Published in final edited form as:

Exp Eye Res. 2016 November ; 152: 94–99. doi:10.1016/j.exer.2016.08.013.

The lens regenerative competency of limbal vs. central regions of mature *Xenopus* cornea epithelium

Paul W. Hamilton^{1,2} and Jonathan J. Henry^{2,*}

¹Department of Biology, Illinois College, 1101 W. College Ave., Jacksonville, IL 62650 United States of America

²Department of Cell & Developmental Biology, University of Illinois, 601 S. Goodwin Ave., Urbana, IL 61801 United States of America

Abstract

The frog, *Xenopus laevis*, is capable of completely regenerating a lens from the cornea epithelium. Because this ability appears to be limited to the larval stages of *Xenopus*, virtually all the work to understand the mechanisms regulating this process has been limited to pre-metamorphic tadpoles. It has been reported that the post-metamorphic cornea is competent to regenerate under experimental conditions, despite the fact that the *in vivo* capacity to regenerate is lost; however, that work didn't examine the regenerative potential of different regions of the cornea. A new model suggests that cornea-lens regeneration in *Xenopus* may be driven by oligopotent stem cells, and not by 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. Using EdU (5-Ethynyl-2'-deoxyuridine), we identified 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. Using this data to identify putative stem cells of the limbal region in *Xenopus*, we tested the regenerative competency of limbal regions and 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. Thus, the regenerative competency in post-metamorphic cornea is not restricted to stem cells of the limbal region, but also occurs in the transit amplifying cells throughout the basal layer of the cornea epithelium.

Keywords

regeneration; lens; cornea epithelium; *Xenopus*; limbus

*Corresponding author. j-henry4@illinois.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflicts of Interest

The authors have no conflicts of interest.

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 report 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 a post-metamorphic eye cup is able to regenerate (Bosco and Willems, 1992).

Development of the *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 competency of the mature cornea that could be related to the specific location of the stem cells of the cornea. We hypothesized that lens regeneration may actually be driven by stem cells and/or their transit amplifying

progeny present throughout the basal layer of the larval cornea epithelium (Perry et al., 2013), and not via 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 expressed by 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. This observation suggests 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 might 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). DNA 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 diluted 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 basal 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.

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 μ l 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 4°C. Corneas were then removed from 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 μ M 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. 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. 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 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 μ m, 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. 1B) and apical layers (Fig. 1C). However, as time passes this signal is lost preferentially in the center of the cornea compared to the peripheral regions (Fig. 1D-O). After three weeks of chase, the EdU signal in the central cornea becomes noticeably diminished (Fig. 1K-O). The basal layer of the central epithelium had very few nuclei retaining label (Fig. 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. 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. 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. 1F, I, J), as well as in some overlying apical layers (Fig. 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. 1F). After ten weeks of chase (Fig. 1P-AA), very few cells of the basal or apical layers retain label in the central cornea (Fig. 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. 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 in the limbal region 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. 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, we tested the regenerative competency 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. 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 μ m 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. 2H): 63.3% positive regeneration from dorsal limbal region (19/30 transplants, Fig. 2B, C); 41.4% from central cornea (12/29 transplants, Fig. 2D, E), and 51.9% from ventral limbal region (14/27 transplants, Fig. 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 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. 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. 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. 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. These post-metamorphic cornea epithelium are still competent to respond to regenerative signals to initiate the conversion of basal cells towards the lens fate; however, the capacity to regenerate a complete lens is not present, consistent with the results of Filoni et al. (1997).

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. 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. 1D). However, there was no significant difference between the three regions tested here (Fig. 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. 2I). These cells likely include limbal stem cells and/or 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 surrounding tissue layers are absent or immature (Fig. 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 retinal 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*. Finally, our data demonstrate that the regenerative competency of the post-metamorphic cornea epithelium is not restricted to the limbal region where the label retaining cells reside, but rather includes other cells found throughout the entire basal layer of the cornea epithelium (e.g, transit amplifying cells). These findings have important implications as far as understanding how lens regeneration takes place in these animals, and calls into question previous hypotheses arguing that this process takes place via transdifferentiation of differentiated cornea cells.

Acknowledgments

This research was supported by NEI grant EY023979 to JJH.

References

- Chung E, Bukusoglu G, Zieske JD. Localization of Corneal Epithelial Stem Cells in the Developing Rat. *Invest Ophthalmol Vis Sci*. 1992; 33:2199–2206. [PubMed: 1607230]
- Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989; 57:201–209. [PubMed: 2702690]
- Davies SB, Chui J, Madigan MC, Provis JM, Wakefield D, Di Girolamo N. Stem cell activity in the developing human cornea. *Stem Cells*. 2009; 27:2781–2792. [PubMed: 19711455]
- Filoni S, Bernardini S, Cannata SM, D'Alessio A. Lens regeneration in larval *Xenopus laevis*: experimental analysis of the decline in the regenerative capacity during development. *Dev Biol*. 1997; 187:13–24. [PubMed: 9224670]
- Fisher RA. On the Interpretation of χ^2 from Contingency Tables, and the Calculation of P. *J. R. Stat. Soc.* 1922; 85:87–94.

- Freeman G. Lens regeneration from the cornea in *Xenopus laevis*. J Exp Zool. 1963; 154:39–65. [PubMed: 14066571]
- Fukui L, Henry JJ. FGF signaling is required for lens regeneration in *Xenopus laevis*. Biol Bull. 2011; 221:137–145. [PubMed: 21876116]
- Graw J. Eye Development. Curr Top Dev Biol. 2010; 90:343–386. [PubMed: 20691855]
- Hamilton PW, Sun Y, Henry JJ. Lens regeneration from the cornea requires suppression of Wnt/beta-catenin signaling. Exp Eye Res. 2016; 145:206–215. [PubMed: 26778749]
- Henry JJ. The cellular and molecular bases of vertebrate lens regeneration, International review of cytology. 2003:195–265.
- Henry JJ, Elkins MB. Cornea-lens transdifferentiation in the anuran, *Xenopus tropicalis*. Dev Genes Evol. 2001; 211:377–387. [PubMed: 11685571]
- Henry JJ, Grainger RM. Inductive interactions in the spatial and temporal restriction of lens-forming potential in embryonic ectoderm of *Xenopus laevis*. Dev Biol. 1987; 124:200–214. [PubMed: 3666306]
- Henry JJ, Mittleman JM. The Matured Eye of *Xenopus laevis* Tadpoles Produces Factors That Elicit a Lens-Forming Response in Embryonic Ectoderm. Dev Biol. 1995; 171:39–50. [PubMed: 7556906]
- Henry JJ, Tsonis PA. Molecular and cellular aspects of amphibian lens regeneration. Prog Retin Eye Res. 2010; 29:543–555. [PubMed: 20638484]
- Hu W, Haamedi N, Lee J, Kinoshita T, Ohnuma S. The structure and development of *Xenopus laevis* cornea. Exp Eye Res. 2013; 116:109–128. [PubMed: 23896054]
- Humason, GL. Animal Tissue Techniques. third. San Francisco: W.H. Freeman and Company; 1972.
- Levis HJ, Daniels JT, Ahmad S. Cultured Limbal Epithelial Stem Cell Therapy for Ocular Surface Diseases. 2013:41–56.
- Nieuwkoop, PD.; Faber, J. Normal Table of *Xenopus laevis* (Daudin). Amsterdam: North Holland Publishing; 1956.
- Perry KJ, Thomas AG, Henry JJ. Expression of pluripotency factors in larval epithelia of the frog *Xenopus*: evidence for the presence of cornea epithelial stem cells. Dev Biol. 2013; 374:281–294. [PubMed: 23274420]
- Salic A, Mitchison TJ. A chemical method for fast and sensitive detection of DNA synthesis in vivo. Proc Natl Acad Sci U S A. 2008; 105:2415–2420. [PubMed: 18272492]
- Slack JMW. Regional biosynthetic markers in the early amphibian embryo. J Embryol Exp Morphol. 1984; 80:289–319. [PubMed: 6747529]
- Thomas AG, Henry JJ. Retinoic acid regulation by CYP26 in vertebrate lens regeneration. Dev Biol. 2014; 386:291–301. [PubMed: 24384390]
- Wolfe AD, Henry JJ. Neuronal leucine-rich repeat 6 (XINLRR-6) is required for late lens and retina development in *Xenopus laevis*. Dev Dyn. 2006; 235:1027–1041. [PubMed: 16456849]
- Yoon JJ, Ismail S, Sherwin T. Limbal stem cells: Central concepts of corneal epithelial homeostasis. World J Stem Cells. 2014; 6:391–403. [PubMed: 25258661]
- Zhao J, Mo V, Nagasaki T. Distribution of label-retaining cells in the limbal epithelium of a mouse eye. J Histochem Cytochem. 2009; 57:177–185. [PubMed: 19001638]

Figure 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 μ m) 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 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 μ m. **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 . Scalebar 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.