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Effect of irritants in the epidermis: The role of kallikrein 5 and 7 and protease activated receptor 2

by

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ABSTRACT

FACULTY OF MEDICINE

Dermatopharmacology

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Joanne Marie Underwood

Contact dermatitis affects 0.5-1.9 cases per 1,000 full-time workers annually, with 80% of cases accounted for by irritant contact dermatitis. The mechanisms underlying irritant contact dermatitis are incompletely understood and there is also a large need in industry for screening tools to identify the irritant potential of novel chemical compounds. The aim of this thesis was to investigate whether disruption of the stratum corneum barrier by irritants induces epidermal serine proteases such as KLKs which act through the PAR2 receptor to cause a pro-inflammatory response. Croton oil (3%) and SDS (5%) were applied to *ex vivo* human skin and expression of KLK5, KLK7 and PAR2 analysed. Both croton oil and SDS increased KLK5 ($p=0.0001$ and 0.0002 respectively), KLK7 ($p=0.0027$ and 0.0009 respectively), and PAR2 ($p=0.0057$ and $p=0.0215$ respectively). In addition, protease activity within the epidermis was concomitantly increased ($p=0.0094$ for croton oil and $p=0.0185$ for SDS).

Although these findings help to provide mechanistic insight, and could have some use as a screening tool, the availability of human skin is a limiting factor in an industrial setting. Using Rho kinase-inhibition to increase the proliferative capacity of adult human keratinocytes to generate 3D epidermal equivalents, it was noted that the resulting stratum corneum was insufficiently resilient to withstand application of SDS, even at reduced concentrations. However, in monolayer cultures it was noted that, following initial screening using qPCR arrays, croton oil and dithranol caused upregulation of IL6, IL36A, CCL5 and CSF2 in Rho kinase-inhibited keratinocytes, and that CCL5 and CSF2 were similarly upregulated in normal human keratinocytes exposed to these compounds. Thus, in Rho kinase-inhibited keratinocytes, CCL5 and CSF2 (and possibly IL6 and IL36A) may form a useful screening tool for potential irritants generated in industry, although further work will be required to test sensitivity and specificity of this approach.

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DECLARATION OF AUTHORSHIP

I, **Joanne Underwood**

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and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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Signed: ……..

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List of abbreviations

2D KM, serum supplemented keratinocyte media for monolayer culture

3D KM, serum supplemented keratinocyte media for culture at air-liquid interface

ACD, allergic contact dermatitis

AD, atopic dermatitis

ALI, air-liquid interface

BCA, bicinchoninic acid protein

BSA, bovine serum albumin

CDSN, corneodesmosin

CnT57, commercially available keratinocyte media

DSC, desmocollin

DSG, desmoglein

ERK, extracellular signal-regulated kinases

FCS, foetal calf serum;

ICAM-1, intercellular adhesion molecule-1

ICD, irritant contact dermatitis

IL, interleukin

$IP_{3,}$ inositol triphosphate

JNK, c-Jun N-terminal kinases

K, keratin

KLK, kallikrein

KO, knock-out

List of abbreviations

LEKTI, lymphoepithelilal kazal type-related inhibitor

LLNA, local lymph node assay

LR, lipid rafts

MAPK, mitogen activated protein kinase

MEST, mouse ear swelling test

MHC, major histocompatibility complex

MMP, matrix metalloproteases

NF-κB, nuclear factor- κB

NHK, normal human keratinocytes

OECD, Organisation for Economic Cooperation and Development

PAR2, protease activated receptor 2

PBS, phosphate buffered saline

PLC, phospholipase C

RhoKIT, rho kinase inhibitor-treated

ROI, region of interest

SDS, sodium dodecyl sulphate

SPINK5, serine protease inhibitor kazal-type 5

SLS, sodium lauryl sulphate

TNFα, tumour necrosis factor-α

TSLP, thymic stromal lymphopoietin

1. Introduction

1.1 Skin structure and function

The skin is the bodies interface with the external environment and it has to perform many protective functions related to dealing with the wide range of noxious environmental factors with which it interacts on a daily basis. One group of skin functions are essentially homeostatic and these include thermoregulation and endocrine functions such as the production of vitamin D (McGrath and Uitto, 2010). However, the major functions of skin relate to protection against potentially damaging physical, chemical and microbial insults or attack to which it is subjected. The skin has to be able to provide protection against normal, everyday physical wear and tear and serious injury, requiring the ability to repair itself. The gross anatomical structure of skin comprises 2 compartments: the outer highly cellular epidermis and the inner matrix rich dermis (McGrath and Uitto, 2010). Epidermal proliferation allows the repair of superficial injuries, while dermal elements orchestrate more complex wound healing responses involving angiogenesis, fibrosis and tissue remodelling (Adzick, 1997).

The defences against environmental insults and injury are fulfilled through a range of properties. First is the formation of the stratum corneum, a mechanical barrier formed from terminally differentiated keratinocytes held together by residual desmosomal attachments, termed corneodesmosomes, which make up the outer layer of the epidermis (Candi et al., 2005). Lipids form layers between the corneocytes, allowing the stratum corneum to form a physical barrier that is substantially impenetrable by microbes. In addition, the stratum corneum is a water impermeable barrier that keeps the aqueous environment and water soluble chemicals out (Nemes and Steinert, 1999). In comparison, organic and lipid soluble chemicals can penetrate through the stratum corneum relatively easily (Elias, 1981). There are therefore metabolic defences in the epidermis including xenobiotic metabolising enzymes (Cytochrome P450), anti-oxidant defences and also reserves of small molecules such as glutathione and thioredoxin, capable of reacting with highly reactive and potentially damaging xenobiotics (Trouba et al., 2002; Ahmad and Mukhtar, 2004).

As well as providing a physical and chemical barrier to keep potentially harmful things out, a crucial protective role of the skin is to participate in the immune response towards substances or microbes with which it interacts. The skin is increasingly recognised as playing a highly active role not only providing direct local protection and defence through possession of a wide range of components of the innate immune response, but also playing an important role in co-ordination and programming of the systemic adaptive immune response (Trinchieri, 1995; Yang et al., 1999; Kupper and Fuhlbrigge, 2004; Pickard et al., 2006; Newell et al., 2009; Novak et al., 2010; Newell et al., 2013). The key cellular elements of the epidermis involved in these responses are the keratinocytes and the intra-epidermal dendritic cells (Langerhans cells); these cells produce cytokines, chemokines and other mediators that are primarily involved in a cross-talk designed to enhance anti-microbial defences (Palucka and Banchereau, 2002; Ziegler and Artis, 2010; Hennen et al., 2011)

If the barrier is compromised or breached, the epidermis is required to rapidly activate defensive mechanisms. These involve simultaneous activation of repair processes required to reconstitute the physical and permeability barriers as quickly as possible, and also activation of the innate immune response designed to deal with any microbes that might invade through the disrupted barrier defences. The innate immune response involves the generation of anti-microbial peptides such as β-defensins and cathelicidins and the activation of processes aimed at rapid recruitment of cellular elements including granulocytes and mononuclear cells (Braff and Gallo, 2006; Kerstin et al., 2010; Reinholz et al., 2012). An extensive range of cytokines, chemokines and adhesion molecules, including IL-1, IL-8, and TNF-α, ICAM-1, E-selectin and VCAM-1 are expressed on endothelial cells and keratinocytes (Nilsen et al., 1998; Gröne, 2002; Makrilia et al., 2009; Čabrijan and Lipozenčić, 2011). Whilst the innate response components may be able to deal with many invading microbes, a second function of the innate response is to activate dendritic antigen presenting cells which in turn activate the adaptive T lymphocyte-mediated immune response (Steinman and Hemmi, 2006). The signals given to the dendritic cells, via cytokines and other mediators, may determine how they programme the differentiation of the T cells with which they will interact (Zhou et al., 2003; Anderson et al., 2004; Dudda et al., 2004).

1.1.1 Programming of the adaptive immune response

The skin is able to programme the epidermal dendritic cells to present antigens to the T cell components of the adaptive response to generate different types of immune response, depending on constitutive factors, such as the presence or absence of the atopic predisposition, and the state of "environmental attack" the skin is under (He et al., 2006; Pickard et al., 2006; Ainscough et al., 2012; Newell et al., 2013). Under normal resting conditions in which the stratum corneum barrier is intact, there is nonetheless a continual flow of exogenous xenobiotics penetrating in low levels which are insufficient to perturb the epidermal environment. The epidermal dendritic cells then appear to generate immunological tolerance mediated by regulatory T cells (Tregs) (Seneschal et al., 2012). An example of this is metallic nickel, which most individuals encounter on a daily basis without clinical reaction to this contact. Such clinically tolerant individuals have been shown to have Tregs which are present in the skin at sites of contact with nickel and which suppress the generation of any effector T cell-mediated inflammatory process (Cavani et al., 2003). However, when the epidermis is perturbed by something that is sensed as "dangerous", such as microbial products or disruption of the permeability barrier, the dendritic cells acquire the ability to activate effector T cells required for anti-microbial immunity (He et al., 2006; Ainscough et al., 2012). When strong contact sensitisers such as dinitrochlorobenzene (DNCB) penetrate into the epidermis, the T cell response generated comprises primarily Th1 and cytotoxic T effector cells (Pickard et al., 2006). However, when the same antigen enters the epidermis of individuals with susceptibility to atopic dermatitis, a skin disorder which is characterised by a red, inflamed scaly skin, the anti-DNCB T cell response is predominantly of Th2 cells (Newell et al., 2009).

1.1.2 Dermis

The overall thickness of the dermis varies depending on the location of the skin, from 5 mm on the back to 0.5 mm on the eyelids (McGrath and Uitto, 2010). It has several functions, including providing nourishment and mechanical support to the cell-rich epidermis. It is split into two layers: the reticular dermis and the papillary dermis. The reticular dermis is the lower layer, constituting 90% of the dermis (McGrath and Uitto, 2010). This layer is composed primarily of thick, densely packed collagen and

elastic fibres providing structure and stability to the upper layers. These collagen and elastin fibres are synthesised by dermal fibroblasts which populate the dermis and serve to maintain the structure of the connective tissue following wounding or injury (Moulin et al., 1996). Elastin and elastin-associated microfibrils make up between 2-4% of the extracellular matrix in the reticular dermis, and provide elasticity and resilience (Rosenbloom et al., 1993). Collagen fibres, primarily type 1 and type 3 (Weber et al., 1984), make up the bulk of the extracellular matrix and provide tensile strength to the skin. The remaining extracellular matrix is provided by ground substance made up of glycosaminoglycan and proteoglycan macromolecules (McGrath and Uitto, 2010). The papillary dermis is a more superficial layer between the reticular dermis and epidermis, consisting of loosely arranged, thin collagen fibres (Meigel et al., 1977).

Adnexal structures of the skin include eccrine sweat glands, apocrine sweat glands, sebaceous glands, and hair follicles. These structures originate and are anchored in the dermis but penetrate the epidermis (Holbrook, 1991). Nutrients and oxygen are provided to the dermis via a series of vascular plexuses present at several layers throughout the dermis. The papillary dermis is fed by a complex of capillary loops which make up the superficial plexus, while the adnexal structures of the reticular dermis are fed by a deeper plexus (Irwin, 1989).

1.1.3 Epidermis

The epidermis is separated from the dermis by a basement membrane, formed by laminins and type IV collagen (Alberts et al., 2002) which are anchored together by nidogen proteins (Fox et al., 1991). This basement membrane allows the epidermis to bind firmly to the loosely arranged fibres of the papillary dermis (Van Agtmael and Bruckner-Tuderman, 2010). The epidermis consists of 4 distinct layers (from the inner surface to the outer surface): stratum basale; stratum spinosum; stratum granulosum; and stratum corneum (**Figure 1.1**). In areas of thickened skin, such as the palms of the hands and the soles of the feet, a stratum lucidum layer is present between the stratum granulosum and stratum corneum (McGrath and Uitto, 2010). The stratum basale is made up of a single layer of keratinocytes anchored to the basement membrane by hemidesmosomes (Kelly, 1966). Keratinocytes migrate from

the stratum basale layers upwards through the layers of the epidermis during their lifespan, flattening until they undergo cornification, whereby the cells undergo terminal differentiation into anucleated corneocytes which organise into a lattice structure (Hohl, 1990). The process of terminal differentiation is regulated by caspase-14, a protease limited to the suprabasal layers of the epidermis, however the exact pathways involved are still not well understood (Eckhart et al., 2000; Rendl et al., 2002)

Keratinocytes generate filaggrin immediately before cornification through phosphorylation and processing of the profilaggrin protein by kinases and serine proteases including caspase-14 (Hoste et al., 2007), calpain 1 (Yamazaki et al., 1997), matriptase (List et al., 2003) and prostasin (Leyvraz et al., 2005). Filaggrin acts as a keratin aggregating protein, binding to keratin intermediate filaments and causing them to align in tightly packed parallel arrays known as macrofibrils (Dale et al., 1985; Sybert et al., 1985). Extensive crosslinking of these macrofibrils by transglutaminases forms a stable, highly insoluble keratin matrix which acts as a protein scaffold for the formation of the stratum corneum by cornified cell envelopes and lipids (Manabe et al., 1991; Steinert and Marekov, 1995). Caspase-14 also degrades the filaggrin into free amino acids in the stratum corneum (Denecker et al., 2007; Hoste et al., 2011) which contribute to the natural moisturising factors which absorb water and maintain hydration of the skin (Rawlings and Harding, 2004).

Immediately before cornification the keratinocytes also secrete large quantities of polar lipids and hydrolytic enzymes from lamellar bodies into the extracellular spaces of the stratum corneum (Bickenbach et al., 1995). The extreme hydrophobicity, intercellular location and organisation of these lipids into broad bilayers accounts for their ability to regulate barrier function (Elias and Friend, 1975). The physical barrier formed by the lattice structure of cornified cells prevents exogenous substances from interacting with the immune system and initiating an immune response (Monash, 1957). The outer layers of the stratum corneum are also sulphur rich (Ogawa et al., 1979), the sulphur forming part of the thiol groups on cysteine as in glutathione, which a recent study has indicated may act as a biochemical buffer against highly reactive chemical sensitisers (Pickard et al., 2009). The stratum corneum has a significantly decreased pH compared with the lower layers of the skin. This ‘acid mantle’ is thought to be important as part of the antimicrobial properties of the

epidermis (Behne et al., 2002; Fluhr and Elias, 2002). As previously mentioned, adnexal structures can create avenues through the epidermal barrier by penetrating the stratum corneum. This may facilitate transepidermal absorption of drugs through the epidermis (Wosicka and Cal, 2010).

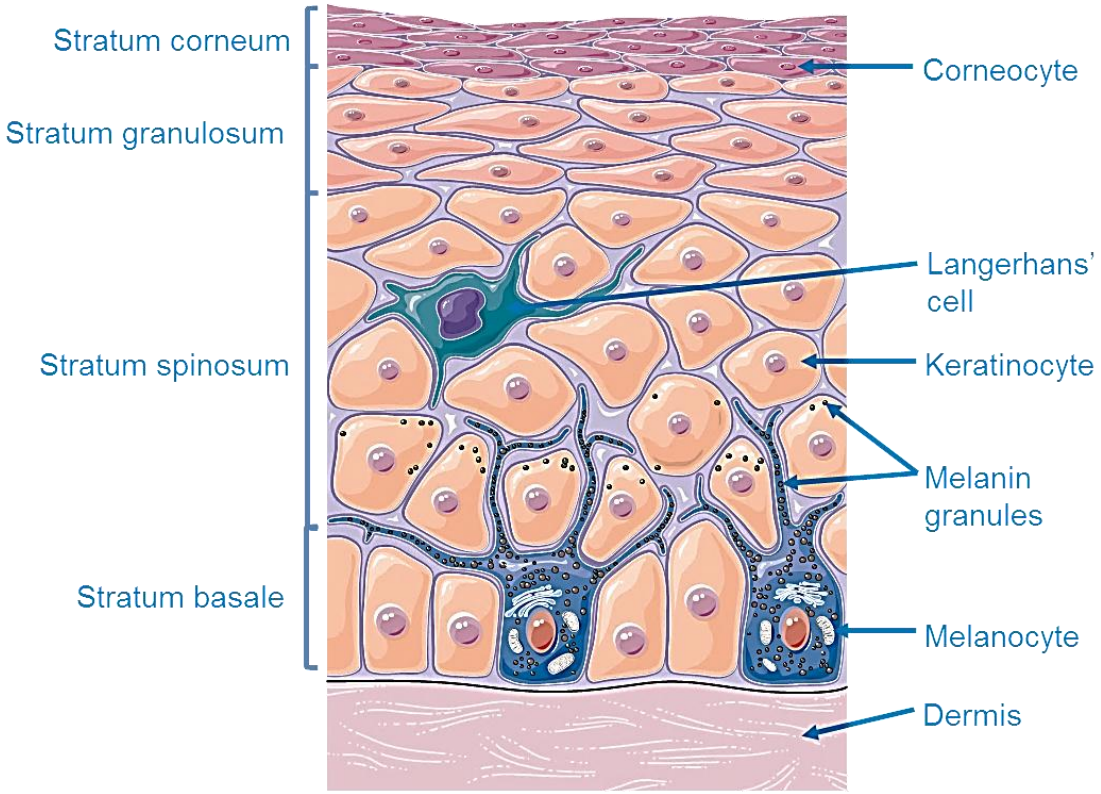


Figure 1.1: Layers of the epidermis. The epidermis primarily consists of keratinocytes, however, Langerhans cells, melanocytes and Merkel cells are also present. The stratum corneum consists of flat polyhedral envelopes surrounding cornified cells. The envelopes, consisting of late envelop proteins, are filled with keratin making the stratum corneum water resistant, and the lattice structure prevents the entry of allergens and other exogenous substances (Nemes and Steinert, 1999). Image used with permission from www.servier.co.uk.

A calcium gradient is maintained across the epidermis, increasing from the stratum basale to the stratum granulosum (Menon et al., 1985). The stratum corneum has a low calcium concentration compared with the stratum granulosum, primarily due to the fact that the relatively dry environment of the corneum means that the corneocytes are not able to dissolve the calcium ions (Elias et al., 2002). This gradient

is required to control the differentiation of keratinocytes and secretion of lamellar bodies, as discussed in section 1.1.6.

1.1.4 Cellular components

While keratinocytes make up 95% of the epidermis, other cell types are also found in the basal and suprabasal layers. These cells contribute to the functions of the skin, including immune surveillance, pigmentation and touch sensation. Melanocytes, Langerhans cells (LC) and Merkel cells make up most of the remaining 5% of the cell population (McGrath and Uitto, 2010). They are found in the basal layer, and in the case of Langerhans cells in the suprabasal layer, of the epidermis, while the stratum corneum is made up entirely of cornified keratinocytes. A small number of T cells (Spetz et al., 1996) and fibroblasts (Hentzer and Kobayasi, 1975) make up the remaining cell population of normal human epidermis.

1.1.5 Melanocytes, Langerhans cells and Merkel cells

Melanocytes are a subset of dendritic cells which produce granules of melanin pigment and package it into sub-cellular organelles - melanosomes. These are then transferred to keratinocytes within the epidermis where the melanin acts to protect the nucleus and the DNA within from damage by UV radiation (Costin and Hearing, 2007; Lin and Fisher, 2007). Melanocytes reside in the basal layer of the epidermis, with approximately one melanocyte for every 36 keratinocytes (Hoath and Leahy, 2003). While there are no significant differences in the numbers of melanocytes between different races or ethnic groups, it is the size of melanosomes and their intracelleular organisation which determines the overall pigmentation of the skin observed between populations (Szabo et al., 1969)

Langerhans cells are a subset of dendritic cells which reside in the stratum basale and stratum spinosum of the epidermis (Romani et al., 2008), extending dendritic processes into the stratum gransulosum. These professional antigen-presenting cells have several important immunological surface receptors including major histocompatibility complex (MHC)-II, CD1a, complement and IgG receptors. They play

an active role in the adaptive immune system by sampling pathogens and microbes which enter the epidermis and presenting antigens to naive T-cells following migration of the Langerhans cells to the lymph nodes (Cumberbatch et al., 1991; Furio et al., 2010; Seneschal et al., 2012).

Merkel cells provide light touch sensation in the skin, forming complexes with somatosensory afferents, which are dermal nerves that carry signals from touch receptors in the skin to the central nervous system (Maricich et al., 2009). While it is known that the Merkel cell-nerve complex acts as a mechanoreceptor, the physiological mechanisms behind this activity are currently undetermined. Merkel cells are present at a far higher concentration in the palms of the hands and soles of the feet compared with the rest of the skin, which supports the theory that Merkel cells are involved in touch perception (Polakovicova et al., 2011). In addition, Merkel cells have been shown to be in contact with the outer root sheath of hair follicles (Narisawa and Hashimoto, 1991; Moll, 1994; Polakovicova et al., 2011), however the exact purpose of this association is unclear.

1.1.6 Keratinocytes

Keratinocytes are the first cells to encounter pathogens, allergens or irritants from the external skin environment. Previously thought to be purely structural cells, keratinocytes have been shown to be immunologically active, playing a significant role in the innate and adaptive immune responses. Upon encountering pathogens keratinocytes are capable of acting as non-professional antigen presenting cells (Bal et al., 1990; Mutis et al., 1993) leading to the activation of T cells and other professional immune cells. Toll-like receptors on the surface of keratinocytes can be activated by various stimuli, leading to the secretion of cytokines and chemokines which recruit professional immune cells including macrophages, T-cells and natural killer cells (Niebuhr et al., 2010; Olaru and Jensen, 2010).

As keratinocytes move up from the basal layer to the stratum corneum they undergo differentiation. Once the keratinocyte begins to migrate upwards from the basal layer they withdraw from the cell cycle and begin to express a variety of additional/different cell surface markers and proteins (Candi et al., 2005) **(Figure 1.2)**.

Most notably, they switch the pattern of keratin expression from keratin 5 (K5) and K14, expressed by basal layer keratinocytes (Fuchs and Cleveland, 1998), to K1 and K10 and involucrin (a precursor of the cornified envelope) expressed by keratinocytes committed to terminal differentiation (Candi et al., 2005). The differentiation process is reflected in structural and metabolic modifications in order to become corneocytes. The process of differentiation is calcium dependent, therefore the establishment and conservation of the calcium gradient from the stratum basale to the stratum granulosum is vital to maintain correct differentiation (Bikle et al., 2001).

Keratinocytes in the upper stratum spinosum contain specialised tubulovesicular secretory granules known as lamellar bodies which contain lipids (glucosylceramides, sphingomyelin, phospholipids and cholesterol), lipid-processing enzymes (sphingomyelinase, secretory phospho-lipase A2) and structural proteins (corneodesmosin) which are required for the formation of the permeability barrier (Elias and Friend, 1975; Odland and Holbrook, 1981; Raymond et al., 2008). Once secreted from differentiated keratinocytes at the stratum granulosum-stratum corneum junction they deliver their contents to the stratum corneum interstices, facilitating the formation of the hydrophobic barrier. Lamellar bodies also contain antimicrobial peptides, proteases and protease inhibitors required for controlled desquamation (Odland and Holbrook, 1981; Freinkel and Traczyk, 1983; Grayson et al., 1985; Menon et al., 1992c).

1.1.7 Desquamation

Desquamation is a process by which corneocytes are actively shed. In normal human skin the number of corneocytes that may be shed ranges from $2x10^8$ to $10x10^8$ per person per day (Roberts and Marks, 1980), and this homeostatic process maintains a balance between *de novo* production of keratinocytes in the basal layer and removal of corneocytes at the surface of the stratum corneum. As discussed in section 1.1.6, keratinocyte proliferation, differentiation and death are associated with expression of specific proteins including keratins and adhesion molecules. In addition, the proteins which maintain adhesion between neighbouring keratinocytes vary from the lower epidermal layers to the upper stratum corneum.

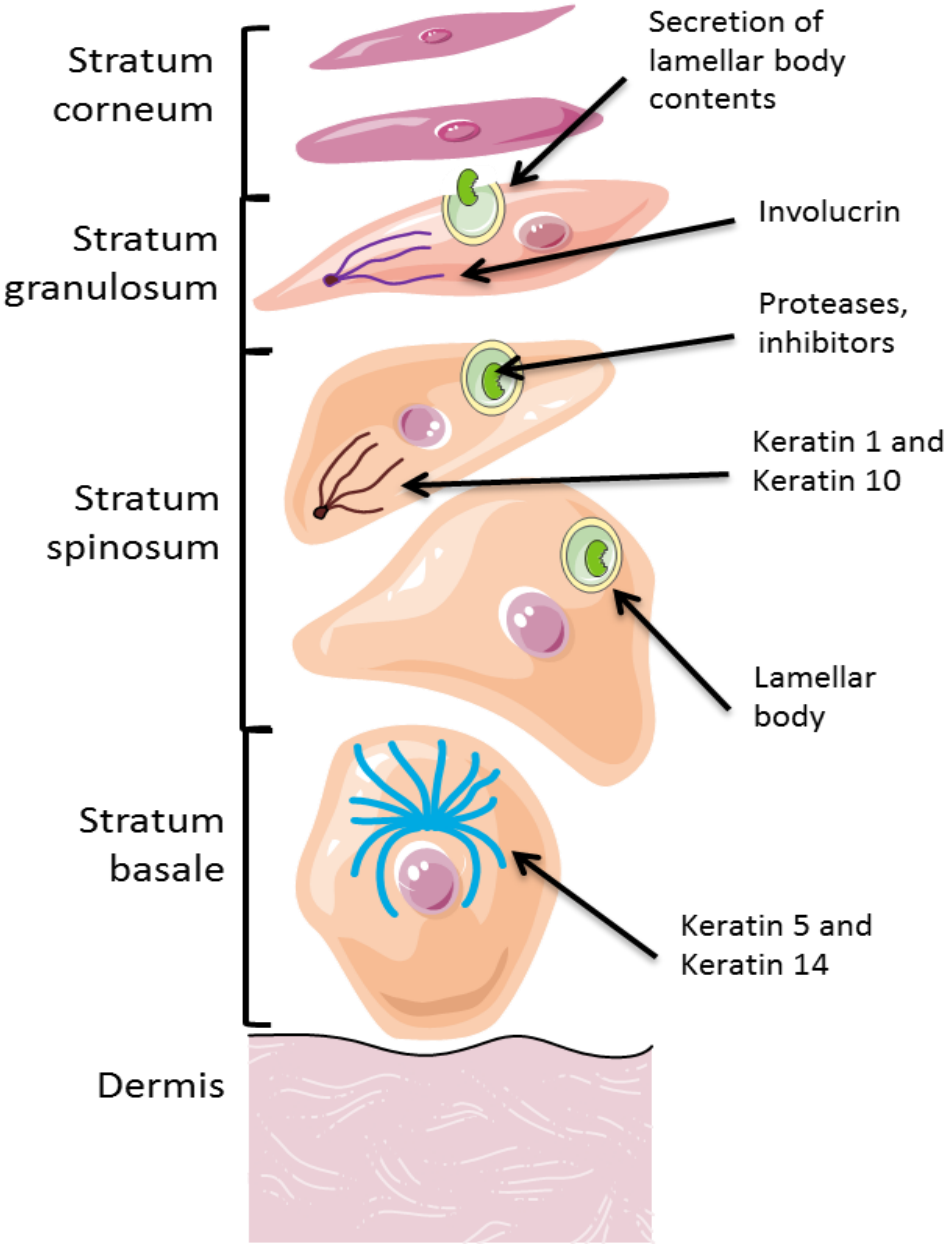


Figure 1.2: Keratinocyte differentiation in the epidermis. Keratinocytes alter the expression of many protein markers throughout the stages of differentiation. Secretion of specific proteins is associated with each stage of differentiation, for example keratins 5 & 14 are expressed in the stratum basale, keratin 1 & 10 are expressed in the late stratum spinosum/stratum granulosum, while involucrin is expressed only when keratinocytes have committed to terminal differentiation. Protein secretion is also altered. Lamellar bodies mature during the later stages of differentiation in the late stratum spinosum and are secreted from the keratinocytes in the stratum granulosum before cornification in the stratum corneum (Elias and Friend, 1975). Original image taken with permission from www..servier.co.uk and adapted to include features such as lamellar bodies and keratins.

In order for desquamation to occur, corenocytes must be separated from the neighbouring cells. Throughout the layers of the epidermis the inter-keratinocyte adhesions are maintained by desmosomes comprising structural adhesion proteins of which 7 types have been identified (desmoglein (DSG) 1-4 and desmocollin (DSC) 1-3). In the lower epidermal layers DSG2, DSG3, DSC2 and DSC3 are highly expressed, whilst DSG1, DSG4 and DSC1 are highly expressed in the stratum corneum (Green and Simpson, 2007). Corneodesmosomes are complexes similar to desmosomes, however they contain corneodesmosin (CDSN), a characteristic adhesive glycoprotein at their surface that forms junctions between keratinocytes in the upper stratum corneum. It has been suggested that non-peripheral corneodesmosomes, i.e those below the outer surface of the stratum corneum, undergo initial pH-dependent proteolysis by epidermal kallikreins (KLK) KLK5 and KLK7. While these proteasesare known to degrade DSG1 and DSC1 corneodesmosomal isoforms (Descargues et al., 2006; Borgono et al., 2007), they appear to have additional roles in activating the innate immune response in damaged epidermis, as discussed in section 1.4.3. Final degradation of the peripheral corneodesmosomes in the superficial stratum corneum leads to shedding of the cells.

1.2 Contact Dermatitis

Occupational contact dermatitis accounts for 30% of all occupational diseases, and more than 95% of work-related dermatoses, with an incidence thought to be in the region of 0.5-1.9 cases per 1,000 full-time workers per year for most countries (Diepgen and Coenraads, 1999). Contact dermatitis can be divided into two sub-categories: irritant contact dermatitis (ICD) and allergic contact dermatitis (ACD).

1.2.1 Irritant Contact Dermatitis

ICD accounts for approximately 80% of contact dermatitis (HSE, 1998), and can be further sub-divided into acute and chronic types. Acute ICD is characterised by redness, burning, stinging, and soreness of affected areas of skin within 48 hours of

contact with the irritant (Wilkinson and Beck, 2010). This is usually caused by an overwhelming exposure or a few brief exposures to strong irritants (English, 2004) including acids, alkalis or irritant solvents such as turpentine. Chronic cumulative ICD may present as dryness, fissuring, hyperkeratosis and increased desquamation lasting for more than 6 weeks which may continue in some cases for years as persistent post-irritant dermatitis following removal of the irritant (Wilkinson and Beck, 2010). Chronic ICD may be caused by repeated exposure to either wet irritants such as detergents, soaps or weak acids or alkalis, or dry irritants such as low humidity air, powders or dust (English, 2004). In the case of chronic ICD an individual may be exposed to concentrations of irritant that cause a subclinical response. However, following a more intense exposure the same low concentration may cause a clinical response in subsequent exposures (English, 2004). ICD is most commonly diagnosed in individuals in wet-work occupations, defined as spending more than 2 hours a day working in a wet environment or wearing occlusive gloves (Lachapelle and Marot, 2011).

1.2.2 Mechanism of irritant contact dermatitis

The mechanism behind ICD is complex and not well characterised, but involves damage to the skin barrier. However, it is a non-antigen specific response with no involvement of immunological memory. The skin barrier can be damaged by either physical disruption, such as tape stripping, or chemical disruption by solvents or surfactants or sensitising compounds with irritant properties. Many different types of chemicals can be irritants, from toxic chemicals such as extreme acids or alkalis (Basketter et al., 2004; Antonov et al., 2012), solvents and surfactants including acetone and sodium lauryl sulphate (SLS) (Zesch, 1983; Nielsen, 2000; Basketter et al., 2004), or organic lipid soluble compounds such as nonanoic acid, dithranol and croton oil (Wahlberg and Maibach, 1980; Wilmer et al., 1994; Schiavi et al., 1996). Studies indicate that each class of irritant may instigate an inflammatory (innate immune) response via different pathways (Willis et al., 1993; Lisby et al., 1995; Grängsjö et al., 1996).

Solvents and surfactants are thought to disrupt the barrier by dissolving the intercellular lipids and the cell membranes, which leads to an inflammatory irritant

response (Novak and Francom, 1984). The skin responds to chemical barrier disruption by initiating repair of the barrier and activation of the immune system (Kobayashi et al., 2003). Some chemicals are very strong irritants to the point of being corrosive to the skin, for example sulfuric acid, hydrochloric acid, hydrofluoric acid and nitric acid (Cartotto et al., 1996; Antonov et al., 2012), whereby the damage is no longer reversible.

The primary response of the epidermis to perturbation and barrier disruption by irritants is to activate innate immune responses involving an inflammatory response mediated by cytokines and chemokines. Keratinocytes are the primary source of cytokines within the epidermis, along with Langerhans cells and melanocytes (Williams and Kupper, 1996). Upon barrier disruption, keratinocytes release interleukin (IL) -1α, which is stored in large quantities as active forms within the keratinocytes (Corsini and Galli, 2000). IL-1α stimulates production of IL-1β, tumour necrosis factor-α (TNFα), IL-6, IL-8 and GM-CSF from keratinocytes (Kupper et al., 1988; Enk and Katz, 1992; Yano et al., 2008). TNFα induces the expression of adhesion molecules in cutaneous vasculature (Osborn et al., 1989; Kyan-Aung et al., 1991), while TNFα and IL-β are required to stimulate the migration of Langerhans cells (Rambukkana et al., 1996; Cumberbatch et al., 1997). IL-8 and IL-18 act as chemokines, recruiting leukocytes including monocytes, macrophages and natural killer cells which contribute to the inflammatory response (Larsen et al., 1989; Barker et al., 1991b; Leung et al., 2001; Terada et al., 2006).

Prevention methods for ICD primarily centre around avoidance of contact with the irritant by use of personal protective equipment such as gloves, replacement of the irritant substance where possible, or the use of barrier creams to prevent the irritants coming into contact with the skin (Slodownik et al., 2008). Severe ICD may be treated with topical corticosteroids which reduce the inflammation by supressing the immune response (Levin et al., 2001).

1.2.3 Allergic contact dermatitis

Epidemiological studies using patch testing for multiple allergens have shown that 12.5-40.6% of people are allergic to at least one allergen (Thyssen et al., 2007), the

most prevalent of which were nickel, thiomersal, fragrance mix and potassium dichromate (Moss et al., 1985; Thyssen et al., 2007). People that are polysensitive (sensitive to 2 or more allergens) are more likely to become sensitised to weak allergens compared with the general population (Schnuch et al., 2008). They have also been shown to be more readily sensitised to the experimental sensitiser DNCB (Moss et al., 1985).

Symptoms of ACD can be indistinguishable from those of ICD; however, the inflammation may sometimes be present in areas that did not come into direct contact with allergen, and there may be a delay of several days between allergen exposure and the rash as it takes at least a week for allergic sensitisation to develop (Friedmann, 1998). Histopathological comparison of allergic and irritant dermatitis show identical patterns of cellular infiltration and inflammatory markers (Willis et al., 1986). Acute irritant reactions in the skin mainly present as epidermal necrosis, however the responses vary depending on the location of the skin and the concentration of irritant (Lachapelle and Marot, 2011).

1.2.4 Mechanism of allergic contact dermatitis

The mechanisms behind ACD have been well characterised. It is a type 4, T cell-mediated hypersensitivity response, which is an antigen specific response mediated by memory/effector T lymphocytes. Lipophilic, low molecular weight chemicals of less than 500 Da which are not immunogenic *per se* are able to penetrate the skin barrier and bind to (haptenate) self-proteins to form neo-antigens (Landsteiner and Jacobs, 1935; Martin, 2004; Simonsson et al., 2011). Simonsson *et al* recently demonstrated that, among others, K5 and K14 are target proteins for haptenation in the epidermis (Simonsson et al., 2011). Haptenation of proteins and/or peptides is required to allow recognition of the hapten by the T cell receptor when it is presented on the carrier peptide lying the groove of the major histocompatibility complex (MHC) (Kaplan et al., 2012). Some chemically inert substances such as substituted benzene derivatives, for example p-Phenylenediamine, are known as prohaptens, as they need to undergo air oxidation or bioactivation in keratinocytes or phagocytes in order to elicit an immune response (Basketter and Liden, 1992; Kawakubo et al., 2000; Pickard et al., 2006; Karlberg et al., 2007; Rudbäck et al., 2012).

Once successful haptenation and, if required, bioactivation has been achieved, the neoantigens are processed and presented by epidermal Langerhans cells and dermal dendritic cells. The Langerhans cells and dendritic cells then migrate to the regional lymph nodes where the neo-antigens are presented to naïve T-cells via the MHC (Grabbe and Schwarz, 1998; Cavani et al., 2001). This process is referred to as sensitisation, or the afferent phase of ACD and no adverse response is elicited at this stage. The priming of the memory T-cells and circulation of antigen specific T-memory cells takes approximately 7 - 10 days from contact with the allergen (Friedmann, 2007; Vocanson et al., 2009). Once this has occurred, any subsequent challenge by the same antigen results in recruitment of effector T-cells to the skin, resulting in the characteristic eczematous inflammation. In order to elicit the specific T cell memory response, a threshold level of allergen exposure is required, however it has been shown that application of unrelated allergens in combination at below-threshold levels can cause a response from both, with one allergen reducing the threshold of the other (McLelland and Shuster, 1990).

Exactly how allergenic chemicals which elicit an allergic contact dermatitis response are sensed as 'dangerous' and induce an immune response is unclear, however, studies have shown that molecular pattern receptors such as Toll-like Receptors (TLRs) are able to sense and respond to some allergens. For example nickel activates human TLR4 by binding directly to histidine residues contained within it (Schmidt et al., 2010). Martin *et al* showed that mice lacking TLR2 and TLR4 are resistant to TNCB-induced contact hypersensitivity (Martin et al., 2008), providing further evidence that TLRs are important in the mounting of an allergic response. Oxidative stress is also thought to be important in the sensing of some allergens as 'dangerous'. It has been shown that contact sensitisers induce the production of reactive oxygen species, and application of antioxidants to the skin prevent a hypersensitivity response to TNCB in mice (Esser et al., 2012). Most strong sensitisers also act as irritants (Coquette et al., 2003) and this irritant effect may be involved in the danger-signalling. There is evidence that irritants can activate common pathways with allergic contact dermatitis (Brasch et al., 1992), however how this is achieved has not yet been elucidated. The irritant response to exogenous chemicals and allergic responses to potential allergens varies widely between individuals, making it difficult to predict potential irritants and allergens (Cua et al., 1990; Muizzuddin et al., 1998).

1.2.5 Susceptibility to contact dermatitis

Several factors contribute to the generation of contact sensitivity to environmental chemicals. These include genetic predisposition, age, gender, race, site of irritant application, the barrier function of the skin, and neurological factors (Wilhelm and Maibach, 1990). Twin studies have shown that there is a genetic risk factor for hand eczema that may be of importance for the development of ICD (Bryld et al., 2003). In a comparison of older skin (approximately 70 years old) against younger skin (approximately 27 years old) it was found that older skin showed a delayed and decreased reaction to irritant application (Schwindt et al., 1998). Men were found to be more reactive than women in a test of 4 irritants and Asian subjects were found to be more sensitive to 3 test chemicals compared with Caucasian subjects (Robinson, 2002). Hicks *et al* have shown that black skin may be more resistant to irritants compared with white skin (Hicks et al., 2003). Dryness of the skin and a history of atopic dermatitis has been shown to cause increased susceptibility to ICD (Tupker et al., 1990). Deficiencies in the filaggrin gene (*FLG*) also lower the threshold for irritant and acute irritant contact dermatitis (Scharschmidt et al., 2009). A case study has shown that of those who suffer from chronic irritant contact dermatitis (CICD), 12.5% have polymorphisms in the *FLG* gene, significantly more than the control group (6.9%) (De Jongh et al., 2008). The same study demonstrated that carriers of the null *FLG* gene had a higher lifetime prevalence of flexural eczema and higher atopy scores than non-carriers, and carriers showed significantly more signs of dermatitis within the control group (non-CICD subjects). Psychosocial and sleep deprivation stress have been shown to disrupt barrier function homeostasis, decreasing barrier function recovery following tape stripping (Altemus et al., 2001). Overall, there are several genetic and environmental factors that contribute to susceptibility to ICD, not the least of which is a predisposition to epidermal barrier damage.

ACD to specific allergens can be tested by application of the suspected allergen to the skin in a process known as patch testing. The allergen is mixed in a suitable base, such as white soft paraffin (Vaseline) and applied to a small aluminium or plastic disc. The disc is then applied to the skin with adhesive tape for 48 hours and the response assessed by a dermatologist (Rive et al., 2013). Using this process large numbers of allergens can be tested at one time.

1.3 The role of the skin barrier in irritant responses

Development of ichthyosis vulgaris, a condition characterised by the formation of dry, flaky scales, has been associated with loss-of-function mutations in *FLG*, the gene encoding filaggrin, and mutations in this gene also represents a strong predisposition to the development of atopic eczema, asthma and peanut allergies (De Jongh et al., 2008; Sandilands et al., 2009; Irvine et al., 2011). Mutations affecting the activity of the transglutaminase family, of which 9 members have been identified to date, are also associated with altered barrier function (Zeeuwen, 2004). Loss of function of transglutaminase 1 causes the development of lamellar ichthyosis (Huber et al., 1995; Russell et al., 1995), another scaly-skin condition affecting the epidermis causing hyperkeratotic skin at birth which cracks and exposes the underlying tissues leading to dehydration. Interestingly, the underlying skin is red indicating a chronic low level inflammatory response, however, there is no research to indicate that sufferers have an increased susceptibility to irritants.

It has long been known that certain genetic skin disorders such as atopic dermatitis (AD), Netherton's syndrome and autosomal recessive congenital ichthyosis , which are characterised by intrinsic defects in the stratum corneum barrier, have an associated inflammatory process (Ruzicka et al., 1986; Rabinowitz and Esterly, 1994; Smith et al., 1995). Previously it was thought that the increased inflammation was due to an increase in exogenous substances breaching the compromised barrier leading to an inflammatory response. However, it has recently been suggested that the break in the barrier itself is causing the inflammatory response by activating the innate immune response (Proksch et al., 2008; Elias and Schmuth, 2009). Studies show that barrier breakage by tape stripping, where the stratum corneum is physically removed, or exposure to acetone, is followed by rapid secretion of lamellar body contents; this can be inhibited by occluding the site of barrier breakage with a water-impervious film, preventing the transepidermal flux of water (Menon et al., 1992b). If the site of exposure is not occluded, the subsequent flux of water causes a rapid drop in local calcium concentration in the stratum granulosum, stimulating the secretion of pre-formed lamellar bodies into the intercellular spaces of the stratum corneum with 15-30 minutes (Menon et al., 1992a; Menon et al., 1992b). A simultaneous rise in pH activates the epidermal serine proteases in the epidermis (Hachem et al., 2003; Fluhr et al., 2004; Hachem et al., 2006b).

1.4 Serine Proteases

The integrity of the stratum corneum and the orderly desquamation of the surface layers requires a carefully regulated balance between proteases and their inhibitors within the epidermis. Proteases are a family of enzymes that cleave peptide bonds; they can be divided into 6 types based on their catalytic domain. These are serine-, threonine-, cysteine-, metallo- glutamic- and aspartate-proteases (Rawlings et al., 2010).

Serine proteases make up approximately 30% of known proteolytic enzymes (Hedstrom, 2002), and have a particularly prominent role in maintaining the epidermal barrier due to their ability to cleave desmosomes and corneodesmosomes, thereby allowing desquamation to occur. It has been shown that acute barrier disruption leads to increased serine protease activity in the epidermis, which can be reversed by topical application of the serine protease inhibitors aprotinin, soybean trypsin inhibitor or trans-4-(Aminomethyl)cyclohexane carboxylic acid (t-AMCHA) (Hachem et al., 2006b). In hairless mice treated with acetone or SDS, or following damage to the barrier via tape stripping, barrier recovery was increased following application of 5% t-AMCHA, 1 mM leupeptin or 1mM TLCK, all of which are serine protease inhibitors (Denda et al., 1997). However, in the same study no increase in barrier recovery has been observed using inhibitors of other types of protease including EDTA (metalloprotease inhibitor), pepstatin (aspartate protease inhibitor) and E64 (cysteine protease inhibitor) (Denda et al., 1997). These data suggest that serine protease play an important role in epidermal barrier recovery.

There are currently 178 known serine proteases in humans, of which 17 are transmembrane enzymes and the remainder are secreted enzymes (Bugge et al., 2009; Rawlings et al., 2012). The serine protease group can be further sub-divided into 13 clans, defined by the catalytic mechanism by which they cleave peptide bonds. Similarities in amino acid sequence are used to separate the clans into 53 families, the largest of which are the kallikreins and the tryptase enzymes (Di Cera, 2009; Rawlings et al., 2012).

Serine proteases are involved in a large number of biological processes, including digestion, blood clotting and aiding the immune system. Several serine proteases are present in the epidermis, including matriptase, prostasin and kallikreins (Ovaere et al.,

2009). These proteases are involved in the maintenance of the skin barrier and the mounting of an immune response following an insult to the skin, and are discussed in further detail later. Protease activity must be controlled in order to prevent disruption to the epidermal barrier. Serine protease inhibitors, termed serpins, play a major role in the control of serine protease activity. These endogenous inhibitors function in the regulation of inflammation, coagulation, wound healing, fibrinolysis and tissue repair by regulating protease activity in these processes (Bauman et al., 2001).

Proteases can also be inhibited by alterations in the pH and trace element availability in the immediate environment. Serine proteases display optimal activity at specific pH ranges, for example matriptase is optimally activated between pH 5.2 and pH 7.2 which corresponds to the pH of the layers of the epidermis where this protease is located (see below) (Lee et al., 2007b; Ovaere et al., 2009). Several metal ions are also able to downregulate the activity of specific proteases including zinc (Schirmeister, 1998), copper (Debela et al., 2007) and titanyl sulphates (Duffy et al., 1998). Taken together, the specific pH and trace element availability of tissues can maintain the activation and inhibition of specific proteases as required for that tissue.

1.4.1 Matriptase

Matriptase is a transmembrane serine protease found in the stratum granulosum and stratum lucidum the uppermost live layers of the epidermis (Netzel-Arnett et al., 2006) (**Figure 1.3**). Studies have shown that matriptase knock-out (KO) mice display incomplete corneocyte differentiation and hyperkeratosis along with abnormal lipid secretions leading to fatal dehydration (List et al., 2002) which indicates that matriptase plays a major role in functional barrier formation. List *et al* used matriptase KO mice to demonstrate that matriptase is involved in barrier formation through control of intercellular lipid secretion and cornified envelope processing via its involvement in the processing of profilaggrin. (List et al., 2003). In addition, matriptase is also able to activate other proteases, including prostasin, KLK5 and KLK7 (Sales et al., 2010).

1.4.2 Prostasin

Prostasin, also known as protease serine S1 family member 8 (Prss8) and channel-activating protease 1 (CAP1), is a trypsin-like serine protease that is present in the epidermis. Prostasin KO mice exhibit an identical phenotype to matriptase KO mice (Leyvraz et al., 2005), which led to investigation of the interactions of matriptase and prostasin. Although previous studies have established the need for matriptase in the activation of prostasin (List et al., 2007; Alef et al., 2008; Szabo et al., 2012), it has recently been shown that prostasin is also required for the activation of matriptase (Buzza et al., 2013; Friis et al., 2013). Friis *et al* established that a reciprocal zymogen activation complex exists between matriptase and prostasin, demonstrating that while prostasin is required for matriptase activation, and matriptase is required for prostasin activation, only the inactive zymogen form of the protease is required in each case. The study showed there is no requirement for the active form of matriptase to activate prostasin, and inversely there is no requirement for the active form of prostasin to activate matriptase.

Activation of prostasin leads to the opening of membrane bound epithelial Na^{2+} channel (ENaC) on keratinocytes (Netzel-Arnett et al., 2006), and the subsequent influx of Na^{2+} leads to polarisation at the apical membrane which causes the voltage gated Ca^{2+} channels to open. The ensuing influx of Ca^{2+} causes several changes to the cells, ultimately leading to terminal differentiation (Netzel-Arnett et al., 2006).

1.4.3 Kallikreins

Kallikreins (KLKs) are a relatively newly classified family of serine proteases that includes the previously known enzymes stratum corneum tryptic enzyme (SCTE, now named KLK5) and stratum corneum chymotryptic enzyme (SCCE, now named KLK7), along with several newly discovered KLKs. KLK5, KLK6, KLK7, KLK8, KLK10, KLK11 and KLK14 have all been identified in the epidermis, though the roles of KLK6, KLK10 and KLK11 have yet to be determined (Kontos and Scorilas, 2012). KLK7 has been identified as an important factor in cornification and desquamation, present in cornified sites of oral mucosa (Sondell et al., 1996) and areas associated with cell shedding such as the luminal parts of the sebaceous gland and hair follicles (Ekholm

and Egelrud, 1998). Studies indicate that KLK5 and KLK7 degrade corneodesmosin, DSG1 and DSC1 (Descargues et al., 2006; Borgono et al., 2007). A second important function of KLKs is in the generation of inflammation through activation of protease activated receptor 2 (PAR2) (Stefansson et al., 2008).

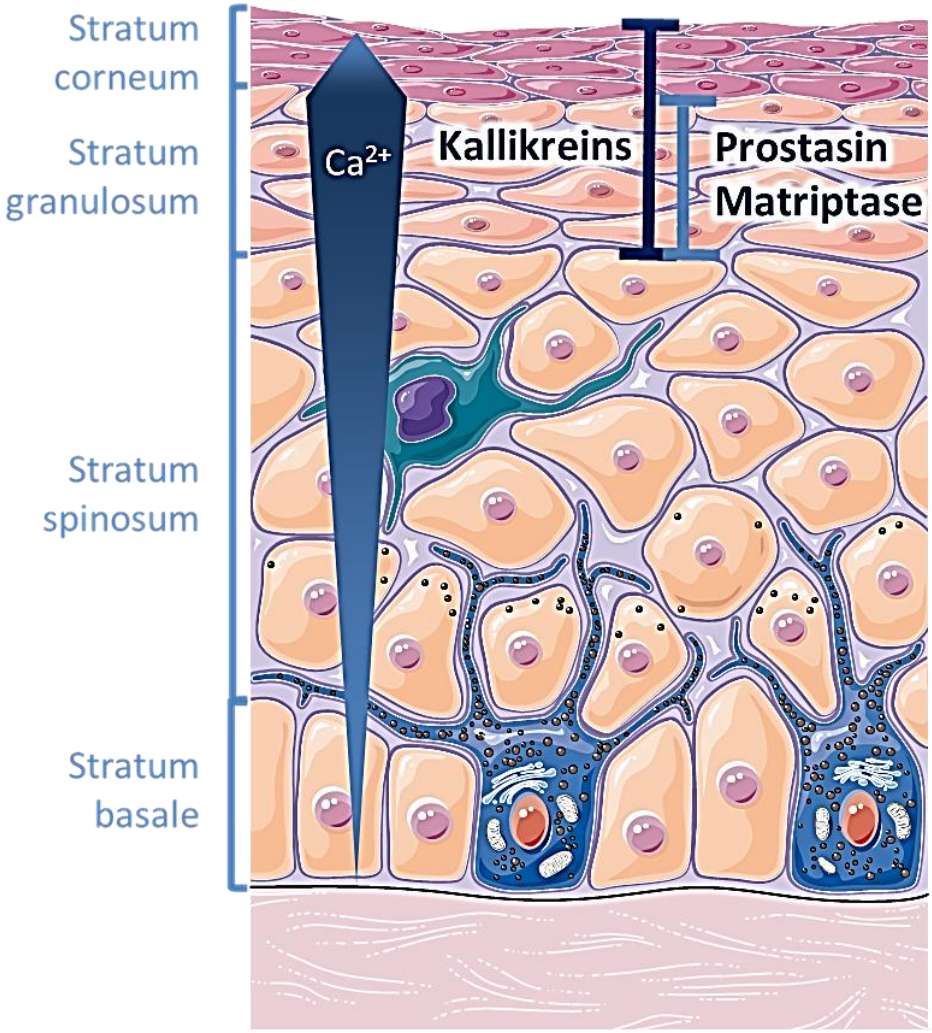


Figure 1.3: **Location of the proteases in the epidermis.** Matriptase and prostasin are found in the granular layer of the epidermis. KLK5, KLK7 and KLK14 are also located in these layers, in addition to the stratum corneum. Calcium concentration increases through the layers of the epidermis, peaking at the interface between the stratum granulosum and the stratum corneum. Secretion of lamellar bodies is a calcium dependent process; therefore the maintenance of a calcium gradient is important to control the level of lipid and protease secretion from differentiating keratinocytes. Image used with permission from www.servier.co.uk.

Like matriptase, KLK5, KLK7 and KLK14 are found in the granular layers of the epidermis (**Figure 1.3**), however unlike matriptase they are also found in the stratum corneum (Ovaere et al., 2009). These KLKs are stored as a pre-pro-enzyme in the lamellar bodies which maintains a separation between both the KLKs and their proposed activators (which include active KLK5 and KLK14), in addition to maintaining a separation from their main substrate corneodesmosin (Ishida-Yamamoto et al., 2005). KLK5, KLK7 and KLK14 are extruded into the intercellular space at the stratum granulosum-stratum corneum interface when the lamellar bodies secrete their contents prior to cornification (**Figure 1.4**a).

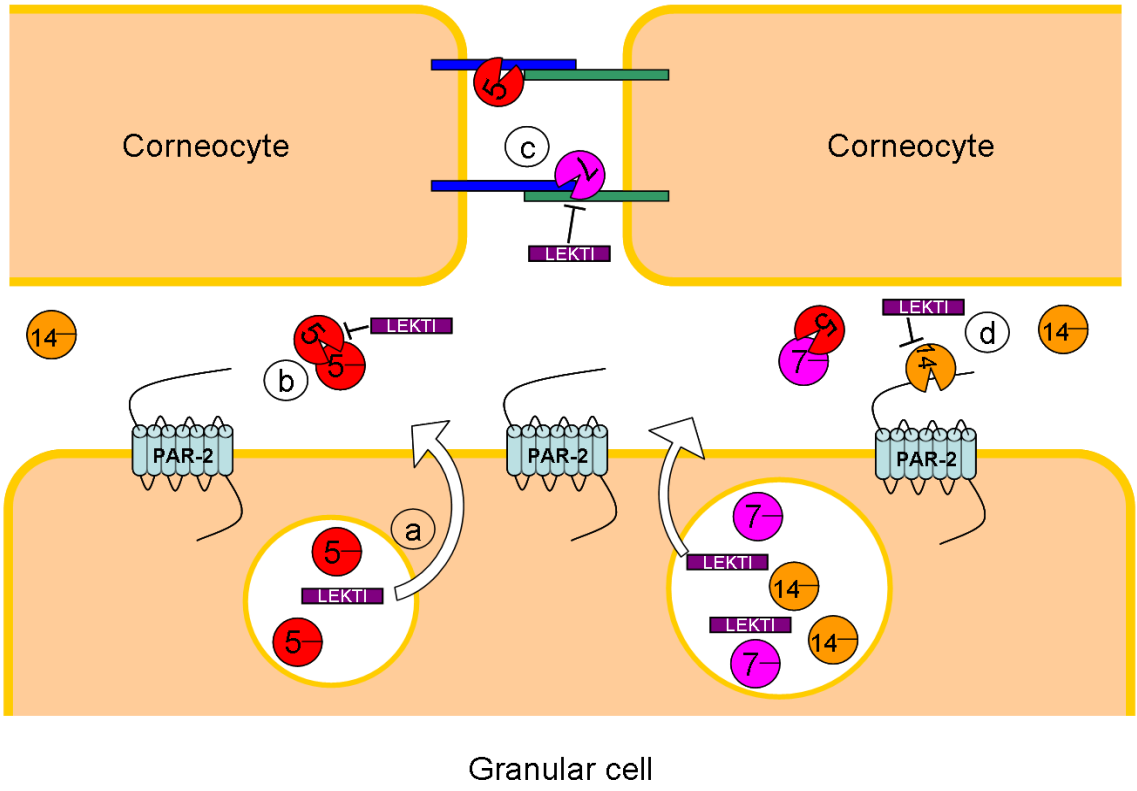


Figure 1.4: Proposed model of kallikrein signalling cascade. Kallikreins are stored as inactive pre-pro-enzymes in lamellar bodies in the granular cells of the stratum granulosum. (a) When the lamellar bodies are extruded into the intercellular space the pre-pro-kallikreins are released. (b) KLK5 can be auto-activated or activated by KLK14, and KLK7 and KLK14 are activated by KLK5. Other activating enzymes have yet to be identified. (c) KLK5 and KLK7 degrade the DSG1 and DCG1 components of the corneodesmosomes, leading to desquamation. (d) KLK5, KLK7 and KLK14 are also able to activate PAR2 by cleaving the N-terminal domain tethered ligand of PAR2. At each step following secretion from the lamellar bodies the activity of the KLK can be inhibited by LEKTI. Image adapted from (Ovaere et al., 2009) Full circles indicate inactive protease, open circles indicate active protease. PAR2 is shown as a 7 transmembrane domain molecule (blue).

While the *in vivo* activators of these KLKs have yet to be conclusively identified, it has been shown that active KLK5 is able to activate pre-pro-KLK5 and the pro-forms of both KLK7 and KLK14 (Brattsand et al., 2005). KLK14 has also been shown to be capable of activating KLK5 (Emami and Diamandis, 2008; Sotiropoulou et al., 2009). Once these pre-pro-KLKs are extruded into the intercellular space they can interact, possibly leading to activation of the KLKs and initiating an amplifying KLK activation loop in the upper layers of the epidermis (**Figure 1.4**b). Activated KLK5 and KLK7 are then able to degrade the DSG1 and DSC1 adhesion proteins of the corneodesmosomes, allowing desquamation to occur (**Figure 1.4**c).

Interestingly, it has recently been reported that KLK5 processes profilaggrin (Sakabe et al., 2013). As previously discussed, the resultant filaggrin is required for the formation of a successful epidermal barrier. This role in profilaggrin processing and subsequent barrier formation is converse to the established role of KLK5 in desquamation. KLK7 has been reported to regulate maturation of caspase-14 through the generation of an intermediate form (Yamamoto et al., 2012). Caspase-14 also processes profilaggrin, and Yamamoto *et al* (2012) concluded that alterations in KLK7 expression in the epidermis may lead to abnormal keratinocyte differentiation and barrier formation.

As previously explained in section 1.1.6, secretion of the lamellar bodies into the intercellular space is a calcium-dependent process. Normal skin displays an increased calcium concentration in the uppermost layers of the stratum granulosum, decreasing again throughout the stratum corneum (**Figure 1.3**) (Menon and Elias, 1991). Maintenance of this calcium gradient is dependent on the integrity of the skin barrier (Menon et al., 1994). Following barrier breakage, the local calcium concentration drops rapidly, decreasing the calcium gradient across the epidermis, causing the contents of the lamellar bodies to be secreted into the intercellular space, thus releasing the KLKs contained within the lamellar bodies (Denda et al., 1997).

KLK5 and KLK14 have optimal activity at a pH of around 9, however KLK5 has at least 50% activity between pH 7 and 10 (Brattsand et al., 2005). KLK14 has at least 50% activity between pH 6.5 and 10, which correlates with findings from studies into barrier breakage showing an increase in pH from around pH 5.5 to around pH 7.0 following an insult to the skin barrier (Elias, 2004; Brattsand et al., 2005). KLK7 enzymatic activity is also retained in a reduced water content such as that observed in

the water restricted stratum corneum intercellular spaces (Watkinson et al., 2001). This also indicates that movement of water out of the stratum corneum following barrier breakage does not affect the activity of KLK7 to a great degree.

KLK5, KLK7 and KLK14 can all be inhibited by the serine protease inhibitor Lymphoepithelial Kazal Type-related Inhibitor (LEKTI). LEKTI is stored in the lamellar bodies and is secreted earlier in the stratum granulosum than KLK5, KLK7 or KLK14, thereby preventing premature degradation of the corneodesmosomal proteins at the stratum granulosum-stratum corneum interface (Ishida-Yamamoto et al., 2005). Inhibition by LEKTI is pH-dependent, with more efficient inhibition occurring at a neutral pH compared with a pH of 4.5-5.5 (Deraison et al., 2007) such as that of the stratum corneum. This is also in line with the increased KLK activity at these pH levels. There are many endogenous inhibitors of KLKs, including zinc, α2-antiplasmin and proteinase C inhibitor (Goettig et al., 2010; Swedberg et al., 2010). KLK7 can also be inhibited by secretory leukocyte peptidase inhibitor (SLPI) (Luo and Jiang, 2006). KLK7 and KLK14 are inhibited by α1-antitrypsin and KLK14 can be inhibited by antithrombin III (Goettig et al., 2010).

In addition, KLKs can be inhibited by exogenous and synthetic inhibitors, although there have been less exogenous inhibitors have been identified compared with endogenous types. KLK5 and KLK14 have been shown to be inhibited by leupeptin, soybean trypsin inhibitor (SBTI) and bovine pancreatic trypsin inhibitor (BPTI) (Brattsand et al., 2005). KLK14 can also be inhibited with chymostatin (Brattsand et al., 2005) and KLK7 can be inhibited by tosyl-phenylalanyl chloromethyl ketone (TPCK) (Debela et al., 2007). There are currently no known inhibitors which are specific to individual KLKs. Most KLK inhibitors act on several proteases, for example the most specific inhibitor known for KLK5 is domain 6 of the LEKTI protein (Egelrud et al., 2005; Borgono et al., 2007), however this protein has also been reported to inhibit trypsin (Kreutzmann et al., 2004) and subtilisin A (Jayakumar et al., 2004). There are currently no studies which investigate the role of this inhibitor on a wider range of proteases.

1.5 Protease activated receptors

In addition to their role in desquamation, KLK5 and KLK14 have been identified as activators of a G-protein coupled receptor known as protease activated receptor 2 (PAR2) (**Figure 1.4**d). Protease activated receptors (PARs), discovered in the 1990's (Vu et al., 1991; Nystedt et al., 1994; Ishihara et al., 1997; Xu et al., 1998), are part of the super-family of G-protein coupled receptors (GPCR). Unlike many other GPCR's they are not activated by a soluble ligand but by proteases which cleave the extracellular amino terminus of the PAR, leading to irreversible removal of a terminal pro-peptide (**Figure 1.5**). This unmasks a 6 amino acid neoepitope which acts as a tethered ligand, activating the receptor and initiating the downstream signal transduction pathway (Maryanoff et al., 2001) (**Figure 1.5**). It is possible to artificially activate these receptors using synthetic peptides that mimic the tethered ligand (**Table 1.1**) (Kawabata et al., 1999; Barry et al., 2006).

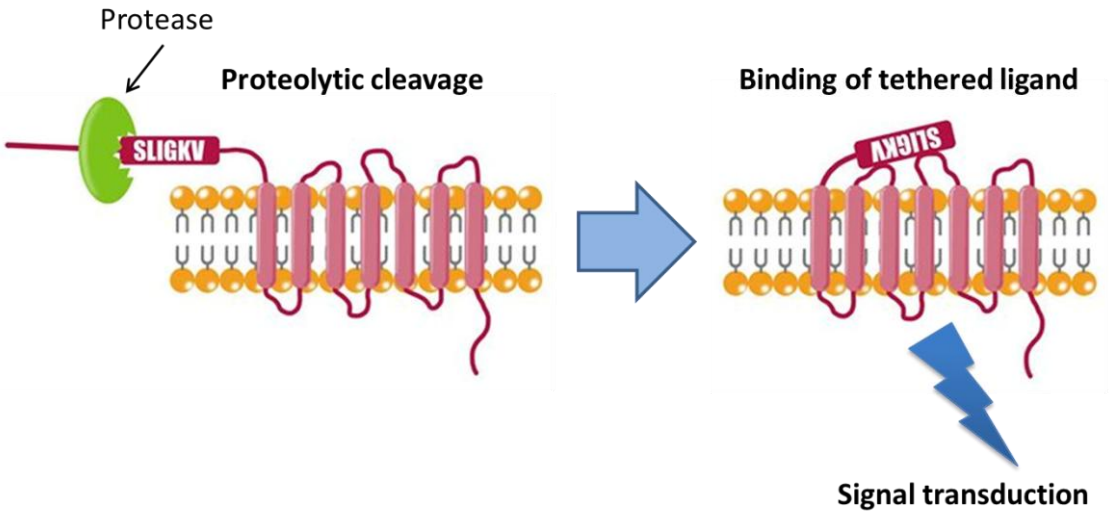


Figure 1.5: Activation of PARs by proteases. Proteases cleave the tethered ligand (SLIGKV), allowing the activating peptide to access the binding site of the PAR receptor on the cell surface. Image by R. Gregory.

To date there are 4 known protease activated receptors (PAR 1-4) (Hollenberg and Compton, 2002), each having a specific location and role in cell maintenance and activity, and each having a specific set of activating and inactivating proteases (**Table 1.1**). Less is known about the activity of PAR3 and PAR4 compared with PAR1 and PAR2. PAR1 is an extensively studied member of the PAR family, with detailed characterisation of the receptor and mechanisms of activation having been established (Dery et al., 1998). PAR1 is located on several cell types, including endothelial cells (Riewald et al., 2002), fibroblasts (Chambers et al., 1998), neurons (Junge et al., 2004) and keratinocytes (Gao et al., 2010). PAR1 is activated by cleavage of its tethered ligand by thrombin, leading to various effects dependent on the cell type, for example platelet aggregation (Andersen et al., 1999), IL-6 secretion from T-cells (Li and He, 2006) and stimulation of keratinocyte migration in wound healing (Gao et al., 2010). PAR3 and PAR4 are also activated by thrombin and have been found in several cell types including platelets (Kahn et al., 1999), macrophages (Colognato et al., 2003), and astrocytes (Wang et al., 2002). Basophils are one of the few cell types that do not express any PARs (Falcone et al., 2005). PARs are highly expressed in the gastrointestinal tract, the CNS and several other cell types throughout the body including endothelial cells, inflammatory cells and fibroblasts (Vergnolle, 2000).

1.5.1 PAR2

PAR2 can be activated by several types of protease, the availability of which is dependent on the type of tissue (**Table 1.2**). It has been shown to be expressed in several cell types, including endothelial cells (Hwa et al., 1996), smooth muscle cells (al-Ani et al., 1995) and osteoblasts (Abraham et al., 2000), as well as immune cells such as T cells (Mari et al., 1996), neutrophils (Howells et al., 1997), mast cells (He et al., 2005) and eosinophils (Dinh et al., 2006). PAR2 is absent on human platelets (Hwa et al., 1996). Ferrell *et al* used PAR2 knockout mouse models to show the importance of PAR2 in chronic and acute inflammation (Ferrell et al., 2003). Freund's complete adjuvant was injected into the joints of normal and PAR2 knockout mice to induce chronic arthritis, and 2% λ carrageenan and 1% kaolin was injected to induce acute inflammation. The PAR2 agonist SLIGRL-NH_2 was also injected into the joints of mice to compare the responses. The PAR2 agonist induced swelling and hyperemia of the

joints of $PAR2^{+/+}$ but not $PAR2^{-/-}$ mice. Both the chronic and acute inflammation was significantly reduced in $PAR2^{-/-}$ compared with both $PAR2^{+/+}$ and $PAR2^{+/-}$ mice. Ferrell *et al* concluded that PAR2 is involved in the orchestration of chronic inflammatory responses. Kelso *et al* have subsequently shown that PAR2 is significantly upregulated in the synovium of rheumatoid arthritis patients compared with control synovial tissue from patients with osteoarthritis or seronegative inflammatory arthritis (Kelso et al., 2007). In this study Kelso *et al* also demonstrated that inhibition of PAR2 using a novel antagonist in the rheumatoid arthritis synovial tissue cultures significantly decreased spontaneous expression of TNFα and IL-1β, which are known to be involved in the inflammatory pathology of rheumatoid arthritis (Fontana et al., 1982; Buchan et al., 1988; Hopkins et al., 1988; Hopkins and Meager, 1988). These data suggest that PAR2 plays an important role in inflammatory responses and innate immune regulation.

PAR2 is expressed abundantly by almost all cells in the skin including the inner root sheath of hair follicles, myoepithelial cells of sweat glands, endothelial cells, dermal dendritic cells, and especially keratinocytes (Santulli et al., 1995; Hou et al., 1998; Steinhoff et al., 1999). It can be cleaved by trypsin or KLK5, KLK7 or KLK14 to expose the tethered ligand SLIGKV at the N terminal. Once cleaved, this ligand is able to bend back and bind to the receptor to initiate a signal cascade that leads to transcription of pro-inflammatory cytokines including IL-8, TNFα and IL-1β (Al-Ani et al., 2002). The exact nature of this cascade has yet to be fully elucidated as most previous studies have focused on PAR1 (Macfarlane et al., 2001; Saban et al., 2007). It is thought that activated PAR2 interacts with G_q/G_{11}, which subsequently leads to the opening of calcium channels via phospholipase C (PLC) and inositol triphosphate (IP_3) (McCoy et al., 2010) (**Figure** 1.6). It was previously reported that PAR2 signalling is not affected by pertussis toxin, which inhibits G_0/G_i signalling, indicating G_0/G_i are not utilised by PAR2 (DeFea et al., 2000), which has since been confirmed by McCoy who showed that PAR2 activates Rho through a G_{12}/G_{13} linked signalling pathway (McCoy et al., 2010)

Table 1.1: Activating and inactivating proteases, synthetic agonists and phenotypes of knockout models for PAR1-4. Highly efficient activators are shown in bold. *Activates both PAR1 and PAR2.

	PAR1	PAR2	PAR3	PAR4
Activating proteases	**Thrombin** (Vu et al., 1991) FXa (Riewald et al., 2001) Granzyme A (Suidan et al., 1994) Trypsin (Vouret-Craviari et al., 1995)	**Trypsin** (Nystedt et al., 1994) Tryptase (Molino et al., 1997) FVIIa (Camerer et al., 1999) FXa (Camerer et al., 2000) Matriptase (Takeuchi et al., 2000b)	**Thrombin** (Ishihara et al., 1997)	**Thrombin** (Xu et al., 1998) Trypsin (Kahn et al., 1998) Cathepsin G (Xu et al., 1998)
Inactivating proteases	Cathepsin G (Parry et al., 1996) Plasmin (Kuliopulos et al., 1999) Elastase (Altrogge and Monard, 2000) Der p 1 (Asokananthan et al., 2002)	Elastase (Ramachandran et al., 2011) Cathepsin G (Ramachandran et al., 2011) Proteinase-3 (Ramachandran et al., 2011)	Cathepsin G (Cumashi et al., 2001) Elastase (Cumashi et al., 2001)	None known
Tethered ligand sequence	SFLLRN (Vu et al., 1991)	SLIGKV (Nystedt et al., 1994)	TFRGAP (Ishihara et al., 1997)	GYPGQV (Kahn et al., 1998)
Synthetic agonist peptide	SFLLRN * (Blackhart et al., 1996) TFLLRN (Hollenberg et al., 1997)	SLIGKV (Nystedt et al., 1994) SFLLRN* (Blackhart et al., 1996)	None	GYPGKF (Kahn et al., 1998) AYPGKF (Faruqi et al., 2000)
Phenotype of knockout	50% embryonic lethality (Connolly et al., 1996)	Impaired allergic inflammation of airway, joints, kidney (Lindner et al., 2000; Ramelli et al., 2010)	Protection against thrombus formation (Weiss et al., 2002)	Protection against thrombus formation (Sambrano et al., 2001)

Table 1.2: PAR2 activators in various tissue types.

Families	Protease	Target tissues
Trypsins	Trypsin (Nystedt et al., 1994)	GI tract (Amadesi and Bunnett, 2004)
Kallikreins	KLK5, KLK14 (Stefansson et al., 2008)	Skin (Briot et al., 2009)
Coagulation proteases	FVIIa (Camerer et al., 2000) Xa (Camerer et al., 2000)	Vascular smooth muscle (Marutsuka et al., 2002)
Leukocyte proteases	Mast cell tryptase (Molino et al., 1997)	Broad tissue distribution
Transmembrane proteases	Matriptase (Takeuchi et al., 2000b) Epitheliasin (Wilson et al., 2005)	Skin (Netzel-Arnett et al., 2006), Prostate (Lin et al., 1999)

Studies by DeFea *et al* have shown that PAR2 signalling through β-arrestins is responsible for the observed activation of ERK1/2 following activation of PAR2 receptors by proteases (DeFea et al., 2000). Studies by Kanke *et al* in human keratinocytes demonstrated that PAR2 agonists also stimulate c-Jun N-terminal kinases (JNK) and p38 mitogen activated protein kinase (MAPK) activation, however the exact pathway utilised is still unclear (Kanke et al., 2001). Hirota *et al* have also shown that PAR2 activation leads to phosphorylation of p38, ERK1/2 and JNK in endometrial stromal cells, leading to production of the inflammatory cytokines IL-6 and IL-8 (Hirota et al., 2005). They also demonstrated that activation of PAR2 led to increased cell proliferation directly proportional to the level of PAR2 activation. Taken together these studies indicate that PAR2 has a role in several cell systems.

As with most GPCRs, activation of PAR2 leads to desensitisation and internalisation of the receptor. Unlike many GPCRs, PAR2 is not recycled due to the proteolysis in the activation process (Ritter and Hall, 2009). Once activated, PAR2 is phosphorylated by protein kinase C (PKC) (Bohm et al., 1996) and the receptor is internalised to the early endosome (Dery et al., 1999). Once the receptor has been trafficked to the late endosome and de-ubiquitinated by the proteases (associated molecule with the Src

homology 3 domain of STAM (signal-transducing adapter molecule) AMSH and ubiquitin-specific protease Y (UBPY) it is processed through the lysosome and degraded. Hasdemir *et al* transfected human epithelial keratinocytes with inactive mutant forms of AMSH and UBPY to show that activated PAR2 accumulates in the early endosome in the absence of deubiquitination, however overexpression of the wild type proteases does not affect PAR2 trafficking (Hasdemir et al., 2009).

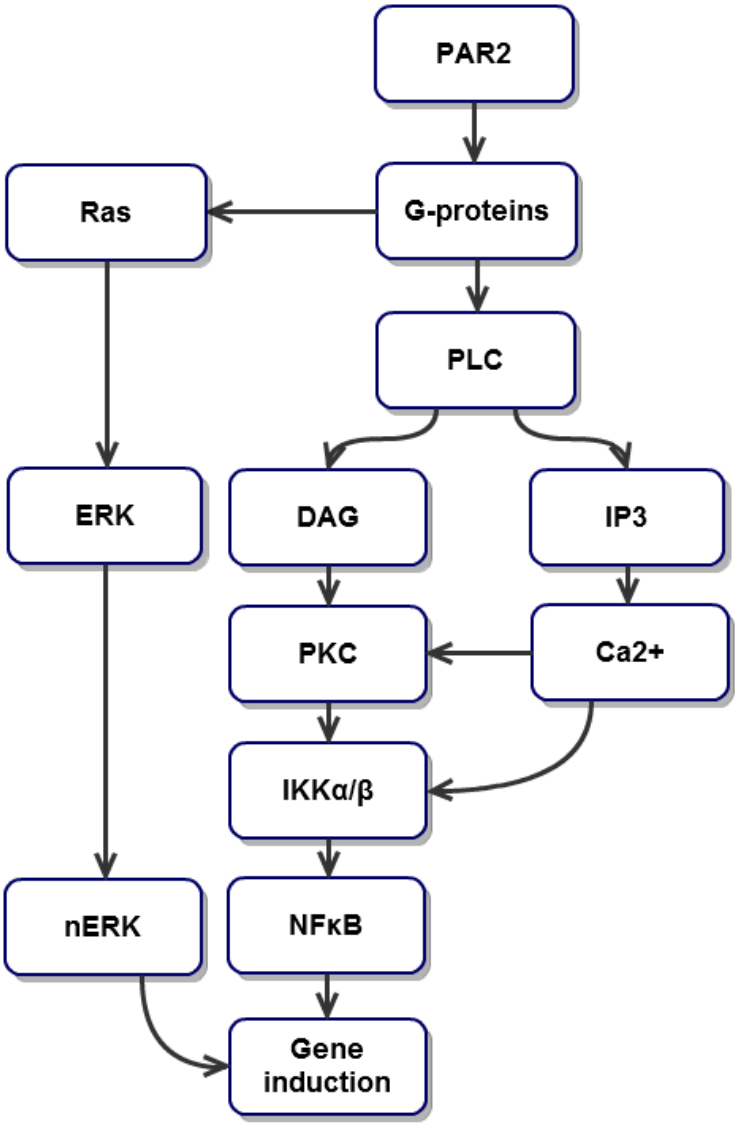


Figure 1.6: **Proposed signalling cascade following PAR2 activation**. Several studies have shown activation of p38, ERK1/2 and NF-κB following stimulation of PAR2 (DeFea et al., 2000; Kanke et al., 2001; Hirota et al., 2005), however none have yet identified the exact pathway linking the receptor with transcription factor activation. For abbreviations see page xix.

1.6 PAR2 activation and its role in barrier homeostasis and skin immunity

Barrier homeostasis is vital for maintaining an operational permeability barrier. If the barrier is damaged, either by a physical mechanism such as tape stripping or abrasions, or by chemicals such as acetone or detergents, several pathways are activated to re-establish the epidermal barrier, and PAR2 has a central role in this (**Figure 1.7**). Removal of the outer stratum corneum allows an efflux of water out of apical surface of the epidermis, decreasing the calcium gradient across the layers of the epidermis which in turn leads to increased secretion of lamellar bodies into the intracellular space (Demerjian et al., 2008). The lipids that are secreted contribute to the reconstitution of the lipid bilayers which make up the 'mortar' of the epidermis (as per the "bricks and mortar" analogy of the epidermis).

In addition to a decrease in the calcium concentration of the stratum granulosum, an increase in the local pH causes increased activity of KLKs in the stratum granulosum-stratum corneum junction (Demerjian et al., 2008). Activated KLK5 is able to cleave the tethered ligand of PAR2. Studies have shown that activation of PAR2 results in a decrease in lamellar body secretion, in turn decreasing the formation of caveolae and lipid rafts (LR) (Hachem et al., 2006b). LR are domains within the plasma membrane that are enriched with cholesterol and sphingolipids. Caveolae are a subclass of LR that contain caveolin as the main structural protein (Stan, 2005). These LR domains play an important role in signal transduction into the cell (Mathay et al., 2011) and it has been hypothesised that caveolae may also play a role in the regulation of terminal differentiation of keratinocytes (Roelandt et al., 2009). Roelandt *et al* compared caveolin-1 (cav-1) knock-out mice to wild-type littermates to show that cav-1 increases terminal differentiation following barrier abrogation (Roelandt et al., 2009), however in the absence of lipid secretions into the extracellular matrix a functional barrier cannot be formed (Demerjian et al., 2008). Application of serine protease inhibitors, including soybean trypsin inhibitor (SBTI), aprotonin and leupeptin, significantly increases barrier recovery rate following barrier disruption, decreasing the time it takes to reform a functional barrier (Denda et al., 1997; Hachem et al., 2006b). Several authors have noted that the balance of lamellar body secretion caused by decreased calcium and increased terminal differentiation caused by PAR2 activation must be carefully controlled in order to achieve successful barrier recovery

(Hachem et al., 2006b; Jeong et al., 2008; Roelandt et al., 2011). Hachem *et al* have proposed that the seemingly opposing effects of lamellar body secretion, which releases activators of PAR2, and PAR2 activation, which inhibits lamellar body secretion, following barrier disruption could be explained by the need for keratinocytes in the stratum granulosum to undergo cornification in order to form a well-structured stratum corneum (Hachem et al., 2006b).

In addition to the roles of PAR2 in barrier repair described above, PAR2 receptors are known to have an important role in activation of inflammatory responses in the innate immune response. Thus, it has been shown that PAR2 activation leads to secretion of IL-6 and GM-CSF from keratinocytes (Wakita et al., 1997), along with IL-8 (Hou et al., 1998). These cytokines and chemokines are known to be involved in the innate immune response to irritants, promoting granulocyte and monocyte recruitment (Hunziker et al., 1992; Wilmer et al., 1994; Holliday et al., 1997). PAR2 activation also induces upregulation of intercellular adhesion molecule-1 (ICAM-1) in human keratinocytes via activation of nuclear factor- κB (NF-κB) (Buddenkotte et al., 2005), which also contributes to the inflammatory response (**Figure 1.8**). This innate immune/inflammatory response will act as a protective mechanism against infectious agents that may penetrate into the skin following barrier disruption. It has become increasingly evident that abnormal expression and activation of proteases and protease receptors plays an important role in the development of several skin disorders, including several types of ichthyosis.

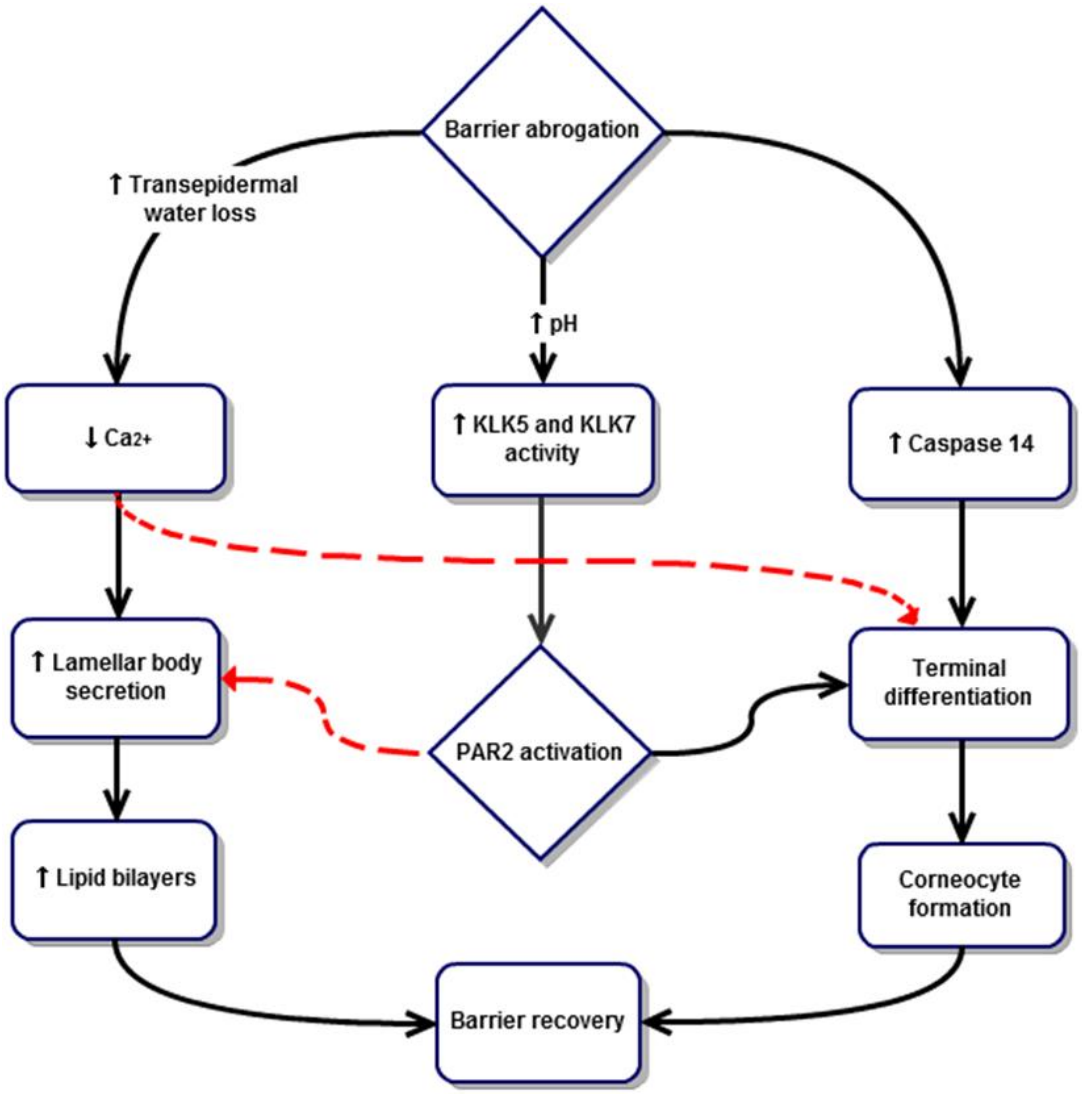


Figure 1.7: Proposed PAR2 involvement in epidermal barrier recovery (previous page). Following barrier abrogation by either chemical or physical damage there is an increase in transepidermal water loss through the epidermis. This causes a decrease in calcium concentration in the upper stratum granulosum which leads to increased secretion of lamellar bodies which contributes to increased formation of lipid bilayers. A simultaneous rise in pH causes increased KLK activity in the stratum granulosum-stratum corneum junction. Subsequent PAR2 activation inhibits lamellar body secretion while increasing terminal differentiation of keratinocytes. These pathways act synergistically to ensure proper barrier recovery. Decreased Ca^{2+} concentration and increased caspase 14 activity in the epidermis caused by barrier abrogation also contributes to the recovery of the barrier. Red lines indicate inhibition. This proposed pathway is supported by several authors (Hachem et al., 2006b; Jeong et al., 2008; Roelandt et al., 2011)

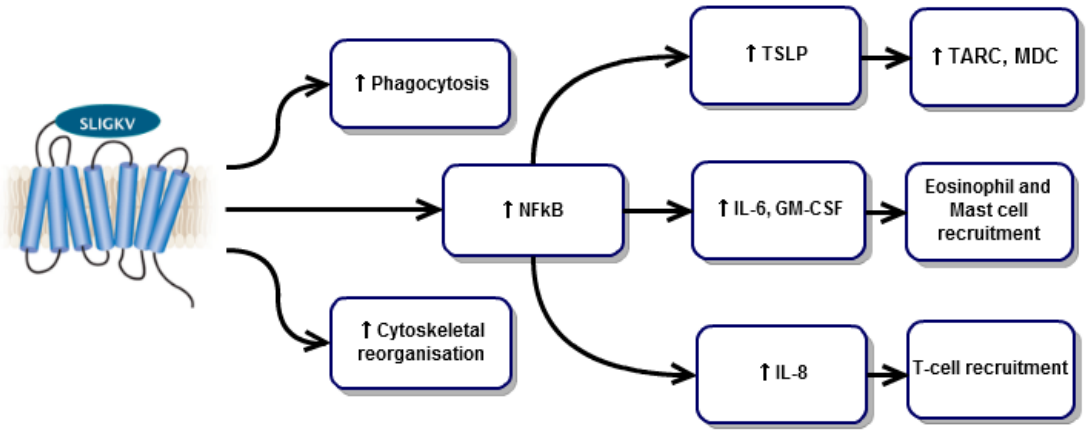


Figure 1.8: PAR2 in inflammation. Activation of PAR2 leads to translocation of NFκB and subsequent transcription of pro-inflammatory cytokines including IL-6, GM-CSF and IL-8 (Wakita et al., 1997). These cytokines recruit T-cells, eosinophils and mast cells to the epidermis (Hunziker et al., 1992). Increased transcription of TSLP follows PAR2 activation, contributing to a Th2-mediated immune response (He et al., 2008; Briot et al., 2009).

1.7 Ichthyosis

The ichthyoses are a family of skin disorders that are characterised by dry, thickened and scaly or flaky skin. The severity of the disorder can range from mild, such as ichthyosis vulgaris, to severe such as Netherton's Syndrome. These are mostly genetic disorders, with only acquired ichthyosis having a non-genetic origin.

Mouse models of ichthyosis have been created by inserting full length prostasin genes under the control of a keratin 14 (K14) promotor (Frateschi et al., 2011). The K14 promoter targets the prostasin to the basal layers of the epidermis. In this study overexpression of prostasin caused scaly skin and up to 2 fold increase in transepidermal water loss (TEWL) in the transgenic mice. This was supported by a later study by Friis *et al* which showed a similar phenotype using prostasin genes under the control of a keratin 5 promoter (Friis et al., 2013). Frateschi *et al* also demonstrated that complete absence of PAR2 ($PAR2^{-/-}$) reverses the effects of prostasin overexpression, in contrast to heterozygous PAR2 mutants ($PAR2^{+/-}$) (Frateschi et al., 2011). This indicates that PAR2 is a downstream effector of prostasin, which is in turn part of a cascade involving matriptase (Netzel-Arnett et al., 2006).

Ichthyosis vulgaris is caused by loss-of-function mutations in the *FLG* gene which encodes for filaggrin (Smith et al., 2006). Filaggrin is a key epidermal protein required for the correct composition and function of the stratum corneum (O'Regan et al., 2008). Specific mutations of the *FLG* gene in ichthyosis vulgaris are also associated with the development of atopic dermatitis (AD) (Marenholz et al., 2006; Ruether et al., 2006; Smith et al., 2006; Stemmler et al., 2006).

1.7.1 Atopic dermatitis

AD affects approximately 20% of the population in the developed world, mainly affecting infants and children (Palmer et al., 2006). It is an inflammatory disease of the skin often linked to asthma and allergic rhinitis (Leung et al., 2004; Weidinger et al., 2006). It is characterised by pruritus and a red, inflamed, scaly rash which runs a course of exacerbation and remission.

Patients with AD exhibit enhanced percutaneous penetration of hydrophilic compounds in addition to increased transepidermal water loss (Seidenari and Giusti, 1995; Leung et al., 2004). While the exact cause has not yet been determined, it has been established that between 30% and 60% of subjects suffering from AD have a loss-of-function mutation in the *FLG* gene (Palmer et al., 2006; Weidinger et al., 2006; Kezic et al., 2012). The characteristically reduced production of anti-microbial peptides (β defensins and cathelicidins) together with the damaged barrier resulting from AD contribute to increased microbial colonisation and infections with *Staphylococcus aureus* in affected patients. The defective barrier allows irritants and allergens to penetrate into the skin (Cork et al., 2009), leading to increased susceptibility to irritants (Goffin and Piérard, 1996; Tabata et al., 1998; de Jongh et al., 2006). Amino acids are released during the degradation of filaggrin in the stratum corneum, which contributes to the pH balance of the skin, therefore alterations in the pH balance caused by decreased levels of filaggrin may alter protease activity in the epidermis and encourage bacterial colonisation (O'Regan et al., 2008).

Skin colonisation by *S. aureus* is present in 80 to 100% of AD patients compared with 5-10% of controls (Leyden et al., 1974; Bunikowski et al., 1999; Breuer et al., 2000). *S. aureus* infection can activate the innate immune response, leading to release of pro-

inflammatory mediators including IL-8 and TNFα via activation of various receptors including toll-like receptor 2 (TLR2) (Takeuchi et al., 2000a), on macrophages and dendritic cells. The adaptive immune response is also activated, leading to recruitment and activation of T-cells (Bunikowski et al., 2000). The activated T-cells produce IL-31 which leads to pruritus and scratching which causes further damage to the epidermal barrier (Sonkoly et al., 2006).

1.7.2 Netherton's syndrome

Netherton's syndrome is a rare inherited type of ichthyosis affecting 1 in 100,000 individuals (Briot et al., 2009). It presents as severe congenital scaly erythroderma and atopic manifestations. These manifestations may be 'atopic-dermatitis'-like lesions which would normally be associated with protein allergens. Netherton's syndrome differs from other types of ichthyosis which cause impairment of the skin barrier but, in contrast to Netherton's syndrome, do not develop constant atopic manifestations (Briot et al., 2010).

Netherton's syndrome is caused by a mutation in the serine protease inhibitor kazal-type 5 (*SPINK5*) gene, which codes for the KLK inhibitor LEKTI (Chavanas et al., 2000). In the epidermal granular layers LEKTI is secreted into the intercellular space and specifically inhibits epidermal KLK5, KLK7 and KLK14 (Egelrud et al., 2005; Schechter et al., 2005; Deraison et al., 2007). Decreased production of LEKTI leads to unrestricted KLK activity in the skin, which in turn causes premature desmosome cleavage in the upper layers of the epidermis and therefore increased stratum corneum shedding (Descargues et al., 2005; Bonnart et al., 2010). More recently, SPINK5 knockout mice have been utilised to show that unrestricted KLK activity induces the expression of proinflammatory cytokines TNFα and IL-8, and the pro-allergic molecule thymic stromal lymphopoietin (TSLP) from keratinocytes by activating PAR2 (Briot et al., 2009). The TSLP produced is able to induce a systemic Th2 response in the absence of exogenous allergens, and initiate eczematous lesions (He et al., 2008). NF-κB mediated transcription of IL-8, TNFα and ICAM-1 also follows PAR2 activation, all of which contribute to an inflammatory response (Briot et al., 2009).

1.8 Current models of measuring sensitizing and irritant potential

Several *in vivo* and *in vitro* methods exist for the determination of sensitizers and irritants using both animal and non-animal methods. During previous decades, animal based models such as the challenge-induced mouse ear swelling test (MEST) (Gad et al., 1986), the local lymph node assay (LLNA) (Kimber et al., 1986) and the integrated model for the differentiation of chemical-induced allergic and irritant skin reactions (Homey et al., 1998) were used as a standard method in cosmetic and medical testing of irritants and sensitisers, however changes to EU regulations which prevent the use of animal testing in all cosmetics have led to an upsurge of research into non-animal models. There are no human based methods currently approved for the testing of novel chemicals for irritant or sensitising potential, and most non-animal models for chemical testing only assess the sensitising, rather than irritant, potential.

1.8.1 *In Silico*, *in chemico* and *in vitro* methods

In silico approaches such as quantitative structure-activity relationship (QSAR) models are used to correlate biological activity such as sensitising or irritant potential, for which there is no detailed understanding of the mechanisms involved, with the structure of the chemicals (Roberts et al., 2008). These structures are then used as a comparison for chemicals whose biological activity is unknown. This approach requires a vast library of known chemical sensitisers, and is limited to chemicals for which the physiochemical properties affecting the sensitizing potential are similar to other chemicals for which the sensitising potential is known (Roberts et al., 2007). The same method can be applied to assess potential irritants (Kodithala et al., 2002), however a lack of chemical descriptions in the comparison library have meant that this method has not been widely accepted.

In chemico approaches analyse the propensity of a chemical to undergo haptenisation with various small peptides and therefore attempt to predict the likelihood of the chemical causing ACD (Gerberick et al., 2007; Vandebriel and Loveren, 2010). This method has several drawbacks, including the removal from the biological environment of the skin, and the restricted peptide range available. *In chemico* methods have failed

to provide significant insight into potential chemical irritants due to the lack of information regarding the reactive chemical properties that make them irritants (Cronin et al., 2009). Better understanding of what makes chemicals irritants, and the development of large databases containing the necessary chemical information, could make *in chemico* methods useful in the longer term, however in the immediate future this method is not likely to be widely used (Cronin et al., 2009).

In vitro approaches have shown the most promise as predictors of sensitising potential. Measurement of the expression of cell surface markers and production of cytokines are used as indicators of dendritic cell maturation caused by sensitising chemicals (Ryan et al., 2004; Gildea et al., 2006; Vandebriel and Loveren, 2010). Expression of CD86 on the surface of dendritic cells is increased following 6 hours exposure to allergens compared with non-sensitising chemicals (Aeby et al., 2004; Hulette et al., 2005) IL-1β secretion is also increased in dendritic cells following allergen exposure. The human monocytic leukaemia cell line (THP-1) have also been utilised to show that the expression of CD86 and CD54 are altered on cells following 24 hours exposure to allergens (Sakaguchi et al., 2006; Hennen et al., 2011). The results from THP-1 cells were not reflected in results using the human histiocytic lymphoma cell line (U-937) in the study by Sakahuchi *et al*. Natsch and Emter used a luciferase transfected human cell line (AREc32) to assess the activation of the Keap1/Nrf2 regulatory pathway following allergen exposure (Natsch and Emter, 2008). They showed that use of a luciferase assay following 24 hours chemical exposure indicated 14 out of 15 strong sensitisers, 31 of 35 moderate sensitisers and 12 or 20 weak sensitisers compared with 4 positive results from 30 non-sensitising chemicals.

These cell based models have been shown to be an inaccurate indicator of sensitising potency in some cases due to the model lacking a barrier. For example differential skin penetration has been shown to drastically alter the sensitising potency of chemicals *in vivo* compared with *in vitro* results (Aeby et al., 2004), indicating that results using the dendritic cell and THP-1 cell models sometimes may not be representative of the *in vivo* effects. More recently, *in vitro* models of irritancy have utilised keratinocytes to create 3D skin models which mimic the physiological environment of the skin.

1.9 3D skin modelling

The Organisation for Economic Cooperation and Development (OECD) work with governments and agencies around the globe, including the United Kingdom, to produce polices which protect the public and aid social and economic development. The only OECD approved non-animal test for irritancy utilises a reconstructed human skin model which mimic human epidermis (Buschmann, 2012). To create these models, keratinocytes are differentiated at an air-liquid interface for up to 21 days, during which time the differentiating cells stratify into the 4 distinct layers of the epidermis (Slivka et al., 1993; Ponec et al., 2000; Netzlaff et al., 2005). It is now possible to purchase pre-prepared 3D skin models from several specialist companies, with new skin models coming to the market each year. Potential irritant chemicals are applied to the skin model for 25 to 60 minutes before being washed off with an aqueous buffer. The reconstructed models are then incubated for 42 hours before cell viability is assessed via an MTT assay. The IL-1β and IL-8 expression profile of 3D models following chemical exposure can be used to determine irritant and sensitising potential (Coquette et al., 2003). According to a study by Coquette *et al*, both IL-1β and IL-8 secretion are increased following irritant exposure, and conversely decreased following exposure to sensitisers.

According to the OECD guidelines the keratinocytes used to create the three dimensional models must be non-transformed, primary keratinocytes. These cells must be extracted from human skin which is limited in its availability, primarily being obtained from surplus tissue following plastic surgery. Although no histological difference is observed between 3D models grown from neonatal and adult keratinocytes (Michel et al., 1997), neonatal keratinocytes grow faster and have a longer lifespan than those extracted from adults (Gilchrest, 1983); therefore foreskin removed during circumcision of infants is preferably used for the construction of three dimensional models.

1.9.1 Immortalised keratinocytes

Primary keratinocytes are, in some cases, a useful *in vitro* model for investigating the properties of irritant and sensitising chemicals. Like most somatic cells, keratinocytes

have a limited lifespan. Over time the proliferative capacity of the cells decreases and eventually the cells cease to divide, a process known as cellular senescence (Hayflick, 1965; Campisi and d'Adda di Fagagna, 2007). Cells undergoing terminal differentiation also cease to proliferate. *In vivo* this limited lifespan may decrease the vulnerability of ageing cells to mutation and disease (Ramirez et al., 2001; Chapman et al., 2010), however *in vitro,* this short lifespan imposes a limitation on laboratory investigations. HaCaT cells are an established, spontaneously immortalised keratinocyte cell line (Boukamp et al., 1988). These cells have undergone mutations in the p53 gene (Lehman et al., 1993), and it was previously thought that they were not able to undergo differentiation into 3D skin equivalents (Boelsma et al., 1999; Schoop et al., 1999). Advances in culture methods and media have meant that these cells can be differentiated in monolayers (Pleguezuelos and Kapas, 2006; Deyrieux and Wilson, 2007), however they have not been shown to create properly stratified epidermal models. Currently the only other spontaneously immortalised keratinocyte cell lines is NIKS (Allen-Hoffmann et al., 2000), however this cell lines was shown to differ in several ways from primary keratinocytes including the addition of a 47th chromosome, and were unable to differentiate into organotypic models. Primary keratinocytes have been shown to successfully differentiate into organotypic models (Stark et al., 1999), however their short lifespan and limited population growth potential restricts their usage to a small window of opportunity following isolation from patient samples. Therefore, increasing the useful lifespan of keratinocytes is desirable.

1.9.2 Rho Kinase inhibitors

Keratinocytes with increased proliferation which retain the ability to differentiate would be advantageous in the production of 3D skin models. Rho GTPases, a sub-family of the RAS superfamily, are thought to play a significant role in the regulation of keratinocyte differentiation (McMullan et al., 2003; Liebig et al., 2009). There are currently 20 known members of the Rho GTPase family. Of these, only 3 have been well characterised: RhoA, Cdc42 and Rac1. RhoA is known to regulate several cellular processes, including cell adhesion and motility, organisation of the actin cytoskeleton and gene expression (Heasman and Ridley, 2008). The Serine/Threonine Rho-associated kinase (ROCK) is one of the principle downstream effectors of RhoA. A

study has shown that culturing keratinocytes with 10 μM Y-27632, a known inhibitor of ROCK, almost completely inhibits suspension-induced differentiation over 24 hours with no increase in cell death (McMullan et al., 2003). Using the same concentration of Y-27632 on adherent cells in culture, it has been shown to increase colony formation (Terunuma et al., 2010) and immortalise human primary keratinocytes from both neonatal foreskin samples (Chapman et al., 2010) and also from adult skin samples (van den Bogaard et al., 2012). These immortalised keratinocytes may have the potential to differentiate into 3D skin models.

1.10 Summary

Overall, the mechanisms of skin irritancy from irritant compounds are poorly understood and it is difficult to predict which new chemical substances will have irritant properties. Recent EU directives prevent the use of animals for testing cosmetic products from 2013 (EU regulation 1223/2009); therefore there is an urgent need to develop new non-animal methods of predicting irritant (and sensitising) potential of a new chemical.

Current methods used to assess irritant and sensitising potential have limitations. Each of these methods analyse individual stages in the process of irritancy or sensitisation, namely skin penetration, haptenisation, KC response, dendritic cell maturation and T-cell activation. In order to fully test a chemical it must be subjected to each of these tests in a lengthy and costly process. The development of a simpler test using a skin model would negate many of the problems and costs involved in this multi-step process.

There are many biological activities associated with irritant stimulus that are not utilised in the current testing of novel compounds. Chemical irritants are thought to damage the barrier of the skin, causing an increase in serine protease activity (Berardesca and Distante, 1994; Torma et al., 2008). Between 1 and 6 hours after an epidermal insult there is a burst of generation of TUNEL-positive “pseudo-apoptotic” cells in the upper stratum corneum, followed by the generation of new corneocytes, possibly due to KLK activity (Demerjian et al., 2008). These TUNEL-positive cells do not show the classic hallmarks of apoptosis, despite their TUNEL-positive status

(Presland, 2009). Lamellar bodies secrete their contents including KLKs, into the intercellular space. KLKs are known to activate PAR2 which has been shown to play a vital role in the control of barrier formation following abrogation. Taken together these mechanisms indicate that following the disruption of the permeability barrier by irritants such as SLS there is a complex co-ordinated early response involving repair pathways, innate immune responses and generation of the early events in inflammation.

This study aims to examine responses to irritants in human skin and identify factors in the stratum corneum that may play a role in skin response to irritants. By determining these factors it may be possible to develop *in vitro* tests as a means of identifying potential irritants amongst novel cosmetic and medicinal products. There may also be the potential to identify novel anti-inflammatory therapies that target these molecules or pathways.

1.11 Hypothesis

The hypothesis to be tested in this thesis is that disruption of the stratum corneum barrier by chemical irritants induces epidermal serine proteases such as KLKs which act through the PAR2 receptor to cause a cutaneous inflammatory response.

1.12 Aims

This project has two broad aims:

- To investigate mechanisms involved in normal skin responses to irritants *in vitro* by using a human skin explant model and primary cells, specifically focusing on the effects of irritants on barrier function and serine protease activity.

- To establish immortalised keratinocytes from skin in order to populate a skin-equivalent model, and to subsequently use this model to examine whether this could serve as a means to test irritant (and potential irritant) substances.

2. Materials and Methods

2.1 Materials

2.1.1 Buffers and gels

All chemicals were purchased from Sigma Aldrich (Poole, UK) unless otherwise stated.

Coomassie blue stain contained 40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) H_2O and 0.1% (w/v) coomassie brilliant blue; **Epidermal lysis buffer** contained 0.1M Triethylammonium bicarbonate, 0.1% SDS; **FACS blocking solution** contained 10% FCS, 0.9% BSA and 0.09% NaN_3 in PBS; **Fluorescent imaging plate reader (FLIPR) assay buffer** contained Tyrodes buffer (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 10 mM D-Glucose and 2 mM $CaCl_2$), 20 mM HEPES, 7.5% NaHCO, pH 7.4; **Hanks buffered saline solution (HBSS)** contained 1.26 mM $CaCl_2$, 811.44 µM $MgSO_4$, 5.37 mM KCl, 440.90 µM KH_2PO_4, 137.00 mM NaCl, 337.3 µM Na_2HPO_4, 5.6 mM D-Glucose and 4.2 mM $NaHCO_3$; **Immunohistochemistry blocking medium** contained Dulbecco's modified Eagles medium containing 20% foetal calf serum (FCS) (Invitrogen, Paisley, UK) and 1% BSA; ***in situ* zymography gels** contained 375 mM Tris, 1% SDS (w/v), 10% bis-acrylamide, 0.4% gelatin (w/v), 0.375% $(NH_4)_2S_2O_8$, and 0.00075% tetramethylethylenediamine (v/v); **Mayers haematoxylin** contained 0.1% haematoxylin, 5% $KAl(SO_4)_2$, 0.1% citiric acid, 5% chloral hydrate (all from Fisher Scientific, Loughborough, UK) and 0.02% $NaIO_3$; **10% neutral buffered formalin** contained 10% formaldehyde (v/v), 4 g/l NaH_2PO_4 (monohydrate) and 6.5 g/l Na_2HPO_4 (anhydrous); **Phosphate buffered saline (PBS)** contained 0.1 M NaCL, 2.7 mM KCl and 1.8 mM KH_4PO_4; **Tris buffered saline (TBS)** contained 4.96 mM Tris and 137.9 mM NaCl; **Tris-Glycine Zymography developing buffer** contained 50 mM Tris–HCl 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% (w/v) Brij 35 (Invitrogen, Paisley, UK); **Tris-glycine zymography sample buffer** contained 63 mM Tris-HCl, 10% glycerol, 2% (w/v) SDS and 0.0025% bromophenol blue, pH 6.8 (Invitrogen, Paisley, UK); **Zymography renaturing buffer** contained 2.7% Triton X-100 (w/v) (Invitrogen, Paisley, UK); **Zymography running buffer** 25 mM Tris base, 192 mM glycine and 0.1% SDS pH 8.3 (Invitrogen, Paisley, UK);

2.1.2 Plasticware

Sterile plastics including tissue culture flasks, pipettes and falcon tubes were purchased from cellstar (Greiner bio-one, Stonehouse, UK).

2.2 Cell culture

2.2.1 Primary keratinocyte extraction

Adult skin samples were obtained from surplus tissue following mastectomy or from skin excised during surgery to assist closure as approved by the Southampton and Southwest Hampshire Research Ethics Committee (LREC 07/Q1704/59). Tissue was stored in PBS for a maximum of 4 hours to reduce cell death. Excess subcutaneous fat present on the skin tissue was removed and discarded. The remaining full thickness skin was cut with a scalpel into strips approximately 2 mm thick and treated in 2 U/ml dispase for 16 hours at 4°C. The epidermis was gently removed using tweezers and the dermis discarded. The epidermis was finely cut with scissors and incubated in a bijou with 0.05% trypsin 0.02% EDTA in HBSS for 6 minutes at 37°C and 5% CO_2. The epidermal sample was agitated every 2 minutes to assist disaggregation of keratinocytes. The trypsin was inactivated with 10% FCS and the epidermis-trypsin mixture filtered through a 70 µm sieve to achieve a single cell suspension. Remaining cells were recovered by washing the bijou with 2 volumes of PBS, which was also used to rinse the 70 µm sieve.

The extracted cells were centrifuged at 200 x g for 5 minutes and were resuspended in either defined keratinocyte medium (CnT57, Cell'n'tech) or in serum supplemented keratinocyte medium (SSKM) (3:1 [v/v] F-12 [Ham]-DMEM, 5% FCS, 0.4 µg/ml hydrocortisone, 5 µg/ml insulin, 8.4 ng/ml cholera toxin, 10 ng/ml epidermal growth factor, 24 µg/ml adenine, 100 U/ml penicillin, and 100 µg/ml streptomycin). Keratinocytes were cultured in SSKM on a feeder layer of irradiated murine NIH/3T3 Swiss albino fibroblasts. In the absence of a feeder layer keratinocytes were cultured in defined keratinocyte medium (CnT57, Cell'n'tech). Following resuspension, cells were seeded into T25 tissue culture flasks in 5 ml of appropriate medium.

2.2.2 Maintenance and subculture of primary keratinocytes

Keratinocytes cultures were washed and fed 24 hours after isolation and then every 48 hours thereafter. Cells were subcultured once they reached 85-90% confluence. Differential trypsinisation was used to remove the 3T3 feeder fibroblasts from the keratinocytes during passaging. The keratinocyte medium was removed and the cultures were rinsed with PBS followed by incubation with 0.05% trypsin 0.02% EDTA in HBSS without $CaCl_2$ or $MgSO_4$ for between 30 seconds and 1 minute at room temperature with close observation by phase microscopy. When the feeder cells (i.e. fibroblasts) showed signs of dissociation from the flask, as indicated by rounding up and detaching, the flasks were tapped and the detached cells were aspirated off. A second PBS rinse was performed followed by incubation at 37°C with 5% CO_2 for a further 3 to 5 minutes in fresh trypsin to remove the keratinocytes.

Gentle pipetting was used to disperse the cells into a single cell solution and 0.1 volumes FCS added to inhibit the trypsin activity. The solution was transferred to a 30 ml universal tube and a further rinse of the flask with PBS was performed to retrieve >90% of the keratinocytes. The cells were centrifuged at 200 x g for 5 minutes and the pellet resuspended in the appropriate keratinocyte media. 10 µl of cell suspension was diluted in 10 µl trypan blue and the cells counted using a haemocytometer. Dead cells were identified by an inability to exclude the blue dye and were not included in the cell count. Following subculture cells were seeded at $4.0x10^4$ per cm^2 in T25 or T75 tissue culture flasks on a fresh feeder layer of γ-irradiated 3T3 fibroblasts where appropriate.

Keratinocytes derived from neonatal foreskin were purchased from Cell'n'Tech (Berne, Switzerland) supplied in a frozen aliquot of $5x10^5$ cells from a pool of at least 3 donors at P2. Upon delivery, the vial was immediately stored in liquid nitrogen until use. The vial was incubated at 37°C in a water bath until only a small crystal of ice remained. The cell solution was added to warmed PBS and centrifuged at 200 x g for 5 minutes, and the pellet resuspended in the appropriate keratinocyte media. Neonatal keratinocytes were maintained in the same way as primary keratinocytes.

2.2.3 Cell line culture and storage

All cells were cultured at 37°C in 5% CO_2. HaCaT cells (Invitrogen) and NIH/3T3 Swiss albino fibroblasts (ECACC, Salisbury, UK) were maintained in DMEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and L-glutamine. Kirsten murine sarcoma virus transformed normal rat kidney (KNRK) cells transfected with human PAR2 (a gift from A. Walls, University of Southampton) were maintained in DMEM with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, L-glutamine and 400 μg/ml G418 sulphate.

2.3 Neutrophil isolation and shape change assay

Neutrophils display increased granularity and size following activation (Watts et al., 1991; Baggiolini and Kernen, 1992). Activation status can be analysed using flow cytometry, with neutrophil activation observed as an increase in forward scatter (Cole et al., 1995).

Blood from healthy volunteers (Ethics approval: SOMSEC0015.07) was taken by venepuncture using a butterfly needle and syringe. Red blood cells were removed from the whole blood by dextran sedimentation. 0.5 volumes of 6% Dextran (150 kDa (Sigma Aldrich, Poole, UK) was mixed thoroughly with the donor blood and incubated in a 50 ml Falcon tube at room temperature for 45 minutes. The dextran binds to the red blood cells causing them to settle at the bottom of the tube, leaving a plasma layer containing leukocytes above. The plasma layer was recovered and centrifuged at 2500 RCF for 5 minutes. The pellet was resuspended in 15 ml ice cold 0.2% NaCl in HBSS and shaken gently for 15 seconds to lyse remaining erythrocytes, followed by addition of 15 ml ice cold 1.6% NaCl to restore isotonicity. The tube was inverted gently to mix and centrifuged at 2300 RCF for 5 minutes. The pellet was resuspended in 5 ml HBSS with 0.2% human serum albumin. 5 ml lymphoprep was layered below the resuspended cells and the tube centrifuged at 1500 rpm for 5 minutes. The pellet containing neutrophils was resuspended in 2 ml HBSS with human serum albumin.

Neutrophils were incubated with either 1 ng/ml lipopolysaccharide (LPS), 1 μg/ml N-formyl-methionine- leucine-phenylalanine (FMLP) or 5 μM interleukin 8 (IL-8) in PBS

for 10 minutes at 37°C with 5% CO_2. Following activation of the neutrophils by these agents, the size and granularity of the neutrophils were assessed by flow cytometry (FACSCalibur (Becton Dickinson, Oxford, UK)). Resting neutrophils were used as a control to compare changes in size and granularity of the neutrophils following activation.

2.4 Human skin explant model

Skin from female breast tissue following mastectomy (surplus to histopathology requirement) was used as an *ex vivo* model for testing protease activity in response to epicutaneous application of irritants. Tissue was obtained from patients undergoing mastectomy at the Princess Anne hospital, Southampton with informed consent and ethical approval (LREC 07/Q1704/59). Tissue was placed in ice cold PBS immediately following removal and stored on ice for a maximum of 2 hours.

The skin was placed into a 60 mm petri dish and the epidermal surface of the skin was carefully blotted dry. Rubber O-rings with a diameter of 8 mm were sealed on to the skin using soft paraffin wax to create wells (**Figure 2.1**). Care was taken to ensure that paraffin wax was applied only to the area where the O-ring made contact with the skin in order to avoid altering the permeability of the skin or the protease activity within the epidermis. In order to prevent the skin from drying out during the incubation period the remaining space within the petri dish was filled with DMEM with 10% FCS at a depth of approximately 2 mm, ensuring no medium touched the epidermal surface of the skin.

50µl test solution (containing irritant substance or appropriate control) was applied to the wells using a 100 µl pipette and the skin incubated at 37°C with 5% CO_2 for the required length of time. Where test compounds were not water soluble, additional relevant vehicle controls were also performed. Following incubation with the test compounds and/or vehicle controls any remaining solution was aspirated off. 6 mm punch biopsies were taken from the treated sites of the skin sample (without removing the rubber O-rings) using sterile biopsy punches. A fresh biopsy punch was used for each treatment to avoid cross-contamination, and the biopsy was removed from within the rubber O-ring using forceps to ensure only treated skin was extracted.

The biopsied tissue was placed in a 1.5 ml eppendorf tube and snap frozen in liquid nitrogen. Samples were stored at -80°C until further investigations.

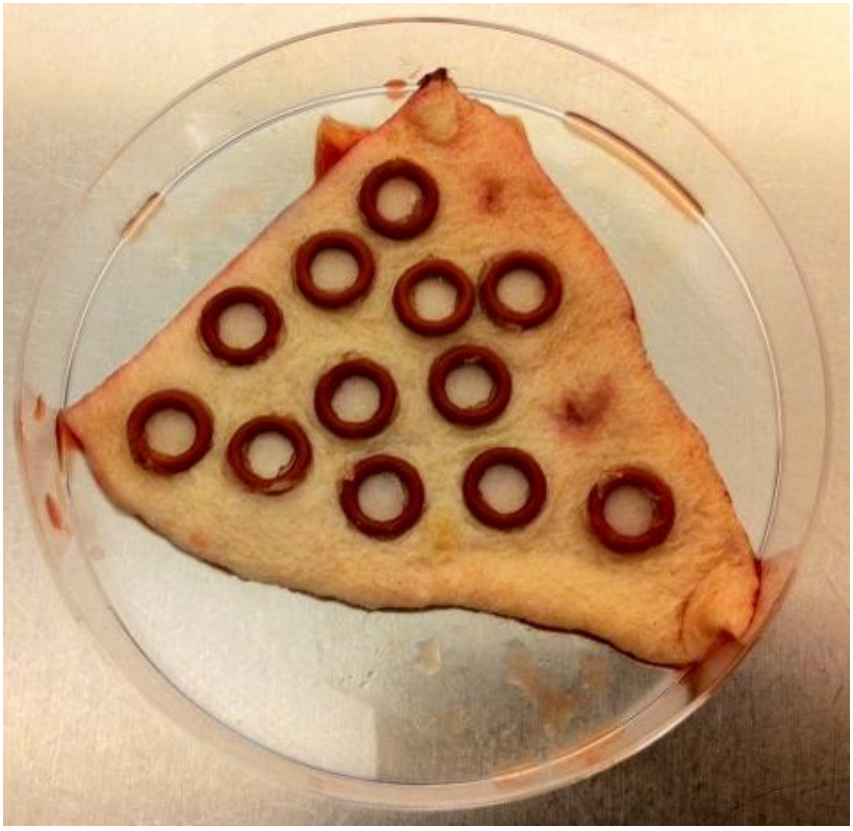


Figure 2.1: Application of irritants to *ex vivo* skin. Skin samples were blotted dry before rubber O-rings were adhered to the skin with soft paraffin wax. The wells formed had a capacity of approximately 55 µl. The surrounding space in the petri dish was filled with DMEM with 10% FCS at a depth of approximately 2 mm before incubation.

2.5 Construction of 3D skin models

2.5.1 Creating 3D skin models using a polycarbonate scaffold

Primary keratinocytes were grown to 80% confluence in tissue culture flasks, trypsinised and viable cells counted using trypan blue exclusion. Cells were seeded onto 12 mm Millicell polycarbonate cell culture inserts at 2 x 10^5 per insert in 500 µl CnT57 or SSKM in 24 well plates (**Figure 2.2**). The surrounding space was filled with 800 µl of the appropriate media and the models incubated at 37°C with 5% CO_2 for 48

hours. To promote differentiation and stratification of the keratinocytes the media was then removed from the apical surface of the model and the basal medium was replaced with the appropriate 3D medium. Models were fed basolaterally at the air-liquid interface for a further 14 – 21 days and then processed as appropriate.

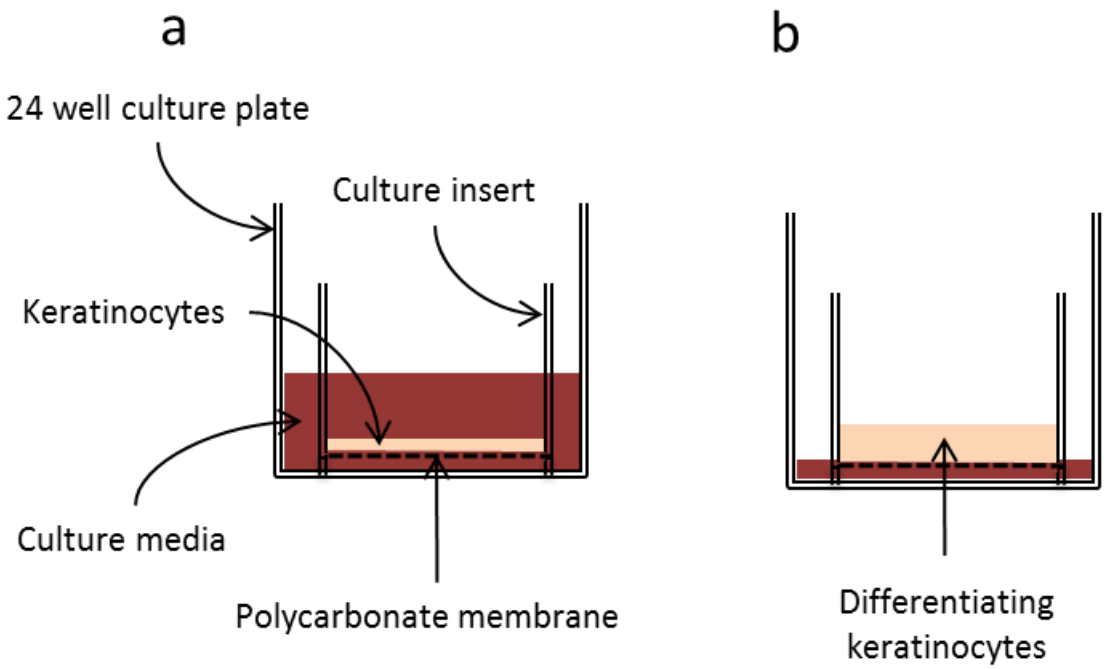


Figure 2.2: Culture of 3D skin models using polycarbonate scaffolds. A) Keratinocytes were grown submerged for 48 hours on culture inserts in 24 well plates. B) Media was removed from apical surface and cultures were fed basolaterally for a further 14 days.

2.5.2 Creating 3D skin models using a collagen scaffold

Collagen scaffolds were prepared in order to create an artificial dermis containing fibroblasts and collagen on which 3D epidermal models could be cultured. To prepare the collagen scaffolds, rat tail collagen type 1 (Invitrogen, Paisley, UK) was stirred with 10% 10x DMEM, 5% FCS and 1 x 10^6/ml NIH/3T3 fibroblasts. The collagen mixture was quickly pipetted into either 12 mm Millicell polycarbonate cell culture inserts or a 30 mm petri dish. The collagen was incubated for 4 hours at 37°C with 5% CO_2 to allow it to polymerise. Primary keratinocytes were trypsinised, counted in a haemocytometer and seeded onto the scaffolds at 2 x 10^5 for those on polycarbonate supports and 1 x10^6 for those in 30 mm petri dishes. Collagen scaffolds on

polycarbonate supports were then grown submerged for 48 hours followed by culture at the air-liquid interface for a further 14 days with basolateral feeding (**Figure 2.3a**). Collagen scaffolds in petri dishes were fed apically for 48 hours and then moved to a wire mesh to be fed basolaterally at the air-liquid interface for 14 days (**Figure 2.3b**).

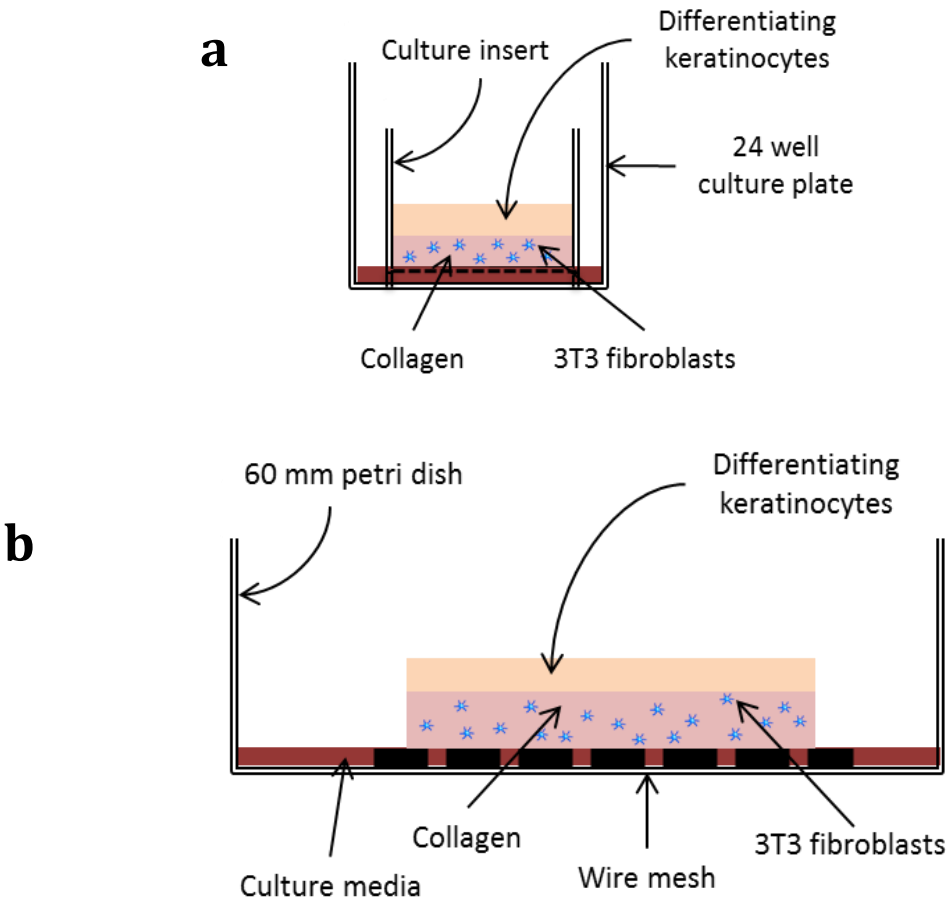


Figure 2.3: Collagen scaffolds for 3D skin culture. A) Collagen scaffolds containing 3T3 fibroblasts formed on polycarbonate membranes were cultured for 4 days prior to seeding of keratinocytes at the apical surface. B) Collagen discs supplemented with 3T3 fibroblasts were cultured in 30 mm petri dishes for 4 days before seeding of keratinocytes to the apical surface. Both collagen scaffold models were cultured submerged in 3D keratinocyte media for 48 hours followed by 14 days at air-liquid interface. Collagen discs were supported by a wire mesh in a 60 mm petri dish to allow basolateral feeding.

2.6 Immunofluorescent staining

2.6.1 Immunofluorescent staining of frozen sections

Frozen 6 mm skin biopsies (as described in section 2.4) were removed from -80°C storage and embedded into Tissue-Tek® optimal cutting temperature medium (OCT) (Sakura Finetek, Thatchem, UK). Transportation of the frozen samples from -80°C storage was performed on dry ice to ensure that the samples did not defrost. 8 µm sections of tissue were cut on a cryostat and allowed to dry onto poly-L-lysine coated glass microscope slides. Tissue was orientated so as to obtain vertical sections, including a cross section of epidermis and dermis, which were cut perpendicular to the epidermal surface to decrease the risk of separation of the skin components. Sections were air-dried for 1 hour at room temperature to ensure firm adhesion to the glass slide then fixed for 10 minutes in ice cold acetone at -20°C.

For immunofluorescence staining, slides were washed with TBS and then blocked for 30 minutes with blocking buffer followed by incubation with the primary antibody in TBS overnight at 4°C. In order to assess the level of non-specific binding, TBS without primary antibody was applied to 1 slide per sample. Following incubation, slides were washed for 3 x 5 minutes in TBS and then incubated with the secondary antibody for 1 hour in the dark. Secondary antibody was removed by washing for 3 x 5 minutes in TBS and nuclear material was stained by incubation with TO-PRO®-3 iodide or DAPI for 10 minutes. A final wash of 3 x 5 minutes in TBS was performed followed by attachment of coverslips with Mowiol, a non-fluorescent mountant which prevents photobleaching of the fluorophore.

Immunofluorescently stained samples were visualised by fluorescence microscopy (Axioskop 2 MOT, Zeiss). Slides were magnified between 100x and 400x using the appropriate light wavelength (**Table 2.1**) and the images digitally recorded (Axiocam, Zeiss). Slides stained in the absence of primary antibody were used to set the exposure levels to reduce background staining. Images were analysed using ImageJ v1.46 software.

Table 2.1: Wavelengths used for specific fluorophores.

Fluorophore	Excitation (nm)	Emission (nm)
FITC	450-490	515-565
DAPI	300-400	420-750
TO-PRO®-3	550-580	590-750

2.6.2 Immunofluorescent staining of cells

Primary keratinocytes or HaCaT cells were grown to 60% confluence in 8 chamber slides or on coverslips in 24 well plates. To fix the cells, the slides or coverslips were rinsed with TBS and then incubated with 4% paraformaldehyde for 10 minutes at room temperature. 50 mM ammonium chloride was added and incubated for 10 minutes at room temperature to permeabilise the cells. The coverslips or slides were then stained as described for skin sections above.

2.6.3 Paraffin embedding and immunohistochemical staining of 3D skin models

Prior to paraffin embedding, 3D skin models were fixed in 10% neutral buffered formalin overnight at 4°C. In the case of 3D skin models on a polycarbonate scaffold the model was carefully excised from the plastic holder using a scalpel and then cut into 2 equal pieces using a scissors. The models were placed onto sponge in an embedding cassette and fixed in place with a second sponge to prevent the sample folding or escaping the cassette during the embedding process. For 3D models on collagen scaffolds, the entire model was placed directly onto a sponge in an embedding cassette and fixed in place with a second sponge. Samples were stored in 70% ethanol for a maximum of 6 hours until embedding.

Embedding was performed by the University of Southampton immunohistochemistry department at Southampton General Hospital. Automated paraffin embedding was performed using a Shandon Hypercentre XP tissue processor (Thermo Scientific, Loughborough, UK). All steps were performed at 37°C with gentle vacuum to aid penetration of the solutions and remove trapped air bubbles in the tissue. Fixed

samples were immersed for 1 hour in 70% alcohol followed by a further 1 hour in 80% alcohol. Samples were then immersed for 1 hour in 90% alcohol followed by 2 x 1 hour in absolute alcohol. A total of 2 subsequent submersions in absolute alcohol were performed for 2 hours each before the samples were immersed in chloroform : absolute alcohol (1:1, v/v) for 1 hour. 2 additional submersions in chloroform for 1.5 hours were followed by immersion in wax for 1.5 hours followed by a final submersion in wax for 3 hours. Processed samples were then embedded in Surgipath histology wax blocks and allowed to cool and solidify before sectioning.

Embedded samples were cut into 5 μM sections using a LeicaRM2135 microtome. Slides were allowed to dry for 24 hours at 40°C before immunohistochemical or tinctotorial staining. Slides were submerged in clearene for 2 x 10 minutes to deparaffinise the sections, followed by submersion in absolute alcohol for 2 x 5 minutes followed by 5 minutes immersion in 70% alcohol to rehydrate the samples. Endogenous peroxidases were inhibited with 0.5% hydrogen peroxide in methanol for 10 minutes, followed by washing in TBS for 3 x 2 minutes. Antigen retrieval was performed by microwaving slides for 25 minutes in 0.01M citrate (pH 6.0). Slides were rinsed with TBS and blocked with 10% FBS in DMEM for 30 minutes. The slides were then rinsed with TBS and coated with the appropriate antibody either for 1 hour at room temperature or overnight at 4°C.

Following incubation with primary antibodies, slides were washed with TBS for 3 x 5 minutes and incubated with secondary antibodies for 30 minutes. Following another washing step for 3 x 5 minutes the slides were coated with avidin biotin-peroxidase complexes and incubated for a further 30 minutes at room temperature. The chromagen DAB was then added for 5 minutes according to manufacturer's instructions. The slides were rinsed in TBS and washed in running water for 5 minutes then submerged in Mayers haematoxylin for 1 minute and subsequently rinsed in running water for 1 minute. The skin tissue was then counterstained by submersion of the slides in eosin for 1 minute. A final wash was performed under running water for 5 minutes followed by mounting of a coverslip over the skin on the glass slide using Pertex.

Slides were visualised by brightfield microscopy (Axioskop 2 MOT, Zeiss) at magnifications between x100 and x400 and the image digitally recorded (Axiocam,

Zeiss). Where required the slides were assessed by a histopathologist (Dr James Chan, University of Southampton).

2.7 Protein analysis

2.7.1 Preparation of skin tissue homogenates for protein analysis

Skin was treated with various irritants or sensitisers for specified times (as detailed in section 2.4 above and in chapter 3, section 3.2) and 6 mm punch biopsies were taken from the treated and untreated sites. The biopsy samples were washed x 3 in PBS and incubated in 1 ml PBS at 56°C for 5 minutes to aid separation of the epidermis from the dermis. The epidermis was removed using fine forceps, finely dissected with a scalpel and sonicated using a Soniprep 150 (MSE, UK) for 3 x 15 seconds in 200 µl lysis buffer. The sample was then centrifuged at 1400 rcf for 5 minutes to remove insoluble proteins. The supernatant containing the soluble proteins was retained and either stored at -20°C for future use or quantified immediately.

2.7.2 Protein quantification

Total protein concentration was determined by bicinchoninic acid protein (BCA) assay (Thermo Scientific Pierce) according to the manufacturer's instructions. In brief, reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide) was mixed with Reagent B (4% cupric sulphate). Sample lysates were diluted 1:5 in lysis buffer. 8 volumes of assay solution was added to the diluted sample lysates in a clear, flat-bottomed 96 well plate and incubated for 30 minutes at 37°C. BSA diluted in lysis buffer was used to create a standard curve between 0.04 and 1.0 mg/ml SBA. Lysis buffer was used as a blank control. All samples and standards were performed in duplicate. Colour change was analysed in a colorimetric plate reader (Spectramax 340pc) using SoftMax Pro software.

2.7.3 Western blotting

Proteins were denatured and reduced by incubation at 80°C for 10 minutes in NuPage lithium dodecyl sulphate sample buffer (pH 8.4) with 50 mM dithiothreitol (Invitrogen). Samples were loaded on 4-12% SDS polyacrylamide gels (Invitrogen) and proteins were separated by gel electrophoresis at 160 volts (v) for 90 minutes. Following a brief rinse in fresh running buffer, a transfer sandwich was assembled containing the gel and a polyvinylidene difluoride (PVDF) membrane.

Following the transfer the PDVF membranes were washed once in methanol followed by double distilled water for 2 x 5 minutes. The membranes were then incubated with blocking buffer (10% Steelhead salmon serum, 0.1% NaN_3 in PBS) (Invitrogen) for 60 minutes at room temperature. Primary antibody was diluted in wash buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% TweenR-20) and applied to each membrane for 12 hours at 4°C. Following 3 x 5 minutes wash in wash buffer (tris buffered saline-tween20) (TBS-T), pH 7.4), membranes were incubated with secondary antibody for 60 minutes at room temperature. A further 3 x 5 minutes wash in wash buffer was followed by incubation with Invitrogen Qdot solution (1 μM solution in 1 M betaine, 50 mM borate pH 8.3 with 0.05% NaN_3) diluted 1/2000 in blocking buffer for 3 hours at room temperature. Membranes were then washed 3 x 5 minutes in wash buffer followed by a final wash in double distilled water for 5 minutes. Membranes were dried and imaged using a BioRad Versadoc using epi-illuminiation. All incubation steps were performed with gentle agitation.

2.8 Zymography

Gelatin zymography was performed by diluting protein samples 1:1 in tris-glycine sample buffer and incubating at room temperature for 10 minutes. 10 μl of sample was loaded into pre-cast tris-glycine poly acrylamide gels containing 0.1% gelatin (Novex© 10% Zymogram, Gelatin, Invitrogen) and proteins were separated by gel electrophoresis at 150 v for 2 hours. Following electrophoresis gels were removed from their casts and washed briefly in distilled water. Following this washing step the gels were incubated in renaturing buffer (Invitrogen) for 30 minutes at room temperature, followed by incubation at 37°C for 30 minutes in developing buffer

(Invitrogen). A further incubation in fresh developing buffer at 37°C overnight was followed by staining in Coomassie blue stain for 30 minutes, followed by destaining in distilled water for 30 minutes. Areas of protease activity appeared as a clear band against a blue background.

2.8.1 *In situ* gelatin zymography

Poly-L-lysine (PLL) coated glass slides were covered with 100 µl of a 10% polyacrylamide gel containing 0.4% gelatin. A coverslip was placed over the liquid polyacrylamide gel in order to achieve an even, plane surface across the gel. Once the gel was set the coverslip was carefully removed and the gel inspected for imperfections. 8 µm cryosections of tissue were placed on the gel and allowed to dry for 10 minutes. The slides were then incubated in a moist chamber at 37°C for 18 hours. Negative controls were incubated at 4°C to inhibit protease activity. After incubation the sections were carefully washed off with PBS and the gel stained with Coomassie blue stain for 30 minutes. Gels were washed in water and photographed using an Axioskop 2 fluorescence microscope (Zeiss)

2.8.2 *In situ* FITC-casein zymography

OCT-embedded samples were cut into 8 µM sections with a cryostat. The sections were applied to poly-L-Lysine coated glass microscope slides and incubated at room temperature for 1 hour to ensure adherence. The slides were washed briefly with 1% PBS-tween followed by application to the sections of EnzChek, a casein derivative heavily labelled with the fluorophore BODIPY FL (Invitrogen, Paisley, UK). The heavy labelling of the casein results in almost complete quenching of the FITC signal, which is restored upon digestion of the casein substrate by proteases. The slides were incubated in humidified chambers for 2 hours at 37°C in the dark. Slides were then rinsed in 1% PBS-tween and coverslips attached using Mowiol mounting medium. After allowing the mounting medium to set for 1 hour the slides were stored at 4°C for up to 24 hours. Slides were imaged using an excitation/emission setting of 488/526 nm. Brightfield images were taken of the same field of view for analysis.

2.9 Assessment of Calcium Flux by FLIPR assay

In order to assess intracellular calcium flux, primary keratinocytes or HaCaT cells were seeded at 1 x 10^4 into black-walled, clear, flat-bottomed 96 well plates. These were incubated at 37°C with 5% CO_2 for 24-48 hours until cells reached 100% confluence. 2.5 mM probenecid and 500 µM brilliant black were added fresh to FLIPR assay buffer on the day of use to make FLIPR loading buffer. Test compounds were diluted in loading buffer to 3 x final concentration in clear V-bottomed 96 well plates.

The cells were incubated for 1 hr at 37°C in loading buffer containing 2 mM Fluo-3 AM. The standard baseline times range from 15-20 seconds, therefore a baseline fluorescence at 480/530 nm was taken for 17 seconds prior to automated addition of 50 µl test compounds using a Flexstation II (Molecular devices, Wokingham, UK). Fluorescence was monitored for a further 150 seconds following addition of the compounds.

2.10 *In situ* cell viability assay

Primary keratinocytes were seeded in 96 well plates at 1.3 x 10^4 per well and incubated for 24 hours at 37°C with 5% CO_2. A range of concentrations of DNCB, DPCP, croton oil and Dithranol (table 1) was applied to the cells which were then incubated overnight at 37°C with 5% CO_2. 70% ethanol was applied to triplicate wells for 5 minutes to provide a 100% cell death control. All treatments were performed in triplicate on primary keratinocytes from 4 separate individuals.

Cells were cultured in additional wells to enable a trypan blue exclusion assay to be performed on untreated cells to confirm 100% cell viability and 100% cell death in 70% ethanol treated wells. The keratinocyte media or 70% ethanol was removed from the wells and 25 µl 50% trypan blue in PBS was applied to wells for 20 seconds. The trypan blue was then removed and the wells rinsed briefly with PBS. The cells were then visualised using phase microscopy and the percentage of cells showing uptake of trypan blue, indicating cells death, was assessed in 4 fields of view per well in triplicate wells for both untreated and 70% ethanol treated cells.

Cell viability was determined with the CellTiter 96® AQ_{ueous} One solution Cell Proliferation Assay (Promega, Southampton, UK). In brief, 5 volumes of serum-supplemented keratinocyte media was added to 1 volume of assay solution and mixed well. Media was removed from all wells and replaced with 100 µl of diluted assay solution. The plate was incubated at 37°C with 5% CO_2 for 2 hours. Cell viability was assessed by a colour change of the medium from yellow to purple, measured with a colourimetric plate reader (Spectramax 340pc) using SoftMax Pro software. Readings from 100% live cells (untreated) and 100% dead cells were used as a standard.

2.10.1 Real time PCR mRNA extraction

Two methods were used to extract mRNA from primary keratinocytes. In most cases RNA was extracted using the RNeasy® Plus Mini Kit (Qiagen) according to manufacturer's instructions. In brief, cells were lysed with 25-50% guanidine thiocyanate and β-mercaptoethanol. Samples may be stored in lysis buffer for up to 3 months at -20°C. To reduce contamination of the extracted RNA by genomic DNA, genomic DNA was bound to a G-eliminator column and 1:1 volume 70% ethanol added to the flow through. RNA was captured in a silica-membrane and washed with 2.5-10% guanidine thiocyanate and 2.5-10% ethanol followed by 75% ethanol. An additional 75% ethanol wash was performed and the membrane centrifuged to remove all ethanol. RNA was eluted from the membrane in 50 µl RNase-free water by centrifugation.

In some cases RNA was extracted by lysing the keratinocytes with TRIZOL. Samples were stored in TRIZOL for up to 3 months at -20°C. 0.2 volumes of chloroform were added to the samples, mixed thoroughly and incubated at room temperature for 15 minutes. The aqueous phase was carefully removed and retained and the organic phase was discarded. 1 volume of isopropanol was added to the retained aqueous phase and incubated for 10 minutes at room temperature. The sample was then centrifuged at 12,000 x g for 10 minutes and the supernatant discarded. The pellet was washed with 70% ethanol in DEPC treated water and vortexed thoroughly. The sample was then centrifuged at 7,500 x g and the supernatant discarded. The pellet was air dried for 5 – 10 minutes followed by resuspension in 30 µl RNAse free water. Complete solubilisation was achieved by heating the sample to 60°C for 10 minutes.

In all cases the concentration and purity of RNA was determined using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, USA). Concentration of RNA was required to be a minimum of 50 µg/µl. RNA was considered to be of acceptable quality if the nucleic acid purity, as measured by 260/230 ratio, was above 1.90 and the RNA purity (free from DNA contamination) as measured by 280/260 ratio, was above 1.95.

2.10.2 Quantification of genes by RT-PCR

cDNA was synthesised from extracted RNA using the RT^2 first strand kit (SABiocience) following manufacturer's instructions. A second genomic DNA elimination step was employed to reduce genomic DNA contamination to negligible levels. 400 ng of RNA was added to 2 µl elimination buffer in a final volume of 10 µl (**Table 2.22**) and incubated at 42°C for 5 minutes followed by 1 minute at 4°C.

Table 2.2: Reagents used in genomic DNA elimination step.

Reagent	**Volume**
RNA	400 ng (χ)
Elimination buffer	2 µl
RNAse free water	(8-χ) µl
Total	**10 µl**

Following elimination of genomic DNA, 2 µl reverse transcriptase and 1 µl control primers were added to the RNA mix as described in **Table 2.33**. 4 µl buffer and 3 µl RNAse free water brought the final volume of solution to 20 µl.

Table 2.3: Reagents used in cDNA synthesis

Reagent	Volume
RNA	10 µl
5x RT buffer	4 µl
Control primers	1 µl
Reverse transcriptase	2 µl
RNAse free water	3 µl
Total	**20 µl**

The reverse transcription solution was incubated at 42°C for 15 minutes followed by 95°C for 5 minutes to inactivate the reverse transcriptase. 90 µl DNA-free water was added and gently mixed. The final cDNA was stored at -20°C until further use.

Quantitative PCR was performed using either pre-coated 384 well PCR plates (PAHS-020A, SABioscience) or manually added primers. A list of the genes analysed is shown in **Appendix 1**. SYBER green master mix which incorporates PCR buffer, hotstart Taq polymerase, nucleotides and ROX® reference dye was added to the cDNA along with primers as appropriate (**Table 2.44**). For pre-coated plates there was no requirement to add primers as these are incorporated into the plate. 10 µl of PCR mix was loaded per well and centrifuged at 300 x g for 1 minute to remove air bubbles before the plate was loaded into the late reader.

In all cases Q-PCR was performed using a 7900HT Fast Real-Time PCR System (Applied Biosystems, CA, USA) and the data collected using SDS 2.4 software (Applied Biosystems, CA, USA). The PCR protocol consisted of an initial cDNA denaturation at 95°C for 10 min, followed by 35 cycles of denaturation at 95°C for 15 seconds and annealing and data collection at 60°C for 60 seconds.

Table 2.4: Reagents used for PCR. For pre-coated 384 well plates the primer pair was substituted with ddH_2O.

Reagent	Volume
cDNA	0.4 µl
RT² SYBER® green qPCR master mix	5.0 µl
DNA free water	4.2 µl
10 µM primer pair stock	2 µl
Total	**20 µl**

For pre-coated plates quality control of the cDNA was performed through the use of a pre-coated RNA quality control plate (SABioscience) to confirm the suitability of housekeeping genes, the efficiency of the reverse transcription steps, the absence of contaminating genomic DNA and the efficiency of the PCR protocol.

The critical threshold (Ct) value is the number of amplification cycles required for the fluorescent signal to cross the background threshold. Ct was set at 0.2 for all experiments. Changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. For pre-coated 384 well plates the average Ct value for 5 housekeeping genes was used for calculating ΔCt value. For Q-PCR using manually added primers ΔCT values were calculated using the Ct value for the housekeeping gene 26S. Each gene was analysed in duplicate wells. ΔCt is calculated by the following equation:

$$\Delta Ct = Ct_{\text{Target gene}} - Ct_{\text{Reference gene}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{Test sample}} - \Delta Ct_{\text{calibrator sample}}$$

$$\text{Fold difference} = 2^{-\Delta\Delta Ct}$$

Analysis of fold change in gene regulation was performed using automated Microsoft Excel analysis tools from SABioscience.

2.11 Statistics

Population doubling levels was calculated as $PDL_n = 3.32*(Log(N^1-N^2) + PDL_{n-1}$ where N^1 is total viable cells at harvest and N^2 is total viable cells at seed. Due to the inability to take accurate cell counts at first seeding (P0), passage 2 (P2) was the first passage at which population doubling could be accurately calculated. The earliest P2 for each pair of cultures was therefore taken as day 1 of culture for analysis.

Data was subjected to a normality test to confirm normal distribution and parametric analysis was performed as appropriate. Where 3 or more samples were being compared analysis of variance (ANOVA) was used. For comparison of 2 samples a student's t-test was performed. Paired or repeated measures analysis applied as appropriate. All statistical analysis was performed using GraphPad 6.0. Values of p less than 0.05 were considered significant.

3. Changes in epidermal serine protease activity and down-stream effects following irritant application

3.1 Introduction

It is becoming increasingly evident that the protective barrier of the epidermis acts not only as a physical barrier to prevent the entry of exogenous substances and to limit the transcutaneous movement of electrolytes and water, but it also plays an active and highly specialised role in immune responses (Feingold et al., 2007; Dunnick and Dellavalle, 2008; Novak et al., 2010). Upon contact with the skin, many foreign substances can activate an innate immune/inflammatory response, referred to as an irritant response. The mechanisms by which chemicals elicit an irritant response have not been well characterised, however previous studies have shown that application of irritants to keratinocytes leads to an upregulation of the proinflammatory cytokines IL-8 and TNFα (Mohamadzadeh et al., 1994; Wilmer et al., 1994; Spiekstra et al., 2005), which has also been shown in *in vivo* studies following application of irritants to human, mouse or rabbit skin (Tsuruta et al., 1996; Coquette et al., 2003; Dickel et al., 2010). The pathway or pathways by which these cytokines are upregulated following irritant exposure has not been fully elucidated. However, more recently studies have indicated a role for serine proteases in regulation of the immune response to barrier abrogation following tape stripping (Hachem et al., 2006b).

Serine proteases and their inhibitors have been shown to play a vital role in the maintenance of a healthy epidermal barrier (Ovaere et al., 2009; Meyer-Hoffert, 2012). As discussed in chapter 1, in humans, serine proteases play a role in several skin disorders such as Netherton's syndrome (Chavanas et al., 2000; Descargues et al., 2005), and animal models have been used extensively to show that deficiencies of specific serine proteases lead to severe impairment of the epidermal barrier (Descargues et al., 2005; Leyvraz et al., 2005; Basel-Vanagaite et al., 2007; Nagaike et al., 2008). Netherton's syndrome is characterised by a scaly epidermis with malformation of the stratum corneum, a constitutive erythematous skin inflammation and immune dysregulation that mimics that of the atopic state, with allergies to

environmental agents including dusts, pollens and foods. The primary genetic defect has been shown to be loss of function mutations in the *SPINK5* gene encoding LEKTI, an inhibitor of serine proteases such as the kallikreins (Chavanas et al., 2000). As a result, there is unregulated activity of kallikrein 5 (KLK5) amongst others, which, acting through the PAR2 receptor, induces increased epidermal production of Thymic Stromal Lymphopoietin (TSLP) (Briot et al., 2009; Briot et al., 2010), a cytokine strongly implicated in the programming of atopic type immune responses (Corrigan et al., 2009; Cheng et al., 2013).

It has been shown that active KLK5 is able to activate KLK7, thereby increasing the overall protease activity within the epidermis (Sotiropoulou et al., 2009). Both KLK5 and KLK7 degrade corneodesmosomes in the stratum corneum (Descargues et al., 2006; Borgono et al., 2007). Subsequently, increased KLK5 activation, and in turn increased KLK7 activation, may contribute to further loss of keratinocytes at the stratum corneum and therefore prevent recovery of the barrier. KLK7 has also recently been shown to at least partially regulate activation of caspase-14, which is also involved in the barrier repair process (Yamamoto et al., 2012). This is in agreement with a study by Hachem *et al* which showed that application of serine protease inhibitors to tape stripped forearms accelerates barrier recovery kinetics (Hachem et al., 2006b). Further, they showed that lamellar body secretion at the stratum corneum-stratum granulosum junction is increased in tape stripped hairless mice when a topical serine protease inhibitor is applied compared with vehicle treated controls. The findings from this study have not yet been verified by any other groups, and the specific serine proteases involved in the activation of PAR2 in human models was not investigated.

Understanding the mechanisms by which irritants cause an innate immune response may lead to identification of biomarkers which may indicate susceptibility, allowing those individuals identified as more susceptible to avoid irritant chemicals. This in turn could reduce the personal, social and economic impact of occupational contact dermatitis, 80% of which is accounted for by ICD. Previous studies have focused on the immune response hours or even days following irritant exposure; however the early stages of the response were undefined. More recent studies have investigated the early irritant-induced responses within cell models (Clemmensen et al., 2010); however these systems ignore the important role of disrupting the permeability barrier in the activation of the innate immune response. In the present study, a full

thickness skin model was used to test the hypothesis that the inflammatory response induced by skin irritants is the result of activation of proteases including kallikreins. It was hypothesised that these proteases in turn activate the innate immune response via the PAR2 receptor which induces release of inflammatory cytokines and chemokines leading to inflammation.

3.2 Methods

3.2.1 Examining gross histological changes

To examine for gross histological changes following application of irritants, H&E staining was performed on skin samples from 8 subjects. Two fields of view were photographed from each sample and the boundaries of the four layers (stratum basale, stratum spinosum, stratum granulosum and stratum corneum) of the epidermis marked on each photographic image (**Figure 3.1**). The accuracy of these boundaries was confirmed by a histologist. Ten vertical regions of interest (ROI's) were measured from each field of view and the contribution from each layer along this measurement was recorded.

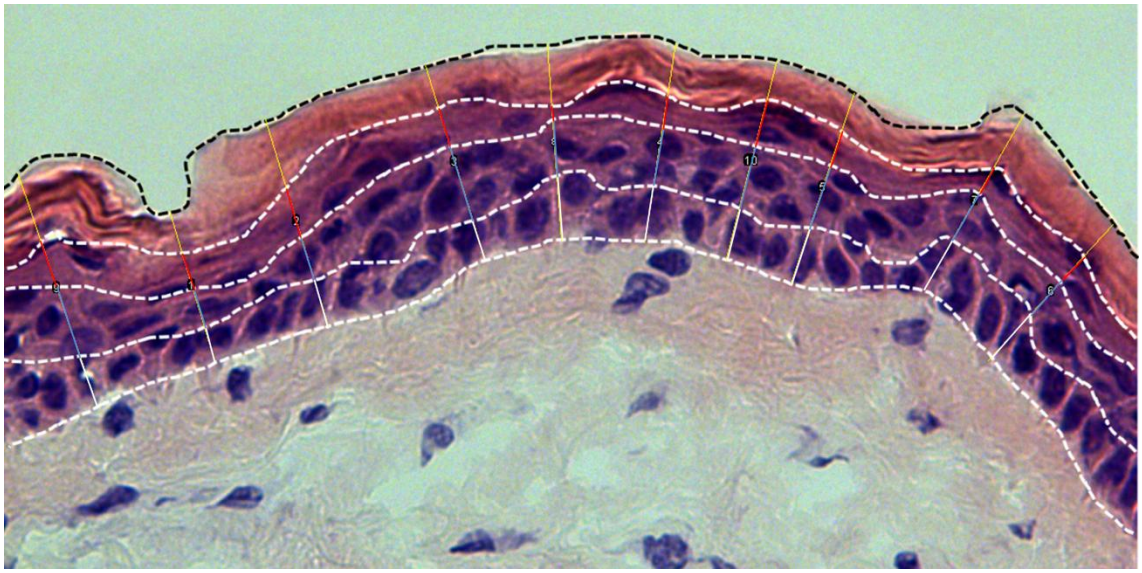


Figure 3.1: Examining gross histological changes in epidermis. The boundary between the 4 layers of the epidermis is marked with white dotted lines. The outer surface of the stratum corneum is marked with a black dotted line. Ten regions of interest were measured and divided between the 4 layers (white – stratum basale, blue – stratum spinosum, red – stratum granulosum and yellow – stratum corneum) and the contribution of each layer to the overall depth of the epidermis was calculated.

3.2.2 Immunofluorescence staining

Immunofluorescence staining was performed as described in chapter 2 using the antibodies described in **Table 3.1.** Age ranges of patient whose skin samples were used for KLK5 and KLK7 staining are shown in **Table 3.2**.

Table 3.1 Primary antibodies used in immunohistochemistry and immunofluorescent staining. FITC conjugated anti-rabbit secondary antibody produced in goat (F9887, Sigma Aldrich, UK) was used at 25 µg/ml.

Target	Source	Host	Concentration
KLK5	Abcam (ab7283)	Rabbit	5 µg/ml
KLK7	Abcam (ab96710)	Rabbit	7 µg/ml
PAR2	Santa Cruz (H99)	Rabbit	1 µg/ml

Table 3.2 Age ranges of patient samples used for immunofluorescence staining. All samples were from female mastectomy patients.

Age group	Number of patients
30 – 40	1
41 – 50	0
51 – 60	1
61 – 70	4
71 – 80	3
81 – 90	2
Total	**11**

3.2.3 Gelatin zymography and activation of recombinant kallikreins

All zymography was performed in chilled electrophoresis tanks on ice. 125 volts was applied to the electrophoresis tanks for 2 hours, as outlined in chapter 2. Images of gels were quantified using ImageJ software.

Recombinant KLK5 was incubated in KLK5 activation buffer overnight at 37°C which allowed it to self-activate.

3.2.4 Neutrophil activation and flow cytometry

Neutrophils were isolated as described in chapter 2 and activated by incubation with either 1 ng/ml lipopolysaccharide (LPS), 1 µg/ml N-formyl-methionine-leucine-phenylalanine (fMLP) or 5 µM interleukin 8 (IL-8) in PBS for 10 min at 37°C. Activated or resting neutrophils were smeared across poly-L-lysine coated glass microscope slides and allowed to dry. Activity of proteases was assessed by application of EnzChek solution (5 µg/ml) after 10min.

3.2.5 *In situ* zymography with EnzChek

Frozen skin sections were cut with a cryostat microtome and allowed to adhere to glass slides for 1 hour. Enzchek solution, diluted to 2 µg/ml in distilled water, was applied to 8 µm skin sections as described in chapter 2. Age ranges of patient samples used for *in situ* zymography are shown in **Table 3.3**.

Table 3.3 Age ranges of patient samples used for *in situ* zymography. All samples were from female mastectomy patients.

Age group	Number of patients
30 – 40	1
41 – 50	0
51 – 60	0
61 – 70	4
71 – 80	2
81 – 90	1
Total	**8**

3.2.6 Calcium flux measured by FLIPR assay

Calcium flux was assessed as described in chapter 2. Trypsin (Sigma-aldrich, Poole, UK) had an activity of 15,450 U/mg. Manufacturers analysis showed 1 mg of soybean trypsin inhibitor (Sigma-aldrich, Poole, UK) inhibited 1 mg trypsin with an activity of 10,000 U/mg.

3.2.7 Statistics

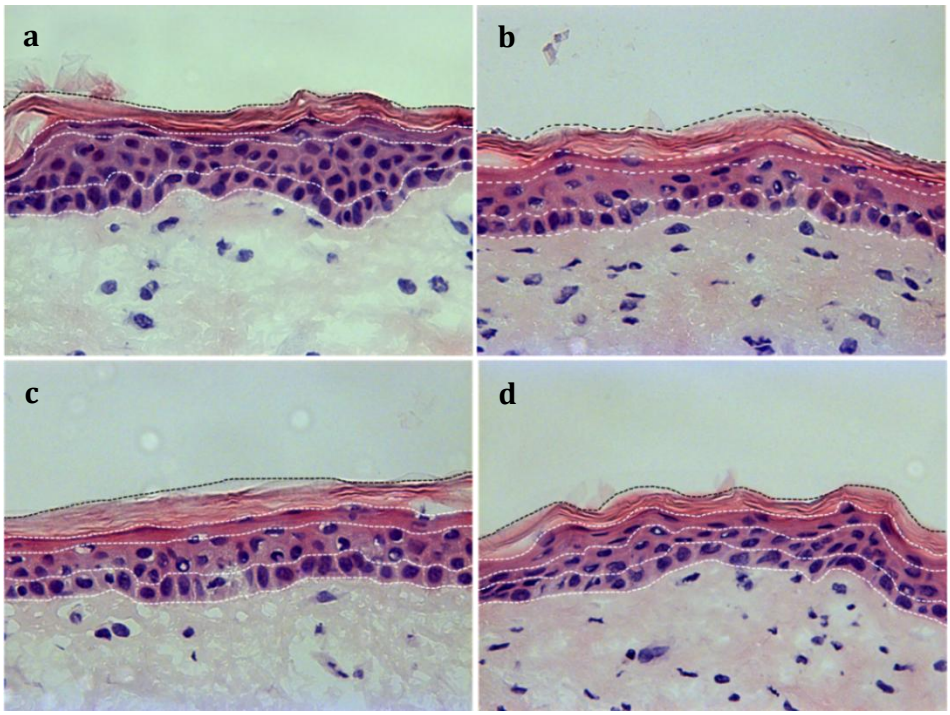
Analysis was of data was performed using GraphPad Prism 6.0 as described in chapter 2, and in the cases of multiple comparisons the Bonferroni correction was applied. Statistical analysis was performed under the advice of Scott Harris, statistician at University of Southampton.

3.3 Results

3.3.1 Effect of irritants on epidermal layers

It is well established that croton oil acts as a skin irritant, inducing cytokine production, hyperkeratosis and dermal infiltration by leukocytes including neutrophils (Pound, 1968; Patrick et al., 1987; Moon et al., 2001). Croton oil is used frequently in studies investigating the effects of irritancy on cellular processes and skin barrier disruption. The detergent sodium lauryl sulphate (SLS), otherwise known as sodium dodecyl sulphate (SDS), is a surfactant (detergent) also known to act as an irritant (Van Der Valk et al., 1984; Willis et al., 1989). It causes damage to the skin barrier by removal of intercellular lipids, which in turn leads to increased proliferation of keratinocytes causing transient hyperkeratosis (Willis et al., 1989; Fartasch, 1997). Therefore, in the current project, skin explants were treated for 30 min with 3% croton oil in acetone or 5% SDS in PBS, as per a previous study from this laboratory (Pickard et al., 2009).

Thirty minutes after application of any/all of the irritants to skin explants, there was no change in the overall thickness of the epidermis compared with PBS-treated skin explants (**Figure 3.2**a-d). However, compared with PBS treatment, there was a significant increase in the thickness of the stratum granulosum in skin treated with acetone ($p=0.0113$), 3% croton oil ($p=0.0335$) and 5% SDS ($p=0.0038$) (paired 2-tailed t-test; **Figure 3.2**e). No other epidermal layer showed a significant change in thickness between any of the treatments and PBS alone. The data is presented as the proportion of the total epidermal thickness of the different cellular layers.



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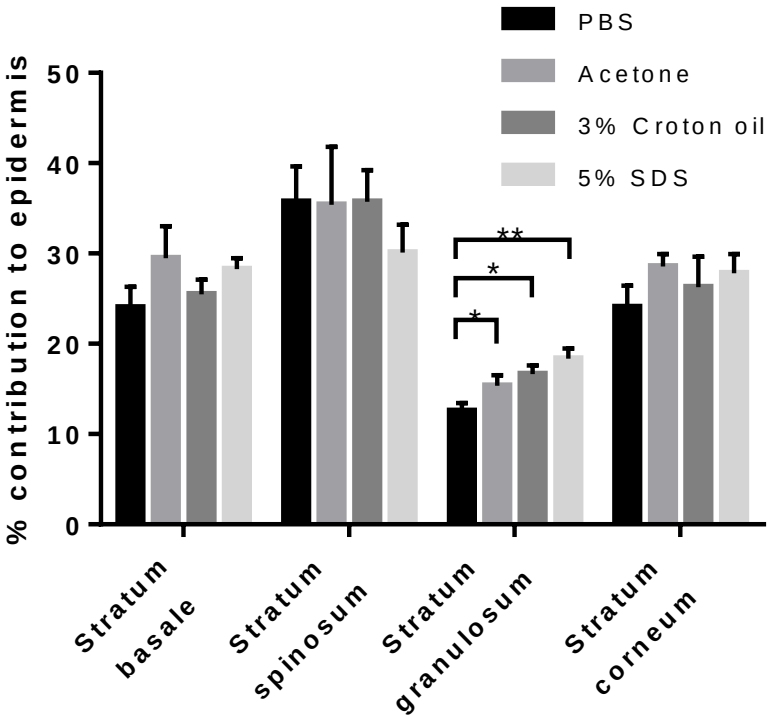


Figure 3.2: Increase in thickness of the stratum granulosum following irritant treatment. H&E stained section of skin with boundaries of epidermal layers marked with white lines for skin treated with PBS (a), acetone (b), 3% croton oil (c) or 5% SDS (d) for 30 minutes. e) Graph of percentage contribution of each layer to overall thickness of the epidermis with PBS, acetone, 3% croton oil and 5% SDS demonstrating that the relative thickness of the stratum granulosum was significantly increased in the skin treated with acetone, 3% croton oil and 5% SDS. *$p<0.05$, **$p<0.01$. Data shown as mean + SE from 9 patients for acetone and 3% croton oil treatment and 7 patients for 5% SDS treatment.

3.3.2 Effects of irritants on KLK5 expression

In order to determine the effects of 3% croton oil and 5% SDS on KLK5 and KLK7 in skin, expression of KLK5 and KLK7 proteins were determined in a semi-quantitative manner by immunofluorescence staining with FITC-labelled antibodies following 30 min treatment of *ex vivo* skin with these irritants.

In general, KLK5 expression was pan-epidermal with the greatest intensity observed at the outermost part of the epidermis. There was minimal variance in the level of KLK5 expression between PBS treated skin samples from different subjects. In all cases, KLK5 appeared to be expressed at higher levels in skin treated with 3% croton oil (n=9) and 5% SDS (n=7) than in PBS-treated skin, as assessed by the intensity of green signal (see **Figure 3.3** for representative examples). A moderate change in KLK5 expression was also observed in acetone-treated samples compared with PBS-treated samples in 2 of 7 cases although there was no change observed in 5 of 7 cases.

For quantification and statistical comparison, epidermal KLK5 expression was compared by repeated measures one-way ANOVA (acetone or 3% croton oil versus PBS) or a 2-tailed paired t-test (5% SDS versus PBS). A significant increase in total KLK5 expression was observed following treatment with acetone ($p=0.0023$) compared with PBS (**Figure 3.4**b). Treatment with 3% croton oil increased KLK5 expression 3.65 ± 2.21 fold ($p=0.0001$) over that seen in PBS treated skin, and this was also significantly higher than the levels observed following acetone treatment ($p=0.0197$). 5% SDS treatment increased KLK5 expression 5.45 ± 2.32 fold ($p=0.0002$) compared with PBS-treated controls.

When the mean KLK5 expression across the epidermis was calculated relative to KLK5 expression in PBS-treated skin, increases remained significant for acetone- ($p=0.0013$), 3% croton oil- ($p=0.0139$) and 5% SDS- ($p=0.0023$) treated skin (**Figure 3.4**a).

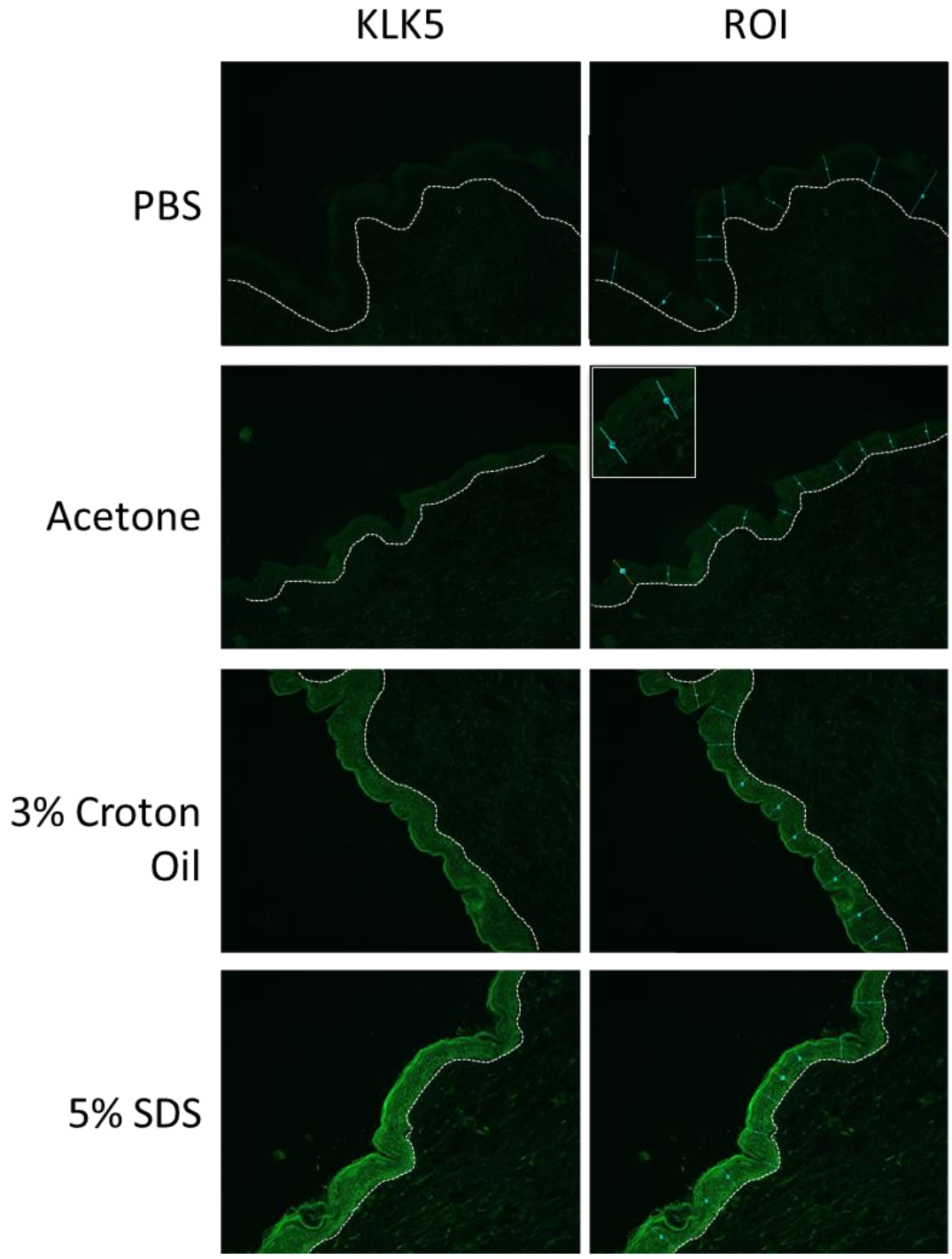


Figure 3.3: KLK5 expression is increased in the epidermis following application of irritants. Immunofluorescence staining of KLK5 shows minimal KLK5 expression in PBS-treated skin (upper left panel). Application of acetone for 30 minutes seemed to cause a mild increase in KLK5 expression, whereas 30 minutes application of 3% croton oil and 5% SDS resulted in a moderate and strong increase respectively in KLK5 expression in the epidermis. The white lines on the images indicate the basement membrane. The images shown are representative of at least 7 experiments and the blue lines on the epidermis on the right hand panels show the region of interest (ROI)(magnified in the second panel on the right) which were used for quantification in **Figure 3.4** and **Figure 3.5**.

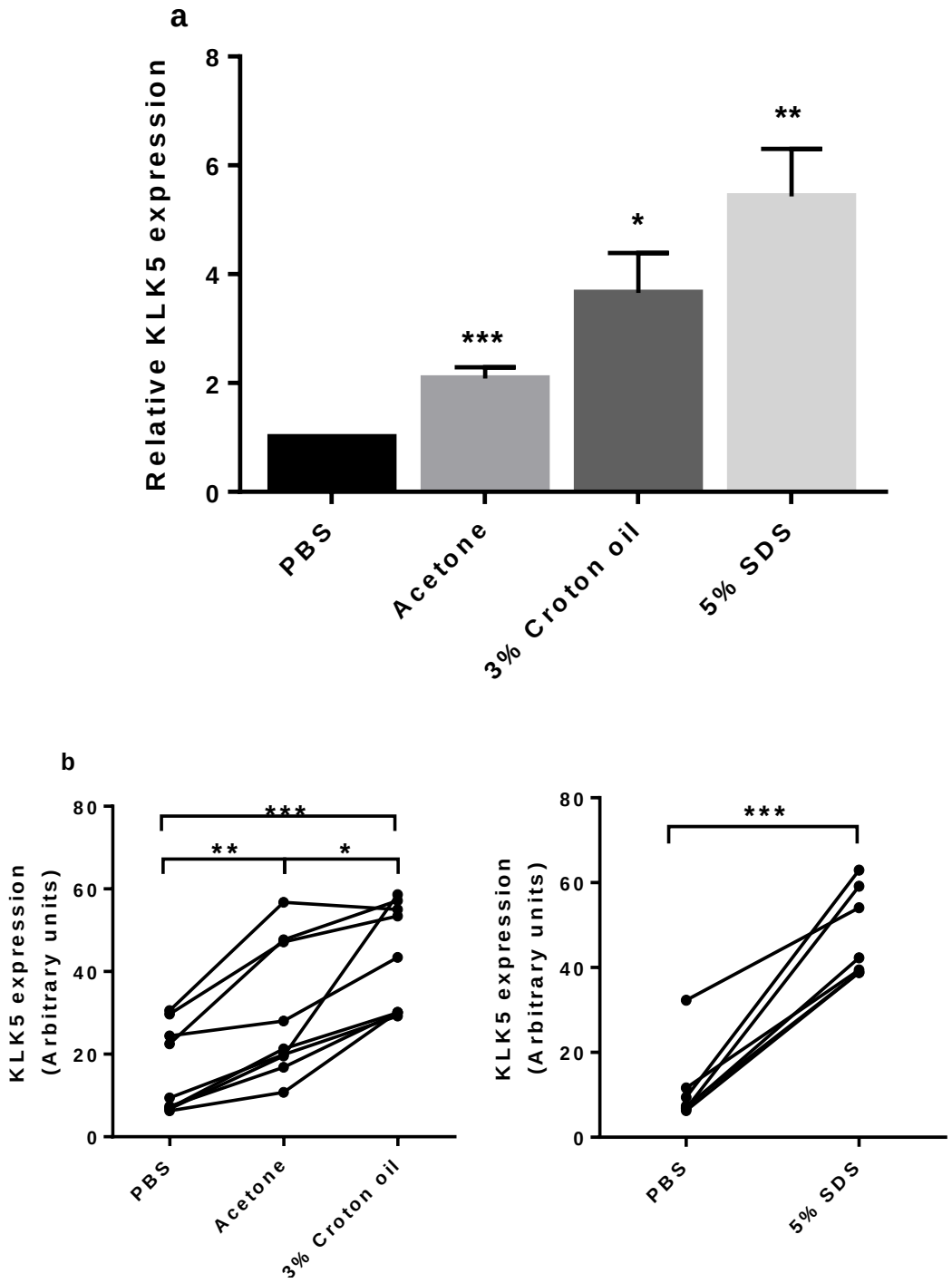


Figure 3.4: Changes in mean fluorescence of epidermis stained for KLK5 following 30 minutes treatment with PBS, acetone, 3% croton oil or 5% SDS. (a) Relative increase in KLK5 expression compared with PBS treated skin. Data shown as mean + SE from 9 patients for acetone and 3% croton oil treatment and 7 patients for 5% SDS treatment. (b) In all cases mean KLK5 expression is increased following acetone, 3% croton oil and 5% SDS as measured by pixel intensity using ImageJ software. There is a significant difference in KLK5 levels between both PBS and 3% croton oil treatments and between 5% SDS treated and PBS-treated skin (**p<0.01, ***p<0.001).

For a more detailed analysis of KLK5 expression within the epidermis the 10 measured ROIs were normalised to 100% and the mean pixel intensity was obtained for every 5% of the length. For each sample the mean intensities were taken for 1 field of view from 3 consecutive sections. The minimum average pixel intensity for a 5% section in the PBS sample was set at 1. All other readings for the sample set were calculated relative to this value. This was performed for each skin sample.

KLK5 expression was approximately equal throughout PBS-treated epidermis with a coefficient of variation of 5.9% across all the measured points. Acetone treated skin also shows little variation in expression, with a relative percentage standard deviation of 8.17%, and no individual point along the mean values from the ROIs showed a significant increase following acetone treatment compared with PBS ($p>0.05$, two-way ANOVA with Bonferroni correction). Conversely, although skin treated with 3% croton oil or 5% SDS for 30 min had a coefficient of variation of 12.60% and 12.25% respectively (**Figure 3.5**), the increase in KLK5 was significant at all points for 3% croton oil and 5% SDS allowing for multiple comparisons ($p<0.05$, two-way ANOVA with Bonferroni correction) (**Table 3.4**).

3.3.3 Effects of irritants on KLK7 expression

Generally, KLK7 expression appeared to be confined to the basal layer and lower stratum corneum/upper stratum granulosum (representative images shown in **Figure 3.6**). There was minimal variation of KLK7 expression between subjects in PBS-treated samples. In all cases of 5% SDS-treated skin, KLK7 expression appeared substantially higher than in PBS-treated skin samples; in acetone-treated skin KLK7 expression was moderately increased. Compared with PBS-, acetone- and 5% SDS-treated skin samples, 3% croton oil-treated skin showed more varied KLK7 expression between subjects, with 2 of 9 cases showing no apparent increase in KLK7 expression in compared with PBS-treated skin, and the remaining 7 were significantly increased.

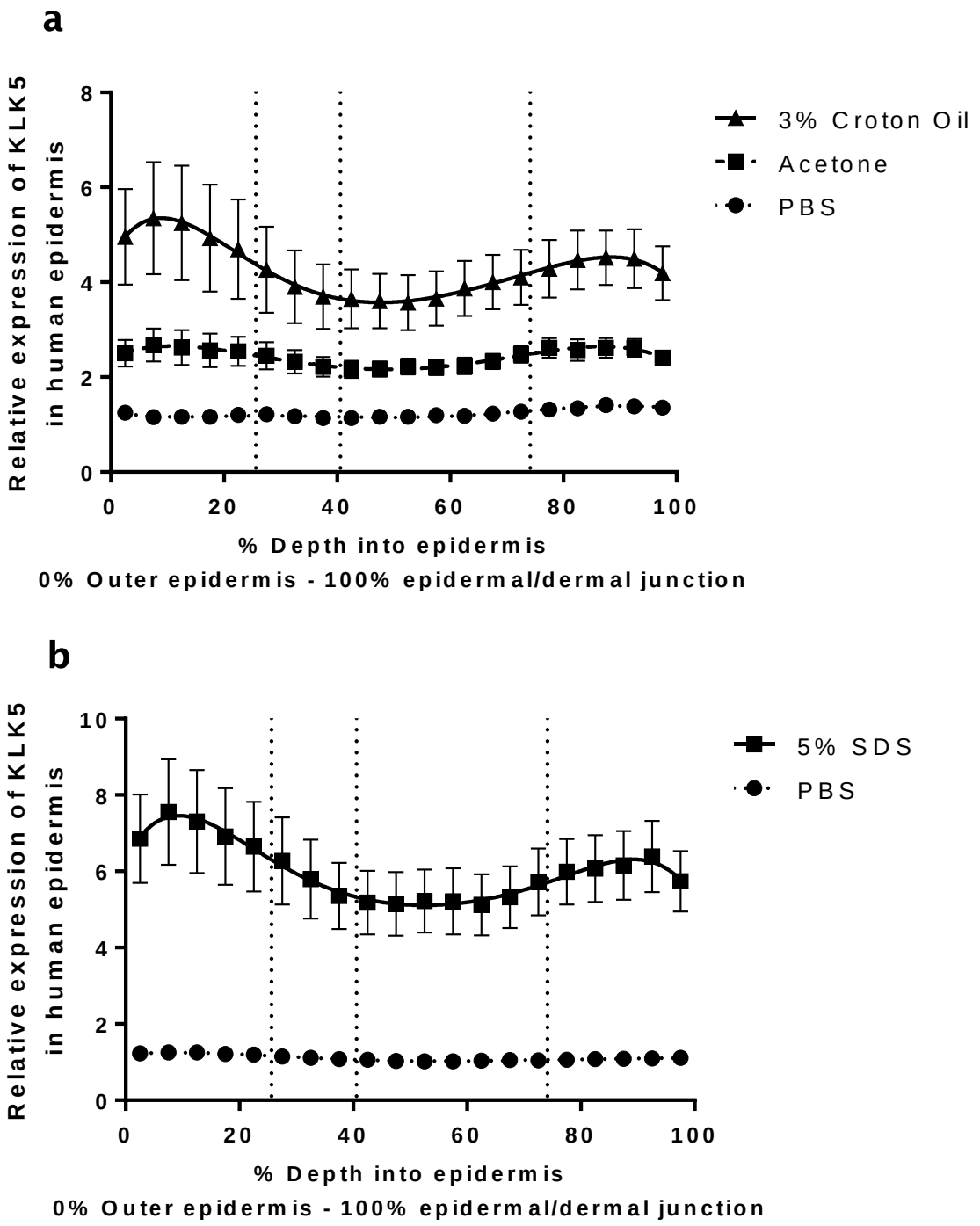


Figure 3.5: Relative expression of KLK5 in *ex vivo* human skin varies throughout the epidermis. Most expression of KLK5 is observed in the stratum corneum and stratum basale regions of the epidermis in skin treated with acetone, 3% croton oil (**a**) or 5% SDS (**b**). PBS-treated skin shows little variation in expression. Results are shown relative to the lowest area of expression within the PBS-treated sample. Vertical lines indicate junction between each epidermal layer.

Table 3.4: Changes in KLK5 expression in the epidermis following 30 minutes treatment with acetone, 3% croton oil or 5% SDS compared with PBS at each point allowing for multiple comparisons. KLK5 expression is not significantly altered in any epidermal layers following 30 minutes treatment with acetone. 30 minutes treatment with 3% croton oil or 5% SDS significantly increased KLK5 expression in all layers of the epidermis. **p<0.01, ***p<0.001, ****p<0.0001. Significance calculated using two-way repeated measures ANOVA with Bonferroni correction.

Epidermal layer	% Depth into epidermis	Acetone	3% croton oil	5% SDS
Stratum corneum	0-5	ns	****	****
	6-10	ns	****	****
	11-15	ns	****	****
	16-20	ns	****	****
	21-25	ns	****	****
Stratum granulosum	26-30	ns	****	****
	31-35	ns	***	***
	36-40	ns	**	***
Stratum spinosum	41-45	ns	**	**
	46-50	ns	**	**
	51-55	ns	**	**
	56-60	ns	**	**
	61-65	ns	**	**
	66-70	ns	***	***
	71-75	ns	***	***
Stratum basal	76-80	ns	***	****
	81-85	ns	****	****
	86-90	ns	****	****
	91-95	ns	****	****
	96-100	ns	***	***

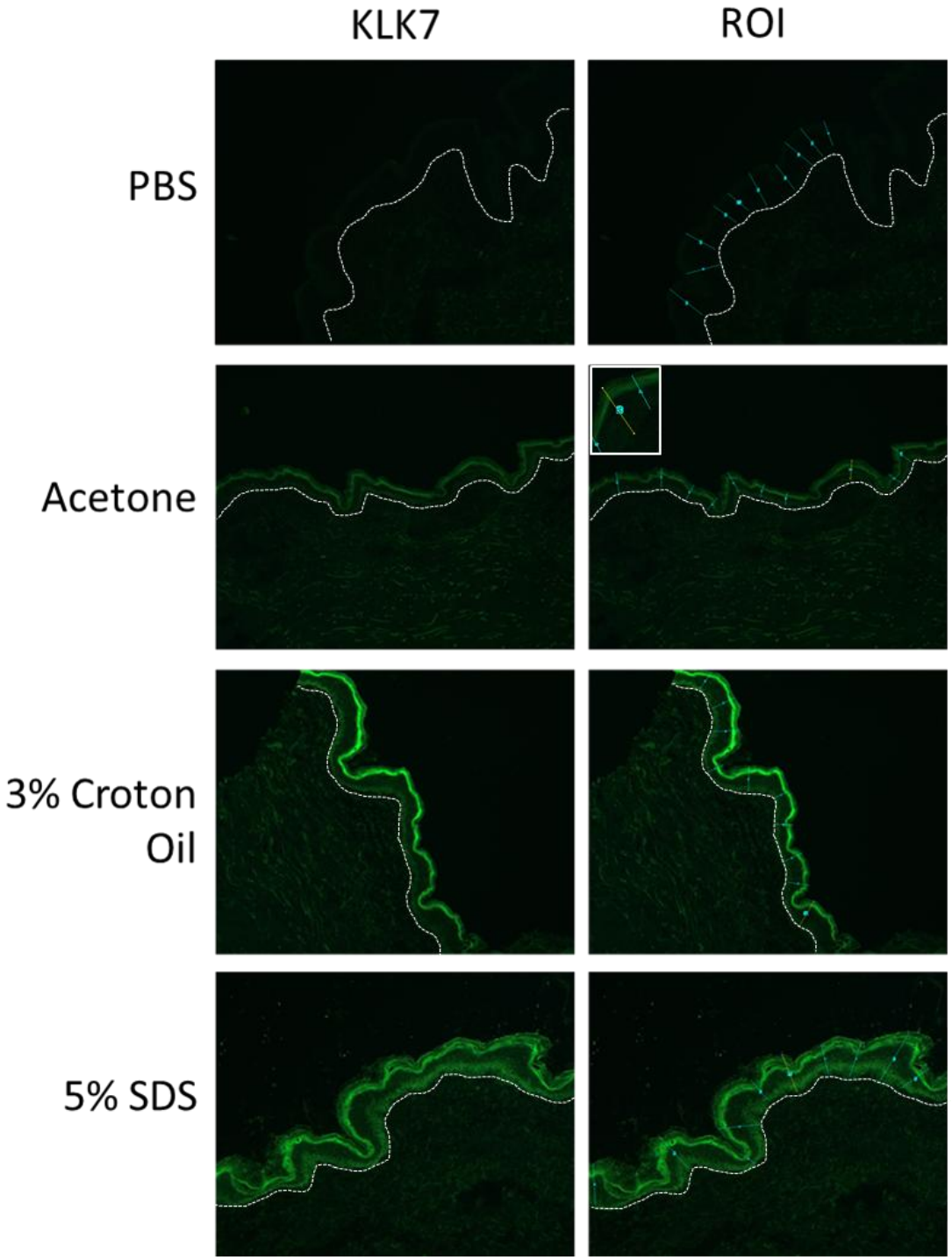


Figure 3.6: KLK7 expression is increased in the epidermis following application of irritants. Staining for KLK7 shows a visible increase in expression of KLK7 in the epidermis following 30 min treatment with 3% Croton Oil or 5% SDS. Very little KLK7 expression was observed in PBS treated skin. Application of acetone appeared to cause a moderate increase in KLK7 expression at the stratum granulosum/stratum corneum junction, while 3% croton oil and 5% SDS treatment both caused a significant increase in KLK7 expression in the stratum basale and stratum granulosum/stratum corneum junction. The white lines on the images indicate the basement membrane. The images shown are representative of at least 7 experiments and the blue lines on the epidermis on the right hand panels show the region of interest (ROI) (magnified in the second panel on the right) which were used for quantification in **Figure 3.7** and **Figure 3.8**.

Analysis of the mean fluorescence across the epidermis by repeated measures one-way ANOVA showed that acetone-treated skin has significantly increased overall KLK7 expression compared with PBS-treated skin (1.61 ± 0.38 fold, p=0.0011). Following application of 3% croton oil for 30 minutes, total KLK7 expression was increased 2.37 ± 0.90 fold (p=0.0027) and 2.97 ± 1.30 fold following application of 5% SDS (p=0.0009, 2-tailed paired t-test) (**Figure 3.7**). 3% croton oil-treated skin has significantly increased KLK7 expression compared with acetone-treated skin (p=0.0351).

As for KLK5 expression, KLK7 expression remained significantly increased when calculated relative to KLK7 expression in PBS-treated samples following acetone- (p=0.0028), 3% croton oil- (p=0.0036) and 5% SDS- (p=0.0072) treatments. Normalisation of results for KLK7 expression shows that in PBS-treated epidermis the relative percentage standard deviation is 16.51% across all the measured points. Acetone-treated skin samples show increased variation in expression with a relative percentage standard deviation of 22.57% compared with PBS-treated skin. This increase in variation is also observed in skin treated with 3% croton oil or 5% SDS for 30 min which has a coefficient of variation of 23.95% and 23.22% respectively (**Figure 3.8**).

Localisation of the expression showed that KLK7 levels are at their minimum within the outer surface of the epidermis and peak at the stratum granulosum-stratum corneum junction, decreasing back to minimum levels in the middle of the stratum spinosum. In the stratum basale, levels are approximately 50% of maximum. In 5% SDS treated skin, KLK7 levels are at 50% maximum at the outer layer of the stratum corneum then follow the pattern described for all other treatments (**Figure 3.8**).

In contrast to the results for KLK5 expression, 30 min acetone treatment causes a significant increase in KLK7 expression at the stratum corneum/stratum granulosum junction, between 16-35% into the outer epidermis, compared with PBS treated skin ($p<0.05$, two-way ANOVA with Bonferroni correction). The increase is significant at all points for 3% croton oil ($p<0.05$, two-way ANOVA with Bonferroni correction), and at all points in the stratum corneum, stratum granulosum and stratum basale for skin treated with 5% SDS (**Table 3.5**).

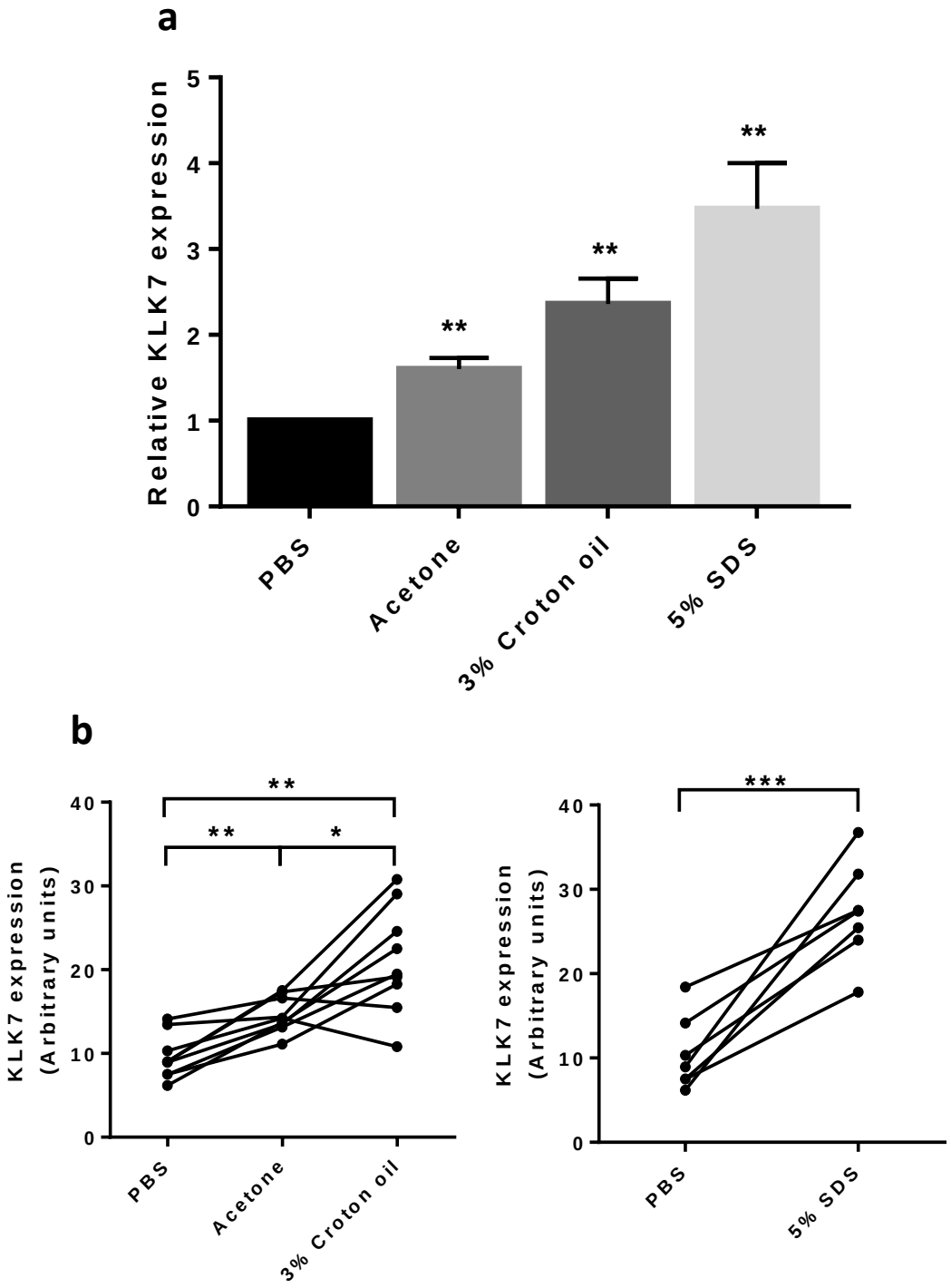


Figure 3.7: KLK7 expression is increased in human epidermis following application of irritants for 30 minutes. (a) Relative increase in KLK7 expression compared with PBS treated skin. Data shown as mean + SE from 9 patients for acetone and 3% croton oil treatment and 7 patients for 5% SDS treatment. (b) Mean KLK7 expression is increased in all cases following 30 minutes application of acetone or 5% SDS, and in 8 of 9 cases following treatment with 3% croton oil compared with PBS-treated skin. There is a significant difference in KLK7 levels between PBS and acetone (n=9) and 3% croton oil treatments (n=9), and in KLK7 levels between PBS-treated and 5% SDS-treated skin (n=7). **p<0.01, ***p<0.001. KLK7 expression measured as green pixel intensity using ImageJ software.

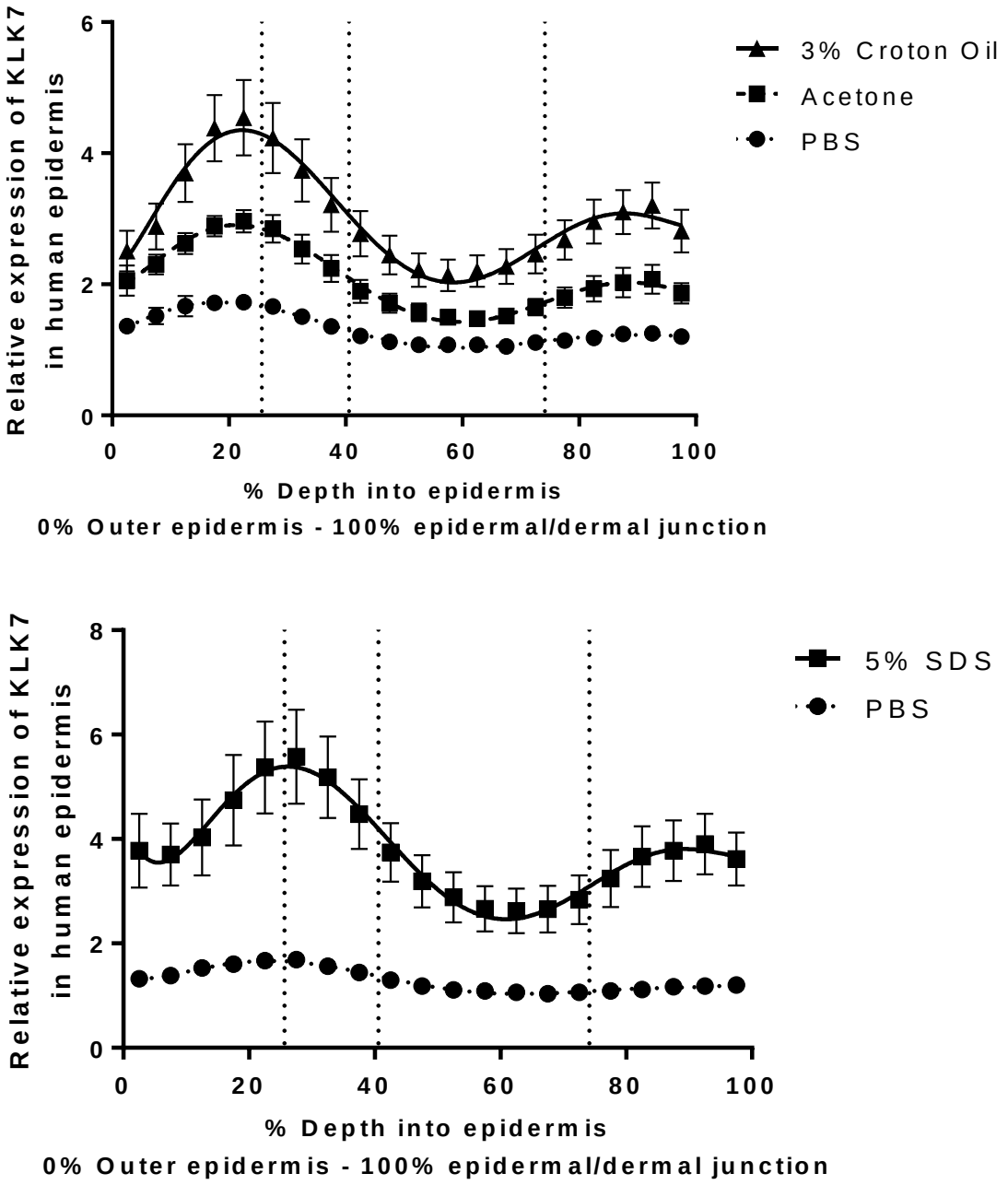


Figure 3.8: KLK7 expression varies throughout the epidermis in *ex vivo* human skin. In PBS, acetone, 3% croton oil (**a**) and 5% SDS-treated (**b**) skin KLK7 expression is highest in the stratum corneum/stratum granulosum junction and the stratum basale. Results are shown relative to the lowest area of expression within the PBS-treated sample. Vertical lines indicate junction between each epidermal layer.

Table 3.5: Changes in KLK7 expression in the epidermis following 30 minutes treatment with acetone, 3% croton oil or 5% SDS compared with PBS at each point allowing for multiple comparisons. 30 minutes treatment with acetone causes a significant increase in KLK7 expression between 16-35% into the epidermis, an area covering the stratum corneum/stratum granulosum junction. KLK7 expression is significantly increased at all points following 30 minutes treatment with 3% croton oil. KLK7 expression is significantly increased in the stratum corneum, stratum granulosum and stratum basale following 30 minutes treatment with 5% SDS. *p<0.05, **p<0.01, p<0.001, ****p<0.0001. Significance calculated using two-way repeated measures ANOVA with Bonferroni correction.

Epidermal layer	% Depth into epidermis	Acetone	3% croton oil	5% SDS
Stratum corneum	0-5	ns	*	**
	6-10	ns	***	**
	11-15	ns	****	**
	16-20	**	****	****
	21-25	**	****	****
Stratum granulosum	26-30	**	****	****
	31-35	*	****	****
	36-40	ns	****	****
Stratum spinosum	41-45	ns	****	**
	46-50	ns	**	*
	51-55	ns	*	ns
	56-60	ns	*	ns
	61-65	ns	*	ns
	66-70	ns	**	ns
	71-75	ns	***	ns
Stratum basal	76-80	ns	****	*
	81-85	ns	****	**
	86-90	ns	****	**
	91-95	ns	****	***
	96-100	ns	****	**

3.3.4 Zymography of irritant treated skin

In order to determine whether the observed increases in KLK5 and KLK7 expression might have led to an increase in protease activity, the epidermis was separated from the dermis and the protein extracted. Gelatin zymography was performed wherein the proteins were separated by electrophoresis and active proteases allowed to digest the gelatin in the gel. Active proteases appear as clear areas in a blue gel and can be identified from their molecular size in comparison with known proteases. Optimisation was performed using recombinant KLK5 and KLK14 (R&D Systems). Recombinant KLK5 and KLK14 are both produced in the pro-KLK forms which have no enzymatic activity. To confirm this, inactivated recombinant KLK5 and KLK14 were applied to the gels in a serial doubling dilution series starting at 1000 pg/well. No activity was detected at any tested concentration for KLK5 or KLK14 (data not shown). Following self-activation of KLK5 overnight at 37°C, activity was detected at quantities as low as 125pg (**Figure 3.9**). Attempts to self-activate KLK14 were not successful resulting in no bands of activity (data not shown), and as the molecular weights of active KLK5 (25 kDa) and active KLK14 (26 kDa) are similar, it was not possible to determine how much activity can be attributed to each protease following the use of KLK5 to activate recombinant KLK14.

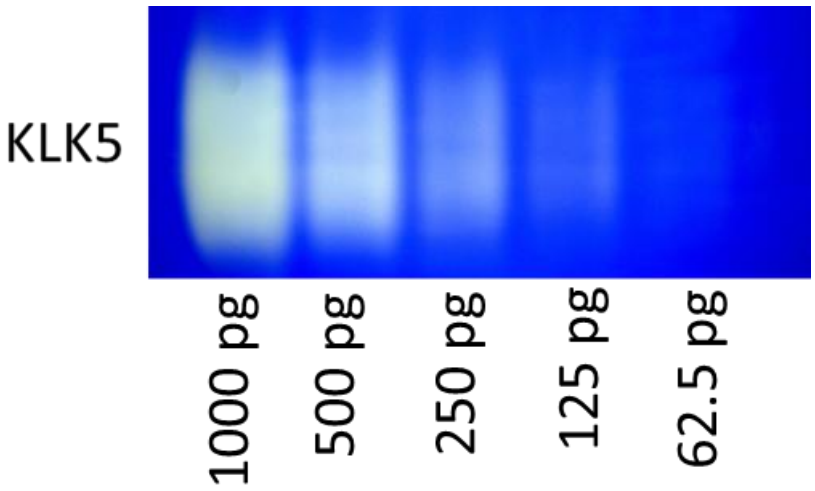


Figure 3.9: Standard zymography using activated recombinant KLK5 at dilutions from 1000 pg to 62.5 pg. Recombinant KLK5 activity was detected at levels as low as 125 pg. Image representative of n=2.

In order to examine KLK5 expression in human skin, skin samples were heated at 56°C for 5 min to facilitate mechanical separation of the epidermis from the dermis. This heating process may cause proteins to become degraded and lead to a loss of protease function. To see whether KLK5 remains active following this extraction process, activated recombinant KLK5 was heated to 56°C for 5 min prior to conducting gel zymography. Analysis of the gel using ImageJ software showed that compared with unheated recombinant KLK5 there was a 34% loss of activity following the heating process (**Figure 3.10**). The lowest concentration at which heated KLK5 was definitely detectable was 250 pg compared with 125 pg for untreated KLK.

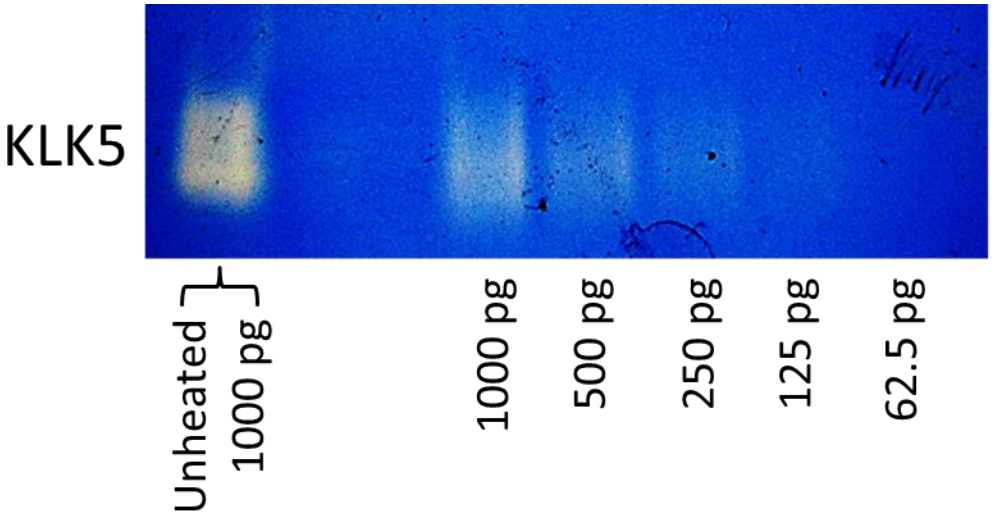


Figure 3.10: Heating protein samples at 56°C decreases KLK5 activity in gelatin zymography. There is a 34% loss of activity following the heating at 56°C for 5 min. Activity of 1000 pg sample is 34% lower than that of the unheated sample (n=2).

The concentration of KLKs in the skin is relatively low compared with other proteases, (e.g. MMP2) (Komatsu et al., 2006). In order to test whether it would be possible to detect KLKs from epidermal samples using standard gelatin zymography, protein was extracted from a piece of skin approximately 3 cm^2. A second similar size piece of skin was split into dermal and epidermal components and the proteins extracted from each. Total protein extracted was determined by bicinchoninic acid assay and serial doubling dilutions made from a starting concentration of 40 µg/10 µl in PBS. Some protease activity was detected in whole skin, dermal and epidermal sections from as

little as 5 µg protein, however all detected activity was at a higher molecular weight than that expected for active KLK's (KLK5 25 kDa, KLK7 28 kDa, KLK14 26 kDa) (**Figure 3.11**). 2 distinct bands of protease activity were detected, the larger of which (approximately 70 kDa) was present in epidermal and, to a lesser extent, whole skin samples, and the smaller of which (approximately 60 kDa) was present in the dermal and whole skin samples. KLKs are not reported to form dimers, therefore these bands most likely correspond to pro and active forms of MMP-2 (72 kDa and 66 kDa respectively), which is constitutively and abundantly expressed in the dermis and epidermis of elderly skin (Ashcroft et al., 1997).

No obvious KLK activity was seen in the skin (**Figure 3.11**), possibly due to a lack of sensitivity of zymography when relatively small amounts (3 cm^2) of skin, dermis or epidermis are used. Furthermore, the *ex vivo* biopsies used earlier in the irritant experiments were 0.28 cm^2, and it was considered necessary to continue with this 0.28 cm^2 size in order to be able to test enough samples. Therefore, it was determined that standard zymography methods were not suitable for detecting KLK activity in this model.

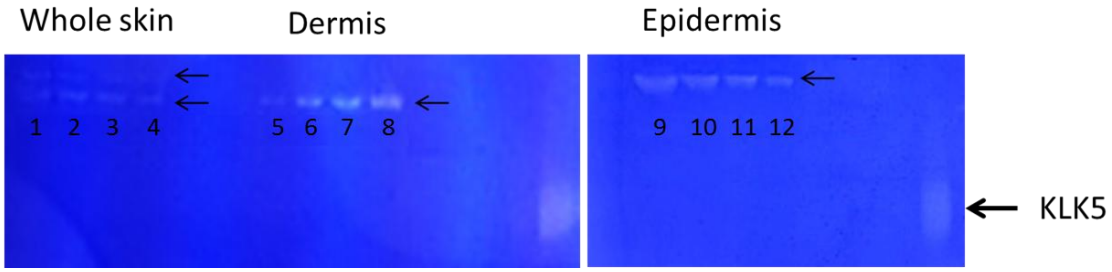


Figure 3.11: Proteolytic activity of split skin. A serial dilution of protein extracts from whole skin, dermal and epidermal skin samples were analysed by gelatin zymography. No KLK activity (25 kDa) was observed for any of the samples. Activity was observed at approximately 70 kDa and 60 kDa in whole skin preparation (black arrows), 60 kDa in dermal preparation and 70 kDa in epidermal preparations. Lanes are marked as follows: Whole skin **Lane 1** 40 µg, **Lane 2** 20 µg, **Lane 3** 10 µg, **Lane 4**: 5 µg; Dermis **Lane** 5 µg, **lane 6** 10 µg, **lane 7** 20 µg, **lane 8** 40 µg; Epidermis **Lane 9** 40 µg, **Lane 10** 20 µg, **Lane 11** 10 µg, **Lane 12**: 5 µg protein loaded per well. Black arrows mark areas of activity. Image representative of n=3 experiments using at least 40 µg protein.

3.3.5 *In situ* gelatin zymography of irritant treated skin

Due to the insensitivity of the standard gelatin zymography method to the KLK proteases in skin samples, and the inability to use this method to identify the precise location of protease activity within the epidermis, attempts were made to create a more sensitive method that also allowed localisation of protease activity within the skin samples.

Using an *in situ* gelatin zymography method to increase the sensitivity of the assay allowed the application of a very thin (<0.05 mm) zymography gel onto full thickness skin sections to localise the activity to specific regions of the skin. Initial experiments were performed on skin treated with 3% croton oil for 30 or 120 min and untreated control skin. Because the tissue section must be removed before staining it is not possible to determine conclusively the location of the activity in relation to the layers of the skin, however transfer of cellular material indicated the edge of the tissue sample allowing estimation of the epidermal region (referred to herein as 'epidermis').

Visual assessment of the slides indicated activity in all the samples as assessed by clear areas on a blue background, and microscopic assessment showed that this activity was mostly accounted for by discrete spots in the dermis. Assessment was performed following blinding of the images. The 'epidermis' of the untreated sample showed minimal activity compared with a diffuse pattern of activity in the 'epidermis' of skin treated for 30 and 120 min (**Figure 3.12**a)

In order to better visualise the areas of activity a binary threshold was set reducing background activity to approximately 5%. These images show a clear increase in activity in the 'epidermis' after treatment with 3% croton oil. After treatment of skin with 3% croton oil there was a time dependent increase in protease activity. This was apparent at 30 minutes, but was much greater at 120 minutes (**Figure 3.12**a).

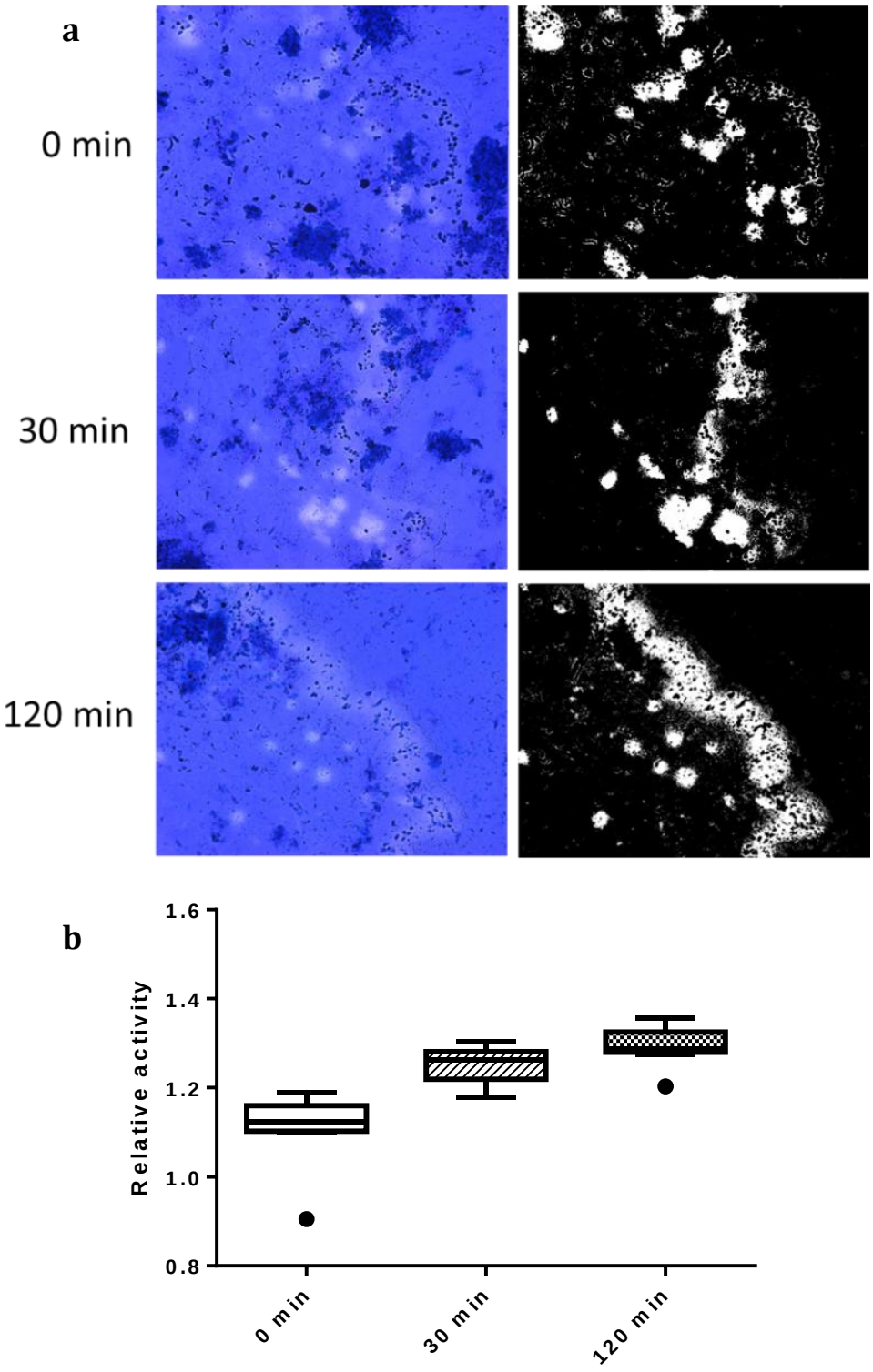


Figure 3.12: Protease activity in human skin as assessed by gelatin *in situ* zymography. (a) *in situ* gelatin zymography of samples treated with 3% croton oil. A lack of markers from the tissue mean it is not possible to localise the activity to specific regions. A binary threshold was set to reduce background activity to approximately 5%. (b) Activity in proposed epidermal region relative to background levels. Images are shown at x200 magnification and are representative of 3 separate experiments.

In order to quantify the activity, pixel intensity was taken for 10 random points within the proposed epidermis and the mean calculated. This was repeated for 4 separate fields of view from 2 sections of each treatment and the relative change in density compared between the 3 treatments.

Analysis of the images indicated that skin treated with 3% croton oil for 30 and 120 min has increased protease activity in the proposed epidermal region compared with the untreated skin (**Figure 3.12**b). However, this method proved to have several problems which prevented accurate assessment of the protease activity in epidermis. Following removal of the tissue, cellular, possibly nuclear, material from the skin section was retained on the gelatin slide, which was stained with Coomassie blue (**Figure 3.13**a). This distorted the view of the protease activity as the Coomassie blue stains the retained proteins and may be concealing an area of activity in the gel below it. This occurred to a much greater extent in samples treated with 5% SDS than in those treated with 3% croton oil (**Figure 3.13**a). When analysing these slides the spots of protein retention were avoided. There was an increase in protease activity after 30 min and 120 min treatment compared with PBS-treated controls. Contrary to the results of 3% croton oil treated skin, there was a decrease in activity between the 30 and 120 min treatments (**Figure 3.13**b). Furthermore, the activity detected may be attributed to MMP2 activity, as it was previously observed that the only activity detected using standard gelatin zymography was at the size expected for MMP2 (**Figure 3.11**)

When using this method of zymography, the tissue is removed before staining, therefore, it is not possible to determine accurately the location of the epidermis. In order to localise the activity to specific regions within the skin, graphite granules were incorporated into the poly-acrylamide gel prior to application to the slide in order to provide landmarks within the gel. Following overnight incubation, the tissue was imaged before removal by washing with PBS (**Figure 3.14**a). These images were used as guides when imaging the stained gels (**Figure 3.14**b). The outer surface of the epidermis and the basement membrane were outlined on the tissue image (**Figure 3.14**c). The outlined tissue and original stained gel images were overlaid and aligned using the graphite markers as guides. The outline was transferred to the stained gel image to demarcate the epidermis (**Figure 3.14**d).

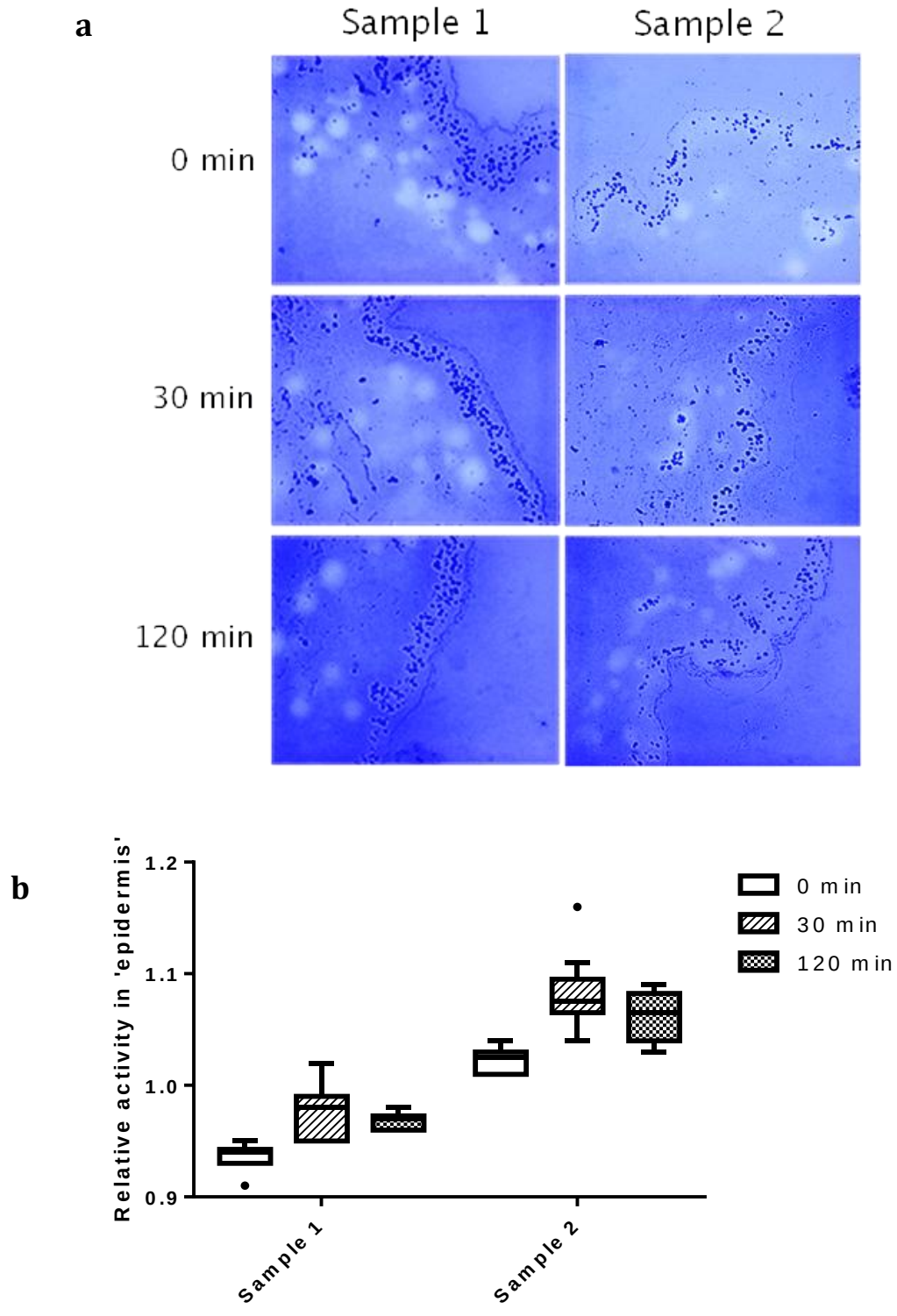


Figure 3.13: Effect of 5% SDS treatment on protease activity detected by *in situ* gelatin zymography. a) *in situ* gelatin zymography of samples treated with 5% SDS for 0, 30 and 120 mins. Following removal of tissue, cellular material has been retained on the gel distorting the activity profile. **b**) Activity in proposed epidermal region relative to background staining levels. 30 min 5% SDS-treated skin has increased activity in the 'epidermis' compared with PBS-treated skin. Images are shown at x200 magnification and are representative of 4 separate experiments.

Results from graphite-gels appeared to show a similar pattern of activity as that seen in previous attempts using graphite-free gel, confirming that the diffuse activity is localised the epidermis with areas of concentrated activity in the dermis. These areas did not appear to be blood vessels or hair follicles, and as it was not possible to perform histological staining of the tissue it cannot be determined whether these areas correspond to leukocytes such as macrophages, dendritic cells or Langerhans cells in the dermis.

It was found that the inclusion of graphite into the poly-acrylamide gel caused a change in the setting properties of the gel, leading to an alteration in the amount of water that was incorporated into the gel. The acrylamide polymerised around the graphite granules, therefore an increase in the concentration of graphite granules led to a decrease in the amount water that was incorporated into the gels causing a change in the density of the gel which could not be quantified.

Titration of the graphite was performed to establish the concentration of graphite at which the gel setting was not affected. It was concluded that in order for there to be no apparent change in gel concentration, determined by the incorporation of water into the gel, graphite granules were too thinly spread throughout the gel to be effective as landmarks.

3.3.6 *In situ* fluorescence zymography of irritant-treated skin tissue

It has been demonstrated in several studies that the Enzchek kit from Invitrogen, designed for detection of serine protease in supernatant samples, can be adapted for *in situ* zymography (Hachem et al., 2006a; Yamasaki et al., 2007; Hachem et al., 2009). Use of this FITC-conjugated casein substrate allows accurate localisation of the activity as the tissue is retained during imaging.

Confirmation of the accuracy and sensitivity of this method was performed using neutrophils which had been activated to varying levels. Several chemo-attractants, including FMLP, LPS and IL-8, are known to activate neutrophils, inducing a virtually instantaneous change in shape (Watts et al., 1991; Baggiolini and Clark-Lewis, 1992; Baggiolini and Kernen, 1992). Activity status was determined by flow cytometry and compared with protease activity measured by EnzChek *in situ* zymography.

The protease activity shown by the EnzChek zymography was proportional to the level of activation of the neutrophils indicated by the alteration in cell size. Protease activity was detected in 45% of control cells, 61% of FMLP activated cells, 71% of LPS activated cells and 100% of IL-8 activated cells (**Figure 3.15** and **Table 3.6**).

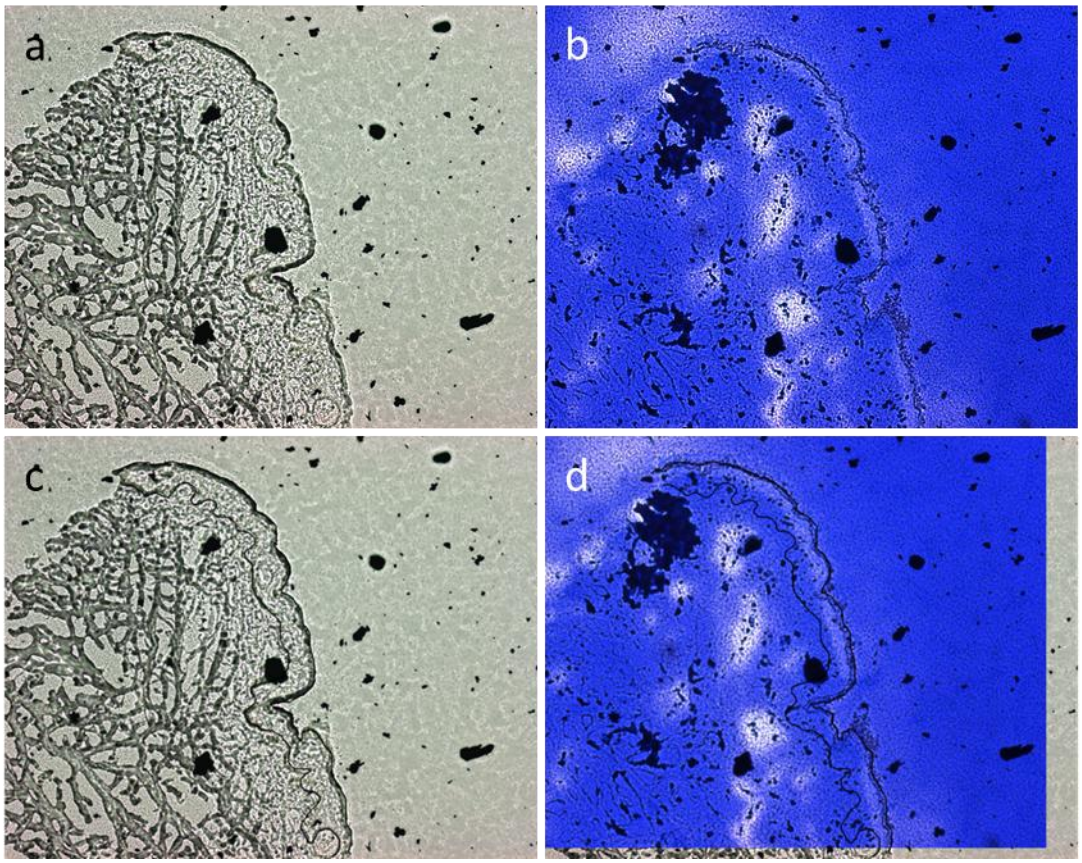


Figure 3.14: Inclusion of graphite granules in the zymography gel allowed localisation of the activity to specific areas of the tissue sample. **a**) Tissue was imaged prior to removal from the gel. **b**) Original image was used to guide imaging of stained gel. **c**) Epidermis was outlined on tissue image. **d**) Graphite granules were used to align stained, tissue free image with original tissue image and the epidermis outline overlaid onto the stained image. Images are shown at x200 magnification and are an example of 6 experiments.

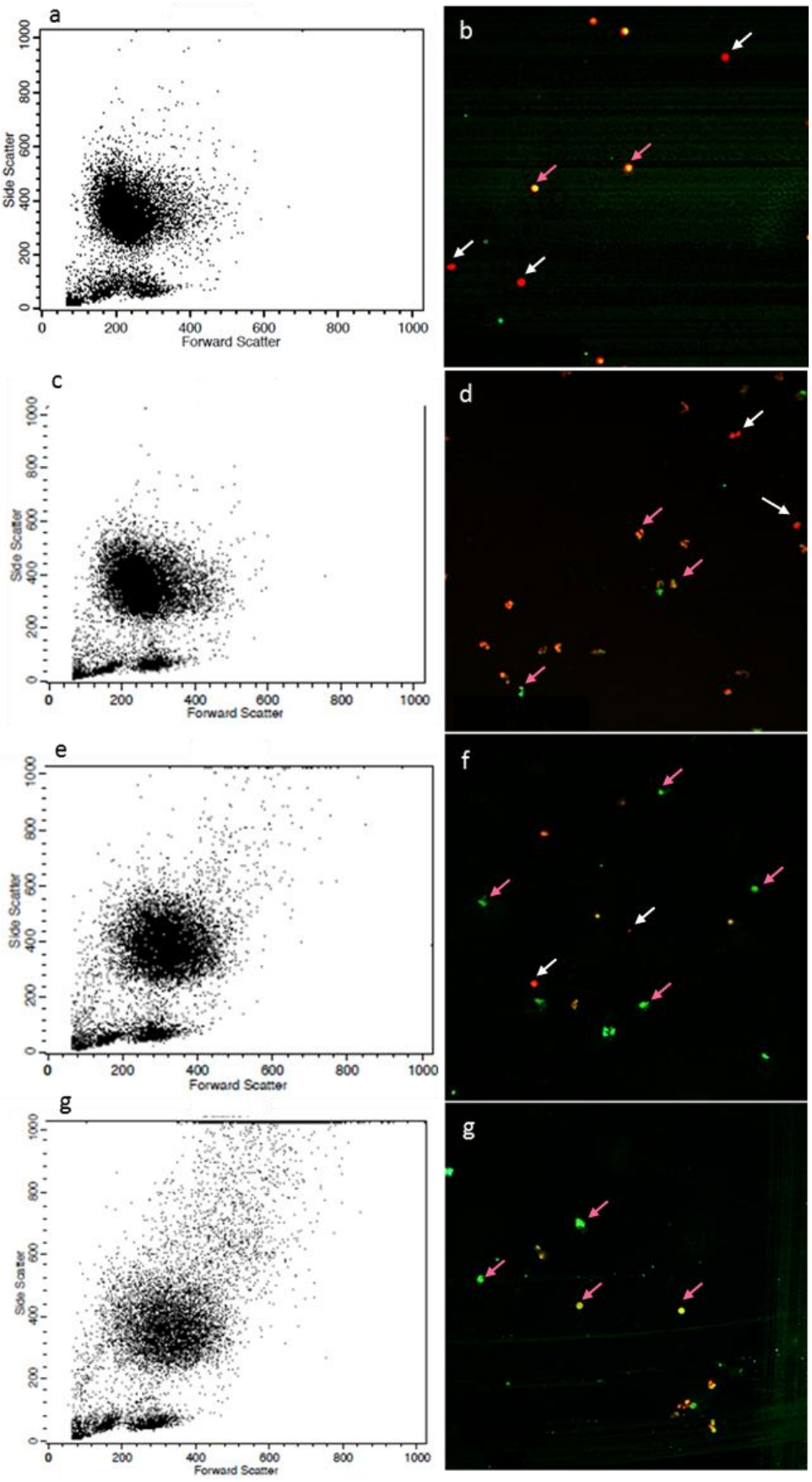

a
b
c
d
e
f
g
g
Side Scatter
Forward Scatter
0
200
400
600
800
1000

Figure 3.15: FACS analysis and *in situ* zymography of resting neutrophils (a+b), and following activation with 1 µg/ml FMLP (c+d), 1 ng/ml LPS (e+f) and 5 nM IL-8 (g+h) (previous page). Activation of neutrophils is indicated by a shift in the FSC and SSC into the top right quadrant (a, c, e, and g). Increasing activation is reflected in increased protease activity measured by EnzChek *in situ* zymography (b, d, f and g). Green indicates protease activity and nuclear material is stained with TOPRO-3 (red). Resting neutrophils are highlighted with white arrows, activated neutrophils are highlighted with pink arrows. Representative of 4 slides per treatment from 2 experiments.

Table 3.6: Increase in neutrophil activation as measured by increased mean forward scatter (FSC) leads to an increase in protease activity as measured by EnzChek. Activity was determined as green fluorescence associated with a nucleus.

Treatment	Mean FSC	% Activity
Control	69.22	45.6
1 µg/ml FMLP	265.57	60.7
1 ng/ml LPS	323.40	70.6
5 nM IL-8	340.19	100.0

Initial microscopic observations showed a wide variation in protease activity between PBS-treated skin samples between, and in 2/8 cases within, subjects. In all cases there was a clearly observable increase in activity around the stratum corneum / stratum granulosum junction following acetone, 3% croton oil or 5% SDS treatment (**Figure 3.16**). An increase was also observed in the lower layers of the epidermis (stratum spinosum and stratum basale), however this was not pronounced in all cases.

Comparison of enzyme activity using raw fluorescence values showed that Acetone ($P=0.0137$) and 3% croton oil ($P=0.0209$) cause a significant increase in protease activity compared with PBS treatment (paired t-test). The increase following treatment with 5% SDS ($n=6$) just failed to reach significance ($p=0.0516$) (**Figure 3.17**). When enzyme activity was normalised to PBS values, acetone showed an almost significant increase in enzyme activity ($p=0.0503$), while 3% croton oil ($p=0.0094$) and 5% SDS treatment ($p=0.0185$) both caused a significant increase in protease activity (**Figure 3.17**).

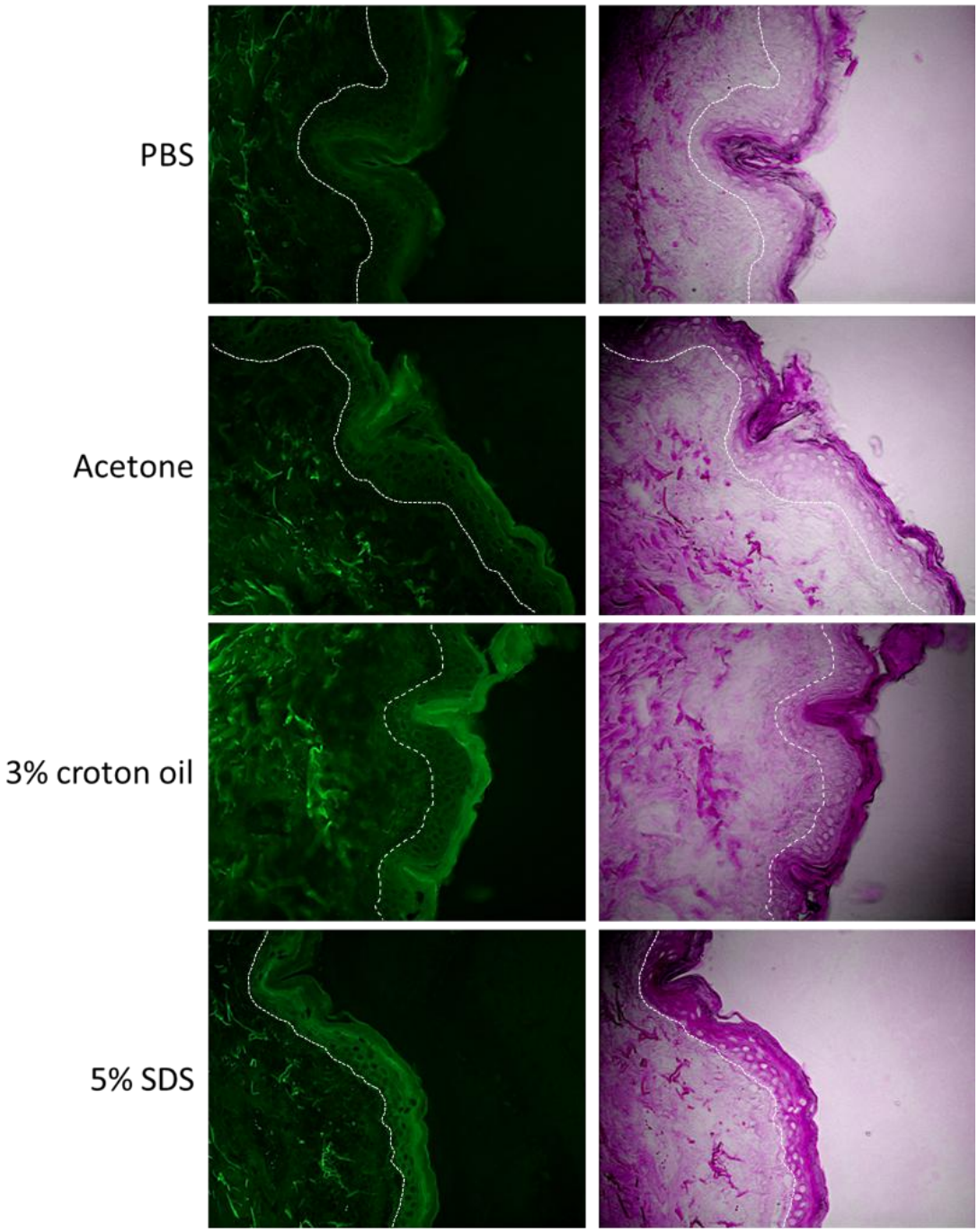


Figure 3.16: An example of protease activity following 30 min epicutaneous application of irritants as detected by fluorescence *in situ* zymography. Protease activity is indicated by green staining. Activity is primarily detected in the stratum corneum of PBS-treated epidermis. Acetone-treated skin shows a similar pattern of staining to PBS-treated skin, whereas 3% croton oil- and 5% SDS-treated skin both show increased protease activity throughout the epidermis. The staining has been overlayed onto the brightfield image of the skin and the colour changed to purple for better visualisation. The white lines on the images indicate the basement membrane. Examples of PBS n=8, acetone n=5, 3% croton oil n=6, 5% SDS n=6.

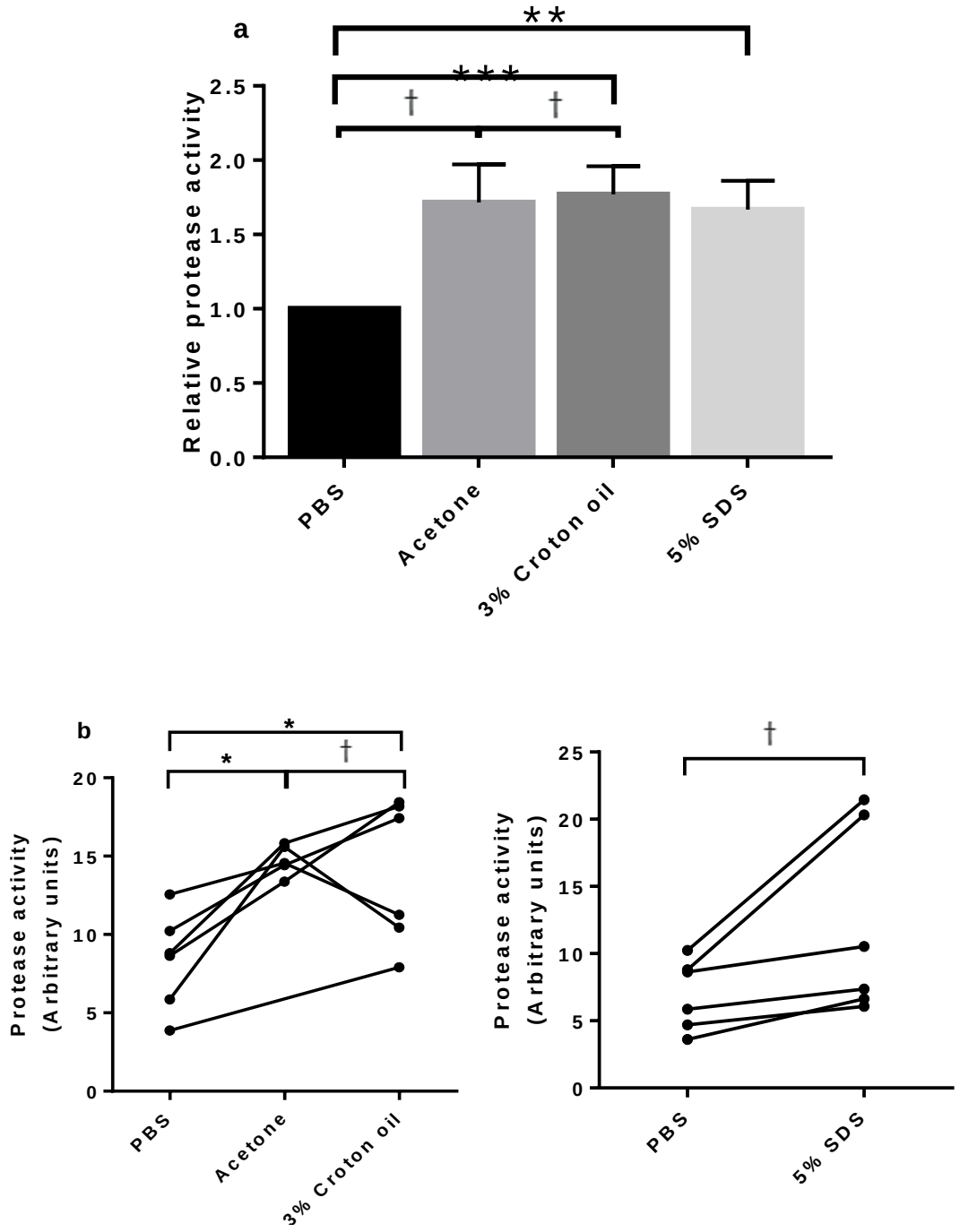


Figure 3.17: Increases in protease activity as measured by Enzchek following 30 min irritant treatment. a) Analysis of relative protease activity showed an increase in protease activity following application of 3% croton oil and 5% SDS, but no significant increase following acetone treatment. b) In all cases activity is increased following acetone and 5% SDS. In 5/6 cases activity was increased following treatment with 3% croton oil. There is a significant increase in protease activity between PBS and both acetone and 3% croton oil treatments. 5% SDS treatment does not cause a significant increase in protease activity compared with PBS. (* = $p<0.05$, † = NS; paired t-test).

The pattern of activity in untreated skin indicates an increased protease activity in the stratum corneum/stratum granulosum junction (**Figure 3.18**) compared to other regions within the epidermis. The initial pattern is similar to that observed for KLK7 expression (**Figure 3.8**), however activity declines steadily toward the stratum basale rather than increasing in the stratum basale as would be expected if protease activity followed the same pattern as that observed for KLK7.

Testing of multiple regions shows that most protease activity is located in the stratum granulosum, extending into the stratum spinosum in the case of skin treated with 5% SDS (**Table 3.7**). Some significant activity is also detected in the outer layers of the stratum corneum in skin treated with 5% SDS, however all significant activity is confined to the stratum granulosum and stratum corneum/stratum granulosum junction in skin treated with acetone and 3% croton oil respectively.

3.3.7 PAR2 activation

It is known that KLK5 is able to activate PAR2 by digesting the tethered ligand. Following the observation that KLK5 expression is upregulated following 30 min treatment with acetone, 3% croton oil or 5% SDS, the expression of PAR2 was investigated.

In keeping with most receptor biology, it would be expected that PAR2 expression would be downregulated following activation due to trafficking to the golgi and degradation. An antibody which binds PAR2 between extracellular regions 2 and 3, encompassing transmembrane domains 6 and 7 and intracellular region 3 was used to examine the expression of PAR2 following application of acetone, 3% croton oil or 5% SDS for 30 minutes. Irritants were applied to *ex vivo* human skin as before, the whole skin biopsy sectioned and PAR2 expression assessed by immunofluorescence staining (**Figure 3.19**).

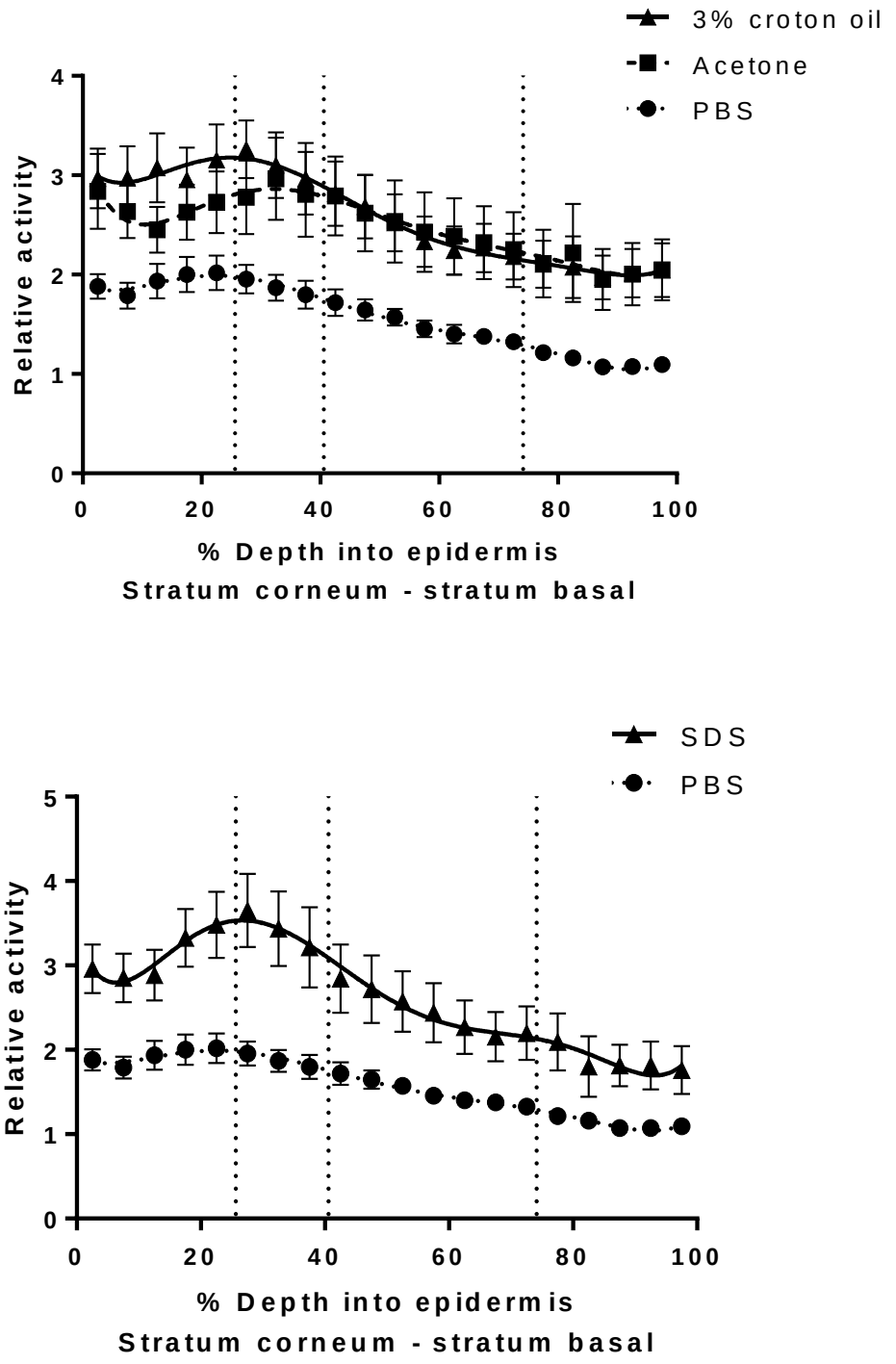


Figure 3.18: Relative protease activity in *ex vivo* human skin varies throughout the epidermis. Most protease activity is observed in the stratum corneum/stratum granulosum junction of the epidermis in skin treated with PBS, acetone, 3% croton oil (**a**) or 5% SDS (**b**). Protease activity was determined using *in situ* zymography, which fluoresces in the presence of protease activity. Results are shown relative to the lowest area of expression (as determined by intensity of fluorescence) within the PBS-treated sample. Vertical lines indicate junction between each epidermal layer.

Table 3.7: Significant change of protease activity compared with PBS at each point allowing for multiple comparisons of relative activity. 30 minutes treatment with acetone causes a significant increase in protease activity in a single section of the epidermis within the stratum granulosum. Treatment with 3% croton oil for 30 minutes increases protease activity at the stratum corneum/stratum granulosum junction. Similarly, 5% SDS treatment also increased protease activity at the stratum corneum/stratum granulosum junction, with the increase extending further into the stratum corneum, through the stratum granulosum and into the stratum spinosum. **p<0.01, p<0.001, ****p<0.0001, two-way ANOVA with Bonferroni correction.

Epidermal layer	% depth into epidermis	Acetone	3% croton oil	5% SDS
Stratum corneum	0-5	ns	ns	*
	6-10	ns	ns	*
	11-15	ns	ns	ns
	16-20	ns	ns	**
	21-25	ns	*	***
Stratum granulosum	26-30	ns	**	****
	31-35	*	*	****
	36-40	ns	ns	***
Stratum spinosum	41-45	ns	ns	*
	46-50	ns	ns	*
	51-55	ns	ns	*
	56-60	ns	ns	*
	61-65	ns	ns	ns
	66-70	ns	ns	ns
	71-75	ns	ns	ns
Stratum basale	76-80	ns	ns	ns
	81-85	ns	ns	ns
	86-90	ns	ns	ns
	91-95	ns	ns	ns
	96-100	ns	ns	ns

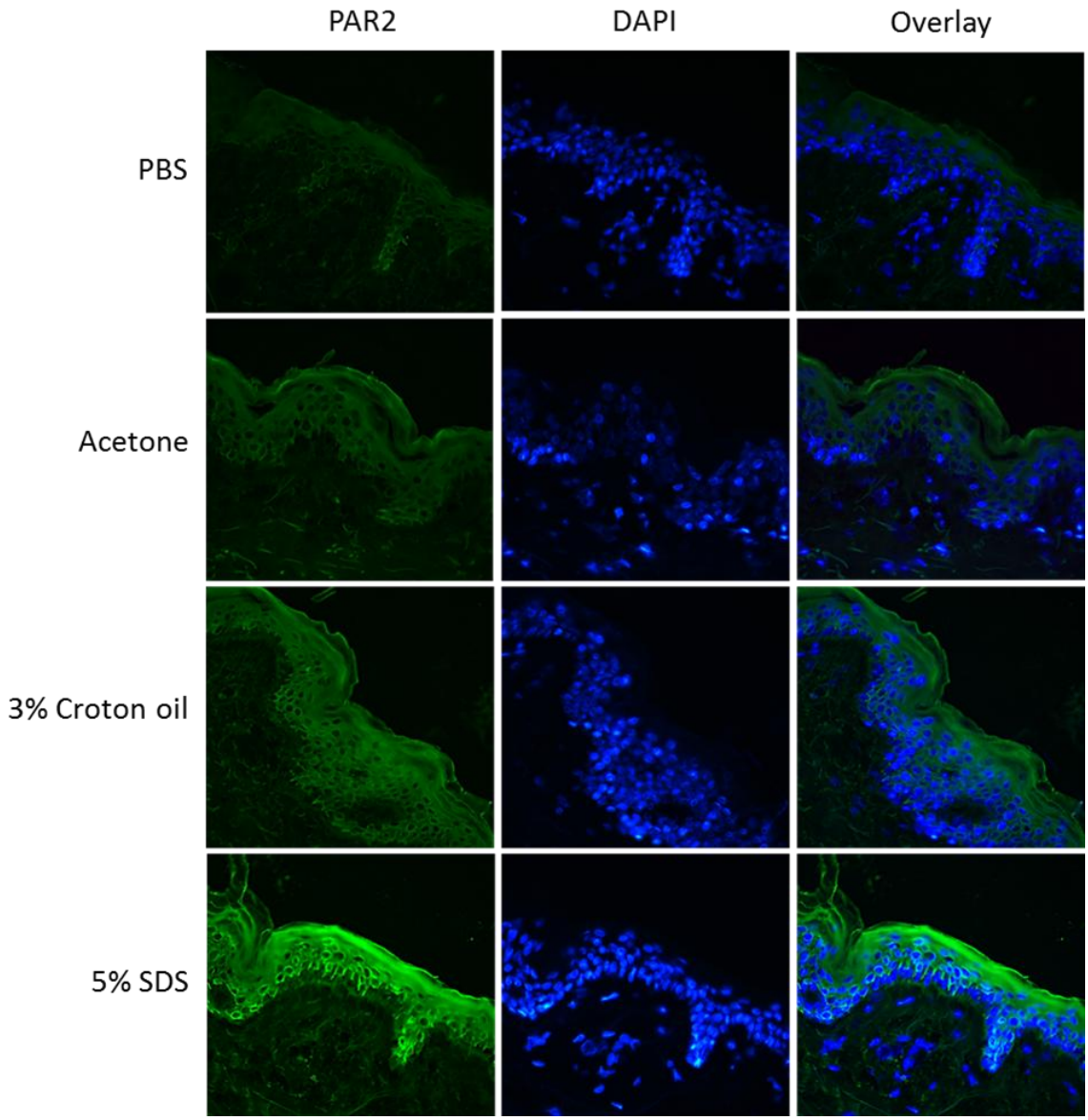


Figure 3.19: PAR2 expression following 30 minutes irritant application. Expression is increased across the entire epidermis, including the stratum corneum, following 30 minutes treatment with acetone, 3% croton oil and 5% SDS. While acetone- and 3% croton oil-treated skin showed moderately increased PAR2 expression, 5% SDS-treated skin appeared to have dramatically increased PAR2 expression across the entire epidermis in most cases. Example images from 6 experiments.

The level of PAR2 expression was analysed using the same method as used for quantifying KLK5 and KLK7 (I.e. 10 regions of interest measured and intensity of staining calculated across the epidermis) (**Figure 3.20**). Comparison of relative overall PAR2 expression showed that application of 3% croton oil causes a significant increase compared with PBS ($p=0.0319$). There was no significant increase in PAR2 expression following 30 minutes treatment with acetone ($p=0.1059$) or 5% SDS ($p=0.0748$) when normalised against PBS-treated samples. However, when raw fluorescence values were used for analysis, 5% SDS treated skin had significantly higher levels of PAR2 expression compared PBS treated samples ($p=0.0215$). 3% croton oil treated samples remained significantly increased compared with PBS treated samples when using raw fluorescence values ($p=0.0057$). Acetone made no difference to PAR2 expression when analysed using raw fluorescence values ($p=0.1514$).

PAR2 expression is most prominent in the stratum corneum, steadily decreasing toward the stratum basale (**Figure 3.21**). The same pattern of expression is observed following acetone, 3% croton oil and 5% SDS treatment as is observed in PBS-treated epidermis. However, in 3% croton oil and 5% SDS-treated skin, PAR2 expression is approximately 1.5 fold greater at all measured points.

Statistical comparison of multiple regions of the epidermis showed that PAR2 expression is significantly increased at all regions following 3% croton oil and 5% SDS treatment compared with PBS-treated skin (**Table 3.8**). 30 minutes treatment with acetone significantly increased PAR2 expression in the stratum basale through to the upper stratum spinosum/lower stratum granulosum. No difference in PAR2 expression was observed at the stratum corneum-stratum granulosum junction or upper stratum corneum following acetone treatment; however expression in the mid-stratum corneum was increased compared with PBS-treated skin.

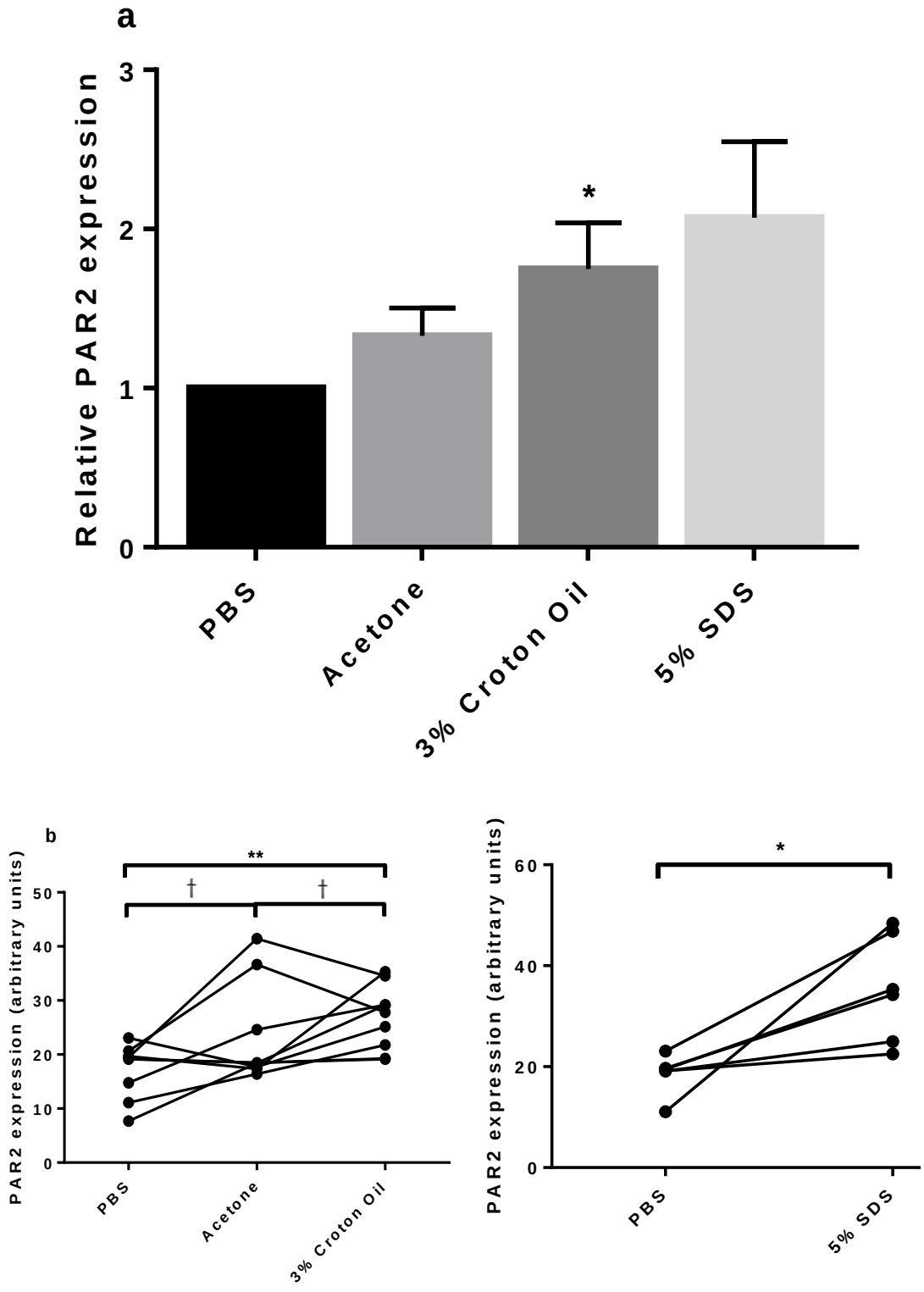


Figure 3.20: Overall PAR2 expression is increased following 30 minutes irritant application. A) Relative PAR2 expression across the entire epidermis. B) PAR2 expression across the epidermis of skin treated with PBS, Acetone or 3% croton oil for 30 minutes for 7 individuals. C) Average PAR2 expression across the epidermis of skin treated with PBS or 5% SDS for 6 individuals.

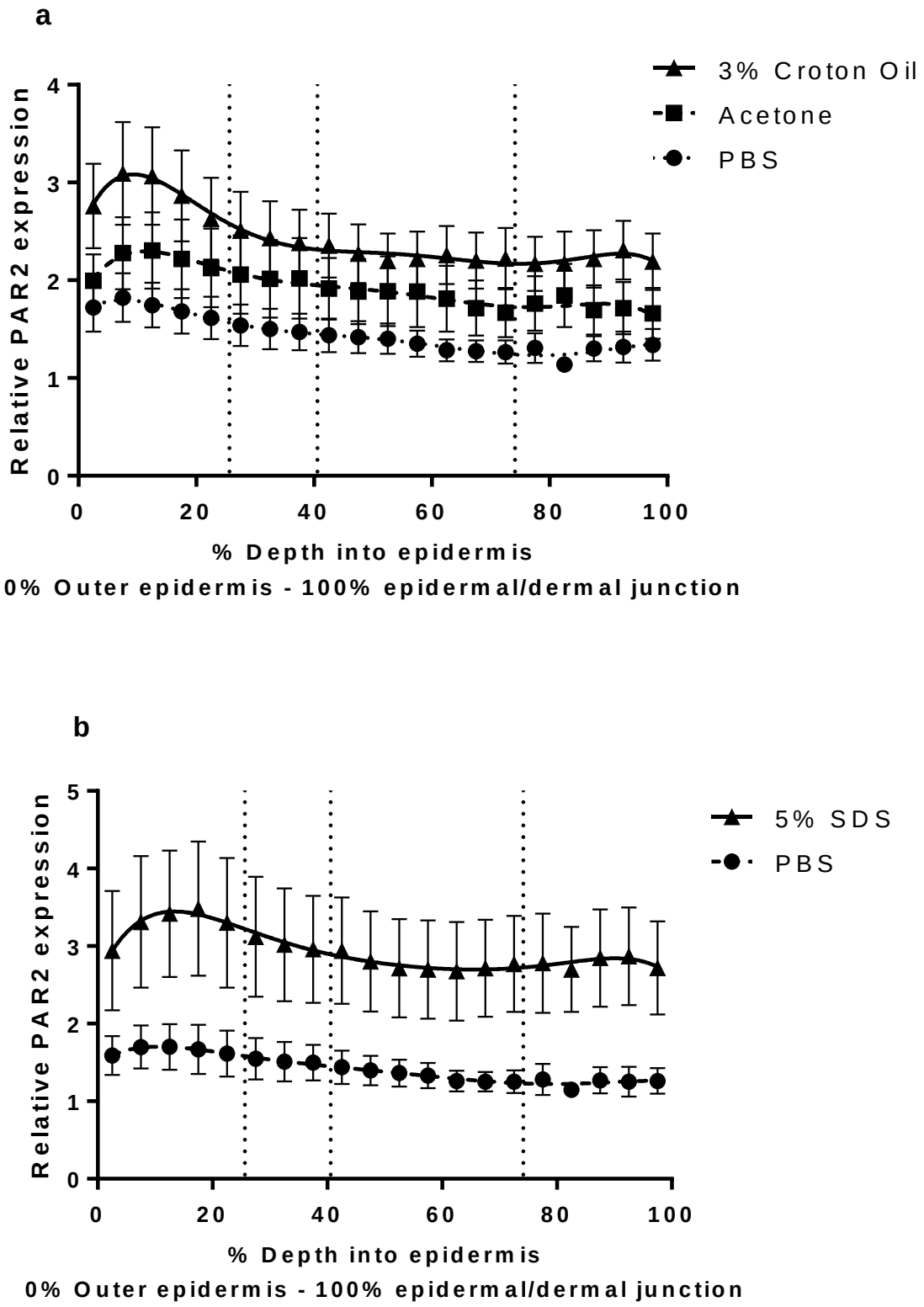


Figure 3.21: Changes in PAR2 expression throughout the epidermis following 30 minutes irritant application. A) PAR2 expression across the epidermis of skin treated with PBS, Acetone or 3% croton oil for 30 minutes for 7 individuals. B) PAR2 expression across the epidermis of skin treated with PBS or 5% SDS for 6 individuals. Results are shown relative to the lowest area of expression within the PBS-treated sample. Vertical lines indicate junction between each epidermal layer.

Table 3.8: Significant change in PAR2 expression compared with PBS at each point allowing for multiple comparisons of relative activity. Both 3% croton oil and 5% SDS treatment caused increased PAR2 expression throughout the entire epidermis. Acetone treatment caused variable PAR2 expression throughout the layers of epidermis, with no difference in stratum corneum/stratum granulosum junction and significant increases in AR2 expression in the stratum basale compared with PBS treatment. **p<0.01, p<0.001, ****p<0.0001, two-way repeated measures ANOVA with Bonferroni correction.

Epidermal layer	% depth into epidermis	Acetone	3% croton oil	5% SDS
Stratum corneum	0-5	ns	****	****
	6-10	*	****	****
	11-15	*	****	****
	16-20	***	****	****
	21-25	ns	****	****
Stratum granulosum	26-30	ns	****	****
	31-35	ns	****	****
	36-40	**	****	****
Stratum spinosum	41-45	**	****	****
	46-50	**	****	****
	51-55	**	****	****
	56-60	**	****	****
	61-65	***	****	****
	66-70	**	****	****
	71-75	**	****	****
Stratum basale	76-80	**	****	****
	81-85	****	****	****
	86-90	**	****	****
	91-95	*	****	****
	96-100	ns	****	****

Whereas the pattern of protease activity mirrored that of KLK7, PAR2 follows a similar pattern of expression across the epidermis with KLK5 (**Figure 3.22**). Both PAR2 and KLK5 expression is increased in the stratum corneum, compared with increases of KLK7 expression and protease activity at the stratum corneum-stratum granulosum junction. Both KLK5 and KLK7 showed increased expression in the stratum basale, which is not observed for PAR2 or protease activity.

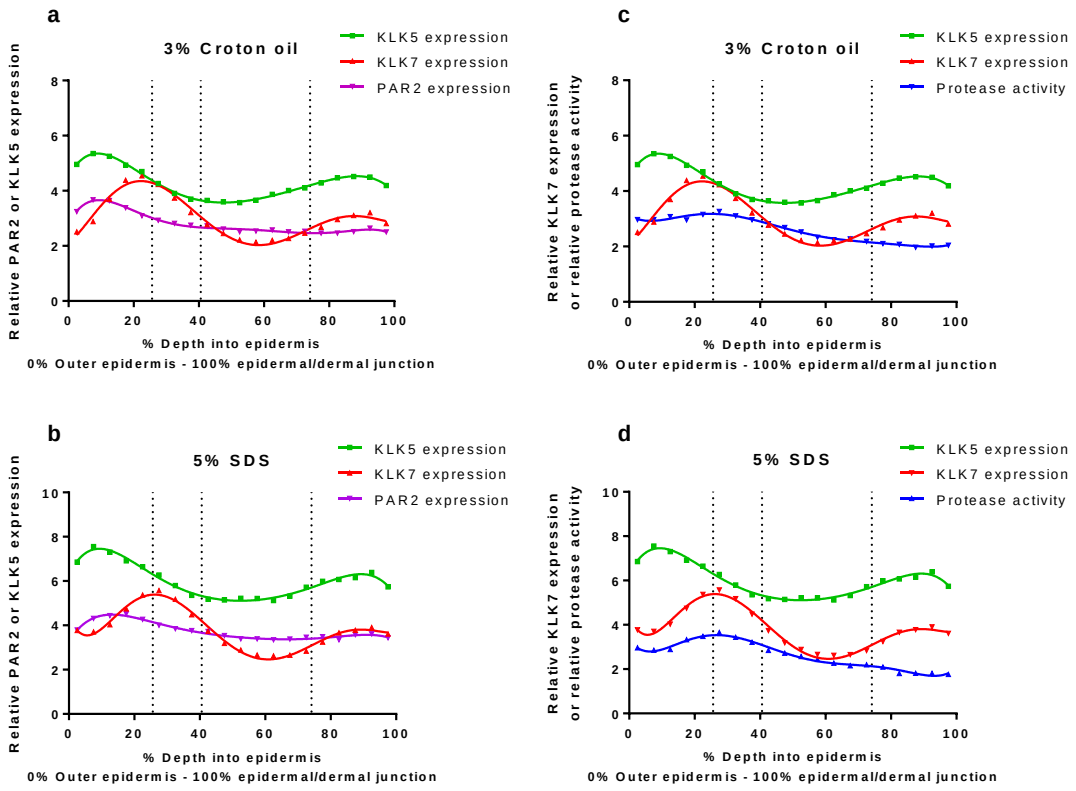


Figure 3.22: KLK7 and KLK5 increase to a greater extent than protease activity following irritant application to skin. PAR2 expression is more closely correlated to KLK5 than KLK7 (a+b), whereas protease activity is more closely correlated to KLK7 activity rather than KLK5 (c+d). Vertical lines indicate junction between each epidermal layer. Images are composite data from (a+b) **Figure 3.5** and **Figure 3.21**, (c+d) **Figure 3.8** and **Figure 3.18**.

3.3.8 Alteration of irritant response by protease inhibitors

The presence of PAR2 on the surface of the primary, undifferentiated keratinocytes was confirmed by stimulation with the synthetic peptide agonist SLIGKV. A dose dependent calcium flux response was observed between 1 and 100 μM SLIGKV-NH2,

with an EC_{50} of 10 μM (**Figure 3.23**). This matched the expected results from the peptide manufacturer. No calcium flux was observed when the scrambled peptide VKGILS-NH2 was applied.

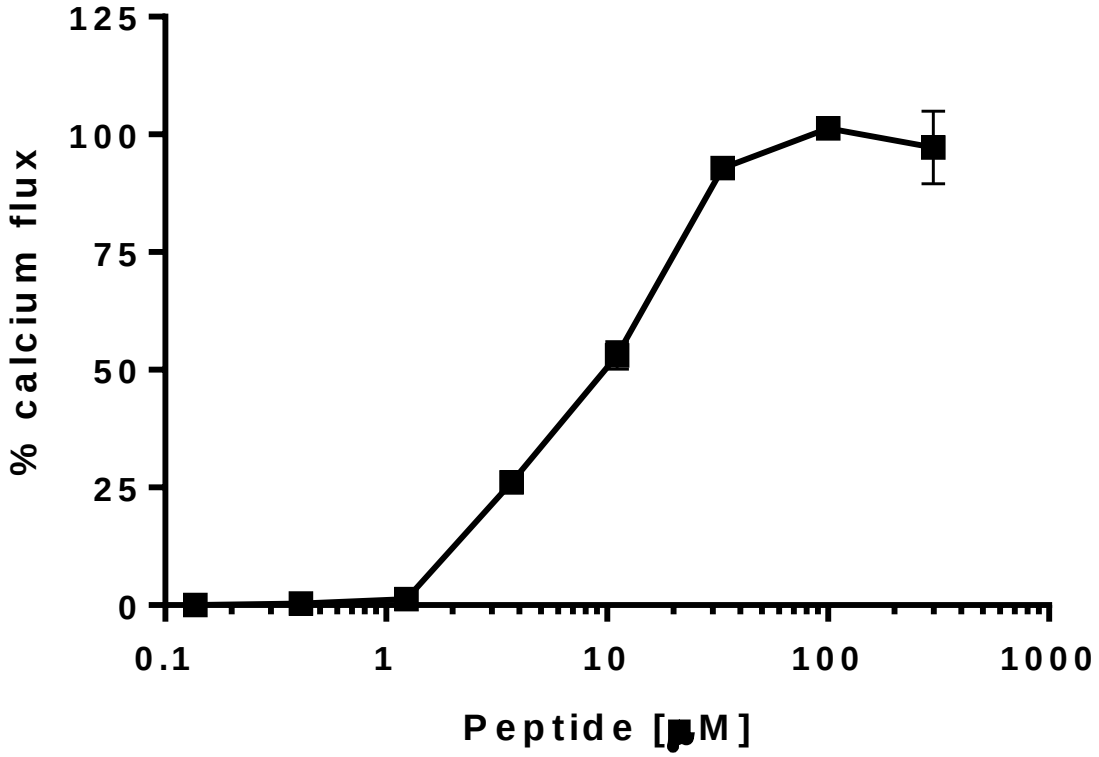


Figure 3.23: Calcium flux following application of PAR2 agonist SLIGKV-NH2. SLIGKV-NH2 was applied to primary human keratinocytes at various concentrations. The EC_{50} was 10 μM in accordance with manufacturer's calculations. Maximal response was stimulated with 100 μM SLIGKV. Graph shows results of 3 experiments ± SEM.

Initial experiments showed that at 0.1% and 0.03% SDS caused cellular damage leading to cell death, therefore a concentration of 0.01% was selected to test the calcium flux response to irritants. The effect of croton oil on calcium flux was tested using a serial dilution from 60 μg/ml to 0.74 μg/ml. It was found the use of this oil-based irritant caused an unpredictable response (**Figure 3.24**), possibly due to formation of an emulsion in the buffer before application to the cells.

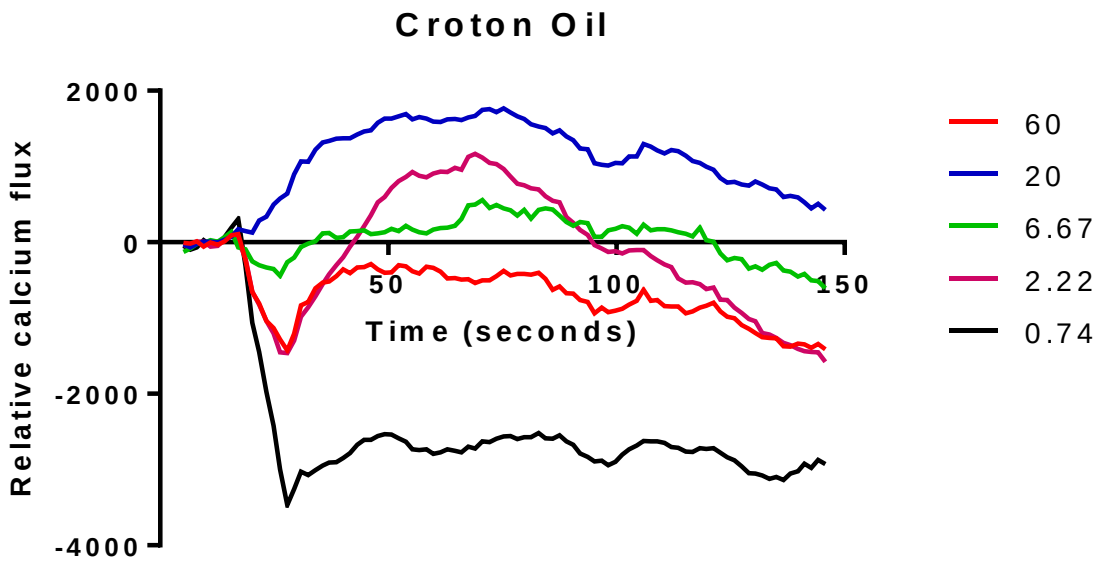


Figure 3.24: keratinocytes do not respond to croton oil in a dose dependent manner. Readings fell below baseline levels when croton oil was applied at the highest (60 µg/m) and lowest (0.74 µg/ml) concentrations, and no difference was detected when the median concentration of 6.67 µg/ml was applied. Representative of 3 experiments.

Trypsin was used as a positive control for PAR2 activation by enzymatic cleavage (as opposed to a synthetic ligand). Preliminary experiments identified 100 nM as the concentration of trypsin causing maximal calcium flux. Compared with 100 µM SLIGKV, 100 nM trypsin gave a slightly higher response, which is in agreement with results from Dulon *et al* using PAR2 transfected KNRK cells, 16HBE and the alveolar epithelial cell line A549 (Dulon et al., 2003). The calcium flux caused by 0.01% SDS reached a maximal peak approximately 25 seconds after application to the keratinocytes. This is compared with a peak approximately 55 seconds after application of 100 nM trypsin or 100 µM SLIGKV-NH2. The calcium response of 0.01% SDS treated keratinocytes also returned almost to baseline within 150 seconds. By contrast, keratinocytes treated with 100 nM trypsin or 100 µM SLIGKV-NH2 maintained an increased calcium level for at least 200 seconds before slowly decreasing to baseline.

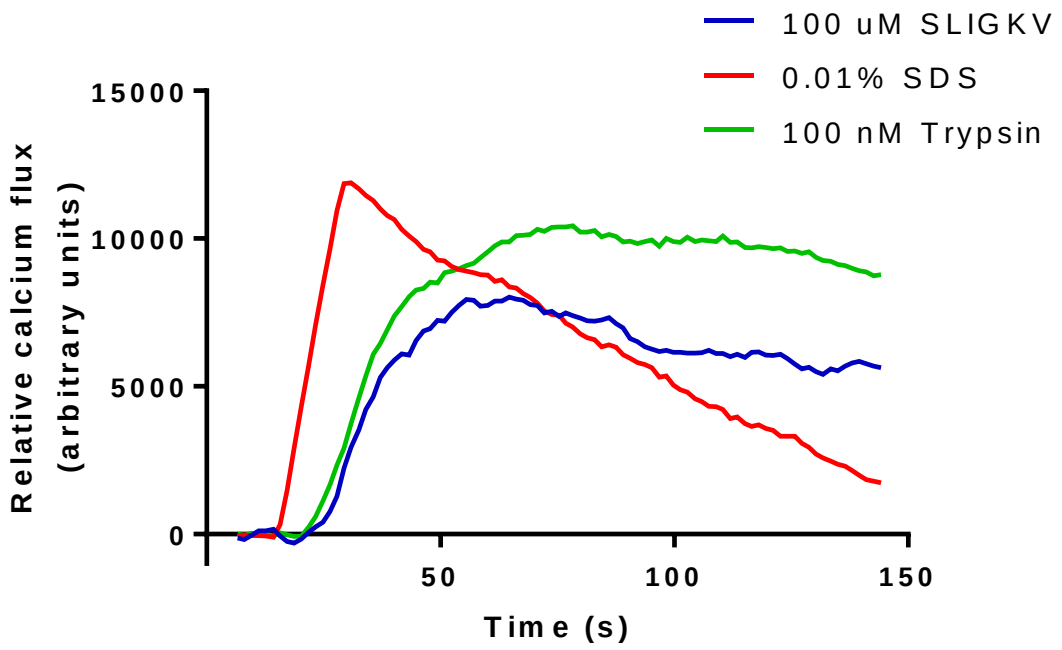
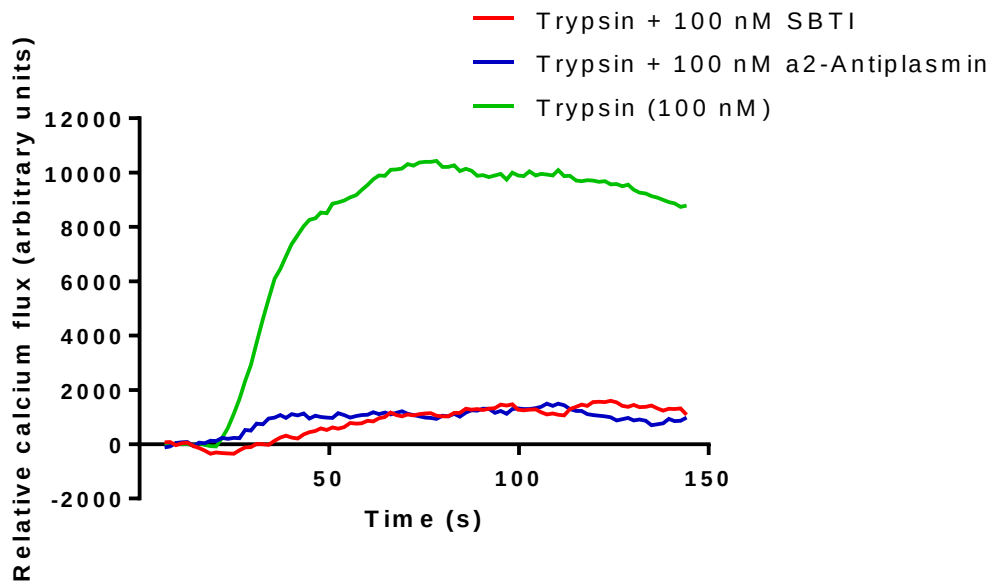
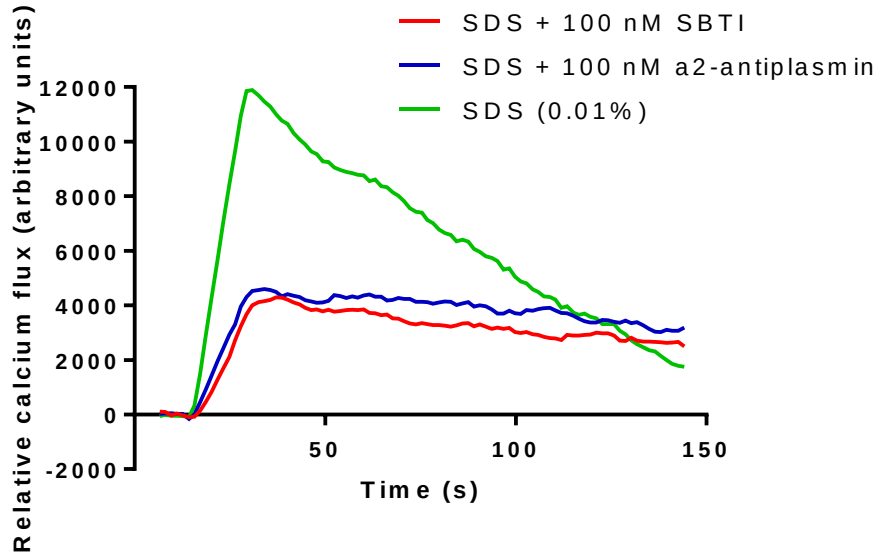


Figure 3.25: 100 nM Trypsin stimulates a similar response to 100 uM SLIGKV. Primary keratinocytes reached a peak calcium response 25 seconds after application of 0.01% SDS, compared with 55 seconds following application of 100 nM trypsin or 100 μM SLIGKV-NH2. All 3 treatments elicited a maximal response, although the response decreased more rapidly following 0.01% SDS compared with both 100 nM trypsin and 100 μM SLIGKV-NH2. Representative of 3 experiments performed in duplicate.

100 nM trypsin was then used to determine the concentration of soybean trypsin inhibitor (SBTI) and α2-antiplasmin which gave >90% inhibition. Serial dilutions of both inhibitors from 1000 nM to 4.12 nM showed that 100 nM was the optimal concentrations for each, to use in subsequent experiments.

The calcium flux initiated by both trypsin and 0.01% SDS was decreased 82.7% (p=0.0076) and 62.8% (p=0.0152) by 100 nM SBTI, and 85.0% (p=0.0079) and 72.6% (p=0.0159) by α2-antiplasmin respectively (**Figure 3.26**).


Trypsin + 100 nM SBTI
Trypsin + 100 nM a2-Antiplasmin
Trypsin (100 nM)
Relative calcium flux (arbitrary units)
12000
10000
8000
6000
4000
2000
0
-2000
50
100
150
Time (s)


SDS + 100 nM SBTI
SDS + 100 nM a2-antiplasmin
SDS (0.01%)
Relative calcium flux (arbitrary units)
12000
10000
8000
6000
4000
2000
0
-2000
50
100
150
Time (s)

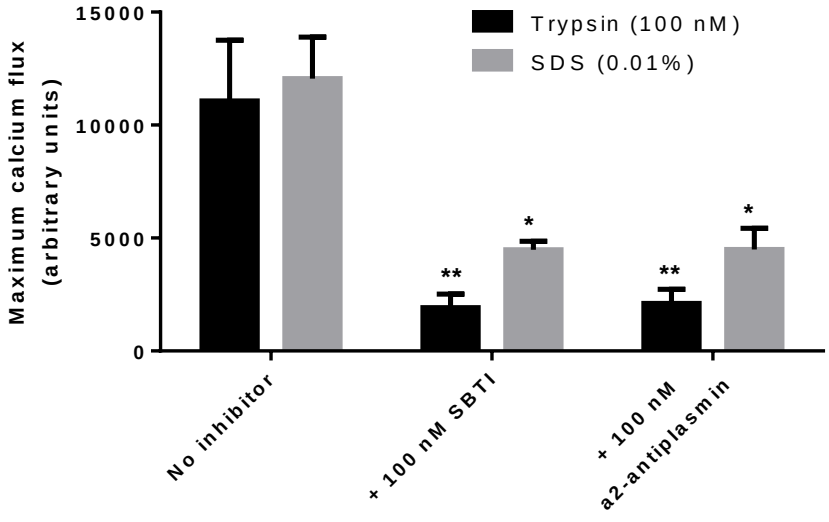

Trypsin (100 nM)
SDS (0.01%)
Maximum calcium flux (arbitrary units)
15000
10000
5000
0
**
*
**
*
No inhibitor
+ 100 nM SBTI
+ 100 nM a2-antiplasmin

Figure 3.26: The calcium flux induced by 0.01% SDS is decreased by trypsin inhibitors (Previous page). Maximal response to 100 μM trypsin was decreased 82.7% by SBTI and 85% by α2-antiplasmin, whereas response to 0.01% SDS was decreased 62.8% and 72.6% by SBTI and α2-antiplasmin respectively. Representative of 3 experiments performed in duplicate ± SEM.

3.3.9 Increased expression of KLK5 and KLK7 at 30 minutes is not due to increased protein synthesis

The previous results have shown that KLK5 and KLK7 expression in the epidermis is upregulated within 30 minutes of exposure to irritants. To determine if this was a reflection of increased mRNA transcription, levels of mRNA were determined within the timeframe 30 mins to 12 hours; RNA was extracted from *ex vivo* human skin following 30 minutes, 1, 3, 6 and 12 hours treatment with PBS, acetone, 3% croton oil or 5% SDS (**Figure 3.27**).

KLK5 mRNA expression was decreased in the first 30 minutes following acetone treatment, but slightly increased in 3% croton oil and 5% SDS treated epidermis. Following 3 hours exposure, *KLK5* mRNA expression was maximal in acetone, 3% croton oil and 5% SDS treated skin, although 5% SDS exposed epidermis had similarly increased KLK5 levels from 30 minutes to 3 hours. For all treatments, *KLK5* mRNA expression was decreased following 12 hours irritant treatment compared to PBS treated epidermis. Skin treated with 5% SDS showed a decrease in *KLK5* mRNA below PBS levels within 6 hours, however it was not possible to test this time point for acetone or 3% croton oil treated skin.

KLK7 mRNA expression remained unaltered in acetone treated skin compared with PBS treated skin. 3% croton oil and 5% SDS treatment gave a similar pattern of expression over 12 hours, with an initial decrease from 30 minutes to 1 hour exposure, increasing to maximal levels at 3 hours before steadily decreasing until 12 hours post-exposure.

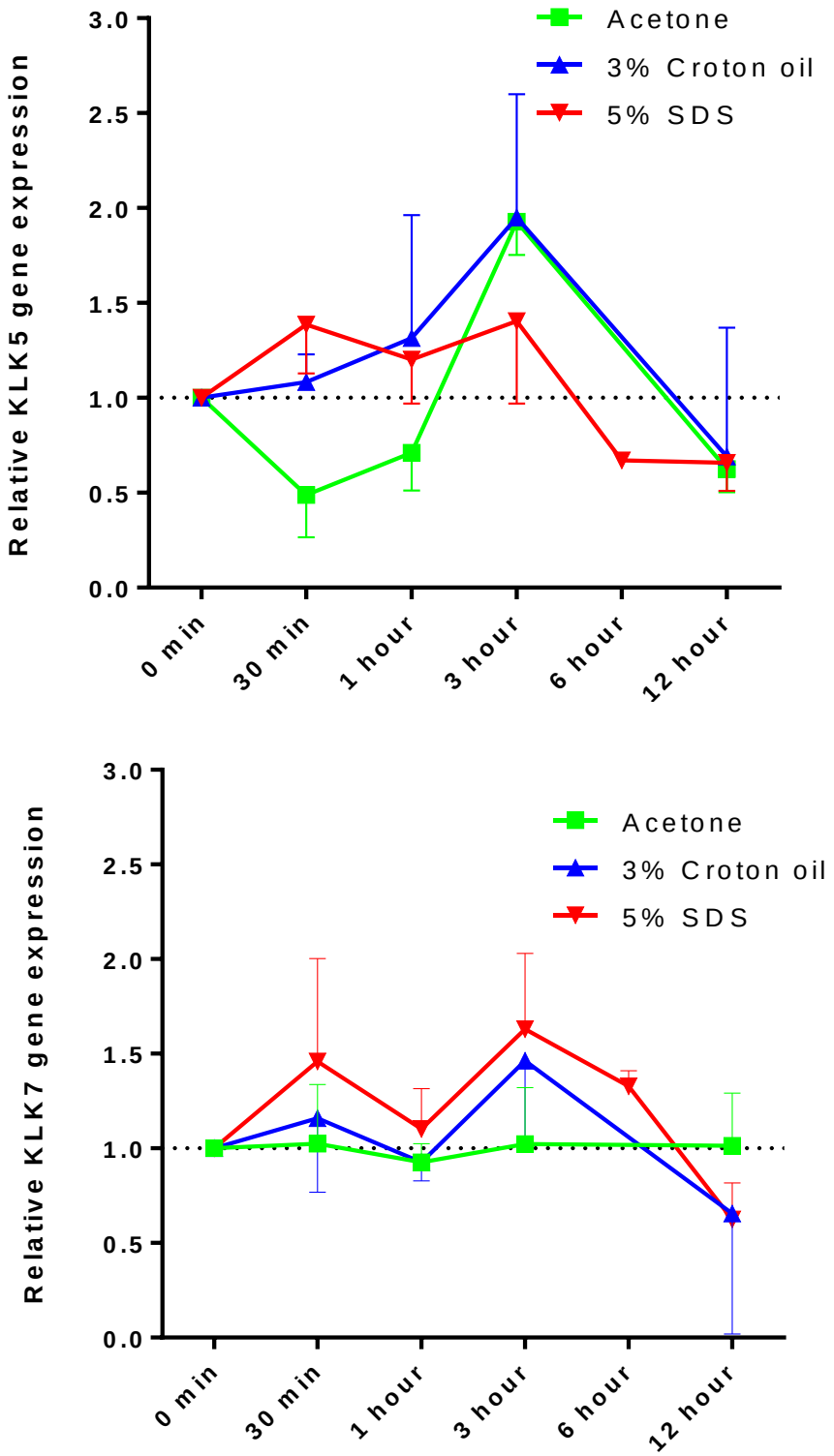


Figure 3.27: *KLK5* and *KLK7* mRNA expression following irritant application at 30 minutes, 1, 3, 6 and 12 hours. 3 hours treatment with acetone, 3% croton oil and 5% SDS increased *KLK5* expression compared with PBS-treated skin, before decreasing below PBS-treated levels at 12 hours. The same is true for *KLK7* expression, however acetone-treated made no difference to *KLK7* expression at any time point compared with PBS treated skin. All data are shown relative to PBS-treated skin.

3.3.10 Proinflammatory cytokines are up-regulated within 3 hours, but not by 30 minutes, of irritant treatment

In addition to KLK5 and KLK7, the expression of IL-8 and TNFα mRNA was also analysed over the period 30 minutes to 12 hours following treatment with PBS, acetone, 3% croton oil or 5% SDS (**Figure 3.28**).

IL8 mRNA expression was not altered by any of the irritant treatments at 30 minutes exposure compared with PBS-treated controls. However, by 3 hours after exposure of skin to acetone, *IL8* mRNA expression was increased compared with 3% croton oil treated skin. Following 12 hours exposure, *IL8* mRNA expression was 3.7 fold greater in 3% croton oil-treated skin than in acetone-treated skin. 5% SDS-treated skin showed dramatic increases in *IL8* mRNA expression from 1 hour of exposure, peaking at 6 hours of exposure before dropping back to levels similar to those seen at 1 hour exposure after 12 hours.

The pattern of expression for *TNFα* mRNA over the course of 12 hours following exposure to irritants differs from that observed for *IL8* mRNA. For acetone-, 3% croton oil- and 5% SDS-treatment, *TNFα* mRNA expression peaks following 3 hours exposure, dropping to levels below that of PBS treated controls by 12 hours of exposure. Although all 3 treatments follow a similar pattern of *TNFα* mRNA expression over 12 hours, exposure to 5% SDS increases expression within 30 minutes, whereas both acetone and 3% croton oil show a small decrease in expression before returning to normal levels within 1 hour and peaking at 3 hours.

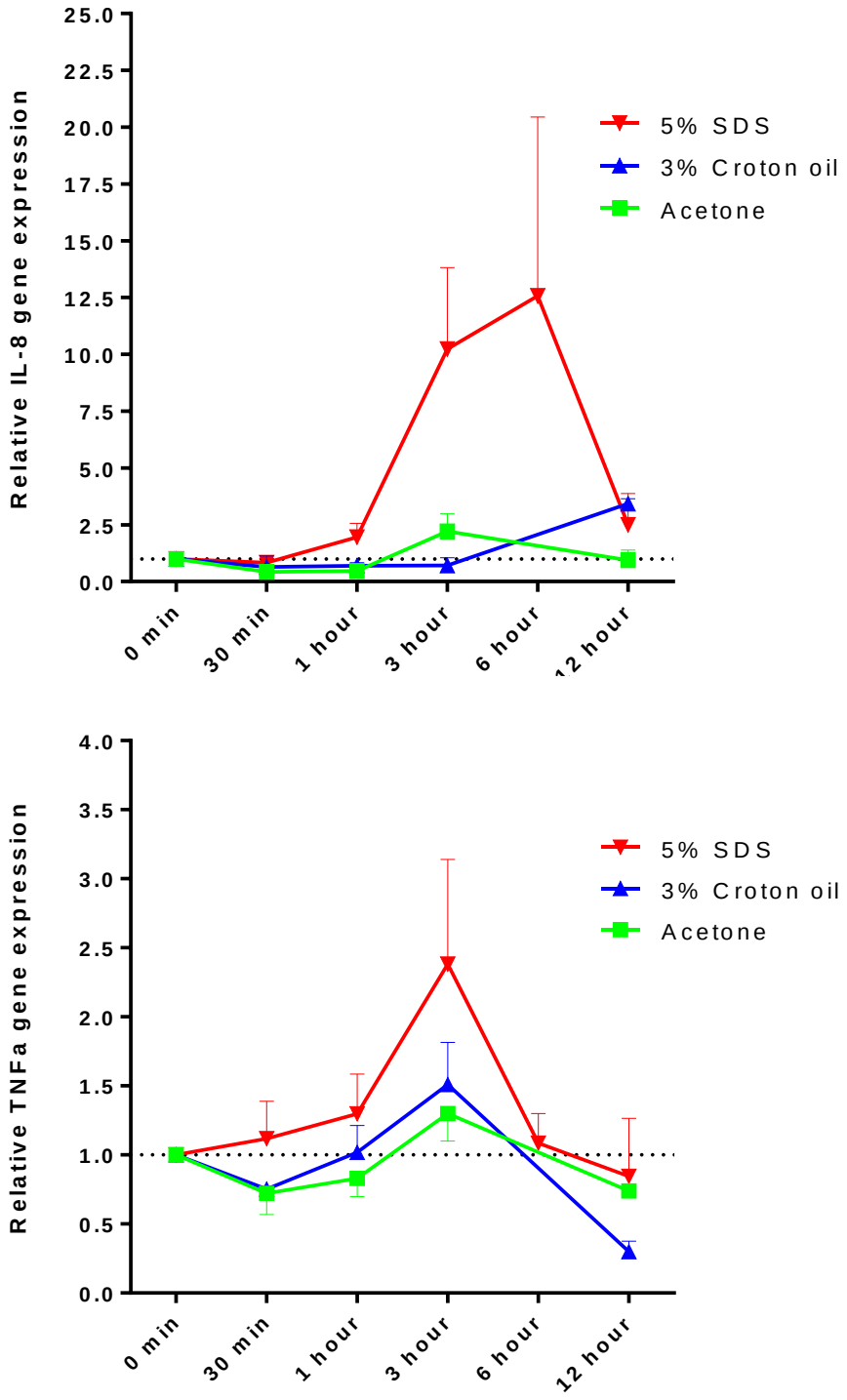


Figure 3.28: *IL8* and *TNFα* mRNA expression following irritant application at 30 minutes, 1, 3, 6 and 12 hours. 6 hours treatment with 5% SDS increased *IL8* mRNA expression compared with PBS-treated skin, with expression remaining more than 2 fold by 12 hours. Acetone treatment increased *IL8* mRNA expression within 3 hours, reducing to normal levels within 12 hours. Treatment with 3% croton oil caused an increase in *IL8* mRNA expression within 12 hours of exposure. All 3 treatments caused an increase in TNF***α*** mRNA expression within 3 hours, falling to below normal levels 6 hours post-exposure. All data are shown relative to PBS-treated skin.

3.4 Discussion

In this chapter the main findings were that expression of KLK5 and KLK7, as reflected by immunostaining, is increased within 30 minutes of exposure to irritants, however, mRNA expression is not increased until 3 hours post-exposure. Protease activity within the stratum corneum-stratum/ granulosum region is also increased within 30 minutes of irritant exposure. PAR2 expression (again, reflected by immunostaining) is increased within 30 minutes, however downstream cytokine transcription is not increased until 3 hours post-exposure. Overall, it appears that the mechanisms are in place to activate the expression of proteases, in particular KLK5 and KLK7 when the epidermal barrier is perturbed by irritants. These in turn may act on PAR2 receptors to activate the transcription of key pro-inflammatory cytokines including IL-8 and TNFα.

Previous studies have utilised mouse skin models or the immortalised human keratinocyte line HaCaTs to investigate the effects of various irritant and sensitising chemicals (Kondo et al., 1994; Mohamadzadeh et al., 1994; Wilmer et al., 1994; Effendy et al., 2000; Park et al., 2010; Hennen et al., 2011; White et al., 2011). While their ease of propagation and distinct keratinocyte morphology make HaCaT cells a good choice for investigating several aspects of keratinocytes activity and differentiation, they have been shown to differ from primary human keratinocytes in several ways. Lewis *et al* showed that HaCaT cells utilize a different signal transduction pathway for UVB-induced NF-κB activation compared with primary normal human keratinocytes (Lewis et al., 2006), and concluded that HaCaTs did not make a suitable surrogate for primary human keratinocytes in studies of UVB effects. Further investigation of HaCaT responses following irritant exposure is required to determine their suitability as models for primary keratinocytes. The irritant potential of large molecular weight chemicals is overestimated using cell-only models due to the absence of the epidermal permeability barrier, which limits the percutaneous penetration of these chemicals (Wilhelm et al., 2001).

By using an *ex vivo* human skin model in this study, we have been able to directly assess changes in KLK and PAR2 expression in human skin following irritant application. This system enables the investigation of innate inflammatory processes caused by irritants in the full thickness skin tissue environment. The skin used for *ex*

vivo models was taken from breast tissue of female patients. Several studies have concluded that there is no significant difference between skin irritant responses in males and females (Lammintausta et al., 1987; Wilhelm and Maibach, 1990; Agner, 1992), however, it has been demonstrated that women have less variation in KLK7 content in the stratum corneum over all age groups (Kishi et al., 2004; Komatsu et al., 2005) compared with men. Keratinocytes extracted from skin from different body sites have a highly conserved proteome, therefore it follows that skin from any body part can be used as a general model for keratinocytes (Sprenger et al., 2013). Patients aged over 70 years have significantly less total KLK protein concentration in the stratum corneum compared with individuals aged 30-50 (Komatsu *et al* 2005), hence patient groups with a range of ages were used wherever possible. One study has indicated that men have a greater susceptibility to irritants (Robinson, 2002), while another shows female dental practitioners report a higher incidence of occupational irritant contact dermatitis compared with their male counterparts (Burke et al., 1995). However, overall there is no conclusive evidence to indicate a significant difference in response to irritants between men and women. These data together indicate breast tissue is a reasonable representative of skin across the body.

Corresponding with results observed by Demerjian *et al* (2008), who showed that nucleated layers are decreased in thickness 30 min following tape stripping, here we have shown that stratum granulosum, and to a lesser extent the stratum corneum, is thicker following irritant application. Increased activity of KLK5 and KLK7, which primarily act to degrade the corneodesmosome complexes at the stratum corneum-stratum granulosum junction may cause an increase in desquamation at the site of irritant exposure. The subsequent alteration in calcium content of the epidermis may lead to increased differentiation and cornification of the keratinocytes of the stratum spinosum and/or stratum granulosum (Bikle et al., 2001). As it is unlikely that these events occur within 30 minutes of irritant exposure, the increased thickness of the stratum granulosum may alternatively be explained by an alteration in the water content of the granular layer due to movement of water from the lower epidermal layers into the upper layers following damage to the permeability barrier. The release of lamellar bodies from the nucleated cells into the stratum corneum-stratum granulosum space may also contribute to the increased thickness of the upper epidermal layers. Demerjian *et al* (2008) also showed that by 3 hours the stratum corneum is increased compared to baseline, which is supported by results from this

study which show that the thickness of the stratum corneum is increasing within 30 minutes.

The antibodies used in the present study to stain for KLK5 and KLK7 were shown to be specific for the respective proteins by use of Western blotting (**Appendix 2**). KLK7 was detected as a single band at the expected size of 27 kDa, while KLK5 was detected in 3 separate bands. 3 bands have been previously reported for KLK5 (Michael et al., 2005) due to different states of glycosylation, and also, according to the manufacturers datasheet, the antibody detects the pre-pro-peptide and pro-peptide forms of the protein as well as the mature peptide. Staining in the epidermis followed the expected pattern for KLK5 (Yamasaki et al., 2007; Oji et al., 2010), whilst KLK7 was detected as expected in the stratum corneum-stratum granulosum junction (Ishida-Yamamoto et al., 2005; Schultz et al., 2013), but also at the stratum basale level. This may be due to increased sensitivity of the antibody used in this study. These data together indicate that the staining observed was specific to KLK5 and KLK7.

The most frequently used irritants in research studies are surfactants and solvents. These have in common the ability to disrupt the stratum corneum permeability barrier (Fulmer and Kramer, 1986; Walters et al., 1988; Abrams et al., 1993; Walters et al., 1993; Kitagawa et al., 2001). However, other chemicals such as nonanoic acid, croton oil and dithranol appear to have pharmacological properties able to directly activate transcription factors which initiate the expression of pro-inflammatory cytokines, resulting in inflammation. Croton oil contains phorbol esters, which are tumour promoters with a capacity to activate the transcription factor AP1 with a multitude of downstream effects on expression of downstream genes (Chun et al., 2004; Lee et al., 2007a), including pro-inflammatory cytokines (Wilmer et al., 1994; Redondo et al., 1997). It has long been known that not all irritant chemicals elicit a response through the same mechanism or common inflammatory pathway (Patrick et al., 1987; Willis et al., 1989). Therefore, it is possible that the response we see from croton oil (a source of phorbol esters) may act through a different pathway to SDS (a surfactant) and acetone (a solvent), both of which disrupt the permeability barrier, however croton oil and SDS both appear to have some activity through the KLK-PAR2 pathway.

The swift increase in protein expression in the absence of increased mRNA expression is most likely due to increased post-translational expression, with transcription increasing between 1 and 3 hours post-exposure. Very few groups have looked at the

early phase of the irritant response, most studies having investigated changes in mRNA expression and cytokine production from between 6 and 24 hours. In 2010, Clemmensen *et al* used a microarray to show that SDS initiates a different response pathway compared to nonanoic acid (an experimental irritant) 30 minutes post-exposure (Clemmensen et al., 2010). The group also analysed changes in histology of the treated skin 30 minutes and 4 hours following a single exposure, and 11 days after repeated exposure to either SDS or nonanoic acid. Despite the differences in the pathways activated, no differences were observed in H&E stained skin at the early time points and sparse differences were observed between the two treatments by 11 days. IL-8 has been shown to upregulated following application of irritants (Mohamadzadeh et al., 1994; Coquette et al., 2003). Together, these data support results observed in the present study which show increased KLK5 and KLK7 expression in all irritant exposed skin, and increased TNFα and IL-8 within 3 hours despite differences in the mechanism utilised by SDS, acetone and croton oil.

Torma *et al* have shown that mRNA expression of KLK5 and KLK7 were decreased 6 hours post-exposure to 1% SDS (Torma et al., 2008), but had returned to normal within 24 hours. However, in that study no earlier time points were investigated. The present study confirms results showing that KLK5 and KLK7 mRNA levels are lowered at 6 hours post-exposure to 5% SDS, however testing at earlier time point showed that mRNA expression is increased by 3 hours, indicating that there is a small window of time when the protein levels are being altered following contact with SDS. This may account for how the level of stored KLK5 and KLK7 proteins are adjusted to normal following increased expression after irritant exposure, something that has not previously been explained. Based on these results, subsequent studies should place more emphasis on early response to irritants rather than focussing only on the response more than 6 hours post-exposure.

Previous studies into the expression of KLKs have focussed on the role of these proteases in skin disease, such as Netherton's syndrome, in which protein levels are constitutively upregulated (Descargues et al., 2005; Briot et al., 2009; Briot et al., 2010). Due to the sustained nature of the increased protein expression it is not possible to identify differences in the rate of protein and mRNA upregulation. Here, we were surprised to observe that protein staining of KLK5 and KLK7 is upregulated within 30 minutes following application of irritants. It is highly unlikely that this

reflects new protein synthesis; this is supported by the observation that there was no corresponding increase in mRNA transcription until 3 hours after the irritants were applied. This indicates that the upregulated protein is due to an increase in post-translational modification as opposed to transcriptional upregulation for both KLK5 and KLK7. KLK5 and KLK7 are both stored in the lamellar bodies as a pre-pro-protein, and are activated by proteolytic cleavage of the precursor proteins following secretion of the lamellar body contents into the intercellular space (Ishida-Yamamoto et al., 2004; Pampalakis and Sotiropoulou, 2007; Debela et al., 2008). Therefore it may be possible that these modifications alter the accessibility of the epitopes recognised by the antibody. The micro-anatomical pattern of increased protease activity in the epidermis following irritant application follows the same pattern as for KLK7 expression, but not that for KLK5, which followed the expression of PAR2. KLK7 expression peaked around the stratum corneum-stratum granulosum junction, while KLK5 expression peaks in the upper layers of the stratum corneum. Caubet *et al* demonstrated that KLK5 is able to degrade CDSN, DSC1 and DSG1, whereas KLK7 was unable to degrade DSG1 (Caubet et al., 2004). This may explain the localisation of KLK7 to a lower compartment of the stratum corneum than KLK5.

The pattern of staining observed for KLK5 indicates that it is present in all layers of the epidermis, however as the antibody is unable to discriminate between the active and inactive forms, we are unable to determine the state of activation at each layer. Similarly for KLK7, there appears to be an increase in expression in both the stratum basale and stratum granulosum-stratum corneum junction; however once again we are unable to discriminate active from non-active KLK7.

Gelatin *in situ* zymography was shown here to be an ineffective tool to investigate protease activity in human skin due to its inability to discriminate between the various layers of the full thickness skin. However, direct application of a fluorescently labelled substrate to tissue sections enabled the identification and separation of the layers of the epidermis during analysis. We have shown that protease activity is increased within 30 minutes of irritant application, in correlation with increased KLK5 and KLK7. However, this increase is only observed in the stratum granulosum-stratum corneum junction, not the stratum basale, which may be indicative of an inactive form of KLK being detected in the stratum basale while the active form is in the stratum granulosum-stratum corneum junction. Our results are in agreement with studies by

Jeong *et al,* who showed that protease activity in mice is increased 10 minutes after tape stripping skin (Jeong et al., 2008). In that study, application of allergens increased this protease activity even further.

It is probable that the increased enzymatic activity observed in the epidermis is due to an increase in activity of several proteases, not just KLK5 and KLK7. However the lack of specific inhibitors for these proteases makes it difficult to identify the exact contribution of each protease to the overall activity in the epidermis. The most specific inhibitor available for KLK5 (LEKTI domain 6) has also been shown to inhibit KLK7, trypsin and subtilisin A (Jayakumar et al., 2004; Kreutzmann et al., 2004; Deraison et al., 2007). In addition, LEKTI is not limited to inhibiting serine proteases, having been shown to inhibit some cysteine proteases. In 2010, Bennett *et al* showed that caspase-14, a cysteine protease, is inhibited by 5 recombinant LEKTI fragments in addition to full length LEKTI (Bennett et al., 2010). All other known inhibitors for KLK5 have been shown to inhibit a range of proteases. For example, SBTI also inhibits trypsin, while α2-antiplasmin inhibits plasmin, KLK2 and KLK14 (Frenette et al., 1997; Michael et al., 2005; Luo and Jiang, 2006; Borgono et al., 2007).

It has been previously reported that plasmin plays a major role in augmenting the kinetics of barrier recovery (Denda et al., 1997). That study used a specific plasmin inhibitor (t-AMCHA) which was shown to increase barrier recovery. Based on those results and the results of the present study, it is likely that the overall increase in barrier recovery observed in that study could be due to decreased activity of several proteases, including plasmin and KLKs. This possibility is further supported by a study which shows that application of SBTI, an inhibitor of KLK5, to the epidermis, increases barrier recovery rates (Hachem et al., 2006b). In the present study is has been shown that KLK5 and KLK7 are significantly increased following irritant application, and that SBTI and α2-antiplasmin, both inhibitors of KLK5, reduce the effects of irritants on primary keratinocytes.

It is well documented that PAR2 is present on the cell surface of primary keratinocytes and internalised following activation (Bohm et al., 1996; Buddenkotte et al., 2005; Macfarlane et al., 2005; Kumar et al., 2007; Ishikawa et al., 2009). Immunofluorescent and immunohistochemical staining in those studies has primarily been performed on cells transfected with FLAG- or GFP- tagged PAR2, followed by detection with standard antibodies against these tags. The obvious drawback to this approach is the

requirement for transfected cell cultures or transgenic animal models, neither of which accurately depict the physiological environment of *in vivo* human skin.

In the present study, immunofluorescent staining using an antibody directed against native human PAR2 indicated that PAR2 is present on the surface of the immortalised keratinocyte cell line HaCaT as expected (Rattenholl et al., 2007), and also on the surface of primary human keratinocytes. Although it was not possible to obtain successful western blots using this antibody, it was shown that the intensity of staining was altered following incubation of keratinocytes with the PAR2 agonist SLIGKV (**Appendix 3**). Hachem *et al* have previously used the H99 antibody to detect PAR2 on the surface of cultured human keratinocytes, and also showed an alteration in detected protein expression following incubation with SLIGKV (Hachem et al., 2006b), similar to that shown here. A recent study by Adams demonstrated that the H99 was specific to PAR2 using murine cells transfected with GFP tagged PAR2 (Adams, 2012). In the present study, PAR2 expression in the epidermis followed the expected pattern, with peak expression in the stratum corneum-stratum/granulosum (Stefansson et al., 2008), and expression was universally increased within 30 minutes of irritant application.

It is possible that PAR2 protein expression may be upregulated in the absence of mRNA upregulation, as it has recently been shown that a keratinocytes overexpressing KLK5 also overexpresses PAR2, with no corresponding increase in mRNA levels (Zhu et al., 2013). As changes in mRNA level are not a requirement for increased protein expression it is not surprising that protein levels are increased within 30 minutes of irritant treatment, during which time KLK5, an activator of PAR2, is also increased. In the present study it was shown that *IL8* and *TNFα* expression was upregulated within 3 hours when skin was treated with acetone, 3% croton oil and 5% SDS. Both of these cytokines are known to be upregulated by PAR2 activation, therefore it is probable that the upregulated PAR2 in the epidermis is due to activation, in part by KLK5, and this leads to subsequent downstream transcription of proinflammatory cytokines.

This timescale of alterations to gene expression was to be expected, based on a mouse study in which TPA (an ingredient of croton oil) was applied to mouse ears (Murakawa et al., 2006). In that study *TNFα* mRNA expression was upregulated 3 hours post-exposure. Similarly, *IL8* expression was expected to increase at the 3 hour time point based on a study in which 20 μg/ml croton oil was applied to primary

keratinocytes (Wilmer et al., 1994). *IL8* mRNA expression was increased within 1 hour, and *TNFα* expression was slightly increased within 30 minutes, and greatly increased by 3 hours post-exposure. It can be expected that the inclusion of the permeability barrier in the *ex vivo* model of the present study, and the lower level of croton oil used (12.5 μg/ml) would increase the time between application of the irritants and altered gene expression.

Most previous studies using human skin models have investigated KLK expression and activity in the stratum corneum layer only, giving a limited insight into the physiological presentation of KLK and PAR2 across the epidermis (Brattsand and Egelrud, 1999; Ekholm et al., 2000; Bernard et al., 2003; Voegeli et al., 2009). Here it has been shown that protease activity is increased not only in the stratum corneum as previously reported (Hachem et al., 2006b; Voegeli et al., 2009), but from the stratum corneum-stratum granulosum junction through to the stratum spinosum. PAR2 is also increased across the entire epidermis, which has not been previously reported. The results shown here also demonstrate the pattern of KLK5 expression following irritant application correlated with PAR2 expression throughout the epidermis, which is to be expected based on evidence that PAR2 is activated by KLK5 but not KLK7 (Stefansson et al., 2008). Conversely, KLK7 expression correlated with increased protease activity across the epidermis within 30 minutes of irritant contact. KLK7 is present in the stratum corneum at a concentration 5.6 fold higher than KLK5 (Komatsu et al., 2006), therefore it is unsurprising that KLK7 should contribute more to overall protease activity than KLK5. The lack of increased PAR2 expression and protease activity in the stratum basale compared with KLK5 and KLK7 may be explained by the detection of inactive forms of the kallikreins at this location.

It has previously been shown that KLK5 is expressed by NHK (Komatsu et al., 2003), therefore it is possible that at least part of the SDS-induced calcium response observed is due to activation of KLK5 , which goes on to activate PAR2 and the subsequent downstream effectors, including calcium flux. Here it was observed that there is a different time course in the activation of the calcium flux induced by 0.01% SDS, 100 nM trypsin and 100 μM SLIGKV-NH2, the SDS-induced response being more rapid than that induced by the known PAR2 activators. It was confirmed that the activation of PAR2 by trypsin could be almost completely abrogated by different protease inhibitors, SBTI and α2-antiplasmin. The activation of the calcium flux by SDS is

clearly more complicated in that, apart from the more rapid onset, the effect of protease inhibitors, while significant, was less complete. There was blockade of the calcium flux to a much lower level and, interestingly, the time course of the recovery of the calcium flux mimicked more closely that following activation by trypsin. These findings could be interpreted as showing that SLS has (at least) 2 effects: first is a direct permeabilsation of the cell membrane, allowing the initial rapid onset of the calcium flux. However second, there is clearly an activation of protease(s) which in turn activate PAR2.

It has been demonstrated that croton oil increases barrier permeability of the skin, most likely due to it causing damage to the barrier (Pickard et al., 2009). Similarly, SDS damages the permeability barrier, which in turn leads to perturbations in the calcium gradient across the epidermis. This may explain how both croton oil and SDS, which have seemingly separate mechanisms of irritation, cause an increase in KLK expression and protease activity in the skin, despite stimulating the difference in the mechanisms utilised. Overall, the results presented in this chapter indicate that PAR2 is potentially being indirectly activated by the irritants through KLK expression and activation.

4. Keratinocyte "pseudo-immortalisation" for expansion of keratinocytes and production of stratified epidermis *in vivo*

4.1 Introduction

Obtaining human skin for *ex vivo* research purposes can be problematic as it is dependent on the availability of surplus tissue following surgeries such as breast reduction, mastectomy and abdominoplasty. The size and quality of these skin samples is highly variable, and lack of availability of suitable tissue samples can cause delays in testing novel chemicals for irritant and/or pro-inflammatory potential. As discussed in chapter 1, there are several *in vitro* methods now being used to test the irritant and sensitising potential of chemicals (Coquette et al., 2003; Ryan et al., 2004; Roberts et al., 2007; Cronin et al., 2009). Keratinocyte monolayers have been extensively used as a model for skin in various studies (Cohen et al., 1991; Eun et al., 1994; Ward et al., 1998; Varani et al., 2007). However, human skin equivalents (HSE) more closely mimic the biological and physiological conditions of the skin, including the presence of a permeability barrier (Bell et al., 1991; Fartasch and Ponec, 1994; Schmook et al., 2001), making them a far more relevant model. Both research and industrial applications which use skin and/or skin equivalents would benefit from tissue engineered *in vitro* to ensure a continuous supply of appropriate skin samples.

Since the 1980's there have been several studies which have shown potential for creating skin models, with barrier functions close to that of *ex vivo* skin, due to improvements in culture conditions and methods (Bell et al., 1981; Asselineau et al., 1986; Bell et al., 1991; Nolte et al., 1993; Fartasch and Ponec, 1994; Ponec et al., 1997; Schmook et al., 2001; Batheja et al., 2009), and there are now several commercially available epidermis-only skin models including Episkin™ (Skinethic, Nice, France), Epiderm™ (MatTek Corporation, Ashland, USA) and RHE™ (Skinethic, Nice, France). There are also commercially available full thickness skin models which include dermal substitutes, including Epiderm FT™ (MatTek Corporation, Ashland, USA), which can be used for the study of wound healing. Keratinocytes for these commercial models are mostly obtained from surplus skin from plastic surgery, which is a limiting factor in the production of the models.

There are several conditions that can be altered to improve or change the quality, structure and functionality of skin models. These include the addition of a dermal scaffold, the presence of fibroblasts within the scaffold, the composition of the differentiation media and inclusion of resident immune cells such as Langerhans cells (Fransson et al., 1998). Keratinocytes must be seeded onto a suitable scaffold which allows basolateral feeding at an air-liquid interface (ALI) in order to differentiate into organotypic models (Lillie et al., 1980; Prunieras et al., 1983; Bernstam et al., 1986; Rosdy and Clauss, 1990). Dermal scaffolds can range from simple polycarbonate membranes (Rosdy and Clauss, 1990) to a collagen matrix populated with feeder fibroblasts to de-epidermalised dermis, or combinations of all three (Lee et al., 2000) (**Figure 4.1**).

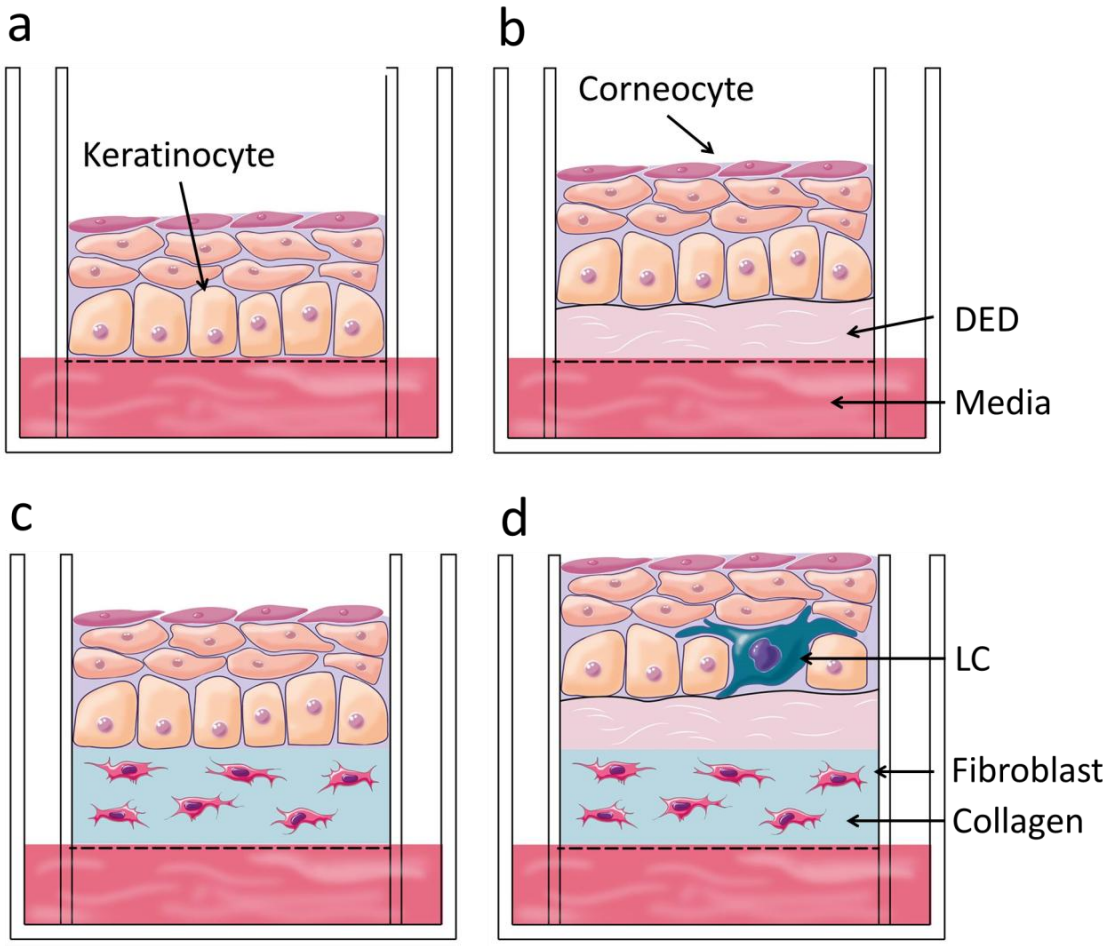


Figure 4.1: Possible conditions for the differentiation of keratinocytes into organotypic models (previous page). Keratinocytes can be differentiated on a simple polycarbonate scaffold (a), on de-epidermalised dermis (DED) (b), on a collagen scaffold infused with fibroblasts (c) or a combination of DED and collagen with fibroblasts (d). These models may also include immune cells such as Langerhans cells (LC) (d). Images provided by R. Gregory.

Cultured primary keratinocytes have a short proliferative lifespan which can be a limitation when using these cells in studies. Primary keratinocytes extracted from neonatal foreskins are capable of undergoing approximately 50 population doublings under ideal culture conditions whereas adult keratinocytes can undergo approximately 20 population doublings before entering senescence (Rheinwald and Green, 1975). In order to obtain a high number of low passage cells, a large tissue sample must be provided, which is not practical in many situations.

Several methods have been used in attempts to create immortal keratinocyte cell lines. Transfection of normal neonatal keratinocytes with a retrovirus overexpressing the catalytic component of telomerase (hTERT) has been shown to increase proliferation to at least 20 population doublings beyond normal levels (Bodnar et al., 1998), however these cells still entered senescence within 100 population doublings (Dickson et al., 2000). Inactivation of p53 and $p16^{INK4a}$, via transduction of keratinocytes with the human papilloma virus oncogenes E6 and E7 in conjunction with hTERT overexpression, resulted in immortalisation of neonatal foreskin keratinocytes (Kiyono et al., 1998). A subsequent study by Ramirez *et al* has shown that overexpression of both Cdk4 and hTERT extended proliferation beyond 300 population doublings, and that these double-transfected keratinocytes were capable of differentiating into organotypic skin models (Ramirez et al., 2003).

More recent studies have shown that the proliferative lifespan of neonatal keratinocytes can be increased indefinitely by supplementing the culture medium with (+)-(R)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl)cyclohexane-carboxamide dihydro-chloride (also known as Y-27632), a cell-permeable, highly potent selective competitive inhibitor of Rho-associated coiled-coil-containing protein kinase (ROCK) I and ROCK II (Chapman et al., 2010; van den Bogaard et al., 2012). Discovered in 1996 (Ishizaki et al., 1996; Leung et al., 1996; Matsui et al., 1996; Nakagawa et al., 1996), ROCK I and II have since been shown to be involved in many signalling pathways, from actin cytoskeletal organisation (Maekawa et al., 1999), cellular adhesion (Ren et al., 1999) and motility (Fukata et al., 1999) to differentiation (McMullan et al., 2003),

apoptosis (Petrache et al., 2001) and cell survival (Han et al., 2005). In addition, stress fibre formation, the rapamycin cascade and ERK signalling pathways, which are all ROCK dependent, can modulate the expression of cyclins and cyclin dependent protein kinases (CDKs), which are major regulators of the cell cycle (Welsh et al., 2001; Roovers and Assoian, 2003; Park et al., 2011; Wedel et al., 2011).

In 2003 McMullan *et al* demonstrated that keratinocyte differentiation is regulated by the Rho and ROCK signalling pathway, observing that inhibition of ROCK I and II resulted in increased keratinocyte proliferation and colony formation (McMullan et al., 2003). In the same study, inhibition of RhoA decreased terminal differentiation as noted by reduced expression of involucrin and transglutaminase. Conversely, McMullan *et al* demonstrated that overexpression of ROCK II results in cell cycle arrest and induces terminal differentiation. It has since been demonstrated that ROCK inhibition can increase proliferation in several other cell types, including astrocytes (Yu et al., 2012) and colon and pancreatic cancer cell lines (Nakashima et al., 2010; Nakashima et al., 2011). By contrast, inhibition of ROCK has been shown to inhibit proliferation of smooth muscle cells (Rees et al., 2003) and induce apoptosis in airway epithelial cells (Moore et al., 2004) including small cell lung cancer cells (Yang et al., 2012).

In the current thesis, it was investigated whether the immortalisation of keratinocytes, through the inclusion of a Rho kinase inhibitor in the culture medium, may allow these cells and/or the resulting epidermal equivalents to be used as models to test the effects of irritant and/or sensitising compounds. However, it is possible that the use of a Rho kinase inhibitor could influence responses to irritants and/or sensitisers, therefore it is necessary to analyse the proinflammatory responses to irritant and sensitising chemicals to determine the suitability of Rho kinase inhibitor treated (RhoKIT) keratinocytes as models of normal keratinocyte responses.

4.2 Methods

4.2.1 Cell culture

Keratinocytes were isolated from both male and female patients aged between 26 and 92 years old (**Table 4.1**) as described in chapter 2.

Table 4.1: Ages of skin donors from which keratinocytes were extracted.

Age group	Number of patients
20 - 30	1
31 – 40	0
41 – 50	4
51 – 60	1
61 – 70	2
71 – 80	1
81 – 90	1
91 - 100	1
Total	**11**

4.2.2 RNA extraction

Cells were lysed in 350 µl of RLT lysis buffer before RNA extraction. RNA extraction was performed as described in chapter 2.

4.2.3 RT-PCR

Real-time PCR was performed as described in chapter 2 using either pre-coated PCR plates or manually added primers (SABioscience). A list of genes analysed is shown in **Appendix 1**. For manually added primers, *26S* and *YWAZ* were used as housekeeping genes. The mean result of the housekeeping genes was used to calculate ΔCt values.

4.2.4 Differentiation of keratinocytes

Keratinocytes were differentiated on polycarbonate membranes or collagen scaffolds as described in chapter 2 in either CnT57.3D (Cell'n'tech), **3D.1 media** (3:1 [v/v]

DMEM-F-12 [Ham], 10% FBS, 0.4 µg/ml hydrocortisone, 5 µg/ml transferrin, 8.4 ng/ml cholera toxin) or **3D.2 media** (3:1 [v/v] DMEM-F-12 [Ham], 10% FBS, 0.4 µg/ml hydrocortisone, 50 U/ml penicillin, 50 µg/ml streptomycin, 24.3 µg/ml adenine, 1 mg/ml L-serine, 2 µg/ml L-carnitine, 6.42 µg/ml palmitic acid, 2.1 µg/ml arachidonic acid, 4.2 µg/ml linoleic acid, 0.4 µg/ml vitamin E, 50 µg/ml ascorbic acid, 580 ng/ml insulin, 1.36 ng/ml tiiodothyronine, 8.4 ng/ml cholera toxin, 5 ng/ml keratinocyte growth factor and 2 ng/ml epidermal growth factor).

4.2.5 Immunohistochemical staining of 3D models

Immunohistochemical staining was performed as described in chapter 2 using the antibodies described in **Table 4.2**. Secondary antibodies targeted against rabbit (Dako, E0431) and mouse (Dako, E0413) were used at 1/400 dilutions.

Table 4.2: Antibodies used for immunohistochemical staining of skin models.

Target	Source	Host	Concentration
KLK5	Abcam (ab7283)	Rabbit	2 µg/ml
KLK7	Abcam (ab96710)	Rabbit	5 µg/ml
PAR2	Santa Cruz (H99)	Rabbit	1 µg/ml
Involucrin	Abcam (ab68)	Mouse	300 ng/ml
Keratin 5	Abcam (ab52635)	Rabbit	1/200 (concentration not given)
Keratin 10	Abcam (ab9026)	Mouse	5 µg/ml

4.2.6 Application of irritants to 3D models

Before application of irritants the 3D skin models were visually assessed to confirm that the surface appeared unbroken and smooth indicating that the epidermal reconstruction was intact and had a visible barrier. Any model that showed evidence of leakage of media from the basolateral compartment onto the apical surface or had a rough surface was discarded.

4.3 Results

4.3.1 Effect of Rho kinase inhibition on cell proliferation

Primary normal human keratinocytes (NHK) were extracted from male and female patients ranging from 26 to 92 years old using trypsin to separate epidermis from dermis as described in chapter 2. Although previous studies using a Rho kinase inhibitor had used a fibroblast feeder layer to culture keratinocytes (Chapman et al., 2010; van den Bogaard et al., 2012), in the current study attempts were made to utilise a keratinocyte culture method already established within our laboratory which allowed the culture of keratinocytes directly onto the plastic surface of tissue culture flasks. For each individual donor, keratinocytes were extracted from tissue ranging in size from 0.25 cm^2 to 5 cm^2 and separated into two 25 cm^2 plastic tissue culture flasks. Previous studies have shown 10 μM to be the optimal concentration of Y-27632 for prolonging the keratinocyte growth phase (Chapman et al., 2010; van den Bogaard et al., 2012) or increasing the number of colony forming keratinocytes from a primary tissue sample (Terunuma et al., 2010). Therefore, keratinocytes were maintained in a commercially available, low calcium keratinocyte medium (CnT57, Cell'n'Tech) in the presence (Rho kinase inhibitor treated, termed RhoKIT) or absence (NHK) of 10 μM Y-27632.

The proliferative capacity of NHK and RhoKIT keratinocytes was determined by the total lifespan and the total population doublings of the cells in culture. The total population doubling of the keratinocyte cultures was taken as the cumulative population doublings from passage 1 until the average daily population doubling fell below 0.2, indicating that the keratinocytes had ceased proliferating (Chapman et al., 2010). Proliferative lifespan was calculated from the keratinocyte extraction date to the date at which keratinocytes entered senescence, determined by the same criteria as for total population doublings (i.e. the point at which average daily population doubling fell below 0.2).

When cultured on plastic in a commercial, defined keratinocyte medium, there was no significant difference in the total population doublings reached by NHK (8.12 ± 4.20) and RhoKIT (12.91 ± 4.50) keratinocytes (n=6 separate patient samples, p=0.135, paired Student's t-test) (**Figure 4.2**). There was also no difference in the proliferative lifespan of these cells (NHK 23.00 ± 11.10 days, RhoKIT 26.67 ± 11.84 days, p=0.592). All cultures ceased proliferating within 40 days of extraction from human skin.

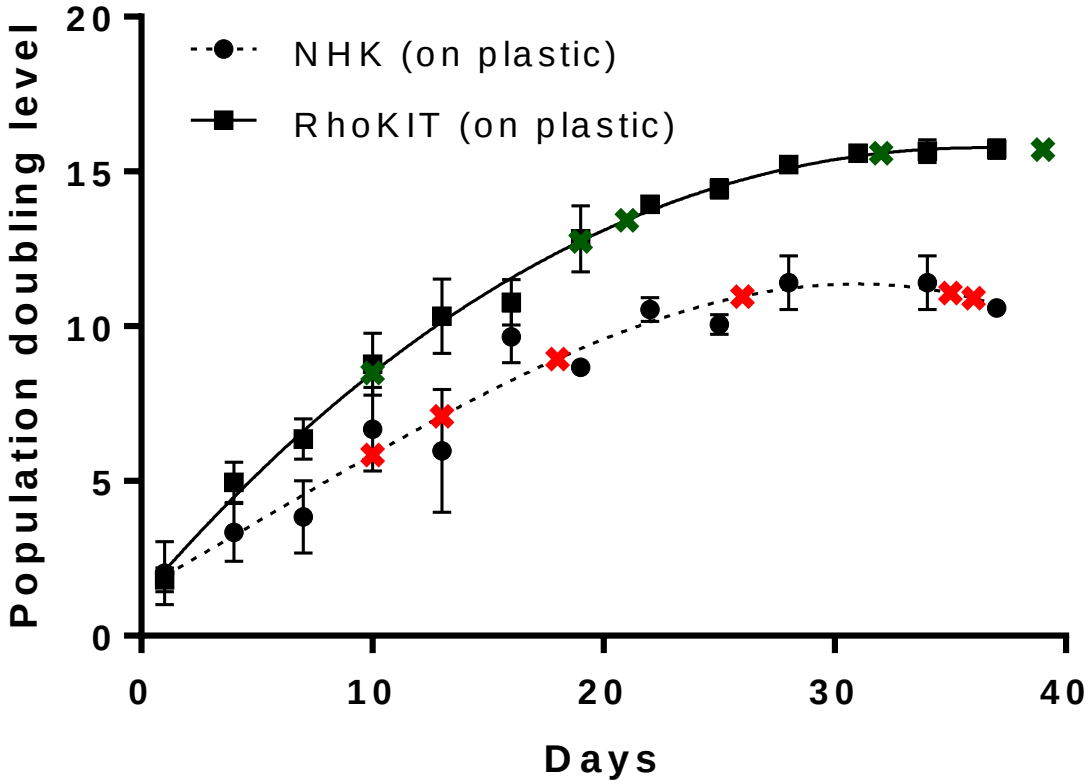


Figure 4.2: Effect of Rho kinase inhibition on primary human keratinocytes grown on plastic. There was no difference in either the total population doublings (p=0.135) or the lifespan (p=0.592) of NHK and RhoKIT keratinocytes. Each point represents mean population doubling over 3 days ± SEM from n=6 separate patient samples. Red (NHK) and green (RhoKIT) crosses indicate days at which individual cultures were deemed to have entered senescence and were discarded.

4.3.2 Varying effects of Rho kinase inhibition in different culture conditions

As described above, when keratinocytes cultured on plastic were treated with a Rho kinase inhibitor, there was no significant increase in total population doublings. This differed from results from a previous study which used keratinocytes from neonatal tissue samples cultured on a feeder layer of 3T3 fibroblasts (Chapman et al., 2010). In order to determine whether the pro-proliferative effect of Y-27632 is dependent on co-culture with fibroblasts, keratinocytes were cultured in the presence of irradiated murine fibroblasts (Swiss-albino 3T3 fibroblasts). When co-culturing keratinocytes with fibroblasts it was necessary to use a serum-supplemented medium (designated 2D-KM), as CnT57 medium does not sustain fibroblast growth.

Compared with NHK cultured on plastic in CnT57 medium, NHK co-cultured with 3T3 fibroblasts in 2D-KM showed an increase in the average lifespan from 23.00 ± 11.10 days to 49.78 ± 19.40 days (p=0.0125, 2-tailed t-test comparison of AUC) (**Figure 4.3**). However, the high level of variation in population growth within these groups meant that there was no significant difference between the total population doublings reached by NHK in CnT57 (8.12 ± 4.20) or 2D-KM (14.15 ± 6.98) conditions (p=0.0898) (**Table 4.3**). RhoKIT keratinocytes cultured on 3T3 fibroblasts in 2D-KM showed significantly increased proliferative capacity compared with RhoKIT keratinocytes cultured on plastic in CnT57 medium. Lifespan was increased 3.23 fold from 26.67 ± 11.84 to 87.73 ± 19.29 days (p<0.0001). Population doublings were increased 4.13 fold from 12.91 ± 4.50 population doublings for CnT-57 conditions to 53.38 ±14.68 populations doublings for 2D-KM conditions (p<0.0001) (**Table 4.4**).

Table 4.3: Effect of Rho kinase inhibition and culture conditions on total lifespan of primary human keratinocytes. Keratinocytes were cultured in the presence or absence of 10 µM Y27632, either on plastic in chemically defined medium (CnT57) or on a feeder layer of 3T3 fibroblasts in serum supplemented medium (2D-KM). Results are shown as mean lifespan in days ± SD. P value was calculated using a paired or unpaired Students' t-test as appropriate .

Culture medium	Lifespan (days)		P value
	NHK	RhoKIT	
CnT57 (cultured on plastic)	23.00 ± 11.10	26.67 ± 11.84	**0.592**
2D-KM (cultured on 3T3)	49.78 ± 19.39	87.73 ± 19.29	**<0.0001**
P value	**0.0125**	**<0.0001**	

Table 4.4: Effect of Rho kinase inhibition and culture conditions on total population doublings of primary human keratinocytes. Keratinocytes were cultured as described for **Table 4.3**. Results are shown as mean lifespan in days ± SD. P value was calculated using a paired or unpaired Students' t-test as appropriate.

Culture media	Population doublings		P value
	NHK	RhoKIT	
CnT57 (cultured on plastic)	8.12 ± 4.20	12.91 ± 4.50	**0.135**
2D-KM (cultured on 3T3)	14.15 ± 6.98	53.38 ± 14.68	**<0.0001**
P value	**0.0898**	**<0.0001**	

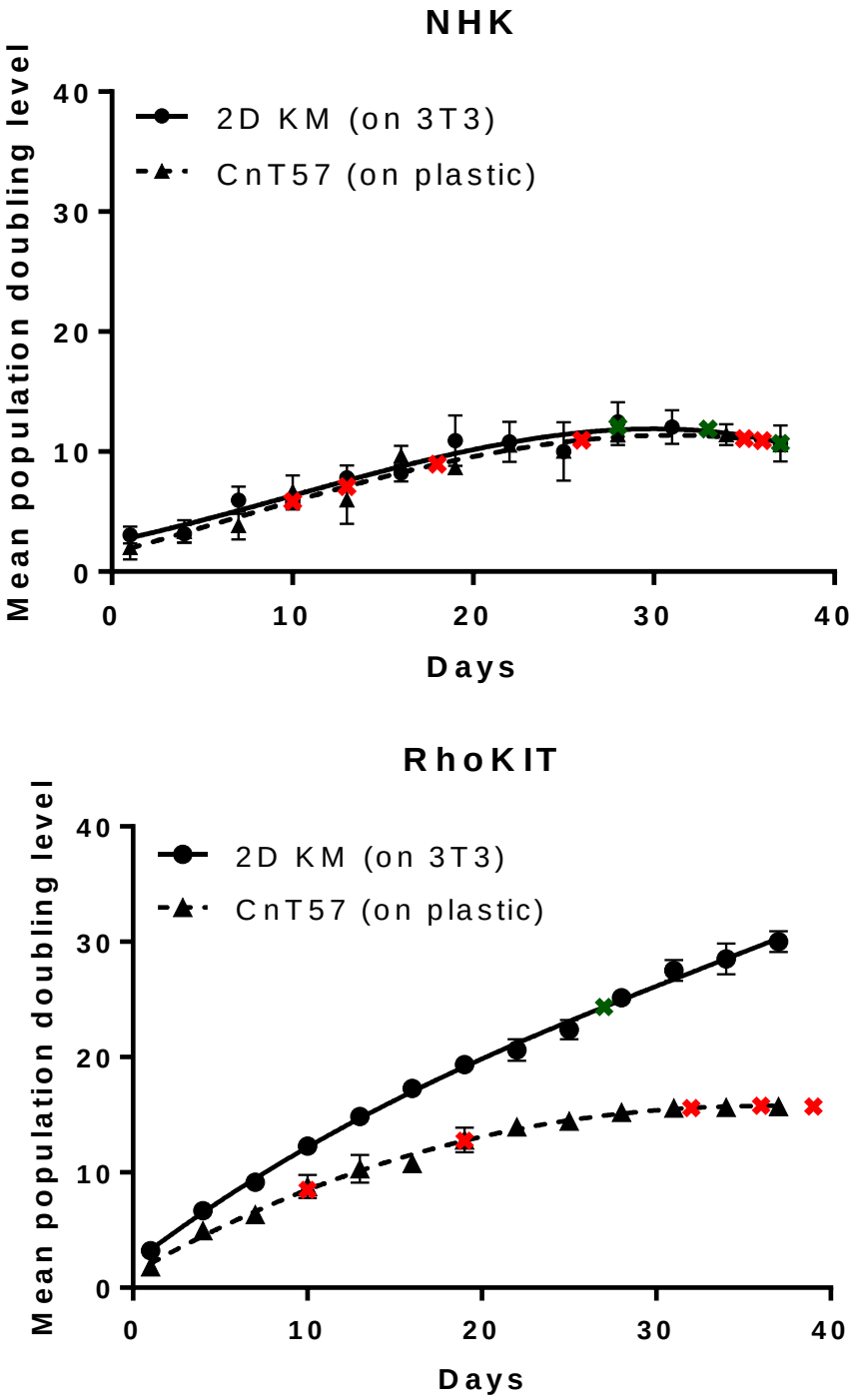


Figure 4.3: Comparison of keratinocyte growth between culture conditions. NHK cultured on plastic showed no difference in total population doublings undergone compared with NHK cultured on 3T3 fibroblasts (p=0.1252), however lifespan was increased 3.23 fold (data beyond 40 days not shown, p<0.0001). RhoKIT keratinocytes cultured on 3T3 fibroblasts showed an increase in both lifespan (p<0.0001) and total population doublings (data beyond 40 days not shown p<0.0001). Red (NHK) and green (RhoKIT) crosses indicate days at which individual cultures were deemed to have entered senescence and were discarded. Results shown are from 6 cultures cultured on plastic in CnT57 media, and 11 cultures cultured on 3T3 fibroblasts in 2D KM media.

When at 90% confluence, the number of keratinocytes cultured on plastic in CnT57 media ($4.36 \times 10^5 \pm 2.61 \times 10^5$ cells) was always less than that of these cells cultured on 3T3 fibroblasts in 2D KM ($1.03 \times 10^6 \pm 3.6 \times 10^5$ cells, p=0.0066). When assessed with an inverted microscope, keratinocytes seeded on 3T3 fibroblasts in the presence of 10 μM Y-27632 appeared to adhere to the tissue culture flasks at a greater density than NHK seeded onto plastic within 4 hours of seeding. NHK and RhoKIT keratinocytes from the same donor were seeded at the same initial density into 3 wells of a 96 well plate in the absence of fibroblasts and incubated in 2D-KM. Culture medium was replaced after 24 hours to remove dead and non-adherent cells. There was no visible difference in the number of dead and non-adherent cells at this stage. As the cell viability assay is dependent on cells undergoing proliferation, a further 48 hours incubation allowed the keratinocytes to fully enter the proliferative stage (Kolly et al., 2005). RhoKIT keratinocytes showed a density of proliferating cells 2.56 fold greater that of NHKs (optical density 0.546 ± 0.28 for RhoKIT and 0.245 ± 0.19 for NHK, p=0.0208), which may be accounted for by the increased adherence at seeding or faster proliferation after adherence of the cells.

In addition to comparing the growth of both NHK and RhoKIT keratinocytes cultured on plastic in CnT57 with growth on 3T3 fibroblasts in 2D KM (**Figure 4.3**), the difference in cell growth potential of NHK and RhoKIT keratinocytes was also compared for keratinocytes cultured on 3T3 fibroblasts. When cultured on feeder fibroblasts in 2D-KM conditions, RhoKIT keratinocytes showed a significant change in both lifespan and total population doublings compared with NHK (**Figure 4.4**). Thus, when media was supplemented with 10 μM Y-27632, lifespan was extended from 49.78 ± 19.39 to 87.73 ± 19.29 days (p=<0.0001) for keratinocytes from the same donor (**Table 4.3**). Total population doubling was increased from 14.15 ± 6.98 for NHK to 53.38 ± 14.68 (P=<0.0001) for RhoKIT keratinocytes (**Table 4.4**). RhoKIT keratinocytes proliferated for a maximum of 137 days and reaching a maximum of 80 population doublings compared with a maximum of 80 days and 21 population doublings for NHK.

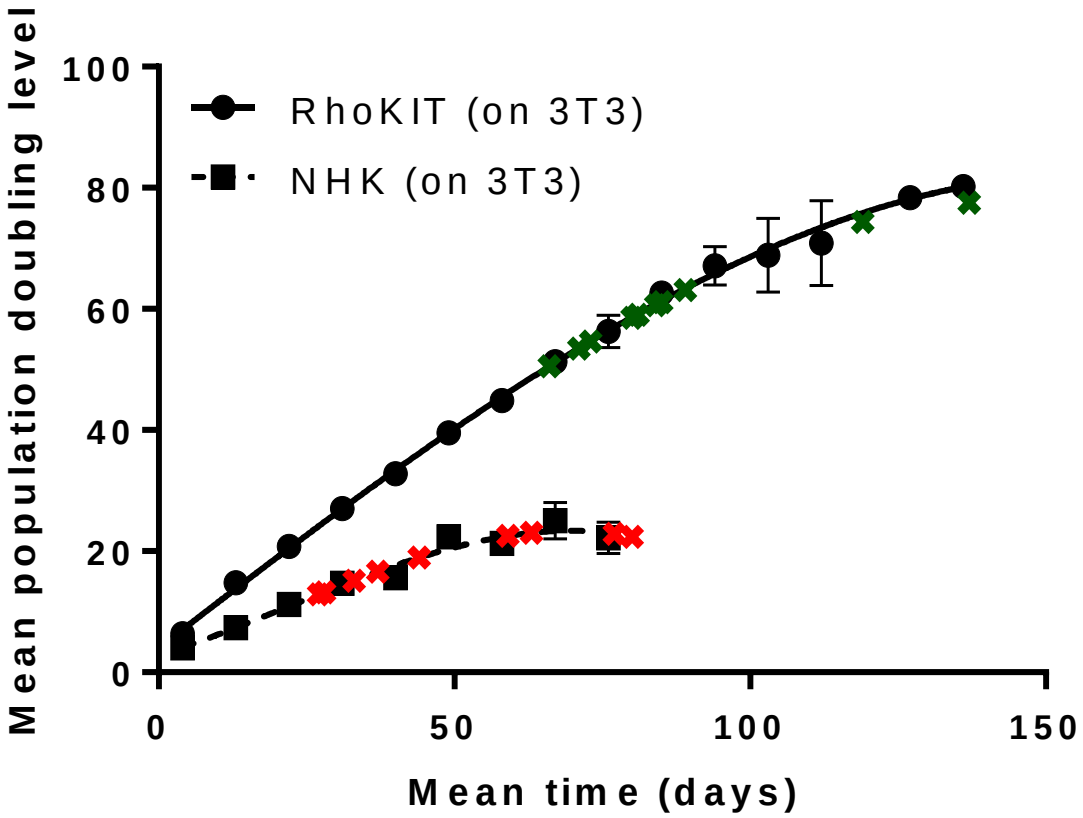


Figure 4.4: Keratinocytes cultured on feeder fibroblasts in the presence of 10 µM Y-27632 show increased proliferative capacity over 150 days. RhoKIT cells demonstrate more population doublings and survive for longer than NHK. Each point represents average population doubling over 9 days ± SEM from 11 separate patient samples. Red (NHK) and green (RhoKIT) crosses indicate days at which individual cultures were deemed to have entered senescence and were discarded.

4.3.3 Effect of Rho kinase inhibition on keratinocyte morphology

Keratinocytes cultured on plastic in CnT57 medium were passaged at 85-90% confluence and imaged before the 1st and 4th passage with an inverted phase-contrast microscope (**Figure 4.5**). RhoKIT cells exhibited an altered morphology compared with NHK at both P0 and P3, developing an elongated or spindly appearance similar to that expected in fibroblasts. The culture medium used was low calcium and serum free and therefore did not sustain fibroblast growth, indicating that abnormal cells observed were keratinocytes rather than contaminating fibroblasts. 10% of RhoKIT cells showed this morphology at P0 compared with <1% of NHK cells.

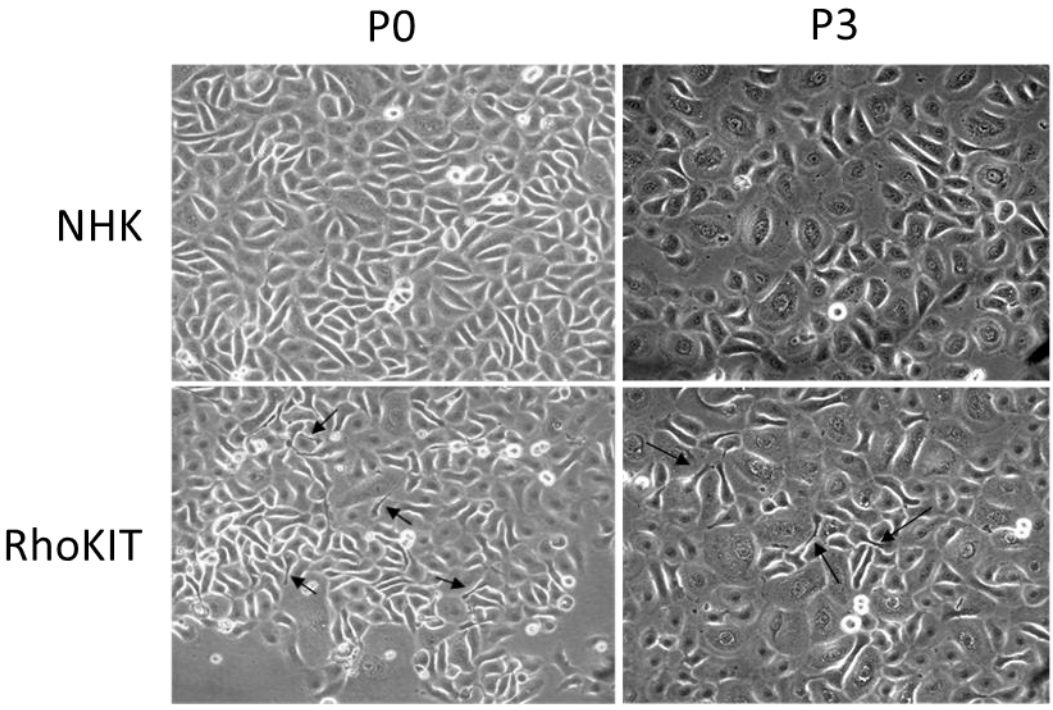


Figure 4.5: Morphological changes in keratinocytes cultured on plastic with Rho kinase inhibition. RhoKIT keratinocytes showed an altered morphology compared with patient-matched NHK when cultured on plastic in defined media (CnT-57). These differences were observed from passage 0 (P0) and were still present at passage 3 (P3). The arrows indicate cells which have developed a more elongated morphology in the RhoKIT cultures. Images representative of cultures from 3 subjects.

Consistent with the reduced cell counts at 90% confluence in keratinocytes cultured on plastic in CnT57 compared with those cultured on 3T3 fibroblasts in 2D KM, cells cultured directly onto plastic had a wider, flattened morphology following passage 1 (**Figure 4.6**). In contrast to keratinocytes cultured on plastic, those cultured on 3T3 feeder fibroblasts maintained a normal 'cobblestone' appearance whether cultured in the presence or absence of a Rho kinase inhibitor. This appearance was maintained for up to passage 30 in the presence of 10 μM Y-27632, while in NHK, drastic changes in morphology were observed between 6-9 passages (**Figure 4.6**), becoming either elongated and granular compared to early passage keratinocytes, or taking on a 'fried egg' appearance, characterised by frilly edges and a large cytoplasmic space. Based on these results and those above, it was determined that culture of keratinocytes would be performed on 3T3 fibroblasts for all subsequent experiments.

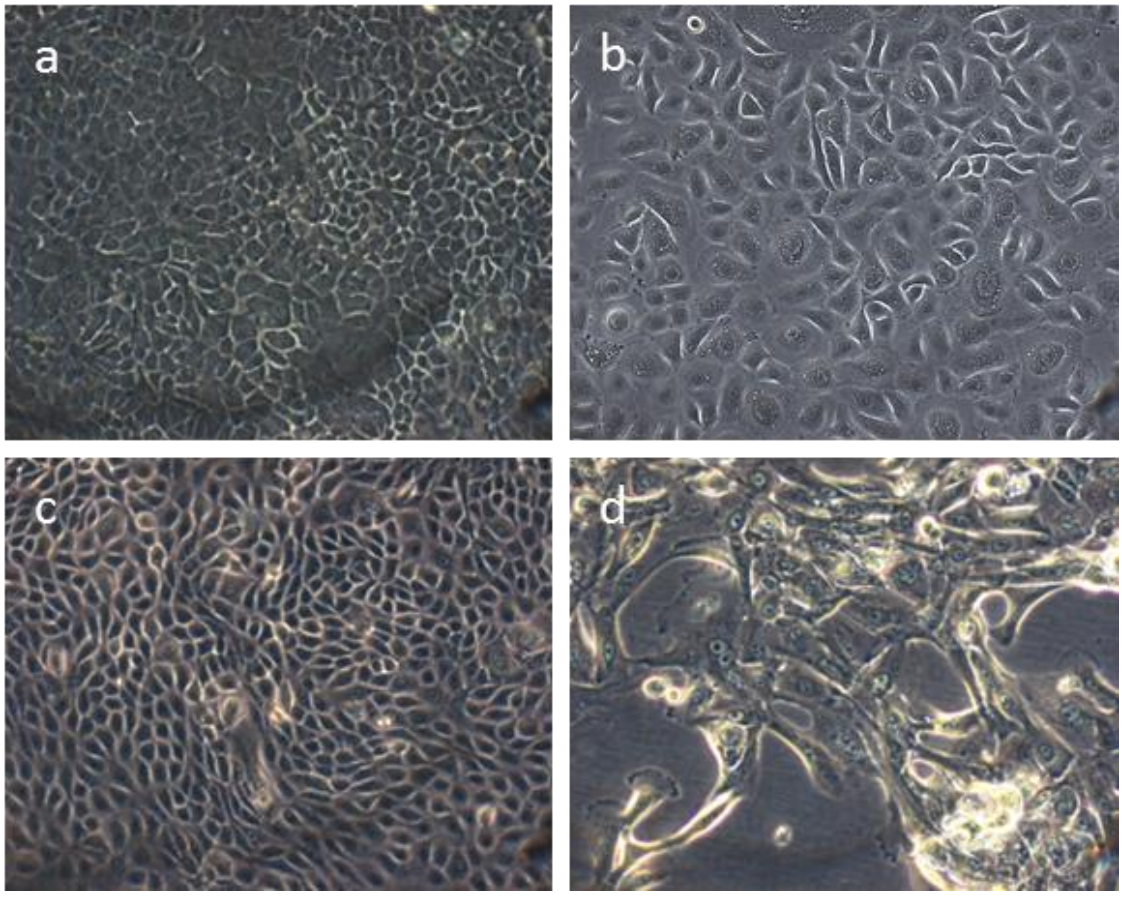


Figure 4.6: Morphological differences of NHK and RhoKIT keratinocytes cultured on 3T3 fibroblasts. a) NHK at passage 0, showing cobblestone like morphology and tightly packed colonies. b) NHK at passage 1 have begun to show a more 'spread out' morphology compared with NHK at passage 0. c) RhoKIT at P13. These cells retain their early passage like morphology. d) NHK at passage 9, which have already started to change morphology and enter senescence. Images representative of n=11 samples.

4.3.4 Effect of age on proliferative capacity of keratinocytes

In the absence of Rho kinase inhibition, the lifespan of NHK from patients aged below 55 years (60.50 ± 15.18 days) was longer than that of NHK from patients older than 55 years (24.20 ± 6.72 days, p=0.0014) (**Figure 4.7**a). In addition, the total population doublings of NHK from patients aged under 55 years (18.81± 5.80) was more than that of NHK from patients aged over 55 years (8.51 ± 3.30, p=0.0097) (**Figure 4.7**b).

The lifespan of keratinocytes isolated from patients aged under 55 years was significantly increased when cultured in the presence of 10 μM Y-27632 (p=0.0480), however, a larger increase in lifespan was achieved in donor matched RhoKIT from patients aged over 55 compared with NHK (p<0.0001) (**Figure 4.7**a). Despite this, the lifespan of RhoKIT keratinocytes from the under 55 age group was greater than that of

the over 55 age group (95.50 ± 22.40 days versus 67.80 ± 7.68 days respectively, p=0.0407). Compared with NHK, population doubling was significantly higher in RhoKIT both from patients aged under 55 (p=0.0006) and over 55 (p=0.0006) (**Figure 4.7**b). There was also a significant difference in the total PD of RhoKIT keratinocytes from the under 55 age group (62.37 ± 13.45) compared with the over 55 age group (42.83 ± 8.48, p=0.0314).

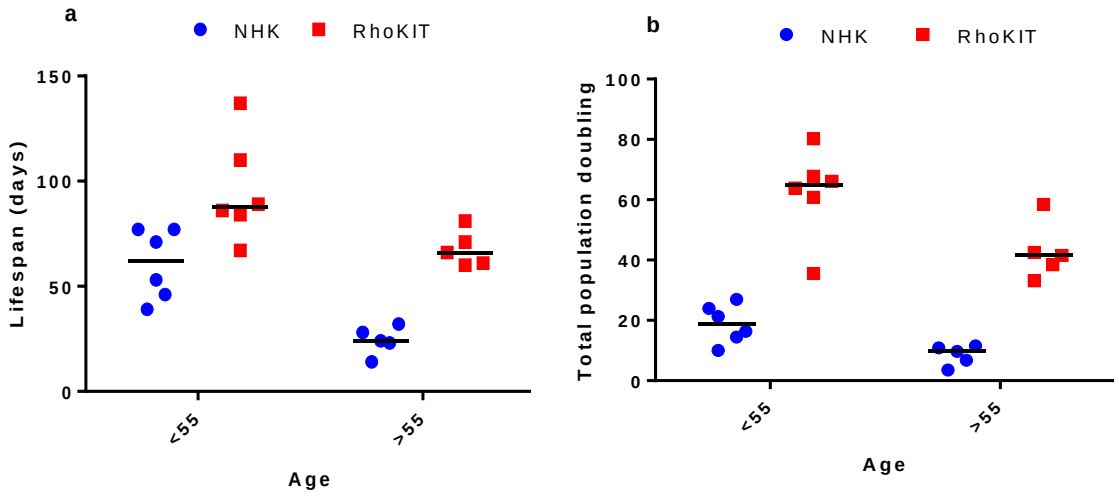


Figure 4.7: Effects of age on NHK and RhoKIT keratinocyte growth. The mean lifespan and total population doublings of NHK from patients aged under 55 years was greater than of NHK from patients aged over 55 years (p=0.0014 and p=0.0097 respectively). Supplementation of culture media with 10 μM Y-27632 increased both the lifespan and total population doublings of under 55 (p=0.0480 and p=0.0006 respectively) and over 55 (p=<0.0001 and p=0.0006 respectively) age groups. Each point represents a single patient. The bars represent medians.

4.3.5 Cell cycle gene expression in NHK and RhoKIT keratinocytes

In order to determine whether the inhibition of Rho kinase alters the expression of genes involved in regulation of the cell cycle, mRNA was extracted from NHK and RhoKIT keratinocytes at passage 1 (designated early stage) and following 12 population doublings (designated late stage). 85 genes involved in the cell cycle and apoptosis control were analysed from early and late stage NHK and RhoKIT keratinocytes, derived from the same donor, for 3 individual patients aged 35, 47 and 79 years. Changes in gene regulation of more than 2 fold compared with controls were considered significant.

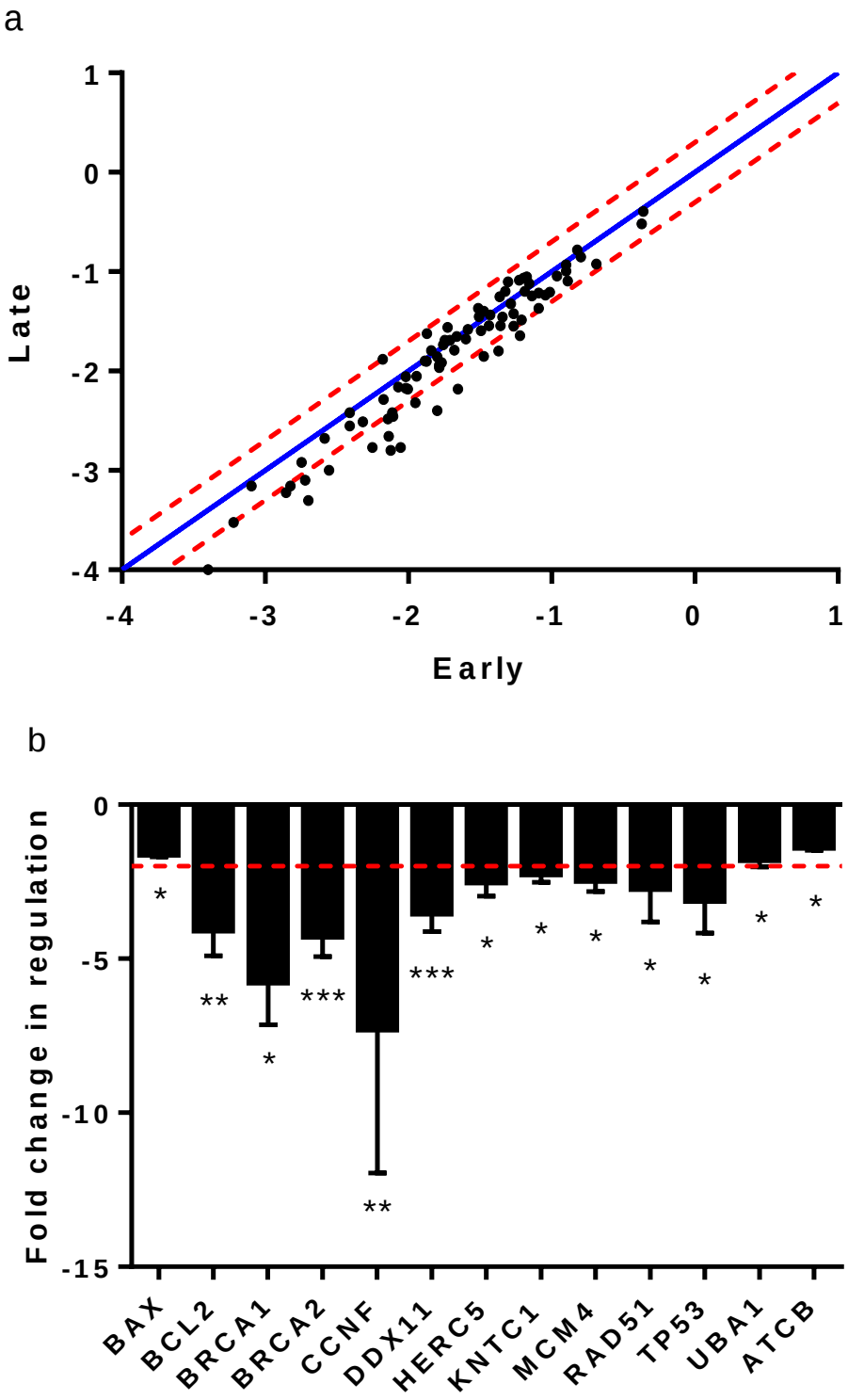


Figure 4.8: Significant changes in cell cycle gene expression of NHK following 12 population doublings. a) log10 of normalised gene expression in early and late stage NHK. 20 genes showed reduced expression following 12 population doublings from passage 1, while a single gene was upregulated. Each point represents the expression of a cell cycle or apoptosis gene in early and late stage NHK (documented as Early and Late on the X and Y axes respectively in upper graph). The blue line indicates no difference in expression; red lines indicate a 2 fold change in expression. b) Ten genes were significantly downregulated more than 2 fold, and 3 genes were significantly downregulated less than 2 fold. Red line indicates a 2 fold change in expression. $*p<0.05$, $**$ $p<0.01$ and $***$ $p<0.0001$ from 3 experiments.

Table 4.5: Known roles for genes which are significantly downregulated more than 2 fold from early to late stage in NHK. Expression was decreased in all genes listed following 12 population doublings from passage 1. Uniprot accession numbers are shown in italics for each protein.

Gene	Protein	Function
BCL2	B - cell lymphoma 2 *(P10415)*	Anti-apoptotic, plays a role in several pathways including regulating cell death by controlling membrane permeability of the mitochondria and inhibition of caspase activity.
BRCA1	Breast cancer type 1 susceptibility protein *(P38398)*	Tumour suppressor required for normal cell cycle progression from G2 to mitosis.
BRCA2	Breast cancer type 2 susceptibility protein *(P51587)*	Tumour suppressor which directly interacts with RAD51 to repair DNA damage.
CCNF	Cyclin-F *(P41002)*	Inhibitor of centrosome reduplification.
DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 11 *(Q96FC9)*	DNA helicase involved in cell proliferation.
HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5 *(Q9U114)*	Mediates ISGylation of protein targets, inhibits viral replication.
KNTC1	kinetochore associated 1 *(P50748)*	Component of the mitotic checkpoint which prevents premature exit from mitosis.
MCM4	minichromosome maintenance complex component 4 *(P33991)*	Component of the MCM complex which is essential for 'once per cell' DNA replication, initiation and elongation.
RAD51	RAD51 *(Q06609)*	Participates in DNA damage response pathways.
TP53	Tumour protein 53 *(P04637)*	Tumour suppressor which negatively regulates cell division.

In NHK, 20 genes were downregulated more than 2 fold following 12 population doublings compared with cells at passage 1 (**Figure 4.8**a). Of these 20, 10 genes showed statistically significant downregulation of greater than 2 fold (**Figure 4.8**b). The roles of these genes are described in **Table 4.5**. Three genes showed statistically significant downregulation of less than 2 fold following 12 population doublings.

In comparison to the 20 genes which were downregulated more than 2 fold in NHK following 12 population doublings, RhoKIT keratinocytes showed an alteration in expression of only 3 genes (**Figure 4.9**a). *CDKN2A* and *DIRAS3* were upregulated, and *GADD45A* which was downregulated more than 2 fold compared with RhoKIT keratinocytes at P1. The only gene which is significantly altered in late stage RhoKIT keratinocytes compared with those at early stage is *GADD45A* (**Figure 4.9**b), which encodes for growth arrest and DNA-damage-inducible protein (GADD45α). A study by Hildisheim *et al* demonstrated that GADD45α induces apoptosis and cell cycle arrest through maintenance of p38 and c-JNK MAPK activation, and that a loss of GADD45α function leads to inadequate p53 activation and subsequent loss of normal activation of G_1 and G_2 checkpoints (Hildesheim et al., 2002).

In order to ascertain whether RhoKIT keratinocytes maintain a similar gene profile to NHK following a single passage, the expression of cell cycle and apoptotic genes in early stage NHK and RhoKIT keratinocytes was compared. 8 genes were identified as having an expression level of more than 2 fold lower in RhoKIT keratinocytes compared with NHK, and 1 gene was more than 2 fold higher (**Figure 4.10**a). By contrast, comparison of late stage RhoKIT keratinocytes with late stage NHK indicated 20 genes were upregulated and 4 genes were downregulated in the RhoKIT cells (**Figure 4.10**b), although not all of these genes reached statistically significant levels.

Of all the genes tested, 17 were identified as having statistically significantly altered expression in early stage RhoKIT keratinocytes compared with NHK. However, of these only 4 showed a change in expression of more than 2 fold (**Figure 4.11**a). *CCND1* and *CCND2*, which encode for cyclin D1 and cyclin D2 were both downregulated. These cyclins are required for G1/S transition and play a role in tumourogensis (Imoto et al., 1997). Conversely, the tumour suppressor genes *CDKN2A* and *CDKN2B*, which encode for cyclin dependent kinase 2a and 2b, were also downregulated (**Table 4.6**). The seemingly opposing nature of these changes in gene expression may be explained by the fact that *CDKN2A* and *CDKN2B* are positively regulated by *CCND1* and *CCND2*

(La Thangue, 1994), therefore it could be expected that altered expression of these regulating genes would lead to similar changes in *CDKN2A* and *CDKN2B* expression.

Five genes (*BCL2*, *CCNF*, *DDX11*, *GTSE1* and *MKI67*) were significantly upregulated more than 2 fold in late stage RhoKIT keratinocytes compared with late stage NHK (**Figure 4.11**b). The affected genes are all anti-apoptotic or pro-proliferative (**Table 4.6**), which correlates with the increased proliferative capacity of RhoKIT keratinocytes.

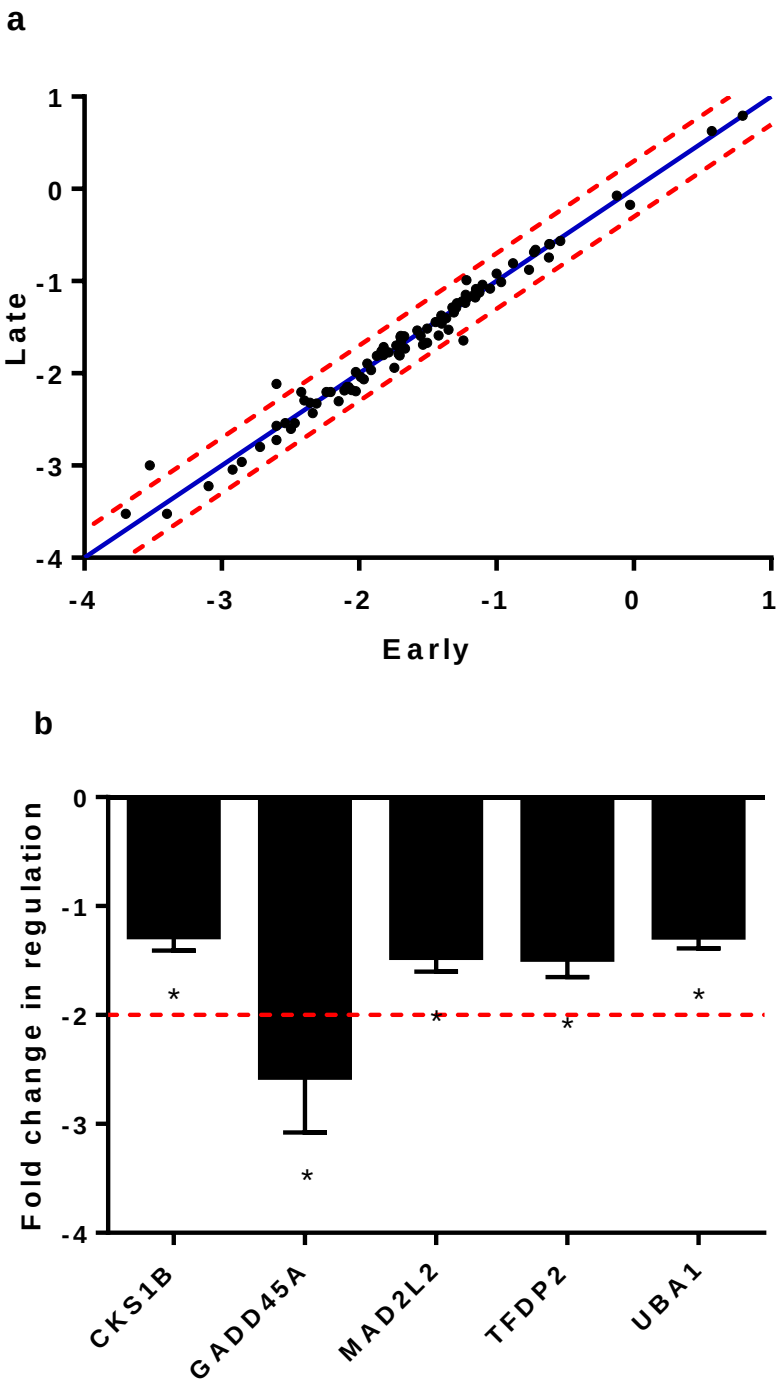


Figure 4.9: Comparison of cell cycle gene expression in RhoKIT keratinocytes over 12 population doublings. a) Log10 of normalised gene expression in early (passage 1) and late (after 12 population doublings) stage RhoKIT. RhoKIT cells show an upregulation of 2 genes and downregulation of a single gene from early stage to late stage. Each point represents the expression of a cell cycle or apoptosis gene in early and late stage RhoKIT keratinocytes. The blue line indicates no difference in expression; red lines indicate a 2 fold change in expression. b) Genes which had significant changes in expression from early stage to late stage. Only GADD45A shows significant downregulation or more than 2 fold in late stage compared with early stage RhoKIT keratinocytes $*p<0.05$ from 3 experiments.

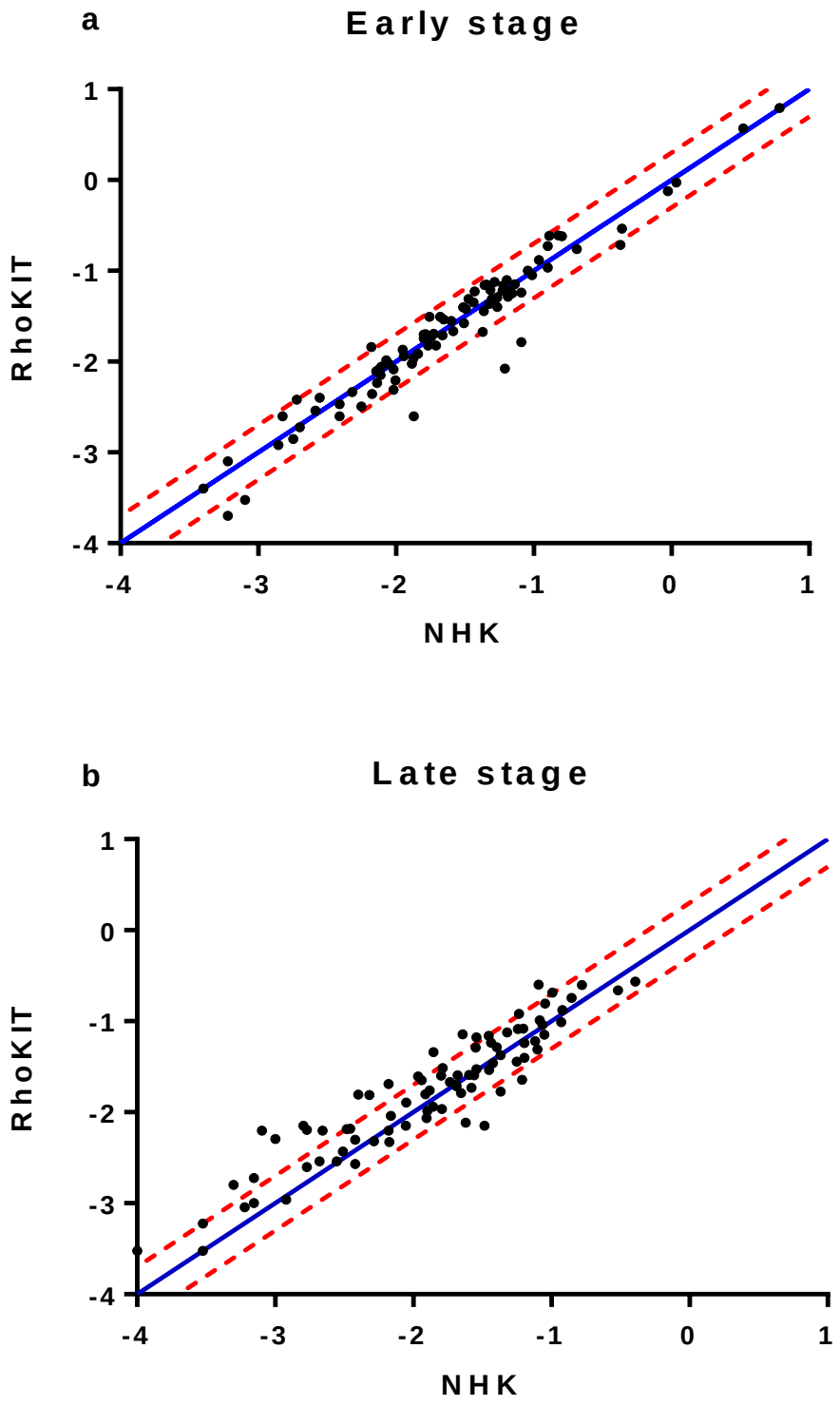


Figure 4.10: Variation in cell cycle gene expression in NHK and RhoKIT. Few differences in cell cycle gene expression were observed between the early passage NHK and RhoKIT samples (a), but there was a large amount of variation in gene expression between the late passage samples (b). Each point represents the log10 of normalised expression of a cell cycle or apoptosis gene in NHK and RhoKIT keratinocytes. The blue line indicates no difference in expression; red lines indicate a 2 fold change in expression. Figure shows results of 3 experiments.

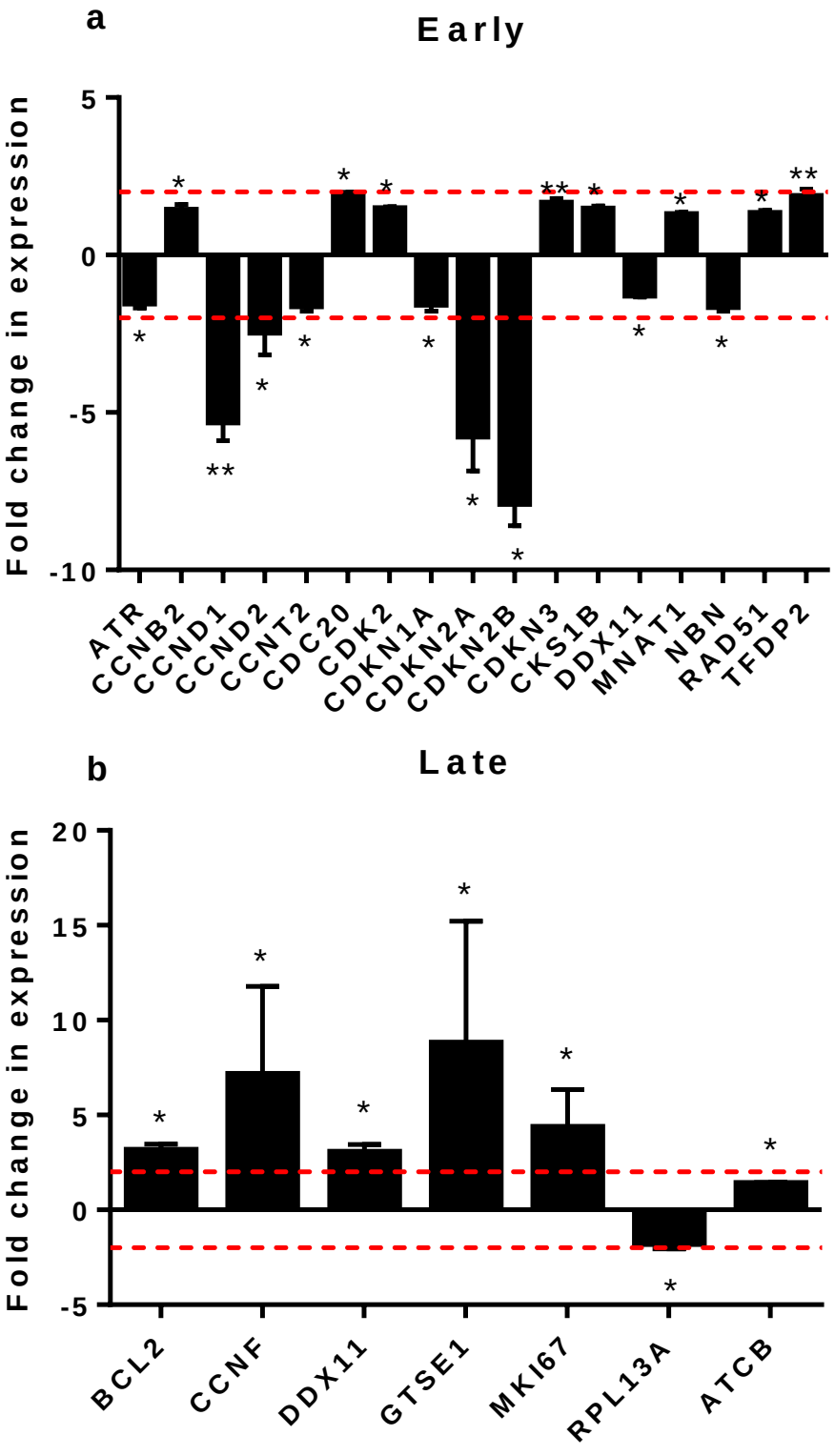


Figure 4.11: Significant changes in gene expression in early and late passage NHK compared with RhoKIT keratinocytes. The red line indicates a threshold of 2 for change in gene expression. ∗ $p<0.05$, ∗∗ $p<0.01$ from paired NHK and RhoKIT keratinocytes cultures from 3 separate donors. Genes are shown on the X axis.

Table 4.6: Altered gene expression in RhoKIT keratinocytes compared with NHK at passage 1 (early) and following 12 population doublings (late passage). At early passage the genes listed were downregulated in RhoKIT keratinocytes compared with NHK. Following 12 population doublings the genes listed were upregulated. Uniprot accession numbers are given in italics.

Early passage (genes downregulated)		**Late passage (genes upregulated)**	
Gene	**Function**	**Gene**	**Function**
CCND1 *(P24385)*	Required for G1/S transition	**BCL2** *(P10415)*	Anti-apoptotic
CCND2 *(P30279)*	Required for G1/S transition	**CCNF** *(P41002)*	Possible regulator of G2-M transition
CDKN2A *(Q8N726)*	Tumour suppressor	**DDX11** *(Q96FC9)*	DNA helicase involved in cell proliferation
CDKN2B *(P42772)*	Tumour suppressor	**GTSE1** *(Q9NYZ3)*	Binds p53 and represses apoptosis
		MKI67 *(P46013)*	Involved in cell proliferation

4.3.6 Normal human keratinocytes from older patients do not form organotypic skin in commercial 3D culture conditions

The results above have shown that RhoKIT keratinocytes can undergo significantly more population doublings than NHK, making them potentially useful in studies requiring a monolayer. In order to test the effects of irritants on these keratinocytes in a tissue structure more similar to normal skin, a human skin equivalent which mimics the barrier properties of the epidermis would need to be produced from the RhoKIT keratinocytes. Although RhoKIT keratinocytes are capable of producing organotypic skin models (Chapman et al., 2010), these have mainly been from younger donors. Therefore, it was first necessary to determine the optimal conditions for differentiation of RhoKIT and NHK keratinocytes from older donors. Optimal conditions using a commercially available system (Cell'n'tech 3D) had previously been used successfully in the Dermatopharmacology Unit, University of Southampton to create fully differentiated 3D skin models from keratinocytes extracted from neonatal foreskin. Therefore, initial experiments were performed using neonatal foreskin

keratinocytes in order to gain experience in this type of tissue culture. The resultant skin models showed distinct, well stratified layers with a well-formed stratum corneum (**Figure 4.12**a).

Following successful differentiation of neonatal keratinocytes, it was next attempted to differentiate NHK from older subjects. Cells were used at passage 1 and treated in the same manner as the neonatal keratinocyte cultures. The resultant models exhibited drastically altered morphology compared with the models using neonatal keratinocytes (**Figure 4.13**a). The keratinocytes of the viable layers lacked definition and was limited to a depth of 2 or 3 cells, compared with 7 or 8 for neonatal keratinocytes. Also, there was evidence of parakeratosis in the older models, with nuclei present in the stratum corneum, which was not present in neonatal models.

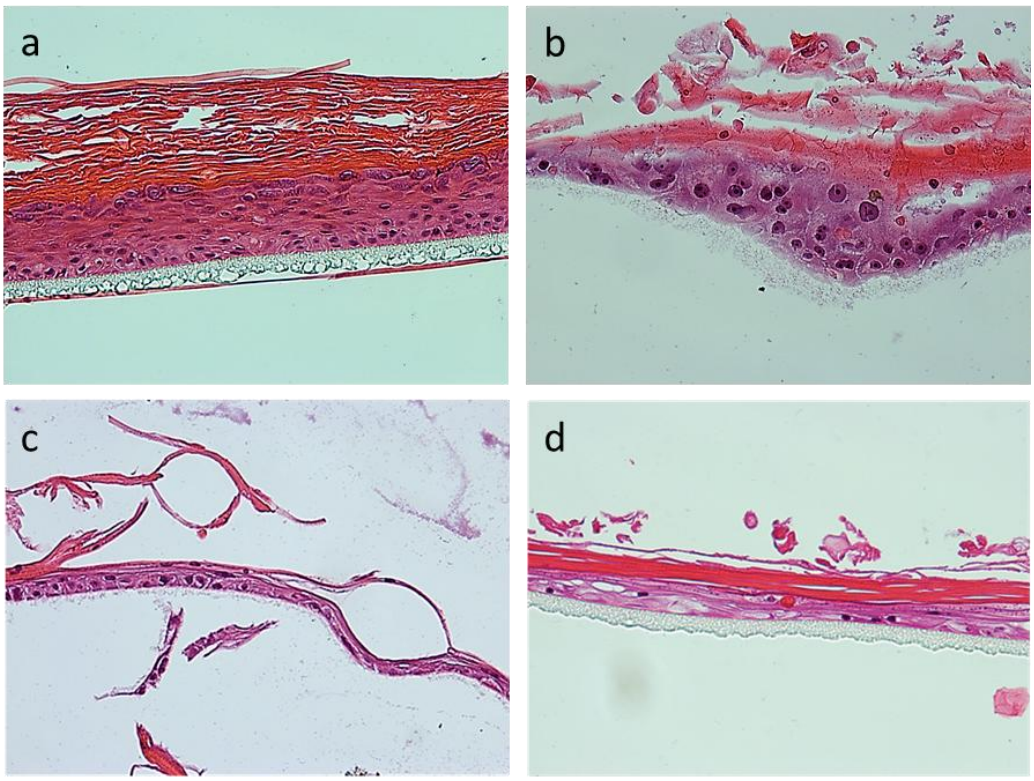


Figure 4.12: NHK from older patients do not differentiate into stratified epithelium in commercial 3D culture conditions (previous page). (a) Neonatal NHK cells and (b) NHK from a 92 year old donor at passage 1 cultured in CnT57 media during 2D culture and CnT57.3D during culture at air-liquid interface (ALI) for 20 days. Neonatal NHK differentiate into well stratified layers including a well-defined stratum basale, stratum spinosum, stratum granulosum and stratum corneum. NHK from a 92 year old donor form an ill-defined stratum corneum, with nucleated keratinocytes visible. No defined layers were visible in the viable compartment of the model below the stratum corneum. (c) RhoKIT keratinocytes from a 92 year old donor at P1 and (d) P5 differentiated into defined stratum corneum, however were still not representative of normal human skin.

Although it was not possible to differentiate NHK at passages higher than 3, RhoKIT keratinocytes grown on 3T3 fibroblasts in 2D culture continued to differentiate at ALI up to passage 5 for patients aged between 45 and 92. However, supplementation of the differentiation media with 10 μM Y-27632 resulted in highly dysplastic NHK models, with the keratinocytes showing abnormal patterns of differentiation throughout the thickness of the epidermis (**Figure 4.13**). It appeared that while some cells had undergone terminal differentiation to form stratum corneum, these cells were not confined to the upper layers of the model. There was little difference in the morphology of NHK and RhoKIT keratinocytes from adult donors differentiated in Y-27632 supplemented CnT57.3D (**Figure 4.13**), with high levels of dysplasia present throughout all the layers of the reconstructed epidermis. The stratum corneum of Y-27632 treated models was scant in comparison to that of the models where the NHK and RhoKIT keratinocytes did not contain Y-27632 during the differentiation stage.

To determine whether the effects of Rho kinase inhibition during differentiation observed in the models using adult keratinocytes were due to the age of the keratinocytes used, neonatal keratinocyte were subjected to Rho kinase inhibition during differentiation (**Figure 4.14**b). Although the overall thickness of the live cell layers within the neonatal epidermal equivalent remained unchanged ($p=0.2266$) (**Figure 4.14**c), the total number of cells contributing to this thickness was significantly lower in 10 μM Y-27632 treated models than controls grown in normal conditions ($p<0.0001$) (**Figure 4.14**d). The cells of the basal layer appear large and vacuolated, however there was still a clear stratum spinosum, stratum granulosum and stratum corneum.

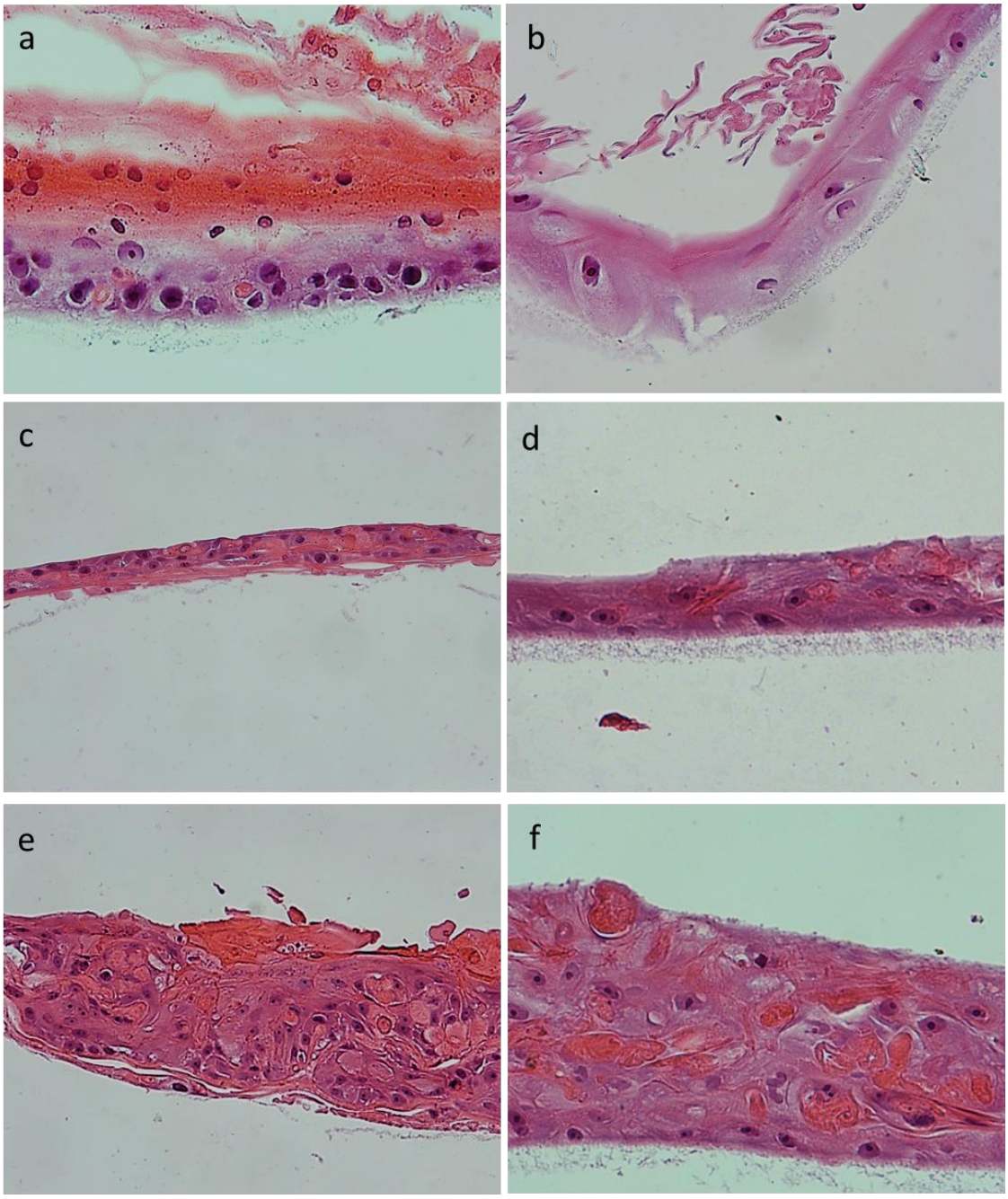


Figure 4.13: Rho kinase inhibition during differentiation alters the morphology of 3D epidermal models. NHK (P1) from a 92 year old donor (a, c+d) and RhoKIT keratinocytes (P1) from an 88 year old donor (b, e+f) differentiated in CnT57.3D (a+b), or CnT57.3D supplemented with 10 μM Y-27632 (c-f). NHK (c+e) and RhoKIT keratinocytes (d+f) differentiated in the presence of 10 μM Y-27632 resulted in both normal thickness (c+d) and abnormally thick models (e+f).

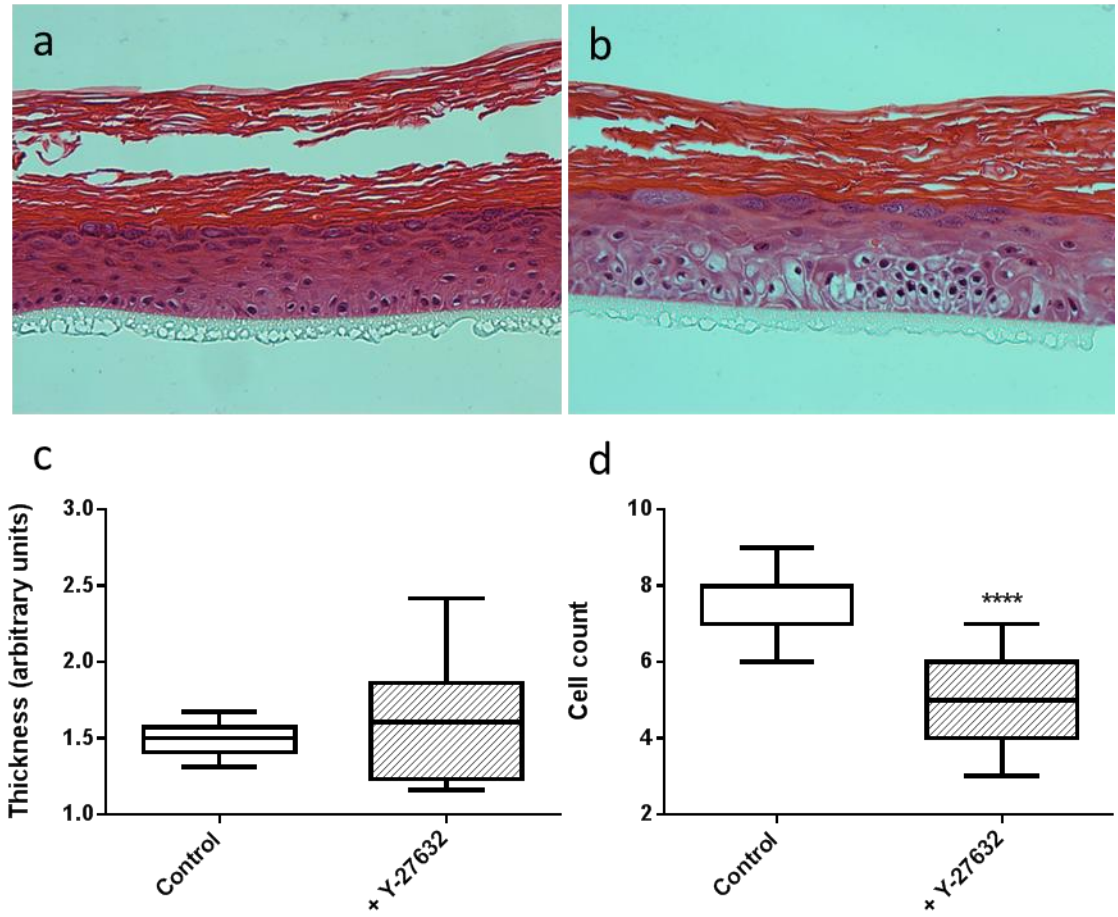


Figure 4.14: Rho Kinase inhibition alters differentiation of NHK in commercial 3D culture conditions (previous page). a) Neonatal NHK cells cultured in CnT57 medium during 2D culture and CnT57.3D (commercial differentiation media) during culture at air-liquid interface for 20 days. b) Neonatal HPEK cells cultured in CnT57 media during 2D culture and CnT57.3D supplemented with 10 μM Y-27632 during ALI culture for 20 days. 10 vertical lines were measured from the stratum basale to the uppermost live layers of the stratum granulsoum to quantify the thickness of the live cell layers (c). The number of cells intersected by the vertical lines used to calculate (c) were counted to quantify the number of cells layers in the viable epidermis component of the epidermal equivalent (d). **** $p<0.0001$.

4.3.7 Neither NHK nor RhoKIT keratinocytes from elderly patients can be successfully cultured on collagen scaffolds

We next attempted to differentiate adult keratinocytes in serum-supplemented differentiation media in the presence of 3T3 fibroblasts. A collagen structure is required to provide a matrix within which to culture the fibroblasts, and serum supplemented medium is required to sustain these cells as CnT57.3D is formulated to prevent fibroblast survival. Collagen scaffolds infused with 3T3 fibroblasts were formed on the surface of polycarbonate membranes. Keratinocytes were seeded onto the surface of the collagen and differentiated in 3D.1 medium, a specifically formulated medium designed to sustain both keratinocyte and fibroblast growth.

Adult RhoKIT keratinocytes differentiated in the absence of Y-27632 showed a significantly altered morphology when differentiated on collagen-membrane scaffolds compared with those cultured on membrane only in CnT57.3D media (**Figure 4.15**). The cells appeared non-viable, and there were no clearly definable layers. However, when the differentiation medium was supplemented with 10 μM Y-27632 it was possible to identify a distinct stratum corneum. A similar morphology was observed in the viable layers of the collagen-membrane and membrane-only models. Despite this, in all cases the epidermis was much thicker at the edges of the collagen scaffold model, and in some cases was absent completely at the central part of the surface.

The inclusion of the polycarbonate membrane in conjunction with the collagen scaffold may have reduced the diffusion of medium to the epidermal cells at the centre of the surface epithelium. To determine if removal of the polycarbonate membrane improved differentiation of keratinocytes, collagen scaffolds infused with 3T3 fibroblasts were incubated on a wire mesh which allowed basolateral feeding of the model. Whereas differentiation of keratinocytes was inconsistent when cultured at ALI on collagen supported by a membrane, when collagen was supported by a wire mesh the resultant epidermal construct was evenly distributed across the entire surface of the collagen matrix (**Figure 4.15**).

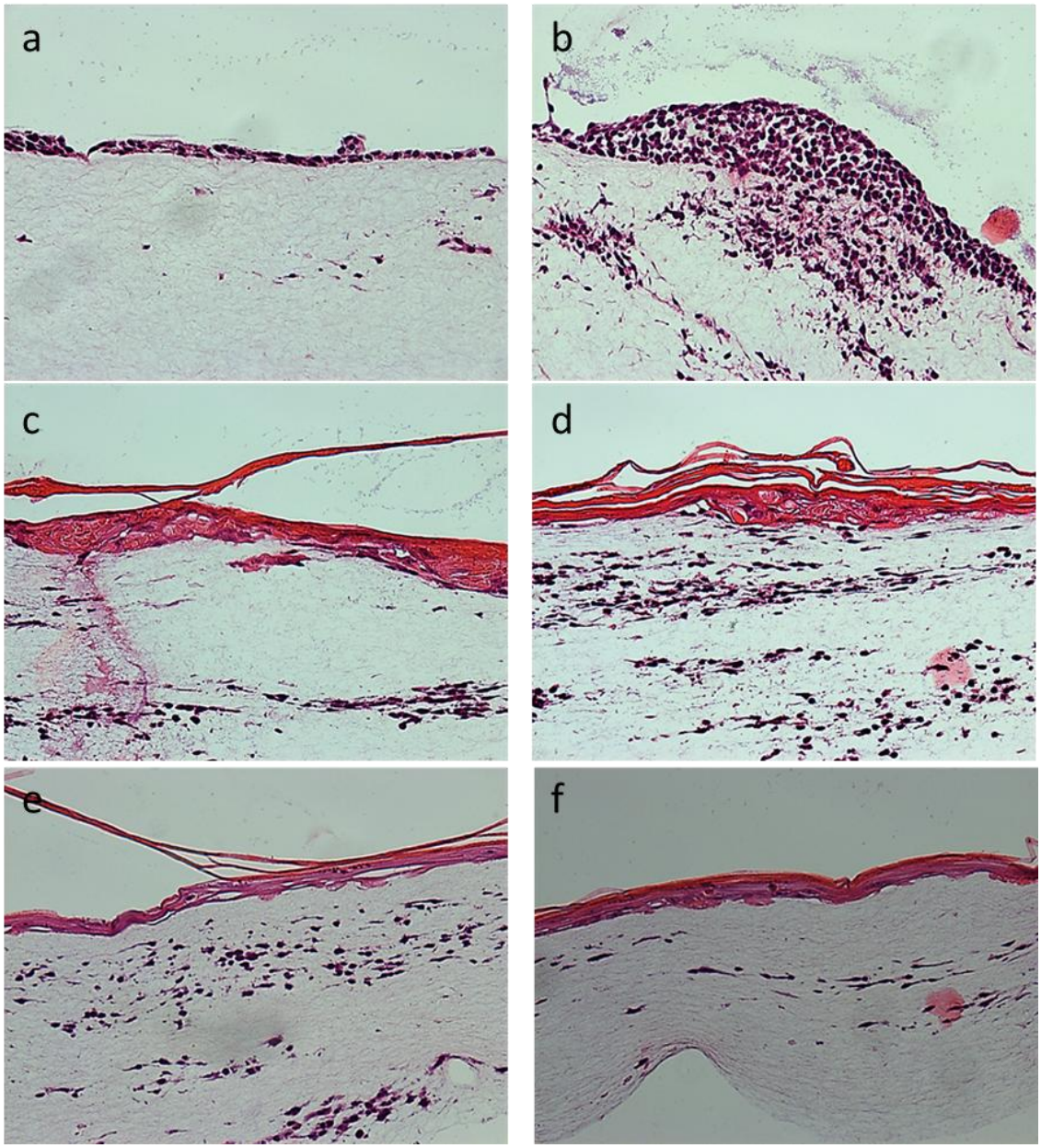


Figure 4.15: RhoKIT keratinocytes from elderly patients did not successfully differentiate on collagen scaffolds. RhoKIT cells (passage 5) cultured on collagen containing 3T3 fibroblasts for 20 days at ALI using 3D.1 media. Collagen was supported by a polycarbonate membrane. (a+b) Models cultured in the absence of Y-27632. Cells show altered morphology to that expected of healthy, normal keratinocytes in 3D culture. No clear layers are visible. (c+d) 3D.1 media supplemented with 10 μM Y-27632 for the duration of the differentiation. A stratum corneum is clearly visible however fewer lower layers can be identified. Keratinocytes extracted from a 64 year old donor (passage 5) were differentiated on a 3T3 infused-collagen disc supported by a wire mesh (e+f). Epidermal growth was consistent across the dermal matrix. Duplicate models are shown. Images representative of 4 models from 2 donors.

4.3.8 Altering ALI culture conditions enables successful 3D differentiation of RhoKIT from elderly patients

As an alternative approach, in order to determine whether altering the differentiation media could improve the stratification of the keratinocytes in the absence of collagen scaffolds, a new formulation of 3D media was used for differentiation of passage 4 RhoKIT keratinocytes on polycarbonate scaffolds. The new media contained several additional amino acids and fatty acids compared with 3D.1 media, including L-serine, linoleic acid, arachidonic acid and L-ascorbic acid (3D.2 media, full formulation shown in 4.2.4. The morphology of the resultant differentiated models was much closer to that expected in normal human epidermis although in some cases the stratum corneum layer exhibited areas of abnormal growth (**Figure 4.16**) which were mostly confined to the edges of the epidermal model.

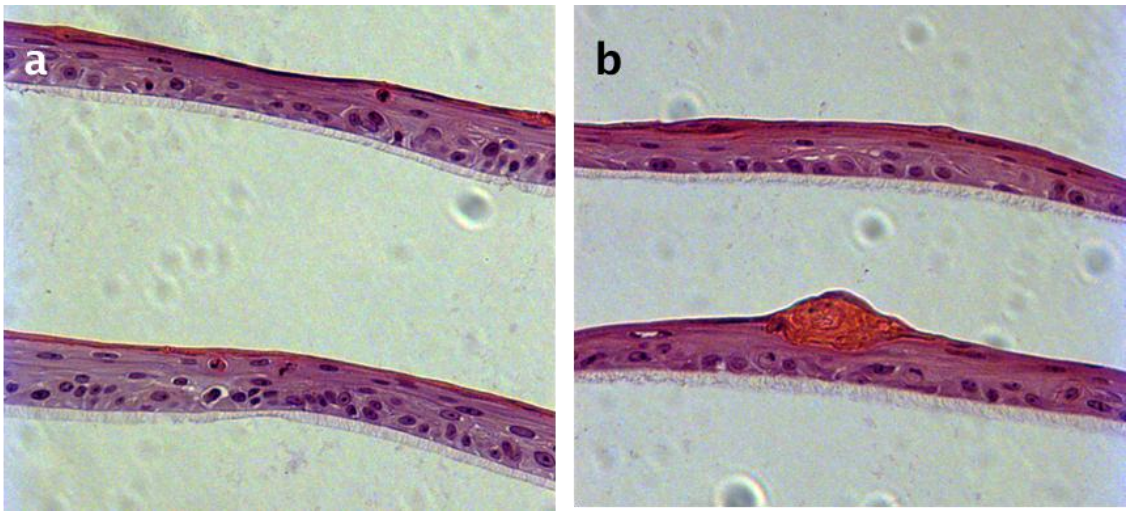


Figure 4.16: **RhoKIT keratinocytes can be differentiated into models which resemble non-dysplastic human epidermis.** RhoKIT cells (passage 4) from an 88 year old donor cultured on polycarbonate scaffolds for 48 hours submerged followed by 10 days at ALI using 3D.2 media (a). (b) 3D.2 media supplemented with 10 μM Y-27632 for the duration of the culture. Example images from 8 experiments.

The level of dysplasia in 5 models grown from adult NHK (NHK-derived) at passage 1 and 8 models grown from adult RhoKIT keratinocytes (RhoKIT-derived) at a minimum of passage 4 and maximum of passage 10 was assessed by a histologist and given a score between 0 (no dysplasia) to 3 (severe dysplasia) (**Table 4.7**). RhoKIT cells were less likely than NHK to result in dysplastic models, with 37.5% of the RhoKIT-derived models scoring 0, compared with 0% of NHK-derived models. Moreover, none of the

RhoKIT-derived models showed severe dysplasia (score of 3), while 20% of the NHK-derived models were severely dysplastic.

The dysplasia of adult NHK- and adult RhoKIT-derived models differentiated in the presence of 10 μM Y-27632 was also assessed. In NHK-derived models, Rho kinase inhibition during the ALI phase decreased the severity of dysplasia observed, resulting in 2/5 models with no dysplasia compared with 0/5 for untreated models. Conversely, Rho kinase inhibition during differentiation of RhoKIT keratinocytes increased the level of dysplasia observed. The number of models with severe dysplasia (grade 3) increased from 0/8 to 2/8 when RhoKIT-derived keratinocytes were differentiated in the presence of 10 μM Y-27632.

Table 4.7: Histological grading of 3D skin models. Dysplasia grade was ranked from 0 (no dysplasia) to 3 (severe dysplasia) by a histologist.

Dysplasia grade	NHK (P1)	NHK (P1) + 3D Y-27632	RhoKIT	RhoKIT +3D Y-27632
0	0	2	3	2
1	1	1	2	3
2	3	1	3	1
3	1	1	0	2

The features contributing to the dysplasia observed were primarily parakeratosis and dyskeratosis. There were minor differences in the presentation of these features between NHK- and RhoKIT-derived models, with all NHK-derived models displaying dyskeratosis. Of the dysplastic RhoKIT-derived models, all presented with dyskeratosis. A single RhoKIT-derived model displayed both dyskeratosis and parakeratosis.

Table 4.8: Features contributing to dysplasia of 3D skin models.

Feature	NHK (P1)	NHK (P1) + 3D Y-27632	RhoKIT	RhoKIT +3D Y-27632
Parakeratosis	0	0	0	3
Dyskeratosis	5	2	4	3
Parakeratosis + dyskeratosis	0	1	1	0
Neither	0	2	3	2

4.3.9 Markers of differentiation are altered in 3D skin models

To assess whether the reconstructed epidermal models mimicked normal human skin, antibodies were used to identify the expression of keratin 5 (K5), keratin 10 (K10) and involucrin in NHK (passage 1) and RhoKIT (passage 2 and passage 4) 3D models and normal human epidermis. K5 expression was similar in normal epidermis and early passage RhoKIT, primarily localising to the stratum basale and stratum spinosum. NHK models showed very little K5 expression, although the limited amount detected was confined to the stratum basale. In addition to the stratum basale and stratum spinosum, later passage (passage 4) RhoKIT models expressed K5 in the stratum granulosum, unlike in normal epidermis. In normal *in vivo* epidermis, keratin 10 (K10) is not present in the basal layer. However, expression of K10 was observed in all layers of the skin models created using both NHK and RhoKIT keratinocytes. In addition, involucrin is normally expressed only in the suprabasal layers of epidermis, but involucrin was observed in all layers of the skin models which used NHK and RhoKIT keratinocytes (**Figure 4.17**).

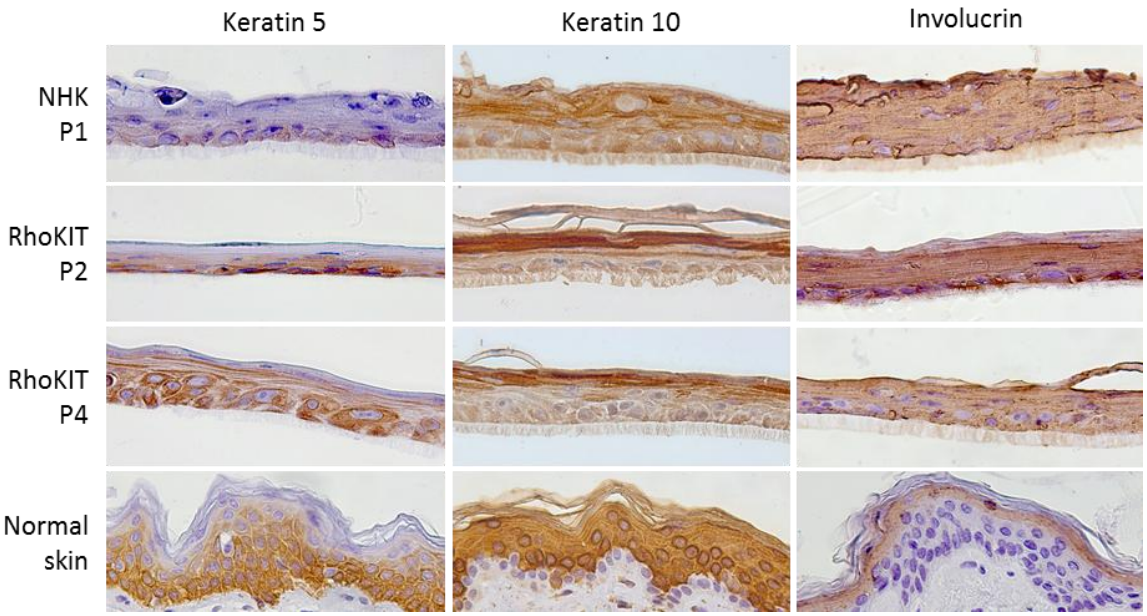


Figure 4.17: Markers of differentiation are altered in skin models. Keratin 5, keratin 10 and involucrin all showed altered expression in skin models using NHK at P1 and RhoKIT keratinocytes at P2 and P4 compared with normal epidermis.

4.3.10 Kallikrein 5 and 7, and PAR2 expression is altered in 3D models compared with normal skin

In normal skin, KLK5 expression is present throughout all layers of the epidermis at low levels (**Figure 4.18**). In the 3D models created using NHK and RhoKIT keratinocytes, KLK5 expression is far greater than that observed in normal epidermis. Similar to normal epidermis, KLK5 expression was evenly distributed across the layers of the 3D models. KLK7 expression was also much greater in 3D models than in normal epidermis. However, KLK7 expression was primarily limited to the stratum corneum and the stratum corneum-stratum granulosum junction (**Figure 4.18**). This distribution was similar in normal epidermis and RhoKIT models, while NHK models appeared to have a less distinct pattern of expression, with greater levels of diffuse staining across the model. This was also true for PAR2 expression, which was mainly restricted to the basale layer and upper stratum granulosum in NHK models with low levels of diffuse staining across the model. In RhoKIT models PAR2 expression was distinctly increased at the stratum corneum-stratum granulosum, with little staining in the lower layers. Normal epidermis showed little to no PAR2 expression in the stratum basale, but moderate PAR2 expression was present from the lower stratum spinosum through to the upper stratum corneum (**Figure 4.18**).

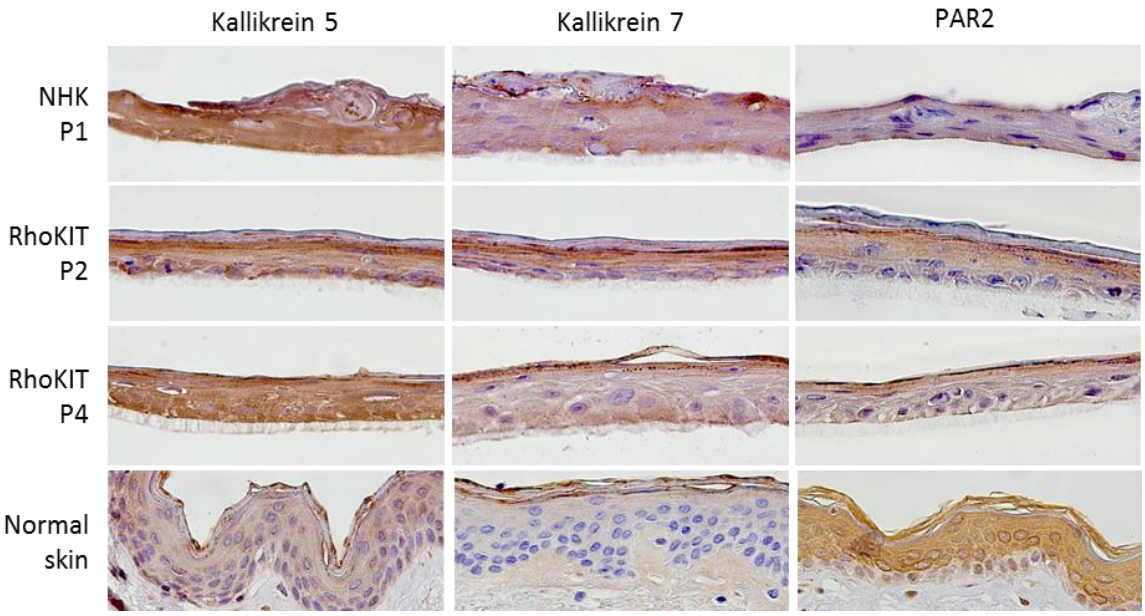


Figure 4.18: Expression of KLK5, KLK7 and PAR2 is altered on 3D skin models compared with normal skin. KLK5 and KLK7 are both upregulated in NHK and RhoKIT models compared with normal skin. PAR2 appears to be distributed more towards the stratum corneum in NHK and RhoKIT models, compared with a more diffuse expression in the normal skin.

4.3.11 3D models do not withstand exposure to moderate irritant concentrations

In order for skin models to be useful in the analysis of irritant responses, it is necessary for the reconstructed epidermis to create a sufficient permeability barrier to withstand application of relevant concentrations of irritant chemicals. To investigate this, 0.001% – 1.0% SDS was applied to reconstructed models for 30 minutes and then removed by careful pipetting. At concentrations above 0.1%, SDS removed the entire surface of the model, leaving only the polycarbonate scaffold. In order to prevent perturbations to the barrier caused by the removal of the SDS solution, subsequent tests were performed by applying 50 µl SDS at various concentrations to the model for 30 minutes, followed by gentle addition of 1 ml 10% neutral buffered formalin to fix the cells and maintain tissue architecture. However, even at concentrations as low as 0.001%, application of SDS for 30 minutes still drastically altered the structure of the epidermal model (**Figure 4.19**), causing it to lose cell layers and overall volume. It must be noted that the application of PBS also caused slight perturbation of the stratum corneum, however this could not be conclusively attributed to the application of the PBS solution because the embedding and sectioning process may have contributed to loss of the stratum corneum layer.

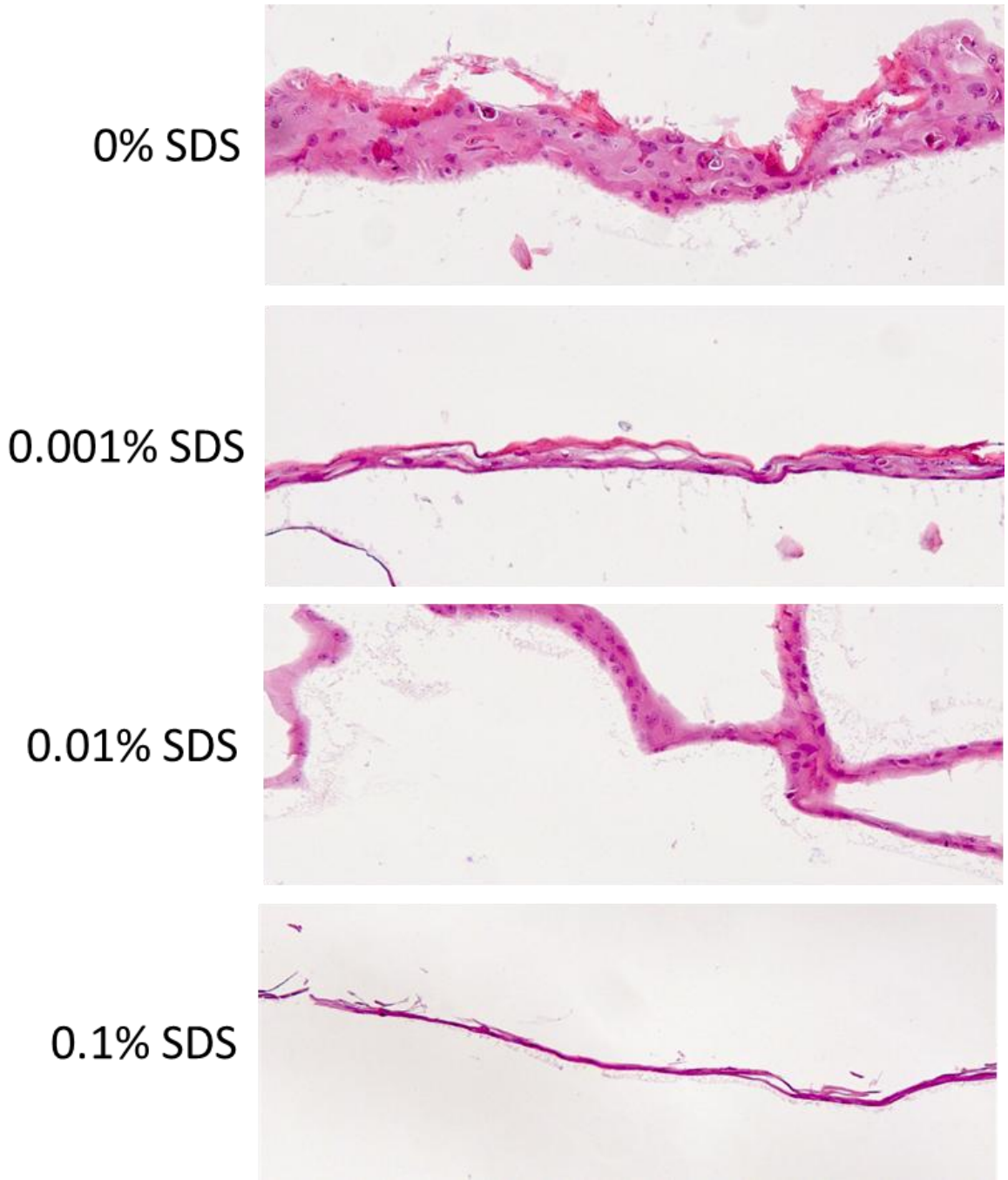


Figure 4.19: Application of even low concentrations of SDS drastically alters the morphology of skin models. Images representative of 4 models treated with 0.1 – 0.001% SDS.

4.3.12 Responses of NHK and RhoKIT keratinocytes to irritants and sensitisers

Having determined that RhoKIT keratinocytes do not differentiate into suitable models for investigation of the effects of irritant compounds such as SDS, it was next investigated whether these RhoKIT keratinocytes in monolayer are suitable for testing the effects of irritants, and separately sensitisers, on cytokine production from a monolayer culture. In order to investigate whether Rho kinase inhibition alters the response of keratinocytes to irritant or sensitising compounds it was first necessary to determine appropriate irritant and sensitiser concentrations for the following test compounds: DNCB (irritant-sensitiser (Friedmann et al., 1983)), DPCP (sensitiser (Wilkerson et al., 1984)), croton oil (irritant (Shaw, 1911)) and dithranol (irritant (Haustein and Lohrisch, 1986)). NHK isolated from 4 individual patients were incubated for 18 hours with varying concentrations of each chemical and cell viability was determined using an MTT assay. In order to reduce the effects caused by toxicity, minimally toxic concentrations of irritant or sensitiser were used. Minimally toxic concentrations were determined as the concentration of each chemical which gave a cell viability of between 75-85% following 18 hours incubation with NHK. Optimal concentrations were: DNCB 2.75 μM (**Figure 4.20**a), DPCP 5.0 μM (**Figure 4.20**b), croton oil 12.5 μg/ml (**Figure 4.20**c) and dithranol 0.2 μg/ml (**Figure 4.20**d).

The expression of 54 inflammatory genes was then analysed in NHK and RhoKIT keratinocytes from 3 patients following 6 hours of treatment with the selected sensitisers and irritants at the optimal concentrations determined above. These genes were mainly in 2 broad groups: oxidative stress response genes and immune activation genes (i.e. cytokines and chemokines). The expression levels of the genes were compared to those from keratinocytes incubated with PBS in place of the sensitising and irritant chemicals. **Figure 4.21** shows genes for which expression was increased or decreased more than 2 fold in at least 2 out of the 3 samples.

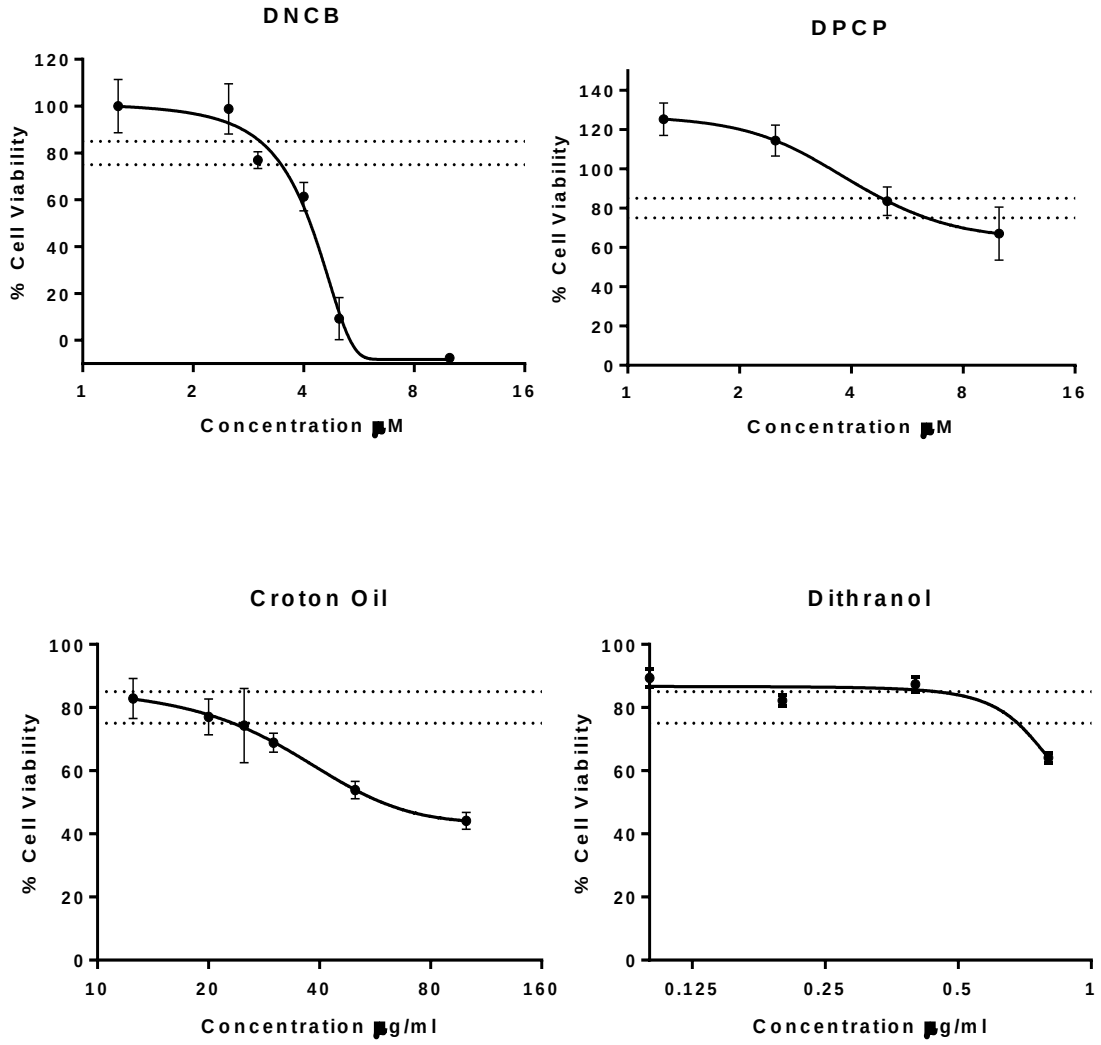


Figure 4.20: Cell viability following application of DNCB, DPCP, Croton Oil and Dithranol. The sensitising chemicals DNCB and DPCP, and the irritants croton oil and dithranol, were applied to keratinocytes at varying concentrations for 18 hours. Cell viability was analysed using the MTT assay and compared to untreated cells (taken as 100% live) and methanol treated cells (taken as 100% dead). Concentrations resulting in 75-85% cell viability were used for subsequent experiments. Results are from triplicate repeats of 4 experiments ± SEM.

There was a clear difference between the expression profiles of both NHK and RhoKIT keratinocytes exposed to croton oil and DPCP, with all altered genes being upregulated in croton oil treated keratinocytes and all but one altered gene being downregulated in DPCP treated keratinocytes. Treatment with the irritant-sensitiser DNCB (Friedmann et al., 1983) gave more varied responses over the 3 samples, possibly due to the dual irritant-sensitising properties of the chemical. Alternatively, the narrow margin between live and dead cells observed in the viability curve following application of various concentrations of DNCB to keratinocytes (**Figure 4.20**a) may mean that while

the concentration used was minimally toxic for keratinocytes from some donors, it may have caused more toxicity in others. *HMOX1* was increased in the DNCB treated, as in croton oil treated, samples; the increased expression of HMOX1 probably reflects the oxidative stress inflicted by DNCB through its rapid depletion of intracellular glutathione reserves (Pickard et al., 2009). *HSPA1A* (encoding heat shock 70 kDa protein 1A) was also increased, as observed in DPCP treated samples. *IL24* was decreased in both DNCB and DPCP treated samples, but remained unchanged in irritant treated samples. *IL24* is in the same locus as *IL10* (Chromosome 1q32), and DNCB causes a profound inhibition of IL10 secretion by monocytes (Pickard et al., 2006), so this may reflect a property of this super-potent sensitiser in that it switches off immunosuppressive cytokine expression. *IL7* expression was increased following treatment with both croton oil and dithranol, but was not altered in the cells exposed to the sensitising chemicals.

Minimal alterations in gene expression were observed following 6 hours treatment with 0.2 μg/ml dithranol. Following further analysis of cell viability results, in which the curve on a graph of cell viability against dithranol concentration showed 10-20% cell death occurred at a higher dithranol concentration than that shown by the individual points, and based on the results of previous studies (Lange et al., 1998; Farkas et al., 2001) the concentration of dithranol used was altered to 0.6 μg/ml for all subsequent experiments.

The results of gene expression changes were then compared between NHK and RhoKIT keratinocytes following incubation with irritants or sensitisers. Overall, 21% of the genes analysed showed a greater than 2 fold difference in expression in all the chemicals tested, 27% showed a difference in 3 out of the 4 chemicals, 20% in 2 and 21% in 1 of the chemicals tested compared with changes in NHK gene expression following the same treatment. Only 9% of the genes analysed showed similar levels of expression in NHK and RhoKIT keratinocytes following treatment with all 4 of the chemicals tested (**Figure 4.22**a). None of the genes tested were consistently altered in sensitisers but not irritants, or vice versa, in either NHK or RhoKIT keratinocytes.

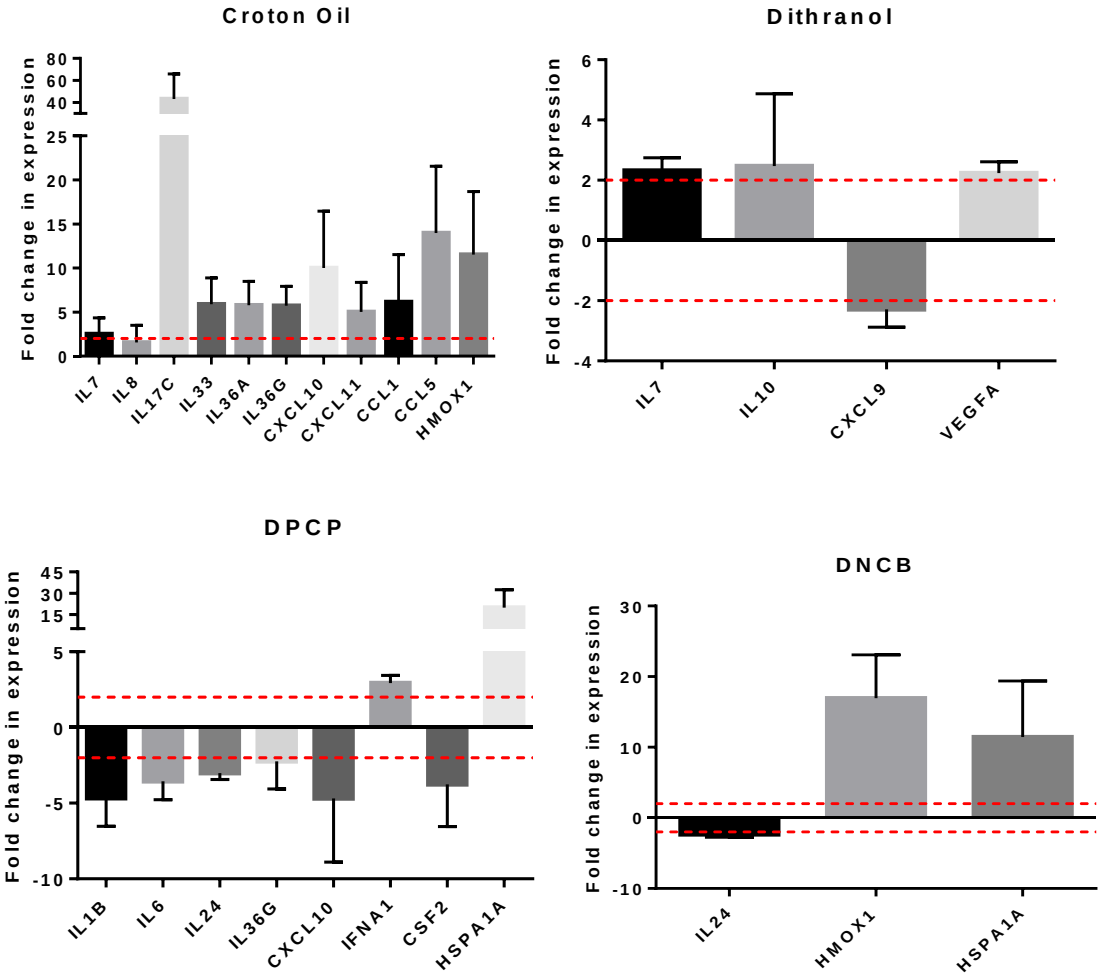


Figure 4.21: Genes showing more than 2 fold change in expression from NHK cells treated with irritants or sensitisers in at least 2 of 3 biological samples. *IL7* expression was increased in keratinocytes treated with either croton oil or dithranol for 6 hours, but remained unchanged in DNCB and DPCP treated cells. Conversely, *IL24* expression was decreased, and *HSPA1A* increased, in DPCP and DNCB treated keratinocytes but not in irritant treated cells. Results shown are for 3 separate experiments ± SEM.

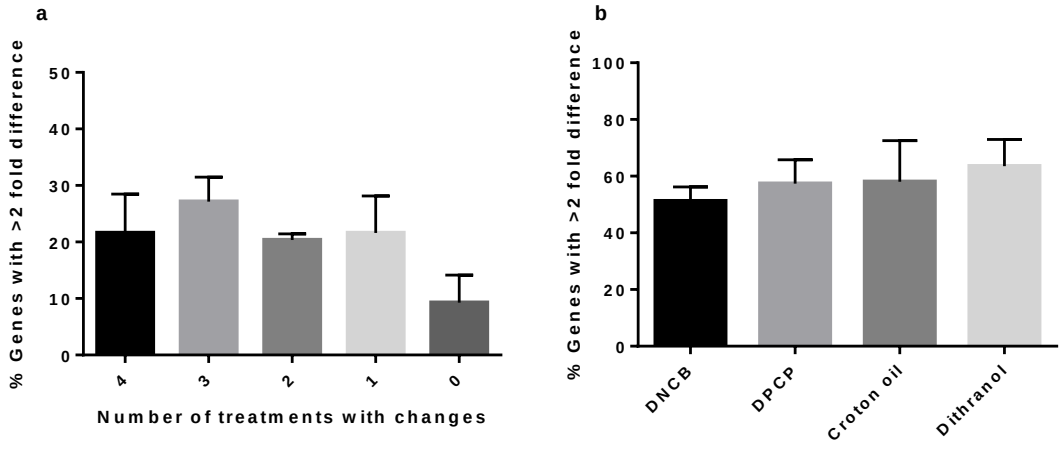


Figure 4.22: Differences in gene expression between NHK and RhoKIT keratinocytes following irritant or sensitiser application. a) Percentage of genes which show greater than 2 fold difference in gene expression in 0, 1, 2, 3, or 4 treatments (DNCB, DPCP, croton oil and dithranol). b) Percentage of 54 tested genes for individual chemicals which showed more than 2 fold difference in expression between NHK and RhoKIT keratinocytes. Data shown as mean of 3 biological replicates ± SEM.

Specific genes for which expression was increased, or decreased, in at least 2 of 3 individuals in NHK were selected for further investigation (*IL6*, *IL7*, *IL33*, *IL36A*, *CCL5* (*RANTES*), *CSF2*, *HMOX1* and *VEGFA*) (**Table 4.9**). In order to test the potential of these genes for use as markers for screening chemicals for possible irritant, or sensitising, properties NHK and RhoKIT keratinocytes from 7 donors were incubated with irritant and sensitising chemicals for 6 hours and changes in gene expression were analysed. *KLK5* and *KLK7* mRNA expression was also examined to determine if Rho kinase inhibition alters the expression of these proteases.

Analysis of the expression of these 10 selected genes from NHK following 6 hours of treatment with 12.5 µg/ml croton oil yielded an expression profile that varied greatly from the expression profile of culture matched NHK following 6 hours incubation with 0.6 µg/ml dithranol (**Figure 4.23**). For both croton oil and dithranol treated NHK, CCL5 was increased more than 2 fold.

Overall, gene expression was not dramatically altered between NHK and RhoKIT keratinocytes following exposure to the two test irritants used. *CCL5* was significantly increased in NHK (41.97 ± 12.72 fold, P=0.0001) and RhoKIT (55.51 ± 20.23 fold, P=0.0060) keratinocytes following exposure to 12.5 µg/ml croton oil. In contrast, CCL5 was increased only 4.34 ± 2.55 fold following application of 0.6 µg/ml dithranol

to NHK, and only 2.75 ± 1.19 in RhoKIT keratinocytes. As opposed to the other genes tested, *IL36A* was expressed at much higher levels in RhoKIT keratinocytes compared with NHK following exposure to both croton oil (P=0.1250) and dithranol (P=0.2500). Overall *IL36A* was expressed at much lower levels than the other cytokines, and there was more variation in its expression. This may have been due to the fact that the Ct was 30-35 cycles rather than 20-25 as for the other genes.

Compared with irritant treated keratinocytes, sensitising chemicals appeared to have little effect on expression of most of the genes tested (**Figure 4.24**). As expected, *IL33* and *HMOX1* showed more than 2 fold decreases in expression in both DNCB and DPCP treated cells, and *IL7* also showed a 2 fold decrease in expression following DPCP treatment. The pattern of gene expression following DPCP exposure was markedly different in RhoKIT keratinocytes compared with NHK, with overall increases in *IL6*, *IL7*, *IL36A*, and *CSF2*, in RhoKIT keratinocytes compared with decreases in NHK, and a much lower increase in *HMOX1* in RhoKIT keratinocytes than in NHK.

In addition, the resting levels of the inflammatory genes were compared between the NHK and RhoKIT keratinocytes to determine whether there were any differences in the constitutive expression of these genes as a result of Rho kinase inhibition (**Figure 4.25**). The resting level of IL-33 was significantly lower in RhoKIT keratinocytes compared with NHK (P=0.0302), although the difference was less than 2 fold (1.74 fold decrease). IL-6 and IL-7 mRNA expression were more than 2 fold lower in RhoKIT keratinocytes compared with NHK, however this difference did not reach significance (P=0.1703 and P=0.4312 respectively). Expression of *CCL5* and *CSF2* were both more than 2 fold higher in RhoKIT keratinocytes, with the difference in *CSF2* expression reaching significant levels (P=0.0248). There were no obvious differences in expression of *IL36A* and *VEGFA* between the two cells types.

Table 4.9: Fold changes in gene expression in NHK following 6 hours incubation with irritant chemicals. NHK were incubated with 12.5 µg/ml croton oil, 0.6 µg/ml dithranol, 2.75 µM DNCB or 5.0 µM DPCP for 6 hours. Expression of 54 proinflammatory genes was assessed by RT-QPCR. Genes which showed a greater than 2 fold change in at least 2 of 3 NHK cultures are shown. Genes which were selected for further testing are shown in bold.

Croton Oil

Gene	Fold change	SEM
IL7	**2.53**	**3.17**
IL17C	28.08	34.65
IL33	**5.90**	**5.14**
IL36A	**5.80**	**4.68**
IL36G	5.74	3.76
CXCL10	10.00	11.17
CXCL11	5.02	5.81
CCL1	6.14	9.34
CCL5	**13.98**	**13.09**
HMOX1	11.53	12.37

DPCP

Gene	Fold change	SEM
IL1B	-4.67	3.21
IL6	**-3.55**	**2.10**
IL24	-3.02	0.71
IL36G	-2.24	3.16
CXCL10	-4.68	7.28
IFNA1	2.93	0.88
CSF2	**-3.76**	**3.63**
HSPA1A	19.89	21.85

Dithranol

Gene	Fold change	SEM
IL7	**2.32**	**0.73**
CXCL9	-2.29	1.03
VEGFA	**2.24**	**0.65**

DNCB

Gene	Fold change	SEM
IL24	-2.33	0.73
HMOX1	**16.95**	**10.61**
HSPA1A	11.44	13.77

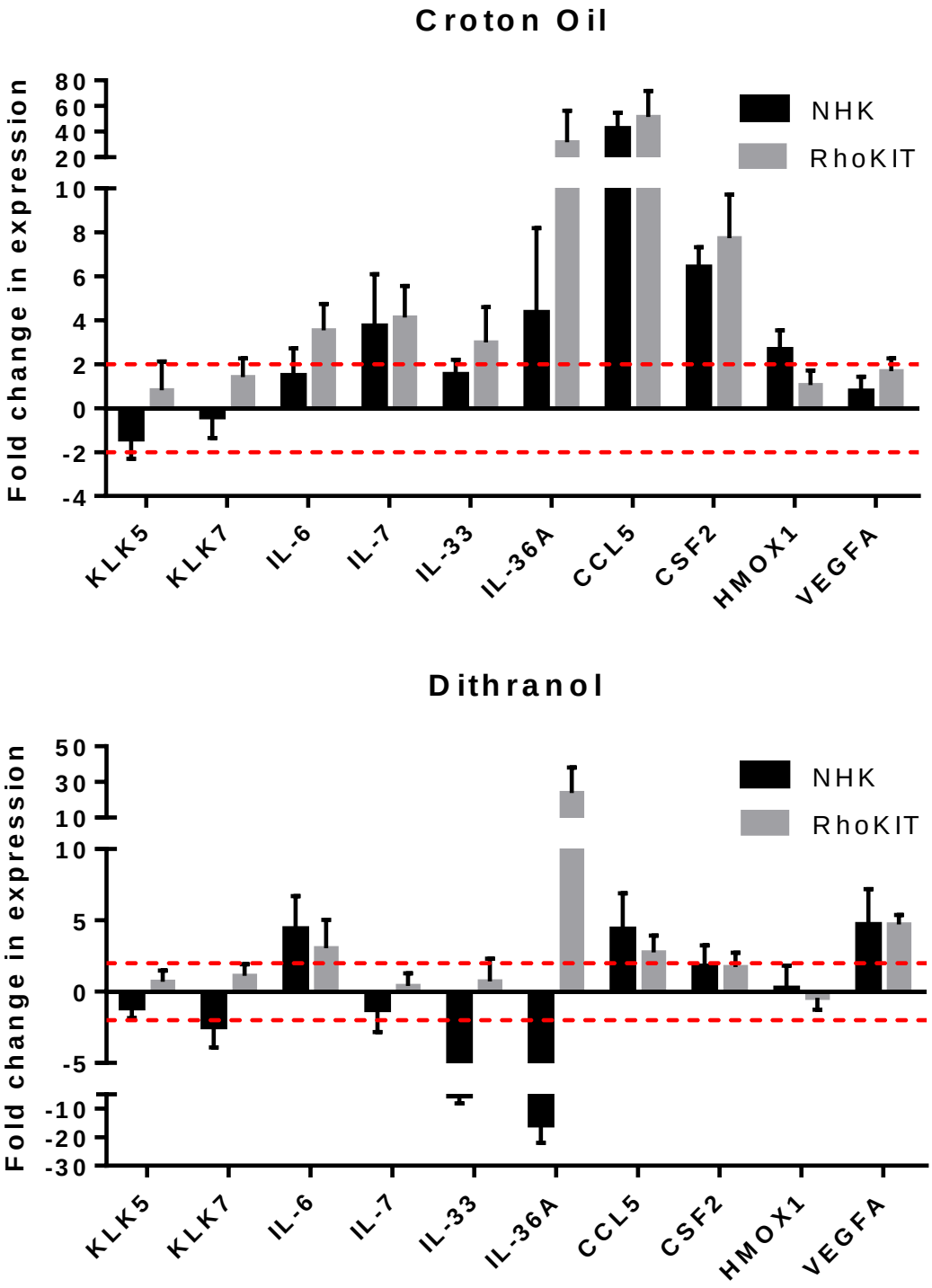


Figure 4.23: Changes in gene expression comparing NHK and RhoKIT treated with irritants. NHK and RhoKIT keratinocytes were treated with 12.5 µg/ml croton oil or 0.6 µg/ml dithranol for 6 hours. Most genes showed a similar change in expression in both NHK and RhoKIT keratinocytes, with the exception of *IL36A*, which had dramatically increased expression in RhoKIT keratinocytes compared with NHK following treatment with either irritant. *IL33* expression was also increased in RhoKIT keratinocytes compared with NHK following incubation with dithranol. Genes are listed along the X axis. Red lines indicated a 2 fold change in gene expression. N=7 ± SEM.

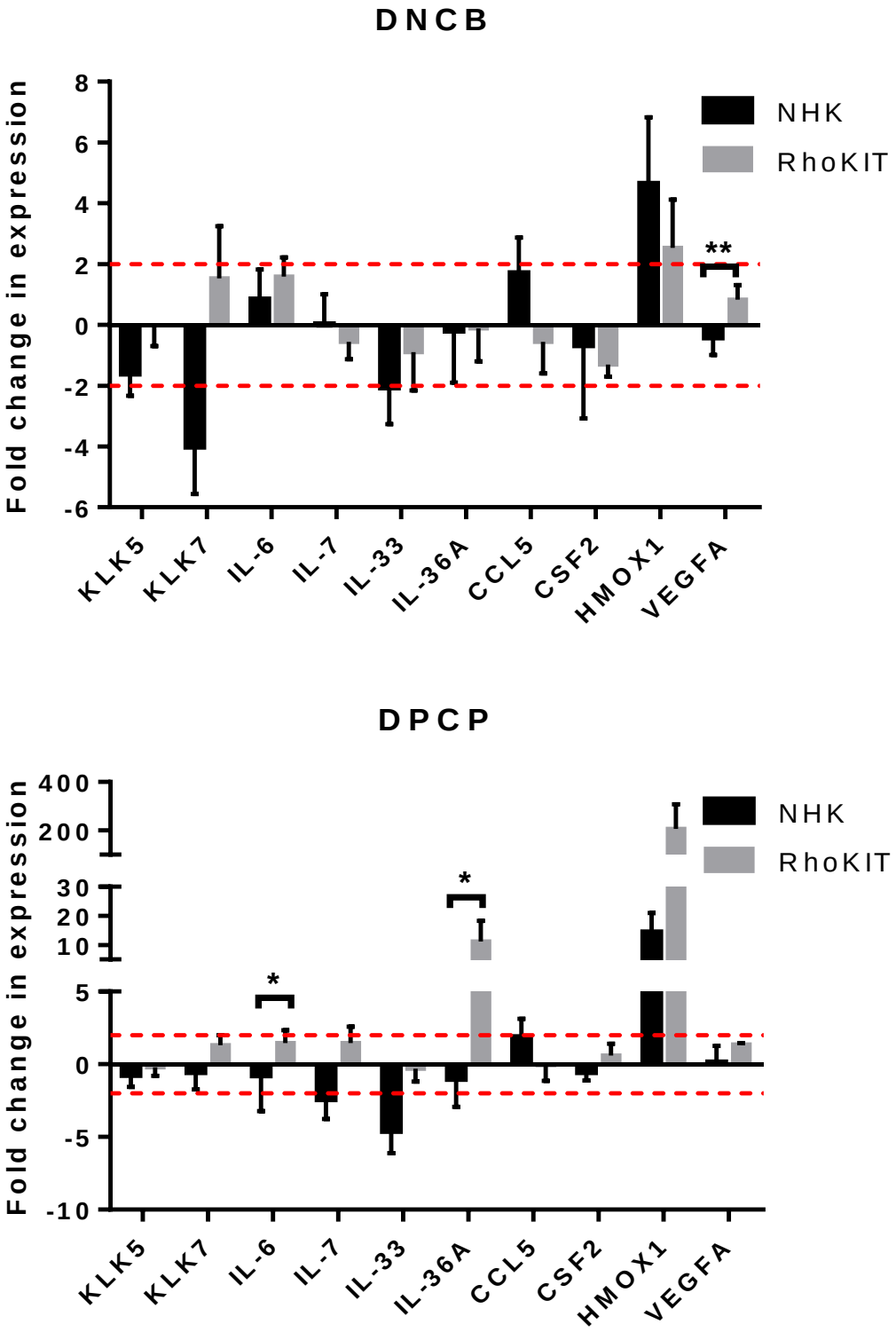


Figure 4.24: Change in gene regulation comparing NHK and RhoKIT treated with sensitisers. NHK and RhoKIT keratinocytes were treated with 2.75 µM DNCB or 5 µM DPCP for 6 hours. There were differences in gene expression from NHK and RhoKIT keratinocytes for several of the genes, significantly *IL6, IL36A*, and *VEGFA* following treatment with either DNCB or DPCP. Genes are listed along the X axis. Red lines indicated a 2 fold change in gene expression. N=7 ± SEM.

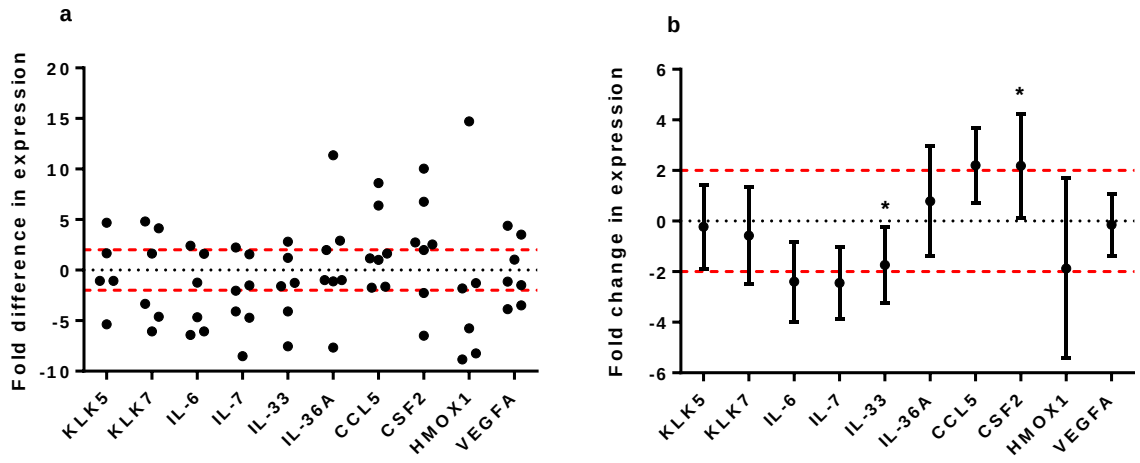


Figure 4.25: Comparison of resting NHK and RhoKIT inflammatory cytokine and chemokine expression. A) The spread of the individual results. B) Summary of the results from NHK and RhoKIT keratinocytes from 7 donors. In both graphs, a result above 0 indicate gene expression is increased in RhoKIT keratinocytes, and results below 0 indicate gene expression is increased in RhoKIT keratinocytes compared with NHK.

Although the constitutive expression of *IL33* and *CSF2* were significantly altered in RhoKIT keratinocytes compared with NHK, there was no difference in the expression of these genes following treatment of the cells with irritants or sensitisers. Overall, the fact that there was no significant difference in the expression of most of the genes tested (*KLK5, KLK7, IL6*, *IL7*, *IL33*, *CCL5* (*RANTES*), *CSF2*, *HMOX1* and *VEGFA*) between NHK and RhoKIT keratinocytes following treatment with the irritants croton oil and dithranol, or the sensitisers DNCB and DPCP, indicates that RhoKIT keratinocytes could be useful as a model to replace NHK in the testing of potentially irritant or sensitising chemicals. Furthermore, *CCL5* and *HMOX1* showed an altered expression pattern when exposed to irritants and sensitisers in both NHK and RhoKIT keratinocytes, therefore these genes may have the potential to be used as biomarkers in conjunction with other markers as a screen to identify irritant and/or sensitising chemicals.

4.4 Discussion

A major limiting factor in research investigating the responses of human skin to environmental insults is the lack of availability of a regular supply of standardised skin samples. Therefore, the purpose of the work presented in this chapter was to attempt to generate immortalised (or "semi-immortalised") keratinocyte cell lines by treating NHK with an inhibitor of Rho kinase. It was then the aim to attempt to generate suitable skin equivalents using these RhoKIT keratinocytes and, as a concomitant approach, to assess the RhoKIT keratinocytes for changes in gene expression that might take place within these cells in order to see whether they could be useful to investigate the effects of irritants and sensitisers in future studies. While the exposure of keratinocytes in submersion cultures to exogenous substances may give some valuable information, it was considered essential to grow them into skin equivalents, with a 3D structure and the differentiation of a permeability barrier, to determine whether the 3D model would have advantages over 2D monocultures.

In this chapter it has been shown that the proliferative capacity of primary adult human keratinocytes is greatly increased by inhibition of Rho kinase in the presence of feeder fibroblasts, and that RhoKIT keratinocytes have an altered expression profile for genes involved in the progression of the cell cycle. The Rho kinase inhibitor Y-27632 was successfully used to increase the proliferative capacity of primary human keratinocytes far beyond their normal levels, corroborating results of previous studies by Chapman *et al* (2010) and van den Bogaard *et al* (2012). The results shown here also confirm that a feeder layer must be used in conjunction with Y-27632 in order to increase the proliferative capacity of keratinocytes, as reported by Liu *et al* (Liu et al., 2012). As in the Chapman study, it was first attempted in the current project to culture the keratinocytes in the absence of fibroblasts using a specifically designed medium. This laboratory has previously noted that CnT57 medium allows significantly greater proliferation of keratinocytes on plastic than that seen with keratinocyte growth media (KGM) (Invitrogen, Paisley) which was used in the Chapman study. It was therefore hypothesised that RhoKIT cells cultured in CnT57 might retain the properties required for indefinite growth without the need for a fibroblast feeder layer which is required when RhoKIT keratinocytes are maintained in KGM, but the results in this chapter show that a feeder layer is also required when CnT57 is used.

Based on the keratinocyte growth observed in this study, supplementation of growth medium with 10 μM Y-27632 in the presence of feeder fibroblasts could potentially provide a final population of 1.17×10^{21} primary adult keratinocytes from an initial population of 1×10^{5} cells. This is compared with a final cell number of 1.02×10^{9} in untreated keratinocytes, a potential increase of 1.15×10^{14} %. Van den Bogaard *et al* (2012) observed increased growth to greater than 30 population doublings in keratinocytes extracted from 3 of 5 adult donors, but the exact ages of the donors used in that study were not reported. Those results correspond well with the data in the present study in which keratinocyte growth, while significantly increased, did not result in unlimited population doublings, i.e. the cells were "semi-immortalised" rather than "immortalised".

This is the first study to examine the differences in growth between different adult age groups, specifically examining the effects of Rho inhibition in keratinocytes taken from younger and older adult donors. By splitting the groups into donors aged under 55 and over 55 years, it was shown that beyond the age of 55 there is a significant decrease in growth of both normal and RhoKIT cells. Although Rho kinase inhibition increased proliferation of keratinocytes in both age groups, the increase was greater in those from donors aged over 55 years. This is most likely due to the low number of population doublings *in vitro* achieved by untreated NHK in this age group; therefore fold increases are more easily achieved.

It has been shown that telomeres are shorter in aged keratinocytes (Matsui et al., 2000), and a previous study which looked at the mechanism by which Rho kinase inhibition increased keratinocyte proliferation investigated alterations to telomerase expression and changes in telomere length between NHK and RhoKIT keratinocytes. That study demonstrated that telomerase expression is progressively increased up to a maximal level at around passage 35, which is maintained up to at least passage 120 in RhoKIT keratinocytes (Chapman et al., 2010). This compares with a decrease in telomerase expression in NHK up to passage 12 (Chapman et al., 2010). Despite this increase telomerase expression, relative telomere length decreases in RhoKIT from passage 5, losing approximately 80% of total length before stabilising at around passage 50 (Chapman et al., 2010).

In the present study, the anti-apoptotic genes *BCL2* and *GTSE1* and pro-proliferative genes *DDX11*, *BRACA1* and *MKI67* were upregulated in RhoKIT keratinocytes following

12 population doublings compared with NHK at the same stage. It is highly likely that these genes contribute to the increased proliferative capacity of RhoKIT keratinocytes. In addition, it has been demonstrated that expression of cyclins CCND1 and CCND2, and the CDKs CDK2A and CKD2B is downregulated by Rho kinase inhibition, which is to be expected since these proteins are regulated by ROCK (Welsh et al., 2001; Roovers and Assoian, 2003; Park et al., 2011). This is in agreement with a previous study which showed that cyclin A and cyclin D1 were reduced in NIH 3T3 murine fibroblasts following incubation with 10 μM Y-27632 (Croft and Olson, 2006). The study by Croft and Olson also indicates that the feeder fibroblasts are affected by Rho kinase inhibition (2006). As this is an immortal cell line there is no observable change in cell proliferation, however it may be that the changes in gene expression within the fibroblasts caused by Rho kinase inhibition may be required for increased keratinocyte proliferation as observed in the present study and by Chapman *et al* (2010).

The estimated frequency of keratinocyte stem cells in the epidermis ranges from 1-10% (Heenen and Galand, 1997; Cotsarelis et al., 1999; Terunuma et al., 2003), but only 0.1-1% of freshly isolated keratinocytes behave as keratinocyte stem cells as measured by colony formation assays (Rheinwald and Green, 1975; Li et al., 1998; Terunuma et al., 2007a; Terunuma et al., 2007b). Terunuma *et al* (2010) concluded that Y-27632 does not boost growth of non-keratinocyte stem cells, and postulated that Rho kinase inhibition increases the number of keratinocyte stem cells that can survive and form healthy colonies in the primary culture.

The two previous studies which have investigated the effects of Y-27632 on keratinocyte differentiation into organotypic models have used scaffolds containing fibroblasts (Chapman et al., 2010; van den Bogaard et al., 2012). These studies both showed normal epidermal morphology when keratinocytes were differentiated in the presence of Y-27632. Contrary to what was observed by Bogaard *et al* and Chapman *et al,* the addition of Y-27632 throughout the differentiation phase in the present study caused changes to the morphology of the 3D models. This difference may be explained by the different ages of the donors, or by the alternative method of differentiation used in the present study. Van den Bogaard *et al* and Chapman *et al* differentiated keratinocytes on a collagen dermal substitute containing 3T3 fibroblasts, whereas organotypic models were created in the present project using a simple polycarbonate

scaffold. It is possible that keratinocytes differentiated in the presence of Y-27632 may require additional factors secreted by fibroblasts, or may need cell-cell contact with fibroblasts, in order to differentiate correctly, thus explaining the altered morphology in situations where fibroblasts are not used during differentiation. While it also possible to use de-epidermalised dermis as a scaffold for full thickness skin models, it was not attempted in the present study because the purpose of the current investigation was to reduce the need for primary human skin (including human dermis) for investigating effects of irritants.

A recent study showed that neonatal NHK cultured in commercial serum-free feeder-free media do not form stratified epithelium in a reconstituted skin model on de-epidermalised dermis (Lamb and Ambler, 2013), however cells cultured in serum-free media were able to differentiate in serum-supplemented media. This effect was observed in adult keratinocytes in the present study, with both NHK and RhoKIT keratinocytes unable to differentiate in commercial serum-free medium (**Figure 4.12**b-d). However, in this study it was possible to correctly differentiate neonatal NHK (**Figure 4.12**a), therefore it is possible that the use of a polycarbonate scaffold in place of de-epidermalised dermis may encourage correct differentiation of younger keratinocytes, whereas adult keratinocytes may need additional factors contained within serum to achieve stratification. The results of the study by Lamb and Ambler also mirrored results of the present study which showed morphological differences in keratinocytes cultured on plastic in serum-free commercial media and those cultured on feeder fibroblasts.

While the reconstructed 3D models showed normal levels of K5 expression, involucrin and K10 were detected across the whole epidermis. As expression of involucrin is a marker of commitment to terminal differentiation (Watt, 1983), and keratinocytes in the stratum basale of normal human epidermis do not express involucrin or K10, it can be concluded that keratinocytes in these reconstructed models do not contain "normal" basal keratinocytes. The barrier was also much more fragile in the reconstructed models compared with normal human skin, as evidenced by the fact that application of SDS at the concentrations used on normal human skin completely removed the organotypic model from the polycarbonate scaffolds. Indeed, it was necessary to use concentrations of SDS which were 20 times more dilute that those used on normal skin in order to retain any of the model on the scaffolds. At

concentrations as low as 0.001% SDS the reconstructed models still showed morphological differences with untreated models, including a dramatic reduction in overall thickness due to decreased numbers of cell layers within the epidermis. This observation means that these models are not yet suitable for testing whether new substances / chemical compounds are potential irritants, however further alterations to the differentiation conditions, including the use of fibroblasts and a dermal substitute may enable the development of a more robust RhoKIT keratinocytes organotypic model. Such as model could be useful for the investigation of the mechanisms by which irritants exert their effects, and/or for high throughput screening of potential irritant or sensitising chemicals.

In addition to looking at genes involved in cell proliferation and apoptosis, the present study investigated whether Rho kinase inhibition altered the response of keratinocytes to chemical irritants and sensitisers. Due to the large difference in the concentrations of these chemicals tolerated by skin and by primary keratinocytes in a monolayer, it was not possible to apply chemicals at the same concentration as that used on *in vivo* or *ex vivo* skin. Therefore, irritant and sensitising chemicals were applied to NHK and RhoKIT keratinocytes at concentrations causing minimal toxic effects and the alterations in gene expression compared, but the relevance of these concentrations to the *in vivo* environment is not known. Although the use of keratinocytes from only 3 donors resulted in highly variable gene expression profiles in the arrays, the subsequent selection of a smaller range of genes tested over 7 individuals gave more reproducible results.

Two main effects were investigated in the qPCR array study: oxidative stress and cytokine production, as these had been shown to be altered following application of irritants or sensitisers to keratinocytes (**Table 4.9**). As expected, it was observed that application of irritants to the keratinocytes for 6 hours caused an increased expression of mRNA for both proinflammatory cytokines and oxidative stress genes. Croton oil and dithranol have tumour promoter properties that stimulate a wide range of cytokines and growth factors via activation of PKC (Anderson et al., 1987; Barker et al., 1991a; Marquardt et al., 1994), therefore a cytokine response would be expected from keratinocytes treated with these chemicals through stimulation of AP1 and NF-κB, which has been reported to result in production of IL-1α, IL-8 and TNFα cytokines (Schreck and Baeuerle, 1990; Lee et al., 1994; Schenk et al., 1994; Corsini et al., 1997).

Dithranol is also a mitochondrial poison (Morliere et al., 1985) and causes oxidative stress through the production of free radicals (Hsieh and Acosta, 1991; Lange et al., 1998), which would explain the large increase in *HMOX1* gene expression observed following dithranol application in this study.

The sensitisers DNCB and DPCP were also applied for 6 hours in this study and the culture conditions lacked Langerhans cells and T cells, so it is unlikely that the antigenicity of the chemicals was responsible for the responses observed. Indeed, the lack of cytokine production caused by the sensitisers supports this interpretation. Similarly, the upregulation of expression of oxidative stress genes following exposure to the sensitisers is more likely to be due to noxious effects of these chemicals rather than as a result of any antigen-specific response.

Overall, the irritants tested seem to elicit a stronger response than sensitisers, and elicit opposing changes in expression for the genes examined (i.e. downregulating instead of upregulating responses), and these responses were generally the same in RhoKIT keratinocytes and NHK with the exception of *IL36A*. Interestingly, RhoKIT keratinocytes incubated with PBS had greater than 2 fold alterations in gene expression for several of the genes analysed compared with NHK, including the proinflammatory cytokines *IL6*, *IL7*, *CCL5* and *CSF2*, but no difference in the oxidative stress gene *HMOX1*. In order to determine if the results observed in the present study are true for all irritants, some irritants or specific to the irritants used, the expression of these genes needs to be tested following application of a range of irritants. If the results are found to be similar for all irritants, it would be necessary to examine whether other, non-irritant, stimuli provoke the same alterations in gene expression profile to confirm whether the results are specific for irritants. Importantly, the current results provide a platform upon which to do perform investigations, and may lead to RhoKIT keratinocytes and expression of these genes being a useful screening tool in an industrial setting in future years to examine whether novel chemical compounds have irritant properties.

5. General discussion

The aim of the work presented in this thesis was to investigate mechanisms that could be responsible for generating the cutaneous inflammatory response induced by irritants. The working hypothesis was that a primary property of irritants is disruption of the stratum corneum permeability barrier, which results in a combination of repair and activation of innate immune defences. Activation of a range of key proteases is likely to be central to these responses. Although there is evidence that caspases are involved in this process (Demerjian et al., 2008), KLKs are a family of proteases important in the formation of the stratum corneum barrier, therefore it was of interest to explore the possibility that these proteases are also involved in the response to irritants. The approach taken was to apply irritants to an *ex vivo* skin model and to primary cells to assess the effects on the expression of KLK5, KLK7 and PAR2, and changes in the potential downstream expression of the inflammatory markers IL8 and TNFα. This investigation specifically focused on the early responses to irritants, examining changes in the profile of these serine proteases and the PAR2 receptor within 30 minutes of contact.

Many chemicals are known to cause disruption to the stratum corneum barrier homeostasis, leading to disorders such as irritant contact dermatitis (Wahlberg and Maibach, 1980; Zesch, 1983; Basketter et al., 2004; Antonov et al., 2012). These disorders have a significant economic impact on industry, particularly in those industries in which wet work is prevalent (English, 2004). Previous methods to identify the potential irritant or sensitising effects of novel chemicals has depended on the use of animal models (Gad et al., 1986; Kimber et al., 1986; Homey et al., 1998), or by identifying similarities in chemical structure with known irritants or sensitisers, or by analysing the potential of a chemical to act as a hapten through binding to proteins (Kodithala et al., 2002; Gerberick et al., 2007; Roberts et al., 2008; Vandebriel and Loveren, 2010). Under new EU regulations from 2013 (EU regulation 1223/2009), animal models are no longer approved for the testing of chemicals for use in cosmetics. It is therefore crucial that alternative, suitable methods are developed which can be used to examine the potential irritant effects of chemicals. Current *in chemico* and *in silico* methods are both limited by a lack of consideration for biological interactions which may occur in the epidermal and dermal environment, and a lack of consideration for the ability of the chemical to cross the skin barrier (Aeby et al., 2004;

Aeby et al., 2010). This limitation is also true of *in vitro* models currently in use, such as those utilising dendritic cells and keratinocytes, in which the cells are treated with the chemical in question and changes in the expression of surface markers or cytokines are analysed (Aeby et al., 2004). In addition, the narrow cytotoxicity ranges of potential sensitisers makes dose selection crucial for their successful identification (Vandebriel and Loveren, 2010).

Prior to this study, the evidence indicated that proteases, and in particular serine proteases, activate the process of innate immunity following barrier disruption (Hachem et al., 2005; Hachem et al., 2006b). The receptor through which these proteases act is likely to be PAR2 (Stefansson et al., 2008). PAR2 is known to play a role in the adaptive immune response to exogenous allergens in the epidermis (Jeong et al., 2008), but more recently it has been shown to be involved in the innate inflammatory response following mechanical barrier abrogation in the epidermis (Roelandt et al., 2011). KLKs are a vital component of the desquamation process and are critical for the maintenance of a functional skin barrier (Descargues et al., 2006; Borgono et al., 2007). Moreover, KLKs have been demonstrated to have increased activity in acute atopic eczematous skin (Voegeli *et al* 2009), and KLK5 has been shown to play a vital role in the pathogenesis of Netherton's syndrome through activation of PAR2. It was therefore hypothesised that irritant chemicals which damage the stratum corneum cause an increase in KLK activity in the epidermis, which in turn increases PAR2 activation and subsequent downstream transcription of proinflammatory cytokines including IL-8 and TNFα.

The present study initially used full thickness *ex vivo* human skin to investigate the effects of irritants applied topically to the skin. There are several advantages to using an *ex vivo* skin model compared with *in vivo* methods in the investigation of irritant responses. The use of an *in vivo* approach would require many biopsies from individual donors, which would have been subjected to local anaesthetic and thus might alter or confound the results. The use of *ex vivo* skin allowed many investigations to be performed on several samples from the same piece of skin, allowing comparisons at various time points and at different chemical concentrations in pilot experiments. However, the *ex vivo* skin model is less suitable for use in industry, where access to volunteers or patients undergoing surgery is more restricted, unless adequate amounts of skin can be sourced from surgical establishments. To date,

ex vivo skin models have frequently been used to assess the percutaneous permeation of molecules when investigating drug delivery methods, or comparing various non-invasive *in vivo* methods such as TEWL measurements with analysis of barrier permeability (Fluhr et al., 2006; Godin and Touitou, 2007), but less commonly used to examine irritant responses.

The results of the present study have indicated that both KLK5 and KLK7 protein expression is upregulated in the epidermis in response to irritant application. Increased KLK7 expression was co-localised with increased protease activity in the epidermis, while KLK5 expression was co-localised with increased PAR2 expression within 30 minutes of irritant application. This co-localisation of increased KLK5 and PAR2 expression is supported by results from Zhu *et al* (2013) who demonstrated that when KLK5 is overexpressed in murine keratinocytes, PAR2 expression is also increased. PAR2 can also be activated by the proteases matriptase and prostasin (Seitz et al., 2007), both of which are present in the epidermis. Due to a lack of specific inhibitors of KLK5 and KLK7, it was not possible in the present study to identify whether these specific proteases were responsible for upregulating, or indeed activating, PAR2 in the *ex vivo* skin.

Previous studies in which protease inhibitors were applied to skin were performed on hairless mice (Hachem et al., 2005) or, in a single study, on human volunteers following tape stripping (Hachem et al., 2006b). General (non-specific) serine protease inhibitors (aprotinin and trans-4-(Aminomethyl)cyclohexane carboxylic acid) were applied directly after acute barrier disruption and the resultant alterations in trans-epidermal water loss were monitored at 0, 3 and 24 hours. Following inhibition of serine proteases, there was an increase in the rate of barrier repair. However, in the absence of either mechanical or chemical barrier damage it is unlikely that the inhibitors would penetrate the epidermis due to their large molecular weights. To date, no studies seem to have been performed in which protease inhibitors were used in relation to irritants on the skin, and it was not possible to do this in the current study because the barrier would have needed to be damaged to allow the protease inhibitors to enter into the skin, and this would have confounded the results. Therefore, in the present study, cultured keratinocytes were used to demonstrate that irritant application caused calcium mobilisation, which was shown to be significantly reduced by inhibitors of trypsin and plasmin, both of which have been shown to

inhibit KLK5 (Goettig et al., 2010; Swedberg et al., 2010). Taken in conjunction with the *ex vivo* skin work in which the irritants increased KLK5, this suggests (but is not conclusive evidence) that KLK5 may have been responsible for the increased calcium mobilisation. Support for this is provided by Oikonomopoulou et al who have reported that KLK5 can trigger a PAR2-mediated cellular calcium response (Oikonomopoulou et al., 2006a; Oikonomopoulou et al., 2006b).

The possible role of KLK5 in the innate immune response is supported by Yamasaki and Gallo (2009), based on investigations into the expression of KLK5 in rosacea skin. They showed that while KLK5 is expressed primarily in the upper epidermal layers of normal skin, in rosacea skin it is expressed in the entire epidermis. The authors concluded from this that KLK5 could play a part of skin inflammatory reactions in rosacea by affecting dermal matrix and vascular remodelling due to the high KLK5 expression in basal cells of rosacea epidermis (Yamasaki and Gallo, 2009). Netherton's syndrome is characterised by over-desquamation of corneocytes due to unrestricted KLK5 activity resulting from the genetically determined lack of LEKTI, the specific inhibitor. Sufferers of Netherton's syndrome exhibit chronic inflammation of the skin, characterised by high levels of proinflammatory cytokines in the epidermis (Descargues et al., 2005). This suggests a possible role for active KLK5 in skin inflammation. Indeed, KLK5 has been reported to induce ICAM1 mRNA, TSLP mRNA and protein, IL8 and TNFα by keratinocytes (Briot et al., 2009).

Although in the present study the two irritants, croton oil and SDS, appeared to initiate a KLK5, KLK7 and PAR2 response within 30 minutes, it will important to examine whether this is true for all or most irritant chemicals. Examples of irritants which would need to be tested include strong acids, strong alkalis and solvents, and dithranol which is used to treat psoriasis (Männistö et al., 1984; Schiavi et al., 1996; Loffler et al., 1999). However, to test sufficient numbers of irritants at a range of concentrations and in repeated experiments would require large amounts of *ex vivo* skin.

There is a need for constant, controllable systems for the investigation of the effects of chemicals on the skin, and obtaining human skin can be problematic as it is dependent on the availability of surplus tissue following surgery such as breast reduction, mastectomy and abdominoplasty. The size and quality of the skin samples is highly variable, and lack of availability of suitable tissue samples can cause delays in testing novel chemicals. Therefore, the possibility of using one or other of the various "skin

equivalent" systems might offer a much more available and consistent test system. Previous studies have shown successful differentiation of keratinocytes on a de-epidermalised dermis or a collagen scaffold containing irradiated fibroblasts (Asbill et al., 2000; Lee et al., 2000), however the formation of a functional stratum corneum varies between methods.

Related to this, commercially available skin equivalents have shown promise as suitable models for testing for irritancy potential. However, there has been some variability in the results obtained with skin equivalents from different providers (Koeper et al., 2007). Previous studies focused on the measurement of cell viability (Fentem et al., 2001) and/or the release of pro-inflammatory markers such as IL-1α (Faller and Bracher, 2002), IL-6 (Bernhofer et al., 1999) and IL-8 (Boelsma et al., 1997) in skin models as possible biomarkers for detection of irritant chemicals.

For the purpose of modelling the effects of irritants, the most important feature of any *in vitro* skin equivalent or model is likely to be the presence of a functionally effective permeability barrier. Although there have yet to be any models which have barriers as effective as that of *in vivo* skin (Asbill et al., 2000; Batheja et al., 2009), advancements in skin models within the past 20 years indicate that there is clearly the potential for high quality models to be created. For the generation of skin equivalents suitable for grafting onto burns, the principal substrate used is a collagen matrix, but the quality of the stratum corneum that results in these models is not fully formed (Batheja et al., 2009). In recent years there have been major advances in the development of scaffolds and substrates for *in vitro* tissue and organ generation (Arun Richard et al., 2011; Kempf et al., 2011; Tchemtchoua et al., 2011; Krishnan et al., 2012). It will be important to explore these advances to search out optimal substrates and scaffolds most appropriate to allow the formation of a fully functional stratum corneum.

The concept of using keratinocytes that have been immortalised in a state in which they are capable of preserving a phenotype and responding similarly to NHKs is attractive for a number of reasons. Firstly, large numbers of cells can be grown from fewer donors. Secondly, keratinocytes could be grown from individuals with particular phenotypes of interest such as atopics with a heightened susceptibility to the effects of irritants (Goffin and Piérard, 1996; Tabata et al., 1998; de Jongh et al., 2006). This would enable the production of enough samples for a robust experiment in an industry setting to test novel chemicals on a model which is representative of sensitive human skin. In addition, in cases where genetic susceptibility to irritant and

sensitising chemicals can be detected in a subject (for example in a subject with a filaggrin mutation (Molin et al., 2009)), or if patients suffer from skin disorders such as atopic dermatitis, it may be possible to use RhoKIT keratinocytes from individual subjects to produce 3D skin models with specific disease phenotypes which could allow investigation of the effects of irritants and/or sensitisers on the biology of the skin relevant to that condition..

The present study has confirmed results from previous studies that the inclusion of a Rho kinase inhibitor in culture media maintains keratinocyte cell growth far beyond normal levels, dependent on coculture with feeder fibroblasts (Chapman et al., 2010; van den Bogaard et al., 2012). The rate of proliferation of keratinocytes cultured on feeder fibroblasts was more consistent between patient samples, for both Rho kinase inhibitor-treated and untreated cells, than those cultured on plastic. This suggests that constitutive predisposing factors, such as age of the donor, are more limiting in cells cultured in defined media in the absence of a feeder layer. It is clear that feeder fibroblasts contribute as yet unidentified factors to enable cells to reach a greater growth rate. There has been further investigation by some research groups into methods which may allow the removal of the feeder layer without the loss of the potentiation of cell growth (Dickson et al., 2000; Ramirez et al., 2001), however this has yet to be successfully achieved.

In the present study it was not possible to create suitable human skin models using RhoKIT keratinocytes; they only showed potential for use in monolayer culture models. However, as discussed previously, it may be possible in future studies to optimise the differentiation conditions to enable stratification and formation a functional barrier by the RhoKIT cells. If it were possible to differentiate the RhoKIT keratinocytes, in order to be a useful model for testing the effects of irritants and sensitisers it would be important that the RhoKIT keratinocytes retain the same gene expression profile as NHK in response to irritants and sensitisers. The observation in this thesis that indicates that this might be possible is the fact that RhoKIT keratinocytes in monolayer culture had a largely similar gene expression response to irritant, and separately sensitising, chemicals as NHK. Within the panel of 9 genes tested, the exceptions to this were *IL36* and *IL33*, which differed in RhoKIT keratinocytes compared with NHK. Interestingly, no difference was observed in the expression of KLK5 or KLK7 in RhoKIT keratinocytes and NHK. Therefore, if successful 3D models can be created, KLK expression following application of

chemicals, as performed on *ex vivo* human skin in this study, may be a useful initial screening tool for irritant potential.

In models in which there is an incomplete or absent stratum corneum it appears it would be necessary to reduce the exposure period of the model to irritant chemicals to detect up- or downregulation of gene expression, because, firstly, longer exposure may be more toxic and, secondly, the interaction with the chemical is more immediate. In the present study, when irritants croton oil or dithranol were applied to keratinocyte monolayers, no changes were observed in expression of *TNFα* or *IL8* mRNA. By contrast, when 3% croton oil or 5% SDS was applied to *ex vivo* skin, *TNFa* mRNA expression was maximally increased within 3 hours, and returned to normal levels within 12 hours. This was similar to results observed in a previous study, which showed that *TNFa* mRNA expression was increased within 3 hours of application of TPA, an active component of croton oil (Murakawa et al., 2006). When SDS was applied to *ex vivo* skin, *IL8* mRNA expression was increased within 6 hours. However, following exposure to 3% croton oil, *IL8* only began to increase from baseline levels after 12 hours. Thus, it is possible that the lack of a stratum corneum barrier in the keratinocyte culture model resulted in a more rapid interaction between the irritant and the cells, and that altered gene expression might have taken place over a shorter time course and have returned to normal by the 6 hour time-point when it was assayed. However, despite this possibility, the lack of change in expression of *TNFα* or *IL8* mRNA in keratinocyte monolayers in response to some of the irritant compounds is somewhat surprising. Croton oil contains TPA which is well known to induce increased expression of TNFα and IL8 through direct activation of relevant transcription factors including AP1 (Marquardt et al., 1994). Therefore, it is clear that it might be beneficial to re-visit some of these experiments to explore the effects of different doses of irritants and a wider range of time course, including earlier time-points than 6 hours, in RhoKIT keratinocytes as well as in NHK. It has been established in previous studies that direct activation of PAR2 leads to increased expression of IL8, TNFα and IL-1β (Hou et al., 1998; Kim et al., 2002; Ishikawa et al., 2009). In addition, *KLK5* and *KLK7* gene expression was not altered in keratinocytes following 6 hours of incubation with irritants, however in *ex vivo* skin these were both shown to be upregulated 3 hours post-irritant exposure. Thus, similar to the TNFα and IL8 experiments, it would be useful to examine the expression of KLK5 and KLK7 in keratinocytes incubated with irritants at several time points up to and including 3

hours to identify the window of time in which the genes are upregulated, if at all, in the RhoKIT and NHK.

5.1 Conclusions and future work

Previously kallikreins have been shown to be involved in the epidermal barrier repair process. The hypothesis that was tested in this thesis proposed that kallikreins have a second role, namely the activation of the innate immune inflammatory response that characterises the response to irritants. Taken together with other literature, the results of this study suggest that KLK5 may activate PAR2 and contribute to the inflammatory response that occurs following exposure to irritants. These data provide evidence for a proposed mechanism of innate immune response initiation by irritants, but it must be accepted that, due to limitations in the availability of small molecule inhibitors which can penetrate into human skin, it was not possible to demonstrate conclusively that KLKs activate PAR2 and result in production of pro-inflammatory cytokines in human skin. Additional limitations to the current study included the lack of specific inhibitors for KLK5 and KLK7. Whilst there are several inhibitors for these proteases available, all of them target at least one other protease in addition to the KLK of interest, thus further research will be required to identify or generate selective inhibitors in order to determine the exact contribution of KLKs to PAR2 activation following exposure to irritants. Although KLK14 is also known to activate PAR2 (Oikonomopoulou et al., 2006a; Stefansson et al., 2008), it only contributes approximately 1% of the total trypsin-like KLK content of the epidermis (Komatsu et al., 2006) therefore it was not tested in the current study. However, future investigations might wish to consider the role of KLK14 in the initiation of the innate immune response to irritants.

It was hoped in the thesis that the use of RhoKIT keratinocytes and the generation of a 3D epidermal model might allow future testing of new chemical compounds for irritant properties. However, the fragility of the stratum corneum in this model means that further work is required in this area; this might include investigations involving RhoKIT cells from younger donors or keratinocytes immortalised by alternative methods. If 3D skin models can be created which can generate an effective barrier as found in in vivo skin, these could also be used to study the mechanistic events involved

in the responses to irritants in much greater detail. It may also be possible to use lentiviral transduction or siRNA approaches to knock-down the expression of specific proteases or PAR2 within the models to assess the role each of these components contribute to the irritant response. Indeed, Van den Bogaard *et al* showed that Y-27632 increases efficacy of lentiviral transduction, which may enable greater manipulation of human skin equivalents produced with RhoKIT keratinocytes (2012). Despite the work in this thesis not culminating in a ready-to-use 3D epidermal model suitable for testing irritants, the qPCR results with the RhoKIT keratinocytes in monolayer provide a foundation upon which to test a wide range of other known irritants in order to determine whether this monolayer approach could be useful as an initial screening tool of the irritant potential of new chemical entities.

6. References

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7. Appendix

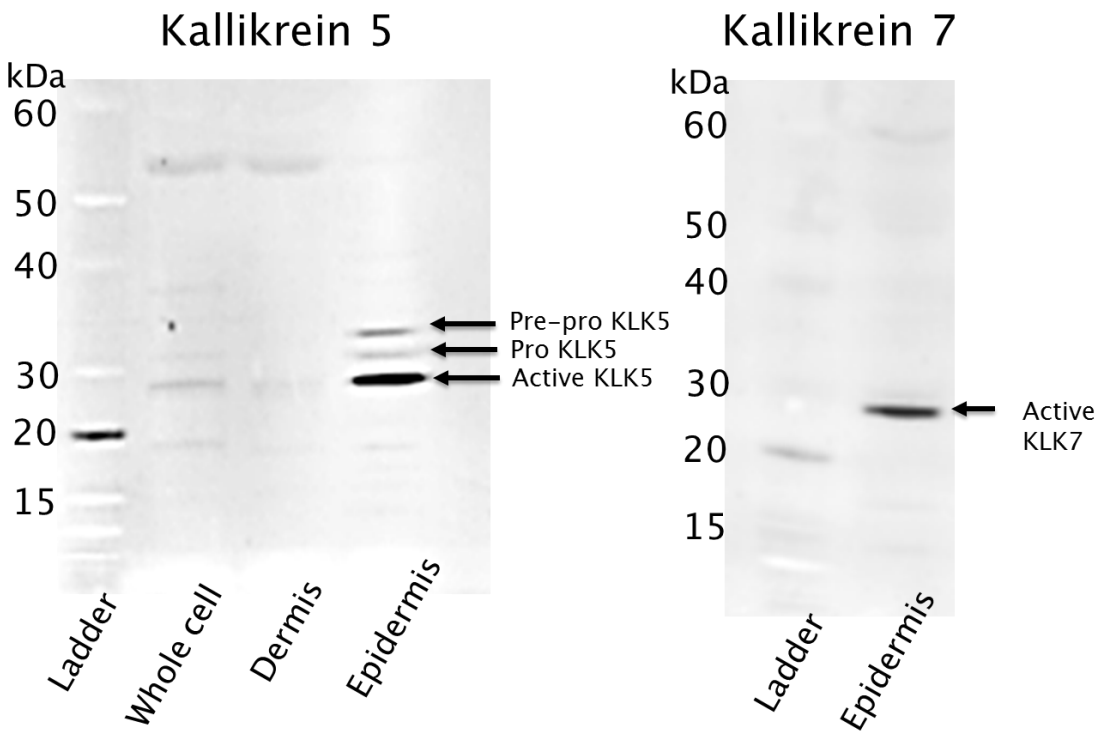
Appendix 1: Genes analysed in custom designed RT-QPCR plates. Custom designed plates were used to assess gene expression following treatment with irritants or sensitisers. Corresponding primers can be found at www.SABiosciences.com using the RT2 catalog numbers listed.

Gene Symbol	Refseq #	Official Full Name	RT2 Catalog Number	
IL1A	NM_000575	Interleukin 1, alpha	PPH00690	
IL1B	NM_000576	Interleukin 1, beta	PPH00171	
IL6	NM_000600	Interleukin 6 (interferon, beta 2)	PPH00560	
IL7	NM_000880	Interleukin 7	PPH00567	
IL8	NM_000584	Interleukin 8	PPH00568	
IL10	NM_000572	Interleukin 10	PPH00572	
IL12A	NM_000882	Interleukin 12A (p35)	PPH00544	
IL12B	NM_002187	Interleukin 12B (p40)	PPH00545	
IL15	NM_000585	Interleukin 15	PPH00694	
IL17C	NM_013278	Interleukin 17C	PPH01074	
IL18	NM_001562	Interleukin 18 (interferon-gamma-inducing factor)	PPH00580	
IL20	NM_018724	Interleukin 20	PPH01078	
IL22	NM_020525	Interleukin 22	PPH01079	
IL23A	NM_016584	Interleukin 23, alpha subunit p19	PPH01688	

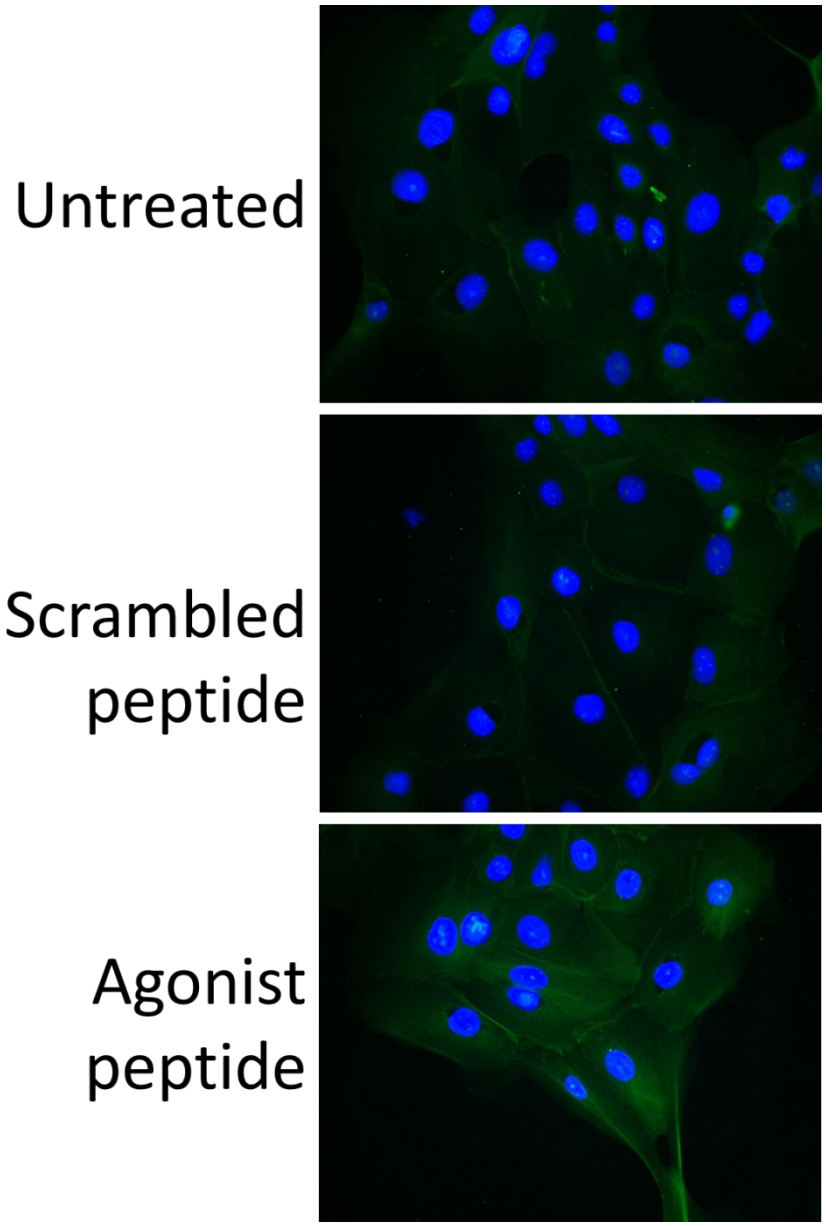
IL24	**NM_006850**	**Interleukin 24**	**PPH01686**	
IL25	**NM_022789**	**Interleukin 25**	**PPH01685**	
IL31	**NM_001014336**	**Interleukin 31**	**PPH60116**	
IL32	**NM_004221**	**Interleukin 32**	**PPH01154**	
IL33	**NM_033439**	**Interleukin 33**	**PPH17375**	
IL36A	**NM_014440**	**Interleukin 36, alpha**	**PPH01683**	
IL36B	**NM_173178**	**Interleukin 36, beta**	**PPH01682**	
IL36G	**NM_019618**	**Interleukin 36, gamma**	**PPH01690**	
CXCL1	**NM_001511**	**Chemokine (C-X-C motif) ligand 1**	**PPH00696**	
CXCL9	**NM_002416**	**Chemokine (C-X-C motif) ligand 9**	**PPH00700**	
CXCL10	**NM_001565**	**Chemokine (C-X-C motif) ligand 10**	**PPH00765**	
CXCL11	**NM_005409**	**Chemokine (C-X-C motif) ligand 11**	**PPH00506**	
CCL1	**NM_002981**	**Chemokine (C-C motif) ligand 1**	**PPH00701**	
CCL2	**NM_002982**	**Chemokine (C-C motif) ligand 2**	**PPH00192**	
CCL5	**NM_002985**	**Chemokine (C-C motif) ligand 5**	**PPH00703**	
CCL11	**NM_002986**	**Chemokine (C-C motif) ligand 11**	**PPH00570**	
CCL13	**NM_005408**	**Chemokine (C-C motif) ligand 13**	**PPH00578**	
CCL17	**NM_002987**	**Chemokine (C-C motif) ligand 17**	**PPH00543**	
CCL18	**NM_002988**	**Chemokine (C-C motif) ligand 18**	**PPH00574**	
CCL20	**NM_004591**	**Chemokine (C-C motif) ligand 20**	**PPH00564**	

CCL22	**NM_002990**	**Chemokine (C-C motif) ligand 22**	**PPH00697**	
CCL26	**NM_006072**	**Chemokine (C-C motif) ligand 26**	**PPH01163**	
CCL27	**NM_006664**	**Chemokine (C-C motif) ligand 27**	**PPH01164**	
TNF	**NM_000594**	**Tumor necrosis factor**	**PPH00341**	
TGFB1	**NM_000660**	**Transforming growth factor, beta 1**	**PPH00508**	
IFNA1	**NM_024013**	**Interferon, alpha 1**	**PPH01321**	
IFNB1	**NM_002176**	**Interferon, beta 1, fibroblast**	**PPH00384**	
IFNG	**NM_000619**	**Interferon, gamma**	**PPH00380**	
CSF2	**NM_000758**	**Colony stimulating factor 2 (granulocyte-macrophage)**	**PPH00576**	
VEGFA	**NM_003376**	**Vascular endothelial growth factor A**	**PPH00251**	
PDGFA	**NM_002607**	**Platelet-derived growth factor alpha polypeptide**	**PPH00217**	
NFE2L2	**NM_006164**	**Nuclear factor (erythroid-derived 2)-like 2**	**PPH06070**	
KEAP1	**NM_012289**	**Kelch-like ECH-associated protein 1**	**PPH09563**	
HMOX1	**NM_002133**	**Heme oxygenase (decycling) 1**	**PPH00161**	
NQO1	**NM_000903**	**NAD(P)H dehydrogenase, quinone 1**	**PPH01546**	
FOSL1	**NM_005438**	**FOS-like antigen 1**	**PPH00145**	
IL1RN	**NM_000577**	**Interleukin 1 receptor antagonist**	**PPH00555**	
HSPA1A	**NM_005345**	**Heat shock 70kDa protein 1A**	**PPH01193**	
POMC	**NM_000939**	**Proopiomelanocortin**	**PPH07105**	

ID2	**NM_002166**	**Inhibitor of DNA binding 2, dominant negative helix-loop-helix protein**	**PPH00414**	
B2M	**NM_004048**	**Beta-2-microglobulin**	**PPH01094**	
HPRT1	**NM_000194**	**Hypoxanthine phosphoribosyltransferase 1**	**PPH01018**	
RPL13A	**NM_012423**	**Ribosomal protein L13a**	**PPH01020**	
GAPDH	**NM_002046**	**Glyceraldehyde-3-phosphate dehydrogenase**	**PPH00150**	
ACTB	**NM_001101**	**Actin, beta**	**PPH00073**	
HGDC	**SA_00105**	**Human Genomic DNA Contamination**	**PPH65835**	
RTC	**SA_00104**	**Reverse Transcription Control**	**PPX63340**	
PPC	**SA_00103**	**Positive PCR Control**	**PPX63339**	



Appendix 2: Confirmation of antibody specificity for KLK5 and KLK7. The antibody for KLK5 detected 3 bands at approximately 34 kDa, 32 kDa and 28 kDa. These most likely correspond to the pre-pro-, pro- and active forms of KLK5. KLK5 was strongly present in the epidermal fraction and faintly detected in the whole skin preparation as expected. KLK7 was strongly detected in the epidermal fraction at the expected size (27 kDa). Results representative of 4 experiments using 100 µg of protein for each lane. No protein was detected when less than 100 µg was used.



Appendix 3: NHK stained for PAR2 with H99 antibody. Staining was increased following activation with the PAR2 agonist SLIGKV-NH_2 compared with untreated cells. No change in staining was observed when NHK were treated with the scrambled peptide VKGILS-NH_2. PAR2 staining is shown in green. DAPI counterstain used for nuclear staining (blue). Images representative of 3 experiments.