INVESTIGATING THE ROLE OF CYP26 AND RETINOIC ACID SIGNALING REGULATION IN VERTEBRATE CORNEA AND LENS REGENERATION

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Cell and Developmental Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2016

Urbana, Illinois

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ABSTRACT

The larvae of *Xenopus laevis* can naturally regenerate a lost lens from the outer cornea epithelium after it is triggered to do so by signals from the neural retina. The signals have been widely studied, and FGFs are reported to play a key role in causing the cornea to transform into a lens. However, the factors that make the cornea itself competent to respond to these signals are unknown. Understanding the factors that underlie regeneration competency is the key to granting otherwise ordinary tissues the ability to regenerate, including in our own bodies. Thus, the focus of this work has been on the cornea, in order to unravel the signaling schemes that operate within it.

The Retinoic Acid (RA) signaling pathway is a major cellular signaling pathway involved in development, organogenesis, and regeneration. It was strongly implicated in the regeneration of the lens in another model system, the newt, where retinal signals trigger the dorsal region of the iris to differentiate and give rise to a lens. Antagonism of RA signaling was shown to inhibit lens regeneration, demonstrating its necessity. We investigated whether the same was true in *Xenopus*. We inhibited RA signaling using inhibitors of RA synthesizing enzymes, and of the RAR nuclear receptors. In all cases we found there to be no effect on regeneration. We validated that the drug treatments were meaningful by observing, via qPCR, a decrease in the expression *cyp26a1*, a well-established marker of RA signaling. We also examined the expression of multiple RA signaling pathway members both in control corneas and in corneas harvested in the first 4 days following lentectomy. In both these normal and regenerating tissues we found the expression of *cyp26* genes, which encode the RA metabolizing enzyme CYP26. In light of this finding, we assessed whether CYP26 was necessary for supporting lens regeneration.

In contrast to the experiment described above, exogenous addition of an antagonist of CYP26 greatly inhibited lens regeneration. Likewise, a synthetic retinoid that activates RA signaling without being metabolized by CYP26 also

inhibited regeneration, as did excess exogenous RA. In all treatments, we observed profound upregulation of the RA signaling marker *cyp26a1*. Taken together, we demonstrated that the action of CYP26 is necessary for lens regeneration, which implies a necessity to attenuate RA signaling by metabolism in order for lens regeneration to occur in *Xenopus*. This represents a species-specific difference in the signaling schemes that underlie lens regeneration, and a previously undescribed role for CYP26 in regeneration. Using immunohistochemistry and a whole-cornea mounting technique, we observed the widespread expression of RALDH and CYP26 enzymes within the corneal layers under a confocal microscope.

We next investigated the possible mechanistic roles of CYP26 that could explain its necessity in lens regeneration. We assessed whether RA signaling regulated cell proliferation in the cornea by quantifying changes in cell division following various treatments. We found that CYP26 antagonism, but not exogenous retinoids, lead to a significant reduction in cell proliferation. This finding lead us to examine the possibility that CYP26 may actively generate RA-metabolites whose absence would lead to reduced corneal cell division, and therefore stunted regeneration. This would suggest a mechanistic role of CYP26 as a ligand generator, rather than simply an RA signaling attenuator. We tested this by supplementing ex vivo cultures with both Liarozole (a CYP26 antagonist), and 4-oxo-RA (a metabolic product of RA). These co-treated cultures failed to regenerate lenses, just like Liarozole-only controls. Additionally, 4-oxo-RA supplementation does not recover the loss of cell division that occurs from Liarozole. We also documented that 4-oxo-RA causes the upregulation of *cyp26a1* in corneas, confirming its transcriptional effects. Taken together, it appears that CYP26 likely plays a role to simply attenuate RA signaling, and does not act to generate novel signaling ligands. We additionally examined the possibility that RA signaling modulates the expression of key corneal stem cell markers like *sox2*, *oct60*, and *p63*. We examined their expression under RA antagonism and stimulation, and found that CYP26 does not likely mediate its effects through such markers. However, we discovered that RA can attenuate the

expression of the eye markers *fgfr2* and *pax6* in the cornea, and CYP26 may prevent this attenuation during regeneration.

Lastly, we looked at whether CYP26 was involved in early, late, or all stages of lens regeneration by varying the timepoints at which compounds were added to ex vivo cultures. We discovered that CYP26 activity is only important during hours 12-48 post-lentectomy. This suggests a role in establishing or maintaining lens-competency in the cornea, rather than lens cell differentiation/maturation. In summary, the principal finding of these studies is that CYP26 is important for *Xenopus* lens regeneration, likely by acting to attenuate RA signaling during early regenerative events.

ACKNOWLEDGEMENTS

I would first like to thank my family, in particular my parents George and Aleykutty Thomas, whose sacrifices in life afforded me the privileges that enabled me to invest—or squander as I saw fit—the entirety of my youth in higher education.

I am grateful to my mentors from my undergraduate years at Illinois, especially Dr. Isaac K O Cann, who welcomed me into his laboratory many years ago. It was under the direct mentorship of Dr. Yuyen "Kent" Lin in his lab that I experienced for the first time both the thrill that comes from pursuing grand scientific questions, and the ineffable satisfaction that comes from obtaining miniscule answers to them. The years I experienced in the Cann lab were my earliest influence to seriously pursue science beyond college.

I also thank my graduate thesis committee members, Drs. Bill Brieher, Stephanie Ceman, Jie Chen, and Lori Raetzmann for their guidance and expertise over the years. Their unwavering skepticism and commitment to excellence not only polished the edges of my graduate work, but they also greatly influenced my vision of what sort of scientist I wish to be in the years ahead.

Above all, I thank my graduate advisor Dr. Jonathan J Henry and all the members of his lab, especially Paul W Hamilton and Kimberly J Perry. By the nature of the work we do, not every day can be enjoyable. But the bitterness of work is sweetened by one's coworkers, and I wish to thank Paul, Kim, Jon, and everyone else that has been part of this team for making my workdays more palatable. Their presence in lab and perspectives on science and all else were an invaluable part of my graduate life. I entered graduate school to become a better thinker and an independent experimentalist. If it can be said that I resemble these things to any degree, I owe credit to the laissez-faire philosophy of mentorship of Dr. Henry, and

the culture he fostered in his lab that was so well suited to my personality. While he has many accomplishments that I hope to mirror in my own professional life, Jon is also something else that I have found to be uncommon in academia—an impressive polymath with a deep and unapologetic interest in a variety of things other than science. More than anything I wish to follow this example, and I hope that my interests outside of lab and career fulfill me, and do not escape from me as I age.

Lastly, I am thankful that I have been able to spend so much time around so many different people of science as part of being at once a PhD candidate and a medical student. I have had several years to pupate in a cocoon of graduate science education, and while it is not yet clear that the process has birthed a fully mature scientist, it has undoubtedly fashioned a better man. I feel wiser and more assured, yet more aware of the magnitude of my ignorance, and more capable of addressing it as needed. I have known the fear that comes with the pursuit of a project with an uncertain ending. I learned how difficult and laborious it is to be sure of even very simple claims about reality. I have discovered within me a capacity and love for teaching that I never knew I had. Importantly, I have realized that professional science is a long stretch of defeat and boredom punctuated with episodes of triumph and beauty—not unlike life itself—and that long spells as such are worth enduring.

So, if all of nature were revealed to us tomorrow, and the whole enterprise of science thus became obsolete, my training here would still have been worthwhile. For that I am grateful; despite the hardship, I consider the years I spent in graduate school to be the most formative period of my life since gastrulation. I am privileged to have spent even a moment of my existence as a scientist, here, and amongst such wonderful people.

TABLE OF CONTENTS

CHAPTER 1

Introduction

A remarkable observation was made by Overton and Freeman in 1960, that if one removed the ocular lens from the frog *Xenopus laevis*, within a few days a new one would grow to replace it that was histologically indistinguishable from the original. At the time, it was thought that the new lens was derived from the animal's iris, but it was soon understood that the source of the regenerated lens was actually the cornea (Freeman, 1963; Freeman and Overton, 1961). Tools for understanding the cytological and histological events behind this observation were limited at that time, but through methodic and diligent observation of thin sections under a light microscope, Freeman could observe that the regenerated lens formed from the innermost layer of the bi-layered outer cornea epithelium (Freeman, 1963). Although the exact term was not coined until 1974 (Selman and Kafatos, 1974), the cornea tissue was said to undergo a process of "transdifferentiation" to form a new lens, meaning events of de-differentiation and re-differentiation of cornea cells precede the genesis of a new lens.

We have since come to learn that the outer cornea epithelium is not simply bi-layered, but actually has three distinct layers when observed under a confocal microscope—a deeper third layer in immediate proximity to the lens was invisible to Freeman's observation methods, and contains a fibrillar matrix and scattered cells (Perry et al., 2013). A modern standard of evidence also disputes whether the lens is truly derived from "transdifferentiated" cornea cells, but rather from resident cornea stem cells. The early studies of *Xenopus* lens regeneration set the stage for a long line of work that would follow, including the work described in these pages, and we are now beginning to understand the molecular signaling events that regulate this process.

1.1 Vertebrate lens regeneration is clinically important

An estimated 38 million people are blind globally, the leading cause of which is the presence of cataracts (Thylefors et al., 1995), accounting for nearly half of all blindness (WHO, 2000). A cataract is the presence of an opaque lens in the eye after the lens has lost its transparency slowly over years of aging, or abruptly due to traumatic injury. In the United States, nearly half of all people will have cataracts by age 75 (NEI, 2010). The amount of money spent on cataracts in the United States is projected to increase dramatically with an increasingly aging population. The chief medical intervention for patients with cataracts is to surgically remove the cloudy lens through an incision made in the cornea, while leaving the fibrous lens capsule that contained the lens cells intact. An artificial lens is then inserted into the lens capsule. The new artificial lens does not attach to the suspensory ligaments of the existing ciliary muscles and is therefore incapable of "accommodation"—the subtle adjustments of lens shape that allow the eye to focus at different focal lengths—but it nonetheless mostly restores useful vision to patients, and has been successfully used for many decades (Asbell et al., 2005; Harper and Shock, 2011). Congenital aphakia (absence of a lens) can affect children born with genetic mutations affecting eye development, or those affected by rubella infection in utero. Intraocular lens implantation in such children frequently leads to adverse effects that warrant further surgery (Group, 2010, 2014). There is thus a pressing need for non-surgical alternatives for pediatric patients with aphakia.

Although the current treatment for cataracts works well, it has been delivered in the absence of a clear model of the pathogenesis of the disease. Despite its ostensibly simple appearance, the lens is a complex structure whose specialized fiber cells rich with crystallin proteins are de-nucleated, elongated, and laid out in a highly regular concentric pattern during embryogenesis, making the structure completely transparent to visible light. The various molecular and cytological changes that occur to lose this transparency over time are largely unknown, and our present understanding is so limited that there is little hope of preventing or

reversing this effect. Due to this lack of understanding, it cannot be asserted with confidence that the increasingly large amount of resources spent on cataract surgeries and artificial lens implantations represent the best possible solution to an ever-expanding public health issue. A deep understanding of the genesis of a proper lens can allow us to develop therapies to slow, halt, or reverse the corruption of a lens. Studying vertebrate lens regeneration affords us the opportunity to do this as we can observe (and intervene at) various stages of generating a lens de novo.

It should be noted that even once we acquire a complete understanding of how to trigger the formation of a new lens in patients with cataracts, the removal of the existing opaque lens would still be necessary, and thus surgery would still be needed. A newly regenerated lens may not even properly attached to ciliary muscles to enable proper focusing of the lens. Thus, it is not likely that the findings from our field of research will directly lead to a therapy that will one day usurp the current treatment of lens removal and artificial lens implantation. Instead, we can lay down the foundation from which future work can build clinical interventions that are rooted in knowledge of how a transparent lens is generated, ensuring in our future both the best quality of outcome possible for patients, and the best use of limited healthcare resources.

1.2 Xenopus lens regeneration is biologically interesting

A fundamental question in biology is why some animals are capable of regenerating certain tissues and others are not. Addressing this question is the key to endowing our own tissues, normally incapable of regeneration, with the capacity to self-renew or regenerate following disease, damage, or loss. Lens regeneration is a relatively uncommon phenomenon amongst vertebrates, and besides species of the frog genus *Xenopus*, it has been observed in some salamanders and one fish. No mammals are known to have the ability to naturally regenerate the intact lens.

Although there are clear advantages to being able to regenerate lost organs like the lens, it is unclear what selective pressures would have directed the evolution of this particular phenomenon in *Xenopus*. There exists a possibility that lens regeneration in this animal is not the product of selective pressures to specifically regenerate this tissue, but is rather an emergent property of the relevant tissues resulting from the way *Xenopus* develops the lens during embryogenesis. The process of lens regeneration from the cornea is remarkably similar to the process of embryonic lens development (see section 1.4). The cornea epithelium from which the regenerated lens is derived originates from the same head ectoderm that the original lens is derived from. In both regeneration and development, molecular signals from the neural retina reach the overlying ectoderm and trigger it to differentiate into a lens. The capacity of the cornea to respond to these retinal signals remains throughout larval, juvenile stages, and even adult stages; thus the inducing nature of the eyecup, and the lentogenic nature of the cornea ectoderm are carried over from embryogenesis. Under this view, lens regeneration in *Xenopus* may simply be a coincidental by-product of the animal's developmental scheme, rather than being an adaptive result of Darwinian selection. So long as the nature of ocular development in the animal is such that the relevant tissues can recapitulate their roles from development, they will remain capable of contributing to regeneration in post-embryonic stages. In other words, the regeneration phenotype need only be as evolutionarily stable as the manner of embryonic organogenesis itself, and thus the ability to regenerate lenses will remain in the population despite the absence of selective pressures that specifically favor it. The phenomenon then is absent in mammals because their developing corneas lose their natural lentogenicity more soon in the course of eye development.

On the other hand, newts and cobitid fish that can regenerate lenses from the iris (termed "Wolffian regeneration") have natural ocular parasites that can damage the lens and render the animal blind in that eye, providing an obvious selective pressure driving the evolution of lens regeneration (Henry, 2003). Although many parasites are known to infect *Xenopus,* none are reported to specifically infect the

eye. This could be because the parasite went extinct suddenly and recently, perhaps due to some human-influenced environmental changes in the African region to which the frog and parasite are native. If so, the ability to regenerate the lens is merely a vestige to the animal, and a scientific opportunity to us. Much of what is known about parasites in *Xenopus* is reported from Californian populations rather than from Africa where the animal evolved (Kuperman et al., 2004), so it may be that we have simply not yet discovered a parasite or agent that drove the evolution of lens regeneration in *Xenopus*. Many mammals including humans harbor a wide variety of ocular parasites (Otranto and Eberhard, 2011), and some parasites are able to infest and damage the mammalian lens (Lester and Freeman, 1976). In light of this it is conceivable that we will one day discover that the ability to naturally regenerate a lens extends into the mammalian class as well, given that they may share this selection pressure in common with urodeles (newts). While no individual experiment described in these pages was set up with the express purpose of probing evolutionary history, our work as a whole nonetheless sheds light on species-specific differences in lens regeneration, and by obtaining a more comprehensive description of what transpires during regeneration, one can formulate better hypotheses aimed at understanding the origins and causes of such regenerative phenomena.

1.3 The history and present status of Xenopus as a model for lens regeneration

Lens regeneration can be easily initiated in *Xenopus* under defined experimental conditions. It begins with the removal of the lens from the eye—termed "lentectomy" in our field, although it is also commonly called "lensectomy" in human medicine. In doing so, the inner cornea epithelium that overlies the pupillary aperture is torn, and molecular factors secreted by the neural retina are free to reach the outer cornea epithelium (**Fig. 1.1**). The original work by Freeman (Freeman, 1963) that characterized *Xenopus* lens regeneration divided the subsequent histological events into 5 distinct stages. In stage 1, the cells of the innermost visible layer of the cornea epithelium change from squamous to cuboidal

in appearance. This change is observed even when the cornea is merely wounded without a lentectomy. In stage 2, basal cells that lie directly over the pupillary aperture undergo aggregation to form a loose clump of cells that are 2-3 layers thick. In stage 3, those cells become reoriented toward each other and begin to separate from the other inner cells of the outer cornea epithelium, forming a vesicle-like structure. In stage 4, the vesicle has enlarged, as have the nuclei of many of the cells. These cells produce few irregular lens fibers, and the cells of the vesicle synthesize lens proteins called "crystallins". Often but not always, the vesicle may be entirely detached from the cornea. In stage 5, lens fiber cells have formed and more emerge from the equatorial zone of the lens epithelium. Fiber cell nuclei begin to disappear, and the lens continues to grow in size. This study also demonstrated that the origin of the regenerated lens is truly from corneal epithelial cells by labeling the cornea with thymidine, and observing the presence of the labeled cells in the newly regenerated lens (Freeman, 1963). Henry and Elkins also showed that when GFP-labeled corneas from transgenic larvae of *X. tropicalis* were transplanted into wild-type eyecups, the regenerated lens would also be GFP positive, demonstrating the corneal origins of the new lens (Henry and Elkins, 2001). The process of the cornea transforming and giving rise to a lens has historically been described as "cornea-lens transdifferentiation" (Cannata et al., 2008; Day and Beck, 2011; Henry, 2003; Henry and Elkins, 2001; Henry and Tsonis, 2010b; Schaefer et al., 1999), implying some form of metaplasia must be occurring to transform corneal cells into lens. Recent work has challenged this idea however, suggesting that a population of oligopotent cornea stem cells might be the source of the regenerated lens (Perry et al., 2013).

Various studies have shown that the presence of the lens and inner cornea act as barriers that block neural factors that trigger lens regeneration from reaching the outer cornea. Under normal circumstances, in the absence of any injury to the lens or inner cornea, these barriers prevent spontaneous lens regeneration events from the outer cornea. Corneal explants placed into the eye cup will form new lenses even in the presence of the existing lens, showing that the lens does not secrete

inhibitory factors to halt regeneration (Bosco et al., 1980). Even when small barriers like cutouts of Millipore filters are placed into the vitreous chamber of a lentectomized eye, in between the outer cornea and neural retina, lens regeneration is prevented by their obstructive presence (Cioni et al., 1982; Filoni et al., 1981). Additionally, if the lens is removed through an incision in the posterior aspect of the eye without puncturing the inner cornea, then regeneration will not commence if the inner cornea is still intact (Bosco et al., 1979). Taken together, it is clear that these structures act as mechanical barriers that physically block the diffusion of retinal signals, and not as tissues that release paracrine signals that inhibit regeneration.

These findings must be reconciled with the observation that although a sheet of lens-competent cornea epithelium is wholly exposed to retinal signals following lentectomy, only one new lens is typically regenerated; regeneration does not occur at multiple foci in the cornea resulting in multiple lenses. This observation is most simply explained by the existence of some secreted factor released in the early stages of regeneration that suppresses the regeneration of more than one lens. Freeman stage 1 can be initiated with corneal damage alone and without lentectomy (Freeman, 1963), so it is unlikely that the suppressive signal would emerge during a stage that is neither unique to, nor a definitive predictor of lens regeneration. A paracrine signal released by the nascent lentoid vesicle that forms during Freeman stage 2 of regeneration could suppress the formation of new vesicles, while permitting the growth of the existing one. However, no published studies have yet demonstrated this possibility explicitly.

The rate at which the inner cornea heals back following lentectomy is the greatest factor that limits the tadpole's ability to naturally regenerate a lens as it matures into late larval stages. Tadpoles approaching metamorphosis have reduced success in regenerating the lens, which progressively worsens as the animal grows (Freeman, 1963). However, if pieces of cornea are inserted into the vitreous chamber of these relatively mature tadpoles, they can form new lenses efficiently,

demonstrating that the effect is neither due to a loss of lens-regenerating competence of the cornea, nor a loss of lens-inducting capability of the retina (Filoni et al., 1997). Moreover, *Xenopus tropicalis* rarely, and *Xenopus borealis* never regenerate lenses naturally following a simple lentectomy because their inner corneas heal too quickly and block the influence of retinal signals. Strikingly, if corneas are placed into the vitreous chamber however, both of these animals can efficiently form new lenses (Filoni et al., 2006; Henry and Elkins, 2001), further demonstrating the occlusive role of the inner cornea.

The necessity of the eyecup for lens regeneration was demonstrated early on, when it was observed that the cornea would not form a lens if the eye was completely removed behind it (Freeman, 1963). Although it was unclear at the time what inductive signals the eyecup released, a large body of work has since emerged regarding the role of the neural retina and ligands that it secretes that leads to lens regeneration. For instance, many studies have demonstrated that a lens can regenerate when cornea is implanted into the vitreous chamber (Bosco et al., 1993a; Bosco et al., 1993b; Bosco et al., 1992; Filoni et al., 1997; Filoni et al., 1981; Henry and Elkins, 2001; Reeve and Wild, 1978). The vitreous chamber is the spherical inner space of the eye bound by the lens and inner cornea-covered pupillary opening anteriorly, and by the neural retina posteriorly. In mammals it contains the "vitreous humour"—a gelatinous mass that maintains the inner pressure of the eye (Ross and Pawlina, 2011). Being in immediate contact with the neural retina, it presumably harbors any secretions from the cells. Taken together with the finding that that corneas exposed to denatured eyecups, whole retinal protein extracts, or retina-conditioned culture media will also form lenses (Filoni et al., 1983), it appears that the signals of the retina are necessary and sufficient to trigger lens regeneration. While it is not entirely clear whether a single molecule or a multitude of signals triggers regeneration in vivo, the Fibroblast Growth Factor (FGF) pathway is known to be important. Not only is FGF signaling necessary for lens regeneration to occur (Fukui and Henry, 2011), but the addition of FGF-1 has been shown to be

sufficient to trigger lens formation from isolated corneas in vitro (Bosco et al., 1994, 1997; Moore, 2015).

Several species of salamanders, as well as the cobitid fish, and chick embryos, are also capable of regenerating lost lenses (reviewed by (Henry, 2003; Henry et al., 2013; Henry and Tsonis, 2010a; Tsonis et al., 2004)). They do so through a process known as "Wolffian regeneration", wherein cells of the pigmented iris become de-pigmented, and then proliferate to form a lens vesicle that grows into a new lens. Studies from these species have synergistically advanced our understanding of the regenerative biology of the lens.

X. laevis offers many advantages over other models to study vertebrate lens regeneration. The adult animals are readily reared in relatively simple aquaria following straightforward and well-established protocols (Henry et al., 2008; Nieuwkoop, 1956). They are poikilothermic animals that tolerate a wide range of temperatures. In the rearing conditions of our own lab, the larvae for experiments are produced in large numbers, and reach stage 48 (the earliest stage we perform lens regeneration studies) within 3-4 weeks, and they develop to suitable larval stages (stages 48-54) for another 3-4 weeks before undergoing metamorphosis (unpublished observations). In vivo experiments require nothing more than an inexpensive buffered solution (Normal Amphibian Media, NAM), or de-chlorinated tap water. Even their cornea-derived cell lines thrive under ambient temperature and gases. The larvae tolerate surgical procedures and prolonged anesthesia extremely well (Hamilton and Henry, 2014). Importantly, the larvae are small enough to carry out experiments in large numbers, but big enough that delicate surgical procedures can be performed by human hands using very simple and widely available tools.

One of the most important experimental designs we implement to study lens regeneration is the use of an ex vivo eye culture system (which we have in the past called an "in vitro eye culture system" (Fukui and Henry, 2011; Thomas and Henry,

2014)). In this system, the cornea can be implanted into the vitreous chamber, while the eye is excised and cultured in small volumes of culture media. This allows the use of pharmacological inhibitors that might normally be toxic to intact live animals, or too expensive to use in large volumes. Under these conditions the effects of the molecules are also limited to the ocular tissues, limiting pleiotropic effects caused by systemic exposure. The whole-eye explants regenerate lenses within 5-7 days and can be examined with standard histology and immunohistochemical protocols. Alternatively, ocular tissues can be isolated for obtaining DNA, RNA, or protein. The media used in ex vivo cultures is nothing more than antibiotic and serum-supplemented L-15 media that has been diluted 3:4. We have also found that *Xenopus* cell lines also prefer this lower osmolarity media (unpublished observations). One limitation of this system however is that the explanted eye often does not retain its shape as robustly as an eye that is present in the head of an animal that has been fixed. The eye often deforms in the course of fixation, histological preparation, and sectioning.

1.4 Embryonic development of the lens and cornea

In the early 20th century, the experimental embryologist Hans Spemann published a seminal body of work establishing the concept of embryonic induction in the development of the amphibian nervous system. His groundbreaking paper from 1924 regarding embryonic grafts introduced the idea of the Spemann-Mangold organizer and that cell-to-cell induction events led to the differentiation of cells and their neighbors, a critical paradigm that guided decades of developmental biology that followed (De Robertis, 2006; Spemann and Mangold, 1924). Although this Nobel Prize-winning work is widely known amongst modern biologists for introducing the concept of tissue induction, Spemann's characterization of induction began much earlier, with his experiments on frog eye development. In 1901, he observed that the unilateral ablation of the presumptive eye rudiment led to a failure of lens development on that side (Saha, 1991; Spemann, 1901), and although it was not obvious at the time, it was one of the first demonstrations of the

importance of induction during embryogenesis. This work and much of his work leading up to the characterization of the Spemann-Mangold organizer over 20 years later was, by Spemann's own account, inspired by the work of his academic contemporary and close friend Gustav Wolff—the namesake of "Wolffian regeneration" who characterized amphibian lens regeneration from the iris (Saha, 1991). The histories of the fields of lens regeneration and eye development are thus closely tied together.

Embryonic tissue is fated for eye contribution well before actual morphogenesis of the eye. Eye development begins as early as the formation of the neural plate. Before it invaginates in the process of neurulation, a subsection of the neural plate specializes into the 'eye field' under the direction of various signaling pathways including BMP, FGF, and Wnt. In *Xenopus*, the eye field is defined by the expression patterns of various transcription factors: *Otx2, Six3, Rax, Tbx3, Lhx2, Tll, Six6*, and *Pax6*, collectively known as Eye Field Transcription Factors (EFTFs) (reviewed by Graw, 2010). Later, the eye field separates into two bilateral 'eye primordia' upon the influence of sonic hedgehog (Shh) signaling, and occurs in concert with the establishment of the midline (Chiang et al., 1996; Li et al., 1997). Each of the twin eye primordia then begin to invaginate to form the 'optic pits'. Mutual inductive signaling interactions occur between the optic pit and the ectoderm that overlies it. This signaling has the effect of further invagination until it becomes the 'optic vesicle'. A wall of ectodermal tissue wraps around this pit as the pit deepens, coming in from two sides and eventually meeting and fusing like a zipper. As these walls have formed around the pit, the optic vesicle has achieved the familiar spheroid shape of the eyecup. The ventral seam at the site of new ectodermal tissue fusion is known as the 'choroid fissure' and is invisible in human eyes, but can be seen as a pigmented raphe in many animals, including *Xenopus*. The optic vesicle has by now also been elevated away from surrounding ectoderm (fated to be brain). The narrow pillar that connects the optic vesicle to the surrounding ectoderm is called the 'optic stalk', and it will eventually become the 'optic nerve',

through which all visual information is transmitted to the brain (Graw, 2010; Paulsen, 2010).

The surface ectoderm overlying the opening of the optic vesicle thickens to form the 'lens placode'. The formation of the lens placode is often considered the first step in lens development, but specification of the lens placode begins even earlier than optic vesicle formation in *Xenopus*, as signals from the anterior neural plate and underlying tissues act as early lens inductors (Henry and Grainger, 1990). Soon, the optic vesicle invaginates and enlarges further to become the 'optic cup'. At the same time, the lens placode over it undergoes a reciprocal process and has invaginated to form a 'lens cup', and later a 'lens vesicle'. Note that Freeman stages 1-3 of lens regeneration are histologically reminiscent of lens vesicle development until this point, insofar as retinal inductive signals lead to the thickening and vesicularization of overlying ectoderm. The lens vesicle has by now also evaginated into the optic cup, and once it detaches from the surface ectoderm, it is known simply as the 'lens'. The region of surface ectoderm from where the lens budded off will eventually become the cornea epithelium, and the inner cells of the optic cup will give rise to the neural retina (Graw, 2010).

The development of the lens and cornea until this stage has been a model example of inductive signaling during embryogenesis, and a large amount of our understanding of this process came from studying *Xenopus* and other amphibians. Not long after Spemann's original eye rudiment ablation experiments, Lewis showed that ectopic implantation of the optic vesicle underneath head ectoderm that was not fated for lens formation can trigger that ectoderm to form a lens (Lewis, 1904). An expansive body of work soon emerged in the early and mid 20th century, variously confirming and challenging the sufficiency of the optic vesicle to induce the formation of a lens (reviewed by Saha et al., 1989). Eventually, work performed with rigorous controls and proper host- and donor-marking systems would essentially confirm Lewis's findings that the optic vesicle can by itself induce lens formation in non-lens fated ectoderm (Liedke, 1955; Reyer, 1958). However, this finding should be understood with the important caveat that a subset of non lens-

fated ectoderm is still nonetheless lens-*biased,* owing to separate inductive events that occur as early as gastrulation, and it is only upon such lens-biased ectoderm that the optic cup can exert its lentogenic influence (Grainger et al., 1988; Henry and Grainger, 1987, 1990). The mature eyecup of *Xenopus* larvae can strongly induce lens formation from isolated embryonic presumptive lens ectoderm, suggesting that the inductive signals are likely the same in both lens development and regeneration (Henry and Mittleman, 1995). Moreover, the eyecup weakly induces lenses from ventral ectoderm that cannot be induced by the optic cup, suggesting that the signaling from the eyecup (such as what occurs during lens regeneration) is more intense than that of the embryonic optic cup (Henry and Mittleman, 1995). Still, the eyecup cannot induce lenses from larval ventral flank ectoderm (Henry and Elkins, 2001), showing that some kind of lentogenic bias (or "lens competence") is still required for lens regeneration, just as it is for lens development. Our understanding of lens regeneration has thus grown concurrently with our growing understanding of lens induction, and it is clear that the inductive paradigm is very similar in both development and regeneration.

Following closure of the lens cup into a lens vesicle, the interior is left with a central cavity. Lens epithelial cells line the anterior face of the now spheroid structure with their basement membrane lying superficial to the cells themselves. Other cells from both the anterior and posterior pole begin to elongate until they meet each other end-to-end towards the center of the sphere and fill up the cavity. These elongated cells are called 'primary lens fiber cells'. Mitotic cells from the lens epithelium then divide and migrate towards the equator of the lens, and upon reaching there they begin to differentiate and elongate. As newer cells arrive at the equator, older ones get pushed centrally and elongation continues. These new epithelium-derived elongated cells are called 'secondary lens fiber cells'. As the tips of elongated secondary fiber cells from opposite poles of the equator begin to meet at the midline, their fused ends form anterior and posterior sutures. In a transverse section of the lens, the cells now appear as concentric circles. As primary and secondary fiber cells near the center of the lens, they also begin to lose their nuclei

and other organelles. Fiber cells always elongate along the anterior-posterior axis, and new cells always come in only from the equator. This assures that a uniform fiber cell polarity is always achieved, forming an organized latticework of cells within the lens that is critical for maintaining transparency and optical quality. Lens maturation in this manner continues after birth, and even primary fiber cells do not become fully denucleated until 2 weeks after opening of the eyelids. Fiber cell elongation and the maintenance of lens polarity (with the lens epithelium facing the anterior only) are the result of sustained signaling from the neural retina, even in adult animals (Coulombre and Coulombre, 1963; Yamamoto, 1976). The basement membrane surrounding the lens vesicle thickens to form the fibrous lens capsule. Fiber cells are never shed from the lens (**Fig 1.2**). Even the original primary fiber cells from embryogenesis are retained in the metabolically quiescent core of the lens for life, entombed within the fibrous lens capsule and many layers of younger cells, and isolated from humoral elements (lens development reviewed by (Graw, 2010; McAvoy et al., 1999; Rao, 2008)).

Lens regeneration essentially recapitulates lens development and maturation perfectly during Freeman stages 3-5, until it culminates in a lens that is indistinguishable from its lens placode-derived counterpart (Freeman, 1963). The chief component of the terminally differentiated fiber cells is a group of proteins known as "crystallins" (which constitute a third of the total cellular mass), the major ones of which are α-, β-, and γ-crystallin (Graw, 2009). Crystallins are key molecular markers that identify differentiated lens cells, and antibodies that stain for lens proteins have been instrumental for positive identification of lenses of varying stages during development and regeneration. As mentioned, the ex vivo eye culture system we use can often fail to preserve the structure of the lens, and lens cells can look very similar to other nearby epithelial cells, depending on the stage of differentiation. Thus, the creation of a polyclonal antibody that broadly stains for *Xenopus* crystallins (Henry and Grainger, 1987) has been a critical tool that enables our use of the ex vivo culture system.

As mentioned above, the ectoderm that overlies the lens vesicle eventually gives rise to the cornea epithelium. As the lens vesicle separates from surface ectoderm, cells of neural crest origin invade the resulting space between them. In mammals, this results in several layers of cells between the cornea epithelium and the lens. They later form a loose arrangement of cells with a lot of extracellular matrix, known as the corneal stroma. The posterior layer of the cells (derived from the neural crest) that lies closest to the lens remains as a tight sheet and is called the lens endothelium (Graw, 2010). *Xenopus* varies from other vertebrates at this stage because of anatomical differences of the pre-metamorphic larval eye. In tadpoles, the cornea epithelium has no stroma formation under it and is usually called the "outer cornea" (or "outer cornea epithelium" or simply "cornea epithelium"). The cornea endothelium remains separated from the epithelium except at the center. Another wave of migrated neural crest cell joins the cornea endothelium to form an "inner cornea" which lies over the pupillary opening, and while it is directly connected to the outer cornea via a small corneal stalk, it remains otherwise separate from it. As the animal undergoes metamorphosis, keratinocytes are drawn towards the corneal stalk and a stroma is deposited, and the cornea thickens until the tissue soon resembles a human cornea (Hu et al., 2013).

1.5 An overview of the Retinoic Acid signaling pathway members[1]

Retinol (Vitamin A) is the biological source of retinoids in animals, which binds to Retinol Binding Protein (RBP4) in the blood before being delivered to the transmembrane protein on the surface cells, Stimulated by retinoic acid (STRA6). In the cytosol, retinol is bound to cytoplasmic retinol binding protein (CRBP1), and is oxidized to retinaldehyde by a suite of dehydrogenases such as RDH10, and is subsequently oxidized to retinoic acid by tissue-specific retinaldehyde dehydrogenases RALDH1/2/3 (Molotkov et al., 2002a; Molotkov et al., 2002b). RA is

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bound by cellular retinoic acid binding protein (CRABP) and transported to the nucleus where it is released. RA exerts its influence on the cell by binding to Retinoic Acid Receptors (RARα/β/γ) and Retinoid X Receptors (RXRα/β/γ), which dimerize and bind to specific genomic elements known as retinoic acid response elements (RAREs), where they act as transcriptional activators or repressors. In the absence of ligand, the aporeceptors generally act to recruit chromatin condensing histone deacetylases, and when RA is bound to the receptors the chromatin is remodeled to enable transcription, via the recruitment of histone acetyl transferases (Niederreither and Dolle, 2008). RA may also form a complex with CRABP and exit the cell, enabling RA signaling to act in both autocrine and paracrine fashions (Cvekl and Wang, 2009).

There is also a cytochrome P450 superfamily enzyme known as CYP26, which metabolizes retinoic acid into polar metabolites such as 4-oxo-retinoic acid in order to be eliminated from the cell. Several early studies using radiolabelled RA had revealed that RA formed various metabolic products that were excreted in animal urine, and that their formation was NADPH dependent (reviewed by (Ross and Zolfaghari, 2011)), suggesting the existence of a heme-containing cytochrome P450-like monooxygenase for degrading RA in vivo. The responsible enzyme however was not identified and characterized until 1996 and was called P450RAI at the time (White et al., 1996). It would later be named CYP26, and it was cloned and characterized in *Xenopus* soon after (Hollemann et al., 1998). As is typical for hepatic cytochrome P450s, there are multiple enzymes that can act upon RA with varying degrees of affinity in liver cells. However, CYP26 has the highest affinity for RA, and it is the principal P450 enzyme involved in attenuating RA signaling in cells and tissues (Ross and Zolfaghari, 2011). The coordinated expression of RA-synthesis (RALDH) and RA-degrading (CYP26) enzymes is used to achieve clearly demarcated boundaries of active RA signaling in developing tissue (Duester, 2008a; Rhinn and Dolle, 2012).

1.6 Retinoic acid signaling in the development of ocular tissues

RA signaling plays important roles in the development of many ocular tissues, including the retina, lens, and cornea (Enwright and Grainger, 2000; Kastner et al., 1994; Wagner et al., 2000). Proper eye morphogenesis itself depends on RA (Hyatt et al., 1996b; Molotkov et al., 2006). Treatment of the presumptive eye field with exogenous RA will result in anophthalmia (absence of eye formation), and later treatments affect the differentiation of dopaminergic cells in the retina (Eagleson et al., 2001). RA-responsive reporter constructs have revealed that RA signaling is active in mice as early as neural fold elevation. Activity is present in the optic vesicle and presumptive lens ectoderm (as well as head mesenchyme and elsewhere) around the time of neural tube closure in the head (Enwright and Grainger, 2000). In zebrafish, exogenous RA treatments lead to aberrant development of the optic stalk, including persistence of the stalk that suggests an increase in ventral characteristics. Localized RA treatment is sufficient to create a pseudo-choroid fissure at the site of treatment. Moreover, a ventral marker *pax(b)* becomes expressed in the dorsal retina, and a dorsal marker *msh(c)* disappears (Hyatt et al., 1996b). RA synthesis inhibition using Citral results in embryos lacking a ventral retina (Marsh-Armstrong et al., 1994). These data suggest that RA is involved in establishing the ventral retina, however RA signaling is not itself responsible for establishing the dorsoventral axis in the retina (Matt et al., 2005; Molotkov et al., 2006). Two phases of RA signaling govern the morphogenesis of the eye; an earlier phase of optic cup formation with RA generated in the periocular mesenchyme, and a later phase of anterior eye formation, with RA made in the neural retina (Duester, 2008b). It is also responsible for differentiation and maturation of photoreceptor rods and cones in zebrafish (Hyatt et al., 1996a). Although RA is produced in the corneal ectoderm by RALDH during mouse eye development, the main target of this RA is the neural crest-derived periocular mesenchyme (Matt et al., 2005). Thus it appears that RA acts primarily as a paracrine signaling molecule within this context. Nonetheless there is evidence that shows that closure of the optic fissure is regulated by RA signaling in periocular mesenchyme as well as ventral optic cup,

and usage of an RAR inhibitor results in ocular coloboma (failure to close choroid fissure) (Lupo et al., 2011). RA may also be involved in late cornea development as it can strongly induce the proliferation and stratification of keratocytes in vitro in addition to stimulating collagen release (Gouveia and Connon, 2013). Additionally, studies in murine contexts have shown that retinoic acid induces crystallin expression in lens cells (Gopal-Srivastava et al., 1998)., and the RA metabolizer CYP26 is highly expressed in the lens epithelium of *Xenopus* embryos, suggesting a necessity of RA signaling ablation in those cells (Hollemann et al., 1998).

Understanding the role of RA signaling in establishing proper tissue patterning has been advanced by work done in regenerative biology. For instance, in the frog *Rana temporaria,* treatment of the regenerating limb bud with RA results in several duplicated limbs (Maden, 1983). In *Xenopus*, treating developing limbs with Vitamin A leads to hypomorphic development, but treating regenerating limbs has different effects, ranging from hypomorphia, to complete suppression, to skeletal duplications (Scadding and Maden, 1986).

As mentioned, fat-soluble Vitamin A (retinol) is the nutritionally acquired precursor to retinoic acid. It is worth noting however that retinol has another very important role in the retina as a photosensitive molecule that participates in the transduction of photons into electrical signals in rod and cone cells—a process known as 'the visual cycle' (Saari, 2012). Popular recommendations of dietary Vitamin A for eye health are mostly related to this functional role of retinol (Evert, 2013; WHO, 2009), which is completely distinct from its role as a precursor to the signaling molecule RA. At the same time, xerophthalmia (excessive corneal dryness) is the leading cause of preventable childhood blindness in the world (WHO, 2009), and while it is related to dietary Vitamin A deficiency, it is treated with topical retinoic acid (Sommer, 1998). Thus, other aspects of eye health may also be related to RA signaling, as many Vitamin A deficiency associated eye diseases have unclear etiologies.

1.7 RA signaling in lens regeneration[2]

RA has been shown to play a necessary role in Wolffian lens regeneration through the use of small molecule inhibitors such as Disulfiram, which inhibits an RA-synthesis enzyme (RALDH), as well as inhibitors of RAR nuclear receptors. While there is almost certainly a redundancy of function of RAR receptor isotypes within the regenerating lens, RARα was specifically implicated when the use of a RARα specific inhibitor, AGN194301, stunted the regenerative capacity of the dorsal iris (Tsonis et al., 2000; Tsonis et al., 2002). In many cases there was a failure to form a dedifferentiated lens vesicle from the dorsal iris, suggesting a role for RA in the earliest events of regeneration. Furthermore, it is interesting to note that the use of a pan-RAR antagonist (AGN193109) resulted in some cases of ectopic lens formation, including one case of lens regeneration from the cornea, reminiscent of that which takes place in *Xenopus*. However, the authors did not use any tissue-marking labeling system and the origin of the lens was determined by observation of histological sections alone. The appearance of a lens directly coming off of some tissue is not adequate criteria for determining lens origin (Grainger et al., 1988; Saha et al., 1989), and the observed result of an ectopic lens from the cornea should be understood with this caveat as they can be fixation and sectioning-related artefacts. The effect was not seen when the RARα specific antagonist was used, suggesting that whatever function RARα might have within the regenerating tissue, it does not involve defining the site of lens formation. *rara* (RARα) is not detected by in situ analysis in unoperated lenses, but it is expressed in the lens epithelial cells and the fiber cells within the regenerating lens, as well as the dedifferentiated lens vesicle that initially buds from the dorsal iris at the start of regeneration (Tsonis et al., 2002). The same expression pattern is true for *RARδ* (the newt *RARγ*), but its functional role has not been specifically investigated in the regenerating lens. Work

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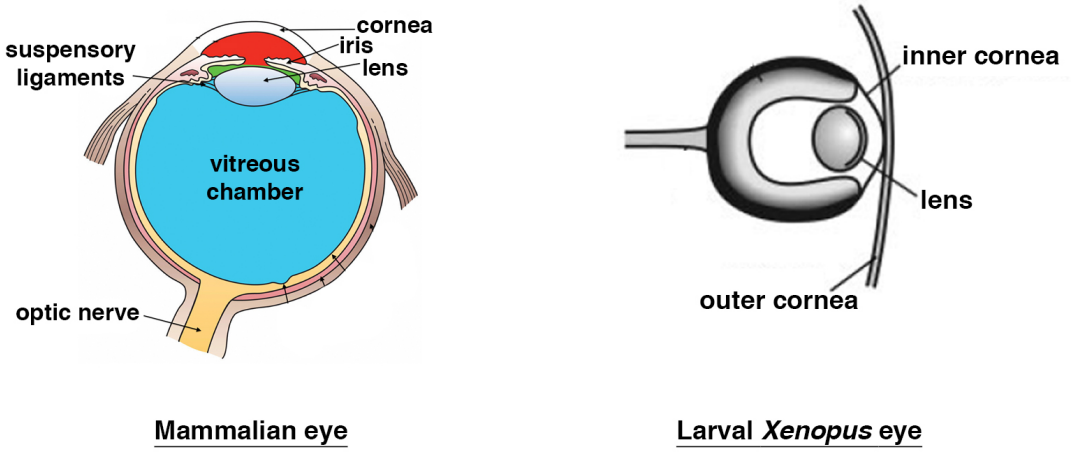
remains to be done to reveal what cellular or molecular mechanisms the various RA nuclear receptors control in the context of vertebrate lens regeneration.

Others have demonstrated that the normally lens-incompetent ventral iris of the newt can be induced to regenerate lenses when transfected with *six-3*, but only when co-treated with retinoic acid (Grogg et al., 2005). These treatments forced the ventral iris to adopt patterns of gene regulation that are normally seen only in the dorsal iris when it undergoes regeneration, and the authors suggest that it is this "dorsalization" that renders the ventral iris capable of transdifferentiation. Since retinoic acid was necessary for this transformation to take place, it can be reasoned that gene regulation by retinoic acid signaling is critical for conferring lens-competence upon lens-incompetent tissue. Taken together with the earlier studies described above, it appears that retinoic acid is important in all phases of Wolffian regeneration—acquiring/maintaining lens competence, dedifferentiation of the iris, and terminal differentiation of lens fiber cells.

The role of retinoic acid in *Xenopus* cornea-lens regeneration has been much less understood. Our lab pulled out *rxrg* (encoding RXRγ) from a subtracted cDNA library enriched for genes upregulated in the first four days of regeneration (Malloch et al., 2009), but there has otherwise been a lack of data regarding either function or expression of any RA signaling members within larval eye tissues. The work outlined in this dissertation fills this void by investigating the role of RA signaling in the context of *Xenopus* lens regeneration, and describes a key role for CYP26 and RA signaling attenuation in the cornea, and ultimately expands our knowledge of the regenerative biology of the vertebrate lens.

Figure 1.1

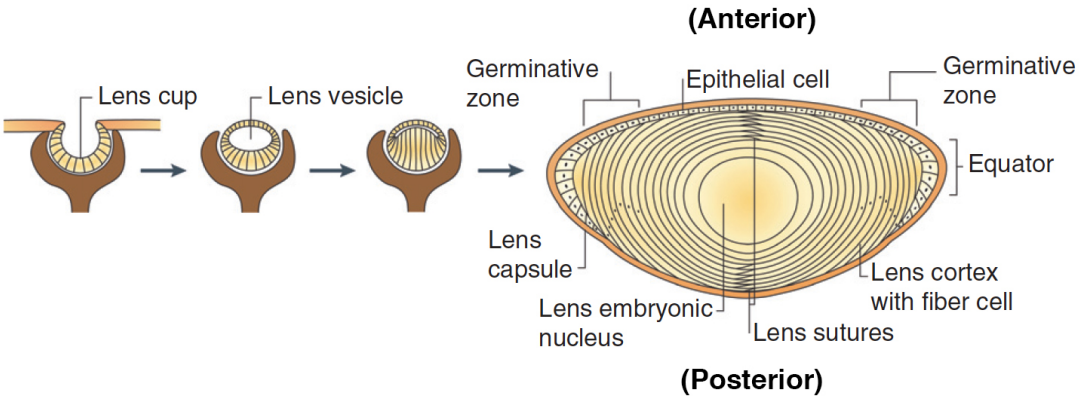


Mammalian eye

Larval *Xenopus* eye

(*Left*) The structure of a typical mammalian eye. (*Right*) The eye of the *Xenopus* larvae have an inner and outer cornea as shown. Adapted from Fukui and Henry, 2011 and Fischer, 2013.

Figure 1.2



The developmental scheme of the vertebrate lens, and the histological structure of a mature lens seen in a transverse cross-section. Adapted from Graw, 2010.

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CHAPTER 2

Retinoic acid regulation by CYP26 in vertebrate lens regeneration[3]

Introduction

Although mammals have a rather limited ability to regenerate lost or damaged tissues, other metazoans exhibit the remarkable capacity to regenerate a variety of tissues, including intact organs. *De novo* regeneration of the lens has been reported in newts, salamanders, a fish, and frogs of the genus *Xenopus* (Freeman, 1963; Henry, 2003). Upon removal of the lens, the outer cornea becomes exposed to molecular factors in the vitreous humor that are secreted by the retina, and these factors induce the cornea to form a new lens. The exact identities of these factors are not clear, but Fibroblast Growth Factors (FGFs) have been implicated as both necessary (Fukui and Henry, 2011) and sufficient (Bosco et al., 1997) for lens regeneration to occur. Additionally, Bone Morphogenic Proteins (BMP) signaling has been shown to be critical for lens regeneration in *Xenopus* (Day and Beck, 2011). However, the molecular factors that support this process and make the cornea competent to respond to these retinal factors are much less understood.

All-trans Retinoic Acid (RA) plays various roles in the development of ocular tissues. Morphogenesis of the eye, as well as the development of the retina, lens, and cornea, have all been shown to be orchestrated by RA signaling (Enwright and Grainger, 2000; Hyatt et al., 1996b; Kastner et al., 1994; Molotkov et al., 2006; Wagner et al., 2000). RA signaling has been implicated in the process of vertebrate lens regeneration as well, when Tsonis and colleagues found evidence that RA

[3] The contents of this chapter has been published in: Thomas, A.G. , Henry. J.J. , 2014. "Retinoic acid regulation by CYP26 in vertebrate lens regeneration". Dev Biol. 2014 Feb 15; 386(2): 291–301. It is excerpted here in its entirety with permission from the publisher.

signaling is necessary for lens regeneration in the newt (Tsonis et al., 2000; Tsonis et al., 2002). In the case of newts and salamanders, lens regeneration occurs via transdifferentiation of the dorsal pigmented iris epithelium. Remarkably, the ventral iris of the newt, which is normally incapable of regenerating a lens, can also give rise to lens cells when they are made to express *six3* in the presence of exogenous RA (Grogg et al., 2005). Although the process of lens regeneration in *Xenopus* has traditionally been described as involving transdifferentiation of the differentiated cornea epithelium, recent studies suggest that a population of multipotent corneal stem cells or their transient amplifying progeny may be the source of the regenerated lens (Perry et al., 2013). Previously, we identified a specific nuclear receptor involved in RA-signaling (*rxrg*) in a subtracted cDNA library representing genes upregulated in the cornea during *Xenopus* lens regeneration (Malloch et al., 2009). The collective data seems to indicate an important role for RA signaling in tissues that regenerate a lens.

The biological source of retinoids in animals is dietary Vitamin A (retinol). Once inside the cell, retinol can be oxidized to retinaldehyde by retinol dehydrogenase enzymes (RDH), and further oxidized into RA by retinaldehyde dehydrogenases (RALDH). RA effects its influence on the cell by binding to Retinoic Acid Receptors (RARα/β/γ) and Retinoid X Receptors (RXRα/β/γ), that can homo- or heterodimerize in limited combinations to bind to specific DNA motifs in the genome known as Retinoic Acid Response Elements (RAREs) (reviewed by Bastien and Rochette-Egly, 2004) . The RA nuclear receptors can act as either transcriptional repressors, or transcriptional activators in different contexts. Moreover, RA can exert its influence at different locations than where it was produced, by binding to Cellular Retinoic Acid Binding Protein (CRABP) and being transported out of these cells. Thus, RA can act as both an autocrine and paracrine signal. A cytochrome P450 superfamily enzyme, CYP26, metabolizes RA within the cell and thereby regulates RA levels in a time and tissue specific manner (Cvekl and Wang, 2009; Niederreither and Dolle, 2008). Careful coordination of RA synthesis and metabolism establishes cell or tissue-specific patterns of RA signaling within an

animal (Duester, 2008; Rhinn and Dolle, 2012). The activity of CYP26 is important for proper embryonic development by establishing boundaries of RA signaling. CYP26 is highly expressed in the lens epithelium of *Xenopus* embryos, suggesting a necessity of RA signaling ablation in those cells (Hollemann et al., 1998), although the reason for that is presently unclear.

Although RA has been implicated in the process of lens regeneration in newts, its role in *Xenopus* cornea-lens regeneration is not understood. In the present study we implicate RA metabolism mediated through CYP26 as a necessary event for lens regeneration to occur in *Xenopus*, using a pharmacological inhibitor of CYP26, as well as exogenous RA, and a synthetic retinoid, TTNPB. Further, we examine the effects of these compounds on cell proliferation, and expression of putative stem cell markers. We also characterize the localization of RALDH and CYP26 proteins within the cornea.

Materials and Methods

Animals

Xenopus laevis adult frogs were acquired from Nasco (Fort Atkinson, WI). *X. laevis* embryos and larvae were raised following Henry and Grainger (1987) , and Henry and Mittleman (1995) . All staging is according to Niewkoop and Faber (1956).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) analysis

X. laevis larvae of stages 48-50 were anesthetized in 1:2000 MS-222 (Sigma, St. Louis, MO) diluted in 1/20 Normal Amphibian Media (NAM; Slack, 1984). Corneas were excised with fine iridectomy scissors and flash-frozen in a dry ice/ethanol bath at -80ºC. “Control” cornea tissue was obtained by collecting and freezing corneas from unoperated animals. For “Regenerating” corneal tissue, stage 48-50 tadpoles were lentectomized, and corneas from the operated eyes were removed and collected at 1, 3, and 5 days post-lentectomy, and pooled together for

RNA extraction. RNA was extracted from the samples by homogenization in TRIzol (Ambion, Grand Island, NY) and processed using Direct-zol RNA MiniPrep columns (Zymo Research, Irvine, CA). Each sample was then treated with DNAse I (New England Bioloabs, Ipswich, MA) to remove any possible genomic DNA contamination, and run through a NucAway Spin column (Ambion, Austin, TX) to remove reaction contaminants. cDNA was synthesized from the RNA using an iScript cDNA synthesis kit (BioRad, Hercules, CA). Half of the RNA from each sample was used for "-RT" control cDNA, which employed the iScript cDNA synthesis kit, but with water in place of the supplied reverse transcriptase enzyme. "Lens" RNA was obtained similarly, from lenses isolated from stage 49-52 tadpoles. As a positive control for all primer sets, "Embryonic" RNA was harvested similarly, using pooled stage 1-33 embryos. PCRs were run in 50µl volumes for 30 rounds using 7ng of input cDNA template. GAPDH was used as a positive control for each sample of cDNA. Sequences of primer pairs and the melting temperatures used for each are listed in Supplemental Table 1. The identity of every PCR band was confirmed by DNA sequencing at the Roy J. Carver Biotechnology Center (University of Illinois, Urbana, Illinois).

In vitro lens regeneration assay

The in vitro lens regeneration assays using pharmacological inhibitors were performed as outlined in Fukui and Henry (2011), with some variations. Briefly, animals were anesthetized in 1:2000 MS-222 (Sigma, St. Louis, MO) diluted in 1/20 NAM. Incisions were made using fine iridectomy scissors in the outer cornea and the lens was removed. The outer cornea was then tucked into the empty vitreous chamber and the whole eye was then excised, and placed into a well of a 24-well tissue culture plate. Eyes were separately cultured for 7 days in 350µl of modified L-15 media (2:3 dilution of L-15 media, with 10% fetal bovine serum, 2.5µg/ml Amphotericin, 10kU/ml penicillin-streptomycin, and 4µg/ml Marbofloxacin) supplemented with the appropriate concentration of pharmacological compound or vehicle (DMSO). The following 6 experimental conditions were tested: 100µM Liarozole Hydrochloride (Tocris, Bristol, UK), 20µM TTNPB (Tocris), 1µM *all-trans*

Retinoic acid (Sigma), 20μM *all-trans* Retinoic acid, 60μM Citral (Sigma), 5μM LE-135 (Tocris). Culture media was changed daily, and after 7 days of culture, eyes were rinsed briefly with PBS and fixed in 3.7% formaldehyde for 2 hours at 25ºC. Eyes were then dehydrated in ethanol, later cleared with xylene, and subsequently embedded in paraffin wax. Eyes were sectioned at a thickness of 9μm and placed onto a positively charged microscope slide (Colorfrost Plus, Thermo Scientific, Kalamazoo, MI) for immunohistochemical staining with a polyclonal anti-lens antibody (Henry and Grainger, 1990). Imaging was done using a Zeiss Axioplan light microscope. The presences of morphologically distinct lentoid structures that positively stained with the antibody were scored as successful cases of lens regeneration. Statistical significance in differences between regeneration rates was established using (two-tailed) Fisher's Exact test.

Quantitative RT-PCR (qPCR)

Corneas were cut around the periphery of the eye from stage 48-51 tadpoles, leaving the cornea epithelium attached to the eye via the central corneal stalk. Then, the whole eye was excised from the animal and placed into modified L-15 culture media containing an appropriate concentration of pharmacological compound or the DMSO. The following conditions were tested: 100μM Liarozole, 20μM TTNPB, 1 μM all-trans Retinoic acid, 20μM all-trans Retinoic acid, with 0.2% DMSO as a control condition. Additionally we tested 60μM Citral and 5μM LE-135, with 0.05% DMSO as a control condition. For each condition, 20-25 eyes were cultured together in small petri dishes containing 3ml of culture media. After 4 days of culture the corneas were surgically removed from each eye and placed into a microcentrifuge tube immersed in ethanol and dry ice, as described above. RNA was extracted and processed from each pool of corneas and cDNA was generated in the same manner as described above.

All qPCR experiments were performed 3-4 times, with technical replicates receiving 10-25ng of input cDNA. SYBR Green reagent (kindly provided by Dr. Jie Chen, University of Illinois at Urbana-Champaign) was used along with 125nM of

forward primers and 500nM of reverse primers for *actb* and *cyp26a1*, and 500nM of both primers for *sox2*, *oct60*, and *p63*. All primers are listed in Supplemental Table 1. *actb* (beta-actin) was used as the internal control gene in every experiment, and the expression of the test gene under each drug-treated condition was normalized to the expression of that same gene in the control condition. Melting-curve analysis was conducted for each experiment. Fold changes of expression were determined using the comparative C_T method (Schmittgen and Livak, 2008). Statistical significance was established using the (unpaired) *t* test.

Immunohistochemistry

Stage 50-51 animals were fixed in 3.7% formaldehyde for 30 minutes at 25ºC. Whole eyes were then excised and processed with the cornea still attached at the corneal stalk. All steps of processing were done in a 1X PBS buffer with 0.2% Triton X-100. Eyes were blocked in a solution of 5% bovine serum albumin and 5% normal goat serum. All primary antibody incubations were done overnight at 4ºC, and secondary antibody incubations were 1-2 hours at 25ºC. Primary antibodies included polyclonal rabbit-anti-CYP26A (1:200 dilution; Abcam, Cambridge, MA), polyclonal rabbit-anti-CYP26B (1:100 dilution; Abcam), and polyclonal mouse-anti-RALDH1 (1:200 dilution; Abcam). Western blot analysis using cornea and embryonic protein extracts revealed appropriately sized bands for each of these antibodies (**Fig. S1**). The following secondary antibodies were used: Alexa Fluor 488 goat-anti-mouse, and Alexa Fluor 488 goat-anti-rabbit (1:500 dilution; Life Technologies, Grand Island, NY). Cell boundaries were visualized with the addition of 1:150 rhodamine-conjugated phalloidin (Cytoskeleton, Denver, CO), added separately after secondary antibody incubation, overnight at 4ºC. Nuclei were visualized by staining the tissue in DAPI for 15 minutes at 25ºC (1µM; Sigma, St.Louis, MO). After processing, the corneas were gently lifted off of the eyes using fine forceps and were placed into a drop of mounting media (ProLong Gold; Invitrogen, Eugene, OR) placed on charged slides (Colorfrost Plus, Thermo Scientific, Kalamazoo, MI). A coverslip was then placed on top of each cornea, and pressed

downward to flatten the tissue before being left to cure overnight at 25ºC. Imaging was done on a Zeiss LSM-700 confocal microscope.

Measuring cell division with phospho-Histone H3 staining

Corneas were cut around the periphery of the eye from stage 48-50 tadpoles, leaving the cornea epithelium attached to the eye via the central corneal stalk. Then, the whole eye was excised from the animal and placed into modified L-15 culture media containing an appropriate concentration of pharmacological compound or DMSO. The following conditions were tested: 100μM Liarozole, 20μM TTNPB, 1 μM all-trans Retinoic acid, 20μM all-trans Retinoic acid, with 0.2% DMSO as a control condition. 3-4 eyes at a time were cultured in 1mL of culture media. After 4 days of culture, the eyes were rinsed briefly in PBS, fixed in 3.7% formaldehyde for 2 hours, and stained using rabbit anti-phospho-Histone H3 (1:200 dilution; Kindly provided by Dr. Craig Mizzen, University of Illinois at Urbana-Champaign) in the same manner described above, with two modifications: nuclei were visualized by staining with Hoechst (1:10,000 dilution; Molecular Probes, Eugene, OR) for 20 minutes, and imaging was done on a Zeiss Axioplan microscope.

Quantification of cell division was performed by first sampling a standardized square area within the cornea in each image to measure the nuclear density of that cornea. Then, the total number of nuclei in each cornea was determined by multiplying the calculated nuclear density by the total area of that cornea, as measured using ImageJ (U.S. National Institutes of Health, Bethesda, MD). Total number of mitotic figures in each cornea was counted manually. The total number of **m**itotic **f**igures per 100 **n**uclei (MFN) was used as a measure of cell division. The MFN was determined for each individual case, and the mean MFN was calculated for every experimental condition. Pericorneal tissue was excluded from the analysis. Statistical significance was established using the (unpaired) *t* test.

Results

Expression of RA regulation related transcripts in *Xenopus* eye tissues

RT-PCR was performed to examine the expression of the transcripts of *aldh1a1/2/3*, which encode the enzyme that acts in the final step of endogenous RA synthesis, and the point at which tissue-specific patterning of an RA presence is generally regulated (see review by Niederreither and Dolle, 2007) . We also examined transcripts of *cyp26a/b/c1*, which metabolize endogenous RA (White et al., 1996). The expression of these members of RA biosynthesis and metabolism allow one to infer changes in the regulation of RA signaling within a given tissue. Three isotypes exist for both enzymes in *Xenopus*, encoded by 6 distinct genes, so primers were designed to examine all 6 independently. *aldh1a1*, *aldh1a2*, and *aldh1a3* (encoding RALDH1, RALDH2, and RALDH3 respectively) are all expressed in "Control" corneas harvested from unlentectomized eyes, as well as "Regenerating" corneas collected and pooled from 1, 3, and 5 days post-lentectomy (**Fig. 2.1A**). *cyp26a1* and *cyp26b1* (encoding CYP26A and CYP26B, respectively), but not *cyp26c1* (CYP26C), are expressed in the cornea in both control and regenerating tissues. The expression of these genes was also examined in harvested lenses, where all of the genes except *aldh1a3* were found to be expressed (**Fig. 2.1B**).

RA signaling activation via CYP26 inhibition or application of exogenous retinoids can both inhibit lens regeneration

In order to investigate the necessity of RA regulation by CYP26, lentectomized eyecups were cultured in the presence of various inhibitors and activators in an in vitro culture system described in Fukui and Henry (2011). Eyes were cultured in the presence of 100μM Liarozole Hydrochloride, an antagonist of CYP26. Treatment with Liarozole profoundly inhibited lens regeneration (p<0.0001). Only 1/23 (4%) eyes regenerated a lens in the drug-treated condition, compared to 13/20 (65%) with DMSO (**Fig. 2.2A, Fig. 2.3 A-D**). This result strongly

implicates a necessary role for CYP26 activity within the eye, suggesting that RA levels need to be reduced in the cornea for lens formation to occur.

To assess whether the diminished regeneration is the result of CYP26 antagonism, we cultured the eyecups with TTNPB, a potent retinoic acid analog (Minucci et al., 1996; Pignatello et al., 1999), which is highly resistant to the metabolizing action of CYP26 (Pignatello et al., 2002). Since the molecular consequence of CYP26 antagonism should lead to a rise of endogenous RA, we assessed whether the addition of CYP26-resistant TTNPB could impart the same consequence on regeneration as Liarozole. Results show that treatment of eyes with 20μM TTNPB greatly reduced lens regeneration (p<0.0001) as only 4/35 (11%) eyes regenerated lenses, compared to 22/32 (69%) with DMSO (**Fig. 2.2A, Fig. 2.3 E-H**). We likewise examined whether exogenous RA would inhibit regeneration. A relatively low concentration (1μM) of RA failed to do so, as 21/30 (70%) treated eyes regenerated, compared with 17/29 (59%) DMSO-treated eyes. The apparent slight increase in regeneration is notably not statistically significant (p=0.40). However, a higher concentration (20μM) of RA very strongly inhibited lens regeneration, as 0/33 (0%) eyes regenerated when treated with 20μM RA, compared to 18/33 (55%) of eyes treated with DMSO (p<0.0001) (**Fig. 2.2A, Fig. 2.3 I-P**).

To demonstrate that Liarozole and retinoid treatments were actually affecting the RA signaling pathway, we performed a drug-treatment validation assay to examine the expression of *cyp26a1*. Cyp26 genes are known to respond robustly to retinoic acid (de Roos et al., 1999; Hollemann et al., 1998; Pavez Loriè et al., 2009), so we checked for overexpression of *cyp26a1* in drug-treated tissue samples to confirm activation of RA signaling. qPCR was performed using RNA extracted from corneas that were treated with either drug or vehicle for 4 days. All compounds used showed a marked increase in *cyp26a1* expression, as Liarozole-treated corneas had 100-fold increase (p=0.0007), and TTNPB had over 1000-fold increase (p=0.0005) compared to control corneas. Additionally, both 1μM and 20μM

RA treatments showed over 1000-fold increase in *cyp26a1* expression (p=0.001 and p=0.008, respectively). There was no statistically significant difference in the expression levels between 1μM and 20μM RA (**Fig. 2.2B**). This result demonstrates that all of the drug treatments have an ultimate molecular consequence of increasing retinoid signaling within the cornea. This increase in RA signaling is correlated above with a failure to regenerate lenses from the cornea epithelium, with the exception of 1μM RA, which despite the increase in RA signaling does not inhibit lens regeneration. This shows that while *cyp26a1* over-expression is a positive readout of increased RA signaling and drug treatment efficacy, elevated expression of *cyp26a1* does not in itself inhibit lens regeneration.

We also treated cases with inhibitors of RA-signaling. Treatment with 60μM Citral, an inhibitor of RA synthesis, did not inhibit lens regeneration (p=0.44), as 12/18 (64%) of Citral-treated eyes regenerated compared to 15/18 (83%) of eyes treated with vehicle alone. Likewise, 5μM of LE-135, an antagonist of the RA nuclear receptors RARα and RARβ, failed to inhibit lens regeneration (p=0.48), as 19/24 (79%) LE-135-treated eyes regenerated lenses, compared to 12/18 (66%) of those treated with DMSO (**Fig. 2.2C, Fig. 2.3 Q-X**). These results are consistent with our hypothesis and suggest that antagonism of RA signaling does not prevent lens regeneration from occurring in the eye.

Additionally we assessed whether these compounds acted upon the RA signaling pathway using qPCR. Corneas treated with LE-135 expressed only half as much *cyp26a1* compared to controls (p<0.0001). This suggests that RA-receptor antagonism does not itself lead to a decrease in lens regeneration. However, *cyp26a1* expression was unaffected for Citral-treated corneas **(Fig. 2.2D)**.

The effect of CYP26 inhibition and exogenous retinoids on cell proliferation in the cornea

We examined the effects of Liarozole, TTNPB, and RA upon cell division corneas by culturing them in the presence of the compounds in vitro for 4 days and measuring cellular division. Phosphorylated Histone H3 is a reliable marker of cell division (Hans and Dimitrov, 2001), so we used an α-phospho-Histone H3 antibody to visualize and count mitotic cells in the corneas. The metric we used in quantifying changes in cell division was to measure the number of **M**itotic **F**igures for every 100 **N**uclei in the cornea (MFN), which allows us to control for the variation in nuclear density and number across individual corneas. Corneas cultured with DMSO had a mean MFN of 1.9 (n=9), and no statistically significant difference was measured for treatment with TTNPB (mean MFN=1.44; n=7), 1μM RA (mean MFN = 1.66; n=8), or 20μM RA (mean MFN= 1.45; n=6) (**Fig. 2.4**). Treatment with Liarozole however reduced cell division in half (mean MFN=0.89; n=9; $p<0.0001$). Since only Liarozole treatment, and not exogenous retinoids, resulted in a reduction of cell division, the decrease in cell division is not due to the rise in endogenous RA that results from CYP26 antagonism. Moreover, a reduction in cell division does not necessarily result from altered RA levels.

The effect of CYP26 inhibition and exogenous retinoids on the expression of putative stem cell markers

We performed qPCR to assess whether the expression of 3 putative corneal stem cell markers—*sox2*, *oct60*, and *p63* (Perry et al., 2013) —was affected by the drug treatments in vitro. A small increase in expression was observed for *sox2* when corneas were treated with TTNPB (3-fold; $p<0.0001$), 1μM RA (2.75-fold; $p<0.0001$), and 20μM RA (2.15-fold; $p<0.0001$). Liarozole treatment did not affect the expression of *sox2*. The expression of *oct60* decreased with 1μM RA (0.6-fold; $p=0.02$) and 20μM RA (0.45-fold; $p=0.0007$). Neither Liarozole nor TTNPB affected the expression of *oct60*. The expression of *p63* decreased with 1μM RA (0.6-fold;

p=0.002) and 20μM RA (0.4-fold; *p*<0.0001). Neither Liarozole nor TTNPB affected the expression of *p63* (**Fig. 2.5**). Since Liarozole inhibited lens regeneration but did not affect the expression of any of these 3 genes, and 1μM RA did not inhibit regeneration but did affect the expression of all 3 genes, it appears that the impact of altered RA signaling on lens regeneration is not directly related to the expression of these 3 genes in the cornea.

Localization of CYP26 and RALDH in the cornea epithelium

Since CYP26 function appeared to be necessary for lens regeneration, we next examined the possible sites of RA metabolism with immunohistochemical staining of CYP26. We recently described that the *Xenopus* cornea is constructed of 3 distinctly identifiable layers of cells (Perry et al., 2013), so we employed whole mount immunohistochemical staining of cornea pelts and confocal microscopy to visualize subcellular localization of proteins within corneal tissue.

Staining with α-CYP26A antibody revealed CYP26A localization in both the outer and basal layers of the cornea epithelium (**Fig. 2.6A, B**). Furthermore, staining was seen in the sparse cells of a deeper fibrillar layer that lies beneath the basal epithelium (**Fig. 2.6C**, arrowheads). In contrast, use of an α-CYP26B polyclonal antibody showed that CYP26B does not localize to the cells of the fibrillar layer (**Fig. 2.6F**, arrowheads) although it does stain the other two cellular layers (**Fig. 2.6D, E**).

Finally we examined the possible site of RA synthesis within the cornea using an α-RALDH1 antibody. Staining revealed RALDH1 localization in cells of the outer, and basal layers, but not the fibrillar layers of cells, in a pattern that was highly similar to that of CYP26B (**Fig. 6G, H, I**).

Discussion

Much of what is currently known about the roles of RA signaling in the development of the vertebrate eye comes from expression analyses and knockout experiments in mouse embryos (Cvekl and Wang, 2009; Duester, 2008; Ross et al., 2000). In *Xenopus*, RA affects eye field and telencephalon field development and has a stronger influence on the ventral aspect of these fields (Eagleson et al., 2001). Regions of RA signaling activity also coincide with regions of Pax6 expression, and *Pax6*-mutant tissues exhibit defective retinoid production. Mice with mutated *Pax6* have reduced RA signaling in the developing eye, with particularly serious effects in the lens (Enwright and Grainger, 2000), and RA induces the expression of *αB-Crystallin*, a marker of murine lens cells, in vitro (Gopal-Srivastava et al., 1998).

In *Xenopus*, it is known that RA, FGF receptor, and Hedgehog signaling pathways all serve to ventralize the developing eye (Lupo et al., 2005). Later in development, the mouse retina has dorsal and ventral zones rich with RA, with a horizontal band of RA-poor cells, all established via coordinated expression of RALDH and CYP26 enzymes (Sakai et al., 2004; Wagner et al., 2000). RA signaling components, including *Raldh* and *Cyp26* genes, are expressed throughout the embryo during *Xenopus* organogenesis (Lynch et al., 2011).

RA signaling has been implicated in the regeneration of several tissues as well. The application of Disulfiram, another RA synthesis inhibitor, inhibits the regeneration of tadpole tails in *Xenopus* as well as limbs in axolotls (Maden, 1998). Application of retinoids such as Vitamin A or TTNPB cause fully formed limbs to sprout in place of a tail in *Rana temporaria* (Maden and Corcoran, 1996). Similarly, application of Vitamin A and RA both result in duplications of limbs when applied during the course of limb regeneration in axolotls (Maden, 1983). In our own lab we have observed that Disulfiram fails to inhibit lens regeneration when live, lentectomized *Xenopus* tadpoles are kept in water containing an even higher concentration (10μM) of the compound than what was used in the above studies

(data not shown). Here we report that RA synthesis inhibition via Citral has the same null effect on lens regeneration in vitro. Likewise, treatment with another RA inhibitor, LE-135, also did not affect lens regeneration (**Fig. 2.2C**).

Lens regeneration in newts (Wolffian lens regeneration) occurs via transdifferentiation of the dorsal iris pigmented epithelial cells, following exposure to factors provided by the retina (Tsonis et al., 2004). RA signaling has been established as a necessary signaling scheme during this process, as application of a RARα inhibitor greatly reduced the regenerative potential of the dorsal iris, and application of a pan-RAR antagonist greatly inhibited regeneration and sometimes caused ectopic lens formation following lens removal. In one case, a lens regenerated from the cornea instead of the dorsal iris, corroborating our finding here that RA signaling attenuation is supportive of lens regeneration from the cornea. Moreover, addition of exogenous retinoids had no effect on newt lens regeneration (Tsonis et al., 2000; Tsonis et al., 2002). The importance of RA in Wolffian regeneration was further demonstrated when RA treatment together with *six3* expression transformed the normally lens-incompetent ventral iris into lens regeneration-competent tissue (Grogg et al., 2005). In contrast, the present study demonstrates that active RA signaling is prohibitive for lens regeneration in *Xenopus*.

RT-PCR data indicate that RALDH1-3 are all present in both control and regenerating corneas. RALDH1 and RALDH2 are both present in the lens, but RALDH3 is absent, suggesting that there might be a reduced need for RA production in lens tissue. CYP26C is also expressed in the lens, unlike control and regenerating corneas, which express only CYP26A and CYP26B, and thus the lens expresses all 3 RA-degrading enzymes (**Fig. 1B**). It is possible that during the course of lens differentiation from corneal tissue, it becomes necessary to reduce the rate of production of RA, and to increase the removal of RA from these tissues, and this is accomplished through regulated expression of the various RALDH and CYP26 enzymes. Although CYP26C can bind and degrade 9-cis-RA, all-trans RA is its

preferred substrate (Taimi et al., 2004). In *Xenopus* embryos the developing lens expresses *cyp26a1*, specifically within the lens epithelium, likely to moderate the effects of RA in that region (Hollemann et al., 1998; Lynch et al., 2011).

Here, immunohistochemical staining revealed the presence of CYP26A and CYP26B in both the outer and basal layers of the cornea epithelium (**Fig. 2.6**). The granular staining patterns observed in these cells is characteristic of the known staining pattern of endosome-localized cytochrome P450 enzymes in the cytoplasm (Makwana et al., 2012; Yague et al., 2004). CYP26A and CYP26B could act to eliminate an unwanted influence of RA in these cells. CYP26A, unlike CYP26B, is also found in the cells of the fibrillar layer. Although the identity and function of these deeper cells is not clear, they may be keratinocytes. Basal keratinocytes of human skin express CYP26A, which carefully modulates the levels of skin RA (Heise et al., 2006). Relative to CYP26B, CYP26A has significantly higher catalytic activity for metabolizing RA into the metabolite 4-oxo-RA (Topletz et al., 2012). The presence of the more highly active CYP26A enzyme in the cells of the fibrillar layer may highlight the urgency of RA disposal in those cells. This is further corroborated by the inducibility of CYP26A by retinoid treatments, as demonstrated through qPCR. Staining of retinoid-treated corneas with anti-CYP26A1 antibody revealed no obvious information as to whether any of the 3 cell layers in particular were more highly responsive to retinoid treatments than the others (data not shown).

Hyatt and colleagues (1996a) prevented the proper development of photoreceptor cells in zebrafish in vivo with the administration of just 3μM Citral. In our experiments, as much as 60μM of Citral had no effect on lens regeneration in vitro. It should be noted however that qPCR data indicated that no decrease occurred in the expression levels of *cyp26a1* following Citral treatment, suggesting the possibility that RA signaling may not have been affected by that treatment. Citral is a competitive inhibitor of the dehydrogenase enzymes responsible for RA synthesis, and there may have been enough RA present in the serum used in the culture media to compensate for the presence of Citral. The usage of LE-135 offers a

better demonstration that inhibition of RA signaling, specifically antagonism of RARα and RARβ, has no consequences upon lens regeneration. *cyp26a1* is strongly downregulated upon treatment with LE-135, indicating that RA signaling has been negatively affected. These results stand in contrast to what has been shown in Wolffian regeneration. Liarozole is a well-established inhibitor of RA metabolism (Pignatello et al., 2002; Stoppie et al., 2000; Takeuchi et al., 2011), and is used clinically for the treatment of skin diseases (Pavez Loriè et al., 2009). Liarozole treatments profoundly inhibited lens regeneration, but our results do not distinguish whether CYP26 acts strictly to clear RA from the cornea, or if it is necessary to generate RA metabolites such as 4-oxo-RA. 4-oxo-RA is known to affect anterior-posterior (AP) axis development in *Xenopus* (Pijnappel et al., 1993), but little else is understood about the potential signaling role for this molecule in other contexts. To corroborate the hypothesis that Liarozole inhibited regeneration due to increased RA signaling that results from the raising of endogenous RA levels, we also treated eyes with TTNPB and all-trans RA, and saw the same effect. TTNPB has been documented as a significantly more potent retinoid than all-trans RA, and is resistant to metabolism by CYP26 (Kistler et al., 1990; Pignatello et al., 1997, 1999). Although TTNPB effectively inhibited lens regeneration, we did not observe a significantly greater inhibition of regeneration with the use of TTNPB compared to all-trans RA. *cyp26a1* expression as examined by qPCR also did not show a significant increase of potency for TTNPB over RA. Treatments with retinoids such as TTNPB are known to cause AP axis defects during *Xenopus* development (Minucci et al., 1996). We treated *Xenopus* gastrulas with the same concentrations of retinoids used in the lens regeneration assays to observe axis defects, and again found that TTNPB was not more potent than RA for inducing defects (data not shown). Therefore, although the reason for the lack of increased potency of TTNPB over all-trans RA in our experiments is unclear, it is not likely that it is due to a unique functional difference of TTNPB in the context of cornea-lens regeneration.

Treatment with 1μM RA did not have an effect on lens regeneration, even though it greatly stimulated the expression of *cyp26a1*, and 20μM of RA both

completely inhibited lens regeneration and stimulated *cyp26a1* expression. This data suggests that there is a limit to the RA-protective activity of CYP26 within these tissues. *cyp26a1* expression is simply an indicator of changes in RA signaling activity and the data shows that its expression is not by itself the cause of the inhibition of lens regeneration.

Cell proliferation occurs throughout the larval cornea epithelium (Perry et al., 2013). Retinoic acid is an inducer of epithelial cell proliferation (Kim et al., 2012; Nabeyrat et al., 1998; Wang et al., 1997) and its popularity in pharmaceutical applications (as Tretinoin) is partly derived from this property. Although it is not yet clear whether the regenerated lens in *Xenopus* is derived from transdifferentiated cornea cells, or multipotent cornea stem cells, a final phase of cellular differentiation is clearly necessary in the course of generating a new lens *de novo*. We assessed whether the presence of RA in corneal tissue may prevent proper differentiation of cells due to changes cell proliferation. We cultured normal, whole corneas in vitro in the presence of Liarozole or retinoids, and examined cell division through immunohistochemical staining of phosphorylated Histone H3. Although our examination was confined to a single snapshot in time, we can nonetheless observe the effects of these compounds on cell proliferation in control and drug-treated corneal tissue. Interestingly, CYP26 inhibition via Liarozole reduced cell proliferation in half, but treatment with the retinoids did not have any measurable effect on proliferation (**Fig. 2.4F**). This suggests that the inhibitory effect of Liarozole on cell division is not likely due to rising levels of endogenous RA levels in these tissues. Rather, it is possible that the metabolite generated by CYP26, 4-oxo-RA, may be a regulator of cell division in the cornea. The action of CYP26 therefore may be necessary as a regulator of cell turnover, and by extension, an enabler of proper differentiation into lens cells. It additionally seems that the inhibitory effect of exogenous retinoids on lens regeneration is not caused by depressed (or elevated) cellular division. This leaves open the possibility that although decreased cell division is coincident with Liarozole treatment, it may not itself be the cause of inhibited lens regeneration. In fact, inhibiting cell proliferation in the *Xenopus*

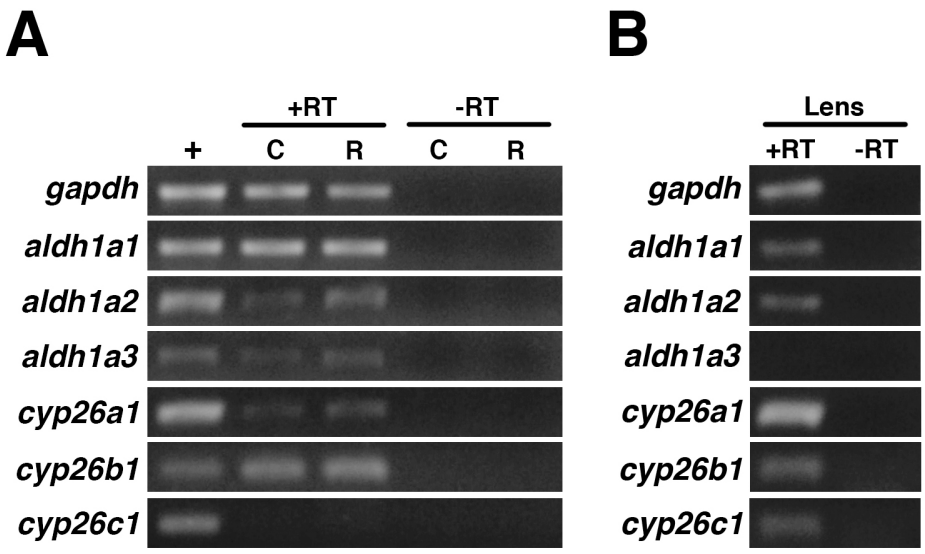
cornea with Mitomycin C does not inhibit lens regeneration (Filoni et al., 1995). Further experiments are necessary to definitively confirm the mechanistic roles and cytological consequences of CYP26 action, and excess retinoids, during lens regeneration.

We have recently provided evidence for a possible stem cell identity for cells of the basal and fibrillar layers (Perry et al., 2013), and CYP26 may act to prevent the differentiating effects of RA in order to maintain cellular multipotency. We tested this possibility by treating corneas with drugs in vitro, to observe how it might affect the expression of *sox2*, *oct60*, and *p63*, three putative stem cell markers expressed within the cornea that are upregulated in the early stages of lens regeneration. Most notably, CYP26 antagonism during tissue culture did not affect the expression of any of these markers (**Fig. 2.5**), even though the antagonism was shown to strongly inhibit lens regeneration (**Fig. 2.2A**). Interestingly, the presence of 1μM RA affected the expression of all 3 genes (**Fig. 2.5**), although it does not affect lens regeneration (**Fig. 2.2C**). Taken together, it appears that altering the expression levels of these particular genes (to the modest degrees seen in our experiments) is not related to lens regenerating competence in the tissue. Notably, this does not provide evidence that the resident stem cell population is irrelevant for lens regeneration. Indeed, the cornea is composed of a heterogeneous cell population, and SOX2 and P63 localize to different subpopulations of cells (Perry et al., 2013). Since it is not yet known which cells are important for lens regeneration and which are important for corneal maintenance and repair, it remains unclear whether and how CYP26 relates to cellular multipotency or lens regenerating competence of the cornea. It should be noted that it is not possible to reliably isolate regenerating corneal tissue once it is inserted into the eyecup and cultured in vitro. Therefore, we were unable to evaluate how the expression of these genes might be affected following lens removal, where the cornea is exposed to signaling factors from the retina, in addition to the pharmacological compounds used in this study. Regardless, it can be inferred from our data that whatever role RA regulation plays

to support cornea-lens regeneration, it is not likely via the modulation of *sox2*, *oct60*, and *p63* transcription.

Considerable effort has been invested into understanding the retinal factors that initiate the process of lens regeneration in vertebrates, and FGF and FGFRs have emerged as important targets of study (Bosco et al., 1997; Fukui and Henry, 2011). However, the nature of the cornea that makes it competent to respond to those retinal signals is poorly understood. *pax6* expression is one factor that has been associated with lens regeneration competence (Gargioli et al., 2008). We presently provide evidence that RA signaling must be attenuated within the cornea epithelium, which could in part be responsible for endowing this tissue with lens-forming competence. Additionally the present study presents for the first time a role for CYP26 in regeneration. It highlights an important deviation from Wolffian lens regeneration, and presents a contrasting view to the regeneration-enabling role historically attributed to retinoic acid (Maden and Hind, 2003). The clear differences between Wolffian and *Xenopus* regenerative processes, as demonstrated here, underscore the importance of investigating animal specific variations in regeneration of the same tissues, and may reveal mechanisms that limit regenerative phenomena.

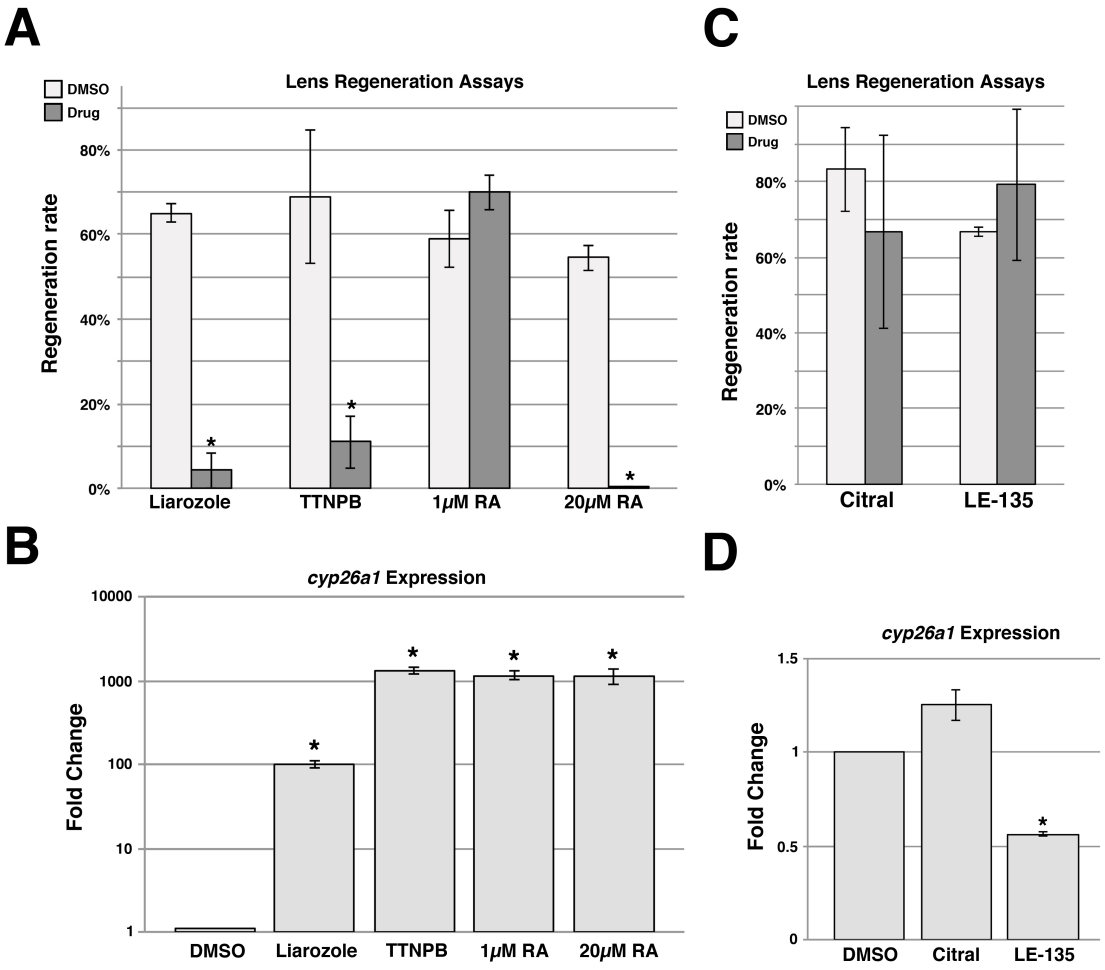
Figure 2.1



Expression of RA signaling related genes.

(**A**) Expression of RA-signaling related genes in control corneas (C) and pooled regenerating corneas from 1, 3, and 5 days post-lentectomy (R). "+RT" group indicates reactions that employed reverse transcriptase for generation of cDNA. "–RT" group indicates reactions that used water in place of reverse transcriptase, as a control for genomic contamination. "+" indicates a positive control reaction performed using embryonic RNA (**B**) Expression of genes in control lenses.

Figure 2.2



RA signaling inhibits lens regeneration, and RA signaling antagonism does not inhibit lens regeneration.

(**A**) Liarozole and TTNPB are potent inhibitors of cornea-lens regeneration. A high concentration of RA very strongly inhibits lens regeneration. * indicates $p< 0.0001$, using two-tailed Fischer's exact test. (**B**) A drug-treatment validation assay using qPCR shows that all of the drugs strongly activate RA signaling, as seen by a profound upregulation of *cyp26a1*, a marker of active RA signaling, in the cornea. * indicates $p<0.01$, using unpaired *t* test. (**C**) Citral and LE-135, inhibitors of RA signaling, fail to inhibit cornea-lens regeneration. (**D**) A drug-treatment validation assay using qPCR shows that LE-135 attenuates expression of *cyp26a1* in the cornea. * indicates $p <0.0001$, using unpaired *t* test. All error bars indicate standard error.

Figure 2.3

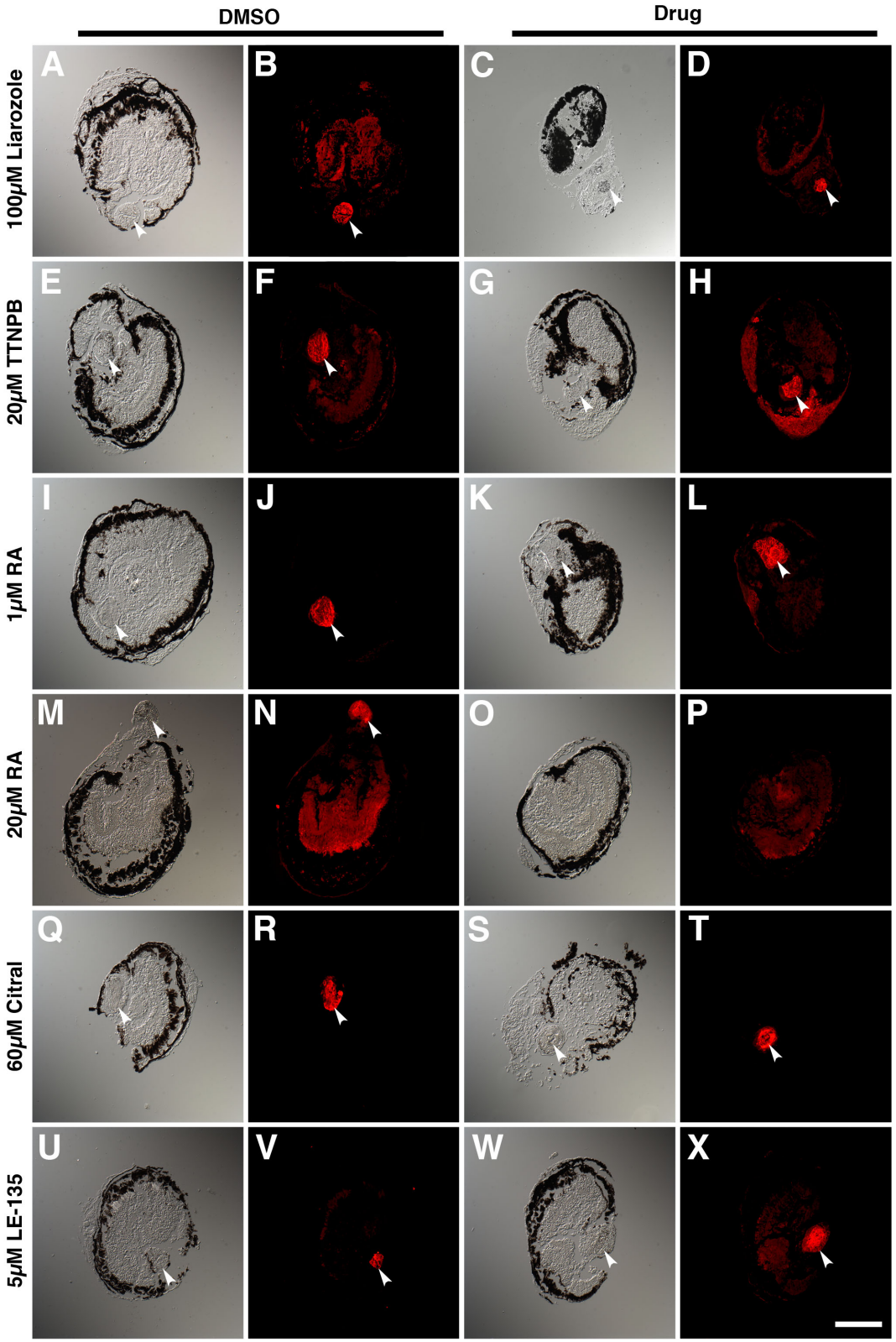
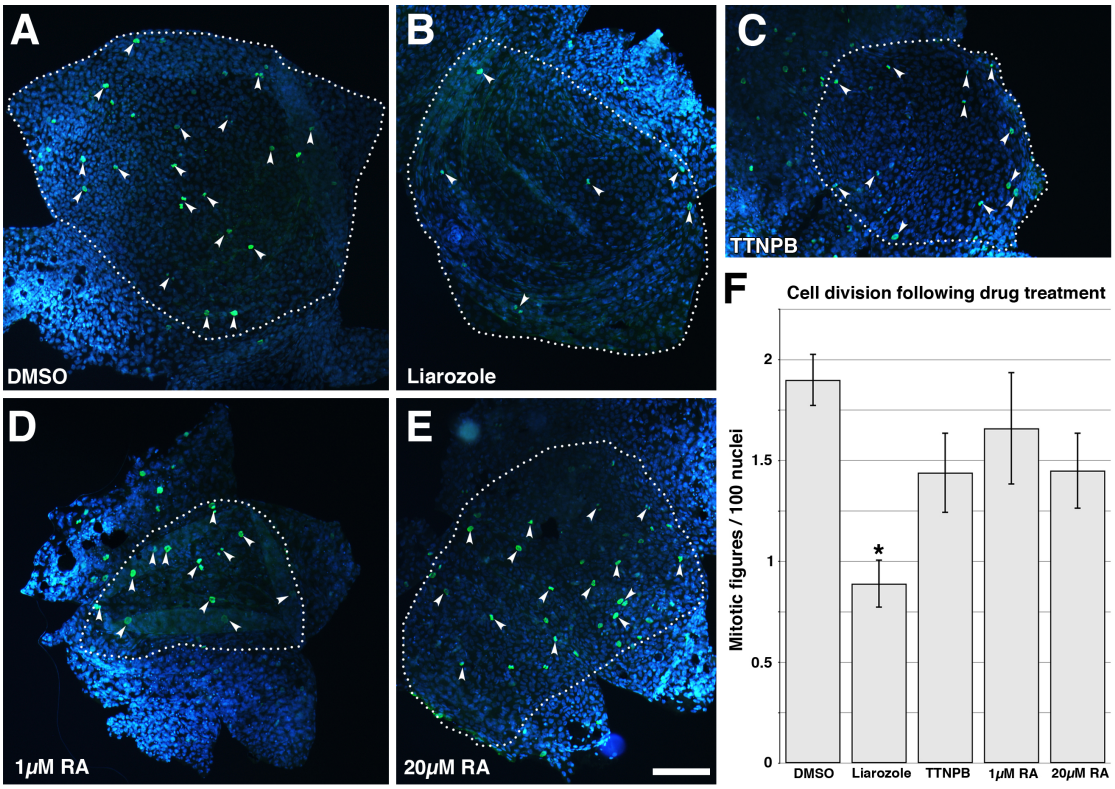

DMSO
Drug
100μM Liarozole
20μM TTNPB
1μM RA
20μM RA
60μM Citral
5μM LE-135
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X

Figure 2.3 (cont.)

Lens regeneration assays.

Representative examples are shown of positive cases of lens regeneration for each condition tested, with the exception of 20μM RA, for which no lenses regenerated. Panels **A, E, I, M, Q, U, C, G, K, Q, S**, and **W** are images taken with a DIC microscope. Panels **B, F, J, N, R, V, D, H, L, P, T**, and **X** are images of fluorescently labeled lenses with an anti-lens antibody (Red). Arrowheads indicate the regenerated lens. Scale bar in **X**= 100μm

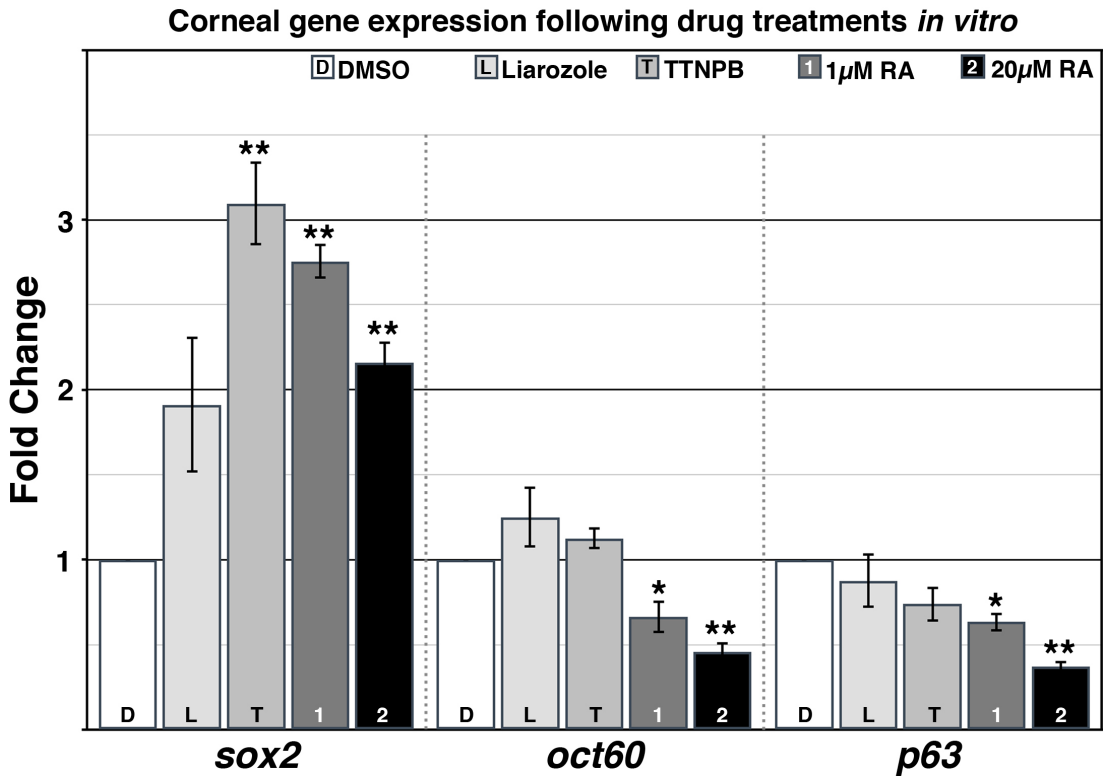
Figure 2.4



The effect of CYP26 inhibition, and exogenous retinoids on cell proliferation in the cornea.

Nuclei are labeled with Hoechst (blue) and mitotic cells are labeled with anti-phospho-Histone H3 antibody (green). When compared to treatment with DMSO in vitro (**A**), treatment with TTNPB (**C**), 1μM RA (**D**), or 20μM RA (**E**) has no effect on the number of mitotic cells at in the cornea. Treatment with Liarozole (**B**) reduced cell division. White arrowheads indicate examples of mitotic figures found in the cornea. Note that not every mitotic figure is pointed out in (A) and (E). The white dotted outline demarcates the cornea proper from pericorneal epithelium. The results are quantified in (**F**). Error bars show standard error. * indicates $p<0.0001$ using unpaired *t*-test. Scale bar in **E**= 50μm

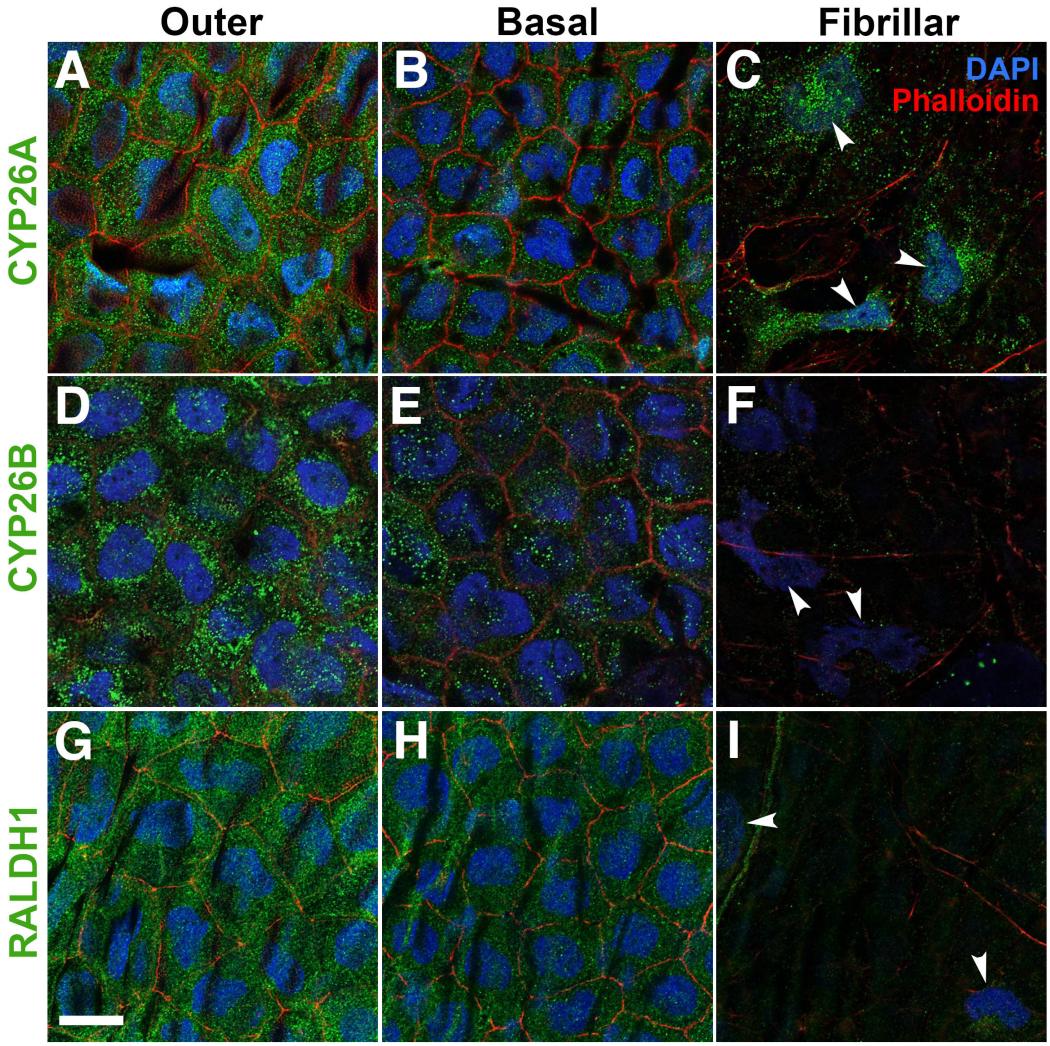
Figure 2.5



qPCR of putative corneal stem cell markers.

Corneas cultured in vitro in the presence of retinoids show upregulation of *sox2*, and downregulation of *oct60* and *p63*. CYP26 inhibition with Liarozole does not affect the expression of any of these genes. Error bars show standard error. * indicates $p<0.05$ and ** indicates $p< 0.001$ using unpaired *t*-test.

Figure 2.6



Cellular localization of CYP26 and RALDH enzymes in the cornea.

Corneal whole-mount immunofluorescence staining was used to visualize the expression of CYP26A (**A-C**), CYP26B (**D-F**), and RALDH1 (**G-I**), in each of the 3 layers of the larval cornea— the outer epithelium (**A, D, G**), the basal layer (**B, E, H**), and the deeper fibrillar layer (**C, F, I**). CYP26A (green) is expressed in all layers, including the sparse cells of the fibrillar layer (arrowheads). CYP26B (green) and RALDH1 (green) are only expressed in the outer epithelial and basal layers, and are not seen in the cells of the fibrillar layer (arrowheads). DAPI (blue), phalloidin (red). Scale bar in **G** = 20μm

Supplemental Table 1

Primer Name	Sequence (5'->3')	T_M (ºC)	Product size (bp)
qpcr_actb_forward	CGCCCGCATAGAAAGGAGAC	60	128
qpcr_actb_reverse	AGCATCATCCCCAGCAAAGC		
qpcr_cyp26a1_forward	GGCTGTCTGTCCAACCTGC	60	181
qpcr_cyp26a1_reverse	GTCGCTTGATGGCGGGATAC		
qpcr_sox2_forward	CCCCGGGCATGTCTCTGGGA	60	195
qpcr_sox2_reverse	GTTGCGACATGTGCAGTCTGCTTTGC		
qpcr_oct60_forward	CAGAAACACAGCCGGACAGA	60	66
qpcr_oct60_reverse	CACCCATAGCAGCACAGCAT		
qpcr_p63_forward	ACAGTGTCACCGCACCATCACC	60	158
qpcr_p63_reverse	CGTCCAGGTGGCTGACTTTGC		
cyp26a1_forward	GGCACTGAAAGAATCCGCAA	50	406
cyp26a1_reverse	CGGCGTTAGAAATCGGTCTG		
cyp26b1_forward	TCCCCAAAGGTTGGAGTGTT	53	244
cyp26b1_reverse	GCAAGCTCAAACCTGCTCAT		
cyp26c1_forward	TACCAGATCCCCAAAGGCTG	53	238
cyp26c1_reverse	TGGCAGTAGTTACCAGCTCC		
aldh1a1_forward	GTGGAAAGAAAGAAGGCGCA	50	377
aldh1a1_reverse	TTCATGCAGGCCATATTCGC		
aldh1a2_forward	TGCCAGCGTTCAGTAGAAAC	50	410
aldh1a2_reverse	GGAAGCACAATACAAGGCGA		
aldh1a3_forward	ATGCCCTGCATACACAGACT	53	420
aldh1a3_reverse	TGTGACAATGGCAATCACGG		
gapdh_forward	CTTGAAGGGAGGTGCCAAGC	50	491
gapdh_reverse	CCAGGATTCCCTTCATTGGGC		

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CHAPTER 3

Understanding the basis of CYP26 mediated regulation of lens regeneration

The ability to fully regenerate a lost lens is a unique phenomenon amongst some animals. No mammal has been identified with this ability, but several vertebrates including salamanders and frogs have been studied for years for their ability to regenerate a lens de novo (Freeman, 1963; Henry, 2003). In the frog *Xenopus laevis*, regeneration begins following the removal of the lens from tadpole larvae. In the absence of the lens and the inner cornea endothelium, molecular factors from the retina cross the vitreous chamber and reach the cornea epithelium ("cornea"), which triggers morphological and cytological changes, and the cornea gives rise to a new lens within a matter of days. Not all of the retinal and corneal factors responsible for this phenomenon have been identified, but Fibroblast Growth Factors (FGFs) from the retina have been implicated as a necessary and sufficient trigger for lens regeneration in *Xenopus* (Bosco et al., 1997; Fukui and Henry, 2011). Our understanding of what makes the cornea, and not any other surrounding ectoderm, capable of responding to these signals is much less clear.

Retinoic acid signaling is known to govern the proper morphogenesis of the eye, including the development of the retina, lens, and cornea (Enwright and Grainger, 2000; Kastner et al., 1994; Molotkov et al., 2006; Wagner et al., 2000). It is mediated through the ligand *all-trans* Retinoic Acid (RA), derived from Vitamin A, which binds to RA receptors in the nucleus to modulate transcription. RA signaling can be regulated both at the level of its synthesis by RALDH enzymes, or metabolism by CYP26, among other means (reviewed by Bastien and Rochette-Egly, 2004). RA is also involved within the cornea to enable lens regeneration. It is required for lens regeneration in newts, as demonstrated when antagonism of RA signaling receptors using pharmacological inhibitors diminished regeneration (Tsonis et al., 2000; Tsonis et al., 2002). In newts, the lens regenerates from the dorsal iris exclusively, instead of the cornea. The ventral iris can be converted into a tissue capable of

generating lens cells if it is made to express *six3* in the presence of exogenous RA (Grogg et al., 2005). We have demonstrated that lens regeneration in *Xenopus* is notably different with regards to RA signaling. Antagonism of RA signaling has no effect on lens regeneration in *Xenopus*, and in fact the activity of the RA-metabolizing, cytochrome P450 enzyme CYP26 is necessary to support regeneration. Two orthologs, CYP26A and CYP26B are both expressed in the cornea, and their antagonism using the molecule Liarozole inhibits lens regeneration just like the addition of excess exogenous RA, or an RA analog that cannot be metabolized (Thomas and Henry, 2014). In developing *Xenopus* embryos, CYP26 is expressed in the lens epithelium, further supporting the need for CYP26 activity in the course of generating a lens (Hollemann et al., 1998). The key question remains however of whether CYP26 acts in the cornea exclusively to clear RA from the cells to attenuate RA signaling, or whether CYP26 additionally acts to actively generate retinoid metabolites, such as 4-oxo-RA, which may be required for signaling. This scenario is further supported by the observation that CYP26 antagonism via Liarozole inhibits cell division in the outer cornea epithelium, but excess exogenous RA and RA analogs do not. Notably, a suspension of cell division does not by itself inhibit lens regeneration in *Xenopus* (Filoni et al., 1995), but the observation suggests that RA itself does not regulate cell division despite the important role of CYP26. Although our work has implicated CYP26 in regeneration, antagonism of CYP26 by itself cannot parse the difference between whether CYP26 is necessary for the tissue to be depleted of RA, or if it is also needed to create RA metabolites that participate in signaling events that permit regeneration. This led to the hypothesis that CYP26 acts to generate an important metabolite like 4-oxo-RA for cell division and lens regeneration. Moreover, the time period during which CYP26 activity was necessary for lens regeneration was previously undetermined. Our hypothesis is that CYP26 activity is required early during lens regeneration as a factor regulating the initial competence to regenerate a lens.

CYP26 eliminates the availability of RA for signaling by metabolizing it, primarily to 4-oxo-RA and 4-OH-RA (White et al., 1996), of which 4-oxo-RA is longer

lived (Topletz et al., 2012).The action of CYP26 is often coordinated with RA synthesis in order to restrict RA signaling within specific tissue boundaries (Duester, 2008; Rhinn and Dolle, 2012). Other potential RA metabolites, like 4-OH-RA, are known to exhibit biological activity in cells (Idres et al., 2002; Reynolds et al., 1993), but the roles of these other metabolites are not well established, especially in developmental and in vivo contexts. We presently focus on 4-oxo-RA, which is known to affect *Xenopus* development (Pijnappel et al., 1993). There is little additional data regarding whether 4-oxo-RA is a biologically relevant signaling molecule, and there is no consensus amongst the few studies in which it has been examined (Niederreither et al., 2002; Pijnappel et al., 1993).

We performed lens regeneration assays using an ex vivo eye culture system to assess the effects of 4-oxo-RA supplementation on regeneration. The ex vivo lens regeneration assays were performed as previously detailed in Fukui and Henry, 2011, and Thomas and Henry, 2014, with some modifications. Animals staged 48-53 (all staging in this study are according to Niewkoop and Faber, 1956) were used throughout the experiments, and they were anesthetized in 1:2000 MS-222 (Sigma, St. Louis, MO) diluted in 1/20 NAM (normal amphibian media), where they remained for the duration of lens removal surgery (lentectomy). Using fine scissors, the outer cornea is cut and the lens is removed through the incision before the whole eye is excised from the head and placed into a well of a 24-well culture plate. Eyes are individually cultured in 350µL of "modified L-15 media" (2:3 dilution of L-15 media, with 10% fetal bovine serum) for 7 days. The media was also supplemented with the appropriate amount of pharmacological compound or the vehicle DMSO (Fisher Scientific, Fair Lawn, NJ), in the same amounts as reported in previous work (Thomas and Henry, 2014). The compounds used are: 100µM Liarozole hydrochloride (Tocris, Bristol, UK), 20µM *all-trans* Retinoic Acid (Sigma), and 20µM *4-oxo*-Retinoic Acid (TRC, Toronto, Canada). The concentration of 4-oxo-RA was chosen to be sufficiently high to elicit a physiological response in ex vivo culture. Culture media was changed every other day, and after 7 days of culture, the eyes were rinsed in PBS and then fixed in 3.7% formaldehyde (Sigma) for 1 hour at

25ºC. Then the eyes were then embedded in paraffin wax for sectioning at a thickness of 10μm, and placed onto a positively charged slide (Colorfrost Plus, Thermo Scientific, Kalamazoo, MI) before immunohistochemical staining with a rabbit polyclonal anti-lens antibody (Henry and Grainger, 1990). The sections were examined, and the presence of a morphologically distinct lentoid structure that positively stained with the anti-lens antibody was scored as a positive case of lens regeneration. Statistical significance in differences between regeneration rates was established using (two-tailed) Fisher's Exact test.

Of DMSO treated eyes, 11/13 (85%) regenerated lenses. When eyes were treated with the CYP26 inhibitor Liarozole, only 2/13 (15%) eyes regenerated lenses, showing greatly diminished regeneration (p=0.0012), as we expected and previously reported (Thomas and Henry, 2014). In order to determine whether CYP26 is relevant simply as an ablator of RA within the corneal tissue, or if it is also an important generator of the RA metabolite 4-oxo-RA to act as a novel signaling ligand in regeneration, we assessed whether lens regeneration could be rescued in cultures co-treated with Liarozole and 4-oxo-RA. The exogenous addition of 20μM 4-oxo-RA alone resulted in only a minor reduction in regeneration (p= 0.046) as 7/14 (50%) regenerated lenses. The addition of 4-oxo-RA and Liarozole together did not significantly increase the rate of regeneration compared to Liarozole alone, and the rate was still significantly lower (2/21, 9.5%; p <0.0001) than DMSO treated eyes (Figure 3.1A). No obvious size differences were noted amongst any regenerated lenses. If it were the case that CYP26 antagonism was inhibiting lens regeneration due to diminished 4-oxo-RA production in the cornea, then the addition of exogenous 4-oxo-RA should have compensated for the loss and rescued regeneration. However, our result shows that this is not the case, providing evidence that 4-oxo-RA is not a relevant signaling molecule in the context of lens regeneration. Note that this does not rule out the possibility that other retinoid metabolites, such as 4-OH-RA, could be involved in regeneration.

To assess whether the addition of 4-oxo-RA to our cultures has any molecular effect on the tissues, we cultured excised eyes in the presence of 4-oxo-RA, harvested the corneas 4 days later, and then extracted RNA to perform qPCR, using previously reported methods (Thomas and Henry, 2014). We specifically examined the expression changes of the gene *cyp26a1*, which encodes CYP26 and is a key positive marker that indicates active RA-signaling (de Roos et al., 1999; Hollemann et al., 1998; Pavez Loriè et al., 2009). Notably, the act of *cyp26a1* upregulation itself has no impact on the rate of lens regeneration, it is simply used as a molecular marker to determine whether RA signaling is active or not (Thomas and Henry, 2014). RNA was extracted from corneas that were cultured with DMSO or 20μM 4-oxo-RA for 4 days. Each technical replicate within each qPCR experiment received 20ng of input cDNA. Fold changes of expression were determined using the comparative C_T method (Schmittgen and Livak, 2008). For the purposes of determining statistical significance and standard error, a single "N" was defined as the whole experiment performed from start to finish, starting with surgical harvest of tissues from live animals, and ending with a qPCR run. Statistical significance was established using the (unpaired) *t* test.

We observed that treatment with 4-oxo-RA profoundly upregulated the expression of *cyp26a1* within the cornea (N=3; $p<0.01$) (Figure 3.1B), demonstrating that within our ex vivo culture system 4-oxo-RA can and does act as a signaling ligand that effects transcriptional events within the corneal tissue. It likely does so through the nuclear retinoic acid receptor RARβ, as it has been shown to have a high affinity for that receptor (Pijnappel et al., 1993). The upregulation of the *cyp26a1* gene is indicative of elevated RA signaling, and this elevation could be the reason for the mild reduction in regeneration observed with 4-oxo-RA treatment alone. Note however that it is not obvious from our experiment whether physiologic levels of 4-oxo-RA generated by endogenous corneal CYP26 can evoke such transcriptional changes. To further validate the activity of the 4-oxo-RA used in experiments, we treated stage 10 *Xenopus* gastrulas with 20μM 4-oxo-RA for about 48 hours and observed them at stage 29. Compared to DMSO-treated gastrulas, all

embryos exhibited severe defects of the anterior-posterior axis (Figure 3.1H), indicating abnormal RA signaling within the developing embryo, just as described by Pijnappel et al. (1993). Altogether, we have shown that exogenous 4-oxo-RA can elicit a molecular response, but whatever the function of CYP26 is within the cornea, it does not appear to act as a generator of 4-oxo-RA to enable lens regeneration.

Earlier work has demonstrated that CYP26 antagonism via Liarozole leads to diminished cell proliferation in the cornea. However this effect of Liarozole is not the cause—or is at least not necessary—for inhibited lens regeneration from the cornea because concentrations of RA that also inhibit lens regeneration fail to impact cell proliferation (Thomas and Henry, 2014), despite the fact that RA generally acts to promote epithelial cell turnover (Nabeyrat et al., 1998; Wang et al., 1997). Thus, even though CYP26 is important for regulating cell division in the cornea, we hypothesized that it must be doing so in an RA-independent manner. Given these observations, we examined whether cell proliferation is reduced in the cornea due to the failure to generate the metabolite 4-oxo-RA upon CYP26 antagonism.

Eyes were excised from stage 49-51 animals, and cultured with attached corneas in media supplemented with the appropriate pharmacological compound or DMSO, just as in the qPCR experiments. After 4 days of culture, explanted eyes were rinsed with PBS and fixed in 3.7% formaldehyde for 1 hour at 25ºC. The explants were stained for phospho-Histone H3, a marker of dividing cells (Hans and Dimitrov, 2001), using a rabbit anti-phospho-Histone H3 antibody (provided by Dr. Craig Mizzen, University of Illinois at Urbana-Champaign). Nuclei were visualized by staining with 1:10,000 Hoechst (Molecular Probes, Eugene, OR) for 20 minutes. After staining, the corneas were carefully detached from the eyes and placed into a drop of Prolong Gold mounting media (Invitrogen) on a glass microscope slide. A coverslip was then placed atop the tissue that was then pressed flat before observation under a Zeiss Axioplan microscope. Each cornea was photographed and the images were used to quantify cell division. In order to quantify cell division, a

standard square area was selected within each image to determine the nuclear density of that cornea. The total number of nuclei in each cornea was calculated by multiplying the nuclear density by the total area of that cornea determined using ImageJ (U.S. National Institutes of Health, Bethesda, MD). The total number of mitotic figures in each cornea was counted manually, and the number of mitotic figures per 100 nuclei (MFN) was used as measure of cell division. For the purposes of determining statistical significance and standard error, each "N" is defined as an individual cornea on which the above analysis was performed. When pericorneal tissue was present in each image, it was excluded from analysis. Statistical significance was determined using the (unpaired) *t* test.

The cell division assay was performed in the presence of DMSO, 100μM Liarozole, 20μM 4-oxo-RA, or both Liarozole and 4-oxo-RA (Figure 3.1 C-G). Liarozole treatment in our experiments diminished the MFN by nearly half (mean MFN=0.77; N=10; p= 0.0159) compared to DMSO-treated corneas (mean MFN=1.34; N=12). Treatment with 4-oxo-RA alone had no significant effect on proliferation (mean MFN= 1.46; N=14). Treatment with both Liarozole and 4-oxo-RA had the same inhibitory effect on cell proliferation as Liarozole treatment alone had (mean MFN = 0.44; N=14; p = 0.0002). As the addition of 4-oxo-RA with Liarozole could not recover the MFN to control levels, it shows that the inhibition of cell proliferation observed with CYP26 antagonism is not explained by diminished 4-oxo-RA production. While proliferating cells in the cornea do contribute to the regenerating lens following lens removal (Perry et al., 2013), a complete arrest of cell division using Mitomycin C reportedly does not stop lens regeneration (Filoni et al., 1995). The exact reason for the effects that Liarozole have is unclear, and work remains to be done to understand the exact relationships between retinoic acid signaling, CYP26 activity, corneal cell proliferation, and lens regeneration.

Next we determined when during regeneration CYP26 is important, as it has remained unclear whether CYP26 activity is relevant during earlier, later, or all stages of lens regeneration. Earlier work had left open the possibility that CYP26

activity could be important in order to maintain a lentogenic bias in the cornea when the cornea tissue first responds to retinal factors (Thomas and Henry, 2014). It may additionally or instead be needed during later stages of regeneration, such as during morphogenesis and growth of the regenerated lens, or differentiation of lens fiber cells and crystallin expression. We tested the timing of CYP26 relevance during lens regeneration by varying the timepoints at which the CYP26 inhibitor Liarozole, or RA was added (Figure 2A). We first setup our controls by reproducing earlier work to show that lens regeneration in ex vivo culture is inhibited by both Liarozole (3/20, 15%; p=0.0063) and RA (1/26, 4%; p<0.0001) when compared to DMSO (16/28, 58%) (Figure 3.2B). In these controls, the compounds are added to the culture media immediately after lens removal, and the tissues are exposed to them throughout 7 days of regeneration.

We simultaneously performed 2 other experiments, where the addition of the compounds were delayed by either 12 or 48 hours following lentectomy (Figure 2B) to examine the effects on regeneration. The results of a 12-hour delay were similar to that of the controls, as regeneration was reduced by both Liarozole (4/22, 18%; p=0.0046) and RA (0/33, 0%; p<0.0001), when compared to DMSO (17/29, 59%). This result demonstrates that CYP26 activity and RA signaling ablation are still necessary after the first 12 hours that follow lens removal. However, when the addition of the compounds was delayed by 48 hours, lens regeneration was unaffected. Both Liarozole (19/22, 86%) and RA (15/19, 79%) treated eyes regenerated at nearly the same rate as DMSO treated ones (27/34, 79%). This result demonstrates that CYP26 activity and RA signaling ablation is not needed beyond the first 48 hours of regeneration. Taken together, it appears that there is window of time within the first 2 days of regeneration during which RA signaling attenuation must be maintained. Past results have shown interesting differences in the results of Liarozole and RA treatment, such as diminished cell division with the former, but not with the latter, and inhibited lens regeneration with either. This suggested that the although the observation of inhibited lens regeneration was common to both treatments, the underlying molecular mechanism that is disrupted in each case

could be different, coincidentally leading to the same result. Given our current finding that regeneration is sensitive to both elevated RA and CYP26 antagonism only in the first 12-48 hours post-lentectomy, the disrupted mechanism or mechanisms, whatever they may be, are likely more related than one would think. For example, a reduced ratio of nucleoli per nucleus is one of the earliest indications of lens regeneration from the cornea, and is seen within 24 hours post-lentectomy in vivo (Freeman, 1963). CYP26 activity and RA signaling attenuation could regulate the different steps that facilitate this histological event. Although there are possible timing differences in ex vivo culture lens regeneration compared to in vivo experiments, lens protein expression is first noted in vivo around days 3-6 post-lentectomy (Day and Beck, 2011; Freeman, 1963), which is consistent with the view that CYP26 is involved only prior to lens cell differentiation.

Given that CYP26 activity is apparently important for the earlier stages of regeneration, we examined whether it could play a role in establishing or maintaining the ability of the cornea to respond to molecular signals that trigger its differentiation—a property known as "lens competence". To this end we assessed the expression of key molecular markers associated with lens competence, *pax6* and *fgfr2*, following CYP26 inhibition and RA addition. *pax6* is a transcription factor that regulates eye and lens development in mammals (Enwright and Grainger, 2000) as well as *Xenopus* (Altmann et al., 1997). *Pax6* is a hallmark of lens competence and initial lens-forming bias in the embryo (Fujiwara et al., 1994; Li et al., 1994; Zygar et al., 1998), and its expression also demarcates the lentogenic area of the cornea from surrounding lens-incompetent ectoderm (Gargioli et al., 2008; Perry et al., 2013). Ectopic misexpression of *pax6* is also sufficient to endow lens-incompetent ectoderm with the ability to respond to retinal factors and to generate a new lens (Gargioli et al., 2008), and work in newts has shown that *pax6* is specifically involved in the early stages of lens regeneration, such as cell proliferation, rather than later stages like lens fiber differentiation (Madhavan et al., 2006). Given that there is a known interaction between *pax6* and RA (Gajovic et al., 1997), we

additionally examined *fgfr2*. FGFR2 is another competence marker that is exclusively expressed in lentogenic ectoderm of *Xenopus* (Arresta et al., 2005).

qPCR analyses were done in the same manner as described above for *cyp26a1*, but the corneas here were treated with either DMSO, 100μM Liarozole, or 20μM RA. We found that the expression of *pax6* was greatly reduced by treatment with Liarozole (35% reduction; N = 4; $p < 0.05$), and by RA (57% reduction; N = 5; $p = 0.0012$) compared to DMSO treatment (N = 5) (Figure 3.2C). This result shows that CYP26 antagonism, and any resultant increases in endogenous RA, will reduce *pax6* expression and thereby transform the cornea into a relatively more lens-incompetent state. Since exogenous RA also decreased the expression of *pax6*, it appears that the effect that CYP26 antagonism had on *pax6* could be due to a rise in tissue RA, rather than a loss of any RA metabolite. Similarly, exogenous RA significantly reduced *fgfr2* expression (27% reduction; N=3; $p<0.001$). While Liarozole treatment appeared to reduce *fgfr2* expression, the effect was not statistically significant (Figure 3.2C). The evidence suggests that the purpose of CYP26 within the cornea could be to maintain a lentogenic state by attenuating RA signaling. Although *pax6* is elsewhere known to have roles in enabling lens fiber cell differentiation and crystallin expression (Madhavan et al., 2006; Shaham et al., 2009), the downregulation of *pax6* observed here is evidently unable to prevent these late events in *Xenopus* lens regeneration. This further suggests a role for CYP26 only in early lens regeneration events only.

The action of CYP26 is necessary within the *Xenopus* cornea in order for lens regeneration to occur, but the precise mechanism remains unclear. The present study however further cements its importance as a retinoic acid metabolizer and ablator of RA signaling, and provides insight into the time period during regeneration in which CYP26 is needed. Understanding the unique mechanisms that allow a species to regenerate lens tissue broadens our understanding of the regenerative biology of the lens, and brings us closer to developing therapies to replace our own damaged or lost lenses.

Figure 3.1

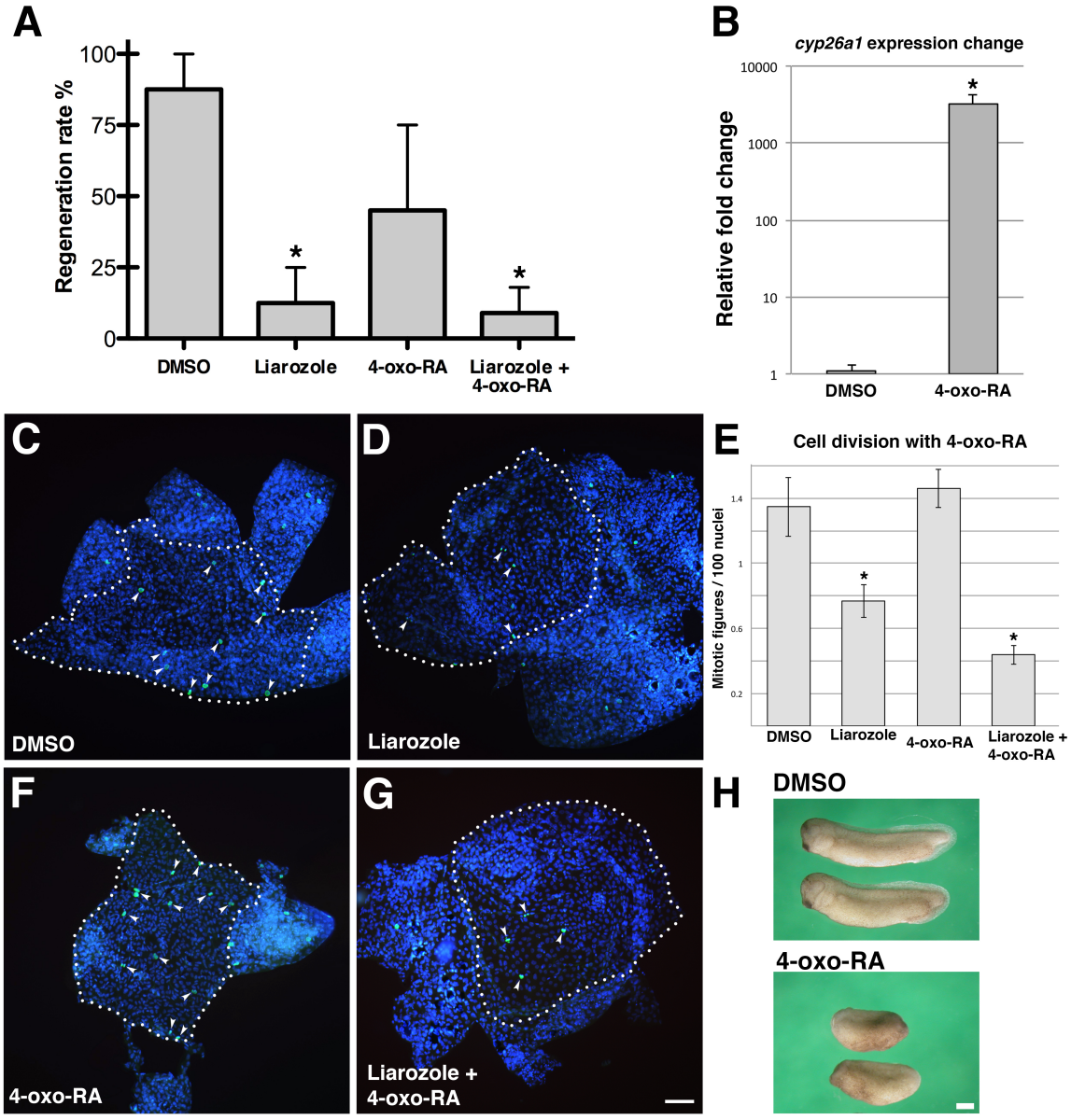
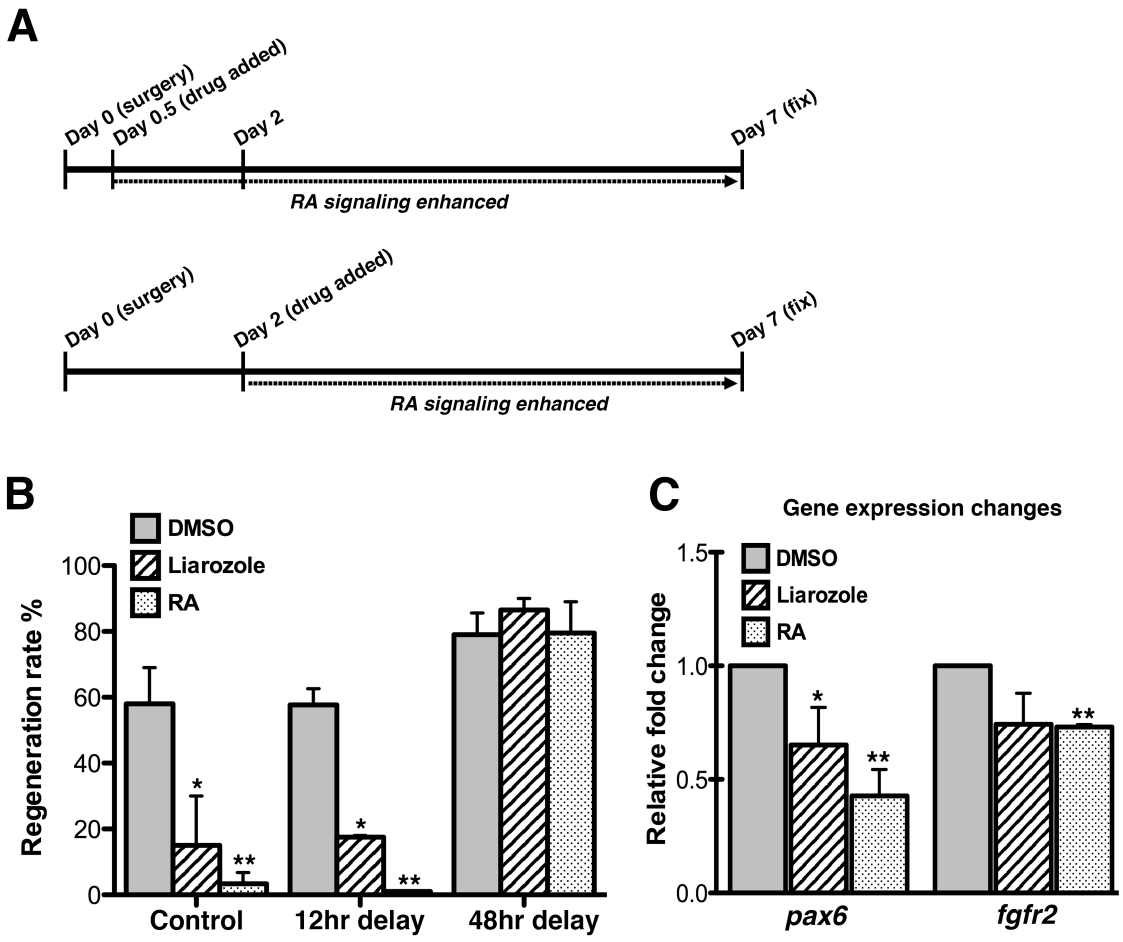

A
Regeneration rate %
100
75
50
25
0
DMSO
Liarozole
4-oxo-RA
Liarozole + 4-oxo-RA
B
cyp26a1 expression change
Relative fold change
10000
1000
100
10
1
DMSO
4-oxo-RA
C
DMSO
D
Liarozole
E
Cell division with 4-oxo-RA
Mitotic figures / 100 nuclei
1.4
1
0.6
0.2
DMSO
Liarozole
4-oxo-RA
Liarozole + 4-oxo-RA
F
4-oxo-RA
G
Liarozole + 4-oxo-RA
H
DMSO
4-oxo-RA

Figure 3.1 (cont.)

(**A**) The addition of 4-oxo-RA with Liarozole does not rescue the inhibitory effects of CYP26 antagonism. * indicates $p \leq 0.001$. (**B**) Treatment of corneal tissue with 4-oxo-RA leads to the upregulation of *cyp26a1*, a marker of RA signaling activity, showing that the molecule has expected molecular effects in the tissue. * indicates $p \leq 0.01$. (**C, D, F, G**) Fixed cornea pelts are labeled with DAPI (blue) and anti-Histone H3 antibody (green) and the number of mitotic figures are counted to determine the amount of cell proliferation in control and drug-treated tissues. White arrowheads indicate mitotic figures. Liarozole treatment leads to diminished cell proliferation (**D**) compared to controls (**C**), and exogenous 4-oxo-RA fails to compensate for this effect (**G**). Treatment of 4-oxo-RA alone has no effect on proliferation (**F**). Scale bar in (**G**) equals 50μm. The results are quantitated in (**E**). * indicates $p \leq 0.01$. (**H**) When stage 10 gastrulas are treated with 4-oxo-RA, they develop defects in the anterior-posterior axis; a classic sign of aberrant RA signaling (Pijnappel et al., 1993). Embryos are photographed at stage 29. Scale bar in (**H**) equals 1mm. All error bars indicate standard error.

Figure 3.2



(**A**) Schematic depicting the timing of events in the experimental design. (**B**) Delaying the addition of the CYP26 antagonist Liarozole or RA yields results nearly identical to that of the control experiment, where the drugs are added immediately following surgery. However, delaying addition by 48 hours results in uninhibited regeneration. * indicates $p < 0.01$, ** indicates $p < 0.0001$. **(C)** Real-time quantitative PCR on cornea-derived RNA shows that Liarozole and RA treatments both significantly lower the expression of the transcription factor *pax6*. Exogenous RA also significantly lowers the expression of *fgfr2*. * indicates $p<0.05$, ** indicates $p < 0.001$. All error bars indicate standard error.

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CHAPTER 4

Conclusions and Future Directions

The chief finding of this work has been to identify that CYP26 is important during *Xenopus* lens regeneration, by acting to inhibit RA signaling. This most likely occurs during the early stages of regeneration. We have described a clear departure from newt regeneration, highlighting the importance of assessing species-specific differences when studying regenerative phenomena. Based on the data, our current operating model for the role of CYP26 and RA signaling in *Xenopus* lens regeneration is that CYP26 metabolizes and eliminates retinoic acid from the cornea early during regeneration. This attenuates RA signaling in the cornea and allows the tissue to adopt a lens-competent state, characterized by the expression of factors such as *fgfr2* and *pax6*. Once this state has been established by the action of CYP26, the cornea is capable of responding to retinal factors that will trigger it to transform into a lens.

Our characterization of RA signaling within this context expands our understanding of the events that underlie corneal transformation into a lens. We have also broadened our knowledge of the functional roles of CYP26 in biology. The three most interesting questions that still remain to be addressed by the field in the years ahead are suggested here.

1: What genes are regulated by RA signaling in the cornea?

RA signaling attenuation is apparently important during lens regeneration, but it is not clear what related events this serves to attenuate or enhance. Investigating which loci are under the control of RA-mediated chromatin remodeling within the regenerating cornea can reveal currently unknown genes or regulatory regions that are a critical part of regenerating lenses de novo. Chromatin-

immunoprecipitation experiments using antibodies that target RAR proteins in cornea-derived extracts would cast light upon such RA-regulated loci.

2: Does CYP26 regulate corneal cell division? Is this function independent of RA metabolism?

Our work demonstrated that CYP26 antagonism would reduce cell division within the cornea, but exogenous RA could not do the same. We also showed that this effect is not due to a reduced production of 4-oxo-RA in the cornea upon CYP26 inhibition. While our work showed that CYP26 antagonism reduced corneal cell division, we have not shown that CYP26 is an endogenous regulator of corneal cell division in vivo. Cornea-wide knockdown of CYP26 and a subsequent analysis of cell division could confirm the relevance of CYP26 in this context. Taken together with the findings from our work, it would suggest a role for CYP26 that is independent from its role as an RA metabolizer and regulator of RA signaling. This hypothesis warrants further biochemical examination, perhaps using mutant CYP26 proteins with inert active sites. A discovery of a novel non-enzymatic role for CYP26 would be a highly significant finding even outside of the field of regenerative biology.

3: Is CYP26 also relevant in *Xenopus* during lens development from surface ectoderm?

While we were able to describe a previously unknown role for CYP26 during lens regeneration, it remains unclear whether the same role is relevant in the course of lens development. In particular, experimental inhibition or knockdown of endogenous CYP26 in the lens placode has not been done to assess whether it is necessary to develop a lens. If interference of CYP26 during lens development should lead to aphakia, the result would be congruent with the findings of our research, and highlight the common ground that exists between lens development and regeneration. Likewise, the opposite finding would reveal a distinction between the two schemes, and highlight the unique processes that underlie regeneration.

While we have learned a great deal about the regenerative biology of *Xenopus*, much work remains to be done to fully understand cornea-lens regeneration, and more work remains to be done before we may translate such findings into clinical medicine.

APPENDIX

Method for an ex vivo lens regeneration assay in *Xenopus laevis* larvae

The use of an ex vivo eye culturing system, first described in 2011 (Fukui and Henry, 2011), allows for the effects of pharmacological agents to be isolated to the cultured tissues, and allows the usage of very small amounts of limited or expensive compounds. In the example protocol described below, we will consider a hypothetical compound "Drug X" and its corresponding control dimethyl sulfoxide (DMSO), the solvent in which Drug X is dissolved.

A.1 Selection of animals

Animals staged 48-52 are ideal for experiments, as they are large enough to perform surgery upon and immature enough to have the ability to regenerate lenses in vivo. Staging is performed according to Nieuwkoop and Faber, 1956. Animals are readily identified at stage 48 when they first acquire an iridescent cover over their abdomen, and at stage 52 when they begin to form digits on their limbs.

When experiments are repeated, the embryos in the repeat experiments are the offspring of a unique pairing of two different parents. Thus, animals used in different experiments are never siblings, but may be half-siblings. Animals with gross defects (e.g., ocular coloboma, albinism, scoliosis, bloating, etc.) are not selected for experiments.

A.2 Preparation of culture media

The culture media used in ex vivo lens regeneration assays is known as modified L-15 (mL15) media. To prepare 500mL of mL15 medial, dissolve 4.18g of L-15 powder (Gibco, Waltham, MA) into approximately 450mL of cell-culture deionized grade water. All mixing should be done in a clean bottle that has never

been exposed to detergents of any kind. It is advisable to reserve a 1L bottle exclusively for this use. Add 50mL of fetal bovine serum (10% of final volume). Adjust the pH of the solution to 7.5-7.6 and adjust the final volume to 500mL. Filter-purify the media using a 0.2µm filter under a laminar flow hood, making sure again that the filtered media goes into a sterile bottle that has never been cleaned with detergents. Aliquot the media into 50mL aliquots in sterile tubes and store them at 4ºC. Media stored in this manner may be used for up to 4 months.

Before initiation of an experiment, add the following antibiotics and antifungal agent to an individual mL15 aliquot so as to achieve final concentrations of: 10kU/mL penicillin-streptomycin, 4µg/mL Marbofloxacin, and 2.5µg/mL Amphotericin B. Once antimicrobials have been added, the media may be used for up to 3 weeks when stored at 4ºC. Before the media may be used in an experiment, it must be brought to room temperature.

A.3 Anesthesia and Surgery

Three to six animals of the appropriate stages are anesthetized at once in a small petri dish filled with 3-5 mLs of anesthetic solution. The anesthetic used is a 1:2000 preparation of MS-222 (Sigma, St.Louis, MO) diluted in normal amphibian media (NAM): 110 mM NaCl, 2 mM KCl, 1 mM $Ca(NO_3)_2$, l mM $MgSO_4$, 0.l mM EDTA, l mM $NaHCO_3$, 2 mM Na phosphate, pH 7.4 (Slack, 1984). Animals are transferred directly from their aquarium into the anesthetic and one must wait until they are completely immobilized (which usually takes 2-4 minutes) before transferring them into the surgical plate for surgery. Animals are transferred from dish to dish using a large bore plastic transfer pipet with the distal end cut off in such a manner to accommodate the size of the animal. Alternatively, a small metal tea strainer can be used as a net. The surgical dish contains soft modeling clay (Van Aken, North Charleston, SC) in which a trough can be molded to stabilize the anesthetized animal on its side while performing surgery on that side. The dish is filled with enough

anesthetic solution to cover the animal, and surgeries are performed under a dissection microscope.

A curved incision is created using precision micro-scissors on the posterior-ventral edge of the outer cornea, taking care not to make cuts outside of the pericorneal area. The pericorneal area is the ring of ectoderm that extends 1.5x the diameter of the eye, and does not immediately overlie the orb of the eye. The incision should span roughly 20-40% of the circumference of the eye. The scissors are then inserted through this incision and used to pierce the inner cornea at the edge of the iris and into the pupillary opening with a downward motion of a single scissor blade. Care must be used for if the iris becomes lacerated with this maneuver, bleeding will occur, in which case the surgery must be aborted and the animal sacrificed.

Once both the outer and inner corneas have been cut, insert fine forceps into the eye and to grab the lens. Pull out the lens through the incisions and discard the lens by wiping the tip of the forceps thoroughly with a Kimwipe, so as to ensure no residual lens cells are left behind on the instrument. Care must be exercised to remove the entire intact lens. Once this is accomplished, cut the outer cornea all the way around the circumference of the eye, again staying within the boundaries of the pericorneal area. The outer cornea should be left attached to the inner cornea via the central stalk. Using forceps, insert the outer cornea into the vitreous chamber of the eye. Make sure the tissue remains inside the vitreous chamber as the forceps are released and removed from the eye. Remove the whole eye from the animal by cutting beneath the eye to sever the optic muscle, artery and nerve connections. Transfer the eye into a 35 mm petri dish containing 2mL of mL15 media (**Fig. A.1**).

The transfer of eyes is done using a P200 micro-pipet with the distal ends of the pipet tip cut off. Barrier pipet tips (Denville Scientific, South Plainfield, NJ) are cut with a razor blade and then autoclaved. The tips are cut to create an opening

large enough to accommodate the size of the eyeballs, but narrow enough to still allow some capillary action, which helps to retain the eye during transfer.

Turn the animal onto the other side and repeat the lentectomy (lens removal from the eye) and enucleation (eye removal from the body) procedures. After the eyes have been transferred into the dish containing mL15 media, transfer them once more into an identical dish of fresh media to "wash" them. After 3-5 minutes, swirl them around and randomly transfer one eye rach each into another 35 mm petri dish containing 2mLs of either DMSO-containing control media or media containing Drug X. This step is to ensure unbiased and random allocation of eyes into experimental and control groups. The wash dishes and media should be replaced after 24-30 eyes have been rinsed in them.

The enucleated animal is euthanized by leaving it in a separate dish of MS-222 for 2 hours, and stored at -20ºC thereafter.

A.4 Ex vivo culturing

After all lentectomized eyes have been collected into the extra small petri dishes with DMSO or Drug X-containing mL15, each eye is transferred into an individual well of a 24-well polystyrene cell culture plate, with each circular well having a radius of 0.77cm. Wells are filled with 350µL of appropriate media and one eye is allocated to each well. Isolation of eyes in this manner prevents fusion of eyes that occurs when cultured in clusters, and it minimizes the spread of potential microbial contamination. Once all eyes are placed into the 24-well plate, the plate is placed inside of a "moisture chamber"— a small, covered, plastic box with a moistened sponge placed inside—to minimize evaporation of the media. The culturing is done at ambient temperature (18-24ºC). If any drug being used is sensitive to light (for instance, retinoids) then the moisture chamber is placed in a dark container or drawer.

The day that surgeries are performed is denoted as “Day 0”, and subsequent days are denoted “Day 1, Day 2…” etc. The culture media is changed every other day (on even numbered days) by pipetting out the 350µL in each well and replacing it with appropriate fresh media. All changes of media should take place under a laminar flow hood, and one should exercise proper asceptic technique. The eyes should be monitored daily for microbial contamination. Contamination can be seen if there is a change in the color of the media to orange, yellow, or purple, if the media appears turbid, or if fungal hyphae are seen growing on or around the eyes. If contamination is present in any well, all contents of that well should be discarded, and all remaining eyes should be transferred to a new, clean 24-well plate. If any eyes appear grossly damaged in the days following surgery, those eyes should be discarded.

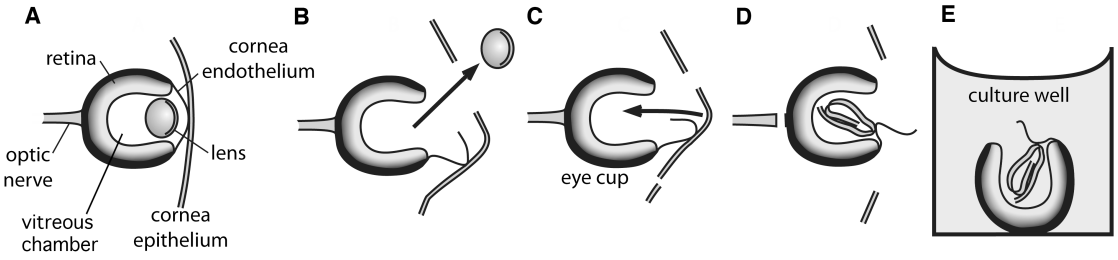

Figure A.1

Illustration from Fukui and Henry, 2011.

A.5 Fixation and histological preparation

Ex vivo culturing is terminated on Day 7 by transferring eyes from their wells into a 35 mm petri dish containing 2mL of PBS. If the eyes have become adhered to the bottom of the culture well, they can be displaced by repeatedly aspirating the media around the eye with a pipet, or they can be gently lifted off the surface using a fine pipet tip. They are swirled in the dish gently for 2-3 seconds to rinse off mL15 media from their surface. They are then immediately transferred into a small glass vial containing 4mL of fixative. The best fixation method to use depends on the

antibody or staining method being used later, and it should be determined empirically.

Typically, eyes are assessed for lens regeneration via an anti-lens antibody (see section A.6), and so they are fixed for 30 minutes in 3.7% formaldehyde at room temperature. They are prepared for thin sectioning by embedding them in paraffin wax, following standard protocols for paraffin-section immunohistochemistry. In brief: the fixative is replaced with PBS and eyes are incubated for 10 minutes at room temperature. These rinses are repeated two more times. The eyes are then carried through a series of dehydrating washes, each step being 10 minutes long and each wash having a volume of about 5mLs: 30% ethyl alcohol (EtOH), 50% EtOH, 70% EtOH, 95% EtOH, 100% EtOH. The alcohol dilutions are made in deionized water. After the final wash, the EtOH is replaced with fresh 100% EtOH and it is incubated at room temperature for 1 hour. Then, it is replaced with a 50/50 mixture of Xylenes and EtOH and incubated for 10 minutes before performing two 10-minutes washes with 100% Xylenes. The Xylenes are replaced with melted paraffin wax (Paraplast Plus) in an oven at 59ºC, and eyes are embedded into blocks of wax and sectioned into 10μm thick sections and placed on charged glass slides (Colorfrost Plus, Thermo Scientific, Kalamazoo, MI) using deionized water. The water is removed from the slides by placing them on a slide-warming tray at 32ºC overnight.

A.6 Immunohistochemistry, and lens scoring criteria

Prior to beginning the staining and scoring of regenerated lenses, each specimen should be anonymized so as to allow an objective and blinded assessment of cases of positive lens regeneration. This is typically done by renaming each paraffin block with a randomly assigned letter, making note of what each letter corresponds to in a separately prepared key. The new letter designation is carried over throughout all subsequent steps in the experiment, blinding the experimenter to the identity of each specimen as they section, stain, and score them. After all

scoring has been done the experimenter can refer to the key to identify how many cases of regeneration are seen in any given experimental or control group.

Immunohistochemical (IHC) staining is performed per standard IHC protocols with slight modifications. The slides are stained with 1:500 polyclonal anti-lens antibody (Henry and Grainger, 1987) for 1 hour at room temperature. It is then stained with an appropriate secondary antibody (typically a goat-anti-rabbit-Alexa 488, 1:300) for 2 hours at room temperature. Coverslips are added using a preparation of 80% glycerol/ 20% PBS. A positive case of lens regeneration is identified when there is a round area of tissue that stains with discernable fluorescent signal. The scoring of each case of regeneration is binary, and is neither qualitative nor quantitative when assessed in paraffin sections.

A.7 Statistical analysis

	No. of eyes that did regenerate a lens	No. of eyes that did NOT regenerate a lens	**Total**
DMSO treatment	A	B	A + B
Small molecule treatment	C	D	C + D
Total	A + C	B + D	N

$$p = \frac{(A+B)!(C+D)!(A+C)!(B+D)!}{A!B!C!D!N!}$$

The Fisher's Exact statistical test is used here to determine the significance of observed deviation between regeneration rates in control, and drug-treated eye cultures. It is better suited than the X^2 test for small sample sizes. Additionally, a X^2 test requires that every value in the table above be greater than 10, as there is 1

degree of freedom in this application of the test (Pagano and Gauvreau, 2000). As such, the test is not suitable for use when an inhibitor is very potent at inhibiting lens regeneration, resulting in fewer than 10 eyes with regenerated lenses. The Fisher's Exact test has no such requirement and can therefore be applied to data from experiments using strong inhibitors. A two-tailed *p*-value calculation will evaluate whether there is a difference between regeneration rates between two groups, without any assumption as to whether the drug-treated group should have a lower or higher regeneration rate than the DMSO-treated control group. A *p*-value <0.05 is considered as statistically significant.

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