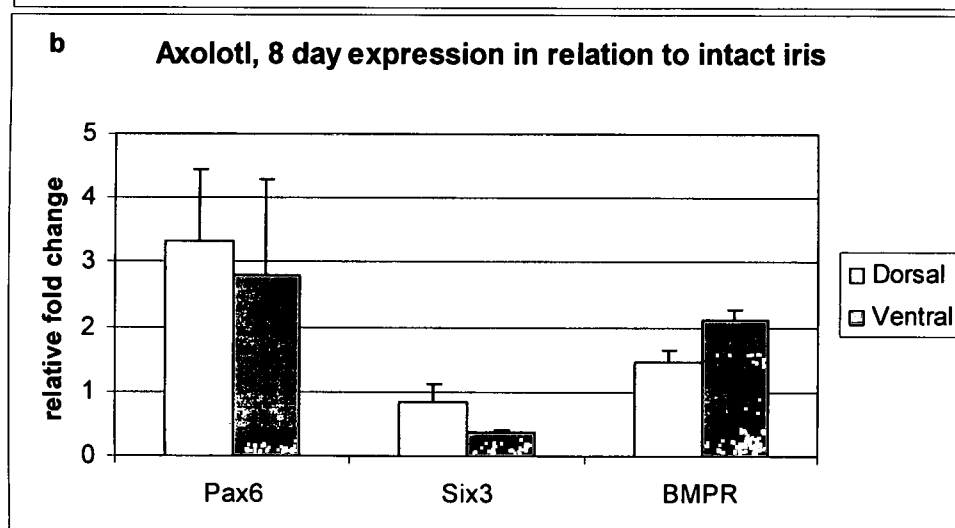
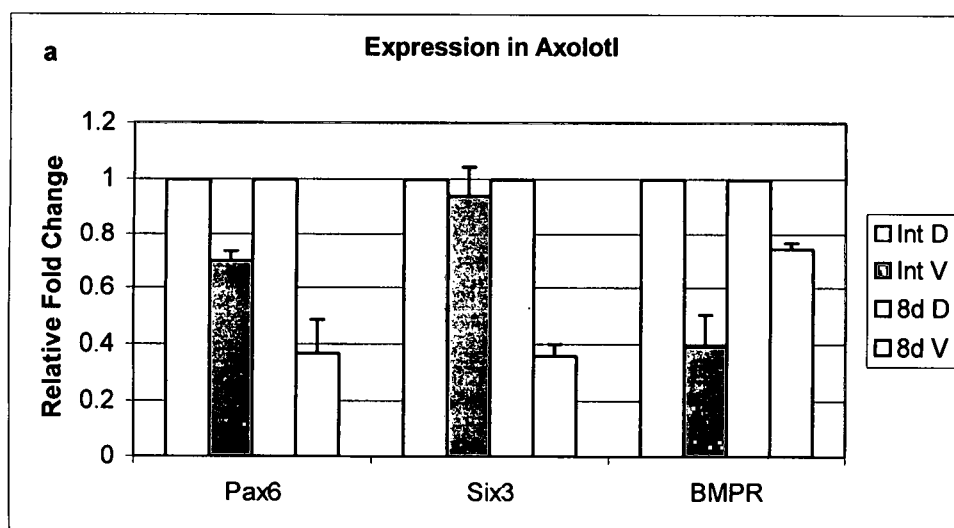


Figure 3. Expression of six-3 and BMPR-1A during regeneration. **a,** Identification of six-3 positive cells in the dorsal and ventral iris 8-day post-lentectomy assessed via immunostaining of serial sections along the nasal-temporal axis (see illustration). No apparent difference in cell numbers or distribution was seen (right panels; results on graphs are means \pm s.d.). **b,** Expression of six-3 and BMPR-1A in both dorsal and ventral iris at early stages (before vesicle formation). Due to heavy pigmentation at these early stages for the BMPR-1A staining sections were bleached. **c,** Expression of six-3 and BMPR-1A at later stages. At 12-day post-lentectomy a definite lens vesicle has been formed. The lens vesicle and the differentiating lens fibers are positive at 15-day and by 25-day only cells in the lens epithelium express six-3. Same patterns can be seen for the BMPR-1A. Negative controls are shown at the bottom. All images are mergers between the dark field and DIC. The stroma (arrows) always shows unspecific fluorescence.

Figure 4. Expression in axolotl. **a**, Expression of *pax-6*, *six-3* and *BMPR-1A* in the axolotl intact and 8-day post-lentectomy dorsal and ventral iris. Comparison between intact dorsal and ventral iris. The values of the dorsal irises have been set to 1 and those of the ventral iris are shown as relative changes. **b**, Expression shown by comparing 8-day irises with intact irises. **c**, Expression in chordin treated iris explants shown as relative fold change to the untreated explants. Results are means \pm s.d.



Supplementary Table 1. Induction of transdifferentiation

Transfected PECs

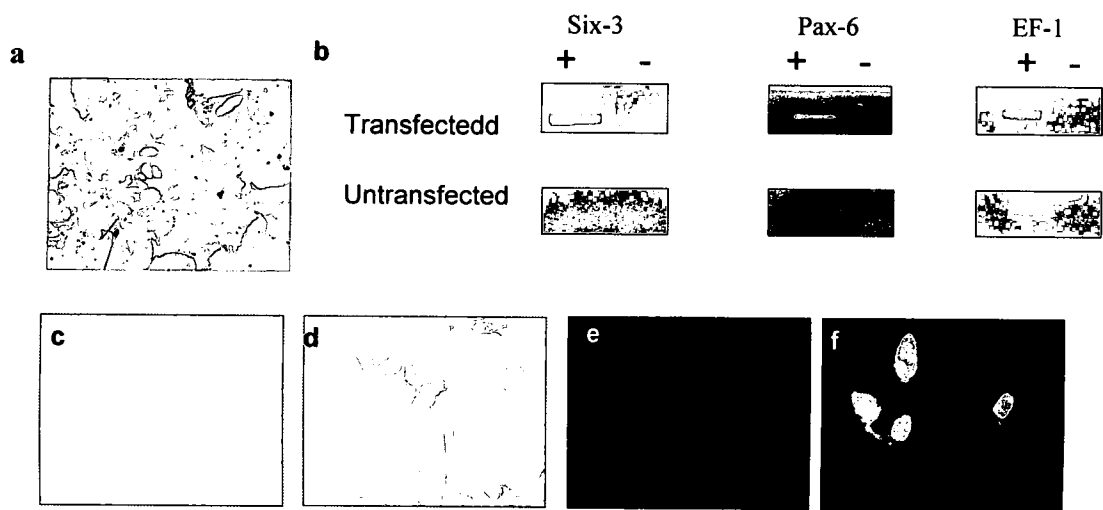
	# lens/host eye	# lens/transplant	# lens/ant transplant
Control Dorsal	10/29 (35%)	10/25 (40%)	10/12 (83%)
Control Ventral	0/15 (0%)	0/12 (0%)	0/11 (0%)
Pax-6 Ventral	0/18 (0%)	0/14 (0%)	0/8 (0%)
Six-3 Ventral	0/20 (0%)	0/17 (0%)	0/10 (0%)
Control Dorsal + RA	3/12 (25%)	3/7 (43%)	3/5 (60%)
Control Ventral + RA	0/16 (0%)	0/11 (0%)	0/9 (0%)
Pax-6 Ventral + RA	0/9 (0%)	0/7 (0%)	0/6 (0%)
Six-3 Ventral + RA	3/15 (20%)	3/6 (50%)	3/4 (75%)

Treated iris explants

Control Dorsal	10/16	10/16	10/16 (62.5%)
BMP-4 Dorsal	5/12	5/12	5/12 (41.6%)
BMP-7 Dorsal	1/12	1/12	1/12 (8.3%)
Control Ventral	0/27	0/27	0/27 (0%)
BMP inhib. Ventral	4/23	4/23	4/23 (17%)

Column one depicts the total number of double lenses per host eye. The second column represents the number of double lenses per case where an aggregate (or an explant) was found. The third column shows the normalized data, which consists of only those aggregates located in the anterior region of the eye.

Supplementary Figure 1. **a**, Detection of beta galactosidase in iris pigment epithelial cells to indicate successful transfection. Note that most of the cells are stained blue. **b**, Expression of transfected genes in cultured PECs detected via RT-PCR. Cultured PECs were transfected with the mouse *six-3*, and the human *pax-6*. RT-PCR was performed on RNA isolated from transfected and untransfected PEC cultures with specific oligos to the transfected cDNAs sequence. Transgene expression is observed only in the transfected iris PECs and not the untransfected. Elongation factor 1 (EF1) was used as the internal control. To ensure no genomic contamination, reactions were performed in the presence or absence of reverse transcriptase (+RT and –RT respectively). **c,d**, Detection of exogenous (human) *pax-6* protein in untransfected and transfected PECs respectively. **e,f**, Detection of exogenous (mouse) *six-3* protein in transfected and untransfected PECs respectively. In **d** and **f** note the nuclear staining.



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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions M.W.G. and M.K.C. contributed equally to this work.

Author Information The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.A.T. (panagiotis.tsonis@notes.udayton.edu). Sequences for *six-3* and *BMPR-1A* were deposited in NCBI GenBank, accession numbers AY799802 and AY795966 respectively.

Chapter III

Conclusions and Future Research

Lens regeneration in the newt involves the process of transdifferentiation. This process is also seen in many other forms of urodelian regeneration such as limb, tail, retina, jaw and brain. One theory behind newt transdifferentiation is that there might be a common signal that acts as a trigger in many of the different regenerative processes that newts perform in order to restore their body parts. It might be that in other organisms incapable of regeneration this signal is blocked and therefore any treatment to induce regeneration will fail until this signal is applied. For example, in the newt eye after lentectomy, both the dorsal and ventral irises undergo early processes. They both re-enter the cell cycle and they both have proliferation. It is possible that our treatments are able to induce lens regeneration because the initial signal for transdifferentiation is already present in the eye. This would also explain why the dorsal iris can regenerate a lens in the regenerating newt limb. The signal for transdifferentiation that is thought to come from the wound epithelium to trigger blastema formation may also trigger the dorsal iris to begin transdifferentiation, followed by the induction of intrinsic properties (signals) within the dorsal iris that allow for regeneration of the lens.

Despite all of the past research that has been conducted on lens regeneration in the newt, several questions still remain unanswered: What is the molecular mechanism behind lens regeneration in the newt? Can lens regeneration be induced from the regeneration incompetent ventral iris? How similar is the ventral iris of the newt to the regeneration incompetent irises of other organisms? And finally, is lens regeneration possible in higher orders such as mammals? Understanding the mechanism of lens regeneration in the newt might involve enormous benefits in addition to the immediate therapeutic upside in dealing with cataract problems worldwide.

We set out to accomplish two goals. The first goal was to induce lens regeneration from the ventral iris in newts. In our attempts at inducing lens regeneration from the ventral iris, we focused on factors that play a role in lens development. The second goal was to examine gene expression of some of the important developmental eye genes during lens regeneration. Both of these goals were met with some level of satisfaction. We were able to induce lens regeneration from the ventral iris by two different methods: 1) Inhibition of the BMP pathway through use of the inhibitor chordin and a truncated form of BMPRII. 2) Over-expression of *Six3* and concomitant treatment with RA. We were also able to show in our study that expression levels of some important developmental eye genes are drastically different than originally thought. The expression levels of *Six3* and *Pax6* are much higher in the intact ventral iris than the intact dorsal iris. However, the levels of *Six3* and *Pax6* become very similar in regenerating ventral iris and

regenerating dorsal iris by eight days post-lentectomy, although the change in expression from intact dorsal to regenerating dorsal was much higher than the change in intact ventral to regenerating ventral. This suggests that a threshold of some sort might exist that must be overcome for the initiation of lens regeneration *in vivo*. Our results also demonstrate the complexity of the molecular mechanism that underlies lens regeneration. Although we were not able to delineate the exact mechanism behind lens regeneration, our study was able to pinpoint key players that are part of a molecular network.

Our studies offer initial insights into the molecular mechanism behind lens regeneration. However, when examining our results several immediate questions come to mind. First and foremost, is there any relation between the two successful treatments? One treatment involves the use of extracellular factors (Chordin and truncated BMPR-IA) involved in blocking a signal transduction pathway while the other employs a transcription factor (*Six3*) and the use of a molecule (RA) that can easily enter the cell and nucleus and bind DNA binding receptors. If both treatments can induce lens regeneration then there is a good possibility that they are connected somehow. In looking for a connection it is good practice to revisit what is known already concerning lens regeneration and lens development. As has already been discussed, many transcription factors such as *Pax6*, *Six3*, *Prox1*, and *Sox2* are important in lens development and have been examined during lens regeneration (see literature review). RA is also known to play a role in eye morphogenesis and lens induction (Eagleson et al., 2001). The BMP pathway seems to play dual

roles in development and regeneration making it a very interesting part of the discussion. On the one hand, BMP4 and BMP7 play very active roles in lens induction and development, but as shown in our study, inhibition of BMP signaling is required for lens regeneration to occur. In some cases it seems that yet undescribed cell signaling factors upstream of these transcription factors may play earlier inductive roles in both regeneration and development. It is possible that the transcription factors drive the differentiation and formation of the lens while the cell signaling factors might drive induction of the dedifferentiation events.

The role of cell adhesion on lens regeneration and cell architecture is a mechanistic process which has not been extensively explored during lens regeneration. *In vitro*, PECs will normally dedifferentiate and transdifferentiate into lentoids. However, collagen substrate will repress dedifferentiation and lens transdifferentiation on PECs in culture (Yasuda, 1979). It has been shown that loss of cell adhesion through focal points is a key process needed for dedifferentiation and proliferation to take place in lens regeneration (Mazaki et al., 1996). If this adhesion is lost through treatment with anti- β 1 integrin antibody, gene expression patterns emerge that resemble those of dedifferentiating PECs (Mazaki et al., 1996). Integrins are transmembrane glycoproteins that attach cells to ECM proteins. Therefore, integrins may play a key role in stabilizing the architecture of PECs both *in vitro* and *in vivo*. It is interesting to note that a molecule, designated 2NI-36, was found that disappears from the dorsal iris during regeneration *in vivo* and

is also not present in actively growing cells *in vitro*, but only witnessed *in vitro* when PECs organize epithelial structures (Eguchi, 1988; Imokawa and Eguchi, 1992; Imokawa et al., 1992). It would be interesting to discern what regulates this molecule and makes it disappear from the dorsal iris during regeneration. This molecule, like integrins, is a glycoprotein and might be the key to why ventral PECs can transdifferentiate *in vitro* but not *in vivo*. Upon examination, integrins might also provide a compelling link between *Six3*, RA, and BMP inhibition.

As discussed earlier, inhibiting the BMP pathway leads to induction of lens regeneration from the ventral iris. The question then becomes what might BMPs be doing to normally block lens regeneration? It is known that BMPs have different downstream targets and that BMPs can both activate and repress transcription of different genes. One such downstream target is integrins. BMP7 is known to up-regulate focal adhesion proteins (Vinall and Reddi, 2001). Therefore, if BMPs up-regulate integrins or other proteins involved in cell adhesion then they might be serving to maintain stability of cellular architecture thereby preventing dedifferentiation of the PECs. Of much significance is the knowledge that BMP4 and *Six3* can mutually antagonize each other (Gestri et al., 2005). *Six3* can directly bind a *BMP4* promoter and repress transcription (Gestri et al., 2005). BMPs are also known to up-regulate a couple of homeobox genes, *Msx1* and *Msx2* (Han et al., 2003). The *Msx* genes are known to play a role in limb development, and *Msx1* is also expressed in the newt eye (Jung et al., 1998). The reason that

Msx1 and *Msx2* may be important is that they are known to be able to bind the *Six3* promoter and repress *Six3* expression (Lengler and Graw, 2001). *Msx2* can also bind a *Sox2* promoter and repress *Sox2* expression (Lengler et al., 2005). *Sox2* is known to regulate crystallin expression and therefore has a role in downstream lens differentiation (Kamachi et al., 1995). Three transcription factors, *Pax6*, *Prox1*, and *Six3* are all known to up-regulate *Sox2* so now *Msx1* and *Msx2* may be pulling double duty and repressing both *Six3* and *Sox2* (Lengler et al., 2005).

So where does RA fit in? Since *Six3* up-regulation is downstream of BMP inhibition, this may explain why RA is needed with *Six3* to induce regeneration. In the absence of BMP inhibitors, RA might provide the necessary signal for induction of dedifferentiation. As it turns out, this may be the case. RA has been shown to both up-regulate integrins (Loeser, 1994) and down-regulate *Msx1* (Yokouchi et al., 1991). This is the key connection in the pathway. If BMP inhibition is at the beginning of the pathway, then that would explain why the inhibitors alone can induce lens regeneration from the ventral iris. BMP inhibitors might perform two functions at once. They might repress integrin expression which would allow for the initial dedifferentiation of the PECs, and also increase *Six3* expression which could then lead to the differentiation into lens. In our other treatment, over-expression of *Six3* alone did not induce lens regeneration from the ventral iris, possibly because *Six3* is needed for a later step. However, adding RA plus the *Six3* over-expression could together provide the two functions that the BMPs inhibitors perform. RA

could thus repress integrins while also blocking *Msx1* repression of *Six3*. Obviously, this is a simplified theory set forth to explain data that is difficult to interpret. Also, it may seem that this model ignores many of the “players” involved in both lens development and lens regeneration, and it should be argued that many of those factors are likely involved at various points in this pathway. There are probably many intermediates not pictured or known in this model, and it should be argued that crosstalk amongst pathways could play a pivotal role in the actual mechanism. Other pathways such as FGF or Shh must either play some role or be able to interfere at some point in the mechanism as it has been shown that they are important for lens differentiation (FGFs) and by affecting these pathways that lens regeneration can be altered (FGFs and Shh). Interestingly, FGFs are known to play a role in lens fiber differentiation and it has been seen that FGFs regulate some integrin expression to allow this differentiation, but that some other factor in the vitreous is also necessary for complete regulation of all of the integrins (Wederell et al., 2005). At the same time, the theory seems very logical based on what is known thus far. Many of the connections will need to be supported with expression data in the future, but the model provides a strong starting point and attempts an explanation of our data.

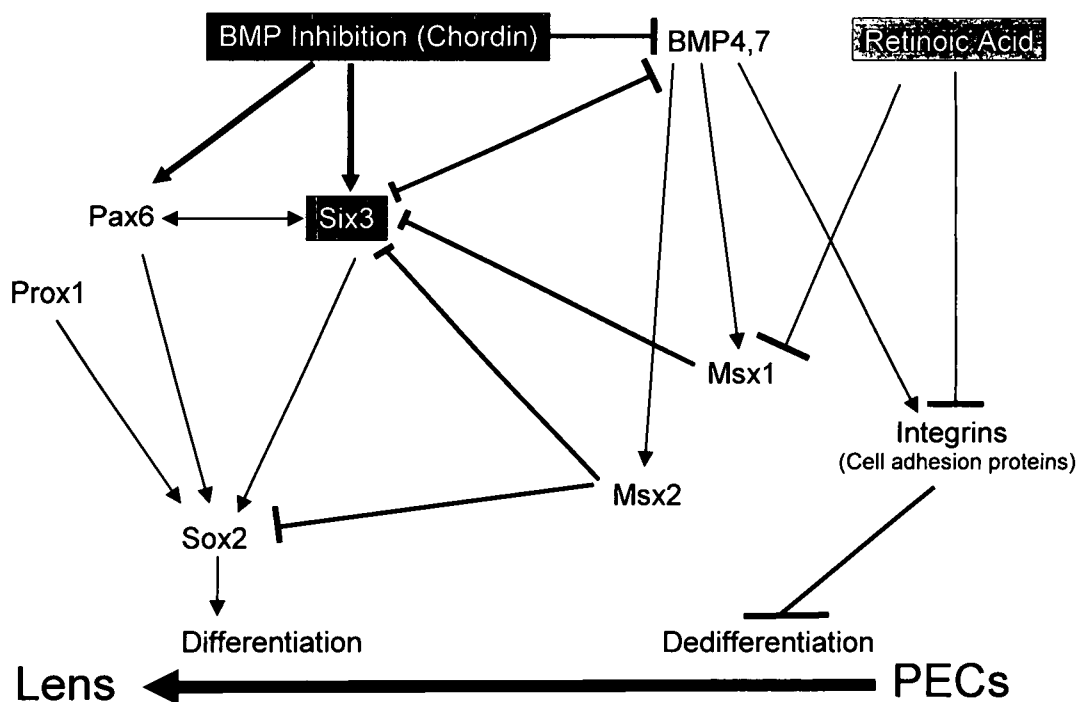


Figure1. Potential molecular mechanism of lens regeneration. Model predicting molecular pathway of lens regeneration based on treatments that induce lens regeneration (in blue). Shows RA decreasing cell adhesion which could allow for dedifferentiation of PECs. Six3 could then regulate differentiation leading to lens formation. BMP inhibition alone could account for both decreased cell adhesion and differentiation to lens cells.

Unlocking the molecular mechanism behind transdifferentiation in iris PECs might provide valuable new insights into the different forms of newt regeneration. A better understanding of newt lens regeneration has the potential to provide new insights into stem cell behavior and answer the age old question asked by many regenerative scientists, just how far back do terminally differentiated cells dedifferentiate and do the cells gain the ability to form fundamentally different cell types? For example, cells found in the

blastema of the regenerating limb come from many different cell types (i.e. muscle, bone) and what was once an osteoblast can now differentiate into muscle. Could that former osteoblast be reprogrammed to form cardiac muscle or other tissue similar to what is being asked of adult stem cells? Understanding the mechanisms behind transdifferentiation in the newt iris PECs could provide a wealth of pharmaceutical and research benefits. Pharmaceutically speaking, it might one day be possible to induce lens regeneration in humans through transdifferentiation, thus eliminating problems with secondary cataracts. Also, uncovering a common trigger for transdifferentiation would be beneficial to all researchers studying regeneration. Discovering the mechanism of lens regeneration in the newt is going to be a very complex and difficult task. The newt genome is not sequenced and therefore any gene expression studies will more times than not involve cloning the gene of interest. Similarly, looking at protein expression with antibodies will require additional characterization in order to determine whether cross-reactivity in the newt is observed. Understanding the molecular mechanism(s) behind newt transdifferentiation might one day allow regenerative scientists to realize their ultimate goal, the translation of this knowledge into other vertebrates and mammals.

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APPENDIX

I.	Lens Regeneration in Mice: Implications in cataracts.....	82
II.	Eye on Regeneration.....	85
III.	Gene Expression and discovery during lens regeneration in mouse: regulation of EMT and lens differentiation.....	92

Letter to the Editor

Lens regeneration in mice: implications in cataracts

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Abstract

Lens regeneration in adult mice is possible when the lens capsule is left behind after lentectomy. The lens is regenerated by the remaining adherent lens epithelial cells, which differentiate to form lens fibres within days, showing normal morphology and bow regions. Epithelial to mesenchymal cell transformation is also seen during the early stages. The mouse, therefore, can become an indispensable animal model for cataract research, surgery and therapy.

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Keywords: lens; regeneration; mouse; cataract

Traditionally, the newt has been hailed as the most powerful animal model for lens regeneration (Del Rio-Tsonis and Tsonis, 2003). True enough adult newts can always replace their lens upon removal. Lens regeneration in newts is achieved by transdifferentiation of the pigment epithelial cells from the dorsal iris. Other amphibia, such as frogs, are capable of lens regeneration by transdifferentiation of the cornea, but only during a short window of time before metamorphosis (Freeman, 1963). The situation in higher vertebrates, especially in mammals, is very different. Lens regeneration has been shown in rabbits, but only if the lens capsule is left behind (Gwon et al., 1990). Obviously, some lens epithelial cells remain attached to the lens capsule and they differentiate to lens fibres to 'regenerate' a lens, which nevertheless is not perfect. Some similar, but limited observations have been seen in cats (Gwon et al., 1993). The studies with rabbits suggest that while lens regeneration does not follow the traditional road of transdifferentiation as in newts, regeneration can nevertheless occur by differentiation of lens epithelial cells remaining on the capsule. Rabbits (or cats), however, are not favorable mammalian animal models for approaching the problem of lens

regeneration with the frontline technology of molecular biology. Therefore, we have turned our attention to mice.

We used three different strains in our study, Balb/c, NZW and MRL/MpJ^{+/+}. The mice were sexually mature (8–12 weeks old) of both sexes. Before operation, mice were anesthetized with ketamine (87 mg kg⁻¹) in combination with xylazine (13 mg kg⁻¹). Two types of operations were performed. In one set, the lens along with the capsule was removed and in the other set, the capsule was left behind (with only part of the anterior capsule damaged). Integrity and restoration of the normal shape of the capsule is important. To remove the lens, a corneal incision was made with a sharp blade. Due to the small size of the mouse eye, we preferred to remove the lens with fine forceps by applying pressure in the eye. Such an operation results, as in the newts, in removal of the lens and the capsule. However, if an incision is made in the anterior lens as well this operation leads to removal of the lens but not the capsule. After such extracapsular extraction, we injected saline solution to clean the capsule. Histological preparations of eyes after lentectomy showed a rather clean capsule with adherent lens epithelial cells. After lentectomy, the mice were collected in time intervals starting at 2 days and ending at 30 days post lentectomy. We found that when the whole lens (with the capsule) was removed no lens regeneration resulted in any case (Table 1). In this respect, and as expected, mice do not regenerate their lens by transdifferentiation of the pigment epithelial cells of the dorsal iris

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Table 1
Lens regeneration in different mouse strains

	With capsule	Without capsule
Balb/c	25/25	0/83
MRL	11/11	0/21
NZW	9/9	0/24
Balb/c with vitamin A	30/30	0/24
MRL with vitamin A	15/15	0/15
NZW with vitamin A	15/15	0/15

Numbers represent lens/eye.

as seen in adult salamanders. On the contrary, to what was previously assumed, intraperitoneal retinol palmitate injections (50 IU mouse^{-1} in $50 \mu\text{l}$ solution) every other day, failed to induce lens regeneration from the dorsal iris (Shekhawat et al., 2001). For this experiment we used the same mouse strains and age as indicated above (Table 1).

However, in eyes where the capsule was left behind, regeneration of the lens was achieved in 100% of the cases

in all strains (Table 1). The growth of the lens was extremely rapid, the capsule filled with differentiated lens fibres within a few days. When the eyes were examined 30 days post-lentectomy, the regenerated lens was of a considerable size (at least half of the intact lens) with morphology displaying an established equator with well differentiated bow regions (Fig. 1(a)–(c)). Differentiation of lens fibres was evident even at day 2 post-lentectomy, as revealed by histology and staining with a lens fiber-specific antibody to β -crystallin (Sawada et al., 1993) (Fig. 1(a),(d)–(f)). This antibody has been shown to be specific for β -crystallin. The same results were received for all three strains and both sexes with or without vitamin A treatment (Table 1). Interestingly, we also observed transformation of lens epithelial cells to mesenchymal cells (EMT) at the posterior part of the capsule. EMT, as judged by the marker smooth muscle α -actin, was evident at early stages, but was diminished considerably after 20 days post-lentectomy (Fig. 1(g)–(i)). This argues that in the beginning there is

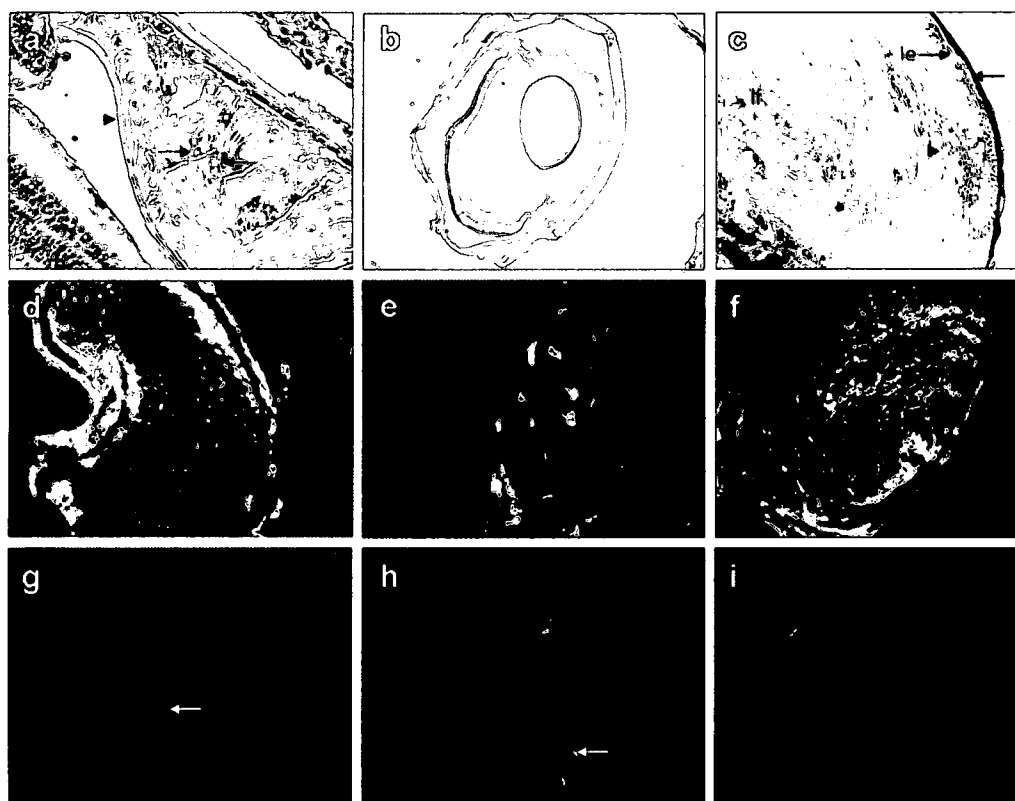


Fig. 1. (a) A histological section through a regenerating lens 2 days post-lentectomy. Note the differentiation of lens fibres (arrows) at the bow region. The posterior capsule is also shown intact (arrowhead) $\times 200$. (b) A regenerated lens 30 days post-lentectomy. Note the normal morphology and size of the regenerated lens $\times 40$. (c) A close-up at the bow region of a regenerated lens 30 days post-lentectomy. Note the normal morphology and differentiation of lens fibres (arrowhead); le, lens epithelium; lc, lens capsule; lf, primary lens fibres $\times 100$. (d–f) Representative sections of regenerating lenses 5, 10 and 20 days post-lentectomy, respectively. Expression of crystallin (green) and type IV collagen (red) (BioDesign) depicting lens fibre differentiation and the lens capsule, respectively, $\times 200$ (d) $\times 400$ (e,f). (g–i) Representative sections of regenerating lenses 5, 10 and 20 days post-lentectomy, respectively. Expression of type IV collagen (red) and smooth muscle α -actin (green) (Sigma). Note that some α -actin positive cells (arrows) can be seen in the posterior part of the regenerating lens during the early stages of lens regeneration, indicating transformation of lens epithelial cells to mesenchymal cells. EMT is largely diminished in the regenerating lens by 20 days post-lentectomy (i) $\times 400$.

a characteristic wound healing response, but later appears to follow a more characteristic differentiation process. Despite the fact that the lens shows normal differentiation, we should stress here that while we call this lens regeneration (as in the case of rabbits) caution should be exercised because of the lack of functional studies.

While several reasons, such as the type of operation (incision of anterior capsule) or age of animals come to mind to explain these positive results, these findings have two major implications. First, they demonstrate that mammals might possess much stronger potential for lens repair than originally thought and, therefore, extending such studies in higher mammals, including humans are now warranted. Indeed, after submission of this manuscript we found out about a similar study using rats (Lois et al., 2003). Second, mouse models might revolutionize cataract research and surgery. The traditional cataract surgery requires that the posterior capsule remains intact to hold the synthetic lens. This, however, could lead to the development of secondary cataracts by opacification of the posterior capsule. This opacification is the result of transformation of the remaining lens epithelial cells to mesenchymal cells (Ibaraki, 1997). Since in our experiments EMT was seen during the early stages but diminished in later ones, mouse lens regeneration could become an indispensable model to study factors that are implicated in the aetiology, inhibition or reversal of EMT. Lens regeneration experiments with knock-out or transgenic mice will open new avenues in this field. Likewise, experimentally induced cataract can be studied

in regeneration models and provide insights about possible regeneration therapy.

Acknowledgements

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Eye on Regeneration

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Lens regeneration in newts is a remarkable process, whereby a lost tissue is replaced by transdifferentiation of adult tissues that only a few organisms possess. In this review, we will touch on the approaches being used to study this phenomenon, recent advances in the field of lens regeneration, similarities and differences between development and regeneration, as well as the potential role stem cells may play in understanding this process. *Anat Rec (Part B: New Anat)* 287B:42-48, 2005. © 2005 Wiley-Liss, Inc.

KEY WORDS: eye; lens; regeneration; transdifferentiation; development

INTRODUCTION

The ability of some organisms to regenerate body parts is one that has both fascinated and plagued scientists for hundreds of years. All animals exhibit some level of regenerative ability, with some able to regenerate entire body parts and organs. Regeneration of body parts in invertebrates and amphibia has been documented for over 200 years. In 1712 Reaumur, perhaps the father of regeneration, noted the talents of crayfish in their ability to regenerate a new limb upon amputation (Dinsmore, 1991). Another major contributor was Trembly, with his elegant studies on Hydra in the 1740s (Dinsmore, 1991). By far the most impressive organisms in regenerative abilities are the amphibians. Many amphibians are capable of

regenerating limbs, tails, nervous tissue, muscle, and in certain species lens and retina. Colucci (1891) and Wolff (1895) first observed independently lens regeneration in the adult newt, which eventually resulted in the term "Wolffian regeneration." This review will focus specifically on newt lens regeneration; why it is advantageous to use as a model for examining regeneration, how it occurs, what approaches have been taken in studying it, and what progress has been made in inducing lens regeneration. We will also touch on stem cells and what help they might possibly provide in solving the regeneration mystery.

WHY STUDY THE LENS?

As discussed above, the procedure of regeneration has been studied for years using different approaches to the problem. During some types of regeneration, terminally differentiated cells lose their characteristics or dedifferentiate and finally differentiate into another cell type. The term that was adopted to denote this change in cell differentiation, that is, a differentiated cell type giving rise to a totally different cell type, is transdifferentiation (Selman and Kafatos, 1974). Depending on the type of regeneration being studied, the number of cell types that needs to be replaced can vary from many cell types to just one. What is even more fascinating is that in some cases of complex regeneration, as in the limb and lens, an exact

replica is formed via the mechanism of pattern formation. For instance, in the process of limb regeneration, all of the tissues of the stump (bone, muscle, cartilage) undergo dedifferentiation. This is followed by the formation of the blastema, which is a region of dedifferentiated mesenchymal cells, and subsequently proliferation and redifferentiation of the lost limb (Tsonis, 1996, 2000). In contrast to limb regeneration, retina regeneration via transdifferentiation, as seen in chicks and amphibia, occurs through the transdifferentiation of a single cell type. That is, the retinal pigment epithelial cells undergo dedifferentiation and redifferentiation to all of the neural retina cell types. In chicks, some neural retina must be left behind or the eye must be treated with fibroblast growth factor (FGF) (Park and Hollenberg, 1989). So far, we have briefly examined two cases of regeneration in which one or more cell types transdifferentiate to form multiple cell types. In order to delineate the mechanism of transdifferentiation, we and others examine the process of lens regeneration in the newt. There are several advantages for using the lens as a model system for studying transdifferentiation. First, the process of lens regeneration occurs via the transdifferentiation of one cell type (iris pigmented epithelial cells) to another cell type (lens cells). Secondly, as will be discussed in more detail below, lens regeneration occurs from only the pig-

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mented epithelial cells (PECs) of the dorsal iris and not the ventral. Therefore, understanding this restriction could help determine why lens regeneration is not possible in other animals. Transdifferentiation of PECs has been studied both *in vivo* and *in vitro* (Eguchi et al., 1974; Mizuno et al., 1999; Tsonis and Del Rio-Tsonis, 2004). While knowledge has been gained through these studies, the exact mechanism of transdifferentiation still eludes scientists today. Today the number of cell types being reported as being capable of transdifferentiation is ever increasing, making it ever more important to delineate the mechanism of transdifferentiation. To help in understanding the mechanism of the transdifferentiation/regeneration process, the rest of this review will focus on the lens.

PROCESS OF LENS REGENERATION

Histological, cellular, and molecular events all take place during the complex process of regeneration. Soon after removal of the lens, the PECs of the dorsal iris dedifferentiate, that is, they lose cellular characteristics such as pigmentation that define their cell type. Macrophages recruited to the area help mediate this process. Dedifferentiation also marks the initiation of cell cycle reentry, which is paramount to proliferation. The first peak of proliferation is observed at about 4 days postlensectomy. At approximately 10 days postlensectomy, the depigmented cells are visible as a vesicle, containing both an inner and an outer layer, at the tip of the dorsal iris (Fig. 1A) (Eguchi, 1963; Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004). The inner layer of the vesicle begins to thicken at 12–16 days postlensectomy as the cells elongate and differentiate into primary lens fiber cells (Fig. 1B). It is also at this time point that the second peak of proliferation is observed and the synthesis of crystallins begins. As regeneration ensues, primary lens fibers continue to form in the inner layer, while nondividing secondary fibers start to form in the external layer of the lens vesicle (days 15–19). At 18–20 days postlensectomy, crystallins are continually synthesized (Fig. 1C). A complete lens is

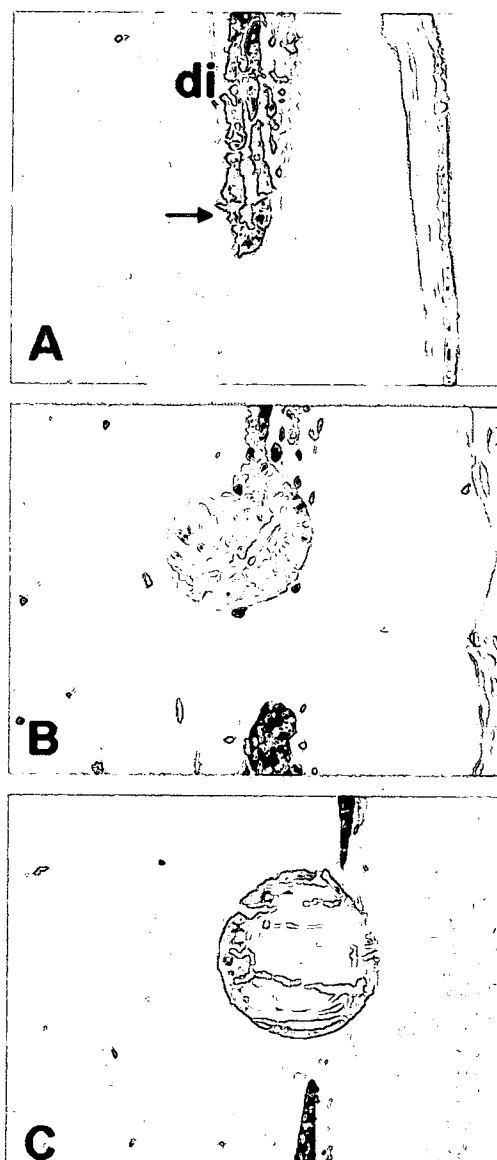


Figure 1. Lens regeneration in the newt stemming from the PECs of the dorsal iris (di). **A:** Ten days postlensectomy. Note the formation of a lens vesicle (arrow). **B:** Fifteen days postlensectomy. Cells are elongating into lens fibers. **C:** Twenty days postlensectomy. The lens is well differentiated with lens fibers.

formed with a layer of lens epithelial cells on the anterior surface and lens fibers cells on the interior of the lens 25 days after lens removal (Eguchi, 1964; Yamada, 1977).

CLASSICAL APPROACHES

The observations of Colucci (1891) and Wolff (1895) led to a new era of studies on lens regeneration. These classical studies, a series of experimental approaches including transplantations attempted in order to understand exactly which cells were

involved in lens regeneration, are some of the most fascinating in regenerative science. Many questions were answered by these studies; however, many more remained. The most basic experiment showed that when grafts of dorsal iris were placed into lentectomized eyes, they gave rise to a lens (Wachs, 1914; Sato, 1930, 1935; Mikami, 1941). When dorsal irises were placed into lentectomized eyes from nonregenerating salamanders, they were also able to form a lens (Ikeda, 1934; Amano and Sato, 1940; Reyer, 1956). Conversely, regeneration-com-

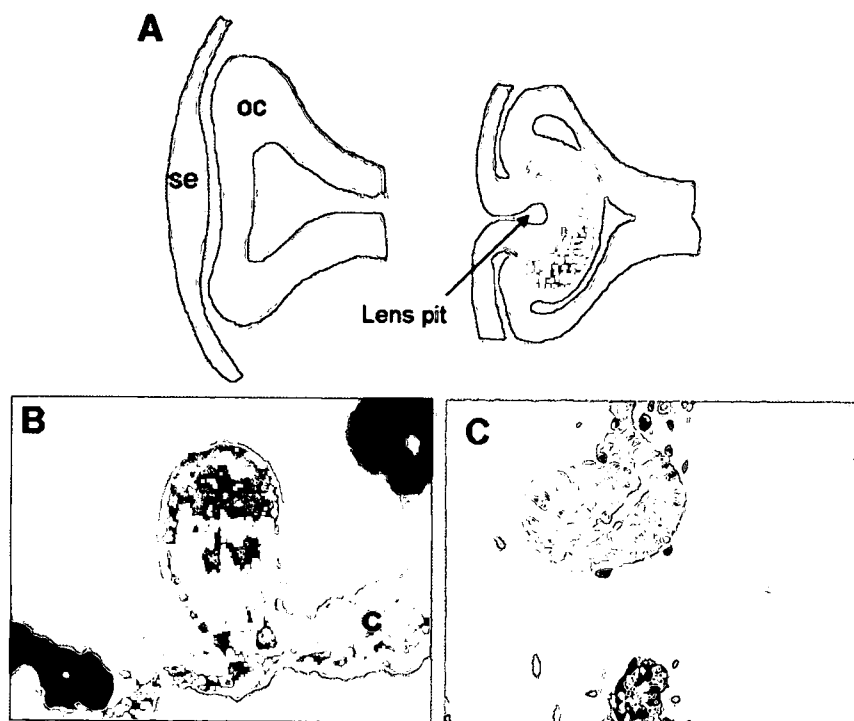


Figure 2. Comparison of lens development with two methods of lens regeneration. **A:** Schematic showing lens development, which involves a series of inductive interactions between the surface ectoderm and the optic cup. The lens pit eventually gives rise to the lens. se, surface ectoderm; oc, optic cup. **B:** Lens regeneration in *Xenopus laevis*. The regenerated lens comes from transdifferentiation of cells in the outer cornea (c). **C:** Lens regeneration in the newt. The regenerated lens comes from transdifferentiation of cells in the dorsal iris.

petent dorsal irises that were implanted into the body cavity or subcutaneously in both lens-regenerating and non-lens-regenerating animals did not form a lens (Ikeda, 1935, 1936; Reyer, 1953, 1954; Stone, 1958a). This suggested that perhaps something else was necessary for lens regeneration to take place. Studies went on to show that the neural retina was sufficient to rescue regeneration of the lens in the previous attempts (Stone, 1958a). It was further shown that by separating the neural retina from the iris, lens regeneration could be prevented (Stone, 1958b). Obviously, one might conclude that there is a certain factor that is provided by the retina that plays a role in lens regeneration. Speculation on the identity of this factor includes FGFs and FGFRs because of their known roles in lens development and polarity. Several classical experiments have shown that the lens itself might provide a factor that is involved. If the lens is removed from lens regeneration-competent animals and then replaced near the dorsal iris,

no regeneration occurs or it is minimal, depending on the distance from the dorsal iris (Eguchi, 1961). The same lack of regeneration is seen from a dorsal iris explant that is placed in the anterior part of the eye and the lens is either left in or removed and then replaced (Reyer, 1961). These classical approaches led to a greater understanding of the parameters of lens regeneration and helped pave the way to the molecular era with many questions looming, mainly that of the actual molecular mechanism.

MODERN APPROACH

In order to understand the molecular aspects of lens regeneration, many scientists begin their quest by examining molecules that are known to play a role in vertebrate lens development. Do the same factors that play a role in developing body parts also play a role in regeneration? In other words, does regeneration recapitulate development? It is in this mode of thinking that some of the strongest data have

been generated. To understand this modern approach, we will first briefly explain the process of lens development.

Vertebrate lens development is brought about by a series of inductive interactions in the embryo, which leads to the initiation of differentiation of the head ectoderm (Coulombre and Coulombre, 1963). Three primordial tissues (ectoderm from the neural tube, surface ectoderm, and mesoderm) are required for formation of the vertebrate eye. The lens as well as the corneal epithelium is derived from the surface ectoderm. In most vertebrates, lens development is initiated by proliferation of the ectodermal cells overlying the optic vesicle, which forms the lens placode. The placode then invaginates along with the optic vesicle to form the lens pit and optic cup, respectively (Fig. 2A). The lens pit deepens and is eventually closed off to form a lens vesicle. The lens vesicle soon separates from the overlying ectoderm forming the lens.

During lens regeneration, events of lens differentiation are initiated after the formation of the lens vesicle. At this point, the processes of lens regeneration and development in the newt are very similar in terms of differentiation and even that of crystallin synthesis. β -crystallin and γ -crystallin proteins are the first to be detected (McDevitt and Brahma, 1982) in the lens vesicle followed by α -crystallin (Takata et al., 1964). It is not until later stages that crystallins are detected in the lens epithelial cells (Takata et al., 1966). The similarities in crystallin gene expression between regeneration and development have been shown via in situ hybridization as well (Mizuno et al., 2002). Another similarity between lens regeneration and lens development is seen in the expression pattern of two important eye genes, *Pax-6* and *Prox-1* (Mizuno et al., 1999). *Pax-6* was expressed soon after lentectomy in a broad region that includes both the dorsal and the ventral iris. As regeneration continues, *Pax-6* expression becomes restricted to the dorsal iris. Eventually *Prox-1* becomes expressed within the *Pax-6* expressing tissue. This sequential expression of *Pax-6* and *Prox-1* is also seen in the lens placode during lens development, indicating that

there may be a common genetic program to both development and regeneration (Mizuno et al., 1999). Due to the apparent similarities between lens development and regeneration, it is quite clear to see why scientists have engaged in the modern approach to this problem.

Retinoids

Retinoids and their receptors play major roles in both lens development and regeneration. Retinoic acid is thought to play a role in inducing the inductive signal during lens development. Retinoic acid deficiency in mouse embryos leads to a failure to form the lens placode and optic vesicle invagination, which leads to a small eye phenotype (Grindley et al., 1995; Bavik et al., 1996). Retinoic acid receptors play a role in transcriptional control of α B and E crystallins (Gopal-Srivastava et al., 1998) during development. It also appears that retinoic acid and its other analogs regulate gene expression in lens cells and plays an important role in maintaining the epithelial layer (Lovicu and Robinson, 2004). Retinoic acid has also been shown to control the fate of neural retinal cells during development (McCaffrey et al., 1993; Wagner et al., 2000). In addition to these roles in development, exogenous retinoic acid has led to the formation of ectopic lens differentiation (Manns and Fritzsche, 1991).

Retinoic acid and retinoic acid receptors not only play a role in development but they also play a crucial role in newt lens regeneration. Treatment of newts, following removal of the lens, with an antagonist to the retinoic acid receptors or with disulfiram (a chemical that inhibits the synthesis of retinoic acid) severely retards the regenerative capability of the dorsal iris. While inhibition was the most prevalent outcome, there were also a few cases of ectopic lenses being formed (Tsonis et al., 2000, 2002).

Fibroblast Growth Factors

Fibroblast growth factors and their receptors are critical for lens development. As was the case for retinoic acid, FGFs play dominant roles in controlling crystallin gene expression and regulating the spatial and tempo-

ral pattern of expression (deIongh et al., 1997; Lang, 1999). In addition, they also play a role in lens fiber differentiation and maintenance. In chicks, FGF-8 expression in the distal optic vesicle leads to the expansion of the lens field (Vogel-Höpkner et al., 2000). Targeted overexpression of FGFs in transgenic mice leads to inappropriate differentiation of the lens epithelium (Robinson et al., 1995, 1998; Lovicu and Overbeek, 1998).

In regeneration, several FGFs and their receptors are expressed but only FGFR-1 was present in the dorsal iris during dedifferentiation (Del Rio-Tsonis et al., 1997, 1998; McDevitt et al., 1997). Further examination showed that FGFR-1 plays a role in regulating lens differentiation. This was shown by inhibiting the function of FGFR-1, which in turn led to inhibition of lens regeneration and lens fiber differentiation (Del Rio-Tsonis et al., 1998).

Homeobox-Containing Genes

Pax-6 has long been known to be one of the most important determinants for eye formation. Mutations in Pax-6 cause aniridia in humans and the "small eye" phenotype in mice and rats. In mice, it is expressed in the head ectoderm early in development and at later stages (E10.0) is expressed in the lens vesicle and optic cup. At E13.5, Pax-6 is expressed in the proliferating anterior epithelial cells, but is not detected in the lens fiber cells beyond this stage (Koroma et al., 1997). Another homeodomain protein important in lens development is Prox-1. Prox-1 is first detected at E9.5 in the lens placode with subsequent expression in the lens vesicle, anterior epithelium, and lens fibers (Oliver et al., 1993; Tomarev et al., 1996; Glasgow and Tomarev, 1998). A Prox-1 mutation in mice leads to death due to lymphatic vessel development. These mutant mice also show a defect in lens differentiation due to a defect in fiber cell elongation, which is in turn due to the absence of crystallin (Wigle et al., 1999). In fact, both Pax-6 and Prox-1 regulate crystallin expression (Cvekl and Piatigorsky, 1996; Tomarev et al., 1996).

During newt lens regeneration, Pax-6 and Prox-1 are expressed in a pattern that is very similar to that seen

in development. Following lentectomy, Pax-6 is expressed in both the dorsal and ventral PECs. However, once dedifferentiation is apparent, the expression of Pax-6 is localized to the dorsal iris PECs and subsequently becomes restricted to the lens epithelium of the regenerating lens (Del Rio-Tsonis et al., 1995; Mizuno et al., 1999). Prox-1 is expressed specifically in the dorsal iris during regeneration and not in the regeneration-incompetent ventral iris (Del Rio-Tsonis et al., 1999).

STEM CELLS

The most recent approach to regeneration is seen in stem cell research. In the past few years, studies have shown that stem cells, those reserved and used for repair, may play more of a role in regeneration than originally thought. The ability to take stem cells and coax them into differentiating into the tissue of choice is very alluring to those in the regenerative medicine field. In this case, the stem cells involved are local (i.e., located in the brain and involved in nervous tissue repair) or nonlocal (i.e., hematopoietic and involved in repair of several tissues such as liver, nervous, or cardiac) multipotent cells, which differ from the urodele repair strategy in that they do not undergo dedifferentiation. It has been hypothesized that there may also be similarities between stem cells and transdifferentiating cells (Tsonis, 2000; Tsonis and Del Rio-Tsonis, 2004). For instance, mesenchymal stem cells located in the bone marrow can differentiate to any number of cells (chondrocytes, myocytes, osteoblasts, or adipocytes), much like that of the cells of the blastema. It has been shown that mammalian myotubes can transdifferentiate through the generation of progenitor cells (Chen et al., 2004). In doing this study, they screened over 50,000 discrete small molecules and found a compound that reversed a terminally differentiated cell into progenitor cells, which were then able to differentiate to osteocytes or adipocytes (Chen et al., 2004; Tsonis, 2004). These findings are of great importance in the regeneration field. If these compounds can induce dedifferentiation in multiple cell types, then it begs

the question of there being a common signal for dedifferentiation. One of the more popular questions these days in the regeneration field asks if cells capable of regeneration such as those in the limb that form the blastema actually revert back to a "younger" state that resembles developing cells or if these cells have a unique quality in which they are multipotent but do not resemble their "younger" selves. Sustar and Schubiger (2005) tackled this question using *Drosophila* imaginal disk cells and found that in fact the cells in the imaginal disks that are capable of transdetermination do not revert back to their "younger" selves, but instead convert into a unique cell type. These findings will have a large impact on the mechanisms of the two strategies of regeneration, that of the urodeles and transdifferentiation and that of recruitment of stem cells.

Lens Regeneration in Other Vertebrates

Lens regeneration is seen predominantly in amphibians, but most spectacularly in some species of urodeles as this feat can occur in an adult organism (as discussed with the newt). The newt is not, however, the only organism that can regenerate its lens. Anurans such as *Xenopus laevis* can also regenerate a lens through the process of transdifferentiation. Unlike the newt, which regenerates the lens through transdifferentiation of the dorsal iris pigmented epithelial cells, *Xenopus* regenerates a lens from the inner layer of the outer cornea (Fig. 2B and C) (Freeman, 1963; Filoni et al., 1997). This process appears to be facilitated by a factor secreted from the retina. Following removal of the lens, this factor is no longer hindered from making contact with the outer cornea (Filoni et al., 1982). Besides the type of cell that undergoes transdifferentiation, another difference exists between the newt and *Xenopus* and that is the stage at which regeneration is possible. As was mentioned earlier, the newt can regenerate a lens throughout its lifespan, which is one of the reasons that scientists use this animal as a model system for studying regeneration. *Xenopus* can only regenerate during early stages of life.

The capacity for regeneration is lost after metamorphosis (Freeman, 1963).

Lens regeneration is not restricted to only amphibians but has been shown to take place in other vertebrates as well. Lens regeneration has been reported to occur from a layer of cells found at the border of the iris near the choroid in the chick embryo (Deth, 1940). These findings, however, are very controversial. The controversy lies in the fact that scientists are not sure if the newly formed lens is a result of transdifferentiation of the cells near the iris or if it is merely an inductive response due to competent ectoderm being left behind after surgery (McKeehan, 1961). The lack of markers to follow the process was a major problem with these studies. Similar to salamanders, some species of adult fish can regenerate a lens through transdifferentiation of cells of the dorsal iris (Sato, 1961; Mitashov, 1966). Other vertebrates, such as mice, rabbits, and cats, can also undergo regeneration. Unlike the other organisms examined, these mammals do not regenerate a lens through the process of transdifferentiation. Instead, regeneration occurs from lens epithelial cells remaining on the capsular bag following removal of the lens. Without the capsular bag, regeneration in these organisms will not take place (Gwon et al., 1989, 1990; Call et al., 2004). As was illustrated above, regeneration occurs via many different mechanisms and even within transdifferentiation different tissues are utilized (cornea in the *Xenopus* and dorsal iris in the newt). With this in mind, is there a common strategy used in regeneration or does each organism employ a unique strategy to regeneration? This question is one that stumps researchers and, of course, the hope would be for a more common mechanism shared among organisms. There is some evidence that organisms that do not normally regenerate still maintain the ability to do so if the right buttons are pushed. The key to answering this must lie in elucidating the mechanism in regeneration-competent animals and then applying those findings to noncompetent ones.

Through the use of in vitro cell culture systems, it has been demon-

strated that PECs from many organisms can undergo the transdifferentiation process to form lentoids, lens-like structures. Tissues that were once thought to lack regenerative capabilities have been shown to have the potential for regeneration through these culturing systems. It has been shown that PECs from the ventral iris of the newt could undergo transdifferentiation to form a lentoid (Eguchi et al., 1974). This potential for transdifferentiation is also seen with retinal PECs of chick embryos (Eguchi and Okada, 1973) as well as in human iris and retinal PECs from adult and fetal eyes (Eguchi, 1988; Tsonis et al., 2001). Knowing that this potential is present in a wide variety of species raises some very interesting questions. Why does transdifferentiation occur in vitro but not in vivo in non-regenerating species? How can we unlock this potential in these species? Perhaps the answers to these questions lie in the cells' extracellular matrix (ECM). The presence of collagen has been shown to inhibit dedifferentiation of PECs (Eguchi, 1979; Yasuda, 1979). In addition, localization of $\beta 1$ integrin at the focal contact sites in PECs is lost during the process of dedifferentiation and is thought to be the result of phosphorylation. Cultured PECs treated with an antibody against $\beta 1$ integrin results in morphological changes as well as changes in the pattern of gene expression. These changes are remarkably similar to the changes observed during dedifferentiation of PECs (Mazaki et al., 1996). Integrins, in addition to other ECM molecules, may be necessary for maintaining the differentiated state of PECs, which may be inhibited or blocked in vivo. A molecule known as 2NI-36 may be responsible for stabilization of the extracellular matrix (Eguchi, 1988). This glycoprotein has been shown to disappear from the dorsal iris of the newt upon regeneration (Eguchi, 1988; Imokawa and Eguchi, 1992; Imokawa et al., 1992). Understanding the role the ECM plays during lens regeneration may provide the key to unlocking the mechanism of lens regeneration. With this key, induction in higher organisms may be one step closer.

INDUCTION OF LENS REGENERATION

It has been clearly established that in vivo lens regeneration in urodele amphibians comes from the dorsal iris only. One of the most exciting prospects in studying lens regeneration is inducing it from the incompetent ventral iris. Treatment with the potent carcinogen MNNG (methyl-nitro-nitrosoguanidine) has shown that such induction is possible. MNNG was added to regenerating eyes and the result was ectopic lenses from the ventral iris (Eguchi and Watanabe, 1973). This was also done on cultured ventral irises in vitro that were treated with the carcinogen and implanted back into a lentectomized eye. Some of the implants produced a lens in this study as well (Eguchi and Watanabe, 1973). While the mechanism is still unknown, it was suggested in that study that MNNG altered the cell surface properties of the ventral PECs. However, this has not been documented. Nevertheless, these experiments clearly show that induction is possible and remains one of the greatest challenges in the field.

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Gene Expression and Discovery during Lens Regeneration in Mouse: Regulation of EMT and Lens Differentiation

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Abstract

Purpose: It has been shown that after extracapsular lens removal by anterior capsulotomy in the mouse, the lens can be regenerated. However, as the capsular bag is filled with fibers, epithelial to mesenchymal transition (EMT), an event which is common after cataract surgery as well, takes place during early stages. This study was undertaken to identify novel regulators and networks in order to more clearly understand secondary cataracts at the molecular level using this unique mouse model.

Methods: We examined global gene expression via microarray analysis of mouse lens regeneration after extracapsular surgery. Gene expression at different time points after surgery was correlated with the processes of EMT, which is seen in the initial stages of regeneration, and lens fiber differentiation, which occurs later.

Results: Several notable patterns were observed from the gene clustering data. It was obvious from the analysis that initially there is a response to injury, extensive matrix remodeling and severe down-regulation of genes encoding lens structural proteins. The patterns returned gradually to normal by week 3 after surgery. New genes were identified from the clustering results that might be potential regulators of EMT and lens differentiation.

Conclusions: With this approach, we demonstrated the utility of a mouse model to study secondary cataracts at the molecular level. Extension of these

studies in mice with known mutations affecting EMT or lens differentiation should allow the identification of the crucial molecular players which could lead to better treatments of secondary cataracts.

Traditionally, the newt has been hailed as the most powerful animal model for lens regeneration [1,2]. True enough, adult newts always replace their lens after removal. Lens regeneration in newts is achieved by transdifferentiation of the pigment epithelial cells from the dorsal iris. Other amphibia, such as frogs, are capable of lens regeneration by transdifferentiation of the cornea, but only during a short window of time before metamorphosis [3]. The situation in higher vertebrates, especially in mammals, is very different. Lens regeneration has been shown in rabbits, but only if the lens capsule is left behind [4]. Obviously, some lens epithelial cells remain attached to the lens capsule and they differentiate to lens fibers to 'regenerate' a lens, which nevertheless is not perfect. Some similar, but limited observations have been seen in cats [5]. The studies with rabbits suggest that while lens regeneration does not follow the same traditional road of transdifferentiation as in newts, regeneration can nevertheless occur by differentiation of lens epithelial cells remaining on the capsule. Rabbits (or cats); however, are not favorable mammalian animal models for approaching the problem of lens regeneration with the frontline technology of molecular biology, and therefore, extensive studies at the molecular level are hindered. We reported previously that when the lens is removed in adult mice, with the capsule remaining in the eye cavity, lens fibers are rapidly differentiating from the adherent lens epithelial cells and fill the capsule within a few weeks and is not limited to Soemmerring's ring only [6]. Such 'regeneration' of the lens is quite remarkable and has been reported in mouse and rats by others as well

[7, 8]. Importantly, during regeneration in mice, epithelial to mesenchymal transition (EMT) is observed at the initial stages, indicating that the process undergoes an initial phase of repair and of lens differentiation.

EMT is a prominent process after cataract surgery. During modern cataract surgery extracapsular lens removal allows the synthetic lens to be placed on the remaining capsule. However, adherent lens epithelial cells (LECs) still persist in most of the cases. The LECs tend to transdifferentiate to mesenchymal cells and this process leads to the so-called Posterior Capsule Opacification (PCO), which requires expensive laser treatment, even though such procedures have been considerably reduced recently because of the state of the art instruments and the techniques applied [9]. The most common experimental procedures to study EMT in LECs are either treating lens epithelial cells *in vitro* or injure lenses *in vivo*. Both rats and mice are commonly used for this assay. The *in vivo* procedure is usually performed by injury of the anterior subcapsular region with a needle. Such injury leads to cataract related changes involving EMT. Culture of capsular bags from humans is another experimental system where proliferation and transdifferentiation of LECs can be studied [10]. In these experiments, the capsular bags from deceased donors can be placed in culture, and the degree and development of EMT can be assessed *in vitro*. Such studies have provided very important information about the factors involved, but are limited

for genetic manipulations. TGF- β is considered an important factor in the initiation of EMT [11-13].

The ability of mice to regenerate the lens under the outlined conditions provides us with a valuable animal model system to study basic biology of EMT at the molecular level and to identify targets that eventually could lead to further understanding of the medical complications and the design of effective treatments. The availability of mutant mice as well as microarray analysis enables us to profile genomic activity during EMT, which is otherwise impossible to carry out. Furthermore, such studies as the ones presented here might elucidate factors that will improve the quality of the regenerated lens, information that will be important in future applications when the lens is accidentally damaged. In the present study, we have examined gene expression during different stages of lens regeneration in mice via microarray hybridization and analysis. Our results provide unique and interesting insights in gene regulation during EMT and lens differentiation.

Methods

Surgical Procedures. C57BL mice of 6-8 months of age were be anesthetized with ketamine [87mg/kg]/xylazine [13mg/kg](IP or SC). Mice were also given the analgesic Buprenorphine [2mg/kg (SC)] pre-emptively. Pupils were dilated with the use of 1% tropicamide and 2.5% phenylephrine hydrochloride. A corneal incision was made and then anterior capsulotomy was performed. The lens was then removed by pushing the eye cavity with forceps. The anterior chamber was then filled with sodium hyaluronate. In our hands this procedure effectively removes the whole lens (we have examined removed lenses and lenticomized eyes by histology) and leaves the capsule behind, eventually with lens epithelial cells. In Fig. 1 we show the morphology of the capsule 1 day after lens removal.

Experimental design. Microarray hybridization methods were used to obtain global gene expression profiles from intact and regenerating eyes after extracapsular lens removal in C57BL mice 8-weeks of age. We examined four time points, time 0 at the time of lens removal and 1, 2, and 3 weeks after surgery. In our previous publication [6] we had presented a histological study of the early stages of these events. During week 1 we can observe fiber differentiation as well as EMT. Week 2 was basically marked by increased fiber differentiation and lower degree of EMT. Finally by week 3 EMT was virtually absent. . Since the goal here was to identify genes that affect EMT

and lens fiber differentiation, these time points are sufficient because both EMT and fiber differentiation occur very rapidly after surgery and follow a particular course.

A 70-mer oligonucleotide library from Operon Technologies, Inc. (Huntsville, AL) representing 24,878 known mouse genes, e.g., genes involved with cell signaling, apoptosis, cell proliferation, etc. (including most if not all of the available known genes involved in regeneration) were used for the microarray experiments.

As depicted in Fig. 2, each regenerating tissue was directly compared to the corresponding intact tissue. RNA from control tissues were obtained from four independent animals and hybridized with RNA obtained from four independent animals at week 1 and three animals at weeks 2 and 3 after the surgery. Biological variation was accounted for by including the multiple biological replicates per experimental condition. Our statistical model does indeed unequivocally factor out the gene-specific dye effect from the estimates of differential expression. This is achieved by fitting a linear statistical model with a "dye" effect, as described in the Data Normalization and Analysis section, to each gene separately. This approach has been demonstrated to work well in unbalanced situations such as the week 2 and 3 comparisons.

Isolation of tissues and total RNA. Total RNA was isolated by standard methods using Tri-Reagent (Molecular Research, Inc). We analyzed the quality of mRNA using an Agilent Bioanalyzer 2100 and NanoDrop 1000.

Target labeling. For each hybridization experiment (a microarray slide), total RNA from two single animals were used. Approximately 10 µg of total RNA was used for each Cy-3 or Cy-5 labeling procedure. cDNA target was synthesized using an indirect labeling method, in which aminoallyl-dUTP (7:3 ratio of aa-dUTP:TTP) was incorporated in the cDNA via an oligo(dT) primed reaction by reverse transcriptase (Superscript III, Invitrogen, Carlsbad, CA). The cDNA was decorated with Cy-3 and Cy-5 (Cy Dye™ Post-Labeling Reactive Dye Packs, Amersham, GE Healthcare, Piscataway, NJ) following the accompanying instructions. When necessary, the RNA was amplified using the Amino Allyl MessageAmp™ kit from Ambion (Austin, TX), which in our hands produced approximately 50-120 µg of amplified RNA (aRNA) with the incorporated amino allyl nucleotides starting from 1 µg of total RNA (10 µg of each aRNA was used per slide). The aRNA is an accurate representation of the original total cellular RNA [14].

Microarray hybridization. The mouse 70-mer oligonucleotides were suspended in 3X SSC at 30 µM and printed at 22°C and 65% relative humidity on aminosilane-coated slides (Cel Associates, Inc.; Pearland, TX; VSA-25C) using a high-speed robotic OmniGrid machine (GeneMachines; San Carlos, CA) with Stealth SMP3 pins (Telechem; Sunnyvale, CA) (Guo et al., 2004; Karyala et al., 2004). The microarray slides were placed in

prehybridization buffer (5x SSC, 0.1% SDS and 1% BSA) and incubated at 48°C for 45-60 min. The slides were washed twice in deionized water and used immediately for hybridization (2X hybridization buffer: 50% formamide, 10X SSC and 0.2% SDS). The Cy-3 and Cy-5 labeled targets were suspended in 9 μ l water and heated at 95°C for 3 min. The following were added to each tube of labeled target to inhibit non-specific hybridization: 8 μ l of 1mg/ml COT1-DNA (Roche Diagnostics, Basel, Switzerland), 2 μ l of 10mg/ml poly(A)-DNA (Sigma, St. Louis, MO), and 2 μ l of 4 mg/ml yeast tRNA (Sigma). Next, 21 μ l of 2X hybridization buffer pre-heated to 48°C was added to the target mixture, mixed well, and centrifuged. The labeled target was applied to the pre-hybridized microarray slides, covered with a 22 x 60 mm glass cover slip, and placed in a sealed hybridization chamber (Corning, Acton, MA). The sealed chamber was placed in a 48°C water bath and incubated for 40-60 hr. For the post-hybridization washes, the coverslips were removed in 1X SSC, 0.1% SDS, and 0.1 mM DTT at 48°C, and the slides were agitated for 15 min. The microarray slides were transferred to a staining dish containing 0.1X SSC, 0.1% SDS, and 0.1 mM DTT at 48°C and agitated for 5 min. The previous wash was repeated two more times. The slides were then washed two times in 0.1X SSC and 0.1 mM DTT at room temperature and agitated for 5 min. The slides were spun dried [15].

Scanning and data generation. Imaging was carried out using a GenePix 4000A and GenePix 4000B (Axon Instruments; Union City, CA) with

GenePixPro 5.0 software. Images were captured in JPEG and TIFF files, and the DNA probes were measured by the adaptive circle segmentation method. Information extraction for a given spot was calculated using the median value for the signal pixels minus the median value for the background pixels to produce a gene set data file for all the DNA spots. The Cy-3 and Cy-5 fluorescence signal intensities were normalized by adjusting total fluorescence levels.

Data normalization and analysis. The data representing raw spot intensities generated by GenePix® Pro version 5.0 was analyzed to identify differentially expressed genes. Data normalization was performed in three steps for each microarray separately [15]. Channel specific local background intensities were subtracted from the median intensity of each channel (Cy-3 and Cy-5). Second, background adjusted intensities were log-transformed and the differences (R) and averages (A) of log-transformed values were calculated as $R = \log_2(X1) - \log_2(X2)$ and $A = [\log_2(X1) + \log_2(X2)]/2$, where X1 and X2 denote the Cy-5 and Cy-3 intensities after subtracting local backgrounds, respectively. Third, data centering was performed by fitting the array-specific local regression model of R as a function of A. The difference between the observed log-ratio and the corresponding fitted value represented the normalized log-transformed gene expression ratio. Normalized log-intensities for the two channels were then calculated by adding half of the normalized ratio to A for the Cy-5 channel and subtracting half of the normalized ratio from A for the Cy-3 channel. A statistical

analysis was performed for each gene separately by fitting the following mixed effects linear model. $Y_{ijk} = \mu + A_i + C_k + T_j + \mu_{ijk}$, where Y_{ijk} corresponds to the normalized log-intensity on the i^{th} array ($i = 1, \dots, 10$), at the j^{th} time point ($j = 1, 2, 3$), and labeled with the k^{th} dye ($k = 1$ for Cy-5, and 2 for Cy-3). μ is the overall mean log-intensity, A_i is the effect of the i^{th} array, T_j is the effect of the j^{th} time point and C_k is the effect of the k^{th} dye. Assumptions about model parameters were the same as described [16] with array effects assumed to be random and treatment and dye effects assumed to be fixed. Statistical significance of differential expression between RNA samples at each time point after the treatment, after adjusting for array and dye effects, was assessed by calculating p -values and applying False Discovery Rates (FDR) multiple hypotheses testing [17,18]. Data normalization and statistical analyses were performed using SAS statistical software package (SAS Institute Inc., Cary, North Carolina).

Cluster analysis. Clustering was performed using Bayesian infinite mixture (BIM) model based clustering for replicated microarray data [19, 20] using replicated normalized \log_2 -ratios from each microarray. BIM model based clustering allowed for the fitting of the statistical mixture model without knowing the number of clusters in the data [20]. The statistical model was fitted using the Gibbs sampler, and hierarchical clustering was produced by treating pair-wise posterior probabilities as the similarity measure and applying the traditional average-linkage principle. The clustering results were displayed using the TreeView program [21].

Functional Clustering. Clusters of co-expressed gene identified by the cluster analysis were correlated with functional groupings defined by Gene Ontologies (GO) [22]. Clusters of genes with significantly over-represented genes from specific GO categories were identified using the EASE software [23]. Statistical significance of over-representation of genes from a cluster in any given GO category was assessed using the Fisher's exact test with the Benjamini-Hochberg adjustment for multiple hypothesis testing [17]. A GO category was considered to be significantly associated with a cluster if it contained more than one gene from the cluster and the adjusted Fisher's exact p-value (i.e. False Discovery Rate) was less than 0.1.

Quantitative real-time polymerase chain reaction (QPCR). RNA was isolated from intact eyes and eyes undergoing lens regeneration using TRI REAGENT[®] (Molecular Research Center, INC.) according to manufacturer's instructions. 0.75 micrograms of RNA was used to synthesize cDNA using iScript[™] cDNA Synthesis Kit (BioRad). All Real-Time PCRs were performed using the iCycler[™] (BioRad). For each Real-Time PCR reaction run in triplicate, 2 microliters of cDNA, 800 nM primers, and iQ[™] SYBR[®] Green Supermix (BioRad) were used. The data were analyzed using the Pfaffl method (Pfaffl, 2001).

Results and Discussion

Labeled target representing mouse mRNA from lens was used to hybridize to arrayed 70-mer probes representing nearly 25,000 mouse genes. Gene expression profiles of regenerating lens (1, 2, and 3 weeks post-lentectomy) were compared to the expression profiles of intact lens (Figure 2). We identified the genes that were significantly differentially expressed during the regeneration process. In all, we identified 2,094 genes that showed regulation during regeneration ($\text{fdr} < 0.05$ in at least one comparison). Six clusters of co-expressed genes defining distinct patterns of expression were significantly correlated with at least one GO category ($\text{fdr} < 0.1$ and more than one gene from a cluster was a member of a given GO category).

A general pattern emerged indicating that during the first week post-lentectomy, there is an increase in RNA levels of genes involved in tissue repair, inflammation, and re-organization of the cytoskeleton and the extracellular matrix (Figure 3). On the other hand, there was a significant decrease in RNA levels of genes encoding lens structural proteins, such as crystallins and other lens-fiber specific markers. As differentiation and growth of the lens ensued, some of the differentially expressed genes gradually returned to control levels of expression. The profile of the crystallins indicated that their synthesis followed the normal developmental program. At the same

time, we observed that some genes never reached control levels. Another interesting and novel discovery from the clustering analysis was that RNA levels decreased for genes involved in transcription and protein synthesis and may be a key early event.

The overall pattern clearly follows two different biological processes that take place after the extracapsular operation. In the initial stages, there is EMT and considerable remodeling of the extracellular matrix. At later stages, a lens differentiation program takes over due to regeneration of lens fibers. This observation is also very clear from a list of the top 50 differentially expressed genes that showed the greatest increase in mRNA levels across the different time points after surgery relative to control lens (Table 1). Thrombospondin-1 precursor showed the greatest fold-change increase. Thrombospondin-1 (TSP-1) is a glycoprotein involved in the activation of TGF- β , which is considered to be the main inducing factor of EMT [24]. TSP-1 has been shown to accumulate during PCO and decline during fiber differentiation [24]. Other highly up-regulated genes encode proteins that are involved in matrix remodeling, such as procollagen, TIMP-1, cathepsin, tenascin C, proteinases, and leucine-rich repeat containing protein (Table 1). Among the 50 genes that showed the greatest decrease in mRNA levels in the regenerating lens relative to the control (Table 2) are genes that encode structural proteins of differentiated lens fibers. The list includes several crystallins, phakinin, beaded filament structural protein, lens fiber membrane intrinsic protein and

lens fiber major intrinsic protein. Also, several regulatory genes, such as the homeo box NKX-2.2, the Kruppel factor 7, the cAMP responsive element binding protein and NFAT are clustered with the lens fiber-specific ones (Table 2). In Tables 1 and 2 the time with the highest (or lowest) regulation is highlighted red. This helps the reader to identify with a glance the times and the genes showing the most regulation. Interestingly, it becomes obvious that at week 2 we have the most severe regulation, positive or negative. These findings may eventually allow us to identify specific gene regulation programs involved in the distinct processes of EMT and fiber differentiation that take place during the process of mouse lens regeneration. Interestingly, the only genes that also coincide with cataract loci are the crystallin genes.

We examined further five general patterns of expression identified by correlating the clusters formed by the cluster analysis of gene expression profiles and functional clusters based on GO categories.

Weak Uniform Increase in RNA levels

In this group, the clustered genes showed a general pattern of a relatively slight increase in RNA levels throughout the regeneration process. In Table 3, the genes are divided according to main GO category, biological process, cellular component and molecular function. Table 4 presents a general feature of this subgroup in that it contains genes involved in defense,

response to injury, and extracellular matrix metabolism and also includes TGF- β and TGF- β -binding proteins, which are known mediators of EMT.

Strong Uniform Increase in RNA levels

As in the previous group, the genes in this cluster showed a general increase in RNA levels but more pronounced. The genes in this group are involved in the immune response, adhesion and remodeling, and processes that mediate injury and re-building of tissues after damage. Thrombospondins and disintegrins are included in the list (Tables 5 and 6).

Strong Delayed Increase in RNA levels

The mRNA levels of the genes in this group showed a sharp increase at week 2. Most of these genes are involved in cytoskeletal organization and negative regulation of transcription (Tables 7 and 8).

Weak Early Decrease in RNA levels

The main characteristic of this group was that the genes are involved in nucleic acid biosynthesis and ribosomal function. This result suggests that during the early events of repair, there a general inhibition of transcriptional and translational events (Tables 9 and 10).

Strong Uniform Decrease in RNA levels

The genes in this cluster are involved in sensory organ development, perception of light, and the structural components of the lens (Tables 11 and 12). mRNA levels for the crystallins and other structural proteins of the lens, such as phakinin, are severely decreased indicating that lens fiber differentiation is not at its final stages during the repair process. Naturally, the drop in RNA levels of some of genes in this group becomes less severe with the later stages of lens fiber differentiation (3-weeks post lentiectomy).

Verification of expression by QPCR

We selected ten genes to verify their expression by QPCR. The selected genes showed different patterns of expression in the microarray experiments. TIMP1 showed a strong uniform increase in RNA levels, lysozyme showed strong increase at week 2, celulo plasmin showed a weak uniform increase and γ B-crystallin showed strong uniform decrease in RNA levels. Others showed not much variation and had lower levels. Ratios observed in microarray experiments for low expressed genes are most likely more variable for overall low expressed genes than for highly expressed genes. However, since we are using the statistical significance as the main criteria for identifying differentially expressed genes, such higher variability will be accounted for. That is, genes with higher variability in observed ratios will have lower statistical significance than genes with low variability ratios. Therefore, the statistical significance of low-expressed genes has been implicitly adjusted in our analysis and the statistically significant genes have

equal chance of being false positives regardless of the overall level of expression. Nevertheless, we also decided to test such genes. As seen in Figure 4, expression of these genes as examined by QPCR was in excellent agreement with the microarray data. The housekeeping gene ATP synthase, epsilon subunit was used as the reference. This gene was found to have no differential expression in our microarray analysis and showed no differential expression in the QPCR experiments as well.

The mouse model for lens regeneration that we have described previously [6] is a valuable one because both EMT and lens fiber differentiation take place. Specifically, while the capsular bag is filled gradually with fibers, EMT is seen during the early stages and diminishes later. This has led us to utilize this model and examine global gene expression in order to associate clustered genes with both processes and identify new genes and networks. The availability of mutant mice will supplement these studies. By extensive genomic studies with mice lacking genes involved in EMT or lens fiber differentiation, the patterns of gene expression reported in this study could be sorted out in order to identify the role and regulation of known and novel genes involved in these processes. Extension of these studies, therefore, will lead to the establishment of databases and will provide indispensable and long-sought animal models for approaching PCO at the genetic level. At the same time, these studies will complement databases related to ocular bioinformatics [25-31].

Table 1. Top 50 up-regulated genes.

Top 50 genes with the greatest increase in relative mRNA expression levels of regenerating lens versus intact control lens after 1, 2, and 3 weeks post-extracapsular surgery.

Gene ID	Gene Name	Fold changes		
		Week 1	Week 2	Week 3
J05605	THROMBOSPONDIN 1 PRECURSOR	1.45	15.23	3.24
X81627	NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN PRECURSOR	4.26	5.27	6.93
X51547	P lysozyme structural	3.39	6.76	3.43
M33960	serine (or cysteine) proteinase inhibitor	5.49	4.13	3.46
AF071068	dopa decarboxylase	2.92	6.17	3.16
BC002069	Lysozyme	3.63	5.29	3.12
AK018742	procollagen, type VIII, alpha 1	1.55	7.62	2.75
M26498	ENDOTHELIN-2 PRECURSOR	5.69	2.26	2.56
	Similar To Cdna Fij10680	1.02	8.41	1.01
BC013651	serine (or cysteine) proteinase inhibitor, clade A, member 3N	3.96	2.44	3.54
D90343	tenascin C	5.24	2.11	1.93
AK003674	collagen triple helix repeat containing 1	4.92	2.40	1.80
BC006783	Connective tissue growth factor	2.64	3.90	2.51
U49430	ceruloplasmin	2.32	4.01	2.39
M17243	tissue inhibitor of metalloproteinase 1	3.27	2.62	2.75
AJ223208	cathepsin S	2.59	3.24	2.62
BC030071	calcitonin/calcitonin-related polypeptide, alpha	2.33	2.89	3.05
X97650	MYOSIN IF	3.37	2.49	2.32
BC016551	macrophage scavenger receptor 2	1.83	3.29	2.83
AF077829	TYRO protein tyrosine kinase binding protein	1.88	3.61	2.40
BC013494	cytochrome P450 4F 18	2.54	2.65	2.68
AF213458	triggering receptor expressed on myeloid cells 2b	1.67	3.42	2.75
L04264	PROTEIN-LYSINE 6-OXIDASE PRECURSOR	4.57	1.69	1.51
AF061272	C-type lectin	2.22	3.42	2.08
X58861	complement component 1, q	1.73	3.56	2.38
X60929	LOW AFFINITY IG GAMMA FC REGION REC. III PRE.	2.28	3.02	2.34
M73490	apolipoprotein E	1.63	3.83	2.15
BC027425	Membrane-spanning 4-domains	3.53	2.43	1.57
BC021539	allograft inflammatory factor 1	3.01	2.75	1.74
AK010252	leucine-rich repeat-containing 2	2.96	2.27	2.24
U47327	histocompatibility 2	1.15	3.76	2.52
AF175282	disintegrin-like and metalloprotease with thrombospondin	2.65	2.69	1.93
U05264	glycoprotein 49 B	2.31	2.55	2.37
AF290914	stabilin 1	2.68	2.85	1.69
X92959	COMPLEMENT C1Q SUBCOMPONENT	2.24	2.76	2.20
D13664	osteoblast specific factor 2	3.61	1.87	1.71
X93035	chitinase 3-like 1	1.89	3.00	2.27
AK004165	regulator of G-protein signaling 5	1.66	3.79	1.69
U42327	VASCULAR CELL ADHESION PROTEIN 1 PRECURSOR	2.63	2.02	2.44
M18524	H-2 CLASS I HISTOCOMPATIBILITY ANTIGEN	1.43	3.16	2.47
AK018713	cytochrome b-245	1.57	2.90	2.57
M69069	histocompatibility 2, D region locus 1	1.11	3.63	2.28
	Brca1 Interacting Protein	1.11	4.31	1.54
M64866	thrombospondin 2	3.79	1.34	1.75
	Similar To Acetyl-CoA Synthetase	1.41	3.61	1.84
Y11758	Biglycan	2.19	2.28	2.35

U83172	paired-Ig-like receptor B	3.35	2.16	1.28
AF063937	SERINE (OR CYSTEINE) PROTEINASE INHIBITOR, CLADE B	4.35	1.39	1.04
L33416	extracellular matrix protein 1	2.74	2.07	1.96
X92960	COMPLEMENT C1Q SUBCOMPONENT, C CHAIN PRECURSOR	2.22	2.27	2.19
AF053757	C3A ANAPHYLATOXIN CHEMOTACTIC RECEPTOR	2.93	2.49	1.20

Table 2. Top 50 down-regulated genes.

Top 50 genes with the greatest decrease in relative mRNA expression levels of regenerating lens versus intact control lens after 1, 2, and 3 weeks post-extracapsular surgery.

Gene ID	Gene Name	Fold changes		
		Week 1	Week 2	Week 3
BC021649	cAMP responsive element binding protein 1	-13.62	-18.28	-13.79
NM_153601	Glutamate-ammonia ligase	-17.18	-15.45	-11.07
	Hypermethylated In Cancer 2 Protein	-7.66	-14.77	-8.49
AF104312	hydroxyacid oxidase 1, liver	-3.94	-14.38	-11.74
S83259	HOMEBOX PROTEIN NKX-2.2	-9.73	-10.93	-8.02
BC004700	Kruppel-like factor 7	-10.64	-10.48	-4.57
	BEADED FILAMENT STRUCTURAL PROTEIN IN LENS-CP94	-7.96	-9.24	-7.53
Y13606		-7.96	-9.24	-7.53
S81982	NITRIC-OXIDE SYNTHASE, BRAIN	-7.95	-8.12	-4.76
M64544	crystallin, gamma C	-15.98	-1.72	-2.21
AF349659	thioredoxin reductase 3	-7.00	-6.86	-5.46
AF047542	cytochrome P450	-4.26	-10.12	-3.32
U21110	signal transducer and activator of transcription 5B	-10.98	-4.58	-1.70
AJ304860	PHAKININ	-5.49	-5.83	-3.85
AF309072	lactase-like	-4.41	-6.68	-3.57
BC025817	FAD-synthetase	-6.18	-4.78	-3.57
AJ272229	crystallin, beta B3	-4.46	-6.07	-3.67
	Prostatic Steroid Binding Protein C1	-1.11	-11.03	-1.97
AF320075	LENS FIBER MEMBRANE INTRINSIC PROTEIN	-4.89	-5.21	-3.65
AJ239052	crystallin, beta A1	-2.24	-8.16	-2.85
NM_172635	Expressed sequence AV312086	-3.68	-5.65	-3.85
AF072881	WD-40-repeat-containing protein	-2.31	-5.98	-4.84
AK053869	crystallin, beta B1	-4.78	-4.99	-3.05
U36576	nuclear factor of activated T-cells	-3.39	-5.66	-3.73
U08095	KERATIN, TYPE I CYTOSKELETAL 12	-8.61	-2.43	-1.69
	Molecule Interacting With Rab13	1.16	-9.99	-3.74
AF334607	DNASE2 LIKE ACID DNASE	-3.90	-6.36	-2.26
AJ224342	GAMMA CRYSTALLIN D	-7.29	-3.29	-1.61
Z22573	GAMMA CRYSTALLIN B	-8.33	-1.53	-2.06
AK003904	Ars component B	-6.75	-3.00	-1.41
AB037890	splicing factor 3b	-4.46	-3.59	-2.54
U03562	heat shock protein 1	-4.09	-2.97	-3.20
AF099938	COMPLEMENT COMPONENT C1Q	-3.38	-2.72	-3.83
AF000143	LENS FIBER MAJOR INTRINSIC PROTEIN	-2.93	-4.93	-1.76
BC032251	brain-specific angiogenesis inhibitor 3	-1.76	-4.57	-3.19
K02586	GAMMA CRYSTALLIN A	-1.83	-5.20	-2.36
BC002008	fatty acid binding protein 5	-3.48	-3.26	-2.42
	Mkiaa1450 Protein	-2.48	-3.00	-3.29
	Sodium Channel	-2.99	-3.29	-2.38
BC022920	Expressed sequence AA408140	-2.22	-3.50	-2.88
AB016768	R-spondin, thrombospondin-1-like domain	-3.03	-3.11	-1.84
X52128	t-complex protein 11	-1.71	-3.99	-2.11
	Translation Initiation Factor	-2.17	-2.93	-2.67
BC024653	O-acyltransferase	-1.81	-3.30	-2.40
NM_172296	Doublesex and mab-3	-1.95	-2.91	-2.46
AF020772	karyopherin (importin) alpha 3	-1.15	-4.75	-1.39
L14569	OLFACTORY RECEPTOR 7B	1.05	-5.11	-3.22
D14423	Tachykinin 2	-2.06	-3.38	-1.81
AF156979	arrestin 3	-2.22	-2.72	-1.80
AY158991	small proline-rich protein 2G	-1.81	-3.02	-1.86
BC012704	carbonic anhydrase 4	-2.21	-2.63	-1.81
AF032115	DnaJ (Hsp40) homolog	-2.32	-2.55	-1.77

Table 3. Gene ontology categories: weak uniform increase. Gene Ontology categories of the gene cluster displaying a weak uniform increase in relative mRNA levels of regenerating lens versus intact control lens.

Main GO Category	Number of Genes in the Category	Gene Category	False Discovery Rate
GO Biological Process	10	defense response	8.2E-03
	10	response to biotic stimulus	1.6E-02
	4	chemotaxis	3.3E-02
	4	taxis	3.3E-02
	3	cellular defense response	5.3E-02
	7	immune response	7.9E-02
	2	antigen processing	7.9E-02
	4	response to wounding	7.9E-02
	4	response to chemical substance	7.9E-02
	11	response to external stimulus	7.9E-02
GO Cellular Component	31	extracellular	3.9E-06
	28	extracellular space	9.1E-06
	7	lytic vacuole	1.7E-04
	7	lysosome	1.7E-04
	7	vacuole	3.1E-04
	2	histone acetyltransferase complex	7.9E-02
GO Molecular Function	3	cytokine binding	3.5E-02
	5	cytokine activity	4.6E-02
	2	interleukin receptor activity	7.9E-02
	2	interleukin binding	7.9E-02
	2	chemokine receptor binding	7.9E-02
	2	chemokine activity	7.9E-02
	3	exopeptidase activity	7.9E-02
	2	chemoattractant activity	7.9E-02
	2	G-protein-coupled receptor binding	8.5E-02
	2	growth factor binding	9.6E-02
	2	carboxypeptidase activity	9.6E-02
	6	receptor binding	9.6E-02

Table 4. Gene cluster: weak uniform increase. The gene cluster displaying a weak uniform increase in relative mRNA levels of regenerating lens versus intact control lens.

Accession	Symbol	Description
BC010275	Arpc1b	actin related protein 2/3 complex, subunit 1B
M10416	B2m	beta-2 microglobulin
U89399	Coro1a	coronin, actin binding protein 1A
S70244	Clu	Clusterin
U56819	Ccr2	chemokine (C-C motif) receptor 2
AF237721	Col9a3	procollagen, type IX, alpha 3
AF039391	Crym	crystallin, mu
S69034	Ctsb	cathepsin B
U74683	Ctsc	cathepsin C
D88689	Flt1	FMS-like tyrosine kinase 1
AF254441	Gcn5l2	GCN5 general control of amino acid synthesis-like 2 (yeast)
BC016431	Leprel2	leprecan-like 2
X62321	Gn	Granulin
AF267747	Gtf2i	general transcription factor II I
BC034217	H13	histocompatibility 13
AB013095	Hebp1	heme binding protein 1
U07741	Hexb	hexosaminidase B
X05429	Ii	Ia-associated invariant chain
U53696	Il10rb	interleukin 10 receptor, beta
M20658	Il1r1	interleukin 1 receptor, type I
D16313	Krt1-15	keratin complex 1, acidic, gene 15
X03491	Krt2-4	keratin complex 2, basic, gene 4
J03881	Lamp1	lysosomal membrane glycoprotein 1
M32018	Lamp2	lysosomal membrane glycoprotein 2
AJ243857	Lhx9	LIM homeobox protein 9
AF139987	Limk1	LIM-domain containing, protein kinase
M89956	Lsp1	lymphocyte specific 1
AF004874	Ltbp2	latent transforming growth factor beta binding protein 2
AK007774	Ltbp3	latent transforming growth factor beta binding protein 3
AJ298054	Blnk	B-cell linker
U47737	Ly6e	lymphocyte antigen 6 complex, locus E
M34094	Mdk	Midkine
L11625	Mertk	c-mer proto-oncogene tyrosine kinase
S77350	Mgla	matrix gamma-carboxylglutamate (gla) protein
M77226	Muc1	mucin 1, transmembrane
AK036379	Ncf2	neutrophil cytosolic factor 2
X57337	Pcolce	procollagen C-proteinase enhancer protein
M74227	Ppic	peptidylprolyl isomerase C
AF065933	Ccl2	chemokine (C-C motif) ligand 2
BC032922	Sla	src-like adaptor
X57413	Tgfb2	transforming growth factor, beta 2
J03299	Trf	Transferrin
BC011182	Zfp39	zinc finger protein 39
X76696	Cd52	CD52 antigen
AF358138	Hcst	hematopoietic cell signal transducer
AJ249987	Taf10	TAF10 RNA polymerase II, TATA box binding protein (TBP)-associated factor
AK018587	Batf	basic leucine zipper transcription factor, ATF-like

AK076162	Litaf	LPS-induced TN factor
AF290973	Ifi30	interferon gamma inducible protein 30
BC005532	Cndp2	CNDP dipeptidase 2 (metallopeptidase M20 family)
AK029988	Cxcl16	chemokine (C-X-C motif) ligand 16
AF219141	Agtpbp1	ATP/GTP binding protein 1
AF393640	Srpx2	sushi-repeat-containing protein, X-linked 2
AK007397	Adh6a	alcohol dehydrogenase 6A (class V)
AF345635	Pir	Pirin
AJ421478	Cnbp2	cellular nucleic acid binding protein 2
BC034522	Oplah	5-oxoprolinase (ATP-hydrolysing)
BC048078	AI428855	expressed sequence AI428855
BC022145	BC022145	cDNA sequence BC022145
BC013712	BC013712	cDNA sequence BC013712
BC027331	P2ry6	pyrimidinergic receptor P2Y, G-protein coupled, 6
AK007226	Atp2c1	ATPase, Ca ⁺⁺ -sequestering
AK028285	Sulf1	sulfatase 1
AK047568	Snph	Syntaphilin
U29501	Zfp75	zinc finger protein 75
BC028661	Myo1g	myosin IG
BC025046	Cdk8	cyclin-dependent kinase 8

Table 5. Gene ontology categories: strong uniform increase. Gene ontology categories of the gene cluster displaying a strong uniform increase in relative mRNA levels of regenerating lens versus intact control lens.

Main GO Category	Number of Genes in the Category	Gene Category	False Discovery Rate
GO Biological Process	17	response to biotic stimulus	1.0E-06
	16	defense response	1.2E-06
	14	immune response	3.3E-06
	7	humoral immune response	3.3E-06
	10	response to pest/pathogen/parasite	2.4E-05
	18	response to external stimulus	3.9E-04
	4	complement activation	1.6E-03
	11	cell adhesion	2.1E-03
	4	humoral defense mechanism (sensu Vertebrata)	2.3E-03
	3	antigen processing	3.3E-03
	11	response to stress	4.7E-03
	3	antigen presentation	5.1E-03
	3	complement activation\, classical pathway	5.1E-03
	4	angiogenesis	6.2E-03
	4	blood vessel development	1.0E-02
	2	antigen presentation\, exogenous antigen	1.2E-02
	2	antigen processing\, exogenous antigen via MHC class II	1.2E-02
	2	cell wall catabolism	1.6E-02
	2	regulation of angiogenesis	3.0E-02
	2	cytolysis	4.9E-02
GO Cellular Component	45	extracellular	4.7E-11
	41	extracellular space	2.0E-10
	13	extracellular matrix	1.1E-06
	2	complement component C1q complex	1.9E-03
GO Molecular Function	10	glycosaminoglycan binding	7.9E-10
	9	heparin binding	5.7E-09
	4	complement activity	4.3E-04
	9	cell adhesion molecule activity	1.9E-03
	6	defense/immunity protein activity	1.9E-03
	2	lysozyme activity	4.2E-03
	3	scavenger receptor activity	9.5E-03
	2	MHC class II receptor activity	1.0E-02
	5	endopeptidase inhibitor activity	1.2E-02
	5	protease inhibitor activity	1.2E-02
	6	enzyme inhibitor activity	1.9E-02
	10	enzyme regulator activity	1.9E-02
	2	integrin binding	4.1E-02
	2	insulin-like growth factor binding	6.7E-02
	2	antimicrobial peptide activity	6.7E-02
	24	signal transducer activity	6.7E-02

Table 6. Gene cluster: strong uniform increase. The gene cluster displaying a strong uniform increase in relative mRNA levels of regenerating lens versus intact control lens.

Accession	Symbol	Description
BC021539	Aif1	allograft inflammatory factor 1
AF212924	Ank3	ankyrin 3, epithelial
U72941	Anxa4	annexin A4
M73490	ApoE	apolipoprotein E
Y11758	Bgn	Biglycan
X58861	C1qa	complement component 1, q subcomponent, alpha polypeptide
X92959	C1qb	complement component 1, q subcomponent, beta polypeptide
X92960	C1qg	complement component 1, q subcomponent, gamma polypeptide
AF053757	C3ar1	complement component 3a receptor 1
BC030071	Calca	calcitonin/calcitonin-related polypeptide, alpha
X07411	Cbr2	carbonyl reductase 2
BC004076	Ccnd3	cyclin D3
X93035	Chi3l1	chitinase 3-like 1
AK052963	Col14a1	procollagen, type XIV, alpha 1
U49430	Cp	ceruloplasmin
BC002072	Cst3	cystatin C
AJ006033	Ctsk	cathepsin K
AJ223208	Ctss	cathepsin S
AK018713	Cyba	cytochrome b-245, alpha polypeptide
AF071068	Ddc	dopa decarboxylase
L33416	Ecm1	extracellular matrix protein 1
M26498	Edn2	endothelin 2
AF135252	Fbln2	fibulin 2
X60929	Fcgr3	Fc receptor, IgG, low affinity III
BC006783	Ctgf	connective tissue growth factor
BC004647	Flot1	flotillin 1
BC004724	Fn1	fibronectin 1
AF149059	Gclm	glutamate-cysteine ligase , modifier subunit
U05264	Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4
V01527	H2-Ab1	histocompatibility 2, class II antigen A, beta 1
M18524	H2-D1	histocompatibility 2, D region locus 1
M69069	H2-D1	histocompatibility 2, D region locus 1
M36939	H2-Eb1	histocompatibility 2, class II antigen E beta
X56790	Cyr61	cysteine rich protein 61
X81627	Lcn2	lipocalin 2
L04264	Lox	lysyl oxidase
BC002069	Lyzs	lysozyme
X51547	Lzp-s	P lysozyme structural
AF061272	Clecsf8	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 8
X97650	Myo1f	myosin IF
U83172	Pirb	paired-Ig-like receptor B
M33960	Serpine1	serine (or cysteine) proteinase inhibitor, clade E, member 1
D37837	Lcp1	lymphocyte cytosolic protein 1
AK002410	Plscr2	phospholipid scramblase 2
U33626	Pml	promyelocytic leukemia
AK079915	Lgals3bp	lectin, galactoside-binding, soluble, 3 binding protein

U57999	Psap	prosaposin
S72304	Rab34	RAB34, member of RAS oncogene family
AK004165	Rgs5	regulator of G-protein signaling 5
U58886	Sh3gl2	SH3-domain GRB2-like 2
BC013651	Serpina3n	serine (or cysteine) proteinase inhibitor, clade A, member 3N
X70946	Serpine2	serine (or cysteine) proteinase inhibitor, clade E, member 2
AK010442	Syngn1	synaptogyrin 1
M64866	Thbs2	thrombospondin 2
M17243	Timp1	tissue inhibitor of metalloproteinase 1
D90343	Tnc	tenascin C
AF077829	Tyrbp	TYRO protein tyrosine kinase binding protein
U42327	Vcam1	vascular cell adhesion molecule 1 a disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif, 8
AF175282	Adamts8	periostin, osteoblast specific factor
D13664	Postn	regulator of G-protein signaling 3
AF350047	Rgs3	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 10
BC023008	Clecsf10	transmembrane 4 superfamily member 7
BC003482	Tm4sf7	interferon induced transmembrane protein 3
AY082484	Ifitm3	ubiquitin-activating enzyme E1-domain containing 1
BC019764	Ube1dc1	alcohol dehydrogenase 6A (class V)
AK007397	Adh6a	membrane-spanning 4-domains, subfamily A, member 6B
BC027425	Ms4a6b	RIKEN cDNA 2310067E08 gene
AK010084	2310067E08Rik	cytochrome P450, family 4, subfamily f, polypeptide 18
BC013494	Cyp4f18	membrane-spanning 4-domains, subfamily A, member 6C
AK008652	Ms4a6c	macrophage scavenger receptor 2
BC016551	Msr2	triggering receptor expressed on myeloid cells 2
AF213458	Trem2	glycoprotein (transmembrane) nmb
BC026375	GpnmB	PC4 and SFRS1 interacting protein 1
AJ308965	Psip1	ankyrin repeat and SOCS box-containing protein 7
AF398968	Asb7	oocyte secreted protein 1
AK033038	Oosp1	stabilin 1
AF290914	Stab1	

Table 7. Gene ontology categories: strong late increase. Gene Ontology categories of the gene cluster displaying a strong increase in relative mRNA levels late during lens regeneration versus intact control lens.

Main GO Category	Number of Genes in the Category	Gene Category	False Discovery Rate
GO Biological Process	6	negative regulation of transcription	7.8E-02
	5	negative regulation of transcription, DNA-dependent	9.7E-02
	9	cytoskeleton organization and biogenesis	9.7E-02

Table 8. Gene cluster: strong late increase. The gene cluster displaying a strong increase in relative mRNA levels late during lens regeneration versus intact control lens.

Accession	Symbol	Description
U76732	Adh7	alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide
U88623	Aqp4	aquaporin 4
L07918	Arhgdib	Rho, GDP dissociation inhibitor (GDI) beta
AC002397	Bcap37	B-cell receptor-associated protein 37
D16432	Cd63	Cd63 antigen
M64278	Chga	chromogranin A
X04591	Ckb	creatine kinase, brain
AB033123	Ctbp2	C-terminal binding protein 2
X94998	Fmod	fibromodulin
S71213	Gnai2	guanine nucleotide binding protein, alpha inhibiting 2
M96645	Gp38	glycoprotein 38
AF027505	Baiap1	BAI1-associated protein 1
D16464	Hes1	hairy and enhancer of split 1 (Drosophila)
M10062	Iap	intracisternal A particles
M60523	Idb3	inhibitor of DNA binding 3
BC003804	Ifit3	interferon-induced protein with tetratricopeptide repeats 3
AK011790	Igfbp2	insulin-like growth factor binding protein 2
X69902	Itga6	integrin alpha 6
M64228	Kcnb1	potassium voltage gated channel, Shab-related subfamily, member 1
AB023656	Kif1b	kinesin family member 1B
AF202892	Kif21a	kinesin family member 21A
D16313	Krt1-15	keratin complex 1, acidic, gene 15
M13805	Krt1-17	keratin complex 1, acidic, gene 17
U55060	Lgals9	lectin, galactose binding, soluble 9
AF367720	Lrp1	low density lipoprotein receptor-related protein 1
X66983	Mak	male germ cell-associated kinase
AF047714	Trpm1	transient receptor potential cation channel, subfamily M, member 1
M36411	Mpv17	Mpv17 transgene, kidney disease mutant
AJ249706	Myo10	myosin X
AK077116	Naga	N-acetyl galactosaminidase, alpha
X61450	Napb	N-ethylmaleimide sensitive fusion protein attachment protein beta
X61455	Napb	N-ethylmaleimide sensitive fusion protein attachment protein beta
S40532	Nhlh2	nescient helix loop helix 2
AF219626	Ninj1	ninjurin 1
AJ006803	Nrxn2	neurexin II
U79523	Pam	peptidylglycine alpha-amidating monooxygenase
AF023529	Pde1a	phosphodiesterase 1A, calmodulin-dependent
BC005661	Pgam1	phosphoglycerate mutase 1
L43371	Ppap2a	phosphatidic acid phosphatase 2a
X58990	Ppib	peptidylprolyl isomerase B
BC004730	Psmb10	proteasome (prosome, macropain) subunit, beta type 10
AB001607	Ptgis	prostaglandin I2 (prostacyclin) synthase
AK088005	Ptma	prothymosin alpha
S52353	Ptn	pleiotrophin
AK050418	Rp2h	retinitis pigmentosa 2 homolog (human)
D43805	Cxcl12	chemokine (C-X-C motif) ligand 12

M74773	Spnb2	spectrin beta 2
U30709	Stat3	signal transducer and activator of transcription 3
U55862	Tia1	cytotoxic granule-associated RNA binding protein 1
M93954	Timp2	tissue inhibitor of metalloproteinase 2
U41741	Usf1	upstream transcription factor 1
U80078	Zfp148	zinc finger protein 148
AJ316580	Mgll	monoglyceride lipase
U43206	Pbp	phosphatidylethanolamine binding protein
AF033116	Zfhx1b	zinc finger homeobox 1b
AK020876	Nubp1	nucleotide binding protein 1
AF128236	Psg18	pregnancy specific glycoprotein 18
AB071988	H2afy	H2A histone family, member Y
AY133242	Ahi1	Abelson helper integration site
AF239886	Espn	espin
AK010636	MGI:1929282	telomerase binding protein, p23
BC007177	Ccnl1	cyclin L1
BC014726	Sertad2	SERTA domain containing 2
AK088173	Nek7	NIMA (never in mitosis gene a)-related expressed kinase 7
AK075861	Sirt3	sirtuin 3 (silent mating type information regulation 2, homolog) 3 (S. cerevisiae)
BC031854	D14Ert449e	DNA segment, Chr 14, ERATO Doi 449, expressed
AK009412	Srp19	signal recognition particle 19
AK017655	Luc7l	Luc7 homolog (S. cerevisiae)-like
BC027328	Bst2	bone marrow stromal cell antigen 2
AK005050	Dhdh	dihydrodiol dehydrogenase (dimeric)
BC052406	Bruno6	bruno-like 6, RNA binding protein (Drosophila)
AY055832	Mtmt2	myotubularin related protein 2
BC002262		
AF130313	Nckipsc	NCK interacting protein with SH3 domain
BC021457	Slmap	sarcolemma associated protein
AF252281	Klhl1	kelch-like 1 (Drosophila)
AY013783	Pcdhb21	protocadherin beta 21
AF296412	Aipl1	aryl hydrocarbon receptor-interacting protein-like 1 diphtheria toxin resistance protein required for diphthamide biosynthesis (Saccharomyces)-like 1
AY078170	Dph21	butyrobetaine (gamma), 2-oxoglutarate dioxygenase 1 (gamma-butyrobetaine hydroxylase)
BC019406	Bbox1	
AF403039	MGI:2183445	SPRY domain-containing SOCS box 4
BC027170	C79267	expressed sequence C79267
BC027407	Zfp472	zinc finger protein 472
BC017130	Pja2	praja 2, RING-H2 motif containing
BC003885	BC003885	cDNA sequence BC003885
BC027194	Golph3l	golgi phosphoprotein 3-like
BC027279	Blvrb	biliverdin reductase B (flavin reductase (NADPH))
AK044042	Cpne5	copine V
AK075830	Usp7	ubiquitin specific protease 7
AK030085	D2Ert485e	DNA segment, Chr 2, ERATO Doi 485, expressed

Table 9. Gene ontology categories: weak early decrease. Gene Ontology categories of the gene cluster displaying a weak decrease in relative mRNA levels early during lens regeneration versus intact control lens.

Main GO Category	Number of Genes in the Category	Gene Category	False Discovery Rate
GO Biological Process	16	macromolecule biosynthesis	2.9E-03
	17	biosynthesis	5.3E-03
	12	protein biosynthesis	6.1E-03
	2	RNA elongation	2.8E-02
	3	purine ribonucleoside triphosphate biosynthesis	5.3E-02
	3	purine nucleoside triphosphate biosynthesis	5.3E-02
	3	ribonucleoside triphosphate biosynthesis	5.3E-02
	7	perception of abiotic stimulus	5.3E-02
	7	sensory perception	5.3E-02
	3	purine ribonucleoside triphosphate metabolism	5.3E-02
	3	ribonucleoside triphosphate metabolism	5.3E-02
	3	nucleoside triphosphate biosynthesis	5.3E-02
	5	perception of chemical substance	5.3E-02
	5	chemosensory perception	5.3E-02
	3	purine nucleoside triphosphate metabolism	5.3E-02
	14	G-protein coupled receptor protein signaling pathway	5.8E-02
	7	perception of external stimulus	5.8E-02
	3	purine ribonucleotide biosynthesis	5.8E-02
	3	nucleoside triphosphate metabolism	5.8E-02
	3	purine nucleotide biosynthesis	6.9E-02
	3	ribonucleotide biosynthesis	7.1E-02
	2	phosphoenolpyruvate-dependent sugar phosphotransferase :	7.6E-02
	3	purine ribonucleotide metabolism	7.6E-02
	3	purine nucleotide metabolism	7.8E-02
	3	ribonucleotide metabolism	7.8E-02
	2	oxidative phosphorylation	8.7E-02
	2	ATP biosynthesis	9.8E-02
	2	nucleoside phosphate metabolism	9.8E-02
GO Cellular Component	10	ribosome	2.0E-03
	12	ribonucleoprotein complex	2.0E-03
	14	mitochondrion	1.2E-02
	3	small ribosomal subunit	5.3E-02
	2	transport vesicle	5.3E-02
	3	organellar ribosome	5.8E-02
	3	mitochondrial ribosome	5.8E-02
	3	mitochondrial matrix	9.8E-02
	2	mitochondrial large ribosomal subunit	9.9E-02
	2	organellar large ribosomal subunit	9.9E-02
GO Molecular Function	10	structural constituent of ribosome	1.1E-03
	13	structural molecule activity	1.7E-02
	10	RNA binding	2.2E-02
	4	hydrogen ion transporter activity	7.8E-02
	4	monovalent inorganic cation transporter activity	7.8E-02
	2	oxidoreductase activity\, acting on heme group of donors\, o:	7.8E-02
	2	cytochrome-c oxidase activity	7.8E-02
	2	oxidoreductase activity\, acting on heme group of donors	7.8E-02
	2	heme-copper terminal oxidase activity	7.8E-02
	2	DNA dependent ATPase activity	8.1E-02
	2	damaged DNA binding	9.7E-02

Table 10. Gene cluster: weak early decrease. The gene cluster displaying a weak decrease in relative mRNA levels early during lens regeneration versus intact control lens.

Accession	Symbol	Description
AF104416	Aqp3	aquaporin 3
BC011291	Arbp	acidic ribosomal phosphoprotein P0
X52940	Cox7c	cytochrome c oxidase, subunit VIIc
X53599	Fmn	fomin
U38498	Gng5	guanine nucleotide binding protein (G protein), gamma 5 subunit
AF319526	Gstm2	glutathione S-transferase, mu 2
U96116	Hadh2	hydroxyacyl-Coenzyme A dehydrogenase type II
D63663	Pwwp1	PWWP domain containing 1
U60001	Hint1	histidine triad nucleotide binding protein 1
U12791	Hmgcs2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2
AK004568	Rps2	ribosomal protein S2
X68193	Nme2	expressed in non-metastatic cells 2, protein
AF102540	Olf161	olfactory receptor 61
AK011242	Rad51	RAD51 homolog (S. cerevisiae)
AK012580	Rpl18	ribosomal protein L18
AK086805	Mrpl23	mitochondrial ribosomal protein L23
L08651	Rpl29	ribosomal protein L29
X80899	Cox7a2l	cytochrome c oxidase subunit VIIa polypeptide 2-like
AF042139	Ssa2	Sjogren syndrome antigen A2
BC010807	Tcea3	transcription elongation factor A (SII), 3
U60150	Vamp2	vesicle-associated membrane protein 2
BC037541	Lynx1	Ly6/neurotoxin 1
AF093260	Homer2	homer homolog 2 (Drosophila)
X76772	Rps3	ribosomal protein S3
AK008036	Atp5l	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit g
BC012241	Atp5o	ATP synthase, H ⁺ transporting, mitochondrial F1 complex, O subunit
AF177399	Dkk1	dickkopf-like 1
BC011162	Ncald	neurocalcin delta
AF119676	Rab25	RAB25, member RAS oncogene family
AK002585	Fxyd1	FXD domain-containing ion transport regulator 1
AK030225	Cyb561d2	cytochrome b-561 domain containing 2
AK014963	Xrcc2	X-ray repair complementing defective repair in Chinese hamster cells 2
AF124425	Cldn10	claudin 10
AB024448	Jph2	junctophilin 2
AK028318	Sv2a	synaptic vesicle glycoprotein 2 a
AK018778	Gng13	guanine nucleotide binding protein 13, gamma
AK003225	Mrpl54	mitochondrial ribosomal protein L54
BC027511	Lsm7	LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)
AF230339	Znrd1	zinc ribbon domain containing, 1
AK003341	Chchd5	coiled-coil-helix-coiled-coil-helix domain containing 5
BC027546	Mrps21	mitochondrial ribosomal protein S21
AK028011	Ndufc1	NADH dehydrogenase (ubiquinone) 1, subcomplex unknown, 1
AK010329	Mrpl11	mitochondrial ribosomal protein L11
BC024346	MGI:1913699	mitochondria-associated protein involved in GMCSF signal transduction
AK088737	Rplp2	ribosomal protein, large P2

AK003223	Dpm3	dolichyl-phosphate mannosyltransferase polypeptide 3
AK003192	Ict1	immature colon carcinoma transcript 1
BC030905	Ascc1	activating signal cointegrator 1 complex subunit 1
AK008201	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11
AB093231	Tln2	talin 2
AY071834	Optn	optineurin
AK005562	Eml2	echinoderm microtubule associated protein like 2
AF319173	Psca	prostate stem cell antigen
AK006855	MGI:1920610	actin-related protein T1
BC016253	Man2c1	mannosidase, alpha, class 2C, member 1
AB046537	Pi16	protease inhibitor 16
AK049110	D15Erd735e	DNA segment, Chr 15, ERATO Doi 735, expressed
AJ421478	Cnbp2	cellular nucleic acid binding protein 2
AK012695	Mgea5	meningioma expressed antigen 5 (hyaluronidase)
AK020927	Rdh12	retinol dehydrogenase 12
AB053477	Abtb1	ankyrin repeat and BTB (POZ) domain containing 1
AJ278462	Mmp1a	matrix metalloproteinase 1a (interstitial collagenase)
X04652	Hist2h4	histone 2, H4
AF425084	Serpnb6c	serine (or cysteine) proteinase inhibitor, clade B, member 6c
AF372838	Rdh9	retinol dehydrogenase 9
BC031759	MGI:2135937	SH3-binding kinase
BC034872	Gpr18	G protein-coupled receptor 18
AK079337	Baalc	brain and acute leukemia, cytoplasmic
AK029205	V1rc15	vomeronasal 1 receptor, C15
AK031458	AI428795	expressed sequence AI428795
AB091829	MGI:2384865	O-acetyltransferase
AK085987	Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase
AY277588	MGI:2668443	retinal short chain dehydrogenase reductase 2
AY317805	Olf684	olfactory receptor 684
AK033012	Npat	nuclear protein in the AT region
AF357887	Dusp15	dual specificity phosphatase-like 15
AY073826	Olf747	olfactory receptor 747
AY073750	Olf1265	olfactory receptor 1265
AY073749	Olf1495	olfactory receptor 1495
AY073644	Olf1231	olfactory receptor 1231
AY073624	Olf1261	olfactory receptor 1261
AY073578	Olf530	olfactory receptor 530
AY073509	Olf1030	olfactory receptor 1030
AY073501	Olf703	olfactory receptor 703
AY073438	Olf1131	olfactory receptor 1131
AY073304	Olf1238	olfactory receptor 1238
AY073177	Olf1377	olfactory receptor 1377
AF171073	Ppia	peptidylprolyl isomerase A
BC034068	BC034068	cDNA sequence BC034068
AK047412	AU020772	expressed sequence AU020772
BC049929	Ddx11	DEAD/H box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)
L38438	Ndufs6	NADH dehydrogenase (ubiquinone) Fe-S protein 6

Table 11. Gene ontology categories: strong uniform decrease. Gene Ontology categories of the gene cluster displaying a strong uniform decrease in relative mRNA levels of regenerating lens versus intact control lens.

Main GO Category	Number of Genes in the Category	Gene Category	False Discovery Rate
GO Biological Process	7	sensory organ development	1.4E-09
	7	peripheral nervous system development	9.6E-09
	5	vision	9.0E-03
	5	perception of light	1.1E-02
	5	response to light	1.1E-02
	5	response to radiation	1.4E-02
	8	neurogenesis	1.5E-02
	2	Mo-molybdopterin cofactor biosynthesis	1.6E-02
	2	Mo-molybdopterin cofactor metabolism	1.6E-02
	2	pteridine and derivative biosynthesis	4.1E-02
	2	pteridine and derivative metabolism	4.8E-02
	2	aromatic compound biosynthesis	5.5E-02
GO Cellular Component	29	cytoplasm	9.5E-02
GO Molecular Function	7	structural constituent of eye lens	9.6E-09

Table 12. The gene cluster displaying a strong uniform decrease in relative mRNA levels of regenerating lens versus intact control lens.

Accession	Symbol	Description
AF072815	Aldh3a1	aldehyde dehydrogenase family 3, subfamily A1
AF087654	Aqp5	aquaporin 5
Y13606	Bfsp1	beaded filament structural protein in lens-CP94
BC012704	Car4	carbonic anhydrase 4
BC021649	Creb1	cAMP responsive element binding protein 1
AJ239052	Cryba1	crystallin, beta A1
AK053869	Crybb1	crystallin, beta B1
AJ272229	Crybb3	crystallin, beta B3
Z22573	Crygb	crystallin, gamma B
M64544	Crygc	crystallin, gamma C
AJ224342	Crygd	crystallin, gamma D
AF032115	Dnajc5	DnaJ (Hsp40) homolog, subfamily C, member 5
AF047542	Cyp2c37	cytochrome P450, family 2, subfamily c, polypeptide 37
AF104312	Hao1	hydroxyacid oxidase 1, liver
S67000	Hdc	histidine decarboxylase
M81659	Hoxa10	homeo box A10
U03562	Hspb1	heat shock protein 1
BC002008	Fabp5	fatty acid binding protein 5, epidermal
AF020772	Kpna3	karyopherin (importin) alpha 3
AJ243857	Lhx9	LIM homeobox protein 9
AF099938	C1qr1	complement component 1, q subcomponent, receptor 1
AK002719	Mocs2	molybdenum cofactor synthesis 2
U36576	Nfatc2	nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 2
S83259	Nkx2-2	NK2 transcription factor related, locus 2 (Drosophila)
S81982	Nos1	nitric oxide synthase 1, neuronal
U85786	Scn1b	sodium channel, voltage-gated, type I, beta polypeptide
BC005434	Serpinb5	serine (or cysteine) proteinase inhibitor, clade B, member 5
AY158991	Sprr2g	small proline-rich protein 2G
U21110	Stat5b	signal transducer and activator of transcription 5B
D14423	Tac2	tachykinin 2
X52128	Tcp11	t-complex protein 11
U90889	Tkt	transketolase
AK031386	D15Mit260	DNA Segment, Chr 15 Massachusetts Institute of Technology 260
AF302077	Mell1	mel transforming oncogene-like 1
AY038025	Ngef	neuronal guanine nucleotide exchange factor
AB021967	Igsf4a	immunoglobulin superfamily, member 4A
AF334607	Dnase2b	deoxyribonuclease II beta
AK003904	Slurp1	secreted Ly6/Plaur domain containing 1
BC004057	Tacc2	transforming, acidic coiled-coil containing protein 2
AF117382	Hic2	hypermethylated in cancer 2
AF072881	Wsb2	WD repeat and SOCS box-containing 2
BC047277	Stk35	serine/threonine kinase 35
AK002226	Lypdc2	Ly6/Plaur domain containing 2
AK006330	Gpr160	G protein-coupled receptor 160
AF320075	Nkg7	natural killer cell group 7 sequence
AK012971	Lrriq2	leucine-rich repeats and IQ motif containing 2

BC003884	Pacsin3	protein kinase C and casein kinase substrate in neurons 3
AB037890	Sf3b1	splicing factor 3b, subunit 1
BC004700	Klf7	Kruppel-like factor 7 (ubiquitous)
BC029689	Tdrd7	tudor domain containing 7
BC022920	Dhx32	DEAH (Asp-Glu-Ala-His) box polypeptide 32
AJ304860	Bfsp2	beaded filament structural protein 2, phakinin
AF391758	Vsx1	visual system homeobox 1 homolog (zebrafish)
AF277385	MGI:2150387	prostatic steroid binding protein C1
AF156979	Arr3	arrestin 3, retinal
AB016768	MGI:2183426	thrombospondin type 1 domain containing gene
BC032251	Bai3	brain-specific angiogenesis inhibitor 3
AK080732	Kcnj14	potassium inwardly-rectifying channel, subfamily J, member 14
M55171	Rho	rhodopsin
BC024653	Oact1	O-acyltransferase (membrane bound) domain containing 1
AK089408	F730023N20	hypothetical protein F730023N20
AK017800	Clic5	chloride intracellular channel 5
AK037164	Eif5b	eukaryotic translation initiation factor 5B
AF309072	Lctf	lactase-like
AK032362	Usp32	ubiquitin specific protease 32
L14569	Olf144	olfactory receptor 144
U08095	Krt1-12	keratin complex 1, acidic, gene 12
BC029696	Dpp10	dipeptidylpeptidase 10

Figure 1. H & E staining of 1 day mouse eye. A histological section through a capsular bag one day after removal of the lens. Note that some cells have remained at the posterior (post) area of the capsule (arrowhead). The anterior (ant) part of the capsule is depicted with the arrow.

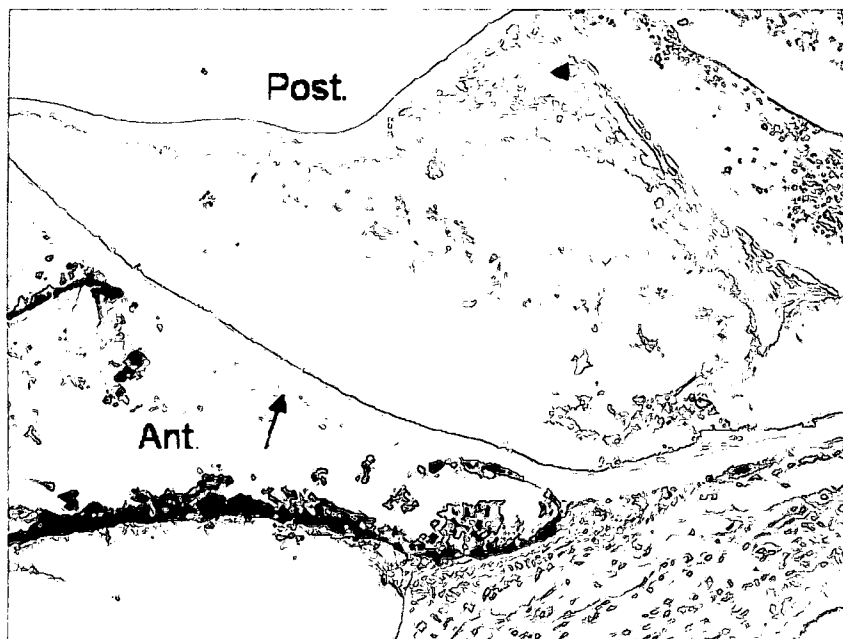


Fig. 2. The experimental design for the microarray studies. The indicated time periods of 1, 2, and 3 represent weeks post-lentectomy from time 0 (Control). Each mouse represents a biological replicate for a given experimental condition. An arrow denotes a microarray slide comparison between a given pair of mice. The oppositely directed arrows for a given experimental comparison represent “dye flips”. Our statistical model does indeed unequivocally factor out the gene-specific dye effect from the estimates of differential expression. This is achieved by fitting a linear statistical model with a “dye” effect, as described in the Data Normalization and Analysis section, to each gene separately. This approach has been demonstrated to work well in unbalanced situations such as the week 2 and 3 comparisons [32].

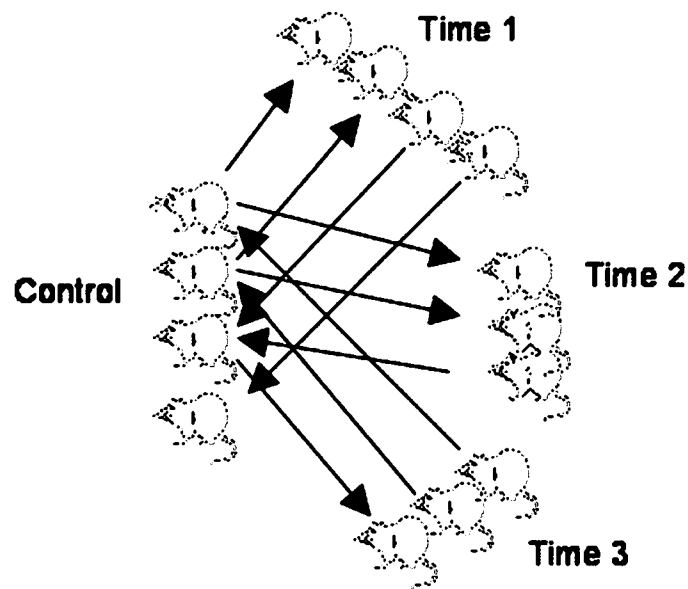


Figure. 3. Heat map from cluster analysis. Cluster analysis of 2,094 differentially expressed genes. The hierarchical clustering of all genes with each line representing expression levels for a gene as compared to control and each column a time point (1, 2 and 3 weeks post-lentectomy). Shades of red indicate increased expression and the shades of green decrease. Genes are grouped according to a particular expression pattern, i.e. up-regulation or down-regulation and within these two groups in other sub-groups, such as strong uniform up-regulation, strong uniform down-regulation, etc. Five clusters of co-expressed genes outlined in the figure were statistically significantly enriched for genes in at least one GO category.

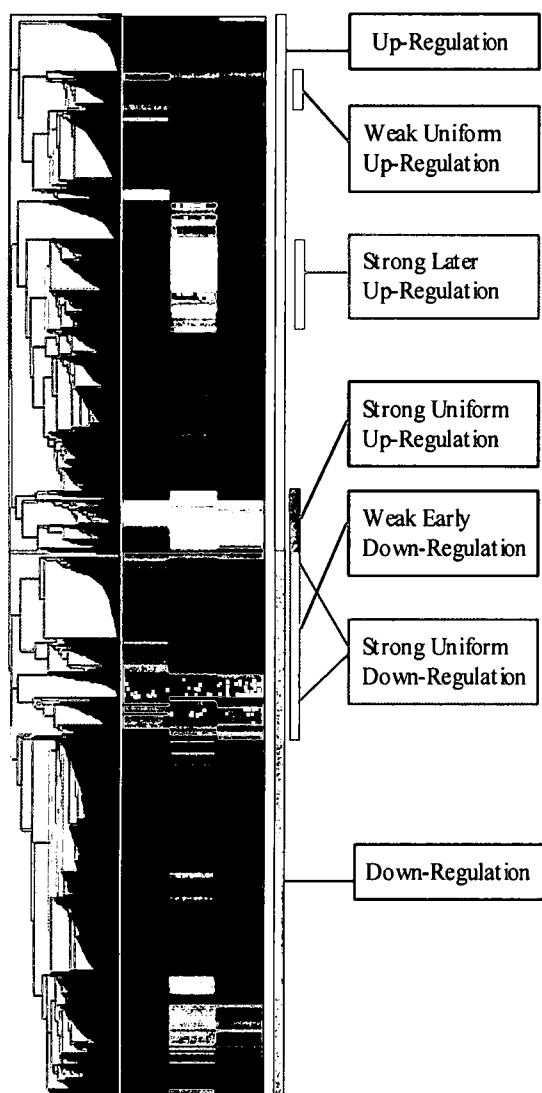
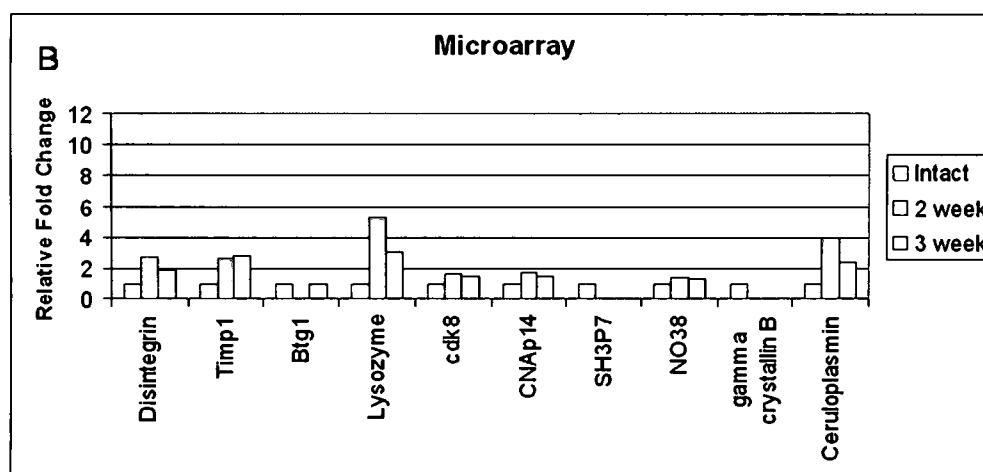
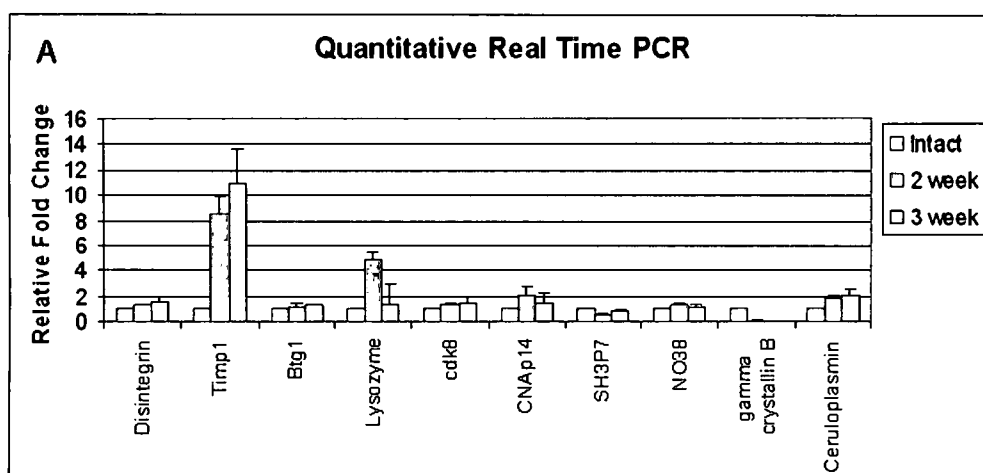


Figure 4. Microarray validation via QPCR. QPCR analysis (A) of ten selected genes and the corresponding data from the microarray analysis (B). The expression values of lens mRNA at 2 and 3 weeks post-surgery are compared with lens mRNA from the non-operated eye (value set at 1). C: the values between the two methods.



C	<u>Microarray</u>			<u>Quantitative Real Time</u>		
	<u>Intact</u>	<u>2 week</u>	<u>3 week</u>	<u>Intact</u>	<u>PCR</u> <u>2 week</u>	<u>3 week</u>
Disintegrin	1	2.69	1.93	1	1.29	1.54
Timp1	1	2.62	2.75	1	8.56	10.87
Btg1	1	-1.3	1.02	1	1.2	1.3
Lysozyme	1	5.29	3.12	1	4.9	1.3
cdk8	1	1.62	1.46	1	1.33	1.49
CNAp14	1	1.73	1.49	1	2.13	1.49
SH3P7	1	-1.88	-1.46	1	0.56	0.73
NO38	1	1.38	1.26	1	1.28	1.14
gamma						
crystallin B	1	-1.53	-2.06	1	0.03	0.007
Ceruloplasmin	1	4.01	2.39	1	1.77	2.04

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