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HGF-MEDIATED C-MET SIGNALING IN HUMAN CORNEAL EPITHELIAL CELLS

By

Kate Elise Tarvestad
B.S. University of Notre Dame, 2020

A Thesis
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A Thesis Approved on

August 11, 2022

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DEDICATION

This thesis is dedicated to my late grandmother, Dr. Margery Neely, a clever, progressive, and witty woman. I know she would be proud of the scientist I am today.

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I would like to thank my mentor, Dr. Brian Ceresa, for his guidance and patience while navigating me through my training. I would also like to thank my other committee members, Dr. Leah Siskind, Dr. Geoff Clark, Dr. Jeff Petruska, and Dr. Patrick Scott for their comments, assistance, and mentoring. I would like to express my thanks to my fiancé, Billy, for his constant understanding and encouragement during stressful times. I would also like to thank my parents, Jill and Larry Tarvestad, for their endless support and cheering me on every step of the process. Lastly, I would like to thank my brothers Luke (his wife, Olivia) and Will Tarvestad.

ABSTRACT

HGF-MEDIATED C-MET SIGNALING IN HUMAN CORNEAL EPITHELIAL CELLS

Kate E. Tarvestad

August 11, 2022

Vision is often regarded as the primary sense of humans. The cornea is the main refractive tissue that permits light through to the retina, allowing a clear image. When the cornea sustains damage, it opens a pathway for infection and blindness through fibrotic processes. Healing the corneal tissue is critical for vision restoration and pain alleviation. Growth factors and their cognate receptors are currently under investigation as tools to restore proper corneal physiology. We hypothesize that manipulating the hepatocyte growth factor (HGF)/ c-Met signaling pathway is one route to promote quality corneal healing. This thesis investigates the signaling, trafficking, and degradation pathway of c-Met following HGF stimulation in corneal epithelial cells. Results indicate that regulating the Cbl proteins, E3 ligases that tag c-Met for degradation, can extend receptor signaling and accelerate re-epithelialization.

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INTRODUCTION TO THE CORNEA AND REPAIR

1. Introduction

Vision is the most important and primary sense of humans. Almost 100 years ago, psychologists noted that our memory and perception are based on visuals, and most research done in cognitive psychology is collected through visual tests. With memory and perception, other senses play a minor role: a study whereby volunteers were presented simultaneously with both an auditory and a visual stimulus showed that people preferentially respond to visual stimuli, with some people reporting to not have heard the auditory stimulus (1-3). The visual cortex, the area of the brain dedicated to processing visual stimuli, is larger than those associated with the other senses, such that almost the entire caudal region of the brain is dedicated to the processing of visual information (4). 40% of all nerve fibers that connect to the brain are linked from the retina, a neuronal covering of the back interior of the eye responsible for converting light into a neural signal [(5), (Figure 1)].

There are at least 2.2 billion individuals worldwide who have impaired vision. The leading causes of vision impairment are uncorrected refractive errors (astigmatism, near and far sightedness), cataracts, glaucoma, retinopathy, and corneal opacities. Of the 2.2 billion, 1 billion of those cases were preventable or

has not yet been treated. The incidence of vision impairment is associated with socio-economic status and income: the prevalence of impaired distance and near vision are estimated to be 4 times and 8 times higher, respectively, in lower income regions than higher income regions (6,7). According to the Center for Disease Control (CDC), vision loss and blindness costs the United States $51.4 billion annually. Most of the individuals impacted by vision loss are working adults over 40, so this statistic rings true: many diseases that impact vision arise with age, like cataracts and age-related macular degeneration (6). Unfortunately, young children can be impacted by degenerative or infectious diseases that may be detrimental to ocular development. As for the aging population, it is only expected that these statistics will worsen.

Corneal blindness is the 4th leading cause of vision loss worldwide, impacting millions of people around the globe. Its etiology ranges from lack of proper nutrition to infection and it can impact individuals of all ages. Unlike diseases such as glaucoma and macular degeneration, corneal blindness is often avoidable and preventable with the proper healthcare (7). Despite these staggering statistics and the widespread impact, there are no FDA-approved drugs that aid in healing and promoting homeostasis of the cornea.

1.2 Introduction to the cornea and associated pathology

The eye is divided into an anterior segment, which is made of the cornea, lens, iris, pupil, aqueous compartment, conjunctiva, and anterior sclera along with oil- and tear-producing glands, and a posterior segment made of the retina, choroid, vitreous humor, posterior sclera and optic nerve (Figures 1 and 3).

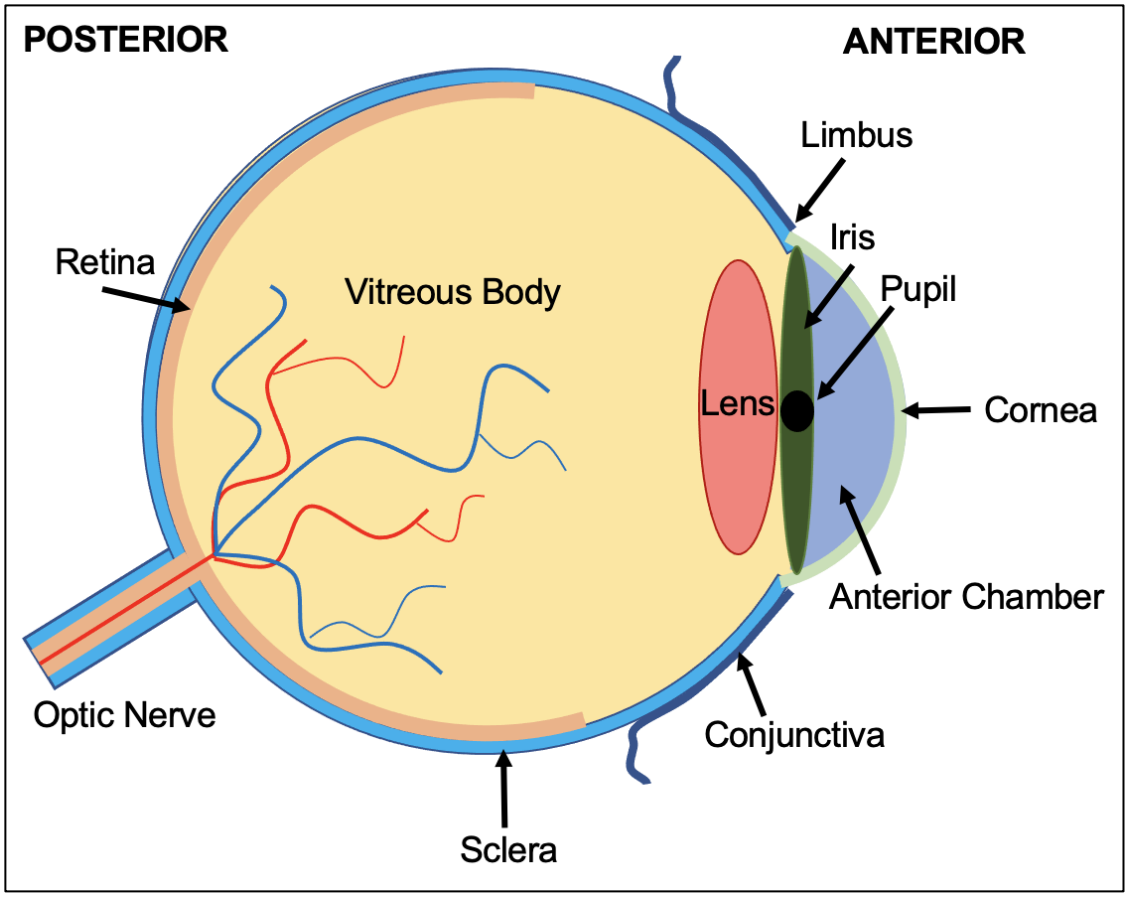

Figure 1. <u>Simplified anatomy of the eye.</u> Light enters through the anterior eye and refracts onto the retina in the posterior eye, which is signals directly to the brain via the optic nerve. Clear vision requires a transparent and healthy cornea to allow passage of light.

The cornea is the most anterior portion of the eye. It is made up of 3 cellular layers (endothelial, stromal, epithelial) and 2 interfaces (Bowman's Layer, Descemet's Membrane) (8). The average thickness of a healthy cornea is about 0.523 mm at the center and 0.660 mm at the periphery (9). Some anterior eye structures, including the cornea, are unique in that they are devoid of vessels to maintain tissue transparency for the passage of light. The lack of lymphatic vessels contributes to the eye's "immune privileged" status (10). Immune privilege was first defined as the ability to graft foreign tissue into an organ and it survive for extended periods of time without evoking an inflammatory response (11). This ability is present in the eye to hinder detrimental inflammation that may impair vision. However, due to this status, invading pathogens that enter through corneal wounds can rapidly lead to infection (12). In addition, trauma to the cornea can lead to fibrosis and opacification to blindness if not properly healed (13,14). Access to healthcare facilities has afforded those with corneal problems the opportunity for treatment, while those who live in areas with less access to clinical centers are less fortunate. For example, the incidence of cornea-related vision loss in some areas of Africa and Asia are 20 times higher than that in developed countries (15,16). Corneal disease often leads to opacification that can result in permanent vision loss without proper treatment.

2. Corneal Anatomy

2.1 Epithelium

The most anterior layer of the cornea is the epithelium. It consists of 5-7 layers of epithelial cells that vary in morphology from anterior to posterior (8). Cells in the

epithelium are in constant motion from the basement membrane to the surface due to the rapid nature of their turnover: it takes about 10 days for the epithelium to completely renew itself (17). The oldest cells, known as squamous cells, are at the surface, most exposed to the environment. Wing cells make up the middle 3-4 layers and reside intermediate to basal and squamous cells. Basal cells are the youngest and attach firmly to the basement membrane. A steady supply of stem cells migrate centripetally along the basement membrane from the Palisades of Vogt to replenish basal cells as they move through their life cycle (18,19). The epithelial layer has numerous functions, though most of its role has to do with protection. It provides a barrier to pathogens and foreign objects to prevent entrance into the cornea (20). Additionally, it protects the cornea from damaging UV radiation from the sun (21,22). There are no spaces between epithelial cells, rather they are anchored together via desmosomes. Desmosomes allow for the passage of nutrients and oxygen but hinder the entrance of unwanted bodies into the cornea (23). Epithelial cells have microvilli that serve to stabilize tear fluid. Another role of the epithelium is to redistribute nutrients and oxygen from the tears to the rest of the cornea (20). The epithelium is penetrated by intraepithelial corneal nerve fibers that extend up parallel to the surface from the subbasal lamina that regulate ocular physiology like the tear and blink reflex [(20), (Figure 2)]. The epithelium is also dotted with resident Langerhans cells, which help mount immune responses since there are no lymphatic vessels (24-26).

2.2 Bowman's Layer

Bowman's Layer is the most anterior noncellular interface of the cornea. It defines the boundary between the stroma and the corneal epithelium. That said, Bowman's Layer has been described as an "acellular condensation of the anterior stroma" (27) because the distinction the two layers is not clearly defined (20). Bowman's Layer is primarily made of randomly oriented collagen I anchored to the epithelial basement membrane through type VII collagen (28). The function of Bowman's Layer is unknown, although there are proposed hypotheses ranging from structural integrity to mediation of cytokine-related interactions (29).

2.3 Stroma

The stroma is the thickest layer in the cornea, accounting for 90% of the total width of the organ (30). It is made up of extracellular matrix (ECM), water, and keratocytes, which are mesenchymal cells that reside in the layers between stromal lamellae. The stroma functions to maintain corneal transparency, strength, and shape (31). Its function heavily relies on the formation and hierarchical organization of collagen I (fibrils to fibers to lamellae); alterations in this lamellae organization has been suggested as the primary etiology of corneal ectasias (31,32). While collagen I the most abundant type, there are numerous other collagens expressed that each have their own function (31). Stromal transparency is maintained by the pump-leak mechanism of endothelial cells and the ordered arrangement of the stromal lamellae (21). Overhydration of just 5% results in increased light scattering and decreased corneal transparency, which in turn can be detrimental to vision (33,34). Keratocytes reside between the stromal lamellae

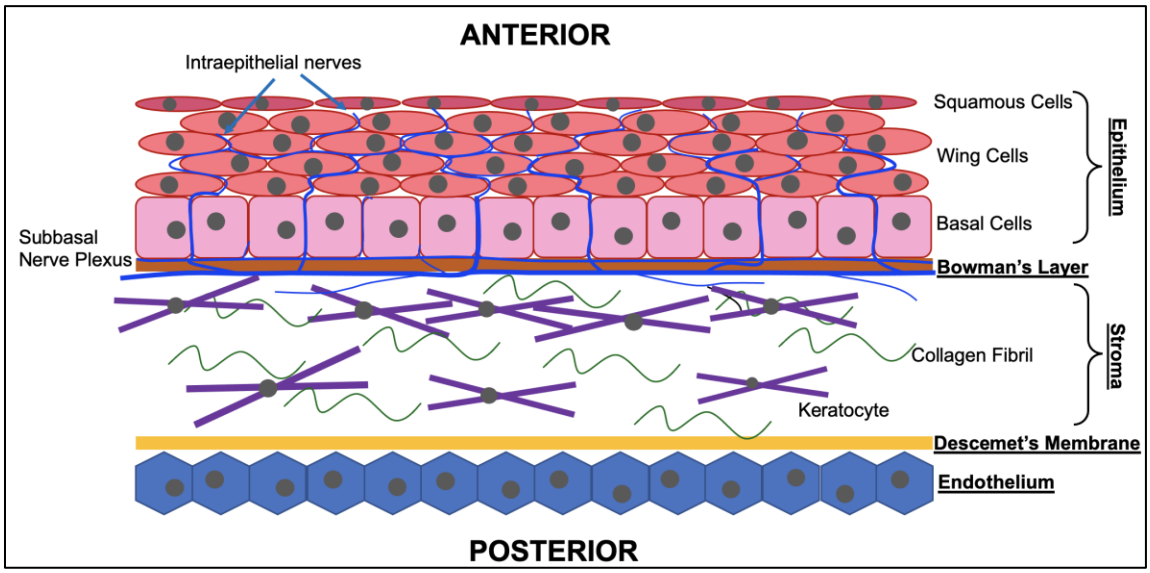

Figure 2. <u>Simplified anatomy of the cornea</u>. The cornea is made up of 3 cellular layers and 2 collagenous interfaces. The endothelial layer is a monolayer of hexagonal shaped cells separated from the stroma by Descemet's Membrane. The stroma makes up the bulk of the cornea and is made primarily of collagen, extracellular matrix, and keratocytes. Keratocytes are located in higher density in the anterior stroma. The epithelial layer is separated from the stroma by Bowman's Layer and the subbasal nerve plexus. Intraepithelial corneal nerves project upward from the nerve plexus through the epithelial cells and synapse at the ocular surface.

(35) and contribute to lamellar stabilization (23). Keratocytes mostly reside in close proximity to Bowman's Layer, located in the anterior stroma [(36), (Figure 2)]. Keratocytes form a syncytium and communicate via gap junctions to play an integral role in the corneal wound healing process (37). Injury to the epithelium or stroma induces keratocytes to differentiate into myofibroblasts, which release ECM to repair damaged tissue [(38-40), (See Section 4)].

2.4 Descemet's membrane

Descemet's membrane is an interface that separates the endothelial layer and the stroma. It is made of Type IV collagen and contains stabilizing proteins that adhere to both the endothelial and stromal layers. This interface has been proposed to play a biomechanical role and provide structural support to the curvature of the cornea (41), though this idea has been challenged and is still under investigation. Nutrients and molecules are able to pass through the membrane in order to reach stromal keratocytes, epithelial cells and nerves (42). Collagen Type VIII is also present and arranged in a hexagonal structure that mirrors the cellular arrangement of the endothelial layer below it. Descemet's membrane also contains perlecan, which can bind to and prevent movement of transforming growth factor-β1 and -β2 (TGF-β1/β2), two cytokines that push keratocytes to mature into fibroblasts. The affinity of perlecan for TGF-β molecules helps maintain homeostasis and regulates fibrotic processes in the stroma following injury (43,44).

2.5 Endothelium

The corneal endothelium is a monolayer of hexagonal cells located at the most posterior portion of the tissue. Endothelial cells are connected via tight and

intermediate junctions (23,45). However, the tight junctions do not fully surround the cells, creating a “leaky” barrier which allows for the flow of solutes into the extracellular space (46). Additionally, ion pumps are present that function to move water out of the endothelial cells and into the aqueous humor (31). This pump-leak system regulates the hydration of the stroma, which in turn ensures that the cornea is transparent (20). Endothelial cells have an abundance of mitochondria to maintain the pump-leak system (47). The leaky barrier also permits the passage of molecules and nutrients from the aqueous humor into the avascular cornea (48). Corneal endothelial cells do not regenerate, which can be detrimental to corneal physiology, but humans are born with a large supply that maintains corneal integrity as we age (49). Endothelial cells deposit and rest upon basement membrane, mainly Type IV collagen, which makes up Descemet’s membrane. This anchors the endothelial cells to the rest of the cornea (20,23).

2.6 Corneal Nerves

The cornea is the most densely innervated tissue in the human body (50), with approximately 7000 nociceptors per mm^2 (51). In the mouse and rabbit corneas, single corneal sensory neurons give rise to 200 and 3000, respectively, individual nerve endings (52-55). Intraepithelial corneal nerves (ICNs) send sensory information from the epithelial surface to the brain to regulate processes like tear production and the blink reflex in response to changes in mechanical, thermal, and painful stimuli [(54),(Figure 3)]. Most corneal nerves are sensory and branch from the trigeminal nerve (cranial nerve V) [(56), (Figure 3)], while there are others that

are autonomic that stem from the oculomotor nerve (cranial nerve III) and help to regulate muscle movement and wound healing (57,58).

Nerves enter the cornea radially at the stromal level and remain parallel to the surface underneath the epithelium, forming the subbasal nerve plexus. In humans, nerves penetrate Bowman's Layer in the peripheral and central cornea (59) with single beaded fibers that project upwards into the epithelium and synapse at the ocular surface (Figure 2). Electrophysiology data and the small size of nerves in the subbasal plexus (0.1-0.5 μm) suggest they are A-δ and C fibers (60-62). Neurons lose their myelin sheaths and perineurium about 1 mm beyond the limbus to maintain transparency of the tissue. Here, the interplay between epithelial cells and intraepithelial corneal nerves is demonstrated by basal epithelial cells acting as surrogate Schwann cells through wrapping themselves around nerve bundles and phagocytosing debris (63). Moreover, the epithelial layer contains one of the highest concentrations of acetylcholine in the body (51), expresses high levels of enzymes that can breakdown choline (64-70), and has many muscarinic receptors on their cell surfaces (71-74). That said, a paracrine signaling mechanism between the epithelial cells and nerves is likely present. Keratocytes may also interact with nerves in the stroma by physically wrapping themselves around axons, but this occurs more often in diseased corneas (51).

Most nerves present in the cornea are sensory (somatic) nerves that contain substance P and/or calcitonin gene-related peptide (CGRP) (51), both of which are implicated in the pain pathway (75). Nerves that express these two molecules typically express vasoactive intestinal polypeptide (VIP), a molecule necessary for

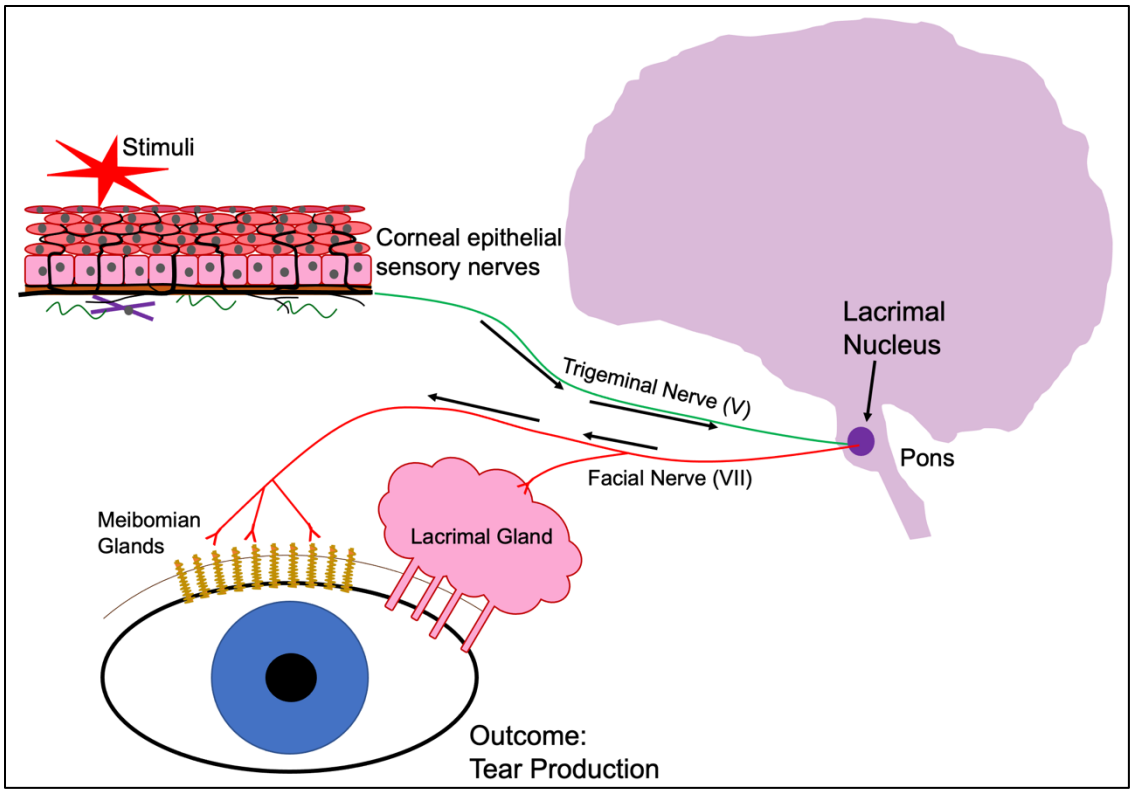

Figure 3. <u>Afferent and efferent pathways of intraepithelial corneal nerves</u>. Intraepithelial corneal nerves have nociceptors that lie on the ocular surface. When a stimulus like temperature change, mechanical touch, a foreign object, or pain is sensed, nerves send signals via the trigeminal nerve to the lacrimal nucleus located in the pons. Here, they synapse with the facial nerve, which sends efferent signals to the lacrimal and meibomian glands to drive tear production to wash away the debris and protect the eye.

growth factor regulation following wounding in the cornea (51,76). There are multiple types of receptors expressed at the free nerve endings, including TRPV1, TRPM8, and Piezo2 cation channels, so nerves can respond to varied stimuli including temperature changes, mechanical/physical touch, and pain (57). In the event of a stimuli presented, afferent fibers from the trigeminal nerve send signals to the lacrimal nucleus in the pons. Here the signal is passed to the facial nerve (cranial nerve VII) which sends efferent signals to both the lacrimal and meibomian glands to stimulate tear and oil production and secretion, respectively, to protect the eye, wet the ocular surface, and wash away debris or foreign bodies [(20,77,78), (Figure 3)].

When corneal nerves are left untreated, neurotrophic keratopathy arises. This can be due to lack of signaling mediators between cells or through loss of regulating neural circuits. Either way, sensitivity to changes in stimuli can diminish, tear production can drop, wound healing can slow, and the epithelium stops getting the important signals it needs from nerves. One outcome of neurotrophic keratopathy is dry eye disease, where the epithelium undergoes erosions, ulcerations, and perforations from lack of tear production. In some extreme cases, the stromal layer can be affected so much so that it melts away and leaves the eye vulnerable to infection and possibly removal (79,80).

2.7 Lacrimal and meibomian glands, tear fluid

The lacrimal and meibomian glands work together to create stable tear fluid made of two main phases: the aqueous and the lipid. The aqueous phase is synthesized and secreted by the lacrimal gland system, making up the majority of the 3-4 μm-

thick tear fluid (20,81,82). The aqueous phase is a liquid containing many components: mucins that reduce friction on the epithelial surface and entrap debris (83,84); proteins that have many roles, but often work to maintain homeostasis of the ocular surface (85-92); electrolytes to regulate the osmolarity of tear fluid (93,94); and other constituents like antioxidants, leukocytes, and debris (85,95,96). The lipid phase of the tears is called meibum, an oily, lipid-rich substance produced by the meibomian glands that helps spread and prevents evaporation of the aqueous phase (93,97). It consists of a plethora of lipids and sits thinly atop the aqueous layer of the tears (0.25 μm thick) (20,98). The meibomian glands line the eyelids parallel to the eyelashes and are compressed with each blink, squeezing meibum out while the eyelid spreads it over the surface as it moves (20). The ocular surface and tear film are deeply intertwined; if there is a homeostatic imbalance in either side, it can promote dry eye disease through tear instability, hyperosmolarity, surface inflammation and epithelial damage (82).

A major focus currently in corneal research is to develop novel therapeutic agents that promote corneal epithelial homeostasis and regeneration to decrease ocular pain, chance of infection, and vision impairment. The critical first step in this process is understanding the underlying structure of the cornea and the mechanisms that regulate its function and repair. The most common corneal epithelial pathologies that compromise the integrity of the corneal surface are described below.

3. *Corneal injury*

Corneal damage can occur in daily life through tasks as simple as putting in contact lenses and cooking. It is prevalent on the battlefield- many active-duty military members report shrapnel, explosions, and chemical burns to the eyes (99). However, many of these injuries sustained during wartime are deeply penetrating wounds that may affect intraocular structure. Superficial epithelial wounds, those that remain in the epithelial layer, can heal in 48-72 hours, although full healing (loss of fibrosis, regeneration of corneal intraepithelial nerves) can take several months to years (100). Some individuals, like diabetic patients and those taking chemotherapeutics (i.e. cetuximab, erlotinib, gefitinib), have recurrent corneal perturbations like punctate keratopathy, dry eye, and trichiasis (101-106), which is extremely painful and lowers their quality of life. Individuals with chronic corneal damage through disease or drug side effects stand to benefit from treatment that helps restore and sustain homeostasis.

3.1 Common superficial wounds

Corneal abrasions are one of the most prevalent ocular diagnoses in primary care facilities in the United States and worldwide (107-110). Common injuries sustained include superficial epithelial physical wounds, which can come from scratches, contact lens damage, and other incidents (99,111). Physical trauma accounts for 1/3 of all cases of corneal blindness (7).

3.2 Infection

Corneal injury can lead to infection from invading pathogens, but some types of bacterium or viruses directly impact the ocular surface. Trachoma (bacterium

chlamydia trachomatis) is the leading cause of infection-driven blindness and ocular morbidity (112). It causes severe scarring on the underside of the eyelid, leading to persistent scratching of the epithelial layer and irreversible blindness. Trachoma is most prevalent in underdeveloped areas that have less access to sanitized water. It mainly affects children (because they rub their eyes often) and women (due to close contact with infected children) (113,114). Herpes Simplex Virus 1 (HSV-1) is a disease that impacts not only the cornea, but also the conjunctiva and retina. It can cause opacification and neovascularization of the cornea if not treated, both pathophysiological properties that may result in irreversible blindness (115).

3.3 Diseases

Some diseases act directly on the corneal epithelium while others have damaging secondary side effects. Primary sources of disease pathology include corneal dystrophies, genetic-based disorders associated with mutations in genes found in certain layers of the cornea. Most epithelial dystrophies are associated with genetic changes in the TGF-β1 gene (116). The corneal dystrophy most prevalent in the clinic is Fuch's endothelial dystrophy, which is passed on through autosomal dominance and characterized by loss of endothelial cells, increased production of ECM, guttae formation in Descemet's membrane, edema, and loss of vision (117,118).

Diseases may also indirectly impact the cornea. Autoimmune diseases like Sjogren's and Grave's diseases present with dry eyes and corneal ulcerations, which can lead to perforation, scarring, infection, and blindness (16,119).

Additionally, diabetic keratopathy manifests secondary to chronic elevated blood glucose, which in turn may lead to recurrent epithelial erosions, ulcers, thinning, low basal cell density, delayed wound healing, and blindness if left untreated (120). Deficiency of vitamin A due to malnutrition can have detrimental effects on the ocular surface because it is crucial for corneal structure and integrity (121,122).

3.4 Iatrogenic cause

Medical intervention to fix eyesight may inadvertently cause corneal injury. Surgeries like laser in situ keratomileusis (LASIK) and photorefractive keratectomy (PRK) cause tissue damage as clinicians create a corneal flap and reshape the stromal layer to allow for better light focusing on the retina. Corneal transplants and lens replacement for cataracts are also common iatrogenic means of ocular damage since they replace critical tissues in the eye. Furthermore, the cornea may be affected through chemotherapy treatment. Many of these drugs are tyrosine kinase inhibitors (TKIs), taken to decrease protein kinase activity, like epidermal growth factor receptor (EGFR) and c-Met. Kinases phosphorylate and activate effectors that most often promote cells to proliferate, migrate, and differentiate. Inhibiting these proteins can prevent over-proliferation and growth of cancerous cells. However, due to the rapid nature of epithelial cell turnover, they express high levels of kinases to maintain homeostasis of the tissue (of the 50+ receptor tyrosine kinases expressed in the human body, the epithelium expresses ~20) (123-125). When these important proteins are inhibited, the epithelium can be subject to detrimental side effects, likely because the cells cannot send or receive the signals

they need. For example, Gefitinib is a chemotherapeutic that targets EGFR and causes recurrent corneal erosions and keratitis (126).

The routes listed above do not make up an exhaustive list. While there are many starting points, the most common injuries often impact the epithelial layer due to its location as the anterior portion of the cornea. Its dense innervation also makes injuries extremely painful. Most superficially damaged corneas (i.e., abrasions) in healthy individuals regenerate well on their own due to the rapid turnover of cells in the corneal epithelium. For these wounds, the standard of care entails topical antibiotics to minimize infection and patching the eye to allow the new epithelial layer form without perturbation by the eyelid. For individuals with superficial scratches, there is little value in regenerative compounds due to the quick healing time of 48-72 hours (100). However, those patients suffering from recurrent corneal erosions either due to disease or as a side effect would benefit from agents that promote epithelial cell migration, proliferation, and differentiation. There is currently ongoing research to better understand signaling pathways that can be manipulated.

4. Corneal wound healing

Many diseases impact the integrity of the corneal epithelium by disrupting the normal homeostatic processes and shifting the corneal epithelium to a pathological state. The cornea is a tissue that must maintain its health and transparency to serve its function. The epithelial layer, being the most anterior and most exposed to the external environment, is responsible for protecting the tissue. The epithelial layer thus must maintain homeostatic conditions so that the entirety of the cornea

can be balanced, healthy and transparent. The epithelial layer receives nutrients, oxygen, proteins, and electrolytes from the tear fluid that help maintain the ocular surface. Homeostasis and healing of the epithelial layer is balanced through cytokines and growth factors present in the tears.

4.1 Growth Factors

Each cellular layer of the cornea has its own assortment of proteins expressed to enable its function. A study performed in 2012 identified 3,250 unique proteins expressed in the cornea. Of these, only about 19% overlapped between all three layers (123). Of the proteins expressed in the cornea, one cytokine, TGF-β, is highly expressed in every layer. This growth factor and others, including epidermal growth factor (EGF), hepatocyte growth factor (HGF), and keratinocyte growth factor (KGF) have an immense role in corneal wound healing and homeostasis. Cytokines are a large category of molecules that are released from one cell and act on another through an autocrine (same cell), paracrine (local cell), or juxtracrine (adjacent cell) manner, though paracrine signaling is the most common (127). Each of these cytokines act on cognate receptors expressed on their target cell.

Basal growth factor expression in the tear fluid and aqueous humor is often sufficient to activate receptors on the epithelial surface to maintain homeostasis. However, expression is upregulated following tissue injury to help recruit inflammatory mediators and begin healing (128). Wilson et al. found that following a central epithelial wound in mice, hepatocyte growth factor (HGF) and keratocyte growth factor (KGF) mRNA expression both increased 7-fold 24 hours post-injury.

Epidermal growth factor (EGF) increased 4-fold. Of their cognate receptors (c-Met, KGFR, and EGFR, respectively), there was a 7.6-, 4.5-, and 1.8-fold increase in expression (128). While these growth factors were examined, there may be others that also change that have yet to be studied. Part of this process may be driven by the IL- 1 family, as IL-1α and IL-1β have both been found to increase expression of HGF and KGF in keratocytes and corneal fibroblasts, a more mature stage of keratocyte life (129-131). While each of these cytokines have unique receptors and activate unique signaling pathways, their stimulation promotes homeostasis of the tissue.

4.2 Healing of Cellular Layers

Each of the three cellular layers of the cornea respond to injury differently. The epithelial layer can regenerate following damage due to a collection of stem cells that reside in the limbal epithelium (132). The stromal layer and endothelial layers are slower to regenerate in general. Up until recently, it was thought that these layers had no renewal capacity at all. In 1996, Wilson et al. demonstrated that after epithelial injury, keratocytes in the stroma began to undergo apoptosis, which set up Zieske et al. in 2001 to show that keratocytes proliferate 12-24 hours following injury, statements that changed the field of corneal biology (133,134). Discoveries continue to be made to find the best treatment options for ocular health.

Corneal wounding is accompanied by sharp pain in the eye and reflex tearing, which increases the availability of growth factors, healing molecules, and inflammatory mediators for the injury. Tearing also allows debris to be washed away. Cytokines like IL-1α and IL-1β are released from the injury site and enter

the stroma, bypassing the damaged Bowman's Layer in high concentration with ease, especially if the epithelial basement membrane is damaged. Here they bind to IL-1 receptors expressed on keratocytes and promote apoptosis of those adjacent to the injury site within minutes (14,129,133,135-139). Greater injuries are associated with increased apoptosis (140). It is hypothesized this response occurs to limit virus infection prior to the immune response that it would elicit from other tissues to prevent over-invasion of leukocytes (14). More posterior and peripheral keratocytes are exposed to lower levels of IL-1, and rather than inducing apoptosis, the IL-1 molecules promote expression of proteins like granulocyte colony stimulating factor (G-CSF), monocyte-derived neutrophil chemotactic factor (MDNCF), and neutrophil-activating peptide (ENA-78) (141). These induced proteins, the IL-1, and other chemokines and cytokines released from the epithelial injury site attract bone marrow-derived cells like monocytes, lymphocytes, and macrophages to the injured location (142-144). Most of the invading cells undergo apoptosis as soon as they enter the cornea and release inner contents, but some remain until the injury is resolved (144). The IL-1α and IL-1β released from the injured tissue also promote expression of metalloproteinases and collagenases in the remaining stromal keratocytes, corneal fibroblasts, and myofibroblasts. These enzymes help in the degradation of disordered ECM and restructuring of new ECM after injury (145-147). However, the stromal response to injury will not fully terminate unless the epithelial basement membrane forms fully again, so healing of the epithelial layer is crucial for full ocular restoration.

The epithelial layer itself heals in a way described as the epithelial tongue (148). A single layer of flattened cells makes up the leading edge of the healing epithelium. As the cells migrate and the wound closes, the basal cells start to regenerate and rebuild the basement membrane. If this step does not occur, the keratocytes in the stroma are subject to continuous TGF-β stimulation, which promotes fibrosis (See section 4.3). After the defect is closed, epithelial cells proliferate to restore the 5-7 layers of wing and squamous cells (14).

4.3 Fibrosis in the cornea

If the epithelial basement membrane is not restored after injury, damaged cells continuously release growth factors like TGF–β and platelet derived growth factor (PDGF) into the stroma. When there is more epithelial basement membrane damage, more of these molecules enter (135,136,149). Usually, TGF–β allowance into the stroma is tightly regulated by the epithelial basement membrane and Bowman's layer; recall that in homeostatic conditions, molecules like perlecan and collagen IV bind to TGF–β molecules and prevent their passage (14). When TGF-β and other cytokines are present in high concentrations in the stroma, they tip the balance and drive keratocytes to corneal fibroblasts, and then to mature myofibroblasts (39,150-155). In non-aberrant conditions, these cells release ECM to restructure injured tissues. In chronic wound healing responses, they persistently release high levels of ECM. This, and the fact that mature myofibroblasts are themselves opaque, opacifies the stromal layer of the cornea and impairs vision through the process of corneal fibrosis (14,39,156). If the basement membrane is restored, TGF–β levels in the stroma decrease,

myofibroblasts undergo apoptosis, and the more posterior keratocytes that didn't mature divide and fill the area. Then, they can restructure the stroma to decrease opacification using enzymatic breakdown of ECM (157-160). Healing and restructuring of the stroma are also necessary for the full restoration of corneal nerves. Myofibroblasts and fibrotic deposition slow the reinnervation process, which is already a slow undertaking which takes weeks to months (161-164). Corneal fibrosis is behind the root cause of corneal blindness.

4.4 Corneal Reinnervation

Since intraepithelial corneal nerves project into the epithelial layer, it makes sense that they also sustain trauma when the epithelium is injured. A fully healed cornea requires neuronal regeneration to ensure proper communication between cell types and corneal layers. There are many clinical studies looking at the progression of healing over time following deep penetrating surgeries like LASIK and PRK that suggest full healing and restoration of the subbasal plexus can take years to heal (70). Nerves can restore axonal and synaptic density by branching or by generation of new nerves. There is evidence that restoration of the subbasal nerve plexus arises from branching stromal nerves following PRK (79), but most literature examines if density increases or not rather than how density increases. Healing of the epithelial layer and corneal nerve regeneration go hand in hand, as both release trophic factors (cytokines, growth factors) for the other to maintain ocular surface homeostasis (14).

5. Bench Practices to study Corneal Epithelial Healing

Investigators use corneal epithelial wound healing as a model for understanding the balance of homeostasis, signaling within cells, and other “macro” processes like fibrosis and neovascularization because the cornea is transparent, easy to access, and can be visualized in a straightforward manner. Wounding the corneal epithelium is reproducible and predictable, and the restoration of it is tractable and quantifiable. Most importantly, the processes involved in wound healing faithfully recapitulate those observed in the maintenance of corneal epithelial homeostasis.

5.1 *in vitro Models*

While it is beneficial for some research to begin with the most complex system, parsing apart small molecular details can be gained from using tissue culture experiments first, in immortalized or primary cells. There are multiple immortalized human corneal epithelial cell lines, but the top three most utilized are SV40 (created by viral vector transfection the sv-40 T antigen (165)), HCLE (created by hTERT reverse telomerase (83)), and hTCEpi (also created by hTERT reverse telomerase (166)). Advantages of using immortalized lines include unlimited passaging abilities allowing for cost-effective experiment repetitions and less cell-to-cell variability. The ability to repeat many experiments at this stage allows the research scientist to optimize treatment conditions before moving on to more clinically relevant models. One major disadvantage of using these is that they are not phenotypically “normal;” they have been engineered to propagate forever, so they are not as physiologically relevant as primary cells.

Primary human corneal epithelial cells can be harvested from donor corneas and cultured for 2-3 passages (100). Using donated cells is advantageous because they are more clinically relevant since they more closely mimic what is seen in patients. Disadvantages include the limited supply of donatable human corneas, variability between patients who donate (age, health history, environment, lifestyle, etc.), and low passaging ability. These issues make it challenging to do many experimental repeats in a cost-effective manner.

The fundamental cell events in corneal wound healing and homeostasis are 1) cell migration to replace cells that have been removed or died and 2) cell proliferation to restore cell numbers. Common assays used in the laboratory include wound healing/scratch assays, transwell migration assays, and proliferation assays. Scratch assays can be done multiple ways, but protocols follow first removing cells or growing cells around barriers to create an acellular space. Cells are usually treated with a molecule or compound of some type and then the rate of wound closure is measured, representing re-epithelialization. Scratch and transwell assays are considered “migration” assays, where the effect of the molecule is measured by cell movement or rate of wound closure. Scratch assays are advantageous because they are both qualitative and quantitative: wounds can be visualized and quantified for change in area. Another component of wound healing, proliferation, can be measured a multitude of ways. A common *in vitro* assay is the Alamar Blue assay, in which cells are treated with a dye that measures mitochondrial function as an output of cell number and growth (more mitochondria = more cells). Measuring proliferation is a way to study re-

epithelialization as well, since the epithelial tongue must first migrate and then proliferate to restore all layers. There are, of course, countless other assays that may or may not be used to study corneal wound healing. Additionally, just because something performs in a tissue culture dish does not mean it will perform that way *in vivo.* Starting small is helpful to answer very specific questions, but any clinical research requires animal usage to understand tissue and systemic impacts rather than just one cell type at a time.

5.2 *ex vivo Models*

ex vivo organ culture is the practice of isolating and culturing a whole tissue, like an entire cornea with all its layers. This approach allows us a better understanding of how drugs, growth factors, or any exogenous substance may affect not only the epithelial layer, but all other layers as well in a more cost-effective way: eyes are usually obtained from a slaughterhouse rather than an in house animal. Culturing corneas is performed by filling the cornea with a mixture of serum rich media and agarose before maintaining the tissue at an air-liquid interface with rocking to mimic tear coverage. *ex vivo* organ culturing recapitulates an intact tissue, in which there is interaction and cross talk between different tissue layers. This is important when studying wound healing, as quality and whole corneal healing it is not contained to just the epithelium. Another advantage of using *ex vivo* wound models is the ability to study reepithelialization over a collagenous surface rather than in a plastic dish. One area of contention is that, unlike *in vivo* work, there are no accessory organs like the tear-producing lacrimal or meibomian glands. However, this provides the researcher the opportunity to control what and when growth

factors or compounds are applied without having to control for endogenous tear production. In addition, there is no immune system that might adversely impact the wound healing process. This model is useful for piloting conditions of treatment, but ultimately the *in vivo* model is required to fully understand the whole system.

5.3 *in vivo Models*

There are multiple animal models used to study the cornea. Most corneal wound healing studies done have been performed in mice and rabbits, though experiments have been carried out in pigs, chickens, rats, cats, and dogs, to name more (100). *In vivo* studies are necessary to carry out because they are the most clinically relevant model you can get. When testing potential therapeutics, models that have fully intact systems allow us to investigate any off-target effects, how the immune system may react to treatment, and finalize concentrations before moving to clinical studies.

There are different practices to study corneal wound healing based on what the research question is. To study the rates of re-epithelialization, debridement protocols are used. Debridement wounds are ones in which the basement membrane underneath the epithelium remains intact. These wounds are made mechanically by demarcating a circular area using a trephine and then removing the epithelial cells via dulled blade, rotating burr, or crescent knife. As a comparison, you would not use debridement to study how the cornea heals after LASIK; there are deeper-penetrating practices to follow for those (100). To study fibrosis and scarring, a common protocol is to create chemical damage by soaking a circular filter in sodium hydroxide (NaOH) and briefly placing it on the cornea.

One last area to mention that studied in the cornea is neovascularization, which is the creation and formation of new blood vessels. This happens in damaged states, some diseases, and genetic disorders (167). Since the cornea is transparent, neovascularization is something that 1) clouds vision when it occurs in humans and 2) can easily be studied in the lab through imaging.

Not all organisms share the same corneal anatomy. The bovine epithelium is almost double in thickness compared to humans, with 8-12 layers of cells (168). As well, rabbits and pigs do not have a Bowman's Layer between the epithelium and the stroma (27). These differences are not thought to be significant and make both animal models viable for research. Lastly, innervation is different in some mammals: sympathetic innervation is scarce in humans and parasympathetic innervation is unclear, though some mammals like cats and rats it is present (169-172). What this means for wound healing practices is unknown.

Each model system goes hand-in-hand and work together to answer big picture questions. *In vitro* work allows for in-depth looks at signaling pathways, *ex vivo* work allows us to see how a whole tissue will respond, and *in vivo* work permits us to understand systemic effects of treatment. Each of these stages are important when designing a drug treatment for wound healing. Current research centers around growth factors and how they modulate quality corneal healing.

6. Hepatocyte growth factor and its cognate receptor, c-Met

6.1 Hepatocyte Growth Factor (HGF)

In 1984, Nakamura et al. identified a serum factor that was upregulated following a 70% hepatectomy in rats. When cultured hepatocytes were treated with this

serum factor, there was an increase in DNA synthesis and cell proliferation, so the group named it hepatocyte growth factor (HGF). They purified HGF from platelets by taking advantage of its affinity for heparin using heparin-affinity chromatography. HGF is formed as a 728 amino acid-long protein in an immature pro-form. After maturation by serine cleavage between residues arginine 494 and valine 495, it has a molecular weight of 84 kDa (173). HGF has two subunits, one of 69 kDa (α) and one of 34 kDa (β) linked by a disulfide bond (174). The full-sized cDNA of human and rat HGF were cloned in 1989 (175).

In 1991, Naldini et al. discovered that HGF is indistinguishable from scatter factor (SF), a protein that is secreted from fibroblasts and promotes epithelial cell dissociation and motility. They demonstrated this by 1) showing that SF and HGF both induced the same level of DNA synthesis; 2) performing radioligand binding assays of SF and HGF and showing that their curves completely overlap; 3) immunoprecipitating the c-Met (cellular-Mesenchymal to Epithelial Transition) receptor (the high-affinity HGF receptor) and showing that both HGF and SF bound to it; 4) revealing that HGF and SF both cause invasion, scattering of cell colonies, and phosphorylation of the c-Met receptor; and 5) cloning and comparing the cDNAs from multiple human tissues and presenting indistinguishable sequences (176). Around the same time, Higashio et al. found an additional factor secreted from fibroblasts, tumor cytotoxic factor, to be the same ligand as HGF and SF (177).

6.2 c-Met/HGF Receptor

The high-affinity receptor for HGF is the c-Met receptor tyrosine kinase. This was discovered after cells were treated with HGF and lysates were measured for phosphotyrosine activation by immunoblotting (178). There was robust tyrosine phosphorylation at 145-kDa, and it was soon found to be the β subunit of the c-Met receptor. c-Met is made of a 50 kDa α–chain and a 145 kDa β–chain (178). The α–chain lies extracellularly, and the β–chain has both transmembrane and intracellular segments. Higuchi et al. demonstrated that after transfection of human c-Met cDNA into COS-7 cells, there are both a high affinity and a low affinity binding site of HGF, with the high affinity site being on the c-Met receptor (k_d= 0.2 nM; tbe low affinity site is heparin (k_d= 2 nM)) (176,179). Activating the receptor with HGF markedly increased DNA synthesis compared to non-transfected cells (179).

c-Met expression levels change throughout different life stages. During human fetal development, for example, it has highest expression in the forming lung tissue. HGF or c-Met knockout mice are embryonically lethal, which shows how important these proteins are for proper development and organ generation (180,181). In adults, c-Met expression is highest in the liver, placenta, lung, and thyroid (182). HGF is implicated in many restorative and regenerative processes. Of course, it has a large role in liver regeneration; it was named following a large removal of rat liver. In addition, when HGF is supplemented to rats with liver cirrhosis, ECM-degrading enzymes (collagenases) are upregulated, which decreases presence of ECM and leads to hepatocyte repair (183).

With its heavy involvement in cell proliferation and tissue regeneration, it is no surprise that the c-Met signaling axis is mutated in several cancers and drives tumor progression forward (184). Not only this, but high c-Met expression is associated with poor prognosis (185-188). There have been c-Met mutations found in breast cancer, non-small-cell lung carcinoma (NSCLC), colorectal cancer, and hepatocellular carcinoma (189). As of 2017, there are 3 tyrosine kinase inhibitors on the market targeting c-Met that are being used for chemotherapy: crizotinib, cabozantinib, and capmatinib (190). Of these, only capmatinib is specific to just c-Met (191), and has been used in the lab to study c-Met inhibition in the lab (192,193).

6.3 c-Met Activation

c-Met is part of the receptor tyrosine kinase (RTK) family. These types of receptors have intrinsic kinase domains that get activated via phosphorylation upon ligand binding and often promote outcomes like cell proliferation, migration, and differentiation. Like many RTKs, inactive c-Met is a monomer embedded the plasma membrane. Upon presentation of HGF, two monomers dimerize in a 2:2 ratio (2 ligands to 2 receptors) and undergo auto-transphosphorylation in which one monomer activates the other and vice versa (194-196). There are multiple tyrosine residues that get phosphorylated, but the main sites serve as either catalytic or docking domains. For c-Met, the residues that get phosphorylated in the kinase domain are tyrosine resides 1234/1235, and the ones that serve as docking sites are tyrosine residues 1349/1356 (197,198). These docking sites allow for adaptor proteins like Gab1, Grb2, and Sos1 to bind (199-201). Most of

the downstream effects of c-Met are mediated through Gab1 (202). This scaffold protein allows for effector proteins that contain the SH2 binding domain to interact with c-Met including: SH2-transforming protein (Shc), phosphoinositide 3 kinase (PI3-K), SH2-domain containing protein tyrosine phosphatase (Shp2), phospholipase Cγ1 (PLCγ1), signal transducer and activator of transcription 3 (STAT3) and Ras GTPase p120 (200,203-205). The effector proteins, once scaffolded onto the c-Met receptor, are phosphorylated by the catalytic domain of the c-Met receptor and transduce signals into the cell (206).

6.4 Outcomes of c-Met signaling

There are multiple outcomes of HGF binding to c-Met, and one of the drivers of differential signaling is distinct adaptor proteins that bind and drive separate signaling pathways. For example, recruitment of Grb2 and SOS by c-Met activates Ras and ERK, which leads to cellular migration, cell cycle progression, and proliferation (207-209) while phosphorylation of Gab1 by c-Met recruits PI3K, PLCγ, and SHP2, promoting morphogenesis and motility (200). Specific to the cornea, HGF treatment can protect corneal epithelial cells from apoptosis by signaling through PI3-K, but not through ERK1/2 (210).

Cell surface interactions and the location of the receptor along its endocytic trafficking pathway are also important mediators of signaling outcomes. c-Met can interact with other growth factor receptors on the cell surface like EGFR and promote drug resistance in multiple cancers (211-215) and wound healing in retinal pigmental epithelial cells (216). c-Met signaling can also activate different pathways based on where it is spatially along its endocytic trafficking route (Figure

4). Briefly, after growth factor stimulation, receptor:ligand pairs are internalized and trafficked through the cell (See Section 6.5, Figure 4). Previously, it was believed that endocytosis was a process specifically for desensitization and turning off receptor activity. However, it has been shown that not only c-Met (217), but other RTKs like EGFR (218), and insulin receptor (219) can also signal from their endocytic pathways. The c-Met receptor's most well-studied influencers of spatial signaling are protein kinase c (PKC) isotypes; PKC-α is required for Met trafficking to perinuclear locations vis microtubules. In contrast, PKC-ε helps accumulation of ERK1/2 into focal zones that aid in Met signaling (220).

No matter how c-Met signals following HGF stimulation, receptor activity is tightly controlled due to the nature of the cellular responses. While short lived activity helps with wound healing and homeostasis, activating mutations in these proteins are often drivers of cancer because they promote cellular proliferation, differentiation, and mobility. Turning off receptor signaling is a necessary step to ensure balance in the tissue.

6.5 Negative regulation of c-Met signaling

Negative regulation of c-Met and other RTKs is hypothesized to be a process that turns off signaling so that cells do not undergo transformation. After stimulation and activation of c-Met by HGF on the cell surface, receptors enter the endocytic pathway in the process of ligand- or receptor-mediated endocytosis (221). The cell membrane begins to invaginate due to clathrin or caveolin redistribution to the inner cell membrane (222,223). As the endocytic compartment forms, dynamin pinches off the vesicle and releases it from the surface (224-227). There are many

proteins involved throughout the endocytic pathway. Some of the main players that aid in turning off receptor signaling are E3 ubiquitin ligase proteins, mainly the Cbl family. These are a family of 3 (c-Cbl, Cbl-b, and Cbl-3) proteins that regulate kinase signaling by tagging active proteins with ubiquitin. Ubiquitin tags have many functions in the cell, including internalization and/or targeting proteins for degradation in the lysosome to terminate receptor signaling (107,228,229). c-Cbl activity is required for the normal kinetics of c-Met degradation following activation (230). It binds with the receptor at c-Met residue tyrosine 1003 and interacts with Grb2 and SOS at the cell surface after receptor stimulation to ubiquitylate the receptor (230,231). The Y1003 residue is absent in the c-Met onco-protein that drives cellular transformation (232). There is still debate on if c-Met is poly- (single lysine residue with a multi-ubiquitin chain) or mono-ubiquitylated (multiple lysines have single ubiquitin molecules). Jeffers et al. found that c-Met is polyubiquitylated within minutes in cell lines generated to express high levels of c-Met (233) while Carter et al. found that c-Met is only monoubiquitylated in HeLa cell extracts (107).

Lysosomal degradation is not the only fate of the receptor. c-Met may also be recycled back to the cell surface for further signaling, though this has mainly been seen in other RTKs, driven via ligand choice (234). Furthermore, desensitization via ligand-mediated endocytosis and ubiquitin-driven degradation are not the only options to turn off receptor signaling. Phosphatases like PTP-1B remove phosphates from the c-Met catalytic domain to turn off signaling (235), and dissociation of the scaffold complex prevents effectors from getting phosphorylated further (206).

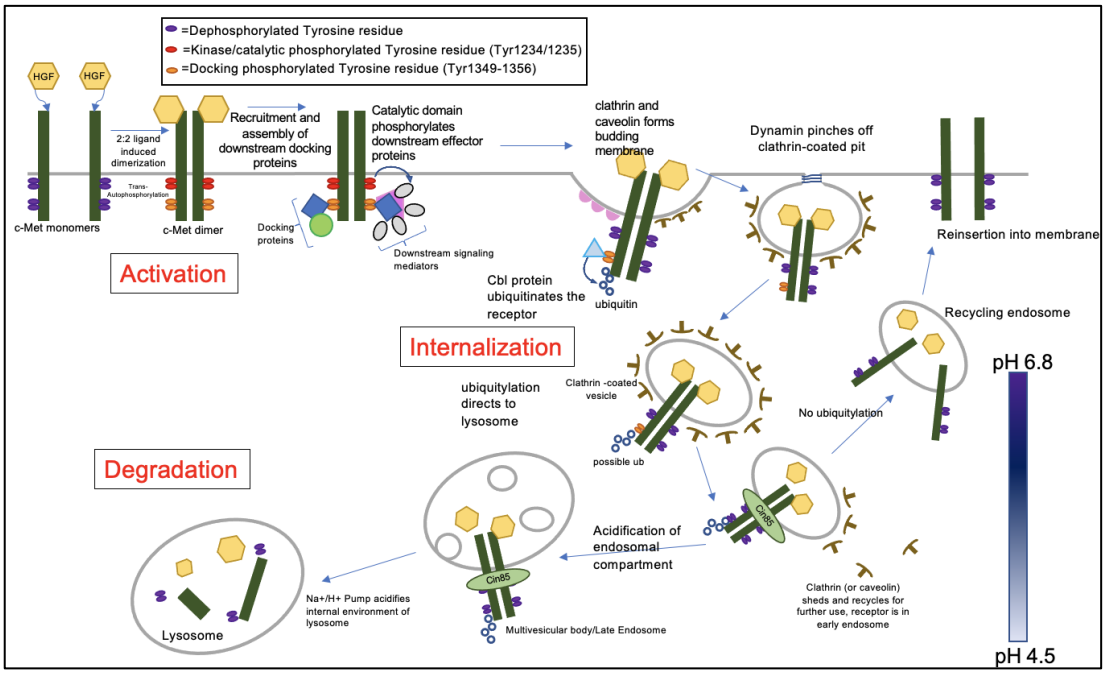

Figure 4. **<u>Trafficking pathway of c-Met following HGF stimulation.</u>** HGF binds to c-Met monomers in a 2:2 ratio and promotes both dimerization and auto-transphosphorylation of the receptor pairs. Recruitment of scaffold proteins brings effector and downstream mediators to the receptor and promotes phosphorylation that propagates signals into the cell. Following stimulation, the receptor gets internalized via ligand-mediated endocytosis in clathrin- or caveolin-covered pits. It is also marked with ubiquitin tags by Cbl proteins, which may drive the receptor and ligand complex to the lysosome for degradation. c-Met can signal throughout its endocytic pathway. It also may be recycled to the surface for further use.

c-Met signaling and regulation of signaling are two processes that aid in corneal epithelial homeostasis and wound healing. Manipulating these pathways by extending signaling or inhibiting proteins from the system present unique opportunities to study tissue restoration.

7. c-Met's role in Wound Healing

Corneal wound healing is an important facet of human health because ocular damage, whether recurring or not, can be detrimental to vision and impact quality of life. Wounds that are left unhealed are vulnerable to infection which can lead to blindness. There is currently ongoing research into targeting growth factors and cytokines to promote corneal wound healing and homeostasis. For example, EGF and its receptor EGFR has been studied as a route for corneal epithelial healing for over 40 years (236). A novel growth factor under investigation for corneal wound healing is HGF. Not only has it shown promise in other tissues, but based on our preliminary studies (See Chapter 3) it is predicted to both quantitatively and qualitatively improve corneal wound healing.

HGF and c-Met have been implicated to have a physiological role in corneal wound healing. Human tear HGF levels increase ~1400% from the pre- to post-photorefractive or phototherapeutic keratectomy (PRK or PTK, respectively) state due to an increase in both HGF production in the lacrimal gland and tear flow rate (237). Additionally, in an *in vivo* murine model, HGF could accelerate corneal epithelial healing by promoting proliferation of epithelial cells and suppressing inflammatory signaling (238). HGF supplemented to mice with nephrotic syndrome

show 4.4-fold higher tubular epithelial proliferation as well as suppression of TGF–β, PDGF, and myofibroblast formation in the kidney (239).

Although re-epithelialization plays a critical role in corneal wound healing, other aspects such as inflammation and nerve regeneration are sometimes overlooked. Inflammation is a process that starts out beneficial, with the influx of cells recruited via cytokines that aid in getting rid of bacteria and clearing debris to allow for proper tissue remodeling. However, inflammation may end up becoming detrimental as opposed to beneficial if this process becomes aberrant and promotes fibrosis, leading to vision impairment. HGF treatment has shown that it can combat inflammation and decrease fibrosis. HGF can inhibit neutrophil invasion in ischemic mouse kidneys by down-regulating adhesion molecules (ICAM-1 and E-selectin) they use to infiltrate tissues (240). HGF can also suppress IL-1, IL-6, and IL-18 (all inflammatory cytokines) that are released from macrophages (241). Additionally, it can promote macrophage differentiation into dendritic cells, which are immunosuppressive to regulatory T-lymphocytes (242,243). HGF also has anti-fibrotic characteristics such that it can down-regulate TGF-β production. The balance between these two molecules often determines the outcome of tissue damage. TGF-β suppresses HGF expression and, as injuries become chronic, TGF–β levels rise and HGF levels decrease, leading to fibrosis if not properly taken care of (173,244). In the cornea, this process can mean sight or no sight. HGF can inhibit TGF-β production by regulating Smad7 (245-247), which in turn prevents myofibroblasts from maturing and releasing more ECM (38-40).

HGF can prevent apoptosis of epithelial cells, a common manifestation in tissue and organ damage. HGF may inhibit caspase-3 (248) or induce anti-apoptotic molecules like Bcl-xL (249). Surprisingly, HGF has been shown to *increase* apoptosis of myofibroblasts via induction of MMP-9, which degrades fibronectin. Without this anchor, cells undergo apoptosis (240). In 2018, Gupta et al. used a combination of HGF and BMP7 gene therapy to selectively promote apoptosis of stromal fibroblasts which resulted in reduced corneal hazing (250).

There is evidence that HGF and c-Met are involved in the regeneration of peripheral nerves and brain health, suggesting that it may also play a regulatory role in corneal nerve homeostasis. Both *in vivo* sciatic nerve pinch models (murine) and *in vitro* growth assays (explants of rat neocortical tissue) suggest that c-Met activity is necessary and sufficient for peripheral nerve outgrowth (251-253). HGF and c-Met have also been implicated in the formation of new synapses (254). Restoring intraepithelial nerves following wounding is a critical step for ensuring complete corneal healing. HGF inhibits neuronal cell death and dysfunction while promoting axonal regrowth in cell cultures from rat hippocampus, cortex, and spinal cords (253,255). As well, Shimamura et al. found that gene transfer of HGF intracisternally to rats with cerebral artery occlusion improved prognosis of memory, increased neurite outgrowth, and promoted synaptogenesis (256). Manipulating the HGF/c-Met axis has also improved rodent model outcomes of neurodegenerative diseases like ALS by delaying progression (257), Parkinson's by protecting dopaminergic neurons (258), and Alzheimer's disease by alleviating Aβ-amyloid induced cognitive impairment (259). There is almost no literature

surrounding c-Met and HGF involvement in intraepithelial corneal nerve communication and wound healing, which is a gap in the field that needs to be addressed.

Given the abundance of evidence that HGF and c-Met *already have a physiological role* in wound healing, it is not surprising that circulating HGF accumulates in injured organs preferentially over uninjured ones even with systemic release following injury. Aside from corneal injury, Mizuno et al. found that recombinant HGF could restore renal dysfunction by promoting tubular repair, down-regulating TGF–β production, and decreasing fibrosis (239). HGF and c-Met also have roles in regeneration of other epithelial tissues including lung (260-262), mucosal GI (263-265), and skin (266). This literature evidence suggests that a drug that works to restore homeostasis of corneal epithelial cells may produce beneficial outcomes in other tissues.

While HGF and other growth factors show promise, clinical data often lacks a convincing response. The effects of HGF and c-Met signaling need to be parsed apart in order to most efficiently promote healing, not only to increase re-epithelialization but also increase quality of healing through lowering inflammation and fibrosis while promoting neuronal regeneration. One theory behind the lack of response to exogenous addition of growth factor is that a high dose may be saturating the receptors and causing desensitization, so finding new strategies to target these receptors is necessary. For example, de Oliviera et al. published that HGF applied to PRK-wounded rabbit corneal epithelium did not accelerate healing and showed no difference compared to control (267). However, this study used a

high amount of HGF (0.1 mg/mL; 50 µL 3x per day for a week), and this may have caused constant c-Met desensitization. One promising route is to extend receptor signaling by manipulating the desensitization pathway and Cbl-mediated degradation. If Cbl proteins can be inhibited, this may allow for extended c-Met signaling and acceleration of the healing process. My overarching hypothesis for the research presented in Chapters 2-4 is two-fold: 1) stimulating immortalized human corneal epithelial cells with HGF will activate the c-Met signaling pathway and accelerate healing itself, and 2) manipulating the c-Met desensitization pathway by knocking out negative regulatory proteins will extend signaling and accelerate the HGF-induced healing response further.

.

CHAPTER 2

MATERIALS AND METHODS

Cells & Cell Culture

Telomerase reverse transcriptase-immortalized human corneal epithelial (hTCEpi, (166)) cells were obtained from Evercyte (Vienna, Australia). To create a c-Cbl/Cbl-b knockout line, hTCEpi cells were edited using CRISPR/CAS9. Briefly, all cells were transduced with a lentivirus encoding CAS9 and the blasticidin-resistance gene. Colonies resistant to blasticidin were isolated and some transfected with tracrRNA and guideRNA against c-Cbl and Cbl-b (tracrRNA: mA*mG*CAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAG UGGCACCGAGUCGGUGCU*mU*mU; c-Cbl crRNA sequence 1: mC*mA*UCUUUACCCGACUCUUUCGUUUUAGAGCUAUG*mC*mU; c-Cbl crRNA sequence 2: mC*mU*AUUCUUUAGCGCCAGCUUGUUUUAGAGCUA UG*mC*mU; Cbl-b crRNA sequence 1: U*mG*CACAGAACUAUCGUACCAGU UUUAGAGCUAUG*mC*mU; Cbl-b crRNA sequence 2: U*mA*AUCUGGUGGAC CUCAUGAGUUUUAGAGCUAUG*mC*mU). Cells were seeded at low density and individual colonies were isolated, amplified, and screened via immunoblot to confirm absence of proteins. Three positive clones of each cell line were identified. All control cells in this study are hTCEpi cells that were only given the Cas9 and blasticidin gene and were never supplemented with the guide RNA; these are referred to as Cas9 cells. All cells were grown in Keratinocyte Basal Medium (KBM)

with growth supplements (Lonza) at 37°C with 5% CO_2. Cells were propagated 2 times per week to maintain normal growth and never allowed to grow more than 90% confluent to prevent differentiation and quiescence of cells. Any other cell lines used (SkBr3, CHO, etc.) were lysates provided from a past laboratory member and were not cultured. These control cell lines were chosen because c-Met is not expressed in SkBr3 and CHO cells (268).

Serum Starvation

Before all experiments, cells were washed twice with phosphate-buffered saline (PBS) pH 7.3 and incubated in serum free media for at least 2 hours.

Immunoblots

To make whole cell lysates, cells were grown to confluency before treatment with HGF. Cells were washed twice in room temperature PBS, followed by equilibration to 4ºC by placing the cells on ice for 5 minutes. Cells were harvested in RIPA butter [150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 50 nM Tris (pH 8.0)] supplemented with protease inhibitor phenylmethylsulfonyl fluoride (PMSF 2mM, Calbiochem) and solubilized by end over end rotation for 10 minutes at 4°C. Insoluble material was pelleted via centrifugation at 15000 RPM for 10 minutes at 4°C. The samples were diluted in 6X SDS buffer/10% β–mercaptoethanol and boiled for 3 minutes prior to gel loading. Equal amounts of protein (20 μg) were loaded and resolved by 10% SDS-PAGE before transfer to nitrocellulose membrane. Membranes were immunoblotted using the indicated antibodies following the manufacturer's instructions: c-Met (#8198), Met phospho-

Y1234/1235 (#3077), MAPK (#4695), MAPK phospho-S202/204 (#9101), c-Cbl (#2747), Cbl-b (#9498) [Cell Signaling Technologies], and α–tubulin (#6199) [Millipore Sigma]. Following incubation with the appropriate horseradish peroxidase-conjugated secondary antibody, enhanced chemiluminescence (ECL) was used to visualize immunoreactive bands in a Fotodyne imaging system. Each experiment was performed at least 3 independent times and representative experiments are shown. Densitometry analysis was performed using NIH ImageJ. GraphPad/Prism was used for generating graphs and performing statistical analysis. Statistics comparing Cas9 and DKO cells were performed using 2-way ANOVA with multiple comparisons.

c-Met Ubiquitylation Assay

hTCEpi-Cas9 cells were grown to confluency and pretreated with 0.1 μM MG-132 for 20 minutes to inhibit the proteosome before treatment with 50 ng/mL HGF for 0, 2', 10', or 30' minutes. Cells were harvested in ubiquitin-lysis buffer [0.5% Triton x-100, Tris pH 7.5, 150 mM NaCl, 1 mM EDTA,1 mM sodium orthovanadate,10 mM sodium fluoride] supplemented with 2 mM PMSF and 16 μM G5 Ubiquitin isopeptidase inhibitor I (Santa Cruz Biotechnology). Cell lysates (~1.10 ug/uL protein) were immunoprecipitated with an antibody that recognizes both mono and polyubiquitylated lysine residues (#ST1200, Millipore Sigma) overnight at 4°C before next-day incubation for 2 hours with protein A/G Agarose beads (Santa Cruz Biotechnology). Immunoprecipitates were washed three times in ubiquitin lysis buffer before dissociation from the beads via boiling in 6X SDS buffer. Proteins were resolved by 7.5% SDS-PAGE and immunoblotted for c-Met

(#8198, Cell Signaling), pY1234/1235 (#3077, Cell Signaling), or ubiquitin (#ST1200, Millipore Sigma).

Indirect Cyto-Immunofluorescence

hTCEpi-Cas9 and hTCEpi-DKO cells were cultured on 12mm round glass coverslips for 2 days. Cells were grown to ~80% confluency and were treated with 50 ng/mL HGF (0-2 hours), washed with PBS++++ (0.5mM CaCl2, 0.5 mM MgCl2, 2% BSA, 0.1 mM glucose, PBS pH 7.3), and fixed in 4% paraformaldehyde/PBS++ at room temperature for 5 minutes and then on ice for 15 minutes. Excess formaldehyde was washed away with PBS++ (0.5 mM CaCl2, 0.5 mM MgCl2, PBS pH 7.3). Cells were then permeabilized and blocked for 20 minutes at room temperature in 0.1% saponin/5% FBS/PBS++ before incubation with primary antibodies. Coverslips were incubated for 1 hour at room temperature with antibodies against c-Met (#8198, Cell Signaling or #AF276, R&D systems) and/or EEA1 (#610456, BD Transduction). After washing to remove left over antibody, coverslips were incubated again at room temperature for 1 hour in the dark, with fluorophore-conjugated secondary antibodies (Alexa-488: #A11055, or #A11070; Alexa-568: #A11004; Thermo-Fisher) in the blocking buffer. After 6 washes in PBS++, coverslips were rinsed in ddH_2O to remove salts from PBS. Excess liquid was removed by gently touching the edge of the slip to a Kimwipe. Coverslips were mounted onto slides using ProLong Gold Antifade Mountant with DAPI (Thermo-Fisher). The slides were cured overnight in the dark and examined the next day using a confocal microscope at 60X objective. Each experiment was repeated 3

times and multiple images acquired from each time point. Representative images are shown.

Indirect Tissue-Immunofluorescence

Human corneas unable to be used for transplantation but approved for research use were obtained from Kentucky Lions Eye Bank (Louisville, KY). Serial sections were fixed and paraffin embedded in July 2010. Presently, corneas were subject to deparaffinization and rehydration through soaks in xylene (3x 5 minutes) and then decreasing solutions of ethanol each for 2x 10 minutes (100%, 95%, 70%, 50%) before rinsing in ddH_2O. Slides were boiled in pH 6.0 sodium citrate buffer for 10 minutes and then cooled for 30 before being rinsed in ddH_2O. Slides were incubated in 3% H_2O_2/MeOH to block peroxidase activity. Corneas were permeabilized and blocked in 1% PBS-Tween and 5% PBS-Tween, respectively. Corneas were incubated for 4 hours at room temperature with c-Met primary antibody (#AF276, R&D systems) diluted in blocking buffer. Slides were washed and then incubated with secondary antibody (#A11055, ThermoFisher) for 1 hour at room temperature in the dark. Slides were washed and a glass cover mounted atop the tissue using ProLong Gold Antifade Mountant with DAPI. Slides were cured overnight and imaged using a confocal microscope.

***in vitro* Wound Healing Assay**

The wound healing assay is a modification of the scratch assay. Silicone elastomer base (Sylgard 184 Elastomer, Dow Corning, Midland MI) was made per manufacturer's instructions and cured for 96 hours. Four 2-mm diameter silicone punch outs were placed directly on the bottom of a 6-well plate and spaced at least

2 mm apart. Cas9 cells were seeded at 400,000 cells per well and DKO cells were seeded at 450,000 to match control healing rate, because they recover slower after being trypsinized. Cells were incubated for 48 hours with a media change at 24 hours to achieve 90% confluency before treatment. The silicone plugs were removed following serum starvation and the cells rinsed with PBS to remove debris. This creates a 2-mm uniform acellular area on the plate. Cells were treated with 0, 10, or 50 ng/mL of HGF and imaged using a BZ-X800 Keyence All-in-One fluorescent microscope at 4X objective. This microscope contains an incubation chamber kept at 37°C with 5% CO_2. “Wounds” were visualized for 24 hours taking pictures every 15 minutes. All wounds were analyzed by finding the area (mm^2) every 4 hours and calculating the percent wound healed. To find the rate of wound closure, the area over time was plotted and the slope between hours 4 and 12 was calculated. GraphPad/Prism was used for statistical analysis and generating graphs. Statistics were performed using the Mann Whitney U test for comparison of Cas9 data (N of experimental =13, N of control = 10), and unpaired t-test for comparison of DKO data (N =3 both experimental and control).

CHAPTER 3

RESULTS

Corneal wound healing is a process that takes place to restore balance and homeostasis to the eye after injury. While most superficial epithelial wounds heal by 3 days, full healing can take months to achieve (100). Corneal injury is among the top ocular reasons why patients visit primary care facilities (107-110). As long as the wound remains open, there is potential for infection and blindness through a multi-step aberrant wound healing response. Despite the high incidence of corneal injury and devastating consequences that may arise if untreated, there are no FDA-approved drugs that help accelerate corneal epithelial wound closure or homeostasis. Many agents can accelerate healing in the context of the laboratory, but often these do not translate clinically, possibly due to desensitization of the receptor. Our overarching hypothesis for these data was two-fold: 1) stimulating hTCEpi-Cas9 cells with HGF will activate the c-Met signaling pathway and accelerate healing itself, and 2) manipulating the c-Met desensitization pathway by knocking out regulatory proteins will extend signaling and accelerate the HGF-induced healing response further.

c-Met is expressed and active in corneal epithelial cells

Each cell type in the body, while containing the same DNA, express their own unique subset of proteins. Immunoblot analysis confirms a robust expression of c-

Met in corneal epithelial cells compared to (Figure 5A). After confirming its presence in corneal epithelial cells, next we confirmed its location. Immunofluorescence of human corneal tissue indicates c-Met is highly expressed in the epithelium and stroma, with particularly high levels the basal epithelial cell surface (Figure 5B). To assess c-Met's activation in human corneal epithelial cells, a dose-response curve was generated (Figure 5C). The K_d of HGF for c-Met is variable in the literature but falls between 17 to 300 pM (176,179,269,270). Ranges from 0-640 pM of HGF were tested and samples blotted against c-Met pY1234/1235 to measure activity. 50 ng/mL, or 0.64 nM, was chosen for further experiments based on activity and to prevent oversaturation and desensitization of the receptor. Results indicate that c-Met is steadily activated in a dose-dependent way.

c-Met phosphorylation peaks 30 minutes after HGF treatment in Cas9 cells

The signaling of c-Met is well-established in other cell lines like HepG2 and Huh7 (271,272). To understand the kinetics of c-Met signaling in human corneal epithelial cells, a time course of HGF treatment was performed (Figure 6). Activation of c-Met can be observed within minutes of HGF stimulation. Phosphorylation peaked around 30 minutes and then slowly dissipated over the next 11 hours. Total c-Met protein levels also decreased over the 12 hours, suggesting that c-Met is degraded and desensitized following activation. The level of pY1234/1235 in the untreated cells establishes the basal level of c-Met phosphorylation. MAPK, or ERK1/2, levels were also tested as an outcome measure of c-Met signaling, as it is established that c-Met signals through the

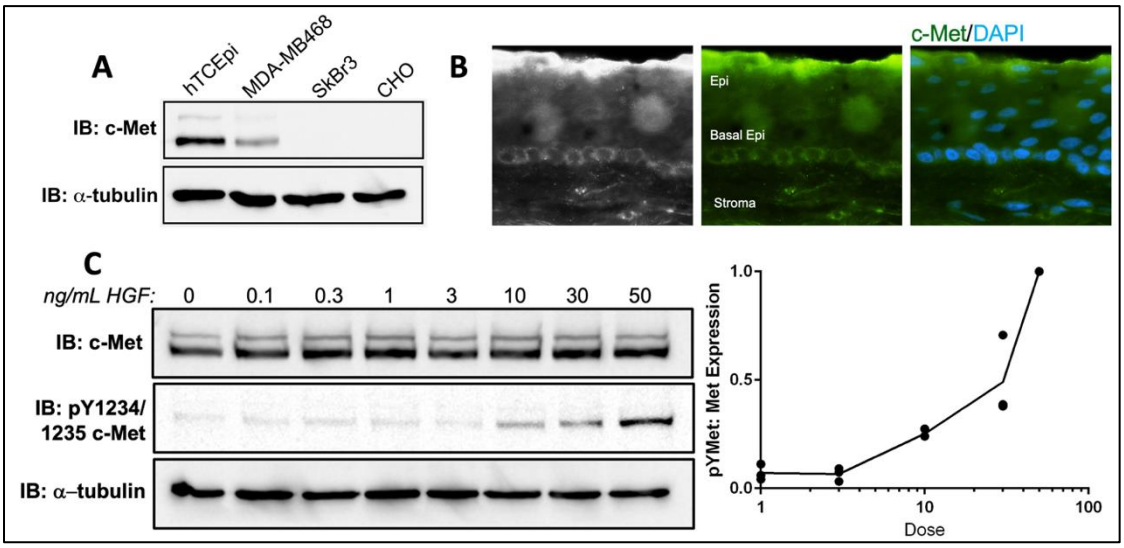

Figure 5. <u>c-Met is expressed and active in corneal epithelial cells.</u> A) hTCEpi-Cas9 cells were harvested without HGF treatment. MDA-MB468, SkBr3, and CHO whole cell lysates were obtained from a past lab member. Lysates were resolved through 7.5% SDS-PAGE and immunoblotted against c-Met. The receptor is robustly expressed in hTCEpi cells. B) Donated human corneas were fixed and paraffin embedded in July 2010. The paraffin was removed, and the tissue rehydrated before staining for c-Met. c-Met is expressed in the epithelium and the stroma, with high cell surface presence in the basal epithelium. C) hTCEpi-Cas9 cells were incubated for 30 minutes with increasing concentrations of HGF before harvesting and resolving through 10% SDS-PAGE. c-Met is activated in a dose-dependent way (measured by pY1234/1235 activity).

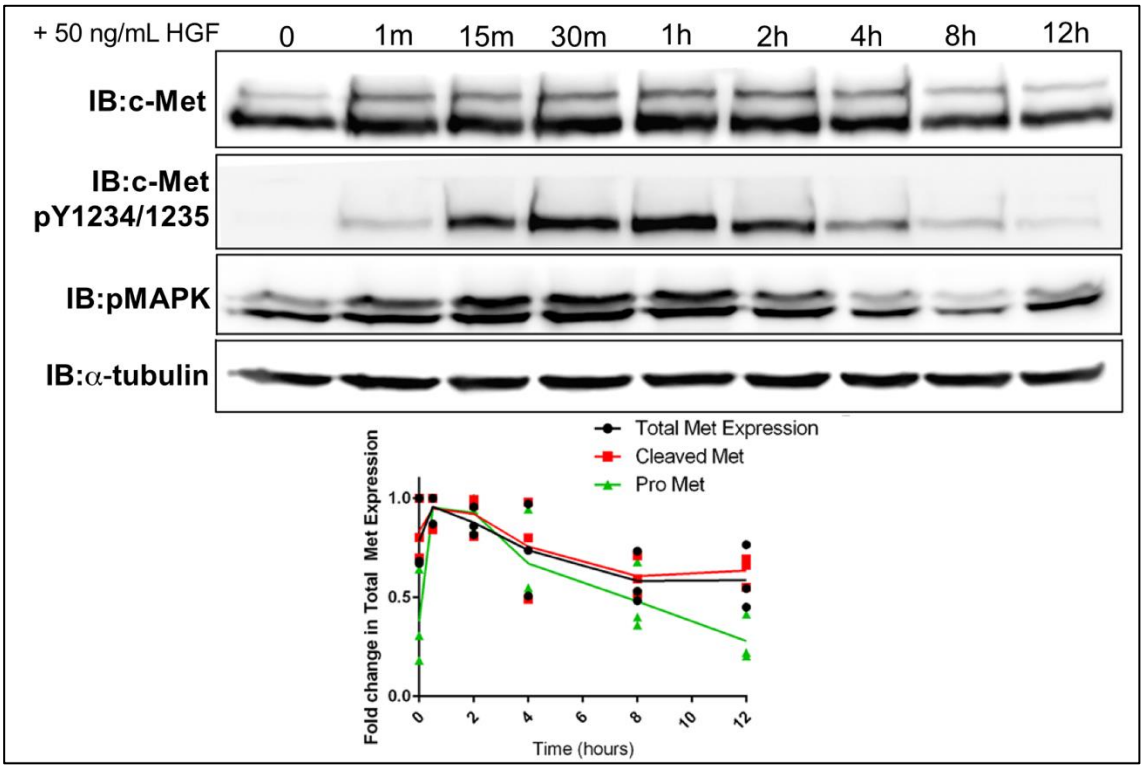

Figure 6. <u>c-Met phosphorylation peaks 30 minutes after HGF treatment in Cas9 cells.</u> hTCEpi-Cas9 cells were treated with one dose of 50 ng/mL HGF for 0-12 hours. Cells were harvested and whole cell lysates resolved through 10% SDS-PAGE. Nitrocellulose membranes were immunoblotted against c-Met, c-Met phosphotyrosine 1234/1235, MAPK phosphothreonine 202/204, and α-tubulin as a loading control. Data shows that c-Met is activated 1 minute after stimulation and peaks around 30 minutes following treatment. Total c-Met levels also decrease over time.

MAPK pathway to promote cell cycle progression, proliferation, and motility (208,273). MAPK activity peaked around 30 minutes before dropping and then returning to baseline.

c-Met is trafficked through the cell following HGF stimulation

c-Met is known to undergo internalization and degradation following stimulation, but the kinetics may be different in different cell lines, based on their respective protein machinery (206). Cyto-immunofluorescence shows that c-Met lies on the cell surface until stimulation with HGF. It redistributes to a more perinuclear location and is co-localized with the early endosome by 30 minutes, indicating that c-Met is moving through its trafficking pathway. After one hour, staining is decreased which suggests degradation. By 4 hours, surface staining has been restored (Figure 7).

c-Met is ubiquitylated following HGF stimulation in Cas9 cells

There is evidence that monoubiquitylation (one ubiquitin molecule on multiple lysine residues) is a tag for internalization while polyubiquitylation (at least 4 ubiquitin molecules on one lysine residue) is a tag for proteasomal degradation (274-277). c-Met has been shown to be both monoubiquitylated (107) and polyubiquitylated (233). In corneal epithelial cells, immunoreactive bands were only revealed when the antibody used to immunoprecipitate could detect monoubiquitylation (Figure 8A). However, further experiments and repeats need to be performed.

c-Met signaling is extended in DKO cells

The overarching research question of this work is to uncover if c-Met signaling can

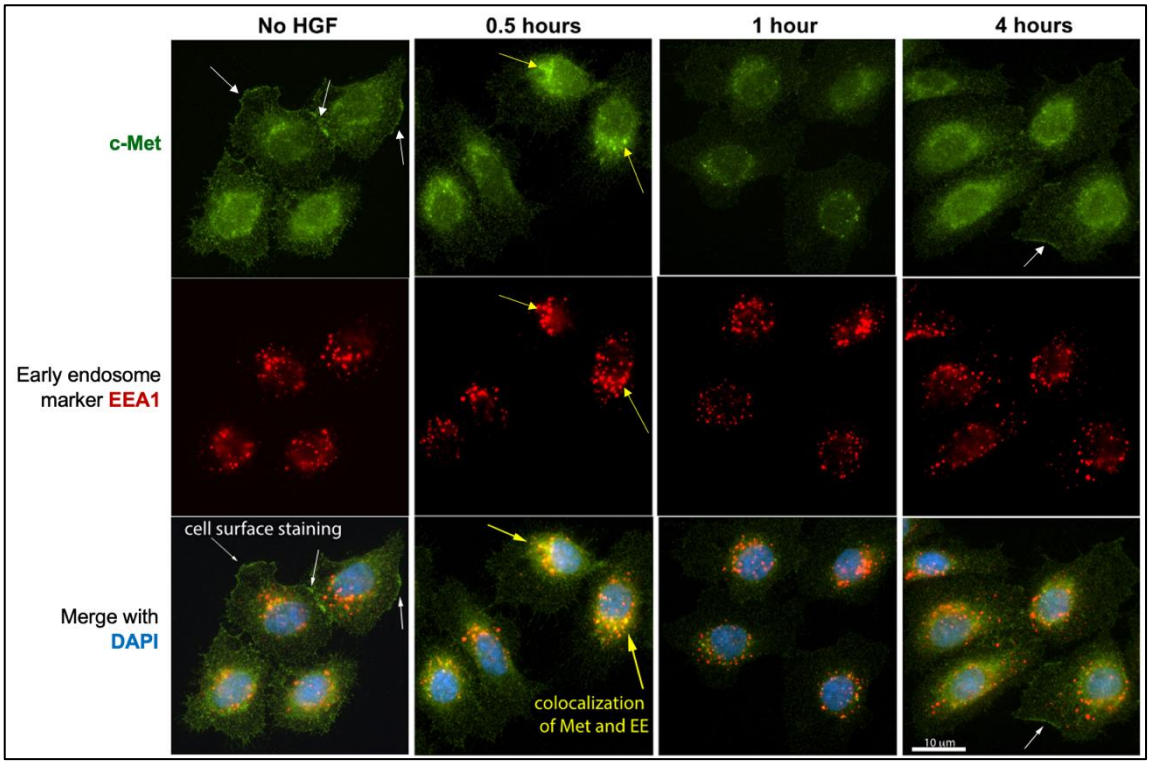

Figure 7. **c-Met is trafficked through Cas9 cells following HGF stimulation.** hTCEpi-Cas9 cells were seeded on 12 mm glass coverslips and treated with 50 ng/mL HGF (0-4 hours) before fixation, permeabilization, and incubation with antibodies against c-Met (#8198, Cell Signaling) and EEA1. After incubation with the appropriate secondary antibody, coverslips were mounted with medium containing DAPI. Slides were imaged at 60X objective using a Nikon fluorescent imaging microscope. Data indicates that c-Met is present on the cell surface (white arrows) without HGF treatment. Following stimulation, c-Met redistributes to a more perinuclear location and colocalizes with the early endosome (indicated by yellow staining). 1 hour into treatment, c-Met staining decreases overall, suggesting degradation of the receptor. 4 hours after treatment, c-Met has been restored to the cell surface. (Scale bar= 10 μm)

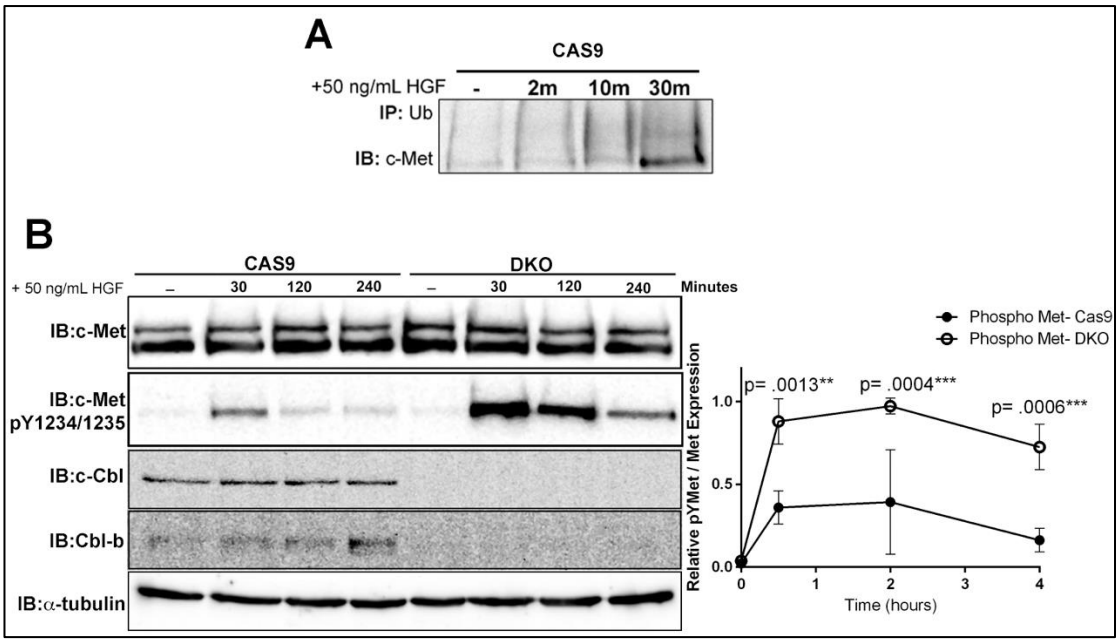

Figure 8. <u>c-Met is ubiquitylated following HGF stimulation in Cas9 cells; without the Cbl proteins c-Met signaling is extended</u>. A) hTCEpi-Cas9 lysates (~1.10 μg/μL protein) were immunoprecipitated with an anti-ubiquitin antibody, resolved through 7.5% SDS-PAGE, and immunoblotted against c-Met. Results indicate that c-Met is ubiquitylated after treatment with HGF. B) Whole cell lysates were created from both Cas9 and DKO cells treated with HGF (0-4 hours). Lysates were resolved through 10% SDS-PAGE and immunoblotted against c-Met (#8198), c-Met pY1234/1235, c-Cbl, Cbl-b, and α-tubulin. Data was normalized to loading control and statistics (2-way ANOVA) were performed between time points and cell lines. In DKO cells, c-Met has significantly higher activity both in magnitude and duration. Area under the curve for the DKO cell line is 173% increased compared to control.

be manipulated to our advantage to accelerate wound healing. Immunoblot data indicates that c-Met signaling is increased in both magnitude and duration in cells where c-Cbl and Cbl-b are not present (Figure 8B). Receptor activity was significantly increased at each time point compared to Cas9 cells (30 minutes p= 0.0013**; 2 hours p=0.0004***; 4 hours p= 0.0006***).

c-Met trafficking is slowed in DKO cells

The effect of ubiquitylation on internalization of c-Met is still up for debate. With the loss of c-Cbl and Cbl-b, the internalization of c-Met is slowed in comparison to Cas9 cells. Immunofluorescence data suggest c-Met remains on the cell surface for longer and can still be seen diffusely through the cell 2 hours post treatment, when almost all is internalized completely in the Cas9 cells (Figure 9).

HGF stimulation accelerates corneal epithelial wound closure; the effect is more dramatic in DKO cells

We tested the re-epithelialization capabilities of 1) loss of c-Cbl and Cbl- b, and 2) HGF treatment. First, in comparing *untreated* Cas9 and DKO cells, basal wound closure rate in DKO cells was faster than Cas9 (Figure 10). Secondly, in cells treated with 50 ng/mL HGF, wounds healed faster than untreated, no matter which cell line. In DKO cells, the HGF stimulation potentiates healing rate even further. Statistics indicate that HGF accelerated healing significantly (p= 0.002 **) from untreated to treated Cas9 cells. There is even more significant healing when comparing untreated treated DKO cells (p= 0.0002 ***). Further repeats need to be performed to carry out statistics between the two cell lines.

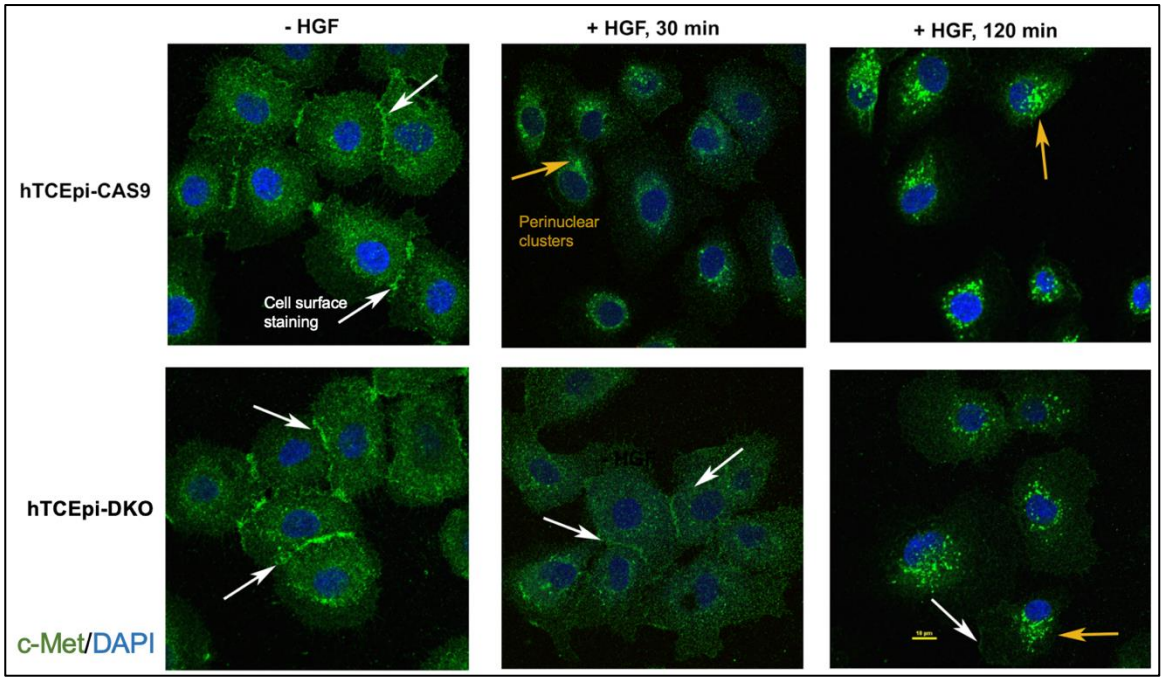

Figure 9. <u>c-Met trafficking is slowed in hTCEpi-DKO cells</u>. Cas9 and DKO cells were seeded on 12 mm glass coverslips. They were treated with 50 ng/mL HGF (0-2 hours) before fixation, permeabilization, and incubation with primary antibodies against c-Met (#AF276, R&D systems). Coverslips were incubated with secondary donkey anti-goat Alexa488-tagged antibodies and mounted in medium containing DAPI. Coverslips were imaged at 60X objective using a confocal microscope. Data indicates that c-Met remains on the surface longer in DKO cells (white arrows) internalization is slowed in DKO cells compared to Cas9. Yellow arrows indicate redistribution to a more perinuclear location. (Scale bar= 10 μm)

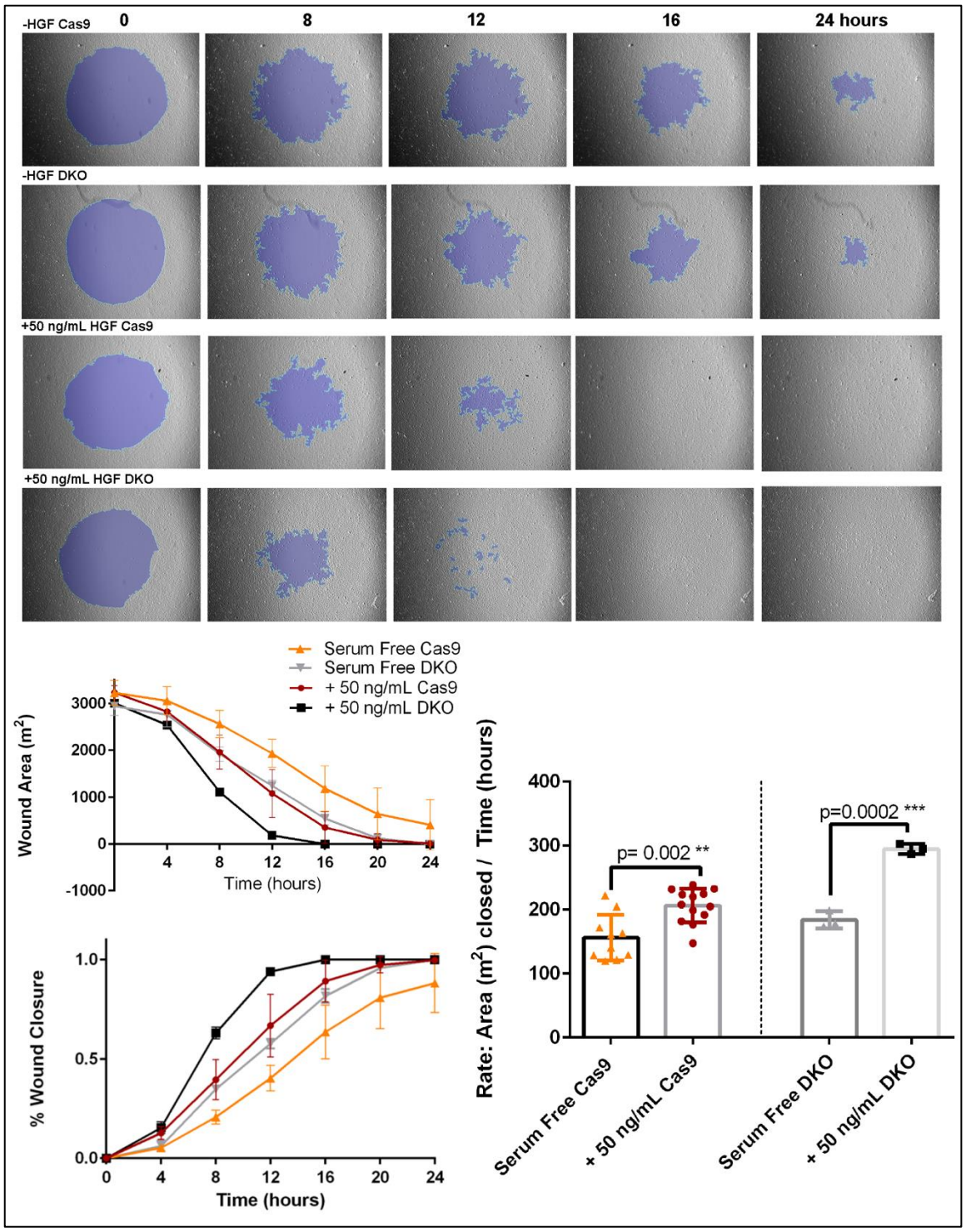

Figure 10. <u>HGF stimulation accelerates corneal epithelial wound closure; the effect is more dramatic in DKO cells.</u> hTCEpi-Cas9 and DKO cells were seeded

around 2mm silicone plugs in 6-well dishes and grown to confluency. The plugs were removed to create acellular areas before treatment with HGF. The plate was placed into the incubation chamber of the Keyence BZ-X800 time lapse imaging microscope and pictures were taken of the healing wounds every 15 minutes over 24 hours. Data shows that HGF treatment accelerates epithelial closure and lack of the Cbl proteins accelerates this response further. Rate of change in: untreated Cas9 cells :0.1563 m^2/hour; untreated DKO cells: 0.1849 m^2/hour; treated Cas9 cells: 0.2062 m^2/hour; treated DKO cells: 0.2118 m^2/hour. Rate was significantly different (p=0.002**) between untreated and treated Cas9 cells. Rate was significantly different (p=0.0209*) between untreated and treated DKO cells. Statistics could not be performed between cell lines because more repetitions need to be completed with DKO cells. The area under the curve of wound area (m^2) over time comparing untreated and treated Cas9 cells decreases 29% with HGF addition. The area decreases another 33% when HGF is added to DKO cells, potentiating the response.

CHAPTER 4

DISCUSSION

The overall purpose of this work was to determine if the HGF/c-Met signaling axis is able to be manipulated to accelerate corneal epithelial wound healing. This signaling axis is involved in many processes in the body, including embryogenesis, organogenesis, cancer progression, tissue healing, and homeostasis. In this thesis, I examined the effect of HGF's role in corneal re-epithelialization and how influencing signaling may accelerate healing. This study was achieved by first performing studies to ensure that c-Met was both expressed where it needed to be and was able to be activated in our cell line hTCEpi (Fig 5A,B). Our results indicate that c-Met is robustly expressed in corneal epithelial cells as compared to other cell lines. Additionally, c-Met was activated in hTCEpi-Cas9 cells in a dose-dependent manner by HGF (Fig 5C). These data taken together indicate that our cell line is a good model system to study c-Met, as it is present, localized where it should be, and is able to be activated. c-Met activity peaked at 30 minutes post HGF treatment and then tapered off over the next 12 hours (Fig 6). Total c-Met levels trended downward over the time frame, consistent with c-Met desensitization and degradation following growth factor stimulation, a process well documented in c-Met and other receptors (206). MAPK also activates after HGF addition, indicating that c-Met can signal to downstream signaling

mediators effectively. When c-Met activity was measured in hTCEpi-DKO cells, those missing c-Cbl and Cbl-b, signaling was increased in both magnitude (more immunoreactive signal) and duration (immunoreactive bands present longer) (Figure 8). These data lead us to the hypothesis that trafficking and/or desensitization are potential molecular mechanisms that may be targeted to extend c-Met signaling.

Ubiquitylation's role in internalization and degradation is still a point of contention in the field of cell biology; there is evidence that ubiquitylation is required for cell-surface protein internalization in yeast, but data varies in animal cells depending on the protein (274,278). Mono vs polyubiquitylation is another gray area surrounding c-Met's trafficking route. Currently, it is thought that monoubiquitylation of proteins mediates internalization and polyubiquitylation mediates proteasomal degradation (280-283), and there is evidence that both ubiquitylation modifications can be added to c-Met (112, 249). The Cbl proteins knocked out of our DKO cell line are also not the only proteins that can ubiquitylate receptors. A third Cbl family member, Cbl-c or Cbl-3, can also do this, along with another protein NEDD4 (279). This being said, c-Cbl and Cbl-b are the primary ubiquitin ligases, so it is thought that lack of these proteins equals lack of ubiquitylation. Immunofluorescence results indicate that c-Met is still internalized without c-Cbl or Cbl-b present; internalization kinetics are just slower (Figure 9). What this means specifically for Met's signaling pathway is yet to be discovered, as c-Met is still able to signal after internalization (217).

Following multiple attempts of immunoprecipitation experiments to pick up polyubiquitylation modifications on the c-Met receptor to no avail, an antibody that could recognize both mono and polyubiquitin additions revealed an immunoreactive band at 145 kDa (Figure 8A). However, these data need to be repeated to ensure results. This immunoprecipitation evidence led us to the hypothesis that slower internalization and degradation due to lack of the Cbl proteins allow c-Met to signal longer.

To study the effect of the HGF/c-Met signaling axis on corneal epithelial healing, *in vitro* wound healing assays were performed. These data suggest that loss of Cbl proteins accelerates the basal healing rate of untreated corneal epithelial cells. When HGF is added to the system, it accelerates healing. Loss of the Cbl proteins potentiates this effect in the DKO cell line. Further experiments must be completed to assess if HGF's effect is on proliferation or migration of the epithelial cells. However, these data reveal ubiquitylation as a unique route to accelerate corneal epithelial wound healing by extending the effects of c-Met through prevention of degradation.

CHAPTER 5

FUTURE DIRECTIONS

Corneal opacities are the 4th leading cause of blindness globally. Despite the high incidence of corneal blindness and wide array of medical issues associated with epithelial damage, there are no FDA-approved drugs that promote corneal wound healing or epithelial homeostasis. To develop these therapies, viable pharmacologic targets need to be identified.

Potential targets include growth factor receptors, or receptor tyrosine kinases. These proteins can be manipulated to induce cell proliferation, migration, and differentiation to promote wound healing. My research centers around the c-Met receptor. When this receptor responds to hepatocyte growth factor (HGF), effector proteins like MAPK begin a phosphorylation cascade that ultimately ends with biochemical or genetic responses that promote accelerated epithelial wound closure. Moreover, HGF has been shown to drive peripheral nerve axonal growth and inhibit inflammatory cytokines like TGF-β, both important facets of quality wound healing. However, c-Met is internalized and degraded following activation, limiting the duration of effect. I propose to examine the signaling pathway to identify regulatory points which may be manipulated to prolong signaling.

The *overall goal* of my research is to gain a better understanding of c-Met receptor signaling and trafficking to find pharmacologic targets that promote quality

corneal wound healing. My *hypothesis* is that the HGF/c-Met signaling axis can not only promote accelerated epithelial healing, but also decrease fibrosis and promote epithelial nerve regeneration. The *rationale* for these studies is that HGF-induced c-Met signaling can produce wound healing phenotypes and nerve restoration while also decreasing inflammation. When these aims are completed, we expect to have 1) identified ways to sustain c-Met signaling and 2) assessed the effect of sustained signaling on corneal epithelial wound healing through *in vitro, ex vivo and in vivo* models. My *approach* is to start at the molecular level and progress through tissue to animal models to reveal how disrupting c-Met trafficking impacts wound healing at multiple levels; my *expectation* is that re-epithelialization will be accelerated and that c-Met regulates multiple aspects of healing (i.e., nerve growth and fibrosis)

My first aim is to determine strategies to optimize c-Met receptor signaling in corneal epithelial cells. The objective of this aim is to elucidate mechanisms that enhance the magnitude and duration of c-Met receptor phosphorylation. Our working hypothesis is that inhibiting processes that negatively regulate c-Met signaling will prolong activity and accelerate wound healing. Using immortalized and primary corneal epithelial cells, I will combine genetic (DKO cells) and pharmacologic (receptor trafficking inhibitors like dynamin and monensin) approaches to inhibit negative regulators of c-Met signaling. *In vitro* assays will be used to determine if these approaches will slow c-Met endocytic trafficking and degradation, resulting in enhanced receptor activity (i.e., amplified receptor and downstream effector phosphorylation) and wound healing phenotypes (i.e.,

increased proliferation (Alamar blue), migration (Transwell), and re-epithelialization (*in vitro* wound healing)**.** Upon completion of this aim, we will have a better comprehension of how c-Met signals in the corneal epithelium, and how disrupting different stages of trafficking impacts healing.

My second aim is to establish how c-Met signaling impacts whole-cornea wound healing and morphology. The objective of this aim is to determine how enhancing c-Met activity affects multiple facets of corneal homeostasis. Our working hypothesis is that enhanced c-Met activity will accelerate re-epithelization, decrease fibrosis and inflammation in the stroma, and promote corneal nerve regeneration. Using *ex vivo* (porcine) and *in vivo* (murine) models, I will examine superficial corneal epithelial wound healing in response to HGF with and without pharmacological modulators of c-Met signaling. Starting with *ex vivo* works allows me to optimize treatment conditions like timing and concentration of dosages before moving onto animal studies. Corneas will be examined at times after wounding and monitored for 1) re-epithelialization and corneal epithelial structure, 3) fibrotic markers, and 4) nerve regeneration (including axon length, density, and branching). Upon completion of this aim, we will have determined how HGF and c-Met signaling impact the cornea's structural physiology during and post-wounding.

The proposed work will allow us a better grasp of the pharmacodynamics of c-Met signaling and its regulation, and how extending signaling impacts the cornea. This research will give us a more educated idea of how to manipulate the system to prolong receptor activity and accelerate corneal epithelial healing.

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CURRICULUM VITAE

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Education:

- **University of Notre Dame**, Notre Dame, IN Fall 2016-Spring 2020
  Bachelor of Science in Neuroscience and Behavior
- **University of Louisville,** Louisville, KY Fall 2020-Present
  Master of Science candidate in Pharmacology and Toxicology

Research Experience:

- **University of Notre Dame,** Notre Dame, IN Fall 2017-Spring 2020
  Research assistant and Lab manager
  - Studied evolved moral development and how western societal raising of children impacts neurobiology, psychology, and decision making as an adult
- **University of Louisville,** Louisville, KY Summer 2019
  Summer Student
  - Investigated how chronic arsenic exposure impacts the ErbB receptor family expression in BEAS-2B lung cells
- **University of Louisville,** Louisville, KY Fall 2020-Present
  Master's candidacy trainee
  - Determining the signaling kinetics of the HGF/c-Met signaling axis in human corneal epithelial cells

Honors and Awards:

2019 2nd place at Research!Louisville undergraduate division

Abstracts and Presentations:

Poster Presentations:

1. March 2019 - University of Notre Dame "Angelman Syndrome"
   a. 10th Annual Conference on Advancing Rare Disease Research, Therapy, and Patient Advocacy
2. August 2019 - University of Louisville "Chronic Arsenic Exposure and its Effect on the ErbB Family Receptor Tyrosine Kinases"
   a. Research!Louisville
3. May 2020 - University of Notre Dame "Associations Among Childhood Basic Needs Fulfillment, Evolved Developmental Niche Provision, and Adult Relational Outcomes"
   a. Association for Psychological Science
4. May 2020 - University of Notre Dame "Growth Mindsets and Peace-Supporting Disposition"
   a. Association for Psychological Science

5. October 2021 – University of Louisville “c-Met’s regulatory role in corneal epithelial homeostasis”
    a. Research!Louisville

Seminars:

1. March 25, 2021: Department of Pharmacology & Toxicology, University of Louisville, “Hepatocyte growth factor receptor regulation of corneal epithelial homeostasis”
2. February 24, 2022: Department of Pharmacology & Toxicology, University of Louisville, “The kinetics of HGF-mediated corneal epithelial homeostasis”

Memberships:

1. Neuroscience Club, University of Notre Dame, Notre Dame, IN 2018-2020
2. Society for Neuroscience, Louisville Chapter, Louisville, KY 2022-Present

Service

1. Brain Day volunteer 2019, 2022