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Dynamics of cell-cell junctions in keratinocytes

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by

Miquella G. Chavez

B.A., Chemistry, University of New Mexico, 2000
B.S., Biology, University of New Mexico, 2000

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

Doctor of Philosophy
Biomedical Sciences

The University of New Mexico
Albuquerque, New Mexico

July 2009

DEDICATION

I DEDICATE MY DISSERTATION TO MY MOM,

WANDA GALINDO CHAVEZ, WHO INSTILLED IN MY THE IMPORTANCE OF EDUCATION AND WHO SACRIFICED SO THAT I MAY HAVE IT. I AM TRULY GRATEFUL FOR ALL SHE HAS DONE AND CONTINUES TO DO FOR ME.

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ABSTRACT

Poor wound healing is a serious medical issue of particular concern in the elderly and people with diabetes. One major obstacle for these patients to achieve complete wound healing is incomplete reepithelialization which is necessary for restoration of barrier function. One molecule that has promising therapeutic value for promoting reepithelialization is epidermal growth factor receptor (EGFR). Using a squamous cell carcinoma cell line (SCC 12F) that expresses moderate levels of EGFR, we investigated the contributions of EGFR activation to reepithelialization, and specifically its roles in modulation of cell-cell junctions. Decreased cell-cell adhesion mediated by adherens junctions and desmosomes is necessary for epithelial outgrowth into the wound area. We find that elevated EGFR levels are necessary for successful reepithelialization in an *in vitro* model. EGFR activation led to junctional and cytoskeletal disruption, nuclear localization of the junctional protein ß-catenin and upregulation of target genes involved in wound healing. We also find desmosomes and adherens

junctions are modulated by different mechanisms. EGF stimulation caused the desmosomal cadherin desmoglein-2 to internalize and enter a recycling pathway, while the adherens junction protein E-cadherin underwent a matrix metalloproteinase-dependent cleavage. We conclude that EGFR can stimulate multiple mechanisms within a given cell type leading to modulation of cadherin function, and that the stimulus is an important determinant in junctional protein fate. Understanding the mechanisms that promote reepithelialization may lead to strategies to improve wound repair in populations predisposed to poor wound healing.

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Commonly used abbreviations

Abbreviation	Full Name	Positive or Negative Regulator of Wound Healing
PDGF	Platelet derived growth factor	Depends on subtype
KGF	Keratinocyte growth factor	Mixed results
MIF	Macrophage migration inhibtory factor	Unknown
IL	Interleukin	Depends on subtype
GM-CSF	Granulocyte-macrophage colony-stimulating factor	Mixed results
TNF-α	Tumor nucrosis factor alpha	Positive
MCP	Monocyte chemoattractant protein	Positive
MIP	Macrophage inflammatory protein	Mixed results
GRO-α	Growth related oncogene alpha	Mixed results
TGF-α	Transforming growth factor -alpha	Positive –reepithelialization
TGF-ß	Transforming growth factor -beta	Yes but excess=scarring
BMP	Bone morphogenetic protein	Negative
EGF	Epidermal growth factor	Positive –reepithelialization
HGF	Hepatocyte growth factor	Positive –granulation
VEGF	Vascular endothelial growth factor	Positive –angiogenesis
NGF	Nerve growth factor	Positive
IGF	Insulin growth factor	Positive
FGF	Fibroblast growth factor	Depends on subtype
HB-EGF	Heparin bound epidermal growth factor growth factor	Positive

1 Introduction

1.1 Skin Structure

The skin is the largest organ in the human body and has many important roles including protection of internal organs from the outside environment, temperature regulation, and fluid retention. The skin is divided into the dermis and the epidermis (Figure 1.1.1). The dermis consists of connective tissue, and is the location of hair follicles, sweat glands and blood vessels (Bolognia *et al.*, 2003). Located within the dermis, there are several appendages that contain niches of stem cells, including the bulge region of the hair follicle, and sebaceous glands (Figure 1.1.1). Connecting the epidermis to the dermis is the basement membrane, which is composed of extracellular matrix proteins such as collagen and laminin. The epidermis has 5 layers and in each layer the epithelial cells, or keratinocytes, have different phenotypes and characteristics based on differentiation status (Figure 1.1.1). The first layer of the epidermis in contact with the basement membrane is the stratum basale, which consists of columnar, proliferating epithelial cells (Bolognia *et al.*, 2003). These cells proliferate for a few cycles, then migrate and differentiate to form the suprabasal layers where they lose their ability to proliferate (Gurtner *et al.*, 2008).

Maintenance of the epidermis depends on intrafollicular stem cells, which are interspersed in the basal layer between hair follicles. Although stem cells also exist within the hair follicle and the bulge region of the hair follicle (Figure 1.1.1), they are responsible for maintenance of the hair follicle and do not contribute to

the intrafollicular epidermis (Watt *et al.*, 2006). However, the existence of intrafollicular stem cells is now under debate. Until recently, intrafollicular stem cells were believed to be the progenitor cells of transit amplifying cells, which are located at the basal layer and undergo several rounds of proliferation before committing to terminal differentiation (Watt *et al.*, 2006). Recent studies support a new model; the committed progenitor cell that proliferates like a stem cell but is committed to terminal differentiation similar to a transit amplifying cell (Clayton *et al.*, 2007; Jones and Simons, 2008).

Whatever their origin, once cells are committed to differentiation, they migrate to the subrabasal layers, beginning with the stratum spinosum, which is directly above the stratum basale (Fig. 1.1.1). The stratum spinosum is composed of several layers of cuboidal epithelial cells that produce large amounts of cytokeratin. The next layer above the stratum spinosum is the stratum granulosum, which is named due to the granules visible by light microscopy and is the uppermost layer containing living cells. The next layer, the stratum lucidum, is only present in thick skin such as the soles of feet and the palms of hands, and is made up of a clear layer of cells that lack internal organelles. The last layer is the stratum corneum, which consists entirely of denucleated keratinocytes. These cells are continuously sloughed off and are replaced as cells differentiate from the proliferating stratum basale up through the strata to the stratum corneum (Bolognia *et al.*, 2003).

Other cells present in the epidermis include melanocytes and Langerhans cells. Melanocytes produce melanin, which lends the skin its pigmentation and

provides partial protection from ultraviolet radiation (Bolognia *et al.*, 2003). Langerhans' cells are the immune cells of the epidermis that upon encountering a foreign substance, break it down into peptides that subsequently enter the lymph system and act as antigens for the immune system (Bolognia *et al.*, 2003). Taken together, the epidermis is a complex tissue with several different cell types that work together to respond to environmental insult in order to maintain skin integrity and function.

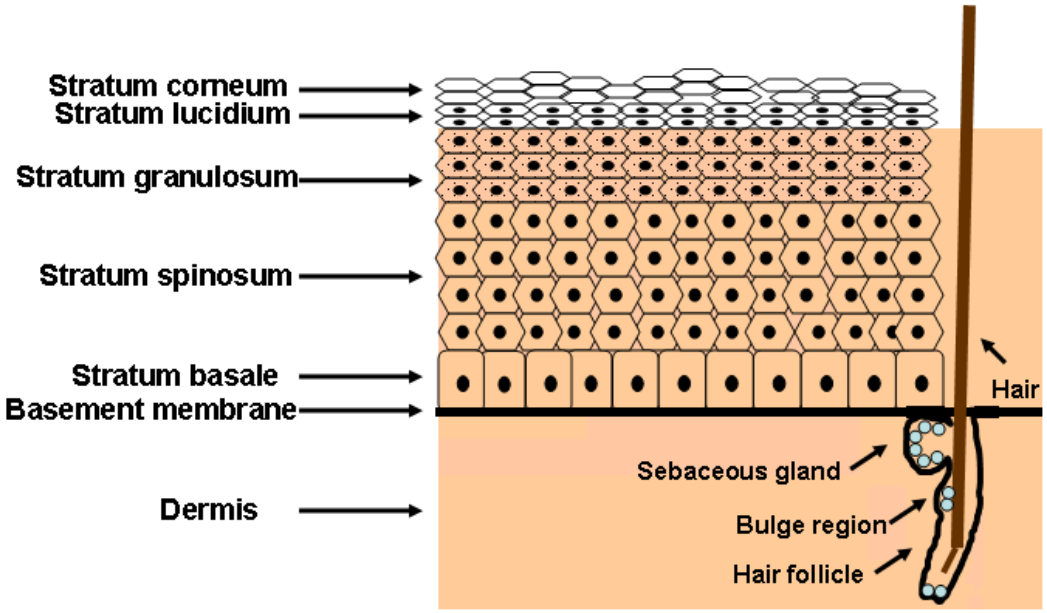


Figure 1.1.1 Layers of the Skins

Schematic representation of the several layers of the epidermis. Starting from the bottom of the figure is the dermis, which contains the hair follicle and sebaceous glands. Directly above the dermis is the basement membrane. Directly above the basement membrane is the proliferating basal layer termed the stratum basale. The stratum spinosum represents the layer in which the keratinocytes start to differentiate and produce cytokeratin. The stratum granulosum is so named for the granular structures present, and is the last layer of fully differentiated, living cells. The stratum lucidium is present only in thick skin, and is named for its clear appearance under the microscope. Finally, the uppermost layer, the stratum corneum, is made up of dead, denucleated keratinocytes, and whose contents are mostly keratin.

1.2 Wound Repair

When a wound is introduced into the skin, it compromises the intact barrier function and opens up the body to threats such as water loss (dehydration) and increased susceptibility to infection (Coulombe, 2003). The progression of wound healing is an intricate interplay of different systems working in concert and has several stages that takes weeks to accomplish (Singer and Clark, 1999). The basic steps include clotting, inflammation, reepithelialization, remodeling of the extracellular matrix, and contraction of the wound (Figure 1.2.1). Within minutes after the initial injury, platelets invade into the wound area and form a clot by releasing thrombin, which cleaves fibrinogen and forms a crosslinked network of fibrin fibers (Dvorak, 1988). It is this resulting clot (Figure 1.2.1, step 1.) that covers the wound area and protects it from further external damage (Martin, 1997). Inflammation and the deposition of granulation tissue follow the appearance of inflammatory cells, fibroblasts and capillaries into the wounded area. Within minutes after wounding, neutrophils enter the wound area and the corresponding pro-inflammatory cytokines are released (Singer and Clark, 1999). It is these neutrophils and platelets that aggregate in the clot and release factors that stimulate cell motility and proliferation in the surrounding keratinocytes and fibroblasts (Eming *et al.*, 2007). The next set of inflammatory cells that invade are macrophages, which also release cytokines and growth factors and phagocytose any outside contamination that may have been introduced into the wound area. The macrophages also phagocytose excess cell and matrix debris that accumulates as part of the wound healing process (Singer and Clark, 1999),

including the neutrophils that are no longer necessary (Eming *et al.*, 2007). The signals and factors produced by macrophages induce both keratinocytes and fibroblasts to produce granulation tissue (Figure 1.2.1, step 2). Keratinocytes produce granulation tissue components collagen IV and laminin-5, whereas fibroblasts produce nidogen and small amounts of collagen IV (Werner *et al.*, 2007). It is this granulation tissue, or matrix, that the epithelial cells will migrate on during reepithelialization.

Reepithelialization, or the formation of a functional epithelial barrier, occurs a few days after the initial injury and involves the proliferation and migration of epithelial cells (keratinocytes) into the wound area (Figure 1.2.1, step 3). During this time period (24 hours to several days) the granulation tissue is remodeled to resemble a traditional extracellular matrix (ECM) (Singer and Clark, 1999).

Capillaries provide blood supply to the granulation site, and new capillaries migrate as branches from intact capillaries in the area around the wound (Baum and Arpey, 2005). Fibroblasts also serve as a contractile mechanism and pull the two opposing sides of the wounded tissue closer together (Figure 1.2.1, step 4) (Singer and Clark, 1999). Platelets release platelet derived growth factor (PDGF) and transforming growth factor beta (TGF-ß), which cause fibroblasts to differentiate into myofibroblasts that have contractile properties used during wound healing (Werner *et al.*, 2007). Finally, it takes weeks to months for the dermis and epidermis to fully mature (Figure 1.2.2), which is accompanied by regeneration of hair and sweat glands (Martin, 1997), a decrease of type I collagen and an increase of type III collagen (Baum and Arpey, 2005).

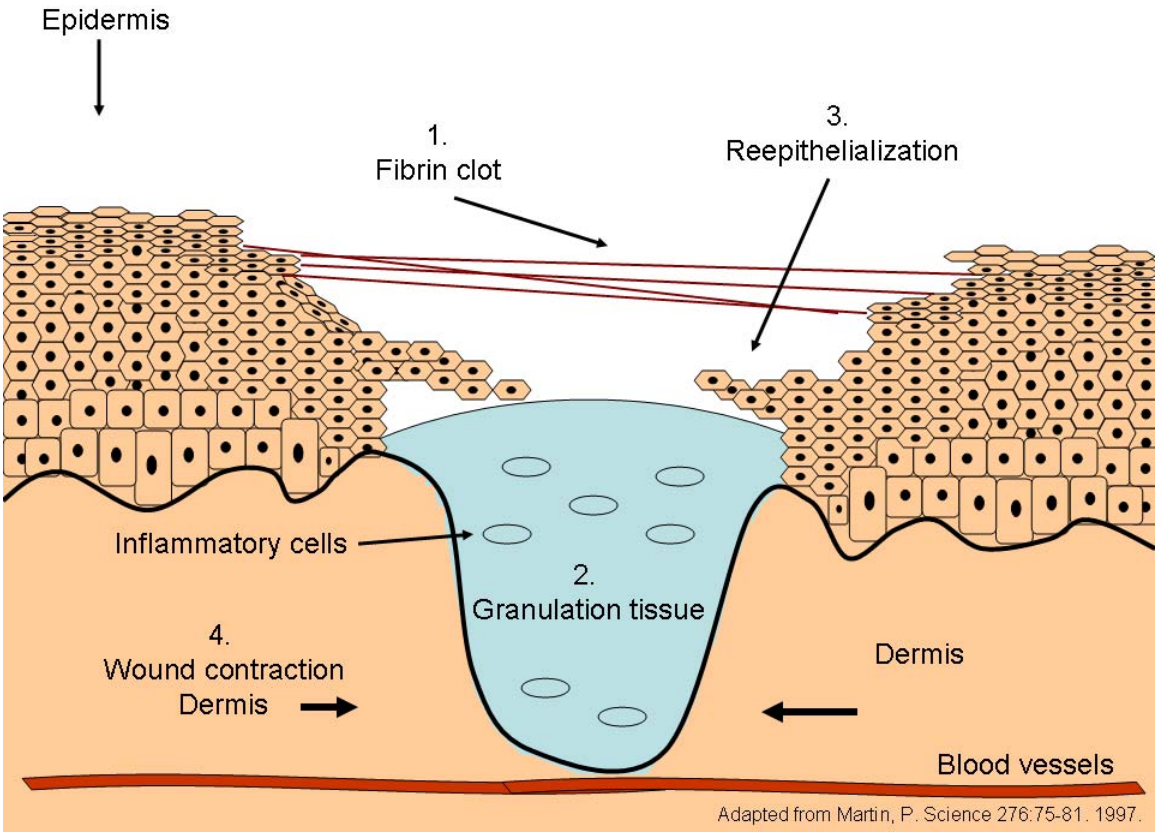
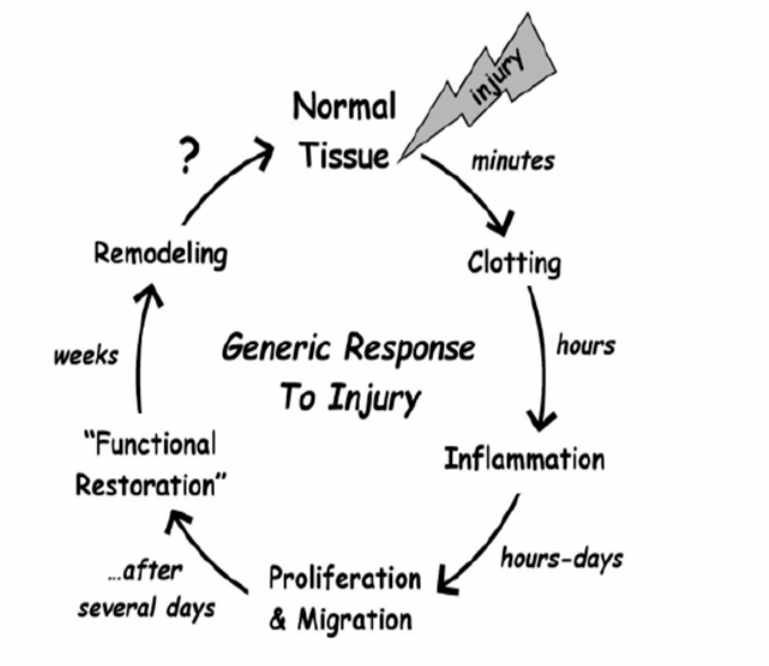


Figure 1.2.1 Reepithelialization of a healing wound

Stages of wound repair begins with (1) production of a fibrin clot to cover the wounded area and protect it from the outside environment. The second stage is (2) invasion of inflammatory cells into the wound area and the formation of granulation tissue. It is on this granulation tissue that the epithelial cells can migrate across in the third stage, reepithelialization (3). Finally, the fibroblasts of the dermis can also participate by contracting the wound area (4), and remodeling of the wound area can continue, for up to weeks after the initial wound. For description of growth factors and cytokines and their roles in promoting wound healing, see figure 1.4.1 and sections 1.3 and 1.4 in the text.



(Coulombe, P.A. 2003. J. Invest. Dermatol. 121:219-30)

Figure 1.2.2 Overview of Wound Healing

Wound healing goes through several different stages, some of them occurring concurrently in order to restore the damaged tissue to a functional epidermis. Within minutes, clotting factors work to provide a temporary barrier. Next, inflammatory cells infiltrate the area and deposit granulation tissue, on which keratinocytes can proliferate and migrate upon in order to produce a "functional restoration" of the epithelial barrier. Weeks to months later, the dermis undergoes remodeling of the granulation tissue in a process that ultimately results in scar tissue formation.

1.3 Modulators of Wound Repair

Throughout the stages of wound healing there are many modulators present that activate various cell types and orchestrate wound repair. These factors include steroid hormones, cytokines and growth factors. Each class of modulators has an impact on several phases of wound healing.

1.3.1 Steroid Hormones

Glucocorticosteroids and the sex hormones are two types of steroid hormones that modulate wound healing. Glucocorticosteroids have been studied for many years, and there are several findings that have come of their use with respect to wound healing. Sex hormones estrogen and testosterone, as well as their precursor DHEA, have not been studied as extensively, but significant findings of these hormones and their effects on wound healing have been revealed in the last 10 years.

Glucocorticosteriods are a class of therapeutics first used in the 1950's for the treatment of several different skin disorders, until severe adverse effects were reported as early as 1955 (Hengge *et al.*, 2006). Adverse cutaneous effects included a desensitization of the skin (tachytphylaxis), increased fragility, easy bruising, and poor wound healing (Hengge *et al.*, 2006). Excess glucocorticosteroids lead to dramatic effects on several areas of wound healing, including less granulation tissue, decreased collagen synthesis, decreased inflammatory response, and a decrease in proliferation of fibroblast and

keratinocytes (Reed and Clark, 1985). The decrease in collagen synthesis led to reduced tensile strength in the wounded skin (Beer *et al.*, 2000a). Specifically, in glucocorticoid treated skin, cytokines interleukin-1 alpha and beta (IL-1α, IL-1ß), and tumor necrosis factor alpha (TNF-α) mRNA levels were decreased at the wound margin (Hubner *et al.*, 1996a), as well as several growth factors including keratinocyte growth factor (KGF) (Brauchle *et al.*, 1995), PDGF, TGF-ß1, and TGF-ß2 (Beer *et al.*, 2000a). Glucocorticosteroid treatment caused chronic wounds in animal studies, which presented ulcers similar to those seen in diabetic patients (Bitar, 1998). Although the exact mechanism of glucocorticoid mediated disruption of wound repair is unknown, studies found that treatment with glucocorticoids compromises epidermal permeability (Kao *et al.*, 2003) and suppressed several genes key in wound healing, including those in the immune response and matrix remodeling as seen by microarray analysis (Stojadinovic *et al.*, 2007).

Sex hormones represent another type of steroid hormones that can have an effect on wound healing. Estrogen is a positive regulator of wound healing that decreases the immune response (Gilliver *et al.*, 2007). Specifically, estrogen decreased an inflammatory response by downregulating the macrophage migration inhibitory factor (MIF) (Ashcroft *et al.*, 2003). Decreasing the immune response is important in wound healing because an increased or prolonged inflammatory period corresponds with poor wound closure (Wang *et al.*, 2006). In addition, estrogen increased keratinocyte migration and induced endothelial cells to form capillary-like structures in cell culture. (Gilliver *et al.*, 2007). A

decrease in the estrogen and androgen hormone precursor dehydroepiandrosterone, (DHEA), correlated with impaired healing in human studies, and addition of DHEA reversed the delay of wound healing seen in aged mice (Mills *et al.*, 2005), via conversion to estrogen and activation of the estrogen receptor. In contrast to estrogen, testosterone had a negative effect on wound healing. Removal of testosterone by the castration of male mice resulted in faster healing compared to that of their control littermates (Ashcroft and Mills, 2002). The metabolically active form of testosterone, 5-α-dihydrotestosterone (DHT) is believed to be responsible for the delays in wound healing, as inhibition of this conversion increased wound healing to the same rate as castrated littermates (Gilliver *et al.*, 2006). In both mice and rats, castration decreased the immune response and collagen deposition, two events that can lead to detrimental effects in wound healing if overstimulated (Gilliver *et al.*, 2007).

Steroid hormones can be both positive and negative regulators of wound healing. Estrogen and its precursor DHEA enhance wound healing which could prove to be a beneficial side effect for postmenopausal women on hormone replacement therapy. Testosterone and glucocorticoids have negative effects on wound healing and caution should be used in prescribing glucocorticoids to populations disposed to poor wound healing. Gender differences in wound repair occur among elderly populations (Ashcroft *et al.*, 1999), and the prevention of the development of chronic wounds occurs in postmenopausal women on hormone replacement therapy (Ashcroft *et al.*, 2003).

1.3.2 Cytokines

Cytokines are small, secreted proteins that can modulate cells of the immune system and represent another group of molecules that regulate wound healing. A brief summary of abbreviations is available for reference on page xi. Proinflammatory cytokines involved in wound repair and specifically reepithelializaton include IL-1, interleukin 6 (IL-6), granulocyte-macrophage colony-stimulating factor (GM-CSF), and TNF-α, all of which are upregulated during the inflammatory phase of wound healing (Werner and Grose, 2003). Chemokines are a subset of cytokines and a number of them are regulated during wound healing, which will be discussed independently in section 1.3.3. The inflammatory response is a major step in wound healing and the activation of this pathway is mediated mainly by proinflammatory cytokines, although reports indicate the importance of anti-inflammatory cytokines as well. Most of these cytokines (IL-1, IL-6, GM-CSF, IL-10) are beneficial to wound healing, but some, such as TNF-α, impair wound healing. The focus in this section will be on those that have a positive or negative effect on reepithelialization and are produced by keratinocytes.

IL-1 is a proinflammatory cytokine that exists in two isoforms, IL-1α and IL-1β. It is produced by keratinocytes, and is normally stored in the cytoplasm at low levels until signaling occurs such as in response to wounding. The keratinocytes then release IL-1 as evidenced by mRNA and protein expression *in vitro* (Hubner *et al.*, 1996a; Sabourin *et al.*, 2000) and *in vivo* wounding studies (Sabourin *et al.*, 2000) into the surrounding environment activated not only

themselves in an autocrine manner, but also activated dermal fibroblasts and endothelial cells (Wood *et al.*, 1996). IL-1 is considered the initiator of the keratinocyte activation cycle that keratinocytes undergo under wounded conditions. Entering this cycle, keratinocytes became hyperproliferative, migratory, and change keratin isoform expression in a process called a keratin switch (Freedberg *et al.*, 2001).

IL-6 is a proinflammatory cytokine produced by several cell types, including keratinocytes, fibroblasts and macrophages (Lin *et al.*, 2003). Transcripts of this cytokine were increased as early as 3 hours after wounding and persisted for over 72 hours in an *in vivo* porcine wounding model (Sabourin *et al.*, 2000). Studies found that IL-6 did not directly induce keratinocyte migration, as was previously believed, but instead acted indirectly by activating STAT-3 phosphorylation in fibroblasts that then caused release of an as yet unknown factor that stimulated keratinocyte migration (Gallucci *et al.*, 2004). In gene deletion studies, lack of this cytokine delayed reepithelialization, which was rescued by adding back recombinant IL-6 one hour prior to wounding (Werner *et al.*, 2007). Recent studies showed that IL-6 acted as an inducer of fibroblast migration into granulation tissue, as IL-6 deficient mice show decreased fibroblast motility (Luckett and Gallucci, 2007).

GM-CSF is a pleiotropic cytokine secreted by keratinocytes in response to wounding (Mann *et al.*, 2001). Clinically, GM-CSF was applied intradermally to leprosy patients with skin lesions that led to increased keratinocyte production and enhanced overall wound healing (Holman and Kalaaji, 2006).

Overexpression of a GM-CSF antagonist in the epidermis resulted in poor wound healing, as evidenced by delayed reepithelialization due to reduced keratinocyte proliferation, as well as reduced neovascularization and collagen deposition (Mann *et al.*, 2006). Similiarily, genetic deletion of GM-CSF also resulted in poor wound healing as evidenced by delayed reepithelializaion and collagen deposition (Fang *et al.*, 2007). These studies indicate the importance of GM-CSF regulation during reepithelialization.

TNF-α is a proinflammatory cytokine whose mRNA is upregulated in wound margin epithelium (Hubner *et al.*, 1996a), but is mainly produced by macrophages in the granulation tissue (Hubner *et al.*, 1996a) and by neutrophils in the first few hours after wounding (Gillitzer and Goebeler, 2001). However, unlike the previously mentioned cytokines, reduced signaling of this cytokine resulted in enhanced wound repair. Gene deletion of the receptor of this cytokine, TNF receptor p55, resulted in decreased leukocyte infiltration into the wound, yet accelerated wound repair (Eming *et al.*, 2007). Consistent with these results, Goren *et al.* used a mouse model *ob/ob* to induce diabetic wound ulcers, and then treated these ulcers with an anti-TNF-α antibody to functionally block TNF-α signaling (Goren *et al.*, 2007). They found increased wound healing and a decrease in the numbers of macrophages in the wound site.

IL-10 is an anti-inflammatory chemokine that in addition to being produced by infiltrating mononuclear cells, is also produced by keratinocytes in the wound epidermis (Sato *et al.*, 1999). IL-10 is a chemokine that works by suppressing the immune response by inhibiting synthesis of several proinfammatory cytokines

in both macrophages and neutrophils (Werner and Grose, 2003). IL-10 mRNA levels were upregulated by 1 hour post wounding in a murine incision model and protein levels were increased in a biphasic response, with the first surge occurring 3 hours post incision and the second occurring 72 hours post wounding (Sato *et al.*, 1999). Use of neutralizing antibodies against IL-10 at the wound margin revealed that IL-10 suppressed the response of several chemokines, including monocyte chemoattractive protein (MCP), macrophage inflammatory protein 1 alpha (MIP1-α), and IL-6. (Sato *et al.*, 1999). This suppression might be required in the later stages of wound healing and for the formation of scar tissue. Inhibition of of IL-6 and IL-8 by IL-10 occured in fetal wound repair, which is scarless (Eming *et al.*, 2007). When IL-10 knockout skin was grafted onto embryos, a large inflammatory response and adult like scarring occurred (Liechty *et al.*, 1998). These studies illustrate the importance of IL-10 in regulating proinflammatory chemokines at the wound site.

The inflammatory response is a step in wound healing that is mediated by several different molecules including the proinflammatory cytokines. Keratinocytes themselves produce cytokines associated with inflammation, which in turn affect keratinocyte migration and modulate the wound environment. Understanding both the positive and negative roles that cytokines play in wound healing will ultimately provide information that is beneficial clinically. Also, recognizing the time frame of wound healing and in particular the release of cytokines in healing wounds will ultimately help in accelerating wound healing and eliminating scar tissue.

1.3.3 Chemokines

Chemokines represent a subset of cytokines expressed during wound repair. Chemokines are cytokines that stimulate chemotaxis of leukocytes during the inflammatory response. Chemokines can be divided into 3 different classes based on their folding and separation of two disulfide bonds between 4 cysteine residues. The CXXC class has two amino acids in between the two disulfide bonds, the CXC class, has one amino acid separating the bonds and the CC class has no amino acid between the two disulfide bonds. Due to the large number of chemokines involved in wound healing, only those that directly bind to or are produced by keratinocytes will be reviewed and these will be further separated by either the positive or negative effect they have on reepithelialization.

Chemokines that are produced in another cell type but affect keratinocytes and reepithelialization include growth related oncogene alpha (GRO-α) and interleukin 8 (IL-8). GRO- α and IL-8 are both members of the CXC class of chemokines. GRO-α, also known as CXCL1, or macrophage inflammatory protein 2 (MIP-2), was elevated after wounding *in vivo* and *in vitro* and was a strong mitogen for keratinocytes (Gillitzer and Goebeler, 2001). GRO-α mRNA expression was upregulated in both human excisional (Engelhardt *et al.*, 1998) and burn wounds, at time points that correlated with keratinocyte migration (Werner and Grose, 2003). However, in human burn patients, the exogeneous application of GRO-α impaired wound contraction, so further study is needed to clarify the positive and negative effects of GRO-α in wound healing. The

receptor for both GRO-α and IL-8 is named CXCR2. This receptor is expressed on keratinocytes, and in gene deletion studies, mice without this receptor showed delayed reepithelialization and neovascularization in response to full thickness excisional wounds (Devalaraja *et al.*, 2000). It appears that both GRO-α and CXCR2 have roles in the inflammatory response, angiogenesis and reepithelialization during the process of wound healing.

IL-8 has no known murine homolog as of yet, but it has been studied *in vitro* and *in vivo* with human keratinocytes and patient samples, respectively. *In vitro* studies showed that IL-8 stimulated both keratinocyte migration and proliferation (Raja *et al.*, 2007). In human wound samples, IL-8 mRNA levels were increased by day one post wounding as visualized by *in situ* hybridization (Gillitzer and Goebeler, 2001). However, high levels of IL-8 were associated with non healing burn wounds, and the same levels inhibited keratinocyte proliferation in culture, (Raja *et al.*, 2007). It appears that strict regulation of this chemokine is necessary during reepithelialization.

There is only one chemokine known to be produced by keratinocytes, the CC chemokine monocyte chemattractant protein (MCP-1), also known as CCL2. Keratinocytes are the major source of MCP-1 in wounded epidermis (Engelhardt *et al.*, 1998). Upregulation of MCP-1 occured in a timeframe that corresponded with macrophage infiltration (Gillitzer and Goebeler, 2001), suggesting a keratinocyte role in macrophage recruitment during wound healing. Treatment of mouse wounds with this chemokine increased the number of macrophages at the wound site, and this number can be reduced by neutralizing antibodies against

MCP-1 (Werner and Grose, 2003). However, transgenic mice lacking MCP-1 had normal numbers of macrophages in the wound area, suggesting that other chemokines might be released to compensate for the lack of MCP-1. Supporting this view, investigators found upregulation of several chemokines and chemokine receptors in wounds when comparing MCP-1 knockouts to wild type mice (Ferreira *et al.*, 2005). Reepithelialization in these mice was delayed, suggesting that MCP-1 plays a role in both macrophage recruitment and reepithelialization.

Overall, there are a number of modulators (steroids, cytokines, chemokines) that play a variety of roles in wound healing, including macrophage recruitment, angiogenesis, reepithelialization, and scar formation. In each category, both positive and negative regulators exist. A clearer understanding of how these and other factors work together in wound healing could provide helpful strategies in both normal wound healing and in chronic wounds.

1.4 Peptide Growth Factors

Peptide growth factors are another large family of molecules that are strongly upregulated in response to wounding. Peptide growth factors involved in wound healing can be divided into tyrosine kinase receptor ligands, and non-tyrosine kinase receptor ligands. This section is limited to those growth factors that are produced by keratinocytes or those that are produced by other cells but directly impact reepithelialization. This includes both non receptor tyrosine kinase ligands, and receptor tyrosine kinase ligands.

1.4.1 TGF-ß Family

The transforming growth factor-beta (TGF-ß) superfamily of ligands represents the best studied and largest family of non tyrosine kinase receptor ligands. TGF-ß family members include TGF-ß, activins, bone morphogenetic proteins (BMPs), Mullerian inhibiting substance, nodals, and inhibins. TGF-ß, activins and BMPs are the classes that have been studied in wound healing, and will be the focus in the following paragraphs.

TGF-ß exists as three isoforms (TGF-ß1, -ß2. -ß3) that are ligands for the TGF-ß receptor complex. The receptor complex consists of two serine/threonine receptors, designated type I and type II, which form a heterodimer, that once bound to ligand, stimulate signaling cascades such as the Smad pathway (Werner and Grose, 2003). TGF-ß3 serves to downregulate the other two isoforms, which are implicated in the formation of scars (Martin, 1997). In addition, TGF-ßs also bind type III receptor, which is non-signaling and functions to present TGF-ß to the type II receptor.

TGF-ß is released from platelets in the wound, and stimulates fibroblast proliferation and differentiation into myofibroblasts (Werner and Grose, 2003). During wounding, TGF-ß causes keratinocytes to switch their integrin profile to allow for migration to occur (Freedberg *et al.*, 2001). Keratinocytes upregulated the TGF-ß receptor complex in response to wounding (Zambruno *et al.*, 1995). The most profound effect of the TGF-ß family on wound healing is their impact on the formation of scar tissue. Wounds in embryonic tissue heal without scarring, and TGF-ß is absent in these wounds (Redd *et al.*, 2004). Addition of

neutralizing antibodies against TGF-ß1 and -ß2 or the addition of exogenous TGF-ß3 at the time of wounding reduced the amount of scarring in a rat wounding model (Shah *et al.*, 1994, 1995). Experiments comparing TGF-ß (1-3) in normal dermal wounds versus oral mucosal wounds, which presented with quicker healing and less scar tissue, revealed a difference in the expression profile of these three isoforms (Schrementi *et al.*, 2008). Oral mucosal wounds had higher levels of TGF-ß3, which functionally blocks the other two isoforms. No differences were seen with TGF-ß2, but TGF-ß1 levels were higher in dermal wounds than in the oral mucosal wounds, again consistent with the idea that increased levels of TGF-ß1 correlates with increased scarring.

Studies to separate the effects of the inflammatory response and TGF-ß are difficult due to the fact that creating TGF-ß null mice causes the mice to die anywhere from 10 days to 3 weeks of age (depending on genetic background) due to a severe wasting syndrome (Grose and Werner, 2004) (Brown *et al.*, 1995). These mice have an extreme inflammatory response that results in tissue necrosis and multiorgan failure shortly after birth. Researchers used the immunosuppressive drug rapamycin to prolong the life of these transgenic mice to 60 days, and another group used an immunodeficient, Scid -/- mouse in order to tackle this problem (Grose and Werner, 2004). Wounding in these two different models led to different results. The rapamycin treated mice had enhanced healing with scabless wounds and increased reepithelialization. The TGFß1 -/- Scid -/- mice, however, had delayed reepithelialization as compared to the TGF ß1 +/+ Scid -/- controls. One possibility for these observed differences

is a positive affect rapamycin could be playing during wound healing on non-immune cells.

Overexpression of TGF-ß1 in mice gave mixed results depending on the method employed. Researchers overexpressed a constitutively active form of TGF-ß1, but found that even though the circulating plasma levels of TGF-ß1 were increased in these mice, there was no increase of TGF-ß1 at the site of wounding (Grose and Werner, 2004). Shah and coworkers overexpressed a latent form of TGF-ß1 under a keratin promoter so that it would be expressed in the skin. Wounding of these mice resulted in higher levels of TGF-ß1 at the wound site, decreased keratinocyte proliferation and delayed reepithelialization as compared to controls (Shah *et al.*, 1999). Recently, overexpressesion of TGF-ß1 under the keratin 5 promoter showed delayed wound healing and increased inflammation during all stages of wound healing (Wang *et al.*, 2006).

Clearly, TGF-ß isoforms have several overlapping and opposing roles during the many processes of wound healing. TGF-ß1 and -ß2 are important in activating the immune response, but extended activity seems to lead to scar formation. TGF-ß3 serves as a checkpoint for this system, by antagonizing the effects of isoforms ß1 and ß2, yet in adult tissue, scarring still occurs. Further research is needed to clarify the roles of TGF-ß in wound healing and maximize the positive effects (keratinocyte migration) while minimizing the negative effects (scarring).

Activins are members of the TGF-ß family, and exist as dimeric proteins, with the monomers linked together by disulfide bonds. There are 3 isoforms of

activin, named activin A with the monomers ßA ßA , activin B, that contains the monomers ßB and ßB, and activin AB, containing the monomers ßAßB (Wankell *et al.*, 2003). The receptors for activins can be separated into type I (ActRI) and type II (ActRII), and there are A and B isoforms of each type. Upon wounding, mRNA of both activin B subunits ßA and to a lesser extent ßB are upregulated in keratinocytes at the migrating wound edge without an increase in their corresponding receptor (Hubner *et al.*, 1996b). Overexpression of the ßA subunit resulted in increased keratinocyte proliferation and enhanced wound healing, particularly in the process of granulation tissue formation (Beer *et al.*, 2000b). These same researchers identified the transcription factor MAD1, a known antagonist of c-Myc, as a novel downstream target of both activin and TGF-ß1 in both keratinocytes and leukocytes post wounding (Werner *et al.*, 2001). Activin knockout mice died shortly after birth, but investigators have found the protein follistatin to be a natural antagonist to activin. Overexpressing the activin antagonist in the skin led to a large reduction in the amount of granulation tissue. Furthermore, the hyperproliferative wound epithelium was thinner, although reepithelialization was not delayed (Wankell *et al.*, 2001). Gene deletion of follistatin in keratinocytes resulted in increased keratinocyte proliferation during the early stages of wound healing, but the overall timeframe of reepithelialization was not affected (Antsiferova *et al.*, 2008). In summary, activins are relatively newly discovered regulators of wound healing, and results so far suggest their contributions to wound healing lie in the formation of granulation tissue, and not in reepithelialization.

Bone morphogenetic proteins (BMPs) are the third class in the TGFß superfamily known to affect wound healing. Although several BMPs (-2, -4, -6, -7) are expressed in the skin (Wankell *et al.*, 2001), only BMP-2 and BMP-6 have shown differences in wound healing experiments (Werner and Grose, 2003). Fetal wound repair is scarless when it occurs in the first two trimesters, and since TGF-ß is known to induce scarring when added exogenously to a fetal wound (Piscatelli *et al.*, 1994), investigators wanted to see if the TGF-ß family member BMP-2 had any deleterious effects on fetal wound repair. Normal uninjured fetal epidermis expressed very low levels of BMP-2, and addition of exogenous BMP-2 to injured fetal tissue resulted in scar formation (Stelnicki *et al.*, 1998). One explanation for these differences between adult and fetal wound healing is the wound area. Fetal wounds tend to have a small surface area, and closure occurs predominantly by contraction of the actin cable surrounding the wound edge in a purse-string mechanism (Martin, 1997). Adult wounds are larger and require epidermal cell migration into the wound area. This process requires signaling events stimulated by numerous growth factors. One consequence of this increased signaling is scarring, which is hypothesized to occur due to an immune response stimulated by these wound-associated factors. In contrast, fetal tissue does not scar due to the lack of a mature immune system (Gurtner *et al.*, 2008).

BMP-6 is a ligand that is upregulated in keratinocytes proximal to the wound edge 3 days after wounding (Kaiser *et al.*, 1998), and to investigate its role, Kaiser et al. overexpressed BMP-6 in the epidermis, then performed wound

healing experiments. BMP-6 overexpressing mice showed delayed wound healing, in particular a delay in reepithelialization (Kaiser *et al.*, 1998). Further research is needed to clarify the role of this ligand in wound healing, since it is upregulated in proximal keratinocytes during wound healing but overexpression delays wound healing.

The TGF-ß superfamily has several members that participate in wound healing, including TGF- ßs, activins and BMPs. Persistent themes across these subfamilies include the modulation of the immune response, contribution to the scarring response and excess of these ligands inhibit reepithelialization. It appears that successful wound healing requires a fine tuning of these ligands, and future research into these ligands appears to be logical route to accomplish scarless adult wound healing.

1.4.2 Receptor Tyrosine Kinases

A number of peptide growth factors elicit their effects through receptor tyrosine kinases (RTKs). There are several classes of RTK's, and only those involved in wound healing will be reviewed (See figure 1.4.1). Growth factors are separated into those produced by keratinocytes, and those produced in other cell types but directly affecting keratinocytes and reepithelialization. The epidermal growth factor (EGF) and its receptor is a growth factor family that is well studied in wound healing and will be discussed in section 1.5.

There are several growth factors produced by keratinocytes that work in a paracrine fashion to affect neighboring cell types to enhance wound healing.

Platelet derived growth factor (PDGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and nerve growth factor (NGF), were first discovered in other cell types, but are also produced by keratinocytes in response to wounding (Matsuda *et al.*, 1998; Cross and Mustoe, 2003; Werner *et al.*, 2007). These growth factors are secreted into the wound area and can stimulate fibroblast proliferation and migration, the production of granulation tissue, and angiogenesis, and keratinocyte proliferation.

Platelet derived growth factor (PDGF), like TGF-ß, is released by platelets in response to wounding minutes after injury. There are five isoforms of the PDGF family named PDGF-AA, -AB, -BB, -CC, and -DD, and three PDGF receptors. PDGF stimulated fibroblasts to differentiate into myofibroblasts and facilitated the dermal contraction of the wound (Werner *et al.*, 2007). It was one of the first growth factors discovered to be important in wound healing, and it was found in wound fluid from pediatric burn patients (McCarthy *et al.*, 1996). Both mRNA levels and protein levels of PDGF ligands and their corresponding receptors were upregulated in porcine (Antoniades *et al.*, 1991), and human (Ansel *et al.*, 1993; Reuterdahl *et al.*, 1993) keratinocytes during wound healing. In a diabetic mouse model mice exhibited chronic, nonhealing wounds. Upon examination, these mice had lower levels of both PDGF-A and its receptor than normal mice (Beer *et al.*, 1997). Upon wounding, expression of PDGF-A decreased even further, suggesting that a lack of this growth factor could contribute to the failure of these wounds to heal. PDGF-B mRNA and protein is upregulated in human keratinocytes after trichloroacetic acid treatment (Yonei *et*

al., 2007). PDGF-B is the only growth factor approved as a drug for chronic wound treatment, and a clinical trial using an adenovirus-PDGF-B vector has shown success in patients with diabetic ulcers (Barrientos *et al.*, 2008).

PDGF-B and B receptor null mice are both embryonically lethal, so in order to study the effects of this deletion in adult mice, investigators irradiated the hematopoietic system of adult mice and replaced these cells with those from PDGF-B null mice (Buetow *et al.*, 2001). No changes in granulation tissue formation occurred in these mice, and vascularization of the wounded area was increased in this system. The lack of PDGF-B in the wound area delivered by endothelial cells could be compensated by production of PDGF-B by macrophages and keratinocytes. However, overexpression of PDGF was evident in hypertrophic scars and keloid tissue (Werner and Grose, 2003),and PDGF-A worked against the positive effects of EGF when both were applied exogenously during wound healing (Gope, 2002). indicating the need for regulation of this growth factor for normal wound repair.

Hepatocyte growth factor (HGF), also known as scatter factor (SF) or plasminogen-related growth factor (PRGF), has only one other known family member, macrophage stimulating protein (MSP), also known as hepatocyte growth factor like protein (HGFL), SF2, or PRGF2. HGF was first discovered in hepatocytes, although it was later found to stimulate keratinocyte migration and proliferation upon binding to its tyrosine kinase receptor cMET (Conway *et al.*, 2007). It is due to this discovery that a role in wound healing was postulated. In a rat wound model, upregulation of HGF and its receptor cMET was observed in

both keratinocytes and in the granulation tissue of healing wounds (Werner and Grose, 2003). HGF appears to play an key role in granulation tissue formation, as overexpression of HGF in transgenic mice revealed enhanced granulation tissue formation after wounding, but did not enhance reepithelialization (Werner and Grose, 2003). In patient samples, expression of HGF, cMET, and their regulators are detected in both acute and chronic wounds, however, differences in the expression levels and areas of expression differed between the two wound types (Conway *et al.*, 2007). It can be concluded that HGF is a vital growth factor in wound healing, but its effects are mostly on granulation tissue formation rather than reepithelialization.

The vascular endothelial growth factor (VEGF) family of ligands is comprised of 6 known members to date, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placenta growth factor (PLGF). They exert their effects on three different receptors, VEGFR-1, VEGFR-2, and VEGFR-3. VEGF-A and PLGF are both produced by keratinocytes (Werner and Grose, 2003), and the VEGFR-1 receptor was upregulated in both murine and human keratinocytes following wounding (Wilgus *et al.*, 2005). VEGF-A neutralizing Ab caused delayed angiogenesis in an abdominal hernia wounding model as observed by decreased wound vessel count and vascular surface area (Howdieshell *et al.*, 2001). Keratinocytes produced PLGF mRNA in response to wounding from day 3 to day 5 post wounding (Failla *et al.*, 2000). PLGF knockout mice displayed poor wound healing due to a delay in angiogenesis (Carmeliet *et al.*, 2001). Although most studies show that VEGF family members primarily effect angiognesis, studies

using a neutralizing antibody against VEGFR-1 showed delayed reepithelialization, possibly by inhibiting keratinocyte proliferation, as the number of proliferating keratinocytes were reduced in the presence of the anti-VEGFR-1 antibody (Wilgus *et al.*, 2005).

Nerve growth factor (NGF) was first thought to participate in wound healing when it was found in high levels in the saliva, and that the licking of wounds enhanced wound contraction (Werner and Grose, 2003). NGF levels in wounded mice occured in both the serum and at the wound site, with maximal serum levels occurring 6 hours post wounding and maximal skin levels occurring 24 hours post wounding (Matsuda *et al.*, 1998). Serum levels were deterimined to originate from salivary gland production, while skin levels of NGF were determined to come from both fibroblasts and keratinocytes (Matsuda *et al.*, 1998). Basal, proliferating keratinocytes were positive for mRNA expression 3 days post wounding, while NGF protein was detected in the superficial, differentiated keratinocytes at this same time point. These investigators then treated non-healing ulcers of diabetic with exogenous NGF or in combination with an anti-NGF Ab. Wound healing was accelerated with exogenous NGF, but these effects were completely abolished with pretreatment with the anti-NGF antibody (Matsuda *et al.*, 1998). Clinically, exogenous application of NGF to non-healing diabetic ulcers have shown slow (5-14 weeks), but positive effects in 15 patients with leg or foot ulcers (Aloe *et al.*, 2008). This represents an interesting new treatment whose mechanism is yet to be revealed.

Receptor tyrosine kinase ligands produced by other cell types but affecting reepithelialization include insulin-like growth factor (IGF) and fibroblast growth factor (FGF). Produced by bone marrow stem cells (IGF) and fibroblasts (FGF), these paracrine growth factors activate their corresponding receptors found on keratinocytes to enhance reepithelialization. Studies using gene deletion in mice as well as looking at clinical patient samples indicate the importance of these two growth factors in wound healing.

The insulin-like growth factor family is a group of ligands released during wound healing. There are two known insulin-like growth factors (IGFs), IGF-I and IGF-II. Both IGFs bind to the type I IGF receptor, while IGF-II can also bind to the IGF type II/mannose-6 phosphate receptor. IGF-I was present in wound fluid in both a porcine and rat model (Steenfos and Jansson, 1992; Marikovsky *et al.*, 1996), and both mRNA levels (Steenfos and Jansson, 1992) and protein levels (Todorovic *et al.*, 2008) of IGF-I were elevated in wounded skin of rats. Both IGF-I and IGF-II mRNA expression occured in porcine wounded and unwounded epithelium (Antoniades *et al.*, 1993). In chronic non-healing wound models using the glucocorticoid stimulated diabetes model, mRNA and protein levels of both IGF-I and IGF-II were decreased, indicating the importance of these molecules to diabetic wound healing (Brown *et al.*, 1997). Clinically, in diabetic patient samples, IGF-I levels were decreased as compared to healthy skin (Blakytny *et al.*, 2000). Using a transgeneic mouse with the IGF-1 gene under the keratin 14 promoter to overexpress IGF-1 in the skin revealed that IGF-1 accelerated reepithelialization (Semenova *et al.*, 2008). Recently, investigators

discovered that bone marrow stem cells enhanced wound closure, produced 49 fold higher levels of IGF-1mRNA, and released 22 fold higher levels of IGF-1 protein into the conditioned medium than dermal fibroblasts (Chen *et al.*, 2008). Although the exact mechanisim of wound closure is as of yet unknown, IGF from bone marrow stem cells represents yet another growth factor able to enhance wound healing and stresses the importance of IGF-1 in normal wound healing, especially in chronic wounds.

Fibroblast growth factors (FGF) stimulate pathways in the developing embryo as well as adult tissues (Grose and Werner, 2004). The FGF family is very complex, with 4 FGF receptors (FGFR1-4), 3 of which have splice variants, and over 22 FGFs (Werner and Grose, 2003). In wound healing of adult tissues, the major role of FGF is to mediate angiogenesis (Grose and Werner, 2004) and were found to be upregulated at day 1 post wounding (Bhora *et al.*, 1995). One FGF family member, FGF-2, is important in reepithelialization, as FGF-2 null mice showed delayed wound healing (Grose and Werner, 2004). This finding was limited to FGF-2, because FGF-1 null mice exhibited normal wound healing, and FGF-1/FGF-2 double mutants had similar wound defects as FGF-2 null mice. In excisional wound models, mRNA levels of several FGFs were upregulated, including FGF-1, FGF-2, FGF-5 and FGF-7 (Werner and Grose, 2003). Protein levels of FGF-2 have also been reported to be upregulated in murine, porcine and human wound models (Werner and Grose, 2003).

One FGF member, keratinocyte growth factor (KGF), also known as FGF-7, has been well studied in wound healing. KGF works in a paracrine fashion,

released by fibroblasts and binding to its receptor, FGF2IIb, on neighboring keratinocytes in response to wounding (Werner *et al.*, 2007). In mouse studies, KGF upregulation at both the protein and mRNA level occurred at the wound margin as early as 1 day post wounding (Grose and Werner, 2004). FGF2IIb receptor null mice showed a marked delay in wound healing, however, in KGF null mice, wound healing was normal (Guo *et al.*, 1996). One explanation for this could be that additional FGF ligands bound the FGF2IIb receptor, namely FGF-10 (KGF-2), and the newly discovered FGF-22. FGF-10 expression remains controversial in the literature, as one group found increased mRNA levels whereas another found no change in response to wounding in mice (Werner *et al.*, 2007). FGF-22 binds to the FGF2IIb receptor, although FGF-22 is expressed mainly by keratinocytes, which is an example of autocrine activation as opposed to the paracrine response elicited by FGF-7 (Werner *et al.*, 2007). Clearly, the FGF family of ligands and receptors has many roles in wound healing that warrant further investigation.

There are several growth factors produced and released in response to a wound. HGF, VEGF, NGF, and PDGF are produced by keratinocytes, and act to promote granulation tissue formation, angiogenesis, keratinocyte proliferation and dermal contraction, respectively. FGFs and IGFs are produced by fibroblasts and bone marrow cells and enhance angiogenesis and enhance wound closure. Collectively, ligands for receptor tyrosine kinases regulate nearly all aspects of wound repair.

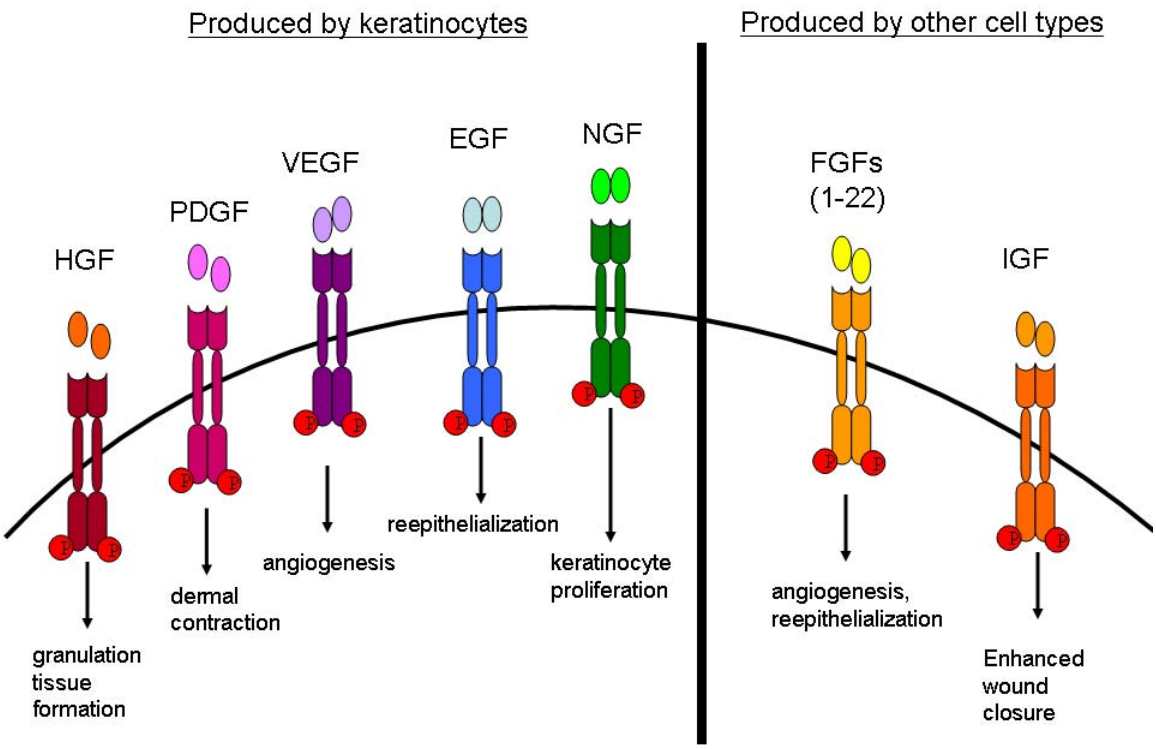


Figure 1.4.1 Receptor Tyrosine Kinase Families

Several receptor tyrosine kinases play roles in wound healing. Ligands are produced by keratinocytes as well as other cell types. Ligands work in both an autocrine and paracrine fashion to execute beneficial effects during several major events of wound healing, including granulation tissue formation, angiogenesis, and reepithelialization.

1.5 The EGF Receptor and Ligands in Wound Repair

Another large family of ligands and receptors are those of the epidermal growth factor (EGF) family (Figure 1.5.1). The EGF receptor is a receptor tyrosine kinase that plays pivotal roles in autocrine regulation of keratinocyte proliferation and migration. Several studies have focused on the effects of the EGF receptor family and ligands on healing.

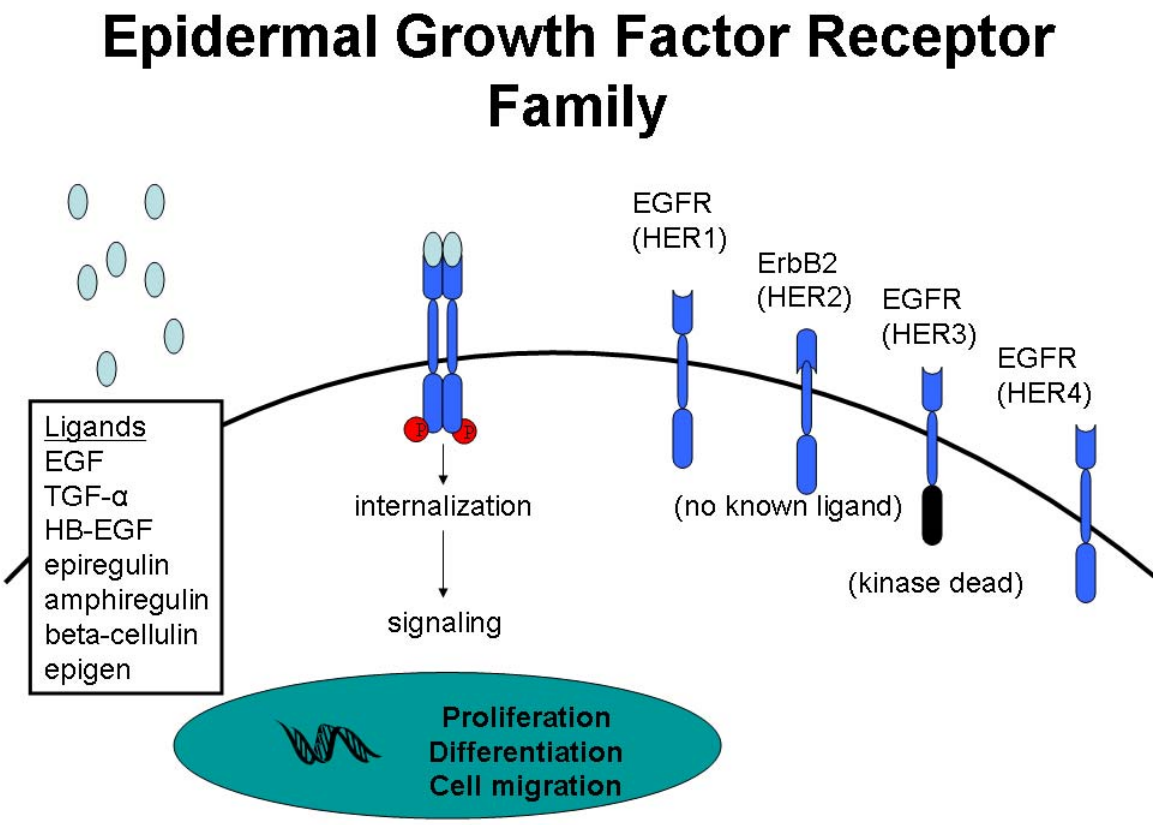


Figure 1.5.1 EGFR and Associated Ligands in Wound Repair

There are 4 EGFR family members EGFR (HER1),ErbB2 (HER2), ErbB3 (HER3), ErbB4 (HER4). EGFR is the first member of the family discovered. ErbB2 cannot bind ligand, but can signal once it forms a dimer with another family partner. ErbB3 cannot signal, but can bind to a ligand and partner with another family member to transduce signal. ErbB4 is not usually found in keratinocytes. Ligands include EGF, TGF-α, HB-EGF, epiregulin, amphiregulin, beta-cellulin, and epigen.

1.5.1 EGF receptor ligands

There are seven known ligands for the EGF receptor (EGFR), EGF, transforming growth factor alpha (TGF-α), heparin bound EGF (HB-EGF), epiregulin, amphiregulin, beta-cellulin and epigen (Schneider *et al.*, 2008) (Figure 1.5.1). Of these EGFR ligands, several have been studied in wound healing, and will be discussed further, namely, epiregulin, TGF-α, HB-EGF, and EGF. It is important to note the other EGFR ligands (beta-cellulin, amphiregulin, and epigen) are mitogenic in different systems, and could possibly play a role in wound healing (Schneider *et al.*, 2008). The EGFR family of ligands can act on several EGF receptors, named EGFR (also known as ErbB1 or HER1), erbB2 (HER2), erbB3 (HER3) and erbB4 (HER4) (Figure 1.5.1). Upon ligand binding, the receptors homodimerize or heterodimerize and then are internalized. ErbB2 has no known ligand, but can participate in signaling via heterodimerizing with other erbB receptors. ErbB3 cannot phosphorylate, due to an inactive kinase domain, but like erbB2, can participate in signaling by forming heterodimers with one of the other EGFR family members. ErbB4 is either present in very low levels or is undetectable in both human and murine epidermis (Plowman *et al.*, 1993; Xian *et al.*, 1997; Kiguchi *et al.*, 2000).

1.5.1.a Animal Studies with EGF

Epidermal growth factor (EGF) is the first discovered and most extensively studied ligand of the EGFR. It was reported in 1962 by Dr. Stanley Cohen that a

factor present in the submaxillary gland of mice, once purified caused early eyelid opening and tooth eruption when injected daily into newborn mice (Cohen, 1962). During the first stage of wound healing, EGF is released by platelets into the wound area, and can also be produced by monocytes, macrophages and keratinocytes (Clark, 1988). EGF as well as other EGFR ligands such as HB-EGF and TGF-α were present in high concentrations in the wound fluid of rats, humans and pigs (Grotendorst *et al.*, 1989; Ono *et al.*, 1995; Marikovsky *et al.*, 1996).

Several studies have looked at the effects of exogenous EGF application in wound healing. Even though the methods of EGF delivery differ, wound healing is improved with exogenous applications of EGF with an increased rate in some aspect of wound repair reported in most cases.

One of the earliest studies of wound repair with EGF used circular punch biopsies on rabbit ears (Franklin and Lynch, 1979). It was a qualitative report of quicker wound healing with EGF treated as compared to control. Histologically, EGF treated epithelium was thicker and had more mature connective tissue than control wounds. Another parameter of wound repair is tensile strength of the epidermis once it has undergone reepithelialization. Tensile strength was 3 times greater with liposome encapsulated EGF 14 days post treatment as compared to saline or liposome alone treated wounds (Brown *et al.*, 1988). Using a gelatin film to protect EGF from proteolytic degradation in partial thickness canine wounds, reepithelialization of the wound area was greater than 50% by 9 days, which was statistically greater than control (Tanaka *et al.*, 2005).

Gelatin sponges with EGF loaded microspheres also showed a marked increase in reepithelialization by 7 days post wounding via punch biopsy in the dorsal area of rabbit skin (Ulubayram *et al.*, 2001).

The above mentioned studies used mice, rats, rabbits and dogs, which have loose skin that contains a panniculus carnosus, a thin sheet of striated muscle. Wound healing in these animals occured mostly by wound contraction of the dermis rather than reepithelialization (Arnoux, 2005). Porcine skin, like human skin, is an example of tight skin that lacks a panniculus carnosus. Wound healing of tight skin relies heavily on reepithelialization for wound closure.

Two studies found that 10 µg/mL EGF significantly improved wound closure as compared to control (Nanney, 1990) or as compared to vehicle alone (Brown *et al.*, 1986) in partial thickness porcine wounds. However, a single treatment of EGF at 10 µg/mL was not effective, indicating the necessity of daily treatment in these studies. In addition to an increased rate of closure, keratinocyte differentiation was also improved with the appearance of a cornified layer only 5 days post injury (Nanney, 1990). Overexpression of EGF in porcine keratinocytes accelerated reepithelialization of full thickness skin wounds as compared to control skin (Vranckx *et al.*, 2007). In summary, several animal models have been used to evaluate the effects of elevated EGF via different delivery methods, all with positive results suggesting clinical benefit for human wound healing.

1.5.1.b Human Studies with EGF

In general, human studies are in agreement with animal models. One indication that EGF rather than other growth factors may provide benefit was provided in a study to measure keratinocyte outgrowth from human skin punch biopsies. The greatest amount of keratinocyte proliferation and epidermal outgrowth occurred following treatment with EGF when compared to IGF or FGF (Bhora *et al.*, 1995).

Clinically, wound healing is improved in patients treated topically with EGF. Silver sulfadiazine cream alone is known to accelerate wound healing when applied daily, but the addition of EGF accelerated the healing to a greater extent when compared to matched skin graft donor sites in a 12 patient double blind study (Brown *et al.*, 1989). However, a study of 44 patients with chronic venous ulcers failed to show a statistically significant improvement with EGF treatment over placebo in a double blind clinical trial (Falanga *et al.*, 1992). These data taken together suggest that there may be differences between wound healing in acute and chronic disease states. Chronic wounds represent a greater health threat to patients, and several additional studies have taken this into account and studied the impact of EGF on treatment of diabetic foot ulcers.

One of the first clinical studies of EGF treatment for chronic wounds occurred in a 2003 study of 61 patients with diabetic foot ulcers. Ulcer grade was determined and patients with grade I or grade II were accepted into the study (Tsang *et al.*, 2003). Patients received either Actovegin alone, a cream made with protein free calf blood extract purported to promote uptake of nutrients into

the wound, or Actovegin in combination with either 0.02% or 0.04% EGF (Tsang *et al.*, 2003). After 12 weeks, the 0.04% EGF group had a rate of healing greater than placebo alone, and this finding was statistically significant. Out of the 21 patients in the 0.04% group, 20 patients achieved full healing after 12 weeks. The 0.02% EGF group did not achieve healing greater than with placebo alone. A recent study used an advanced moist dressing alone or in combination with EGF on a total of 89 patients with diabetic foot ulcers and 76% of wounds healed with the advanced dressing (Hong *et al.*, 2006). While the Tsang study limited the patients to a severity of grade II, Hong *et al.* allowed both grade II and grade III patients in this trial. Of the combined grade II and grade III cases, 24% improved with advanced dressing alone; while 58% of the total cases improved with the advanced dressing containing 0.001% EGF (Hong *et al.*, 2006). A Phase III study was conducted in India with the use of REGEN-D (150 µg/g recombinant EGF), and the results were compared to a post-marketing follow up study with the same medication 2 years later (Mohan, 2007). A total of 135 patients with grade I and II lesions were assessed. The post marketing study found that 92% of patients achieved full wound closure by week 10, as compared to 69% at the same timepoint in the Phase III study, which had a smaller sample size of 60 subjects. The investigators stressed that the results demonstrated the efficacy of the product. Intralesional injections of Citoprot-P (recombinant EGF; available as either 25 µg/mL or 5 µg/mL) were given as 5 mL injections to patients with either grade III or grade IV ulcers, and at serious risk for foot amputation. After 5 weeks, 74% of the 25 µg/mL treatment group achieved a

complete response, which was defined as coverage of the ulcer surface with granulation tissue and 50% of the 5 µg/mL treatment group achieved a complete response. These response numbers increased to 83% and 61%, respectively after 8 weeks.

Collectively, there is good experimental and clinical data for the use of EGF in both acute and chronic wounds. Studies that started in the early 1990's in animal models have led to successful clinical trials in less than 20 years. As the delivery systems become more sophisticated, advances in wound care will continue to provide better treatment for future patients.

1.5.2 Other EGFR ligands in wound repair

EGF is not the only ligand for EGFR, and it stands to reason that the other ligands can also contribute to wound repair. Since the discovery of EGF and its benefits in wound repair, other EGFR ligands have been investigated for their benefits to wound repair. Examples of ligands that positively affect wound healing include epiregulin, TGF-α, and HB-EGF.

Epiregulin (EPR) is a member of the EGF family of growth factors with 24-50% homology to other EGF ligands (Draper *et al.*, 2003b). Although the EPR protein levels are low in the skin, 2.5 fold upregulation of EPR mRNA occurred after wounding normal mouse keratinocytes, but only 1.5 fold upregulation occurred in human keratinocytes (Shirakata *et al.*, 2005). Topical application of EPR significantly improved healing of murine excisional wounds, as compared to control and EGF treated animals (Draper *et al.*, 2003b). Further studies from this

group extended the findings to show that EPR accelerated wound repair more effectively than either TGF-α or EGF (Draper *et al.*, 2003a). However, EPR null mice showed no defects in wound healing (Schneider *et al.*, 2008), so the functional importance of epiregulin in wound healing is still unclear. It is possible that other EGFR ligands were able to compensate for the lack of epiregulin in the epidermis.

TGF-α is a growth factor that is upregulated in wound tissue soon after injury (Grose and Werner, 2004). TGF-α is expressed in several layers of the epidermis, including the basal, spinous and granular layers (Schneider *et al.*, 2008) and was found in wound fluid of rats (Grotendorst *et al.*, 1989). During wound healing, TGF-α is released from platelets during the clotting phase, and macrophages produce TGF-α during the inflammatory phase (Clark, 1988), suggesting that TGF-α release is important during early stages of wound healing. Exogenous application of TGF-α enhanced reepithelialization of burn wounds in pigs. In this study, a statistically significant increase of reepithelialization occurred with 0.1μg/mL TGF-α as compared to placebo or the same concentration of EGF, although earlier papers determined that the optimal concentration of EGF to be 10μg/mL (Schultz *et al.*, 1987).

Two groups looked at the effect of the loss of TGF-α in wound repair in the early 90's. Luetteke *et. al.* used the waved-1 mice, which have a genetic mutation in the TGF-α gene, and were first described in 1933 by Frances A.E. Crew (Schneider *et al.*, 2008). Mann *et. al*. deleted the TGF-α gene and studied the effects of wound healing in these mice. In both cases, very similar phenotypes

were described, including curly whiskers and hair, as well as corneal defects (Luetteke *et al.*, 1993; Mann *et al.*, 1993). Both sets of investigators saw no change in wound healing with the TGF-α deficient mice as compared to heterozygous or wild type controls. Mann *et al*. looked at homozygous null mice as compared to heterozygous mice, and found no change when observing wound healing of clipped tails. Luetteke *et al.* used two different methods, a full thickness excision in the backskin as well as punch biopsy of the ear and saw no change in healing in the homozygous null mice as compared to wild type controls. Investigators concluded that EGF or some other EGFR ligand could be compensating for the lack of TGF-α in these models.

TGF-α null mice show no difference in wound healing in earlier studies, but a more recent study found a large decrease in reepithelialization of partial thickness ear wounds 3 days post wounding as compared to control mice (Kim *et al.*, 2001). This statistically significant reduction in reepithelialization was extended to day 5 post wounding, yet healing of wounds in both sets of animals was complete by 8 days. Ear wounding solely requires reepithelialization, and no granulation tissue formation. In order to study the effects of granulation tissue, wound healing from a head wound, which requires both reepithelialization and granulation tissue formation was performed. There were no differences in the rate of reepithelialization in the head wounds from TGF-α null mice and the control mice. The authors of this study concluded that the formation of granulation tissue compensated for the lack of TGF-α in the full thickness model in their experiments and the previous reported experiments. TGF-α is also found

at the leading edge of migrating epithelium in human burn patient samples (Wenczak and Nanney, 1993), indicating the importance of this ligand in human reepithelialization.

Heparin bound EGF (HB-EGF) is another EGFR ligand that is present in wound fluid (Marikovsky *et al.*, 1996; McCarthy *et al.*, 1996) and protein expression was found in keratinocytes at the wound margin of burn patients (McCarthy *et al.*, 1996). In normal skin, HB-EGF is found in hair follicle epithelial cells and in proliferating keratinocytes. Both mRNA and protein levels of HB-EGF increased at the leading edge of *in vitro* and *in vivo* wounds (Shirakata *et al.*, 2005; Mathay *et al.*, 2008). The use of CRM197, an HB-EGF inhibitor, inhibited *in vitro* wound healing in a human corneal epithelial cell line (Boucher *et al.*, 2007). HB-EGF null mice died postnatally due to heart defects (Schneider *et al.*, 2008). However, a conditional mutant mouse strain was generated that limited the gene deletion to keratinocytes, and in these mice, reepithelialization, and specifically keratinocyte migration independent of proliferation, was impaired (Shirakata *et al.*, 2005).

Looking at the similarities in EGFR ligands with respect to their enhancement of wound healing suggests a functional redundancy, however, differences in function could be due to timing of expression during wound healing. While TGF-α expression occurs at the onset of wounding, EPR and HB-EGF occured at later timepoints. Differences in wound healing models make it difficult to draw conclusions between studies on the exact roles of each EGFR

ligand, but it is clear that collectively EGFR ligands contribute to the reepithelialization phase of wound repair.

1.5.3 EGFR in wound repair

Numerous EGFR ligands are present in wound fluid, suggesting that EGFR plays an important part in wound healing. Transactivation of EGFR is an alternate method of stimulating a response without ligand, and examples of this can be found in wound healing. Several factors have been shown to transactivate the EGFR, and these include pathways stimulated by G protein coupled receptors (GPCRs) (Filardo, 2002; Yahata *et al.*, 2006), cytokines (Tanida *et al.*, 2004; Itoh *et al.*, 2005), antimicrobial peptides (Tokumaru *et al.*, 2005; Carretero *et al.*, 2008), crosstalk by other tyrosine kinases (Higashiyama and Nanba, 2005; Xu *et al.*, 2006; Xu and Yu, 2007; Nanba *et al.*, 2008), nucleotides (Boucher *et al.*, 2007) and extracellular matrix components (Cabodi *et al.*, 2004; Tran *et al.*, 2004). These examples represent the large variety of non ligands that researchers have found to cause transactivation of the EGFR. Many of these factors transactivate the EGFR by inducing shedding of HB-EGF, which in turn activates the EGFR. Non-ligand transactivation of the EGFR pathway can occur by other factors present during wound healing, including angiotensin II, catecholamines, and inflammatory markers TNF-α and IFN-γ (Pastore *et al.*, 2008). These studies highlight the importance of the wound milieu and the ability of non EGF ligands to cause stimulation of the EGFR pathway.

There are several mechanisms for activation of the EGFR, including ligand and non-ligand activation that lead to enhanced wound repair. There is also evidence that levels of EGFR have an impact on wound healing. Tape stripping induces a wound that is limited to the epidermis, and using this protocol a maximum of 5 fold increase in the levels of the EGFR receptor was seen from 1 to 2 days post wounding at the margins of healing wounds that returned to baseline levels after day 4 post wounding (Stoscheck *et al.*, 1992). This same group of investigators extended their focus to EGFR in *in vivo* wounds. In patients with either partial thickness or full thickness burns, upregulation of EGFR was seen in the advancing epithelium adjacent to the burn (Wenczak *et al.*, 1992). This upregulation was limited to the keratinocytes, as the underlying dermis showed no EGFR staining.

Further evidence for the importance of the EGFR in wound healing is revealed by EGFR null mice. Although EGFR null mice do not live more than a few days after birth (Threadgill *et al.*, 1995), skin from these newborns can be grafted onto adult mice to study the impact of EGFR on wound healing. Wound healing was defective in EGFR null skin (Repertinger *et al.*, 2004). Whereas wound healing was complete within 5 days in wild type skin grafts, repair of EGFR null skin took up to 14 days to complete (Repertinger *et al.*, 2004). Conversely, overexpression of the EGFR by gene gun transfection increased wound healing in a porcine skin model (Nanney *et al.*, 2000). In our laboratory, we have shown keratinocyte cell motility increased with increased EGFR activation (McCawley *et al.*, 1997).

Most studies have focused on the contributions of the EGFR on wound repair. Recently, the ErbB3 receptor was implicated in wound healing. In a porcine wound model, partial thickness wounds were created, and adenovirus with ErbB3 gene was deposited in the wound bed (Okwueze *et al.*, 2007). Various EGFR ligands (EGF, HB-EGF, epiregulin, or heregulin) were then added in a gel matrix once daily. Significant increases in reepithelialization occurred with all ligands in wounds with elevated ErbB3 as compared to the LacZ adenovirus control. However, ligand alone also caused an increase in reepithelialization, and the only combination of ErbB3 and ligand to cause a statistical increase over ligand alone was HB-EGF and ErbB3, indicating involvement of the EGFR in a EGFR:ErbB3 heterodimer because HB-EGF does not bind to ErbB3. The activity of ErbB3 in wound healing represents an exciting new area of research that has previously gone unstudied.

Regulation of both ligands for the EGFR and the levels of receptor itself appear to be important for reepithelialization. Expression levels must be appropriate, as disruption of receptor levels delays wound repair, as seen in the null receptor studies. It is possible that several different mechanisms for both direct activation and transactivation exist in order to regulate appropriate expression during wound healing. Further studies on the different receptor family members and their coordinate activation during wound healing could represent an interesting avenue for enhancing wound repair.

Little is known about the essential processes regulated by the EGFR that may be critical for wound repair, but in order to migrate during reepithelialization,

keratinocytes must detach from neighboring cells, which requires the cells to lose or modify their adhesive properties and obtain migratory properties. The loss of these adhesive properties includes cell-cell junctions, such as adherens junctions and desmosomes as well as cell-matrix interactions. The cell must also loosen its adhesion to the extracellular matrix below in order to migrate. These activities are very similar to what happens in epithelial to mesenchymal transition (EMT), which is important in the developing embryo and is major event for metastasis in cancer.

1.6 Epithelial to mesenchymal transition (EMT)

Epithelial to mesenchymal transition (EMT) is a process in which epithelial cells undergo profound changes to resemble a mesenchymal cell type. Classical EMT was first studied in embryonic development, and occurs as epithelial cells take on a migratory fibroblastic like phenotype and are capable of invading extracellular matrix (ECM). Developmental EMT occurs in two phases of development, gastrulation and migration of the neural crest cells from the neural tube (Hay, 2005). Gastrulation is the migration of the primitive endoderm cells (epithelial origin) to invaginate within the egg cylinder and become mesodermal mesenchymal cells (Thiery, 2002). Once this process is complete, organogenesis begins.

In vertebrates, neural crest cells are those epithelial cells that are at the border of the neural tube and the epithelial ectoderm (Newgreen, 2005). These cells migrate from the neural crest and are the precursors to an entire subset of cell lineages that exist throughout the body, from connective tissue to skin

pigment cells (Newgreen, 2005). Developmental EMT is characterized by changes in gene expression that serve as a molecular definition of the process.

1.6.1 Classical Hallmarks of EMT

A switch from keratin to vimentin based intermediate filaments and the loss of cell-cell junctions are two markers that are traditionally used to define an EMT event (Thiery, 2003). The application of these markers to define EMT in adult tissue, such as a full EMT in tumor metastasis or a partial EMT in wound repair is still controversial. However, a switch from keratins to vimentin, an appearance of stress fibers, a loss of E-cadherin, and a gain of N-cadherin are a few of these classical hallmarks that are widely accepted to indicate EMT in normal and tumorigenic adult tissues.

When cells take on a mesenchymal phenotype, in general, they assume a biopolar, spindle shaped morphology. The cells extend filopodia from the front, leading end, and are able to invade neighboring extracellular matrix (ECM). One switch that may help these cells become more invasive is the switch from a keratin based cytoskeleton to a vimentin based cytoskeleton. Increased vimentin is common in highly invasive tumor cells (Heatley *et al.*, 1993; Gilles *et al.*, 1996; Zajchowski *et al.*, 2001), and there is experimental evidence that vimentin expression fosters increased migration (Ramaekers *et al.*, 1989; Gilles *et al.*, 1999; McInroy and Maatta, 2007). In contrast, although there is some evidence for increased vimentin at wound margins (Brem *et al.*, 2007), dramatic changes in keratin profiles are evident in reepithelialization.

Upon injury, keratinocytes change their keratin profile again in a process called the keratinocyte activation cycle. During this cycle, IL-1 initiates activation of keratinocytes and expression K6, K16 and K17 (Freedberg *et al.*, 2001). The most well studied activator of keratinocytes is the EGF receptor. EGFR activation upregulated both K6 and K16 expression (Jiang *et al.*, 1993). Deactivation of keratinocytes is achieved by TGF-ß expression from dermal fibroblasts once reepithelialization is complete (Freedberg *et al.*, 2001). This switch of keratin profile after wounding is very similar to the cytoskeletal switch that other cell types undergo during a full EMT.

Another well studied classical marker of EMT is the loss of cell-cell junctions. The mechanisms of cell-cell junction disruption, including the events that occur during EMT, will be discussed in detail in section 1.7.1. There is a striking similarity between mechanisms of migration by cells in development, and wound repair, although reepithelialization appears to represent a partial EMT.

1.7 Cell-cell junctions

Disruption of cell-cell junctions is a hallmark of EMT. Keratinocytes maintain their integrity by forming cell-cell contacts with neighboring cells. There are three types of cell-cell contacts between cells: tight junctions, adherens junctions and desmosomes.

Tight junctions form a seal around cells and prevent the passage of small molecules through the epithelial barrier. This also functions as a way to separate the basal from apical membrane proteins, thus regulating polarity (Tsukita *et al.*, 2008). Tight junction membrane proteins that directly mediate adhesion are

claudin and occludin, which form a network of ridges (Figure 1.7.1, part A). Intracellularly, they bind scaffolding zonula occludens proteins (ZO1-3), which connect to the cytoskeleton via actin.

Adherens junctions are calcium dependent junctions that the most well studied of all cell-cell junctions. Adherens junctions are comprised of several proteins, including cadherins and catenins. E-cadherin is a calcium dependent cell surface protein with intracellular and extracellular domains. The extracellular domains interact with E-cadherin on neighboring cells, thereby creating adhesion between cells (Figure 1.7.1, part B). ß-catenin binds to the intracellular domain of E-cadherin and along with other scaffolding proteins, stabilizes the junction to the actin cytoskeleton. ß-catenin can also function in the Wnt signaling pathway. Plakoglobin (γ-catenin) is analogous to ß-catenin in its scaffolding function, and can participate in adherens junctions as well as in desmosomes. Plakoglobin, however, is a minor member of adherens junctions and is found preferentially in desmosomes. It is currently unknown if plakoglobin can participate in signaling pathways. Another adherens junctional protein is p120, which can bind to the intracellular domain of E-cadherin, and like ß-catenin and plakoglobin, can enter the nucleus, bind and regulate the transcriptional partner Kaiso, although it is still unknown if it can act as a transcriptional activator on its own (Daniel, 2007) . Alpha-catenin can bind to either ß-catenin and plakoglobin, and connects the adherens junction to the cytoskeleton by binding to actin.

Desmosomal structure is similar to adherens junctions. The adhesion molecules in desmosomes that mediate intercellular adhesion are also

cadherins, named desmoglein and desmocollin (Chitaev and Troyanovsky, 1997) (Figure 1.7.1, part C). Scaffolding proteins of desmosomes include the aforementioned plakoglobin, as well as desmosomal specific scaffolding proteins desmoplakin and plakophilin. The final attachment intracellularly of desmosomes is plakophilin to intermediate filaments, connecting the desmosome to the cytokeratin network and stabilizing the complex. Desmosomes are very abundant in skin epithelial cells, and it is from the abundance of keratin that the cells get their alternate name, keratinocytes.

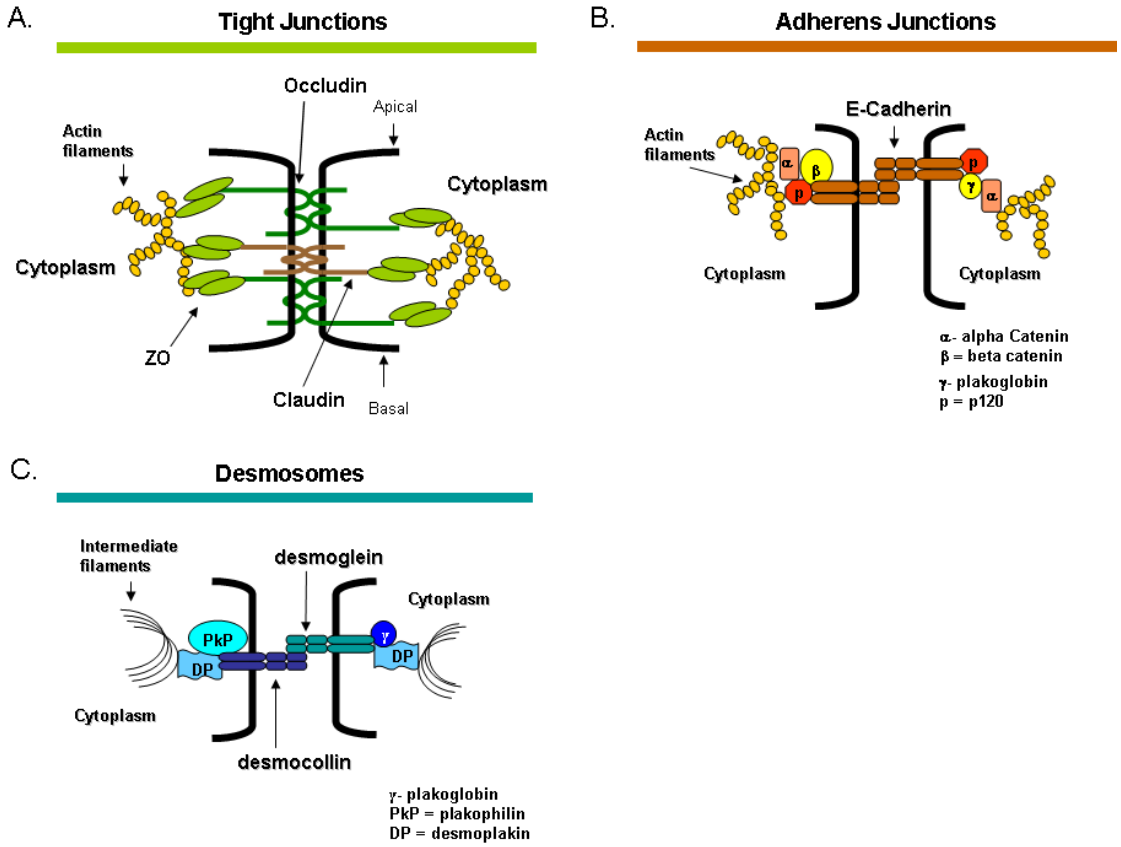


Figure 1.7.1 Cell-cell Junctions

A. Tight junctions function in polarity and to keep the skin impervious to water. Occludin and claudin are the two proteins that form dimers across cells, and bind ZO family members intracellularly, which then form a stable connection to the actin cytoskeleton.
B. Adherens junctions also use the actin cytoskeleton for stabilization, adhesion to which is mediated by α-catenin. Other adaptor proteins include p120, ß-catenin, and plakoglobin, which bind the transmembrane protein E-cadherin, which mediates cell-cell adhesion across cells by forming a homodimer with E-cadherin from neighboring cells.
C. Desmosomes, the predominant cell-cell junction in keratinocytes, have their own desmosomal cadherins, desmoglein and desmocollin, which form heterodimers across neighboring cells. Intracellularly, they bind plakoglobin, plakophilin, and desmoplakin, which attach to intermediate filaments for junctional stabilization.

1.7.1 Cell-cell junctions in EMT

Cell-cell junctional modulation occurs during formation of the primitive streak in development and in invasive carcinomas. Downregulation of junctions also occurs during wound healing, and will be discussed in section 1.7.2.

During development, in particular gastrulation, cells migrate and start to form the primitive streak. The primitive streak is the linear area at which the epiblast undergoes EMT activity to form mesenchyme and endoderm under the epithelial cells that remain to form the outlying ectoderm (Hay, 2005). At this point, cells migrating into the primitive streak have no desmosomes but extensive tight junctions (Shook and Keller, 2003). Once cells migrate into the primitive streak, desmosomes are detected as evidenced by staining for desmoplakin on the periphery but not throughout the streak (Franke *et al.*, 1982). The adherens junction protein E-cadherin is also lost at the primitive streak (Damjanov *et al.*, 1986), in a process that has recently been found to be dependent on FGF signaling (Ciruna and Rossant, 2001). The loss of this E-cadherin alone is sufficient to induce many of the changes associated with EMT (Baum *et al.*, 2008).

The adherens junction cadherin E-cadherin has been extensively studied for its role in tumor-associated EMT. A inverse relationship between E-cadherin expression and tumor invasiveness has been established in experimental systems and tumor tissues (Jeanes *et al.*, 2008), although there are notable exceptions. E-cadherin and N-cadherin are maintained in well differentiated

ovarian cancers, and a increase in tumor invasiveness correlates with a loss of E-cadherin but not of N-cadherin (Hudson *et al.*, 2008). In breast cancer tumor lymphovascular emboli, there is an increase in E-cadherin that allows the tumor to persist in the lymphovasculature, and the tumor can be eliminated with the use of anti-E-cadherin antibodies (Tomlinson *et al.*, 2001). However, there is also a strong correlation between loss of E-cadherin and higher cancer grade and metastasis in cancers of the head and neck (Kramer *et al.*, 2005), bladder (Baumgart *et al.*, 2007), breast (Heimann *et al.*, 2000), gastric tissue (Mayer *et al.*, 1993), nasopharyngeal tissue (Zheng *et al.*, 1999), ovary (Davidson *et al.*, 2000), pancreas (von Burstin *et al.*, 2009), prostate (Mol *et al.*, 2007) ,and colorectal tissue (Delektorskaya *et al.*, 2005).

Although less well studied, there is also evidence for downregulation of desmosomes in invasive tumors. Interestingly, the modulation of desmosomes in cancer has been investigated sporadically since the early 1980's as a possible tumor marker in not only skin cancer, but other epithelial cancers as well. Early studies looked at total desmosome numbers in carcinomas by using electron microscopy. A reduced number of desmosomes were reported in several invasive carcinomas (Pauli *et al.*, 1978; Alroy *et al.*, 1981; Kocher *et al.*, 1981; Schindler *et al.*, 1982), although one group reported that the desmosomes that remained were larger and occupied a larger amount of cell surface area (Pauli *et al.*, 1978). A later study used rat bladder carcinoma cells that normally had an epithelial phenotype but could be induced to an EMT fibroblast phenotype with the addition of "inducing medium". This study found the internalization (or

abnormal expression) of desmoglein, desmoplakin, and plakoglobin upon inducing conditions, as well as increased migratory activity, although the migratory capabilities were not dependent on the internalization of desmosomes (Boyer *et al.*, 1989). This suggests that downregulation of desmosomes may be associated with EMT-like events.

The downregulation of cell-cell junctions, along with the rearrangement of the cytoskeleton and change in morphology from a stationary epithelial cell to a migratory mesenchymal like cell that occurs during EMT is very similar to what happens at the leading edge of a wound, Indeed, a partial EMT iis essential for reepithelialization, as well as the reversion back to an epithelial phenotype once reepithelialization is complete.

1.7.2 Cell-cell junctions in wound healing

The dissolution of both hemidesmosomes, which connect the cell to the extracellular matrix, and desmosomes (Krawczyk and Wilgram, 1973; Sciubba *et al.*, 1978) is a key step in wound healing. The Krawczyk and Wilgram study followed keratinocytes after suction induced blister wounding in mice for 12 to 18 hours and found the restoration of both hemidesmosomes and desmosomes during reepithelialization occurs in several steps. First, the appearance of fibrils that connect the keratinocytes, which we now know are cadherins. Second, the attachment plate develops at the inner leaflet of the membrane. We can propose that this is the assembly of the rest of the junctional components. Finally, tonofilaments insert into the attachment plate, which we now call intermediate

filaments, attaching to the intracellular junctional proteins. This three stage process held true for both desmosomes, which maintain cell-cell attachments, and hemidesmosomes, which attach cells to the basement membrane. Scuibba and coworkers extended this finding in rats, comparing the healing of oral mucosa, which is known to heal very rapidly, to the healing of epidermis (Sciubba *et al.*, 1978). In this case, hemidesmosomes followed the same formation sequence as in the Krawczyk and Wilgam study, but occurred by 4 hours in mucosa as compared to 12 hours in rat epidermis. Additional studies in rabbit skin saw a decrease in the number of desmosomes and intermediate filaments at the wound margin 8 days post wounding as compared to control skin, and at 10 days post wounding, intermediate filaments were still retracted away from the plasma membrane (Gabbiani *et al.*, 1978). The reappearance of desmosomes is now considered a marker of the end of reepithelialization.

Adherens junctions are intimately linked to desmosomes in that their formation precedes desmosome assembly (Lewis *et al.*, 1997). In mouse epidermis, a decrease in E-cadherin was seen at day 3 post wounding in both full thickness incisional or excisonal type wound model. E-cadherin expression was restored at day 5 in the incisional model but took up to 10 days in the excisional model (Kuwahara *et al.*, 2001). Antibody blocking studies show that functionally blocking E-cadherin led to uneven wound margin and disruption of the reorganizing actin cytoskeleton in mouse epidermis (Danjo and Gipson, 1998). A decrease in E-cadherin staining was detected at the migrating corneal epithelia in a canine eye wound model (Chandler et al., 2007). In contrast, E-cadherin was

apparent within all epithelial cells throughout reepithelialization in a rat corneal wound healing model (Suzuki *et al.*, 2000). Desmogleins-1 and -2, however, in agreement with the earlier mentioned studies, were decreased immediately after laser ablation and did not reappear until the basement membrane, as indicated by laminin-1 staining, was reestablished (Suzuki *et al.*, 2000).

In a rat corneal wound model, investigators observed tight junctions throughout the 72 hour reepithelialization process and found that the tight junction proteins occludin and ZO-1 were present in epithelial cells throughout all stages of reepithelialization (Suzuki *et al.*, 2000; Hutcheon *et al.*, 2007). One explanation for the maintenance of tight junctions throughout wound healing is to serve as a scaffold for assembly and localization of myosin cables during wound repair. Using Madin-Darby canine kidney (MDCK) epithelial cells, cells were observed using live cell microscopy after laser ablation of the epithelial monolayer and a continuous association between myosin and ZO-1 occurred as quickly as 5 minutes post wounding (Tamada *et al.*, 2007). In human epidermis, the trend continued, as ZO-1 and occludin were present in migrating keratinocytes 6 days post wounding (Malminen *et al.*, 2003).

Clearly, cell-cell junctions undergo different fates during reepithelialization. While tight junctions remain intact, adherens junctions and desmosomes are, with the exception of a few cases, downregulated after the initial wound. The mechanisms for this downregulation as of yet remain unclear, but candidate mediators for this deregulation exist in the wound environment.

1.7.3 Mechanisms of junctional modulation

There are several possible mechanisms within the wound environment for junctional modulation. The decrease of divalent cations in the wound environment, phosphorylation, proteolysis, internalization and transcriptional repression are some of the reported processes for modulation cell-cell junctional proteins (Figure 1.7.2).

Cadherins can only maintain adhesion in the presence of calcium. Normal calcium levels (1.2-1.8 mM) are also necessary for proper keratinocyte differentiation and stratification (Hennings *et al.*, 1980; Boyce and Ham, 1983; Hennings and Holbrook, 1983). Calcium levels in wound fluid is quickly decreased at the onset of wounding, and remain low 24 hours post wounding (Grzesiak and Pierschbacher, 1995). Restoration of normal calcium levels is seen after 48 hours. Therefore, one well employed technique to study junctional disruption is the removal of this divalent cation in the wound environment. Under low calcium conditions (0.05-0.1 mM calcium), large intracellular spaces between keratinocytes, a lack of desmosomes, and a retraction of intermediate filaments from the cell border and their perinuclear accumulation was observed (Hennings and Holbrook, 1983). Upon restoration of normal calcium levels in this system, desmosomal plaques were visible as early as 5 minutes, and full desmosomes were formed by 1-2 hours after calcium addition (Hennings and Holbrook, 1983). Keratinocytes that were cultured and allowed to differentiate and stratify were unable to migrate in a wound scratch assay (Magee *et al.*, 1987). Once placed

in low calcium conditions, a reduction in desmosomes occurred and the cells were able to migrate (Magee *et al.*, 1987).

E-cadherin mediated adhesion is also calcium dependent, so it stands to reason that adherens junctions are also disrupted by low calcium conditions. Indeed, vinculin and actin, two members of the actin cytoskeleton, were retracted from the plasma membrane when cells are placed in low calcium, which could be reversed as quickly as one hour after return to normal calcium levels (O'Keefe *et al.*, 1987). It is interesting to note that in this study, the appearance of desmosomes was not visible until 2 hours after calcium restoration, indicating that adherens junction reformation occurred before that of desmosomes. The breakdown of adherens junctions with low calcium can be overcome by activation of protein kinase C (PKC), indicating the importance of this signaling pathway in adherens junction regulation (Lewis *et al.*, 1994). Deletion studies revealed that the formation of desmosomes required the preformation of adherens junctions (Lewis *et al.*, 1997).

Although tight junction adhesion molecules occludin and claudin are not calcium dependent, tight junctions were also disrupted by calcium removal (Rothen-Rutishauser *et al.*, 2002). Experiments with antibodies against E-cadherin showed that the reformation of adherens junctions, desmosomes and tight junctions with calcium addition were impaired when antibodies blocking E-cadherin were added (Gumbiner *et al.*, 1988). During the initial phase of wound healing, transient low levels of calcium could account for disruption of cell-cell adhesion. However, calcium levels are restored to normal by 24 hours after

wounding, while junctions remain downregulated, so other mechanisms must account for this persistent downregulation.

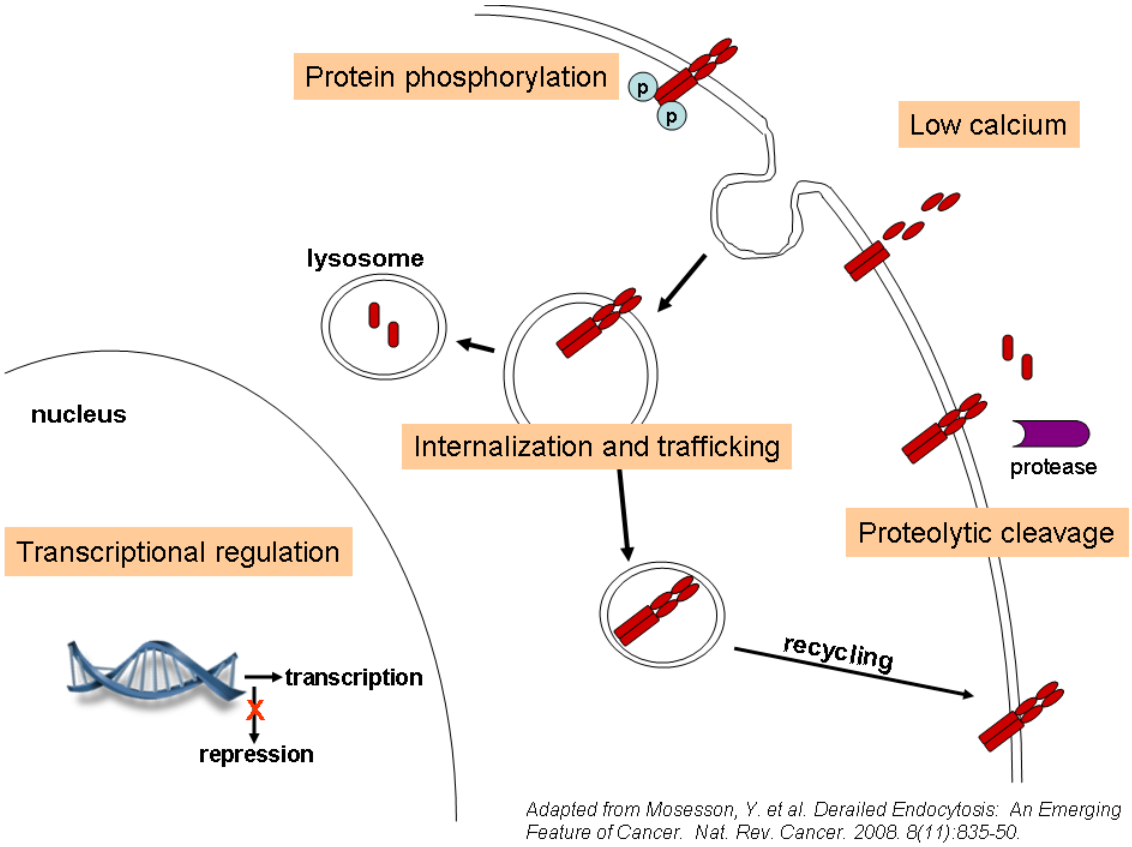


Figure 1.7.2 Multiple Mechanisms of Cadherin Disruption
There are multiple pathways that can lead to cadherin downregulation and junctional disruption. Protein phosphoryation is one event that can cause quick dissociation from the cell-cell junctions. Low calcium conditions can decrease adhesion, as these junctions are calcium dependent. Proteolytic cleavage is an alternate mechanism to cause loss of adhesion and junction instability. Internalization and trafficking of junctional proteins can also disrupt junctions, and finally, transcriptional downregulation is a slow process to rid the cell of proteins necessary to build cell-cell junctions

1.7.3.a Protein phosphorylation

Another mechanism for junctional modulation is that of phosphorylation. Several desmosomal and adherens junctional proteins are phosphorylated in response to different stimuli (See Table 1.7.1). Phosphorylation of junctional components occurs in junctional assembly and function, as several proteins, once phosphorylated, cannot bind the necessary junctional partners. For example, phosphorylation of ß-catenin and plakoglobin causes dissociation from junctional complexes, where they can then participate in other pathways such as transcriptional activation and/or repression (see section 1.7.3b). Phosphorylation of ß-catenin increased migratory activity in *in vitro* wound assays that can be inhibited with the addition of protein tyrosine phosphatases (Muller *et al.*, 1999). Addition of tyrosine phosphatases, as expected, reverted cells to an epithelial phenotype and increased cell-cell adhesion (Taddei *et al.*, 2002; Yan *et al.*, 2006).

Phosphorylation events also occur in tight junctions and regulates their assembly and disassembly. Overexpression of Src revealed the persistence of tight junctions but the absence of both adherens junctions and desmosomes (Takeda and Tsukita, 1995). Src activation in MDCK cells causes tyrosine phosphorylation of tight junction proteins occludin and claudin as well as the adherens junction protein p120 (Palovuori *et al.*, 2003). Ras transformed cells, which have a fibroblastic phenotype and few cell-cell junctions were used to study the effect of inhibition of the MAPK pathway on tight junctions. MAPK

inhibition caused tyrosine phosphorylation of occludin and restoration of tight junctions, indicating that this phosphorylation event participates in tight junction assembly (Chen *et al.*, 2000).

Phosphorylation is an acute signal in the wound environment that can decrease adhesion between cells by targeting critical molecules through phosphorylation and subsequent relocalization or degradation. Most junctional proteins can undergo phosphorylation/desphosphorylation events, leading to either junctional breakdown or stabilization. Regulation of assembly and disassembly is essential in wound repair, so phosphorylation events are likely an important in the reversible modulation of junctions that occurs during wound repair.

Table 1.7.1 Juntional Component Phosphorylation

Protein Reference	Phosphorylation	Stimulus	Effect
Cadherins			
E-cadherin (Fujita *et al.*, 2002)	Tyrosine	Src, c-Met	Endocytosis
E-cadherin (Serres *et al.*, 2000)	Serine	CK2	Stabilized junction
Desmoglein-2 (Lorch *et al.*, 2004)	Tyrosine	EGFR	Decreased junctions
Linker proteins			
ß-catenin (Kinch *et al.*, 1995)	Tyrosine	Ras OE	E-cadherin dissociation
ß-catenin (Piedra *et al.*, 2001)	Tyrosine (Tyr-654)	Mutants	E-cadherin dissociation
ß-catenin (Piedra *et al.*, 2003)	Tyrosine (Tyr-142)	Ras OE	α-catenin dissociation
ß-catenin (Hu *et al.*, 2001; Hu *et al.*, 2003)	Tyrosine	Phosophatase inhihbitor	E-cadherin, α-catenin dissociation, Nuclear localization
p120 (Mariner *et al.*, 2004)	Tyrosine (Tyr-288)	EGFR	Localized to AJ,lamellopodia
p120 (Mariner *et al.*, 2001)	Tyrosine	Src	Necessary for Shp-1 interaction
p120 (Brown *et al.*, 2009)	Serine (Ser-879)	PDGFR	PKC dependent
Plakoglobin (Gaudry *et al.*, 2001)	Tyrosine (Tyr-692, Tyr-724, Tyr-729)	EGFR	Desmoplakin dissociation
Plakoglobin (Lorch *et al.*, 2004; Yin *et al.*, 2005)	Tyrosine	EGFR	Junctional disruption
Plakoglobin (Hu *et al.*, 2001; Hu *et al.*, 2003)	Tyrosine	Phosphatase inhibitor	E-cadherin, α-catenin dissociation; Nuclear localization
Desmoplakin (Amar *et al.*, 1999)	Serine	TPA	Junctional disruption

Key: CK = OE= overexpressor, AJ = Adherens junctions, Shp-1: SH2 containing nonreceptor tyrosine phosphatase, PKC = protein kinase C, TPA = 12-O-tetradecanoylphorbol-13-acetate

1.7.3.b Transcriptional regulation

Another mechanism for regulating cell-cell junctions is by transcriptional repression, which is often seen in invasive cancers. The best studied example is the transcriptional repression of the adherens junction protein E-cadherin. E-cadherin has several known transcriptional repressors, including Snail, E47, Slug, SIP-1, deltaEF1 (ZEB1), ZEB2 and Twist (Bolos *et al.*, 2003; Eger *et al.*, 2005; Peinado *et al.*, 2007). Snail was one of the first transcription factors discovered to directly bind E-boxes present in the E-cadherin promoter and cause transcriptional repression (Batlle *et al.*, 2000). Overexpression of Snail in epithelial cells causes an invasive phenotype (Cano *et al.*, 2000), and introduction of siRNA against Snail in an invasive cell line restores E-cadherin at cell-cell junctions (Batlle *et al.*, 2000). Twist is a known developmental transcription factor and is also upregulated in many cancers. Recently Twist was found to mediate transcriptional downregulation of E-cadherin in several breast cancer cell lines (Vesuna *et al.*, 2008).

ZEB-2, (SIP-1) is another E-box binding transcription factor that overlaps with the E-cadherin promoter area that Snail binds. ZEB-2 can be induced by TGF-ß, and causes E-cadherin repression and increased invasion in MDCK cells (Comijn *et al.*, 2001). Another ZEB family member, ZEB-1 (δEF1) is upregulated in response to Snail overexpression (Guaita *et al.*, 2002), and can directly repress E-cadherin and cause a EMT like phenotype (Eger *et al.*, 2005).

Less is known about transcriptional repression of desmosomal cadherins but a member of the Snail family of transcription factors Slug/Snai2 is one

candidate. Slug/Snai2 was first discovered in development as being necessary for neural crest development (Nieto *et al.*, 1994; del Barrio and Nieto, 2002). It also plays a role in EMT like responses in cancer development and wound repair. Slug is necessary for reepithelialization during wound repair (Savagner *et al.*, 2005). Slug mRNA and protein levels are upregulated at wound margins *in vivo, ex vivo, and in vitro*, along with a concomitant decrease in desmosomes as evidenced by loss of desmoplakin (Savagner *et al.*, 2005). Overexpression of Slug in a keratinocyte cell line accelerates reepithelialization in an *in vitro* scratch assay, and these cells have decreased desmosomes as evidenced by a decrease in desmoplakin and desmoglein-3 expression (Savagner *et al.*, 2005). Similarly, expression of Slug was associated with a decrease in the desmosomal components desmoplakin and desmoglein, but not E-cadherin in rat bladder epithelial cells (Savagner *et al.*, 1997).

Transcriptional repression is one mechanism for downregulation of junctional proteins. Little is known about the role of this mechanism during wound repair.

1.7.3.c Proteolytic cleavage

Proteolytic cleavage is a common mechanism for modulation of cell membrane proteins function, as there are several proteases in the extracellular environment including matrix metalloproteases (MMPs), a disintegrin and metalloproteases (ADAMs) and plasmin and plasminogen activators. MMPs can be activated by several growth factors that are present during wound healing, including TGF-ß, PDGF, TNF-α, IL-1, FGF, EGF and KGF (Toriseva and Kahari,

2009). During wound healing, several MMPs are expressed by keratinocytes at the migrating wound edge, including, collagenase MMP-1, gelatinase MMP-9, and stromelysins MMP-3 and MMP-10 (Madlener *et al.*, 1998). Other MMPs that are expressed during wound healing but not necessarily by migrating edge keratinocytes include MMP-3, MMP-8, MMP-12, MT1-MMP, MMP-19, MMP-26 and MMP-28 (Toriseva and Kahari, 2009). ADAMs are transmembrane proteins known to cleave ectodomains of membrane bound proteins and ADAM substrates in wound repair are likely to be EGFR ligands (Sahin *et al.*, 2004; Sahin and Blobel, 2007). Plasminogen is the precursor to plasmin, which is important for fibrin homeostasis as well as angiogenesis, two essential steps in wound healing. Cleavage of plasminogen can occur by tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA), kallikrein and coagulation factors XIa and XIIa. During wound healing, uPA is expressed by basal and suprabasal keratinocytes (Romer *et al.*, 1991), while its receptor, uPAR, is expressed at the leading wound margin (Romer *et al.*, 1994). Cleavage of junctional components can be accomplished by various members of all three of these proteolytic systems, and are described below.

Extracellular cleavage of E-cadherin is a well studied mechanism for junctional disruption, and several proteases, including MMPs: MMP-3, MMP-7 (Davies *et al.*, 2001; Noe *et al.*, 2001), MMP-9 (Symowicz *et al.*, 2007; Cowden Dahl *et al.*, 2008), MT1-MMP, ADAM10 (Maretzky *et al.*, 2005) and ADAM15 (Najy *et al.*, 2008), as well as plasmin (Ryniers *et al.*, 2002), and kallikrein 7 (Johnson *et al.*, 2007) calpain (Rios-Doria *et al.*, 2003) and γ-secretase

(Marambaud *et al.*, 2002; Ferber *et al.*, 2008) are reported to cleave E-cadherin. The protease γ-secretase can cleave E-cadherin, and the cytoplasmic domain of E-cadherin can then translocate to the nucleus and interact with DNA, possibly in the regulation of apoptosis (Ferber *et al.*, 2008).

Extracellular cleavage of desmosomal cadherins occurs in response to pemphigus autoantibodies, apoptosis, and by MMPs and ADAMS. Pemphigus vulgaris is an autoimmune disease in which patients develop antibodies against desmoglein-3. The appearance of a 60 kD fragment of desmoglein-3 occurs *in vitro* in keratinocytes treated with patient sera with pemphigus vulgaris (Cirillo *et al.*, 2008a). In apoptosis, both desmoglein-2 and desmoglein-3 undergo cleavage events, by MMP-9 (Cirillo *et al.*, 2007) and caspase-3 (Cirillo *et al.*, 2008b), respectively.

Other junctional proteins occasionally undergo proteolytic cleavage as well. The tight junctions protein occludin is proteolytically cleaved by metalloproteinases in endothelial cells after inhibition of tyrosine phosphatases (Wachtel *et al.*, 1999). No other tight junction or adherens junctional proteins were affected, although desmosomal proteins were not investigated in this system. The adherens junction protein ß-catenin is cleaved by the calcium dependent protease calpain in both breast and prostate cancer cell lines upon induced calcium influx (Rios-Doria *et al.*, 2004). In keratinocytes, both ß-catenin and plakoglobin are cleaved after UV exposure, by caspases-3 and -8 (Hung *et al.*, 2006), probably due to activation of an apoptotic pathway, as cleavage of

these catenins by caspases is seen in endothelial cells undergoing apoptosis as well (Herren *et al.*, 1998).

Cleavage of all junctional proteins can be mediated by one or all of the above mentioned proteolytic systems, although the main targets seem to be the transmembrane cadherins. Many proteinases are found in wound fluid, and this is a plausible mechanism of cadherin downregulation in wound healing. Downregulation of cadherins by proteolytic cleavage is possible during the partial epithelial to mesenchymal transition which occurs during wound healing and as of yet has not been examined.

1.7.3.d Internalization and Trafficking

Internalization of proteins from the cell membrane occurs during routine protein turnover, as well as in response to signal transduction pathways. Transmembrane proteins can be internalized via micropinocytosis, clathrin dependent or independent endocytosis, and caveolin dependent or independent endocytosis (Figure 1.7.3). Once internalized, there are different possible itineraries for the protein. Generally, internalized proteins enter a recycling pathway, in which they are shuttled back to the cell membrane, or a degradation pathway, in which they are destroyed either by the lysosome or proteosome. Cadherins can undergo different types of internalization depending on the cell type and stimulus presented.

The internalization of E-cadherin has been studied in several models and clathrin-dependent (Le *et al.*, 1999; Ivanov *et al.*, 2004; Izumi *et al.*, 2004; Bryant *et al.*, 2005; Palacios *et al.*, 2005; Troyanovsky *et al.*, 2006; Miyashita and

Ozawa, 2007b), clathrin independent (Akhtar and Hotchin, 2001; Paterson *et al.*, 2003), and caveolae-dependent (Lu *et al.*, 2003) internalization of E-cadherin have been described, which vary according to the stimulus presented (Lu *et al.*, 2003; Bryant and Stow, 2004; D'Souza-Schorey, 2005). In basal conditions with no stimulus, E-cadherin undergoes clathrin mediated endocytosis, then is recycled back to the cell surface (Le *et al.*, 1999). Once internalized with low calcium, recycling of E-cadherin back to the plasma membrane after restoration of normal calcium levels involved direct interaction with phosphatidylinositol phosphate kinase and the AP1 complex (Ling *et al.*, 2007). The scaffold protein PALS1 is normally associated with tight junction formation, but also functions in E-cadherin transport to the plasma membrane (Wang *et al.*, 2007). The tight junction protein occludin is also continuously recycled in a process dependent on Rab13 (Morimoto *et al.*, 2005). Junctional protein recycling is a possible overarching mechanism for junctional regulation at basal conditions.

Various treatments of cells cause different results. Clathrin dependent endocytosis (Figure 1.7.3, labeled in green) can occur in response to HGF treatment (Palacios *et al.*, 2002), low calcium conditions (Ivanov *et al.*, 2004; Ling *et al.*, 2007) or the tyrosine kinase Src (Palacios *et al.*, 2005). Researchers using a cell free system also found clathrin dependent endocytosis of E-cadherin that was inhibited by Rac activation (Izumi *et al.*, 2004). E-cadherin cointernalizes into early endosomes (Figure 1.7.3, labeled in green) with the tyrosine kinase receptors c-Met and FGFR1 in response to HGF and FGF treatments, respectively (Kamei *et al.*, 1999; Bryant *et al.*, 2005). The use of E-cadherin

mutants revealed that E-cadherin that cannot bind p120 colocalizes with both early and recycling endosomes (Miyashita and Ozawa, 2007b), and protein kinase C (PKC) activation can also induce endocytosis and recycling of E-cadherin (Le *et al.*, 2002). E-cadherin that cannot bind ß-catenin is targeted for destruction via the lysosome (Miyashita and Ozawa, 2007a). Lysosomal degradation also occurs after TGF-ß treatment, Raf-1 overexpression (Janda *et al.*, 2006), low calcium (Shen *et al.*, 2008), and Src activation (Palacios *et al.*, 2005).

Clathrin independent endocytosis of E-cadherin is reported to occur after various treatments as well. EGF treatment is reported to cause micropinocytosis (Figure 1.7.3, labeled in red) of E-cadherin (Bryant *et al.*, 2007) as well as caveolar dependent internalization (Figure 1.7.3, labeled in yellow) (Lu *et al.*, 2003). Low calcium and Rac overexression also causes clathrin independent, caveolar independent internalization of E-cadherin (Akhtar *et al.*, 2000). Surface labeled E-cadherin was found to be internalized by a clathrin independent, dynamin dependent mechanism (Paterson *et al.*, 2003).

The well studied E-cadherin can undergo various modes of internalization after presentation of different stimuli (see Figure 1.7.3). Newly synthesized E-cadherin is transported to the plasma membrane from the Golgi via recycling endosomes, and cycles through this recycling itinerary. Several stimuli, including low calcium disruption of junctions, growth factors such as HGF, FGF and TGF-ß, as well as perturbance of molecules indicated in trafficking, such as Src or ARF, can cause the clathrin dependent internalization and eventual degradation

of E-cadherin in the lysosome. The same stimuli in other systems have been found to cause non clathrin dependent internalization, including low calcium and EGF causing caveolar internalization, whereas EGF has also been reported to internalize E-cadherin by micropinocytosis and low calcium also causing clathrin dependent internalization. It appears that the fate of E-cadherin is dependent on both stimulus and cell type.

In contrast, very little is known about desmosomal cadherin regulation. Early studies on desmosomal assembly focused on disruption of junctions by low calcium conditions, then reassembly after restoration of calcium levels. The half life of desmogleins 1-3 and desmoplakins 1-2 are decreased rapidly after placement in low calcium (Penn *et al.*, 1987) but continue to assemble what was described as “half desmosomes” (Duden and Franke, 1988) that were constitutively endocytosed in vesicles that associate with intermediate filaments. Further studies found that desmosomes in low calcium were internalized in a clathrin independent manner and are accumulated in cytoplasmic vesicles that do not colocalize with lysosomal markers (Holm *et al.*, 1993; Burdett and Sullivan, 2002). The Burdett group found evidence of desmoglein-1 in endosomes that did not label for early endosomes. These unknown endosomes were able to fuse with late endosomes. After the switch from low calcium to normal calcium levels the desmosomal cadherins desmoglein-1 and desmocollin-2 were transported from the Golgi to the plasma membrane in two stages, first in 60 nM vesicles, then in 200 nM vesicles (Burdett and Sullivan, 2002).

Factors other than low calcium can cause the internalization of desmosomal proteins. Desmoglein-1 is reported to be downregulated in response to c-Met activation by HGF (Li *et al.*, 2001), but it was not reported if it was by a cleavage or internalization mechanism. Desmoglein-3, in response to pemphigus antibody, an autoantibody produced in patients with the autoimmune disease pemphigus vulgaris, was internalized and degraded in lysosomes in as little as 1 hour, with complete loss of surface desmoglein-3 by 6 hours (Calkins *et al.*, 2006). However, when studying earlier timepoints (1 hour), the Kowalczyk lab found this internalization to be a clathrin, dynamin, and caveolin independent mechanism (Figure 1.7.4) most likely mediated by lipid rafts (Delva *et al.*, 2008).

Desmosomal cadherins are less well studied, and although internalization of desmosomes by low calcium appears to be a clathrin independent mechanism that proceeds to late endosomes but not lysosomes, these studies were done with electron microscopy techniques that recognize the electron dense desmosome but not the individual members of the desmosome involved. However, more recent studies focusing on desmoglein-3 internalization after addition of autoantibodies against it also showed a clathrin independent internalization that shuttled to endosomes (Figure 1.7.4), but in this case lysosomal degradation did occur. Due to the small amount of information available for desmosomal internalization, we focused on desmoglein-2, a very abundant desmosomal cadherin, in response to EGF stimulation in the context of wound healing.

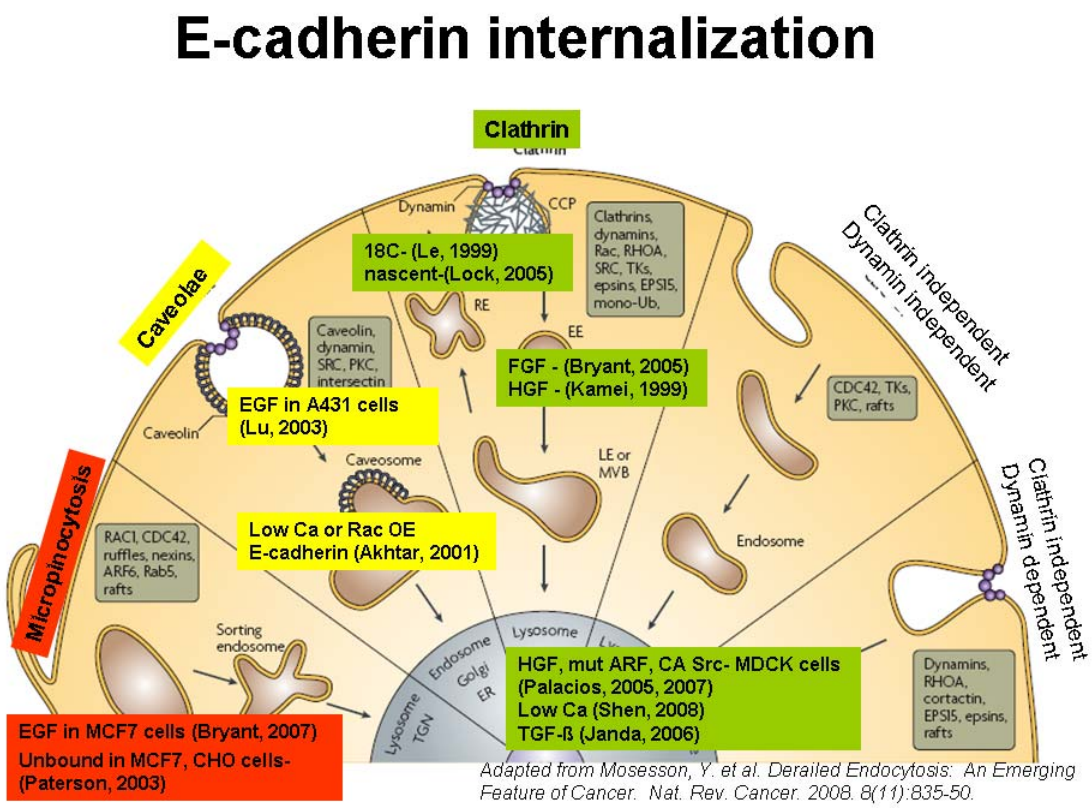
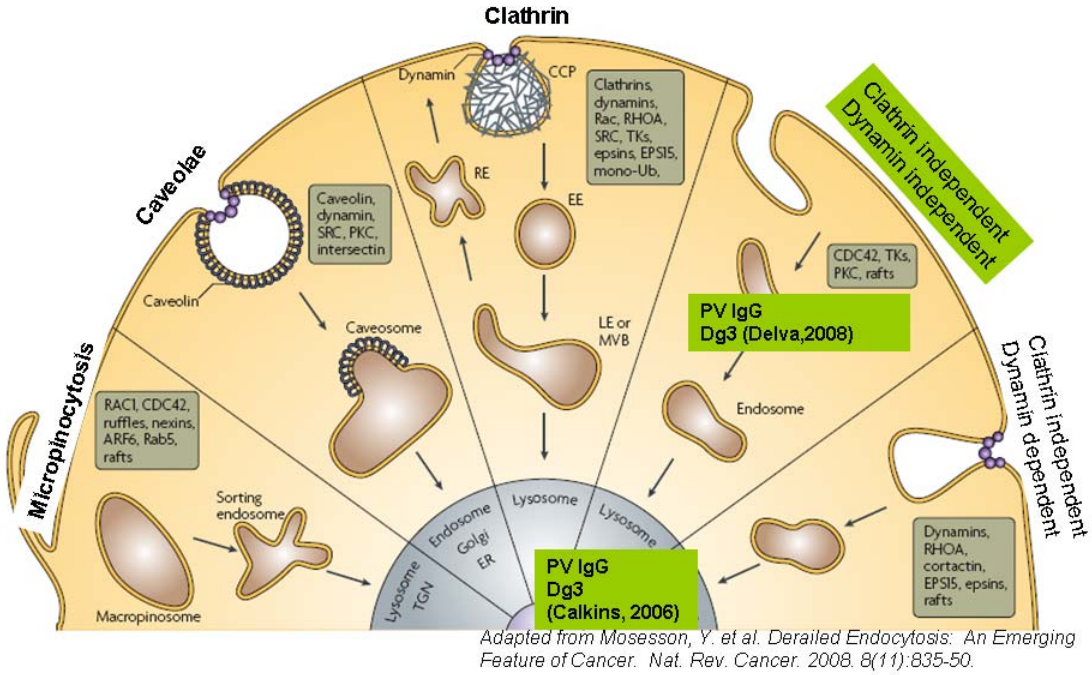


Figure 1.7.3 E-cadherin Internalization

Internalization of E-cadherin has been reported to occur by various mechanisms depending on the stimulus. The majority of studies have shown clathrin dependent endocytosis, and either recycling to the plasma membrane via recycling endosome or transfer to late endosomes and lysosomal degradation. Other pathways such as micropinocytosis and caveolar dependent endocytosis are less well studied but have been reported to occur. CCP = clathrin coated pit, RE = recycling endosome, EE = early endosome, LE = late endosome, MVB = multivesicular body.

Desmosomal cadherin internalization



Adapted from Mosesson, Y. et al. Derailed Endocytosis: An Emerging Feature of Cancer. Nat. Rev. Cancer. 2008. 8(11):835-50.

Figure 1.7.4 Desmosomal Cadherin Internalization

Desmosomal cadherin internalization is less well studied than that of E-cadherin. The only stimulus studied was the effect of pemphigus vulgaris autoantibody induced internalization of desmoglein-3. In two separate studies by the Kowalczyk lab, desmoglein-3 was internalized in a clathrin independent, dynamin independent pathway that is postulated to occur via lipid rafts. After internalization, desmoglein-3 is transferred to the endosomal pathway where it eventually undergoes lysosomal degradation.

1.8 Hypothesis and rationale

Although it is clear that junctions are modulated during reepithelialization, the underlying mechanisms are not well understood. One possible candidate for being a cell-cell junctional modulator is EGFR. EGFR levels are upregulated at wound margins *in vivo* (Wenczak et al., 1992) and due to the elevated EGFR levels, the abundant EGFR ligands in the wound environment, and evidence that EGFR activation can disrupt junctions (Cowden Dahl *et al.*, 2008; Klessner *et al.*, 2008; Shen *et al.*, 2008) the impact of the EGFR on two major adhesive junctions is the focus of this dissertation. First, I examined the effects of elevated EGFR on cadherin based cell-cell junctions in an *in vitro* model of reepithelialization (Section 3.1). I hypothesized that elevated EGFR levels will disrupt cadherin based cell-cell junctions after extended EGF treatment, causing a release of transcriptional activators that will then cause upregulation of gene transcripts involved in EMT-like events.

I then focused on the effects of EGFR activation on the individual components of cell-cell junctions and their cytoskeletal counterparts in the context of wound repair (Section 3.2). I hypothesized that EGFR activation will cause disruption of both cell-cell junction components as well as disrupt their cytoskeletal counterparts during wound healing. Any differences seen in junctional components after EGFR activation could be due to either downregulation of gene expression or post-transcriptional regulation. In order to investigate what mechanisms are involved, I tested for differences in production

of junctional components, including RNA and protein production in an EGFR overexpressing cell line (Section 3.3). I compared the results of EGFR activation to that of low calcium, a well studied mechanism of modulating junctions (Section 3.3). I hypothesized that EGFR will downregulate several junctional proteins and that this will differ from what is seen with low calcium.

Due to the lack of information on cadherin regulation during wound healing, I focused on the junctional cadherins E-cadherin and desmoglein-2, and their fates after EGFR activation (Sections 3.4 and 3.5). I hypothesized that both cadherins will undergo downregulation after EGFR activation.

Understanding the mechanisms of junctional modulations during wound healing could reveal possible avenues of treatment for patients with non-healing, chronic wounds. Identifying the junctional and cytoskeletal components modulated during wound healing after EGFR activation will help us to identify the mechanism by which EGF therapies are beneficial and could eventually help us fine tune these treatments for increased efficacy.

2 Methods

2.1 Cell Line and Reagents

Squamous cell carcinoma (SCC) 12F cells and SCC 13 cells were originally derived from tumors of the facial epidermis and were generously provided by Dr. William A. Toscano, Jr. (University of Minnesota, Minneapolis, MN) and Dr. Kathleen Green (Northwestern University, Chicago, IL), respectively. SCC 12F cells were maintained in 10cm^2 plates in Dulbecco's modified Eagle's medium: Ham's F-12 nutrient mixture (DMEM:F-12) containing 5% (v/v) iron-supplemented defined calf serum (HyClone Laboratories, Inc., Logan UT), 2mM L-glutamine and antibiotics (penicillin, 100U/ml, streptomycin, 50μg/ml). For all experiments involving growth factor addition, SCC 12F cells were placed into DMEM:F-12 containing 0.1% (w/v) bovine serum albumin (BSA) for 24 hours prior to growth factor addition.

For low calcium experiments, cells were placed into DMEM:F12 depleted of divalent cations using Chelex 100 Resin (BioRad, Hercules, CA) with addition of 50 μM $CaCl_2$ and 1mM $MgCl_2$ to the culture medium. SCC 13 cells stably expressing control vector or EGFR were maintained as for SCC 12F except for the addition of 300 μg/ml G418 to maintain selection (McCawley *et al.*, 1997). Murine epidermal growth factor (EGF) was obtained from Biomedical Technologies Inc. (Stoughton, MA). GM6001X was purchased from Chemicon (Temecula, CA). DMEM:F-12, BSA, Penicillin/Streptomycin and L-glutamine were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2 Immunoblotting of protein and conditioned medium

Cells were lysed either with SDS collection buffer (10mM Tris pH 7.5, 1% SDS, 5mM EDTA, 2mM EGTA, 1mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A) to collect total protein, or by subcellular fractionation. For subcellular fractionation, cells were lysed with 0.05% saponin collection buffer (10mM Tris pH 7.5, 140mM NaCl, 0.05% saponin, 5mM EDTA, 2mM EGTA, 1mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A), then centrifuged at 14,000 rpm, and the supernatant was called the saponin, or cytoplasmic, fraction. The pellet was then fully resuspended in 1% triton collection buffer (10mM Tris pH 7.5, 140mM NaCl, 1% Triton X-100, 5mM EDTA, 2mM EGTA, 1mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A), then centrifuged, and the resulting supernatant was labeled the triton soluble, or membrane fraction. Finally, the remaining pellet was fully resuspended in 1% SDS collection buffer, and this was labeled the triton insoluble, or cytoskeletal fraction. For experiments using nuclear extracts, the protocol used is a modified Dignam protocol (Dignam *et al.*, 1983). Cells were collected in ice cold PBS, centrifuged, and the pellet was resuspended in buffer A (1M Hepes-KOH, pH 7.9, 1M $MgCl_2$, 1M KCl). After vortexing, the mixture was centrifuged at 1700 rcf then supernatant was aspirated. The remaining pellet (nuclear extract) was resuspended in buffer C (1M Hepes-KOH pH 7.9, 25% glycerol (v/v), 1M KCl, 5M NaCl, 1M $MgCl_2$, 0.5M EDTA).

Equal amounts of total protein were determined by bicinchoninic acid (BCA) colormetric assay (Pierce, Rockford, IL). Equal quantities of protein were

fractionated on 8% (w/v) SDS polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes.

For experiments using conditioned medium, cells were treated, and conditioned medium was collected from 6 well plates (1 mL total volume), then concentrated using a centrifugal filter device (Millipore, Bedford, MA) with a 30,000 molecular weight cutoff. The retentate was collected and resolved on an 8% (w/v) SDS polyacrylamide gel, then transferred onto a PVDF membrane. Immunoblotting was performed as described below. Membranes were blocked in 5% (w/v) non-fat dry milk in Tris buffered saline with 0.05% Tween-20 (TBST) for 1 hour at room temperature before the addition of primary antibody. Primary antibodies were used at a 1:1000 dilution and included: ß-catenin (Santa Cruz, Biotechnology, Santa Cruz, CA), plakoglobin (Santa Cruz Biotechnoloogy, Santa Cruz, CA), Activated ß-catenin (Upstate, Temecula, CA), E-cadherin (clone NCH-38, Dakocytomation, Carpinteria, CA), E-cadherin (Santa Cruz Biotechnology, Santa Cruz, CA), desmoglein-2 (Invitrogen, Carlsbad, CA) and alpha-catenin (Chemicon, Temecula, CA). Primary antibody was added, and the blot was incubated at room temperature for 1 hour. The membranes were washed with TBST 3 times, and secondary antibody horseradish peroxidase labeled goat anti-mouse from Promega (Madison, WI), at a dilution of 1:10,000 in 5% (w/v) milk, was added for 1 hour at room temperature. The membranes were washed with TBST 3 times and developed using the Supersignal chemiluminescent detection system (Pierce, Rockford, IL). Visualization and densitometry of the blots was

obtained with the Kodak Image Station 440 System (New England Nuclear, Boston, MA).

2.3 Immunofluorescence

SCC 13 or 12F cells were seeded in a Lab Tek II chamber slide system (Nalge Nunc International, Naperville, IL). Cells were transferred to serum-free medium and then treated with 20 nM EGF for various times, or pretreated with the broad spectrum MMP inhibitor GM6001X for 30 minutes prior to addition of EGF. To probe for junctional proteins, cells were fixed with cold dry methanol for 2 minutes or alternatively with freshly prepared paraformaldehyde (3.7%) for 10 minutes. To look for cytoplasmic staining, paraformaldehyde slides were permeabilized with 0.01% (w.v) Triton X-100 (Sigma, St. Louis, MO) for an additional 5 minutes. The slides were then blocked in 3% (w/v) BSA in complete PBS (phosphate buffered saline containing 0.8 mM magnesium chloride and 0.18 mM calcium chloride) at 37º C in a humidified chamber. For lysosomal detection, Lysotracker (Invitrogen, Carlsbad, CA) was added at a 1:250 dilution to live cells 2 hours before fixation.

Primary antibodies were used at a 1:100 dilution and included: ß-catenin (Chemicon, Temecula, CA), plakoglobin, caveolin-1, pancytokeratin (Santa Cruz, Santa Cruz, CA), E-cadherin (HECD-1 clone), desmoglein-1, desmoglein-2 (Invitrogen, Carlsbad CA), EEA1 (Affinity Bioreagents, Golden, CO), epidermal growth factor receptor (EGFR) (Upstate, Chicago, Il.), rab11 (clone p71, NM275, rabbit polyclonal Ab) and rab7 (clone , both kindly provided by Dr. Angela Wandinger-Ness, University of New Mexico). Primary antibody was allowed to

incubate for 1 hour at 37ºC in a humidified chamber. Slides were washed 3 times in complete PBS, then a fluorophore conjugated secondary antibody (Invitrogen, Carlsbad, CA) was added at a 1:300 dilution, and was further incubated for 1 hour at 37ºC in a humidified chamber. Actin staining was obtained by incubating with TRITC-labeled Phalloidin (0.5 μg/ml in PBS, Sigma, St. Louis, MO) for 30 minutes at room temperature. Slides were washed 3 times in complete PBS then mounted with a coverslip using Vectashield mounting medium (Vector Labs, Burlingame, CA). Images were obtained with an inverted microscope (Olympus IX70, Melville NY) and MagnaFire software 2.1 (Optronics, Goleta, CA) or with a Zeiss confocal Microscope (Zeiss, Thornwood, NY).

2.4 Wound Healing Assay

For evaluation of *in vitro* reepithelialization, confluent cell monolayers were deprived of serum and growth factors for 24 hours, and a cell-free area was introduced by scraping the monolayer with a standard dimension blue pipette tip (USA Scientific, Ocala, FL) followed by extensive washing to remove cellular debris. *In vitro* reepithelialization was monitored by repopulation of the cleared area (wound width typically between 200–300 mm) with cells over time either in the presence or absence of 5 μM of the EGFR inhibitor AG1478 (Alexis/Axxora Inc., San Diego, CA). Quantification of wound area was obtained by measuring area before and after reepithelialization using digital pictures and ImagePro (Media Cybernetics, Inc., Bethesda MA) software.

2.5 Polymerase Chain Reaction (PCR)

RNA was isolated using 0.5 ml Trizol (Invitrogen Life Technologies, Carlsbad, CA), following the manufacturer's instructions. cDNA was synthesized from total RNA. All PCR reagents were purchased from Promega (Madison, WI). Primers were ordered from Sigma-Genosys (The Woodlands, TX) and included the following: E-cadherin (Forward, GGGTGACTACAAAATCAATC, Reverse, GGGGGCAGTAAGGGCTCTTT), desmoglein-2 (Forward, CACTATGCCACCAACCACTG, Reverse, TTAGGCATGGCCAGAGTAGG), and 18s rRNA (Forward, AAACGGCTACCACATCCAAG, Reverse, CCTCCAATGGATCCTCGTTA), Slug (Forward, CCCTGAAGATGCATATTCGGAC, Reverse, CTTCTCCCCCGTGTGAGTTCTA), tenascin-C (Forward, GGCATTGAGCTGACCTACGG, Reverse, CTTTGGCTGGGTTGCTTGAC). E-cadherin amplification was performed with an initial denaturation step at 94ºC for 4 minutes, followed by 38 cycles with a denaturing step at 94º for 30 seconds, an annealing step of 55ºC for 30 seconds and an extension step of 72ºC for 30 seconds, and a final extension step of 72 ºC for 4 minutes. Desmoglein-2 amplification was performed as above, with the annealing step of 64ºC for 30 seconds, with a total of 30 cycles. 18s rRNA amplification was performed with an initial denaturation step at 94ºC for 4 minutes, followed by 20 cycles with a denaturing step at 94º for 30 seconds, an annealing step of 55ºC for 30 seconds and an extension step of 72ºC for 30 seconds, and a final extension step of 72 ºC for 4 minutes. Slug amplification was performed with an initial denaturation step at 94ºC for 3 minutes, followed by

36 cycles with a denaturing step at 94º for 30 seconds, an annealing step at 60ºC for 30 seconds and an extension step at 72ºC for 60 seconds, and a final extension step of 72 ºC for 7 minutes. Tenascin-C amplification was performed with an initial denaturation step at 94ºC for 4 minutes, followed by 35 cycles with a denaturing step at 94º for 30 seconds, an annealing step at 67ºC for 30 seconds and an extension step at 72ºC for 30 seconds, and a final extension step of 72 ºC for 7 minutes.

GoTaq Flexi products (Promega, Madison, WI) were used for PCR. The resulting PCR products were loaded onto a 3% agarose gel (EMD Chemicals, San Diego, CA). SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA) was added for staining purposes, and the gel was imaged on a Kodak Image Station 440 System (New England Nuclear, Boston, MA).

2.6 Statistical Analysis

Due to the small samples sizes in my studies (n=3), I cannot test for normal distribution, so a nonparametric test is needed to analyze my results (Daniel, 1987). Due to the small sample size, I also cannot assume equal variance (Motulsky, 1999). Results from different treatment groups in immunoblotting experiments were compared by using the nonparametric Welsh's t test, and the value for statistical significance was considered at $p<0.05$.

3 Results

3.1 Elevated EGFR required for junctional disruption

Since the EGFR is elevated at the edge of wound margins *in vivo*, it is possible that increased receptor expression alters keratinocyte function. To test this hypothesis, the first goal was to identify potential differences in junctional disruption due to contributions of the EGF receptor. I used the squamous carcinoma cell line SCC 13, a cell line from a tumor of the facial epidermis that normally expresses low levels of the EGFR (~40,000 receptors/cell), as well as SCC 13 cells that were transfected to moderately overexpress (about 5 fold, ~200,000 receptors/cell) EGFR (Hudson and McCawley, 1998) . EGF dependent morphological differences are apparent between the parental cells and the EGFR overexpressing cells. SCC13 cells transfected with vector alone maintain an epithelial morphology, exemplified by their round shape, cobblestone appearance, and tight colony formation following exposure to EGF. In contrast, EGFR overexpressing cells have an elongated, spindle-like morphology, typical of an EMT-like transition to a fibroblastic-like phenotype (Figure 3.1.1). The transition occurs approximately 8 hours post EGF treatment, and extends throughout 24-48 hours of treatment in EGFR overexpressing cells. In contrast, even after 48 hours of continuous EGF stimulus, vector control cells maintain their epithelial morphology and remain in tight colonies.

In order to determine if the lack of response by the SCC 13 vector control cells was due to the concentration of EGF, the effects of different concentrations

was investigated. Cells were treated either with vehicle alone (control), 10 nM EGF, or 100 nM EGF for 24 hours (Figure 3.1.2). At 24 hours, EGFR cells were elongated and spindle shaped in response to EGF treatment. Vector control cells, however, maintained their tight colonies at both the 10 nM and 100 nM EGF concentrations. This indicates that vector control cells, with low levels of the EGFR are resistant to ligand dependent scattering regardless of both time (Figure 3.1.1) and EGF concentration (Figure 3.1.2). However, under low calcium conditions in which junctions are already disassembled, both vector control and EGFR cells migrate in response to EGF (McCawley *et al.*, 1997). This indicates that colony disruption under conditions of intact junction (normal calcium levels) in the SCC 13 cell line is dependent on elevated EGFR levels and that the predominant effect of elevated EGFR is on the modulation of junctions rather than cell migration.

Although the cell scattering indicates a loss of cell-cell junctions, confirmation requires immunofluorescent staining for individual junctional components. ß-catenin was used as a marker for adherens junctions, while desmoglein-2 was used as a marker of desmosomes. After treating both the vector control cells and the EGFR overexpressing cells for various timepoints up to 24 hours with EGF, differences between the two cells lines became evident. Vector control cells maintained both adherens junctions and desmosomes at the cell-cell border, indicative of intact junctions (See figures 3.1.3 and 3.1.4, left panels). However, cells overexpressing the EGFR had a decrease in cell border staining of both the adherens junction protein ß-catenin and the desmosomal

protein desmoglein-2 around 6 hours post EGF treatment (Figures 3.1.3 and 3.1.4, right panels). By 24 hours, most of the desmoglein-2 and nearly all of the ß-catenin was no longer present at cell-cell junctions (Figures 3.1.3 and 3.1.4, white arrows), indicating that elevated EGFR is necessary for junctional dissolution, as suggested by the colony disruption findings. The characteristic change in morphology was also seen after 24 hours post EGF treatment (comparte 3.1.1 with 3.3.4). Differences in staining intensities were observed between the vector control cells and the EGFR overexpressing cells for both ß-catenin and desmoglein-2. This may be an artifact of the convential microscopy technique, because no differences in intensities were seen with confocal microscopy for ß-catenin staining (Figure 3.1.7).

In order to evaluate protein levels for junctional components after EGF treatment, cells were collected in SDS protein lysis buffer and processed for immunoblotting to detect the junctional cadherins E-cadherin and desmoglein-1. While it appears that cadherin levels decrease in EGFR overexpressing cells after EGF treatment, there were no statistically significant differences for either cadherin 24 hours after EGF exposure (Desmoglein-1, vector cells control vs. EGF ($p=0.2987$); EGFR cells, control vs. EGF ($p=0.6444$) in a total of 3 independent experiments) (Figure 3.1.5). Similar results were observed for E-cadherin. No statistically significant difference was detected in vector cells control vs. EGF ($p=0.1772$) and the EGFR cells ($p=0.2987$) in a total of 3 independent experiments (Figure 3.1.5). The trend toward cadherin decrease after 24 hours of EGF treatment, even though not statistically significant, combined with the

immunofluorescence data revealing loss of protein from the cell-cell junctions (Figure 3.1.3 and 3.1.4) indicates a possible role for elevated EGFR levels and junctional breakdown specifically via cadherin modulation.

Because the EGFR has been reported to modulate ß-catenin, levels of this protein were evaluated following EGF treatment in vector control and EGFR overexpressing cells. No EGF dependent changes in ß-catenin were detected between the 0h and 24 hour timepoints in both the vector alone cells ($p=0.99$), and in the EGFR overexpressing cells ($p=0.7955$) (Figure 3.1.6). This finding suggests that EGF-stimulated junctional breakdown is targeted at the cadherin portion of the junction.

ß-catenin not only functions as a part of the junctional complex, but can also act as a transcriptional regulator when translocated to the nucleus. Two classes of ß-catenin target genes have been identified. The first, or classical ß-catenin targets, are genes important in cell survival, proliferation and apoptosis (Moon *et al.*, 2002). The second, or non-classical ß-catenin target genes, are those important in EMT, such as Slug, tenascin-C, survivin, and MMP14 (Brabletz *et al.*, 2005). To test whether there may be differences in ß-catenin signaling, cells were treated with EGF, and nuclear localization of ß-catenin was examined. Vector control cells showed intense cytoplasmic staining for ß-catenin (Figure 3.1.6) with 48 hours EGF treatment, although not all the nuclei of cells stain positive for ß-catenin. However, EGFR overexpressing cells have both cytoplasmic and nuclear staining of ß-catenin. This indicates that overexpression of the EGFR causes a redirection of at least a portion of the total

ß-catenin pool to the nucleus. Similar results were observed following 5.5 hours of EGF treatment, as EGFR overexpressing cells had a portion of the total population staining positive for nuclear ß-catenin (Figure 3.1.7, white arrows). This asynchronous nuclear localization of ß-catenin is apparent at several timepoints in EGFR overexpressing cells after EGF addition, and suggests that ß-catenin nuclear localization could be important throughout reepithelialization.

Another way to further investigate the impact of elevated EGFR levels on keratinocyte function is to use a cell line that has endogenously elevated EGFR levels. The SCC 12F cell line is originally of head and neck origin, and has endogenous overexpression of EGFR levels, similar to the levels seen in the transfected SCC 13 EGFR cell line (McCawley *et al.*, 1997). The response of SCC 12F cells treated with EGF for various timepoints was comparable to that of the EGFR overexpressing SCC 13 cells. While it appears that there is a decrease in ß-catenin protein in SCC 12F cells (Figure 3.1.8), no statistically significant changes occurred between control and 24 or 48 hours post treatment (24h p=0.5117, 48h p=0.5150). ß-catenin mRNA levels were investigated to determine if elevated EGFR levels might be causing transcriptional repression of ß-catenin (Figure 3.1.9). No changes in ß-catenin transcript levels were seen, indicating that the junctional protein is not being modulated by this mechanism.

Nuclear localization of ß-catenin in SCC 12F cells was apparent around 4 hours post treatment (Figure 3.1.10, white arrow), but is transient, as no nuclear localization of ß-catenin was evident at 6 hours post treatment. This timecourse is in agreement with the SCC 13 EGFR cells short time course. In order to

confirm the presence of ß-catenin in the nucleus, nuclear extracts were collected after an EGF timecourse from 2-48 hours. Using an antibody that recognized the activated form of ß-catenin, which is dephosphorylated on Ser-37 or Thr-41, an increase in ß-catenin is apparent after 2 hours EGF treatment and is persistent at 24 and 48 hours post EGF treatment (Figure 3.1.11).

However, this nuclear localization of transcription factors is not global, as the ß-catenin family member plakoglobin did not undergo nuclear localization at the same timepoints (Figure 3.1.12). Although plakoglobin mirrored ß-catenin in regards to protein (Figure 3.1.8) and transcript levels (Figure 3.1.9) after EGF treatments, at 5.5 hours post EGF treatment, both vector control cells and the EGFR overexpressors maintained plakoglobin at cell-cell junctions, indicating that EGFR overexpression preferentially relocalizes ß-catenin to the nucleus, but not its family member plakoglobin.

The next step was to investigate the possibility of altered gene expression as a function of ß-catenin nuclear localization due to increased EGFR levels. Non-classical downstream targets of ß-catenin include the transcriptional factor Slug and the extracellular protein tenascin-C, both of which have been associated in cells with an EMT-like phenotype in development and cancer. In wound healing, tenascin-C is upregulated early, and has been shown to be produced by keratinocytes (Latijnhouwers *et al.*, 1997). Slug, as mentioned earlier, is required for EGFR dependent reepithelialization (Savagner *et al.*, 2005). In order to look at transcriptional upregulation of these ß-catenin target genes, RT-PCR of Slug and tenascin-C was performed (Figure 3.1.13) from RNA

collected after different timepoints of EGF treatment. An induction of the Slug transcript is visible after 1 hour of EGF treatment, and is persistent up to 4 hours post treatment. Tenascin-C is upregulated 24-48 hours post EGF treatment. This indicates that two known ß-catenin transcriptional targets are upregulated after EGFR activation in a SCC cell line that moderately overexpresses the EGFR.

Overall, these data provide evidence that increased availability of ligand and transient increase of EGFR that is present at the wound margin leads to keratinocyte modification of its cell-cell junctions. An increase in EGFR levels is necessary for junctional disruption in keratinocytes and this disruption likely leads to ß-catenin dependent transcription events that may regulate classical targets, such as cell proliferation genes cyclin-D, as well as non-classical targets important in EMT events, including the transcription factor Slug and the extracellular matrix protein tenascin-C. Regulation of ß-catenin target genes could be a contributing factor to cell migratory behavior during reepithelialization.

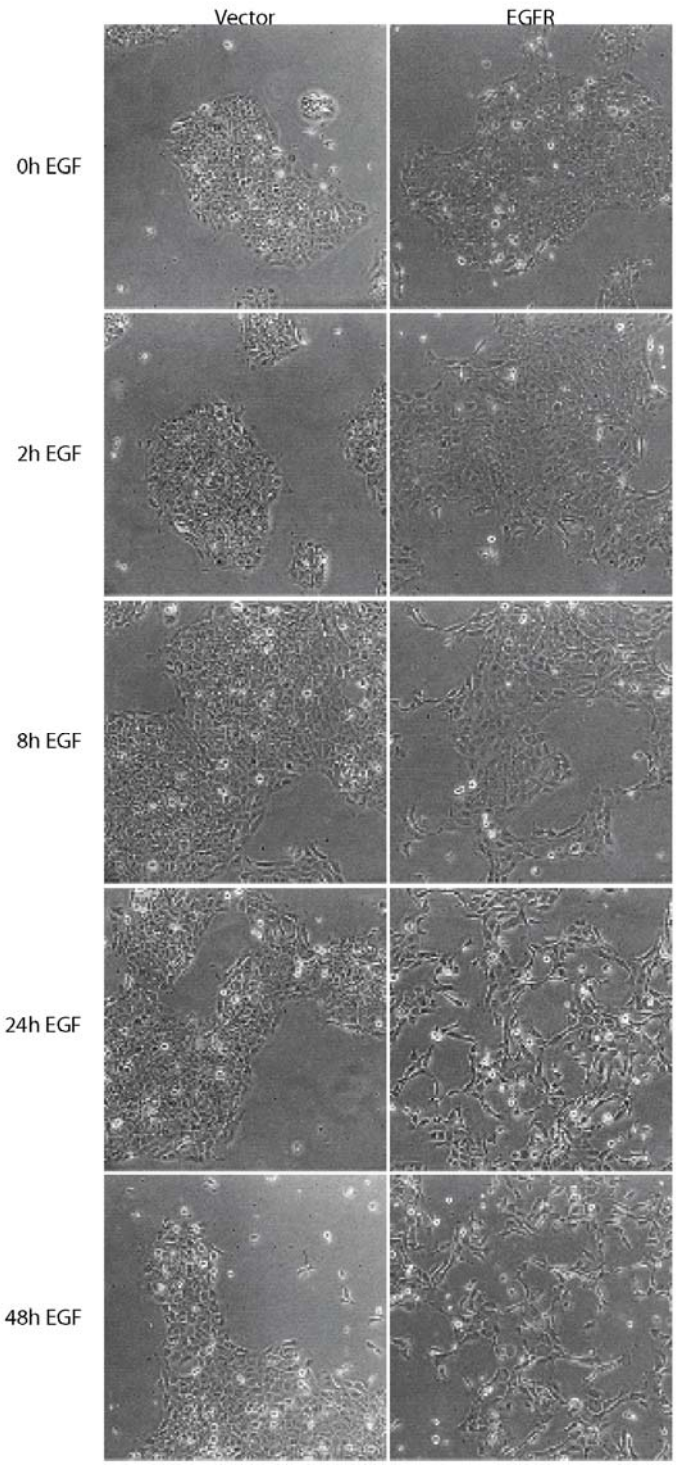


Figure 3.1.1 Elevated EGFR levels required for cell scattering.
EGF timecourse comparison of SCC13 cells transfected with vector alone, or with a vector containing EGFR. With no treatment both sets of cells display an epithelial morphology that is retained up to 4 hours post EGF treatment. However, from 8-48 hours post EGF treatment, EGFR overexpressing cells scatter, typical of a loss of cell-cell junctions in an EMT-like event. Vector control cells with low levels of EGFR maintain their epithelial shape and do not scatter in response to EGF.

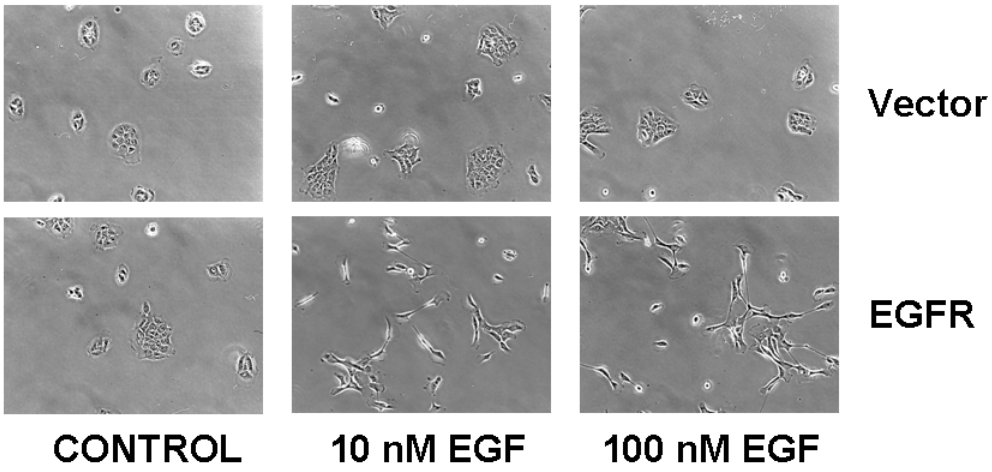


Figure 3.1.2 SCC 13 Vector control cells do not migrate in response to increased levels of EGF

SCC 13 Vector control cells and EGFR overexpressing cells were serum starved overnight, then treated with either 10 nM EGF or 100 nM EGF for 24 hours. EGFR overexpressing cells scatter at both concentrations. Vector control cells remain in colonies regardless of the concentration of EGF present.

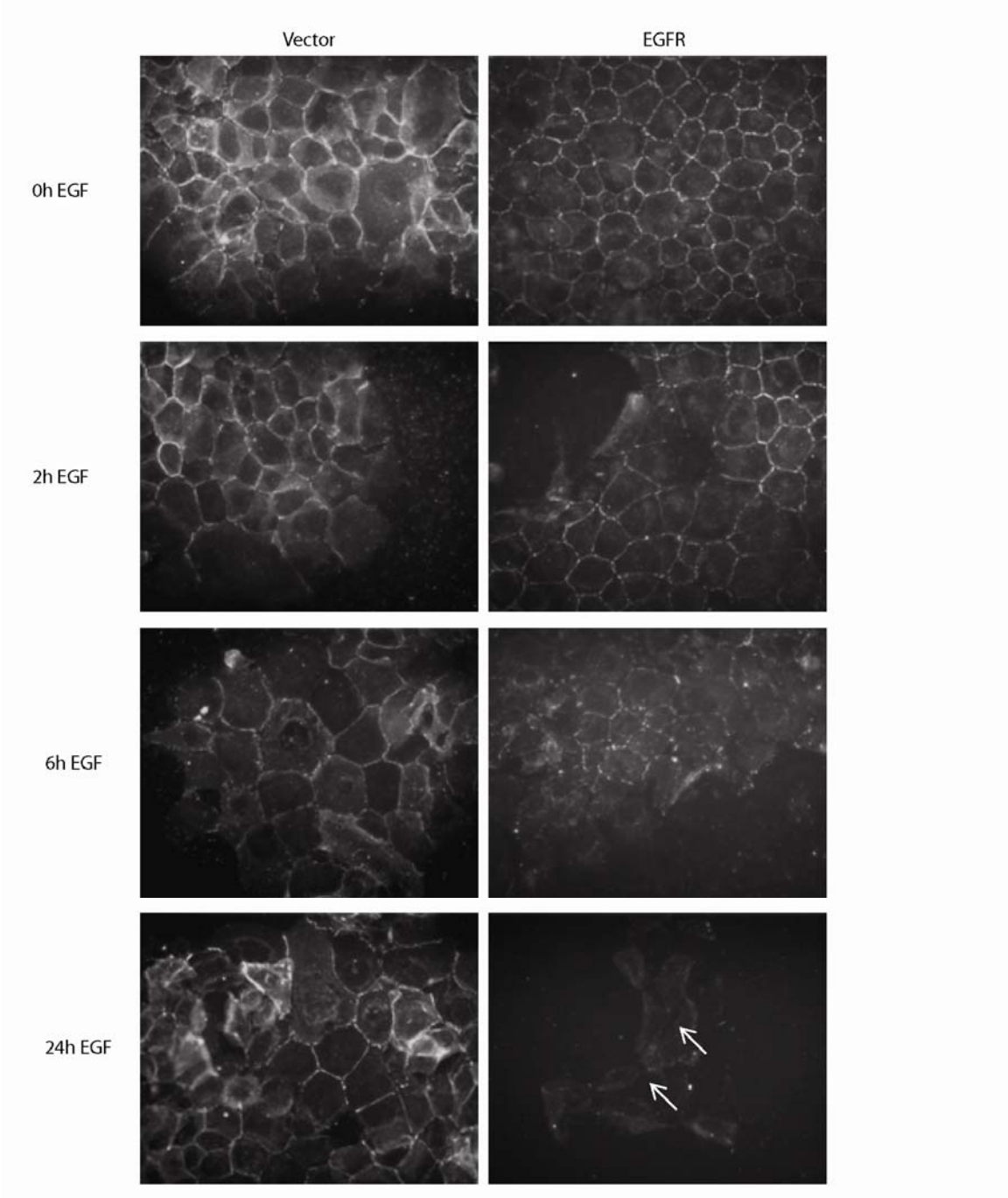


Figure 3.1.3 Elevated EGFR necessary for adherens junction disruption
SCC13 cells transfected with vector alone or with EGFR were treated for increasing times with 20 nM EGF. Cells were then fixed and immunofluorescently labeled for the adherens junction protein ß-catenin. Adherens junction disruption is visible by the loss of ß-catenin staining (white arrows) in the EGFR overexpressing cells, while vector control cells maintain junctions for at least 24 hours post EGF treatment.

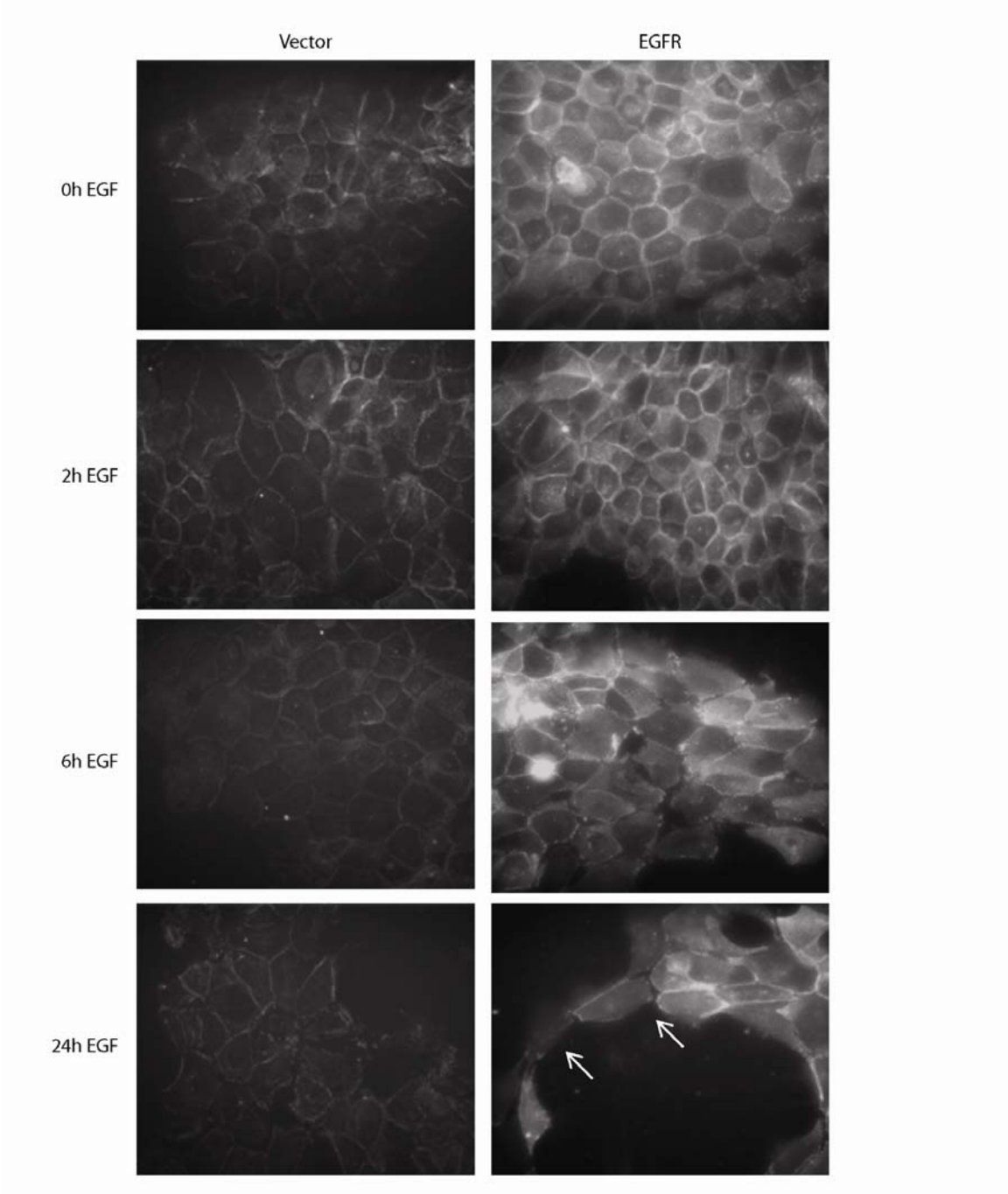


Figure 3.1.4 Elevated EGFR is required for desmosomal disruption

SCC13 cells transfected with vector alone or with EGFR were treated for increasing times with 20 nM EGF. Cells were then fixed and immunofluorescently labeled for the desmosomal protein desmoglein-2. Desmosomal junction disruption is visible by the loss of desmoglein staining (white arrows) in the EGFR overexpressing cells, while vector control cells maintain junctions for at least 24 hours post EGF treatment. The shape change in morphology, similar to what is seen in Figures 3.1.1. and 3.1.2 is also visible in the EGFR overexpressing cells in this experiment.

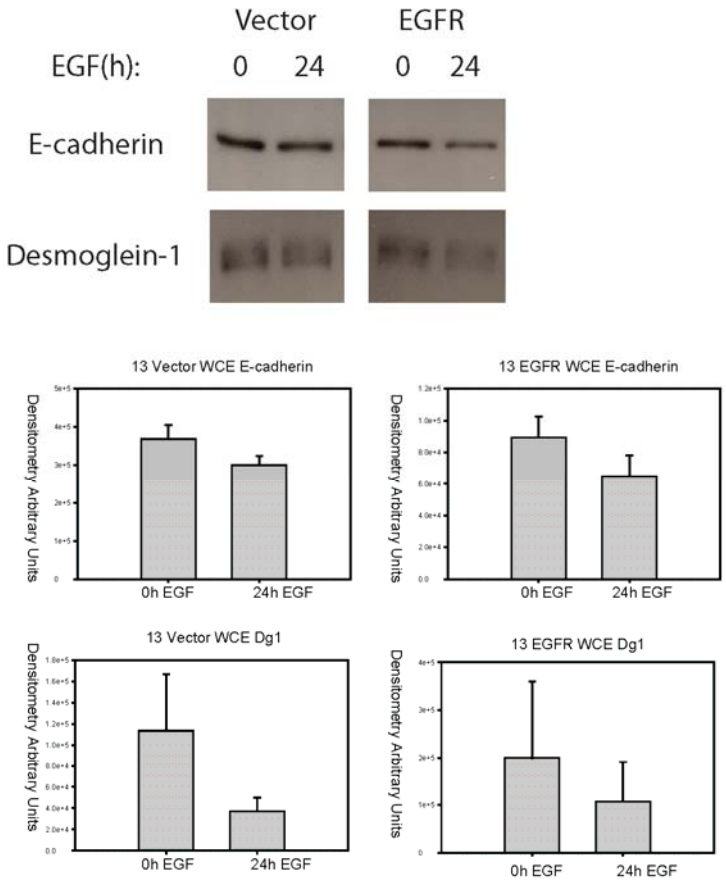


Figure 3.1.5 Elevated EGFR does not significantly decrease total cadherin protein levels
SCC13 cells transfected with either vector alone or with EGFR were serum starved overnight. Total protein was collected after no treatment or treatment with 20 nM EGF for 24 hours. Both E-cadherin and desmoglein-1 appear to decrease after EGF treatment in EGFR overexpressing cells, but not to a statistically significant degree as measured by densitometry.

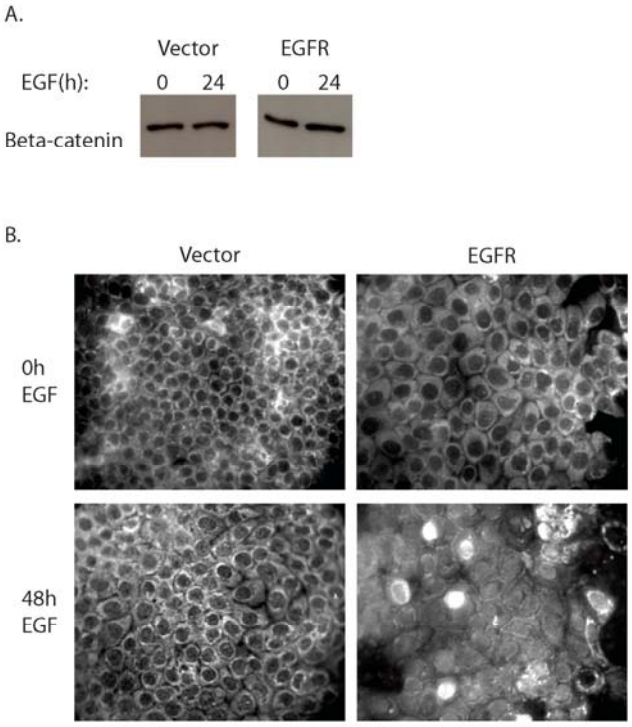


Figure 3.1.6 Localization of β-catenin differs between control and EGFR overexpressing cells without alterations in total protein levels

A. SCC13 cells transfected with either vector alone or with EGFR were serum starved overnight. Total protein was collected after no treatment or treatment with 20 nM EGF for 24 hours. There is no change in ß-catenin protein levels between the untreated and 24 hour treated cells for either the vector alone cells nor the EGFR overexpressing cells. B. SCC13 vector cell line and EGFR cell line were serum starved for 24 hours, then treated with either vehicle (0h) or EGF for 48 hours. After cell fixation, cells were permeabilized and stained for ß-catenin. Cytoplasmic ß-catenin is visible in both vector alone and EGFR cells. However, 48 hours post EGF treatment, nuclear localization of ß-catenin is visible only in the EGFR transfected cells.

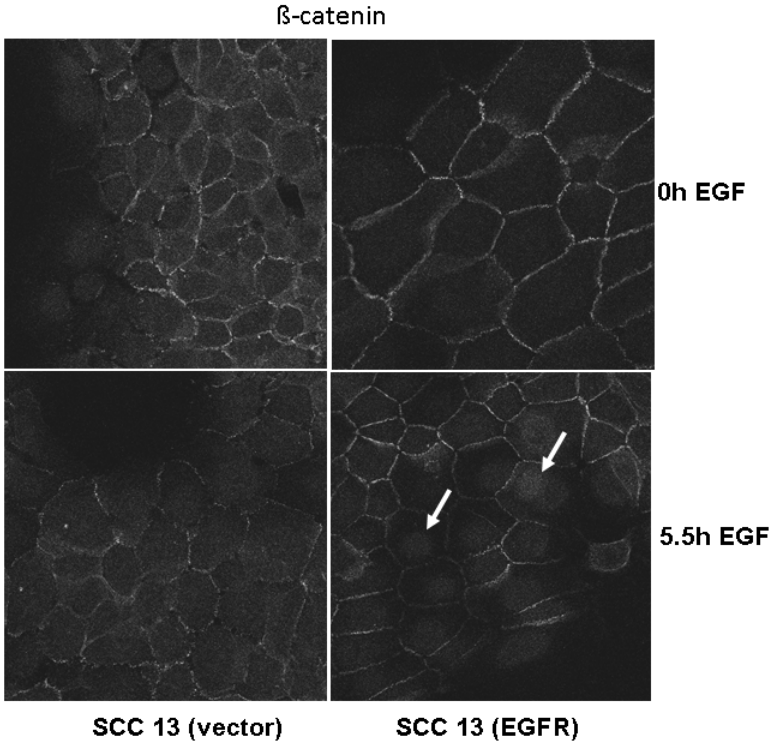


Figure 3.1.7 Nuclear localization of ß-catenin after EGF stimulation requires elevated EGFR levels

SCC13 cells transfected with either vector alone or with EGFR were serum stared overnight then treated with vehicle (0h) or with 20 nM EGF for 5.5 hours. After fixation and permeabilization, cells were stained for ß-catenin. While vector control cells show no change in border staining for ß-catenin, populations within the EGFR cells show nuclear staining after 5.5 hours of EGF treatment.

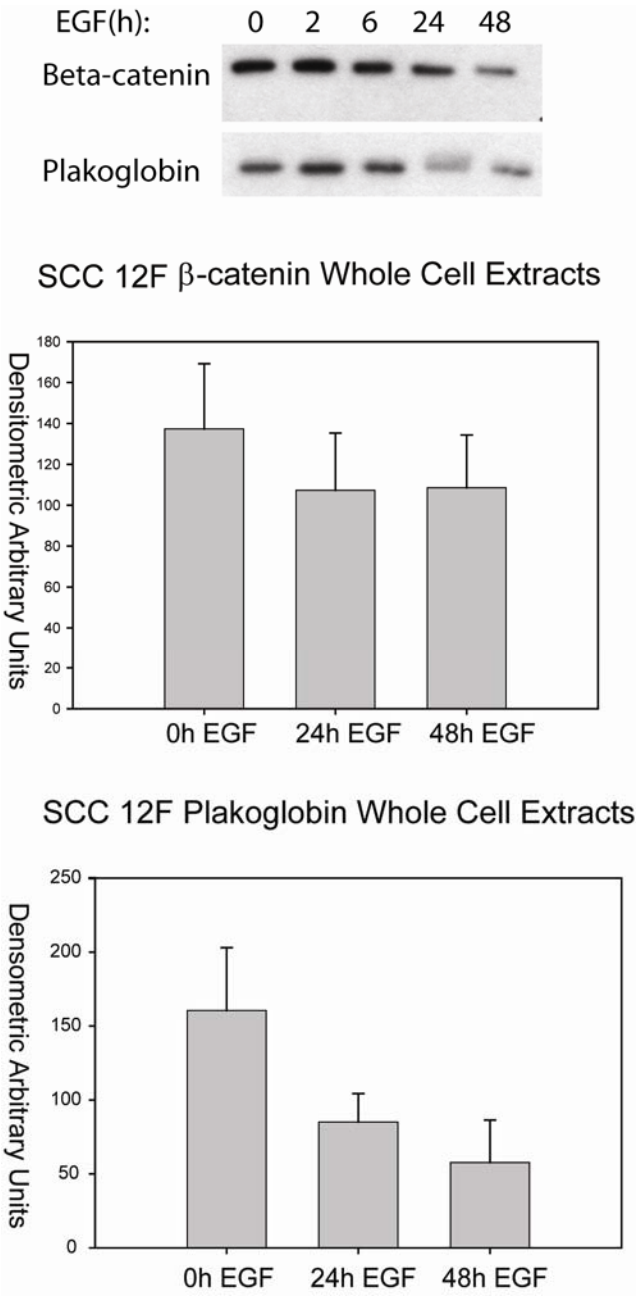


Figure 3.1.8 Catenins show decreasing trend after EGF treatment in SCC 12F cell line. Cells were grown to subconfluence, placed in serum free medium overnight, and then treated with EGF for various timepoints. Although both ß-catenin and plakoglobin decrease upon extended time with EGFR activation, the observed decrease was not statistically significant. Results are representative of a minimum of three separate experiments. Bar graphs represent the densitometric quantification of each lane normalized to no treatment control.

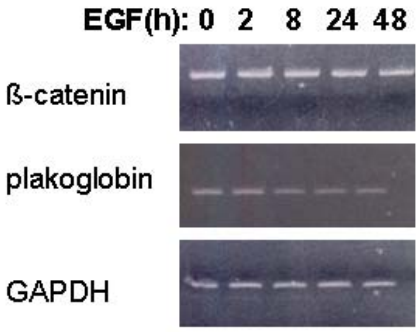


Figure 3.1.9 Neither β-catenin nor plakoglobin are transcriptionally regulated following EGF treatment

SCC 12F were serum starved and treated with 20 nM EGF for various timepoints. Total RNA was collected and RT-PCR performed. No change in ß-catenin or plakoglobin transcription occurs between 2 to 48 hours post EGF treatment. GAPDH represents loading control.

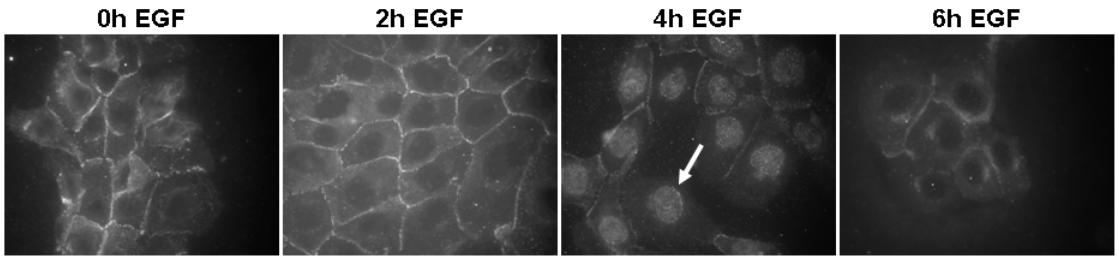


Figure 3.1.10 SCC 12F cells display transient nuclear localization of ß-catenin following short term EGF exposure.

SCC 12F cells were serum starved overnight and treated for 2-6 hours with 20 nM EGF. Cells were then fixed, permeabilized, and stained for ß-catenin. A transient nuclear localization of ß-catenin is apparent at 4 hours post EGF treatment (white arrow), similar to the timeframe for the SCC 13 EGFR cells (see Figure 3.1.7).

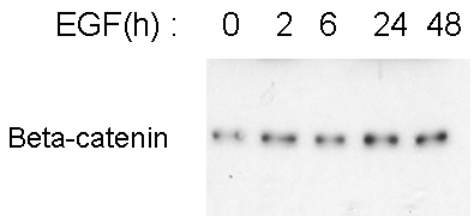


Figure 3.1.11 ß-catenin protein is increased in nuclear extracts following extended EGF treatment.

SCC 12F cells were serum starved overnight and treated with 20 nM EGF for the indicated times. Cells were scraped and the nuclear extract was isolated. Using an antibody specific for active ß-catenin, we see an increase in the total amounts of ß-catenin found in the nucleus upon extended EGF treatment.

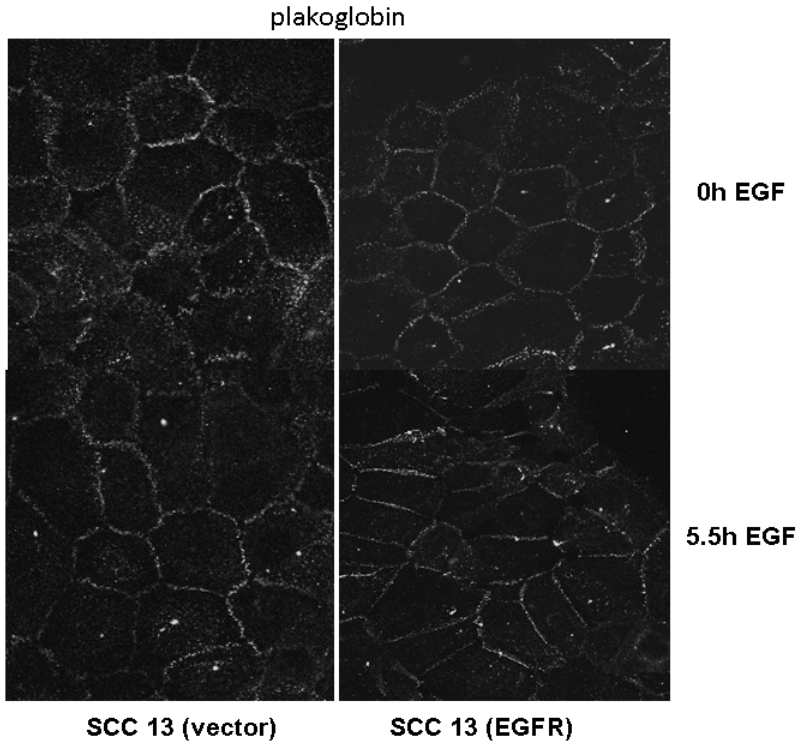


Figure 3.1.12 Neither vector nor EGFR overexpressing cells have nuclear plakoglobin following short term EGF treatment

SCC 13 cells transfected with either vector alone or with EGFR were serum starved overnight and then treated with vehicle (0h) or with 20 nM EGF for 5.5 hours. After fixation and permeabilization, cells were stained for plakoglobin. Neither vector control cells nor EGFR cells show any change in border staining for plakoglobin.

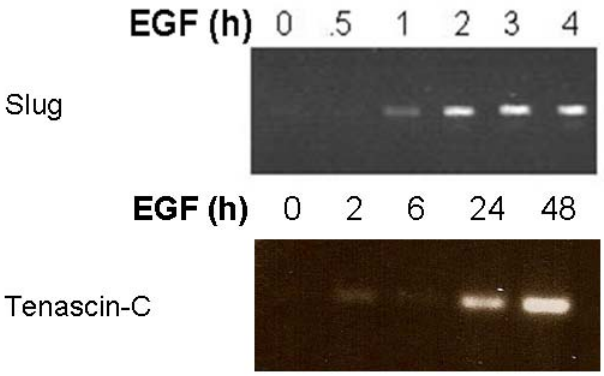


Figure 3.1.13 Non-classical ß-catenin targets are upregulated after EGF treatment in SCC 12F cell line

SCC 12F cells were serum starved and treated with 20nM EGF for the indicated times. Total RNA was isolated and RT-PCR against Slug and tenascin-C transcripts reveals a modest upregulation in Slug transcript by 1 hour post EGF treatment that increases at 2 hours and remains persistent up to 4 hours post treatment. Tenascin-C transcript is upregulated after 2 hours at 24 hours post EGF treatment and persists up to 48 hours.

3.2 Effects of elevated EGFR at wound margins on junctional and cytoskeletal components

EGF receptor activation has been reported to stimulate wound repair *in vivo* (Nanney *et al.*, 2000; Repertinger *et al.*, 2004) and disrupt junctional complexes *in vitro* (Bhora *et al.*, 1995; Hudson and McCawley, 1998). We conducted *in vitro* wound closure assays using SCC 12F cells, a well-differentiated squamous cell carcinoma line (Hudson *et al.*, 1986) to investigate the status of desmosomal and adherens junctions at an *in vitro* wound border upon EGFR inhibition (Figure 3.2.1). The goal of this experiment was to investigate the contributions of EGFR activation under basal (serum free conditions), serum stimulated conditions, as well as EGF stimulated conditions. Contributions of the EGFR were singled out by using AG1478, an inhibitor that affects the catalytic domain of the receptor. Wound closure in serum free conditions (addition of BSA) occurs to a small degree (around 15%) even in the absence of exogenous EGF, however, the increase in wound closure is greatly increased with the addition of EGF (Figure 3.2.1). Migration into the wound area was substantially inhibited by inclusion of EGFR tyrosine kinase inhibitor AG1478 in the culture medium, indicating that activated EGFR is involved in the response (Figure 3.2.1). Inhibition of the EGFR also decreases migration in serum containing conditions, indicating the contribution of the EGFR even in the presence of other serum proteins. Again, inhibition of the EGFR with AG1378 attenuates the migration of cells regardless of the presence of EGF. An

interesting finding in this set of wound closure assays was the decline of the basal, unstimulated response of these cells upon EGFR inhibition both in the serum free and serum containing experiments. Autocrine production of EGF in keratinocytes has been reported (Pittelkow *et al.*, 1993) and could be responsible for both the unstimulated response and the corresponding decrease seen with EGFR inhibition in the serum free conditions. The decrease in migration in the presence of serum upon EGFR inhibition indicates the necessity of this pathway, even when migration is being stimulated by ligands other than EGF.

EGFR activity upon EGF stimulation was responsible for cell scattering and eventual junctional disruption in cells with elevated EGFR (Section 3.1). In addition, junctional disruption was detected at wound margins as evidenced by the loss of catenin border staining for both ß-catenin and plakoglobin (Figure 3.2.2, arrows). Positive catenin staining at the wound edge in serum free, unstimulated conditions with EGFR inhibition suggests that junctional integrity was retained once the EGFR was no longer active (Figure 3.2.2, arrowheads). It is likely that both desmosomal and adherens junctional components are sensitive to EGFR activity. As might be expected, cadherin staining was also altered at the wound margin (Figure 3.2.3). Whereas cells with no treatment and presumably elevated EGFR activity had disrupted cadherin staining (Figure 3.2.3, white arrows), cells under conditions of EGFR inhibition (+AG1478) had stronger cell staining at cell borders at the wound margin (Figure 3.2.3, white arrowheads).

In order to confirm that the loss of junctional proteins from the cell surface results in disruption of a functional junction, we used confocal microscopy to look

at the scaffolding proteins at the junction in relationship to their associated cytoskeletal attachments in the context of wound healing. The actin cytoskeleton is indicated by the marker phalloidin (Figure 3.2.4), and the intermediate filaments are indicated by staining for cytokeratin (Figure 3.2.5). Both sets of cytoskeletal markers were shown to lose their organization at the wound edge 1 day post wounding (right panels). Upon EGFR inhibition, this disorganization was less apparent, as the cells appeared to maintain their cytoskeletal structure right up to the wound margin. Cells at the confluent interior (left panels) had no such defects in structure after wounding. Actin stress fibers and the keratin intermediate filament network were evident in the confluent monolayer and showed a great deal of organization as evidenced by the filamentous extentions across the entirety of the cell up to the cell borders, although it did appear that with EGFR inhibition, less overall stress fibers were evident (Figure 3.2.4, bottom right panel), although junctional integrity was maintained.. These results indicate that cytoskeletal disruption, and in effect, junctional disruption, are localized to the wound margin, are dependent on EGFR activity, and do not consist of a global signaling throughout the monolayer.

Reepithelialization represents a partial and reversible EMT *in vivo,* so the timeline of junctional disruption in our system was further investigated. Cells were allowed to grow out into the wounded area from 1 to 3 days. After 3 days, junctional proteins plakophilin (Figure 3.2.6) and ß-catenin (Figure 3.2.7) were apparent at the borders of cells at the wound margins. Both cytokeratin staining (3.2.6) and the actin cytoskeleton (Figure 3.2.7) have restored their organization

in cells at the wound margin, which is now identical to the staining found in cells in the confluent interior. This indicates that the modulation of the cell-cell junctions and their corresponding cytoskeletal components by the EGFR is a transient and reversible phenomenon.

These data collectively demonstrate EGFR dependent junctional disruption, cytoskeletal reorganization, and cell migration, even in the absence of exogenous EGF. These phenomena are limited to the wound margin, as cells distant to the wound edge are unaffected. Indeed, a recent study found that patients with chronic, non-healing wounds have a marked reduction of EGFR levels in the ulcerated epidermis as compared to adjacent, nonulcerated skin (Brem *et al.*, 2007), indicating the clinical relevance to our *in vitro* studies.

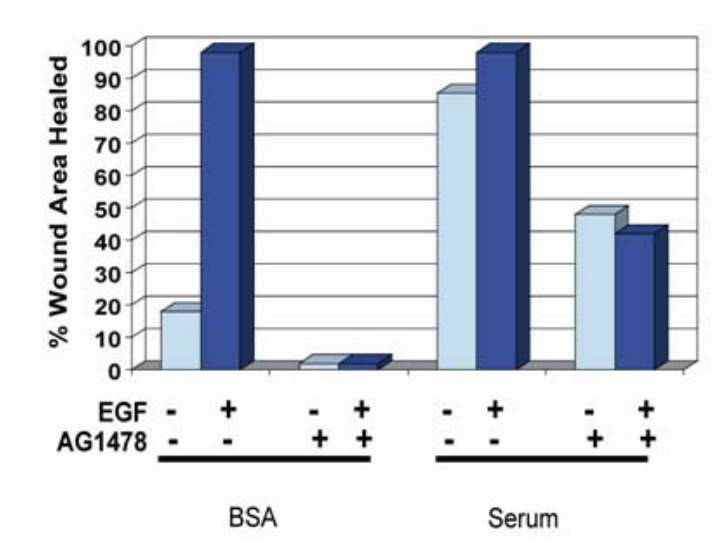


Figure 3.2.1 SCC12F cells in vitro reepithelialization highlights necessity of EGFR for wound closure

SCC 12F cells were grown to confluence, placed in serum free medium overnight, and then a wound was introduced with a pipette tip. Cells were washed 3 times with PBS and then returned to serum free medium +/- 5 uM of the selective EGFR inhibitor AG1478. The area of wound closure was measured using ImagePro software and compared with the inclusion or absence of serum in the medium concurrently in the presence of EGF or AG1478. Upon EGFR inhibition, migration into the wounded area is impaired. This inhibition occurs in both serum free medium and in serum containing medium, indicating the importance of EGFR activation in cell migration.

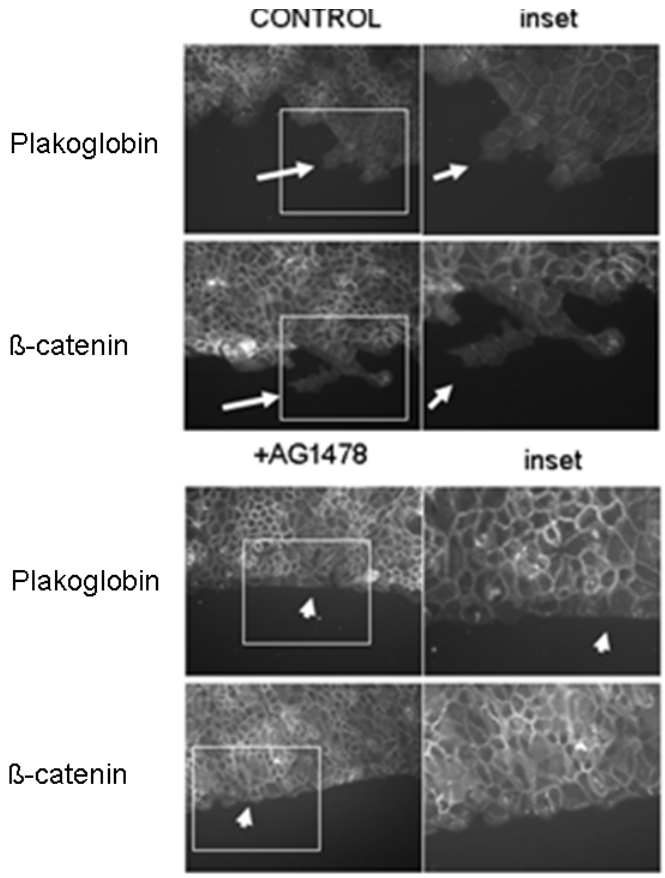


Figure 3.2.2 Catenin Immunofluorscence at wound edge shows junctional protection
After wounding, SCC 12F cells were washed 3 times with PBS, then placed back in serum free medium +/- 5 uM of the selective EGFR inhibitor AG1478. After 24 hours, cells were fixed and immunostained for either plakoglogin or ß-catenin. Note the loss of border staining is limited to the wound edge (arrows). Upon EGFR inhibition, junctional proteins are apparent at the wound edge (arrowheads).

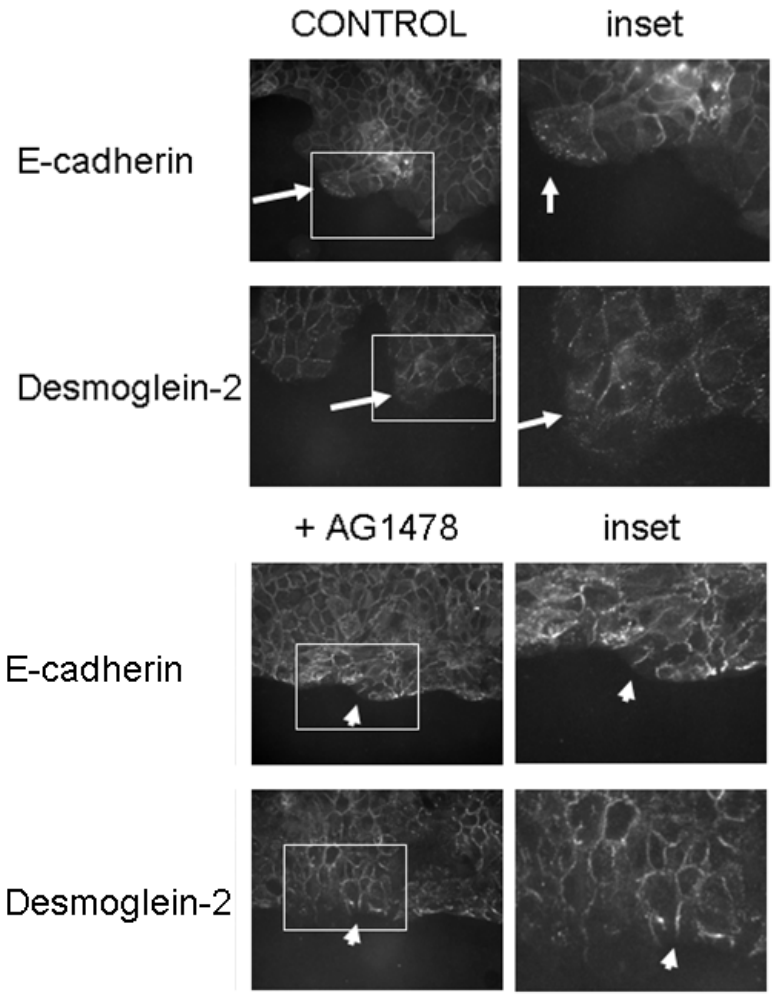


Figure 3.2.3 **Cadherin immunofluorescence at wound margin reveals junctional protection with the EGFR inhibitor AG1478**

After wounding, SCC 12F cells were washed 3 times with PBS, then placed back in serum free medium +/- 5 uM of the selective EGFR inhibitor AG1478. After 24 hours, cells were fixed and immunostained for either E-cadherin or desmoglein-2. Note the loss of border staining is limited to the wound edge (arrows). Upon EGFR inhibition, junctional proteins are apparent at the wound edge (arrowheads).

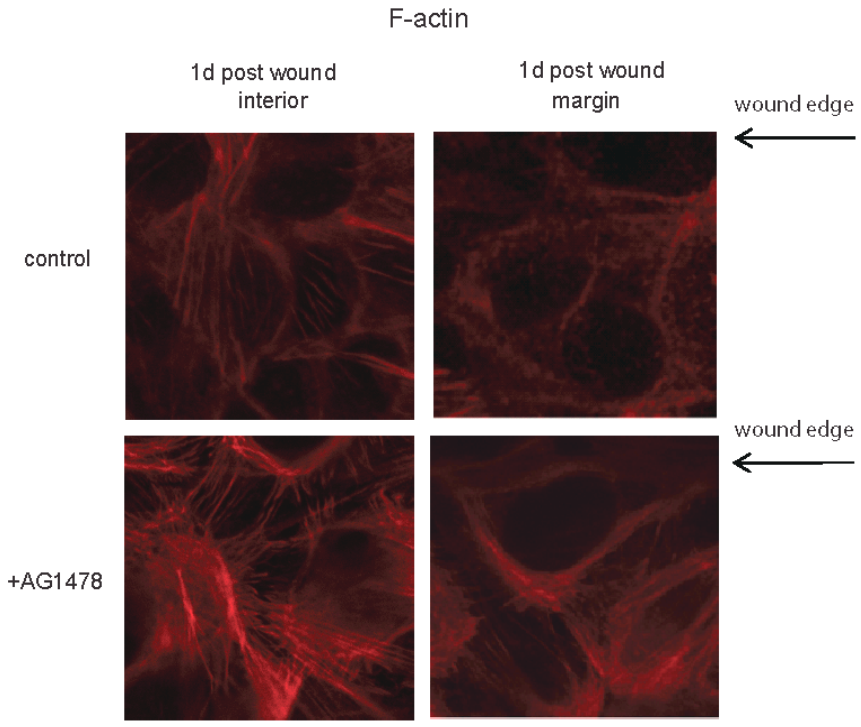


Figure 3.2.4 Actin cytoskeleton maintained at wound margin with inhibition of EGFR activity
SCC 12F cells were grown to a confluent monolayer, and then a mechanical wound was introduced. The cells were then grown in low serum media with or without the addition of AG1478 for 24 hours then fixed and stained for cytoskeletal marker F-actin. Disregulation of the actin cytoskeleton is visible in the upper right hand panel, in which the EGFR is not inhibited, and disorganization is restricted to the wound edge area, as the interior of the wound (left hand panels) are undisturbed.

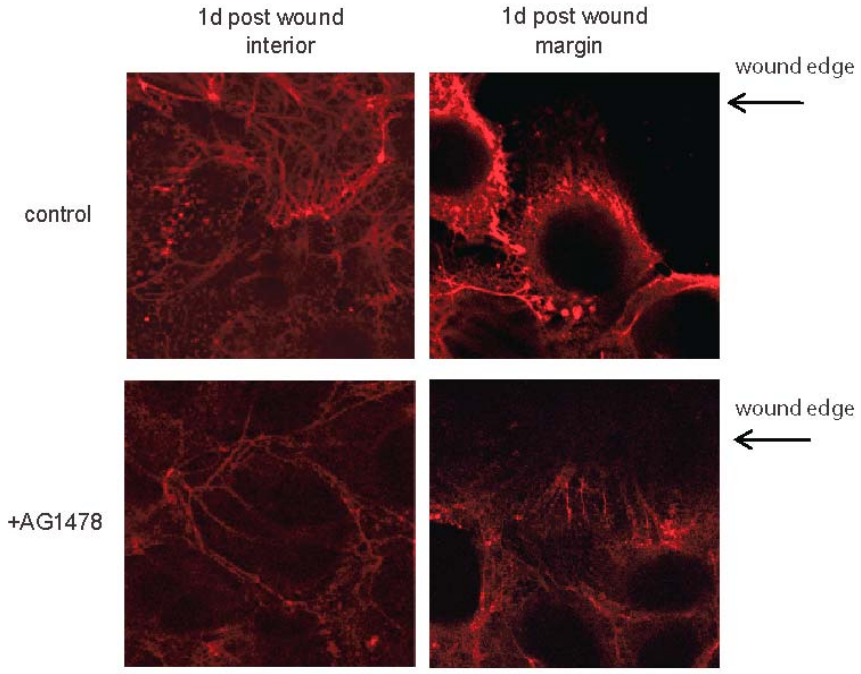


Figure 3.2.5 Cytokeratin organization maintained at wound edge with inhibition of EGFR activity

SCC12F cells were grown to a confluent monolayer, and then a mechanical wound was introduced. The cells were then grown in low serum media with or without the addition of AG1478 and stained for interfilament marker cytokeratin proteins. Similar to the results for the actin cytoskeleton, intermediate filaments as the wound edge are disturbed when the EGFR is not inhibited. This disruption is localized to the wound edge, as the confluent interior (left panels) shows no defects in organization.

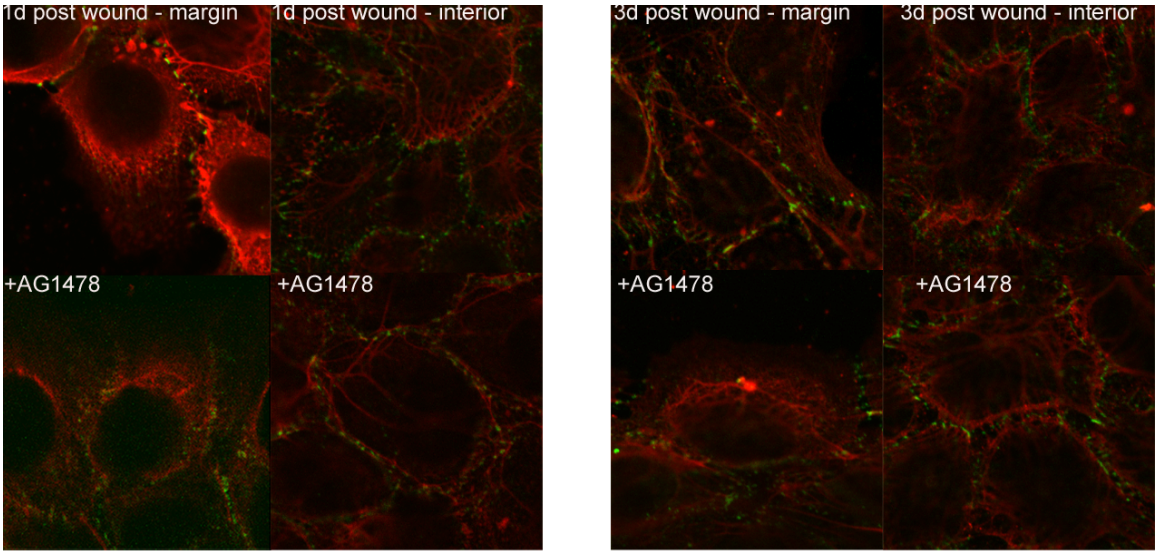


Figure 3.2.6 Disruption of cytokeratin cytoskeleton at the wound margin is transient, and restored 3 days post wounding.

SCC 12F cells were grown to a confluent monolayer, and then a mechanical wound was introduced. The cells were then grown in low serum media with or without the addition of AG1478 for 24 hours then fixed and stained for the desmosomal junctional protein plakophilin (green staining) and the intermediate filament protein cytokeratin (red staining). 3 days post wounding, junctions are restored, as evidenced by plakophilin-2 (Pkp2) staining at the wound margin and reorganization of the actin cytoskeleton.

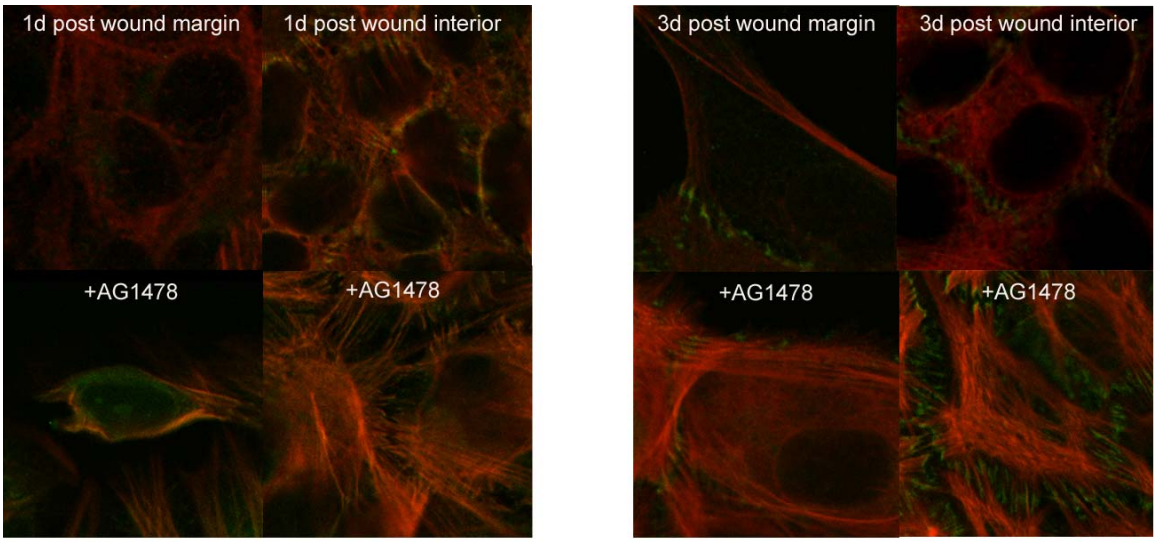


Figure 3.2.7 Disruption of actin cytoskeleton at the wound edge is transient, restored after 3 days post wound

SCC 12F cells were grown to a confluent monolayer, and then a mechanical wound was introduced. The cells were then grown in low serum media with or without the addition of AG1478 for 24 hours then fixed and stained for the adherens junction protein ß-catenin (green staining) and the cytoskeletal marker F-actin (Red staining). 3 days post wounding, junctions are restored, as evidenced by ß-catenin staining at the wound margin and reorganization of the actin cytoskeleton.

3.3 Differences in EGF stimulated changes in Adherens Junctions versus Desmosomes

EGFR has been reported to modulate intracellular junctional proteins, namely the catenins, by various mechanisms, such as phosphorylation, leading to down-regulation and protein degradation (See Section 1.7.3, Table 2). As the catenins are very well studied in response to EGFR activation, we decided to focus on the cadherin responses to EGFR activation by EGF. Cadherins are also regulated in different systems, with varied routes of regulation possible (Figure 1.7.2). Whole cell extracts were collected and examined to determine if differences existed between the SCC 12F cells and the earlier mentioned SCC 13 cells, which showed no statistically significant differences in the cadherin levels after 24 hours of EGF treatment (Figure 3.1.5). Although SCC 12F cells have similar amounts of EGFR as the SCC 13 EGFR cell line (McCawley *et al.*, 1997), we see a statistically significant decrease in desmoglein-2 cadherin levels after 24 hours EGF treatment as compared to the SCC 13 EGFR cell line (Figure 3.3.1 A vs. Figure 3.1.5). No change was seen in desmoglein-1 cadherin after 24 hours in the SCC 13 cell line, which may be due to differences in the cell line or in the kinetics of regulation between the two desmoglein isoforms. E-cadherin levels decreased to a statistically significant amount after 48 hours of EGF treatment (Figure 3.3.1 A). SCC 13 E-cadherin protein levels appeared to be decreasing at 24 hours, but it was not statistically significant ($p<0.05$). Total cadherin protein levels in the SCC 12F cells decreased after EGF treatment, but

it was not clear if certain pools were preferentially targeted. Using sequential detergent extraction, I was able to compare protein levels of membrane-associated (triton soluble) versus junction-bound (triton insoluble) cadherins. I found that extended exposure to EGF redistributed both the desmosomal cadherin desmoglein-2 and adherens junction protein E-cadherin from the cell membrane (Figure 3.3.1 B) as well as from preformed junctions (Figure 3.3.1 C). Interestingly, not all junctional components were affected, as there was no decrease in total protein of the adherens junctional linker protein α-catenin (Figure 3.3.1).

Although both cadherins were being redistributed in response to EGF, I saw a difference in the time dependent loss of individual cadherin proteins in response to EGF in the membrane-associated and junction-associated protein pools. The triton soluble, membrane-associated fraction showed a decrease in desmoglein-2 protein as early as 6 hours, whereas a statistically significant decrease in E-cadherin levels does not occur until 24 hours (Figure 3.3.1 B, asterisks). Similar to the triton soluble fraction, the triton insoluble fraction, which contains intact junctions, did not show statistically significant decrease in E-cadherin until 24 hours post EGF treatment. Desmoglein-2, however, had a statistically significant decrease as early as 2 hours post EGF treatment (Figure 3.3.1 C, asterisks). In all cases, we saw that cadherins were lost from cell membrane pools in response to EGF and that desmoglein-2 was preferentially lost first. This suggests that desmosomes are modulated by EGF exposure through different mechanisms than adherens junctions.

Since transcriptional repression of E-cadherin is one reported mechanism of down-regulation (26, 27), we examined mRNA levels for E-cadherin and desmoglein-2 in response to EGF (Figure 3.3.2). In a time course that spanned 2-48 hours, no statistically significant changes in either E-cadherin or desmoglein-2 transcript levels were evident. We also saw no change in E-cadherin transcript levels using quantitative real time PCR (data not shown). This result indicates that cadherin expression is being regulated at the protein level.

In order to elucidate the mechanism for downregulation, we examined cadherins by immunofluorescence at timepoints that showed statistically significant decreases in protein levels. In a timecourse that spanned 24 hours, we saw redistribution of cadherins from the sites of cell-cell contacts. E-cadherin staining was retained at the cell periphery for up to several hours after EGF treatment (Figure 3.3.3, upper panel arrow), and evidence of redistribution from cell borders occurred after 24 hours of exposure. In contrast, internalization of desmoglein-2 was evident within 6 hours of EGF treatment as determined by punctate intracellular staining (Figure 3.3.3, lower panel arrow). This supports the hypothesis that EGF exposure modulates desmosomes earlier than adherens junctions through distinct mechanisms.

Immunofluorescence staining of the corresponding cytoskeleton partners showed disruption of the desmosomal-associated cytokeratin network at 6 hours, the same time frame as that of desmosomal cadherin internalization (Figure 3.3.4). The adherens junction-associated actin cytoskeleton remained intact at

this timepoint, consistent with E-cadherin localization remaining at cell-cell junctions at the plasma membrane, disruption of the actin skeleton was seen at 24 hours post treatment (data not shown). This indicates a functional disruption of desmosomes that precedes that of adherens junctions.

In order to determine if these observed differences in the kinetics of junction disruption were due to intrinsic differences in the junctions themselves or if the differences were stimulus driven, we compared EGF-induced cadherin internalization against another known disruptor of junctions, low calcium conditions. In response to low calcium, E-cadherin protein levels were decreased by 15-30 minutes post removal and undetectable after 24 hours (Figure 3.3.5). Desmoglein-2 protein was decreased within 2 hours and also was undetectable after 24 hours of low calcium. This loss of junction due to reduced extracellular calcium was more rapid compared to the EGF timecourse (Figure 3.3.1 A), where E-cadherin protein levels remained unchanged at 6 hours post EGF treatment and decreased at 24 hours post treatment. EGF stimulation did not cause a significant decrease in desmoglein-2 protein levels until 24 hours post treatment. This suggests not only that the pathways for junctional modulation differs between EGF stimulation and low calcium removal, but that the kinetics for junctional disruption differs under both sets of stimuli.

In response to low calcium, both E-cadherin and desmoglein-2 localization was completely intracellular, and mostly perinuclear at 2 hours post removal (Figure 3.3.6). When normal calcium levels were restored, however, E-cadherin was visible at cell borders within 4 hour of restoration (Figure 3.3.6, upper panel

arrows), while desmoglein-2 was not visible at cell borders until 24 hours post calcium restoration, indicating that while E-cadherin is rapidly restored to the cell surface, the kinetics of desmoglein-2 restoration is markedly slower. When treated with EGF for 24 hours, E-cadherin became very diffuse and almost undetectable in the cytoplasm (Refer to Figure 3.3.3). Removal of EGF and the return of cells to basal conditions showed a slower restoration of E-cadherin to the cell surface than with low calcium (compare to Figure 3.3.6), returning 6 hours after removal of EGF and was still incomplete after 24 hours (Figure 3.3.7, upper panel, arrows). Restoration of E-cadherin to cell-cell borders was finally detectable in some cells by 48 hours post EGF reversal (Figure 3.3.8). Desmoglein-2, however, showed a similar pattern of slow restoration to the cell surface as in the low calcium reversal (compare to Figure 3.3.6), but only started to reappear 24 hours after removal of EGF in some cells (Figure 3.3.7, lower panel, arrowheads). By 48 hours post EGF removal, nearly all cell-cell borders stained for desmoglein-2. An antibody that recognizes 2 isoforms of the desmosomal cadherin desmocollin (desmocollin-2, desmocollin-3) revealed essentially no staining after 48 hours in EGF free conditions. This suggests that EGF treatment does not have the same effect on the different cadherins. Collectively, these data support the hypothesis that different mechanisms for cadherin internalization from the cell surface occur between low calcium and EGF exposure and that the kinetics between cadherins differ in both disassembly and reassembly even with two different stimuli.

These data, taken together, suggest a differential regulation of E-cadherin and desmoglein-2 by EGF, exemplified by the kinetics in protein downregulation, corresponding cytoskeletal disruption and decrease in overall cell border staining. When compared to another stimulus, low calcium, again we saw differences between cadherins in kinetics of low calcium disruption of junctions and junctional reassembly once calcium was restored. When compared to reassembly after EGF removal, we saw not only differences in kinetics between junctional reassembly, but differences in the kinetics between stimuli. Overall, these data suggest different itineraries and timelines for cadherins depending on the individual cadherin in question and the stimuli presented. The variety of ligands and other stimuli present at the wound margin could be working on junctional modulation with different results depending on the timing and the cadherin in question.

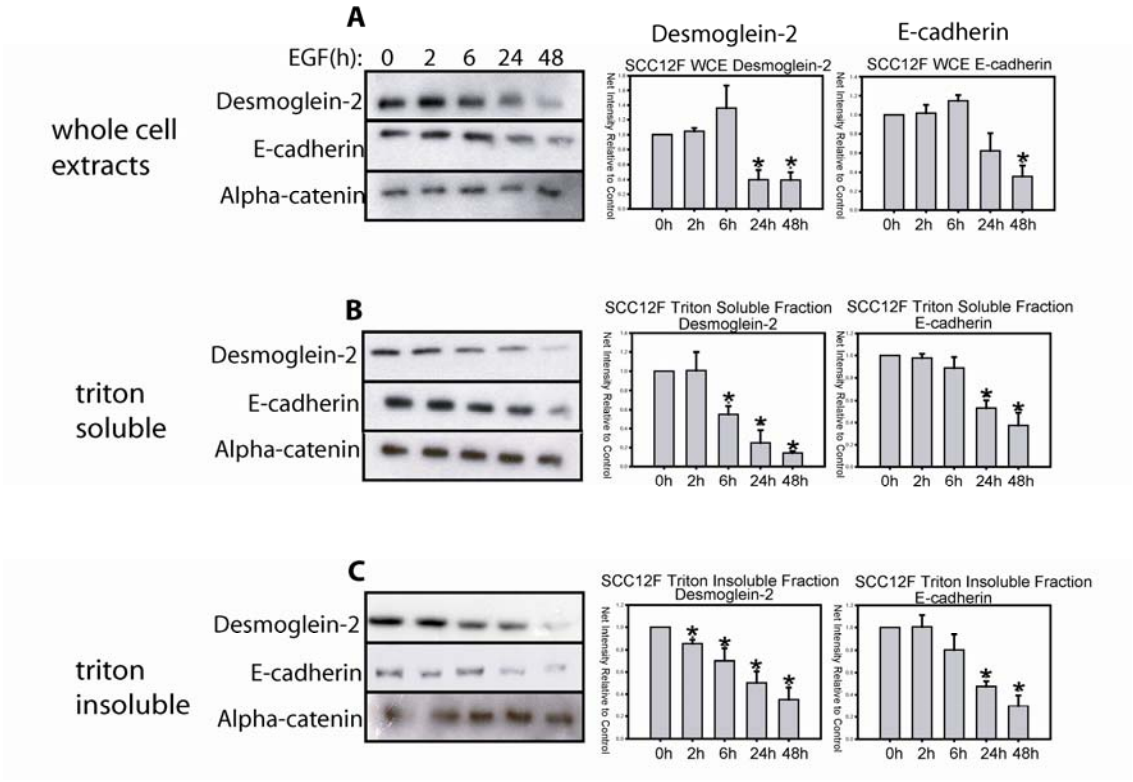


Figure 3.3.1 Cadherin protein levels decrease from membrane pools upon extended EGF treatment

SCC 12F cells were grown to subconfluence, placed in serum free medium overnight, and then treated with EGF for various timepoints. A. Whole cell lysates reveal a decrease in both E-cadherin and desmoglein-2 at 24 to 48 hours post EGF treatment. α-catenin, an adherens junction protein, showed no change. Sequential detergent extraction separates the triton soluble, or cytoplasmic fraction (B), from the triton insoluble, or membrane associated fraction (C). A decrease in total desmoglein-2 is evident as early as 6 hours post treatment in the triton soluble, membrane associated pool, but as early as 2 hours in the triton insoluble, preformed junction pool. E-cadherin levels decrease by 24 hours in the both fractions. Results are representative of a minimum of three separate experiments. Bar graphs represent the densitometric quantification of each lane normalized to no treatment control, with asterisks indicating statistical significance. ($p<0.05$)

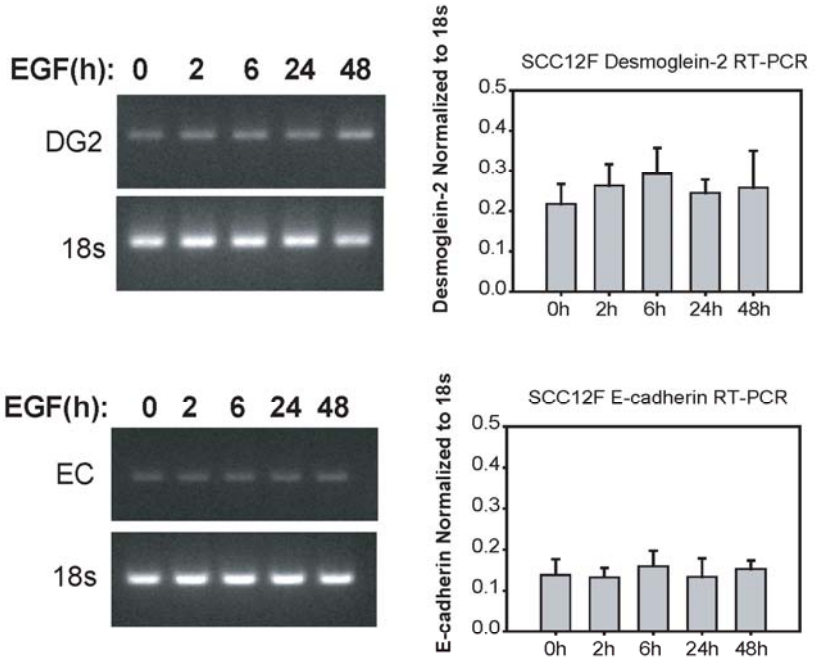


Figure 3.3.2 Cadherins are not transcriptionally regulated in response to EGFR activation.

SCC 12F cells were treated with EGF for various times from 2 hours up to 48 hours. Control cells had no EGF treatment. Bar graphs represent the densitometric quantification of bands normalized to no treatment control. No changes in desmoglein-2 or E-cadherin mRNA levels are seen, suggesting that cadherin protein loss from the junctions is due to a protein downregulation and not a change in mRNA levels of the genes.

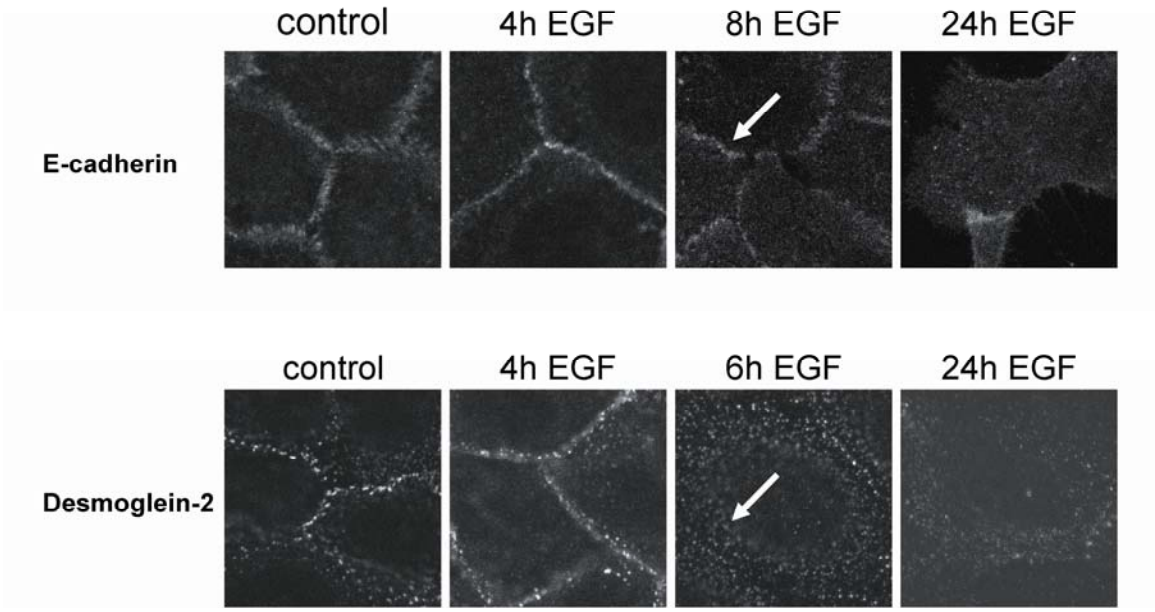


Figure 3.3.3 EGF dependent relocalization of cadherins timeline differs
SCC 12F cells were treated with EGF for the indicated timepoints. Cells were fixed, and then probed with E-cadherin or desmoglein-2 antibodies. E-cadherin maintains strong border staining at 8 hours post EGF treatment (upper panel arrow). Loss of E-cadherin staining from cell borders is not evident until 24 hours post EGF treatment. In contrast, relocalization of the desmosomal cadherin desmoglein-2 from cell borders occurs following treatment with EGF for 4-6 hours (lower panel arrow).

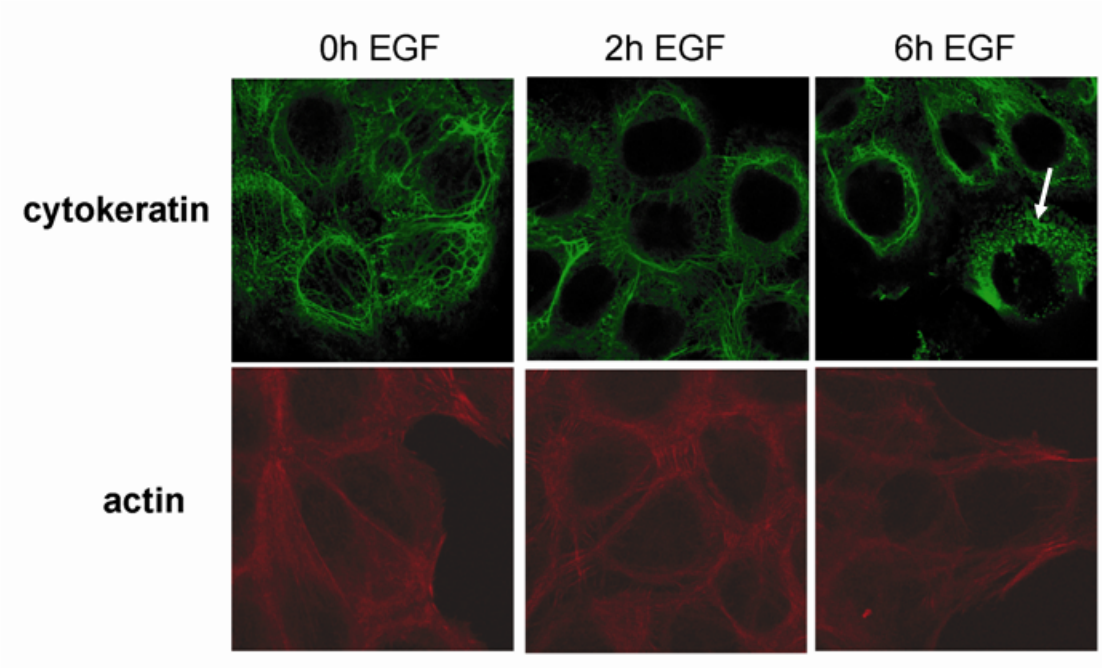


Figure 3.3.4 Cytoskeletal changes with EGF treatment

The cytoskeletal counterparts cytokeratin and actin also respond differently to EGF treatment. SCC 12F cells were treated with EGF, fixed, then stained with phalloidin to stain the actin cytoskeleton, or with the antibody pan-cytokeratin, which labels keratin filaments. Disorganization of the keratin network is seen at 6 hours (white arrow), while the actin cytoskeleton remains intact at 8 hours post treatment.

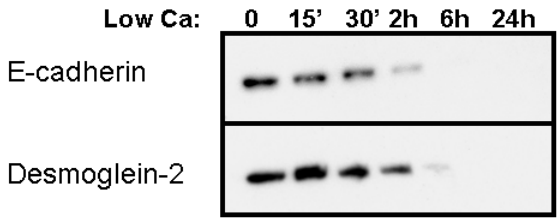


Figure 3.3.5 Cadherins downregulated after low calcium treatment as soon as 2 hours post calcium removal.

SCC 12F cells were grown to subconfluence, placed in serum free medium overnight, and placed in low calcium media for various timepoints. A decrease in E-cadherin protein can be seen as early as 2 hours in low calcium medium, and is completely undetectable after 6 hours in low calcium. A decrease in desmoglein-2 is visible as early as 2 hours post treatment, and is undetectable at 24 hours post low calcium exposure.

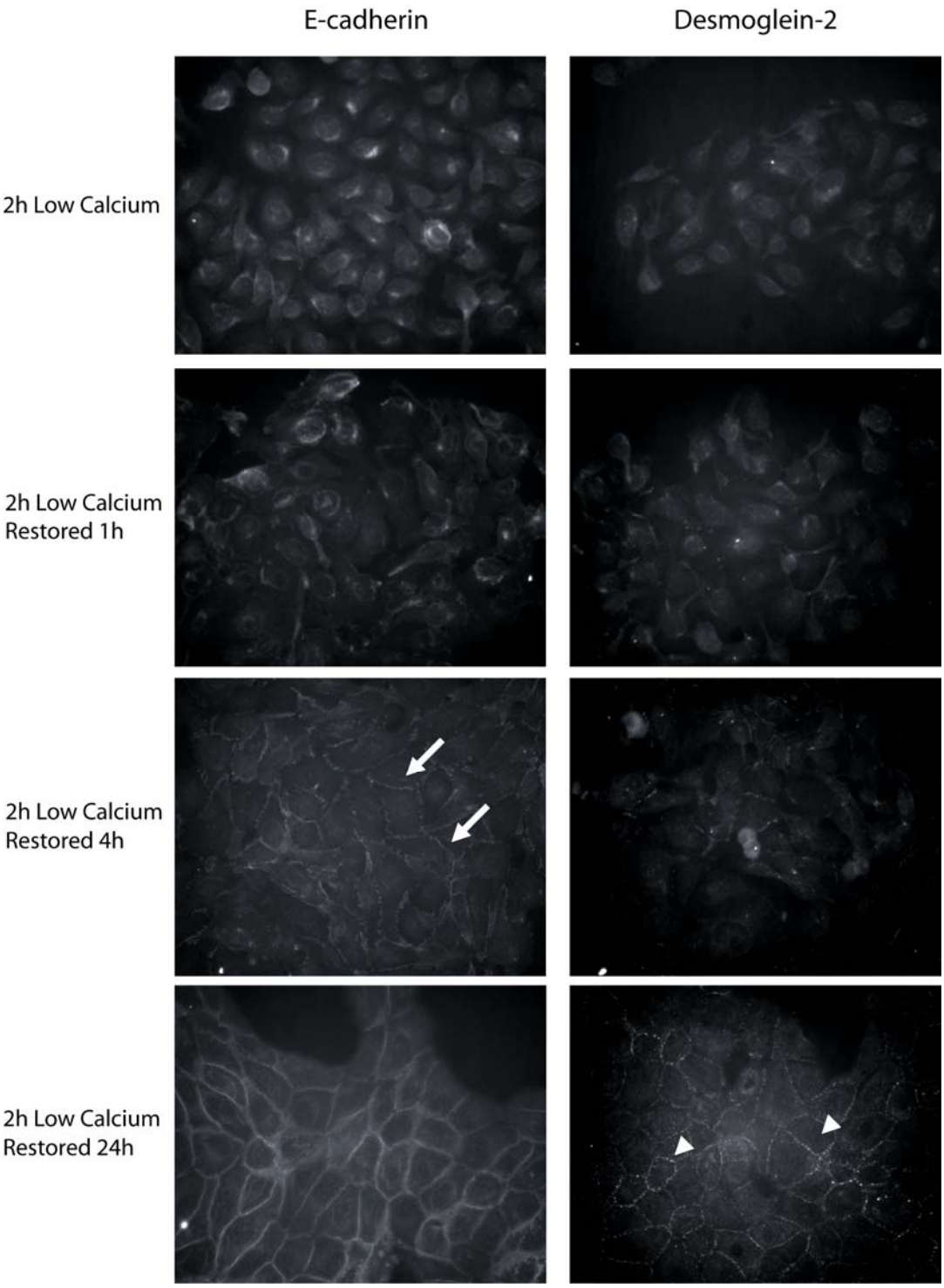


Figure 3.3.6 Short term low calcium reversal presents different kinetics for E-cadherin and desmoglein-2

SCC 12F cells were serum starved then placed in low calcium media (0.5 mM) for 2 hours. After 2 hours, cells were placed in calcium containing serum free media. E-cadherin is visible at the cell surface 4 hours after calcium addition, while desmoglein-2 is not visible at the cell surface until 24 hours after calcium addition, indicating different kinetics responsible for junctional restoration after disruption by low calcium.

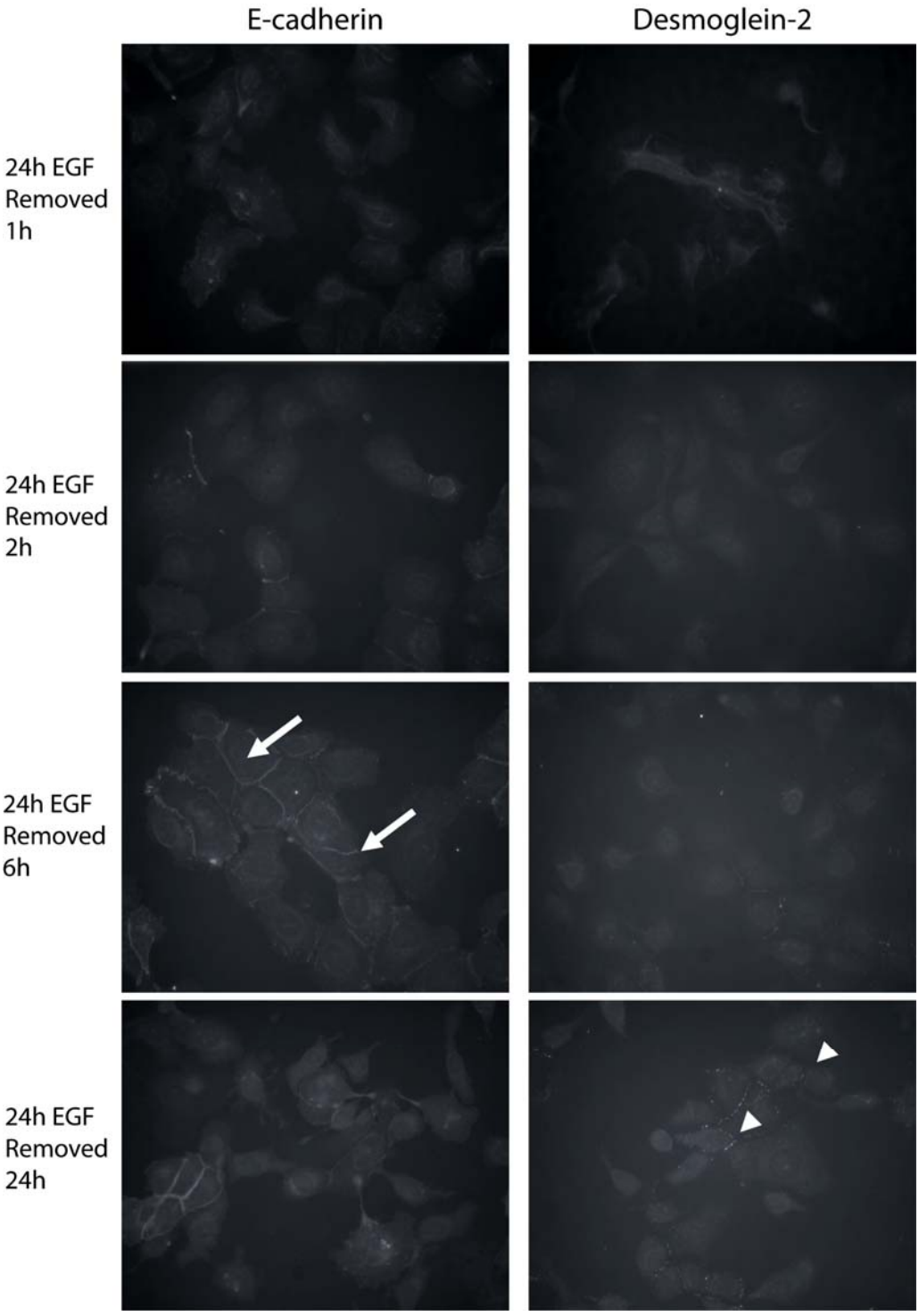


Figure 3.3.7 Short term reversal from EGF exposure finds different kinetics of restoration between E-cadherin and desmoglein-2.

SCC 12F cells were serum starved in control lanes, then treated with 20 nM EGF for 24 hours. After 24 hours, stimulus was removed, by washing away EGF and placing cells in serum free media for the indicated times. E-cadherin starts to reappear at cell borders by 6 hours of EGF removal (arrows), while desmoglein-2 starts to reappear at the cell surface at 24 hours after EGF removal (arrowheads), indicating different kinetics responsible for individual cadherin restoration.

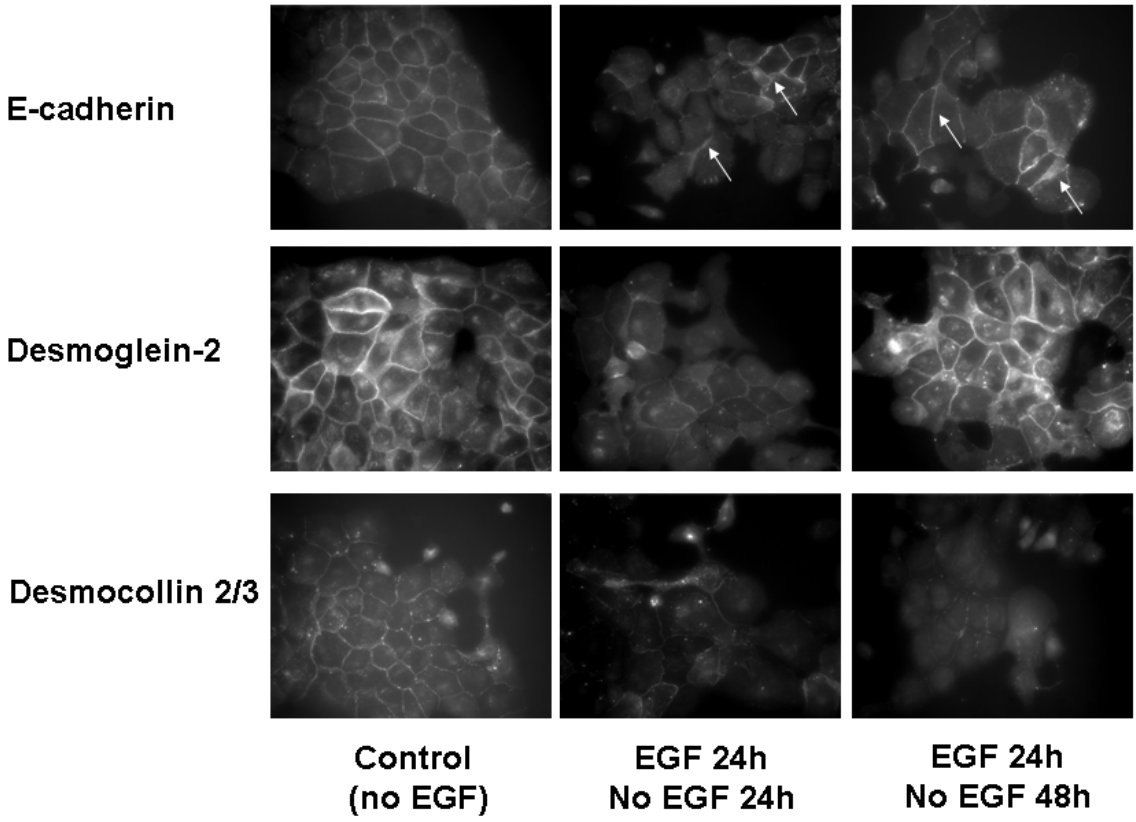


Figure 3.3.8 Long term reversal of EGF stimulus shows differences in between the different cadherins

SCC 12F cells were serum starved in control lanes, then treated with 20 nM EGF for 24 hours. After 24 hours, stimulus was removed, by washing away EGF and placing cells in serum free media. E-cadherin and desmoglein-2 were restored to the cell surface by 48 hours after EGF removal, while desmocollins fail to appear at the cell borders, indicating different kinetics responsible for individual cadherin restoration.

3.4 EGF Stimulated Cadherin Trafficking Itineraries

Investigation into the possibility of cadherin internalization was based on the preliminary indication that in response to EGF, desmoglein-2 staining was punctate and cytoplasmic (Figure 3.3.3). Although there have been several studies of E-cadherin internalization in response to various growth factors (Kamei *et al.*, 1999; Palacios *et al.*, 2002; Bryant *et al.*, 2005; Bryant *et al.*, 2007), EGF-stimulated trafficking of desmosomal cadherins has not been as well described. Internalization of desmoglein-3 in response to pemphigus antibody was found to undergo a clathrin independent internalization and subsequent lysosomal degradation (Calkins *et al.*, 2006; Delva *et al.*, 2008). EGFR inhibition has been shown to restore desmosomal cadherins to the cell surface and prevents a cleavage event seen in the highly motile squamous cell carcinoma line (Klessner *et al.*, 2008), highlighting the importance of the EGFR regulation for junctional integrity. EGFR activation stimulates multiple trafficking pathways including clathrin dependent and independent (Lu *et al.*, 2003; Bryant *et al.*, 2007; Ning *et al.*, 2007) trafficking itineraries. EGFR itself undergoes a clathrin dependent route of internalization upon stimulation with EGF(Huang *et al.*, 2004; Sigismund *et al.*, 2008), although it can undergo clathrin independent internalization under certain situations such as oxidative stress (Khan *et al.*, 2006).

Although cadherin modulation is an EGFR-stimulated event, we found that neither E-cadherin nor desmoglein-2 co-localized with the EGFR following EGFR activation. EGFR internalization preceded that of desmoglein-2 by several hours (Figure 3.4.1, white arrows). This suggests that while the internalization of

desmoglein-2 is due to EGFR activation, it does not internalize with the receptor itself. Dual immunofluorescence staining for both desmoglein-2 and E-cadherin revealed that desmoglein-2 colocalized with E-cadherin at the cell surface with no treatment, but did not colocalize with E-cadherin in the cytosol post EGF treatment (Figure 3.4.2, white arrows), in agreement with our earlier observation of different staining patterns with single staining over prolonged EGF treatment, further indicating distinct fates for the two cadherins.

After 6 hours of EGF treatment, desmoglein internalization was evident by immunofluorescence. To determine whether this internalization occurs through the classical endosomal pathway or through other internalization pathways, we used several markers of the classical endosomal pathway (EEA-1, Rabs) as well as markers for caveolar dependent internalization (caveolin-1). Interestingly, portions of E-cadherin at the cell membrane colocalized with caveolin-1 at the cell surface (Figure 3.4.3). However, there was no intracellular co-localization of either E-cadherin (Figure 3.4.3, arrows) or desmoglein-2 (Figure 3.4.4, arrows) with caveolin-1, a marker for the caveosomal dependent trafficking. EGF dependent caveolin-1 endocytosis has been reported previously by our laboratory, in an ovarian cancer cell line, which mediated integrin α2 internalization (Ning *et al.*, 2007). Desmoglein-2 differs from E-cadherin at the cell surface as no membrane colocalization was found between desmoglein-2 and caveolin-1. This suggests that while some E-cadherin colocalized in caveolin-1 rich domains, neither it nor desmoglein-2 internalized with caveolin-1 upon EGF stimulation.

The next pathway we investigated was the classical endosomal pathway. EEA-1 is a marker of early endosomes, and while no colocalization of E-cadherin with EEA-1 was evident at any timepoint, a small portion of desmoglein-2 was present in EEA-1 positive early endosomes at 6 hours post EGF addition (Figure 3.4.6, white arrows). This indicates that a while a small portion of desmoglein-2 is found in early endosomes, the majority of desmoglein-2 and E-cadherin is not found in early endosomes after EGF addition.

Although a small amount of desmoglein-2 was detected in early endosomes, there was no evidence of desmoglein colocalization with rab7 (Figure 3.4.7), a late endocytic vesicle marker, and only a small amount colocalized with lysotracker, a marker of lysosomes, at 12 hours post EGF treatment (summarized in Table 3.4.1). The majority of desmoglein-2 co-localized with the recycling marker Rab11 (Figure 3.4.8). In timepoints from 2-6 hours post EGF treatment, desmoglein-2 and rab11 colocalized in small punctate vesicles both on the plasma membrane and in the cytosol (Figure 3.4.8, white arrows). This represents a novel internalization pathway for desmoglein-2. Upon EGF treatment, the desmoglein-2 protein undergoes a predominantly recycling pathway rather than a lysosomal degradation pathway.

E-cadherin, however, did not colocalize at any timepoint (up to 10 hours post EGF treatment) with the late endosomal protein Rab-7 (Figure 3.4.9), the recycling protein Rab-11 (Figure 3.4.10), nor was it found in the lysosome (Table 3.4.1). We can conclude from these findings that another mechanism other than

internalization and trafficking is responsible for the downregulation of E-cadherin after EGFR activation.

In contrast to desmoglein-2, EGF treatment revealed a gradual decrease in the intensity of E-cadherin staining at cell-cell borders without an emergence of punctate intracellular staining indicative of internalization (see Figures 3.4.2, 3.4.3, 3.4.5, 3.4.9 and 3.4.10). Therefore I tested if E-cadherin could be undergoing a cleavage event at the plasma membrane. Immunoblotting revealed a time dependent increase in an 80 kD E-cadherin fragment in the conditioned medium upon extended EGF treatment that was statistically significant at 18 hours and 24 hours post treatment (Figure 3.4.11). When stripped and probed for desmoglein-2, we saw no evidence of cleavage products (data not shown). We then tested if this cleavage event could be prevented by pretreatment with a broad spectrum MMP inhibitor. I employed an antibody against an intracellular epitope for immunofluorescent detection of E-cadherin within the cells. I found loss of E-cadherin staining at the cell borders 19 hours post EGF treatment in control cells. Cells pretreated with the broad spectrum MMP inhibitor GM6001, however, retained junctional E-cadherin staining at the plasma membrane (Figure 3.4.12). The addition of the MMP inhibitor maintained adherens junction integrity even upon EGF stimulation, indicating an MMP-dependent process for adherens junction disruption. Conversely, this protection was not extended to the desmosomal cadherin desmoglein-2, as internalization occurred independently of the MMP inhibitor (Figure 3.4.13). Thus we can conclude that the decrease in E-cadherin staining is due at least in part to cleavage events by

MMPs. This MMP cleavage is not extended to all cadherins, as desmoglein-2 is not protected from internalization with the addition of inhibitor. This demonstrates distinct fates for the desmosomal cadherin, desmoglein-2, as compared to the classical cadherin, E-cadherin, upon EGF stimulation in a keratinocyte cell line. Protein collected after 24 hours EGF treatment revealed E-cadherin protein levels were decreased as would be expected from Figure 3.3.1, but when pretreated with the MMP inhibitor GM6001, E-cadherin levels were restored to control levels (Figure 3.4.1). Desmoglein-2, although partially protected from downregulation with GM6001, was not restored to control levels. This suggests that the majority of EGF-dependent E-cadherin downregulation is due to MMP cleavage. It also indicates that downregulation of desmoglein-2 may be complex, as a portion of desmoglein-2 protein is protected with the use of the MMP inhibitor.

Collectively, these data reveal differential fates for E-cadherin and desmoglein-2 in response to EGF stimulation. While E-cadherin is found in caveolin rich regions at the cell surface, it does not internalize with caveosomes or within the classical endosomal pathway. Other investigators have found E-cadherin to undergo micropinocytosis after EGF treatment in a breast cancer cell line (Bryant *et al.*, 2007) and cleavage after calcium influx (Rios-Doria *et al.*, 2003) in the same MCF-7 cell line. We find E-cadherin undergoes a cleavage event at the cell membrane mediated by a member of the MMP family of proteases. Desmoglein-2, however, takes a different route upon EGF stimulation, entering the classical endosomal pathway into recycling

compartments for several hours post EGF treatment. Recognizing the different fates of cadherins in response to EGF as well as the response of cadherins to other stiumuli are two parts in understanding the effective mechanisms necessary for successful reepithelialization.

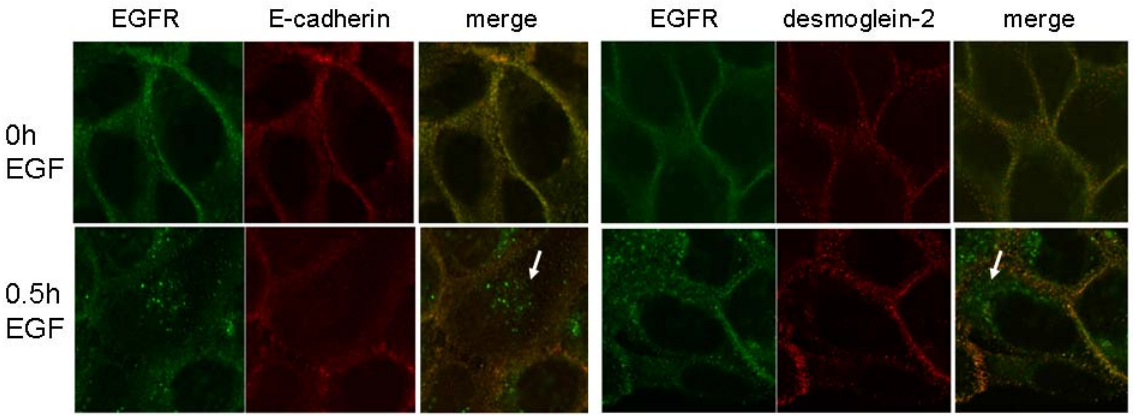


Figure 3.4.1 Cadherin internalization does not follow the same timecourse as EGFR internalization

SCC 12F cells were serum starved, then treated with 20 nM EGF for 30 minutes, fixed, and probed for the EGFR (green) or junctional cadherin (red). White arrows indicate the internalized EGF receptor in the cytoplasm. Both junctional cadherins, E-cadherin (left panels) and desmoglein-2 (right panels) remain intact and at the cell surface for up to 30 minutes post EGF treatment.

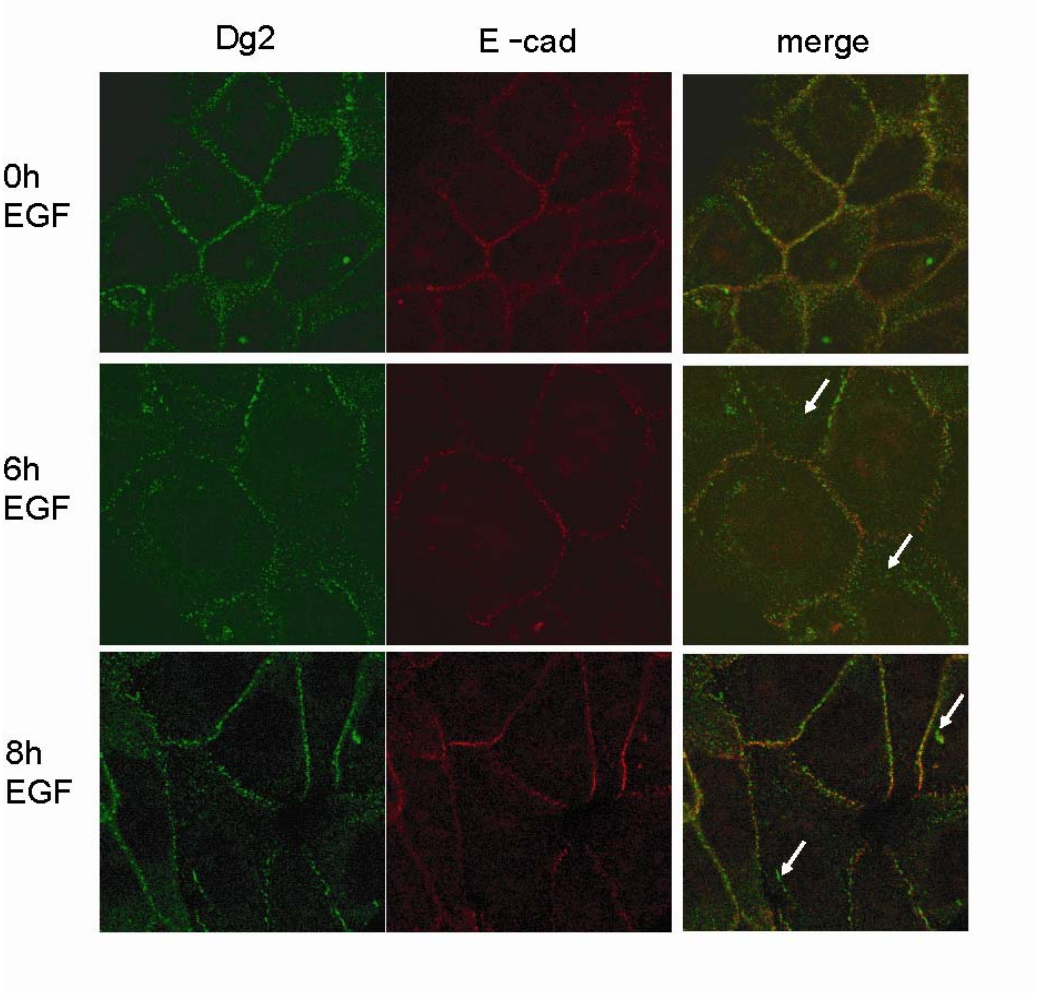


Figure 3.4.2 Desmoglein-2 does not internalize with E-cadherin. Time dependence for EGF-stimulated junctional disruption.

SCC 12F cells were treated with 20 nM EGF for the indicated timepoints. Cells were fixed, then probed with E-cadherin or desmoglein-2 antibodies. The desmosomal cadherin desmoglein-2 is relocalized from the cell borders following treatment with EGF by 6-8 hours. Note punctate cytoplasmic staining (white arrows). This pattern differs from that observed for the adherens junctional cadherin, E-cadherin, where strong border staining is evident at 6 hours post EGF treatment.

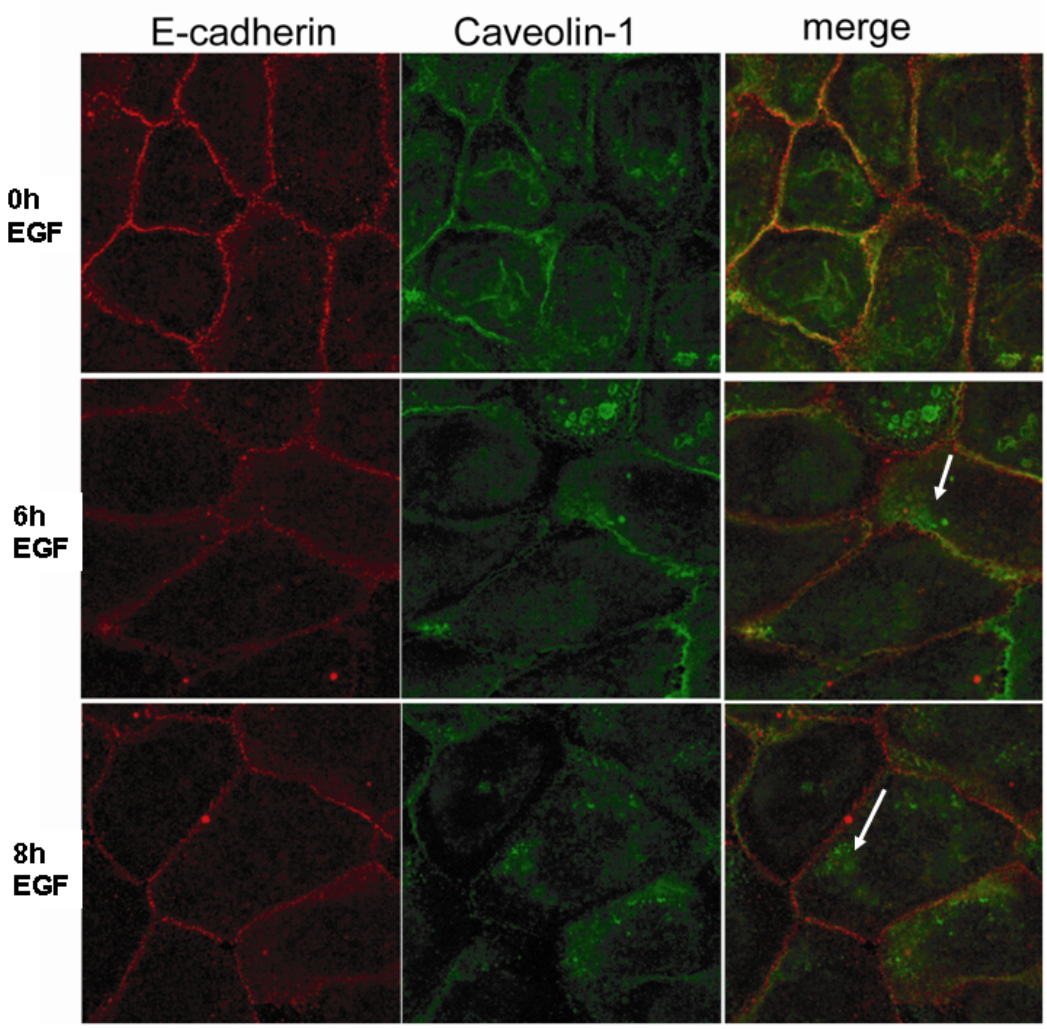


Figure 3.4.3 E-cadherin does not colocalize intracellularly with caveolin-1
SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated timepoints, fixed, and probed for the E-cadherin (red) or caveolin-1 (green). E-cadherin colocalizes partially with caveolin-1 at the cell membrane in control cells, but after EGF stimulation, E-cadherin does not colocalize with internalized caveolin-1 (white arrows) up to 8 hours post EGF treatment.

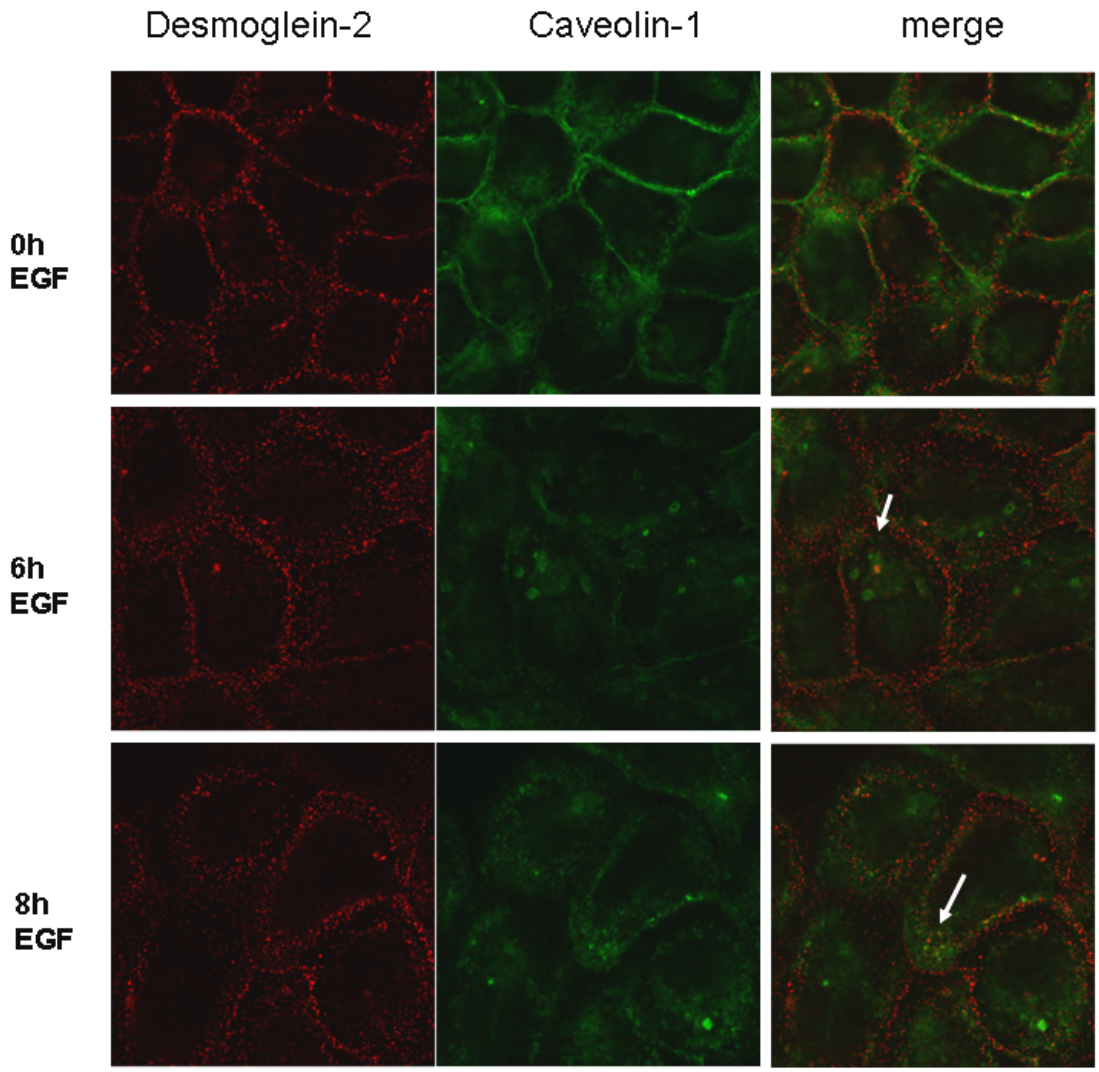


Figure 3.4.4 Desmoglein-2 does not internalize with caveolin-1

SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated times, fixed, and probed for desmoglein-2 (red) or caveolin-1 (green). Desmoglein-2 does not colocalize with caveolin-1 in control cells with no treatment nor at 6-8 hours post EGF stimulation, when both proteins are internalized.

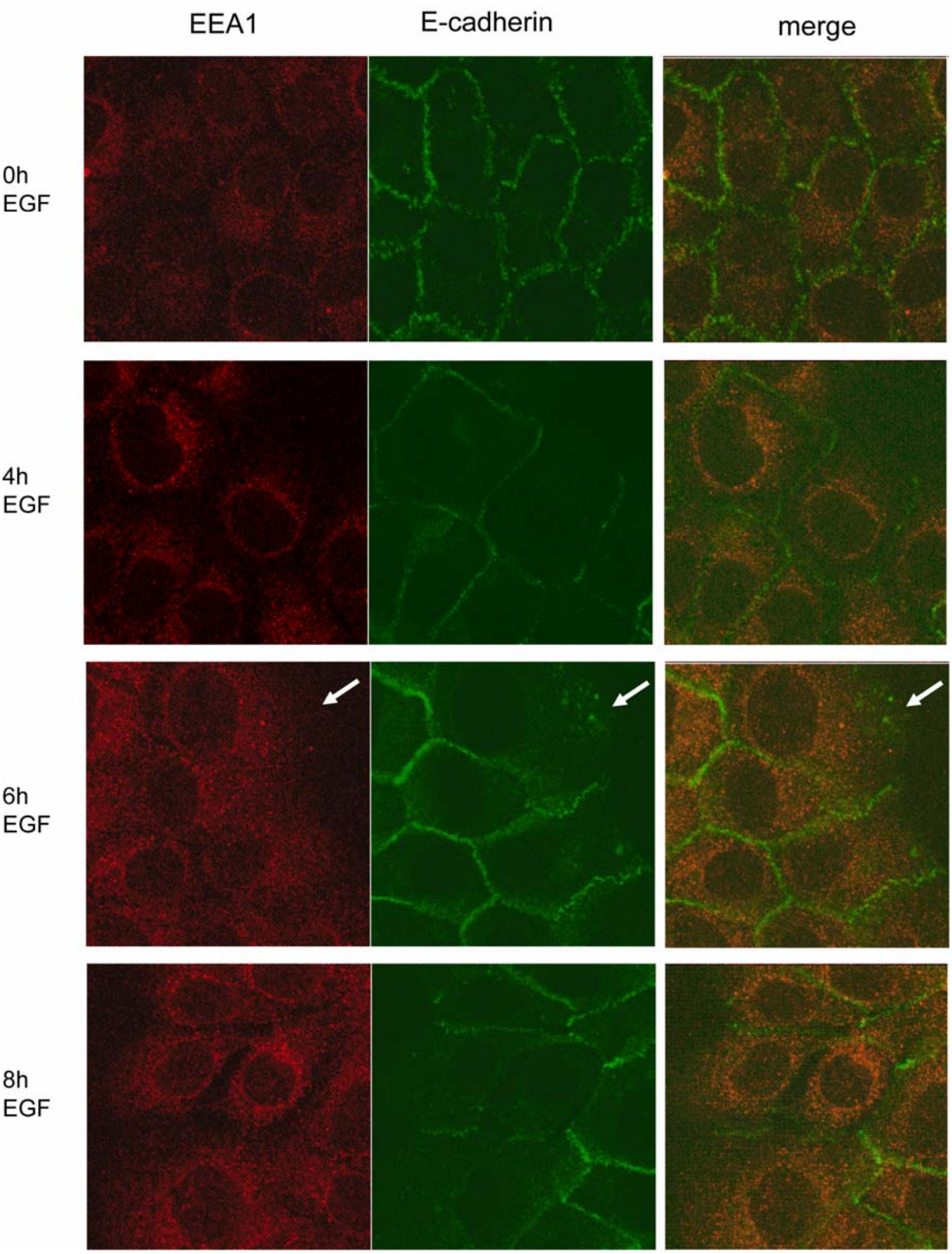


Figure 3.4.5 E-cadherin does not internalize with the early endosome marker EEA1 after EGF stimulation

SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated times, fixed, and probed for EEA-1 (red) or E-cadherin (green). E-cadherin does not colocalize with EEA-1 in control cells or after EGF stimulation.

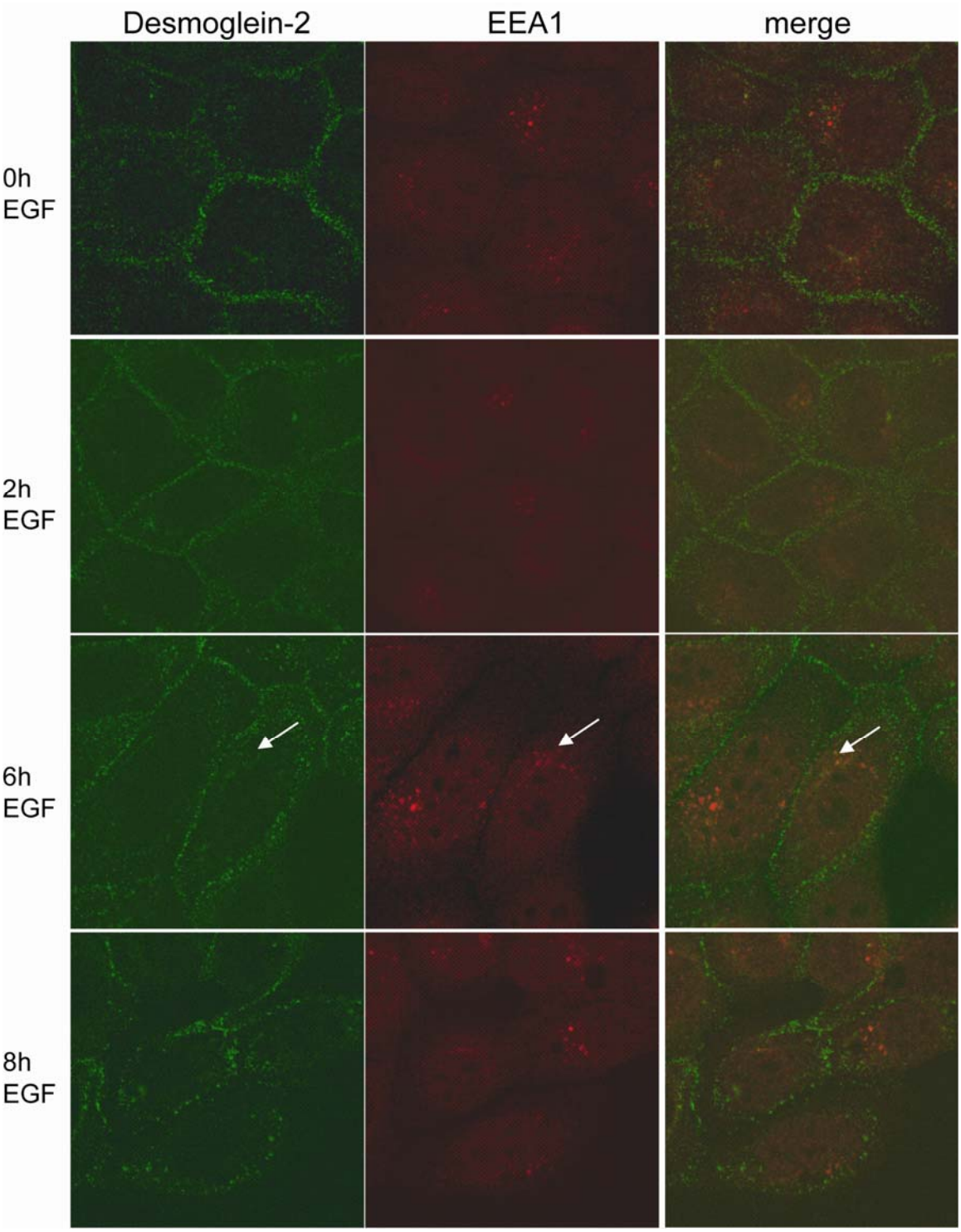


Figure 3.4.6 Only a small portion of desmoglein-2 colocalizes with the early endosome marker EEA1

SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated times, fixed, and probed for desmoglein-2 (green) or EEA-1 (red). A small amount of desmoglein-2 colocalizes with EEA-1 after 6 hours EGF stimulation (white arrows).

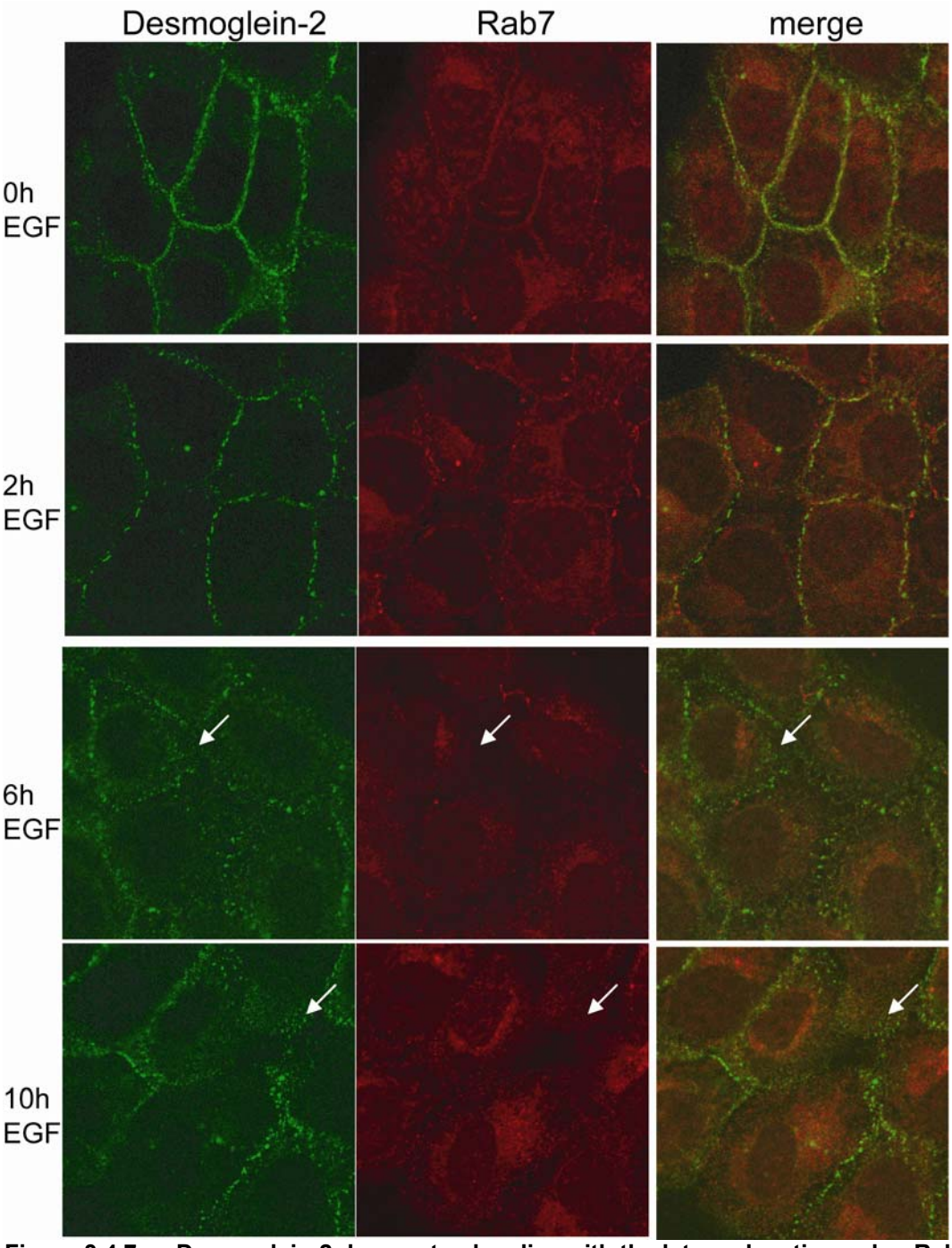


Figure 3.4.7 Desmoglein-2 does not colocalize with the late endocytic marker Rab7 after EGF stimulation.

SCC 12F cells were serum starved, then treated with EGF for various times, fixed, and probed for desmoglein-2 (green) or Rab-7 (red). Although both proteins are cytoplasmic after EGF activation, no colocalization is seen from 6-10 hours EGF stimulation (white arrows).

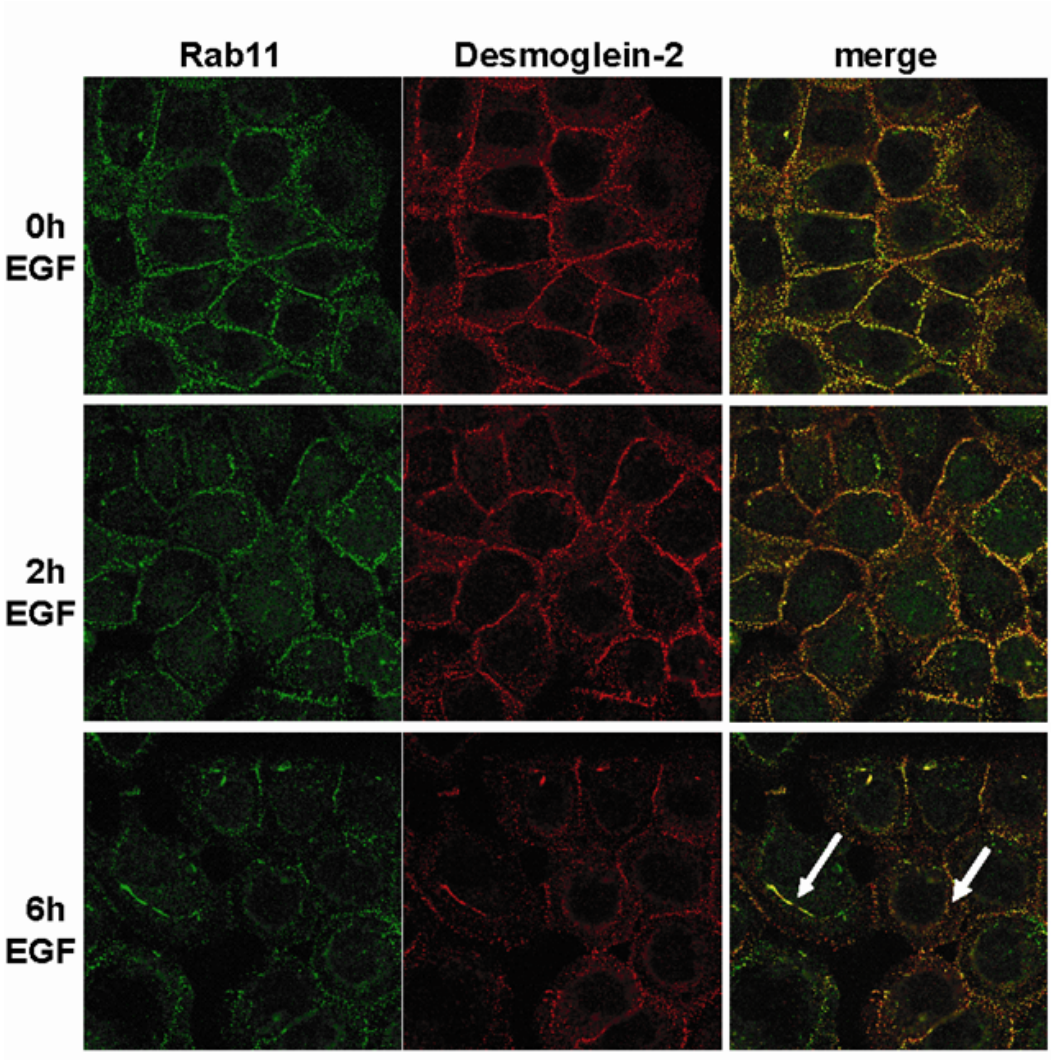


Figure 3.4.8 Desmoglein-2 colocalizes with the recycling marker Rab-11
SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated timepoints, fixed, and probed for desmoglein-2 (green) or Rab-11 (red). Desmoglein-2 colocalizes with Rab-11 both at the cell membrane and in the cytoplasm 6 hours post EGF stimulation (white arrows).

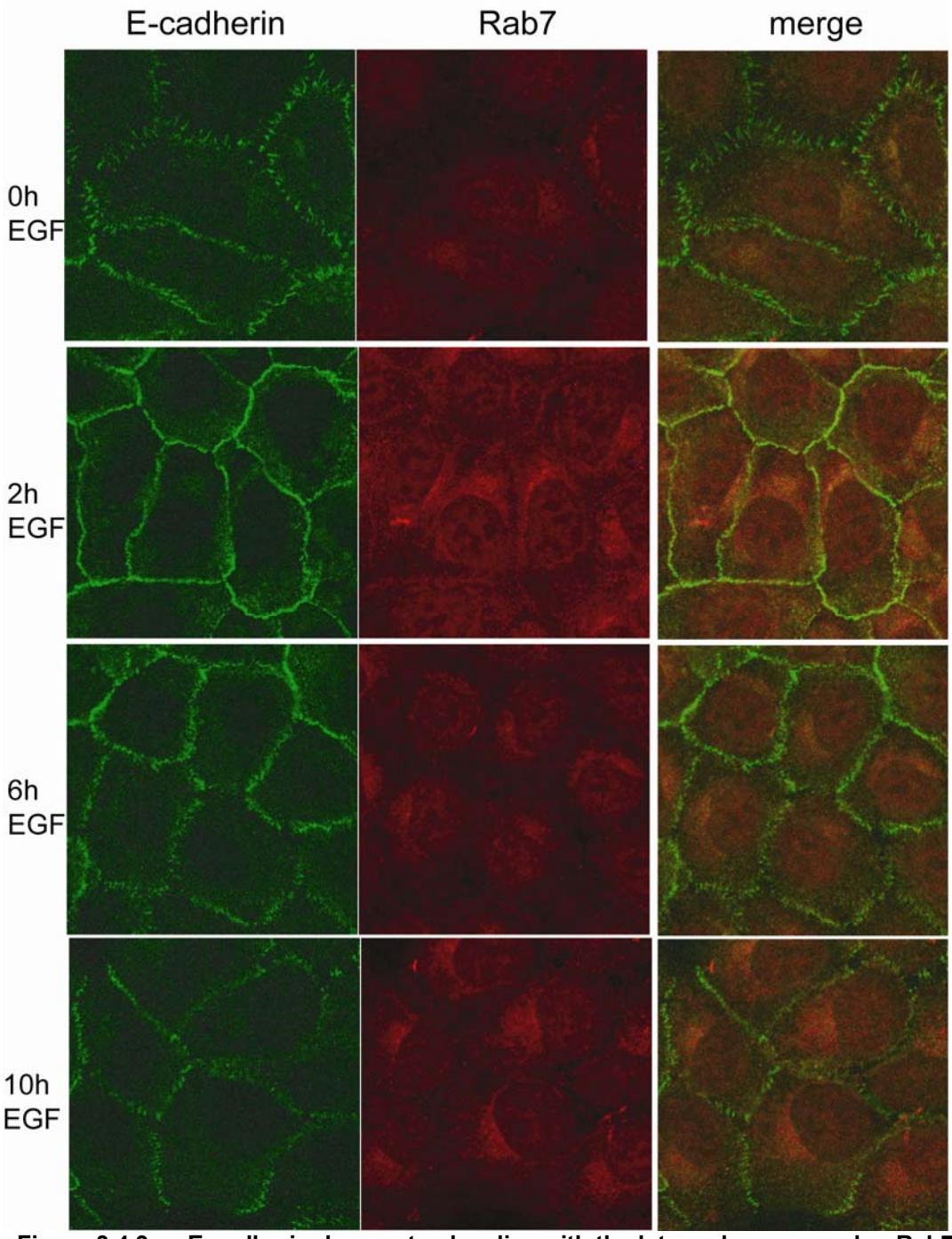


Figure 3.4.9 E-cadherin does not colocalize with the late endosome marker Rab7 after EGF stimulation.

SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated timepoints, fixed, and probed for E-cadherin (green) or Rab-7 (red). No colocalization was seen between the two proteins at any timepoints.

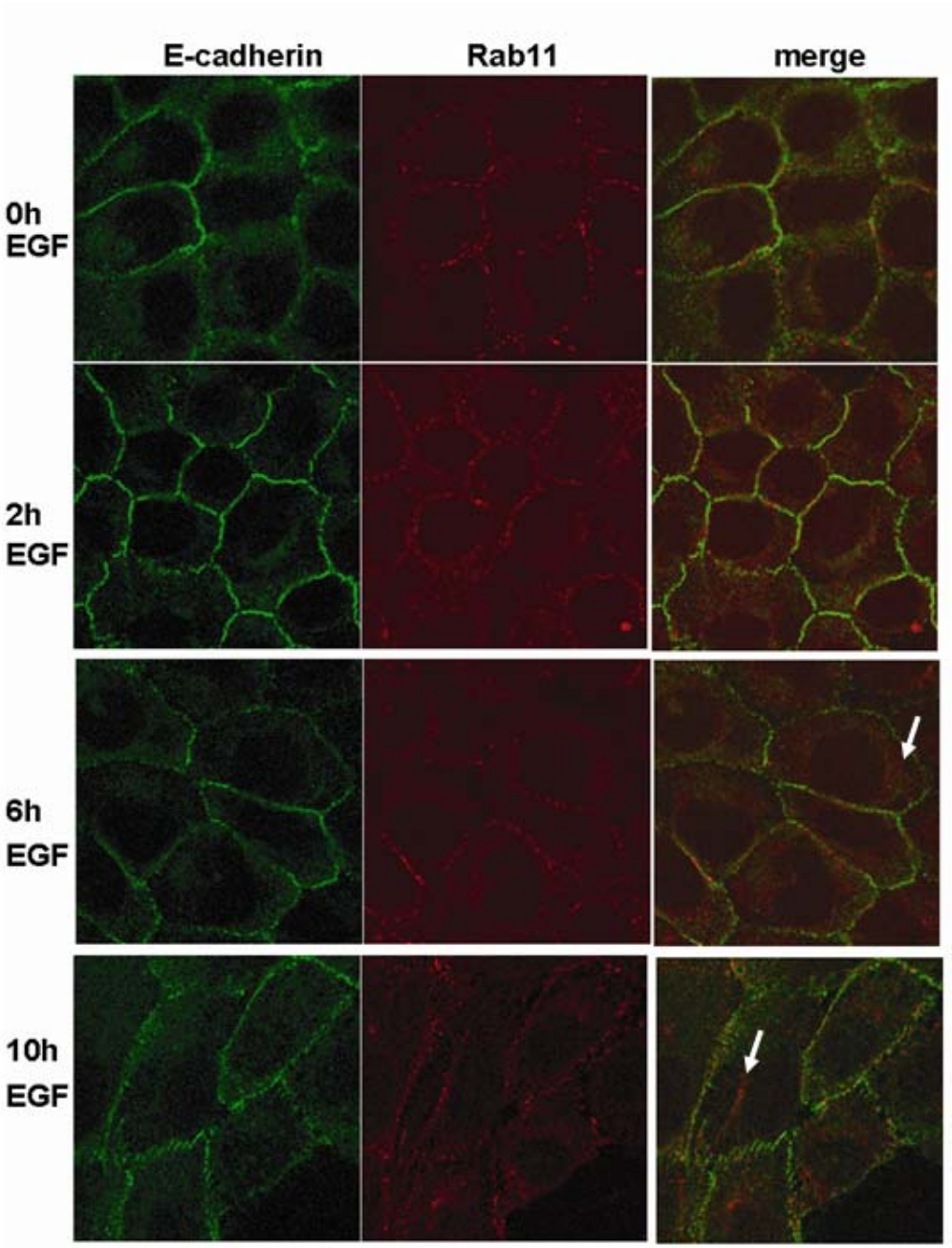


Figure 3.4.10 E-cadherin does not colocalize with the recycling marker Rab11 upon EGF stimulation

SCC 12F cells were serum starved, then treated with 20 nM EGF for indicated timepoints, fixed, and probed for E-cadherin (green) or Rab-11 (red). No colocalization was seen between the two proteins at any timepoints.

Table 3.4.1 Summary of colocalization experiments

	Desmoglein-2 colocalization	E-cadherin colocalization
EEA-1 (Early endosome)	(+)	(-)
Rab-11 (Recycling endosome)	(+++)	(-)
Rab-7 (Late endosome)	(-)	(-)
Lysotraker (Lysosome)	(+)	(-)
Caveolin-1 (caveosome)	(-)	(+/-)

Key: Colocalization experiments were carried out with immunofluorescence techniques described above. EGF timecourse ranged from 2-12 hours, and were done a minimum of 3 times. (-) indicates no colocalization at any timepoint; (+/-) indicates colocalization at the plasma membrane but not in cytoplasm; (+) indicates colocalization in vesicles at at least one timepoint; (+++) indicates colocalization in vesicles at several timepoints.

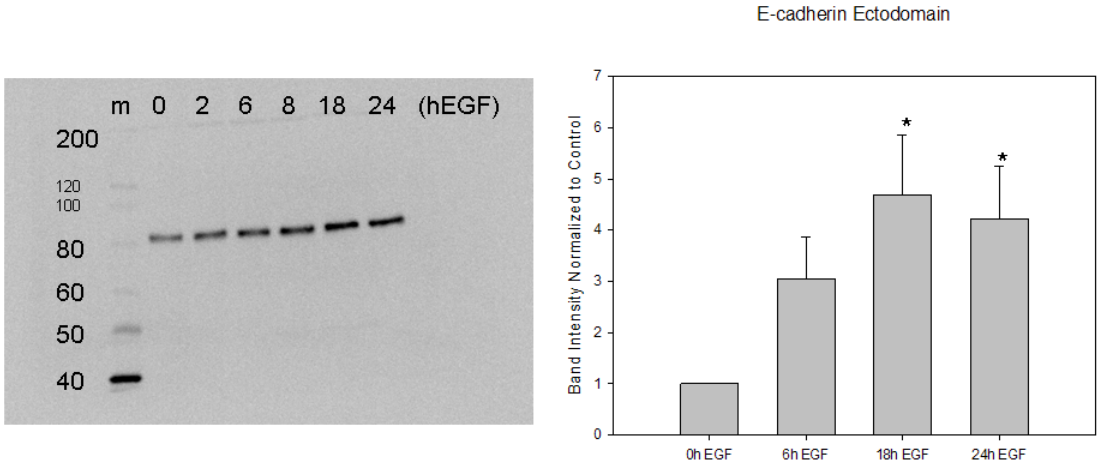


Figure 3.4.11 Evidence of an 80 kD E-cadherin ectodomain fragment in the conditioned cell culture medium after EGF treatment.

SCC 12F cells were serum starved overnight in medium without 0.1% BSA, then stimulated with 20 nM EGF for the indicated times. Medium was collected, spun down to remove cellular debris, then processed as normal on Western blot. Membrane was probed with E-cadherin antibody that recognized the extracellular portion of the protein. A statistically significant increase in E-cadherin ectodomain was seen at 18 hours post EGF treatment (n=3, $p<0.05$).

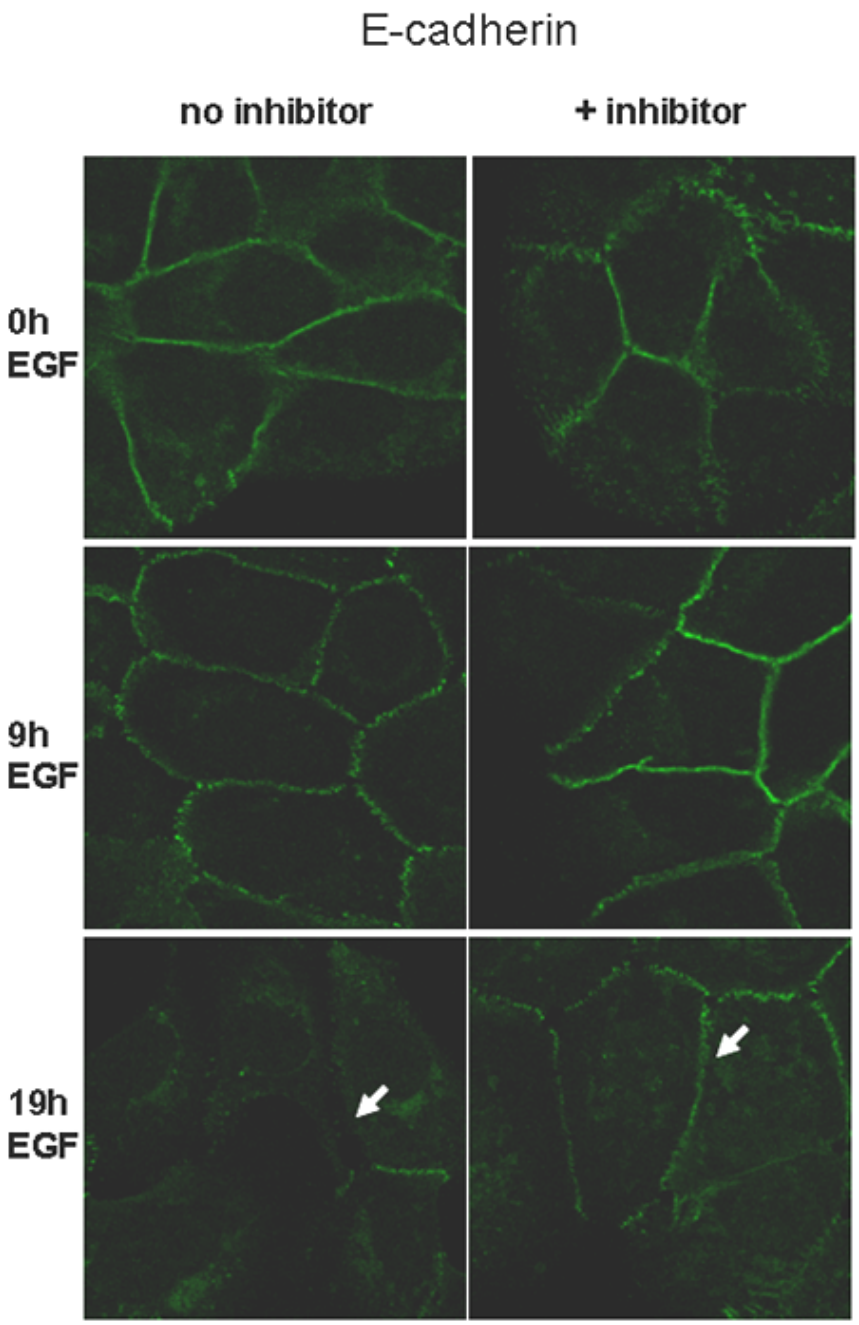


Figure 3.4.12 Maintenance of cell-cell junctions after EGF treatment with the addition of broad spectrum MMP inhbitor

SCC 12F cells were serum starved overnight, then treated with 20 nM EGF for indicated timepoints, either with or without pretreatment with 50 μM GM6001, an MMP inhibitor. Cells were then fixed, and probed with an antibody that recognizes the intracellular domain of E-cadherin (green). After 19 hours post EGF treatment, E-cadherin is no longer visible at the cell borders and the intracellular domain of E-cadherin is detected in the cytoplasm (white arrow, left panel). However, the presence of GM6001 maintains E-cadherin at the cell borders (white arrow, right panel).

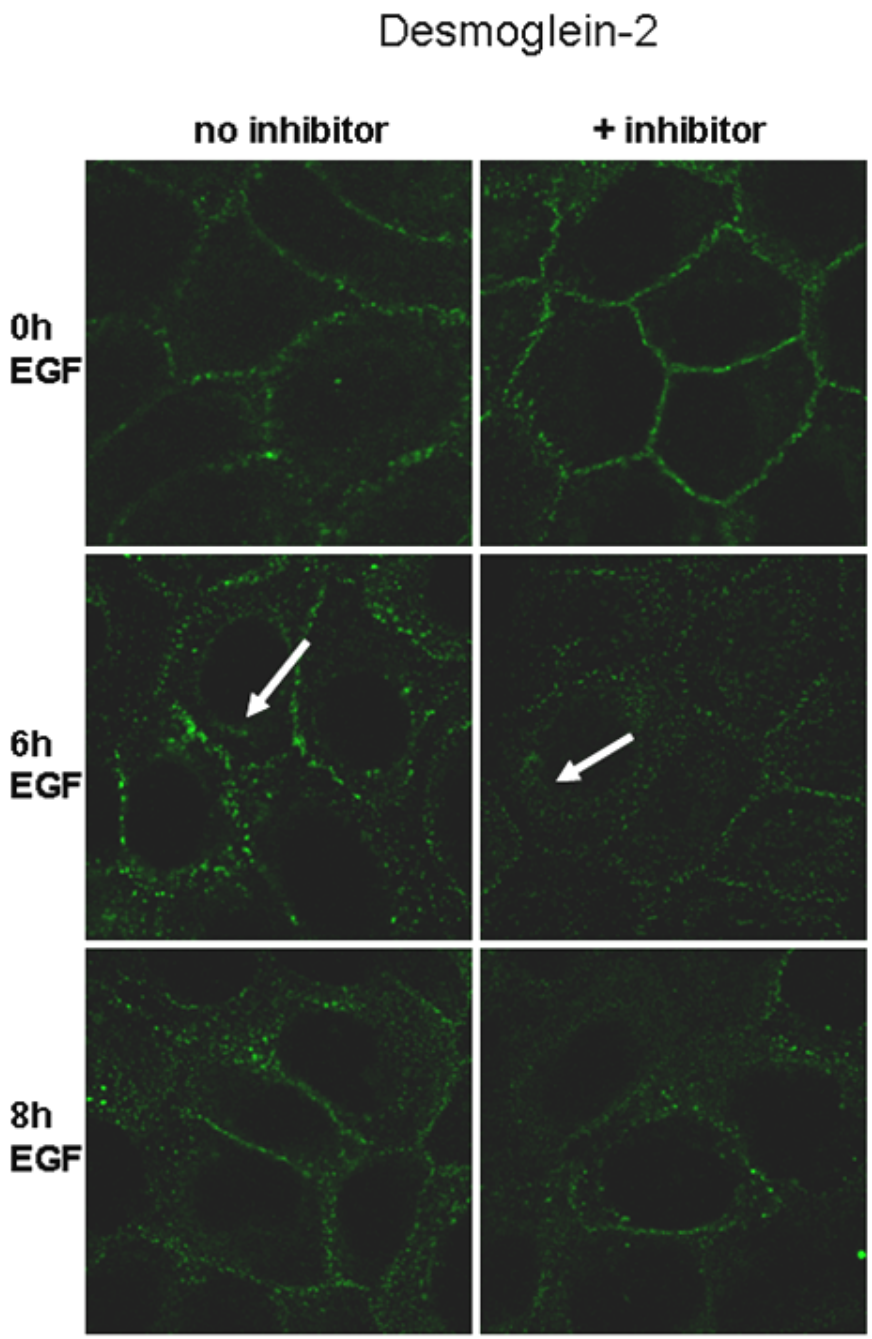


Figure 3.4.13 Desmoglein-2 internalization is not prevented with the addition of the broad spectrum MMP inhibitor GM6001.

SCC 12F cells were serum starved overnight and treated with 20 nM EGF for indicated timepoints, either with or without pretreatment with 50 μM GM6001, an MMP inhibitor. Cells were then fixed, and probed with an antibody that recognizes the intracellular domain of E-cadherin (green). After 6 hours post EGF treatment, desmoglein is no longer visible at the cell borders and is detected in the cytoplasm (white arrow, left panel). The pretreatment with GM6001 does not prevent internalization of desmoglein-2 from occuring (white arrow, right panel).

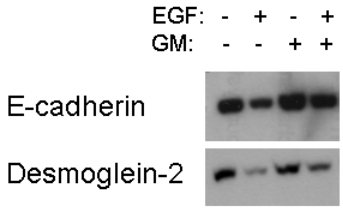


Figure 3.4.14 E-cadherin, and to a smaller degree, desmoglein-2, are protected from downregulation by the use of global MMP inhibitor GM6001.
SCC 12F cells were serum starved then treated with 20 nM EGF for indicated timepoints, either with or without pretreatment with 50 μM GM6001, an MMP inhibitor. Both E-cadherin and desmoglein-2 decrease after EGF treatment, but only E-cadherin levels are restored to control levels with pretreatment with the MMP inhibitor. Demsoglein-2 levels are partially restored, indicating partial protection from cleavage with the MMP inhibitor.

4 Discussion

This study has investigated consequences of EGFR activation in keratinocytes to better understand events that occur at the margins of healing wounds during reepithelialization. I found that elevated EGFR is required for EGF dependent junctional modulation, including cytoskeletal rearrangement and translocation of junctional components to initiate changes in gene transcription. Additionally, I have identified that the mechanisms involved in EGF-dependent junctional disruption differs for adherens junctions and desmosomes. While desmoglein-2 enters a recycling pathway after EGF treatment, E-cadherin undergoes a cleavage event that is MMP dependent. I have found further differences in the temporal sequence of junctional modulation between two stimuli, low calcium and EGF. The significance of these findings is discussed below.

4.1 Elevated EGFR at wound margins and effects

Dynamic regulation of both EGFR and ligand levels occurs at the wound edge. When EGFR levels are elevated, an autocrine release of EGFR ligand is produced by keratinocytes that promotes migration. This autocrine regulation of *in vitro* repithelializtion cannot be fully compensated by the growth factors and serum upon EGFR inhibition, indicating that EGFR activation is key in a full reepithelialization response. In support of this, studies have shown that wound repair is greatly decreased in mice lacking EGFR (Repertinger *et al.*, 2004).

Elevated EGFR at wound margins also results in junctional disruption that releases ß-catenin and a causes a change in the transcription that is consistent with the needs of reepithelialization. A recent study highlights the importance of elevated EGFR for successful reepithelialization in humans. Patients with chronic wounds have a decreased amount of EGFR at the margins as compared to their own adjacent, healthy skin (Brem *et al.*, 2007). This indicates the physiological and pathophysiological role for EGFR regulation at wound margins. Understanding the mechanisms of EGFR dependent reepithelialization could have clinical benefits for patients with non-healing wounds.

The observation that elevated EGFR leads to junctional disruption and nuclear localization of ß-catenin has significant implications. ß-catenin can activate a number of genes necessary for cell survival, proliferation and migration. Investigators have separated these downstream targets into two categories, classical and non-classical targets. Classical targets of ß-catenin include cyclin-D, Myc, and survivin, which are important in cell survival and proliferation. Non-classical target genes include MMP-14, Slug and tenascin-C, genes important for migration and an EMT response. Brabletz and coworkers suggest that in a cancer model, low levels of ß-catenin in the nucleus are sufficient to stimulate the classical pathway of transcription (Brabletz *et al.*, 2005). In order to activate the non-classical target genes, however, a second stimulus, or change in the microenvironment is needed. Examples mentioned that can induce EMT are HGF, EGF and TGF-ß. This concept originated for cancer has striking similarities to wound healing. In both tumor metastasis and

wound healing, there is a need for cell migration and an overall EMT event. We find that a change in the microenvironment, wounding, elevates EGFR and ligand levels which result in translocation of ß-catenin and activation of non-classical target genes Slug and tenascin-C, both of which are required for effective wound repair. Differences in tumor metastasis and wound healing involve the fact that wound healing represents a partial EMT (Arnoux, 2005), and reversion is needed back to an epithelial state, although some tumors do dedifferentiate to epithelial status after invasion (Jeanes *et al.*, 2008). Further studies are needed to fully understand the range of genes affected by EGFR activated ß-catenin transcriptional regulation.

Questions that remain unanswered are: 1.) why is EGFR upregulated at wound margins and 2.) what are the signals that initiate this upregulation. The answers to these questions are currently unknown, but one possibility is that adhesion serves as a cue and disruption of adhesion starts signaling cascades that leads to EGFR upregulation. One example in the literature that supports this hypothesis is the fact that soluble E-cadherin fragments were able to bind ErbB2 and stabilize its association with ErbB3 and increase downstream Erk signaling (Najy *et al.*, 2008). Other possibilites are the myriad of other factors that are present in the wound microenvironment released by inflammatory cells. Future experiments to investigate the roles of these other factors available during wound healing could give insight into regulation of modulation of cell-cell junctions during reepithelialization

4.2 Mechanisms of EGF dependent modulation differ between E-cadherin and desmoglein-2

Another significant finding is that different mechanisms are responsible for EGF dependent modulation of E-cadherin and desmoglein-2. I investigated the effects of EGFR activation on the fate of the adherens junction protein E-cadherin and the desmosomal cadherin desmoglein-2 *in vitro*. Upon extended EGF treatment, desmoglein-2 is internalized and enters a recycling pathway that lasts up to 8 hours post stimulus. Other studies have shown internalization of E-cadherin in response to EGF treatment (Bryant *et al.*, 2007), as well as a variety of other stimuli in different cell types (Table 4.2.1). However, we find that in SCC 12F cells, E-cadherin remains at cell-cell borders until 18-19 hours post EGF treatment. After 18-19 hours post EGF treatment, E-cadherin ectodomain can be found in the medium and cell border staining is decreased, in a process that can be prevented with a MMP inhibitor. This suggests a direct link between MMPs and E-cadherin cleavage after EGFR stimulation. While there are several examples of MMP dependent E-cadherin cleavage (Davies *et al.*, 2001; Noe *et al.*, 2001; Symowicz *et al.*, 2007; Cowden Dahl *et al.*, 2008), this is the first example of a desmosomal cadherin entering a recycling pathway in response to growth factor activation. Wound repair is a dynamic process and rapid reassembly after reepithelialization is complete is crucial to the restoration of a functional barrier, so it stands to reason that desmosomal cadherins entering a recycling pathway would be beneficial to cells undergoing reepithelialization.

There are many different mechanisms reported for junctional disruption, including low calcium (discussed in the previous section give section number)

(1), transcriptional repression (2), phosphorylation events (3), internalization and trafficking (4) and proteolytic cleavage (5) (See Figure 4.2.1).

Transcriptional repression (Figure 4.2.1, part 2) of E-cadherin, ß-catenin, desmoglein-2 or plakoglobin was not observed under our conditions, however, transcriptional repression of cadherins represents an important mechanism in invasive cancers. Transcriptional repression is a mechanism employed in human breast cancer samples for desmosomal downregulation. In 32 tumor samples, 72% had decreased mRNA expression of desmocollin-3 and of these, 56% had hypermethylated promoter regions, a known epigenetic mechanism of gene silencing (Oshiro *et al.*, 2005). A recent study revealed that the repression of desmocollin-2 gene was due to transcription factors CDX1 and CDX2, repression of these transcription factors restored a normal epithelial morphology (Funakoshi *et al.*, 2008).

Several mediators can activate a Slug induced repression of E-cadherin, including activation of the Notch pathway (Leong *et al.*, 2007), calreticulin (Hayashida *et al.*, 2006), estrogen (Park *et al.*, 2008), TGF-ß (Choi *et al.*, 2007), and FGF-1 (Savagner *et al.*, 1997). Slug/Snai2 represses E-cadherin in tumors (Uchikado *et al.*, 2005; Come *et al.*, 2006) and overexpression of Slug in a rat bladder carcinoma cell line led to an EMT like response (Savagner *et al.*, 1997). The absence of Slug is associated with a retention of adherens junction protein at the wound margin (Chandler *et al.*, 2007).

We did not investigate if phosphorylation events (Figure 4.1 part 3) occur in our system due to the fact that phosphorylation typically induces a rapid (within

minutes) downregulation of proteins, and we do not see changes in cadherins for several hours. Many others have reported growth factor addition to cause phosphorylation of cadherins. In MDCK cells, HGF treatment caused Src activation, which led to tyrosine phosphorylation of E-cadherin, ubiquitination by the E3 ubiquitin ligase Hakai and internalization by 4 hours post treatment (Fujita *et al.*, 2002). PKC-α can modulate desmosomal components directly, via serine phosphorylation of desmoplakin (Amar *et al.*, 1999), which serves to stabilize the junction. Tyrosine phosphorylation of desmoglein-2 is prevented by inhibition of the EGFR in a invasive SCC cell line, which results in reassembly of desmosomal junctions and overall increased intracellular adhesion (Lorch *et al.*, 2004). Phosphorylation events, therefore, may be more important for initial assembly and reassembly than for junctional disruption.

The internalization of cadherins and cadherin trafficking has been studied in several models (Figure 4.2.1, part 4). We find the desmosomal cadherin desmoglein-2 to enter a recycling pathway after EGF stimulation. Although this is the first desmosomal cadherin shown to enter this pathway after growth factor treatment, E-cadherin has been shown in to enter a recycling pathway at basal conditions. Overexpression of a GFP tagged E-cadherin showed colocalization of the cadherin with Rab11, the endocytic recycling marker (Lock and Stow, 2005), and nascent, post-Golgi E-cadherin uses the recycling pathway to sort to the plasma membrane in MDCK cells (Lock and Stow, 2005). The use of E-cadherin mutants revealed that E-cadherin that cannot bind p120 colocalizes with both early and recycling endosomes (Miyashita and Ozawa, 2007b).

We found no evidence of E-cadherin entering the lysosome for degradation, but a small portion of desmoglein-2 was found in lysosomes after extended timepoints of EGF treatment. This could indicate a similar process in which after being in the recycling pathway for an extended period of time, desmoglein-2 is shuttled to the lysosome for degradation. However, since only a small amount of desmoglein-2 was found in the lysosome, we can assume that either only a portion of desmoglein undergoes recycling and degradation or that the recycling and degradation of total desmoglein occurs asynchronously.

Another mechanism of cell-cell junction disruption is proteolytic cleavage (Figure 4.2.1, part 5). We found evidence of MMP dependent cleavage of E-cadherin that was not extended to the desmosomal cadherin desmoglein-2, although it is possible that cleavage of desmoglein-2 occurred that was below our detection limits. Desmosomal components have been reported to undergo cleavage in response to different stimuli. Desmoglein-3, in response to autoimmune antibodies produced in patients with pemphigus vulgaris caused internalization and lysosomal degradation by 3 hours post treatment (Calkins *et al.*, 2006). Desmoglein-3 fragmentation in response to apoptosis occurs by MMP-9 (Cirillo *et al.*, 2007). The same laboratory found cleavage products of desmoglein-3 in response to pemphigus vulgaris autoantibodies (Cirillo *et al.*, 2008a). In a highly invasive squamous cell line that forms sparse cell cell junctions, an 100 kD desmoglein-2 fragment was detected in low calcium (.09mM) conditions that was decreased upon both EGFR and MMP inhibition (Lorch *et al.*, 2004). Using the same system, the Green laboratory showed

production of the 100 kD fragment of desmoglein-2 was blocked upon inhibition of the protease ADAM17 (Klessner *et al.*, 2008). ADAM17 was predicted to cleave several substrates, including desmoglein-2, in proteomic analysis (Bech-Serra *et al.*, 2006). Collectively, these studies suggest that some desmosomal constituents may be sensitive to cleavage as a mechanism of junctional disruption. This is consistent with our findings that a small but detectable amount of desmoglein-2 was protected with the use of an MMP inhibitor (Figure 3.4.14).

During wound healing, several MMPs are expressed by keratinocytes at the migrating wound edge, including, collagenase MMP-1, gelatinase MMP-9, and stromelysins MMP-3 and MMP-10 (Madlener *et al.*, 1998). Of these MMPs, two are reported to cleave E-cadherin in response to TPA treatment (Noe *et al.*, 2001) and EGF (Cowden Dahl *et al.*, 2008). In SCC 12F cells, we found MMP production and cell motility to be stimulus dependent, as EGF, TGF-α, and HGF/SF can cause colony dispersion and increased MMP production while other growth factors such as KGF and IGF-1 had no effect (McCawley *et al.*, 1998).

Although there is evidence for transcriptional regulation, phosphorylation, internalization and proteolytic cleavage of cadherins in various different cells, we find that upon EGF stimulation, E-cadherin undergoes a MMP dependent cleavage event and desmoglein-2 enters a recycling pathway in human keratinocytes. Multiple mechanisms can occur with a given cell type for the same cadherin. A good example is the in the well studied Madin-Darby canine kidney (MDCK) epithelial cell line, in which various fates of E-cadherin (Figure 4.2.2) have been reported. As mentioned earlier, E-cadherin in MDCK cells undergo

recycling under basal conditions (Lock and Stow, 2005). However, two independent investigators have found E-cadherin to colocalize with caveolin-1 at the cell membrane in MDCK cells (Galbiati *et al.*, 2000; Palacios *et al.*, 2002), and both E-cadherin and ß-catenin have also been found to colocalize and coimmunoprecipitate with caveolin-1 under basal conditions. In low calcium, MDCK cells internalize E-cadherin into a recycling endosome and addition of calcium back to the media restores E-cadherin to the cell surface unless a recycling inhibitor is present (Le *et al.*, 1999). Others have reported addition of low calcium shuts E-cadherin from a recycling pathway to degradation in the lysosome (Shen *et al.*, 2008). Clathrin dependent endocytosis (Palacios *et al.*, 2002), lysosomal degradation (Palacios *et al.*, 2005), cleavage by plasmin (Ryniers *et al.*, 2002), and tyrosine phosphorylation (Fujita *et al.*, 2002) has also been reported to occur to E-cadherin in MDCK cells (Figure 4.2.2). Transcriptional repression by Slug and SIP-1 are 2 other mechanisms for E-cadherin downregulation in MDCK cells (Comijn *et al.*, 2001; Bolos *et al.*, 2003).

Multiple mechanisms can occur within a given cell type for the same protein. E-cadherin was reported to undergo internalization, phosphorylation and transcriptional regulation in MDCK cells. One explanation for the differences is the initial stimulus presented to the cells. Wound reepithelialization is a dynamic, multifaceted process in which signals in the microenvironment change in a temporal sequence. Although certain responses were not detected in this study, it does not preclude them from being possible contributors in the overall process of reepithelialization.

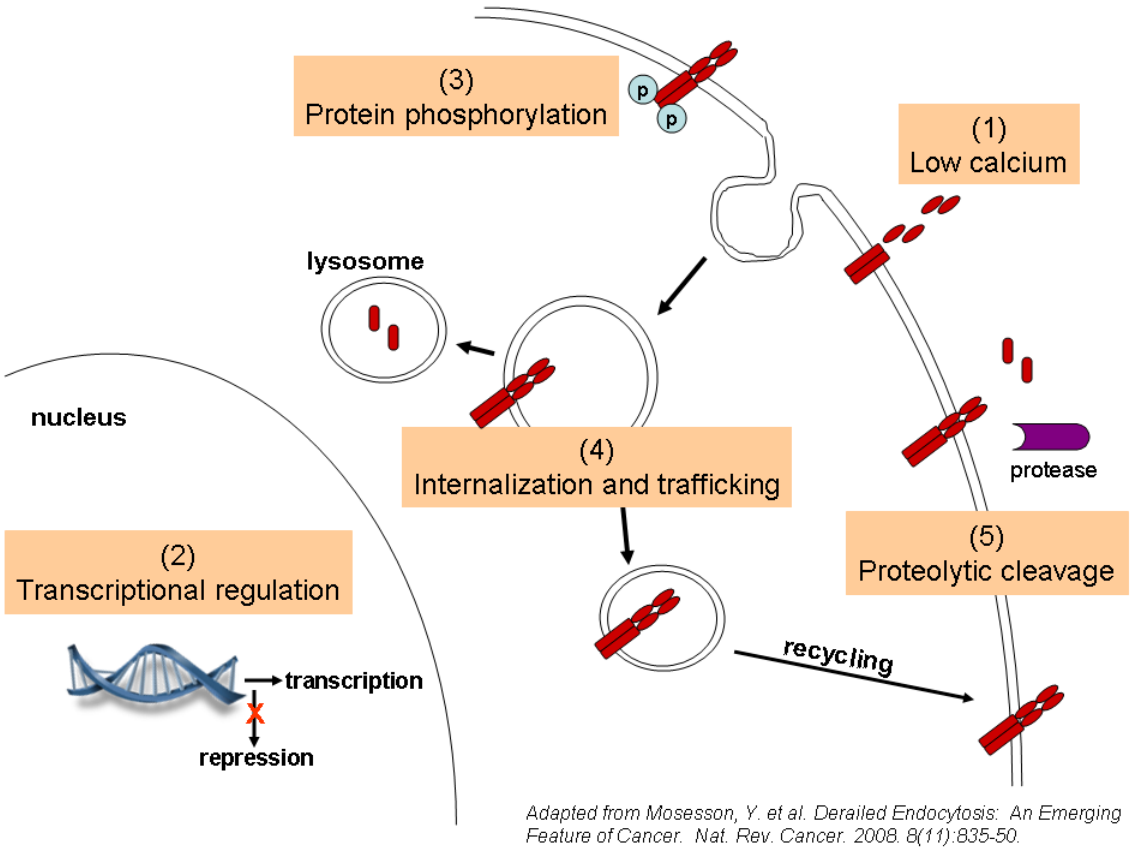


Figure 4.2.1 Mechanisms of junctional disruption

Several reported mechanisms of junctional disruption. All events listed have been reported to occur to cadherins as a mechanism for downregulation.

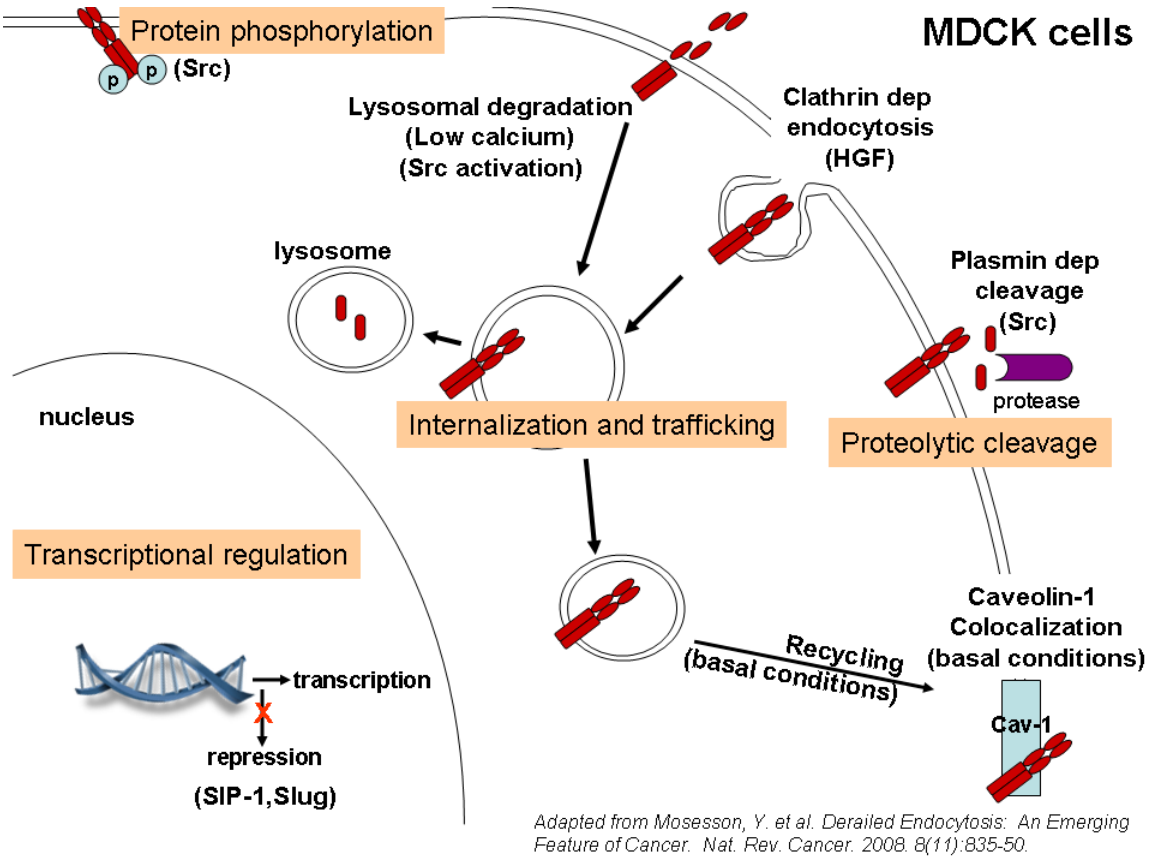


Figure 4.2.2 E-cadherin trafficking in response to stimuli in MDCK cells
Examples of differential regulation of E-cadherin after various stimuli in the MDCK cell line. Note that some stimuli, such as Src activation has been reported to have several different effects on E-cadherin in the same cell line.

4.3 Stimulus dependent regulation of cadherins assembly and disassembly

A related finding to the differential regulation of E-cadherin and desmoglein-2 is the differences in kinetics for each junctional cadherin with both EGF stimulation and low calcium, and the differences that occur after reversal of each stimulus. Not much is known about junctional modulation at wound margins, however, there are some early studies conducted on assembly of desmosomes and there is evidence of modulation in invasive cancers.

Early studies on desmosomal disassembly/reassembly focused on disruption of junctions by low calcium conditions, then reassembly after restoration of calcium levels. The half life of desmogleins 1-3 and desmoplakins 1-2 are decreased rapidly after placement in low calcium (Penn *et al.*, 1987) but continue to assemble what was described as “half desmosomes” (Duden and Franke, 1988) that were constitutively endocytosed in a clathrin independent manner in cytoplasmic vesicles that do not colocalize with early endosomes or lysosomal markers, but do colocalize with late endosomes (Holm *et al.*, 1993; Burdett and Sullivan, 2002). The desmosomal cadherins desmoglein-1 and desmocollin-2 were found to be transported from the Golgi to the plasma membrane in two stages, first in 60 nM vesicles, then in 200 nM vesicles (Burdett and Sullivan, 2002). When MDCK cells were returned from low calcium levels to high calcium levels, desmoplakin associated with intermediate filaments, which were aligned with the actin cytoskeleton in mouse primary keratinocytes (Green *et al.*, 1987). The Holm group found that actin depolymerization, but not

microtubule disruption, inhibited low calcium desmosomal disassembly. Actin disruption, the cytoskeletal component linked to adherens junctions inhibiting desmosomal disassembly is an interesting finding when we compare disassembly to assembly. E-cadherin and preformation of adherens junctions are required for desmosome assembly (Lewis *et al.*, 1997), so it appears that the two junctions are intertwined at both assembly and disassembly. Desmosomes cannot internalize if the adherens junctions and cytoskeletal network are disturbed, and desmosomes cannot reform once broken until adherens junctions are in place. Our studies are in agreement with these findings, as we find desmosomal disruption to occur before that of adherens junctions, as well as reassembly of desmosomal junctions after both low calcium and EGF reversal to occur after that of adherens junctions.

Another way to look at reassembly is to take invasive cancer cells with few cell-cell junctions and modulate their signaling to increase adhesion, such as inhibition of EGFR. Inhibition of the EGF receptor allows accumulation of desmoglein-2 and desmoplakin at cell-cell borders in an oral SCC line that overexpresses EGFR (Lorch *et al.*, 2004). These cells also express a 100kD cleavage product of desmoglein-2, which could be reversed by inhibition of the EGFR and several members of the sheddase family of ADAMs, including ADAM17 (Klessner *et al.*, 2008). Our disassembly studies do not suggest desmoglein-2 cleavage event, cleavage products of desmoglein-2 were undetected in the conditioned medium. It is possible amounts could be present that are below our detection limit. Using the MMP inhibitor GM6001 did not

inhibit the internalization of desmoglein-2, yet it is possible that other proteases could be mediating cleavage. It appears that adherens junctions and desmosomes are linked in reassembly and disassembly, but mechanistic differences are responsible depending on the method of stimulus, be it a calcium switch method or EGFR inhibition in cells with few junctions. In agreement with this hypothesis, we find temporal differences in both internalization and downregulation of junctions in response to low calcium versus EGF treatment.

Stimulus then, seems to dictate the type of junctional modulation in both reassembly and disassembly. When we look at the various types of stimuli in and the resulting effects on E-cadherin, we see a variety of effects (See Table 4.3.1). Basal E-cadherin associates with caveolin at the plasma membrane (Galbiati *et al.*, 2000; Palacios *et al.*, 2002), but is also seen to enter clathrin dependent recycling pathway in MDCK cells (Le *et al.*, 1999). Low calcium conditions cause clathrin dependent endocytosis (Ivanov *et al.*, 2004), recycling (Ling *et al.*, 2007), caveolar dependent endocytosis (Akhtar and Hotchin, 2001) and lysosomal degradation (Shen *et al.*, 2008) depending on the cell type. In response to overexpression of the tyrosine kinase Src, clathrin dependent endocytosis and lysosomal degradation was observed in MDCK cells (Palacios *et al.*, 2005). EGF treatment is reported to cause micropinocytosis of E-cadherin (Bryant *et al.*, 2007) in MCF-7 cells and caveolar dependent internalization (Lu *et al.*, 2003) in A431. HGF treatment in these cells resulted in both dynamin dependent endocytosis (Palacios *et al.*, 2002) and co-internalization into early endosomes with the tyrosine kinase receptors c-Met in response to HGF

treatment (Kamei *et al.*, 1999). TPA induced activation of protein kinase C (PKC) induced endocytosis and recycling of E-cadherin (Le *et al.*, 2002). Clearly, there are several different internalization mechanisms available to E-cadherin depending on the cell type and stimulus.

In my studies with the SCC 12F cell line with the Src inhibitor SU6656, I find that desmoglein-2 internalization occurs independent of Src (data not shown). Although many groups have reported E-cadherin internalization dependent on Src (Palovuori *et al.*, 2003; Palacios *et al.*, 2005; Shen *et al.*, 2008), in support of my findings, another group found that Src activation was not necessary for the overall breakdown of tight junctions and transformation to a mesenchymal phenotype (Maeda *et al.*, 2006). This strengthens our hypothesis of differential fates of junctional cadherins dependent on both the stimulus and the junction in question. One unanswered question is the reason for time delay in between the initial EGF stimulation and cadherin response. One possibility is the necessity for new protein synthesis or RNA synthesis to occur. One glimpse that this might be possible came by work in our lab by Jennifer Halblieb, who used cycloheximide, a protein synthesis inhibitor before EGF treatment, and found E-cadherin remained at the cell surface after 24 hours. New protein synthesis of MMPs could be required for E-cadherin cleavage to occur. I used cycloheximide and treated cells for 6 hours with EGF, and internalization of desmoglein-2 still occurred (data not shown), indicating that no new protein synthesis is necessary for the internalization of desmoglein-2. It would have been interesting to see what the results of EGF reversal and junctional reassembly

would be, since E-cadherin and adherens junction assembly is required for desmosomal assembly. I would predict that without new protein synthesis of MMPs, E-cadherin would already be assembled at adherens junctions and the restoration of desmosomes would occur much more quickly.

Table 4.2.1 Stimulus dependent internalization of E-cadherin

Stimulus	MDCK cells Reference	Other cells Reference
None	Caveolar association at PM; clathrin dependent recycling (Galbiati *et al.*, 2000; Palacios *et al.*, 2002); (Le *et al.*, 1999)	Clathrin independent dynamin dep (MCF-7, CHO) (Paterson *et al.*, 2003))
Low calcium	clathrin dependent endocytosis, recycling (Ivanov *et al.*, 2004; Ling *et al.*, 2007)	caveolar dep (SCC12F); lysosomal degradation (MCF-7) (Akhtar and Hotchin, 2001); (Shen *et al.*, 2008)
Src	clathrin dependent endocytosis; lysosomal degradation (Palacios *et al.*, 2005)	
HGF	dynamin dependent endocytosis; Early endosomes with receptor (Palacios *et al.*, 2002); (Kamei *et al.*, 1999)	
TGF-ß	Recycling (Le *et al.*, 2002)	
EGF		micropinocytosis (MCF-7); caveolar endocytosis (A431) (Bryant *et al.*, 2007); (Lu *et al.*, 2003)
FGF		Early endosomes with receptor (MCF-7) (Bryant *et al.*, 2005)
TPA		lysosomal degrdation (Shen *et al.*, 2008)

Key: PM = plasma membrane, CHO= chinese hamster ovary cells, HGF = hepatocyte growth factor, TGF-ß = transforming growth factor beta, EGF = epidermal growth factor, FGF = fibroblast growth factor, TPA = 12-O-tetradecanoylphorbol-13-acetate

4.4 Conclusions

Overall, junctional modulation is a dynamic process. Several variables, such as cell type, stimulus presented as well as the junction in question affect the route or eventual fate of the junctional component. Regulation of junctional modulation during wound healing is likely due to specific extracellular signals and these extracellular signals are dynamic, and change within the time sequence of wound healing. We find distinct pathways of junctional modulation that occurs with elevated EGFR levels and activation with EGF. Although in this cell line, with this stimulus, we see E-cadherin cleavage and desmoglein-2 recycling, other signals at different timepoints could lead to very different results. Future studies should involve 1) mechanisms that cause elevation of EGFR at the wound margin, 2) dissection of the temporal sequence of signals other than EGF that are also present in the wound microenviroment and their effects on junctional modulation, and 3) how different signals change the fate and itinerary of junctional cadherins. These questions will need to be addressed before we have a full picture of junctional dynamics during reepithelialization.

5 References

Akhtar, N., and Hotchin, N.A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. Mol Biol Cell *12*, 847-862.

Akhtar, N., Hudson, K.R., and Hotchin, N.A. (2000). Co-localization of Rac1 and E-cadherin in human epidermal keratinocytes. Cell Adhes Commun *7*, 465-476.

Aloe, L., Tirassa, P., and Lambiase, A. (2008). The topical application of nerve growth factor as a pharmacological tool for human corneal and skin ulcers. Pharmacol Res *57*, 253-258.

Alroy, J., Pauli, B.U., and Weinstein, R.S. (1981). Correlation between numbers of desmosomes and the aggressiveness of transitional cell carcinoma in human urinary bladder. Cancer *47*, 104-112.

Amar, L.S., Shabana, A.H., Oboeuf, M., Martin, N., and Forest, N. (1999). Involvement of desmoplakin phosphorylation in the regulation of desmosomes by protein kinase C, in HeLa cells. Cell Adhes Commun *7*, 125-138.

Ansel, J.C., Tiesman, J.P., Olerud, J.E., Krueger, J.G., Krane, J.F., Tara, D.C., Shipley, G.D., Gilbertson, D., Usui, M.L., and Hart, C.E. (1993). Human keratinocytes are a major source of cutaneous platelet-derived growth factor. J Clin Invest *92*, 671-678.

Antoniades, H.N., Galanopoulos, T., Neville-Golden, J., Kiritsy, C.P., and Lynch, S.E. (1991). Injury induces in vivo expression of platelet-derived growth factor (PDGF) and PDGF receptor mRNAs in skin epithelial cells and PDGF mRNA in connective tissue fibroblasts. Proc Natl Acad Sci U S A *88*, 565-569.

Antoniades, H.N., Galanopoulos, T., Neville-Golden, J., Kiritsy, C.P., and Lynch, S.E. (1993). Expression of growth factor and receptor mRNAs in skin epithelial cells following acute cutaneous injury. Am J Pathol *142*, 1099-1110.

Antsiferova, M., Klatte, J.E., Bodo, E., Paus, R., Jorcano, J.L., Matzuk, M.M., Werner, S., and Kogel, H. (2008). Keratinocyte-derived follistatin regulates epidermal homeostasis and wound repair. Lab Invest.

Arnoux, V., Come, C., Kusewitt, D., Hudson, L., Savagner, P. (2005). Cutaneous Wound Reepithelializaton: A partial and reversible EMT In: Rise and Fall of Epithelial Phenotype: Concepts of Epithelial-Mesenchymal Transition, ed. S. P., Berlin: Springer, 111-134.

Ashcroft, G.S., Greenwell-Wild, T., Horan, M.A., Wahl, S.M., and Ferguson, M.W. (1999). Topical estrogen accelerates cutaneous wound healing in aged humans associated with an altered inflammatory response. Am J Pathol *155*, 1137-1146.

Ashcroft, G.S., and Mills, S.J. (2002). Androgen receptor-mediated inhibition of cutaneous wound healing. J Clin Invest *110*, 615-624.

Ashcroft, G.S., Mills, S.J., Lei, K., Gibbons, L., Jeong, M.J., Taniguchi, M., Burow, M., Horan, M.A., Wahl, S.M., and Nakayama, T. (2003). Estrogen modulates cutaneous wound healing by downregulating macrophage migration inhibitory factor. J Clin Invest *111*, 1309-1318.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., and Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. Wound Repair Regen *16*, 585-601.

Batlle, E., Sancho, E., Franci, C., Dominguez, D., Monfar, M., Baulida, J., and Garcia De Herreros, A. (2000). The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. Nat Cell Biol *2*, 84-89.
Baum, B., Settleman, J., and Quinlan, M.P. (2008). Transitions between epithelial and mesenchymal states in development and disease. Semin Cell Dev Biol *19*, 294-308.
Baum, C.L., and Arpey, C.J. (2005). Normal cutaneous wound healing: clinical correlation with cellular and molecular events. Dermatol Surg *31*, 674-686; discussion 686.
Baumgart, E., Cohen, M.S., Silva Neto, B., Jacobs, M.A., Wotkowicz, C., Rieger-Christ, K.M., Biolo, A., Zeheb, R., Loda, M., Libertino, J.A., and Summerhayes, I.C. (2007). Identification and prognostic significance of an epithelial-mesenchymal transition expression profile in human bladder tumors. Clin Cancer Res *13*, 1685-1694.
Bech-Serra, J.J., Santiago-Josefat, B., Esselens, C., Saftig, P., Baselga, J., Arribas, J., and Canals, F. (2006). Proteomic identification of desmoglein 2 and activated leukocyte cell adhesion molecule as substrates of ADAM17 and ADAM10 by difference gel electrophoresis. Mol Cell Biol *26*, 5086-5095.
Beer, H.D., Fassler, R., and Werner, S. (2000a). Glucocorticoid-regulated gene expression during cutaneous wound repair. Vitam Horm *59*, 217-239.
Beer, H.D., Gassmann, M.G., Munz, B., Steiling, H., Engelhardt, F., Bleuel, K., and Werner, S. (2000b). Expression and function of keratinocyte growth factor and activin in skin morphogenesis and cutaneous wound repair. J Investig Dermatol Symp Proc *5*, 34-39.
Beer, H.D., Longaker, M.T., and Werner, S. (1997). Reduced expression of PDGF and PDGF receptors during impaired wound healing. J Invest Dermatol *109*, 132-138.
Bhora, F.Y., Dunkin, B.J., Batzri, S., Aly, H.M., Bass, B.L., Sidawy, A.N., and Harmon, J.W. (1995). Effect of growth factors on cell proliferation and epithelialization in human skin. J Surg Res *59*, 236-244.
Bitar, M.S. (1998). Glucocorticoid dynamics and impaired wound healing in diabetes mellitus. Am J Pathol *152*, 547-554.
Blakytny, R., Jude, E.B., Martin Gibson, J., Boulton, A.J., and Ferguson, M.W. (2000). Lack of insulin-like growth factor 1 (IGF1) in the basal keratinocyte layer of diabetic skin and diabetic foot ulcers. J Pathol *190*, 589-594.
Bolognia, J.L., Jorizzo, J.L., and Rapini, R.P. (2003). Dermatology. Mosby: London.
Bolos, V., Peinado, H., Perez-Moreno, M.A., Fraga, M.F., Esteller, M., and Cano, A. (2003). The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. J Cell Sci *116*, 499-511.
Boucher, I., Yang, L., Mayo, C., Klepeis, V., and Trinkaus-Randall, V. (2007). Injury and nucleotides induce phosphorylation of epidermal growth factor receptor: MMP and HB-EGF dependent pathway. Exp Eye Res *85*, 130-141.
Boyce, S.T., and Ham, R.G. (1983). Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. J Invest Dermatol *81*, 33s-40s.
Boyer, B., Tucker, G.C., Valles, A.M., Franke, W.W., and Thiery, J.P. (1989). Rearrangements of desmosomal and cytoskeletal proteins during the transition from

epithelial to fibroblastoid organization in cultured rat bladder carcinoma cells. J Cell Biol *109*, 1495-1509.
Brabletz, T., Jung, A., Spaderna, S., Hlubek, F., and Kirchner, T. (2005). Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer *5*, 744-749.
Brauchle, M., Fassler, R., and Werner, S. (1995). Suppression of keratinocyte growth factor expression by glucocorticoids in vitro and during wound healing. J Invest Dermatol *105*, 579-584.
Brem, H., Stojadinovic, O., Diegelmann, R.F., Entero, H., Lee, B., Pastar, I., Golinko, M., Rosenberg, H., and Tomic-Canic, M. (2007). Molecular markers in patients with chronic wounds to guide surgical debridement. Mol Med *13*, 30-39.
Brown, D.L., Kane, C.D., Chernausek, S.D., and Greenhalgh, D.G. (1997). Differential expression and localization of insulin-like growth factors I and II in cutaneous wounds of diabetic and nondiabetic mice. Am J Pathol *151*, 715-724.
Brown, G.L., Curtsinger, L., 3rd, Brightwell, J.R., Ackerman, D.M., Tobin, G.R., Polk, H.C., Jr., George-Nascimento, C., Valenzuela, P., and Schultz, G.S. (1986). Enhancement of epidermal regeneration by biosynthetic epidermal growth factor. J Exp Med *163*, 1319-1324.
Brown, G.L., Curtsinger, L.J., White, M., Mitchell, R.O., Pietsch, J., Nordquist, R., von Fraunhofer, A., and Schultz, G.S. (1988). Acceleration of tensile strength of incisions treated with EGF and TGF-beta. Ann Surg *208*, 788-794.
Brown, G.L., Nanney, L.B., Griffen, J., Cramer, A.B., Yancey, J.M., Curtsinger, L.J., 3rd, Holtzin, L., Schultz, G.S., Jurkiewicz, M.J., and Lynch, J.B. (1989). Enhancement of wound healing by topical treatment with epidermal growth factor. N Engl J Med *321*, 76-79.
Brown, M.V., Burnett, P.E., Denning, M.F., and Reynolds, A.B. (2009). PDGF receptor activation induces p120-catenin phosphorylation at serine 879 via a PKCalpha-dependent pathway. Exp Cell Res *315*, 39-49.
Brown, R.L., Ormsby, I., Doetschman, T.C., and Greenhalgh, D.G. (1995). Wound healing in the transforming growth factor-beta-deficient mouse. Wound Repair Regen *3*, 25-36.
Bryant, D.M., Kerr, M.C., Hammond, L.A., Joseph, S.R., Mostov, K.E., Teasdale, R.D., and Stow, J.L. (2007). EGF induces macropinocytosis and SNX1-modulated recycling of E-cadherin. J Cell Sci *120*, 1818-1828.
Bryant, D.M., and Stow, J.L. (2004). The ins and outs of E-cadherin trafficking. Trends Cell Biol *14*, 427-434.
Bryant, D.M., Wylie, F.G., and Stow, J.L. (2005). Regulation of endocytosis, nuclear translocation, and signaling of fibroblast growth factor receptor 1 by E-cadherin. Mol Biol Cell *16*, 14-23.
Buetow, B.S., Crosby, J.R., Kaminski, W.E., Ramachandran, R.K., Lindahl, P., Martin, P., Betsholtz, C., Seifert, R.A., Raines, E.W., and Bowen-Pope, D.F. (2001). Platelet-derived growth factor B-chain of hematopoietic origin is not necessary for granulation tissue formation and its absence enhances vascularization. Am J Pathol *159*, 1869-1876.
Burdett, I.D., and Sullivan, K.H. (2002). Desmosome assembly in MDCK cells: transport of precursors to the cell surface occurs by two phases of vesicular traffic and involves

major changes in centrosome and Golgi location during a Ca(2+) shift. Exp Cell Res *276*, 296-309.
Cabodi, S., Moro, L., Bergatto, E., Boeri Erba, E., Di Stefano, P., Turco, E., Tarone, G., and Defilippi, P. (2004). Integrin regulation of epidermal growth factor (EGF) receptor and of EGF-dependent responses. Biochem Soc Trans *32*, 438-442.
Calkins, C.C., Setzer, S.V., Jennings, J.M., Summers, S., Tsunoda, K., Amagai, M., and Kowalczyk, A.P. (2006). Desmoglein endocytosis and desmosome disassembly are coordinated responses to pemphigus autoantibodies. J Biol Chem *281*, 7623-7634.
Cano, A., Perez-Moreno, M.A., Rodrigo, I., Locascio, A., Blanco, M.J., del Barrio, M.G., Portillo, F., and Nieto, M.A. (2000). The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. Nat Cell Biol *2*, 76-83.
Carmeliet, P., Moons, L., Luttun, A., Vincenti, V., Compernolle, V., De Mol, M., Wu, Y., Bono, F., Devy, L., Beck, H., Scholz, D., Acker, T., DiPalma, T., Dewerchin, M., Noel, A., Stalmans, I., Barra, A., Blacher, S., Vandendriessche, T., Ponten, A., Eriksson, U., Plate, K.H., Foidart, J.M., Schaper, W., Charnock-Jones, D.S., Hicklin, D.J., Herbert, J.M., Collen, D., and Persico, M.G. (2001). Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. Nat Med *7*, 575-583.
Carretero, M., Escamez, M.J., Garcia, M., Duarte, B., Holguin, A., Retamosa, L., Jorcano, J.L., Rio, M.D., and Larcher, F. (2008). In vitro and in vivo wound healing-promoting activities of human cathelicidin LL-37. J Invest Dermatol *128*, 223-236.
Chandler, H.L., Colitz, C.M., Lu, P., Saville, W.J., and Kusewitt, D.F. (2007). The role of the slug transcription factor in cell migration during corneal re-epithelialization in the dog. Exp Eye Res *84*, 400-411.
Chen, L., Tredget, E.E., Wu, P.Y., and Wu, Y. (2008). Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. PLoS ONE *3*, e1886.
Chen, Y., Lu, Q., Schneeberger, E.E., and Goodenough, D.A. (2000). Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells. Mol Biol Cell *11*, 849-862.
Chitaev, N.A., and Troyanovsky, S.M. (1997). Direct Ca2+-dependent heterophilic interaction between desmosomal cadherins, desmoglein and desmocollin, contributes to cell-cell adhesion. J Cell Biol *138*, 193-201.
Choi, J., Park, S.Y., and Joo, C.K. (2007). Transforming growth factor-beta1 represses E-cadherin production via slug expression in lens epithelial cells. Invest Ophthalmol Vis Sci *48*, 2708-2718.
Cirillo, N., Campisi, G., Gombos, F., Perillo, L., Femiano, F., and Lanza, A. (2008a). Cleavage of desmoglein 3 can explain its depletion from keratinocytes in pemphigus vulgaris. Exp Dermatol *17*, 858-863.
Cirillo, N., Femiano, F., Gombos, F., and Lanza, A. (2007). Metalloproteinase 9 is the outer executioner of desmoglein 3 in apoptotic keratinocytes. Oral Dis *13*, 341-345.
Cirillo, N., Lanza, M., De Rosa, A., Cammarota, M., La Gatta, A., Gombos, F., and Lanza, A. (2008b). The most widespread desmosomal cadherin, desmoglein 2, is a novel target of caspase 3-mediated apoptotic machinery. J Cell Biochem *103*, 598-606.

Ciruna, B., and Rossant, J. (2001). FGF signaling regulates mesoderm cell fate specification and morphogenetic movement at the primitive streak. Dev Cell *1*, 37-49.
Clark, R.A.F. (1988). The Molecular and Cellular Biology of Wound Repair. Plenum Press: New York.
Clayton, E., Doupe, D.P., Klein, A.M., Winton, D.J., Simons, B.D., and Jones, P.H. (2007). A single type of progenitor cell maintains normal epidermis. Nature *446*, 185-189.
Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new-born animal. J Biol Chem *237*, 1555-1562.
Come, C., Magnino, F., Bibeau, F., De Santa Barbara, P., Becker, K.F., Theillet, C., and Savagner, P. (2006). Snail and slug play distinct roles during breast carcinoma progression. Clin Cancer Res *12*, 5395-5402.
Comijn, J., Berx, G., Vermassen, P., Verschueren, K., van Grunsven, L., Bruyneel, E., Mareel, M., Huylebroeck, D., and van Roy, F. (2001). The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. Mol Cell *7*, 1267-1278.
Conway, K., Ruge, F., Price, P., Harding, K.G., and Jiang, W.G. (2007). Hepatocyte growth factor regulation: an integral part of why wounds become chronic. Wound Repair Regen *15*, 683-692.
Coulombe, P.A. (2003). Wound epithelialization: accelerating the pace of discovery. J Invest Dermatol *121*, 219-230.
Cowden Dahl, K.D., Symowicz, J., Ning, Y., Gutierrez, E., Fishman, D.A., Adley, B.P., Stack, M.S., and Hudson, L.G. (2008). Matrix metalloproteinase 9 is a mediator of epidermal growth factor-dependent e-cadherin loss in ovarian carcinoma cells. Cancer Res *68*, 4606-4613.
Cross, K.J., and Mustoe, T.A. (2003). Growth factors in wound healing. Surg Clin North Am *83*, 531-545, vi.
D'Souza-Schorey, C. (2005). Disassembling adherens junctions: breaking up is hard to do. Trends Cell Biol *15*, 19-26.
Damjanov, I., Damjanov, A., and Damsky, C.H. (1986). Developmentally regulated expression of the cell-cell adhesion glycoprotein cell-CAM 120/80 in peri-implantation mouse embryos and extraembryonic membranes. Dev Biol *116*, 194-202.
Daniel, J.M. (2007). Dancing in and out of the nucleus: p120(ctn) and the transcription factor Kaiso. Biochim Biophys Acta *1773*, 59-68.
Daniel, W.W. (1987). Biostatistics: A Foundation for Analysis in the Health Sciences. John Wiley & Sons: New York.
Danjo, Y., and Gipson, I.K. (1998). Actin 'purse string' filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound, providing coordinated cell movement. J Cell Sci *111 (Pt 22)*, 3323-3332.
Davidson, B., Gotlieb, W.H., Ben-Baruch, G., Nesland, J.M., Bryne, M., Goldberg, I., Kopolovic, J., and Berner, A. (2000). E-Cadherin complex protein expression and survival in ovarian carcinoma. Gynecol Oncol *79*, 362-371.
Davies, G., Jiang, W.G., and Mason, M.D. (2001). Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. Clin Cancer Res *7*, 3289-3297.

del Barrio, M.G., and Nieto, M.A. (2002). Overexpression of Snail family members highlights their ability to promote chick neural crest formation. Development *129*, 1583-1593.
Delektorskaya, V.V., Perevoshchikov, A.G., Golovkov, D.A., and Kushlinskii, N.E. (2005). Expression of E-cadherin, beta-catenin, and CD-44v6 cell adhesion molecules in primary tumors and metastases of colorectal adenocarcinoma. Bull Exp Biol Med *139*, 706-710.
Delva, E., Jennings, J.M., Calkins, C.C., Kottke, M.D., Faundez, V., and Kowalczyk, A.P. (2008). Pemphigus vulgaris IgG-induced desmoglein-3 endocytosis and desmosomal disassembly are mediated by a clathrin- and dynamin-independent mechanism. J Biol Chem *283*, 18303-18313.
Devalaraja, R.M., Nanney, L.B., Du, J., Qian, Q., Yu, Y., Devalaraja, M.N., and Richmond, A. (2000). Delayed wound healing in CXCR2 knockout mice. J Invest Dermatol *115*, 234-244.
Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res *11*, 1475-1489.
Draper, B.K., Komurasaki, T., Davidson, M.K., and Nanney, L.B. (2003a). Epiregulin is more potent than EGF or TGFalpha in promoting in vitro wound closure due to enhanced ERK/MAPK activation. J Cell Biochem *89*, 1126-1137.
Draper, B.K., Komurasaki, T., Davidson, M.K., and Nanney, L.B. (2003b). Topical epiregulin enhances repair of murine excisional wounds. Wound Repair Regen *11*, 188-197.
Duden, R., and Franke, W.W. (1988). Organization of desmosomal plaque proteins in cells growing at low calcium concentrations. J Cell Biol *107*, 1049-1063.
Dvorak, H.F., Kaplan, A.P., and Clark, R. A. F. (1988). Potential Functions of the Clotting System in Wound Repair. In: The Molecular and Cellular Biology of Wound Repair, ed. R.A.F. Clark, Henson, P. M. , New York: Plenum Press, 57-85.
Eger, A., Aigner, K., Sonderegger, S., Dampier, B., Oehler, S., Schreiber, M., Berx, G., Cano, A., Beug, H., and Foisner, R. (2005). DeltaEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells. Oncogene *24*, 2375-2385.
Eming, S.A., Krieg, T., and Davidson, J.M. (2007). Inflammation in wound repair: molecular and cellular mechanisms. J Invest Dermatol *127*, 514-525.
Engelhardt, E., Toksoy, A., Goebeler, M., Debus, S., Brocker, E.B., and Gillitzer, R. (1998). Chemokines IL-8, GROalpha, MCP-1, IP-10, and Mig are sequentially and differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. Am J Pathol *153*, 1849-1860.
Failla, C.M., Odorisio, T., Cianfarani, F., Schietroma, C., Puddu, P., and Zambruno, G. (2000). Placenta growth factor is induced in human keratinocytes during wound healing. J Invest Dermatol *115*, 388-395.
Falanga, V., Eaglstein, W.H., Bucalo, B., Katz, M.H., Harris, B., and Carson, P. (1992). Topical use of human recombinant epidermal growth factor (h-EGF) in venous ulcers. J Dermatol Surg Oncol *18*, 604-606.

Fang, Y., Gong, S.J., Xu, Y.H., Hambly, B.D., and Bao, S. (2007). Impaired cutaneous wound healing in granulocyte/macrophage colony-stimulating factor knockout mice. Br J Dermatol *157*, 458-465.
Ferber, E.C., Kajita, M., Wadlow, A., Tobiansky, L., Niessen, C., Ariga, H., Daniel, J., and Fujita, Y. (2008). A role for the cleaved cytoplasmic domain of E-cadherin in the nucleus. J Biol Chem *283*, 12691-12700.
Ferreira, A.M., Rollins, B.J., Faunce, D.E., Burns, A.L., Zhu, X., and Dipietro, L.A. (2005). The effect of MCP-1 depletion on chemokine and chemokine-related gene expression: evidence for a complex network in acute inflammation. Cytokine *30*, 64-71.
Filardo, E.J. (2002). Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. J Steroid Biochem Mol Biol *80*, 231-238.
Franke, W.W., Grund, C., Kuhn, C., Jackson, B.W., and Illmensee, K. (1982). Formation of cytoskeletal elements during mouse embryogenesis. III. Primary mesenchymal cells and the first appearance of vimentin filaments. Differentiation *23*, 43-59.
Franklin, J.D., and Lynch, J.B. (1979). Effects of topical applications of epidermal growth factor on wound healing. Experimental study on rabbit ears. Plast Reconstr Surg *64*, 766-770.
Freedberg, I.M., Tomic-Canic, M., Komine, M., and Blumenberg, M. (2001). Keratins and the keratinocyte activation cycle. J Invest Dermatol *116*, 633-640.
Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H.E., Behrens, J., Sommer, T., and Birchmeier, W. (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat Cell Biol *4*, 222-231.
Funakoshi, S., Ezaki, T., Kong, J., Guo, R.J., and Lynch, J.P. (2008). Repression of the desmocollin 2 gene expression in human colon cancer cells is relieved by the homeodomain transcription factors Cdx1 and Cdx2. Mol Cancer Res *6*, 1478-1490.
Gabbiani, G., Chaponnier, C., and Huttner, I. (1978). Cytoplasmic filaments and gap junctions in epithelial cells and myofibroblasts during wound healing. J Cell Biol *76*, 561-568.
Galbiati, F., Volonte, D., Brown, A.M., Weinstein, D.E., Ben-Ze'ev, A., Pestell, R.G., and Lisanti, M.P. (2000). Caveolin-1 expression inhibits Wnt/beta-catenin/Lef-1 signaling by recruiting beta-catenin to caveolae membrane domains. J Biol Chem *275*, 23368-23377.
Gallucci, R.M., Sloan, D.K., Heck, J.M., Murray, A.R., and O'Dell, S.J. (2004). Interleukin 6 indirectly induces keratinocyte migration. J Invest Dermatol *122*, 764-772.
Gaudry, C.A., Palka, H.L., Dusek, R.L., Huen, A.C., Khandekar, M.J., Hudson, L.G., and Green, K.J. (2001). Tyrosine-phosphorylated plakoglobin is associated with desmogleins but not desmoplakin after epidermal growth factor receptor activation. J Biol Chem *276*, 24871-24880.
Gilles, C., Polette, M., Piette, J., Delvigne, A.C., Thompson, E.W., Foidart, J.M., and Birembaut, P. (1996). Vimentin expression in cervical carcinomas: association with invasive and migratory potential. J Pathol *180*, 175-180.
Gilles, C., Polette, M., Zahm, J.M., Tournier, J.M., Volders, L., Foidart, J.M., and Birembaut, P. (1999). Vimentin contributes to human mammary epithelial cell migration. J Cell Sci *112 (Pt 24)*, 4615-4625.

Gillitzer, R., and Goebeler, M. (2001). Chemokines in cutaneous wound healing. J Leukoc Biol *69*, 513-521.
Gilliver, S.C., Ashworth, J.J., and Ashcroft, G.S. (2007). The hormonal regulation of cutaneous wound healing. Clin Dermatol *25*, 56-62.
Gilliver, S.C., Ashworth, J.J., Mills, S.J., Hardman, M.J., and Ashcroft, G.S. (2006). Androgens modulate the inflammatory response during acute wound healing. J Cell Sci *119*, 722-732.
Gope, R. (2002). The effect of epidermal growth factor & platelet-derived growth factors on wound healing process. Indian J Med Res *116*, 201-206.
Goren, I., Muller, E., Schiefelbein, D., Christen, U., Pfeilschifter, J., Muhl, H., and Frank, S. (2007). Systemic anti-TNFalpha treatment restores diabetes-impaired skin repair in ob/ob mice by inactivation of macrophages. J Invest Dermatol *127*, 2259-2267.
Green, K.J., Geiger, B., Jones, J.C., Talian, J.C., and Goldman, R.D. (1987). The relationship between intermediate filaments and microfilaments before and during the formation of desmosomes and adherens-type junctions in mouse epidermal keratinocytes. J Cell Biol *104*, 1389-1402.
Grose, R., and Werner, S. (2004). Wound-healing studies in transgenic and knockout mice. Mol Biotechnol *28*, 147-166.
Grotendorst, G.R., Soma, Y., Takehara, K., and Charette, M. (1989). EGF and TGF-alpha are potent chemoattractants for endothelial cells and EGF-like peptides are present at sites of tissue regeneration. J Cell Physiol *139*, 617-623.
Grzesiak, J.J., and Pierschbacher, M.D. (1995). Shifts in the concentrations of magnesium and calcium in early porcine and rat wound fluids activate the cell migratory response. J Clin Invest *95*, 227-233.
Guaita, S., Puig, I., Franci, C., Garrido, M., Dominguez, D., Batlle, E., Sancho, E., Dedhar, S., De Herreros, A.G., and Baulida, J. (2002). Snail induction of epithelial to mesenchymal transition in tumor cells is accompanied by MUC1 repression and ZEB1 expression. J Biol Chem *277*, 39209-39216.
Gumbiner, B., Stevenson, B., and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. J Cell Biol *107*, 1575-1587.
Guo, L., Degenstein, L., and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. Genes Dev *10*, 165-175.
Gurtner, G.C., Werner, S., Barrandon, Y., and Longaker, M.T. (2008). Wound repair and regeneration. Nature *453*, 314-321.
Hay, E.D. (2005). EMT Concept and Examples from the Vertebrate Embryo. In: Rise and Fall of Epithelial Phenotype, ed. P. Savagner, New York: Plenum Press, 1-11

Hayashida, Y., Urata, Y., Muroi, E., Kono, T., Miyata, Y., Nomata, K., Kanetake, H., Kondo, T., and Ihara, Y. (2006). Calreticulin represses E-cadherin gene expression in Madin-Darby canine kidney cells via Slug. J Biol Chem *281*, 32469-32484.
Heatley, M., Whiteside, C., Maxwell, P., and Toner, P. (1993). Vimentin expression in benign and malignant breast epithelium. J Clin Pathol *46*, 441-445.
Heimann, R., Lan, F., McBride, R., and Hellman, S. (2000). Separating favorable from unfavorable prognostic markers in breast cancer: the role of E-cadherin. Cancer Res *60*, 298-304.

Hengge, U.R., Ruzicka, T., Schwartz, R.A., and Cork, M.J. (2006). Adverse effects of topical glucocorticosteroids. J Am Acad Dermatol *54*, 1-15; quiz 16-18.
Hennings, H., and Holbrook, K.A. (1983). Calcium regulation of cell-cell contact and differentiation of epidermal cells in culture. An ultrastructural study. Exp Cell Res *143*, 127-142.
Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K., and Yuspa, S.H. (1980). Calcium regulation of growth and differentiation of mouse epidermal cells in culture. Cell *19*, 245-254.
Herren, B., Levkau, B., Raines, E.W., and Ross, R. (1998). Cleavage of beta-catenin and plakoglobin and shedding of VE-cadherin during endothelial apoptosis: evidence for a role for caspases and metalloproteinases. Mol Biol Cell *9*, 1589-1601.
Higashiyama, S., and Nanba, D. (2005). ADAM-mediated ectodomain shedding of HB-EGF in receptor cross-talk. Biochim Biophys Acta *1751*, 110-117.
Holm, P.K., Hansen, S.H., Sandvig, K., and van Deurs, B. (1993). Endocytosis of desmosomal plaques depends on intact actin filaments and leads to a nondegradative compartment. Eur J Cell Biol *62*, 362-371.
Holman, D.M., and Kalaaji, A.N. (2006). Cytokines in dermatology. J Drugs Dermatol *5*, 520-524.
Hong, J.P., Jung, H.D., and Kim, Y.W. (2006). Recombinant human epidermal growth factor (EGF) to enhance healing for diabetic foot ulcers. Ann Plast Surg *56*, 394-398; discussion 399-400.
Howdieshell, T.R., Callaway, D., Webb, W.L., Gaines, M.D., Procter, C.D., Jr., Sathyanarayana, Pollock, J.S., Brock, T.L., and McNeil, P.L. (2001). Antibody neutralization of vascular endothelial growth factor inhibits wound granulation tissue formation. J Surg Res *96*, 173-182.
Hu, P., Berkowitz, P., O'Keefe, E.J., and Rubenstein, D.S. (2003). Keratinocyte adherens junctions initiate nuclear signaling by translocation of plakoglobin from the membrane to the nucleus. J Invest Dermatol *121*, 242-251.
Hu, P., O'Keefe, E.J., and Rubenstein, D.S. (2001). Tyrosine phosphorylation of human keratinocyte beta-catenin and plakoglobin reversibly regulates their binding to E-cadherin and alpha-catenin. J Invest Dermatol *117*, 1059-1067.
Huang, F., Khvorova, A., Marshall, W., and Sorkin, A. (2004). Analysis of clathrin-mediated endocytosis of epidermal growth factor receptor by RNA interference. J Biol Chem *279*, 16657-16661.
Hubner, G., Brauchle, M., Smola, H., Madlener, M., Fassler, R., and Werner, S. (1996a). Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. Cytokine *8*, 548-556.
Hubner, G., Hu, Q., Smola, H., and Werner, S. (1996b). Strong induction of activin expression after injury suggests an important role of activin in wound repair. Dev Biol *173*, 490-498.
Hudson, L.G., and McCawley, L.J. (1998). Contributions of the epidermal growth factor receptor to keratinocyte motility. Microsc Res Tech *43*, 444-455.
Hudson, L.G., Toscano, W.A., Jr., and Greenlee, W.F. (1986). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) modulates epidermal growth factor (EGF) binding to basal cells from a human keratinocyte cell line. Toxicol Appl Pharmacol *82*, 481-492.

Hudson, L.G., Zeineldin, R., and Stack, M.S. (2008). Phenotypic plasticity of neoplastic ovarian epithelium: unique cadherin profiles in tumor progression. Clin Exp Metastasis *25*, 643-655.
Hung, C.F., Chiang, H.S., Lo, H.M., Jian, J.S., and Wu, W.B. (2006). E-cadherin and its downstream catenins are proteolytically cleaved in human HaCaT keratinocytes exposed to UVB. Exp Dermatol *15*, 315-321.
Hutcheon, A.E., Sippel, K.C., and Zieske, J.D. (2007). Examination of the restoration of epithelial barrier function following superficial keratectomy. Exp Eye Res *84*, 32-38.
Itoh, Y., Joh, T., Tanida, S., Sasaki, M., Kataoka, H., Itoh, K., Oshima, T., Ogasawara, N., Togawa, S., Wada, T., Kubota, H., Mori, Y., Ohara, H., Nomura, T., Higashiyama, S., and Itoh, M. (2005). IL-8 promotes cell proliferation and migration through metalloproteinase-cleavage proHB-EGF in human colon carcinoma cells. Cytokine *29*, 275-282.
Ivanov, A.I., Nusrat, A., and Parkos, C.A. (2004). Endocytosis of epithelial apical junctional proteins by a clathrin-mediated pathway into a unique storage compartment. Mol Biol Cell *15*, 176-188.
Izumi, G., Sakisaka, T., Baba, T., Tanaka, S., Morimoto, K., and Takai, Y. (2004). Endocytosis of E-cadherin regulated by Rac and Cdc42 small G proteins through IQGAP1 and actin filaments. J Cell Biol *166*, 237-248.
Janda, E., Nevolo, M., Lehmann, K., Downward, J., Beug, H., and Grieco, M. (2006). Raf plus TGFbeta-dependent EMT is initiated by endocytosis and lysosomal degradation of E-cadherin. Oncogene *25*, 7117-7130.
Jeanes, A., Gottardi, C.J., and Yap, A.S. (2008). Cadherins and cancer: how does cadherin dysfunction promote tumor progression? Oncogene *27*, 6920-6929.
Jiang, C.K., Magnaldo, T., Ohtsuki, M., Freedberg, I.M., Bernerd, F., and Blumenberg, M. (1993). Epidermal growth factor and transforming growth factor alpha specifically induce the activation- and hyperproliferation-associated keratins 6 and 16. Proc Natl Acad Sci U S A *90*, 6786-6790.
Johnson, S.K., Ramani, V.C., Hennings, L., and Haun, R.S. (2007). Kallikrein 7 enhances pancreatic cancer cell invasion by shedding E-cadherin. Cancer *109*, 1811-1820.
Jones, P., and Simons, B.D. (2008). Epidermal homeostasis: do committed progenitors work while stem cells sleep? Nat Rev Mol Cell Biol *9*, 82-88.
Kaiser, S., Schirmacher, P., Philipp, A., Protschka, M., Moll, I., Nicol, K., and Blessing, M. (1998). Induction of bone morphogenetic protein-6 in skin wounds. Delayed reepitheliazation and scar formation in BMP-6 overexpressing transgenic mice. J Invest Dermatol *111*, 1145-1152.
Kamei, T., Matozaki, T., Sakisaka, T., Kodama, A., Yokoyama, S., Peng, Y.F., Nakano, K., Takaishi, K., and Takai, Y. (1999). Coendocytosis of cadherin and c-Met coupled to disruption of cell-cell adhesion in MDCK cells--regulation by Rho, Rac and Rab small G proteins. Oncogene *18*, 6776-6784.
Kao, J.S., Fluhr, J.W., Man, M.Q., Fowler, A.J., Hachem, J.P., Crumrine, D., Ahn, S.K., Brown, B.E., Elias, P.M., and Feingold, K.R. (2003). Short-term glucocorticoid treatment compromises both permeability barrier homeostasis and stratum corneum integrity: inhibition of epidermal lipid synthesis accounts for functional abnormalities. J Invest Dermatol *120*, 456-464.

Khan, E.M., Heidinger, J.M., Levy, M., Lisanti, M.P., Ravid, T., and Goldkorn, T. (2006). Epidermal growth factor receptor exposed to oxidative stress undergoes Src- and caveolin-1-dependent perinuclear trafficking. J Biol Chem *281*, 14486-14493.
Kiguchi, K., Bol, D., Carbajal, S., Beltran, L., Moats, S., Chan, K., Jorcano, J., and DiGiovanni, J. (2000). Constitutive expression of erbB2 in epidermis of transgenic mice results in epidermal hyperproliferation and spontaneous skin tumor development. Oncogene *19*, 4243-4254.
Kim, I., Mogford, J.E., Chao, J.D., and Mustoe, T.A. (2001). Wound epithelialization deficits in the transforming growth factor-alpha knockout mouse. Wound Repair Regen *9*, 386-390.
Kinch, M.S., Clark, G.J., Der, C.J., and Burridge, K. (1995). Tyrosine phosphorylation regulates the adhesions of ras-transformed breast epithelia. J Cell Biol *130*, 461-471.
Klessner, J.L., Desai, B.V., Amargo, E.V., Getsios, S., and Green, K.J. (2008). EGFR and ADAMs Cooperate to Regulate Shedding and Endocytic Trafficking of the Desmosomal Cadherin Desmoglein 2. Mol Biol Cell.
Kocher, O., Amaudruz, M., Schindler, A.M., and Gabbiani, G. (1981). Desmosomes and gap junctions in precarcinomatous and carcinomatous conditions of squamous epithelia. An electron microscopic and morphometrical study. J Submicrosc Cytol *13*, 267-281.
Kramer, R.H., Shen, X., and Zhou, H. (2005). Tumor cell invasion and survival in head and neck cancer. Cancer Metastasis Rev *24*, 35-45.
Krawczyk, W.S., and Wilgram, G.F. (1973). Hemidesmosome and desmosome morphogenesis during epidermal wound healing. J Ultrastruct Res *45*, 93-101.
Kuwahara, M., Hatoko, M., Tada, H., and Tanaka, A. (2001). E-cadherin expression in wound healing of mouse skin. J Cutan Pathol *28*, 191-199.
Latijnhouwers, M., Bergers, M., Ponec, M., Dijkman, H., Andriessen, M., and Schalkwijk, J. (1997). Human epidermal keratinocytes are a source of tenascin-C during wound healing. J Invest Dermatol *108*, 776-783.
Le, T.L., Joseph, S.R., Yap, A.S., and Stow, J.L. (2002). Protein kinase C regulates endocytosis and recycling of E-cadherin. Am J Physiol Cell Physiol *283*, C489-499.
Le, T.L., Yap, A.S., and Stow, J.L. (1999). Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. J Cell Biol *146*, 219-232.
Leong, K.G., Niessen, K., Kulic, I., Raouf, A., Eaves, C., Pollet, I., and Karsan, A. (2007). Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. J Exp Med *204*, 2935-2948.
Lewis, J.E., Jensen, P.J., Johnson, K.R., and Wheelock, M.J. (1994). E-cadherin mediates adherens junction organization through protein kinase C. J Cell Sci *107 (Pt 12)*, 3615-3621.
Lewis, J.E., Wahl, J.K., 3rd, Sass, K.M., Jensen, P.J., Johnson, K.R., and Wheelock, M.J. (1997). Cross-talk between adherens junctions and desmosomes depends on plakoglobin. J Cell Biol *136*, 919-934.
Li, G., Schaider, H., Satyamoorthy, K., Hanakawa, Y., Hashimoto, K., and Herlyn, M. (2001). Downregulation of E-cadherin and Desmoglein 1 by autocrine hepatocyte growth factor during melanoma development. Oncogene *20*, 8125-8135.
Liechty, K.W., Crombleholme, T.M., Cass, D.L., Martin, B., and Adzick, N.S. (1998). Diminished interleukin-8 (IL-8) production in the fetal wound healing response. J Surg Res *77*, 80-84.

Lin, Z.Q., Kondo, T., Ishida, Y., Takayasu, T., and Mukaida, N. (2003). Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. J Leukoc Biol *73*, 713-721.
Ling, K., Bairstow, S.F., Carbonara, C., Turbin, D.A., Huntsman, D.G., and Anderson, R.A. (2007). Type Igamma phosphatidylinositol phosphate kinase modulates adherens junction and E-cadherin trafficking via a direct interaction with mu 1B adaptin. J Cell Biol *176*, 343-353.
Lock, J.G., and Stow, J.L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. Mol Biol Cell *16*, 1744-1755.
Lorch, J.H., Klessner, J., Park, J.K., Getsios, S., Wu, Y.L., Stack, M.S., and Green, K.J. (2004). Epidermal growth factor receptor inhibition promotes desmosome assembly and strengthens intercellular adhesion in squamous cell carcinoma cells. J Biol Chem *279*, 37191-37200.
Lu, Z., Ghosh, S., Wang, Z., and Hunter, T. (2003). Downregulation of caveolin-1 function by EGF leads to the loss of E-cadherin, increased transcriptional activity of beta-catenin, and enhanced tumor cell invasion. Cancer Cell *4*, 499-515.
Luckett, L.R., and Gallucci, R.M. (2007). Interleukin-6 (IL-6) modulates migration and matrix metalloproteinase function in dermal fibroblasts from IL-6KO mice. Br J Dermatol *156*, 1163-1171.
Luetteke, N.C., Qiu, T.H., Peiffer, R.L., Oliver, P., Smithies, O., and Lee, D.C. (1993). TGF alpha deficiency results in hair follicle and eye abnormalities in targeted and waved-1 mice. Cell *73*, 263-278.
Madlener, M., Parks, W.C., and Werner, S. (1998). Matrix metalloproteinases (MMPs) and their physiological inhibitors (TIMPs) are differentially expressed during excisional skin wound repair. Exp Cell Res *242*, 201-210.
Maeda, M., Shintani, Y., Wheelock, M.J., and Johnson, K.R. (2006). Src activation is not necessary for transforming growth factor (TGF)-beta-mediated epithelial to mesenchymal transitions (EMT) in mammary epithelial cells. PP1 directly inhibits TGF-beta receptors I and II. J Biol Chem *281*, 59-68.
Magee, A.I., Lytton, N.A., and Watt, F.M. (1987). Calcium-induced changes in cytoskeleton and motility of cultured human keratinocytes. Exp Cell Res *172*, 43-53.
Malminen, M., Koivukangas, V., Peltonen, J., Karvonen, S.L., Oikarinen, A., and Peltonen, S. (2003). Immunohistological distribution of the tight junction components ZO-1 and occludin in regenerating human epidermis. Br J Dermatol *149*, 255-260.
Mann, A., Breuhahn, K., Schirmacher, P., and Blessing, M. (2001). Keratinocyte-derived granulocyte-macrophage colony stimulating factor accelerates wound healing: Stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization. J Invest Dermatol *117*, 1382-1390.
Mann, A., Niekisch, K., Schirmacher, P., and Blessing, M. (2006). Granulocyte-macrophage colony-stimulating factor is essential for normal wound healing. J Investig Dermatol Symp Proc *11*, 87-92.
Mann, G.B., Fowler, K.J., Gabriel, A., Nice, E.C., Williams, R.L., and Dunn, A.R. (1993). Mice with a null mutation of the TGF alpha gene have abnormal skin architecture, wavy hair, and curly whiskers and often develop corneal inflammation. Cell *73*, 249-261.

Marambaud, P., Shioi, J., Serban, G., Georgakopoulos, A., Sarner, S., Nagy, V., Baki, L., Wen, P., Efthimiopoulos, S., Shao, Z., Wisniewski, T., and Robakis, N.K. (2002). A presenilin-1/gamma-secretase cleavage releases the E-cadherin intracellular domain and regulates disassembly of adherens junctions. Embo J *21*, 1948-1956.
Maretzky, T., Reiss, K., Ludwig, A., Buchholz, J., Scholz, F., Proksch, E., de Strooper, B., Hartmann, D., and Saftig, P. (2005). ADAM10 mediates E-cadherin shedding and regulates epithelial cell-cell adhesion, migration, and beta-catenin translocation. Proc Natl Acad Sci U S A *102*, 9182-9187.
Marikovsky, M., Vogt, P., Eriksson, E., Rubin, J.S., Taylor, W.G., Joachim, S., and Klagsbrun, M. (1996). Wound fluid-derived heparin-binding EGF-like growth factor (HB-EGF) is synergistic with insulin-like growth factor-I for Balb/MK keratinocyte proliferation. J Invest Dermatol *106*, 616-621.
Mariner, D.J., Anastasiadis, P., Keilhack, H., Bohmer, F.D., Wang, J., and Reynolds, A.B. (2001). Identification of Src phosphorylation sites in the catenin p120ctn. J Biol Chem *276*, 28006-28013.
Mariner, D.J., Davis, M.A., and Reynolds, A.B. (2004). EGFR signaling to p120-catenin through phosphorylation at Y228. J Cell Sci *117*, 1339-1350.
Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. Science *276*, 75-81.
Mathay, C., Giltaire, S., Minner, F., Bera, E., Herin, M., and Poumay, Y. (2008). Heparin-binding EGF-like growth factor is induced by disruption of lipid rafts and oxidative stress in keratinocytes and participates in the epidermal response to cutaneous wounds. J Invest Dermatol *128*, 717-727.
Matsuda, H., Koyama, H., Sato, H., Sawada, J., Itakura, A., Tanaka, A., Matsumoto, M., Konno, K., Ushio, H., and Matsuda, K. (1998). Role of nerve growth factor in cutaneous wound healing: accelerating effects in normal and healing-impaired diabetic mice. J Exp Med *187*, 297-306.
Mayer, B., Johnson, J.P., Leitl, F., Jauch, K.W., Heiss, M.M., Schildberg, F.W., Birchmeier, W., and Funke, I. (1993). E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. Cancer Res *53*, 1690-1695.
McCarthy, D.W., Downing, M.T., Brigstock, D.R., Luquette, M.H., Brown, K.D., Abad, M.S., and Besner, G.E. (1996). Production of heparin-binding epidermal growth factor-like growth factor (HB-EGF) at sites of thermal injury in pediatric patients. J Invest Dermatol *106*, 49-56.
McCawley, L.J., O'Brien, P., and Hudson, L.G. (1997). Overexpression of the epidermal growth factor receptor contributes to enhanced ligand-mediated motility in keratinocyte cell lines. Endocrinology *138*, 121-127.
McCawley, L.J., O'Brien, P., and Hudson, L.G. (1998). Epidermal growth factor (EGF)- and scatter factor/hepatocyte growth factor (SF/HGF)- mediated keratinocyte migration is coincident with induction of matrix metalloproteinase (MMP)-9. J Cell Physiol *176*, 255-265.
McInroy, L., and Maatta, A. (2007). Down-regulation of vimentin expression inhibits carcinoma cell migration and adhesion. Biochem Biophys Res Commun *360*, 109-114.

Mills, S.J., Ashworth, J.J., Gilliver, S.C., Hardman, M.J., and Ashcroft, G.S. (2005). The sex steroid precursor DHEA accelerates cutaneous wound healing via the estrogen receptors. J Invest Dermatol *125*, 1053-1062.
Miyashita, Y., and Ozawa, M. (2007a). A dileucine motif in its cytoplasmic domain directs beta-catenin-uncoupled E-cadherin to the lysosome. J Cell Sci *120*, 4395-4406.
Miyashita, Y., and Ozawa, M. (2007b). Increased internalization of p120-uncoupled E-cadherin and a requirement for a dileucine motif in the cytoplasmic domain for endocytosis of the protein. J Biol Chem *282*, 11540-11548.
Mohan, V.K. (2007). Recombinant human epidermal growth factor (REGEN-D 150): effect on healing of diabetic foot ulcers. Diabetes Res Clin Pract *78*, 405-411.
Mol, A.J., Geldof, A.A., Meijer, G.A., van der Poel, H.G., and van Moorselaar, R.J. (2007). New experimental markers for early detection of high-risk prostate cancer: role of cell-cell adhesion and cell migration. J Cancer Res Clin Oncol *133*, 687-695.
Moon, R.T., Bowerman, B., Boutros, M., and Perrimon, N. (2002). The promise and perils of Wnt signaling through beta-catenin. Science *296*, 1644-1646.
Morimoto, S., Nishimura, N., Terai, T., Manabe, S., Yamamoto, Y., Shinahara, W., Miyake, H., Tashiro, S., Shimada, M., and Sasaki, T. (2005). Rab13 mediates the continuous endocytic recycling of occludin to the cell surface. J Biol Chem *280*, 2220-2228.
Motulsky, H. (1999). Analyzing Data with GraphPad Prism. GraphPad Software, Inc: San Diego, CA.
Muller, T., Choidas, A., Reichmann, E., and Ullrich, A. (1999). Phosphorylation and free pool of beta-catenin are regulated by tyrosine kinases and tyrosine phosphatases during epithelial cell migration. J Biol Chem *274*, 10173-10183.
Najy, A.J., Day, K.C., and Day, M.L. (2008). The ectodomain shedding of E-cadherin by ADAM15 supports ErbB receptor activation. J Biol Chem *283*, 18393-18401.
Nanba, D., Inoue, H., Shigemi, Y., Shirakata, Y., Hashimoto, K., and Higashiyama, S. (2008). An intermediary role of proHB-EGF shedding in growth factor-induced c-Myc gene expression. J Cell Physiol *214*, 465-473.
Nanney, L.B. (1990). Epidermal and dermal effects of epidermal growth factor during wound repair. J Invest Dermatol *94*, 624-629.
Nanney, L.B., Paulsen, S., Davidson, M.K., Cardwell, N.L., Whitsitt, J.S., and Davidson, J.M. (2000). Boosting epidermal growth factor receptor expression by gene gun transfection stimulates epidermal growth in vivo. Wound Repair Regen *8*, 117-127.
Newgreen, D.F.a.M., S. J. (2005). The Neural Crest: A Model Developmental EMT. In: Rise and Fall of Epithelial Phenotype, ed. P. Savagner, New York: Plenum Press, 29-39.
Nieto, M.A., Sargent, M.G., Wilkinson, D.G., and Cooke, J. (1994). Control of cell behavior during vertebrate development by Slug, a zinc finger gene. Science *264*, 835-839.
Ning, Y., Buranda, T., and Hudson, L.G. (2007). Activated epidermal growth factor receptor induces integrin alpha2 internalization via caveolae/raft-dependent endocytic pathway. J Biol Chem *282*, 6380-6387.
Noe, V., Fingleton, B., Jacobs, K., Crawford, H.C., Vermeulen, S., Steelant, W., Bruyneel, E., Matrisian, L.M., and Mareel, M. (2001). Release of an invasion promoter E-cadherin fragment by matrilysin and stromelysin-1. J Cell Sci *114*, 111-118.

O'Keefe, E.J., Briggaman, R.A., and Herman, B. (1987). Calcium-induced assembly of adherens junctions in keratinocytes. J Cell Biol *105*, 807-817.
Okwueze, M.I., Cardwell, N.L., Pollins, A.C., and Nanney, L.B. (2007). Modulation of porcine wound repair with a transfected ErbB3 gene and relevant EGF-like ligands. J Invest Dermatol *127*, 1030-1041.
Ono, I., Gunji, H., Zhang, J.Z., Maruyama, K., and Kaneko, F. (1995). Studies on cytokines related to wound healing in donor site wound fluid. J Dermatol Sci *10*, 241-245.
Oshiro, M.M., Kim, C.J., Wozniak, R.J., Junk, D.J., Munoz-Rodriguez, J.L., Burr, J.A., Fitzgerald, M., Pawar, S.C., Cress, A.E., Domann, F.E., and Futscher, B.W. (2005). Epigenetic silencing of DSC3 is a common event in human breast cancer. Breast Cancer Res *7*, R669-680.
Palacios, F., Schweitzer, J.K., Boshans, R.L., and D'Souza-Schorey, C. (2002). ARF6-GTP recruits Nm23-H1 to facilitate dynamin-mediated endocytosis during adherens junctions disassembly. Nat Cell Biol *4*, 929-936.
Palacios, F., Tushir, J.S., Fujita, Y., and D'Souza-Schorey, C. (2005). Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. Mol Cell Biol *25*, 389-402.
Palovuori, R., Sormunen, R., and Eskelinen, S. (2003). SRC-induced disintegration of adherens junctions of madin-darby canine kidney cells is dependent on endocytosis of cadherin and antagonized by Tiam-1. Lab Invest *83*, 1901-1915.
Park, S.H., Cheung, L.W., Wong, A.S., and Leung, P.C. (2008). Estrogen regulates Snail and Slug in the down-regulation of E-cadherin and induces metastatic potential of ovarian cancer cells through estrogen receptor alpha. Mol Endocrinol *22*, 2085-2098.
Pastore, S., Mascia, F., Mariani, V., and Girolomoni, G. (2008). The epidermal growth factor receptor system in skin repair and inflammation. J Invest Dermatol *128*, 1365-1374.
Paterson, A.D., Parton, R.G., Ferguson, C., Stow, J.L., and Yap, A.S. (2003). Characterization of E-cadherin endocytosis in isolated MCF-7 and chinese hamster ovary cells: the initial fate of unbound E-cadherin. J Biol Chem *278*, 21050-21057.
Pauli, B.U., Cohen, S.M., Alroy, J., and Weinstein, R.S. (1978). Desmosome ultrastructure and biological behavior of chemical carcinogen-induced urinary bladder carcinomas. Cancer Res *38*, 3276-3285.
Peinado, H., Olmeda, D., and Cano, A. (2007). Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype? Nat Rev Cancer *7*, 415-428.
Penn, E.J., Burdett, I.D., Hobson, C., Magee, A.I., and Rees, D.A. (1987). Structure and assembly of desmosome junctions: biosynthesis and turnover of the major desmosome components of Madin-Darby canine kidney cells in low calcium medium. J Cell Biol *105*, 2327-2334.
Piedra, J., Martinez, D., Castano, J., Miravet, S., Dunach, M., and de Herreros, A.G. (2001). Regulation of beta-catenin structure and activity by tyrosine phosphorylation. J Biol Chem *276*, 20436-20443.
Piedra, J., Miravet, S., Castano, J., Palmer, H.G., Heisterkamp, N., Garcia de Herreros, A., and Dunach, M. (2003). p120 Catenin-associated Fer and Fyn tyrosine kinases regulate beta-catenin Tyr-142 phosphorylation and beta-catenin-alpha-catenin Interaction. Mol Cell Biol *23*, 2287-2297.

Piscatelli, S.J., Michaels, B.M., Gregory, P., Jennings, R.W., Longaker, M.T., Harrison, M.R., and Siebert, J.W. (1994). Fetal fibroblast contraction of collagen matrices in vitro: the effects of epidermal growth factor and transforming growth factor-beta. Ann Plast Surg *33*, 38-45.
Pittelkow, M.R., Cook, P.W., Shipley, G.D., Derynck, R., and Coffey, R.J., Jr. (1993). Autonomous growth of human keratinocytes requires epidermal growth factor receptor occupancy. Cell Growth Differ *4*, 513-521.
Plowman, G.D., Culouscou, J.M., Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G., and Shoyab, M. (1993). Ligand-specific activation of HER4/p180erbB4, a fourth member of the epidermal growth factor receptor family. Proc Natl Acad Sci U S A *90*, 1746-1750.
Raja, Sivamani, K., Garcia, M.S., and Isseroff, R.R. (2007). Wound re-epithelialization: modulating keratinocyte migration in wound healing. Front Biosci *12*, 2849-2868.
Ramaekers, F.C., Verhagen, A.P., Isaacs, J.T., Feitz, W.F., Moesker, O., Schaart, G., Schalken, J.A., and Vooijs, G.P. (1989). Intermediate filament expression and the progression of prostatic cancer as studied in the Dunning R-3327 rat prostatic carcinoma system. Prostate *14*, 323-339.
Redd, M.J., Cooper, L., Wood, W., Stramer, B., and Martin, P. (2004). Wound healing and inflammation: embryos reveal the way to perfect repair. Philos Trans R Soc Lond B Biol Sci *359*, 777-784.
Reed, B.R., and Clark, R.A. (1985). Cutaneous tissue repair: practical implications of current knowledge. II. J Am Acad Dermatol *13*, 919-941.
Repertinger, S.K., Campagnaro, E., Fuhrman, J., El-Abaseri, T., Yuspa, S.H., and Hansen, L.A. (2004). EGFR enhances early healing after cutaneous incisional wounding. J Invest Dermatol *123*, 982-989.
Reuterdahl, C., Sundberg, C., Rubin, K., Funa, K., and Gerdin, B. (1993). Tissue localization of beta receptors for platelet-derived growth factor and platelet-derived growth factor B chain during wound repair in humans. J Clin Invest *91*, 2065-2075.
Rios-Doria, J., Day, K.C., Kuefer, R., Rashid, M.G., Chinnaiyan, A.M., Rubin, M.A., and Day, M.L. (2003). The role of calpain in the proteolytic cleavage of E-cadherin in prostate and mammary epithelial cells. J Biol Chem *278*, 1372-1379.
Rios-Doria, J., Kuefer, R., Ethier, S.P., and Day, M.L. (2004). Cleavage of beta-catenin by calpain in prostate and mammary tumor cells. Cancer Res *64*, 7237-7240.
Romer, J., Lund, L.R., Eriksen, J., Pyke, C., Kristensen, P., and Dano, K. (1994). The receptor for urokinase-type plasminogen activator is expressed by keratinocytes at the leading edge during re-epithelialization of mouse skin wounds. J Invest Dermatol *102*, 519-522.
Romer, J., Lund, L.R., Eriksen, J., Ralfkiaer, E., Zeheb, R., Gelehrter, T.D., Dano, K., and Kristensen, P. (1991). Differential expression of urokinase-type plasminogen activator and its type-1 inhibitor during healing of mouse skin wounds. J Invest Dermatol *97*, 803-811.
Rothen-Rutishauser, B., Riesen, F.K., Braun, A., Gunthert, M., and Wunderli-Allenspach, H. (2002). Dynamics of tight and adherens junctions under EGTA treatment. J Membr Biol *188*, 151-162.

Ryniers, F., Stove, C., Goethals, M., Brackenier, L., Noe, V., Bracke, M., Vandekerckhove, J., Mareel, M., and Bruyneel, E. (2002). Plasmin produces an E-cadherin fragment that stimulates cancer cell invasion. Biol Chem *383*, 159-165.
Sabourin, C.L., Petrali, J.P., and Casillas, R.P. (2000). Alterations in inflammatory cytokine gene expression in sulfur mustard-exposed mouse skin. J Biochem Mol Toxicol *14*, 291-302.
Sahin, U., and Blobel, C.P. (2007). Ectodomain shedding of the EGF-receptor ligand epigen is mediated by ADAM17. FEBS Lett *581*, 41-44.
Sahin, U., Weskamp, G., Kelly, K., Zhou, H.M., Higashiyama, S., Peschon, J., Hartmann, D., Saftig, P., and Blobel, C.P. (2004). Distinct roles for ADAM10 and ADAM17 in ectodomain shedding of six EGFR ligands. J Cell Biol *164*, 769-779.
Sato, Y., Ohshima, T., and Kondo, T. (1999). Regulatory role of endogenous interleukin-10 in cutaneous inflammatory response of murine wound healing. Biochem Biophys Res Commun *265*, 194-199.
Savagner, P., Kusewitt, D.F., Carver, E.A., Magnino, F., Choi, C., Gridley, T., and Hudson, L.G. (2005). Developmental transcription factor slug is required for effective re-epithelialization by adult keratinocytes. J Cell Physiol *202*, 858-866.
Savagner, P., Yamada, K.M., and Thiery, J.P. (1997). The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. J Cell Biol *137*, 1403-1419.
Schindler, A.M., Amaudruz, M.A., Kocher, O., Riotton, G., and Gabbiani, G. (1982). Desmosomes and gap-junctions in various epidermoid preneoplastic and neoplastic lesions of the cervix uteri. Acta Cytol *26*, 466-470.
Schneider, M.R., Werner, S., Paus, R., and Wolf, E. (2008). Beyond wavy hairs: the epidermal growth factor receptor and its ligands in skin biology and pathology. Am J Pathol *173*, 14-24.
Schrementi, M.E., Ferreira, A.M., Zender, C., and DiPietro, L.A. (2008). Site-specific production of TGF-beta in oral mucosal and cutaneous wounds. Wound Repair Regen *16*, 80-86.
Schultz, G.S., White, M., Mitchell, R., Brown, G., Lynch, J., Twardzik, D.R., and Todaro, G.J. (1987). Epithelial wound healing enhanced by transforming growth factor-alpha and vaccinia growth factor. Science *235*, 350-352.
Sciubba, J.J., Waterhouse, J.P., and Meyer, J. (1978). A fine structural comparison of the healing of incisional wounds of mucosa and skin. J Oral Pathol *7*, 214-227.
Semenova, E., Koegel, H., Hasse, S., Klatte, J.E., Slonimsky, E., Bilbao, D., Paus, R., Werner, S., and Rosenthal, N. (2008). Overexpression of mIGF-1 in keratinocytes improves wound healing and accelerates hair follicle formation and cycling in mice. Am J Pathol *173*, 1295-1310.
Serres, M., Filhol, O., Lickert, H., Grangeasse, C., Chambaz, E.M., Stappert, J., Vincent, C., and Schmitt, D. (2000). The disruption of adherens junctions is associated with a decrease of E-cadherin phosphorylation by protein kinase CK2. Exp Cell Res *257*, 255-264.
Shah, M., Foreman, D.M., and Ferguson, M.W. (1994). Neutralising antibody to TGF-beta 1,2 reduces cutaneous scarring in adult rodents. J Cell Sci *107 (Pt 5)*, 1137-1157.

Shah, M., Foreman, D.M., and Ferguson, M.W. (1995). Neutralisation of TGF-beta 1 and TGF-beta 2 or exogenous addition of TGF-beta 3 to cutaneous rat wounds reduces scarring. J Cell Sci *108 (Pt 3)*, 985-1002.
Shah, M., Revis, D., Herrick, S., Baillie, R., Thorgeirson, S., Ferguson, M., and Roberts, A. (1999). Role of elevated plasma transforming growth factor-beta1 levels in wound healing. Am J Pathol *154*, 1115-1124.
Shen, Y., Hirsch, D.S., Sasiela, C.A., and Wu, W.J. (2008). Cdc42 regulates E-cadherin ubiquitination and degradation through an epidermal growth factor receptor to Src-mediated pathway. J Biol Chem *283*, 5127-5137.
Shirakata, Y., Kimura, R., Nanba, D., Iwamoto, R., Tokumaru, S., Morimoto, C., Yokota, K., Nakamura, M., Sayama, K., Mekada, E., Higashiyama, S., and Hashimoto, K. (2005). Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. J Cell Sci *118*, 2363-2370.
Shook, D., and Keller, R. (2003). Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. Mech Dev *120*, 1351-1383.
Sigismund, S., Argenzio, E., Tosoni, D., Cavallaro, E., Polo, S., and Di Fiore, P.P. (2008). Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. Dev Cell *15*, 209-219.
Singer, A.J., and Clark, R.A. (1999). Cutaneous wound healing. N Engl J Med *341*, 738-746.
Steenfos, H.H., and Jansson, J.O. (1992). Gene expression of insulin-like growth factor-I and IGF-I receptor during wound healing in rats. Eur J Surg *158*, 327-331.
Stelnicki, E.J., Longaker, M.T., Holmes, D., Vanderwall, K., Harrison, M.R., Largman, C., and Hoffman, W.Y. (1998). Bone morphogenetic protein-2 induces scar formation and skin maturation in the second trimester fetus. Plast Reconstr Surg *101*, 12-19.
Stojadinovic, O., Lee, B., Vouthounis, C., Vukelic, S., Pastar, I., Blumenberg, M., Brem, H., and Tomic-Canic, M. (2007). Novel genomic effects of glucocorticoids in epidermal keratinocytes: inhibition of apoptosis, interferon-gamma pathway, and wound healing along with promotion of terminal differentiation. J Biol Chem *282*, 4021-4034.
Stoscheck, C.M., Nanney, L.B., and King, L.E., Jr. (1992). Quantitative determination of EGF-R during epidermal wound healing. J Invest Dermatol *99*, 645-649.
Suzuki, K., Tanaka, T., Enoki, M., and Nishida, T. (2000). Coordinated reassembly of the basement membrane and junctional proteins during corneal epithelial wound healing. Invest Ophthalmol Vis Sci *41*, 2495-2500.
Symowicz, J., Adley, B.P., Gleason, K.J., Johnson, J.J., Ghosh, S., Fishman, D.A., Hudson, L.G., and Stack, M.S. (2007). Engagement of collagen-binding integrins promotes matrix metalloproteinase-9-dependent E-cadherin ectodomain shedding in ovarian carcinoma cells. Cancer Res *67*, 2030-2039.
Taddei, M.L., Chiarugi, P., Cirri, P., Buricchi, F., Fiaschi, T., Giannoni, E., Talini, D., Cozzi, G., Formigli, L., Raugei, G., and Ramponi, G. (2002). Beta-catenin interacts with low-molecular-weight protein tyrosine phosphatase leading to cadherin-mediated cell-cell adhesion increase. Cancer Res *62*, 6489-6499.
Takeda, H., and Tsukita, S. (1995). Effects of tyrosine phosphorylation on tight junctions in temperature-sensitive v-src-transfected MDCK cells. Cell Struct Funct *20*, 387-393.
Tamada, M., Perez, T.D., Nelson, W.J., and Sheetz, M.P. (2007). Two distinct modes of myosin assembly and dynamics during epithelial wound closure. J Cell Biol *176*, 27-33.

Tanaka, A., Nagate, T., and Matsuda, H. (2005). Acceleration of wound healing by gelatin film dressings with epidermal growth factor. J Vet Med Sci *67*, 909-913.
Tanida, S., Joh, T., Itoh, K., Kataoka, H., Sasaki, M., Ohara, H., Nakazawa, T., Nomura, T., Kinugasa, Y., Ohmoto, H., Ishiguro, H., Yoshino, K., Higashiyama, S., and Itoh, M. (2004). The mechanism of cleavage of EGFR ligands induced by inflammatory cytokines in gastric cancer cells. Gastroenterology *127*, 559-569.
Thiery, J.P. (2002). Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer *2*, 442-454.
Thiery, J.P. (2003). Epithelial-mesenchymal transitions in development and pathologies. Curr Opin Cell Biol *15*, 740-746.
Threadgill, D.W., Dlugosz, A.A., Hansen, L.A., Tennenbaum, T., Lichti, U., Yee, D., LaMantia, C., Mourton, T., Herrup, K., Harris, R.C., and et al. (1995). Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype. Science *269*, 230-234.
Todorovic, V., Pesko, P., Micev, M., Bjelovic, M., Budec, M., Micic, M., Brasanac, D., and Ilic-Stojanovic, O. (2008). Insulin-like growth factor-I in wound healing of rat skin. Regul Pept *150*, 7-13.
Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., Yang, L., Higashiyama, S., Yoshimura, A., Sugai, M., and Hashimoto, K. (2005). Induction of keratinocyte migration via transactivation of the epidermal growth factor receptor by the antimicrobial peptide LL-37. J Immunol *175*, 4662-4668.
Tomlinson, J.S., Alpaugh, M.L., and Barsky, S.H. (2001). An intact overexpressed E-cadherin/alpha,beta-catenin axis characterizes the lymphovascular emboli of inflammatory breast carcinoma. Cancer Res *61*, 5231-5241.
Toriseva, M., and Kahari, V.M. (2009). Proteinases in cutaneous wound healing. Cell Mol Life Sci *66*, 203-224.
Tran, K.T., Griffith, L., and Wells, A. (2004). Extracellular matrix signaling through growth factor receptors during wound healing. Wound Repair Regen *12*, 262-268.
Troyanovsky, R.B., Sokolov, E.P., and Troyanovsky, S.M. (2006). Endocytosis of cadherin from intracellular junctions is the driving force for cadherin adhesive dimer disassembly. Mol Biol Cell *17*, 3484-3493.
Tsang, M.W., Wong, W.K., Hung, C.S., Lai, K.M., Tang, W., Cheung, E.Y., Kam, G., Leung, L., Chan, C.W., Chu, C.M., and Lam, E.K. (2003). Human epidermal growth factor enhances healing of diabetic foot ulcers. Diabetes Care *26*, 1856-1861.
Tsukita, S., Yamazaki, Y., Katsuno, T., Tamura, A., and Tsukita, S. (2008). Tight junction-based epithelial microenvironment and cell proliferation. Oncogene *27*, 6930-6938.
Uchikado, Y., Natsugoe, S., Okumura, H., Setoyama, T., Matsumoto, M., Ishigami, S., and Aikou, T. (2005). Slug Expression in the E-cadherin preserved tumors is related to prognosis in patients with esophageal squamous cell carcinoma. Clin Cancer Res *11*, 1174-1180.
Ulubayram, K., Nur Cakar, A., Korkusuz, P., Ertan, C., and Hasirci, N. (2001). EGF containing gelatin-based wound dressings. Biomaterials *22*, 1345-1356.

Vesuna, F., van Diest, P., Chen, J.H., and Raman, V. (2008). Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer. Biochem Biophys Res Commun *367*, 235-241.
von Burstin, J., Eser, S., Paul, M.C., Seidler, B., Brandl, M., Messer, M., von Werder, A., Schmidt, A., Mages, J., Pagel, P., Schnieke, A., Schmid, R.M., Schneider, G., and Saur, D. (2009). E-cadherin Regulates Metastasis of Pancreatic Cancer in Vivo and Is Suppressed by a SNAIL/HDAC1/HDAC2 Repressor Complex. Gastroenterology.
Vranckx, J.J., Hoeller, D., Velander, P.E., Theopold, C.F., Petrie, N., Takedo, A., Eriksson, E., and Yao, F. (2007). Cell suspension cultures of allogenic keratinocytes are efficient carriers for ex vivo gene transfer and accelerate the healing of full-thickness skin wounds by overexpression of human epidermal growth factor. Wound Repair Regen *15*, 657-664.
Wachtel, M., Frei, K., Ehler, E., Fontana, A., Winterhalter, K., and Gloor, S.M. (1999). Occludin proteolysis and increased permeability in endothelial cells through tyrosine phosphatase inhibition. J Cell Sci *112 (Pt 23)*, 4347-4356.
Wang, Q., Chen, X.W., and Margolis, B. (2007). PALS1 regulates E-cadherin trafficking in mammalian epithelial cells. Mol Biol Cell *18*, 874-885.
Wang, X.J., Han, G., Owens, P., Siddiqui, Y., and Li, A.G. (2006). Role of TGF beta-mediated inflammation in cutaneous wound healing. J Investig Dermatol Symp Proc *11*, 112-117.
Wankell, M., Munz, B., Hubner, G., Hans, W., Wolf, E., Goppelt, A., and Werner, S. (2001). Impaired wound healing in transgenic mice overexpressing the activin antagonist follistatin in the epidermis. Embo J *20*, 5361-5372.
Wankell, M., Werner, S., Alzheimer, C., and Werner, S. (2003). The roles of activin in cytoprotection and tissue repair. Ann N Y Acad Sci *995*, 48-58.
Watt, F.M., Lo Celso, C., and Silva-Vargas, V. (2006). Epidermal stem cells: an update. Curr Opin Genet Dev *16*, 518-524.
Wenczak, B., and Nanney, L.B. (1993). Correlation of transforming growth factor-alpha and epidermal growth factor receptor with proliferating cell nuclear antigen in human burn wounds. Wound Repair Regen *1*, 219-230.
Wenczak, B.A., Lynch, J.B., and Nanney, L.B. (1992). Epidermal growth factor receptor distribution in burn wounds. Implications for growth factor-mediated repair. J Clin Invest *90*, 2392-2401.
Werner, S., Beer, H.D., Mauch, C., Luscher, B., and Werner, S. (2001). The Mad1 transcription factor is a novel target of activin and TGF-beta action in keratinocytes: possible role of Mad1 in wound repair and psoriasis. Oncogene *20*, 7494-7504.
Werner, S., and Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. Physiol Rev *83*, 835-870.
Werner, S., Krieg, T., and Smola, H. (2007). Keratinocyte-fibroblast interactions in wound healing. J Invest Dermatol *127*, 998-1008.
Wilgus, T.A., Matthies, A.M., Radek, K.A., Dovi, J.V., Burns, A.L., Shankar, R., and DiPietro, L.A. (2005). Novel function for vascular endothelial growth factor receptor-1 on epidermal keratinocytes. Am J Pathol *167*, 1257-1266.
Wood, L.C., Elias, P.M., Calhoun, C., Tsai, J.C., Grunfeld, C., and Feingold, K.R. (1996). Barrier disruption stimulates interleukin-1 alpha expression and release from a pre-formed pool in murine epidermis. J Invest Dermatol *106*, 397-403.

Xian, W., Rosenberg, M.P., and DiGiovanni, J. (1997). Activation of erbB2 and c-src in phorbol ester-treated mouse epidermis: possible role in mouse skin tumor promotion. Oncogene *14*, 1435-1444.
Xu, K.P., Yin, J., and Yu, F.S. (2006). SRC-family tyrosine kinases in wound- and ligand-induced epidermal growth factor receptor activation in human corneal epithelial cells. Invest Ophthalmol Vis Sci *47*, 2832-2839.
Xu, K.P., and Yu, F.S. (2007). Cross talk between c-Met and epidermal growth factor receptor during retinal pigment epithelial wound healing. Invest Ophthalmol Vis Sci *48*, 2242-2248.
Yahata, Y., Shirakata, Y., Tokumaru, S., Yang, L., Dai, X., Tohyama, M., Tsuda, T., Sayama, K., Iwai, M., Horiuchi, M., and Hashimoto, K. (2006). A novel function of angiotensin II in skin wound healing. Induction of fibroblast and keratinocyte migration by angiotensin II via heparin-binding epidermal growth factor (EGF)-like growth factor-mediated EGF receptor transactivation. J Biol Chem *281*, 13209-13216.
Yan, H.X., Yang, W., Zhang, R., Chen, L., Tang, L., Zhai, B., Liu, S.Q., Cao, H.F., Man, X.B., Wu, H.P., Wu, M.C., and Wang, H.Y. (2006). Protein-tyrosine phosphatase PCP-2 inhibits beta-catenin signaling and increases E-cadherin-dependent cell adhesion. J Biol Chem *281*, 15423-15433.
Yin, T., Getsios, S., Caldelari, R., Godsel, L.M., Kowalczyk, A.P., Muller, E.J., and Green, K.J. (2005). Mechanisms of plakoglobin-dependent adhesion: desmosome-specific functions in assembly and regulation by epidermal growth factor receptor. J Biol Chem *280*, 40355-40363.
Yonei, N., Kanazawa, N., Ohtani, T., Furukawa, F., and Yamamoto, Y. (2007). Induction of PDGF-B in TCA-treated epidermal keratinocytes. Arch Dermatol Res *299*, 433-440.
Zajchowski, D.A., Bartholdi, M.F., Gong, Y., Webster, L., Liu, H.L., Munishkin, A., Beauheim, C., Harvey, S., Ethier, S.P., and Johnson, P.H. (2001). Identification of gene expression profiles that predict the aggressive behavior of breast cancer cells. Cancer Res *61*, 5168-5178.
Zambruno, G., Marchisio, P.C., Marconi, A., Vaschieri, C., Melchiori, A., Giannetti, A., and De Luca, M. (1995). Transforming growth factor-beta 1 modulates beta 1 and beta 5 integrin receptors and induces the de novo expression of the alpha v beta 6 heterodimer in normal human keratinocytes: implications for wound healing. J Cell Biol *129*, 853-865.
Zheng, Z., Pan, J., Chu, B., Wong, Y.C., Cheung, A.L., and Tsao, S.W. (1999). Downregulation and abnormal expression of E-cadherin and beta-catenin in nasopharyngeal carcinoma: close association with advanced disease stage and lymph node metastasis. Hum Pathol *30*, 458-466.