The Role of Apoptosis in UVB-Induced Clearance of Psoriasis

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Abstract

Ultraviolet (UV) B therapy can induce complete clearance of psoriasis and often leads to prolonged remission following a treatment period. UVB phototherapy is a commonly used, highly effective treatment modality, but despite this its mechanism of action remains poorly understood. This thesis investigates the importance of keratinocyte apoptosis in UVB-induced clearance of psoriasis using a novel approach, and employs a mathematical model to explore the effects of this on epidermal homeostasis.

The *in vivo* effects of two wavelengths of UVB were compared, one of which is clinically effective in clearing psoriasis (311nm), and one which is ineffective (290nm) even at high doses. This allowed investigation of which effects of UVB are relevant to plaque clearance; eliminating 'bystander' effects such as erythema. The study showed significant keratinocyte apoptosis in lesional psoriatic epidermis following 311nm UVB compared to 290nm UVB; peaking 16-24h post irradiation. To determine clinical significance, a computational model of psoriatic epidermis was created utilising histological and kinetic parameters. The model predicted that apoptosis should occur in both stem and TA cells to account for plaque remodelling. This was confirmed and quantified experimentally, with real-time assays determining the rate of keratinocyte apoptosis. These data were fed back into the model and demonstrated that the observed level of keratinocyte apoptosis was sufficient to account for complete remodelling of psoriatic plaques in response to therapeutic UVB.

The data was supported by gene array studies, which showed differential regulation of apoptotic genes at early time-points following 311nm rather than 290nm UVB. Finally, the wavelength dependence of UVB-induced apoptosis was examined, and only wavelengths which can clear psoriasis (i.e. greater than 300nm & less than 320nm UVB) induced significant epidermal apoptosis.

In summary, this thesis demonstrates that keratinocyte apoptosis is a key mechanism of psoriatic clearance in response to UVB.

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Abbreviations

AGER (RAGE)	Advanced glycosylation end product-specific receptor
AIF	Apoptosis-inducing factor
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
APC	Allophycocyanin
APES/ TESPA	3-Aminopropyltriethoxysilane
ATF	Activating transcription factor
ATG	Autophagy related gene
ATP	Adenosine triphosphate
AURKA	Aurora kinase A
BrDU	Bromodeoxyuridine
BSA	Bovine serum albumin
BUB1	Budding uninhibited by benzimidazoles 1 homolog
CASP3	Caspase-3
CCL	Chemokine (C-C motif) ligand
CDKN1A (p21/ WAF1)	Cyclin-dependent kinase inhibitor 1A
CK	Cytokeratin
CO_2	Carbon dioxide
COX	Cyclooxygenase

CREM	Cyclic AMP response element gene
Ct	Cycle threshold. The point at which PCR fluorescence crosses a given threshold
DASL	cDNA-mediated annealing, selection, extension and ligation
DC	Dendritic cell
ddH2O	Double-distilled water
DEFB	Human beta-defensin gene
DEVD	(The amino acid sequence) Asp-Glu-Val-Asp
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
DUSP	Dual specificity phosphatase
EC50	Median effective concentration (required to induce 50% effect)
EGF	Epidermal growth factor
ELOVL	Elongation of very long chain fatty acids gene
EndoG	Endonuclease G
FACS	Fluorescence-activated cell sorting
FADS	Fatty acid desaturase gene
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLM	Fraction of labelled mitoses
FOSL	FBJ murine osteosarcoma viral oncogene homolog-like antigen

FRET	Fluorescence-resonance energy transfer
FSC	Forward-scatter
GDF15 (MIC-1)	Growth differentiation factor 15
GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAS	Genome wide association scan
h	Hours
HBD	Hemoglobin delta
HLA	Human leukocyte antigen
ICAD	Inhibitor of caspase-activated DNase
IFN	Interferon
IFNγ	Interferon γ
IHC	Immunohistochemistry
IL	Interleukin
IPA	Ingenuity pathway analysis
IQR	Interquartile range
KGF (FGF-7)	Keratinocyte growth factor (fibroblast growth factor-7)
KRT	Keratin
LCE	Late cornified envelope
LL37 (CAMP)	cathelicidin antimicrobial peptide
MAF	Musculoaponeurotic fibrosarcoma
MAPK	Mitogen-activated protein kinase

MDM2	Mouse double minute 2 human homolog of p53 binding protein
MED	Minimal erythema dose
Min	Minute
NaOH	Sodium hydroxide
NEK2	NIMA (never in a mitosis gene a)-related protein
NFKB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells
NGF/ NGRR	Nerve growth factor/ nerve growth factor receptor
NHEK	Normal human epidermal keratinocytes
NHS	National Health Service
NK cell	Natural killer cell
nm/ ng	Nanometer/ nanogram
NOS2	Nitric oxide synthases 2
NOXA	NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activator
NSAID	Non-steroidal anti-inflammatory drug
OCT medium	Optimal cutting temperature medium
PARP	Poly (ADP-ribose) polymerase
PASI	Psoriasis area and severity index
PBMNs	Polymorphonuclear leukocytes
PBS	Phosphate buffered saline

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDC	Plasmacytoid dendritic cell
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PGE_2	Prostaglandin E2
PI	Propidium iodide
PI3	Peptidase inhibitor 3 (skin derived)
PIK3C2G	Phosphoinositide-3-kinase class 2, gamma polypeptide
PSORS	Psoriasis susceptibility locus
PUVA	Psoralen and Ultraviolet A
RIN	RNA integrity number
RNA	Ribonucleic acid
ROI	Region of interest
rtp	Room temperature
RUNX1 (PSORS 2)	Runt-related transcription factor 2
S100A	S100 calcium binding protein
SCID	Severe combined immunodeficient
SD	Standard deviation
SED	Standard erythema dose

SELP	Selectin P
SEM	Standard error of the mean
SNP	Single-nucleotide polymorphism
SPHK	Sphingosine kinase
SRB	sulphorhodamine
SPRR	Small proline-rich protein gene
SSC	Side-scatter
STAT	Signal transducer and activator of transcription
TA	Transit amplifying
TEM	Transmission electron microscopy
TGF-α/β	Transforming growth factor alpha/ beta
TH cells	T helper cells
TNFα	Tumour necrosis factor α
TNIP	TNFAIP3 (TNF alpha-inducing protein-3) interacting protein
TP53	p53
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling
UVA/B/C	Ultraviolet A/B/C
UVR	Ultraviolet radiation
VEGF	Vascular endothelial growth factor
μm/ μl	Micrometer/ microlitre

1. Introduction

Psoriasis is a common disorder with an estimated lifetime prevalence of 1.5-2.2% in the adult population in the Western world (Stern, Nijsten et al. 2004; Gelfand, Weinstein et al. 2005), with 75% of patients presenting before age 40 (Barker, Palmer et al. 2006). The term is derived from the Greek word ‘psora’ meaning itch or rash, and was first formally described in 1808 (Plumbe 1824).

Psoriasis can manifest in different clinical forms, including plaque, guttate, pustular and erythrodermic. Chronic plaque psoriasis is the most common form, and is characterised by thick, erythematous, scaly plaques which can appear at any site on the skin. It causes symptoms ranging from itching and bleeding to loss of limb function, with significant social morbidity and occupational disability (Smith and Barker 2006). It is also a major economic burden, and in the US has been estimated to cost $1500 per patient/ year, with work loss accounting for 40% of this (Fowler, Duh et al. 2008).

Current treatment options are relatively limited. Patients with mild-to-moderate disease may be adequately treated with topical therapies. However, these are time consuming, can be messy, smelly and stain skin or clothing, and have limited efficacy in many patients. Patients with moderate to severe psoriasis often require potentially toxic treatments to control their disease, including ciclosporin, methotrexate, and anti-TNFα-therapies. In contrast, UVB (ultraviolet B) phototherapy is a relatively safe (Young 1995) and effective treatment option for many patients. The efficacy of the most commonly used systemic treatments (Pearce, Nelson et al. 2006) is shown in Figure 1-1, however it should be noted that these results are drawn from separate studies which will not have been carried out using identical methodologies. Moreover, current measurement of treatment success (e.g. Psoriasis Area and Severity Index scoring (PASI) see appendix C) doesn’t distinguish remodelling of existing plaques back to normal from switching off active disease (i.e. the ability for psoriasis to flare up again). As psoriasis may flare during a course of treatment due to external effects (e.g. concurrent infection) the improvement in disease may be underestimated as measured by the current scoring systems, making it more difficult to compare efficacy of different treatments as this cannot be controlled for.

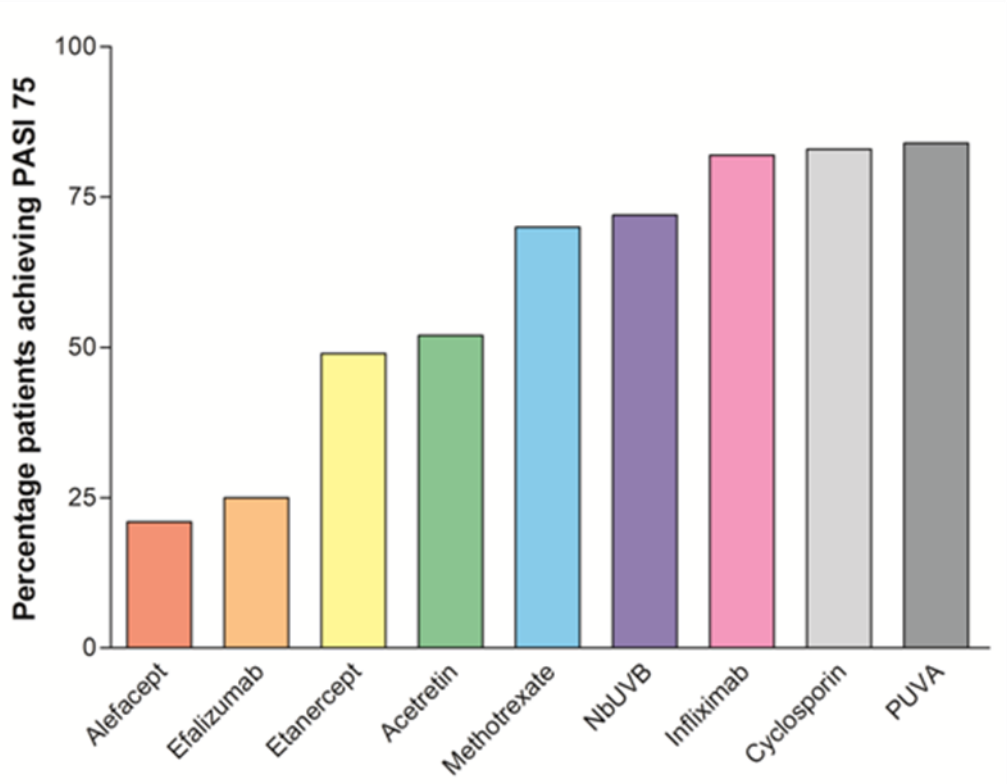


Figure 1-1. Comparison of the efficacy of psoriatic therapies. Data show a meta-analysis of the percentage of patients achieving improvement in PASI score by at least 75% over a 12 week period. The studies will have had different protocols and this should be considered when interpreting the results. Adapted from (Pearce, Nelson et al. 2006).

Systemic treatments can be highly effective in many patients, although side-effects and dosage regimes can limit their usefulness. At present there are no known indicators to suggest which patients are likely to respond to which treatment, and a trial and error approach is often employed. Phototherapy with UVB is a safer approach to treatment, and is generally very well tolerated. UVB wavelengths of around 311nm are highly effective in clearing psoriasis (Coven, Burack et al. 1997; Gordon, Diffey et al. 1999; Kirke, Lowder et al. 2007), but wavelengths just 20nm lower, around 290nm, are ineffective (Parrish and Jaenicke 1981). This thesis describes a novel approach to examine the mechanism of action of UVB in the clearance of psoriasis *in vivo* by comparing the cellular effects of clinically effective (311nm) and ineffective (290nm) wavelengths of UVB. Thus, changes specific to the therapeutic wavelength can

be detected whilst eliminating non-specific effects of UVB. An overview of the use of UVB in psoriasis is presented, and the possible mechanisms by which it may work are discussed. Understanding the mechanisms by which UV acts to clears psoriasis, may provide insight into the pathology that drives the disease, and help with development of treatments which are more targeted, leading to greater efficacy and less side-effects.

1.1. The Epidermis

The skin is the largest organ in the body, exceeding $2m^2$ in the average adult, yet at the majority of sites are no more than 2.5mm in thickness. Structurally, it is principally divided into two layers: the epidermis which is 75-150µm thick, but may be up to 600µm on palms and soles; and dermis which is usually less than 2mm, but may be up to 4.5mm on sites such as on the back (Tobin 2006). The skin is the primary barrier to exogenous insults such as UV radiation, chemical exposure, mechanical stresses and infection. It also has important homeostatic functions including maintenance of temperature and fluid balance, providing sensory input, and plays an important role in psychosexual communication.

The epidermis forms a continuous sheet of stratifying keratinizing epithelium, of which keratinocytes are the principal cell type (approximately 80%) (Savill 2003). Other cell types include melanocytes, T cells, Langerhans' cells and monocytes. The epidermis is conventionally divided into four layers (Figure 1-2).

1. The *stratum corneum* which comprises anucleated differentiated squames which are eventually exfoliated
2. The *stratum granulosum* which contains the majority of differentiating cells
3. The *stratum spinosum* which includes proliferating cells, but these will be committed to differentiation (transit amplifying cells)
4. The basal layer which contains the keratinocyte stem cell population

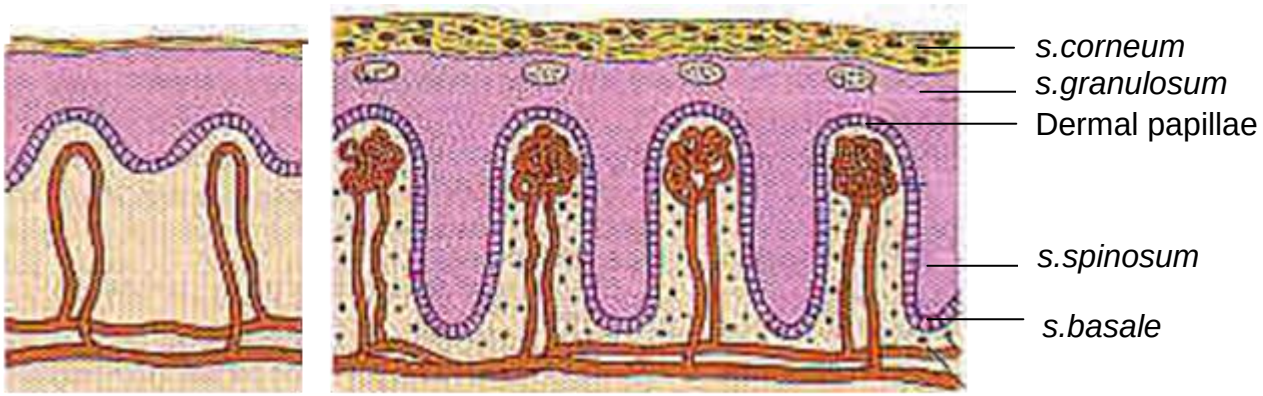


Figure 1-2. Histology of normal and psoriatic skin. In psoriasis (right) there is elongation of the rete ridges, with thinning above the dermal papillae and an increase in dermal vasculature. The number of epidermal keratinocytes is markedly increased and the *stratum corneum* is thickened. Adapted from Hunter (Hunter 1995).

In psoriasis the epidermis becomes hyperkeratotic, with elongation of the rete ridges and marked thickening of the *stratum corneum* and *stratum spinosum* (Figure 1-2). Epidermal stem cells are found within the basal layer, and have been shown to be of greatest density adjacent to the tips of the dermal papillae (Jones, Harper et al. 1995; Ghali, Wong et al. 2004). Stem cells are also located in the bulge region of the hair follicle, however evidence suggests that these have a transient role in regenerating epidermis in response to wound healing, rather than having a role in epidermal homeostasis (Ito, Liu et al. 2005). Stem cells divide within the basal layer, therefore an increase in their activity may explain the elongation of the rete ridges classically found within psoriasis. Other changes in psoriatic epidermis include a reduced or absent *stratum granulosum* in proliferating plaques, and significant growth of superficial capillaries within plaques leading to tortuous elongated capillaries in the dermal papillae. The latter is thought to be due to release of mediators such as vascular endothelial growth factor and platelet derived growth factor from keratinocytes (Liu, Krueger et al. 2007).

1.2. Pathogenesis of psoriasis

Traditionally psoriasis was considered to be caused by a primary abnormality of keratinocyte hyperproliferation and reduced turnover time. However, with the realisation that ciclosporin cleared psoriasis, and that plaques contained large numbers of T cells which 'disappeared' as plaques resolved, it became widely accepted that psoriasis could be due to a primary abnormality within the T cell compartment. In normal skin, T cells are not present in the epidermis at all. However in psoriasis, T cells (predominantly CD8+ cells) can be found in high numbers throughout the epidermis, and T cells are increased within the dermis (predominantly CD4+ cells). There is also an increase in the number of CD11c+ dendritic cells within the lower epidermis, and Langerhans' cells (which are usually present uniformly within the epidermis) migrate up to the *stratum spinosum* (Liu, Krueger et al. 2007). More recently evidence has once again challenged dogma suggesting that primary abnormalities may also exist within the keratinocyte compartment (Section 1.2.3). Although the primary abnormality in psoriasis is still debated, what is clear is that psoriasis represents a complex interplay between the innate and adaptive immune system, environmental triggers and susceptibility genes. The evidence for these is examined below.

1.2.1.1. Genetics of psoriasis

The incidence of psoriasis varies considerably between both geographical location and race, and is quoted as approximately 2% in Caucasians and 0.1% in the Chinese population (Sun, Li et al. 2007). The genetic basis for psoriasis is generally accepted, although it is complicated by the fact that the penetrance of genes associated with psoriasis is so variable, and environmental triggers are required to stimulate phenotypic changes. Overall, a family history of psoriasis is only found in a third of patients (Elder, Nair et al. 2001). The concordance of psoriasis in monozygotic twins is estimated at 65-72%, and 15-30% in dizygotic twins (Bowcock and Cookson 2004).

Two peaks in age at presentation have been described: the first is in individuals aged 20-30 years (individuals presenting below the age of 40 are defined as having type 1 psoriasis) and a second smaller peak at 50-60 years (type 2 psoriasis). It is postulated that these two peaks may have differing aetiologies, and indeed evidence for genetic association is stronger in the group with earlier age of presentation. Epidemiological studies have suggested that the relative risk of developing psoriasis in a first degree relative of an affected individual with type 1 disease is 10, with the risk falling to 2 in relatives of type 2 disease (Elder, Nair et al. 2001).

The genetics of psoriasis are multifactorial, and linkage studies examining the genetics of psoriasis have often yielded inconsistent results (Nair, Henseler et al. 1997; Trembath, Clough et al. 1997; Samuelsson, Enlund et al. 1999; Lee, Ruschendorf et al. 2000) . Psoriasis susceptibility 1 (PSORS1) is an exception to this, and is the most extensively studied locus. It is located in the major histocompatibility complex (HLA) class1 region, on the short arm of chromosome 6. The HLA-Cw6 allele is thought to be the most likely candidate within this, and is carried by two thirds of psoriatic patients, although 10% of unaffected individuals also express this (Elder, Nair et al. 2001). Elucidation of the genetics of psoriasis and other complex genetic disorders are confounded by the high population frequency of disease alleles (Risch and Merikangas 1996), but the development of HapMap has made large-scale genome wide association studies (GWAS) possible . These allow typing of 100,000 to 100,000,000 SNPs in thousands of individuals to identify susceptibility loci, which can be consistently reproduced between studies. The major association loci identified by GWAS to date include SNPs located within 19 regions including: HLA-C, IL12B, IL23R, IL23A, IL4/IL13, TNFAIP3, TNIP1, LCE3B, LCE3C, ZNF313, PTPN22, CDKAL1 (Cargill, Schrodi et al. 2007; Capon, Bijlmakers et al. 2008; Liu, Helms et al. 2008; Nair, Duffin et al. 2009; Zhang, Huang et al. 2009), and seven of these have been consistently replicated between studies (IL23R, IL13, TNIP1, IL12B, HLA-c, TNFAIP3 and IL23A) (Elder, Bruce et al. 2010).

IL23R, a gene identified by GWAS, encodes the IL-23 receptor gene (a newly defined cytokine which induces IFN-γ) (Cargill, Schrodi et al. 2007). IL-23 comprises two subunits, p19 and p40, and possesses structural similarity to IL-12. Human keratinocytes produce both the IL-12 and IL-23 subunits (Piskin, Sylva-Steenland et al. 2006), and can thereby promote the type 1 response. Lesional psoriatic keratinocytes express an enhanced level of IL-12 and IL-23 mRNA and protein compared to non-lesional or normal skin (Yawalkar, Karlen et al. 1998; Piskin, Sylva-Steenland et al. 2006; Cargill, Schrodi et al. 2007), which can then directly modulate T cell IFN-γ secretion by T cells. Enhanced IL-23 expression has also been shown in macrophages, dermal dendritic cells and Langerhans' cells (Piskin, Sylva-Steenland et al. 2006). Recently Th17 cells have been described and these have been strongly implicated in psoriasis (Di Cesare, Di Meglio et al. 2009). IL23 stimulates proliferation and survival of Th17 cells, which produce IL-17 and IL-22 (Haider, Lowes et al. 2008), this enhances the expression of keratinocyte antimicrobial peptides, and promote the psoriatic phenotype. Therapeutic treatment options such as narrowband UVB have been shown to suppress lesional psoriatic IL-23 down to levels seen in normal and non-lesional skin (Piskin, Tursen et al. 2004).

Drugs which target monoclonal antibodies against TNFα such as Infliximab can clear psoriasis. Polymorphisms in the genes whose products work downstream of TNFα to regulate NFκB (TNFAIP3 and TNIP1), have been identified as possible psoriasis susceptibility loci (Nair, Duffin et al. 2009). TNFAIP3 encodes a protein (A20) which inhibits NFκB and temporarily limits immune responses. In a mouse model, the region containing TNFAIP3 has been shown to promote psoriasis through TNFα (Wang, Kess et al. 2008), and interestingly the same region of the gene is associated with an increased risk of atherosclerosis (Idel, Dansky et al. 2003); a co-morbidity with a higher prevalence in patients with psoriasis (Gelfand, Neimann et al. 2006). However, common polymorphisms in this region have also been associated with rheumatoid arthritis and systemic lupus erythematosis in other studies (Plenge, Cotsapas et al. 2007; Graham, Cotsapas et al. 2008; Musone, Taylor et al. 2008).

Further genes of interest identified by GWAS include the late cornified envelope genes LCE3B and LCE3C. Homozygous deletion of these are associated with an increased risk of psoriasis, and the LCE genes are strongly up-regulated in psoriasis (de Cid, Riveira-Munoz et al. 2009; Coto, Santos-Juanes et al. 2010; Huffmeier, Bergboer et al. 2010). This supports the evidence described in section 1.2.3, that psoriatic keratinocytes are abnormal.

1.2.2. **Role of T cells in psoriasis**

T cells play an important role in the pathogenesis of psoriasis (Abrams, Lebwohl et al. 1999; Bos and De Rie 1999). This is illustrated in animal models where engrafted non-lesional human psoriatic skin develops into psoriasis, but is preventable by inhibition of T cells proliferation (Boyman, Hefti et al. 2004). Clinical observation also supports the role of T cells in psoriasis, for example, bacterial superantigens exacerbate or unmask psoriasis (Valdimarsson, Baker et al. 1995), bone marrow transplantation from psoriatic donors has been reported to trigger psoriasis in recipients who have not previously had psoriasis (Gardembas-Pain, Ifrah et al. 1990), T cell targeted therapies can treat psoriasis (Chaudhari, Romano et al. 2001; Lebwohl, Tyring et al. 2003; Leonardi, Powers et al. 2003) and cytokine-based treatments can exacerbate psoriasis (Asnis and Gaspari 1995).

There has been much interest in studying the depletion of T cells following psoriatic treatments including UVB (Krueger, Wolfe et al. 1995; Ozawa, Ferenczi et al. 1999), although whether these clear as a cause or effect phenomenon is unclear. Psoriasis is sometimes labelled as an autoimmune disease although no potential antigen has ever been discovered. Perhaps the most convincing evidence for T cells as the primary abnormality in psoriasis comes from a study in which superantigen-activated psoriatic immunocytes were injected into uninvolved psoriatic human skin grafted onto severe combined immunodeficient (SCID) mice (Wrone-Smith and Nickoloff 1996).

This resulted in the ‘symptomless’ skin changing into a psoriatic phenotype. A caveat to this study is that activation with non-antigenic substances, such as nerve growth factor (NGF), have been shown to cause a similar phenotypic change in engrafted normal skin (Nickoloff, Schroder et al. 2000). This suggests that local factors within the skin may also activate lesional T cells, inducing the observed phenotypic changes.

The T cell model of psoriasis fails to explain several features of psoriasis. In particular, the symmetrical nature of psoriasis, the Koebner phenomenon, and the resolution of psoriasis at sites of anaesthesia (Farber, Lanigan et al. 1990; Raychaudhuri and Farber 1993). The latter has been linked with up-regulation of NGF (which is expressed by keratinocytes) and its receptor (NGF-R) in lesional and non-lesional psoriasis (Fantini, Magnoni et al. 1995). NGF activates T cells, recruits inflammatory cells, promotes proliferation of keratinocytes and protects keratinocytes from apoptosis (Farber and Raychaudhuri 1999). It has been hypothesised that NGF has a role in wound healing, and its up-regulation may drive an inflammatory response and keratinocyte proliferation, possibly explaining why the Koebner phenomenon occurs in psoriasis (Nickoloff, Schroder et al. 2000). Moreover, it is now realised that drugs which have immunosuppressive effects on T cells, e.g. ciclosporin and tacrolimus, also exert direct independent effects on both neutrophils and keratinocytes (Fisher, Duell et al. 1988; Reynolds, Voorhees et al. 1998; Al-Daraji, Grant et al. 2002). Furthermore, psoriasis often relapses almost immediately after these drugs are stopped, suggesting that they are suppressing the disease rather than inducing remission.

1.2.3. **Role of keratinocytes in psoriasis**

Non-lesional psoriatic keratinocytes differ from normal keratinocytes as evidenced by abnormally increased rate of proliferation (Weinstein, McCullough et al. 1984) and an abnormal intracellular calcium signal in response to

physiological agonists (Karvonen, Korkiamaki et al. 2000; Ross 2005). Moreover they have altered intracellular signalling pathways including activated STAT3 (Pasparakis, Courtois et al. 2002; McKenzie and Sabin 2003; Sano, Chan et al. 2005; Arthur and Darragh 2006). Transgenic mice with constitutively active STAT3 develop a psoriatic phenotype, but require activated T cells to be present for this to occur (Sano, Chan et al. 2005). Similarly when non-lesional psoriatic skin is grafted onto SCID mice, the psoriatic phenotype develops only when activated T cells are introduced (Wrone-Smith and Nickoloff 1996; Gilhar, Yaniv et al. 2006).

An inducible JunB double-knockout mouse model has been described by Zenz et al (Zenz, Eferl et al. 2005). JunB is located in the PSORS6 psoriasis susceptibility region and is involved in regulation of cell proliferation, differentiation and cytokine expression. Deletion of JunB and c-jun from epidermal keratinocytes resulted in mice with the psoriatic phenotype and arthritic lesions. The authors concluded that changes in keratinocytes were sufficient to initiate psoriasis-like skin changes. However, conflicting data exists about whether JunB is reduced or increased in psoriatic plaques (Zenz, Eferl et al. 2005; Haider, Duculan et al. 2006).

LL37 is an antimicrobial peptide which can be transiently expressed by keratinocytes following skin injury (Dorschner, Pestonjamasp et al. 2001; Heilborn, Nilsson et al. 2003). In psoriasis LL37 expression has been shown to be persistently high (Frohm, Agerberth et al. 1997), and can bind to self-DNA (Lande, Gregorio et al. 2007). This activates plasmacytoid dendritic cells and triggers TLR9, producing IFN-α (Lande, Gregorio et al. 2007). LL37 has been shown to be the principal trigger for IFN-α production, which is central to the development of psoriasis (Nestle, Conrad et al. 2005). In this way, T cells are further stimulated and a positive feedback loop is initiated (Nickoloff and Griffiths 1990). However unlike in normal skin, this effect is maintained long after the initial stimulus is withdrawn.

Further evidence in support of keratinocytes being the primary abnormality in psoriasis includes the fact that psoriasis can occur linearly within Blaschko lines

(migratory patterns of epidermis) which suggests that the responsible genetic defect lies within this compartment (Moss 1999). Finally, proliferation of stem cells from non-lesional psoriasis can be stimulated using supernatant and T cells derived from psoriatic plaques. However, T cells do not stimulate proliferation of stem cells derived from non-psoriatic patients (Bata-Csorgo, Hammerberg et al. 1995).

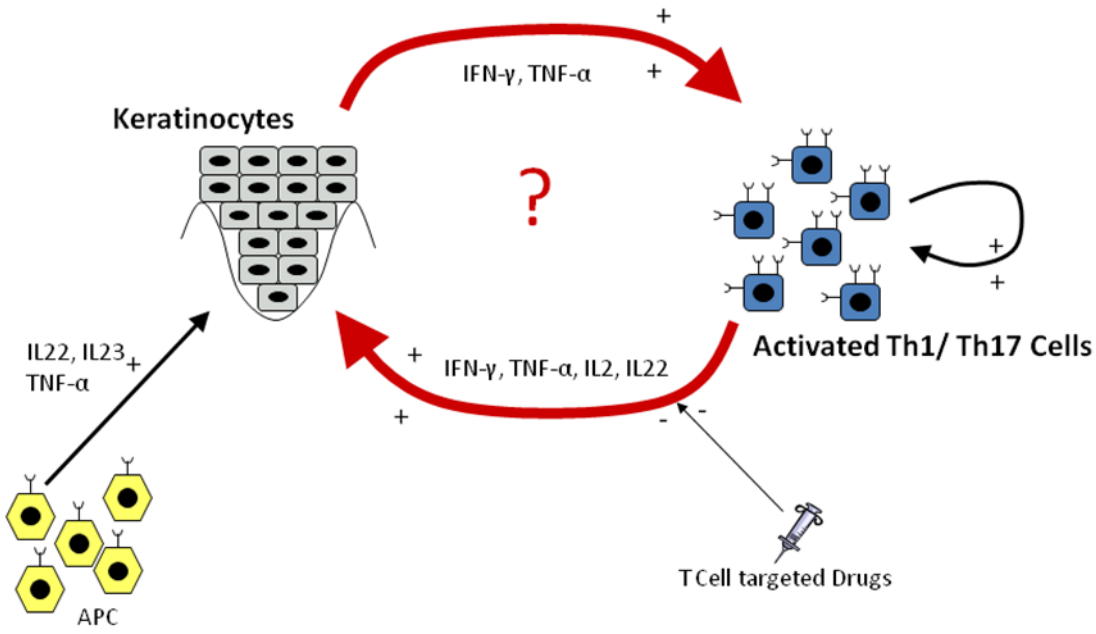


Figure 1-3. Pathogenesis of psoriasis. T cells, keratinocytes and antigen presenting cells (APCs) secrete proinflammatory cytokines which can act in a positive feedback loop, augmenting the development of psoriasis. Th17 cells and APC secrete IL22 which up regulates STAT3 and induces antimicrobial peptide production by keratinocytes which contribute to the psoriatic phenotype. Whether the primary abnormality lies with keratinocytes or T cells is unknown.

If keratinocytes represent a primary abnormality in psoriasis, then T cells are pivotal in amplifying abnormal cytokine production to drive and maintain plaque formation. Psoriatic keratinocytes secrete higher levels of chemo-attractant factors such as interleukin 1 and TNFα which induce epidermal T cells migration and activation. Once activated, a positive feedback loop can exist

whereby T cells will induce hyperproliferation of keratinocytes through release of stimulating cytokines including GM-CSF and IFN-γ (Bata-Csorgo, Hammerberg et al. 1995) (Figure 1-3). TNFα production stimulates further TNFα releasing cytokines from keratinocytes, dendritic cells, T cells, neutrophils, monocytes, macrophages and NK T cells; making it difficult to identify the primary trigger.

1.2.4. **Role of dendritic cells in psoriasis**

Dendritic cells (DCs) are involved in tolerance and generate specific T cell activation in response to environmental signals. Normal skin comprises Langerhans' cells and dermal dendritic cells. The latter are increased in psoriasis, and two other DC subsets are also present: plasmacytoid DCs and inflammatory dendritic epidermal cells. Interestingly, dermal DCs are activated in both lesional and non-lesional psoriasis (Zhou, Krueger et al. 2003), and produce TNFα (Nickoloff, Karabin et al. 1991). When activated, DCs transform into stimulatory APCs, but under conditions of steady-state they present peptides with insufficient stimulation leading to the development of T cell anergy or regulatory T cell production. It has been postulated that psoriasis can be triggered when this balance of immune reactivity versus tolerance is disturbed (Bos, de Rie et al. 2005).

Plasmacytoid dendritic cells (PDC) secrete IFNα, and are key effector cells in the innate immune system in response to viruses, and in doing so provide a link between the innate and adaptive immune system. IFNα therapy can exacerbate psoriasis (Downs and Dunnill 2000), and the IFNα signalling pathway has been shown to be activated in psoriasis (van der Fits, van der Wel et al. 2004). A study by Nestle et al demonstrated that IFNα derived from PDCs had an essential role in the local activation and expansion of T cells in grafted psoriatic plaques on AGR-/- mice, and suggested that these are key cells in driving psoriasis development (Nestle, Conrad et al. 2005).

1.3. Ultraviolet radiation

UV radiation is part of the electromagnetic spectrum, lying between the visible and x-ray region, and can be divided into three regions according to its wavelength (Figure 1-4) although the accepted waveband definitions do differ slightly between countries:

UVA: 320- 400 nm;

UVB: 290- 320 nm;

UVC: 200- 290 nm.

Approximately 5% of terrestrial summer solar radiation comprises energy within the UV spectrum, with the majority of this being UVA, and a small proportion (approximately 6%) being UVB. UVA alone is not effective in clearing psoriasis, although can be very effective when combined with a psoralen (PUVA). The increased risk of burning and skin cancers associated with this treatment modality, limits the number of exposures to PUVA therapy that any patient can safely be given (Slaper, Schothorst et al. 1986; McKenna, Patterson et al. 1996).

There is a wide variation in biological effects of UV, dependent on wavelength. Longer wavelengths (UVA) penetrate deeper into the skin, and may cause effects within the dermis, whereas shorter wavelengths show less penetration. Average transmission of UV through skin on the lower back follows an exponential curve, with maximal change between 275 and 290nm. In normal forearm skin, wavelengths of 290nm have been shown to penetrate into the lower epidermis approximately 14 times less than 314nm (Meinhardt, Krebs et al. 2009). UVB and UVC have both been shown to induce apoptosis of normal keratinocytes *in vitro*. However, UVC appears to cause significantly more (6-4) photoproducts and cyclobutane pyrimidine dimers than UVB in cultured keratinocytes (Takasawa, Nakamura et al. 2005). The authors of this study also found a greater release of mitochrondrial apoptotic pathway factors (such as cytochrome C) with UVC rather than UVB, but UVB achieved equal rates of

apoptosis through activation of the extrinsic pathway (see appendix A). This suggests that effects of UV on keratinocytes are wavelength specific, irrespective of differences in penetration.

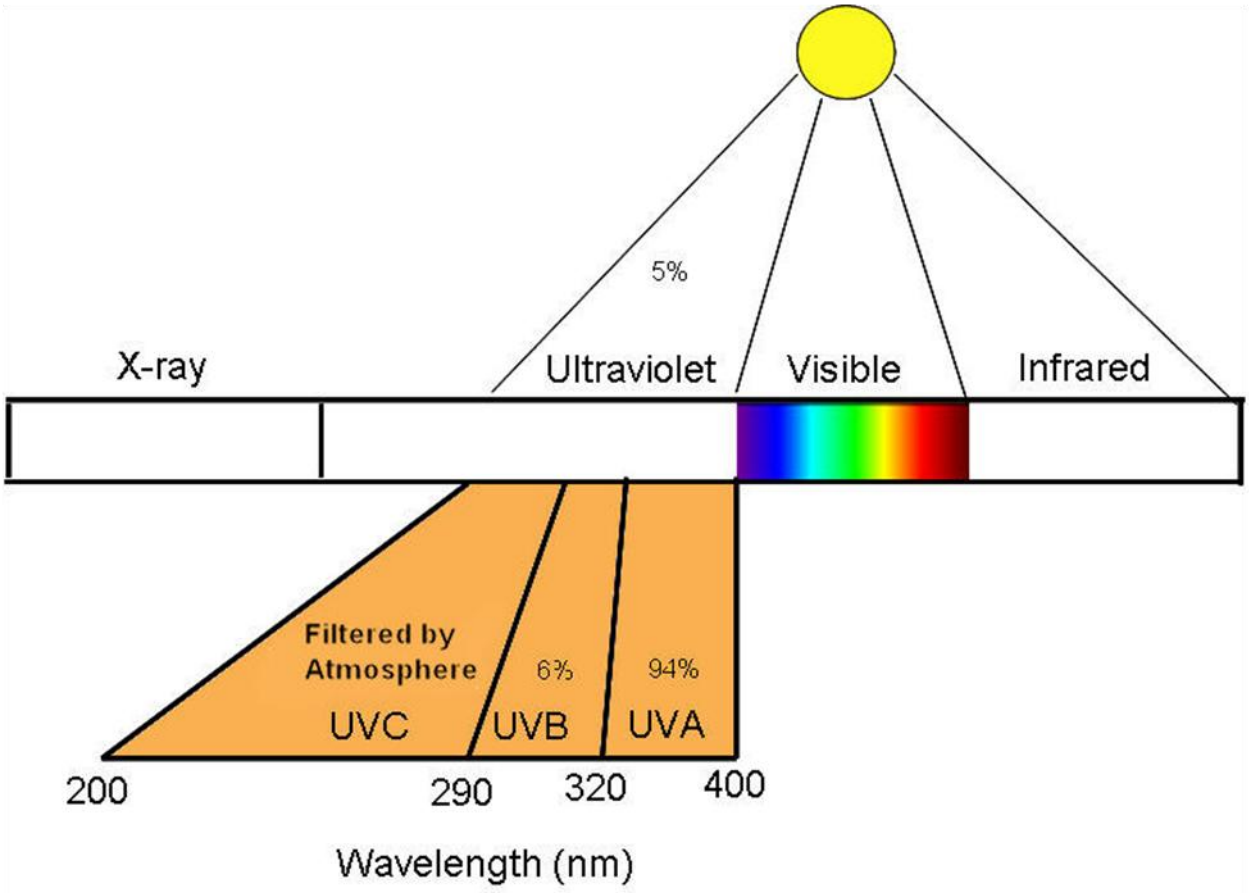


Figure 1-4. Diagrammatic illustration of the UV spectrum showing the proportion of UV reaching the Earth's surface. The ultraviolet spectrum comprises 5% of solar radiation with the majority of terrestrial UV radiation being UVA (94%). UVB comprises just 6%.

The mechanism of action of UVB in the clearance of psoriasis is unknown. However, even in normal skin it causes a plethora of cellular effects within the epidermis, including apoptosis (Kulms and Schwarz 2000), DNA damage (Ichihashi, Ueda et al. 2003; Cadet, Sage et al. 2005), cell cycle arrest (Athar, Kim et al. 2000; Pavey, Russell et al. 2001), immunosuppression (Schwarz 2002), cellular proliferation (Del Bino, Vioux et al. 2004) and cytokine release (Schwarz and Luger 1989).

Apoptosis (programmed cell death) is a highly regulated, conserved, energy requiring process that eliminates damaged, infected or superfluous cells. In normal skin, keratinocytes can undergo apoptosis following UV irradiation at doses of around or greater than the minimal erythemal dose (Murphy, Young et al. 2001). These cells have a characteristic morphology consisting of shrunken eosinophilic cytoplasm, membrane blebbing, chromatin condensation and DNA fragmentation, leading to formation of pyknotic nuclei and apoptotic bodies (Claerhout, Van Laethem et al. 2006). The mechanism of 'sunburn cell' formation is dependent on the degree of cellular DNA damage, and is controlled by the tumour suppressor gene p53, which triggers the intrinsic apoptotic pathway. DNA is a chromophore within the epidermis, directly absorbing UVB which leads to formation of cyclobutane dimers and (6-4) photoproducts. UVA can produce mutations indirectly, through mechanisms such as oxidative stress and photosensitisation reactions (Ichihashi, Ueda et al. 2003), but can also generate UVB-type photoproducts (Douki, Reynaud-Angelin et al. 2003). There is evidence that UV may also trigger apoptosis through the extrinsic pathway by directly activating the death receptors or inducing release of their ligands (Murphy, Young et al. 2001) (for an overview of apoptotic pathway see appendix A). Apoptosis of these cells is an important regulatory mechanism to reduce the associated risk of malignant transformation.

Interestingly, cumulative UV-induced mutations secondary to chronic sun exposure leads to a reduced apoptotic response (Claerhout, Van Laethem et al. 2006). This implies either a down-regulation in pro-apoptotic stimulus, or an overactive anti-apoptotic pathway, e.g. survivin over-expression (Aziz, Ghotra et al. 2004).

UV dose can be expressed either as MEDs (minimal erythemal dose) or SEDs (standard erythemal dose). An MED is a visible indicator of individual biological response, and is defined as the lowest amount of radiation required to achieve just perceptible reddening of the skin 24h after irradiation. This measure is widely used in clinical practice, although it will be greatly affected by many variables. These include the wavelength of the UV source, the irradiated field size, intrinsic characteristics of the skin (such as pigmentation and

anatomical location) and observational factors such as the ambient light used to read the measurement etc. An SED is a standardised measure of erythemogenic UV exposure, with one SED being equal to an erythemal effective exposure of 100 J/m^2 (Diffey 2002). This erythemally weighted quantity is useful when describing an administered dose, but does not take into account an individual's erythemal sensitivity.

1.3.1. **UVB as a treatment for psoriasis**

For years it has been well recognised that exposure to sunlight can improve psoriasis in some patients. Accordingly, UV lamps have been developed commercially, which are effective in clearing psoriasis in approximately 70% of patients (Coven, Burack et al. 1997; Gordon, Diffey et al. 1999; Kirke, Lowder et al. 2007), often with prolonged remission (Markham, Rogers et al. 2003). Patients attend for treatment 2-3 times per week for approximately 6-8 weeks, with an average starting dose of 0.7 times their MED. However, some patients don't respond at all, and others relapse immediately after completion of therapy. Presently, we are unable to predict who is likely to respond well to treatment, and do not know why it is so effective for some patients, but others remain less susceptible to its effects.

Different wavebands of UVB have been used to clear psoriasis. Most commonly used is narrowband UVB and less so broadband UVB (280-315nm). Evidence for the efficacy of specific wavelengths is based on work by Parrish and Jaenicke (Parrish and Jaenicke 1981) in which four patients with psoriasis were irradiated with seven different UV wavelengths, from 254nm to 313nm. They showed that wavelengths below 296nm cause erythema but are not effective in clearing psoriasis, even when used at in multiples of up to 28 times the minimal erythema dose; however, wavelengths from 300 to 313nm were effective. Monochromatic excimer lasers (308nm) are now also used as a treatment for localised psoriasis (Asawanonda, Anderson et al. 2000; Bianchi,

Campolmi et al. 2003; Gerber, Arheilger et al. 2003; Kollner, Wimmershoff et al. 2005) and have been demonstrated to be as effective as 311nm in clearing psoriasis (Kollner, Wimmershoff et al. 2005).

In response to Parrish and Jaenicke's work which suggested that wavelengths around 313nm were more effective at clearing psoriasis than the nearest tested wavelength of 304nm (Parrish and Jaenicke 1981), fluorescent lamps (narrowband UVB) were developed commercially. The phosphor coating on these bulbs produces a peak emission of 311nm, and have also found to be highly effective for clearing psoriasis. The efficacy of 311nm UVB in clearing psoriasis has not been directly compared to 313nm.

It has been hypothesised that UVR induces apoptosis of lymphocytes *in vivo*. In part this is because epidermal T cells deplete as psoriasis improves with UV treatment. Studies have examined skin biopsies from patients before treatment and after resolution of psoriasis at a non-disclosed time interval from the last dose of radiation (Krueger, Wolfe et al. 1995; Ozawa, Ferenczi et al. 1999). We know from previous work that keratinocyte apoptosis in normal skin appears around 8-12h, peaks 24-48h after UV exposure, and will not be detected after 60-72h (Woodcock and Magnus 1976; Murphy, Mabruk et al. 2002; Mass, Hoffmann et al. 2003), although the former study was performed in mice. If psoriasis has virtually resolved, it is arguably too late to know which cells have been important in causing this to occur and which cells are altered as a bystander effect of psoriatic resolution.

The mechanism of UVB-induced clearance of psoriasis has been the focus of numerous studies. However, previous work has looked for changes occurring following therapeutic UVB (Bianchi, Campolmi et al. 2003; Abou El-Ela, Nagui et al. 2010; Johnson-Huang, Suarez-Farinas et al. 2010). No previous studies have compared the direct effects of therapeutic versus ineffective wavelengths of UVB, which would allow identification important contributors to psoriasis clearance rather than bystander effects.

UV-induced T cell apoptosis has been demonstrated with the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling)

technique (Krueger, Wolfe et al. 1995; Bianchi, Campolmi et al. 2003). However, studies have shown that TUNEL gives false positive results in psoriatic epidermis because of an increased number of DNA nicks, caused by the high rate of DNA replication (Wrone-Smith, Mitra et al. 1997). This technique is therefore unreliable, and there is no other direct evidence to suggest that large numbers of T cells undergo apoptosis within treated psoriatic epidermis. *In vitro* data from four UVR sources demonstrated peripheral T cells apoptosis with UV wavelengths ranging from 290- 311nm (Novak, Berces et al. 2004). However, in support of the theory that T cells may not be the principal target cells in effective UV treatment, they demonstrated that 290nm is three times more effective at inducing T cell apoptosis than 311nm. The fact that 290nm is known to be clinically ineffective in clearing psoriasis suggests that T cells may not be as important in the mechanism of action of this particular therapeutic modality.

1.4. Apoptosis

Apoptosis is a key physiological event in both developmental biology and tissue homeostasis, and is a normal physiological response in reaction to stimuli such as irreparable DNA damage. The physical characteristics of apoptotic cells described above (section 1.3) are well characterised, although the pathways involved are more complicated. Apoptosis can occur through the intrinsic (mitochondrial) pathway or extrinsic (death receptor) pathway (see appendix A). The former can be triggered by DNA damage or cytotoxic drugs, and is mediated by the bcl-2 family of proteins. The extrinsic pathway is initiated by the recruitment of Fas-associated death domain to the death domain receptors of the TNF Superfamily, leading to downstream activation of caspase 8 (Wallach, Boldin et al. 1997). The ‘caspase cascade’ is usually common to

both pathways (see below), and consists of the executioner caspases 3, 6 and 7, which are activated by proteolytic cleavage. These caspases are key mediators of DNA fragmentation via cleavage of a wide range of cytoplasmic and nuclear proteins (Duncan, Turowec et al. 2010). In particular, cleaved caspase-3 translocates to the nucleus, where it cleaves substances including the DNA repair enzymes ICAD (inhibitor of caspase-activated DNAse) and PARP (poly (ADP-ribose) polymerase), leading to DNA fragmentation. A caspase independent pathway has also been shown to exist following mitochondrial release of EndoG (endonuclease G) and AIF (apoptosis inducing factor), which can directly translocate to the nucleus and induce DNA fragmentation independently (Bajt, Cover et al. 2006).

1.4.1. **Methods of apoptosis detection**

Apoptosis is a dynamic process which is often measured by end-point assays, such as immunohistochemistry and flow cytometry. Morphological changes including DNA-fragmentation and cytoplasmic shrinkage (as opposed to swelling of the cell associated with necrosis) can be observed following nuclear staining and examination by light/ electron microscopy. Techniques based on detection of DNA fragmentation (e.g. TUNEL (see section 1.3.1), in situ nick translation, in situ hybridisation and PARP detection) are not specific for apoptosis, and therefore need to be interpreted cautiously (Stadelmann and Lassmann 2000). Annexin V is used to detect early apoptosis by flow cytometry as it precedes DNA fragmentation (Martin, Reutelingsperger et al. 1995), but cannot be used for *in vivo* detection as it binds to phosphatidylserine transposed to the outer membrane during apoptosis (van Engeland, Nieland et al. 1998) and these are disrupted during processing (van den Eijnde, Luijsterburg et al. 1997).

Antibodies against various members of the apoptotic pathways exist. However, detection of early proteins such as p53, cytochrome C and the Bcl-2

family do not imply that the apoptotic pathway will complete, and cells expressing these markers may recover completely. In contrast, antibodies to caspase-3 are sensitive and specific for apoptosis *in vivo* and *in vitro*, and once activated the cell is destined to undergo apoptosis (Stadelmann and Lassmann 2000; Huerta, Goulet et al. 2007). A caspase-3 substrate is available for real time imaging, and can be used in primary cells (Cen, Mao et al. 2008) (see section 2.2.2) and techniques such as FRET (fluorescence-resonance energy transfer) can be a useful for measuring the rate of apoptosis, but can only be applied to transfected cells (Tyas, Brophy et al. 2000).

1.4.2. **Apoptosis in psoriasis**

Wrone-Smith et al studied the effects of incubating cultured psoriatic and non-psoriatic keratinocytes in a semisolid medium (methylcellulose) for up to 24h and found significantly less apoptosis in the psoriatic keratinocytes. They surmised that psoriatic keratinocytes had an increased capacity to resist apoptosis (Wrone-Smith, Mitra et al. 1997), and this is often quoted as evidence that psoriatic keratinocytes are generally resistant to apoptosis. Apoptosis as a mechanism of plaque clearance has since been largely overlooked. However, a few studies have indicated that apoptosis may be a relevant mechanism of plaque clearance.

Low dose methotrexate has been shown to induce apoptosis of keratinocytes using epidermal explants comprising normal human keratinocytes, and the authors suggested that this may explain the reduction in epidermal hyperplasia in psoriasis treated with methotrexate (Heenen, Laporte et al. 1998). Dithranol (anthralin) is another effective treatment for psoriasis, and has also been shown to induce apoptosis in normal keratinocytes (McGill, Frank et al. 2005). Moreover, ongoing work in our laboratory has shown that keratinocyte apoptosis can be detected *in vivo* following routine dithranol treatment to psoriatic plaques (Leech et al, unpublished). Apoptosis of psoriatic

keratinocytes has also been shown *in vivo* following treatment with infliximab (Markham, Mathews et al. 2006; Raho, Vena et al. 2010), although the importance of this in plaque clearance has not yet been demonstrated.

1.5. Mathematical modelling

Mathematical modelling allows prediction of how a biological system will behave based on available evidence, without the need for time-consuming and sometimes impossible experiments (Diffey 2004). It also allows further understanding of the possible mechanisms of specific processes. Modelling does not replace the need for laboratory based experimentation, but complements it by focusing on what is likely to be important, and which mechanisms or pathways fit best with the known evidence.

A model is a simplification of the *in vivo* process, and can be used to examine the relationship between cause and effect; for example the effects of UVB on the keratinocyte compartment of psoriatic skin. This requires temporary exclusion of the non-essential details, and by striping the model down to essential components only, it is possible to examine whether the process under investigation acts directly on the compartment under examination. If a direct relationship is not found, this suggests that other factors are likely to be of greater importance and should also be considered. Furthermore modelling highlights gaps in knowledge, which may lead the researcher to go back ‘to the bench’ and design further relevant experiments which may be fundamental to understanding the system under investigation. Finally, modelling allows a more predictive approach which can then be tested, allowing for considerable savings in time and money (Kirkwood, Boys et al. 2003).

Predominantly two types of computational models exist: stochastic and deterministic. Stochastic models are useful when random events may occur and may have important influences on the behaviour of the system. Each run of the model will give a different result, and for this reason numerous stimulations are required. The advantage of stochastic models is that they allow the user to test a range of possibilities which fall within and outside the expected range of probability, and can be used to determine whether the model assumptions hold true. They may however, be either very complex computationally, or be seen to overly simplify the problem being tested, by ignoring possible contributing factors. If a model still holds true when a particular factor is excluded it suggests that any relationship between this factor and the modelled biological process is indirect. Likewise, if it doesn't hold true, it would suggest that the factor has a direct effect on the process being processed.

Deterministic models, also known as compartmental models, give fixed reproducible results and take no account of individual random variation. They are useful when working with large populations and attempt to describe the average of what happens to the population by categorising individuals into separate subgroups (Trottier and Philippe 2001). In this project, only stochastic modelling is used as the remodelling effect of UVB on psoriatic patients is variable, and individuals with similar demographic data or type of psoriasis do not react to UVB in a similar or typical manner.

To create a successful model it is important to include as many known and relevant parameters as possible, and this is largely done through collation and evaluation of previously published experimental work. These parameters can then be inserted into the model and tested to see if the model holds true within a given range of values. This exercise in itself will often highlight relevant gaps in the literature, and can guide the researcher to undertake further relevant bench work to inform the model.

1.5.1. Keratinocyte behaviour

The keratinocyte compartment can be further subdivided into the stem cell, TA cell and differentiating cell compartments (Dover and Wright 1991; Webb, Li et al. 2004) and this can be helpful when considering the physical location of the cells within the epidermis, their role in proliferation or differentiation and their different kinetic behaviour patterns. Each of these is addressed below.

1.5.1.1. Stem cells

Epidermal keratinocytes comprise stem cells, transit amplifying (TA) cells and differentiating cells. Non-follicular stem cells are located on the basement membrane and although distributed throughout the length of the basement membrane, are thought to have a greater density towards the tips of the dermal papillae in normal skin (Jones, Harper et al. 1995; Ghali, Wong et al. 2004). Stem cells have the potential to divide indefinitely (Potten and Loeffler 1990), and can either divide symmetrically to produce two stem cells, or asymmetrically giving one stem cell and one TA cell. During times of homeostasis asymmetrical division occurs, but in times of stress e.g. wound repair, symmetrical division may occur, and this may lead to epidermal acanthosis (Fuchs 2008).

The proportion of proliferating cells within the normal epidermis has been estimated to be as high as 43-66% (Bata-Csorgo, Hammerberg et al. 1993; Jones, Harper et al. 1995), with putative stem cells accounting for approximately 3-12% of epidermal cells (Webb, Li et al. 2004; Youn, Kim et al. 2004). The proportion of stem cells is likely to be variable depending on body site (Jones, Harper et al. 1995; Webb, Li et al. 2004). Estimates of the proportion of epidermal stem, TA and differentiating cells and how many of them are actively dividing, comes predominantly from flow cytometry, immunohistochemistry and *in vivo* labelling techniques. Reliable evidence has been hampered by lack of good keratinocyte stem cell markers, and such cells are usually identified as being ‘stem-cell-like’ but in fact maybe early TA cells;

this may explain why the quoted ranges in the older literature are so variable. However, stem cells have been shown to express β1 integrin at levels which are up to 2-3 times higher (β1 integrin-bright) than in TA cells (β1 integrin-dim) (Jones and Watt 1993), although early TA cell will have a similar expression profile to stem cells. Some studies have attempted to overcome this problem by comparing properties of the α6-integrin bright (proliferating) cells and identifying CD71 (transferin) negative cells amongst these as stem-cell like, and CD71 positive cells as TA cells (Hayashi, Yamato et al. 2008). However, these antibodies enrich the cells rather than specifically identify them.

Stem cells are thought to pass through the cell cycle at a slower rate than TA cells (Dover and Wright 1991), and have been estimated to have a cell cycle time of 100-200 hours in normal epidermis (Potten and Bullock 1983; Potten 1986; Morris and Hopewell 1990), although cell cycle times as slow as 400h have been described in human epidermis (Weinstein and Frost 1968). However, it is likely that cell cycle times will vary considerably both within and between different individuals (Dover and Wright 1991).

1.5.1.2. TA cells

TA cells retain a limited ability to proliferate but are committed to differentiate at some stage. They are located in the basal/ suprabasal layer, dividing approximately three to five times (Watt 1998; Savill 2003) before migrating upwards, and eventually differentiating. Actively dividing cells are located basally or suprabasally, possibly because younger cells have a higher binding affinity to the basement membrane or because successful cell division requires a given concentration of soluble factors released from the basement membrane/ dermis (e.g. keratinocyte growth factor). What makes the basement membrane length increase in conditions such as psoriasis is unclear, although arguably if cells are actively dividing and require certain basement membrane or dermis derived soluble factors to do this, an increased number of

dividing TA cells may induce the basement membrane to expand in order to accommodate this.

TA cells have been shown to divide either symmetrically (giving rise to two TA cells), or asymmetrically (giving rise to one TA cell and one differentiating cell) and it is likely that they can switch between these in certain states. This has been demonstrated in mouse skin (Clayton, Doupe et al. 2007), and in other tissue types including cortical development and haemopoeitic cells (Mione, Cavanagh et al. 1997; Estivill-Torrus, Pearson et al. 2002; Bullock, Wen et al. 2007). This has not yet been studied in human skin, although it can be assumed that certainly in disease states such as psoriasis, symmetrical division occurs since differentiating cells are not scatted throughout the lower epidermis; instead they are situated above the TA cells in the upper epidermis. This physical distinction implies that TA cells will continue to migrate up the epidermis and will either differentiate when they reach a certain age, or after a certain number of divisions. This has been demonstrated within the eye, and it is thought that a gradient may exist which determines whether cells remain as transit amplifying cells or differentiate (as they progress further towards the surface of the eye) (Revoltella, Papini et al. 2007).

In normal epidermis the turnover time for proliferating cells has been estimated at 152-580h (Epstein and Maibach 1965; Weinstein and McCullough 1973; Allegra and De Panfilis 1974; Potten 1986), and the total epidermal turnover time in normal human epidermis has been estimated as 672-840h using heavy water labelling of keratin *in vivo* (Lindwall, Hsieh et al. 2006). Cell turnover can be defined as the time taken to replace all the cells within a compartment and if the birth rate and death rate are equal, and when the epidermis is in equilibrium, can be defined as:

$$\text{Turnover rate} = \frac{\text{Number of cells within the defined compartment}}{\text{Birth/ Death rate}}$$

As the rate of cell proliferation increases, there is a corresponding increase in the number of cells replaced within the compartment, and so epidermal turnover time is decreased (Iizuka, Honda et al. 1999).

TA cells may be resting or cycling, but when cycling, are thought to progress through the cell cycle faster than stem cells (Potten and Bullock 1983). The cell cycle time has been estimated using the FLM (fraction of labelled mitosis) technique extended over 45h to minimize the effects of diurnal variation or cell synchronization. The FLM curve allows analysis of the time of cell cycle by flash labelling of the pre-mitotic phase of proliferating cells with Thymidine and repeat analysis. This technique has shown a mean cell cycle time of 56h with a 75% coefficient of variation (Duffill, Wright et al. 1976), with other estimates ranging from 50-65h (Chopra and Flaxman 1974; Goodwin, Hamilton et al. 1974; Bauer and Grood 1975; Wright 1976).

1.5.1.3. Differentiating cells

It is thought that the overall proportion of nucleated cells in normal epidermis within the differentiating compartment is 40-66% (Weinstein, McCullough et al. 1984; Bata-Csorgo, Hammerberg et al. 1993). Differentiated cells continue to migrate up the epidermis until they die and become part of the *stratum corneum*. The transit time is the time taken for a cell to migrate from the bottom of a compartment to the top, and in normal epidermis this is estimated to be 240-336 hours for cells within the differentiating compartment (Epstein and Maibach 1965; Weinstein and Van Scott 1965; Allegra and De Panfilis 1974).

What defines when a TA cell differentiates is unclear, however histological evidence showing differentiation markers such as keratin 10 and S100A proteins, suggest differentiation may be due to an inverse gradient arising from the basement membrane (Figure 1-5).

1.5.2. **Altered kinetics in psoriatic epidermis**

In psoriasis there is epidermal hyperproliferation. In general, hyperproliferation can arise by either:

1. increasing epidermal turnover time,
2. increasing the proportion of proliferating cells
3. altering the rate of the cell cycle

Following epidermal wounding of normal skin either by repeated tape stripping or incision, there is a delay of 15-65h before proliferation is observed (Dover and Wright 1991). The rate of proliferation peaks, and then settles over the following 3-4 days. Such a period of latency is consistent with stimulation of cells in the G0 phase, and may suggest that resting stem cells or TA cells are recruited into the cell cycle. Tape stripping of uninvolved psoriatic skin however, produces over-expression of TGFα in contrast to normal epidermis, and a significantly exaggerated proliferative response occurs (Hatta, Takata et al. 1997). This may explains how plaques can develop over a matter of days in previously normal looking skin. Interestingly, the hyperproliferative response is seen following wounding which physically removes cells from the system (e.g. tape stripping), but not where the cells are damaged in situ (e.g. burning) (Denekamp and Fowler 1976). This suggests that proliferation can be driven in response to local cell barrier disruption, and this may be partly explained by disruption of the calcium gradient (Elias, Ahn et al. 2002).

The epidermal turnover time in normal human epidermis is estimated at 28-35 days (approximately 19 days for proliferative compartment and 10-14 days for the differentiating compartment) in normal epidermis excluding *stratum corneum* (Epstein and Maibach 1965; Weinstein and Frost 1971; Bergstresser and Taylor 1977; Lindwall, Hsieh et al. 2006), whereas psoriatic epidermis has a reduced estimated turnover time of 7-10 days (Goodwin and Fry 1974; Laptev and Nikulin 2005). This reduction in turnover time suggests that alteration in turnover time is not the cause of hyperproliferation seen in psoriasis, since if cells are removed from the epidermis at a quicker rate (but all other factors e.g.

cell cycle length remain the same) the epidermis would be expected to be thinner. This is clearly not the case in psoriasis. Cell cycle times in psoriasis have a large coefficient of variation which has been estimated at 75% (Duffill, Appleton et al. 1977), but studies suggest that there is no reduction in the cell cycle time of psoriatic keratinocytes (Chopra and Flaxman 1974; Bauer and Grood 1975). In normal human epidermis, studies have shown that up to 80% of cells in the basal layer (stem and TA cells) are not actively dividing (Gelfant 1976). Although proliferation within normal epidermis is generally thought to be confined to the basal layer, Penneys et al demonstrated that approximately 30% of cycling cells (identified by flash labelling) were located suprabasally in normal epidermis (Penneys, Fulton et al. 1970). The following differences have been reported in psoriatic epidermis compared to normal skin:

- An increase the number of proliferating cells is approximately six times normal (Doger, Dikicioglu et al. 2007), and in total epidermal mitosis of 10-23% (Weinstein and Frost 1968; Goodwin, Hamilton et al. 1974; Duffill, Wright et al. 1976). However, evidence exists to show that the cell cycle time does not change in psoriasis compared to normal skin (van Ruissen, de Jongh et al. 1996).
- The turnover time for psoriatic epidermis is up to four times faster than in normal skin (Weinstein and Van Scott 1965).
- The overall number of epidermal cells increases by up to two to five times normal (Vanscott and Ekel 1963; Weinstein, McCullough et al. 1984; Weinstein, McCullough et al. 1985).
- The estimated turnover time for the proliferating compartment is reduced from 150-600h in normal skin to 36-114h in psoriasis (Weinstein and Frost 1968; Wright 1976).
- The estimated transit time for the differentiating compartment is reduced from 240-330h in normal epidermis to down to as rapid as 48h in psoriasis (Weinstein and Van Scott 1965; Weinstein and Frost 1968).
- Several lines of evidence indicate gradients of differentiation markers within epidermis (Korver, van Duijnhoven et al. 2006),(Ishida-Yamamoto and Iizuka 1995; Martinsson, Yhr et al. 2005) (Figure 1-5). Although the

molecular signals that drive this process remain to be fully elucidated, the histological pattern of induction of keratinocyte differentiation is not consistent with a gradient emanating from a fixed distance beneath the *stratum corneum*, but rather indicates a gradient arising from the basal layer or dermis (Figure 1-5).

- In psoriatic epidermis, a six to sevenfold increase in the number of cells in the S/G2M phase of cell cycle was found among CD29+ K1/K10- cells (the putative stem cell population). Furthermore, all lesional putative stem cells showed high PCNA positivity, indicating that these cells had all been induced into the cell cycle recently (Bata-Csorgo, Hammerberg et al. 1993); therefore providing evidence that the proportion of actively cycling stem cells in markedly increased in psoriasis.
- Cells with the highest proliferation potential will have greater adhesion to the basement membrane and /or other cells of higher proliferative potential (Fuchs 2008).

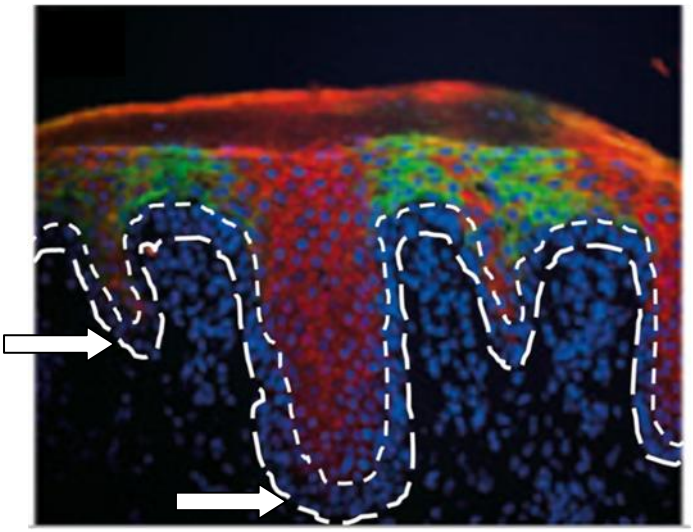


Figure 1-5. Epidermal section showing characteristic distribution of keratin 10 (red) and keratin 6 (green). Short dashed line shows outline of differentiation markers, and the long dashed line shows the basement membrane. Note the greater gap between the 2 lines at the bottom of the rete ridges (arrows), than at the top; consistent with a gradient produced from the basement membrane (see Figure 2-8). Adapted from Korver et al (Korver, van Duijnhoven et al. 2006).

1.5.3. Existing mathematical models of the epidermis

1.5.3.1. Models of normal epidermis

A mathematical model has been proposed to examine how the hierarchical cell populations maintain equilibrium within the normal epidermis (Savill 2003). This model examined the effects of various parameters on epidermal homeostasis including variation in both cell cycle time and the number of transit generations produced. Reduced cell cycle time has been proposed as a possible cause of hyperproliferation seen in psoriasis, but Savill showed that reducing cell cycle time doesn't necessarily lead to hyperproliferation. Furthermore his model demonstrates that increasing the TA cell cycle time (assuming that number of TA divisions remains constant, and the cell spends the same amount of time within the TA compartment) leads to an increase in cell density, but doesn't affect the birth rate. In contrast he showed that increasing the number of TA divisions has a substantial impact on increasing birth rate. In conclusion, Savill showed that hierarchical cell populations need to be modelled by taking into account properties of different cell compartments within the epidermis, e.g. different cell cycle times for stem and TA populations, and variation in the number of transit generations produced.

A computational model of epidermal homeostasis has been developed using a multi-agent systems approach (Grabe and Neuber 2005; Sutterlin, Huber et al. 2009). This allows each cell to follow a set of rules which will determine how it will interact with other cells within the model. The advantage of this type of model is that it allows the modeller to directly visualise what the effects of altering specific cellular parameters can have on interactions with neighbouring cells and on the model system as a whole. The purpose of Grabe and Neuber's model was to computationally show how cells could get from an undulating basal layer to form a flat epidermal surface following agent-based rules about morphology, kinetics and calcium flow. They included an arbitrary maximum age a cell can reach (1000h) which is integral to the model, and specified that TA cells proliferate for the first 10% of their life time. They assumed a cell cycle of 62h for both stem and TA cells and would only permit a cell to divide if there was room for it to do so within a fixed epidermal structure. The epidermis has a vertical calcium ion gradient, with low calcium in the basal layers and higher

levels at the top of the epidermis (Elias, Ahn et al. 2002). As the gradient reaches a given threshold they will differentiate, and this is reflected in the Grabe model. With the above parameters they predicted an epidermal turnover time averaging 672h (27 days), which is fairly consistent with existing literature quoting 28-35 days (Epstein and Maibach 1965; Weinstein and Frost 1971; Bergstresser and Taylor 1977; Lindwall, Hsieh et al. 2006). However, many of the above assumptions are not supported by experimental evidence, yet are pivotal to the models function; therefore results need to be interpreted cautiously.

Recently a 3D computational model of normal epidermis has been developed to investigate the effects of cell proliferation and migration on wound healing (Adra, Sun et al. 2010). TGF-β1 was used to explore the “subcellular rules” of cultured keratinocytes which cause cells to alter their behaviour pattern in response to external events such as wound healing. The authors used FLAME (Flexible Large-scale Agent Modelling Environment) (Coakley 2007) and COPASI (Complex PAthway SImulator) (Hoops, Sahle et al. 2006) modelling environments to incorporate intracellular signalling pathways into an agent-based model. A 3D model was produced based on a flat surface to which stem cells attach. As cells migrate upwards they progress to become TA cells and then differentiated cells. This model did not consider cell cycle times, the mechanism of TA cell division (e.g. symmetrical or asymmetrical), and assumes that progression from TA cell to differentiation occurs due to a cell reaching a set distance from the stem cells. It does however provide a framework to investigate the roles of growth factors and signalling pathways within the epidermis.

1.5.3.2. Models of psoriatic epidermis

There has only been one published computational model of psoriasis morphology to date (Grabe and Neuber 2007). However, this model published in 2007, claimed that psoriasis could be simulated by increasing the fractional time period during which TA cells proliferate from 8% to 100% (Grabe and Neuber 2007). This one change led to an increase in both the germinative and differentiated compartments, with a greater increase in the former, and a reduction in turnover time by approximately 40% to 450h (19 days). This compares to an estimated turnover time of down to 7-10 days in other published studies (Goodwin, Hamilton et al. 1974; Laptev and Nikulin 2005). However, the Grabe model showed a relatively modest increase in total cell numbers of 50%, although evidence suggests that the total cell number is increased by 4-5 times in psoriatic epidermis compared to normal skin (Iizuka, Ishida-Yamamoto et al. 1996). Furthermore this model required that all cells within the proliferating compartment are actively cycling, whereas previous experimental studies for psoriasis estimate that the proliferative indices increased by 10-23% (Weinstein and Frost 1968; Goodwin, Hamilton et al. 1974; Duffill, Wright et al. 1976).

Iizuka et al created a non-computational geometric model of psoriasis based on quantitative analysis of psoriatic epidermis (Iizuka, Honda et al. 1999).They suggested that “while the differentiating compartment increases its size by increasing its volume (cell number), the proliferative compartment increases by increasing the size (area) of dermo-epidermal interface”. They argue that this is the explanation for the typical morphology observed in psoriatic plaques, and that the total epidermal volume is determined by the size of the proliferative compartment; thus the shape of the basement membrane may predict the number of proliferating cells within the compartment. As the rate of proliferation is increased in the epidermis, the turnover time reduces accordingly. Iizuka et al later showed that psoriasis with a granular layer (i.e. resolving lesions) had a turnover time at least 1.28 times longer than plaques without this layer (Iizuka, Takahashi et al. 2004), although it was still increased compared to normal skin.

1.6. Gene Arrays

The ability to study changes in gene expression has revolutionised our understanding of many disease processes. Different disease states are associated with up or down regulation of a variety of genes, with consequent altered levels of protein production which directly influences cellular activity. Therapeutic options may work by directly modulating gene expression returning it to a normal state, and the ability to detect these changes can help us understand which mechanisms to target when designing or modifying treatment options.

1.6.1. Gene array studies in psoriasis

A large gene profiling study has recently been published examining gene expression profiles between untreated lesional/ non lesional psoriasis and control patients without psoriasis (Gudjonsson, Ding et al. 2009). Whole punch biopsies were analysed (i.e. epidermis and dermis) following a short washout period of just two weeks for systemic psoriatic treatment and one week for topical treatment. Results showed a marked difference in expression profile between lesional and non lesional psoriasis, but the difference between non-lesional and control skin was more subtle (maximum fold change of 24-134 in the former and 1.8-1.9 in the latter) although this still amounted to 179 statistically significant differentially regulated genes. Some of the most strongly up-regulated genes included those encoding for epidermal differentiation such as SPRR2B, SPRR2G and SPRR3, and genes involved in innate immunity such as S100A family, DEFB4 (encoding human beta defensin-2), RNASE7 (with broad spectrum anti-microbial activity) and IL1F9 (encoding IL-1$^{\varepsilon}$ the proinflammatory cytokine). Down-regulated genes were predominantly lipid biosynthesis genes such as ELOVL3 and FADS1. Interestingly, none of the down-regulated genes had been identified by other studies (Bowcock, Shannon

et al. 2001; Zhou, Krueger et al. 2003; Kulski, Kenworthy et al. 2005; al Yacoub, Romanowska et al. 2008) and S100A7 was the only up-regulated gene in common to have been previously identified (Zhou, Krueger et al. 2003); although the concordance between these studies was higher for gene changes between lesional and non-lesional psoriatic skin.

Gene expression profiling has also been used to analyses differences between punch biopsies taken from untreated lesional and non-lesional psoriasis in seven Japanese patients (Kulski, Kenworthy et al. 2005). The authors showed that lesional psoriatic plaques contained up-regulated genes coding for apoptotic stress signals, with other affected genes encoding for a range of functions including interferon mediation, immunity, cell adhesion, signal transduction and cytoskeletal restructuring.

Biomarkers can prove a highly useful tool in predicting patient's response to treatment. One small study has used microarray data from peripheral blood mononucleocytes (PBMCs) of 16 patients with psoriasis to identify candidate genes which may predict response to treatment with Alefacept (Suarez-Farinas, Shah et al. 2010). The authors identified 23 candidate genes which may be useful markers, but in particular responders had up-regulation of genes such as cAMP response element (CREM) which is associated with reduced IL-2 and T cell anergy, and MAFF which is involved in the cellular stress response. The relevance of this will become clearer following completion of prospective studies.

1.6.2. **The effects of UVB on the epidermis**

UVB radiation has been shown to suppress the adaptive immune system contributing to photocarcinogenesis, but enhance the innate immune response. In particular antimicrobial peptides (AMPs), which are secreted by keratinocytes in the *stratum granulosum* (Zasloff 2002), have been shown to have enhanced

expression following exposure to UVB in primary keratinocytes *in vitro* (Glaser, Navid et al. 2009). AMPs are located in the *stratum corneum* and are an effective defence against bacteria, viruses, fungi and protozoa. In contrast to eczema where their levels are suppressed, their expression is markedly over-expressed in psoriasis. At high levels AMPs can induce necrosis by physically disrupting the cell membrane, or if internalised, can induce apoptosis by disruption of intracellular organelles (Zasloff 2002; Barlow, Li et al. 2006). UVR also induces production of LL-37 (cathelicidin) by keratinocytes, which protects keratinocytes from apoptosis when expressed at high levels by regulation of cycloxygenase-2 (COX2) and inhibitor of apoptosis protein (IAP-2) (Chamorro, Weber et al. 2009). It has been suggested that inhibition of PGE_2 would lead to LL-37 induced apoptosis of basal keratinocytes (Zasloff 2009), which may be enhanced by drugs which inhibit COX-2 such as non-steroidal anti-inflammatory agents (NSAIDs).

1.7. Study hypothesis

The study hypothesis was that keratinocytes are of primary importance in the pathogenesis of psoriasis, and that apoptosis of psoriatic keratinocytes is induced by therapeutic wavelengths of UVB but not by non-therapeutic wavelengths. Moreover, it was hypothesised that UVB-induced apoptosis of lesional psoriatic keratinocytes leads directly to clearance of psoriatic plaques.

1.8. Study aims

The overall aims of this study were:

1. To compare the *in vivo* effects of clinically effective and ineffective wavelengths in psoriatic epidermis. This provides a novel way to distinguish which of the many effects of UVB are important in clearing psoriasis, and which occur as a non-specific response to UVB irradiation.
2. To investigate the mechanisms of clearance of psoriasis following therapeutic UVB irradiation, and in particular to increase our understanding of the role of keratinocyte apoptosis in this process.
3. To test dynamically whether the rate of apoptosis observed in psoriatic epidermis is sufficient to account for remodelling of psoriatic plaques back to normal.
4. To examine the apoptotic response of a range of wavelengths within the UVB spectrum to see which wavelength induces the greatest amount of apoptosis. If keratinocyte apoptosis is an important mechanism of psoriatic clearance, this could potentially indicate a more effective treatment wavelength.

1.9. Objectives

1. To obtain ethical approval.
2. Recruit patients to the study if they had been prescribed narrowband UVB for their psoriasis.
3. Obtain written informed consent from patients.
4. Calculate MEDs.
5. Irradiate matched psoriatic plaques on the patients' lower back with equi-erythemogenic doses of UVB.
6. Biopsy irradiated plaques at defined time-intervals after irradiation.
7. Analyse biopsies for cellular differences including apoptosis, proliferation, and markers of other cell types such as T cell and Langerhans' cells.
8. Confirm the presence of apoptosis by electron microscopy.
9. Create an interactive mathematical model of normal and psoriatic epidermis
10. Integrate experimental data into mathematical model and use this as a tool to assess the likely impact of the observed number of apoptotic cells on overall tissue homeostasis.
11. Look for early changes in gene expression levels in biopsies of irradiated plaques, and assess whether or not genes controlling apoptosis are differentially regulated following irradiation with clinically effective UVB rather than clinically ineffective UVB.

2. Materials and Methods

2.1. Volunteers

Volunteers were recruited from tertiary referral clinics at the Royal Victoria Infirmary, Newcastle-upon-Tyne. All patients had been prescribed narrowband UVB for their psoriasis, were given detailed information about the study, and gave written permission to be included (appendix B). Ethical approval was obtained from County Durham and Tees Valley Research Ethics Committee. Each patient had a maximum of four 6mm punch biopsies following irradiation to small areas (15mm diameter) of their skin, with specific wavelengths within the UVB waveband.

Patient demographics were collected including age of onset of psoriasis, presence of joint or nail involvement and skin type. A detailed history and examination of each patient was performed prior to entry into the trial, and clinical response measured using PASI scores taken prior to treatment. All patients were then given their routine UVB treatment course, and PASI assessments were made at weekly intervals during the routine weekly irradiation UVB treatment. Routine treatment consisted of three times weekly whole body exposure to narrowband UVB using a starting dose of 70% of the MED. The dose was increased after alternate treatments by 40%, decreasing stepwise to 5% by the 18^{th} treatment.

2.1.1. Patient recruitment

Patients were recruited if they met the following criteria:

- Given informed consent to the study
- Had chronic plaque psoriasis and had been routinely prescribed narrowband UVB treatment
- Over 18 years of age

- Had not used immune modifying drugs, systemic psoriatic treatments, UVB, PUVA, sun beds, or received significant exposure to sunlight for three months (e.g. sunny holidays etc) prior to inclusion in the trial

- Had not used topical treatments for two weeks prior to inclusion with the exception of emollients

Patients were recruited to the study sequentially into 1 of 6 groups:

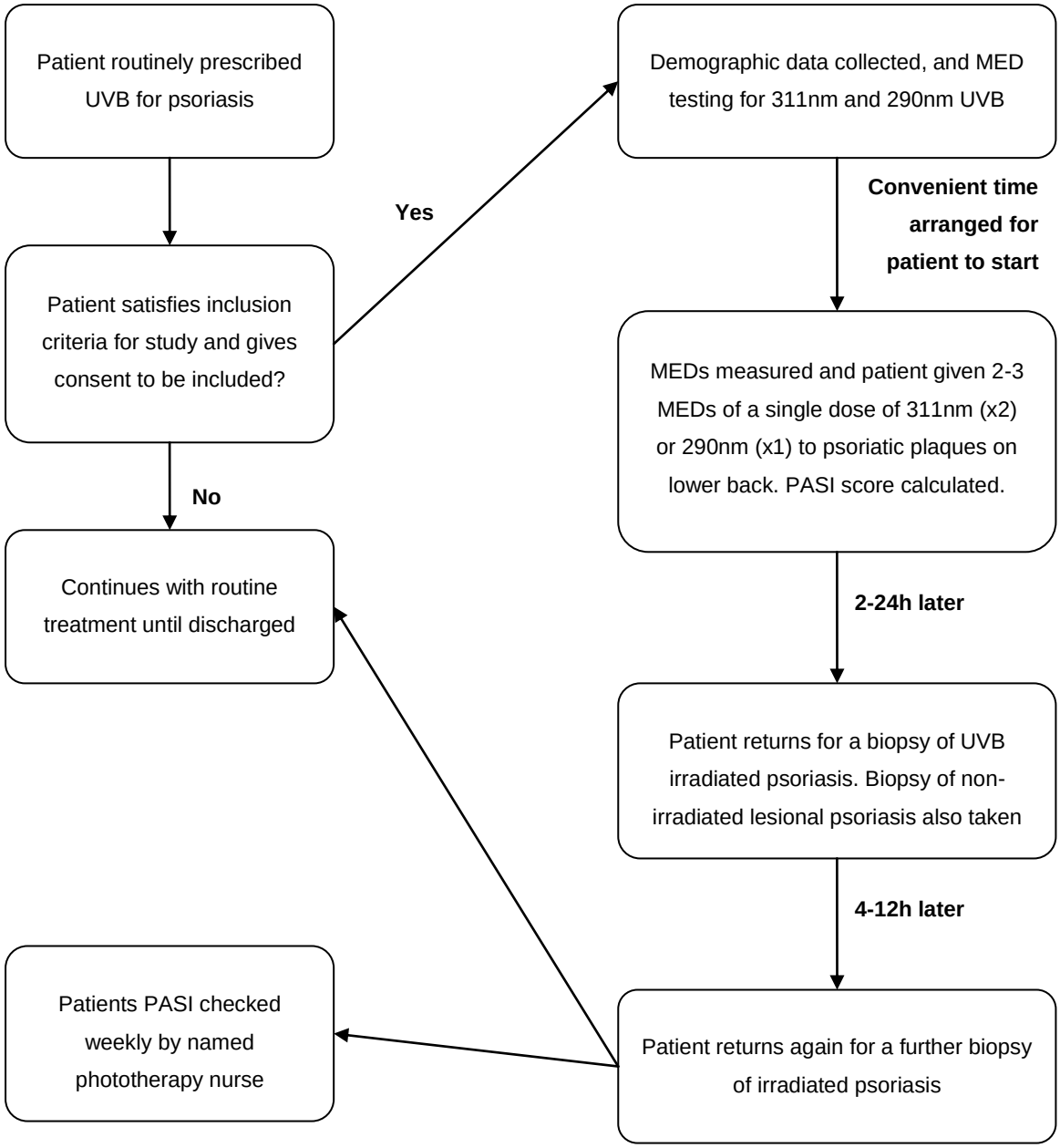
1. The first group was to establish a time-course of apoptosis following single exposure to a medium dose (2-3 MED) of 311nm and/ or 290nm UVB (n=36)
2. To examine whether apoptosis is detectable following a single low dose of 311nm UVB (0.75-1 MEDs) which is more frequently used in clinical practice (n=11)
3. To examine whether apoptosis occurs following repeated low dose irradiation of 311nm, as occurs in routine phototherapy (n=5)
4. To examine intra-patient variation 24h following 311nm-irradiation to neighbouring matched plaques (n=6)
5. To examine early gene changes which occur within plaques following irradiation with 311 and 290nm UVB (n=6)
6. To examine the effects of other wavelengths within the UVB spectrum to determine whether any of these may cause a greater apoptotic effect, which may potentially improve patient treatment (n=18).

Group sizes were determined according to pilot data obtained, and proof of principle. It was previously estimated that approximately three out of five patients showed a 311nm-induced apoptotic response above baseline, however with ethical approval, group sizes were modified from the original study design according to the level of apoptosis seen in the individual patients recruited. Furthermore, several of the groups overlap, and patients' results were therefore

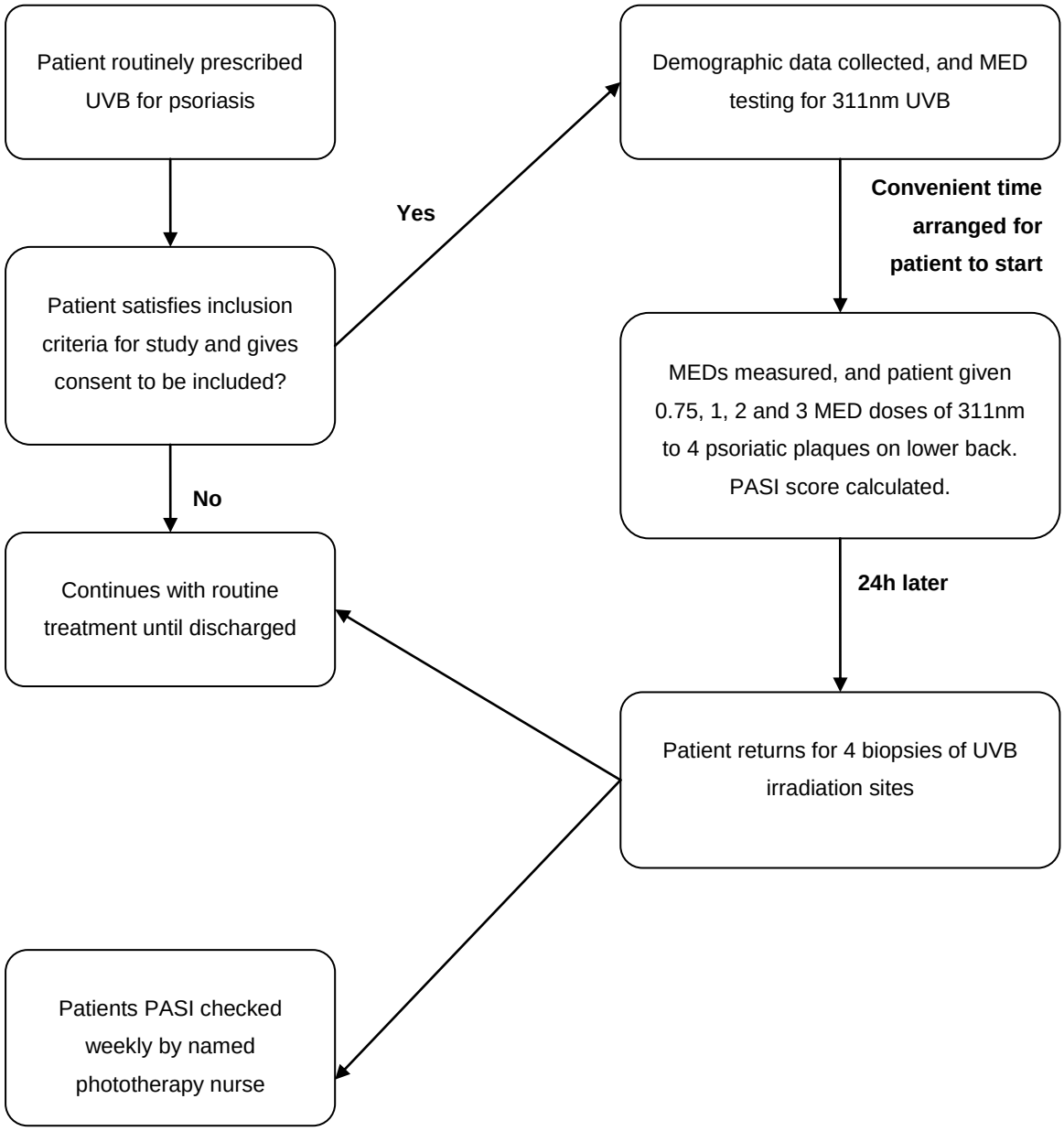
analysed together where possible (e.g. most patients had a biopsy 18-24h post 3 MED 311nm, and this was analysed in the time-course study as well as in the subgroup for which they were recruited).

The flowcharts below show the patients' journey following recruitment into each group:

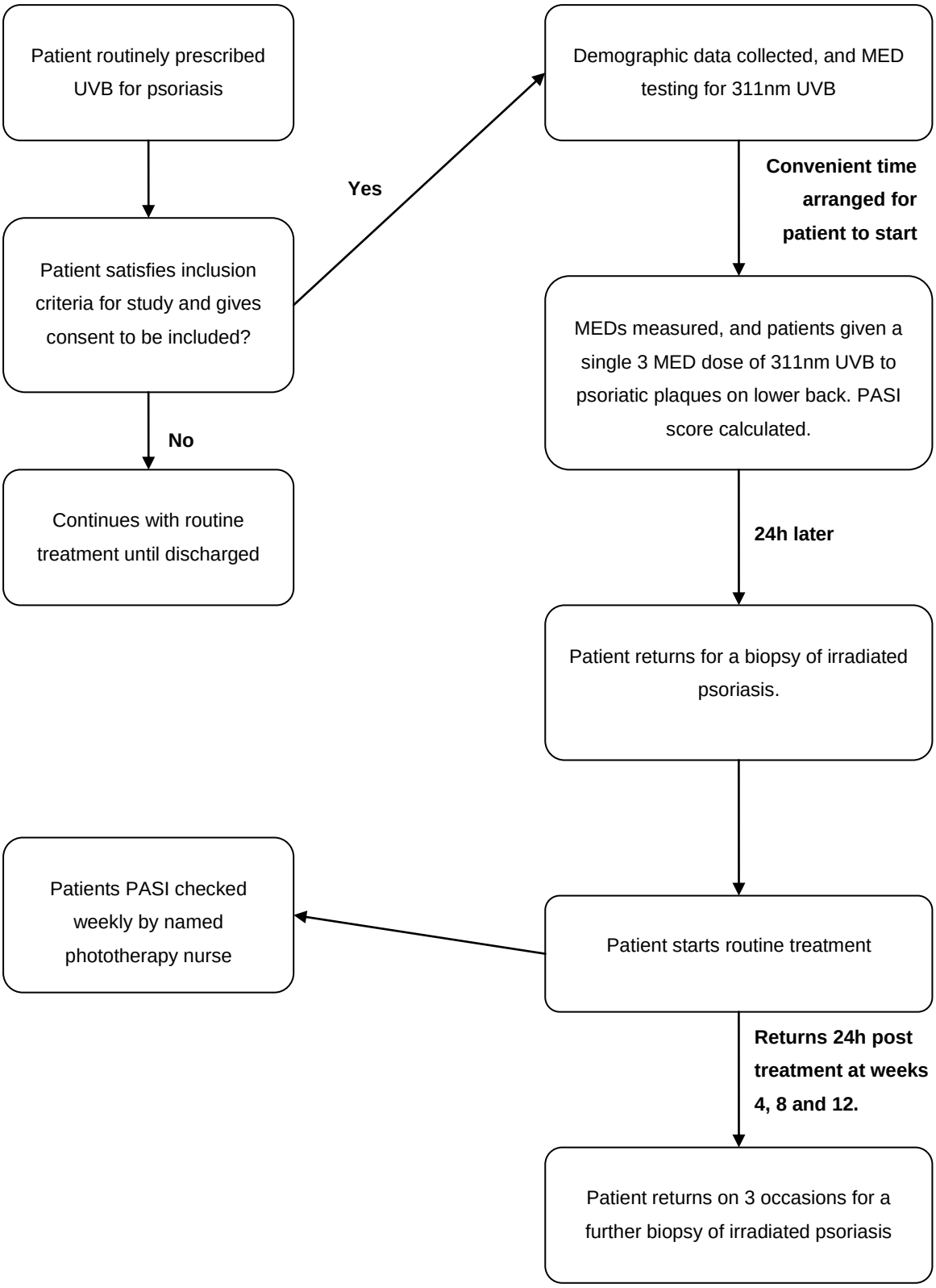
Group 1: Time course of 311nm and 290nm UVB.



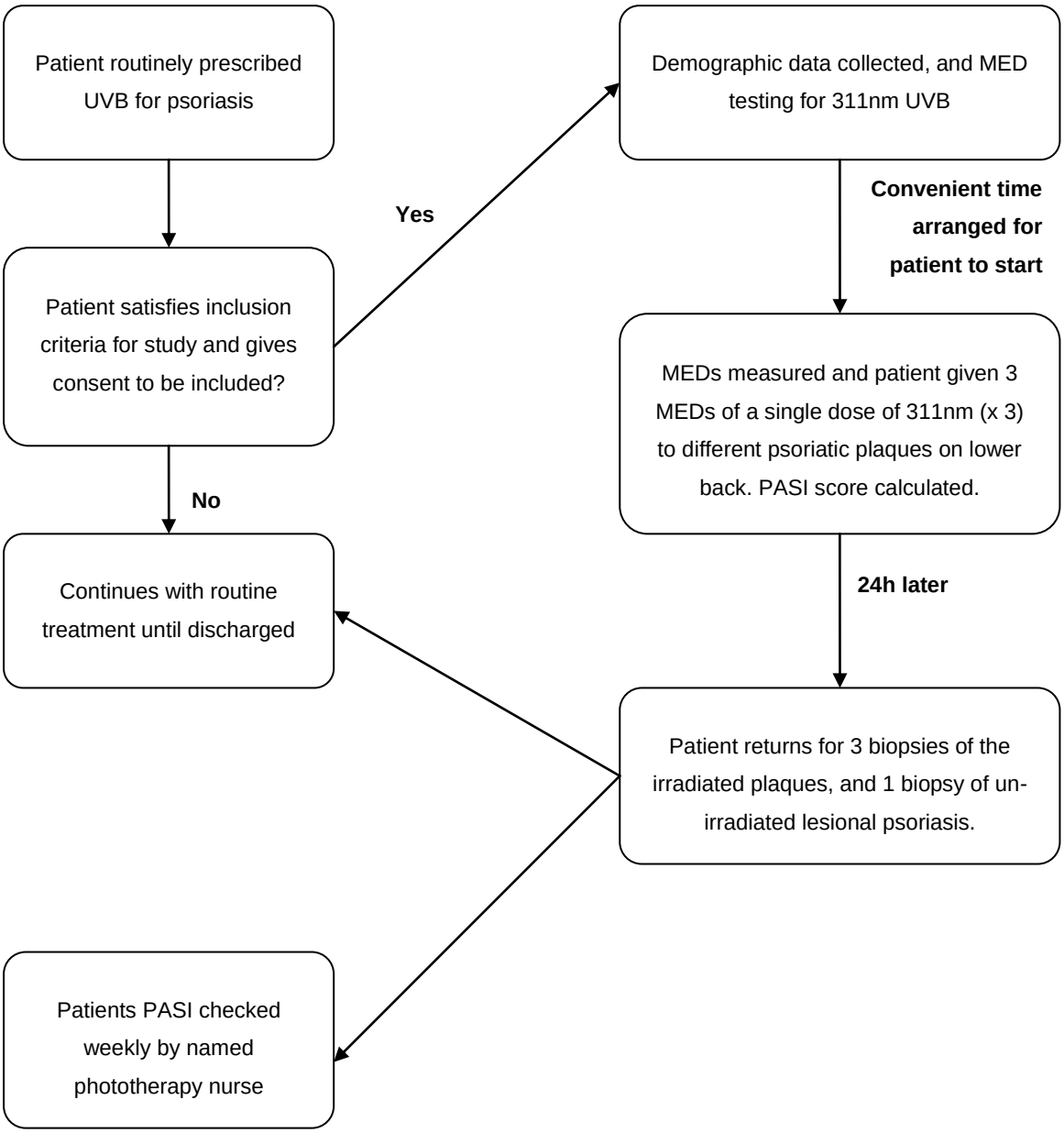
Group 2: Apoptotic effect of sub-erythemogenic doses of 311nm UVB.



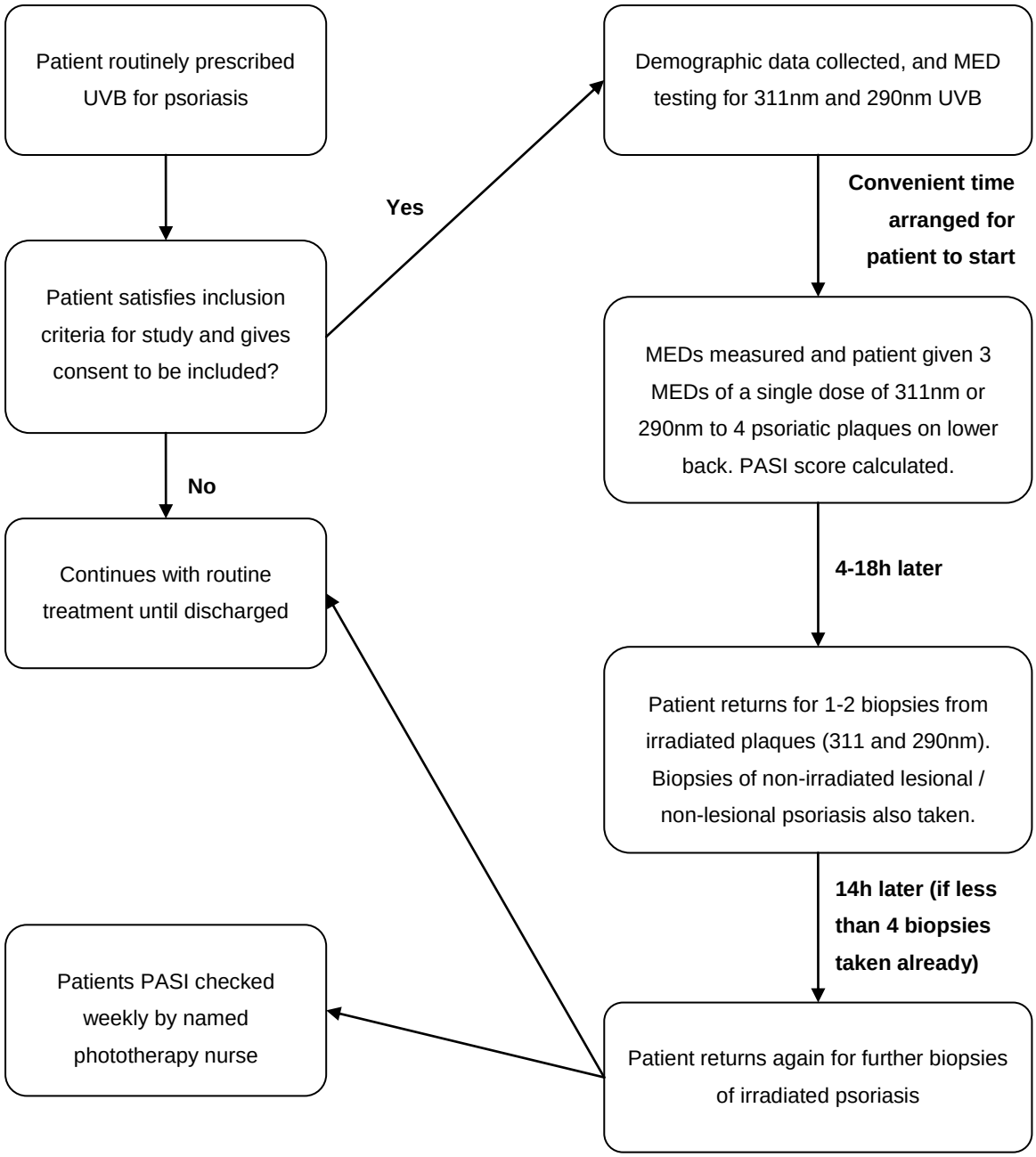
Group 3: Apoptotic effect of repeated 311nm UVB in clinical treatment



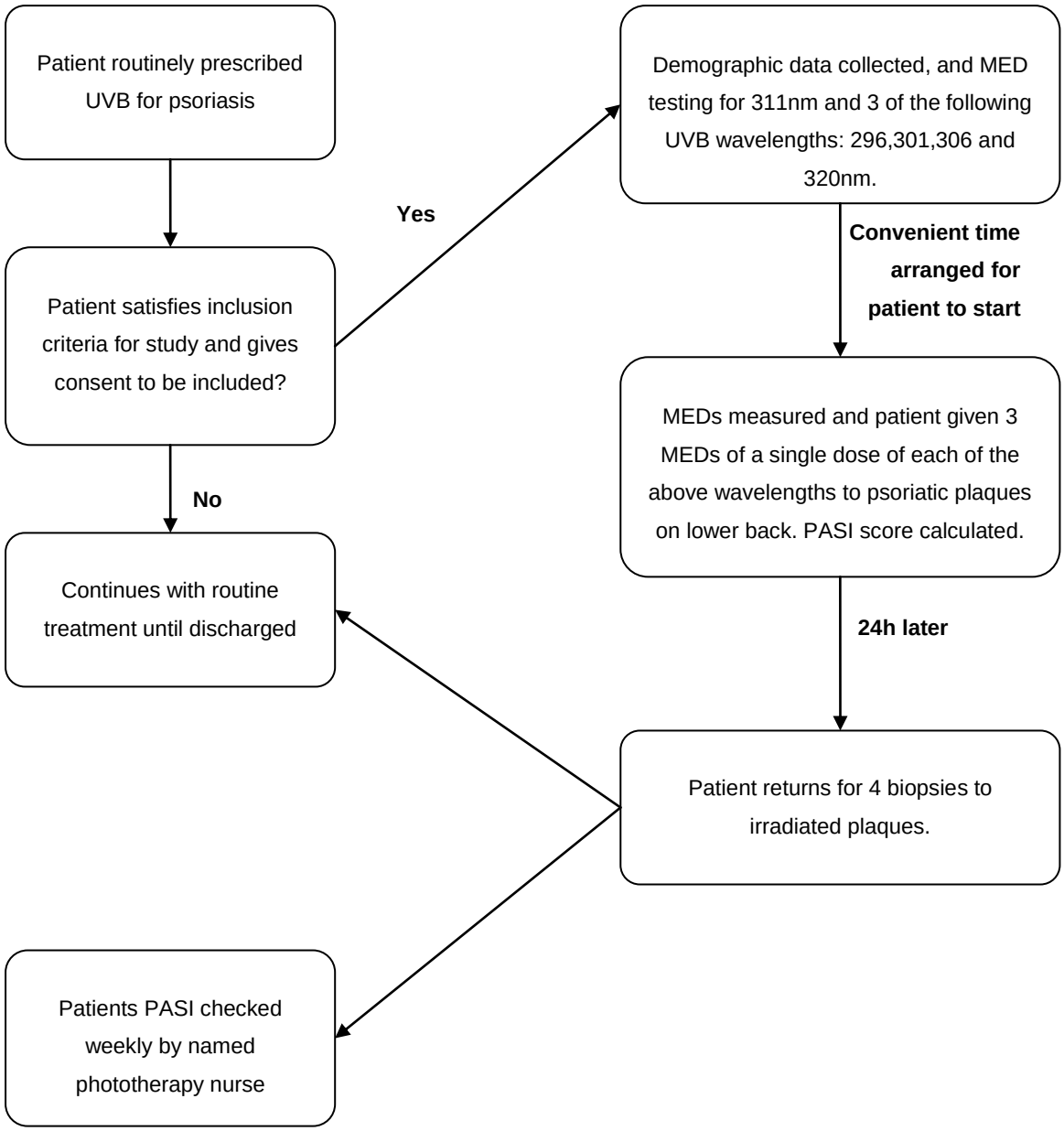
Group 4: Intra-patient variation in apoptotic effect.



Group 5: Early effects of UVB on gene regulation within psoriatic plaques.



Group 6: Apoptotic effects of other UVB wavelengths (296-320nm).



2.1.2. **Patient irradiation**

Patients had MED testing to narrow-band UVB (narrowband UVB; TL01 lamp; herein referred to as 311nm) and other wavelengths of UVB used as appropriate, on sun-protected non-lesional skin of the lower back. All UV irradiation was given at least 3cm from the midline of the vertebral column, as the midline is known to be less sensitive to UV (Farr and Diffey 1984) and could lead to spurious results. The MED was taken as the lowest dose required to induce just perceptible erythema 24h after irradiation with a specific wavelength (Gordon, Saunders et al. 1998). A handheld 10 aperture TL01 lamp was used for narrowband UVB irradiation. In front of each aperture was a metal grid with a regular pattern of holes. The size of the grid hole varies such that the ratio of irradiance between adjacent grids is the square root of two. This allows the patient to be exposed to geometric series of doses with a single application of the device. An irradiation monochromator was used for 290, 296, 301, 306 and 320nm wavelengths, set at a bandwidth at half maximum intensity of 5nm for wavelengths of 306nm and below, and 10nm for 320nm (Figure 2-1). Both the monochromator and the TL01 lamp had a 10min warm up-time before use and output was checked with a radiometer before and after patient exposure.

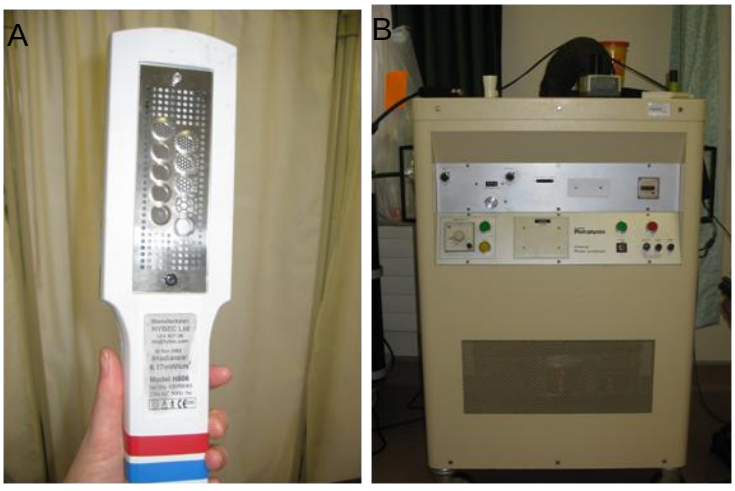


Figure 2-1. Devices used to administer specified wavelengths of UVB. a) Showing hand-held TL01 lamp to determine MEDs; and b) A monochromator used to administer 290nm wavelength.

After measuring the MED at 24h, each patient was irradiated with predefined multiples of the MED for each wavelength using a 15mm UV guide from a monochromator or suitable irradiation source (e.g. handheld narrowband UVB lamp). The edges of matched target-plaques were irradiated, along with non-lesional skin adjacent to each of these (Figure 2-2) so that the erythema induced could be objectively measured with the erythema meter. This allowed confirmation that patients had received equi-erythemogenic doses of UVB when different wavelengths had been used.

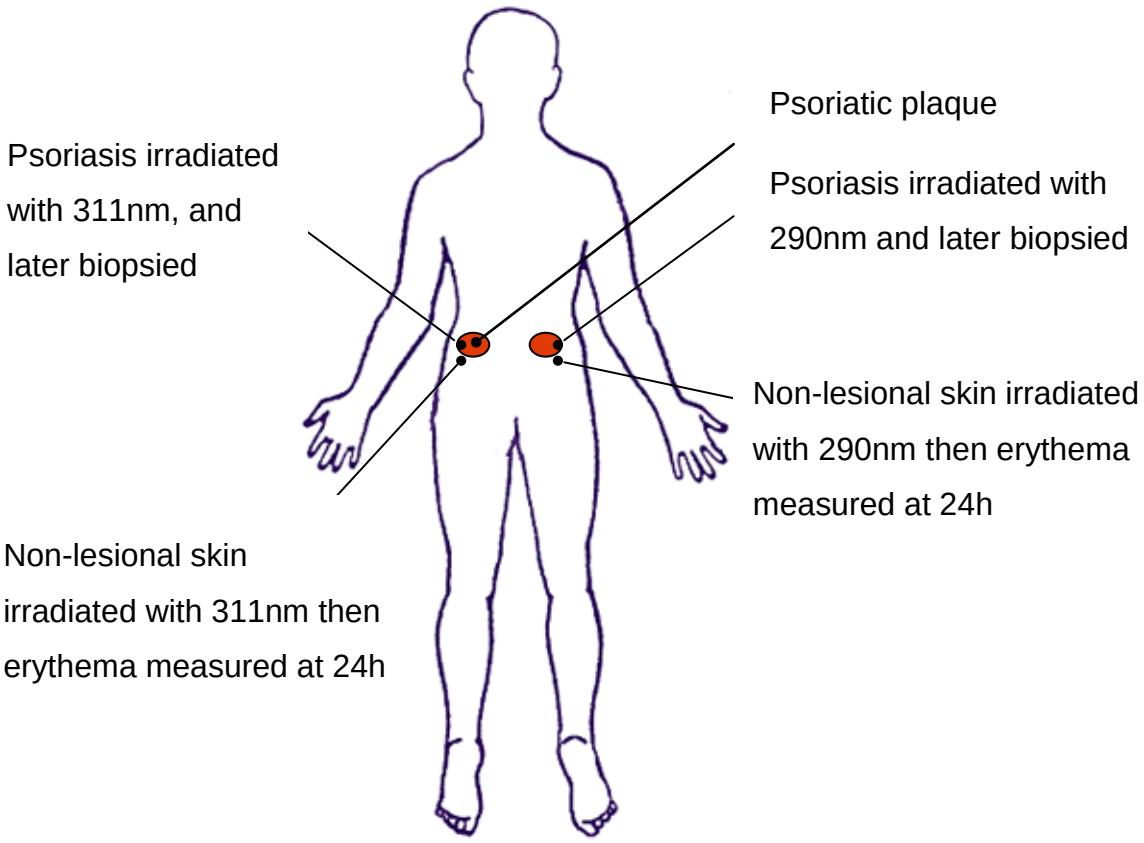


Figure 2-2. Diagram of typical position of UV irradiation within psoriatic plaques. Fifteen millimetre areas at the edge of a plaques (red) were irradiated, along with adjacent non-lesional skin, which was used as a control to ensure equi-erythemogenic doses had been delivered.

2.1.3. **Patient biopsies**

Six-millimetre punch biopsies were then taken from up to four matched sites on the patients' back, at pre-specified time intervals between 2-48h post UV exposure. A suture was used at each site to aid healing. The patients then received their normal UV treatment regime, and the suture was removed a week later in the phototherapy department. Previous reports have suggested that there may be cellular differences within individual psoriatic plaques, with the active edge of a plaque having an increased vascular component (Goodfield, Hull et al. 1994), and biopsies were therefore taken from small plaques (where the majority of the plaque was removed) or the edge of larger plaques. All biopsies were taken from the lower back, at least 3cm from the spine. Punch biopsies were snap frozen in liquid nitrogen, mounted in OCT (Raymond A Lamb) embedding medium then stored at –80°C until analysed.

2.1.4. **Immunohistochemical methods**

Frozen tissue was cut into 6µm sections using a Leica CM 1900 cryostat (Leica Microsystems, Milton Keynes, UK), and mounted on APES/ TESPA coated glass slides (2% aminopropyltriethoxy-silane in dry acetone). Tissue sections were defrosted at room temperature for 15 mins, and fixed. The fixation method was optimised for key antibodies using either paraformaldehyde (4% in PBS + 1mM NaOH), 100% acetone, 100% methanol or a combination of acetone and methanol (1:1) for 10 minutes (see appendix D). A Dako pen (Dako, Cambridgeshire, UK) was used to delineate the tissue section on the slide.

Following fixation, sections were permeabilised with 0.2% Triton X-100 for 10 mins and blocked with normal goat serum (1:60 dilution, 30 mins). They were incubated with anti-active caspase-3 (1:500 for 60 mins, R and D Systems, Oxfordshire, UK) at room temperature. Stored biopsies from patients with lichen

planus were used as a positive control for epidermal apoptosis. Cells were secondarily labelled with goat-anti-rabbit conjugated to Oregon Green 488 for 45 mins (1:300; Molecular Probes, Paisley, UK).

If double labelling was performed, sections were incubated with goat serum again for 20 mins, and then labelled with an antibody raised in a different species, and incubated with the appropriate secondary flurochrome. Finally, Toto-3 (1:7000 for 10 mins; Invitrogen, Paisley, UK) was added as a nuclear stain. Between each of the above steps, sections were washed three times with Tween 20 (0.05%) in PBS. Sections were then washed a further three times with PBS followed by a rinse with double-distilled water and then ethanol, and allowed to air-dry. A coverslip was then fixed in place with Mowiol mounting medium (see appendix E). All antibodies, serums and flurochromes were dissolved in 2% bovine serum albumin (BSA) in PBS.

2.1.4.1. Identification of Cell Type

Double labelling was performed with the following antibodies for 45 mins, secondarily labelled with Alexa Fluor 568 goat anti-mouse (1:300) secondary antibody (where appropriate) (see appendix E). Concentrations of each were optimised, and different fixation methods (see appendix D) were tried for each antibody to ensure the optimal antibody detection.

- AE1-AE3 (1:50; Abcam, Cambridge, UK), a monoclonal broad cytokeratin marker which identifies cytokeratins 1-7, 10, 14, 15, 16 and 19, but excludes simple epithelial markers such as cytokeratin 8 and 18. To ensure even staining, cytokeratin 14 (1:200; Novocastra, Newcastle-upon-Tyne, UK) was added.

- Anti CD3 (1:100; Dako, Cambridgeshire, UK), a pan T cell monoclonal antibody which is specific to T cells when detected in the skin. It is cytoplasmic in immature T cells within the thymus, and becomes membrane bound as the cell matures.

- Anti CD8 (1:100; Dako, Cambridgeshire, UK), a monoclonal antibody which detects the transmembrane glycoprotein CD8. This is present on mature cytotoxic/ suppressor T cells, which is the most common type of T cell infiltrate in the psoriatic epidermis.

- Anti CD68 (1:2000; Dako, Cambridgeshire, UK), a monoclonal antibody which identifies a 110 kD transmembrane glycoprotein which is highly expressed in monocytes, macrophages, Langerhans cells and plasmacytoid dendritic cells .

- Anti langerin (1:500; Beckman, Buckinghamshire, UK): a monoclonal antibody specific for Langerhans' cells, and expressed on the membrane and in cytoplasm of cells within the epidermis.

- Anti Cd1A (1:50; BD PharMingen, Cambridge, UK); a monoclonal antibody to Langerhans' cells and other myeloid dendritic cells.

- MelanA (1:100; Abcam, Cambridge, UK): a monoclonal melanocytes antibody recognising the protein MART-1 (melanoma antigen recognised by T cells), a subcellular fraction found in melanosomes.

- Anti Bax (1:500; Axxora, Nottingham, UK): a monoclonal pro-apoptotic protein which is over-expressed in psoriasis (Kocak, Bozdogan et al. 2003), and up regulated after UV exposure in normal skin peaking in expression at 33h (Murphy, Mabruk et al. 2002)

- Anti-p53 (1:100; Novocastra, Newcastle-upon-Tyne, UK): a monoclonal antibody to p53. p53 accumulates in response to DNA damage, and induces growth arrest and/ or apoptosis (intrinsic pathway).

- Anti Ki67 (1:200; Novocastra, Newcastle-upon-Tyne, UK): a marker of cells active in the cell cycle. It is detected in G1, S, M and G2, but not in G0. Localised to the nucleus.

- Cyclin B1 (1:200; Dako, Cambridgeshire, UK), which is maximally expressed in late G2/M and then degraded during anaphase.

- Anti-p63 (1:200; Abcam, Cambridge, UK), which is associated with cells with a high proliferative potential, and absent from those undergoing terminal differentiation (Parsa, Yang et al. 1999).

2.1.5. **Microscopy of the epidermis**

For colocalisation experiments, a Leica TCP SP2 scanning confocal microscope was used to visualise cells, using sequential scanning. Oregon Green was excited at 488nm with the Argon ion laser using a detection window of 497-535, Alexa red was excited at 543nm with the krypton laser set at a detection window of 580-620 and Toto-3 excited at 633nm with the Helium red neon laser set at a detection window of 645-689. Cells were visualised using a x60 oil objective.

For biopsy cell counts a BD^{TM} Pathway 850 Bioimaging system was used with a x40 objective. This allowed the entire biopsy to be imaged in 2-3 montages of 9 adjacent images, and allowed all cells within the biopsy to be imaged, for counting. The Pathway microscope is a non-laser system that utilises dual-mercury metal halide lamps and a series of 16 excitation filters to allow fluorescence imaging from 340 to 750nm, therefore fluorescence was detected using alexa-488 and cy5 settings for anti-active caspase-3 and Toto-3 respectively.

Volocity 4.01 image analysis software was used to quantify apoptotic cells within the epidermis, determine the strength of colocalisation, and to express apoptotic cells as a proportion of the total cell count. A region of interest (ROI) line was manually drawn around the epidermis, and the following protocol was created which allowed automated counting of cells:

Find Objects Using SD (select voxels) Intensity (intensities are represented as standard deviations from the mean intensity)

↓

Exclude Objects Not Touching ROI

↓

Exclude objects > 1000 μm^2

↓

Exclude objects < 50 μm^2

For caspase-3 activity a maximum of 0.65 apoptotic cells per 1000 nucleated epidermal cells was used as the cut-off point for a normal baseline, with anything greater than 1.75 positive cells per 1000 defined as convincingly positive. This value was chosen as it represents the upper limit of apoptosis observed within un-irradiated psoriatic epidermis.

To measure the thickness of psoriatic epidermis consecutive measurements were made from each biopsy by measuring the maximum epidermal thickness including the *stratum corneum*, as shown in Figure 2-3.

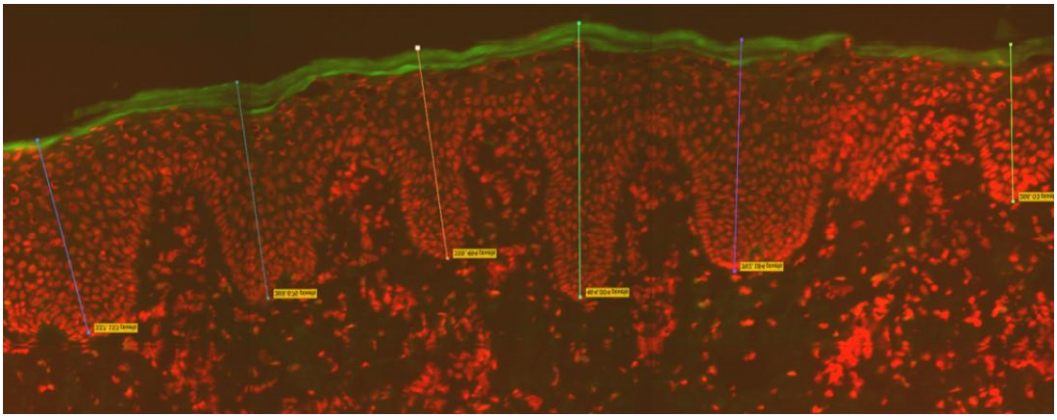


Figure 2-3. Measuring the maximal thickness of the epidermis using Volocity software. This allows inter-and intra-patient comparison of plaque thickness.

2.1.6. **Electron microscopy**

Epidermal sections of 2mm diameter were processed for transmission electron microscopy, following fixation in 2% gluteraldehyde in Sorensons phosphate buffer, and secondary fixation in 1% osmium tetraoxide. Samples were then dehydrated in acetone and embedded in resin before analysis. Microscopy was performed using a Philips CM100 TEM with Compstage and a high resolution digital image capture. Images were scanned in a sequential manner and apoptotic cells identified morphologically by changes including cell shrinkage and condensation of chromatin. Cell typing was possible by identification of features unique to specific cell types, e.g. keratin filaments and hemi-desmosome connections between neighbouring keratinocytes.

2.2. Cell culture

Primary keratinocytes derived from human skin were obtained from routine circumcision procedures carried out within the Newcastle Hospitals NHS Trust. Ethical committee approval was obtained, and each patient or their parents gave informed consent as appropriate.

Skin samples were rinsed in PBS and excess subcutaneous tissue was trimmed away. A blade was then used to mark the surface of the epidermis in a grid-like manner, and skin was incubated in dispase overnight at 4^0C. The following day, the epidermis was gently peeled away from the dermis, and incubated in 5ml of Trypsin at 37^0C for 5 minutes. Cells were then shaken, 500µl FCS was added, and then centrifuged at 1250rpm for five minutes and washed. Cells were seeded in a 75ml tissue culture flask, with 10ml medium (described below); a further 300µl FCS was added to the cells for the first 24h of culture to aid attachment to the flask. To grow cells, Epilife® medium (Invitrogen, Paisley, UK) was used, supplemented with bovine pituitary extract 0.2%, EGF (0.2 ng/ml), hydrocortisone (0.18 µg/ml), insulin (5 µg/ml),

transferrin (5 µg/ml), penicillin G (50 U/ml) and streptomycin (50 µg/ml). Cells were incubated at 37ºC with 5% CO_2.

2.2.1. **Seeding Cells**

Cells were washed twice with PBS, and detached using trypsin over the minimum amount of time required (approximately 10 mins). Trypsin was neutralised with the addition of 10ml medium and 1ml FCS, and transferred to a universal container. A cell pellet was obtained by centrifugation at 1250rpm for five minutes. The pellet was re-suspended in 10ml medium, and cell counts were obtained using a haemocytometer. When seeding into 24 well sensoplates for UV irradiation, 60 000 cells were allocated per well to allow confluency within 48h. Each well was made up to a volume of 400µl with Epilife® medium. As Epilife is slightly coloured, medium was temporarily replaced with PBS during the irradiation process, to ensure that this did not affect the dosing regimen. Cells were used up to and including passage two only, as higher passages yielded very variable results.

2.2.2. **UV Irradiation of Cells**

Cells were grown to 70-80% confluency and then irradiated with increasing doses of UVB (2-10 SED) using either four TL01 lamps (311nm) mounted in a wooden ventilated light box positioned 29cm above the irradiation surface or an irradiation monochromator for 290nm UVB (set at a 5nm bandwidth). An SED is a measure of effective radiant exposure (section 1.4), with approximately three SEDs being equivalent to one MED. The weighted doses used were 20-100mj/cm^2 for each wavelength (total unweighted doses of 20-100mj/cm^2 for 290nm UVB and 350-1750mj/cm^2 for 311nm UVB). Spectral irradiance was

measured using a Bentham Instruments portable monochromator, calibrated to National Physical Laboratory standards, and for 290nm irradiation). For 290nm irradiation, a 15mm input optic (attached to a light guide) was positioned directly above the cells, which gave a uniform 15mm diameter field of irradiation (equivalent to 1 well in a 24-well plate). A calibrated radiometer was used to ensure consistent irradiation between experiments. Where different SEDs were given to individual wells within a single 24-well plate, wells were covered appropriately to prevent irradiation from reaching the cells. The plates used for irradiation (Sensoplates, Greiner bio-one, Germany) had black opaque sides to each well, which prevent stray UV from being transmitted. A control well was included in each experiment.

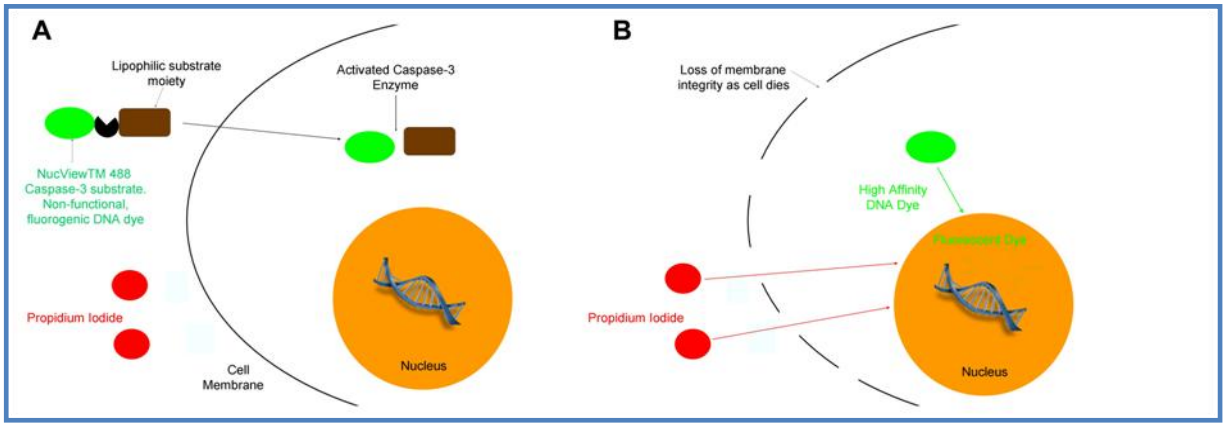


Figure 2-4. Diagram of real-time caspase-3 assay in NHEK cells. (a) The caspase-3 substrate enters through an intact cell membrane. If active caspase-3 is present within the cell, the moiety is cleaved, and the substrate fluoresces green in the nucleus. (b) when the cell dies the membrane integrity is lost, and PI can enter (cell fluoresces red). The time between green and red fluorescence can be used to estimate the rate of keratinocyte apoptosis.

Following irradiation, NucViewTM 488 Caspase-3 assay kit for live cells (Biotium, Cambridge, UK) was added at a concentration of 1μM, to allow visualisation of cells undergoing apoptosis. This is a live cell membrane-permeable fluorogenic substrate which detects caspase-3 activity within cells (Figure 2-4). It can be used in real time, and will show the onset of caspase-3

activity, but will remain positive after activity has ceased and the cell has died. Therefore Propidium Iodide (PI; 1µM) was used as a marker of cell death, allowing an estimation of the time that a live cell will remain caspase-3 positive for before dying (at which point it would be phagocytosed *in vivo*). Control wells included the addition of a specific caspase-3 inhibitor (supplied as part of the Biotium kit), non-irradiated cells, and wells containing either anti-active caspase-3 alone or PI alone. Etoposide (5µM) was used as a positive control for caspase-3 activity. Total cell counts were obtained at the end of experiment by the addition of a membrane permeable dye with a high affinity for dsDNA, such as Hoescht 33258 (Invitrogen, Paisley, UK; 45µM) or DRAQ5 (Biostatus, Leicestershire, UK; 5µM). Incubation with either of these dyes from the start of the experiment (even at the lowest possible concentration for detection) was found to enhance both caspase-3 and PI detection in cells after approximately 4h, indicating that these induce toxicity over time, and this was therefore avoided.

2.2.3. **Live Cell Imaging**

Live cells were imaged within an hour following irradiation, until a maximum of 48 hours post-UV exposure. A BD^{TM} Pathway Bioimager 850 microscope was used with a x20 objective, which was set up to autofocus, and images taken in time-lapse every 10-15 mins from each well using a predefined macro. Each image consisted of a montage of 9 adjacent images, allowing approximately 100 cells to be tracked over time. The cell conditions during imaging were kept constant, with the temperature set to 37^0C, and CO_2 at 5%, and the cells kept in a darkened environment.

2.2.4. **Image Analysis**

An algorithm was developed using Volocity 4.01 software to automatically analyse these results, allowing calculation of a median time of caspase-3 positivity prior to the onset of PI detection (signalling cell death). This was done by allowing Volocity to track individual cells over time, and measuring the time-points of first detectable green fluorescence (caspase-3) and the first detectable red fluorescence (PI entry) within each cell. These were subtracted from each other to give an estimate of how long cells remain apoptotic for before membrane integrity is breached and they die.

2.3. Flow cytometry

Flow cytometric analysis was employed to examine differential susceptibility of cells to apoptosis following irradiation with six SEDs of 311nm or 290nm UVB. Furthermore, apoptosis occurring within different stages of cell cycle and with different proliferative ability (i.e. stem, TA and differentiating cells) was examined following irradiation with six SEDs of 311nm UVB. All experiments described below were repeated a minimum of three times (and in triplicate within each experiment for cultured cells), although numerous similar experiments were performed as part of the optimisation process. A BD LSR11 four colour flow cytometer was used to process the cells, and analysis was performed using VenturiOne^{TM} software.

2.3.1. Flow Cytometric analysis of cultured cells

Primary keratinocytes were grown to 90% confluency in six well plates, then incubated with PBS and irradiated as described below. The cell medium was changed immediately after this process and cells were incubated with NucViewTM 488 caspase-3 substrate (1µM) for 24h. After this time, the medium was removed and stored, and the remaining cells were detached from each well by trypsinisation. To neutralise the trypsin, FCS was added to the cells as previously described and these were centrifuged with their previous medium, and washed. PI (1µM) was then added to each sample. Controls included an irradiated well with PI alone, and an un-irradiated well incubated for 4h with caspase-3 substrate and Etoposide (5µM) only.

Approximately 30 000 cells were collected for each sample, and analysed using VenturiOne^{TM} software. Doublet cells (approximately 5-8% of total cells) were gated out, then the proportion of caspase-3 positive and PI positive cells were analysed.

2.3.1.1. Comparison of the apoptotic effect of 311nm and 290nm UVB.

Primary keratinocytes were irradiated in 6-well plates with six SEDs of 311 or 290nm UVB, 24h prior to analysis. For 311nm irradiation, a bank of four TL01 lamps were used as described in section 2.2.2, and for 290nm UVB the light guide was positioned 47mm above to 35mm diameter wells, and this provided a uniform irradiation field to the well (total unweighted doses were 20-100mj/cm^2 for 290nm and 350-1750mj/cm^2 for 311nm UVB). Three control (un-irradiated) wells were used as a comparison for the effects of UVB. The numbers of anti-active caspase-3 and PI positive cells induced by both wavelengths were compared.

2.3.1.2. Cell cycle analysis

An automated cell cycle analysis on the dead (PI positive) cells. The proportion of dead caspase-3 positive cells in the G1, S and G2M phase were compared between the irradiated and mock irradiated cells both within experiments and between experiments.

2.3.2. Proliferation analysis

2.3.2.1. Cell sorting

To identify the scatter location of keratinocytes, and ensure other epidermal cell types were not being analysed, cells were analysed according to their scatter distribution. Four scatter locations were identified (Figure 2-5) and cells were collected from each using a FACSDiva cell sorter. A minimum of 10 000 cells were collected from each area and allowed to air-dry on slides. These were then fixed in methanol: acetone (1:1), stained for markers of cell type (section 2.1.4.1), and identified using a BD^{TM} Pathway 850 Bioimaging system.

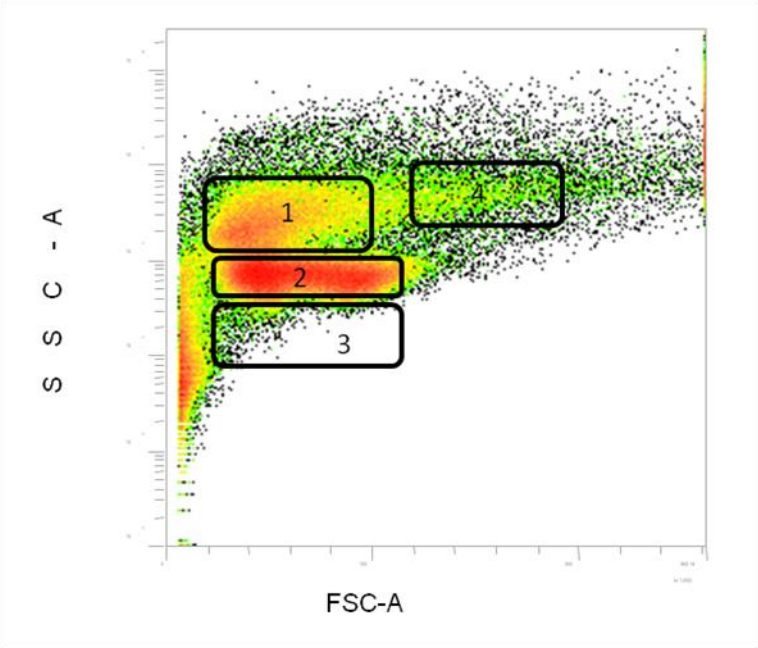


Figure 2-5. Scatter plot showing regions of sorted epidermal cells. Cells collected from each area were labelled with markers of cell origin by immunohistochemistry, to identify which areas contained keratinocytes, and which were cells derived from other lineages.

2.3.2.2. Identifying apoptosis in cells of different proliferative potential

Fresh human foreskins were divided into two sections and prepared for dispase-separation of the epidermis from the dermis. Prior to overnight incubation, half of the intact epidermis was irradiated (epidermal-side up) in PBS with six SEDs 311nm UVB (TL01 lamp). After 18h cells the epidermis was peeled off the dermis and incubated in trypsin for five minutes. The trypsin was then inactivated with fetal calf serum and the cells washed twice. The keratinocytes were then incubated with PE-CD49f (α6-integrin; BD Pharmingen, Oxford, UK) and APC-CD71 (BD Pharmingen, Oxford, UK) for 45 minutes. Cells were washed and incubated with FITC-annexin V for 15 minutes (Molecular Probes), and 20 000 cells per sample collected for analysis. Following initial collection, PI was added and cells re-examined.

Spectral overlap exists between the excitation of PI and PE (Figure 2-6), therefore analysing these two dyes together can lead to confusion. For this reason, most of the analysis was done without PI. However, it is useful to know the proportion of dead cells within each sample, and therefore to partially circumvent this problem PI was added after initial analysis, and PI positivity was assessed using the Hoescht channel (which will detect only the 1st excitation peak of PI; approximately 350nm). All cells which are PI positive can then be gated out of the analysis leaving live cells only. This is not ideal however, as PI entry is not an all or nothing event, and cells which may have been slightly (but not fatally) damaged will contain small amounts of PI (but not enough to be gated out as dead cells), and may be falsely analysed as PE positive.

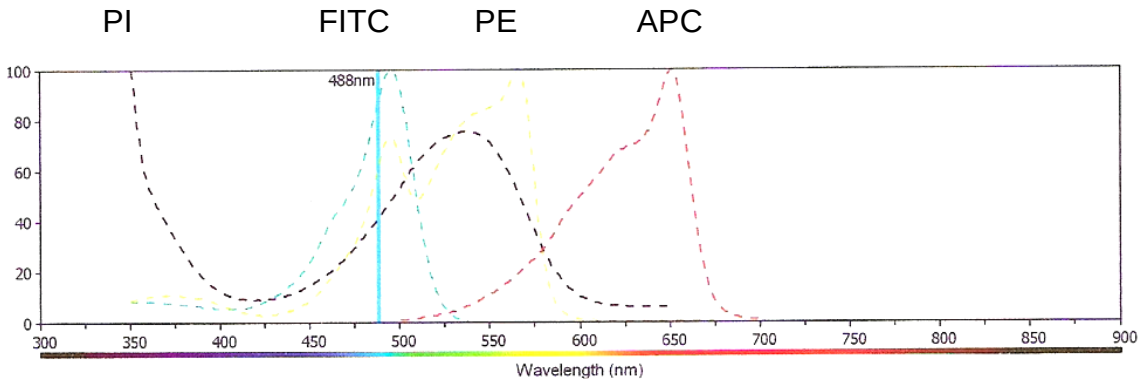


Figure 2-6. Excitation spectrum of the 4 flurochromes used. Note that PI has 2 excitation peaks, the second of which overlaps with PE. Diagram modified from the BD fluorescence spectrum viewer (http://www.bdbiosciences.com/external_files/media/spectrumviewer/index.jsp)

Analysis was performed by first gating cells which lie within the region consistent with intact keratinocytes. The number of annexin V positive cells was compared between irradiated and un-irradiated samples. Expression levels of α6-integrin and CD71 antibodies within the cells were examined on a scatter plot, and cells with the high levels of α6-integrin and CD71 expression were deemed TA cells, those with high levels of α6-integrin but low CD71 expression

were deemed putative stem cells, and those with low levels of α6-integrin were deemed differentiating cells. The proportion of annexin-V positive cells within each of these groups was then compared.

2.4. Mathematical modelling

2.4.1. Modelling environment

A model was creating using NetLogo version 4.1, which is an agent-based modelling environment. Agent-based modelling can be used to simulate the effects of cells (agents) on each other and their local environment, which can lead to prediction of higher level behaviour. This theory behind this is that complex biological processes are dependent on the regulation of relatively few cellular mechanisms (Sun, Adra et al. 2009). Agent-based modelling allows each type of cell to be given a list of instructions and then operate independently, allowing the effects of micro-level behaviour to be observed at the macro-level. NetLogo (Wilensky 1999) is written in Java and runs as an independent application; it can be downloaded as freeware from http://ccl.sesp.northwestern.edu/netlogo/download.shtml. Netlogo was developed out of StarLogoT in 1999 because of the need to expand the language, make it more user-friendly, more powerful and to support the HubNet (multiuser participatory simulation environment) architecture. Hubnet allows simulations to be run by human interaction rather than just according to a set of rules, therefore allowing participation by the user.

In NetLogo the ‘world’ appears as a grid, the size of which can be determined by the user. The grid comprises patches, each of which can be identified by its x-y coordinates, and turtles which are the agents that move and interact on top of the patches. Each turtle has its own unique identity tag (known as ‘who’) allowing the user to track the progress of individual turtles using their ‘who’

number. All commands are directed specifically at either ‘the observer’ (an overview of the whole model), the patches or the turtles. The interface allows direct modification of turtle/ patch behaviour using buttons, switches and sliders, and observation of output using monitors or graphical displays. The model can be visualised in 2- or 3-dimensions and can output detailed results which can be analysed using Excel.

Programming in NetLogo is fairly straight-forward and does not require a programming background. Basic commands are used such as ‘ask’, ‘if’, ‘set’, ‘move-to’, ‘stop’ and ‘die’ and a series of brackets identifies what each command refers to. As the model is dynamic, the program automatically calculates an arbitrary measure of time with each unit of time referred to as a ‘tick’. At the beginning of the model a list of parameters used within the code are declared under the headings ‘turtles-own’, ‘patches-own’ or ‘globals’, so that the model can correctly assign the parameters as it runs. NetLogo has a useful ‘check’ facility which can be used to ensure that the code is accurate and all brackets are correctly positioned prior to running. This helps prevents the model from ‘crashing’ and helps the user identify any problem that needs addressing.

2.4.2. **Components of the model**

The model is divided into a series of procedures, the first of which is ‘setup’ which puts all patches and turtles in their initial positions. The ‘go’ command (which can be accessed as a button from the interface) will then allow sequential execution of all other procedures according to the parameters set by the user on the interface (Figure 2-7). The model can be stopped at any time and restarted by clicking the ‘go’ button on the interface or reset by clicking ‘setup’.

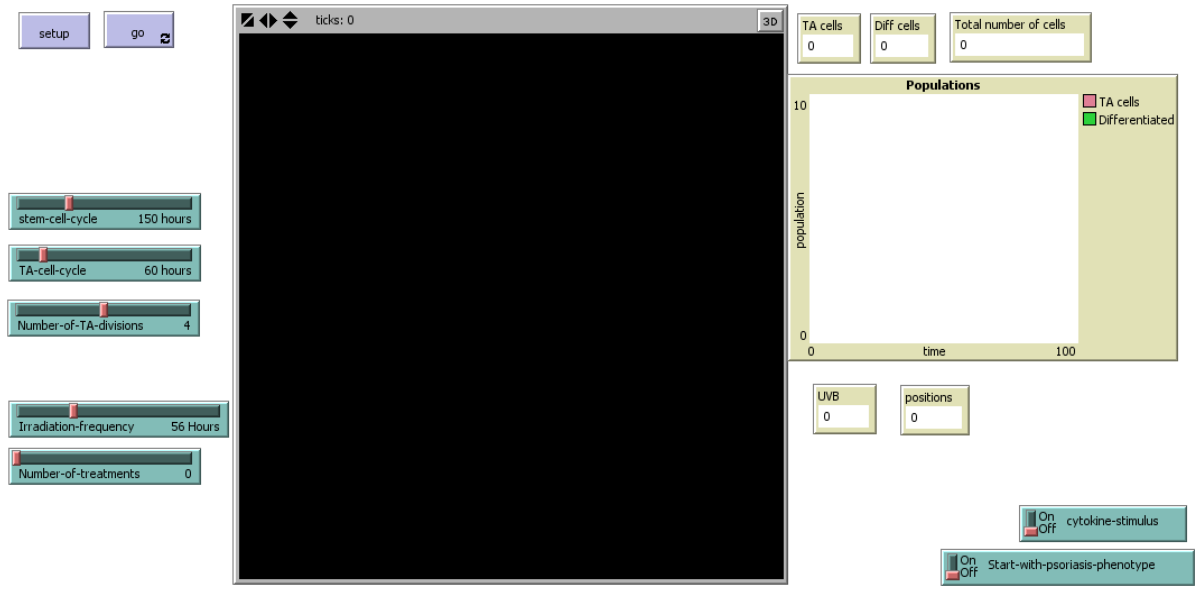


Figure 2-7. The interface for the model created in Netlogo. The two buttons at the top allow the epidermis to be setup and the programme to be run. There are a series of sliders (blue) which allow easy adjustment of parameters, and monitors (grey) to visualise what is going on when the model runs. The 'ticks' at the top of the model equate to the number of hours since the simulation began. The number of cells present over time is also represented graphically in the 'populations' window.

Kinetic data describing epidermal homeostasis is not absolute, and it is likely that parameters vary substantially both between and within patients and cells. To allow exploration of the effects of altering some of these parameters a series of sliders are provided on the interface allowing the user to test the effects of altering the following (permissible range shown in brackets):

- The cell cycle time of dividing stem cells (1-500h)
- The cell cycle time of dividing TA cells (1-400h)
- The potential number of rounds a TA cell can divide (1-8)
- The frequency of UV administration (1-96h intervals)
- The number of UV treatments administered (0-40)

- Whether the patches display a normal or psoriatic epidermal phenotype initially
- Whether there has been a “cytokine stimulus” which would trigger an increase in the proportion of stem and TA cells actively proliferating (e.g. a super-antigen response). Therefore, leading to the development of psoriasis.

The following monitors allow quantification of the model, and are also displayed on the interface:

- Number of TA cells present (cycling and non-cycling)
- Number of differentiating cells present
- Total number of cells (includes stem, TA and differentiating cells)
- Number of UV treatments administered
- Position of basement membrane (‘1’ (normal), through to ‘7’ (thickest psoriasis modelled))

2.4.3. **Model assumptions**

The model makes the following assumptions

- At a specified distance above the dermal papillae, differentiated cells are incorporated into the *stratum corneum* (not represented in the model) resulting in a ‘flat’ surface to the epidermis
- Under normal conditions each stem cell divides asymmetrically to produce one stem cell and one daughter TA cell

- The daughter cell will divide symmetrically for the user-defined number of times at the beginning of its life. It will gradually migrate up the epidermis.

- A gradient arising from the basal layer determines when cells differentiate. This was created to be consistent with the pattern of differentiation observed with immunohistochemical analysis (Korver, van Duijnhoven et al. 2006) (Figure 1-5), while keeping the proportion of differentiating: proliferating cells consistent with published evidence (Weinstein, McCullough et al. 1984; Bata-Csorgo, Hammerberg et al. 1993).

- Stem cells always remain in the basal layer and do not move.

- If the actively dividing TA cells become too numerous to fit within the specified area for division, the basement membrane stretches (i.e. the rete ridges elongate) to accommodate the cells. Conversely, if too few non-dividing TA cells are within this area, the rete ridges will reduce in length.

- To create 'psoriasis' the number of actively dividing stem cells were increased by a factor of four and the number of times a TA cell can divide was increased from an average of 4 times to 4.5 times.

- The model assumes that lesional psoriasis contains twice the number of nucleated epidermal cells compared to non-lesional skin, representing psoriasis of moderate severity (typical of the patients who are treated with UVB), however the model could be used to examine thicker psoriasis; which would take longer to clear.

Unlike previous models (Grabe and Neuber 2005; Grabe and Neuber 2007; Sutterlin, Huber et al. 2009) there is no maximum age specified for individual cells, and no cell is destined to spend a set period of time in either the proliferating or differentiating compartment. Actively dividing cells are always situated on the basement membrane or suprabasal layers, although this area is greater in the rete ridges than adjacent to the dermal papillae as it is determined by a gradient diffusing from the basement membrane/ dermis (Figure 2-8). Several lines of evidence indicate gradients of differentiation

markers within epidermis (Ishida-Yamamoto and Iizuka 1995; Martinsson, Yhr et al. 2005; Korver, van Duijnhoven et al. 2006). Although the molecular signals that drive this process remain to be fully elucidated, the histological pattern of induction of keratinocyte differentiation is not consistent with a gradient emanating from a fixed distance beneath the *stratum corneum*, but rather indicates a gradient arising from the basal layer. Cells with the highest proliferation potential will have greater adhesion to the basement membrane and /or other cells of higher proliferative potential (Fuchs 2008), and will therefore migrate from the basal layer to the epidermal surface as they age.

An *in silico* gradient is produced by giving each patch in the basement membrane an arbitrary value. This is diffused across the epidermal patches by allowing each neighbouring patch to 'share' a proportion of the value assigned to a given patch. This spreads over all patches producing a gradient along which cells can move. For the purposes of the model, a cut off point was chosen whereby cells would be deemed far enough away from the basal layer to differentiate. This gradient was kept constant for both normal and psoriatic epidermis (Figure 2-8), and was chosen to reflect the *in vivo* expression of differentiation markers such as keratin 10 / S100A proteins seen within normal and psoriatic skin (Ishida-Yamamoto and Iizuka 1995; Martinsson, Yhr et al. 2005; Korver, van Duijnhoven et al. 2006) (Figure 1-5). If the actively dividing TA cells become too numerous to fit within the specified area for division, the basement membrane stretches (i.e. the rete ridges elongate) to accommodate the cells. Conversely, if too few non-dividing TA cells are within this area, the rete ridges will reduce in length.

Psoriatic epidermis varies according to the severity of disease, and the depth of a plaque exists on a continual spectrum from normal to several times thicker than normal skin. To reflect this, and to computationally allow the epidermal phenotype to remodel from "psoriatic" back to "normal", the model contains a number of "positions" allowing plaque thickness to gradually return to normal. The seven positions vary from position '1' (normal) to '7' (thickest psoriasis modelled) (Figure 6-13). The latter allowed the epidermis to expand to contain twice the number of cells compared to position '1'. The *stratum corneum* is not

represented, as the hyperkeratosis seen in psoriasis is an effect of hyperproliferation rather than a cause. Although psoriatic skin can clinically be thicker than the model allows *in vivo*, the model aims to represents a typical plaque.

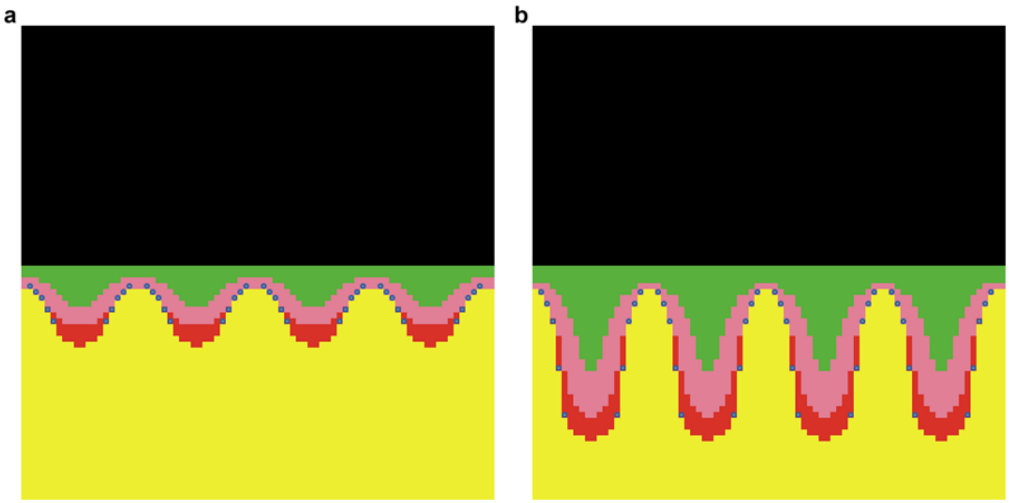


Figure 2-8. Gradient of patches within the model represented with a colour scheme. a) represents normal epidermis and b) represents psoriatic epidermis. Two inverted gradients were applied within the model arising from the basement membrane; one maps to the pattern of keratin-10 immunostaining (green) with a specified threshold determining the onset of keratinocyte terminal differentiation; a second gradient contains actively dividing TA cells and stem cells (red) and regulates expansion/ contraction of the basement membrane. Yellow represents the basement membrane.

2.5. Gene Array

2.5.1. **Samples**

Changes in gene expression were examined in psoriatic plaques at 4h and 18h post three MEDs of 311nm or 290nm UVB. Six patients (24 samples) were initially recruited and had four biopsies each (Table 2-1). Samples were analysed without knowing whether the patient would respond to their routine treatment or would have high levels of apoptosis on IHC. As the patients response to treatment/ apoptotic levels became known it became clear that a larger sample number would be needed to extrapolate meaningful results. Therefore, a further 24 samples taken from other patients within the study were analysed; attempting to make the number of patients in each treatment group equal (although in several cases this was not possible as insufficient number of biopsies were taken at the 4h time points). Total numbers of samples within each group is shown at the bottom of Table 2-1.

Patients	Un-irradiated non-lesional	Un-irradiated lesional	311nm		290nm	
			4h	18h	4h	18h
1	✓	✓		✓		✓
2	✓	✓		✓		✓
3	✓	✓		✓		✓
4		✓	✓	✓	✓	
5		✓	✓		✓	✓
6		✓	✓	✓	✓	
Overall	3	11	9	11	6	8

Table 2-1. Patients' biopsies for gene array studies. Each patient had a maximum of four biopsies taken, at least three of which were from lesional psoriatic skin. The above design allowed at least three samples to be collected for each time-point and wavelength from the initial six patients (24 samples). A further 24 samples were later analysed, and the total number of samples for each group is expressed.

Frozen tissue was mounted in OCT and kept at -80^{0}C as previously described. Three to four 35µm sections were cut using a Leica CM 1900 cryostat, and collected onto glass PEN membrane slides (Leica Microsystems, Milton Keynes, UK). The slides were kept at -20^{0}C in the cryostat during preparation, although local re-warming of a small area of the slide with a finger tip was necessary to allow adhesion of each section. Slides were stored at -80^{0}C until micro-dissection. For each array batch, all sections were processed on the same day for both epidermal micro-dissection and also RNA extraction, in order to minimise handling variation.

2.5.2. **Epidermal micro-dissection**

Slides were kept on dry ice prior to fixation, then dipped sequentially for one second into 50ml falcons containing ethanol at the following concentrations: 100%, 100%, 70%, 50%, then in 0.5% toluidine blue containing 6.1mM nitrotetrazolium blue, then ethanol again at 50%, 70%, 100% and 100%. All dilutions were made with double distilled autoclaved water. Slides were air dried briefly then the epidermis was micro-dissected using a scalpel (with a new sterile blade for each sample) on a dissection microscope, and placed in the cap of an inverted 0.5ml microcentrifuge tube containing 10µl extraction buffer (PicoPureTM RNA Isolation kit, Arcturus Biosciences, USA). Tubes were briefly centrifuged then incubated at 42^{0}C for 30 minutes, then stored at -80^{0}C until RNA extraction.

2.5.3. **RNA extraction**

Prior to RNA extraction 1µl of Polyinosinic Acid Potassium salt (Sigma, Poole, UK; 200ng/ml) was added to each cell extract, to reduce the quantity of RNA sticking to the microcentrifuge tube. The standard protocol supplied with the PicoPureTM isolation kit was followed as below:

- The RNA purification columns were preconditioned by pipetting 250µl conditioning buffer onto the purification column filter membrane, and incubated for five mins at room temperature. The columns were then centrifuged at 16 000x g for one min.
- 10µl of 70% ethanol was added to each RNA extract and mixed by pipetting up and down.
- RNA was bound by centrifuging for two mins at 100x g, immediately followed by centrifugation at 16 000x g for 30 secs to remove flow through.
- 100µl wash buffer 1 was pipetted into each purification column and centrifuged for one min at 8 000x g.
- 100µl wash buffer 2 was then pipetted into each purification column and centrifuged for one min at 8 000x g.
- A further 100µl wash buffer 2 was pipetted into each purification column and centrifuged for two mins at 16 000x g.
- The purification column was transferred to a new 0.5ml microcentrifuge tube, and 11µl elution buffer was pipetted directly onto the membrane of the purification column, and incubated for one min at room temperature.
- The columns were centrifuged for one min at 1000x g to distribute the buffer in the column, then for a further minute at 16 000x g to elute the RNA. The entire sample was then stored at -80^0C.

Quantity of eluted RNA was measured using a Nanodrop spectrophotometer, and RNA quality was checked using a Bioanalyser (Agilent technologies). Total RNA required for each sample was 10-100ng.

2.5.4. **Array processing**

Samples were processed using Illumina cDNA-mediated Annealing, Selection, Extension, and Ligation (DASL) whole genome array technology. This permits RNA profiling of more than 24 000 transcripts per sample using partially degraded RNA, allowing frozen tissue sections to be analysed with an improved sensitivity. This assay begins by converting RNA to cDNA using random primers and annealing the biotinylated cDNA to the DASL assay pool, which consists of small (50 base-pair) probes specifically designed to the target within the transcripts to be investigated. Oligos are then extended and amplified by PCR before being deployed on a HumanRef-8 BeadChip. The latter allows eight samples to be processed in parallel, therefore reducing experimental variation. These BeadChips have been shown to have high concordance and reproducibility (r^2>0.86) between hybridisation replicates, with each BeadChip having a 30-fold redundancy to increase accuracy (http://www.illumina.com/technology/whole_genome_dasl_assay.ilmn). The assays were performed on two separate occasions as described as above, using Aros Technologies, Denmark (http://www.arosab.com/inhouse.htm) and Cambridge Genomic Services, UK (http://www.cgs.path.cam.ac.uk/), following the steps outlined below (Figure 2-9).

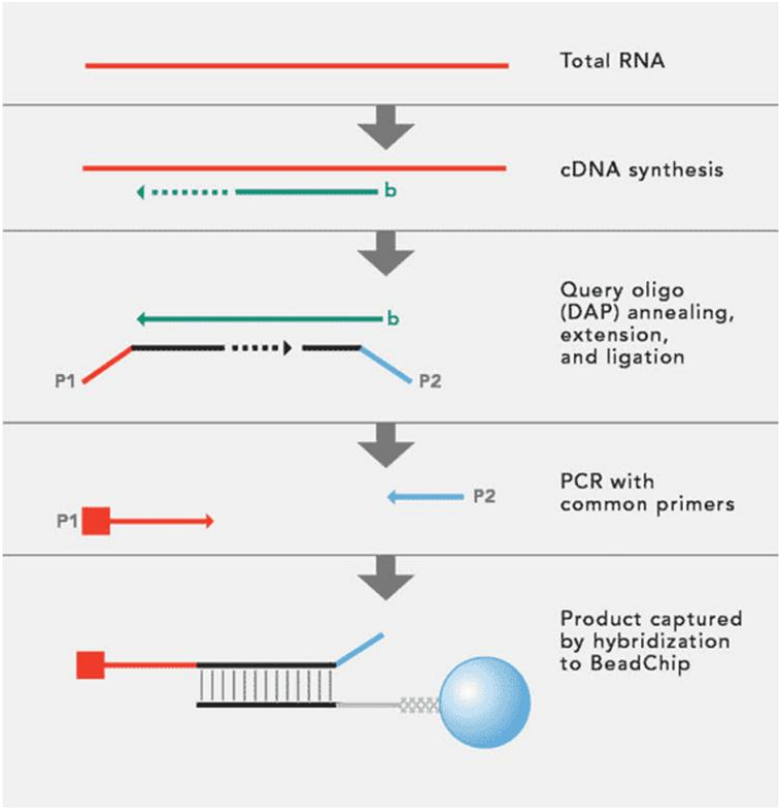


Figure 2-9. The sequence of events for RNA profiling with the whole-genome DASL assay. The above is performed over a three day period; firstly involving synthesis and PCR amplification of cDNA, then binding of the PCR product and hybridisation to the BeadChip, and finally washing and analysis of the array. Diagram modified from the Illumina website (http://www.illumina.com/technology/whole_genome_dasl_assay.ilmn).

2.5.5. Array analysis

Expression values were derived from importing raw data into GeneSpring GX 11.0 (Agilent technologies). Expression values were log transformed (log base 2) and normalised using quantile normalisation with a baseline transformation to the median of all samples. Batch variability between the two sets of microarray data generated were removed using the empirical Bayes method implemented in Combat.R (Johnson, Li et al. 2007) prior to analysis. Differential expression between groups was performed using the RankProducts package (Breitling, Armengaud et al. 2004) in BioConductor (Gentleman, Carey et al.

2004). Genes considered to be significantly different between groups were selected with a p-value of <0.05 after 100 permutations. GeneSpring GX software allowed visualisation of the median and IQR for differentially regulated genes and further assesses the likely relevance of the observed fold changes. Further analysis was performed using Ingenuity Pathway Analysis (IPA) 8.7 software and comparisons of gene expression were made within the following groups of psoriatic skin:

1. Untreated lesional v untreated non-lesional
2. Untreated lesional vs 4h post 311nm irradiation
3. Untreated lesional vs 18h post 311nm irradiation
4. Untreated lesional vs 4h post 290nm irradiation
5. Untreated lesional vs 18h post 290nm irradiation
6. 4h post irradiation with 311nm vs 290 UVB
7. 18h post irradiation with 311nm vs 290 UVB
8. Irradiation with 311nm after 4h v 18h
9. Irradiation with 290nm after 4h v 18h

IPA allows modelling and analysis of gene-to-gene interactions by creating pathways which link genes by identifying relationships using mechanisms, functions and relevant connections e.g. upstream/ downstream relationships. This allows the build up of novel connections which can identify gene pathways that may be relevant to the system under investigation. Each of the above datasets underwent a functional (biological) analysis to assess the most significant biological functions (with a p-value cut-off of 0.05). These networks typically contain molecules involved in several pathways and do not have directionality, but do show known interactions between the different genes of interest. Canonical (classical activation) pathways were also investigated. These may be either metabolic or signalling pathways, do have directionality, and are not changed upon data input (i.e. based on the literature rather than generated in response to inputted data). These canonical pathways are derived from journal articles, review articles and text books and KEGG Ligand.

To investigate which relevant genes may be differentially regulated by 311nm and not 290nm UVB, the function of the top 200 genes was manually checked using pubmed and Entrez Gene, and canonical and biological pathways were created using all significantly expressed genes to find associations. Furthermore, possible interactions of genes of interest were examined by exploring known connections and pathways between these using the My Pathway function in IPA.

2.6. Real-time PCR

Real-time PCR analysis was used to confirm the gene array data. Five of the most highly differentially regulated genes identified from the array analysis were selected for PCR. The chosen genes represented significant up and down regulation of genes following irradiation with either 311nm or 290nm UVB, some reaching significance at early time-point (4h and 18h) post irradiation. These genes were CDKN1A, DEFB103A, FOSL1, GDF15, and KRT77 with GAPDH as a loading control; their sequences are shown in Table 2-2.

Probe	Sequence	Amplicon Length
CDKN1A	GCAGACCAGCATGACAGATTTCTAC	66
DEFB103A	TGGTGCCTGTTCCAGGTCATGGAGG	93
FOSL1	CAGCCCAGCAGAAGTTCCACCTGGT	100
GDF15	CGCCAGAAGTGCGGCTGGGATCCGG	78
KRT77	GACTACAAGAGCAAGTATGAGGATG	101
GAPDH	CGCCTGGTCACCAGGGCTGCTTTTA	122

Table 2-2. Probes used in PCR, showing amino-acid sequence and amplicon length.

2.6.1. **CDNA preparation**

cDNA was initially prepared from RNA using a Superscript^{TM} III first-strand synthesis system for RT-PCR, based on the standard protocol shown in Table 2-3, and was then stored at -20^{0}C until real-time PCR analysis

Prior to PCR all cDNA was quantified using a Quant-IT ssDNA kit (Invitrogen). cDNA was then diluted in water to a standard concentration of 10ng/µl.

Summary of Procedure	Reagents	Amount per reaction (µl)	Temp (^{0}C)	Time (mins)
Denature	RNA 50ng/µl random hexamers dNTP DEPC-treated water	5 (max) 1 1 to 10	65	5
			4	1
Anneal	To RNA/ primer mix add.. 10x RT buffer 25nM $MgCl_2$ 0.1M DTT RNaseOUT^{TM} (40 U/µ) SuperScript^{TM} III RT (200 u/µl)	 2 4 2 1 1	25	10
cDNA Synthesis			50	50
Terminate Reaction			85	5
Remove RNA	RNase H	1	37	20

Table 2-3. Summary of cDNA preparation prior to PCR.

2.6.2. **Real time PCR processing**

TaqMan® Gene Expression Assay probes were purchased from Applied Biosystems using probes which span an exon junction and do not detect genomic DNA. A reporter dye (FAM^{TM}) is linked to the 5' end of the probe and binds to DNA allowing quantification in the PCR reaction. A minor grove binder (MGB) is attached to the 3' end of the probe and allows an increase in melting temperature without increasing the probe length. A non-fluorescent quencher dye is also attached to the probe; cleavage by DNA polymerase separates the reporter and quencher dyes leading to increased fluorescence by the reporter, which only occurs if the target sequence is complementary to the probe. The genes shown in Table 2-4 were selected for PCR analysis, and GAPDH used as a reference gene for "loading control". cDNA from 4 different patients was examined (where obtained quantities of cDNA were sufficient) for each wavelength and time-point, and all experiments were done in duplicate. Negative control wells (containing no DNA) were used for each probe to check for contamination.

A gene expression assay mix was made for each probe by combining the following:

1. 20x probe mix (1µl per reaction)
2. 2x Taqman universal mastermix (1µl per reaction)
3. H_20 (4µl per reaction)

For each reaction a 15µl aliquot of the assay mix was added to well in a 96 well plate, with 5µl of the prepared cDNA, making a total volume of 20µl per well. Standard PCR cycle conditions were run as follows using a Chromo4 qPCR continuous fluorescence detector:

1. 50^0C for 2 minutes
2. 95^0C for 10 minutes
3. 95^0C for 15 seconds
4. 60^0C for 60 seconds
5. Plate read

6. Repeat steps 3-5 x40 times
7. Incubate at 10^0C for 10 minutes

Where heating DNA to 95^0C denatures the DNA allowing the two strands to separate and cooling to 60^0C allows primers to anneal to complementary strands. The process repeats 40 times, and at each repeat the primer is used allowing with the short copy made in the previous rounds of synthesis allowing exponential amplification. The reaction will typically plateau around 31-35 cycles giving over $1.5x10^9$ copies of the amplified DNA.

Gene	**Samples examined**			**Expression change to be demonstrated**
CDKN1A	Un-irradiated psoriasis	311nm at 4h	290nm at 4h	Increased with 311nm only at 4h
DEFB103A	Un-irradiated psoriasis	311nm at 18h	290nm at 18h	Increased with 311nm only at 18h
FOSL1	Un-irradiated psoriasis	311nm at 4h	290nm at 4h	Increased with 311nm only at 4h and 18h
GDF15	Un-irradiated psoriasis	311nm at 4h	311nm at 18h	Increased with 311nm only at 4h and 18h
KRT77	Un-irradiated psoriasis	311nm at 4h	290nm at 4h	Decreased with 290nm only at 4h
GAPDH	All samples used for the above			Baseline reading

Table 2-4. Taqman® gene expression assay probes used to validate the gene array assay. The probes were chosen to demonstrate expression changes relevant to apoptosis and proliferation effects following irradiation with 311nm and 290nm UVB compared to un-irradiated psoriasis.

A Ct (cycle threshold) value was measured for each reaction, and GADPH was used to calculate a fold change correction for each sample. The Delta-Delta Ct method was used for analysis (i.e. the difference between the two Ct values), after correction of the loading control (Livak and Schmittgen 2001). This difference was expressed as a fold change (equivalent to $2^{\text{Delta-Delta Ct}}$).

2.7. Statistical analysis

Means and standard error of the mean were used to describe normally distributed data, and medians and inter-quartile ranges used for non-normally distributed data.

The repeatability of measurements of apoptotic cells was calculated using Measurement error (intra-patient standard deviation). This test requires transformation of the data so that the inter-patient variances are unrelated to the magnitude of the measurement. The difference between the biopsies measurement and true value is expected to be 1.96 x sw (square root of the variance) 95% of the time. This is used to calculate the acceptable range in which the intra-patient variation should lie if intra-patient variation < inter-patient variation. Significance was determined at a level of $p<0.05$. Further explanation has been published by Bland (Bland 1996).

Paired data was analysed with a paired T-test, and non-parametric paired data analysed using Mood's median. Mann Whitney-U tests were used to compare non-parametric data such as the number of apoptotic cells induced by 311 and 290nm, and a one-way ANOVA was used to compare differences between groups with two or more factors. Ordinal logistic regression was used to analyse demographic associations in section 3.2.1. All calculations have been carried out in Minitab statistical software v15.

3. Effects of 311nm and 290nm UVB on psoriasis *in vivo*

3.1. Introduction

To distinguish which cellular effects may be important in plaque clearance, the *in vivo* effects of 311nm irradiation were compared to another UVB wavelength (290nm) which is known to be clinically ineffective in clearing plaques, even at doses of up to 28 times the MED (Parrish and Jaenicke 1981). Although clinically ineffective for psoriasis, 290nm UVB causes erythema of the skin, allowing equal erythemogenic doses of the wavelengths to be compared. Biopsies of treated lesional skin at time-points between 2h and 48h post irradiation allowed investigation of any cellular changes in the psoriatic epidermis following each wavelength; thereby allowing identification of specific effects which may be relevant to plaque clearance.

Psoriatic keratinocytes have been reported to be relatively resistant to apoptosis *in vitro* (Wrone-Smith, Mitra et al. 1997), and perhaps as a result, keratinocyte apoptosis has not been studied as a possible mechanism of plaque clearance in psoriasis. Apoptosis of T cells has been proposed as a mechanism of plaque clearance, although this was based on results obtained using TUNEL (Ozawa, Ferenczi et al. 1999) which is known to show false positives in psoriasis (Wrone-Smith, Mitra et al. 1997), circumstantial evidence that T cells deplete during plaque clearance (Krueger, Wolfe et al. 1995; Ozawa, Ferenczi et al. 1999), and observation that direct irradiation of peripheral T cells instigates their apoptosis (Novak, Berces et al. 2004). However, this chapter clearly demonstrates that significant keratinocyte apoptosis occurs in response to the clinically effective wavelength (311nm) but not to the ineffective wavelength (290nm), at doses which are relevant to clinical practice.

3.2. Aims

- Identify the apoptotic cell types within psoriatic epidermis following irradiation with clinically effective UVB (311nm)
- Determine an apoptotic time-course following irradiation with 290nm and 311nm UVB in psoriatic epidermis *in vivo*
- Measure the apoptotic effect of increasing doses of 311nm UVB on psoriatic epidermis
- Assess whether epidermal apoptosis occurs in psoriatic epidermis during a routine phototherapy course
- Examine the differential apoptotic response following UVB irradiation within individual plaques of the same patients
- Measure the differential effect of 290nm and 311nm UVB on epidermal T cells and proliferation markers *in vivo*
- Correlate overall PASI improvement with maximal epidermal apoptotic levels

3.3. Results

3.3.1. Patient demographics

Eighty four patients were recruited overall (54 males and 30 females), with one patient dropping out prior to the start of the study due to transport problems. The majority of patients who were asked to join the study consented to do so, and for those who did not, the reasons given were almost always either not having the time to attend for extra appointments or not wanting biopsies. No patients had any symptomatic burning due to UVB administered for the study, although one patient went on to develop repeated burning during his routine course of treatment, resulting in the course being discontinued. Two patients developed infection in a biopsy site out of a total of 322 biopsies overall, but these settled quickly with antibiotics. No other complications occurred. All patients met the study entry criteria described in section 2.1.1, and details of their demographic data are given in Table 3-1.

Patient characteristics	**Number at baseline**	**N**
Mean age and SD (years)	45± 15	83
Median disease activity (PASI) with IQR	9 (7-13)	68
Number with joint involvement	53 (75%)	71
Number with nail involvement	45 (63%)	71
Number of patients with onset of psoriasis below 40 yrs	66 (80%)	81
Median 311nm MED with range (mJ/cm^2)	459 (172-1459)*	83
Median 290nm MED with range (mJ/cm^2)	12.6 (5-50.4)*	36

Table 3-1. Baseline demographic data and clinical characteristics of patients. Key: SD= standard deviation; IQR= inter-quartile range; N= number of patients with details recorded. * Refers to un-weighted dose (equates to three MEDs at each wavelength).

Recruited patients were Fitzpatrick skin type 1-5, with the following number of patients in each group (Table 3-2).

Skin type	Definition	Number of patients
1	Burns easily, doesn't tan	6 (7%)
2	Usually burns, sometimes tans	39 (48%)
3	Sometimes burns, usually tans	26 (32%)
4	Rarely burns, tans easily	10 (12%)
5	Naturally pigmented skin, tans easily	1 (1%)

Table 3-2. Recruited patients according to their Fitzpatrick skin type. The majority of patients were skin type 2 and 3.

Ordinal multiple regression analysis showed no association between the number of apoptotic cells from each patient and their age (according to decade), sex, skin type, age of onset of psoriasis or nail/ joint involvement ($p=0.667$). Patients given a larger dose of UVB due to higher MEDs had a positive trend towards a greater yield of apoptotic cells ($p<0.01$), although results for individual patients were variable (Figure 3-1).

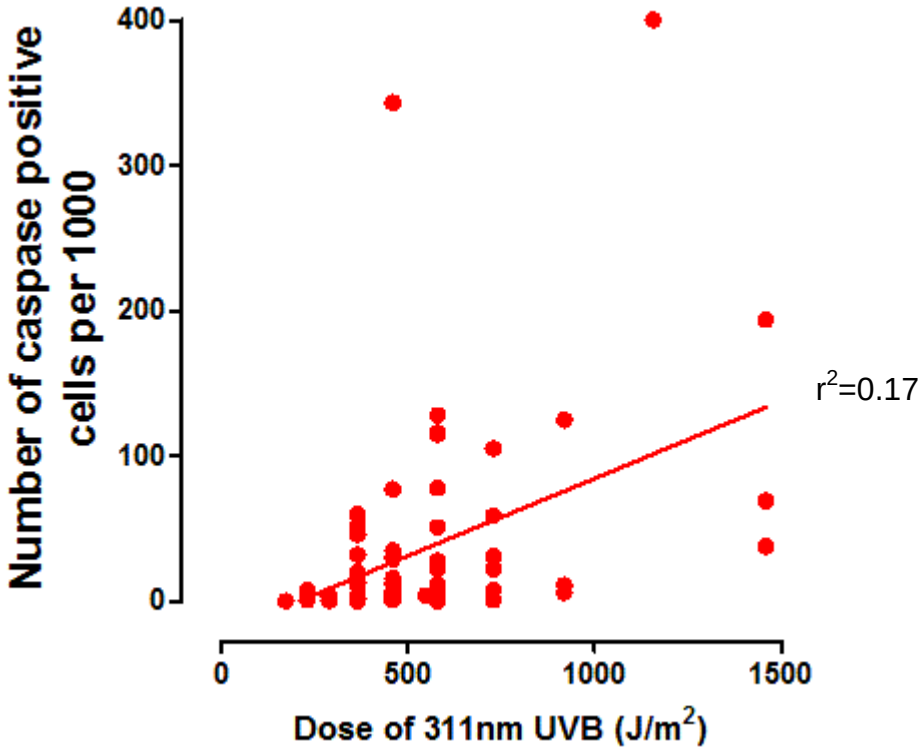


Figure 3-1. Relationship between the actual dose of UVB applied to an individual plaque (three MEDs) and the resultant number of apoptotic cells 24h post irradiation. Note that this data is skewed by a few outliers resulting in a low r^2 value, although there is a positive association even when these are excluded ($p<0.05$, $n=73$). There was no significant effect of dose on apoptotic response at lower doses (below 900 J/m^2); $p=0.2$, $n=66$).

3.3.2. Induction of apoptosis with 311nm but not 290nm UVB in psoriatic epidermis

Apoptotic cells were identified by positive anti-active caspase-3 expression, and were predominantly located in the lower epidermis. Dermal apoptotic cells were rarely seen. Equal erythemogenic doses of UVB which is clinically effective in clearing psoriasis (311nm) and UVB which is clinically ineffective (290nm) were compared, but only 311nm caused a significant apoptotic response in psoriatic epidermis *in vivo* (Figure 3-2).

As expected, the background number of apoptotic cells in un-irradiated lesional psoriatic skin (n=29) was very low (median 0.1 cells per 1000 nucleated epidermal cells), consistent with previous literature (Laporte, Galand et al. 2000). In contrast, a significant increase in the number of apoptotic cells were seen in lesional epidermal cells between 16h and 48h following irradiation with equally erythemogenic (three MED) doses of 311nm but not 290nm UVB ($P<0.001$) as shown in Figure 3-2 and Figure 3-3.

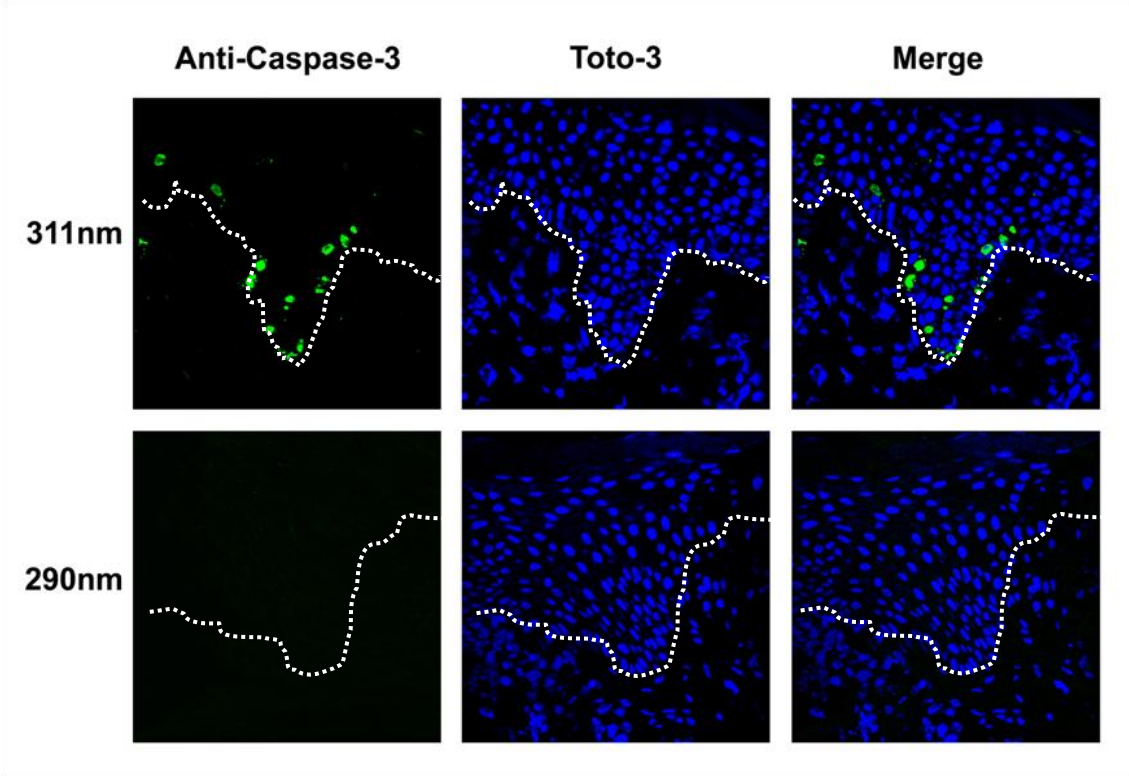


Figure 3-2. Representative confocal image of immunostained lesional psoriatic epidermis 18h post *in vivo* irradiation. Anti-active caspase-3 (apoptosis) is stained green and Toto-3 (nuclear dye) is blue. Dotted line shows the basement membrane.

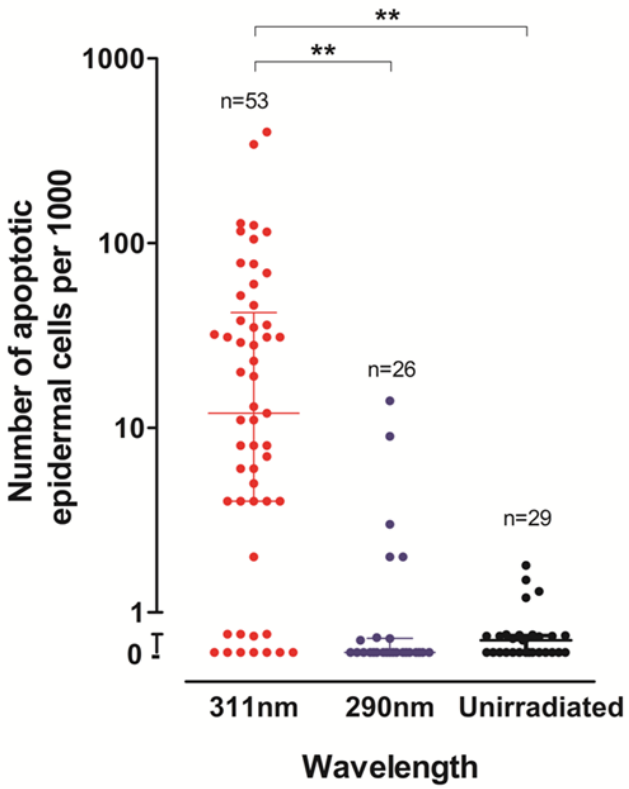


Figure 3-3. Significant apoptosis following irradiation of psoriatic plaques with 311nm UVB *in vivo*. Number of anti-active caspase-3 (apoptotic) cells within lesional psoriatic epidermis seen 16-48h following irradiation with either 311nm or 290nm UVB compared with un-irradiated psoriasis. Lines show medians (12/1000 and 0/1000 epidermal cells for 311nm and 290nm UVB respectively) and inter-quartile range. **P<0.01.

3.3.3. Identification of apoptotic cell type

To identify which epidermal cell type was undergoing apoptosis, a minimum of 100 apoptotic cells from at least five different patients 24h post irradiation with 311nm were triple labelled with the following monoclonal antibodies: anti-CD3 (a pan T cell marker), Langerin (specific for Langerhans' cells), Melan A (specific for melanocytes) and CD68 (a marker of monocytes/ macrophages) (Figure 3-4). To identify keratinocytes, the pan keratinocyte marker AE1-AE3 was combined with CK14 to improve previously suboptimal basal layer staining

(Figure 3-5). The majority of apoptotic cells colocalised with keratinocyte markers (94.5%), with occasional apoptotic T cells (0.38%) and CD68 positive cells (1.7%). Apoptotic melanocytes and Langerhans' cells were not observed (Table 3-3). Apoptotic keratinocytes were confirmed by transmission electron microscopy (n=3) in biopsies taken 24h post irradiation with 311nm but was not seen following irradiation with 290nm UVB (Figure 3-6).

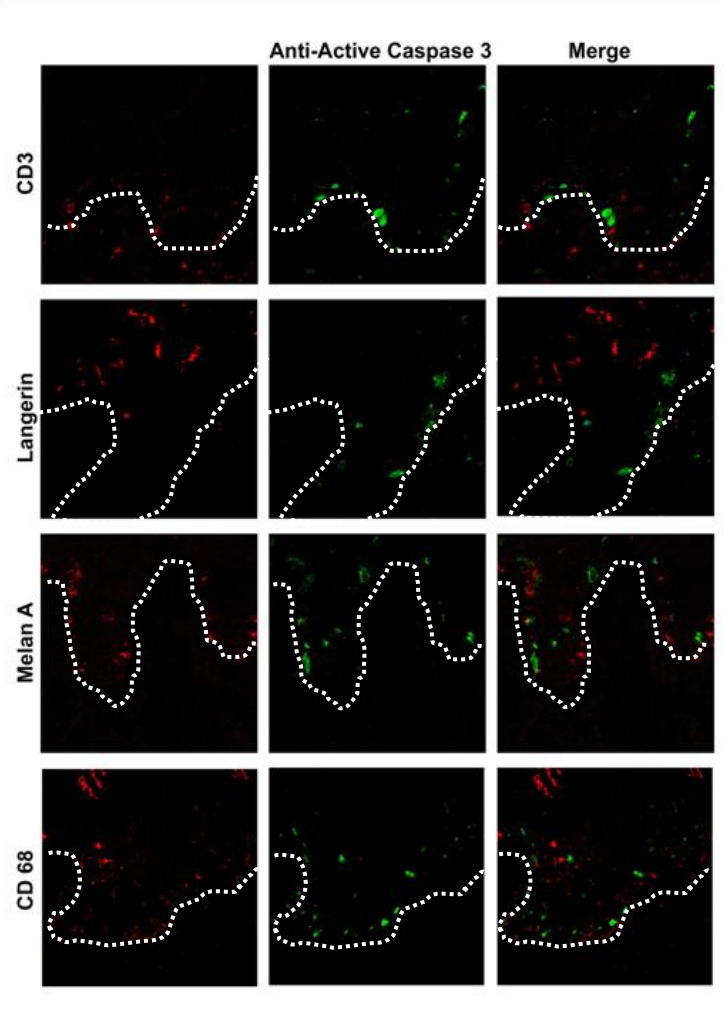


Figure 3-4.Colocalisation of apoptotic cells within lesional psoriatic epidermis with markers specific for epidermal cell types 24h post irradiation with 311nm UVB. Mid Z-section of a confocal image showing colocalisation of apoptotic cells (green) with CD3 is a marker for T cells, Langerin for Langerhans' cells, Melan A for melanocytes and CD68 for monocyte/ macrophages. Dotted lines represents the basement membrane.

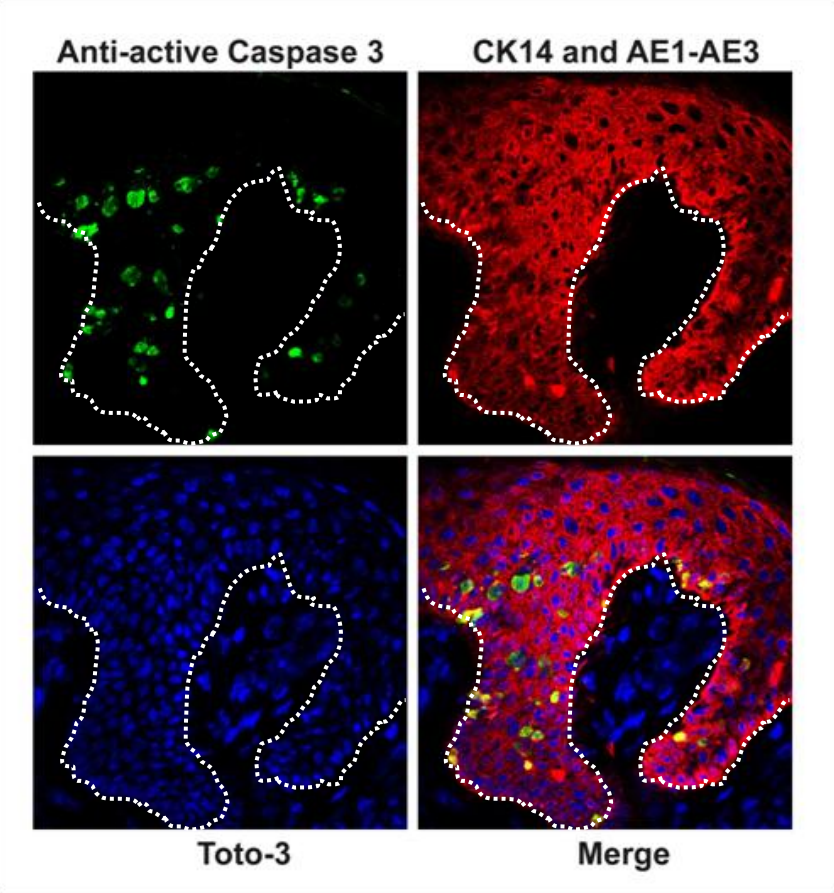


Figure 3-5. Colocalisation of apoptotic cells within lesional psoriatic epidermis with markers specific for epidermal cell types 24h post irradiation with 311nm UVB. Mid Z-section of a confocal image showing colocalisation of apoptotic cells (green) with keratinocyte markers and other epidermal cell types (red).

Epidermal cell type	Colocalisation coefficient with anti-active caspase-3
Keratinocytes	0.945
T cells	0.0038
Melanocytes	0
Langerhans' cells	0
Monocytes/ macrophages	0.017

Table 3-3. Table summarising the colocalisation coefficients for apoptotic cells and the specific epidermal cell markers. Note that a small number of cells (3.4%) could not be identified by colocalisation. These cells were presumed to be a very late stage of apoptosis, and had lost their surface markers.

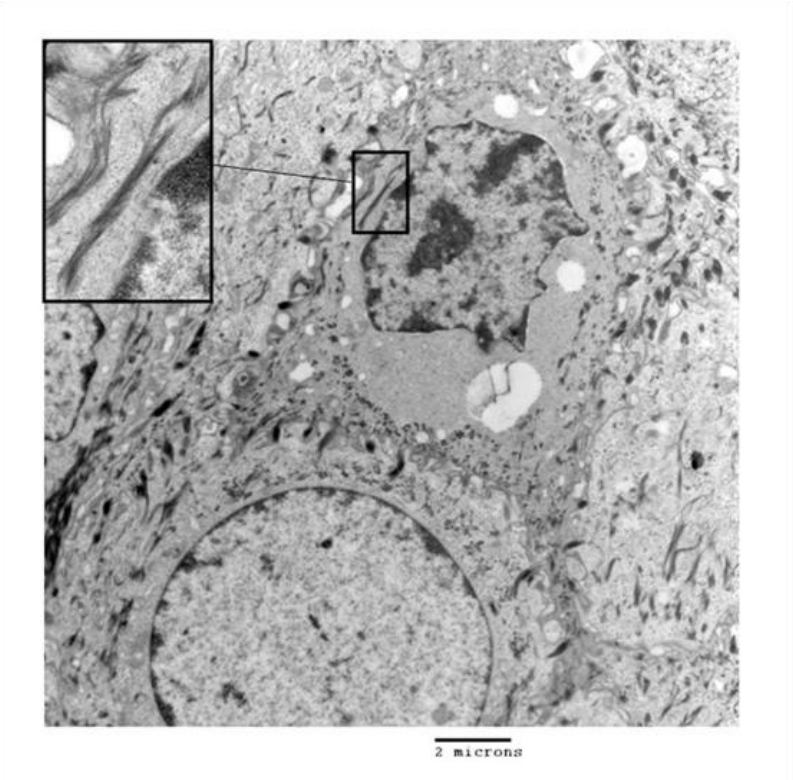


Figure 3-6. Transmission electron micrograph showing an apoptotic keratinocyte 24h post irradiation with 311nm UVB in psoriasis. Insert shows keratin filaments. Desmosomal junctions between the cells were also seen, confirming cells are keratinocytes.

3.3.4. **Time-course of apoptosis following UVB irradiation**

Time-course studies showed that the peak time of epidermal apoptosis was 16-24h post irradiation with 311nm UVB in psoriasis *in vivo*, although apoptosis was detected in significantly increased levels from 8h post irradiation. Low levels of apoptosis occurred following irradiation with equal (three MEDs) doses of 290nm, but this was not significantly different to un-irradiated psoriasis. Furthermore, a comparison was made between 311nm and 290nm UVB, and did not show a differential time-course of apoptosis between the two wavelengths (Figure 3-7), and at no time points were significantly increased numbers of apoptotic cells seen following 290nm UVB irradiation.

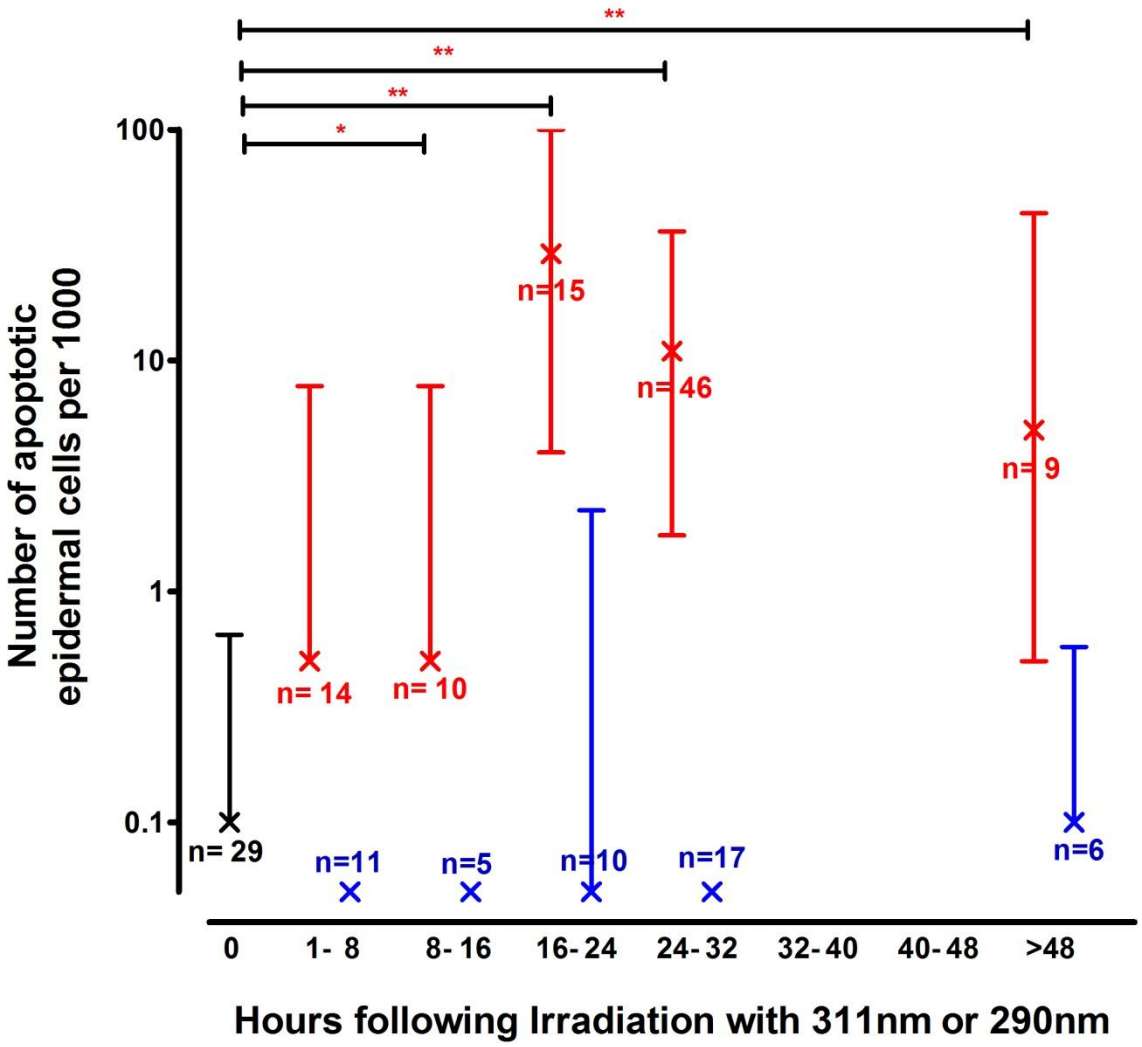


Figure 3-7. Time-course showing number of apoptotic cells in lesional psoriatic epidermis post UVB. Median number of apoptotic cells per 1000 epidermal nucleated cells (crosses), and inter-quartile ranges shown. Red bars represent irradiation with 311nm UVB, blue represents 290nm UVB (note median and inter-quartile range is zero at time-points 0-8h, 8-16h and 24-32h) and black bar is un-irradiated psoriatic epidermis. Unweighted doses given at different time-points were similar (p=0.75, median 1377mj/cm^2 for 311nm and p=0.83, median 37.8mj/cm^2 for 290nm UVB). Significant differences were shown between un-irradiated psoriasis and at the time-points identified, where * p<0.05, ** p<0.01 following irradiation with 311nm UVB only.

Apoptotic cells were predominantly located in the basal and suprabasal epidermis consistent with an effect on the proliferative compartment. Despite the count of apoptotic cells declining after 24h, apoptotic cells still localised to

the lower epidermis rather than migrating up to the *stratum corneum* (Figure 3-8). This suggests that these cells are being phagocytosed within the lower epidermis, and that this process is complete within a matter of hours. Moreover, the number of apoptotic cells were counted in patients who had biopsies over several time points. Following the peak in apoptosis, the median time for number of apoptotic cells to declined by 50% was 2.14h (inter-quartile range 1.32-6.25; n=11), again suggesting that the cells were being removed within a few hours (Figure 3-9). Consequently, in order to show maximal apoptotic response, biopsies were taken at 18h-24h post UVB for the remaining parts of the study (with the exception of the biopsies for gene array as described in chapter seven).

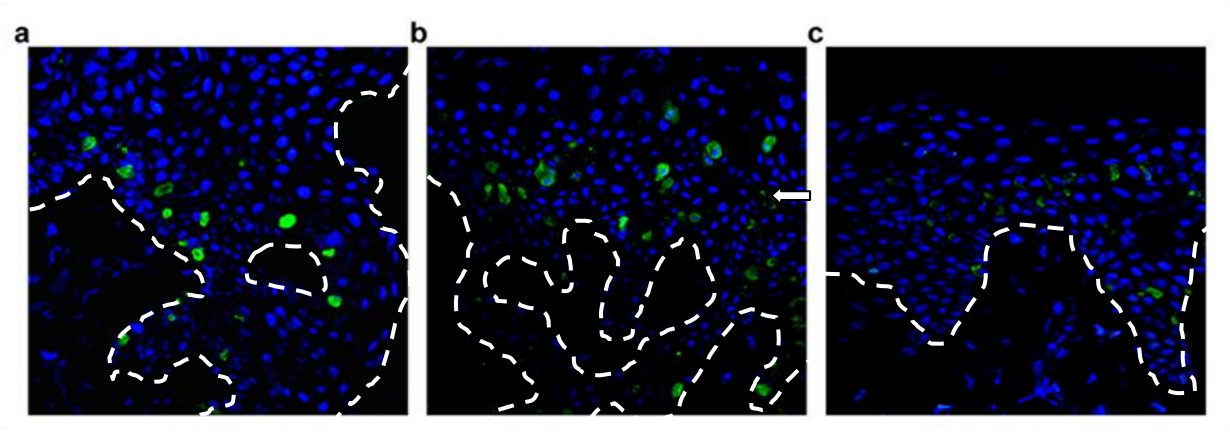


Figure 3-8. Apoptotic cells are located in the lower epidermis between 18h and 48h, without appearing to migrate into the upper epidermis. Apoptotic cells (green) are located in the basal and suprabasal layers at different time points. Figure shows three biopsies taken from a typical individual at three time points: a) 18h, b) 24h, and c) 48h post in vivo irradiation with 311nm. Toto-3 (blue) used as a nuclear dye, and basement membrane shown as white line. Arrow shows fragmented apoptotic cells.

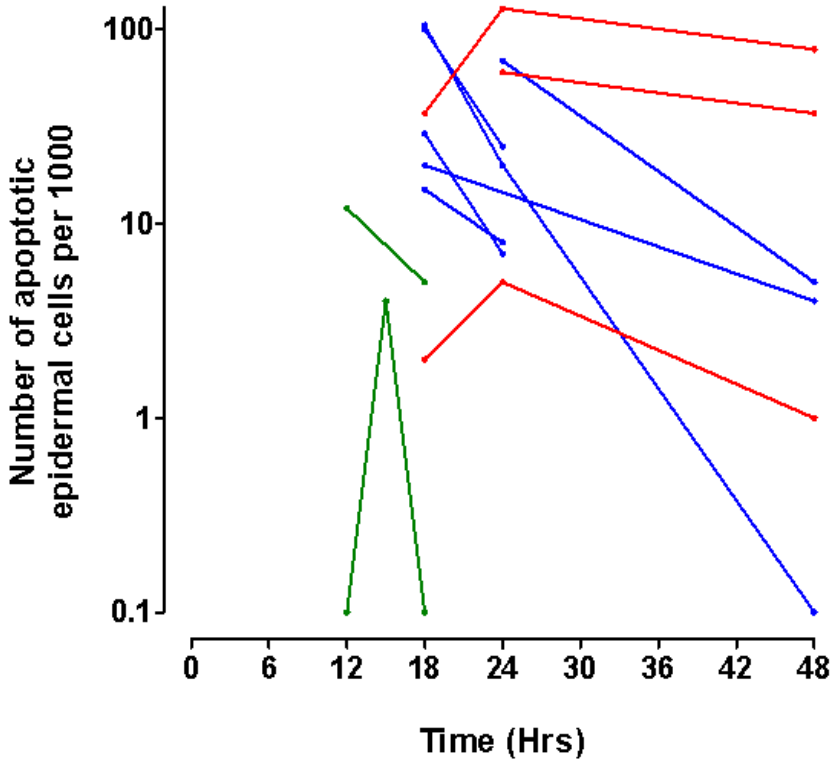


Figure 3-9. Individual's time-courses following irradiation with 311nm UVB in 11 patients. Timing of the peak number of apoptotic cells varied from less than 18h (green) to 24h (red) in different patients. Note the rapid decline in the number of apoptotic cells detected with time, consistent with these cells being removed from the *in vivo* epidermis within a matter of hours.

3.3.5. Apoptotic effect of UVB in routine treatment

Higher doses (three MEDs) of UVB have been used in this study to augment the effects of smaller clinical doses, which may otherwise be difficult to detect. To test whether the responses observed following erythemogenic doses of 311nm are representative of lower routine clinical doses, apoptosis was measured in matched plaques biopsied 24h following irradiation with single doses of 0.75, 1, 2 and 3 MEDs *in vivo* (Figure 3-10). As expected, 2-3 MEDs of 311nm induced a significantly higher rate of apoptosis than 0.75 and 1 MEDs

(p<0.001). Importantly though, apoptosis induced by 0.75 and 1 MED was significantly greater than un-irradiated psoriasis (p<0.001).

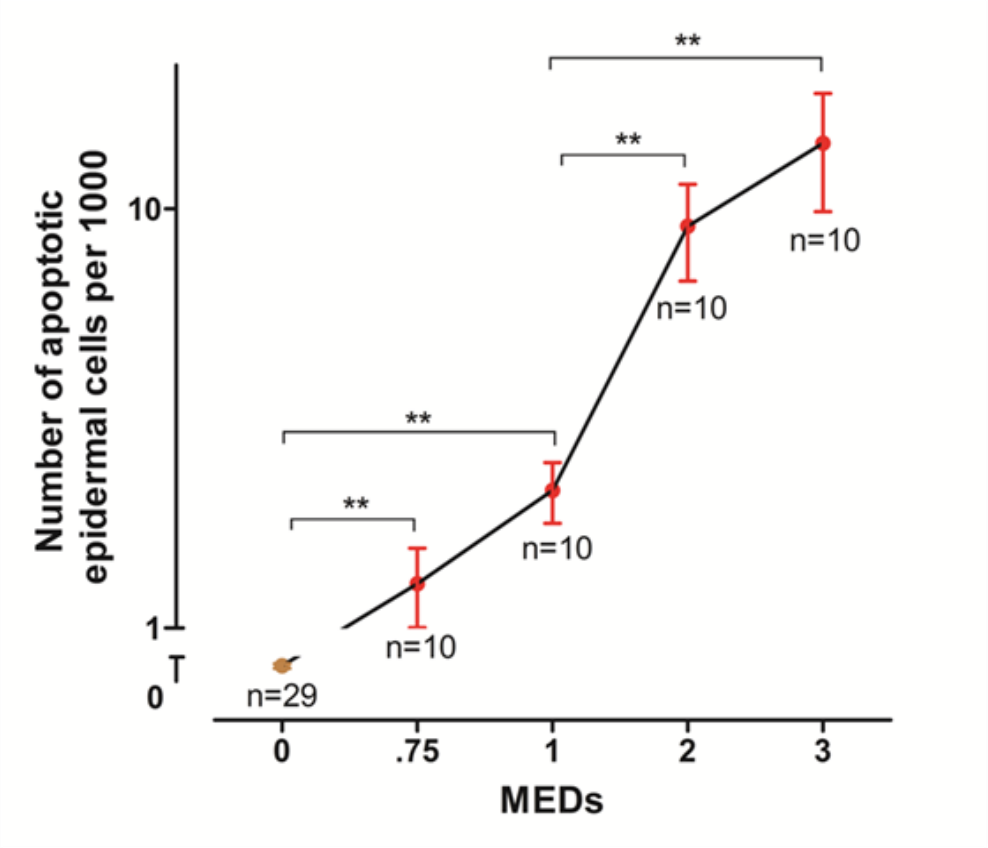


Figure 3-10. Number of apoptotic cells in lesional psoriatic epidermis in 10 patients 24h post 311nm UVB irradiation. Increasing apoptotic response is seen with increasing doses of UVB (means of 1.3, 2.1, 9.1 and 14.3 per 1000 epidermal cells respectively), which is significantly different to un-irradiated psoriasis at all doses. Mean and SEM shown. ** p<0.01.

Routine clinical treatment of psoriasis uses repeated sub-erythemogenic doses of UVB three times per week for approximately eight weeks. To confirm that epidermal apoptosis occurred during low dose repeated irradiation (as in clinical therapy), apoptotic cells were counted following biopsies of plaques taken 24h following the 4th, 8th and 12th routine treatment (n=5). Apoptosis

was significantly greater (mean of 2.5 cells per 1000 epidermal cells) compared to un-irradiated psoriasis (p=0.001; n=29) (Figure 3-11). Linear regression analysis did not show a significant correlation between the number of 311nm UVB-induced apoptotic cells within the epidermis and maximal plaque thickness (p=0.079, r^2=0.06, n=43; representing the patients with apoptosis detected 16-48h post irradiation) following three MEDs of 311nm UVB.

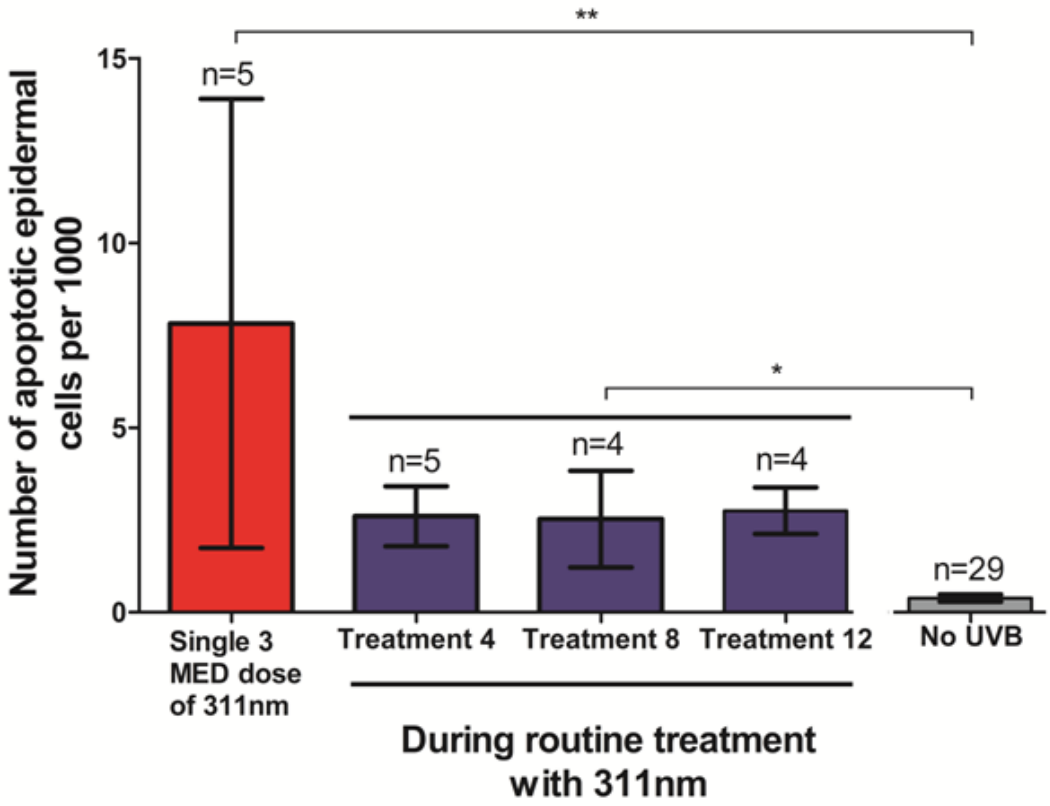


Figure 3-11. Apoptotic response in psoriatic plaques following routine clinical UVB treatment. A single three MED dose of 311nm UVB was administered to a psoriatic plaque in vivo and biopsied 24h later. This was compared to the apoptotic response seen 24h after the 4th, 8th and 12th dose during a routine treatment course in the same patients (n=5). One patient dropped out after the 4th treatment due to burning. Mean and standard error of the mean shown. Note the number of apoptotic cells induced by the three MED dose was not significantly different to values obtained during routine treatment. * P<0.05, **P<0.01.

3.3.6. **Effects of epidermal thickness on apoptotic response.**

As described in section 3.3.4, apoptotic cells were predominantly basal or supra-basal. The thickness of psoriatic epidermis is very variable both between patients and at different stages of the evolution of plaques, and this may impact on the proportion of UVB that penetrates down into the lower epidermis. The majority of apoptosis seen was observed to be in the rete ridges rather than adjacent to the dermal papillae, therefore the maximum epidermal thickness (including and excluding the *stratum corneum*) was measured in up to 12 areas per patient (three sites in up to four biopsies per patient), and the mean maximal thickness was correlated to the maximum number of apoptotic cells observed 18-24h post 311nm UVB irradiation; n=53. There was no correlation between plaques thickness and the number of apoptotic cells whether the *stratum corneum* was included in the measurement (p=0.0695) (Figure 3-12) or not (p=0.0748). Moreover, there was no correlation between overall PASI improvement during routine treatment and initial plaque thickness (p=0.795).

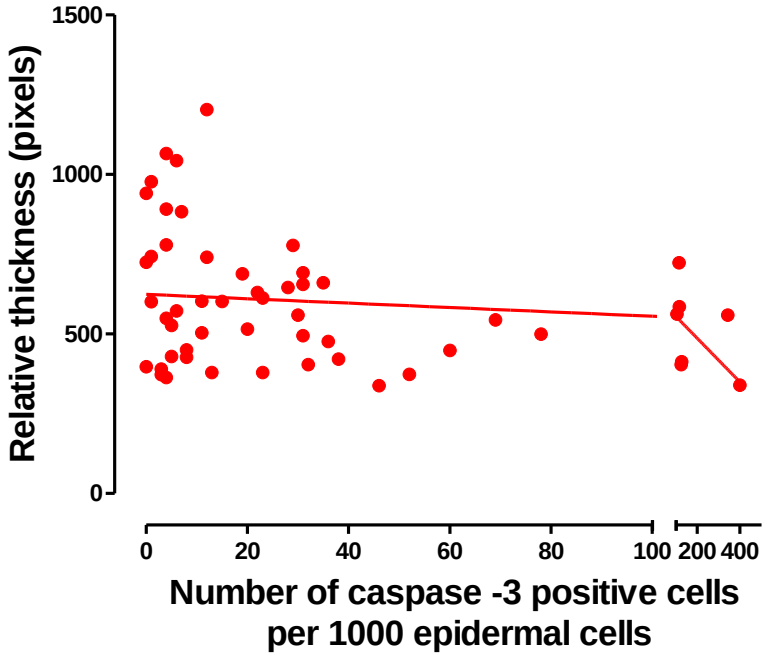


Figure 3-12. No significant correlation exists between the maximal epidermal thickness of psoriasis (including the stratum corneum) and apoptotic response 18-24h after 311nm UVB. Linear regression line shown. Note the split axis. n=53.

3.3.7. Intrapatient variation of apoptosis in psoriatic plaques

Throughout the study, biopsies were taken from the edge of psoriatic plaques, and where possible, small plaques were biopsied as there is evidence to suggest that plaques have a leading edge (Goodfield, Hull et al. 1994), which may behave differently in response to treatment. To test whether apoptosis in a single biopsy was representative of other similarly irradiated biopsies taken from the same patient, six patients had biopsies taken from different plaques on their lower back 24h post irradiation with three MEDs of 311nm UVB. A further biopsy was taken from un-irradiated psoriasis in each patient, as a control. No apoptosis was seen in untreated psoriasis except in one patient, who had 1.3 apoptotic cells per 1000 epidermal cells (patient 2) (Figure 3-13).

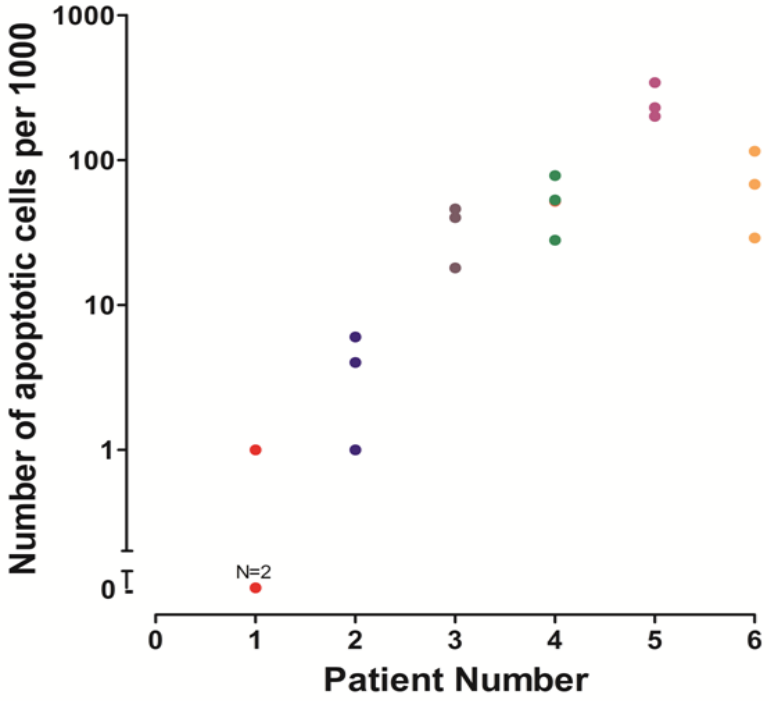


Figure 3-13. The number of apoptotic cells produced following a single three MED dose of 311nm UVB to lesional psoriatic skin *in vivo* shows intra-patient variability but is reproducible. Biopsies from different psoriatic plaques within individuals taken 24h post 311nm irradiation, a measurement error test showed significantly greater interpatient variation than intra-patient variation ($P<0.05$). Matched un-irradiated psoriatic plaques in each patient did not show any apoptosis, confirming no pre-existing apoptosis.

Measurement error testing was used to assess repeatability of the count of apoptosis in different biopsies from the same individual. As the counts included zero and had a range of variances, all values were log transformed using the equation y= log (x + 1). Repeatability is taken as 2.77 x square root of the mean variance (Bland 1996), and gave an overall value of 0.58; the difference between the three measurements from the same individual was within 0.58 in each case, suggesting that any variation in the number of apoptotic cells observed between biopsies from the same individual can be explained by the expected natural variation within the subject or processing error, rather than being a true difference between different plaques within the same individual. Therefore, a single biopsy can be assumed to be representative of the patient, and any interpatient variability in apoptosis count will be greater than any intra-patient variability.

3.3.8. **Effect of 311nm and 290nm on epidermal T cells**

To assess the effect of UVB on epidermal T cells, the count of T cells present in plaques irradiated with 290nm UVB were compared to those irradiated with 311nm UVB at time points from 4-48h. Patients with paired biopsies following irradiation with equal doses of 290nm and 311m taken at the same time-point were compared. The variation in number of T cells present ranged from 0.5- 21 per 1000 epidermal cells. A paired T test showed no significant trend towards reduced T cell numbers following 311nm UVB compared to 290nm UVB ($p=0.129$; $n=14$), and this is illustrated in Figure 3-14. When divided into time periods following irradiation the p values were 0.413, 0.787 and 0.179 for 4-12h, 18-24h and 48h respectively; therefore no significant difference occurred at any time point.

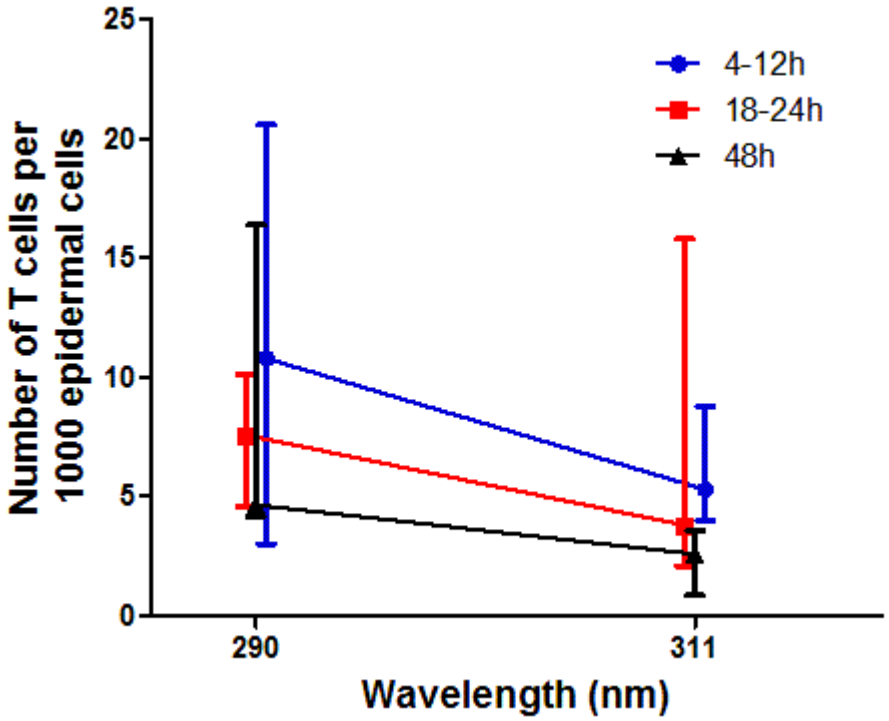


Figure 3-14. Change in epidermal T cell count at time-points between 4 and 48h post irradiation with 311 or 290nm UVB in paired plaques *in vivo*. Median and inter-quartile ranges shown. Blue line represents time points between 4-8h, red line 18-24h and black line is 48h. Overall, there was no significant difference in T cell numbers following either wavelength; p=0.129 (n=14).

Overall the average number of epidermal T cells in biopsies taken between 16h and 48h post UVB irradiation was 4.64 per 1000 epidermal cells, and the proportion of apoptotic T cells at these time-points comprised 0.38% of all epidermal apoptotic cells (Table 3-3). As the ratio of T cells: keratinocytes is 1:214 of all epidermal cells, and the ratio of apoptotic T cells: apoptotic keratinocytes is 1:249, this suggests that the proportion of T cells undergoing apoptosis is similar to that in keratinocytes. Therefore, no preferential apoptotic susceptibility has been demonstrated.

3.3.9. **Effect of 311nm and 290nm on proliferation markers**

UVB causes cell cycle arrest which may contribute to the therapeutic effects of UVB in the clearance of psoriatic plaques. To investigate any potentially significant effect on cell cycle, the numbers of epidermal Ki67+ cells were counted at time points between 4h and 48h following irradiation with 311nm or 290nm UVB in matched plaques within the same individuals (n=21).

A one-way ANOVA showed no significant difference in the number of Ki67+ cells observed following irradiation with 311nm or 290nm compared to un-irradiated lesional skin (p=0.091 and p=0.339 respectively), although a paired T test showed significantly higher Ki67+ counts were observed following irradiation with 290nm than 311nm UVB (p=0.026). However, when this was subdivided according to time following irradiation (Figure 3-15) there was no significant difference in the number of Ki67+ cells at any time-point following irradiation with 290nm and 311nm UVB at 4-12h (p=0.727, n=4), 18-24h (p=0.105, n=11) or 30-48h (p=0.131, n=6). The mean numbers of Ki67+ cells were 143, 162 and 111 per 1000 epidermal cells for un-irradiated, 290nm and 311nm UVB-irradiated lesional skin respectively.

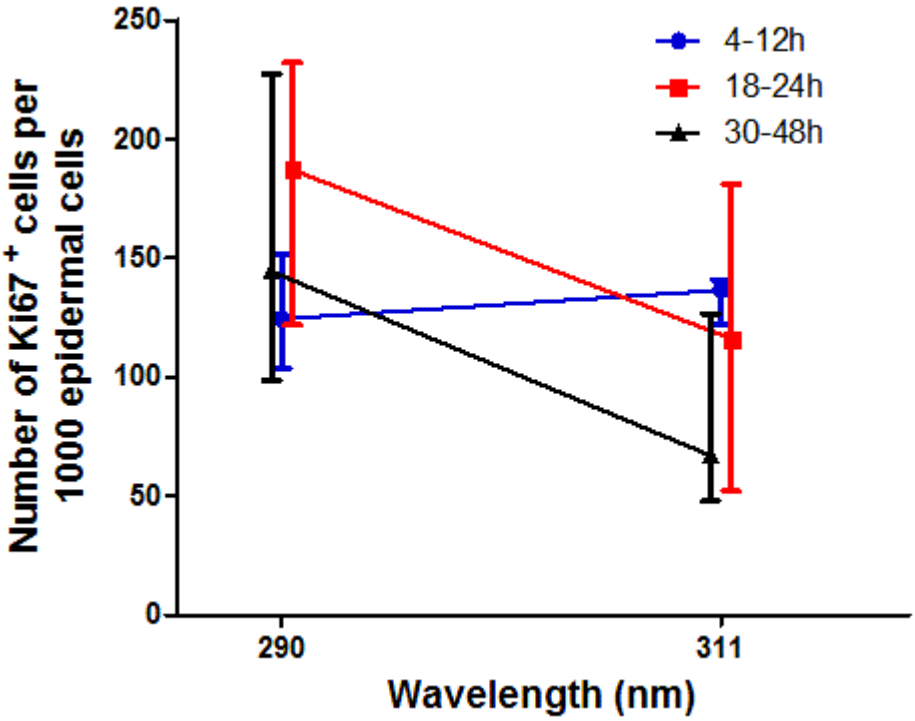


Figure 3-15. Change in epidermal Ki67+ cell count at time-points between 4 and 48h post irradiation with 311nm or 290nm UVB in paired plaques *in vivo*. Median and inter-quartile ranges shown. Blue line represents time points of 4-12h, red line 18-24h and black line 30-48h. Overall, higher numbers of Ki67+ cells were observed following irradiation with 290nm rather than 311nm (p=0.026, n=21), although this was not significant when subdivided according to time.

3.3.10. Correlation of PASI score to measured variables

Patients PASI scores were measured at baseline and at weekly intervals by a named nurse while attending for their routine treatments, and the median percentage PASI score improvement at the end of treatment was 95% (IQR 85-99); n=66. There was no correlation between baseline PASI score and apoptotic response 16-48h following irradiation with 311nm UVB (p=0.386). Overall, three patients did not achieve a PASI 50. In two cases the patients stopped attending treatment after two or six weeks of treatment respectively and in the third case the patient experienced a flare up during the treatment

course. PASI 75 was achieved by 56/66 (85%) patients, PASI 90 by 43/66 (65%) and total clearance by 18/66 (27%) patients. However, 26/66 (39%) of patients experienced a flare of their psoriasis during their phototherapy course, and 10/66 (15%) did not complete their course (defined as attending for less than six weeks of treatment but still improving until this point, or stopped within the first 2-3 weeks of treatment). Overall patients attended for a median of eight weeks in total (range 2-11 weeks). The best PASI score improvement obtained within a one week period was used to calculate patient's overall response to treatment, as shown in Figure 3-16.

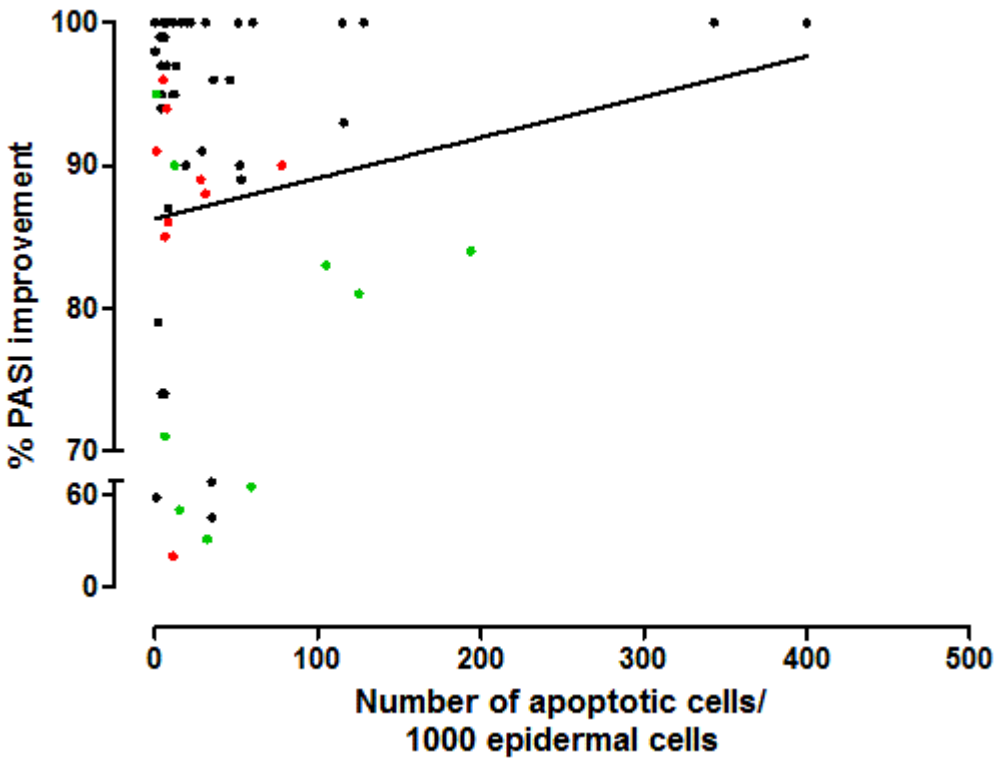


Figure 3-16. The observed number of apoptotic cells did not predict patients' overall improvement in PASI scores. Green dots represent patients who did not complete their prescribed treatment course, and red dots represent patients whose psoriasis flared up towards the end of their UVB treatment. Linear regression did not show a significant association; p=0.35 (line shown represents overall regression).

Linear regression analysis did not show any significant association between PASI improvement and caspase-3 levels (p=0.38), however, interpretation of this is hampered by the very low number of patients who did not achieve a PASI change of less than 50% overall. When analysed by weekly PASI improvement, it was observed that patients who exhibited rapid improvements in their PASI score during the first month of treatment (i.e. PASI reduced by 50% or greater within a one week period), were significantly more likely to have higher numbers of apoptotic cells (median of 25 per 1000 epidermal cells; n=30) compared to gradual responders (median of 7 per 1000 epidermal cells; n=27); p=0.013 (Figure 3-17).

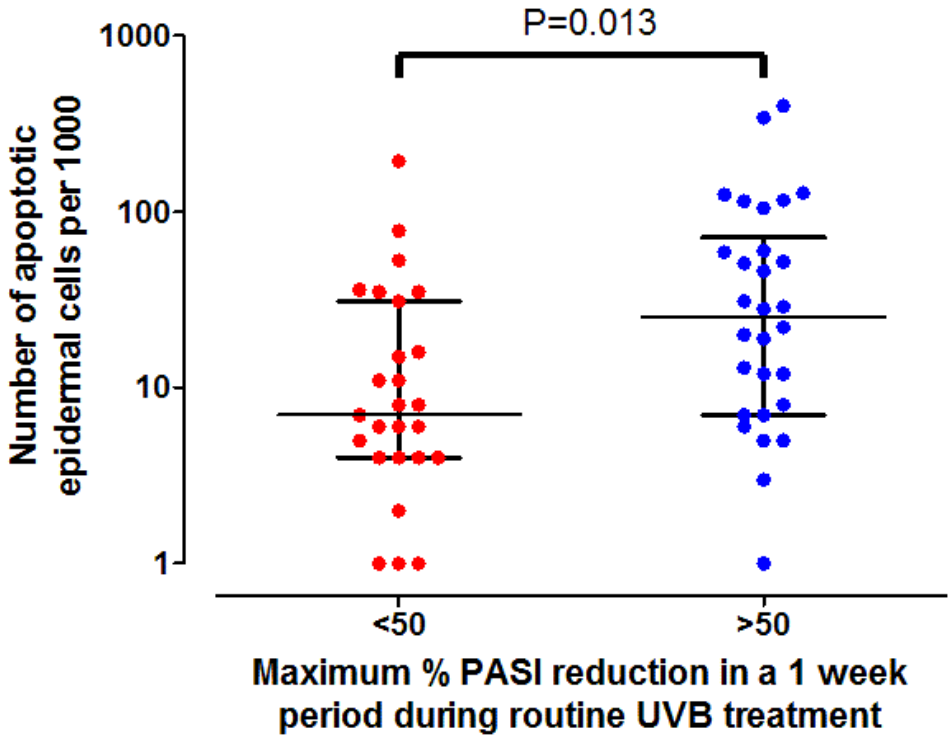


Figure 3-17. Maximum percentage improvement in PASI score occurring within a 1 week period during the first 4 weeks of routine UVB treatment. Patients with a PASI improvement of greater than 50% had a significantly higher number of apoptotic cells. Median and inter-quartile ranges shown. Mann Whitney U test was used for statistical analysis.

3.4. Discussion

UVB wavelengths of 300-313nm are highly effective at clearing psoriasis (Parrish and Jaenicke 1981), and phototherapy is one of the few treatments which can induce periods of complete remission of psoriasis after therapy has been withdrawn (Dawe, Wainwright et al. 1998). In this chapter the differential cellular effects of two wavelengths of UVB (311nm and 290nm) have been compared *in vivo*; both of which cause erythema of the skin, but only 311nm is clinically effective in clearing psoriasis. The results clearly show that 311nm induces keratinocyte apoptosis, and this is significantly different to the very low background levels seen in un-irradiated lesional psoriasis or psoriasis irradiated with 290nm UVB.

Of the 84 patients recruited to the study, nearly two thirds were male which is likely to reflect the ratio of men: women seen during the recruitment period. In this patient group 63% had nail involvement, which is slightly higher than the expected proportion of effected patients from published data (Reich 2009); 75% had joint involvement which is the upper limit of previously estimated levels of 30-73% of patients (Sadek, Abdel-Nasser et al. 2007; Reich 2009); and 80% had manifested psoriasis for the first time below the age of 40, which is broadly consistent with previous reports (Henseler and Christophers 1985). The majority of recruited patients were skin type 2 and 3 (70%), reflecting both the local population and the higher incidence of psoriasis amongst Caucasians. Patients were recruited sequentially into one of six groups within the study. Further details of how patients were subdivided are described in section 2.1. However, most patients had at least one biopsy 18-24h following irradiation with three MEDs of 311nm UVB, and where some overlap existed between groups, all relevant biopsy results were included in the analysis.

Each patient had a maximum of four biopsies taken in accordance with the ethical permission obtained for the study. This did cause some limitations for the study however, as it was not possible to follow the effects of UVB in detail over time. To define a time course, it would be preferable to biopsy non-irradiated lesional psoriasis, and then re-biopsy the same patient at regular

intervals over 48h; however, this would cause maximum disruption for patients (in particular as all patients in this study were outpatients), and even if biopsies were only taken every 6h, would require nine biopsies from each patient. Therefore the results taken from all patients were pooled, but this in itself causes problems. Figure 3-9 shows how the rate of apoptosis differs between patients, and more importantly the peak in apoptosis also varies from under 12h post UVB until 24h or later; perhaps explaining why some patients who have been irradiated with 311nm UVB do not appear to have an apoptotic response when measured at 18-24h (Figure 3-3). Reasons for these variations are likely to be numerous, but may include how actively proliferating the plaques are, and the stage of cell cycle that these proliferating cells are in when they are irradiated. These results may also contribute to the lack of correlation with plaque thickness and overall change in PASI, and these issues are addressed in the following chapters.

Equal erythemogenic doses of both wavelengths were used throughout the study; however it is unclear how far UV penetrates into the epidermis in order to induce the erythema response. If this occurs in the lower epidermis, the biologically effective dose of UVB reaching the lower epidermis will be comparable between wavelengths, but this will not be the case if it is due to a superficial response; however, as all plaques are likely to vary in thickness, it is difficult to find another way of ensuring equal doses are given. Meinhardt et al showed that 314nm UVB penetrates into the lower epidermis approximately 14 times more than 290nm in forearm skin (Meinhardt, Krebs et al. 2009). If the erythema response occurs superficially, it would be likely that 290nm is not reaching the target cells *in vivo* accounting for the differential apoptotic response. This has been partially addressed in the next chapter, although further work would include irradiation with 14 times the dose of 290nm compared to 311nm UVB and examining the variation in apoptotic response. In this study however, the effect of penetration of 311nm was examined, and no significant correlation was found between the thickness of the epidermis (including *stratum corneum*) and the count of apoptotic cells using linear regression.

Previous studies have suggested that keratinocyte apoptosis in normal human and mouse skin is first observed 8-12h post irradiation, peaks at 24-48h and is undetectable at 60-72h post UVB (Woodcock and Magnus 1976; Murphy, Mabruk et al. 2002; Mass, Hoffmann et al. 2003). This study suggested that apoptosis in psoriatic epidermis peaks slightly earlier at 18-24h following 311nm UVB. No evidence was found to suggest that irradiation with 290nm UVB induces a different time-course of apoptosis, although biopsies were only examined up until 48h post UVB. This aspect has been taken further in chapter five, where the time course was more closely examined with *in vitro* work.

Two different methods have been used to confirm that the apoptotic cells observed are predominantly keratinocytes, although a small number of apoptotic T cells and CD68+ cells also identified. Overall 96.6% of all apoptotic cells were identified by cell type; the remaining cells could not be identified and are likely to represent cells in the final stages of apoptosis; at this stage cells may have lost their surface markers which identify their origin. T cells have previously been reported to be apoptotic following UVB irradiation in psoriasis (Krueger, Wolfe et al. 1995), and interestingly the relative proportion of T cells undergoing apoptosis was similar to that of keratinocytes, suggesting that there may not be a differential susceptibility between cells of different origins.

Throughout this study patients were given higher multiples of their MED than is routinely used in clinical practice. This was done to augment any small but relevant response which may be missed using sub-erythemogenic doses. To ensure this is relevant to low clinical doses, small groups of patients were assessed in a dose response study, and patients having routine UVB treatment were also biopsied to assess apoptotic response. In both these groups significant levels of apoptosis were detected in contrast to the previous reports that psoriatic epidermis is relatively resistant to apoptosis (Wrone-Smith, Mitra et al. 1997). It should be noted however, that the conclusions of Wrone-Smith et al were based on apoptosis in cultured keratinocytes. More recent clinical practice has included the use of 308nm-excimer lasers in clearing psoriatic plaques, and these are used effectively at doses of 3-6 MEDs. Although these lasers use a monochromatic wavelength slightly below 311nm, they have been

shown to be of similar efficacy to both the 308nm and 311nm lamps (Kollner, Wimmershoff et al. 2005; Goldinger, Dummer et al. 2006). It can be predicted that the epidermal keratinocytes in plaques treated with medium-high dose lasers are also undergoing apoptosis, although it was beyond the scope of this study to test this hypothesis.

There are many factors which influence whether a patient's psoriasis will respond to UVB treatment, and interestingly the same patients can respond well on some occasions and inadequately at other times. This suggests that external factors are important in determining clearance, and it is interesting to note that in the group of patients in this study, weekly PASI score indicated that nearly 40% of all patients experienced a flare in their psoriasis during the treatment period. This suggests that some external trigger was stimulating the disease, which could have reduced the efficacy of phototherapy treatment. In other words, it is important to distinguish treatment modalities that suppress disease initiation (or flare) from those that induce clearance. These observations suggest that UVB is not effective at preventing disease flare-ups. The other obvious confounding issue is patients not attending for all their treatments (15%), as they need to attend the department three times weekly for a period of 6-8 weeks. Surprisingly, only three patients did not achieve a PASI 50 by the end of their treatment, and in two cases this as because the patient dropped out of treatment and in the third the patient had slowly been improving but then experienced a flare-up. This is very encouraging as the treatment has been effective, but it does not allow analysis of whether or not the level of observed apoptosis may reflect the chance of plaques clearing in response to UVB. This study has shown that patients may have a peak in their apoptotic response at variable times, and that this level can decline over a few hours. It is therefore likely that the peak in apoptotic response is underestimated in many patients as the biopsy may have been taken at a suboptimal time. It is therefore unsurprising that when a patient has had a low apoptotic response, it does not predict whether or not they are likely to clear with treatment. For this reason, patients' weekly PASI improvement was analysed, and it was found that patients' PASI improvement was often much greater in one or two weeks and

gradual in others. In general, patients often had a moderate level of improvement for some weeks then a sudden improvement over a one week period. Those who responded with over 50% PASI improvement in score from one week to the next had a significantly higher apoptotic score than those who did not. This sudden change in PASI may suggest that there is a threshold response for apoptosis, or it may indicate that UVB has had another effect such as synchronising cell cycle, which may make larger numbers of actively proliferating cells susceptible to irradiation if this occurs during specific phases of the cycle. Further work is needed to investigate this, but is beyond the scope of this thesis.

3.5. Conclusions

The main conclusions from this chapter are:

- Significant numbers of psoriatic keratinocytes undergo apoptosis in response to 311nm UVB, but not 290nm UVB or in un-irradiated lesional skin despite similar weighted doses.
- The time-course of apoptosis following 311nm UVB shows a peak at 16-24h after irradiation.
- Individual patients have a very variable apoptotic response, both in terms of time course, and also the level of apoptosis achieved.
- The intra-patient variability in apoptotic response was smaller than the inter-patient variability.
- A dose response was seen with increasing doses of 311nm UVB associated with higher apoptotic counts.
- There is a positive relationship between apoptotic response and PASI improvement over the first four weeks of treatment, but measured apoptotic response cannot be used to predict overall response to treatment.

4. Apoptotic effects of other UVB wavelengths

4.1. Introduction

Knowledge of the action spectrum of UVB in the clearance of psoriasis is important as it allows optimisation of treatment regimes. Wavelengths of around 311nm can be highly effective in clearing psoriasis (Coven, Burack et al. 1997; Gordon, Diffey et al. 1999; Kirke, Lowder et al. 2007), and narrowband UVB lamps (which have a peak output at 311nm UVB) are routinely used in clinical practice and are effective in around 70% of patients (Coven, Burack et al. 1997; Gordon, Diffey et al. 1999; Kirke, Lowder et al. 2007). A landmark study examining efficacy of UVB in psoriasis was done by Parrish and Jaenicke in 1981, in which they showed that wavelengths between 300nm and 313nm UVB were effective, although this work was based on just four patients (Parrish and Jaenicke 1981). Over recent years, the 308nm excimer laser has been used to effectively clear individual plaques using doses of up to 3-6 MEDs (Menter, Korman et al. 2010). Irradiation of matched psoriatic plaques in 15 patients with the 308nm excimer laser, 308nm UVB lamp and 311nm UVB lamp has shown a similar efficacy over a 10 week period with all three irradiation sources, with a mean of 24 treatments required for clearance (Kollner, Wimmershoff et al. 2005). A further study compared the 308nm excimer laser and 311nm UV lamp in 16 patients in an open single blinded right/left comparison trial, and again found similar efficacy overall although nine patients responded better to the 308nm excimer laser and four to the 311nm lamp (Goldinger, Dummer et al. 2006). Therefore although these treatments are highly effective in clearing psoriasis, they clear psoriasis to varying extents in different patients and don't work at all in some patients.

If keratinocyte apoptosis is an important mechanism of UVB-induced psoriasis clearance, we may be able to predict which wavelengths are optimal for clearing psoriatic plaques *in vivo* by comparing the apoptotic response induced by a single 2-3 MED dose of different wavelengths of UVB. Results from chapter three demonstrate a lack of apoptotic response following irradiation with 290nm UVB, and a significant apoptotic effect following 311nm UVB. Therefore wavelengths were examined for apoptosis following irradiation

of psoriatic plaques *in vivo* at a 5nm interval from 296nm to 311nm as well as 320nm UVB.

4.2. Aims

- Compare the differential apoptotic effect of 290nm, 296nm, 301nm, 306nm 311nm and 320nm UVB
- Identify any demographic factors which may affect which wavelength induces maximal apoptosis in individual patients

4.3. Results

Eighteen patients were recruited to (and completed) this arm of the study. Each patient had MEDs calculated for four different wavelengths of UVB. Four matched psoriatic plaques were chosen on the lower back of each patient and each was irradiated with a single dose of UVB wavelengths between 296nm and 320nm, making up the following total:

- 10 plaques irradiated with 296nm UVB (5nm bandwidth)
- 18 plaques irradiated with 301nm UVB (5nm bandwidth)
- 18 plaques irradiated with 306nm UVB (5nm bandwidth)
- 18 plaques irradiated with 311nm UVB (5nm bandwidth)
- 8 plaques irradiated with 320nm UVB (10nm bandwidth with WG305 filter to remove unwanted wavelengths below 305nm).

Irradiation times varied from 12s (296nm) to 50 mins (320nm). Where long irradiation times were required due to high MED readings, all four of the 'test' plaques in these patients were irradiated with two multiples of the MED at each dose instead of three, therefore keeping the irradiation times within a more acceptable limit for the patient. In total 10 patients therefore received three MEDs of 296nm, 301nm, 306nm and 311nm UVB and eight received two MEDS of 301nm, 306nm, 311nm and 320nm UVB. A biopsy was taken from each plaque 24h later, and this was then analysed for keratinocyte apoptosis. Previous results had shown no significant apoptosis 18-24h following irradiation of plaques with three MEDs of 290nm UVB (n=26) (section 3.2.2), and this was used as a baseline to show whether each wavelength induced significant levels of apoptosis.

4.3.1. Apoptotic effects of wavelengths between 296 and 320nm UVB *in vivo*

Compared to the unmatched patients described in section 3.2.2 (n=26), a Mann Whitney-U test showed that wavelengths between 301nm and 311nm induced significant apoptosis ($p<0.001$), but those above and below this did not (296nm $p=0.16$, 320nm $p=0.39$) (Figure 4-1). A regression analysis did not show a statistically significant difference in the number of apoptotic cells induced following irradiation with 301nm, 306nm or 311nm UVB ($p=0.17$), and there was no association with dose administered and apoptosis overall (Figure 4-2). Interestingly, different patients responded with higher levels of apoptosis following irradiation with different wavelengths, as shown below. No apoptosis was detected 24h following irradiation with any of the UVB wavelengths in one patient, and in another, only very low levels of apoptosis occurred at similar levels following 306nm and 311nm irradiation. Interestingly the peak wavelength for apoptosis induction varied between patients, with 50% (n=9) of patients having maximum levels of apoptosis following irradiation with 311nm UVB, compared to 17% (n=3) and 22% (n=4) of patients following irradiation with 301nm and 306nm of UVB respectively (Figure 4-3). This variation between individuals suggests that the differential effect is not due to absorption by a chromophore, but other confounding factors (e.g. epidermal thickness) may be contributing.

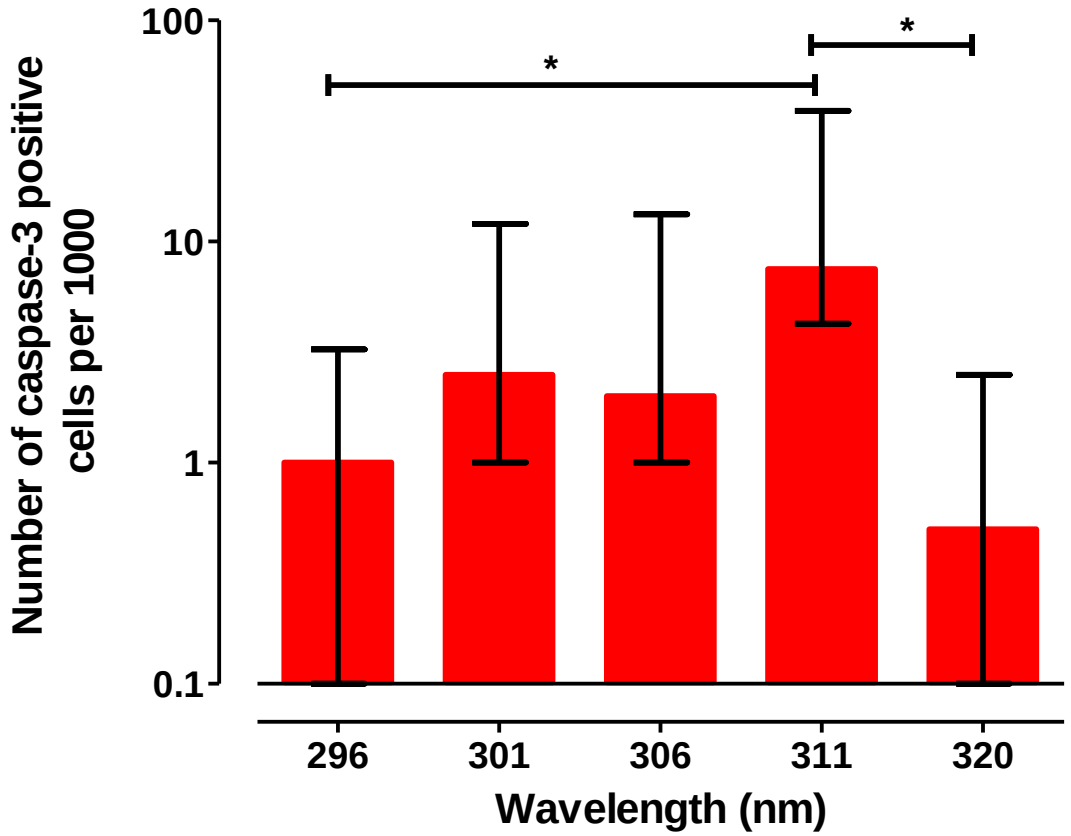


Figure 4-1. Significant apoptosis occurs following irradiation with 301nm, 306nm and 311nm UVB ($p<0.001$) compared to un-irradiated psoriasis or psoriasis irradiated with 290nm. Median and inter-quartile ranges shown. No significant difference was seen in number of apoptotic cells following irradiation with either 296nm or 320nm UVB and un-irradiated psoriasis. Patients were irradiated with either three MEDs of 296, 301, 306 and 311nm (n=10), or two MEDs of 301, 306, 311 and 320nm UVB (n=8). Note the y-axis is a log scale * $p<0.05$.

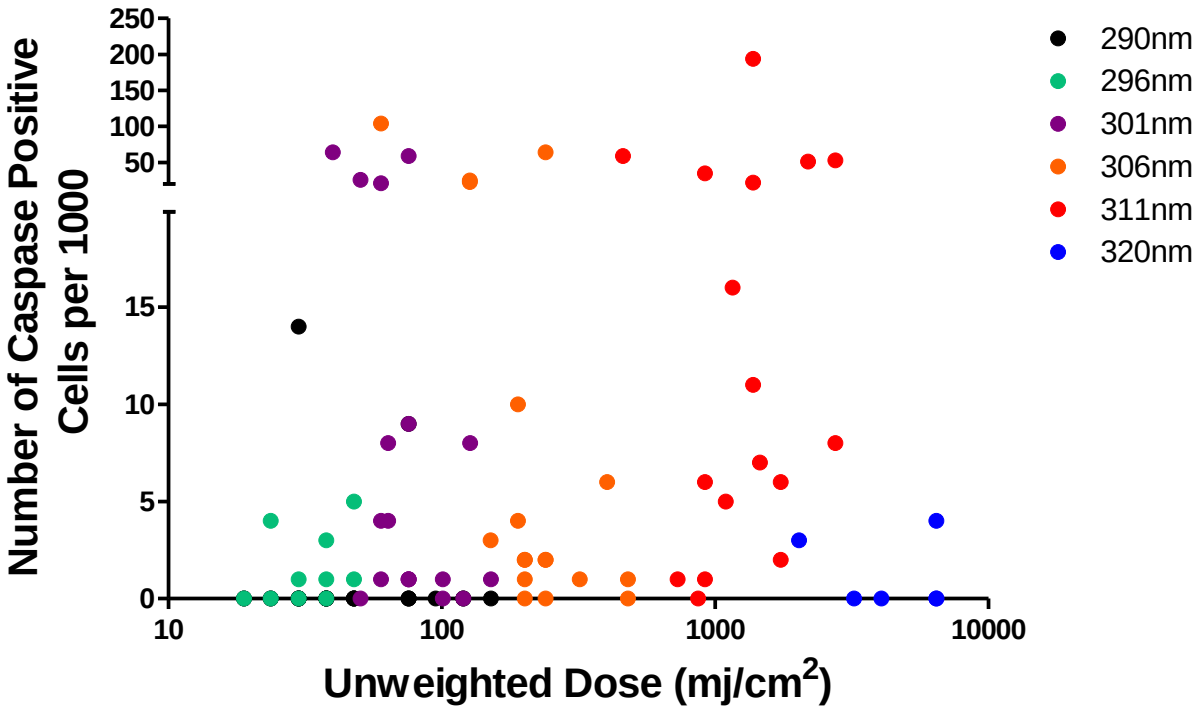
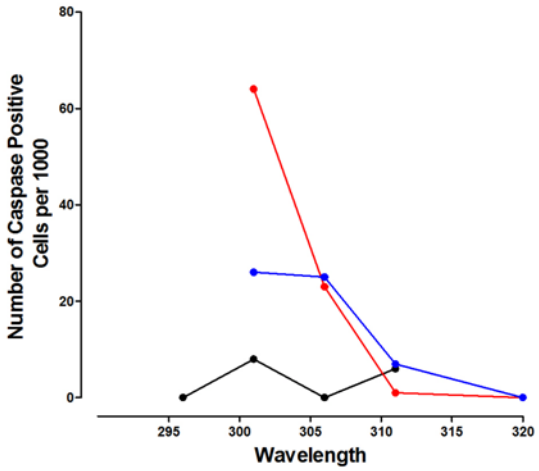
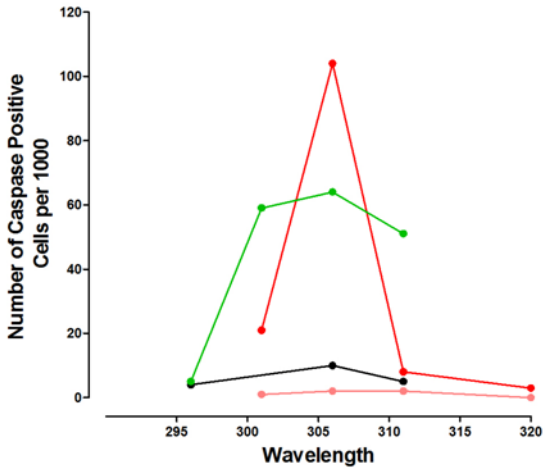


Figure 4-2. No dose response was found between unweighted dose administered and apoptosis for the six different wavelengths (p=0.57). Note the log scale on the x-axis.

a



b



c

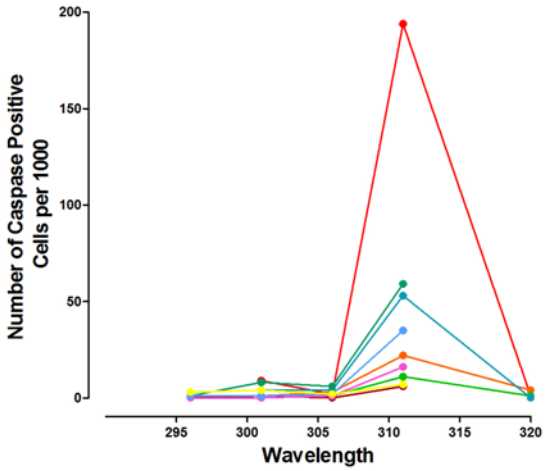


Figure 4-3. Individual patients' action spectra for UV-induced apoptosis in psoriatic plaques. Each line represents an individual patients' response. Note the increasing maximum number of apoptotic cells as the peak wavelength increases from 301nm (a) to 306nm (b) and then 311nm (c). Maximal number of apoptotic cells were seen following irradiation with 301nm (three patients), 306nm (four patients) and 311nm (nine patients).

To explore whether these differences are affected by wavelength penetration, the epidermal thickness of each plaque was measured as described in section 2.1.5, and related to the quantity of apoptosis measured as described below.

4.3.2. Factors affecting variation in apoptotic response

4.3.2.1. Consistency of intra-patient plaque thickness

To exclude inadvertent plaque selection bias (i.e. thinner plaques being irradiated with wavelengths of 311nm UVB, which is known to be effective), intra-patient plaque comparisons were made. The mean of 10 consecutive measurements of rete ridge depth were compared between the four irradiated biopsies taken from each patient. Results are shown in **Figure 4-4** and a measurement error test showed significantly greater inter-patient variability than intra-patient variability ($p<0.05$) for 13/18 patients, confirming that plaques selected for irradiation were of similar thickness in most patients.

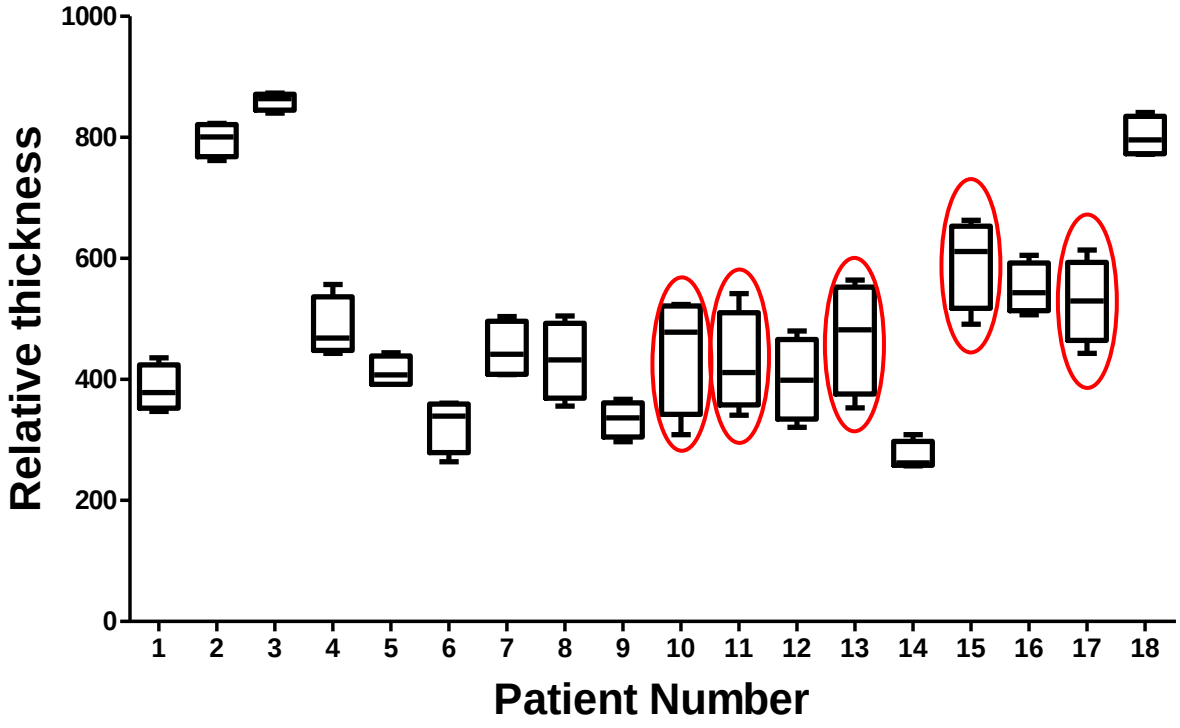


Figure 4-4. Intra-patient variation in mean epidermal plaque thickness. Bars show mean value and standard deviation for four biopsies, with whiskers showing maximum and minimum values. Measurement error testing showed significantly greater inter-patient variability than intra-patient variability in the majority of patients, confirming that plaques were of similar thickness in each patient. Patients circled red had at least one irradiated plaque with a significantly different epidermal thickness.

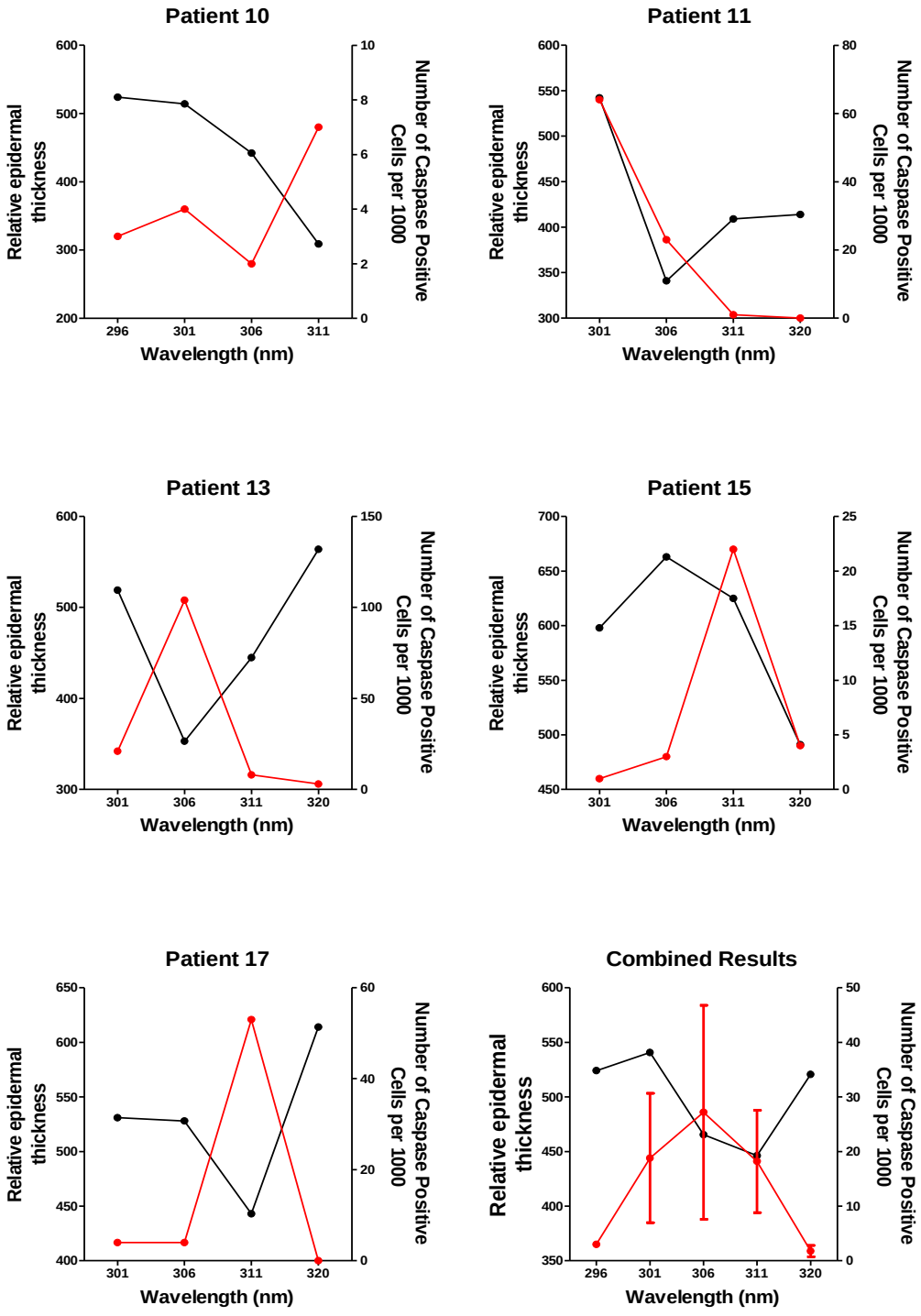


Figure 4-5. Individual plots for the five patients with significantly varying intra-patient plaque thickness. Red lines show apoptosis (error bars show standard error of the mean) and black lines show mean epidermal thickness of rete ridges (i.e. maximal thickness). There does not appear to be a correlation between thinner plaques and increased rate of apoptosis overall.

In the other five patients, at least one of the four chosen plaques was significantly thinner or thicker than the others, but a closer examination showed no overall correlation between thinner plaques and apoptosis (Figure 4-5).

The observed optimal wavelength for apoptosis induction in individual patients did tend towards a positive correlation with epidermal thickness 24h after irradiation (i.e. patients with a thinner epidermis had a peak apoptosis-induction following irradiation with shorter wavelengths of UVB down to 301nm, and patients with a thicker epidermis had a peak apoptosis induction 24h following irradiation with longer wavelengths up to 311nm), although this was not significant overall (p=0.17, Figure 4-6).

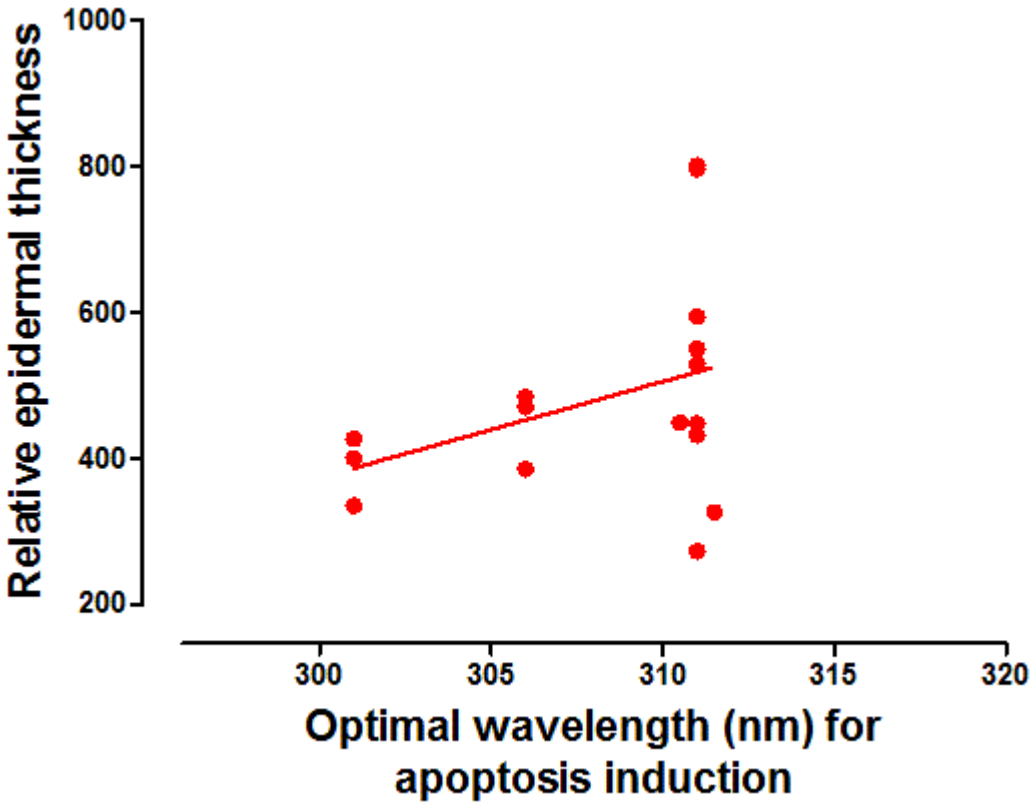


Figure 4-6. Correlation between optimal wavelength for apoptosis induction and relative epidermal thickness of the irradiated psoriatic plaque. A positive correlation was seen, although this was not significant (p=0.17, r^2=0.13).

4.3.2.2. The effect of skin type and dose on optimal wavelength for apoptosis induction

No significant correlation was seen between skin type and peak wavelength for apoptosis (p=0.83) (Figure 4-7) or administered dose and peak apoptotic wavelength (p=0.71).

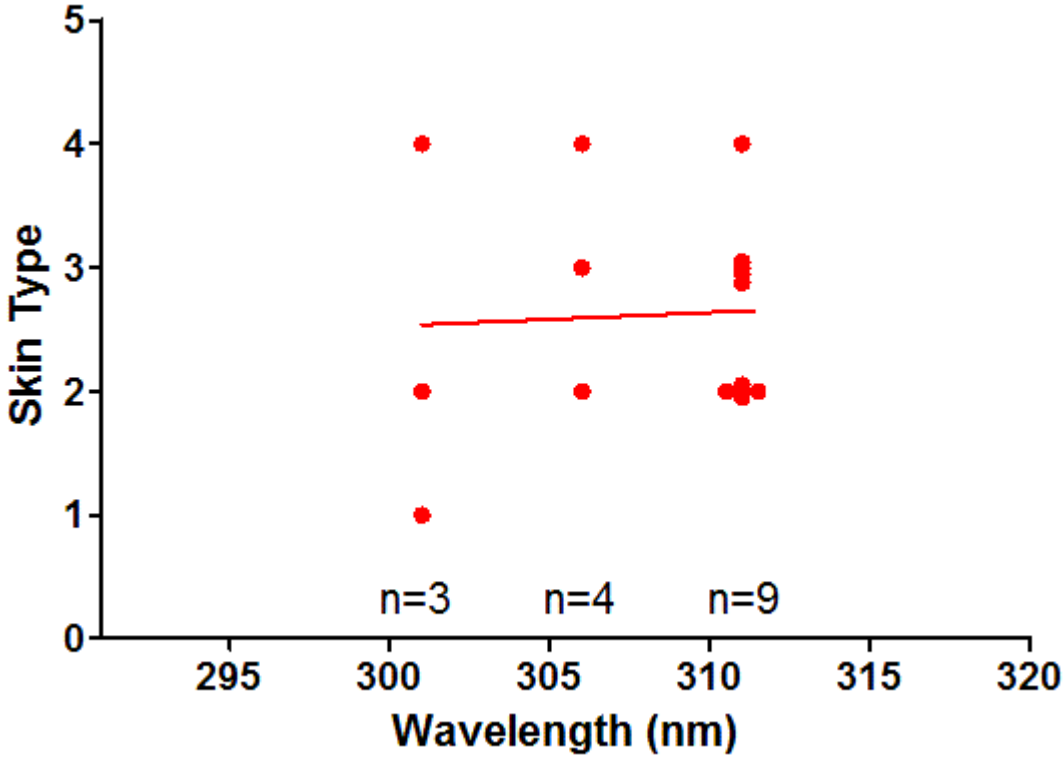


Figure 4-7. No significant relationship was seen between skin type and peak wavelength for apoptosis. Linear regression line shown (p=0.85, r^2=0.003).

4.3.2.3. Clinical outcome and optimal wavelength for apoptosis induction

Unsurprisingly a Mann Whitney U test showed no correlation between end PASI improvement (PASI 90) and which wavelength was optimal for induction of apoptosis (p=0.431). This is as expected because most patients had significant apoptosis seen following irradiation with 311nm even if the apoptotic response was higher following irradiation with 301nm or 306nm UVB. However, it remains to be seen whether or not these individuals may have had a better or quicker clinical response if irradiation was given with a wavelength which has been ‘tailored’ to plaque thickness.

4.4. Discussion

The erythema action spectrum of UVB in skin is similar to the action spectrum of DNA damage (Setlow 1974) and non-melanoma skin cancer induction (Setlow 1974; de Gruijl and van der Leun 1994), but appears distinct from the therapeutic action spectrum of UVB in the clearance of psoriasis (Parrish and Jaenicke 1981). Ideally, a large randomised study is required to examine the efficacy of different wavelengths of UVB, however, as patients have such a large variation in response to treatment anyway (both between different patients, and within individual patients following courses of UVB at different times) very large numbers of patients would be required to confidently show which wavelength is optimal. However, reducing the peak wavelength by just 11nm (from 311nm to 300nm) changes an effective treatment into a completely ineffective treatment (Parrish and Jaenicke 1981), therefore subtle changes in wavelength may have profound clinical importance.

In this study, five wavelengths within the UVB spectrum ranging from 296nm to 320nm, were used to irradiate localised plaques in 18 patients (four wavelengths per patient), and apoptosis was used as a 'biomarker' of response. Interestingly, peak apoptotic response in 50% of the patients was at 311nm, but in 17% of patients the peak was at 301nm and in 22% of patients the peak was following 306nm UVB. As longer wavelengths penetrate deeper into the epidermis, and apoptosis is seen in the lower epidermis, it could be argued that the optimal wavelength for apoptosis induction needs to be matched to epidermal thickness of the plaque. However, although a positive correlation was found between epidermal thickness and optimal wavelength for induction of apoptosis, this was not statistically significant. Apoptotic response was also independent of irradiation dose given and patients skin type. It is therefore unclear from this study why some patients have a differential apoptotic response to wavelengths which do induce epidermal apoptosis. One explanation could be that this arises due to chance (sample error), and larger numbers of patients would be required to reduce this potential bias.

If results shown here are representative of the general population of psoriatic patients, it could be assumed that UVB lamps with emission in the 301-311nm range and no significant emission of highly erythemal wavelengths < 301nm, would induce the most apoptosis, and therefore potentially have the best clinical outcome for patients if apoptosis is an important mechanism of psoriasis clearance. Broadband UVB lamps used to treat psoriasis may be “selective” (UV6), i.e. with little emission at 300nm or shorter wavelengths, or “conventional” (TL12), i.e. with significant emission < 300nm. It may therefore be predicted that narrowband UVB or selective broadband UVB would be more effective for a given erythemal dose than conventional broadband UVB, as a greater proportion of the output is within the apoptosis-inducing spectrum. Kirke et al found no significant difference in psoriasis clearance in a randomised study of 100 patients comparing TL01 and UV6 (Kirke, Lowder et al. 2007). Several small half-body studies have shown a significantly greater efficacy for narrowband rather than conventional broadband UVB (van Weelden, Young et al. 1980; Storbeck, Holzle et al. 1993; Coven, Burack et al. 1997; Walters, Burack et al. 1999). These data are consistent with the study hypothesis.

Throughout this study it has been assumed that peak erythema occurs 18-24h following UVB irradiation in psoriatic epidermis, and it has previously been demonstrated that the peak in the erythemal time-course is similar in normal skin for UVA, UVB and UVC (Diffey, Farr et al. 1987; Farr, Besag et al. 1988). Ideally an erythema time-course would be obtained at each wavelength to exclude a different time-course in psoriatic skin, however when patients were seen back for their biopsies at 48h following initial MED irradiations there was no suggestion of a delayed erythemal response for any of the tested wavelengths.

In clinical practice, wavelengths below 300nm UVB do not clear psoriasis, and if apoptosis is a marker of clinical response, then this is consistent with the results shown here as no apoptosis was seen following irradiation with wavelengths below 301nm UVB. Although other studies have not looked at the clinical efficacy of wavelengths of around 320nm UVB, these would present a

practical obstacle in clinical treatment as the irradiation time is long, and likely to be too excessive for patients in the absence of a very marked benefit.

4.5. Conclusions

- Wavelengths between 301nm and 311nm induced apoptosis in psoriatic epidermis
- Differential apoptotic response did positively correlate with epidermal thickness, but larger numbers of patients are required to demonstrate significance
- Overall irradiation with UVB of around 311nm appeared to induce maximal apoptosis in psoriatic epidermis

5. Effects of 311nm and 290nm on primary keratinocytes

5.1. Introduction

Apoptosis is a dynamic process but is routinely measured by end-point assays such as immunohistochemistry and flow cytometry. These assays allow a ‘snap-shot’ of the process but will not give any information about the rate at which apoptosis is occurring. However, this rate is fundamental to understanding the overall impact of the apoptosis on tissue homeostasis. For example, if 100 apoptotic cells are visualised within the epidermis at a particular time-point, the effect on tissue homeostasis would be far greater if each apoptotic cell is detectable for just 10 mins (i.e. the turnover of apoptotic cells is fast), compared to if they are present for 10h. Therefore, to interpret the *in vivo* data in chapter three, this chapter examines the rate of apoptosis in human keratinocytes using time-lapse imaging *in vitro*.

Shorter wavelengths of UVB do not penetrate as deeply into the epidermis, and as the majority of UVB-induced epidermal apoptosis occurs in the basal and suprabasal layers, this is arguably a reason why 290nm UV-induced irradiation rarely elicits any keratinocyte apoptosis. To test this, primary cultured human keratinocytes were directly irradiated with either wavelength and the apoptotic responses compared. This was assessed with both time-lapse imaging and flow cytometry, and gives an understanding of the contribution of wavelength penetration to the apoptotic effects seen.

Understanding why some cells undergo apoptosis when their immediate neighbours remain unaffected may help us understand why patients respond very differently to the same treatment. To explore this further, the differential susceptibility of cells to apoptosis in different stages of the cell cycle was examined, as well as the effect of the cells proliferative potential (i.e. if it is a putative-stem cell, TA cell or differentiating cell) on its chance of undergoing apoptosis.

Together these data support the clinical results described in chapter three, and further our understanding of whether or not the quantity of apoptosis seen

could be sufficient to directly affect plaque remodelling and clearance of psoriasis, using mathematical modelling techniques (described in chapter six).

5.2. Aims

- Compare the apoptotic effects of 311nm and 290nm UVB on primary cultured keratinocytes *in vitro* and define a time-course of apoptosis for each of these
- Investigate the time taken for individual cells to complete the apoptotic process
- Examine the effects of cell cycle and proliferative capacity on susceptibly to apoptosis

5.3. Results

5.3.1. Live cell imaging of apoptosis following irradiation with 311nm and 290nm

To investigate the apoptotic effects of increasing doses of UVB on human keratinocytes, cells were irradiated with 2-10 SEDs of 311nm or 290nm UVB. Cells were initially incubated with DEVD-NucViewTM caspase-3 substrate, PI and a nuclear marker (Draq5 or Hoescht 33258) for total cell count. However, although SRB (sulphorhodamine) assays, which measure cytotoxicity, had shown that these nuclear markers did not induce additional toxicity over the 24h incubation period. Live-cell imaging experiments repeatedly showed significant cell death in all wells within 4-8h of the onset of imaging. Spectrophotometer measurements of the spectral output from the Pathway Bioimager excluded any unexpected emissions (in particular in the UV range), and all experiments were carried out in a dark environment. Hoechst 33258 (a bis-benzimidazole derivative compound), binds to the DNA and requires UV excitation for detection. Cells were being imaged every 30-60 mins, and even if very low doses of UV are required for excitation, this may in theory contribute to apoptosis. In practice however, no difference was noted in the overall number of apoptotic cells detected when cells were imaged every 30 mins versus every 60 mins. To eliminate any potential confounding effects, experiments were carried out by incubating with DEVD-NucViewTM caspase-3 substrate and PI alone, then adding the nuclear marker at the end of 24h to count the total number of cells present.

Repeated experiments (n=3) showed that the cumulative number of apoptotic cells reached a plateau by approximately 18h, although cells continued to be imaged at 30 min intervals until 24h post irradiation. Maximal cumulative apoptotic response occurred after irradiation with six and eight SEDs of 311nm, with medians of 49% (inter-quartile range 44- 53%) and 55% (inter-quartile range 52- 58%) respectively (Figure 5-1). However, examining each of these doses in isolation showed that higher doses of 311nm or 290nm UVB (6-8

SEDs) induced apoptosis earlier than lower doses (2-4 SED) (Figure 5-2 and Figure 5-4), with an overall EC50 of 14.5h and 16h respectively (n=3), although these were not significantly different at the 95% confidence interval. The highest dose of 311nm used, 10 SEDs, was not as effective at inducing apoptosis as irradiation with 8 SEDs ($p<0.001$), although there was no significant difference in the percentage of PI+ cells following 8 and 10 SEDs ($p=0.591$), suggesting that cells may be undergoing necrosis following the 10 SED dose.

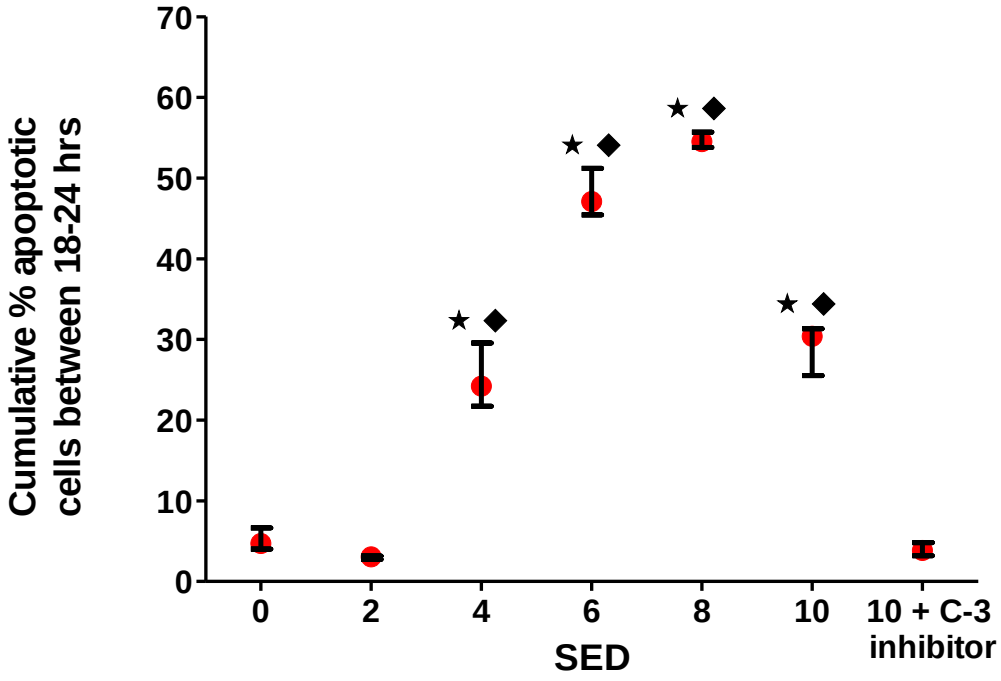


Figure 5-1. Cumulative apoptosis in NHEKs is maximal following eight SEDs of 311nm at 18-24h time-points. A membrane-permeable caspase-3 substrate and live cell imaging was used to detect the onset of apoptosis in normal human epidermal keratinocytes (NHEK). Minimal apoptosis was seen in un-irradiated cells, following two SEDs of UVB and when a specific caspase-3 inhibitor was incubated with irradiated cells. Median and inter-quartile ranges shown for hourly time-points between 18 and 24h in cells derived from three patients. A significant difference in the cumulative proportion of apoptosis was seen between indicated doses and sham irradiated cells (*) or irradiated cells incubated with caspase-3 and inhibitor (♦) ($p<0.05$).

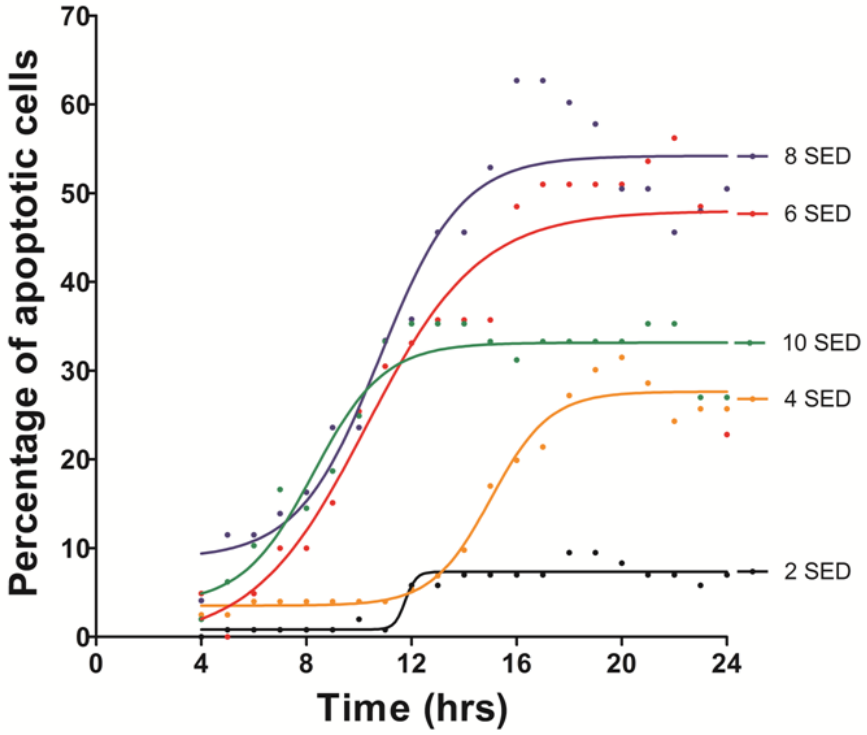


Figure 5-2. Apoptosis occurs following irradiation with 311nm UVB in primary foreskin derived keratinocytes. A membrane-permeable caspase-3 substrate and live-cell imaging was used for detection of onset of apoptosis in normal human epidermal keratinocytes (NHEK) between 4 and 24h. Cumulative percentage of apoptosis following irradiation with increasing doses of 311nm shown. Note the earlier onset of apoptosis with doses causing higher overall apoptotic effect.

Irradiation with 290nm showed maximal cumulative apoptosis following irradiation with eight SEDs at the 24h time-point (Figure 5-3), which peaked at approximately 24h (Figure 5-4). The cumulative percentage of apoptotic cells induced by irradiation with 311nm and 290nm (Figure 5-1and Figure 5-3) were compared at each dose, and no significant difference ($p<0.05$) was found between the two wavelengths at each dose using a Mann Whitney U test.

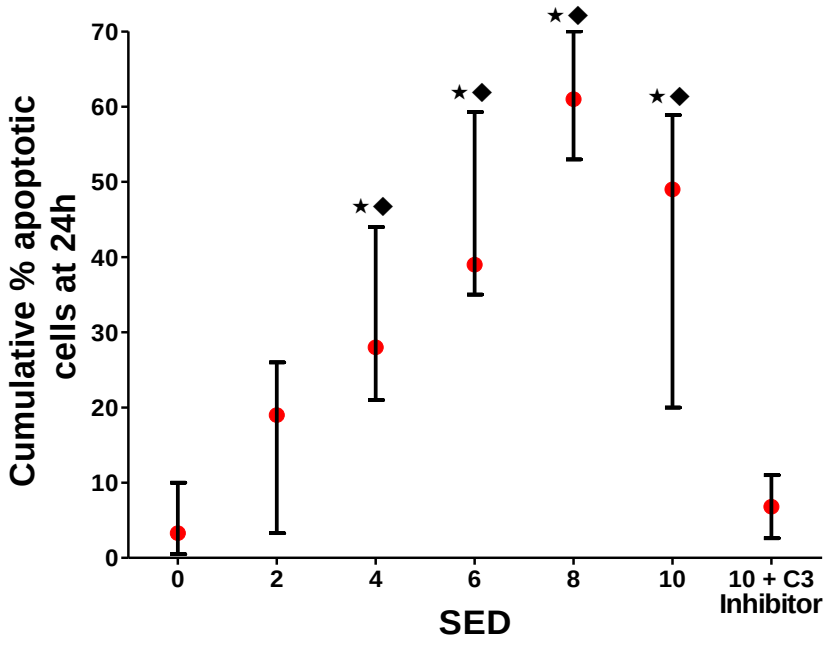


Figure 5-3. Cumulative apoptosis in NHEKs is maximal following eight SEDs of 290nm at 24h. A membrane-permeable caspase-3 substrate and live cell imaging was used for detection of onset of apoptosis in normal human epidermal keratinocytes (NHEK). Minimal apoptosis was seen in un-irradiated cells, and when a specific caspase-3 inhibitor was incubated with irradiated cells. Median and inter-quartile ranges shown for a single 24h time-point in cells derived from three different patients. A significant difference in the cumulative proportion of apoptosis was seen between indicated doses and sham irradiated cells (*) or irradiated cells incubated with caspase-3 and inhibitor (♦) ($p<0.05$).

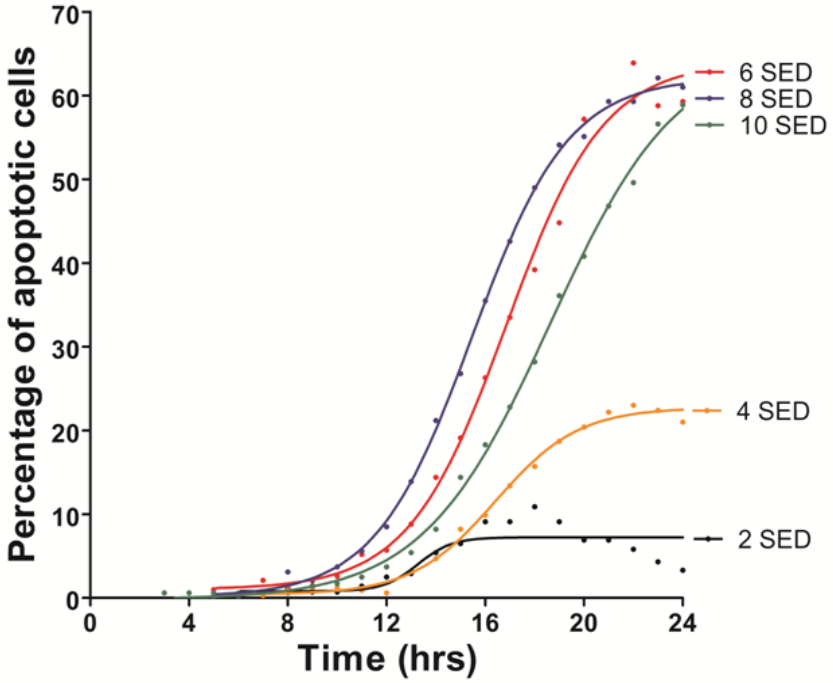


Figure 5-4. Apoptosis occurs following irradiation with 290nm UVB in primary foreskin derived keratinocytes. A membrane-permeable caspase-3 substrate and live cell imaging was used for detection of onset of apoptosis in NHEKs. Cumulative percentage of apoptosis following irradiation with increasing doses of 290nm UVB shown. Note the earlier onset of apoptosis with doses causing higher overall apoptotic effect.

5.3.1.1. The duration of apoptosis in individual cells

Over 300 cells from three separate experiments were automatically tracked using Volocity 4.01 software, and individually analysed to calculate the time between the onset of caspase-3 fluorescence, and PI entry into the cell. As shown in Figure 5-5, there was considerable variation in cellular response. The duration of apoptosis prior to loss of cell membrane integrity ranged from less than 10 mins to over 9.5h, and was randomly distributed. Analysis of each individual experiment and the results from all three experiments combined gave a median time of 20 mins for the onset of caspase-3 activity until breach of membrane integrity, however 28% of cells had completed the apoptotic process, turning from green to red fluorescence, in less than 10 mins, the minimal time-frame imaged.

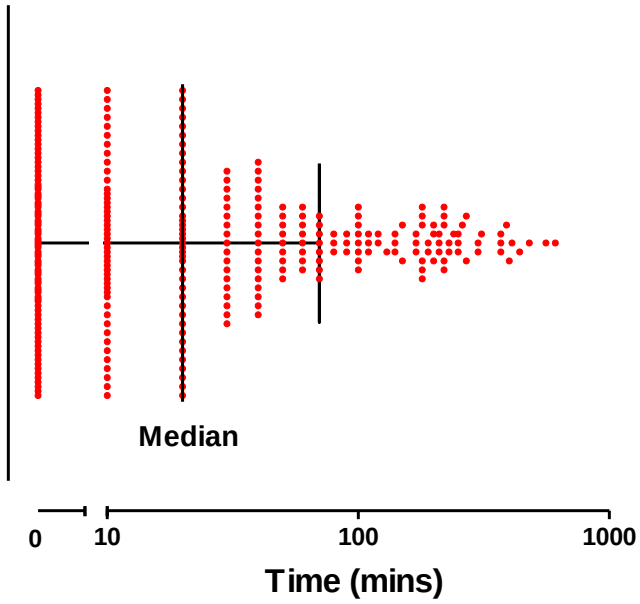


Figure 5-5. Time taken for apoptotic cells to die following the onset of caspase-3 activity, induced within the first 24h following six SEDs of 311nm UVB. Three hundred cells are shown here (100 from each of three separate experiments). The median time of apoptosis was 20 mins in each separate experiment and 20 mins overall, (inter-quartile range 10-70 mins).

5.3.2. FACS analysis of apoptosis following irradiation with 311 and 290nm

To support the theory that lack of 290nm induced apoptosis *in vivo* may be at least in part, due to reduced penetration of 290nm compared to 311nm, flow cytometry was also used to compare the proportion of apoptotic cells induced by direct irradiation of primary keratinocytes. Cells were then incubated with DEVD-NucViewTM caspase-3 substrate and PI for 24h in the dark, thus any augmentation of apoptosis induced by the imaging process of the Pathway were eliminated (section 5.3.1). Figure 5-6 shows a result from a representative experiment, and although both 311nm and 290nm UVB caused a significant increase in the proportion of apoptotic cells observed (Figure 5-7), the difference between the two wavelengths was not significant with repeated experiments ($p<0.05$).

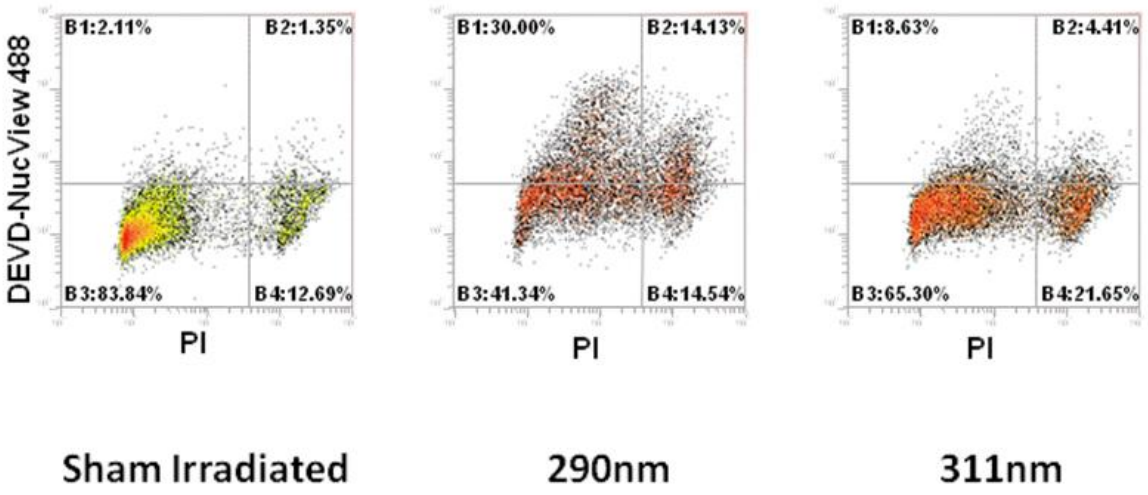


Figure 5-6. Comparison of the apoptotic effects of 290 and 311nm UVB 24h irradiation of NHEKs. Flow cytometry scatter plots showing that irradiation with 290nm UVB induces a greater proportion of apoptotic (caspase-3+) and dead (PI+) cells than 311nm, at 24h. Cells in top left quadrant are early apoptotic, and those in the top right quadrant are late apoptotic.

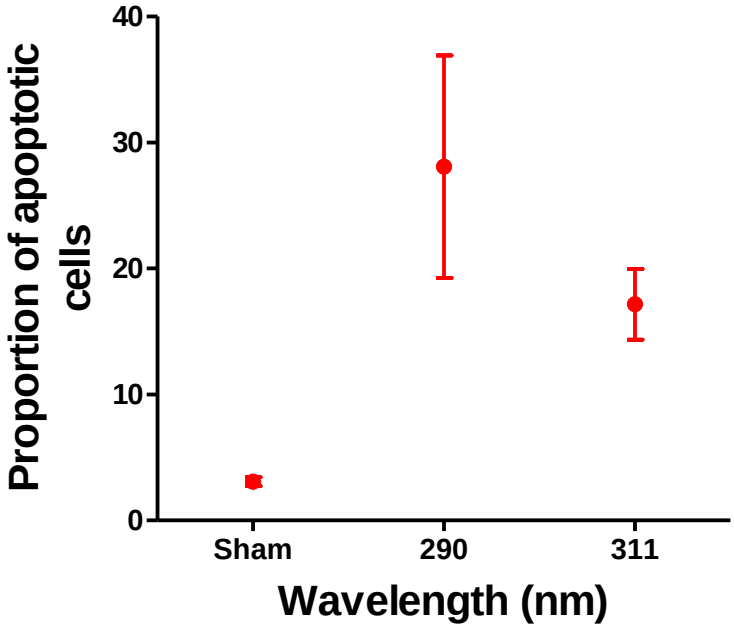


Figure 5-7. Comparison of the apoptotic effects of 290nm and 311nm UVB 24h irradiation of NHEKs versus sham irradiated cells. The proportions of apoptotic cells detected by flow cytometry are compared. Each experiment was repeated in triplicate, and the average of these are shown (n=3). No significant difference was observed in apoptosis following irradiation with 290nm versus 311nm UVB ($p=0.44$), although these wavelengths were both significantly more apoptotic than sham-irradiation ($p<0.05$).

5.3.3. Effects of cell cycle on susceptibility to apoptosis

One explanation for the observed variation in the rate and time of onset of apoptosis following UVB irradiation could be that cells have differential sensitivity to apoptosis in different stages of the cell cycle. To investigate this further, PI+ /caspase-3+ cells (late stage apoptosis) were analysed 24h after irradiation with 311nm UVB and the number of cells in the G1, S and G2M phases of the cell cycle were compared to un-irradiated cells. Figure 5-8 shows a significantly greater number of cells die in G2M following UVB, with a similar number of cells dying in G1 and S phase as un-irradiated controls.

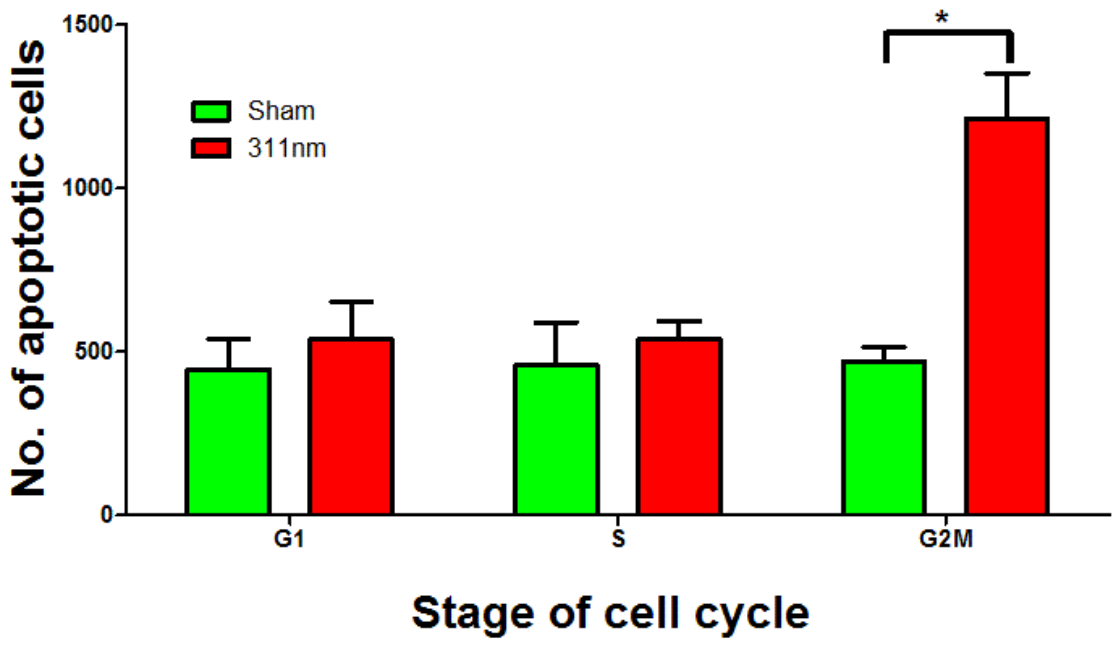


Figure 5-8. A greater number of cells undergo apoptosis in G2M. Primary keratinocytes were sham irradiated or irradiated with 311nm UVB, incubated with caspase-3 and PI, and analysed by flow cytometry at 24h. Analysis of the caspase-3+ dead (PI+) cells showed a significantly greater proportion of cells died in G2M compared to G1 or S phase following 311nm irradiation but not sham-irradiated cells. Mean ± SEM shown. *P<0.05. n=3 with each experiment repeated in triplicate.

5.3.4. Effects of proliferative potential on susceptibility to apoptosis

To determine whether cells of differing proliferative potential had altered susceptibility to apoptosis, intact normal epidermis was irradiated *ex vivo*, and analysed at 24h for apoptotic stem, TA and differentiating cells. As the epidermis comprises more than just keratinocytes, and the scatter of epidermal cells showed distinct clustering, cells were initially sorted (as shown in Figure 5-9) according to their scatter distribution and then analysed by immunocytochemistry using markers for keratinocytes, T cells, melanocytes, and Langerhans' cells. Material with a very low forward scatter was cell

fragments/ debris and excluded from further analysis (see Figure 5-9). Keratinocytes were found in all areas, and comprised a mean of 99% cells in areas 1, 98% in area 4, 86% in area 2 and 9% in area 3. Melanocytes were seen in areas 2 (2%), 3 (5%) and 4 (2%), CD3+ T cells were confined to area 1 (<1%), and CD1a+ Langerhans' cells were not seen. The majority of cells in area 3 could not be identified with the above epidermal markers, and were also negative for Ki67, a marker of cell proliferation. It was therefore assumed that these cells are likely to be dying and had lost their original cell surface markers. Area 2 was not present in over 50% of the epidermis samples studied. These data were used to inform gate locations in the experiments described below with areas 2 and 3 excluded from analysis.

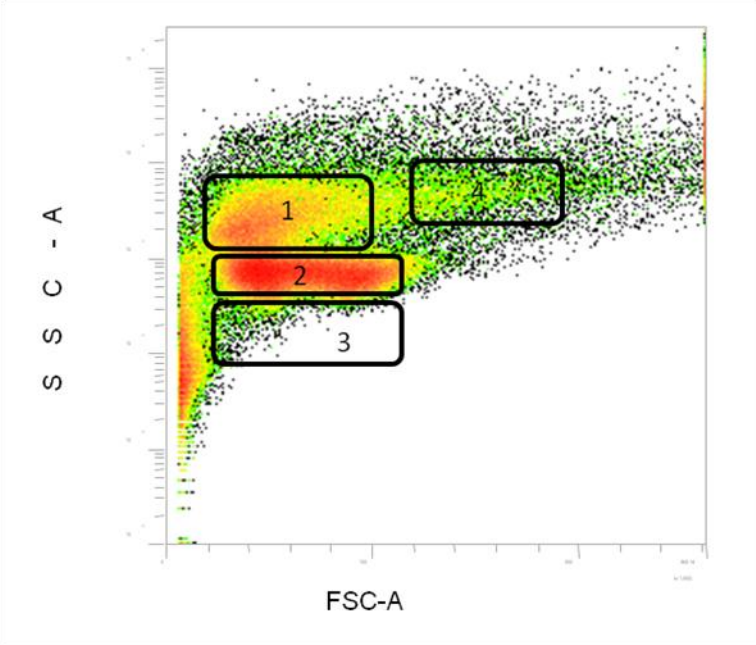
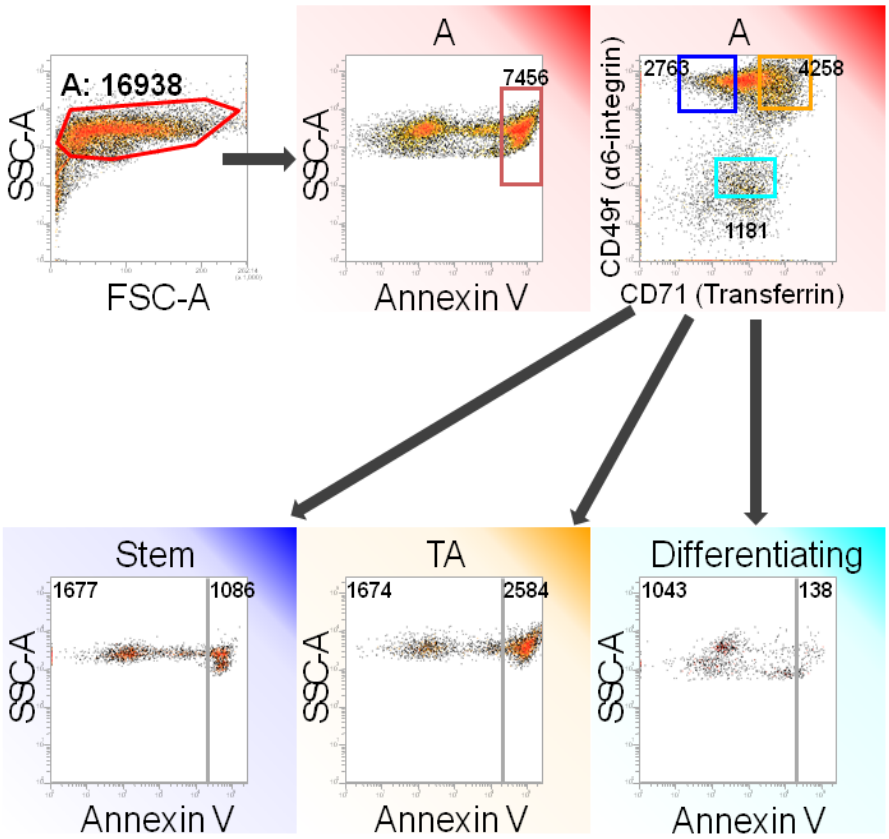


Figure 5-9. Cells were harvested from fresh intact human epidermis and isolated by cell sorting according to their scatter profile. Analysis of the scatter distribution of cells was performed to ensure that keratinocytes could be identified. Cells showed distinct clustering on the SSC (side scatter) and FSC (forward scatter) indicating different sizes. Cells clustering on the far left were identified as debris. A sample of cells from each cluster (1-4) was isolated by cell sorting and further analysed to identify the cell types at each scatter point. n=3.

Apoptotic keratinocytes (Annexin V+) were subdivided according to their expression of proliferative markers, α6-integrin (CD49f; positive in cells of proliferative potential, but not differentiating cells) and transferrin (CD71, positive in TA cells but not stem cells). At least 20 000 cells were collected and the change in number of apoptotic putative-stem, TA and differentiating cells between sham-irradiated and 311nm-irradiated epidermis was compared at 24h. Figure 5-10 shows that there was an increase in the total number of apoptotic cells in each compartment following 311nm UVB, but this was most noticeable for TA cells, which accounted for the majority of Annexin-V+ cells (Figure 5-11). When each compartment was analysed individually, 20% of TA cells were found to be apoptotic, in contrast to 13% of stem cells and 9% of differentiating cells (Figure 5-12). However, these results represent a 'snap-shot' of what is occurring in the epidermis 24h after irradiation *ex vivo*, and do not take into account the fact that many of these cells are dividing *in vivo*, and therefore many of these cells would have already been replaced 24h after an epidermal insult.

a


A: 16938
SSC-A
FSC-A
A
7456
SSC-A
Annexin V
A
2763
4258
1181
CD49f (α6-integrin)
CD71 (Transferrin)
Stem
1677
1086
SSC-A
Annexin V
TA
1674
2584
SSC-A
Annexin V
Differentiating
1043
138
SSC-A
Annexin V

b

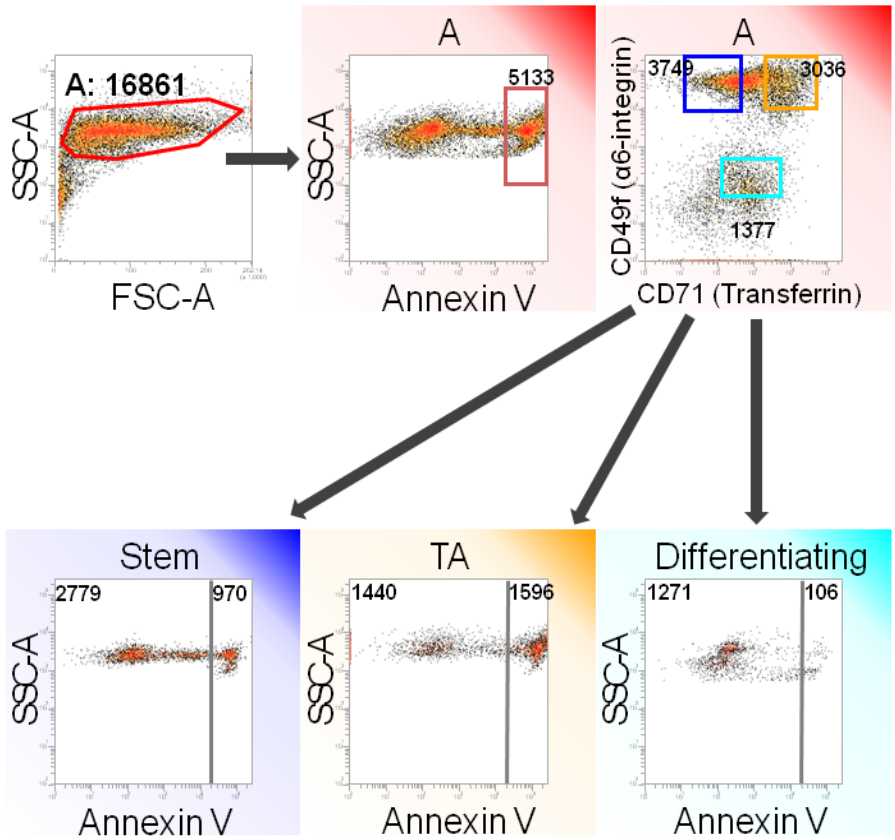

A: 16861
SSC-A
FSC-A
A
5133
SSC-A
Annexin V
A
3749
3036
1377
CD49f (α6-integrin)
CD71 (Transferrin)
Stem
2779
970
SSC-A
Annexin V
TA
1440
1596
SSC-A
Annexin V
Differentiating
1271
106
SSC-A
Annexin V

Figure 5-10. Apoptosis induced by 311nm UVB occurs in cells of all proliferative potential. Flow cytometric analysis of the apoptotic effect of a single six SED irradiation of 311nm UVB on normal human skin *ex vivo*. Keratinocytes were harvested from fresh skin 18h after irradiation and stained for apoptosis (Annexin-V-FITC), and markers of proliferative potential; CD49f-PE and CD71-APC. Putative stem cells were identified as CD49f+ APC – (blue gate), TA cells as CD49f+ APC + (orange gate), and differentiating cells as CD49f – (aqua gate). The top left panels show all the cells, and the gate of nucleated cells (A) was used for the analysis. The top middle and right panels show the actual number of apoptotic cells, and differential expression of proliferative markers from all the nucleated cells gated in A. The lower panels show that actual numbers of apoptotic cells according to the expression of proliferation markers. (a) Representative example of sham irradiated epidermis, and (b) skin irradiated *ex vivo* with six SEDs of 311nm UVB in the same patient.

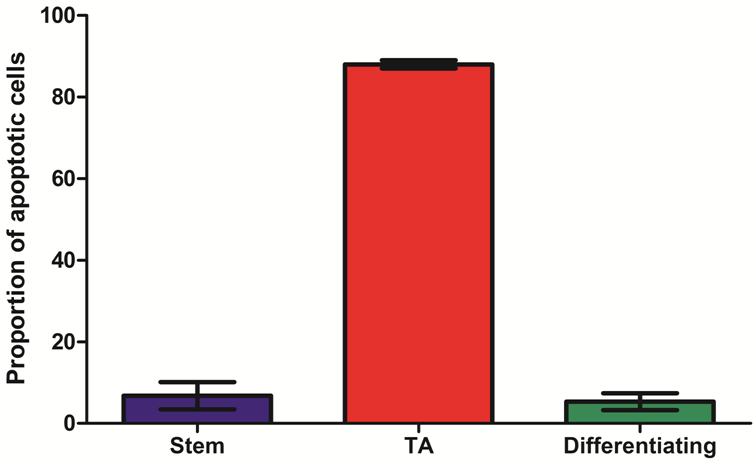


Figure 5-11. The majority of apoptotic cells are TA cells. Apoptotic cells in irradiated epidermis were divided into three categories according to their α6-integrin/ transferring expression, and the proportion of cells in each group is shown here. Stem cells (α6-integrin+/ transferrin-) accounted for a mean of 7%, TA cells (α6-integrin+/ transferrin+) 88%, and differentiating cells (α6-integrin-) for 5% of all Annexin-V+ cells. A significantly higher number of apoptotic cells expressed surface markers consistent with TA cells ($p<0.001$), rather than stem or differentiating cells.

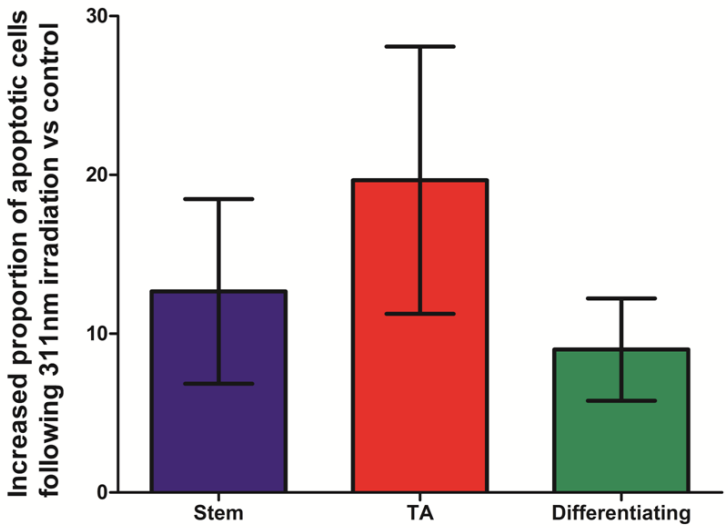


Figure 5-12. Cells of differing proliferative potential (proliferative compartments) have a similar susceptibility to apoptosis following UVB-irradiation *ex vivo*. The increase in the actual number of apoptotic cells within each compartment was compared following a single six SED dose of 311nm UVB and sham-irradiated controls, and expressed as a proportion in each experiment (n=3). Mean and standard error of the mean are shown. There was no significant difference between the three groups (p=0.51).

5.4. Discussion

These experiments indicate that 6-8 SEDs of both 311nm and 290nm UVB induce greater total apoptosis and an earlier onset of apoptosis than lower doses. The latter may be because apoptosis is occurring in response to a 'threshold' level of damage accumulating in cells, and it would therefore follow that cells exposed to higher doses of UVB should accumulate DNA damage faster (Li and Ho 1998). It was also observed that the highest dose of UVB used (10 SED) appeared to induce less apoptosis than 6-8 SEDs in both wavelengths, although a similar proportion of cells were PI+ indicating that cells may be dying via other pathways e.g. necrosis. The data presented here demonstrate by two different methods: live cell imaging and flow cytometry, that 311nm and 290nm induce a similar degree of apoptosis in primary (monolayer) human keratinocytes. This suggests that when equal erythemal doses of these wavelengths reach a cell they can induce similar apoptotic effects when penetration is not a confounding factor. In normal forearm skin, 290nm has been shown to penetrate into the lower epidermis approximately 14 times less than 314nm (Meinhardt, Krebs et al. 2009). This suggests that differential penetration of 290nm and 311nm UVB contributes, at least in part, to the differences in number of apoptotic cells observed in psoriatic epidermis after 290nmand 311nm irradiation. 290nm UVB clearly penetrates sufficiently to induce DNA damage (Setlow 1974) and non-melanoma skin cancer (Setlow 1974; de Gruijl and van der Leun 1994). Indeed, evidence suggests that 293nm is a more potent inducer of non-melanoma skin cancer in humans than longer UVB wavelengths, although this data is extracted from mouse data (de Gruijl and van der Leun 1994) , suggesting that differential penetration may not be the only explanation for our findings or a different chromophore is involved.

These *in vitro* studies were performer in normal human keratinocytes derived from neonates/ children. Ideally adult psoriatic keratinocytes would have been studied, but successful culture psoriatic keratinocytes remains difficult; this has yet to be achieved within our laboratory. Culture of adult keratinocytes was performed however, but this yielded very few stem cells and results obtained

were extremely variable. All experiments were therefore carried out using donations from neonates/ children.

Toxicity of live-cell nuclear dyes over time is an under-reported phenomenon, but is known to occur due to the formation of oxygen-dependent free-radicals, generated during the light-induced excitation of nuclear dyes with surrounding cellular components (Frigault, Lacoste et al. 2009). The primary purpose of using a nuclear dye in live-cell imaging is to provide a reliable cell marker for auto-focussing over a prolonged time period. Without a nuclear marker, the auto-focussing had to be dependent on PI, as minimal caspase-3 activity was detectable for the first 6-8h. This resulted in suboptimal measurement on occasions as the image was sometimes out of focus. The secondary reason for using a dye was to count the total number of cells imaged within a montage, allowing the proportion of apoptotic cells to be calculated. To contend with the latter issue, the dye was added at the end of 24h to allow accurate cell counts. As exclusion of these dyes until the end of the experiments resulted in a stable environment with only very low levels of apoptosis occurring in non-irradiated controls, it was concluded that both Draq-5 and Hoescht 33258 become toxic to cells when combined with the visible light or UV used to measure fluorescence over time.

The data showed a wide variation in the time for a cell to turn caspase-3 positive until its membrane was breached, indicating cell death. Cells are more likely to undergo UV-induced apoptosis whilst in G2M of the cell cycle. Arguably cells may be more susceptible to apoptosis if they are already in G2M and may therefore complete the apoptotic process quicker in this situation. Alternatively, cells may get damaged at any stage of the cell cycle and start the apoptotic process, but continue cycling and only die when they reach G2M. These experiments only addressed late stage apoptosis as PI was used to analyse the cell cycle. Various techniques were used to fix all cells prior to analysis (including freezing in 100% methanol, permeabilising with 0.5% triton X and fixation with 3.75% formaldehyde) to allow inclusion of early stage apoptosis in the analysis (using a live -dead cell marker to distinguish early and late apoptosis), however analysis was hindered by poor PI entry with all of these

methods. Further studies are needed to address this, but are beyond the scope of this thesis.

The cell sorting experiments (Figure 5-9) showed that the majority of the epidermal cells isolated had cell surface markers consistent with keratinocytes as expected, and that the purest keratinocyte population is located in areas one and four. Only cells from these areas were therefore analysed. In 2010 we currently still do not have any reliable stem cell markers for the human epidermis. However, evidence exists to suggest that proliferating human keratinocytes express high levels of α6-integrin and these can further be subdivided into those expressing high levels of transferrin (TA cells) and those with low levels (putative stem cells) (Tani, Morris et al. 2000; Youn, Kim et al. 2004; Hayashi, Yamato et al. 2008). However, cells express these markers as part of a continuum rather than all or nothing expression. For this reason, analysis of putative stem, TA and differentiating cells was performed by examining the cells which fitted most neatly into each category, rather than trying to subdivide all cells. Surprisingly only a small proportion of the cells isolated by flow cytometry were classified as differentiating cells, although the literature suggests that 40- 60% of all epidermal cells are differentiating (Weinstein, McCullough et al. 1984; Bata-Csorgo, Hammerberg et al. 1993). It is possible that differentiated cells may be differentially susceptible to cell damage during trypsinisation, or that these larger cells undergo higher levels of shear stress during centrifugation, resulting in a higher proportion of these larger cells fragmenting, and are therefore not included in the analysis.

DEVD-NucView caspase-3 substrate allowed more accurate counting of apoptotic cells over a prolonged period as this is freely membrane permeable and cells retain this marker even after death. However, the fluorescence of this marker has a wide excitation spectrum, and it is therefore not suitable for use with other fluorescent markers such as PE and APC (which were used here to delineate proliferative potential of cells). Therefore this was substituted by Annexin V in further experiments which binds to phosphatidyl serine and has a more distinct excitation spectrum. During early apoptosis and necrosis, changes occur within the plasma membrane as phosphatidyl serine

translocates from the inner to outer leaflet of the lipid bilayer. However, in contrast to necrosis, the cell membrane remains intact during apoptosis, therefore Annexin V in combination with a marker of cell membrane integrity can distinguish apoptosis from other methods of cell death. As a result, dead (PI+) Annexin V+ cells were excluded from analysis resulting in a type 2 bias, i.e. any dead cell that had previously undergone apoptosis was discounted.

In all, this chapter shows for the first time that UVB induced keratinocyte apoptosis takes a median time of just 20 mins to complete, and that a wide intracellular variation was seen. Reasons for this variation were explored. Altogether, these results can be integrated with the *in vivo* data obtained in chapter three to inform the mathematical model described later (chapter six).

5.5. Conclusions

- Both 311nm and 290nm of UVB induce similar amounts of apoptosis in primary human keratinocytes using weighted equally erythemogenic doses. This suggests that poorer penetration of 290nm into the lower epidermis may account, at least in part, for the difference in apoptotic effect observed *in vivo* when skin is irradiated with these two wavelengths.

- Draq5 and Hoescht 33258 are toxic to primary keratinocytes over time when repeatedly exposed to visible light or UV during imaging.

- The median rate of UVB-induced apoptosis in primary keratinocytes is 20 mins, with a large intercellular variation which may reflect different cell cycle stage of DNA damaged cells.

- The majority of UV-induced apoptotic cells are TA cells, although a similar proportion of the putative stem cell and TA cell population are induced into apoptosis by 311nm UVB.

6. Mathematical modelling of psoriatic epidermis

6.1. Introduction

Although the preceding chapters showed that keratinocyte apoptosis occurs in psoriasis following the clinically effective but not the ineffective UVB wavelength, the overall relevance of this to clearing psoriasis was not directly addressed. Mathematical modelling allows dynamic investigation and testing of the experimental data to determine whether they may be sufficient to directly explain the observed clearance in patients.

A mathematical model of normal epidermis was created using a stochastic agent-based modelling environment as previously described (section 2.4.1). Until now, no dynamic *in silico* model of psoriasis development has been described, but development of such a model has allowed exploration of the contribution of specific quantifiable parameters (such as the analysis of cell cycle time for both stem and TA cells and the proportion of stem and TA cells actively dividing) on the overall homeostasis of the epidermis. Uniquely, the model combined known kinetic parameters with characteristic histological features of normal and psoriatic epidermis, and iterative stimulation allowed rigorous testing and establishment of robust boundaries within which the model remained stable.

To assess the contribution of keratinocyte apoptosis to remodelling of psoriatic epidermis back to normal, the measured apoptotic effects of UVB on psoriatic epidermis as determined by both the *in vivo* and *in vitro* data described above were inserted into the model. This showed that apoptosis of keratinocytes is sufficient to result in remodelling of psoriasis back to normal epidermis within the clinically expected time-frame for the dose given. Cell cycle arrest was also explored as a possible alternative mechanism for clearance of psoriasis. The model was used to predict the relative importance of apoptosis in cells of differential proliferative ability (i.e. stem, TA and differentiating cells).

6.2. Aims

Specifically, the aims of this chapter where to:

1. Create a robust model of normal and psoriatic epidermis using observed histological features and kinetic parameters derived from the literature.
2. Test whether the numbers of apoptotic cells observed in UV-irradiated psoriatic plaques in vivo were sufficient to account for plaque clearance following a series of repeat irradiations, which would be consistent with observed clinical clearance.
3. Use the model to predict which cells of different proliferative potential were more likely to undergo apoptosis in response to UVB, assuming that this is an important mechanism of clearance.

6.3. Results

Using the criteria defined in section 2.4, a model was created with the Netlogo 4.1 programming environment (appendix F). The model was designed to allow user friendly adjustment of parameters derived from the literature, via a series of sliders adjacent to the model window (Figure 6-1). This enabled testing of multiple scenarios which may account for the hyperproliferative state seen in psoriasis.

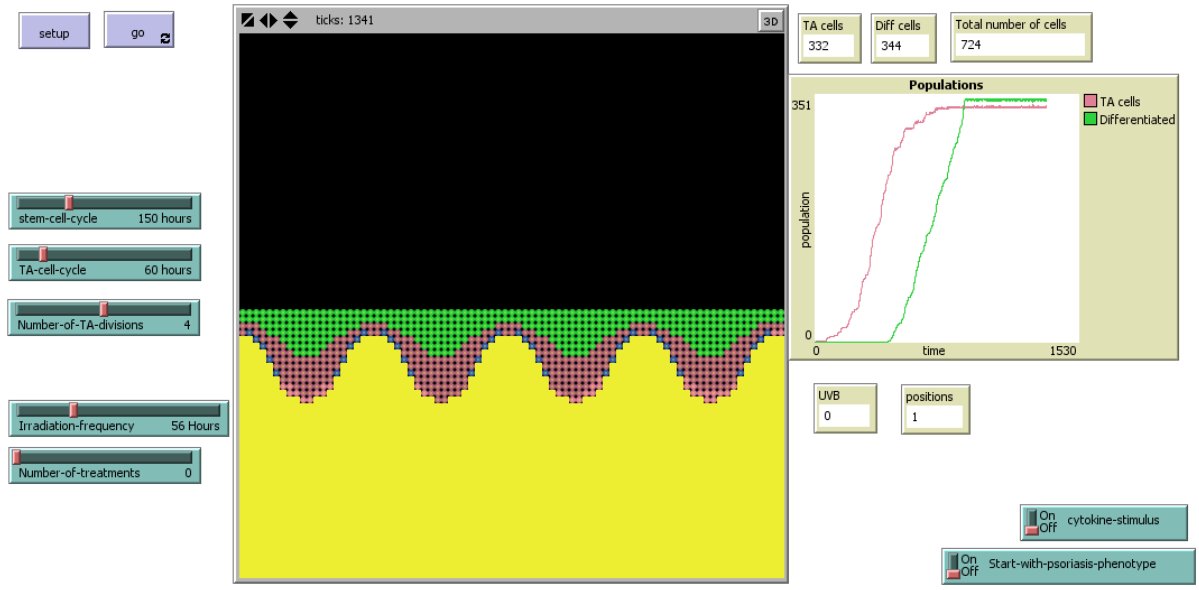


Figure 6-1. The model interface consists of buttons, sliders, and monitors to allow easy adjustment of parameters by the user, and observation of the result of these adjustments. Each 'tick' represents one hour, and is displayed at the top of the interface. As each turtle is born into the model its sequential and unique identifying number is displayed at the bottom of the interface in the observer panel. 'Start-with-psoriasis-phenotype on/ off' allows the user to choose whether to start the model as normal or psoriatic phenotype, but does not affect the running of the model in any way. The positions monitor goes from '1' (normal) to '7' (maximal psoriatic phenotype for this model), and will change according the number of cells and proliferation rate within the model. 'Cytokine-stimulus' allows the user to choose to 'switch on a stimulus' to increase the proportion of actively proliferating stem and TA cells. Note that it takes over 900h for the model to start to reach equilibrium (as shown in the overview display window). Yellow patches represent the basement membrane, dermis and below. Stem cells are shown as blue, TA cells as pink (light pink when actively dividing and darker pink when resting), and green cells are differentiating.

6.3.1. **Model of normal epidermis**

The keratinocyte compartment of normal epidermis was modelled *in silico* by setting the parameters outlined below, which represent typical values derived from the literature (see section 1.5.1 and Table 6-1):

Parameter used in the model of normal epidermis	**Evidence**
Stem cell cycle is 150h	Estimated to be 100-200h (Potten and Bullock 1983; Potten 1986; Morris and Hopewell 1990)
TA cell cycle is 60h	Estimated to be 50-65h (Chopra and Flaxman 1974; Goodwin, Hamilton et al. 1974; Bauer and Grood 1975; Wright 1976)
TA cells start to age as they are born, but will divide four times before becoming 'resting' TA cells	Estimated to divide 3-5 times (Watt 1998; Savill 2003)
TA cells differentiate when they move out of the lower epidermis along a gradient, and reach a defined gradient from the basal layer	Demonstrated in the eye (Revoltella, Papini et al. 2007) and supported by histological evidence in skin (Ishida-Yamamoto and Iizuka 1995; Martinsson, Yhr et al. 2005; Korver, van Duijnhoven et al. 2006)
Differentiating cells die when they are pushed beyond a boundary of the top of the epidermis	This is an assumption based to histological evidence that the surface of the *stratum corneum* is flat, and the upper epidermal layer forms a continuous sheet
The *stratum corneum* is not represented in this model as it is the consequence not the cause of the underlying epidermis, and removing this *in vivo* does not affect the rest of the epidermis	The stratum corneum consists of dead cells therefore they cannot undergo apoptosis. The model doesn't attempt to deal with penetration of UVB.

Table 6-2. Parameters used for stochastic modelling and the evidence to support their inclusion.

Applying the above parameters creates an epidermis which fulfils the criteria set out in section 2.4.3. Specifically, the epidermis is 15 cells thick, contains

proliferating: differentiating cells in a ratio of approximately 1:1, has an epidermal turnover time of approximately 29 days (706 hours) and a transit time of 336h (14 days) for differentiating cells (Figure 6-2).

To test the stability of the epidermis, each of the following was tested by altering each parameter independently: rate of the stem cell cycle; proportion of stem cells dividing; rate of the TA cell cycle; and number of TA divisions occurring. Manipulation of each of these parameters was used to try to induce a psoriatic phenotype as described below.

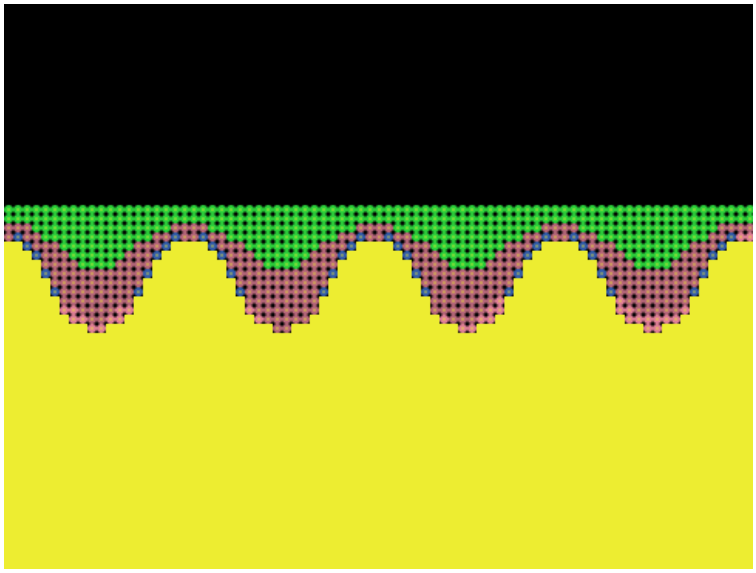


Figure 6-2. The normal epidermis *in silico*. Yellow patches represent the basement membrane and area below this, blue cells represent stem cells (only a small proportion of these will be cycling at any time), TA cells are shown in pink (dark pink cells are no longer cycling), and differentiated cells are shown in green. This epidermis maintained its stability over a tested period of 5000h.

6.3.2. **Model of psoriatic epidermis**

6.3.2.1. Altering the stem cell cycle time

Lengthening the stem cell cycle from 100 to 175h increased the overall turnover time by 16 days while still maintaining a stable epidermis. If the time taken for stem cell division was shortened to 60h the epidermis expanded to position 2 in the model, and the turnover time for the epidermis reduced to a total of 12 days (Figure 6-3). Therefore a shortened stem cell cycle time gives a turnover time that would be in keeping with psoriasis, but with a very modest increase in total cell numbers of just 16%.

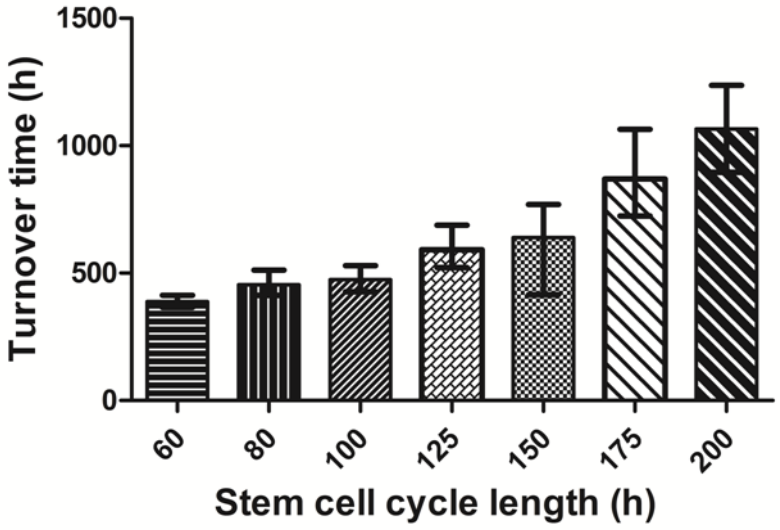


Figure 6-3. Turnover time of the epidermis gets longer as the stem cell cycle increases in length. Stochastic modelling shows that altering the stem cell cycle between 80h and 100h does not affect the total turnover time of the epidermis ($p=0.21$), but other changes were significant ($p<0.05$). Median and inter-quartile ranges shown (n=50).

6.3.2.2. Altering the TA cell cycle time

TA cell cycle adjustments of 20h intervals were tested within the model, to see if this would affect the turnover rate of the epidermis (Figure 6-4). Adjusting the TA cell cycle between 20h and 60h had no significant effect on turnover rate (p=0.307). This is as expected, because TA cells have a period of time where they rest within the epidermis, before being pushed too far from the basal layer and differentiating. After 100h the number of proliferating cells is too great and the basement membrane would need to expand in order to contain the cells, although total time for cell turnover increases (900h; 37.5 days), rather than shortens as is seen in psoriasis.

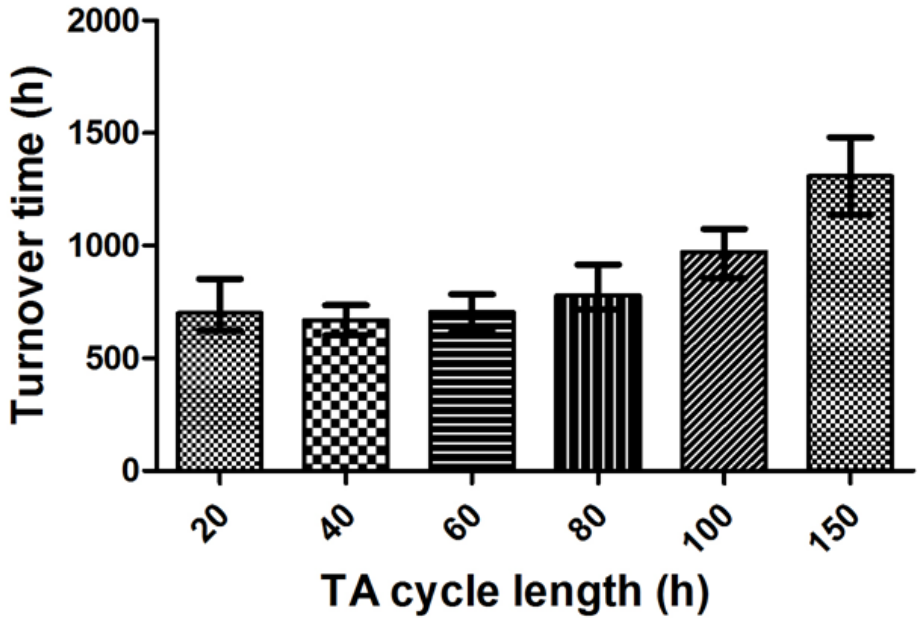


Figure 6-4. Turnover time of the epidermis increases as the TA cell cycle increases in length. Note that altering the TA cell cycle between 20h and 80h does not affect the total turnover time of the epidermis. Median and inter-quartile ranges shown (n=50).

6.3.2.3. Altering the number of TA cell divisions

After a TA cell is produced by a stem cell, it will normally divide four times before differentiating. Increasing the number divisions from four to five resulted in an increase in proliferation within the epidermis. The basement membrane elongated to position '2' of the model and the turnover time reduced to approximately 430h (18 days) but with a modest increase in total cell numbers of 12%. Increasing the number of TA-divisions to six resulted in the model reaching position '7', with a doubling of the total number of epidermal cells and a shortening the turnover time to 12-13 days. However, because each stem cell division then resulted in a total of 64 new TA cells (rather than 16 cells if the TA cells divided four times) the pattern of proliferation within the epidermis became patchy, with minimal proliferation occurring in some areas while too many cells are dividing in others (Figure 6-5). *In vivo* however, there is little evidence to suggest that the proliferative rate is so variable throughout the epidermis.

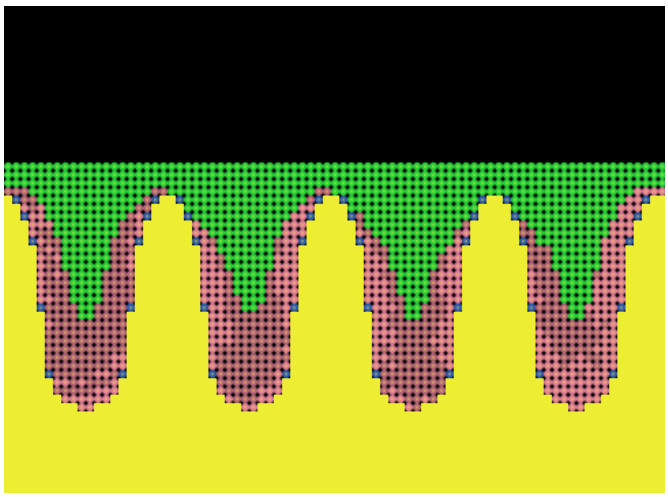


Figure 6-5. Increasing the number of TA cell divisions from four to six within the epidermis results in patchy proliferation. Note the high number of proliferating TA cells (bright pink) in the fourth rete ridge which extend well outside the suprabasal layer, whereas there are very few proliferating cells in the first rete ridge (dark pink). This patchy configuration is not seen clinically in psoriasis.

6.3.2.4. Altering the relative percentage of stem cells actively dividing

The baseline normal model contained 48 stem cells. To maintain stability of normal epidermis, 20% of these cells cycle at any time. If this proportion is increased by 20% intervals (while maintaining all other parameters as described above), the turnover time decreases as shown in Figure 6-6.

Although the turnover time gets quicker by increasing the proportion of dividing stem cells, it is not reduced as far as would be expected if this was the only change required to cause the increase in cell numbers in psoriasis. However, if the average number of TA cell divisions increases from 4 to 4.5 and the proportion of dividing stem cells increases from 20% to 80% then the turnover time reduces from 29 days (706h) to 11 days (270h) in the model of psoriatic skin. Furthermore the transit time of differentiated cells *in silico* reduces from 336h (14 days) to 100h (4 days), and this may explain how psoriatic plaques can develop over a period of just days following a known trigger such as a streptococcal throat infection. If the proliferative change occurred only at the stem cell level, then even if all stem cell start to divide at once it would take at least six days (i.e. approximately the length of the stem cell cycle) to have any noticeable clinical effect, and a further 8-10 days for the TA cells to complete four rounds of cell divisions; therefore maximal plaque thickness would take a minimum of 14 days after exposure. Clinical evidence suggests that the onset of plaques is much faster than this, often within four days of the onset of a sore throat (Gudjonsson, Thorarinsson et al. 2003). The model predicts that approximately four times the number of stem cells would need to be actively cycling, along with an increase in the number of rounds of TA cell division from four to five in at least 50% of TA cells, to induce this change in turnover time and a psoriatic phenotype (Figure 6-7 and Figure 6-8).

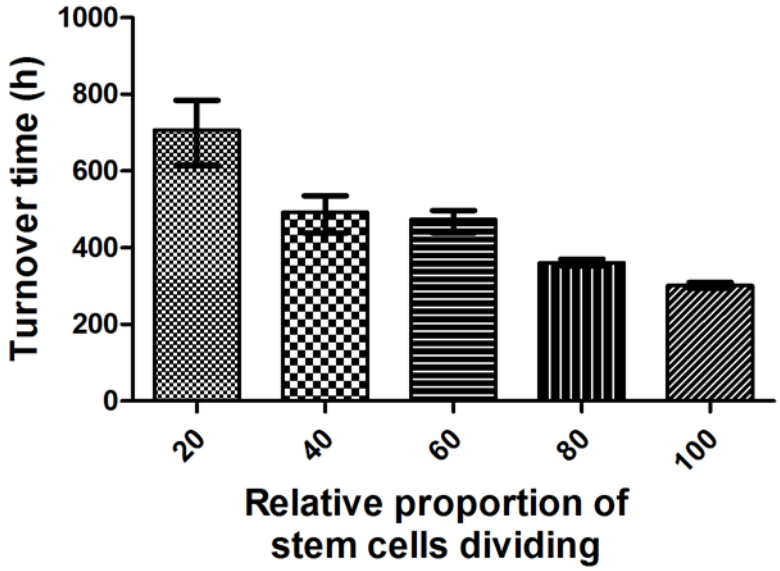


Figure 6-6. Turnover time of the epidermis increases as the proportion of actively dividing stem cells increases. Median and inter-quartile ranges shown (n=50).

6.3.3. Model of normal and psoriatic epidermis

A range of theories were tested which may explain the hyperproliferative keratinocyte compartment seen in psoriatic epidermis. Each was initially tested by altering just one parameter from the model described in section 6.3.1 above. The model was designed to allow flexibility of the basement membrane (as described in section 2.4.3), therefore elongation of the rete ridges can be seen as the total number of cells within the epidermis increases (Figure 6-13). This reflects the morphological pattern observed in psoriasis.

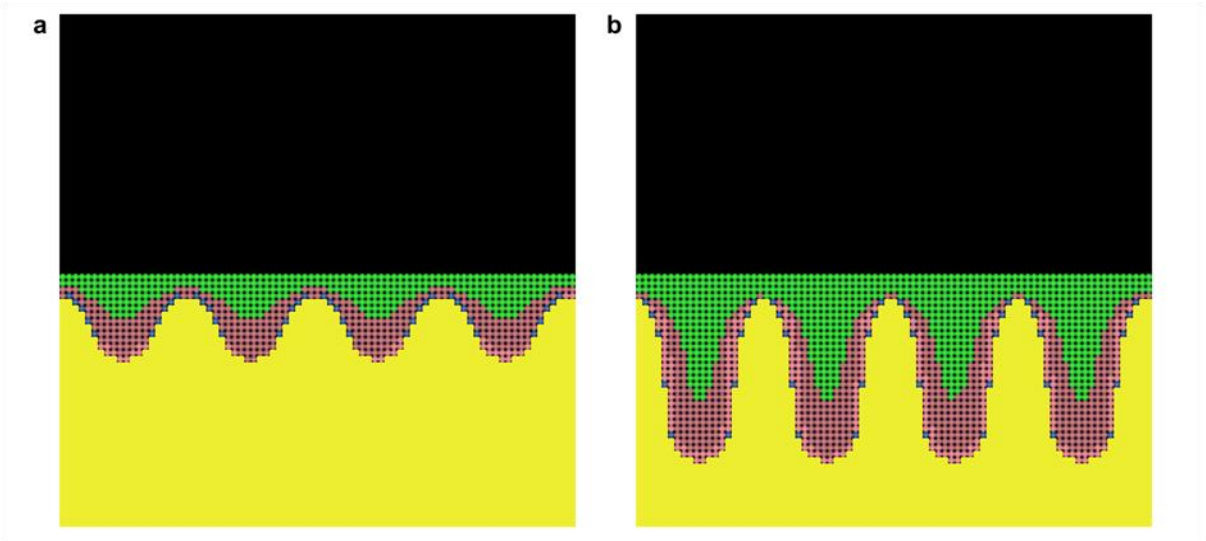


Figure 6-7. Relative increase in cycling stem cells from 20% to 80%, and an average number of rounds of TA cell divisions to 4.5, causes changes consistent with psoriasis. a) 'normal' epidermis with a total turnover time of 29 days for all cells, and transit time of 14 days for differentiating cells; and b) psoriatic' epidermis with a turnover time of 11 days and transit time of 4 days. Yellow patches represent the basement membrane and area below this, blue cells represent stem cells, pink cells are actively dividing TA cells (which are shown as dark pink when they are no longer dividing) and green cells represent differentiating cell.

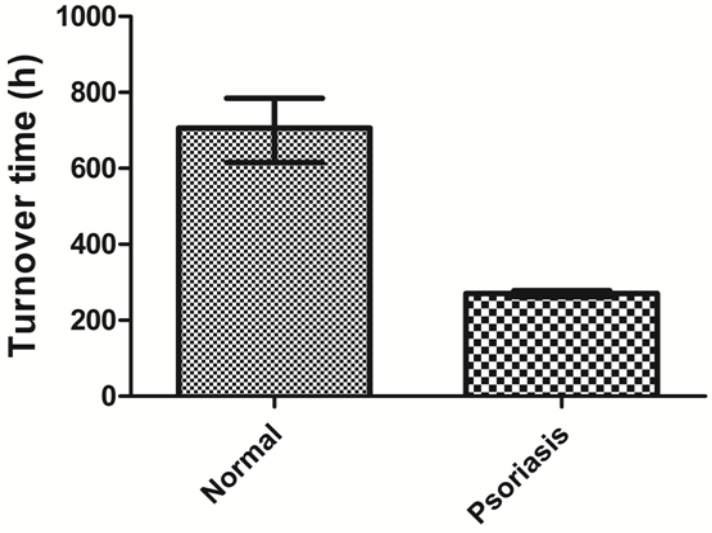


Figure 6-8. Turnover time for normal and psoriatic epidermis *in silico*. The mean (706h (29 days) in normal and 270h (11 days) in psoriatic epidermis) and SEM are shown for repeated simulations (n=50).

The model incorporated a threshold switch in the proportion of stem and TA (transit-amplifying) cells that proliferate, consistent with an immunological stimulus initiating psoriasis through cytokine signals that induce keratinocyte proliferation. Iterative simulation of the model allowed rigorous testing and establishment of robust boundaries within which the model remained stable (Figure 6-3- Figure 6-6). In contrast to adjusting cell cycle times, the model showed that increasing both the proportion of actively dividing stem and TA cells within 'normal' epidermis were key events contributing to the development of psoriasis. The model showed excellent concordance with existing data (sections 1.5.2) including an absolute increase in both the proliferative and differentiating compartments to levels found in psoriasis, and a reduction in total epidermal turnover time and the transit time of differentiating cells. Therefore to attain the above parameters the model predicted that an absolute increase in both the number of proliferating stem and TA cells was required to simulate psoriasis.

6.3.4. **Model of UV treatment to psoriatic plaques**

To model whether UV-induced keratinocyte apoptosis could directly impact on epidermal remodelling, it was necessary to first design experiments to determine the rate and duration of keratinocyte apoptosis as well as the differential sensitivity of stem, TA and differentiating cells to apoptosis. Apoptosis detection in human tissue *in vivo* is limited by the requirement for tissue sampling to determine the time course of events and the clearance rate of apoptotic cells; for example, immunochemistry can only provide a 'snapshot' of what is occurring in the skin at any one time. As real-time imaging of individual cells within human epidermis is not possible *in vivo*, further experiments were designed to address this question *in vitro* using primary keratinocytes (section 5.3.1), and these data were combined with observations from the *in vivo* data (section 3.3.4).

6.3.4.1. Estimate of number of apoptotic cells induced by a single exposure to UVB

Apoptosis of keratinocytes *in vivo* approximated to a Gaussian distribution, which can be described by the equation (TutorVista 2010):

$$f(x) = ae - \frac{(x-b)^2}{2c^2}$$

Where:

a = height of the peak

b = mean (time)

c = standard deviation

e = Euler's number (2.718)

Integration of this equation gives the area under the curve, which can be used to approximate the number of cells induced by a single three MED dose of 311nm UVB, and is expressed as:

$$ae - \frac{(x-b)^2}{2c^2}\,dx = ac\sqrt{(2\pi)}$$

This study has shown that a single six SED irradiation of 311nm to whole epidermis *ex vivo* causes apoptosis in 13% of stem cells, 15% of TA cells and 8% of differentiating cells (section 4.2.4). Section 5.3.1.1 shows that it takes around 20 mins from the onset of caspase-3 activation to cell membrane disruption, however it is unknown how quickly apoptotic cells are removed by phagocytosis within the epidermis. Data from this study indicate that the true figure is between 20 mins and 6 hours (the *in vivo* analysis showed significant differences between individual's number of apoptotic cells between 18h and

24h post irradiation). If it is assumed that each apoptotic cell remains detectable within the epidermis for 3-6h (sections 3.3.4), this would predict that 120-240 epidermal cells per 1000 cells undergo apoptosis over a 56h period following a single three MED irradiation (not including the number of new cells which would be produced by the epidermis during this time). Approximately seven 3 MED irradiations would then be required *in silico* to remodel psoriatic epidermis back to normal (Figure 6-12). If apoptosis also peaks at 18h (with a similar standard deviation to the observed three MED dose) for sub-erythemogenic doses of 311nm UVB, it would be possible to predict the number of apoptotic cells after different MED doses. Section 3.3.5 showed that in 10 patients the median number of apoptosis cells is 14.3 per 1000 epidermal cells 24h after three MEDs of 311nm, and that this reduces to 1.3 per 1000 epidermal cells 24h following 0.75 MED of 311nm (i.e. ratio of 11: 1). The 18h peak number of apoptotic cells following three MEDs is 29, therefore it follows that the 18h peak for 0.75 MED would be around 2.6 per 1000 epidermal cells. Using these data, the area under the curve calculation for 0.75 MED gives an estimate of 65 per 1000 cells undergoing apoptosis per single 0.75 MED irradiation. The model predicts that 25-30 irradiations of this dose of 311nm would be required if apoptotic cells remain detectable in the epidermis for an average of approximately 3h. This fits well with clinical observation.

6.3.4.2. Modelling proliferative potential of apoptotic cells

Section 5.3.4 describes the probability of cells of differing proliferative potential undergoing apoptosis in response to a single three MED dose of 311nm UVB. To test the individual effect of each of these within the model, the model was run sequentially, with UVB targeting either stem (Figure 6-9), TA cells (Figure 6-10) or differentiating cells (Figure 6-11) alone, or in combination (Figure 6-12).

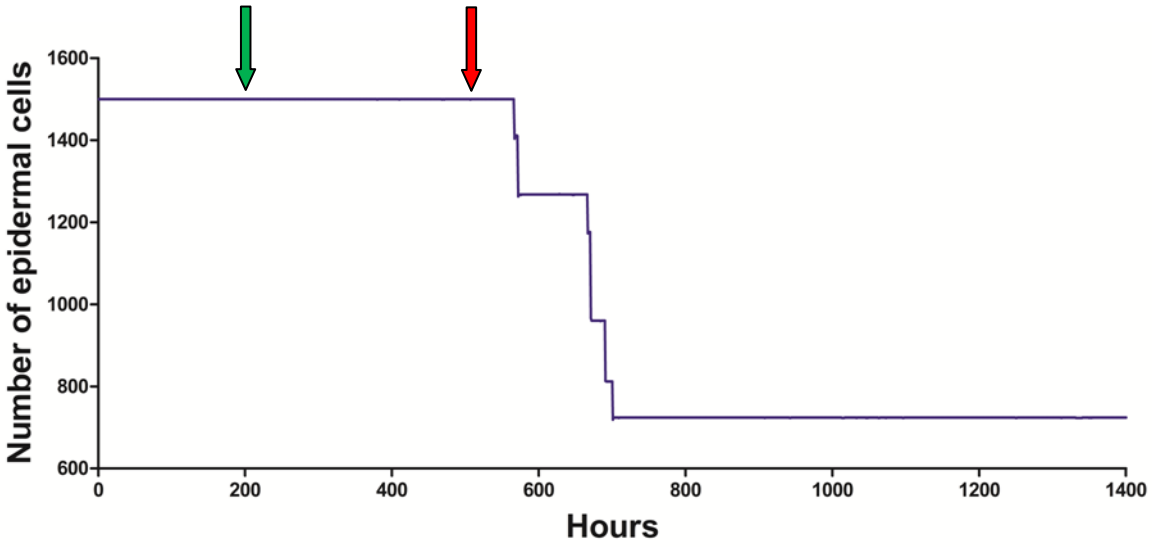


Figure 6-9. Model simulations of seven UVB treatments (three MEDs) if apoptosis occurs in only stem cells. In this model 13% of stem cells undergo apoptosis with each UV-irradiation. The TA cell cycle is 60h and the stem cell cycle is 150h. There is a delay of over 500h between the first irradiation and the beginning of plaque clearance. Green arrow shows time of first irradiation, and red arrow shows the final treatment.

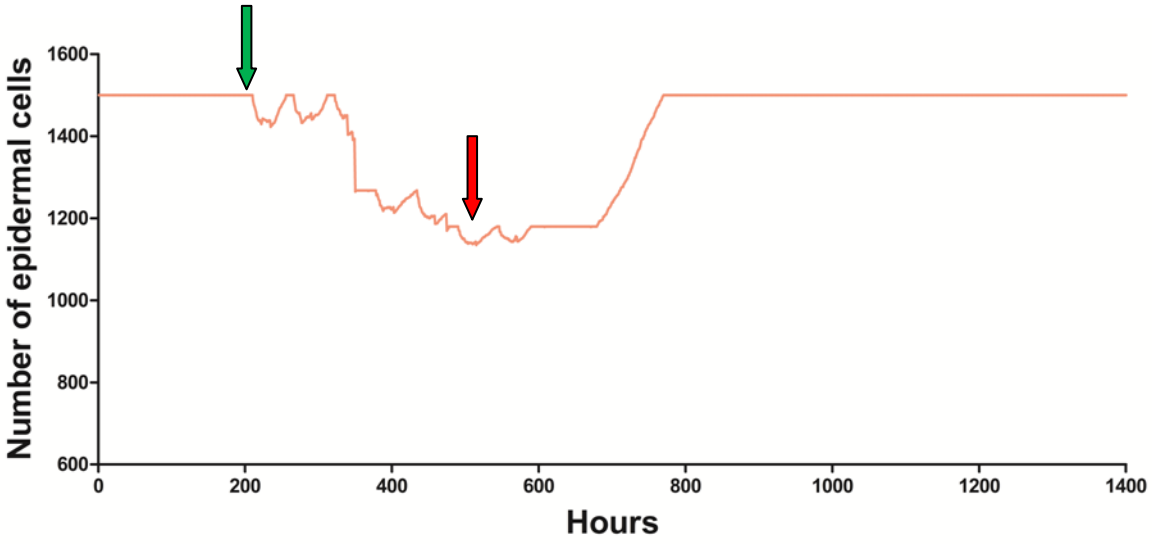


Figure 6-10. Model simulations of seven UVB treatments (three MEDs) if apoptosis occurs in only TA cells. In this model 20% of TA cells undergo apoptosis with each UV-irradiation. The TA cell cycle is 60h and the stem cell cycle is 150h. Although there is a fairly rapid 'clinical' response seen with this model, remodelling of the plaque is limited and temporary. Green arrow shows time of first irradiation, and red arrow shows the final treatment.

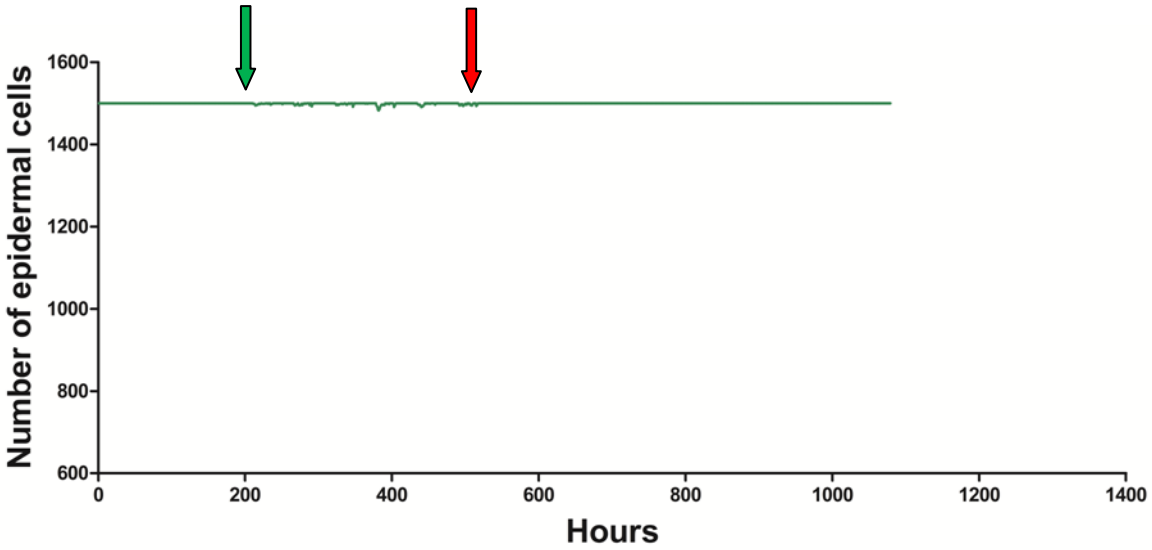


Figure 6-11. Model simulations of seven UVB treatments (three MEDs) if apoptosis occurs in differentiating cells alone. Nine percent of differentiated cells undergo apoptosis with each UV-irradiation. The TA cell cycle is 60h and the stem cell cycle is 150h. In this model, there is minimal impact on the epidermis, even when over 20 irradiations were given (not shown here). Green arrow shows time of first irradiation, and red arrow shows the final treatment.

6.3.4.3. Model assumptions

The following data were used in the mathematical model to simulate UVB irradiation, based on the above findings:

- An area under the curve calculation using data from Figure 3-7 was combined with the data shown in Figure 5-5 (duration of apoptosis in individual cells) to calculate an estimate of the rate of apoptosis.
- Apoptosis occurs at least 12h following apoptosis, with a mean time of 18h post-irradiation.
- Following a single exposure of three MEDs of 311nm UVB, 13% of stem cells and 20% of TA cells (see Figure 5-12) randomly undergo apoptosis. Differentiating cells do not undergo apoptosis in the model as this does not fit with the distribution of apoptosis seen *in vivo* (Figure 3-2 and Figure 3-8), and does not affect the overall outcome of the model (Figure 6-11).
- As the number of proliferating cells decreases in response to treatment, it is assumed that an average of 4.5 rounds of TA divisions back to four will occur gradually (as immunocytes and keratinocytes produce cytokines which drive the proliferative process) and this is reflected in the model.
- If a stem cell undergoes apoptosis it will be replaced over time by symmetrical division of a neighbouring stem cell. This new stem cell will divide 20% of the time (as for normal epidermis). Therefore the same number of keratinocyte stem cells would be expected in the epidermis before and after a course of phototherapy.

As cells undergo apoptosis, the basement membrane gradually contracts back to its 'normal' size (Figure 6-13); this can be seen as a movie at: http://research.ncl.ac.uk/psoriasis. The movie starts showing normal epidermis (position '1') which is stable over time. At 1400h (hours are shown as 'ticks' at the top of the movie) a 'cytokine stimulus' is triggered (i.e. there is an increase in proliferation inducing cytokines due to an external trigger), and the basement membrane expands in a serial manner from position '1'-'7'. The stimulus is

withdrawn at 1900h but the psoriasis remains stable, and the epidermis maintains homeostasis in this state. At 2300h seven doses of three MEDs 311nm UVB are administered (as described above), and these are given at 56h intervals (equivalent to three times per week). This returns the epidermis to the normal state, where homeostasis is retained until a further stimulus is received. Note that:

- The thickness of the epidermis is not affected after the first UV doses are administered; most effects occur towards the middle/ end of the course.
- The *stratum corneum* is not modelled here as it is assumed to be a consequence of the underlying epidermal changes, but in clinical practice this would result in a hyperkeratotic surface.

The program interface allows the user to adjust the rate of stem and TA cell division, as well as the number of irradiations and their frequency. The model can either be started in the 'normal' position, or as 'psoriasis'.

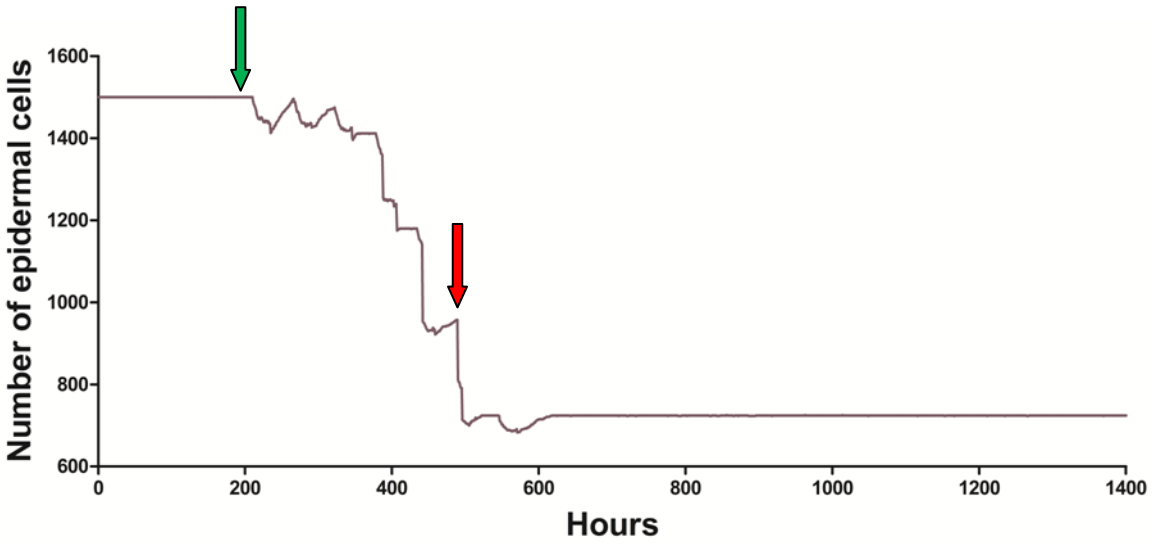


Figure 6-12. Model simulations of seven UVB treatments (three MEDs) if apoptosis occurs in both stem and TA cell without an effect on differentiating cells. In this model 13% of stem cells and 20% of TA cells undergo apoptosis with each UV-irradiation. The TA cell cycle is 60h and the stem cell cycle is 150h. This model fits well with the clinical situation, as the initial treatments have little impact on plaque remodelling, but complete clearance can be achieved at the end of the last (7th) treatment, and can be maintained. Green arrow shows the time of first irradiation, and the red arrow shows the final treatment.

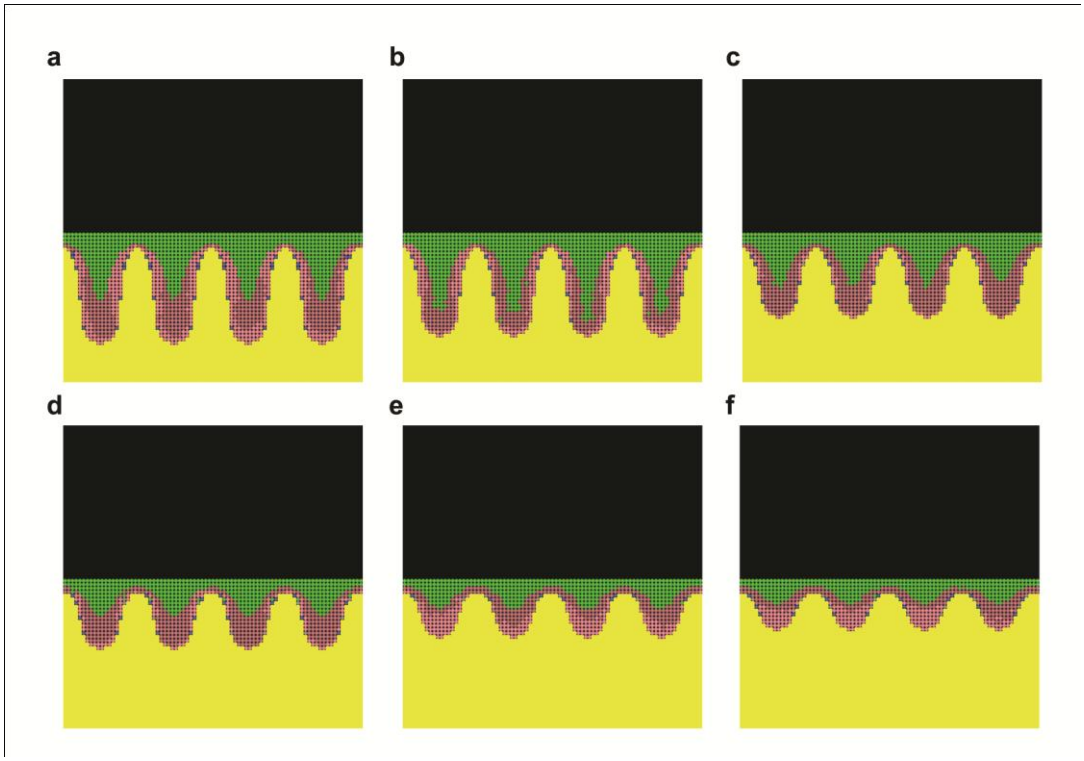


Figure 6-13. Remodelling of psoriatic epidermis back to normal, showing progressive reduction in depth of rete ridges following UV-induced apoptosis, in response to reduced cell numbers. Eight 3 MED treatments are given in this example. Six of the seven possible positions of the basement membrane are shown, where (a) is the maximal psoriatic phenotype and (f) represents normal (non-lesional) skin.

If cell cycle arrest rather than apoptosis was the mechanism of clearance following UVB, the model shows that psoriasis would continue to improve for a period of 32 days (Figure 6-14) after a UVB treatment course had completed. In clinical practice further clearance of psoriatic plaques is not seen after completion of a course of UVB, therefore if the model is correct, it is unlikely that UVB clears psoriasis through cell cycle arrest.

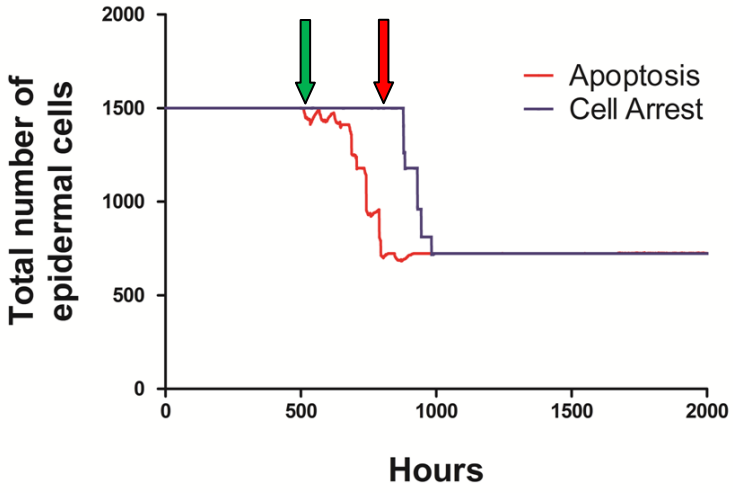


Figure 6-14. Comparing time taken to remodel psoriatic epidermis during and following a course of three MED of 311nm UVB (UVB given every 56h, representing three times per week). In this model 13% of stem cells and 20% of TA cells undergo apoptosis (red) or cell cycle arrest (blue) with each UV-irradiation. The TA cell cycle is 60h and the stem cell cycle is 150h. First irradiation was given at 500h (green arrow) and last at 836h (red arrow). Note that if the mechanism of clearance was cell cycle arrest, no change in epidermal thickness would be seen until after the treatment course was completed, with a lag time of 336h (14 days) post completion of the treatment course to clearance.

6.4. Discussion

The purpose of the model was to test the hypothesis that the observed rate of UVB-induced keratinocyte apoptosis in psoriatic epidermis could directly account for plaque clearance, and that the number of repeat irradiations required for clearance in the model was consistent with clinical observation. The results obtained in the modelling experiments were consistent with both these hypotheses. Moreover, if UV-induced cell cycle arrest was substituted for UV-induced apoptosis in the model then plaque clearance was not achieved.

The model created is based on parameters previously described in the literature, but differs from previous models as it also utilises observed histological features of psoriasis, and has not included fixed arbitrary parameters (previous models have used uninformed values such as cell's maximal lifespan or time spent within any defined compartment which have major consequences on the model outcome (Grabe, Pommerencke et al. 2006; Grabe and Neuber 2007; Sutterlin, Huber et al. 2009)).

The model provides evidence that the increase in the number of dividing cells may be directly responsible for the elongation of the rete ridges as seen in psoriasis, assuming that dividing cells need to be within a given distance of the basal layer. This is supported by immunohistochemical analysis of normal and psoriatic skin in which proliferating cells always appear in the basal or suprabasal layers, and analysis of differentiation markers such as keratin 10, S100A7 and involucrin show a similar pattern of distribution to that shown by the 'gradient' hypothesis proposed here (Ishida-Yamamoto and Iizuka 1995; Martinsson, Yhr et al. 2005; Korver, van Duijnhoven et al. 2006). In this model cells with the greatest proliferative ability are located closest to the basement membrane as has previously been described (Fuchs 2008), and are gradually pushed up to the surface as their proliferative ability diminishes (Figure 2-8). A vertical calcium gradient exists within the epidermis which is highest at the epidermal surface and lowest in the basal layer, and has been shown to affect differentiation (Elias, Ahn et al. 2002). However, as the histological pattern of differentiation within the epidermis is undulating rather than a flat layer (Korver,

van Duijnhoven et al. 2006), this cannot be solely responsible for inducing cell differentiating. Elucidation of the identity of soluble growth factor/ cytokine gradients arising from the dermis or basal layer are beyond the scope of this thesis, but speculation would include dermis-derived growth factors such as TGF-β1 or KGF (FGF-7), or that cells with the highest proliferative capacity have a greater number of receptors for these. The former has been shown to induce differentiation along an inverse gradient in skin culture (Ellis, Grey et al. 1992; Sun, Adra et al. 2009), and the latter has been suggested as a mechanism of FGF control of branching morphogenesis in lacrimal and salivary glands (Makarenkova, Hoffman et al. 2009).

The model demonstrates that a psoriatic phenotype could be achieved by either an increase in the proportion of stem cells dividing or an increase in the number of times a TA cell divides or both. However it is unlikely that the psoriatic phenotype is caused by just an increase in the number of TA divisions as this would lead to patchy areas within the epidermis of very high proliferation rates and areas of very little proliferation. This would result in neighbouring rete ridges varying in size extensively within a plaque, and this is not seen in plaques histologically (Figure 6-5). If stem cells alone were affected, it would take longer than is clinically observed for plaques to develop after the onset of a known trigger such as a streptococcal throat infection, and a course of UVB would not show any clinical effect until after the entire course of treatment (Figure 6-9); which does not fit with clinical observation. However, combining an increase in both stem and TA cell proliferation satisfies all criteria and leads to the expected epidermal turnover time and differentiating cell transit time.

When UVB phototherapy clears psoriasis, prolonged remission can be obtained suggesting that the mechanism of clearance may be different to other treatment modalities in which remission is not achieved (e.g. immunocyte targeted therapy). If the mechanism of UV clearance is directly due to keratinocyte apoptosis, this suggests that the psoriatic phenotype is due to an increase in birth rate rather an effect on cell cycle length or increased life span of individual cells.

Finally the model is presented as an interactive tool, whereby the user can control variables such as stem cell cycle time and can directly observe the effects of this on the epidermis over time. This will allow further application of the model in the future, and if sufficient data (including rate and magnitude of effect) describing other mechanisms of action for plaque clearance (e.g. autophagy) are described at a later date, the model could be utilised to assess whether these effects could be directly responsible for plaque clearance. The model therefore has the potential to provide further insight into psoriatic plaque development and the impact of treatment modalities.

6.5. Conclusions

- The model created is based using both observed histology and previously elucidated kinetic data for normal and psoriatic epidermis, and fits well with established data.

- Iterative stimulation of the model showed the model remained stable when tested within expected boundaries.

- When the experimental evidence from chapters three and five are used within the model, psoriatic epidermis is shown to remodel back to normal within approximately seven exposures of UVB using doses of three MEDs, and 25-30 exposures using sub-erythemogenic doses consistent with routine clinical treatment. Both these fit with published clinical observation.

- The model demonstrates that keratinocyte apoptosis can directly account for plaque clearance.

- If cell cycle arrest is substituted for apoptosis, plaque clearance will occur as for apoptosis but with a lag time of approximately 32 days; this does not fit with clinical observations, and suggests that no clinical improvement is likely to occur until after a course of medium dose UVB is completed.

7. Gene array

7.1. Introduction

Psoriasis has a complex genetic basis, and with the exception of HLA-Cw6, each of the underlying alleles only makes a small contribution to the overall phenotype. It is therefore important to study gene expression and gene interactions in order to understand the likely contribution of individual factors to the disease process. Array technology has transformed the field, for example, Illumina arrays allow high throughout analysis of over 46 000 genes simultaneously and can be used to assess up- and down-regulation of affected genes. Where these genes have a common function, further understanding can be obtained about the relevance of these.

Some previous gene array studies in normal human primary keratinocytes have shown that UVB up-regulates pro-apoptotic genes e.g. NOXA and MAPK (Takao, Ariizumi et al. 2002; Nys, Van Laethem et al. 2010) which activate the intrinsic apoptotic pathway. In contrast however, Li et al did not find any differential regulation in apoptotic genes within cultured keratinocytes at any time point up to 24h post irradiation with broadband UVB source (Li, Turi et al. 2001). It is unclear why these studies show different results although the latter used keratinocytes at one day post-confluency (rather than 80% confluent) which may have given cells greater protection from UVB and resistance to apoptosis; furthermore the cells used by Li et al were 3rd passage (rather than 1st) and may therefore have had a significantly smaller stem cell population . It has been demonstrated that the response to UVB differs between *in vivo* human epidermis and cultured keratinocytes, with the profile of the former biased towards DNA repair (even after doses of four MEDs) and the profile of the latter towards genes associated with cell cycle arrest and apoptosis (Enk, Jacob-Hirsch et al. 2006). For the purposes of this study, it was therefore decided to perform a gene array study of psoriatic plaques *in vivo* following irradiation of clinically effective and ineffective wavelengths of UVB.

There have been few gene array studies examining differential gene regulation in response to therapeutic intervention in psoriasis, and just one has looked at UVB. In the latter, 116 genes were differentially regulated following a

complete course of narrowband UVB, but no control wavelength was used to differentiate the bystander effects of erythema etc from therapeutic effects (Hochberg, Zeligson et al. 2007). Furthermore, arrays taken at the end of the course do not provide information about the mechanism of clearance that occurred earlier in the treatment course when the disease was very active, as expression of genes rapidly changes and only a small therapeutic effect is occurring within the skin at the end of a treatment course. Of the other interventional studies, the first looked at difference in gene expression in lesional psoriasis *in vivo* following treatment with ciclosporin in three patients at weeks 1, 4, 8 and 12 after commencing treatment (two responders and one non-responder) or an experimental immunomodulatory cytokine, rhIL-11 at the same time-points (five responders and one non-responder) (Oestreicher, Walters et al. 2001). This study found differential regulation of genes corresponding to psoriasis susceptibility loci and also related to aberrant IFNγ expression within the skin, which returned to normal levels prior to clinical improvement. However, further analyses of the results are difficult to interpret with such a small control group of non-responders. Two further studies have the compared the differential effects of biologic drugs between clinical responders and non-responders at least a week after commencing treatment (Zaba, Suarez-Farinas et al. 2009; Suarez-Farinas, Shah et al. 2010). The first of these examined biopsies of psoriatic plaques in 15 patients and found that only clinical responder's (n=11) down-regulated (back to baseline) genes associated with the IL17 pathway (Zaba, Suarez-Farinas et al. 2009), and the second examined PBMNs in 16 patients and showed that in responders (n=9) 23 genes were differentially regulated between the two groups although they did not attempt to group these genes functionally (Suarez-Farinas, Shah et al. 2010).

In this chapter, the effects of UVB on gene regulation were studied at early time-points (4h and 18h) following irradiation of psoriasis with a single exposure of either 311nm or 290nm UVB *in vivo*, and compared to each other and to non-irradiated epidermis. Moreover, the epidermis was studied in isolation by micro-dissection of frozen tissue, and there have been no studies looking at the gene profile of the psoriatic epidermis only. Comparing the *in vivo*

effects of clinically effective and ineffective UVB wavelengths on psoriatic epidermis (using equal erythemogenic doses), allows elimination of the large number of non-specific changes that occur in response to UVB irradiation.

7.2. Aims

- To investigate the differential gene response following irradiation with clinically effective (311nm UVB) and ineffective (290nm UVB) wavelengths of UVB, at early (4h and 18h) time-points post irradiation. In particular to examine for changes of apoptosis, and which apoptotic pathway is predominantly affected.
- To investigate differential effects of 311nm versus 290nm UVB on other pathways
- To validate the array findings using real-time PCR.

7.3. Results

Array analysis was performed in 48 micro-dissected epidermal samples from a total of 20 patients (maximum of 4 biopsies/ patient), using 30µm cryosections. These biopsies included involved untreated psoriatic epidermis, and psoriasis biopsied at 4h or 18h post irradiation with a single dose of 311nm or 290nm UVB (see Table 2-1). Counts of caspase-3 positive cells were performed in parallel for each biopsy using cryosections for IHC analysis. RNA is partially degraded by freezing biopsy specimens making gene array analysis more difficult when derived from frozen tissue. However, the recent addition of the whole-genome DASL (c-DNA-mediated annealing, selection, extension and ligation) micro-array assays to the Illumina platform (as discussed in section 2.5.4) has made RNA analysis from such tissue possible, with highly sensitive and reproducible results (http://www.illumina.com/products/whole_genome_dasl_assay_kit.ilmn).

RankProducts analysis in Bioconductor was used to identify genes within psoriatic epidermis which were significantly differentially expressed following irradiation with either: 311nm vs 290nm UVB, 311nm UVB vs un-irradiated, or 290nm UVB vs un-irradiated; at the 4h and/ or 18h time-point post irradiation ($p<0.05$). This amounted to a total of 389 genes 4h post UVB-irradiation, and 768 genes at 18h. These are described in more detail below.

7.3.1. Altered gene expression following irradiation with 311nm UVB compared to irradiation with 290nm UVB and untreated psoriasis

Using the analysis detailed in section 2.5.5, expression of differentially regulated genes induced at both 4h and/ or 18h post irradiation with 311nm UVB (compared to post irradiation with 290nm UVB or un-irradiated psoriatic epidermis) were quantified. At the 4h time-point, 46 genes were found to be significantly differentially regulated (n=9) and at 18h, 164 genes were significantly differentially regulated (n=11) ($p<0.05$). The Venn diagrams below illustrate the number of genes differentially regulated in each comparison (Figure 7-1). The genes differentially regulated by 311nm UVB and not 290nm UVB or un-irradiated psoriasis (i.e. in the purple areas of the venn diagrams) are listed in appendix G. At 4h, 12% (46/389) of the genes were dysregulated after irradiation with 311nm compared to 290nm UVB/ un-irradiated psoriasis, increasing to 21% (159/768) at 18h. The genes identified in the purple section of these diagrams are of greatest potential interest, as the study aimed to identify genes which are differentially regulated within lesional epidermis by therapeutic rather than non-therapeutic wavelengths of UVB, compared to un-irradiated lesional epidermis.

Data mining using Ingenuity pathway analysis (section 2.5.5) identified the differentially regulated genes of interest in this study, and these have been analysed in more detail below. A summary of their differential regulation compared to either 290nm UVB-irradiated or un-irradiated psoriasis is given in Table 7-1.

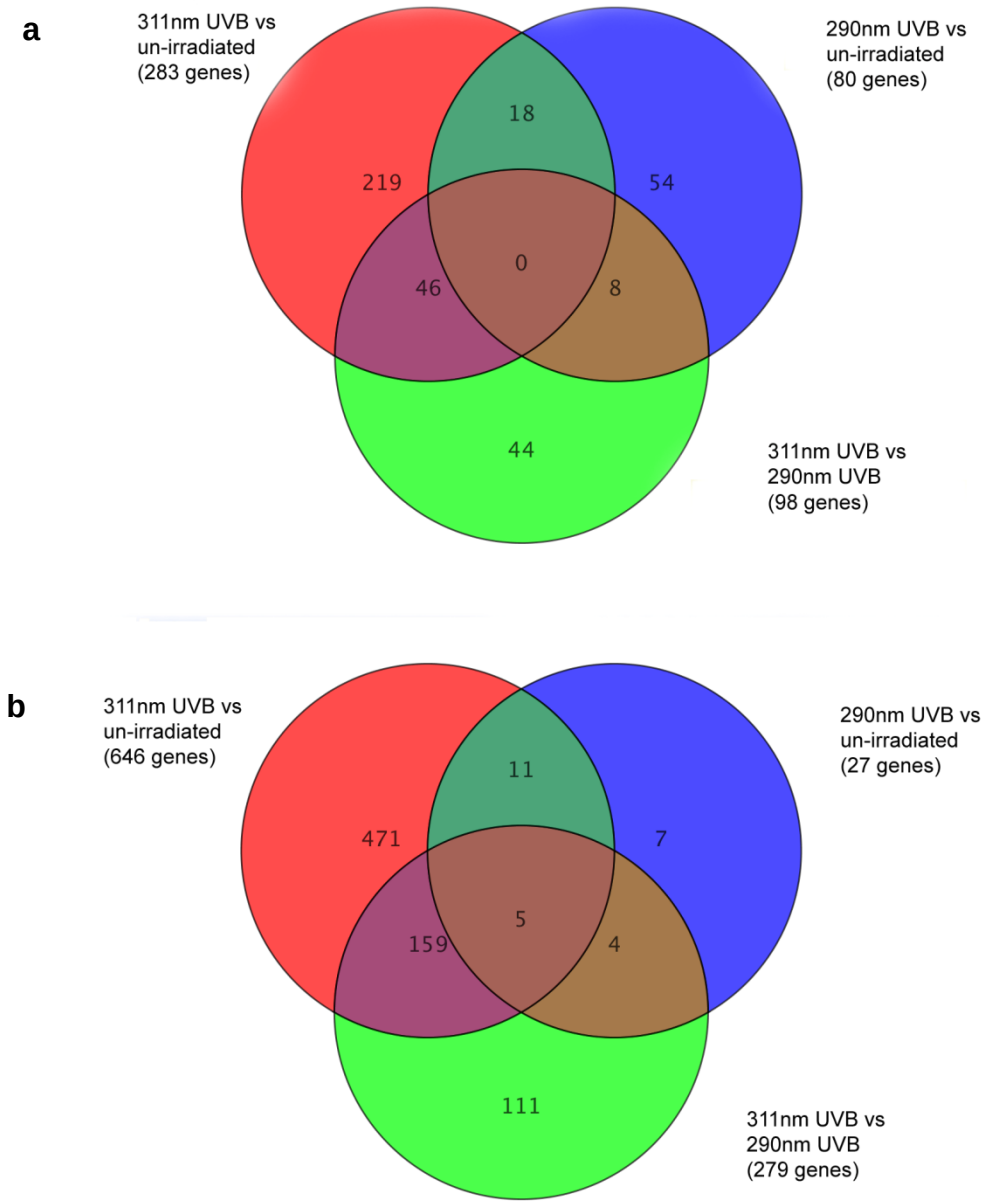


Figure 7-1. Venn diagrams showing the number of differentially regulated genes in psoriatic plaques following irradiation with 311nm and 290nm UVB compared with un-irradiated psoriasis. a) 4h post irradiation and b) 18h post irradiation. 311nm UVB differentially up-regulates 46 genes after 4h (n=9) and 159 after 18h (n=11), compared to irradiation with 290nm UVB and un-irradiated psoriasis.

Gene Name	Differential regulation in psoriatic epidermis following 311nm UVB compared to 290nm irradiation at 4h or 18h (p<0.05)	Differential regulation in psoriatic epidermis following 311nm UVB compared to un-irradiated psoriasis (p<0.05)	Major functional roles*
CDKN1A/ P21/ WAF1	↑	↑	Apoptosis Cell cycle regulation
MDM2	-	↑	Apoptosis Cell cycle regulation
GDF15	↑	↑	Apoptosis Differentiation
IL24/ MDA7	↑	↑	Apoptosis Differentiation
PIK3C2G	↓	↓	Apoptosis Cell proliferation Cell migration IL17 pathway
JUNB	↑	↑	Cell proliferation Differentiation Apoptosis
FOSL1	↑	↑	Cell proliferation Differentiation Apoptosis
MAPK10	↓	↓	Apoptosis IL17 pathway
IRF2	-	↓	Cell proliferation Cell cycle regulation Apoptosis
BUB1	-	↓	Cell cycle regulation Apoptosis
MMP3	↑	↑	Apoptosis Wound repair Tumour formation IL17 pathway
IL6	↑	↑	Inflammation Cell proliferation Cell cycle regulation Apoptosis

AURKA	-	↓	Cell cycle regulation Tumour formation
DLGAP5	-	↓	Cell cycle regulation Tumour formation
NEK2	↓	↓	Cell cycle regulation
DUSP4	↑	↑	Cell proliferation Differentiation
DUSP6	↑	↑	Cell proliferation Differentiation
AKT2	-	↑	IL17 pathway Tumour formation Angiogenesis Insulin signalling
NOS2A	↓	↓	IL17 pathway Antimicrobial peptide
IL19	↓	↓	IL17 pathway Apoptosis
IL8	-	↑	IL17 pathway Neutrophil activation
LCE2A	↑	↑	Precursor of cornified envelope of *stratum corneum*
LCE2C	↑	↑	Precursor of cornified envelope of *stratum corneum*
LCE3D	↑	↑	Precursor of cornified envelope of *stratum corneum*
LCE3E	↑	↑	Precursor of cornified envelope of *stratum corneum*
DEFB103A/ HBD-3	↑	↑	Antimicrobial peptide
DEFB4/ HBD-2	-	↑	IL17 pathway Antimicrobial peptide
PI3	-	↑	Antimicrobial peptide Atherogenesis
S100A12/ EN-RAGE	-	↑	Cell cycle regulation Differentiation Antimicrobial peptide Inflammation

PDGF-D	-	↓	Angiogenesis Cell proliferation Differentiation Cancer
CCL21	-	↑	Angiogenesis Chemotaxis
KRT77	↓*	↓*	Keratinocyte structure

**Data from Ingenuity pathway analysis*

Table 7-1. Summary of differential gene expression within psoriatic epidermis 4/18h post irradiation with a single dose of three MEDs of 311nm UVB compared to 290nm UVB or un-irradiated psoriasis. The major known functional roles are listed for each gene. * see Figure 7-11**.**

7.3.2. **Differentially regulated pathways**

The pathways of interest which were differentially regulated included apoptosis, control of cell cycle and the antimicrobial peptides. Of note, autophagy genes (such as p63 and Beclin 1) were not differentially regulated at the studied time-points.

At 4h post 311nm UVB the highest gene expression group compared to un-irradiated lesional psoriasis was cell death (p=6.33 x 10-7), followed by cell cycle (p=9.81 x 10-6) (Figure 7-2). Although expression profiles in these groups were still significantly altered at 18h post 311nm compared to un-irradiated lesional psoriasis, expression of these groups then ranked 21st (p=3.04 x 10-4) and 16th (p=6.01 x 10-5) respectively. Expression of antimicrobial peptides ranked 54th at the 4h time-point and 58th at 18h post 311nm UVB compared to un-irradiated psoriasis, with significance levels of p=5.74 x 10-3 and 5.6 x10-3 respectively.

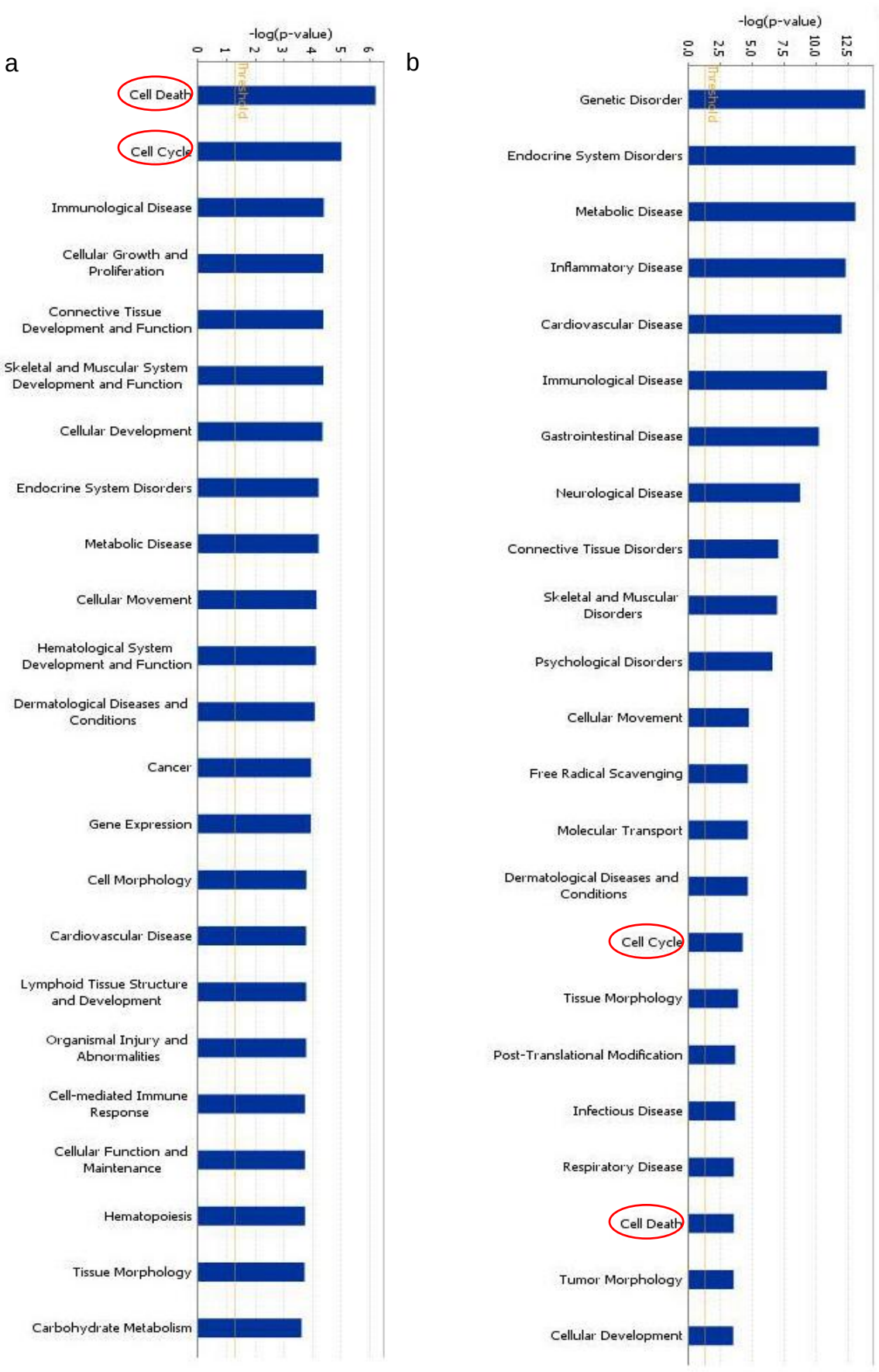

a
-log(p-value)
0
1
2
3
4
5
6
Threshold
Cell Death
Cell Cycle
Immunological Disease
Cellular Growth and Proliferation
Connective Tissue Development and Function
Skeletal and Muscular System Development and Function
Cellular Development
Endocrine System Disorders
Metabolic Disease
Cellular Movement
Hematological System Development and Function
Dermatological Diseases and Conditions
Cancer
Gene Expression
Cell Morphology
Cardiovascular Disease
Lymphoid Tissue Structure and Development
Organismal Injury and Abnormalities
Cell-mediated Immune Response
Cellular Function and Maintenance
Hematopoiesis
Tissue Morphology
Carbohydrate Metabolism
b
-log(p-value)
0.0
2.5
5.0
7.5
10.0
12.5
Threshold
Genetic Disorder
Endocrine System Disorders
Metabolic Disease
Inflammatory Disease
Cardiovascular Disease
Immunological Disease
Gastrointestinal Disease
Neurological Disease
Connective Tissue Disorders
Skeletal and Muscular Disorders
Psychological Disorders
Cellular Movement
Free Radical Scavenging
Molecular Transport
Dermatological Diseases and Conditions
Cell Cycle
Tissue Morphology
Post-Translational Modification
Infectious Disease
Respiratory Disease
Cell Death
Tumor Morphology
Cellular Development

Figure 7-2. Ingenuity pathway analysis of functional datasets differentially regulated at different time-points post irradiation of 311nm UVB to psoriatic plaques *in vivo*. Expression profiles are shown and values depict log mean values compared to un-irradiated lesional psoriasis. a) after 4h (n=9), and b) after 18h (n=11). Only the first 23 groups are shown in each case, with the yellow threshold line indicating significance at $p<0.05$ (i.e. all the datasets shown are above the significance level). Sixty genes are included in the cell death dataset and 34 in the cell cycle dataset at 4h, compared to 59 and 29 respectively at 18h.

7.3.3. Apoptosis and cell cycle regulatory genes

Some of the other most significantly differentially regulated genes following 311nm but not following 290nm UVB or un-irradiated psoriasis, were genes with important apoptotic effects. Many pro-apoptotic genes are also important in the control of cell cycle and some are described further below including CDKN1A, MDM2, DUSP6, IL6, AURKA AND BUB1. All biopsies were analysed in each case; i.e. at 4h post 311nm irradiation n=9, 18h post 311nm irradiation n=11, 4h post 290nm irradiation UVB n=6 and 18h post 290nm irradiation UVB n=8.

7.3.3.1. Differentially regulated genes at 4h post UVB-irradiation

Ingenuity analysis identified 23 significantly differentially regulated apoptotic genes which are directly or indirectly linked to each other in biological pathways leading to apoptosis (Figure 7-4).

CDKN1A (p21/ WAF1) was the most significantly up-regulated gene occurring at 4h post 311nm (with a 23.7 and a 27.5 fold up-regulation following irradiation with 311nm compared to un-irradiated or 290nm UVB-irradiated psoriasis respectively ($p<0.05$)) (Figure 7-3). The expression of CDKN1A is tightly controlled by p53 (Herbig, Jobling et al. 2004), and is selectively induced prior to apoptosis (Gomez-Manzano, Fueyo et al. 1997). CDKN1A is specifically cleaved by caspase-3-like enzymes, which in turn activates CDK2 leading to apoptosis (Gervais, Seth et al. 1998). This occurs via Bax activation within the mitochondrial pathway (Gomez-Manzano, Fueyo et al. 1997). CDKN1A is located on chromosome six, and acts as a regulator of cell cycle progression at G1 by binding to and inhibiting cyclin-CDK2 or –CDK4 complexes. It also regulates DNA replication and damage repair in S phase by interaction with proliferating cell nuclear antigen (PCNA). CDKN1A is controlled by MDM2 expression (see below). CDKN1A also has p53 independent effects on cell

cycle and apoptosis through interactions with other proteins (Murphy, Mabruk et al. 2002), as shown in Figure 7-4.

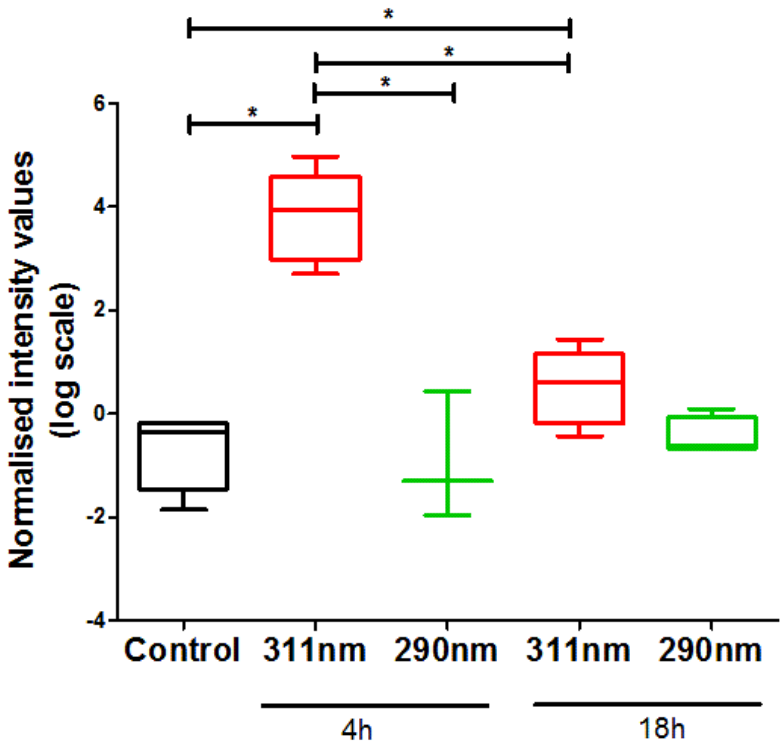


Figure 7-3. Expression fold change of genes in response to 311nm UVB and 290nm UVB at 4h and 18h post irradiation, compared to un-irradiated psoriasis for CDKN1A. Median and inter-quartile range shown, with whiskers indicating minimum and maximum values. Graphs shows normalised values centred around zero. Red plots represent irradiation with 311nm UVB and green plots represent irradiation with 290nm UVB. *Significant difference shown ($p<0.05$) (N.B comparison between 311nm at 4h and 290nm at 18h (and vice versa) not done).

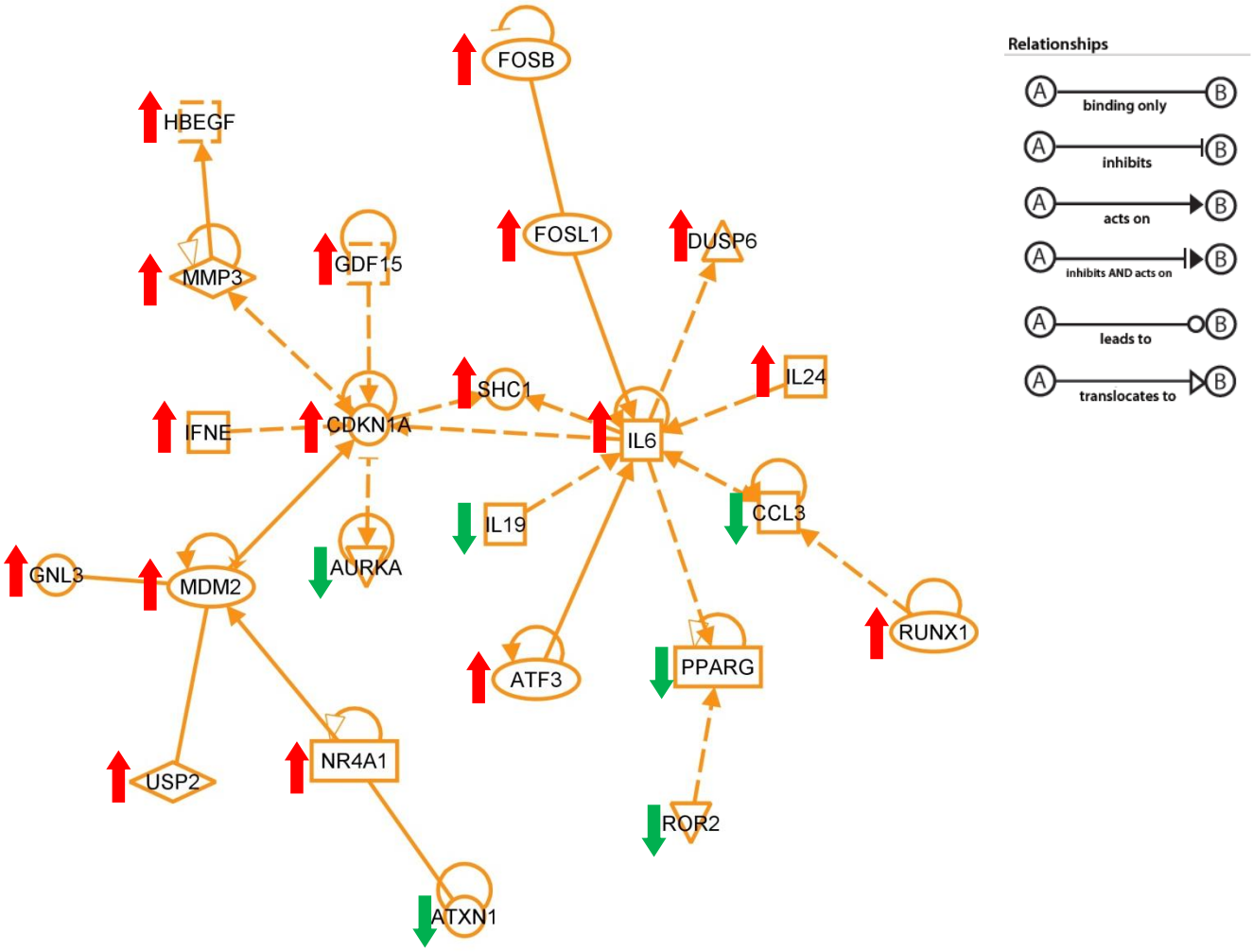


Figure 7-4. Interactions of the apoptotic genes, differentially expressed in psoriatic epidermis 4h post 311nm UVB irradiation *in vivo* compared to non-irradiated lesional epidermis. Ingenuity analysis revealed the above biological connections. Direct interactions are shown with uninterrupted lines, and indirect connections with dashed lines. Inverted triangles represent kinases, up-right triangles are phosphatase, squares are cytokines, interrupted squares are growth factors, diamonds are peptidases, rectangles are ligand-dependent nuclear receptors, ovals are transcription regulators and circles are other molecules. Red arrows indicate significant up-regulation, and green arrows indicate significant down-regulation of the genes compared to un-irradiated psoriasis. The number and close interactions of expressed genes seen in this network is highly suggestive that it will translate into biological changes of apoptosis.

Figure 7-5 shows the relationship of p53 (TP53) and caspase 3 (CASP3) to some of the key apoptotic genes identified. Interestingly RUNX1 is biologically connected to this pathway and has been identified as a psoriasis susceptibility locus (PSORS2) (Helms, Cao et al. 2003; Stuart, Nair et al. 2006); this was up-regulated 5.7 fold compared to un-irradiated psoriasis at the 4h time-point post irradiation with 311nm UVB, but not differentially regulated at 18h compared to un-irradiated psoriasis, or compared to irradiation with 290nm UVB at either time-point.

CDKN1A influences cell cycle progression at G1 and S phase, and appears to be the central node which directly interacts with the differentially regulated genes listed above, with altered expression following 311nm but not 290nm UVB (Figure 7-4 and Figure 7-5). Genes involved in checkpoint control at G2M were generally down-regulated, while those influencing G1 arrest were up-regulated. Genes associated with arrest in cell cycle featured prominently in the psoriatic epidermis taken 4h post 311nm irradiation with an overall significance of $p=1.35 \times 10^{-4}$. The following genes had altered expression in response to 311nm UVB in psoriatic epidermis (see Table 7-1):

- MDM2 is located on chromosome 12 and encodes a nuclear phosphoprotein which acts as a regulator of p53 and induces G1 arrest. Traditionally MDM2 was considered a negative regulator of p53, but recent evidence suggests that this gene may also promote p53 activity (Naski, Gajjar et al. 2009). This was up-regulated by 4.8 fold 4h following 311nm UVB compared to un-irradiated psoriasis ($p<0.05$), but this effect was not significantly different to irradiation with 290nm UVB (Figure 7-6). Therefore regulation of MDM2 is not specific to the therapeutic wavelength of UVB, and may be a bystander effect.

- IRF2 (interferon regulatory factor 2; a candidate for PSORS3, a previously identified psoriasis susceptibility locus) is situated on chromosome 4q. It promotes cell cycle proliferation, and is involved in cell cycle regulation by binding histone H4 during S phase, and may regulate a critical cell cycle checkpoint at G2M (Xie, van Wijnen et al. 2002). IRF2 has been shown to

up-regulate the anti-apoptotic protein BCL-2 (Wang, Liu et al. 2007). In this experiment IRF2 was down-regulated by 2.8 fold only by 311nm at 4h compared to un-irradiated psoriasis ($p<0.05$) but again was not significantly differentially regulated compared to 290nm UVB (Figure 7-6).

- BUB1: is involved in the spindle-assembly checkpoint, and also binds to p53 preventing apoptosis. BUB1 was down-regulated 4-fold at 4h following 311nm UVB compared to un-irradiated psoriasis ($p<0.05$) (Figure 7-6), consistent with in increased susceptibility to apoptosis (Beeharry and Yen 2009) but was not significantly differentially regulated compared to 290nm UVB.

- AURKA: encodes a protein required for mitosis, including spindle assembly and mitotic check-point control. This was down-regulated by 4.1 fold 4h following 311nm UVB compared to un-irradiated psoriasis ($p<0.05$), but not following irradiation with 290nm UVB (Figure 7-6).

- DLGAP5 (HURP/ DLG7) is important in spindle assembly in mitosis, and is down-regulated 2.8 fold 4h post 311nm UVB compared to un-irradiated psoriasis ($p<0.05$) but was not significantly differentially regulated compared to 290nm UVB.

- IL6: is cytokine has been shown to be up-regulated in response to UV induced DNA-damage in primary keratinocytes (Petit-Frere, Clingen et al. 1998). UVC was shown to up-regulate IL6 to a greater extent that UVB in the latter study, although the data shown here suggests IL6 is up-regulated by 3.6 fold following 311nm UVB compared to un-irradiated psoriasis and 4.5 fold compared to psoriasis irradiated with 290nm UVB ($p<0.05$) (Figure 7-6). IL6 induces cell proliferation and appears to be important for progression through G1 (Loyer, Ilyin et al. 1996), prevents apoptosis (Tassone, Neri et al. 2005), and augments the Th17 pathway which leads to an enhanced inflammatory response; the latter is further discussed in section 7.3.4.

- NEK2 encodes a protein kinase which forms part of the mitotic spindle-assembly checkpoint. It is located on chromosome one and is down-regulated by 4.4 fold 4h following 311nm irradiation compared to un-irradiated psoriasis ($p<0.05$), and 6.7 fold compared to psoriasis irradiated with 290nm UVB ($p<0.05$).

- JUNB (candidate gene for PSORS6) is located on chromosome 19 and is a core member of the activator protein-1 (AP-1) transcription complex which consists of dimers of the JUN, FOS, activating transcription factor (ATF) and musculoaponeurotic fibrosarcoma (MAF) families (Zenz and Wagner 2006). Enhanced activity (induced by activation of MAPK) regulates cell proliferation, differentiation, attenuates apoptosis via the mitochondrial pathway (Son, Heo et al. 2010) but promotes apoptosis via the death receptor pathway (Baumann, Hess et al. 2003). Down-regulation of JUNB is associated with hyperproliferation in psoriasis (Zenz, Eferl et al. 2005). Irradiation with 311nm UVB induced a 3.4 fold up-regulation of this gene at 4h compared to un-irradiated psoriasis ($p<0.05$) and 5.7 fold up-regulation compared to 290nm UVB-irradiated psoriasis ($p<0.05$).

- FOSL1 encodes a leucine zipper protein which can dimerise with proteins from the Jun family, forming the transcription factor AP-1, and has a role in cell proliferation, differentiation and transformation. It is located on chromosome 11 and is up-regulated by 8.5 fold 4h following 311nm irradiation compared to un-irradiated psoriasis ($p<0.05$) and 4.4 fold compared to 290nm UVB ($p<0.05$), and remains up-regulated at 18h (4.5 fold compared to un-irradiated psoriasis, and 4.4 fold compared to irradiated with 290nm UVB ($p<0.05$)) (Figure 7-6). Up-regulation of FOSL1 and JUNB are associated with reduced proliferation by targeting cyclin A2 (Pan, Nakade et al. 2010) which affects both G1/S and G2/M, and may promote apoptosis (see JUNB above).

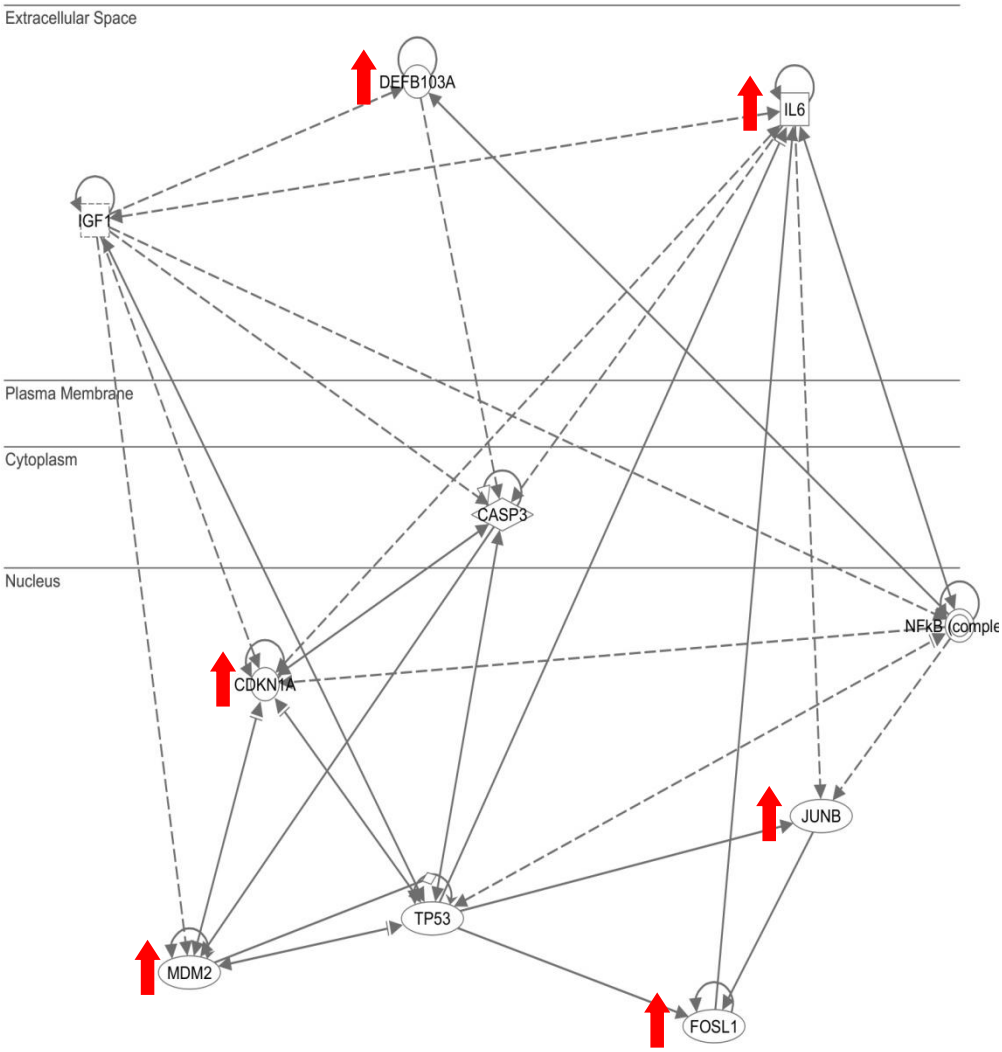
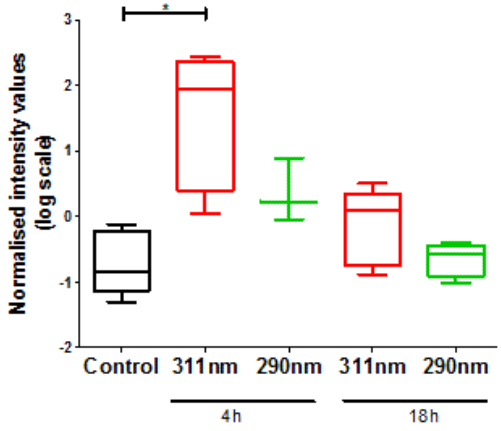


Figure 7-5. Canonical apoptotic pathway connecting key identified pro-apoptotic genes to p53 and caspase 3. Ingenuity analysis revealed the above interactions. Direct connections are shown with uninterrupted lines, and indirect connections with dashed lines. Squares represent cytokines, interrupted squares are growth factors, diamonds are peptidases, ovals are transcription regulators and circles are other molecules. Red arrows show genes significantly up-regulation compared to un-irradiated psoriasis. Where no arrow exists the gene was not differentially regulated in the dataset, although related proteins for 2 of these (TP53 (p53)-inducible nuclear protein and IGFL1) were both up-regulated, suggesting that these genes may be activated rather than differentially regulated, or differentially regulated at a different time-point. Note this also links in with the antimicrobial peptide DEFB103A (HBD-3), insulin-like growth factor 1 (IGF-1/ somatomedin-c) and the NFκB complexes.

a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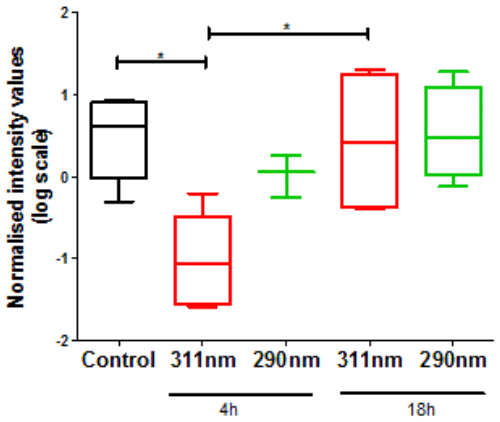
311nm
290nm
311nm
290nm
4h
18h

b


Normalised intensity values (log scale)
Control
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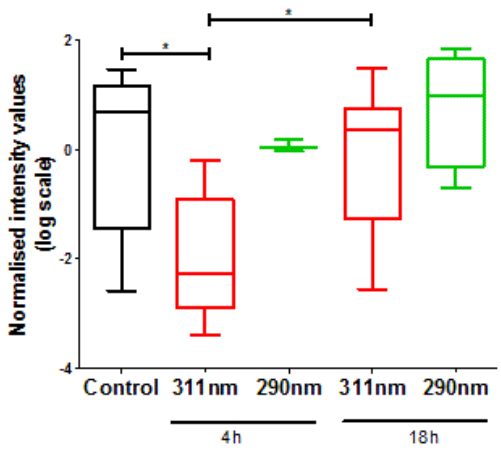
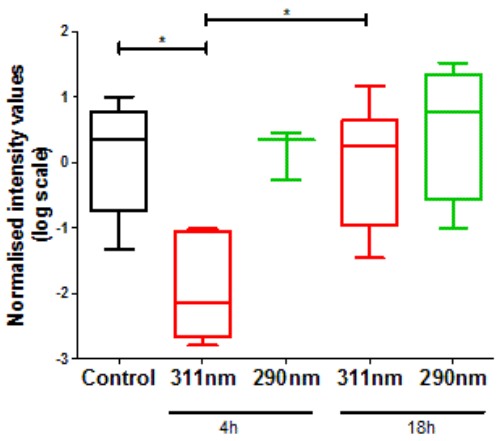
290nm
311nm
290nm
4h
18h

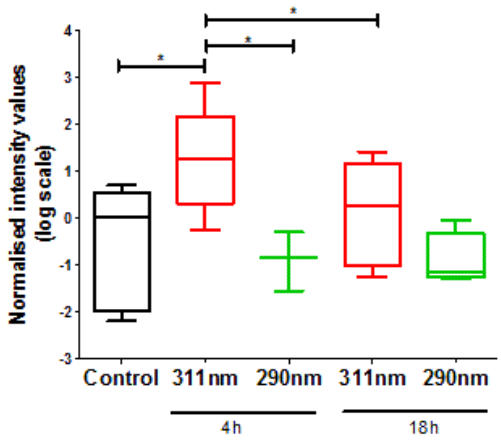
c


Normalised intensity values (log scale)
Control
311nm
290nm
311nm
290nm
4h
18h

d


Normalised intensity values (log scale)
Control
311nm
290nm
311nm
290nm
4h
18h

e


Normalised intensity values (log scale)
Control
311nm
290nm
311nm
290nm
4h
18h

f

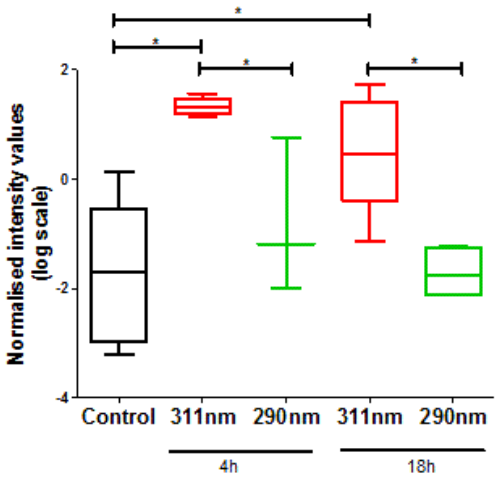

Normalised intensity values (log scale)
Control
311nm
290nm
311nm
290nm
4h
18h

Figure 7-6. Expression fold change of genes in response to 311nm UVB and 290nm UVB at 4h and 18h post irradiation, compared to un-irradiated psoriasis for a) MDM2, b) IRF2, c) BUB1, d) AURKA, e) IL6 and f) FOSL1. Median and inter-quartile range shown, with whiskers indicating minimum and maximum values. Graphs shows normalised values centred around zero. Red plots represent irradiation with 311nm UVB and green plots represent irradiation with 290nm UVB. *Significant difference shown ($p<0.05$) (N.B comparison between 311nm at 4h and 290nm at 18h (and vice versa) not done).

7.3.3.2. Gene expression maximally differentially regulated at 18h post 311nm UVB irradiation

The following genes were maximally differentially expressed at the 18h time-point, and are pro-apoptotic.

- GDF15 (growth differentiation factor 15; also known as MIC-1 or macrophage inhibitory cytokine-1) is a member of the Transforming Growth Factor beta superfamily and is located on chromosome 19. It is a transcriptional target gene for p53, and induces apoptosis via the MAPK signalling pathway (Yang, Choi et al. 2009). Interestingly it is thought to be a good prognostic marker for coronary artery disease (Schlittenhardt, Schober et al. 2004; Sun, Huang et al. 2010). It was persistently up-regulated between 4h and 18h post 311nm UVB compared to un-irradiated psoriasis (5.9-6.4 fold), and 290nm UVB (5.9-6.0 fold) ($p<0.05$) (Figure 7-7).

- MMP3 is expressed by keratinocytes and encodes a protein from the matrix metalloproteinase family which are involved in the normal breakdown of extracellular matrix. This enzyme plays a central role in apoptosis and is thought to interrupt extracellular matrix survival signalling, therefore accelerating the apoptotic process (Levkau, Kenagy et al. 2002). It also has functions in wound repair and tumour formation, and is located on chromosome 11. It was up-regulated following 311nm irradiation at 4h and 18h compared to un-irradiated psoriasis and 290nm irradiated psoriasis ($p<0.05$), but was maximal at 18h (9.5 and 10.7 fold change respectively).

- IL24 (MDA7) is a member of the IL10 family of cytokines. It is located on chromosome one and over-expression leads to elevated levels of several GADD genes, down-regulating the anti-apoptotic BCL-2 proteins resulting in apoptosis (Sarkar, Su et al. 2002). IL24 is up-regulated following 311nm irradiation at both 4h and 18h compared to un-irradiated and 290nm-irradiated psoriasis ($p<0.05$), but maximally at the latter time-point (3.2 and 5.4 fold respectively).

- PIK3C2G encodes a protein belonging to the phosphoinositide 3-kinase (PI3K) family. These proteins coordinate a diverse range of cell functions including proliferation and cell survival. PI3K activation leads to activation of AKT, which in turn phosphorylates BAD enhancing survival, therefore acting on the mitochondrial apoptotic pathway. This gene is located on chromosome 12 and is down-regulated 4.6 fold 18h post 311nm compared to un-irradiated psoriasis and 5.5 fold post 290-irradiated psoriasis ($p<0.05$), therefore promoting apoptosis.

- DUSP4 and 6 (dual specificity protein phosphatase) encodes a protein located on chromosome 8 and 12 respectively and are both up-regulated 4h following 311nm irradiation 3.1 and 7.5 fold respectively compared to un-irradiated psoriasis ($p<0.05$), and 6.3 and 8.8 fold respectively compared to psoriasis irradiated with 290nm UVB ($p<0.05$). These negatively regulate the MAPK (mitogen-activated protein kinase) superfamily which are associated with cell proliferation and differentiation (e.g. MAPK, SPHK1/ JNK (MAPK8) and p38. MAPK was significantly down-regulated 18h following 311nm in this experiment). Specifically, both inactivate ERK2 (MAPK1), and DUSP4 also inactivates ERK1 (MAPK3) and JNK (MAPK8), all of which are key regulators in cell differentiation and proliferation.

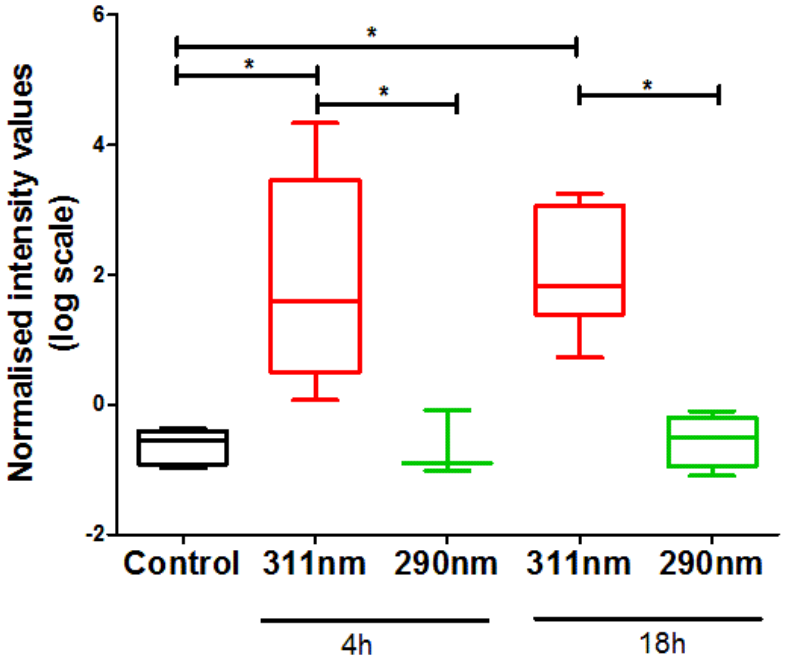


Figure 7-7. Expression fold change of genes in response to 290nm UVB at 4h and 18h post irradiation, compared to un-irradiated psoriasis for GDF15. Median and inter-quartile range shown, with whiskers indicating minimum and maximum values. Graph shows normalised values centred around zero. Red plots represent irradiation with 311nm UVB and green plots represent irradiation with 290nm UVB. *Significant difference shown ($p<0.05$) (N.B comparison between 311nm at 4h and 290nm at 18h (and vice versa) not done).

7.3.4. Adaptive immune system

Cytokines including TNFα, IL17 and IL22 are secreted by Th17 cells and are important in the development of psoriasis. At 4h post 311nm UVB four genes were differentially regulated from the IL17 canonical pathway compared to un-irradiated psoriasis: AKT2, IL6 and MMP3 were up-regulated and IL19 was down-regulated (IL6 and MMP3 were also significantly differentially regulated compared to irradiation with 290nm UVB) ($p<0.05$). At 18h, eight genes were significantly differentially regulated compared to un-irradiated psoriasis, of which three genes were up-regulated (DEFB4, IL8 and MMP3) and five genes were down-regulated (MAPK10, PIK3C2G, IL19, IL17f and NOS2A) ($p<0.05$).

Of these MMP3, MAPK10, PIK3C2G, IL19 and NOS2A were also significantly differentially regulated compared to irradiation with 290nm at 18h. Therefore at these early time-points the Th17 pathway appears to be induced.

7.3.5. **Innate immune system**

LCE2a, 2c, 3d and 3e were all significantly up-regulated in response to 311nm UVB ($p<0.05$); in particular at the 18h time-point (4.2, 3.6, 4.9 and 6.2-fold respectively compared to un-irradiated psoriasis and 4.1, 4.6, 3.1 and 3.7 fold respectively compared to 290nm irradiated psoriasis), consistent with previous data which showed group up-regulation in response to UVB in NHEKs and in skin biopsies from four patients (Jackson, Tilli et al. 2005). It is thought that this may occur as a protective mechanism, although further research is necessary. de Cid et al recently showed reduced copy number of LCE3C and LCE3B in psoriatic skin compared to controls (de Cid, Riveira-Munoz et al. 2009), suggesting that these genes may influence the psoriasis phenotype.

The gene with the largest fold change 18h following irradiation was the antimicrobial peptide DEFB103A (up-regulated by 10.8 fold 18h post 311nm irradiation compared to un-irradiated psoriasis, and 4.8 fold compared to psoriasis irradiated with 290nm UVB ($p<0.05$)) (Figure 7-8). DEFB103A, also known as HBD-3 (human beta defensin 3), is secreted by keratinocytes, and contain three disulphide bridges. It has a broad-spectrum antimicrobial activity but is preferentially active against gram-positive bacteria (Buchau and Gallo 2007). HBD-2 (also known as DEFB4) was up-regulated by 4.6 fold 18h post 311nm UVB irradiation compared to un-irradiated psoriasis, but not significantly differentially regulated compared to 290nm UVB-irradiation. Both human defensins are located on chromosome eight, are inducibly expressed by keratinocytes, and are strongly expressed in the psoriatic epidermis. Their presence has been shown to stimulate release of the proinflammatory cytokines IL8, IL18 and IL20 from primary keratinocytes (Niyonsaba, Ushio et al. 2005).

Associations of HBD-3 and apoptosis/ cell cycle regulatory genes are shown below (Figure 7-9).

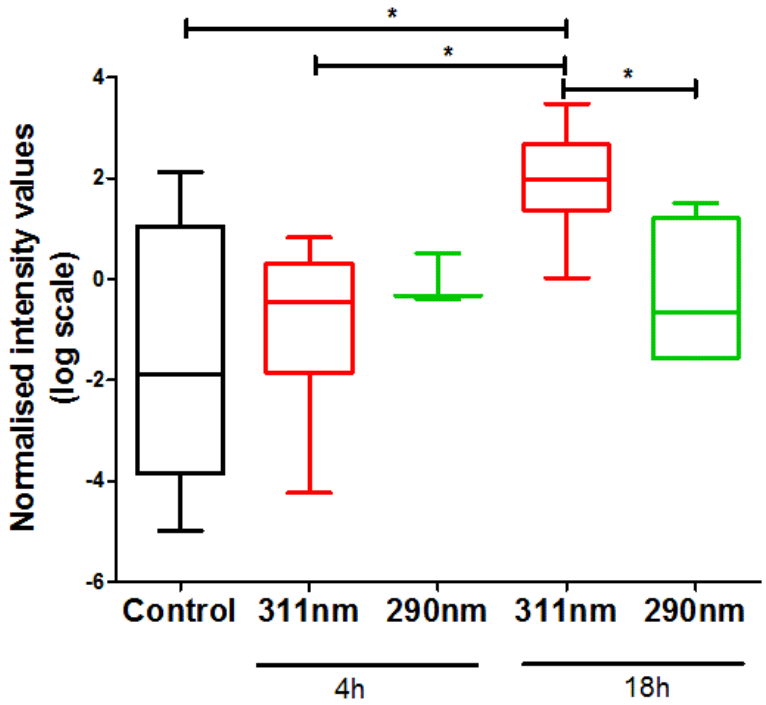


Figure 7-8. The human beta defensin-3 gene (HBD-3/ DEFB103A) is up-regulated by 311nm UVB at 18h compared to un-irradiated psoriasis and psoriasis irradiated with 290nm UVB. Median and inter-quartile range shown, with whiskers indicating minimum and maximum values. Graph shows normalised values centred around zero. Red plots represent irradiation with 311nm UVB and green plots represent irradiation with 290nm UVB. *Significant difference shown ($p<0.05$) (N.B comparison between 311nm at 4h and 290nm at 18h (and vice versa) not done).

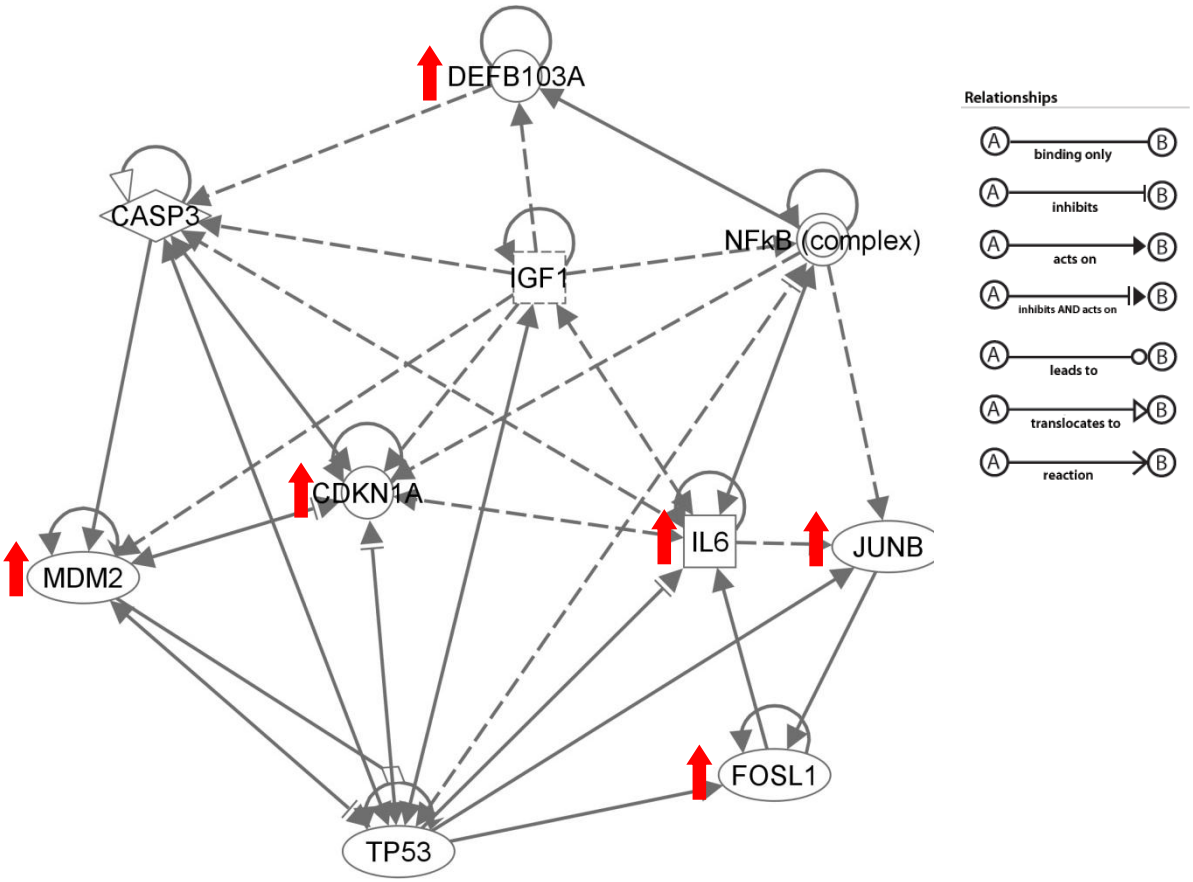


Figure 7-9. Functional network showing interactions between the antimicrobial peptide DEFB103A (HBD-3), and genes associated with apoptosis and cell cycle control. Red arrows show genes that were significantly up-regulated compared to un-irradiated psoriasis.

Other antimicrobial peptides to be differentially regulated by UVB were:

- PI3 encodes an elastase-specific inhibitor that functions as an antimicrobial peptide against Gram-positive and negative bacteria. It is located on chromosome 20 and was up-regulated by 4.1 fold 18h post 311nm UVB compared to un-irradiated psoriasis ($p<0.05$), this was not significantly differentially regulated compared to 290nm UVB.

- S100A12 (Calgranulin C/ ENRAGE) is located on chromosome one and was up-regulated by 2.3 fold 18h post 311nm UVB compared to un-irradiated psoriasis ($p<0.05$), this was not significantly differentially

regulated compared to 290nm UVB. It is a proinflammatory cytokine expressed by basal and suprabasal keratinocytes (Eckert, Broome et al. 2004), and is over-expressed in psoriatic skin (Mirmohammadsadegh, Tschakarjan et al. 2000). It activates AGER (RAGE) which is located within the MHC class three region on chromosome six, which can drive cellular dysfunction. It is not known if this affects apoptosis.

- NOS2A (nitric oxide synthesis 2; iNOS) is located on chromosome 17, and is down-regulated 2.1 fold at 18h post irradiation with 311nm UVB compared to un-irradiated psoriasis, and 2.1 fold compared to irradiated with 290nm UVB ($p<0.05$).

7.3.6. **Insulin resistance**

There has been much interest in the recently discovered association between psoriasis and the metabolic syndrome, which leads to an increased risk of cardiovascular disease (Prodanovich, Kirsner et al. 2009). This is thought to be due to the effects of insulin resistance, and may be regulated by genes affecting platelet aggregation such as p-selectin. In particular up-regulation of Peptidase inhibitor-3 (PI3) has been shown to be anti-atherogenic and in contrast to MAPK which is pro-atherogenic (Madonna, Pandolfi et al. 2004). In this study cardiovascular genes ranked 5th in the functional datasets at the 18h time-point following irradiation with 311nm UVB compared to un-irradiated psoriasis. Of the 149 genes significantly affected, 96 were associated with atherosclerosis, and 78 of these were down-regulated. This experiment showed that PI3 (anti-atherogenic) is up-regulated by four fold compared to un-irradiated psoriasis but is not differentially regulated by 290nm UVB, Platelet derived growth factor-D (PDGF-D) is down-regulated 2.3 fold and MAPK (pro-atherogeic) is down-regulated by 1.9 fold in biopsies taken 18h following 311nm compared to un-irradiated psoriasis, but not following irradiation with 290nm. Figure 7-10 shows interactions between the main genes involved.

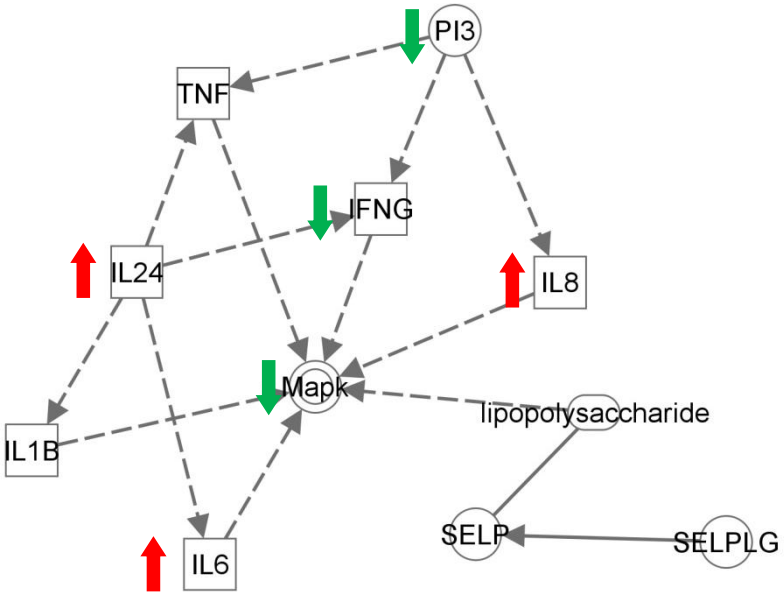


Figure 7-10. Simplified diagram showing interaction of genes involved in cardiovascular risk factors. Squares represent cytokines, ovals are transcription regulators and double circles are complexes, and circles are other molecules. Uninterrupted lines show direct relationships and broken lines show indirect relationships. SELP (P-selectin) was not differentially regulated in the analysis. Red arrows show genes significantly up-regulated and green arrows show significant down-regulation compared to un-irradiated psoriasis

7.3.7. Other genes of interest

It has previously been demonstrated that VEGF (which is released by keratinocytes) is elevated in psoriasis, and this decreases with treatment (Coimbra, Oliveira et al. 2010). In this study, CCL21 (which is an inhibitor of VEGF-c) was elevated 18h post treatment with 311nm UVB compared to un-irradiated psoriasis ($p<0.05$) but was not significantly differentially regulated compared to 290nm UVB. VEGF was not differentially regulated at the time-points studied. Therefore the data here is consistent with previous studies.

7.3.8. Differential regulation of genes by 290nm UVB-irradiation compared to 311nm UVB or un-irradiated psoriasis.

In chapter five the concept of reduced penetration of 290nm UVB with respect to 311nm UVB was partly addressed. However, results from this gene array study clearly showed that some genes were preferentially affected following *in vivo* irradiation with 290nm rather than 311nm (Figure 7-1) (e.g. the keratin gene KRT77 (Figure 7-11) and the ATPase enzyme transporter gene ATP6v0A4), indicating that sufficient quantities of 290nm UVB penetrate into the epidermis to have significant biological effects.

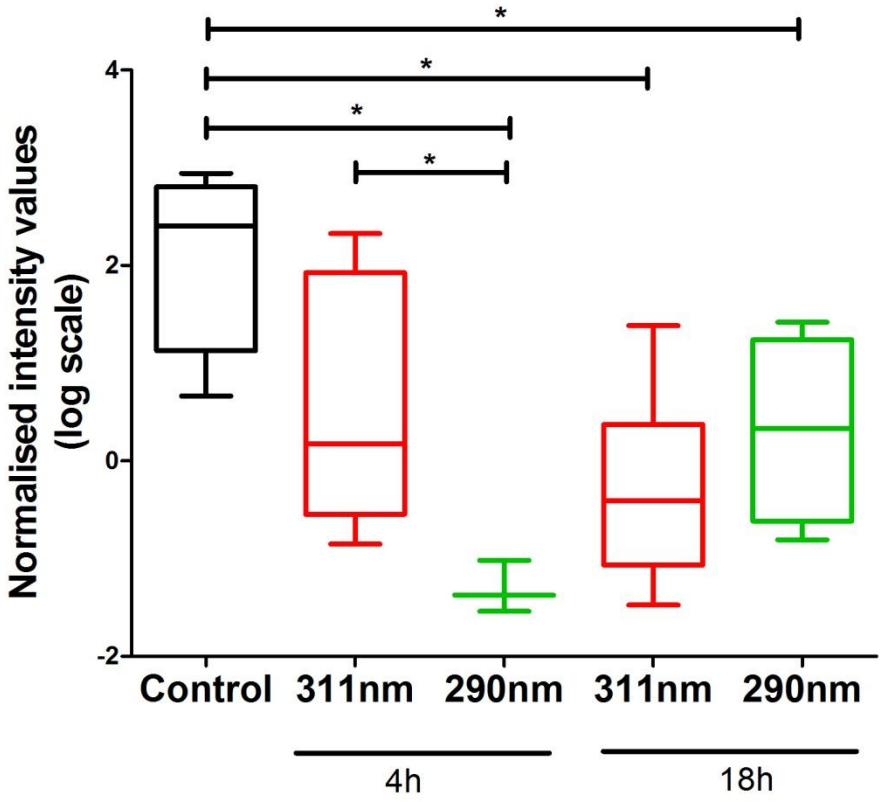
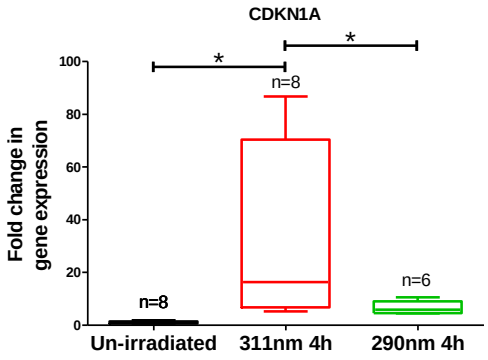


Figure 7-11. The Keratin 77 gene (Krt77) is down-regulated by both 311nm and 290nm UVB at 4h and 18h compared to un-irradiated psoriasis. Down-regulation is most marked at 4h following 290nm. Median and inter-quartile range shown, with whiskers indicating minimum and maximum values. Graph shows normalised values centred around zero. Red plots represent irradiation with 311nm UVB and green plots represent irradiation with 290nm UVB. *Significant difference shown ($p<0.05$) (N.B comparison between 311nm at 4h and 290nm at 18h (and vice versa) not done).

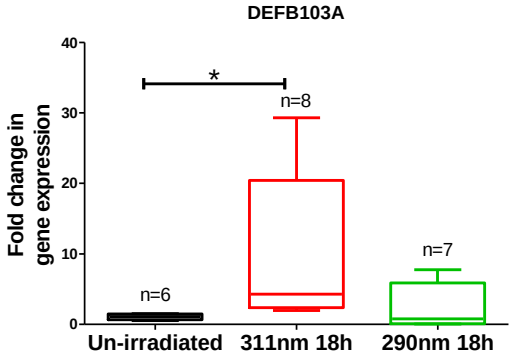
7.3.9. Real-time PCR validation of 311nm UVB induced gene expression changes

Five genes were chosen to validate the above arrays using a two-step real-time PCR reaction. These were chosen to represent differential regulation of genes at 4h and 18h post irradiation and following 311nm, and differential regulation between 290nm and 311nm UVB (section 2.6). The majority of DNA would be expected to amplify after 20-35 rounds of PCR if present in sufficient quantities, and would then reach a plateau with minimal further amplification. PCR which significantly amplifies DNA after 35 rounds is difficult to interpret as this suggests that very small quantities of the relevant gene were present within the DNA sample. As the quantity of each DNA sample has been standardised initially against an internal control (GADPH), it was known that the overall quantity of DNA was consistent between reactions, but if the gene is expressed at very low levels an accurate quantification is not possible unless the sample is run with a much larger DNA content (i.e. double the sample is required to amplify the DNA by just one Ct). As RNA had been extracted from micro-dissected epidermis, relatively small quantities were available for PCR. Consequentially, the quantity of DNA obtained was insufficient in some samples were the gene is expressed at very low levels, and as a result baseline levels (i.e. un-irradiated lesional psoriasis) of some genes (such as GDF15 and FOSL1) were not quantified. In these cases, the differential regulation of genes following irradiation with 311nm and 290nm UVB are directly compared. Overall these data correlated well with the above gene array results (Figure 7-12 and Table 7-2), although overall significance was not achieved for most of the PCR reactions due to small sample numbers.

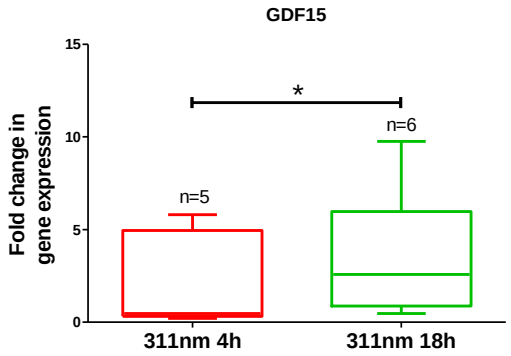
a



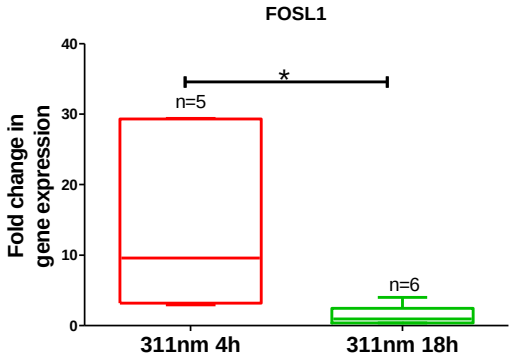
b



c



d



e

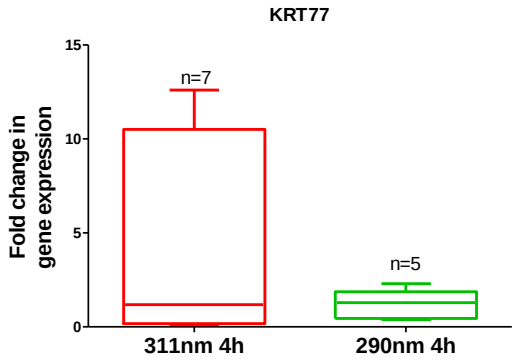


Figure 7-12. PCR results for 5 differentially regulated genes within psoriatic epidermis. Up-regulation of a) CDKN1A at 4h post 311nm UVB and b) DEFB103A at 18h post 311nm UVB respectively compared to un-irradiated or 290nm-irradiated psoriasis. c) GDF15 up-regulation 18h following 311nm UVB compared to the 4h time-point; d) FOSL1 up-regulation at 4h following 311nm UVB compared to 18h; e) Keratin 77 is up-regulated 4h following 311nm UVB compared to 290nm UVB-irradiation. Median and IQR shown with whiskers indicating maximum and minimum values. * indicates significance at $p<0.05$ using a Mann Whitney-U test.

Gene name	Fold change following irradiation with 311nm measured by gene array	Fold change following irradiation with 311nm measured by PCR
CDKN1A	↑ x **4** at 4h compared to 290nm UVB; ↑ x **28** compared to un-irradiated	↑ x **2.8** at 4h compared to 290nm UVB; ↑ x **19** compared to un-irradiated
DEFB103A	↑ x **4.8** at 18h compared to 290nm; ↑ x **10.9** at 18h compared to un-irradiated	↑ x **5.4** at 18h compared to 290nm; ↑ x **3.8** at 18h compared to un-irradiated
GDF15	↑ x **1.1** at 18h compared to 4h post 311nm UVB	↑ x **5.5** at 18h compared to 4h post 311nm UVB
FOSL1	↑ x **3** at 4h compared to 18h post 311nm UVB	↑ x **10** at 4h compared to 18h post 311nm UVB
KRT77	↓ x **3.5** fold at 4h with 290nm compared to 4h post 311nm UVB	↓ x **2.5** fold at 4h with 290nm compared to 4h post 311nm UVB

Table 7-2. Comparison of gene expression changes as measured by gene array and PCR following irradiation of psoriatic epidermis *in vivo*. Results generally show good concordance, validating the array process. GADPDH was used as a control probe for each sample in the real time PCR reactions, and standard internal controls were used for the Illumina DASL arrays.

7.4. Discussion

Analysing the differential effects of a clinically effective and ineffective psoriatic treatment provides a novel way to interrogate which changes in the transcriptome may be important in clearing psoriasis. This study has focussed on early time-points (4h and 18h) after a single irradiation with 290 or 311nm UVB in order to discover genes which may have a causal role in clearing psoriasis, rather than those whose expression mirrors clinical improvement. The major pathways affected were apoptosis and cell cycle regulation, and interestingly these pathways overlap considerably at the gene level. The most highly differentially up-regulated apoptosis-inducing genes following 311nm UVB-irradiation (compared to 290nm UVB-irradiated or un-irradiated psoriasis) included CDKN1A (p21/ WAF1), MMP3, GDF15 and IL24. These genes act via the mitochondrial route to induce apoptosis. The observed apoptosis induction at the gene level is consistent with the detected UVB-induced keratinocyte apoptosis seen *in vivo* following irradiation with 311nm UVB to psoriatic plaques, and described in chapter three.

DASL arrays (see section 2.5.4) were used in these experiments which are specially designed to cope with RNA extracted from processed tissue specimens, by using short primers spanning approximately 50 bases. For the PCR reactions, specific primers containing approximately 60-100 base pairs were used. The results confirm that RNA processing was satisfactory as gene levels are reasonably consistent between patients; despite the expected effects on gene expression which may be present due to factors such as age, sex and previous UV exposure. Real-time PCR was performed using five of the above differentially regulated genes and GAPDH as a control. These reactions were run using some of the samples processed for the gene arrays, and successfully validate the array data.

The array data showed that 159 genes are differentially regulated by therapeutic UVB (18h) compared to un-irradiated and 290nm-irradiated psoriasis, and 46 following effective UVB (4h) compared to un-irradiated and 290nm-irradiated psoriasis. Therefore over three times as many differentially

regulated genes were identified18h post 311nm UVB than at the 4h time-point, supporting the observed apoptosis demonstrated in previous chapters.

It is well known that UVB induces cell cycle arrest and these results suggest that arrest may occur in G1, with reduced checkpoint control in G2M consistent with reduced ability to repair damaged cells. Cells which are damaged beyond repair will undergo apoptosis in due course. However, it is interesting to note that many of the cell cycle regulatory genes were not differentially regulated by 290nm compared to 311nm UVB, suggesting that they are not wavelength specific effects and may not directly contribute to clearance of psoriasis at the time-points studied. It would have been interesting to study a further time-point around 8-12h, to see whether a significant differential effect is observed between the two wavelengths, as the lack of differential effect could be explained by sampling the wrong time-point.

Other major pathways to be differentially regulated include inflammation, in particular the Th17 pathway, which was up-regulated after 311nm UVB. This is in-keeping with erythema seen post irradiation, in particular as the peak erythemal response seen following 311nm and 290nm UVB follow a similar time-course (Farr, Besag et al. 1988), although the difference in slope of the dose-response curves means that for a given multiple of the MED, erythema induced by 311nm will be of greater intensity than 290nm UVB (Farr and Diffey 1985).

Attention has focused on antimicrobial peptides over the recent years. In particular LL37 which is expressed in keratinocytes and has previously been shown to be induced by UVB (Mallbris, Edstrom et al. 2005). It was therefore surprising that this peptide was not significantly differentially regulated in this study. However, in Mallbris study of eight patients (without psoriasis) biopsies were taken 24-28h post UVB, perhaps explaining the difference between the studies. The above data showed up-regulation of HBD-2, HBD-3, PI3 and S100A12 at the 18h time-point post 311nm UVB exposure compared to un-irradiated psoriasis, consistent with published data which showed up-regulation of HBD-2 and HBD-3 24h post 313nm UVB both in normal epidermis *in vivo*

and in cultured keratinocytes (Glaser, Navid et al. 2009). Previous studies have confirmed that these anti-microbial peptides are expressed in higher levels in psoriatic skin than normal epidermis (Buchau and Gallo 2007), but whether further enhancement of expression of these peptides occur with UV exposure has not previously been elucidated. Interestingly, enhancing antimicrobial peptides further has been shown to induce apoptosis for LL37 (Barlow, Li et al. 2006; Zasloff 2009) but the effects of high levels of other antimicrobial peptides on apoptosis have not yet been examined, and further work is necessary to investigate whether or not this may influence the apoptotic response. The known interactions between HBD-3, apoptotic genes and cell cycle regulatory genes is summarised in Figure 7-9.

Notably a few key genes were perhaps surprisingly not differentially regulated in this study, in particular NFκB (which is central to activation of the apoptotic pathway) and the effecter gene caspase-3. As such a significant level of apoptosis was seen within psoriatic epidermis at 18h post 311nm irradiation, it was expected that NFκB would be significantly differentially regulated compared to irradiation with 290nm UVB and un-irradiated psoriasis. The two most likely reasons for this are: firstly these genes were differentially expressed at different time-points and could therefore missed be because biopsies were only analysed at 4h and 18h post UVB; or secondly, that the gene levels are not affected, but may be cleaved/ phosphorylated etc therefore activating the gene to cause down-stream effects. Further analysis of biopsies taken at time-points between 8h and 24h are required to study this further, but are beyond the scope of this thesis.

Finally, it is interesting to note that even at these early time-points, before any clinical improvement in psoriasis is observed, pro-atherogenic genes are down-regulated and anti-atherogenic genes are up-regulated, consistent with previous studies which have linked good control of psoriasis with improved cardiovascular risk factors, for example post treatment with methotrexate (Prodanovich, Ma et al. 2005).

7.5. Conclusions

- Pro-apoptotic genes are expressed in psoriatic epidermis at early time-points (4h) following irradiation with 311nm but not 290nm UVB.
- UVB induces G1 arrest and reduces check-point control at G2M, but this effect is not specific to 311nm UVB-irradiation at the time-points studied.
- Antimicrobial peptides appear to be further up-regulated by UVB at the 18h time-point.
- UVB induces anti-atherogenic genes at the time-points studied.
- The PCR studies successfully backed up the data obtained by gene arrays.

8. Concluding remarks

This thesis unquestionably demonstrated the presence of keratinocyte apoptosis following *in vivo* irradiation of psoriatic plaques with a clinically effective dose of UVB (311nm), but not following an ineffective wavelength (290nm), using two different methodologys. It has previously been observation that irradiation of psoriasis with high doses (up to 28 times the MED) of UVB with wavelengths below 300nm did not improve psoriasis (Parrish and Jaenicke 1981), and this provided a neat and novel way to distinguish which of the many effects of UVB may be important in clearing psoriasis.

A rigorous *in vivo* approach demonstrated that the observed differences in lesional keratinocyte apoptosis were not due to a differential time-course. The apoptotic time-course was also defined here for both wavelengths using primary human keratinocyte cultures. This was shown to be similar in both wavelengths, although 290nm UVB was actually a more potent inducer of apoptosis than 311nm *in vitro*. A further observation of interest was that keratinocyte apoptosis was primarily suprabasal, and apoptotic cells were often located within the rete ridges rather than above the dermal papillae. This suggested that the chromophore for UVB-induced apoptosis may be within the basal/ suprabasal layers (containing proliferating cells). Reduced penetration by wavelengths below 300nm may therefore account for the very low numbers of apoptotic cells seen following irradiation with these wavelengths *in vivo* but not *in vitro*.

Real-time experiments were designed to track primary keratinocytes in culture following exposure to both 311nm and 290nm UVB. This demonstrated that actively proliferating keratinocytes were more likely to undergo UVB-induced apoptosis. Cells did not appear to influence their neighbours, suggesting that regulation by keratinocyte induced cytokines etc was unlikely. Cultured cells also had a wide range of time-of-onset for apoptosis following the initial irradiation, and a wide variation in the time taken for each cell to complete the apoptotic process was observed. The latter may be due to the extent of DNA damage received by the cell, and therefore the degree of attempted cell repair that occurs. These data were used in combination with observed numbers of apoptotic cells within the psoriatic plaques to estimate an

approximate rate of apoptosis. Flow cytometry experiments demonstrated that proliferating cells were indeed more likely to undergo apoptosis, and this applied to both stem and transit amplifying cells (although predominantly the latter) following irradiation of epidermis *ex vivo*. Further studies are required to confirm this observation in psoriatic epidermis.

The wavelength-dependence for UVB-induced apoptosis was examined by irradiating psoriatic plaques with equi-erythemogenic doses of UVB using a range of wavelengths, from 290nm to 320nm. This showed that significant keratinocyte apoptosis was induced using wavelengths between 301nm and 311nm, but none at 296nm (or below) or at 320nm. Although the numbers of patients recruited did not allow demonstration that one of the three apoptosis-inducing wavelengths (301nm, 306nm and 311nm) was significantly more effective than the others, the data suggested that 311nm UVB is likely to be equally effective, if not the most effective apoptosis-inducing wavelength tested. However there may be inter-patient variation suggesting the possibility of tailored UV phototherapy for the future.

To assess the overall contribution of UVB-induced keratinocyte apoptosis to psoriatic plaque remodelling, a mathematical model was created. A unique model of both normal and psoriatic epidermis was developed by combining histological observation with published data from the literature. Iterative testing of the model using established data ensured its robustness. The model predicted that if apoptosis was an important mechanism in clearing psoriasis, the majority of affected cells would be proliferating, and that both stem and transit amplifying cells would be affected. This was confirmed with experimental data. The model was used to dynamically demonstrate that the observed rate of apoptosis was sufficient to clear psoriasis following a number of repeated irradiations consistent with what is observed in clinical practice. This is the first time systems biology has been used to predict the effect of a psoriatic treatment, and may be applied to other treatments in the future.

The genes which may be important in triggering the apoptotic process were investigated at an early time-point (4h) prior to any histological suggestion of

apoptosis, and at 18h when apoptosis is just observed in biopsy specimens, but no apoptotic/ plaque clearing effect would be clinically apparent *in vivo*. Interestingly a number of important pro-apoptotic genes were found to be significantly up-regulated from 4h (such as CDKN1A/ p21/WAF1) following 311nm but not 290nm to psoriasis *in vivo*, and several anti-apoptotic genes were significantly down-regulated following 311nm but not 290nm compared to un-irradiated psoriasis. These genes all induce apoptosis via the mitochondrial pathway, consistent with previous work in the lab on dithranol (McGill, Frank et al. 2005). Importantly however, 290nm UVB differentially regulated several genes compared to un-irradiated psoriasis and 311nm UVB, suggesting that sufficient 290nm UVB penetrates into the epidermis to induce a biologically significant effect, although this may be predominantly within the upper epidermis. Unexpectedly, genes controlling atherosclerosis were also significantly differentially regulated, and anti-microbial peptides were also significantly differentially up-regulated compared to un-irradiated and 290nm-irradiated psoriasis. The latter will be further investigated in future work.

Finally, monitoring patients' weekly PASI scores during their routine narrowband phototherapy treatment allowed analysis of the rate of patient clearance, irrespective of whether or not they complete the course. This also partly deals with patients' psoriasis flaring up part way through a treatment course, which may confound the end effect of a treatment course. For example, a patient may be clearing well, but then have a streptococcal throat infection during their phototherapy course, leading to a super-antigen response and consequent deterioration in their psoriasis. Measuring a single PASI score at the beginning and end of a treatment course does not consider such flares, and as such a course of UVB may be deemed to be unsuccessful despite an initially good response to treatment. Assessment of weekly PASI scores can highlight such a flare, and this can be taken into account when determining patient response. This study found that 35% of patients did not achieve a PASI 90 overall, consistent with previous reports (see sections 1.3.1 and 3.3.10). However, further analysis revealed that this reduces to 17% when patients' with a disease flare-up / had dropped out before at least six weeks of treatment,

were excluded. An interesting extension to this work would therefore be to monitor weekly PASI scores in a larger number of patients undergoing routine therapy, so see if many of the ‘poor responders’ are actually patients that experience a flare-up of their disease. This would suggest that patients may benefit from concurrent therapy aimed at suppressing cytokine production.

Overall this thesis demonstrates that keratinocyte apoptosis can directly account for clearance of psoriatic plaques, and challenges the previous *in vitro* observations that psoriatic keratinocytes are relatively resistant to apoptosis (Wrone-Smith, Mitra et al. 1997). This has important implications in the design of future therapeutic options for psoriasis, and suggests that treatments either directly targeting apoptosis or acting synergistically with them may be highly effective in clearing disease. Importantly, the model predicts that clearance induced by keratinocyte apoptosis can induce long term remission, although in contrast to T cell/ cytokine therapies, it would not prevent a new trigger (e.g. super-antigen exposure) from inducing further psoriasis.

Further work which arises from this thesis includes analysis of which part of the cell cycle leaves cells most susceptible to apoptosis, and whether or not this can be utilised in the clinical situation to enhance the apoptotic effect of therapeutic wavelengths of UVB in psoriasis clearance. For example, using a topical agent to induce cell cycle arrest in G2M, combined with phototherapy. This may lead to shorter treatment courses (and therefore quicker clinical response), with an improved safety profile by reducing total UV exposure.

Separating patients into good and poor responders (according to PASI response) will allow further insight into the mechanism of UVB-induced clearance of psoriasis, and indicate which pathways are differentially regulated when patients respond well to treatment. Further data-mining of the gene array analysis generated here may identify common pathways and potential biomarkers which are differentially regulated in the good versus poor responders. This will help identify why some patients’ psoriasis responds better to UVB phototherapy, and will provide greater insight into the mechanisms of UVB clearance of plaques. Understanding these mechanisms will allow further

optimisation of phototherapy and potentially other therapeutic modalities in clinical practice.

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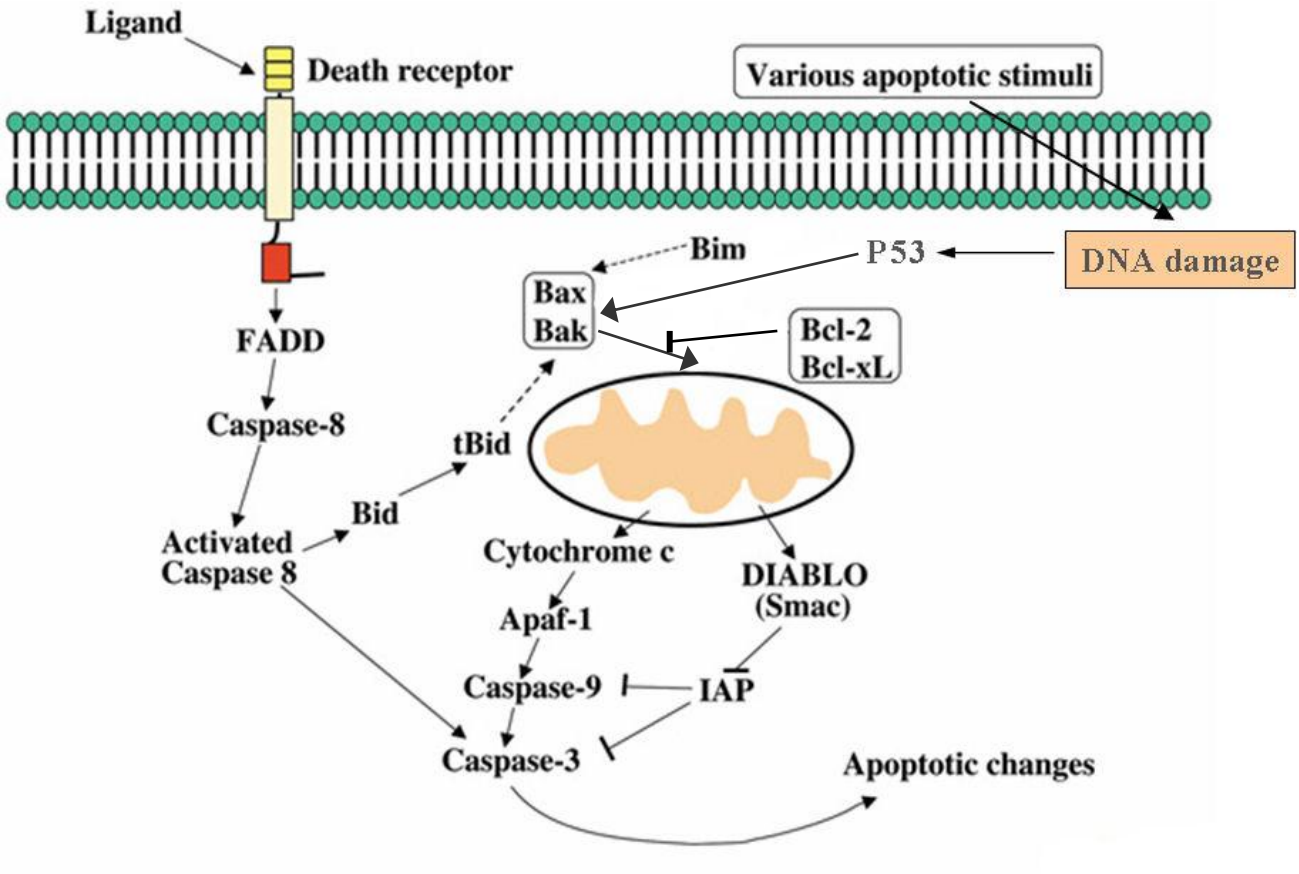
10. Appendices

11. Appendix A: Apoptosis pathways



Adapted from Mak and Yeh (Mak and Yeh 2002)

12. Appendix B: Patient forms

The Newcastle Upon Tyne Hospitals

NHS Trust

Royal Victoria Infirmary

Newcastle Upon Tyne

NE1 4LP

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PATIENT INFORMATION SHEET A[1]

EFFECTS AND MECHANISMS OF ULTRAVIOLET B (UVB) IN PSORIASIS.

We would like to invite you to participate in a research study. Before you decide whether you will participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about this study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Light therapy, particularly UVB (ultraviolet B) therapy is an effective method of treating psoriasis. Although we know that this can be very effective in many patients, we don't know exactly how it works, or which wavelengths of light will be most effective. This study is designed to address both these issues. We will look at samples of skin before and after exposure to different wavelengths of UVB, to see whether special proteins

[1] V3. Date 01/10/06

have been altered. We will also look at the effects of different wavelengths of light, as described below. The benefit of finding out how light treatments work and which part of light is most helpful, is that it may enable us to design new treatment regimes in the future, which may work more effectively and have less side effects. The maximum amount of time you could be involved with this study is 6- 8 weeks (i.e. the duration of your UVB course).

This research will form the basis of a thesis for a PhD.

Why have I been asked to help?

Your doctor has prescribed UVB as part of the treatment for your psoriasis. You will therefore be given this treatment routinely, and will be required to attend 3 times per week for treatment. If you decide to participate in this trial you will have up to 4 small areas of psoriasis and 4 areas of uninvolved skin treated with a separate light source, as explained below. Following this, treatment to all areas will continue as normal.

In total we are asking approximately 120 patients to participate in this study (into 1 of 6 groups). We are now recruiting patients to group A; this is explained below.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you are considering taking part you will be given this information sheet to keep, and will be asked to sign a consent form if you decide to go ahead (you will be given a copy of this to keep). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part? (Group A)

You will be involved in the research for up to 4 days before starting your UVB treatment. We are examining the effects of UV light both at wavelengths which are, and wavelengths which are not helpful in treating psoriasis. This will help us work out why UV clears psoriasis, and may help improve UV treatment in the future. We will fully explain to you what will be involved before you decide to participate. Your normal treatment will not be affected.

Visit 1

On day 1 we will test how sensitive your skin is to UV by shining increasing amounts of the UV to be tested onto your skin. Skin sensitivity testing is done routinely whether or not you participate.

Visit 2 (approximately 45 minutes)

We then see you as usual on day 2 (24 hours later) to read the results. At this visit, we will also shine a specific light on up to 4 small areas (15mm) of normal skin and on up

to 4 similar areas of psoriatic skin. Occasionally this may cause a slight redness or burning to that area of skin, although this will be temporary.

Visits 3/4 (approximately 15 minutes each)
You will be asked to attend on 2 further visits for this study. These will be between 12 hours and 3 days after the UV (you will be informed when this will be before deciding whether to participate). This time intervals will have been chosen to give us the most helpful results, and will mean that your time and effort in participating in the study will be put to the most use.

We need to examine biopsies from normal and involved skin in patients with psoriasis. Skin biopsies are small samples of skin (4-6mm across) which are taken following the injection of a small amount of local anaesthetic. Local injection is painful for the first 4-5 seconds but following this the area of skin is not sensitive to pain. We usually insert 1or 2 stitches after the biopsy has been taken, although on occasions a stitch may not be required. If a stitch is required, this will need to be removed two weeks later either by the practice nurse at your GP surgery, or by the phototherapy nurses when you attend for your treatment. In total we would ask you to have up to 4 skin biopsies during your involvement in this study.

You will be asked to provide a blood sample for later analysis, and this will be stored. This is entirely voluntary.

We will also examine your skin before treatment and at weekly intervals when you attend for your routine UV treatment; this will take approximately 10 minutes on each occasion, and will not cause discomfort. Photographs may be taken of selected areas of your skin to show clearance of psoriasis, although you will not be identifiable from these.

Expenses and payments
You will be asked to make 3 extra visits to hospital as part of your involvement in the study. Extra visits will be kept to a minimum and every effort will be made to make the timing of visits as convenient as possible for participants. We will reimburse expenses

for additional visits, e.g. bus fare, petrol, parking etc. However, if you need to take a taxi to make the journey please inform us of this in advance.

What do I have to do?

Your involvement in this study will not affect your day-to-day activities. However, following a biopsy we will ask you to keep this area clean and dry for at least 2 days, and to contact us on the telephone number below if you have any concerns about the wound.

What are the side effects of any treatment received when taking part?

As with your normal light treatment, it is possible that you may get some transient reddening of your skin in the areas treated (similar to a mild sunburn). This should not require any specific treatment, although we would ask you to let us know if this does occur.

Biopsies, just like any small cut in the skin, may bleed a little, although this should stop if you apply firm and continuous pressure to the area for 5-10 minutes. The anaesthetic used to numb your skin will wear off after a few hours, and it may therefore be helpful to take some simple painkillers like paracetamol, when you get back home. The risk of infection in the wound is small, however the risk will increase if you leave a wet dressing in place. You will therefore be given spare dressings to take home, and asked to contact us or your GP if you have any concerns about this. Finally, biopsies will leave a small scar; and although this tends to fade with time, there is usually a small permanent mark.

What are the possible benefits of taking part?

Although we believe that this work will help us understand and improve the treatment of psoriasis, it is important to realise that we do not expect you to benefit directly by volunteering for this study.

What if there is a problem?

Any complaint about the way you have been dealt with during this study or side effects you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information.

If you require further information about the trial, Dr S Weatherhead, Prof P Farr or Prof N Reynolds can be contacted in the Dermatology department on 0191 2824214. If you have any concerns during your involvement in the study, 24 hour help can be received by calling 0191 2825599.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

The Newcastle Upon Tyne Hospitals

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PATIENT INFORMATION SHEET B

EFFECTS AND MECHANISMS OF ULTRAVIOLET B (UVB) IN PSORIASIS.

We would like to invite you to participate in a research study. Before you decide whether you will participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about this study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Light therapy, particularly UVB (ultraviolet B) therapy is an effective method of treating psoriasis. Although we know that this can be very effective in many patients, we don't know exactly how it works, or which wavelengths of light will be most effective. This study is designed to address both these issues. We will look at samples of skin before and after exposure to different wavelengths of UVB, to see whether special proteins have been altered. We will also look at the effects of different wavelengths of light, as described below. The benefit of finding out how light treatments work and which part of light is most helpful, is that it may enable us to design new treatment regimes in the future, which may work more effectively and have less side effects. The maximum

amount of time you could be involved with this study is 6- 8 weeks (i.e. the duration of your UVB course).

This research will form the basis of a thesis for a PhD.

Why have I been asked to help?

Your doctor has prescribed UVB as part of the treatment for your psoriasis. You will therefore be given this treatment routinely, and will be required to attend 3 times per week for treatment. If you decide to participate in this trial you will have up to 4 small areas of psoriasis and 4 areas of uninvolved skin treated with a separate light source, as explained below. Following this, treatment to all areas will continue as normal.

In total we are asking approximately 120 patients to participate in this study (into 1 of 6 groups). We are now recruiting patients to group B; this is explained below.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you are considering taking part you will be given this information sheet to keep, and will be asked to sign a consent form if you decide to go ahead (you will be given a copy of this to keep). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

You will be involved in the research for up to 4 days before starting your UVB treatment. We are examining which of the many effects of UV light are important for psoriasis clearance, and this may help improve UV treatment in the future. We will fully explain to you what will be involved before you decide to participate. Your normal treatment will not be affected.

Visit 1

On day 1 we will test how sensitive your skin is to UV by shining increasing amounts of the UV to be tested onto your skin (this is done routinely whether or not you participate).

Visit 2 (approximately 60 minutes)

We then see you as usual on day 2 (24 hours later) to read the results. At this visit, we will shine different doses of UV onto 4 small areas (15mm) of normal skin and onto 4

similar areas of psoriatic skin. Occasionally this may cause a slight redness or burning to that area of skin, although this will be temporary.

Visit 3 (approximately 30-40 minutes)
You will be asked to attend on 1 further occasion for this study. These will be between 12 hours and 3 days after the UV (you will be informed when this will be before deciding whether to participate). This time interval will have been chosen to give us the most helpful results, and will mean that your time and effort in participating in the study will be put to the most use.

We need to examine biopsies from normal and involved skin in patients with psoriasis. Skin biopsies are small samples of skin (4-6mm across) which are taken following the injection of a small amount of local anaesthetic. Local injection is painful for the first 4-5 seconds but following this the area of skin is not sensitive to pain. We usually insert 1or 2 stitches after the biopsy has been taken, although on occasions a stitch may not be required. If a stitch is required, this will need to be removed two weeks later either at the practice nurse by your GP surgery, or by the phototherapy nurses when you attend for your treatment. In total we would ask you to have up to 4 skin biopsies during your involvement in this study.

You will be asked to provide a blood sample for later analysis, and this will be stored. This is entirely voluntary.

We will also examine your skin before treatment and at weekly intervals when you attend for your routine UV treatment; this will take approximately 10 minutes on each occasion, and will not cause discomfort. Photographs may be taken of selected areas of your skin to show clearance of psoriasis, although you will not be identifiable from these.

Expenses and payments
You will be asked to make at 2 extra visits to hospital as part of your involvement in the study. Extra visits will be kept to a minimum and every effort will be made to make the timing of visits as convenient as possible for participants. We will reimburse expenses for additional visits, e.g. bus fare, petrol, parking etc. However, if you need to take a taxi to make the journey please inform us of this in advance.

What do I have to do?

Your involvement in this study will not affect your day-to-day activities. However, following a biopsy we will ask you to keep this area clean and dry for at least 2 days, and to contact us on the telephone number below if you have any concerns about the wound.

What are the side effects of any treatment received when taking part?

As with your normal light treatment, it is possible that you may get some transient reddening of your skin in the areas treated (similar to a mild sunburn). This should not require any specific treatment, although we would ask you to let us know if this does occur.

Biopsies, just like any small cut in the skin, may bleed a little, although this should stop if you apply firm and continuous pressure to the area for 5-10 minutes. The anaesthetic used to numb your skin will wear off after a few hours, and it may therefore be helpful to take some simple painkillers like paracetamol, when you get back home. The risk of infection in the wound is small, however the risk will increase if you leave a wet dressing in place. You will therefore be given spare dressings to take home, and asked to contact us or your GP if you have any concerns about this. Finally, biopsies will leave a small scar; and although this tends to fade with time, there is usually a small permanent mark.

What are the possible benefits of taking part?

Although we believe that this work will help us understand and improve the treatment of psoriasis, it is important to realise that we do not expect you to benefit directly by volunteering for this study.

What if there is a problem?

Any complaint about the way you have been dealt with during this study or side effects you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information.

If you require further information about the trial, Dr S Weatherhead, Prof P Farr or Prof N Reynolds can be contacted in the Dermatology department on 0191 2824214. If you have any concerns during your involvement in the study, 24 hour help can be received by calling 0191 2825599.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

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PATIENT INFORMATION SHEET C

EFFECTS AND MECHANISMS OF ULTRAVIOLET B (UVB) IN PSORIASIS.

We would like to invite you to participate in a research study. Before you decide whether you will participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about this study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Light therapy, particularly UVB (ultraviolet B) therapy is an effective method of treating psoriasis. Although we know that this can be very effective in many patients, we don't know exactly how it works, or which wavelengths of light will be most effective. This study is designed to address both these issues. We will look at samples of skin before and after exposure to different wavelengths of UVB, to see whether special proteins have been altered. We will also look at the effects of different wavelengths of light, as described below. The benefit of finding out how light treatments work and which part of light is most helpful, is that it may enable us to design new treatment regimes in the

future, which may work more effectively and have less side effects. The maximum amount of time you could be involved with this study is 6- 8 weeks (i.e. the duration of your UVB course).

This research will form the basis of a thesis for a PhD.

Why have I been asked to help?

Your doctor has prescribed UVB as part of the treatment for your psoriasis. You will therefore be given this treatment routinely, and will be required to attend 3 times per week for treatment. If you decide to participate in this trial you will have up to 4 small areas of psoriasis treated with a separate light source, as explained below. Following this, treatment to all areas will continue as normal.

In total we are asking approximately 120 patients to participate in this study (into 1 of 6 groups). We are now recruiting patients to group C; this is explained below.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you are considering taking part you will be given this information sheet to keep, and will be asked to sign a consent form if you decide to go ahead (you will be given a copy of this to keep). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

You will be involved in this research for up to 2 days before starting your UVB treatment, and then during the first 2 ½ weeks of routine treatment. We are examining which of the many effects of UV light are important for psoriasis clearance, and this may help improve UV treatment in the future. We will fully explain to you what will be involved before you decide to participate. Your normal treatment will not be affected. You will be asked to attend on 1 additional occasion as part of your involvement in this.

Visit 1

On day 1 we will test how sensitive your skin is to UV by shining increasing amounts of the UV to be tested onto your skin (this is done routinely whether or not you participate).

Visit 2

We then see you as usual on day 2 (24 hours later) to read the results. At this visit, you will start your routine treatment but with 4 small areas (15mm) of psoriasis on covered up. These areas will be treated with UV just before or just after your routine treatment,

and are likely to clear much quicker than the rest of your psoriasis. Occasionally this may cause a slight redness or burning to that area of skin, although this will be temporary.

Visits alongside routine treatment: (approximately 30-60 minutes per visit)
You will be asked to attend for normal phototherapy 3 times per week. Over the next 7 occasions we will repeat the extra doses of UV (as for visit 2). On each occasion we will treat the same small areas of skin with UV, although we ask that you keep the areas we are assessing covered during your standard treatment, until your involvement in the study is completed.

Additional visit: (approximately 30 minutes)
Four skin biopsies (see below) will be taken on 1 occasion between 12 hours and 3 days after the UV (you will be informed when this will be before deciding whether to participate). This time interval will have been chosen to give us the most helpful results, and will mean that your time and effort in participating in the study will be put to the most use.

Skin biopsies are small samples of skin (4-6mm across) which are taken following the injection of a small amount of local anaesthetic. Local injection is painful for the first 4-5 seconds but following this the area of skin is not sensitive to pain. We usually insert 1or 2 stitches after the biopsy has been taken, although on occasions a stitch may not be required. If a stitch is required, this will need to be removed two weeks later either by the practice nurse at your GP surgery, or by the phototherapy nurses when you attend for your treatment. In total we would ask you to have up to 4 skin biopsies during your involvement in this study.

You will be asked to provide a blood sample for later analysis, and this will be stored. This is entirely voluntary.

We will also examine your skin before treatment and at weekly intervals when you attend for your routine UV treatment; this will take approximately 10 minutes on each occasion, and will not cause discomfort. Photographs may be taken of selected areas of your skin to show clearance of psoriasis, although you will not be identifiable from these.

Expenses and payments

You will be asked to make at least 1 extra visit to hospital as part of your involvement in the study. Extra visits will be kept to a minimum and every effort will be made to make the timing of visits as convenient as possible for participants. We will reimburse expenses for additional visits, e.g. bus fare, petrol, parking etc. However, if you need to take a taxi to make the journey please inform us of this in advance.

What do I have to do?

Your involvement in this study will not affect your day-to-day activities. However, following a biopsy we will ask you to keep this area clean and dry for at least 2 days, and to contact us on the telephone number below if you have any concerns about the wound.

What are the side effects of any treatment received when taking part?

As with your normal light treatment, it is possible that you may get some transient reddening of your skin in the areas treated (similar to a mild sunburn). This should not require any specific treatment, although we would ask you to let us know if this does occur.

Biopsies, just like any small cut in the skin, may bleed a little, although this should stop if you apply firm and continuous pressure to the area for 5-10 minutes. The anaesthetic used to numb your skin will wear off after a few hours, and it may therefore be helpful to take some simple painkillers like paracetamol, when you get back home. The risk of infection in the wound is small, however the risk will increase if you leave a wet dressing in place. You will therefore be given spare dressings to take home, and asked to contact us or your GP if you have any concerns about this. Finally, biopsies will leave a small scar; and although this tends to fade with time, there is usually a small permanent mark.

What are the possible benefits of taking part?

Although we believe that this work will help us understand and improve the treatment of psoriasis, it is important to realise that we do not expect you to benefit directly by volunteering for this study.

What if there is a problem?

Any complaint about the way you have been dealt with during this study or side effects you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information.

If you require further information about the trial, Dr S Weatherhead, Prof P Farr or Prof N Reynolds can be contacted in the Dermatology department on 0191 2824214. If you have any concerns during your involvement in the study, 24 hour help can be received by calling 0191 2825599.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

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PATIENT INFORMATION SHEET D

EFFECTS AND MECHANISMS OF ULTRAVIOLET B (UVB) IN PSORIASIS.

We would like to invite you to participate in a research study. Before you decide whether you will participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about this study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Light therapy, particularly UVB (ultraviolet B) therapy is an effective method of treating psoriasis. Although we know that this can be very effective in many patients, we don't know exactly how it works, or which wavelengths of light will be most effective. This study is designed to address both these issues. We will look at samples of skin before and after exposure to different wavelengths of UVB, to see whether special proteins have been altered. We will also look at the effects of different wavelengths of light, as described below. The benefit of finding out how light treatments work and which part of light is most helpful, is that it may enable us to design new treatment regimes in the

future, which may work more effectively and have less side effects. The maximum amount of time you could be involved with this study is 6- 8 weeks (i.e. the duration of your UVB course).

This research will form the basis of a thesis for a PhD.

Why have I been asked to help?

Your doctor has prescribed UVB as part of the treatment for your psoriasis. You will therefore be given this treatment routinely, and will be required to attend 3 times per week for treatment. If you decide to participate in this trial you will have up to 2 small areas of psoriasis treated with a separate light source, as explained below. Following this, treatment to all areas will continue as normal.

In total we are asking approximately 120 patients to participate in this study (into 1 of 6 groups). We are now recruiting patients to group D; this is explained below.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you are considering taking part you will be given this information sheet to keep, and will be asked to sign a consent form if you decide to go ahead (you will be given a copy of this to keep). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

You will be involved in this research for up to 2 days before starting your UVB treatment. We are examining the effects of UV light both at wavelengths which are, and wavelengths which are not helpful in treating psoriasis. This will help us work out why UV clears psoriasis, and may help improve UV treatment in the future. We will fully explain to you what will be involved before you decide to participate. Your normal treatment will not be affected.

Visit 1

On day 1 we will test how sensitive your skin is to UV by shining increasing amounts of the UV to be tested onto your skin. Skin sensitivity testing is done routinely whether or not you participate.

Visit 2 (maximum 3h)

We then see you as usual on day 2 (24 hours later) to read the results. At this visit, we will shine 2 different wavelengths of light onto small areas (15mm) of your psoriasis. In total this process takes approximately 45 minutes. Occasionally this may cause a slight

redness or burning to that area of skin, although this will be temporary. Four and 18 hours later, we will need to take 2 skin biopsies from your psoriasis (this will take an additional 20 minutes), to work out what effects the different wavelengths of light have had on your skin.

Skin biopsies are small samples of skin (4-6mm across) which are taken following the injection of a small amount of local anaesthetic. Local injection is painful for the first 4-5 seconds but following this the area of skin is not sensitive to pain. We usually insert 1or 2 stitches after the biopsy has been taken, although on occasions a stitch may not be required. If a stitch is required, this will need to be removed two weeks later either by the practice nurse at your GP surgery, or by the phototherapy nurses when you attend for your treatment. In total we would ask you to have up to 2 skin biopsies during your involvement in this study.

You will be asked to provide a blood sample for later analysis, and this will be stored. This is entirely voluntary.

Expenses and payments

It is possible that you will be asked to make an extra visit to hospital as part of your involvement in the study. Extra visits will be kept to a minimum and every effort will be made to make the timing of visits as convenient as possible for participants. We will reimburse expenses for additional visits, e.g. bus fare, petrol, parking etc. However, if you need to take a taxi to make the journey please inform us of this in advance.

What do I have to do?

Your involvement in this study will not affect your day-to-day activities. However, following a biopsy we will ask you to keep this area clean and dry for at least 2 days, and to contact us on the telephone number below if you have any concerns about the wound.

What are the side effects of any treatment received when taking part?

As with your normal light treatment, it is possible that you may get some transient reddening of your skin in the areas treated (similar to a mild sunburn). This should not require any specific treatment, although we would ask you to let us know if this does occur.

Biopsies, just like any small cut in the skin, may bleed a little, although this should stop if you apply firm and continuous pressure to the area for 5-10 minutes. The anaesthetic used to numb your skin will wear off after a few hours, and it may therefore be helpful to take some simple painkillers like paracetamol, when you get back home. The risk of infection in the wound is small, however the risk will increase if you leave a wet dressing in place. You will therefore be given spare dressings to take home, and asked to contact us or your GP if you have any concerns about this. Finally, biopsies will leave a small scar; and although this tends to fade with time, there is usually a small permanent mark.

What are the possible benefits of taking part?

Although we believe that this work will help us understand and improve the treatment of psoriasis, it is important to realise that we do not expect you to benefit directly by volunteering for this study.

What if there is a problem?

Any complaint about the way you have been dealt with during this study or side effects you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information.

If you require further information about the trial, Dr S Weatherhead, Prof P Farr or Prof N Reynolds can be contacted in the Dermatology department on 0191 2824214. If you

have any concerns during your involvement in the study, 24 hour help can be received by calling 0191 2825599.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

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PATIENT INFORMATION SHEET E

EFFECTS AND MECHANISMS OF ULTRAVIOLET B (UVB) IN PSORIASIS.

We would like to invite you to participate in a research study. Before you decide whether you will participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about this study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Light therapy, particularly UVB (ultraviolet B) therapy is an effective method of treating psoriasis. Although we know that this can be very effective in many patients, we don't know exactly how it works, or which wavelengths of light will be most effective. This study is designed to address both these issues. We will look at samples of skin before and after exposure to different wavelengths of UVB, to see whether special proteins have been altered. We will also look at the effects of different wavelengths of light, as described below. The benefit of finding out how light treatments work and which part of light is most helpful, is that it may enable us to design new treatment regimes in the

future, which may work more effectively and have less side effects. The maximum amount of time you could be involved with this study is 6- 8 weeks (i.e. the duration of your UVB course).

This research will form the basis of a thesis for a PhD.

Why have I been asked to help?

Your doctor has prescribed UVB as part of the treatment for your psoriasis. You will therefore be given this treatment routinely, and will be required to attend 3 times per week for treatment. If you decide to participate in this trial you will have up to 4 small areas of psoriasis and 4 areas of uninvolved skin treated with a separate light source, as explained below. Following this, treatment to all areas will continue as normal.

In total we are asking approximately 120 patients to participate in this study (into 1 of 6 groups). We are now recruiting patients to group E; this is explained below.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you are considering taking part you will be given this information sheet to keep, and will be asked to sign a consent form if you decide to go ahead (you will be given a copy of this to keep). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

You will be involved in this research for up to 4 days before starting your UVB treatment. We are examining the effects of UV light both at wavelengths which are, and wavelengths which are not helpful in treating psoriasis. This will help us work out why UV clears psoriasis, and may help improve UV treatment in the future. We will fully explain to you what will be involved before you decide to participate. Your normal treatment will not be affected.

Visit 1

On day 1 we will test how sensitive your skin is to UV by shining increasing amounts of the UV to be tested onto your skin. Skin sensitivity testing is done routinely whether or not you participate, but we will also test your sensitivity to the other wavelengths that we are using.

Visit 2

We then see you as usual on day 2 (24 hours later) to read the results. At this visit, we will shine 4 different wavelengths of light onto 4 small areas (15mm) of your psoriasis, and onto your normal skin. In total this process takes approximately 60 minutes. Occasionally this may cause a slight redness or burning to that area of skin, although this will be temporary.

Visit 3

You will be asked to attend once more to complete your involvement in the study. In order to make this as convenient as possible, this visit will usually coincide with your first routine treatment in the phototherapy department, and will take an additional 30 minutes.

We will need to examine biopsies from your normal and involved skin, to work out what effects the different ranges of light have had on your skin. Skin biopsies are small samples of skin (4-6mm across) which are taken following the injection of a small amount of local anaesthetic. Local injection is painful for the first 4-5 seconds but following this the area of skin is not sensitive to pain. We usually insert 1or 2 stitches after the biopsy has been taken, although on occasions a stitch may not be required. If a stitch is required, this will need to be removed two weeks later either by the practice nurse at your GP surgery, or by the phototherapy nurses when you attend for your treatment. In total we would ask you to have up to 4 skin biopsies during your involvement in this study.

You will be asked to provide a blood sample for later analysis, and this will be stored. This is entirely voluntary.

We will also examine your skin before treatment and at weekly intervals when you attend for your routine UV treatment; this will take approximately 10 minutes on each occasion, and will not cause discomfort. Photographs may be taken of selected areas of your skin to show clearance of psoriasis, although you will not be identifiable from these.

Expenses and payments

It is possible that you will be asked to make an extra visit to hospital as part of your involvement in the study. Extra visits will be kept to a minimum and every effort will be made to make the timing of visits as convenient as possible for participants. We will reimburse expenses for additional visits, e.g. bus fare, petrol, parking etc. However, if you need to take a taxi to make the journey please inform us of this in advance.

What do I have to do?

Your involvement in this study will not affect your day-to-day activities. However, following a biopsy we will ask you to keep this area clean and dry for at least 2 days, and to contact us on the telephone number below if you have any concerns about the wound.

What are the side effects of any treatment received when taking part?

As with your normal light treatment, it is possible that you may get some transient reddening of your skin in the areas treated (similar to a mild sunburn). This should not require any specific treatment, although we would ask you to let us know if this does occur.

Biopsies, just like any small cut in the skin, may bleed a little, although this should stop if you apply firm and continuous pressure to the area for 5-10 minutes. The anaesthetic used to numb your skin will wear off after a few hours, and it may therefore be helpful to take some simple painkillers like paracetamol, when you get back home. The risk of infection in the wound is small, however the risk will increase if you leave a wet dressing in place. You will therefore be given spare dressings to take home, and asked to contact us or your GP if you have any concerns about this. Finally, biopsies will leave a small scar; and although this tends to fade with time, there is usually a small permanent mark.

What are the possible benefits of taking part?

Although we believe that this work will help us understand and improve the treatment of psoriasis, it is important to realise that we do not expect you to benefit directly by volunteering for this study.

What if there is a problem?

Any complaint about the way you have been dealt with during this study or side effects you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information.

If you require further information about the trial, Dr S Weatherhead, Prof P Farr or Prof N Reynolds can be contacted in the Dermatology department on 0191 2824214. If you have any concerns during your involvement in the study, 24 hour help can be received by calling 0191 2825599.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

The Newcastle Upon Tyne Hospitals

NHS Trust

Royal Victoria Infirmary

Newcastle Upon Tyne

NE1 4LP

Tel: 0191 2824214

PATIENT INFORMATION SHEET F

EFFECTS AND MECHANISMS OF ULTRAVIOLET B (UVB) IN PSORIASIS.

We would like to invite you to participate in a research study. Before you decide whether you will participate, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully. Talk to others about this study if you wish.

- Part 1 tells you the purpose of this study and what will happen to you if you take part
- Part 2 gives you more detailed information about the conduct of the study

Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Part 1

What is the purpose of this study?

Light therapy, particularly UVB (ultraviolet B) therapy is an effective method of treating psoriasis. Although we know that this can be very effective in many patients, we don't know exactly how it works, or which wavelengths of light will be most effective. This study is designed to address both these issues. We will look at samples of skin before and after exposure to different wavelengths of UVB, to see whether special proteins have been altered. We will also look at the effects of different wavelengths of light, as described below, and try to work out why some people respond better than others. The benefit of finding out how light treatments work and which part of light is most helpful,

is that it may enable us to design new treatment regimes in the future, which may work more effectively and have less side effects. The maximum amount of time you could be involved with this study is 6- 8 weeks (i.e. the duration of your UVB course).
This research will form the basis of a thesis for a PhD.

Why have I been asked to help?

Your doctor has prescribed UVB as part of the treatment for your psoriasis. You will therefore be given this treatment routinely, and will be required to attend 3 times per week for treatment. If you decide to participate in this trial you will be examined before treatment and part of the way through your treatment course, to see how you are improving. We may take up to 2 small skin biopsies from an area of your psoriasis to try to find out what is going on in your skin to clear your psoriasis, or why it is not responding.

In total we are asking approximately 120 patients to participate in this study. We are now recruiting the final group of patients to this study, group F; this is explained below.

Do I have to take part?

No. It is up to you to decide whether or not to take part. If you are considering taking part you will be given this information sheet to keep, and will be asked to sign a consent form if you decide to go ahead (you will be given a copy of this to keep). If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

What will happen to me if I decide to take part?

You will be involved in this research for an assessment prior to starting your UVB treatment, and will then be seen again after you have attended several routine treatments. We are examining why some patients with psoriasis respond well to UV light but others don't. We will fully explain to you what will be involved before you decide to participate. Your normal treatment will not be affected.

Visit 1 (approximately 10 minutes)

On day 1 we ask you some routine questions about your psoriasis and examine your skin. You psoriasis will be graded according to how red, thick, scaly and extensive it is, and this will be used later to assess how much better you have got with treatment. This visit will coincide with your routine 'photo-testing' visit.

Visit 2 (approximately 20 minutes)
We then see you during your routine therapy after you have had several treatments, but before you have started to respond significantly to the UV. We will ask you have 2 skin biopsies (see below) from your psoriasis, which may help us work out what has happened in your skin to make you respond /not respond to phototherapy.

Skin biopsies are small samples of skin (4-6mm across) which are taken following the injection of a small amount of local anaesthetic. Local injection is painful for the first 4-5 seconds but following this the area of skin is not sensitive to pain. We usually insert 1or 2 stitches after the biopsy has been taken, although on occasions a stitch may not be required. If a stitch is required, this will need to be removed two weeks later either by the practice nurse at your GP surgery, or by the phototherapy nurses when you attend for your treatment.

You will be asked to provide a blood sample for later analysis, and this will be stored. This is entirely voluntary.

We will also examine your skin before treatment and at weekly intervals when you attend for your routine UV treatment; this will take approximately 10 minutes on each occasion, and will not cause discomfort. Photographs may be taken of selected areas of your skin to show clearance of psoriasis, although you will not be identifiable from these.

Expenses and payments
It is possible that you will be asked to make an extra visit to hospital as part of your involvement in the study. Extra visits will be kept to a minimum and every effort will be made to make the timing of visits as convenient as possible for participants. We will reimburse expenses for additional visits, e.g. bus fare, petrol, parking etc. However, if you need to take a taxi to make the journey please inform us of this in advance.

What do I have to do?
Your involvement in this study will not affect your day-to-day activities. However, following a biopsy we will ask you to keep this area clean and dry for at least 2 days,

and to contact us on the telephone number below if you have any concerns about the wound.

What are the side effects of any treatment received when taking part?

As with your normal light treatment, it is possible that you may get some transient reddening of your skin in the areas treated (similar to a mild sunburn). This should not require any specific treatment, although we would ask you to let us know if this does occur.

Biopsies, just like any small cut in the skin, may bleed a little, although this should stop if you apply firm and continuous pressure to the area for 5-10 minutes. The anaesthetic used to numb your skin will wear off after a few hours, and it may therefore be helpful to take some simple painkillers like paracetamol, when you get back home. The risk of infection in the wound is small, however the risk will increase if you leave a wet dressing in place. You will therefore be given spare dressings to take home, and asked to contact us or your GP if you have any concerns about this. Finally, biopsies will leave a small scar; and although this tends to fade with time, there is usually a small permanent mark.

What are the possible benefits of taking part?

Although we believe that this work will help us understand and improve the treatment of psoriasis, it is important to realise that we do not expect you to benefit directly by volunteering for this study.

What if there is a problem?

Any complaint about the way you have been dealt with during this study or side effects you might suffer will be addressed. The detailed information on this is given in Part 2.

Will my taking part in this study be kept confidential?

Yes. All the information about your participation in this study will be kept confidential. The details are included in Part 2.

Contact for further information.

If you require further information about the trial, Dr S Weatherhead, Prof P Farr or Prof N Reynolds can be contacted in the Dermatology department on 0191 2824214. If you have any concerns during your involvement in the study, 24 hour help can be received by calling 0191 2825599.

This completes Part 1 of the Information Sheet.

If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making a decision.

Part 2

What will happen if I don't want to carry on with the study?

You can withdraw from the study at any time. Any stored biopsies or blood may still be analysed in the event of you withdrawing but will be coded and only the researchers will know that they are yours. Others will not have access to this information.

What if there is a problem?

Complaints.

If you have a concern about any aspect of this study, you should speak with the researchers who will do there best to answer your questions (0191 2824214). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

Will my taking part in this study be kept confidential?

If you decide to participate we will document this in your medical notes and inform your GP. Your confidentiality will be maintained by anonymising your details and keeping them on a secured database. The information will be kept for up to 6 years to allow the data to be analysed, forming the basis for publication in an appropriate medical journal. You will not be personally identified in any publication.

What will happen to any samples I give?

Biopsy samples will be given a unique code known only to be researchers, and will be stored in the laboratory in Dermatological Sciences, University of Newcastle-upon-Tyne. These will be analysed at a later time within the laboratory, and destroyed at the end of the study. Samples will be accessed by the researchers only.

Will any genetic tests be done?

We may use your skin samples to examine how UV therapy affects the proteins expressed in your skin as genes are switched on or off due to the UV. This can help us to understand what drives psoriasis. Similarly your blood sample will be used to analyse any genetic differences which may make you more or less likely to respond well to UV treatment. We will only look for genetic changes relevant to the clearing of

your psoriasis. You will not be told the results of this, and it will not be recorded in your medical records. This part of the study is optional.

Who is funding the study?

This study is funded by the Wellcome Trust. The investigators will not receive any financial benefit as a result of your participation in this trial.

Who has reviewed this study?

This study has been given favourable ethical opinion by the Local Research Ethics Committee.

Thank you for your interest and help.

The Newcastle Upon Tyne Hospitals

NHS Trust

Phototherapy Dept

Royal Victoria Infirmary

Newcastle Upon Tyne

NE1 4LP

Consent Form **Tel: 0191 2824214**

Effects and Mechanisms of Ultraviolet B (UVB) in Psoriasis

Researchers: Dr S Weatherhead, Prof P Farr and Prof N Reynolds

I confirm that I have read and understood the information sheet dated 01/10/06 for the above study and have had the opportunity to ask questions ☐

I understand that my participation is voluntary and that I'm free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

I understand that sections of my medical notes may be looked at by responsible individuals from the Dermatology department where it is relevant to me taking part in research. I give permission for these individual to have access to my records. ☐

I understand that photographs will be taken of my skin during the study, and that these will be used as part of the assessment of my response to the light. These photographs will remain confidential and I will not be recognisable to others from the pictures. ☐

I understand that my blood and skin samples may be stored for further analysis including genetic (DNA) testing. However, this will be for research purposes and will not affect me personally. ☐

I agree to take part in the above study. ☐

_____	_____	_____
Name of Patient	Date	Signature

_____	_____	_____
Name of Person taking consent	Date	Signature

(if different from researcher)

_____	_____	_____
Researcher	Date	Signature

1 for patient; 1 for researcher, 1 to be kept with hospital notes

The Effects and Mechanisms of UVB in Psoriatic Clearance

Patient code: ……..

Tel. No: ……

Appoint. Times: ……..

Age: ……. Sex: ……..

Date of entry into trial: …… Group: …….. ST: ……..

Age of onset of psoriasis: ……..

Previous psoriasis treatment?

Recent: ……

UVR: ……...

Other Medications: ……

Relevant PMH: ……..

Large/ medium/ small plaques Scalp/ nails/ joint involvement

PASI score:…… Target plaque: ……

No. MED given: …3……. Wavelengths: …TL01/290…….

MED for treatment: ……..

TL01

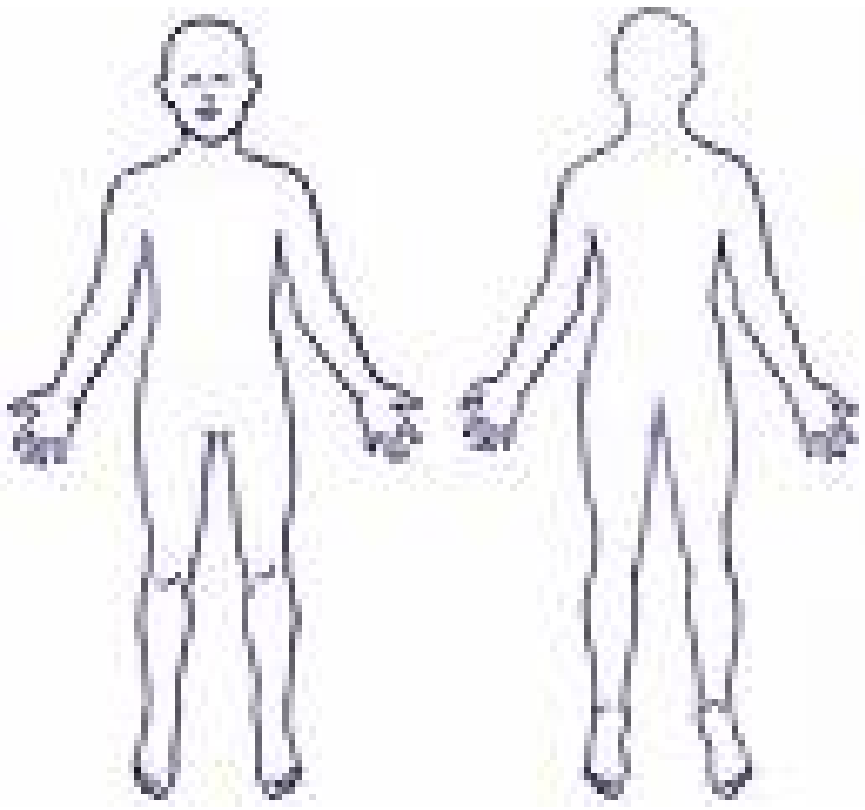
Irradiance:……

	ST 2-4	ST1
1	1459	1094
2	1158	868
3	919	689
4	729	547
5	579	434
6	459	345
7	365	273
8	289	217
9	230	172
10	182	137

290

Irradiance: …….

	ST 2-4	ST1
1	40	20
2	31.7	15.9
3	25.2	12.6
4	20	10
5	15.9	7.9
6	12.6	6.3
7	10	5.0
8	7.9	4.0
9	6.3	3.1
10	5	2.5



Biopsy date and times: ……Sample Codes: ……

13. Appendix C: PASI scoring sheet

Erythema/ thickness/ scale

0= none, 1= slight, 2= moderate, 3= striking, 4= exceptional

Area

0= none, 1=<10%; 2=10- <30%; 3=30-<50%; 4= 50-<70%; 5=70-<90%; 6=>90%

HEAD

E ☐ + T ☐ + S ☐ = ☐ = ☐

A ☐ x 0.1 = ☐

TRUNK

E ☐ + T ☐ + S ☐ = ☐ = ☐

A ☐ x 0.3 = ☐

UPPER LIMBS

E ☐ + T ☐ + S ☐ = ☐ = ☐

A ☐ x 0.2 = ☐

LOWER LIMBS

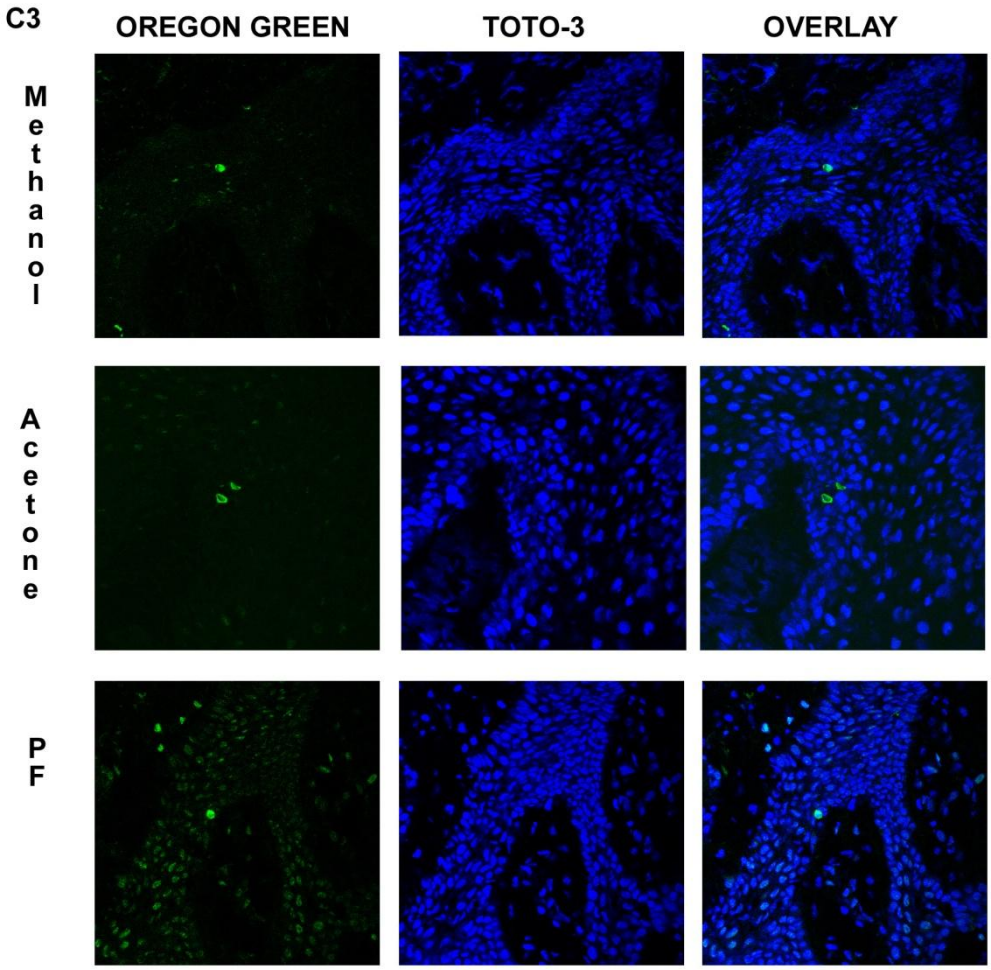
E ☐ + T ☐ + S ☐ = ☐ = ☐

A ☐ x 0.4 = ☐

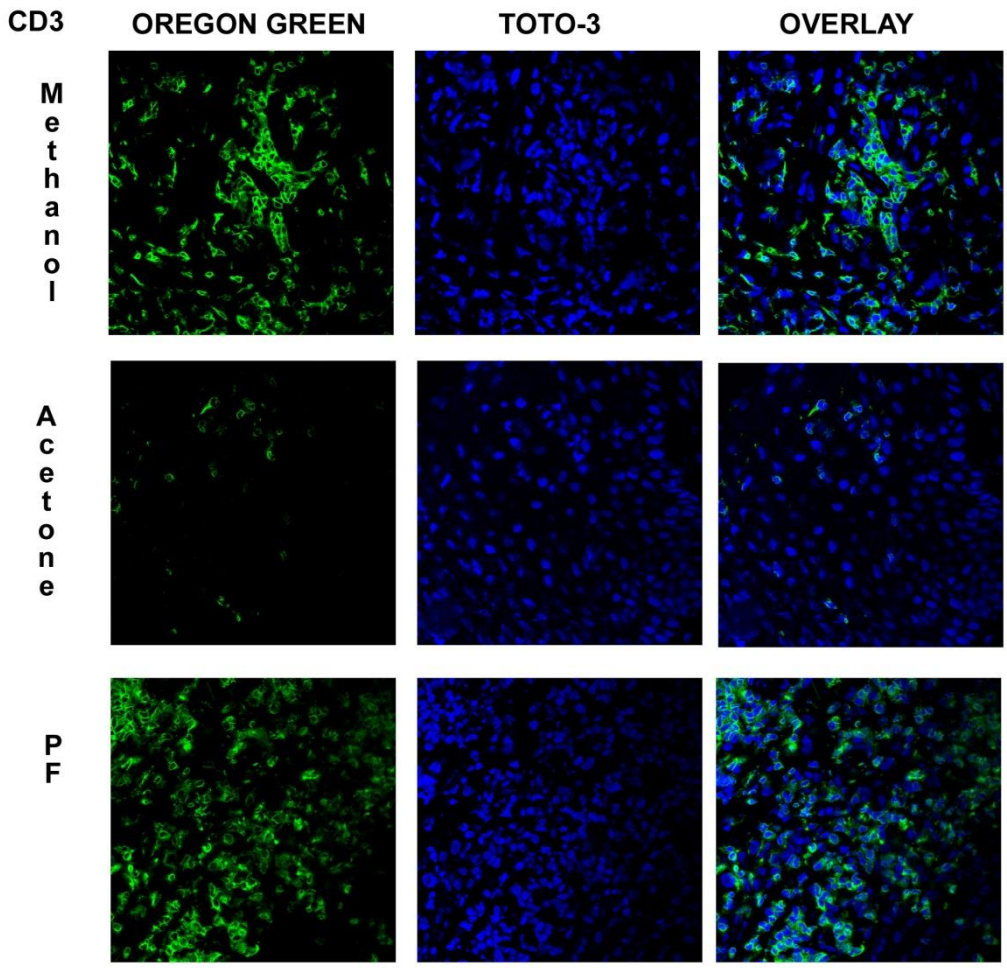
= ☐

14. Appendix D: Fixation methods

Examples of differential antibody staining with different fixation methods.



Anti-active caspase-3 detection in tissue sections with different fixation techniques. Serial sections of psoriatic epidermis, taken from a patient 24h post a single 3 MED irradiation of 311nm UVB. Cryosections were fixed in either 100% methanol, 100% acetone or 4% paraformaldehyde, and prepared as in section 2.1.4. Double labelled was performed with anti-active-caspase-3 (Oregon green secondary antibody) and Toto-3 (blue). It can be seen that fixation with paraformaldehyde gives a more intense background, and it can be difficult to determine which cells are positive. Methanol and or acetone (or indeed both together in a 1:1 ratio- not shown here) are therefore the preferred fixation method for this antibody.



T cell detection in tissue sections with different fixation techniques. Serial sections of psoriatic epidermis. Cryosections were fixed in either 100% methanol, 100% acetone or 4% paraformaldehyde, and prepared as in section 2.1.4. Double labelled was performed with anti-CD3 (Oregon green secondary antibody) and Toto-3 (blue). It can be seen that fixation with acetone gives a significantly reduced staining pattern.

15. Appendix E: Protocols

Mowiol

- To a 100ml beaker add 3.84g Mowiol 40-88 and 12g glycerol
- Mix with a stir bar for 2h
- Add 12ml ddH_20 and stir overnight
- Add 20ml Tris-HCL (pH 8.5), and continue stirring. Heat occasionally to 50^0C (maximum) in a water-bath for 10 mins. Continue to stir until all dissolved
- Centrifuge at 5000x g for 15 mins to clarify the solution
- Remove supernatant
- Optional: add 2.5% 1,4 diazabicyclo [2,2,2] octane (DABCO) to reduce fading
- Aliquot and store at -20^0C for up to 6 months or -80^0C for longer term storage
- Apply around edge of air-dried immuno-stained tissue sections and press cover firmly in place; removing any small air bubbles
- Keep in fridge overnight, then store in dark area at RTP

Protocol for Immunohistochemistry: Sequential Method

1. Fix for 10 mins with either:
   a. Acetone (dry)
   b. Methanol
   c. Acetone: Methanol (1:1)
   d. Paraformaldehyde (0.4g in 10ml PBS +2ul 5mmol NaOH)
2. Wash with PBS and Tween
3. Triton x 0.2% 10 min
4. Wash 3x 1 min
5. Block with goat serum in 2% BSA 1:60. Incubate for 30 mins at rtp
6. Tip off
7. Primary antibody: e.g. caspase-3 in 2% BSA 1:500. Incubate for 60 mins at rtp
8. Wash x3
9. Goat anti-rabbit (488) in 2% BSA 1:300. Incubate for 45 mins
10. Wash x3
11. Goat serum 1:60. Incubate for 20 mins
12. 2^{nd} Antibody in 2% BSA. Incubate for 45mins
13. Wash x3
14. Goat anti-mouse (568) in BSA 1:300. Incubate for 30 mins
15. Wash x3
16. Toto-3 1:7000 12 mins
17. Wash x3
18. Wash x1 with distilled water
19. Wash x1 with 100% ethanol
20. Dry at rtp
21. Mowicol around edges of section then press coverslip firmly in place
22. Leave overnight in fridge, then store in dark area

16. Appendix F: Netlogo modelling code

```
turtles-own
[
  stem?          ;; true for stem cells, false for TA or diff
  TA?            ;; true for TA cells, false for others
  diff?          ;; true for differentiating cells, false for others
  arrest?        ;; cells that arrest but don't die after UV
  dermis?        ;; turtle to draw the dermis
  stem-factor ;;level of factor x within cell, determining whether the cell can divide or not
  time-as-differentiated ;; no. hours since cell derived  TA as a differentiated cell
  stem-clock    ;;hours for stem cell division
  TA-clock      ;;hours for a TA to divide
  time-since-stem ;; hours since stem for TA
  age-of-cell    ;; turover time for the cell
  m              ;; turtle to move
  divide         ;; number of divisions for each TA cell
  add            ;; for increasing bm
  minus          ;; for decreasing bm
  grey-clock     ;; time from apoptosis for stem cell
  dying?         ;; cells undergoing apoptosis
]

patches-own
[
  gradient       ;; cytokine gradient from the basal layer/ top of the epidermis
  ?              ;; altering variable in epidermis set-up
]

globals
[
  TA-count       ;; count of TA cells
  diff-count     ;; count of diff cells
  stem-count     ;; count of stem cells
  total-count    ;; count of all cells
  treatments     ;; number of UVB epidsodes
  UV-gap         ;; hours between UV irradiations
  positions      ;; position of the basement membrane
  psos           ;; psoriasis phenotype
  clock          ;; count (hrs) since last change in position of the basement membrane
  change         ;; indicates that the basement membrane will need to shift up or down
  shift-up       ;; basement membrane reduces in size
  add-           ;; adds the number of actively dividing TA cells which are not close
```

```
                    enough to the basement membrane (may need to expand bm)
  minus-            ;; adds the number of non-dividing cells near the bm (may need to
                    reduce the bm)
  active-TA         ;; a TA cell which is still in the dividing phase of its life
  adding            ;; number of dividing TAs present/ total number of places in the
                    dividing zone
  reducing          ;; extra number of non-dividing cells in the dividing zone/ total
                    number of places in the dividing zone
  percent-stem      ;; percentage of stem cells actively dividing at any time
  turn-clock        ;; count down (hrs) since irradiation
]


to setup
;; setup the basement membrane and create a gradient
  clear-all
  ask patches
    [
    set pcolor black
    ]
  setup-epidermis
  set-default-shape turtles "circle"
  set-stem
  ask patches with [pcolor = yellow]
     [
     set gradient 10000
     ]
  ask patches with [pycor = max-pycor]
     [
     set gradient 0
     ]
  repeat 80
     [
     diffuse gradient 0.25
     ]
   ask patches with [pycor = -2]
     [
       set gradient 100
     ]
   ask patches with [pycor = -1]
     [
       set gradient 50
     ]
   ask patches with [pycor >= 0]
     [
       set gradient 0
     ]
  setup-plot
  evaluate-params
```

```
  set treatments 0
  set UV-gap 0
  set clock 0
  set turn-clock 0
;;movie-start "psoriasis-movie1.mov"
;;movie-set-frame-rate 10
 end


to setup-epidermis
;; create the epidermis as either the normal or psoriatic morphology
  clear-all
  let normal    [0 0 1 1 2 2 2 1 0 1 0 -1 0 -1 -2 -2 -2  -1 -1  0 ]
  let pso       [0 1 2 3 8 8 2 1 0 1 0 -1 0 -1 -2 -8 -8 -3 -2 -1 ]
  ifelse Start-with-psoriasis-phenotype
     [
     set percent-stem 80
     set positions 7
     crt 1
     let newx -1
     let newy -5
     ask patches with [pxcor = 80]
       [if pycor <= -5
          [
          set pcolor yellow
          ]
       ]
     ask turtles
         [
         let q 0
         loop
            [
            foreach pso
               [
               set newx newx + 1
               set newy newy - ?
               setxy newx newy
               facexy newx min-pycor
               let r 1
               set pcolor yellow
               while [r <= world-height]
                  [
                  fd 1
                  set pcolor yellow
                  set r r + 1
                  ]
               ]
            set q q + 1
            if (q > 3) [stop]
```

```
            ]
          ]
        ]
        [
        set percent-stem 20
        set positions 1
        crt 1
        let newx -1
        let newy -5
        ask patches with [pxcor = 80]
          [if pycor <= -5
             [
             set pcolor yellow
             ]
          ]
 ask turtles
           [
           let q 0
           loop
              [
              foreach normal
                 [
                 set newx newx + 1
                 set newy newy - ?
                 setxy newx newy
                 facexy newx min-pycor
                 let r 1
                 set pcolor yellow
                 while [r <= world-height]
                    [
                    fd 1
                    set pcolor yellow
                    set r r + 1
                    ]
                 ]
              set q q + 1
              if (q > 3) [stop]
             ]
          ]
        ]

end


to set-stem
;;create stem cells
   crt 50
   ifelse Start-with-psoriasis-phenotype
      [
```

```
set positions 7
ask turtles
   [
  if (who = 3)  [setxy 1 -5]
  if (who = 4) [setxy 2 -7]
  if (who = 5) [setxy 3 -10]
  if (who = 6)or (who = 43) [setxy 4 -18]
  if (who = 7) [setxy 5 -26]
  if (who = 8) [setxy 14 -26]
  if (who = 9)or (who = 44) [setxy 15 -18]
  if (who = 10) [setxy 16 -10]
  if (who = 11)  [setxy 17 -7]
  if (who = 12) [setxy 22 -7]
  if (who = 13) [setxy 23 -10]
  if (who = 14)or (who = 45) [setxy 24 -18]
  if (who = 15) [setxy 25 -26]
  if (who = 16) [setxy 34 -26]
  if (who = 17)or (who = 46) [setxy 35 -18]
  if (who = 18) [setxy 36 -10]
  if (who = 19)[setxy 37 -7]
  if (who = 20) [setxy 38 -5]
  if (who = 21) [setxy 18 -5]
  if (who = 22) [setxy 21 -5]
  if (who = 23) [setxy 41 -5]
  if (who = 24) [setxy 42 -7]
  if (who = 25) [setxy 43 -10]
  if (who = 26)or (who = 47) [setxy 44 -18]
  if (who = 27) [setxy 45 -26]
  if (who = 28) [setxy 54 -26]
  if (who = 29)or (who = 48) [setxy 55 -18]
  if (who = 30) [setxy 56 -10]
  if (who = 31) [setxy 57 -7]
  if (who = 32) [setxy 58 -5]
  if (who = 33) [setxy 61 -5]
  if (who = 34) [setxy 62 -7]
  if (who = 35) [setxy 63 -10]
  if (who = 36)or (who = 49) [setxy 64 -18]
  if (who = 37) [setxy 65 -26]
  if (who = 38) [setxy 74 -26]
  if (who = 39)or (who = 50) [setxy 75 -18]
  if (who = 40) [setxy 76 -10]
  if (who = 41) [setxy 77 -7]
  if (who = 42) [setxy 78 -5]
  set size 1
  set stem-clock random 150
  set stem? true
  set TA? false
  set diff? false
  set dermis? false
```

```
    set color 85
    set dying? false
    if (who = 1)[die]
    if (who = 2) [die]
    if (who = 0) [die]
    ]
]
[set positions 1
 ask turtles
    [
    if (who = 3) [setxy 1 -4]
    if (who = 4) [setxy 2 -5]
    if (who = 5) [setxy 3 -6]
    if (who = 6)or (who = 43) [setxy 4 -8]
    if (who = 7) [setxy 5 -10]
    if (who = 8) [setxy 14 -10]
    if (who = 9)or (who = 44) [setxy 15 -8]
    if (who = 10) [setxy 16 -6]
    if (who = 11) [setxy 17 -5]
    if (who = 12)[setxy 22 -5]
    if (who = 13) [setxy 23 -6]
    if (who = 14)or (who = 45) [setxy 24 -8]
    if (who = 15)[setxy 25 -10]
    if (who = 16) [setxy 34 -10]
    if (who = 17)or (who = 46) [setxy 35 -8]
    if (who = 18) [setxy 36 -6]
    if (who = 19) [setxy 37 -5]
    if (who = 20) [setxy 38 -4]
    if (who = 21) [setxy 18 -4]
    if (who = 22) [setxy 21 -4]
    if (who = 23) [setxy 41 -4]
    if (who = 24) [setxy 42 -5]
    if (who = 25)[setxy 43 -6]
    if (who = 26)or (who = 47) [setxy 44 -8]
    if (who = 27) [setxy 45 -10]
    if (who = 28) [setxy 54 -10]
    if (who = 29)or (who = 48) [setxy 55 -8]
    if (who = 30) [setxy 56 -6]
    if (who = 31) [setxy 57 -5]
    if (who = 32) [setxy 58 -4]
    if (who = 33) [setxy 61 -4]
    if (who = 34) [setxy 62 -5]
    if (who = 35) [setxy 63 -6]
    if (who = 36)or (who = 49) [setxy 64 -8]
    if (who = 37) [setxy 65 -10]
    if (who = 38) [setxy 74 -10]
    if (who = 39)or (who = 50) [setxy 75 -8]
    if (who = 40) [setxy 76 -6]
    if (who = 41) [setxy 77 -5]
```

```
        if (who = 42) [setxy 78 -4]
        set size 1
        set stem-clock random 150
        set stem? true
        set TA? false
        set diff? false
        set arrest? false
        set dermis? false
        set color 85
        set dying? false
       if (who = 1)[die]
       if (who = 2) [die]
       if (who = 0) [die]
       ]
   ]
 end


to go
;; run the model
;; Rechecks the number of dividing  cells adjacent to the basal layer, and expands /
          contracts the epidermis accordingly.
no-display
if  (count turtles with [pycor = -4] > 75) and (ticks > 400) and
     ((count patches with [(pycor < -1 and pcolor = black) and count turtles-here > 0] /
          count patches with [pycor < -1 and pcolor = black]) >= 0.98)
      [
      bm
      ask-concurrent (turtles with [stem?])
        [
        ;;ask stem cells to move down if a space has been created below them by a shift
          in the basement membrane
          ask patch-at-heading-and-distance 180 1
             [
             if pcolor = black
               [
               ask turtles-on patch-at-heading-and-distance 0 1
                 [
                 shift-down

                 ]
               ]
             ]

          ]

      ]
ask turtles
   [
```

```
  if any? neighbors with [(count turtles-here = 0) and ([pycor] of self < [pycor] of myself)]
      [
      check-position
      ]
  ]

ask turtles
 [
  ;; allow normal cell division to occur, with cells differentiating when they reach a
        threshold gradient
  set-stem-division
  set-TA-division
  set-diff
  let nb5 max-one-of neighbors with [pcolor = black][gradient]
  if (TA? or diff?) and (count turtles-on nb5 = 0)
      [
      move-to nb5
      ]
  if stem?
     [
     set stem-clock stem-clock + 1
     set grey-clock grey-clock + 1
     ]
  if TA?
     [
     set TA-clock TA-clock + 1
     set time-since-stem time-since-stem + 1
     set age-of-cell age-of-cell + 1
     ]
  if diff?
     [
     set time-as-differentiated time-as-differentiated + 1
     set age-of-cell age-of-cell + 1
     ]
  if TA? and (divide > TA-cell-cycle) and ([gradient] of patch-here < 1800)
     [
     set TA? false
     set diff? true
     set color 65
     set time-as-differentiated 0
     ]
  if color = pink
       [
         check-position
       ]
 ]
 ;; irradiate the epidermis at the specified frequency and time interval
 if (irradiation-frequency > 0) and (UV-gap >= irradiation-frequency) and (Number-of-
        treatments > treatments)
```

```
 [
 uv
 ask turtles
  [
  ;; check that the youngest most actively dividing cells have not been displaced from
        the basal and suprabasal layers by cells with less proliferative ability
   check-position
  ]
 ]
;; close any gaps that appear in the epidermis, especially after irradiation
repeat 5
 [ask-concurrent turtles
   [
   if (TA? or diff?)
     [
     let shifting turtles-here
     ask patch-at-heading-and-distance 180 1
        [
        if (pcolor = black) and (count turtles-here = 0)
           [
           ask shifting
              [
              shift-down
              ]
            ]
        ]
     ]
    ]
  ]
;; stop cells piling-up on top of each other
ask turtles with [stem?]
           [
            if count turtles-here with [TA?] > 0
               [
                 ask turtles-here with [TA?]
                   [
                    relocate
                   ]
               ]
           ]
ask turtles with [TA?]
 [
 if count turtles-here > 2
   [
   relocate
   ]
 ]
set clock clock + 1
```

```
;;set the counter for when to give the next irradiation
set uv-gap uv-gap + 1

if turn-clock = 1 [if any? turtles with [dying?]
                   [
                    ask turtles with [dying?]
                      [
                        die
                      ]
                    ]
                  ]
if (turn-clock > 0)
    [
      set turn-clock turn-clock - 1
    ]
if (turn-clock > 0) and (turn-clock <= 24)
    [
                ask turtles with [dying?]
                  [
                    let s random 24
                    if s < 2
                      [
                        die
                      ]
                  ]
      ]
ask turtles with [(diff?) and (pycor > -4)]
  [
    if count turtles-on patch-at-heading-and-distance 180 1 = 0
      [
        ask turtles-on patch-at-heading-and-distance 0 1
          [
            set heading 180
            fd 1
          ]
       ]
  ]
;; adjust the cytokine level according to the total keratinocyte number
divide-change
;; turtles die as they fall off the top of the epidermis
surface
display
evaluate-params
tick
if ((ticks >= 1200) and (ticks mod 2 = 0)) [movie-grab-interface]
;;if ticks >= 2300 [set cytokine-stimulus "off"]
if ticks >= 3200 [movie-close stop]
end
```

```
to surface
;; if lowest level is top of the epidermis, don't go higher
if any? turtles with [pycor > -1]

        [
         ask turtles-on patches with [pycor > -1]
           [
             set heading 180
             fd 1
             if pycor > -1 [fd 1]
           ]
        ]
  if count turtles-on patches with [pycor = -1] > 80
            [
              ask turtles-on patches with [(count turtles-here > 1) and (pycor = -1)]
                [
                  ask min-one-of turtles-here [time-since-stem]
                    [
                     stop
                    ]

                 roll
                ]
            ]
 if count patches with [(pycor = -1) and (count turtles-here > 0)] < 80
            [
              ask patches with [(count turtles-here > 1) and (pycor = -1)]
                 [
                   ask max-one-of turtles [time-since-stem]
                     [
                       roll
                     ]
                 ]
            ]
end


to roll
let x (count turtles-on patches with [pycor = -1]) - 81
if x >= 1
  [
   ask n-of x turtles-on patches with [(pycor = -1) and (count turtles-here > 1)] [die]
  ]
if ((count turtles-here > 1) and (pycor = -1))
   [
     ask max-one-of turtles-here [time-since-stem]
        [
          set heading 90
```

```
          fd 1
          roll
          if count turtles-here <= 1 [stop]
        ]
  ]
end


to check-position
;; check the most proliferative cells are closest to the basal layer
if stem?
        [
        stop
        ]

if ((TA?) or (diff?))
   [
   repeat 25
          [
          let friends-patch max-one-of neighbors with [(pcolor = black) and ((count turtles-
        here = 0) or ( [time-since-stem] of min-one-of turtles-here [time-since-stem] >
        [time-since-stem] of myself))] [gradient]
          ifelse friends-patch = nobody
              [
              ask patch-at-heading-and-distance 180 1
                   [
                     if (pcolor = black) and ((count turtles-here = 0) or ( [time-since-stem] of
        min-one-of turtles-here [time-since-stem] > [time-since-stem] of myself))
                        [
                          ask turtles-on patch-at-heading-and-distance 0 1
                            [
                              set heading 180
                              fd 1
                              relocate
                            ]
                        ]
                   ]
              ]
              [
              if turtles-on friends-patch != stem?
                [
                move-to friends-patch
                relocate
                ]
              ]
          ]
    ]
end
```

```
to divide-change
;; if cytokine-stimulus is on, the maximum cytokine production occurs in keeping with
        psoriasis,
;; if not the amount of cytokines produced is relative to the total keratinocyte load
;; and this affects the proportion of TA cells whicvh divide 5 times instead of 4
ifelse cytokine-stimulus
  [
  ask turtles with [color = pink]
      [
          let s random 99
          ifelse s < 50
            [
              set Number-of-TA-divisions 5
            ]
            [
              set Number-of-TA-divisions 4
            ]
          ]
  ask turtles with [color = 85]
    [
    set percent-stem 80
    ]
   ]
   [
  ask turtles
     [
   if (count turtles with [color = pink] >= 200)
        [
        ask turtles with [(color = pink) ]
          [
          let s random 99
          ifelse s < 50
            [
              set Number-of-TA-divisions 5
            ]
            [
              set Number-of-TA-divisions 4
            ]
          ]
        ]

    if (count turtles with [color = pink] < 200) and (count turtles with [color = pink] >= 150)
        [
        ask turtles with [(color = pink) ]
          [
          let s random 99
          ifelse s < 37.5
            [
```

```
            set Number-of-TA-divisions 5
          ]
          [
            set Number-of-TA-divisions 4
          ]
        ]
      ]
    if (count turtles with [color = pink] < 150) and (count turtles with [color = pink] >= 100)
      [
      ask turtles with [(color = pink) ]
        [
        let s random 99
        ifelse s < 25
          [
            set Number-of-TA-divisions 5
          ]
          [
            set Number-of-TA-divisions 4
          ]
        ]
      ]
    if (count turtles with [color = pink] < 100) and (count turtles with [color = pink] >= 75)
      [
      ask turtles with [(color = pink) ]
        [
       let s random 99
        ifelse s < 12.5
          [
            set Number-of-TA-divisions 5
          ]
          [
            set Number-of-TA-divisions 4
          ]
        ]
      ]
  if (count turtles with [color = pink] < 75)
      [
      set percent-stem 20
      ask turtles with [(color = pink)]
        [
        set Number-of-TA-divisions 4
        ]
      ]
    ]
  ]
end
```

```
to bm
;; changes size of basement membrane according to the number of actively dividing
          cells
 ask turtles
    [
    set add 0
    set minus 0
    if (TA?)  and (color = pink)
     [
     if ([gradient] of patch-here <= 4000)
            [
             set add 1
             set minus 0
            ]
      ]
     ask patches with [gradient > 4000]
         [
         ask turtles-here with [(color = 134) or diff?]
           [
           set minus 1
           set add 0
           ]
       ]
     ]
set change 0
set shift-up 0
if count turtles with [add  = 1] = 0
  [
  stop
  ]
let k (active-TA - (count turtles with [add = 1]) + (count turtles with [minus = 1]))
set adding (active-TA / (k))
set reducing (((count turtles with [minus = 1]) - (count turtles with [add = 1])) / k)
if  adding > 1.20
       [
       set positions positions + 1
       set shift-up 1
       set change 1
       if positions > 7
           [
           set positions 7
           set change 0
          ]
      ]
if  reducing > 0.4
       [
        set positions positions - 1
         set change 1
         if positions < 1
```

```
        [
        set positions 1
        set change 0
        ]
     ]

if change = 0
    [
    stop
    ]
cp
crt 1
    [
    set TA? false
    set diff? false
    set stem? false
    set dermis? true
    ]
let newx -1
let newy -5
ask patches with [pxcor = 80]
       [if pycor <= -5
          [
          set pcolor yellow
          ]
       ]
if positions = 7
    [
set psos    [0 1 2 3 8 8 2 1 0 1 0 -1 0 -1 -2 -8 -8 -3 -2 -1 ]
ask turtles
   [
   if dermis?
       [
       let q 0
       loop
          [
          foreach psos
             [
             set newx newx + 1
             set newy newy - ?
             setxy newx newy
             facexy newx min-pycor
             let r 1
             set pcolor yellow
             while [r <= world-height]
                [
                fd 1
                set pcolor yellow
                set r r + 1
```

```
                ask patches with [pcolor = yellow and pxcor = newx]
                    [
                     if any? turtles-here with [TA? or stem? or diff?]
                         [
                       ifelse (shift-up = 1)
                           [
                           ask turtles-here with [TA? or stem? or diff?]
                               [
                               shift-down
                               ]
                           ]
                           [
                           ask turtles-here with [TA? or stem? or diff?]
                               [
                               shift
                               ]
                           ]
                       ]
                    ]
                ]
            ]
          set q q + 1
          if (q > 3)
                [
                 stop
                ]
            ]
        ]
    ]
  ask turtles
     [
     if dermis?
       [
       die
       ]
     ]
         ]
if positions = 6
    [
set psos     [0 1 2 3 7 7 2 1 0 1 0 -1 0 -1 -2 -7 -7 -3 -2 -1 ]
ask turtles
   [
   if dermis?
      [
      let q 0
      loop
         [
         foreach psos
             [
```

```
                set newx newx + 1
                set newy newy - ?
                setxy newx newy
                facexy newx min-pycor
                let r 1
                set pcolor yellow
                while [r <= world-height]
                   [
                   fd 1
                   set pcolor yellow
                   set r r + 1
                   ask patches with [pcolor = yellow and pxcor = newx]
                       [
                        if any? turtles-here with [TA? or stem? or diff?]
                            [
                           ifelse (shift-up = 1)
                               [
                               ask turtles-here with [TA? or stem? or diff?]
                                   [
                                   shift-down
                                   ]
                               ]
                               [
                               ask turtles-here with [TA? or stem? or diff?]
                                   [
                                   shift
                                   ]
                               ]
                           ]
                       ]
                   ]
               set q q + 1
               if (q > 3)
                     [
                      stop
                     ]
                ]
             ]
          ]
      ask turtles
         [
         if dermis?
           [
           die
           ]
         ]
      ]
if positions = 5
```

```
[
set psos [0 1 2 2 6 6 2 1 0 1 0 -1 0 -1 -2 -6 -6 -2 -2 -1 ]
ask turtles
   [
   if dermis?
     [
     let q 0
     loop
       [
       foreach psos
         [
         set newx newx + 1
         set newy newy - ?
         setxy newx newy
         facexy newx min-pycor
         let r 1
         set pcolor yellow
         while [r <= world-height]
           [
           fd 1
           set pcolor yellow
           set r r + 1
           ask patches with [pcolor = yellow and pxcor = newx]
              [
               if any? turtles-here with [TA? or stem? or diff?]
                   [
                  ifelse (shift-up = 1)
                      [
                      ask turtles-here with [TA? or stem? or diff?]
                          [
                          shift-down
                          ]
                      ]
                      [
                      ask turtles-here with [TA? or stem? or diff?]
                          [
                          shift
                          ]
                      ]
                  ]
              ]
           ]
       ]
     set q q + 1
     if (q > 3)
          [
           stop
          ]
       ]
```

```
        ]
    ]
    ask turtles
        [
        if dermis?
            [
            die
            ]
        ]
    ]
if positions = 4
 [
 set psos  [0 1 2 2 5 5 2 1 0 1 0 -1 0 -1 -2 -5 -5 -2 -2 -1 ]
  ask turtles
    [
    if dermis?
        [
         let q 0
         loop
           [
            foreach psos
               [
                set newx newx + 1
                set newy newy - ?
                setxy newx newy
                facexy newx min-pycor
                let r 1
                set pcolor yellow
                while [r <= world-height]
                   [
                    fd 1
                    set pcolor yellow
                    set r r + 1
                    ask patches with [pcolor = yellow and pxcor = newx]
                        [
                         if any? turtles-here with [TA? or stem? or diff?]
                              [
                            ifelse (shift-up = 1)
                                [
                                ask turtles-here with [TA? or stem? or diff?]
                                    [
                                    shift-down
                                    ]
                                ]
                                [
                                ask turtles-here with [TA? or stem? or diff?]
                                    [
                                    shift
                                    ]
```

```
                        ]
                    ]
                ]
            ]
        ]
        set q q + 1
        if (q > 3)
            [
             stop
            ]
        ]
    ]
  ]
  ask turtles
    [
    if dermis?
    [
    die
    ]
  ]
]
if positions = 3
 [
 set psos  [0 0 1 2 4 4 2 1 0 1 0 -1 0 -1 -2 -4 -4 -1 -2  0 ]
 ask turtles
   [
   if dermis?
      [
       let q 0
       loop
         [
          foreach psos
            [
             set newx newx + 1
             set newy newy - ?
             setxy newx newy
             facexy newx min-pycor
             let r 1
             set pcolor yellow
             while [r <= world-height]
               [
                fd 1
                set pcolor yellow
                set r r + 1
                ask patches with [pcolor = yellow and pxcor = newx]
                   [
                    if any? turtles-here with [TA? or stem? or diff?]
                        [
                      ifelse (shift-up = 1)
```

```
                        [
                        ask turtles-here with [TA? or stem? or diff?]
                            [
                            shift-down
                            ]
                        ]
                        [
                        ask turtles-here with [TA? or stem? or diff?]
                            [
                            shift
                            ]
                        ]
                    ]
                ]
            ]
        ]
        set q q + 1
        if (q > 3)
            [
             stop
            ]
       ]
    ]
   ]
     ask turtles
       [
       if dermis?
         [
         die
         ]
       ]
]
if positions = 2
 [
 set psos [0 0 1 1 3 3 2 1 0 1 0 -1 0 -1 -2 -3 -3 -1 -1  0 ]
 ask turtles
   [
   if dermis?
     [
      let q 0
      loop
        [
         foreach psos
           [
            set newx newx + 1
            set newy newy - ?
            setxy newx newy
            facexy newx min-pycor
            let r 1
```

```
                set pcolor yellow
                while [r <= world-height]
                  [
                   fd 1
                   set pcolor yellow
                   set r r + 1
                   ask patches with [pcolor = yellow and pxcor = newx]
                      [
                       if any? turtles-here with [TA? or stem? or diff?]
                           [
                          ifelse (shift-up = 1)
                              [
                              ask turtles-here with [TA? or stem? or diff?]
                                  [
                                  shift-down
                                  ]
                              ]
                              [
                              ask turtles-here with [TA? or stem? or diff?]
                                  [
                                  shift
                                  ]
                              ]
                           ]
                      ]
                  ]
              ]
          set q q + 1
          if (q > 3)
                [
                 stop
                ]
           ]
        ]
    ]
    ask turtles
       [
        if dermis?
          [
            die
          ]
       ]
   ]
if positions = 1
  [
  set psos  [0 0 1 1 2 2 2 1 0 1 0 -1 0 -1 -2 -2 -2  -1 -1  0 ]
   ask turtles
     [
     if dermis?
```

```
    [
    let q 0
    loop
      [
      foreach psos
          [
          set newx newx + 1
          set newy newy - ?
          setxy newx newy
          facexy newx min-pycor
          let r 1
          set pcolor yellow
          while [r <= world-height]
             [
             fd 1
             set pcolor yellow
             set r r + 1
             ask patches with [pcolor = yellow and pxcor = newx]
                [
                 if any? turtles-here with [TA? or stem? or diff?]
                     [
                    ifelse (shift-up = 1)
                        [
                        ask turtles-here with [TA? or stem? or diff?]
                            [
                            shift-down
                            ]
                        ]
                        [
                        ask turtles-here with [TA? or stem? or diff?]
                            [
                            shift
                            ]
                        ]
                    ]
                ]
          ]
      set q q + 1
      if (q > 3)
            [
             stop
            ]
       ]
   ]
]
ask turtles
    [
    if dermis?
```

```
            [
            die
            ]
          ]
  ]
 ;;reset the gradient
 ask turtles
  [
  set add 0
  set minus 0
  ]
 ask patches with [pcolor = black]
    [
    set gradient 0
    ]
  ask patches with [pcolor = yellow]
    [
    set gradient 10000
    ]

  repeat 80
    [
    diffuse gradient 0.25
    ]
   ask patches with [pycor = -2]
    [
      set gradient 100
    ]
   ask patches with [pycor = -1]
    [
      set gradient 50
    ]
   ask patches with [pycor >= 0]
    [
      set gradient 0
    ]
surface
end


to shift-down
;; if there is a gap in the epidermis, close it
set heading 180
fd 1
ask patch-at-heading-and-distance 180 1
   [
   if (pcolor = black) and (count turtles-here = 0)
        [
         ask turtles-on patch-at-heading-and-distance 0 1
```

```
            [
            shift-down
            ]
        ]
    ]
end


to shift
;; change the cell position if basement membrane changes shape (called by BM)
if stem? or TA? or diff? or arrest?
    [
    set heading 0
    fd 1
    if any? other turtles-here
       [
       ask other turtles-here
          [
          shift
          ]
       ]
    ]
end


to set-stem-division
;; stem cells each have a % probability of dividing according to the user defined setting
          on the interface
if (color = 85) and (stem-clock > stem-cell-cycle)
      [
      let s random 99
      if s < percent-stem
          [
          hatch 1
             [
            show ""
            set stem? false
            set TA? true
            set diff? false
            set dermis? false
            set color pink
            set TA-clock -10 + random 15
            set time-since-stem 1
            set age-of-cell 0
            set divide 0
            relocate
            ]
        ]
```

```
        set stem-clock 0
      ]
;; grey cells are stem cells which have undergone UV-induced apoptosis and have later
        been replaced by symmetrical stem cell division
  if (color = grey) and (stem-clock > stem-cell-cycle) and (percent-stem = 20)
      [
      let s random 99
       if s < 20
          [
          hatch 1
            [
            show ""
            set stem? false
            set TA? true
            set diff? false
            set dermis? false
            set color pink
            set TA-clock -10 + random 15
            set time-since-stem 1
            set age-of-cell 0
            set divide 0
            relocate
            ]
         ]
         set stem-clock 0

      ]
 end


to set-TA-division
;; cell divides specified number of times then rests (turns dark pink)
if TA? and color = 134
  [
    stop
  ]
 if (TA?) and (divide >= Number-of-TA-divisions)
            [
            set color 134
            set time-since-stem time-since-stem + 1000
            ]
 if (TA?) and (((TA-clock >= TA-cell-cycle) and (divide < Number-of-TA-divisions)) and
         not (dying?))
      [
      set divide divide + 1
      set age-of-cell 0
      set TA-clock -10 + random 15
      hatch 1
              [
```

```
            show ""
            set TA? true
            set TA-clock -10 + random 15
            set stem? false
            set diff? false
            set dermis? false
            set color pink
            set age-of-cell 0
            relocate
            stop
            ]
        stop
      ]
end


to set-diff
;; cells differentiate when they reach a defined threshold in the gradient of the
        underlying patches
  if (color = 134) and ([gradient] of patch-here < 1900)
    [
    set TA? false
    set stem? false
    set diff? true
    set dermis? false
    set color 65
    set time-as-differentiated 0
    set time-since-stem time-since-stem + 5000
    set add 0
    set minus 0
    stop
    ]
end


to relocate
;; only allows 1 turtle per patch, moving cells away from a single patch after division
;; don't do anything if you've reached the top of the world
if pycor = max-pycor
   [
   stop
   ]
if count turtles-on patch-here < 2
   [
   stop
   ]
 ;; to assign each turtle a different moving priority
 ifelse (max-one-of turtles-here([time-since-stem]) = (min-one-of turtles-here [time-
        since-stem]))
```

```
 [
 set m (min-one-of turtles-here [who])
 if m = stem?
   [
   set m (max-one-of turtles-here [time-since-stem])
   ]
 ]
 [
 set m max-one-of turtles-here [time-since-stem]
 ]
 if count turtles-on patch-here < 2
 [
 stop
 ]
;; move to neighbour with highest gradient and not occupied
let nb max-one-of neighbors with [(pcolor = black) and (count turtles-here = 0)]
        [gradient]
if nb != nobody
;;get the correct turtle to move
  [
  ifelse [gradient] of nb < [gradient] of patch-here
      [
      ask m
        [
        move-to nb
        check-position
        stop
        ]
      ]
      [
      ifelse turtles-on patch-here = stem?
        [
        set m min-one-of turtles-here [time-since-stem]
        ask m
          [
          move-to nb
          ]
        ]
        [
        move-to nb
        ]
      ]
      if count turtles-here < 1
        [
        stop
        ]
      if any? turtles-here = stem?
        [
        if count turtles-here = 2
```

```
          [
          stop
          ]
        ]
        relocate
    ]
if count turtles-here < 2
  [
  stop
  ]
;; turtle to move to patch with highest gradient, black and older than myself
let aged [time-since-stem] of m
if any? neighbors with [(pcolor = black) and ([time-since-stem] of min-one-of turtles-
      here [time-since-stem] > aged) ]
  [
  let nb2 max-one-of neighbors with [(pcolor = black) and ([time-since-stem] of min-
      one-of turtles-here [time-since-stem] > aged)] [gradient]
  if nb2 != nobody
     [
      if ([gradient] of patch-here > [gradient] of nb2) and (turtles-on nb2 != stem?)
       [
       ask m
          [
           move-to nb2
           ask other turtles-here
               [
               relocate
                ]
            stop
            ]
         ]
      ]
      check-position
   ]
if count turtles-here < 2
    [
     check-position
    stop
    ]
;; otherwise look at lowest gradient patch and move here
let nb3 min-one-of neighbors with [pcolor = black] [gradient]
if m != nobody
 [
 ask m
    [
    if stem?
       [
       stop
       ]
```

```
      if [gradient] of patch-here > [gradient] of nb3
         [
          move-to nb3
          relocate
          stop
         ]
      ]
      check-position
  ]
end


to uv
;; irradiate the epidermis
  set UV-gap 0
  set treatments treatments + 1
  set turn-clock 36
   ask turtles with [(color = pink) or (color = 134) ]
      [
      let s random 99
      if s < 20
         [
          set dying? true
         ]
      ]
  ask turtles with [color = 85]
      [
      let t random 99
      if t < 13
            [
           set color grey
           set grey-clock 0
            ]
      ]
end



to setup-plot
 set-current-plot "Populations"
end


to evaluate-params
  set TA-count count turtles with [color = pink or color = 134 or color = white]
  set diff-count count turtles with [color = 65]
  set stem-count count turtles with [color = 85]
  set add- count turtles with [add = 1]
  set minus- count turtles with [minus = 1]
```

```
  set total-count count turtles
  set active-TA count turtles with [color = pink]
  set-current-plot-pen "TA cells"
  plot count turtles with [color = pink or color = 134 or color = white]
  set-current-plot-pen "Differentiated"
  plot count turtles with [color = 65]
  let file "ps-80 params"
  file-open file
  file-print ""
  file-write ticks
  file-write stem-count
  file-write TA-count
  file-write diff-count
  file-write treatments
  file-write total-count
  file-write add-
  file-write minus-
  file-close
end
```

17. Appendix G: Differentially regulated genes

The following lists contain the genes whose expression was significantly differentially regulated following irradiation with 311nm UVB compared to 290nm UVB or un-irradiated psoriatic epidermis, at 4h (46 genes) and 18h (159 genes).

At 4h:

AMPD1
AOC2
AOC2
ATF3
C10orf113
C19orf48
C6orf122
CDKN1A
CDKN2A
CYP8B1
DUSP4
DUSP6
EGR3
EGR3
EPHB2
FLJ45139
FOSB
FOSL1
GDF15
GGT1
HAS1
HAS3
HEXIM1
IL6
JUNB
KIAA1967
LOC131149
MRPL27
MT1A

At 18h

ALAS2
ANXA2
ANXA9
APOBEC3A
APOL3
BAT2
BCAS3
BIRC5
C16orf74
C1orf56
C6orf142
C7orf10
C9orf93
CADPS2
CATSPERB
CCDC46
CCNB3
CD68
CD8A
CDH16
CHRNA9
CLDN8
CLYBL
CNTNAP3
COL4A6
CST6
CST6
CXCR5
CYP4F2
HBEGF
HIST1H2BD
HIST1H3B
HMCN1
HOMER2
HOXA9
IGFL2
IL19
IL1F10
IL24
IMMP2L
JUP
KEL
KIAA1328
KLK12
KLK6
KLK9
KRT75
LCE1B
LCE1D
LCE2A
LCE2C
LCE3C
LCE3D
LCE3E
LNX1
LOC284751
LY6H
LY6H
PSORS1C2
PYDC1
RAD51L1
RGS20
RGS22
RHOD
RIMBP2
RPL29
RPS7
RUNDC3A
SAA2
SCN2A
SDS
SEMA7A
SERPINA1
SERPINA12
SERPINB7
SERPINE1
SHANK2
SLC25A21
SLC35E4
SLC5A10
SLC6A1
SLCO4A1
SMOX
SMTN
SMYD3
SORCS1
SOX6

NAT5
NEK2
NEU4
NOL6
NR4A1
OSR2
PHC2
PIM3
PREI3
PSPN
TFAP2A
TSSK4
USMG5
USP2
WTAP
ZHX2
ZSWIM3

DBC1
DEFB103A
DEGS2
DMBT1
DNASE1L2
DOCK3
DPP6
EDA2R
EFCAB2
ELF3
EPHA5
ETV3L
EXOC4
FAM155B
FHIT
FHOD3
FOSL1
GABRB1
GAL
GCGR
GDF15
GPHN
GPRC5A
HAP1

MAPK10
MBD5
MED24
METRNL
MMP1
MMP10
MMP3
MSRA
MYBPHL
NEFH
NLGN1
NME1-NME2
NODAL
NOS2A
NPTX1
NRG4
ODZ3
PACRG
PCDH11Y
PGM5
PIK3C2G
PNMA3
PROM1
PRSS3

SPINK6
SPINK7
SPRR1B
SPRR3
SPRR4
SULT2B1
SUPT3H
SYT17
TAGLN3
TBC1D5
TF
TH
THRSP
TLN2
TM4SF19
TM4SF19
TMEM93
TPSAB1
TREX2
TSPO
VGF
VWCE
WFDC12
ZHX2