The Epidermal Growth Factor Receptor Regulates Cell Autonomous Pro-inflammatory Signalling in Keratinocytes

Hayday, Tom

Awarding institution:
King's College London

The Epidermal Growth Factor Receptor Regulates Cell Autonomous Pro-inflammatory Signalling in Keratinocytes

A thesis submitted to King's College London for the Degree of Doctor of Philosophy, 2019

By

Thomas Hayday

Acknowledgments

Without a doubt the first person I would like to thank is my superb supervisor, Professor Maddy Parsons. Without her constant support and expertise this would not have been possible. It has been a joy to work with Maddy from day 1. I would also like to thank my second supervisor, Dr. John McGrath, for his breadth of knowledge on all things skin and of course, all of the jokes. I am also indebted to all of the other much more capable and experienced scientists that helped me along this journey from the Randall, the wider KCL and elsewhere. I would also like to thank anyone who has encouraged me on this journey either in the lab or in the pub!

I would also like to specifically thank Grace, Willow and Yonis. We all began this journey together and I truly couldn't of done it without you guys. I would also like to thank all the members of the Parsons lab past and present, Claudia, Sofia, Tom P, Salwa, Campbell, Elena, Jake, Brooke, Rosie, Sweta, Penny, Mag, and Karin. It's been a pleasure to work with all of you. I guess I am also required to thank Fuad, the honorary Parsonite. I would also like to thank all the members of the Randall as a whole, as the comradery and collaborative spirit has been truly special.

Finally, I would like to thank my family for supporting me in every way through every step of this journey. My Mum has given me so much support and encouragement throughout despite my testing her to the limit at times! My Dad has been a great source of inspiration and expertise while not putting pressure on me to be anything but myself. I'd also like to thank Greg, Lara and Anoushka for their emotional support, as well as the odd south of France escape!

Lastly, I have to thank my partner Carly for pretty much everything. Thank you for putting up with me during this time and always being there during the best and the worst times. I couldn't have gotten through it without you.

Abstract

The Epidermal Growth Factor Receptor (EGFR) is a fundamental regulator of epidermal integrity. We recently reported the first loss of function mutation in EGFR in a patient with skin fragility and inflammation. Similar inflammatory phenotypes have been reported in cancer patients following treatment with EGFR inhibitors, sometimes with severe consequences. This inflammation has been suggested to be due to defects in barrier integrity induced by EGFR deficiency, facilitating microbial challenge that promotes inflammatory responses. Here we provide evidence that loss of EGFR function in basal keratinocytes in culture is sufficient to induce a profound pro-inflammatory phenotype characterised by increased bioactive chemokine release that promotes leukocyte migration. We further show this depends on EGFR-dependent control of a balance of active STAT3 and SHP2 that control pro-inflammatory cytokine production at the transcriptional level. Moreover, our data demonstrates that that these changes are not attributable to loss of cell-cell interactions within keratinocyte monolayers. We therefore conclude that EGFR functions under homeostatic conditions to maintain a gene expression programme that limits the expression of pro-inflammatory genes and their products. These roles of EGFR are in addition to its regulation of epithelial cell proliferation and differentiation, and may be important for maintaining the a normal, proliferative epidermis. This has implications for the clinical use of EGFR inhibitors, and suggests with the combined use of anti-inflammatory, e.g. STAT3 inhibitors, but not necessarily anti-microbial inhibitors, may benefit patients exhibiting pro-inflammatory responses. Our data also provides evidence that primary epithelial defects may be the causal drivers of inflammatory skin disease.

Table of Contents

List of Figures

Abbreviations

AJ	Adherens Junction
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CCL	C-C motif chemokine
CHO	Chinese Hamster Ovary
CXCL	C-X-C motif chemokine
DC	Dendritic Cell
DETC	Dendritic Epidermal T Cell
DMSO	Dimethyl Sulfoxide
EB	Epidermolysis Bullosa
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ERK	Extracellular signal-Regulated Kinase
FA	Focal Adhesion
FACS	Fluorescence Activated Cell Sorting
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
FN	Fibronectin
GFP	Green Fluorescent
GMCSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanine Triphosphate
IFN	Interferon
IL	Interleukin
ILC	Innate-like Cell
KGF	Keratinocyte Growth Factor
KO	Knockout
LB	Lysogeny broth
mAb	Mono-clonal Antibody
MACS	Magetic Activated Cell Sorting
MMP	Matrix Metalloproteases
MT	Microtubule
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
PTB	Phosphotyrosine-binding domain
SH2	Src Homology 2
SkInFs	Skin Inflammatory Factors
TGF	Transforming Growth Factor
Th1	T-helper 1

Th2	T-helper 2
TJ	Tight Junction
TNF	Tumor Necrosis Factor
TRITC	Tetramethylrhodamine
UVA/B	Ultraviolet A/B
WT	Wild type

1. Introduction

1.1 Skin

1.1.1 Structure of the skin

The skin is the largest organ in the body and can be separated into three distinct sections. The outmost layer, or epidermis, is a relatively thin layer that is mostly waterproof and its main function is as the body's barrier to the outside world. Under the epidermis lies the dermis, which predominantly comprises collagen, elastin and fibrillin, and gives our skin its elasticity and strength. The dermis also contains nerve endings, sebaceous glands, blood vessels and hair follicles. The innermost layer of the skin is a fatty layer of subcutaneous tissue that is essential for body insulation, padding and as an energy store. Figure 1.1 shows a schematic of the structure of the epidermis.

The main cell type of the epidermis are the keratinocytes. These epithelial cells originate in the basal layer, 'Stratum basal', and differentiate upwards through the epidermis forming its further layers. The basal layer is the only site at which keratinocytes proliferate and thus is responsible for the replenishment of the skin. From here, older keratinocytes are pushed up into the 'stratum spinosum', differentiating becoming spinier in shape, changing nuclear and chemical composition, and assembling into a more rigid arrangement. Above this in the 'stratum granulosum', the keratinocytes are more granular, owing to the production of large quantities of keratin. From here, the keratinocytes further differentiate into the 'stratum lucidum' where they begin to die off, finally forming the protective 'stratum corneum' of dry, de-nucleated, keratin-filled, lipid-rich corneocytes. Basal keratinocytes can be identified by expression of both keratin 5 and 14. The epidermis also contains other cell types such as melanocytes, Merkel cells, Langerhans cells amongst a plethora of other skin-resident immune cells.

Basal keratinocytes anchor the epidermis to the basement membrane via adhesions to extra cellular matrix (ECM) proteins, such as collagen and laminin, which are produced by both keratinocytes as well as fibroblasts in the dermis. The two most common of these adhesions are focal adhesions and hemidesmosomes. Basal

keratinocytes also form strong cell-cell adhesions, such as adeherens junctions which are responsible for the structure and integrity of the epidermis and many cell-cell junctional proteins regulate cellular polarity and enable the correct differentiation of keratinocytes into the complete epidermal architecture.

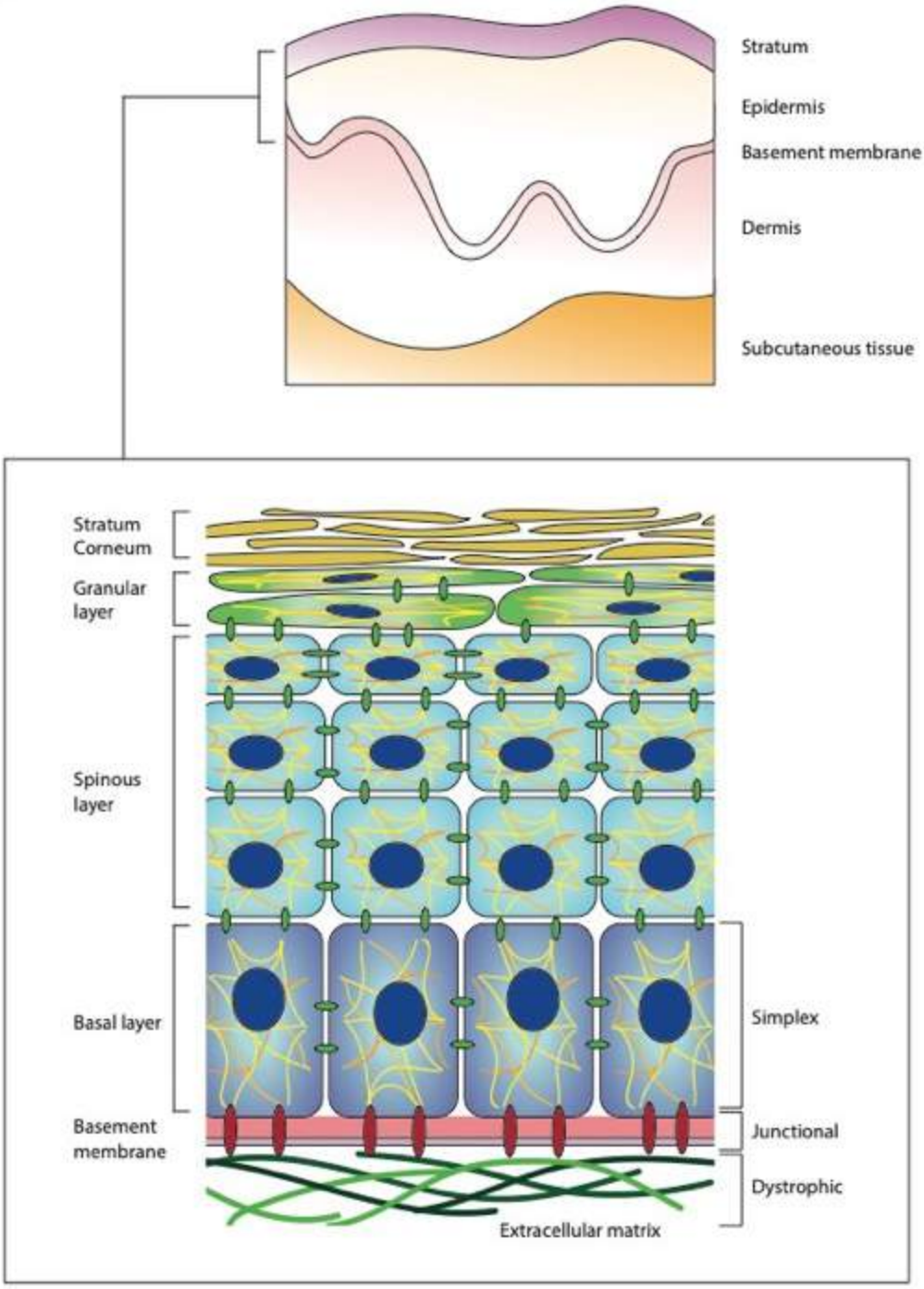


Figure 1.1 Schematic representation of the epidermis: Top: The basic structure of the skin. Bottom: The organisation of the epidermis .

1.1.2 Basement membrane and extra cellular matrix remodeling

The basement membrane (BM) is a very thin ECM structure that mechanically and functionally separates the epidermis from the dermis (Figure 1.1) (Van Agtmael and Bruckner-Tuderman 2010) predominantly providing structure to the epidermis. The two major components of the BM structure are type IV collagen isoforms and laminins (LeBleu, MacDonald, and Kalluri 2007). Type IV collagen and laminin self-assemble into suprastructures bridged by both nidogen and perlecan, conferring stability to the BM (Pöschl et al. 2004). These BM proteins express many biding sites for cell adhesion molecules required for the proper anchoring of basal keratinocytes.

Matrix-metallopeptidases or MMPs play important roles in ECM/BM restructuring, and composition by degrading gelatin, collagen, fibronectin, elastin, laminin and several other protein substrates (Ram, Sherer, and Shoenfeld 2006). MMPs are mostly produced by basal keratinocytes and dermal fibroblasts and their expression is regulated by a number of secreted factors including TGF-β, TNF-α, and histamine produced by mast cells in the upper dermis (Ichiyama et al. 2006). By restructuring the extracellular matrix, MMPs can drive immune cell infiltration, and the release of non-covalently bonded cytokines and growth factors (Bergers et al. 2000; Page-McCaw 2008).

1.1.3 Barrier function of the skin

The primary function of the skin is to provide a barrier to the outside world. The most critical aspects of the barrier are protection against UV radiation, antioxidants, antimicrobial function, sensory barrier, and the permeability barrier. Different cell compartments are critical for the formation and regulation of each of these barrier functions.

Micro-organisms in the skin represent one of the largest microbe population in humans (Gallo 2017). In order to control the growth and limit the invasiveness of the

microbe colony, cells within the skin including keratinocytes are able to produce a number of anti-microbial peptides (AMPs) such as β-defensins (BD) (Harder et al. 1997; L. Liu et al. 1998), cathelicidins (human hCAP18/LL37, or its mouse equivalent CRAMP) (Frohm et al. 1997), lactoferrin (Cumberbatch et al. 2000), lysozyme (Marchini et al. 2002), dermcidin (Schittek et al. 2001), α-Defensins (Harwig, Ganz, and Lehrer 1994), and perforin (Stenger et al. 1998).

Defence against UV radiation is another critical aspect of the skin's barrier function as UVA and UVB radiation can lead to DNA photodamage having cytotoxic and mutagenic effects (De Gruijl 2000). Protection against UV radiation is the primary function of melanocytes in the epidermis. These cells produce melanin in response to UV radiation in ovoid organelles known as melanosomes and then accumulates keratinocytes and melanocytes via transport along melanocyte dendrites in the epidermis (FITZPATRICK and BREATHNACH 1963). Melanin is capable of dissipating up to 99% of UV radiation dependent of melanin concentration in the skin (Meredith and Riesz 2004).

The physical barrier function of the skin is responsible for skin structure and permeability and is primarily controlled by the adhesions that are formed between cells of the epidermis, adherens junctions and desmosomes, and the adhesions formed between basal layer keratinocytes and the basement membrane, focal adhesions and hemidesmosomes.

1.2 Keratinocyte Adhesions

1.2.1 Cell-cell adhesions

An important property of keratinocytes is to form cell-cell adhesions with adjacent keratinocytes and this plays a critical role in the formation and homeostasis of the epidermal barrier. Primarily cell-cell adhesions are crucial for the maintaining of epidermal architecture and integrity as well as being involved in the barrier function preventing water loss from the body (Sumigray and Lechler 2015). This is achieved by helping to organise the cytoskeleton across adjacent cells and conferring cross-talk via intracellular signaling pathways. Cell-cell junctions come in three major types in keratinocytes; adherens junctions, desmosomes and tight junctions. The primary cell-cell adhesion in basal keratinocytes are adherens junctions with desmosomes and tight junctions being found further up the epidermis in the granular layer (Morita et al. 1998; Furuse et al. 2002; Schlüter et al. 2004).

1.2.2 Adherens junctions

As previously mentioned, adherens junctions are the primary cell-cell adhesion in the basal layer of the epidermis. They are primarily made of cadherins and catenins which form structures that connect the cell-cell junctions to the intracellular actin cytoskeleton (Niessen and Gottardi 2008; Čabrijan and Lipozenčić 2011). The formation of adherens junctions in the epidermis is primarily driven by the calcium dependent function of epithelial cadherin, E-cadherin (Kim et al. 2011). Calcium binding to E-cadherin induces a conformational change allowing for the interaction of the large extra cellular domain with the extra-cellular domain of other cadherins on adjacent cells (Figure 1.2, Top). (Tomschy et al. 1996; Ozawa 2002). The importance of E-cadherin and adherens junctions in the skin is demonstrated by the loss of E-cadherin and adherens junctions leading to malfunction in differentiation of keratinocyte and loss of hair follicles (Tinkle et al. 2004).

The intracellular domain of E-cadherin is where cadherin interacts with catenins. Stability of E-cadherin at cell-cell junctions is regulated and maintained by β-catenin by preventing degradation (Huber et al. 2001). β-catenin allows the interaction of the cell-cell adherens junction with the actin cytoskeleton via α-catenin as in Figure 1.2 (Aberle et al. 1994). α-catenin can drive F-actin cytoskeleton assembly with the assistance of a number of actin binding proteins including vinculin (Izard et al. 2004; Sumigray and Lechler 2015).

Adherens junctions are not static structures and have been shown to undergo dynamic changes during cell migration and play an active role in force and tension sensing (Yonemura et al. 2010; Gumbiner 2005).

1.2.3 Desmosomes

Unlike adherens junctions, desmosomes are predominantly associated with suprabasal keratinocytes. Desmosomes are made up of 3 main proteins; desmosomal cadherins (desmocollin and desmogleins) which form the junctional contact between adjacent cells (Figure 1.2, Bottom); armadillo proteins (plakoglobin and plakophilins) which allow for intracellular signal transduction from the junction recruits plakin family members, such as desmoplakin, into desmosomal plaques; and desmoplakins form the link between desmosomes and the IF networks (Stappenbeck and Green 1992; Stappenbeck et al. 1993) Depletion of desmoplakin disrupts the organisation of the IF network (Gallicano et al. 1998; Vasioukhin et al. 2001). Desmasomes play an important role in skin barrier homeostasis as demonstrated by epidermal-specific knockout of desmoplakin causing skin blistering in mice (Vasioukhin et al. 2001), which is also seen after human desmoplakin mutations (Cheong, Wessagowit, and McGrath 2005).

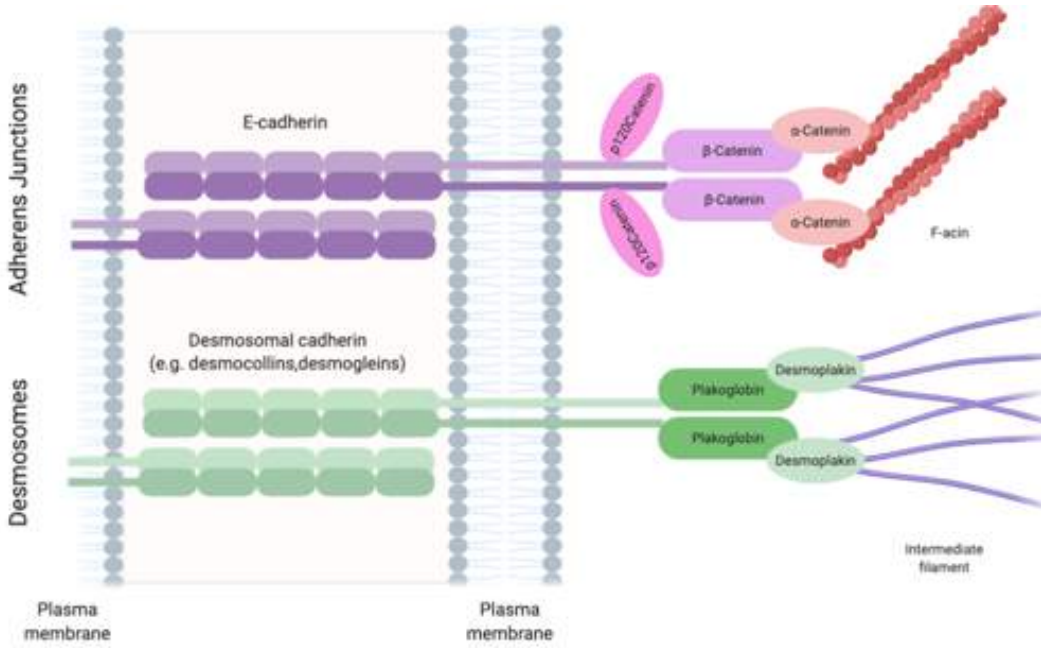


Figure 1.2: Structural organisation of adherens junctions and desmosomes. Adherens junctions connects between adjacent keratinocytes through E-cadherin. It can recruits β-catenin and p120-catenin, which can then recruit α-catenin to bind the actin cytoskeleton. Desmosomal junctions connects through desmocollins and desmogleins. Desmosomes recruit plakoglobin and desmoplakins to bind intermediate filaments.(Ohashi, Fujiwara, and Mizuno 2017).

1.2.4 Role of cell-matrix adhesions in keratinocyte function

Cell-matrix adhesions are essential for many cellular functions, including migration and proliferation. They are particularly important after skin injuries, such as mechanical trauma and burns, to promote re-epithelisation of the wound (Hopkinson et al. 2014). The two main types of cell-matrix adhesions are focal adhesions and hemidesmosomes.

1.2.5 Focal adhesions

Focal adhesions are the primary cell-matrix adhesion in the basal layer of the epidermis and crucial for the regulating of epithelial homeostasis, basal keratinocyte proliferation, and re-epithelialisation after wounding (Duperret and Ridky 2013). The structure of a focal adhesion can be seen in Figure 1.3. Focal adhesions are formed via the clustering of integrins followed by the recruitment of talin which binds to the actin cytoskeleton (Nagano et al. 2012), which is then followed by actin-binding proteins such as vinculin and actinin to further link the extra-cellular matrix to the cytoskeleton (Parsons, Horwitz, and Schwartz 2010).

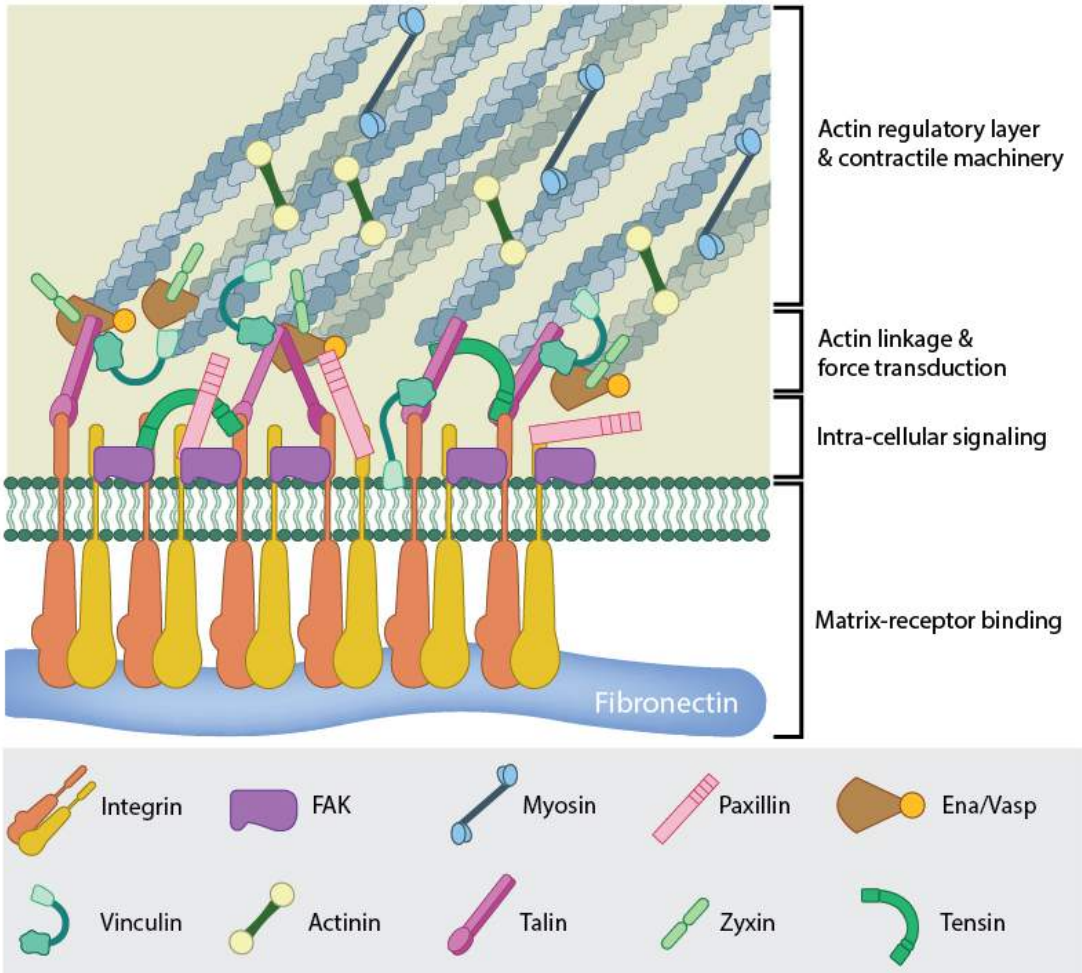


Figure 1.3: Structure of mature focal adhesions: Schematic shows integrin clusters binding to the extra cellular matrix. Inside the cell, talin is bound to the cytoplasmic tails of integrin proteins and bridges them to actin while also recruiting a number of other proteins such as paxillin actinin and tensin. (https://www.mechanobio.info/what-is-mechanosignaling/what-is-the-extracellular-matrix-and-the-basal-lamina/what-are-focal-adhesions/what-are-mature-focal-adhesions-composed-of/#what-are-mature-focal-adhesions-composed-of)

1.2.6 Vinculin

One of the most important components of focal adhesions is the actin binding protein Vinculin. Vinculin is made up of a head and tail domain joined via a linker region as seen in Figure 3. (Bays and DeMali 2017; Ziegler, Liddington, and Critchley 2006). The domains of vinculin have specific functions with the head domain interacting with talin and the tail domain interacting primarily with actin, playing an important role in linking the ECM with the cytoskeleton (Carisey and Ballestrem 2011; Bays and DeMali 2017).

The importance of vinculin in cell adhesion formation has been previously shown where depletion of vinculin leads to less stable focal adhesions and increased focal adhesion turnover (Ziegler, Liddington, and Critchley 2006). One of the most important roles of vinculin in the focal adhesion is its role in mechanosensing. Vinculin has been shown to be recruited to focal adhesions in response to mechanical stress which is potentially mediated via talin (Gingras et al. 2005). Vinculin cleavage is also critical to the correct disassembly of focal adhesions after inactivation by PIP_2 (Franco et al. 2004; Saunders et al. 2006).

1.2.7 Hemidesmosomes

As well as focal adhesions, hemidesmosomes are also structures that link basal layer keratinocytes to the basement membrane. The epidermis expresses Type I hemidesmosomes which consist of α6β4 integrins, BPAG2 and tetraspanin protein CD151 (Tsuruta et al. 2011; Walko, Castañón, and Wiche 2014). BPAG2 and α6β4 integrins interact with basement membrane protein laminin-332 initiating hemidesmosome formation (Tsuruta et al. 2011). The cytoplasmic tail of integrins interacts with keratin 5 and 14 forming a link to intermediate filaments (Walko, Castañón, and Wiche 2014). Hemidesmosomes are critical in the maintenance of epidermal integrity and homeostasis, as mutations of hemidesmosomal proteins have been shown to lead to blistering disease. (Tsuruta et al. 2011).

1.2.8 Collective cell migration

Collective cell migration is an important collective cellular behavior that occurs in keratinocytes in a plethora of processes, including wound healing re-epithelisation , cancer, and morphogenesis (De Pascalis and Etienne-Manneville 2017; Friedl and Gilmour 2009).

This process is defined as a group of cells attached by stable cell-cell adhesions, migrating as a single 'supra-cellular unit'. Dysregulation of collective cell migration has many negative impacts such as driving cancer as well as chronic wound healing conditions. During epidermal wound healing, ultra-proliferative keratinocytes at the wound edge form lead cells that extend protrusions in the direction of migration to close the wound. These protrusions, lamellipodia or filopodia, promote the assembly of cell-matrix adhesions such as focal adhesions, which are crucial for generating cellular traction allowing the 'supra-cellular' unit to migrate in a coordinated fashion (Zaidel-Bar et al. 2007; Haeger et al. 2015).

1.3 Inflammation in the skin

1.3.1 Epidermal Inflammation

The skin provides the first immunological defense against infection. The crosstalk between epidermal cells and the cells of the immune system is crucial for the regulation of tissue homeostasis and tissue repair. Inflammation in the skin is regulated by the tissue resident immune cells, infiltrating immune cells, and the inflammatory properties of other skin epithelial cells as seen in Figure 1.4.

The skin resident immune cells include skin mast cells which play a role in acute bacterial infection and wound healing (Ng 2010); Langerhans cells, which are skin-resident dendritic cells (DCs) responsible for antigen presentation from the skin to the

adaptive immune system (Ginhoux et al. 2006); dendritic epidermal T-cells (DETCs) , comprising tissue-resident γδ cells that appear soon after birth (Vantourout et al. 2014), and αβ cells that enter the skin after priming against skin infections in the local lymph nodes including resident CD8+ T cells involved in the cytotoxic killing of antigen presenting epithelial cells (Black et al. 2007). Innate lymphoid cells (ILCs) represent a heterogeneous population of immune cells responsible for a plethora of tissue inflammatory functions, and have been demonstrated to be activated by keratinocytes during inflammatory skin conditions such as psoriasis (Mjösberg and Eidsmo 2014). Additionally, keratinocytes actively participate in skin immunity (Streilein 1983), an example of epithelial driven inflammation. Skin associated immune cells are summarised in Figure 4.

Under normal conditions, keratinocytes produce interleukin (IL)-1, IL-7, and transforming growth factor (TGF)-β (Graham et al. 2004). After keratinocyte stimulation via microbes such as *P. acnes* or after other trauma such as physical wounding, there is an increase in production of cytokines IL-8, IL-1α, tumor necrosis factor (TNF)-α, IL-6, IL-15, IL-18, IL-36 and granulocyte macrophage colony-stimulating factor (GM-CSF)(Foster et al. 2014; Mizutani, Black, and Kupper 1991). Some of these cytokines are known to be pro-inflammatory, whilst others are promoters of T-cell activation and some are modulators of Langerhans cell phenotype. Keratinocytes are also actively involved in leukocyte trafficking having been shown to produce CCL17, CCL27, CXCL12, CCL5, CCL20 and CCL2 (Galkowska, Wojewodzka, and Olszewski 2006), blockade of which drastically inhibits leukocyte migration into the skin in mouse models (Reiss et al. 2001). Keratinocytes also produce IL-33 which has been shown to be increased in many allergic dermatoses such as rosacea (Suhng et al. 2018). A summary of a number of important keratinocyte derived cytokines and their proposed functions is listed in Table 1.

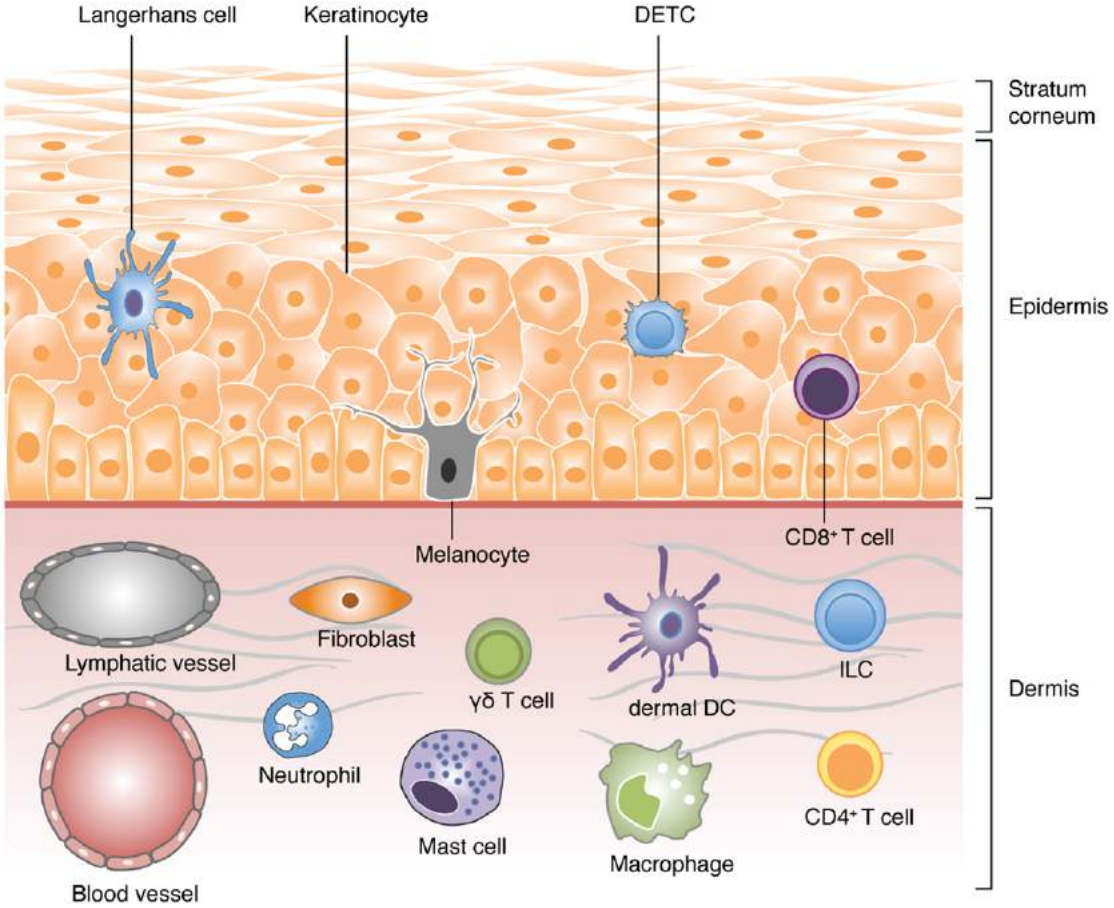


Figure 1.4: A schematic view of the different cell types populating the skin.
Schematic shows the localisation of major epidermal resident immune cell types such as Langerhans cells, DETCs and CD8 T cells within the epidermis. Other immune cells can be seen residing in the dermis either as resident cells such as dermal dendritic cells (DC) and innate like cells (ILC), or as recruited, circulating CD4 T cells or neutrophils. Other cells like macrophages require recruitment into the epidermis via chemotaxis. (Chong, Evrard, and Ng 2013)

Table 1: Keratinocyte derived cytokines and their roles.

CYTOKINE	ROLE	REF
IL-1	Plays a role in wound healing and leukocyte recruitment	(Sauder 1990; Kupper et al. 1987)
IL-7	Growth factor for DETCs: Increases keratinocyte migration	(Heufler et al. 1993; Takashima et al. 1995)
IL-6	Increase keratinocyte proliferation and plays a role in wound healing and hyperplasia	(Grossman et al. 1989; Sugawara et al. 2001)
IL-10	Expressed after UV radiation and plays an immunomodulatory role. Predominantly expressed in mouse keratinocytes.	(Nishigori et al. 1996)
IL-15	A Growth factor for DETCS and plays role in migration of inflammatory cells through the dermis.	(Döbbeling et al. 1998; Han et al. 1999)
IL-18	Augments Th1 responses by enhancing IL-12 induced IFN-γ production and plays a role in early wound healing.	(Kämpfer et al. 2000)
IL-33	Induces other cytokine production by keratinocytes and Mast cells	(Balato et al. 2012)
IL-36	Overexpression leads to hyperplasia, also plays a role in wound healing in other epithelium	(Hashiguchi et al. 2018)
GM-CSF	Accelerates wound healing and stimulates keratinocyte proliferation	(Mann et al. 2001)
TNF α	Plays a role in early response to UVB radiation and plays a role in wound healing	(Köck et al. 1990; Bashir, Sharma, and Werth 2009)
CCL2	Plays a role in leukocyte recruitment	(Purwar et al. 2006)
CCL5	Increases immune infiltrate into the epidermis and plays a role in the migration of immature Langerhans cells from the dermis to the epidermis	(Jie Li et al. 1996; Ouwehand et al. 2012)
CCL17	Chemoattractant for Th2 cells and T cells expressing the cutaneous homing receptor	(Gilet et al. 2009)
CCL20	Plays a role in the maintenance and recruitment of Th17 cells in the skin as well as recruitment of CCR6+ dendritic cells	(Harper et al. 2009; Le Borgne et al. 2006)
CCL27	Accelerates wound healing and recruits T cells	(V. Huang et al. 2008)

1.3.2 Cross-talk between keratinocytes and immune cells during wound healing

Epidermal wound healing is a dynamic process that involves the complex interaction of many tissue components, resident immune cells, infiltrating immune cells and soluble mediators, with the goal of returning to homeostasis and epidermal integrity (Singer and Clark 1999).

Tissue injury by wounding leads to the onset of an acute inflammatory response. The traditional thinking has been that this inflammatory response and cells of the immune system are the primary sources of growth factor and cytokines that are required for the repair of damaged tissue (Simpson and Ross 1972). However as mentioned previously, it is clear that other skin cells also play a predominant role in this process. Keratinocytes play a key role during wound healing in the release of cytokines that recruit immune cells such as macrophages to the epidermis (Mann et al. 2001)

Macrophage infiltration into the skin after wounding is regulated by growth factors, proinflammatory cytokines, and chemokines such as CCL5 derived by keratinocytes (Luisa A. DiPietro et al. 1998)(Badiu, Vasile, and Teren 2011). Macrophages at the wound site are important for the resolution of wound healing by promoting generation of growth factors that play a role in driving keratinocyte proliferation and induce production of ECM proteins in keratinocytes and fibroblasts (L. A. DiPietro and Polverini 1993). As mice models depleted for macrophages demonstrate delayed wound healing (Leibovich and Ross 1975). This demonstrates the importance of keratinocytes role in macrophage recruitment for wound healing to be successful.

During the final stages of wound healing, the inflammatory response is primarily orchestrated by normal epidermal resident immune cells and T-cells, which remain at the wound site after the closure of the wound, during tissue remodelling phase (Fishel et al. 1987). These T cells confer their wound healing function via cell-cell interactions with keratinocytes and fibroblasts. This crosstalk is partially achieved via CD40 expressed on both cell types. Importantly, ligation of CD40 expressed on keratinocytes leads to inhibited proliferation and induces keratinocyte differentiation, which is

required for the generation of healthy skin (Péguet-Navarro et al. 1997). As chemokines produced by keratinocytes are crucial to maintaining T cell populations in healing skin, this further demonstrates the importance of keratinocytes in the inflammatory wound healing process.

Skin resident γδ T cells or γδDETCs, play an important role in epidermal inflammation during wound healing and are in constant contact with keratinocytes. γδDETCs are a key source of growth factors that play a role in the regulation of keratinocyte proliferation and differentiation (Jameson et al. 2002; Sharp et al. 2005). γδDETCs also play an important role in immune surveillance. It has been demonstrated that γδDETCs expressing NKG2D can sense keratinocyte stress by recognition of upregulated stress proteins such as Rae1, MICA and ULBP2 on keratinocytes after UVB radiation or cellular damage (Vantourout et al. 2014). However, keratinocytes themselves play an important role in maintenance of γδDETCs by their expression of SKINT1, which regulates the homing and maintenance of skin resident γδDETCs in the epidermis (Barbee et al. 2011).

Successful tissue repair after wounding is crucial to survival and it is clear that crosstalk between not only immune cells but the inflammatory potential of keratinocytes is required for wound healing to resolve.

1.4 Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor (EGFR) was the first receptor tyrosine kinase to be discovered in 1978 (Carpenter, King, and Cohen 1978). It belongs to the ErbB family of receptor tyrosine kinase, with other members being ErbB2/HER2, ErbB3/HER3 and ErbB4/HER4. Unlike EGFR, HER2 does not contain a ligand-binding domain, whereas HER3 does not have a kinase domain.

1.4.1 EGFR structure

EGFR contains an extracellular receptor domain, a transmembrane domain and a cytosolic tyrosine kinase domain. There are four subdomains (I-IV) within the extracellular domains, where domain I and III are members of the leucine-rich repeat family and domain II and IV being homologous to cysteine-rich domains (Ullrich et al. 1984; Ferguson 2008). Three sites within domain I and III have been characterized to be involved in ligand binding, whereas a loop within domain II (dimerisation arm) mediates EGFR dimerisation (Ogiso et al. 2002). The structure of EGFR is shown in Figure 1.5.

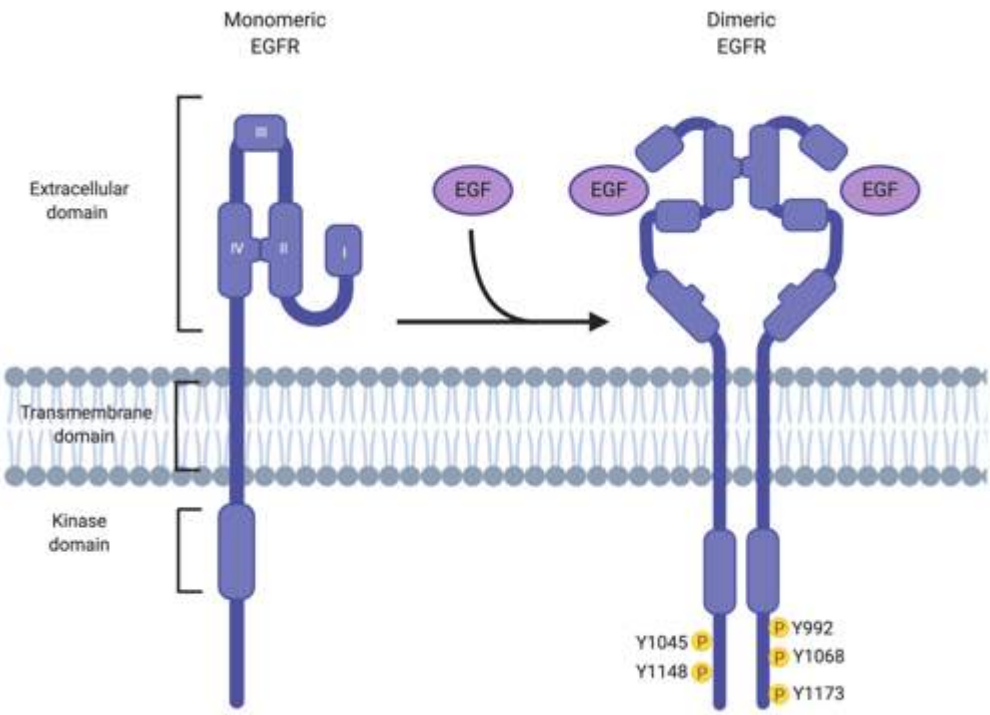


Figure 1.5: Structure and activation of EGFR. EGFR contains an extracellular domain with four subdomain (I-IV), a transmembrane domain and a intracellular kinase domain. EGFR exists in a closed conformation, where ligand binding induces conformational change of EGFR, exposing the dimerisation arm in subdomain II for dimerisaton. This is followed by trans-autophosphorylation of the cytoplasmic tails to activate downstream signaling pathways. (Seshacharyulu et al. 2012)

1.4.2 EGFR Signaling

EGFR is a very important mediator in a plethora of functions such as cell differentiation, proliferation, survival and migration. To date, many activating ligands for EGFR have been reported including epidermal growth factor (EGF), transforming growth factor alpha (TGFα), amphiregulin, betacellulin, eigen, epiregulin, heparin-binding EGF and neuregulin 2β (Henriksen et al. 2013). These EGFR ligands are usually expressed as integral-membrane proteins that can be cleaved via metalloproteinases, releasing soluble ligands (Mill et al. 2009).

The EGFR signaling mechanism stimulated by ligand binding has been studied extensively (Figure 1.6). Dimerization allows the regulatory domain to stabilize the tyrosine kinase domain in the active conformation. Importantly, different ligands cause the phosphorylation of distinct sets of EGFR tyrosine residues but the specific mechanisms are still unclear. Approximately 10 EGFR tyrosine residues are phosphorylated following receptor dimerization (Schulze, Deng, and Mann 2005). These residues act as binding sites for a series of cytosolic proteins containing Src homology 2 (SH_2) domains or phospho-tyrosine binding (PTB) motifs. Ligand binding to EGFR results in the activation of number of signaling pathways, including Ras, MAPK/ERK, Src, JAK/STAT, PLCγ, PKC, and PI3-kinase (Figure 1.6) (M. A. Lemmon, Schlessinger, and Ferguson 2014). Thus, phosphorylation of the receptor via the tyrosine kinase domain leads to the recruitment of downstream effectors effecting proliferation, survival and differentiation (Normanno et al. 2006). These downstream effects can be ligand specific (Wilson et al. 2009). Receptor activation eventually leads to internalization, which is followed by either degradation or recycling to the cell surface (L. Yue et al. 2006). EGFR internalization is thought to be predominantly clathrin-mediated (Ebner and Derynck 1991). However, more recent studies have shown that in the absence of clathrin, EGFR stimulation with specific ligands can induce clathrin-independent internalization (Hinrichsen et al. 2003). Furthermore, it was recently shown that ligand concentration can affect internalization pathways, for example, stimulation with high concentrations of EGF can result in internalization *via* micropinocytosis (Sigismund et al. 2005). The downstream molecules that negatively

modulate the receptor are also stimulated by EGFR ligands. For example, phosphorylation of EGFR Tyr974 triggers EGFR endocytosis and that of EGFR Tyr1045 triggers Cbl-dependent EGFR ubiquitination and proteasomal degradation (Wilson et al. 2009). Therefore, EGFR ligands do not only positively regulate the EGFR, but are crucial for negative feedback and receptor regulation.

The EGFR also hetero-dimerises with other ErbB family members , and this results in the modulation of EGFR signaling. In fact, ErbB2/EGFR heterodimers can form through a ligand-independent mechanism, resulting in ligand-independent EGFR signaling while increasing affinity for available EGF (Mark A. Lemmon 2009). The hetero-dimerization of ErbB2 with EGFR also alters EGFR endocytosis and intracellular trafficking (Hendriks, Wiley, and Lauffenburger 2003). Another recent study demonstrated that EGFR hetero-dimerization with a variant of ErbB4 can protect the receptor from ligand induced proteasomal degradation (Kiuchi et al. 2014).

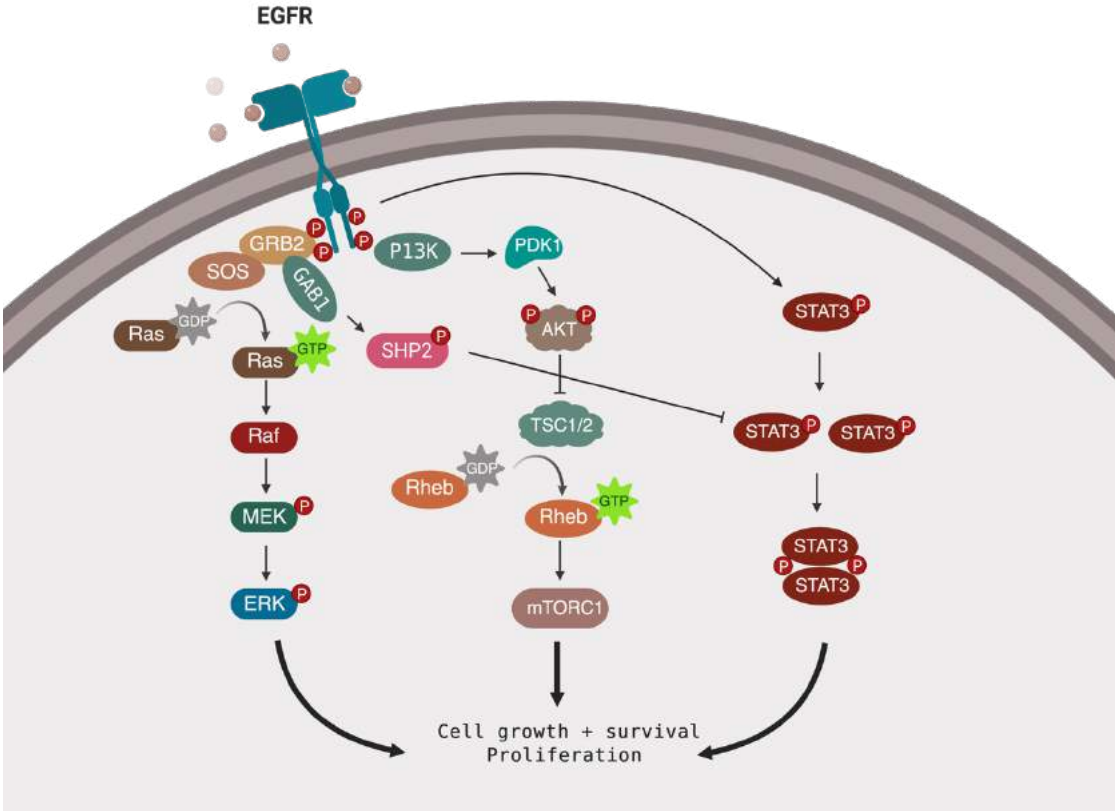


Figure 1.6: EGFR phosphorylation and signalling: Phosphorylation of tyrosine residues on EGFR provide binding sites for SH2- or PTB-containing proteins which leads to the recruitment and activation of various downstream signalling pathways (e.g. Erk/MAPk, PI3K/Akt, STAT3) to regulate cellular processes, such as proliferation and and survival.

1.4.3 EGFR and integrin cross-talk

Previous studies have suggested that the cross-talk between integrin and EGFR signaling is an important pathway in the regulation of cellular processes, such as proliferation, migration and adhesion. For cross-talk to occur local association of EGFR and integrins is essential. For example, EGFR interacts with β1 integrins during early cell-matrix adhesion formation, as well as at cell-cell adhesions (Figure 1.7)(Moro *et al.*, 1998; Wang *et al.*, 1998). β3 integrin and EGFR association has been detected after adhesion to fibronectin (Laura Moro et al. 2002; Cabodi et al. 2004).

Cell-matrix adhesions are required to drive EGFR phosphorylation that is in turn required for anchorage-dependent proliferation and cell survival fibroblasts, endothelial cells, intestinal epithelial cells and smooth muscle cells (Cybulsky, McTavish, and Cyr 1994; L Moro et al. 1998)(Kuwada and Li 2000; Jones, Crack, and Rabinovitch 1997).

EGFR and integrin cross-talk have also been demonstrated to regulate cell-cell adhesions. E-cadherin recruits EGFR and integrin in response to an increased in intercellular tension. This leads to the recruitment of vinculin into cadherin complexes at cell-cell junctions (Sehgal et al. 2018).

Integrins have been demonstrated to be partially responsible for directly regulating EGFR phosphorylation in a ligand indapendant manner. Cell-matrix adhesions can promote EGFR localistation to β-integrins in a macromolecular complex containing p130Cas and c-Src (Figure 1.7) (L Moro et al. 1998; Laura Moro et al. 2002; Cabodi et al. 2004). This triggers the ligand-independent phosphorylation of EGFR by c-Src (Bill et al. 2004; Miyamoto et al. 1996; Laura Moro et al. 2002; X. Yu, Miyamoto, and Mekada 2000).

There is observed bi-directionality in the co-operation between integrin and EGFR as EGFR has also been shown to be important in the regulation of integrin function. For example, EGFR has been shown to regulate focal adhesion turnover in corneal

keratinocytes after EGF-stimulation (Eberwein et al. 2015), and EGF-stimulation can trigger the phosphorylation of β4 integrin leading to disassembly of hemidesmosomes (Mariotti et al. 2001; Wilhelmsen, Litjens, and Sonnenberg 2006; Mainiero et al. 1996).

The signalling pathways downstream of EGFR and integrin cross-talk are unclear. One protein that has been shown to be regulated by EGFR and integrin is calpain-2 which promotes talin turnover during breast cancer cell migration (Schwartz et al. 2018). FAK has recently been suggested as the bridge between EGFR and integrin signalling as FAK has been described to interact with phosphorylated EGFR promoting EGF-stimulated cell migration in fibroblast (Sieg et al. 2000) however the complete mechanism of this is still unclear.

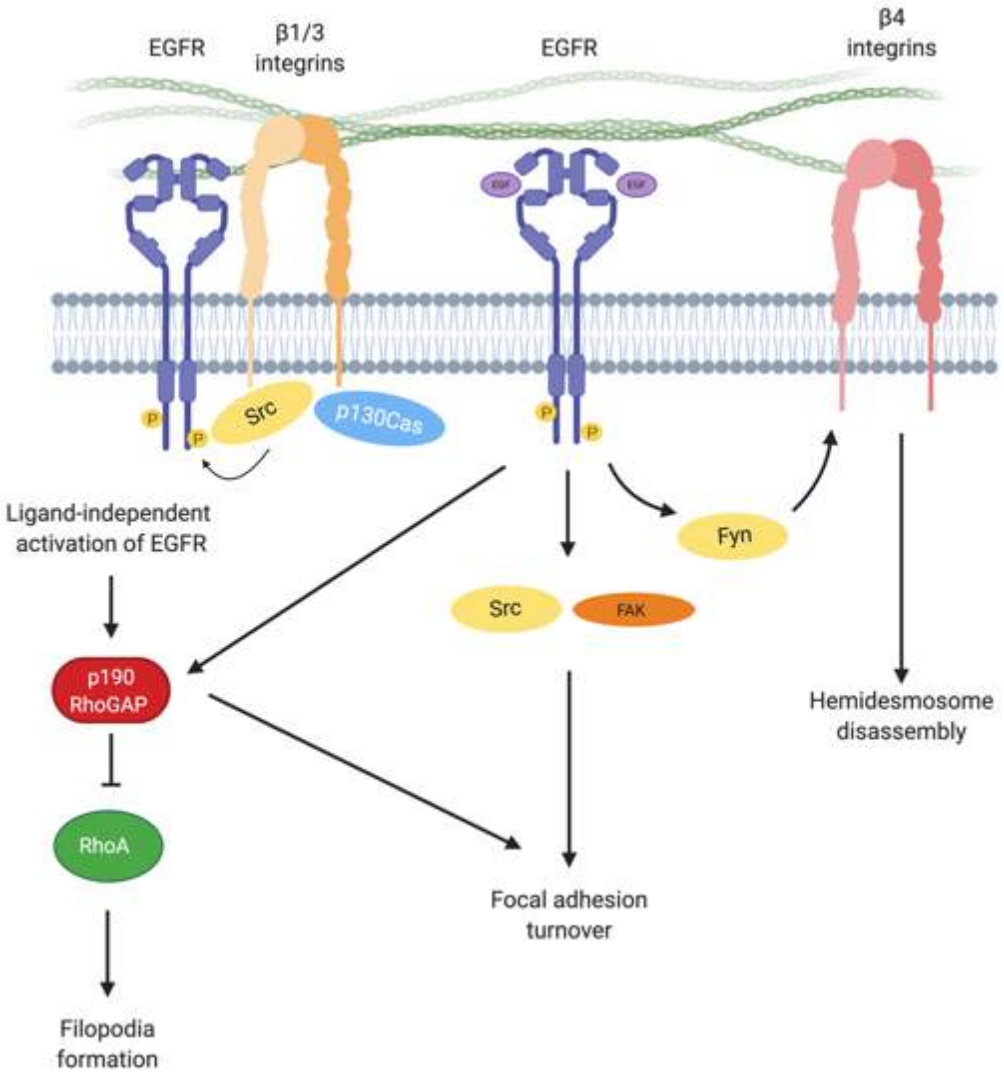


Figure 1.7: Schematic of EGFR integrin cross-talk. β1/β3 integrin can form a macromolecular complex with Src and p130Cas driving ligand-independent activation of EGFR. Ligand-independent activation of EGFR can activate p190RhoGAP, leading to inhibition of RhoA to promote filopodia formation. On the other hand, EGF-stimulated activation of EGFR can phosphorylate p190RhoGAP, Src and FAK to promote focal adhesion turnover. EGF-stimulation can also promote Fyn-mediated phosphorylation of β4 integrin triggering hemidesmosome disassembly.

1.4.4 EGFR in inflammation

EGFR plays a direct role in regulating the immunological properties of the epidermis. It was recently demonstrated that the EGFR is required for the full induction of IL-1α in keratinocytes infected with *S. aureus* (Simanski et al. 2016). EGFR activation is involved in the control of chemokine expression in human keratinocytes. In particular, EGFR activation by TGFα or EGF potently down-regulates the levels of TNFα or IFNγ induced CCL5 and CCL2, potentially abrogating the recruitment of neutrophils, T cells and monocytes/macrophages into the skin (Francesca Mascia et al. 2003). EGFR has been shown to have immune regulatory functions such as the impairment of T-cell migration due to suppression of the CXCR3 ligand IP-10 by normal EGFR signaling. Also, EGFR inhibition has been shown to significantly reduce the upregulation of the NKG2D ligand MIC-A normally caused by stress factors such as UV radiation (Vantourout et al. 2014), providing evidence for a role for EGFR in immune surveillance.

Conversely, EGFR-dominant negative mutations show strong epidermal macrophage-driven inflammatory responses (Hansen et al. 1997), extended EGFR inhibition can cause keratinocyte cell death leading to more acute and longer term immune cell activation and infiltration (J Li et al. 2001). Cultured keratinocytes displayed up-regulation of T-cell chemo-attractants when EGFR was blocked (Pastore et al. 2005). In agreement with these findings, mouse models of inflammatory skin conditions demonstrate that EGFR/ERK inhibition can lead to acute skin inflammatory response, consisting of massively upregulated levels of proinflammatory cytokines and chemokines as well as large numbers of infiltrating T-cells and macrophages. Outside of the skin, enhanced expression of pro-inflammatory molecules, including the T-cell-selective chemo-attractants CXCL9, CX3CL1, and CXCL18, was observed in cervical carcinoma epithelial cells treated with small-molecule EGFR inhibitors (Woodworth et al. 2005). Despite these many implications of EGFR in skin immune activity, not all effects may be direct. For example, in atopic dermatitis abrogated EGFR signaling may be responsible for a decrease in epidermal rigidity that allows for easier infiltration of inflammatory immune cells (Boguniewicz and Leung 2011).

1.4.5 EGFR in Cancer

EGFR was the first receptor to be directly associated to human cancer (de Larco and Todaro 1978). Genetic modulation and dysregulation of the EGFR is very common in cancers throughout the body including the head and neck, colon, and breast cancer (Yarden 2001), and it has been shown that cancer cells express between 10-20 times the amount of EGFR on their surface compared with normal cells.

There have been many reports indicating changes in EGFR in cancer. Firstly, it was shown that the hypoxic microenvironment caused by tumors could induce EGFR overexpression by increasing the translation of EGFR mRNA (Franovic et al. 2007). Furthermore, EGFR overexpression can result in high levels of autocrine production of TGFα and EGF (Yarden and Shilo 2007). Various activating EGFR mutations have been reported in tumor samples as contributors to these phenomena. Truncation mutants due to gene rearrangement have been reported in glioblastoma, the most common of which is EGFRvIII where amino acids 6–273 are deleted from the gene (Frederick et al. 2000). Truncation mutants such as EGFRvIII are also reported in other types of tumors, including breast, ovarian and non-small-cell lung cancer (Moscatello et al. 1995; Wikstrand et al. 1995). Additionally, point mutations and missense mutations in the ectodomain of the EGFR can result in increased phosphorylation of the kinase domain. This increase in activity has been shown to drive tumorigenicity in 3T3 cells (K. Zhang et al. 1996). Mutations have also been described in the kinase domain itself, and these have been shown to also hyper-activate the receptor in tumors.

Due to the role of EGFR in driving cancer, various types of EGFR targeting treatments have been employed. Monoclonal antibodies (mAbs) and small-molecule tyrosine-kinase-inhibitors (TKIs) are the most widely used treatments to target EGFR. mAbs bind to the extracellular domain of EGFR and compete with endogenous ligands, thereby blocking the ligand-induced EGFR tyrosine kinase activation (Garrett et al. 2002). Small-molecule (TKIs) compete reversibly with adenosine 5’ triphosphate and inhibit EGFR autophosphorylation and downstream signaling. Despite many of these treatments having been approved, and others being in phase II and III of clinical trials,

they commonly result in adverse, highly inflamed skin toxicities somewhat similar to epidermolysis bullosa (EB) (H. B. Liu et al. 2013). These findings offer another link between EGFR and inflammation.

1.4.6 A G428D EGFR mutation leads to epithelial blistering

Recently, the Parsons and McGrath groups identified a novel homozygous missense mutation in EGFR from a male infant with extensive skin inflammation resembling EB (Campbell et al. 2014). This work reported on the first loss-of-function mutation in EGFR and made the link between this mutation and an EB-like disorder raising the key question of how altered EGFR function causes a severe inflammatory phenotype.

When interrogated by immunofluorescence microscopy, the distribution of EGFR in the patients skin was greatly altered compared to that of healthy controls (Figure 1.8). The EGFR, which is normally localized predominantly to the cell membrane, was reportedly dispersed throughout the cytoplasm and perinuclear space. The mutation was a single amino acid replacement within the linking region of the EGFR. The location of this mutation is novel in respect to the more common hyper-activating mutations found in tumors that are most commonly found within the tyrosine kinase domain (Kumar et al. 2008). Aside from ligand binding, the area around the mutation has been postulated to be involved with EGFR dimerization and receptor activation (Dawson et al. 2005; Ogiso et al. 2002).

Microarray data from the patients skin revealed modulations in gene expression of a wide range of different genes, including extra-cellular matrix (ECM) protein expression, immune processes, and anti-microbial peptide expression. Importantly, there was little alteration in EGFR at the RNA level. Many of the most highly enriched networks amongst up-regulated transcripts were inflammatory networks as seen in Table 2. Some genes relating to skin inflammation were highly upregulated such as IL1F9 (IL-36), NFKB2 (NF-kB), JNK1, and CCL2 the last of which plays a role in regulating monocytic/macrophage infiltrates into the skin and has been shown to be abrogated in EGFR knockout mice models (Lichtenberger et al. 2013). Interestingly, there was

profound down-regulation in IL-8 (CXCL8), an inducer of neutrophil chemotaxis, which was in keeping with the low numbers of neutrophils present in the inflamed skin biopsies. Many gene components that are involved in the epidermal chemical barrier to microbes were also up regulated, specifically CXCL12, SERPINA1 (Alpha-1-antitrypsin), CYP1A1 (Cytochrome P450) and IRAK2, which is and indicator of increased TLR activation. As this data was generated using total skin biopsy, there is a high level of value in studying the effects of EGFR down-regulation in keratinocytes alone, to determine the effect of EGFR on the inflammatory potential of keratinocytes.

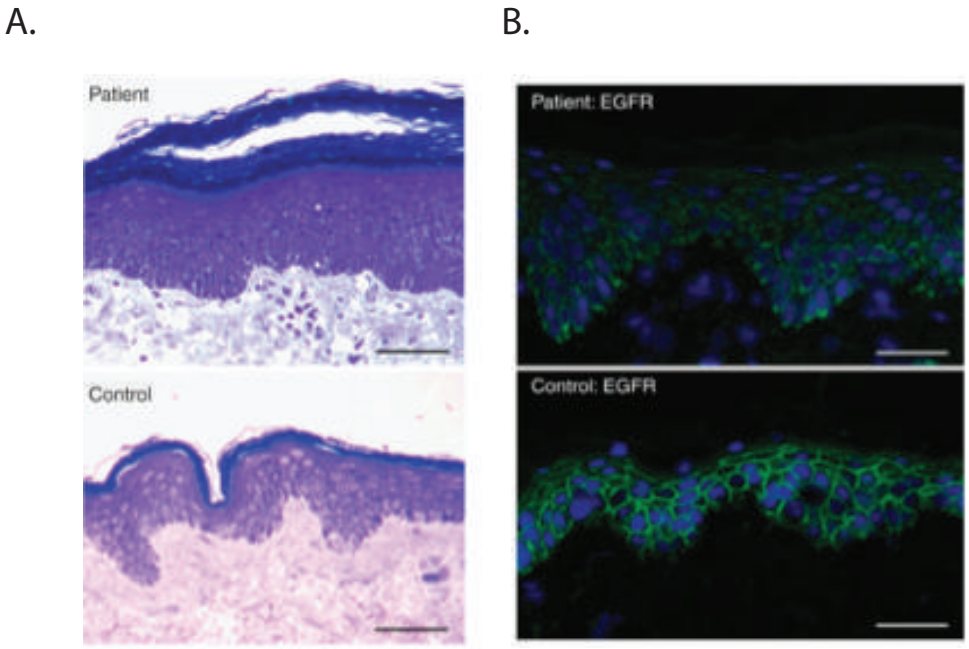


Figure 1.8 - Characterisation of G428D EGFR in the epidermis. A. Skin section of the G428D EGFR patient, exhibiting slight epidermal thickening. B. Immunostaining of EGFR in patient epidermis shows that G428D EGFR shows reduced EGFR localisation to cell periphery. (Campbell et al. 2014)

\

#	Networks	p-Value
1	Cytoskeleton_Spindle microtubules	1.33e – 06
2	Inflammation_Innate inflammatory response	1.67e – 06
3	Signal transduction_Neuropeptide signaling pathways	1.55e – 05
4	Inflammation_MIF signaling	1.62e – 05
5	Cell cycle_Mitosis	7.53e – 05
6	Inflammation_Amphoterin signaling	1.48 – 04
7	Cell cycle_S phase	2.04e – 04
8	Inflammation_Kallikrein-kinin system	3.70e – 04
9	Inflammation_Protein C signaling	1.06e – 03
10	Cell cycle_Core	1.11e – 03

Table 2: Enriched networks amongst up-regulated transcripts from G428D patient skin biopsy. (Campbell et al. 2014)

Hypothesis

The hypothesis of this thesis is that EGFR plays a role in the regulation of the inflammatory function of keratinocytes and that this may not be coupled to a breakdown of the epidermal barrier.

Aims

The experiments in this thesis are designed to address the following aims:

- To investigate the effect of EGFR knockout or G428D mutant re-expression in the regulation of basal keratinocyte barrier formation

- To analyse the effect of EGFR knockout or G428D mutant re-expression on the immune function of basal keratinocytes in a clean, otherwise unperturbed, environment

- To identify the signalling pathways downstream of EGFR that may play a role in the keratinocyte phenotype caused by EGFR knockout or G428D mutant re-expression

2. Materials and Methods

2.1 Reagents

Table 3: Cell culture Reagents

Reagent	Source
35mm quad µDish	Ibidi
35mm high µDish	Ibidi
Collagen Type 1	Corning
DMSO (Dimethyl sulphoxide)	Sigma Aldrich
Eagle's Minimal Essential Medium (EMEM)	Lonza
EGF (Epidermal Growth Factor)	PeproTech
Fetal Bovine Serum (FBS)	Hyclone
Fibronectin	Millipore
HEPES	Sigma-Aldrich
High Glucose Dulbecco's Modified Eagle's Media (DMEM)	Sigma-Aldrich
Liothyronine	Sigma-Aldrich
Lipofectamine2000	Thermofisher
OPTIMEM	Thermofisher
PBS (Phosphate Buffered Saline)	Sigma-Aldrich
Penicillin/Streptomycin	Sigma-Aldrich
Polybrene (hexadimethrine bromide)	GE Healthcare
Polyethylenimine (PEI)	AKfra Aesar
Trypsin/ EDTA	Sigma-Aldrich
RPMI-1640	Sigma-Aldrich
0.1g/L sodium bicarbonate	Sigma-Aldrich

Table 4: Molecular biology reagents

Reagent	Source
Ampicillin	Sigma-Aldrich
Kanamycin	Sigma-Aldrich
Luria-Bertani Agar and Broth	Sigma-Aldrich
Midiprep Kit	Qiagen
Miniprep Kit	Qiagen
OneShot TOP10 Chemically Competent E. coli	Thermofisher
RNeasy Mini Kit	Qiagen

Table 5: Biochemical assay reagents

Reagent	Source
1.5 mm Cassettes	Thermofisher
2-mercaptoehtanoesulfonic acid sodium salt (MesNA)	Sigma-Aldrich
30% Acrylamide/Bis solution	Biorad
Agarose resin	ThermoFisher
Ammonium persulphate (APS)	Sigma-Aldrich
BSA (Bovine Serum Albumin)	Sigma-Aldrich
Dithiothreitol (DTT)	Sigma-Alrich
ECL Plus Western blotting detection system	GE Healthcare
Glycerol	VWR International
Glycine	Sigma-Aldrich
Hybond ECL Nitrocellulose Membrane	Amersham Bioscience
Immersion 5101 Immersion oil	Zeiss
Magnesium chloride	Sigma-Aldrich
Methanol	Sigma-Aldrich
Milk Powder	Sigma-Aldrich
Nitrocellulose	GE Healthcare
PBS Tablets	Thermo Scientific

PeqGOLD Protein Marker V	Thermo Scientific
PFA (Paraformaldehyde)	PeqLab
Phosphatase Inhibitor Cocktail Set II (Stock 100x) containing: 200 mM Imidazole, 100 mM Sodium Fluoride, 115 mM Sodium Molybdate, 100 mM Sodium Orthovanadate, 400 mM Sodium Tartrate, dyhydrate	Millipore
Pierce ECL Western Blotting Substrate	Alpha Diagnostic International
Protease Inhibitor Cocktail set I (Stock 100x) containing: AEBSF, Hydrochloride - 500 µM Aprotinin, Bovine lung, crystalline – 150 nM E-64 Protease Inhibitor - 1 µM EDTA Disodium – 0.5 mM Leupeptin, Hemisulphate – 1 µM	Millipore
Sodium chloride	Sigma-Aldrich
Sodium Dodecyl Sulphate	Sigma-Aldrich
Sodium Fluoride	Acros organics
Sodium Orthovanadate (Vanadate)	New England Biolabs
Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris-Base	Sigma-Aldrich
Tris-HCl	Sigma-Aldrich
Triton X-100	Sigma-Aldrich
Tween-20	Calbiochem

Table 6: Materials and solutions for biochemical assays

Buffer/ Solution	Composition
12% Stacking Acrylamide Gel	12% 30%-acrylamide mix, 400 mM Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.05% TEMED
10% Stacking Acrylamide Gel	10% 30%-acrylamide mix, 400 mM Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.05% TEMED
8% Stacking Acrylamide Gel	8% 30%-acrylamide mix, 400 mM Tris (pH 8.8), 0.1% SDS, 0.1% APS, 0.05% TEMED
Running Buffer (10x)	0.25 M Tris base, 1.92 M glycine, 1% SDS
SDS Sample Buffer 2x	60mM Tris-HCl (pH 6.8), 25% Glycerol, 2.5% SDS, 0.02% Bromophenol blue, 2% β-mercaptoethanol
SDS Sample Buffer 5x	250 mM Tris-HCl (pH6.8), 10% SDS, 30% Glycerol, 0.02% Bromophenol blue, 5% β-mercaptoethanol
TBS-Tween (10x)	20 mM Tris-base (pH 7.5), 150mM NaCl, 0.1% Tween- 20
Transfer Buffer (10x)	0.25 M Tris base, 1.86 M glycine, 10% methanol

Table 7: Inhibitors

Compound	Source
AG1478	Tocris
SHP009	Sigma-Aldrich
GM6001	Tocris
5,15-DPP	Tocris

Table 8: Antibodies

	Reagent	Species	Dilution	Source
Primary	Anti-E-cadherin	Mouse	1:400 (IF)	Abcam
	Anti-EGF Receptor	Mouse	1:1000 (WB)	Santa Cruz
	Anti-EGF Receptor	Rabbit	1:2000 (WB)	Cell Signalling
	Anti-ERK	Rabbit	1:1000 (WB)	Cell Signalling
	Anti-GAPDH	Mouse	1:5000 (WB)	Chemicom
	Anti-HSC70	Rabbit	1:5000 (WB)	Sigma-Adrich
	Anti-Phospho-EGF Receptor (Tyr1173)	Rabbit	1:1000 (WB)	Cell Signalling
	Anti-Phospho-ERK 1/2 (Thr202/Tyr204)	Rabbit	1:1000 (WB)	Cell Signalling
	Anti-STAT3	Mouse	1:500 (WB)	Cell Signalling
	Anti-Phospho-STAT3 (Tyr705)	Rabbit	1:500 (WB)	Cell Signalling
	Anti-SHP2	RAbbit	1:1000 (WB)	Abcam
	Anti-Phospho-SHP2 (Tyr542)	Rabbit	1:1000 (WB)	Abcam
	Anti-Vinculin	Mouse	1:400 (IF)	Sigma-Aldrich
	Anti-β-Catenin	Rabbit	1:500 (IF)	Santa Cruz
	Il-33	Mouse	1:500 (IF)	Invitrogen
Secondary	Anti-Mouse Alexafluor 488	Goat	1:500 (IF)	Molecular probe
	Anti-Mouse Alexafluor 568	Goat	1:500 (IF)	Molecular probe
	Anti-Mouse-HRP	Goat	1:5000 (WB)	Dako
	Anti-Rabbit Alexafluor 488	Goat	1:500 (IF)	Molecular probe

	Anti-Rabbit Alexafluor 568	Goat	1:500 (IF)	Molecular probe
	Anti-Rabbit-HRP	Goat	1:5000 (WB)	Dako
	Phalloidin 488	N/A	1:500 (IF)	Invitrogen
	Phalloidin 568	N/A	1:500 (IF)	Invitrogen
	Phalloidin 647	N/A	1:500 (IF)	Invitrogen
Flow Cytometry	Il-4 Alexa-Fluor 488	N/A	1:500	BioLegend
	CD4 APC	N/A	1:500	BioLegend
	CCR4 PE	N/A	1:400	BioLegend
	CCR6 PE-Cy7	N/A	1:400	Biolegend
	GATA3 BV421	N/A	1:400	BD
	L/D Aqua	N/A	1µl per 1ml stain media	ThermoFisher

Table 9: Plasmids

Plasmid	Type	Insert and tag	Original Reference/ Source
eGFP (SFFV)	Lentiviral	GFP tag	(Demaison et al. 2002; Jayo et al. 2016)
EGFR-eGFP (SFFV)	Lentiviral	Human EGFR, eGFP tag	Generated in house by G. Chan based on eGFP plasmid
G428D-eGFP (SFFV)	Lentiviral	G428D mutant EGFR,, eGFP tag	Generated in house by G. Chan based on eGFP plasmid
LV-Cre pLK0.1	Lentiviral	Cre recombinase	Addgene (Beronja et al. 2001)

Table 10: Materials for bioassays and functional studies

Reagent	Source
TRITC Dextran 20kDa	Sigma -Aldrich
CellEvent™ Caspase-3/7 Green Detection Reagent	Thermofisher
QCM™ Gelatin Invadopodia Assay (Red)	Merk
MS Tubes	Miltenyi Biotec
MiniMACS Separator	Miltenyi Biotec
CD3ε MicroBead Kit, mouse	Miltenyi Biotec
Monocyte Isolation Kit (BM), mouse	Miltenyi Biotec
IL4 Recombinant Mouse Protein	Thermofisher
Recombinant Mouse IL-6 (carrier-free)	Biolegend
Dynabeads Mouse T-Activator CD3/CD28	Thermofisher
BD GolgiStop™	BD
Brefeldin A 1000x	Biolegend
CCL2 Neutralising antibodies	RnD Systems
CCL5 Neutralising antibodies	RnD Systems
IL-33 Neutralising antibodies	RnD Systems
CXCL10 Neutralising antibodies	RnD Systems

Table 11: Materials for RNAseq and QPCR

Reagent	Source
TaqMan® Fast Advanced Master Mix 5ml	Thermofisher
Eukaryotic 18S rRNA Endogenous Control 125rxns	Thermofisher

MicroAmp® Fast Optical 96-Well Reaction Plates	Thermofosher
RevertAid First Strand cDNA Kit	Thermofisher
Next rRNA Depletion kit	New England BioLabs

Table 12: QPCR Probes (All Thermofisher)

Probe	ID
COL1A1	Mm00801666
COL4A6	Mm00474735
CCL2	Mm00441242
CCL5	Mm01302427
CCL20	Mm01268754
CCL27	Mm04206819
CXCL10	Mm00445235
C3	Mm01232779
IL1B	Mm00434228
MMP9	Mm00442991
MMP10	Mm01168399

Table 13: ELISA Kits

Kit	Source
Mouse IL-33 DuoSet ELISA	RnD Systems
Mouse CCL5/RANTES DuoSet ELISA	RnD Systems
Mouse CCL2/JE/MCP-1 DuoSet ELISA	RnD Systems
Mouse CXCL10/IP-10/CRG-2 DuoSet ELISA	RnD Systems
Mouse CCL20/MIP-3 alpha Quantikine ELISA Kit	RnD Systems

2.2 Methods

2.2.1 Bacterial transformation

Chemically-competent E.coli was thawed on ice, followed by addition of DNA into the Top10 bacterial cells. After cells were incubated on ice for 30 minutes, they were heat-shocked at 42°C for 30-45 seconds. They were then incubated on ice for 2 minutes before 1 ml of S.O.C. medium or LB was added into the cells. This was followed by incubation for 1 hour at 37°C before spreading onto LB agar plates with the appropriate antibiotic (Ampicillin 100µg/ml or Kanamycin 50 µg/ml). Plates were incubated at 37°C overnight. Single colonies could then be picked using a sterile p100 pipette tip and grown overnight in LB broth shaken at 37°C at appropriate volume for DNA preparation.

2.2.2 Midiprep of DNA plasmids

QIAGEN Plasmid Plus Midi Kit was used to extract DNA plasmid from bacteria. 100 ml of bacterial culture was centrifuged at 4000 rpm for 15 minutes at 4°C and the supernatant discarded. Pellet was then resuspended in 4ml of Buffer P1. 4ml of Buffer P2 was added into the bacteria and was incubated at room temperature for 3 minutes. 4ml of Buffer S3 was added to stop the cell lysis. The lysate was transferred to a QIAfilter cartridge and was incubated at room temperature for 10 minutes. Afterwards, the lysate was filtered and 2 ml of Buffer BB was added. After mixing, the solution was added to a QIAGEN Plasmid Plus Midi spin column. Vacuum pressure was used to filter the solution through the column. The column was then washed with 700 µL of Buffer ETR and Buffer PE. This was followed by centrifugation for 1 minute at 13,000 rpm to remove any excess wash buffer. After the column was transferred to a clean Eppendorf tube, 200µL of Buffer EB was added to the column to collect DNA in the flow-through. DNA concentration and quality was analysed using a Nanodrop Spectrophotometer.

2.2.3 Generation of lentiviruses from HEK-293T cells

HEK-293T cells were plated to 40-50% confluent the night before transfection. A transfection mixture containing 2.1 µg pCMV8.91, 0.7 µg pMD.G and 3.75 µg of the appropriate lentivirus constructs was mixed in 500 µL of OPTIMEM. This was followed by the addition of 22.5 µL of PEI transfection reagent into the transfection mixture. The mixture was left for 15 minutes at room temperature before adding to the cells with media without antibiotics. Media was then replaced by OPTIMEM after incubation with the transfection mixture for 4-5 hours at 37°C. Viruses were harvested after 48 hours, where the media was removed and centrifuged at 1200 rpm for 3 minutes to remove any HEK-293T. The viruses were then filtered through 0.4 µm and stored in 1 mL aliquots at -80°C.

2.2.4 Lentiviral infection to generate stable cell lines

CHO, EGFR-2, and mouse keratinocytes were plated into T-25cm^2 to 40% confluency. 8 µg /mL polybrene was added into media to increase the efficiency of viral infection. 1 mL of lentiviral particle solution was added to the cells and left to incubate at 37°C for 24-48 hours. The media was changed afterwards and cells were grown and passaged.

2.2.5 Establishing cell lines from EGFR$^{-/-}$ mice

The following mice were received from the Maria Sibilia lab in Vienna based on the EGFR$^{-/-}$ mouse. Cell-line establishment was performed by Simon Broad from the Fiona Watt lab at the Centre for Stem Cells & Regenerative Medicine, King's College London.

1x male EGFR KO P14
1x female EGFR KO P14
1x male WT P14
1x female WT P14

Skin was removed from the mice separately and sterilised in 1% iodine (Pevidone). Skin was incubated in surgical scrub solution for 10s followed by two rinses in 70% ethanol for 5s each then placed in Hanks' BSS (Sigma-Aldrich). Skins were spread out on a 90mm perti dish and excess fat and connective tissue removed mechanically. Trypsin (0.25%) solution was added and the skin was incubated at 37°C for 1 hour. The fragments of epidermis were then scraped away from the dermis and resuspended in growth medium containing FBS. The mixture was filtered through a nylon strainer to remove hair fragments. The resulting cell suspension was then washed twice in growth medium and seeded onto a feeder layer of mitotically inactivated 3T3 cells. After 8-10 days the cells became confluent and were trypsinised and reseeded onto a fresh layer of 3T3 fibroblasts. This process was repeated until keratinocytes became immortalised. Basal layer keratinocytes were validated by keratin 5 and 14 expression.

2.2.6 EGFR-2 mouse keratinocytes

EGFR-2 cells are immoratalised basal keratinocytes derived from the EGFR$^{fl/fl}$ mouse Cells were provided by Laura Hansen at the Department of Biomedical Sciences, Creighton University, Omaha, NE, USA.

2.2.7 Cell Culture

Human Embryonic Kidney-293T (HEK293T) and CHO cells were cultured in DMEM supplemented with 10% (v/v) FBS, 100 unit/ml penicillin, 0.1 mg/ml streptomycin and 2 mM L-Glutamine.

KO/WT mouse keratinocytes and EGFR-2 cell lines were cultured in normal growth media consisting of EMEM with 2.2% (v/v) chelexed FBS , 4.7% (v/v) unchelexed FBS, 5000 unit/ml penicillin and 5 mg/ml streptomycin. All cell lines were maintained at 37°C in a 5% CO2 humidified atmosphere.

Isolated primary mouse T cells and monocytes were cultured in RPMI-1640 with 2mM L-glutamine, 0.1g/L sodium bicarbonate, supplemented with 100U/mL penicillin and 0.1mg/mL Streptomycin, and 10mM Hepes.

All cells, except primary mouse immune cells, were passaged when 80-90% confluent by washing once with PBS (without Calcium or Magnesium), followed by trypsinisation using trypsin in EDTA (0.05% concentration). After cells detached, normal growth media was used to wash and collect the cells. Cells were then centrifuged for 3 minutes at 1200 RPM, where the supernatant was removed and the pellet was resuspended in media to be plated into tissue culture flasks.

Cells were also frozen down for future use where cell pellets were resuspended in freezing media, which consists of 40% normal media, 50% FBS and 10% DMSO. The cells were transferred to -80°C and then to liquid nitrogen for long term storage. To thaw frozen stocks, cells were thawed at 37°C before adding into normal media. They were then centrifuged and resuspended into normal media before plating into tissue culture flasks.

2.2.8 Recombinant KGF and EGF treatment

EGFR-2 or mouse keratinocytes were serum starved in OptiMEM for 12-24 hours at 37°C. Recombinant KGF or EGF was added to the cells at a final concentration of 10 ng/ml or 100ng/ml and incubated at 37°C for the required period of time.

2.2.9 Drug treatments

EGFR-2 cells and mouse keratinocytes were plated in the relevant tissue culture plates and once at the correct confluence, treated with reagents as described in Table 8. DMSO treated cells were used as a control.

Table 14: Reagent treatments

Reagent	Role	Concentration	Duration	Control
AG1478	Inhibitor of EGFR	5 µM	1-24 hours	DMSO
SHP099	Inhibitor of SHP2 activation	50 µM	48 hours	DMSO
5,15-DPP	Inhibitor of STAT3 phosphorylation	1 µM	1-24 hours	DMSO
GM6001	Pan-inhibitor of MMPs	25mM	Up to 16hrs	DMSO

2.2.10 RNA extraction

Total RNA was isolated using the RNeasy Qiagen kit according to the manufacturer's instructions. Cells were trypsinised and pelleted before being resuspended with buffer RLT. Afterwards, the lysate was transferred to RNeasy spin column and was centrifuged for 15 seconds. Flow-through was discarded, and 700 µL of Buffer RW1 was added to wash the column. After, the column was washed twice with Buffer RPE before the column was centrifuged to remove any excess liquid. Afterwards, the column was transferred into a new Eppendorf before 50 µL of RNase-free water was used to elute the RNA. The resulting RNA was used for cDNA synthesis using the RevertAid First Strand cDNA Synthesis Kit as per manufacturers instruction.

2.2.11 QPCR

qPCR was conducted using 7900HT Fast Real-Time PCR System (ThermoFisher Scientific). Using probes, listed in Table 10, multiplexed with a 18S probe (all Thermofisher) in TaqMan® Fast Advanced Master Mix as per manufacturer's instructions.

2.2.12 RNA sequencing

EGFR-2 cell lines were plated in 6-well plates at high density and harvested when confluent monolayers formed. RNA was isolated using RNeasy Qiagen kit according to the manufacturer's instructions. Library preparation was completed using NEBNext Ultra Directional RNA Library Prep Kit for Illumina. Depletion of ribosomal RNA was performed using Next rRNA Depletion kit as per manufacturers instruction. RNA quality was confirmed by bioanalyser (Agilent 2100 Bioanalyzer G2938B), resulting in a mean RIN score of 8.2, ranging from 7.5-8.6. Paired-end sequencing was then conducted using the HiSeq 2500 platform (Illumina). Raw data was checked for quality using FASTQC. Processing of the raw data involving alignment and annotation were done using Partek. After annotation, reads per million normalised data were then used for statistical analysis. Inclusion criteria for significantly differentially expressed genes was a false discovery rate of <0.05 and a fold change of greater than 1.5x. Subsequent processing and visualisation of the data was completed in RStudio or Morpheus (Broad Institute, Boston, MA).

2.2.13 SDS-PAGE analysis

To evaluate protein expression based on their molecular weights, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gels with 8-12% (v/v) polyacrylamide resolving layer and a 4% (v/v) stacking layers. After cells were lysed in sample buffer containing DTT to denatures the proteins from tertiary structure to a primary amino acid structure, proteins were separated under an

SDS-PAGE system. Protein Marker V was also ran alongside. A constant voltage of 80V was running through the stacking layer for 20 minutes before the voltage was then increased to 180V until the gel band-front had moved through the separating gel.

2.2.14 Western blot

To look for specific protein expression, the proteins were transferred to nitrocellulose for 1.5 hours at 20V using a transfer kit (Invitrogen) in Transfer Buffer. The membranes were blocked using blocking buffer (5% (w/v) skim milk powder or Bovine Serum Albumin in TBS, 0.1% (v/v) Tween) depending on target, for one hour at room temperature. This was followed by incubation with primary antibodies, as listed in Table 6, in blocking buffer overnight at 4°C. Membranes were then washed three times for 10 minutes in TBST before incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, also listed in Table 6, for 1 hour at room temperature. After washing three times with TBST, proteins were detected by ECL chemiluminescence kit (BioRad) and directly imaged using the BioRad imager digital imaging system. Blots were analysed and processed using BioRad Image Lab and FIJI.

2.2.15 Immunofluorescence microscopy

Cells were plated into appropriate optical plastic dishes and incubated in normal growth media. Cells were fixed in 4% (v/v) PFA in PBS, pH 7.4 for 15 minutes on ice. For monolayer staining, cells were fixed with 4% (v/v) PFA in PBS, pH 7.4 with 0.01% Triton X-100 for 15 minutes. Dishes were washed once with cold PBS before permeabilisation with 0.1% Triton X-100/ PBS or ice-cold methanol for 5 minutes. After three washes with PBS, dishes were blocked with blocking buffer (5% (w/v) BSA in PBS or TBST) for 1 hour at room temperature before incubating with various primary antibodies, as listed in Table 6, in blocking buffer for 1-2 hours at room temperature, or overnight at 4°C. Dishes were washed three times with PBS before incubating with appropriate secondary fluorescent conjugated antibody, Hoechst and phalloidin (if required) for at least 1 hour at room temperature in the dark.

Dishes were then washed 3 times with PBS and then PBS added and kept in dishes for imaging.

2.2.16 Confocal microscopy

Images of fixed cells were acquired on a Nikon A1R inverted confocal microscope (Nikon Instruments UK) with an environmental chamber maintained at 37°C. Images were taken using a 60x or 100x Plan Fluor oil immersion objective (numerical aperture of 1.4). Excitation wavelengths of 488nm (argon laser), 561nm (diode laser) or 640nm (diode laser) were used. Images were acquired using NIS-Elements imaging software (v4) and were saved in Nikon Elements .nd2 format. Image processing was performed in FIJI processing software.

2.2.17 Flow Cytometry

For intracellular cytokine detection, cells were cultured in the presence of Brefeldin A, and GolgiStop, for the final 3 hours of incubation. For surface staining, cells were harvested and incubated with live/dead Aqua for 15 minutes in phosphate buffered saline (PBS), followed by the appropriate volume of antibody diluted in 0.5% bovine serum albumin (BSA) in PBS for 20 minutes, all at 4°C. Cells were then washed and fixed in 3% paraformaldehyde for 15 minutes at room temperature. For intracellular cytokine staining, cells were incubated with the appropriate volume of antibody, diluted in 0.1% saponin in 0.5% BSA in PBS for 45 minutes at room temperature or 4°C overnight. Cells were acquired using a BD FACSCanto II, and analysis conducted using FlowJo V.10.1 software (Tree Star Inc.).

2.2.18 ELISA Assay

Sandwich ELISA was used to detect supernatant analytes. Cell-free supernatants were extracted from wells 24 hours after cell monolayers had formed and kept at -20°C until analysis. ELISA kits, Table 11, were used and conducted according to

manufacturer's protocols and detected on a Victor 1420 multilabel counter (Perkin Elmer) quantifying concentrations drawn from a standard curve on each plate.

2.2.19 Spleen/Lymph node digestion into single cell suspension

Both spleen and lymph nodes were isolated from culled WT CD1 mice. Spleens and lymph nodes were digested to a single cell suspension using the "Spleen dissociation/digestion protocol" from the International Mouse Phenotyping Consortium.
https://www.mousephenotype.org/impress/ProcedureInfo?action=list&procID=732

2.2.20 Isolation of CD3+ T cells and CD14+ monocytes from mouse tissue digestions

Both spleen and lymph node digestions were isolated by magnetic cell sorting using mouse monocyte isolation kit and using the column isolate from a mouse CD3 positive selection kit, MACS MS columns and MiniMACS magnetic separator as per manufacturers instruction. Cells were then counted and used immediately in necessary assays.

2.2.21 Chemotaxis Assay

For chemotaxis assays, modified Boyden assays were used. 10,000 isolated primary CD3+ T cells or CD14+ monocytes were plated in the top of a transwell with pore size of 3.0um. 600ul of conditioned media taken from EGFR-2 cells and WT and KO keratinocytes was then added to the lower well beneath the transwell membrane. After 4 hours, the transwells were removed and the plates were then centrifuged for 3 minutes at 1200 RPM. Plates were then left for a further 2 hours in order to allow for optimal cell adhesion to the well. Cells were then fixed with 4% (v/v) PFA and incubated with Hoechst for 10 minutes. Hoechst positive cells were then counted using the DAPI channel on an EVOS.

2.2.22 Gel degradation assay

24-well optical plastic plates were coated with Cy3-labelled gelatin using a QCM™ Gelatin Invadopodia Assay (Red) kit as per the manufacturer's instructions. EGFR-2 cells and mouse keratinocytes were then seeded at high density onto the coated wells in normal growth media with or without the pan-MMP inhibitor GM6001 at 25mM for 16hrs. Cells were carefully washed 3 times with PBS as not to disturb the cell monolayer or gealtin. Wells were then fixed with 4% (v/v) PFA.

2.2.23 Gel degradation analysis

To analyse the total gelatin degradation per well and per cell, the cells were stained with Hoechst and phalloidin and imaged via confocal for Hoechst, phallodin and Cy3-gelatin. In FIJI, channels were separated and cell area was measured by thresholding phalloidin channel and degradation was measured by calculating the inverse of the thresholded Cy3 channel.

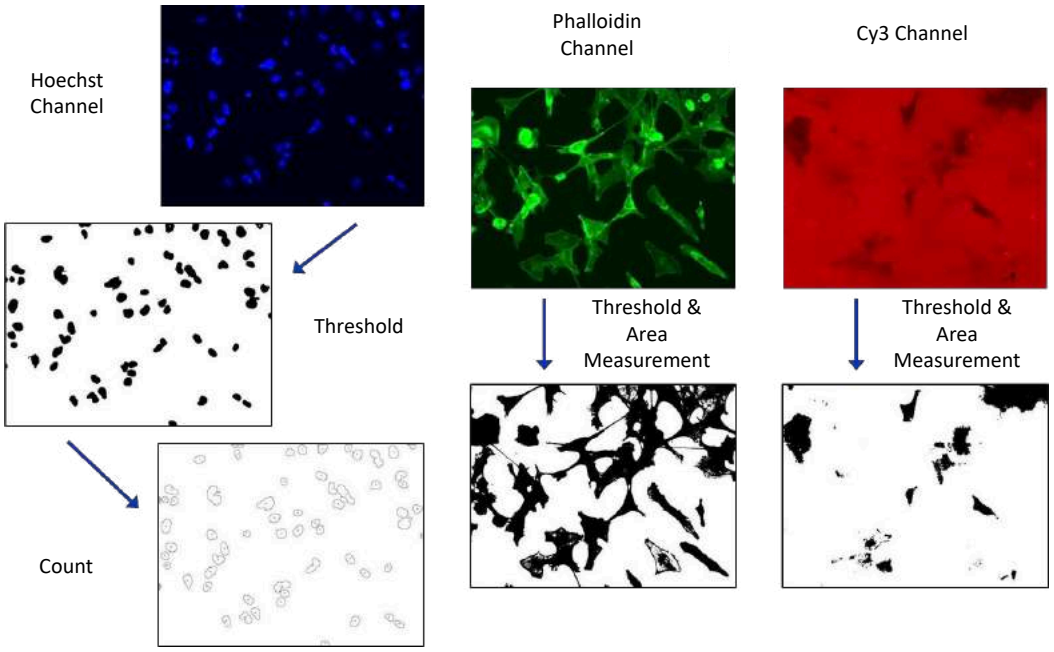


Figure 2.1 – Method for gelatin degradation analysis. An example of image analysis using FIJI software Hoechst signal is thresholded for high intensities, then analysed as "particles" to provide a nuclear (cell) count. Similarly, phalloidin signal is thresholded for high intensities to allow measurement of cell area. Conversely, fluorescent Cy3 gelatin signal is thresholded for low intensities to enable degradation area quantification

2.2.24 Apoptosis assay

WT and KO mouse keratinocytes were cultured in normal growth media in 24 well plates. After monolayers had formed between 24-48 hours, growth media was removed and CellEvent™ Caspase-3/7 Green Detection Reagent diluted in PBS with 5% FBS was added to wells for 30 minutes as per manufacturer's protocol. Wells were then washed with PBS and normal growth media added to wells with or without appropriate growth factors for 24hr culture. Cells were then fixed with 4% (v/v) PFA and images captured using the GFP channel on an EVOS microscope. Green cells were then counted using FIJI.

2.2.25 Proliferation assay

EGFR-2 and mouse keratinocytes were plated at 1000 cells/well in 24-well plates in normal growth media. One well per cell line was fixed at 12, 24 and 48 hours after plating. Cells were incubated with Hoechst for 30mins and then total Hoechst positive cells per well were counted using the DAPI channel on an EVOS.

2.2.26 Dextran permeability assay

EGFR-2 and Mouse keratinocytes were plated at 10,000 cells per well in 0.2ml normal growth media in the upper chamber of 0.4um pore size transwells pre coated with collagen-1, with 0.6ml of growth media in the lower well. After 24hrs, wells were checked for the formation of complete cell monolayers. At this point, media was removed from the upper and lower wells and replaced with fresh growth media with or without 5mM EDTA and incubated for 30 minutes at 37°C. 10µl of TRICT-dextran (20 KDa) solution was then added to the upper chamber of all wells and plates placed back in the incubator. 100µl of media was collected from the lower chamber after 2 hours of incubation. The media fluorescence was then measured using a fluorescence plate reader.

2.2.27 Wound healing assay

EGFR-2 and mouse keratinocytes were plated at high density and incubated for 4-24 hours. Wells were then washed with PBS and cells cultured in the presence of 2 mM Calcium for 4hrs. Confluent monolayers were wounded with a 10-µl pipette tip scratched across the middle of the wells. Cells were washed in 2mM calcium containing growth media, to remove scraped cells and excess debris. Cell migration into the wound was captured using an EVOS every 30 minutes or every hour over 24 hours. Wound closure was analysed using FIJI.

2.2.28 Focal adhesion analysis

To analyse the number and area of focal adhesion in cell monolayers, cells were fixed and stained with antibody against vinculin to label focal adhesions. Focal adhesion was thresholded and the number and area of focal adhesions were analysed using the analyse particle option in FIJI.

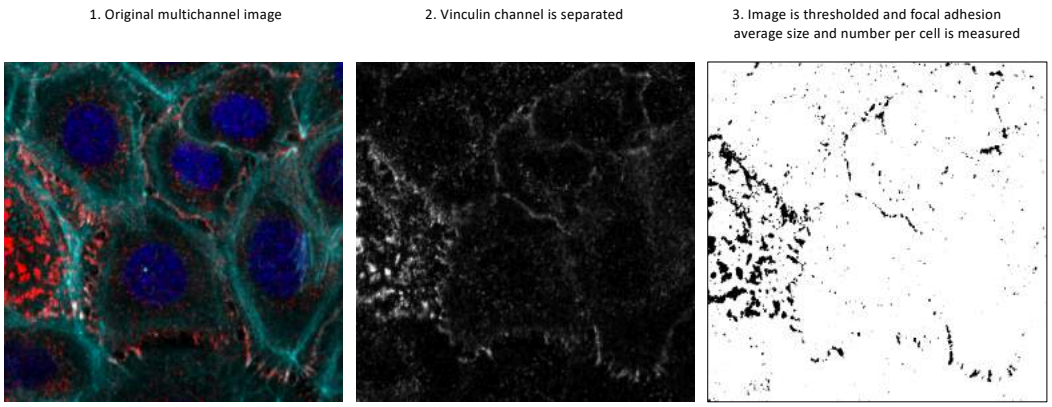


Figure 2.2: Method for focal adhesion analysis. 1. Multichannel images were used showing vinculin, phalloidin and nuclear stain. 2 & 3. The vinculin staining channel was isolated for thresholding to isolate the focal adhesions from background noise and particle analysis in ImageJ was used to measure the number and area of focal adhesions.

2.2.29 Statistical analysis

Data is represented as the mean ± standard error of the mean (s.e.m.). All statistical tests were carried out using GraphPad Prism. The Student's t-test was performed for comparing two groups for statistical analysis. Analysis of variance (ANOVA) with Tukey's post hoc test was used for multiple comparisons. $P < 0.05$ was considered as statistically significant. Specific statistical tests and P numbers are indicated within figures.

3. Generation and Characterisation of EGFR Knockout and G428D Mutant Expressing Cell Lines

3.1 Introduction

The basal layer is the only site at which keratinocytes divide and thus is responsible for the replenishment of the skin. Dysfunction in these basal keratinocytes has been identified as a major contributing factor to many skin diseases such as epidermolysis bullosa simplex (EBS) (Coulombe, Kerns, and Fuchs 2009). Basal keratinocytes express high levels of EGFR and this is required for their proliferation and normal function in homeostasis.

As previously mentioned, Parsons and McGrath groups identified a novel homozygous missense mutation in EGFR, p.Gly428Asp (G428D) from a male infant with extensive skin inflammation resembling EBS (Campbell et al. 2014). This work reported on the first loss-of-function mutation in EGFR and made the link between this mutation and an EBS-like disorder raising the key question of how altered EGFR function causes a severe inflammatory phenotype. This mutation has since been reported on in other cases (Ganetzky et al. 2015).

The aim of this chapter was to interrogate the role of EGFR in regulating the inflammatory phenotype as observed in patients harbouring the homozygous G428D mutation. To do so, it was essential to generate cell lines lacking endogenous EGFR as well as those re-expressing the G428D EGFR variant to determine the effects on cell behaviour. A useful cell line for the study of the function of EGFR are CHO cells as they do not express endogenous EGFR, although they do express other HER family receptors. This provides a suitable host for testing the expression and function of the EGFR constructs to be used in this study, however CHO cells do not provide any use in studying the role of EGFR in skin inflammation. The study of EGFR negative keratinocytes is technically challenging as EGFR is such an important receptor for processes such as proliferation and cell growth. To overcome this, we chose to use the EGFR-2 keratinocyte cell line generated from an *EGFR*$^{fl/fl}$ mouse (Hammiller et al. 2015). This would allow us to knockout endogenous EGFR by expressing Cre-recombinase, generating EGFR null background keratinocytes in which to re-express WT and G428D constructs. A benefit of using this cell line is that they have been shown

to be competent and proliferative in the absence of EGFR when supplemented with KGF rich media in lieu of the more commonly supplemented EGF. Along with the CHO cells and EGFR-2 cells, we also established immortalized mouse keratinocytes derived from a male *EGFR*$^{-/-}$ mouse (Sibilia and Wagner 1995) at P14 and a matched *WT* mouse. These cells provide a useful addition to the other cell lines as these EGFR-KO keratinocytes have not had to be infected in order to express specific constructs or to knockout any endogenous receptor.

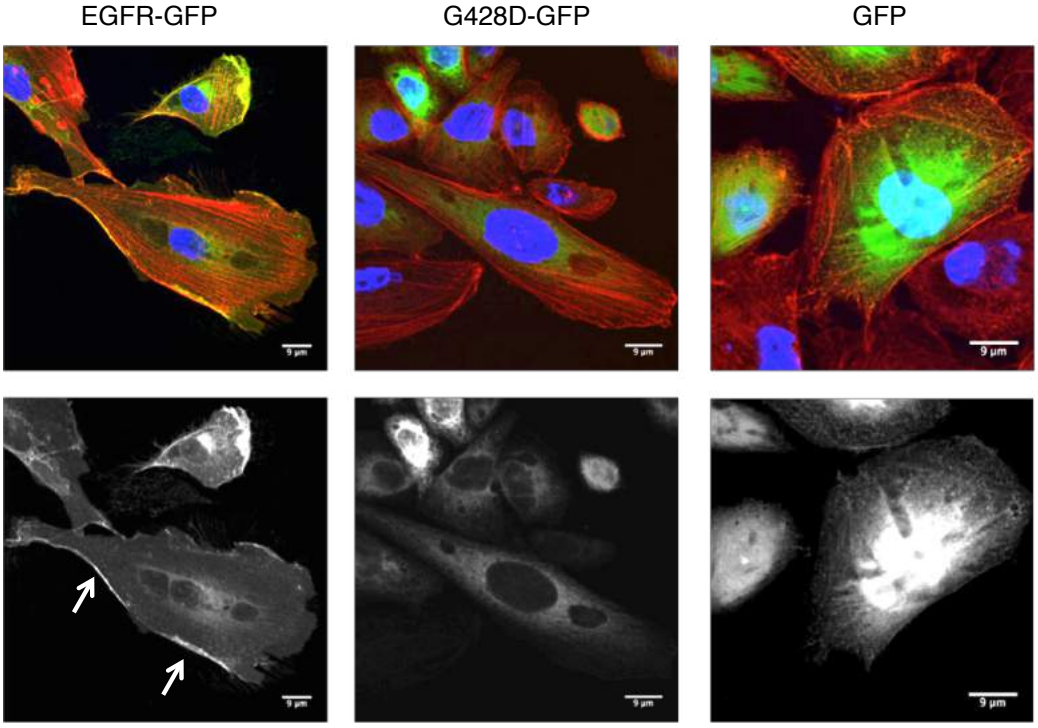
3.2. Results

3.2.1 G428D EGFR in CHO cells show loss of plasma membrane localisation and cannot be activated by EGF

In order to first characterise the effect of expressing wild-type (WT) or G428D EGFR in a naturally EGFR null cell line, lentivirus was generated from plasmids encoding WT human EGFR tagged to GFP (EGFR-GFP), p.Gly428Asp mutated EGFR tagged to GFP (G428D-GFP) or GFP alone (GFP) and used to infect CHO-K1 cells. One-week post infection, cells were plated on coverslips, fixed and stained for F-actin (Phalloidin) and DAPI and analysed by confocal microscopy. Resulting images showed successful expression of GFP-tagged constructs (Fig 3.1). Cells expressing EGFR-GFP demonstrated clear surface localisation of the receptor as indicated by arrows, whereas G428D-GFP lacked distinct membrane localisation and was predominantly cytoplasmic, similar to GFP alone (Fig 3.1A). During normal cell culture, no obvious differences were observed in growth rates of any of the CHO cell lines.

To determine whether expression of WT or G428D EGFR altered EGF-dependent activation of the receptor, CHO cells were stimulated with 10ng/ml EGF for 10 and 30 minutes. Lysates were then extracted from the cells and prepared for SDS-PAGE followed by blotting for EGFR, phospho-EGFR-Tyr-1173 (pEGFR), and GAPDH as a loading control. Resulting blots demonstrated that both EGFR-GFP and G428D-GFP were expressed at similar levels and only cells expressing EGFR-GFP showed an increase in EGF-induced pEGFR (Fig 3.1 B).

A



(Top row: GFP, DAPI, ACTIN. Bottom Row GFP only)

B

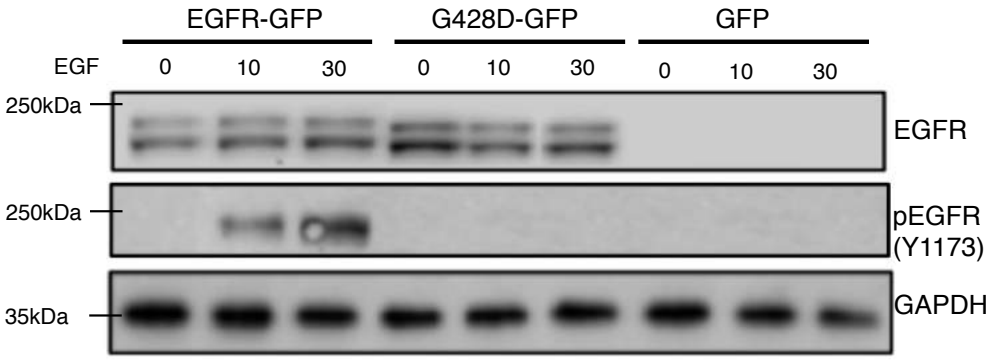


Figure 3.1: G428D EGFR in CHO cells shows loss of plasma membrane localisation and cannot be activated by EGF (A) Confocal images of CHO cells expressing GFP, WT-EGFR-GFP or G428D-GFP (green) stained for F-actin (red) and DAPI (blue). Arrows on bottom panels highlight EGFR plasma membrane localisation. Images representative of 3 independent experiments. (B) Western blots showing EGFR and pEGFR levels in CHO cells after stimulation with 10ng/ml of EGF for 0, 10 and 30 minutes. Representative of 3 experiments.

3.2.2 G428D EGFR expression in EGFR null keratinocytes shows loss of plasma membrane localisation and cannot be activated by EGF

Having demonstrated that G428D EGFR resulted in an inactive receptor in CHO cells, the next goal was to determine whether this was also the case in keratinocytes depleted of endogenous EGFR. EGFR-2 cells were infected with the EGFR-GFP, G428D-GFP and GFP lentiviruses. Two weeks post infection, cell lines were again infected with lentivirus generated to include a Cre-recombinase (CRE) construct to knock out the endogenous EGFR gene that, in EGFR-2 cells, is sited between two LoxP sites allowing for removal. Before infecting with the CRE virus, the cells were grown in low calcium growth media supplemented with KGF in lieu of EGF as is normal for keratinocyte *in vitro* culture. This allowed for growth and proliferation after the removal of endogenous EGFR.

Infection with CRE virus, followed by two weeks of growth, led to the generation of 4 distinct EGFR-2 cell lines: **GFP** expressing GFP and endogenous EGFR; **EGFR-GFP** expressing WT-EGFR-GFP with endogenous EGFR removed, **G428D-GFP**, expressing G428D EGFR with endogenous EGFR removed and **KO-GFP** expressing GFP only with endogenous EGFR removed.. Confocal imaging of fixed cells stained for F-actin (phalloidin) and DAPI showed similar localisation of WT-EGFR as in CHO cells, and G428D-GFP was predominantly cytoplasmic (Fig 3.2A). Western blotting was also used to assess the ability of the EGFR-GFP variants to respond EGF stimulation and as seen with the CHO cell lines, only the EGFR-GFP cells showed EGFR phosphorylation following EGF stimulation (Fig 3.2B). This blot also demonstrated very near complete loss of EGFR in the KO-GFP cells, confirming successful removal of endogenous receptor.

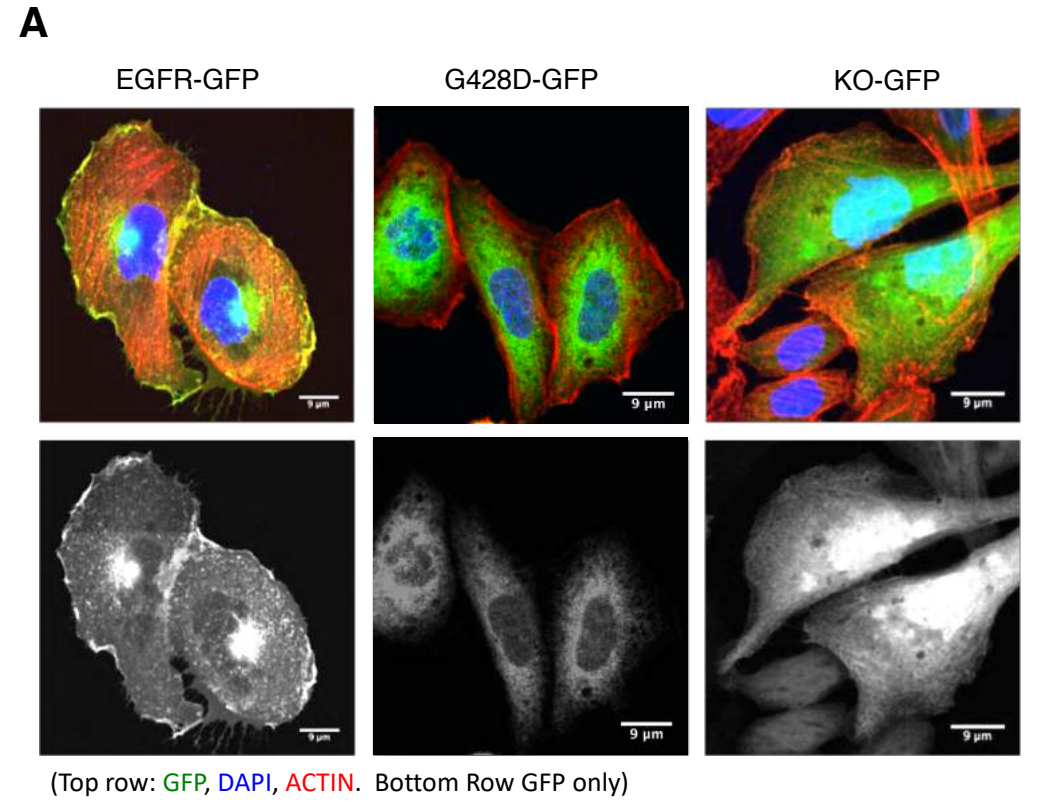


Figure 3.2: G428D EGFR expression in EGFR null keratinocytes shows loss of plasma membrane localisation and cannot be activated by EGF (A) Confocal images of EGFR-2 cells expressing WT-EGFR-GFP, G428D-GFP and GFP after knockout of endogenous EGFR. Cells were stained for F-actin (red) and DAPI (blue). Arrows highlight plasma membrane localization of WT-EGFR-GFP. Images representative of 3 experiments. (B) Western blots of EGFR and pEGFR expression in EGFR-2 cells after knockout of endogenous EGFR by CRE-recombinase and stimulation with 10ng/ml of EGF for 0, 10 and 30 minutes. Representative

3.2.3 EGFR knockout or re-expression of G428D EGFR results in reduced keratinocyte proliferation which can be partially rescued by growth in KGF rich media.

Under normal culture conditions, mouse keratinocytes are grown in growth media supplemented with EGF. This is crucial for the normal growth and survival of the cells and indicates the importance of the EGFR in these processes. The EGFR-2 cell lines generated in this chapter are grown in media supplemented with KGF instead of EGF to retain cell growth in culture. In order to confirm that loss of EGFR has a detrimental effect on cell growth, equal numbers of GFP, EGFR-GFP, G428D-GFP and KO-GFP cells were cultured in growth media with or without 2mM KGF and were then fixed at 1hr, 24hrs and 48hrs post-plating follow by staining with DAPI. Images were then captured for the entire well using tile scans and total cell numbers calculated. Data demonstrated that G428D-GFP and KO-GFP lines showed significantly lower growth rates that GFP and EGFR-GFP cell lines (Figures 3.3A,B). The data also indicated that G428D-GFP and KO-GFP cells grown in KGF rich media proliferated more than with either EGF or DMSO, and that this increase was significant in the case of the G428D-GFP line. To confirm these findings in an alternative model system, the same analysis was also performed on keratinocytes isolated from WT or EGFR-/- mice (termed WT and KO from hereon in). The KO cells proliferated less than the WT cells under all conditions except the DMSO condition (Figures 3.3C,D). However, as with the EGFR-2 cell lines, the addition of KGF significantly increased proliferation of KO cells compared to when treated with EGF or DMSO (Figure 3.3D).

To determine whether the apparent reduced proliferation in the absence of EGFR was also due to enhanced apoptosis, cells were incubated with CellEvent Caspase 3/7 Green detection reagent. This cell permeable reagent is intrinsically non-fluorescent, however in the presence of activated caspase-3 or caspase-7, the reagent is able to bind to DNA and produces green fluorescence upon illumination with 488nm light. Due to the fact that the assay relies on the production green fluorescence, only the WT and KO cell lines could be used as the EGFR-2 cell lines already expressed GFP tagged constructs.

Cells were grown in KGF+, KGF-, and KGF-EGF+ growth media in the presence of CellEvent and fixed at 24h, followed by analysis of green fluorescence using fluorescence microscopy. Data demonstrated that similar levels of caspase-positive cells were seen in both WT and KO lines in KGF+ media, and whilst overall levels of caspase-positive cells increased in KGF- growth media, no significant differences were observed between WT and KO cells (Figures 3.3D,E). However, EGFR KO cells showed a higher level of apoptosis compared to WT cells when cultured in KGF-EGF+ media (Figure 3.3F). This data demonstrates that loss of EGFR leads to lower proliferation and increased apoptosis in cells cultured in standard keratinocyte growth media.

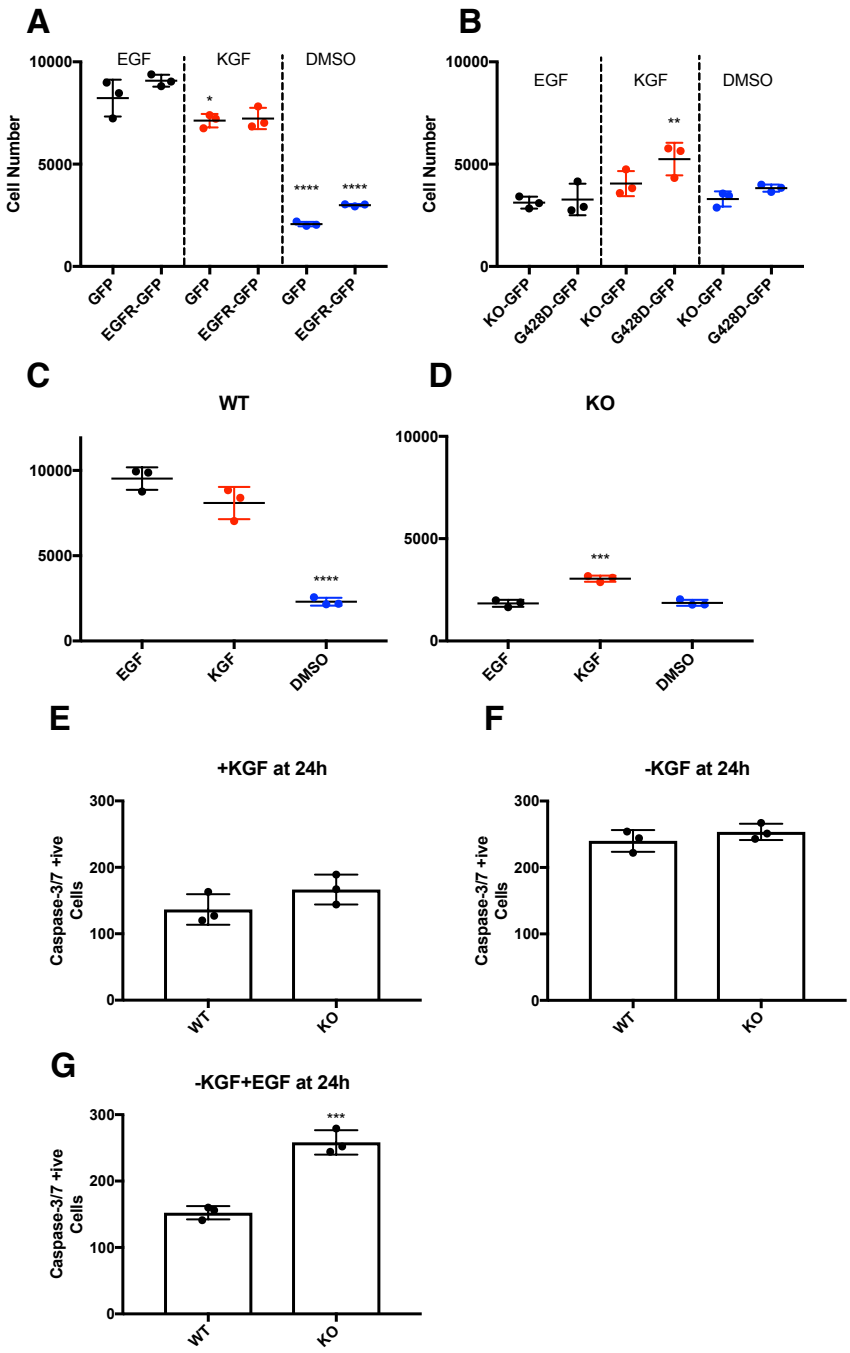


Figure 3.3: KGF increases proliferation after EGFR knockout or re-expression of G428D mutant EGFR (A,B,C,D) Graphs showing proliferation at 48 hours in EGFR-2 cells expressing WT-EGFR-GFP, G428D-GFP and GFP after knockout of endogenous EGFR or of WT or EGFR KO keratinocytes, in +EGF, +KGF and DMSO containing growth media. (E,F,G) Graphs showing the number of caspase-3/7 positive WT or EGFR KO cells after 24 hours grown in +KGF, -KGF and –KGF+EGF containing growth media. For all graphs n=3 and data points represent the mean of duplicate technical repeats. Means +/- SEM are shown. Statistics performed using unpaired t-test. * P<0.01, ** P<0.001, *** P<0.0001, ****P<0.00001

3.2.4 EGFR knockout or re-expression of G428D EGFR does not alter assembly of E-cadherin or β-catenin positive cell-cell adhesions

The next aim was to assess the impact of and the loss of EGFR or G428D mutation on the formation of keratinocyte monolayers and cell-to-cell adherens junctions. To analyse this, EGFR-2 cell lines were grown into confluent monolayers in normal growth media in basal conditions. Once confluent, growth media was removed and replaced with growth media containing 2mM calcium for 2hrs in order to enable assembly of intact cadherin-positive junctions. The cells were then fixed and stained for either E-cadherin or β-catenin, F-actin (phalloidin) and DAPI and analysed by confocal microscopy.

Resulting confocal images demonstrated that E-cadherin localised in the junctional regions between cells with clear cortical F-actin cables assembled in all cell lines with no visible differences in staining pattern observed (Figure 3.4). A similar localisation pattern was also seen for β-catenin, and this was also unchanged across the different cell lines (Figure 3.5).

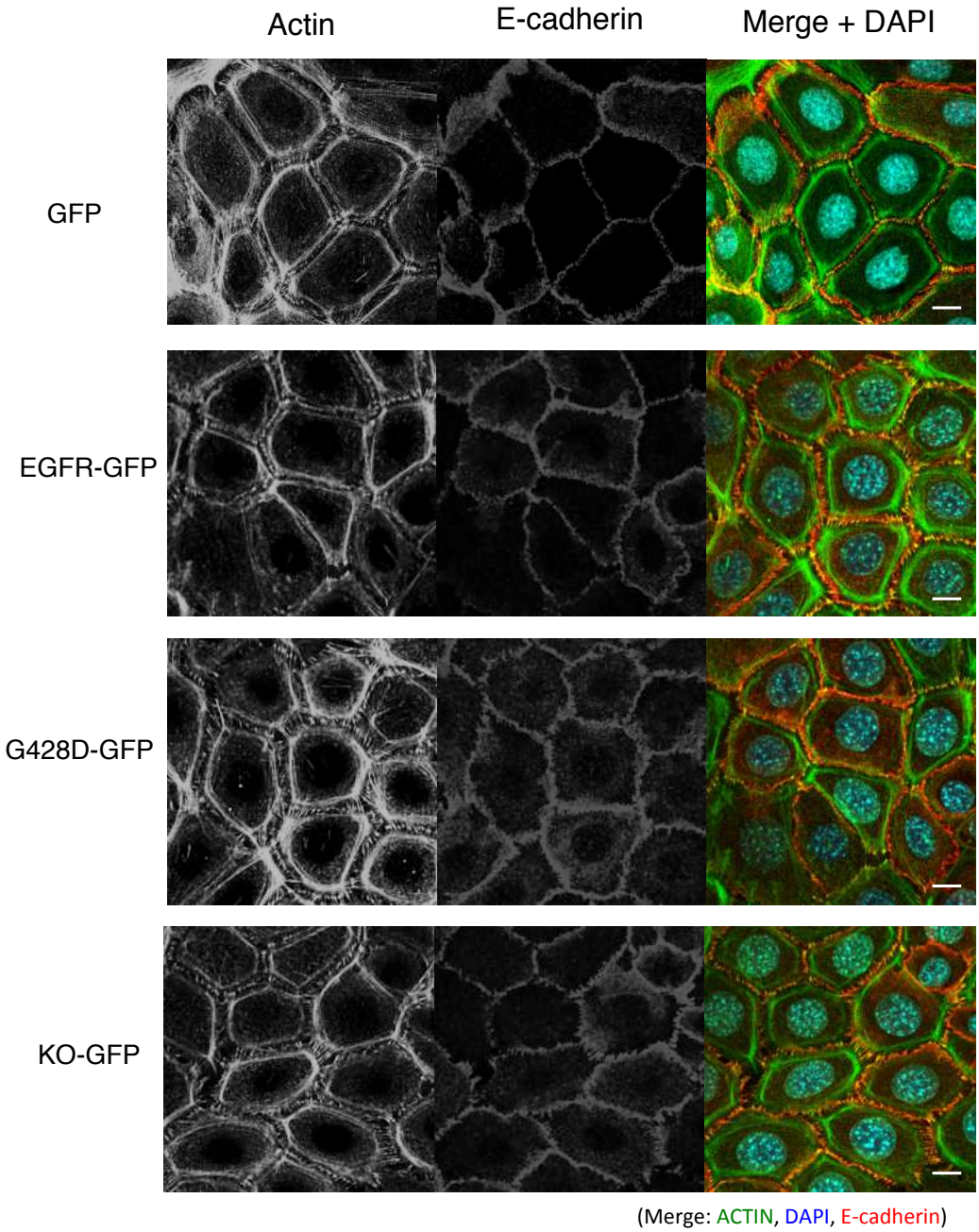


Figure 3.4: EGFR knockout or re-expression of G428D EGFR does not alter assembly of E-cadherin positive cell-cell adhesions Confocal images of EGFR-2 cell line monolayers cultured in basal conditions after addition of 2mM calcium for 2h, fixed and stained for actin (green), E-cadherin (red) and DAPI (blue). Cell lines are labelled to the left. Images are representative of 3 separate experiments and 6 frames per experiment. Scale bars are 8um.

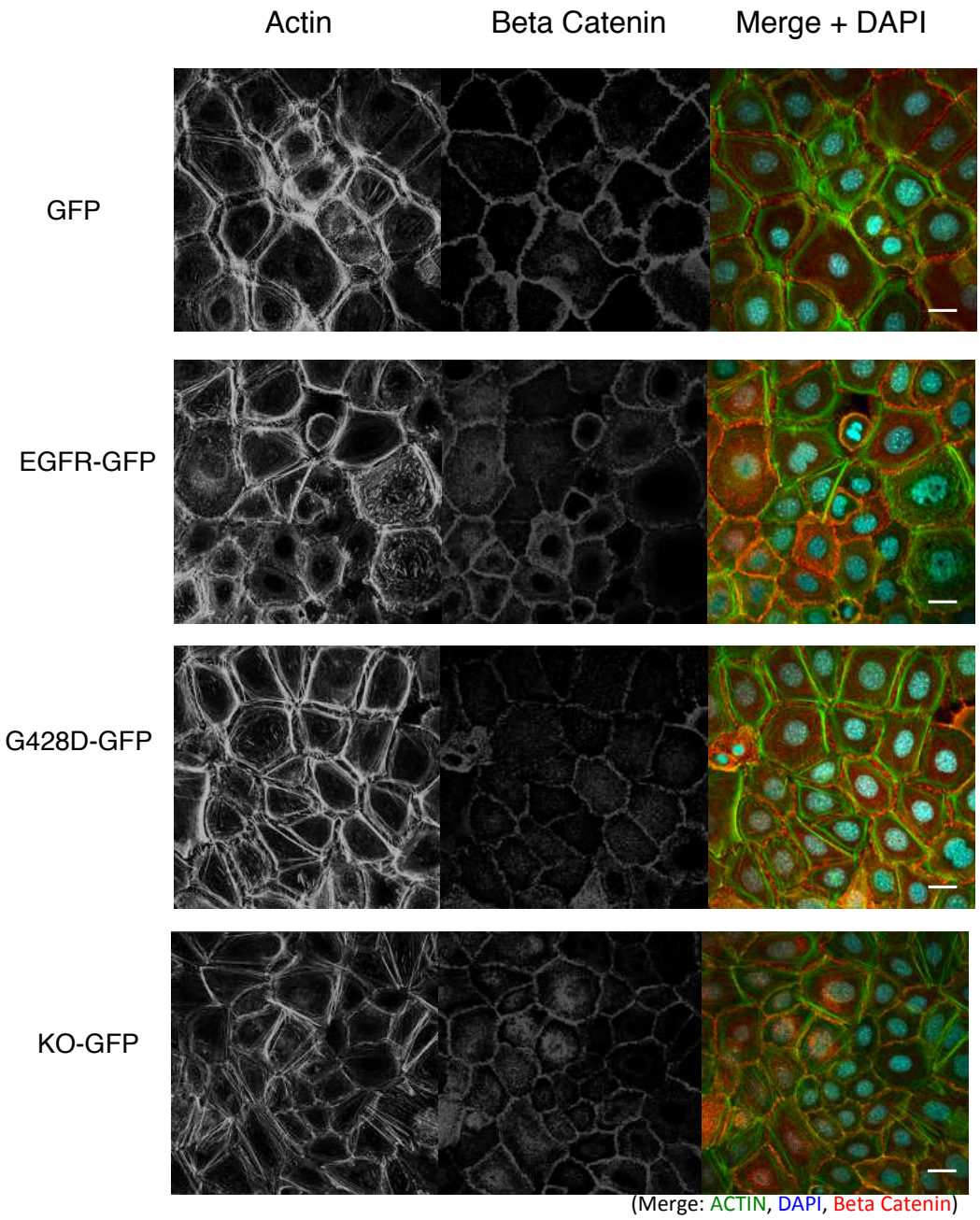


Figure 3.5: EGFR knockout or re-expression of G428D EGFR does not alter assembly of β-catenin positive cell-cell adhesions Confocal images of EGFR-2 cell line monolayers cultured in basal conditions after addition of 2mM calcium for 2h fixed and stained for actin (green), β-catenin (red) and DAPI (blue). Cell lines are labelled to the left. Images are representative of 3 separate experiments and 6 frames per experiment. Scale bars are 12um.

3.2.5 EGFR knockout or re-expression of G428D EGFR does not alter keratinocyte monolayer permeability

Whilst immunostaining did not reveal any clear changes to cell-cell adhesion assembly, a dextran assay was used to determine whether any differences in permeability of the monolayers, as a readout of barrier formation, could be detected.

EGFR-2, KO and WT cells were grown into monolayers on 0.4μm pore transwell membranes under basal conditions. Once monolayers were formed, cells were treated with 2mM calcium containing growth media with or without 5mM EDTA. EDTA is a chelating agent that reduces the effectiveness of calcium to induce the formation of strong cell-to-cell junctions and therefore acted as a positive inducer of permeability and a control for these experiments. TRITC Dextran (20kDa) was then added to the media on top of the monolayers and left for 2hrs. At this time, media from below the transwells was removed and TRITC fluorescence intensity was measured on a fluorescence plate reader.

EGFR-2 monolayers treated with calcium alone showed significantly less dextran passage than monolayers treated with calcium and EDTA (Figure 3.6A). The same was also seen in WT and KO cells (Figure 3.6B). There was no observable difference in monolayer permeability between EGFR-2 cell lines treated with calcium alone (Figure 3.6A, right hand bars) There was also no difference in permeability between WT and KO cells treated with calcium alone (Figure 3.6B, right hand bars). Taken together, these data suggest that EGFR dysregulation has no impact on the strength of cell-to-cell junctions or responsiveness to Calcium and no effect on the permeability of keratinocyte monolayers.

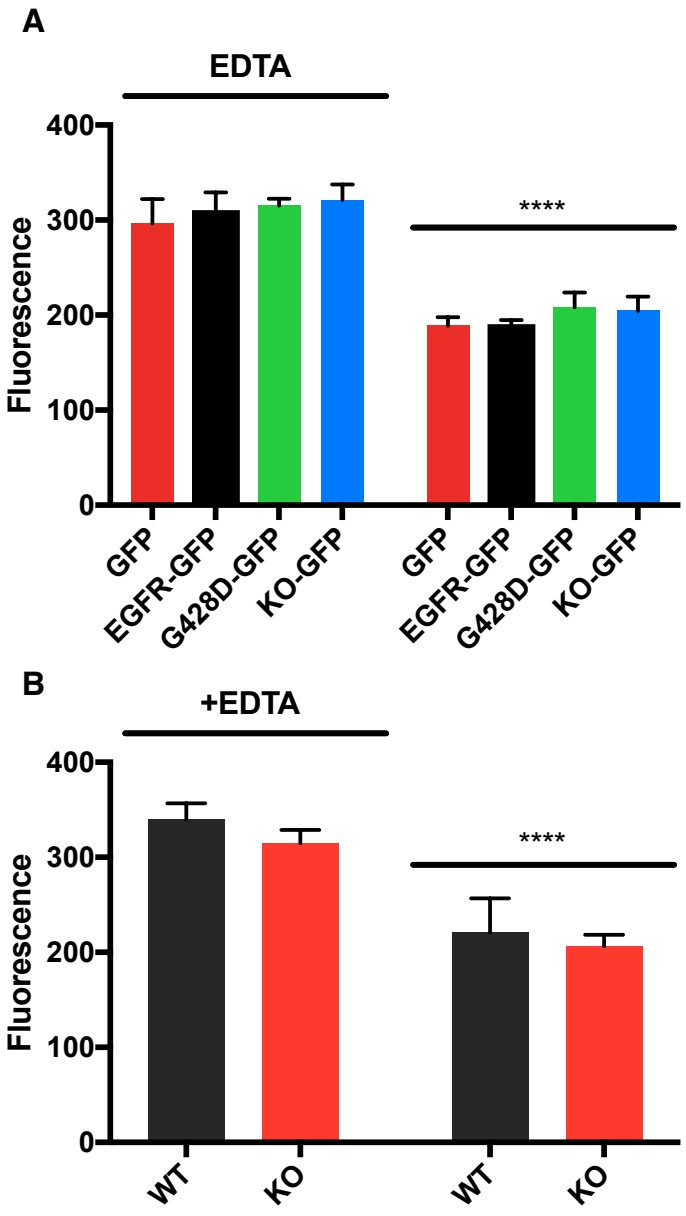


Figure 3.6: EGFR knockout or re-expression of G428D EGFR does not alter keratinocyte monolayer permeability (A) Graph showing fluorescence intensity of media obtained from dextran permeability assay performed on EGFR-2 cell lines. Cells were cultured in basal conditions with 2mM calcium for the duration of the assay, with or without 5mM EDTA as a positive control. (B) Permeability of WT and EGFR KO mouse keratinocytes. Cells were cultured in basal conditions with 2mM calcium for the duration of the assay, with or without 5mM EDTA as a positive control. All data pooled from 3 independent experiments with 2 sample replicates per experiment. Data shown is pooled from all experiments and shows mean +/-SEM. Statistics measured by 2way ANOVA, **** P <0.0001. Statistics show comparison between EDTA+ calcium and calcium alone for all cell lines

3.2.6 EGFR knockout or re-expression of G428D EGFR leads to assembly of larger focal adhesions

As one of the phenotypes of patients with the G428D mutant EGFR was epithelial fragility, this suggested that EGFR may play a role in cell-matrix adhesion. To analyse this, EGFR-2 cells were grown into monolayers under basal conditions and treated with 2mM calcium for 2hrs, then fixed and stained for the focal adhesion protein vinculin, as well as F-actin (phalloidin) and DAPI, followed by imaging by confocal microscopy.

Resulting images demonstrate that all EGFR-2 cell lines were able to assemble focal adhesions (Figure 3.7). However, the focal adhesions in G428D-GFP and KO-GFP cells appeared to be larger than those seen in GFP and EGFR-GFP cells (Figure 3.7). To analyse this in more detail, images were quantified using FIJI and resulting data demonstrated that G428D-GFP expressing cells assembled significantly fewer focal adhesions/cell than other cells analysed (Figure 3.8A). Moreover, both G428D-EGFR and KO-GFP cells had larger adhesions than both GFP and EGFR-GFP cells (Figure 3.8B). However, despite the changes in number and size of the focal adhesions, there was no significant change in the number of focal adhesions when corrected for cell area, due to the smaller size of the G428D-EGFR and KO-GFP cells (Figure 3.8 C). This data demonstrates that dysregulation of EGFR has an effect on the formation of focal adhesions in keratinocyte monolayers.

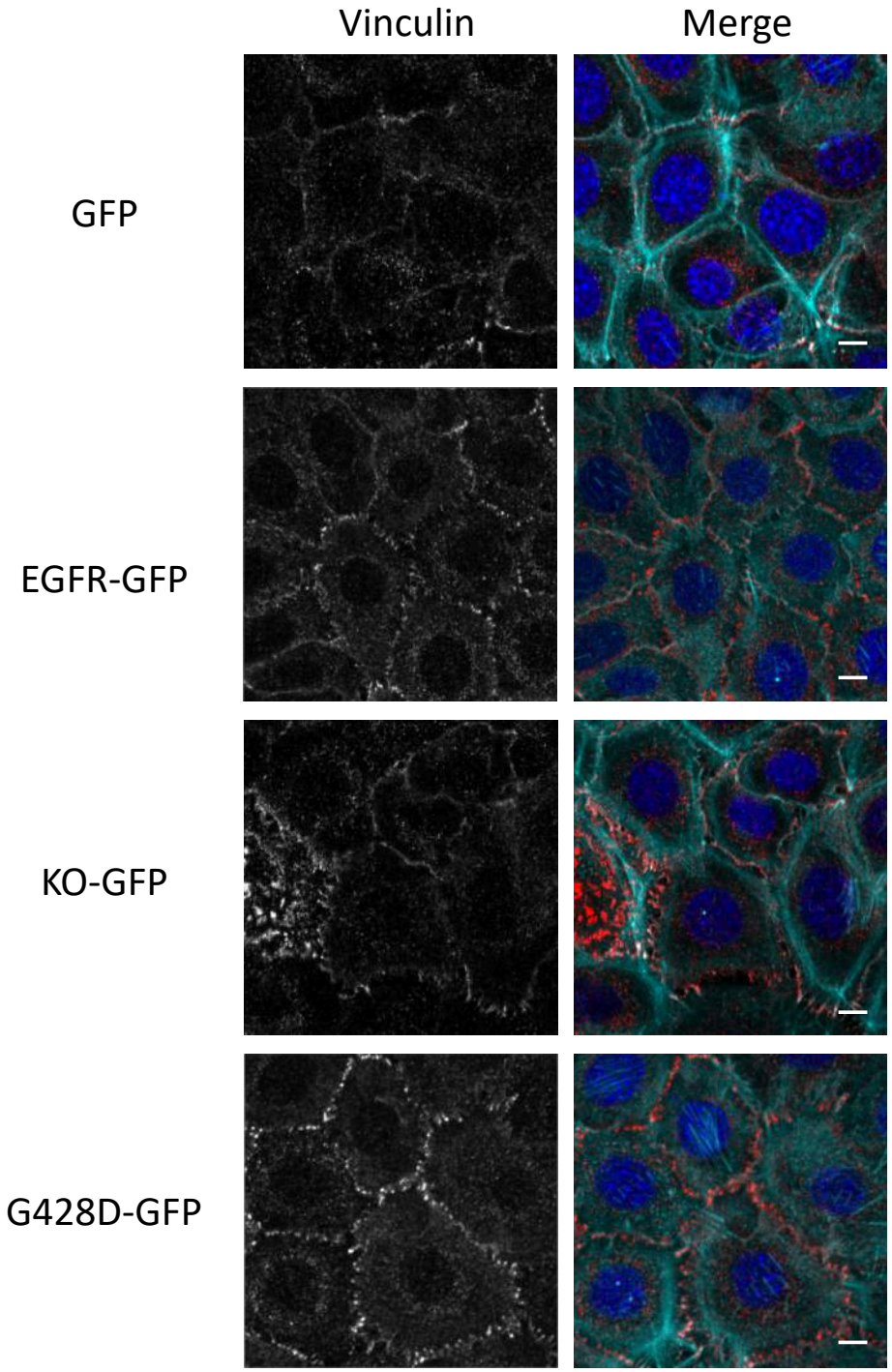


Figure 3.7: EGFR knockout or re-expression of G428D EGFR leads to assembly of larger focal adhesions EGFR-2 cell monolayers were grown in basal conditions followed by the addition of 2mM calcium for 2hrs before fixing and staining for vinculin (red), F-actin (cyan) and DAPI (blue). Images are representative of three independent experiments. Scale bars are 8um

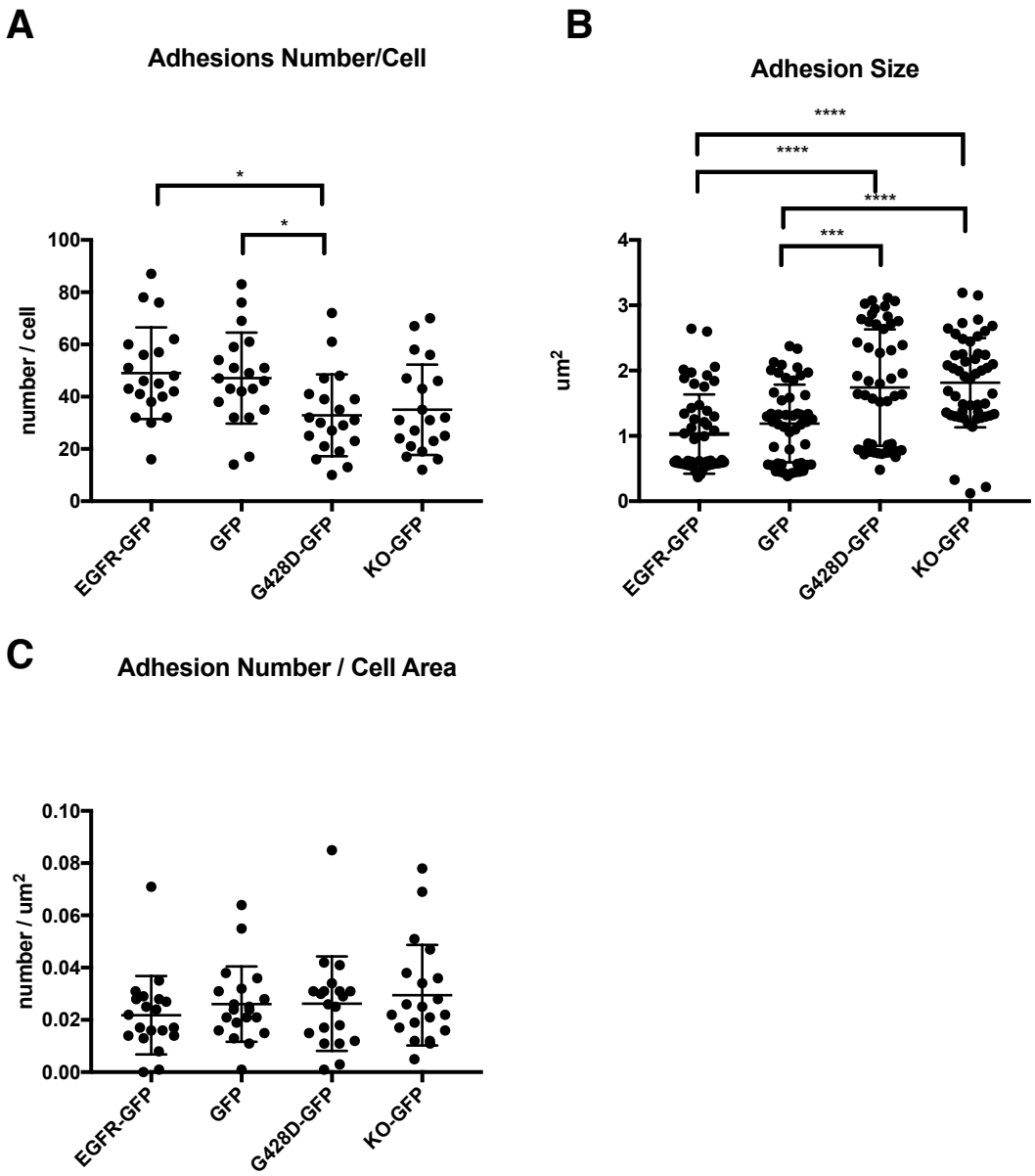


Figure 3.8: EGFR knockout or re-expression of G428D EGFR leads to assembly of larger focal adhesions. Cell monolayers were grown in basal conditions followed by the addition of 2mM calcium for 2hrs before fixing and staining for vinculin ad F-actin. Focal adhesions were measured using vinculin fluorescence. (A) Number of adhesions per cell, with cell boundary identified by actin staining. (B) Adhesion size in um^2 (C) Number of adhesions per um^2 within cell area. Calculated for 50 randomly selected cells per cell line, over 3 independent experiments. One representative experiment is shown, also showing means and SEM. n=3 Statistics measured by 2way ANOVA. For **** $P < 0.0001$ *** $P < 0.001$ * $P > 0.05$

3.2.7 EGFR knockout or re-expression of G428D EGFR has no significant effect on collective migration speeds

Given that EGFR was shown to play a role in the formation of focal adhesions, for a potential role for EGFR in collective cell migration was assessed by wound healing assays. EGFR-2, WT and KO cells were grown into monolayers on wells coated with collagen. Monolayers were then scratched using a pipette tip in the presence of 2mM calcium with or without EGFR inhibitor AG1478, and imaged using phase-contrast microscopy after 0.5, 12 and 24 hours. The sizes of the remaining 'wounds' were then compared at each time point. Figure 3.9A shows an example of the images acquired.

Resulting quantification demonstrated that there was no significant difference in wound closure between cell lines although a trend towards lower rates of migration were seen in G428D-GFP and KO-GFP cells compared to GFP and EGFR-GFP cells (Figure 3.9B). This trend was also seen between WT and KO cells (Fig 3.9C). The graphs also suggested that there is a reduction in wound closure in all cell lines when treated with AG1478, although again no significant differences were seen. This data suggests that EGFR dysregulation has little if any effect on collective keratinocyte migration in 2D.

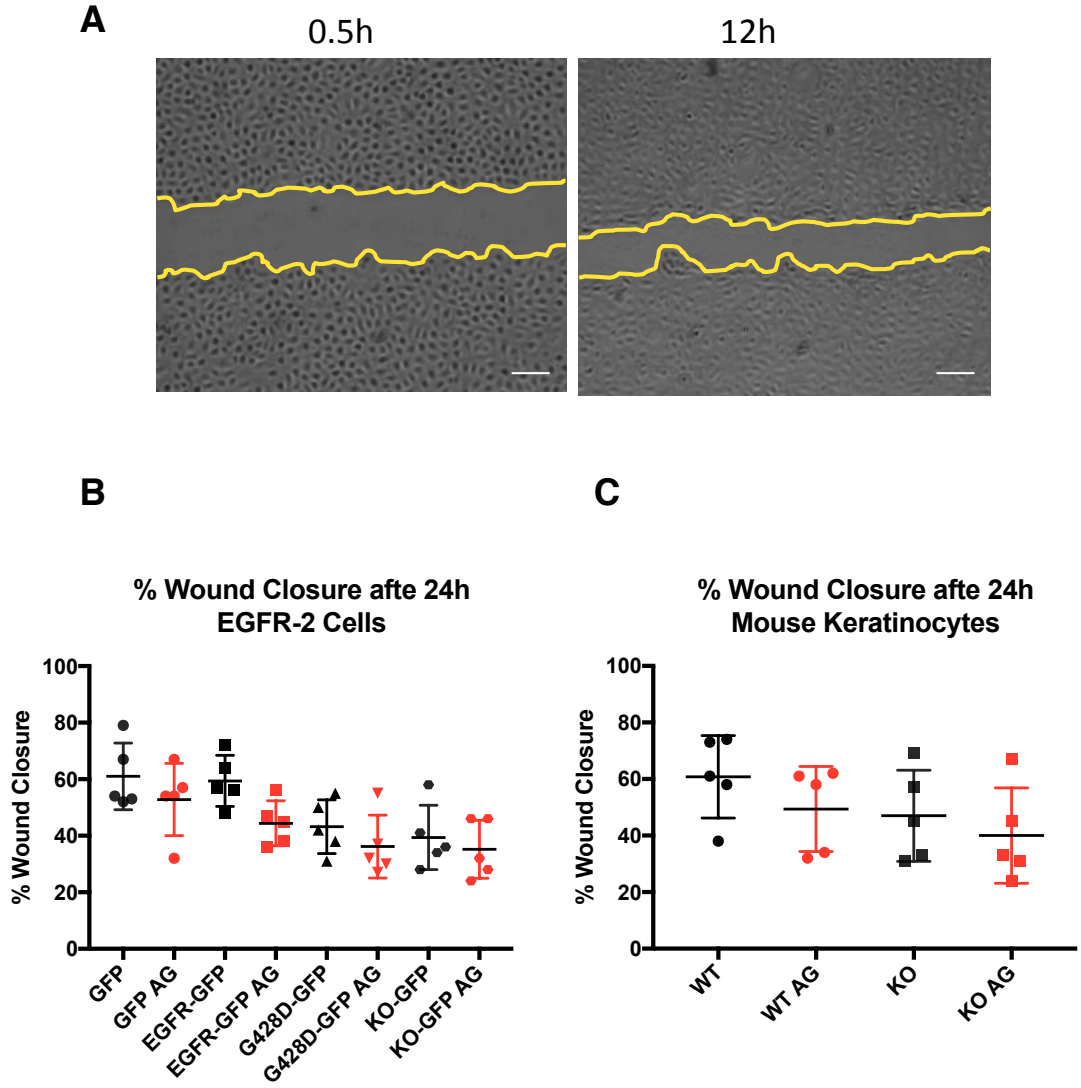


Figure 3.9: EGFR knockout or re-expression of G428D EGFR has little effect on 2D wound closure

(A) Representative images of scratch wound healing assays performed on EGFR-2 and WT/EGFR KO keratinocytes. Cells were grown to monolayers in 24 well plates in basal conditions. Wound border shown in yellow. Wounds were imaged after 0.5 and 24 hours. Scale bars are 185um. (B) Graph showing % wound closure at 24h in EGFR-2 cells with or without addition of EGFR inhibitor AG1478 (red) (C) Graph showing % wound closure in WT vs EGFR KO keratinocytes at 24h with or without addition of EGFR inhibitor AG1478 (red). Each data point shown is from one experiment, and 5 independent experiments were performed. Lines denote mean. Statistical analysis was performed by one-way ANOVA and multiple comparison tests with no significance reported.

3.2.8 EGFR knockout cells are sensitised to undergo apoptosis in response to UVB radiation.

As the skin, and keratinocytes, are constantly being challenged by numerous sources of stress, the potential role of EGFR in regulating keratinocyte responses to UV irradiation were assessed. To do so, WT and KO keratinocytes were irradiated with UVB at 10 and 20 mJ/cm^2 and apoptosis was measured using the CellEvent reporter as in Figures 3.3 D-F.

Data demonstrates that 4hrs after UVB irradiation, an increase in WT cell apoptosis was seen at both radiation levels compared to basal conditions, with a trend towards higher apoptosis in 20 mJ/cm^2 UV treated cells (Figure 3.10A). This response was also seen in KO cells, however similar levels of apoptosis were detected at both 10mJ and 20mJ/cm^2 (Fig 3.10 B). This suggests that knockout of EGFR can potentially sensitise keratinocytes to undergo apoptosis in response to UVB radiation.

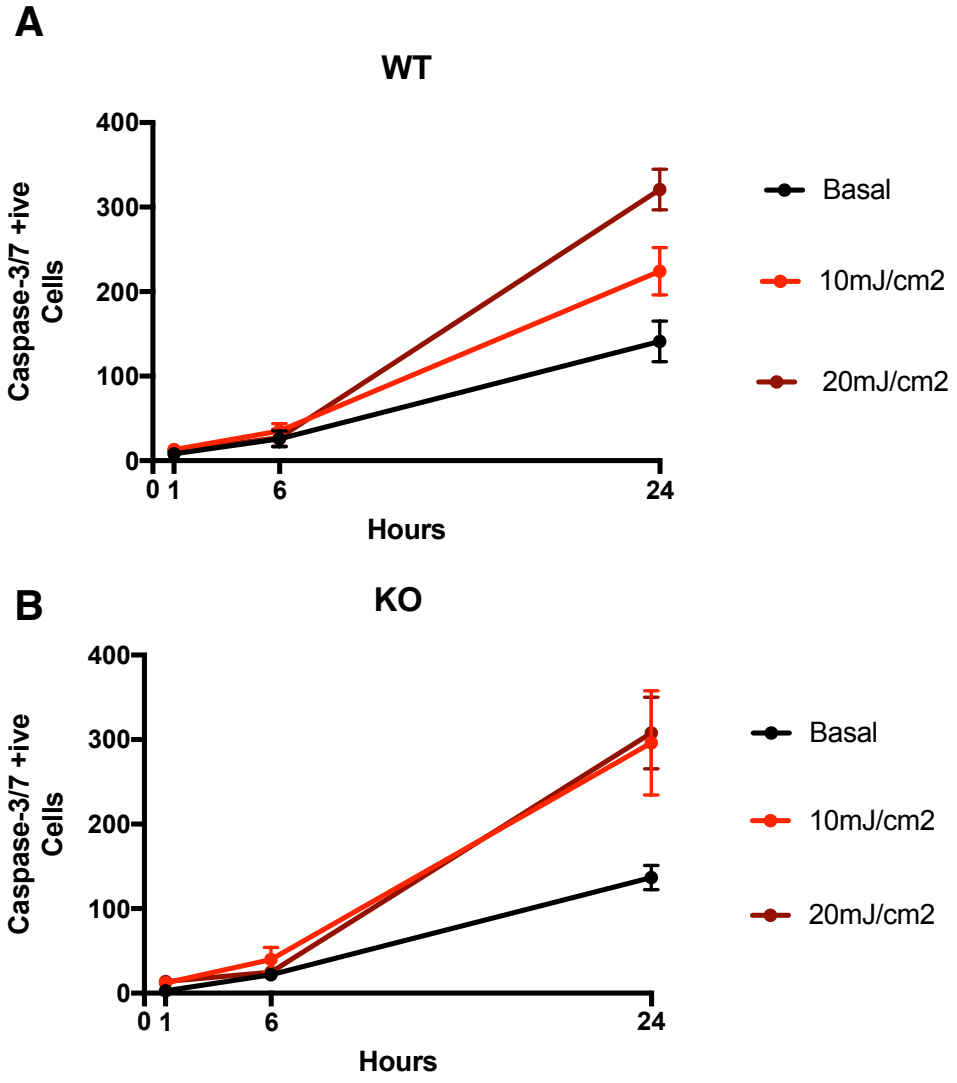


Figure 3.10: EGFR knockout cells are sensitised to undergo apoptosis in response to UVB

Graphs of caspase-3/7 positive mouse keratinocytes monolayers under basal conditions after UVB irradiation at basal, 10 or 20mJ/cm2 for (A) WT and (B) KO cells. Caspase-3/7 was measured at 1, 6 and 24 hours after irradiation. Pooled from 3 independent experiments and the mean of duplicate technical repeats. Data shows means +/-SEM. One-way ANOVA was performed between treatments at 24h with no significance recorded.

3.3 Discussion

This chapter provides the data that characterises an *in vitro* cellular model with which to study the effects of EGFR knockout or G428D mutant re-expression in mouse keratinocytes. To this point, most studies of EGFR loss-of-function have been done by using EGFR inhibitory drugs as well as useful but limited conditional *in vivo* models. The data demonstrates that by supplementing keratinocytes with KGF, it is possible to culture EGFR knockout or G428D mutant re-expression cells *in vitro* with little observable alterations in 2D monolayer structure. The data also indicates that the G428D mutant has similar functional and localisation properties as previously shown *in vitro* and from patient biopsies (Campbell & Morton 2014). This chapter also indicates at a potential role of EGFR in regulating response to stress induced by UVB radiation.

3.3.1 G428D cellular phenotype is consistent across cell lines and shows cytoplasmic/perinuclear localisation.

The data in Figure 3.1 further validates previous findings (Campbell et al. 2014) that the G428D mutant fails to effectively localise the cell surface as is the case for both endogenous and WT construct EGFR. Importantly, Figure 3.1A demonstrates this in CHO cells that do not express any endogenous EGFR. The data in this chapter also shows that this loss of membrane localisation is consistent in EGFR-2 mouse keratinocytes (Figure 3.2A). As well as localisation defects, the G428D mutant receptor also fails to respond to EGF treatment as assessed by EGFR phosphorylation (Figure 3.1B, 3.2B). Again, this is consistent with previously published data on the mutation (Campbell et al. 2014; Ganetzky et al. 2015). As EGFR signalling is crucial for many processes in keratinocytes such as proliferation and growth and differentiation (Olayioye 2000), this data suggests that these processes would be compromised in the cell models presented.

The data suggests that the reason the G428D mutant fails to respond to EGF signalling is that there is no receptor on the cell surface able to bind to ligand, driving receptor

dimerisation and phosphorylation. The G428D mutant is defined by a single point mutation in the junctional region of the EGFR protein monomer, and this chapter holds no information on the mechanism by which this mutation confers an inability for the protein to properly express at the cell surface. However, unpublished work from our lab has suggested that this mutation plays a role in protein folding, glycosylation and trafficking. A key finding is that the G428D mutant EGFR monomer displays a reduced ability to homo-dimerise and hetero-dimerise with other HER family receptors. Additionally, there is a high level of co-localisation between the G428D mutant and proteins of the endoplasmic reticulum, suggesting an inability for the receptor to properly traffic within the cell. These findings were made using siRNA knockdown of endogenous EGFR in normal, human keratinocytes, NHKs.

3.3.2 EGFR knockout or G428D mutant re-expression reduces proliferation in keratinocytes and this can be partially rescued by incubating cells in KGF rich media.

The demonstrated loss of EGFR signalling caused by EGFR knockout or G428D mutant re-expression presents a difficulty in the long-term culture and study of cells with these characteristics. EGFR-2 cells are suggested to be grown in KGF rich media in lieu of EGF when knocking out endogenous EGFR by Cre-recombinase (Hammiller et al. 2015). In order to properly characterise the cellular models generated in this chapter, it was important to assess the effects of KGF on cell proliferation and apoptosis.

The data presented indicates that cells expressing either endogenous or WT re-expression EGFR fail to properly proliferate in the absence of either EGF or KGF but are able to proliferate in the presence of either of the growth factors (Figure 3.3A, Figure 3.3C). This corroborates other literature demonstrating that KGF plays a role in keratinocyte growth and differentiation (Yang, Fu, and Li 2002; Marchese et al. 1990). As expected, all cell lines with EGFR knockout or G428D mutant re-expression proliferate less than WT EGFR expressing cells Figure 3.3B, 3.3D). However, when grown in KGF rich media, WT EGFR lacking cells were able to proliferate only slightly more than without KGF. Although the increase in proliferation is small, it is sufficient to keep the cells alive and proliferative in culture although they grow significantly

slower than cells expressing WT EGFR even when both are grown in KGF rich media in the absence of EGF. A reason for this may be due to the role of autocrine EGFR signalling in the WT expressing cell lines. Keratinocytes express EGFR ligands such as EGF tethered to the cell surface which are shed in response to ADAM family protease activity (Sahin et al. 2004). In cells expressing WT EGFR, these keratinocyte derived EGFR ligands could easily bind to EGFR and drive downstream processes whereas this would not happen in EGFR knockout or G428D mutant re-expression cell lines. As well as partially restoring cell proliferation in the absence of EGFR signalling, this chapter has also demonstrated that KGF can protect keratinocytes from increased apoptosis (Figure 3.3E, 3.3F, 3.3G). This has been previously demonstrated both under basal conditions and in response to stress and toxins (Braun et al. 2006).

3.3.3 EGFR dysregulation effects barrier function via cell-ECM interactions although cell-to-cell barrier function appears unaffected.

Having generated EGFR knockout and G428D mutant re-expression cell lines and demonstrated that they are viable *in vitro* if cultured in the presence of KGF in lieu of EGF, the next aim of this chapter was to assess the effect of EGFR dysregulation on basal keratinocyte monolayer formation and structure as barrier deficiency is a hallmark of skin inflammation.

The images in Figure 3.4 and 3.5 show clearly that all EGFR-2 keratinocyte cell lines form 2D monolayers regardless of EGFR status. Furthermore, it appears that there is little variation in cell-cell interactions as assessed by the junctional localisation of the proteins E-cadherin and β-catenin. E-cadherin has been shown to control adherins junctions in the epidermis (Young et al. 2003). β-catenin binds to the intracellular domain of E-cadherin. This interaction is crucial for the coupling of actin cytoskeletons of neighbouring cells in the epidermis (Aberle et al. 1994; Fuchs and Raghavan 2002; Jamora and Fuchs 2002). Interestingly, EGFR signalling has been shown to trigger tyrosine phosphorylation of the E-cadherin/β-catenin complex resulting in adherens junction disruption (Fujita et al. 2002). Other studies have also shown that e-cadherin

mediated adhesions can inhibit EGFR signalling (Qian et al. 2004). Despite this bidirectional regulation between EGFR and e-cadherin, the data presented in this chapter suggests that EGFR knockout or G428D mutant re-expression has little effect on the formation of calcium dependant E-cadherin/β-catenin mediated adherens junctions. As well the cell-cell junction structural similarity, there was also no effect on the barrier functionally as demonstrated by the data in Figure 3.6.

As well as assessing cell-cell interactions, the data presented in this chapter shows that EGFR knockout or G428D mutant re-expression leads to the development of larger focal adhesions (Figure 3.7, Figure 3.8). This suggests that these cells could adhere more to the basement membrane. In addition, focal adhesions and in particular the focal adhesion protein vinculin have been shown to be important in mechano-sensing at the cell-ECM junction (Hayakawa, Tatsumi, and Sokabe 2012). This suggests that EGFR signalling may have a functional relationship with the mechano-sensing dynamics in keratinocytes. In order to fully understand this relationship, it would be useful to study other focal adhesion proteins such as paxillin and talin in respect to EGFR dysregulation. It would also be of interest to interrogate vinculin/focal adhesion dynamics using fluorescence recovery after photobleaching (FRAP) in response to EGFR dysregulation either by knockout and g428D mutant receptor re-expression or acute EGFR inhibition.

Despite the appearance of larger focal adhesions in EGFR knockout and G428D mutant re-expression cells, there was no significant difference in collective migration between any cell lines. Vinculin have been demonstrated to play a role in collective cell behaviour when located at focal adhesions but also at the site of cell-cell adhesions (Seddiki et al. 2018). It would be of interest to assess the localisation of vinculin in respect to adherens junctions to see if this was consistent across cell lines.

3.3.4 EGFR may protect keratinocytes from overactive response to stress.

The skin barrier function is essential in protecting the body from external challenges and stresses such as pathogen, mechanical, radiation and toxic stress. It has been shown that EGFR activation is a downstream effect of UVB radiation and that UVB induced overactivation of EGFR can lead to specific skin conditions (Iordanov et al. 2002)(El-Abaseri, Putta, and Hansen 2006). The data in Figure 3.10 demonstrates that EGFR KO keratinocytes become more readily apoptotic in response to UVB radiation than WT keratinocytes. This may be due to the UVB transient activation of EGFR in WT cells driving cell growth and proliferation. This suggests that EGFR may confer a certain level of protection from UVB induced cell death and that under normal conditions, UVB driven EGFR activation is important for barrier epidermal stability under UVB stress. Interestingly, it has been previously published that EGFR expression is positively correlated with NKG2D receptor ligand expression (Vantourout et al. 2014). NKG2D ligands are often expressed in response to stress and their expression regulates immune visibility. This makes it clear that the effect of EGFR dysregulation of the stress response of keratinocytes requires further study despite the findings in this chapter.

In summary, data presented in this chapter has demonstrated that loss of EGFR in basal keratinocytes results in little alteration of the structure and stability of 2D monolayers. Cell-cell interactions appear to be intact and cells are proliferative enough to be grown in culture. This validates the EGFR-2 cell line model as useful for the study of EGFR dysregulation in the epidermis and will thus be used in the rest of this study to assess the effects of EGFR dysregulation on epidermal inflammation.

4. EGFR Knockout and G428D Mutant Re-Expression Drives an Inflammatory Phenotype in Keratinocytes

4.1 Introduction

As previously discussed, microarray performed on DNA obtained from total skin biopsy from a patient harbouring the G428D EGFR mutation had a significant upregulation in a number of inflammatory pathways (Campbell et al. 2014). This result was consistent with the prominent EBS-like inflammatory skin condition displayed by the patient, which was the first case of such a skin condition being linked to a genetic mutation in EGFR. Interestingly there are many similarities between the patient's skin phenotype with the phenotype of those on EGFR inhibitory anti-cancer treatment courses (Ranson 2004). This EGFR inhibition associated skin phenotype is used clinically as an indicator of good prognosis suggesting that the inflammation only occurs when EGFR is significantly inhibited (Herbst and Shin 2002). The severity of the skin inflammation is however much more severe in the G428D mutant harbouring patient. Despite these findings, the relationship between epidermal inflammation and EGFR has been scantily studied. One of the main unanswered questions in this topic is whether or not EGFR has a direct impact on epidermal inflammation via epithelial derived inflammatory mediators, or whether EGFR dysregulation results in epidermal barrier breakdown that in turn drives inflammatory skin conditions.

The impact of EGFR knockout and loss-of-function mutation expression on epidermal inflammation is poorly understood primarily due to the systems available. Having generated EGFR-2 cell lines expressing G428D mutation and EGFR knockout, these cells will act as a system by which to study inflammation under basal conditions in a clean environment *in vitro.* Hallmarks of inflammation in the skin are elevated levels of specific cytokines and chemokines as well as the recruitment of various immune cells to the site of inflammation (Richmond and Harris 2014). These responses are crucial in the skins response to pathogenic microorganisms as well as in wound healing (Cañedo-Dorantes and Cañedo-Ayala 2019).

A characteristic of the inflammation observed during EGFR-I treatment and in the pathology of persons harbouring the G428D mutation is a relatively 'clean' inflammation signature, with no obvious trigger outside of the loss-of-function of

EGFR. For this reason, the aim of this chapter was to assess the impact of EGFR mutation and knockout on inflammatory factors generated by keratinocytes under basal conditions. To do this we first took a genetic approach using RNAseq and QPCR assays, followed by a series of assays to assess the inflammatory factors at the protein level and their bioactivity *in vitro.*

4.2 Results

4.2.1 EGFR knockout or re-expression of G428D EGFR leads to differential expression of pro-inflammatory genes

The microarray data of the original patient harbouring the G428D EGFR mutation was performed using whole skin biopsies. For this reason, the originating cell type or the specific contribution of different cell types, of this altered profile could not be determined. In order to analyse whether G428D mutant re-expression or EGFR knockout leads to altered pro-inflammatory responses in vitro, RNA-sequencing was performed using cDNA generated from RNA of EGFR-2 cell lines GFP, EGFR-GFP, G428D-GFP and KO-GFP that were grown to confluent mono-layers under basal conditions. Each cell line was tested at different 2 passages as an internal control. Paired-end sequencing was then conducted using the HiSeq 2500 platform. Raw data was checked for quality using FASTQC. Processing of the raw data involving alignment (STAR) and annotation (mouse Ensembl) were done using Partek. After annotation, reads per million normalised data were then used for statistical analysis. Inclusion criteria for significantly differentially expressed genes was a false discovery rate of <0.05 and a fold change of greater than 2.0x. Subsequent processing and visualization of the data was completed in RStudio, Morpheus (Broad Institute, Boston, MA) or CytReg.

Principal component analysis (PCA) demonstrated that the main variation in the transcriptional profile was dependent on the expression of functional EGFR, with both passages from GFP and EGFR-GFP cells forming one cluster and G428D-GFP and KO-GFP cells forming another cluster (Figure 4.1A). This clustering was further confirmed

by the lack of variation between GFP and EGFR-GFP cells by volcano plot (Figure 4.1B). A list of genes with a fold increase or fold decrease greater than 5% vs GFP cells is in Appendix Table 1.

The list of differentially expressed genes (DEGs) of both G428D-GFP and KO-GFP cells showed up-regulation of many genes involved in inflammatory pathways. Because of this, the DEGs were compared to human and mouse libraries of genes that are known/likely skin inflammation factors (SkInFs). These SkInFs were derived from mining published literature on skin inflammation. From the total DEGs, 48 common SkInFs were found. Using pathway mapping, of these 48 differentially expressed SkInFs, a number were potential downstream targets of EGFR with the most potent regulators being STAT3 (Figure 4.1C). The most prominent DEGs regulated are shown in Figure 4.1D.

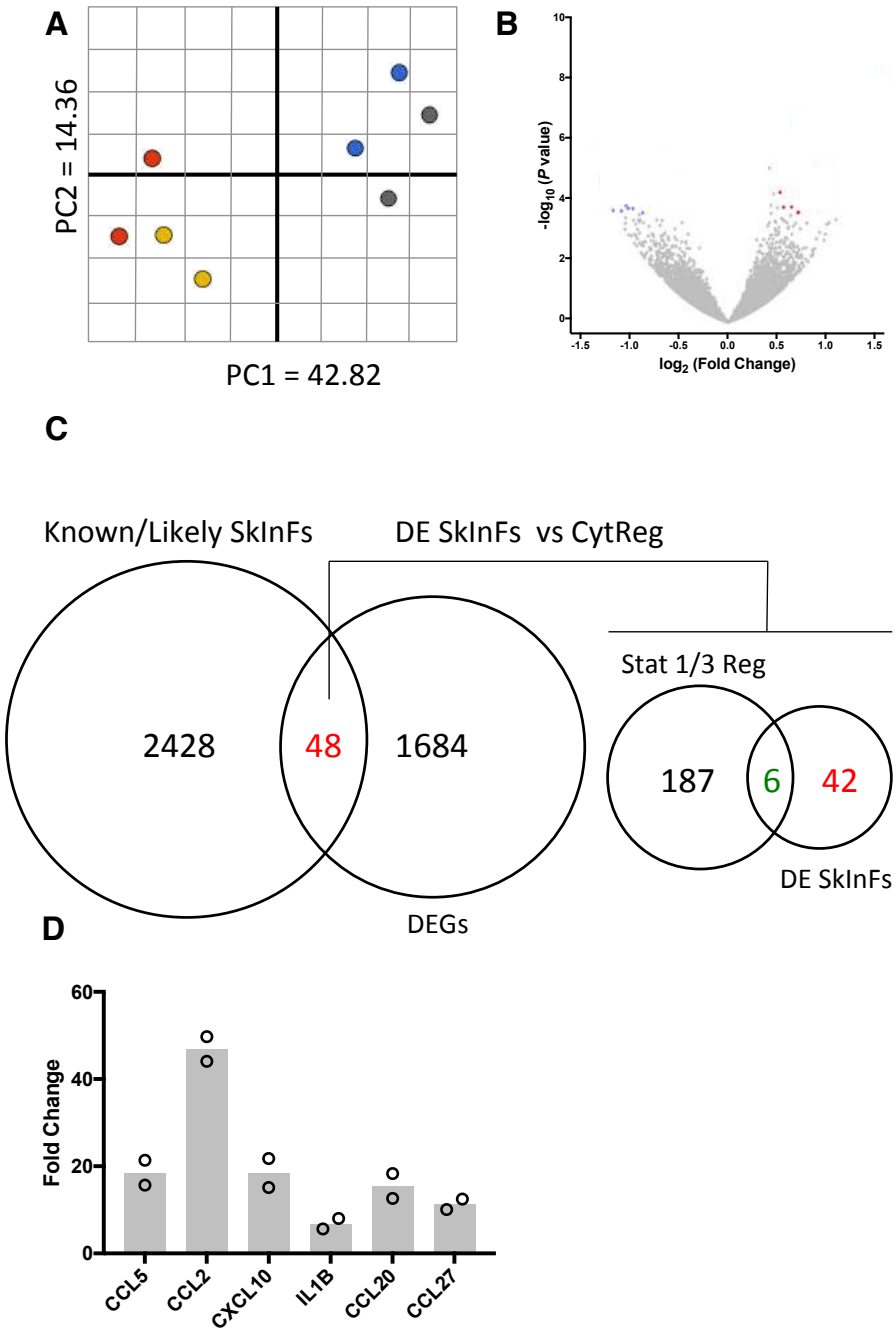


Figure 4.1: EGFR knockout or re-expression of G428D EGFR leads to a differential gene expression phenotype that includes a number of genes involved in epidermal inflammation and regulated downstream of Stat 1/3 (A) Principal component analysis of EGFR-2 cell lines with analysis performed on total normalised counts in an unsupervised manner (n=2 per cell line) (Red = GFP, Yellow = EGFR-GFP, Blue = G428D-GFP, Grey = KO-GFP). (B) Volcano plot showing the differential gene expression of EGFR-GFP cells vs GFP cells. (Red= up-regulated, Blue= down-regulated, Grey = not significant) (C) Workflow for the identification of putative to skin inflammation factors (SkInFs). First, differentially expressed genes were cross referenced with known or likely human and mouse SkInFs, and the subsequently identified differentially expressed transcription factors were cross referenced with previously validated Stat 1/3 mediated genes using CytRef. These genes are shown in (D).

4.2.2 EGFR knockout or re-expression of G428D EGFR leads to upregulation of pro-inflammatory cytokine expression.

After identifying the DEGs involved in skin inflammation, it was then important to validate the RNAseq data using QPCR against a number of chosen target genes. CCL2, CCL5, CXCL10, CCL20 and CCL27 as well as the cytokine IL1β, the matrix metallopeptidases 9 and 10, and ECM proteins col1a1 and col4a6 were chosen for follow-up analysis.

The graphs in Figure 4.2 show the expression of these genes by QPCR in EGFR-2 KO-GFP, EGFR-GFP and G428D-GFP cells, all presented relative to GFP cells. The graphs demonstrate that no significant change in gene expression was detected in EGFR-GFP cells compared to GFP cells, confirming the RNA-seq data. Moreover, all genes analysed by QPCR showed similar significant changes in expression to those seen in RNAseq data.

4.2.3 EGFR knockout or re-expression of G428D EGFR leads to reduced Collagen I and IV expression

After validating target DEGs using QPCR it was then important to investigate whether or not these changes were observable at the protein level. EGFR-2 cells were grown under basal conditions to confluent monolayers before being lysed and lysates analysed by western blotting for Collagen I or IV, and HSC70 as a loading control.

The resulting blots and quantification showed a clear decrease in both collagen IV (Figure 4.3A,B) and Collagen I (Figure 4.4A,B) in both G428D-GFP and KO-GFP cells in comparison to GFP and EGFR-GFP cells. This data confirms that synthesis of these ECM proteins is reduced upon loss of EGFR or re-expression of the G428D EGFR mutant.

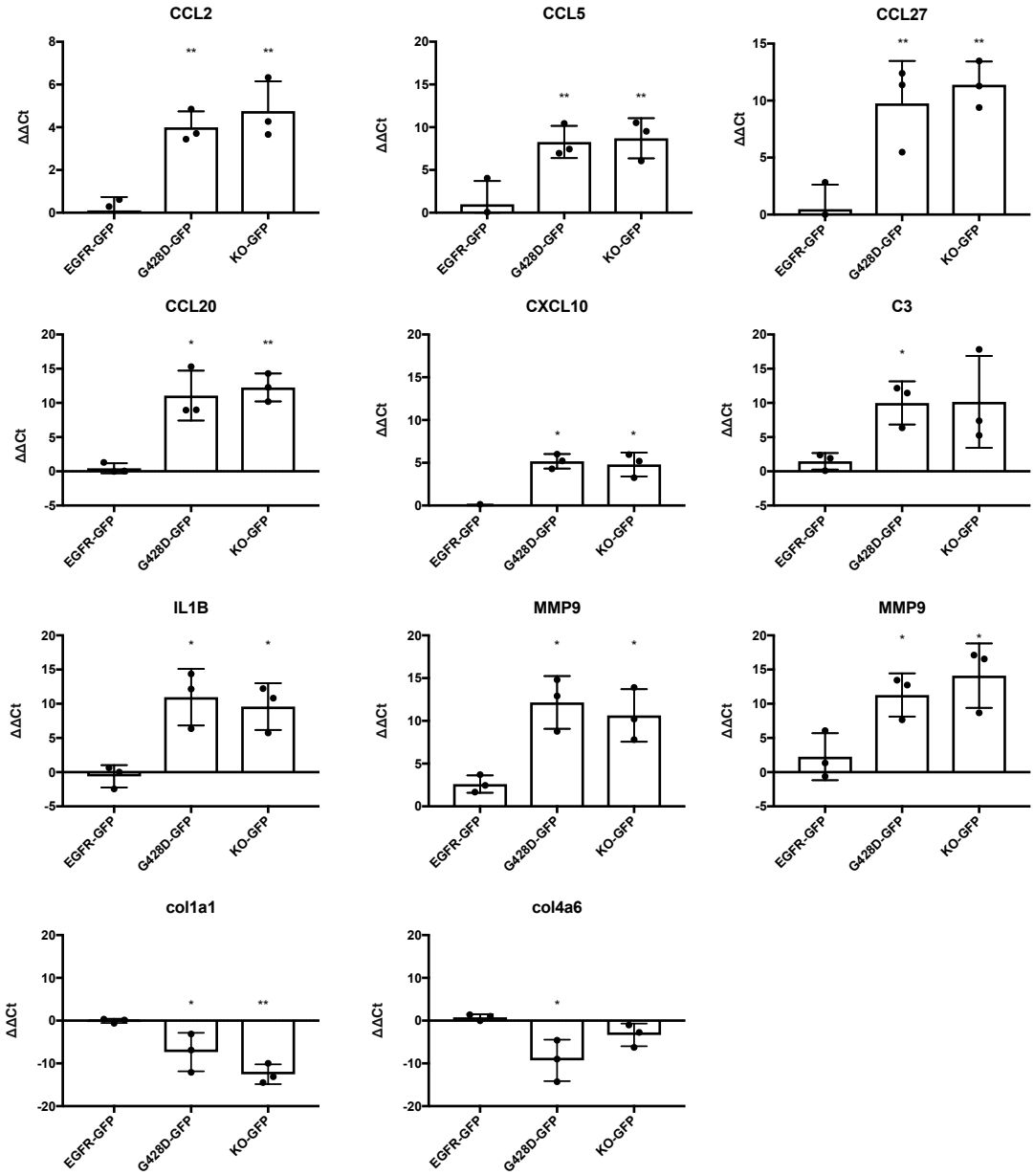


Figure 4.2: EGFR knockout or re-expression of G428D EGFR leads to upregulation of pro-inflammatory cytokine expression Graphs showing qPCR analysis for specified targets in EGFR-2 cell lines. RNA expression ddCt values of specified target proteins was normalised to EGFR-2 GFP expressing cells. n=3 data points represent the mean of triplicate wells per experiment. Data is shown as mean +/- SEM. Statistical analysis performed by one-way ANOVA are indicated as follows, * $P < 0.05$, ** $P < 0.005$.

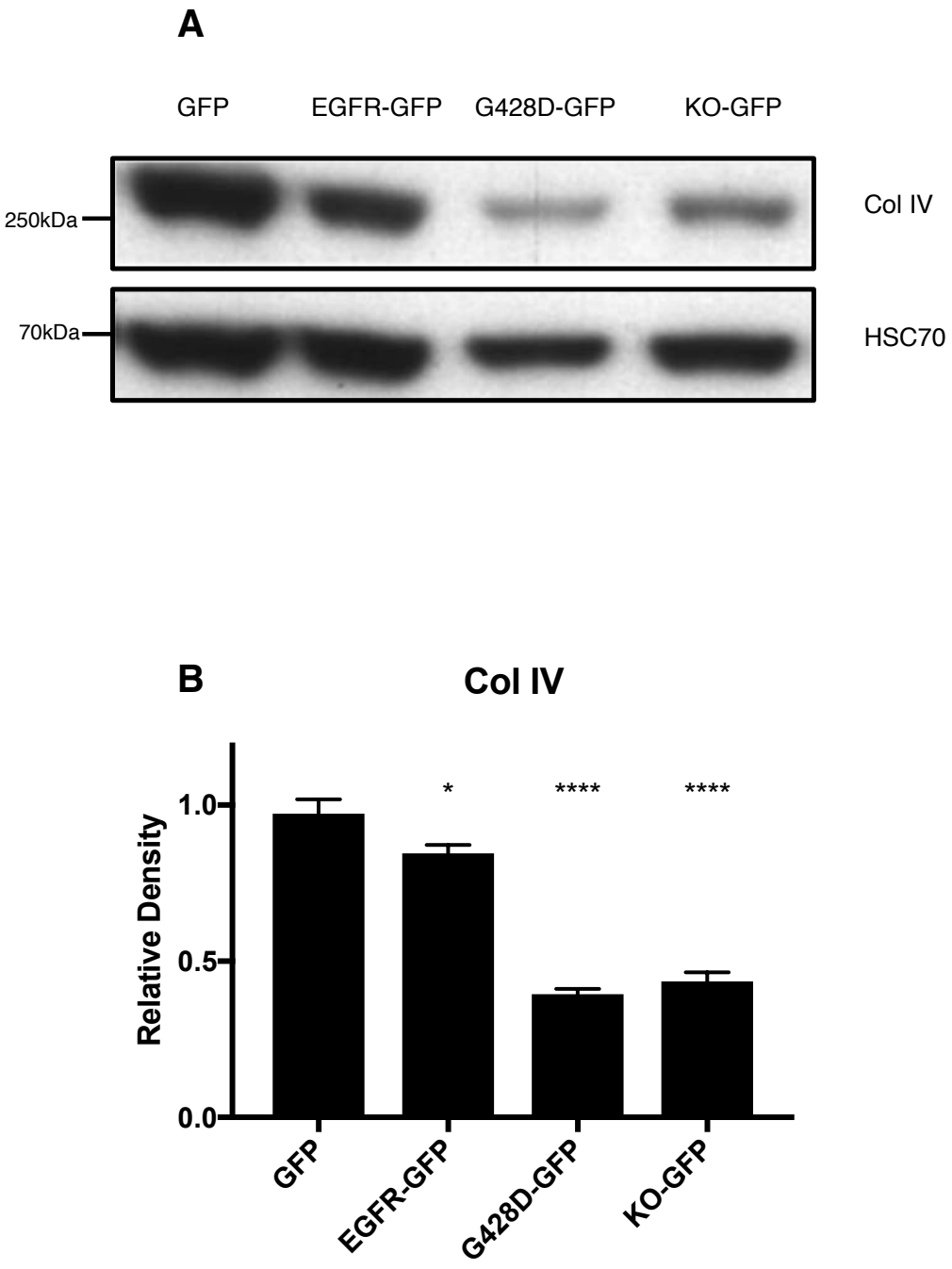


Figure 4.3: EGFR knockout or re-expression of G428D EGFR leads to reduced Collagen IV expression (A) Western blot showing Collagen IV protein expression in EGFR-2 cell lines. Lysates extracted from cell monolayers cultured under basal conditions. Representative of three independent experiments (B) Graph showing quantification of densitometry on western blots normalised to HSC70 loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA are indicated as follows, * $P < 0.05$, **** $P < 0.0001$.

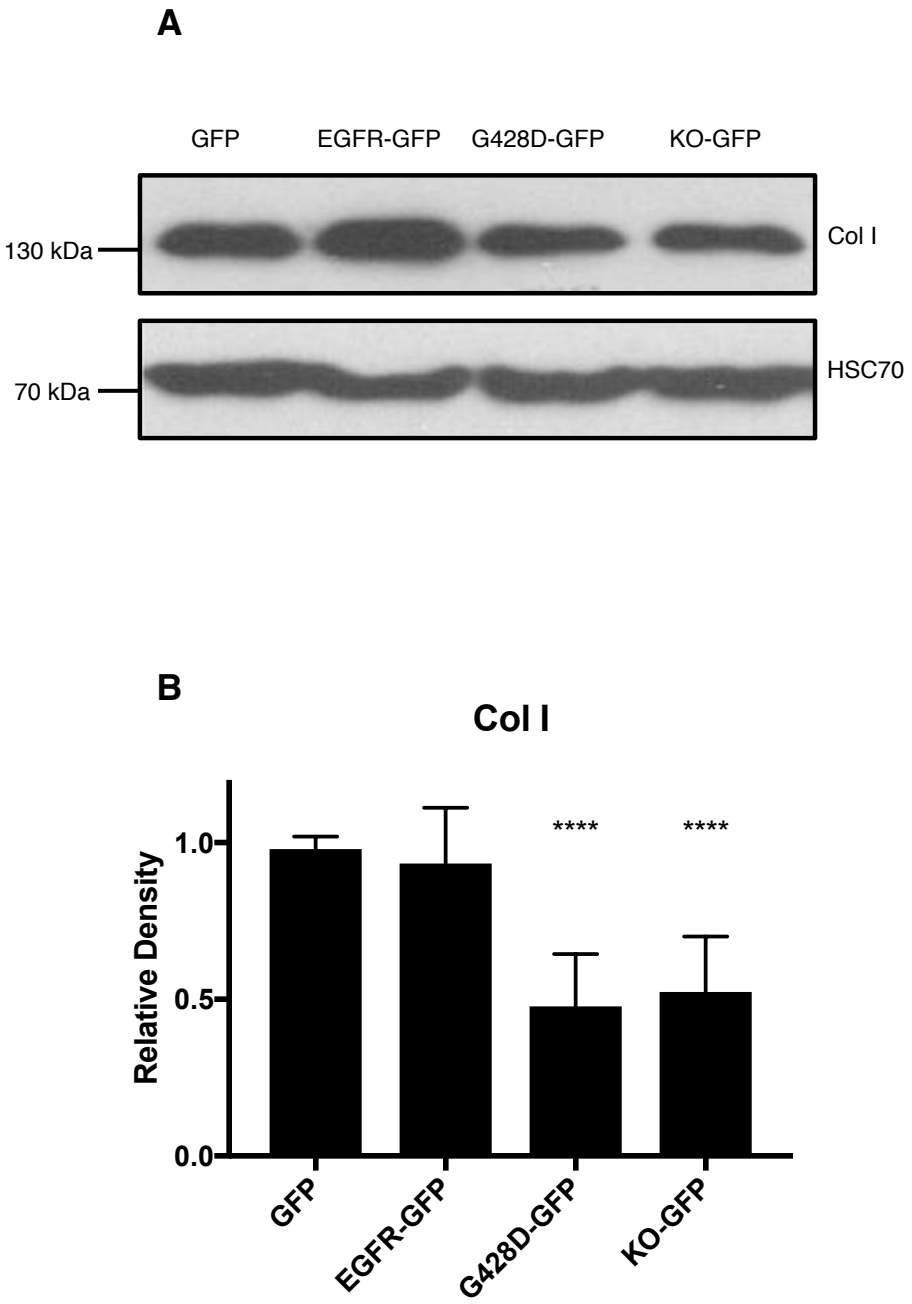


Figure 4.4: EGFR knockout or re-expression of G428D EGFR leads to reduced Collagen I expression (A) Western blot showing Collagen I protein expression in EGFR-2 cell lines. Lysates extracted from cell monolayers cultured under basal conditions. Representative of three independent experiments (B) Graph showing quantification of densitometry on western blots normalised to HSC70 loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA are indicated as **** $P < 0.0001$.

4.2.4 EGFR knockout or re-expression of G428D EGFR leads to up-regulation of pro-inflammatory cytokine secretion

In order to examine the changes in cytokine/chemokine levels identified in the RNAseq and QPCR experiments, ELISA assays were carried out to measure the level of the target proteins secreted by EGFR-2 cell lines as well as KO and WT keratinocytes.

For this, all keratinocyte lines were grown to confluent monolayers under basal conditions at which point the growth media was replaced and harvested after 24hrs. The harvested supernatants were then used alongside protein standards to determine the concentrations of secreted cytokines.

Resulting data (Figure 4.5) showed a significant increase in the concentration of CCL2, CCL5, CXCL10 and CCL20 in G428D-GFP and KO-GFP EGFR-2 cells in comparison with GFP cells. A similar significant increase in these cytokine was also detected in KO keratinocytes compared to WT counterparts as shown on the right hand side of the graphs in Figure 4.5. These data confirm that the increased cytokines detected at mRNA level translate to increased secretion of these cytokines in EGFR knockout or G428D EGFR expressing cells.

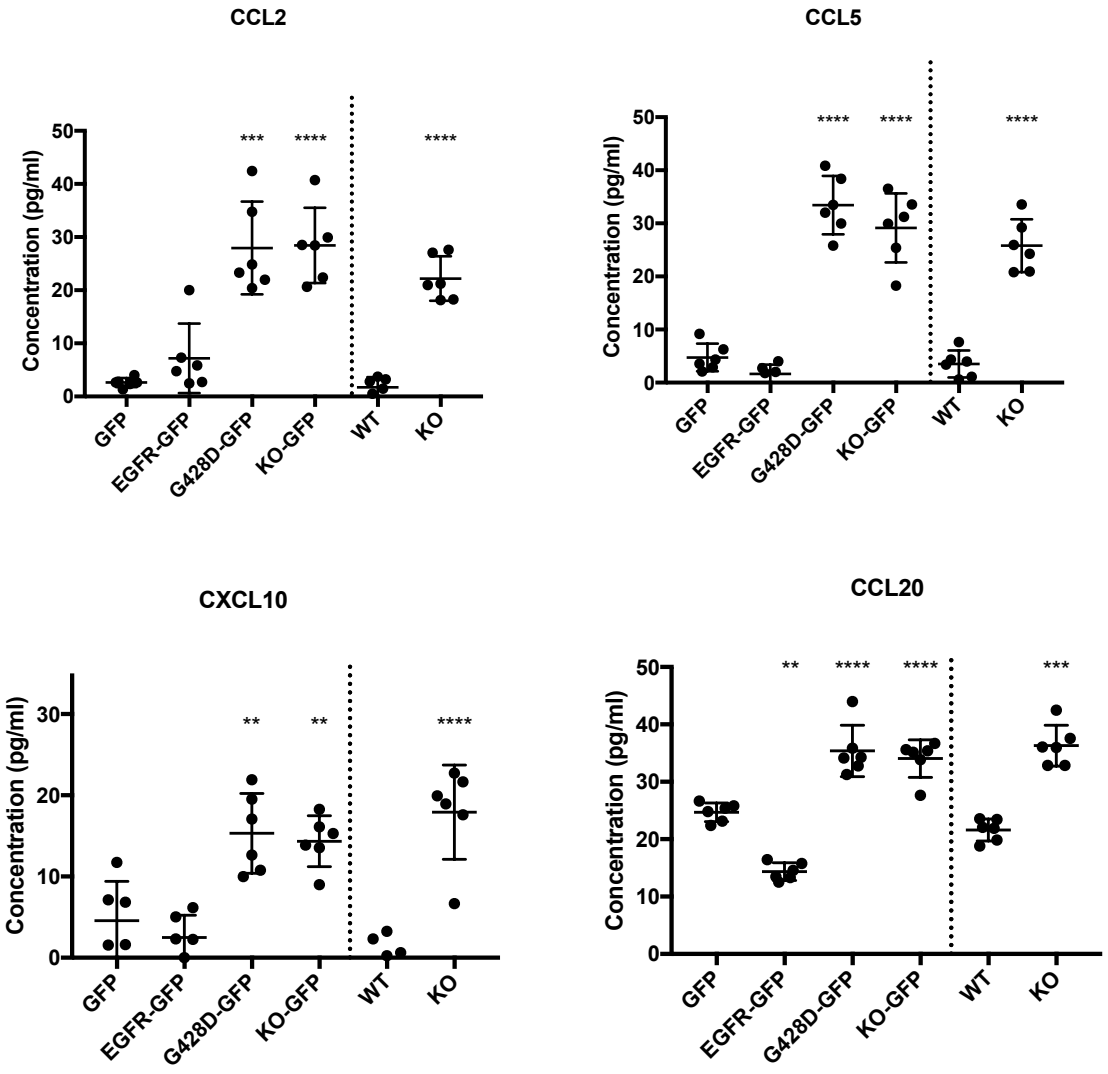


Figure 4.5: EGFR knockout or re-expression of G428D EGFR leads to up-regulation of pro-inflammatory cytokine secretion Graphs showing ELISA assays for specified targets performed using supernatant from EGFR-2 and WT/EGFR KO mouse keratinocyte cell line monolayers grown under basal conditions. Data is pooled from 5 independent experiments with each data point representing the means of 2 duplicate wells per experiment. Significance was measured in comparison to GFP cells for EGFR-2 cell lines and to WT for mouse keratinocytes. Statistical analysis performed by one-way ANOVA are indicated as ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

4.2.5 Increased CCL5, CXCL10 and CCL2 secretion in EGFR knockout or re-expression of G428D EGFR promotes immune cell chemotaxis.

Having determined that there was an increase in secreted chemokine concentration after EGFR knockout and G428D re-expression, it was then necessary to investigate whether the observed concentrations were enough to promote chemotaxis in various immune cell subsets. To measure chemotaxis, modified Boyden assays were used. Primary CD3+ T-cells or CD14+ monocytes were isolated from healthy WT CD1 mouse spleens and lymph nodes and then grown in the top of a transwell with pore size of 3.0um, large enough to allow active migration through the membrane, but not too large to permit non-specific migration of cells to the bottom chamber. Conditioned media taken from EGFR-2 cells and WT and KO keratinocytes was then added to the lower well beneath the transwell membrane, enabling the determination of whether factors secreted by keratinocytes could drive the chemotaxis of T-cells across the membrane. To determine specificity of responses, experiments were also performed with or without the presence of specific chemokine neutralising antibodies.

Resulting data demonstrated that media from G428D-GFP and KO-GFP cells promoted significant chemotaxis of T cells, whereas media from GFP and EGFR-GFP cells did not (Figures 4.6A,B). The addition of CCL5 neutralising mABs led to a significantly reduction in the amount of T-cell migration compared to media alone, but did not fully block chemotaxis (Figure 4.6A, grey bars). The addition of CXCL10 neutralising mABs also significantly reduced the migration of T cells in response to G428D-GFP cell media but was insufficient to fully block chemotaxis (Figure 4.6B, grey bars). Media from KO keratinocytes was also able to drive T cell migration in comparison to WT keratinocyte media (Fig 4.6 C). Addition of CCL5 neutralising mABs again led to a significant reduction in T cell migration, and whilst a similar trend to reduced chemotaxis was also seen in the presence of a CXCL10 mAB, this was not seen to be significant (Figure 4.6C). Media from G428D-GFP and KO-GFP cells was able to drive monocyte chemotaxis (Fig 4.7A). Addition of CCL2 neutralising mABs were able to significantly reduce monocyte migration. Similar data was obtained using media form KO keratinocytes in comparison to WT cells (Fig 4.7B). These data combined demonstrate that the elevated chemokines and cytokines seen in EGFR knockout or G428D EGFR expressing cells are bioactive and able to enhance immune cell migration.

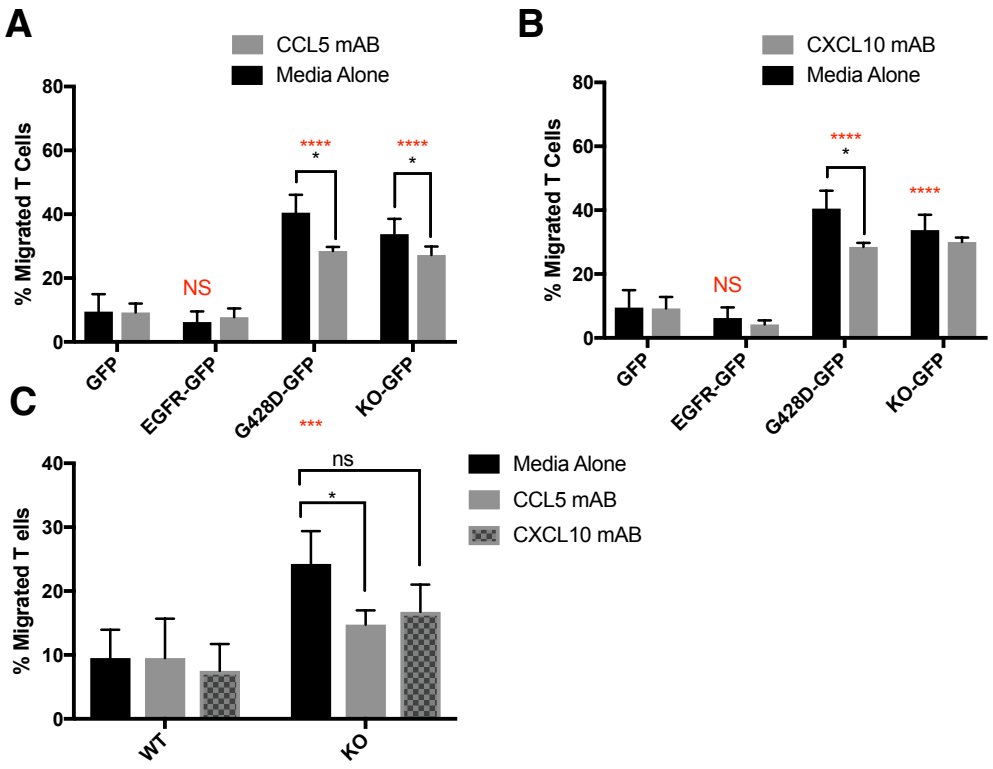


Figure 4.6: Increased CCL5 and CXCL10 secretion in EGFR knockout or re-expression of G428D EGFR promotes T cell chemotaxis Primary mouse CD3+ T cell chemotaxis in response to conditioned media from EGFR-2 and WT/EGFR KO mouse keratinocyte cell lines in a modified Boyden assay. Conditioned media was used with or without specific chemokine neutralising antibodies. (A) EGFR-2 media without (black bars) or with (grey bars) CCL5 antibody. (B) EGFR-2 media without (black bars) or with (grey bars) CXCL10 antibody. (C) Mouse keratinocyte WT/EGFR KO media without (black bars) or with CCL5 (grey bars) or CXCL10 (hatched bars) antibody. Data is pooled from 3 independent experiments, means +/- SEM are shown. Statistical analysis performed by two-way ANOVA are indicated as * $P < 0.01$ *** $P < 0.0001$ **** $P < 0.00001$. * indicates media alone conditions compared with GFP or WT cell lines.

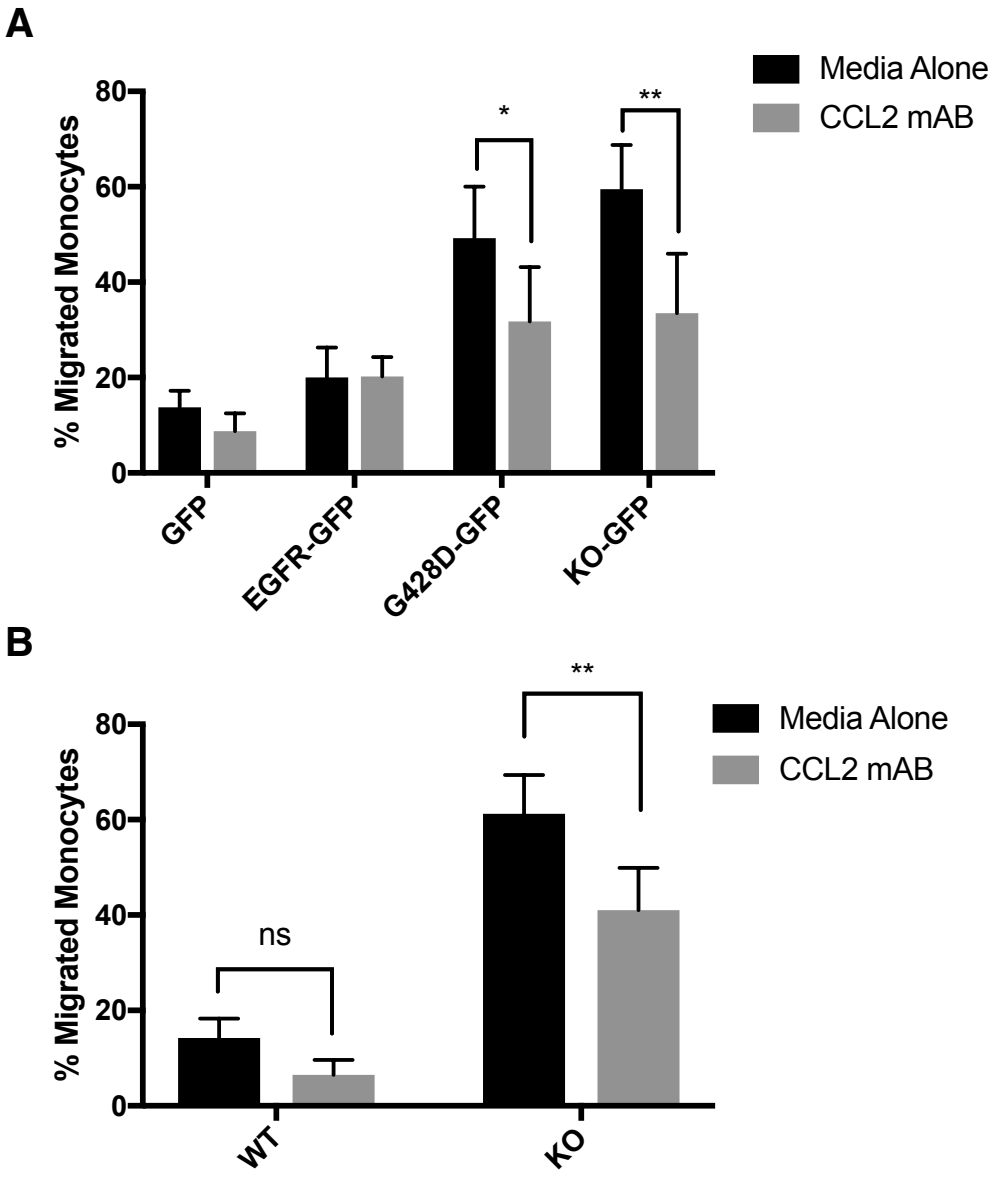


Figure 4.7: Increased CCL2 secretion in EGFR knockout or re-expression of G428D EGFR promotes monocyte cell chemotaxis Mouse CD14+ monocyte chemotaxis in response to conditioned media from EGFR-2 and WT/EGFR KO mouse keratinocyte cell lines in a modified Boyden assay. Conditioned media was used with or without CCL2 neutralising antibodies. (A) EGFR-2 media without (black bars) or with (grey bars) CCL2 antibody. (B) WT/EGFR KO mouse keratinocyte media without (black bars) or with (grey bars) CCL2 antibody. Data is pooled from 3 independent experiments, means +/- SEM are shown. Statistical analysis performed by two-way ANOVA. ** indicates $P < 0.005$

4.2.6 EGFR knockout or re-expression of G428D EGFR leads to enhanced gelatin degradation

As both MMP9 and MMP10 were seen to be increased in EGFR knockout/G428D EGFR cells by RNAseq and QPCR analysis, these targets were analysed as further potential functional regulators of the epithelial fragility and inflammation phenotypes associated with these EGFR defects. Both these MMP's are known to degrade collagen and basement membrane proteins. In order to determine whether the increased transcripts of these genes translated to functionally active proteases, a functional gelatin degradation assay was carried out.

Cy-3 labelled gelatin was coated onto optical plastic chambers. EGFR-2, WT and KO keratinocytes were then seeded at high density and grown to monolayers on the dyed gelatin for 16hrs under basal conditions with or without the pan-MMP inhibitor GM6001 (indicated as GM on graphs). Cells were then fixed and stained for f-actin (phalloidin) and DAPI, and Cy3-gelatin was visualised using confocal microscopy and the amount of degradation (as defined by black spots in the gelatin-Cy3 monolayer) was analysed using FIJI software. Example images of cy3-gelatin degradation by WT and KO cells is shown in Figure 4.8A.

Quantification of multiple fields of view revealed a significant increase in the total gelatin degradation by G428D-GFP and KO-GFP cells in comparison to GFP and EGFR-GFP cells (Figure 4.8B). A similar result was also seen in KO keratinocytes compared with WT cells (Figure 4.8C). This increase in total degradation area was also correlated with an increase in the number of degraded gelatin punctae per cell (Figure 4.8D) Moreover addition of GM6001 significantly reduced the amount of gelatin degradation in all cases (Figures 4.8B,C; red bars). These data combined demonstrate that knockout of EGFR or expression of G428D EGFR leads to increased active MMP secretion, resulting in enhanced degradation of the underlying extracellular matrix.

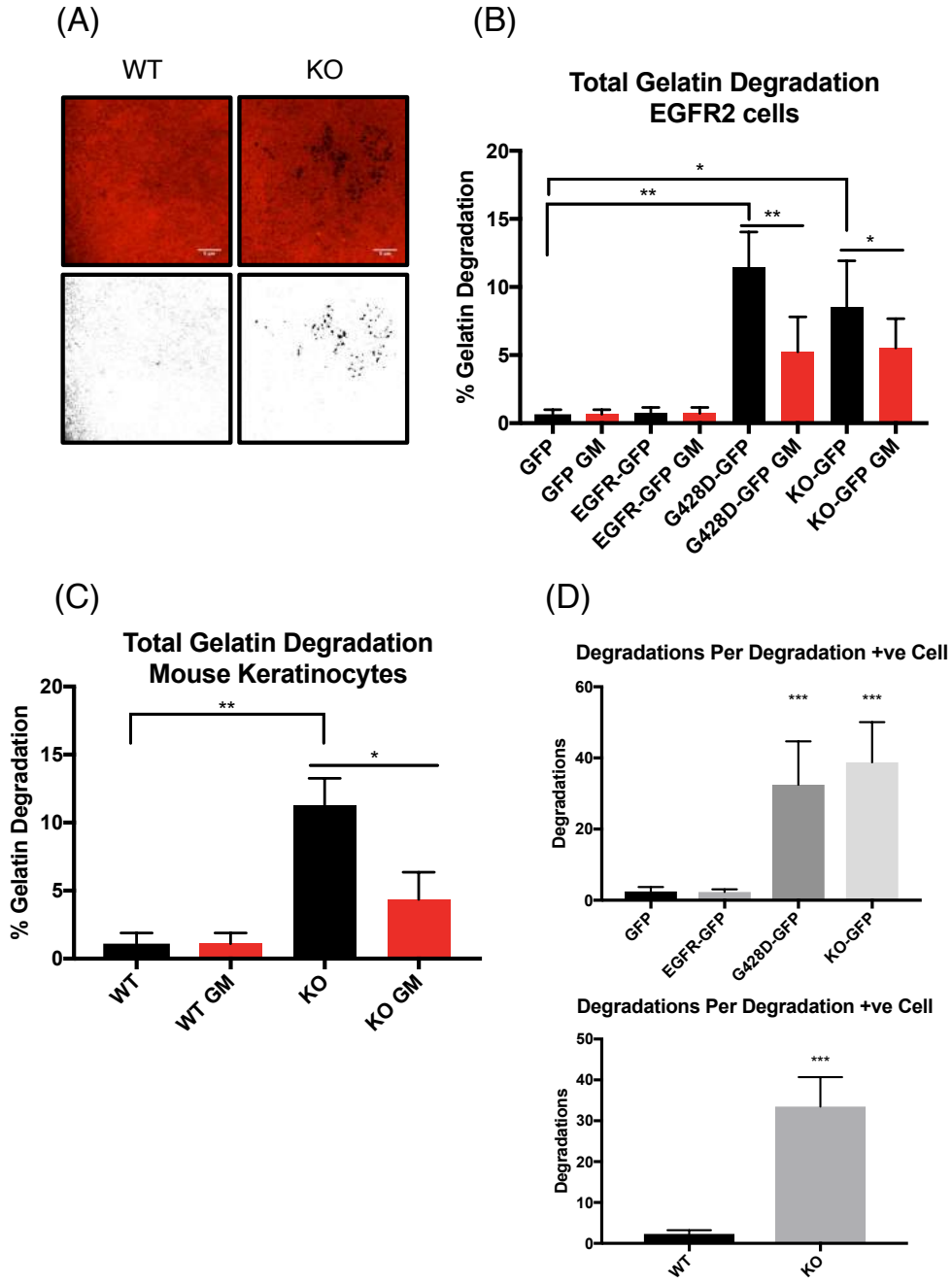


Figure 4.8: EGFR knockout or re-expression of G428D EGFR leads to enhanced gelatin degradation (A) Example confocal images of Cy-3 dyed gelatin after 24hr culture with WT/EGFR KO mouse keratinocytes. Top row: Cy-3 Gelatin, bottom row: binarized images showing measurable degradation points. Scale bars are 9um (B) Analysis of gel degradation as % of image tiles by EGFR-2 cell lines (C) Analysis of gel degradation as % of image tiles by WT/EGFR KO mouse keratinocytes. (D) Average number of degradation points per cell area in EGFR-2 cells (top graph) and WT/EGFR KO mouse keratinocytes (bottom graph). Data is pooled from 3 independent experiments with duplicate wells per experiment, means +/- SEM are shown. Statistical analysis performed by two-way ANOVA. * $P<0.05$ ** $P<0.005$ *** $P<0.0005$

4.2.7 EGFR knockout or re-expression of G428D EGFR leads to enhanced IL-33 secretion and altered localisation

IL-33 was another DE SkInFs that was significantly increased in EGFR knockout and mutant samples. IL-33 is increasingly studied in epithelial inflammation and allergy, and as such made an interesting target for follow-up. As an alarmin cytokine, the localisation of IL-33 is an important factor in assessing the function of this molecule in context. To analyse localisation, WT and KO keratinocytes were fixed and stained for IL-33, DAPI and F-actin (phalloidin). Resulting images demonstrated that IL-33 showed strong nuclear/peri-nuclear localisation in WT keratinocytes (Figure 4.9A, left panels). However, this localisation was lost in KO keratinocytes and IL-33 instead demonstrated diffuse cytoplasmic localisation (Figure 4.9A, right panels).

Western blotting for IL-33 from cell lysates showed a significant reduction in cell-associated IL-33 in EGFR-2 cell lines compared with GFP line. There was no significant change in cell-associated IL-33 between WT and KO cells (Figure 4.9B, 4.9C). Analysis of secreted IL-33 using ELISA assays also demonstrated a significant increase in secreted IL-33 from G428D-GFP and KO-GFP EGFR-2 cells in comparison with GFP and EGFR-GFP cells (Figure 4.9D). A similar increase in secreted IL-33 was also seen in KO compared to WT keratinocytes (Figure 4.9C, far right on graph). These data combined demonstrate that knockout or mutation of EGFR leads to reduced sequestration of IL-33 within the peri-nuclear region and enhanced IL-33 secretion.

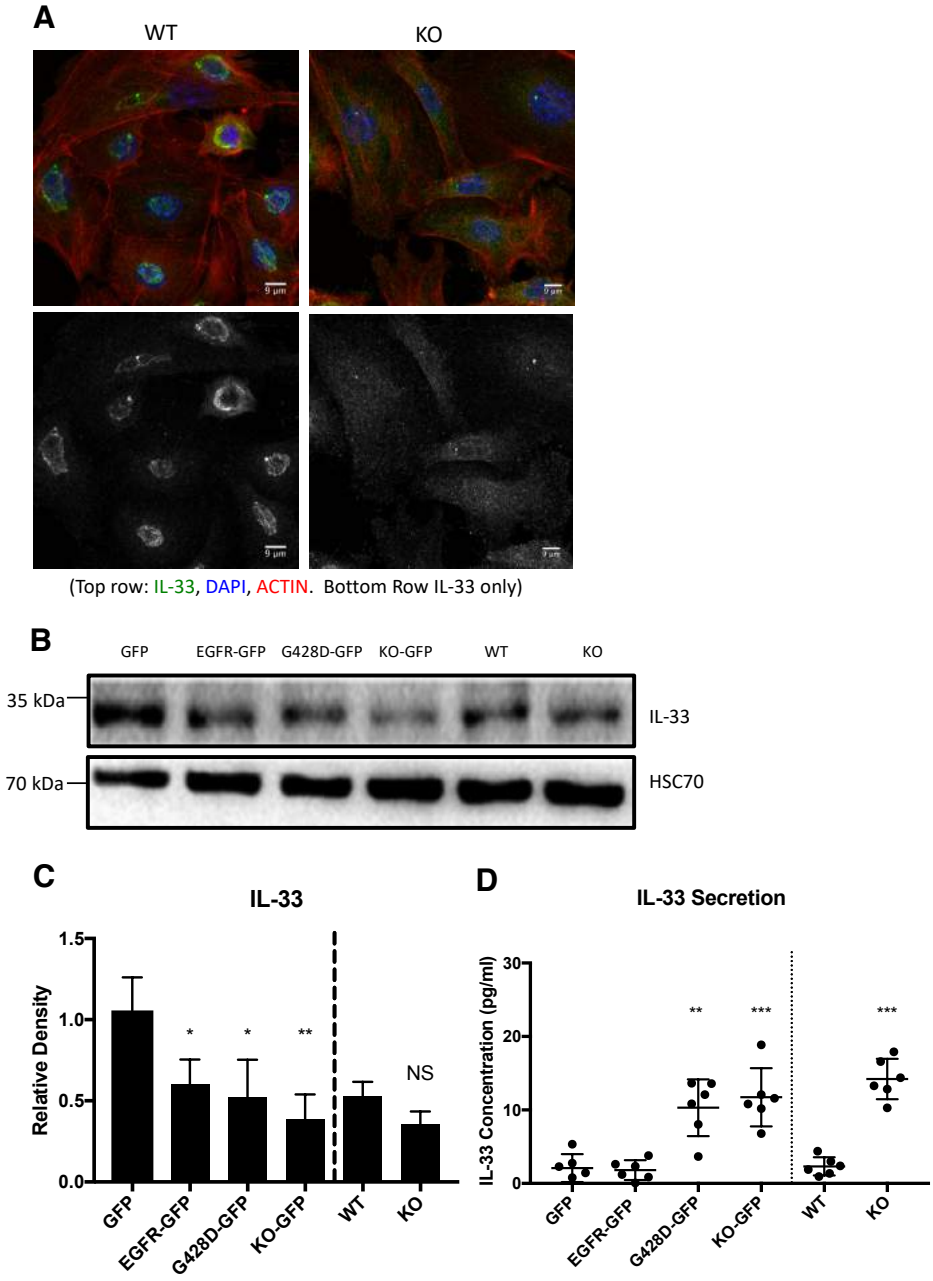


Figure 4.9: EGFR knockout or re-expression of G428D EGFR leads to enhanced IL-33 secretion and altered localisation (A) Example confocal images of IL-33 localisation (green) in WT/EGFR KO mouse keratinocytes stained for F-actin (red) and DAPI (blue). Scale bars are 9µm. (B) Western blot analysis of IL-33 expression from lysates of EGFR-2 cells and WT/EGFR KO mouse keratinocytes grown in basal conditions. Blots representative of 3 experiments. (C) Graph showing quantification of densitometry on western blots normalised to HSC70 loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA are indicated as * P <0.01 * P <0.001 (D) Concentration of IL-33 in supernatants from EGFR-2 and mouse keratinocytes grown under basal conditions measured by ELISA. Data is pooled from 5 independent experiments with individual data points representing the mean of triplicate experimental repeats; means +/- SEM are shown. Statistical analysis performed by one-way ANOVA using GFP and WT as controls for respective cell lines. * P<0.05 ** P<0.005

4.2.8 Enhanced cytokine expression in EGFR knockout keratinocytes promotes increased cytokine expression in WT keratinocyte

Keratinocytes express many cytokine receptors as well as large amounts of ST2, the IL-33 receptor. For this reason, the next experiments were aimed at investigating whether the increased levels of cytokines secreted after EGFR knockout or G428D re-expression could feedback autonomously onto keratinocytes to alter inflammatory potential.

To analyse this, media from confluent monolayers of KO keratinocytes was harvested and added to WT keratinocytes for 6hrs in the presence of either DMSO or IL-33 neutralising mAbs. The WT keratinocytes were then harvested for QPCR to analyse levels of cytokine transcripts. Resulting data demonstrated a significant increase in CCL5, CCL27, CCL20 and CXCL10 RNA expression in WT keratinocytes treated with media from KO keratinocytes, whereas CCL2 mRNA levels were unaffected (Figure 4.10). There was no significant difference in cytokine expression between cells treated with KO media containing DMSO or IL-33 neutralising mAbs (Figure 4.10). This data suggests that increased cytokine production by EGFR knockout keratinocytes can act in an autocrine fashion to further exacerbate a pro-inflammatory phenotype in the epithelium.

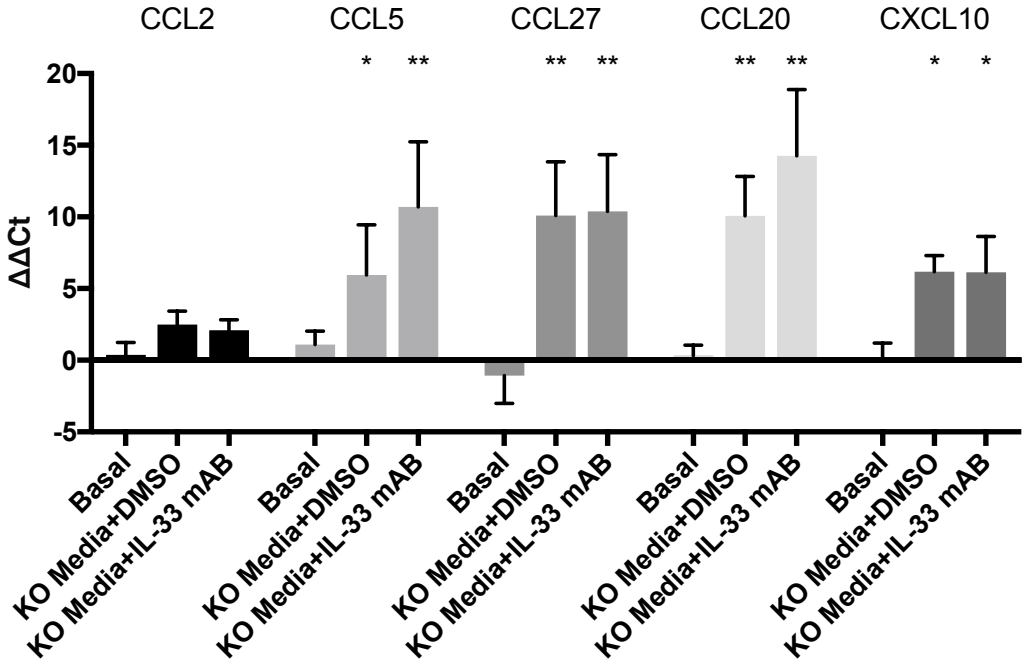


Fig 4.10: Enhanced cytokine expression in EGFR knockout keratinocytes promotes increased cytokine expression in WT keratinocyte QPCR data for GFP expressing EGFR-2 cells. GFP cells were cultured as monolayers under basal conditions followed by culture in media from KO-GFP cells including DMSO or IL-33 neutralising antibodies for 6h. Pooled from 3 independent experiments with 2 technical replicates per experiment; means +/- SEM are shown. Statistical analysis performed by one-way ANOVA compared to basal for each cytokine. * P<0.05 ** P<0.005

4.2.9 Acute inhibition of EGFR mimics loss of EGFR expression in mouse keratinocytes

As data in this chapter demonstrated that EGFR knockout or G428D mutant re-expression can lead to an inflammatory phenotype in mouse keratinocytes, it was important to determine whether these phenotypes were due to activation of EGFR through the canonical auto-phosphorylation and dimerization pathway. To analyse this, GFP expressing EGFR-2 cell monolayers were grown under basal conditions followed by treatment with the EGFR kinase inhibitor AG1478 for 4hrs, a time during which no observable change in cell health occurred. RNA from the AG1478 treated cells was then used to perform QPCR measuring the levels of the differentially expressed SkInFs analysed in previous figures.

Data revealed that treatment with AG1478 was sufficient to induce significantly increased expression of CCL5, CCL20, CCl27 and IL-1b, with other cytokines showing a trend towards increased expression that was not statistically significant (Figure 4.11A). However, treatment of cells with AG1478 had no effect on IL-33 expression levels in GFP and EGFR-GFP cells (Fig 4.11B).

To determine whether kinase inhibition of EGFR was also sufficient to enhance MMP activity, gelatin degradation assays were carried out (as in Fig. 4.8) using GFP and EGFR-GFP cells treated with either DMSO or AG1478. Analysis revealed that AG1478 treatment led to a significant increase in gelatin degradation by EGFR-2 keratinocytes (Fig 4.11C) although not to the extent seen in G428D-GFP or KO-GFP cells (Fig 4.8B). These data demonstrate that blocking EGFR activity acutely is sufficient to induce a pro-inflammatory phenotype in keratinocytes.

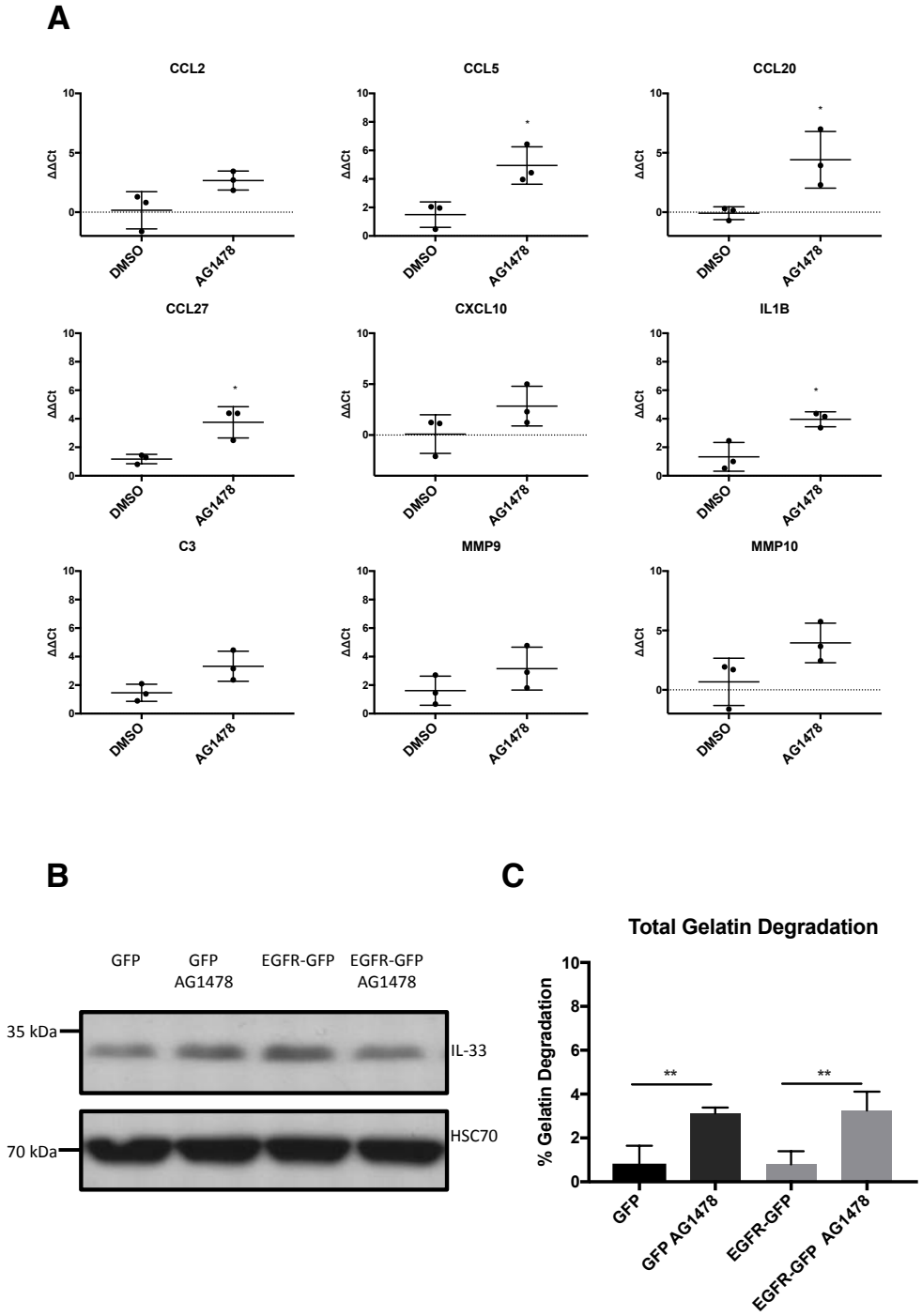


Fig 4.11: Acute inhibition of EGFR mimics loss of EGFR expression in mouse keratinocytes (A) QPCR data for specified targets from GFP expressing EGFR-2 cells cultured as monolayers under basal conditions with DMSO or EGFR inhibitor AG1478 for 4hrs. Data is pooled from 3 independent experiments with 2 technical replicates per experiment. (B) IL-33 expression in lysates from GFP and EGFR-GFP expressing EGFR-2 cells treated with or without AG1478 for 4hrs. Representative of 3 independent experiments. (C) Analysis of gelatin degradation by GFP and EGFR-GFP expressing EGFR-2 cells with or without AG1478 for 16hrs. n = 3. Means +/- SEM are shown throughout. Statistical analysis performed by T-Test compared to respective controls. * P<0.05 ** P<0.005

4.2.10 Conditioned media from KO-GFP cells is insufficient to drive IL-4+ CD4 T cells

A number of the cytokines and chemokines shown to be up-regulated after EGFR knockout or G428D mutant re-expression have been shown to play an important role in the driving and maintenance of a Th2 immune response such as CCL2 (Chensue et al. 1996), CCL5 (Zhang et al. 2015), CCL20 (Weckmann et al. 2007), CCL27 (Chen et al. 2006) and IL-33 (Murukami-Satsutani et al. 2014). Thus, it was of interest to determine whether or not these secreted factors in conditioned growth media taken from KO-GFP cells could drive the expansion of Th2 T-cells *in vitro*.

To analyse this, CD4+ T cells were isolated from the lymph nodes of WT CD1 mice using magnetic activated-cell sorting. The T cells were then activated by incubating with CD3/28 beads and grown in either normal T cell media or in conditioned media from KO-GFP EGFR-2 keratinocytes with or without IL-4 (10ng/ml), a known driver of Th2 differentiation. This was performed in the presence of brefeldin and monensin to block cytokine secretion. The T-cells were then fixed and stained for CD4, CCR4, CCR6, GATA3 and IL-4 and analysed by flow cytometry.

Flow cytometric analysis revealed that incubation of T-cells with IL-4 was able to significantly increase the number of IL-4+ T-cells compared with unstimulated or activated CD4 T cells (Figure 4.12A, 4.12B). However, treatment with conditioned media from KO-GFP EGFR-2 keratinocytes had no effect on the population of IL-4+ T cells with or without IL-4 stimulation.

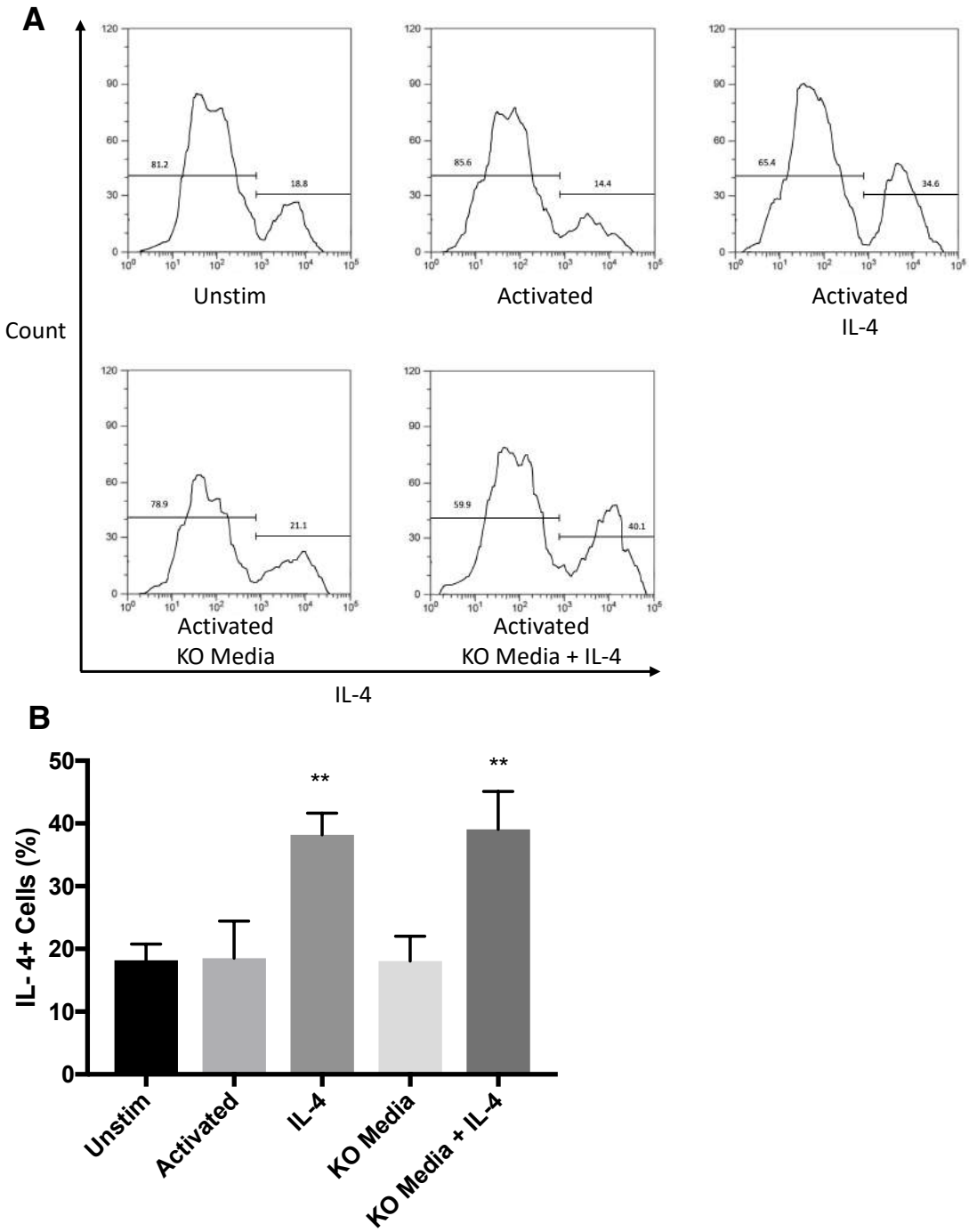


Fig 4.12: Conditioned media from KO-GFP cells is insufficient to drive IL-4+ CD4 T cells (A) Expression of intracellular IL-4 in isolated murine primary CD4+ T cells. CD+ T cells were pre-gated for CCR4+, CCR6-. Cells were activated with CD3/28 beads with or without recombinant IL-4 in normal growth media or conditioned media from KO-GFP EGFR-2 cells supplemented with recombinant IL-2. Histograms are representative of 3 separate experiments. (B) Analysis of IL-4 expression between stimulation variants. Statistical analysis performed by one-way ANOVA with multiple comparisons against unstimulated control. ** $P < 0.001$

4.3 Discussion

The data presented in this chapter demonstrates that under basal conditions, knockout or G428D mutant re-expression is sufficient to drive an inflammatory phenotype basal layer keratinocytes *in vitro*. This is indicated by the up-regulation of many inflammatory mediators at both the transcript and protein level. RNAseq revealed thousands of differentially expressed genes after EGFR knockout or G428D mutant re-expression. Furthermore, the data shows that EGFR knockout and G428D mutant re-expression can be considered phenocopies in comparison to cells expressing endogenous EGFR or WT EGFR re-expression. Additionally, the data demonstrates that the level of up-regulated cytokines, chemokines and MMPs are sufficiently bioactive when assessed using a number of bioassays such as T cell/monocyte chemotaxis and gelatin degradation analysis.

4.3.1 The Inflammatory phenotype has commonalities with EGFR-I patient phenotypes as well as with patients harbouring G428D mutation.

Although the data demonstrated that EGFR knockout or G428D mutant re-expression act as phenocopies, there are a number of inconsistencies between these data and the microarray data obtained from the G428D mutant patient (Campbell et al. 2014). This is not unexpected as the data presented in this chapter is derived from pure populations of mouse keratinocyte lines compared with the patient data which was obtained from whole skin biopsies. Despite this, there are a number of commonalities within the immune compartment between the data presented in this chapter and the data generated from the patient. This includes significant up-regulation of CCL2, CCL5, CCL20 and CCL27. This may suggest that the keratinocytes are major contributors to this component of the clinical phenotype. However, the patient data also shows significant up-regulation of IL-8, a major chemoattractant for the recruitment of neutrophils (Zeilhofer and Schorr 2000), which is not seen in the data presented here. This is consistent with the elevated levels of neutrophil skin infiltration seen in conditional EGFR skin knockout mice. This difference between data sets suggests that

the contribution of IL-8 to the *in vivo* phenotype is likely not primarily derived from keratinocytes, or at least requires other cell-cell interactions to occur. It has also been previously shown that cancer patients treated with EGFR inhibitor drugs such as Gefitinib have higher levels of skin CCL2 and CCL5 compared with healthy skin (Yamaki et al. 2010).

4.3.3 The inflammatory phenotype has a functional effect on ECM degradation, which could be linked to immune cell recruitment

Having demonstrated that EGFR knockout or G428D mutant re-expression was able to drive an inflammatory phenotype in keratinocytes, the next aim of this chapter was to determine whether or not this phenotype was sufficient to drive inflammatory processes in other cell types.

Figure 4.5 showed that a number of cytokines up-regulated at the transcript level were also secreted at higher levels by KO-GFP and G428D-GFP cells in comparison to GFP and EGFR-GFP cells. The data in Figure 4.6 and 4.7 demonstrate that these secreted chemokines are able to induce chemotaxis in primary mouse T cells and monocytes. This result suggests that under normal, homeostatic conditions, EGFR signalling is able to supress the secretion of chemokines that could go on to induce immune infiltration into the skin via chemotaxis. As increased immune cell infiltrate has been shown to occur after conditional EGFR knockout (F Mascia et al. 2013), and EGFR inhibitory treatment (Holcmann and Sibilia 2015), the data presented in this chapter suggests that the keratinocytes may play a primary role in promoting this infiltration.

As well as cytokines and chemokines, other pro-inflammatory factors such as MMPs were also upregulated, in particular MMP-9 and MMP-10. These proteases have been implicated in the breakdown of ECM proteins involved in immune infiltration into a number of tissues (Deryugina et al. 2014; Jedryka et al. 2012). The data presented in Figure 4.8 indicates that the elevated levels of MMP production by G428D-GFP, KO-GFP EGFR-2 cells and EGFR KO cells was able to cause high levels of gelatin degradation compared to cells expressing WT EGFR. Taken together with the increase in chemokine

expression and the ability for this to drive immune cell chemotaxis, the data presented in this chapter proposes a major role for EGFR in the regulation of keratinocyte-driven immune infiltration into the skin.

4.3.4 Portions of the inflammatory phenotype suggest a Th2 motif which has been linked to wound healing phenotypes in the skin.

The immune phenotype uncovered in this chapter does not correspond perfectly to well-categorised inflammatory compartments such as Th1, Th2 and Th17 inflammation. However, there are many similarities to Th2 immune response especially in the context of wound healing. Although the phenotype presented in this chapter does not include expression of IL-4, a major indicator or Th2, many other factors are involved in Th2 immune responses. Th2 immune response is known to be required in acute wound healing (Ellis, Lin, and Tartar 2018) and in particular, CCL2 (Wood et al. 2014), CCL20 (Kennedy-Crispin et al. 2012) and CCL27 (Inokuma et al. 2006) have all been shown to play critical roles in the initiation of wound healing processes. This suggests that EGFR signalling may play a regulatory role in the production of Th2/wound healing associated immune mediators. Both CCL2 and CCL5 have been shown to be involved in chronic wound healing conditions (Ridiandries, Tan, and Bursill 2018). This is important as the EBS-like phenotype displayed by the G428D mutant expressing patient shows hallmarks of chronic wound healing.

Although not a complete, canonical Th2 phenotype, it was important to try and determine if the secreted factors were able to drive the differentiation of Th2 T cells *in vitro*. Figure 4.12 demonstrates that conditioned media from KO-GFP cells was unable to increase the population of IL-4+ Th2 T-cells. This was only possible with the inclusion of recombinant IL-4, however this had no synergistic effect with the conditioned media. This suggests that although the phenotype is Th2-like, there are many other factors necessary to induce a full Th2 response which are likely supplied not by basal layer keratinocytes. Other reasons for this result may be due to technical difficulties with this particular assay. It would be more suitable to be able to co-culture the keratinocytes with the T cells however this proves lethal for either cell type

depending on the growth media used. This is unfortunate as direct cell-cell interaction between the epithelium and immune cells has been shown to effect immune cell phenotype (Peeters, Wouters, and Reynaert 2015). An experimental model that could be used to further study this is using co-culture in a 3D microenvironment (Van Den Bogaard et al. 2014), however this would need to be further optimisation to be performed using the EGFR-2 cell line. Another explanation for the results of this assay may be due to other secreted factors that are not associated with Th2 immune response. One of these is the chemokine CXCL10 which, along with its receptor CXCR3, has been shown to primarily drive Th1 responses in the skin (Kuo et al. 2018).

Taken together, the data from this chapter has shown that both EGFR knockout and G428D-mutant re-expression induce a proinflammatory, Th2-like phenotype in keratinocytes and that this phenotype is sufficient in driving immune cell chemotaxis and ECM degradation suggesting a major role in epidermal immune infiltration.

5. The inflammatory phenotype in EGFR knockout and G428D mutant re-expression cells is partly driven by altered STAT3 and SHP2 signalling

5.1 Introduction

The EGFR signalling pathway controls many downstream transcription factors, contributing to a plethora of cellular processes. A number of the key downstream proteins such as ERK and AKT have been shown to exhibit reduced activation after EGFR inhibition, knockout, or G428D mutant re-expression, however the status of the subsequent pathways activated by key upstream regulators, such as STAT1/3, remains unclear.

STAT1/STAT3 are involved in the regulation of many of the SkInFs identified in chapter 2, hence this was a primary candidate to investigate as a potential mediator of the pro-inflammatory signature observed in EGFR manipulated cells. STAT3 is known to be involved in many cellular processes such as survival and proliferation in keratinocytes. However, depending upon the activation level, STAT3 has been shown to drive both inflammatory and anti-inflammatory phenotypes in different cells in different contexts.

Some of the identified up-regulated cytokines regulated by STAT3 include, CCL2 (Griesinger et al. 2015), CCL5 (Zhou et al. 2016), CCL20 (Bae et al. 2018), and CCL27 (Karakawa et al. 2014). CXCL10, an interferon induced chemokine, has been shown to be regulated in part by STAT1 however the role of STAT3 in CXCL10 regulation is unclear (Burke et al. 2013). Interestingly, the receptors for many of these cytokines also activate STAT3 initiating a positive feedback loop of high STAT3 phosphorylation unless otherwise regulated (H. Yu, Pardoll, and Jove 2009). In some conditions, STAT3 activation is partly regulated by the protein-tyrosine-phosphatase (PTP) SHP2 (Y. Huang et al. 2017; Zehender et al. 2018)(Huang et al. 2017). As both SHP2 and STAT3 are activated downstream of EGFR under normal conditions, the aim of this chapter was to dissect the effects of STAT1/3 and SHP2 activation on the inflammatory phenotype seen in EGFR-2 cells after EGFR knockout or G428D mutant re-expression.

5.2 Results

5.2.1 STAT3 is over active in EGFR knockout and G428D re-expression EGFR-2 cells grown under basal conditions.

To investigate the role of STAT3 signalling in regulating the inflammatory phenotype in keratinocytes, the activation state of STAT3 under basal conditions in EGFR-2 cells was analysed. EGFR-2 cells were grown into confluent monolayers and either left in normal growth media or starved for 24hrs. Lysates were then extracted from the cells and run on SDS-page gels before being transferred and blotted for total STAT3, pSTAT3 and GAPDH as a loading control.

Resulting blots and quantification revealed that under basal conditions, G428D-GFP and KO-GFP cells expressed higher levels of pSTAT3 than both GFP and EGFR-GFP cells (Figure 5.1A lanes 1,2,5,6; Figure 5.1B). All starved cell lines show significantly lower pSTAT3 levels than cell lines under basal conditions. Under starved conditions, GFP and EGFR-GFP cells have significantly lower expression of pSTAT3 compared with KO-GFP. Starved G428D-GFP also have a significantly reduced pSTAT3 expression compared to KO-GFP although to a lower extent than the GFP and EGFR-GFP cells.

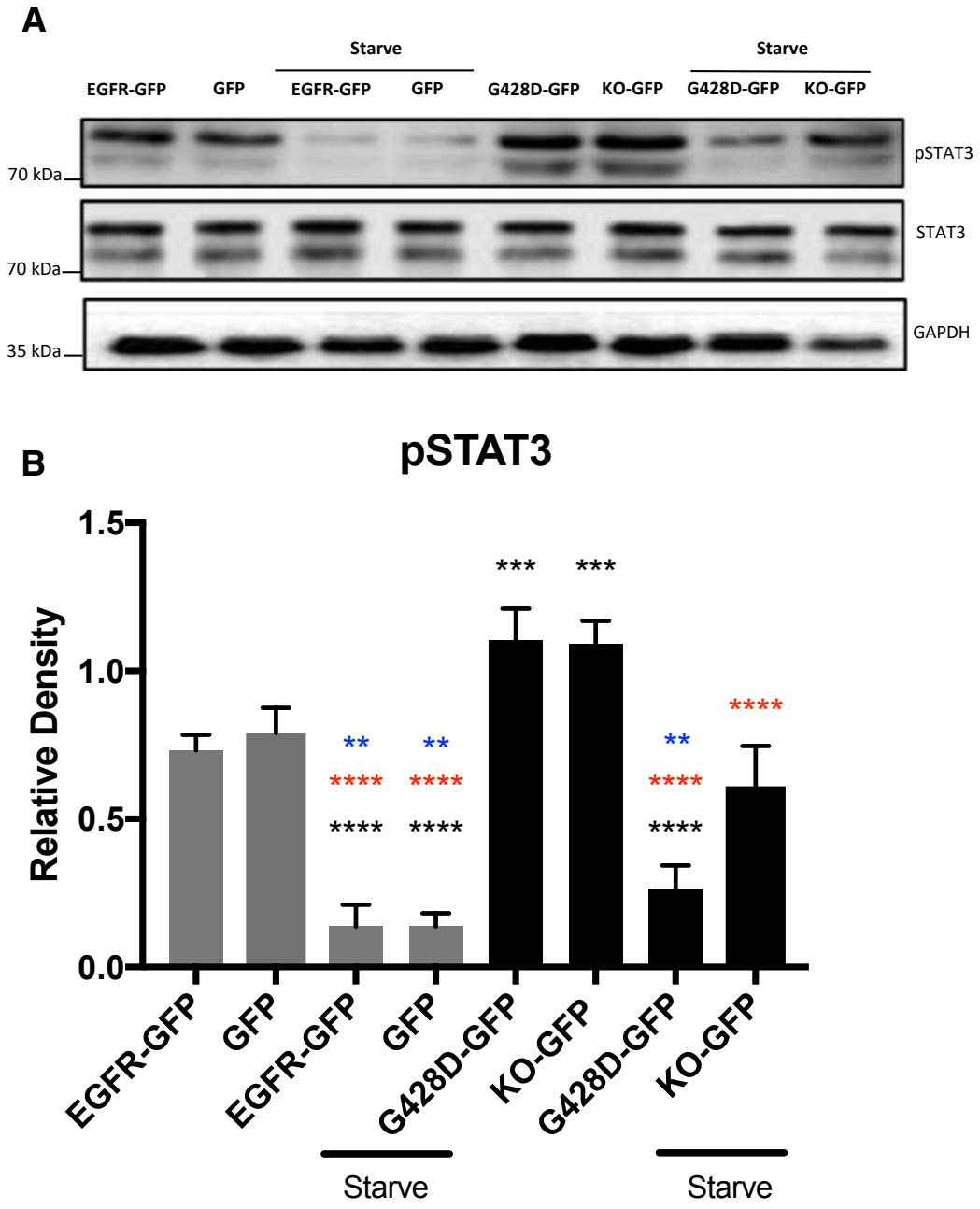


Figure 5.1: STAT3 is over active in EGFR Knockout and G428D re-expression EGFR-2 cells grown under basal conditions (A) Western blot showing phospho-STAT3 and STAT3. Lysates extracted from confluent monolayers of EGFR-2 cells grown under basal or starved conditions. Representative of four independent experiments (B) Graph showing quantification of densitometry on western blots normalised to GAPDH loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA indicated as follows, *** P < 0.0001, **** P < 0.00001. * represents comparison against basal GFP expressing cells. * represents comparison within cell lines between basal and starved conditions. * represents comparison of starved cell lines against starved KO-GFP.

5.2.2 IL-6 stimulation leads to increased pSTAT levels in all EGFR-2 cell lines

As pSTAT3 was significantly increased after EGFR knockout or G428D mutant re-expression we wanted to understand whether pSTAT3 levels were at the maximal level in these cells, and whether this result could be replicated after actute EGFR inhibition using AG1478.

To determine the maximal activation level of pSTAT3, EGFR-2 cells were stimulated with 10 ng/ml recombinant IL-6, a cytokine known to strongly activate STAT3 in many cell types including keratinocytes (Wnag et al. 2004), and lysates probed for pSTAT3. Resulting blots and quantification revealed that IL-6 stimulation of GFP and EGFR-GFP cells led to the expected increase in pSTAT3 (Figure 5.2A, lanes 1-4; Figure 5.2B) but that this increase was at higher levels than observed in KO-GFP and G428D-GFP cells under basal conditions (Figure 5.2B). Moreover, KO-GFP and G428D-GFP cells stimulated with IL-6 showed higher still levels of pSTAT3 (Figure 5.2A, lanes 5-8) demonstrating that EGFR knockout or G428D re-expression does not lead to maximal activation of STAT3 in EGFR-2 keratinocytes.

AG1478 was then used to actuley inhibit EGFR in GFP or EGFR-GFP cells to determine whether the increase in pSTAT3 levels seen in KO-GFP and G428D-GFP cell lines could be reproduced by kinase inhibition (Figure 5.2C, D). Data demonstrated a significant reduction in pSTAT3 was seen after acute EGFR inhibition to levels seen in GFP cells. AG1478 did not reduce pSTAT levels in GFP cells (Figure 5.2C, D).

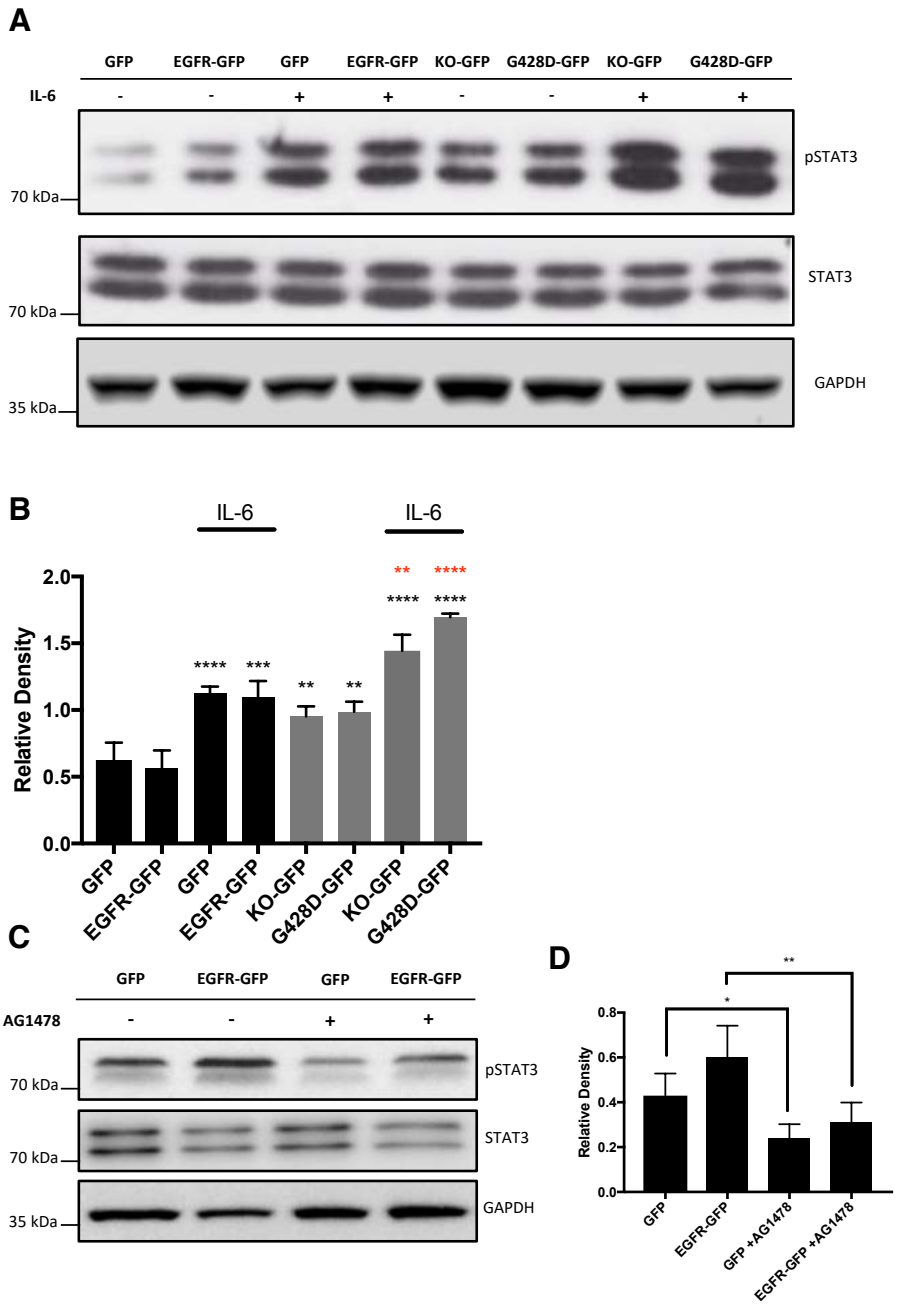


Figure 5.2: IL-6 stimulation leads to increased pSTAT3 levels in all EGFR-2 cell lines whereas acute EGFR inhibition leads to reduced pSTAT3 levels (A) Western blot showing pSTAT3 and STAT3. Lysates extracted from confluent monolayers of EGFR-2 cells grown under basal conditions stimulated with or without 10ng/ml recombinant IL-6 for 30mins. Representative of three independent experiments (B) Graph showing quantification of densitometry on western blots normalised to GAPDH loading control. (C) Western blots showing pSTAT3 and STAT3. Lysates extracted from confluent monolayers of EGFR-2 cells grown under basal conditions treated with or without AG1478 for 4hrs. Representative of three independent experiments. (D) Graph showing quantification of densitometry on western blots normalised to GAPDH loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way and two-way ANOVA are indicated as follows, * $P < 0.01$, ** $P < 0.001$, **** $P < 0.00001$. * indicates comparison to basal GFP expressing cells. * indicates comparison between IL-6 treated cell lines against GFP expressing cells treated with IL-6.

5.2.3 SHP2 activation is abrogated in EGFR knockout, inhibited or G428D re-expression EGFR-2 cells

Having demonstrated that STAT3 activation is altered down stream of EGFR knockout or G428D mutant re-expression, we next turned to SHP2 activation state, a known regulator of STAT3 activation.

EGFR-2 cells were grown to confluent monolayers and then left in normal growth media or starved for 24hrs. Lysates were then run on SDS-page gels before being transferred and blotted for total SHP2, pSHP2 at Y542 and loading control GAPDH. Y542 is a major phosphorylation site of SHP2 and has been shown to interact with Grb2 directly downstream of EGFR (Ahmed 2019). The resultant blots showed significantly higher levels of pSHP2 in GFP and EGFR-GFP cells compared with KO-GFP and G428D-GFP (Figure 5.3A,B). In order to determine whether the altered active SHP2 levels were also seen following acute EGFR inhibition, cells were treated with AG1478 (as in Figure 5.2). Data demonstrated that pSHP2 levels were also significantly reduced in KO-GFP and G428D-GFP cells compared to EGFR-expressing cells. These data combined demonstrate that loss or inhibition of EGFR, or expression on G248D-EGFR, all result in reduced active SHP2.

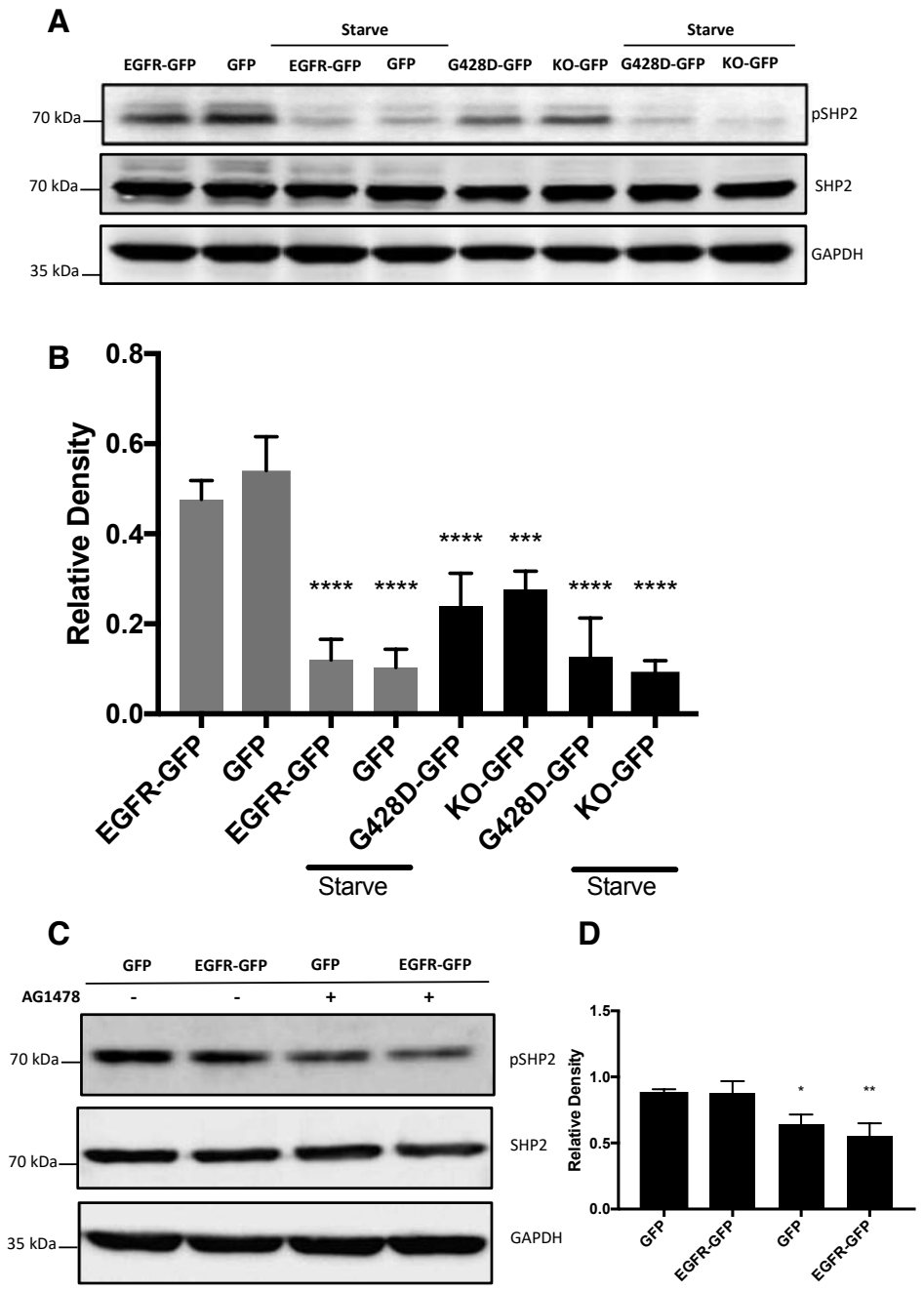


Figure 5.3: SHP2 activation is abrogated in EGFR knockout and G428D re-expression EGFR-2 cells grown under basal conditions and after acute EGFR inhibition. (A) Western blot showing phospho-SHP2 and SHP2. Lysates extracted from confluent monolayers of EGFR-2 cells grown under basal or starved conditions. Representative of three independent experiments (B) Graph showing quantification of densitometry on western blots normalised to GAPDH loading control. (C) Western blots showing pSTAT3 and STAT3. Lysates extracted from confluent monolayers of EGFR-2 cells grown under basal conditions treated with or without AG1478 for 4hrs.Representative of three independent experiments. (D) Graph showing quantification of densitometry on western blots normalised to GAPDH loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way and two-way ANOVA are indicated as follows, * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$ **** $P < 0.00001$.

5.2.4 STAT1 activation is unaffected by EGFR-knockout or G428D mutant re-expression.

The analysis of the RNAseq data suggested that the inflammatory factors up-regulated after EGFR-knockout or G428D mutant re-expression, could be regulated by STAT1 as well as STAT3. For this reason, active STAT1 levels were also investigated in the EGFR-2 cell lines.

Confluent monolayers of EGFR-2 cell lines were grown in either normal growth media or starved for 24hrs before having lysates extracted and analysed by western blotting for total STAT1, pSTAT1 and GAPDH loading control.

Resulting blots and densitometry analysis demonstrated a significant reduction in pSTAT1 in starved conditions compared to growth conditions in all four EGFR-2 cell lies (Figure 5.4A,B). However no significant difference in the levels of pSTAT1 were seen between GFP/EGFR-GFP cells and KO-GFP/G428D-GFP cells. This suggests that STAT1 is differentially regulated to STAT3 upon EGFR knockout or G428D-EGFR in keratinocytes.

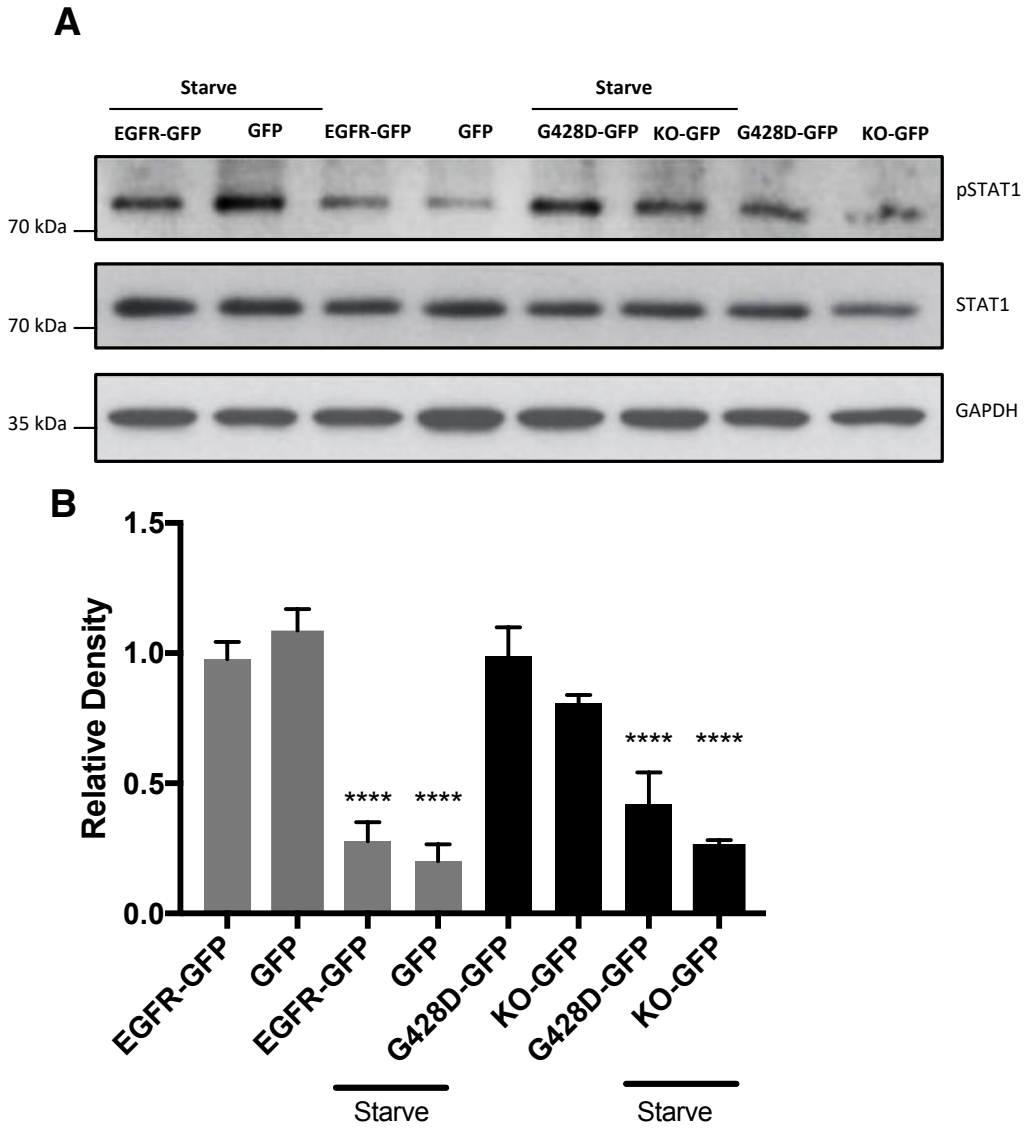


Figure 5.4: STAT3 activation is unaffected by EGFR-knockout or G428D mutant re-expression. (A) Western blot showing phospho-STAT1 and total STAT1. Lysates extracted from confluent monolayers of EGFR-2 cells grown under basal or starved conditions. Representative of three independent experiments (B) Graph showing quantification of densitometry on western blots normalised to GAPDH loading control. Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA. **** $P < 0.00001$

5.2.5 STAT3 inhibition rescues the inflammatory phenotype in EGFR null keratinocytes.

As data in the previous chapter showed a number of up-regulated SkInFs after EGFR knockout and G428D mutant re-expression, and this is coupled with higher levels of active STAT3, the next experiments were aimed to test whether EGFR-dependent STAT3 activation contributed to the increase in observed altered transcripts.

In order to determine this, the specific STAT3 inhibitor 5,15-DPP was used, that inhibits STAT3 dimerization *via* Src homology 2 (SH2) domains, preventing nuclear translocation and DNA binding. Importantly, 5,15-DPP has a negligible effect on STAT1 and does not affect Grb2 (Uehara et al. 2009).

EGFR-2 cell lines were grown to confluent monolayers under growth conditions and treated with either DMSO or 5mM 5,15-DPP for 2hrs. RNA was then extracted and used for QPCR analysis of target genes identified in the previous chapter (CCL2/5/20/27, CXCL10, MMP9 and MMP10). Resulting data revealed that treatment of EGFR-GFP cells with 5,15-DPP had no effect on any of the target genes analysed (Figure 5.5A). However, a significant reduction in levels of CCL2, CCL5, CXCL10 and CCL20 were seen in both G428D-GFP and KO-GFP cells after 5,15-DPP treatment (Figure 5.5A) Interestingly, 5,15-DPP treatment had no effect on the levels of MMP9 and MMP10 in any EGFR-2 cell lines (Figure 5.5A). Analysis of lysates from control or treated cells confirmed that 5,15-DPP treatment effectively inhibits pSTAT3 in GFP expressing EGFR-2 cells (Figure 5.5B).

The Blots in Figure 5.5 (C) demonstrate that 5,15-DPP has no effect on active pSHP2 or total SHP2 levels in EGFR-2 cell lines. This data suggests that there is no negative feedback on active SHP2 levels after STAT3 inhibition.

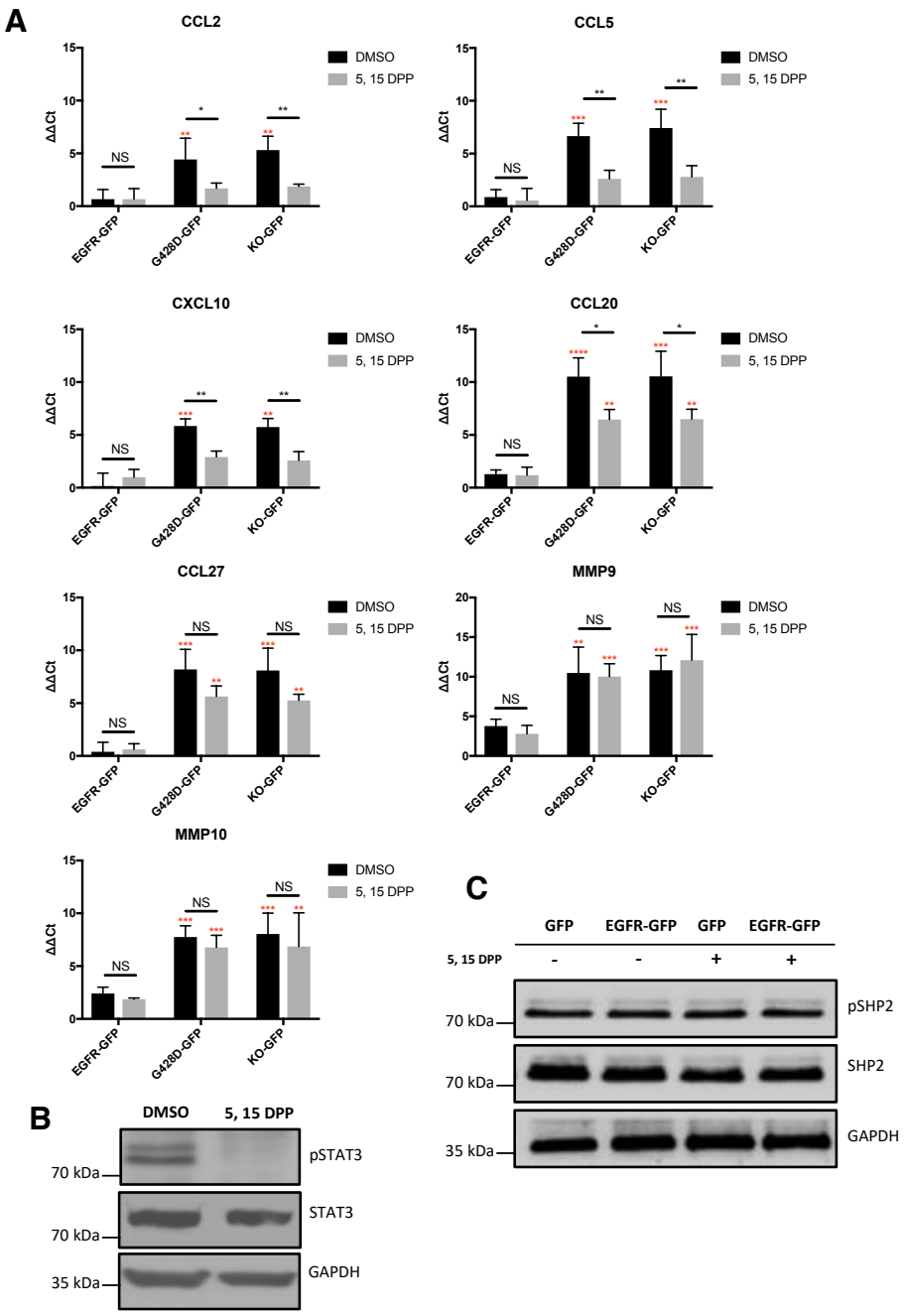


Figure 5.5: STAT3 inhibition rescues non-inflammatory normal phenotype in EGFR-2 keratinocytes. (A) Graphs showing qPCR analysis for specified targets in EGFR-2 cell lines treated with or without STAT3 inhibitor 5, 15-DPP. RNA expression ddCt values of specified target proteins was normalised to EGFR-2 GFP expressing cells. (B) Western blot showing pSTAT3, total STAT3 and GAPDH loading control. Lysates extracted from confluent monolayers of GFP expressing EGFR-2 cells treated with either DMSO or 5, 15-DPP (C) Western blot showing pSHP2, total SHP2 and GAPDH loading control. Lysates extracted from confluent monolayers of EGFR-2 cells treated with either DMSO or 5, 15-DPP. Representative of three independent experiments Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA * $P < 0.01$, ** $P < 0.001$. * indicates significant change over GFP expressing cells.

5.2.6 SHP2 inhibition leads to an inflammatory phenotype and increased levels of pSTAT3 in keratinocytes

Data thus far has demonstrated that loss of EGFR activity leads to higher active pSTAT3 and an increased inflammatory gene signature, that can be partly inhibited by blocking STAT3 activation. The next experiments were aimed at determining whether SHP2 played a role in the regulation of STAT3 in EGFR-2 cells and the resulting inflammatory phenotype of EGFR knockout and G428D mutant re-expressing EGFR-2 cells.

In order to test this possibility, EGFR-2 cell lines were grown to confluent monolayers under basal conditions and treated with either DMSO or 10µM SHP099 for 2hrs. SHP099 is small molecule inhibitor that stabilizes SHP2 in an auto-inhibited conformation leading to loss of action of the enzyme upon the target substrates. Following treatment with SHP099, RNA was extracted and used for QPCR to quantify levels of the same transcripts analysed in Figure 5.5. Resulting data demonstrated that EGFR-GFP cells treated with SHP099 showed significantly increased levels of CCL2, CCL5, CXCL10 and CCL20 (Figure 5.6A). Conversely, SHP099 had no effect of the levels of SkInFs in either G428D-GFP or KO-GFP cell lines. In all EGFR-2 cells, SHP099 treatment had no effect on the expression of SkInFs (Figure 5.6A). The blots in Figure 5.6 (B) showed that SHP099 effectively inhibited pSHP2 in GFP expressing EGFR-2 cells.

It was then important to test whether or not this inhibition of SHP2 has an impact on the levels of pSTAT3 in all EGFR-2 cell lines. The blots in Figure 5.6 (C) shows that both GFP and EGFR-GFP cells treated with SHP099 exhibited increased levels of pSTAT3 compared to DMSO control, indicating that blocking SHP2 activity has a positive impact on STAT3 activation.

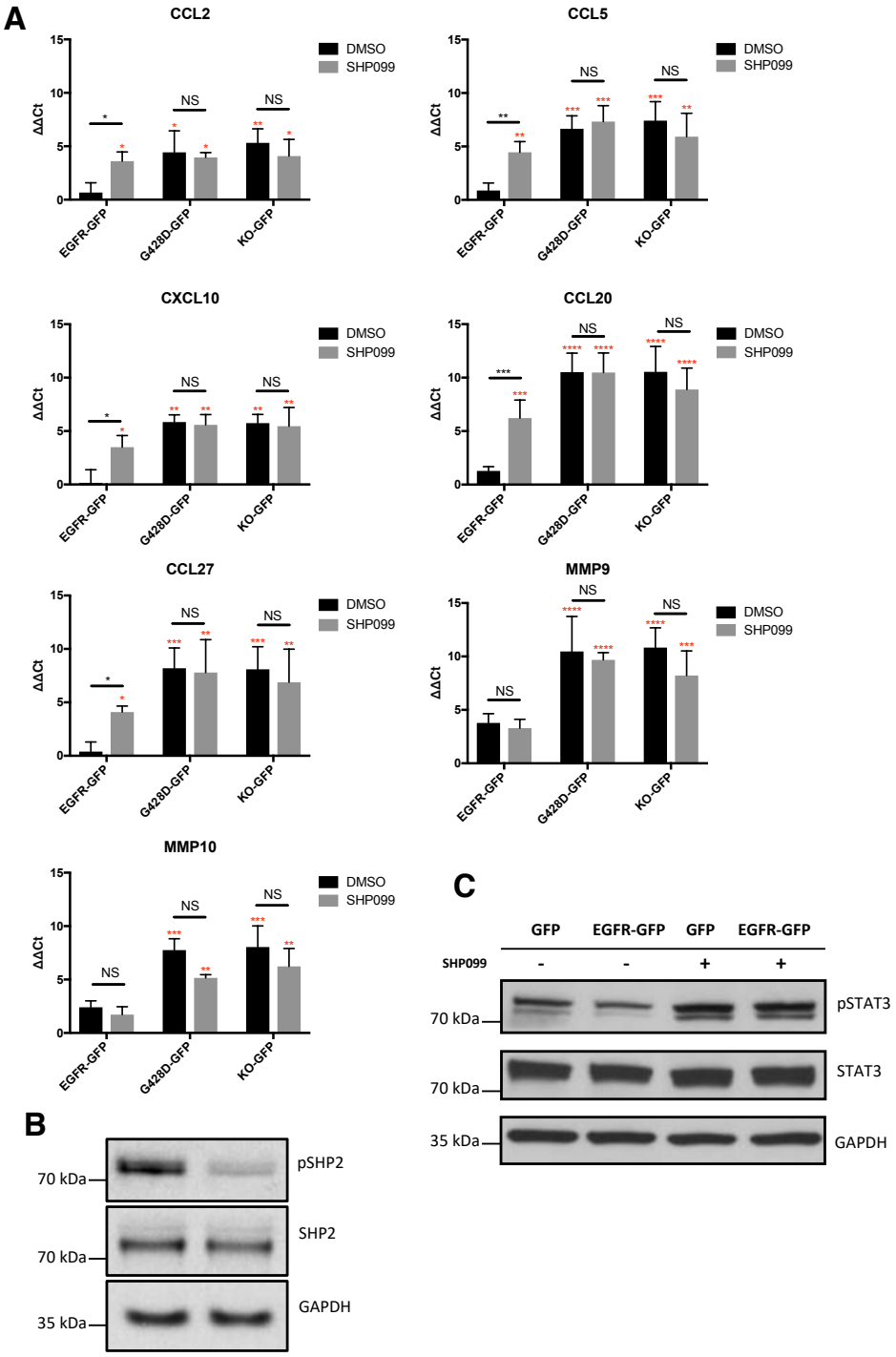


Figure 5.6: SHP2 inhibition drives inflammatory phenotype and increased levels of pSTAT3 in WT EGFR expressing cells. (A) Graphs showing qPCR analysis for specified targets in EGFR-2 cell lines treated with or without SHP2 inhibitor SHP099. RNA expression ddCt values of specified target proteins was normalised to EGFR-2 GFP expressing cells. (B) Western blot showing pSHP2, total SHP2 and GAPDH loading control. Lysates extracted from confluent monolayers of EGFR-2 cells treated with either DMSO or SHP099. (C) Western blot showing pSTAT3, total STAT3 and GAPDH loading control. Lysates extracted from confluent monolayers of EGFR-2 cells treated with either DMSO or SHP099. Representative of three independent experiments Data is presented as mean +/- SEM. Statistical analysis performed by one-way ANOVA * P < 0.01, ** P < 0.001, *** P < 0.0001 **** P < 0.00001. * indicates significant change over GFP expressing cells.

5.2.7 KO-GFP cell conditioned media increases the level of active STAT3 in keratinocytes

Previous data in this chapter showed that pSHP2 and pSTAT3 levels are differentially affected by EGFR knockout or G428D mutant re-expression in EGFR-2 keratinocytes. The next question to address was whether the secreted SkInFs that were up-regulated in G428D-GFP and KO-GFP cells could contribute to the inflammatory phenotype by altering pSTAT3 and pSHP2 levels in an autocrine fashion.

To test this hypothesis, growth media containing secreted SkInFs from KO-GFP cell monolayers was removed after 24hrs of culture and used to stimulate GFP expressing EGFR-2 cells for 4hrs. This was performed in the presence of either DMSO or SHP099 to determine any potential contributions from SHP2 to the activation of STAT3. Cells were then lysed and subjected to western blotting analysis of pSHP2 and pSTAT3.

Resulting blots and quantification demonstrated that in all conditions, using SHP099 led to significantly decreased pSHP2 levels (Figure 5.7A,B). However, the addition of KO cell media had no effect on pSHP2 levels in these cells. In contrast, pSTAT3 levels were significantly increased following stimulation with KO media and this increase was also observed in cells treated with both KO media and SHP099 (Figure 5.7A and C). These data combined demonstrate that factors secreted by EGFR knockout keratinocytes can promote enhanced levels of active STAT3 in WT keratinocytes.

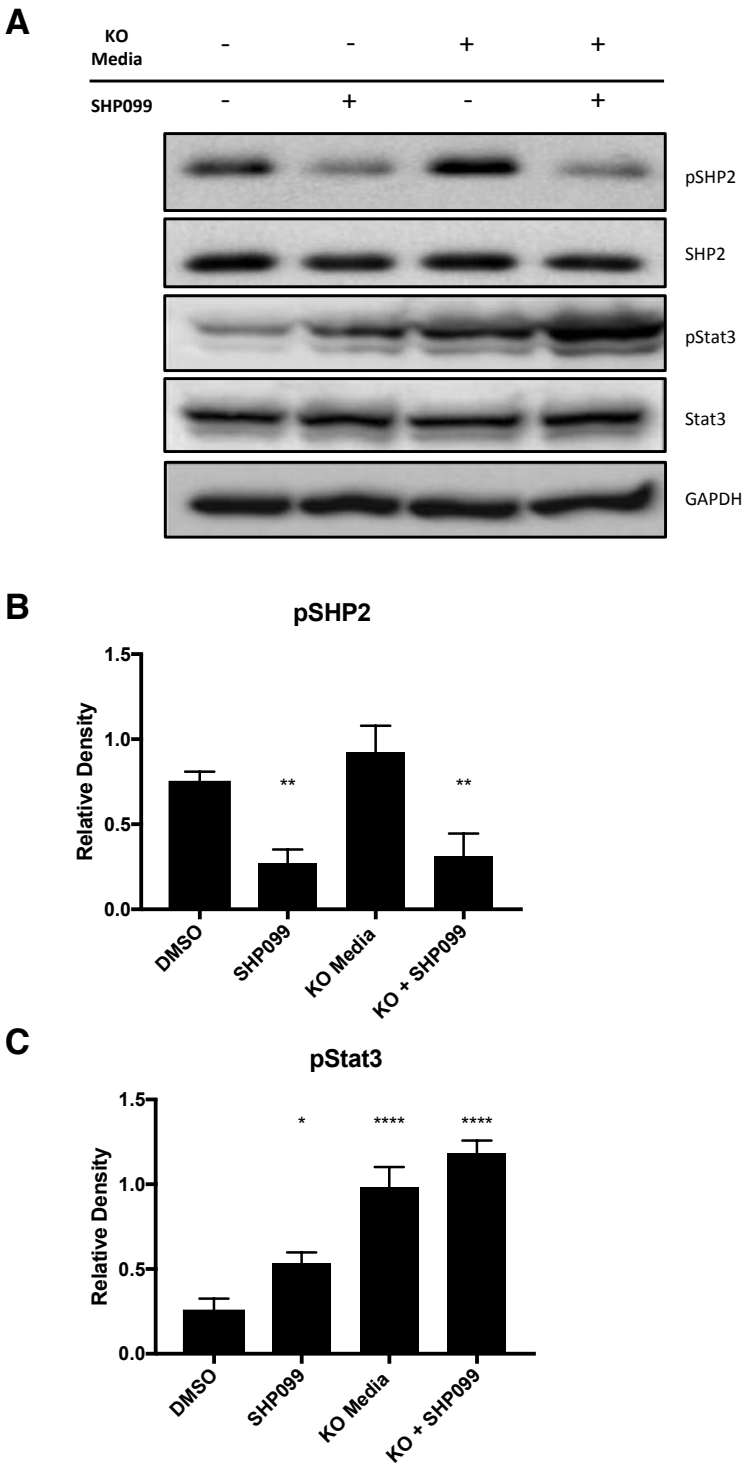


Figure 5.7: Conditioned media from KO-GFP cells increases the level of active pSTAT3 in GFP expressing EGFR-2 cells (A) Western blot showing pSTAT3, total STAT3, pSHP2, total SHP2 and GAPDH loading control. Lysates extracted from confluent monolayers of GFP expressing EGFR-2 cells treated with either DMSO, KO cell Media, SHP099 or a combination. Representative of three independent experiments. (B) Densitometry plot for pSHP2 blot. (C) Densitometry plot for pSTAT3 blot. Data is presented as mean +/- SEM. STATistical analysis performed by one-way ANOVA * P < 0.01, ** P < 0.001, **** P < 0.00001

5.3 Discussion

Data presented in this chapter demonstrated that EGFR knockout or G428D mutant re-expression in EGFR-2 keratinocytes leads to an increase in pSTAT3 levels and a decrease in pSHP2 levels under basal conditions. Furthermore, acute inhibition of STAT3 was sufficient to significantly reduce the expression of inflammatory cytokines in KO-GFP and G428D-GFP cell lines, while acute inhibition of SHP2 was sufficient to significantly increase the expression of inflammatory cytokines in GFP and EGFR-GFP expressing cells. In addition, EGFR knockout or G428D mutant re-expression had little effect on the activation state of STAT1. Data in this chapter also demonstrated that media taken from KO-GFP cells was sufficient to induce an increase in pSTAT3 in GFP expressing EGFR-2 keratinocytes.

5.3.1 Chronic EGFR dysregulation leads to an increase in STAT3 activation that is not seen after acute inhibition of EGFR

Data in this chapter has shown that under basal conditions, there are elevated levels of pSTAT3 in EGFR knockout and G428D mutant re-expression keratinocytes (Figure 5.1). This is an interesting and unexpected finding as it is known that acute inhibition of EGFR leads to a reduction in pSTAT3 in keratinocytes (W. J. Wang et al. 2017). However, due to the cell toxicity of AG1478, the majority of available literature where this is used to inhibit EGFR only tests pSTAT3 levels between approximately 20 minutes up to a few hours. This decrease in pSTAT3 has also been an expected outcome due to STAT3 phosphorylation being downstream of EGFR signalling, amongst other receptors. In the cells used in this thesis, where EGFR is stably knocked out with or without G428D mutant re-expression, allowed us to define that chronic lack of EGFR signalling leads to increased levels of pSTAT3, which has not been previously reported.

Interestingly, in cases where STAT3 is constitutively activated *in vivo* in mouse models, there was observable skin hyperplasia and up-regulation of some terminal

differentiation markers in isolated primary keratinocytes (Orecchia et al. 2015). These features closely resemble phenotypes of the skin during chronic inflammation where elevated STAT3 levels have been shown (Yu et al. 2009). Moreover, these features are shared in the skin of the patients harbouring the G428D mutation (Campbell et al. 2014). It would be interesting to interrogate pSTAT3 levels in primary keratinocytes isolated from mice with conditional epidermal EGFR knockout that have been used in other studies (F Mascia et al. 2013). It would also be of interest to study the levels of pSTAT3 in the keratinocytes of mice treated with clinical EGFR inhibitors on a similar drug course as prescribed to human cancer patients as these patients also demonstrate mild epidermal hyperplasia and inflammation (Holcmann and Sibilia 2015).

Another interesting area of study would be to understand at what point the level of pSTAT3 begins to increase after the initial decrease following EGFR inhibition or knockout. This was not possible in our system as immediately following infection with CRE lentivirus, the EGFR-2 cell lines become very unhealthy and mildly senescent, requiring between 5-10 days to fully recover and begin to proliferate in KGF-enriched media. As this also happens to the EGFR-2 cells when infecting with non-CRE lentiviruses, it is not possible to say whether this senescence and lack of proliferation is a direct effect of EGFR knockout or an off target broad effect of lentiviral infection.

5.3.2 Increased STAT3 activity may be driven by the loss of active SHP2 after EGFR knockout or G428D mutant re-expression, as seen by SHP2 inhibition in WT EGFR expressing cells.

As the elevated levels of STAT3 was a surprising find downstream of EGFR knockout and G428D mutant re-expression, it was important to elucidate whether or not EGFR dysregulation had an effect on any STAT3 regulators. Previous published data has shown that SHP2, which is activated downstream of EGFR, can potently de-phosphorylate STAT3 and plays an active role in attenuating normal STAT3 activation downstream of IL-6 signalling (Ohtani et al. 2000).

The data presented in Figure 5.3 demonstrates that level of active SHP2 is decreased following EGFR knockout or G428D mutant re-expression. Previous studies have also demonstrated that active SHP2 levels are decreased after acute EGFR inhibition (Y. C. Wang et al. 2018). The data presented here also shows this pSHP2 decrease following EGFR inhibition using AG1478. Interestingly it has also been shown that inhibition of SHP2 abrogates EGFR signalling as SHP2 has been shown to prolong EGFR activation and downstream signalling (Sun et al. 2017). Despite this, data in this chapter has demonstrated that the loss of pSTAT3 dephosphorylation after SHP2 inhibition outweighs any potential attenuation of EGFR signalling in regulating pSTAT3 levels. To date, there have been no publications that have studied the feedback loop between SHP2 and STAT3 activation specifically downstream of EGFR in the skin.

Data in this chapter also shows that inhibition of SHP2 in cells expressing wild-type EGFR leads to elevated levels of pSTAT3 under basal conditions (Figure 5.7). This is the first indication that SHP2 may play a homeostatic role in regulating over-activation of STAT3, and that this is downstream of basal EGFR signalling. However, constitutively active EGFR mutations in cancers have been shown to lead to both increased levels of active SHP2 and STAT3 (Furcht et al. 2014), demonstrating that this potential feedback loop can be manipulated by both loss-of and gain-of function in EGFR.

5.3.3 The inflammatory phenotype driven by EGFR dysregulation can be partially rescued by STAT3 inhibition

Data in the previous chapter demonstrated that EGFR dysregulation via EGFR knockout or G428D mutant re-expression was sufficient to drive an inflammatory phenotype in EGFR-2 keratinocytes and primary keratinocytes from an $EGFR^{-/-}$ mouse in the form of upregulation of many inflammatory mediators such as cytokines and chemokines. As the data in this chapter uncovered a significant increase in pSTAT3 after EGFR dysregulation, it was important to test whether or not this was helping to drive this inflammatory phenotype.

The data in Figure 5.5 illustrates that upon STAT3 inhibition with 5,15-DPP, there is a partial rescue of EGFR KO or mutant cells back to a homeostatic phenotype. CCL2, CCL5, CCL20 and CCL27 levels are all increased in KO-GFP and G428D-GFP cell lines under basal conditions and these levels are significantly decreased after STAT3 inhibition. Although this decrease is not to the expression levels seen in GFP and EGFR-GFP cell lines, this may be due to the timing of the interrogation after 5,15-DPP treatment. Unfortunately, due to the toxicity of the inhibitor it was impossible to get trustworthy results at longer time points. This finding is in line with current publications that also demonstrate that STAT3 inhibition drives downregulation of these chemokines in other cell types such as neurons (Fletcher et al. 2019), vascular smooth muscle cells (Daniel et al. 2012) and T-cells (Ikeda et al. 2016) amongst others.

Interestingly, the data also demonstrated that STAT3 inhibition decreased CXCL10 expression in KO-GFP and G428D-GFP cells. This was a surprising finding as previous literature has suggested that in STAT3 knockout keratinocytes there is a prolific upregulation of CXCL10 (Archer et al. 2017) as well as in CD8+ T-cells (C. Yue et al. 2015). The data in Figure 5.5A does show that although not significant, there is an increase in CXCL10 in EGFR-GFP cells after STAT3 inhibition. This suggests that the decrease in CXCL10 may be caused by a cooperative effect STAT3 inhibition but also EGFR knockout or G428D mutant re-expression. Alternatively, this could suggest that another regulator of CXCL10 may be regulated by EGFR that was not tested in this study and competes with STAT3 in the regulation of CXCL10 expression. Previous publications have shown that STAT1 can drive CXCL10 expression (Tomita et al. 2017). However, the data in this chapter reveals that there is no change in STAT1 activation driven by EGFR dysregulation. Other studies have established that overexpression and activation of EGFR in cancers leads to increased STAT1 activity (Cheng et al. 2018), demonstrating that EGFR knockdown is unlikely to simply be the reverse of overexpression.

Data presented in this chapter also established that the overexpression of both MMP-9 and MMP-10 in KO-GFP and G428D-GFP cells is unaffected by STAT3 inhibition. This was surprising as STAT3 has been shown to upregulate MMP-9 (Jia et al. 2017) and

MMP-10 (X. Zhang et al. 2009), however both of these studies were in cancer models. As the relationship between STAT3 and MMP-9/10 has not been widely researched in keratinocytes, the data presented here demonstrates that although EGFR dysregulation clearly upregulates both proteases, STAT3 is unlikely to play a key role in this process.

Conversely to STAT3 inhibition, SHP2 inhibition with SHP099 had no effect on the chemokine levels in EGFR knockout or G428D mutant re-expression cells. This was expected as the data has demonstrated that pSHP2 levels are already reduced in these cells. However, the data in Figure 5.6 shows that SHP2 inhibition in WT EGFR expressing cells does increase levels of these chemokine. Taken together with the data from Figure 5.5, this suggests that SHP2 inhibition in WT EGFR expressing keratinocytes increases pSTAT3 which drives chemokine expression.

5.3.4 Secreted SkInFs by EGFR knockout and G428D mutant re-expression cells further drive the inflammatory phenotype in a cell-autonomous fashion.

As previously published, the receptors for CCL2 (Tian et al. 2017), CCL5 (Tang, Jiang, and Liu 2015), CCL20 (Yamazaki et al. 2008) and CCL27 (Davila et al. 2016) all signal downstream via STAT3. For this reason, it was important to determine whether the cytokines secreted by KO-GFP cells could potentially drive the inflammatory phenotype. The data demonstrates that the addition of KO-GFP media to GFP cells does indeed increase pSTAT3 levels. Importantly, this increase is exacerbated by the addition of SHP2 inhibitor. This suggests two co-operative features enable this inflammatory phenotype to persist when EGFR is dysregulated. Firstly, dysregulation of EGFR leading to a loss of active SHP2 leading to an increase in pSTAT3. Then in turn, increased chemokine expression acting back on the keratinocytes in a cell-autonomous fashion to further drive pSTAT3 expression. It would be of interest to study the effect of recombinant chemokines in driving the mRNA expression of the other SkInFs in the cell models used here along with SHP2 and or STAT3 inhibition.

In conclusion, data in this chapter has demonstrated that the inflammatory phenotype driven by EGFR dysregulation is in part regulated by a STAT3/SHP2 axis. However, other transcription factors are very likely either effected directly downstream of EGFR, or potentially downstream of cytokines or chemokines that may be responsible for further characteristics of the inflammatory phenotype such as the MMP-9/10 overexpression.

6. Discussion

6.1 Final Discussion

Data in this thesis has demonstrated that loss of EGFR signalling or protein expression in basal keratinocytes leads to a pro-inflammatory epithelial cell phenotype and that this is driven in the absence of any major barrier defects *in vitro* apart from the appearance of larger focal adhesions (Summarised in Figure 6.1).

The phenotype has been shown to include the up-regulation of many chemokines and cytokines involved in Th2 inflammation such as IL-33, CCL2, CCL5, CCL20 and CCL27. The data also shows upregulation of MMPs and their active ability to break down the ECM. This suggests that under normal conditions, EGFR signalling or expression is required for the regulation of epithelial-derived immune mediators in the epidermis. Additionally, data presented demonstrates that loss of EGFR signalling or expression leads to an accumulation of active pSTAT3 driven by a loss of active SHP2, and that this drives the upregulation of many of the inflammatory chemokines (Summarised in Figure 6.2). The combined data therefore demonstrates that the EGFR is a critical receptor in the regulation of epithelial inflammation and demands further study into its potential role as a drug target in the treatment of certain inflammatory skin conditions.

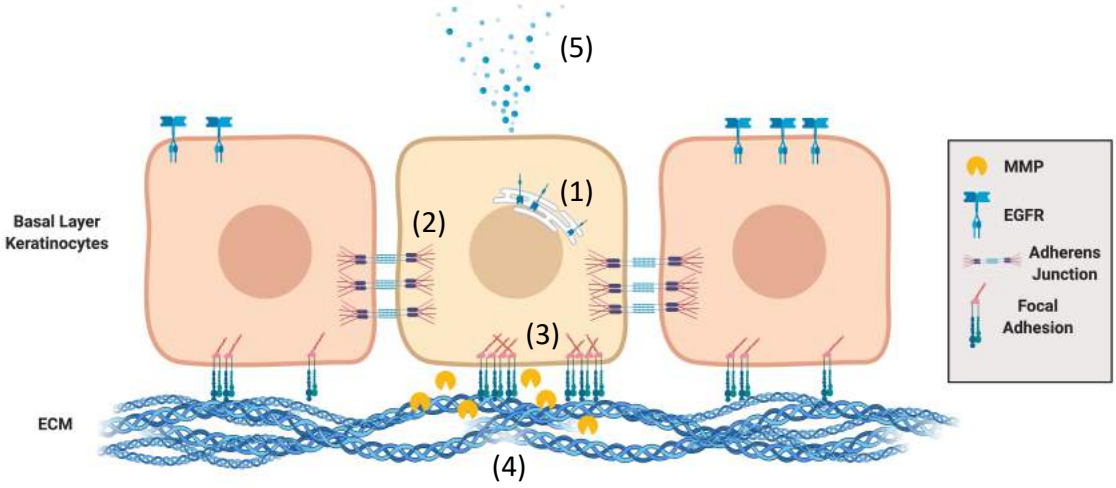


Figure 6.1: Model of the effect of EGFR knockout or G428D mutant re-expression in basal keratinocytes. Middle cell represents WT-EGFR knockout cell expressing G428D mutant receptor, flanked by cells expressing WT-EGFR. (1) Cell expresses no surface EGFR and G428D mutant receptor is primarily located in the ER. (2) Adherens junctions between cells are unaffected in both structure and measured by monolayer porosity. (3) Larger focal adhesions are present in knockout and G428D expressing cells compared with normal keratinocytes. Active MMP molecules are up-regulated compared to normal cells and these contribute to the degradation of the ECM. (5) Knockout or G428D mutant expressing cells have increased secretion of a plethora of pro-inflammatory chemokines/cytokines.

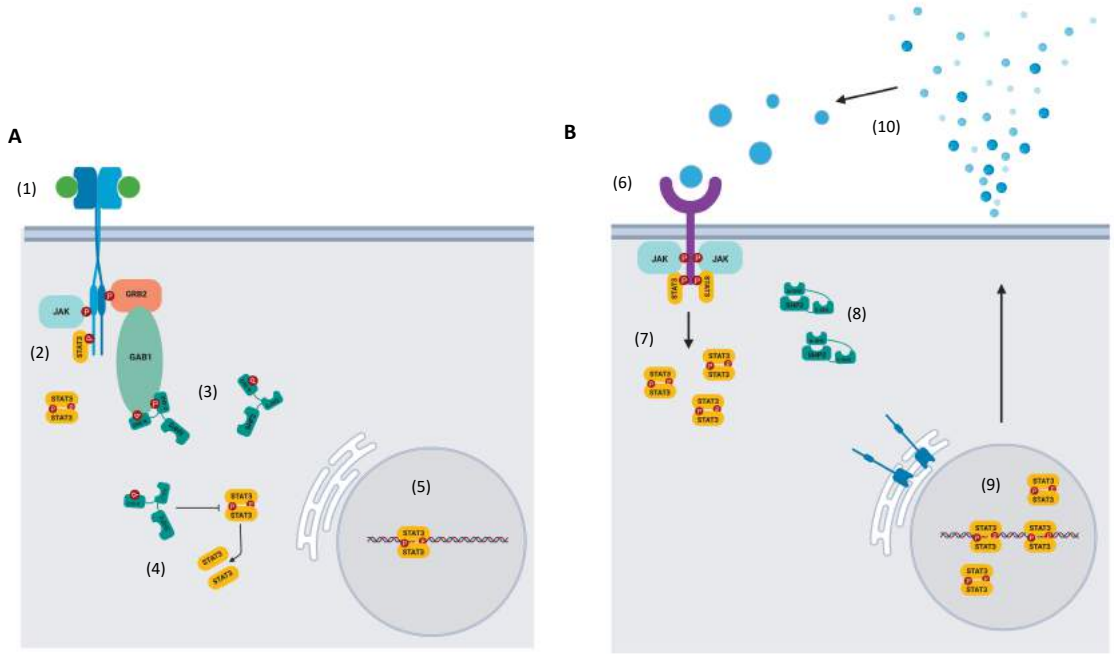


Figure 6.2: Breakdown of homeostatic SHP2/STAT3 feedback loop drives expression of inflammatory mediators in basal keratinocytes. (A) In a WT basal keratinocytes there is normal surface expression of EGFR (1) allowing the receptor to bind to ligand and drive downstream signalling. (2) EGFR phosphorylation leads to STAT3 phosphorylation via JAK. EGFR phosphorylation also leads to the phosphorylation of GAB1 via adapter protein GRB2. (3) GAB1 phosphorylation drives the phosphorylation of SHP2 into its active state. (4) Active SHP2 potently inhibits pSTAT3 dimers resulting in dephosphorylation. (5) Limited amounts of pSTAT3 are able to localise to the nucleus for gene transcription leading to cell processes such as survival and cell growth. (B) Keratinocytes expressing no EGFR or expressing G428D mutant have no surface EGFR receptor available for ligand binding. (6) Various receptors, including chemokine/cytokine receptors, bind to ligand and drive intracellular signalling. (7) Many of these receptors drive the upregulation of pSTAT3. (8) The absence of EGFR leads to a reduction in active SHP2, with most SHP2 locked in its auto-inhibitory state. (9) Loss of active SHP2 leads to over-accumulation of pSTAT3 and an excess of pSTAT3 activity. Excessive pSTAT3 drives alternative gene transcription and cellular processes such as over-expression and secretion of pro-inflammatory mediators. (10) Secreted inflammatory mediators further exacerbate the phenotype in a cell-autonomous fashion.

6.1.1 EGFR regulates inflammation in basal layer keratinocytes with no evidence of barrier function defects

Data presented here demonstrates that normal EGFR expression and/or signalling regulates expression of inflammatory mediators in basal keratinocytes. Previous studies have demonstrated that the level of cytokines such as CCL2, CCL5 and CXCL10 that are up-regulated during inflammation can be reduced in the epithelium by stimulation of EGFR with EGFR-ligand (Yamaki et al. 2010; Francesca Mascia et al. 2003). It has been suggested that the up-regulation of these chemokines are driven by elevated levels of IFNγ and TNFα produced by immune cells at the site of inflammation (Van Den Bogaard et al. 2014). Taken together with the data presented here, this suggests that under normal conditions EGFR signalling negatively regulates keratinocyte chemokine expression and that IFNγ and/or TNFα are necessary to overcome this negative regulation. It also suggests that epidermal barrier breakdown is a precursor to epidermal inflammation due to the requirement of immune infiltration to drive epithelial inflammatory mediators. However, data presented in this thesis demonstrates that in the absence of any major barrier malfunction or the presence of immune cells, EGFR dysregulation is sufficient to drive an inflammatory phenotype in keratinocytes.

Despite the lack of any barrier malfunction in the data presented here, it is likely that EGFR knockout or G428D mutant re-expression does have an effect of the epidermal barrier. Patient biopsies from G428D mutant harbouring patients show clear thickening of the upper layers of the epidermis as well as widening of spaces between keratinocytes (Campbell et al. 2014; Ganetzky et al. 2015). The phenotype closely resembles that of inherited abnormality of desmosomes (Petrof, Mellerio, and McGrath 2012). As this study focused on basal layer keratinocytes, desmosomal abnormalities were impossible to detect as epidermal desmosomes are primarily expressed between suprabasal keratinocytes (White and Gohari 1984; Wan et al. 2003). Interestingly, genes encoding for proteins associated with desmosomes such as desmogleins, desmocollins, desmoplakin, plakoglobin, and plakophilins (Garrod and Chidgey 2008) were not differentially expressed in KO-GFP or G428D-GFP cell lines.

Additional investigations would need to be carried out to determine the role of EGFR in desmosome formation. Assays involving the generation of 3D epidermal *in vitro* models were attempted for this however EGFR-2 cells failed to differentiate much further than the basal layer and thus this would require more optimisation. Interestingly another study has demonstrated that acute EGFR inhibition actually promotes desmosome assembly and can strengthen adherens junctions in squamous cell carcinoma cells suggesting that the relationship between EGFR and barrier integrity is a complex one and potentially cell type specific (Lorch et al. 2004).

Another reason it is likely that EGFR dysregulation can perturb epidermal barrier formation is the relationship between E-cadherin and EGFR in the control of tissue polarisation and tight junction formation. Tight junctions in the epidermis are crucial for epithelial polarisation and the barrier function of the outer layers of the epidermis and previous studies have demonstrated that trans-epithelial-resistance, a measure of tissue barrier function, is lowered after EGFR overactivation (Singh and Harris 2004). Interestingly it has also been demonstrated that acute inhibition of EGFR leads to a reduction of tight junctions in the epidermis (Rübsam et al. 2017). E-cadherin plays a major role in the formation of these tight junctions and has also been shown to co-localise at junctions and e-cadherin can transactivate EGFR (Fedor-Chaiken et al. 2003).

Despite the evidence that EGFR likely plays a role in barrier function and formation in the epidermis, it is clear from the data presented in this thesis that the observed pro-inflammatory phenotype is not driven as a result of barrier malfunction but is driven purely by the loss of EGFR expression or signalling. This goes against the common notion that epidermal inflammation is preceded by barrier malfunction. This notion has been questioned before in the case of psoriasis where it has been demonstrated that the deletion of Jun proteins in keratinocytes is sufficient to drive psoriasis-like skin conditions in mice (Zenz et al. 2005).

6.1.2 The activity of STAT3 and SHP2 play a role in driving the inflammatory phenotype downstream of EGFR and could be targeted to combat EGFR inhibition induced epidermal inflammation.

Although EGFR knockout and inhibition has been shown to lead to epidermal inflammation, the downstream pathway controlling this is not well understood. Data form this thesis has shown a novel finding that after EGFR knockout or long-term expression of G428D mutant receptor, there is a striking accumulation of pSTAT3. Most studies concerning inhibition of EGFR utilise EGFR inhibitors in order to acutely inhibit the receptor due to the lethality of EGFR knockout. This acute EGFR inhibition characteristically involves a drop in pSTAT3 however the data presented here demonstrates that chronic EGFR dysregulation leads to a novel increase in pSTAT3 not seen before. By inhibiting pSTAT3, it was possible to reduce the expression of pro-inflammatory mediators suggesting that in the case of EGFR inhibition associated inflammation, STAT3 may be driving much of the phenotype. This data also demonstrates that this overactivity of STAT3 may be due to a loss of SHP2 activity downstream of EGFR as inhibiting SHP2 was sufficient to partially induce an inflammatory phenotype in WT keratinocytes and able to increase pSTAT3. Interestingly, many cancers harbour EGFR- gain-of-function mutations and/or EGFR is overexpressed. In these cancer cells, there is increased pSTAT3 and this is linked to the increased survival rate of the cancer cells (Quesnelle, Boehm, and Grandis 2007; Lo et al. 2005). This suggests that at a certain point SHP2 is insufficient in inhibiting STAT3 phosphorylation. In many of these cases STAT3 also plays a role in the driving of tumour associated inflammatory pathways (Nguyen et al. 2013). Combined with the data presented here, it can be surmised that EGFR is a crucial regulator of STAT3 activity in epithelial cells and that both too much EGFR or too little, is enough to hyperactivate STAT3 and drive inflammation.

In terms of epidermal inflammation associated with EGFR inhibition, the work in this thesis suggests that combined EGFR and STAT3 or JAK inhibition may alleviate the inflammatory side-effect. In fact, there has been a clinical trial using combination erlotinib (EGFR inhibitor) and ruxolitinib (JAK inhibitor) in the treatment of breast

cancer (H. A. Yu et al. 2017). Although the combined treatment has little effect on cancer progression, the study was based on using JAK inhibitors to reduce resistance to erlotinib. As erlotinib resistant patients rarely display any inflammatory skin conditions this trial failed to answer whether or not STAT3 inhibition could alleviate said side effects in erlotinib reactive patients.

6.1.3 Potential relevance to the role of EGFR in inflammatory skin conditions.

The role of EGFR in skin inflammation is somewhat limited to that of the skin rash associated with EGFR inhibitors and the patients harbouring loss-of-function EGFR mutations. STAT3 however has been studied extensively due to its role in inflammatory skin conditions and in particular, psoriasis. Due to the coupling of EGFR activity and STAT3 activity to the inflammatory phenotype of keratinocytes presented in this thesis, it would be of interest to study EGFR in the context of other inflammatory skin conditions.

STAT3 has been demonstrated to be over active in many cases of psoriasis (Calautti, Avalle, and Poli 2018). Elevated levels of active STAT3 have been shown to drive psoriasis in mouse models (Sano et al. 2005). Interestingly, in these cases, STAT3 overactivation is linked to the upregulation of many Th17 cytokines and chemokines including IL-23, IL-22 and IL-17 in the skin, none of which were upregulated in the model presented here. This suggests that STAT3 overactivation downstream of EGFR loss-of-function drives a unique epidermal inflammatory phenotype. The immune mediators greatly up-regulated in this study fall roughly into a Th2 compartment, with the caveat that there is no IL-4. Th2 inflammation in the skin is primarily related to the early and more commonly the late stages of wound healing (Loke et al. 2007; Allen and Wynn 2011). IL-33 in particular has been demonstrated to rapidly accelerate cutaneous wound healing (Yin et al. 2013). This is somewhat contradictory to the exhibited inflammatory conditions by both EGFR inhibition patients and G428D harbouring patients. In the case of the G428D mutation, the skin presents an epidermolysis bullosa simplex like phenotype. However, the phenotype presented in

this thesis may suggest a role for EGFR in regulating normal wound healing dynamics and that this is partially triggering the observed epidermal inflammatory response.

More broadly, the data presented suggests that in healthy individuals, expressing functional WT EGFR, the EGFR may be playing a role in protecting the epidermis from over-active immune responses. However, inflammatory skin conditions are commonly occurring diseases and there is little evidence to suggest there is any aberrant EGFR function in patients. This may suggest that the underlying cause of inflammatory skin disease is potent enough to overcome any protective function of EGFR. This means that EGFR likely plays a role in skin homeostasis as opposed to directly playing a role in the causality of many inflammatory diseases.

In sum, data from this thesis has explored the relationship between EGFR and epidermal inflammation and demonstrated that EGFR signalling regulates Th2-like inflammatory mediators in keratinocytes. The data has also demonstrated that it is possible to drive epidermal inflammation in the absence of any barrier malfunction, stress, or macrobiotic challenge. The data suggests that EGFR could be an important receptor to further study in the context of inflammatory disease and that STAT3/SHP2 may be potential co-targets in combatting the EGFR inhibition driven epidermal inflammation.

6.2 Future Directions

6.2.1 Investigate the role of EGFR dysregulation on suprabasal epidermal barrier function

As previously mentioned, the main barrier defects observed in the skin of G428D mutant harbouring patients is in the suprabasal layers of the epidermis. For this reason it would be of interest to further attempt to generate *in vitro* epidermal models using the EGFR-2 cell lines generated in this thesis. By doing this, it would be possible to observe the structure of desmosomes and the loacalisation of specific desmosomal proteins in relation to EGFR dysregulation. Importantly, this could be done in a clean environment lacking and immune infiltrate. 3D models would also allow a more in-depth study of wound healing in relation to EGFR dysregulation using published methods of studying re-epithelialisation stage wound healing *in vitro* (Deshayes et al. 2018).

6.2.2 Investigate the role of STAT3/SHP2 in epidermal inflammation *in vivo*

As the majority of $EGFR^{-/-}$ global knockout mice are still born and the rest die within a few days, many studies looking into EGFR related epidermal inflammation utilise the conditional epidermal EGFR knockout mouse model (Lichtenberger et al. 2013). Using this mouse model ,it would be possible to treat the mice with STAT3 inhibitors to see if it is possible to recapitulate the data presented here demonstrating a slight rescue in abrogating the inflammatory phenotype. We could also use this model to interrogate the activation state of both SHP2 and STAT3 in the skin pre and post EGFR knockout induction.

6.2.3 To understand the pathway controlling the upregulation of MMPs downstream of EGFR dysregulation

Although the data demonstrated that STAT3 was responsible for the up-regulation of a number of chemokines in keratinocytes, both MMP-9 and MMP-10 were not affected by STAT3 or SHP2 inhibition, suggesting that there is another downstream

regulator of these proteases. Interestingly, published studies have demonstrated that MMP-9 is sometimes downregulated after acute EGFR inhibition (Cowden Dahl et al. 2008; Hudson, Moss, and Stack 2009). In order to tease out what factors may be involved in the upregulation of MMPs observed in this thesis. PEA3 and AP-1 have been shown to be regulators of MMPs and that they are often in turn regulated by MAPKS such as ERK, JNK and p38, all of which are downstream of EGFR (Ellerbroek et al. 2001; Dahl, Zeineldin, and Hudson 2007). By interrogating PEA3 an AP-1 in our cell lines it may be possible to get an idea of where this MMP upregulation is coming from.

6.2.4 To understand the molecular basis for the changes in localisation and secretion of IL-33

An interesting finding in this thesis is the altered localisation and probable active secretion of IL-33 by keratinocytes after EGFR knockout or G428D mutant re-expression. Il-33 is becoming known as a major Th2 driving cytokine but is normal observed as an alarmin, only released from the cell body after cellular damage. The model generated here would be useful for the co-staining of IL-33 with a plethora of exosomal markers in order to figure out what secretory pathway may be responsible for its active release. By expressing fluorescently tagged IL-33 in keratinocytes it would be possible to observe the trafficking of Il-33 within the cell giving further clues to its secretory mechanism.

6.2.5 Define the effects of EGFR inhibition on immune cells in the skin

Although this thesis has focused entirely on the role of EGFR dysregulation on keratinocyte derived inflammation it is important to look further into its role on immune cells themselves. EGFR is almost ubiquitously expressed and has been shown to play a role in a number of immune cell processes such as the licensing of Th2 T cells to produce IL-13 in response to IL-33 during infection (Minutti et al. 2017). This is of particular interest in the case of those patients harbouring the G428D mutation as this mutation would also be present in the immune cells. This could be done by establishing a number of immune cell lines generated from the $EGFR^{fl/fl}$ mouse and

removing EGFR with Cre-recombinase, or alternatively using siRNA against EGFR in readily developed proliferative immune cell lines.

Appendix

Appendix Table 1. Genes up or down regulated in KO-GFP and G428D-GFP cells vs GFP cells with old increase or deacrease over 5x.

GENE	KO-GFP	G428D-GFP
ACOT4		-5.441
ADAMTS1	-14.76	-18.41
ADAMTSL4	16.782	
ADM	11.231	17.931
AKR1C3	5.564	12.264
ALDH3A1	5.322	17.022
ALG10B	-6.122	
ATF3	7.864	14.564
BCL6	10.045	5.825
BTG1	23.881	49.661
C1S	5.693	11.357
C3	18.703	24.367
CALM1	-17.885	-21.535
CCDC28A	-7.677	0.067
CCL2	44.075	49.739
CCL20	7.389	13.053
CCL27A	12.644	22.308
CCL5	15.688	21.352
CCNB1	-41.365	-45.015
CCND1	-6.871	-10.521
CCNF	-34.129	-37.779
CD276	-15.123	-18.773
CDC20	-28.746	-32.396
CDC25A	-5.002	-8.652
CDKN1C	5.401	14.065
CENPF	-12.448	-11.228
CLDN4		7.984
COL1A1	-22.37	-21.15
COL1A1	-26.193	-24.973
COL4A6	-20.896	-19.676
CSF2	6.023	8.687
CTPS	-5.002	-5.782
CXCL10	15.101	21.765
CXCL14	89.49	25.154
CXCL15	-5.576	-7.974
CYP1A1	34.957	40.621
CYP1A2	31.498	37.162

DAPL1	5.461	11.125
DIRC2		-7.53
DST	5.667	5.331
DUSP1	5.135	10.799
EGR1	-12.112	-10.892
F3	-8.987	-11.385
FERMT1	-11.774	-18.172
FLNB	-11.349	-13.747
FOXM1	-34.587	-36.985
GABARAPL1	6.781	12.445
GALC	-6.009	
GNB3	5.069	
GPN1	NA	-10.276
H2BC12	19.124	15.124
H2BC21	11.897	7.897
H2BC4	14.047	10.047
HAS3	-11.005	-13.403
HBP1	7.344	13.008
HS3ST2	-5.001	-7.399
ID3	5.123	12.733
IFI27	29.686	27.296
IFIT1	12.478	10.088
IL1B	8.002	5.612
IL1F11	14.001	5.611
IL1F9	5.981	20.426
IL1R2	26.783	14.393
IL1RN	13.928	11.538
IL24	-6.001	-8.399
IRAK2	41.881	39.491
IRF1	32.189	29.799
ITGA5	-5.104	-7.502
KIF14	-8.987	-11.385
KIF20A	-15.123	-17.521
KIF2C	-9.465	-8.187
KLK6		12.164
LUM	18.456	16.066
LYAR	-5.001	-5.732
MAP3K8	12.236	9.846
METTL7A1	5.002	
MFSD11		-5.013
MMP10	31.467	36.897
MMP1A	28.122	33.552
MMP9	45.682	30.112

MRVI1	11.324	
MYC	-5.104	-6.22
MYCBP	-26.489	
NCOA7	6.401	11.831
NFKB2	26.004	29.434
NRG1	-12.112	-15.512
NUPR1	6.077	11.507
OGG1	11.221	
PCDHGA11	-16.791	-20.191
PCDHGA7	-12.471	-15.871
PCDHGB5	-10.103	-13.503
PHLDA1	-22.37	-25.77
PHLDA2	-26.193	-23.523
PIK3IP1	12.884	18.314
PLEK2	-15.238	-12.568
PLK1	-17.998	-15.328
PPRC1	-11.005	-8.335
PSMA2	-14.122	
PTK6	-15.889	-13.219
PTPRA	-5.034	
PUS1	-5.001	-9.581
RAB9	14.788	20.218
RGMA	14.892	17.322
RNASE4	22.301	27.731
RWDD4A		-12.355
SERPINA1C	14.509	
SLC26A2	7.044	
SOX4	13.205	18.635
SSRP1	-5.576	-10.156
STAT2	48.102	23.532
SUMO2		14.567
SYNGR3	20.117	
SYNJ2BP	5.003	
TC2N		-17.5
TGFA	-9.465	-14.045
THBS2	-15.889	-20.469
TOP2A	-6.001	-10.581
TPX2	-22.587	-27.167
UGT1A6A	15.823	21.253
ULK1	12.434	17.864
VAV3	13.785	19.215
VTCN1	82.268	97.698

7. Bibliography

Aberle, H, S Butz, J Stappert, H Weissig, R Kemler, and H Hoschuetzky. 1994. "Assembly of the Cadherin-Catenin Complex in Vitro with Recombinant Proteins." *Journal of Cell Science* 107 (Pt 1 (December): 3655–63.

Agtmael, Tom Van, and Leena Bruckner-Tuderman. 2010. "Basement Membranes and Human Disease." *Cell and Tissue Research*. https://doi.org/10.1007/s00441-009-0866-y.

Allen, Judith E., and Thomas A. Wynn. 2011. "Evolution of Th2 Immunity: A Rapid Repair Response to Tissue Destructive Pathogens." *PLoS Pathogens*. https://doi.org/10.1371/journal.ppat.1002003.

Badiu, Diana, Monica Vasile, and Ovidiu Teren. 2011. "Regulation of Wound Healing by Growth Factors and Cytokines." In *Wound Healing: Process, Phases and Promoting*.

Bae, H C, S H Jeong, J H Kim, H Lee, W I Ryu, M G Kim, E D Son, T R Lee, and S W Son. 2018. "RIP4 Upregulates CCL20 Expression through STAT3 Signalling in Cultured Keratinocytes." *Exp Dermatol* 27 (10): 1126–33. https://doi.org/10.1111/exd.13750.

Balato, Anna, Serena Lembo, Martina Mattii, Maria Schiattarella, Rita Marino, Amato De Paulis, Nicola Balato, and Fabio Ayala. 2012. "IL-33 Is Secreted by Psoriatic Keratinocytes and Induces pro-Inflammatory Cytokines via Keratinocyte and Mast Cell Activation." *Experimental Dermatology*. https://doi.org/10.1111/exd.12027.

Barbee, Susannah D., Martin J. Woodward, Gleb Turchinovich, Jean Jacques Mention, Julia M. Lewis, Lynn M. Boyden, Richard P. Lifton, Robert Tigelaar, and Adrian C. Hayday. 2011. "Skint-1 Is a Highly Specific, Unique Selecting Component for Epidermal T Cells." *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1010890108.

Bashir, M. M., M. R. Sharma, and V. P. Werth. 2009. "TNF-α Production in the Skin." *Archives of Dermatological Research*. https://doi.org/10.1007/s00403-008-0893-7.

Bays, Jennifer L, and Kris A DeMali. 2017. "Vinculin in Cell-Cell and Cell-Matrix

Adhesions." *Cellular and Molecular Life Sciences : CMLS* 74 (16): 2999–3009. https://doi.org/10.1007/s00018-017-2511-3.

Bergers, Gabriele, Rolf Brekken, Gerald McMahon, Thiennu H. Vu, Takeshi Itoh, Kazuhiko Tamaki, Kazuhiko Tanzawa, et al. 2000. "Matrix Metalloproteinase-9 Triggers the Angiogenic Switch during Carcinogenesis." *Nature Cell Biology*. https://doi.org/10.1038/35036374.

Bill, Heather M, Beatrice Knudsen, Sheri L Moores, Senthil K Muthuswamy, Vikram R Rao, Joan S Brugge, and Cindy K Miranti. 2004. "Epidermal Growth Factor Receptor-Dependent Regulation of Integrin-Mediated Signaling and Cell Cycle Entry in Epithelial Cells." *Molecular and Cellular Biology* 24 (19): 8586–99. https://doi.org/10.1128/MCB.24.19.8586-8599.2004.

Black, Antony P.B., Michael R. Ardern-Jones, Victoria Kasprowicz, Paul Bowness, Louise Jones, Abigail S. Bailey, and Graham S. Ogg. 2007. "Human Keratinocyte Induction of Rapid Effector Function in Antigen-Specific Memory CD4+ and CD8+ T Cells." *European Journal of Immunology*. https://doi.org/10.1002/eji.200636915.

Bogaard, Ellen H. Van Den, Geuranne S. Tjabringa, Irma Joosten, Mieke Vonk-Bergers, Esther Van Rijssen, Henk J. Tijssen, Mirthe Erkens, Joost Schalkwijk, and Hans J.P.M. Koenen. 2014. "Crosstalk between Keratinocytes and T Cells in a 3D Microenvironment: A Model to Study Inflammatory Skin Diseases." *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.2013.417.

Boguniewicz, M, and D Y Leung. 2011. "Atopic Dermatitis: A Disease of Altered Skin Barrier and Immune Dysregulation." *Immunol Rev* 242 (1): 233–46. https://doi.org/10.1111/j.1600-065X.2011.01027.x.

Borgne, Marie Le, Nathalie Etchart, Anne Goubier, Sergio A. Lira, Jean Claude Sirard, Nico Van Rooijen, Christophe Caux, et al. 2006. "Dendritic Cells Rapidly Recruited into Epithelial Tissues via CCR6/CCL20 Are Responsible for CD8 + T Cell Crosspriming in Vivo." *Immunity*. https://doi.org/10.1016/j.immuni.2006.01.005.

Braun, Susanne, Monika Krampert, Enikö Bodó, Angelika Kümin, Christiane Born-Berclaz, Ralf Paus, and Sabine Werner. 2006. "Keratinocyte Growth Factor Protects Epedermis and Hair Follicles from Cell Death Induced by UV Irradiation,

Chemotherapeutic or Cytotoxic Agents." *Journal of Cell Science*. https://doi.org/10.1242/jcs.03259.

Burke, S J, M R Goff, D Lu, D Proud, M D Karlstad, and J J Collier. 2013. "Synergistic Expression of the CXCL10 Gene in Response to IL-1beta and IFN-Gamma Involves NF-KappaB, Phosphorylation of STAT1 at Tyr701, and Acetylation of Histones H3 and H4." *J Immunol* 191 (1): 323–36. https://doi.org/10.4049/jimmunol.1300344.

Cabodi, S, L Moro, E Bergatto, E Boeri Erba, P Di Stefano, E Turco, G Tarone, and P Defilippi. 2004. "Integrin Regulation of Epidermal Growth Factor (EGF) Receptor and of EGF-Dependent Responses." *Biochemical Society Transactions* 32 (Pt3). https://doi.org/10.1042/BST0320438.

Čabrijan, Leo, and Jasna Lipozenčić. 2011. "Adhesion Molecules in Keratinocytes." *Clinics in Dermatology* 29 (4): 427–31. https://doi.org/10.1016/j.clindermatol.2011.01.012.

Calautti, Enzo, Lidia Avalle, and Valeria Poli. 2018. "Psoriasis: A STAT3-Centric View." *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms19010171.

Campbell, Patrick, Penny E. Morton, Takuya Takeichi, Amr Salam, Nerys Roberts, Laura E. Proudfoot, Jemima E. Mellerio, et al. 2014. "Epithelial Inflammation Resulting from an Inherited Loss-of-Function Mutation in EGFR." *Journal of Investigative Dermatology* 134 (10): 2570–78. https://doi.org/10.1038/JID.2014.164.

Cañedo-Dorantes, Luis, and Mara Cañedo-Ayala. 2019. "Skin Acute Wound Healing: A Comprehensive Review." *International Journal of Inflammation*. https://doi.org/10.1155/2019/3706315.

Carisey, Alex, and Christoph Ballestrem. 2011. "Vinculin, an Adapter Protein in Control of Cell Adhesion Signalling." *European Journal of Cell Biology* 90 (2–3): 157–63. https://doi.org/10.1016/j.ejcb.2010.06.007.

Carpenter, Graham, Lloyd King, and Stanley Cohen. 1978. "Epidermal Growth Factor Stimulates Phosphorylation in Membrane Preparations in Vitro [21]." *Nature*. https://doi.org/10.1038/276409a0.

Cheng, Chun Chia, Hsin Chi Lin, Kaun Jer Tsai, Ya Wen Chiang, Ken Hong Lim, Caleb

Gon Shen Chen, Ying Wen Su, et al. 2018. “Epidermal Growth Factor Induces STAT1 Expression to Exacerbate the IFNr-Mediated PD-L1 Axis in Epidermal Growth Factor Receptor-Positive Cancers.” *Molecular Carcinogenesis*. https://doi.org/10.1002/mc.22881.

Cheong, J. E. Lai, V. Wessagowit, and J. A. McGrath. 2005. “Molecular Abnormalities of the Desmosomal Protein Desmoplakin in Human Disease.” *Clinical and Experimental Dermatology* 30 (3): 261–66. https://doi.org/10.1111/j.1365-2230.2005.01736.x.

Chong, Shu Zhen, Maximilien Evrard, and Lai Guan Ng. 2013. “Lights, Camera, and Action: Vertebrate Skin Sets the Stage for Immune Cell Interaction with Arthropod-Vectored Pathogens.” *Frontiers in Immunology*. https://doi.org/10.3389/fimmu.2013.00286.

Coulombe, Pierre A, Michelle L Kerns, and Elaine Fuchs. 2009. “Epidermolysis Bullosa Simplex: A Paradigm for Disorders of Tissue Fragility.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI38177.

Cowden Dahl, Karen D., Jaime Symowicz, Yan Ning, Elisa Gutierrez, David A. Fishman, Brian P. Adley, M. Sharon Stack, and Laurie G. Hudson. 2008. “Matrix Metalloproteinase 9 Is a Mediator of Epidermal Growth Factor-Dependent E-Cadherin Loss in Ovarian Carcinoma Cells.” *Cancer Research*. https://doi.org/10.1158/0008-5472.CAN-07-5046.

Cumberbatch, M., R. J. Dearman, S. Uribe-Luna, D. R. Headon, P. P. Ward, O. M. Conneely, and I. Kimber. 2000. “Regulation of Epidermal Langerhans Cell Migration by Lactoferrin.” *Immunology*. https://doi.org/10.1046/j.1365-2567.2000.00014.x.

Cybulsky, A V, A J McTavish, and M D Cyr. 1994. “Extracellular Matrix Modulates Epidermal Growth Factor Receptor Activation in Rat Glomerular Epithelial Cells.” *The Journal of Clinical Investigation* 94 (1): 68–78. https://doi.org/10.1172/JCI117350.

Dahl, Karen D Cowden, Reema Zeineldin, and Laurie G. Hudson. 2007. “PEA3 Is Necessary for Optimal Epidermal Growth Factor Receptor-Stimulated Matrix Metalloproteinase Expression and Invasion of Ovarian Tumor Cells.” *Molecular Cancer Research*. https://doi.org/10.1158/1541-7786.MCR-07-0019.

Daniel, Jan Marcus, Jochen Dutzmann, Wiebke Bielenberg, Rebecca Widmer-Teske, Dursun Gündüz, Christian W. Hamm, and Daniel G. Sedding. 2012. “Inhibition of STAT3 Signaling Prevents Vascular Smooth Muscle Cell Proliferation and Neointima Formation.” *Basic Research in Cardiology*. https://doi.org/10.1007/s00395-012-0261-9.

Dawson, J.P., M.B. Berger, C.-C. Lin, J. Schlessinger, M.A. Lemmon, and K.M. Ferguson. 2005. “Epidermal Growth Factor Receptor Dimerization and Activation Require Ligand-Induced Conformational Changes in the Dimer Interface.” *Molecular and Cellular Biology* 25 (17): 7734–42. https://doi.org/10.1128/MCB.25.17.7734-7742.2005.

Deryugina, Elena I., Ewa Zajac, Anna Juncker-Jensen, Tatyana A. Kupriyanova, Lisa Welter, and James P. Quigley. 2014. “Tissue-Infiltrating Neutrophils Constitute the Major In Vivo Source of Angiogenesis-Inducing MMP-9 in the Tumor Microenvironment.” *Neoplasia*. https://doi.org/10.1016/j.neo.2014.08.013.

Deshayes, Nathalie, Fabienne Bloas, Florian Boissout, Jennifer Lecardonnel, and Maryline Paris. 2018. “3D In Vitro Model of the Re-Epithelialization Phase in the Wound-Healing Process.” *Experimental Dermatology*. https://doi.org/10.1111/exd.13390.

DiPietro, L. A., and P. J. Polverini. 1993. “Role of the Macrophage in the Positive and Negative Regulation of Wound Neovascularization.” *Behring Institute Mitteilungen*.

DiPietro, Luisa A., Marie Burdick, Quentin E. Low, Steven L. Kunkel, and Robert M. Strieter. 1998. “Mip-1α as a Critical Macrophage Chemoattractant in Murine Wound Repair.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI1020.

Döbbeling, Udo, Reinhard Dummer, Elisabeth Laine, Natascha Potoczna, Jian Zhong Qin, and Günter Burg. 1998. “Interleukin-15 Is an Autocrine/Paracrine Viability Factor for Cutaneous T-Cell Lymphoma Cells.” *Blood*.

Duperret, Elizabeth K, and Todd W Ridky. 2013. “Focal Adhesion Complex Proteins in Epidermis and Squamous Cell Carcinoma.” *Cell Cycle (Georgetown, Tex.)* 12 (20): 3272–85. https://doi.org/10.4161/cc.26385.

Eberwein, Philipp, Dougal Laird, Simon Schulz, Thomas Reinhard, Thorsten Steinberg,

and Pascal Tomakidi. 2015. “Modulation of Focal Adhesion Constituents and Their Down-Stream Events by EGF: On the Cross-Talk of Integrins and Growth Factor Receptors” 1853 (10): 2183–98. https://doi.org/10.1016/j.bbamcr.2015.06.004.

Ebner, R, and R Derynck. 1991. “Epidermal Growth Factor and Transforming Growth Factor-Alpha: Differential Intracellular Routing and Processing of Ligand-Receptor Complexes.” *Cell Regulation* 2 (8): 599–612. https://doi.org/10.1091/mbc.2.8.599.

El-Abaseri, Taghrid B., Sumanth Putta, and Laura A. Hansen. 2006. “Ultraviolet Irradiation Induces Keratinocyte Proliferation and Epidermal Hyperplasia through the Activation of the Epidermal Growth Factor Receptor.” *Carcinogenesis*. https://doi.org/10.1093/carcin/bgi220.

Ellerbroek, Shawn M., Jennifer M. Halbleib, Mario Benavidez, Janel K. Warmka, Elizabeth V. Wattenberg, M. Sharon Stack, and Laurie G. Hudson. 2001. “Phosphatidylinositol 3-Kinase Activity in Epidermal Growth Factor-Stimulated Matrix Metalloproteinase-9 Production and Cell Surface Association.” *Cancer Research*.

Ellis, Samantha, Elaine J. Lin, and Danielle Tartar. 2018. “Immunology of Wound Healing.” *Current Dermatology Reports*. https://doi.org/10.1007/s13671-018-0234-9.

Fedor-Chaiken, Mary, Patrick W. Hein, Jane C. Stewart, Robert Brackenbury, and Michael S. Kinch. 2003. “E-Cadherin Binding Modulates EGF Receptor Activation.” *Cell Communication and Adhesion*. https://doi.org/10.1080/cac.10.2.105.118.

Ferguson, Kathryn M. 2008. “Structure-Based View of Epidermal Growth Factor Receptor Regulation.” *Annual Review of Biophysics* 37: 353–73. https://doi.org/10.1146/annurev.biophys.37.032807.125829.

Fishel, R. S., A. Barbul, W. E. Beschorner, H. L. Wasserkrug, and G. Efron. 1987. “Lymphocyte Participation in Wound Healing. Morphologic Assessment Using Monoclonal Antibodies.” *Annals of Surgery*.

FITZPATRICK, T. B., and A. S. BREATHNACH. 1963. “DAS EPIDERMALE MELANIN-EINHEIT-SYSTEM.” *Dermatologische Wochenschrift*.

Fletcher, J S, M G Springer, K Choi, E Jousma, T A Rizvi, E Dombi, M O Kim, J Wu, and N Ratner. 2019. "STAT3 Inhibition Reduces Macrophage Number and Tumor Growth in Neurofibroma." *Oncogene* 38 (15): 2876–84. https://doi.org/10.1038/s41388-018-0600-x.

Foster, Alexander M., Jaymie Baliwag, Cynthia S. Chen, Andrew M. Guzman, Stefan W. Stoll, Johann E. Gudjonsson, Nicole L. Ward, and Andrew Johnston. 2014. "IL-36 Promotes Myeloid Cell Infiltration, Activation, and Inflammatory Activity in Skin." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1301481.

Franco, Santos J., Mary A. Rodgers, Benjamin J. Perrin, Jaewon Han, David A. Bennin, David R. Critchley, and Anna Huttenlocher. 2004. "Calpain-Mediated Proteolysis of Talin Regulates Adhesion Dynamics." *Nature Cell Biology* 6 (10): 977–83. https://doi.org/10.1038/ncb1175.

Franovic, A, L Gunaratnam, K Smith, I Robert, D Patten, and S Lee. 2007. "Translational Up-Regulation of the EGFR by Tumor Hypoxia Provides a Nonmutational Explanation for Its Overexpression in Human Cancer." *Proc Natl Acad Sci U S A* 104 (32): 13092–97. https://doi.org/10.1073/pnas.0702387104.

Frederick, L, G Eley, X Y Wang, and C D James. 2000. "Analysis of Genomic Rearrangements Associated with EGRFvIII Expression Suggests Involvement of Alu Repeat Elements." *Neuro Oncol* 2 (3): 159–63. http://www.ncbi.nlm.nih.gov/pubmed/11302336.

Friedl, Peter, and Darren Gilmour. 2009. "Collective Cell Migration in Morphogenesis, Regeneration and Cancer." *Nature Reviews Molecular Cell Biology* 10 (7): 445–57. https://doi.org/10.1038/nrm2720.

Frohm, Margareta, Birgitta Agerberth, Ghasem Ahangari, Mona Ståhle-Bäckdahl, Sture Lidén, Hans Wigzell, and Gudmundur H. Gudmundsson. 1997. "The Expression of the Gene Coding for the Antibacterial Peptide LL-37 Is Induced in Human Keratinocytes during Inflammatory Disorders." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.272.24.15258.

Fuchs, E, and S Raghavan. 2002. "Getting under the Skin of Epidermal Morphogenesis." *Nat Rev Genet* 3 (3): 199–209. https://doi.org/10.1038/nrg758.

Fujita, Yasuyuki, Gerd Krause, Martin Scheffner, Dietmar Zechner, Hugo E.Molina

Leddy, Jürgen Behrens, Thomas Sommer, and Walter Birchmeier. 2002. “Hakai, a c-Cbl-like Protein, Ubiquitinates and Induces Endocytosis of the E-Cadherin Complex.” *Nature Cell Biology*. https://doi.org/10.1038/ncb758.

Furcht, C M, J M Buonato, N Skuli, L K Mathew, A R Munoz Rojas, M C Simon, and M J Lazzara. 2014. “Multivariate Signaling Regulation by SHP2 Differentially Controls Proliferation and Therapeutic Response in Glioma Cells.” *J Cell Sci* 127 (Pt 16): 3555–67. https://doi.org/10.1242/jcs.150862.

Furuse, Mikio, Masaki Hata, Kyoko Furuse, Yoko Yoshida, Akinori Haratake, Yoshinobu Sugitani, Tetsuo Noda, Akiharu Kubo, and Shoichiro Tsukita. 2002. “Claudin-Based Tight Junctions Are Crucial for the Mammalian Epidermal Barrier.” *The Journal of Cell Biology* 156 (6): 1099–1111. https://doi.org/10.1083/jcb.200110122.

Galkowska, Hanna, Urszula Wojewodzka, and Waldemar L. Olszewski. 2006. “Chemokines, Cytokines, and Growth Factors in Keratinocytes and Dermal Endothelial Cells in the Margin of Chronic Diabetic Foot Ulcers.” *Wound Repair and Regeneration*. https://doi.org/10.1111/j.1743-6109.2006.00155.x.

Gallicano, G. Ian, Panos Kouklis, Christoph Bauer, Mei Yin, Valeri Vasioukhin, Linda Degenstein, and Elaine Fuchs. 1998. “Desmoplakin Is Required Early in Development for Assembly of Desmosomes and Cytoskeletal Linkage.” *The Journal of Cell Biology* 143 (7): 2009–22. https://doi.org/10.1083/jcb.143.7.2009.

Gallo, Richard L. 2017. “Human Skin Is the Largest Epithelial Surface for Interaction with Microbes.” *Journal of Investigative Dermatology*. https://doi.org/10.1016/j.jid.2016.11.045.

Ganetzky, Rebecca, Erin Finn, Atrish Bagchi, Ornella Zollo, Laura Conlin, Matthew Deardorff, Margaret Harr, et al. 2015. “EGFR Mutations Cause a Lethal Syndrome of Epithelial Dysfunction with Progeroid Features.” *Molecular Genetics & Genomic Medicine* 3 (5): 452–58. https://doi.org/10.1002/mgg3.156.

Garrett, Thomas P.J., Neil M. McKern, Meizhen Lou, Thomas C. Elleman, Timothy E. Adams, George O. Lovrecz, Hong-Jian Zhu, et al. 2002. “Crystal Structure of a Truncated Epidermal Growth Factor Receptor Extracellular Domain Bound to Transforming Growth Factor α.” *Cell* 110 (6): 763–73.

https://doi.org/10.1016/S0092-8674(02)00940-6.

Garrod, David, and Martyn Chidgey. 2008. “Desmosome Structure, Composition and Function.” *Biochimica et Biophysica Acta - Biomembranes*. https://doi.org/10.1016/j.bbamem.2007.07.014.

Gilet, Jules, Ying Chang, Cécile Chenivesse, Benjamin Legendre, Han Vorng, Catherine Duez, Benoît Wallaert, Henri Porte, Stéphanie Senechal, and Anne Tsicopoulos. 2009. “Role of CCL17 in the Generation of Cutaneous Inflammatory Reactions in Hu-PBMC-SCID Mice Grafted with Human Skin.” *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.2008.333.

Gingras, Alexandre R, Wolfgang H Ziegler, Ronald Frank, Igor L Barsukov, Gordon C K Roberts, David R Critchley, and Jonas Emsley. 2005. “Mapping and Consensus Sequence Identification for Multiple Vinculin Binding Sites within the Talin Rod.” *Journal of Biological Chemistry* 280 (44): 37217–24. https://doi.org/10.1074/jbc.M508060200.

Ginhoux, Florent, Frank Tacke, Veronique Angeli, Milena Bogunovic, Martine Loubeau, Xu Ming Dai, E. Richard Stanley, Gwendalyn J. Randolph, and Miriam Merad. 2006. “Langerhans Cells Arise from Monocytes in Vivo.” *Nature Immunology*. https://doi.org/10.1038/ni1307.

Graham, G. M., M. D. Farrar, J. E. Cruse-Sawyer, K. T. Holland, and E. Ingham. 2004. “Proinflammatory Cytokine Production by Human Keratinocytes Stimulated with Propionibacterium Acnes and P. Acnes GroEL.” *British Journal of Dermatology*. https://doi.org/10.1046/j.1365-2133.2004.05762.x.

Griesinger, A M, R J Josephson, A M Donson, J M Mulcahy Levy, V Amani, D K Birks, L M Hoffman, et al. 2015. “Interleukin-6/STAT3 Pathway Signaling Drives an Inflammatory Phenotype in Group A Ependymoma.” *Cancer Immunol Res* 3 (10): 1165–74. https://doi.org/10.1158/2326-6066.CIR-15-0061.

Grossman, R. M., J. Krueger, D. Yourish, A. Granelli-Piperno, D. P. Murphy, L. T. May, T. S. Kupper, P. B. Sehgal, and A. B. Gottlieb. 1989. “Interleukin 6 Is Expressed in High Levels of Psoriatic Skin and Stimulates Proliferation of Cultured Human Keratinocytes.” *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.86.16.6367.

Gruijl, Frank R. De. 2000. “Photocarcinogenesis: UVA vs UVB.” *Methods in*

Enzymology.

Gumbiner, Barry M. 2005. “Regulation of Cadherin-Mediated Adhesion in Morphogenesis.” *Nature Reviews Molecular Cell Biology* 6 (8): 622–34. https://doi.org/10.1038/nrm1699.

Haeger, Anna, Katarina Wolf, Mirjam M. Zegers, and Peter Friedl. 2015. “Collective Cell Migration: Guidance Principles and Hierarchies.” *Trends in Cell Biology* 25 (9): 556–66. https://doi.org/10.1016/J.TCB.2015.06.003.

Hammiller, B O, T B El-Abaseri, A A Dlugosz, and L A Hansen. 2015. “A Method for the Immortalization of Newborn Mouse Skin Keratinocytes.” *Front Oncol* 5: 177. https://doi.org/10.3389/fonc.2015.00177.

Han, Gang Wen, Keiji Iwatsuki, Masayuki Inoue, Takashi Matsui, Akiko Nishibu, Hitoshi Akiba, and Fumio Kaneko. 1999. “Interleukin-15 Is Not a Constitutive Cytokine in the Epidermis, but Is Inducible in Culture or Inflammatory Conditions.” *Acta Dermato-Venereologica*. https://doi.org/10.1080/000155599750011679.

Hansen, L A, N Alexander, M E Hogan, J P Sundberg, A Dlugosz, D W Threadgill, T Magnuson, and S H Yuspa. 1997. “Genetically Null Mice Reveal a Central Role for Epidermal Growth Factor Receptor in the Differentiation of the Hair Follicle and Normal Hair Development.” *Am J Pathol* 150 (6): 1959–75. http://www.ncbi.nlm.nih.gov/pubmed/9176390.

Harder, J., J. Bartels, E. Christophers, and J. M. Schroder. 1997. “A Peptide Antibiotic from Human Skin [6].” *Nature*. https://doi.org/10.1038/43088.

Harper, Erin G., Changsheng Guo, Heather Rizzo, Joseph V. Lillis, Stephen E. Kurtz, Iliyana Skorcheva, David Purdy, Erin Fitch, Mihail Iordanov, and Andrew Blauvelt. 2009. “Th17 Cytokines Stimulate CCL20 Expression in Keratinocytes in Vitro and in Vivo: Implications for Psoriasis Pathogenesis.” *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.2009.65.

Harwig, Sylvia S.L., Tomas Ganz, and Robert I. Lehrer. 1994. “Neutrophil Defensins: Purification, Characterization, and Antimicrobial Testing.” *Methods in Enzymology*. https://doi.org/10.1016/0076-6879(94)36015-4.

Hashiguchi, Yuriko, Rikio Yabe, Soo-Hyun Chung, Masanori A. Murayama, Kaori Yoshida, Kenzo Matsuo, Sachiko Kubo, et al. 2018. “IL-36α from Skin-Resident

Cells Plays an Important Role in the Pathogenesis of Imiquimod-Induced Psoriasiform Dermatitis by Forming a Local Autoamplification Loop." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.1701157.

Hayakawa, Kimihide, Hitoshi Tatsumi, and Masahiro Sokabe. 2012. "Mechano-Sensing by Actin Filaments and Focal Adhesion Proteins." *Communicative and Integrative Biology*. https://doi.org/10.4161/cib.21891.

Hendriks, Bart S., H. Steven Wiley, and Douglas Lauffenburger. 2003. "HER2-Mediated Effects on EGFR Endosomal Sorting: Analysis of Biophysical Mechanisms." *Biophysical Journal*. https://doi.org/10.1016/S0006-3495(03)74696-7.

Henriksen, Lasse, Michael Vibo Grandal, Stine Louise Jeppe Knudsen, Bo van Deurs, and Lene Melsæther Grøvdal. 2013. "Internalization Mechanisms of the Epidermal Growth Factor Receptor after Activation with Different Ligands." Edited by Christophe Lamaze. *PLoS ONE* 8 (3): e58148. https://doi.org/10.1371/journal.pone.0058148.

Herbst, Roy S., and Dong M. Shin. 2002. "Monoclonal Antibodies to Target Epidermal Growth Factor Receptor-Positive Tumors a New Paradigm for Cancer Therapy." *Cancer*. https://doi.org/10.1002/cncr.10372.

Heufler, Christine, Gerda Topar, Alfred Grasseger, Ursula Stanzl, Franz Koch, Nikolaus Romani, Anthony E. Namen, and Gerold Schuler. 1993. "Interleukin 7 Is Produced by Murine and Human Keratinocytes." *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.178.3.1109.

Hinrichsen, Lars, Jens Harborth, Lars Andrees, Klaus Weber, and Ernst J. Ungewickell. 2003. "Effect of Clathrin Heavy Chain- and α-Adaptin-Specific Small Inhibitory RNAs on Endocytic Accessory Proteins and Receptor Trafficking in HeLa Cells." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M307290200.

Holcmann, Martin, and Maria Sibilia. 2015. "Mechanisms Underlying Skin Disorders Induced by EGFR Inhibitors." *Molecular and Cellular Oncology*. https://doi.org/10.1080/23723556.2015.1004969.

Hopkinson, Susan B, Kevin J Hamill, Yvonne Wu, Jessica L Eisenberg, Sho Hiroyasu, and Jonathan C R Jones. 2014. "Focal Contact and Hemidesmosomal Proteins in Keratinocyte Migration and Wound Repair." *Advances in Wound Care* 3 (3):

247–63. https://doi.org/10.1089/wound.2013.0489.

Huang, Victor, Anke S. Lonsdorf, Lei Fang, Takashi Kakinuma, Vivian C. Lee, Emily Cha, Hong Zhang, et al. 2008. “Cutting Edge: Rapid Accumulation of Epidermal CCL27 in Skin-Draining Lymph Nodes Following Topical Application of a Contact Sensitizer Recruits CCR10-Expressing T Cells.” *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.180.10.6462.

Huang, Y, J Wang, F Cao, H Jiang, A Li, J Li, L Qiu, et al. 2017. “SHP2 Associates with Nuclear Localization of STAT3: Significance in Progression and Prognosis of Colorectal Cancer.” *Sci Rep* 7 (1): 17597. https://doi.org/10.1038/s41598-017-17604-7.

Huber, Andrew H., Daniel B. Stewart, Douglas V. Laurents, W. James Nelson, and William I. Weis. 2001. “The Cadherin Cytoplasmic Domain Is Unstructured in the Absence of β-Catenin.” *Journal of Biological Chemistry* 276 (15): 12301–9. https://doi.org/10.1074/jbc.M010377200.

Hudson, Laurie G., Natalie M. Moss, and M. Sharon Stack. 2009. “EGF-Receptor Regulation of Matrix Metalloproteinases in Epithelial Ovarian Carcinoma.” *Future Oncology*. https://doi.org/10.2217/fon.09.10.

Ichiyama, Takashi, Madoka Kajimoto, Naoko Suenaga, Shinji Maeba, Tomoyo Matsubara, and Susumu Furukawa. 2006. “Serum Levels of Matrix Metalloproteinase-9 and Its Tissue Inhibitor (TIMP-1) in Acute Disseminated Encephalomyelitis.” *Journal of Neuroimmunology*. https://doi.org/10.1016/j.jneuroim.2005.10.010.

Ikeda, Sho, Akihiro Kitadate, Mitsugu Ito, Fumito Abe, Miho Nara, Atsushi Watanabe, Naoto Takahashi, Tomomitsu Miyagaki, Makoto Sugaya, and Hiroyuki Tagawa. 2016. “Disruption of CCL20-CCR6 Interaction Inhibits Metastasis of Advanced Cutaneous T-Cell Lymphoma.” *Oncotarget*. https://doi.org/10.18632/oncotarget.6916.

Inokuma, Daisuke, Riichiro Abe, Yasuyuki Fujita, Mikako Sasaki, Akihiko Shibaki, Hideki Nakamura, James R. McMillan, Tadamichi Shimizu, and Hiroshi Shimizu. 2006. “CTACK/CCL27 Accelerates Skin Regeneration via Accumulation of Bone Marrow-Derived Keratinocytes.” *Stem Cells*. https://doi.org/10.1634/stemcells.2006-0264.

Iordanov, Mihail S., Remy J. Choi, Olga P. Ryabinina, Thanh-Hoai Dinh, Robert K. Bright, and Bruce E. Magun. 2002. “The UV (Ribotoxic) Stress Response of Human Keratinocytes Involves the Unexpected Uncoupling of the Ras-Extracellular Signal-Regulated Kinase Signaling Cascade from the Activated Epidermal Growth Factor Receptor.” *Molecular and Cellular Biology* 22 (15): 5380–94. https://doi.org/10.1128/MCB.22.15.5380-5394.2002.

Izard, Tina, Gwyndaf Evans, Robert A. Borgon, Christina L. Rush, Gerard Bricogne, and Philippe R. J. Bois. 2004. “Vinculin Activation by Talin through Helical Bundle Conversion.” *Nature* 427 (6970): 171–75. https://doi.org/10.1038/nature02281.

Jameson, Julie, Karen Ugarte, Nicole Chen, Pia Yachi, Elaine Fuchs, Richard Boismenu, and Wendy L. Havran. 2002. “A Role for Skin Γδ T Cells in Wound Repair.” *Science*. https://doi.org/10.1126/science.1069639.

Jamora, Colin, and Elaine Fuchs. 2002. “Intercellular Adhesion, Signalling and the Cytoskeleton.” *Nature Cell Biology*. https://doi.org/10.1038/ncb0402-e101.

Jedryka, M, A Chrobak, A Chelmonska-Soyta, D Gawron, A Halbersztadt, A Wojnar, and J Kornafel. 2012. “Matrix Metalloproteinase (MMP)-2 and MMP-9 Expression in Tumor Infiltrating CD3 Lymphocytes from Women with Endometrial Cancer.” *Int J Gynecol Cancer* 22 (8): 1303–9. https://doi.org/10.1097/IGC.0b013e318269e27b.

Jia, Zan Hui, Yan Jia, Feng Jun Guo, Jun Chen, Xi Wen Zhang, and Man Hua Cui. 2017. “Phosphorylation of STAT3 at Tyr705 Regulates MMP-9 Production in Epithelial Ovarian Cancer.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0183622.

Jones, P L, J Crack, and M Rabinovitch. 1997. “Regulation of Tenascin-C, a Vascular Smooth Muscle Cell Survival Factor That Interacts with the Alpha v Beta 3 Integrin to Promote Epidermal Growth Factor Receptor Phosphorylation and Growth.” *The Journal of Cell Biology* 139 (1): 279–93. https://doi.org/10.1083/jcb.139.1.279.

Kämpfer, H., H. Mühl, M. Manderscheid, U. Kalina, D. Kauschat, J. Pfeilschifter, and S. Frank. 2000. “Regulation of Interleukin-18 (IL-18) Expression in Keratinocytes (HaCaT): Implications for Early Wound Healing.” *European Cytokine Network*.

Karakawa, M, M Komine, Y Hanakawa, H Tsuda, K Sayama, K Tamaki, and M Ohtsuki. 2014. “CCL27 Is Downregulated by Interferon Gamma via Epidermal Growth

Factor Receptor in Normal Human Epidermal Keratinocytes." *J Cell Physiol* 229 (12): 1935–45. https://doi.org/10.1002/jcp.24643.

Kennedy-Crispin, Milène, Erika Billick, Hiroshi Mitsui, Nicholas Gulati, Hideki Fujita, Patricia Gilleaudeau, Mary Sullivan-Whalen, Leanne M. Johnson-Huang, Mayte Suárez-Farĩas, and James G. Krueger. 2012. "Human Keratinocytes' Response to Injury Upregulates CCL20 and Other Genes Linking Innate and Adaptive Immunity." *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.2011.262.

Kim, S. A., C.-Y. Tai, L.-P. Mok, E. A. Mosser, and E. M. Schuman. 2011. "Calcium-Dependent Dynamics of Cadherin Interactions at Cell-Cell Junctions." *Proceedings of the National Academy of Sciences* 108 (24): 9857–62. https://doi.org/10.1073/pnas.1019003108.

Kiuchi, Tai, Elena Ortiz-Zapater, James Monypenny, Daniel R. Matthews, Lan K. Nguyen, Jody Barbeau, Oana Coban, et al. 2014. "The ErbB4 CYT2 Variant Protects EGFR from Ligand-Induced Degradation to Enhance Cancer Cell Motility." *Science Signaling*. https://doi.org/10.1126/scisignal.2005157.

Köck, Andreas, Thomas Schwarz, Reinhard Kirnbauer, Agatha Urbanski, Patricia Perry, John C. Ansel, and Thomas A. Luger. 1990. "Human Keratinocytes Are a Source for Tumor Necrosis Factor α: Evidence for, Synthesis and Release upon Stimulation with Endotoxin or Ultraviolet Light." *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.172.6.1609.

Kumar, A, E T Petri, B Halmos, and T J Boggon. 2008. "Structure and Clinical Relevance of the Epidermal Growth Factor Receptor in Human Cancer." *J Clin Oncol* 26 (10): 1742–51. https://doi.org/10.1200/JCO.2007.12.1178.

Kuo, Paula T., Zhen Zeng, Nazhifah Salim, Stephen Mattarollo, James W. Wells, and Graham R. Leggatt. 2018. "The Role of CXCR3 and Its Chemokine Ligands in Skin Disease and Cancer." *Frontiers in Medicine*. https://doi.org/10.3389/fmed.2018.00271.

Kupper, T. S., A. O. Chua, P. Flood, J. McGuire, and U. Gubler. 1987. "Interleukin 1 Gene Expression in Cultured Human Keratinocytes Is Augmented by Ultraviolet Irradiation." *The Journal of Clinical Investigation*. https://doi.org/10.1172/JCI113090.

Kuwada, S. K., and X. Li. 2000. “Integrin Alpha 5/Beta 1 Mediates Fibronectin-Dependent Epithelial Cell Proliferation through Epidermal Growth Factor Receptor Activation.” *Molecular Biology of the Cell* 11 (7): 2485–96. https://doi.org/10.1091/mbc.11.7.2485.

Larco, J E de, and G J Todaro. 1978. “Epithelioid and Fibroblastic Rat Kidney Cell Clones: Epidermal Growth Factor (EGF) Receptors and the Effect of Mouse Sarcoma Virus Transformation.” *J Cell Physiol* 94 (3): 335–42. https://doi.org/10.1002/jcp.1040940311.

LeBleu, Valerie S., Brian MacDonald, and Raghu Kalluri. 2007. “Structure and Function of Basement Membranes.” *Experimental Biology and Medicine*. https://doi.org/10.3181/0703-MR-72.

Leibovich, S. J., and R. Ross. 1975. “The Role of the Macrophage in Wound Repair. A Study with Hydrocortisone and Antimacrophage Serum.” *American Journal of Pathology*.

Lemmon, M. A., J. Schlessinger, and K. M. Ferguson. 2014. “The EGFR Family: Not So Prototypical Receptor Tyrosine Kinases.” *Cold Spring Harbor Perspectives in Biology* 6 (4): a020768–a020768. https://doi.org/10.1101/cshperspect.a020768.

Lemmon, Mark A. 2009. “Ligand-Induced ErbB Receptor Dimerization.” *Experimental Cell Research*. https://doi.org/10.1016/j.yexcr.2008.10.024.

Li, J, A Perry, C D James, and D H Gutmann. 2001. “Cancer-Related Gene Expression Profiles in NF1-Associated Pilocytic Astrocytomas.” *Neurology* 56 (7): 885–90. http://www.ncbi.nlm.nih.gov/pubmed/11294925.

Li, Jie, Grenham W. Ireland, Paula M. Farthing, and Martin H. Thornhill. 1996. “Epidermal and Oral Keratinocytes Are Induced to Produce RANTES and IL-8 by Cytokine Stimulation.” *Journal of Investigative Dermatology*. https://doi.org/10.1111/1523-1747.ep12345482.

Lichtenberger, B M, P A Gerber, M Holcmann, B A Buhren, N Amberg, V Smolle, H Schrumpf, et al. 2013. “Epidermal EGFR Controls Cutaneous Host Defense and Prevents Inflammation.” *Sci Transl Med* 5 (199): 199ra111. https://doi.org/10.1126/scitranslmed.3005886.

Liu, H B, Y Wu, T F Lv, Y W Yao, Y Y Xiao, D M Yuan, and Y Song. 2013. “Skin Rash Could Predict the Response to EGFR Tyrosine Kinase Inhibitor and the Prognosis

for Patients with Non-Small Cell Lung Cancer: A Systematic Review and Meta-Analysis." *PLoS One* 8 (1): e55128. https://doi.org/10.1371/journal.pone.0055128.

Liu, Lide, Lina Wang, Hong Peng Jia, Chengquan Zhao, Henry H.Q. Heng, Brian C. Schutte, Paul B. McCray, and Tomas Ganz. 1998. "Structure and Mapping of the Human β-Defensin HBD-2 Gene and Its Expression at Sites of Inflammation." *Gene*. https://doi.org/10.1016/S0378-1119(98)00480-6.

Lo, Hui Wen, Sheng Chieh Hsu, Mohamed Ali-Seyed, Mehmet Gunduz, Weiya Xia, Yongkun Wei, Geoffrey Bartholomeusz, Jin Yuan Shih, and Mien Chie Hung. 2005. "Nuclear Interaction of EGFR and STAT3 in the Activation of the INOS/NO Pathway." *Cancer Cell*. https://doi.org/10.1016/j.ccr.2005.05.007.

Loke, P'ng, Iain Gallagher, Meera G. Nair, Xingxing Zang, Frank Brombacher, Markus Mohrs, James P. Allison, and Judith E. Allen. 2007. " Alternative Activation Is an Innate Response to Injury That Requires CD4 + T Cells to Be Sustained during Chronic Infection ." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.179.6.3926.

Lorch, Jochen H., Jodi Klessner, J. Ken Park, Spiro Getsios, Yvonne L. Wu, M. Sharon Stack, and Kathleen J. Green. 2004. "Epidermal Growth Factor Receptor Inhibition Promotes Desmosome Assembly and Strengthens Intercellular Adhesion in Squamous Cell Carcinoma Cells." *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M405123200.

Mainiero, F, A Pepe, M Yeon, Y Ren, and F G Giancotti. 1996. "The Intracellular Functions of Alpha6beta4 Integrin Are Regulated by EGF." *The Journal of Cell Biology* 134 (1): 241–53. https://doi.org/10.1083/jcb.134.1.241.

Mann, Amrit, Kai Breuhahn, Peter Schirmacher, and Manfred Blessing. 2001. "Keratinocyte-Derived Granulocyte-Macrophage Colony Stimulating Factor Accelerates Wound Healing: Stimulation of Keratinocyte Proliferation, Granulation Tissue Formation, and Vascularization." *Journal of Investigative Dermatology*. https://doi.org/10.1046/j.0022-202x.2001.01600.x.

Marchese, C., A. Messina, A. Faggioni, M. R. Torrisi, L. Frati, D. Ron, J. Rubin, and S. A. Aaronson. 1990. "Human Keratinocyte Growth Factor Activity on Proliferation and Differentiation of Human Keratinocytes: Differentiation Response

Distinguishes KGF from EGF Family." *Journal of Cellular Physiology*. https://doi.org/10.1002/jcp.1041440219.

Marchini, Giovanna, S. Lindow, H. Brismar, B. Stàbi, V. Berggren, A. K. Ulfgren, S. Lonne-Rahm, B. Agerberth, and G. H. Gudmundsson. 2002. "The Newborn Infant Is Protected by an Innate Antimicrobial Barrier: Peptide Antibiotics Are Present in the Skin and Vernix Caseosa." *British Journal of Dermatology*. https://doi.org/10.1046/j.1365-2133.2002.05014.x.

Mariotti, A, P A Kedeshian, M Dans, A M Curatola, L Gagnoux-Palacios, and F G Giancotti. 2001. "EGF-R Signaling through Fyn Kinase Disrupts the Function of Integrin Alpha6beta4 at Hemidesmosomes: Role in Epithelial Cell Migration and Carcinoma Invasion." *The Journal of Cell Biology* 155 (3). https://doi.org/10.1083/jcb.200105017.

Mascia, F, G Lam, C Keith, C Garber, S M Steinberg, E Kohn, and S H Yuspa. 2013. "Genetic Ablation of Epidermal EGFR Reveals the Dynamic Origin of Adverse Effects of Anti-EGFR Therapy." *Sci Transl Med* 5 (199): 199ra110. https://doi.org/10.1126/scitranslmed.3005773.

Mascia, Francesca, Valentina Mariani, Giampiero Girolomoni, and Saveria Pastore. 2003. "Blockade of the EGF Receptor Induces a Deranged Chemokine Expression in Keratinocytes Leading to Enhanced Skin Inflammation." *The American Journal of Pathology* 163 (1): 303–12. https://doi.org/10.1016/S0002-9440(10)63654-1.

Meredith, Paul, and Jennifer Riesz. 2004. "Radiative Relaxation Quantum Yields for Synthetic Eumelanin¶." *Photochemistry and Photobiology*. https://doi.org/10.1562/0031-8655(2004)079<0211:rcrqyf>2.0.co;2.

Mill, Pleasantine, Angela W.S. Lee, Yuko Fukata, Ryouhei Tsutsumi, Masaki Fukata, Margaret Keighren, Rebecca M. Porter, Lisa McKie, Ian Smyth, and Ian J. Jackson. 2009. "Palmitoylation Regulates Epidermal Homeostasis and Hair Follicle Differentiation." *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1000748.

Minutti, Carlos M., Sebastian Drube, Natalie Blair, Christian Schwartz, Jame C. McCrae, Andrew N. McKenzie, Thomas Kamradt, et al. 2017. "Epidermal Growth Factor Receptor Expression Licenses Type-2 Helper T Cells to Function in a T Cell Receptor-Independent Fashion." *Immunity*.

https://doi.org/10.1016/j.immuni.2017.09.013.

Miyamoto, S, H Teramoto, J S Gutkind, and K M Yamada. 1996. “Integrins Can Collaborate with Growth Factors for Phosphorylation of Receptor Tyrosine Kinases and MAP Kinase Activation: Roles of Integrin Aggregation and Occupancy of Receptors.” *The Journal of Cell Biology* 135 (6 Pt 1): 1633–42. https://doi.org/10.1083/jcb.135.6.1633.

Mizutani, H., R. Black, and T. S. Kupper. 1991. “Human Keratinocytes Produce but Do Not Process Pro-Interleukin-1 (IL-1) Beta Different Strategies of IL-1 Production and Processing in Monocytes and Keratinocytes.” *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI115067.

Mjösberg, J., and L. Eidsmo. 2014. “Update on Innate Lymphoid Cells in Atopic and Non-Atopic Inflammation in the Airways and Skin.” *Clinical and Experimental Allergy*. https://doi.org/10.1111/cea.12353.

Morita, Kazumasa, Masahiko Itoh, Mitinori Saitou, Yuhko Ando-Akatsuka, Mikio Furuse, Kozo Yoneda, Sadao Imamura, Kazushi Fujimoto, and Shoichiro Tsukita. 1998. “Subcellular Distribution of Tight Junction-Associated Proteins (Occludin, ZO-1, ZO-2) in Rodent Skin.” *Journal of Investigative Dermatology* 110 (6): 862–66. https://doi.org/10.1046/j.1523-1747.1998.00209.x.

Moro, L, M Venturino, C Bozzo, L Silengo, F Altruda, L Beguinot, G Tarone, and P Defilippi. 1998. “Integrins Induce Activation of EGF Receptor: Role in MAP Kinase Induction and Adhesion-Dependent Cell Survival.” *EMBO J* 17 (22): 6622–32. https://doi.org/10.1093/emboj/17.22.6622.

Moro, Laura, Laura Dolce, Sara Cabodi, Elena Bergatto, Elisabetta Boeri Erba, Monica Smeriglio, Emilia Turco, et al. 2002. “Integrin-Induced Epidermal Growth Factor (EGF) Receptor Activation Requires c-Src and P130Cas and Leads to Phosphorylation of Specific EGF Receptor Tyrosines.” *The Journal of Biological Chemistry* 277 (11): 9405–14. https://doi.org/10.1074/jbc.M109101200.

Moscatello, D K, M Holgado-Madruga, A K Godwin, G Ramirez, G Gunn, P W Zoltick, J A Biegel, R L Hayes, and A J Wong. 1995. “Frequent Expression of a Mutant Epidermal Growth Factor Receptor in Multiple Human Tumors.” *Cancer Res* 55 (23): 5536–39. http://www.ncbi.nlm.nih.gov/pubmed/7585629.

Nagano, Makoto, Daisuke Hoshino, Naohiko Koshikawa, Toshifumi Akizawa, and

Motoharu Seiki. 2012. “Turnover of Focal Adhesions and Cancer Cell Migration.” *International Journal of Cell Biology* 2012 (January): 1–10. https://doi.org/10.1155/2012/310616.

Nate Archer, Steven K Lee, Roger V Ortines, Yu Wang, Haiyun Liu, Robert J Miller, Carly A Dillen, Mark Marchitto, Alyssa G Ashbaugh, Angad Uppal, Sarah Cai and Lloyd S Miller. 2017. “Interplay between Keratinocyte STAT1 and STAT3 Signaling Controls Skin Inflammation and T-Cell Development in a Mouse Model of Psoriasis.” *J Immunol* 198 (1).

Ng, Michael Fy. 2010. “The Role of Mast Cells in Wound Healing.” *International Wound Journal*. https://doi.org/10.1111/j.1742-481X.2009.00651.x.

Nguyen, Andrew V., Yuan Yuan Wu, Qiang Liu, Donghai Wang, Stephanie Nguyen, Ricky Loh, Joey Pang, et al. 2013. “STAT3 in Epithelial Cells Regulates Inflammation and Tumor Progression to Malignant State in Colon.” *Neoplasia (United States)*. https://doi.org/10.1593/neo.13952.

Niessen, Carien M., and Cara J. Gottardi. 2008. “Molecular Components of the Adherens Junction.” *Biochimica et Biophysica Acta (BBA) - Biomembranes* 1778 (3): 562–71. https://doi.org/10.1016/j.bbamem.2007.12.015.

Nishigori, Chikako, Daniel B. Yarosh, Stephen E. Ullrich, Arie A. Vink, Corazon D. Bucana, Len Roza, and Margaret L. Kripke. 1996. “Evidence That DNA Damage Triggers Interleukin 10 Cytokine Production in UV-Irradiated Murine Keratinocytes.” *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.93.19.10354.

Normanno, N, A De Luca, C Bianco, L Strizzi, M Mancino, M R Maiello, A Carotenuto, G De Feo, F Caponigro, and D S Salomon. 2006. “Epidermal Growth Factor Receptor (EGFR) Signaling in Cancer.” *Gene* 366 (1): 2–16. https://doi.org/10.1016/j.gene.2005.10.018.

Ogiso, Hideo, Ryuichiro Ishitani, Osamu Nureki, Shuya Fukai, Mari Yamanaka, Jae Hoon Kim, Kazuki Saito, et al. 2002. “Crystal Structure of the Complex of Human Epidermal Growth Factor and Receptor Extracellular Domains.” *Cell* 110 (6): 775–87. https://doi.org/10.1016/S0092-8674(02)00963-7.

Ohashi, Kazumasa, Sachiko Fujiwara, and Kensaku Mizuno. 2017. “Roles of the Cytoskeleton, Cell Adhesion and Rho Signalling in Mechanosensing and

Mechanotransduction." *Journal of Biochemistry* 161 (3): mvw082. https://doi.org/10.1093/jb/mvw082.

Ohtani, T, K Ishihara, T Atsumi, K Nishida, Y Kaneko, T Miyata, S Itoh, et al. 2000. "Dissection of Signaling Cascades through Gp130 in Vivo: Reciprocal Roles for STAT3- and SHP2-Mediated Signals in Immune Responses." *Immunity* 12 (1): 95–105. https://www.ncbi.nlm.nih.gov/pubmed/10661409.

Olayioye, M. A. 2000. "NEW EMBO MEMBERS' REVIEW: The ErbB Signaling Network: Receptor Heterodimerization in Development and Cancer." *The EMBO Journal*. https://doi.org/10.1093/emboj/19.13.3159.

Orecchia, V, G Regis, B Tassone, C Valenti, L Avalle, S Saoncella, E Calautti, and V Poli. 2015. "Constitutive STAT3 Activation in Epidermal Keratinocytes Enhances Cell Clonogenicity and Favours Spontaneous Immortalization by Opposing Differentiation and Senescence Checkpoints." *Exp Dermatol* 24 (1): 29–34. https://doi.org/10.1111/exd.12585.

Ouwehand, Krista, Sander W. Spiekstra, Taco Waaijman, Melanie Breetveld, Rik J. Scheper, Tanja D. de Gruijl, and Susan Gibbs. 2012. "CCL5 and CCL20 Mediate Immigration of Langerhans Cells into the Epidermis of Full Thickness Human Skin Equivalents." *European Journal of Cell Biology*. https://doi.org/10.1016/j.ejcb.2012.06.004.

Ozawa, Masayuki. 2002. "Lateral Dimerization of the E-Cadherin Extracellular Domain Is Necessary but Not Sufficient for Adhesive Activity." *Journal of Biological Chemistry* 277 (22): 19600–608. https://doi.org/10.1074/jbc.M202029200.

Page-McCaw, Andrea. 2008. "Remodeling the Model Organism: Matrix Metalloproteinase Functions in Invertebrates." *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2007.06.004.

Parsons, J. Thomas, Alan Rick Horwitz, and Martin A. Schwartz. 2010. "Cell Adhesion: Integrating Cytoskeletal Dynamics and Cellular Tension." *Nature Reviews. Molecular Cell Biology* 11 (9): 633. https://doi.org/10.1038/NRM2957.

Pascalis, Chiara De, and Sandrine Etienne-Manneville. 2017. "Single and Collective Cell Migration: The Mechanics of Adhesions." Edited by Valerie Marie Weaver. *Molecular Biology of the Cell* 28 (14): 1833–46.

https://doi.org/10.1091/mbc.e17-03-0134.

Pastore, Saveria, Francesca Mascia, Feliciana Mariotti, Cristina Dattilo, Valentina Mariani, and Giampiero Girolomoni. 2005. "ERK1/2 Regulates Epidermal Chemokine Expression and Skin Inflammation." *Journal of Immunology (Baltimore, Md. : 1950)* 174 (8): 5047–56. https://doi.org/10.4049/jimmunol.174.8.5047.

Peeters, Paul M., Emiel F. Wouters, and Niki L. Reynaert. 2015. "Immune Homeostasis in Epithelial Cells: Evidence and Role of Inflammasome Signaling Reviewed." *Journal of Immunology Research*. https://doi.org/10.1155/2015/828264.

Péguet-Navarro, Josette, Catherine Dalbiez-Gauthier, Corinne Moulon, Odile Berthier, Alain Réano, Martine Gaucherand, Françoise Rousset, Daniel Schmitt, and Jacques Banchereau. 1997. "CD40 Ligation of Human Keratinocytes Inhibits Their Proliferation and Induces Their Differentiation." *Journal of Immunology*.

Petrof, G., J. E. Mellerio, and J. A. McGrath. 2012. "Desmosomal Genodermatoses." *British Journal of Dermatology*. https://doi.org/10.1111/j.1365-2133.2011.10640.x.

Pöschl, Ernst, Ursula Schlötzer-Schrehardt, Bent Brachvogel, Kenji Saito, Yoshifumi Ninomiya, and Ulrike Mayer. 2004. "Collagen IV Is Essential for Basement Membrane Stability but Dispensable for Initiation of Its Assembly during Early Development." *Development*. https://doi.org/10.1242/dev.01037.

Purwar, Rahul, Miriam Wittmann, Jörg Zwirner, Martin Oppermann, Michael Kracht, Oliver Dittrich-Breiholz, Ralf Gutzmer, and Thomas Werfel. 2006. "Induction of C3 and CCL2 by C3a in Keratinocytes: A Novel Autocrine Amplification Loop of Inflammatory Skin Reactions." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.177.7.4444.

Qian, Xiaolan, Tatiana Karpova, Allan M. Sheppard, James McNally, and Douglas R. Lowy. 2004. "E-Cadherin-Mediated Adhesion Inhibits Ligand-Dependent Activation of Diverse Receptor Tyrosine Kinases." *EMBO Journal*. https://doi.org/10.1038/sj.emboj.7600136.

Quesnelle, Kelly M., Amanda L. Boehm, and Jennifer R. Grandis. 2007. "STAT-Mediated EGFR Signaling in Cancer." *Journal of Cellular Biochemistry*.

https://doi.org/10.1002/jcb.21475.

Ram, Maya, Yaniv Sherer, and Yehuda Shoenfeld. 2006. “Matrix Metalloproteinase-9 and Autoimmune Diseases.” *Journal of Clinical Immunology*. https://doi.org/10.1007/s10875-006-9022-6.

Ranson, M. 2004. “Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors.” *British Journal of Cancer*. https://doi.org/10.1038/sj.bjc.6601873.

Reiss, Yvonne, Amanda E. Proudfoot, Christine A. Power, James J. Campbell, and Eugene C. Butcher. 2001. “CC Chemokine Receptor (CCR)4 and the CCR10 Ligand Cutaneous T Cell-Attracting Chemokine (CTACK) in Lymphocyte Trafficking to Inflamed Skin.” *Journal of Experimental Medicine*. https://doi.org/10.1084/jem.194.10.1541.

Richmond, Jillian M., and John E. Harris. 2014. “Immunology and Skin in Health and Disease.” *Cold Spring Harbor Perspectives in Medicine*. https://doi.org/10.1101/cshperspect.a015339.

Ridiandries, Anisyah, Joanne T.M. Tan, and Christina A. Bursill. 2018. “The Role of Chemokines in Wound Healing.” *International Journal of Molecular Sciences*. https://doi.org/10.3390/ijms19103217.

Rübsam, Matthias, Aaron F. Mertz, Akiharu Kubo, Susanna Marg, Christian Jüngst, Gladiola Goranci-Buzhala, Astrid C. Schauss, et al. 2017. “E-Cadherin Integrates Mechanotransduction and EGFR Signaling to Control Junctional Tissue Polarization and Tight Junction Positioning.” *Nature Communications*. https://doi.org/10.1038/s41467-017-01170-7.

Sahin, Umut, Gisela Weskamp, Kristine Kelly, Hong Ming Zhou, Shigeki Higashiyama, Jacques Peschon, Dieter Hartmann, Paul Saftig, and Carl P. Blobel. 2004. “Distinct Roles for ADAM10 and ADAM17 in Ectodomain Shedding of Six EGFR Ligands.” *Journal of Cell Biology*. https://doi.org/10.1083/jcb.200307137.

Sano, Shigetoshi, Keith Syson Chan, Steve Carbajal, John Clifford, Mary Peavey, Kaoru Kiguchi, Satoshi Itami, Brian J. Nickoloff, and John DiGiovanni. 2005. “Stat3 Links Activated Keratinocytes and Immunocytes Required for Development of Psoriasis in a Novel Transgenic Mouse Model.” *Nature Medicine*. https://doi.org/10.1038/nm1162.

Sauder, Daniel N. 1990. “The Role of Epidermal Cytokines in Inflammatory Skin

Diseases." *Journal of Investigative Dermatology*. https://doi.org/10.1111/1523-1747.ep12505705.

Saunders, Ruth M., Mark R. Holt, Lisa Jennings, Deborah H. Sutton, Igor L. Barsukov, Andrey Bobkov, Robert C. Liddington, Eileen A. Adamson, Graham A. Dunn, and David R. Critchley. 2006. "Role of Vinculin in Regulating Focal Adhesion Turnover." *European Journal of Cell Biology* 85 (6): 487–500. https://doi.org/10.1016/j.ejcb.2006.01.014.

Schittek, Birgit, Rainer Hipfel, Birgit Sauer, Jürgen Bauer, Hubert Kalbacher, Stefan Stevanovic, Markus Schirle, et al. 2001. "Dermcidin: A Novel Human Antibiotic Peptide Secreted by Sweat Glands." *Nature Immunology*. https://doi.org/10.1038/ni732.

Schlüter, Holger, Roger Wepf, Ingrid Moll, and Werner W Franke. 2004. "Sealing the Live Part of the Skin: The Integrated Meshwork of Desmosomes, Tight Junctions and Curvilinear Ridge Structures in the Cells of the Uppermost Granular Layer of the Human Epidermis." *European Journal of Cell Biology* 83 (11–12): 655–65. https://doi.org/10.1078/0171-9335-00434.

Schulze, Waltraud X, Lei Deng, and Matthias Mann. 2005. "Phosphotyrosine Interactome of the ErbB-receptor Kinase Family." *Molecular Systems Biology*. https://doi.org/10.1038/msb4100012.

Schwartz, A.D., C.L. Hall, L.E. Barney, C.C. Babbitt, and S.R. Peyton. 2018. "Integrin A6 and EGFR Signaling Converge at Mechanosensitive Calpain 2." *Biomaterials* 178 (September): 73–82. https://doi.org/10.1016/J.BIOMATERIALS.2018.05.056.

Seddiki, Rima, Gautham Hari Narayana Sankara Narayana, Pierre Olivier Strale, Hayri Emrah Balcioglu, Grégoire Peyret, Mingxi Yao, Anh Phuong Le, et al. 2018. "Force-Dependent Binding of Vinculin to α-Catenin Regulates Cell-Cell Contact Stability and Collective Cell Behavior." *Molecular Biology of the Cell*. https://doi.org/10.1091/mbc.E17-04-0231.

Sehgal, Poonam, Xinyu Kong, Jun Wu, Raimon Sunyer, Xavier Trepat, and Deborah Leckband. 2018. "Epidermal Growth Factor Receptor and Integrins Control Force-Dependent Vinculin Recruitment to E-Cadherin Junctions" 131 (6): jcs206656. https://doi.org/10.1242/jcs.206656.

Seshacharyulu, Parthasarathy, Moorthy P Ponnusamy, Dhanya Haridas, Maneesh

Jain, Apar K Ganti, and Surinder K Batra. 2012. “Targeting the EGFR Signaling Pathway in Cancer Therapy.” *Expert Opinion on Therapeutic Targets* 16 (1): 15–31. https://doi.org/10.1517/14728222.2011.648617.

Sharp, Leslie L., Julie M. Jameson, Gabrielle Cauvi, and Wendy L. Havran. 2005. “Dendritic Epidermal T Cells Regulate Skin Homeostasis through Local Production of Insulin-like Growth Factor 1.” *Nature Immunology*. https://doi.org/10.1038/ni1152.

Sibilia, M, and E F Wagner. 1995. “Strain-Dependent Epithelial Defects in Mice Lacking the EGF Receptor.” *Science* 269 (5221): 234–38. https://doi.org/10.1126/science.7618085.

Sieg, D J, C R Hauck, D Ilic, C K Klingbeil, E Schaefer, C H Damsky, and D D Schlaepfer. 2000. “FAK Integrates Growth-Factor and Integrin Signals to Promote Cell Migration.” *Nature Cell Biology* 2 (5): 249–56. https://doi.org/10.1038/35010517.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P. P. Di Fiore, and S. Polo. 2005. “Clathrin-Independent Endocytosis of Ubiquitinated Cargos.” *Proceedings of the National Academy of Sciences* 102 (8): 2760–65. https://doi.org/10.1073/pnas.0409817102.

Simanski, M, F Rademacher, L Schroder, R Glaser, and J Harder. 2016. “The Inflammasome and the Epidermal Growth Factor Receptor (EGFR) Are Involved in the Staphylococcus Aureus-Mediated Induction of IL-1alpha and IL-1beta in Human Keratinocytes.” *PLoS One* 11 (1): e0147118. https://doi.org/10.1371/journal.pone.0147118.

Simpson, D. M., and R. Ross. 1972. “The Neutrophilic Leukocyte in Wound Repair a Study with Antineutrophil Serum.” *The Journal of Clinical Investigation*. https://doi.org/10.1172/JCI107007.

Singer, Adam J., and Richard Clark. 1999. “Mechanisms of Disease - Cutaneous Wound Healing.” *The New England Journal of Medicine*.

Singh, Amar B., and Rayrnond C. Harris. 2004. “Epidermal Growth Factor Receptor Activation Differentially Regulates Claudin Expression and Enhances Transepithelial Resistance in Madin-Darby Canine Kidney Cells.” *Journal of Biological Chemistry*. https://doi.org/10.1074/jbc.M308682200.

Stappenbeck, T. S., E A Bornslaeger, C M Corcoran, H H Luu, M L Virata, and K J Green. 1993. “Functional Analysis of Desmoplakin Domains: Specification of the Interaction with Keratin versus Vimentin Intermediate Filament Networks.” *The Journal of Cell Biology* 123 (3): 691–705. https://doi.org/10.1083/jcb.123.3.691.

Stappenbeck, T. S., and K J Green. 1992. “The Desmoplakin Carboxyl Terminus Coaligns with and Specifically Disrupts Intermediate Filament Networks When Expressed in Cultured Cells.” *The Journal of Cell Biology* 116 (5): 1197–1209. https://doi.org/10.1083/jcb.116.5.1197.

Stenger, Steffen, Dennis A. Hanson, Rachel Teitelbaum, Puneet Dewan, Kayvan R. Niazi, Christopher J. Froelich, Tomas Ganz, et al. 1998. “An Antimicrobial Activity of Cytolytic T Cells Mediated by Granulysin.” *Science*. https://doi.org/10.1126/science.282.5386.121.

Streilein, J. W. 1983. “Skin-Associated Lymphoid Tissues (SALT): Origins and Functions.” *Journal of Investigative Dermatology*.

Sugawara, Tadaki, Randle M. Gallucci, Petia P. Simeonova, and Michael I. Luster. 2001. “Regulation and Role of Interleukin 6 in Wounded Human Epithelial Keratinocytes.” *Cytokine*. https://doi.org/10.1006/cyto.2001.0946.

Suhng, Eunah, Bo Hee Kim, You Won Choi, Hae Young Choi, Hyunjin Cho, and Ji Yeon Byun. 2018. “Increased Expression of IL-33 in Rosacea Skin and UVB-Irradiated and LL-37-Treated HaCaT Cells.” *Experimental Dermatology*. https://doi.org/10.1111/exd.13702.

Sumigray, Kaelyn D, and Terry Lechler. 2015. “Cell Adhesion in Epidermal Development and Barrier Formation.” *Current Topics in Developmental Biology* 112: 383–414. https://doi.org/10.1016/bs.ctdb.2014.11.027.

Sun, Y J, Z L Zhuo, H P Xian, K Z Chen, F Yang, and X T Zhao. 2017. “Shp2 Regulates Migratory Behavior and Response to EGFR-TKIs through ERK1/2 Pathway Activation in Non-Small Cell Lung Cancer Cells.” *Oncotarget* 8 (53): 91123–33. https://doi.org/10.18632/oncotarget.20249.

Takashima, A., H. Matsue, P. R. Bergstresser, and K. Ariizumi. 1995. “Interleukin-7-Dependent Interaction of Dendritic Epidermal t Cells with Keratinocytes.” In *Journal of Investigative Dermatology*. https://doi.org/10.1038/jid.1995.10.

Tang, Qiu, Jun Jiang, and Jian Liu. 2015. “CCR5 Blockade Suppresses Melanoma

Development Through Inhibition of IL-6-Stat3 Pathway via Upregulation of SOCS3." *Inflammation*. https://doi.org/10.1007/s10753-015-0186-1.

Tian, Dai Shi, Jiyun Peng, Madhuvika Murugan, Li Jie Feng, Jun Li Liu, Ukpong B. Eyo, Li Jun Zhou, Rochelle Mogilevsky, Wei Wang, and Long Jun Wu. 2017. "Chemokine CCL2-CCR2 Signaling Induces Neuronal Cell Death via STAT3 Activation and IL-1β Production after Status Epilepticus." *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.0315-17.2017.

Tinkle, C. L., T. Lechler, H. A. Pasolli, and E. Fuchs. 2004. "Conditional Targeting of E-Cadherin in Skin: Insights into Hyperproliferative and Degenerative Responses." *Proceedings of the National Academy of Sciences* 101 (2): 552–57. https://doi.org/10.1073/pnas.0307437100.

Tomita, Kyoko, Ayano Kabashima, Brittany L. Freeman, Steven F. Bronk, Petra Hirsova, and Samar H. Ibrahim. 2017. "Mixed Lineage Kinase 3 Mediates the Induction of CXCL10 by a STAT1-Dependent Mechanism During Hepatocyte Lipotoxicity." *Journal of Cellular Biochemistry*. https://doi.org/10.1002/jcb.25973.

Tomschy, A, C Fauser, R Landwehr, and J Engel. 1996. "Homophilic Adhesion of E-Cadherin Occurs by a Co-Operative Two-Step Interaction of N-Terminal Domains." *The EMBO Journal* 15 (14): 3507–14.

Tsuruta, Daisuke, Takashi Hashimoto, Kevin J Hamill, and Jonathan C R Jones. 2011. "Hemidesmosomes and Focal Contact Proteins: Functions and Cross-Talk in Keratinocytes, Bullous Diseases and Wound Healing." *Journal of Dermatological Science* 62 (1): 1–7. https://doi.org/10.1016/j.jdermsci.2011.01.005.

Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, et al. 1984. "Human Epidermal Growth Factor Receptor CDNA Sequence and Aberrant Expression of the Amplified Gene in A431 Epidermoid Carcinoma Cells." *Nature* 309 (5967): 418–25. https://doi.org/10.1038/309418a0.

Vantourout, Pierre, Carrie Willcox, Andrea Turner, Chad M. Swanson, Yasmin Haque, Olga Sobolev, Anita Grigoriadis, Andrew Tutt, and Adrian Hayday. 2014. "Immunological Visibility: Posttranscriptional Regulation of Human NKG2D Ligands by the EGF Receptor Pathway." *Science Translational Medicine*. https://doi.org/10.1126/scitranslmed.3007579.

Vasioukhin, Valeri, Ethan Bowers, Christoph Bauer, Linda Degenstein, and Elaine Fuchs. 2001. “Desmoplakin Is Essential in Epidermal Sheet Formation.” *Nature Cell Biology* 3 (12): 1076–85. https://doi.org/10.1038/ncb1201-1076.

Walko, Gernot, Maria J. Castañón, and Gerhard Wiche. 2014. “Molecular Architecture and Function of the Hemidesmosome.” *Cell and Tissue Research* 360 (3): 529–44. https://doi.org/10.1007/s00441-014-2061-z.

Wan, Hong, Michael G. Stone, Cathy Simpson, Louise E. Reynolds, John F. Marshall, Ian R. Hart, Kairbaan M. Hodivala-Dilke, and Robin A.J. Eady. 2003. “Desmosomal Proteins, Including Desmoglein 3, Serve as Novel Negative Markers for Epidermal Stem Cell-Containing Population of Kiratinocytes.” *Journal of Cell Science*. https://doi.org/10.1242/jcs.00701.

Wang, Fei, Valerie M. Weaver, Ole W. Petersen, Carolyn A. Larabell, Shoukat Dedhar, Per Briand, Ruth Lupu, and Mina J. Bissell. 1998. “Reciprocal Interactions between B1-Integrin and Epidermal Growth Factor Receptor in Three-Dimensional Basement Membrane Breast Cultures: A Different Perspective in Epithelial Biology.” *Proceedings of the National Academy of Sciences* 95 (25).

Wang, Wei Jan, Chien Feng Li, Yu Yi Chu, Yu Hui Wang, Tzyh Chyuan Hour, Chia Jui Yen, Wen Chang Chang, and Ju Ming Wang. 2017. “Inhibition of the EGFR/STAT3/CEBPD Axis Reverses Cisplatin Cross-Resistance with Paclitaxel in the Urothelial Carcinoma of the Urinary Bladder.” *Clinical Cancer Research*. https://doi.org/10.1158/1078-0432.CCR-15-1169.

Wang, Yao Chen, De Wei Wu, Tzu Chin Wu, Lee Wang, Chih Yi Chen, and Huei Lee. 2018. “Dioscin Overcome TKI Resistance in EGFR-Mutated Lung Adenocarcinoma Cells via down-Regulation of Tyrosine Phosphatase SHP2 Expression.” *International Journal of Biological Sciences*. https://doi.org/10.7150/ijbs.22209.

White, F. H., and K. Gohari. 1984. “Some Aspects of Desmosomal Morphology during Differentiation of Hamster Cheek Pouch Epithelium.” *Journal of Submicroscopic Cytology*.

Wikstrand, C J, L P Hale, S K Batra, M L Hill, P A Humphrey, S N Kurpad, R E McLendon, et al. 1995. “Monoclonal Antibodies against EGFRvIII Are Tumor Specific and React with Breast and Lung Carcinomas and Malignant Gliomas.”

Cancer Res 55 (14): 3140–48. http://www.ncbi.nlm.nih.gov/pubmed/7606735.

Wilhelmsen, Kevin, Sandy H M Litjens, and Arnoud Sonnenberg. 2006. "Multiple Functions of the Integrin {alpha}6{beta}4 in Epidermal Homeostasis and Tumorigenesis." *Molecular and Cellular Biology* 26 (8): 2877–86. https://doi.org/10.1128/mcb.26.8.2877-2886.2006.

Wilson, Kristy J., Jennifer L. Gilmore, John Foley, Mark A. Lemmon, and David J. Riese. 2009. "Functional Selectivity of EGF Family Peptide Growth Factors: Implications for Cancer." *Pharmacology & Therapeutics* 122 (1): 1–8. https://doi.org/10.1016/j.pharmthera.2008.11.008.

Wood, Stephen, Vijayakumar Jayaraman, Erica J. Huelsmann, Brian Bonish, Derick Burgad, Gayathri Sivaramakrishnan, Shanshan Qin, et al. 2014. "Pro-Inflammatory Chemokine CCL2 (MCP-1) Promotes Healing in Diabetic Wounds by Restoring the Macrophage Response." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0091574.

Woodworth, C D, E Michael, D Marker, S Allen, L Smith, and M Nees. 2005. "Inhibition of the Epidermal Growth Factor Receptor Increases Expression of Genes That Stimulate Inflammation, Apoptosis, and Cell Attachment." *Mol Cancer Ther* 4 (4): 650–58. https://doi.org/10.1158/1535-7163.MCT-04-0238.

Yamaki, Mayuko, Kazumitsu Sugiura, Yoshinao Muro, Yoshie Shimoyama, and Yasushi Tomita. 2010. "Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitors Induce CCL2 and CCL5 via Reduction in IL-1R2 in Keratinocytes." *Experimental Dermatology*. https://doi.org/10.1111/j.1600-0625.2010.01108.x.

Yamazaki, Tomohide, Xuexian O. Yang, Yeonseok Chung, Atsushi Fukunaga, Roza Nurieva, Bhanu Pappu, Natalia Martin-Orozco, et al. 2008. "CCR6 Regulates the Migration of Inflammatory and Regulatory T Cells." *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.181.12.8391.

Yang, Y, X Fu, and J Li. 2002. "Effect of Keratinocyte Growth Factor-2 on Proliferation of Human Adult Keratinocytes." *Chin J Traumatol* 5 (6): 342–45. https://www.ncbi.nlm.nih.gov/pubmed/12443574.

Yarden, Y. 2001. "The EGFR Family and Its Ligands in Human Cancer. Signalling Mechanisms and Therapeutic Opportunities." *Eur J Cancer* 37 Suppl 4: S3-8. http://www.ncbi.nlm.nih.gov/pubmed/11597398.

Yarden, Y, and B Z Shilo. 2007. “SnapShot: EGFR Signaling Pathway.” *Cell* 131 (5): 1018. https://doi.org/10.1016/j.cell.2007.11.013.

Yin, Hui, Xiangyong Li, Shilian Hu, Tao Liu, Baohong Yuan, Hongbiao Gu, Qian Ni, Xiaofan Zhang, and Fang Zheng. 2013. “IL-33 Accelerates Cutaneous Wound Healing Involved in Upregulation of Alternatively Activated Macrophages.” *Molecular Immunology*. https://doi.org/10.1016/j.molimm.2013.05.225.

Yonemura, Shigenobu, Yuko Wada, Toshiyuki Watanabe, Akira Nagafuchi, and Mai Shibata. 2010. “α-Catenin as a Tension Transducer That Induces Adherens Junction Development.” *Nature Cell Biology* 12 (6): 533–42. https://doi.org/10.1038/ncb2055.

Young, P., Oreda Boussadia, Hartmut Halfter, Richard Grose, Philipp Berger, Dino P Leone, Horst Robenek, Patrick Charnay, Rolf Kemler, and Ueli Suter. 2003. “E-Cadherin Controls Adherens Junctions in the Epidermis and the Renewal of Hair Follicles.” *The EMBO Journal* 22 (21): 5723–33. https://doi.org/10.1093/emboj/cdg560.

Yu, H, D Pardoll, and R Jove. 2009. “STATs in Cancer Inflammation and Immunity: A Leading Role for STAT3.” *Nat Rev Cancer* 9 (11): 798–809. https://doi.org/10.1038/nrc2734.

Yu, Helena A., Leslie Perez, Qing Chang, Sizhi P. Gao, Mark G. Kris, Gregory J. Riely, and Jacqueline Bromberg. 2017. “A Phase 1/2 Trial of Ruxolitinib and Erlotinib in Patients with EGFR-Mutant Lung Adenocarcinomas with Acquired Resistance to Erlotinib.” In *Journal of Thoracic Oncology*. https://doi.org/10.1016/j.jtho.2016.08.140.

Yu, X., S. Miyamoto, and E. Mekada. 2000. “Integrin Alpha 2 Beta 1-Dependent EGF Receptor Activation at Cell-Cell Contact Sites.” *Journal of Cell Science* 113 (12).

Yue, C, S Shen, J Deng, S J Priceman, W Li, A Huang, and H Yu. 2015. “STAT3 in CD8+ T Cells Inhibits Their Tumor Accumulation by Downregulating CXCR3/CXCL10 Axis.” *Cancer Immunol Res* 3 (8): 864–70. https://doi.org/10.1158/2326-6066.CIR-15-0014.

Yue, L, J Cheng, Pf Zhang, G Lin, Z Yi, and R Zhang. 2006. “[Expression and Its Significance of STAT3, STAT5, Survivin and Ki67 in Nasal NK/T Cell Lymphoma].” *Lin Chuang Er Bi Yan Hou Ke Za Zhi* 20 (20): 931-933,936.

https://www.ncbi.nlm.nih.gov/pubmed/17225522.

Zaidel-Bar, Ronen, Shalev Itzkovitz, Avi Ma'ayan, Ravi Iyengar, and Benjamin Geiger. 2007. "Functional Atlas of the Integrin Adhesome." *Nature Cell Biology* 9 (8): 858–67. https://doi.org/10.1038/ncb0807-858.

Zehender, A, J Huang, A H Gyorfi, A E Matei, T Trinh-Minh, X Xu, Y N Li, et al. 2018. "The Tyrosine Phosphatase SHP2 Controls TGFbeta-Induced STAT3 Signaling to Regulate Fibroblast Activation and Fibrosis." *Nat Commun* 9 (1): 3259. https://doi.org/10.1038/s41467-018-05768-3.

Zeilhofer, Hanns Ulrich, and Walter Schorr. 2000. "Role of Interleukin-8 in Neutrophil Signaling." *Current Opinion in Hematology*. https://doi.org/10.1097/00062752-200005000-00009.

Zenz, Rainer, Robert Eferl, Lukas Kenner, Lore Florin, Lars Hummerich, Denis Mehic, Harald Scheuch, Peter Angel, Erwin Tschachler, and Erwin F. Wagner. 2005. "Psoriasis-like Skin Disease and Arthritis Caused by Inducible Epidermal Deletion of Jun Proteins." *Nature*. https://doi.org/10.1038/nature03963.

Zhang, K, J Sun, N Liu, D Wen, D Chang, A Thomason, and S K Yoshinaga. 1996. "Transformation of NIH 3T3 Cells by HER3 or HER4 Receptors Requires the Presence of HER1 or HER2." *J Biol Chem* 271 (7): 3884–90. http://www.ncbi.nlm.nih.gov/pubmed/8632008.

Zhang, Xiaoying, Peiji Yin, Dongmei Di, Guanghua Luo, Lu Zheng, Jiang Wei, Jun Zhang, Yuanping Shi, Jichen Zhang, and Ning Xu. 2009. "IL-6 Regulates MMP-10 Expression via JAK2/STAT3 Signaling Pathway in a Human Lung Adenocarcinoma Cell Line." *Anticancer Research*.

Zhou, Bo, Chaoyang Sun, Na Li, Wanying Shan, Hao Lu, Lili Guo, Ensong Guo, et al. 2016. "Cisplatin-Induced CCL5 Secretion from CAFs Promotes Cisplatin-Resistance in Ovarian Cancer via Regulation of the STAT3 and PI3K/Akt Signaling Pathways." *International Journal of Oncology*. https://doi.org/10.3892/ijo.2016.3442.

Ziegler, Wolfgang H, Robert C Liddington, and David R Critchley. 2006. "The Structure and Regulation of Vinculin." *Trends in Cell Biology* 16 (9): 453–60. https://doi.org/10.1016/j.tcb.2006.07.004.