MECHANISMS OF EPIDERMAL GROWTH FACTOR RECEPTOR ACTIVATION AFTER EPITHELIAL WOUNDING

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Wounding disrupts the primary function of an epithelium, which is to provide a barrier to the outside environment. The longer the epithelial defect remains unhealed, the greater the risks of morbidity and mortality from infection, loss of tissue homeostasis, and fibrosis. Normally, epithelial cells restore barrier function by becoming highly motile and migrating to cover the defect, but in many situations cells do not move fast enough to prevent tissue malfunction. This is especially true in the cornea, where even minor wounds can impair vision. Therefore, there is considerable therapeutic interest in identifying signals that induce epithelial migration. Activation of the epidermal growth factor receptor (EGFR) is a key signaling event that promotes cells to move and cover wounds in many epithelia. The broad goal of this dissertation research was to identify mechanisms of wound-induced EGFR activation so that therapies may be developed to improve normal and pathological healing.

I hypothesized that mechanisms of EGFR activation may differ with respect to distance from the wound, so I developed wounding models to analyze signaling specifically in cells near to or far from "wounds" in a human corneal epithelial cell line. I have examined the involvement of extracellular ATP, phospholipase D, Src-family kinases (SFKs), and the focal adhesion kinase Pyk2, all of which are signals that have been hypothesized to be stimulated by environmental cues related to wounding and to activate the EGFR.

I have found that the proximal mechanism of EGFR activation is the proteolytic release of membrane-bound ligands, which is regulated by activation of SFKs. After wounding, multiple pathways converge on SFKs to regulate EGFR activation and cell motility. In one pathway, extracellular ATP transactivates the EGFR through phospholipase D2. In a distinct pathway that functions specifically near the wound edge, Pyk2 triggers SFK and EGFR activation. Finally, my data suggest the presence of a third distinct pathway that promotes SFK and EGFR activation in response to a physically unconstrained edge. By delineating signaling pathways that stimulate EGFR activation, I have identified potential therapeutic targets for modulating EGFR signaling and cell motility in wound healing and other pathologies.

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PREFACE

ACKNOWLEDGMENTS

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This research has been aided by a number of others who have shared reagents and facilities: Joseph C. Loftus (Mayo Clinic, Scottsdale, AZ) supplied Pyk2- and PRNK-expressing adenovirus; Sylvain G. Bourgoin (Université Laval, Quebec, QC, Canada) supplied anti-PLD2 antibodies; adenovirus encoding tet-OFF was from Ora A. Weisz (University of Pittsburgh, Pittsburgh, PA); adenovirus encoding GFP was from Michael P. Czech (University of Massachusetts, Worcester, MA); Ilene K. Gipson (Harvard Medical School, Boston, MA) supplied the HCLE cells; Bridget M. Deasy and Steven M. Chirieleison (University of Pittsburgh, Pittsburgh, PA) helped with the time-lapse microscopy. This work was supported by the National Institutes of Health Grants EY013463, EY08098, and T32 EY017271 and grants from Research to Prevent Blindness and The Eye and Ear Foundation (Pittsburgh, PA).

It is an honor to thank my family for all they have done for me over the years. My parents, Bruce and Marian, have given me every opportunity to succeed, but even when I haven't, they have loved and been proud of me. My father-, mother-, and brother-in-law, Ali, Audrey, and Will, have taught me so much in just a short time, have encouraged me, and have made me feel that my research is important and exciting. My sister, Adrienne, in whose footsteps I followed in studying biology, has given me unyielding support, endless reasons to laugh, and the inspiration to do good in my life. My wife, Azi, aka Dr. Block, PhD, has simply saved my life. Her energy, intelligence, work ethic, compassion, and love have given me the strength to persevere, the motivation to succeed, and the confidence to believe in myself. Finally, I thank our two-month-old son, Jonah, for filling my life with joy. I have never before looked so positively toward the future. To all of you, my family, I dedicate this dissertation.

ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
AG	Tyrphostin AG 1478 (EGFR inhibitor)
APY	Apyrase
AR	Amphiregulin
CBX	Carbonexolone
Cx	Connexin
DAG	Diacylglycerol
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ErbB	Erythroblastic leukemia viral oncogene homolog
ERK	Extracellular signal-regulated kinase
FAK	Focal Adhesion Kinase
FFA	Flufenamic acid
GA	Glycerrhetinic acid
GPCR	G-protein-coupled receptor
HB-EGF	Heparin binding EGF-like growth factor
HCLE	Human Corneal Limbal Epithelial
HGF	Hepatocyte growth factor
LASIK	Laser-assisted in situ keratomileusis

LDH	Lactate dehydrogenase
LPA	Lysophosphatidic acid
LY	Lucifer yellow
MAPK	Mitogen-activated protein kinase
MARCKS	Myristoylated alanine-rich C kinase substrate
MMP	Matrix metalloprotease
P2Y	Type 2-Y purinergic receptor
PA	Phosphatidic acid
PBS	Phosphate-buffered saline
PIP2	Phosphatidylinositol (4,5)-bisphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PLD	Phospholipase D
polyHEMA	Poly(2-hydroxyethyl methacrylate)
PP2	Src-family kinase inhibitor PP2
PRK	Photorefractive keratectomy
PRNK	Pyk2-related non-kinase
Pyk2	Proline-rich tyrosine kinase 2, or focal adhesion kinase 2
RB2	Reactive Blue 2
SFK	Src-family kinase
SKI	Src-family kinase inhibitor I
TGFα	Transforming growth factor α

1.0 INTRODUCTION

1.1 EPITHELIAL WOUND HEALING

The fundamental role of an epithelium is to provide a barrier to the outside environment. Wounding severely disrupts the epithelial barrier, leaving the organism prone to infection, fluid loss, and other consequences of unregulated permeability. The longer a wound takes to heal, the greater the risk to life and limb. Although powerful and efficient mechanisms have evolved to restore barrier function rapidly after wounding, many wounds heal inefficiently, resulting in malfunctioning tissue, and some wounds do not heal at all.

Wounds come in a variety of forms: wounds to the "external" epithelia such as the skin and the cornea can be caused by traumatic abrasions, chemical burns, or surgery; wounds to the "internal" epithelia such as the gastrointestinal tract and the airway are typically caused by inflammatory diseases. Genetic, molecular, and cellular approaches have shown that wound repair is accomplished through a complex series of events that may involve varying degrees of participation by not only epithelial cells, but also fibroblasts in the underlying stroma, platelets, nerves, immune and inflammatory cells, and endothelial cells (Cole et al., 2001; Grose and Werner, 2004; Gurtner et al., 2008; Jacinto et al., 2001; Jane et al., 2005; Martin, 1997).

The role of non-epithelial cells in the healing process varies greatly depending on the cause and location of the wound, but the normal response of epithelial cells themselves is quite

similar: cells become highly motile and migrate as a collective sheet to close the epithelial defect (Friedl and Gilmour, 2009; Martin and Parkhurst, 2004). Epithelial cells do eventually divide to replace lost cells, but the quickest fix to re-epithelialization is cell migration.

When an epithelium is wounded, cells at the edge undergo profound phenotypic changes and subsequently acquire an ability to migrate rapidly. Cell motility is a complex multistep process that is accomplished through repeated cycles of 1. protrusion of the leading edge into the free space, 2. adhesion of these protrusions to the extracellular matrix, 3. de-adhesion from the matrix at the trailing edge, and 4. contraction of the cell body. This biomechanical feat is accomplished through highly integrated processes involving the modulation of substrate adhesions and rearrangements of the dynamic actin cytoskeleton (Jacinto et al., 2001; Kaverina et al., 2002; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996; Nobes and Hall, 1999; Thiery and Sleeman, 2006).

The mechanics of cell motility during wound healing have been studied extensively, but the cellular signals that induce these dramatic phenotypic changes after wounding are poorly understood. The broad goal of this dissertation research was to clarify the cellular signals that promote the induction of motility after wounding so that therapies may be developed to ameliorate normal and pathological healing.

1.1.1 Wound healing in the cornea

Of all epithelial wounds, those inflicting the cornea are perhaps the most prone to causing major morbidity, even for small wounds in otherwise healthy individuals. The reason for this is simply anatomical (Figure 1). The cornea is the most anterior tissue in the eye; it is the first tissue through which light travels on its path through the lens and finally to the retina, where

photostimuli are transduced into neural impulses. The cornea is normally transparent and is responsible for roughly two thirds of the eye's refractive power. The integrity and health of the cornea are critical for the maintenance of transparency, so wounding can lead to low vision and even blindness. Corneal pathologies are the second leading cause of blindness worldwide and complications from corneal wounds account for an estimated one to two million new cases of monocular blindness every year (Whitcher et al., 2001).

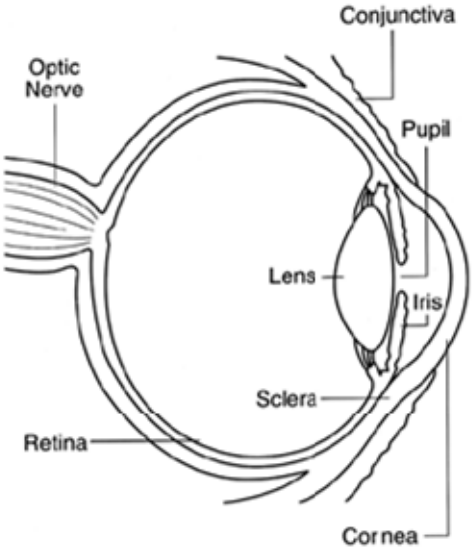

Figure 1 Anatomy of the eye

The cornea is the most anterior tissue and is the first to refract light as it travels to the retina. The limbus (not marked) is the area where the cornea meets the sclera.

The transparency and refractive power of the cornea are a result of the highly organized collagen/proteoglycan lattice that comprises the corneal stroma. As seen in the human corneal cross-section in figure 2, the stromal layer makes up about 90% of the cornea's thickness. It is interspersed with a syncytium of specialized mesenchymal cells, keratocytes, which regulate the production and organization of the collagen lattice (Funderburgh, 2002; Funderburgh et al., 2003). The most anterior layer of the cornea is a stratified layer of corneal epithelial cells. The epithelium is bathed in the tear film, which contains mucous, aqueous (ions, growth factors, etc), and lipid components (Gipson, 2007). An endothelial monolayer separates the stroma from the aqueous humor and regulates hydration and nutrition of stromal components. Aiding

transparency even further, the cornea is normally avascular and plays host to very few resident immune cells. The cornea is, however, heavily innervated. These nerves secrete neurotropic factors such as substance P that support the health of epithelial cells and their stimulation signals tearing responses by the lacrimal glands (Davis and Dohlman, 2001; Tsubota, 1998).

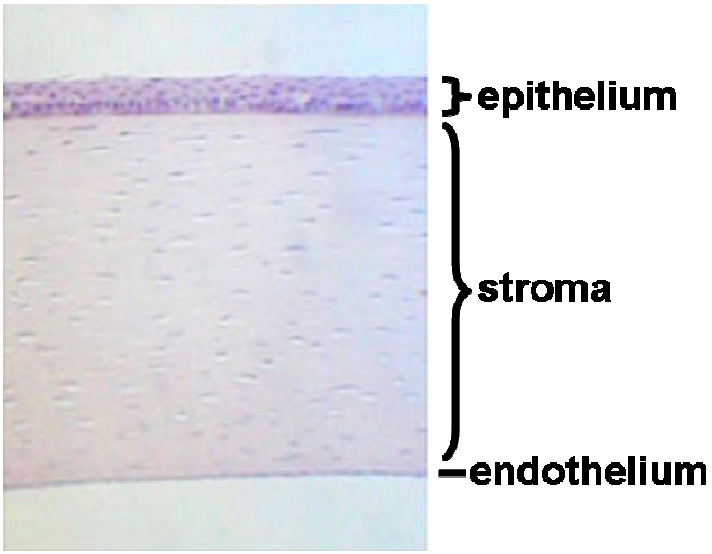

Figure 2 The cornea has a simple layered structure

The three cellular layers of the cornea: the stratified epithelium, the stroma with interspersed keratocytes (dark spots), and the endothelial monolayer. The cornea is normally avascular and heavily innervated (not visible).

Epithelial wounds and defects heal through tightly regulated and highly coordinated phenotypic changes to the corneal cell layers (Bazan, 2005; Dupps and Wilson, 2006; Fini and Stramer, 2005; Netto et al., 2005; Wilson et al., 2001; Wilson et al., 2003). Immediately after wounding, cytokines released from wounded epithelial cells such as interleukin-1α and tumor necrosis factor-α induce apoptosis of stromal keratocytes proximal to the sight of injury. This may serve as a safety mechanism for inhibiting the replication of infectious agents. Simultaneously, in the epithelium, growth factors, such as epidermal growth factor (EGF) and hepatocyte growth factor (HGF) in the tear film begin to stimulate cell motility and re-epithelialization (Baldwin and Marshall, 2002; Imanishi et al., 2000; Klenkler and Sheardown, 2004; Klenkler et al., 2007; Yu et al., 2009).

As re-epithelialization progresses, the corneal stroma commences repair processes of its own. Immune cells are summoned by chemokines to clear debris and fight infection. Also, the keratocytes in the stroma migrate to the wound site and undergo a dramatic phenotypic transformation into myofibroblasts, in large part due to the actions of transforming growth factor-β (Saika, 2004). Myofibroblasts remodel and repair the collagen lattice and promote migration of epithelial cells.

While these repair processes are necessary for proper healing, they are also potentially disastrous to vision. Excess numbers of immune cells and myofibroblasts can impair vision because they are less transparent than keratocytes. Also, excessive stimulation of immune cells can lead to further inflammation and even corneal vascularization. Excessive myofibroblast stimulation can lead to the deposition of disorganized extracellular matrix (fibrosis), leading to corneal "haze" or scaring (Netto et al., 2006). The signals that can hyper-stimulate the immune and keratocyte responses are present as long as re-epithelialization is incomplete (Netto et al., 2005), so clearly, the faster epithelial cells migrate to cover the wound, the lesser the chance of poor clinical outcomes.

Causes and clinical outcomes of corneal wounds are highly variable. Accidental abrasions that do not penetrate the epithelial basement membrane typically heal rapidly and without complication (Wilson and Last, 2004). Wounds that penetrate into the stroma or through the endothelium take longer to heal and are therefore more prone to infection and scarring. The cornea can also be wounded by chemical, thermal, and electrical burns, which are further complicated by persistent modification of the basement membrane that can lead to corneal ulcers (Kato et al., 2006).

Elective vision-correcting surgeries represent a class of increasingly common corneal wounds (Kuo, 2004; Schallhorn et al., 2006). During laser-assisted in situ keratomileusis (LASIK) an incision is made around almost the entire circumference of the cornea and this corneal flap is lifted to allow correction of the stromal surface beneath. Epithelial cells must heal around the edges when the flap is replaced. During photorefractive keratectomy (PRK), the underlying stroma is accessed by complete debridement of the central cornea, and peripheral epithelial cells must migrate to cover the denuded stroma.

A number of underlying medical conditions can also cause corneal defects, which are similar to wounds in that they result in the breakdown of the epithelial barrier due to loss of epithelial cells. Some of the most common of these include viral infection (Kaye and Choudhary, 2006), fungal infection (Thomas, 2003), dry eye (the most common presenting ocular disorder that is a collection of diseases affecting the volume and/or composition of the tear film) (Gipson, 2007; Tsubota, 1998) and neurotropic keratitis (the loss or disease of corneal nerves) (Bonini et al., 2003; Nishida and Yanai, 2009; Pushker et al., 2001).

Not only do these underlying conditions cause epithelial defects, but along with diabetes (Chikama et al., 2007), prior corneal transplant (Obata and Tsuru, 2007), and corneal dystrophies (Das et al., 2005) they also impair healing of even minor surface abrasions. The damage to corneal nerves from LASIK and PRK causes defects in tears and neurotropic factors that impedes re-epithelialization not only during recovery from surgery but also after future wounds (Belmonte, 2007).

The longer a corneal wound or defect remains open, the higher the risks of scaring, infection, and loss of sight, yet there is currently no commonly-used therapy for increasing healing rates of the corneal epithelial cells (Cameron, 2007; Duane et al., 2002; Wilson and Last,

2004). Treatment of corneal wounds consists of antibiotics, and when applicable, treatment of the underlying corneal pathology. The great clinical need for therapies that increase epithelial healing rates has inspired investigators for decades to study signal transduction pathways that regulate cell motility and to apply their findings in the clinic.

1.2 THE EPIDERMAL GROWTH FACTOR RECEPTOR

One molecular signal that has been identified as being important for epithelial motility is activation of the EGF receptor (EGFR, also ErbB1) (Jorissen et al., 2003; Warren and Landgraf, 2006; Wells, 1999). Activation of the EGFR tyrosine kinase is stimulated by wounding and is absolutely required for enhanced cell migration and healing of numerous epithelia, including the gut, airway, epidermis, and cornea (Goodlad and Wright, 1995; Puddicombe et al., 2000; Repertinger et al., 2004; Zieske et al., 2000).

The EGFR is a receptor tyrosine kinase of the ErbB family that also includes ErbB2 (HER2/Neu), ErbB3, and ErbB4. Unliganded ErbB receptors are held in an autoinhibited conformation, but upon ligand binding, allosteric conformational changes release autoinhibition, increase receptor dimerization, and elevate kinase activity (Zhang et al., 2006). Trans- and autophosphorylation of cytoplasmic tyrosine residues after activation can be detected by Western Blotting. I routinely use antibodies directed against the EGFR phosphorylated on tyrosine-1173 as a marker for receptor activation.

1.2.1 EGFR signaling and epithelial motility

The tyrosine-phosphorylated EGFR activates a myriad of signaling cascades that promote epithelial cell motility (Figure 3). Phosphotyrosine (pY) residues serve as docking platforms for numerous substrates, enzymes, and adaptors. For example, pY1173 is the docking site for phospholipase C (PLC)-γ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) and stimulates calcium signaling, as well as for the adaptor protein Shc, which couples EGFR activation to signaling by the mitogen-activated protein kinases (MAPKs) Extracellular signal-Regulated Kinases (ERK1/2, or p44/42 MAPK) (Katz et al., 2007; McKay and Morrison, 2007; Rubinfeld and Seger, 2005). Other pY residues stimulate activation of phosphatidylinositol 3'-kinase and Src kinase signaling (Jorissen et al., 2003; Yarden and Sliwkowski, 2001).

The myriad of signaling cascades activated downstream of the EGFR work in concert to stimulate motility through a number of effector pathways that directly regulate the biomechanics of motility. Importantly, EGFR activation, at times through ERK1/2, leads to the dissolution of cell/matrix bonds that stabilize epithelial cells in place and mobilizes actin-based protrusions and contractions that propel the cell forward (Chen et al., 1996; Dieckgraefe et al., 1997; Dise et al., 2008; Fitsialos et al., 2007; Glading et al., 2000; Hudson and McCawley, 1998; Jorissen et al., 2003; Wells et al., 1998; Xie et al., 1998).

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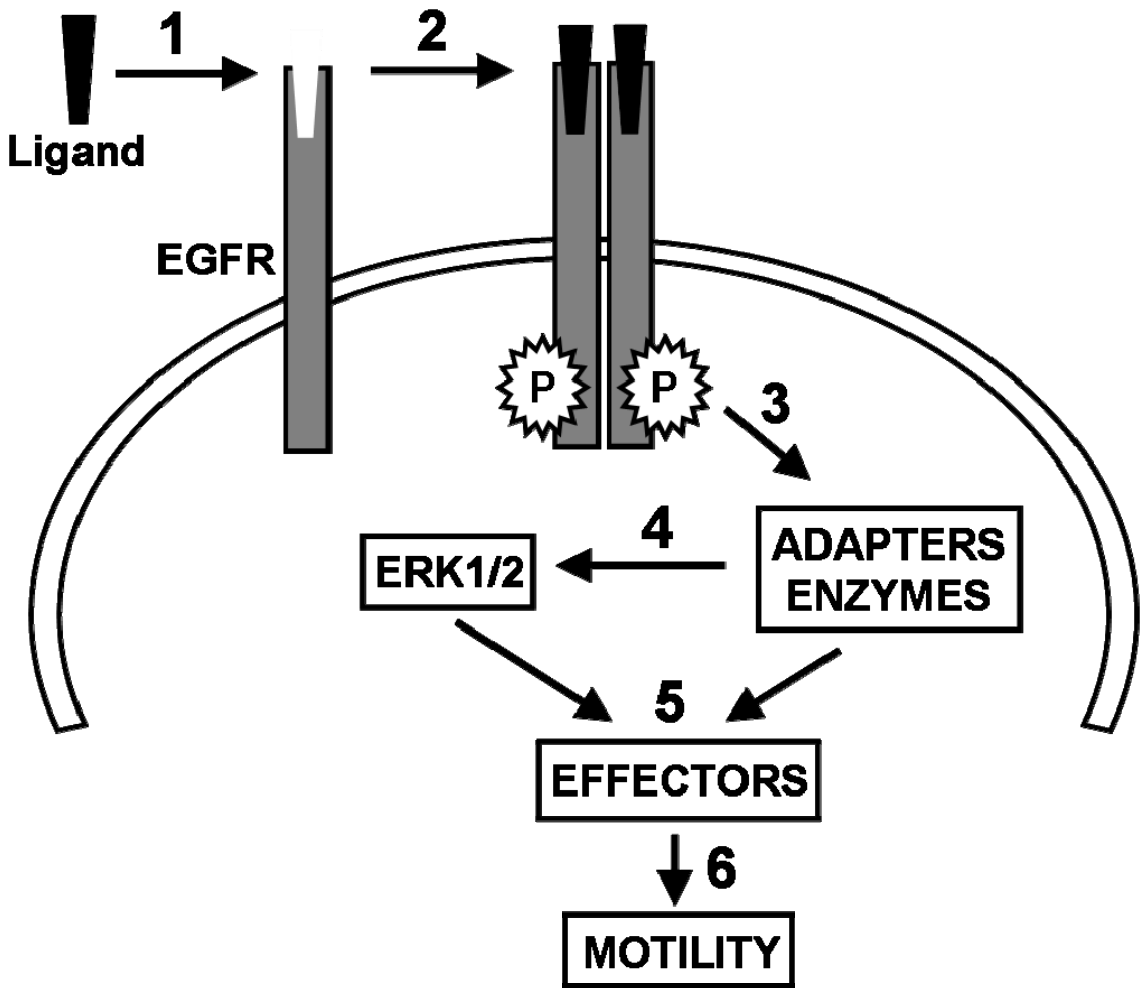

Figure 3 EGFR activation stimulates signaling cascades that promote motility

An EGFR ligand binds the receptor leading to receptor dimerization and transphosphorylation of cytoplasmic domain tyrosines. Phosphotyrosines present docking sites for enzymes and adaptors that initiate numerous signaling cascades, including ERK1/2, that promote motility through various effector pathways. Depicted as a unidirectional linear pathway for simplicity. See text for details.

1.2.2 Wound-induced EGFR activation

ErbB ligands such as amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), and transforming growth factor-α (TGFα) are synthesized as membrane-bound precursors

(Harris et al., 2003; Higashiyama et al., 2008). The EGFR can be activated by a variety factors that stimulate the release of these ligands, which is dependent on proteases of the matrix metalloprotease (MMP) and A Disintegrin And Metalloprotease (ADAM) families (Blobel, 2005; Daub et al., 1996; Fischer et al., 2003; Ohtsu et al., 2006; Sanderson et al., 2006). EGFR activation by proteolytic ligand release, first described following stimulation with G-protein-coupled receptor (GPCR) agonists, has been termed the "triple membrane-passing signaling" (TMPS) mode of EGFR activation and is now known to occur following stimulation by a variety of stimuli such as ultraviolet radiation and receptor tyrosine kinase activation (Figure 4) (Higashiyama et al., 2008; Prenzel et al., 2000).

The proximal event for wound-induced EGFR activation in numerous epithelia such as the skin, gut, airway, and cornea is this very TMPS mechanism (Block et al., 2004; Davies et al., 1999; Myhre et al., 2004; Shirakata et al., 2005; Tokumaru et al., 2000; Xu et al., 2004). Methods to study the TMPS mechanism include the use of inhibitors of the ligand proteases, anti-ligand neutralizing antibodies, anti-EGFR antibodies that bind to and block the ligand binding domain, and conditional knockouts of EGFR ligands. In the corneal epithelium, EGFR activation and the induction of cell motility after wounding is regulated by the ligands HB-EGF and AR (Block et al., 2004; Mazie et al., 2006; Spix et al., 2007; Xu et al., 2004).

The intracellular signals that regulate activation of EGFR ligand proteases are poorly understood, but recent data suggest the involvement of Src-family kinases (SFKs) (Zhang et al., 2004). The Src family includes the ubiquitously expressed c-Src, Fyn, and Yes isoforms. These kinases regulate numerous cellular events related to cytoskeletal dynamics, cell/matrix interactions, and the transduction of extracellular signals (Abram and Courtneidge, 2000; Ingley, 2008; Roskoski, 2005; Thomas and Brugge, 1997). It is likely that SFKs also control EGFR

ligand shedding after wounding because they are activated after wounding and their activity is necessary for EGFR activation (Xu et al., 2006; Yamada et al., 2000). Therefore, deciphering mechanisms of wound-induced EGFR activation may go hand-in-hand with understanding mechanisms of SFK activation.

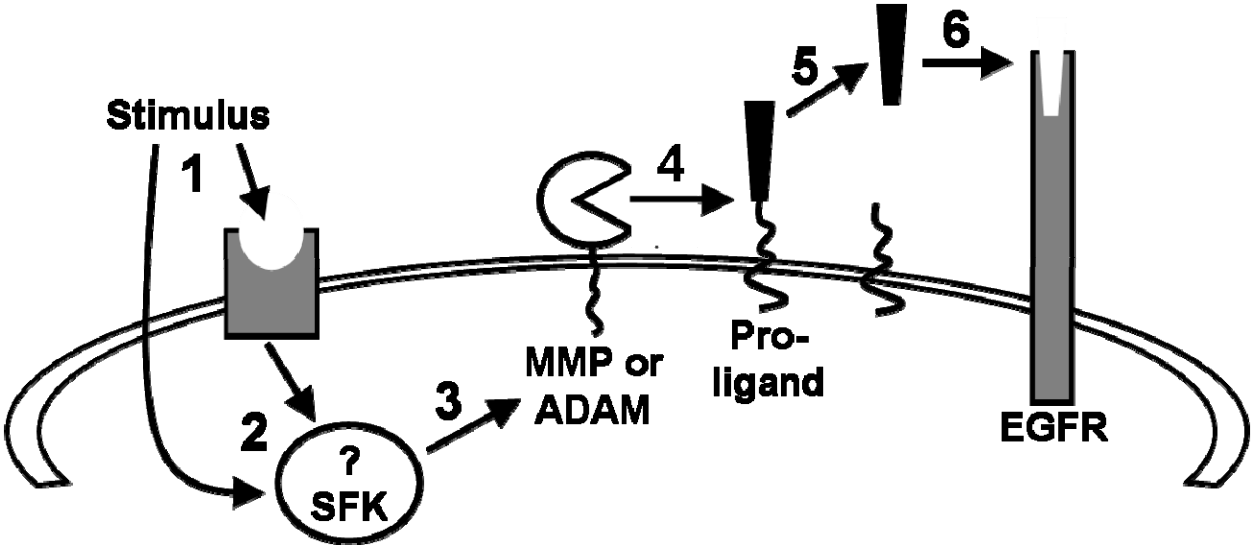

Figure 4 Triple Membrane-Passing Signaling (TMPS) mechanism for EGFR activation

Extracellular stimuli such as a GPCR agonist or UV irradiation trigger activation of poorly understood intermediate signals that include SFKs. Consequently, a protease of the MMP or ADAM family is activated, which cleaves an EGFR pro-ligand. The released ligand then binds and activates the EGFR.

Signaling by extracellular ATP, by phospholipase D (PLD), and by the focal adhesion kinase Pyk2, among others, have been recently hypothesized to contribute to wound-induced EGFR activation because they are known to be active after wounding and to stimulate SFK and/or EGFR signaling in other systems (Boucher et al., 2007; Klepeis et al., 2004; Mazie et al., 2006; Yang et al., 2004; Yin et al., 2007; Yu et al., 2009)

Recently, a number

1.2.3 EGFR signaling in the clinic

The overwhelming experimental evidence indicating that EGFR activation regulates wound closure suggests an obvious and simple clinical solution for corneal wounds: treatment with EGF. The actual clinical implementation and success of such treatments, however, has proven to be not so simple. Whereas a number of studies have indicated that EGF accelerates corneal wound healing in a variety of animal models and in humans (Baldwin and Marshall, 2002; Imanishi et al., 2000; Klenkler and Sheardown, 2004; Lu et al., 2001; Pastor and Calonge, 1992), the overall clinical utility of EGF has been hampered by a number of problems. The biggest hurdle to EGF therapy has been one of drug delivery. EGF and most soluble, non-cell-permeable factors are rapidly washed away from the epithelial surface in the tear film after blinking, so frequent high doses, exceeding five applications of 10 μg/ml EGF solutions per day, have been required. Another complication with EGF therapy has been activation of myofibroblasts in the corneal stroma, which is exposed after wounding. Activated myofibroblasts can cause low vision by promoting corneal haze through the aberrant production and distribution of extracellular matrix (He and Bazan, 2008). As a result of these complications, EGF is not commonly used and, as stated earlier, there remains no established therapy for hastening the corneal healing process (Cameron, 2007).

The broad goal of this dissertation was to identify molecular mechanisms of wound-induced EGFR activation. It is expected that such information will allow new therapeutic strategies to be developed for improving normal and pathological healing of many tissues. For example, the identification of factors upstream of proteolytic ligand release could suggest therapies that overcome many of the hurdles encountered by EGF therapy by promoting

increased and long-lasting shedding of endogenous EGFR ligands. Additionally, delineating signaling pathways leading to EGFR activation may help to identify mechanisms by which conditions such as diabetes, infection, and dry eye inhibit healing (Kaye and Choudhary, 2006; Klenkler et al., 2007; Wakuta et al., 2007).

1.3 MODELS TO STUDY WOUND SIGNALING

1.3.1 The cornea and corneal cell culture

For decades, the cornea has been a popular model to study wound healing. The first reason for this is clinical: as detailed in the previous section, it is necessary to understand the cellular mechanisms of corneal epithelial wound healing to treat wounds and improve surgical outcomes. The second reason is practical: the cornea is easily accessible for study in intact animals as well as in organ culture (of whole globes or excised corneas) and in cell culture. The final reason is that the corneal epithelium serves as a model for epithelial cell motility in general. The cornea has a simple layered structure (Figure 4) and is normally avascular and wounds heal by the collective migration of epithelial sheets. One can apply what is learned from corneal healing, which is typically efficient and scar-free, to the normal and pathological motility of many epithelia.

Cell culture is a relatively cost-efficient yet powerful method for studying epithelial healing. Although some aspects of wound healing are lost, such as interactions with vascular and lymphatic elements, the most basic and essential aspect of wound healing, that of epithelial migration, can be closely approximated in cell culture. The signals that stimulate a wounded

epithelial sheet to migrate have been studied extensively in cell culture. Many growth factors, including EGF, were observed to enhance migration of cultured cells, leading to the development of promising therapies (Hori et al., 2007; Imanishi et al., 2000; Pastor and Calonge, 1992; Watanabe et al., 1987).

Cultured primary corneal epithelial cells and cell lines have been used extensively. Human corneal epithelial cells grown from primary tissue explants (Ebato et al., 1987) most closely resemble cells *in vivo*, but donor tissue is scarce and highly variable due to age and genetic background. Primary cells grown from rabbit explants are less scarce and less variable, but are incompatible with many human-specific reagents. Human corneal epithelial cells immortalized by the SV40 large T-antigen have been established and are frequently used (Ali and DeCaprio, 2001; Kahn et al., 1993). Most of the experiments described here have been performed in a different cell line, Human Corneal Limbal Epithelial (HCLE) cells, which have been immortalized by abrogation of $p16^{INK4A/Rb}$ and p53 functions and overexpression of the catalytic subunit of the telomerase holoenzyme (Gipson et al., 2003). They express markers of the differentiated corneal epithelium such as the corneal epithelial-specific keratins K3 and K12, and serve as a model for native corneal epithelial cells.

1.3.2 Minimizing cell damage

Wound healing has traditionally been studied in cultured cells by use of the scratch assay (Liang et al., 2007): a confluent cell sheet is scratched with a pipet tip or similar implement, scraping away a swath of cells, and cell migration is monitored over time. In response to the scratch, epithelial cells migrate into the denuded area as a collective sheet. The scratch assay has proved

to be an excellent method for studying collective cell migration, a process distinct from unicellular (amoeboid or mesenchymal) migration (Friedl, 2004; Friedl and Gilmour, 2009).

To study the cellular signaling caused by wounding, the scratch assay has again been an effective tool. Following singular or repeated scratches, signals are detected either by various imaging techniques or by preparing extracts of the remaining cells for biochemical analysis. Scratch-wounding of cultured cells closely approximates an *in vivo* scratch wound and is an essential tool for understanding the breadth of epithelial responses to wounding.

A scratch wound is a complex stimulus: cells are challenged with the loss of cell/cell contacts, cell stretch, cell lysis, and the sudden availability of free space, among other perturbations. Each of these factors stimulates a host of cellular signals that regulate the numerous epithelial responses to wounding: cells rupture and die, they undergo apoptosis, they repair damaged membranes, they proliferate, they increase phagocytosis of cell debris, and they increase production of extracellular signals that affect inflammation or vascularization. To complicate matters, these processes may vary greatly depending on the tissue or the type of wound.

Of all the epithelial responses to wounding, the only one that is absolutely required for re-epithelialization is the induction of motility. Identifying the precise cues that induce motility after wounding is a major focus of wound research in general (Block et al., 2004; Martin and Parkhurst, 2004; Tschumperlin, 2004; Vermeer et al., 2003; Xu et al., 2006; Yin et al., 2007), and of this dissertation in particular. From a practical standpoint, it is therefore necessary to eliminate some of the signaling "noise" caused by wounding so that only the signals that regulate the transition to motility can be studied.

To minimize the signaling effects of cell damage and the loss of cell/cell contacts, we have previously developed a method for "wounding" sheets of cells by the removal of agarose barriers: cells are cultured to confluence around a thin agarose strip to which they do not adhere, and the cell sheet is subsequently "wounded" by the removal of this strip. We do not detect cell damage after wounding cell sheets in this manner, and importantly, gaps close at the same rate as when wounds are made by scraping (Block et al., 2004). Similar observations have since been made using a thin film of polydimethylsiloxane instead of agarose (Poujade et al., 2007). Therefore, the signals that are necessary for normal wound healing in culture are elicited simply by the removal of physical barriers.

I use the term "wounding" to describe the stimulation of cell sheets by the removal of agarose barriers, but I recognize that it is somewhat misleading, given that there is no (or minimal) actual damage to the cells. Since the removal of physical barriers stimulates cells with a subset of the stimuli induced by conventional wounding while promoting motility to a similar degree, I feel that this research is directly applicable to wound healing and that the terminology is at least less cumbersome, if not justified.

1.3.3 Signaling at the edge

A number of studies have reported variations in cell signaling with respect to distance from the wound. For example, wound-induced increases in intracellular calcium concentrations near the wound edge depend on extracellular calcium, but increases far from the wound edge depend on intracellular calcium (Klepeis et al., 2001). Also reactive oxygen species have been observed in cells near the wound edge but not farther away (Nikolic et al., 2006). This may be explained by the obvious hypothesis that cells near the edge of a wound are subjected to a number of spatial

and mechanical perturbations while cells far from a wound are more influenced by the release of soluble factors. Many studies also assert that the cells near the wound edge are vital for healing because it is in edge cells where activation of the cellular migration machinery is necessary (Fenteany et al., 2000) and where increased velocities have been measured (Farooqui and Fenteany, 2005; Poujade et al., 2007). It is therefore important to study spatial aspects of signaling and to characterize signaling specifically in near-edge cells.

To monitor the spatial aspects of EGFR signaling in my own studies, I have developed methods to biochemically analyze cells that are either near or far from wounds. To prepare extracts of cells far (>3 mm) from wounds for biochemical analysis, reactions are slowed on ice before removing cells near the wound, and then prepare extracts from the cells that remain (Figure 5 and for details see Methods 8.5). As a control, cells are scraped away from a confluent, unwounded culture.

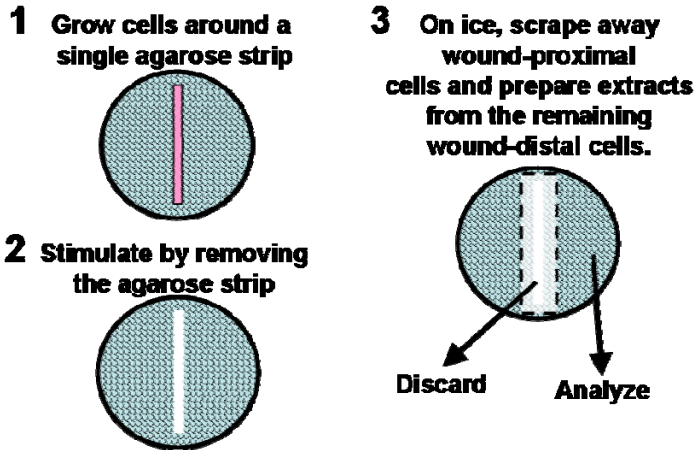

Figure 5 A method for detecting signaling selectively in wound-distal cells

Agarose is shown in pink. After stimulation, 3 mm from each side of the wound is removed.

Due to low yield, the cells that are removed from near the wound are difficult to analyze. To selectively analyze cells near the wound edge, we have previously developed a method for culturing cells around agarose droplets, and then "wounding" the cells by removing the droplets

(Block et al., 2004) (Figure 6 and for details see Methods 8.4). As a control, agarose droplets are left in place or removed on ice.

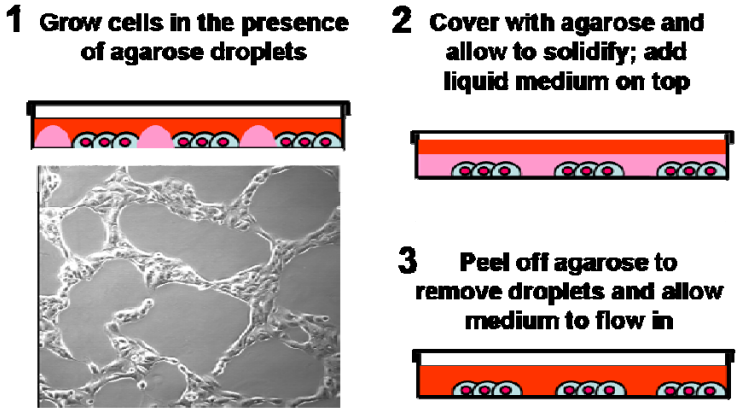

Figure 6 A method for detecting signaling selectively in wound-proximal cells

Agarose is shown in pink. Phase contrast image is of cells grown around agarose droplets.

Wounding by the removal of agarose droplets minimizes cell damage in comparison to scrape-wounding (see Chapter Four), but cells are still stimulated by numerous factors such as free extracellular matrix and the loss of physical constraints. Because physically unconstrained, or "free" edges are inherently present in all wounds, we have also developed a model to study the signaling caused by the presence of a free edge in the absence of cell damage or leading-edge matrix adhesions. In this model, cells are cultured on thin plastic strips that sit on polyHEMA, a compound to which cells do not adhere (Figure 7 and for details see Methods 8.6). Cultures prepared on plastic strips have many free edges, while control cultures have virtually none. A major difference between this and the agarose methods is that cells cultured on plastic strips are not stimulated acutely. Therefore, the plastic strip method may be more applicable to studying the signaling from cells in migrating epithelial sheets, which occur hours after wounding or during development.

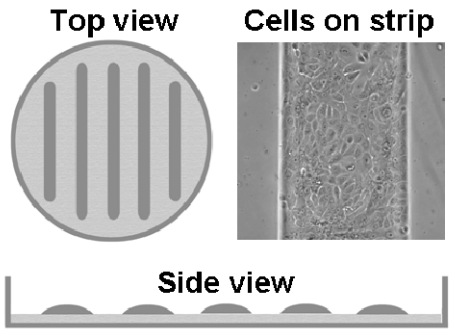

Figure 7 A method for culturing cells with many free edges

Schematic of plates covered with polyHEMA and plastic strips (not to scale). Light grey is polyHEMA; dark grey is plastic. Phase contrast image is of cells grown on plastic strips.

The models described above minimize signaling noise, yet still allow the study of signals that induce migration. Additionally, these models allow me to determine the spatial range of a signal and to study signaling specifically at the wound edge.

1.4 SUMMARY, GOAL, AND HYPOTHESES

The function of a wounded tissue and the health of an organism is jeopardized as long as an epithelial wound remains open. Wounds to the cornea can lead to impairment or even loss of vision. Cell migration is absolutely required for the closure of epithelial wounds, and EGFR activation has been identified as an essential signal for the induction of motility after wounding. To study EGFR activation, I have developed novel wounding models that minimize cell damage and allow biochemical analysis of cells specifically near the wound edge.

The goal of this dissertation was to gain a better understanding of the cellular signals that induce migration after epithelial wounding. By clarifying mechanisms of EGFR activation after wounding, my findings are expected to suggest new therapeutic avenues for improving normal and pathological healing. To that end, I have tested the following primary hypotheses (and see diagram in Figure 8):

1. Mechanisms of EGFR activation vary with respect to distance from a wound.

2. Extracellular ATP stimulates wound-induced EGFR activation through Phospholipase D.

3. ATP is released from the wound edge through connexin hemichannels.

4. EGFR activation near the wound edge is regulated by Src-family kinases and Pyk2.

5. EGFR activation is stimulated by the presence of a free edge.

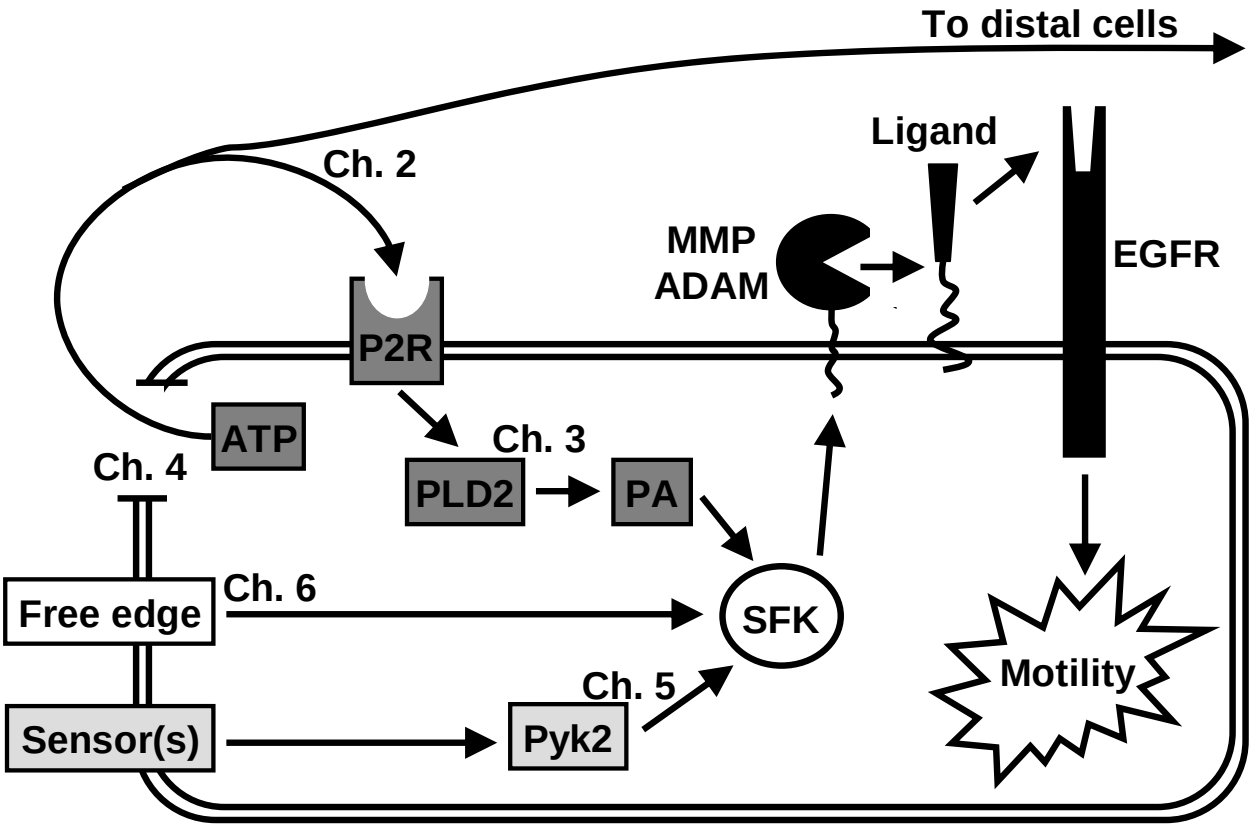

Figure 8 A hypothetical model for mechanisms of EGFR activation after epithelial wounding

A number of hypotheses have been tested in this dissertation to clarify mechanisms of wound-induced EGFR activation. The chapters describing the testing of these hypotheses are shown near their respective pathways. P2R, type-2 purinergic receptor; PLD2, phospholipase D2; PA, phosphatidic acid; Pyk2, the focal adhesion kinase Pyk2; SFK, Src-family kinase; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; EGFR, epidermal growth factor receptor.

2.0 DUAL MECHANISMS ACTIVATE THE EGFR AFTER EPITHELIAL WOUNDING

2.1 INTRODUCTION

Activation of the EGFR is absolutely required for the induction of epithelial motility after wounding. Recent research suggests that extracellular ATP may regulate wound-induced EGFR activation (Boucher et al., 2007; Yin et al., 2007). In addition to their numerous intracellular functions, ATP and other nucleotides function as extracellular signaling molecules (Burnstock, 2006; Schwiebert and Zsembery, 2003). Extracellular ATP signals by binding to the P2X class of cation channels and the P2Y class of G-protein coupled receptors (Abbracchio et al., 2006). ATP may also be metabolized by ecto-enzymes to adenosine, which activates the P1 class of receptors. ATP is released from epithelial cells following mechanical perturbation and is released from corneal epithelial cells after wounding (Boucher et al., 2007). Treatment with extracellular ATP promotes EGFR activation and healing of wounds in corneal epithelial cells through P2Y receptor activation (Boucher et al., 2007; Klepeis et al., 2004; Yang et al., 2004; Yin et al., 2007).

In this section, I have tested the hypothesis that signaling by extracellular ATP is necessary for wound-induced EGFR activation. Furthermore, since extracellular ATP is freely diffusible, I was interested in analyzing the spatial aspects of EGFR activation in wounded

epithelial cell sheets. Numerous studies have concluded that cell signaling and phenotype vary in relation to distance from the wound (Fenteany et al., 2000; Klepeis et al., 2001; Nikolic et al., 2006; Omelchenko et al., 2003; Poujade et al., 2007), and that the major driving forces for epithelial migration appear to be derived mainly from the first few rows of cells from the wound edge (Farooqui and Fenteany, 2005; Fenteany et al., 2000). Therefore, I determined whether the signals that induce EGFR activation vary as a function of distance from the wound edge, and whether ATP signaling is necessary for the induction of cell motility following wounding. Portions of this chapter were published previously, ASCB copyright 2008, in http://www.molbiolcell.org/cgi/content/full/19/11/4909 (Block and Klarlund, 2008) and have, in some cases, been modified.

2.2 RESULTS

2.2.1 EGFR activation in cells near the wound edge proceeds independently of signaling by ATP.

Mechanically wounding sheets of epithelial cells results in robust release of ATP, presumably mostly as a result of leakage from damaged cells (Klepeis et al., 2001; Yang et al., 2004; Yin et al., 2007). To test whether ATP is released by our wounding model of removing agarose droplets (see Chapter One, Figure 5 and Methods), I collected conditioned media from cells wounded by this procedure and performed an ATP bioluminescence assay. As is seen in Figure 9, wounding by this procedure also results in significant release of ATP.

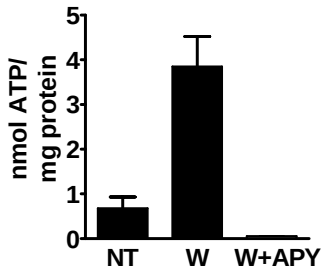

Figure 9 Wounding induces ATP release

ATP contents in conditioned media were determined in HCLE cell cultures that were unwounded (NT), wounded and incubated with medium for 10 minutes (W), or treated similarly in the presence of 5 U/ml apyrase (W+APY).

To test whether extracellular ATP is necessary for wound-induced EGFR activation, I wounded HCLE cells in the presence of apyrase, which dephosphorylates extracellular ATP and ADP to AMP. AMP is not known to signal, but can be further metabolized to adenosine, which signals through P1 purinergic receptors. To assess activation, I performed Western Blotting for the phosphorylated and total forms of the EGFR and of Extracellular signal-Regulated Kinases 1 and 2 (ERK1/2), which are down-stream targets of the EGFR that are important for wound-healing (Dieckgraefe et al., 1997; Fitsialos et al., 2007; Glading et al., 2000; Katz et al., 2007; McKay and Morrison, 2007; Rubinfeld and Seger, 2005). Although apyrase reduced ATP concentrations in the conditioned medium to below detectable limits (Figure 9), wound-induced activation of EGFR and ERK1/2 were unaffected by apyrase (Figure 10A). Extracellular ATP is known to stimulate protein kinase C (PKC) activity by binding P2Y and P2X purinergic receptors at the cell surface (Burnstock, 2006; Schwiebert and Zsembery, 2003). Therefore, as a further control to verify that the apyrase was functional, I immunoblotted for the phosphorylated forms of Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS), an indicator of PKC activity (Aderem, 1992) and observed that PKC activation was indeed blocked by apyrase (Figure 10A).

EGFR activation after wounding depends on the proteolytic release of ligands such as amphiregulin (AR) and HB-EGF (Block et al., 2004; Xu et al., 2004). I was able to detect

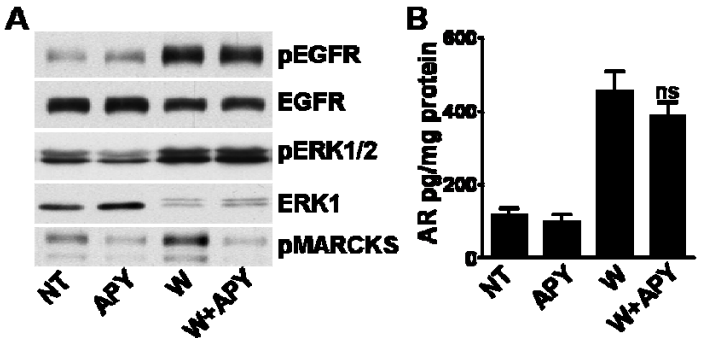

Figure 10 EGFR activation near the edge is independent of extracellular ATP

A. Western Blots for activation of EGFR, ERK1/2 and MARCKS after no treatment (NT), wounding (W), and/or treatment with 30 U/ml Apyrase (APY). **B**. Quantities of AR in conditioned media from cells treated as in A.

AR, but not HB-EGF, by ELISA of media conditioned by HCLE cells. AR release from cells near the edge was stimulated by wounding, and this was not affected by treatment with apyrase (Figure 10B). These results indicate that EGFR and ERK1/2 activation proceeds independently of extracellular ATP in cells near the wound edge.

2.2.2 ATP functions as a long-range messenger that activates the EGFR.

Because ATP is freely diffusible, I next examined whether extracellular ATP mediates EGFR activation in cells at a distance from the wound edge. To do this, I prepared extracts from cells >3 mm from the site of agarose strip removal (for details of the procedure, see Chapter One, Figure 6). Inclusion of apyrase in the medium had no detectable effect on basal levels of EGFR activity, but it abolished wound-induced stimulation (Figure 11). Similarly, wound-induced ERK activation was abrogated by treatment with apyrase. Although I did not measure ATP concentrations following agarose strip removal, the presence of apyrase-sensitive signals indicates that ATP is released from cells in this wounding model. As a control, I noted that treatment with apyrase did not reduce the activation of EGFR and ERK1/2 kinases after addition of EGF (Figure 12).

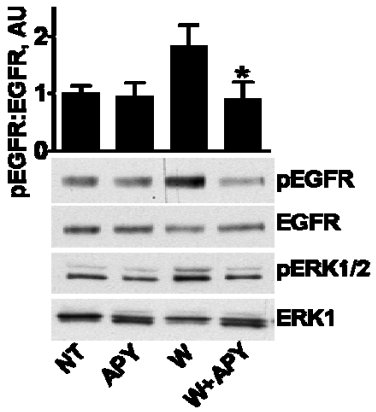

Figure 11 Extracellular ATP is necessary for EGFR activation near the wound edge

Western Blots for activation of EGFR and ERK1/2 after no treatment (NT), wounding (W), and/or treatment with 30 U/ml Apyrase (APY). * indicates significant reduction from wounded controls ($p<0.001$).

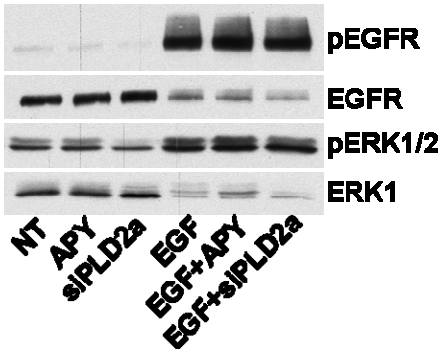

Figure 12 EGFR and ERK1/2 activation by EGF is not inhibited by apyrase or PLD2 siRNA

Western Blots for activation of EGFR and ERK1/2 after no treatment (NT), 10 min incubation with 10 ng/ml EGF, and/or treatment with 30 U/ml Apyrase (APY) or transfection with 50 nM PLD2 siRNA (see Chapter Three).

To visualize the extent of EGFR signaling induced by extracellular ATP directly, I performed immunofluorescence microscopy following stimulation by removal of an agarose strip. I was not able to identify an antibody to the activated EGFR that generated a satisfactory read-out, but phospho-ERK1/2 antibodies produced good signals. ERK1/2 were activated at least up to 5 mm from the wound edge (Figure 13). The specificity of the immunofluorescence signals was verified by the fact that UO126, which inhibits ERK1/2 activation, quenched the signals, and the EGFR-dependence of the signals was verified by the observation that they were blocked by the EGFR tyrosine kinase inhibitor AG 1478. Inclusion of apyrase in the medium resulted in quenching of the signals at a distance from the wounds, but ERK1/2 activation was still clearly detected within 250 μm of the wound edge. Together, these results indicate that

extracellular ATP is necessary for EGFR activation >250 μm from the wound edge, but that extracellular ATP-independent mechanisms function in cells near the wound edge.

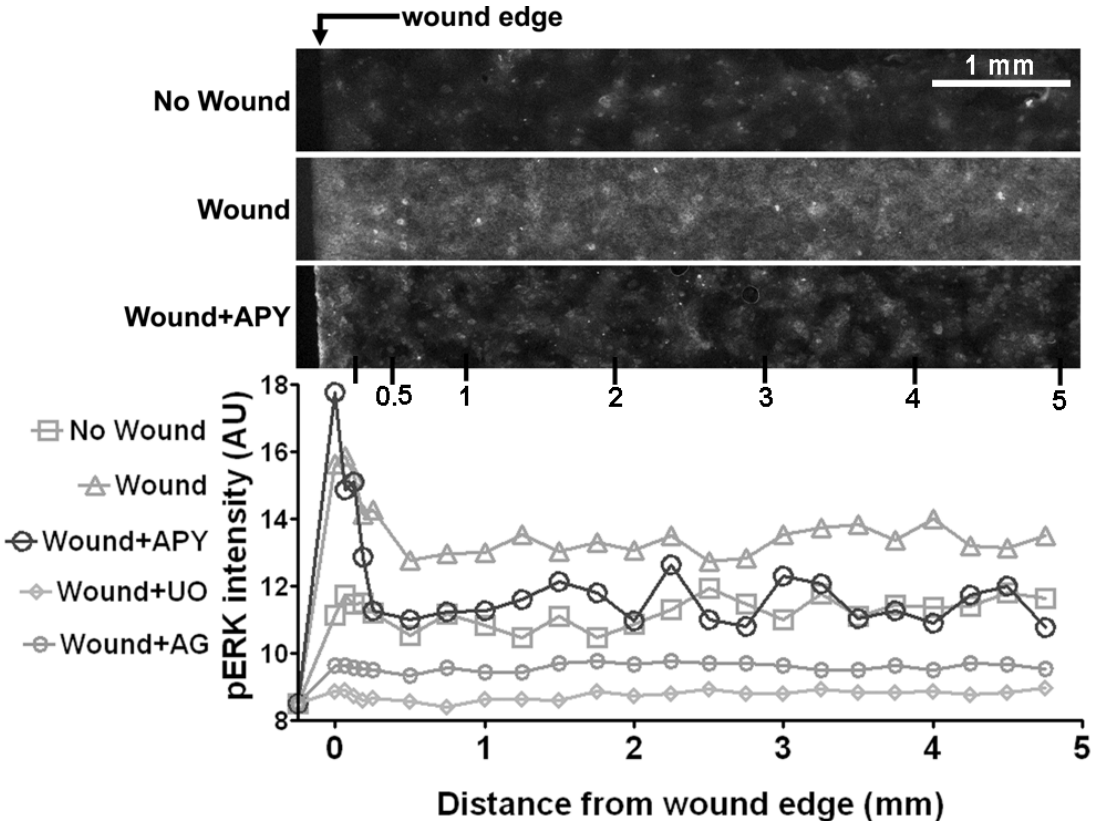

Figure 13 Activation of the EGFR/ERK1/2 pathway is dependent on signaling by ATP in cells far from, but not near wounds

Sheets of HCLE cells were cultured around an agarose strip and were left unwounded (No Wound), wounded (Wound), or wounded in the presence of 30 U/ml apyrase (Wound +APY), fixed 10 minutes later, and stained with antibodies recognizing the activated forms of ERK1/2. Results of quantitative analysis are shown below the immunofluorescence images, in the same scale. Images of cells wounded in the presence of 10 μM UO126 (Wound + UO) or 1 μM tyrphostin AG 1478 (Wound +AG) were also analyzed.

2.2.3 Wounds heal in the absence of signaling by extracellular ATP.

Cells within monolayers typically move spontaneously, and according to some models, cells do not move into wounds as a result of increases in speeds of migration, but rather as a result of the lack of mechanical constraints in the denuded area (Bindschadler and McGrath, 2007; Sherratt and Dallon, 2002). According to such models, it is not necessary to postulate the existence of any biochemical signals that affect cell behavior. To determine whether wounding induces increased velocities, positions of cells were recorded at various times by time-lapse microscopy. As is seen in Figure 14, cells within 50 μm from the agarose barrier exhibited basal motility irrespective of induction of wounds. However, wounding induced significant increases of the velocities of the cells at the edge of wounds, and this was not affected by the presence of apyrase. I conclude that wounding, by simply removing a physical barrier, does indeed elicit a biological response in the HCLE cells in that they move faster.

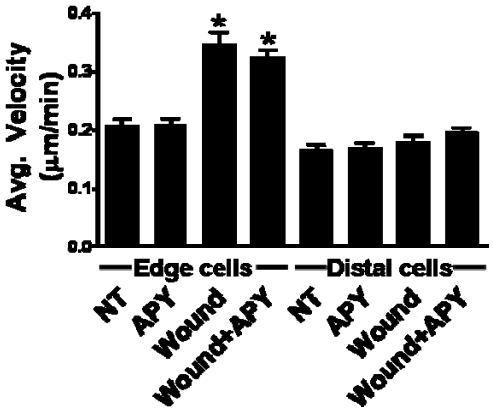

Figure 14 Increased velocities of cells near the wound edge are not dependent on extracellular ATP.

Agarose strips were left in place (NT) or removed (Wound) in the presence of 30 U/ml apyrase (APY), as indicated. The velocities of individual cells within 50 μm of the wound edge (Edge cells) or greater than 2 mm from the wound edge (Distal cells) were calculated. * indicates significant increases from unwounded groups ($p<0.001$).

I next tested whether signaling by ATP is necessary for healing of wounds by examining cell migration in the presence of 30 U/ml apyrase, which reduced extracellular ATP to

undetectable levels (Figure 9) and inhibited wound-induced PKC activation (Figure 10A). As shown in Figure 15A, no significant difference in wound healing was observed between the untreated control and apyrase-treated groups. Similar results were obtained with secondary cultures of rabbit corneal epithelial cells (Figure 15B) and with cultured rabbit corneas (Figure 15C).

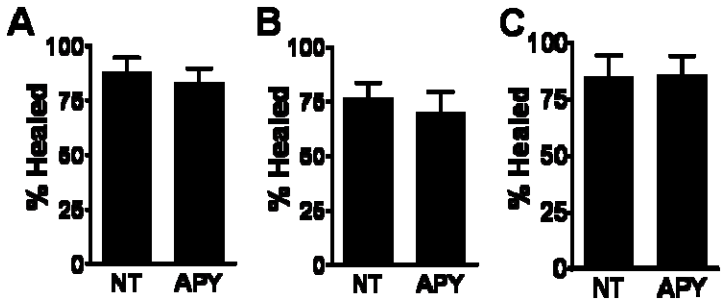

Figure 15 Extracellular ATP is not necessary for wound healing

Healing of **A**. HCLE cells, **B**. secondary cultures of rabbit corneal epithelial cells, and **C**. cultured rabbit corneas were monitored in the presence of 30 U/ml apyrase (APY), as indicated. No significant differences were observed.

To verify that the apyrase was functional, I tested its ability to degrade [α-^{32}P]-ATP and [α-^{32}P]-ADP. As little as 0.3 U/ml apyrase was sufficient to degrade ATP and ADP completely, and 30 U/ml was fully active even after 14 hours in cell culture (Figure 16). From these data, I conclude that extracellular ATP signaling is not necessary for wound healing.

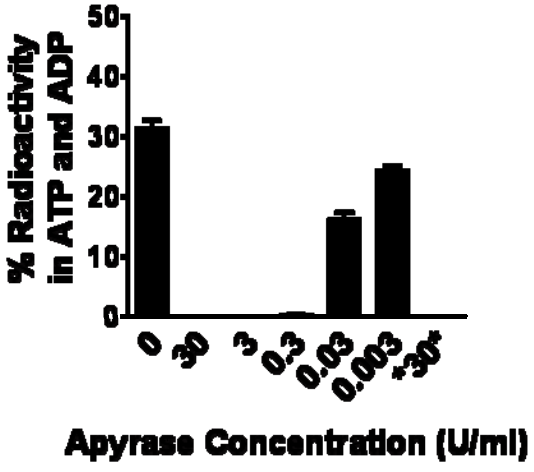

Figure 16 Apyrase was active throughout the healing assays

Wounded sheets of HCLE cells were incubated with the indicated concentrations of apyrase and the degradation of [α-^{32}P]-ATP was measured for 5 min. The 5-minute degradation of ATP was also evaluated in the medium of cells cultured for 14 hours with 30 U/ml apyrase (*30*).

Other reports have suggested that ATP signaling is critical for wound healing because healing and wound-induced EGFR activation are inhibited by the general P2 receptor antagonist reactive blue 2 (RB2) (Boucher et al., 2007; Klepeis et al., 2004; Yin et al., 2007). I have confirmed these findings but I have also observed that the same concentration of RB2 inhibited activation of the EGFR by AR, which makes RB2 an unsuitable reagent for this type of study (Figure 17).

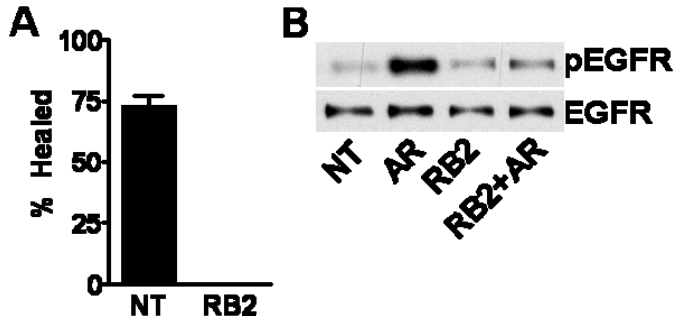

Figure 17 RB2 inhibits healing and EGFR activation by AR.

A. Healing of wounds in HCLE cells with no treatment (NT) or 100 μM RB2. **B**. Cells received no treatment (NT) or 10 ng/ml AR for 10 minutes (AR), following 30 minute pre-treatment with 100 μM RB2 as indicated.

2.3 DISCUSSION

Previous reports have shown that wounding sheets of corneal epithelial cells results in release of ATP, and that extracellular ATP can activate the EGFR (Boucher et al., 2007; Yin et al., 2007). The data presented in this section indicate that ATP is necessary for wound-induced EGFR and ERK1/2 activations in cells far (>250 μm) from wounds. Additionally, I report here the existence of a distinct mode of activation of the EGFR after wounding, which is not dependent on ATP signaling, based on the following observations: 1, activation of the EGFR in cells near the wound edge is unaffected by removal of extracellular ATP with apyrase; 2, visualization by

immunofluorescence shows that the EGFR/ERK1/2 pathway is stimulated in cells near wounds in the presence of apyrase; 3, the enhanced velocity of cells near wounds is not affected by the presence of apyrase.

Elimination of extracellular ATP with apyrase did not affect the rate of wound healing, suggesting that extracellular ATP signaling pathways are not required for healing of wounds. This is in agreement with one previous report describing similar results with Madin-Darby canine kidney epithelial cells (Farooqui and Fenteany, 2005), but differs from the conclusions reached by the use of the purinergic receptor antagonist RB2 (Boucher et al., 2007; Klepeis et al., 2004; Yin et al., 2007). Importantly, I have found that RB2 also blocks EGFR activation by exogenous ligands. RB2 is therefore not a suitable reagent for determining the role of purinergic signaling in healing of wounds in corneal epithelial cells because EGFR activation is an absolute prerequisite for the induction of motility. Numerous P2Y receptors have been found in the corneal epithelium (Klepeis et al., 2004), but to date, no knockout studies have addressed whether these receptors regulate wound healing *in vivo*.

Differences in signaling based on distance from the wound edge have been described previously. For instance, in human corneal epithelial cells immortalized by the SV-40 T-antigen, increased cytosolic [Ca^{2+}] in edge cells is dependent on extracellular calcium but the propagation of a calcium wave to distal cells is dependent on intracellular calcium (Klepeis et al., 2001). Also, in Madin-Darby canine kidney epithelial cells, the presence of reactive oxygen species and the activation of ERK1/2 was observed only in near-edge cells 10 minutes after wounding (Nikolic et al., 2006). Methods that allow analysis of signaling and physiology selectively in either wound-proximal or –distal cells will continue to help define the functions of these distinct cell populations in wound healing.

A notable difference in the two EGFR activation pathways is their range of action. ATP is diffusible, and I found both by direct immunoblotting of cells at a distance from wounds and by immunofluorescence studies that ATP signaling activates the EGFR up to at least 5 mm from the wound edge. ATP has previously been shown to mediate intracellular [Ca^{2+}] increases over similar distances in mechanically stimulated liver and alveolar epithelial cells and wounded corneal epithelial cells (Frame and de Feijter, 1997; Isakson et al., 2001; Klepeis et al., 2001). Also, ATP is found in conditioned media after wounding at a concentration of 1-2 μM, which is sufficient to activate the EGFR (Yin et al., 2007).

In contrast to ATP signaling, the extracellular ATP-independent signaling pathway appears to act only in cells near wounds. EGFR ligands such as AR and HB-EGF are not expected to mediate strong activation of the EGFR in wound-distal cells because of their limited diffusion. HB-EGF and AR bind to not only the EGFR but also to cell surface glycosaminoglycans and the tetraspannin CD9, resulting in an active ligand that remains mostly cell-associated (Harris et al., 2003; Iwamoto et al., 1994). I detected AR in wound-conditioned media at concentrations below that which induces detectable activation of the EGFR (<100 pg/ml), yet the EGFR was highly activated after wounding. However, even when EGFR activation cannot be detected by immunoblotting, the EGFR may still transduce potent biological effects, especially when the ligand can limit receptor internalization (Iyer et al., 2008; Reddy et al., 1996). Thus, after wounding, AR acts predominantly in a local manner while ATP signals over distances.

2.3.1 Summary and Model

In summary, wounding activates the EGFR by dual mechanisms: by extracellular ATP signaling and by an unknown mechanism near the wound edge (see diagram in Figure 18). The mechanism by which ATP transactivates the EGFR is explored in Chapter Three, and mechanisms of ATP release from wound edge cells are discussed in Chapter Four. Mechanisms of extracellular ATP-independent EGFR activation near the wound edge are the subjects of Chapters Five and Six.

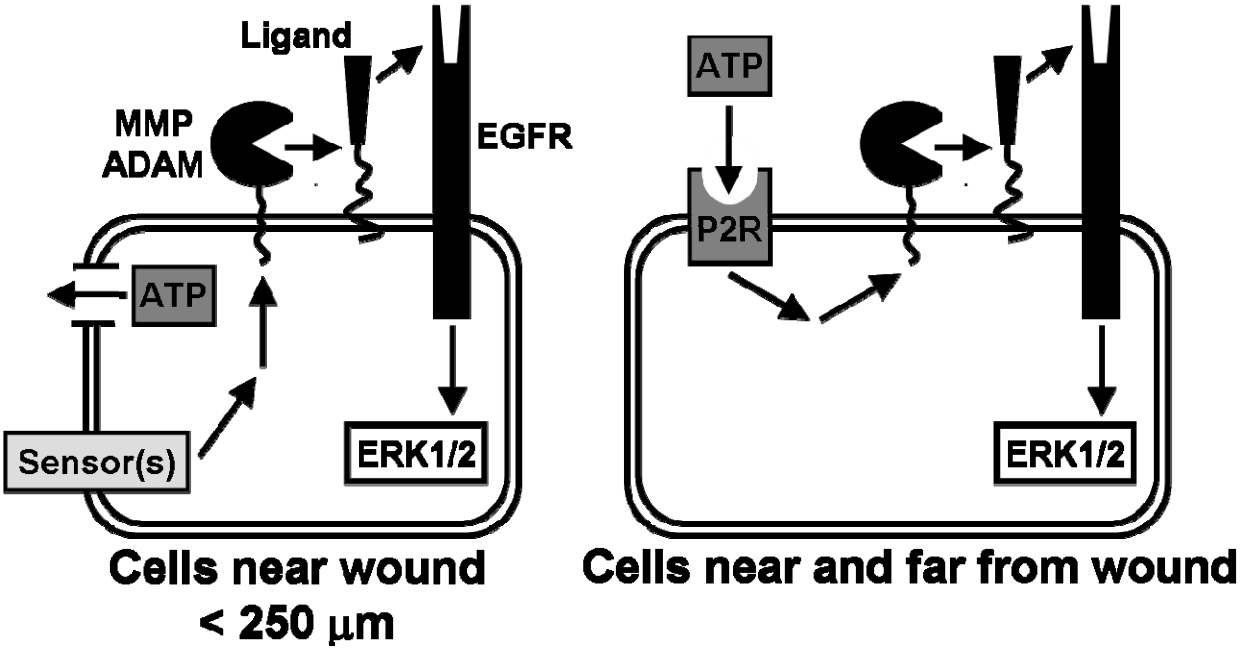

Figure 18 A hypothetical model for the role of extracellular ATP in wound-induced EGFR activation

See text for details. P2R, type-2 purinergic receptor; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinases 1 and 2.

3.0 ATP TRANSACTIVATES THE EGFR THROUGH PHOSPHOLIPASE D2 SIGNALING

3.1 INTRODUCTION

In Chapter Two, I confirmed that extracellular ATP transactivates the EGFR after wounding. Similarly to signaling by extracellular ATP, signaling by Phospholipase D (PLD) has recently been identified as a potential regulator of wound-induced EGFR activation (Mazie et al., 2006). PLD catalyzes the hydrolysis of phosphatidylcholine, an abundant membrane phospholipid, to free choline and the second-messenger phosphatidic acid (PA) (Cazzolli et al., 2006; Exton, 2002; Jenkins and Frohman, 2005; McDermott et al., 2004). PLD is activated rapidly after wounding sheets of corneal epithelial cells and treatment with PA activates the EGFR (Mazie et al., 2006). In fact, the EGFR transactivation processes induced by exogenously added ATP and PA both depend on proteolytic ligand release, and ATP is known to induce activation of phospholipase D (PLD) in many systems (el-Moatassim and Dubyak, 1992; Gargett et al., 1996; Kusner and Adams, 2000; Perez-Andres et al., 2002; Pochet et al., 2003; Sun et al., 1999). This led me to test the hypothesis that ATP signals through PLD to activate the EGFR.

The observation that healing rates of HCLE and rabbit corneal epithelial cells (Figure 15) and of Madin Darby canine kidney epithelial cells (Farooqui and Fenteany, 2005) are not affected by treatment with apyrase suggests that EGFR transactivation by extracellular ATP has

a biological role in addition to enhancing cell motility. Although wounds heal due to cell migration, a normal response to wounding includes replacing lost cells via cell division, which is also regulated by EGFR activity (Nakamura et al., 2001). In the corneal epithelium, wounding induces cell division in the limbus (see Figure 1) (Barrandon, 2007; Daniels et al., 2006; Revoltella et al., 2007; Zieske et al., 2001), an anatomical region that is typically distal from the point of injury. The observation that extracellular ATP stimulates cell division in skin keratinocytes (Braun et al., 2006) therefore suggests that EGFR transactivation by ATP may be a wound-induced mitogenic signal. In this chapter, I have examined whether PLD mediates EGFR transactivation by extracellular ATP and whether extracellular ATP promotes cell division after wounding. Portions of this chapter were published previously, ASCB copyright 2008, in http://www.molbiolcell.org/cgi/content/full/19/11/4909 (Block and Klarlund, 2008) and have, in some cases, been modified.

3.2 RESULTS

3.2.1 ATP is released after wounding and activates PLD.

I have previously shown that wounding sheets of HCLE cells by the removal of agarose droplets results in the release of ATP (Figure 9) and in activation of PLD (Mazie et al., 2006). To determine whether PLD is activated as a consequence of the released ATP, cell sheets were wounded in the presence of apyrase. Apyrase eliminated ATP effectively from the medium (Figure 9), and apyrase reduced wound-induced PLD activity to unwounded levels (Figure 19), which implies that activation of PLD is stimulated by the ATP that is released after wounding.

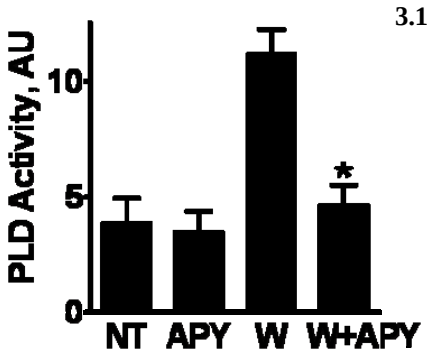

Figure 19 ATP released after wounding activates PLD

Assay for activity of PLD after no treatment (NT), wounding (W), and/or treatment with 30 U/ml apyrase (APY) and incubation for 5 minutes. * indicates significant reduction from wounded controls ($p<0.001$).

3.2.2 The PLD2 isoform is activated by wounding and by treatment with ATP.

No satisfactory chemical inhibitor of PLD activity was commercially available so I employed a short interfering RNA (siRNA)-based approach to evaluate whether PLD is necessary for ATP-stimulated EGFR activation. Mammalian cells express two PLD isoforms, PLD1 and PLD2, which are both ubiquitously expressed. Distinct intracellular localizations, mechanisms of activation, and downstream effectors have been described for each isoform (Hiroyama and Exton, 2005; Kusner et al., 2002; Morris, 2007; Oude Weernink et al., 2007; Preininger et al., 2006). SiRNA oligonucleotides targeted to PLD1 and PLD2 were transfected into HCLE cells and whole cell extracts were prepared 3 days after transfection, and as illustrated by the immunoblots in Figure 20A, the siRNA-mediated knock-downs of the PLD1 or PLD2 isoforms were efficient and specific. Quantitation of the intensities of the bands shows that expression of PLD2 was reduced by 80% and PLD1 by 70% (Figure 20B). PLD activation after wounding was greatly diminished in cells transfected with one of the PLD2 oligonucleotides (PLD2 siRNA-a) but was unaffected in cells transfected with PLD1 siRNA, and knock-down of both isoforms had no greater inhibitory effect than knock-down of the PLD2 isoform alone (Figure 20C). A second PLD2 siRNA oligonucleotide (PLD2 siRNA-b), which was made to control for off-target effects, also reduced the level of PLD2 expression. These results suggest that wound-induced PLD

activation is predominantly determined by the PLD2 isoform. In the following experiments with siRNAs, PLD1 and 2 were verified to be downregulated to levels similar to that shown in Figure 20A.

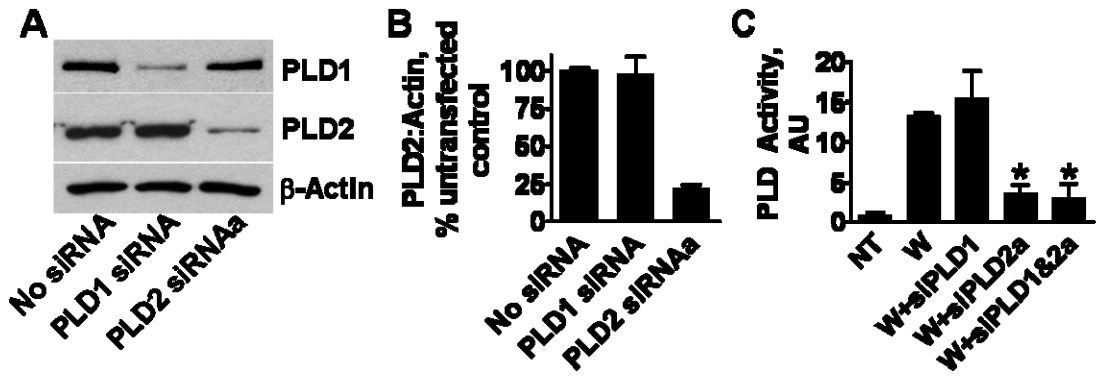

Figure 20 Wounding selectively activates the PLD2 isoform

A. Western blots of whole cell extracts of HCLE cells untransfected (No siRNA) or transfected with siRNA against PLD1 or PLD2 cultured around agarose droplets. **B**. Densitometry of immunoblots transfected with the indicated siRNAs. **C**. Assay for activity of PLD after no treatment (NT), wounding (W), and/or transfection with siRNAs against PLD1 or PLD2 . * indicates significant reduction from wounded controls ($p<0.001$).

Exogenously added ATP stimulated PLD activity in HCLE cells (Figure 21A), as it does in many other cell types (el-Moatassim and Dubyak, 1992; Gargett et al., 1996; Kusner and Adams, 2000; Perez-Andres et al., 2002; Pochet et al., 2003; Sun et al., 1999). Similarly to wound-stimulated PLD activity, ATP-stimulated PLD activity was blocked in HCLE cells transfected with PLD2 siRNA-a but not in cells transfected with PLD1 siRNA (Figure 21A). Stimulation with apyrase-treated ATP or with 50 μM adenosine did not activate PLD (Figure 21B), indicating that activation is a direct effect of ATP. Addition of 10 μM of the EGFR kinase inhibitor tyrphostin AG 1478 did not inhibit activation of PLD by ATP (Figure 21B) although it

completely inhibited EGFR activation by ATP in parallel incubations, indicating that the observed PLD activation is not a result of EGFR signaling.

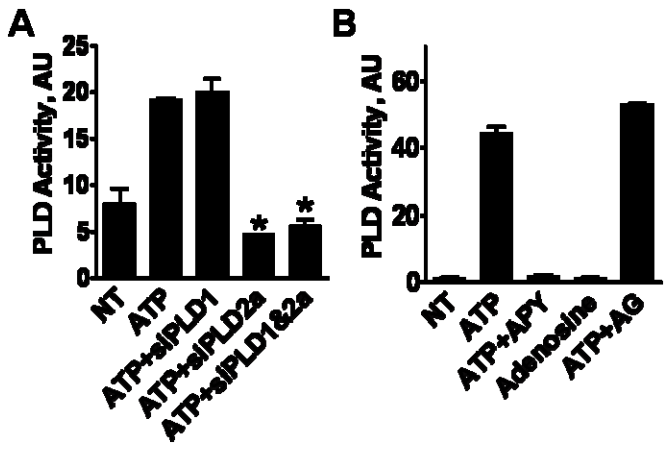

Figure 21 Extracellular ATP activates PLD2

A. PLD activities in confluent HCLE cells not treated (NT) or transfected with siRNA against PLD1, PLD2, or both and then treated for 5 minutes with 50 μM ATP. * indicates significant reduction from ATP-treated controls ($p<0.001$). **B**. Assay for activity of PLD after no treatment (NT), 5 minute incubation with 50 μM ATP (ATP), 50 μM ATP that had been pre-incubated for 15 minutes with 5 U/ml apyrase (ATP+APY), or 50 μM ATP after 15 minute pre-treatment of cells with 10 μM tyrphostin AG 1478 (ATP+AG).

3.2.3 Extracellular ATP transactivates the EGFR via PLD2 signaling.

To test the role of PLD signaling in ATP-stimulated EGFR activation, cells were transfected with siRNA oligonucleotides. Activation was monitored by Western Blotting with an antibody that recognizes the EGFR phosphorylated on tyrosine 1173. Both PLD2 siRNA-a and siRNA-b blocked EGFR activation, whereas PLD1 siRNA did not (Figure 22A and B). Transfection of PLD2 siRNA-a did not block activation of the EGFR by EGF (Figure 12). This provides direct evidence that activation of the EGFR by ATP is mediated through PLD2 signaling.

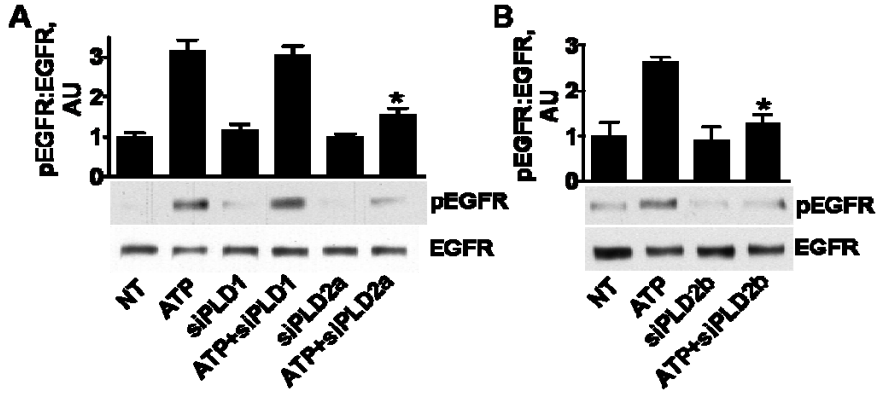

Figure 22 ATP activates the EGFR through PLD2 signaling

Western Blotting and densitometry for EGFR activation after no treatment (NT) or treatment with 50 μM ATP for 10 minutes (ATP). Cells were transfected with siRNA against **A**. PLD1 or PLD2 (siPLD2a) or **B**. a different PLD2 siRNA (siPLD2b). * indicates significant reduction from ATP-treated controls ($p<0.001$).

Extracellular ATP transactivates the EGFR through stimulation of proteases at the cell surface that cleave precursors of ligands for the receptor (Boucher et al., 2007; Yin et al., 2007). To establish a measure of the transactivation process, I assayed one such ligand, amphiregulin (AR), and found that wounding, addition of ATP, and stimulation with a water-soluble analog of phosphatidic acid 1,2-octanoyl-sn-glycero-3-phosphate (C_8-PA), all increased AR release (Figure 23). Furthermore, neutralization experiments indicated that AR contributes to EGFR activation

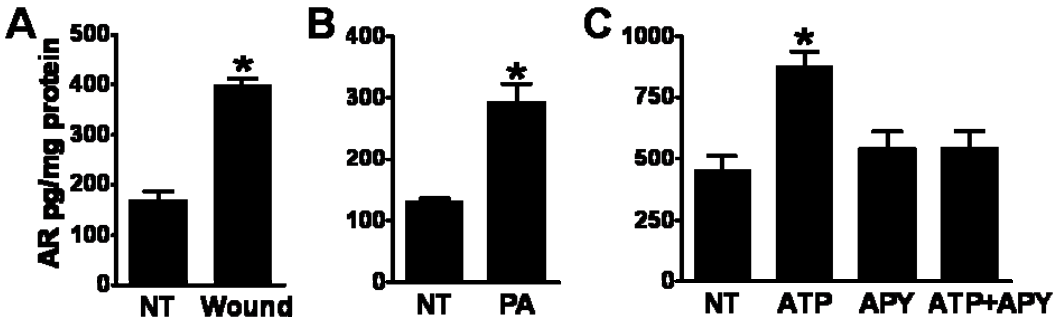

Figure 23 AR is released after wounding or treatment with PA or ATP

Quantities of AR in conditioned media were determined in the 10 minutes following no treatment (NT) or **A**. wounding, **B**. treatment with 25 μM C_8PA (PA), **C**. treatment with 50 μM ATP, and or 30 U/ml apyrase. * indicates significant increase from untreated controls ($p<0.001$).

in response to these treatments (Figure 24).

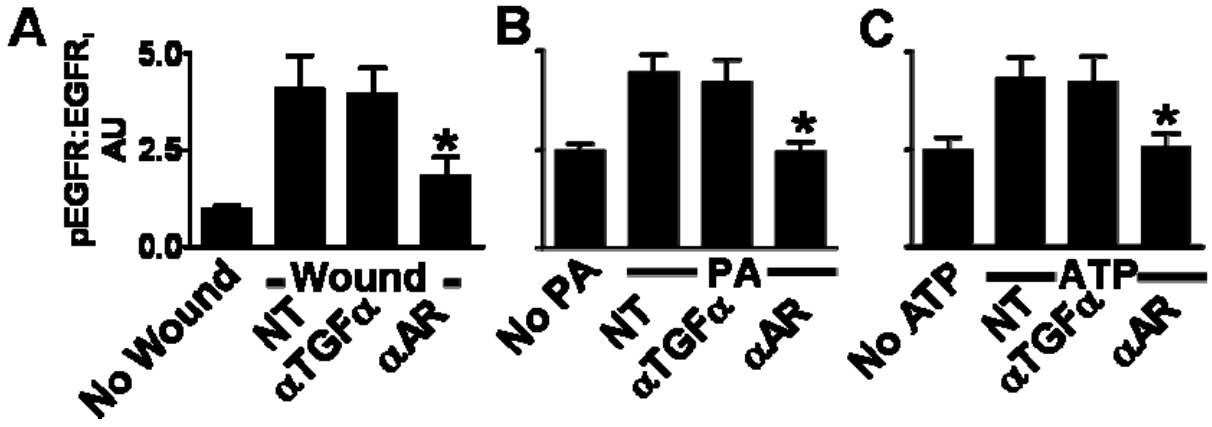

Figure 24 AR contributes to EGFR activation after wounding or treatment with PA or ATP

Activation of the EGFR was determined by densitometry of Western Blots. Cells were stimulated for 10 minutes after **A**. wounding, **B**. treatment with 25 μM C_8PA (PA), or **C**. treatment with 50 μM ATP. As indicated, cells were incubated for 2 hr with 20 μg/ml neutralizing antibodies against TGFα (isotype control) or AR. * indicates significant decrease from stimulated controls ($p<0.001$).

Because ATP activates the EGFR through PLD2, reduction of expression of PLD2 was expected to decrease secretion of AR in response to ATP. Reducing the level of PLD2 resulted in reduction of basal and ATP-stimulated secretion of AR, whereas interfering with the cellular levels of PLD1 had no effect (Figure 25A). The reduction of PLD2 by either siRNA oligonucleotide correlated directly with AR release, suggesting that the reduction of AR release is the result of knock-down of PLD2 rather than of off-target effects (Figure 25B).

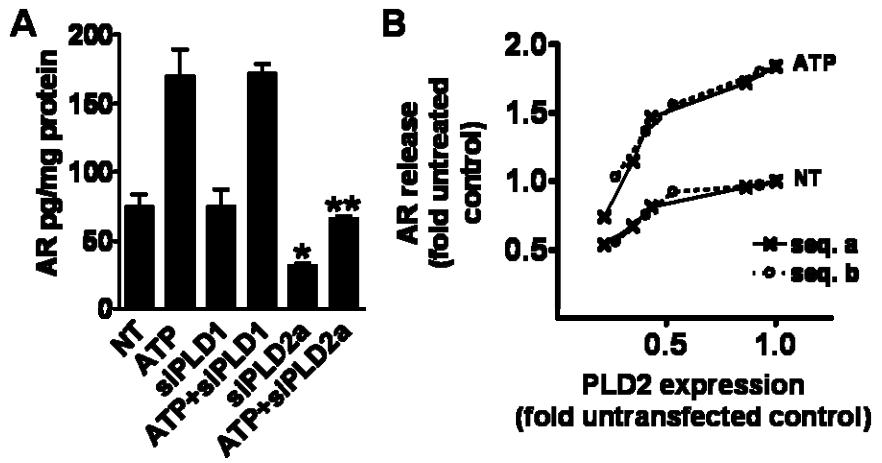

Figure 25 ATP-stimulated AR release depends on PLD2

A. Quantities of AR in conditioned media were determined in the 10 minutes following no treatment (NT) or treatment with 50 μM ATP. As indicated, cells were transfected with siRNA against PLD1 or PLD2. * indicates significant decrease from untreated controls and ** indicates significant decrease from ATP-treated controls ($p<0.001$). **B**. HCLE cells were untransfected (data points farthest to the right) or transfected with PLD2 siRNAa or b at 50, 16, 5.0, or 1.6 nM (data points from left to right). Cells were then left untreated (NT) or were treated with 50 μM ATP before determination of AR concentration in conditioned medium and cellular PLD2 expression.

3.2.4 PLD2 activation is not required for wound-proximal EGFR activation

Together with results from Chapter Two, data presented in this section suggest that ATP/PLD2 signaling is necessary for EGFR transactivation in cells >250 μm from the wound edge. To determine if PLD2 is necessary for signaling near the edge, I examined EGFR activation in cells transfected with PLD2 siRNA. As shown in Figure 26, knockdown of PLD2 did not inhibit EGFR activation near the wound edge, confirming my observations from Chapter Two (Figure 11) that EGFR activation in wound-edge cells proceeds independently of extracellular ATP signaling.

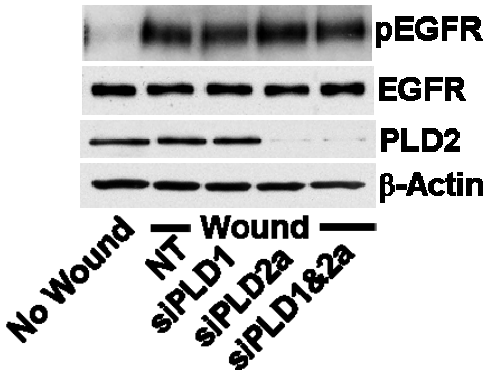

Figure 26 EGFR activation near the edge is independent of signaling by PLD

Western Blots for wound-induced activation of EGFR in the presence of no treatment (NT) or transfection with siRNAs against PLD1 and/or 2.

3.2.5 The mechanism of PLD activation by extracellular ATP

The mechanism of PLD activation by extracellular ATP is not entirely clear. PLD activity requires binding to phosphatidylinositol 4,5-bisphosphate and activity of protein kinase C (PKC) (Exton, 2002; Jenkins and Frohman, 2005; McDermott et al., 2004). Extracellular ATP binds to the P2X class of cation channels and the P2Y class of G-protein coupled receptors (Abbracchio et al., 2006). ATP may also be metabolized by ecto-enzymes to adenosine, which activates the P1 class of receptors. Since apyrase abrogated activation of PLD after wounding and exogenously-added adenosine had no effect on PLD activity, P1 receptors are not likely involved in PLD2 activation. Agonist and antagonist profiling has indicated that PLD is activated through $P2X_7$ and $P2Y_2$ receptors that signal through phospholipase C (PLC) and protein kinase C (PKC) (el-Moatassim and Dubyak, 1992; Katzur et al., 1999; Purkiss et al., 1992). In preliminary studies, I have observed that wound-induced PLD activation and ATP-stimulated AR release are both sensitive to the classical PKC inhibitor bisindolylmaleimide I (Figure 27), and that wounding activates PKC through extracellular ATP signaling (Figure 10), which is consistent with the hypothesis that extracellular ATP activates PLD through PLC and PKC signaling.

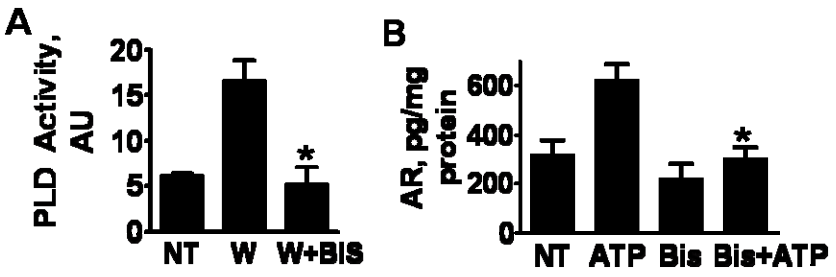

Figure 27 PKC activity is necessary for wound-induced PLD activation and ATP-induced AR release

Assays for **A**. PLD activity or **B**. AR release following no treatment (NT), wounding (W), or treatment with 50 μM ATP. 10 μM of the Classical PKC inhibitor bisindolylmaleimide I (Bis) was added 30 minutes prior to stimulations. * indicates significant decrease from ATP-treated controls ($p<0.001$).

3.2.6 PLD2 mediates EGFR activation through SFKs

Recent research has indicated that EGFR transactivation by GPCR ligands (such as ATP) is dependent on activity of the Src-family kinases (SFKs) (Zhang et al., 2004). To determine whether SFK activity is necessary for EGFR transactivation by extracellular ATP in the present system, I incubated cells with PP2 or Src Kinase Inhibitor-I (SKI), two structurally dissimilar SFK inhibitors that are known to have distinct non-specific interactions (Bain et al., 2007). Either SFK inhibitor blocked EGFR activation by treatment with ATP (Figure 28A) but not by treatment with EGF (Figure 28B). Similarly, the SFK inhibitors blocked ATP-stimulated AR release (Figure 28C). These results suggest that ATP requires SFK activity to transactivate the EGFR. I next determined whether SFK signaling acts upstream of PLD2. The SFK inhibitors did not reduce PLD activation by wounding (Figure 29A) or by treatment with ATP (Figure 29B). Together, these results indicate that SFK activity acts downstream of PLD2 to mediate EGFR transactivation by ATP.

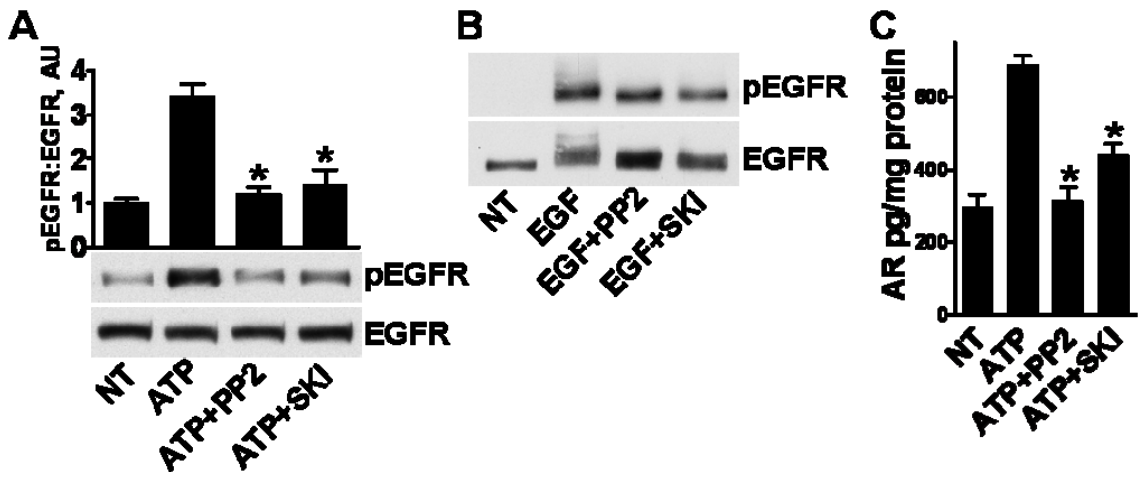

Figure 28 Activation of SFKs is necessary for EGFR transactivation by extracellular ATP

A. Western Blotting and densitometry for activation of the EGFR after no treatment (NT) or 10 minute treatment with 50 μM ATP. **B**. Cells were treated as in A but received 10 ng/ml EGF instead of ATP. **C**. AR quantities were determined in conditioned media from cells treated as in A. * indicates significant decrease from ATP-treated controls ($p<0.001$).

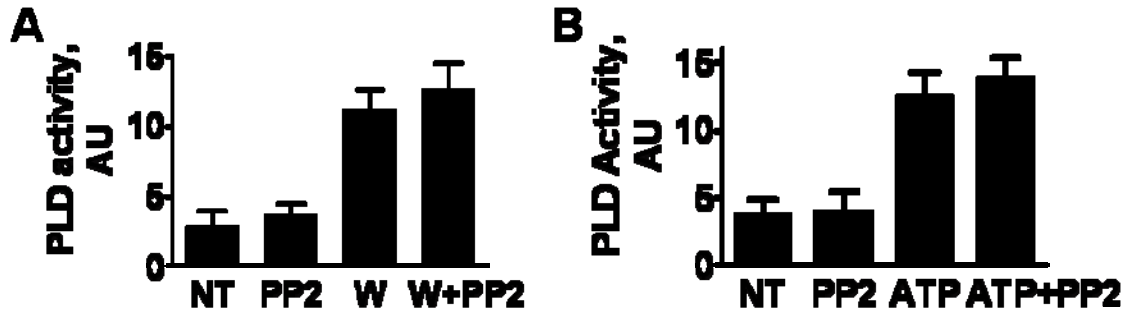

Figure 29 Wound- and ATP-induced activation of PLD proceeds independently of SFK activation

Assay for PLD activity following no treatment (NT), **A**. wounding (W), or **B**. treatment with 50 μM ATP. 10 μM PP2 or was added 30 minutes prior to stimulations. Wound- or ATP-stimulated groups were significantly increased from unstimulated controls but were not significantly affected by PP2.

PLD can affect signaling through intermolecular interactions (Kim et al., 2006; Lee et al., 2006) or through the direct actions of PA (Fang et al., 2001; Frank et al., 1999; Lehman et al., 2007; Zhao et al., 2007). Since PA stimulated AR release and EGFR activation (Figures 23 and

24), PLD2 signaling likely stimulates EGFR activation through the actions of PA. Treatment of cells with PA stimulated activation of SFKs, as indicated by Western Blotting for the kinases autophosphorylated on Tyr-419 (Figure 30A), and SFK inhibitors blocked EGFR activation by treatment with PA (Figure 30B). These results are consistent with the hypothesis that after wounding, ATP transactivates the EGFR through the sequential activation of PLD2 and SFKs.

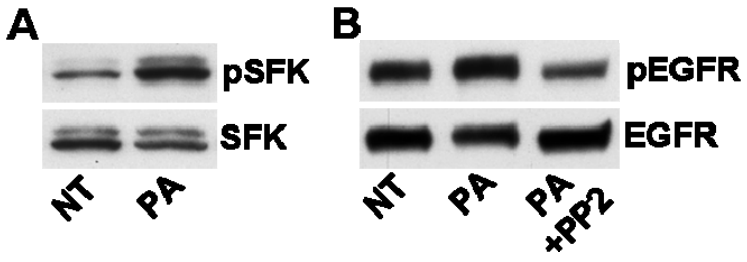

Figure 30 PA activates the EGFR through SFK signaling

Western Blotting for activation of **A**. SFKs or **B**. the EGFR following no treatment (NT) or treatment with 25 μM C_8PA (PA). 10 μM PP2 was added 30 minutes prior to stimulation.

3.2.7 Wound-induced cell division depends on signaling by EGFR but not extracellular ATP.

Transactivation of the EGFR by extracellular ATP can promote cell motility, but it is unclear whether this signaling module serves any additional biological role after wounding. EGFR activation can stimulate cell division in many cell types, and regulates wound-induced proliferation in the corneal epithelium (Nakamura et al., 2001). Furthermore, PLD signaling is mitogenic (Foster and Xu, 2003) and in cultured keratinocytes, extracellular ATP promoted cell division (Braun et al., 2006). Therefore, I investigated whether extracellular ATP regulates cell division after wounding. To detect cell division, I immunostained for Ki67, a protein present in the nuclei of dividing cells (Brown et al., 2002).

To determine whether EGFR transactivation by ATP is necessary for cell division, I examined Ki67 immunostaining after wounding sheets of HCLE cells. Wounding caused

increased Ki67 staining in cells near the wound margin at 8 and 24 hours, and was most increased at 48 hours, when the wounds had fully healed (Figure 31). Wounding did not alter the number of Ki67-positive cells in areas >1 mm (~50 rows of cells back) from the wound edge, which was similar to that observed near the edges of unwounded controls. Treatment with the EGFR kinase inhibitor AG 1478 reduced Ki67 staining, but treatment with apyrase had no effect on either the number or location of Ki67-positive cells (Figure 31). These results indicate that cell division stimulated by wounding is regulated by activation of the EGFR, independently of signaling by extracellular ATP.

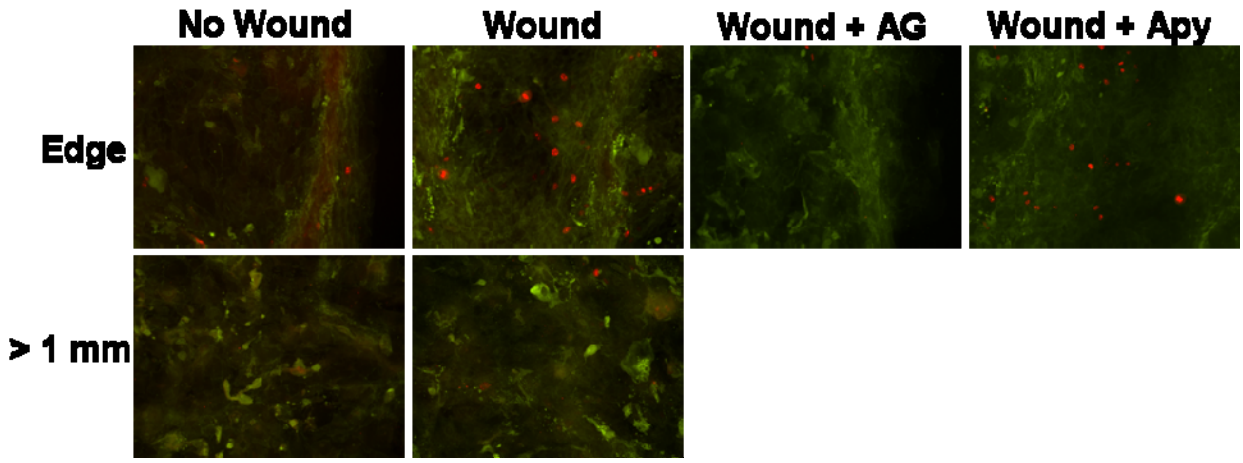

Figure 31 Wound-induced cell division depends on signaling by the EGFR but not extracellular ATP

Immunofluorescence images taken from HCLE cell cultures 48 hours after wounding. Cells were stained for E-cadherin (green) and Ki67 (red). As indicated, cells were treated with 1 μM of the EGFR inhibitor AG 1478 (AG) or 25 U/ml apyrase (Apy) during healing. The wound edge is visible in the "No Wound" and "Wound + AG" conditions.

3.3 DISCUSSION

PLD activation and release of extracellular ATP have both been suggested to be up-stream events that lead to EGFR transactivation after wounding (Boucher et al., 2007; Klepeis et al., 2001; Klepeis et al., 2004; Mazie et al., 2006; Yin et al., 2007). In this chapter I have reported that these two signaling events are related by showing that ATP signals through PLD2 to activate the EGFR. I have based this conclusion on the following evidence: 1, wound-induced PLD activation is abrogated by apyrase; 2, wounding and treatment with ATP both activate the PLD2 isoform; 3, treatment with ATP or PA similarly stimulates EGFR activation through AR and SFK signaling; and 4, knock-down of PLD2 expression inhibits ATP-induced EGFR activation and AR release.

ATP/PLD2 signaling is necessary for EGFR activation in cells >250 μm from the wound. This pathway contributes to the induction of motility since addition of extracellular ATP or PA has been shown to enhance wound healing in many different epithelial cell lines (Dignass et al., 1998; Klepeis et al., 2004; Mazie et al., 2006; Sponsel et al., 1995; Wesley et al., 2007; Yin et al., 2007). I was unable to use siRNA to test whether the activity of PLD2 is necessary for wound healing. Only stratified cultures of HCLE cells heal efficiently and PLD2 expression in siRNA-transfected cells was restored to control levels after the stratification process. An alternate approach to examine the effects of PLD signaling on cell migration would be to use a class of recently-described chemical inhibitors that were not available at the time of this research (Lavieri et al., 2009; Lewis et al., 2009). Traditionally, primary alcohols such as 1-butanol have been used to inhibit PLD activities, despite numerous non-specific and toxic effects.

Signaling by the EGFR, PLD, and extracellular ATP are known to stimulate cell division (Braun et al., 2006; Foster and Xu, 2003; Nakamura et al., 2001). However, apyrase had no

effect on wound-induced cell division (Figure 31), indicating that extracellular ATP is not necessary for cell division after wounding. In vivo, wounding stimulates corneal epithelial cell division within the wound margin (Zieske et al., 2001), as I observed in Figure 31, and far from the wound in the limbus, which is the niche for epithelial stem and transit amplifying cells (Daniels et al., 2006). I attempted to investigate whether extracellular ATP regulates cell division in the limbus, but I was unable to reproduce the phenomenon of wound-induced limbal cell division in mouse and rat whole eye organ cultures. In the future, one could treat mouse eyes with apyrase *in vivo* or use a gene knockout approach to test the hypothesis that ATP/PLD2 signaling regulates cell division in the limbus. Potential mechanisms of ATP release and additional biological outcomes of extracellular ATP signaling are discussed in Chapter Four.

My observation that treatment with PA induces SFK activation (Figure 30) is in agreement with the observation that PLD2 over-expression increased SFK activity (Ahn et al., 2003). However, the mechanism whereby PA activates SFKs is unknown. SFK activity is stimulated by intermolecular interactions and de-phosphorylation of the Tyr-527 residue. A number of candidate phosphatases have been identified as mediating SFK activation by de-phosphorylating Tyr-527 including PTP1B, Shp1 (Src homology 2 domain-containing tyrosine phosphatase 1), and Shp2 (Roskoski, 2005). Interestingly, PA has been shown to bind and promote the activity of Shp1 (Frank et al., 1999). The hypothesis that PA activates SFK through the activation of Shp1 could, in the future, be tested by measuring Shp1 activity in response to PA and by inhibiting Shp1 activity with siRNA.

The results reported here are to my knowledge the first descriptions of a role for PLD in ATP-stimulated EGFR transactivation. There is a growing list of cytokines and growth factors that stimulate EGFR transactivation (Higashiyama and Nanba, 2005; Ohtsu et al., 2006;

Sanderson et al., 2006) and many of these, such as angiotensin II, bradykinin, endothelin-I, and lysophosphatidic acid also stimulate PLD activity (Bollag et al., 1990; Liu et al., 1992; Martin et al., 1989; van der Bend et al., 1992). A role for PLD2 has previously been reported for angiotensin II-mediated transactivation of the EGFR (Li and Malik, 2005), and I have also observed that AR release stimulated by phorbol 12-myristate 13-acetate (PMA or TPA) is dependent on PLD2. It therefore seems reasonable to hypothesize that PLD2 mediates EGFR transactivation by stimuli other than ATP.

3.3.1 Summary and model

In summary, ATP is released from cell sheets after wounding by the removal of agarose barriers. Extracellular ATP activates the PLD2 isoform leading to the production of PA, which in turn activates SFK, thereby stimulating ligand release and EGFR activation (see diagram in Figure 32). EGFR activation, but not extracellular ATP, is necessary for wound-induced cell division.

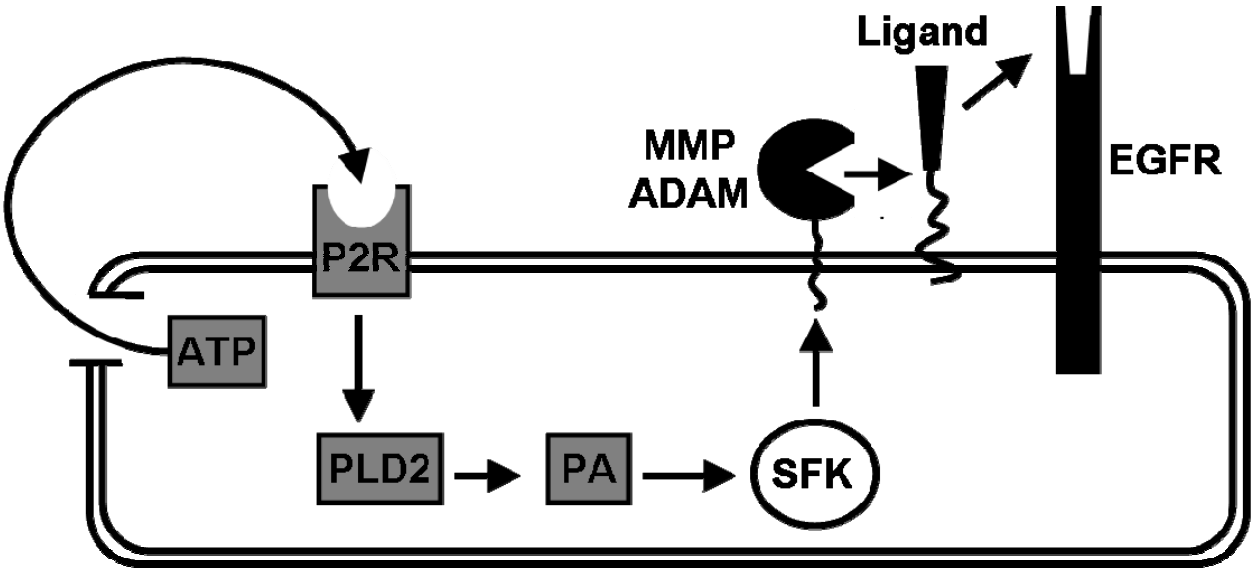

Figure 32 A hypothetical model for the mechanism of EGFR trans-activation by extracellular ATP after epithelial wounding

See text for details. P2R, type-2 purinergic receptor; PLD2, phospholipase D2; PA, phosphatidic acid; SFK, Src-family kinase; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; EGFR, epidermal growth factor receptor.

4.0 ATP IS RELEASED PERSISTENTLY FROM WOUNDS AND FREE EDGES IN EPITHELIAL SHEETS

4.1 INTRODUCTION

Data presented in Chapters Two and Three and from other laboratories show that ATP is released from wounded epithelia and transactivates the EGFR (Block and Klarlund, 2008; Boucher et al., 2007; Yin et al., 2007). I have not observed a necessity for extracellular ATP in wound healing or cell division. However, extracellular ATP can promote epithelial cell motility (Dignass et al., 1998; Klepeis et al., 2004; Sponsel et al., 1995; Wesley et al., 2007; Yin et al., 2007) and may additionally have effects on wound healing and ocular physiology that cannot be observed in cultured epithelial cells. ATP is now recognized as an extracellular messenger with numerous functions, and has the potential of influencing processes related to wound healing such as induction of inflammation, regeneration of nerves, alteration of tear composition, and communication with the underlying stroma (Belliveau et al., 2006; Bours et al., 2006; Burnstock, 2006; Burnstock, 2007; Chen et al., 2006; Jumblatt and Jumblatt, 1998). It is therefore important to understand the kinetics and mechanisms of ATP release from wounded sheets of epithelial cells.

The concentration of ATP is roughly 1000-fold higher inside of cells, so there is a large outwardly directed gradient. Consequently, ATP is released into the extracellular space from

ruptured cells after wounding. Additionally, wounding models that minimize cell damage have indicated that ATP may be released after wounding independently of cell rupture (Figures 9 and 11). ABC-family transporters such as P-glycoprotein and CFTR, fusion and release of ATP-loaded vesicles, pannexin channels, and gap junction hemichannels have all been described as conduits for ATP release (Lazarowski et al., 2003; Praetorius and Leipziger, 2009; Schwiebert and Zsembery, 2003; Spray et al., 2006).

Gap junctions are cell surface structures that allow the transfer of small molecules (less than 1000 Da) between cells (Evans and Martin, 2002). Each cell expresses half of the channel, a hemichannel, which is itself a hexamer of proteins of the connexin family. Unpaired hemichannels, or connexons, are expressed at the cell surface and secrete small molecules such as ATP in regulatable fashion (Evans et al., 2006; Goodenough and Paul, 2003; Li et al., 1996). The corneal epithelium expresses functional gap junctions that are composed of the connexins (named according to molecular weight) Cx43, Cx31.1, Cx30, and Cx26 (Chen et al., 2006; Shurman et al., 2005; Williams and Watsky, 2002). Evidence for ATP release through hemichannels includes a number of studies employing both gain-of-function (connexin expression) and loss-of-function (connexin inhibition) methodologies in HeLa and other cell lines (Cotrina et al., 1998), astrocytes (Stout et al., 2002), corneal endothelial cells (Gomes et al., 2005), cochlear-supporting cells (Zhao et al., 2005), and osteocytes (Genetos et al., 2007).

Since I have detected ATP release from wounded cell cultures containing mostly wound-proximal cells ((Block and Klarlund, 2008) and Figure 9), I expect that ATP is released from cells near the wound edge. Here, I used multiple wounding models to examine the kinetics of ATP release from cells near the wound edge, and I correlated this with markers of cell rupture. I

have also conducted preliminary experiments to test the hypothesis that ATP is released from cells near the wound edge through connexin hemichannels.

4.2 RESULTS

4.2.1 ATP is released from cells near edges in epithelial sheets chronically and independently of damage.

Using wounding models that minimize cell damage, I have observed that ATP is released acutely after wounding epithelial sheets ((Block and Klarlund, 2008), Figures 9 and 11), but even minimal cell damage can lead to ATP leaking from cells. Cells respond to sublethal damage by a variety of repair mechanisms that re-seal the plasma membrane (Bement et al., 2007; Idone et al., 2008; McNeil and Kirchhausen, 2005; Woolley and Martin, 2000), but until repair is complete, lactate dehydrogenase (LDH) leaks out of cells (Loo and Rillema, 1998). Increased amounts of LDH were detected in culture supernatants in the ten minutes immediately following the removal of agarose droplets (Figure 33A). For comparison, I note that levels of LDH released following agarose removal were roughly 10% of those observed following scrape-wounding. Cells were washed and fresh conditioned media were collected at various later time points. Increased LDH was detected after 20-30 minutes, but was no different from unwounded controls after one or 24 hours (Figure 33A). The same conditioned media were analyzed to determine quantities of extracellular ATP, which can be measured with a bioluminescence assay (see Methods 8.19). As shown in Figure 33B, elevated ATP concentrations were observed at all time points. Together,

these data indicate that cells near wound edges secrete ATP persistently and independently of cell damage.

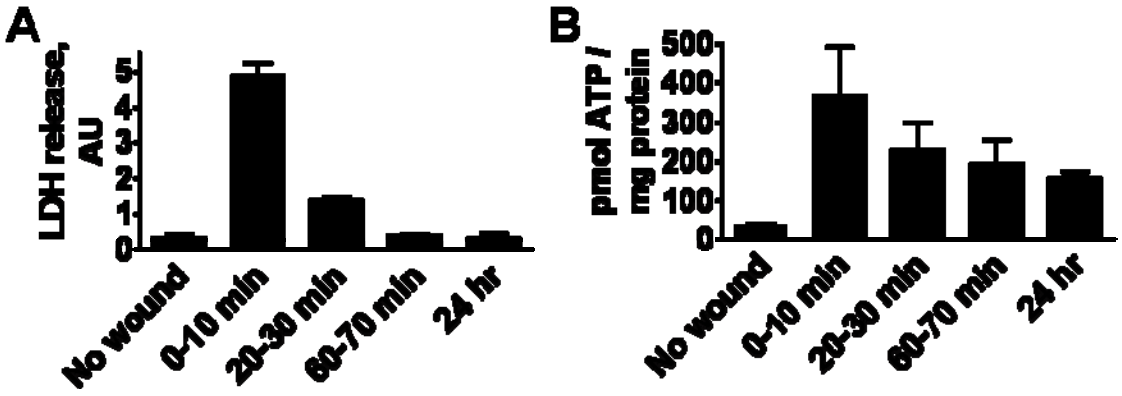

Figure 33 ATP is released persistently after wounding.

Assays for quantities of **A**. LDH or **B**. ATP in conditioned media from HCLE cell sheets collected at the indicated times after wounding by removal of agarose droplets.

Although I could not detect increased LDH in the supernatants of cultures 24 hours after wounding, these cells had experienced some degree of rupture initially. Therefore, to verify that cells near wound edges secrete ATP independently of cell damage, I employed a different model for analyzing cells near edges in epithelial sheets. To produce a cell culture with many free edges, HCLE cells were cultured on thin plastic strips above a non-adhesive surface of polyHEMA (see Figure 7 and Methods 8.6). As controls, cells were cultured on polyHEMA-coated dishes that had been completely covered with plastic. Levels of lactate dehydrogenase in the supernatants of cells grown in the two conditions were low and similar, indicating that growth of cells on the strips does not promote cell lysis (Figure 34A). Importantly, ATP concentrations were significantly higher in supernatants from cells grown on strips (Figure 34B). These results corroborate the data gathered from wounded cell sheets indicating that cells near the wound edge secrete ATP persistently and in the absence of cell damage.

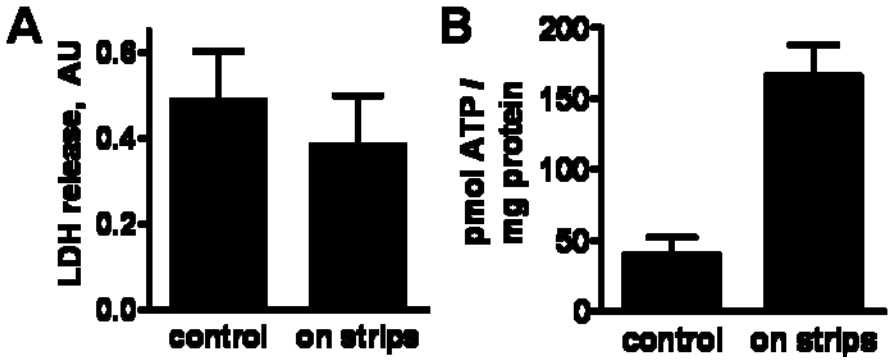

Figure 34 Cells cultured with many free edges release ATP independently of cell rupture

Assays for quantities of **A**. LDH or **B**. ATP in conditioned media from HCLE cells cultured on plastic strips or controls.

4.2.2 A drug screen implicates gap junction hemichannels in ATP release.

A number of conduits for damage-free ATP release from cells have been identified including ABC-family transporters, fusion and release of ATP-loaded vesicles, pannexin channels, and gap junction hemichannels (Lazarowski et al., 2003; Praetorius and Leipziger, 2009; Schwiebert and Zsembery, 2003; Spray et al., 2006). To examine mechanisms of transport, I treated cells grown on plastic strips with various chemical inhibitors and measured ATP release (Figure 35). Drugs that target ABC-family transporters (glibenclamide and verapamil), nucleotide transporters (atractyloside), or exocytosis (brefeldin A), did not inhibit ATP release. One inhibitor of gap junction permeability, 18β-glycerrhetinic acid (GA), did not inhibit ATP release, but another, flufenamic acid (FFA), dramatically reduced ATP release.

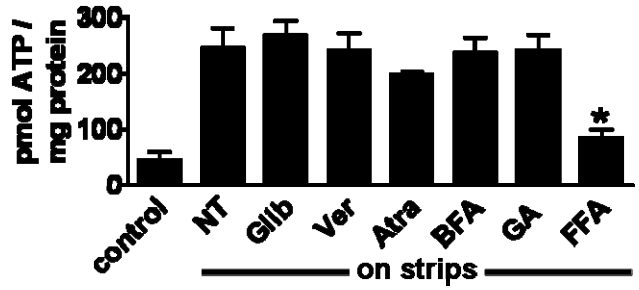

Figure 35 A drug screen for inhibitors of ATP release

ATP concentrations were determined in conditioned media from cells cultured on plastic strips following no treatment (NT), treatment with 10 μM glibenclamide (Glib), 10 μM verapamil (Ver), 10 μM atractyloside (Atra), 10 μM brefeldin A (BFA), 100 μM 18β-glycerrhetinic acid, or 50 μM flufenamic acid (FFA). * indicates significant decrease from NT condition ($p<0.001$).

Flufenamic acid (FFA) reversibly inhibits gap junction channels through a mechanism that does not involve PKC, changes in intracellular calcium concentrations, changes in pH (Harks et al., 2001), or calcium binding (Eskandari et al., 2002). As shown in Figure 36, FFA also inhibited ATP release from cells 24 hours after wounding cell sheets by the removal of agarose barriers. I therefore could not rule out a role for gap junction hemichannels in ATP release from epithelial edges.

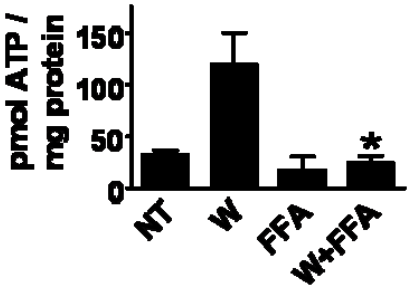

Figure 36 FFA inhibits the persistent release of ATP after wounding

Assay for quantities of ATP in conditioned media from cells 24 hours after no treatment (NT) or wounding (W). 50 μM FFA was added to cells 30 min prior to measurements.

If HCLE cells secrete ATP through gap junction hemichannels, they are expected to express functional intercellular gap junctions. This was tested by examining cell coupling with the gap junction-permeable dye Lucifer yellow (LY) (Abbaci et al., 2008; Stewart, 1978). The

dye was introduced into monolayers of HCLE cells by scraping the cells with a pipet tip and allowing the dye to enter damaged cells and diffuse through gap junctions into neighboring cells during a five-minute incubation. Cells were simultaneously incubated with rhodamine-conjugated dextran, which is excluded from gap junctional transport due to its large size. Fluorescence microscopy clearly showed LY- and rhodamine-dextran-positive cells adjacent to the scratch, but only LY was found in cells distal from the scratch, indicating the presence of functional gap junctions. When cells were incubated with 50 μM FFA prior to dye loading, LY-positive cells were restricted to the rhodamine-dextran positive cells, indicating that FFA can inhibit dye coupling through gap junctions. Incubations with 100 μM GA however did not inhibit dye coupling, indicating that this compound may not inhibit gap junction permeability in my hands. This may explain the lack of effect of GA relative to FFA in the drug screen (Figure 35).

FFA can inhibit permeability of channels formed by not only connexins, but also by pannexins, a recently described family of proteins that is structurally homologous to connexins. Like connexins, pannexins form plasma membrane channels (pannexons) that are permeable to small molecules such as ATP, but unlike connexons, they are principally non-junctional channels (D'Hondt et al., 2009; Panchin et al., 2000). Permeability of connexons is more sensitive to FFA than another inhibitor, carbonexolone (CBX), while pannexons are more sensitive to CBX (Bruzzone et al., 2005; D'Hondt et al., 2009). As shown in Figure 37, ATP release from cells cultured on plastic strips was more sensitive to FFA than to CBX. These results are consistent with the hypothesis that connexin hemichannels mediate ATP release after wounding.

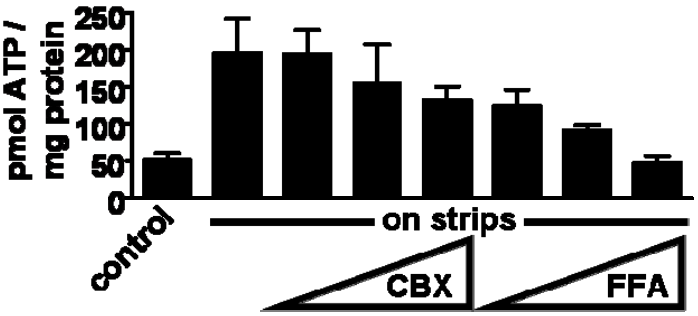

Figure 37 ATP release is sensitive to inhibitors of connexons and pannexons

ATP concentrations were determined in conditioned media from cells cultured on plastic strips following no treatment (NT), treatment with 5, 50 or 500 μM carbonexolone (CBX), or 5, 50, or 500 μM flufenamic acid (FFA).

4.2.3 Connexin mimetic peptides do not inhibit ATP release.

I next examined ATP release in the presence of inhibitors that more specifically target connexons. Connexin mimetic peptides have been designed and are used routinely to inhibit permeability of gap junctions and connexin hemichannels (Evans and Boitano, 2001; Evans et al., 2006; Kwak and Jongsma, 1999; Leybaert et al., 2003). These peptides, 10-15 amino acids in length, bind to extracellular regions specific to particular connexins, and mimic connexin/connexin binding at gap junctions. Long term (>2 hour) treatment of cells with these peptides can prevent the formation of gap junctions, but short-term treatment (10-30 minutes) can prevent hemichannel opening and ATP release, perhaps by functioning as a plug in the connexon pore (Leybaert et al., 2003).

I investigated the effects of the mimetic peptides “Gap26” and “Gap27” (Boitano and Evans, 2000) on the release of ATP from cells cultured on strips. I could not use Gap27 because it was soluble only at a low pH that interfered with the bioluminescence assay. Treatments with

Gap26, however, were feasible. As shown in Figure 38, incubation with 300 μM of Gap26 for 30 min, 3 hours, or 24 hours did not inhibit ATP release from cells cultured on strips. However, LY dye transfer was unaffected by 3-hour treatment with Gap26, indicating that the Gap26 may not have successfully blocked connexon permeability. Therefore, I cannot conclude from these mimetic peptide studies whether connexons contribute to ATP release.

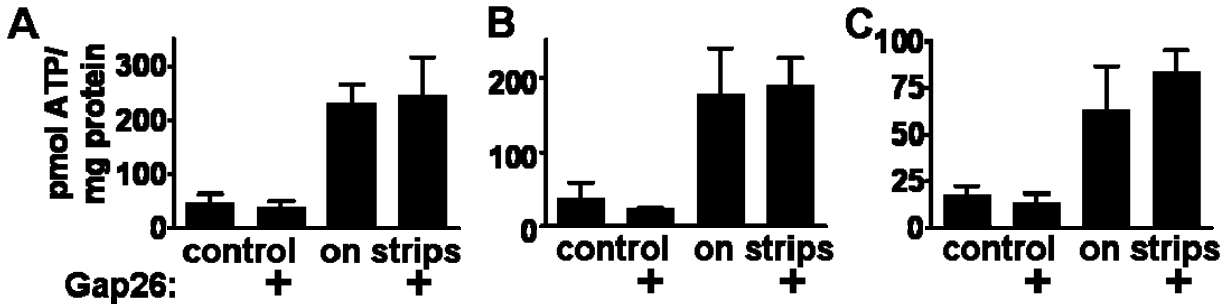

Figure 38 The mimetic peptide Gap26 does not inhibit ATP release

Assay for quantities of ATP in conditioned media from cells cultured on plastic strips or controls. As indicated, cells were incubated with 300 μM of Gap26 and media were assayed after **A**. 30 min, **B**. 2 hr, and **C**. 24 hr.

4.3 DISCUSSION

Wounding is known to cause cell damage that results in the rapid release of intracellular components such as ATP. In this chapter, I have shown that wounding also causes the persistent release of ATP independently of cell damage. I have also conducted preliminary studies to identify the mechanism by which ATP is secreted. ATP secretion was inhibited by treatment with the gap junction inhibitor FFA, but not by treatment with the connexin mimetic peptide Gap26.

Using two distinct damage-minimizing models, I have made the novel observation that ATP is persistently released from cells near the wound edge. In migrating neutrophils, ATP has been found to be released from the leading edge of the cell surface (Chen et al., 2006), but whether ATP is released from the leading edge of migrating epithelial sheets remains to be determined. Various imaging techniques based on performing bioluminescence assays *in situ* under dark-field microscopy could shed light on this issue (Beigi et al., 1999; Wang et al., 2000).

In both wounding models used, concentrations of ATP in conditioned media increased four-fold, from roughly 25 nM to 100 nM. Because ATP activates the EGFR in HCLE and other epithelial cells in the 5-50 μM range (concentrations that are typically observed in conditioned media minutes after wounding), the persistent release of ATP may have functions that are independent of EGFR signaling. Low nM concentrations of extracellular ATP are sufficient to induce type-2 purinergic receptor activation and stimulate increases in intracellular calcium concentrations, but the precise role of persistent ATP release in facilitating wound healing is unclear (Abbracchio et al., 2006; Geyti et al., 2008).

Extracellular ATP concentrations in the nM range are consistent with a mechanism of ATP release that is independent of cell damage. My experiments do not support a role for ABC-family transporters, nucleotide transporters, or exocytosis of ATP-loaded vesicles because their inhibitors did not block ATP release in my studies as they have in others (Knight et al., 2002; Lazarowski et al., 2003; Maroto and Hamill, 2001). ATP release was, however, sensitive to inhibition by FFA and CBX, which block permeability of channels formed by the connexin and pannexin protein families. Inhibition of ATP release was more sensitive to FFA than to CBX, suggesting that release is a function of connexon, not pannexon, permeability. Incubations with the connexin mimetic peptide Gap26 did not inhibit ATP release (Figure 38) or LY dye transfer,

so these studies should be repeated in the future using higher purity peptides. Gap26 can bind and inhibit Cx43, the dominant connexin expressed in the corneal epithelium, but it is unknown whether it inhibits the actions of other corneal connexins (Shurman et al., 2005; Williams and Watsky, 2002). Based on amino acid sequence analysis, Gap26 is expected to inhibit all corneal connexins except Cx30.

Although I cannot conclude that wounding stimulates ATP release through connexin hemichannels, these preliminary studies can serve as a foundation for more decisive studies. For example, imaging of connexon-permeable dyes (Stout et al., 2002), approaches involving loss-of-function (siRNA or dominant-negative) methodologies in HCLE cells, and gain-of-function methodologies in HeLa cells (that do not express connexins or pannexins) could determine whether the persistent release of ATP is indeed regulated by connexin hemichannels.

5.0 CHARACTERIZATION OF THE ATP/PLD2-INDEPENDENT MECHANISM OF WOUND-INDUCED EGFR ACTIVATION: ROLES OF SRC-FAMILY KINASES AND PYK2

5.1 INTRODUCTION

Cells at the edges of wounds undergo profound phenotypic changes and acquire the ability to migrate rapidly (Block and Klarlund, 2008; Farooqui and Fenteany, 2005; Fenteany et al., 2000; Omelchenko et al., 2003; Poujade et al., 2007; Tamada et al., 2007). Activation of the EGFR is absolutely required for these changes to occur (Block et al., 2004; Zieske et al., 2000), and I have previously identified the presence of dual mechanisms of wound-induced EGFR activation: one that depends on signaling by extracellular ATP that is dispensable for wound healing, and another that functions specifically in cells near the wound edge ((Block and Klarlund, 2008) and Chapters Two and Three).

Recent evidence indicates that Src-Family Kinases (SFKs) control wound-induced EGFR activation by regulating EGFR ligand shedding after wounding (Block and Klarlund, 2008; Xu et al., 2006). SFKs contain Src-Homology-2 and -3 (SH2 and SH3) domains that bind to consensus phosphotyrosine and proline-rich domains, respectively, which can lead to activation by loss of inhibitory intramolecular interactions (Roskoski, 2005). The signals that control wound-induced SFK activation, and ultimately stimulate EGFR signaling and healing, are not known.

The focal adhesion kinase (FAK) family of non-receptor tyrosine kinases includes FAK and Pyk2 (a product of the PTK2B gene; also called Related Adhesion Focal Tyrosine Kinase (RAFTK) and Cell Adhesion Kinase β (CAKβ)), and is known to bind and activate SFKs (Avraham et al., 2000; Dikic et al., 1996; Schlaepfer et al., 1999). Also, various stimuli that cells encounter near the wound edge, such as integrin/matrix interactions, physical stretch, and calcium influx are known to activate FAK and/or Pyk2 (Assoian and Klein, 2008; Avraham et al., 2000). In this chapter, I have tested the hypothesis that after epithelial wounding, the FAK family stimulates SFK activity, which in turn activates the EGFR and promotes motility. Portions of this chapter have been submitted for publication (see Appendix A).

5.2 RESULTS

5.2.1 Wounding stimulates EGFR ligand release and EGFR activation in the absence of extracellular ATP.

EGFR activation in cells near the wound edge is regulated by extracellular ATP and by an extracellular ATP-independent mechanism (Block and Klarlund, 2008). To characterize the mechanism of EGFR activation in the ATP/PLD2-independent pathway, I have used a model for detecting signaling in wound-proximal cells (Figure 5) and performed most of the following experiments in the presence of 25 U/ml apyrase. I have previously shown that similar amounts of apyrase hydrolyze extracellular ATP to undetectable levels (Figure 9) and inhibit activation of PKC (Figure 10) and PLD (Figure 19) in cells near the wound edge.

Inclusion of the general protease inhibitor GM 6001 inhibited activation of the EGFR after wounding (Figure 39), although it did not inhibit activation by exogenous ligand (Block et al., 2004), which is consistent with the notion that EGFR activation is the result of a proteolytic event. Also, the LA1 antibody, which blocks activation of the EGFR by extracellular ligands, and neutralizing anti-AR antibodies inhibited EGFR activation (Figure 39). Together with the previous observation that AR is released in the presence of apyrase (Figure 10) these data support that the extracellular ATP-independent pathway activates the EGFR though a triple membrane-passing mechanism (Figure 4).

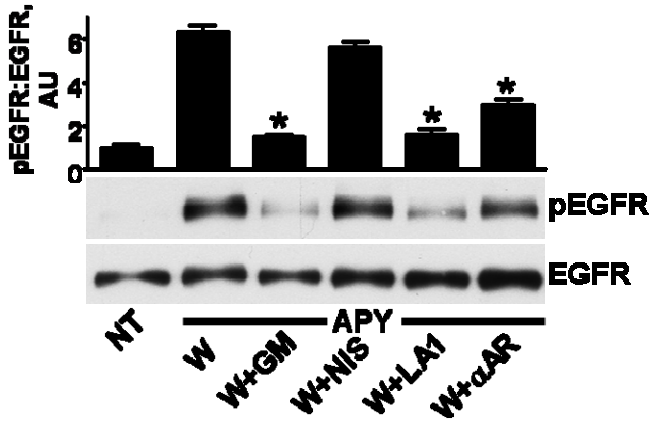

Figure 39 EGFR activation by proteolytic ligand release in the absence of extracellular ATP

Western Blots for activation of EGFR after no treatment (NT), wounding (W), and/or treatments with 25 U/ml Apyrase (APY), 1 μM GM 6001 (GM), 20 μg/ml non-immune serum (NIS), 20 μg/ml anti-EGFR antibody (LA1) and 20 μg/ml AR neutralizing antibody (αAR).* indicates significant decrease from wounded controls ($p<0.001$)

5.2.2 Src-family kinases regulate EGFR activation near the wound edge.

The Src-family kinases (SFKs) have been suggested to act upstream of EGFR activation after wounding (Xu et al., 2006) and after treatment with ATP (Chapter Three). To examine their possible role in the ATP/PLD2-independent pathway, I first noted that elimination of extracellular ATP with apyrase did not abolish activation of SFK as monitored by immunoblotting with an antibody that recognizes the SFKs phosphorylated on Tyr-419

(Roskoski, 2005) (Figure 40A). Blocking EGFR kinase activity with tyrphostin AG 1478 did not block SFK activation in HCLE cells, in agreement with the notion that SFKs are upstream of EGFR activation (Figure 40B). To assess whether SFKs might also be instrumental in activation of the EGFR in the ATP/PLD2-independent pathway, I incubated cells with two structurally dissimilar SFK inhibitors that are known to have distinct non-specific interactions (Bain et al., 2007). Inclusion of either PP2 or Src Kinase Inhibitor-I (SKI) inhibited activation of the EGFR and the release of AR in the presence of apyrase (Figure 40C and D), although they did not inhibit EGFR activation by exogenous ligand (Figure 28B), indicating that SFKs are required for EGFR activation in this pathway.

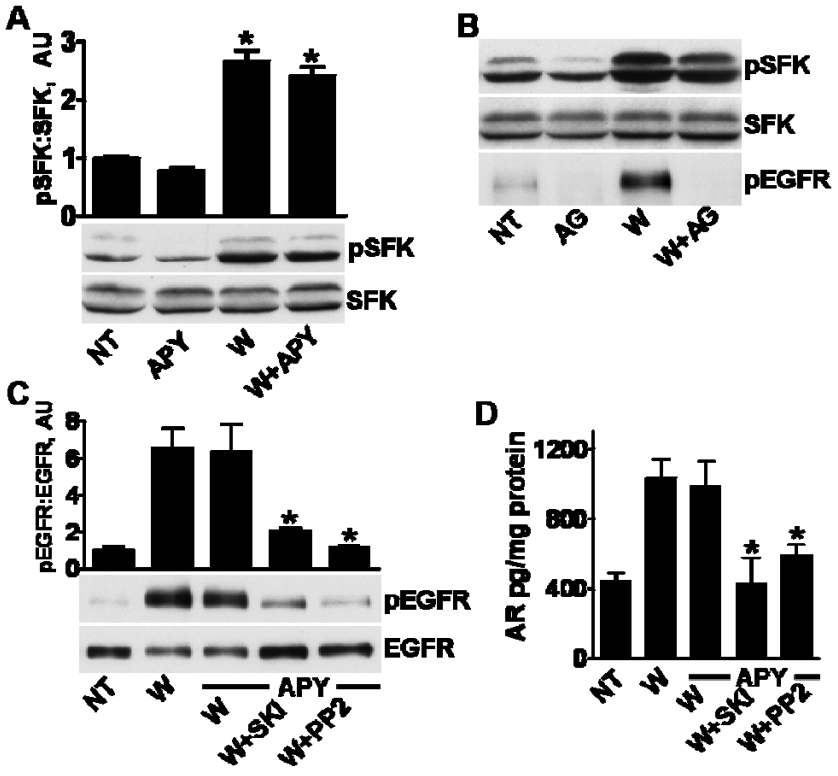

Figure 40 SFKs regulate wound-induced EGFR activation in the absence of extracellular ATP

A. Western Blots and densitometry for activation of SFK 5 min after no treatment (NT), wounding (W), and/or treatment with 25 U/ml Apyrase (APY). * indicates significant increases from unwounded controls ($p<0.001$). **B**. Cells were treated as in A but 1 μM AG 1478 (AG) was added 30 min prior to stimulations. **C**. Activation of EGFR

was monitored in the presence of 1 μM Src Kinase Inhibitor-I (SKI) or 10 μM PP2. **D**. AR quantities in conditioned media from cells treated as in C were determined. For C and D, * indicates significant decrease from wounded controls ($p<0.001$).

5.2.3 Pyk2, but not FAK, is activated rapidly after wounding, independently of signaling by extracellular ATP, EGFR, or SFK.

To determine whether the Focal Adhesion Kinase family plays a role in initiating SFK and EGFR activation, I first tested whether FAK or Pyk2 is activated after wounding. Pyk2, but not FAK, was activated five minutes after wounding as determined by Western Blotting for an autophosphorylation site on either kinase (Figure 41A). Pyk2 and FAK activation was observed after treatment of cells with 100 nM of the phosphatase inhibitor sodium orthovanadate (Figure 41A), indicating the immunoblotting procedure was capable of detecting both kinases when autophosphorylated. Since FAK was not activated detectably after wounding, I concluded that FAK signaling is not likely to regulate SFK activity in this context, so I therefore focused on Pyk2.

I determined that Pyk2 activation proceeds independently of extracellular ATP because Pyk2 activation was not inhibited by 25 U/ml apyrase (Figure 41B). In parallel studies, 25 U/ml apyrase did inhibit wound-induced activation of PKC, indicating that the treatment was effective (Figure 41C). In some systems, Pyk2 is activated downstream of the EGFR (Park et al., 2007; Schauwienold et al., 2008; Shi and Kehrl, 2004). However, activation of Pyk2 was not reduced in the presence of 100 nM of the EGFR tyrosine kinase inhibitor AG 1478, which effectively blocked EGFR autophosphorylation (Figure 41B). Pyk2 contains tyrosine residues that can be phosphorylated by the SFKs, and some studies indicate that SFK-mediated phosphorylation

regulates Pyk2 activity (Dewar et al., 2007; Duong et al., 1998; Sieg et al., 1998). However, wound-induced Pyk2 autophosphorylation was unaffected by treatment with 1 μM of the SFK inhibitor SKI (Figure 41D). The SFK inhibitor blocked EGFR phosphorylation of Tyr-845, which is catalyzed by the SFKs, demonstrating the efficacy of the inhibitor (Figure 41D). Together, these results indicate that Pyk2 is activated independently of the EGFR and SFKs.

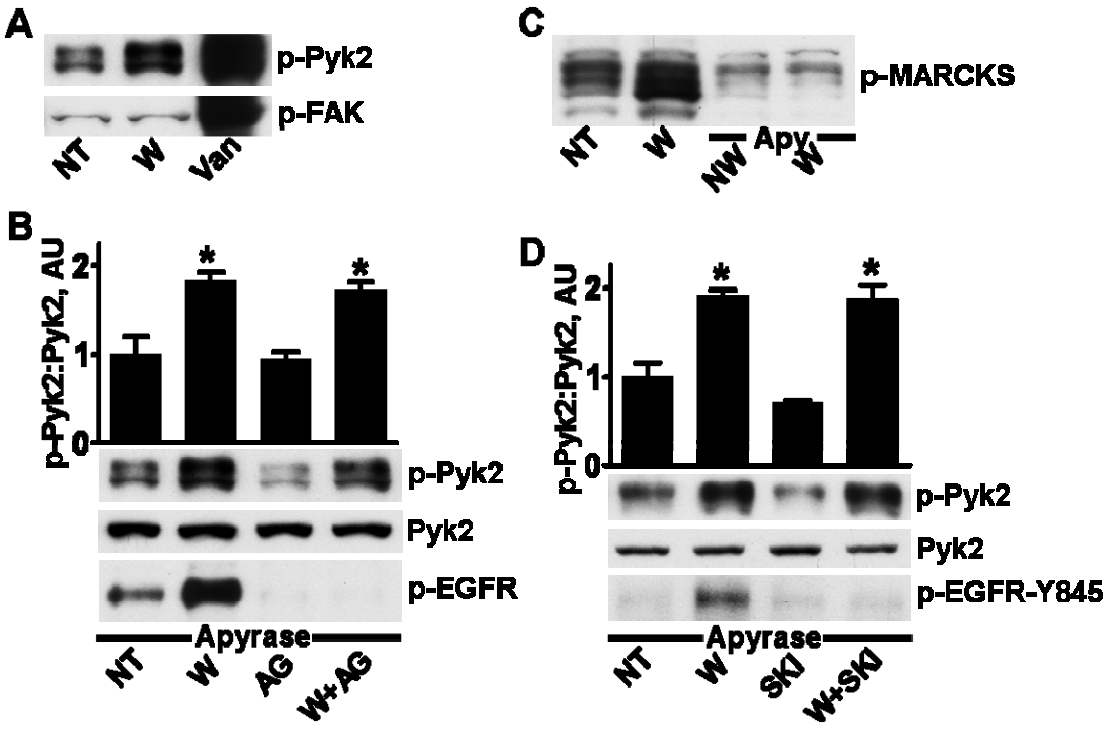

Figure 41 Pyk2, not FAK, is activated rapidly after wounding

A. HCLE cells were given no treatment (NT) or were wounded (W) and incubated for 5 minutes and cell extracts were analyzed by Western Blotting for Pyk2 phosphorylated on Tyr-402 (p-Pyk2) or for FAK phosphorylated on Tyr-397 (p-FAK). As a positive control for the antibodies, 100 nM Na_3VO_4 was added to the cells for 30 minutes prior to analysis (Van). **B**. In the presence of 25 U/ml apyrase, cells received no additional treatment (NT) or were wounded (W) and incubated for 5 minutes. Where indicated, cells were treated with 100 nM of AG 1478 (AG) for 30 min prior to and during wounding. Western Blots were probed for p-Pyk2 and were then stripped and re-probed for total levels of Pyk2 and EGFR phosphorylated on Tyr-1173 (p-EGFR). The ratio of p-Pyk2:Pyk2 was determined by densitometry and * denotes significant differences from unwounded groups ($p<0.001$). **C**. Cells

were treated as indicated and Western Blots were probed for phosphorylated MARCKS (p-MARCKS), which is a marker of PKC activity. **D**. Cells were treated as in B, except 1 μM of Src Kinase Inhibitor-I (SKI) was added for 30 min prior to and during wounding, where indicated. The blots were analyzed with an antibody that recognizes the EGFR phosphorylated on Tyr-845 (p-EGFR-Y845). Densitometry was performed as in B.

5.2.4 C-Src is activated and associates with Pyk2 after wounding.

Since the pharmacological studies suggested that Pyk2 may be activated upstream of SFK, I proceeded with various studies to determine whether Pyk2 signaling directly causes SFK activation after wounding, the first of which was to determine whether Pyk2 and SFKs interact. Pyk2 autophosphorylation on Tyr-402 presents binding sites for SFK SH2 domains (Dikic et al., 1996). Thus, SFK can be activated by binding to autophosphorylated Pyk2.

I was able to immunoprecipitate the major SFK isoforms c-Src and Fyn from my cells (Figure 42) and analyze their activation states with an antibody that recognizes activated forms of the SFKs. Both c-Src (Figure 43A) and Fyn (Figure 43B) were activated within five minutes after wounding. SFKs are activated downstream of the EGFR in some systems (Abram and Courtneidge, 2000), however the presence of 100 nM tyrphostin AG 1478, which effectively blocked EGFR activity (Figure 41B), had no effect on c-Src or Fyn activation, indicating that c-Src and Fyn are activated upstream of the EGFR.

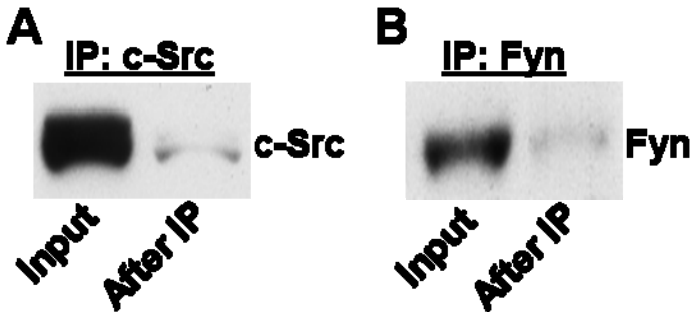

Figure 42 The SFK isoforms c-Src and Fyn were immunoprecipitated from HCLE cells

Depletion of SFK isoforms from cell extracts is shown by Western Blots for **A**. c-Src and **B**. Fyn before (Input) and after immunoprecipitations (IP).

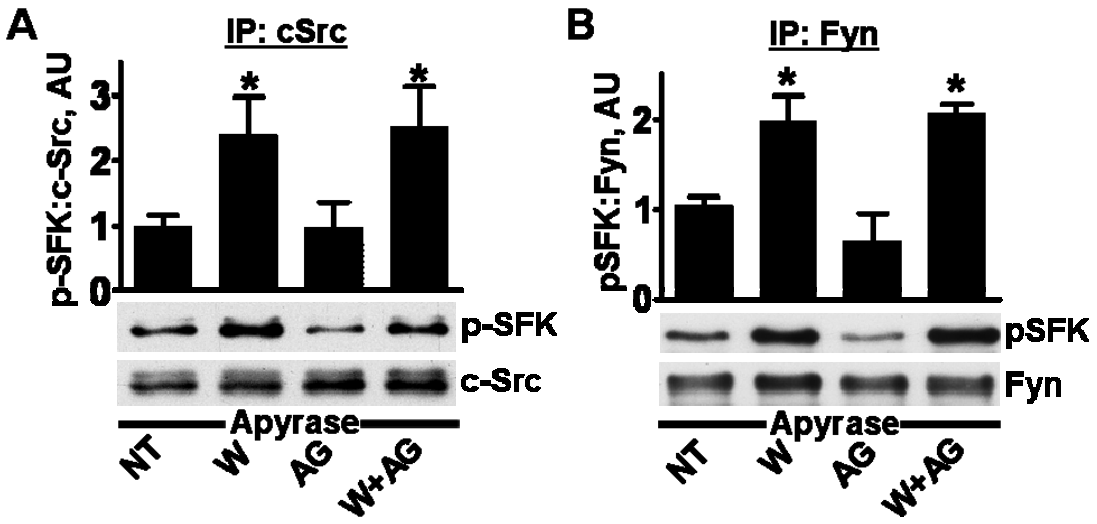

Figure 43 C-Src and Fyn are activated after wounding

Cells received no treatment (NT) or were wounded (W) and incubated for 5 minutes; all conditions in the presence of 25 U/ml apyrase. Where indicated, cells were treated with 100 nM of the EGFR inhibitor AG 1478 (AG) for 30 min prior to and during wounding. The extracts were precipitated with **A**. c-Src and **B**. Fyn antibodies, and the precipitates were subjected to Western Blotting for SFK phosphorylated on Tyr-419 (p-SFK) and subsequently for total levels of c-Src or Fyn. Ratios were determined by densitometry and * denotes significant differences from unwounded groups ($p<0.001$).

The SFKs can be activated by binding to Pyk2 after it is autophosphorylated (Dikic et al., 1996). Analysis of immunoprecipitates of c-Src showed the presence of increased levels of Pyk2 after wounding, suggesting an increased level of association of the two molecules (Figure 44A). The specificities of the signals were confirmed by performing mock precipitations with non-immune IgG (Figure 44B). The interaction did not depend on EGFR activation since co-immunoprecipitation was not blocked by the presence of tyrphostin AG 1478 (Figure 44A). A similar analysis with c-Fyn was not possible because the signals were too faint. Together, these results indicate that wounding stimulates activation of multiple SFK isoforms and promotes formation of a c-Src/Pyk2 signaling complex.

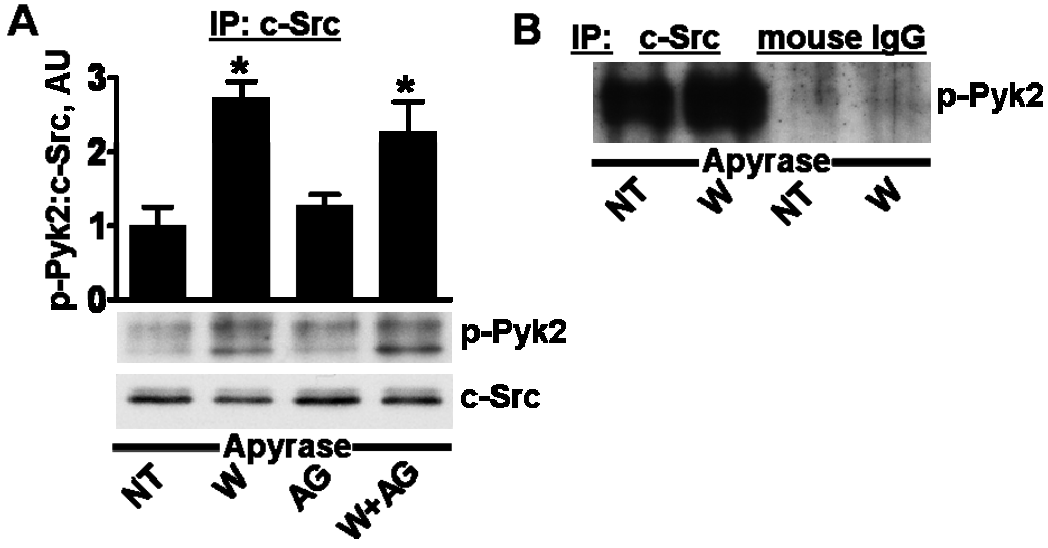

Figure 44 C-Src interacts with Pyk2 after wounding

A. Cells received no treatment (NT) or were wounded (W) and incubated for 5 minutes; all conditions in the presence of 25 U/ml apyrase. Where indicated, cells were treated with 100 nM of the EGFR inhibitor AG 1478 (AG) for 30 min prior to and during wounding. The extracts were precipitated with c-Src antibodies and the precipitates were subjected to Western Blotting for Pyk2 phosphorylated on Tyr-402 (p-Pyk2) and subsequently for total levels of c-Src. Ratios were determined by densitometry and * denotes significant differences from unwounded

groups ($p<0.01$). **B**. Lysates were subjected to immunoprecipitation with anti-c-Src antibodies or non-immune mouse IgG; immunoprecipitates were subjected to Western Blotting for p-Pyk2. Over-exposed film shows the absence of signal from the non-immune control.

5.2.5 Pyk2 signaling is necessary for SFK and EGFR activation after wounding.

To determine whether Pyk2 is necessary for wound-induced SFK activation, I employed multiple approaches to modulate Pyk2 signaling. I initially examined the effects of reducing Pyk2 expression with siRNA prior to wounding. Cells were transfected with 10 nM of a pool of Pyk2-targeted siRNA oligonucleotides before wounding. Pyk2 siRNA transfection caused 70% reduction in Pyk2 expression compared to controls and resulted in inhibition of wound-induced SFK and EGFR activation (Figure 45). As a confirmation and to better control for off-target effects, I repeated these studies using 10 nM of a single Pyk2-targeted siRNA oligonucleotide. The Pyk2 siRNA caused approximately 75% reduction in Pyk2 expression (Figure 46A) and inhibited significantly SFK (Figure 46B) and EGFR (Figure 46C) activation after wounding. Treatment of Pyk2 siRNA-transfected cells with EGF yielded equivalent EGFR activation as treatment of control-transfected cells (Figure 46D), indicating that the siRNA did not interfere with the normal kinase activity of the EGFR.

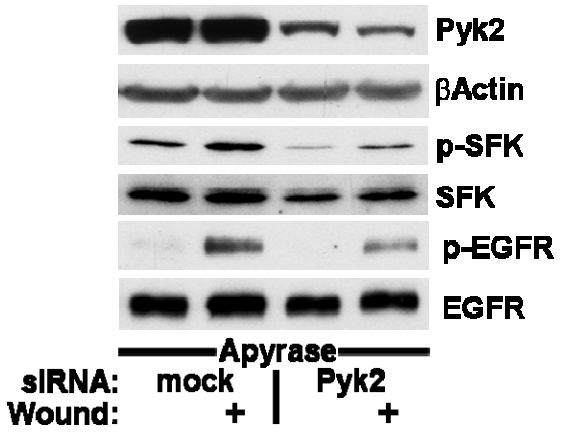

Figure 45 Knockdown of Pyk2 with siRNA inhibits SFK and EGFR activation after wounding

HCLE cells mock-transfected or transfected with a pool of Pyk2 siRNA oligonucleotides were wounded as indicated. 25 U/ml apyrase was present in all conditions. Western Blots were probed for activation of SFK and EGFR and for verification of Pyk2 knockdown.

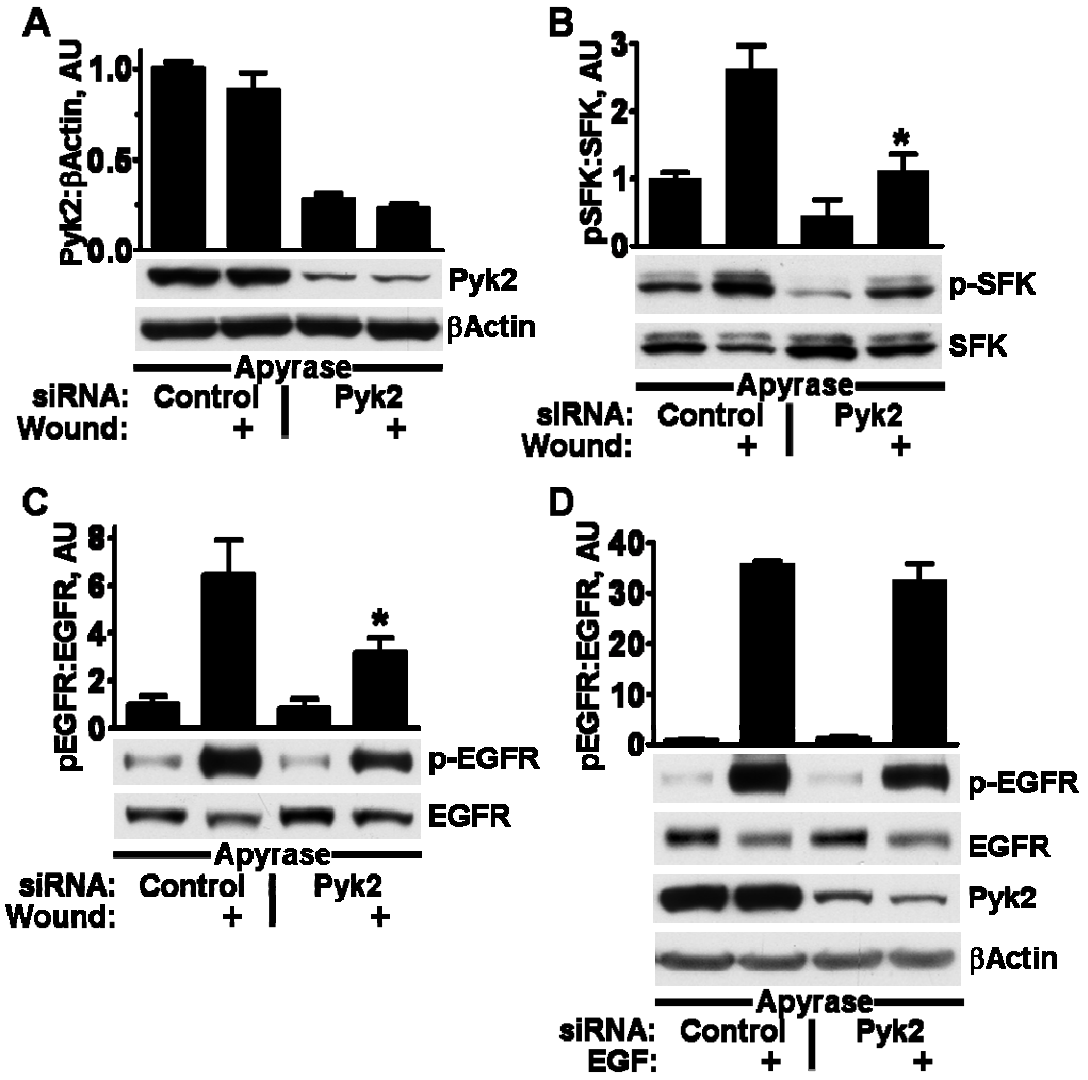

Figure 46 Knockdown of Pyk2 with siRNA inhibits SFK and EGFR activation after wounding but not after stimulation with EGF

HCLE cells transfected with 10 nM of control or Pyk2 siRNA were treated with apyrase and were wounded as indicated. Western Blots were probed for **A**. total levels of Pyk2 and βActin, **B**. SFK phosphorylated (p-SFK) and non-phosphorylated (SFK) on Tyr-419, and **C**. EGFR phosphorylated on Tyr-1173 (p-EGFR) and total levels of EGFR. Ratios were determined by densitometry and * denotes significant decrease from wounded controls ($p<0.001$). **D**. HCLE cells transfected with control or Pyk2 siRNA were treated with 10 ng/ml EGF for 5 min where indicated prior to Western Blotting and densitometry.

As a complement to these studies, I tested whether Pyk2 is necessary for wound-induced SFK and EGFR activation by wounding sheets of HCLE cells that express Pyk2-Related Non-kinase (PRNK), which is a truncated version of Pyk2 that consists of the C-terminal 120 amino acids and lacks the kinase domain. It can act in a dominant-negative fashion to inhibit endogenous Pyk2 activation and signaling (Xiong et al., 1998). PRNK (~30 kDa) was clearly expressed in HCLE cells infected with an adenovirus coding for PRNK, and its presence blocked Pyk2 activation after wounding as expected (Figure 47A). Importantly, activation of SFKs (Figure 47B) and the EGFR (Figure 47C) were also significantly inhibited by the presence of PRNK. EGF-induced EGFR activation was not inhibited by PRNK expression (Figure 47D), indicating that PRNK did not affect the EGFR directly.

I also examined the effects of blocking Pyk2 signaling on the individual SFK isoforms. C-Src activation was inhibited when Pyk2 signaling was disrupted by siRNA (Figure 48A) or PRNK (Figure 48B). Similarly, activation of the Fyn isoform was inhibited by Pyk2 siRNA (Figure 48C) or PRNK (Figure 48D). Therefore, although I could not determine the degree to which Fyn and Pyk2 interact after wounding, Pyk2 signaling does appear to be necessary for Fyn activation. Together, the results from the studies with siRNA and PRNK support that Pyk2 mediates activation of the SFKs and the EGFR after wounding.

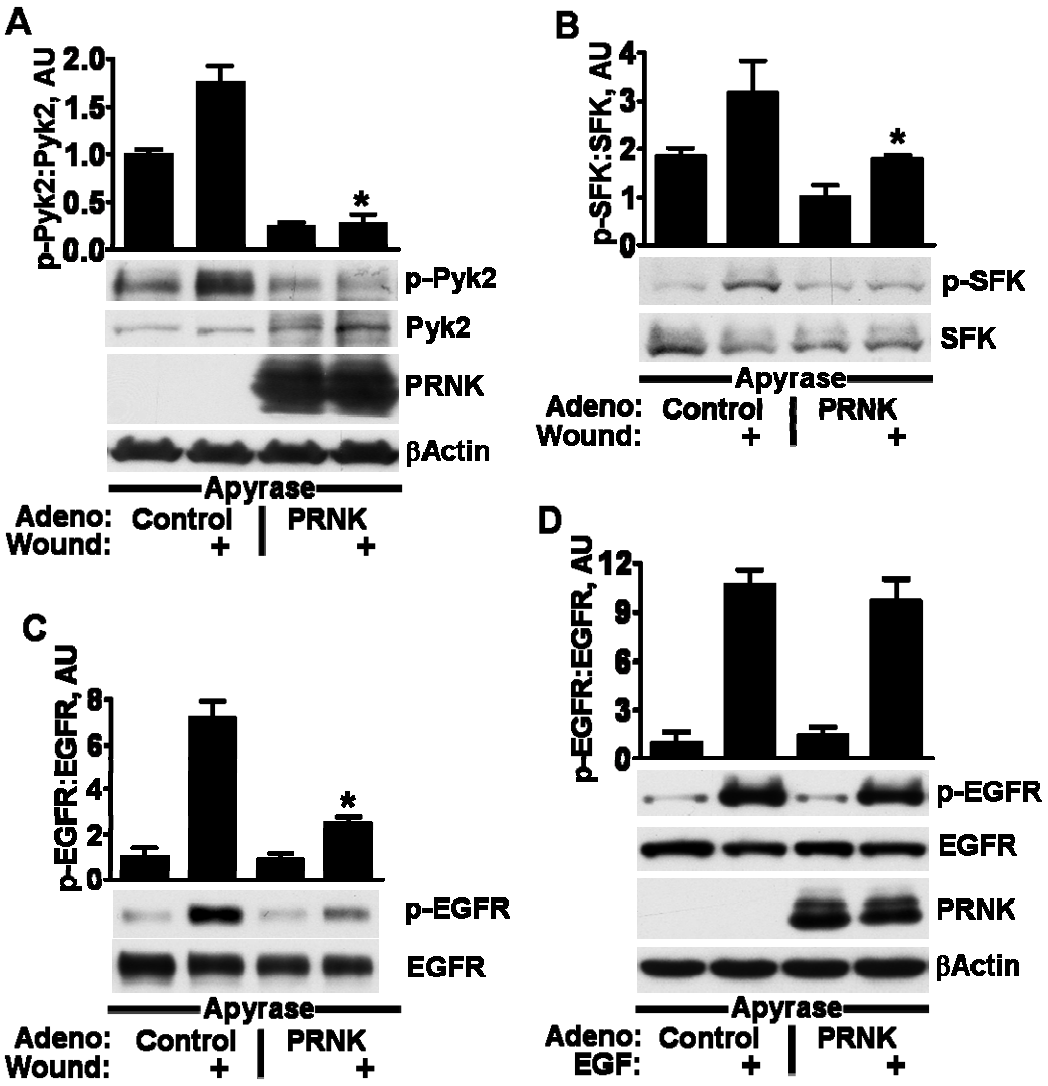

Figure 47 Expression of a Pyk2 dominant-negative mutant inhibits SFK and EGFR activation after wounding but not after stimulation with EGF

HCLE cells infected with control adenovirus or adenovirus coding for PRNK were incubated with apyrase and were wounded as indicated. Western Blots were probed for **A**. Pyk2 phosphorylated on Tyr-402 (p-Pyk2), total levels of Pyk2, PRNK, and βActin, **B**. SFK phosphorylated (p-SFK) and non-phosphorylated (SFK) on Tyr-419, and **C**. EGFR phosphorylated on Tyr-1173 (p-EGFR) and total levels of EGFR. Ratios were determined by densitometry and * denotes significant decrease from wounded controls ($p<0.001$). **D**. HCLE cells infected with control adenovirus or adenovirus coding for PRNK were treated with 10 ng/ml EGF for 5 min where indicated prior to Western Blotting and densitometry.

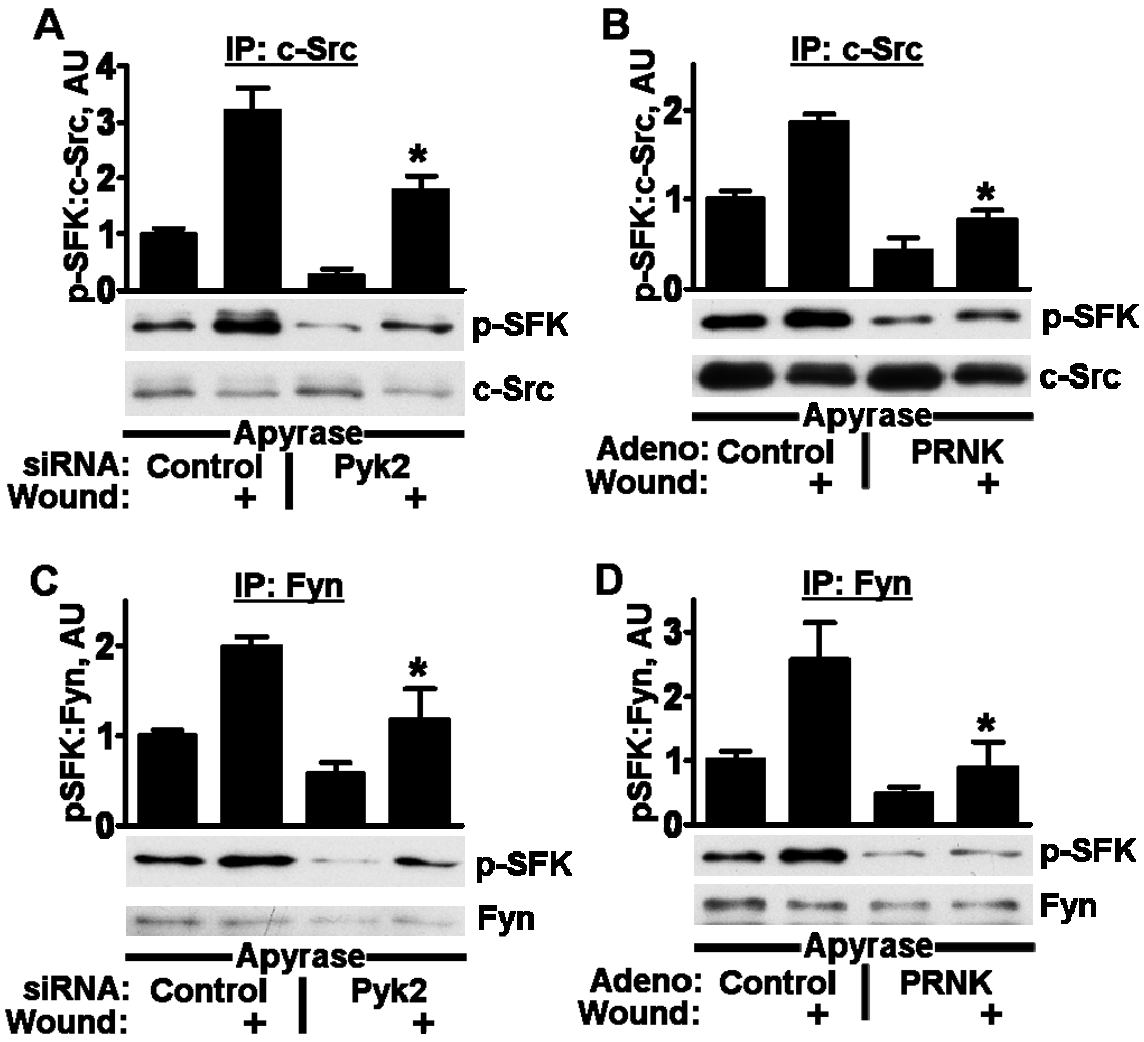

Figure 48 Disruption of Pyk2 signaling by siRNA or PRNK inhibits wound-induced activation of c-Src and Fyn

HCLE cells transfected with 10 nM of control or Pyk2 siRNA (**A** and **C**) or infected with control adenovirus or adenovirus coding for PRNK (**B** and **D**) were treated with apyrase and were wounded as indicated. C-Src immunoprecipitates were immunoblotted for SFK phosphorylated on Tyr-419 (p-SFK) and for total levels of c-Src (**A** and **B**). Fyn immunoprecipitates were immunoblotted for SFK phosphorylated on Tyr-419 (p-SFK) and for total levels of Fyn (**C** and **D**). Ratios were determined by densitometry and * denotes significant decrease from wounded controls ($p<0.001$).

To test whether increasing Pyk2 signaling promotes SFK and EGFR activation, I infected cells with adenovirus encoding wild-type Pyk2, which caused highly increased levels of activated Pyk2, perhaps because the over-expression overwhelmed Pyk2 phosphatases or other negative regulators. Pyk2 over-expression caused highly increased levels of activated SFKs (Figure 49A), which was expected due to the direct association of the two kinases. Importantly, Pyk2 overexpression also induced significant increases in the levels of activated EGFR (Figure 49B) in agreement with a role for Pyk2 as an upstream activator. The activation of SFKs by Pyk2 is direct, but many signaling intermediates are required for EGFR activation by SFKs, so relative to SFKs, the EGFR was only modestly activated by Pyk2 expression.

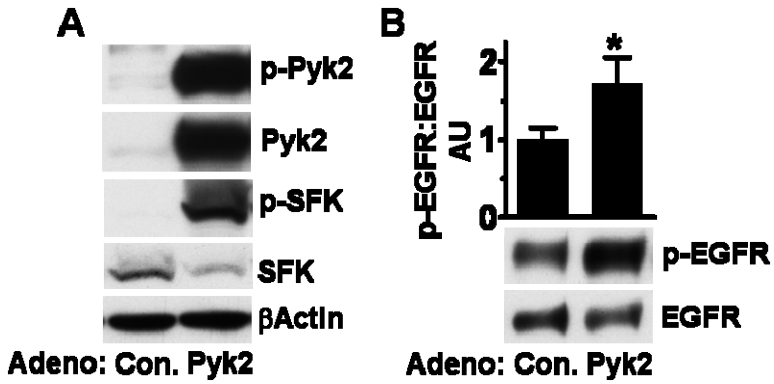

Figure 49 Over-expression of wild-type Pyk2 stimulates SFK and EGFR activation

HCLE cells infected with control adenovirus (Con.) or adenovirus coding for Pyk2 were subjected to Western Blotting for **A**. Pyk2 phosphorylated on Tyr-402 (p-Pyk2), total levels of Pyk2, SFK phosphorylated (p-SFK) and non-phosphorylated (SFK) on Tyr-419, and β-actin, and **B**. EGFR phosphorylated on Tyr-1173 (p-EGFR) and total levels of EGFR. Ratios were determined by densitometry and * denotes significant increase from controls ($p<0.01$).

5.2.6 Pyk2 activation is necessary for wound healing.

Since Pyk2 regulates SFK and EGFR activation, I expected that modulation of Pyk2 signaling would alter healing rates in our cell culture wound healing assay. Transfection with 10 nM of a

single Pyk2 siRNA oligonucleotide prior to wounding reduced Pyk2 expression by ~60% and retarded wound healing by 40% compared to transfection with control siRNA (Figure 50A). The effect of Pyk2 siRNA on healing was not due to differences in cell division or death because similar cell densities were observed in control and Pyk2 siRNA-transfected cultures (Figure 50B). Treatment with 10 ng/ml EGF during healing resulted in similar accelerated healing rates in both control and Pyk2 siRNA-transfected conditions (Figure 50A), indicating that the effects of Pyk2 siRNA are upstream of the EGFR.

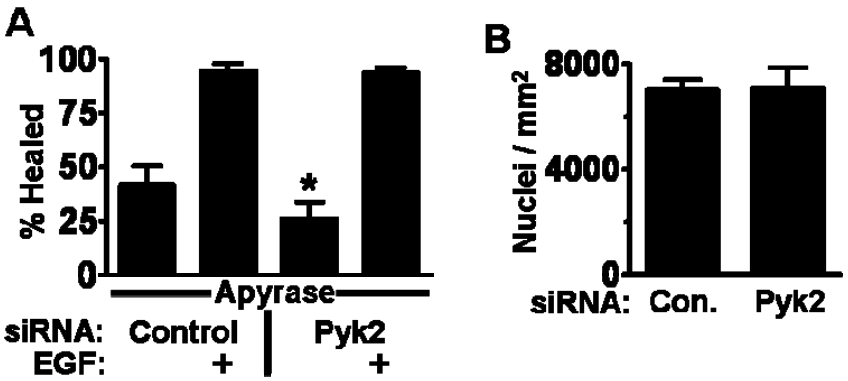

Figure 50 Pyk2 activation is necessary for wound healing

HCLE cells transfected with 10 nM of control or Pyk2 siRNA were subjected to wound healing assays in the presence of 25 U/ml apyrase. Where indicated, cells were incubated with 10 ng/ml EGF during healing. **A**. The percentages of the areas covered during 18 hour periods of healing are shown. * indicates significant difference from untreated controls ($p<0.01$). **B**. Cells were fixed, stained with DAPI, and the average number of nuclei/mm^2 in ten random fields was determined.

Infection of cells with PRNK-expressing adenovirus reduced wound healing by 60% compared to infection with control virus (Figure 51A). Cell densities were similar in control and PRNK-infected cultures (Figure 51B). The PRNK mutant used was tagged with the FLAG epitope, so PRNK expression could be monitored by immunofluorescence microscopy. Interestingly, in fields where PRNK expression was not induced in all of the cells, the FLAG-negative cells had migrated further than the FLAG-positive cells (Figure 51C). These data are

consistent with the hypothesis that Pyk2 activation is necessary for cell motility. EGF stimulated healing in PRNK-expressing cultures 20% less than it stimulated healing in controls (Figure 51A), which indicates that PRNK expression in itself causes a slight defect in wound healing. Pyk2 and PRNK interact with paxillin (Li and Earp, 1997), a component of focal adhesions, so I monitored localization of paxillin by immunofluorescence confocal microscopy. In both FLAG-negative and FLAG-positive cells, paxillin was observed in small puncta near the base of the cell that co-localized with termini of actin fibers (Figure 51D), indicating that PRNK expression does not cause any major perturbation of focal adhesions.

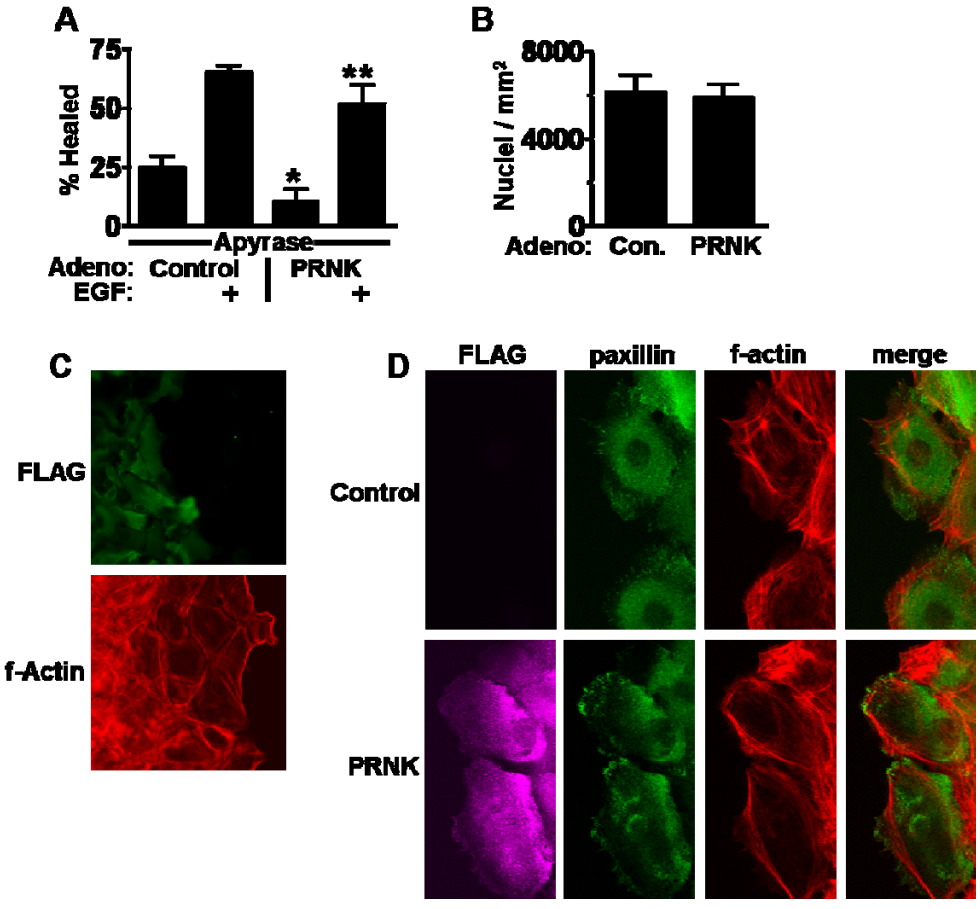

Figure 51 Pyk2 activation is necessary for wound healing

A. Healing and **B**. density of cells infected with control adenovirus or adenovirus coding for FLAG-tagged PRNK was measured as for Figure 47. * ($p<0.001$) indicates significant differences from untreated controls and ** ($p<0.05$) indicates significant difference from EGF-treated controls. Immunofluorescence images of cells from A taken with a **C**. 20X or **D**. 60X objective. Only PRNK-expressing cells stain for FLAG.

Since inhibition of Pyk2 signaling by either siRNA or the dominant negative PRNK resulted in inhibition of healing, I conclude that induction of cell motility in epithelial cells is dependent on Pyk2 and its downstream signaling to the SFKs and the EGFR.

5.3 DISCUSSION

Activation of the EGFR in cells near the wound edge is a decisive signal for the induction of motility, but the signals that trigger EGFR activation after wounding are not completely known. In this chapter, I have shown that EGFR activation is dependent on SFKs that signal to the EGFR through a triple membrane-passing mechanism (Figure 4). I have also identified Pyk2 as a key regulator of wound-induced SFK and EGFR activation that promotes cell migration. Evidence for this conclusion includes: 1, Pyk2 was activated rapidly after wounding, and pharmacological inhibition of signaling by the SFKs or the EGFR did not abrogate Pyk2 activation; 2, association of c-Src and Pyk2 was increased after wounding; 3, over-expression of wild-type Pyk2 stimulated EGFR activation, and 4, inhibition of Pyk2 signaling with siRNA or a dominant-negative mutant inhibited SFK and EGFR activation and healing of wounds in cell sheets.

In the present study, I have observed partial, but not complete inhibition of EGFR activation and wound healing by Pyk2 siRNA or by expression of PRNK. This may be due to incomplete inhibition of Pyk2 signaling or alternatively, it may suggest the presence of additional mechanisms of SFK and EGFR activation after wounding. Wounding a sheet of epithelial cells is a complex event that challenges cells with numerous stimuli that have been hypothesized to promote EGFR activation, including exposure to extracellular matrix, physical stretch, loss of polarity, and loss of physical constraints (Iwasaki et al., 2000; Moro et al., 1998;

Vermeer et al., 2003), and full activation of the EGFR may therefore depend on several signals. Alternate mechanisms of wound-induced EGFR receptor activation are discussed further in Chapter Six.

The mechanism of wound-induced Pyk2 activation is presently unknown. Pyk2 activity is sensitive to numerous environmental cues including integrin/matrix binding, calcium concentration, growth factors and cytokines, reactive oxygen species, and actin cytoskeletal dynamics (Avraham et al., 2000; Schlaepfer et al., 1999). Within minutes of wounding, calcium influx, reactive oxygen species generation, and integrin adhesions have all been observed in cells near the wound edge (Klepeis et al., 2001; Nikolic et al., 2006; Sammak et al., 1997; Zaidel-Bar et al., 2003), but the extent to which these signals contribute to Pyk2, SFK, or EGFR activation in the present system is unclear.

Some evidence indicates that Pyk2 activation is stimulated by EGFR signaling (Park et al., 2007; Schauwienold et al., 2008; Shi and Kehrl, 2004). I have observed Pyk2 activation in HCLE cells treated with EGF, which suggests the presence of positive feedback loops in the wounded epithelium. Indeed, inhibition of EGFR signaling with tyrphostin AG 1478 resulted in the slight reduction of Pyk2 activation after wounding (Figure 41), although these results were not statistically significant.

Many studies investigating GPCR signaling have suggested through indirect means that Pyk2 mediates EGFR transactivation (Burdick et al., 2006; Kanno et al., 2003; Montiel et al., 2007; Park et al., 2006), although some have indicated that Pyk2 and EGFR activation are unrelated events (Kodama et al., 2002; Zwick et al., 1999). In this report, I have tested the hypothesis directly that Pyk2 signaling is necessary for wound-induced EGFR activation by using Pyk2-targeted siRNA oligonucleotides (Figure 46) and by expression of the Pyk2

dominant negative, PRNK (Figure 47). Two other studies have also tested directly whether Pyk2 signaling is necessary for EGFR phosphorylation: EGFR transactivation by GPCR agonists was inhibited in Pyk2-deficient fibroblasts (Andreev et al., 2001), and EGFR transactivation by thiazolidinediones was inhibited by Pyk2-targeted siRNA in a liver epithelial cell line (Dewar et al., 2007). Therefore, data presented here add to the mounting evidence for an important connection between Pyk2 and the EGFR. This generates interest in Pyk2 as a therapeutic target for not only wound healing, but also EGFR-related pathologies such as certain cancers and cardiac hypertrophy (Behmoaram et al., 2008; Hirotani et al., 2004; Iiizumi et al., 2008; Menashi and Loftus, 2009; Roelle et al., 2008; Sun et al., 2008).

Most studies are in accord with a positive role for Pyk2 in promoting motility, as I have shown here in HCLE cells. Decreasing Pyk2 expression inhibited motility of vascular smooth muscle cells (Soe et al., 2009), glioma cells (Lipinski et al., 2005) and macrophages (Owen et al., 2007). Furthermore, Pyk2 over-expression increased motility of glioma (Lipinski et al., 2005) and hepatocellular carcinoma (Sun et al., 2008) cells and expression of a constitutively active Pyk2 mutant enhanced motility of endothelial (Kuwabara et al., 2004) and breast cancer (Zrihan-Licht et al., 2000) cells. However, a few studies are at variance with this conclusion: Pyk2 siRNA stimulated motility of prostate epithelial cells (de Amicis et al., 2006), and a kinase-dead Pyk2 mutant had no effect on motility of breast cancer cells (Zrihan-Licht et al., 2000). Pyk2 knockout mice (Okigaki et al., 2003) would be an excellent model to further examine the role of Pyk2 in epithelial migration and repair. My conclusion that Pyk2 is an upstream activator of the EGFR provides a rationale for a pro-migratory role in cell motility because the EGFR is widely recognized to stimulate cell migration.

5.3.1 Summary and model

In summary, the results presented here identify Pyk2 activation as a critical signal that leads to SFK and EGFR activation in cells near the wound edge. By stimulating SFKs and the EGFR, Pyk2 activation is necessary for healing of epithelial wounds (see diagram in Figure 52).

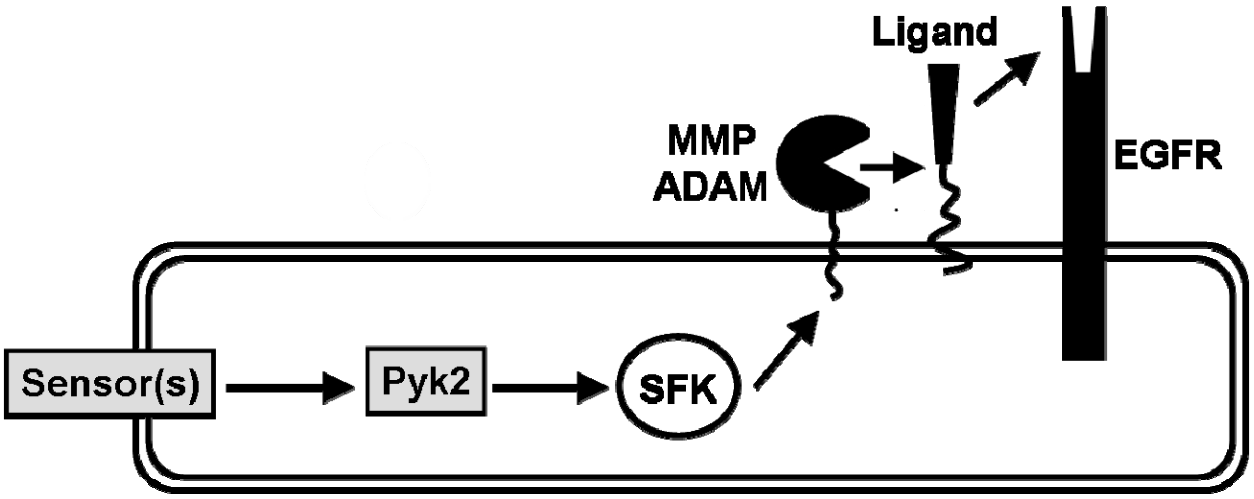

Figure 52 A hypothetical model for the role of Pyk2 in wound-induced EGFR activation and cell migration

See text for details. Pyk2, the focal adhesion kinase Pyk2; SFK, Src-family kinase; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; EGFR, epidermal growth factor receptor.

6.0 EPITHELIAL FREE EDGES TRIGGER ACTIVATION OF THE EGFR

6.1 INTRODUCTION

Wounding is a complex event that presents epithelial cells with numerous stimuli that could promote EGFR activation and the ensuing transition to the motile state. The relative importance of these stimuli may vary based on the type of wound, the distance from the wound, or the time after wounding. For example, as I have shown in Chapters Two and Four, ATP is released rapidly after wounding and can stimulate EGFR activation in cells far from the wound, but hours later, ATP is secreted at levels too low to induce EGFR activation. As another example, alkali burns disrupt the corneal basement membrane differently than scratch wounds, thereby altering cell/matrix interactions that may affect signaling (DeMali and Burridge, 2003; Guo and Giancotti, 2004; Moro et al., 1998; Saika et al., 1993). Whatever the manner of infliction or time after incidence, a common factor shared by all wounds is the presence of a physically unconstrained, or free, edge.

Free edges in epithelial sheets are not exclusive to wounds but are in fact found during embryonic development. In a mammalian model of epithelial morphogenesis, eyelid closure in mice, epithelial sheet movement is dependent on the proteolytic release of HB-EGF that activates the EGFR (Mine et al., 2005). Therefore, not only are the cellular processes that regulate epithelial movements during morphogenesis and wound healing similar, but the signals that

induce this motility are similar as well (Friedl and Gilmour, 2009; Jacinto et al., 2001; Martin and Parkhurst, 2004).

Identifying signaling mechanisms that regulate the activation of the EGFR has been a major focus of recent wounding research (Yu et al., 2009). The majority of this research has focused on signaling that occurs minutes after wounding, but studies of the epidermal and corneal epithelia have indicated that EGFR activation persists as long as a free edge is present (Shirakata et al., 2005; Stoscheck et al., 1992; Zieske et al., 2000). To study signaling that results from free edges, I have employed a model for comparing cell cultures containing numerous edges with cultures containing no edges (see Figure 7 and Methods 8.6). Under the culture conditions, cell damage and access to unoccupied adjacent extracellular matrix is negligible. Using this and other models, I have tested the hypothesis that a free edge alone is sufficient to trigger EGFR activation. Portions of this chapter have been submitted for publication (see Appendix A).

6.2 RESULTS

6.2.1 Chronic EGFR activation is necessary for epithelial migration.

Studies in the epidermal and corneal epithelia have indicated that wounding stimulates prolonged activation of the EGFR, which may lead to its downregulation near the wound edge (Shirakata et al., 2005; Stoscheck et al., 1992; Zieske et al., 2000). To examine the kinetics of EGFR activation near the edges of wounded HCLE cell sheets, I monitored EGFR activation for hours after the removal of agarose droplets (Figure 53A). Rapid activation of the EGFR was followed

by downregulation of total levels of the receptor. 24 hours after wounding, the ratio of active to total EGFR remained elevated relative to controls (Figure 53B). Similarly to the EGFR, activation of ERK1/2 persisted for hours (Figure 53A and B).

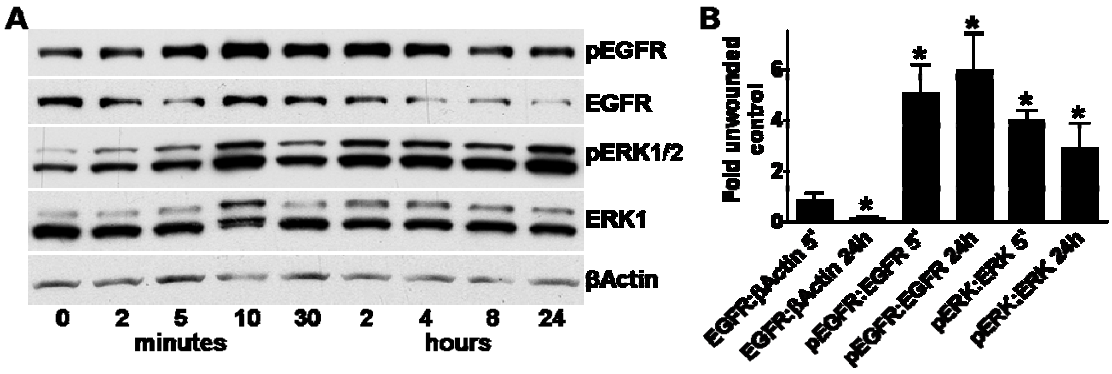

Figure 53 Wounding causes persistent EGFR and ERK1/2 activation

A. HCLE cell sheets were wounded by the removal of agarose droplets and the activation of EGFR and of ERK1/2 was monitored for 24 hr by Western Blotting. **B**. Ratios were determined by densitometry of Western Blots from 5 minutes (5') or 24 hours (24h) after wounding. * indicates significant difference from unwounded controls ($p<0.001$).

The EGFR inhibitor AG 1478 blocked ERK1/2 activation at both the early and late time points (Figure 54; this figure, as well as figures 55, 56B, and 64 were not prepared by the author, see acknowledgments), indicating that the EGFR is actively signaling even when total levels are downregulated. In wound healing assays, addition of AG 1478 at any time during healing slowed cell migration (Figure 55). These data indicate that chronic EGFR activation is necessary for the migration of epithelial sheets.

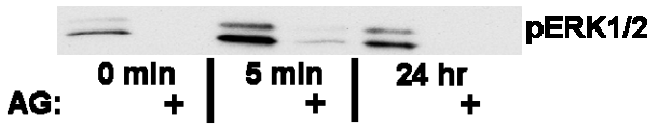

Figure 54 EGFR signaling regulates ERK1/2 activation after wounding

Western Blotting for activation of ERK1/2 at 0 or 5 minutes or 24 hours after wounding. As indicated, 1 μM of the EGFR inhibitor AG 1478 (AG) was added 30 min prior to cell lysis. Figure prepared by Mike Tolino.

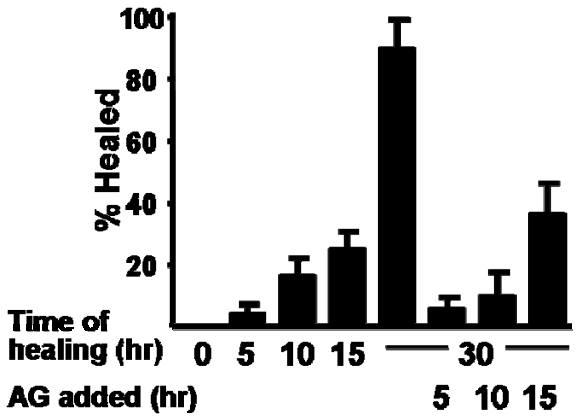

Figure 55 EGFR activity is necessary throughout healing

Wounded HCLE cell sheets were fixed at 0, 5, 10, 15, or 30 hours. For comparison, 1 μM of the EGFR inhibitor AG 1478 (AG) was added at the indicated times, and healing was allowed to progress over the full 30 hours before fixation. Figure prepared by Mike Tolino.

6.2.2 A cell culture model for detecting signaling by free edges.

Many of the stimuli associated with wounding could cause chronic EGFR activation in cells near the wound edge. We developed a model to examine the consequences of a stimulus that is by definition ubiquitous in wounds, the presence of a free edge (see Figure 7, and Methods). To produce a cell culture with many free edges, HCLE cells were cultured on thin plastic strips above a non-adhesive surface of polyHEMA. In xz sections generated by confocal microscopy, I observed that cells at the free edge were not physically constrained by the polyHEMA and that their membranes extended over the edges (Figure 56A).

Rates of thymidine uptake were reduced similarly in cells grown on strips and in control cultures when they reached confluence (Figure 56B). Levels of lactate dehydrogenase in the supernatants of cells grown in the two conditions were low and similar, indicating that growth of cells on the strips does not promote cell lysis (Loo and Rillema, 1998) (Figure 34A). These results indicate that differences in cells grown on strips or controls are unlikely to be due to differences in proliferation states or cell lysis.

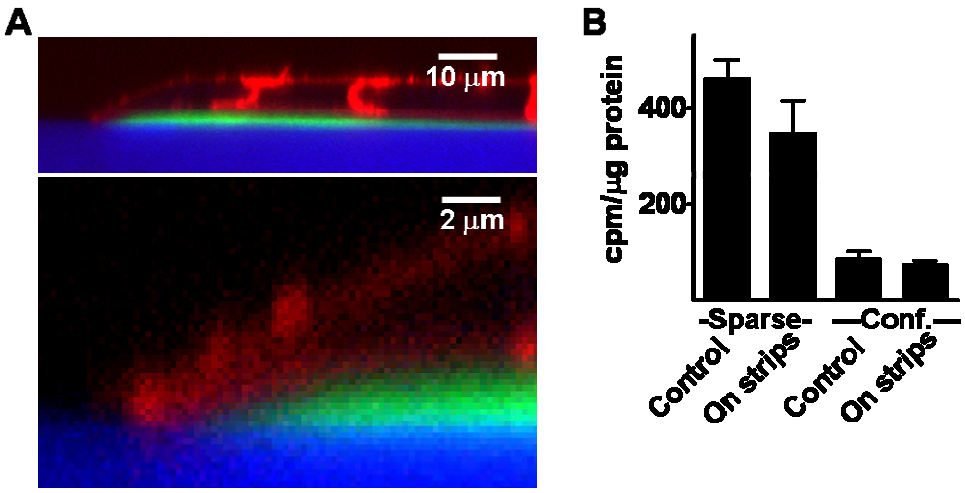

Figure 56 Cells grown on plastic strips have a physically unconstrained edge and slow proliferation when they reach confluence

A. Cross-sections of cells grown on plastic strips were generated by confocal microscopy. The plastic strips and polyHEMA were labeled with fluorophores (shown in green and blue, respectively), and the cells were labeled with the membrane dye Vybrant® DiD (depicted in red). **B**. ^{3}H-thymidine uptake by cells grown on plastic strips or controls at sparse or confluent densities. No significant differences were detected.

6.2.3 EGFR activation requires physically unconstrained edges.

I performed Western Blotting on extracts from HCLE cells cultured on plastic strips and found that relative to controls, the EGFR was down-regulated but there was an increased ratio of active to total EGFR (Figure 57A and B). These results are similar to those observed in cells near the edge 24 hours after wounding (Figure 53). I also analyzed Madin-Darby canine kidney (MDCK) cells grown on plastic strips and found increased EGFR phosphorylation, although EGFR down-regulation was less pronounced (Figure 58). ERK1/2 were also activated in cells cultured on plastic strips (Figure 59A) and this activation was dependent on EGFR signaling because it was

abrogated by the inclusion of the EGFR inhibitor AG 1478 (Figure 59B). Similar results were obtained when agarose was used as the non-adhesive substrate instead of polyHEMA.

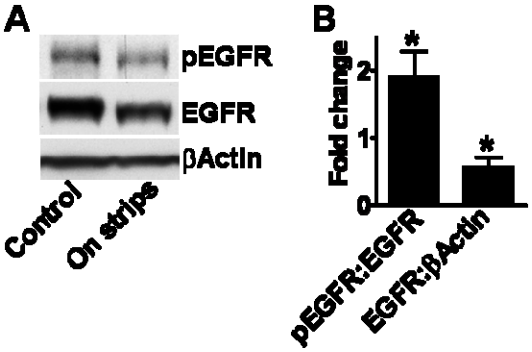

Figure 57 Activation of the EGFR by free edges in HCLE cell sheets

A. Immunoblot of extracts of HCLE cells with an antibody against EGFR phosphorylated on Tyr-1173. The blots were stripped, and re-probed for total amounts of the EGFR and β-actin as a load control. **B**. Quantitation of Western Blots by densitometry. * indicates significant differences from controls ($p<0.001$).

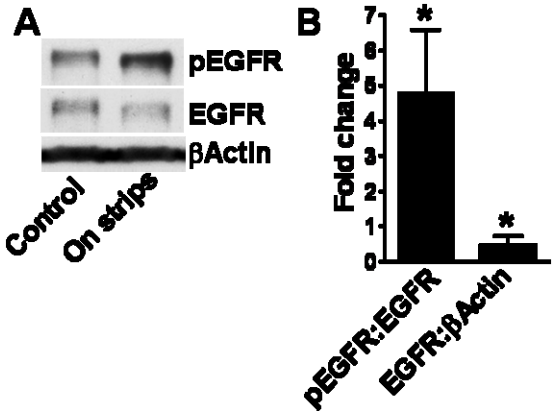

Figure 58 Activation of the EGFR by free edges in MDCK cell sheets

A. Immunoblot of extracts of MDCK cells was performed as for Figure 53. **B**. Quantitation of Western Blots by densitometry. * indicates significant differences from controls ($p<0.001$).

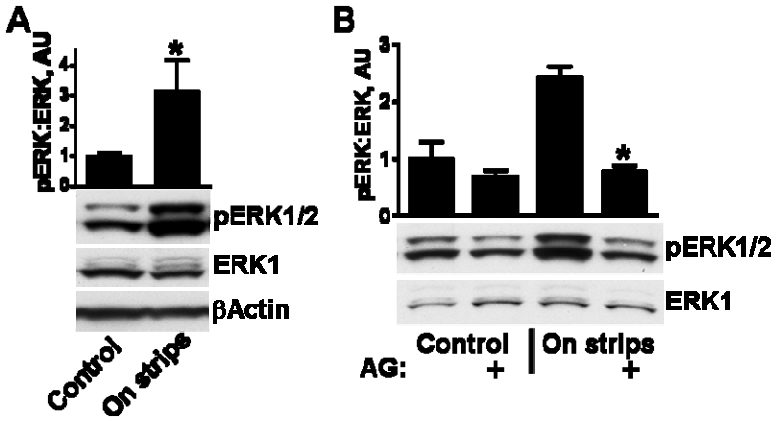

Figure 59 Activation of ERK1/2 by free edges

A. Western Blotting and densitometry for active ERK1/2 and β-actin as a load control. * indicates significant difference from controls ($p<0.001$). **B**. Cells cultured on strips or controls were treated for 30 min with 1 μM of the EGFR inhibitor AG 1478 (AG). * indicates significant difference from untreated cells on strips ($p<0.001$).

To determine how far from an edge activation occurs, cells were seeded on polyHEMA-coated plates that were half-covered with plastic, and therefore contained a single edge (see Methods 8.7). Immunoblotting extracts of cells located at various distances from the edge revealed that activation occurred locally within the most proximal 5 mm from the edge (Figure 60). Treatment of cells cultured on re-dissolved plastic with the MAPK inhibitor UO126 led to quenching of pERK1/2 activation as detected by Western Blotting but not as detected by immunofluorescence microscopy. Therefore, although microscopy could be a useful tool for examining the penetrance of activation, I concluded that culture on re-dissolved plastic creates some artifactual signal that subverts the utility of immunofluorescence microscopy.

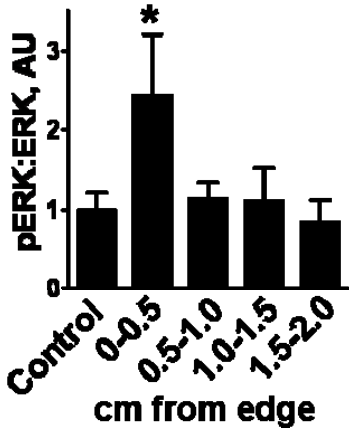

Figure 60 ERK1/2 activation is limited to near the free edge

Cells were cultured with one free edge by growing on polyHEMA-coated plates that were half covered in plastic. On ice, 0.5 cm strips were sequentially scraped away from the edge and were analyzed individually. Densitometry of Western Blots is shown. * indicates significant differences from all other groups ($p<0.001$).

To determine whether a physical barrier can inhibit EGFR activation in strips of epithelial cells, I analyzed cells grown in thin strips around agarose droplets (see Figure 5 and Methods). In xz sections generated by confocal microscopy, I observed that cells were physically constrained by the agarose droplets (Figure 61A). Levels of EGFR activation, downregulation, and ERK1/2 activation were similar in these and control confluent cultures lacking agarose (Figure 61B and C). Addition of EGF to cells grown either as uninterrupted sheets or with agarose droplets resulted in similar levels of EGFR activation (Figure 61D). I

conclude that free edges in epithelial cell sheets induce EGFR activation while edges that are physically constrained by an inert material such as agarose do not.

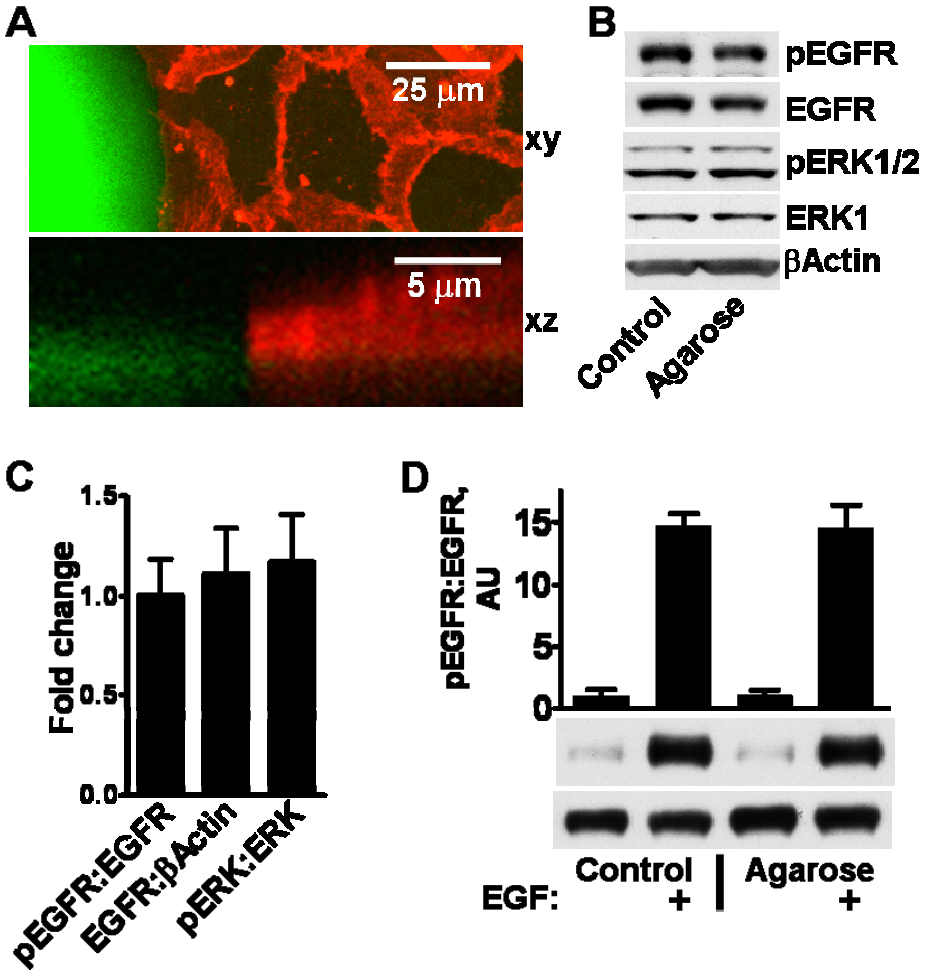

Figure 61 Edges are physically constrained do not activate the EGFR

A. Cells were labeled with the membrane dye Vybrant® DiD (red) and agarose droplets were labeled with fluorescein (green). The plastic below the cells and agarose droplets was left unlabeled for clarity. **B**. Western Blots of extracts of HCLE cells cultured without and with agarose droplets. **C**. Quantitation of Western Blots by densitometry. No significant differences from controls were found. **D**. EGFR activation was monitored after stimulation of HCLE cells grown either as uninterrupted sheets or around agarose droplets with 10 ng/ml EGF for 10 min.

6.2.4 Free edges stimulate EGFR activation through SFK-mediated proteolytic ligand release.

Wounding activates the EGFR rapidly by the proteolytic release of membrane-bound ligands (Block et al., 2004; Xu et al., 2004). To determine whether EGFR activation induced by free edges is regulated by a similar mechanism, I incubated cells with various inhibitors of signaling by EGFR ligands and monitored activation of ERK1/2, which are downstream of the EGFR. ERK1/2 activation was abrogated by treatment with GM6001, which inhibits EGFR pro-ligand shedding, and by the anti-EGFR antibody LA-1, which inhibits extracellular ligands from binding the EGFR (Figure 62). Therefore, I conclude that free edges activate the EGFR through proteolytic ligand release.

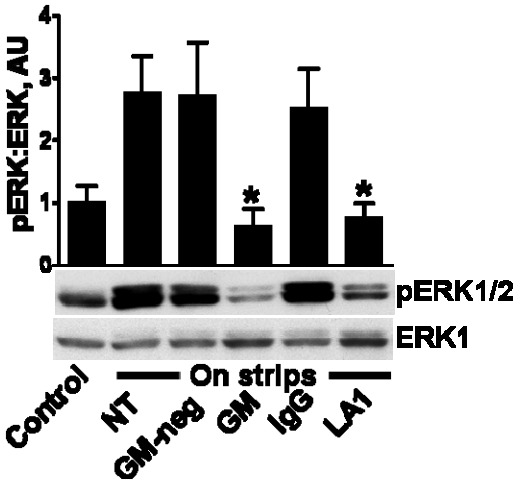

Figure 62 Free edges activate ERK1/2 through proteolytic release of EGFR ligands

Western Blots and densitometry for activation of ERK1/2 in cells cultured on strips after no treatment (NT), or treatment with 1 μM GM negative control (GM-neg), 1 μM GM 6001 (GM), 20 μg/ml non-immune IgG (IgG), or 20 μg/ml anti-EGFR antibody (LA1). * indicates significant difference from untreated cells on strips.

Src-family kinases (SFKs) regulate EGFR ligand release after wounding ((Block and Klarlund, 2008) and Chapter Five), so I expected that SFKs also regulate EGFR activation stimulated by free edges. I observed strong downregulation of SFKs using antibodies that

recognize active or inactive forms of the kinases (Figure 63A). Highly activated forms of SFKs are targeted for degradation, so SFK down-regulation is likely a result of chronic SFK activation (Ingley, 2008). I next monitored ERK1/2 activation in the presence of two structurally dissimilar SFK inhibitors that are known to have distinct non-specific interactions, PP2 and Src Kinase Inhibitor-I (SKI) (Bain et al., 2007). Inclusion of either SFK inhibitor abrogated ERK1/2 activation (Figure 63B). Together, these results suggest that free edges activate the EGFR through SFK signaling.

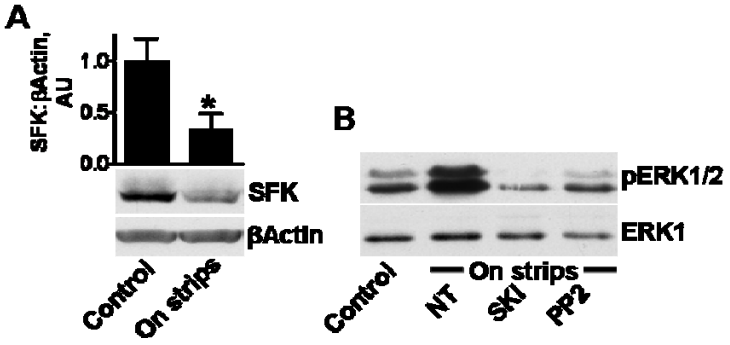

Figure 63 Activation of SFKs by free edges is necessary for ERK1/2 activation

A. Western Blotting and densitometry of extracts with an antibodies against SFKs and β-actin as a load control. * indicates significant differences from controls ($p<0.001$). **B**. ERK1/2 activation was monitored in cells on strips after 30 min treatment with 1 μM Src Kinase Inhibitor-I (SKI) or 10 μM PP2.

6.2.5 Free edges stimulate EGFR/ERK activation independently of signaling by ATP, Pyk2, or loss of polarity

SFK and EGFR activation after wounding can be regulated by extracellular ATP ((Block and Klarlund, 2008; Yin et al., 2007), Chapters Two and Three). However, the amount of ATP in supernatants from cells grown on plastic strips, approximately 100 nM (Figure 34), is too low to induce measurable activation of the EGFR and ERK1/2 (Yin et al., 2007), and addition of apyrase did not block activation of the EGFR/ERK1/2 pathway in cells grown on strips (Figure

64). These data strongly suggest that activation occurs through a mechanism that is independent of signaling by extracellular ATP.

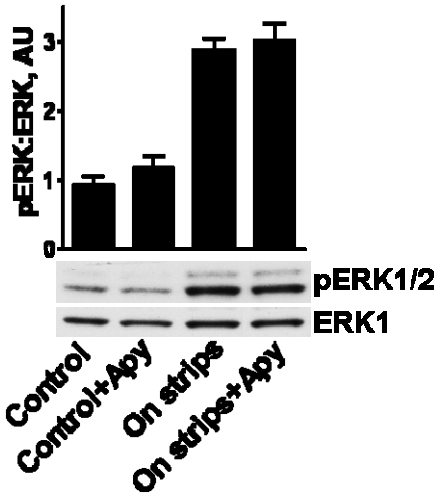

Figure 64 Free edges activate ERK1/2 independently of extracellular ATP signaling

Western Blots and densitometry for activation of ERK1/2 in cells cultured on strips or controls and treated with 30 U/ml apyrase (Apy) as indicated.

SFK and EGFR activation after wounding can also be regulated by Pyk2 (Chapter Five). I did not observe Pyk2 activation in cells grown on strips, relative to controls (Figure 65A). Furthermore, in preliminary studies, activation of the EGFR and ERK1/2 were unaffected by knockdown of Pyk2 by siRNA (Figure 65B). These data suggest that free edges stimulate EGFR activation by a mechanism independent of signaling by Pyk2.

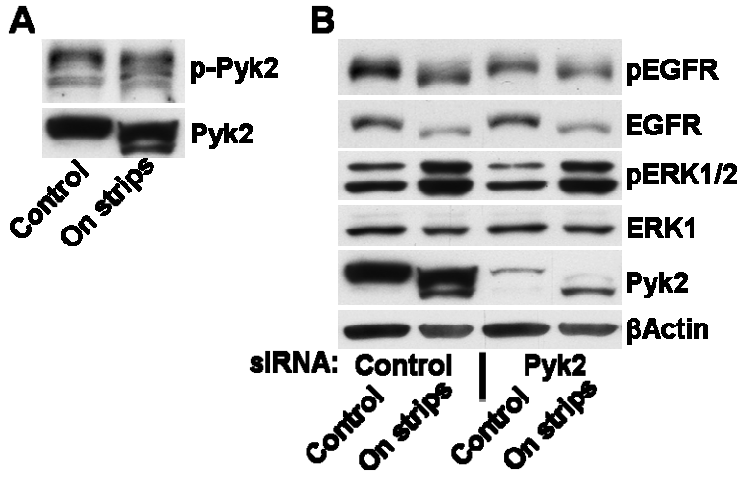

Figure 65 Free edges activate the EGFR independently of signaling by Pyk2

A. Western Blots for activation of Pyk2 in cells cultured on strips and controls. Similar results were obtained in four independent experiments. **B**. Activation of EGFR and ERK1/2 and expression of Pyk2 in cells cultured on strips and controls was monitored after transfection with 10 nM of Pyk2 siRNA. Due to splice variants and post-translational modifications, multiple Pyk2 bands can be resolved by Western Blotting.

In the airway epithelium, ErbB2-3, which are related to the EGFR, are activated by a mechanism that is based on segregation of the apical ligand (heregulin) from the basolateral receptors (Vermeer et al., 2003). Wounding breaks down the barrier function of the epithelium allowing heregulin and the receptors to interact. For such a mechanism to operate, the epithelial cells must provide effective separation of apical and basolateral compartments, which is mainly due to tight junctions. Specialized procedures are required for epithelial cells to form fully-developed tight junctions in tissue culture and as expected they were incomplete in HCLE cells grown on redissolved plastic (Figure 66A). Furthermore, EGF added apically to cells grown under control conditions potently activated the EGFR in basolateral membranes (Figure 66B).

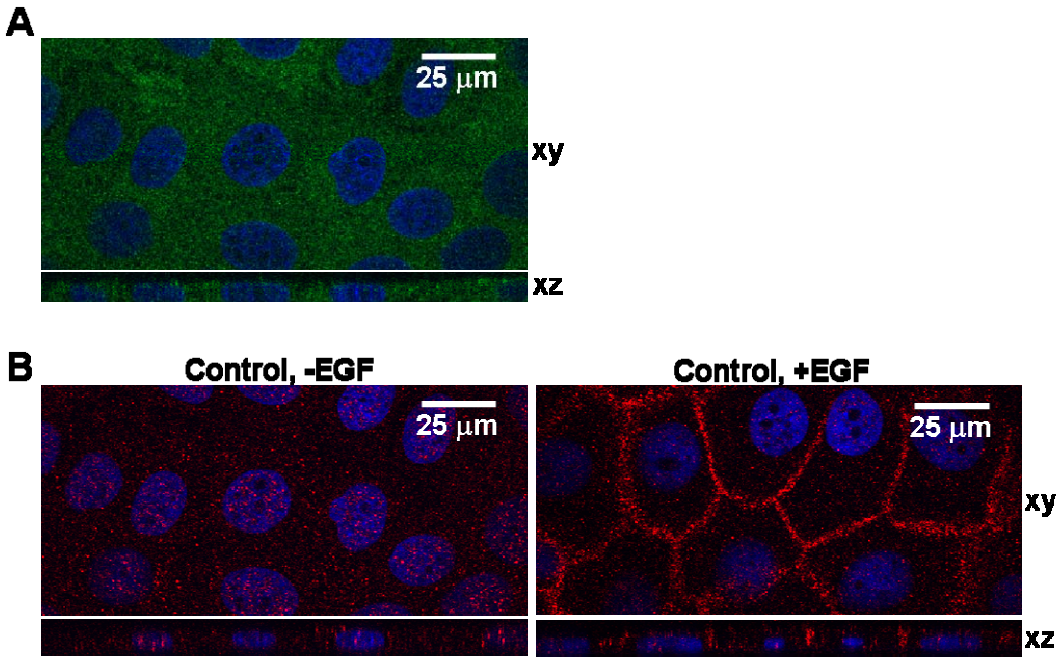

Figure 66 EGFR ligands are not segregated from receptors

Xy and xz projections were generated by confocal microscopy of HCLE cells cultured on plastic-covered polyHEMA and stained for **A**. ZO-1 or **B**. phosphorylated EGFR. As indicated, cells were treated with 100 ng/ml EGF for 2 minutes.

Since the HCLE cells do not provide an effective barrier between apical and basolateral compartments, activation of the EGFR by the presence of free edges cannot be due to its disruption.

6.3 DISCUSSION

In this chapter, I have investigated the signaling caused by an element common to migrating epithelial sheets found during development in the embryo and wound repair in the adult: the presence of a free edge. I have shown that the EGFR is persistently activated in cells near the free edge and that an epithelial sheet requires persistent EGFR activation to migrate. To determine whether a free edge is sufficient to induce this chronic activation, we developed methods to compare cultures of cells with many free edges, many constrained edges, and virtually no edges. I have concluded that free edges trigger EGFR activation based on two key observations: 1, the EGFR and ERK1/2 were activated similarly in cells near the free edge after either wounding or culture on plastic strips and 2, constraining the free edge with agarose blocked activation of the EGFR and ERK1/2.

There are marked similarities in the mechanisms of epithelial movement during the healing of wounds and during development (Friedl and Gilmour, 2009; Jacinto et al., 2001; Martin and Parkhurst, 2004). For example, cells near the leading edge are propelled forward by a combination of lamellipodial crawling and "purse-string"-like contractions of the actin cytoskeleton (Danjo and Gipson, 1998; Mitchison and Cramer, 1996; Tamada et al., 2007). For decades, researchers have speculated that a free edge may be a signal that induces these and other changes, but there has been little hard evidence (Martin and Parkhurst, 2004; Rand, 1915).

Here I have made the observation that free edges induced EGFR activation in not only HCLE cells, but also in MDCK cells (Figure 59), a prototypical epithelial model, which suggests that the ability to detect free edges is a universal mechanism that promotes epithelial motility.

I was unable to examine directly whether a free edge induces EGFR signaling acutely after wounding. In Chapter Five, I observed that blocking signaling by both Pyk2 and extracellular ATP resulted in only partial inhibition of wound-induced EGFR activation. Because free edges are created suddenly by the removal of agarose barriers, I cannot exclude the possibility that the presence of a free edge is a stimulus for rapid EGFR activation as well. If, in the future, I find molecular or pharmacological approaches that inhibit SFK activation by free edges (see Chapter Seven), I will be able to use these same approaches to determine whether free edges contribute to acute EGFR activation after wounding.

I have found that the mechanism of EGFR activation by free edges involves the SFK-mediated release of EGFR pro-ligands (Figure 63). Other proposed mechanisms of wound-induced EGFR activation suggest that activation occurs not as a result of increased SFK-mediated ligand shedding, but rather as a result of basal ligand shedding having a greater effect due to increased access to receptors (Chu et al., 2005; Tschumperlin et al., 2004; Vermeer et al., 2003). The observation that a free edge stimulates SFK activation suggests that free edges actively stimulate signaling upstream of the EGFR.

The observation that edges do not cause activation when they are constrained by agarose is significant, because it strongly suggests that suppression of activation is not due to any specific interaction between cells. For instance, contact inhibition of cell locomotion is usually thought to result from activation of receptors on cells recognizing ligands on adjacent cells (Abercrombie, 1979; Carmona-Fontaine et al., 2008). More likely, activation is repressed in epithelial cell

sheets by mechanical forces that the cells exert on one another (Bindschadler and McGrath, 2007; Farooqui and Fenteany, 2005). At unconstrained edges, cells do not encounter opposing forces, and tensions in the cells would be relaxed. As is discussed further in Chapter Seven, ongoing research is exploring the possibility that the free edge sensor may involve the actin cytoskeleton, which is a keen sensor of tensile stress that signals through SFKs (Ingber, 2006; Ingley, 2008).

6.3.1 Summary and model

In summary, the results presented here indicate that free edges in epithelial cell sheets stimulate signals that activate SFKs and, subsequently, the EGFR (see diagram in Figure 67).

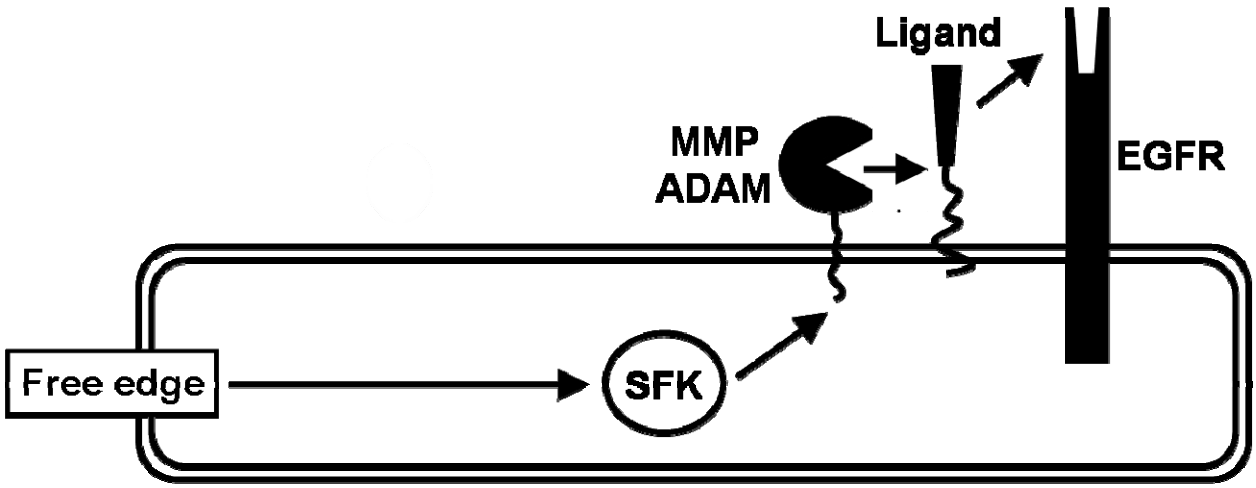

Figure 67 A hypothetical model for the role of free edges in wound-induced EGFR activation

See text for details. SFK, Src-family kinase; MMP, matrix metalloprotease; ADAM, a disintegrin and metalloprotease; EGFR, epidermal growth factor receptor.

7.0 CONCLUSIONS

7.1 A MODEL FOR MECHANISMS OF WOUND-INDUCED EGFR ACTIVATION

The goal of the research presented in this dissertation was to gain a better understanding of the cellular signals that induce migration after epithelial wounding. Specifically, I have tested hypotheses relating to mechanisms of wound-induced EGFR activation. The data presented in Chapters Two through Six, combined with previously published reports from our and other laboratories (Block and Klarlund, 2008; Block et al., 2004; Boucher et al., 2007; Mazie et al., 2006; Xu et al., 2006; Yin et al., 2007; Zieske et al., 2000), have led to the development of a working model for mechanisms of wound-induced EGFR activation (Figure 68). The proximal mechanism of EGFR activation is the proteolytic release of membrane-bound ligands, which is regulated by activation of Src-family kinases (SFKs). I have found that multiple pathways converge on SFK signaling to regulate EGFR activation. One pathway is dependent on signaling by extracellular ATP: wounding causes the release of ATP from cells near the wound edge via cell rupture and unspecified channels; ATP then binds type-2 purinergic receptors (P2R) at the cell surface, which signal to SFKs through the actions of phospholipase D2 (PLD2) and its product, phosphatidic acid (PA). Since ATP is diffusible, ATP/PLD2 signaling can stimulate EGFR activation at a distance from wounds. In a distinct pathway that functions solely in cells near (<250 μm from) the wound edge, an undefined wound sensor stimulates activation of the

focal adhesion kinase Pyk2. Activated Pyk2 then binds to and activates SFKs, leading to EGFR activation. Finally, my data suggest the presence of a third distinct pathway, which triggers SFK and EGFR activation in response to a physically unconstrained, or free edge.

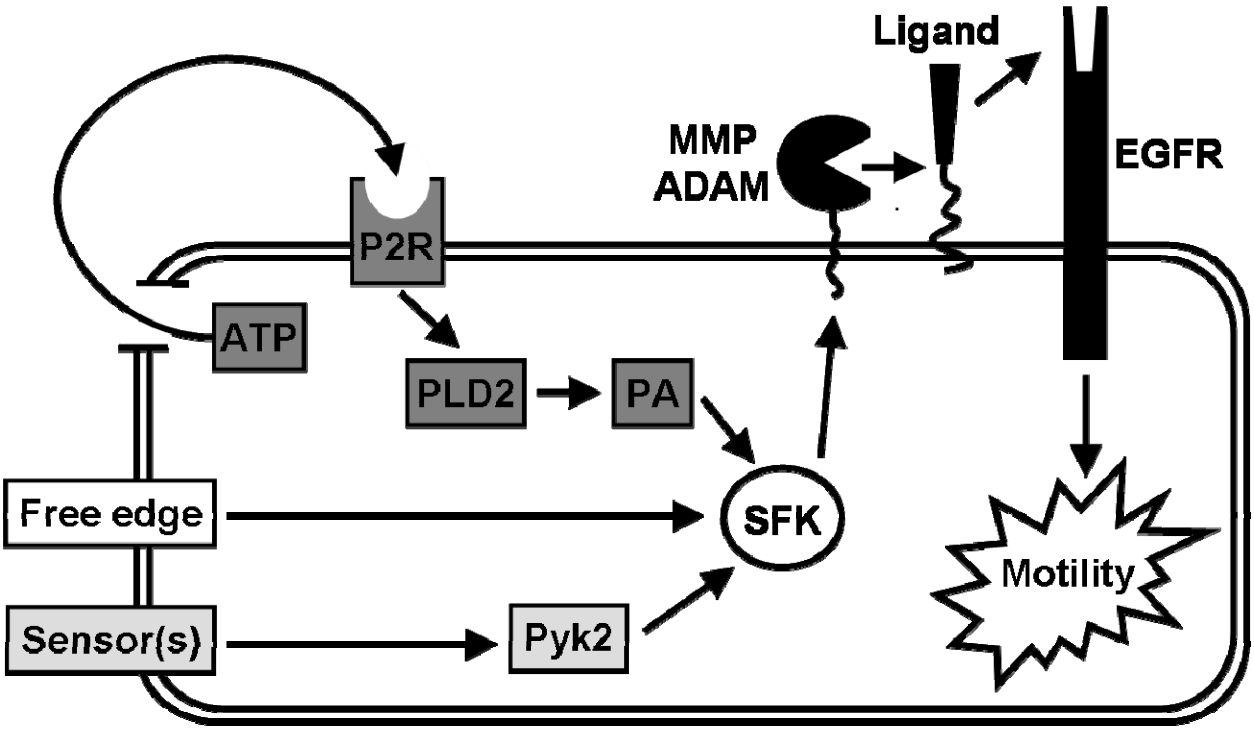

Figure 68 A working model for mechanisms of EGFR activation after epithelial wounding

Evidence presented in this dissertation indicates that after epithelial wounding, multiple signaling pathways trigger activation of the EGFR via SFK-mediated proteolytic ligand release. ATP (dark grey pathway) is released from ruptured cells and unspecified membrane channels. Extracellular ATP binds type-2 purinergic receptors (P2R) and stimulates SFK and EGFR activation through phospholipase D2 (PLD2) and phosphatidic acid (PA) signaling. Pyk2 (light grey pathway) is activated by undefined wound sensors and binds to and activates SFKs. Finally, the presence of a free edge stimulates SFK activation through unspecified mechanisms.

All good working models stimulate more questions than they answer. In this chapter, I will discuss the validity of this model: what are the shortcomings of this research that may have confounded my interpretations? I will also discuss key areas for future research, specifically,

what is the molecular nature of the wound sensors that activate Pyk2 and that respond to a free edge. Finally, I will discuss the implications of this model for wound healing in the clinic, and for other pathologies that are affected by EGFR signaling.

7.2 SHORTCOMINGS

The major shortcoming of these studies leads us to question whether the model depicted in Figure 63 is generally applicable to all epithelial wounds: my conclusions have been based almost entirely on experiments with a human corneal epithelial cell line, and furthermore, I have used unconventional wounding models. Are these conclusions relevant to actual wounds in any epithelium?

First, the question of wounding models: in place of traditional scrape wounding, I have used wounding models based on the removal of agarose barriers from the midst of otherwise confluent epithelial sheets (Figures 5 and 6). By so doing, I have minimized the signaling input from cell damage, which is not necessary for the induction of motility (Block et al., 2004; Poujade et al., 2007) (although it may be necessary for other aspects of the normal healing response). I therefore believe that our models allow the detection of signals that induce motility.

But are the signals I detect the same as those created by scrape-wounding? Using the agarose methods, we have previously reported that the EGFR is activated after wounding through the actions of HB-EGF (Block et al., 2004). Almost simultaneously, another report was published that reached the same conclusions by using scrape-wounding (Xu et al., 2004). The activation of Pyk2 after scrape-wounding has not been reported. Therefore, to increase

confidence that our agarose methods do not induce signaling unrelated to wounding, my studies might have benefited from comparisons to scrape-wounding experiments.

Do the signals I detect after agarose removal have the same relative impact on EGFR activation as they would after scrape-wounding? Scrape-wounding causes 5-10 fold higher amounts of ATP to be released than does the removal of agarose barriers. Therefore, extracellular ATP should have a much greater relative signaling role during scrape-wounding studies. Elimination of ATP with apyrase did not alter healing rates, even after scrape-wounding of corneas in organ culture (Figure 15C), so I believe that the increased signaling influence of ATP during scrape-wounding experiments masks other signals that are necessary for EGFR activation and healing.

In this dissertation, I have applied our wounding models almost exclusively to the study of a human corneal epithelial cell line, HCLE cells. While these are a good model for native corneal epithelial cells (Gipson et al., 2003), I have in two instances used additional cell types to strengthen my conclusions: I used secondary cultures of rabbit corneal epithelial cells and rabbit corneal organ cultures to increase confidence in my conclusion that extracellular ATP is not necessary for wound healing (Figure 15), and I used MDCK cells to increase confidence in my conclusion that free edges in epithelial sheets activate the EGFR (Figure 59). Some of the other major findings of this dissertation (such as Figures 13, 22, 33, and 47) could have benefited from confirmation in human neonatal foreskin epidermal keratinocytes (HEKn) or in MDCK cells. Similar findings in these other cell types would raise confidence that the mechanisms of EGFR activation shown in figure 68 are applicable to wounds in other tissues.

While it is clear that wounding activates the EGFR by proteolytic ligand release *in vivo* (Shirakata et al., 2005; Tokumaru et al., 2000; Zieske et al., 2000), it is unclear whether the

upstream mechanisms I have identified operate *in vivo* as well. One would expect that mechanisms of EGFR activation would vary in cell culture compared to living tissue and also from one tissue to another, in large part due to highly variable physical and biochemical microenvironments. For example, cultured cells are bathed in media that may be more conducive to signaling by soluble factors than is the *in vivo* environment. In the same respect, signaling by extracellular ATP may be less important in the skin than it is in the fluid environment of the cornea. Detecting the release of ATP into the tear film or other wound fluids should be feasible.

Whether signaling molecules such as ATP, PLD2, and Pyk2 regulate epithelial wound healing *in vivo* could be tested using gene knockout strategies. Pyk2 knockout mice have been bred (Okigaki et al., 2003) and live to adulthood. Wound healing in their corneas, skin, or any epithelia could be compared to that in wild-type mice. Healing stimulated by EGF should also be assessed to confirm that the loss of Pyk2 signaling creates no general defect in cell motility. Live animal studies were beyond the scope of this research, but my findings in cultured cells have generated testable hypotheses for future study.

A final minor experimental shortcoming also deserves mention. I have measured the release of amphiregulin (AR) from HCLE cells by ELISA of tissue culture supernatants. As discussed in Chapter Two, the amounts of AR detected by the ELISA are far too low, perhaps 100-fold too low, to induce measurable activation of the EGFR, although EGFR activation clearly depends on AR (Figure 24). This implies that I am only detecting a fraction of the bioactive AR that may or may not be representative of the entire bioactive pool. In experiments where I have measured EGFR activation and AR release in parallel, AR quantities fluctuated similarly with EGFR activation, suggesting that I am indeed measuring a representative fraction

of the active AR pool. On the other hand, AR is captured from the culture medium by the EGFR, which is internalized after activation (Wiley, 2003), suggesting that AR levels detected after wounding may over-estimate the degree of ligand release. I have attempted to address this issue in two ways. First, I have tried to use high salt concentrations to remove bioactive AR from the cell surface prior to analysis. Even after removal of the salt, which interfered with the ELISA, I could not detect increased AR. A better approach would have been to measure AR concentrations in the presence of an EGFR antibody that blocks ligand binding and thus greatly inhibits capture from the culture medium (Oehrtman et al., 1998). Second, I have attempted to measure AR release by Western Blotting with two different C-terminal-directed antibodies that detect both the inactive (full-length) and active (cleaved) forms. Unfortunately, I was not able to detect AR in HCLE cells by Western Blotting with either antibody.

7.3 WHAT ARE THE WOUND SENSORS?

In this dissertation, I have identified three signaling pathways that promote EGFR activation after wounding (Figure 68): Signaling by extracellular ATP, by Pyk2, and by free edges. My data indicate that these signals induce motility in wounded epithelia, but what are the molecular signals, the "wound sensors," that kick-start these three pathways? Some possibilities will be discussed in the next sections.

7.3.1 Wound sensors in the extracellular ATP-dependent pathway

In the case of signaling by extracellular ATP, cell damage is clearly a wound sensor because cell damage is responsible for the bulk of ATP released immediately after wounding. However, in Chapter Four, I have shown that ATP, albeit at lower concentrations, is released independently of cell damage. This implies that there is a second wound sensor that regulates ATP release through an undefined channel. Experiments attempting to identify the ATP channel were difficult to interpret, but the additional experiments suggested in Chapter Four could more rigorously test the hypothesis that connexin hemichannels regulate ATP release. Release through the undefined channel may be regulated by gene expression or membrane trafficking, or by channel gating, either through mechanical factors such as stretch, or through cell signaling.

7.3.2 Wound sensors in the Pyk2 pathway

The identification of a Pyk2-dependent pathway for EGFR activation near the wound edge is significant because it allows us to work upstream from Pyk2 to identify a wound sensor. Well-characterized proximate causes for Pyk2 activation include signaling by integrins, by calcium, and by reactive oxygen species (Avraham et al., 2000; Schlaepfer et al., 1999).

7.3.2.1 Integrin signaling

Integrins are heterodimeric adhesive receptors, comprised of α and β chains, that mediate cell-cell and cell-extracellular matrix interactions. Twenty-four different receptors are formed by the 18 α and 8 β subunits described in mammals, and they are named according to subunit composition (e.g. αvβ1). Integrins are allosterically regulated by intracellular binding proteins

that prime the integrins for extracellular ligand binding ("inside-out" signaling) and by extracellular ligands that induce intracellular signals ("outside-in" signaling) (Humphries et al., 2003; Hynes, 2002; Luo et al., 2007; Shattil and Newman, 2004). Integrins also serve as a transmembrane linkage between the extracellular matrix and the actin cytoskeleton (Blystone, 2004). A subset of α/β chain combinations have been described in the corneal epithelium, primarily combinations that bind fibronectin, laminin, and collagen (Stepp, 2006).

A well-known intracellular signaling pathway stimulated by integrin adhesion is the activation of Src-family kinases (Mitra and Schlaepfer, 2006; Playford and Schaller, 2004; Reichelt, 2007), which has been reported to link adhesion to EGFR activation (Cabodi et al., 2004; Kuwada and Li, 2000; Moro et al., 2002; Moro et al., 1998).

After wounding, integrin-based adhesions are formed rapidly at the edge of the wound (Zaidel-Bar et al., 2003), but even cells in confluent monolayers exhibit basal cell motilities that require replacing old integrin adhesions with new. It is therefore unclear whether a cell can sense a wound based on integrin binding alone. Activation of FAK is an early signaling event following integrin/matrix binding (Schlaepfer et al., 1999). I observed elevated FAK activities in HCLE cells after attachment from suspension, but I did not observe FAK activation after wounding (Figure 41A), suggesting that integrin/matrix binding is not a significant wound sensor. Consistent with this conclusion, I have observed that treatments with integrin-blocking antibodies and peptides did not inhibit wound-induced EGFR activation, even though they blocked migration in wound healing assays.

7.3.2.2 Calcium signaling

Intracellular calcium concentrations are tightly regulated and many signaling molecules, such as Pyk2, SFKs, and the EGFR can be activated by increased calcium concentration (Avraham et al.,

2000; Horiuchi et al., 2007; Schlaepfer et al., 1999; Thomas and Brugge, 1997). Minutes after wounding, calcium increases have been observed in cells near the wound edge (Isakson et al., 2001; Klepeis et al., 2001; Sammak et al., 1997), which coincides temporally and spatially with the Pyk2-mediated activation of the EGFR. Calcium may therefore be a signaling intermediate connecting wounding with Pyk2 and the EGFR. A number of future studies could explore this possibility and eventually determine the proximate cause of increased calcium concentrations, which could be a wound sensor.

To determine whether wound-induced Pyk2 activation occurs as a result of calcium signaling, calcium signaling could be blocked by the intracellular calcium chelator BAPTA-AM. Calcium activates Pyk2 by associating with calmodulin and binding near the Pyk2 N-terminus (Kohno et al., 2008; Schaller, 2008). Therefore, as a complimentary approach, I could test whether expression of the Pyk2 N-terminus (lacking the kinase domain) (Lipinski et al., 2006) blocks endogenous Pyk2 activation by wounding. If BAPTA-AM and the Pyk2 N-terminus block wound-induced Pyk2 activation, I would next determine whether the source of calcium is intracellular stores (the ER) or extracellular fluid by wounding cells in the presence of the extracellular calcium chelator EGTA or the Ca^{2+}/ATPase inhibitor thapsigargen. I would use this approach to examine wound-induced Pyk2 activation as well as changes in calcium concentration measured by fluorometric dyes.

Studies in corneal, alveolar, and intestinal epithelial cells and bovine endothelial cells have indicated that calcium signaling in cells near the wound edge is due to the influx of extracellular calcium (Isakson et al., 2001; Klepeis et al., 2001; Rao et al., 2001; Sammak et al., 1997). Furthermore, calcium influx is necessary for epithelial migration (Rao et al., 2001; Rao et al., 2006). Calcium influx in cells near the wound edge was found to occur independently of cell

rupture, suggesting the presence of a wound-sensing calcium channel. Calcium channels can be voltage-gated or non-voltage-gated, and calcium influx in non-excitable cells occurs primarily through store-operated or stretch-activated channels, both of which have been shown to contribute to cell motility (Duncan and Collison, 2002; Lee et al., 1999; Maroto et al., 2005; Rao et al., 2006; Yamada et al.). Store-operated channels supply increased cytosolic calcium to replenish ER stores following IP3 signaling. If inhibitors of PLC, which generates IP3, do not inhibit calcium influx, store-operated channels are not likely responsible for calcium influx. Using fluorometric calcium indicators, pharmacological and kinetic analyses of calcium influx could implicate a particular type of channel that could be further targeted through siRNA or dominant-negative approaches (Jaffe, 2007; Trollinger et al., 2002).

7.3.2.3 Reactive oxygen species

Like signaling by calcium, signaling by reactive oxygen species (ROS) also can activate Pyk2 and coincides temporally and spatially with wound-induced Pyk2 activation (Nikolic et al., 2006; Schlaepfer et al., 1999). ROS is the general term given to molecules such as hydrogen peroxide and superoxide that catalyze the reversible oxidation of cellular proteins. ROS can be generated as a byproduct of aerobic respiration, by the NADPH oxidase complex, or by arachidonic acid metabolism (Rhee, 2006; Rhee et al., 2005). ROS can be detected directly by fluorescent dyes and indirectly by Western Blotting for reduced and oxidized forms of proteins. Active-site thiols of protein tyrosine phosphatases are especially prone to oxidation and inactivation by ROS, which couples cellular ROS production to tyrosine kinase signaling networks. ROS activate Pyk2 by deactivating the phosphatase PTP-PEST (Lyons et al., 2001). Interestingly, ROS generation by NADPH oxidase or arachidonic acid metabolism can be stimulated by the small GTPase Rac1 (Gregg et al., 2003). Consequently, Rac1 can generate localized ROS signaling

and PTP-PEST inactivation that contributes to lamellipodia formation and cell migration (Wu et al., 2005).

To determine whether ROS generation is the wound sensor for the Pyk2 pathway, I would first apply various imaging and immunoblotting techniques to determine if ROS are generated after wounding by our methods. For example, ROS can increase the fluorescence of the cell-permeable dye, dichlorodihydrofluorescein diacetate, which could be visualized before and after wounding. If ROS are generated, I would then wound cells in the presence of antioxidants and measure Pyk2 activation. I could then proceed with various experiments to determine the source of the ROS, and whether Rac1 is involved in their generation.

A major theoretical kink in the "ROS as wound sensor" hypothesis is that ROS can induce activation of the EGFR and of SFKs, either directly or through phosphatase deactivation (Nakashima et al., 2005; Peus et al., 1998; Zhang et al., 2001), which would seem to circumvent the requirement for Pyk2 I have observed in Chapter Five. Still, pursuing this hypothesis may uncover localized signaling mechanisms, as has been observed in lamellipodia (Wu et al., 2005), that could explain this apparent contradiction.

7.3.3 The free edge sensor

In Chapter Six I presented data indicating that the presence of a free, physically unconstrained edge stimulates activation of the EGFR, while a constrained edge does not. The proximate cause for EGFR activation in response to a free edge is SFK-mediated ligand release, but the mechanism of SFK activation and the molecular nature of the free edge "sensor" are unknown. Because edges constrained by acellular barriers block activation, the free edge sensor is unlikely to be the absence of a particular cell/cell interaction (Abercrombie, 1979; Carmona-Fontaine et

al., 2008; Desai et al., 2009; Lauffenburger et al., 1998). More likely, cells sense free edges due to the lack of mechanical force opposing the free edge.

Biological systems are acutely sensitive to mechanical forces in large part due to the tension cells maintain in the cytoskeleton to stabilize cell shape and subcellular organization (Ingber, 2006). Mechanical stimuli alter conformation of numerous proteins (mechanosensors) either directly or by challenging the rigidity of the cytoskeleton, and thereby stimulate signaling cascades (del Rio et al., 2009; Friedland et al., 2009; Giannone and Sheetz, 2006; Orr et al., 2006; Tamada et al., 2004). Cells can also sense mechanical signals based on the tension of the plasma membrane, with low tension promoting both endocytosis and actin-based protrusions (Sheetz and Dai, 1996). SFKs are activated by various mechanosensors and thus transduce mechanical signals into cellular responses (Han et al., 2004; Ingley, 2008; Na et al., 2008).

To determine if a mechanosensor is responsible for free edge-stimulated EGFR activation, a logical first approach would be to study the cytoskeleton at the free edge. I could microscopically examine the actin and microtubule cytoskeletons in cells cultured on plastic strips and compare it to control cultures. Since cytoskeletal rearrangements occur downstream of EGFR signaling, I would expect to see them in the presence of the EGFR inhibitor AG 1478 if they are involved with the free edge sensor. Actin-based protrusions such as filopodia can act like sensory organelles (De Joussineau et al., 2003; Weber, 2006), raising the possibility that a lack of physical constraints could cause actin-based protrusions to stimulate signaling. Pharmacological inhibition of the actin and/or microtubule cytoskeletons can inhibit mechanotransduction (Baker et al., 2004; Craig et al., 2008), so I would determine the effects of drugs such as colchicine and cytochalasin D on free edge-induced EGFR activation. I could also determine how free edges alter activities of proteins that control cytoskeletal rearrangements,

like the small GTPases Rho, Rac, and Cdc42 (Welch and Mullins, 2002). Identifying a single mechanosensory molecule will be difficult, but if its expression or phosphorylation is altered by free edges, I may be able to find reasonable candidates following genomic and proteomic analyses.

7.3.4 Redundancy with a purpose

My data suggest the presence of three distinct mechanisms for wound-induced EGFR activation, and each of these suggests its own set of hypothetical wound sensors. The possibilities for wound sensors I have discussed (cell damage, ATP release, integrin signaling, calcium signaling, ROS signaling, and cytoskeletal signaling) are not mutually exclusive. Instead, I assert that many wound sensors contribute to EGFR activation, and that they can vary in importance depending on the tissue and type of wound.

Because EGFR activation is so important for the induction of motility, many mechanisms have evolved to ensure that the EGFR is activated following a variety of injuries. Many of these mechanisms are present in the epithelium alone, but many more exist at the whole tissue level. In the cornea for example, the EGFR is activated by EGF from tears, by EGF secreted from infiltrating immune cells, and the EGFR is transactivated by HGF secreted from keratocytes in the stroma (Klenkler and Sheardown, 2004; Klenkler et al., 2007; Spix et al., 2007). Additionally, wounding may affect many of the systems that modulate EGFR avidity or signaling, such as receptor trafficking (Curto et al., 2007; Gipson, 2004; Partridge et al., 2004; Wiley, 2003), electrical currents (Zhao, 2009; Zhao et al., 1999), or EGFR oligomerization with other signaling elements (Li et al., 2001; Warren and Landgraf, 2006; Yu et al., 2009).

7.4 IMPLICATIONS FOR WOUND HEALING THERAPY

Even with all of the mechanisms in place to ensure robust activation and efficient signaling of the EGFR after wounding, there are still many situations when wounds do not heal fast enough, or at all. The broad goal of this dissertation was to identify molecular mechanisms of wound-induced EGFR activation so that new therapeutic strategies may be developed for improving normal and pathological healing.

The observation that stimulation of growth factor receptors, such as the EGFR, promote epithelial restitution is not new, but growth factor therapies have met with limited clinical success, primarily due to the poor stability of the proteins in the wound environment (Chen et al., 1997; Hardwicke et al., 2008; Shell, 1982). HB-EGF and AR, ligands that have been found to induce autocrine EGFR activation after wounding, adhere to cell surface glycosaminoglycans after release, leading to a pool of active ligands that remains cell-associated (Harris et al., 2003). Thus, unlike treatment with EGF that can act in "hit and run" fashion, treatment of wounded epithelia with factors that promote autocrine ligand release leads to long-lasting and efficient signaling (Shvartsman et al., 2002).

By investigating mechanisms of wound-induced EGFR activation, I have identified signaling pathways that could be therapeutically stimulated to promote autocrine EGFR ligand release. Treatment with the non-hydrolysable nucleotide analog ATPγS or the water soluble phosphatidic acid analog C_8-PA warrant further study in animal models to determine if they can induce long-lasting EGFR activation and healing. Additionally, factors known to stimulate Pyk2 or PLD2, such as acetylcholine or glial cell derived neurotropic factor (GDNF), might be considered for future wound healing studies (Avraham et al., 2000; Exton, 2002).

Recently, many advances in drug delivery and gene therapy have made promising strides for wound healing therapies (Hardwicke et al., 2008; Mohan et al., 2005; Torchilin, 2006). The corneal epithelium is an attractive model for gene therapy because it is easily accessed and transgenic cells will eventually be lost due to natural sloughing. Other novel therapies include proteins that are chemically modified or polymer-impregnated to achieve controlled release at the wound site (Hardwicke et al., 2008; Hori et al., 2007). Similar controlled release techniques with ATPγS and C_8-PA, and gene therapy techniques with PLD2 and Pyk2 should be examined to test their safety and efficacy in promoting wound healing.

In addition to suggesting therapeutic targets for enhancing cell motility, the mechanistic insights gained from my studies should help to understand how underlying pathologies disrupt the normal healing process. For example, the loss of corneal nerves may impair healing due to the loss of secreted factors such as GDNF and acetylcholine, which can stimulate PLD and/or Pyk2 signaling (Cheng et al., 2003; Montiel et al., 2007; Nishida and Yanai, 2009; Sariola and Saarma, 2003; You et al., 2001). Also, factors such as elevated tear osmolarity in dry eye and high glucose in diabetes that affect EGFR signaling could be investigated for their affects on PLD and Pyk2 signaling (Akhtar et al., 2009; Fischer et al., 2004; Lezama et al., 2005; Tomassen et al., 2004; Xu et al., 2009).

Another clinical benefit of clarifying signals that control wound-induced EGFR activation is the ability to identify risks of various ocular and systemic drugs. Cancer-fighting anti-EGFR therapies cause persistent corneal epithelial and epidermal defects (Forster et al., 2008; Garibaldi and Adler, 2007; Johnson et al., 2009), so drugs that interfere with signaling upstream of the EGFR may have similar side-effects. Epithelial health, especially after

wounding, will need to be closely monitored in patients receiving new anti-cancer drugs that target SFKs, Pyk2, and PLD2 (Bagi et al., 2008; Kim et al., 2009; Scott et al., 2009).

7.5 IMPLICATIONS FOR EGFR-RELATED PATHOLOGIES

The mechanisms I have identified in regulating EGFR activation (Figure 63) may not be limited to wounded epithelia, but may also be relevant in other biological systems. I expect my findings to be significant for pathologies that feature dysregulation of EGFR signaling, such as cancer and cardiac hypertrophy.

7.5.1 Cancer

Many of the cellular processes that regulate epithelial migration after wounding also regulate the motility of cancer cells (Friedl and Gilmour, 2009). EGFR activation is especially important for cell migration during metastasis, the process by which cancer cells exit the primary tumor site, enter the blood or lymphatic system, and take residence in other tissues (Xue et al., 2006; Yamaguchi and Condeelis, 2007). As in wounds, the EGFR can be activated in cancer cells by SFK-mediated proteolytic ligand release stimulated by GPCR agonists or other environmental cues (Bhola and Grandis, 2008; Fischer et al., 2003; Higashiyama et al., 2008).

The intracellular signaling pathways I have identified as being mediators of EGFR ligand release, PLD2 and Pyk2, have both been implicated in promoting cell migration and invasion in a variety of cancers (Behmoaram et al., 2008; Foster and Xu, 2003; Huang and Frohman, 2007; Iiizumi et al., 2008; Roelle et al., 2008; Scott et al., 2009; Sun et al., 2008; Zheng et al., 2006).

Despite the importance of EGFR signaling in cancer, only three studies I know of have investigated whether either PLD or Pyk2 signaling can trigger EGFR activation (Andreev et al., 2001; Dewar et al., 2007; Li and Malik, 2005). Their findings support my conclusion that PLD2 and Pyk2 promote cell motility through EGFR signaling. Thus, cancers over-expressing Pyk2 or PLD2 may be sensitive to anti-EGFR therapies. Recent reports indicate that inhibiting signaling pathways, such as SFKs and the EGFR, in combination is more effective than inhibiting either alone (Egloff and Grandis, 2008). Therefore, inhibitors of PLD2 or Pyk2 should be tested in combination with EGFR-blocking drugs.

7.5.2 Cardiac Health

Activation of the EGFR by proteolytic ligand release is important for the development and function of the cardiovascular system (Booz and Baker, 1995; Eguchi et al., 2003; Iwamoto and Mekada, 2006). In fact, most HB-EGF null mice die before weaning due to severe heart valve defects (Iwamoto et al., 2003; Jackson et al., 2003). In the adult heart, EGFR ligand signaling is important for cardiac remodeling, which is a constant dynamic process that responds to hypertension, atherosclerosis, and heart attack. In cases of excessive cardiac remodeling, EGFR signaling causes cardiac hypertrophy and fibrosis, leading to poor heart function and death. I expect that my findings may be relevant to the study of cardiac hypertrophy.

A major regulator of myocardial EGFR activation, especially in response to hypertension, is angiotensin II (Ang II). In this context, Ang II promotes cardiac hypertrophy through transactivation of the EGFR by proteolytic ligand release (Chan et al., 2006; Higuchi et al., 2007; Kagiyama et al., 2002; Smith et al., 2004; Thomas et al., 2002; Touyz and Berry, 2002), but the intracellular signals that connect the Ang II receptor with EGFR ligand shedding are not clear.

Interestingly, Ang II stimulates PLD activity (Bollag et al., 1990) and inhibition of the PLD2 isoform by overexpression of a dominant-negative mutant inhibited Ang II-stimulated hypertrophy in cultured vascular smooth muscle cells (Parmentier et al., 2006). Similarly, Ang II signaling activates Pyk2, which contributes to the hypertrophic phenotype (Hirotani et al., 2004; Menashi and Loftus, 2009; Touyz and Berry, 2002). Taken together with my findings (Figure 68), these results suggest the hypothesis that Ang II stimulates the EGFR through PLD2 and/or Pyk2 signaling. Also, therapies that antagonize PLD2 or Pyk2 signaling may prevent cardiac hypertrophy.

7.6 SUMMATION

The function of a wounded tissue and the health of an organism is jeopardized as long as an epithelial wound remains open. This is especially true in the cornea, where even subtle inflammation or scaring can impair vision. Cell migration is absolutely required for the closure of epithelial wounds, and EGFR activation has been identified as an essential signal for the induction of motility after wounding. Re-epithelialization rates determine clinical outcomes, yet there are few effective therapies for enhancing cell migration. The broad goal of this dissertation research was to clarify the cellular signals that stimulate EGFR activation after wounding so that therapies may be developed to ameliorate normal and pathological healing.

To study EGFR activation, I have developed novel wounding models that minimize cell damage and allow biochemical analysis of cells specifically near to, or far from, the wound edge. I have found that multiple pathways converge on SFK signaling to regulate EGFR activation. In cells near and far from wounds, extracellular ATP stimulates EGFR activation through PLD2

signaling. In distinct pathways that functions solely in cells near the wound edge, Pyk2 and an undefined free edge sensor trigger SFK and EGFR activation. These findings lay the groundwork for future studies to identify wound sensors and suggest new therapeutic targets for modulating EGFR signaling and cell motility.

8.0 MATERIALS AND METHODS

8.1 MATERIALS

Antibodies against phospholipase D2 were the generous gift of Dr. Sylvain G. Bourgoin (Université Laval, Quebec, QC, Canada). Antibodies (followed by catalog number) against phospholipase D1 (sc-28314), EGFR (sc-03), EGFR phosphorylated on Tyr-1173 (sc-12351R), ERK1 (sc-93), ERK1/2 phosphorylated on Tyr-204 (sc-7383), Pyk2 phosphorylated on Tyr-402 (sc-11767R), FAK phosphorylated on Tyr-397 (sc-11765R), and c-Src (mouse monoclonal used for immunoprecipitation, sc-8056) were from Santa Cruz Biotechnology (Santa Cruz, CA); antibodies against EGFR phosphorylated on Tyr-845 (2231), SFK phosphorylated on Tyr-419 (2101), SFK non-phosphorylated on Tyr-419 (2102), and c-Src (rabbit polyclonal used for immunoblotting, 2109) were from Cell Signaling Technology (Danvers, MA); antibodies against E-cadherin (610182), Pyk2 (610549), and Fyn (610163) were from BD Biosciences; antibodies against MARCKS phosphorylated on serines 152 and 156 (442709) were from EMD Chemicals (San Jose, CA); antibodies against paxillin and ZO-1 were from Invitrogen (Carlsbad, CA); antibodies against β-Actin (A5316) and FLAG were from Sigma-Aldrich (St. Louis, MO). Antibodies against Ki67 (RM-9106) were from Thermo Fisher Scientific (Waltham, MA). Neutralizing antibodies against AR, HB-EGF, and TGFα were from R&D Systems (Minneapolis, MN). Tyrphostin AG 1478, GM 6001, GM 6001 negative control, PP2, and Src

Kinase inhibitor-I were from EMD Chemicals (Gibbstown, NJ). ATP, grade VII apyrase from potato, type V adenosine deaminase from bovine spleen, reactive blue 2, and polyHEMA were from Sigma-Aldrich. Phosphatidic acid (1,2-dioctanoyl-s*n*-glycero-3-phosphate) and phosphatidylinositol standards were from Avanti Polar Lipids (Alabaster, AL). Vybrant® DiO, DiD, and Alexa Fluor®-conjugates were from Invitrogen. The peptides Gap-26 (VCYDKSFPISHVR) and Gap-27 (SRPTEKTIFII) were custom synthesized and provided at 70% purity by GenScript (Piscataway, NJ). Cell culture reagents were from Mediatech (Manassas, VA) and other reagents and supplies were from ISC BioExpress (Kaysville, UT) or Thermo Fisher, unless noted.

8.2 CELL CULTURE

Human corneal limbal epithelial (HCLE) cells have been immortalized by abrogation of p16INK4A/Rb and p53 functions and overexpression of the catalytic subunit of the telomerase holoenzyme (Gipson et al., 2003). HCLE cells were cultured in Keratinocyte Serum-Free Medium (KSFM, Invitrogen) supplemented with 0.3 mM $CaCl_2$, 25 µg/ml bovine pituitary extract, 0.1 ng/ml human recombinant EGF, 50 IU/ml penicillin, and 50 µg/ml streptomycin. At least 16 hours prior to stimulations, cells were washed and cultured in the same medium without pituitary extract and EGF. HCLE monolayers were used for signaling studies, while stratified cultures of HCLE cells were used for wound healing assays (see below). For some experiments, I used secondary rabbit corneal epithelial cells. These cells were prepared from limbal epithelial explants from rabbit eyes from Pel-Freez Biologicals (Rogers, AR) according to established protocols (Ebato et al., 1987).

8.3 WOUND HEALING ASSAY

A modification of the traditional scrape wounding assay was used as described previously (Block et al., 2004). Molds for casting agarose strips were made by cutting 1 x 10 mm strips from rectangles of vinyl vehicle lettering from the Letterz-Numberz™ Kit (Chroma International Inc., Alcoa, TN). Vinyl pieces were used right out of the package but were not sterilized. The rectangles were attached to the growing surfaces of tissue culture plates by static cling and 2.5% agarose with 0.6% glycerol was pipetted into the mold and allowed to dry overnight. The molds were removed and cell suspensions added according to standard tissue culture procedures. HCLE cells were grown to confluence around the agarose strips and were induced to differentiate into a stratified epithelium (Gipson et al., 2003). Cells were transferred to Dulbecco's Modified Eagle Medium:F-12 1:1 with 0.5%-2% newborn calf serum, the agarose strip was removed, and healing was allowed to progress 14-24 hours before fixation or preparation of cell extracts. Micrographs of cultures were obtained before and after healing and wound areas were quantified using the region tracing utility in MetaMorph® software (MDS Analytical Technologies, Sunnyvale, CA). Experiments with mitomycin C have previously demonstrated that wounds in HCLE cells heal as a result of cell migration.

8.4 WOUNDING MODEL FOR BIOCHEMICAL ANALYSIS OF SIGNALING IN CELLS NEAR THE WOUND EDGE

A method for detecting signaling in cells 1-5 rows from the wound edge was used as described previously (Block et al., 2004). A solution of 1% agarose was applied in a ring around the

periphery of tissue culture dishes to prevent cells from growing up the sides of the dishes. A molten agarose solution (0.25% agarose with 0.6% glycerol) was then applied to the tissue dishes on ice/water baths using an atomizer from Suave® Naturals™ Ocean Breeze Spray Gel (Suave, Chicago, IL) that generated a fine mist of agarose droplets. After drying the dishes at 65ºC for 30 minutes, cells were plated by standard procedures and were allowed to grow to confluence around the droplets. To stimulate cells, fluid medium was replaced with semi-solid medium containing 0.70% agarose. This top agarose layer was allowed to bond to the droplets and solidify for 30 minutes in a 37ºC incubator followed by 15 minutes at room temperature. 37ºC medium was added on top of the agarose and using a metal spatula, the top agarose (with droplets attached) was gently lifted off as fluid medium flowed underneath. Stimulated cells were then incubated for various times before biochemical analysis.

8.5 WOUNDING MODEL FOR BIOCHEMICAL ANALYSIS OF SIGNALING IN CELLS FAR FROM THE WOUND EDGE

The HCLE cell populations were grown to confluence around a single agarose strip as described for *wound healing assay* above. Cells were stimulated by the removal of this strip and reactions were stopped 10 minutes later by plunging the bottom of the tissue culture dish into ice water and replacing the medium with ice-cold PBS. Cells proximal to the wound were scraped and discarded using a polyethylene cell lifter (Corning Inc., Corning, NY) trimmed to 7 mm, effectively removing approximately 100 rows of cells from each side of the wound, and the remaining cells were lysed directly in 1% SDS buffer.

8.6 PRODUCTION OF POLYHEMA PLATES WITH PLASTIC STRIPS.

Tissue culture dishes (3.5 cm) were coated with 1 ml of 10% polyHEMA in 95% ethanol and dried overnight at 37^{o}C. The bottom parts of tissue culture dishes (Corning) were broken into pieces and a 5% (weight/volume) solution in chloroform was prepared in 50 ml conical tubes on an end-over-end rotator. Strips of the plastic solution were applied to the surface of the polyHEMA with a narrow-bore 200 µl pipette tip. Strips were roughly 0.5 mm wide, fitting 10-20 rows of cells. As controls, the entire polyHEMA-coated surface was covered with a thin layer of dissolved plastic. The plates were dried at 65^{o}C overnight. HCLE cells were then seeded according to standard procedures.

8.7 ANALYSIS OF SIGNALING AT VARIOUS DISTANCES FROM FREE EDGES

Populations of HCLE cells were seeded onto polyHEMA-coated tissue culture plates that were half covered with dissolved plastic (with a single edge down the center). Reactions were stopped by plunging the bottom of the tissue culture dish into ice water and replacing the medium with ice-cold PBS. 0.5 cm-wide regions of sheets of cells were sequentially removed by scraping with thin strips of autoradiography film, starting at the plastic/polyHEMA interface. A fresh 0.5 ml of ice-cold PBS was used to collect each subsequent region and corresponding regions from 3 dishes were pooled. Cells were collected by centrifugation before analysis.

8.8 WESTERN BLOTTING

Following stimulations, cells were washed in ice-cold phosphate-buffered saline (171 mM NaCl, 10.1 mM Na_2HPO_4, 3.35 mM KCl, and 1.84 mM KH_2PO_4, pH 7.2) and lysed in SDS (1% in H_2O). The material was sonicated and protein contents were normalized after BCA assay (Thermo Fisher). Lysates were loaded on SDS poly-acrylamide gels in the Mini-PROTEAN 3 gel apparatus (Bio-Rad, Hercules, CA). Immunoblotting was performed according to standard procedure using optimized concentrations of antibodies. Multiple exposures of Western Blots were collected, and densitometry of appropriate images was performed using Quantity One software (Bio-Rad).

8.9 IMMUNOPRECIPITATION

Following treatment and washing in ice-cold PBS, cells were lysed in a non-denaturing lysis buffer (50 mM Tris-Cl, 260 mM NaCl, 0.02% NaN_3, 5 mM EDTA, 1% Triton X-100, 0.5 mM Na_3VO_4, 50 mM NaF, 1 tablet/10 ml of protease inhibitor cocktail (Roche Diagnostics GmbH), and 5 µM pepstatin A, pH 7.5). Approximately 250 µg of cellular protein was incubated with 30 µl of protein-A sepharose slurry and 1 µg of antibody on an end-over-end rotator at 4°C overnight. Bead pellets were washed twice with immunoprecipitation lysis buffer and three times with lysis buffer made with 0.1% Triton X-100. SDS-PAGE sample buffer was added to beads prior to Western Blotting.

8.10 IMMUNOCYTOCHEMISTRY

Cells were fixed by adding 3.7% formaldehyde directly to the culture medium and incubating for 30 minutes. After three washes with PBS, the cells were permeabilized with 0.5% Triton X-100 in PBS with 10% calf serum (blocking buffer) for at least 1 hour. Subsequently, samples were incubated overnight with appropriate dilutions of antibodies in blocking buffer. After washing, samples were incubated with 1:500 dilutions of fluorophore-conjugated secondary antibodies for 1 hour. After three washes, coverslips were applied with 90% glycerol in PBS and 2.5% 1,4-diazabicyclo-(2,2,2)-octane.

8.11 CONFOCAL FLUORESCENCE MICROSCOPY

Following immunocytochemistry, data were captured with an Olympus IX81 confocal microscope equipped with a 60X oil objective (NA 1.4). Images were generated and analyzed using Fluoview® software (Olympus, Center Valley, PA).

8.12 EPIFLUORESCENCE MICROSCOPY

Following immunocytochemistry, data were captured with a Nikon TE2000E microscope equipped with a 20X oil objective (NA 0.75). Images were generated and analyzed using Elements® software (Nikon Instrumets Inc., Melville, NY).

8.13 GENERATION OF IMAGES CONTAINING BOTH WOUND-PROXIMAL AND WOUND-DISTAL CELLS

Following immunocytochemistry, seven contiguous aligned fields (a total of 5 mm from the wound edge) per sample were captured on a Nikon TE2000E automated microscope with a 10X objective (NA 0.3) using the MetaMorph® scanslide utility. For quantitation of phospho-ERK signal intensities, images were acquired at identical exposures and the average intensities of 250 μm- or 62.5 μm-wide regions were recorded for the entire 5 mm width of the image using MetaMorph®. Debris and other technical artifacts from sample preparation were excluded from quantitation.

8.14 IMMUNOFLUORESCENCE MICROSCOPY OF CELLS ON PLASTIC STRIPS OR NEXT TO AGAROSE DROPLETS

As rhodamine-dextran was serendipitously found to bind strongly to polyHEMA, polyHEMA-coated plates were stained by incubation with 1 mg/ml rhodamine-dextran in PBS for 1 hour. The plates were then washed 5 times with PBS and dried at room temperature. 10 μl/ml Vybrant® DiO (Invitrogen) was added to the plastic solution before application as strips. Agarose was conjugated to fluorescein by dissolving 8 mg agarose in 200 μl DMSO at 100^{o}C, and reacting overnight with 4 mg fluorescein isothiocyanate (Sigma-Aldrich) at room temperature. It was diluted to 1.6 ml in water, chilled to 0^{o}C, and the solidified agarose washed extensively with water. The conjugate was diluted to 0.25% before application to plastic-covered plates. The cells were fixed with 3.7% formaldehyde in PBS and cell membranes were stained with 100 μl/ml of

Vybrant® DiD (Invitrogen) in PBS overnight. Images of the cultures were captured with an Olympus IX81 confocal microscope equipped with a 60X oil objective (NA 1.4) and xy and xz projections were generated and analyzed using Fluoview® software (Olympus).

8.15 LIVE CELL MICROSCOPY AND DETERMINATION OF CELL VELOCITIES

HCLE cells were stratified around agarose strips in a 12-well dish and medium was changed to 1:1 DMEM:F-12 with 10% NCS 24 hours prior to experiment. Cells received no treatment or 30 U/ml apyrase and agarose strips were left in place or removed, such that N=3 for each condition. Cell migration was monitored for the subsequent 24 hours with a Live Automated Cell Imager (Schugar et al., 2009): cells were maintained in an environmentally controlled chamber (37^{o}C and 5% CO_2) on a robotic stage and were visualized with an inverted Nikon Eclipse TE 2000 U microscope equipped with a 10X objective and Photometric ES Cool Snap CCD camera. Time-lapse images were created and cell velocities were calculated using MetaMorph® by manually tracking individual cells. In each well, four cells from each of two regions at the wound edge and four cells from each of two regions 2 mm from the wound were analyzed. Cells were chosen arbitrarily, prior to viewing the time-lapse movie. The analysis was blinded to apyrase treatment, and in cells distal to the edge, the analysis was blinded to wounding. Due to errors in stage movement and manual tracking, the velocity of a fixed object was determined to be 0.1 μm/min which was subtracted from calculated cell velocities.

8.16 RABBIT AND RAT CORNEAL ORGAN CULTURE

To inflict wounds in rabbit eyes for organ culture, a 7.5 mm diameter mark was made with a trephine, and the epithelium was removed by means of an Algerbrush II (Alger Equipment Co., Lago Vista, TX) and a sharp forceps. The wounded corneas were excised with a 2-3 mm scleral rim and placed on hemispheric supports made from the round end of transfer pipets (Samco Scientific Corp., San Fernando, CA) in 12-well dishes. The wound was briefly stained with 0.1% fluorescein and photographed. They were subsequently incubated submerged at 37^{o}C in Ham's F12 Medium:Dulbecco's Modified Eagle's Medium (1:1) with 0.3% gentamycin for 60 hours. I prefer a submerged culture technique because it allows continuous access of inhibitors. The corneas were then stained with 1% alizarin red in phosphate buffered saline and photographed again. The percentage of healing was calculated using MetaMorph® software based on the areas of the wounds before and after incubation. Whole rat eyes (Pel-Freez Biologicals) were wounded by abrasion with an Algerbrush and cultured in the presence of no treatment or 30 U/ml apyrase, and fixed after 24-48 hours in culture. Corneas were excised from globes and the expression of Ki-67 was examined by immunohistochemistry of whole mounts.

8.17 ASSAY FOR PHOSPHOLIPASE D ACTIVITIES

Cells were labeled with 10-20 μCi [^{3}H]myristic acid for 4 hours, stimulated, and 1-butanol added to a final concentration of 0.5% for 5 minutes. Reactions were stopped and lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). The dried samples were resuspended

in chloroform:methanol (2:1), and the phospholipids resolved on oxalate-treated silica gel G/UV TLC plates (Whatman) by developing with $CHCl_3$:methanol:glacial acetic acid (50:15:2). The plates were air-dried, treated with EN^3HANCE® (PerkinElmer, Waltham, MA), and exposed to a Kodak Biomax XAR film at –80°C. For quantitation, phosphatidyl-butanol and phosphatidylcholine spots were cut from the plates, and the radioactivity measured in a Beckman LS3800 ß counter. Labeled phosphatidyl-butanol was identified by co-migration with authentic phosphatidyl-butanol, by its absence without addition of 1-butanol, and by its increase upon stimulation of cells with phorbol 12-myristate 13-acetate (PMA or TPA). Labeled phosphatidylcholine was identified by co-migration with authentic phosphatidylcholine. The amount of radioactivity in phosphatidyl-butanol was normalized to the amount in phosphatidylcholine for each sample.

8.18 AMPHIREGULIN MEASUREMENT

Concurrent with wounding or hormonal stimulation, HCLE cells were cultured in freshly added keratinocyte serum-free medium (KSFM) without EGF and pituitary extract for 10 minutes. Conditioned medium was collected and subjected to centrifugation for 2 minutes at 5,000 x g. The cell-free supernatants were aliquotted and stored at -20°C until further processing. Amphiregulin was measured in the supernatants using the DuoSet® ELISA (R&D Systems) according to manufacturer's protocol. For normalization purposes, protein content of whole cell extracts was determined by the BCA protein assay (Thermo Fisher).

8.19 ATP MEASUREMENT

Concurrent with wounding or hormonal stimulation, HCLE cells were cultured in freshly added KSFM without EGF and pituitary extract for 10 minutes. Conditioned medium was collected and subjected to centrifugation for 2 minutes at 5,000 x g. The cell-free supernatants were aliquotted and stored at -20°C until further processing. ATP was measured in the cell-free tissue culture supernatants with the ATP Bioluminescent Assay Kit (Sigma-Aldrich). Independent standard curves were created for the drug-treated groups to control for effects of the drug or altered pH on the assay. For normalization purposes, protein content of whole cell extracts was determined by the BCA protein assay (Thermo Fisher).

To detect ATP degradation in situ, 10 μCi [α-^{32}P]-ATP was added to the cultures and an aliquot was collected after 5 minutes for analysis by thin-layer chromatography as described (Hagmann et al., 1999). ATP and ADP were excised from the thin-layer chromatography plate, subjected to scintillation counting, and normalized to total radioactivity in the sample.

8.20 LUCIFER YELLOW AND RHODAMINE-DEXTRAN UPTAKE

Lucifer yellow and rhodamine-conjugated dextran were diluted to 0.5% in culture medium and added to live HCLE cells for five minutes with desired treatments. After removal of dyes, cells were washed three times with PBS and fixed with 3.7% formaldehyde in PBS before fluorescence microscopy.

8.21 SIRNA TRANSFECTIONS

SiRNA was transfected into subconfluent cells at the time of seeding using the siPORT™ *NeoFX*™ lipid-based reverse transfection reagent (Applied Biosystems, Foster City, CA). Two days after transfections, cells were re-seeded to generate confluent cultures and were used the following day. For PLD experiments, the following oligonucleotides were designed according to published sequences and standard rules (Pei and Tuschl, 2006) and were synthesized by Applied Biosystems (sense strand): PLD1, GUUAAGAGGAAAUUCAAGCdTdT; PLD2 sequence a, UGGGGCAGGUUACUUUGCUdTdT; PLD2 sequence b, AGUCUUGAUGAGGUCUGCUCdTdT. For Pyk2 studies, multiple siRNA oligonucleotides were used: CACAUGAAGUCCGAUGAGAdTdT (Sigma-Aldrich) and a Pyk2 *SMART*pool that contains at least four duplexes of undisclosed sequence (Millipore, Billerica, MA). As a control for Pyk2 siRNA, I used MISSION® siRNA Universal Negative Control #1 (Sigma-Aldrich). BLAST searches (Altschul et al., 1990) with siRNA sequences revealed significant sequence homologies with only the targeted mRNAs. For each experiment, the expression of target protein was monitored by Western Blotting.

8.22 ADENOVIRAL INFECTIONS

FLAG-tagged Pyk2 and FLAG-tagged PRNK cloned into adenovirus vectors were obtained from Dr. Joseph C. Loftus (Mayo Clinic, Scottsdale, AZ). Adenovirus encoding the tet-OFF transactivator (used as adenovirus control) was from Dr. Ora A. Weisz (University of Pittsburgh, Pittsburgh, PA) and adenovirus encoding GFP was from Dr. Michael P. Czech (University of

Massachusetts, Worcester, MA). Preliminary experiments using GFP-encoding virus helped to determine the multiplicity of infection necessary for achieving expression in ~100% of cells. For signaling studies, cells were infected at a multiplicity of 10 for 1 hr and were used the following day. For wound healing studies, cells were infected at a multiplicity of 20 in a small volume (1 x 10^5 cells/μl) for 1 hr, with gentle agitation every 15 min. After washing and centrifugation, cells were seeded for wound healing assay and were used 2 days later.

8.23 STATISTICAL ANALYSIS

All experiments reported were repeated a minimum of three times with similar results. For some figures, representative Western Blots are shown below densitometry from at least four replicates. Quantitative data were plotted (means +/- S.D.) and analyzed by t-test or one-way ANOVA followed by Bonferroni's multiple comparisons test using Prism (GraphPad Software, La Jolla, CA).

APPENDIX A

LIST OF PEER-REVIEWED PUBLICATIONS CO-AUTHORED BY ETHAN R. BLOCK

Block,E.R, Tolino, M.A., and Klarlund, J.K. (2010). "Pyk2 Activation triggers EGF receptor signaling and cell motility after wounding sheets of epithelial cells. Submitted.

Block, E.R., Tolino, M.A., Lathrop, K.L., Sullenberger, R.S., Mazie, A.R., and Klarlund, J.K. (2010). Edges in epithelial cell sheets stimulate epidermal growth factor receptor signaling. Submitted.

Block, E. R., and Klarlund, J. K. (2008). Wounding sheets of epithelial cells activates the epidermal growth factor receptor through distinct short- and long-range mechanisms. *Mol Biol Cell* **19,** 4909-17.

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Block, E. R., Matela, A. R., SundarRaj, N., Iszkula, E. R., and Klarlund, J. K. (2004). Wounding induces motility in sheets of corneal epithelial cells through loss of spatial constraints: role of heparin-binding epidermal growth factor-like growth factor signaling. *J Biol Chem* **279,** 24307-12.

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