Reconstitution of a Multi-layered, Differentiated Cornea by HTERT-Immortalized Corneal Epithelial Cells Transduced with Thymidine Kinase Transplanted onto Denuded Mouse Corneas

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by

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TABLE OF CONTENTS

PRIOR PRESENTATIONS

- "Case Report: Vogt-Koyanagi-Harada Disease in a Postpartum Patient." **JP Kalangara**, J Thomas.
    - Presented at the 2009 University of Texas Southwestern Medical Center Ophthalmology Grand Rounds
- "Tissue Engineered Universal Artificial Corneal Epithelium." **JP Kalangara**, DM Robertson, RB Baucom, SI Ho, HD Cavanagh.
    - Presented at the 2009 University of Texas Southwestern Medical Student Research Forum
- "A Tissue Engineered Universal Artificial Corneal Epithelium." Danielle M. Robertson, OD, PhD; **Jerry Kalangara**; Rebecca Baucom; H. Dwight Cavanagh, MD, PhD, FAAO.
    - Presented at the 2008 American Academy of Optometry Anaheim Conference
- "Reconstitution of a Multi-layered, Differentiated Cornea by HTERT-Immortalized Corneal Epithelial Cells Transduced with Thymidine Kinase Transplanted onto Denuded Mouse Corneas." **JP Kalangara**, DM Robertson, RB Baucom, SI Ho, HD Cavanagh.
    - Presented at the 2008 Association for Research in Vision and Ophthalmology National Conference

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE
Introduction

The ocular surface is a vital component of the eye and consists of the conjunctiva, cornea, and limbus (Figure 1, pg.9).[1] All three of these regions have stratified, squamous epithelia on their surfaces. This epithelium sits on a basement membrane that is anchored to an underlying connective tissue stroma.[1,2] The connective tissue provides the necessary nutritional and structural support for the ocular surface.

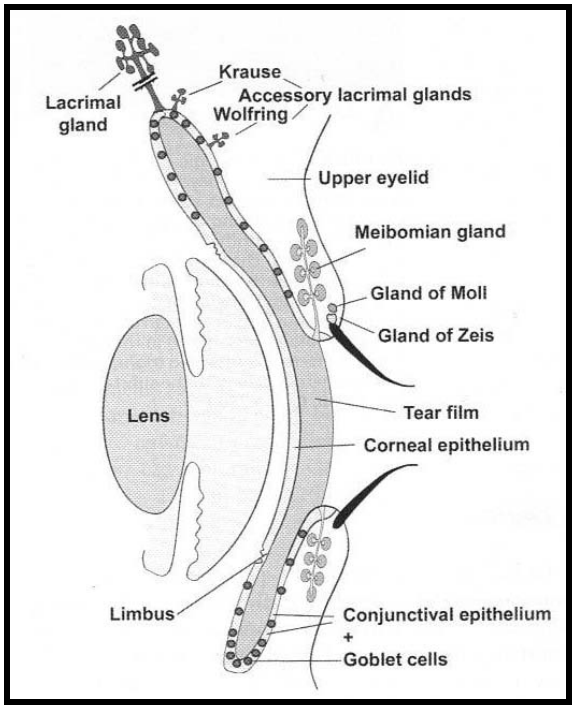


Figure 1: Structures of the ocular surface. Adapted from *Smolin and Thoft's The Cornea: Scientific Foundations and Clinical Practice.*[1]

The three different regions of the ocular surface serve multiple important functions. The conjunctival epithelium protects the surface through the production of membrane-associated mucins (MUC1, MUC4, MUC16).[3] These mucins form a tear film mucous layer that acts as a hydrophilic, negatively charged barrier at the epithelial surface. It prevents the adherence of

epithelial surfaces by providing lubrication and prevents pathogen entrance into the eye.[4] The conjunctiva also contains goblet cells intercalated between surface epithelial cells. These goblet cells form a large gel-forming mucin (MUC5AC), which cleans debris and pathogens from the surface of the eye.[3,4] The eye's immunogenic response to pathogens is primarily due to the conjunctival substantia propria. This region houses an abundance of immune cells including lymphocytes, neutrophils, and plasma cells that can neutralize infectious insults.[5]

The cornea is an avascular area of the eye that is about 1 mm thick peripherally and 0.5 mm thick centrally.[6] It consists of epithelium, stroma, and endothelium, which are arranged in a uniform fashion to allow for the precise refraction of light. The corneal epithelium is transparent so it can allow for the transmission of light. It is composed of five to seven layers and has consistent thickness over the entire cornea.[6] Its apical surface is smooth and wet serving as the major refractive surface of the eye. The corneal epithelium also functions as a barrier to pathogen entrance via membrane-spanning mucins at the apical surface. Tight junctions form the epithelial barrier which regulates fluid loss and resistance to abrasive pressures.[7] To maintain these important functions, the cornea is tightly regulated by homeostatic mechanisms governing proliferation, differentiation, and apoptosis.

The corneal epithelium is constantly shedding and renewing itself, undergoing complete turnover every 5-7 days.[8] However, the source for regeneration does not directly come from the cornea, but rather it comes from the limbus, a transitional zone between the cornea and conjunctiva. The limbal epithelium contains a subpopulation of adult or tissue-specific stem cells within its basal layer.[8] These limbal stem cells are capable of asymmetric cell division, allowing one daughter cell to remain a stem cell, while the other enters the path of terminal differentiation; the latter is termed a transient amplifying cell.[9] Transient amplifying cells

centripetally migrate from the peripheral limbal region to the basal cell layer of the central cornea (Figure 2, pg.11).[10-12] They have a high capacity for proliferation and undergo vertical terminal differentiation to replenish the corneal epithelium. Thus, limbal stem cells have an integral role in the maintenance of the corneal epithelium.

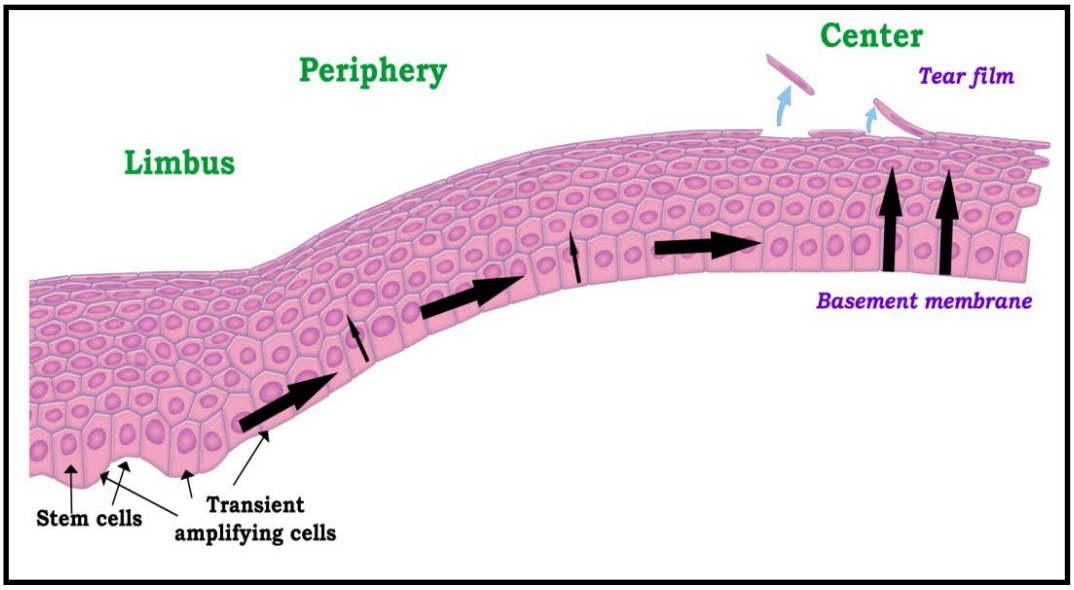


Figure 2: The Regeneration of the Corneal Epithelium. Adapted from Ladage et al.[12]

Severe damage to limbal stem cells can be caused by several ocular conditions such as chemical injury, pterygia, and Stevens-Johnson syndrome (Table 1, pg.12).[1,13,14] If these conditions lead to total loss of limbal stem cells, then limbal stem cell deficiency (LSCD) results. Primary LSCD is due to inherent deficiencies in the limbal region resulting in decreased stem cell function, while secondary LSCD occurs when a pathogenic process or injury leads to the destruction of limbal stem cells.[14,15] LSCD has a worse prognosis when it is due to chronic inflammatory conditions as opposed to acute, non-inflammatory causes. LSCD can present with a variety of symptoms: decreased vision, photophobia, recurrent pain, tearing; further, it can

present with clinical signs of persistent corneal epithelial defect, neovascularization, and scarring.[16-18] Ultimately, LSCD prevents the regeneration of the corneal epithelium, leading to the peripheral conjunctival epithelium centripetally invading the central cornea causing opacification and blindness.[9]

Primary limbal stem cell deficiency
Aniridia
Congenital erythrokeratodermia
Keratitis associated with multiple endocrine deficiencies
Neurotrophic keratopathy
Chronic limbitis
Idiopathic
Secondary limbal stem cell deficiency
Atopy
Chemical, thermal, or mechanical injury
Iatrogenic (medication toxicity or surgical trauma)
Ultraviolet or ionizing radiation exposure
Pterygia/pseudopterygia
Stevens-Johnson syndrome
Advanced ocular cicatricial pemphigoid
Collagen vascular diseases
Contact lens-induced epitheliopathy
Extensive microbial infection
Chronic keratoconjunctivitis
Cryotherapy
Squamous cell carcinoma

Table 1: Causes of Limbal Stem Cell Deficiency. Adapted from *Smolin and Thoft's The Cornea: Scientific Foundations and Clinical Practice.*[1]

LSCD can be treated with transplantation of autogenic or allogenic tissue from the conjunctival-limbal region (Figure 3, pg.13).[19] With autogenic transplantation, donor tissue is harvested from the contralateral healthy eye. This method is preferred as there is no risk of immune rejection and no need for immunosuppressive therapy. It has shown excellent success

rates over the past several years.[20] Unfortunately, autogenic transplantation is only a viable option with unilateral LSCD and even then caution must be used as harvesting tissue from the donor eye has resulted in complications such as microperforation, donor filamentary keratitis, pseudopterygium, and even damage to the stem cell compartment.[21,22]

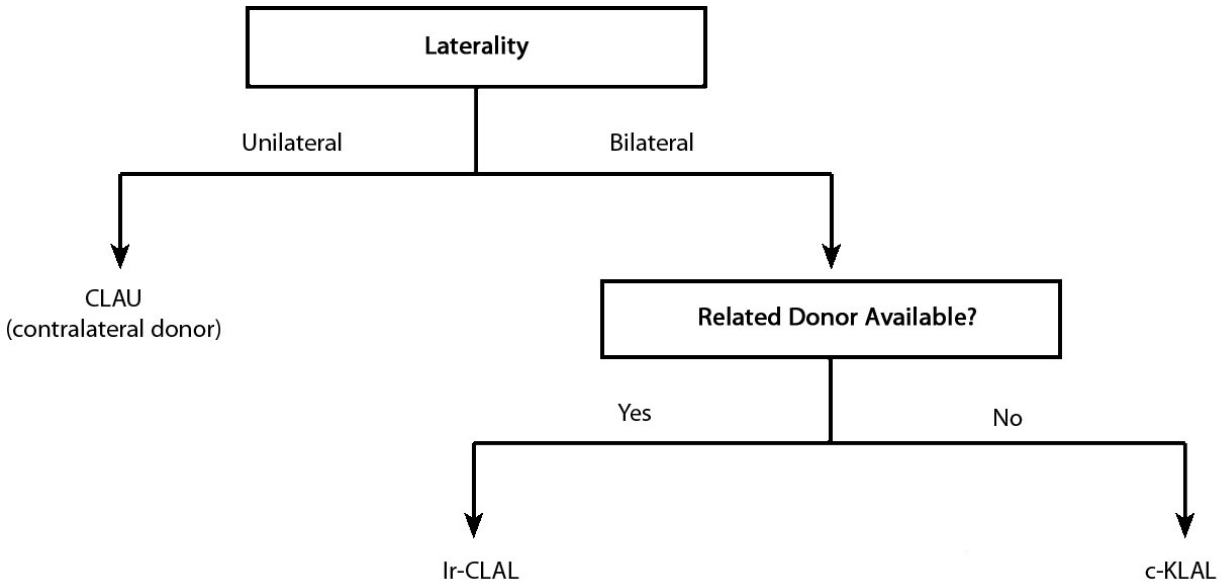


Figure 3: Surgical Treatment Options for Diffuse Limbal Stem Cell Deficiency.
CLAU – conjunctival-limbal autograft; Ir-CLAL – living-related conjunctival-limbal allograft; c-KLAL – cadaveric keratolimbal allograft. Adapted from Lee et al.[19]

Recently, limbal stem cell transplantation has included the use of cell culture techniques to expand limbal epithelial cell populations ex vivo. Using this technique, only a limited number of progenitor cells are required and this allows for harvesting of less donor tissue. Pellegrini et al. successfully used ex vivo expanded limbal autografts (EVELAU) to treat patients with LSCD.[23] Although conjunctival-limbal autograft transplantation is the treatment of choice for unilateral LSCD, EVELAU is an excellent second option if cell culture capabilities are available.

In 1995, Kim and Tseng promoted the use of amniotic membrane in combination with limbal stem cell transplantation.[24] The amniotic membrane consists of a single layer of

epithelium and an underlying stroma. It enhances corneal epithelial healing by reducing inflammation and scarring; it also provides a basement membrane that allows for the growth and proper differentiation of corneal epithelial cells.[24,25]

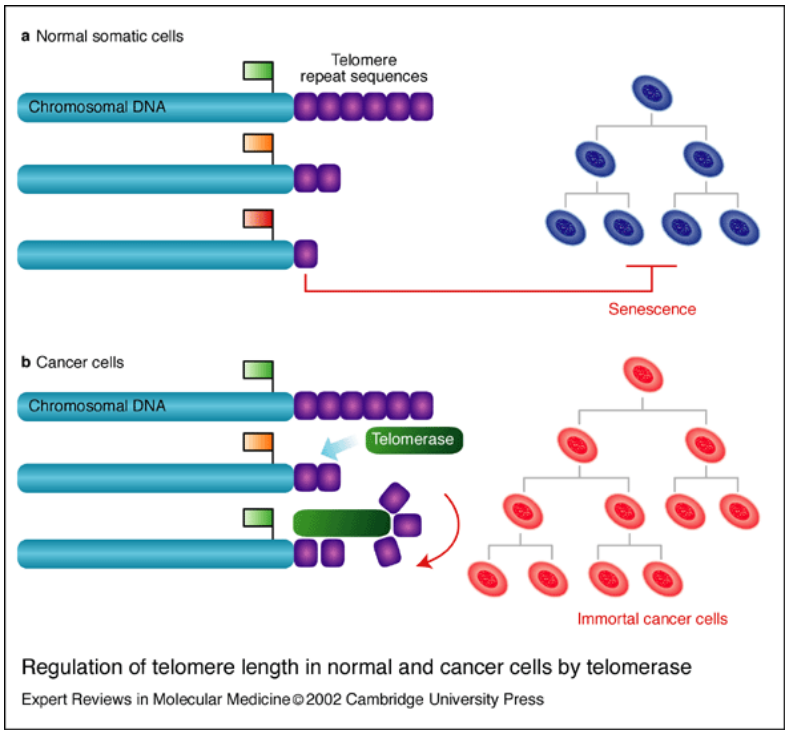
When LSCD is bilateral, allogenic transplantation is necessary. In this case, the donor tissue is harvested from a living relative or cadaver. It is preferred that the donor tissue comes from a relative that has similar human leukocyte antigen (HLA) typing. HLA are proteins on most cells of the body that the immune system recognizes as its own; the immune system attacks cells expressing different HLA.[26] Thus, during transplantation, donor tissue from the closest HLA match is preferred to reduce the risk of immune rejection. Since the donor tissue is not an exact HLA-match, life-long systemic immunosuppression is still required. Advances in immunosuppressive therapy have led to decreased graft loss in the early post-transplant phase; however, chronic rejection still remains an obstacle for long-term graft viability.[26] Even with immunosuppressive therapy, the rate of rejection when using cadaveric keratolimbal allografts has been reported to be as high as 72.7-76.7% after three years.[27,28] Due to the lower success rate of the allogenic transplants, alternatives are currently being explored. Some studies have worked with oral mucosal epithelial cells as a source for transplantation.[2,9] These cells would be autologous and there would be no risk of immune rejection, but unfortunately, these cells are morphologically and functionally distinct from corneal epithelium. The corneal epithelium is transparent partly due to the relative sparse accumulation of organelles within it; whereas, the oral mucosal cells are much thicker with an irregular surface.[9,29] More importantly, these cells do not transdifferentiate to corneal cells. Consequently, the oral mucosal epithelial cells may not be the answer to LSCD. Instead, the development of a tissue-engineered universal donor corneal

epithelial cell line may provide a potential therapeutic alternative for patients with bilateral LSCD.

CHAPTER TWO
The Study

BACKGROUND

The corneal epithelial cell line used in these studies was created using harvested epithelial cells from the limbal region of a 62-year old white male donor.[7] These cells were then immortalized by infection with human telomerase enzyme reverse transcriptase (hTERT), which contains telomerase. Telomerase is an enzyme that prevents the shortening of telomeres, which are short nucleotide repeats at the ends of chromosomes (Figure 4, pg.15).[7,30] Normal somatic cells do not contain telomerase and thus the telomeres progressively shorten after each mitotic division. Once a critical length threshold is reached, the shortened telomeres signal the cell to stop replication. Infection of a cell line with hTERT leads to activation of telomerase and prevents the shortening of telomeres and thus immortalizes the cell line.



Regulation of telomere length in normal and cancer cells by telomerase
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Figure 4: Regulation of telomere length in normal and immortalized cancer cells. Adapted from Keith et al.[30]

Once this hTERT-immortalized corneal epithelial cell line (hTCEpi) was established, studies performed in vitro confirmed that the cell line displayed cell cycle kinetics, differentiation, and gene expression profiles resembling normal human corneal epithelial cells.[7] In addition, these cells exhibited contact-inhibited growth. Based on this data, the hTCEpi cell line looked appealing as a source of transplantation in vivo. However, the application of this cell line for ocular surface reconstruction is limited due to the high levels of telomerase expressed by hTCEpi cells, creating a possible chance for oncogenic transformation.

As a fail-safe for unwanted proliferation, we used gene directed enzyme pro-drug therapy (GDEPT). GDEPT involves transferring a drug susceptibility gene into a cell and then applying that drug later to kill the cell.[31] There are various GDEPT systems, but the one we selected is the hygromycin phosphotransferase gene fused with the herpes simplex virus type 1 thymidine kinase gene (termed HyTK gene).[32,33] This system provides hygromycin resistance to all cells transduced with the viral vector containing the gene. Cells also have an active thymidine kinase that can phosphorylate and activate the drug, ganciclovir (Figure 5, pg.16).[32-34] Thus when ganciclovir is applied to these cells, it can cause cell death by inhibiting DNA replication. Several studies have shown that GDEPT using the HyTK gene/ganciclovir system induces cellular suicide in rapidly proliferating cells.[34] Using this strategy, hTCEpi cell line was transduced with a viral vector containing the HyTK gene giving rise to the HyTK cell line.

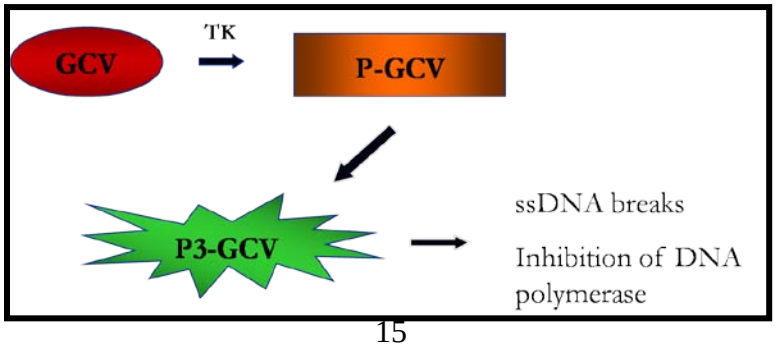

Figure 5: Activation of ganciclovir via GDEPT system.

PURPOSE

The purpose of this study was to determine the feasibility of using HyTK cells for the reconstruction of a multi-layered, differentiated corneal surface, and to evaluate the efficacy of ganciclovir in maintaining proliferative control over the HyTK cell line in vitro.

METHODS

Culture of HyTK Cells

The hTCEpi cells were routinely grown in Keratinocyte Growth Medium-2 (KGM-2) serum-free culture media (Lonza, Walkersville, MD). KGM-2 media also contained penicillin/ streptomycin/amphotericin B mixture and KGM-2 Single Quots Kit (Lonza, Walkersville, MD), which consisted of 2 mL of bovine pituitary extract, 0.5 mL gentamicin sulfate amphotericin B, 0.5 mL hydrocortisone, 0.5 mL epinephrine, 0.5 mL transferrin, 0.5 mL rhEGF, and 0.5 mL insulin per 500 mL of media. Cells were cultured on T175 cm^2 tissue culture flasks (Thermo Fisher Scientific, Rochester, NY), incubated at 37°C in 5% CO_2, and passaged every 5 to 7 days. The HyTK cell line was established by transduction of the hTCEpi cell line with a retroviral vector encoding the fusion gene of hygromycin phosphotransferase and the herpes simplex virus type 1 thymidine kinase under hygromycin selection – media containing 0.1896 mM hygromycin (EMD Biosciences, Inc., San Diego, CA).

CMFDA (5-chloromethylfluorescein diacetate) Staining

To establish the efficacy of HyTK cell adherence to the denuded mouse cornea, HyTK cells were stained with 25 µg/mL Cell Tracker Green CMFDA (Molecular Probes, Eugene, OR) for 45 minutes. Cells were washed twice with media and suspended in 100 µL of KGM-2 media prior to transplantation.

LSCD Mouse Model

BALB/cAnNCr-nu/nu female mice aged 6 weeks (Charles River Laboratories International, Inc., Voluntown, CT) were used as transplant recipients. This particular mouse

strain, which is incapable of T-cell mediated immune rejection, was used to avoid the complications of immune rejection at this time. All experiments received approval from the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas, Texas, USA. Experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmologic and Vision Research. Mice were anesthetized with an intraperitoneal injection of ketamine hydrochloride (Butler Animal Health Supply, Dublin, OH) and xylazine hydrochloride (Anased, Shenandoah, IA) at concentrations of 100 mg/ kg of body weight and 10 mg/ kg of body weight, respectively. Initially, a single drop of 0.5% proparacaine hydrochloride (Bausch & Lomb, Rochester, NY) was topically applied to each mouse eye and a sterile plastic tube was fitted around each eye (Figure 6, pg.19). Each plastic tube was then filled with 20 µL of 0.5 M EDTA. After 20 minutes, the plastic tubes were removed and each mouse cornea was scraped with a cellulose surgical spear (Braintree Scientific, Inc., Braintree, MA). Mechanical debridement was directly visualized with a dissecting microscope. Afterwards, 0.25% mitomycin C (Sigma, St. Louis, MO) was applied to each cornea for 20 minutes; at this concentration, mitomycin C is cytotoxic and inhibits proliferation of any remaining cells. Thus, residual epithelium was easily removed using a cellulose spear. Following corneal epithelial removal, each cornea was thoroughly rinsed with phosphate-buffered saline (PBS).

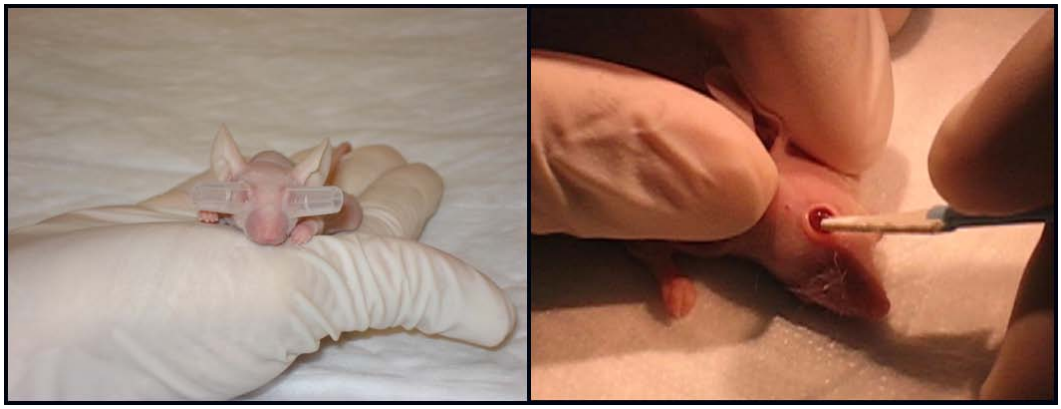

Figure 6: (Left) Plastic tubes fitted on mouse eyes prior to epithelial removal.
(Right) Mechanical debridement of corneal epithelium with cellulose surgical spear.

Transplantation of HyTK Cells

After 48 hours, the right cornea was once again fitted with a plastic tube and a suspension of HyTK cells (5-8 X 10^6 cells/ 200 µL) in KGM-2 media were put into the tube and allowed to adhere for 1 hour. The plastic tube was then removed and the eye was closed using a single suture. The left denuded eye was not transplanted and served as a control.

Tissue Harvesting

Mouse corneal tissue was harvested at 4 hours, 24 hours, and 7 days post-transplantation. Prior to tissue harvesting, mice were anesthetized as mentioned previously and euthanized by cervical dislocation. Entire mouse globes were fixed with 1% paraformaldehye (Electron Microscopy Sciences, Hatfield, PA), enucleated, and chilled at 4°C for 5 minutes. Afterwards, the globes were placed in plastic molds containing embedding medium (Jung, Nussloch, Germany), snap frozen in liquid nitrogen, and stored at -80°C.

Immunohistochemistry

Frozen corneal tissue was sectioned (16µm thick) using the Leica Cryostat (Leica Microsystems, Wetzlar, Germany), and placed onto 4% gelatin-coated slides (Thermo Fisher Scientific, Waltham, MA). All sections were permeabilized with acetone for 5 minutes, washed three times with PBS, and blocked with 10% donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 30 minutes at 37°C in a humid chamber prior to antibody application.

To evaluate for the presence of basement membrane following epithelial removal, slides were incubated in 25 µg/mL rabbit polyclonal anti-laminin (Sigma-Aldrich, St. Louis, MO) in

PBS for one hour at room temperature. To assess the adherence of transplantation, mouse corneal tissue transplanted with HyTK cells labeled with CMFDA prior to transplant were evaluated at 4 hours and 1 day post-transplantation.

Mouse corneal tissues were also evaluated at day 7 post-transplantation for stratification and a differentiated corneal epithelial phenotype. To detect f-actin and stratification, slides were incubated in FITC-conjugated Alexa Fluor 488 Phalloidin (Molecular Probes, Eugene, OR) in PBS for 2 hours at 37°C in a humid chamber. To assess for keratin 3, tissues were incubated in 2.5 µg/mL mouse monoclonal anti-human keratin 3 (Serotec Ltd., Kidlington, Oxford, UK) in PBS overnight at 4°C. To determine the proportion of proliferating cells, tissues were incubated in 1 µg/mL mouse monoclonal anti-Ki-67 (DBS, Pleasanton, CA) in PBS overnight at 4°C.

Following primary antibody staining, FITC-conjugated or rhodamine-conjugated antibody fragments (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) were used as secondary antibodies in a concentration of 28 µg/mL in PBS for 1 hour at 37°C. All specimens were counterstained with either Propidium iodide (Sigma-Aldrich, St. Louis, MO), or DRAQ 5 (Alexis Biochemicals, San Diego, CA) to label epithelial nuclei. Following a washing cycle with PBS, the slides were mounted in glycerol, and visualized using a Leica SP2 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

Ganciclovir Cytotoxicity Assay

To measure the cytotoxic effects of ganciclovir in vitro, HyTK cells suspended in KGM-2 medium with hygromycin B were plated at a density of 5 $X10^5$ cells/ cm^2 on collagen-coated glass coverslips (Thermo Fisher Scientific, Waltham, MA) in a 24-well culture plate (Thermo Fisher Scientific, Waltham, MA); the remaining wells were plated with hTCEpi control cell line suspended in KGM-2 medium. After allowing 24 hours for adherence, ganciclovir was applied

to wells at varying concentrations of 0.1, 0.5, 1.0, 5.0, and 10.0 μM. Following 72-hour treatment with ganciclovir, coverslips were stained using Live-Dead Viability/ Cytotoxicity kit for mammalian cells according to manufacturer instructions (Molecular Probes, Eugene, OR). For optimal staining conditions, 4 mM calcein AM and 2 mM ethidium homodimer-1 were diluted to 2 μM and 4 μM with PBS, respectively; these reagents were combined to form a 10 mL total working solution. After washing coverslips twice with PBS, the working solution was placed to cover each coverslip and allowed to incubate for 45 minutes at room temperature. Following another wash cycle with PBS, coverslips were mounted on slides using glycerol. The slides were visualized using a Leitz Diaplan Fluorescent microscope. Live cells absorb the relatively non-fluorescent cell-permeant calcein AM and use ubiquitous intracellular esterase to convert it to fluorescent green calcein. Dead cells with damaged membranes allow ethidium homodimer-1 to enter; once inside, it binds to nucleic acids, which causes a bright red fluorescence. For each ganciclovir concentration, both HyTK and hTCEpi cells were counted as alive or dead using five respective visual fields for data collecting. This experiment was replicated three times.

Statistical Analysis of Ganciclovir Cytotoxicity Assays

Data from ganciclovir cytotoxicity assay was statistically analyzed using a two-way ANOVA test to compare the percentage of live cells at each ganciclovir concentration tested for both cell lines. Following this, a Holm-Sidak multiple comparison test was used to make comparisons between the HyTK and hTCEpi cell lines at the varying concentrations of ganciclovir.

RESULTS

The corneal epithelium was successfully removed using 0.5 M EDTA (Table 2, pg. 23). After epithelial debridement, almost all epithelial cells were removed from the corneal surface when compared to the normal cornea; this was visualized with a dissecting microscope.

Date	Number of Mice Used	Method of Epithelial Removal	Epithelium Removed
9/24/2006	4	2.5 mM EDTA	No
10/3/2006	3	2.5 mM EDTA	No
10/10/2006	2	2.5 mM EDTA	No
10/10/2006	2	0.25 M EDTA	No
10/17/2006	2	0.25 M EDTA	No
10/18/2006	2	0.5 M EDTA	Yes
11/1/2006	1	0.5 M EDTA	Yes
11/2/2006	1	0.5 M EDTA	Yes

Table 2: Debridement of Native Mouse Corneal Epithelium. The concentration of 0.5 M EDTA was determined to successfully remove corneal epithelial cells. The epithelium removal was visualized with a dissecting microscope.

Epithelial regeneration from any residual cells was inhibited by application of 0.25% Mitomycin C (Table 3, pg. 23). Seven days after Mitomycin C treatment, mouse tissue sections were visualized using LSCM; corneal epithelial cells were not visualized at this time point indicating that no re-growth of corneal epithelium occurred. Thus, a successful LSCD mouse model was created using both 0.5 M EDTA and 0.25% Mitomycin C.

Date	Number of Mice Used	Mitomycin C Concentration (%)	Epithelial Re-growth
11/7/2006	2	0.02	Yes
11/21/2006	1	0.02	Yes
11/21/2006	1	0.1	Yes
11/29/2006	4	0.1	Yes
12/18/2006	1	0.25	No
12/18/2006	1	0.25	No
12/27/2006	1	0.25	No

Table 3: Prevention of Native Mouse Corneal Epithelium Regeneration. Epithelial re-growth defined as visualizing corneal epithelial cells on mouse tissue sections using LSCM. The concentration of 0.25% Mitomycin C was found to prevent epithelial re-growth.

Labeling for laminin was performed in order to assess the integrity of the basement membrane following epithelial removal.[35] Immunofluorescence with anti-laminin on cornea cross-sections showed staining throughout the entire corneal surface (Figure 7, pg.24).

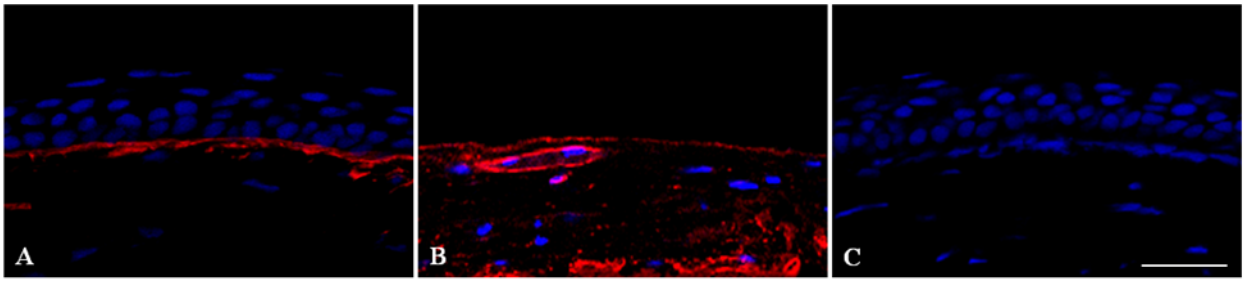

Figure 7: Double-labeling with Laminin (red) and DRAQ5 (blue). **A:** Normal mouse cornea. **B:** After epithelial removal, laminin staining confirms the presence of an intact basement membrane. **C:** Negative control, 1º Laminin Antibody omitted. (20X) Scale 29.93µm.

After corneal epithelial removal, HyTK cells successfully adhered to the mouse cornea within four hours as visualized by LSCM. Transplanted mouse corneas were imaged at 4 hours and 1 day post-transplantation; the presence of CMFDA staining confirmed the attachment of HyTK cells to the corneal surface (Figure 8, pg. 25). HyTK cells were dispersed in a relatively uniform fashion. As cells continued to grow on the mouse corneal surface, they began to flatten out.

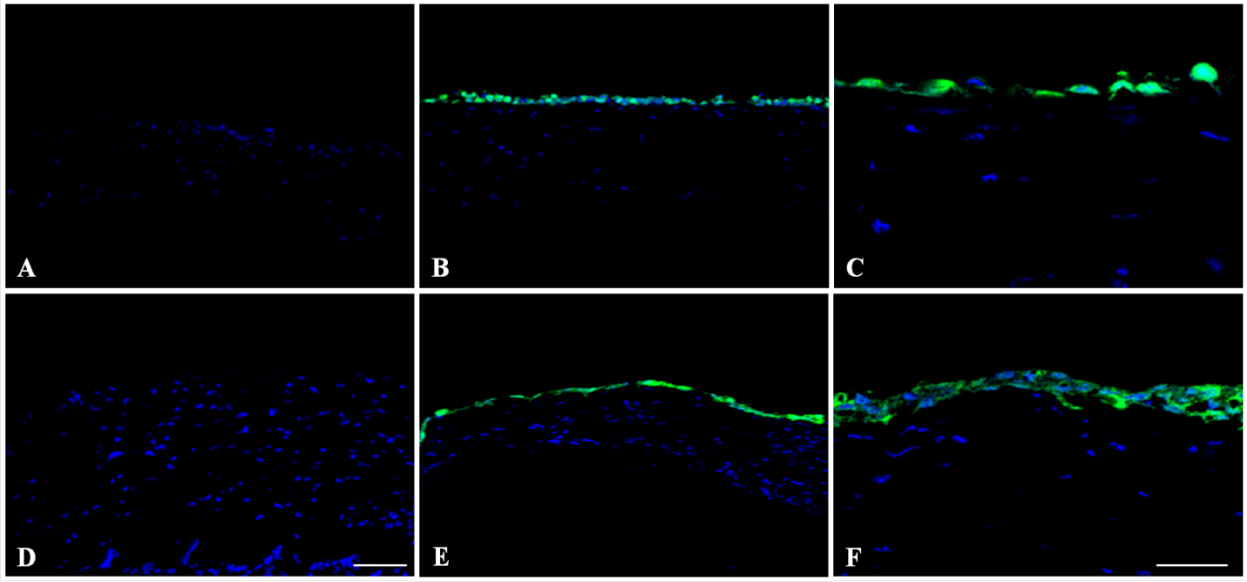

Figure 8: CMFDA staining (green) and DRAQ5 (blue). **A,B,C:** 4 hours post transplantation. **A:** Denuded cornea without transplanted cells (20X). **B:** The presence of CMFDA staining (green) indicates that HyTK cells adhered to the denuded mouse cornea (20X). 8 million cells were used for transplantation. Scale: 80.00 μm. **C:** HyTK cells adhered to the denuded mouse cornea (63X). Scale: 40.00 μm.
D,E,F: 24 hours post transplantation. **D:** Denuded cornea without transplanted cells (20X).
E: The presence of CMFDA staining (green) indicates that HyTK cells adhered to the denuded mouse cornea (20X). Scale: 80.00 μm. **F:** HyTK cells adhered to the denuded mouse cornea (63X). Scale: 40.00 μm.

HyTK cell transplantation experiments were replicated several times (Table 4, pg. 26). A successful transplantation was defined as when at least one layer of corneal epithelium was visualized with LSCM; this occurred regularly when using 5-8 million cells.

Date	Number of Mice Used	Number of Cells Transplanted (millions)	Successful Transplantation
1/8/2007	1	1	No
1/8/2007	1	1	No
1/16/2007	1	2	No
2/12/2007	1	2	No
2/21/2007	1	4	No
3/9/2007	1	8	Yes
3/28/2007	1	8	No
4/3/2007	2	14	Yes
4/20/2007	1	25	Yes
6/1/2007	1	8	Yes
6/7/2007	1	6	Yes
6/12/2007	1	5	Yes
6/21/2007	2	10	Yes
6/26/2007	1	7	Yes

Table 4: Transplantation of HyTK cells onto denuded mouse cornea. Successful transplantation was defined as visualization of at least one layer of corneal epithelial cells using LSCM; this was observed when 5-8 million cells were used.

Seven days post transplantation, cross-sections of mouse corneas were imaged to assess for stratification via immunofluorescence (IF) with phalloidin-FITC and PI. By 7 days, the transplanted HyTK cells had stratified into a multi-layered epithelium in most samples (Figure 9, pg. 27). The multilayered epithelium consisted of three to four cell layers including a cuboidal basal layer, one to two polygonal wing cell layers, and one squamous cell layer. Histologically, the total reconstructed corneal surface morphologically resembled native cornea. Control eyes (left eyes) showed no signs of re-epithelialization (Figure 9, pg. 27).

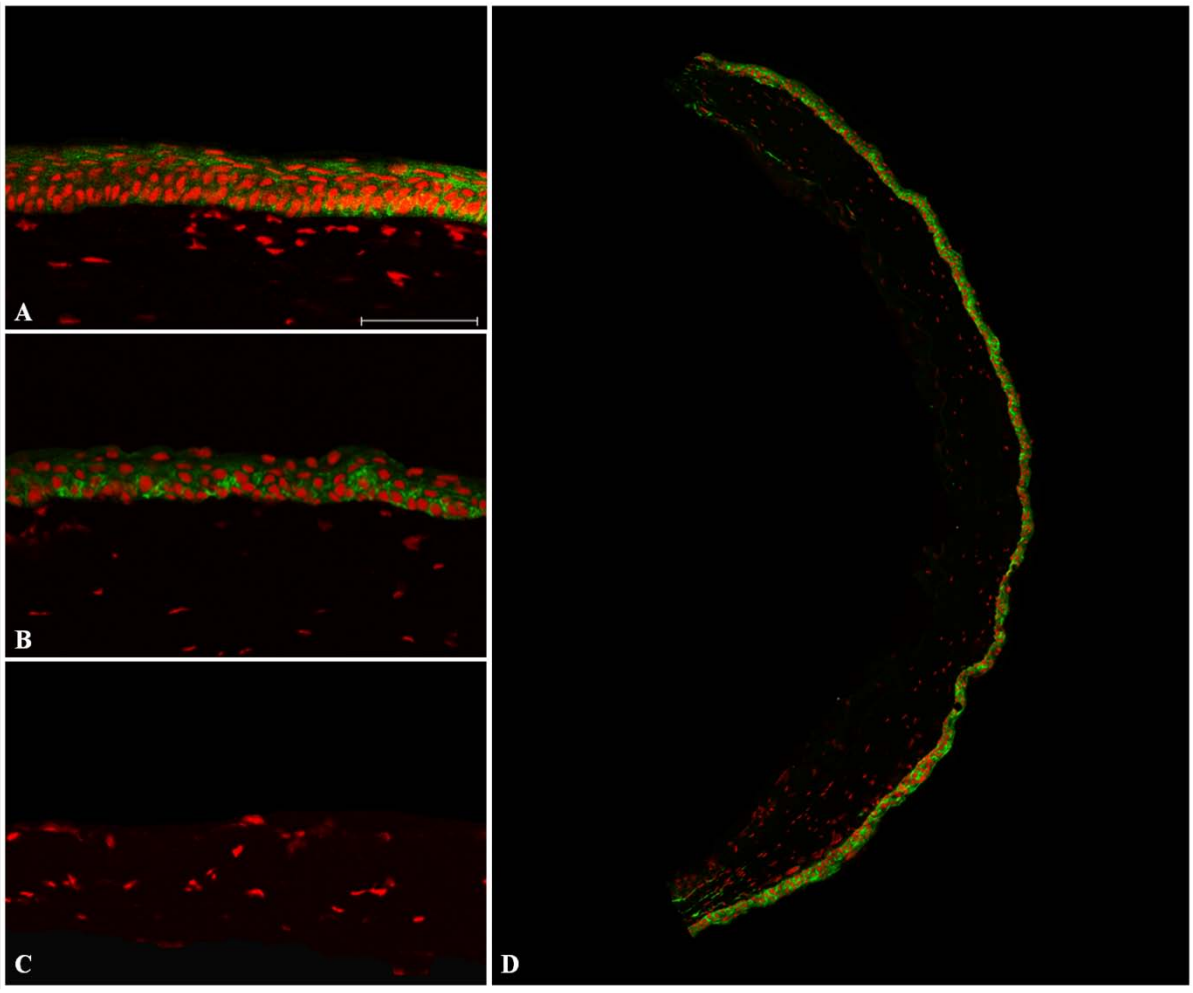

Figure 9: Cryosections Double-labeled with FITC-conjugated Phalloidin (green) and PI (red). **A:** Normal mouse cornea. **B:** HyTK cells grown for 7 days on denuded mouse cornea.

C: Denuded mouse cornea without transplanted cells. Scale: 74.82µm. (63X)
D: Montage of reconstituted corneal epithelium. (20X)

Following the successful transplantation of HyTK cells, mice were followed over seven days and assessed for multi-layering and stratification (Table 5, pg. 28).

Date	Number of Mice Used	Transplantation Successful
7/5/2007	2	Yes
7/10/2007	1	Yes
7/12/2007	1	No
7/18/2007	2	Yes
7/26/2007	2	Yes
7/27/2007	1	Yes
8/4/2007	2	Yes
8/5/2007	2	Yes
8/11/2007	1	No
8/19/2007	3	Yes
11/9/2007	1	Yes
1/29/2008	2	Yes
2/20/2008	2	Yes
2/26/2008	1	No
2/27/2008	1	Yes
3/2/2008	2	Yes
3/9/2008	1	Yes
3/16/2008	1	Yes
3/23/2008	1	Yes

Table 5: Seven-day transplantation experiments with HyTK Cells. Successful transplantation was defined as visualization of at least one layer of corneal epithelial cells using LSCM.

At the 7-day time point, IF with anti-keratin 3, the accepted cornea specific differentiation marker, identified the presence of keratin 3 on corneal epithelial cross-sections (Figure 10, pg. 29). Keratin 3 expression localized to the cytoplasm of basal, wing, and squamous cells suggestive of a normal differentiated phenotype.

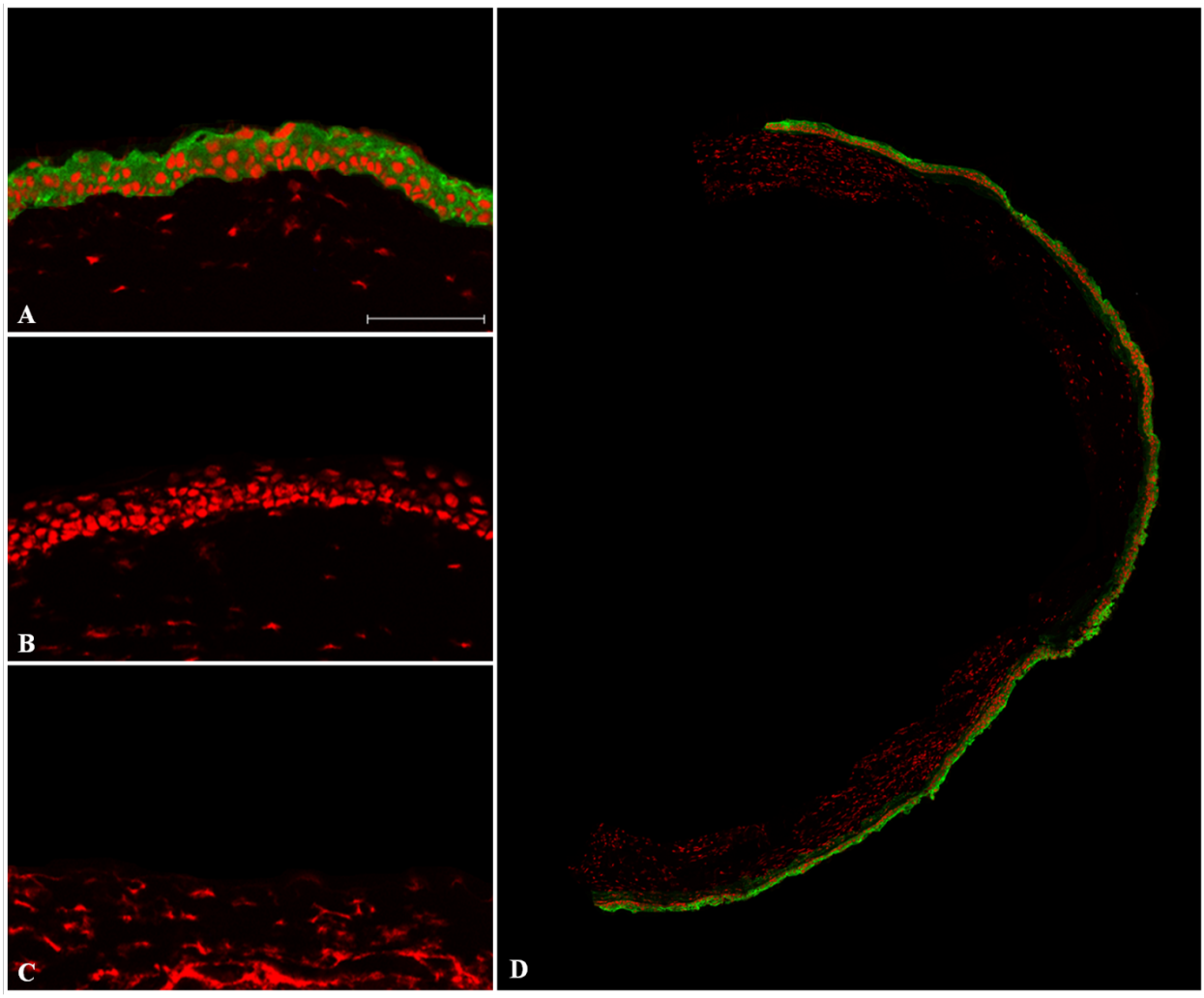

Figure 10: Double-labeling with K3 (green) and PI (red). **A:** HyTK cells grown for 7 days. K3 localized to the cytoplasm of all basal and suprabasal epithelial cells.
B: Negative control, 1°Antibody omitted.
C: Denuded mouse cornea without transplanted cells. Scale: 74.82µm. (63X)
D: Montage of reconstituted corneal epithelium. (20X)

To evaluate cellular proliferation of transplanted HyTK cells growing on mouse cornea for 7 days, cryo-sections were labeled with Ki-67 and DRAQ5 (Figure 11, pg. 30). The normal corneal epithelium shows Ki-67 in the basal cell layer.[36] Preliminary imaging shows Ki-67 predominantly in the basal layer of the reconstituted epithelium. Further studies are planned to confirm these findings; this suggests that the HyTK cells are exhibiting a growth down-regulated phenotype similar to normal human cornea.

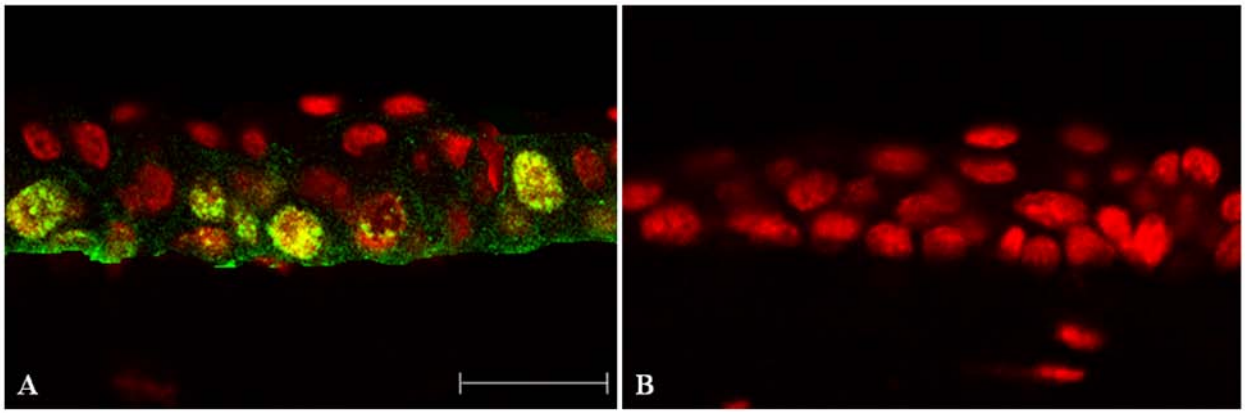

Figure 11: HyTK cells grown for 7 days that are double-labeled with Ki-67 (green) and DRAQ5 (red). **A:** Ki-67 localized primarily to HyTK cells in the basal layer and was absent in suprabasal layers. **B:** Negative control, 1°Antibody omitted. (63X) Scale: 23.8μm.

To assess the cytotoxic effects of ganciclovir on HyTK cells in vitro, Live/dead assay was used to determine cell death after ganciclovir treatment. Statistical analysis showed that ganciclovir treatment induced a cytotoxic effect on HyTK cells in vitro and had no significant effect on the hTCEpi control cell line (Figure 12, pg. 31). The cytotoxic effect was significant at 0.5µM with $p < 0.05$. Further, the potency of the cytotoxic effect increased as the concentration of ganciclovir used increased.

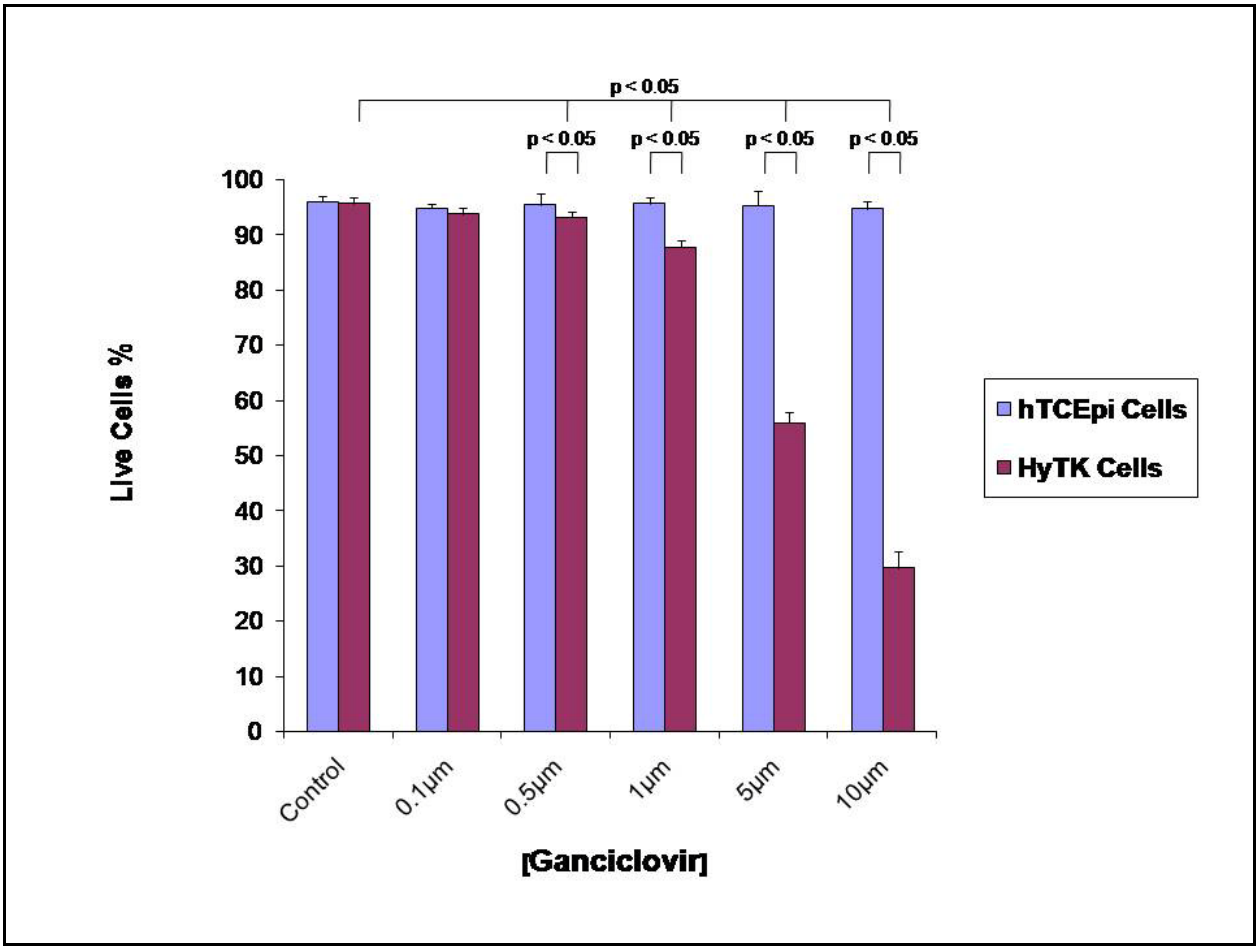

Figure 12: Percentage of Calcein positive (live cells) assessed by Live Dead Assay following treatment with ganciclovir. Significant cell death was seen in HyTK cells at concentrations beginning at 0.5 µM ($p < 0.05$, Two-way ANOVA; results of Holm-Sidak multiple comparison test shown on graph.

DISCUSSION

These studies demonstrated the development of an animal model for limbal stem cell deficiency. Further, these data show for the first time, the successful reconstitution of a multi-layered, differentiated corneal epithelium by HyTK cells in a murine model. HyTK cells were chosen as the source for ocular surface reconstruction for a number of reasons. The HyTK cell line was developed from the hTCEpi cell line, which has already been shown to be a genomically stable corneal epithelial cell line that differentiates normally in vitro.[7] The hTCEpi cell line displays normal 24-hour population doubling-time. Further, an air-lifting technique was used to study the cell line's behavior at the air-liquid interface, which resembles its biological niche within the eye. This method made use of organotypic constructs, which allowed for the basal side of the cell layer to be submerged in liquid, while the apical side was exposed to air. After use of the air-lifting technique, hTCEpi cells stratified into a multilayered epithelium with normal expression of actin cytoskeleton and ZO-1, an apical tight junction protein.[7] In addition, there was a high expression of K5/K14 (stratification markers) in the submerged culture, which is consistent with the upregulation of K5/K14 seen during in vivo corneal epithelial regeneration in rabbits.[7,37] After air-lifting, the K5/K14 expression tapers and K3, a cornea differentiation marker, is upregulated; this finding is to be expected since the cells begin to differentiate after air-lifting.[7] These in-vitro studies encouraged us to use the HyTK cell line for ocular surface reconstruction.

Another advantage the HyTK cell line holds is that it is an immortalized cell line, as defined by more than 100 population doublings, and has an essentially unlimited supply. Its ability to proliferate indefinitely in culture is very useful when studying molecular pathways in

the in-vitro setting. In addition, with the scarcity of donor tissue for the treatment of LSCD, a potential alternative supply is high in demand.[28,38] These characteristics make this cell line an ideal candidate in ocular surface reconstruction.

To create the LSCD mouse model, the native mouse corneal epithelium and limbal stem cells of the ocular surface needed to be eliminated. Several different methods of epithelial removal were tested. Initially, application of n-heptanol to the mouse eye was used. This method was previously used by Homma et al. In their experiments, a 2-mm diameter paper wiper soaked in n-heptanol was placed on the cornea for 1 minute and successfully injured the corneal epithelium.[11] This method failed to completely remove the corneal epithelium in our experiments. Epithelial debridement was also attempted by the use of a gelatin-coated slide as previously described by Zhao and Nagasaki.[39] In their experiments, the epithelium was removed by repeated touching of the eye with a dried gelatin-coated slide.[39,40] This method was unsuccessful in our experiments as well. We found EDTA, often used in treating calcific band keratopathy, to be the most effective in softenening the epithelium for removal with a cellulose surgical spear.[41] Once the epithelium was successfully removed, we needed to be certain that no epithelial regeneration takes place. Mitomycin C, an alkylating agent that inhibits protein synthesis and DNA/RNA replication and thus inhibits cell proliferation was used for this step.[42] Clinically, Mitomycin C (at a concentration of 0.02%) has been used in photorefractive keratectomy (PRK) due to its ability to block myofibroblast formation and thus prevent a corneal haze that some patients experience after PRK.[42,43] Initially, a concentration of 0.02% Mitomycin C was used to inhibit corneal epithelial regeneration; however, corneal epithelial re-growth was seen. Fleuckiger et al. observed that Mitomycin C at a concentration of 0.1% had a toxic effect

on porcine eyes.[44] Consequently, we increased the concentration of Mitomycin C and found that 0.25% completely prevented corneal epithelial regeneration.

In creating the LSCD mouse model, it was a priority to have an intact basement membrane remaining after epithelial debridement, as a roughened stromal surface would hinder attachment of the overlying epithelia. The corneal epithelium is normally anchored to the basement membrane through hemidesmososmes; and thus, an intact basement membrane is a necessity for HyTK cell attachment to the ocular surface.[45] Laminin IF staining confirmed an intact basement membrane following epithelial debridement.

The application of a 5-8 million HyTK cell suspension to the denuded mouse cornea was successful; the CMFDA staining seen on the transplanted corneas confirmed that HyTK cells were adherent. Nearly 75% of the corneal surface was covered by the transplanted HyTK cells. Following transplantation, the HyTK cells appeared to multi-layer and differentiate in vivo, as measured by phalloidin and K3, similar to our previously reported in vitro studies.[7] The localization of Ki-67 to HyTK cells in the basal layer and its absence in the suprabasal cell layers further suggests a growth down-regulated phenotype.

Additional studies evaluating cornea-specific epithelial differentiation markers are in progress to assess and validate the use of the HyTK cell line in vivo. Keratin-12, a recognized cornea-specific epithelial differentiation marker, is of particular interest.[46] Membrane proteins such as ZO-1, an apical tight junction protein normally seen in the corneal epithelium will also be evaluated.[7] In addition, studies assessing the longevity of the transplanted HyTK cells are also in progress; these studies will assess transplanted HyTK cells at later time points including: 2 and 4 weeks post-transplantation.

Due to the high levels of telomerase in the hTCEpi cell line, we implemented GDEPT as a potential safeguard to control for unwanted proliferation. Under non-differentiated conditions in vitro, topical ganciclovir effectively killed almost 70% of proliferating HyTK cells while having no effect on the viability of hTCEpi cells. It still remains to be tested in vivo; however, the use of the HyTK/ganciclovir system looks promising in eliminating any potential risk of oncogenic transformation.

In order to extend the HyTK cell line to the human ocular surface in vivo, further studies evaluating critical immune pathways are needed. With an immunosuppressed murine model in place, a transition to an immunocompetent model can be made to explore the immune rejection process further. The incorporation of contemporary gene therapy techniques may provide a useful strategy to dampen the rejection process at the cellular level. Specifically, class two transcriptional activator (CIITA), IL-1, and IL-10 will all be explored as potential targets for genetic manipulation. CIITA is a gene that encodes for class II major histocompatibility complex transactivator.[47] It is important in the activation of $CD4^+$ T cells. Consequently, its deficiency causes the bare lymphocyte syndrome, which leads to severe immunodeficiency and recurrent infections.[48] Thus, CIITA gene modification may provide an avenue to dampen the rejection process as part of a multi-strategy approach. IL-1 and IL-10 are pro-inflammatory and anti-inflammatory cytokines respectively. They can play an important role in the rejection process as well and so alteration in their expression levels may provide useful with immunosuppression.

CHAPTER THREE
CONCLUSION

In conclusion, a mouse model of LSCD was created onto which HyTK cells were successfully grafted demonstrating the successful reconstitution of a multi-layered, differentiated corneal epithelium by HyTK cells in a nude mouse model. Following transplantation, the HyTK cells appear to stratify and differentiate in vivo similar to native tissue. In order to extend the HyTK cell line to the human ocular surface in vivo, further studies evaluating critical immune pathways, additional corneal phenotypic markers, and longevity of HyTK transplantation are needed. Our findings do suggest that the HyTK cell line looks promising as a model for a universal donor artificial corneal epithelium.

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VITAE

Jerry P. Kalangara was born in Dallas, Texas on July 6, 1982 to George and Licy Kalangara. He grew up in Carrollton and graduated from Jesuit College Preparatory School as Salutatorian in 2000. He attended Austin College in Sherman, Texas for his undergraduate studies. At Austin College, he was selected as a Presidential Scholar and received multiple achievements including M.D. Bryant Fellowship for Excellence in Biology, Phi Beta Kappa Society, and Alpha Chi National Honor Scholastic Society. Jerry graduated *summa cum laude* from Austin College with a Bachelor of Arts degree in biology in 2004.

Jerry matriculated at the University of Texas Southwestern Medical School in 2004. Following his second year of medical school, Jerry participated in a pilot project with Dr. H. Dwight Cavanagh and Dr. Danielle Robertson involving the transplantation of a tissue-engineered corneal cell line. At the conclusion of his project, Jerry's work resulted in poster presentations at the 2008 Association for Research in Vision and Ophthalmology Conference and the 2008 American Academy of Optometry Conference. He was also selected to give an oral presentation at the Medical Student Research Forum at the University of Texas Southwestern Medical School in 2009. After graduation from medical school, Jerry plans to pursue residency training in Anesthesiology.