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IMMUNOLOCALIZATION OF ALPHA SMOOTH MUSCLE ACTIN AND TYPE IV  
COLLAGEN DURING LENS REGENERATION IN THE

NEWT, *NOTOPHTHALMUS*

*VIRIDESCENS*

Thesis

Submitted to

The College of Arts and Sciences of the

**UNIVERSITY OF DAYTON**

In Partial Fulfillment of the Requirements for

The Degree

**Master of Science in Biology**

By

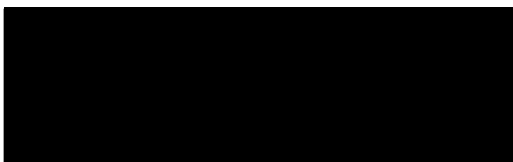
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**UNIVERSITY OF DAYTON**

Dayton, OH

May, 2005

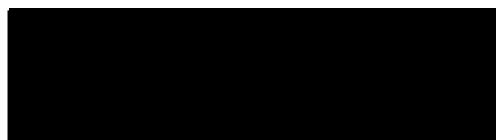
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## ABSTRACT

### IMMUNOLOCALIZATION OF ALPHA SMOOTH MUSCLE ACTIN AND TYPE FOUR COLLAGEN DURING LENS REGENERATION IN THE NEWT, *NOTOPHTHALMUS VIRIDESCENS*

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University of Dayton, 2005

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Alpha smooth muscle actin is considered to be a marker for epithelial-mesenchymal transition (EMT). EMT occurs in a variety of tissues, including the lens after cataract surgery. In humans, a cataract of the lens is usually treated by surgical removal of the affected lens, while the lens capsule is left behind. In many cases, lens epithelial cells remain in the capsule during surgery. These epithelial cells can differentiate to form lens fibers and undergo EMT, which causes the formation of a secondary cataract. EMT is also observed during mouse lens regeneration, which occurs from lens epithelial cells left in the lens capsule after lentectomy. Because secondary cataract formation is not seen in newt lens regeneration, we decided to investigate the expression of  $\alpha$  smooth muscle actin ( $\alpha$ -SMA), which is used as a marker for EMT, and type IV collagen, a marker for the capsular bag, by using immunofluorescence microscopy to localize the proteins during regeneration. Our results showed the presence of  $\alpha$ -SMA in the lens vesicle and lens epithelium all throughout regeneration, while type

IV collagen was not present until 30 days post lentectomy. These data indicate that the lens vesicle and, later, lens epithelial cells may possess the potential to differentiate into either epithelial or mesenchymal cells. However, this bi-potency of lens epithelial cells is not expressed in newts and is only the property of lens epithelial cells after cataract surgery.

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## LIST OF ABBREVIATIONS

AEC	Apical epidermal cap
AER	Apical ectodermal ridge
ALK	Activin receptor-like kinase
$\alpha$ -SMA	$\alpha$ -Smooth muscle actin
BCA	Bicinchoninic acid
BMP	Bone morphogenetic protein
BMPR	Bone morphogenetic protein receptor
DIC	Differential interference contrast microscopy
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
FITC	Fluorescein isothiocyanate
Ggf	Glial growth factor
HRP	Horse radish peroxidase
Ihh	Indian hedgehog

LECs	Lens epithelial cells
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MSC	Mesenchymal stem cells
OFZ	Organelle free zone
PBS	Phosphate-buffered saline solution, pH 7.2
PBST	PBS and Triton X-100
PCO	Posterior capsular opacification
PDGF	Platelet-derived growth factor
PECs	Pigmented epithelial cells
Ptc	Patched (hedgehog receptor)
RA	Retinoic acid
RAR	Retinoic acid receptor
Rb	Retinoblastoma protein
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
Shh	Sonic hedgehog
TGF- $\beta$	Transforming growth factor- $\beta$

## Introduction

In humans, lens epithelial cells (LECs) are often left behind during cataract surgery, when the lens is removed from the lens capsule. An injury response is seen in these cases, where the LECs will proliferate to attempt to repair the damage (Rakic et al., 1997, 2000). In many cases, these cells will undergo epithelial-mesenchymal transition (EMT), a process where the cells transdifferentiate from LECs to mesenchymal cells called myofibroblasts. EMT normally plays critical roles in embryonic development, and in many types of cancer (Zavadil et al., 2004). A number of factors have been identified as playing various roles in the process of EMT (and thus the formation of posterior capsular opacifications). These factors include lumican (Saika et al., 2003), FGF family members (Wormstone, 2002; Tanaka et al., 2004), TGF $\beta$  superfamily members (Saika et al., 2001), and transferrin and EGF (Wormstone, 2002). A standard marker used to identify this process is  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) (Wormstone, 2002). The myofibroblasts that differentiate as a result of EMT cause a secondary opacification that requires a second surgery.

There are no such complications during lens regeneration in the newt. Following lentectomy, the dorsal iris tissue, but not the ventral iris, begins to dedifferentiate. After approximately 10 days, an undifferentiated lens vesicle has formed. The vesicle cells will differentiate into LECs, and the LECs begin to proliferate and differentiate into lens fibers expressing crystallin proteins. By 15 days post lentectomy, lens fibers have accumulated, and the entire lens is beginning to grow in size. At 25-30 days post

lentectomy, the lens has grown to full size, and is considered fully regenerated (Tsonis, 2000b).

A second type of regeneration, regeneration of the limb, is of interest because the dedifferentiated cells produced during regeneration are mesenchymal cells. After amputation, adult tissue in the limb stump, such as bone muscle and cartilage, dedifferentiates to form the mesenchymal blastema (Tsonis, 1996). In muscle tissue (the tissue most studied in dedifferentiation), the process involves signaling from the activated form of thrombin, which has been shown to be necessary for muscle fiber cells to reenter the cell cycle (Tanaka et al., 1999). Also of importance is the retinoblastoma (Rb) protein. The phosphorylated form of this protein, not seen in differentiated myofibers, is inactivated, and thus allows for the cells of the myofibers to reenter the cell cycle (Tam et al., 1995; Tanaka et al., 1997).

We chose to examine the expression of  $\alpha$ -SMA in regenerating and intact newt eyes, as this has never been previously investigated, and because it may prove to be informative in understanding the process of posterior capsular opacification. We also examined expression of collagen type IV, which is a marker for the lens capsule, which has also never been previously investigated. It was expected that the newt would not express  $\alpha$ -SMA, as EMT and the resulting opacification are not seen in newt regeneration. We examined expression in developing newt lenses to compare with the regenerating lens. We also chose to examine the expression of  $\alpha$ -SMA in regenerating newt limbs since blastema cells are mesenchymal cells. Finally, we used western blot

analysis to confirm that our antibody was binding the correct protein. Our experiments revealed that regenerating newt lenses, but not intact or embryonic ones, expressed  $\alpha$ -SMA, indicating that the epithelial cells may act as bipotent stem cells with the potential to differentiate to a mesenchymal cell or a lens fiber.

## **Literature Review**

### **I: Regeneration**

#### **A. Regeneration Overview**

Regeneration is the process by which an organism can replace damaged tissue or a lost body part. All organisms are capable of tissue regeneration; some, however, possess more spectacular regenerative abilities than others. In rats with nervous system injuries, simple axonal outgrowth can be induced. In many mammalian species, tissues can be repaired via the proliferation of the cells in the remaining tissue, or by the proliferation and differentiation of tissue specific stem cells, such as hematopoietic stem cells. The most complex types of regeneration involve the reproduction of complex lost body parts through the process of dedifferentiation (Tsonis, 2000a).

Urodele amphibians have this ability to regenerate lost body parts. This includes the regeneration of limbs, tail, jaw, lens, retina, and heart. Other amphibians, the anurans, also possess regenerative abilities. However, these abilities, such as limb and lens regeneration, decrease with age, and have disappeared by the end of metamorphosis. The chick is also capable of retina regeneration, but only during development. The urodele amphibians are unique in that their regenerative ability remains throughout life (Tsonis, 2000a).

## B. Limb Regeneration

Limb regeneration is impressive because the animal is regenerating multiple tissue types (the same is true for tail regeneration). Upon amputation, epidermal cells from the skin near the amputation site cover the wound, forming a specialized wound epithelium. The adult tissues of the limb stump, which includes cartilage, muscle, Schwann cells, and dermal and muscle fibroblasts dedifferentiate, and form a blastema, that will proliferate and form the new limb (Brockes, 1997; Tsonis, 2000a).

The process by which this dedifferentiation occurs has been studied, especially in regards to the breakdown of multinucleated muscle fibers into mononucleated cells. Research by Tanaka et al. (1999) has shown that activated thrombin plays a critical role in this process. They treated cultured newt myotubes with sera taken from several animal sources, and found that they all stimulated the myotubes, which usually do not undergo mitosis, to reenter the cell cycle. In comparison, mouse myotubes remained in their differentiated state upon treatment. Muscle precursor cells from both species, were stimulated to divide by the same treatments. Separation of the sera components revealed that thrombin was the critical component, although it did require other serum factors to work.

A second important molecular event in the breakdown of differentiated myotubes is the phosphorylation of the Rb protein, which helps regulate the cell cycle. Differentiated myotubes possess the non-phosphorylated form of Rb, which prevents entry into S phase of the cell cycle. When myotubes are treated with serum, the Rb

protein becomes phosphorylated, and thus inactive, and the myotubes are able to reenter the cell cycle, and dedifferentiate (Tanaka et al., 1997). Further studies indicate that the phosphorylated form of Rb is regeneration specific, as mouse myotubes only possess the non-phosphorylated form of Rb (Tam et al., 1995). Transfection with *msx-1* does lead to mouse myotubes reentering the cell cycle (Odelberg et al., 2000).

Once the blastema has dedifferentiated, there are numerous factors that play critical roles in the regeneration process. Following amputation, the blastema is reinnervated. Denervation inhibits regeneration ability (Brockes, 1984). There are several neural factors that also play a role in limb regeneration. These include Fgf2, Ggf-2, substance P, and transferrin. These four factors are located in either axons or cell bodies of limb nerves. They are also thought to be neural mitogens for blastema cells (reviewed in Nye et al., 2003).

There are many paracrine-signaling factors that play roles in limb regeneration. Members of the fibroblast growth factor (Fgf) family appear to be critical for limb regeneration. *Fgfs 1* and *2* are expressed in the apical epidermal cap (AEC) of the blastema, as is *Fgf receptor (Fgfr) 1* (Boilly et al., 1991; Mullen et al., 1996; Christensen et al., 2002; Dungan et al., 2002; Poulin et al., 1993). Both Fgf1 and Fgf2 are known to have mitogenic effects on the blastema, and Fgfr1 is the primary receptor for those molecules.

*Fgf 8* is also expressed in the AEC, and expression appears to be dependent upon *Fgf 10* expression in the underlying mesenchyme. During development, where the apical

ectodermal ridge (AER) functions in a similar manner to the AEC, Martin (1998) reported that *Fgf 10* knockout mice do not develop limbs, indicating that *Fgf 10* expression is necessary for limb outgrowth. In the African-clawed frog, *Xenopus*, which undergoes a regenerative decline as they approach metamorphosis, Fgf 10 treatment rescues the regenerative ability in limbs just after metamorphosis (Yokoyama et al., 2001). Treatment allows for regeneration of the limb up to the foot structures, along with reestablishing expression of *Fgf 8*. Treatment with Fgf 8 gave only a limited improvement in regeneration, and Fgf 2 did not improve regeneration at all.

Research by D'Jamoos et al. (1998) also indicated a critical role for Fgfs, by showing that *Fgfr1* and *Fgfr2* are expressed in the wound epidermis and blastema of pre-metamorphic frogs, while expression was not seen in regeneration-deficient limbs. Also, inhibition of Fgfrs led to the inhibition of regenerative ability.

Another set of genes known to play critical roles in both development and regeneration are the *Hox* genes. The *Hox* genes code for a family of transcription factors that feature the homeobox, a DNA binding domain. The homeobox is strongly conserved evolutionarily. In vertebrates, the basic set of homeobox genes has been duplicated three times, giving a total of four different gene groups, which has allowed for the evolution of new features (Tsonis, 2003).

Different members of the *Hox* family are known to play different roles in limb development, and consequently also in regeneration. Specifically, members from the A and D cluster are known to play roles in limb pattern formation. In chick limb

development, *HoxA 10, 11*, and *13* are expressed proximal to distal, in overlapping domains. *HoxD 9-13* are also expressed in overlapping fashion, from the anterior proximal region of the limb bud, to the posterior distal region (McGinnis and Krumlauf, 1992).

During limb regeneration, the Hox D and A cluster genes are also expressed from proximal to distal in the limb, moving from 3' to 5' along the DNA sequence, with *D10* and *A9* associated with proximal limb structures, and *D 13* and *A 13* associated with distal structures (Nye et al., 2003). Just after amputation, *HoxA 9* and *HoxA 13* are both expressed in the blastema, and do not exhibit the spatial restrictions, with both genes localizing to the stump. Once the blastema has formed, *HoxA 9* remains expressed in the stump tissue as well as in the blastema. *HoxA 13* expression is lost in the adult tissue, and is found only in the blastema. As regeneration progresses, *HoxA 9* expression is seen throughout the blastema. *HoxA 13*, on the other hand, becomes restricted to the distal portion of the blastema, within the *HoxA 9* domain (Gardiner et al., 1995).

During limb regeneration *HoxD* genes are re-expressed in the limb. *HoxD 8* and *HoxD 10* are expressed immediately in the limb stump. Expression for both genes appears in the early blastema, but has disappeared by palate stage. Expression of *HoxD 11* is not seen until early bud stage blastema, and is seen mostly in the posterior distal region of the blastema. When regeneration reaches the late bud stage, distal expression is no longer seen, and a band of expression across the anterior to posterior axis is formed. Expression is strongest in the posterior, and absent in the most anterior. As digits begin

to form, expression remains proximally in the wrist and zeugopod (forelimb) (Torok et al., 1998).

Finally, retinoic acid (RA) appears to play a role in maintaining the proximal-distal axis during limb regeneration. Treating a regenerating limb with RA leads to the duplication of the limb along the proximal-distal axis, i.e., the limb regenerates as if it were amputated at a level more proximal than the actual amputation (Maden, 1982). Experiments by Gardiner et al. (1995) indicate that this effect may be due to the RA altering the expression pattern of *Hox* genes during regeneration. After one day of treatment with RA, the limb blastemas showed a decrease in *HoxA13* expression in the distal tip. Treatment for three days increased the effect, as very little *HoxA13* expression is seen. The increased amount of RA treatment corresponded with an increase in the frequency of limb pattern alterations. When *HoxA9* expression was examined, it was found that there was no difference in the expression pattern. This indicates that the RA reestablished the limb pattern to a region that was more proximal than the original limb stump.

### C. Lens Regeneration

The entire process of newt lens regeneration occurs over approximately 30 days, and involves the transdifferentiation of dorsal iris cells into LECs (Tsonis, 2002). During the first 10 days, molecular signaling events occur that lead to the dorsal iris cells de-pigmenting, and taking on an undifferentiated state. At 10 days a de-pigmented early lens vesicle, consisting of lens epithelial cells, can be observed. Over the next few days that vesicle grows, and by 15 days post lentectomy the cells of the lens epithelium can be seen to be differentiating into crystalline-expressing lens fibers. From 20 to 25 days post lentectomy, the new lens continues to grow, as more and more lens fibers differentiate, and by day 30, the new lens has reached the size of the original lens (Tsonis, 2000).

There are many molecular factors that play roles in lens regeneration. The first examined was the paired domain and homeobox domain containing gene, *pax6*, which is known to play critical roles in eye development (Gilbert, 2000). Experiments by Del Rio-Tsonis et al. (1995) reveal that expression of *pax6* is seen throughout the adult newt eye, in the neural retina, lens, and cornea epithelium. These expression patterns were also seen in the lens of regenerating newt eyes at all stages.

Because lens regeneration specifically occurs from the dorsal iris tissue, identifying dorsal specific factors is important. Research by Del Rio-Tsonis et al. (1999) identified *prox-1*, a homolog of the *Drosophila prospero* gene, as such a factor. In situ hybridization revealed *prox-1* transcripts in both the dorsal and ventral irises, as well as

in the regenerating lens. Western blot analysis revealed that prox-1 protein was only present in the dorsal iris, but not in the ventral.

The role of retinoic acid (RA) has been examined in lens regeneration because of the roles it is known to play in eye development, and its roles in gene regulation. Treatment with different types of RA (all-trans RA, 9-cis RA, and retinol palmitate) revealed no effect on regeneration (Tsonis et al, 2000). Inhibiting RA signaling, however, did have an effect. Disulfiram, an inhibitor of retinal aldehyde dehydrogenase, stops the production of RA, and caused a retardation of the lens regeneration process. Inhibition was also performed using the RA receptor (RAR) antagonist, 193109. This treatment also led to the retardation of normal lens regeneration. Most interesting, however, was that these inhibitory treatments led to the transdifferentiation of ectopic lenses from the cornea, ventral iris, and a second lens from the dorsal iris. These ectopic lenses, while rare, were significant in that this effect had never been seen previously (Tsonis et al., 2000).

Research has been done on the roles of Fgf family members in lens regeneration. Localization experiments showed that *Fgf1* and *Fgfr 2* and *Fgfr 3* are expressed in the newt retina and in the regenerating lens. Expression was also seen in pigmented iris tissue. Interestingly, immunohistochemistry revealed that both receptors were seen in both dorsal and ventral irises (Del Rio-Tsonis et al., 1997).

Further investigation revealed a significant difference in the expression of *Fgfr 1*. Del Rio-Tsonis et al. (1998) reported that, while expression was mostly similar to the

expression seen for *Fgfr 2* and *Fgfr 3*, *Fgfr 1* expression was restricted to the dorsal iris (or that ventral expression was much lower). The same pattern was seen using the *Fgfr1* antibody. This indicates that *Fgfr1* may play a role in the restriction of regenerative ability to the dorsal iris.

Hayashi et al. (2004) further implicated Fgf signaling by treating intact eyes with Fgf2. A single dose of Fgf2 (along with other members of the Fgf family) was injected into intact newt eyes. These Fgf2 injections led to the dorsal iris tissue transdifferentiating a new lens (none of the other treatments yielded this result). Reverse transcriptase polymerase chain reaction (RT-PCR) revealed that this injection upregulated genes that are upregulated during normal regeneration, indicating that Fgf2 plays a role in this process.

Recent work from the Tsonis laboratory has indicated roles for members of the bone morphogenetic protein (BMP) pathway and for the transcription factor, *six-3* during regeneration (unpublished data). Transfection of cultured newt ventral iris pigmented epithelial cells (PECs) with *six-3* failed to allow the ventral PECs to transdifferentiate, as did treatment with RA. However, when the two treatments were combined, transdifferentiation was observed at a rate similar to what is seen for transdifferentiation of cultured dorsal PECs. Treatment of ventral iris explants with either the BMP antagonist, chordin, or with a soluble competitor for the BMP receptor (BMPR) 1A also led to transdifferentiation, although it was at a much lower rate.

Real time RT-PCR analysis of intact and regenerating newt eyes revealed an interesting pattern of expression of *six-3*, *pax-6*, and *BMPRIA* (Tsonis Lab, unpublished data). In intact eyes, all three genes were expressed, but with higher ventral expression. At 8 days post lentectomy, when transdifferentiation has begun, *six-3* expression was greatly up regulated in the dorsal iris, but not in the ventral. *Pax-6* and *BMPRIA* were slightly up regulated. When ventral PECs and explants were treated as before and analyzed using real time RT-PCR, they revealed a pattern of expression similar to the regenerating dorsal iris.

Experiments have also been performed showing that sonic hedgehog (*Shh*) plays a role in newt regeneration. Using in situ hybridization Tsonis et al. (2004) started by examining the presence of *Shh*, *Indian hedgehog* (*Ihh*), and the two hedgehog receptors, *patched (ptc) 1* and *ptc 2*. *Shh* and *Ihh* were not seen in the intact lens, while *ptc1* was present. During regeneration, all three were seen in the early vesicle and in the 15-day lens. By the time the primary and secondary lens fibers had differentiated, *Ihh* and *ptc1* were seen throughout the lens, while *Shh* was restricted to the lens epithelium and secondary fibers. *Ptc2* was never seen in the eye using in situ hybridization.

In order to observe expression in the iris tissue, and to confirm the in situ hybridization results, RT-PCR was utilized, looking at intact (dorsal iris, ventral iris, lens, retina), regenerating (dorsal iris, ventral iris, lens), and developing embryonic lens. *Shh* was not found in the intact lens or iris, but *Ihh* was found to be in the intact iris tissue, and both *ptc 1* and *2* were found in the lens and iris tissues. All genes examined were

found in the two iris tissue types and the lens of regenerating eyes, as well as the developing lens.

To finally confirm that the hedgehog pathway does play a role in lens regeneration, Tsonis et al. (2004) used two different hedgehog inhibition methods to inhibit the pathway. It was found that both methods caused interference with the regeneration process. This was first confirmed by observing that both treatments led to the down regulation of *ptc-1*, their target in the pathway. Secondly a BrdU assay was used to observe that cellular proliferation was significantly decreased following inhibition (indicating that hedgehog signaling plays a role in proliferation). Finally, crystallin staining was performed on the regenerating vesicles of inhibited eyes.  $\beta$ -crystallin was not observed in the treated vesicle. This confirmed that hedgehog inhibition is responsible for the loss of regeneration.

In addition to signaling molecules that might be expected to play roles in regeneration, there are some unexpected players in regeneration. Research by Kimura et al. (2003) has indicated that complement pathway members 3 and 5 may play a role in limb and lens regeneration. Complement proteins are best known for playing a role in the body's latent immune response. C3 was not seen in the regenerating lens, but was observed in iris pigmented epithelium and in the cornea. C5 was observed in the regenerating lens and cornea. These antibody-staining results were confirmed by in situ hybridization. Expression of both genes is seen in the regenerating lens vesicles. Neither protein was ever seen in the intact lens.

Another interesting treatment that could induce lens regeneration is the carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Experiments by Eguchi and Watanabe (1973) were performed by either placing an MNNG crystal into the lentectomized eye for at least one hour, or by treating dorsal and ventral iris explants with MNNG solution, and implanting the iris pieces into lentectomized eyes. Treatment with the MNNG crystal yielded multiple supernumerary lenses (normal regeneration, from the central portion of the dorsal iris was not affected). Interestingly, if a secondary lentectomy was performed, where both lenses were removed, regeneration was seen for both lenses, indicating that the MNNG-induced competency of the iris tissue remained for at least 12 months. When iris pieces were treated with MNNG, it was found that the dorsal iris pieces transdifferentiated normally, while a small number of the ventral pieces also were able to transdifferentiate into new lenses.

During lens regeneration, expression of the *crystallin* genes (specifically  $\alpha A$ ,  $\beta B1$ , and  $\gamma$  crystallins) appears to resemble the expression pattern, seen in normal development. Crystallin expression was first detected approximately 12-15 days post lentectomy, and expression of all three genes was seen in the primary lens fibers. When secondary lens fibers began to appear, expression of  $\alpha$  and  $\beta$  crystallins are seen in those secondary fibers, while decreasing in the primary fibers.  $\gamma$ -crystallin is not seen in the secondary fibers, but does decrease in the primary fibers, the same as  $\alpha$  and  $\beta$  (McDevitt and Brahma, 1982, 1990; Mizuno et al., 2002).

A second important step in lens regeneration is the degradation of the lens fiber organelles in cells that light will pass through, so that those organelles will not scatter light. During development, this process begins in the core of the lens and creates an organelle free zone (OFZ) (Bassnett and Beebe, 1992). There is an initial phase of degradation that occurs rapidly. This includes fragmentation of the mitochondria and endoplasmic reticulum. The degradation of the nucleus is relatively slower, and involves the nucleus taking on a more spherical shape, the two nucleoli fusing, and condensation of the DNA. Finally, the nuclear membrane begins to fragment, and the filamentous cytoplasmic components intermix with the nuclear remnants. The condensed DNA eventually is broken down (although in some species it persists for a long time) (Bassnett, 2002).

## II. Secondary Cataracts

### A. Formation of Secondary Cataracts Following Surgery

Understanding the process of lens regeneration is important for the implications that it holds in regards to the treatment of cataracts in humans. When cataract surgery is performed, the lens capsule, which holds the lens in place, is left behind, and a synthetic lens is inserted (Wormstone, 2002). During removal of the original lens, LECs are inadvertently left behind in the capsule. In approximately 20 to 40% of patients, these remaining LECs will migrate into the space where the lens used to be and will either proliferate or differentiate into mesenchymal cells. This differentiation leads to posterior capsular opacification (PCO), referred to as secondary cataracts, and will often require a second surgery to correct (Wormstone et al., 2002). Research has shown that the LECs need to be able to migrate along the lens capsule for EMT to occur (Nagamoto et al., 2000).

In the rabbit (Gwon et al., 1990), and mouse (Call et al., 2004), it has been shown that the wound healing response from cataract surgery (i.e., the differentiation of the epithelial cells left in the capsule) can lead to the regeneration of the lost lens. Call et al. (2004) reported that lens fiber differentiation was observed as early as 2 days post lentectomy, and that by 30 days post lentectomy a significant lens (at least half the size of an intact lens) was seen. Lens fiber differentiation was confirmed by positive staining with a  $\beta$ -crystallin antibody. Interestingly, they also observed  $\alpha$ -SMA antibody staining

from 10 to 20 days post lensectomy, indicating that EMT was occurring.  $\alpha$ -SMA was observed in cells located by the lens capsule, marked by antibody staining for collagen type IV.

EMT is the process that generally leads to PCO in cataract patients. EMT leads to the wrinkling of the capsule that, in conjunction with the production of extracellular matrix (ECM) by the mesenchymal cells, leads to the observed opacification (Apple et al, 1992). In embryonic development, where it plays many critical roles, EMT is characterized by a loss of cell-cell contacts, reorganization of cytoskeletal elements, the expression of mesenchymal cell markers, and migratory properties (Hay, 1995). In addition to being required for embryonic development, EMT is also observed in many advanced cancer phenotypes (Zavadil et al., 2004).

The exact cause of this process is not well understood. Studies have been performed to try to observe when the increased levels of cell proliferation that participate in the opacification occur. Rakic et al. (1997) first observed that when lens fibers were separated from the lens epithelium and cultured, there was a large increase in cell proliferation, as compared to control lenses in culture. Further research has shown that this increase in cell proliferation is stopped by the differentiation of new lens fibers. In cases of PCO, cell proliferation levels matched levels seen in normal eyes. However, when the synthetic lens was removed, proliferation levels increased to the levels seen in previous studies, indicating that the initial burst of proliferation only lasts until new lens fibers are formed (Rakic et al., 2000).

In addition to this physical trigger of the proliferation seen in PCO, several key molecular components have been identified. Proteins such as transferrin, EGF, and members of the FGF and TGF- $\beta$  families have been shown to play various roles in the healing process following an injury to the lens (Wormstone, 2002). These will be discussed below.

TGF- $\beta$  family members are believed to play significant roles in the process of PCO. Studies have shown that the addition of TGF- $\beta$  protein to rat LECs induces them to transdifferentiate into mesenchymal cells, as marked by the expression of  $\alpha$ -SMA (reviewed in Wormstone, 2002). Saika et al. (2001) have shown that injury to the mouse anterior lens capsule will lead to the nuclear translocation of the proteins, smad 3 and 4. The smad proteins are a downstream target of TGF- $\beta$  signaling, and indicate that endogenous levels of TGF- $\beta$  molecules, such as TGF- $\beta$ 2 play a role in the wound healing process that leads to PCO.

Lee and Joo (1999) reported on the effect of TGF- $\beta$ 1 on the expression of genes that are markers for EMT. In cultured bovine and rabbit LECs, they discovered that treatment with TGF- $\beta$ 1 increased expression of fibronectin and type I collagen (bovine) and  $\alpha$ -SMA (rabbit). These results compared well with human lenses that exhibited anterior polar cataracts, which are morphologically similar to secondary cataracts formed by EMT.

Research by Wormstone et al. (2002) investigated the role played by TGF- $\beta$ 2, the major TGF- $\beta$  isoform found in the eye. They found that TGF- $\beta$ 2 was present in cultured

lens capsules, but that this increased expression was mostly not significant. Treatments of cultured capsules with TGF- $\beta$ 2 lead to the observable wrinkling of the capsule that is seen in secondary cataracts. In addition, treatment of the capsules with both TGF- $\beta$ 2 and CAT-152, an anti-TGF- $\beta$ 2 neutralizing antibody did not yield wrinkling. Treatments with TGF- $\beta$ 2 also caused an increase in matrix metalloproteinases 2 and 9, which indicate that changes to the cellular matrix that characterize EMT are occurring.

The activin receptor-like kinases (ALK) can act as receptors for TGF- $\beta$ . Desgrosellier et al. (2005) reported that misexpression of constitutively active *ALK2* can induce EMT in heart tissue during development. The authors misexpressed both *ALK2* and *ALK5* in regions of the developing heart where EMT does not normally occur. It was found that *ALK2* but not *ALK5* induced EMT. In addition, they also overexpressed *Smad6* in regions of the heart where EMT normally occurs. They found that *Smad6* overexpression decreased the amount of EMT seen.

Other evidence, however, indicates that the expression of  $\alpha$ -SMA following injury to the lens may be independent of TGF- $\beta$  signaling. Nagamoto et al. (2000) showed that even in the absence of TGF- $\beta$ , cultured mammalian lens epithelial cells (LECs) express  $\alpha$ -SMA and form the multilayered cellular aggregates that are the hallmark of PCO. This indicates that there are other factors involved in PCO.

FGFs also appear to play a role in the wound healing process after cataract surgery. Members of the FGF family have been shown to play roles in the proliferation, migration, and differentiation of lens epithelial cells that remain in the capsular bag

(reviewed in Wormstone, 2002). Evidence from Tanaka et al. (2004) indicates that *Fgf2* is critical for the proliferation of lens epithelial cells. However, this particular FGF member does not play a role in differentiation, as *Fgf2* null mice show no difference in  $\alpha$ -SMA expression.

Mansfield et al. (2004) indicated that *Fgf2* plays a critical role in the process. TGF- $\beta$ , in addition to inducing the EMT process, is known to induce apoptosis. It is also known that *Fgf2* exacerbates the formation of anterior subcapsular cataracts. Experiments were performed where cultured LECs from rats were treated with FGF2 and TGF- $\beta$ , which showed an increase in cell survival over cells just treated with TGF- $\beta$ .

Other factors, such as lumican and transferrin, are also necessary for EMT to occur. In mice that have normal lumican expression,  $\alpha$ -SMA expression is seen as it normally is during the wound repair following a lens injury. However, in lumican knock-out mice, LECs visually appear to keep their epithelial shape following injury, and expression of  $\alpha$ -SMA is delayed past the point it normally begins (Saika et al., 2003). The secreted protein transferrin, an iron-transport protein, is thought to be an epithelial cell survival factor (Davidson et al., 1998).

### III. Mesenchymal Stem Cells

#### A. Mesenchymal Stem Cells and Smooth Muscle Actin

Mesenchymal stem cells (MSCs) were first identified as cells with bone forming ability. Peled et al. (1991) identified a set of cells, termed bone marrow stromal cells, that they described as expressing  $\alpha$ -SMA, but that were not hematopoietic in nature. Friedenstein (1991) was the first to describe a method of isolating these cells for study. They were later classified as stem cells when it was discovered that they were capable of differentiating into multiple tissue types, including osteoblasts, chondrocytes, endothelial cells, and neuronal-like cells. They have been distinguished from hematopoietic stem cells by their negative staining for hematopoietic markers such as CD34, CD45, CD14, and others. In contrast, MSCs are positive for markers such as CD105, CD166, among others (Kassem et al., 2004).

Research by Pittenger et al. (1999) has confirmed that MSCs from adult humans have this potential to differentiate into several different cell types. They isolated MSCs from adult bone marrow samples, and cultured them in conditions favorable for MSC growth, confirming the purity of their samples using the available markers. They then found that these adult MSCs could be induced, under appropriate conditions, to differentiate into adipocytes, chondrocytes, and osteocytes. Normal adult fibroblasts were not capable of differentiation. They also concluded that these cells were multipotent by inducing differentiation, from a single parent cell, into different cell types.

Research has shown that these cells express the contractile actin isoform,  $\alpha$ -SMA, as has been seen in mouse, canine, and human models ( $\alpha$ -SMA is primarily seen, as the name suggests, in smooth muscle, and is critical for the muscle's contractile properties). The  $\alpha$ -SMA co-localizes with stress fibers that are characteristically seen in MSCs (Bonanno et al., 1994; Cai et al., 2001; Peled et al., 1991). Western blot analysis and immunohistochemical analysis by Kinner et al. (2002) has confirmed the co-localization. Kinner et al. (2002) also showed that, when these adult MSCs were grown on a collagen-glycosaminoglycan matrix, contraction of the matrix pores, mediated by the  $\alpha$ -SMA expressing cells, could be observed. An increase in  $\alpha$ -SMA expression was seen when cells were treated with TGF- $\beta$ 1, and expression decreased when treated with platelet-derived growth factor (PDGF)-BB. Contraction of the cells was also seen to decrease with the PDGF treatment.

#### IV. Statement of the Problem

In humans, following cataract surgery, LECs remain in the lens capsule, and can undergo EMT. This change in cell type leads to the lens capsule wrinkling, and causes the lens capsule to become opacified, requiring a second surgery to correct the problem. In mice, cataract surgery can be performed without the implantation of a synthetic lens, which allows the LECs to differentiate into lens fiber cells, regenerating the lost lens. Beginning 5 days post lentectomy, EMT is seen as marked by staining for  $\alpha$ -SMA, with the lens capsule marked by staining collagen type IV.

Because the newt is capable of regeneration without complication from EMT, I decided to examine for the presence of  $\alpha$ -SMA in the newt during regeneration by antibody staining. I also examined for the presence of collagen type IV since the attachment to the lens capsule is required for EMT. I also examined  $\alpha$ -SMA and collagen type IV expression in embryonic newt lenses to compare and see if  $\alpha$ -SMA is a feature of the newt lens, and examined  $\alpha$ -SMA staining in two week regenerating newt limbs to compare with another regenerating tissue.

## Materials and Methods

**Animals:** Adult red spotted newts, *Notophthalmus viridescens*, were obtained from Mike Tolley Newt Farm (Donelson, TN) and from Charles D. Sullivan, Co. Inc. (Nashville, TN). The newts were anesthetized in 0.5% general anesthesia (ethyl 3-amino benzoate methane sulfonic acid in 1x phosphate buffer). Some newts were lentectomized by cutting the cornea and removing the lens. Other newts were anesthetized and their limbs were amputated at the mid forelimb level. A final set of newts was euthanized and kidney tissue was dissected out and frozen.

Adult (6 weeks or older) Balb/CJ mice, *Mus musculus*, were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were euthanized and kidney tissue was dissected out and frozen.

**Histology:** Lentectomized newts were maintained for 10-, 15-, 20-, 25-, or 30-days post lentectomy. The animals, along with others not lentectomized (for intact eyes), were euthanized, and the eyeballs dissected out. The eyes were prepared for histology by fixing with 4% paraformaldehyde overnight at 4°C. They were then washed in phosphate-buffered saline (PBS) for 30 minutes at 4°C, 0.85% saline solution for 30 minutes at 4°C, and a 1 to 1 saline-ethanol mix for 15 minutes at room temperature. The eyes were then dehydrated using an ethanol series (70% ethanol for 15 minutes twice, 85% ethanol for 30 minutes, 95% ethanol for 30 minutes, and 100% ethanol for 30 minutes twice, all at room temperature), and washed in xylene twice for 30 minutes at room temperature. The eyes were then soaked in a 1 to 1 xylene-paraffin mixture for 45

minutes at 60°C, followed by a soak in paraffin wax three times for 20 minutes at 60°C, before being embedded in paraffin wax.

Amputated newts were maintained for 2 weeks. The limbs were then collected, and prepared for histology using the same procedure as the newt eyes.

Newt embryos, fixed in Dent's solution (20% DMSO in methanol) and approximately stage 40 (Khan et al., 1999), were obtained from Dr. Hans-George Simon (Northwestern University), and prepared for histology as above.

Newt eyes, limbs, and embryos were cut into 10µm sections and placed on Vectabond-coated slides (Vector Labs, Burlingame, CA). Five sections were used per slide to provide for experimental replication.

**Antibodies:** Three different primary antibodies were used for this study: a rabbit anti-mouse collagen type IV IgG (Biodesign International, Saco, ME), a monoclonal mouse anti- $\alpha$ -SMA IgG (Sigma), and a mouse anti- $\beta$ -crystallin hybridoma. Type IV collagen antibody was diluted to 1:100, and  $\alpha$ -SMA antibody was diluted to 1:400, in 0.2% Triton X-100 and 10% horse serum in PBS. The  $\beta$ -crystallin hybridoma (cell media from the mouse cell culture) was applied directly with no dilution.

Two different fluorescein-conjugated secondary antibodies were used: a fluorescein-conjugated goat anti-mouse IgG, and a fluorescein-conjugated goat anti-rabbit IgG (Both from Vector Labs, Burlingame, CA). Each antibody was diluted 1:100 in PBS with 5% horse serum.

**Immunohistochemistry:** Slides were prepared for immunostaining by melting the paraffin wax at 55°C for 30 minutes, washing in xylene twice for 10 minutes each at room temperature, and rehydrating using a graded ethanol series (100%, 95%, 80%, 70%, and 30% ethanol solutions for one minute each at room temperature). The slides were then rinsed in water for one minute, washed in PBS three times for 5 minutes each at room temperature, and fixed in 4% paraformaldehyde for 10 minutes at room temperature. Antigen retrieval was performed using 1% SDS for 5 minutes at room temperature, followed by PBS washes three times for 5 minutes at room temperature. The slides were blocked using 10% horse serum for one hour at room temperature. The primary antibodies were added to the slides and incubated overnight at 4°C. Negative controls were set up incubating overnight with PBS instead of primary antibody.

The slides were then washed three times in PBS for 5 minutes at room temperature, and the secondary antibody was applied, incubating for 90 minutes at 37°C. The slides were then washed in PBST (PBS and 0.2% Triton X-100) for 5 minutes three times, and PBS for 5 minutes three times, and cover slips were applied with Vectashield (Vector Labs, Burlingame, CA). The staining was observed using fluorescence and confocal microscopy, both using FITC filters.

**Hematoxylin and Eosin Staining:** The paraffin was melted by heating the slides at 55°C for 30 minutes. The slides were then washed in xylenes twice for 15 minutes, followed by hydrating the slides through a graded ethanol series (100% ethanol, 95%, 80%, 70%, and 30%) for one minute each. The slides were washed in distilled water for

one minute, and stained in hematoxylin for two minutes. The slides were rinsed in tap water for one minute, and distilled water for two minutes. The slides were then dehydrated through another graded ethanol series (30%, 70%, 80%, and 95%) for one minute each. The slides were stained in eosin for one minute, followed by a one-minute wash in 95% ethanol, and a two-minute wash in 100% ethanol. Finally, the slides were again washed in xylenes for 10 minutes. Cover slips were affixed using Permount (Fisher Scientific, Pittsburgh, PA), and the slides were observed using brightfield microscopy.

**Western Blotting:** The frozen kidney tissue from newt and mouse was weighed, finely diced, and placed in a tube with lysis buffer (150 mM NaCl, 50 mM Tris basic, 5 mM EDTA, 1% Triton x-100, and 10% SDS, pH 8.0, 3 mL per gram tissue), and protease inhibitor (10  $\mu$ L per mL lysis buffer). The mixture was then homogenized on ice, and centrifuged at 10,000G at 4°C. The supernatant, containing the protein extract, was collected and stored at -70°C.

The protein concentration of the extract was determined using a BCA™ Protein Concentration Assay Kit (Pierce Biotechnology, Rockford, IL).

Approximately 1.5 to 2 mg/ml of protein extract from each sample was separated using SDS-PAGE, with a 10% polyacrylamide gel (The protein was loaded in 1x Laemmli sample buffer, diluted from 2x buffer: 4ml 10%SDS, 2ml glycerol, 1.2 ml 1M Tris, 2.8 ml water, 0.01% bromphenol blue. The gel was run in 1x Laemmli running buffer, diluted from 10x stock: 30.3g Tris base, 144.2g glycine, 10g SDS, water to 1L,

pH 8.3). Electrophoresis was run at 100 volts until the sample buffer reached the separating gel, when the voltage was increased to 150 volts. The electrophoresis was run until the sample buffer reached the bottom of the gel, ensuring proper separation. The proteins were then transferred to a nitrocellulose membrane in ice cold transfer buffer (25mM Tris, 192mM glycine, 20% v/v methanol with deionized H<sub>2</sub>O to 1L). The transfer was run at 100 volts and 200 mA for one hour. The membrane was blocked overnight in 5% non-fat dry milk with 0.05% Tween-20 in PBS at 4°C.

The primary antibody, anti-  $\alpha$ -smooth muscle actin, was diluted 1:400 in PBS with 0.2% Triton x-100 and 0.5% non-fat dry milk. The antibody was added to the membrane and incubated for one hour at room temperature. The membrane was washed twice in PBS for 10 minutes each. The secondary antibody (HRP conjugated goat anti-mouse IgG, Santa Cruz Biotechnology, Santa Cruz, CA), was diluted to 1:10,000 in PBS with 0.2% Triton X-100. The secondary antibody was added and incubated at room temperature for 1 hour. The membrane was washed twice in PBST for 10 minutes each and the HRP labeling was revealed using a SuperSignal® West Pico Chemiluminescent Substrate kit (Pierce Biotechnology, Rockford, IL) according to the manufacturers instructions. The membrane was then exposed to Kodak scientific imaging film for 30 seconds, and developed using an automatic developer. The western blotting was repeated three times.

## Results

$\alpha$ -SMA was absent from the intact newt eye. Staining was observed in the different stages of regeneration (Fig. 1). At 10 days post lentectomy,  $\alpha$ -SMA was seen throughout the depigmented lens vesicle, where dedifferentiation occurred. At 15 days post lentectomy, expression could be seen in the stalk connecting the regenerating lens to the dorsal iris. In the lens, where crystallin expression and fiber differentiation has begun, expression was restricted to the epithelial cells. At 20 and 25 days, the expression remained restricted to the lens epithelial cells. By 30 days post lentectomy, expression appeared to be decreasing in the posterior, but remained strong in the rest of the epithelium.  $\alpha$ -SMA was never seen in the differentiated dorsal iris tissue, or in the ventral iris (Fig. 1).

Because these data were unexpected, we decided to examine  $\alpha$ -SMA expression in developing newt lenses. As a positive control (and to confirm that our antibody staining method worked in tissues fixed in Dent's fixative), we stained for  $\beta$ -crystallin protein.  $\beta$ -crystallin staining was positive in the developing lens, whereas  $\alpha$ -SMA staining was absent (Fig 2). Type IV collagen staining was also negative (data not shown).

We also decided to examine  $\alpha$ -SMA expression in the regenerating newt limb. Since blastema cells are mesenchymal, it was expected that we would see  $\alpha$ -SMA staining. Staining was present in the two-week-old blastema. Staining was especially

noted in the dedifferentiating tissues at the edge of the blastema, specifically the bone and muscle (Fig. 3).

In order to confirm that the  $\alpha$ -SMA antibody was actually binding  $\alpha$ -SMA, a western blot using protein isolated from newt kidney, and mouse kidney as a positive control was analyzed (The secondary antibody is anti-mouse, and as such, will bind to all mouse tissue). The antibody bound to a protein at approximately 40 kilodaltons, the expected size of  $\alpha$ -SMA (Fig. 4).

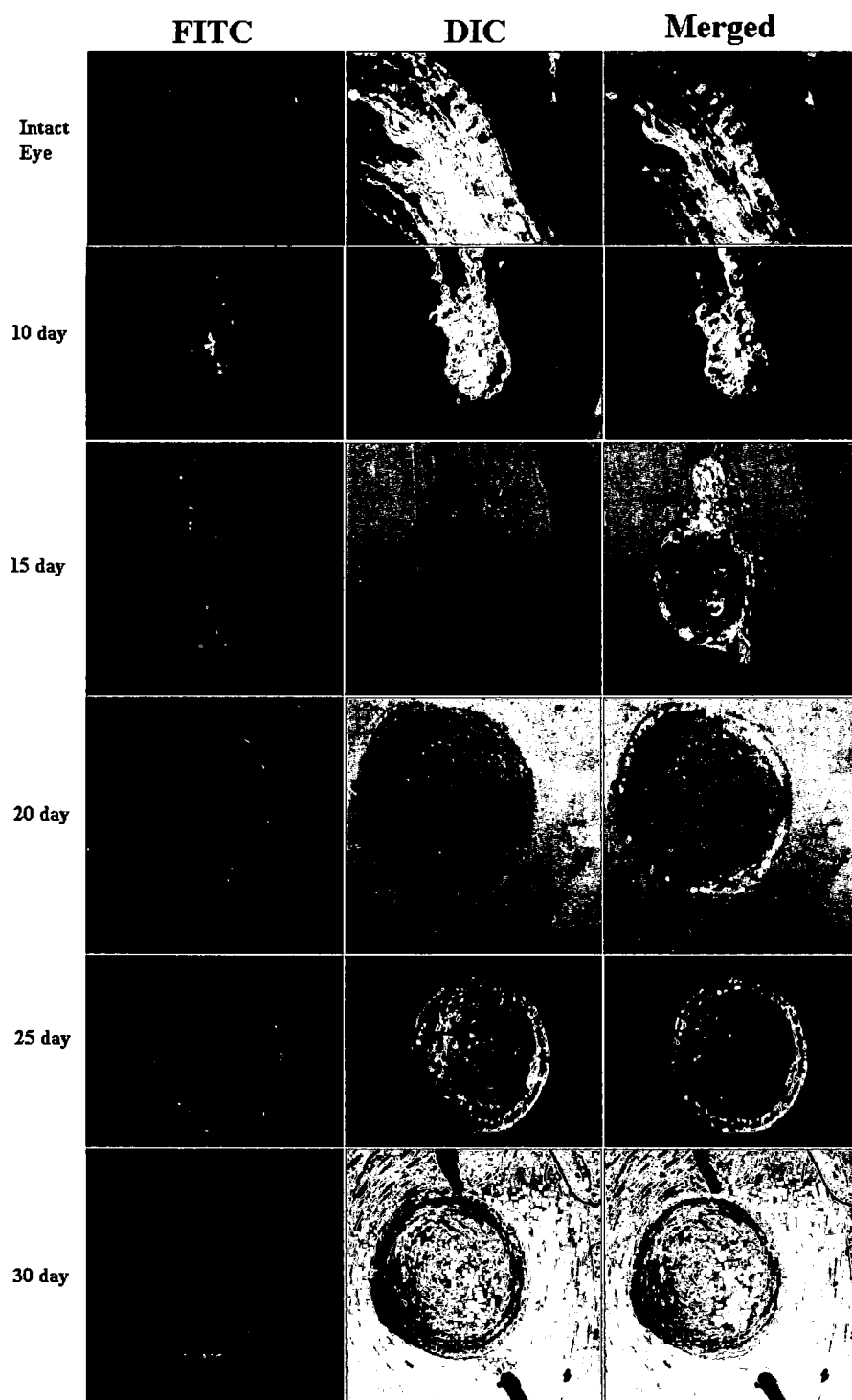
As expected, type IV collagen expression was seen in the intact lens in the collagen-rich lens capsule. Collagen type IV expression was not seen in the regenerating lens until 30 days post lentectomy, when the LECs appeared to be secreting collagen type IV (as seen from close up images taken using confocal microscopy), reestablishing the capsule (Fig. 5).

### Figure 1

Staining of  $\alpha$ -smooth muscle actin in adult intact and regenerating newt eyes. Column 1 shows FITC fluorescence micrographs of  $\alpha$ -SMA staining. Column 2 shows differential interference contrast (DIC) micrographs. Column 3 shows the merged micrographs.

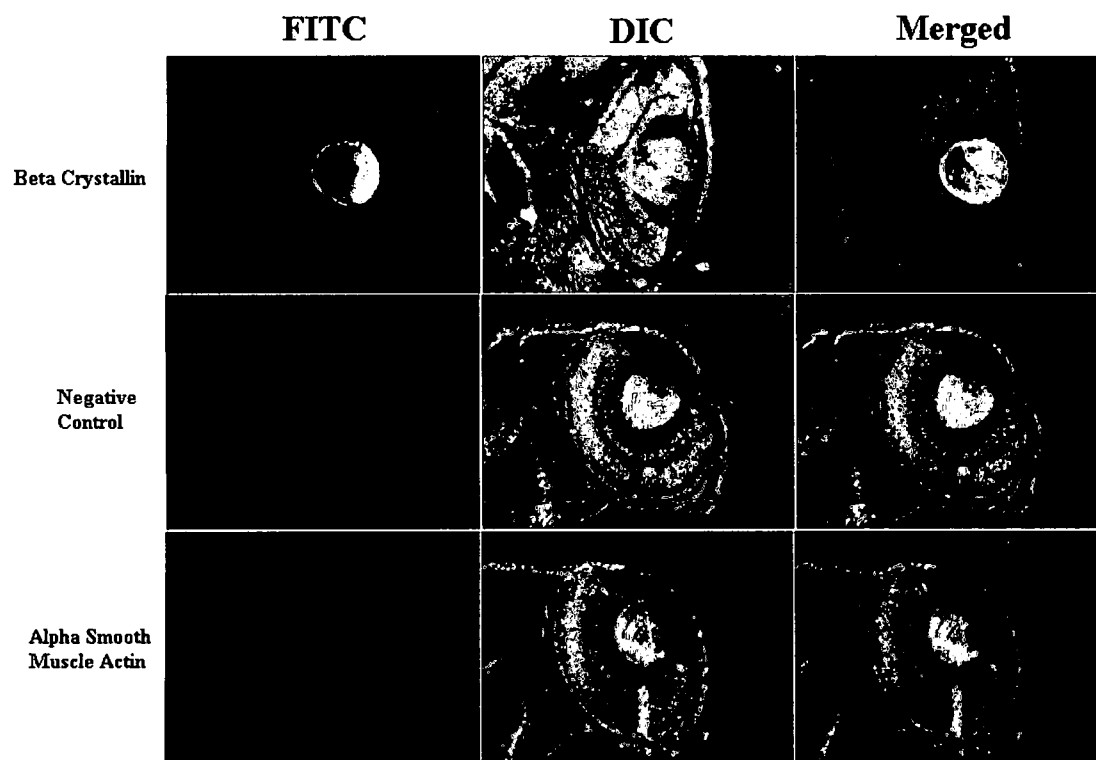
Intact and 10 day micrographs taken with 40x objective; 15, 20 and 25 day micrographs taken with 20x objective; 30 day micrographs taken with 10x objective. Intact, 10-, and 25-day micrographs were taken using an Olympus BX51 fluorescence microscope. 15-, 20-, and 30-day micrographs were taken using an Olympus confocal fluorescence microscope. The anterior eye is to the right of the image.  $\alpha$ -SMA expression is not seen in the intact eye. Staining is seen in the 10-, 15-, 20-, 25-, and 30-day post lentectomy eyes. In 10-day eyes the expression is seen throughout the lens vesicle. At 15 days post lentectomy, expression is seen in the stalk connecting the vesicle to the iris, and in the differentiating lens epithelium. In 20, 25, and 30 days, the expression is restricted exclusively to the lens epithelium.

Figure 1:



## Figure 2

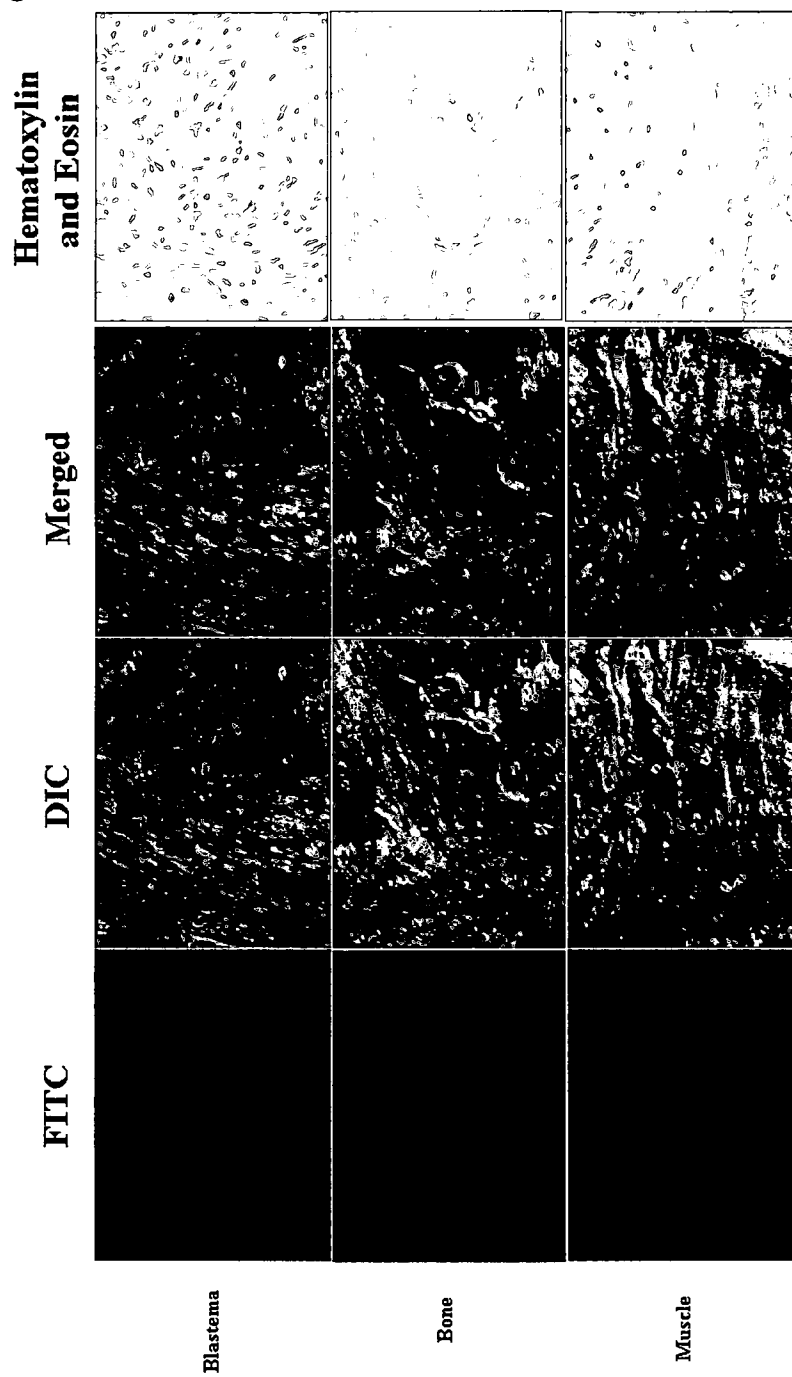
Staining of  $\beta$ -crystallin (row 1) and  $\alpha$ -smooth muscle actin (row 3) in embryonic newt lenses.  $\beta$ -crystallin staining was positive in the lens, while  $\alpha$ -SMA staining was negative, as the fluorescence level is comparable to the negative control (row 2). All micrographs were taken with the 10x objective using an Olympus BX51 fluorescence microscope. The anterior of the eye is to the right.

**Figure 2:**

### Figure 3

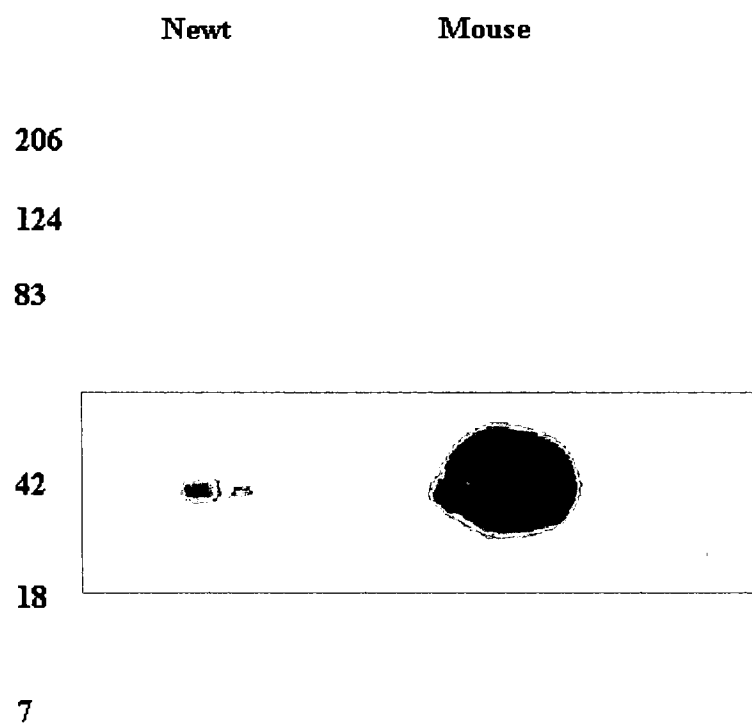
Antibody ( $\alpha$ -smooth muscle actin) and histological (hematoxylin and eosin) staining in 2 week regenerating newt limbs. Expression is clearly seen in the blastema of the 2 week limb (top row), but is not seen in the epithelium of the limb (not shown). Closer examination of the limb also reveals that expression is seen in the limb regions where adult bone (row 2) and muscle (row 3) are dedifferentiating to become part of the blastema. Hematoxylin and eosin staining, taken using bright field microscopy, confirms that the tissues seen are bone and muscle. All micrographs were taken with the 20x objective using an Olympus BX51 microscope. The distal end of the limb is to the left, and the epithelium, which stained negative for  $\alpha$ -SMA, is not shown.

Figure 3:



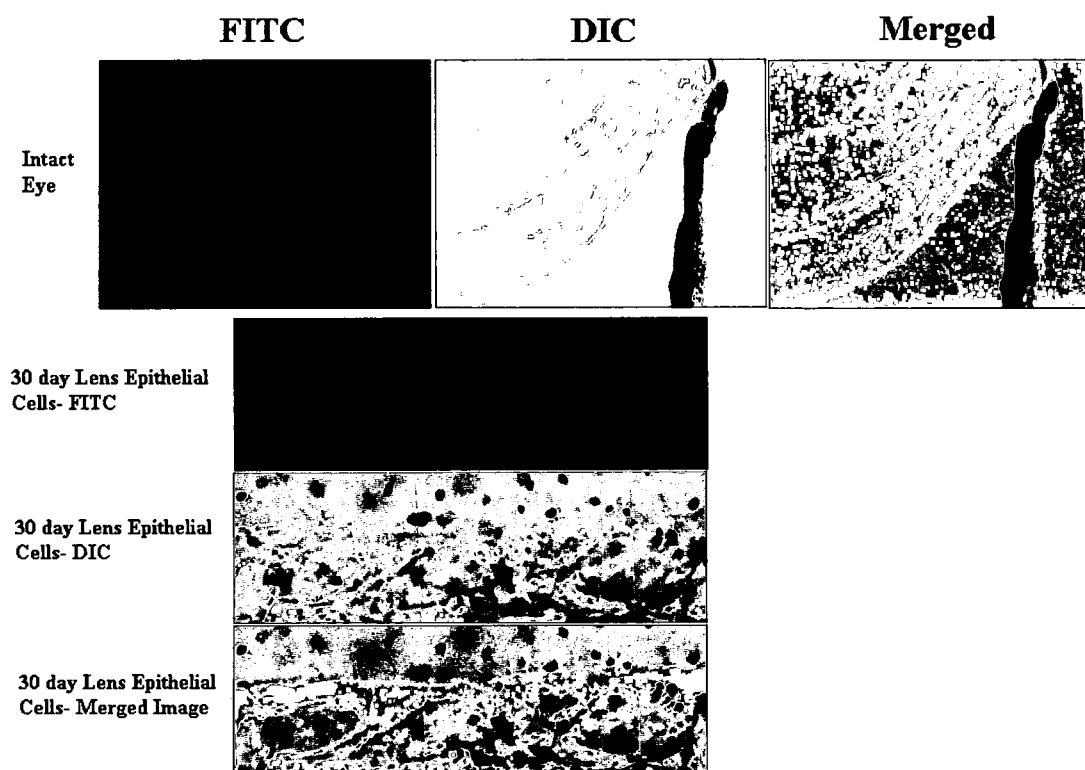
#### Figure 4

Western blot showing protein bands expressing  $\alpha$ -smooth muscle actin. The first lane was loaded with a protein extract from newt kidney, and the second lane was loaded with a protein extract from mouse kidney. The band in the newt lane is approximately 40 kD in size, the expected size of  $\alpha$ -SMA. Numbers at the left indicate the positions of the molecular weight markers.

**Figure 4:**

## Figure 5

Staining for collagen type IV in intact and regenerating newt lenses. Collagen type IV staining is seen in the lens capsule of the intact eye. Collagen type IV is being secreted by the lens epithelial cells in the 30-day post lentectomy eye. Staining for collagen type IV was not seen in any of the other stages of lens regeneration. Intact eye micrographs were take at 40x using an Olympus BX51 fluorescence microscope. Anterior is to the right. 30-day lentectomy micrographs are high magnification views of the lens epithelium (the outside of the lens is up), taken with an Olympus confocal microscope. The black spots are dirt artifacts on the slide.

**Figure 5:**

## Discussion

$\alpha$ -SMA was seen throughout the different stages of regeneration, beginning in the 10-day lens vesicle. By 15 days post lentectomy, the  $\alpha$ -SMA was seen exclusively in the lens epithelial cells, as examined through 30 days. Expression of  $\alpha$ -SMA is not seen in the ventral iris, or in the undifferentiated dorsal iris. This indicates that the expression of  $\alpha$ -SMA is initially restricted to the undifferentiated lens vesicle cells, and later to the differentiated LECs.

$\alpha$ -SMA is normally expressed in mesenchymal cells, but never seen in epithelial cells. The presence of  $\alpha$ -SMA indicates that these cells may be a bi-potent stem cell capable of differentiating into either LECs or into mesenchymal cells. Following cataract surgery, LECs that remain in the capsule undergo EMT, differentiating into myofibroblasts that express  $\alpha$ -SMA (Wormstone, 2002). It is possible that there is some newt specific mechanism that prevents these cells from differentiating into myofibroblasts.

During the wound healing process, the remaining LECs will migrate to cover the lens capsule before EMT occurs (Wormstone, 2002), and other research has shown that cellular migration is required for  $\alpha$ -SMA expression (Nagamoto et al., 2000). Antibody staining revealed that type IV collagen, a marker for the lens capsule, was not present during regeneration until 30 days post lentectomy, after the vesicle cells had differentiated into LECs. It is possible that this prevented the cells from being able to migrate and differentiate into myofibroblasts. Rakic et al. (1997) indicated that the physical separation of the lens fibers from LECs triggered some of the mitotic events that

precede EMT. Since that separation does not occur after the reestablishment of the capsule at 30 days, EMT does not occur at that stage.

After seeing expression of  $\alpha$ -SMA in regenerating newt lens, we wanted to see if  $\alpha$ -SMA expression was seen in another type of regeneration. In the regenerating limb, adult tissues from the amputated limb stump (bone, cartilage and muscle among others) dedifferentiate to form an undifferentiated blastema. The cells of that blastema are like fibroblast cells, and they secrete an extracellular matrix similar to that seen in mesenchymal cells. The cells of the blastema will proliferate and differentiate to form an exact duplicate of the lost limb, which consists of many different mesenchymal derivatives.

We examined expression of  $\alpha$ -SMA in the newt blastema two weeks post amputation. The blastema cells, which are mesenchymal, stained positive for  $\alpha$ -SMA. Closer examination of the blastema where adult bone and muscle tissue could be seen dedifferentiating revealed increased levels of expression. This indicates that these cells are dedifferentiating into mesenchymal cells that will have the ability to differentiate back into a mesenchymal derived tissue, such as bone, muscle or cartilage.

The expression of  $\alpha$ -SMA in the blastema cells is likely a feature of the cells fibroblastic nature. Since  $\alpha$ -SMA is seen in both lens and limb regeneration, it would be interesting to examine other regenerating tissues, such as tail or retina to see if they also expressed  $\alpha$ -SMA.

We also wanted to examine the presence of  $\alpha$ -SMA in developing newt lenses, to see if  $\alpha$ -SMA was a feature of lens development as well as regeneration. Embryonic newts, approximately stage 40, were obtained, embedded, and sectioned for immunohistochemistry. The staining for  $\alpha$ -SMA was negative, indicating that  $\alpha$ -SMA is not present in the developing eye. Staining for type IV collagen was also negative in the developing newt lens, indicating that the collagen lens capsule has not yet been established at this stage.

As stated previously, collagen type IV is not seen until 30 days post lentectomy. This indicates that our lentectomy surgery completely removes the newt lens capsule. It also indicates that the lens epithelial cells do not begin secreting collagen type IV to reestablish the lens capsule until approximately 30 days after lentectomy. Close up images of the LECs of a 30-day lens clearly shows that the cells are secreting the collagen needed to help reestablish this membrane.

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