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Lens regeneration and epithelial to mesenchymal transition in the mouse, *Mus musculus*

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**LENS REGENERATION AND EPITHELIAL TO MESENCHYMAL TRANSITION
IN THE MOUSE, *MUS MUSCULUS***

Dissertation

Submitted to

The College of Art and Sciences of the
UNIVERSITY OF DAYTON

In Partial Fulfillment of the Requirements for

The Degree

Doctor of Philosophy in Biology

By

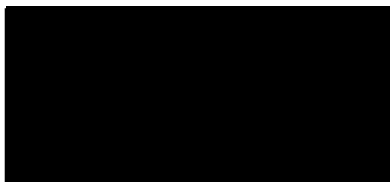
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UNIVERSITY OF DAYTON

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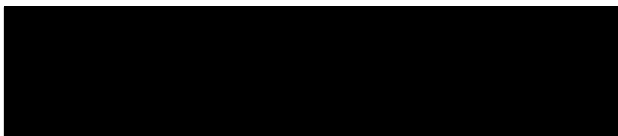
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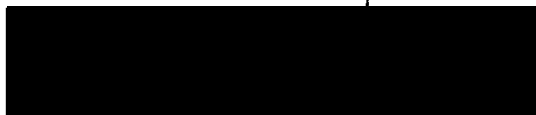
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ABSTRACT

LENS REGENERATION AND EPITHELIAL TO MESENCHYMAL TRANSITION IN THE MOUSE, *MUS MUSCULUS*

Mindy Kay Call
University of Dayton, 2006

Advisor: Dr. Panagiotis Tsonis

Lens regeneration in mammals was first shown to occur in New Zealand albino rabbits in 1825 following removal of the lens. Since that time, research has shown that the regenerative capabilities of the rabbit were the result of a wound healing event and not the classical regenerative process of transdifferentiation as seen in the adult newt. Lens regeneration in mammals occurs when several lens epithelial cells, which remain attached to the capsule following removal of the lens, migrate to the posterior and differentiate into mesenchymal cells. This differentiation of cell types is termed epithelial to mesenchymal transition (EMT). EMT is the leading cause of posterior capsule opacification (PCO). PCO or secondary cataract occurs when lens epithelial cells left behind after cataract surgery migrate posteriorly and occupy the region between the capsule and the intraocular lens (synthetic lens). These cells undergo EMT, which over time will result in a fibrotic plaque leading to

opacification and vision loss. PCO is the most prevalent complication seen during cataract surgery.

The first study described here examines the ability of mice to undergo lens regeneration. We found that mice can indeed undergo lens regeneration if the capsule is left behind following removal of the lens. Our data also show that EMT diminishes at the later stages of regeneration making this an excellent model system in which to study the role of EMT in PCO. Due to the genetic and molecular tools available for the mouse, we also examined global gene expression at various stages during the regeneration process. Microarray analysis revealed that initially there is a response to injury, extensive matrix remodeling, and severe down-regulation of genes encoding lens structural proteins. These patterns gradually return to normal three weeks after surgery. Together these studies represent the first steps to identify genes and gene networks involved in EMT and provide insights into lens regeneration therapy.

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I thank Dr. Tsonis for all of his guidance and help throughout the years. He hired me as a technician in his lab five years ago and was instrumental in encouraging me to get my Ph.D. I've learned many things from him throughout the years, but most of all I learned to be a critical thinker and an overall better scientist. I thank Dr. Del Rio-Tsonis who is not only a member of my committee but is also the individual who showed me the ropes when I first started working in the lab. She has been a tremendous help over the past five years and is someone I can always depend on to discuss the complexities of research. I also thank the other members of my graduate advisory committee, Dr. Krane, Dr. Hofmann, and Dr. Wright. They have all helped me achieve this goal by guiding and supporting me during committee meetings, examinations, and my defense.

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LIST OF ABBREVIATIONS

α -SMA	Alpha-Smooth Muscle Actin
aRNA	Amplified RNA
ASC	Anterior Subcapsular Cataract
BMP	Bone Morphogenetic Protein
ECM	Extracellular Matrix
EMT	Epithelial to Mesenchymal Transition
FDR	False Discovery Rates
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
LECs	Lens Epithelial Cells
MMP	Matrix Metalloproteinases
PECs	Pigmented Epithelial Cells
PCO	Posterior Capsule Opacification
SLRP	Small Leucine Repeat Proteoglycan
TGF β	Transforming Growth Factor beta
YAG	Yttrium Aluminum Garnet

CHAPTER I

Review of Literature

I. Anatomy of the Eye

Layers

The eye, while being relatively small in size, provides us with one of the most important of the five senses, vision. The eye is a complex organ consisting of multiple parts working together to ultimately convert light into electrical energy, which is transferred via electrical impulses to the optic nerve, where the brain processes the information into an image. The eye is composed of three layers: cornealscleral coat (outer), uvea (middle), and the retina (inner) (Snell and Lemp, 1989) (Figure 1). The outer layer consists of the cornea, sclera, and optic nerve. The cornea is the first structure that light encounters as it enters the eye and therefore the cornea must be transparent and convex. The cornea itself is composed of five layers, the corneal epithelium, Bowman's membrane, stroma, Descement's membrane, and corneal endothelium (Oliveira-Soto and Efron, 2001). The corneal endothelium is an important layer of the cornea in that it provides the metabolic exchange between the cornea and aqueous humor, which will be discussed below (Waring et al., 1982). The sclera is the second major constituent of the outer layer. The sclera functions as the attachment site for intrinsic muscles of the eye and is composed of dense connective tissue

(Oyster, 1999). The middle layer or uvea of the eye has very important functions and consists of the choroid, ciliary body, and the iris. The iris functions as the contractile diaphragm anterior to the lens. The pupil is the central aperture in the iris, which changes in size in response to varying light intensities (Loewenfeld and Newsome, 1971). The second member of the middle layer is the choroid. The choroid is a vascular layer that provides nutrients to the retina. The choroid also functions to absorb excess reflected light in order to minimize glare and prevent blurring of vision. The final part of the uvea is the ciliary body. The ciliary body is located in the anterior region of the eye between the iris and the choroid and functions in lens accommodation, secretion of aqueous humor, and anchoring of zonular fibers that form the suspensory ligament of the lens (Escribano and Coca-Prados, 2002). The third layer of the eye is the retina. The retina is a complex structure containing both neural tissue and retinal pigmented epithelial cells (PECs). The retina functions in converting reflected light into chemical and finally electrical signals, which are sent to the brain for processing via the optic nerve (Wassle and Boycott, 1991). Another major component of the eye is the crystallin lens. The lens is a transparent, avascular, biconvex structure that is suspended between the edges of the ciliary body by zonule ligaments (Figure 1). The lens functions in accommodation to bend light rays so that they focus on the retina. The lens is composed of the lens capsule, a thick basal lamina of anterior lens cells; subcapsular epithelium, simple cuboidal epithelium present only on the anterior surface; and lens fibers, highly

elongated, thin, flattened structures without nuclei and other organelles (Figure 2). The fiber cells are filled with crystallins (Duke-Elder, 1958).

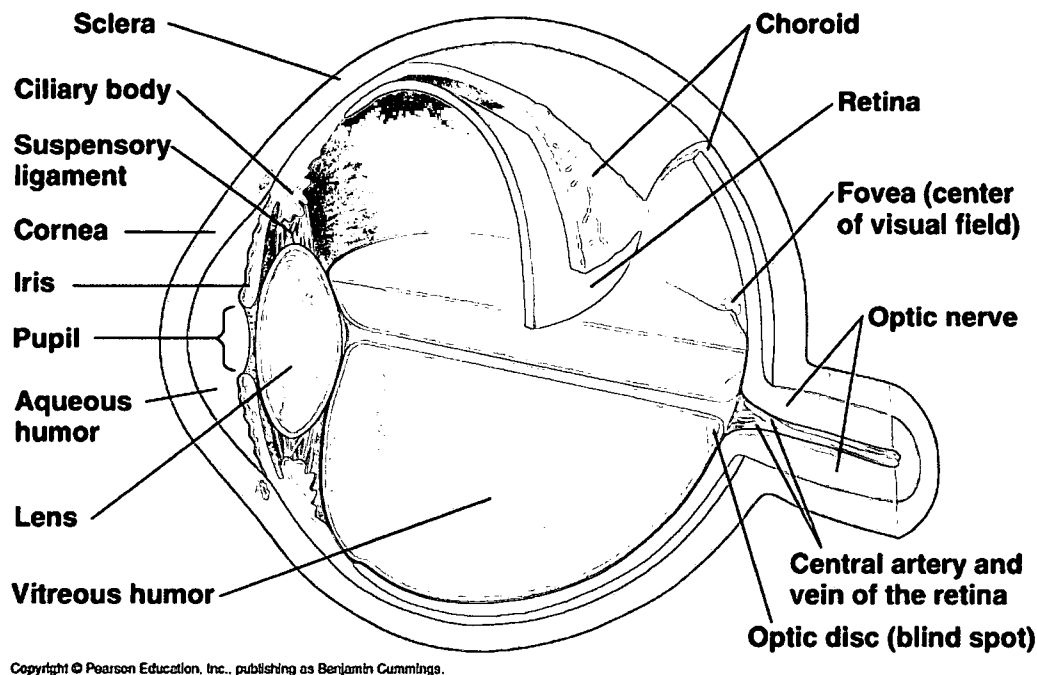


Figure 1. Anatomy of the eye. Diagram depicting the various structures of the human eye. Adapted from Pearson Education, Inc., Benjamin Cummings.

Chambers

The eye is divided into three chambers: anterior chamber, posterior chamber, and vitreous space. The anterior chamber occupies the space between the cornea and iris while the posterior chamber occupies the space between the posterior surface of the iris and the anterior surface of the lens. Both the anterior and posterior chamber are composed of a watery fluid called aqueous humor. The aqueous humor is secreted by the ciliary body and functions in maintaining intraocular pressure, provides the substrates glucose,

oxygen, and electrolytes for metabolic activity of the avascular lens and cornea, and removes metabolic products such as carbon dioxide, pyruvate, and lactate (To et al., 2002). The third chamber is the vitreous space, which occupies the space between the posterior surface of the lens and the neural retina. The vitreous space is composed of the vitreous humor, which occupies about 80% of the volume of the eyeball. The vitreous humor is a clear gel composed of water (99%), collagen fibrils, hyaluronic acid, inorganic salts, sugar, and secreted factors from the retina (Hyatt and Bebee, 1993; Schultz et al., 1993).

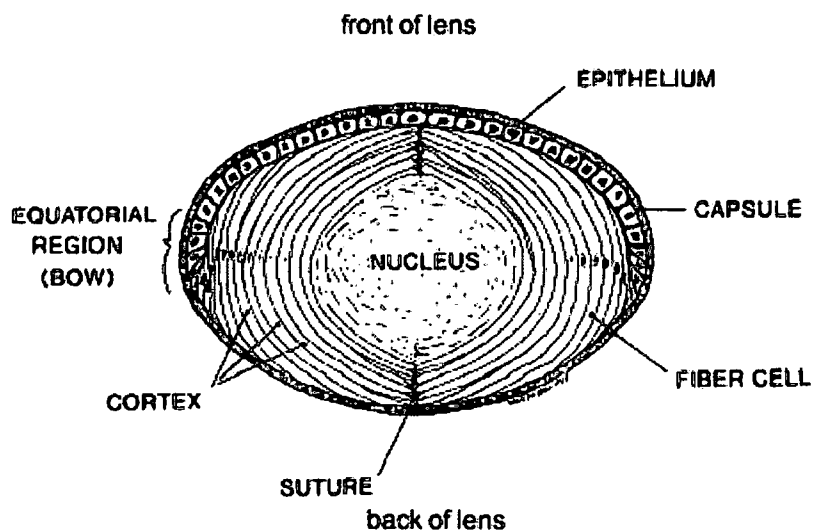


Figure 2. Anatomy of the lens. Diagram depicting the lens epithelial cells, lens fiber cells, and the capsule. Adapted from Kenneth P. Mitton, 2001.

II. Lens Development

The lens develops through a series of inductive interactions and has therefore been a major model system for studying mechanisms of development. The four main processes of lens development include induction, morphogenesis, differentiation, and growth (Grainger et al., 1992; Grainger, 1996; Sullivan et al., 2004).

The lens is derived from the head surface ectoderm in vertebrate embryos. Optic vesicles (outgrowths from the forebrain) are closely associated with the surface ectoderm (Figure 3). Following this close association, lens morphogenesis begins and the ectoderm thickens to form the lens placode. Subsequently the lens placode and the optic vesicle invaginate forming the lens pit and optic cup, respectively (Coulombre, 1965; Jacobson, 1966; Fisher and Grainger, 2004; Lang and McAvoy, 2004). The optic cup differentiates into the retinal pigment epithelium and the neural retina. The lens pit continues to deepen and finally breaks away from the ectoderm forming the lens vesicle and eventually the lens (Reviewed in Lang, 2004).

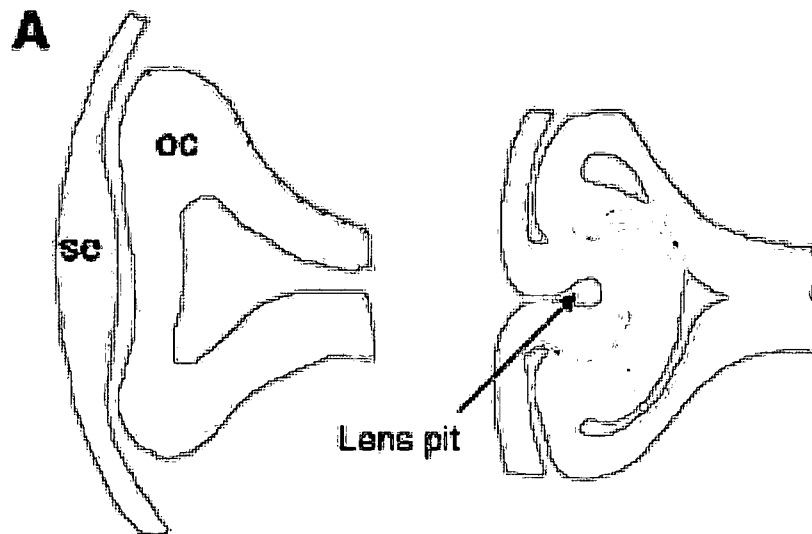


Figure 3. Lens formation during development. Schematic showing lens development, which involves a series of inductive interactions between the surface ectoderm and the optic cup. The lens pit eventually gives rise to the lens. se, surface ectoderm; oc, optic cup.

Over the years, research has investigated the molecules responsible for the induction, morphogenesis, and differentiation processes of lens development. A homeobox gene, *Pax6*, is sufficient and critical for the process of lens development. *Pax6* in the presumptive lens ectoderm makes the ectoderm competent to respond to inductive signals produced by the optic vesicle (Fujiwara et al., 1994). Members of the fibroblast growth factor (FGF) (McAvoy et al., 1991; McAvoy et al., 1999; Lang and McAvoy, 2003) and the bone morphogenetic protein (BMP) (Dudley et al., 1995; Luo et al., 1995; Futura and Hogan, 1998) family of paracrine factors are two such inducers secreted by the optic vesicle. These inducers up-regulate genes such as *L-*

Maf, Sox-2 and Sox-3, which are essential for lens induction as well as crystalline synthesis (Lang, 2004).

Morphogenesis and differentiation of the lens start when the lens vesicle differentiates into two types of lens cells, epithelial and fiber cells. Cells in the anterior differentiate forming the lens epithelium, while the cells in the posterior elongate and differentiate to form primary lens fibers (Blixt et al., 2000). As the lens fiber cells elongate they also make large quantities of crystallins. Crystallins are transparent proteins and are the primary structural component the lens fibers (Boyle and Takemoto, 2000). Primary lens fibers soon lose their nuclei and other cellular organelles and become inert structures (Bassnett and Beebe, 1992). Cells just posterior to the equator (transitional zone) of the lens begin to elongate forming secondary lens fiber cells. As secondary lens fibers continue to be formed, a concentric pattern of lens fibers forms with the primary fibers forming the core or nucleus of the lens. The epithelial cells in the anterior of the lens continue to proliferate in the germinative zone, which is just anterior to the equator. This continual proliferation allows the anterior surface of the lens to be constantly covered by a single layer of epithelial cells even as the lens continues to grow in size (Harding et al., 1971; McAvoy, 1978 a, b). The differentiation of the lens cells establishes a polarity that is maintained throughout the life of the organism. This distinct polarity is maintained primarily by FGF signaling. High concentrations of FGFs are found in the vitreous humor, whereas low concentrations are found in the aqueous humor. The low concentration of

FGFs in the aqueous humor may be due to FGF inhibition by Wnt signaling (Lovicu and McAvoy, 2005). High concentrations of FGFs activate p57kip2, a cell cycle inhibitor. This results in the exit from the cell cycle, migration, and elongation into fiber cells. Low FGF signaling allows for proliferation and the maintenance of the epithelial layer (Lovicu and McAvoy, 2005).

As demonstrated above the development of the lens is an intricate process involving many inductive interactions and signaling factors. Complications at any stage of the developmental process can lead to eye abnormalities such as cataracts. The natural aging process also leads to the formation of cataracts and is the leading cause of visual loss among the elderly.

III. Cataracts

A cataract is the clouding of the eye's natural lens (Chylack et al., 1993). The lens is comprised of mainly water and proteins and functions to focus light onto the retina. The proteins are arranged in a precise way that keeps the lens clear and allows light to pass through it. As we age many individuals will develop age-related cataracts, in fact cataracts are the leading cause of visual loss among adults over the age of 55. In the United States, 50% of those between the ages of 65 and 74, and 70% of those older than 75, will have a cataract. This will result in an estimated 1.5 million cataract surgeries performed yearly in the United States (Hodge et al., 1995).

Cataracts are generally classified into one of three categories, nuclear, cortical, and subcapsular (Chylack Jr. et al., 1993). Nuclear cataracts are the most common. They form within the nucleus and are typically due to the aging process, specifically the clumping of protein. When proteins in the lens clump, it clouds the lens and reduces the light that is able to reach the retina (Costello et al., 1992). The second most common type of cataract is the cortical cataract. This type of cataract is often associated with diabetes. As the name suggests, this cataract is formed within the cortex (periphery) of the lens and is due to disruption in the order of fiber cells. Gaps created due to this disorganization fill with water and debris resulting in light scattering (Leske et al., 1991). The final and least prevalent form of cataract is the subcapsular cataract. This type of cataract forms at the back of the lens and is seen predominantly in individuals with diabetes, retinitis pigmentosa, or those taking high doses of steroids (Hiller et al., 1986).

Cataract removal is one of the most common operations performed in the United States (Michelson, 2005). The two types of cataract surgery performed are phacoemulsification and extracapsular surgery. The former method is quickly becoming the most prevalent method of cataract surgery in the United States, as it has a quicker recovery time and fewer post-operative complications. Briefly, in phacoemulsification a small incision is made on the side of the cornea. A probe that emits ultrasound waves is inserted into the eye in order to soften and break up the lens. The pieces of the lens are then removed by suction. During extracapsular surgery, a longer incision is made

across the cornea through which the cloudy core of the lens is removed in one piece. The remainder of the lens is then removed by suction. In both types of cataract surgery, the capsular bag must be left in place in order to hold the intraocular lens (synthetic) in the appropriate position for vision to occur. Despite recent advances in surgical technique, a major problem with cataract surgery still exists, namely that lens epithelial cells (LECs) remain on the anterior capsule following removal of the lens. These cells proliferate, migrate and eventually accumulate between the capsule and the prosthetic lens, where they form fibrotic plaques (Wormstone, 2002). This transformation is often referred to as epithelial to mesenchymal transition (EMT).

EMT creates posterior capsule opacification (PCO) or secondary cataracts, which is the most common complication of cataract surgery. PCO develops in a significant proportion of patients to such an extent that a secondary loss of vision occurs which requires corrective laser surgery to fix (Schaumburg et al., 1998). The effects of PCO are not noticed directly following surgery. It takes several years for the opacification to block vision. The incidence of PCO is 41% four years after surgery. Studies have shown that there is an age-related factor in determining the rate of PCO (Tetz and Nimsgern, 1999). In patients greater than 60 years of age, the incidence of PCO is 37%; however, the incidence increases to 70% in patients under the age of 40. Children have the most rapid rate of PCO development (Knight-Nanan et al., 1996).

IV. Epithelial to Mesenchymal Transition

Transforming Growth Factor β

Both *in vitro* and *in vivo* models have been used to study the factors regulating the process of EMT (Liu et al., 1994; Hales et al., 1999). The regulators are expressed in the lens cells and other ocular tissues. These regulators are involved in autocrine and paracrine signaling. One of the major paracrine factors involved in both PCO and EMT are members of the TGF β superfamily (Saika et al., 2001). The lens expresses both TGF β 1 and TGF β 2. TGF β s are also abundant in the ocular media (Gordon-Thomason et al., 1998). Under normal conditions members of the TGF β superfamily are tightly regulated in the eye. Inhibitors of TGF β are found in both the vitreous humor, α_2 -macroglobulin, (Schultz et al., 1996) and the lens epithelium, serine proteases HtrA1 and HtrA3, (Tocharus et al., 2004). Receptors for other members of the TGF β superfamily such as activins and BMPs are also expressed in the lens (Obata et al., 1999; de longh et al., 2001, 2004). TGF β is thought to promote normal lens fiber differentiation, but its effect on lens epithelial cells is quite different (de longh et al., 2001, 2004). Studies have shown that TGF β can promote an aberrant differentiation pathway (e.g. EMT), which can lead to the formation of cataracts (de longh et al., 2005).

Liu et al. (1994) were the first to show the effects of TGF β on LECs *in vitro*. Treatment of LECs with TGF β 1 induced the cells to adopt an abnormal spindle shape and produce aberrant extracellular matrix (ECM). Other

studies utilizing explants have shown that TGF β induces localized wrinkling (Liu et al, 1994; Hales et al., 1995) and induction of alpha-smooth muscle actin (α -SMA), a marker for the epithelial to mesenchymal transition. *In vitro* studies using whole lens cultures showed that TGF β was able to induce lens opacities, which corresponded to localized subcapsular plaques of spindle shaped cells (Hales et al., 1995). These TGF β -induced plaques accumulated ECM proteins (collagens type I and III, tenascin, and fibronectin), which are not normally expressed in the lens. In addition to ECM proteins, the plaques also expressed abnormal intermediate filament proteins, desmin, and α -SMA as well as heparan sulfate proteoglycans and laminin all of which are not normally present in the lens (Hales et al., 1995; Lovicu et al., 2002).

In vivo studies have also lent support to the fact that TGF β is a major contributor to EMT and the formation of lens opacities. Hales et al. (1999) injected TGF β into the vitreous chamber of rat eyes and showed that the LECs at the equator migrated abnormally and formed subcapsular plaques. These plaques were comprised of spindle-shaped cells that expressed α -SMA. Transgenic mice, which over-express constitutively active TGF β 1 have anterior subcapsular plaques that are positive for α -SMA (Srinivasan et al., 1998). These transgenic mice also show marked down-regulation of epithelial markers such as Pax-6, E-cadherin, and α -crystallin (Lovicu et al., 2004 a,b). Other *in vivo* studies have shown that a penetrating injury to the mouse lens results in the stimulation of the TGF β signaling pathway as indicated by nuclear translocation of smad3 and smad4 (Saika et al., 2001).

Following injury, the LECs undergo an epithelial to mesenchymal transition and express markers such as α -SMA, lumican collagens type I and III, and the transcription factor snail (Saika et al., 2001, 2003, 2004). Snail is a transcriptional repressor that has been shown to regulate the EMT (Oloumi et al., 2004).

The morphological and molecular features of the TGF β -induced plaques are similar to those found in human anterior subcapsular cataracts (ACS) (Lovicu et al., 2004b) and PCO (Wormstone, 2002). Human ASC is characterized by plaques of fibrotic cells underneath the anterior capsule, which result in light scattering.

While TGF β appears to be a key player in the EMT, it is not the only factor needed for this transition. Research has shown that mammalian LECs in culture can form multilayered cellular aggregates even in the absence of TGF β , indicating that there are other factors involved in PCO (Schulz et al., 1996). In addition, TGF β induces lens cells to undergo apoptosis; however, a key feature of PCO is the prolonged survival of the TGF β -induced cells making it unlikely that PCO development is the result of TGF β alone (Maruno et al., 2002).

Fibroblast Growth Factor

FGFs are another set of signaling molecules that are important not only during lens development but also during PCO. Using cultured human

lens capsular bags, Wormstone et al. (2001) discovered the presence of FGF and its receptor FGFR-1 via RT-PCR. To test the functional significance of FGF signaling in the formation of PCO, the pathway was blocked by using a specific inhibitor, SU5402, to FGFR-1. The inhibition of FGFR-1 caused a retardation of growth.

Tanaka et al. (2004) examined the role of endogenous FGF2 in injury-induced proliferation of LECs. Following an injury to the lens, the level of FGF2 increased 10 fold when compared to an uninjured lens. In addition to the marked accumulation of FGF2 following injury, FGF2 was also found to increase proliferation of mouse LECs *in vitro*. To further elucidate the role of FGF2 in EMT, knockout mice were created. These mice showed a decrease in injury-induced proliferation, but did not show any effect on the EMT as shown by the expression of α -SMA (Tanaka et al., 2004). A study by Mansfield and co-workers (2004) also examined the role of FGF2 in the formation of PCO. Using rat lens epithelial explants treated with TGF β 2 and FGF2, it was found that FGF2 was able to prolong survival of TGF β -treated cells. In addition, the FGF2 treated cells no longer expressed Pax6, a lens epithelial marker, but did express non-lens markers such as α -SMA, collagen type I, and fibronectin, molecules that are all associated with PCO (Mansfield et al., 2004).

Extracellular Matrix Proteins

The lens capsule plays a significant role in the regulation of PCO. It is composed primarily of laminin and collagen type IV; however, during the process of PCO many additional ECM proteins such as collagen type I are up-regulated (Wunderlich et al., 2000). Migration of LECs is a significant step in the formation of PCO. The presence of ECM proteins on the capsular bag enhances the migration of LECs to the posterior. Integrins link LECs to the underlying matrix. Anterior capsular discs removed from patients at the time of cataract surgery show expression of $\alpha 2$, $\alpha 3$, $\alpha 5$, $\beta 1$, and $\beta 2$ integrins (Nishi et al., 1997; Zhang et al., 2000). Nishi and colleagues (1997) showed a decreased number of viable LECs when these cells were cultured on collagen or laminin dishes following treatment with a monoclonal antibody to $\beta 1$ integrin.

Matrix metalloproteinases (MMPs) are another family of proteins that regulate the extracellular matrix during PCO (Tamiya et al., 2000; Duncan et al., 2001). MMPs are enzymes which are involved in physiological processes such as morphogenesis. The most common MMPs found in the eye are the gelatinases, which are found in both the aqueous and vitreous chambers (Vaughan-Thomas et al, 2000). To date, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have not been detected in native human lenses (Smine and Plantner, 1997); however, high levels of these MMPs have been detected via gelatin zymography following a sham cataract operation (Tamiya et al., 2000). Additional work by Duncan et al. (2001) confirmed the expression of MMP-2

and MMP-9 during PCO by showing the TGF β 2 could sustain elevated levels of these proteins in human capsular bags.

Lumican, a small leucine repeat proteoglycan (SLRP), is present in the ECM (Ying et al., 1997). In addition to being a member of the ECM, lumican also plays a role in wound healing and neoplasm based on its ability to modulate cellular behavior such as proliferation and migration (Hardingham and Fosang, 1992; Iozzo and Murdoch, 1996). Anterior subcapsular cataract and PCO in humans are positive for lumican expression (Saika et al., 2003). Based on this evidence, Saika and colleagues (2003) utilized lumican knockout mice to examine its role in EMT. Loss of lumican was found to perturb EMT of mouse LECs (Saika et al., 2003), further implicating the role of ECM in the formation of posterior capsular opacification.

V. Lens Regeneration

Mammals are not typically thought of as being proficient at regeneration. Amphibians on the other hand are quite adept at regeneration (Stocum, 2004). Amphibians are capable of regenerating not only their lenses, but also their tails, retinas, limbs, and muscle. Colucci (1891) and Wolf (1895) were the first to document in detail the phenomenon of lens regeneration in amphibians, which is sometimes referred to as Wolffian regeneration. In newts, the lens regenerates through a process known as transdifferentiation (Eguchi, 1963). In this process, the dorsal iris PECs dedifferentiate, proliferate, and finally differentiate into a completely new cell

type, the lens cells (Eguchi, 1963; Tsonis, 2000; Tsonis and del Rio-Tsonis, 2004). *Xenopus laevis* is another amphibian capable of lens regeneration via the process of transdifferentiation, but unlike the newt, *Xenopus* regenerates via transdifferentiation of the inner layer of the outer cornea and not the dorsal iris PECs (Freeman, 1963; Filoni et al., 1997). Over the years, researchers have extensively examined the role of lens regeneration in amphibians; however, not much has been studied in mammals.

Mammalian lens regeneration was first documented in New Zealand albino rabbits and to a limited extent in dogs in 1825 (Cocteau and D'Etoille, 1827). In these very early experiments, an incision was made in the capsule and the lens was removed with a curette. Six months following surgery, lenses similar in consistency and volume were extracted. In the 19th century, researchers identified the conditions necessary for mammals to successfully regenerate a lens to such an extent that it resembled the normal lens. It was discovered that the lens capsule as well as LECs had to be present in order for regeneration to take place. If the lens capsule along with the lens were removed, no regeneration takes place (Randolph, 1900). Several other conditions that need to be met in order for regeneration to take place in rabbits are an uninjured posterior capsule, a relatively intact anterior capsule, and no adherence between the capsules (Mayer, 1832; Middlemore, 1832; Milliot, 1872).

These early experiments also demonstrated that the form of the capsule dictated the form of the regenerated lens (Randolph, 1900). More

recent research has shown that this is indeed the case. Utilizing a rabbit model system, Gwon et al. (1993) found that by placing a collagen patch over the capsule followed by filling the capsular bag with air or Healon resulted in rapid lens regeneration with 100% of the capsular bag being filled by the lens. It is thought that the air prevents the anterior and posterior capsules from adhering to one another thereby allowing for an uninhibited regenerative process to take place (Gwon et al., 1993).

Following these early observations of mammalian lens regeneration, other conditions were shown to be important in the regenerative process. One of these conditions was the age of the animal. The extent of regeneration was shown to occur more quickly and to a fuller extent in younger animals (Gwon et al., 1992). The ability of younger animals to regenerate a lens more quickly is also supported by clinical observations, where younger patients develop PCO more frequently and sooner than older patients (Knight-Nanan et al., 1996). Another condition that has been suggested to aid in the regenerative process is the inflammatory response. Studies indicate that severe inflammation can prevent lens regeneration, while mild inflammation enhances the growth of the lens (Middlemore, 1832; Stewart, 1962). This suggests that there may be an inflammatory molecule, which is capable of mediating the lens regeneration response.

Mechanism of Regeneration

Regeneration of the mammalian lens is thought to proceed in a similar manner to that of embryological lens development. In rabbits, Gwon et al. (1990) observed LECs migrating and proliferating along the posterior and anterior capsule one week following lens removal. Approximately 2-4 weeks following surgery, the posterior LECs begin to elongate and by 1 month the anterior cells begin to migrate. Cell differentiation was only seen in the equatorial region 2 months post operation, as is the case in the adult rabbit lens (Gwon et al., 1990). To date, all of the lenses regenerated from mammals have some extent of irregularity in the patterning of lens fibers (Gwon et al., 1999; reviewed in Gwon, 2006). As stated earlier, proper patterning of fiber cells is crucial to maintain the function of the lens, which is to allow for light refraction as a result of its transparent nature. Transparency is achieved through the spacing and alignment of fiber cells during development and growth. Since the rate of proliferation and differentiation is not consistent throughout the capsular bag, the early fiber cells are misaligned and improperly spaced. This can lead to impaired refractive capabilities and hence a decrease in optical clarity (Mayer, 1832; Milliot, 1872; Gwon et al., 1999).

VI. Statement of Problem

While many advances have been made in determining the mechanism underlying mammalian lens regeneration, there is still a significant amount of work that needs to be done. Most of the information regarding mammalian lens regeneration has come from research utilizing rabbits. However, the rabbit is a poor model system in that there are few molecular and genetic tools available for the study of lens regeneration in this organism. The initial objective of this research was to examine the potential of mice to undergo lens regeneration focusing on lens differentiation and EMT. We hypothesized that, like other mammalian organisms, the mouse would be able to undergo lens regeneration if the capsule was left in place following removal of the lens. In addition, we hypothesized that the presence of an EMT would be significantly different than that seen during the formation of PCO in cataract patients. This research is significant because the availability of genetic and molecular tools for the mouse will allow this organism to be an excellent model system in which to study cataracts and the formation of secondary cataracts as a result of EMT.

Chapter II examines the regenerative potential in mice and the presence of EMT. In order to study these processes in the mouse, we utilized immunohistochemical analysis at various stages of lens regeneration. Specifically we examined crystallin (lens fibers), collagen type IV (capsule), and α -SMA (marker for EMT). Chapter III discusses the use of microarray

technology in order to examine the global expression of genes involved in lens differentiation and EMT as seen during mouse lens regeneration.

The appendix contains published papers, which discuss the ability of the newt to undergo both limb and lens regeneration. These papers deal with regeneration through a process of transdifferentiation, which is different than the differentiation of lens cells seen during mouse lens regeneration. These papers will give the reader some insight into transdifferentiation and possible applications for lens regeneration therapy in mammalian organisms.

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CHAPTER II

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 lentiectomy. The lens is regenerated by the remaining adherent lens epithelial cells, which differentiate to form lens fibers within days. The regenerated lens is of good size and morphology. Epithelial to mesenchymal cell transformation is also seen during the early stages. The mouse, therefore, can become an indispensable animal model for caratact research, surgery and therapy.

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 fibers 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 (87mg/kg) in combination with xylazine (13mg/kg). 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 as seen in adult salamanders. Contrary to what was previously assumed, intraperitoneal retinol palmitate injections (50 IU/mouse in 50 μ 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 fibers 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 (Figure 1a-c). Differentiation of lens fibers 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) (Figure 1a, 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 (Figure 1g-i). This argues that in the beginning there is 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.

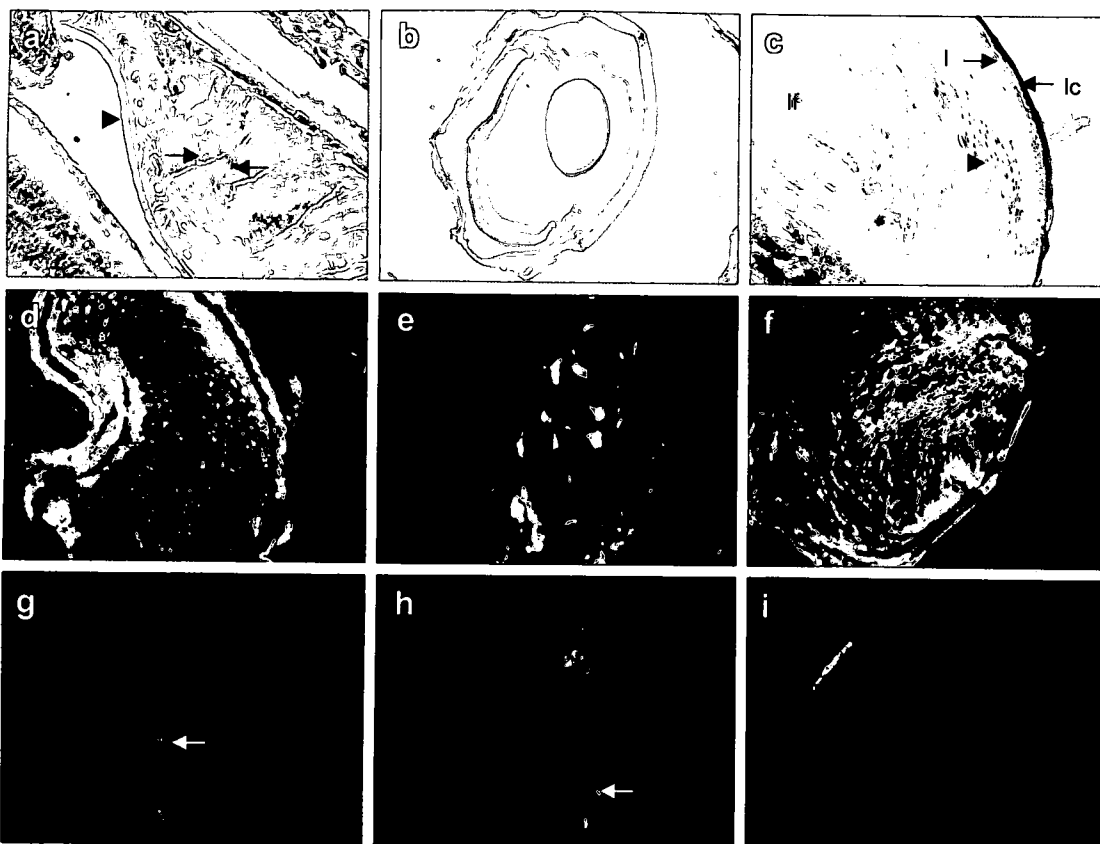
While several reasons, such as the type of operation (incision of anterior capsule) or age of animals come to mind to explain these positive results, these findings have two major implications. First, they demonstrate that mammals might possess much stronger potential for lens repair than originally 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.

Table 1. Lens regeneration in different mouse strains
(numbers represent lens/eye)

	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

Figure 1. Immunohistochemistry of the Regenerating Mouse Lens

a: A histological section through a regenerating lens 2 days post-lentectomy. Note the differentiation of lens fibers (arrow) at the bow region. The posterior capsule is also shown intact (arrowhead). b: A regenerated lens 30 days post-lentectomy. Note the normal morphology and size of the regenerated lens. c: A close-up at the bow region of a regenerated lens 30 days after lentectomy. Note the normal morphology and differentiation of lens fibers. d, e, f: Representative sections of regenerating lenses 5, 10 and 20 days post-lentectomy respectively. Expression of crystallin (green) and type IV collagen (red) depicting lens fiber differentiation and the lens capsule respectively. g, h, 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). Note that some α -actin positive cells 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).



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Chapter III

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 μ g 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 μ g of amplified RNA (aRNA) with the incorporated amino allyl nucleotides starting from 1 μ g of total RNA (10 μ g 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 μ M 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. Trombospondin-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 Flj10680	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			
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	Grn	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	Mglap	matrix gamma-carboxyglutamate (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	Transferring
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	Tyropb	TYRO protein tyrosine kinase binding protein
U42327	Vcam1	vascular cell adhesion molecule 1
AF175282	Adamts8	a disintegrin-like and metalloprotease (repolysin type) with thrombospondin type 1 motif, 8
D13664	Postn	periostin, osteoblast specific factor
AF350047	Rgs3	regulator of G-protein signaling 3
BC023008	Clecsf10	C-type (calcium dependent, carbohydrate recognition domain) lectin, superfamily member 10
BC003482	Tm4sf7	transmembrane 4 superfamily member 7
AY082484	Ifitm3	interferon induced transmembrane protein 3
BC019764	Ube1dc1	ubiquitin-activating enzyme E1-domain containing 1
AK007397	Adh6a	alcohol dehydrogenase 6A (class V)
BC027425	Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B
AK010084	2310067E08Rik	RIKEN cDNA 2310067E08 gene
BC013494	Cyp4f18	cytochrome P450, family 4, subfamily f, polypeptide 18
AK008652	Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C
BC016551	Msr2	macrophage scavenger receptor 2
AF213458	Trem2	triggering receptor expressed on myeloid cells 2
BC026375	Gpnmb	glycoprotein (transmembrane) nmb
AJ308965	Psip1	PC4 and SFRS1 interacting protein 1
AF398968	Asb7	ankyrin repeat and SOCS box-containing protein 7
AK033038	Oosp1	oocyte secreted protein 1
AF290914	Stab1	stabilin 1

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	lap	intracisternal A particles
M60523	ldb3	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	Zfx1b	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	Ccn1	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	Mtmr2	myotubularin related protein 2
BC002262		
AF130313	Nckipsd	NCK interacting protein with SH3 domain
BC021457	SImap	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	Dph2l1	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	formin
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	Olf61	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	D15ErtD735e	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	Serpinb6c	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	vomer nasal 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	Olfr684	olfactory receptor 684
AK033012	Npat	nuclear protein in the AT region
AF357887	Dusp15	dual specificity phosphatase-like 15
AY073826	Olfr747	olfactory receptor 747
AY073750	Olfr1265	olfactory receptor 1265
AY073749	Olfr1495	olfactory receptor 1495
AY073644	Olfr1231	olfactory receptor 1231
AY073624	Olfr1261	olfactory receptor 1261
AY073578	Olfr530	olfactory receptor 530
AY073509	Olfr1030	olfactory receptor 1030
AY073501	Olfr703	olfactory receptor 703
AY073438	Olfr1131	olfactory receptor 1131
AY073304	Olfr1238	olfactory receptor 1238
AY073177	Olfr1377	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	Spr2g	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	LctI	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.

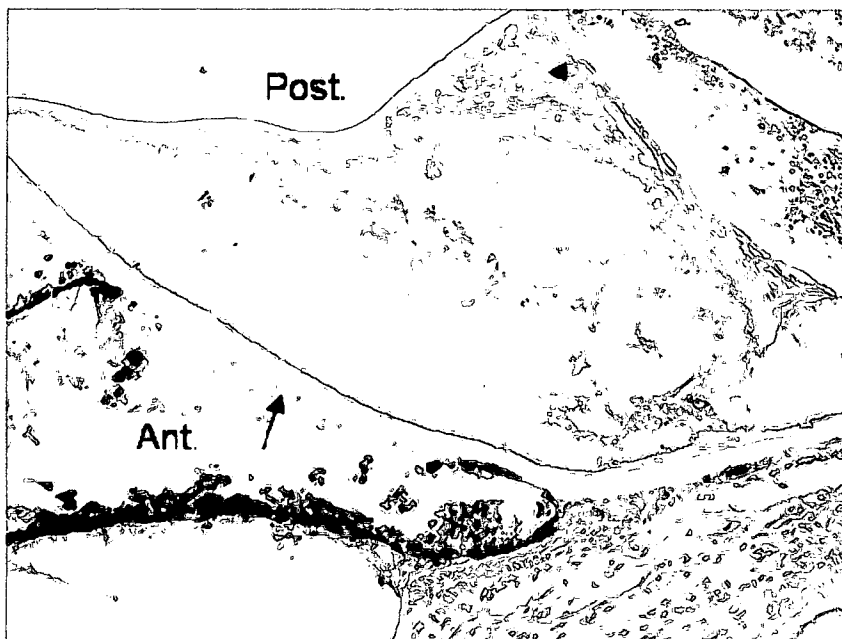


Fig. 2. The experimental design for the microarray studies. The indicated time periods of 1, 2, and 3 represent weeks post-lentectomy from time 0 (Control). Each mouse represents a biological replicate for a given experimental condition. An arrow denotes a microarray slide comparison between a given 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].

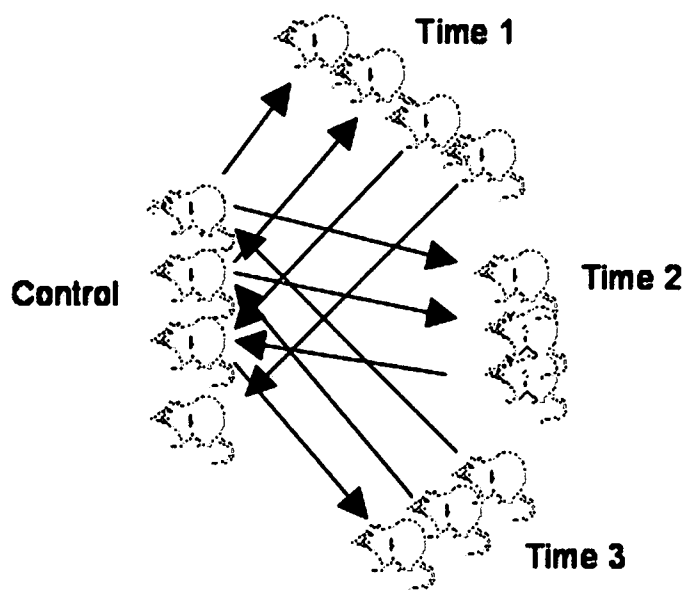


Figure. 3. Heat map from cluster analysis. Cluster analysis of 2,094 differentially expressed genes. The hierarchical clustering of all genes with each line representing expression levels for a gene as compared to control and each column a time point (1, 2 and 3 weeks post-lentectomy). Shades of red 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.

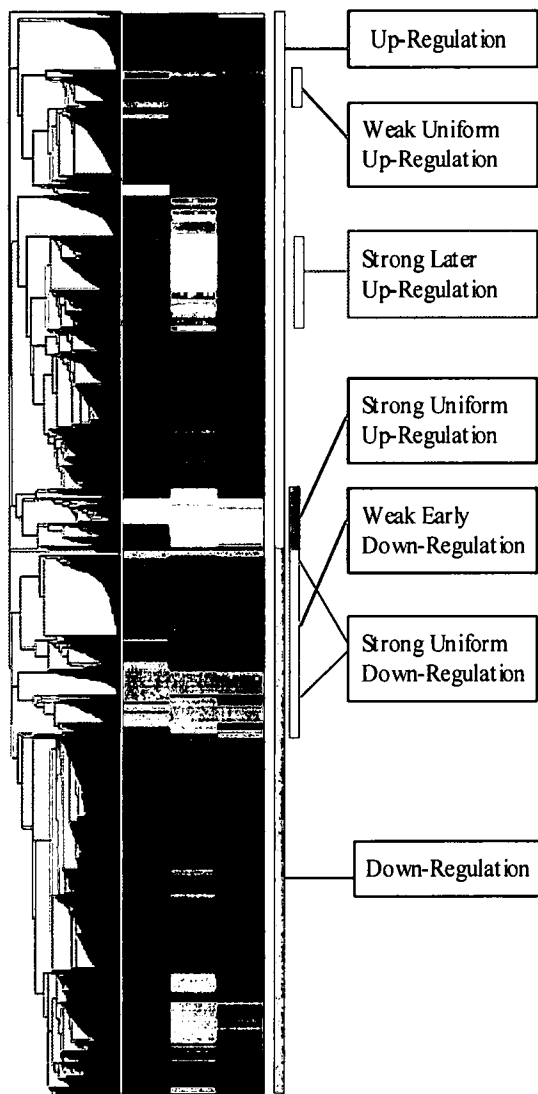
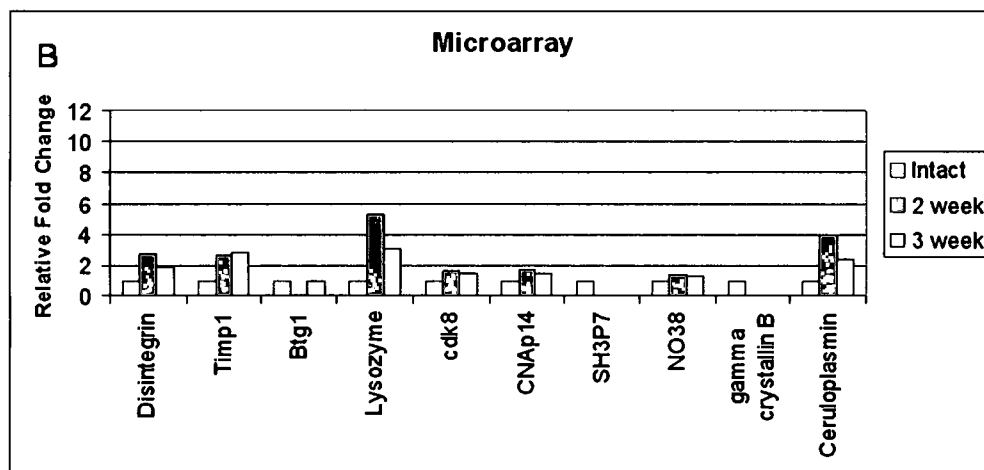
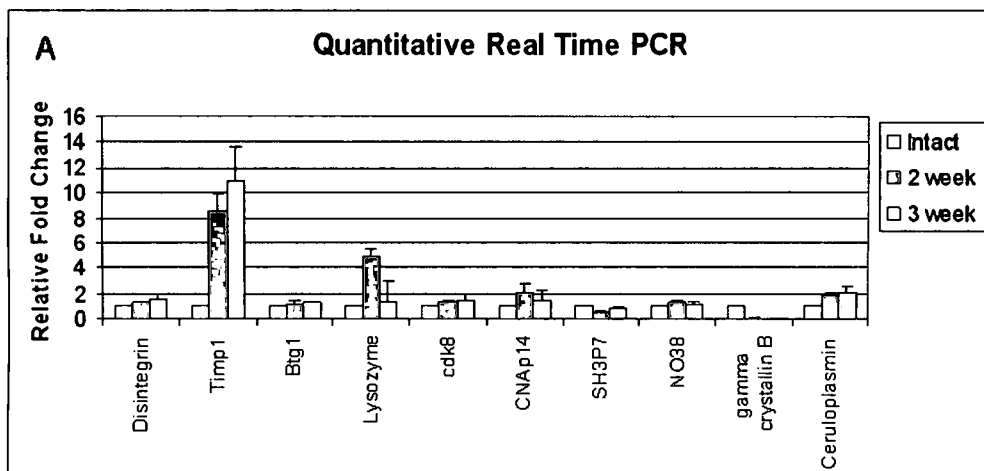


Figure 4. Microarray validation via QPCR. QPCR analysis (A) of ten selected genes and the corresponding data from the microarray analysis (B). The expression values of lens mRNA at 2 and 3 weeks post-surgery are compared with lens mRNA from the non-operated eye (value set at 1). C: the values between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

Conclusions

We have shown through immunohistochemical staining and microarray analysis that the mouse makes an excellent model system in which to study mammalian lens regeneration and the epithelial to mesenchymal transition associated with posterior capsule opacification.

We have shown that during the early stages of lens regeneration, the eye undergoes a typical wound healing response. In this response lens epithelial cells left behind on the capsule migrate to the posterior and transition into mesenchymal cells. Interestingly, this transition ceases at later stages of regeneration, whereby the eye switches from a wound healing response to a lens differentiation response. This feature of the mouse model makes it an extremely useful system in which to study posterior capsule opacification. Posterior capsule opacification or secondary cataract is the leading complication seen following cataract surgery. The secondary cataract is a result of the migration and differentiation of lens epithelial cells to mesenchymal cells, which results in the formation of a fibrotic plaque.

In addition to establishing the mouse model of regeneration, we have started the initial stages of identifying genes involved in both lens fiber differentiation and the epithelial to mesenchymal transition seen during mammalian lens regeneration. These initial studies have identified a large

number of genes that are differentially expressed during various stages of lens regeneration. These include genes involved in repair, inflammation, lens differentiation, and reorganization of the cytoskeleton and extracellular matrix.

With the data obtained from these initial studies and the genetic and molecular tools available for the mouse, research can be done to study factors that may reverse or inhibit the epithelial to mesenchymal transition that can ultimately lead to a secondary cataract. As mentioned earlier secondary cataracts affect approximately 40% of the patients who have had a successful cataract surgery. In order to remove the secondary cataract, another surgery utilizing the Yttrium Aluminum Garnet (YAG) laser system must be employed. This treatment not only runs the risk of creating other eye injuries such as retinal detachment, but also costs a significant amount of money. The financial burden is quite significant and many individuals in under-developed countries simply cannot afford to undergo laser surgery to remove the secondary cataract. Unfortunately, these individuals will again lose their vision despite the fact that they had a successful cataract surgery. In order to eliminate secondary cataracts, a way in which to prevent the EMT is imperative.

The data obtained in these studies make apparent two possible mechanisms in which to prevent an EMT and secondary cataract formation. The first makes use of the global pattern of gene expression seen at various stages of lens regeneration. At approximately 3 weeks post lentectomy, the mouse switches from an EMT response to a lens differentiation response.

Utilizing the microarray data we can now try to identify genes that are differentially expressed during the switch from EMT to lens differentiation. These genes can subsequently be studied to see if their removal or addition can inhibit or reverse the progression of secondary cataracts. Use of knock-out mice will be a valuable tool in the discovery of such factors. Microarray analysis performed on mice lacking genes thought to be involved in the EMT such as lumican or smad3 can be used to filter out the most important genes (from the original 2100) involved in the reversal of EMT.

The second mechanism for preventing secondary cataracts is to study the lens regeneration potential in humans. All mammals studied thus far seem to have the potential for lens regeneration if the capsular bag is left behind following lentectomy. If human lens regeneration is possible, perhaps a similar reversal of phenotypes will be seen as was the case for the mouse. This phenotype switch would not allow for the formation of PCO because the cells will have diverted into a lens differentiation response. For this type of regeneration therapy to be successful, work must be done to ensure that the regenerated lens forms normally and functions accurately as visual acuity is of the utmost importance.

In addition to studying lens regeneration in mammals through the mechanism of differentiation, we can also consider lens regeneration therapy via a process of transdifferentiation as seen in the newt. The newt regenerates its lens when PECs from the dorsal iris transdifferentiate into lens cells (Appendix). This is different than the mouse because the cells that form

the regenerated lens in mice are lens cells and therefore do not involve the conversion of one cell type into another. Recent work has shown that the ventral iris of the newt, which never regenerates a lens, can be induced to transdifferentiate into a lens by inhibiting the bone morphogenetic protein pathway or by treatment with *six-3* and retinoic acid (Appendix). In addition, *in vitro* studies have shown that PECs taken from the newt ventral iris, mouse iris, or human iris can form a lens like structure (lentoid). This suggests that PECs from iris tissue have the potential to regenerate a lens but *in vivo* this capability is lost. The treatment used to induce lens regeneration from the newt ventral iris can now be applied to other organisms with the hope that the newt ventral iris behaves similarly to mammalian iris tissue, as is suggested from the *in vitro* studies. Not only will this help in eliminating cataracts and PCO but it will also aid individuals that have significant damage to their lens and capsular bag.

This initial research has created many avenues by which to study cataracts, EMT, and the potential for lens regeneration therapy. Future research utilizing this mouse model of lens regeneration has the potential to significantly impact medicine and human health, by drastically reducing the need for secondary cataract surgery.

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Expression and role of retinoic acid receptor alpha in lens regeneration

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The role of retinoids in eye development has been well studied. Retinoids and their receptors regulate gene expression and morphogenesis of the eye. In this study, a highly specific antagonist of retinoic acid receptor (RAR)- α was used in an attempt to study its function in lens regeneration. It was found that this antagonist inhibited lens regeneration and lens fiber differentiation. It was also shown that RAR- α is expressed in the lens during the process of regeneration. These results indicate that different RAR might have unique as well as redundant effects and patterns of expression in the regenerating lens.

Key words: lens, regeneration, retinoic acid receptor alpha.

Introduction

The process of lens regeneration in adults occurs in only a few species of salamanders. Upon removal of the lens, these animals regenerate a complete, new lens through the transdifferentiation of the pigment epithelial cells (PEC) from the dorsal iris. Such an event might hold clues for the process of differentiation and genetic reprogramming, as well as to why other animals are not able to regenerate the lens (Tsonis *et al.* 2000, Tsonis 2000a, 2000b).

In the past few years, research into the molecular biology of this phenomenon has revealed the participation of several genes, but the mechanism that could be applied in other animals is still elusive (Del Rio-Tsonis *et al.* 1995, 1997, 1998, 1999). Our previous investigation has led us to believe that retinoic acid and its receptors might be involved in lens regeneration (Tsonis *et al.* 2000). While we found that exogenous retinoids did not affect the process of lens regeneration, inhibition of retinoid production or inhibition of their receptors showed some interesting results. Specifically, we were able to show that lens regeneration was affected by an antagonist to retinoic acid receptors (RAR). In most cases, lens regeneration was inhibited or retarded by the antagonist, but in a few spectacular cases the lens was regenerated

from sites other than the normal dorsal site. We continued this research to more clearly delineate the components of such an effect. Since knockout experiments are impossible in newts, inhibiting the action of proteins by specific antagonists is an excellent method for our purpose. In a previous study we had used an antagonist that could inhibit the action of all RAR. In this study, we used an antagonist specific to RAR- α to determine the specific roles of RAR- α in the process of lens regeneration. We report here that RAR- α is expressed in the eye tissues of the newt and that its specific inhibition affects lens regeneration.

Materials and Methods

Animal operations

Newts (*Notophthalmus viridescens*) were lentectomized under general anesthesia by using 1% 3-aminobenzoic acid ethyl ester (Sigma, St Louis, MO, USA). Eyes were collected at different stages of lens regeneration for either histological staining or for expression studies.

Treatment with the retinoic acid receptor alpha antagonist

In our previous study we had established that the method of administration that produced the best results for inhibition or ectopic lens regeneration was to absorb the antagonist (20 mg/mL) on AGI-X2 formate beads (100–200 mesh; Bio-Rad, Hercules,

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CA, USA). Such treatment resulted in inhibition of lens regeneration from the dorsal iris and in ectopic lens regeneration from other sites. We therefore have selected this method and concentration of administration to examine and compare the effects of a specific antagonist to RAR- α . The beads were soaked in the solution and one of them was inserted in the eye cavity immediately upon lentiectomy. The antagonist, AGN 194301 (Allergan, Irvine, CA, USA) was dissolved in dimethylsulfoxide (DMSO). Control animals received a bead soaked in DMSO only. AGN 194301 is a specific antagonist to RAR- α . This compound is particularly effective in inhibiting retinoic acid-induced gene transcription at RAR- α (Teng *et al.* 1997).

In situ hybridization

Expression of RAR- α during lens regeneration was studied by *in situ* hybridization as described in detail in previous publications (Del Rio-Tsonis *et al.* 1997, 1998). The newt RAR- α probe was a gift from Dr J. P. Brockes (University College London, UK; Ragsdale *et al.* 1992).

Results and Discussion

Lens regeneration in the adult newt begins with proliferation and dedifferentiation of dorsal iris PEC. By dedifferentiation we mean the loss of characteristics that define PEC, such as pigmentation.

Dedifferentiation initiates molecular events, such as re-entering the cell cycle, which are necessary for cell proliferation and the subsequent regeneration of the lens. At approximately 10 days post-lentiectomy, a lens vesicle is formed from the depigmented dorsal PEC. Approximately 12–16 days post-lentiectomy, the internal layer of the lens vesicle thickens and synthesis of crystallins begins. This marks the beginning of primary lens fiber differentiation. During days 15–19, proliferation and depigmentation of PEC slows down. In the internal layer, the lens fiber complex is formed, and in the margin of the external layers, non-dividing secondary lens fibers appear. By 18–20 days, the PEC have stopped proliferating and the lens fibers continue to accumulate crystallins. Lens regeneration is considered complete by day 25–30 (Tsonis 1999, 2000a, 2000b). Lens regeneration therefore is possible by transdifferentiation, which is the transformation of one cell type to another (in this case PEC to lens cells). The processes of dedifferentiation and transdifferentiation have been proven beyond any doubt in lens regeneration. These processes can also be observed when single PEC cells are placed in culture (Eguchi *et al.* 1974; Tsonis *et al.* 2001). As the PEC proliferate, they become depigmented and then transdifferentiate to lens cells. Therefore, while in many other regenerative tissues stem cells may play a role, such a possibility is very unlikely in lens regeneration.

Histological examination of the eyes 20 days after lentiectomy indicated that in 30% of cases (seven out

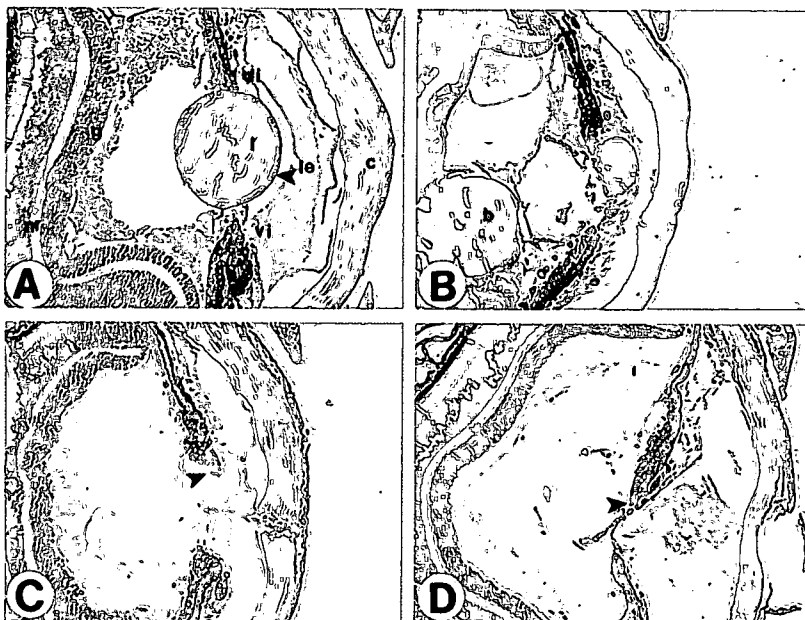


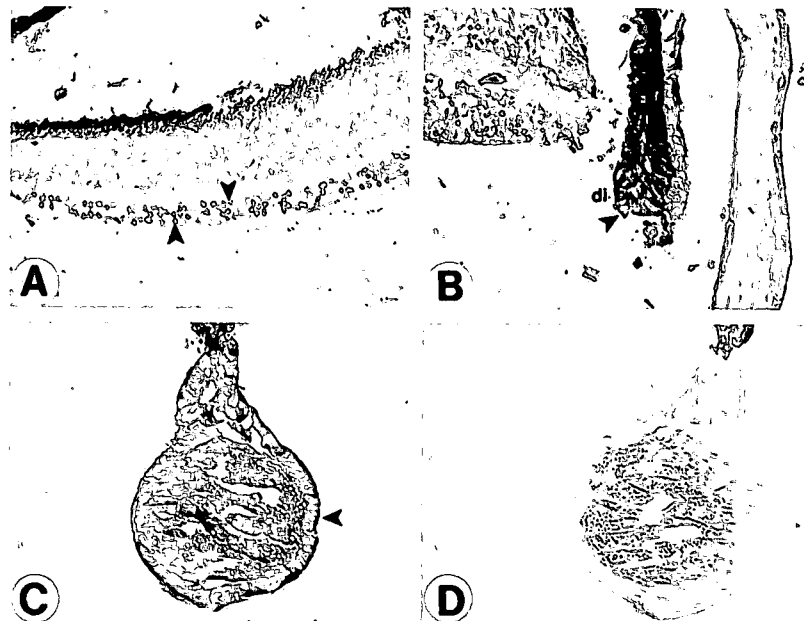
Fig. 1. Effects of retinoic acid receptor (RAR)- α antagonist on lens regeneration. (A) Section through an eye with a normally regenerated lens 20 days after lentiectomy. The lens (l) occupies the area between the dorsal iris (di; from where it was originated) and the ventral iris (vi). The lens fibers are surrounded by the lens epithelium (le). The neural retina (nr), ganglion cell layer of the neural retina (g) and cornea (c) are also denoted. (B–D) Sections through lentiectomized eyes treated with the antagonist 20 days after lentiectomy. Note the varying degrees of lens regeneration. In (B), a small lens has regenerated from the dorsal iris. This lens has differentiated fibers. The bead (b) is also visible in this section. In (C) and (D), however, there is only a small lens vesicle (arrowheads) without apparent lens fiber differentiation.

of 23) the lens was affected. In three of those the lens was completely absent and in the remaining four the lens was smaller (to varying degrees). Examples of such cases can be seen in Figure 1. Figure 1A shows a section through an eye with a regenerating lens 20 days post-lentectomy. This section is from a control eye (treated in DMSO solution) and shows a normally regenerated lens. No effects were seen in 31 control cases. The lens (fibers) is surrounded by the lens epithelium and is of normal size, filling the space between the dorsal and ventral iris. In Figure 1B–D we can observe the effects of the antagonist on lens differentiation and regeneration; in Figure 1B, a smaller lens has regenerated with apparent fiber differentiation in the interior. The bead can also be seen in this section. In Figure 1C and D, lens differentiation and regeneration has been completely inhibited. We can, however, see a small lens vesicle at the tip of the dorsal iris. In our previous study (Tsonis *et al.* 2000) we showed that an antagonist to all RAR was able to affect lens regeneration in more than 60% of cases. Also in that study, regeneration was observed from sites other than the dorsal iris. In the present study, treatment with a specific RAR- α antagonist resulted in fewer affected lenses. The differences in these effects should not be attributed to the concentration of the two different antagonists because the same method and concentration was used for administration. Rather, this might indicate that the different RAR might act in a redundant fashion or that different RAR can contribute to different stages of

lens formation (or both). Redundancy in RAR is a well-established phenomenon (Kastner *et al.* 1994). Obviously, by using specific antagonists to RAR (as they become available) we will be able to delineate their exact function in lens regeneration. One obvious conclusion from the present study is that RAR- α is involved in the proliferation and differentiation process of the lens vesicle, but is not involved in the mode of regeneration as it pertains to the site of origin in the eye. Such effects could be mediated via the specific synthesis of retinoic acid along the dorsoventral axis in the eye (Manns & Fritzsche 1991; McCaffery *et al.* 1992, 1993; Wagner *et al.* 2000). Such spatial distribution patterns are known to affect eye morphogenesis as well as gene expression (Enwright & Grainer 2000).

Expression of RAR- α was consistent with such effects. RAR- α was expressed weakly only in the ganglion layer of the retina in the intact eye. The intact lens was negative for RAR- α (Fig. 2A). However, as the PEC from the dorsal iris dedifferentiated and produced the lens vesicle, RAR- α transcripts were detected in the dedifferentiated cells and in the subsequent transdifferentiated lens epithelial cells (Fig. 2B). The expression in the epithelial cells was prominent even by day 20 post-lentectomy. However, expression in the differentiating lens fibers was also strong (Fig. 2C). In this respect, RAR- α shows similar patterns to the previously reported expression patterns for RAR- δ (Tsonis *et al.* 2000). Such patterns of expression of RAR are quite consistent with their

Fig. 2. Expression of retinoic acid receptor (RAR)- α in intact and regenerating lens. (A) Expression in the intact eye. Signal was detected only in the ganglion layer of the retina (arrowheads). (B) Expression in a regenerating lens vesicle 10 days after lentectomy. Expression is obvious in the dedifferentiated vesicle (arrowhead) from the dorsal iris (di). (C) Expression in a regenerated lens 20 days after lentectomy. Note expression in the lens epithelium (arrowhead) and the lens fibers. (D) Negative control, a section with a regenerated lens 20 days after lentectomy hybridized with the sense probe. Note the absence of signal.



roles. It is conceivable that blocking the function of RAR by specific antagonists would inhibit lens formation, as the differentiation of the lens fibers depends on the lens epithelium. Related to this, it has been shown before that retinoid receptors regulate crystallin synthesis in the lens (Gopal-Srivastava *et al.* 1998). The fact that we see inhibition of lens formation can then be explained by the blocking of RAR function. We believe that while the function of different RAR can be redundant, it can be complementary or unique as well. Extension of these studies with other specific antagonists of retinoid function might lead to a better understanding of the mechanisms of lens regeneration and its uniqueness in some urodele amphibia.

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Unique Expression Patterns of the Retinoblastoma (*Rb*) Gene in Intact and Lens Regeneration-Undergoing Newt Eyes

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ABSTRACT

Based on the role of retinoblastoma (*Rb*) in lens development and in the cell cycle reentry of muscle cells during limb regeneration, we have analyzed expression of *Rb* patterns in intact and lens regeneration-undergoing newt eyes. We find that in intact newt eye *Rb* is expressed in the retina as a gradient with higher levels in the photoreceptor layer and virtually no expression in the ganglion layer. In addition, a second gradient was detected within the photoreceptor layer with expression diminishing at the dorsal and ventral regions. In the intact lens, *Rb* is expressed in the lens epithelium and in the differentiating lens fibers at the bow region. During lens regeneration, *Rb* is expressed very strongly in the differentiating lens fibers, but not in the lens epithelium. Using an antibody specific to the hyperphosphorylated form of *Rb*, we detected the inactive protein only in the pigment epithelial cells of the iris. These distinct patterns might be related to the regenerative potential of the lens in the newt. Anat Rec Part A 271A:185–188, 2003. © 2003 Wiley-Liss, Inc.

Key words: newt; lens regeneration; retina; retinoblastoma

Lens regeneration in the adult vertebrates is only possible in some newts and begins with dedifferentiation and proliferation of dorsal iris pigment epithelial cells (PECs). By dedifferentiation we mean the loss of characteristics that define the pigment epithelial cells, such as pigmentation. Dedifferentiation initiates molecular events, such as reentering the cell cycle, which is necessary for cell proliferation and the subsequent regeneration of the lens. The first peak of cell proliferation in the dorsal iris is observed between 4 and 6 days postlentectomy (Yamada, 1977). At about 10 days postlentectomy, a lens vesicle is formed from the depigmented dorsal PECs. Around 12 to 16 days postlentectomy, the internal layer of the lens vesicle thickens and synthesis of crystallins begins. From 12 to 15 days postlentectomy, a second peak of cell proliferation is observed in the dorsal iris (Yamada, 1977). This marks the beginning of primary lens fiber differentiation. During days 15 to 19, proliferation and depigmentation of PECs slow down. In the internal layer of the regenerating vesicle, the lens fiber complex is formed and in the margin of the external layers nondividing secondary lens fibers

appear. By 18 to 20 days the PECs have stopped proliferating, and the lens fibers continue to accumulate crystallins. Lens regeneration is considered complete by day 20 to 25 (Tsonis, 2000, 2001). Lens regeneration, therefore, is possible by transdifferentiation, which is the transformation of one cell type to another (in this case, pigment epithelial cells to lens cells). The process of transdifferentiation has been proven beyond any doubt in this system. When single PEC cells are placed in culture the process of

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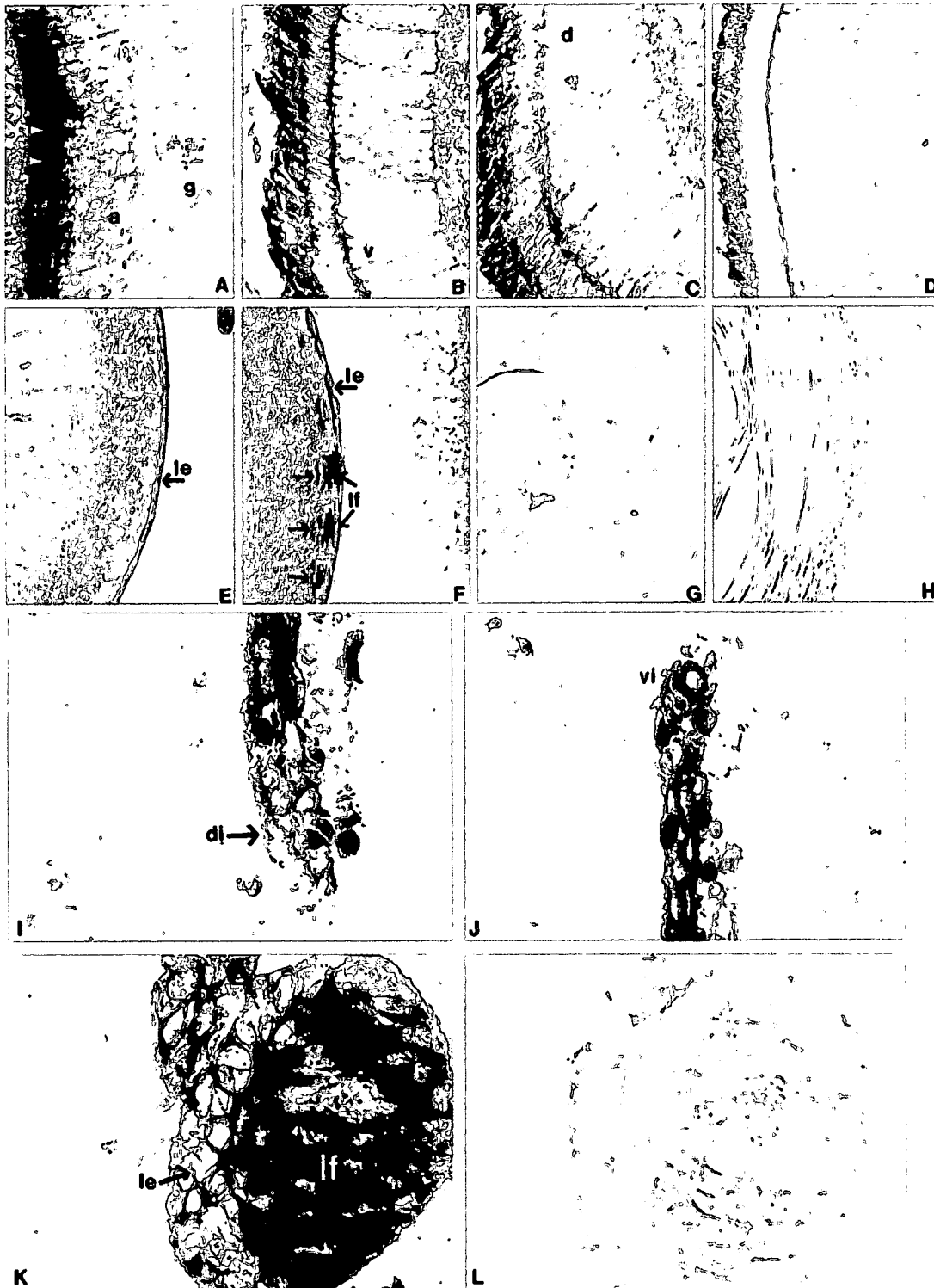


Fig. 1. Expression of *Rb* in intact adult newt eye. **A:** Expression as a gradient in the neural retina. Note strong expression in the photoreceptor (arrowheads) layer and part of the amacrine layer (a). No expression is seen in the ganglion layer (g). **B:** Expression in the photoreceptor layer is higher in the center and lower in the ventral part (v) of the retina. **C:** Expression is lower in the dorsal part (d) of the retina. **D:** Negative control; hybridization with the sense probe. **E:** Expression in the anterior lens epithelial cells (le). **F:** Expression in the lens epithelial cells at the bow region (le) and the differentiating fibers (lf, arrows). **G:** Lack of

expression in the lens fibers at the center of the lens. **H:** Negative control; a portion of the anterior lens hybridized with the sense probe. **I-L:** Expression of *Rb* during lens regeneration. **I:** Lack of expression in the dedifferentiated tip (arrow) of the dorsal iris (di) 10 days postlens-tomy. **J:** Lack of expression in the tip of the ventral iris (vi). The dark color is due to the heavy pigmentation of the pigment epithelial cells. **K:** Strong expression in the lens fibers (lf) of a regenerating lens 20 days postlens-tomy. Note that the lens epithelial cells (le) are rather negative. **L:** Negative control using a similar section as in C and the sense probe.

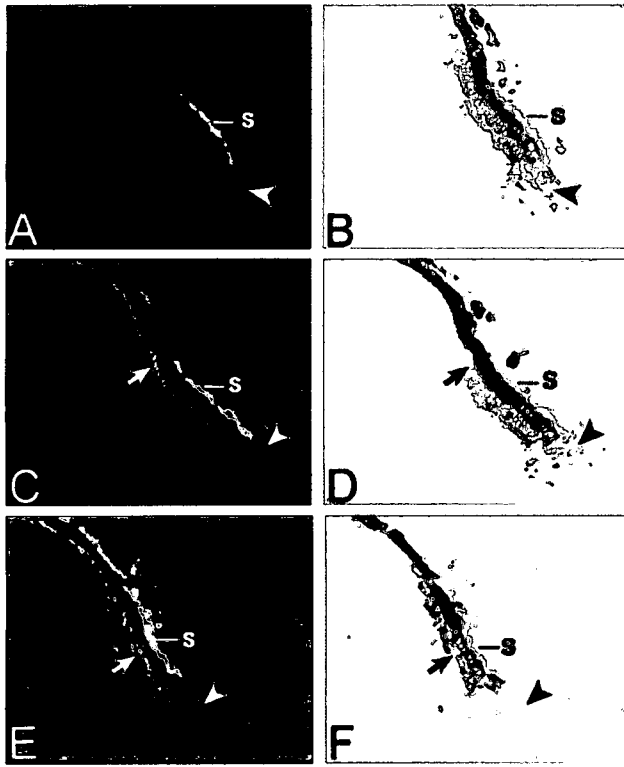


Fig. 2. Rb protein detection by immunofluorescence in the pigment epithelial cells (PECs) of the newt iris. **A:** Negative control; incubation with the primary antibody was omitted. This is a section through a regenerating eye 10 days postlentectomy. **C:** A section through a regenerating eye ten days postlentectomy. Presence of Rb in the PECs of the dorsal iris (arrow). The lens vesicle (arrowhead) is negative. **E:** A section through a regenerating eye 15 days postlentectomy. Note presence of Rb in the PECs (arrow) and absence in the lens vesicle (arrowhead). Stroma (S) always shows unspecific autofluorescence. **B, D, F:** Corresponding differential interference contrast (DIC) images of A, C, and E, respectively.

transdifferentiation can be observed (Kodama and Eguchi, 1995; Tsonis, 2001). As the PECs proliferate, they become depigmented and then transdifferentiate to lens cells.

The presence of retinoblastoma (*Rb*) and the regulation of its phosphorylation is an important event for reentry to the cell cycle. Such an event is especially important during lens development and controls proliferation and apoptosis that lead to lens fiber differentiation (Morgenbesser et al., 1994). In addition, *Rb* phosphorylation is correlated with the ability of newt myotubes to reenter the cell cycle, an important step in the dedifferentiation process that leads to limb regeneration (Tanaka et al., 1997). Because the newt lens can be regenerated by the transdifferentiation, it is paramount to examine mechanisms of cell cycle reentering. Given the important role of *Rb* in cell cycle regulation we decided to examine its expression patterns in the intact and lens regeneration-undergoing eye of the adult newt.

Animals

Adult newts, *Notophthalmus viridescens*, were lentectomized under anesthesia, 0.1% MS222 (Sigma, St. Louis,

MO), and their eyes were collected in time intervals up to 20 days postlentectomy. The eyes were processed for expression studies using in situ hybridization and immunohistochemistry.

In Situ Hybridization

This was carried out as described previously (Del Rio-Tsonis et al., 1997, 1999). The newt *Rb* probe was a gift from Dr. J.P. Brockes (Tanaka et al., 1997).

Immunofluorescence

The mouse monoclonal antibody 51beta7 directed against the hyperphosphorylated form of *Rb* (Rubin et al., 2001) was used in immunohistochemistry with eye sections. Frozen sections were collected from different stages during lens regeneration. The sections were air-dried for 30 min at room temperature (RT) and fixed in 4% paraformaldehyde for 10 min. The sections were then permeabilized in 100 mM glycine pH 7.0, 0.2% Triton X-100, incubated with 10% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS) for 5 min, and washed in 1× PBS/0.2% bovine serum albumin (BSA) 3× for 5 min each. Sections were then blocked with 0.2% BSA, 0.1% Tween-20, 10 mM NaF, 10 mM B-glycerolphosphate for 10 min. Following blocking, sections were incubated with primary antibody at 37°C for 1 hr, or at 4°C overnight in 0.2% BSA, 0.1% Tween-20, 10 mM NaF, 10 mM B-glycerolphosphate, and the *Rb* antibody at 20 µg/ml. Subsequently, sections were washed in PBS/0.2% BSA 3×, 5 min each, and then were incubated with secondary antibody (0.2% BSA/PBS, 1:1,000 dilution of Fluorescein) 2×, 30 min, at RT. Finally, sections were washed in PBS/0.2% BSA 3×, 5 min each, and mounted with gel mount.

RESULTS AND DISCUSSION

In the adult intact eye, *Rb* was expressed in both the neural retina and the lens. In the neural retina, *Rb* was expressed as a gradient with higher expression levels in the photoreceptor layer and no expression in the ganglion cell layer (Fig. 1A). In addition, this expression was most pronounced in the central part of the photoreceptor layer and not in the dorsal or ventral part (Fig. 1B,C). These expression patterns are quite different from the ones observed during development of mouse eye, where expression was exactly opposite, with the signal mostly seen in the ganglion cell layer (Jiang et al., 1997) or from adult human retina where *Rb* was found in all cell layers (Nork et al., 1994). *Rb* was expressed in the intact lens as well, both in the lens epithelium and in the differentiating lens fibers at the bow region (Fig. 1E,F) but not in the fibers at the center of the lens (Fig. 1G).

After lentectomy, PECs from the dorsal iris dedifferentiate and reenter the cell cycle. They lose their pigments and produce a vesicle that is destined to become the lens epithelium, which subsequently differentiates to lens fibers (Tsonis, 2000, 2001). The lens fibers are postmitotic and *Rb* inactivation (as shown during mouse development) is sufficient to cause proliferation of lens fibers. In this case, *Rb* is needed for withdrawal of fiber cells from cell cycle before their end-stage differentiation (Morgenbesser et al., 1994). During the process of regeneration, we found that *Rb* transcripts were absent during the early events of vesicle formation (Fig. 1I,J). During the formation of the regenerating lens, *Rb* was expressed very

strongly in the lens fibers but it was virtually absent in the lens epithelium (Fig. 1K). This is an interesting difference between the intact and regenerating lens, because the lens epithelium of the intact lens was rather positive for *Rb*. This indicates a fundamental difference between the genetic activity of lens epithelium during regeneration, which might reflect the fact that other factors, not belonging to the *Rb* family, might be involved in lens epithelium cell proliferation (Nguyen et al., 2002). Expression in the retina of the eyes undergoing lens regeneration was virtually the same as in the retina of the intact eyes. Due to heavy pigmentation in the iris, we were unable to detect expression of *Rb* using in situ hybridization in the PECs. Among the several *Rb* antibodies that we tried, we were able to detect a signal with a specific *Rb* antibody directed against the hyperphosphorylated form of mouse *Rb* (Rubin et al., 2001). This form is inactive and leads to dissociation from E2F and entering of the cell cycle. We found that this form was only expressed in the pigment epithelial cells of the iris, especially of the dorsal iris (Fig. 2). Expression was not detected when the sections were treated with phosphatase (not shown). This pattern is novel and consistent with a role of *Rb* in allowing these cells to reenter the cell cycle, proliferate, and contribute to the dedifferentiation process. The absence of signal in any other tissue, especially the intact and regenerating lens, suggests that the hypophosphorylated form of *Rb* might be the predominant one. This is consistent with its association with cell cycle withdrawal (Morgenbesser et al., 1994; Rampalli et al., 1998).

Overall, expression of *Rb* seems to be unique in the newt eye and such patterns might be correlated with their regenerative properties. Further studies with specific inhibitors of *Rb* phosphorylation might shed light to its role in lens transdifferentiation.

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Expression of Complement 3 and Complement 5 in Newt Limb and Lens Regeneration^{1,2}

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Some urodele amphibians possess the capacity to regenerate their body parts, including the limbs and the lens of the eye. The molecular pathway(s) involved in urodele regeneration are largely unknown. We have previously suggested that complement may participate in limb regeneration in axolotls. To further define its role in the regenerative process, we have examined the pattern of distribution and spatiotemporal expression of two key components, C3 and C5, during limb and lens regeneration in the newt *Notophthalmus viridescens*. First, we have cloned newt cDNAs encoding C3 and C5 and have generated Abs specifically recognizing these molecules. Using these newt-specific probes, we have found by in situ hybridization and immunohistochemical analysis that these molecules are expressed during both limb and lens regeneration, but not in the normal limb and lens. The C3 and C5 proteins were expressed in a complementary fashion during limb regeneration, with C3 being expressed mainly in the blastema and C5 exclusively in the wound epithelium. Similarly, during the process of lens regeneration, C3 was detected in the iris and cornea, while C5 was present in the regenerating lens vesicle as well as the cornea. The distinct expression profile of complement proteins in regenerative tissues of the urodele lens and limb supports a nonimmunologic function of complement in tissue regeneration and constitutes the first systematic effort to dissect its involvement in regenerative processes of lower vertebrate species. *The Journal of Immunology*, 2003, 170: 2331–2339.

The complement system is comprised of several serum proteins, membrane-bound receptors, and regulatory proteins that constitute a phylogenetically ancient mechanism of innate immunity (1–2). The functions of the complement system in host defense and inflammation are mediated mainly through the sequential activation and proteolytic cleavage of serum proteins.

Complement activation occurs through three distinct pathways (classical, alternative, and lectin), all of which converge in the activation of C3, the third component of complement. C3 can interact with a wide spectrum of factors and therefore is able to mediate a wide variety of functions (3–5). In addition to complement proteins, C3 fragments interact with several proteins that are involved in differentiation, such as fibronectin and integrins (6, 7). Other complement factors share homologies with domains of extracellular matrix proteins, such as collagen binding domains, which might indicate that complement factors could be involved in such interactions in the extracellular matrix (8). Therefore, some of the functions of complement are apparently not immunologic. For

example, C3 is expressed in myoblasts (9, 10) and is also associated with proliferation and growth of B cells in vitro (11).

C5, the fifth component of complement, has also been found to have novel noninflammatory functions in various tissues. Studies in a human neuroblastoma cell line have suggested that C5a (a fragment of C5) participates in apoptotic signal transduction pathways through its binding to the neuronal C5aR (12, 13). It has also been shown that the terminal complex system C5b-9 (membrane attack complex), in sublytic doses, can induce DNA synthesis and cell proliferation in cultured mouse fibroblasts (14), human aortic smooth muscle cells (15), oligodendrocytes (16), and glomerular epithelial cells, in the absence of other growth factors (17). Also, sublytic concentrations can activate monocytes and induce cytokine release through activation of NF- κ B signaling pathways, which are critical for the cell cycle transition into DNA synthesis (18).

We have reported previously that C3 is expressed during limb regeneration in the axolotl (19) in the blastema, a cell population that gives rise to the various cell types that reconstitute the limb, through processes that involve dedifferentiation, transdifferentiation into different phenotypes, and extensive tissue remodeling. C3 was not expressed in the intact or developing limb, indicating its specificity for the regeneration process. Furthermore, we were able to show expression of C3 in dedifferentiated newt muscle cells in vitro. These findings were the first to indicate that complement might have a novel, possibly nonimmunologic, role in regenerative processes. This potential role for complement in tissue regeneration was supported by our recent observation that C5-deficient mice exhibit defective liver regeneration after acute toxic injury (20). In mice, the liver can regenerate by proliferation of the remaining hepatic cells. Mice lacking C5 are not able to repair their liver properly unless they are reconstituted with C5a (20).

To expand our understanding of this newly discovered role of the complement system in regenerative processes, we decided to use the newt *Notophthalmus viridescens* because of its extensive repertoire of regenerative capabilities. The newt can regenerate its

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limbs, tail, jaw, some of its internal organs, and its eye tissues, including the lens and the retina. In many cases, research in species other than humans is hampered by the lack of a wide range of tools to approach a specific problem. Abs represent one such tool that is a prerequisite for our studies addressing the function of newt C3 and C5. As a first step in our research, we decided to systematically generate the necessary tools (C3- and C5-specific probes and Abs) in the newt, so that they could be used to explore the role of complement in regeneration in this species. In this study, we describe the specific and robust expression of complement components C3 and C5 in regenerating tissues of the newt limb and lens, both at the mRNA and protein levels. In the effort to characterize the expression profile of these components, we isolated and characterized newt C3 and C5 cDNA and generated specific Abs against newt C3 and C5. The expression patterns of C3 and C5 suggest that both complement proteins are involved in the process of regeneration.

Materials and Methods

Animals

Newts (*N. viridescens*) were purchased from Mike Tolley Newt Farm (Nashville, TN). Newt limbs were amputated at the ulnar-radial plane, and the regenerated limbs were collected 7, 14, and 21 days after amputation. Lenses were removed from adult newts, and regenerating eyes were collected 5, 10, 15, and 20 days thereafter. The collected tissues were either immersed in OCT (Andwin Scientific, Warner Center, CA) and quick-frozen to be processed for immunohistochemical staining, or they were fixed in 4% paraformaldehyde overnight and then processed for paraffin embedding for subsequent in situ hybridization analysis. Blood was collected through a capillary tube after amputating the tail proximal to the body. Before these procedures, all animals were anesthetized with 3-aminobenzoic acid hydrochloride (MS222; Sigma-Aldrich, St. Louis, MO) and were handled in compliance with the regulations of the Institutional Animal Care and Use Committee.

Purification of newt C3

Newt C3 was purified from newt plasma by a combination of polyethylene glycol (PEG)⁴ precipitation and anion exchange and gel filtration chromatography (Fig. 1A). The total recovery of purified C3 from 2 ml of plasma was 0.8 mg, which suggested that the concentration of C3 in newt plasma is similar to that found in other species. Purified C3 protein was analyzed by 7.5% SDS-PAGE under reducing and nonreducing conditions. To obtain partial protein sequence for C3 primer design, the purified protein was electrophoresed and electroblotted onto a polyvinylidene difluoride (PVDF) membrane, and the α -chain was subjected to Edman degradation.

Cloning of newt C3 and C5

First-strand cDNA was synthesized from newt liver total RNA using the Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. A degenerate primer, 5'-AARGCNGGNGC NAARGC-3' (N = G, A, T, and C; R = A and G), was designed based on the sequence KAGAK which represents the C3 sequence KAGK with the insertion of A between residues G and K which is conserved in the C5 of various species; this primer was used to obtain both C3 and C5 cDNA. In addition, a sense primer, 5'-GNTGYGGNGARCARAAATG-3' (Y = T and C), and an antisense primer, 5'-CATRTTYTGYTCNCCRCANC-3', were designed from a conserved sequence in the thioester region GCGEQNM as well as the primer 5'-ACRTANGCNGTNARCCA-3', which corresponds to the conserved sequence WLTAIV (21). Different combinations of primers were used to obtain the desired cDNA sequences. PCR were performed as follows: 30 cycles of denaturation at 94°C for 0.5 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min. The amplified PCR products were gel-purified and subcloned into the pGEM-T Easy vector (Promega) and sequenced.

Abbreviations used in this paper: PEG, polyethylene glycol; PVDF, polyvinylidene difluoride; DAPI, 4',6'-diamidino-2-phenylindole; WE, wound epithelium; DIC, differential interference contrast.

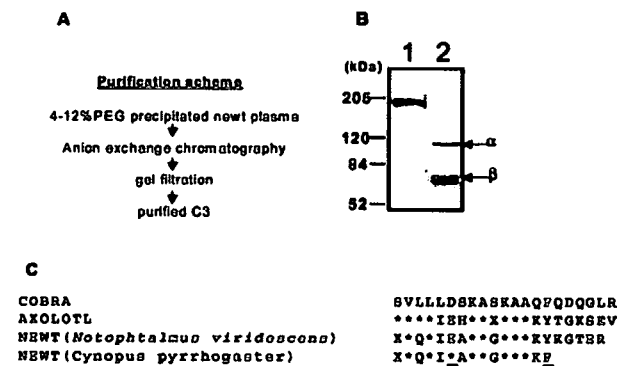


FIGURE 1. Purification and characterization of newt C3. A, Schematic of C3 purification. Newt plasma (4–12% PEG precipitated) was applied to a Mono Q HR 10/10 anion exchange chromatography column, and fractions were eluted with a 50–350 mM salt gradient. Based on SDS-PAGE analysis under reducing and nonreducing conditions, C3-containing fractions were concentrated and subjected to gel filtration on a Superose 12PG column. B, Coomassie-purified C3 was run on 7.5% SDS-PAGE under reducing (lane 2) and nonreducing (lane 1) conditions. C, Amino acid sequence comparison of the newt C3 α -chain N terminus to cobra and axolotl C3. An asterisk denotes identical residues. The amino acid sequence of newt C3 was obtained by N-terminal sequencing of purified C3.

Expression and characterization of newt C3a and C5a

The newt C3a fragment corresponding to residues 8–76 of the C3 sequence (Fig. 2A) was amplified by PCR from the cloned C3 fragment using the primers 5'-GGATCCAAAGCTGGCAAAGCT-3' and 5'-AAGCTTTTATCTTGACAAAAACAGTTG-3'. These primers added a *Bam*HI site at the beginning of the C3 sequence and a *Hind*III site at the end of the cDNA. For convenience, the fragment was cloned into pGEM-T Easy vector, and the sequence was verified. The fragment was excised with *Bam*HI and *Hind*III restriction enzymes and cloned into the expression vector pQE-30 (Qiagen, Studio, CA).

The C3a protein was expressed and purified from the *Escherichia coli* strain M15 (pREP4; Qiagen) according to the manufacturer's instructions. The *E. coli* was first treated with a lysogen solution (1 mg/ml), and then the pellet was resuspended with a solution of 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl containing 10 mM 2-ME and 8 M urea (pH 8.0). The cleared lysate was mixed with Ni-NTA agarose (Qiagen) for 1–2 h and loaded onto a disposable column. The column was washed with 0.1 M NaH₂PO₄ and 0.01 M Tris-HCl containing 8 M urea (pH 8.0), followed by the same solution at pH 6.3 and then pH 5.9, and finally eluted at pH 4.5. The recombinant protein was refolded by dialysis overnight into 0.1 M Tris-HCl with 2 mM reduced glutathione, 0.2 mM glutathione, and 0.005% Tween 80 (22). Contaminating proteins were removed by a reversed-phase column using a RESORSE RPC 3-ml column (Amersham Pharmacia Biotech, Piscataway, NJ) as previously described (23). The purity and identity of recombinant newt C3a protein was assessed by SDS-PAGE and mass spectrometry. The newt C5a fragment (residues 9–79) (Fig. 2B) was prepared using the sense primer 5'-GGATCCATAGAGGCATTAAACTCT-3' and the antisense primer 5'-AAGCTTTTATCGAGCCAGTTCCAG-3' and was expressed and purified in the same fashion as recombinant newt C3a.

Preparation of anti-C3a- and anti-C5a-specific Abs

Rabbits were immunized by repeated s.c. injection at multiple sites with 30 μ g of purified C3a or C5a emulsified in CFA, followed by a booster injection at 3 wk. The IgG fraction of immune sera was prepared by a combination of caprylic acid and ammonium sulfate precipitation and was extensively dialyzed against PBS. The anti-C3a Ab was further purified by affinity chromatography using a C3a-Sepharose 4B column. The specificity of generated Abs was assessed by SDS-PAGE followed by Western blotting. Briefly, 0.25 μ l of normal newt serum treated with or without cobra venom factor were incubated at 37°C for 30 min and used for the detection of C3 and C5, respectively. Samples were subjected to SDS-PAGE on 7.5% gels under nonreducing conditions and electroblotted onto a PVDF membrane. The membrane was blocked with PBS containing 10% milk-0.05% Tween and incubated with the anti-C3a or anti-C5a IgG (5 μ g/ml) for 40 min. After washing with PBS-0.05% Tween, the membrane was incubated for 30 min with HRP-conjugated goat anti-rabbit IgG (1:1000).

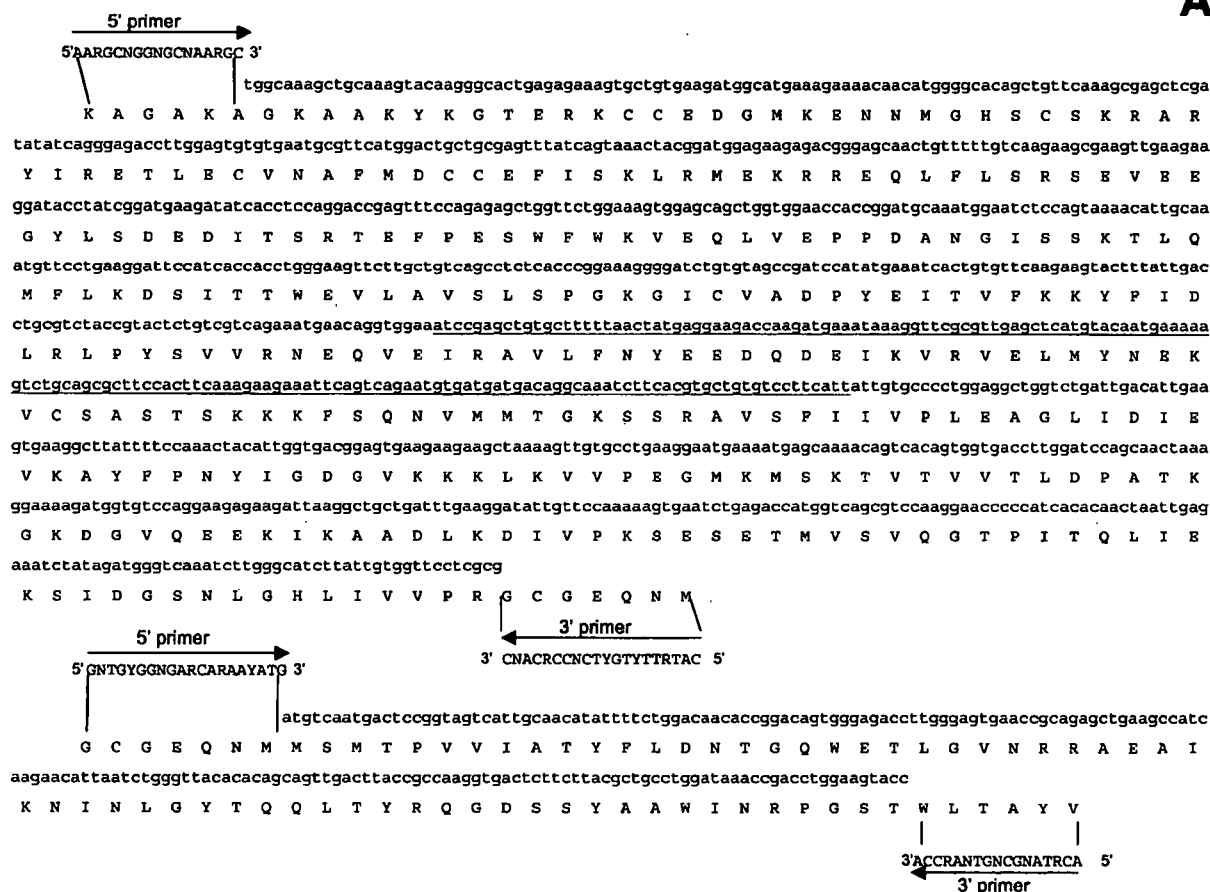
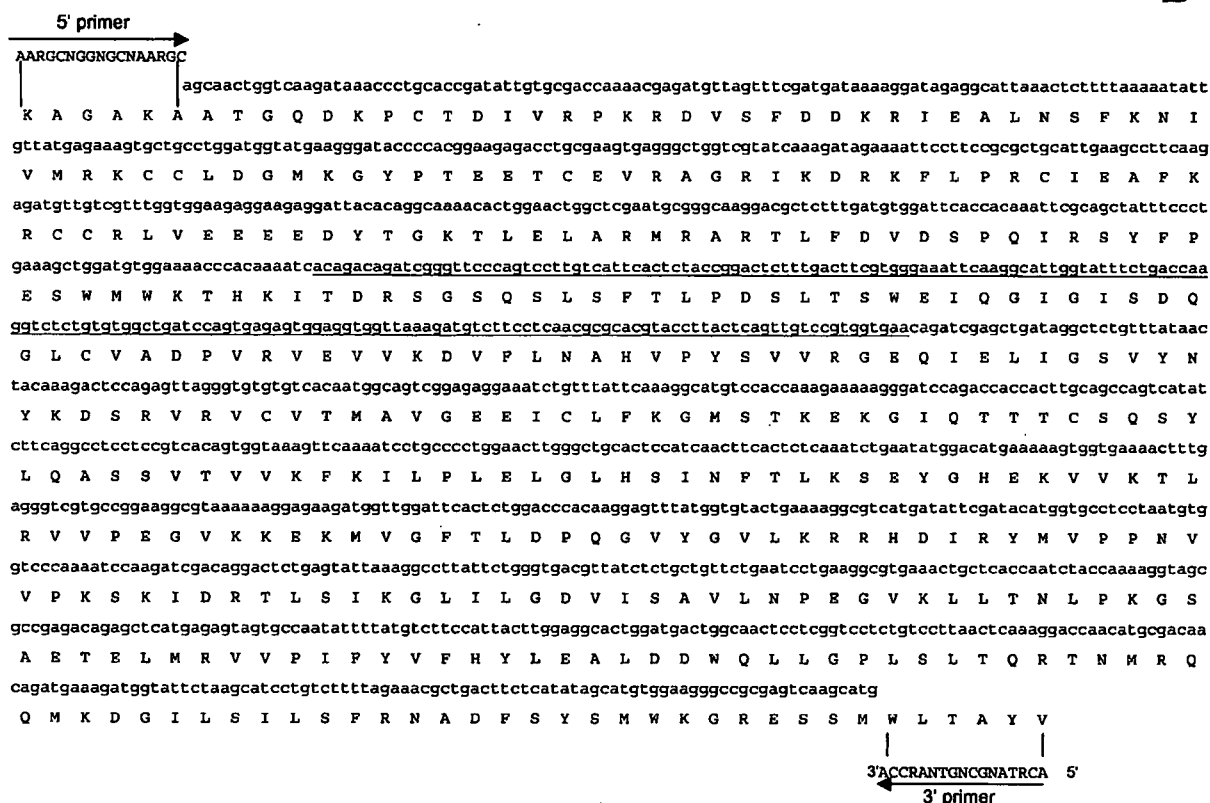
A**B**

FIGURE 2. Nucleotide and deduced amino acid sequences of C3 and C5. The translated amino acids of C3 (A) and C5 (B) are in single-letter code. Arrows indicate the sequence of primers used in the RT-PCR. The underlined sequences indicate sequences of probes used for the in situ hybridization.

The membrane was washed and developed using ECL solution (Amersham, Arlington Heights, IL).

Immunohistochemistry

Frozen samples were sectioned and processed for immunohistochemistry as follows: frozen sections from the various stages of limb and lens regeneration, including intact limbs as well as intact lenses, were preincubated with 10% goat serum in 1× PBS for 1 h at room temperature, then with anti-C3 or anti-C5 Ab (1:100) in 1× PBS/10% goat serum/0.3% Triton X-100 (PBSGST) for 1 h at room temperature. After several washings in 1× PBS, the sections were incubated with a rhodamine-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 in PBSGST for 2 h at 37°C. The sections were then washed in PBSGST followed by 1× PBS and then counterstained with 4',6'-diamidino-2-phenylindole (DAPI; Roche, Indianapolis, IN), mounted with Vectashield (Vector Labs, Burlingame, CA), and examined under a fluorescence microscope.

In situ hybridization

In situ hybridizations were conducted essentially as previously described for newt tissues (19). Slides containing paraffin sections were deparaffinized in xylene and then hydrated through an ethanol series. The slides were rinsed in 1× PBS and then fixed in 4% paraformaldehyde for 15 min. After a rinse with 1× PBS, the slides were incubated with 250 µg/ml pepsin at 37°C for 11–15 min. They were again rinsed with 1× PBS and then treated with 0.1 M triethanolamine/0.25% acetic anhydride. The slides were then washed with 1× PBS and dehydrated through an ethanol series. After 1 h of air drying, the sections were hybridized at 58°C for 16 h with hybridization solution (10 mM Tris-HCl (pH 7.5) containing 50% formamide, 1 mM EDTA, 600 mM NaCl, 0.25% SDS, 10% PEG 6000, 1 Denhart's, 200 µg/ml tRNA, and 250 ng/ml digoxigenin-labeled probes). The next day, the slides were washed with 4× SSC, followed by treatment with 50 µg/ml RNase at 37°C for 1 h. The slides were incubated in 2× SSC at 63°C two times for 30 min each, then in 0.1× SSC at 63°C two times for 30 min each. For immunological detection, the slides were rinsed in buffer 1 (0.1 M Tris-HCl (pH 7.5) with 0.15 M NaCl) and then incubated in buffer 2 (buffer 1 with 1% blocking reagent (Roche, Indianapolis, IN)) for 1 h at room temperature. The sections were then incubated with alkaline

phosphatase-conjugated anti-digoxigenin Ab in buffer 2 at 1:2500 for 1 h at room temperature. After three washes with buffer 1 for 30 min each, the slides were incubated in buffer 3 (0.1 M Tris-HCl (pH 9.5) with 0.1 M NaCl and 50 mM MgCl₂) for 10 min, then in the same solution plus nitroblue tetrazolium salt/5-bromo-4-chloro-3-indolyl phosphate for 16–24 h. The reaction was stopped with Tris-EDTA, and sections were mounted with Crystal Mount (Biomedica, Foster City, CA).

Results

Cloning of newt C3 and C5

To obtain partial amino acid sequence for newt C3, the protein was purified from newt serum. SDS-PAGE analysis of the purified C3 protein under nonreducing conditions showed a single 200-kDa band. Under reducing conditions, a 120-kDa α-chain and a 70-kDa β-chain were detected (Fig. 1B). To assess whether the purified protein was indeed C3, it was electrophoresed and electroblotted onto a PVDF membrane, and the α-chain was subjected to Edman degradation. The obtained sequence (Fig. 1C) showed high sequence similarity to C3 from other species. These data suggest that the isolated protein is indeed C3.

Partial cDNA sequences of newt C3 and C5 were obtained via RT-PCR as described in *Materials and Methods* (Fig. 2, A and B). The deduced amino acid sequence of newt C3 was 55% identical with that of human C3, 58% identical with that of *Xenopus* C3, and 62% identical with that of chicken C3. Likewise, the newt C5 amino acid sequence had 48% sequence identity with human C5, 46% with mouse C5, and 42% with trout C5.

Expression of recombinant C3a and C5a in E. coli and production of specific anti-C3 and anti-C5 Abs

Given the partial cDNA sequences of newt C3 and C5 that we had obtained, we decided to subclone and express putative C3a_{8–76} and C5a_{9–79} fragments in *E. coli* (22) (Fig. 3A). The observed mass of

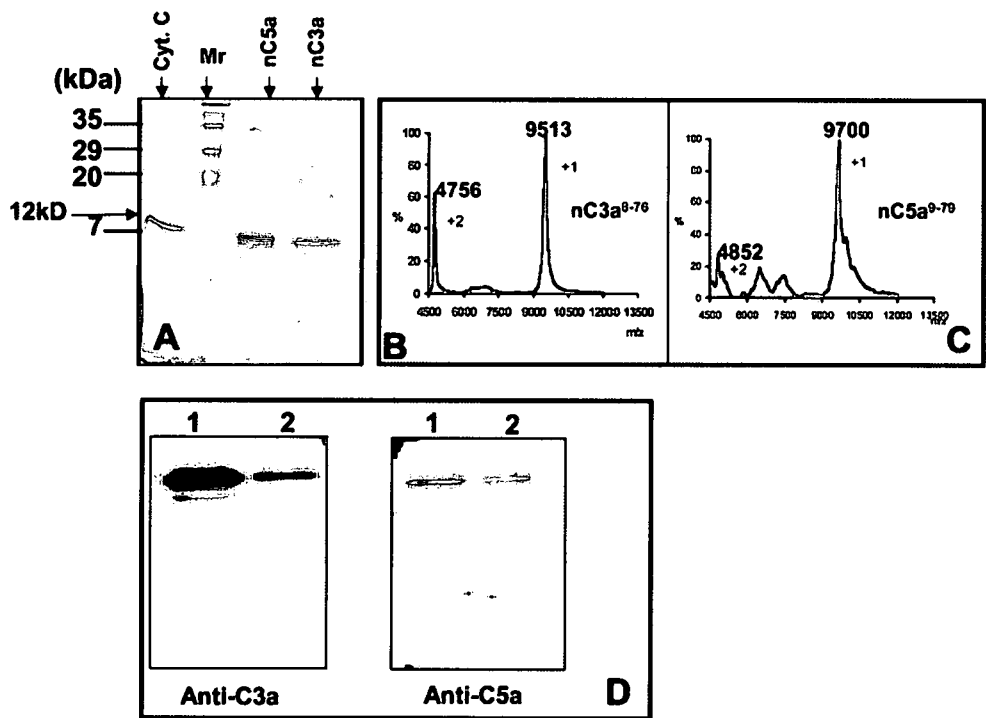
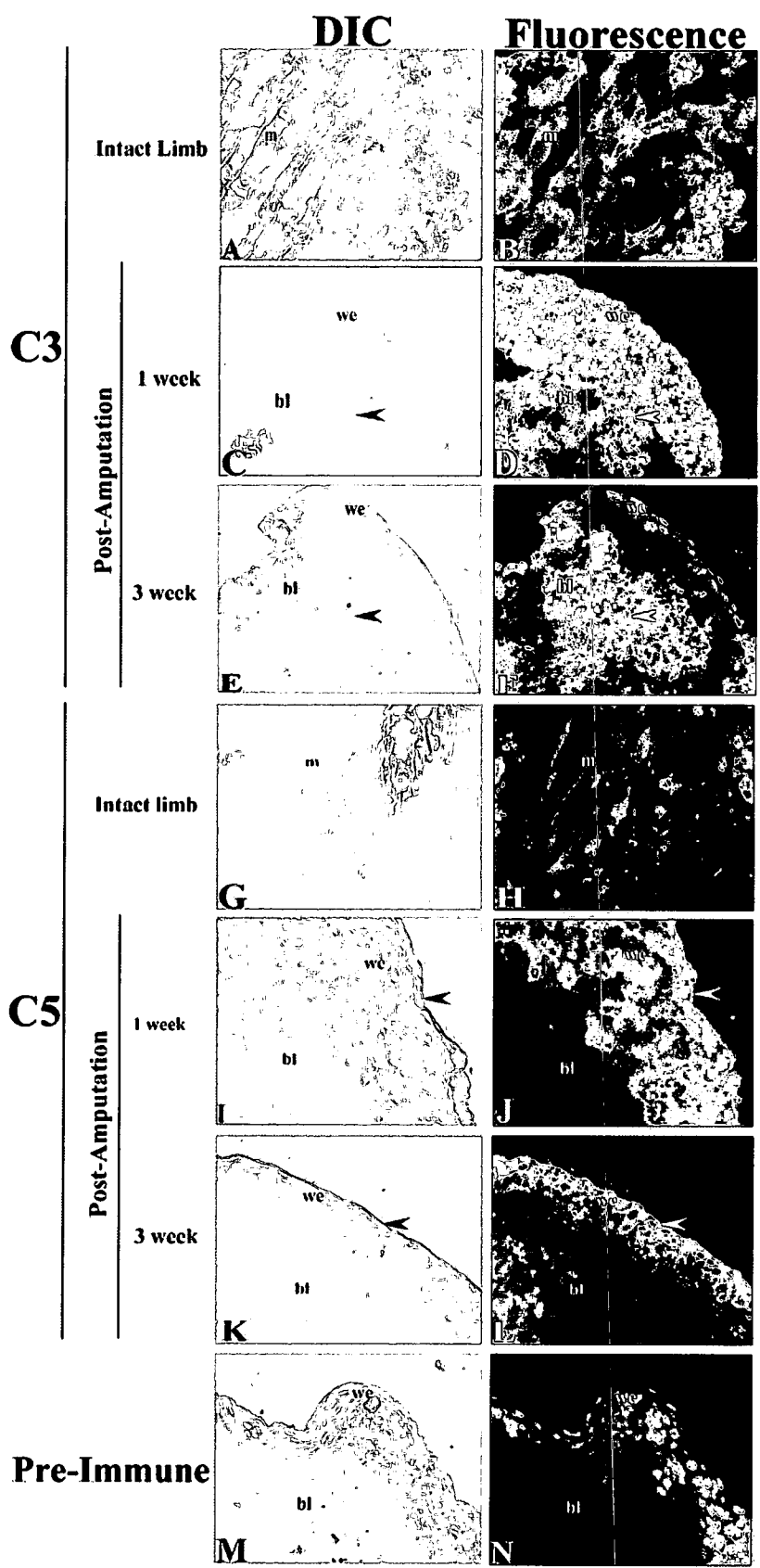


FIGURE 3. Characterization of expressed newt (n)C3a_{8–76} and nC5a_{9–79} and of Abs to these proteins. A, Coomassie-stained 16% SDS-PAGE gel. Lanes 1, 1 µg cytochrome c; 2, markers; 3, 1 µg nC5a_{9–79}; and 4, 1 µg nC3a_{8–76}. Mass spectrometric analysis of nC3a (B) and nC5a (C). D, Specificity of anti-C3 and anti-C5 Abs. Normal (lane 1) or cobra venom factor-treated (lane 2) serum was subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel under nonreducing conditions, blotted onto PVDF membrane, and then incubated with polyclonal anti-C3a (left panel) or anti-C5a Ab (right panel) followed by anti-rabbit IgG HRP-conjugated Ab and ECL solution.

FIGURE 4. Expression of newt C3 and C5 proteins during limb regeneration. Expression of C3 (A–F) and C5 (G–N) proteins during limb regeneration. Column one contains differential interference contrast (DIC) images (magnification, $\times 20$); column two shows the corresponding fluorescence images. Intact muscle in the limb (A and B) shows no C3 expression. C3 (red) is predominantly expressed in the blastema as early as 1 wk (C and D) postamputation and is maintained even at 3 wk postamputation (E and F). C5 is not expressed in intact muscle (G and H) but is expressed in the WE of the regenerating limb at 1 wk (I and J) and 3 wk (K and L) postamputation. Using preimmune serum instead of anti-C3 or anti-C5 Ab yields no fluorescent signal (M and N). Sections have been counterstained with DAPI (blue). bl, Blastema; m, muscle; and we, WE. Arrowheads indicate areas of C3 or C5 protein expression.



expressed C3a protein is 9513 (Fig. 3B) and of newt C5a is 9700 (Fig. 3C), which are very close to the theoretical mass values of 9514 and 9704, respectively. Polyclonal Abs were then raised

against these recombinant proteins. These Abs specifically recognized newt C3 and C5, respectively, but only under nonreducing conditions (Fig. 3D).

Expression of C3 and C5 during limb regeneration

After the newt limb is amputated, the first event that takes place is the closure of the wound by a specialized epithelium, the wound epithelium (WE). This epithelium provides the necessary signals to the underlying differentiated tissues, such as muscle and bone, to dedifferentiate. During dedifferentiation, these tissues lose the characteristics of their tissue of origin (i.e., muscle fibers become mononucleated), and by division, they produce a population of undifferentiated cells called the blastema. After ~2 wk, the blastema starts to differentiate into the various tissues that comprise the limb and to reconstitute the lost parts with exact replicas of the missing parts (24).

To analyze the expression patterns of C3 and C5 in this process, we examined sections taken from the blastema 1 or 3 wk after amputation. These stages represent an early and a late blastema. Immunohistochemical staining of regenerating limbs using the newt-specific anti-C3 and anti-C5 Abs indicated that C3 and C5 had nonoverlapping expression patterns. Even though C3 was present in the WE, most of the protein was found in the blastema, as was evident in the early regeneration stage, the 1-wk blastema (Fig. 4, C and D). This pattern continued in later stages of limb regeneration (Fig. 4, E and F). In contrast, C5 was present exclusively in the WE, with no expression in the blastema at any stage of regeneration (Fig. 4, I–L). Neither protein was found in the intact limb (Fig. 4, A and B (C3), and 4, G and H (C5)). In addition, in situ hybridization for C3 and C5 demonstrate that these molecules are being made locally, which suggests that systemic activation of complement is not a prerequisite for its involvement in limb regeneration. In early (1 wk; Fig. 5, A and D) and late (3 wk; Fig. 5, B and E) regenerates, both of these molecules are expressed in the WE and the blastema. This supports previously reported expression for C3 during axolotl limb regeneration (19).

Expression of C3 and C5 during the process of lens regeneration

After removal of the lens, the pigment epithelial cells of the dorsal iris dedifferentiate (lose their pigment) and form a small vesicle. At ~10 days after lentiectomy, this vesicle begins to synthesize crys-

tallins. As the vesicle continues to grow, it differentiates into lens epithelial cells and lens fibers. This differentiation continues for ~2 wk, and at ~25–30 days, the lens has completely regenerated (25, 26).

C3 was not seen in the regenerating lens vesicle at any stage of lens regeneration (Fig. 6, D–I). However, it was present in the stroma and pigmented epithelium of the iris and in the cornea. In contrast, C5 was present in the regenerating vesicle at 10 (Fig. 6, M–O), 15 (not shown), and 20 days (Fig. 6, P–R) postlentiectomy. C5 was also present in the cornea at all stages of regeneration (not shown). C3 and C5 were absent from the intact lens (Fig. 6, A–C (C3) and J–L (C5)); however, they were present in the corneal tissue (not shown). No reactivity was detected in the retina at any stage with either of the Abs (not shown).

To confirm that C3 and C5 were made locally in the eye tissues, we performed in situ hybridization studies using both C3- and C5-specific probes. Both C3 and C5 mRNA were found to be present in the regenerating eyes. C3 and C5 also showed similar expression patterns in the regenerating lens vesicle at all stages from 10–20 days postlentiectomy (Fig. 7, B–E (C3), and G–J (C5)). C3 was also expressed in the retina (not shown), while C5 was exclusively expressed in the regenerating lens. No expression of either C3 or C5 was found in the intact lens (Fig. 7, A and F).

Discussion

We have previously reported that C3, a central component of the complement system, is expressed in the regenerating blastema of the amputated limb in axolotls. This finding suggested that complement may exert a novel, nonimmunologic role in complex developmental processes such as limb regeneration in urodeles. We have previously demonstrated by in situ hybridization that a newt blastema cell line expresses C3 mRNA, suggesting that these cells are actually synthesizing C3 locally (19). In the same report, we showed that C3 mRNA and protein were also expressed in the regenerating limb of another salamander species, the axolotl, which does not regenerate eye tissues. Extending this intriguing

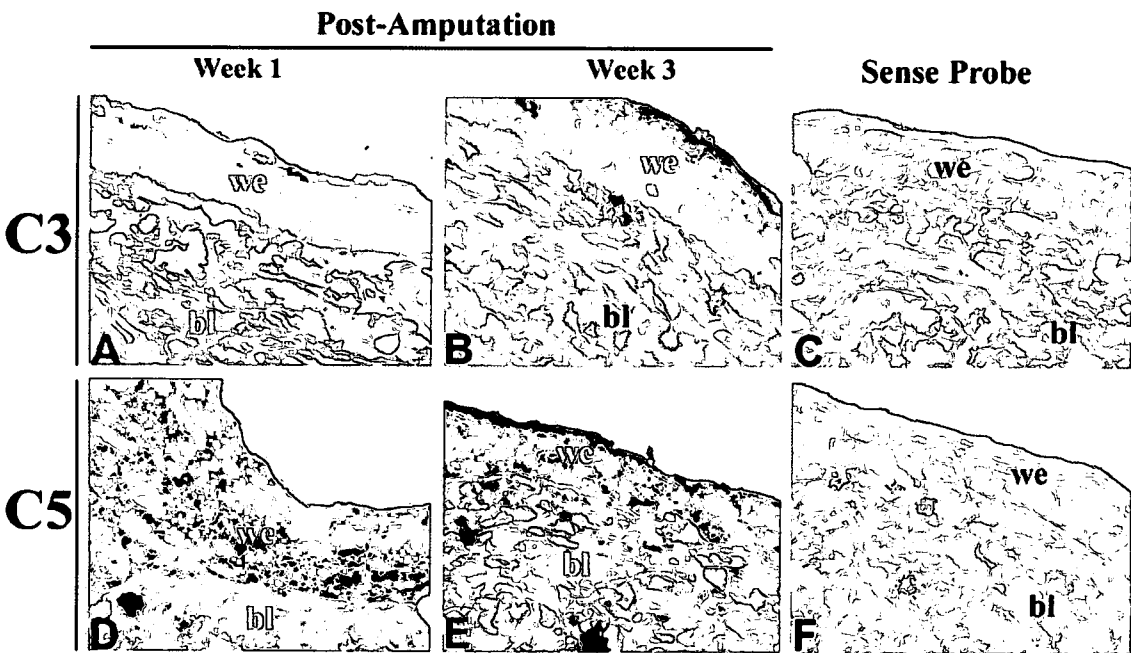


FIGURE 5. Expression of C3 and C5 mRNA during limb regeneration. In situ hybridization shows that C3 (A–C) and C5 (D–F) mRNA are expressed in both WE (we) and the blastema (bl). Images are $\times 40$.

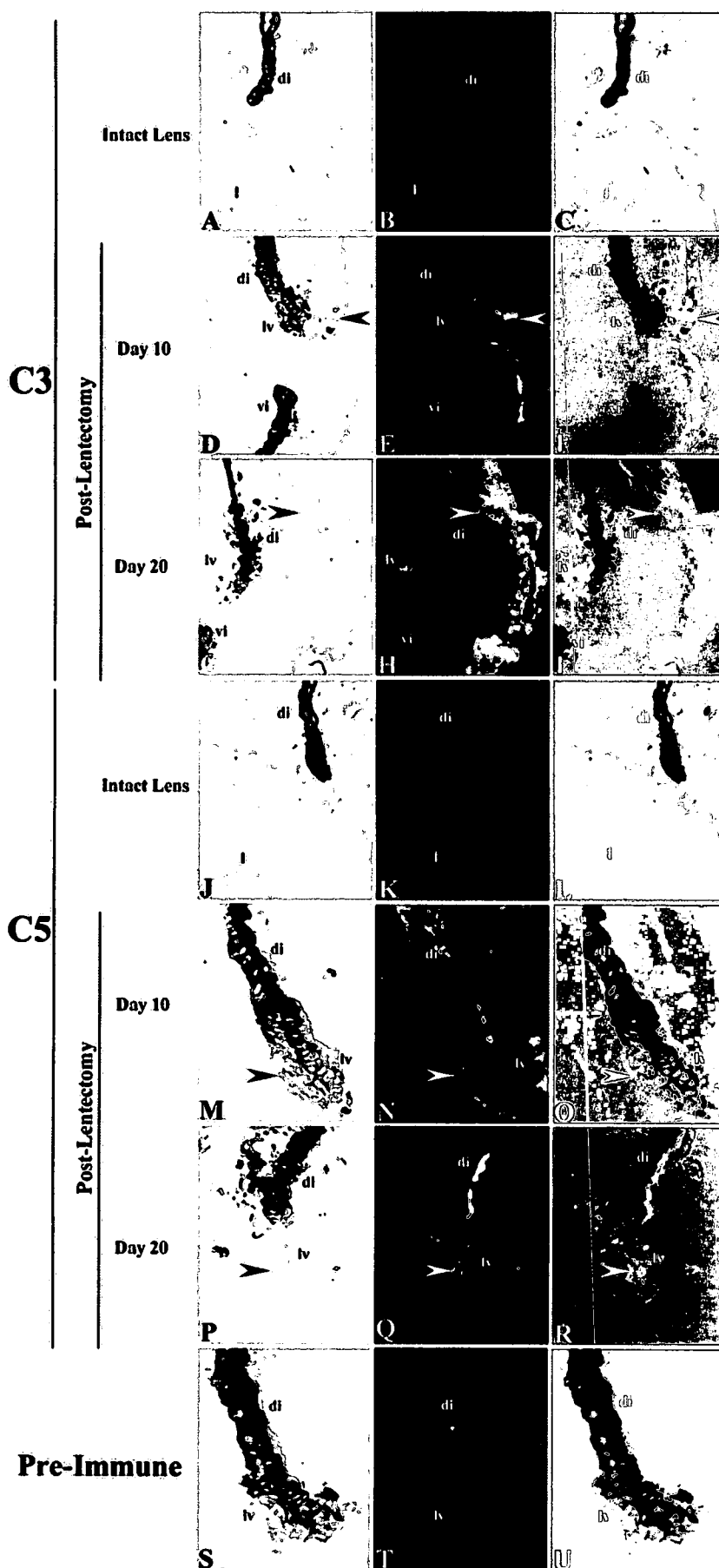


FIGURE 6. Protein expression of C3 and C5 during lens regeneration. The first column contains DIC images (magnification, $\times 20$ in *A-L* and $\times 40$ in *M-U*) of the sections in columns two and three. Column two contains fluorescence images, while the pictures in column three are overlays of the DIC and fluorescence images in columns one and two. The intact lens shows no expression of C3 protein (*A-C*). C3 (red) is expressed in the stroma and pigmented cells of dorsal iris at days 10 (*D-F*) and 20 (*G-I*) postlentectomy. Sections have been counterstained with DAPI (blue) to visualize nuclei. C5 protein expression (red) is shown in *J-R*. There is no C5 expression in the lens of an intact eye (*J-K*). C5 expression is seen in the dorsal iris and lens vesicle at days 10 (*M-O*) and 20 (*P-R*) postlentectomy. There was no fluorescent signal when preimmune serum was used instead of either anti-C3 or anti-C5 Ab (*S-U*). c, Cornea; di, dorsal iris; l, lens; lv, lens vesicle; and vi, ventral iris. Arrowheads indicate areas of C3 or C5 protein expression.

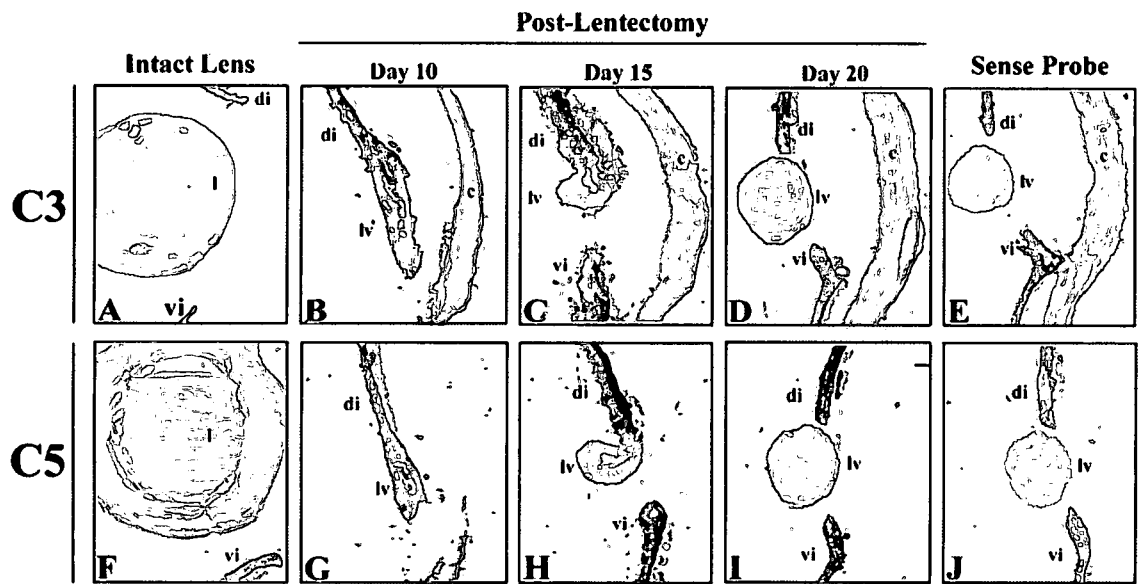


FIGURE 7. Expression of C3 and C5 mRNA during lens regeneration. In situ hybridization shows that C3 (A–D) and C5 (F–I) are expressed in the regenerating lens vesicle. C3 mRNA is not expressed in the intact lens (A), but C3 expression is seen in the lens vesicle at day 10 postlentectomy (B). A similar pattern is seen at days 15 (C) and 20 (D) postlentectomy. A day 20, regenerating lens vesicle probed with the sense probe gives no signal (E). Similarly, an intact lens does not show any C5 expression (F), but lens vesicles at days 10 (G), 15 (H), and 20 (I) postlentectomy express C5 mRNA. There is no staining when a C5 sense probe is used (J). c, Cornea; di, dorsal iris; l, lens; lv, lens vesicle; and vi, ventral iris.

observation to another urodele species that possesses the remarkable capacity to regenerate several of its body parts, we investigated the involvement of complement in urodele regeneration in a more rigorous and systematic manner. In that respect, we focused on dissecting the role of two key components of the complement activation cascade, C3 and C5, in two distinct regenerative processes, limb and lens regeneration.

We have now shown that C3 protein is present throughout the process of limb regeneration in the newt *N. viridescens* and is found mainly in the blastema, with some staining of the WE as well, matching the pattern that we have reported previously for the axolotl, but with a more distinct pattern in the blastema. In contrast, we observed that C5 (not previously reported) is strongly expressed in the WE at all stages of regeneration. This differential distribution of complement proteins is indicative of a specific role in the process of limb regeneration.

During lens regeneration in the newt, the distribution pattern was again complementary, even though both molecules were present in the cornea. C3 was found in the stroma and pigmented epithelial cells of the iris, while C5 was mostly found in the regenerating lens vesicle. No expression was observed in the intact lens. We also show in this study that complement components are locally being synthesized in the eye during the process of regeneration (Fig. 7). It is interesting to note that, even though both C3 and C5 mRNA were expressed in the regenerating lens vesicle, we detected only the C5 protein. There seems to be a regulatory mechanism in place that controls C3 translation, or a rapid degradation or processing of the protein product occurs. However, another possibility is that, in addition to the locally produced C3 and C5, circulating complement molecules move to these tissues during the process of regeneration.

It is possible that these complement molecules play a role in the proliferation and establishment of the blastema (limb) and lens vesicle (lens). It is known that complement components play a role in mitogenic events, as has been reported for C3 during the proliferation of a macrophage-monocyte progenitor lineage (20) and

during the proliferation and growth of B cells in vitro (9). Several studies have suggested that the terminal complexes (C5b-9) can exert a mitogenic effect on various cell types, including mouse fibroblasts (14), human aortic smooth muscle cells (15), and glomerular epithelial cells (17), when administered in sublytic doses (18). Moreover, the anaphylatoxic peptide C5a has been shown to be mitogenic for undifferentiated human neuroblastoma cells (27).

It is also possible that these molecules play a role in early differentiation processes, or even later processes such as differentiation. Reica et al. (28) have found expression of several complement receptors and complement components in normal human early stem/progenitor cells as well as in lineage-differentiated hematopoietic cells. Functional studies should further clarify the role/roles of these molecules in these regenerative processes. For now, it is important to stress that, in other systems, C5 has been shown essential for the process of liver regeneration, because C5 knockout mice exhibit defective liver regeneration after acute toxic injury (20).

Although our studies point to a possible role of the complement system in regenerative processes, its actual role in a pure developmental sense, such as pattern formation, remains unclear. In this respect, it is interesting to note that a molecule with homology to CD59, a protein that binds and inhibits membrane attack complex formation, has recently been labeled as an indicator of blastemal cell identity during limb regeneration (29), i.e., its expression depended on the position of the cells along the proximo-distal axis of the limb. Such discoveries, in association with our data, further support a new role for the complement system in regenerative processes.

Finally, it is interesting to speculate that complement components might be involved in tissue regeneration by binding to cell surface receptors and triggering signaling pathways that modulate cell-cell interactions and/or adhesion. All these could lead to proliferation, differentiation, or positional identity. Alternatively, cleavage of C3 and C5 could generate C3a and C5a which could stimulate the vascular and cellular elements of local inflammation.

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Effects of a CDK inhibitor on lens regeneration

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Lens regeneration in adult newts is always initiated from the dorsal iris by transdifferentiation of the pigment epithelial cells. One of the most important early events should be the ability of pigment epithelial cells to dedifferentiate and re-enter the cell cycle. As a first step in an attempt to study this event, we have decided to examine the effects of a cyclin-dependent kinase-2 inhibitor on lens regeneration. At the appropriate concentration, this inhibitor completely abolished the ability of pigment epithelial cells to form a new lens, but it did not stop them from dedifferentiating and forming a small lens vesicle. The effects of this inhibitor seem to be mediated by its opposite effects on cell proliferation and apoptosis. The inhibitor significantly reduced cell proliferation and enhanced apoptosis of pigment epithelial cells both in vitro and in vivo and of the regenerating lens in vivo. (WOUND REP REG 2004;12:24-29)

Lens regeneration, as occurring in adult salamanders, is controlled by events that are intimately linked to the cell cycle. Upon removal of the lens, the pigment epithelial cells (PECs) from the dorsal iris undergo a dedifferentiation process. These terminally differentiated cells are capable of re-entering the cell cycle, proliferating, and finally transdifferentiating into lens epithelial cells, which consequently will give rise to lens fibers and to the complete regenerated lens.^{1,2} The differentiation of the lens epithelial cells to fibers is also dominated by the loss of organelles and by apoptosis.^{3,4} However important these events of proliferation and apoptosis are for the process of lens regeneration, little is known about the mechanisms and the molecules involved. We have begun to address these issues and as a first step we have decided to

Brdu	5-bromodeoxyuridine
CDK	Cyclin-dependent kinase
CKI	CDK inhibitor
DMSO	Dimethylsulfoxide
PBS	Phosphate buffered saline solution
PEC	Pigment epithelial cells
Rb	Retinoblastoma protein
TBS	Tris buffered saline solution
TUNEL	Terminal deoxynucleotidyl transferase nick end labeling

examine the role of a specific inhibitor of cyclin-dependent kinases (CDKs), which are largely known as important regulators of proliferation, apoptosis, and differentiation in lens development.⁵

CDKs control the progression through the G1 phase of the cell cycle and the initiation of DNA synthesis (S phase). The activities of CDKs are in turn regulated by specific inhibitors called CDK inhibitors (CKIs). There are two classes of CKIs. The first includes the INK4 proteins, named as such because they inhibit CDK4 and also CDK6. These proteins are the p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4c}. The other class contains the inhibitors of the Cip/Kip family, which affect the activities of cyclin D-, E-, and A-dependent kinases. This class includes the proteins p21^{Cip1}, p27^{Kip1} and p57^{Kip2}.⁵⁻⁷ One of the targets of these inhibitors is (via inhibition of CDKs) phosphorylation of the retinoblastoma protein (Rb), which has been shown to be important in lens differentiation as well.^{8,9} When Rb is hypophosphorylated it is active and

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this results in lens fiber differentiation and fiber denucleation. This pathway of events does not require p53. However, when Rb is hyperphosphorylated (inactive), this leads to proliferation and via the activation of p53 to apoptosis in the lens.³ Obviously, the control of Rb phosphorylation, which is mediated by CDK inhibitors p27 and p57, is paramount in establishing the stage for the interplay between proliferation and differentiation of lens cells.⁹

Given the importance of the mechanisms involved in cell proliferation and apoptosis, we have decided to probe the effects of a specific CDK inhibitor on the process of lens regeneration. This inhibitor is 1.8-fold and 9-fold more selective for cdk2 than cdk4 and cdk1, respectively.¹⁰

MATERIALS AND METHODS

A novel cdk2-selective inhibitor, SU9516 from SUGEN (South San Francisco, CA) was used. SU9516, 3-[1-(3*H*-imidazol-4-yl)-meth-(*Z*)-ylidene]-5-methoxy-1,3-dihydroindol-2-one, is a novel 3-substituted indolinole compound.¹⁰ For use in our experiments SU9516 was dissolved in dimethylsulfoxide (DMSO).

Animals and operations

The newt, *Notophthalmus viridescens*, was used in our studies. After lentectomies, the animals were placed in inhibitor solutions. Several concentrations, ranging from 1 μ M-100 μ M, were examined in this study. Control animals were treated with appropriate DMSO solutions. The newts were kept for 20 days, at which time the eyes were collected and examined histologically. All operations were performed under anesthesia according to animal care protocols approved by the University of Dayton IACUC. MS222 (Sigma Chemical, St. Louis, MO) was used as anesthesia.

BrdU incorporation in cultured cells

To examine the effects of SU9516 on cell proliferation, we treated cells with the compound (10 μ M for 24 hours and pulsed them with 5-bromodeoxyuridine [BrdU] for 60 minutes). For these studies, we used a rat cell line (LacZ rat gliosarcoma, CRL-2200) and primary cultures of PECs isolated from the dorsal iris. For this procedure, we used the In Situ Cell Proliferation Kit, FLUOS, Cat. no. 1810740 from Roche Molecular Biochemicals (Indianapolis, IN). To the culture medium, 1/10 volume of BrdU labeling solution was added, and incubated in a humidified chamber at 25 °C for 60 minutes. After that, cells were washed three times with phosphate buffered saline solution (PBS) and fixed with 3 volumes 50 μ M glycine solution pH 2.0 with 7 volumes 100 percent ethanol at 4 °C for 30 minutes. Following fixation, the cells were washed with PBS and HCl-

denaturation solution was added at room temperature for 20 minutes. Cells were then rinsed three times with PBS and were incubated with incubation buffer at room temperature for 10 minutes. Anti-BrdU-FLUOS antibody working solution was added to cells and incubated in a humidified chamber at 37 °C for 45 minutes. Cells were then washed twice with PBS, covered with GEL/MOUNT (Biomedica Corp., Foster City, CA), and allowed to dry before analysis. Several areas in different slides were selected and positive vs. negative cells were counted.

Apoptosis analysis in vitro

The same cells used for the proliferation assays were also examined for the effects of the inhibitor on apoptosis using the terminal deoxynucleotidyl transferase nick end labeling (TUNEL) procedure. The TdT-FragEL™, DNA Fragmentation Detection Kit, Cat# QIA33 from Oncogene Research Products (Boston, MA) was used. After treatment (10 μ M, 24 hours) cells were fixed with 4 percent formaldehyde (in PBS) at room temperature for 10 minutes. Cells were then pelleted by centrifugation for 5 minutes and resuspended in 80 percent ethanol, fixed (100 μ l) onto poly L-lysine coated glass slides and stored at 4 °C. Slides were rehydrated by immersion in tris-buffered saline solution (TBS) for 15 minutes at room temperature. Sections were incubated with 20 μ g/ml proteinase K at room temperature for 5 minutes and then the slides were rinsed two to three times with TBS. To inactivate endogenous peroxidase the slides were treated with 3 percent H₂O₂, incubated at room temperature for 5 minutes, and rinsed two to three times with TBS. For labeling, sections were covered with 1X TdT Equilibration Buffer and incubated at room temperature for 30 minutes followed by TdT Labeling Reaction Mixture, which was placed onto sections. Each specimen was covered with a piece of parafilm and the slides were incubated in a humidified chamber at 37 °C for 1.5 hours. After that time, parafilm was removed from the slides, and slides were rinsed two to three times in TBS. To terminate the reaction, sections were incubated with Stop Solution at room temperature for 5 minutes and then rinsed two to three times with TBS. For detection, sections were covered with Blocking Buffer at room temperature for 10 minutes. The blocking buffer was blotted from the sections and immediately sections were covered with diluted 1X conjugate and incubated in a humidified chamber at room temperature for 30 minutes. Sections were then incubated with diaminobenzidine solution (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and incubated for 15 minutes at room temperature, rinsed with deionized H₂O and covered with Crystal/Mount (Biomedica Corp.) and allowed to dry before observation. Several areas in different slides were selected and

positive vs. negative cells were counted. The student's *t*-test was used for statistical analysis.

in vivo proliferation and apoptosis determination

Animals were treated with the CDK inhibitor (25 μ M) for 5 days between day 12 and 15 postlentectomy. After the treatment (the 16th day), eyes were injected with BrdU and were collected 1 day later. For analysis of apoptosis, animals were treated the same way and eyes were collected at the end of the treatment. We selected this treatment and not a continuous one because continuous treatment does not result in any regenerating lens (see results), and thus we would not be able to observe proliferation and cell death patterns in the regenerating lens. The protocols for BrdU and cell death detection were similar to the ones used for cultured cells, but from different kits. The In Situ Cell Death detection kit (TMR-red (Roche) was used for apoptosis and the BrdU labeling and detection kit I (Roche) was used for proliferation. After collection, the eyes were fixed in 4 percent formaldehyde for 10 minutes and then were treated with 30 percent sucrose for cryoprotection. The whole eyes were sectioned and proliferation and apoptosis profiles were observed in selected sections, which represented the same area unit in all samples. The student's *t*-test was employed for statistical analysis.

RESULTS AND DISCUSSION

Lens regeneration in the adult newt begins with dedifferentiation and proliferation of dorsal iris PECs. By

dedifferentiation we mean the loss of characteristics that define the PECs, such as pigmentation. Dedifferentiation initiates molecular events, such as re-entering the cell cycle, which is necessary for cell proliferation and the subsequent regeneration of the lens. The first peak of cell proliferation in the dorsal iris is observed between 4 and 6 days postlentectomy.¹¹ At about 10 days postlentectomy, a lens vesicle is formed from the depigmented dorsal PECs. Around 12–16 days postlentectomy, the internal layer of the lens vesicle thickens and synthesis of crystallins begins. From 12 to 15 days postlentectomy, a second peak of cell proliferation is observed in the dorsal iris.¹¹ This marks the beginning of primary lens fiber differentiation (Figure 1a). During days 15–19, proliferation and depigmentation of PECs slow down. In the internal layer of the regenerating vesicle, the lens fiber complex is formed and in the margin of the external layers nondividing secondary lens fibers appear. By 18–20 days the PECs have stopped proliferating and the lens fibers continue to accumulate crystallins (Figure 1b). Lens regeneration is considered complete by day 20–25.^{1,2}

Therefore, lens regeneration is possible by transdifferentiation, which is the transformation of one cell type to another (in this case PECs to lens cells). The process of transdifferentiation has been proven beyond any doubt in this system. When single PEC cells are placed in culture the process of transdifferentiation can be observed.^{1,12} As the PECs proliferate, they become depigmented and then transdifferentiate to lens cells.

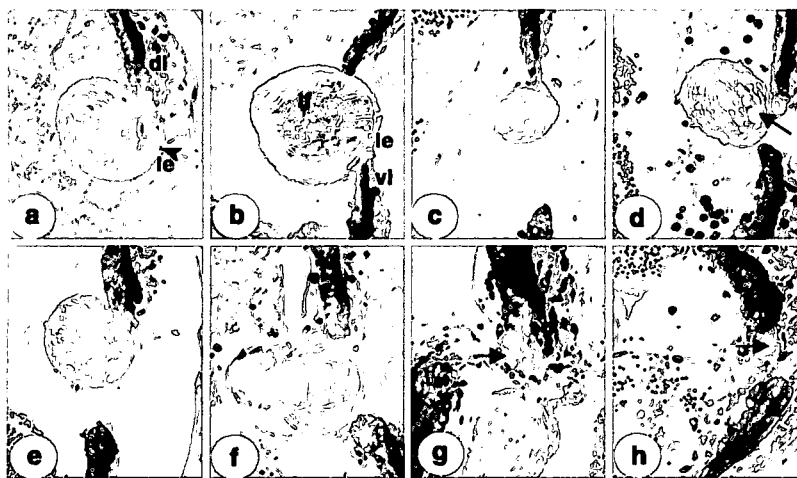


FIGURE 1. Histologic appearance of lens tissue following *in vivo* treatment of newts with a CDK inhibitor. (a) Normal lens regeneration 15 days postlentectomy. Note the lens fiber differentiation from the posterior part of the lens (arrow); le: lens epithelium, di: dorsal iris. (b) Normal lens regeneration 20 days postlentectomy. Note that the lens has been regenerated and is packed by lens fibers (lf); le: lens epithelium, vi: ventral iris. (c) A regenerated lens 15-days postlentectomy after treatment with the CDK inhibitor during days 4–6 postlentectomy. Note that the lens is smaller. (d) Same treatment as in (c), but the lens was examined 20 days postlentectomy. Note that the lens is vacuolated (arrow). (e, f) Regenerated lens 20 days after lentectomy following treatment with the CDK inhibitor during days 15 postlentectomy. In (e) we can see a retarded lens and in (f), regeneration of two lenses. (g, h) Two cases of inhibited lens regeneration, but with a small dedifferentiated vesicle (arrows) in eyes treated continuously with 25 μ M of the CDK inhibitor. The eyes were examined at day 20 postlentectomy. (Original magnification for all sections $\times 40$)

Therefore, while in many other regenerative tissues stem cells may play a role, such a possibility is very unlikely for lens regeneration.

Several treatments with the CKI were performed (see Table 1 for a summary of the treatments and effects). Continuous treatment at 1 μM concentration did not affect lens regeneration at all. Also, treatment at 100 μM was lethal for the animals. The most dramatic effects that were not lethal were observed when the animals were treated at 25 μM concentration. When animals treated during the first peak of proliferation (4–6 days postlentectomy) were examined, only two lenses out of 14 were affected. These lenses were of a good size (even though smaller than the untreated control), but they were vacuolated (Figure 1c, d). When animals treated during the second peak of proliferation (12–15 days postlentectomy) were examined, three out of 16 lenses were affected. Of these three lenses, two were retarded and the other case was a double lens (Figure 1e, f). When animals were treated continuously (from day 1 postlentectomy), examination of 18 eyes showed that in eight eyes lens regeneration was completely inhibited and in the remaining ten lenses regeneration was severely impaired with only a small vesicle differentiated from the dorsal iris (Figure 1g, h). The major point of these results is that a continuous treatment is necessary for the CKI to exert its effect on the morphogenesis of the regenerated lens. Short treatments can affect regeneration somewhat, but obviously the process is able to recover. The vacuolated lenses are reminiscent of cataracts observed in lenses. The only case of double lens is, of course, interesting. Such a result is very rare and we have never seen it during normal regeneration. The only other cases of such double lenses we have seen were obtained by the use of exogenous fibroblast growth factor.¹³ We believe that cells at the equator change polarity and differentiate to fiber cells on the posterior part of the eye as well as creating two lenses. Abnormal proliferation patterns could be the reason for such an effect. The fact that the majority of the cases treated continuously at the 25 μM concentration were totally inhibited or only showed early vesicle differentiation is very interesting. Obviously, the inhibitor does not completely inhibit the dedifferentiation process, but it does inhibit the fiber differentiation process in 100 percent

of the cases. This might be explained by the effects of this inhibitor on cell proliferation and apoptosis (see below).

Having established the effects of this CKI on morphogenesis of the regenerating lens, we decided to examine its effects on cell proliferation and apoptosis. For this, we analyzed profiles of treated cells in vitro and during lens regeneration in vivo. For the in vitro studies, we used an established rat cell line as well as primary cultures of PECs isolated from the dorsal iris of the adult newt. The rat cell line was used only as a reference point. When cells were treated with the inhibitor, incorporation of BrdU was much lower than in cells treated with only DMSO for both cell types (Figure 2A). The effects on apoptosis were opposite, with the inhibitor treatment resulting in a significant increase of apoptosis (Figure 2B). The figures for apoptosis were higher for the rat cell line than the PECs, but this is probably due to the

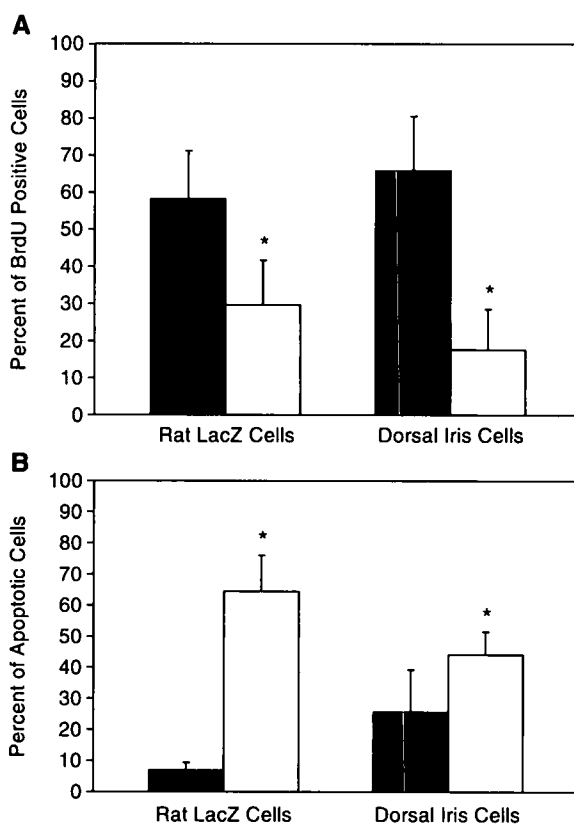


FIGURE 2. Effect of in vitro CKI inhibitor treatment on cell proliferation and apoptosis. (A) Effects of the cdk-2 inhibitor on cell proliferation. Cells were treated in vitro and analysis was performed 24 hours later. Treatment of cells significantly inhibited proliferation as indicated by the incorporation of BrdU in the cell line Rat LacZ and in primary dorsal iris cells from the newt. (B) Effects of the cdk-2 inhibitor on apoptosis of the same cells as in (A). Treatment resulted in an increased rate of cell apoptosis. The differences between control (black bars) and treated cells (white bars) are statistically significant (asterisks indicate $p < 0.001$; t -test, standard deviation). Five different areas were examined and from the total cell number, the percentage of the positive cells was calculated.

Table 1. Number of affected regenerated lenses due to the CKI treatment

Duration of treatment	CKI concentration (μM)		
	1	25	100
4–6 days postlentectomy	No effect	2/14	lethal
12–15 days postlentectomy	No effect	3/16	lethal
Continuous	No effect	18/18*	lethal

*In 8/18 complete inhibition was observed and in 10/18 only a small lens vesicle was formed.

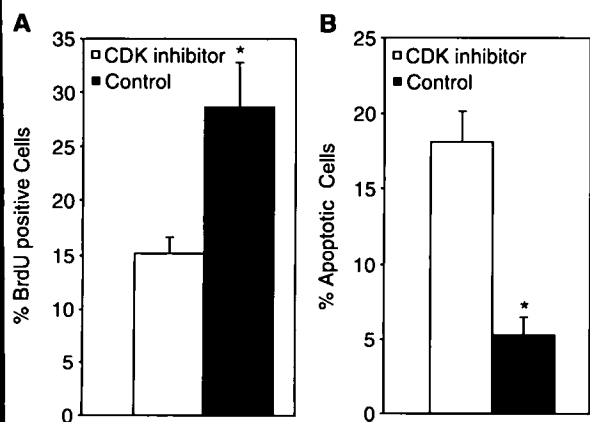


FIGURE 3. Effect of in vivo CKI treatment on cell proliferation and apoptosis. (A) Effects of the CKI on cell proliferation as measured by BrdU incorporation. The percentage of positive cells was taken from counting lens cells from seven eyes treated with the inhibitor and five control eyes. (B) Effects of the CKI on apoptosis in the regenerating lens. The same number of eyes was examined as in the proliferation assay. From all lens sections, we selected the cells from the center as the area unit. The same area unit was considered in all eyes. Asterisk indicates $p < 0.001$ (two tailed, two sample equal variance t -test between corresponding data sets).

fact that the PECs are primary cells. Similar effects were obtained in previous studies using different cancer cell lines.¹⁰ When proliferation and apoptosis profiles were examined during lens regeneration in vivo it was found that the CKI reduced cell proliferation and increased apoptosis in the regenerating lens.

As explained in the Methods section, we selected short treatments between 12 and 15 days postlentectomy because continuous treatments do not result in a visible differentiated lens and because at the selected period of treatment (12–15 days) there is active differentiation of the lens fibers and proliferation. We examined seven eyes from treated animals and five eyes from controls. The positive cells (either BrdU-labeled, or TUNEL-positive) were counted and the percentage was calculated with respect to the total cell number in selected area units (the area unit was the two most center sections from a lens). As in the case with the cultured cells, we found that the CKI decreased proliferation and increased apoptosis in the regenerating lens. These data are shown in Figure 3. Representative sections showing the effects of the inhibitor on proliferation and apoptosis are shown in Figure 4. Green fluorescence shows incorporation of BrdU in control

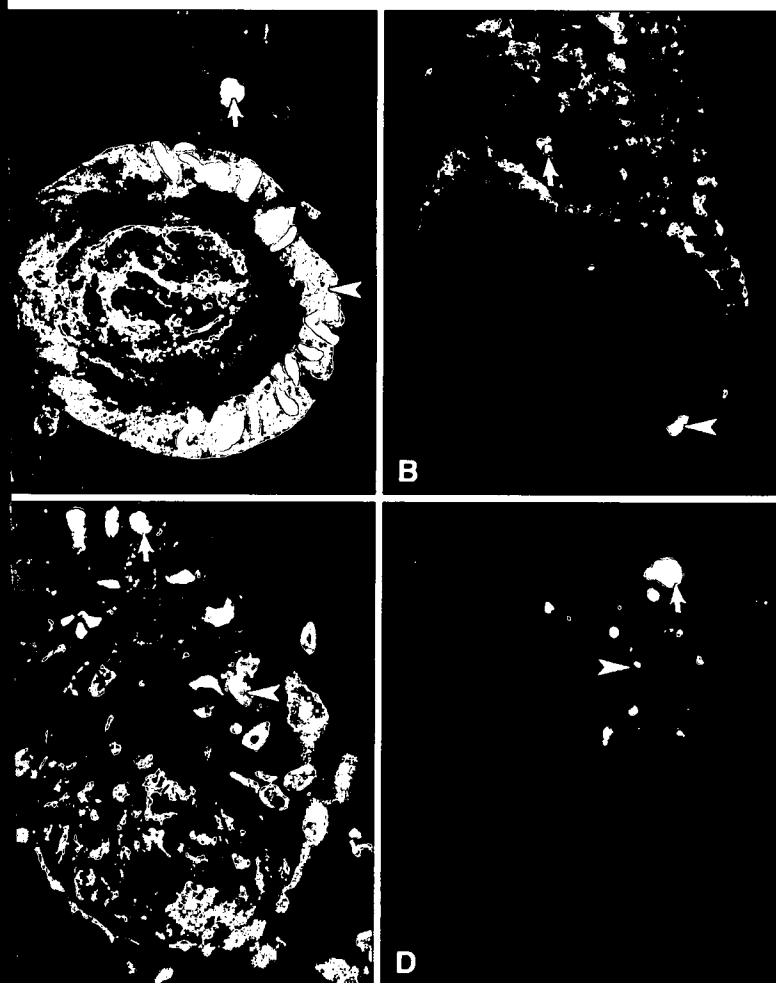


FIGURE 4. Effects of the CKI on cell proliferation and apoptosis during lens regeneration. (A) BrdU incorporation in the dorsal iris PECs (arrow) and in the regenerating lens (arrowhead) in a control eye 16 days postlentectomy. (C) BrdU incorporation in the dorsal iris PECs (arrow) and in the regenerating lens (arrowhead) in a CKI-treated eye 16 days postlentectomy. Note the dramatic decrease of positive cells in the lens epithelium of the treated eyes. (B) Apoptosis in a control regenerating lens 16 days postlentectomy. Note that there is one apoptotic cell in the dorsal iris (arrow) and one in the regenerating lens (arrowhead). (D) Apoptosis in a CKI-treated regenerating lens 16 days postlentectomy. Note a dramatic increase in cells with fragmented DNA.

DMSO-treated (Figure 4A) and CKI-treated (Figure 4C) eyes undergoing lens regeneration. Red fluorescence shows fragmented DNA in apoptotic cells in control (Figure 4B) and CKI-treated (Figure 4D) samples. Note the marked decrease of BrdU-positive cells and increase of apoptotic cells due to the influence of the CKI.

The present results strongly suggest that lens regeneration could be regulated by specific interplay of mechanisms involved in cell proliferation and cell death. The downstream targets of this inhibitor (and of course of cdk2, and cdk4) in lens regeneration are not at the present known, but eventually such CKIs as the one tested in the present work should prove valuable in the future. One possible candidate might be Rb, because it is expressed during lens regeneration¹⁴ and the inhibitor has been shown to reduce Rb phosphorylation in human colon carcinoma cell lines.¹⁰ It is interesting to note here that the same effects seen by the use of SU9516 can be seen in cell lines with the use of E2F-derived oligopeptides.¹⁵ These peptides block the phosphorylation site of cyclin A/cdk2 and cyclin E/cdk2 complexes. E2F is a protein that binds Rb but dissociates upon Rb phosphorylation. It is believed that the apoptotic effects of SU9516 may relate to its effects on the cyclin A/cdk2 complex rather than cyclin E/cdk2. This is because cyclin A/cdk2 can negatively regulate E2F and thus inhibition of cdk2 allows an increase in free E2F, which can induce both cell proliferation and apoptosis.^{16,17}

Proliferation also occurs in the ventral iris after lentectomy, but is short lived. This may mean that regulation of proliferation and apoptosis might be vital to ensure that the ventral iris does not transdifferentiate to lens as its dorsal counterpart. Therefore, detailed studies on control of proliferation and apoptosis using the factors discussed in this paper might shed light on this important regulative process and on the molecular mechanisms of lens regeneration.

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A novel role of the hedgehog pathway in lens regeneration

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Abstract

Lens regeneration in the adult newt is a classic example of replacing a lost organ by the process of transdifferentiation. After lens removal, the pigmented epithelial cells of the dorsal iris proliferate and dedifferentiate to form a lens vesicle, which subsequently differentiates to form a new lens. In searching for factors that control this remarkable process, we investigated the expression and role of hedgehog pathway members. These molecules are known to affect retina and pigment epithelium morphogenesis and have been recently shown to be involved in repair processes. Here we show that *Shh*, *Ihh*, *ptc-1*, and *ptc-2* are expressed during lens regeneration. The expression of *Shh* and *Ihh* is quite unique since these genes have never been detected in lens. Interestingly, both *Shh* and *Ihh* are only expressed in the regenerating and developing lens, but not in the intact lens. Interfering with the hedgehog pathway results in considerable inhibition of the process of lens regeneration, including decreased cell proliferation as well as interference with lens fiber differentiation in the regenerating lens vesicle. Down-regulation of *ptc-1* was also observed when inhibiting the pathway. These results provide the first evidence of a novel role for the hedgehog pathway in specific regulation of the regenerating lens.

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Keywords: Lens; Transdifferentiation; Regeneration; *Shh*; *Ihh*; *ptc-1*; *ptc-2*; HIP; KAAD

Introduction

Celebrated as the champion of regeneration research for hundreds of years, the adult newt is the prime paradigm for regenerating entire body parts by transdifferentiation of terminally differentiated cells (Brockes and Kumar, 2002; Tsonis, 2000,2002). Adult newts are able to regenerate their limbs, tail, retina, and lens among other body parts. We have concentrated on lens regeneration because it involves a transformation from one cell type to another and therefore can be regarded as the simplest system for studying regeneration via transdifferentiation. Following lentiectomy, a new lens is regenerated by transdifferentiation of the pigmented epithelial cells of the dorsal iris. These cells proliferate as they lose their pigments (dedifferentiation) and eventually

differentiate into lens cells. The ventral iris does not contribute to this event, even though it does initially reenter the cell cycle (Del Rio-Tsonis and Tsonis, 2003). Conveniently, the ventral iris can be used as a negative control within the lens regeneration process and can allow for comparisons at the molecular level with the dorsal iris. The restriction implies specific gene expression unique to either the dorsal or the ventral iris. We believe that this restriction must be related to cell signaling, cell–cell communication, and cell interactions since newt ventral irises or cells from both the dorsal and the ventral irises of species unable to regenerate a lens *in vivo* are able to transdifferentiate *in vitro* (Eguchi, 1998; Tsonis et al., 2001).

In pursuing experiments along these lines, we decided to examine the expression and role of molecules involved in the hedgehog pathway. Members of the hedgehog (Hh) gene family are key signaling molecules important in many developmental processes in vertebrates. The products of these genes are secreted proteins that act as short- or long-range signals (Drossopoulou et al., 2000; Gritli-Linde et al.,

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2001; Lewis et al., 2001; Panman and Zeller, 2003; Zeng et al., 2001). Sonic hedgehog (Shh) has been found to regulate the dorsoventral patterning of the neural tube and the somites and the anteroposterior axis of the developing limb bud (Drossopoulou et al., 2000; Ekker et al., 1995; Ericson et al., 1996; Panman and Zeller, 2003; Riddle et al., 1993; Schauerte et al., 1998; Wijgerde et al., 2002; Yang et al., 1997). In addition, Shh activity from the ventral forebrain regulates the spatial expression of Pax-6 and therefore plays a crucial role in the development of the midline and consequently of the two eyes (Ekker et al., 1995; Macdonald et al., 1995).

In the developing eye, *Shh* is expressed in the ganglion cell layer of the retina (Perron et al., 2003; Wallace and Raff, 1999; Zhang and Yang, 2001a,b). It has been shown that Shh plays an important role in the differentiation of photoreceptors in the developing eye as well as in controlling the ganglion cell population (Levine et al., 1997; Neumann and Nusslein-Volhard, 2000; Zhang and Yang, 2001b). Shh signaling from the retinal ganglion cells is also required for normal laminar organization in the vertebrate retina (Wang et al., 2002).

The importance of Shh in eye development has been illustrated by interfering with normal Shh activity. Defective Shh mutants, overexpression of Shh, and inhibition of the hedgehog pathway in several animal models result in eye defects and/or 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, 2001a). These data suggest that the hedgehog pathway is indeed vital in eye morphogenesis. Overexpression of *Shh* in zebrafish and *Xenopus* embryos reduces the expression of *Pax-6* and affects eye morphogenesis (Macdonald et al., 1995; Perron et al., 2003; Sasagawa et al., 2002). Specifically in *Xenopus*, this overexpression affects the dorsal–ventral and proximodistal axis of the retina (Perron et al., 2003; Sasagawa et al., 2002). In addition, perturbing the hedgehog pathway in embryos that have an eye field established results in severe defects in retinal pigment epithelial cell differentiation (Perron et al., 2003). In chicks, overexpression of Shh causes retina cells to switch fates and become retinal pigment epithelial cells, while inhibition of Shh transforms pigment epithelial cells to neural retina (Zhang and Yang, 2001a).

Indian hedgehog (*Ihh*) and Desert hedgehog (*Dhh*) are other members of the hedgehog family that are expressed in the retina. While *Ihh* has been exclusively detected in the retinal pigmented epithelial cells, *Dhh* has been found in both the retinal pigmented epithelium (RPE) and the neural retina (Levine et al., 1997; Perron et al., 2003; Takabatake et al., 1997). The functions of hedgehog molecules are mediated by binding to their receptors patched 1 (*ptc-1*) and patched 2 (*ptc-2*) (Carpenter et al., 1998; Marigo et al., 1996; Stone et al., 1996), which are also expressed in the eye (Perron et al., 2003;

Takabatake et al., 1997; Zhang and Yang, 2001b). Despite the role of the hedgehog pathway in neural retina and RPE development and differentiation and its role in the establishment of the dorsal–ventral and proximodistal axis of the eye, this pathway has not been clearly associated with lens development and differentiation (Levine et al., 1997; Neumann and Nusslein-Volhard, 2000; Perron et al., 2003; Sasagawa et al., 2002; Zhang and Yang, 2001a,b; Wang et al., 2002). An interesting report, however, has shown that in zebrafish *Gli-2* (a downstream effector of the Hh pathway) mutants, the adenohipophysis transdifferentiates to lens (Kondoh et al., 2000). In addition, data have been presented in the literature to indicate that *Shh*, *Ihh*, and their receptors are not expressed in the lens (Levine et al., 1997; Takabatake et al., 1997). However, we have found that *Shh*, *Ihh*, and their receptors are expressed during lens development and regeneration. Our functional studies suggest that the hedgehog pathway is involved in regulating the regenerative process of the lens. These results indicate a novel function of hedgehog members that might bear significance in controlling this unique regenerative process.

Materials and methods

Animals

Adult newts (*Notophthalmus viridescens*) were obtained from Mike Tolley Newt Farm (Nashville, TN). For surgical procedures and euthanasia, the animals were anesthetized using a 0.1% 3-aminobenzoic ethyl ester solution. Eye tissues were collected for histology, in situ hybridization, immunohistochemistry, BrdU staining, and RNA collection. Fixed newt embryos were obtained from Dr. H-G. Simon (Northwestern University, Chicago, IL).

In situ hybridization

In situ hybridizations were carried out as previously described for newt tissues (Del Rio-Tsonis et al., 1999). Probes used were made from newt clones provided by Dr. J. P. Brockes (*Ihh*), Dr. K. Takeshima (*Shh*, *ptc-1*, and *ptc-2*), and Dr. Y. Imokawa (*Shh*).

Total RNA isolation

Total RNA was extracted from intact dorsal iris, ventral iris, retina, and lens, as well as from these tissues during different stages of regeneration and from developing lenses that were carefully isolated from fixed newt embryos at stages 39–44 according to Khan et al. 1999. Whole iris or whole eyes were also collected. When tissue was abundant, RNA was extracted using TRIzol reagent (Gibco, Grand Island, NY, USA) following the manufacturers instructions. When the amount of tissue was small, RNA isolation was

performed using the NucleoSpin® RNA and Virus Purification Kit (BD Biosciences, Palo Alto, CA). RNA yield was determined by UV spectrophotometry.

Reverse transcription

Up to one microgram of total RNA was used for reverse transcription. For RNA isolated using TRIzol reagent, RNA was incubated with 1 unit of RQ1 DNase (Promega, Madison, WI) at 37°C for 20 min. One microliter, 0.5 mM EGTA was added to inactivate the DNase followed by incubation at 65°C for 10 min. The reverse transcriptase steps were performed using a standard protocol using Superscript™ II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA). For reverse transcription of RNA isolated with the NucleoSpin® RNA and Virus Purification Kit, no DNase treatment was included, as it was done during the isolation.

Amplification of cDNA in lens tissue

The Super SMART™ PCR cDNA Synthesis Kit (BD Bioscience) was used to reverse transcribe and amplify 21-day regenerating lens RNA isolated with the NucleoSpin® RNA and Virus Purification Kit following the manufacturer's instructions. PCR was then carried out as described.

PCR primers

PCR primer sequences used include the following: *EF-1α* forward 5'-ATC GAC AAG AGA ACC ATC GA-3' reverse 5'-GTG ATC ATG TTC TTG ATC AA-3'; *Shh* forward 5'-ACC TCC TCT TTG TAG GCC AGG C-3' reverse 5'-GTG CCA CTT ACA GAC TTC AGT-3'; *Ihh* forward 5'-GTG CCA CTT ACA GAC TTC AGT-3' reverse 5'-CCA CAG CAA AGC AGG ATA CGA-3'; *ptc-1* forward 5'-AAC AAA AAT TCA ACC AAA CCT C-3' reverse 5'-TGT CTT CAT TCC AGT TGA TGT G-3'; and *ptc-2* forward 5'-CAC CTC TGT CGA TGG CTT TA-3' reverse 5'-CAG TTC CTC CTG CCA GTG CA-3'. Resulting PCR product for *EF-2α* is 203 bp, *Shh* is 278 bp, *Ihh* is 198 bp, *ptc-1* is 243 bp, and *ptc-2* is 223 bp.

PCR

PCR reactions were initially incubated at 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, annealing temperature (*EF-1α* 53°C, *Shh* 53°C, *Ihh* 55°C, *ptc-1* 55°C, *ptc-2* 50°C) for 45 s, and extension at 72°C for 1 min. A final extension cycle of 72°C for 5 min was included. *EF-1α* was used as a positive control, and no RT was added to the negative controls. The PCR product was prestained with an equal volume of a 1:250 dilution of Sybr-green (Molecular Probes, Eugene, OR) for at least 30 min and then separated on a 2% agarose gel and visualized by using the Storm Scanner 1500 (Amersham Biosciences, Piscataway, NJ).

Primary iris culture treated with or without KAAD to determine *ptc-1* regulation

Newt irises from 8-day regenerating eyes (irises express *ptc-1* throughout regeneration) were isolated and cultured in vitro with or without KAAD at a concentration of 20 μM. Control samples were treated with the same amount of ethanol present in the KAAD samples. Irises were incubated at room temperature for 24 h and then collected for RNA isolation and processed for examination of *ptc-1* expression.

HIP treatment to determine *ptc-1* regulation in iris tissue

Due to differences in culturing conditions (especially temperature), newt irises could not be cultured in vitro with mammalian HIP-expressing cells effectively. Therefore, pellets of HIP-expressing cells or pellets of control GFP-expressing cells were implanted in the newt eye cavity 3 days postlensectomy (see section on inhibition studies using HIP for details on cell pellet preparation). The animals were kept in normal conditions for two more days, and then they were sacrificed for iris collection. RNA was extracted from the iris tissue as described above to examine *ptc-1* expression (as mentioned above, irises express *ptc-1* throughout regeneration; therefore, the timing of this experiment is not critical).

Semiquantitative PCR to determine *ptc-1* regulation

To determine relative levels of *ptc-1* mRNA, cDNAs were reverse transcribed from total RNAs isolated from iris tissue incubated with either KAAD or 100% ethanol (see primary iris culture) or from irises dissected from eyes treated with HIP- or GFP-expressing cells as mentioned above. The NucleoSpin® RNA and Virus Purification Kit was used for RNA isolations. To perform a semiquantitative comparison, both *ptc-1* and *EF-1α* were optimized for cycle number, annealing temperature, and cDNA amount (data not shown). *EF-1α* was used as an internal control to normalize *ptc-1* expression levels. Increasing amounts of cDNA were used for both *Ptc-1* and *EF-1α* to demonstrate that values used for quantification were taken from the exponential phase of the PCR and were not obtained from a saturated PCR reaction. PCR was carried out using one cycle of 95°C for 5 min, followed by 34 cycles of 95°C for 45 s, 53°C (*EF-1α*) or 55°C (*ptc-1*) for 45 s, 72°C for 1 min, and a final synthesis cycle at 72°C for 5 min. PCR products were separated on a 2% agarose gel and poststained for 1 h with Sybr-green (Molecular Probes). Images were captured using the Storm Scanner 1500 and quantified using Imagequant software (Amersham Biosciences).

Inhibition studies using KAAD

Heparin beads were incubated in a 200-μM solution of KAAD (a potent cyclopamine derivative: 3-keto, *N*-amino-

ethyl aminocaproyl dihydrocinnamoyl, generous gift from Dr. James Chen and Dr. Philip Beachy) in 100% ethanol for 2 h at 4°C. Control beads were processed in the same way but incubated in 100% ethanol. Fifty-six eyes were lentectomized and KAAD beads were introduced in the eyes. At 5 days postlentectomy, a second set of KAAD beads were placed in the eyes. At the same time, 23 eyes were used as controls following the same procedure but introducing control beads with ethanol into the eyes. The eyes were collected at 20 days postlentectomy and processed for histology. The sections were stained with hematoxylin and eosin.

Inhibition studies using HIP

Mammalian 293 cells were transiently transfected with either a Myc-HIP expression vector or with a control plasmid with GFP (Chuang and McMahon, 1999; Zeng et al., 2001). A hanging drop protocol was followed to pellet the cells. EDTA-treated cells were concentrated to 5×10^7 cells/ml and subsequently aliquoted in 30 μ l drops that were placed in a Petri dish, which was inverted and incubated for

3 h at 37°C and 5% CO₂. The same surgical procedure, described for the KAAD experiment, was followed on 18 newt eyes, but this time cell pellets expressing HIP were introduced in the eye cavity instead of beads at 0 and 5 days postlentectomy. Seventeen eyes were used as controls, using cell pellets transfected with the control plasmid. The eyes were collected 20 days postlentectomy and processed for histology as described above.

BrdU experiments and immunohistochemistry

Heparin beads incubated with either KAAD (200 μ M) or with 100% ETOH (controls) were introduced into eyes of newts that had been lentectomized 12 days prior. Likewise, HIP- or GFP-expressing cells (controls) were implanted in a parallel set of experiments. In animals used for studying cell proliferation, 1 μ l of BrdU solution (10 mM) was then injected into the eye. Twenty-four hours later, the eyes were collected and fixed in 4% formaldehyde solution. The samples were then embedded in OCT (Andwin Scientific, Warner Center, CA) and sectioned at 10 μ m. For the β -crystallin expression study, the tissues

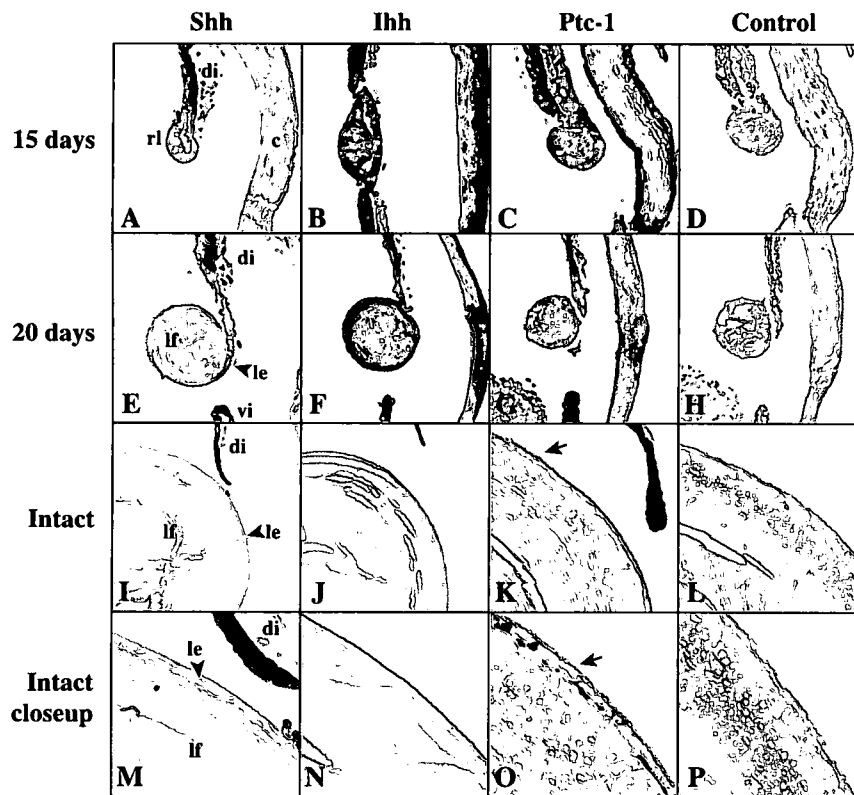


Fig. 1. Expression of *Shh*, *Ihh*, and *ptc-1* in the regenerating lens detected by in situ hybridization. Selected sections from eyes at 15 (A–D), 20 days (E–H) after lentectomy, and of intact eyes (I–P). Note expression of all genes in the regenerating lens vesicle (A–C). As the regenerate developed, the expression of *Shh* and *Ptc-1* was in both lens epithelium (le) and lens fibers (lf) (F and G), but mostly in the lens epithelium and secondary fibers for *Shh* (E). In the intact lens, only expression of *ptc-1* was detected in the lens epithelium (K and O; arrow). Controls are representative hybridizations with the sense probe for *Shh* (D, H, L, and P), but all samples had similar background. M–P represent a closeup of the intact lenses (I–L), respectively. Arrowheads in I and M point to the lens epithelium (le). di: Dorsal iris; vi: ventral iris; c: cornea; rl: regenerating lens; le: lens epithelium; lf: lens fibers.

were collected and processed, using the same protocol, 3 days after the beads were implanted. Nine KAAD-treated eyes and eight control eyes were used for the BrdU experiment, as well as six HIP-treated eyes and five GFP control eyes. BrdU was detected using a 1:100 dilution of the primary anti-BrdU antibody (Sigma, St. Louis, MO). To study the expression of β -crystallin, 14 KAAD-treated and 10 control eyes were processed. Ten-micron sections were incubated overnight at room temperature with primary antibody (anti- β -crystallin; designated $\beta 6$; Sawada et al., 1993) (diluted 1:10 in blocking solution). A 1:10 dilution of FITC conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used to detect the primary antibody. Vectashield (Vector Labs, Burlingame, CA) was used to protect the fluorescence of the samples.

Results and discussion

Lens regeneration in the adult newt begins with proliferation and dedifferentiation of dorsal iris pigment epithelial cells (PECs). Dedifferentiation is the loss of characteristics that define the pigment epithelial cells, such as pigmentation. At about 10 days postlentectomy, a lens vesicle is formed from the depigmented dorsal PECs. Around 12–16 days postlentectomy, the internal layer of the lens vesicle thickens and synthesis of crystallins begins, marking the onset of primary lens fiber differentiation. During days 15–19, proliferation and depigmentation of PECs slows down while primary lens fibers migrate to the center of the lens and nondividing secondary lens fibers appear in the periphery. By 18–20 days, the PECs stop proliferating and the lens fibers continue to accumulate crystallins. Lens regeneration is considered complete by days 25–30 (Del Rio-Tsonis and Tsonis, 2003; Tsonis, 2000).

Shh is specifically turned on during lens regeneration

In our studies, we observed expressions of *Shh*, *Ihh*, and *ptc-1* via in situ hybridization during different stages of lens regeneration. Expression of these genes was evident at the lens vesicle stage and continued throughout all stages of regeneration. In Fig. 1, we show in situ hybridizations using representative stages of lens regeneration. *Shh* and *Ihh* were absent in the intact lens (Figs. 1I, J, M, and N); however, *ptc-1* was expressed in the lens epithelium of the intact lens (Figs. 1K and O). During the process of lens regeneration, all three genes *Shh*, *Ihh*, and *ptc-1* were expressed in the early regenerating lens vesicle (not shown) as well as later stages, including 15 days postlentectomy where the expression patterns were similar (Figs. 1A–D). At a later stage when the lens vesicle has differentiated into distinct layers that include lens fibers and lens epithelium (Figs. 1E–H), the expres-

sion seemed more homogenous for *Ihh* and *ptc-1* (Figs. 1F and G, respectively) in the lens epithelium as well as in all lens fibers, whereas *Shh* was mainly expressed in the lens epithelium and secondary lens fibers (Fig. 1E). It is important to note that the conditions for the in situ hybridization studies were optimized for each of the molecules used and hence the differences in background levels and expression levels. Because the iris is heavily pigmented and quite compacted, we were unable to distinguish expression patterns in the iris using in situ hybridization. Also, expression of *ptc-2* was not readily detectable with this method. To corroborate the presence of these genes in the newt eye, the sensitive method of RT-PCR was used. This method was in fact more informative when we examined expression in the iris. We thus examined expression of *Shh*, *Ihh*, *ptc-1*, and *ptc-2* in the following tissues from intact eyes and from eyes undergoing lens regeneration: lens, dorsal iris, ventral iris, and retina. Specific primers for each of the genes were made to avoid possible cross hybridization. *EF-1 α* was used as a positive control. The results are presented in Fig. 2. In agreement with the in situ studies, *Shh* and *Ihh* were not found in the intact lens. In addition, *Shh* was not found in the dorsal and ventral irises of the intact eye, while *Ihh* was found in both. However, both receptors *ptc-1* and *ptc-2* were detected in the intact lens and irises. During regeneration, *Shh* and *Ihh* transcripts were found in the regenerating lens and in both dorsal and ventral irises. The fact that activation of *Shh* during regeneration occurs in

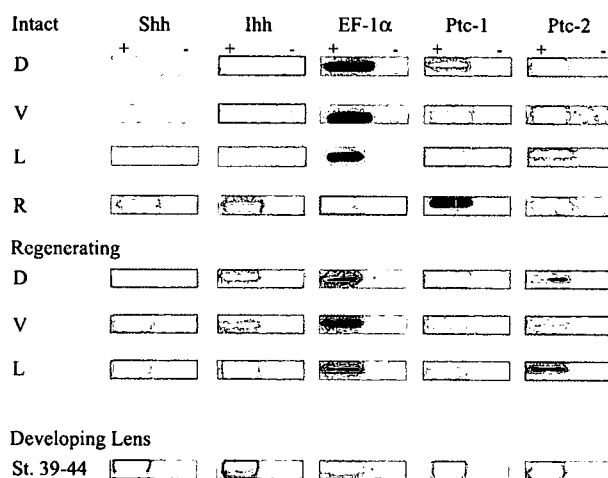


Fig. 2. Expression of *Shh*, *Ihh*, *ptc-1*, and *ptc-2* in tissues of adult (intact and regenerating) and developing newt eyes. Expression studies were performed by RT-PCR. Note that *Shh* and *Ihh* are absent in the intact lens but present in the regenerating lens 21 days postlentectomy. The rest of the panel shows presence or absence of the transcripts in irises and retina of intact and developing eyes (stages 39–44, according to Khan et al., 1999) as well as in eyes undergoing regeneration, representing a range of regeneration stages from 3 to 21 days postlentectomy. *Shh* is the only gene absent in the dorsal and ventral iris of the intact eye. *EF-1 α* was used as a control for the RTPCR. + or – indicates the presence or absence of reverse transcriptase (RT).

Table 1

Treatment	Affected	Normal
KAAD ^a	16/56 = 28.6%	40/56 = 71.4%
KAAD control	0/23 = 0%	23/23 = 100%
HIP ^a	6/18 = 33.3% ^b	12/18 = 66.7%
HIP control	0/17 = 0%	17/17 = 100%

Regenerating eyes treated with KAAD beads and their respective control beads as well as HIP- and GFP (control)-transfected cells collected 20 days postlentectomy for histological analysis. Affected = vesicles that were 70% or less of the size of a normal regenerating lens, considering differentiation of lens fibers as measure of more mature or larger vesicles.

^a Indicates that this group shows a statistically significant difference from its corresponding control group ($P < 0.01$) using a chi-square test.

^b 3/18 had no lens = 17%.

both dorsal and ventral irises deserves some attention, especially as it pertains to cell proliferation (see later section). It is possible that *Shh* expression in the irises is related to the activation of the cell cycle since it is known that both dorsal and ventral irises reenter the cell cycle upon lens removal, even though the rate of proliferation is much higher in the dorsal iris (Reyer, 1977).

We also examined the expression of these genes in the lens of newt embryos (stages 39–44, according to Khan et al., 1999). Interestingly, we found expression for all the genes examined (Fig. 2). Expression of *Shh* and *Ihh* has never been reported in the developing or mature lens in other animals, such as chick or mouse. Therefore, it seems that in newts, these genes might be uniquely expressed during lens formation, get turned off in the mature lens, but can be reactivated during regeneration. In all, our expression studies clearly show that *Shh* is transcriptionally activated in the postlentectomy iris and in the regenerating lens, while *Ihh* is activated in the regenerating lens.

Inhibition of the hedgehog pathway interferes with the process of lens regeneration

Having made this initial observation, we decided to examine the effects of inhibition of the hedgehog path-

way on the process of lens regeneration. One of most widely used methods to inhibit the hedgehog pathway is to treat cells or organisms with cyclopamine (Cooper et al., 1998; Incardona et al., 1998; Taipale et al., 2000). This steroidal compound interferes with the downstream factor Smoothened (Chen et al., 2002; Taipale et al., 2000) and is a standard choice in inhibiting the pathway. Another method consists of implanting mammalian cells transiently expressing hedgehog interacting protein (HIP). This protein binds hedgehog molecules and prevents access to their receptors, thus interfering with the downstream signaling targets (Chuang and McMahon, 1999; Zeng et al., 2001).

We used both methods during lens regeneration. HIP-expressing cells or KAAD-soaked beads were implanted into the eye cavity at zero and five days postlentectomy (see Materials and methods). KAAD is a synthetic form of cyclopamine that is 10–20 times more potent and less toxic (Taipale et al., 2000). In control eyes, transfected cells with a control plasmid or ETOH-soaked beads were implanted. Out of the 18 eyes treated with HIP-expressing cells, close to 33% of the eyes were affected showing smaller regenerating lens vesicles (vesicles were considered affected if they were 70% or less in size of a normal regenerating lens, considering as a measure the degree of lens fiber differentiation), including about 17% of the cases with no lens regeneration at all. All 17 eyes treated with control plasmid-expressing cells had normal regenerating lenses (Table 1 and Figs. 3A and B). Of 56 eyes examined in the KAAD experiment, lens morphogenesis was affected in nearly 30% of the cases, even though no cases of complete absence of vesicles were observed (Table 1 and Fig. 3C). This difference between the two treatments is probably attributed to the method of delivery or because these compounds have different modes of action. In addition, KAAD effects seem to be reversible; therefore, the method of delivery is critical. Heparin beads do not allow for a prolonged or slow delivery of the substance being used; thus, we applied KAAD beads at least two

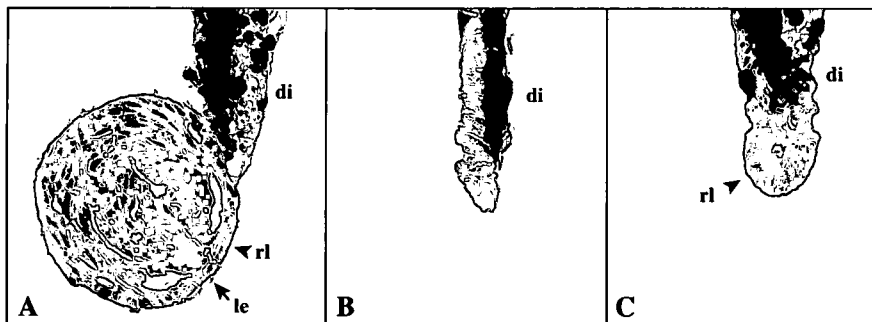
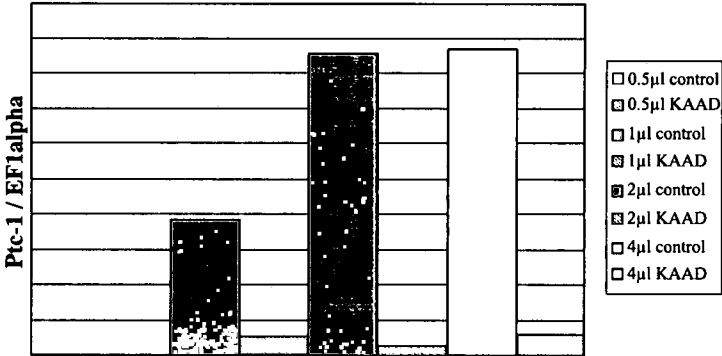


Fig. 3. Representative sections from control, HIP, and KAAD-treated lentectomized eyes showing the effects on the morphology of the regenerating lens. The eyes were collected 20 days postlentectomy and processed for histology; the sections were stained with hematoxylin and eosin. (A) Control regenerated lens, with extensive differentiation of lens fibers (lf). (B) HIP-treated eye showing no lens regeneration from the dorsal iris (di). Only a small depigmented tip is shown, similar to what the ventral iris is capable of at this stage. (C) KAAD-treated eye showing a small lens vesicle without apparent fiber differentiation. di: Dorsal iris; rl: regenerating lens; lf: lens fibers; le: lens epithelium.

A

Effect on <i>ptc-1</i> expression by KAAD treatment									
	EF-1α				Ptc-1				Negative Control
	(.5μl)	(1μl)	(2μl)	(4μl)	(.5μl)	(1μl)	(2μl)	(4μl)	
Control Treated Iris									
KAAD Treated Iris									

Relative *Ptc-1* mRNA levels in KAAD treated vs. control regenerating lens



B

Effect on <i>ptc-1</i> expression by HIP treatment									
	EF-1α				Ptc-1				Negative Control
	(1μl)	(2μl)	(4μl)	(8μl)	(1μl)	(2μl)	(4μl)	(8μl)	
Control GFP Treated Iris									
HIP Treated Iris									

Relative *Ptc-1* mRNA levels in HIP treated vs. control regenerating lens

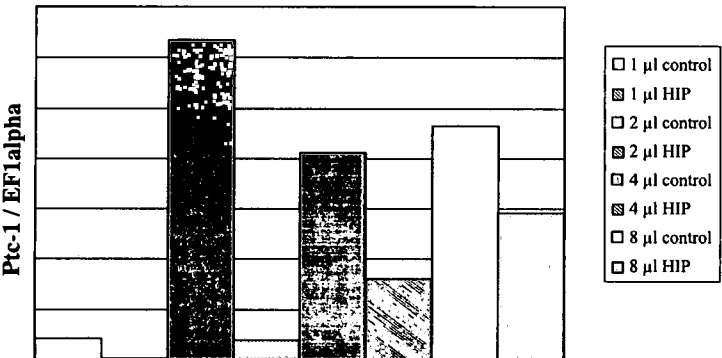


Fig. 4. Semiquantitative RT-PCR showing the effects of KAAD or HIP treatment on *ptc-1* expression. The expression of *ptc-1* in each experiment was determined in relation to *EF-1α* (internal control). (A) Note that treatment of irises undergoing regeneration with KAAD nearly abolished the expression of *ptc-1* (top panel). This was confirmed when quantified using Imagequant software (bottom panel; also see Methods). (B) Similar results were observed on the semiquantitative RT-PCR for the HIP-treated irises, where the expression of *ptc-1* was significantly reduced compared to that of the control experiment (top panel). This was also confirmed using Imagequant software (bottom panel). The negative controls contain no reverse transcriptase. The ratios of *ptc-1*/*EF-1α* for the lowest doses of the inhibitors in both experimental and control (A) and in experimental (B) were too small to be visible; therefore, scale bars are not presented.

times during the regeneration process. Consequently, considering the issues associated with the methods of delivery, the rate of 30–33% was shown to be significant ($P < 0.01$) using the chi-square test (see Table 1), especially when in the control experiments lens regeneration was normal in 100% of the cases.

However, established molecular tests can corroborate the specificities of these treatments. For example, a commonly used evaluation for cyclopamine effect on the hedgehog pathway is down-regulation of *ptc-1*, which is a downstream target of hedgehog molecules. Therefore, if the KAAD treatment truly affected the pathway, we should observe down-regulation of *ptc-1* in this system. Because our in vivo experiments showed only 30% effect, we developed a controlled and quantitative assay for *ptc-1* regulation. Irises undergoing the process of lens regeneration were isolated 8 days postlentectomy (see Materials and methods) and cultured in vitro for 24 h with a controlled amount of KAAD. Under these optimal conditions, we should be able to tell if KAAD affects *ptc-1* expression. Indeed, when we examined regenerating irises subjected to KAAD treatment and compared for *ptc-1* expression levels with nontreated regenerating irises, *ptc-1* expression was nearly abolished in the treated irises (Fig. 4A). In parallel experiments, we also examined the effects of HIP on regulation of *ptc-1*. As explained in the Materials and methods, for these experiments, cells were implanted into eyes 3 days postlentectomy and irises were collected 48 h later. Inhibition of *ptc-1* expression was shown in these experiments as well (Fig. 4B).

Cell proliferation and lens fiber differentiation are affected if the hedgehog pathway is inhibited during lens regeneration

To test if inhibiting the hedgehog pathway affected cell proliferation during lens regeneration, we treated day 12 regenerating eyes with KAAD and assayed for BrdU incorporation over the next 24 h. This time period was chosen because during normal lens regeneration, cell proliferation in the regenerating vesicle is high (Eguchi and Shingai, 1971). Taking into consideration that the effects of KAAD cannot last over prolonged periods of time (and this can explain the 30% effect seen in our in vivo assays; see discussion above), we decided to treat at this critical time and only for a period of 24 h. Thus, this assay is more likely to accurately measure the effects of KAAD treatments on cell proliferation. The labeled cells in all treated eyes were counted. Indeed, regenerating eyes treated under these conditions showed that cell proliferation in the lens vesicle was significantly affected ($P < 0.001$) (Fig. 5A). Parallel experiments with implantation of HIP-expressing cells showed that proliferation in the lens vesicle was affected with this treatment as well ($P < 0.005$) (Fig. 5B). These results strongly indicate that the hedgehog pathway regulates cell division in this

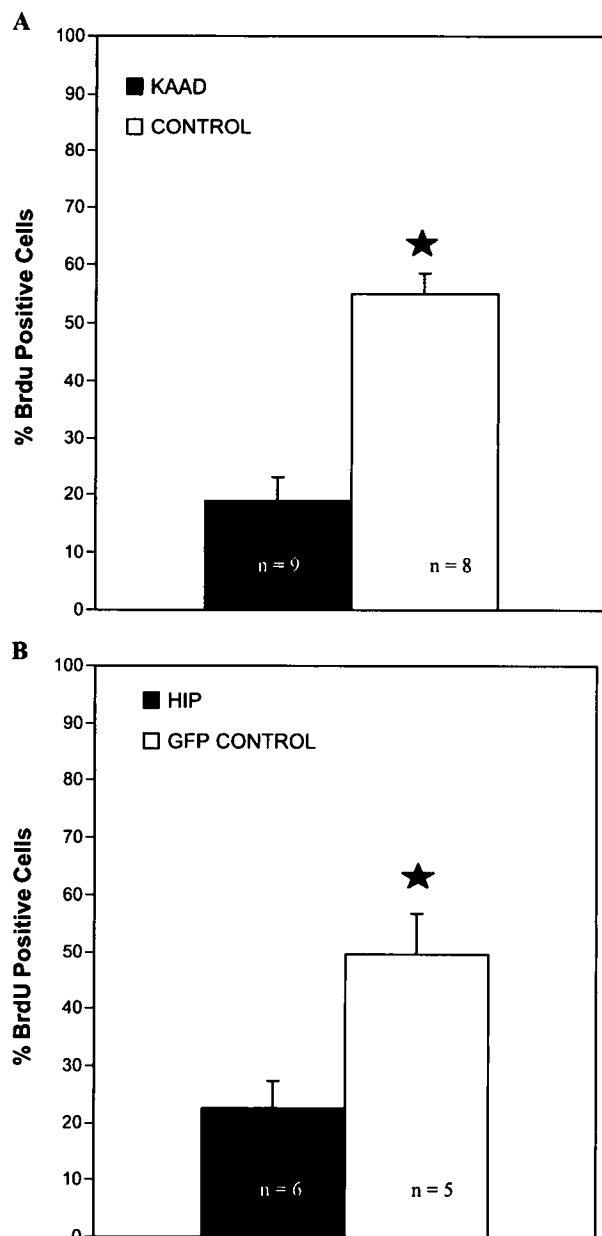


Fig. 5. (A) Effects on cell proliferation in KAAD-treated eyes undergoing lens regeneration. Cells in the lens vesicle showed close to 55% BrdU positive cells compared to the KAAD-treated eyes, where the BrdU positive cells dropped to nearly 20%. Error bars are standard error of mean. All cells in the central portion of each lens were counted; control lens had an average of 54 cells but similar regions of KAAD-treated eyes had only 19 cells on an average. *Denotes statistical significance ($P < 0.001$) using Student's *t* test. (B) HIP inhibits cell proliferation in eyes undergoing lens regeneration. Cells of the regenerating lens that were treated with control GFP-expressing cells have approximately 50% of their cells labeled with BrdU, whereas eyes treated with HIP-expressing cells have only 22% of their cells labeled with BrdU. Error bars are standard error of mean. An average of 31 cells per eye per section were counted for HIP and control-treated eyes. *Denotes statistical significance ($P < 0.005$) using Student's *t* test.

process. As it was mentioned above, Shh might be an important early player in the activation of proliferation in both dorsal and ventral iris. Obviously, other factors are additionally involved to restrict regeneration only from the dorsal iris.

In addition, we assayed for lens fiber differentiation by examining for the presence of β -crystallin in regenerating vesicles. Eyes undergoing lens regeneration were treated with KAAD at day 12 and assayed for crystallin expression at day 15. We observed that β -crystallin was not synthesized in eyes that were most affected by KAAD (Figs. 6C and D). Control-regenerating eyes taken at the same stage (day 15 postlentectomy) showed a normal pattern of β -crystallin protein expression, indicating lens

fiber differentiation (Figs. 6A and B). We also assayed a 12-day regenerating eye that was not treated with KAAD to compare if at this stage β -crystallin expression had initiated. Indeed at this stage, lens fiber differentiation was evident (Figs. 6E and F). The size of the vesicle at this stage (Fig. 6E) was comparable to the one that had been formed in eyes that were treated with KAAD at 12 days and collected at 15 days postlentectomy (Fig. 6C). The β -crystallin antibody we used is a lens fiber-specific marker (Sawada et al., 1993), suggesting that inhibition of hedgehog proteins affects the differentiation of the regenerating lens fibers as well. We found no evidence (via tunnel assays) that apoptosis increased during KAAD treatment (data not shown).

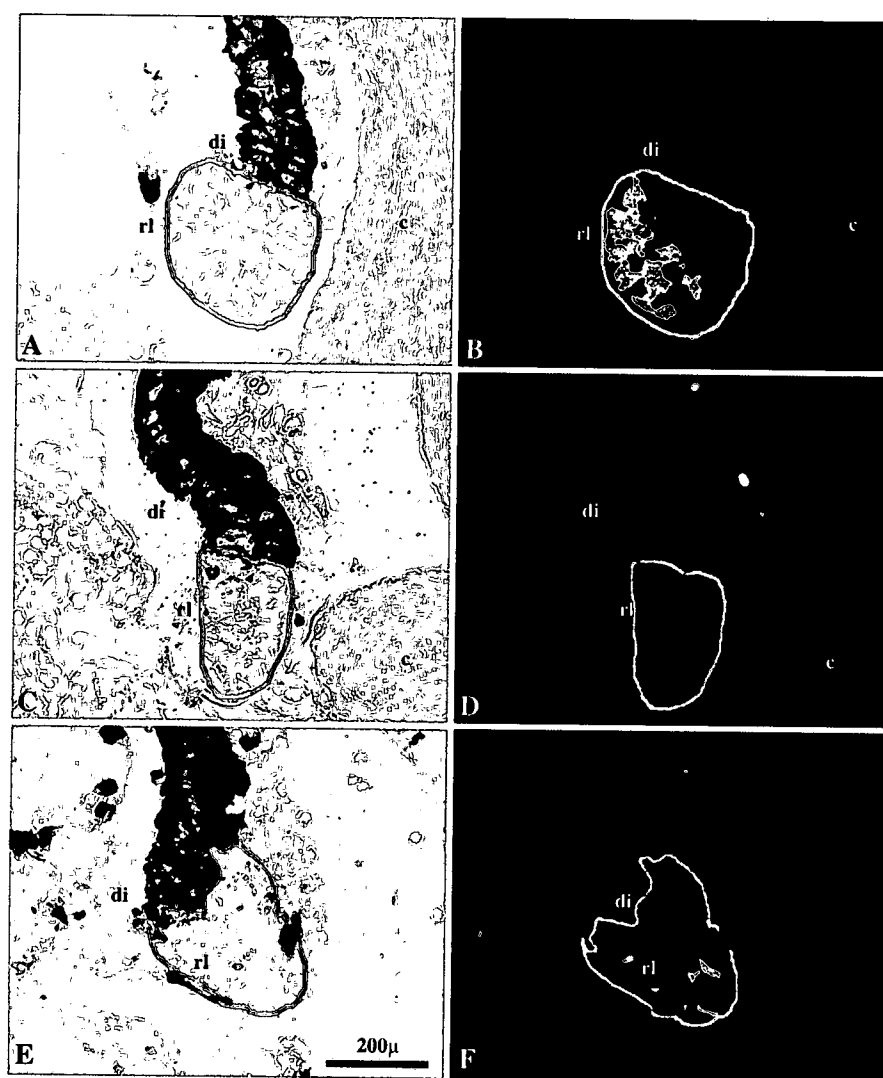


Fig. 6. Crystallin expression in KAAD-treated regenerating eyes. (B) Control-regenerating lens 15 days postlentectomy. Note expression of β -crystallin at the posterior region of the regenerating lens (rl). The cornea (c) is always at the anterior region of the eye. (D) A regenerating lens vesicle from a KAAD-treated eye (15 days postlentectomy) showed no β -crystallin expression. (F) A regenerating control lens vesicle from an untreated eye at 12 days of regeneration showing crystallin expression is included as a comparative control. This lens vesicle was at a similar stage as the one shown in C and D. The blue line outlines the regenerating lens vesicle. (A, C, and E) DIC images of B, D, and F, respectively. di: Dorsal iris; rl: regenerating lens; c: cornea.

In this paper, we provide proof that members of the hedgehog family and their receptors are expressed and involved in the morphogenesis and differentiation of the regenerating lens of the newt. This is the first report to indicate that these genes are expressed in the lens and that they might affect its growth and differentiation. Despite the plethora of data dealing with expression of *Shh*, *Ihh*, and *Patched* in retina and pigment epithelium, expression of these molecules has not been described or focused on during lens development. When the hedgehog pathway is manipulated by overexpressing *Shh* in chick embryos and *Xenopus*, lens morphogenesis is affected. In the first case, the lens is malformed and appears to lack lens fiber differentiation (Zhang and Yang, 2001a), and in the other case, the lens appears smaller than the controls (Sasagawa et al., 2002). Both reports, however, do not elaborate or show any details on the possible effects on lens morphogenesis. Our results strongly suggest that indeed, hedgehog molecules affect lens morphogenesis and that these molecules are recruited for the process of lens regeneration. Other reports support the role of hedgehog molecules in regenerative processes. Studies on limb and fin regeneration have shown that hedgehog molecules are not only expressed during regeneration but are also implicated in the process (Endo et al., 1997; Imokawa and Yoshizato, 1997; Laforest et al., 1998; Quint et al., 2002; Roy and Gardiner, 2002; Roy et al., 2000; Stark et al., 1998; Torok et al., 1999). It is interesting to note here that hedgehog proteins also play a role during tissue repair (Ferguson et al., 1999; Ito et al., 1999; Murakami and Noda, 2000; Vortkamp et al., 1998). Our results indicate that the utilization of the hedgehog pathway is reserved for lens regeneration in newts. The pathway regulates the proliferation and differentiation of the regenerating lens cells. Cell proliferation and differentiation of specific cell types have been shown to be regulated by the hedgehog pathway in other systems (Ingham and McMahon, 2001; Lai et al., 2003; Rowitch et al., 1999; Wetmore, 2003; Yu et al., 2002; Zhang and Yang, 2001a). The novel role of the hedgehog pathway in lens regeneration might bear significance in delineating the mechanisms of such a unique phenomenon.

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Vertebrate Limb Regeneration

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Summary

In this chapter, we have touched upon some of the key processes of limb regeneration from the formation of the wound epithelium to pattern formation, to provide a picture of the many complex and intricate facets of this system. Our synthesis incorporates recent advances in molecular biology, which has revealed some important factors related to the initiation, induction and patterning in limb regeneration.

Overview

Regeneration of the adult limb is a fascinating phenomenon, which occurs only in the urodele amphibians. What makes these species so unique in their ability to regenerate its limbs? Why is this mechanism lost in higher vertebrates such as mammals? Understanding the intricate process of amphibian limb regeneration will hopefully shed some light on these questions. The process of limb regeneration requires a complex set of events leading to the formation of many different tissue types, such as bone, muscle, nerves, and blood vessels. At the end of this amazing process, an exact replica of the missing part is built. This chapter will examine the process of limb regeneration primarily in urodele amphibians. Attention will be also paid to comparisons with limb regeneration in anuran amphibians and mammals.

Formation of the Wound Epithelium

Following amputation of the limb, the surface of the wound is covered rapidly with epithelial cells forming what is called the wound epithelium (WE). Amputation obviously induces within minutes rapid signal transduction events (Tsonis et al., 1991). The WE influences the underlying cells to dedifferentiate and re-enter the cell cycle. During this stage muscle, cartilage, and connective tissue lose their characteristics and become blastema cells. The blastema cells will proliferate and from the blastema, a region of dedifferentiated mesenchymal cells, which will ultimately give rise to the new limb (Figure 1). The formation of the WE is a critical event, for without it regeneration will not take place (Thornton, 1957). The wound epithelium is distinct from that of the normal epithelium and is formed by the migration of epidermal cells at the edge of the amputation surface (Figure 2; Tsonis, 1996). Due to this distinction, it is believed that the expression of key molecules within the WE is paramount for regeneration. These key molecules may be important factors signaling the underlying tissues to re-enter the cell cycle and differentiate. One of them the WE3 is probably related to secretion (Tassava et al, 1986). We will now examine several molecules that are predominantly expressed within the wound epithelium.

Collagen is a molecule that is expressed in the WE. Following the covering of the amputation surface, the cells of the WE begin to synthesize proteins essential for the basement membrane such as laminin and collagen

type IV (Del Rio-Tsonis et al., 1992). Collagen type XII has also been shown to be expressed within the wound epithelium. Expression of collagen type XII was first found 3 days post-amputation in the basal layer of the wound epithelium. By day 10 both the basal layer of the WE and the mesenchymal cells were expressing collagen type XII. As regeneration progressed expression of collagen type XII continued to change until becoming restricted to the perichondrium in late digit stage (Wei et al., 1995).

Other important factors that may be necessary for the proper formation of the wound epithelium are the matrix metalloproteinases (MMPs). Matrix metalloproteinases play a pivotal role in matrix degradation, which is an important step in formation of the wound epithelium. It has been suggested that MMPs may be involved in the initial dedifferentiation of cells preparing for regeneration by breaking down the extracellular matrix (Miyazaki et al., 1996). One of them the newt MMPe seems to be unique to newts and is specifically expressed in the apical epidermal cap and the wound epidermis (Kato et al., 2003). Other MMPs, such as MMP3/10-b is expressed specifically at the basal layer of the AEC. MMP9 at a very early stage (2 hours post-amputation is localized in the wound epithelium and later is synthesized by cartilage and distributed in the basement membrane of the bone and the extracellular matrix of the blastema (Yang et al, 1999; Kato et al., 2003). During limb regeneration within *Xenopus* (at early stages), gelatinase B (*Xmmp-9*) is expressed in the ectoderm and mesoderm at the tip of the amputated limb. This expression occurs very early during the process

of limb regeneration, within 6-24 hours after amputation (Carinato et al., 2000). It is important to note that *Xenopus* can regenerate their limbs only during early larval stages but lose this capacity after metamorphosis occurs.

Expression of complement factors shows an intriguing pattern. Complement component 3 (C3) was found present in the blastema and complement component 5 (C5) was exclusively found in the wound epithelium (Kimura et al, 2003). This might indicate that complement components might have non-immunologic functions in regenerative processes. Indeed, mice deficient in C5 show no liver regeneration (Mastellos et al, 2001).

We will see later (in relation to stimulation of limb regeneration) that growth factors, such as fibroblast growth factors (FGFs), have also been shown to be expressed within the wound epithelium. It is thought that these factors may provide the signals necessary for dedifferentiation and blastema formation.

Dedifferentiation

The formation of the WE appears to be what leads to the initiation of dedifferentiation and the formation of the blastema. Dedifferentiation and blastema formation are key events for successful limb regeneration. Fritsch (1911) showed that the regenerate comes from the production of undifferentiated blastema cells (Figure 2). Since this time, numerous studies have been performed examining the process of dedifferentiation during limb regeneration. The strongest evidence for dedifferentiation came from Hay

and Fischman (1961). In their study, they observed the transition of muscle cells to mononucleated cells. These mononucleated cells contained large nuclei and ribonuclear granules both of which are indicative of protein synthesis. This knowledge proved that proteolysis, one of the dominating theories of the time, was not a contributing factor in the formation of blastema cells, since proteolysis does not involve active protein synthesis.

Transplantation Evidence

Transplantation into X-ray irradiated limbs proved to be quite instrumental in showing the process of dedifferentiation. Irradiation has been shown to inhibit regeneration. Dunis and Namenwirth (1977) transplanted triploid cells into the limbs of irradiated diploid animals. The triploid cells participated in the regeneration process giving rise to cartilage, connective tissue, and fibroblasts. Steen (1973) showed that triploid skin transplanted into irradiated diploid axolotls was able to form cartilage, connective tissue, dermis, and epidermis. In another set of transplantation experiments, Thornton (1938) showed that metaplasia, transformation of one differentiated cell type into another, plays a role during regeneration. By removing the humerus and subsequently amputating through the area of the existing bone, limb regeneration took place with all skeletal elements distal to the humerus present. Regeneration took place despite the fact that the humerus was not present to be the source of the skeletal elements. These and other transplantation experiments help to solidify the importance of dedifferentiation

in the formation of the blastema and ultimately the regenerate (for more details see Tsonis, 1996).

Cellular Evidence

Dedifferentiation has also been shown *in vitro* using cultured myotubes. Blastema explants in culture show both dedifferentiation and metaplasia. ^3H -thymidine treated polynucleated cells, from explants, have been shown to go through dedifferentiation. When these cells were introduced into the blastema, they became mononucleated cells (contained ^3H -thymidine). In addition labeled cells were also found to contribute to the regenerate, as ^3H -cells were also present in cartilage, indicating metaplasia (LO et al., 1993; Tsonis et al., 1995). Obviously these cells were able to re-enter the cell cycle. Retinoblastoma (Rb) has been found to be involved in such re-entry regulation and so is thrombin activation (Tanaka et al, 1997; Tanaka et al., 1999). To examine cell cycle re-entry and dedifferentiation/differentiation independently, cell cycle progression was inhibited through X-irradiation of the cells or transfection with p16 (CDK 4/6 inhibitor). It was found that post-mitotic newt myotubes generate mononucleated cells in the absence of cell cycle progression (Velloso et al., 2000).

It has also been shown that mammalian post-mitotic nuclei re-enter the cell cycle. This was specifically shown when hybrid myotubes were created by the fusion of mouse C2C12 and newt A1 myogenic cells. Upon

serum stimulation the C2C12 nuclei re-enter the cell cycle in the hybrids but not when grown alone (Velloso et al., 2001). Other work examining mammalian myotube dedifferentiation has shown that treatment of post-mitotic mammalian myotubes with an extract generated from newt regenerating limbs allowed for dedifferentiation. This indicates that when stimulated with the appropriate factors, mammalian myotubes can dedifferentiate, providing some light on the restriction of mammalian cells to regenerate (McGann et al., 2001). Also, mammalian myotubes re-enter the cell cycle after transfection with the homeo box-containing gene *msx-1* (Odelberg et al., 2000).

Stimulation of Regeneration

Fibroblast growth factors

In addition to being expressed within the wound epithelium, fibroblast growth factors (FGFs) and their receptors (FGFRs) are thought to play a key role in providing important cues for proliferation and induction of limb regeneration. FGF-10 has been shown to be present in the mesenchymal cells and is correlated with regeneration in *Xenopus* (Yokoyama, 2000). Further investigation, showed that introduction of FGF-10 protein into regeneration-deficient *Xenopus* limb buds was able to stimulate the expression of several genes including *shh*, *msx-1*, and *fgf-10*. In addition to gene expression, limb structures also formed at the regeneration-deficient stage following FGF treatment (Yokoyama, 2001). Along similar lines,

previous research has shown that FGFR-1 and FGFR-2 are expressed in the blastema mesenchyme and wound epidermis of regeneration-competent *Xenopus* hindlimbs but not in the wound epidermis of regeneration-deficient hindlimbs. Inhibitors of these receptors suppress regeneration in regeneration-competent *Xenopus* hindlimbs (D'Jamoos et al., 1998).

FGF-2 has been proposed to be the elusive neurotrophic factor. Older research has shown that limb regeneration is dependent on an adequate nerve supply, suggesting that the nerves release a neurotrophic factor required for the proliferation of blastema cells (Singer and Mutterperl, 1963; Singer, 1965). In axolotl limb regeneration, it has been shown that upon denervation expression of FGF-2 and *Dlx-3* (a homolog of the homeobox-containing distal less; see below), is down regulated in the epidermis. FGF-2 replacement, which is expressed in the nerves and the apical epidermal cap re-establishes the normal expression of *Dlx-3* (Mullen et al., 1996). FGF-2 has also been shown to be an endogenous mitogenic factor responsible for blastema formation in frogs.

In *Xenopus* early larvae, however, hindlimb regeneration can proceed in the absence of nerves, while in late larvae this is not the case. It seems that following denervation, FGF-2 levels increase in regeneration-competent *Xenopus* limb buds but not in regeneration-deficient limb buds. This nerve-independent regeneration seen during the larval stages is due to the presence of FGF-2, which is serving as a neurotrophic-like factor. This suggests that the low level of FGF-2 present in the late *Xenopus* limb buds

(regeneration deficient) is the reason for the onset of nerve dependence and ultimately why there is a lack of blastema formation at this stage (Cannata, 2001).

Another implication of FGF2 as a factor in blastema formation came from a study in which the regeneration rates of two urodele species were examined. In this study, FGF-2 expression was examined in *T. carnifex* and *T.v. meridionalis*. Both species have the ability to regenerate their limbs, but the regeneration process is much faster in *T.v. meridionalis* than in *T. carnifex*. The rate of regeneration correlated with delayed expression of FGF-2 in *T. carnifex*. The delayed expression of FGF-2 resulted in the delayed formation of the apical ectodermal cap (AEC), which is necessary for blastema formation (Giampaoli et al., 2003).

***Msx* genes**

Another set of genes being implicated in playing an essential role during limb regeneration is the *msx* genes. The *msx* genes are transcription factors expressed in regions of epithelial-mesenchymal interactions during embryogenesis of developing appendages. Numerous studies implicate these genes in the maintenance of embryonic tissues in an undifferentiated, proliferative state. Carlson et al. (1998) examined the expression of *Msx-2* during limb development and limb regeneration in the axolotl. They found *Msx-2* expression to be downregulated at late stages of limb development, but re-expressed within one hour of limb amputation. These results indicate

that *Msx-2* may have an important role in initiating the limb regeneration cascade. Earlier studies like the one described above have shown that, similar to *Msx-2*, *Msx-1* is also upregulated in response to limb amputation in the newt (Crews et al., 1995; Simon et al., 1995).

Recent evidence has come about supporting the role of *msx* genes in mammalian digit regeneration. Mice as well as humans have the capacity to regenerate their digits upon amputation. Regeneration of the lost tip depends on the treatment given as well as the level of amputation. If the amputation is distal to the last interphalangeal joint and nothing is done to close the wound, regeneration will be successful (Illingworth, 1974; Borgens, 1982). The area of digit regeneration seems to correspond to a region associated with the nail bed. Previous studies have shown that both *Msx-1* and *Msx-2* are expressed in association with the nail organ of neonatal digits as well as in developing digits (Reginelli et al., 1995). During development it has been shown that *Msx-1* mediates a BMP signaling pathway that leads to the induction of the apical ectodermal ridge (AER). In a study by Han et al. (2003), the role of *Msx-1* and BMP4 were examined. It was found that *Msx-1* mutant mice display a regeneration defect. Part of this defect included the downregulation of *BMP4* expression. Treatment with exogenous BMP4 was able to rescue the regeneration defect. These experiments provided the first functional evidence linking *Msx-1* and BMP signaling to the control of digit tip regeneration in mammalian fetal tissue (Han et al., 2003). As mentioned above the role of *msx-1* in dedifferentiation of mammalian myotubes

strengthens its importance in regulating the process of dedifferentiation and blastema formation in urodeles (Odelberg et al., 2000).

Pattern Formation

So far, we have seen the importance of dedifferentiation and blastema formation in the process of limb regeneration. Now we will look at the process of pattern formation to gain an understanding of how an exact replica of the missing part is produced. During pattern formation, all three axes (anterior-posterior, proximal-distal, and dorsal-ventral) must be properly attained. Several theories have been suggested as to how pattern formation takes place and we will briefly mention them next.

Positional Information

Wolpert (1969) proposed the idea of positional information stating that every cell possesses intrinsic information regarding its body position. During embryogenesis, cells acquire sets of coordinates that tell them where they are within the limb. Different ideas regarding the interpretation of these coordinates into positional information have been proposed and will be discussed briefly.

Boundary model

This model proposed by Meinhardt (1983) states that a diffusible morphogen produced at the boundary of two tissues can offer positional

information. At the apical ectodermal ridge, the boundary between the anterior-posterior (AP) and the dorsal-ventral (DV) axes, the morphogen is produced and ultimately sets the proximal-distal (PD) axis.

Polarizing zone model

This model was proposed primarily due to the discovery of the zone of polarizing activity (ZPA) during chick limb development (Saunders and Gasseling, 1968). The ZPA is a region found in the posterior limb bud that is able to specify skeletal development ultimately playing a role in AP patterning. This model proposed by Tickle et al. (1975) states that positional information is acquired due to the amount of time spent in the polarizing zone.

Imokawa and Yoshizato (1997) suggested that the newt blastema contains a ZPA through their work on *Sonic hedgehog* (*shh*). *In situ* hybridization experiments indicated that *shh* was expressed within the mesenchymal cells of the posterior region of regenerating blastemas of newt limbs. Grafting experiments in which forelimbs were amputated at the mid-upper arm level supported this notion. At mid-bud stage the blastema was removed and grafted onto the original stump with a 180° rotation (reversal of the AP/DV axes). To reverse the AP axis, the blastema was grafted onto the contra-lateral stump without rotation. The grafted blastemas formed supernumerary limbs and expressed *shh* ectopically in a newly induced ZPA. These experiments suggest that AP patterning seen during limb regeneration is due to the regulation of *shh*-ZPA, similar to what is seen during limb

development. Another study examining the role of *shh* during axolotl limb regeneration also found *shh* to have a role similar to that seen in development (Roy et al., 2000). In this study, *shh* was ectopically expressed using the vaccinia virus. Ectopic expression caused supernumerary digits as well as other hand and foot elements.

Polar coordinate model

In this model, there is no diffusible factor required to obtain the positional information. Rather, coordinates regarding positional information are obtained through interactions with neighboring cells (Bryant et al., 1977; Bryant et al., 1981). According to this model each cell possesses positional information with respect to radius and circumference. Two cells which come in contact with one another due to wound healing or grafting will fill in the missing positional values (both radial and circumferential) creating a properly patterned limb. This model made spectacular and accurate predictions on limb morphogenesis during regeneration (for details see Tsonis, 1996).

Retinoic Acid and Pattern Formation

Numerous studies have been performed examining the role of vitamin A derivatives such as retinol and *all-trans* retinoic acid on limb regeneration. Maden (1982) found that a vitamin A derivatives, retinol palmitate or *all-trans*-retinoic acid, affected the proximal-distal axis. Limbs amputated at the wrist, which would normally regenerate only the hand elements regenerated more

proximal structures. He also found that the proximalizing effect of vitamin A was dose dependent. At a high concentration, vitamin A became inhibitory to limb regeneration. Similar proximalization results were also obtained by using an analogue of all-*trans*-retinoic acid, 9-*cis*-retinoic acid. In fact, 9-*cis*-retinoic acid had a more potent morphogenetic effect when compared to all-*trans*-retinoic acid (Tsonis et al., 1994).

Based on this work and others it was thought that positional information could be obtained depending on a chemical gradient. In this case a gradient of vitamin A along the proximal-distal axis, with a higher concentration in the proximal tissues. This gradient ultimately results in the proximalization effect of vitamin A. Treatment with vitamin A following amputation at the wrist would expose these tissues to a higher concentration of vitamin A. This increase in vitamin A would result in the formation of more proximal elements.

In fact, vitamin A has been found to alter chick limb bud patterning in a similar fashion to the ZPA. Recall from earlier, that the ZPA is a region found in the posterior limb bud that specifies skeletal development. Saunders and Gasseling (1968) discovered that transplantation of the ZPA into the anterior limb bud resulted in skeletal duplication along the AP axis. The same result was seen by Tickle et al. (1982) after retinoic acid was inserted into the anterior margin of a developing chick limb bud.

Of interest is the distribution of retinoids in the newt regenerating blastema. Scadding and Maden (1994) have shown that especially all-*trans*

retinoic acid is present as a gradient along the anteroposterior axis with less at the anterior region and more at the posterior (as also seen in developing chick limb buds, Thaller and Eichele, 1987). Interestingly, there is more all-trans RA in the distal blastema as compared to the proximal, but this is valid only for a particular stage of regeneration. In Figure 3 we present illustrations depicting the relative (qualitative) distribution of retinoids in newts and also compare with the absence of gradients in *Xenopus* limbs that are not able to regenerate. These studies suggest that a graded distribution of all-trans RA is correlated with the process of limb regeneration.

Hox Genes and Limb Regeneration

Hox genes are homeobox-containing genes, which control pattern formation and segmentation. These genes are nuclear and contain a helix-turn-helix motif as their DNA binding domain, similar to that seen in bacterial repressors. This DNA binding domain is termed the homeodomain because it was found in many homeotic genes. The homeodomain is 60 amino acids in length and is highly conserved among different species. The Hox genes are arranged in clusters and are expressed in similar patterns in species ranging from *Drosophila* to mammals. Due to two duplications in vertebrates, there are four clusters of Hox genes, A, B, C, and D. The 3' Hox genes mark the anterior parts of the body whereas the 5' Hox genes mark the posterior parts. Each cluster contains several genes denoted as A1, A2...A13 (this varies slightly depending on the cluster) (Figure 4). During limb development the

most 5' genes (Hox D13) are expressed most posteriorly and distally with Hox D12, -11, -10 appearing sequentially with Hox D10 being the most anterior and proximal (Izpisua-Belmonte et al., 1991).

Several Hox genes have been shown to be specific to or upregulated within the blastema during limb regeneration. (Brown and Brockes 1991; Beauchemin and Savard, 1992; Beauchemin et al., 1994, 1998; Gardiner et al., 1993; Carlson et al., 2001; Christen et al., 2003). In Figure 4 we can see the specific regulation of some of these Hox genes. The horizontal arrows indicate regulation in the proximal versus distal regions (or anterior-posterior) of the limb and the tip of the arrow indicates higher levels of expression. The perpendicular arrows indicate specific regulation in the regenerating limb. This is the case of HoxC10 upregulation (long transcript), which otherwise is absent from the developing and intact limb (Carlson et al., 2001). Likewise, the arrow for A13 shows down regulation by retinoic acid, while A9 is unaffected (Gardiner et al., 1995). Also, re-expression of A9 during regeneration is synchronous with A13, arguing against colinearity in the expression of Hox genes during regeneration, which is the case during limb development. Interestingly, some of the posterior/distal Hox genes (D10, D11, A11) show higher expression in the proximal blastemas. This might bear significance because during regeneration the pattern has to be respecified, however, from an already established adult limb and not an embryonic limb. In addition to the expression of Hox genes that are members of the clusters other non-clustered Hox genes have been found to be

expressed during limb regeneration. One of the best examples is the *msx* genes mentioned in an earlier section. Others include *Emx-2*, with a clear higher distal/lower proximal distribution in the epidermis and wound epithelium (Beauchemin et al, 1998), and *distal-less* (Beauchemin and Savard, 1992).

Figure 1: Stages in the process of limb regeneration. A: A section through a blastema 2 weeks post-amputation, showing the accumulation of undifferentiated blastema cells. The dotted line denotes the plane of amputation. B: A section through a regenerating limb 5 weeks after amputation. Note the differentiating cartilage composing the ulna and radius. C: a section through a regenerated limb 2 months after amputation at the elbow (e) level. Note the regenerated skeletal elements and the correct formation of the four fingers. u: ulna, r: radius, c: carpals, m:metacarpals.

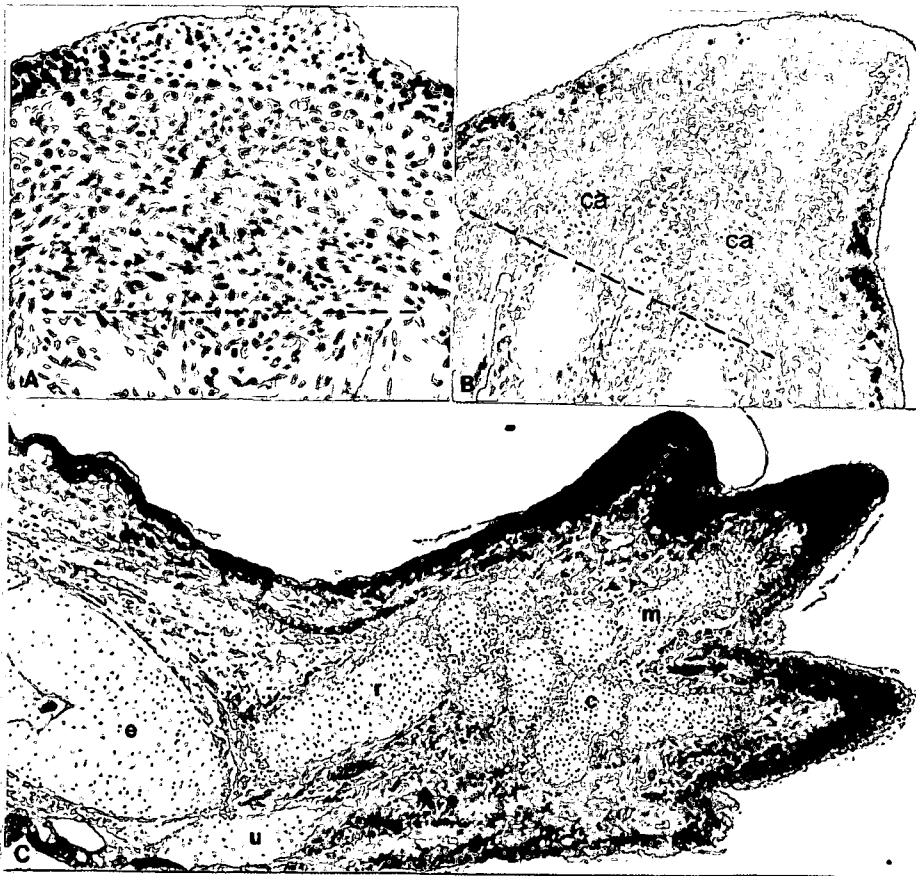


Figure 2: Scanning electron microscopy of wound closure and blastema cells. A: Covering of the stump by the migrating cells (arrows) of the wound epithelium, 2 days post-amputation (X500). B: Higher magnification of A, showing the adhering cell on collagen fibers (cf) (X1800). C: Blastema cell population one week after amputation, indicating the fibroblast-like morphology of the blastema cells and the extensive extracellular matrix fibers (arrows).

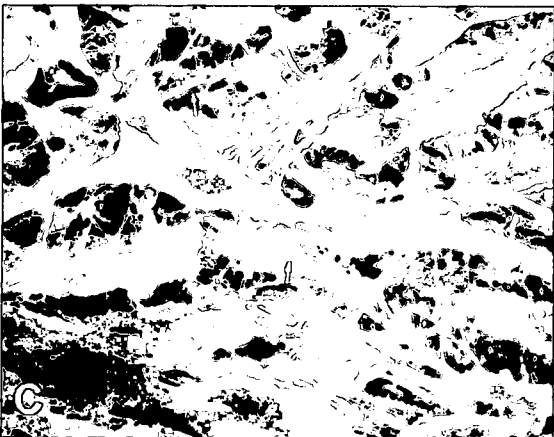
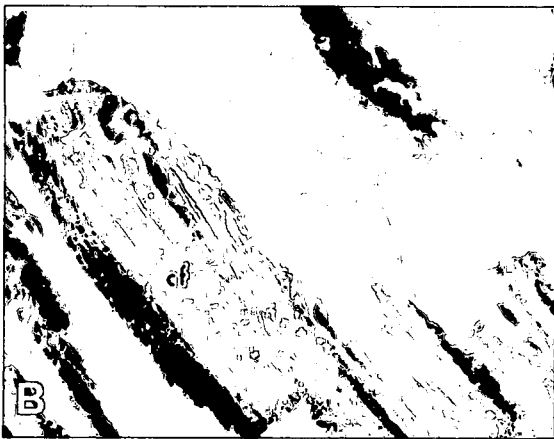
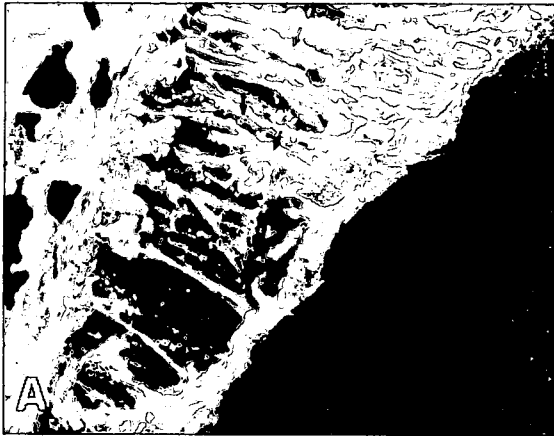


Figure 3. Illustrations showing the distribution of different retinoids in the newt and *Xenopus* blastema. The different shades indicate graded distribution ranging from low (white) to dark blue (high). The shading is meant to show the qualitative difference and the presence of gradients and is not quantitative in regard of absolute values of retinoid concentrations.

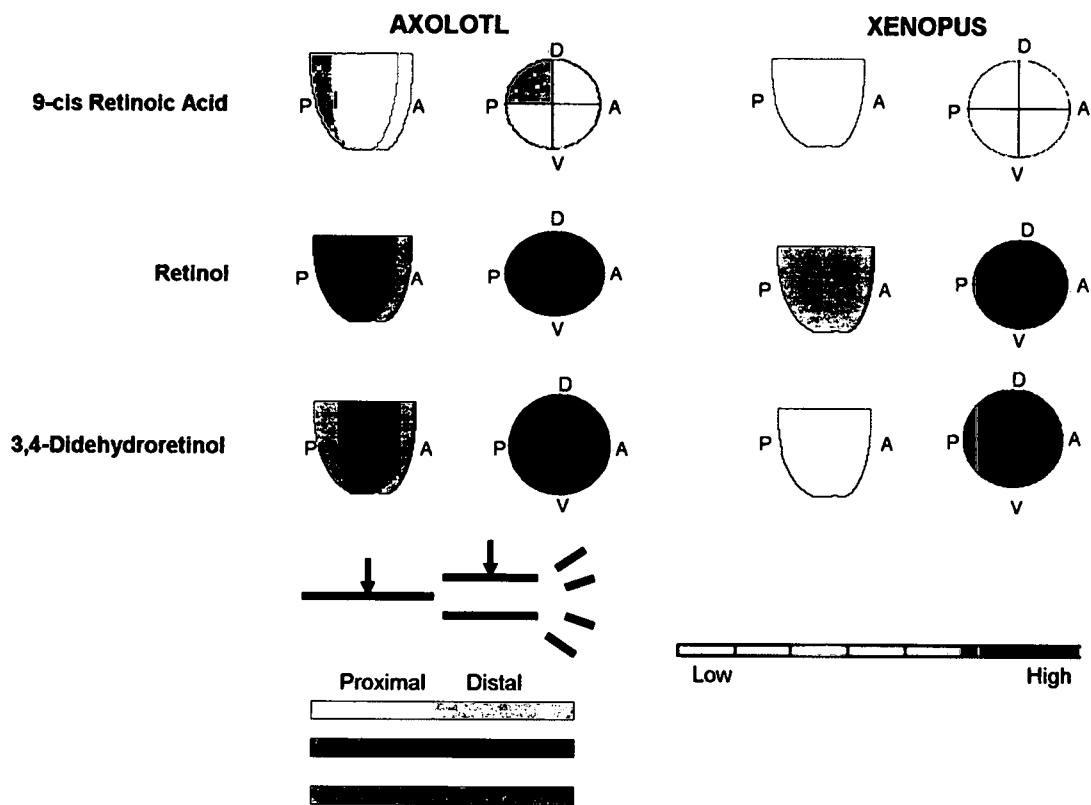
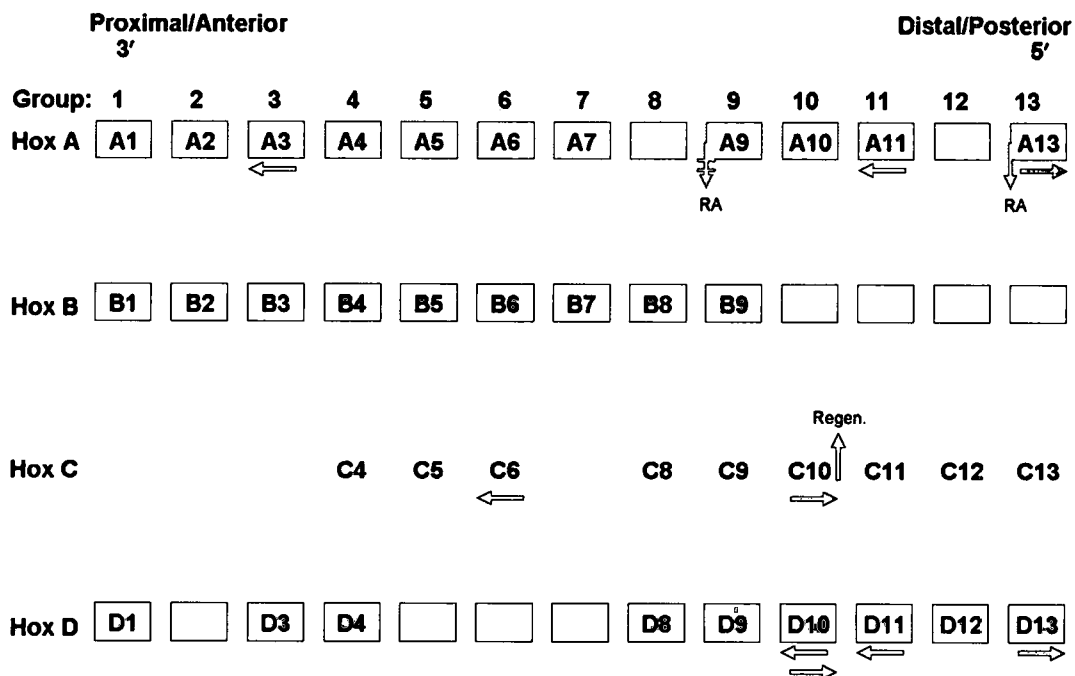


Figure 4: An illustration indicating the organization of the four Hox gene clusters. Horizontal arrows show graded expression along the proximal-distal axis (blue) or along the anterior-posterior axis (purple). The tips of the arrows indicate the direction of the gradient from low to high. Perpendicular arrows (red) indicate specific regeneration expression of C10 and regulation by retinoic acid (gray).



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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.

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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 Trembley, 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 postlens removal. At approximately 10 days postlens removal, 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 postlens removal 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 postlens removal, crystallins are continually synthesized (Fig. 1C). A complete lens is

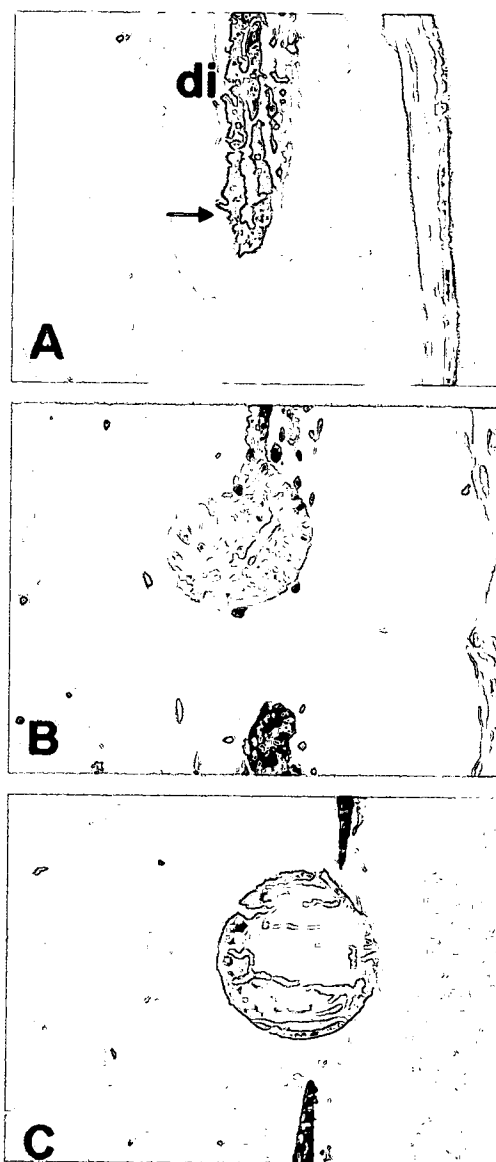


Figure 1. Lens regeneration in the newt stemming from the PECs of the dorsal iris (di). **A:** Ten days postlens removal. Note the formation of a lens vesicle (arrow). **B:** Fifteen days postlens removal. Cells are elongating into lens fibers. **C:** Twenty days postlens removal. The lens is well differentiated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

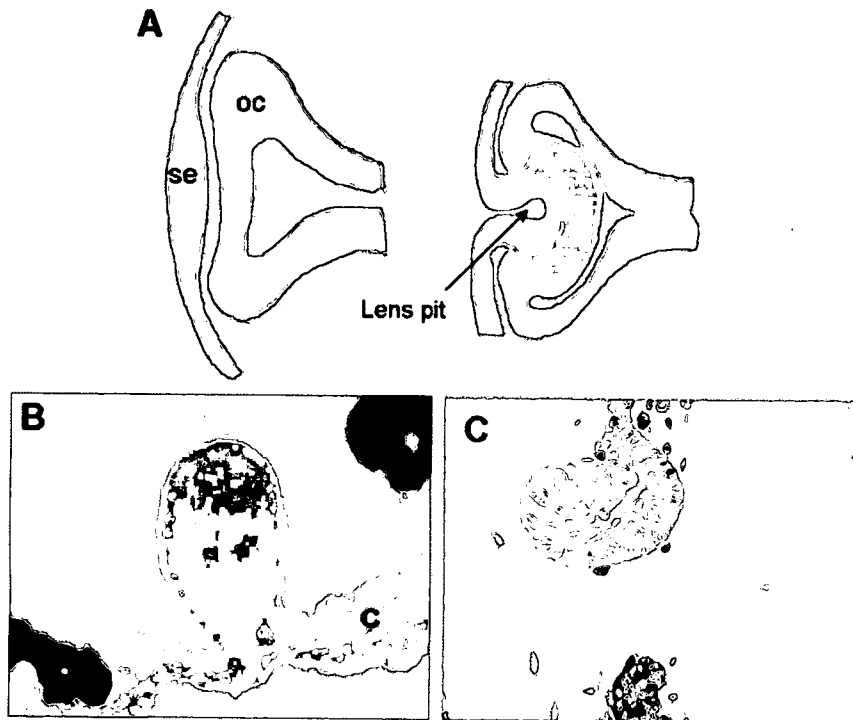


Figure 2. Comparison of lens development with two methods of lens regeneration. **A:** Schematic showing lens development, which involves a series of inductive interactions between the surface ectoderm and the optic cup. The lens pit eventually gives rise to the lens. se, surface ectoderm; oc, optic 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 lentiectomy 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 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 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 (deJongh 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 lenticectomy, 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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LETTERS

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

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Lens regeneration in adult newts is a classic example of how cells can faithfully regenerate a complete organ through the process of transdifferentiation^{1–6}. After lens removal, the pigment epithelial cells of the dorsal, but not the ventral, iris dedifferentiate and then transdifferentiate to form a new lens. Understanding how this process is regulated might provide clues about why lens regeneration does not occur in higher vertebrates. The genes *six-3* and *pax-6* are known to induce ectopic lenses during embryogenesis^{7,8}. Here we tested these genes, as well as members of the bone morphogenetic protein (BMP) pathway that regulate establishment of the dorsal–ventral axis in embryos⁹, for their ability to induce lens regeneration. We show that the lens can be regenerated from the ventral iris when the BMP pathway is inhibited and when the iris is transfected with *six-3* and treated with retinoic acid. In intact eyes, *six-3* is expressed at higher levels in the ventral than in the dorsal iris. During regeneration, however, only expression in the dorsal iris is significantly increased. Such an increase is seen in ventral irises only when they are induced to transdifferentiate by *six-3* and retinoic acid or by BMP inhibitors. These data suggest that lens regeneration can be achieved in noncompetent adult tissues and that this regeneration occurs through a gene regulatory mechanism that is more complex than the dorsal expression of regeneration-specific genes.

To determine the role of *six-3* and *pax-6* in the induction of transdifferentiation of the ventral iris, ventral iris cells were transfected in the presence or absence of retinoic acid with the appropriate constructs and examined for induction by using an *in vitro* transfection and *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 limb^{13–16}. In addition, dorsal or ventral iris explants were treated with soluble BMP-4, BMP-7, chordin and a soluble competitor for the receptor BMPR-IA. After transfection and implantation of aggregated pigment epithelial cells (PECs), scores of eyes were examined (see Supplementary Information). As a rule, untransfected dorsal PEC aggregates transdifferentiate to lens, whereas the ventral ones do not. Under the conditions outlined in the Methods, short-term culturing of cells does not interfere with the potential for lens transdifferentiation. Dorsal aggregates produced a lens in over 83% of tests (10/12), whereas the ventral ones, as expected, did not (0/11; Fig. 1a–c). It has been shown, through β -galactosidase staining, that the lens is indeed derived from the aggregate¹². Dorsal aggregates transfected with the constructs with retinoic acid treatment also transdifferentiated to lens (data not shown). With ventral PECs, however, only one protocol—namely, transfection of PECs with *six-3* in the presence

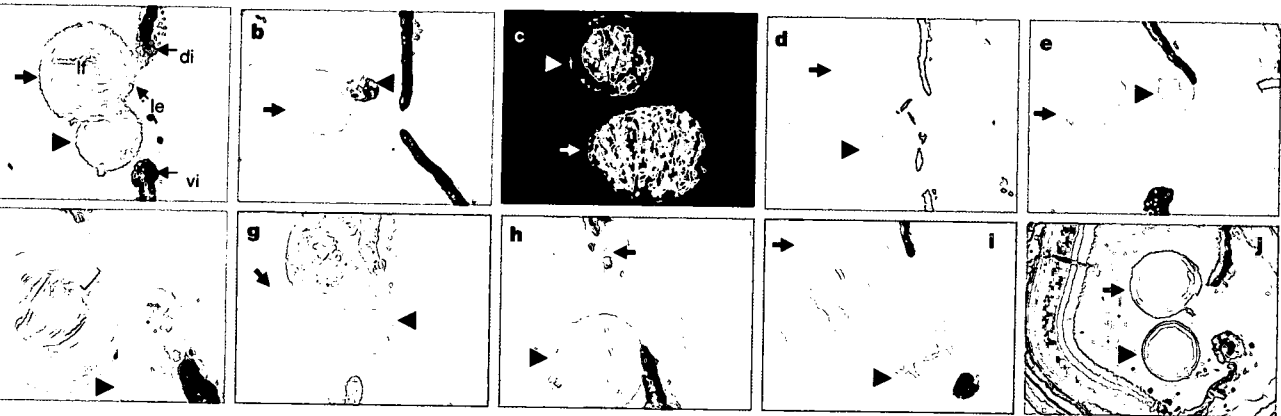


Figure 1 | Lens induction from ventral PECs. **a–f**, Lens induction by transplantation of PEC aggregates examined 30 d after transplantation. Black arrows indicate the host regenerated lens; arrowheads indicate the PEC aggregate or the lens induced from the PEC aggregate. **a**, A control transfected dorsal PEC aggregate that has transdifferentiated to lens. **le**, lens epithelium; **lf**, lens fibres; **di**, dorsal iris; **vi**, ventral iris. **b**, A control transfected ventral PEC aggregate (arrowhead) that 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 fibre-specific antibody to β -crystallin²³. **d–f**, Induced lenses from ventral PEC aggregates transfected with *six-3* and treated with retinoic acid (RA). **g–j**, Induced lenses from ventral iris explants treated with BMPR-IA inhibitor (**g–i**) and with chordin (**j**).

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of retinoic acid—led to the induction of lens transdifferentiation (Fig. 1d–f). This induction occurred at a rate (3/4; 75%) comparable to that seen in the dorsal aggregates. Neither treatment with retinoic acid alone nor transfection of ventral PEC cultures with *six-3* alone induced transdifferentiation.

In the BMP series we found that inhibition of the pathway by either the BMPR-IA competitor or chordin resulted in the induction of a lens from the ventral explants (3/15 and 1/8 respectively; Fig. 1g–j). The incidence of induction was low (17%); however, we regard this as highly significant because no untreated ventral explants differentiated 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 inhibiting the binding of BMPs to receptors results in dorsalization⁹. Notably, treatment of the dorsal iris explants with BMP-7, and to a lesser degree with BMP-4, considerably inhibited their ability to transdifferentiate to lens (1/12 (8.3%) and 5/12 (41.6%), respectively). Such results clearly indicate that BMPs maintain ventral identity and that inhibition of the pathway dorsalizes the ventral iris, allowing transdifferentiation.

To probe further the mechanism of induction, we undertook gene expression profiling of *six-3* and *BMPR-IA* during lens regeneration and during the experimental treatments that lead to the induction of lens regeneration from the ventral iris. Expression of *pax-6* was also assessed because of its known association with *six-3*. We examined samples of iris isolated 2, 4 and 8 d after lentectomy, as 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. Because these genes are also expressed in the differentiating lens, their induction-related expression might be ‘contaminated’. Several unexpected points emerged from the expression patterns that 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. Analysis of the data to compare expression between the dorsal and ventral iris showed that the three genes were expressed more highly in the intact ventral iris. This pattern was maintained at day 8 but with a lesser relative fold change (Fig. 2a). However, analysis of the data to compare expression in the 2-, 4- and 8-d dorsal iris relative to the intact dorsal iris, and the 2-, 4- and 8-d ventral iris relative to the intact ventral iris, to correlate expression with the process of regeneration, revealed another pattern. The expression of *six-3* was increased only in the dorsal iris and seemed comparable at day 8 to that in the ventral iris. *BMPR-IA* and *pax-6* were also slightly upregulated (Fig. 2b–d). Upregulation of *six-3* in the dorsal iris started at day 4 (Fig. 2c), whereas that of *pax-6* and *BMPR-IA* started at day 8 (Fig. 2d). Thus, an increase in *six-3* seems to be important during the dedifferentiation process in the dorsal iris. Because regeneration occurs only from the dorsal iris and because the ventral iris also expresses these genes, our data suggest that gene regulation associated with the competency for lens regeneration aims to increase expression over a particular threshold and not simply to render a regulatory gene as dorsal-specific. Such a pattern for *six-3* is clearly shown when its expression at the different time points is directly compared with that in the intact dorsal iris (Fig. 2e).

Treatment of ventral iris cells with *six-3* and retinoic acid, which resulted in induction of transdifferentiation, showed a similar pattern of upregulation of *six-3*, *pax-6* and *BMPR-IA* when compared with the untransfected ventral cells (Fig. 2f). Treatment of the cells with retinoic acid alone or transfection of *six-3* alone, which did not induce the irises to differentiate to lens, did not show such a pattern (data not shown). Similarly, treatment of ventral iris explants with chordin, which also resulted in induction, invoked a marked upregulation of *six-3*, *pax-6* and, to a lesser degree, *BMPR-IA* in the treated ventral irises, as compared with the increase in untreated irises (Fig. 2g). *BMPR-IA* transcriptional regulation might not be that important for the induction. Notably, the rate of increase (as a relative fold change) in the treated ventral irises was comparable to

the increase in the regenerating 8-d dorsal iris. Thus, the treated ventral irises that transdifferentiated to lens adopted a gene expression profile (especially for *six-3*) that was seen only in the dorsal iris during dedifferentiation and regeneration. This observation indicates that when the ventral irises are coaxed to mimic patterns of regulatory events seen in the dorsal iris they become ‘dorsalized’ and thus can transdifferentiate into lens.

These expression patterns of *six-3* led us to examine whether there are subpopulations of cells in the dorsal or ventral iris that might account for these differences by using 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 8-d dorsal and ventral irises examined without apparent differences in their distribution (Fig. 3a), showing that the upregulation of *six-3* is not due to its expression in more cells. When the aggregates (or explants) transdifferentiated to lens, nearly all cells participated, also arguing against the existence of *Six-3*-expressing subpopulations (Fig. 1). We also examined the expression of *Six-3* and *BMPR-IA* throughout the lens regeneration process. Figure 3b shows expression in dorsal and ventral iris at early stages (before vesicle formation) and Fig. 3c shows expression in

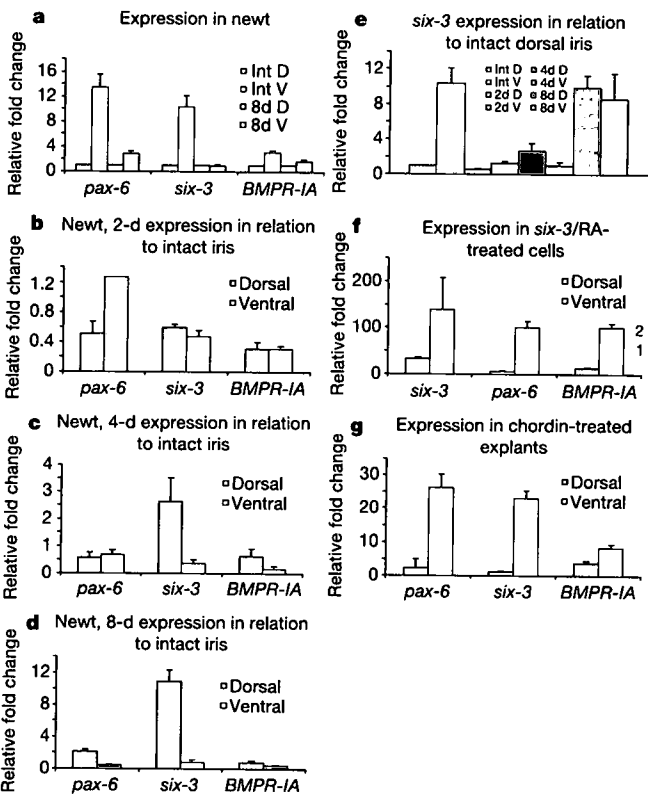


Figure 2 | Expression during lens regeneration and induction. **a**, Comparison of *pax-6*, *six-3* and *BMPR-IA* expression between the newt dorsal and ventral iris. Expression is shown in intact irises and 8 d after lentectomy. Values in the dorsal iris have been set to 1 and those in the ventral iris are shown as relative fold changes. **b–d**, Comparison of *pax-6*, *six-3* and *BMPR-IA* expression in 2-, 4- and 8-d irises with that in intact irises. **e**, Comparison of *six-3* expression at all time points with that in the intact dorsal iris. **f**, Expression in PECs treated with *six-3* plus retinoic acid (RA) relative to untransfected cells. Note the considerable increase in exogenous *six-3* levels in the ventral PECs. Note that the levels of *pax-6* and *BMPR-IA* are much lower, as indicated by the numbers on the right y axis (used to accommodate the very high fold increase in *six-3*). **g**, Expression in chordin-treated iris explants relative to untreated explants. Data show mean \pm s.d.

rsal iris at later stages (in vesicles or regenerating lens). Expression
ventral iris at later stages was also positive (data not shown). The
presence of Six-3 in dorsal and ventral iris is consistent with the
polymerase chain reaction (PCR) data; however, the immuno-
nining data are not quantitative.
We also considered whether the above 'treatments' are unique to
the newt or whether they could induce lens transdifferentiation in
species from other vertebrates by testing another salamander, the
axolotl, which possesses the ability to regenerate limbs and tail, but
not the lens. None of the treatments induced transdifferentiation,
indicating that the treatments are most probably 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 in the newt but
from the cornea in the pre-metamorphic frog—two strategies that
differ from the embryonic induction of lens development and that
could argue against absolute conservation of the inductive
mechanisms.

To gain insight into the axolotl data, we examined gene expression
in intact irises, in irises 8 d after lentectomy and in treated irises. In
contrast to what was observed in the newt, the expression profiles in
the axolotl differed considerably in both intact and 8-d irises (Fig. 4a).
Expression in the intact and 8-d ventral irises was not higher than in
the dorsal ones. Moreover, *six-3* was severely downregulated in the
irises 8 d after lentectomy as compared with the intact irises. *pax-6*
and *BMPR-IA* 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 negative regulation
of *six-3*, as well as regulation in establishing thresholds, might be
responsible for the lack of lens regeneration in the axolotl. So, why
did the treatments fail to induce lens transdifferentiation? We think
that it was because repression of *six-3* persisted even after the
treatments. Indeed, treatment with chordin, which mediated
upregulation of *six-3* and *pax-6* in the induced newt ventral irises,
did not do so in the axolotl (compare Fig. 4c with Fig. 2g). Several
explanations can account for the axolotl results. First, *six-3*

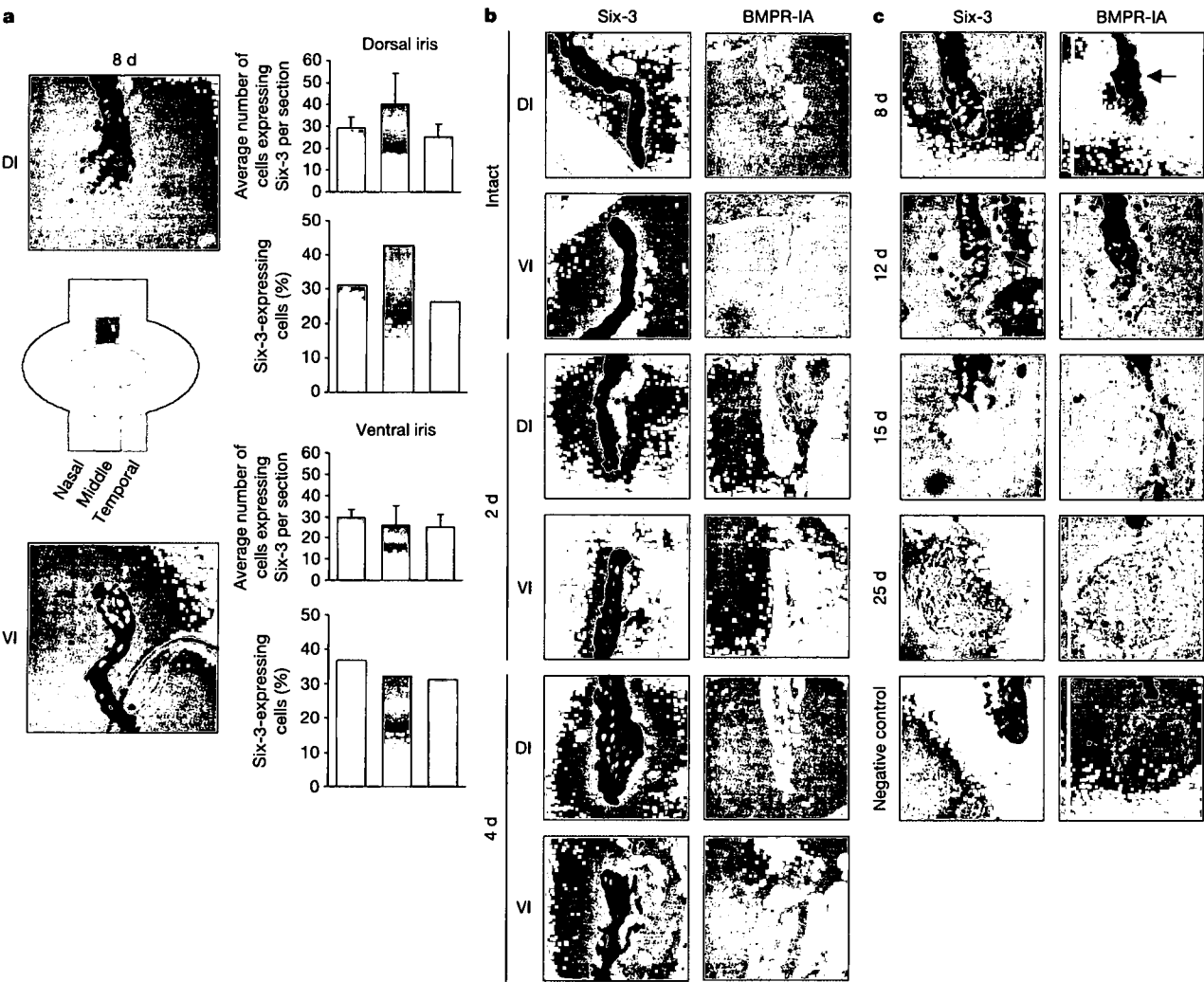


Figure 3 | Expression of Six-3 and BMPR-IA during regeneration. Identification of Six-3-positive cells in the dorsal (DI) and ventral (VI) iris 8 d after lentectomy, as assessed by immunostaining of serial sections along the nasal-temporal axis (see drawing). No apparent difference in cell numbers or distribution was seen (right). Graphical data are the mean \pm s.d. **b**, Expression of Six-3 and BMPR-IA in dorsal and ventral iris at early stages (before vesicle formation). Owing to heavy pigmentation at these early stages for the BMPR-IA staining, sections were bleached.

c, Expression of Six-3 and BMPR-IA at later stages. At 12 d after lentectomy, a definite lens vesicle has been formed. The lens vesicle and the differentiating lens fibres are positive at 15 d, and by 25 d only cells in the lens epithelium express Six-3. The same patterns can be seen for BMPR-IA. Negative controls are shown at the bottom. All images shown are mergers of the dark field and DIC images. The stroma (arrows) always shows unspecific fluorescence.

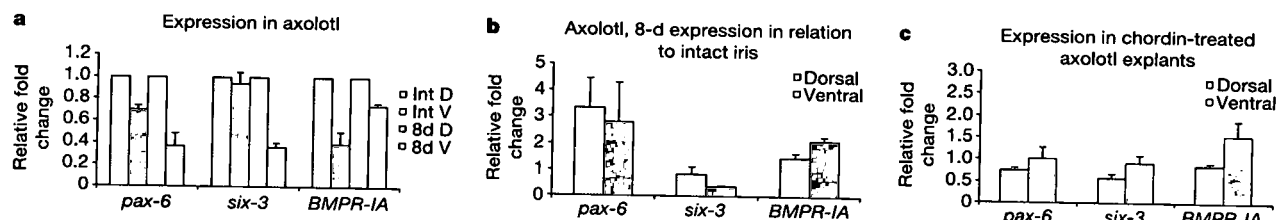


Figure 4 | Expression in axolotl. **a**, Comparison of *pax-6*, *six-3* and *BMPR-IA* expression between the axolotl dorsal and ventral iris. Expression is shown in intact irises and 8 d after lentectomy. Values in the dorsal iris have been set to 1 and those in the ventral iris are shown as relative fold

changes. **b**, Comparison of expression in 8-d irises with intact irises. **c**, Expression in chordin-treated iris explants relative to untreated explants. Data show mean \pm s.d.

repression could be regulated more tightly in the axolotl 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.

Notably, *pax-6* did not induce transdifferentiation of the ventral iris, even though it was shown to be upregulated in the chordin-treated ventral irises. However, *six-3* was not upregulated in *pax-6*-transfected cells (data not shown), which might be the reason why *pax-6* could not induce transdifferentiation. On the basis of other results from our laboratories, we now consider that *pax-6* is involved in later events of lens regeneration, such as the proliferation of PECs in both the dorsal and ventral iris and the control of crystallin synthesis (unpublished data). The fact that retinoic acid was also necessary for the induction most probably indicates that other factors regulated by retinoic acid are involved, a synergism that has been shown in other studies^{16–18}. Notably, both inhibitors of the BMP pathway and the *six-3*–*pax-6* loop are part of a network identified during induction of eye development¹⁹. The upregulation of *six-3* and *pax-6* in chordin-treated iris explants (Fig. 2g) suggests that the BMP signalling is upstream of the *six-3*–*pax-6* regulatory loop.

Previous work 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^{20,21}. This expression goes against the commonly held belief that regulatory genes involved in lens regeneration should be dorsal-specific. However, our detailed quantitative studies suggest that previously unknown regulatory events are involved in the induction of lens regeneration. Collectively, our data show that induction of lens regeneration can be achieved in noncompetent adult tissues—an important finding because ectopic lens formation has not been previously shown in adults and it opens new avenues in the field of vertebrate lens regeneration.

METHODS

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

Cloning of newt *six-3* and *BMPR-IA* partial cDNAs. *BMPR-IA* was cloned with RNA isolated from newt forelimb blastema (~2 weeks after amputation) using Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. Dorsal PECs were used to clone a partial cDNA for *six-3*. We used 1 μ g of RNA to synthesize cDNA using an iScript cDNA synthesis kit (BioRad). For PCR, a portion of the DNA was used along with Taq polymerase, 100 μ M dNTPs and 800 nM primers. We used the following primers: *BMPR-IA*, forward (5'-TGCTGATGCTGAYTTGG-3') and reverse (5'-GGRTCATTCACCA-3'); *six-3*, forward (5'-CACTACCAGGAGGCCGAGAA-3') and reverse (5'-TCCTGAAGCAGTGGCTCTT-3'). DNA was purified with a MinElute gel extraction kit (Qiagen). The fragment was cloned by the pGEM-T easy vector system (Promega) and sequenced.

Immunostaining. Affinity-purified polyclonal antibodies were made against *six-3* and *BMPR-IA* peptides. Antibody against *six-3* was made in rabbit (New England Peptide) and antibody against *BMPR-IA* in chick (Cocalico). Newts were anaesthetized and the lens was removed through a slit in the cornea. The newts were killed at 2, 4, 8, 12, 15 and 25 d after lentectomy. The eyeballs were nucleated and fixed in 4% formaldehyde for 4 h, washed in 1 \times PBS buffer,

cryoprotected in 30% sucrose, embedded in OCT (Andwin Scientific), frozen and sectioned at 10 μ m. Slides with frozen serial sections were washed several times in PBS and 1% saponin (Sigma) and incubated in 10% goat serum in PBS. Occasionally, to reduce pigmentation, sections were bleached in 0.1% potassium permanganate for 10 min, immersed in 0.5% oxalic acid for 5 min and then rinsed in PBS.

The samples were incubated at 4 $^{\circ}$ C overnight with primary antibody against newt *six-3* diluted 1:10 in blocking solution or against newt *BMPR-IA* diluted 1:100 in blocking solution, washed in 0.3% PBST and PBS, and then incubated with the following secondary antibody for 2 h at 37 $^{\circ}$ C: Alexafluor 546-conjugated goat anti-rabbit (Molecular Probes) for *six-3*, or FITC-conjugated rabbit antibody against chicken (Sigma) for *BMPR-IA*, diluted 1:200 in 10% goat serum in PBST. The sections were washed with PBST and PBS, and covered with coverslips using Vectashield (Vector Labs). Images were taken by confocal microscopy.

Real-time PCR. RNA was isolated from iris tissue and PECs by using Tri Reagent (Molecular Research Center) according to the manufacturer's instructions. We used the following tissues and cells: intact dorsal and ventral iris; 2-, 4-, 8-d dorsal and ventral iris; cells isolated from dorsal and ventral iris; dorsal and ventral iris cells transfected with *six-3* and treated with retinoic acid, transfected with *six-3* alone, or treated with retinoic acid alone; explants from dorsal and ventral iris; dorsal and ventral iris explants treated with chordin or *BMPR-IA*; axolotl intact and 8-d dorsal and ventral iris; and chordin-treated axolotl dorsal and ventral iris. The RNA isolated was used to evaluate expression of *six-3*, *BMPR-IA* and *pax-6* (along with a suitable reference gene) by real-time PCR, as well as by RT-PCR to verify that the correct fragment was amplified. Appropriate negative controls were included in all sets.

We used 0.75 μ g of RNA to synthesize cDNA using an iScript cDNA synthesis kit (BioRad). All real-time PCRs were done with an iCycler (BioRad). For each real-time PCR reaction run in triplicate, 2 μ l 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-IA* and from a published sequence of *pax-6*. The new primers were *rpL27*, forward (5'-TACAACCACTTGATGCCA-3') and reverse (5'-CAGTCTGTATCGTTCCTCA-3'); *pax-6*, forward (5'-CTGGCCAGGTATTACGAG-3') and reverse (5'-GTCTCTGATTCCCAAGGC-3'); *six-3*, forward (5'-CAAGAAGTCCCGCTGC-3') and reverse (5'-GGTAGGGTCTGTAGGTAC-3'); *BMPR-IA*, forward (5'-TGCTGTATTGCTGATTAGG-3') and reverse (5'-ATAGGTATCAAGCAAGCAGTA-3'). The axolotl primers were *RP*, forward (5'-CATCAGATCAAGCAAGCAGTA-3') and reverse (5'-CCAATGCAGCAGTTTAGATG-3'); *pax-6*, forward (5'-GAGTGCTCCGC AACCTG-3') and reverse (5'-ATTCGTGTTCTCGCCTCC-3'); *six-3* (same as newt); *BMPR-IA*, forward (5'-CAGTGTGCTGATTGCTGAT-3') and reverse (5'-GGTACTTCCCAATAACC-3').

For each real-time PCR the basic program was as follows: denaturation at 95 $^{\circ}$ C, annealing at 50.6 $^{\circ}$ C and extension at 72 $^{\circ}$ C (40 cycles). To minimize the background caused by primer-dimer formation, an extra step was added (78 $^{\circ}$ C for 6 s) at the end of each cycle. The readings were taken during this step. Data analysis was done with the Pfaffl method²². The reference genes were *rpL27* for newt and *RP* for axolotl, which both encode ribosomal proteins.

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Author Information Sequences for *six-3* and *BMPR-1A* have been deposited in GenBank under accession numbers AY799802 and AY795966, respectively. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to P.A.T. (panagiotis.tsonis@notes.udayton.edu).

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