UNDERSTANDING CORNEA STEM CELLS DURING HOMEOSTASIS
AND WOUND HEALING IN XENOPUS

BY
MOHD TAYYAB ADIL

DISSEMINATION
Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Cell and Developmental Biology
in the Graduate College of the
University of Illinois Urbana-Champaign, 2021

Urbana, Illinois

Doctoral Committee:
Professor Jonathan J Henry, Chair and Director of Research
Associate Professor William M Brieher
Associate Professor Rachel Smith-Bolton
Professor Jing Yang
Corneal development, homeostasis and wound healing are supported by specialized stem cells, that include the cornea epithelial stem cells (CESCs). Damage to or loss of these cells results in blindness and other debilitating ocular conditions. Here, we focus on cornea epithelial stem cell deficiency (commonly called limbal stem cell deficiency, LSCD). Fundamental developments have been made in LSCD therapies by utilizing epithelial stem cell transplants to restore normal vision in these patients. However, effective repair and recovery depends on many factors, such as the source and concentration of donor stem cells, and maintain the proper conditions to support these transplanted cells. We do not yet fully understand how CESCs heal wounds or how transplanted CESCs are able to restore transparency in LSCD patients, and questions still remain about the basic biology of CESCs, including their precise cell lineages and behaviors in the cornea. A major hurdle has been the lack of adequate vertebrate models to study CESCs. Here, we utilized a short treatment with Psoralen AMT (a DNA cross-linker), immediately followed by UV treatment (PUV treatment), to establish a novel frog model that recapitulates the characteristics of cornea Stem Cell Deficiency (SCD), such as pigment cell invasion from the periphery, corneal opacity, and neovascularization. These PUV treated whole corneas do not regain transparency. Moreover, PUV treatment leads to appearance of the Tcf7l2 labeled subset of apical skin cells in the cornea region. PUV treatment also results in increased cell death, immediately following treatment, with pyknosis as a primary mechanism. Furthermore, we show that PUV treatment causes depletion of p63 expressing basal epithelial cells, and stimulates mitosis in surviving cells within the cornea region. To study the response of CESCs, we created localized PUV damage by focusing the UV radiation on one half of the cornea. These cases initially develop localized SCD characteristics and showed increased pyknosis on the treated side. The localized PUV treatment is capable of stimulating some mitosis and results in changes in the percentages of p63 expressing cells in the untreated (control) half of those corneas. Unlike the whole treated corneas, the treated half is ultimately able to recover and corneal transparency is restored, thus providing an excellent model system for studying these rare and debilitating diseases.

system to study healing and the contributions of CESC
ts. To label and lineage trace the response of these strategically spared cells in the untreated half of the cornea, we utilized a transgenic line carrying a heat-shock inducible H2B-mCherry reporter, which can be activated in a localized cluster of cells at a specific location on the cornea. We showed that in contrast to the minimal displacement observed in the labeled cells in control corneas, the cells labeled in the untreated half underwent rapid displacement into the PUV treated half. This displacement did not continue at later time points (7 day post PUV onwards). As one of the few studies utilizing lineage tracing to study SCD, this work provides insights into the corneal cell response following stem cell depletion, their displacement and their mitosis, and establishes Xenopus as an excellent model using lineage tracing for studying CESC
ts, stem cell deficiency, and other cornea diseases. This work will also be valuable for understanding the nature of transplanted CESC
ts, leading to progress in improving transplant therapies for LSCD. Ultimately, a more thorough understanding of these cornea cells will advance our knowledge of stem cell biology and lead to better cornea disease therapeutics.
ACKNOWLEDGEMENTS

I wish to express my deepest gratitude to all the people who were fundamental to my accomplishments, and have made a big difference in my doctorate journey and my future. Firstly, I want to thank my family, in particular my parents, whose hard-work, determination, and sacrifices in life made it possible for me to pursue higher education. The early encouragement and inspiration I received from them, instilled in me a love for science. I cannot thank them enough for their unending love and support.

Most of all, I am deeply grateful to my advisor Dr. Jon Henry, for giving me the opportunity to study stem cells and wound healing in his lab. I owe my scientific development and independent research thinking to his mentorship, where I could always venture and explore new avenues for my projects. Jon’s unwavering support and his guidance has made all the difference, and I could not have asked for a better mentor. His energy, enthusiasm, and spirit of curiosity are truly inspiring, and if I could match just half of it in my career, I would consider my career to be successful.

I would also like to express my sincere gratitude to my graduate thesis committee members Dr. Bill Brieher, Dr. Rachel Smith-Bolton, and Dr. Jing Yang for their guidance throughout my PhD. Their expertise and unique perspectives were invaluable in providing new ideas and refining my thesis work, and I am grateful for all of their insightful feedback, which helped me sharpen my thinking.

I am especially grateful to Dr. Meelad Dawlaty, my mentor during the summer research opportunity at the Jaenisch lab. As an undergraduate, fresh after my junior year, his training was crucial to instilling in me the foundations of being a scientific researcher, and the curiosity of the scientific pursuit, inspiring me to pursue a PhD. I am thankful for his continued encouragement, support, and wishes throughout my graduate school years.
I would like to extend my sincere thanks to our department’s Office Admin Laura Martin. I found myself reaching out to her for advice on a variety of topics including thesis, visa, finances, and I was always glad that I did, for her valuable advice made all the difference.

I am also grateful for my lab mates Kim Perry and Surabhi Sonam, for their unmatched support and help with everything, ranging from methodologies and protocols, to helping me improve my papers and presentations. I also want to thank our previous lab members - Paul Hamilton and Alvin Thomas who showed me the ropes and helped me learn new methodologies when I was a fresh grad student, and were a constant source of wise counsel and inspiration; Claire Simons, undergrad research mentee who helped me with experiments for my thesis research; Maryna and Marty for their valuable advice and feedback; and everyone on the animal care staff for keeping our frogs and tadpoles happy and healthy.

I am glad to have friends like Vishnu, Shafi, Mayank, Ishan, Swami, Surbhi, Samarth, Jay, Phil, Nilmani, who were an integral part of my professional and personal life, with whom I could discuss my science, brainstorm ideas, seek out advice, play badminton and AOE, party and travel, and simply hang out for coffee breaks when experiments wouldn’t work. The scientific endeavor and graduate school in particular, can be full of uncertainties, and usually consists of a lot more defeats than triumphs, and all my friends have been the best support system throughout the ups and downs of PhD.
# TABLE OF CONTENTS

**CHAPTER 1:** INTRODUCTION ..........................................................................................................................1

**CHAPTER 2:** UNDERSTANDING CORNEA HOMEOSTASIS AND WOUND HEALING USING A NOVEL MODEL OF STEM CELL DEFICIENCY IN *XENOPUS* ........................................58

**CHAPTER 3:** LINEAGE TRACING TO STUDY CORNEAL WOUND HEALING IN THE PUV MODEL IN *XENOPUS* .......................................................................................................121

**CHAPTER 4:** LENS REGENERATION FOLLOWING PUV TREATMENT IN THE STEM CELL DEFICIENCY MODEL IN *XENOPUS* .............................................................................................142

**CHAPTER 5:** CONCLUSIONS AND PERSPECTIVES ......................................................................................151

**APPENDIX:** UNDERSTANDING THE BASIS OF CYP26 MEDIATED REGULATION OF LENS REGENERATION USING EX VIVO EYE CULTURES AND 4-OXO-RA ..................157
1.1. The Corneal Epithelium and Epithelial Stem Cells

Corneal integrity and transparency are indispensable for clear vision, owing to the crucial role of the cornea in refracting and transmitting light to the retina. Significantly, this outermost tissue focuses the light that enters our eyes (by ~66% or 40 diopter). Furthermore, the cornea protects the underlying tissues of the eye from external environmental factors, such as dust particles, airborne toxins and various pathogens (DelMonte & Kim, 2011). The integrity and maintenance of the cornea epithelium is conferred by its resident population of stem cells, which includes Cornea Epithelial Stem Cells (CESCs) that reside in the basal layer of the cornea epithelium. The CESC s are somatic stem cells that have the ability to self-renew and ultimately give rise to differentiated corneal cells. Human cornea epithelial cells turnover in about 3-10 days (Medical Advisory, 2008), and also support wound healing, being essential for maintaining normal vision (Cotsarelis, Cheng, Dong, Sun, & Lavker, 1989). During these processes, CESCs undergo asymmetric divisions to generate Transit Amplifying Cells (TACs), which undergo more rapid and numerous cell divisions, and ultimately form the terminally differentiated cells located in the more superficial apical layers (Beebe & Masters, 1996; Thoft & Friend, 1983). These differentiated cells eventually undergo senescence and are sloughed off.

1.2. Stem cell niches

Stem cells must maintain themselves in an undifferentiated state, and strictly regulate their proliferation and differentiation (Jones & Wagers, 2008). To support this, somatic stem cells reside in specialized niches. When stem cells are removed from their niche they typically die or undergo terminal differentiation (Scadden, 2006). Cornea epithelial stem cells are regulated by signals from adjacent stromal niche cells, the ECM, cell adhesion components, soluble factors, neural inputs, and the surrounding vascular network (Schofield, 1978). Frequently, such niches are organized as specialized,
crypt-like structures that in some cases may protect the stem cells from damage, such as environmental toxins (Gordon, Schmidt, & Roth, 1992), or ultraviolet irradiation (Tumbar et al., 2004). Failure of proper niche formation during development can lead to these stem cells being exposed to environmental damage or the mis-regulation of stem cell maintenance, causing stem cell related disorders. Some examples include microvilli in the intestine (Barker, 2014), the hair follicle bulge (Cotsarelis, Sun, & Lavker, 1990), or in the case of CESC, the Palisades of Vogt in the limbus of the cornea (Townsend, 1991).

Although it is generally accepted that CESC reside in the basal layer of the peripheral cornea or limbus (Amitai-Lange et al., 2015; Davanger & Evensen, 1971; Di Girolamo, 2011; Dua, Shanmuganathan, Powell-Richards, Tighe, & Joseph, 2005; Pellegrini et al., 1999; Schermer, Galvin, & Sun, 1986; Shortt, Secker, Munro, et al., 2007; Townsend, 1991; J. Zhao, Mo, & Nagasaki, 2009), the location of the CESC has been a topic of debate in the field, and some studies suggest CESC may reside throughout the cornea (Chang, Green, McGhee, & Sherwin, 2008; Dua, Miri, Alomar, Yeung, & Said, 2009; F. Majo, Rochat, Nicolas, Jaoude, & Barrandon, 2008). These differences may be related to the species being studied or the developmental age of the tissues that were examined. Studies reveal that putative CESC are present throughout the cornea during early development, being more highly restricted to the periphery at later developmental stages (Davies et al., 2009; Hamilton & Henry, 2016; Pajoohesh-Ganji, Ghosh, & Stepp, 2004).

Some insight into the role of the surrounding cellular niche was gained when rabbit cornea epithelial cells were recombined with mouse embryonic dermis. This resulted in a loss of the corneal phenotype and differentiation of these cells into epidermal keratinocytes and hair follicle cells. This was accompanied by increased expression of basal keratinocyte markers K5/K14, and downregulation of Pax6 and the cornea specific marker K3/K12 (Pearton, Yang, & Dhouailly, 2005). In contrast, hair follicle stem cells can be induced to differentiate into corneal epithelial cells and start expressing cornea specific markers, such as K12 and Pax6, when cultivated in a limbal-like microenvironment comprised of laminin-5 and limbal stromal fibroblast media (Blazejewska et al., 2009). Further analysis of CESC will improve our understanding of their precise locations/niches and the factors that control their regulation.
1.3. Limbal stem cell deficiency and its characteristics

Corneal diseases represent a major cause of blindness worldwide, second only to cataracts (Whitcher, Srinivasan, & Upadhyay, 2001). Corneal opacity (a subset of these diseases) is considered a priority eye disease, and a major cause of blindness globally, with 1.5-2 million new cases each year. Additionally, the World Health Organization reported that about 11% of blindness cases result from corneal scarring and neovascularization. Corneal opacity, scarring (epithelial defects), and vascularization, with consequent visual impairment or blindness, constitute the hallmark symptoms and clinical diagnostic features of Limbal Stem Cell Deficiency (LSCD) (Figure 1.1A) (Sacchetti, Rama, Bruscolini, & Lambiase, 2018; Sejpal, Bakhtiari, & Deng, 2013). LSCD is a debilitating corneal disease that results from functional or anatomical loss of the cornea epithelial stem cell population. In LSCD, the transparent cornea epithelium cannot be renewed, the corneal epithelial barrier that separates the cornea and the conjunctiva cannot be maintained (Kubilus, Zapater, & Linsenmayer, 2017), and the cornea is replaced by surrounding conjunctival epithelial cells (conjunctivalization) (Figure 1.1A). Pterygium appears to be a related condition, in which fibrovascular tissue grows from the conjunctiva onto the cornea (T. Liu, Liu, Xie, He, & Bai, 2013). Blood vessels from the conjunctiva may also appear in the cornea region (Figure 1.1A) (Dua, 1998; Fini & Stramer, 2005), which is normally free of vasculature (Azar, 2006). Other epithelial defects, such as erosions, inflammation, and scarring are commonly seen. Although not used for clinical diagnosis currently, centripetal migration of pigment cells from the periphery of the cornea into the central cornea has also been observed during the process of LSCD in rabbits, guinea pigs, and even humans (Cafaro et al., 2009; Cowan, 1963; Davanger & Evensen, 1971; Henkind, 1965; Mann, 1944; Michaelson, 1952; Wolosin, Xiong, Schutte, Stegman, & Tieng, 2000). Furthermore, LSCD may be associated with other ocular surface abnormalities.

1.4. Severity and extent of LSCD

Clinical manifestations of LSCD vary based on the severity and extent of involvement of the cornea tissue (Dua & Azuara-Blanco, 2000). There can be partial LSCD with some conjunctivalization, in which only a segment of the limbus is involved and residual CESC may be present. In contrast, extensive cases are referred to as total LSCD, and involve conjunctivalization of the entire corneal surface due to a complete loss of CESC.

3
Physicians and scientists have established a global consensus that classifies the stages of LSCD based on clinical presentation, which will be a helpful benchmark in LSCD diagnoses and research (Deng et al., 2019). Typically, LSCD can be categorized into mild, moderate, and severe cases, depending on the degree of epithelial opacity, epithelial defects, and vascularization. (Aravena et al., 2019; Le, Xu, & Deng, 2018; Sacchetti et al., 2005; Shortt, Secker, Notara, et al., 2007). In mild cases, patients may be asymptomatic initially, as long as there is enough stem cells to sustain the cornea (J. J. Chen & Tseng, 1991; Huang & Tseng, 1991; Tseng, 1989). As the disease progresses, symptoms such as ocular discomfort, conjunctival redness, tearing, dryness, pain, photophobia, and decreased vision may appear. If left untreated, more severe cases of LSCD can present with symptoms that include subepithelial fibrosis, fibrovascular pannus, stromal scarring, neovascularization, and recurrent epithelial defects (Deng et al., 2019; Lim et al., 2000).

Another difference in LSCD presentation that plays a major role in terms of its treatment, is whether the LSCD is unilateral, where only one eye is affected, or bilateral, where both eyes are affected. For unilateral cases, cells/tissues for transplant therapy can be obtained from the healthy cornea, whereas for bilateral LSCD cases, donor tissue must be obtained from allogenic sources, which carries a risk of transplant rejection (discussed further in Section 3 below).

1.5. Etiologies of LSCD

The severity and extent of LSCD depends on its wide range of etiologies, which play a crucial role in its prognosis, and complicates the diagnosis and treatment of LSCD. This is because secondary afflictions, such as dry eye syndrome, inflammation, infection, and underlying immune or hereditary diseases can tremendously reduce the success of the transplanted tissues or cells used to treat LSCD.

Ranging from devastating events like chemical burns to something as simple as daily contact lens wear, many factors can cause LSCD. The etiologies for LSCD can be categorized as acquired (from external factors that destroy CESCs) or genetic. As subsets of the external factors, chemical or thermal burns, are the most common cause of LSCD (about 60-75% of total cases) (Baylis, Figueiredo, Henein, Lako, & Ahmad, 2011). These cases may have better prognoses, as the underlying secondary complications are reduced (unlike those related to genetic or auto-immune diseases) (Fatima, Iftekhar, Sangwan, & Vemuganti, 2008; Pellegrini et al., 1997; Schwab, 1999; T. H. Tsai et al., 2014). Certain immune-mediated diseases, such as Stevens-Johnson syndrome (Choi, Kim, & Oh, 2019; Gomes et al.,
Contact lens wear has become yet another cause of LSCD (C. C. Chan & Holland, 2013; Clinch, Goins, & Cobo, 1992; Rossen et al., 2016), as they may cause corneal scratches/abrasions, infections, and dry eyes (Bobba et al., 2017).

LSCD may also arise from iatrogenic causes (where the diagnostic procedure or treatment for one ailment can result in further complications). These include several major procedures, such as various chemotherapies: hydroxyurea (Ellies, Anderson, Topuhami, & Tseng, 2001), S-1 (K. H. Kim & Kim, 2015), hydroxycarbamide (Ding, Bishop, Herzlich, Patel, & Chan, 2009), Mitomycin C (Lichtinger, Pe'er, Frucht-Pery, & Solomon, 2010); Radiation therapies (Fujishima, Shimazaki, & Tsubota, 1996); eye surgeries such as surgery for pterygium (Atallah, Palioura, Perez, & Amescua, 2016), the use of 5-fluorouracil in glaucoma surgeries (Pires, Chokshi, & Tseng, 2000); and something as common as the use of preservatives (e.g., benzalkonium chloride) in widely used eye medications (Lin et al., 2013; Pisella, Pouliquen, & Baudouin, 2002).

Among the genetic causes of LSCD, is the developmental disorder aniridia, which is characterized by mutations in the PAX6 gene, leading to abnormal iris development with partial or complete absence of the iris, among other conditions (Holland, Djalilian, & Schwartz, 2003; Lee, Khan, & O'Keefe, 2008; Shortt et al., 2014). PAX6 also plays key role in the development of the corneal epithelium, and controls CESC lineages in corneal development and diseases (Sasamoto, Y. et al., 2016; G. Li et al., 2015). Mutations in PAX6 can also affect CESC regulation and the proper development of the CESC niche, resulting in LSCD. Some other genetic causes include dyskeratosis congenita (Aslan & Akata, 2010), ectodermal dysplasia (Di Iorio et al., 2012; Merenmies & Tarkkanen, 2000), keratitis caused by endocrine deficiencies (Mohammadpour & Javadi, 2006), and epidermolysis bullosa (Thanos, Pauklin, Steuhl, & Meller, 2010). These genetic changes usually result in LSCD, as a result of structural damage, damage to the corneal niche, chronic corneal surface erosion, or repeated inflammation. Several reviews can be consulted about the many etiologies of LSCD (Deng et al., 2019; Haagdorens et al., 2016; Shortt, Secker, Notara, et al., 2007).
1.6. Diagnosis of LSCD

Early diagnosis plays an important role in timely treatment, and prevents the progression and severity of LSCD. Slit-lamp microscopy has been one of the oldest and most common diagnostic tools, and provides some indication about the corneal surface, and signs of damage to the cornea or limbal palisades. However, visualization under white-light, provides very limited information and has poor resolution. The application of fluorescein, which collects in areas of epithelial thinning or irregularity, followed by visualization under a cobalt blue light in slit-lamp microscopy, is useful for detecting LSCD (Donisi, Rama, Fasolo, & Ponzin, 2003; Dua & Azuara-Blanco, 2000; Dua, Gomes, & Singh, 1994). Fluorescein is beneficial for diagnosis, as it cannot penetrate normal corneal epithelial cells that are interconnected by tight junctions, whereas, it can penetrate the relatively loose cell-cell contacts in the conjunctival epithelium, which have greater permeability (Chandra et al., 2008).

For a confirmatory diagnosis of LSCD, corneal impression cytology (CIC) (Nesti et al., 2008) has been frequently employed to check the most superficial layer of the cornea for goblet cells, epithelial loss, or evidence of ectopic conjunctival epithelium (Puangsricharern & Tseng, 1995). The superficial cells are sampled for study by placing a filter paper on the corneal surface. The sensitivity of CIC can be further improved by using immunocytochemistry to detect cytokeratin markers expressed by cornea (e.g., CK3/CK12) vs. conjunctiva cells (e.g., CK19) (Le et al., 2018; Sacchetti et al., 2005). CIC has some major drawbacks including reliability concerns owing to inconsistent/inadequate removal of cells, and post-procedure pain as patients may have epithelial defects and an unstable corneal surface.

Advances in confocal microscopy provide valuable diagnostic tools in the form of In-Vitro Confocal Microscopy (IVCM), which has higher resolution and allows visualization at the cellular level (Barbaro et al., 2010; Vera et al., 2009). Besides visualizing goblet cells and vascularization, IVCM can provide details of basal epithelial cells, and can differentiate between corneal cells and conjunctival cells based on their morphology (Dua et al., 2009; Kobayashi, Yoshita, & Sugiyama, 2005; Le et al., 2018). IVCM studies have revealed a decreased basal cell density in the cornea and limbus in patients diagnosed with LSCD (E. H. Chan, Chen, Rao, Yu, & Deng, 2015). Drawbacks of IVCM are that it only allows for parallel-to-corneal surface views, morphological appearance of CESC's goblet cells can be difficult to identify, and most importantly, the technique becomes less effective if the corneal opacity is high (Haagdorens et al., 2016).
Anterior Segment Optical Coherence Tomography (AS-OCT) is another imaging technique that allows scanning in variable orientations (e.g. en face sections, cross sections, and sections parallel to the limbus), with the trade-off being lower resolution than IVCM (Banayan et al., 2018). AS-OCT is quicker, more convenient, provides for a larger field of view, and permits the diagnosis of LSCD induced epithelial thinning by measuring the corneal epithelial thickness, corneal epithelial irregularity, and structural changes in the limbus (Le et al., 2018; Liang, Le, Cordova, Tseng, & Deng, 2020; Mehtani, Agarwal, Sharma, & Chaudhary, 2017). Also, AS-OCT angiography can be used to measure neovascularization (Binotti et al., 2020).

1.7. Limbal Stem Cell Deficiency therapeutics - Stem cell transplants

Contemporary treatments for Limbal Stem Cell Deficiency (LSCD) involve epithelial stem cell transplants. With significant advances made in corneal transplant therapies in the last two and a half decades, these stem cell transplants have become a benchmark treatment for patients with LSCD (Figure 1.1B). Initially, LSCD therapy was limited to autologous (in the case of unilateral LSCD) (Kenyon & Tseng, 1989; Kruse, 1994) or allogenic (cadaveric or living) donor tissues transplants (Turgeon, Nauheim, Roat, Stopak, & Thoft, 1990; Weise, Mannis, Vastine, Fujikawa, & Roth, 1985). Autologous grafts are preferred because graft-versus-host disease or tissue rejection is a major concern with allogenic transplants. However, autologous transplants are only possible in unilateral cases. More importantly, large autologous limbal grafts, or even allogenic grafts from a living donor, can potentially compromise the healthy donor eye, resulting in LSCD. Allogenic grafts are also less likely to succeed, even with immune suppression.

A major breakthrough allowed for the cultivation and expansion of a small amount of limbal tissue containing autologous limbal stem cells, which could be utilized for transplant therapies (Figure 1.1B) (Lavker, Tseng, & Sun, 2004; Pellegrini et al., 1997; Rama et al., 2010). The limbal tissue for this procedure is obtained by small limbal biopsy, and then ex-vivo expanded in labs before transplantation. The harvested tissue can be tested for the presence of CESCs using various markers (Rama et al., 2010). The transcription factor p63 is a commonly used marker, but detects all proliferative cells (CESCs and TACs) (Arpitha, Prajna, Srinivasan, & Muthukkaruppan, 2008; Di Iorio et al., 2005; L. Liu et al., 2018; Pellegrini et al., 2001). Studies are still ongoing to find suitable markers to identify and isolate pure populations of CESCs for transplantation (Pajoohesh-Ganji, Pal-Ghosh, Tadvalkar, & Stepp, 2012;
Sonam, Srnak, Perry, & Henry, 2019), and a specific marker to differentiate stem cells from TACs would be very beneficial (Sacchetti et al., 2018). One drawback is the requirement of a sophisticated lab facility to properly process and expand the harvested limbal cells, which greatly increases the treatment costs (Sangwan, Basu, MacNeil, & Balasubramanian, 2012).

Another major advance came in the form of utilizing amniotic membranes (AM) as the carrier/substrate for cultivating and expanding harvested cells for these transplants (J. C. Kim & Tseng, 1995; R. J. Tsai, Li, & Chen, 2000; Utheim et al., 2018). Both intact and denuded (de-epithelialized) AMs have been used for transplants (Grueterich, Espana, Touhami, Ti, & Tseng, 2002; Koizumi et al., 2001; Sangwan, Vemuganti, Iftekhar, Bansal, & Rao, 2003; Utheim et al., 2018). Certain modifications of the AM, like crosslinking, can improve its mechanical and thermal stability (Utheim et al., 2018). Alternative cell carriers have also been used, such as collagen (He & McCulley, 1991), fibrin (Rama et al., 2010), hydrogel contact lenses, etc. (Di Girolamo et al., 2009; Haagdorens et al., 2016). Growing the limbal epithelial cells directly onto a specialized contact lens can be particularly beneficial, as it eliminates the need for 3T3 feeder cells and these cells can be transplanted by simply inserting the contact lens onto the eye. A modified technique involving in-vivo expansion, where the harvested donor limbal tissue is divided into smaller pieces and merely applied to the eyes along with an AM, reduces the high cost of the ex-vivo expansion of the donor limbal tissue (Sangwan et al., 2012). Combined, these breakthroughs have propelled the field of LSCD therapeutics. Very little healthy tissue is now required, which can be obtained autologously in many cases, and reduces the need for chronic immunosuppression.

These advances, however, still do not resolve the problem of immune rejection of certain transplanted cells, as bilateral LSCD cases require allogenic cells. This often results in poor success rates, particularly in patients with pre-existing immunological disorders. The advent of oral mucosal culture transplants may have solved this problem. A subset of the cultivated epithelial transplants, this technique utilizes epithelial cells from the inner buccal mucosa, which are expanded on AM and transplanted onto the cornea (Bains, Fukuoka, Hammond, Sotozono, & Quantock, 2019; Nakamura, Inatomi, Sotozono, Koizumi, & Kinoshita, 2004; K. Nishida et al., 2004). A number of novel and fascinating sources of stem cells are being explored to treat LSCD, such as stem cells from hair-follicles (Meyer-Blazejewska et al., 2011), dental pulp (Gomes et al., 2010), the umbilical cord (Reza, Ng, Gimeno, Phan, & Ang, 2011), and induced Pluripotent Stem Cells (Hayashi et al., 2012; J. Yu et al.,
The discovery of these novel sources of corneal cells highlight the developmental plasticity of various cell types and improve our understanding of the regulation of cornea stem cells.

1.8. Limbal Stem Cell Deficiency therapeutics - Keratoprotheses

Keratoprotheses (artificial corneas) are suitable for patients who don’t qualify for transplants, either because immunosuppression is not an option for these patients or previous stem cell transplants have failed. The Boston KPro type 1 is one such prothesis that involves supporting titanium backplate and a front plate with an optical stem, which can be transplanted onto the damaged eye (Hou, de la Cruz, & Djalilian, 2012; Sejpal, Yu, & Aldave, 2011). They are not free of complications, however, which can include recurrent epithelial defects, retroprosthetic membrane formation, sterile melts, secondary glaucoma, endophthalmitis and retinal detachment (Atallah et al., 2016). The Modified Osteo-Odonto Keratoprosthesis (MOOKP) (Falcinelli, Falsini, Taloni, Colliardo, & Falcinelli, 2005; Strampelli, 1963), and the Temprano keratoprosthesis (Michael et al., 2008), use dental and bone lamina from the patient’s tooth and bone surrounding the tooth, and tibia, respectively, to support an optical cylinder that allows for light to enter the eye and reach the retina. A layer of oral buccal mucosa is applied on the lamina-cylinder complex to protect it. Both these prostheses have good retention rates and vision recovery, but can cause complications such as oroantral fistula, trophic mucosal alterations, lamina exposure, mucous membrane overgrowth, hypotony, expulsion of the optic cylinder, endophthalmitis glaucoma, sterile vitritis, and retinal detachment (Iyer, Michalet, Chang, & Weiss, 2010). Other modification of the Boston KPro type prostheses such as Boston KPro type 2, and LVP keratoprosthesis have also been explored (Atallah et al., 2016; Basu, Sureka, Shukla, & Sangwan, 2014).

1.9. Gaps in our knowledge and problems that need to be addressed

Although these remarkable therapies are continuously being improved, they are associated with complications, such as recurrent epithelial defects, conjunctivalization, keratitis, and subsequent bacterial/fungal infections (Basu, Ali, & Sangwan, 2012; Shimazaki et al., 2002). Additionally, eye surgeries in general can increase the risk of infection, retinal detachment, or glaucoma, and are one of the causes of LSCD (Atallah et al., 2016; Shimazaki, Shimmura, & Tsubota, 2004). Immunosuppressive
drugs can cause other issues, such as anemia, hyperglycemia, infections, and renal/liver problems (Krakauer, Welder, Pandya, Nassiri, & Djalilian, 2012; Shimazaki et al., 1999). Animal infections from the 3T3 fibroblast or Fetal Bovine Serum used for cultured transplants are yet another problem (T. Nishida et al., 1996). Long term outcomes for allogenic transplants are not optimal, even with prolonged immunosuppression (Biber, Skeens, Neff, & Holland, 2011; Holland, 2015; Ilari & Daya, 2002). Reoccurrence of LSCD is a persistent problem after stem cell transplants, and many patients require a secondary keratoplasty. Complications from various stem cell transplantation methods have been reviewed in detail by Yin and Jurkunas (2018). Complications can also develop in the donors (Miri, Said, & Dua, 2011).

While epithelial stem cell transplants offer promising success to many patients, effective repair and recovery can be limited owing to several factors. Varying success rates depend on etiologies, conditions to support the transplant (e.g. wet environment, normal eyelid function, etc.), methods used to culture the donor cells, the substrate/matrix used to expand/cultivate the harvested cells, etc. (Atallah et al., 2016; Baylis et al., 2011; Ti, Anderson, Touhami, Kim, & Tseng, 2002). Finding suitable donors is also difficult, with cadaveric tissues typically giving poorer results. Source and concentration of donor stem cells, and the amount of tissue required, is also an important consideration. One study observed that cultures in which p63 expressing cells constituted more than 3% of the total number of clonogenic cells were generally associated with successful transplantation (Rama et al., 2010). However, some cultures containing less than 3% p63 expressing cells were also successful, and some cultures containing the required number of holoclones failed, leaving into question the minimum number of transplanted stem cells that are required for successful outcomes (Pellegrini et al., 2013). Additionally, there is some debate over whether it is the stem cells, the TACs, or a combination of both that yield the best results in these cases.

One important outstanding question in the field is whether transplanted donor stem cells truly integrate and repopulate the patient’s corneal tissue (i.e., the reintegration hypothesis) or merely stimulate patients’ endogenous stem cell populations (i.e., the biological bandage hypothesis) (Shortt, Tuft, & Daniels, 2011). Several studies have tried to answer this question by analyzing donor vs. recipient DNA after transplantation, generally by PCR or FISH. A study conducted by Henderson and colleagues (Henderson, McCall, Taylor, & Noble, 1997) utilized the ameleogenin gene probe and Y-specific DNA probes to detect the presence of surviving donor cells, but were ultimately unable to show survival of those cells. A recent study involving 16 patients who received allogenic ex-vivo expanded
cells on amniotic membranes, showed no donor DNA in a majority of the cases (Campbell et al., 2019). On the other hand, a study that used polymerase chain reaction restriction fragment length polymorphism was able to detect long term survival of donor-derived cells following an allogenic transplant in 78% of the cases (Shimazaki et al., 1999). Another study was able to detect donor cells 3.5 years after limbal allograft transplantation in a small sampling of 3 cases (Djalilian et al., 2005). In another study, just 3 months after transplantation, donor epithelial cells were replaced by the recipients epithelial cells in all 52 cases examined, and only donor stromal and endothelial cells remained (Lagali et al., 2009). The proportion of surviving cells was highly variable in this study (4%-95% for stromal and 6%-95% for endothelial cells). Indeed, if stem cell transplants had succeeded, as planned, some cells of the donor tissue should be found in the recipient’s cornea. In the absence of those donor cells, the mechanism for corneal restoration becomes unclear. This is further complicated by studies showing survival of apparently healthy corneal epithelium even in cases with total LSCD (Dua et al., 2009), and that healing may be independent of limbal stem cell influence (Chang et al., 2008) (discussed further in Section 6.6). The mystery of the interplay between the recipient cornea and transplanted tissue at the cellular level is, therefore, unsolved.

Although new techniques are continuously evolving with various sources of stem cells (Haagdorens et al., 2016; Sasamoto, Ksander, Frank, & Frank, 2018), the emerging theme from these therapies is that they are focused towards obtaining stem cells for the transplants from a different/novel source, and do not necessarily lead us towards a better understanding of LSCD pathology, the specific behavior of CESC, or how transplanted CESC restore transparency in LSCD patients. More than a 100 years after the first successful human corneal transplant by Eduard Zirm (for English translated reference see Zirm (1989)), the exact mechanism underlying corneal restoration via corneal transplants still evades us.

One avenue towards understanding these mechanisms is to study the cellular and molecular factors that regulate proliferation, migration, and differentiation of transplanted CESC during repair. The involvement of SMADs and MMPs has been reported in cornea repair (Fini & Stramer, 2005; Pal-Ghosh et al., 2011), and may be suitable as drug targets. Tcf712 may be another interesting drug target, as it has been implicated in maintaining human CESC and its localization (activity) switches from the cytoplasm during homeostasis, to the nucleus during wounding (Lu et al., 2012). In addition, retinoic acid has been used for treatment of partial LSCD (Chew & Watson, 2016). One interesting approach has been the use of autologous serum, which contains a mix of proteins, cytokines, growth factors, etc., that
can regulate the proliferation, differentiation, and maturation of CESC to treat various ocular surface disorders and corneal epithelial defects (Geerling, Maclennan, & Hartwig, 2004; Poon, Geerling, Dart, Fraenkel, & Daniels, 2001; Young et al., 2004). A recent study showed that aggressive treatment with autologous serum derived from LSCD patients, when applied to their corneas, showed reversal of severe contact lens induced LSCD (Yeh, Chu, Cheng, Wu, & Tsao, 2020). The identities of key components in this serum are unknown. Studies suggest a role of certain growth factors, cytokines, and exosomes in improving and accelerating corneal healing (Castro-Munozledo, Ozorno-Zarate, Naranjo-Tackman, & Kuri-Harcuch, 2002; Han, Tran, Chang, Azar, & Zieske, 2017; Tamariz-Dominguez, Castro-Munozledo, & Kuri-Harcuch, 2002; Thomasen, Pauklin, Steuhl, & Meller, 2009). Therefore, a thorough understanding of LSCD pathology and the mechanism of stem cell transplant restoration, will not only improve or complement the existing transplant therapies, but may open up potential new approaches, possibly bypassing the need for transplanted cells.

1.10. Mammalian models to study stem cell deficiency in the cornea

Animal models are vital for studying the disease process at the cellular and molecular level, and also for pre-clinically testing the effectiveness of treatments being developed for patients. Suitable animal models to study a human disease can advance our understanding of the field. As such, several models have been developed to study CESC and LSCD.

Since CESC are generally thought to reside in the peripheral limbus (Schermer et al., 1986), the early methods to study LSCD involved removal of this peripheral tissue by mechanical or surgical methods. Surgical removal of limbal epithelium (J. J. Chen & Tseng, 1991), scraping with a dull blade (Gipson & Kiorpes, 1982) or a blunt spatula (Amirjamshidi et al., 2011; Pal-Ghosh, Tadvalkar, Jurjus, Zieske, & Stepp, 2008), or limbal peritomy using a crescent knife (Galindo et al., 2017) have all been used to create LSCD. In these procedures, different size trephines (dulled to prevent deeper stromal penetration) have been used to demarcate the area where tissue will be removed (Stepp et al., 2014). The size of debridement determines the extent of a response. For example, in the mouse cornea, the wound needs to be larger than 2mm in diameter to recreate the morphological changes seen in LSCD (Pajoohesh-Ganji et al., 2012; Pal-Ghosh, Pajoohesh-Ganji, Brown, & Stepp, 2004). Some more specific keratectomy procedures have also been used to generate varying degrees of wounding to study corneal repair. Penetrating keratectomy to remove epithelium, stroma, and endothelium in mice (Stramer,
Zieske, Jung, Austin, & Fini, 2003) and rabbits (Cintron, Schneider, & Kublin, 1973; West-Mays et al., 1997), and Photorefractive Keratectomy (laser surgery for vision correction, can be used to remove corneal layers) to create wounds in rabbits, rats, and mice (Azar, Tuli, Benson, & Hardten, 1998; Kato, Chang, & Azar, 2003; R. R. Mohan, Stapleton, Sinha, Netto, & Wilson, 2008; Singh et al., 2011) are common approaches. However, these methods to create LSCD are difficult to standardize, technically challenging, and less reproducible from lab to lab. More importantly, most of the debridement type methods typically only create partial LSCD, as only the superficial layers are affected and the basement membrane is typically left intact (Sta Iglesia & Stepp, 2000), and surgical removal may risk damaging the stroma, associated niche structures or cause anterior chamber perforations.

The use of a rotating burr tool, (e.g. AlgerBrush II, Alger Equipment Company Inc., Lago Vista, TX), is the contemporary, preferred method to create corneal wounds (Ferrington et al., 2013; Pal-Ghosh et al., 2011; Yang et al., 2013) and have been used to cause LSCD in mice (Afsharkhamseh et al., 2016; Ksander et al., 2014; Meyer-Blazejewska et al., 2011) and rabbits (F. J. Li et al., 2016). Burr tools can be beneficial over scraping tools, as they can also remove the basement membrane, although some studies show that they may not do so completely (Boote et al., 2012; Stepp et al., 2014). Another advantage of the AlgerBrush II over other mechanical methods is the ease and consistency of creating wounds in the hands of less experienced researchers. However, several factors such as the speed of the burr, the pressure applied on the cornea, the angle of burr placement, residence time of the burr, etc., are crucial to getting consistent results (Stepp et al., 2014). This requires more experience and care, and can be a potential source of variability and lack of reproducibility between different labs. Furthermore, this method may only create partial LSCD. For example, LSCD characteristics persisted after 5 weeks in a rabbit model created using the AlgerBrush II, but the severity was only about 40% (F. J. Li et al., 2016). Another study had to repeat the procedure multiple times, for more complete removal of the limbal cells (Afsharkhamseh et al., 2016). One interesting method to recreate thermal burn induced LSCD is via ophthalmic cautery to damage cells in the cornea (F. Majo et al., 2008). This method has also been combined with the AlgerBrush II to create LSCD in mice (Afsharkhamseh et al., 2016).

Chemical methods are also used to create LSCD. The most commonly used method has been alkali damage using NaOH (Gomes et al., 2010; Luengo Gimeno et al., 2007). Wounding is done by topical application, typically using a filter paper soaked in NaOH (Bargagna-Mohan et al., 2012). These wounds are expected to be similar to some accidental forms of chemical damage seen in LSCD patients; however Kethiri et al. (2019) compared the alkali burn (NaOH) model of LSCD in mice and rabbits to
chemical burn induced LSCD corneas from humans, and found significant differences in the extent of vascularization, inflammation, epithelial thickness, and goblet cell formation. Another chemical, sulfur mustard, which is a vesicating agent, has been used to cause LSCD in a mouse model (Ruff et al., 2013), and delayed-onset LSCD in a rabbit model (Kadar et al., 2013; Kadar et al., 2011), reproducing the ocular injuries reported in soldiers during several wars. Interestingly, a common preservative in eyedrops, benzalkonium chloride, which has been shown to cause LSCD in humans, has also been used to create a mouse model of LSCD (Lin et al., 2013; Pisella et al., 2002). Chemical treatments can also be combined with surgical removal of the limbus. A common standard protocol is a short application of n-heptanol to remove the corneal epithelium followed by surgery to remove the limbus (Hirst, Kenyon, Fogle, Hanninen, & Stark, 1981; Reinshagen et al., 2011). An important drawback of chemical methods is the variability (ranging from mild to severe cases) and unpredictability in the results, owing to difficulties in controlling the extent (depth) of chemical damage and of the injury/pathology they caused. There is also a risk of causing perforation or damage to the deeper stroma or the surrounding conjunctiva.

Besides the mechanical and chemical methods used to create LSCD, there is also the genetic mouse model carrying a pax6 knockdown to study LSCD. In this study, the authors showed that Pax6 controls limbal stem cell lineages in cornea development and diseases (G. Li et al., 2015). Additionally, Pax6 levels are known to be elevated during wound healing (Sivak et al., 2000), and it also upregulates MMP9, which plays an important role in remodeling of the extracellular matrix during corneal repair (R. Mohan et al., 2002). Other genes involved in corneal dystrophies have also been studied in zebrafish models (Bibliowicz, Tittle, & Gross, 2011; Boisset, Polok, & Schorderet, 2008). Some genetic models are used less frequently, due to difficulty in generating and rearing specific transgenic animals, as their long term survival may be an issue. Also, LSCD has fewer genetic etiologies, and presentation of LSCD caused by mutations may be less severe.

A challenge to the study of LSCD and CESCs has been a lack of more convenient vertebrate models. The above animal models to study LSCD and CESCs are limited mainly to a few mammals (mouse, rat, and rabbit). The mouse model is advantageous particularly because a wide array of experimental tools and reagents are available. However, the mouse corneal limbus differs from the human corneal limbus in its thickness and the location of Bowman’s membrane where the limbus starts (Kinoshita et al., 2001; Pajoohesh-Ganji et al., 2012). Additionally, the mouse cornea doesn’t have the same crypt-like limbal structures (Molon-Noblot S, 1991). Given these differences in mice corneas, as
compared to humans (Grieve et al., 2015), caution has been recommended for extrapolating data from mouse to humans (Richardson, Wakefield, & Di Girolamo, 2016). Furthermore, the large number of specimens required for each study can be expensive for mice, especially if transgenics have to be created. The rabbit is an excellent pre-clinical model as their eye sizes are very similar to humans; however, they are more expensive than mouse models, and fewer experimental tools and reagents are available. Research to study LSCD in other model organisms has been slow so far, and additional animal models to study LSCD and CESC s can help us to outstanding questions in this field. An examination of other vertebrate models can also enlighten us about any species-specific differences in the mechanisms of CESC s and LSCD.

1.11. Lineage tracing to study corneal stem cells and wound healing

Therapies for LSCD hinge on a comprehensive understanding of corneal wound healing. This entails the study of cell migration, proliferation, and differentiation, as these pertain to barrier function, scarring, vascularization, conjunctivalization, and inflammation. Progress has been made in research identifying cellular and molecular regulatory mechanisms underpinning corneal wound healing, including key growth factors and signaling pathways (Gonzalez, Sasamoto, Ksander, Frank, & Frank, 2018; Mort et al., 2012; Stepp et al., 2014; F. S. Yu, Yin, Xu, & Huang, 2010). However, several questions still remain unanswered. What is the behavior of the remaining corneal cells following LSCD? How does transplanted tissue heal LSCD? What are the migratory paths of the cells responsible for healing? Do the transplanted cells integrate into the host tissue or remain at the implantation site? How long do transplanted cells survive after transplantation? Do transplanted CESC s/TAC s repopulate the depleted stem cell pool? How many transplanted CESC s/TAC s are required for effective healing? What molecular cues determine these responses? Lineage tracing studies have made significant contributions to answering some of these questions.

Lineage tracing is particularly important to study stem cells, as cells can be marked, and their behaviors can be observed, allowing discovery of both the short-term and long-term fate of stem cells and their progenies. A particular advantage conferred by in-vivo lineage tracing is the ability to study these cells while they are in their natural microenvironment, eliminating the disadvantages associated with ex-vivo cultures, or transplantation of cells into a new environment. For example, controversies regarding the renewal of the epidermis by hair follicle stem cells were resolved by lineage tracing.
Studies revealed that hair follicle stem cells contribute to epidermal wound repair, but not epidermal homeostasis (Claudinot, Nicolas, Oshima, Rochat, & Barrandon, 2005). Likewise, lineage-tracing studies showed that there are two types of stem cells that maintain the intestinal crypts and villi epithelium, including the crypt base columnar stem cells (CBC) at the crypt base and the +4 “reserve” stem cells (occupying the fourth position from the crypt base), which restore the CBC stem cells following injury (Barker et al., 2007). Another study used the Lgr5 promoter driven Confetti construct to show that the initially multicolor labeled cells in small intestinal crypts eventually became monochromatic, indicating that homeostasis is supported by Lgr5 expressing stem cells (Snippert et al., 2010).

These techniques becomes even more useful for studying wound healing, which is a highly dynamic process in which cells are deployed over long distances and distinct periods of time. Spatiotemporal information obtained for the cells being tracked, combined with in-vivo imaging in live animals, provides for a powerful and elegant way to study wound healing in real time.

The natural transparency and exposed location of the cornea makes it well-suited for lineage tracing. While the stem cells in the cornea were believed to reside exclusively in the limbus, there has been a long-standing controversy about their location. Several studies in clinical patients and animal models (Chang et al., 2008; Dua et al., 2009; F. Majo et al., 2008), suggest the presence of progenitor populations in the central cornea capable of repairing the corneal epithelium independent of LESC. This led to numerous studies utilizing lineage tracing to locate these corneal stem cells. Irrespective of the controversy regarding the location of corneal stem cells, or the possibility of a secondary population of stem cells, it is well accepted that the depletion or loss of corneal stem cells result in LSCD, and that corneal stem cells and their TACs play a crucial role in wound healing.

1.12. Natural pigmentation and dyes for lineage tracing during corneal wound healing

The first corneal wound healing studies utilized the naturally occurring melanin in the peripheral corneas in humans and rabbits to monitor cell migration in response to corneal injuries (Mann, 1944). Pigmented cells were shown to migrate following epithelial debridement or exposure to mustard gas, and it was found that the position, size, depth, and type of wound were all factors that affected the rate and direction of cell movement. The migration of pigmented limbal cells was also observed by Davanger and Evensen (Davanger & Evensen, 1971) after corneal wounding in humans and guinea pigs.
During re-epithelialization, cell displacement occurred via circumferential “tongue-shaped projections”, which met along the limbus before migrating centripetally to close the wound (Dua & Forrester, 1990). These early studies laid the foundation for our contemporary understanding of corneal wound healing.

Lineage tracing studies can also be conducted using dyes or cell autonomous tracers. India ink (in mixture with thorium dioxide), was used by Buck (1985), to label peripheral cells in the mouse cornea and to observe centripetal migration of corneal epithelial cells. Buck also used this dye to study repair of wounds in mice corneas, showing that 1-1.4 mm diameter wounds closed in 12-16 hr, thus calculating speeds of epithelial migration (30-60um/hr) in the mouse cornea (Buck, 1979). Cell membrane labeling dyes, DiI or DiO, have been used routinely in live animals owing to their low toxicity, and labeled cells can be tracked for long periods owing to their photostability. In one study, DiI labeled cells were cultured on amniotic membranes for transplantation onto cat corneas, which was helpful in evaluating efficacy of this treatment method (Fan et al., 2013). Another study utilized DiI labeling of corneal and conjunctival cells during development of chick eyes, and showed the formation of a boundary between corneal and conjunctival cells during development (Kubilus et al., 2017). A more recent method using Quantum dot nanocrystals (Qdots) has been used to track cultured limbal epithelial cells for transplantation (Genicio, Gallo Paramo, & Shortt, 2015). In this study, cells marked with Qdots could be detected for 2 weeks post-transplantation and no toxicity or dysfunction was observed in the labeled cells. Dilution over several generations and exocytosis are some of the major concerns using such labels as Qdots. Nevertheless, this is a promising tool to label and track cells and its utilization is expected to increase in the future.

### 1.13. DNA labeling for lineage tracing during corneal wound healing

Cells can also be labeled by incorporating modified nucleic acid analogues into the DNA of dividing cells in pulse-chase experiments. This is particularly useful in studying stem cells, as the nucleic acid gets incorporated into the DNA of progenitor cells (during the pulse phase), and can be tracked as they divide, differentiate, or migrate (during the chase phase). Tritiated thymidine or BrdU (and similar compounds such as EdU/IdU/CldU etc.) are the most commonly used analogues. One of the earliest studies utilized tritiated thymidine to determine epithelial cell turnover and migratory rates in uninjured human, mouse, rat, guinea pig and dog corneas (Hanna, Bicknell, & O'Brien, 1961; Hanna & O'Brien, 1960), and the proliferation and migration of corneal epithelial cells during wound healing in
rats and rabbits (Hanna, 1966). Tritiated thymidine was also used in the benchmark study by Cotsarelis et al. (1989), which showed a population of rare, slow cycling cells in the basal limbus in mice corneas during a 4-week chase period (presumably the CESC). Additionally, the authors showed that these slow-cycling cells could be stimulated to proliferate following wounding, and appeared in the central cornea in response to wounding or application of the tumor promoter 12-O-tetradecanoylphorbol 13-acetate (TPA) (Cotsarelis et al., 1989).

BrdU labeled slow-cycling cells were observed in the basal limbal epithelium in whole-mount rat corneas, and these labeled cells were tracked and studied during their division and differentiation (Beebe & Masters, 1996). The authors showed that the BrdU labeled cells stayed in the basal layer for a variable time period, and synchronously underwent terminal differentiation upon leaving the basal layer. In another study using BrdU labeling, Lehrer and colleagues (Lehrer, Sun, & Lavker, 1998) showed that the slow cycling limbal stem cells and their transit amplifying progeny increased their replication rate in response to wounding or TPA application. This study also highlighted the differences in the proliferative capacity of different corneal cells, as repeated TPA applications caused a reduction in proliferation of central corneal cells, but the putative LSCs and peripheral corneal epithelial cells continued to proliferate (Lehrer et al., 1998). Furthermore, BrdU labeling in a Pax6+-/- mouse model of aniridia showed faster basal to suprabasal movement in Pax6+-/- compared to WT mice, an observation helping to explain the corneal epithelial cell loss in aniridia-induced LSCD (Douvaras et al., 2013).

More recently, an innovative study utilized consecutive labeling with multiple thymidine analogues to determine cell cycle time and proliferation dynamics during wound healing in mouse corneas (Sagga, Kuffova, Vargesson, Erskine, & Collinson, 2018). The authors showed that the percentage of LESC entering the S-phase increased significantly within 24 hours of wounding (~33%, a 7 fold rise in activation compared to unwounded cornea), and an even further increase was observed 48 hours after wounding (80-90% LESC in S-phase) (Sagga et al., 2018). Curiously, 24-48 hour after wounding, LESC were observed to re-enter S-phase in the contralateral unwounded eye as well, which was attributed to an unidentified systemic effect of corneal wounding (Sagga et al., 2018).

A major pitfall of these approaches is that the animals need to be euthanized and fixed to visualize the labeled cells, which precludes continuous monitoring in the same specimen over multiple time points. Additionally, these DNA labeling studies are highly dependent on the delivery method, and the incorporation of 3H-TdR or BrdU, which depends on cells being in S-phase, and this can vary between individual specimens.

The biggest advancements in lineage tracing studies came with the use of transgenic animals. While a lot of early genetic constructs were not inducible, and associated with problems due to embryonic lethality and congenital defects, they were quickly replaced by cell/tissue specific expression constructs and inducible constructs (Kao, 2006). Tissue specific constructs restrict expression to one type of tissue, distinguishing the labeled tissue in a lineage-restricted manner, and provide for fine spatial control. Inducible constructs can be activated to provide temporal control, and most utilize Cre recombinase (Sauer & Henderson, 1988).

Initially, lot of studies involved transgenic animals carrying LacZ for lineage tracing, driven by promoters for various genes to study their expression (Soriano, 1999). Collinson et al. (2002) and Mort et al. (2009) utilized chimeric and X-inactivation mosaic mice expressing LacZ, and observed randomly oriented patches of LacZ expressing cells in the cornea at 5 and 3 weeks respectively, suggesting random distribution of progenitor cells in the cornea at these early stages. The LacZ expressing cells developed into streaks extending from the limbus to the central cornea at 10-weeks, suggesting a consolidation of progenitors to the limbus.

Mort et al. (2009) further used this model to study wound healing and showed that central wounds healed by centripetal movement of cells converging at the corneal center (Figure 1.2A, 1.2C, 1.2E), but peripheral wounds involved both centripetal and centrifugal cell movements, which formed a second point of convergence within those wounds (Figure 1.2B, 1.2D, 1.2F). The centrifugal movement of cells during healing of peripheral wounds indicated that population pressure from the cells originating at the limbus that moved centripetally is not the main driving force for healing these wounds. Interestingly, the stripes from around the wound edge extended at different rates, healing the wound asymmetrically, with the second point of convergence not coinciding with the center of the peripheral wound (Figure 1.2G, 1.2H). This provides clues to the healing mechanisms and suggests that either the population pressure from surrounding cells is unequal or cells are attracted to a specific region in the wound area that may not be the center of the wound. The authors also used Pax6-GFP mice to confirm the results and showed increased (40-fold) centripetal migration of labeled cells during corneal wound healing, as compared to the homeostatic condition (Mort et al., 2009). Both the LacZ and Pax6-GFP
stripes do not mix during wound healing and the cell movement was clonal, indicating a coordinated healing response. A different approach of using the putative stem cell marker K5 (B. Chen, Mi, Wright, & Connon, 2010), as a promoter to drive the LacZ transgene to label and track cells, was adopted by Douvaras et al. (2012). The authors showed patches of K5 labeled cells throughout the cornea, in addition to streaks extending from the limbus to the central cornea.

Another approach involved the use of the ubiquitous CAGG promoter to drive LacZ (Dora, Hill, Collinson, & West, 2015), which is advantageous because it is unbiased and stochastically labels all corneal cell types, including stem cells in the limbus and throughout the cornea. This can be beneficial over using mosaic expression or a presumptive LSC marker to drive expression, which only labels a specific subset of these cells. Using this approach for lineage tracing, the authors showed labeled patches throughout the cornea at early time points (Figure 1.3A). At later time points, the authors observed centripetal streaks, but did not observe long-lived clones in the central cornea (Figure 1.3B) (Dora et al., 2015). The observation of discontinuous streaks in these experiments was attributed to LSCs alternating between quiescent and active phases, and/or loss of these LSC streaks followed by replacement with unlabeled neighboring LSCs (the “neutral drift model”) (Doupe et al., 2012).

An elegant strategy was applied by Tanifuji-Terai et al. (2006) to study corneal development using a combination of LacZ and AP reporters in Krt12(Cre/Cre)/ZAP bitransgenic mice. The construct was designed such that undifferentiated corneal cells in these mice that do not express K12, will express the LacZ gene. On the other hand, the differentiated corneal cells that express K12, will also express Cre recombinase, which excises the LacZ in these cells, and results in expression of Alkaline Phosphatase. After epithelial debridement, maturation of the cornea was accelerated, and a mosaic pattern of K12-negative cells was observed, suggesting presence of CESCs in the central cornea, which were activated after injury. The exact role of these K12-negative basal epithelial cells with regards to self-renewal was not specifically examined in that study.

Contrary to the generally accepted limbal location of stem cells, one study used LacZ lineage tracing to demonstrate the presence of a secondary population of stem cells capable of corneal regeneration within the entire cornea epithelium (F. Majo et al., 2008). The authors of that study also grafted the labeled limbal and central corneal tissues onto the limbal region of immunocompromised mice, and showed that transplanted limbal cells migrated only as a wound healing response, whereas cornea homeostasis was maintained independent of limbal cells. Studies utilizing lineage tracing in mice have shown centripetal migration of cells (Amitai-Lange et al., 2015; Di Girolamo et al., 2015; Ksander
et al., 2014; West, 2015), and attributed these results to disturbance of the normal corneal state by the surgical sutures used during transplantation.

On the other hand, some studies have proposed different mechanisms to explain these results. For example, the leakage of stem cells from the limbal niche due to overcrowding, or simultaneous symmetric divisions of two stem cells to fill a vacant stem cell location, causing one stem cell’s progeny to be ejected from the niche, could explain the presence of stem cells in the central cornea (Lobo et al., 2016). Amitai-Lange et al. (2015) concluded that LSC progeny (i.e., new TACs) that exited the limbus could have a long-term proliferative capacity, and may replenish the cornea for several months, providing an explanation for corneal self-maintenance seen for months after removal of the limbus (F. Majo et al., 2008). The dedifferentiation of differentiated corneal epithelial cells into LSCs facilitated by an intact limbal niche (Nasser et al., 2018), could explain the results by Majo et al. (2008), as heat induced wounding in that study likely did not damage the deeper cells in the limbal niche. These studies can also explain the clinical findings where survival of a healthy corneal epithelium was described even in cases with total LSCD (Dua et al., 2009). Both centrifugal and centripetal migration of cells during repair of a “donut” shaped circular wound have also been shown in human cornea organ culture models (Chang et al., 2008). This group also showed that central corneal epithelial cells were capable of regeneration, at least up to 12 hours after laser ablating the limbus (Chang et al., 2008), owing to the presence of a secondary clonogenic population of cells within the central cornea (Chang, McGhee, Green, & Sherwin, 2011).

1.15. Genetic approaches: Fluorescent reporter systems for lineage tracing during corneal wound healing

The major drawback of LacZ models is the requirement of fixing the specimens and harvesting the tissues before staining with the colorimetric substrate (X-gal), eliminating the possibility of in-vivo live imaging of the same specimen over time. Constructs with fluorescent reporters are thus advantageous, as they can be readily visualized in live specimens. The transgenic animals for these latter studies typically use putative marker proteins’ promoters to drive expression of fluorescent reporters in the cell type under examination.

The keratin K12 promoter has been used to drive tissue specific gene expression in corneal cells (Chikama et al., 2005; Kao et al., 1996). This model was utilized for lineage tracing to study corneal
wound healing in the mouse (Kasetti et al., 2016). Repair of 1.5mm diameter wounds in the central cornea involved GFP expressing stripes extending to the wound during the early healing period (1 day), and these stripes remained connected to the limbus throughout ~4 weeks. In a larger, 2.5mm diameter wound the GFP stripes were still observed but some stripes were discontinuous and detached from the limbus. From these studies, the authors concluded that wound repair was carried out by early TACs derived from the limbus, which have limited proliferation potential that is exhausted during more extensive wound healing processes (Kasetti et al., 2016).

Another study involved Abcb5/Cre reporter mice crossed with tdTomato mice (Gonzalez et al., 2018). tdTomato-positive ABCB5-derived progeny were found scattered across the entire mouse cornea. These ABCB5-expressing precursors gave rise to self-renewing corneal epithelium during development and regeneration, highlighting their role as corneal stem cells (Gonzalez et al., 2018). The crucial role of these corneal stem cells in repair is further supported by studies showing impaired corneal wound healing in Abcb5 knockout mice (Ksander et al., 2014). Besides being a marker for LSCs, ABCB5 functions as a regulator of differentiation (Frank et al., 2003). However, in these wounding studies, transplantation of cultured ABCB5 expressing cells restored K12 expression in only about 30% of the cornea (Ksander et al., 2014). Nevertheless, ABCB5 expression can be particularly useful to sort cells for transplants, and obtain a population of progenitors without immunogenic cells, which can cause transplant rejection. Additionally, ABCB5 expressing cells co-express PD-1, which is involved in maintaining the immune privileged state of the cornea. These two factors help prevent graft-versus-host disease following allogenic transplantation (Gonzalez et al., 2018).

The development of Brainbow constructs (Lichtman, Livet, & Sanes, 2008; Livet et al., 2007; Snippert et al., 2010) added resolution to lineage tracing studies by providing a range of inheritable fluorescent colors in different cells. The stochastic recombination of Lox sites in these constructs allows combinatorial expression of the fluorescent reporters, resulting in numerous different color combinations. Since neighboring cells rarely have the same combination of reporters, it facilitates tracking of individual clones labeled with specific colors (Buckingham & Meilhac, 2011; Kretzschmar & Watt, 2012). The zebrafish version of brainbow, “Zebrabow”, was used for long-term lineage tracing in the cornea to show that embryonic corneal epithelial clones get replaced by centripetally expanding cells from the limbus, which form large wedge shaped clones (Pan et al., 2013). The authors also showed that the peripheral clones become scattered and interspersed during the early time points from 10-15 day post fertilization, and the appearance of rosette-like structures.
K14 has been widely used as a putative limbal stem cell marker (B. Chen et al., 2010). One study involved K14CreERT2:R26R-confetti mice for lineage tracing combined with confocal imaging of corneas harvested at various time intervals up to 17 weeks (Amitai-Lange et al., 2015). The authors demonstrated that the confetti labeled patches observed at 10 days (Figure 1.4A), formed thin multicolored stripes extending between the limbus and the cornea, which represented clones of cells emerging from the limbus and colonizing the corneal epithelium that reach the center by about 17 weeks (Figure 1.4B) (Amitai-Lange et al., 2015). Furthermore, DMSO was applied for creating corneal injuries, resulting in partial to acute loss of corneal transparency one week following application (in both moderate and severe cases). This was associated with epithelial thinning and increased proliferation. Interestingly, healing of mild wounds only involved an increase in the size of the corneal clones (Figure 1.4C), whereas following moderate and severe wounding, cell migration from the limbus was also accelerated (about eight times faster for severe wounds) as compared to cell migration occurring during homeostasis (Figure 1.4D) (Amitai-Lange et al., 2015). Additionally, stripes formed during healing were less organized and occasionally projected in other directions (Figure 1.4D) (Amitai-Lange et al., 2015).

Another group used the K14 driven confetti construct and tracked K14 expressing clones in live mice (Di Girolamo et al., 2015; Richardson et al., 2016). Labeled cells started appearing around 3 weeks post activation (Figure 1.4E) and extended centripetally from the limbus to the center of the cornea by 18 weeks (Figure 1.4F). These cells formed mono-colored stripes that were present through the full-depth of the cornea epithelium. The authors concluded that the clonal expansion observed in their experiments suggests that all cells in a stripe originated from a single precursor at the limbus (Di Girolamo et al., 2015; Richardson et al., 2016).

Both studies of Amitai-Lange et al. (2015) and Di Girolamo et al. (2015) show extensive K14 driven expression of fluorescence in the limbus, and both observed additional small patches of fluorescent clones in the central cornea. Amitai-Lange et al. (2015) reported the presence of K14 expression in most basal corneal epithelial cells, which was present even before the fluorescent stripes developed from the limbus. On the other hand, Di Girolamo et al. (2015) reported much less K14 expression in the basal corneal epithelial cells, and the K14 expression was reported only for later stages.

More recently, the K14 driven confetti construct was employed to study LSCD in mice corneas (Richardson, Park, Watson, Wakefield, & Di Girolamo, 2018). Corneal tissues obtained from K14
confetti transgenic mice were transplanted onto wild type mouse eyes with Algerbrush II induced LSCD, and lineage traced thereafter (Richardson et al., 2018). The confetti grafts generated clones at 2 weeks post injury in the peripheral cornea (Figure 1.4G), which were evenly distributed across the cornea at 6 weeks (Figure 1.4H) (Richardson et al., 2018). The number of these clones decreased overtime, which was attributed to a significant TAC population in the transplanted tissue with limited proliferative capacity (Lehrer et al., 1998). The corneal epithelium and K12 expression were not restored in these experiments. Another study from the same group conducted lineage tracing following annular injuries at different locations on the cornea (Park et al., 2019). The authors reported elevated proliferation in the periphery but not in the center. The repair primarily occurred via centripetally migrating K14 expressing limbal epithelial cells that eventually replaced the central corneal epithelium, suggesting a vital role of these cells in wound healing.

In another study, K15 driven GFP lineage tracing (expressed in the limbal cells) was performed in a wounding model where an Algerbrush II was used to remove limbal epithelium, marginal conjunctiva and the marginal corneal periphery (Nasser et al., 2018). 48 hours after injury the limbus healed, and 30 days after injury, K15-GFP expression recovered in the limbus. The authors argued that repair appeared to occur via differentiated corneal cells, which migrated centrifugally to repair the limbus and then dedifferentiated into functional LESC. The authors further showed that in these experiments, when the limbal niche was damaged, the corneas and K15-GFP expression did not recover (Nasser et al., 2018). These results highlight a potential therapeutic mechanism for the recovery from LSCD, by restoring the connection between the niche and the LSCD affected cornea.

1.16. Overview of subsequent chapters

In chapter 2 of this thesis, we describe a novel model to study Stem Cell Deficiency (SCD) in *Xenopus* that utilizes the DNA crosslinker, Psoralen 4-amino-methyl trioxsalen (AMT) hydrochloride, and UVA illumination to create SCD (the PUV model (Adil, Simons, Sonam, & Henry, 2019). This frog PUV model recapitulates many of the hallmarks of human LSCD, including corneal opacity, corneal neovascularization, and a rough/irregular corneal epithelium. Furthermore, cellular changes following the PUV treatment such as cell death, mitosis, and decrease in the number of p63 expressing basal epithelial cells, have been examined. An additional benefit of this model is the ease with which the PUV damage can be localized to any part of the cornea. In these cases, the treated portion is ultimately able
to recover and corneal transparency is restored. This allowed us to study the response of the remaining cells in the undamaged part of the cornea following SCD and during repair.

In chapter 3, we present a novel model to study healing following PUV treatments that combines lineage tracing (using a lab reared transgenic line carrying a heat-shock inducible H2B-mCherry) in the PUV frog model of SCD (Adil et al., 2019). Since SCD can be restricted to one half of the cornea, this approach strategically spares proliferative cells in the other half, which can be labeled by activating localized mCherry expression using a heat-shock probe, and the labeled cells (nuclei) can subsequently be traced to study the healing process.

Since the PUV model of cornea epithelial stem cell deficiency depletes and damages corneal oligopotent stem cells, and the surrounding skin invades the corneal region, we tested if lenses regeneration was still possible in these treated corneas. Furthermore, by performing a reverse ex-vivo lens regeneration assay (where a control untreated cornea was implanted into a PUV treated eyecup), we were able to determine if this treatment causes any potential damage to the retina or its molecular signaling required to trigger lens regeneration. This is discussed in chapter 4 of this thesis.

Finally, in chapter 5 of this thesis we close with the main conclusions of this work and its future implications on the field of cornea stem cells and limbal stem cell therapeutics. Possible directions for studies based on this work are also be proposed.
Figure 1.1: Limbal stem sell deficiency and stem cell transplantation in humans, and stem cell deficiency and corneal restoration in *Xenopus*

(A) Example of a human cornea with total limbal stem cell deficiency after receiving a chemical burn with involvement of the corneal stroma. Corneal opacification (black arrows) and neovascularization (black arrowheads) is apparent. (B) Example of another patient who received a cultivated autologous limbal stem-cell graft, and penetrating keratoplasty (in some cases, this secondary corneal transplant from an allogenic cornea is necessary for improving visual acuity), showing successful restoration of a transparent corneal epithelium (black arrow). Vascularization is now restricted to the conjunctiva (black arrowheads). Dotted black circle outlines the cornea region. Image in (A) is from Sacchetti et al. (2018), Limbal Stem Cell Transplantation: Clinical Results, Limits, and Perspectives, Copyright © (2018), open access article distributed under the Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/). Image in (B) is from The New England Journal of Medicine, Paolo Rama, Stanislav Matuska, Giorgio Paganoni, et al., Limbal stem-cell therapy and long-term corneal regeneration, 363(2), 147-155. Copyright © (2010) Massachusetts Medical Society, Reprinted with permission from Massachusetts Medical Society.
Figure 1.2: Wound healing and cell tracking in the mouse Pax6-GFP model

(A, C, E) Images from time-lapse confocal microscopy showing healing of a 1 mm diameter central wound in a PAX6-GFP mosaic corneal epithelium (the white areas are GFP-positive). The
Figure 1.2 (cont.):
wound completely closes by 18.75 hours (h) after wounding. (B, D, F) Healing of a peripheral wound showing closure and the formation of a secondary whorling pattern. By 18.75h this wound has not closed completely. Dotted circles show the extent of the wounds. (G, H) High magnification views and graphical representation detail the healing of a central wound in a PAX6-GFP cornea. (G) View of the initial 1mm wound and the future routes taken by tracked cell stripes (numbered 1 to 6). (H) After 18 hours of healing the wound is completely closed, but the stripes do not meet at the center of the original wound, and do not extend equally. Scale bars in (A) to (F) equal approximately 1mm. Scale bar in H equals 500μm and applies to (G) and (H). Images are from Mort et al. (2009), Mosaic analysis of stem cell function and wound healing in the mouse corneal epithelium, Copyright © 2009, Mort et al; licensee BioMed Central Ltd., open access article distributed under the Creative Commons Attribution 4.0 International License 4.0 (http://creativecommons.org/licenses/by/4.0/).
Figure 1.3: Lineage tracing in mice corneas

(A, B) Lineage tracing in CAGG driven LacZ mice. Eyes were stained for β-gal activity after a 5 week (wk) chase period. (A) shows small β-gal positive patches throughout the cornea and conjunctiva. (B) After a 14 week chase period stripes extend radially towards the center of the cornea. Images in (A, B) are from Dora et al. (2015), Lineage tracing in the adult mouse corneal epithelium supports the limbal epithelial stem cell hypothesis with intermittent periods of stem cell quiescence, Copyright © 2015, Dora et al., published by Elsevier, open access article distributed under the Creative Commons Attribution License 4.0 (http://creativecommons.org/licenses/by/4.0/).
Figure 1.4: Lineage tracing in K14-confetti mice during homeostasis, following DMSO induced wounding, and in corneal epithelial cell transplants

(A-B) Lineage tracing during homeostasis. (A) Flattened corneas ten days after tamoxifen induction showed the appearance of sporadic small clusters of cells expressing different fluorophores.
Figure 1.4 (cont.):

(B) Four months after tamoxifen induction, radial stripes of migrating cells emerged from the limbus into the center of the cornea, along with some isolated clusters of labeled cells. (C, D) Lineage tracing during wound healing. Tamoxifen induction was carried out by topical application with DMSO to create the corneal wound. (C) One week after mild wounding by single topical application of DMSO, numerous sporadic clusters of cells expressing the different fluorophores appeared. (D) One week after severe wounding, created by 3 successive daily applications of DMSO, multiple wide streaks of fluorescent cells were observed, some of which were less organized and projected in various directions. (E-F) Lineage tracing during homeostasis. Intravital fluorescence microscopy shows expansion and migration of K14+ fluorescent clones in Confetti transgenic mice. Different colored cells were observed in the peripheral cornea at 3 weeks post-tamoxifen induction (E), which developed into discrete clones (stripes) extending towards the central cornea to the apex by 18 weeks (F). (G, H) WT mice that were subjected to total corneo-limbal epithelial debridement, received a graft consisting of fibrin and fluorescent corneo-limbal epithelial cells from Confetti mice. Clones (stripes) from confetti grafts in the cornea at 2 weeks (G), and 6 weeks (H) post injury observed using intravital fluorescence microscopy. Dashed white line in (A-D) marks the limbal-corneal border. White dotted circle in (E) encloses the lens. CFP, cyan fluorescent protein; GFP, green fluorescent protein; RFP, red fluorescent protein; YFP, yellow fluorescent protein; W, weeks. Scale bars equal 1mm in (A-D) and 400μm in (E-H). Images in (A-D) are from Amitai-Lange et al. (2015), Lineage Tracing of Stem and Progenitor Cells of the Murine Corneal Epithelium, published in Stem Cells, Copyright © 2014 AlphaMed Press, Ruby Shalom-Feuerstein, Beatrice Tiosano, Noora Dbayat, et al., Published by John Wiley and Sons. Images in (E, F) are reprinted from The Ocular Surface, 14 /2, Alexander Richardson, Denis Wakefield, Nick Di Girolamo, Fate Mapping Mammalian Corneal Epithelia, 82-99, Copyright © (2016), with permission from Elsevier. Images in (G, H) are from Richardson, A., Park, M., Watson, S. L., Wakefield, D., & Di Girolamo, N. (2018). Visualizing the Fate of Transplanted K14-Confetti Corneal Epithelia in a Mouse Model of Limbal Stem Cell Deficiency. Invest Ophthalmol Vis Sci, 59(3), 1630-1640, under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (https://creativecommons.org/licenses/by-nc-nd/4.0/).
1.18. References


dermal fibroblast and corneal limbal epithelium. *PLoS One, 7*(9), e45435. doi:10.1371/journal.pone.0045435


47


54


CHAPTER 2. UNDERSTANDING CORNEA HOMEOSTASIS AND WOUND HEALING USING A NOVEL MODEL OF STEM CELL DEFICIENCY IN 
XENOPUS

2.1. Introduction

The basal cornea epithelium includes a population of Corneal Epithelial Stem Cells (CESCs) that are responsible for corneal homeostasis, wound healing, and for maintaining corneal transparency, which is essential for normal vision (Cotsarelis et al., 1989). During homeostasis, CESC undergo asymmetric divisions to generate Transit Amplifying Cells (TACs), which undergo more rapid and numerous cell divisions, and ultimately form terminally differentiated cells of the more superficial layers. Superficial cells are eventually sloughed off from the surface as they die (Beebe and Masters, 1996; Thoft and Friend, 1983). It is generally accepted that CESC reside in the basal layer of the peripheral cornea or limbus, in crypt-like structures called Palisades of Vogt (Amitai-Lange et al., 2015; Davanger and Evensen, 1971; Di Girolamo, 2011; Dua et al., 2005; Pellegrini et al., 1999; Schermer et al., 1986; Shortt et al., 2007a; Townsend, 1991, Zhao et al., 2009). However, some studies suggest they may reside throughout the cornea in certain species (Chang et al., 2008; Dua et al., 2009; Majo et al., 2008).

Limbal Stem Cell Deficiency (LSCD) is a debilitating corneal disease resulting from functional or anatomical loss of the cornea epithelial stem cell population. In LSCD, the transparent cornea epithelium cannot be renewed and is ultimately replaced by conjunctival epithelial cells. Clinical features for diagnosis of LSCD include epithelial defects, corneal opacity and vascularization, with consequent visual impairment or blindness (Sejpal et al., 2013). Centripetal migration of pigment cells into the central cornea has also been observed during this process in rabbits, guinea pigs, and even humans (Cafaro et al., 2009; Cowan, 1963; Davanger and Evensen, 1971; Henkind, 1965; Mann, 1944; Michaelson, 1952; Wolosin et al., 2000). Clinical manifestations of LSCD vary based on the severity and extent of involvement of the cornea tissue (Dua and Azuara-Blanco, 2000). There can be partial LSCD, in which only a segment of the limbus is involved, or more severe cases, involving total LSCD.

---

3 This chapter was originally published as Adil MT, Simons CM, Sonam S, Henry JJ. (2019) Understanding cornea homeostasis and wound healing using a novel model of corneal epithelial stem cell deficiency in Xenopus. Exp Eye Res. 187:107767, and has been reproduced with permission from copyright owner.
Remarkable therapies have been developed to treat LSCD by utilizing epithelial stem cell transplants (Atallah et al., 2016; Galindo et al., 2017; Grueterich et al., 2002; Haagdorens et al., 2016; Holland 2015; Koizumi et al., 2001; Le and Deng, 2019; Ljubimov and Saghizadeh, 2015; Rama et al., 2010; Saghizadeh et al., 2017; Sasamoto et al., 2018; Shortt et al., 2007b; Utheim et al., 2018; Yazdani et al., 2019). However, effective repair and recovery depends on many factors, such as the source and concentration of donor stem cells, and the proper conditions to support these transplanted cells (Atallah et al., 2016; Ti et al., 2002). Our understanding of LSCD pathology, and the specific behavior of CESCs, is still rather limited. We do not fully understand mechanisms underlying how CESCs heal wounds or how the transplanted CESCs restore transparency in LSCD patients (Castro-Munozledo, 2013). A major hurdle to studying CESCs and LSCD has been a lack of convenient vertebrate models.

One excellent vertebrate model is the frog *Xenopus*. In the early larval stages (Nieuwkoop and Faber, 1994), the *Xenopus* cornea epithelium is a two-cell layer thick structure, comprised of an outer apical layer and a basal layer. A few scattered keratocytes lie below the basal layer. The cornea endothelium is not initially fused to the basal layer; the two layers being connected only at the center of the cornea by a structure called the corneal stalk or stroma attracting center (Hu et al., 2013). As the cornea develops, more keratocytes appear, the stroma develops, and additional structures are added (Bowman’s layer, and Descemet’s membrane) (Hu et al., 2013). In *Xenopus*, both cornea morphology and development are largely conserved with that of humans (DelMonte and Kim, 2011; Hu et al., 2013), which makes it a suitable model system to study the cornea epithelium, its stem cells, and potentially the pathology of corneal diseases.

One of the methods used to identify stem cells is via retention of incorporated thymidine analogs (BrdU, EdU). Unlike the highly proliferative TACs, the more quiescent stem cells retain the incorporated thymidine analogs for longer durations and are referred to as Label Retaining Cells (LRCs) (Yoon et al., 2014). Previous studies conducted in our lab show that while these LRCs are restricted to the peripheral limbal region in the adult frog cornea, (Hamilton and Henry, 2016) the LRCs appear to be distributed throughout the basal layer in the larval frog cornea (Perry et al., 2013). Interestingly, in both the larval and adult frog cornea, p63 (conventionally accepted as one of the CESC/TAC markers (Pellegrini et al., 2001)) labels all nuclei throughout the basal layer of larval and adult corneas (Perry et al., 2013; Sonam et al., 2019).

Here, we used a photoactivatable DNA cross-linker, Psoralen 4-amino-methyl trioxalen (AMT) hydrochloride, in combination with UVA exposure (PUV), to establish a novel frog model of stem cell
deficiency (SCD) that mimics many of the changes seen in human LSCD. This combination of Psoralen and UVA is routinely used in labs to generate feeder cells (McGarry et al., 2009) for tissue culture, in clinical treatments of psoriasis (Stern, 2007), and to inactivate leukocytes for preventing transfusion-associated graft-versus-host disease (Grass et al., 1998). We show that treatments done on whole corneas result in cases of SCD, and these corneas do not regain transparency. Furthermore, we have characterized cellular/molecular events, including: mitosis, cell death, cell density, appearance of the Tcf7l2 labeled subset of apical skin cells, and changes in the percentage of p63 positive basal cells (as a potential indicator of basal proliferative cells, e.g., CESC and TAC), over a period of up to 32 days following these treatments. We showed that PUV treatment results in cell death and a conversion to the skin phenotype. On the other hand, corneas subject to treatments on only one half of the cornea are eventually able to heal and restore their transparency.

2.2. Materials and methods

2.2.1. Animals

Adult *Xenopus laevis* frogs were obtained from Nasco (Fort Atkinson, WI). Tadpole larvae were reared following established protocols (Henry and Grainger, 1987; Henry and Mittleman, 1995). Tadpole stages were assessed using those defined by Nieuwkoop and Faber (1994). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Animal Resources (DAR) at the University of Illinois.

2.2.2. Creating LSCD via photoactivatable (UV), chemical treatments (Psoralen AMT)

To deplete cornea stem cells we used Psoralen AMT (Sigma-Aldrich, St. Louis, MO). Psoralen is a planar tricyclic compound, which passes freely through cell and nuclear membranes, where it intercalates into the DNA strands (Cimino et al., 1985). It is a light-sensitive drug that absorbs ultraviolet light (long wave-UVA) to form monoadducts and diadducts (crosslinks) between thymine bases (Cimino et al., 1985; Deans and West, 2011; McGarry et al., 2009). Therefore, treatment with UVA forms covalent bonds and irreversible DNA inter-strand crosslinks (Cimino et al., 1985). Inter-strand crosslinking with Psoralen interferes with DNA replication, arrests cell division, and can cause cell death, effectively preventing proliferation of treated cells. This novel application of Psoralen/UV (PUV) treatment, when applied to the cornea, creates a state of stem cell deficiency within the cornea.
2.2.3. Topical application of Psoralen (Construction of the reservoir pipette tip)

A standard 1ml pipette tip (Catalog No. 1111-2021, Tip One, USA Scientific Inc.) was used to construct a modified reservoir tip for localized application of Psoralen to the cornea. The tip was cut at approximately 5mm from the dispensing end at a 45° angle (Figure 2.1A, 2.1B). This results in an oval opening with inner diameters of approximately 1.7mm by 1.4mm at the end of the pipette (Figure 2.1C). A notch of approximately 9mm in length was also cut into one side of the pipette, about 7mm above this opening (Figure 2.1B, 2.1C). This creates an open reservoir with a holding volume of about 40µl. The reservoir opening is sufficiently large to permit addition of Psoralen solution, without disturbing or moving the pipette when it’s placed tightly against the cornea. Lastly, the back end of the pipette was cut in half, about 20mm from its opening (Figure 2.1B), and half of the pipette was removed so that it can be attached to a metal rod held by a Narishige joystick hydraulic micromanipulator, using a small binder clip (Figure 2.1D, 2.1E).

2.2.4. Psoralen-UV (PUV) treatment procedure

*Xenopus* tadpoles were treated at developmental stages 51-53 (Nieuwkoop and Faber, 1994). The tadpoles were first anaesthetized (~1-2 minutes) in MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, St. Louis, MO) diluted 1:2000 in 1/20x Normal Amphibian Media (NAM) (Slack, 1984). A 60mm petri dish was layered with anesthesia-moistened Kimwipes to form a trough to restrain the tadpole during treatment (Figure 2.1E). The tadpole was gently maneuvered into the trough with a small fire polished glass rod to position it on one side, such that the left lateral side was facing upwards toward the microscope objective. The reservoir pipette tip was maneuvered using the Narishige hydraulic micromanipulator, to lower it to the surface of the cornea, where the modified tip establishes a sealed, leak-free pocket with the small reservoir directly over the cornea (Figure 2.1E).

A fine gel loading pipette tip (Catalog No. 02-707-181, Fisher Scientific, Hampton, NH) was used to deliver 20µl of the Psoralen solution into the reservoir. After the treatment time had passed, the Psoralen was removed from the reservoir and the pipette tip was retracted from the eye. The tadpole, was immediately illuminated by UVA light (wavelength=346nm) for the required amount of time, using an upright fluorescence microscope (Zeiss Axioplan) with a 10X objective (Figure 2.1F). The area of UV exposure was constrained to the approximate diameter of the eye and the thin ring of skin immediately surrounding the eye by using the fluorescence microscope’s adjustable iris. In some cases
only the posterior half of the cornea was irradiated. To accomplish this, a mask was placed over the anterior half of the eye that consisted of a rectangular sheet of aluminum foil, approximately 24mm X 15mm. One edge was folded back along the long axis to prepare a final size of 24mm X 11mm. This was done since the trimmed edges might be sharp, and therefore only the smooth, folded edge was allowed to contact the cornea.

After illumination, the tadpoles were transferred to recovery bowls containing 1/20x NAM for about 30 minutes, and the bowls were placed on a rocker set at 20 rocks per minute to permit quick recovery of the tadpole from the anesthesia (Hamilton and Henry, 2014). The tadpoles were next transferred into a bowl with dechlorinated tap water. Corneas from the untreated right sides of these tadpoles were used as internal controls.

2.2.5. Phenotypic assessment of LSCD

In both treated and control cases, morphological changes were observed, including the invasion of pigment cells from the peripheral limbus towards the center of the cornea, changes in opacity, and vascularization of the cornea. Images for phenotypic assessment were taken under a compound microscope (Zeiss Axioplan) using external epi-illumination from a gooseneck fiber-optic light source. A SPOT camera (Spot Imaging, Sterling Heights, MI) was used to capture images at multiple focal planes for each specimen. These images were then merged (flattened) using Helicon Focus software (Helicon Soft Ltd., Kharkov, Ukraine). FIJI (National Institutes of Health, Bethesda, MD, USA) was used for image analyses. The area of the cornea was approximated in these images by drawing a circle around the peripheral region overlying the diameter of the eye, and calculating the area inside this circle (boundary demarcated in Figure 2.1G).

The number of pigment cells were then counted in this area, and normalized to a standard area (1mm² for whole cornea treatments and 0.5mm² for each half following half cornea treatments). The normalized number of pigment cells were then plotted and compared to controls. Opacity and vascularization were scored on a scale of 0, 1, and 2, based on the degree of opacity or vascularization (see Table 2.1 for the scoring criteria), which are based on similar criteria reported in the literature (Le et al., 2018; Shortt et al., 2014).

Animals were reared for a period of up to 32 days. Challenges were associated with rearing and imaging the live animals, which are subjected to repeated anesthetization and observation. Some animals
do not tolerate repeated, frequent anesthesia to permit daily observations. Therefore, the time intervals between repeated observations were lengthened and varied for different specimens.

2.2.6. Optimization of Psoralen treatment concentration and duration

A stock solution of 5mM Psoralen AMT was first prepared in DMSO and diluted into 1/20xNAM (Slack, 1984), to make the required working solutions. A wide variety of conditions, including different Psoralen concentrations (between 5 and 25µM), treatment times (1 to 5 minutes), and UVA exposure times (between 60 and 75 seconds), were tested in preliminary experiments to determine a set of conditions that produced consistent results (preliminary data not shown). In some cases, the treatments were even repeated a second time on the same animals. It was found that a single application of Psoralen, at a concentration of 20µM, for 2 minutes, 45 seconds, with UVA exposure of 65 seconds, represented the lowest concentration and time interval with the most consistent effects, where 100% of the specimens exhibited some opacity at 32 days post treatment (dpt). 88% of these cases exhibited signs of neo-vascularization by 32dpt. Furthermore, of these cases, 92% of the specimens exhibited some signs of opacity and neovascularization by 15/16dpt (Figure 2.4B, 2.4C, Table S2.1A, S2.1B). With lower concentrations and shorter exposure times the percentage of cases with these effects was smaller. Higher concentrations and longer treatment times were found to cause the eyeball to undergo a slight rotation away from the surface in some cases. Interestingly, when these same conditions were tested for the half-cornea treatments, the effects were found to be somewhat less severe. For example, only 50% of the cases showed some moderate opacity and only 50% of the cases showed some moderate neovascularization at 15dpt on the treated side. The other cases appeared to be normal. Therefore, a slightly higher concentration of Psoralen (25µM) with a longer application time (3 minutes, 30 seconds), and longer UVA exposure (75 seconds) were ultimately chosen for those half cornea treatments. These conditions resulted in some opacity in 100% of the specimens, and neo-vascularization in 86% of the specimens at 15dpt (Figure 2.4E, 2.4F, Table S2.1C, S2.1D). Even though this treatment was greater than that used on whole corneas, most of these half-treated corneas were eventually able to recover by 28dpt (unlike whole treated corneas, see Results below).

2.2.7. TUNEL assay

The TUNEL staining protocol was adapted from Hensey and Gautier (1998) with the following changes. Tadpoles were fixed in 3.7% formaldehyde for 2.5 to 3 hours at room temperature (RT).
Eyeballs were removed and rinsed with 1X Phosphate Buffered Saline (1XPBS), 3 times for 15 minutes at RT. Permeabilization was done by incubating eyeballs in 1XPBS and 0.2% Triton X-100 (PBT) for 2 washes of 10 minutes each. The samples were then washed in 1XPBS 2 times for 15 minutes each, before incubating in 1X Terminal deoxynucleotidyl Transferase (TdT) buffer (Promega, Madison, WI) for 30 minutes at RT. Recombinant TdT (Promega, Madison, WI) was added at a concentration of 150 U/ml with 0.5µM digoxigenin-11-dUTP (Roche), and samples were incubated overnight at RT. Reactions were terminated with 1XPBS and 1mM EDTA at 65°C (two washes of one hour each). EDTA was removed with four one-hour washes in 1XPBS at RT. The chromogenic detection reactions have been adapted from Harland (1991) with the following changes. After the 1XPBS washes from the previous step, the eyes were washed in PBT for 15 minutes at RT, blocked in PBT with 20% goat serum, for one hour at RT, and incubated overnight at 4°C in Fab fragments of anti-digoxigenin-alkaline-phosphatase (Roche, Basel, Switzerland) diluted 1:2000 in PBT with 20% goat serum. Reactions were rinsed with PBT at RT for 6 washes of one hour each, and finally at 4°C overnight. The samples were briefly washed 2 times for 10 minutes each with alkaline phosphatase (AP) buffer (0.1M Tris, pH 9.5 and 0.05M MgCl₂), before the staining was developed using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate substrates, each diluted 1:300 in AP buffer. The chromogenic reaction developed at RT for approximately 25 minutes and was terminated by washing in 1XPBS once at RT for 5 minutes, followed by 2 washes of 1XPBS at RT for 30 minutes each to remove any leftover chromogenic reagents.

2.2.8. Immunofluorescence

The processed samples from TUNEL assays were washed in PBT, 2 times for 15 minutes each at RT. Blocking was done for one hour at RT with the blocking mixture prepared as follows: 1% Bovine Serum Albumin and 10% Goat Serum, diluted in PBT. The samples were then transferred into primary antibodies, which are all diluted in the immunofluorescence blocking solution, and incubated overnight at 4°C. The p63 antibody (1:125, ab735, Abcam) was used to detect basal cells (CESCs/TACs), and the phospho-Histone H3S10P antibody (1:400, sc-8656-R, Santa Cruz) was used to detect the subset of cells undergoing mitosis. Samples were rinsed in PBT 5 times for 10 minutes each before incubation in secondary antibodies (1:300, diluted in immunofluorescence blocking solution, Alexa Fluor goat anti-mouse 488 or Alexa Fluor goat anti-rabbit 546, Life Technologies) for 1.5 hours at RT. Next, samples were washed 2 times in PBT for 10 minutes each at RT. Nuclei were visualized by staining with
Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) (2µg/ml in 1XPBS) for 30 minutes at RT. Lastly, samples were rinsed 2 times in PBT for 10 minutes each at RT, and once in 1XPBS for 10 minutes at RT.

For Tcf7l2 immunostaining, animals were fixed in Dent’s fixative (20% DMSO, 80% methanol) overnight at 4°C, followed by 2 washes with 100% methanol for 10 minutes each at RT. Eyeballs were removed and washed with 1X PBS and 0.5% Tween 20 once for 5 minutes at RT, followed by incubation in Dent’s blocking solution (1X PBS, 0.5% Tween 20, and 10% Goat Serum) for 2 hours at RT. Samples were incubated in the Tcf7l2 primary antibody (anti-TCF4, D-4: sc-166699, Santa Cruz Biotechnology, Dallas, TX) at 1:200 dilution in Blocking solution, overnight at 4°C. Afterwards, samples were washed with 1X PBS and 0.5% Tween 20, 6 times for 10 minutes each at RT, before incubating in secondary antibody (1:300, Alexa Fluor Goat anti-mouse 488, Life Technologies) for 1.5 hours at RT. The samples were then washed with 1X PBS and 0.5% Tween 20 four times for ten minutes each. Nuclei were visualized by counterstaining with Hoechst 33342 (1µg/ml in 1XPBS) (Thermo Fisher Scientific) for ten minutes at RT. Finally, samples were rinsed 2 times in PBT for ten minutes each at RT, before mounting the corneas on microscope slides, as described above.

Following the staining, the corneas were gently detached from the eyes by lifting their edges using fine forceps, and cutting the cornea free from the corneal stalk (Figure 2.1G). The detached corneas were placed in a 60mm petri dish filled with 1XPBS. Ultra-fine iridectomy scissors were used to make 4-5 small cuts in each cornea from the periphery towards the center, which helps to flatten their spherical structure. The corneas were then placed into a drop of SlowFade Gold Antifade mountant (Thermo Fisher Scientific, Waltham, MA) on a glass microscope slide. Coverslips were placed on the samples and gently pressed to flatten the specimens.

2.2.9. Hematoxylin and Eosin Staining

PUV treated specimens were euthanized and fixed in 3.7% formaldehyde or Dent’s fixative as described in section 2.2.7 and 2.2.8 above. The samples were then dehydrated in ethanol for 10 minutes each, cleared in Xylene, and embedded in Paraplast Plus (McCormick Scientific LLC, St Louis, MO). The samples were sectioned at a thickness of 9µm, and stained with Harris Hematoxylin (Fisher Scientific, Hampton, NH), and Eosin, based on the established protocols (Humason, 1972; Wolfe and Henry, 2006).
2.2.10. Microscopy and image analysis

Samples were observed under a Zeiss Axioplan microscope, and imaged using a Axiocam 503-mono camera controlled by ZEN software (Carl Zeiss, Munich, Germany). The number of labeled nuclei were determined using Image J (U.S. National Institutes of Health, Bethesda, MD). To quantify the total number of nuclei (Hoechst labeled nuclei), p63 labeled nuclei, and cell death (TUNEL positive nuclei, and more highly condensed, fragmented Hoechst labeled pyknotic nuclei) three random areas (minimum area = 70µm X 70µm and maximum area = 345µm X 260µm (area of the entire 40X image)) were selected on PUV treated whole corneas, untreated controls, Psoralen only (without UV) controls, and UV only (without Psoralen) controls. For the half cornea PUV treated samples, and half cornea UV only treated controls, two random areas (as above) were selected on both the treated and untreated halves of these corneas. The number of nuclei were counted in these areas, and expressed as nuclei per unit area, to calculate the nuclear density for Hoechst, p63, TUNEL assays and pyknotic nuclei. Typically, the TUNEL positive nuclei were sporadically distributed across the cornea, so for all these cases the value was obtained by counting all the TUNEL positive nuclei within the cornea samples. In all cases, the total area of the cornea was calculated and the TUNEL positive nuclear density was then expressed as total TUNEL positive nuclei divided by total cornea area. To quantify the cells undergoing mitosis (H3S10 positive nuclei), all the labeled nuclei present in the cornea were counted.

To quantify pyknotic nuclei, Hoechst labeled speckles (HLS) smaller than approximately 3µm (about one third to one half of the usual size of a healthy nucleus) were considered as pyknotic nuclei. Closely spaced clusters of HLS were counted as one pyknotic nucleus. A distinct pyknotic nucleus was also scored when the distance between HLS was greater than approximately 3µm. Whenever the appearance of pyknotic nuclei was not well-defined (due to a greater degree of nuclear fragmentation) confirmation was obtained using the appearance of nuclear blisters in the corresponding DIC images, where multiple closely situated HLS, appearing in one nuclear blister, were counted as a single pyknotic nucleus. The Hoechst nuclear density was normalized to a standard area of 70µm x 70µm. Furthermore, the number of p63, H3S10, TUNEL positive, and pyknotic nuclei were divided by the total number of nuclei, to determine the number of respective positive nuclei per total number of nuclei present within the cornea or standard area. Expressing these numbers as a percentage of the total nuclei in the cornea allows us to control for the variation in cell density of the cornea and variations among individual specimens. Since there are very few mitotic nuclei, as compared to Hoechst labeled nuclei, mitosis was expressed as the number of mitotic figures per 1000 Hoechst nuclei. Pericorneal tissue, where
pigmentation is present (Figure 2.1G), was excluded from analysis for all control samples. Likewise, in PUV treated samples where pericorneal tissue (skin cells/pigment cells) ultimately appeared over the edge of the eye to replace the cornea, only the area located directly over the eyeball was included in these analyses. For statistical analysis and error calculation each “N” is defined as the number of individual corneas analyzed for that particular set of conditions. Statistical analysis has been done by comparing standard error of the means in respective cases, and evaluated using the unpaired t-test.

2.3. Results

2.3.1. Characterization of corneal phenotypes after PUV treatment

We examined the effects of the PUV treatment on corneas to evaluate whether the phenotype recapitulated characteristics of LSCD seen in mammals, e.g., changes in corneal opacity, corneal neo-vascularization, and even the presence of pigment cells (melanocytes) in the cornea. Untreated control corneas never showed signs of SCD, and appeared completely transparent, and maintained a regular, intact, smooth, curved surface (Figure 2.2A, 2.2A’). Corneas that received PUV treatment started showing moderate effects, and appeared translucent by 3-7dpt (Figure 2.4B, Table S2.1A). Dorsal views also show that the PUV treated corneas developed a bumpy appearance, with an irregular epithelial surface (Figure 2.2B, 2.2B’). A few blood vessels were also seen in the cornea, but were mostly restricted to the peripheral regions (Figure 2.4B, Table S2.1A). Pigment cells started appearing in the cornea region by 7dpt, and by 15dpt the pigment cells were scattered over the entire cornea (Figure 2.2C, 2.2C’). At even later stages (25 days post treatment), pigment cells were still present throughout the cornea and blood vessels extended further inward into the central cornea region (Figure 2.2D’), both signs of a more severe SCD phenotype. None of the treated corneas returned to normal appearance and continued to show these phenotypes over the course of 32 days post treatment (described in more detail below).

A feature which is unique to aquatic vertebrates is a group of sense organs within the lateral line organ system (LLOS) that helps these animals navigate through water by detecting movements, vibrations, and pressure changes. These lateral lines are located at various positions along the body. Two of these, the supraorbital and infraorbital canals, extend around the eye (white arrows, Figure 2.3A, 2.3A’). In an untreated control cornea, these parts of the LLOS were restricted to the epidermis surrounding the cornea (Figure 2.3A, 2.3A’). In PUV treated corneas, the supraorbital and infraorbital...
canals became displaced towards the center of the cornea region that overlies the eye, together with pigment cells (Figure 2.3B, 2.3B’).

Interestingly, over time, we also observed that PUV treated eyes would appear somewhat smaller in size compared to the untreated control eyes (Figure 2.2A, 2.2A’ vs. Figure 2.2C, 2.2C’). Measurements taken over time showed that PUV treated eyes showed continued growth that was delayed between 1-11dpt, compared to the untreated control eyes, before resuming similar growth rates to controls (Figure S2.1). We examined these eyes more closely in sections to determine if the smaller size might be caused by damage to the retina or the cells residing in the ciliary marginal zone. Histological examinations revealed no morphological differences between the untreated and the treated eyes at 15dpt and 30dpt, and these eye tissues appeared to be normal (Figure S2.2).

In order to examine how untreated cornea tissue may repair damage following PUV treatment, we restricted the treatments to the posterior half of the cornea. Superficially, the untreated halves showed a phenotype similar to untreated control (whole corneas); and changes in opacity or increases in pigmentation were not observed from 1-28dpt (Figure 2.2E, 2.2E’). On the other hand, the PUV treated half initially developed a phenotype similar to the PUV treated whole corneas, as described above (Figure 2.2E, 2.2E’). However, when the half-cornea treatment animals were followed through 28dpt, most of the treated posterior halves of the corneas appeared to restore their normal appearance and looked similar to the anterior untreated halves (Figure 2.2F, 2.2F’). Interestingly, some changes were initially observed in both the treated and untreated halves of these corneas in response to PUV treatments, which are described in greater detail, below.

2.3.2. The effect of PUV treatment on pigmentation

The cornea is typically devoid of pigment cells. To quantify the effects of PUV treatment on pigmentation in the cornea, changes in pigment cell counts were quantified. The natural pigment in these melanocytes allows for ready visualization of these cells and for tracing their movements, and provides one sign for the influx of cells from the epidermis surrounding the cornea.

The untreated control corneas showed a minimal number of pigment cells, with only 1-4 pigment cells in the standard 1mm² area for 1 to 22dpt time points, and an average of 6 pigment cells at the 32dpt time point, which may be related to the larger size of these eyes (Figure 2.4A). These few melanocytes were always observed close to the peripheral boundary of the cornea, and never extended into the central cornea region. The pigment cell counts in the PUV treated corneas were similar to the untreated control
corneas at 1 to 5dpt, but started increasing at 7dpt. In contrast to the untreated control corneas, the pigment cell counts kept increasing over time (average of 53 pigment cells in the standard area) at 15dpt (Figure 2.4A). The highest number of pigment cell counts was observed at 32dpt (average of 141 pigment cells in the standard area), when the eyeball appears to be completely covered by epidermis (Figure 2.3B, 2.3B’). This sharp increase may also be influenced by the process of metamorphosis, which is accompanied by normal remodeling of the skin, head/skull, and retraction of the eyeball within the orbit (Figure 2.3B, 2.3B’).

In cases where PUV treatment was restricted to the posterior halves, the untreated control halves of these corneas showed minimal numbers of pigment cells between 1-28dpt (average of 1-4 pigment cells in a standard area). In the treated half of these corneas, the pigment cell counts were higher at 5dpt and continued to increase until 15dpt (average of 29 pigment cells in a standard area) (Figure 2.2E, 2.2E’, Figure 2.4B). After 15dpt, the number of pigment cells decreased at 22 and 28dpt (Figure 2.4B). The corneas didn’t become completely free of pigment cells over this time interval, but the number of pigment cells was greatly reduced (average of 5 pigment cells in a standard area) (Figure 2.2F, 2.2F’).

2.3.3. The effect of PUV treatment on corneal opacity

The untreated control corneas showed no signs of corneal opacity for 1 to 32dpt (Figure 2.4B, Table S2.1A). The PUV treated corneas did not show opacity at 1dpt, but started showing moderate opacity starting at 3dpt (Figure 2.4B, Table S2.1A). As the PUV damage progressed, an increased number of specimens showed moderate opacity at 7dpt (Figure 2.4B, Table S2.1A). The severity of opacity, as well as the percentages of specimens showing severe opacity, increased as the time points progressed, and 100% of the specimens exhibited some opacity at 32dpt (Figure 2.4B, Table S2.1A) (Figure 2.3B, 2.3B’). A few specimens examined at 11, 15, and 22dpt did not show signs of corneal opacity (Figure 2.4B, Table S2.1A), which suggests that the accumulation of the PUV damage was delayed in those cases.

In cases that received PUV treatment only on the posterior halves, the untreated control halves of the corneas were clear and transparent, and showed no signs of opacity at any of the time points examined (Figure 2.4E, Table S2.1C). The treated posterior halves of the cornea initially did not show opacity at 1 and 3dpt, but started showing moderate corneal opacity starting at 5dpt (22% cases) (Figure 2.4E, Table S2.1C). The number of specimens that showed moderate to severe opacity increased from 5 to 11dpt, and peaked at 15dpt (57% moderate opacity, 43% severe opacity) (Figure 2.4E, Table S2.1C).
(Figure 2.2E, 2.2E’). After 15dpt, the corneal opacity decreased at 22 and 28dpt. Some samples (40%) showed no signs of opacity at 22dpt (Figure 2.4E, Table S2.1C), and most cases were transparent by 28dpt (83%, Figure 2.4E, Table S2.1C) (Figure 2.2F, 2.2F’), indicating that most specimens underwent significant repair.

2.3.4. The effect of PUV treatment on vascularization

The untreated control corneas did not show signs of vascularization in most cases. In some specimens, slight vascularization was observed very close to the peripheral boundary of the cornea, but these vessels never extended into the central cornea region (Figure 2.4C, Table S2.1B). In PUV treated corneas, the number of specimens showing moderate vascularization started increasing at 3dpt, where either the supraorbital and/or the infraorbital artery would extend branches into the peripheral cornea (Figure 2.4C, Table S2.1B). The severity of vascularization also increased from 5 to 11dpt. Starting at 15dpt the ratio of specimens graded severe vs. moderate was higher, indicating increased severity of neo-vascularization at these time points (Figure 2.4C, Table S2.1B). In severe cases, multiple blood vessels branching from either the supraorbital and/or the infraorbital arteries could be seen extending into the cornea region (Figure 2.3C, 2.3C’). In a few cases, other arteries like the supratrochlear artery, that runs further away from the cornea was also seen to extend branches into the tissue over the eye. Only a few specimens of those examined at 15, 22, and 32dpt did not show signs of vascularization (Figure 2.4C, Table S2.1B).

In cases that received PUV treatment only on the posterior halves, some of the specimens, interestingly, showed some moderate vascularization in the untreated anterior halves of the corneas at 3 to 28dpt (Figure 2.4F, Table S2.1D). However, vascularization was never found to be severe in the untreated half, and blood vessels were restricted close to the peripheral boundary of those corneas. The treated posterior halves did not show any vascularization at 1dpt, but some specimens started showing moderate vascularization at 3dpt (Figure 2.4F, Table S2.1D). The number of specimens that showed vascularization increased as the time progressed from 3 to 15dpt, with 57% showing moderate and 29% showing more severe vascularization at 15dpt (Figure 2.4F, Table S2.1D) (Figure 2.2E’). The number of specimens exhibiting vascularization decreased at 22dpt. Finally, at 28dpt, 67% of the cases examined showed no signs of vascularization and 33% cases showed moderate vascularization (Figure 2.4F, Table S2.1D) (Figure 2.2F’).
2.3.5. The effect of PUV treatment on expression of Tcf7l2 (whole cornea treatments)

Tcf7l2 is a HMG box transcription factor, known to be a downstream effector in the canonical Wnt signaling pathway. Tcf7l2 has been implicated in maintaining human corneal epithelial stem cells and is localized to the cytoplasm during normal homeostasis, being translocated to the nucleus in response to wounding (Lu et al., 2012). Tcf7l2 has also been shown to be essential in long term maintenance and wound repair of skin epithelia (Nguyen et al., 2009). A recent study from our lab (Sonam et al., 2019) discovered that Tcf7l2 expression serves as a good marker for a subset of apical epithelial cells within the frog skin (Figure 2.5A-2.5F), which are not normally present in the cornea (Figure 2.5E’, 2.5F’).

Expression of Tcf7l2 in the peripheral epidermis and the cornea were compared in both untreated controls and PUV treated corneas at 12 days post treatment (Figure 2.5E-2.5H, and 2.5E’-2.5H’, respectively). In contrast to the Tcf7l2 expression that is normally restricted to the peripheral epidermis (Figure 2.5E, 2.5F, 2.5E’, 2.5F’), Tcf7l2 expression in the PUV treated cornea is observed both in the peripheral epidermis and in cells extending into the central cornea region (Figure 2.5G, 2.5H, 2.5G’, 2.5H’). Interestingly, these Tcf7l2 positive cells in the cornea region and peripheral epidermis of PUV treated cases (Figure 2.5G, 2.5H, 2.5G’, 2.5H’) appear to be smaller and more compactly arranged when compared to the Tcf7l2 positive cells found in the peripheral epidermis of untreated control cases (Figure 2.5E, 2.5F).

2.3.6. The effect of PUV treatment on cell death via pyknosis

To examine cell death, the percentage of pyknotic nuclei were evaluated in the PUV treated corneas, and compared to the percentage of pyknotic nuclei in untreated control corneas. The control corneas exhibited a minimal level of cell death via pyknosis at all time points from 1 to 15dpt (Figure 2.6A, Figure S2.3A-S2.3C). On the other hand, the corneas that received PUV treatment showed significant increases in cell death at 1dpt (p<0.0001) and at 3dpt (p=0.0018) (Figure 2.6A, Figure S2.3A’-S2.3B’). At 5dpt, cell death levels declined to control levels and no significant increases in the percentages of pyknotic nuclei were observed for the 7, 11, and 15dpt time points, the last time points recorded, when compared to controls (Figure 2.6A, Figure S2.3C’).

In the corneas that received localized PUV treatment, the treated halves of the corneas showed a significant increase in the percentage of pyknotic nuclei at 1 and 3dpt (9.5%, p=0.0039 at 1dpt; 3.1%, 71
p=0.0336 at 3dpt) (Figure 2.6C, Figure S2.6A’, S2.6B’), as compared to the untreated halves of the corneas, which showed a minimal percentage of pyknotic nuclei (Figure 2.6C, Figure S2.6A, S2.6B). The percentage of pyknotic nuclei in the treated halves of the cornea decreased to levels seen in the untreated halves starting at 5dpt (Figure 2.6C, Figure S2.6C, S2.6C’).

2.3.7. The effect of PUV treatment on cell death (TUNEL assays)

The effects of PUV treatment on cell death were also examined by TUNEL assays. In the control corneas, a baseline percentage of TUNEL positive cells was detected (average of 3% at 1dpt and 3.5% at 3dpt) (Figure 2.6B, Figure S2.4A column i, S2.4B column i). However, at 5dpt, an increase in the percentage of TUNEL positive cells was observed in some of the samples, and the individual samples showed variability in the percentage of cell death (minimum = ~0.1% to maximum = ~45.6%) (Figure 2.6B, Figure S2.4C column i). The percentage of cell death appeared to be similar at 7 and 11dpt, with a slightly lower variability among individual samples (Figure 2.6B). The percentage of cell death and the variability among individual specimens declined at 15 and 20dpt, before an increase was observed at 25 and 30dpt time points (Figure 2.6B). Increases in cell death at later stages are typically observed as animals approach metamorphosis (Ishizuya-Oka et al., 2010; Nakajima et al., 2005; Nieuwkoop and Faber, 1994).

When compared to control corneas, the PUV treated corneas exhibited a significant increase in the percentage of cell death at 1dpt (mean cell death = 7.6%, p=0.0198) (Figure 2.6B, Figure S2.4A’ column i). The percentage of cell death in PUV treated corneas appeared to be similar to control corneas at 3 and 5dpt (Figure 2.6B, Figure S2.4B’ column i, S2.4C’ column i). At 7dpt, an increase in the median percentage of cell death was observed in PUV treated corneas; however, the mean was not statistically different than the controls. The percentage of cell death decreased at 11-20dpt, and showed an increase at 25 and 30dpt, similar to control corneas (Figure 2.6B).

Corneas in which PUV treatment was restricted only to the posterior half also showed a significant increase in cell death in the treated half (7.9%, p=0.036) as compared to the untreated control half at 1dpt (Figure 2.6D, Figure S2.7A column i, S2.7A’ column i). The levels of cell death declined at 3dpt in both the treated vs. untreated sides (Figure 2.6D, Figure S2.7B column i, S2.7B’ column i). At 5dpt, a marked increase in the levels of cell death was observed in both the halves (Figure 2.6D, Figure S2.7C column i, S2.7C’ column i). From 7 to 15dpt, the levels of cell death declined in both the halves, before showing an increase at the 20 to 30dpt time points (Figure 2.6D, Figure S2.7D column i,
Noticeable differences in cell death levels between the treated and the untreated halves of the corneas were only seen at 1dpt, while later time points (3 to 30dpt) showed no significant differences.

2.3.8. The effect of PUV treatment on the expression of the basal epithelial cell marker, p63

The transcription factor p63 is widely used as a marker for epithelial stem cells and TACs (Arpitha et al., 2008; Di Iorio et al., 2005; Liu et al., 2018; Pellegrini et al., 2001). Furthermore, p63 has been shown to localize to the basal epithelium of the *Xenopus* cornea, which is where proliferative cells are localized (Perry et al., 2013). The p63 antibody detects the delta form of p63 (Perry et al., 2013; Tomimori et al., 2004). Here, the effects of PUV treatment on the expression of this marker were examined in the cornea (Di Iorio et al., 2005; Perry et al., 2013; Sonam et al., 2019).

In the control corneas, the percentage of p63 labeled nuclei, (which is localized only to nuclei in the basal cells) remained fairly constant, ranging between 46.9% to 57.7% of all cells present between 1 to 30dpt time points (Figure 2.7A, Figure S2.5A column i – S2.5E column i). In PUV treated corneas, a diminished percentage of p63 positive nuclei was observed at 1dpt (~10% p63 positive nuclei, p<0.0001) (Figure 2.7A, Figure S2.5A’ column i). The percentage of p63 nuclei appeared to increase at 3dpt, and returned to control levels at 7dpt (Figure 2.7A, Figure S2.5B' column i, S2.5C’ column i). The lower percentage of p63 labeled nuclei may be attributed to a greater initial effect on the proliferating population of cornea cells as expected from the PUV treatment. Compared to the controls, the percentage of p63 nuclei were still significantly lower at 3dpt (p<0.0001) and 5dpt (p=0.0295). From 11 to 30dpt, a slight decline was observed in the percentage of p63 positive cells in the PUV treated corneas. The percentage of p63 positive cells in PUV treated corneas was significantly lower than control corneas at 15 and 20dpt (Figure 2.7A, Figure S2.5D’ column i), but not at 25 and 30dpt, reflecting the higher variation among individual samples at these later time points (Figure 2.7A, Figure S2.5E’ column i).

In cases where only the posterior half of the cornea received the PUV treatment, the percentage of p63 positive nuclei in the treated half was slightly lower when compared to the untreated half of the cornea at 1dpt (p=0.2754) (Figure 2.7A’, Figure S2.8A column i, S2.8A’ column i). The percentage of p63 positive cells appeared to increase at 3dpt in both the treated halves and the untreated halves of the corneas (Figure 2.7A’, Figure S2.8B column i, S2.8B’ column i). A decline in the percentage of p63 nuclei was observed at 5dpt in both the halves of the corneas, followed by an increase at 7dpt (Figure 73
As compared to the untreated controls (whole corneas) (Figure 2.7A), both the untreated and treated halves showed a lower percentage of p63 from 1 to 7dpt (Figure 2.7A’). At 11 and 15dpt, the percentage of p63 nuclei increased in both the untreated and treated halves (Figure 2.7A’, Figure S2.8D column i, S2.8D’ column i). From 5 to 15dpt, the slight differences in the percentage of p63 positive cells between the treated and untreated halves were not statistically significant. Interestingly, at 20dpt, the percentage of p63 positive cells declined in both the halves, and the percentage of p63 positive cells in the treated half was significantly lower than the percentage of p63 positive cells in the untreated half (p=0.0085) (Figure 2.7A’). The percentage of p63 positive cells in the untreated half of the corneas remained consistent for the 25 and 30dpt time points, and was similar to the treated half of the corneas (Figure 27A’, Figure S2.8E column i, S2.8E’ column i).

2.3.9. The effect of PUV treatment on mitosis in the cornea

Anti-phosphorylated-histone-H3S10 antibody, was used as a reliable marker to detect mitotic cells (Hans and Dimitrov, 2001; Perry et al., 2010; Thomas and Henry, 2014; Walter et al., 2008). The untreated control corneas showed an increase in mitosis from 1 to 5dpt (Figure 2.7B, Figure S2.5A column ii – S2.5C column ii). The mitotic nuclei counts then decreased at 7 and 11dpt, before increasing at 15dpt (Figure 2.7B, Figure S2.5D column ii). These represent the basal mitosis levels of the CESC and TACs in the untreated control cornea, over this period of larval development. Additionally, an increase in mitosis was observed at 25 and 30dpt in the untreated control corneas (Figure 2.7B, Figure S2.5E column ii). These fluctuations are an indicator of natural variations associated with differences in growth rates and ages/stages of larval development.

In contrast to the untreated control corneas, the mitotic nuclei counts were significantly decreased at 1dpt in PUV treated corneas (Figure 2.7B, Figure S2.5A’ column ii). This decrease may be correlated with changes in cell death and the likelihood that cells become arrested in response to the DNA crosslinking from the PUV treatment (Cuddihy and O’Connell, 2003). Subsequently, a significant increase was observed in mitosis at 3dpt in the PUV treated corneas as compared to the control corneas (Figure 2.7B, Figure S2.5B’ column ii), suggesting that the surviving CESC/TACs are stimulated to undergo mitosis and may be responding to mitigate the damage caused by PUV treatment. In contrast to the untreated control corneas, the mitotic counts continued to remain higher at 5, 7, and 11dpt in the PUV treated corneas (Figure 2.7B). At 15dpt, the mitotic counts declined to levels observed in untreated
controls (Figure 2.7B, Figure S2.5D’ column ii). The mitotic counts subsequently dropped further at 20dpt in the PUV treated corneas and remained somewhat lower than the mitotic counts in untreated control corneas at 25 and 30dpt, respectively (Figure 2.7B, Figure S2.5E’ column ii).

In cases where the PUV treatment was restricted to the posterior half of the cornea (Figure 2.7B’), the treated half of the cornea initially showed a statistically significant decrease in mitosis compared to the untreated (control) half at 1dpt (Figure 2.7B’, Figure S2.8A column ii, S2.8A’ column ii). Both the treated and untreated halves of the cornea showed a sharp increase in mitosis at 3 and 5dpt (Figure 2.7B’, Figure S2.8B column ii, S2.8C column ii, S2.8B’ column ii, S2.8C’ column ii). The mitotic count in both the treated half and the untreated half stayed elevated at 7 and 11dpt, before declining at 15 and 20dpt (Figure 2.7B’, Figure S2.8D column ii, S2.8D’ column ii). Furthermore, both the treated and untreated halves showed increased mitosis at 25 and 30dpt. (Figure 2.7B’, Figure S2.8E column ii, S2.8E’ column ii). While changes in mitosis were observed, the differences between the treated and untreated halves for time points from 3 to 30dpt were not found to be statistically significant.

2.3.10. The effect of PUV treatment on total cell density in the cornea

In control corneas, the total cell density remained fairly consistent for the time points from 1 to 15dpt, except for a slight decrease seen at 3dpt (Figure 2.7C, Figure S2.5A column iii – S2.5D column iii). During the time points from 20 to 30dpt, an increase was observed in the total cell density (Figure 2.7C, Figure S2.5E column iii). At these time points the animals are at stage 58-66, which corresponds to the developmental stages approaching metamorphic climax (Nieuwkoop and Faber, 1994), where the corneal epithelial cells appear more condensed, and the cornea epithelium is known to thicken (Hu et al., 2013) and finally mature into a multilayered structure.

A significant decrease in the total cell density was observed in the PUV treated cases at 1dpt (p=0.0094) (Figure 2.7C, Figure S2.5A’ column iii), which is related to the increased levels of cell death at this time point. The cell density in PUV treated corneas showed an increase at 3dpt. From 5 to 20dpt, the cell density in PUV treated corneas continued to increase and became significantly higher when compared to the control corneas (Figure 2.7C, Figure S2.5C’ column iii, S2.5D’ column iii). This is likely due to a combination of increased mitosis of any surviving cornea cells, and primarily the invasion of skin cells from the peripheral epidermis. As mentioned earlier, we noted a difference in the morphology of Tcf7l2 positive cells within the PUV treated cornea (Figure 2.5G’, 2.5H’) vs. that
normally seen in the peripheral epidermis (Figure 2.5E, 2.5F). This appears to be due to differences in the thickness of these tissues, because the tissue that replaces the cornea after the PUV treatment is thicker and these cells become more columnar in shape. The observed increase in total cell density in PUV treated corneas supports this conclusion. Although the total cell density in PUV treated corneas at 25 and 30dpt was higher than the control corneas, this difference was not statistically significant, which reflects the higher variation found among individual samples at these later time points (Figure 2.7C).

For those cases in which half of the cornea was treated, no statistically significant differences were observed in the cell density of the untreated halves vs. the treated halves (Figure 2.7C’, Figure S2.8A’ column iii – S2.8E’ column iii). The cell density in both the untreated and the treated halves showed a slight decrease at 3dpt, followed by an increase from 5 to 15dpt (Figure 2.7C’, Figure S2.8A column iii – S2.8D column iii). Cell density in both the halves of the cornea declined at 20 and 25dpt before an increase in cell density was observed at 30dpt (Figure 2.7C’, Figure S2.8E column iii). Interestingly, the cell density in both the untreated and the treated halves of these corneas appeared to follow a similar trend as compared to the cases that received PUV treatment on the whole corneas (Figure 2.7C).

2.3.11. The effects of Psoralen only and UV only treated controls

The effects of treatment with Psoralen without UV exposure (Psoralen only controls), and UV without Psoralen (UV only controls) were also examined. Both the whole cornea Psoralen only controls (Figure 2.2G, 2.2G’) and the UV only controls (Figure 2.2H, 2.2H’) showed no signs of SCD.

A minimal number of pigment cells were observed in the contralateral right (untreated) corneas, with an average of ~1-3 pigment cells in standard area (Figure 2.8A). Only slightly elevated levels of pigmentation were observed in the Psoralen only treated controls (average of ~2-6 pigment cells in standard area) from 1-15dpt. A significant increase in the Psoralen only treated controls was only observed at 7dpt time point. UV only controls showed a significant increase in pigment cell counts (average of ~3-7 pigment cells), as compared to their contralateral right (untreated) corneas (average of ~1-4 pigment cells) at 5 to 15dpt time points (Figure 2.8B). However, the pigmentation in the UV only treated corneas was still minimal and was restricted to the peripheral edge of the cornea. Pigment cells were never seen in the central cornea region.

Both the Psoralen only controls and the UV only controls did not show any signs of opacity (Figure 2.9A, 2.9B, Table S2.2A, S2.2C). In Psoralen only controls and UV only controls slight
vascularization was observed very close to the peripheral boundary of the cornea in some specimens, but these vessels never extended into the central cornea region (Figure 2.9D, 2.9E, Table S2.2B, S2.2D).

Both the Psoralen only controls and the UV only controls showed minimal levels of pyknotic cell death that were similar to the untreated control corneas (Figure 2.10A). This verifies that Psoralen or comparable doses of UV alone do not trigger a significant increase in cell death.

TUNEL assays showed variations among individual specimens. However, many time points showed no significant differences between the Psoralen only controls vs. untreated controls, and UV only controls vs. untreated controls. Significantly lower levels of cell death, as compared to untreated controls, were detected for: Psoralen only controls at 5dpt (p = 0.0486) and 15dpt (p = 0.0415); and for UV only controls at 15dpt (p = 0.0281) (Figure 2.10B).

No significant differences were observed in the levels of mitosis and the percentage of p63 positive cells in both the Psoralen only controls and the UV only controls, which were similar to those seen in the untreated control corneas (Figure 2.11A, 2.11B). Changes in the total cell density in both the Psoralen only controls and the UV only controls followed similar trends, as compared to the untreated controls (whole corneas) (Figure 2.11C). However, both the Psoralen only controls and the UV only controls showed a higher total cell density as compared to the untreated controls, which was significant at 7 to 15dpt for the Psoralen only controls, and at 1 to 15dpt for the UV only controls.

2.3.12. The effects of aluminum foil mask and localized UV only control treatments

To test whether localized UV irradiation alone, and the presence of the aluminum foil mask could be affecting the cornea, localized UV treatments were performed without Psoralen on half of the cornea (UV only half controls). The aluminum foil mask was placed on the anterior half of the corneas, and the posterior half of the corneas was exposed to UVA, for the same duration of 75 seconds as in the PUV half cornea treated cases.

Both the UV only treated and the untreated halves of the corneas had a minimal number of pigment cells, with an average of ~1-6 pigment cells in the standard area for all time points from 1 to 15dpt (Figure 2.8C). The slight increase in the number of pigment cells in the treated halves when compared to the untreated halves was only found to be significant at 3dpt (p = 0.037). Changes in corneal opacity were not observed in any of the specimens for both the treated and the untreated halves from 1 to 15dpt (Figure 2.9C, Table S2.2E). Both halves also did not show vascularization in most cases. However, in some specimens among both the halves, slight vascularization was observed very
close to the peripheral edge of the cornea, but these vessels never extended into the central cornea region (Figure 2.9F, Table S2.2F).

Both the treated and the untreated halves of the corneas showed very minimal pyknotic cell death throughout the time points examined (Figure 2.10C), and no significant differences were observed between the UV treated half vs. the aluminum foil covered untreated half of the cornea. TUNEL assay results were varied among individual specimens, but the mean levels of cell death in the untreated half of the corneas did not show any significant differences when compared to the treated half of the corneas (Figure 2.10D). Additionally, the mitosis levels, percentage of p63 positive cells, and the total cell density showed similar levels in the treated vs. untreated halves of the corneas and no significant differences were observed between them (Figure 2.11A’-2.11C’). These findings reveal that the placement of the aluminum foil mask does not trigger significant changes in the cornea.

2.4. Discussion

Though remarkable therapies have been developed to treat LSCD using epithelial stem cell transplants (Atallah et al., 2016; Galindo et al., 2017; Grueterich et al., 2002; Haagdorens et al., 2016; Holland 2015; Koizumi et al., 2001; Le and Deng, 2019; Ljubimov and Saghizadeh, 2015; Rama et al., 2010; Saghizadeh et al., 2017; Sasamoto et al., 2018; Shortt et al., 2007b; Utheim et al., 2018; Yazdani et al., 2019), our understanding of LSCD pathology, and the specific behavior of CESCs, is still rather limited. Convenient vertebrate models to study stem cell deficiency in the cornea will help us answer these questions. In this study, we have established a new frog model to study CESCs. This model recapitulates the hallmarks of LSCD in humans and other vertebrates. Combined with significant experimental advantages afforded by the Xenopus model system, PUV treatments provide a robust, versatile, and tractable system to readily prepare animals to study SCD, with a rapid, single treatment. Furthermore, the combination of highly localized application of Psoralen and restricted UV irradiation using the microscope pinhole iris and aluminum foil masks, allows one to target specific regions of the cornea to create localized damage. This allows us to further study the response of the cells in the undamaged region of the cornea. Additionally, we have characterized various cellular changes associated with these PUV treatments, such as cell death, mitosis, total cell density, and presence of Tcf7l2 expressing subset of apical skin cells and p63 positive basal epithelial cells.
2.4.1. Changes in Cornea Morphology/Histology

The cornea is normally avascular owing to its anti-angiogenic properties (Azar, 2006) and a corneal epithelial barrier is thought to separate the epithelial cells of the cornea and conjunctiva (Kubilus et al., 2017). When the CESC/TACs are depleted in the case of LSCD, structural mechanisms and cell signals that maintain this boundary are disrupted (Batlle and Wilkinson, 2012; Dahmann et al., 2011). As a result, blood vessels and cells of the conjunctiva begin to appear in the cornea region (Fini and Stramer, 2005) (Dua, 1998). Related events are observed in the case of Pterygium (Liu et al., 2013).

Likewise, following PUV treatments in the frog, we observed branches of the supraorbital and infraorbital arteries had entered the cornea region, (Figure 2.4B, 2.4C, Table S2.1A, S2.1B) (Amitai-Lange et al., 2015; Kadar et al., 2013; Le et al., 2018) (Figure 2.2B, 2.2C, 2.2D’, 2.2E’, 2.3C, 2.3C”). The larval frog eye does not possess an obvious conjunctival epithelium, rather the surrounding skin directly abuts the outer edge of the cornea. Following PUV treatments we found that skin cells ultimately invade the cornea region. This includes the pigmented melanophores. In fact, studies conducted in rabbits (Mann, 1944; Michaelson, 1952; Wolosin et al., 2000), guinea pigs (Cafaro et al., 2009; Davanger & Evensen 1971; Henkind, 1969), and also humans (Cowan, 1963; Davanger & Evensen, 1971; Mann, 1944; Wolosin et al., 2000) reported that pigment cell migration can accompany LSCD. In the present study, pigment cells started appearing in the cornea region seven days following PUV treatments, and the number of these cells was found to increase over time (Figure 2.4A). Likewise, we observed that cells of the adjacent lateral line organ system enter the cornea region following PUV treatments (Figure 2.3B, 2.3B’). Finally, the appearance of Tcf7l2 positive cells in the PUV treated corneas confirmed that apical skin cells had also invaded the cornea in response to changes caused by PUV treatments (Figure 2.5G’, 2.5H’). Together, these observations suggest that the surrounding epidermis eventually replaces the cornea tissue.

2.4.2. Changes in the Growth of the Eye

Although the eye continues to grow larger, its growth is initially delayed following PUV treatment compared to the contralateral, untreated control eyes (Figure S2.1). On the other hand, we did not observe any abnormal histological changes in the retina or the ciliary marginal zones (Figure S2.2). One possibility is that the cornea normally sends signals to deeper eye tissues to regulate their growth, and these signals may have been disrupted as a result of the PUV treatments slowing eye growth. In fact,
it is known that pre-lens, placodal ectoderm (from which both the lens and cornea develop) sends signals to the optic vesicle to control its normal development (Hyer et al., 2003).

### 2.4.3. Changes in Cell Death

A significant amount of cell death is seen very early, within 1-3dpt. Most of this appears to result from pyknosis, as a primary mechanism of cell death, rather than apoptosis (Figure 2.6A). The stimuli that causes apoptosis can also induce necroptosis (a programmed form of necrosis) and other forms of cell death can also occur independently of apoptosis (Tait et al., 2014). While TUNEL detects both apoptosis and necrosis, apoptosis is detected more generally (Gold et al., 1994), and necrotic cells can go undetected by TUNEL assays. Interestingly a wound healing study conducted in chicken corneas detected necrotic cell death in the absence of delayed cell death or apoptosis (Ritchey et al., 2011).

Beyond the first day, we did not observe any significant differences in percentage of TUNEL positive cells in the PUV treated corneas vs. the untreated control corneas (3dpt to 30dpt), and the results of TUNEL assays showed variations in the rate of cell death among the samples (Figure 2.6B). The short window of detection of DNA fragmentation by TUNEL (typically 1-3 hours from initiation to termination) (Gavrieli et al., 1992), and natural variations among specimens developing at different rates, may have contributed to these variations.

TUNEL positive cells in the PUV treated corneas were most pronounced around 5dpt-7dpt, but were not significantly higher than the untreated control corneas (Figure 2.6B). This was surprising because the inter-strand crosslinks caused by the PUV treatment might be expected to cause a delayed onset of apoptotic cell death in moderately damaged surviving cells, as they attempt to undergo proliferation. Such delayed responses to wounding, and delayed loss of CESCs have been previously reported in rabbits (Kadar et al., 2011).

When the tadpoles are undergoing metamorphosis around 25dpt-30dpt, another increase in apoptosis was observed (Figure 2.6B). Apoptosis is commonly seen during metamorphosis when tissue remodeling occurs (Ishizuya-Oka et al., 2010; Nakajima et al., 2005). Consistent with our results, increases in epithelial cell death have previously been reported in *Xenopus* corneas around metamorphosis (Hu et al., 2013). The results suggest that apoptotic cell death does not appear to be the most significant driver of cell death following PUV treatment.
2.4.4. Changes in p63 Expression

The transcription factor p63, has been widely used as a marker for proliferative epithelial cells (stem cells and TACs) (Arpitha et al., 2008; Di Iorio et al., 2005; Liu et al., 2018; Pellegrini et al., 2001). It has also been used to distinguish stem cells for transplants in patients with LSCD (Rama et al., 2010). Similar to humans and mice (Collinson et al., 2002; Dua et al., 2003), p63 antibody labeling is detected throughout the nuclei of all basal cells of the cornea in both larval and adult Xenopus (Perry et al., 2013; Sonam et al., 2019).

PUV treatment resulted in a marked decrease in the fraction of p63 labeled cells in the corneal epithelium at 1dpt (Figure 2.7A). This agrees with the results from other studies that reported a decreased basal cell density in the cornea and limbus in patients diagnosed with LSCD (Chan et al., 2015), and in the rabbit model of LSCD (Galindo et al., 2017). A combination of enhanced mitosis in any surviving cells and the influx of cells from the peripheral epidermis likely contributes to the observed subsequent increase in the percentage of p63 labeled cells in the PUV treated corneas at 3dpt-7dpt. However, since p63 labels all basal cells in both the epidermis and the cornea (Li et al., 2015; Mills et al., 1999; Perry et al., 2013; Yang et al., 1999), the contribution of the p63 cells from epidermal stem cells/TACs in the skin, vs. those remaining in the cornea, cannot be distinguished. So far, there is no marker that can differentiate the stem cells/TACs of the cornea from those of the epidermis in Xenopus (Sonam et al., 2019).

2.4.5. Changes in Mitosis

Phospho-histone H3S10 is a widely used marker for mitosis (Thomas and Henry, 2014; Walter et al., 2008). The PUV treatment significantly diminished mitosis by 1dpt, suggesting that PUV treatment was capable of affecting the proliferation within the cornea epithelium. However, the increased mitosis seen at 3dpt to 11dpt (Figure 2.7B), suggests that a subset of cells survived PUV treatments. This population of cells ultimately appears to respond to the PUV damage by undergoing increased levels of mitosis, possibly compensate for the damage. Our results appear similar to those showing that CESC/TACs can be stimulated to undergo increased mitosis in response to other forms of wounding (Richardson et al., 2018; Sagga et al., 2018). The increased mitoses, however, did not appear to mitigate the PUV damage to restore cornea transparency. Note that mitosis of invading epidermal stem cells or epidermal TACs that repopulate the cornea region likely also contribute to the observed increases in
mitosis. Mitotic counts were lower in the PUV treated corneas at later time points (15dpt to 30dpt) (Figure 2.7B), presumably because the tissue had been converted to skin.

2.4.6. Repair Occurs Following Localized PUV Treatments

We showed that localized PUV treatments restricted to half of the cornea, resulted in a transient response on the treated side, and subsequently this damage is restored, most likely from undamaged cells on the untreated side. Compared to whole cornea treatments, the severity of PUV damage was somewhat lower in half-treated corneas. The appearance of pigment cells in the treated half of the cornea region was also delayed. While the number of pigment cells continued to increase following whole cornea treatments, this number eventually returned to levels closer to those seen in the untreated (control) halves of the corneas (Figure 2.4B). In half cornea treatments, fewer samples showed severe opacity, as compared to the whole cornea treatments. Changes in opacity also appeared at later time points, as compared to respective time points when PUV treatment was performed on the whole cornea. Likewise, neo-vascularization was observed in fewer specimens at both earlier and later time points, as compared to the whole cornea treatments. Ultimately, many of these half-treated corneas regained a normal appearance. Following repair, although the anterior and posterior halves of the cornea looked similar in the lateral views (Figure 2.2F), closer observation from the dorsal side (Figure 2.2F’) revealed that the treated half did not regain the same curved profile of control corneas in some cases. Although this could represent a permanent change in cornea morphology, it may mean that full restoration of the normal structural phenotype may take longer than 28 days.

We have shown that the damage caused by PUV treatment and the overt phenotypic changes observed in whole treated corneas were mainly restricted to the treated half of the cornea. Interestingly, however, the untreated half was found to exhibit some cellular changes. This includes initial signs of some moderate neo-vascularization, increases in the levels of mitosis, increases in cell density, and changes in the percentage of p63 positive cells (Figure 2.7A’-C’). These latter changes are suggestive of a compensatory response that may be aiding repair of the treated side (such as the increased levels of mitosis). Ultimately, these numbers return back to levels seen in control corneas. The initial changes observed in the untreated half could also be the result of a more generalized inflammatory response within the eye that resulted from PUV treatments and the damage created on the treated side. This could be related to the release of interleukins and/or cytokines associated with immune responses accompanied by the arrival of various immune cells such as macrophages, etc., that causes the untreated half to
undergo these changes. The initial signs of some moderate neo-vascularization on the untreated side seem to support these arguments.

2.4.7. Variations in the Response to PUV treatments

While the end results of PUV treatments appear to be similar, the rate of advancement of LSCD and appearance of the skin phenotype differs among the treated cases. The rate of cornea restoration was also different in half-treated specimens. These variations are likely due to the fact that these animals vary somewhat in overall size and developmental stage, and do not develop or grow at the same rate, which is subject to natural variation and competition for food when animals are cultured in groups (for example there is always a great deal of variation in overall animal size in lab reared specimens). Variability could also arise from the specimens being derived from different clutches/parents. Human cases of CESD also do not follow the same trajectories (Haagdorens et al., 2016; Shortt et al., 2014).
2.5. Figures and Tables

Figure 2.1: Apparatus and procedure for the PUV treatment.

(A) Standard 1ml pipette tip, which is modified as (B) a special reservoir applicator pipette using a razor blade, according to the dimensions shown. The broad end of the pipette (mmc) is cut in half so that it can be attached to the metal rod held by the micromanipulator using a small binder clip. (C) Top view of the reservoir pipette showing the internal diameters (d1 and d2) of the pipette opening and side port or reservoir opening. (D-F) PUV-treatment procedure. (D) Anesthetized tadpole is placed in a petri dish on Kimwipes soaked in anesthetic solution. Reservoir pipette tip is affixed to a metal rod held by the micromanipulator using a small binder clip. The tip is gently placed around the cornea to form a sealed chamber. Psoralen solution is then added into the reservoir and allowed to contact the cornea. (E) Higher magnification view of area shown in panel D. (F) UV exposure using a 10X objective. UV is localized to the cornea, (bright blue spot), using the microscope iris diaphragm. (G) Schematic diagram of larval Xenopus eye. Structures are as labeled. cer, corneal end of reservoir; mmc, micromanipulator clamp end; ro, reservoir opening.
Figure 2.2: Comparison of corneal phenotypes.
Phenotypes in untreated control corneas (A, A’), PUV treated whole corneas (B-D, B’-D’), PUV treated half corneas (E, F, E’, F’), Psoralen only controls (whole cornea) (G, G’), and UV only controls (whole cornea) (H, H’). Images were taken at various time points (dpt), as indicated. (A, A’) Control
Figure 2.2 (cont.): cornea is clear and pigment cells are restricted to the edges of the peripheral cornea. (B, B’, C, C’) Cornea after PUV treatment starts showing increased opacity and vascularization (white arrows). Pigment cells (white arrowheads) are also entering the cornea. (D, D’) Cornea is still opaque 25dpt after whole cornea PUV treatment. The normal corneal phenotype is lost, and pigment cells completely cover the cornea (white arrowheads). (E, F, E’, F’) Dotted blue lines indicate the boundary between the PUV treated half and the untreated (control) half. (E, E’) At 15dpt after PUV treatment, the treated half shows opacity and increased pigmentation (white arrowheads), whereas the untreated half is devoid of pigment cells and appears transparent. (F, F’) At 28dpt after PUV treatment on half of the cornea, the treated half has become clear of pigment cells and the normal corneal phenotype and transparency are restored. (G, G’, H, H’) Psoralen only treated corneas and UV only treated corneas, respectively, are clear and pigment cells are restricted to the edges of peripheral cornea. Green arrowheads in (A’, E’ to H’) show transparent cornea. Blue arrowheads point to wrinkled irregular epithelium. an, anterior side of the tadpole; dr, dorsal side of the tadpole; ps, posterior side of the tadpole; vr, ventral side of the tadpole. Scale bar in H’ equals 500µm.
Figure 2.3: Severe phenotypes in whole cornea PUV treated cases.

PUV treated cases showing the more severe Stem Cell Deficiency (SCD) phenotypes. (A, A’) Control corneas depicting normal transparent cornea morphology. These corneas are free of vasculature, pigment cells, and the lateral line organs (white arrows) are restricted to the skin. (B, B’) PUV treated cases on the opposite sides of the control corneas shown in (A, A’) at 32 and 29 days post treatment, respectively, depicting the loss of the normal transparent corneal phenotype. The cornea is completely opaque and covered with epidermal tissue similar to that surrounding the eye. Skin cells, including pigment cells and the lateral line organs (white arrows), have invaded the corneal region. (C, C’) PUV treated cases at 15dpt, depicting vascularization with multiple blood vessels passing into the cornea (white arrowheads). an, anterior side of the tadpole; dr, dorsal side of the tadpole; ps, posterior side of the tadpole; vr, ventral side of the tadpole. Scale bar in C’ equals 200µm for (C, C’), and 665µm for (A, A’, B, B’).
Figure 2.4: Quantification of pigmentation, opacity, and vascularization following PUV treatment on whole corneas and half corneas.

(A) Control corneas show very minimal levels of pigment cells (black line), whereas corneas that received PUV treatment show increased pigmentation at the corresponding time points (red line).
(B) Control corneas show no opacity at all time points examined (silver columns), whereas opacity increased in PUV treated corneas as the time points progressed (orange and red columns). (C) A majority of control corneas showed no vascularization at all time points examined (silver columns),
Figure 2.4 (cont.):
however, some corneas showed slight vascularization very close to the peripheral boundary of the cornea (gray columns). Vascularization scores in the PUV treated corneas increased as the time points progressed (orange and red columns). (D) In the cases where only half of the cornea received PUV treatment, the untreated half (blue line) shows minimal pigmentation, whereas the treated half (red line) shows an increase in pigmentation, through 15dpt, after which it starts declining as the pigment cells are lost from the cornea. (E) No corneal opacity was detected in the untreated half of the cornea (silver columns), whereas corneal opacity in the treated half of the cornea increased until 15dpt (orange and red columns), and then declined by 28dpt (blue column). (F) A majority of untreated halves of the corneas were scored 0 for vascularization at all time points examined (silver columns), however, some corneas showed slight vascularization very close to the peripheral boundary of the cornea (gray columns). Vascularization scores in the PUV treated corneas increased until 15dpt (orange and red columns), and then declined by 28dpt (blue and orange columns). Error bars represent standard error of the mean. Statistical analysis has been done for untreated control corneas vs. PUV treated corneas in (A), and for untreated control halves vs. the PUV treated halves of the corneas in (D). N = 7-12 for time points in (A-C), except for control (whole cornea) at 1dpt (N = 4). N = 5-9 for time points in (D-F). *** = p-value ≤ 0.0002. ** = p-value ≤ 0.008; * = p value ≤ 0.05.
Figure 2.5: Tcf7l2 immunostaining showing appearance of Tcf7l2 expressing skin cells in the cornea region following whole cornea PUV treatments.

(A-D) Normal skin tissue obtained from the flank region of a representative tadpole. (A) DIC image showing pigment cells, which are a characteristic of skin tissue. (B) Skin tissue shows positive immunostaining for Tcf7l2, which labels a subset of apical epithelial skin cells that are not normally present in the cornea. (C, D) are the corresponding Hoechst and merged images for (A, B), respectively. (E-H’) Control and PUV treated corneas obtained from the left and right sides of the same tadpole, respectively. (E, F) shows the peripheral epidermis region (skin that surrounds the cornea), where Tcf7l2 staining is normally observed. (E’, F’) shows the cornea region of the control (untreated) cornea, where no Tcf7l2 staining is normally observed. (G, H) is the peripheral skin region, and (G’, H’) is the cornea region overlying the eye and of a PUV treated cornea collected at 12dpt. The presence of Tcf7l2 expressing cells in the cornea region shows the presence of these skin cells. Note that these Tcf7l2 positive cells appear smaller and more compacted than those found in the surrounding skin. (F, F’, H, H’) are the corresponding merged (Tcf7l2+Hoechst) images of (E, E’, G, G’), respectively. Scale bar in D equals 100µm and applies to A-D and equals 40µm in H’ and applies to E-H’.
Figure 2.6: Quantification of pyknotic nuclei and apoptotic cell death following PUV treatment on whole corneas and half corneas.

(A, B) Counts of pyknotic nuclei and TUNEL positive nuclei, respectively, in the untreated controls (whole cornea) (black line) and the PUV treated whole corneas (red line). (C, D) Counts of pyknotic nuclei and TUNEL positive nuclei, respectively, in the untreated (control) halves (blue line) vs. the PUV treated halves of the corneas (red line) of the same corneas. Control (whole cornea) from (A) has been included in (C) for convenience of comparison (black line). The observations have been taken at the time points indicated on the X-axis (dpt). The pyknotic nuclei and TUNEL positive nuclei are represented as a percentage of the total Hoechst nuclei. (A, C) Error bars represent standard error of the mean. (B, D) Boxes represent lower and upper quartiles; whiskers and outliers represent Tukey range. Statistical analysis has been done for untreated control corneas (black) vs. PUV treated corneas (red) in (A, B), and for untreated control halves (blue) vs. the PUV treated halves (red) of the corneas in (C, D). Outliers have been included in calculating p-values in (B, D). N = 9 for all time points in (A). N = 9-13 for time points in (B). N = 10 for all time points in (C). N = 10-15 for time points in (D). *** = p ≤ 0.001; ** = p ≤ 0.01; * = p ≤ 0.05.
Figure 2.7: Quantification of p63 positive nuclei, mitosis, and total cell density following PUV treatment on whole corneas and half corneas.

(A-C) Percentage of p63 positive nuclei, mitotic nuclei counts (H3S10), and total cell density, respectively, in the untreated controls (whole cornea) (black lines) vs. the PUV treated whole corneas (red lines). (A’-C’) Percentage of p63 positive nuclei, mitotic nuclei counts (H3S10), and total cell density in the untreated control half (blue lines) vs. the PUV treated half (red lines) of the cornea. The controls (whole cornea) from (A-C) have been included in (A’-C’), respectively, for convenience of comparison (black lines). The observations have been taken at the time points indicated on the X-axis, as
Figure 2.7 (cont.):
days post treatment (dpt). Error bars indicate standard error of the mean. Statistical analysis has been done for untreated control corneas (black lines) vs. PUV treated corneas (red lines) in (A-C), and for untreated control halves (blue lines) vs. the PUV treated halves (red lines) of the corneas in (A’-C’). N = 7-10 for time points in (A). N = 10-15 for time points in (A’). N = 7-13 for time points in (B). N = 9-15 for time points in (B’). N = 9-13 for time points in (C). N = 10-15 for time points in (C’). *** = p ≤ 0.001; ** = p ≤ 0.01; * = p ≤ 0.05.
Figure 2.8: Quantification of pigmentation in Psoralen only (whole cornea), UV only (whole cornea), and UV only (half cornea) controls.

(A) The contralateral control corneas (black line) show a minimal level of pigmentation. Only slightly elevated levels of pigmentation were observed in Psoralen only whole cornea treatment controls (blue line). A significant increase was only observed at 7dpt. (B) When compared to the contralateral untreated corneas (black line), UV only treatment on whole corneas (red line) appears to cause a significant increase in the levels of pigmentation at 5, 7, and 15dpt. However, pigmentation was still minimal in the UV only treated controls at these time points. (C) In the cases where only half of the
Figure 2.8 (cont.): cornea received UV treatment, the treated halves (red line) show minimal increases in pigmentation at 1-7dpt. Error bars indicate standard error of the mean. Statistical analysis has been done for control cornea vs. Psoralen only treated (A), control cornea vs. UV only treated (B), and untreated halves vs. the UV only treated halves of the corneas (C). N = 4 for all time points in (A-C). *** = p ≤ 0.001; ** = p ≤ 0.01; * = p ≤ 0.05.
Figure 2.9: Opacity and vascularization in Psoralen only (whole cornea), UV only (whole cornea), and UV only (half cornea) controls.

Stacked column graphs of corneal opacity and vascularization are shown. For each time point examined, the analysis has been done for the Psoralen only treated control corneas vs. their contralateral corneas (A, D), respectively, the UV only treated control corneas vs. their contralateral corneas (B, E), respectively, and the untreated half vs. the treated half of the UV only half treated control corneas (C, F), respectively. None of the controls showed any signs of opacity at any of the time points examined (silver and blue columns in A, B, E). Whereas a majority of control specimens showed no signs of vascularization (silver and blue columns in D, E, F), some control specimens showed slight vascularization very close to the peripheral boundary of the cornea (gray and orange columns in D, E, F), however, these vessels never extended into the central cornea region.
Figure 2.10: Quantification of pyknotic nuclei and apoptotic cell death in Psoralen only (whole cornea), UV only (whole cornea), and UV only (half cornea) controls.

(A) Similar to the untreated control corneas (black line), both the Psoralen only controls (blue line) and the UV only controls (red line) showed minimal levels of pyknotic cell death at all time points examined. (B) Compared to the untreated control corneas (black line), the average percentage of TUNEL positive nuclei was lower in both the Psoralen only (blue line) and UV only (red line) controls at all time points. (C, D) At each time point, both the untreated halves (blue lines) and the UV only treated halves (red lines) of the corneas showed similar minimal levels of pyknosis (C), and apoptosis (D). The untreated controls (whole cornea) from (A, B) have been included in (C, D), respectively, for convenience of comparison. Error bars represent standard error of the mean. (B, D) Boxes represent lower and upper quartiles; whiskers and outliers represent Tukey range. Statistical analysis has been done for control (whole cornea) vs. Psoralen only treated, and control (whole cornea) vs. UV only treated in (A, B), and untreated halves vs. the UV only treated halves of the corneas in (C, D). Outliers have been included in calculating p-values in (B, D). N = 6-7 for Psoralen only, UV only, and UV only half treated corneas at all time points in (A-D). N = 9 for control (whole cornea) for all time points in (A-D). * = p ≤ 0.05 (displayed for UV only).
Figure 2.11: Quantification of p63 positive nuclei, mitosis, and total cell density in Psoralen only (whole cornea), UV only (whole cornea), and UV only (half cornea) controls.

At each time point observed, the percentage of p63 positive nuclei (A), and mitotic nuclei counts (B) were similar among the untreated controls (black line), Psoralen only controls (blue line), and UV only controls (red line), respectively. (C) Compared to the untreated controls (black line), the total cell density is significantly higher at 7 and 15dpt in Psoralen only controls (blue line), and significantly higher at all time points in UV only controls (red line). Percentage of p63 positive nuclei (A’), mitotic nuclei counts (B’), and total cell density (C’) in both the untreated (blue line) and treated (red line) halves of the UV only treated half cornea controls show similar levels, respectively. The controls (whole
Figure 2.11 (cont.):
cornea) from (A-C) have been included in (A’-C’), respectively, for convenience of comparison. Error bars indicate standard error of the mean. Statistical analysis has been done for controls (whole cornea) vs. Psoralen only treated, and controls (whole cornea) vs. UV only treated in (A-C), and untreated halves vs. the UV only treated halves of the corneas in (A’-C’). N = 6-7 for Psoralen only treated, UV only treated, and UV only half treated at all time points in (A-C, A’-C’). N = 9 for controls (whole cornea) for all time points in (A, C, A’, C’). N = 7-9 for controls (whole cornea) for time points in (B, B’). *** = p ≤ 0.001; ** = p ≤ 0.01; * = p ≤ 0.05 (for UV only vs. controls (whole cornea)). # = p ≤ 0.05 (for Psoralen only vs. controls (whole cornea)).
<table>
<thead>
<tr>
<th>Score</th>
<th>Corneal Opacity</th>
<th>Vascularization</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>- Cornea is transparent.</td>
<td>- No blood vessels present.</td>
</tr>
<tr>
<td>(No signs)</td>
<td>- Outlines of xanthophores, iridiophores and melanophores in the iris are clearly visible.</td>
<td>- Example: Figure 2A, 2A’</td>
</tr>
<tr>
<td></td>
<td>- Pupillary edge (free edge of the iris) is well defined and distinctly visible.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Example: Figure 2A, 2A’</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>- Cornea appears hazy, with a grayish translucency.</td>
<td>- Blood vessels seen at the cornea boundary, but stay restricted to the peripheral cornea region.</td>
</tr>
<tr>
<td>(Moderate)</td>
<td>- Outlines of xanthophores, iridiophores and melanophores in the iris are blurry.</td>
<td>- Example: Figure 2B’</td>
</tr>
<tr>
<td></td>
<td>- Pupillary edge is discernable but blurry.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>- Example: Figure 2B’</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>- Cornea appears opaque.</td>
<td>- Multiple blood vessels are present.</td>
</tr>
<tr>
<td>(Severe)</td>
<td>- Outlines of xanthophores, iridiophores and melanophores in the iris are not visible.</td>
<td>- Blood vessels extend into the central cornea.</td>
</tr>
<tr>
<td></td>
<td>- Pupillary edge is indistinguishable.</td>
<td>- Example: Figure 2D’, Figure 3B, 3B’</td>
</tr>
<tr>
<td></td>
<td>- Example: Figure 2D, 2D’, Figure 3B, 3B’</td>
<td>- Example: Figure 3C, 3C’</td>
</tr>
</tbody>
</table>

**Table 2.1: Scoring system used to grade corneal opacity and vascularization.**

This table shows scoring system based on a scale of 0 (no signs), 1 (moderate), and 2 (severe). The criteria used to assign the score for corneal opacity and vascularization are described, and some examples of these assigned scores shown in various figures are also listed.
Supplementary Figure 2.1 (Figure S2.1): Quantification of cornea area following PUV treatment on whole corneas.
(supplements data in Figure 2.2A-2.2D, 2.2A’-2.2D’)

Control corneas that received no PUV treatment show a steady increase in cornea area (black line). Corneas that received PUV treatment (red line) showed continued growth that was initially delayed compared to the control cornea. Error bars represent standard error of the mean. Statistical analysis has been done for untreated control corneas vs. PUV treated corneas. N = 7-12 for the time points, except for control cornea at 1dpt (N = 4). *** = p-value ≤ 0.0003. ** = p-value ≤ 0.005; * = p value ≤ 0.05.
Supplementary Figure 2.2 (Figure S2.2): Histological examination of eye tissue morphology. (supplements data in Figure 2.2A, 2.2C, 2.2D, 2.2A’, 2.2C’, 2.2D’)

Representative images of Hematoxylin and Eosin stained eye cross sections are shown (Column i), along with the higher magnification images of their retina (Column iii) and ciliary marginal zones (Column ii and Column iv). (A, B) The untreated control and the PUV treated corneas, respectively, at 15 days post treatment (dpt). (C, D) The untreated control and the PUV treated cornea at 30dpt. No differences were observed in overall eye morphology between the untreated control and the PUV treated corneas, which includes the tissues of the retina and ciliary marginal zones. Scale bar in D column iv equals 50µm for images in Column ii – Column iv, and 200µm for images in Column i.
Supplementary Figure 2.3 (Figure S2.3): Cell death analysis via pyknotic nuclei assessment following PUV treatment on whole corneas.
(supplements the data in Figure 2.6A)

Representative images showing Hoechst staining for 1 to 5 days post treatment (dpt) as labeled. (A-C) and (A’-C’) correspond to 1-5dpt for untreated control corneas and PUV treated corneas, as indicated. (A-C) Control corneas show only a minimal number of pyknotic nuclei. (A’-C’) An increase in the number of pyknotic nuclei is observed at 1 and 3dpt in PUV treated corneas, after which the number of pyknotic nuclei is similar to control corneas at 5dpt. Red arrowheads indicate representative pyknotic nuclei. White dotted circles enclose single fragmented pyknotic nuclei. Scale bar in C’ is 50µm.
Supplementary Figure 2.4 (Figure S2.4): TUNEL assay for cell death analyses following PUV treatment on whole corneas.
(supplements the data in Figure 2.6B)

Representative brightfield images of TUNEL positive nuclei are shown (Column i), alongside corresponding Hoechst images (Column ii), for the time points indicated as days post treatment (dpt). (A-D) and (A’-D’) correspond to 1, 3, 5, 15dpt for untreated control corneas and PUV treated corneas, as indicated. A significant increase in apoptosis levels in PUV treated corneas, as compared to control corneas was only observed at 1dpt. No significant differences were observed at other time points. Note presence of invading pigment cells in the PUV treated case shown in (D’). Red arrowheads indicate representative TUNEL positive nuclei and black arrows indicate pigment cells, which are larger and have a distinct color and irregular shape. Scale bar in D’ column ii is 50µm.
Supplementary Figure 2.5 (Figure S2.5): Percentage of p63 positive nuclei, mitotic nuclei, and total cell density following PUV treatment on whole corneas.

Representative images of p63 positive nuclei are shown in the green fluorescence channel (Column i), mitotic nuclei (H3S10) are shown in the red fluorescence channel (Column ii), alongside corresponding Hoechst counterstain in the blue (UV) fluorescence channel (Column iii), for the time points indicated as days post treatment (dpt). (A-E) and (A’-E’) correspond to 1, 3, 5, 15, 25dpt for untreated control corneas and PUV treated corneas, as indicated. Percentage of p63 remains fairly consistent in untreated control corneas. PUV treatment reduced the percentage of p63 nuclei at 1dpt, and the nuclei appear damaged. The mitotic nuclei counts were decreased at 1dpt in PUV treated corneas, while an increase was observed in mitosis at 3 and 5dpt in the PUV treated corneas. After the initial increase, the mitotic counts declined at 15dpt to control levels, and remained lower than the mitotic counts in untreated control corneas at 25dpt. Total cell density remains fairly consistent from 1 to 15dpt in untreated control corneas, and an increase in the total cell density was observed at 25dpt. Total cell density decreased at 1dpt in PUV treated corneas, while increased total cell density was observed at 3, 5, 15, 25dpt. Scale bar in E’ column iii is 50µm.
Supplementary Figure 2.6 (Figure S2.6): Cell death analyses of pyknotic nuclei following PUV treatment on half corneas.
(supplements the data in Figure 2.6C)

Representative images showing Hoechst staining for 1 to 5 days post treatment (dpt). (A-C) and (A’-C’) correspond to 1-5dpt for untreated control halves and PUV treated halves of the corneas, as labeled. (A-C) Untreated control halves shows a minimal number of pyknotic nuclei. (A’-C’) An increase in the number of pyknotic nuclei was observed at 1 and 3dpt in the PUV treated halves of the corneas, after which the number of pyknotic nuclei is similar to control halves of the corneas at 5dpt. Red arrowheads indicate representative pyknotic nuclei. White dotted circles enclose single fragmented pyknotic nuclei. Scale bar in C’ is 50µm.
Supplementary Figure 2.7 (Figure S2.7): TUNEL assay for cell death analyses following PUV treatment on half corneas.
(supplements the data in Figure 2.6D)

Representative brightfield images of TUNEL positive nuclei are shown (Column i), alongside corresponding Hoechst images (Column ii), for the time points indicated as days post treatment (dpt). (A-D) and (A’-D’) correspond to 1, 3, 5, 15dpt for untreated control halves and PUV treated halves of the corneas, as indicated. An increase was observed in apoptosis at 1dpt in the treated half of the cornea vs. the untreated control half of the cornea, whereas no significant differences were observed at other time points. Red arrowheads indicate representative TUNEL positive nuclei. Scale bar in D’ column ii is 50µm.
Supplementary Figure 2.8 (Figure S2.8): Percentage of p63 positive nuclei, mitotic nuclei, and total cell density following PUV treatment on half corneas. (supplements the data in Figure 2.7A’-2.7C’)

Representative images of p63 positive nuclei are shown in the green fluorescence channel (Column i), mitotic nuclei (H3S10) are shown in the red fluorescence channel (Column ii), alongside corresponding Hoechst counterstain in the blue (UV) fluorescence channel (Column iii), for the time points indicated as days post treatment (dpt). (A-E) and (A’-E’) correspond to 1, 3, 5, 15, 25dpt for untreated control half of the corneas and the PUV treated half of the corneas, as indicated. PUV treatment reduced the percentage of p63 nuclei at 1dpt in both the halves, which recovered at 15 and 25dpt. The treated half of the cornea initially showed a decrease in mitosis compared to the untreated half at 1dpt. Similar levels of mitosis were observed between the treated and untreated halves for 3, 5, 15, 25dpt, where both the halves showed an increase in mitosis at 3, 5, 25dpt. The total cell density in the treated half of the cornea, appeared similar to the untreated halves of these corneas for the respective time points, and showed an increase starting at 5dpt. Scale bar in E’ column iii is 50µm.
Supplementary Table 2.1 (Table S2.1): Changes in corneal opacity and vascularization following PUV treatment on whole corneas and half corneas. Corneal opacity (A, C) and vascularization (B, D) have been graded on a scale from 0 to 2, as described in Table 1. This table shows the individual scores for corneal opacity and vascularization for each time point examined. The analysis has been done for
Supplementary Table 2.1 (Table S2.1) (cont.):
untreated control corneas, PUV treated whole corneas; the untreated halves and the treated halves of the same corneas. Control corneas do not show significant changes in opacity and vascularization in any of the cases examined. Cases that received PUV treatment on the whole cornea show increased opacity and increased vascularization. The treated halves of the corneas show increases in opacity and vascularization up to 15dpt, after which the opacity and vascularization starts decreasing and most cases are restored by 28dpt.
### A) Corneal opacity following Psoralen only treatment

<table>
<thead>
<tr>
<th>Days post treatment (dpt)</th>
<th>Control cornea</th>
<th>Psoralen only treated</th>
<th>Cases scored 0</th>
<th>Cases scored 1</th>
<th>Cases scored 2</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
</tbody>
</table>

### B) Corneal vascularization following Psoralen only treatment

<table>
<thead>
<tr>
<th>Days post treatment (dpt)</th>
<th>Control cornea</th>
<th>Psoralen only treated</th>
<th>Cases scored 0</th>
<th>Cases scored 1</th>
<th>Cases scored 2</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
</tbody>
</table>

### C) Corneal opacity following UV only treatment

<table>
<thead>
<tr>
<th>Days post treatment (dpt)</th>
<th>Control cornea</th>
<th>UV only treated</th>
<th>Cases scored 0</th>
<th>Cases scored 1</th>
<th>Cases scored 2</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
</tbody>
</table>

### D) Corneal vascularization following UV only treatment

<table>
<thead>
<tr>
<th>Days post treatment (dpt)</th>
<th>Control cornea</th>
<th>UV only treated</th>
<th>Cases scored 0</th>
<th>Cases scored 1</th>
<th>Cases scored 2</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
</tbody>
</table>

### E) Corneal opacity following UV only half treatment

<table>
<thead>
<tr>
<th>Days post treatment (dpt)</th>
<th>Untreated half (aluminum foil covered)</th>
<th>UV only treated half</th>
<th>Cases scored 0</th>
<th>Cases scored 1</th>
<th>Cases scored 2</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
</tbody>
</table>

### F) Corneal vascularization following UV only half treatment

<table>
<thead>
<tr>
<th>Days post treatment (dpt)</th>
<th>Untreated half (aluminum foil covered)</th>
<th>UV only treated half</th>
<th>Cases scored 0</th>
<th>Cases scored 1</th>
<th>Cases scored 2</th>
<th>Total cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>4 (100%)</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td>3 (75%)</td>
<td>1 (25%)</td>
<td>0 (0%)</td>
<td>4</td>
</tr>
</tbody>
</table>

Supplementary Table 2.2 (Table S2.2): Changes in corneal opacity and vascularization following Psoralen only (whole cornea), UV only (whole cornea), and UV only (half cornea) treatments.

Corneal opacity and vascularization have been graded on a scale from 0 to 2, as described in Table 1.
Supplementary Table 2.2 (Table S2.2) (cont.):
This table shows the individual scores for corneal opacity and vascularization for each time point examined. The analysis has been done for the Psoralen only treated control corneas vs. their contralateral corneas (A, B), respectively, the UV only treated control corneas vs. their contralateral corneas (C, D), respectively, and the untreated half vs. the treated half of the UV only half treated control corneas (E, F), respectively. None of the controls showed any signs of opacity at any of the time points examined. Some control specimens showed slight vascularization very close to the peripheral boundary of the cornea, however, these vessels never extended into the central cornea region.
2.6. References


Collinson, J.M., Morris, L., Reid, A.I., Ramaesh, T., Keighren, M.A., Flockhart, J.H., Hill, R.E.,
activity, and cell movement during the development and maintenance of the murine corneal epithelium.
Dev Dyn 224, 432-440.

limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial
stem cells. Cell 57, 201-209.


Cytol 222, 99-140.

Dahmann, C., Oates, A.C., Brand, M., 2011. Boundary formation and maintenance in tissue

Davanger, M., Evensen, A., 1971. Role of the pericorneal papillary structure in renewal of

467-480.

37, 588-598.


DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. Proc Natl Acad Sci
U S A 102, 9523-9528.

Dua, H.S., 1998. The conjunctiva in corneal epithelial wound healing. The British journal of
ophthalmology 82, 1407-1411.


Dua, H.S., Joseph, A., Shanmuganathan, V.A., Jones, R.E., 2003. Stem cell differentiation and
the effects of deficiency. Eye (Lond) 17, 877-885.

corneal epithelial maintenance: testing the dogma. Ophthalmology 116, 856-863.


CHAPTER 3. LINEAGE TRACING TO STUDY CORNEAL WOUND HEALING IN THE PUV MODEL IN *XENOPUS*\(^4\)

3.1. Introduction

Contemporary treatments for LSCD, which utilize transplantation of cells or tissues, have benefitted greatly from animal model studies to understand the process of LSCD and corneal healing mechanisms. However, these studies have revealed several potential pitfalls. Transplanted cells can fail to integrate into the host tissue, and the surgeries also create further complications from their own perturbations. This can affect successful outcomes and the mechanisms involved in healing, by influencing the behavior of the transplanted cells (Di Girolamo, 2015; West, 2015). These factors may explain contradictory results reported in wound healing experiments by Majo et al. (2008). Surgical sutures can cause neovascularization (Kather & Kroll, 2014). Due to sutures being anchored near eyelid skin folds, Confetti labeled donor cells were sometimes found to be present in the eyelid epidermis of recipient mice (Richardson et al., 2018). These factors can, therefore, confound observations of corneal epithelial cell movements (J. Y. Zhao, Wang, Sun, & Zhang, 2011). A nonsurgical equivalent to study LSCD healing, where cells can be labeled without surgical/transplant methods, has obvious advantages (West, 2015), yet experiments examining wound healing during LSCD that avoids transplantation are currently lacking.

In this section, we present a novel model to study healing following SCD that combines lineage tracing with our previous PUV frog model of SCD (Adil et al., 2019). Since SCD can be restricted to one half of the cornea, this approach strategically spares proliferative cells in the other half, which can be labeled and traced to study the healing process, thereby bypassing the requirement for transplanted tissues. Additionally, the frog model provides the cost-effective advantage offered by this aquatic animal. Well established transgenic procedures in *Xenopus* allow one to generate hundreds of transgenic F0 frogs at a fraction of the cost of a single transgenic mouse (Amaya & Kroll, 1999; Smith, Fairclough, Latinkic, Sparrow, & Mohun, 2006), making it one of the most studied and powerful vertebrate systems (Paredes, Ishibashi, Borrill, Robert, & Amaya, 2015; Satoh, Sakamaki, Ide, & Tamura, 2005).

---

\(^4\) This chapter was originally published as a part of Adil MT, Henry JJ. (2021) Understanding cornea epithelial stem cells and stem cell deficiency: Lessons learned using vertebrate model systems. Genesis 59:1-2. © 2021 Wiley Periodicals LLC, and has been reproduced with permission from copyright owner.
The transgenic construct used in our study involves a heat-shock inducible promoter driving the expression of H2B-mCherry and membrane GFP (GFP-GPI) (Figure 3.1D) (Hamilton & Henry, 2014). The use of the heat-shock promoter provides a fairly unbiased approach, unlike a putative stem cell marker to drive fluorescent protein expression, which selectively labels the cell population, and may miss any CESCs/TACs populations that do not express that particular marker (Di Girolamo, 2015; West, 2015). Additionally, since this system couples mCherry to H2B histone, it allows us to label the nuclei of proliferative cells. The addition of GFP driven by a lens gamma crystallin promoter allows easy sorting of the transgenic vs. non-transgenic embryos (Figure 3.1E, 3.1E’). The expression of H2B-mCherry is achieved by using a fine thermal probe to induce heat shock in a cluster of cells on the cornea, to provide both temporal and spatial control (Figure 3.1F). Therefore, a cluster of cells can be labeled in the undamaged half of the cornea at any required time point. The use of external heat-shock for inducing H2B-mCherry expression also prevents delayed recombination (Di Girolamo, 2015), variability in expression, and corneal toxicity (Muftuoglu, Ucakhan, & Kanpolat, 2006; Noureddin et al., 1999; Tarafdar, Lim, Collins, & Ramaesh, 2012) associated with other methods, such as those using tamoxifen. Additionally, real-time live, or time-lapse imaging can be performed to track labeled cells, circumventing the need to euthanize animals to study these tissues.

3.2. Methods

3.2.1. Wild type Animals

Adult wild-type Xenopus laevis frogs were obtained from Nasco (Fort Atkinson, WI). Tadpole larvae were reared following established protocols (Henry and Grainger, 1987; Henry and Mittleman, 1995). Tadpole stages were assessed using those defined by Nieuwkoop and Faber (1994). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Animal Resources (DAR) at the University of Illinois.

3.2.2. Transgenic Animals

Transgenic animals were created using the sperm nuclear injection method (Smith et al. 2006), with the transgenic construct (Figure 3.1D) described in Hamilton and Henry (2014). Sperm nuclei stock was provided by Dr. Paul Hamilton (Illinois College, Jacksonville, IL). The transgene construct was mixed with the sperm nuclei stock and sperm dilution buffer as described in Smith et al. (2006). A Kent Scientific Genie syringe pump (Kent Scientific, Torrington, CT) was used with a 2.5ml Hamilton
syringe and Tygon tubing (R3603, Saint Gobain Performance Plastics) filled with mineral oil (Sigma, St. Louis, MO). Drummond microcap needles (#1-000-0500, Drummond Scientific Company, Broomall PA) were pulled using a PUL-1 Micropipette Needle Puller (World Precision Instruments, Sarasota, FL), set at delay = 1 and heat = 10. The tip of the pulled needles were cut so that the needle diameter at the injection end was about 40µm. The needles were held by a Narishige micromanipulator, connected to the Tygon tubing from the syringe pump, and were used to inject the aqueous transgene mixture into the eggs obtained by inducing female adult frogs obtained from Nasco (Fort Atkinson, WI) with Chorulon HCG (142021 R2, Merck, Kenilworth, NJ). The F0 transgenic animals created using this technique were reared to sexual maturity (~ 20 months) and utilized as founder lines. These F0 transgenic animals were mated to obtain F1 transgenic animals to be utilized for the experiments described here. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Animal Resources (DAR) at the University of Illinois.

3.2.3. Assembly of the nichrome filament heat-shock probe

An adjustable voltage Enercell power supply (Catalog no. 2730462), with 700mA output, set at 7.5V DC, is used to power the heat-shock probe. The heat-shock apparatus consists of a 100 ohms 10 turn potentiometer with on-off SPST switch, and an “M” style adaptaplug with its tip set to be positive (Figure 3.1G). The potentiometer dial can be adjusted to regulate the current flow to the nichrome filament at the end of the probe, and hence the temperature of the filament. It is important to start the unit using a higher resistance (low current) and then gradually lower the resistance to increase the current to get the desired temperature at the probe.

The filament temperature is detected by a K-type thermocouple (Omega Engineering Inc., Norwalk, CT). The fine thermocouple is glued to the end of the spade shaped, pointed filament using a white thermal adhesive (Arctic Alumina AATA-5G, Arctic Silver, Visalia, CA), and connected to a Super MCJ thermocouple-to-analog converter (Omega Engineering Inc., Norwalk, CT) (Figure 3.1F, 3.1G). The converter is connected to a voltmeter to read the millivolt output, which is the equal to the temperature, as the converter output is linear (1mV/degree). Before operating the apparatus, it is important to ensure that the delicate thermocouple wires are neither touching each other, nor any metallic surfaces, which would create a short-circuit. This can be achieved by separating the exposed thermocouple wires using a small piece of Kimwipe. Note that it is extremely important that the power is switched off before taking the probe out of the water, to prevent burning out the filament, and
detachment of the thermocouple. As opposed to air, the water removes a great deal of heat from the filament.

### 3.2.4. Heat-shock via a Nichrome filament probe

The tadpoles were first anesthetized in MS-222 (ethyl 3-aminobenzoate methanesulfonate, Sigma-Aldrich, St. Louis, MO) for (~1-2 minute) diluted 1:2000 in 1/20x Normal Amphibian Media (NAM) (Slack, 1984). A clay lined 60mm petridish was filled with a more diluted solution of MS-222 (1:4000) in 1/20x NAM, and a fire polished glass ball tool was used to create a trough in the clay. The anesthetized animals were placed in the trough and clay was gently moved over the animal, to immobilize it in an orientation such the left lateral side of the animal was facing up towards the microscope objective. It is important that the volume of 1/20x NAM is enough to completely submerge the end of the nichrome probe, when it is in touch to the cornea. The nichrome filament probe was maneuvered using the Narishige hydraulic micromanipulator, to lower it to the surface of the cornea at the desired location of the heat-shock. Next, the power supply to the probe is switched on, and the dial is adjusted to obtain a probe temperature of 35-37 degree Celsius. After 6 minutes of heat-shock, the power supply is switched off and the probe is removed from the cornea. The animal was then removed and placed in a bowl filled with 1/20x NAM placed on a rocker until awakened, at which point it was transferred into a bowl filled with dechlorinated tap water (see Hamilton and Henry, 2014).

### 3.2.5. Psoralen-UV treatment:

Psoralen-UV treatment was performed, as described in Adil et al. (2019), with the following modifications. For the UV illumination period, a 22mm square glass coverslip was broken into half and placed on the moist cornea to avoid drying out of the tissue. Drying of the cornea can also activate some expression in the cornea. Similarly, during half cornea treatments, the aluminum foil was placed on top of the coverslip described above, along its longer side. The edges of aluminum foil were folded downward underneath the edge of the coverslip to keep the foil from moving. The aluminum foil covered coverslip was then placed on the cornea such that the aluminum foil part of the coverslip was on the anterior half of the cornea, and the uncovered part of the coverslip was on the posterior half of the cornea, thus PUV treating the posterior half (Figure 3.1C).
3.2.6. Time lapse Imaging of animals

The tadpoles were first anesthetized and then immobilized in a clay lined petridish, as described in Section 3.2.4 above. Images were taken under a compound microscope (Zeiss Axioplan), using a SPOT camera (Spot Imaging, Sterling Heights, MI) at multiple focal planes, which were then merged/flattened using Helicon Focus software (Helicon Soft Ltd. Kharkov, Ukraine). Besides visualization of mCherry expression, corresponding brightfield images were taken at all the required time points, to align the eyes from one time point to the next utilizing the location of the choroid fissure, and any other obvious landmarks.

3.2.7. Image analysis

Analysis of images flattened using Helicon focus software (Helicon Soft Ltd., Kharkov, Ukraine) was done using FIJI (National Institutes of Health, Bethesda, MD, USA). A circle was drawn over the eyeball to approximate the area of the eye, which was then used to calculate the diameter of the eye using Area = \((\pi \times d^2)/4\) (the eyes are approximately circular). Free hand outline of the H2B-mCherry expressing heat-shock labeled cell clusters was traced. The centroid of both the eye and the heat-shock labeled cluster was determined using the measure function of FIJI, and the distance between these two points was calculated using \(\sqrt{((x_1 - x_2)^2 + (y_1 - y_2)^2)}\), where \(x_1\) and \(y_1\) are the coordinates of the centroid of the eye, and \(x_2\) and \(y_2\) are the coordinates of the centroid of the cluster. This distance was then divided by the diameter of the eye to normalize for any changes in eye size that occurs due to growth over the time intervals examined. This procedure was repeated for all time points.

A vertical line passing through the choroid and dividing the treated and the untreated halves, was assigned as the “zero” line, such that clusters towards the left (representing the untreated side) of this line were denoted as negative displacements (distances) and the clusters positioned towards the right (representing the treated side) as positive displacements (distances). Relative displacement of the heat-shock labeled clusters between two time points was then calculated by subtracting their respective distances from the center. Note that it is assumed that the eye is growing uniformly around its center, which could vary among specimens. Therefore, this analysis only provides a general measure of the relative displacement.
3.3. Results and Discussion

3.3.1. Response of cornea cells during homeostasis and to localized PUV treatment

We examined the response of cells in control corneas by heat-shock labeling the nuclei in a cluster of cells located in the anterior half of control corneas. Images were taken immediately before heat-shock to ascertain the presence of any background or leaky H2B-mCherry expression in the cornea, as some scattered labeled nuclei may be present (Figure 3.2A). At 1-day post heat-shock (dphs), a discrete cluster of labeled nuclei can be visualized (Figure 3.2A’). Note that it takes approximately 1 day for mCherry expression to reach high levels of expression and fluorescence intensity. At 7dphs, no apparent displacement in the labeled nuclei was detected in the cornea (Figure 3.2A’’, Figure 3.5A). The shape and size of the labeled clusters appear largely similar from 1 to 7dphs, and any minor changes can be attributed to the increased eye size due to larval growth over this time period. Additionally, at 7dphs some sporadically distributed label may appear near the periphery of the cornea, which we believe is due to leaky expression from the hsp promoter (Figure 3.2A’’’). A disadvantage of the transgenic animals used here, is that some specimens can show leaky expression and background fluorescence (Figure 3.2C-3.2C’’’). Nevertheless, heat-shock can be done in a discrete cluster of cells for easy distinction, and most specimens only show minimal and sporadic leaky/background expression (Figure 3.2A’’, 3.2B-3.2B’, Figure 3.3B-3.3E).

To determine the response of corneal cells after PUV treatment, heat-shock labeling was performed in the anterior halves of the transgenic corneas, as above. Images were taken at 1dphs showing the initial location of heat-shock labeled mCherry expressing nuclei (Figure 3.2B). These specimens then received PUV treatment restricted to the posterior halves using aluminum foil masks at 1dphs (Figure 3.1C). Rapid displacement of labeled nuclei towards the treated half of the cornea was observed within 1-day post treatment (1dpt) or 2dphs, accompanied with some changes in the overall shape of the labeled clusters (Figure 3.2B’, Figure 3.5B). At 3dpt (4dphs), fewer labeled nuclei can be seen (Figure 3.2B’’), likely due to increased cell division and dilution of the label at 3 days post PUV treatment (4dphs). We have previously shown that the untreated half of the cornea responds to the PUV treatment on the treated half, and results in increased mitosis (Adil et al., 2019). In that study, we also showed that the highest increase in mitosis was observed during early time points (3 and 5dpt), which is consistent with the dilution and disappearance of mCherry expressing heat-shock labeled nuclei over 1-6dpt (Figure 3.2B-3.2B’’, 3.2C-3.2C’’’, Figure 3.3B). A similar increase in mitosis has been observed
in other lineage tracing models involving different types of wounding (Hanna, 1966; Lehrer et al., 1998; Sagga et al., 2018). Furthermore, since heat-shock labeled nuclei represent a subset of proliferative cells in the cornea, the displacement of these labeled nuclei from the untreated half towards the treated half may explain the changes in the distribution of p63 expressing cells observed in our previous study (Adil et al., 2019). Rarely, in some specimens, labeled nuclei can be seen at 3dpt in the treated half of the cornea (Figure 3.2C’’). Note that the region containing labeled nuclei gets smaller and changes shape as the cells are displaced and undergo mitosis to repair the damaged cornea tissue (Figure 3.2B’, 3.2B’’’, 3.2C’, 3.2C’’’).

Some labeled nuclei appear to be displaced more slowly, and remained close to where the heat-shock was performed (Figure 3.2B’’, 3.2C’, 3.2C’’’). However, close examination reveals that these nuclei appear different from the more rounded nuclei of the cornea epithelial cells, and are actually deeper cells of the keratocyte layer located below the basal cornea epithelium (Sonam et al. 2019).

To help visualize the heat-shock labeled mCherry expressing epithelial cells that get displaced into the PUV treated half, corneas from the specimens fixed in formaldehyde at 1dpt were removed from the eye, counterstained with Hoechst, and mounted as described previously (Adil et al., 2019). Higher magnification showed that the heat-shock labeled mCherry expressing cells were present in both halves of the cornea at 1dpt (Figure 3.2D, 3.2D’, 3.2D’’’).

3.3.2. Response of cornea cells at later time points after localized PUV treatment

To determine how long cells in the untreated half may continue to respond to localized PUV damage in the other half, we examined their behavior at different time points. Specimens received PUV treatment on the posterior halves of the corneas, and heat-shock labeling was performed in the anterior halves at 2, 6, 11, and 15dpt (Figure 3.3A). Nuclei that were labeled at 2dpt in the untreated half, showed rapid displacement into the treated half through the next 3-6dpt (Figure 3.3B, Figure 3.5C), similar to the displacement at 1-3dpt described above. Additionally, the size of the labeled cluster and intensity of the labeled nuclei appears to be reduced, indicating some dilution of the labeled nuclei via mitosis (see above). Interestingly, we observed that some of the labeled cells (nuclei) may leave the cornea and enter the peripheral skin at 6dpt (Figure 3.3B column iv). This could be due to a disruption of the cornea-skin boundary from the PUV treatment (Adil et al., 2019). Note that since heat-shock expression generally takes approximately 1 day to be clearly visualized, the initial location of the labeled
nuclei cannot be directly imaged, and the labeled nuclei may have already started to become displaced during that time period (as described in the previous section). This may contribute to some labeled nuclei being seen early in the treated side of the cornea at 3dpt (Figure 3.3B column i). Similar to the specimens in the section above, these specimens also showed the presence of some labeled nuclei at the initial location of the heat-shock in the untreated half.

In cases where nuclei were labeled at 6dpt, and observed through 7-10dpt, only a small relative displacement was observed in some cells located closest to the treated half (Figure 3.3C, Figure 3.5D). Cases that received heat-shock at 11 or 15dpt, and observed at 12-15dpt and 16- 28dpt, respectively, showed minimal displacement, if any (Figure 3.3D, 3.3E, Figure 3.5E, 3.5F).

Our results show that while the cells from the undamaged half of the cornea are quickly displaced into the PUV treated half of the cornea, this behavior does not continue through later time points (Figure 3.3B-3.3E, Figure 3.5B-3.5F). This reveals that cells from the undamaged half quickly repopulate the damaged tissue.

3.3.3. Response of cornea cells after increased UV exposure following localized PUV treatment

In the above experiments we observed that, unlike our previous study (Adil et al., 2019), those specimens that received localized PUV treatment recovered more quickly. This was likely due to the addition of a glass coverslip that was placed on the cornea to prevent it from being dried out. This also reduces the resultant UV exposure to the cornea. To see if this might be the case, and to test if the response of the cornea cells at later stages was dependent on the severity of wounding, the UV exposure time was increased by 20%. These corneas showed the typical PUV damage phenotype in 9 out of 11 cases at 15dpt (see Adil et al., 2019). Nuclei were then heat-shock labeled in the untreated halves of the corneas in all 11 of these specimens at 15dpt. Like the experiments described above, no displacement of cells was observed at the time points imaged from 16-28/30dpt in these corneas (Figure 3.4A-3.4D, Figure 3.5G). Transparency was ultimately restored in 7 out of the 9 cases that showed a PUV phenotype at 15dpt. However, a few pigment cells did remain in some of these corneas (Figure 3.4D, 3.4D').

In comparison to the control corneas, where no apparent displacement was observed (Figure 3.2A-3.2A'', Figure 3.5A), the rapid displacement of cells following wounding (Figure 3.2B-3.2B'', 3.2C-3.2C'', Figure 3.3B, , Figure 3.5B, 3.5C) is consistent with other studies showing that the migration of cells increased sharply immediately following wounding (Amitai-Lange et al., 2015; Chang et al., 2008;
Mort et al., 2009; Richardson et al., 2018). The rate of displacement of labeled cells (nuclei) in our experiments showed variation among specimens, and is likely dependent on the severity of the injury, as individual specimens can show varied responses to the PUV treatment (Adil et al., 2019). Additionally, the change in shape of the labeled cluster of cells after wounding appears to be indicative of a directional movement towards the treated half, which could be a localized response to signals resulting from the PUV damage. Similar changes in the direction of limbal streaks during wound healing have been observed in other systems (Amitai-Lange et al., 2015; Mort et al., 2009).

One possibility regarding the displacement of labeled cells is that these cells are simply displaced into the “vacuum” created by the cell death occurring in the treated half (Danjo & Gipson, 2002; M. Zhao, Song, Pu, Forrester, & McCaig, 2003). However, there is a change in the shape of labeled cells (Figure 3.2B-3.2B″, 3.2C-3.2C″, Figure 3.3B), and their displacement continues at later time points (Figure 3.3B), during which no increased cell death was observed in our previous study (Adil et al., 2019). Further experiments are required to distinguish active vs. passive cell displacement.

Additionally, note that in Figure 3.5, the labeled clusters appear to move backwards (showing some retrograde, negative displacement) likely due to the ever growing eye sizes. Note that the largest negative displacement is observed when samples were imaged through longer time periods (controls shown in Figure 3.5A and cases shown in Figure 3.5F, 3.5G), where eyes have grown more extensively over time. As cells undergo mitosis the H2B-mCherry label eventually becomes diluted. Additionally, labeled cells located nearer to the center that have undergone mitosis may tend to be sloughed off more quickly, and may not be re-populated by sufficient labeled cells with brighter labeled nuclei in those clusters. Note also that these experiments were done in larval frogs. Unlike the adults, where the corneal epithelial stem cells appear to mainly reside in the peripheral limbal region, larval stem cells reside throughout the cornea. Thus, there may not be a pronounced centripetal flux of cells pushing larval cornea epithelial cells toward the center under normal growth conditions (Hamilton & Henry, 2016; Perry et al., 2013; Sonam et al., 2019).
3.4. Figures

Figure 3.1: Localized PUV treatment and heat-shock apparatus to label cells in the *Xenopus* cornea

(A-C) Localized PUV-treatment procedure. (A) MS-222 anesthetized tadpole is placed in a petri dish on Kimwipes soaked in anesthetic solution. Reservoir pipette tip is affixed to a metal rod held by the micromanipulator using a small binder clip. The tip is gently placed around the cornea to form a sealed...
Figure 3.1 (cont.):
chamber. Psoralen solution is added into the reservoir and allowed to contact the cornea. (B) Higher magnification view of area shown in panel (A). (C) Image through the 10X objective showing aluminum foil covering the anterior half of the cornea, restricting UV illumination to the posterior half. Aluminum foil was first placed on top of a glass coverslip, which was then placed on the moistened cornea to avoid drying out of the tissue (We found that drying of the cornea can activate some expression in these cells). (D) Transgenic animals contain a transgenic construct that uses the hsp70 promoter for heat-shock inducible H2B-mCherry expression in nuclei and GFP-GPI expression in cell membranes. The second portion of the construct contains a gamma-crystallin promoter driving GFP expression in the lenses of these transgenic animals. The transgenic animals carrying this construct were created by the sperm nuclear injection method of transgenesis (Smith et al., 2006), which were then reared to sexual maturity and mated to obtain F1 transgenic animals to be utilized for these experiments (see section 3.2.2. for details). (E, E’) Convenient sorting of the transgenic vs. non-transgenic animals is made possible by observing the presence vs. absence of the GFP expressing lenses, respectively. (F) Heat-shock probe to induce localized expression in a specific cluster of cells. The probe is attached to a micromanipulator and the sharpened nichrome filament is gently touched to the desired location on the cornea. (G) Complete set-up of the heat-shock apparatus showing various components, as indicated. Tadpoles at stages 51-53 (Nieuwkoop & Faber, 1994) immobilized in a clay lined petridish containing 1/20x Normal Amphibian Media, after anesthetizing, as described by Adil et al. (2019). Electric current is applied to the filament to achieve the desired temperature of 35-37°C for 6 minutes. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Animal Resources (DAR) at the University of Illinois. Images in (A, B) are reprinted from Experimental Eye Research, 187, 107767, Mohd Tayyab Adil, Claire M. Simons, Surabhi Sonam, Jonathan J. Henry, Understanding cornea homeostasis and wound healing using a novel model of stem cell deficiency in Xenopus, Copyright (2019), with permission from Elsevier. Image in (D) is based on Hamilton and Henry (2014), Prolonged in vivo imaging of Xenopus laevis, Jonathan J. Henry, Paul W. Hamilton, published in Developmental Dynamics by John Wiley and Sons.
Figure 3.2: Response of cornea cells during homeostasis and following localized PUV treatment in *Xenopus*

(A – A’’) Control transgenic corneas that did not receive PUV treatment, showing unlabeled cornea immediately before localized heat-shock (A), and corneas showing labeled nuclei at 1 day (A’)

132
Figure 3.2 (cont.):
and 7d post heat-shock (A’’). Over time, the labeled cluster of cells has become somewhat elongated.
(B-B’’) Corneas showing the initial location of heat-shock labeled nuclei (B), and their rapid
displacement towards the treated side of the cornea 1 day after localized PUV treatment (B’). These
labeled nuclei presumably divide quickly to dilute the fluorescence by 3dpt as fewer labeled nuclei can
be seen (B’’, and inset). (C-C’’) Another example showing displacement of heat-shock labeled nuclei
from 1-3dpt. This particular specimen has extensive leaky background expression from the hsp70
promoter in deeper cells of the iris/retina. Nevertheless, the cluster of heat-shock labeled cornea cells is
distinctly detectable, and has become displaced to the PUV treated side by 3dpt. Note that the dotted
area gets smaller as the labeled nuclei are displaced and undergo mitosis. (D) Excised flat mount of a
cornea that received PUV treatment on one half and heat-shock to label the nuclei in cells on the
untreated half. Labeled nuclei are present in both halves at 1dpt. (D’, D’’) Magnified images of the
boxed areas in (D). Dotted white vertical line passes through the center of the cornea. White dotted
enclosed area indicates heat-shock activated H2B-mCherry expressing nuclei. HS = heat-shock; dphs =
days post heat-shock; PUV = Psoralen UV treatment; dpt = days post treatment. Number of cases
examined (N) = 6 for control cases; and for PUV treated cases, N = 19 for 1dpt, 9 for 2dpt, 10 for 3dpt.
Scale bar in (D’’) equals 50μm for (D’, D’’), 100μm for (D), and 200μm for (A-C’’).
Figure 3.3: Response of cornea cells at later time points after localized PUV treatment in *Xenopus*.

(A) Schematic of experimental outline. Specimens received PUV treatment on one half of the cornea. At 2, 6, 11, and 15dpt, specimens received heat-shock to a cluster of cells on the untreated half.
Figure 3.3 (cont.):

of the corneas, and representative mCherry images are shown at various time points, as indicated (N = 11 for time points in column i-column iii; N greater than or equal to 8 for time points in column iv). (B) Corneas showing rapid displacement of nuclei that were labeled initially at 2dpt. (C, D, E) Corneas showing minimal movement in the nuclei that were labeled at 6, 11, and 15dpt, respectively. Dotted white vertical line passes through the center of the cornea. White dotted enclosed area indicate clusters of heat-shock activated H2B-mCherry expressing nuclei. PUV = Psoralen UV treatment; dpt = days post treatment. Scale in (E column iv) equals 200μm.
Figure 3.4: Response of labeled cells to increased UV exposure during localized PUV treatment in *Xenopus*

(A-D) Typical example showing nuclei labeled at 15dpt in the untreated half of the cornea in specimens that received PUV treatment in the other half. (see data in Figure 5G). Representative mCherry images are shown at the time points indicated after PUV treatment. Labeled nuclei show minimal movement from 16-28/30dpt. (A’-D’) Corresponding brightfield images of the specimen in (A-D) at respective time points. A similar response was seen as compared to cases shown in Figure 3E above. Dotted white vertical line passes through the center of the cornea. White dotted enclosed area indicates cluster of heat-shock activated H2B-mCherry expressing nuclei. dpt = days post treatment. Scale in (D’) equals 200μm.
Figure 3.5: Displacement of the heat-shock labeled cell clusters in larval Xenopus corneas

Column graphs representing the relative normalized displacement between specific time points, as indicated. All specimens received localized heat-shock labeling in one half of the cornea to label a
Figure 3.5 (cont.):

cluster of cells expressing H2B-mCherry in their nuclei. (A) Normalized displacement is shown for control corneas, where the H2B-mCherry expressing clusters of cells showed only a small displacement away from the center (negative displacement) between 1-7 days after heat-shock. (B) Other specimens received localized heat-shock labeling in one half of the cornea and were PUV treated in the opposite half of the cornea 1d after the heat-shock. Within 1d post PUV treatment a sharp increase in normalized displacement is observed, as the labeled cluster was displaced towards the treated half of the cornea. These labeled clusters showed continued displacement between 1d-2d post PUV treatment. (C) When the heat-shock was performed in the untreated half of the corneas at 2 days post PUV treatment, elevated displacement of the labeled cluster was observed for all subsequent time points, as indicated. (D-G) When the heat-shock was performed at 6, 11, and 15d post PUV treatment, or 15d post PUV treatment with 20% longer UV exposure, (see main text, F), minimal displacement away from the center (negative displacement) was observed, similar to controls. Number of cases are as indicated for each time point. PUV = Psoralen UV treatment; dpt = days post treatment; dphs = days post heat-shock.
3.5. References


Danjo, Y., Gipson, I.K., 2002. Specific transduction of the leading edge cells of migrating epithelia demonstrates that they are replaced during healing. Exp Eye Res 74, 199-204.


CHAPTER 4. LENS REGENERATION FOLLOWING PUV TREATMENT IN THE STEM CELL DEFICIENCY MODEL IN XENOPUS

4.1. Introduction

*Xenopus* is one of the few animal models capable of an extraordinary and uncommon phenomenon of de novo regeneration of an intact lens upon removal of the original lens (Freeman, 1963; Freeman and Overton, 1961). Unlike newts and cobitid fish that regenerate their lenses from the iris (called “Wolffian regeneration”), *Xenopus* is unique in this aspect as the source of the regenerated lenses is from the innermost layer of the bi-layered outer cornea epithelium (Freeman, 1963; Freeman and Overton, 1961, Henry and Elkins, 2001). While initially believed to be a cornea trans-differentiation phenomenon like Wolffian regeneration, *Xenopus* lens regeneration likely involves a population of oligopotent cornea stem cells, which are located in the basal layer of the larval cornea epithelium and express the transcription factor p63 (a putative marker for proliferative cells of the cornea) (Perry et al., 2013).

The lens regeneration process is triggered when signaling factors from the neural retina come into contact with the outer cornea, which are normally blocked by the presence of the lens and the inner cornea endothelium. Note that lens does not secrete inhibitory factors to halt regeneration (Bosco et al., 1980), and any other barriers (such as millipore filters) placed into the vitreous chamber of a lentectomized eye, can also prevent lens regeneration (Cioni et al., 1982; Filoni et al., 1981). The necessity of signaling factors from the neural retina was demonstrated by several studies showing that a lens can regenerate when the cornea is implanted into the vitreous chamber (the spherical inner space of the eye located posterior to the lens) (Bosco et al., 1993a; Bosco et al., 1993b; Bosco et al., 1992; Filoni et al., 1997; Filoni et al., 1981; Henry and Elkins, 2001; Reeve and Wild, 1978). Additionally, lenses can also regenerate when corneas are exposed to denatured eyecups, whole retinal protein extracts, or retina-conditioned culture media (Filoni et al., 1983). The lentogenic cornea can therefore regenerate lenses in the presence of the inducer(s) provided by the neural retina. Interestingly, in *Xenopus* tadpoles, the lentogenic region of the cornea extends to twice the diameter of eyecup (Freeman, 1963), and has been linked with the expression of *Pax6* in this region (Gargioli et al., 2008).
In the PUV model of cornea epithelial stem cell deficiency, these corneal oligopotent stem cells are depleted/damaged, and the surrounding skin invades the corneal region: therefore, we tested if the lenses regeneration was still possible in these corneas. We tested this by utilizing a pioneering experimental design to study lens regeneration via an ex vivo eye culture system, which allows the PUV treated corneas to be tucked into untreated control eyecups (Fukui and Henry, 2011; Thomas and Henry, 2014), and the presence of regenerated lenses to be examined via a polyclonal antibody for Xenopus crystallins (Henry and Grainger, 1987). Furthermore, by performing the reverse ex-vivo assay (a control untreated cornea into PUV treated eyecup) we were able to determine if this treatment causes any potential damage to the retina or its molecular signaling following the PUV treatment.

4.2. Methods

4.2.1. Animals

Adult Xenopus laevis frogs were obtained from Nasco (Fort Atkinson, WI). Tadpole larvae were reared following established protocols (Henry and Grainger, 1987; Henry and Mittleman, 1995). Tadpole stages were assessed using those defined by Nieuwkoop and Faber (1994). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Animal Resources (DAR) at the University of Illinois.

4.2.2. Lentectomy and ex-vivo eye culture

The lentectomy and ex-vivo eye culture systems described by Fukui and Henry (2011) and Thomas et al. (2014), were adopted for these experiments. Animals were anesthetized and placed in a petri dish lined with clay, such that the eye to be operated on is positioned towards the dissection/stereo microscope objective, as described in section 3.2.4, above. Ultra-fine iridectomy scissors were used to make a small incision (20-40% of the circumference) at the edge of the outer cornea (cornea epithelium). Using fine forceps or the iridectomy scissors, the cornea endothelium is pierced at the edge of the iris, from which the lens is grabbed, removed, and discarded. Care must be taken to remove the entire lens intact (with the lens capsule). Additionally, puncturing/lacerating of other eye tissues must be avoided. The outer cornea is then excised using the scissors, and gently tucked into the empty vitreous chamber of an eye cup using forceps. It is essential that one ensure close proximity to the retinal tissue. Care must be taken to ensure that the cornea stays tucked in the vitreous chamber, as the forceps are removed from the eyecup, and to ensure that the cornea does not get dislodged during any of the further
processing. The entire eyes can then be removed by cutting the optic muscles, artery, and nerve connections beneath the eyecup. Using a sterilized standard 200μl micro-pipette tip cut at its distal end to accommodate the eye, the eyes are then transferred to a 35mm petri dish filled with modified L-15 media (mL15) containing 61% L-15 powder (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (Invitrogen, Carlsbad, CA), along with the following antibiotics/antifungals: 100 U/ml of penicillin, 100 μg/ml of streptomycin (Mediatech, Manassas, VA), 2.5 μg/ml Amphotericin B (Sigma, St. Louis, IL), and 4 μg/ml Marbofoloxacin (Sigma, St. Louis, IL). The eyes are transferred again to a second 35mm petri dish filled with the mL15 media to ensure removal of anesthetic and any contaminants. Finally, the eyes are transferred and cultured in a 24 well plate filled with the same mL15 media, and the culture media is replaced every other day. During culture, the samples are stored at RT in a moisture chamber (a plastic box lined with moist Kimwipes or a sponge) to prevent evaporation of the media. The animals were euthanized after the procedure using 1:2000 diluted MS-222. All surgeries were performed in a designated sterilized area according to procedures approved by the Institutional Animal Care and Use Committee (IACUC) and the Division of Animal Resources (DAR) at the University of Illinois.

4.2.3. Immunostaining

After 7 days of culture, the eye tissues were fixed in 3.7% formaldehyde in PBS (Sigma, St. Louis, IL) for 30 minutes at RT. The samples were then dehydrated in ethanol for 10 minutes each, cleared in Xylene, and embedded in Paraplast Plus (McCormick Scientific LLC, St Louis, MO), before sectioning at a thickness of 9μm. The serially sectioned tissues that were mounted on charged glass slides (Colorfrost Plus, Thermo Scientific, Kalamazoo, MI), using deionized water. The water was removed by placing the slides on a slide warmer at 32 degrees overnight, after which they were dewaxed by soaking in Xylene. The slides were then soaked in 1X PBS for 5 minutes at RT before soaking in 0.1M glycine in 1X PBS for 30 minutes at RT. After another 1X PBS wash for 5 minutes at RT, the slides were blocked for 45 minutes using 5% dry milk in 1X PBS. The tissues were then stained with anti-lens antibody (Henry and Grainger, 1987), diluted 1:200 in 5% milk 1XPBS solution overnight at 4 degrees. After 2 washes with 1X PBS for 10 minutes each at RT, Alexa Flour goat anti-rabbit 546 secondary antibody (Life Technologies ) was used, diluted 1:200 in 5% milk 1XPBS solution for 1.5 hours at RT. One 10 minute 1X PBS wash at RT was done, followed by incubation in 2μg/ml in 1X PBS Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) for 30 minutes at RT. Finally, after 2 washes with 1X PBS for 10 minutes each at RT, a few drops of 80% glycerol solution in 1X PBS were
placed on the slide, followed by gently lowering a clean coverslip onto the tissues. Weights were placed on the coverslips overnight at RT.

4.2.4. Imaging

Samples were observed under a Zeiss Axioplan microscope, and imaged using a Axiocam 503-mono camera controlled by ZEN software (Carl Zeiss, Munich, Germany). Positive cases of lens regeneration were identified based on the presence of distinct round areas of tissue (lentoids) that were positively stained with the anti-lens antibody. The scoring of lens regenerating cases is binary (regenerated or not regenerated).

4.2.5. Statistical analysis

The number of positive cases of lens regeneration were expressed as a percentage of total cases examined, and averaged across three separate sets of experiments. Fisher’s exact test was used to determine the statistical significance of the average percentage of successfully regenerated lenses. Two tailed p-values were calculated and considered significant for p-values less than 0.05.

4.3. Results and Discussion

PUV treatments were performed on full corneas as described in Adil et al. (2019). First, to determine the control levels of regeneration, the contralateral untreated corneas from the PUV treated animals were tucked inside their respective control eye cups following lentectomies, and these cases were used as an internal controls for lens regeneration. The average lens regeneration rate was observed to be 83% among the cases examined (N = 29, see Figure 4.1A, 4.1D, 4.1E, Figure 4.2). Next, to evaluate the impact of PUV treatment on lens regeneration in an unbiased manner, the PUV treated corneas were implanted into untreated control eyecups from a different set of untreated animals. The average lens regeneration rate dropped to 47% in these cases (p = 0.0135, N = 19, see Figure 4.1A’, 4.1D’, 4.1E’, Figure 4.2). Furthermore, to determine the impact of PUV treatment on the retina and its factors that induce lens regeneration, untreated control corneas were implanted into the eyecups obtained from the PUV treated eyes of the animals. The lens regeneration in these cases was similar to the control cases, with an average lens regeneration rate of 80% among the cases examined (N = 27, see Figure 4.1A”, 4.1D”, 4.1E”, Figure 4.2). Representative images showing a positive case of lens regeneration
from each of the three experimental sets are shown in Figure 4.1. Note the smaller eye size in Figure 4.2B"’ due to delayed growth of the PUV treated eye (see section 2.3.1. and 2.4.2. for details).

The reduced lens regeneration from the PUV treated corneas is consistent with our findings in Chapter 2, further establishing PUV treatment as an effective method to damage the stem cells and TACs of the cornea. Interestingly, approximately half of the cases were still able to successfully regenerate lenses from the PUV treated corneas. One reason for this residual lens regeneration capacity could be that the lentogenic area that is capable of regenerating lenses extends into the pericorneal epidermis and up to twice the diameter of the eyecup (Freeman 1963; Gargioli et al., 2008). These pericorneal epidermal cells are outside the PUV treated region, and invade the cornea region after PUV treatment (see Chapter 2 for details), and therefore could be the source of the regenerated lenses. Indeed, Pax6 expression, which is associated with lens regenerating competence, has also been shown to extend beyond the cornea region into the pericorneal epidermis (Gargioli et al., 2008; Sonam et al., 2019). Another possible factor could be the severity of the PUV treatment. Since some cases show a more moderate PUV phenotype (see section 2.3.1. - 2.3.4. and section 2.4.7.), they may possess more viable proliferative cells and still be capable of some level of lens regeneration.

The eyecups obtained from the PUV treated eye of the animals showed similar lens regeneration as controls in our reverse ex-vivo lens regeneration assay involving untreated corneas. This supports the histological examinations in section 2.3.1. and 2.4.2., which showed no morphological changes in the retina or the ciliary marginal zones, and suggest that the retinal signaling that is required for lens regeneration is unaffected following PUV treatment.

The ex-vivo eye culture approach provides us with a good opportunity to study the PUV treated cornea in a control eye cup, and a control cornea in a PUV treated eyecup, thus allowing the evaluation of the effects of PUV treatment separately on the cornea and the eyecup in an unbiased manner, without other tissues impacting the results.
4.4. Figures

**Figure 4.1: Representative cases showing positive cases for lens regeneration**

(A-A’’) Cross section of representative red fluorescence channel images showing lentoids labeled by anti-lens antibodies. (B-B’’) Corresponding DIC images showing the eye, where neural and pigmented retinal tissue can be visualized. (C-C’’) Corresponding Hoechst images to visualize the nuclei. (D-D’’) Merged images of both the red channel and DIC images showing the position of the lens relative to the eyecup. (E-E’’) Merged images of all three channels, including Hoechst. White arrows point to the regenerated lentoids.
Figure 4.2: Analysis of lens regeneration rate

Bar graphs showing the percentage of cases that were scored positive for lens regeneration (y-axis) for the three categories as indicated on the x-axis. Control corneas when tucked into their respective control eyecups showed lens regeneration in 83% of the cases. PUV treated corneas when tucked into an eyecup from a control eye showed reduced lens regeneration (47% positive cases). When control corneas were tucked into eyecups from PUV treated corneas, they regenerated lenses in 80% of the cases. N represents total cases examined across the three separate cohorts. Error bars indicate standard error evaluated from the standard deviation among the three cohorts for each category. * = p < 0.05.
4.5. References


CHAPTER 5. CONCLUSIONS AND PERSPECTIVES

Remarkable LSCD therapies have been developed since the first corneal transplants, including culturing of isolated corneal cells, the use of amniotic membranes and other cell carriers to propagate these cells, and even novel sources of epithelial stem cells to repair the cornea. However, several questions surrounding corneal restoration still remain, such as the ultimate fates of the transplanted stem cells, their integration and survival in the host tissue, and the nature of their movements as they heal the cornea, etc. The focus of this work was, therefore, to understand some of these mechanisms in a novel model of Stem Cell Deficiency (SCD) and lineage tracing in the *Xenopus* (frog) cornea.

In this study, we developed the SCD model using a rapid, single treatment with Psoralen (a DNA crosslinker) and focused UVA illumination (the PUV model). The PUV model recapitulates the hallmarks of the human disease, including corneal opacity, corneal neovascularization, and a rough/irregular corneal epithelium, and provides a robust, versatile, and tractable system for depleting proliferating cells and creating SCD in the cornea. Many models to study LSCD largely remove all types of cells in a particular region including CESCs, adjacent niche cells, as well as differentiated corneal cells. On the other hand, due to its mechanism of action, Psoralen and UV treatment (PUV treatment) specifically targets and destroys the proliferating cells of the cornea (see chapter 2 for details). The greater specificity in depleting the proliferating CESCs and TACs, as opposed to creating a physical, mechanical or chemical wound, makes this model highly beneficial in providing specific insights into the mechanisms of SCD, as opposed to a more generalized knowledge of wound healing. Next, we characterized the cellular changes in this model, which revealed increased pyknotic cell death immediately following the PUV treatment, depletion of the number of p63 expressing basal epithelial cells (a marker for specific epithelial cells including stem cells and transit amplifying cells), and stimulation of mitosis in a subset of the surviving cells.

A huge advantage of this model is the ease with which SCD can be restricted to one half of the cornea by restricting UV irradiation, using an aluminum foil mask, strategically sparing proliferative

---

cells in the other half to be studied as they respond to PUV damage and during the healing process. These cases initially developed SCD characteristics and increased pyknotic cell death on the treated half, but the cornea was subsequently restored, thus providing an excellent system to study corneal healing. We also showed that cells on the untreated half respond to the PUV damage on the treated half, resulting in increased mitosis, as well as changes in the percentage of p63 expressing basal epithelial cells.

To further understand the role of these strategically spared cells in the untreated half, we deployed the PUV model in a lab reared heat-shock inducible transgenic line, which allows us to specifically label a cluster of cells in the untreated half and trace them as they respond to SCD and during corneal repair. Using live animal in-vivo imaging to track and observe these cells, we showed that the cells from the undamaged half of the cornea are quickly displaced into the PUV treated half of the cornea, but this behavior does not continue through later time points. This system provides spatiotemporal information about the responding corneal cells, and a powerful and elegant way to study wound healing in real time. A unique aspect of this approach is that it bypasses the requirement for transplanted tissues or cells, which is beneficial as studies involving transplants can create complications affecting the outcomes and mechanisms of healing (West, 2015)

As a result of the PUV treatments, the corneal oligopotent stem cells (also thought to be the source of regenerated lens in *Xenopus*) are depleted/damaged, and the surrounding skin invades the corneal region. Therefore, we tested whether lens regeneration can still occur in this SCD model using an ex-vivo eye culture system that allows the PUV treated corneas to be tucked into an untreated control eyecup. We showed that the lens regeneration rate in treated corneas was significantly reduced, as compared to control corneas. Furthermore, we also conducted a reverse ex-vivo lens regeneration assay (a control untreated cornea tucked into eyecup of a PUV treated cornea) to show that the PUV treatment does not damage the retina or its molecular signaling that is required to trigger lens regeneration. Histological examination also revealed that PUV treatment does not damage deeper tissues such as the retina/ciliary marginal zone, nor cause any other obvious morphological changes in the eye.

As one of the few studies utilizing lineage tracing to study SCD (Richardson et al., 2018), this work provides insights into the proliferative behavior and displacement of cornea cells, and establishes an excellent system to tease apart corneal healing mechanisms. This system will provide valuable opportunities to investigate interesting questions that are still outstanding in the field, which are presented below.
What signaling pathways and molecular regulation underlies healing following SCD?

Visualizing and tracking the corneal cells is critical to gaining insights about their roles in corneal wound healing, yet, there are relatively few studies analyzing corneal healing following LSCD. This *Xenopus* PUV-lineage tracing model in combination with genetic or pharmacologic tools will be useful in identifying and studying the roles of specific signaling pathways and the molecular regulatory mechanisms that underlie stem cell behavior during wound healing, and may be targeted for treating SCD. For example, the role of YAP1 in maintaining the proliferative potential of limbal and corneal basal cells was identified in a lineage tracing model (Kasetti et al., 2016), and the role of Twist2 for corneal keratocyte proliferation was identified by tracing the progeny of Twist2 expressing cells (Weaving et al., 2010). Likewise, lineage tracing following different wounding perturbations in this system will bring new insights into the involvement of signaling pathways in regulating corneal cell fates. This knowledge may allow us to circumvent the need for externally transplanted cells/tissues, to develop more advanced LSCD therapeutics.

What’s the mechanism of repopulation of the proliferative cell pool?

Our work showed that cells from the untreated areas respond to the PUV damage in the treated side by increasing mitosis, and are displaced continually up to 6 days post PUV treatment. This would be expected to reduce the percentage of proliferative cells in the untreated half, which is also indicated by the changes in the percentages of p63 expressing cells in our results. Understanding the mechanism by which these cells are able to undergo increased mitosis to repopulate and maintain the proliferative cell pool, and stimulating the surviving host cells would contribute to improvements in LSCD therapeutics. It will be interesting to determine whether the responding cells switch from asymmetric to increased symmetric divisions, or reduce the cell cycle times to achieve these changes. Additionally, how do these cells relocate into the PUV damaged half of the cornea? What molecular regulation underlies their displacement? Is their displacement an active or a passive phenomenon? Answering these questions will reveal mechanisms that can potentially be utilized to facilitate the integration of transplanted stem cells into the host tissue.

Does the displacement of cells occur exclusively in a centripetal direction during wound healing?

We did not observe appreciable centripetal migration during homeostasis in control larval corneas, however there was increased displacement towards the wounded half in the PUV treated
corneas. Further lineage tracing experiments can be done by damaging tissues at different locations to understand general rules and routes. For example, one can damage peripheral cornea, central cornea, or create a donut shaped wound to evaluate whether the corneal cells can move centrifugally, centripetally, or by both routes, respectively (Amitai-Lange et al., 2015; Chang et al., 2008; Dua et al., 2009; Majo et al., 2008; Mort et al., 2009). This PUV-lineage tracing method provides an excellent system to study the movement of cells and test various models. Additionally, labeled cells can be evaluated regarding their movement towards the apical layer and their differentiation during healing.

**What is the precise location of stem cells in the *Xenopus* larval and adult cornea?**

In light of the controversy regarding the location of stem cells in the cornea, investigations utilizing this lineage tracing system to locate proliferating cells (CESCs and transient amplifying cells (TACs)) will be extremely valuable. The heat-shock inducible H2B-mCherry can be activated specifically in the proliferative cells throughout the cornea, and provides one an opportunity to perform label retention studies, and to identify the precise corneal niche. This particular system becomes advantageous, as live imaging can be repeatedly performed on animals without fixing/processing the tissues. This will provide further insight into the dynamics of the developing/establishing stem cell niche, and how the proliferating cells may become localized to a specific region of the cornea during later development and through metamorphosis to an adult stage. Are the stem cells getting actively displaced towards a peripheral niche as the frog undergoes metamorphosis? Do the stem cells in the central region undergo symmetric divisions to form TACs and terminally differentiated cells? Questions such as these can be answered using the label retention studies coupled with immunostaining for EMT markers and differentiation markers. The external mode of development in *Xenopus* makes such studies highly convenient.

**How is the response to PUV in the adult *Xenopus* cornea different from the larval cornea?**

We have performed our lineage tracing-PUV experiments in larval tadpoles. While it yields significant insight into the response of cells after PUV wounding and during healing, similar studies undertaken using adult frogs, which has similarities with the adult human cornea, may be more directly applicable to clinical applications. Previous studies in the lab examining EdU retention suggest that the label retaining cells are present only in the limbus in the adult (e.g., post-metamorphic cornea), as opposed to being distributed throughout the basal layer in the case of the larval cornea (Hamilton and
Henry, 2016). Therefore, it will be interesting to determine if the response to PUV damage, and healing events are conserved between larvae and adults. Interesting questions remain. What’s the contribution of these peripheral stem cells in the niche vs. the more centrally located TACs? Does it take longer for the adult cells to undergo displacement? Another experiment to evaluate the relative contributions of peripheral vs. central cornea cells could involve combining restricted PUV treatments on one half of the corneas, and removal of the peripheral corneal tissue on the untreated sides. If the peripheral corneal cells are indeed a major contributor to healing, one can expect that restoration of the treated half of the corneas should be unsuccessful or incomplete, as compared to cases where the peripheral tissue was left intact. On the other hand, if the contribution of peripheral cornea cells towards healing is similar to central cornea cells, the restoration of the treated half of the corneas should largely be similar to cases where the peripheral tissue was left intact.

The field of corneal stem cells and limbal stem cell deficiency has made tremendous progress. We anticipate that this work will facilitate future studies to determine the exact functions and specific importance of the pathways/regulatory mechanisms involved in cornea homeostasis and wound healing, and allow us to translate these findings into clinical medicine to advance LSCD therapeutics.

5.1. References


APPENDIX: UNDERSTANDING THE BASIS OF CYP26 MEDIATED REGULATION OF LENS REGENERATION USING EX VIVO EYE CULTURES AND 4-OXO-RA

This work was conducted in collaboration with Dr. Alvin G. Thomas, who is the primary author of this work. The abstract of this work is provided below, and the full manuscript is available, (see the reference provided in the footnote).

ABSTRACT:

PURPOSE: *Xenopus* has the remarkable ability to regenerate a lens from the basal cornea epithelial cells in response to signals from the retina. Previous work demonstrated that the Retinoic Acid (RA) metabolizing enzyme CYP26 is expressed in the cornea, and that its activity is required for lens regeneration. Gaps remain in our knowledge as to whether CYP26 is needed only to attenuate RA signaling via RA elimination, or whether it also acts to generate retinoid metabolites, such as 4-oxo-RA, to act as signaling ligands. Other key questions are why CYP26 antagonism, but not exogenous retinoids, can reduce cell division in the cornea, and when during regeneration CYP26 is important.

MATERIALS AND METHODS: *Ex vivo* cultures supplemented with RA, 4-oxo-RA, or the CYP26 inhibitor Liarozole were used to assay the effects of these compounds on lens regeneration. Similarly, corneas were explanted, cultured in the presence of these compounds, and assayed for mitotic changes by counting anti-Histone H3 positive nuclei. qPCRs validated responsiveness to these compounds.

RESULTS: *Ex vivo* cultures showed that when the media was supplemented with the RA metabolite 4-oxo-RA in addition to Liarozole, lens regeneration was still inhibited. 4-oxo-RA also does not rescue the loss of cell division in the cornea that is observed upon CYP26 antagonism. Liarozole inhibited regeneration when added 12 hours after lentectomy, but not when added 48 hours after.

CONCLUSIONS: These data show that the necessity of CYP26 is not explained as a generator of 4-oxo-RA for regeneration. Moreover, Liarozole-induced mitotic reduction is not explained by 4-oxo-RA.

---

6 This pre-print of this appendix is available as: Thomas AG, Adil MT, Henry JJ. (2019) Understanding the basis of CYP26 mediated regulation of lens regeneration using *ex vivo* eye cultures and 4-oxo-RA. bioRxiv 631994; https://doi.org/10.1101/631994. The full text can be obtained at: https://www.biorxiv.org/content/10.1101/631994v1.
deficiency. These results support a model of RA-independent mitotic regulation by CYP26, though other retinoid metabolites may be active. Finally, CYP26 activity is only needed between 12 and 48 hours post-surgery, showing that its action is required only during the earliest stages of lens regeneration.