NEW INSIGHTS INTO CORNEA-LENSES REGENERATION IN XENOPUS LAEVIS: THE ROLE OF WNT/BETA-CATENIN SIGNALING AND THE REGENERATIVE CAPACITY OF THE LIMBAL REGION

BY

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DISSERTATION

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ABSTRACT

One of the large outstanding questions in the field of developmental biology is how some tissues and organs of certain species are able to regenerate while others cannot. It is only by understanding the molecular mechanisms that drive residual cells in a damaged or diseased tissue to proliferate and differentiate to replace lost structures that we will have the knowledge to attempt to recapitulate these regenerative processes in other species, including our own. Towards this end, the focus of this work is centered on understanding the cell and molecular mechanisms of lens regeneration in the frog, *Xenopus laevis*, which possesses a high capacity to regenerate larval tissues, such as the complete regeneration of the lens from the cornea epithelium.

To fill in a large void in our current knowledge of the cell signaling pathways necessary for regeneration, we investigated the Wnt/β-catenin signaling pathway in the context of lens regeneration (Chapter 2) as it has been shown to be important in both embryonic lens development as well as in Wolffian lens regeneration that takes place in newts and salamanders; however, it had not been functionally studied in the context of cornea-lens regeneration in *Xenopus* despite being implicated to be involved in the early events of lens regeneration from two independent studies. We examined the expression of frizzled receptors and wnt ligands in the frog cornea epithelium. Numerous frizzled receptors (*fzd1, fzd2, fzd3, fzd4, fzd6, fzd7, fzd8, and fzd10*) and wnt ligands (*wnt2b,a, wnt3a, wnt4, wnt5a, wnt5b, wnt6, wnt7b, wnt10a, wnt11, and wnt11b*) are expressed in the cornea epithelium, demonstrating that this tissue is transcribing many of the components of the Wnt signaling pathway. When compared to flank epithelium, which is lens regeneration incompetent, only *wnt11* and *wnt11b* are different (expressed only in the cornea epithelium), identifying them as potential regulators of cornea-lens regeneration. To detect changes in canonical Wnt/β-catenin signaling occurring within the cornea epithelium, *axin2* expression was measured over the course of regeneration. *axin2* is a well-established reporter of active Wnt/β-catenin signaling, and

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its expression shows a significant decrease at 24 hours post-lentectomy. This decrease recovers to normal endogenous levels by 48 hours. To test whether this signaling decrease was necessary for lens regeneration to occur, regenerating eyes were treated with either 6-bromoindirubin-3'−oxime (BIO) or 1-azakene 37 −oxime - both activators of Wnt signaling - resulting in a significant reduction in the percentage of cases with successful regeneration. In contrast, inhibition of Wnt signaling using either the small molecule IWR-1, treatment with recombinant human Dickkopf-1 (rhDKK1) protein, or transgenic expression of Xenopus DKK1, did not significantly affect the percentage of successful regeneration. Together, these results suggest a model where Wnt/β-catenin signaling is active in the larval cornea epithelium and needs to be suppressed during early lens regeneration in order for these cornea cells to give rise to a new lens. While this finding differs from what has been described in the newt, it closely resembles the role of Wnt signaling during the initial formation of the lens placode from the surface ectoderm during early embryogenesis of the vertebrate eye.

This similarity between larval lens regeneration and embryonic lens development may not be surprising when one looks at the histological structure of the larval cornea epithelium which is similar to that of the fetal cornea in humans. However, as larvae mature through metamorphosis, the cornea epithelium (and underlying layers of the cornea) matures to become structurally very similar to our own. In light of a new model suggesting that cornea-lens regeneration in the frog, Xenopus, may be driven by oligopotent stem cells and not transdifferentiation of mature cornea cells, we investigated the regenerative potential of the limbal region in post-metamorphic cornea, where the stem cells of the cornea are thought to reside. It has been reported that the mature cornea is competent to regenerate under experimental conditions, despite the fact that the in vivo capacity to regenerate is lost; however, that work did not examine the regenerative potential of different regions of the cornea. Using the thymidine-analog 5-Ethynyl-2′-deoxyuridine, we identify long-term label retaining cells in the basal cells of peripheral post-metamorphic Xenopus cornea, consistent with slow-cycling stem cells of the limbus that have been described in other vertebrates. Additionally, the pattern of label being lost from the central cornea is consistent with a model of centripetal migration of cornea.
epithelial cells away from the limbal region and into more superficial layers. Using this data to identify putative stem cells of the limbal region in *Xenopus*, we tested the regenerative capacities of the dorsal and ventral limbal regions, and compared that to the central cornea. All three regions showed a similarly high ability for the cells of the basal epithelium to express lens proteins when cultured in proximity to larval retina. This indicates that the regenerative capacity in post-metamorphic cornea is not restricted to stem cells of the limbal region, but also occurs in the transit amplifying cells located throughout the basal layer of the cornea epithelium. In contrast, there was no clear evidence that apical differentiated cells are contributing to lens regeneration.

Finally, in order to more precisely monitor *in vivo* cell behavior during regenerative phenomena in future studies, we developed a new prolonged imaging technique (Chapter 4). While live imaging of embryonic development over long periods of time is a well-established method for embryos of the frog *Xenopus laevis*, once development has progressed to the swimming stages (when most regenerative phenomena that are studied currently occur), continuous live imaging becomes more challenging because the tadpoles must be immobilized. Current imaging techniques for these advanced stages generally require bringing the tadpoles in and out of anesthesia for short imaging sessions at selected time points, severely limiting the resolution of the data. Here we demonstrate that creating a constant flow of diluted tricaine methanesulfonate (MS-222) over a tadpole greatly improves their survival under anesthesia. Based on this result, we describe a new method for imaging stage 48 to 65 *X. laevis* (when lens regeneration occurs), by circulating the anesthetic using a peristaltic pump. This supports the animal during continuous live imaging sessions for at least 48 hr. The addition of a stable optical window allows for high quality imaging through the anesthetic solution and provides for the first time a method for continuous observations of developmental and regenerative processes in advanced stages of *Xenopus* over 2 days. Together this work provides new insights into the cell signaling mechanisms during larval regeneration, and sets the stage for using new imaging techniques *in vivo* in future studies of the regenerative process and how it may change as the cornea epithelium develops through metamorphosis.
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CHAPTER 1. INTRODUCTION

It has long been the dream of the medical community to unlock the secrets of regeneration in human tissue for the obvious clinical benefits to human health and longevity. However, to master that level of cellular reprogramming in a living organism is an intuitively difficult task. Take for instance the 1980 movie Star Wars: Episode V The Empire Strikes Back. Near the end of this film Luke Skywalker loses a hand during an iconic lightsaber duel with Darth Vader. Afterwards, the movie concludes with a scene on a spaceship where Luke receives a robotic prosthesis to replace the hand he just lost. To a regenerative biologist, this scene is a bit frustrating, because the implication is that in this fantasy universe where technology has developed to the point of achieving interstellar space travel and building moon-sized space stations that can blow up planets, using regenerative medicine to replace a lost structure, like a hand, still appears to be far, far away.

Despite this particular observation that human regeneration of a complete structure like a hand is too fantastical for a fantasy movie like Star Wars, regeneration of a limb is quite possible in several animals, such as the newt. In fact, animals from a variety of phyla have evolved varying degrees of regenerative capacity (Brockes and Kumar, 2008). Through the expansion of our knowledge of these systems, it is possible that one day we may be able to recapitulate some of these regenerative mechanisms in our own tissues. There are numerous model systems used for studying regeneration in the laboratory, and while they each have their own unique strengths and shortcomings, all are important to study if we are ever to understand the principles that dictate why some tissues and organs in specific organisms are able to regenerate, while others cannot. The African clawed frog, *Xenopus laevis*, provides an excellent system for studying

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regenerative phenomena, and is the focus of this work. The larval stages (i.e., tadpoles) of X. laevis are capable of regenerating numerous tissues of the body including the tail, limb bud, and lens (Beck, 2012), and a single mating between adult frogs can produce hundreds of larvae for experimental analysis. In addition, X. laevis has been well studied in the context of development due to the size of its large externally developing embryo, providing extensive background knowledge about the development of this organism and a wide assortment of molecular tools that can be used for experimental analysis, such as transgenesis. This work focuses on the ability of X. laevis to regenerate the lens of the eye from the cornea epithelium. Compared to the number of tissues present in the regeneration of a tail or limb bud, lens regeneration is a relatively simple system due to the limited number of tissues involved, which makes experimentally isolating variables easier than in other regenerative systems.

1.1. Development of the Cornea and Lens

The vertebrate eye is composed of three primary tissues: cornea, lens, and retina, each playing a critical role in sight (Fig. 1). The cornea serves to protect the deeper tissues of the eye from the external environment, but is also a critical focuser of light. The amount of light that enters the eye after passing through the cornea is regulated by the iris, which can constrict or dilate to adjust the diameter of the pupil. Light that enters through the pupil is then precisely focused by the lens of the eye. The lens is able to accommodate, or change its shape, to adjust the focus of light based on its attachment to surrounding ciliary muscles. Finally, light is focused onto the neural retina where photoreceptors are activated and send signals to the central nervous system to be processed into an image.

During embryonic development, the first significant morphological evidence of eye development occurs during the formation of the optic vesicle from the evaginating diencephalon as it approaches the overlying surface ectoderm (Graw, 2010). Interactions between the optic vesicle and the surface ectoderm lead to the induction of the lens (Fig. 1.2 A). This induction is regulated by a host of transcriptional factors, of which Pax6 is a central regulator (Ashery-Padan and Gruss, 2001). The surface ectoderm (presumptive
lens ectoderm) thickens to form the lens placode, marking the earliest morphological evidence of the emerging lens (Fig. 1.2 B). In typical vertebrate development, the lens placode then invaginates to form the lens cup, or lens pit (Fig. 1.2 C). As the lens cup closes, it detaches from the overlying surface ectoderm to form a lens vesicle (Fig. 1.2 D). It is important to note that this differs slightly in *Xenopus*, as lens formation does not involve invagination of the lens placode. As the lens develops, cells of the posterior portion of the lens vesicle elongate and differentiate to form primary fiber cells that fill the vesicle from the posterior portion of the lens, and the cells of the anterior portion remain epithelial (Fig. 1.2 E). Finally, cells divide and differentiate from the equator of the lens and move inwards, forming secondary fiber cells, a process which continues to occur as the lens grows (Fig. 1.2 F). Meanwhile, the invaginating optic vesicle will continue to become the optic cup which will differentiate into the retina. The inner layer of the optic vesicle differentiates into the amacrine cells, ganglion cells, horizontal cells, bipolar cells, and photoreceptor cells of the neural retina, and the outer layer becomes the pigmented retina epithelium.

The mature cornea in both humans and *Xenopus* is a multi-layered tissue with 3 primary cellular layers (Fig. 1.2 I). The outermost layer is the cornea epithelium, and like many epithelial tissues it is maintained by a population of somatic stem cells that serve to replenish the cells of this stratified layer as they are lost. Deep to the epithelium (and the acellular Bowman’s layer) is the corneal stroma which is a thick layer of collagen fibers and distributed keratocyte cells. It is the precise patterning of the collagen fibers which gives the cornea much of its, strength, transparency and optical properties. Deep to the stroma (and Deschment’s membrane, another acellular layer) is a single layer of cells called the cornea endothelium, which regulates fluid and nutrient transport between the aqueous fluid and the rest of the cornea, as the cornea is an avascular tissue. Interestingly, each of these three basic cellular layers of the cornea originate from different embryonic tissues: the cornea epithelium from the surface ectoderm, cornea stroma from mesoderm, and cornea endothelium from neural crest cells (Harada et al., 2007).
To date, the vast majority of the work that has been done in *X. laevis* lens regeneration has focused on larvae ranging from Stage 46 – Stage 56 (Nieuwkoop and Faber, 1956), which are the stages that Freeman (1963) reported are capable of regenerating. However, during these stages the cornea is not yet fully mature, and the cornea epithelium is structurally more like that of a human fetus of 5-24 weeks of gestation (Eghrari et al., 2015; Hu et al., 2013). At developmental Stage 25 in *Xenopus* the cornea epithelium exists as a two cell layer thick tissue (Hu et al., 2013; Perry et al., 2013). It is from the basal cells of this epidermal layer that the lens is induced (Freeman, 1963). Around Stages 37-39, the cornea endothelium begins to form from invading mesenchymal cells (neural crest) that form a monolayer that lies over the optic cup and separates the lens and vitreous from the overlying cornea epithelium (Hu et al., 2013). By Stages 43-45, the cornea epithelium and cornea endothelium are established and are connected by a small central stalk that is surrounded by presumptive keratocytes (Hu et al., 2013). This central point of connection (stroma-attracting center; Hu et al., 2013) appears to be at the center of stromal development between the cornea epithelium and endothelium as development continues through Stages 55-60, when more typical stroma exists between the two cornea layers. By the time the animal has reached post-metamorphic adult hood, all 5 layers of the cornea (epithelium, Bowman’s layer, corneal stroma, Descemet’s membrane, and endothelium) are observable, and the mature cornea of the frog is structurally similar to that of humans (Hu et al., 2013).

### 1.2. Lens Regeneration

Several vertebrates are capable of regenerating a lens, however the source tissue for these regenerative processes differs between different systems. As a result there are at least three reported mechanisms to restore lost lens tissue, which will be discussed below. The first is the replacement of part of the lens by residual stem cells of the original lens (i.e., cells of the lens epithelium). A second mechanism is to regenerate the lens from dorsal iris tissue, which is derived from a different embryologic source than the original lens (neuroepithelium vs. surface ectoderm, respectively). Finally, cornea epithelium is also competent to regenerate a lens and is also derived from the surface ectoderm from which the embryonic lens is formed.
Mammals, such as mice and rabbits are able to regenerate the lens of the eye (Call et al., 2004; Gwon, 2006; Gwon et al., 1990). In these systems lenses reform from residual lens epithelial stem cells left behind in the lens capsule. In fact, this form of lens regeneration also occurs in macaques, and has recently been described experimentally for the first time in humans, showing great promise for treating cataracts in children (Lin et al., 2016). However, there are other forms of lens regeneration that occur in frogs, newts, and one fish where the lens is regenerated de novo, that is to say that no residual lens tissue is left behind and so the new lens regenerates from another source. In “Wolffian lens regeneration” in newts, named after Wolff (1895), the de novo regeneration of the lens occurs from the iris. This form of regeneration is considered an example of transdifferentiation, as the pigmented epithelial cells of the iris that serve as the source for the new lens must observably dedifferentiate (and lose their pigment) before differentiating towards the lens fate. In most instances of Wolffian regeneration in newts, it is the dorsal iris specifically that is competent to regenerate a lens, and not the ventral iris (Henry and Tsonis, 2010; Tsonis et al., 2004). More recent work has shown that axolotls have greater regenerative plasticity of the iris, as both dorsal and ventral iris are capable of regenerating a new lens under certain conditions (Suetsugu-Maki et al., 2012). In addition to several species of newt, one fish in the Cobitidae family of true loaches, *Misgurnus anguillicaudatus*, is also capable of regenerating a lens from the iris (Sato, 1961), although studies in this organism have been very limited. Finally, the cornea epithelium is also capable of regenerating a lens in the salamander *Hynobius unnangso* (Ikeda, 1936) frogs of the genus, *Xenopus* (Filoni et al., 2006; Freeman, 1963; Henry and Elkins, 2001).

1.3. Cornea-Lens Regeneration in *Xenopus*

In 1960, Jane Overton and Gary Freeman first reported on the ability of the frog, *Xenopus laevis*, to regenerate a lens. Initially, the process was described as another example of Wolffian iris-derived regeneration, although in subsequent reports this was corrected, as they discovered that the lens is actually derived from the cornea (Freeman and Overton, 1961, 1962). This was later confirmed as both iris and retina tissue were ruled out as the source of the regenerating lens (Bosco et al., 1981). Freeman (1963) later
published a detailed characterization of the process, dividing the process into five stages that are still used by the field today. The first visible change to the cornea occurs when cells of the basal (or inner) layer of the cornea epithelium change their shape from squamous to cuboidal, and the number of nucleoli per nucleus reduces (Stage 1 – Day 1). Next, an aggregate of cells forms in the basal layer of the cornea epithelium (Stage 2 – Day 2). The cells of the aggregate then form a vesicle, which will ultimately detach from the cornea epithelium (Stage 3 – Day 4 & 5). By the sixth day, these lenses are synthesizing lens specific proteins, as primary lens fibers emerge (Stage 4). After 10 days, secondary lens fibers emerge and a lens with normal histological morphology now exists, although it will continue to increase its size (Stage 5). Another important conclusion from this seminal work was that between developmental stage 46 to stage 56 (Nieuwkoop and Faber, 1956), above 50% of larvae are able to regenerate lenses in vivo. However, as animals approach the completion of metamorphosis (Stage 66), this ability to regenerate a lens is lost (Freeman, 1963). To date most of the work in *Xenopus* lens regeneration has been carried out in *X. laevis*, although other related species such as *X. tropicalis* and *X. borealis*, are also capable of regenerating lenses (Filoni et al., 2006; Henry and Elkins, 2001).

The cornea to lens transition is initiated when the cornea epithelium is exposed to the neural retina upon perforation of the cornea endothelium and complete removal of the lens (Bosco et al., 1979; Filoni et al., 1982; Reeve and Wild, 1978, 1981). Disruption of these two barriers that separate the cornea epithelium from the neural retina results in the initiation of lens regeneration. In fact, lens regeneration can be inhibited simply by placing a mechanical barrier such as a Millipore filter disk into a lentectomized eye because it prevents the inducing neural factors from reaching the cornea epithelium (Cioni et al., 1982). Additionally, while isolated corneas ectopically transplanted into tail tissue (in the absence of the retina) are not capable of regenerating lenses (Waggoner, 1973), lenses are capable of regenerating from the cornea in ectopic eyes (including retina tissue) that have been transplanted into larval tails (Reeve and Wild, 1977). Again, these cut-and-paste experiments demonstrate that the signals that initiate this regenerative phenomenon are produced by the neural retina. Based on this observation, an *ex vivo*
culture system was developed where the cornea epithelium is tucked tightly into the eyecups of excised eyes that have been lentectomized and culture them for seven days to regenerate lenses in culture (Fukui and Henry, 2011; Hamilton et al., 2016; Thomas and Henry, 2014). This technique has yielded a great deal of knowledge, and is ideal for testing small molecular modulators of cellular signaling, something that can be expensive and have numerous off target effects when used to treat whole animals. It has also been reported that lens regeneration will initiate in isolated larval cornea epithelium that is cultured in retina-conditioned medium (Bosco et al., 1997a).

In the past twenty years, efforts in the field have shifted to understanding the molecular mechanisms involved in cornea-lens regeneration and how it relates to lens development. One of the early investigations into particular genes in the lens regenerative process, examined lens specific crystallins αA, βB1, and γ (Mizuno et al., 1999a). This study found that there were differences between embryonic lens development and regeneration in regards to the timing of when these genes are expressed, with γ-crystallin expressing later than what occurs during lens development. However, other work demonstrated that the timing and patterns of expression of transcription factors like pax6 and prox1 in Xenopus cornea during lens regeneration is very similar to what is observed during embryonic lens development (Mizuno et al., 1999b; Schaefer et al., 1999). These works raise several interesting points. The first is that lens regeneration not only mimics embryonic lens development on a morphological level, but on a molecular level as well. However, there can be subtle differences in the timing and expression of individual genes, so one cannot presume to understand the role of any molecule or signaling pathway based on what is known from one system or the other. The work of Schaefer et al. (1999) and Mizuno et. al (1999b) also shows that despite being in different organisms and being derived from different tissues, lens regeneration may have fundamental mechanisms that are necessary to form a lens, regardless of the specific molecular strategies employed. Mizuno and colleagues (1999b) compared the expression of pax6 and prox1 cornea-derived lens regeneration in Xenopus to that of iris-derived lens regeneration in newt (Cynops). In both systems it was shown that pax6
expresses first and more broadly, and then *prox1* expresses specifically in the lens forming region (Mizuno et al., 1999b).

A breakthrough in the field occurred when Jonathan Henry created a subtracted cDNA library in order to identify genes upregulated during early (first four days) cornea-lens regeneration. (Henry et al., 2002). This library identified a large array of candidate genes involved in the early events of regeneration, including numerous factors involved in several common cell signaling pathways: Fibroblast growth factor (FGF), Retinoic acid (RA), int/Wingless (Wnt), Transforming growth factor beta (TGF-β), Hedgehog, as well as others (Henry et al., 2002; Malloch et al., 2009). This candidate screen also helped identify genes involved in other aspects of lens regeneration, such as the role of the matrix metallopeptidase *mmp9* during cornea wounding (Carinato et al., 2000). As a result of this screen, many of these cell signaling pathways have been investigated (Henry et al., 2013). FGF signaling has been shown to be both necessary and sufficient for cornea cells to differentiate into lens cells (Arresta et al., 2005; Bosco et al., 1997b; Fukui and Henry, 2011). This appears to be similar to observations made in Wolffian regeneration that FGF is both necessary and sufficient for lens regeneration to occur (Del Rio-Tsonis et al., 1998; Hayashi et al., 2002; Hayashi et al., 2004). As FGF signaling has a well-established role in establishing lens-forming competence, as well as in lens fiber formation (Robinson, 2006), it appears that FGF signaling may be a fundamentally important pathway to forming a lens regardless of the specific tissue of origin (i.e. cornea vs. iris). However, not every signaling pathway has shown a conserved role between Wolffian lens regeneration and cornea-lens regeneration in *Xenopus*. RA signaling through CYP26 needs to be reduced in the cornea in order for regeneration to occur (Thomas and Henry, 2014), whereas in Wolffian lens regeneration RA signaling is necessary for lens regeneration to occur (Tsonis et al., 2000; Tsonis et al., 2002). This highlights the importance of studying lens regenerative phenomena in a variety of systems, so that the field can ascertain what signaling mechanisms are inherently important for lens formation, and what pathways may be more dependent on the specific organism or tissue that is being studied. The Bone Morphogenetic Protein (BMP) signaling pathway has also been investigated and has been shown to be necessary for lens
regeneration to occur (Day and Beck, 2011). In this same work, Caroline Beck published an independent list of genes upregulated during lens regeneration using microarray analysis of corneas that had been regenerating for 3 days (Day and Beck, 2011). For the second time, various members of the Wnt signaling pathway were shown to be expressed during cornea lens regeneration, corroborating the original implication of Wnt signaling involvement during early lens regeneration and making it a prime suspect for further study (Day and Beck, 2011; Malloch et al., 2009).

1.4. Wnt Signaling

Wnt signaling occurs via different signaling pathways: the canonical Wnt/β-catenin pathway and the non-canonical pathways, such as the Planar Cell Polarity (PCP) pathway and the Wnt/calcium pathway. The canonical Wnt/β-catenin pathway is centered on the regulation of β-catenin. In the absence of Wnt ligand, the β-catenin degradation complex, comprised of Glycogen synthase kinase 3 (GSK3), Casein kinase 1 (CK1), and the scaffolding protein Axin, come together to phosphorylate β-catenin, leading to its degradation via the proteasome. However, when a Wnt ligand binds to the Frizzled (FZ)/Low density lipoprotein receptor-related protein (LRP) co-receptor, signaling is activated. This leads to the recruitment of Axin to the receptor, through the protein Dishevelled (DVL). This recruitment of Axin to the membrane inhibits the ability of the degradation complex to phosphorylate β-catenin, allowing it to accumulate in the nucleus. Once in the nucleus, it is able activate transcription through T cell factor/Lymphoid enhancer factor (TCF/LEF) complexes (Logan and Nusse, 2004; Clevers, 2006; MacDonald et al., 2009).

Wnt signaling can also activate two non-canonical pathways. In the PCP pathway, FZ and DVL remain involved; however, upon ligand binding, DVL recruitment results in the activation of either Rho or Rac. These small GTPases lead to either cytoskeletal changes through Rho-associated protein kinase (ROCK) or gene transcription through the Jun N-terminal kinase (JNK) cascade (Katoh 2005; Jones and Chen, 2007). In the Wnt/calcium pathway, activation by the ligand leads to an increase in intracellular calcium. This results in an increase in the activation of the calcium binding proteins,
Calcium calmodulin-dependent protein kinase II (CamKII) and Protein kinase C (PKC), ultimately feeding into either the Nuclear factor of activated T-cells (NFAT) or PKC pathways (Kohn and Moon, 2005).

While the vast majority of work in regards to the role of Wnt signaling in vertebrate lens development/regeneration has focused on the canonical pathway, the PCP pathway is emerging as an important signaling pathway for lens morphogenesis (Sugiyama et al., 2011). In Looptail (Lp) mice, which contain mutations in key PCP genes, lenses initially develop normally with proper differentiation of the lens epithelial and fiber cells; however, lenses have irregular flatter shapes and misaligned fiber cells (Sugiyama et al., 2010). More recently, in a microarray analysis of genes expressed during X. laevis lens regeneration, Day and Beck (2011) found the Wnt/PCP pathway was represented. The authors point out that this was due primarily to an overrepresentation of a single Frizzled-7 receptor, but it would certainly make sense that PCP is playing a role during lens regeneration, possibly organizing fiber cells in later stages. In regards to the other non-canonical Wnt pathway, the Wnt/calcium pathway, virtually nothing is known about its involvement during lens development or regeneration.

1.5. Wnt/β-catenin Signaling in Lens Development

Most of the emphasis on the role of Wnt signaling in the context of lens development has focused on the canonical Wnt/β-catenin signaling pathway. Wnt/β-catenin signaling is involved in many processes during eye organogenesis, including the lens (Fuhrmann, 2008). In the early stages of vertebrate lens development canonical Wnt signaling in the presumptive lens ectoderm needs to be inhibited in order for lens formation to occur (Kreslova et al., 2007; Machon et al., 2010; Miller et al., 2006; Smith et al., 2005). This comes from a collection of experiments that demonstrate that β-catenin loss-of-function does not affect the ability of the surface ectoderm to form a lentoid (Kreslova et al., 2007; Smith et al., 2005). However, if canonical Wnt signaling is held in an active state in the surface ectoderm, then lens formation is greatly inhibited (Miller et al., 2006; Smith et al., 2005). Interestingly, inactivation of β-catenin function
in murine periocular and nasal ectoderm one can induce lentoid formation in these tissues that normally do not give rise to a lens (Kreslova et al., 2007; Smith et al., 2005). Thus, the role of Wnt/β-catenin signaling in the surface ectoderm during embryonic lens development appears to be restricting the field of ectoderm that is normally competent to respond to the inductive signals being produced by the underlying diencephalon. However, as the lens itself begins to form, the canonical Wnt signaling becomes necessary for proper development of the lens (Fuhrmann, 2008). During later stages of lens development, canonical Wnt signaling becomes required for proper differentiation of the lens epithelium (Stump et al., 2003) and lens fiber cells (Chen et al., 2006). The expression patterns of various components and regulators of Wnt signaling in the lens have been well characterized and include many of the wnt ligands, frizzled receptors, dickkopfs, and secreted frizzled related proteins (Ang et al., 2004; Chen et al., 2004), the latter two being antagonists of the Wnt signaling pathway.

1.6. Wnt/β-catenin Signaling in Wolffian Lens Regeneration

While much is known about the role that Wnt signaling plays during initial lens development, very little is known about the involvement of this signaling pathway during lens regeneration. The sole functional analysis of Wnt signaling during the process of lens regeneration was carried out by Hayashi et al. (2006) in the newt. Using RT-PCR of iris tissue collected at various time points during regeneration, Hayashi and colleagues observed the expression of wnt2b, wnt5a, fz2, and fz4. Two of these genes, wnt2b and fz4, showed elevated expression levels in late lens regeneration, specifically in the dorsal iris (day 8 and day 12, respectively). Lens regeneration rates were dramatically reduced when cultured in the presence of FGF2 and the Wnt signaling antagonists DKK1 and SFRP1 (Fig. 3). When WNT3A (a canonical Wnt) was added with FGF2 in culture medium, larger lenses regenerated from the dorsal iris and limited lens regeneration was even observed from the ventral iris, which is not normally capable of regeneration. The findings of this study suggest that canonical Wnt signaling is necessary for lens regeneration of the dorsal iris and is sufficient for lens regeneration to occur in ventral irides that have already undergone the early steps of lens regeneration triggered by FGF2. While a differential screen of genes expressed during the initiation of depigmentation and
proliferation of Wolffian lens regeneration did not produce any ESTs of Wnt pathway members (Maki et al., 2010) the authors point out this could have been due to the particular stage of the irides collected or may have been a caveat of the cloning methods. Additionally, it should also be mentioned that the Tsonis lab reported unsuccessful attempts to perturb lens regeneration via small molecule activation or inhibition of Wnt signaling when iris explants were implanted into lentectomized newt eyes (unpublished data; see Grogg et al., 2006). One possible explanation for the different results could simply be a difference in the in vivo newt culture system vs. in vitro culturing of irides in the presence of FGF2.

1.7. Wnt/β-catenin Signaling in Cornea-Lens Regeneration

Studies examining the role of Wnt signaling in cornea-lens regeneration in Xenopus have been very limited. However, screens have been carried out to characterize gene expression during the process of cornea-lens regeneration. Henry et al. (2002) created a subtracted cDNA library of genes expressed during the process of lens regeneration in X. laevis using control corneas and corneas in the first four days of lens regeneration. From this study several ESTs were identified as members of the Wnt signaling pathway: the ligand Wnt7b, as well as the Wnt antagonists secreted frizzled-related proteins 3 and 5 (sfrp3 and sfrp5; Malloch et al. 2009), suggesting the involvement of Wnt signaling during this process. In a more recent study using a X. laevis Affymetrix GeneChip, Day and Beck (2011) identified the differential expression of numerous components of the Wnt signaling pathway during cornea-lens regeneration. Further qRT-PCR analysis of two of these genes, Fzd7 and Wnt7a, revealed significant changes (upregulation and downregulation, respectively) in expression during regeneration when compared to sham operated control corneas. Interestingly, sfrp2 was also shown to be upregulated during early regeneration (Day and Beck, 2011). Taken together, the presence of both positive and negative regulators of Wnt signaling from two independent screens to identify genes upregulated during the early events of lens regeneration raises several key questions that will be addressed in Chapter 2 about the role of Wnt signaling during Xenopus lens regeneration and whether it is necessary as has
been described in Wolffian lens regeneration, or needs to be suppressed as it does during embryonic lens development.

1.8. Cornea Stem Cells and Regeneration

Over the last several years a new model has emerged for the process of lens regeneration in X. laevis. For a long time regeneration in Xenopus has been considered transdifferentiation, a process in which differentiated cells of the larval cornea epithelium dedifferentiate into earlier progenitor cells and then differentiate towards the lens fate. This was likely influenced by the longer-studied Wolffian lens regeneration system in the newt that has an observable dedifferentiation step, where pigment in the PECs (pigmented epithelial cells) of the iris is lost (Henry and Tsonis, 2010; Tsonis et al., 2004). Thus, CLT (cornea-lens transdifferentiation) became the conventional way to refer to Xenopus lens regeneration in the field, despite the lack of direct evidence of dedifferentiation during the process. This terminology is now being challenged as a result of work in the field that provided evidence that the larval cornea epithelium may contain stem cells that possess an oligopotent ability to differentiate into cornea epithelial cells or lens cells (Perry et al., 2013). In this work, it was demonstrated that numerous pluripotency genes are transcriptionally expressed in the cornea epithelium, and that stem cell proteins like SOX2 and P63 can be observed in the cornea using immunofluorescence. In fact, the entire basal layer of the larval cornea epithelium express the transcription factor p63 (Perry et al., 2013), which is one of the best putative markers for cornea epithelial stem cells (O'Sullivan and Clynes, 2007). As it appears to be the basal layer of the cornea epithelium from which new lenses regenerate (Freeman, 1963), this is consistent with a model where the basal layer of the cornea epithelium is stem cell like in nature and is serving as the source of the new lens.

Like most epithelial tissues, the cornea epithelium has a protective function as it serves as a barrier between the external environment and the underlying eye tissues, and dead squamous cells of the most superficial layer are constantly being lost. Therefore, a population of somatic stem cells must exist in the cornea epithelium to replenish this tissue. Based on the observation of pigment migrating into the cornea from the limbal
region of the eye in guinea-pigs, it was initially proposed that the palisades of Vogt are the niches where the cornea stem cells reside (Davanger and Evensen, 1971). These undulating crypts in the basal layer of the cornea epithelium lie at the junction where the cornea meets the sclera of the eye. Like many somatic stem cells, basal cells of the limbal cornea epithelium are slow cycling under homeostatic conditions, and therefore are referred to as label retaining cells (LRCs) as they retain DNA labels over long periods of time (Cotsarelis et al., 1989).

In Freeman’s (1963) publication, the lens regenerative capacity of the post-metamorphic (mature) cornea was tested, and it was found that after metamorphosis the cornea is no longer able to regenerate a lens in vivo. However, a later study demonstrated that the cornea epithelium of the post-metamorphic cornea is still competent to respond to regenerative signals if it is excised and put into larval retina (Filoni et al., 1997), and so it appears that the loss of regenerative capacity observed by Freeman in the post-metamorphic frog is due to the inductive signals secreted by the retina being blocked by the more rapid healing of the cornea endothelium during metamorphosis. This was an exciting finding that so far has largely been ignored by the field, likely due to the ease of working with larvae which take only weeks to develop to the appropriate stages for experimental analysis, as opposed to post-metamorphic froglets, which take much longer to develop (Nieuwkoop and Faber, 1956). The reason this is an interesting result is because it is actually the mature post-metamorphic cornea that most resembles our own structurally (Hu et al., 2013), while the 2-cell layer thick larval cornea epithelium is more equivalent to an immature human cornea epithelium at 24 weeks of development (Secker and Daniels, 2008). As access to mammalian fetal cornea epithelium is clinically challenging, regenerative therapies designed off of our understanding of larval lens regeneration may not necessarily apply to the mature cornea epithelium of our own eyes. Therefore it is important that we gain an understanding of the regenerative mechanisms of the cornea epithelium in the mature post-metamorphic cornea that Filoni et al. (1997) described. In light of our new model that suggests the entire basal layer of the larval cornea epithelium is stem cell-like, and that it is from these stem cells and/or their transit amplifying progeny that lenses regenerate, one immediately begins to question whether
this is also true in the post-metamorphic cornea. Chapter 3 addresses this question, by analyzing the regenerative potential of the limbal region of post-metamorphic cornea, where label retaining cells have been identified to reside in the basal periphery of the cornea, and compares it to that of central cornea which is devoid of label retaining cells in the basal layer.

In preparation for future studies to examine cornea stem cells and their role in cornea epithelial maintenance and regeneration, we created a new technology that can be used to examine cellular behavior and regenerative phenomena in larval *Xenopus* by allowing for continuous live cell imaging for up to 48 hours (Chapter 4). Before its creation, *in vivo* imaging of development in *Xenopus* was limited to embryos before they reach the swimming stages, however, it is during the swimming stages that most of the studied regenerative processes in *Xenopus* occur. Taking advantage of an observation made in the laboratory that tadpoles recover from anesthesia with higher success rates when rocked, we developed a technology that uses flowing anesthetic to keep tadpoles alive and immobilized for up to 48 hours, through all stages of development and metamorphosis. When used in conjunction with transgenic reporter systems it allows for the first time, the ability to use continuous *in vivo* imaging to study development, tissue homeostasis, wound healing, or regeneration of the cornea epithelium, revealing many of the dynamic processes that are lost in more static data.

Finally, this work will conclude with a summarization of the main conclusions of this work and the implications on the field of lens regeneration moving forward (Chapter 5). Additionally, we propose several new future directions based off of the new questions raised by this work.
1.9. Figures

Figure 1.1. Sagittal section through vertebrate eye showing basic anatomy. Three forms of lens regeneration occur in vertebrates: one where lens regeneration occurs from residual lens cells (e.g. rabbit), one where lens regeneration occurs from the iris (e.g. newt), and one where lens regeneration occurs from the most superficial layer of the cornea, the cornea epithelium (e.g. frog). Image is credited to the National Eye Institute, National Institutes of Health (www.flickr.com/photos/nationaleyeinstitute/7544655864).
Figure 1.2. Development of the vertebrate cornea and lens. (A) Surface ectoderm which is the embryonic source of both the cornea epithelium and lens (green), comes into close proximity with the diencephalon, inducing formation of the optic vesicle. (B) Signals from the optic vesicle, which will become the retina, induce thickening of the surface ectoderm to form the lens placode. (C) Invagination of the lens placode to form the lens vesicle. Note: lens development in the frog does not involve invagination of the lens placode. (D) Lens vesicle separates from presumptive cornea epithelium. (E) Mesenchymal cells begin to migrate in to establish cornea endothelium and stroma, and primary lens fiber differentiation occurs from the posterior lens. (F) Secondary lens fiber differentiation. (G) Mesenchymal cells continue to establish presumptive stroma and cornea epithelium. Final lens structure is complete but will continue to grow. (H) Structure of the mature cornea is now established, but epithelium is still only 1-2 cell layers thick. (I) Structure of the mature cornea, complete with stratified squamous epithelium. Adapted from Swamynathan (2013).
1.10. References


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CHAPTER 2. LENS REGENERATION FROM THE CORNEA REQUIRES SUPPRESSION OF WNT/BETA-CATENIN SIGNALING

2.1. Introduction

While examples of regeneration are widespread among the invertebrate population, few vertebrates possess the ability to regenerate complete organs lost to either damage or disease (Brockes and Kumar, 2008). In vertebrates, one organ capable of complete regeneration is the lens of the eye. However, the capacity to replace this structure is restricted to certain species of frogs, one fish, and some newts and salamanders (Henry et al., 2013; Henry and Tsonis, 2010). Newts are able to regenerate a lens via transdifferentiation of the dorsal pigmented iris epithelium, in a process referred to as Wolffian regeneration (see Henry and Tsonis, 2010). The frog *Xenopus laevis* is also capable of regenerating a lens, but instead of the iris, the lens is regenerated from the basal layer of the cornea epithelium (Freeman, 1963). This process is initiated when signals from the neural retina are able to reach the cornea epithelium, upon perforation of the cornea endothelium and removal of the lens (Freeman, 1963; Reeve and Wild, 1978). While cornea-lens regeneration has traditionally been described as transdifferentiation of the cornea, a different model has emerged suggesting that the regenerated lens may instead be derived from a population of basal stem cells or transit amplifying cells in the cornea which possess an oligopotent capacity to give rise to a new lens (Perry et al., 2013). However, the cellular signaling events needed to initiate lens regeneration in these cornea cells is not understood.

In recent years, several signaling pathways have been shown to be important for cornea-lens regeneration such as the Fibroblast Growth Factor (FGF, Bosco et al., 1997; Fukui and Henry, 2011), Retinoic acid (Thomas and Henry, 2014), and Bone Morphogenetic Protein (BMP) signaling pathways (Day and Beck, 2011). The involvement of multiple pathways suggests that this process is regulated by a complex signaling network, and it is possible that other pathways may also be involved. One

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pathway that has been implicated as playing a role in cornea-lens regeneration in two independent screens for genes expressed during early regeneration is the Wnt signaling pathway (Day and Beck, 2011; Henry et al., 2013; Malloch et al., 2009). In the canonical Wnt/β-catenin signaling pathway, wnt ligands bind to corresponding frizzled receptors and associated co-receptors in order to inhibit the downstream β-catenin degradation complex (reviewed in Logan and Nusse, 2004; MacDonald et al., 2009). Inhibition of this degradation complex allows β-catenin to accumulate and translocate to the nucleus where it activates transcription through T-cell factor/lymphoid enhancer factor (TCF/LEF). In the absence of an appropriate wnt signal, the degradation complex remains in an active state and begins to degrade β-catenin. The role of Wnt signaling has been well studied during the development of the eye, and is important for the initial formation of the vertebrate lens (Fuhrmann, 2008; Graw, 2010). During vertebrate lens development, Wnt/β-catenin signaling needs to be suppressed in the ectoderm overlying the eye in order to initiate a lens placode (Kreslova et al., 2007; Miller et al., 2006; Smith et al., 2005). Since the cornea is derived from this surface ectoderm, it could be that the larval cornea retains some of these same signaling mechanisms deployed during development, as there appear to be many similarities between lens regeneration and initial lens development (Henry, 2003; Henry and Mittleman, 1995).

However, from observations made during Wolffian lens regeneration in the newt, active Wnt/β-catenin signaling is necessary for lens regeneration to occur (Hayashi et al., 2006). While Wolffian lens regeneration and cornea-lens regeneration occur in different organisms and from different tissues, it appears that some signaling (e.g. FGF signaling) is somewhat conserved between these two systems (Fukui and Henry, 2011; Hayashi et al., 2004). It is possible that the role of Wnt/β-catenin signaling in cornea-lens regeneration may also be conserved. On the other hand, recent work from our lab has shown that while Retinoic acid signaling appears to be necessary for Wolffian lens regeneration, it must be reduced during lens regeneration in *Xenopus*, which differs from observations in newt lens regeneration (Thomas and Henry, 2014). This raises the following questions: What is the role of Wnt/β-catenin signaling in cornea-lens regeneration, and how does it compare to that of Wolffian lens regeneration?
In this study we present evidence that Wnt/β-catenin signaling needs to be suppressed in order for cornea-lens regeneration to occur. We identified a wide assortment of wnt ligands and frizzled receptors that are expressed within the cornea epithelium and compared the expression of these genes to lens-incompetent flank epithelium. Using axin2 expression as a readout for active Wnt/β-catenin signaling, we demonstrated that there is a natural suppression of Wnt/β-catenin signaling in the cornea epithelium that occurs 24 hours into the course of lens regeneration and recovers by 48 hours. Additionally, we functionally tested the necessity of this inhibition by holding the cornea in a state of active Wnt/β-catenin signaling using 6-bromoindirubin-3’-oxime (BIO) and 1-azakenpaullone, and we found that the percentage of successfully regenerating cases significantly decreased in the presence of these compounds. Finally, we inhibited Wnt/β-catenin signaling using IWR-1, recombinant human DKK1, and transgenic expression of Xenopus DKK1 and found no significant effect on lens regeneration. Together these data suggest a model where Wnt/β-catenin signaling is active in the cornea epithelium and needs to be suppressed during early lens regeneration in order for a new lens to form. This finding differs from what has been described in the newt (Hayashi et al., 2006), but resembles the role of Wnt signaling during the initial formation of the lens placode from the surface ectoderm during early embryogenesis (Kreslova et al., 2007; Miller et al., 2006; Smith et al., 2005).

2.2. Materials and Methods

2.2.1. Animals

Xenopus laevis adults were acquired from Nasco (Fort Atkinson, WI). Larvae were generated and reared as previously described (Henry and Grainger, 1987; Schaefer et al., 1999) and were developmentally staged according to Nieuwkoop and Faber (1956). All lentectomies were performed as described in Henry and Mittleman (1995) on stage 48-53 animals in a 1:2000 dilution of the anesthetic MS 222 (ethyl 3-aminobenzoate methanesulfonate, Sigma, St. Louis, MO) in 1/20x normal amphibian media (NAM; Slack, 1984). The animal care and use in this work was approved and overseen by the University of Illinois Institutional Animal Care and Use Committee and monitored by the staff of the Division of Animal Resources at the University of Illinois.
2.2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Cornea epithelial tissue and flank epithelial tissue was collected from multiple st. 48-53 X. laevis. Like tissues were pooled together in microcentrifuge tubes, flash frozen in a dry ice/ethanol bath, homogenized in TRIzol (Invitrogen, Carlsbad, CA), and RNA was purified using Direct-zol RNA Miniprep (Zymo Research, Irvine, CA) according to the manufacturer’s protocol. The resulting RNA was treated with DNaseI (New England Biolabs, Ipswich, MA) to ensure removal of genomic contamination. cDNA was generated from purified cornea RNA and flank RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). PCR reactions were conducted using the newly synthesized cDNA as a template, along with the primers listed in Table 2.1. For this study, only Xenopus laevis wnt ligands and frizzled receptors from the National Center for Biotechnology Information’s Reference Sequence database were examined. Negative –RT (“−”, Fig. 2.1) controls were generated by conducting PCR on iScript reactions that did not contain reverse transcriptase (RT). As a positive control (“+”, Fig. 2.1), PCR was carried out on cDNA generated from pooled embryos ranging in stages from 11-39, when these transcripts are known to be present.

2.2.3. Ex vivo eye culture

Eyes were lentectomized and cultured in an ex vivo system (formerly referred to as in vitro culture), as described in Fukui and Henry (2011). In the ex vivo culture system, eyes are lentectomized and the cornea epithelium is tucked into the eyecup to ensure close proximity to the retina. Eyes are then removed and placed into culture medium, consisting of the following: 61% L-15 powder (Invitrogen, Carlsbad, CA); 100 U/ml of penicillin and 100 ug/ml of streptomycin (Mediatech, Manassas, VA); 10% fetal bovine serum (Invitrogen, Carlsbad, CA); 2.5 ug/ml Amphotericin B (Sigma, St. Louis, IL); and 4 ug/ml Marbofloxacin (Sigma, St. Louis, IL). 1-azakenpaullone (A.G. Scientific, San Diego, CA) was diluted to a final concentration of 10 µM from a 10 mM stock in dimethyl sulfoxide (DMSO). BIO (6-bromoindirubin-3’-oxime, Tocris Bioscience, Bristol, UK) was diluted to a final concentration of 1 µM from a 1 mM stock in DMSO. IWR-1 (Inhibitor of Wnt Response-1; Sigma, St. Louis, IL) was diluted to a final concentration of 10 µM from a 10 mM stock in DMSO. Recombinant human DKK1
(rhDKK1; R&D Systems, Minneapolis, MN) was diluted to a final concentration of either 200 ng/ml or 500 ng/ml from a 100 μg/ml stock in PBS + 0.1% bovine serum albumin (BSA). An equal volume of DMSO was added to the control medium for experiments using IWR-1, 1-azakenpaullone, or BIO. For the rhDKK1 controls, an equal volume of Phosphate-buffered saline (PBS) + 0.1% BSA was added to the culture media. Culture media were changed daily. After 7 days of culture (sufficient time for a lentoid to regenerate, see Fukui and Henry, 2011; Thomas and Henry, 2014), eyes were fixed for 3 hours in 3.7% formaldehyde, embedded in Paraplast Plus (McCormick Scientific, Richmond, IL) and serially sectioned at 8 μm (as described by Humason, 1972). To identify regenerated lenses, immunohistochemistry was carried out on serial sections using a polyclonal anti-lens antibody (Henry and Grainger, 1987). Positive cases of regeneration were scored based on the presence of morphologically distinct lentoids that were positively stained by the anti-lens antibody.

### 2.2.4. HGEM-DKK1 Transgenesis

All F0 HGEM-DKK1 transgenic tadpoles were generated using sperm nuclear injection, following the protocol of Smith et al. (2006). The HGEM-DKK1 transgene (Fig. 2.5A) was kindly provided by Dr. Jonathan Slack (University of Minnesota) and has been successfully used in *Xenopus* tail regeneration studies (Lin and Slack, 2008). Only transgenic animals displaying robust GFP expression throughout the lenses of both eyes were used. Eyes were lentectomized and cultured in modified L-15 media in the *ex vivo* culture system, which allowed for individual transgenic animals to contribute one eye to the experimental group and one eye to the control group. The experimental groups received daily heat-shocks for 40 minutes at 34°C, while the control groups were maintained at room temperature (20-24°C). Eyes were then fixed, sectioned, and stained with an anti-lens antibody. Western blots were performed on HGEM-DKK1 tail tissue collected and heat-shocked in culture. Western blotting followed standard protocols (Henry et al., 2008), using the following primary antibodies: anti-β-tubulin (Sigma, T8328; expected ~50 kDa) and anti-β-catenin (Sigma, C2206; expected size 94 kDa).
2.2.5. Quantitative polymerase chain reaction (qPCR) Methods

For the drug validation experiments, eyes were removed from st. 48-53 tadpoles leaving the cornea epithelium attached to the underlying cornea endothelium via the central stalk. It is important to note that the lens and cornea endothelium are both undisturbed, so as not to induce regeneration. Eyes were then cultured in the appropriate drug or control medium (as described above) for 24 hours, at which point cornea epithelial tissue was collected and cDNA was generated as previously described. For the axin2 regenerating time course, eyes were lentectomized, removed from the animal, and cultured in the culture medium (no drugs present) for either 24, 48, 72, or 96 hours. Cornea epithelial tissue was then collected, pooled, and cDNA was generated for qPCR as previously described. Control corneas for the time course experiment are simply cornea epithelial tissue that is not regenerating and has not been wounded. All qPCR experiments were performed a minimum of three times, each with triplicate technical replicates. Reactions contained SYBR Green reagent kindly provided by Dr. Jie Chen (University of Illinois at Urbana-Champaign), 500 nM of both forward and reverse primers, and 10-25 ng of input cDNA. The following primers were used: actb (F: 5’-CGCCCGCATAGAAAGGAGAC-3’; R: 5’-AGCATCATCCCCAGCAAAGC-3’; Thomas and Henry, 2014), axin2 (F: 5’-TGCAGCCAGTATCAACGACAG-3’; R: 5’-CAAAGACACTTGTCCATTGGC-3’; Myers et al., 2014), and odc (F: 5’-GCCATTGTGAAGACTCTCTCCATTC-3’; R: 5’-TTGGGTGATTCCTTGCCAC-3’; Heasman et al., 2000). Changes in relative expression were determined using the comparative CT method (Schmittgen and Livak, 2008), and normalized to a reference gene, either ornithine decarboxylase or beta-actin.

2.2.6. Statistical Tests

Statistical significance of the percentage of cases that successfully regenerated was determined using Fisher’s exact test (Fisher, 1922), under the two-tailed condition. Statistical significance of qPCR experiments was determined using an unpaired t test. Statistical differences were considered significant if the p-values were less than 0.05. All error bars represent standard error.
2.3. Results

2.3.1. Expression of fzd receptors and wnt ligands in lens competent and incompetent tissue

To identify frizzled (fzd) receptors and wnt ligands that are expressed in the cornea, RT-PCR was performed on cornea epithelial tissue (Fig. 2.1). Of the nine frizzled receptors investigated, eight appear to be transcriptionally expressed at some level: fzd1, fzd2, fzd3, fzd4, fzd6, fzd7, fzd8, and fzd10. Only fzd5 was found to be absent in the larval cornea epithelium. Numerous wnts are also expressed in cornea epithelium: wnt2b.a, wnt3a, wnt4, wnt5a, wnt5b, wnt6, wnt7b, wnt10a, wnt11, and wnt11b. Of those examined, only wnt1, wnt8a, and wnt8b do not appear to be expressed in this larval tissue.

In order to better understand any differences in the expression of these genes in an epithelial tissue that is not competent to regenerate a lens, we reanalyzed the same genes in st. 48-53 flank epithelium, which is lens regeneration incompetent after stages 30/31 (Arresta et al., 2005). Flank expressed many of the same transcripts including: fzd1, fzd2, fzd3, fzd4, fzd6, fzd7, fzd8, fzd10, wnt2b.a, wnt3a, wnt4, wnt5a, wnt5b, wnt6, wnt7b, and wnt10a (Fig. 2.1). Others, including fzd5, wnt1, wnt8a, wnt8b, wnt11, and wnt11b were not expressed in flank. Only two of these genes were found to be different between flank and cornea epithelium: wnt11 and wnt11b, which are present only in the cornea epithelium.

2.3.2. Time Course of axin2 expression during lens regeneration

As the cornea epithelium expresses many of the appropriate signals and receptors to be participating in Wnt signaling, we wanted to quantitatively assess the activity of canonical Wnt/β-catenin signaling in the cornea over the first four days of regeneration. To do this, axin2 expression was examined using quantitative polymerase chain reaction (qPCR), as its expression is regulated by Wnt/β-catenin signaling and is commonly used as a readout for active canonical signaling (Jho et al., 2002; Myers et al., 2014). Larvae were lentectomized and allowed to regenerate for either 24, 48, 72, or 96 hours, at which point cornea epithelial tissue was isolated for qPCR analysis. Expression was normalized to beta-actin (actb) and regenerating expression levels were compared to the expression
level in control cornea epithelium that had not been wounded and was not regenerating. At 24 hours post-lentectomy there is a 46.6% reduction in axin2 expression, representing a statistically significant decrease \( (p=0.0006, \textbf{Fig.~2.2}) \). This is a particularly strong effect, considering that not all cells of the cornea epithelium are thought to respond to the retinal signals to initiate a new lens (Freeman, 1963). By 48 hours post-lentectomy, the levels of axin2 return to that of the control and are maintained at that level through 96 hours post-lentectomy (\textbf{Fig.~2.2}).

2.3.3. Activation of Wnt signaling significantly reduces successful lens regeneration

To test whether the suppression of Wnt/\( \beta \)-catenin signaling observed by qPCR was functionally significant, lentectomized eyes were treated with activators of Wnt signaling to see if holding the eyes in an active state of Wnt/\( \beta \)-catenin signaling would result in a failure to regenerate a lens. The Wnt signaling activators used were 1-azakenpaullone and BIO (6-bromoindirubin-3’:oxime), which both activate Wnt/\( \beta \)-catenin signaling by inhibiting Glycogen synthase kinase 3\( \beta \) (Gsk3\( \beta \); Kunick et al., 2004; Meijer et al., 2003). Gsk3\( \beta \) is an important member of the \( \beta \)-catenin degradation complex, responsible for the phosphorylation and subsequent degradation of \( \beta \)-catenin via the proteasome (see MacDonald et al., 2009). Thus, inhibition of Gsk3\( \beta \) prevents the degradation of \( \beta \)-catenin, permitting the activated Wnt/\( \beta \)-catenin signaling pathway. Eyes were lentectomized and cultured \textit{ex vivo}, in the presence of either 1-azakenpaullone or BIO for seven days. In the \textit{ex vivo} system, lentectomized eyes are removed and the cornea epithelium is tucked inside of the eyecup to ensure close proximity to the neural retina (Fukui and Henry, 2011; Thomas and Henry, 2014). Positive cases of regeneration are histologically scored based on the presence of morphologically distinct lentoids that are positively stained by an anti-lens antibody. A 10 \( \mu \)M treatment of 1-azakenpaullone reduced regenerative success from 94.4\% (34/36 eyes; control) to 26.5\% (9/34 eyes; treated). This difference represents a statistically significant decrease in lens regeneration \( (p<0.0001; \textbf{Fig.~2.3A, D-G}) \). Although not as dramatic as the effect seen with 1-azakenpaullone, a 1 \( \mu \)M treatment of BIO also resulted in a statistically significant difference \( (p=0.0031) \), decreasing regenerative success from 95.5\% (21/22 DMSO treated eyes) to 55.0\% (11/20 BIO treated eyes; \textbf{Fig.~2.3B, H-K}). To confirm that these
compounds were having the desired effect on Wnt/β-catenin signaling, the relative expression levels of *axin2* were measured using qPCR (Fig. 2.3C). Corneas treated for 24 hours in either 10 μM of 1-azakenpuallone or 1 μM of BIO showed a statistically significant increase in the relative expression levels of *axin2*, as expected (*p*=0.0226 and *p*<0.0001, respectively).

### 2.3.4. Inhibition of Wnt Signaling does not prevent lens regeneration

It has been shown that Wnt/β-catenin signaling is necessary for lens regeneration to occur in Wolffian lens regeneration (Hayashi et al., 2006), and it could be that perturbing Wnt signaling in either direction could affect lens regeneration, so we also assessed lens regeneration under Wnt signaling inhibition using three different approaches. First, we challenged regeneration in the presence of the small molecule Inhibitor of Wnt Response-1 (IWR-1). This molecule has been shown to be a potent inhibitor of Wnt/β-catenin signaling by stabilizing the β-catenin degradation complex (Lu et al., 2009). It has also been used to inhibit Wnt signaling in several *Xenopus* specific applications (Borday et al., 2012; Myers et al., 2014). Using the *ex vivo* eye culture system, lentectomized eyes were treated in 10 μM IWR-1 continuously for seven days and were then assessed for the presence of lenses. This concentration is sufficient to inhibit tail fin regeneration in zebrafish (Chen et al., 2009). Eyes cultured in the control medium successfully regenerated 77.8% (14/18 eyes) of the time, while the IWR-1 treated eyes regenerated at a very similar percentage of 76.9% (10/13 eyes; Fig. 2.4A, D-G). While these experiments showed no effect on the percentage of regenerative success (*p*=1.0000), this concentration of IWR-1 did successfully suppress Wnt signaling, as measured by *axin2* levels using qPCR on cornea tissue treated with 10 μM of IWR-1 (*p* < 0.0001; Fig. 2.4C)

Next, we treated eyes in the same *ex vivo* lens regeneration system in the presence of recombinant human Dickkopf1 (rhDKK1; Glinka et al., 1998) protein. DKK1 inhibits Wnt signaling by binding to the low density lipoprotein receptor-related proteins (Lrp), Lrp5/6, which serve as critical co-receptors in canonical Wnt signaling (Niehrs, 2006). At concentrations of 200 ng/ml, treated eyes (14/14; 100%) were still able to regenerate
as well as the control eyes (13/14; 92.9%, Fig. 2.4B, H-K). Increasing the concentration of rhDKK1 to 500 ng/ml gave the same result, with treated eyes (19/19; 100%) regenerating at percentages near the control eyes (17/19; 89.5%, Fig. 2.4B, L-O). Neither of these differences was statistically significant (p=1.0000 and p=0.4865, respectively), although both treated groups showed an increase in the percent of successful regenerates. Suppression of Wnt signaling using rhDKK1 was also confirmed using qPCR of axin2 expression (p=0.0081, Fig. 2.4C).

Finally, we transgenically expressed *Xenopus* DKK1 under the control of a heat shock inducible hsp70 promoter (Lin and Slack, 2008; Fig. 2.5A). This “Heat-shock Green-Eyed Monster” (HGEM) construct also contains a lens-specific γ-crystallin promoter driving the expression of GFP, providing an easy way to screen for transgenic tadpoles (Fig. 2.5B, C). HGEM-DKK1 F0 transgenic tadpoles had both lentectomized eyes removed and cultured *ex vivo* in modified L-15 medium, either in the control group (no heat-shock) or the experimental group (daily heat-shocks). Eyes were then fixed, sectioned, and stained with a polyclonal anti-lens antibody to identify regenerated lenses (Fig. 2.5D, G). Inhibition of Wnt/β-catenin signaling in the cornea did not significantly affect the percentage of successful regeneration (p=0.3706) between heat-shocked eyes (22/32; 68.8%, Fig. 2.5H) and control eyes (23/28; 82.1%, Fig. 2.5H). In order to confirm that DKK1 had the desired effect on Wnt/β-catenin signaling, Western blots of β-catenin (CTNNB1) were carried out on either heat-shocked or control tissue collected from the tails of HGEM-DKK1 tadpoles. As expected, levels of β-catenin were greatly diminished in the transgenic tail tissue expressing DKK1 after heat-shock (Fig. 2.5I).

2.4. Discussion

Wnt signaling is known to be an important regulator in the development of the vertebrate lens (Fuhrmann, 2008). Within the developing lens itself, active Wnt signaling is needed for proper differentiation of lens fiber cells and lens epithelial cells (Chen et al., 2006; Stump et al., 2003), as well as for the proper growth and orientation of lens fiber cells (Chen et al., 2008; Sugiyama et al., 2011). However, earlier in development, active Wnt signaling in the presumptive lens ectoderm prevents this tissue from giving rise to a
lens (Kreslova et al., 2007; Smith et al., 2005). This comes from observations that holding the surface ectoderm in a state of active Wnt signaling results in the loss of lens formation (Miller et al., 2006; Smith et al., 2005). Additionally, β-catenin loss-of-function has no effect on the ability of a lentoid to form and is actually sufficient to induce lentoid formation in murine nasal and periocular ectoderm (Kreslova et al., 2007; Smith et al., 2005).

Far less is known about the involvement of Wnt signaling during lens regeneration. The Tsonis lab challenged lens regeneration in the newt by treating iris explants with either an activator or inhibitor of Wnt signaling and then implanting the treated iris into lentectomized newt eyes, but no effect on lens regeneration was reported (unpublished data discussed in Grogg et al., 2006). However, Hayashi and colleagues (2006) were able to demonstrate that the addition of media conditioned with either Xenopus DKK1 or human sFRP1 (both inhibitors of Wnt signaling) to cultures of dorsal iridies results in the inability to regenerate a lens in the newt. Interestingly, the addition of WNT3A, in conjunction with FGF2, was able to induce lentoid formation in the ventral iris, which does not usually give rise to a lens (Hayashi et al., 2006). These experiments demonstrated a necessity for active Wnt signaling during Wolffian lens regeneration, but it was unclear if this finding was broadly applicable to all lens regeneration or if it was specific to the Wolffian system.

In cornea-lens regeneration in Xenopus, even less is known about the role of Wnt signaling. Wnt signaling has been implicated in two independent screens for genes expressed during early lens regeneration. From a cDNA library screen for genes upregulated during the process of lens regeneration, three genes specific to the Wnt signaling pathway were identified, including two inhibitors and one ligand: sfrp3, sfrp5, and wnt7b (Malloch et al., 2009). Another study of global transcriptional expression during this process also revealed many components of the Wnt signaling cascade, including, fz7, fz8, wnt2, wnt3, wnt5b, wnt6, wnt7a, and other components further down the signaling cascade (Day and Beck, 2011). Interestingly, Day and Beck (2011) also found the inhibitor sfrp2 to be upregulated during lens regeneration. The RT-PCR
data from the present study shows that the cornea epithelium is normally transcribing a wide assortment of frizzled receptors and wnt ligands, demonstrating that this tissue expresses the appropriate signals and receptors involved in active Wnt signaling (Fig. 2.1). It is important to note that the cornea epithelium is not made up of a homogenous population of cells, so it is possible that not every cell in this tissue expresses all of these genes.

In order to identify any differentially expressed genes between cornea and other skin that is not competent to regenerate a lens, we looked at the expression of the same genes in flank epithelium (Arresta et al., 2005). Flank epithelium expresses most of the same wnts and frizzleds, with the notable difference being \textit{wnt11} and \textit{wnt11b}, which were both expressed in the cornea epithelium but not in the flank (Fig. 2.1). While nothing is known about these genes in the context of lens regeneration, \textit{wnt11} has been shown to be specifically expressed in the limbal region of human corneas, where the population of stem cells that replenish the mature cornea reside (Nakatsu et al., 2011). This is interesting since there is now evidence that the basal layer of the larval cornea epithelium, which serves as the source of regenerated lenses in \textit{Xenopus} (Freeman, 1963), also appears to contain a population of oligopotent stem cells and their transit amplifying cells (Perry et al., 2013). It could be that \textit{wnt11} and/or \textit{wnt11b} help to maintain the oligopotency of these cells, either through a non-canonical or canonical mechanisms. \textit{wnt11} has traditionally been defined as a non-canonical wnt (Rao and Kühl, 2010) but there has also been a report in \textit{Xenopus} of \textit{wnt11} working in a canonical fashion (Tao et al., 2005), so its specific involvement in Wnt signaling cascades has some dependency on the biological context.

To better understand how the levels of active Wnt/β-catenin signaling in the cornea epithelium might be changing during the early events of lens regeneration, we analyzed the expression levels of \textit{axin2} by qPCR. \textit{axin2} is a commonly used readout for the level of active canonical Wnt/β-catenin signaling, as its expression is regulated through TCF/LEF (Jho et al., 2002). qPCR analysis of \textit{axin2} during cornea-lens regeneration revealed a significant reduction in \textit{axin2} expression at 24 hours post-
lentectomy, representing a reduction in Wnt/β-catenin signaling during that time (Fig. 2.2). This timing coincides with Freeman lens regeneration Stages 1 and 2, when the cells of the inner layer of the cornea epithelium transition from squamous to cuboidal to form a thickened placode (Freeman, 1963). Additionally, the recovery of axin2 expression levels at 48 hours suggests that the observed decrease in Wnt/β-catenin signaling is occurring within the cornea and not lens tissue, as 48 hours is not sufficient time for the formation of the lens vesicle or fiber cells (Freeman, 1963). While this result is specific to the canonical Wnt/β-catenin signaling pathway, it does not rule out a possible role for non-canonical Wnt signaling, such as Wnt/Planar Cell Polarity (PCP) during this regenerative process. In fact, as the new lens continues to form it seems likely that Wnt/PCP signaling could be necessary for the proper orientation of differentiated lens fiber cells (Sugiyama et al., 2011).

To functionally test the observed inhibition from the qPCR experiment, we impeded the ability of the cornea epithelium to decrease Wnt signaling by culturing regenerating eyes ex vivo in the presence of an activator of Wnt/β-catenin signaling. Both 1-azakenpaullone and BIO treatments resulted in a statistically significant decrease in the percentage of cases that successfully regenerated (Fig. 2.3). These data suggest that suppression of Wnt/β-catenin signaling is necessary in order for the cornea epithelium to transition towards the lens fate to regenerate a new lens. This is not the only regenerative system where Wnt/β-catenin signaling needs to be inhibited for regeneration to occur, as both retina regeneration in the chick (Zhu et al., 2014) and head regeneration in planaria (Liu et al., 2013; Sikes and Newmark, 2013; Umesono et al., 2013) display a similar phenomenon. It is important to note that because retinal tissue is a necessary component of the ex vivo culture system, it is impossible to rule out the possibility that treatment of the neural retina may be contributing to the observed results. Regardless of whether or not the required suppression is specific to the cornea epithelium, these date demonstrate that the role of Wnt signaling differs from what has been described during Wolffian lens regeneration in the newt, where active Wnt signaling is necessary for lens regeneration (Hayashi et al., 2006).
In fact, to confirm this difference, we inhibited Wnt signaling using IWR-1 treatment, rhDKK1 treatment, and transgenic expression of *Xenopus* DKK1, and none of these resulted in a statistically significant change in regeneration (Fig. 2.4 and 2.5). As these two forms of lens regeneration occur in completely different organisms, and the lens is regenerating from different tissues (cornea epithelium vs. dorsal iris) within these systems, this result may not be surprising. A recent study concluded that Retinoic acid (RA) signaling differs between the newt and cornea-lens regeneration systems (Thomas and Henry, 2014). In the newt, RA signaling has been shown to be necessary for lens regeneration (Tsonis et al., 2000; Tsonis et al., 2002), while Thomas and Henry (2014) demonstrated that in *Xenopus* RA signaling must be suppressed. These differences help illustrate the importance of studying regenerative mechanisms in a variety of organisms, as there appear to be distinct molecular pathways to regenerate a lens.

While different from Wolffian lens regeneration, our findings do appear to resemble what is known about the role of Wnt signaling during the initial development of the vertebrate lens. Holding Wnt signaling in an active state prevents the ability of the surface ectoderm to form a lens (Miller et al., 2006; Smith et al., 2005), which is similar to what was observed when cornea epithelium (which is derived from the surface ectoderm) is cultured in the presence of Wnt signaling activators (Fig. 2.3). In contrast, disruption of β-catenin through loss-of-function experiments during lens development, has no effect on the ability of the surface ectoderm to form a lentoid (Kreslova et al., 2007; Smith et al., 2005). Again, this matches our results from inhibiting Wnt signaling in regenerating eyes (Fig. 2.4 and 2.5). In fact, β-catenin loss-of-function in murine periocular and nasal ectoderm is sufficient to induce lentoid formation in these tissues during development (Kreslova et al., 2007; Smith et al., 2005). We find it particularly interesting that in the rhDKK1 experiments, both the 200 ng/ml and 500 ng/ml treatments resulted in 100% of the cases regenerating lenses, which was not observed in any other experiment (Fig. 2.4B). While this did not represent a statistically significant increase from the controls, which regenerated well themselves, it is a tantalizing thought that this inhibition may have been so effective that it helped promote lens formation as has been observed in periocular and nasal ectoderm (Kreslova et al., 2007; Smith et al., 2005).
Taken together, these data suggest that the involvement of Wnt signaling during lens regeneration may parallel that of early embryonic lens development.

In light of the findings from this study, it is also interesting that both of the screens for genes involved in cornea-lens regeneration found secreted frizzled-related proteins (sfrp2, sfrp3, and sfrp5), known inhibitors of wnt signaling, to be upregulated in the cornea early during regeneration (Day and Beck, 2011; Malloch et al., 2009). Perhaps these are key inhibitory factors responsible for diminishing canonical Wnt signaling in the cornea during this process. As not every cell of the cornea epithelium responds to the retinal signals to regenerate a lens (Freeman, 1963), it is unlikely that there is a global upregulation of these inhibitors. Instead, it seems more likely that sfrp upregulation would occur in a localized subset of cells which ultimately give rise to the lens.

One possible explanation as to why Wnt/β-catenin signaling would need to be suppressed in the cornea epithelium is that it could be helping to maintain the oligopotency of stem cells and/or their transit amplifying progeny that exist in the basal layer of the larval cornea epithelium (Perry et al., 2013). Studies of Wnt/β-catenin signaling in human cornea cells have indicated that the pathway may help to maintain cornea epithelial stem cells (Lu et al., 2011; Lu et al., 2012; Nakatsu et al., 2011). Active Wnt/β-catenin signaling has been observed in the human cornea in a subset of basal epithelial cells that reside in the limbus (Nakatsu et al., 2011) and helps to regulate proliferation of these human limbal stem cells. Additionally, studies have shown that active Wnt/β-catenin signaling helps to maintain human corneal epithelial cells in a less differentiated state (Lu et al., 2011; Lu et al., 2012). It is possible that the larval cornea epithelium in Xenopus also possesses active Wnt/β-catenin signaling that helps to maintain cornea stem cells (or their transit amplifying progeny) in the basal layer that are capable of giving rise to a new lens. However, in order for the cornea tissue to ultimately respond to the lens-forming cues released from the retina, these cells, or at least a subset of cells, must first reduce the level of Wnt signaling.
2.5. Figures and Table

Figure 2.1. Expression of wnts and frizzled receptors in larval cornea and flank epithelium. RT-PCR was conducted on RNA isolated from larval cornea epithelium (“C”) or flank epithelium (“F”). “+” denotes a positive control from pooled embryonic RNA. “C-” and “F-” are reverse transcriptase negative controls. Primers and corresponding product sizes are listed in Table 2.1.
Figure 2.2. *axin2* expression decreases early during lens regeneration. *axin2* transcriptional levels in cornea epithelium regenerating for either 24, 48, 72, or 96 hours. Control expression is from cornea epithelium that is not undergoing regeneration. *axin2* expression was normalized to beta-actin (*actb*). Asterisk denotes a statistically significant decrease (*p* value = 0.0006). Error bars indicate standard error.
Figure 2.3. Activation of Wnt signaling reduces lens regeneration rates. Lens regeneration rates for treatment with Wnt signaling activators, including 10 µM 1-azakenpaullone (A) or 1 µM BIO (B), compared to DMSO treated controls. (C) Relative expression levels of axin2 in corneas treated with 10 µM 1-azakenpaullone or 1 µM BIO. Asterisks indicate statistical significance (p < 0.05) and error bars indicate standard error. (D-K) Representative examples of positive cases of lens regeneration for BIO or 1-azakenpaullone treated eyes. D, F, H, and J show histological eye sections labeled using an anti-lens antibody (red fluorescence). Arrowheads indicate regenerated lenses. Sometimes higher background signal is observed in retinal tissue (H and J), but lenses are easily identified due to their distinct morphology. E, G, I, and K show an overlay of anti-lens staining with the corresponding DIC images. Dark tissue is retinal pigment epithelium. All images are at the same scale, scale bar in K is 200 µm.
**Figure 2.4. Wnt inhibition has no effect on lens regeneration.** Lens regeneration rates for *ex vivo* eyes treated with either 10 µM IWR-1 (A) or rhDKK1 protein at either 200 ng/ml or 500 ng/ml (B). Neither of these experiments showed statistically significant differences. (C) Both IWR-1 and rhDKK1 treatment significantly decreased *axin2* transcriptional levels in treated ocular tissue, verifying the Wnt inhibitors had the desired effect on Wnt signaling. Asterisks indicate statistical significance (*p*<0.05), and error bars indicate standard error. (D-O) Representative examples of positive cases of lens regeneration for IWR-1 or rhDKK1 treated eyes. D, F, H, J, L, and N show fluorescent (red) labeling of histological eye sections using an anti-lens antibody. Arrowheads indicate regenerated lenses. Sometimes higher background signal is observed in retinal tissue (H and J), but lenses are easily identified due to their distinct morphology. E, G, I, K, M, and O show anti-lens staining overlaid with the corresponding DIC images. Dark tissue is retinal pigment epithelium. All images are at the same scale, scale bar in O is 200 µm.
Figure 2.5. Transgenic expression of DKK1 does not inhibit lens regeneration. (A) A diagram of the HGEM-DKK1 bi-cistronic transgene. The heat-shock inducible HSP70 promoter drives expression of *Xenopus* DKK1 and a gamma-crystallin promoter drives GFP expression specific to the lens. (B-C) HGEM-DKK1 transgenic eye showing constitutive expression of GFP in the lens (C). (D-G) Anti-lens antibody staining of regenerated lenses in a sectioned control eye kept at room temperature (“Cont.”, D and E) and a heat-shocked eye (“HS”, F and G). Arrowheads indicate regenerated lenses. Dark tissue in DIC images is retinal pigment epithelium (D and F). All images are at the same scale, scale bar in K is 200 µm. (H) Regeneration rates of HGEM-DKK1 eyes are not significantly different (p=0.1674). Error bars represent standard error. (I) Depletion of β-catenin (CTNNB1; 94 kDa) in western blots of HGEM-DKK1 transgenic tissue. β-tubulin (TUBB; 50 kDa) is the loading control.
Table 2.1 Oligonucleotide primers used for RT-PCR. Primers were designed from the NCBI reference sequences (accession numbers provided) of the target genes, and were purchased from Integrated DNA Technologies (Coralville, Iowa). Primer pairs for \( fzd8 \) and \( fzd10 \) recognize both isoforms of the gene, and do not distinguish between \( fzd8a \) and \( fzd8b \), and \( fzd10a \) and \( fzd10b \), respectively. For each primer set, the expected band size in base pairs (bp) is provided.

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2.6. References


CHAPTER 3. THE REGENERATIVE CAPACITY OF LIMBAL VS. CENTRAL REGIONS OF MATURE *XENOPUS* CORNEA EPITHELIUM

3.1. Introduction

The frog, *Xenopus laevis*, is capable of completely regenerating the lens of the eye from the cornea epithelium (Freeman, 1963). This occurs following lentectomy, when regeneration inducing factors secreted by the neural retina are able to reach the cornea epithelium after perforation of the cornea endothelium (for review see Henry, 2003; Henry and Tsonis, 2010). Thus far, most of the effort to understand the molecular mechanisms driving cornea-lens regeneration has been focused on larval stages of *Xenopus*, as the ability of the cornea epithelium to give rise to a new lens *in vivo* was reported to be lost as larvae progress through metamorphosis (Filoni et al., 1997; Freeman, 1963). However, one group claimed that the mature cornea of the post-metamorphic frog is still competent to initiate the regenerative process (Filoni et al., 1997). Mature corneas of post-metamorphic froglets (15-30 days post-metamorphosis) appear to retain some level of competency to differentiate lens cells, if they are excised and implanted into the vitreous chamber of stage 56 tadpoles (Filoni et al., 1997). It has also been shown that the post-metamorphic retina still produces the regeneration inductive factors necessary to initiate lens regeneration, as larval cornea epithelium that is transplanted into post-metamorphic eye cup is able to regenerate (Bosco and Willems, 1992).

The development of *Xenopus* cornea through metamorphosis has been recently characterized by Hu et al. (2013). During embryonic lens development, the surface ectoderm overlying the optic cup subsequently gives rise to both the lens and the cornea epithelium (see Graw, 2010). In the developing *Xenopus* embryo, the embryonic ectoderm is comprised of two cell layers thick with both a pigmented (apical) and unpigmented sensorial (basal) layer (Hu et al., 2013; Nieuwkoop and Faber, 1956). As development proceeds to the stages most commonly used in cornea-lens regeneration studies (Nieuwkoop and Faber (1956) stages 46-54), the immature cornea epithelium remains primarily two cell layers, making it reminiscent of the surface ectoderm from
which the lens and cornea were originally derived (Hu et al., 2013; Perry et al., 2013). In fact, in both cases the lens forms from the deeper layer of the cornea epithelium or surface ectoderm, respectively (Freeman, 1963; Nieuwkoop and Faber, 1956). During stages 46-54, the cornea epithelium and underlying cornea endothelium are not fused to one another, except for a small point of connection at the center of the cornea (the “stroma-attracting center”), and there is no stroma located between the layers (Hu et al., 2013). Beyond stage 55, stroma gradually fills in between the epithelium and endothelium, and the epithelium begins to add additional superficial layers (Hu et al., 2013). By the time the cornea has matured it is structurally identical to the human cornea with a multilayered epithelium, thick stroma containing keratocytes, an endothelium, and intervening acellular layers including Bowman’s layer and Descemet’s membrane (Hu et al., 2013).

Filoni et al. (1997) concluded that the failure to regenerate in vivo is primarily the result of rapid healing of the inner cornea endothelium that prevents inductive retinal signals from reaching the cornea epithelium. A similar conclusion was reached in larval X. tropicalis cornea-lens regeneration, where rapid healing of the cornea endothelium explained the lower regeneration success observed by Henry and Elkins (2001). However, Filoni et al. (1997) did not address regional differences in the regenerative capacity of the mature cornea that could be related to the specific location of the stem cells of the cornea. We have hypothesized that lens regeneration may actually be driven by stem cells and/or their transit amplifying cells present throughout the basal layer of the larval cornea epithelium (Perry et al., 2013), and not from transdifferentiation of cornea cells into lens cells, as has been previously assumed to account for this process (Henry, 2003). During both human and rat cornea development, it is thought that stem cells initially exist throughout the basal layer of the cornea epithelium, and that as the cornea continues to mature, the stem cells that serve the cornea become restricted to the limbus at the periphery of the cornea (Chung et al., 1992; Davies et al., 2009). In Xenopus larvae, the transcription factor p63 (a common marker of cornea epithelial stem cells; Pellegrini et al., 2001) is expressed throughout the entire basal layer of the larval cornea epithelium, making it similar to developmental rat and human models (Perry et al., 2013).
Little is known about the stem cells of the mature cornea in the frog, but Hu et al. (2013) recently proposed the existence of a limbus from observations that the mature *Xenopus* cornea possesses a “wavy structure” in the peripheral cornea that may be analogous to the Palisades of Vogt observed in humans and that cells in this region also stained positively for p63. Hu et al. (2015) suggested that, like mammalian models, the mature frog cornea is maintained by a population of limbal stem cells. Here we hypothesize that the cornea epithelium of the post-metamorphic frog is maintained by a population of limbal stem cells, and that these cells and their transit amplifying progeny may also be able to support lens regeneration. If the regenerative competency of the mature cornea is restricted to cornea epithelial stem cells, then lower regenerative success would be expected from central cornea fragments as opposed to peripheral cornea fragments containing the limbal region. Alternatively, if the regenerative competency also extends to transit amplifying cells as well, then it would be expected that the center of the cornea would have similar regenerative success as the limbal region.

One of the proposed characteristics of limbal stem cells is that they are slow cycling cells and will therefore retain label over long periods of time (Yoon et al., 2014). Label retention techniques have been successfully used to identify slow cycling cells of the basal epithelium in the limbal region of the cornea, as labeled thymidine or thymidine analogs will be incorporated into the DNA of replicating cells and then dilute through subsequent mitotic divisions (Cotsarelis et al., 1989; Zhao et al., 2009). Therefore, as transit amplifying cells and their progeny divide and migrate centripetally and apically towards the center of the cornea, this nuclear signal is lost in the central cornea, but retained in the slowly cycling peripheral stem cells (Cotsarelis et al., 1989). In the larval *Xenopus* cornea, single doses of the thymidine-analog EdU have previously been used in pulse-chase experiments in the cornea epithelium, and over the course of four weeks only a few label retaining cells remained in the cornea with no obvious bias towards the periphery of the cornea (Perry et al., 2013). This data seems to suggest that there is no centripetal migration of cells from the limbal region in the larval cornea; however, it remains unknown if there is a functional limbus in the mature frog cornea. If the mature frog does possess limbal stem cells, then it would follow that these stem cells may be
slow cycling and located in the peripheral cornea like their mammalian counterparts (Cotsarelis et al., 1989; Zhao et al., 2009). In the present study we used label retention of the thymidine analog, EdU, to assess whether a population of label retaining cells exists in the peripheral Xenopus cornea, consistent with current models of mammalian limbal stem cells. We then conducted region specific transplants of central cornea versus peripheral limbal regions, to assess the regenerative potential of different areas of the mature cornea.

3.2. Materials and Methods, Results, and Discussion

In order to identify label retaining cells in the mature cornea epithelium, post-metamorphic X. laevis froglets (approximately 15-30 days after the completion of metamorphosis) were repeatedly injected with the thymidine analog EdU (5-Ethynyl-2’-deoxyuridine, Invitrogen, Carlsbad, CA). Froglets were raised from fertilized embryos from matings of adult X. laevis frogs acquired from Nasco (Fort Atkinson, WI) and following previously described husbandry procedures (Henry and Grainger, 1987; Henry and Mittleman, 1995). All animal care and use was approved and overseen by the University of Illinois Institutional Animal Care and Use Committee and the Division of Animal Resources at the University of Illinois. Using a 27 gauge needle and a Hamilton microliter syringe, 2 µl of 5 mM EdU diluted in PBS was injected through the peritoneum on the ventral side of each animal. All injections were done under anesthesia (1:2000 MS-222, Sigma, St. Louis, IL), and after injection animals were allowed to recover for 24 hours in 1/20x NAM (Slack, 1984) before returning to standard animal care. To ensure that the EdU pulse would be long enough to incorporate into slow-cycling cells, injections were repeated daily over the course of seven days. We did observe some natural variation between the robustness of EdU incorporation into the cornea between individual animals; however, the patterns of label retention were consistent regardless of the number of cells initially labeled with EdU. After chase periods of 5 days, 3 weeks, and 10 weeks following the last injection, froglets were euthanized and fixed in 3.7% formaldehyde overnight at 4ºC. Corneas were then removed off of each animal and EdU was visualized using either Alexafluor 488 or 594 azide (Invitrogen, Carlsbad, CA) following the strategy of Salic and Mitchison (2008). Each cornea was stained with 1 µM DAPI for 15 minutes, washed with
PBS, and mounted onto a glass slide in ProLong Gold Antifade Mountant (Fisher Scientific, Pittsburgh, PA). To help the corneas lay flat on the glass slide, three to four cuts were made into the periphery of each cornea prior to mounting (Fig. 3.1D, E, P, and Q). Confocal microscopy (LSM 700, Carl Zeiss, Munich, Germany) was used to determine the layers of the cornea epithelium that retained signal. Orthogonal image projections (Fig. 3.1F, K, R, W) were generated by creating a Maximum Intensity Projections from confocal data using ZEN software (Carl Zeiss, Munich, Germany).

The structure of the post-metamorphic cornea is shown in Figure 3.1A. For sectioning, post-metamorphic froglets were euthanized and fixed in 3.7% formaldehyde overnight at 4°C. The eyes were removed, and dehydrated in EtOH, cleared in Xylene, embedded in Paraplast Plus (Fisher Scientific, Pittsburg, PA), sectioned at a thickness of 8 µm, and stained in Harris hematoxylin/Eosin (Fisher Scientific, Pittsburg, PA) according to previously published protocols (Humason, 1972; Wolfe and Henry, 2006). Five days after the conclusion of the EdU labeling pulse (as defined by the final injection), cells throughout the cornea epithelium are labeled in both the basal (Fig. 3.1B) and apical layers (Fig. 3.1C). However, as time passes this signal is lost preferentially in the center of the cornea compared to the peripheral regions (Fig. 3.1D-O). After three weeks of chase, the EdU signal in central cornea becomes noticeably diminished (Fig. 3.1K-O). The basal layer of the central epithelium had very few nuclei retaining label (Fig. 3.1K, N, O), and most of the remaining signal that does exist in the central cornea lies in the flattened nuclei of the squamous cells of the more apical layers of the epithelium (Fig. 3.1K, L, M). This finding is consistent with a net apical migration of cells from the basal epithelium, as cells divide and differentiate to maintain these epithelial layers. Occasionally, labeled macrophage-like cells can be observed in the basal layer of the cornea epithelium (Fig. 3.1K, N; Perry et al., 2013). In contrast, at the periphery of a 3 week chase cornea, label is retained more abundantly in the regularly spaced and rounded nuclei of the basal epithelium (Fig. 3.1F, I, J), as well as in some overlying apical layers (Fig. 3.1F, G, H). Interestingly, the observed occurrence of label retaining cells of the basal layer quickly taper off in a direction working towards the central cornea in both number and fluorescence intensity (arrow in Fig. 3.1F). After ten
weeks of chase (Fig. 3.1P-AA), very few cells of the basal or apical layers retain label in the central cornea (Fig. 3.1W-AA). However, in the peripheral cornea, label can still be observed robustly in the nuclei of cells of the basal epithelium, as well as in some of the overlying apical cells (Fig. 3.1R-V). Taken together, these observed patterns of label retention are consistent with the model of centripetal migration where progeny from slow cycling stem cells at the limbus move centripetally and superficially (apically) as they replenish lost cells of the cornea epithelial surface. Additionally, in many of our cases there was an increased bias in label retention towards the ventral periphery (Fig. 3.1D).

One possible explanation for this involves that fact that post-metamorphic Xenopus only possess a lower eyelid (Hu et al., 2013). In mammals it has been proposed that limbal stem cells are more concentrated in the limbus beneath the eyelids, as the eyelid may help protect the stem cell niche in the cornea from various factors like ultraviolet light and desiccation (Levis et al., 2013; Yoon et al., 2014). While these are not likely concerns in this aquatic species of frog, the recess of the eyelid may be providing other support to the stem cell niche that could bias peripheral stem cells in the frog towards the ventral side. Because of this observation, we decided to test the regenerative capacity of both the dorsal and the ventral limbal region.

Having identified label retaining cells in the periphery of the cornea epithelium, which is consistent with a limbal stem cell model, we tested the regenerative capacity of the dorsal and ventral limbus compared to that of central cornea. Here central cornea is defined as existing only above the pupillary space, where little label retention is seen in the basal layer after a three week chase. Tissue from the dorsal and ventral limbal regions was collected starting from approximately the outer edge of the pupil to the outer edge of the eye, at the transition between the transparent cornea and the pigmented skin (Fig. 3.2A). Regional pieces of mature cornea were collected from post-metamorphic froglets and transplanted into the eyecups of larval hosts (Stages 54-57). Larval host eyes were lentectomized and to ensure that any observed regeneration was derived from the donor tissue and not the cornea epithelium of the larval host, cornea epithelia were completely removed from larval eyes (including the point of attachment to the underlying cornea endothelium). Transplant fragments were then collected and immediately placed
into the larval eyecups in an *ex vivo* eye culture system (see Fukui and Henry, 2011; Hamilton et al., 2016; Thomas and Henry, 2014). These eyes were then removed from the animals, and placed into culture media consisting of: 61% L-15 powder (Invitrogen, Carlsbad, CA); 100 U/ml of penicillin and 100 µg/ml of streptomycin (Mediatech, Manassas, VA); 10% fetal bovine serum (Invitrogen, Carlsbad, CA); 2.5 µg /ml of Amphotericin B (Sigma, St. Louis, IL); and 4 µg /ml of Marbofloxacin (Sigma, St. Louis, IL). Culture media was changed every 2 to 3 days. After 10 days of regeneration eyes were fixed for 3 hours in 3.7% formaldehyde, dehydrated, cleared, infiltrated, and embedded in paraffin wax, and serially sectioned at 8 µm for histological analysis. Lens regeneration was assessed by carrying out immunohistochemistry on the histological sections using a polyclonal anti-lens antibody (see Henry and Grainger, 1987). Positive lens regeneration was scored by the presence of positive antibody staining in the transplanted tissue.

All three regions of the post-metamorphic cornea showed a similar ability to initiate the process of lens regeneration (*Fig. 3.2H*): 63.3% positive regeneration from dorsal limbal region (19/30 transplants, *Fig. 3.2B, C*); 41.4% from central cornea (12/29 transplants, *Fig. 3.2D, E*), and 51.9% from ventral limbal region (14/27 transplants, *Fig. 3.2F, G*). Differences between any two groups were not statistically significant as measured by Fisher’s Exact Test (Fisher, 1922). The observed regenerative rates were higher than what Filoni et al. (1997) observed; however, our experiments were carried out in an *ex vivo* culture system where the cornea is tucked directly into the optic cup so that it is in close contact with the neural retina, typically leading to high rates of regeneration (Fukui and Henry, 2011; Hamilton et al., 2016; Thomas and Henry, 2014). *Figure 3.2I*, shows a portion of mature cornea collected from the dorsal limbal region of a post-metamorphic froglet, tucked inside of a larval eyecup. The post-metamorphic cornea can be easily distinguished by the distinctive multi-layered epithelium and thick stroma with keratocytes (*Fig. 3.2J*). Furthermore, basal cells of the mature cornea epithelium are distinguished by their cuboidal shape, and have rounded nuclei, and the more apical cells of the stratified epithelium are squamous and have flattened nuclei (*Fig. 3.2J*; Hu et al., 2013). In those sections that clearly showed the multi-layered cornea
epithelium positive lens protein expression was seen specifically in the basal layer of that tissue (Fig. 3.2K). This is consistent with observations from larval cornea-lens regeneration, showing that the basal layer of the cornea epithelium gives rise to the new lens (Freeman, 1963). Though differentiated lens cells expressing lens protein were observed, no morphologically normal lenses were observed to have regenerated over the course of these experiments. Though the post-metamorphic cornea epithelium is still competent to respond to regenerative signals to initiate the conversion of basal cells towards the lens fate, the capacity to regenerate a complete lens is not present.

The results from our transplant experiments eliminate the possibility that this regenerative competency is restricted to any stem cells located in the limbal region of the peripheral cornea, because central cornea epithelium appears to be just as responsive (Fig. 3.2). Likewise, if the regenerative competency were restricted to stem cells in the limbal region, one might expect to see greater regenerative success in implants derived from the ventral limbus, as this region was found to contain a greater density of label retaining cells (Fig. 3.1D). However, there was no significant difference between the three regions tested here (Fig. 3.2H). Additionally, it appears that the entire post-metamorphic cornea is still competent to form lens cells, as the entire basal epithelium of each transplant appeared to be capable to respond, and not just a subset of cells within the layer (Fig. 3.2I). These cells likely include limbal stem cells and their transit amplifying progeny. While the cells of the basal epithelium seem to initiate the process of lens regeneration to express lens proteins ex vivo, their association with Bowman’s layer, the stroma, as well as the more superficial differentiated layers of the mature cornea may provide a steric hindrance on the ability of the basal cells to organize a lens vesicle, unlike the case present during embryonic lens development and larval lens regeneration when these tissues are absent or immature (Fig. 3.2K).

The EdU label retention that we observed in the peripheral cornea is consistent with animal models that possess limbal stem cells that send progeny centripetally towards the central cornea (Chung et al., 1992; Zhao et al., 2009). This data goes beyond the structural similarity between frog and mammalian corneas, and shows cellular evidence
of a limbal stem cell model in *Xenopus*, expanding the known occurrences in vertebrates. However, we still have much to learn about the specific location and cell and molecular characteristics of the stem cells that serve the mature cornea in the frog. Additionally, we need a better understanding of the molecular properties of the stem cells and transit amplifying cells of the basal epithelial during regeneration, as these cells are responding to the factors responsible for inducing lens regeneration. It is actually surprising that we currently know almost nothing about the molecular signaling pathways involved during post-metamorphic cornea-lens regeneration or how it relates to larval regeneration or lens development. Due to the structural similarity between the mature frog cornea and our own, answering these questions in the post-metamorphic cornea should provide further insights into future regenerative therapies of our own ocular tissues. Perhaps our cornea epithelium is also capable of initiating regenerative mechanisms, but successful regeneration of a lens is prevented by the steric hindrance of the surrounding ECM and cellular layers. Future experiments are needed to determine if weakening or removing the stroma from underneath the cornea epithelium helps release these cells to be able to regenerate a complete lens *in vivo*. 
3.3. Figures

**Figure 3.1.** EdU label retention in post metamorphic corneas. A) Histological structure of a post-metamorphic cornea. Boxes with dotted lines indicate putative limbal regions, and box with solid line indicates central cornea (above pupillary space). After a 5 Day chase, EdU (red) can be observed throughout the basal layer of the epithelium (B) and the
more superficial apical layers (C). Scale bar (100 µm) for both B and C is located in C. DAPI labeled nuclei are blue. D-E) EdU label (red) in 3 week (3W) cornea pelt. Boxes indicate locations of central and peripheral images in F-O. F-J) Corneas after 3 week chase show label retention in the peripheral cornea. F) An orthogonal projection of confocal data taken from cornea shown in D reveals the layers of the cornea epithelium (“CE”) that are retaining signal. The stroma (“S”) lies beneath the cornea. Arrow indicates the direction to the center of the cornea from the periphery. Arrowheads identify label retaining cells of the basal epithelium. G-H) Apical cell layers from the Z-stack used to create the orthogonal projection in (F). I-J) Basal cell layers from the Z-stack used to create the orthogonal projection in (F). K-O) Central cornea after 3 week chase has very little signal in basal layers, but some signal is still present in apical layers. * denotes macrophage-like cell commonly observed in basal layer of cornea epithelium (K, N). After 10 weeks of chase (P-AA), corneas possess label retaining cells in peripheral cornea (R-V), while the central cornea is relatively devoid of signal and the signal that remains is weak (W-AA). B, C, H, J, M, O, T, V, Y, and AA show an overlay of EdU (red) and DAPI (blue). Scale bar for D, E, P, and Q is 1 mm and is located in Q. Scale bar for G-J, L-O, S-V, and X-AA is 50 µm and is located in AA. Dorsal (“D”) Ventral (“V”) axis is shown in D and P.
Figure 3.2. Regenerative capacity of the mature cornea. A) Zones of post-metamorphic donor corneas. Black dotted line represents putative limbal region. Scale bar in A is 1 mm. B-G) Representative cases of regeneration as defined by positive staining using an anti-lens antibody. Scale bar in B-G is 200 µm. H) Percent regenerative success as determined by the percentage of examined cases expressing lens proteins. Error bars indicate standard error. I) Mature cornea tucked inside larval retina is expressing lens proteins. J-K) Higher magnification showing expression in the same eye as (I) where lens crystallin protein expression is restricted to the basal layer of the cornea epithelium. Scale bar in I is 200 µm. Scale bar in J and K is 100 µm. J) Structure of mature cornea inside larval eyecup. Abbreviations: DL, dorsal limbal region; VL, ventral limbal region; CC, central cornea; S, stroma; CE, cornea epithelium; LR, larval retina; K, keratocytes; BE, basal cells of cornea epithelium; AE, apical cells of cornea epithelium.
3.4. References


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CHAPTER 4. PROLONGED IMAGING OF XENOPUS CORNEA EPITHELium

4.1. Introduction

The larval form of the African clawed frog, *Xenopus laevis*, provides an ideal system to study developmental and regenerative processes *in vivo* (Beck & Slack, 2001; Henry et al., 2008). Embryos are relatively large and can be genetically manipulated through the use of Morpholinos, synthetic mRNAs, and transgenes (Henry et al., 2008; Mimoto & Christian, 2011; Takagi et al., 2013). In order to better understand changes in gene expression and function using these techniques, methods of imaging live embryos in real-time have been established and are now invaluable tools in many laboratories (Keller, 1978; Kieserman et al., 2010; Wallingford, 2010; Danilchik, 2011). However, these techniques are limited to the early stages of development, before the tadpole gains locomotion. It is during these more advanced stages of development that *Xenopus* larvae display remarkable regenerative abilities in the limb, tail, spinal cord, and parts of the eye (Henry et al., 2008; Slack et al., 2008; Beck et al., 2009). In addition, many other important developmental processes continue during these advanced stages, such as those of the limb, heart, and nervous system (Nieuwkoop & Faber, 1956; Tschumi, 1957; Warkman & Krieg, 2007). Currently, to monitor expression and functional changes during these important developmental and regenerative processes, one must either fix specimens at a few selected time points, or repeatedly bring the animals in and out of anesthesia for brief individual imaging sessions. In either case, this greatly reduces the resolution of these experiments.

The current anesthetic of choice for *X. laevis* is tricaine methanesulfonate (commonly known as MS-222), which is widely accepted and used for procedures in fish and amphibians (Popovic et al., 2012; Guénette et al., 2013). When *Xenopus* tadpoles are immersed in a buffered solution of MS-222 (typically a 1:2,000 dilution), the anesthetic is absorbed quickly and acts to inhibit sensory function, motor function, and consciousness (Lalonde-Robert et al., 2012). While this quick activity makes it an ideal

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anesthetic agent for short procedures, *X. laevis* larvae are not able to live when kept in this anesthetic for long periods of time, also making it a widely used choice as a euthanizing agent at higher concentrations (Leary et al., 2013). The consequence is that any live imaging sessions of anesthetized tadpoles are limited to a short period of time before their health begins to fail. One question that can be raised is whether this failing is due to the direct effect of the anesthetic agent itself, or is instead the result of poor respiration due to immobilization. This question is raised from observations in fish, where it is thought that one of the primary causes of death for adult fish kept in continuous MS-222 is asphyxiation due the cessation of buccal pumping and gill irrigation (Soivio et al., 1977; Cornish & Moon, 1986). As a result, fish are often supported using an artificial gill irrigation system during long anesthesia to promote gas exchange with the gills (Carter et al., 2011). Since *Xenopus* tadpoles also use buccal pumping for gill irrigation, it is possible that they also become hypoxic when left in MS-222 for long periods of time. Additionally, since frogs are bimodal breathers and are able to undergo gas exchange through their skin (Emilio & Shelton, 1974; Boutilier & Shelton, 1986), it is possible that being immobilized in static anesthetic also affects their ability to properly exchange gas through these tissues.

In this study, we found the simple act of rocking *Xenopus* tadpoles in a dilute anesthetic solution of MS-222 (1:7,000) significantly increases their longevity from hours up to days. By using a peristaltic pump to create a similar flow over the animals, tadpoles can remain anesthetized and immobilized to allow for live imaging over long periods of time. Here we describe the construction of an apparatus that accomplishes this task, and we demonstrate the power of this technique by showing the development of the hindlimb over the course of 48 hours. Additionally, we demonstrate the usefulness of this technique when combined with transgenesis by imaging cellular divisions in the cornea epithelium using a transgenic histone H2B-mCherry nuclear reporter. This technique opens the door for new imaging of a variety of developmental and regenerative processes in *Xenopus* and should provide new insights into these key areas of research.
4.2. Materials and Methods

4.2.1. Animals.

*Xenopus laevis* adults were acquired from Nasco (Fort Atkinson, WI). Larvae were generated and reared as previously described (Henry & Grainger, 1987; Schaefer et al., 1999). All larvae were developmentally staged according to Nieuwkoop and Faber (1956). The anesthetic used in all experiments was MS-222 (Sigma, St. Louis, MO) diluted 1:7,000 (0.14 mg/ml) in 1/20X normal amphibian media (NAM; Slack, 1984); and all animals were euthanized at the end of the designated 48-hour time interval. Transgenic animals were generated following the protocol described by Smith et al. (2006). The mG-HGEM transgenic construct was created by adding the sequences for histone H2B-mCherry and membrane bound GFP (mG) into the HGEM (heat-shock green-eyed monster; Beck et al., 2003) construct downstream of a heat-shock inducible *hsp70* promoter (the membrane bound GFP signal was not utilized in this particular study). This transgenic cassette uses a gamma-crystallin promoter to drive GFP expression in the lens as a method for screening transgenic animals. Heat shocks were carried out on the whole animals by bath immersion in water at 34°C for 30 minutes. The animal care and use in this work has been overseen by the University of Illinois Institutional Animal Care and Use Committee and monitored by the staff of the University of Illinois Division of Animal Resources (DAR).

4.2.2. Rocking vs. Stationary Anesthesia.

Tadpoles were fully anesthetized in 1:7,000 MS-222 for 2 minutes before being individually transferred into small glass bowls containing 50 ml of the anesthetic solution. Cases were placed either onto a Labnet Rocker 25 (Labnet International, Edison, NJ) set to 20 rocks per minute (rpm) with a tilt range of +/- 7°, or sibling controls were left stationary beside the rocker. Anesthetic solution was changed every 12 hours. Heart rates were determined by manually counting heartbeats viewed through a dissecting microscope (Stemi 2000, Zeiss) to determine heart rate per minute. Death was defined at the point at which the calculated heart rate was observed to be zero beats per minute (bpm). Statistical tests and figures were generated using Prism software (GraphPad Software, Inc., La Jolla, CA).
4.2.3. Respiratory Chamber.

The respiratory chamber was constructed as detailed in Figure 4.3. 1:7,000 MS-222 solutions were made in 350 ml batches (a quantity sufficient to recirculate in our system) and changed every 24 hours. Silastic tubing was used to connect the anesthetic solution source to the respiratory chamber (Fig. 4.4A) via a variable speed medium flow peristaltic pump (Cat No. 73160-32, Cole-Parmer, Vernon Hills, IL) set to a flow rate of 20 ml/min (tubing of 127 mm length and 4.8 mm inner diameter that was capable of an adjustable range of 17.0-60.0 ml/min was used). Tadpoles were held in place using non-toxic modeling clay (Van Aken International, Rancho Cucamonga, CA). To create a recirculating system, the respiratory chamber was then placed into a shallow plexiglass overflow tray, which drained back into the anesthetic source solution. In order to maintain a constant fluid level, wicks cut from Whatman filter paper were used to pull solution out of the respiratory chamber.

4.2.4. Imaging.

All images were taken using a Zeiss Axioplan microscope. In order to protect the microscope and to drain the overflow of anesthetic solution, a custom overflow tray was built out of plexiglass to fit precisely onto the microscope stage. Tadpoles were placed into the respiratory chamber as previously described, and the respiratory chamber was placed into the overflow tray. To provide a consistent optical surface for imaging, a coverslip was then placed on the pedestals with small pieces of clay. The top of the coverslip was treated with Rain-X Original Glass Treatment (Rain-X, Huston, TX) to create a hydrophobic surface to prevent anesthetic solution from spilling over the top of the coverslip. Images were taken automatically at 5 minute intervals using a SPOT Flex camera and the SPOT Advanced software (Diagnostic Instruments, Sterling Heights, MI). A shutter was used to control the light source via the TTL output on the SPOT Flex camera, so that the animal was only exposed to light while the image was being captured. For hindlimb imaging, a motorized stage (Prior Scientific, Rockland, MA) was used to take multiple focal planes, which were then flattened into a single image using Helicon Focus (Helicon Soft Ltd). During imaging sessions, heart rates were occasionally checked to monitor the health of the animal. This was accomplished by switching to a
lower power objective (2.5x) during the interval between captured images. Time-lapse movies (Movies 4.1 and 4.2) were generated in ImageJ (NIH, Bethesda, MD).

4.3. Results

4.3.1. Rocking tadpoles in MS-222 significantly increases survivability.

We hypothesized that part of the reason tadpoles expire in the continuous presence of MS-222 is due to poor oxygenation resulting from immobilization in the anesthetic. In order to test this hypothesis, we set up a simple experiment where tadpoles were placed in an anesthetic solution of MS-222 on a common lab rocker in order to aerate and circulate the solution to promote better gas exchange. All of the control cases (n=8) kept stationary in the anesthetic expired within 8 hours, with 50% expiring within the first 4 hours. Those that did survive past a few hours were opaque and pale in color, and often displayed signs of heart failure such as arrhythmia, slow blood flow, and signs of fluid accumulation and swollen tissue, particularly noticeable over the heart. Remarkably, 75% of the rocked cases (n=8) survived for 48 hours. This difference represents a statistically significant increase in survivability (p-value < 0.0001; Fig. 4.1A). It is important to note that some tadpoles naturally open their mouths when anesthetized, which could allow for some flow into the buccal cavity and over the gills while rocking. However, this did not appear to be necessary as it was observed that animals with closed mouths were also able to survive for the full 48 hours. This observation suggests that it is cutaneous gas exchange that is sufficient to increase survivability.

Throughout the experiments the heart rate of each animal was determined as a quantitative assessment of the vitality of the tadpoles in the anesthetic solution. Of the six cases that survived in the anesthetic solution for the full 48 hours, a slight decrease in the heart rate over time was observed (Fig. 4.1B,D,F). This was true regardless of the stages used. While the baseline heart rate of the older and larger tadpoles was lower than that of the younger tadpoles (as described by Hou & Burrgren, 1995), the trend between the two groups was very similar with an approximate reduction of 4 to 4.5 beats per minute every 6 hours.
4.3.2. Prolonged anesthesia of immobilized tadpoles.

In order to see if gill irrigation could be used to support a tadpole held in a fixed position, we created an artificial gill irrigation system (similar to those used in fish), using a small funnel to direct flow through the open mouth and over the gills; however, this method produced variable survivability results (data not shown). Since directed flow through the open mouth did not appear to be necessary to promote effective gas exchange as observed in the rocker experiments, we designed a more general system to create flow over the entire animal in order to more closely recreate those conditions (Fig. 4.2). A custom manifold was created from plexiglass and placed into a petri dish along with three pedestals to hold a coverslip as the solution current impacts the ability to clearly view the animal (Fig. 4.3 and 4.4A). Non-toxic modeling clay was placed between the pedestals to hold tadpoles gently, but securely, in place. Minimal contact was maintained between the animals and clay to promote flow of the anesthetic solution around the tadpoles. However, it is necessary to provide enough contact to prevent the animals from moving in the flow. It is also important to note that great care was taken to specifically avoid pushing any clay against the heart or abdomen, so as not to interfere with heart contractions or peristalsis of the gut.

Once the tadpole was properly placed in the clay, the respiratory chamber was placed into an overflow tray to catch the runoff from the continuously pumped anesthetic solution. This tray drained into a reservoir bottle containing the anesthetic solution. A variable-speed peristaltic pump was used to pump the anesthetic solution from the reservoir bottle through the slot on the manifold and over the tadpole at a rate of 20 ml/min, creating a recirculating system. While we did not carry out extensive trials, we found that lower flow rates of approximately 10 ml/min were insufficient, as tadpoles did not survive past six hours at these rates (n=2). However, a flow rate of 20 ml/min worked well, supporting tadpoles ranging from stage 48 to stage 55 for 48 hours (n=5; Fig. 4.1A). For younger and older tadpoles, one may need to empirically determine an appropriate flow rate that works well.
4.3.3. *In vivo* Imaging.

Once it was demonstrated that tadpoles survive well while kept stationary in the respiratory chamber, we adapted the system to be used with an upright microscope for time-lapse imaging (Fig. 4.4). A custom plexiglass overflow tray was built to attach to the microscope stage for collection of the anesthetic runoff to be recirculated (Fig. 4.4B). The respiratory chamber was then placed underneath the objective, with the tadpoles mounted in clay and a stationary coverslip secured on top of three pedestals using clay (Fig. 4.4C). The coverslip is necessary to maintain an optically transparent and flat surface for imaging, as without it, the circulating air-water interface distorts the image due to water tension and subtle changes in fluid volume. It is also important to make the top surface of the coverslip hydrophobic, to prevent anesthetic solution from flowing over the top of the coverslip. However, if a water immersion objective were to be used, then the coverslip would no longer be necessary.

Using this setup, we anesthetized wild-type tadpoles and imaged them for up to 48 hours. As an example, we imaged 48 hours of hindlimb development. This is made possible by using the automatic image acquisition capabilities of the SPOT Flex microscope camera and software (Diagnostic Instruments, Sterling Heights, MI). Figure 4.5 shows the development of a St. 53-54 left hindlimb. During this time interval we were able to observe the emergence and migration of melanophores in the limb, vascular development in the digital plate, and elongation of the digits. Particularly noticeable is the elongation of digit 4, which corresponds to the protrusion of the marginal vein at that digit (Fig. 4.5). The full time-lapse video of these events can be viewed in Movie 4.1. This video also shows melanophores on the tail and limb as they respond to changes in illumination and other physiological conditions. In addition, one can see continued peristalsis of the gut and the expulsion of waste products. These events continue throughout the anesthetic treatment, indicating that these physiological events appear to be unaffected by the prolonged anesthesia.
One of the powerful uses of this technique is in combination with transgenic expression of fluorescently labeled proteins in living tissues. We generated F0 transgenic tadpoles, expressing the mG-HGEM transgene (Fig. 4.6A), which carries a heat-shock inducible histone H2B-mCherry fusion protein, in order to fluorescently label cell nuclei (Beck et al., 2003; Takagi et al., 2013). This nuclear labeling allows for monitoring divisions and tracking cellular movements within a tissue. Using the \textit{in vivo} imaging setup, we imaged fluorescently labeled nuclei in the cornea epithelium. \textbf{Figure 4.6 (B-G)} follows an individual cell of the cornea epithelium through the phases of mitotic division and the migration of the daughter cells away from each other. The entire division can be viewed as a time-lapse movie in \textbf{Movie 4.2}.

\textbf{4.4. Discussion.}

One of the most significant advantages of this system is the relative ease with which one can set up imaging experiments and collect very complete sets of data. It takes only a few minutes to prepare the specimen, and the time interval and focal planes between successive images can be customized as required by the experiment. Currently, the only manual work that must be done during the imaging session is daily changes of the anesthetic solution and adjustment of the microscope focus to compensate for any possible drift or growth of the specimen. In setups where one has an automated motorized stage, focal adjustment is less of a concern since multiple focal planes can be captured as a stack and flattened into a single image using appropriate software. This ensures that the area of interest remains in focus for the duration of the time-lapse experiment.

One concern with this technique is the potential physiological effect of the MS-222 on the anesthetized animal. In both the leopard frog \textit{Rana pipiens} and adult zebrafish, it has been reported that MS-222 decreases heart rates during anesthesia (Cakir \& Strauch, 2005; Sun et al., 2009). However, recent work in \textit{X. laevis} reports very little effect on heart rates during anesthesia of adults in MS-222 (Lalonde-Robert et al., 2012), so it was unclear whether or not MS-222 would affect heart rates in larval \textit{Xenopus}. In an attempt to minimize any potential effects, we used a 1:7,000 dilution of MS-222, which is
sufficient to anesthetize the tadpole. While the concentrations used for surgical applications are generally higher than this (e.g. 1:2,000 in Perry et al., 2013), dilutions as low as 1:10,000 have been reported as effective (Hou & Burggren, 1995; Sakaguchi et al., 1997). At the concentrations used here, we did observe a gradual reduction in heart rate over time (Fig 4.1B), suggesting that MS-222 does have some effect on the cardiovascular system of larval *Xenopus*. However, the heart rates of these tadpoles never deviated from what would otherwise be considered a normal range for *Xenopus* larvae of those sizes and stages of development (Hou and Burggren, 1995).

One limitation with this technique is the length of time that the imaging can be carried out. We terminated our experiments after a predetermined 48 hours, at which time tadpoles still appeared to be in good health. This means it may be possible for even longer imaging sessions to be carried out using this method; however, care must be taken to consider experimental artifacts that may be introduced in studies greater than 48 hours. For instance if the heart rate continues to decline, at some point there may not be enough blood circulation to maintain the health of the tissues of interest. Also, the lack of food for multiple days will likely affect metabolism, and potentially the observed results.

In summary, this technique offers a new approach to imaging developmental and regenerative processes in living tissues. Until now, this has not been feasible due to the inability to maintain *X. laevis* tadpoles for long periods of time in an anesthetic solution. For instance, the development of the limb, eye, heart, and nervous system are all easily imaged through the transparent larval skin and can now be more effectively studied *in vivo*. It is anticipated that the adoption of this imaging system by other laboratories will help provide new insight into the development of many systems and tissues, and aid our current understanding of cellular biology in the context of the living organism.
4.5. Figures and Movies

**Figure 4.1.** Survivability of anesthetized tadpoles. (A,C,E) Kaplan-Meier survival curve of tadpoles anesthetized in 1:7,000 MS-222. Tadpoles kept either stationary (dashed-red) or on the rocker (solid-blue). Both the St. 50-51 group and the St. 53-54 group demonstrated a statistically significant difference (p<0.0001; Mantel-Cox test) between the groups. The St. 47-48 group did not display a statistically significant difference (p=0.3218). (B,D,F) Mean heart rates of the rocker tadpoles from the survival curves. Error Bars show standard deviation. Dotted lines are linear regression lines.
Figure 4.2. Schematic drawing of the respiratory chamber and pump system (or XICU: Xenopus Intensive Care Unit). An anesthetic solution of MS-222 is pumped to the respiratory chamber via a peristaltic pump. The respiratory chamber contains a manifold that provides continuous flow of anesthetic solution over the tadpole, which is held in place with clay. Three fixed pedestals hold a coverslip to provide a transparent and flat surface for imaging. The respiratory chamber is placed into a shallow plexiglass overflow tray to catch the anesthetic solution. The overflow tray collects anesthetic runoff to a waste container that can be discarded or recirculated, as needed by the experiment.
Figure 4.3. Specifications of manifold pieces. A 0.5 inch endmill was used to create a slot in a piece of 0.25 inch thick plexiglass (Piece 1), and an inlet hole was drilled into the top to connect with the slot, as shown. Piece 1 was fixed on top of Piece 2 using 1,2-dichloroethane. A piece of a 1 ml plastic serological pipette (Falcon 7506, Becton Dickinson Labware, Lincoln Park, NJ) was bent at approximately 90° (Piece 3) over a Bunsen burner flame and was fixed (using 1,2-dichloroethane) into the hole of Piece 1. Three pedestals (Piece 4) were cut from plexiglass rod, to be used to hold a coverslip. The assembled manifold and the three plexiglass rods were then affixed to a 100x15 mm petri dish to create the respiratory chamber using 1,2-dichloroethane (see Figure 4.4A). Specification diagrams were generated in AutoCad. Measurements are in inches, with millimeters provided in parenthesis.
Figure 4.4. *Xenopus* Live Imaging Setup. **A)** The *Xenopus* respiratory chamber. Anesthetic solution is pumped into the manifold and out through a slot to generate flow across the tadpole. Three pedestals are used to hold a coverslip to allow for a smooth optical surface for imaging. **B)** Tadpoles are mounted in clay using a rounded glass rod. Arrows denote the primary trench for the animal. A secondary waste trench aids in waste removal. **C)** Kaplan-Meier survival curve of anesthetized tadpoles (ranging from St. 48-65) maintained by the pump. **D)** The microscopy setup. Anesthetic solution is pumped using a peristaltic pump to the manifold. The overflow tray collects the run-off and either returns it to the source for a recirculating setup, or can go to separate waste collection bottle (not shown). **E)** Respiratory chamber under the objective. Filter paper wicks are used to accelerate drainage and maintain a low fluid level at the top of the dish.

Abbreviations used are: anesthetic solution (an), clay (cl), coverslip (cs), drain hole (dh), glass rod (gr), line-in (li), line-out (lo), manifold (ma), overflow tray (ot), petri dish (pd), pedestal (pe), pump (pu), manifold slot (sl), waste trench (wt), and filter paper wick (wi).
**Figure 4.5.** Time-lapse imaging of hindlimb development. (A-E) Growth of a stage 53-54 left hindlimb at 24 hour intervals over 48 hours. During this time melanophores can be seen migrating and expanding on the ventral edge of the left hind limb (arrowheads indicate the expanding range of these cells), in addition to vascularization and digit development in the digital plate. The shape of the marginal vein can be seen changing as digit 4 elongates (asterisk indicates the location of digit 4). A dashed line has been included as a reference mark for this elongation. This plate corresponds to Movie 4.1. Relative time is denoted in hours:minutes. Scale bar in E is 250 μm.
Figure 4.6. Micrographs of cell division in *Xenopus* cornea epithelium. A) The mG-HGEM transgenic construct carrying a heat-shock inducible histone H2B-mCherry protein. It also carries a membrane GFP protein separated by a 2A peptide sequence (GFP expression was not imaged in these experiments). As a screening tool the construct uses the lens-specific gamma-crystallin promoter to drive GFP expression. (B-G) Imaging H2B-mCherry expression in a cornea epithelial cell going through mitosis (arrowheads). (B) Pre-mitotic cell. (C) Prophase. (D) Metaphase. (E) Anaphase. (F) Telophase. (G) The resulting daughter cells. This particular division occurred over an unusually long distance. A movie of this division can be seen in Movie 4.2. Relative time is denoted in hours:minutes. Scale bar in G is 20 μm.
**Movie 4.1.** Movie of left hindlimb development. Relative time is denoted in hours:minutes. Frame rate is 21 frames per second. Scale bar = 250 μm

**Movie 4.2.** Movie of a mitotic division in cornea epithelium. Relative time is denoted in hours:minutes. Frame rate is 7 frames per second. Scale bar = 20 μm
4.6. References


Cakir Y, Strauch SM. 2005. Tricaine (MS-222) is a safe anesthetic compound compared to benzocaine and pentobarbital to induce anesthesia in leopard frogs (*Rana pipiens*). Pharmacol Rep 57:467-474.


CHAPTER 5. CONCLUSIONS AND PERSPECTIVES

This dissertation work focuses on understanding the cell and molecular mechanisms driving the process of the cornea-lens regeneration in the frog, *Xenopus laevis*. One of the key findings of this work is that Wnt/β-catenin signaling needs to be suppressed during larval cornea-lens regeneration. This necessary reduction in signaling appears to occur 24 hours into the process of lens regeneration, when there is an observable decrease in *axin2* that recovers by 48 hours. Our finding contributes to a growing body of work that cornea-lens regeneration in *Xenopus* possesses its own unique set of molecular signaling pathways compared to that of iris-derived (Wolffian) lens regeneration in the newt. Additionally, this requirement for reduced Wnt signaling in the cornea mirrors the observed role of Wnt signaling in the surface ectoderm during embryonic lens development. Taken together these results highlight two important points to be considered as we think about this system going forward. The first is that the molecular mechanisms driving cornea-lens regeneration in larval *Xenopus* appear to be very similar to the original embryonic development of the lens. This suggests that the observed regenerative phenomenon in larvae may be more the result of residual signaling mechanisms left over in the immature cornea from when the embryonic lens was originally formed, rather than being a unique pathway that has independently evolved to replace lost lenses. From this perspective, it is possible that our own fetal cornea epithelium may have some capacity to regenerate a new lens as well, but that the window of opportunity quickly closes as the cornea matures in utero. Another important point from this work is that it is critical that the field studies regenerative mechanisms in as many systems as possible. Despite the fact that newts and frogs are both capable of regenerating a lens, the molecular mechanisms used to achieve this end are clearly distinct, which may not be surprising considering they come from two different tissues with different developmental origins.

So if we take the stance that larval cornea-lens regeneration is primarily a redeployment of mechanisms used during embryonic lens development, it raises the question of how does the maturation of tissues, as tadpoles progress through
metamorphosis, affect the regenerative capacity of the cornea. A previous study reported that the post-metamorphic cornea epithelium is still competent to respond to regeneration inducing molecules, but that the mature cornea has lost its capacity to regenerate a lens in vivo. However that study did not address the possibility that regional differences in stem cell populations of the cornea could also be playing a role. One outstanding question in the field is the role of stem cells and their transit amplifying cells in cornea-lens regeneration, as we have proposed that stem cells serve as the source of the regenerated lens in larvae. Therefore, we hypothesized that the regenerative potential could become more restricted to the limbus of the mature cornea, where the stem cells of the cornea are thought to reside in humans. EdU label retention in the mature cornea, revealed a distinct bias towards the basal and peripheral cornea over 10 weeks, which is consistent with a population of slow-cycling stem cells in the limbus that send centripetally migrating transit amplifying cells in to replenish the central cornea. Based on this finding, we tested the regenerative potential of the central cornea and peripheral limbal region (both dorsal and ventral) and observed that all three regions are equally capable of initiating regeneration. Additionally, we found that the cells of the basal layer of the epithelium appear to be the most responsive to regenerate, matching what is observed in larval cornea-lens regeneration. Together, these results suggest that both limbal stem cells and the more broadly distributed transit amplifying cells in the basal layer of the mature cornea epithelium are able to respond to regeneration cues. However, more work is needed as we still haven’t identified the precise location of the individual stem cells that serve the Xenopus cornea, nor exactly how they and their progeny behave during cornea homeostasis and lens regeneration. Additionally, we propose that the steric hindrance of the basement membrane and the stroma may also be contributing to the inability of the mature cornea to regenerate. The basal cells of the cornea epithelium may still be capable of receiving the signals that initiate lens regeneration. However, the responding cells are stuck between stroma and the multiple differentiated apical layers that have developed, and so there may simply be no room for these cells to proliferate to form a new lens. It is interesting to speculate if this could be the case in mature human corneas.
In order to ultimately understand cornea epithelial behavior, we developed a new technique that allows for the continuous imaging of *Xenopus* over 48 hours. This technique offers a powerful new method to directly observe cell behaviors in the living cornea tissue. In conjunction with the power of transgenesis, it is now possible to lineage trace cells as they move through the cornea during cornea epithelial maintenance, wound healing, and lens regeneration. We expect that this technique will shed numerous insights into the field of cornea stem cell biology and regeneration, as we continue to investigate the cornea epithelium and its ability to regenerate a lens. Several future directions remain that need to be investigated, and they are discussed below.

**What are the molecules responsible for the observed inhibition of Wnt/β-catenin signaling?**

Our data suggests that within the first 48 hours of lens regeneration there is a necessary inhibition of Wnt signaling in the cornea epithelium. However, we still do not know the endogenous molecules that are responsible for this inhibition. Previous work in the field has identified three inhibitors of Wnt signaling that are upregulated during the first several days of lens regeneration (*secreted frizzled related proteins: sfrp2, sfrp3, and sfrp5*), making them ideal candidates for further functional analysis in this process. Perhaps some of these *sfrps* are an important component of the molecules secreted by the retina to induce lens regenerations. Additionally, our hypothesis would suggest that these same *sfrps* may be important for initiating embryonic lens development. Future work is needed to examine the location and timing of expression of these genes in the process of lens regeneration and development, as well as their functional significance.

**Is Wnt/β-catenin signaling in the cornea epithelium regulating cornea epithelial stem cells?**

From our work investigating Wnt/β-catenin signaling in lens regeneration, we demonstrated that the signaling pathway needs to be suppressed in order for lens regeneration to occur. While we don’t know exactly what Wnt signaling is doing in the cornea, one hypothesis we proposed is that it is helping to hold the cells of the basal layer of the cornea epithelium in an oligopotent stem cell state (see Chapter 2.4). If this is the
case, then coaxing a subset of those cells to differentiate towards the lens fate could require lifting Wnt signaling in those cells. Experiments to see if Wnt signaling is positively regulating the various pluripotency factors that have been shown to be expressed in *Xenopus* cornea would help shed light on this issue. Additionally, if this hypothesis is correct then functional experiments that maintain the pluripotency of the cornea epithelium would be expected to result in a failure of lenses to regenerate.

**Where is the precise location of the stem cells in the *Xenopus* cornea?**

Our EdU label retention in the mature (post-metamorphic) cornea strongly suggests the existence of limbal stem cells and that there is centripetal migration of cornea epithelial cells (transit amplifying cells) from the peripheral cornea. Using transgenic lineage tracers in conjunction with the prolonged *in vivo* imaging system we created would help us directly observe centripetal migration in the living tissue, and would contribute to a better understanding of where the stem cells are that serve the cornea.

**Can we reduce the mechanical inhibition of the stroma and/or cornea endothelium to restore *in vivo* lens regeneration in the mature frog?**

As we think about coaxing the cornea to regenerate a new lens in organisms that aren’t capable of doing so naturally, it appears that in the mature frog at least, the trick lies in getting the stroma and cornea endothelium out of the way. Future strategies should focus on *in vivo* approaches that mechanically disrupt the mature stroma and cornea endothelium to create room for the cornea epithelial cells to proliferate and form a new lens. Alternatively, as it appears that the entire basal layer of the mature cornea is competent to respond to regenerate, isolating these cells may allow for advances in *in vitro* regeneration approaches. It is very possible that there may also be molecular differences between the basal cells in the larval cornea and the mature cornea which are themselves responsible for the reduced potential to regenerate, but so far molecular studies in the post metamorphic regenerative process are essentially non-existent.
How have the roles of molecular signaling networks (e.g., Wnt/β-catenin signaling) changed during regenerative events in the post-metamorphic cornea epithelium compared to the larval cornea epithelium?

While the field has spent most of its effort understanding the molecular mechanisms that drive larval cornea-lens regeneration, it may actually be that the mature post-metamorphic cornea will yield insights that are more directly applicable to lens regeneration in a clinical setting. Nothing is currently known about the molecular signaling that is occurring during post-metamorphic regenerative events and how this signaling relates to larval lens regeneration. For instance, it is unknown whether the post-metamorphic cornea requires the same suppression of Wnt/β-catenin signaling that we observed in larval cornea. If the basal cells of the post-metamorphic cornea are unable to respond to suppressors of Wnt signaling, then it is possible that this could contribute to the inability of the mature cornea to regenerate a fully formed lens. Then again, if the signaling mechanisms between larval and post-metamorphic regenerative events are conserved, it would suggest that the failure to regenerate may be more of an issue of steric hindrance rather than one of cellular signaling. It is time that we take a second look at the post-metamorphic cornea and gain a better understanding of both the cell and molecular mechanisms that prevent regeneration from occurring in a mature cornea. These insights will also help us answer the question of why some animals are capable of cornea-lens regeneration while others are not, as cornea structure is well conserved among many vertebrates.
APPENDIX: DETAILED METHODS AND PROTOCOLS

STANDARD LENTECTOMY PROCEDURE

Solutions:

10x Normal Amphibian Media (NAM) - (Slack, 1984)

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<tr>
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<th>Final</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>1.11 M</td>
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<td>KCl</td>
<td>20.1 mM</td>
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<tr>
<td>Ca(NO₃)₂•4H₂O</td>
<td>10.16 mM</td>
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<tr>
<td>MgSO₄•7H₂O</td>
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<td>Na₂EDTA•2H₂O</td>
<td>993.98 mM</td>
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<td>dH₂O</td>
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- Autoclave

0.1 M NaPO₄ pH 7.5 – 1000 ml

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>Na₂PO₄ anhydrous</td>
<td>84.42 mM</td>
<td>11.9 g</td>
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<tr>
<td>NaH₂PO₄•H₂O</td>
<td>15.94 mM</td>
<td>2.2 g</td>
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<tr>
<td>dH₂O</td>
<td>800 ml</td>
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- pH to 7.5
- Bring volume up to 1000 ml
- Autoclave

0.1 M NaHCO₃ – 50 ml

<table>
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<th>Final</th>
<th>Quantity</th>
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<tbody>
<tr>
<td>NaHCO₃</td>
<td>100 mM</td>
<td>0.42 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>50 ml</td>
<td></td>
</tr>
</tbody>
</table>

- Mix well and aliquot 500 µL into 1.5 ml eppendorf tubes
- Store at -20°C
## 1/20x NAM

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x NAM</td>
<td>1/20x</td>
<td>5 ml</td>
</tr>
<tr>
<td>0.1 M NaPO₄ pH 7.5</td>
<td>2 mM</td>
<td>20 ml</td>
</tr>
<tr>
<td>0.1 M NaHCO₃</td>
<td>0.05 mM</td>
<td>500 µl</td>
</tr>
<tr>
<td>dH₂O</td>
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<td>Up to 1000 ml</td>
</tr>
</tbody>
</table>

- Shake before use

## Anesthetic Solution (1:2000 MS-222) – 50 ml

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<th>Final</th>
<th>Quantity</th>
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<tr>
<td>MS-222 (Sigma, St. Louis, MO)</td>
<td>1:2000</td>
<td>0.025 g</td>
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<tr>
<td>1/20x NAM</td>
<td></td>
<td>50 ml</td>
</tr>
</tbody>
</table>

1. Anesthetize tadpoles
2. Transfer tadpole into MS-222 anesthetic solution (about 3 mL in a 60 x 15 mm round Tissue Culture Dish) until tadpole stops moving.
3. Prepare the surgical dish. Surgical dish is a 60 x 15 mm round Tissue Culture Dish half full with non-toxic modeling clay (green; Van Aken International, Rancho Cucamonga, CA). The surgical dish with clay is then filled with anesthetic solution. Using a rounded glass probe created by heating the end of a 5 ¾” glass Pasteur pipette, carve out a tadpole shaped pit in the modeling clay, so that the tadpole can rest down the clay with one side up (conventionally we lentectomize the left side).
4. Looking through a stereomicroscope, gently place the anesthetized tadpole into the pit in the modeling clay of the surgical dish. Gently secure the tadpole by using the rounded glass probe to fold small projections of clay over the tadpole. It is important not to press clay down into the animal, especially in the region of the gut or heart, and care should also be taken not to damage the tail.
5. Using customized scissors made by Jonathan Henry from Dumont #5 forceps, make one cut into the dorsal-posterior portion of the cornea epithelium with the scissors. This cut is typically about 1/3 up to 1/2 of the circumference of the eye.
6. Using the scissors, insert the forceps under the outer cornea and make a cut in the posterior side of the inner cornea, at the edge of the pupillary space. Take care not to damage the pupil or underlying lens/retina.

7. Using sharpened Dumont #5 forceps, remove the lens of the eye through the hole made into the cornea endothelium. NOTE: It is critical that the entire lens be removed. If the lens is damaged, falling apart, or not spherical, discard the animal and repeat as residual lens cells left behind from an incomplete lentectomy could interfere with experimental results.

8. Care should be taken not to break the central point of attachment between the inner and outer corneas during this procedure.

9. For in vivo regeneration, animals are immediately placed into a solution of 1/20x NAM to recover for 24 hours, before returning to standard animal care. Alternatively the Ex Vivo Eye Culture Assay can be carried out (see below).

EX VIVO EYE CULTURE ASSAY

Adapted from Fukui and Henry (2011)

Solutions:

Modified L-15 Culture Medium (mL15) – 500 ml

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<th>Ingredient</th>
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<th>Quantity</th>
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<tr>
<td>Leibovitz’s L-15 Powder</td>
<td>60 %</td>
<td>4.18 g</td>
</tr>
<tr>
<td>Fetal Bovine Serum (FBS)</td>
<td>10 %</td>
<td>50 ml</td>
</tr>
<tr>
<td>dH2O</td>
<td></td>
<td>Up to 500 ml</td>
</tr>
</tbody>
</table>

- Dissolve L-15 Powder in approximately 450 ml dH2O
- Add FBS and pH to 7.6
- Adjust volume up to 500 ml with dH2O
- Filter purify medium using 0.2 µm filter in sterile laminar flow hood
- Divide into 50 ml aliquots and store at 4°C
**Working mL15 Medium (contains antibiotics) – 15 ml**

<table>
<thead>
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<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>mL15 Medium</td>
<td>---</td>
<td>14.64 ml</td>
</tr>
<tr>
<td>Penicillin/Streptomycin (100x)</td>
<td>100 U/ml, 100 µg/ml</td>
<td>150 µl</td>
</tr>
<tr>
<td>Amphotericin B (250 µg/ml)</td>
<td>2.5 µg/ml</td>
<td>150 µl</td>
</tr>
<tr>
<td>Marbofloxacin (1 mg/ml)</td>
<td>4 µg/ml</td>
<td>60 µl</td>
</tr>
</tbody>
</table>

- This solution should be prepared under sterile conditions in a Laminar Flow Hood
- Working solutions are made fresh every time
- Pen/Strep is a general antibiotic to prevent bacterial contamination, Amphotericin B is an antifungal used to prevent fungal contamination, and Marbofloxacin is used to specifically prevent *Pseudomonas* contamination that plagued our experiments.
- This recipe can be scaled up or down to match the volume needed for an experiment

1. In a laminar flow hood, prepare an appropriate amount of Working mL15 Media as dictated by the experimental needs. Eyes are typically cultured in 500 µl of Working mL15 per well of a 24-well cell culture plate (for a 24-well plate, 12 ml of Working mL15 is needed). It is best to keep one eye per well, but up to 5 eyes can be placed in a single well if necessary.

   a. One major advantage of the ex vivo culture system is that it allows for treatment of eye tissues in small volumes, to reduce the amount (and therefore cost) of small molecule inhibitor or exogenous protein necessary to conduct an experiment. To ensure consistency between all of the wells, master aliquots should be made for the treated wells (adding a small molecule inhibitor or exogenous protein to Working mL15), or control wells (adding the % v/v of the solvent that the inhibitors or proteins are dissolved in, typically DMSO, to Working mL15), and the wells of the 24-well plate should be filled from the master aliquots.
b. 3 wash plates should also be made in order to dilute any possible
contaminates or debris that is transferred from the surgical dish. Each wash
plate is a 35 x 10 mm petri dish containing approximately 3 ml of the
Working mL15 medium. If conducting a drug treatment experiment, a set
of treated wash dishes, and control wash dishes are needed containing the
appropriate medium. If the drug is cost prohibitive, this step may need to be
modified to reduce the amount of treated medium that is being used in the
experiment.

c. Wash plates 2 and 3, as well as the prepared 24-well plate remain in the
laminar flow hood, while wash plate 1 is taken to the dissecting microscope.

2. Carry out Steps 1-9 of the **Standard Lentectomy Procedure** (described above).

3. Using the fine scissors, cut all the way around the cornea epithelium, leaving the
cornea epithelium attached to the cornea endothelium via the central stalk.

4. Using sharpened forceps, gently tuck the cornea epithelium into the neural retina into
the eye. It is helpful to take care to leave some cornea endothelium over the pupillary
space during the initial lentectomy, as it helps to hold the cornea epithelium inside the
eye.

5. Using the fine scissors, cut the optic nerve and vessel and surrounding ocular tissues
(muscles) to remove the eye from the tadpole.

6. Using a cut 200 µl pipette tip (cut large enough to accommodate the eye), gently draw
the eye into the pipette tip and transfer it into the Treated Wash 1 dish. It is important
to transfer the eye in as little volume as possible.

   a. Cut pipette tips are made by taking SHARP® Precision Barrier Tips,
   Standard Length for P-200, 200 µl (Denville Scientific Inc.; Cat # P1122)
   and cutting the tip using a sterile razor blade at the first gradation line. Cut
   pipette tips are then replaced into the original holder and autoclaved to
   sterilize them. A fresh tip is used for each transfer, so it is important to
   always have a box or two prepared before beginning an experiment.

7. If using the *ex vivo* culture system for a drug treatment experiment, it is important that
one animal provide both an experimental eye and a control eye. Using the rounded
glass probe, gently release the tadpole from the clay and turn it over so that its right
side is now facing up. Repeat the surgical process for this eye, and place it into the Control Wash 1 dish.

8. After all eyes have been lentectomized, removed and placed into their respective Wash 1 plates, transfer all eyes into the second wash plate using a cut 200 µl pipette tip. Gently swirl the eyes around and let sit for 5-15 minutes. This should occur in the laminar flow hood under sterile conditions.

9. Repeat Step 8, transferring eyes to the third wash plate.

10. Finally transfer eyes into the prepared 24-well plate. Ideally only one eye will be in each well, but if the drug is cost prohibitive, up to 5 eyes can be cultured in one well.

**HISTOLOGY PROTOCOL**

*Adapted from Humason (1972)*

**Solutions:**

**10x Phosphate Buffered Saline (PBS) – 1 L**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
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<tbody>
<tr>
<td>NaCl</td>
<td>1.4 M</td>
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<td>KCl</td>
<td>26.8 mM</td>
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<tr>
<td>Na₂HPO₄</td>
<td>101.4 mM</td>
<td>14.4 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>17.6 mM</td>
<td>2.4 g</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>Up to 1000 ml total volume</td>
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</tbody>
</table>

- Check that pH is about 7.4
- Autoclave

**1x Phosphate Buffered Saline (PBS) – 1 L**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PBS</td>
<td>1x PBS</td>
<td>100 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>900 ml</td>
</tr>
</tbody>
</table>
1. Fix 3 hours in 3.7% formaldehyde in 1x PBS (or overnight at 4°C)
   a. For excised eyes use 3 hours
   b. For tadpoles or froglets overnight at 4°C
2. Wash 5-10 minutes in 1x PBS
3. Wash 5-10 minutes in 1x PBS
4. Wash 5-10 minutes in 1x PBS
   a. For tadpoles or froglets, tissue of interest can be removed at this time to reduce volumes needed for the remaining procedure
5. 10 min wash in 30% EtOH (all washes carried out in 8 ml Wheaton Glass Specimen Vials)
6. 10 min wash in 50% EtOH
7. 10 min wash in 70% EtOH
8. 10 min wash in 95% EtOH
9. 10 min wash in 100% EtOH
10. 10 min wash in 100% EtOH
11. 10 min wash in 1:1 xylene/100% EtOH (in chemical hood)
12. 10 min wash in 100% xylene (in chemical hood)
13. 10 min wash in 100% xylene (in chemical hood)
14. Reduce volume of Xylene to about 1/3 of 8 ml Wheaton Glass Specimen Vials
15. Place into 60°C oven for 10 minutes to allow xylene to warm (be sure vial is capped)
16. Add warm Paraplast 1:1 to xylene
   a. Paraplast Plus Tissue Embedding Medium (McCormick Scientific, St. Louis, MO)
   b. Incubate 6 hours (or overnight) in oven at 60°C
17. Remove xylene/Paraplast and add fresh 100% Paraplast
   a. Incubate 6 hours (or overnight) in oven at 60°C
18. Fill empty embedding molds with fresh 100% Paraplast
   a. Peel-A-Way Disposable Embedding Molds (T-12) Truncated, 22 mm square top tapered to 12 mm bottom (Polysciences, Warrington, PA)
19. Transfer tissue into embedding molds
   a. Wait 6 hours (or overnight) in oven at 60°C
20. Using a heated dissecting needle, orient specimens in the bottom of the embedding molds, making sure that tissue is centered and away from the edges of the embedding mold
   a. Let blocks harden at room temperature
   b. Store molds at 4°C until ready to mount and section
21. Remove the embedding mold using a razor blade by slitting the corners of the mold and peeling away from the wax block. Using a metal spatula heated over a flame, melt the freshly cut side of the mold and place it onto a wooden tissue block (the bottom side of the mold that contains the tissue should be out and away from the block).
22. Trim the embedded block face with the tissue to remove excess Paraplast away from the tissue. It is important to leave a small amount of Paraplast around the tissue, but generally the tighter to the tissue you can trim, the more sections you can fit on a single slide. It is also critical to keep the edges of the trimmed potion parallel to one another. If they are off by even a little bit, as sectioning occurs the ribbon will curve, reducing the number of sections you can easily put on a single slide.
23. Clamp the wooden tissue block into the microtome. Make sure to align one trimmed face of the embedded mold that contains the tissue with the blade. It should be square in the X, Y, and Z planes.
24. Section specimens at a thickness of 8 µm, until a ribbon of sections of the desired length is achieved.
25. Using fine paint brushes, gently remove the ribbon and orient and place on the slide.
26. Continue to place ribbons of tissue on the slide, maintaining the same orientation, so that the maximum number of sections have been placed on the slide.
27. Using a glass Pasteur pipet, gently dispense distilled water underneath the sections so that they are floating on the water. This ensures that the sections stretch to remove wrinkles and folds.
28. Slowly remove the water so that the tissue lies flat on the glass slides, then place on a slide warmer at 37°C until all of the water has evaporated.
29. Slides can be stored at 4°C.
ANTI-LENS ANTIBODY STAINING OF SECTIONED EYES

1. Let slides reach room temperature
2. 10 minute wash in 100% Xylene (preferred) or Histo-clear (this dewaxes slides)
3. 10 minute wash in 100% Xylene or Histo-clear
4. Air dry the slides at RT. Should take less than 1 minute for Xylene, more for Histo-clear. Make sure the slides are completely clear.
5. 5 minute wash in PBS
6. 30 minute wash in 0.1 M glycine in PBS
   a. 50 ml PBS
   b. 0.375 g glycine
7. 5 minute PBS wash
8. 45 minutes in 5% dry milk in PBS
   a. 50 ml PBS
   b. 2.5 g Dry milk
9. Put slides in a moisture chamber and add 200-300 µl of Primary Antibody (Rabbit anti-lens)
10. Incubate slides in moisture chamber for at least 2 hours (RT) or overnight (4°C)
11. Collect the primary antibody
12. 10 minute wash in PBS
13. 10 minute wash in PBS
14. Dilute fluorescent secondary antibody 1:500 (Goat anti-Rabbit) in 5% dry milk and PBS
15. Add 200 µl of secondary antibody solution to slides in a moisture chamber
16. Incubate in dark in secondary antibody for at least 1 hour (RT) or overnight (4°C).
17. 10 minute wash in PBS
18. 10 minute wash in PBS
19. Mount in 80% glycerol / 20% PBS
20. Store slides at 4°C
POLYMERASE CHAIN REACTION (PCR)

Standard PCR was carried out using *Taq* DNA Polymerase (NEB, Cat # M0273S) and recommended manufacturer’s protocols.

**Standard PCR Reaction Mix**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
<th>50 µl Reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Standard Taq Reaction Buffer</td>
<td>1x</td>
<td>5 µl</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>200 µM</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 µM Forward Primer</td>
<td>200 nM</td>
<td>1 µl</td>
</tr>
<tr>
<td>10 µM Reverse Primer</td>
<td>200 nM</td>
<td>1 µl</td>
</tr>
<tr>
<td>Template</td>
<td>variable</td>
<td>variable</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>0.25 µl</td>
<td>1.25 units</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>up to 50 µl</td>
<td>N/A</td>
</tr>
</tbody>
</table>

**Standard Thermocycler conditions**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Duration</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Cycle (30-40 cycles)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Annealing</td>
<td>Variable*</td>
<td>30 seconds</td>
</tr>
<tr>
<td>Extension</td>
<td>68°C</td>
<td>1 minute/kb</td>
</tr>
<tr>
<td>Final Extension</td>
<td>68°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>Forever</td>
</tr>
</tbody>
</table>

*Annealing temperature is determined using online calculators such as the NEB Tm Calculator (https://www. http://tmcalculator.neb.com/#!). Gradient PCR can be done to optimize the optimal annealing temperature.
REVERSE TRANSCRIPTASE PCR (RT-PCR)

1. Homogenize tissue in TRIZol
   a. 50 µl TRIZol
   b. Homogenize using fitted pestle
   c. Add 300 µl TRIZol
   d. Vortex 1 minute
   e. Add 350 µl 100% EtOH
   f. Vortex 1 minute
2. Load 700 µl TRIZol solution onto Direct-zol Miniprep column
   a. Follow manufacturer’s protocol
   b. Elute in 45 µl
3. DNase Treatment (in PCR tube)
   a. 45 µl RNA elution
   b. 5 µl 10x Buffer (NEB)
   c. 1 µl DNase I (NEB)
   d. In Thermocycler
      i. 37°C for 10 min
      ii. Add 1 µl 0.5M EDTA
      iii. 75°C for 10 min
4. Nuc-Away Cleanup
   a. Follow manufacturer’s protocol
5. Quantify RNA concentration and purity using Nanodrop
6. iScript cDNA Synthesis (in PCR tube)
   a. + RT Reactions
      i. 4 µL 5x Buffer
      ii. 1 µL RT
      iii. 15 µL RNA + water (10-25ng cDNA per reaction)
   b. – RT
      i. 4 µL 5x Buffer
      ii. 16 µL RNA + water (10-25ng cDNA per reaction)
SPERM NUCLEI TRASGENESIS

Adapted from Smith et al. (2006)

1. Prepare Solutions for Transgenesis

**10x Marc’s Modified Ringer (MMR) – 1 L**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1 M</td>
<td>58.443 g</td>
</tr>
<tr>
<td>KCl</td>
<td>20 mM</td>
<td>1.491 g</td>
</tr>
<tr>
<td>MgCl₂*6H₂O</td>
<td>10 mM</td>
<td>2.0331 g</td>
</tr>
<tr>
<td>CaCl₂*2H₂O</td>
<td>20 mM</td>
<td>2.9403 g</td>
</tr>
<tr>
<td>HEPES</td>
<td>50 mM</td>
<td>11.915 g</td>
</tr>
</tbody>
</table>

- pH to 7.5 with NaOH
- Autoclave

**1x Marc’s Modified Ringer (MMR) – 1 L**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x MMR</td>
<td>1x MMR</td>
<td>100 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>900 ml</td>
</tr>
</tbody>
</table>

- Autoclave

**0.1x Marc’s Modified Ringer (MMR) – 1 L**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x MMR</td>
<td>1x MMR</td>
<td>100 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>900 ml</td>
</tr>
</tbody>
</table>

- Autoclave
Injection Plates (1% Agarose in 0.4x MMR plates) – 200 ml

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x MMR</td>
<td>0.4x MMR</td>
<td>80 ml</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td></td>
<td>120 ml</td>
</tr>
<tr>
<td>Agarose</td>
<td>1% (w/v)</td>
<td>2.0 g</td>
</tr>
</tbody>
</table>

- Melt agarose in microwave
- Pour approximately 14 ml into each of 14 60mm x 15mm petri dishes
- Place a small weigh boat (1 5/8 sq. in.) in the center of the dish
- Place a weight inside the center of the weigh boat
- Allow agarose to cool (approximately 20-30 minutes)
- Wrap each boat in parafilm and store at 4ºC (good for weeks)
- Warm to 16ºC overnight before use

Injection Solution - 0.4x MMR, 4% ficoll, and 10 ug/ml gentamicin – 50 mL

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x MMR</td>
<td>0.4x MMR</td>
<td>20 ml</td>
</tr>
<tr>
<td>Ficoll</td>
<td>4% w/v</td>
<td>2g</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10 ug/ml</td>
<td>10 ul (50 mg/ml stock)</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td></td>
<td>Up to 50 ml total volume</td>
</tr>
</tbody>
</table>

- Filter Sterilize
- Store @ 4ºC
- Warm to 16ºC overnight before use
Sperm Dilution Buffer (SDB)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>250 mM</td>
<td>1.67 ml (1.5 M stock)</td>
</tr>
<tr>
<td>KCl</td>
<td>75 mM</td>
<td>375 µl (2 M stock)</td>
</tr>
<tr>
<td>Spermidine trihydrochloride</td>
<td>0.5 mM</td>
<td>0.5 ml (10 mM stock)</td>
</tr>
<tr>
<td>Spermine tetrahydrochloride</td>
<td>0.2 mM</td>
<td>0.2 ml (10 mM stock)</td>
</tr>
<tr>
<td>HEPES (pH 7.5)</td>
<td>200 µM</td>
<td>20 µl (100 mM stock)</td>
</tr>
<tr>
<td>H₂O</td>
<td></td>
<td>7.2 ml</td>
</tr>
</tbody>
</table>

- Check that solution is 7.3-7.6, if necessary adjust
- Divide into 500 µl aliquots
- Store at -20ºC

2. Pull needles for injection
   a. Pull 50 µl Drummond microcaps (Drummond Scientific, Cat # 1-000-0500) using World Precision Instruments PUL-1 Micropipette Needle Puller with the following pull settings: Delay = 1, Heat = 10

3. Inject female *Xenopus laevis* with human chorionic gonadotropin (hCG)
   a. Dilute 10,000 U hCG in 5.2 ml of sterile water (or PBS)
   b. Inject each female frog with roughly 1400 U of hCG (0.75 ml)
      i.Injection should be made into the dorsal lymph sac (dorsal side of each frog just beside the lateral line system near the hind leg).
      ii. Be sure that all air bubbles have been removed out of syringe
   c. Leave female frogs in the dark overnight
   d. Females can start laying as early as 8 hours post injection
   e. NOTE: 2 females are typically injected to ensure at least one is producing enough eggs for the experiment.
4. Preparing oocytes for injection
   a. Retrieve Sperm nuclei stock from -80°C freezer, keep in ice
   b. Warm up the transgene @ 65°C for 5 minutes
   c. While transgene is warming, prepare 200 ml of 2.5% cysteine in 1xMMR

   **2.5% cysteine in 1x MMR**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>2.5% w/v</td>
<td>5 g</td>
</tr>
<tr>
<td>1x MMR</td>
<td></td>
<td>200 ml</td>
</tr>
</tbody>
</table>
   - Adjust pH with NaOH to 7.8 – 8.0

   d. Place transgene in ice for 1 min
   e. Add 1 µl transgene (200 - 300 ng) to 4 µl sperm nuclei stock
   f. Mix by manually stirring with pipet tip
      - DON’T PIPET UP AND DOWN! – This will damage sperm nuclei
   g. Incubate sperm nuclei/transgene mix for 5 minutes at room temperature
   h. Add 22 µl Sperm Dilution Buffer to sperm/transgene mix, stirring with pipet tip
   i. Incubate for 15 minutes at room temperature
      - While waiting, collect first batch of eggs
   j. Dilute sperm nuclei/transgene using 300 µl of Sperm Dilution Buffer
   k. Using a 200 µl cut pipette tip, pipette up and down 30x to mix
   l. Keep Sperm nuclei/transgene in 16°C Incubator

5. Collecting oocytes for injection
   a. Dejelly eggs in 2.5% cysteine in 1x MMR
      - Only takes a few minutes, done when oocytes are just touching one another
   b. Wash 3 times in 1x MMR, remove any obviously bad eggs
   c. Pour Injection solution into Injection plates
   d. Transfer eggs into injection plates (usually 2 dishes per collection)
   e. Incubate at 16°C for 5 minutes
6. Injections
   a. Set up apparatus and turn flow on to 0.6 µl/min
      - This may need to be adjusted to match your personal injection speed. If you see most of your embryos have abnormal cleavage early, you may be injecting too much material and need to slow the pump down (or inject faster).
   b. Break injection needle tip to approximately 50 µm using razorblade under stereo microscope. These needles tend to naturally break at approximately this size after being gently pierced one time through a stretched KimWipe.
   c. Using cut 200 µl tips and a P-200 pipette, mix the transgene solution by pipetting up and down several times and then draw up 70 µl of sperm nuclei/transgene
   d. Use capillary action to pull the nuclei mixture into the blunt end of the injection needle by placing it into the cut pipette tip (save any remaining transgene mix)
   e. Remove bubbles from the injection rig line by advancing the slider, then attach the needle
   f. Quickly inject each oocyte a single time into the animal pole.
      - Timing is critical. If the needle is in the embryo too long, you may deliver two sperm nuclei and the embryo will not cleave properly. If you are in and out too fast and you may not get fertilization. You can always adjust the flow rate of the injection pump to help adjust to your natural injection timing.
   g. Replace agarose plate with injected oocytes back into 16ºC
   h. Wait 3-4 hours for first sorting (4-cell stage).

7. Change 0.1x MMR daily and remove any dead or malformed embryos.
8. On day 5-7 post injections the embryos are ready to sort for the presence of GFP positive lenses. A “full transgenic” animal will express robust GFP throughout the lenses of both eyes. “Partial transgenics” may only express GFP in a single lens, or it may be mosaic with in the lens(es). Discard nontransgenic animals.

9. 7 days after injections, transgenic animals can be moved to general tadpole care and fed yeast for 7 days following standard rearing practices.

**EdU INJECTION AND LABELING IN XENOPUS CORNEA**

*Adapted from Perry et al. (2013) & Salic and Mitchison (2008)*

**EdU Injection**

<table>
<thead>
<tr>
<th>EdU for Injection – 50 µl</th>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mM EdU</td>
<td>5 mM</td>
<td>10 µl</td>
</tr>
<tr>
<td></td>
<td>Filtered PBS</td>
<td>---</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

1. Anesthetize a froglet in 100 ml of 1:2000 MS-222 (as described above) in a 100 x 50 mm crystallizing dish dedicated to live animal use (i.e. has never contained toxic chemicals, fixatives, or been exposed to detergents).
   a. Anesthesia takes longer in froglets than it does in larvae.

2. Place 2-3 Kim Wipes soaked in 1:2000 MS-222 into a 60 x 15 mm petri dish to create the froglet injection dish.

3. Once the froglet is completely anesthetized, remove it from the MS-222 solution and place it ventral side up in the froglet injection dish. The MS-222 soaked Kim Wipes can be slightly shaped to help keep the froglet secure and anesthetized. Place the froglet injection dish under a stereomicroscope.

4. Using fine scissors that have been custom made from Dumont #5 forceps, cut a small incision through the ventral skin of the froglet in the abdominal region. This is easiest if the scissors are used to gather a small fold of skin, and then cut the fold.
5. Using a 10 µl Hamiton Syringe with a 27 gauge needle, inject 2 µl of EdU into the intraperitoneal space of the froglet.

6. Let the froglet rest for approximately 1 minute to give the EdU time to disperse so that it doesn’t all immediately leak out of the injection site.

7. After 1 minute rest, gently transfer the froglet into 1/20x NAM in a 100 x 50 mm crystallizing dish rocking gently on a table top rocker.

8. Once the froglet has recovered from the anesthesia, place it into a large bowl in 1/20x NAM for 24 hours before returning it to standard animal care.

**EdU Labeling**

### 10x Tris Buffered Saline (TBS) – 1 L

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>0.5 M</td>
<td>60.6 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>1.54 M</td>
<td>90 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>---</td>
<td>Up to 1000 ml</td>
</tr>
</tbody>
</table>

- pH to 8.4 using HCl
- Autoclave

### 1x TBS – 1 L

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TBS</td>
<td>1x</td>
<td>100 ml</td>
</tr>
<tr>
<td>dH₂O</td>
<td>---</td>
<td>900 ml</td>
</tr>
</tbody>
</table>

### 1 M Tris – 1 L

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>1.0 M</td>
<td>121.14 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>---</td>
<td>Up to 1000 ml</td>
</tr>
</tbody>
</table>

- pH to 8.5 using HCl
**0.5 M CuSO₄ – 20 ml**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuSO₄</td>
<td>0.5 M</td>
<td>1.596 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>---</td>
<td>Up to 20 ml</td>
</tr>
</tbody>
</table>

**0.5 M Ascorbic Acid – 50 ml**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascorbic Acid</td>
<td>0.5 M</td>
<td>4.403 g</td>
</tr>
<tr>
<td>dH₂O</td>
<td>---</td>
<td>Up to 50 ml</td>
</tr>
</tbody>
</table>

**Click-it Solution – 1.0 ml (add in order)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Final</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH₂O</td>
<td>---</td>
<td>697 µl</td>
</tr>
<tr>
<td>1 M Tris</td>
<td>100 mM</td>
<td>100 µl</td>
</tr>
<tr>
<td>0.5 M CuSO₄</td>
<td>1 mM</td>
<td>2 µl</td>
</tr>
<tr>
<td>Fluorescent Azide (10 mM)</td>
<td>10 µM</td>
<td>1 µl</td>
</tr>
<tr>
<td>0.5 M Ascorbic Acid</td>
<td>100 mM</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

1. Euthanize froglets in MS-222
2. Fix froglets in 50 ml of 3.7 % formaldehyde diluted in 1x TBS overnight at 4ºC
3. Wash 3 times in TBS for 5-10 minutes on bench-top rocker
4. Surgically remove eyes from animals. This can be done using the custom scissors made out of Dumont #5 forceps (similar to Lumsden BioScissors, BioRad). As the fixed froglet tissues are tougher to cut through than larval tissues, use a pair of scissors that have good cutting action on the sides. It is much less important for them to have good tip cutting action.
5. Once eyes have been removed, cut the neural retina and surrounding tissues to cut the eye in half, removing the posterior portion from the anterior portion of the eye.
6. Using Dumont #5 forceps, remove the lens from the anterior portion of the eye.
7. Using a combination of forceps and scissors, carefully trim back the non-cornea tissue on the anterior portion of the eye. It is important to leave a ring of pigmented skin around the cornea, as this is a clear boundary for where the peripheral cornea ends.

8. Using the forceps, gently peel away the neural retina and pigmented retina epithelium. Be careful not to damage the cornea during this procedure. The fixed tissue typically pulls out together in large tissues.

9. Once corneas are relatively free of extraneous tissues and the pigmented ocular tissues have been removed, corneas are placed into 1x TBS in 1.5 ml Eppendorf tubes.

10. Repeat one more wash of 1x TBS for 5-10 min on rocker.

11. Incubate corneas for 20 minutes in 1x TBS + 0.5% Triton X-100 at room temperature

12. Wash 2 times in 1x TBS for 5-10 minutes, rocking
   a. During this time assemble the Click-it solution. It should always be freshly made.

13. Incubate corneas in the Click-it solution, for 30 min at room temperature and in the dark
   a. NOTE: From this point on, all steps should be carried out in the dark.

14. Wash 3 times in 1x TBS + 0.5% Triton X-100 for 5-10 minutes, rocking in the dark
   a. If high background is an issue, an additional wash overnight at 4°C may be beneficial.

15. Wash 3-4 times in 1x TBS at room temperature

16. Incubate in 1:10,000 Hoechst diluted in TBS for 30 min

17. Wash 2-3 times in 1x TBS

18. Mount specimen in either ProLong Gold Antifade Mountant (Thermo Fisher Scientific, which will preserve the signal better, but tissue can’t be easily recovered for other staining) or in 80% glycerin/20% TBS (or PBS works, too).
A.1. References


