Mechanism of action of cyclosporine A in atopic eczema and role of cyclophilin B as a regulator of human keratinocyte growth and differentiation

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Thesis submission for the degree of

Doctor of Medicine (MD)

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Submitted August 2013

ABSTRACT

Atopic eczema has a profound negative impact on patients and their families and improved therapeutic options are required. Although, the pathogenesis is still not fully understood, evidence of a primary barrier defect predisposing to increased stimulation of the innate and adaptive immune system increases.

Cyclosporine A (CsA) is an effective therapy for inflammatory skin disease. CsA binds to cyclophilin B (CypB) with high affinity, mediating T-cell immunosuppression. CsA also exerts T-cell independent effects in skin. The Reynolds lab has previously shown that CypB is expressed and secreted by normal human epidermal keratinocytes (NHEK). In this work we aimed to investigate the functional role of CypB in the epidermis.

Confocal analysis of normal human skin revealed CypB expression within the granular cell layer. Transduction of NHEK with retroviral vectors containing $CypB_{WT}$-GFP, $CypB_{W128A}$-GFP (a mutant reducing CsA binding by 97%) positively regulated keratinocyte differentiation (transglutaminase promoter luciferase and enzyme activity). In addition, transduction of NHEK with $CypB_{WT}$ and $CypB_{W128A}$ significantly increased colony formation and keratinocyte proliferation compared to empty vector. Furthermore, conditioned medium from NHEKs transduced with $CypB_{WT}$ or $CypB_{W128A}$ increased proliferation of naïve NHEK. Conversely, knockdown of $CypB_{WT}$ reduced NHEK proliferation. Moreover, NHEK transduced with $CypB_{WT}$ formed thicker epidermal equivalents compared with empty vector controls.

Analysis of eczematous skin treated with CsA, showed changes in expression of CypB before and following 2 weeks of CsA treatment. An increase in filaggrin expression in eczematous skin during these early phases of treatment with CsA, suggests that CsA may act by positively repairing impaired barrier function. These studies provide an increased understanding of the physiological and pathological role of CypB in keratinocytes, further insight into the mechanism of action of CsA and may identify CypB as a novel drug target for atopic eczema.

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ABBREVIATIONS

AE	Atopic eczema
AMP	Antimicrobial peptide
AP1	Activating protein-1
AP2	Activating protein-2
bp	Base pairs
BCA	Biocinchoninic acid
Ca^{2+}	Calcium
$[Ca^{2+}]_i$	Intracellular calcium
$[Ca^{2+}]_o$	Extracellular calcium
CAML	Calcium modulating cyclophilin B ligand
CBP	Calcium binding proteins
CE	Cornified envelope
CsA	Cyclosporine A/ ciclosporin
CypB	Cyclophilin B
CMV	Cytomegalovirus
DLR^{TM}	Dual Luciferase® Reporter Assay System
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
EDC	Epidermal differentiation complex
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
FCS	Fetal calf serum
FKBP	FK506 binding proteins
FLG	Filaggrin
GAG	Glycosaminoglycans
G-CSF	Granulocyte colony-stimulating factor
GFP	Green Fluorescent Protein

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK3β	Glycogen synthase kinase 3 beta
HCV	Hepatitis C Virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HeBS	HEPES buffered saline
HIV-1	Human immunodeficiency virus-1
ICAM	Intercellular adhesion molecules
IFN α	Interferon alpha
IFN□	Interferon gamma
IL	Interleukin
INV	Involucrin
IP$_3$	Inositol trisphosphate
IV	Ichthyosis vulgaris
JAK kinases	Janus family tyrosine kinases
K1,4,5,10	Keratins 1,4,5,10
KGF	Keratinocyte growth factor
KHG	Keratohyalin granules
KIF	Keratin intermediate filaments
KSC	Keratinocyte stem cell
LEP	Late envelope proteins
LAR	Luciferase Assay Reagent
Luc	Luciferase
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony-stimulating factor
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T cells
NFkB	Nuclear factor kappa B
NLS	Nuclear localisation sequence
OCT	Optimal cutting temperature (medium)
OD	Optical Density
PBS	Phosphate buffered saline
PLC	Phospholipase C
PPIase	Peptidyl-prolyl cis-trans isomerise activity
Pol	Polymerase

PUVA	Psoralen + UVA
PRL	Prolactin
RAR	Retinoic acid receptors
RIPA	Radioimmunoprecipitation
ROC	Receptor operated channels
RPA cells	Retroviral phoenix amphoteric cells
RVI	Royal Victoria Infirmary
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
shRNA	Short hairpin RNA
shRNAmir	microRNA-adapted shRNA
SASSAD	Six Area, Six Sign, Atopic Dermatitis (severity scoring)
SOC	Store operated channels
Sp1	Sp1 transcription factor
SPRs	Small proline rich proteins
SRB	Sulforhodamine B
STAT	Signal transducers and activators of transcription
TBS	Tris-buffered saline
TG	Transglutaminase
TGFα	Transforming growth factor-alpha
TGFβ	Transforming growth factor-beta
Th1	T helper cell type 1
Th2	T helper cell type 2
TNFα	Tumour necrosis factor-alpha
TPA	12-O-tetradecanoly-phorbol-13-acetate
TRC	Texas Red® Cadeverine
UVA	Ultraviolet A rays (wavelength: 400-315 nm)
UVB	Ultraviolet B rays (wavelength: 315-280 nm)
VOC	Voltage operated channels

INTRODUCTION

Atopic eczema and atopic disease asthma, hayfever and allergy, are increasing in frequency, affecting approximately 22-25% of children worldwide (Odhiambo et al., 2009). The reported lifetime prevalence of atopic eczema in adults is 8-17% before the age of 60 and this cohort represents a more severe and persistent group of patients (Hoare *et al*., 2000; Montnemery *et al*., 2003). The development of atopic eczema is often seen prior to the development of asthma and allergic rhinitis, with half of atopic eczema patients developing asthma and two thirds developing allergic rhinitis, a sequence known as the atopic march (Spergel and Paller, 2003).

Atopic eczema has a profound negative impact on quality of life, not only for patients but also for the whole family (Baron et al., 2006). 30% of consultations in primary care are concerned with skin disease and 10-20% of referrals to a dermatologist are for atopic eczema (Charman et al., 2003).

The precise pathogenesis of atopic eczema remains incompletely understood with complex genetic and environmental components contributing to its pathogenesis. Although atopic eczema was for many years primarily considered to be an immunological disease, there is a increasing evidence of a primary epithelial barrier defect (S. J. Brown *et al*., 2008). A complex and carefully orchestrated program of proliferation and differentiation occurs in order for the epidermis to achieve its primary role in barrier function which begins when the basal cells switch from cell division to a program of terminal differentiation to form corneocytes (Eckert et al., 1997). Studies have shown genetic linkage between the epidermal differentiation complex (EDC) on Chromosome 1q2 and atopic eczema suggesting a role for barrier function and repair in this disorder (Sugiura et al., 2005). More recently, mutations in the filaggrin (FLG) gene have been shown to predispose to childhood eczema (Palmer *et al*., 2006; Sandilands *et al*., 2007) and are strongly associated with a more severe course persisting into adult life (Barker et al., 2007).

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Moderate to severe atopic eczema can be difficult to manage and often requires UVB phototherapy or second-line systemic treatments (S. Brown and Reynolds, 2006).

Ciclosporin (CsA), an immunosuppressant agent, revolutionised organ transplant surgery due to its ability to block the activation of T-lymphocytes and prevent graft rejection (M. Schreiber et al., 1997). Its mechanism of action on T-cells has been well established: CsA binds specifically to cyclophilins, the intracellular receptors for CsA, of which Cyclophilin B (CypB) has the highest affinity. This interaction subsequently prevents T-cell activation via the calcineurin/NFAT pathway (Rao et al., 1997).

CsA is extremely effective in the treatment of atopic eczema (Sowden et al., 1991). It works quickly and significantly improves quality of life but its use is limited due to long term side effects (Salek et al., 1993). There is good evidence indicating that CsA exerts effects in the skin that are independent of its action on T-cells (Fisher et al., 1988; Taylor et al., 1993; Prens et al., 1995; Reynolds et al., 1998).

The cyclophilins belong to a group of enzymes known as immunophilins that possess a highly conserved ligand binding domain and peptidyl-prolyl cis-trans isomerase (PPIase) activity, important for protein folding, assembly and trafficking (Michael T G Ivery, 2000b; Al-Daraji et al., 2002). The cyclophilins are the specific internal receptors for CsA. Cyclophilin B (CypB) has a high affinity for CsA and is able to mediate the immunosuppressive effects of CsA (Allain et al., 1996).

CypB binds to CsA with 10 times higher affinity than CypA. The W128 residue which is found in the very conserved 3_{10} helix of CypB, and all eukaryotic cyclophilins is essential for CsA binding. Substituting alanine for tryptophan at residue 128 in the CsA-binding region reduces CsA binding by 97%, but does not have a significant effect on its PPIase activity (Carpentier et al., 1999). The CypB-CsA complex binds to calcineurin and has a higher inhibitory effect on calcineurin activity compared to the CypA-CsA complex due to particular

residues (G77, D155 and D158) found on CypB which are not found in CypA (Carpentier *et al.*, 2000).

CypB has also been shown to interact with two types of receptors known as Type I (CD147) and Type II (glycosaminoglycans) binding sites which are involved in signalling events and biological activity, respectively. The Type I binding site is situated within the CsA binding pocket of the molecule of CypB and the Type II site is found within its N-terminal and are thus positioned on opposite sides of the molecule. Interaction via the Type II site increases the local concentration near the Type I receptor (Carpentier *et al.*, 2002).

We have found that CypB is expressed in the granular layer of normal human epidermis, suggesting that it may play a role in epidermal differentiation. The Reynolds' lab has shown that components of the cyclophilin pathway are expressed and functional in human epidermis and keratinocytes (Al-Daraji et al., 2002) and that CypB is expressed and secreted by normal human epidermal keratinocytes (Fearon et al., 2011).

The role of CypB in the epidermis requires further investigation and by studying the mechanism of action of therapeutic agents such as CsA we aim to gain further understanding of the pathogenesis of atopic eczema and potentially develop new therapeutic targets and safer and more effective treatment strategies.

Chapter 1: Normal Skin

1.1 The Structure and Function of Normal Skin

Human skin consists of an outer epidermis, an underlying dermis and a subcutis. Adult skin weighs approximately 6% of our body weight and covers $2m^2$ in area (Tobin, 2005).

The epidermis is a stratified, non-vascularised epithelium measuring between 75-150µm on the face and trunk and increasing to 600µm on the palms and soles. The epidermis consists of four distinct layers: the outer stratum corneum, followed by the stratum granulosum, stratum spinosum and stratum basale (Tobin, 2006) . Beneath this is the dermis, an underlying connective tissue providing strength, elasticity and nutrition and measuring between less than 2mm – 4mm. The rete pegs of the epidermis interlock with the dermal papillae which increases the surface area in contact with each other. Within the dermis, fibroblasts produce collagen, elastin and glycosaminoglycans (GAGs) as well as other components which make up the fibrous and elastic components of the extracellular matrix (ECM) (Tobin, 2006). The dermis consists of an upper papillary layer with thin collagen fibres and a lower reticular part where the collagen fibres are thicker and more haphazardly arranged. Within the dermis lie skin appendages derived from epithelial germ cells which form the hair follicles, sebaceous glands and apocrine and eccrine sweat glands. Blood vessels, lymphatics and nerves are all found throughout the dermis (Fig 2). The nails are also an important skin appendage providing protection to the distal phalynx and improving fine touch and manipulation (Tobin, 2006).

Between the epidermis and dermis lies the dermo-epidermal junction, a highly complex interface that has a key role in cell anchorage, adhesion, migration and differentiation, acting as a barrier and filter and involved in signalling between the ECM and the basal cells of the epidermis. Below the dermis lies the sub-cutis or hypodermis (Tobin, 2006).

Human skin provides the protective interface between the harsh external environment and the complexities of the human body. It is a physical barrier protecting us against electrolyte and fluid imbalance, the entry of microorganisms, mechanical and chemical damage and ultraviolet radiation (Tobin, 2006). It is a sensory organ detecting touch, vibration, pressure, temperature variations, pain and pruritus. It has important autonomic functions maintaining cutaneous homestasis by regulating vasomotor functions, sweat gland secretion and pilomotor activity. In addition, it is important in secretory activity, for physiologic function, and excretory activity, for elimination of waste products of metabolism (Tobin, 2006). It has an important immunological role - with keratinocytes playing a significant part as well as the resident immune Langerhans' cells, circulating lymphocytes, neutrophils and other cells of the immune system which together with cutaneous blood vessels, lymphatics and lymph nodes collectively make up the skin immune system (Tobin, 2006). Finally, its role in sociosexual communication is extremely important.

1.1.1 The Epidermis

The epidermis is a highly compartmentalised structure that provides us with a physical and permeable barrier. It is continuously renewed throughout life, in order to replace cells lost through normal exposure to the environment, via a carefully orchestrated process of proliferation, differentiation and apoptosis (Eckert *et al.*, 1997; Chaturvedi *et al.*, 1999). The keratinocyte is the major cell that makes up 95% of the epidermis. These cells travel from their attachment to the basement membrane from the basal layer (stratum basale) through each layer to become anucleated corneocytes – dead but metabolically active flattened cells known as squames (Figure 1).
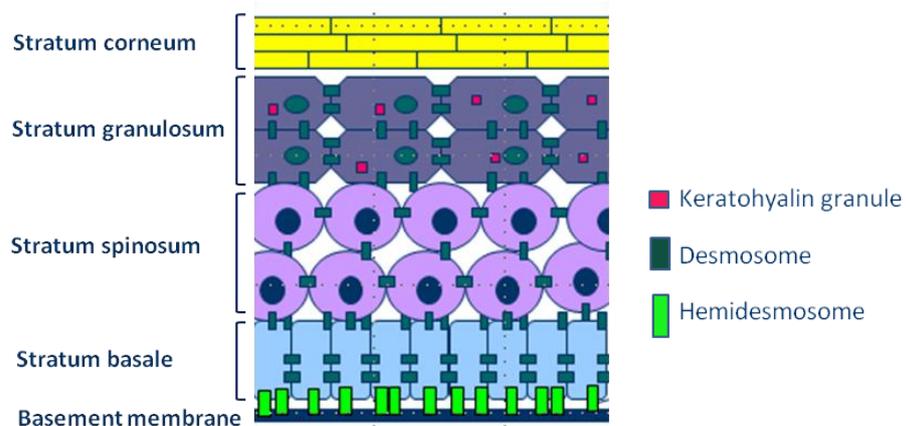
Figure 1. The four layers of the epidermis.

Corneocytes have a tough insoluble cornified cell envelope (CE) that replaces the plasma membrane and are cross linked by intercellular lipid to make up the stratum corneum. This process of terminally differentiating keratinocytes is known as cornification or keratinisation (Candi et al., 2005).

Keratinocytes are linked via several types of intercellular junctions: desmosomes which allow cells to withstand trauma; gap junctions that connect the cytoplasm of adjacent keratinocytes and other cells; adherens junctions concerned with cell motility, changes in shape and cell interactions; and tight junctions which regulate permeability and cellular polarity (Green and Simpson, 2007; Mese *et al.*, 2007; Niessen, 2007). The other important cells residing within the epidermis are melanocytes, Langerhans' cells and Merkel cells.

1.1.2 Epidermal Proliferation and Differentiation

The process of epidermal differentiation begins when basal stem cells switch from cell division to a program of terminal differentiation to form corneocytes (or squames), a process which takes approximately 60 days (Hunter et al., 1995). In normal epidermis, the rate of proliferation of the basal keratinocytes is precisely balanced by the desquamation of the corneocytes at the surface of the skin in order to form a functional barrier (Candi et al., 2005).

Three populations of keratinocyte stem cells (KSC) have been identified – the interfollicular epidermal stem cells in the epidermal basal layer, the hair follicle stem cells of the bulge and the sebaceous gland stem cells located immediately above the hair bulge. These three populations appear to function independently of each other, although may be able to replicate any skin structure if the epidermis is stressed (Eckert et al., 2012).

Stratum Basale: The basal layer is protected in the epidermis from dehydration, chemical and mechanical damage and irradiation (Eckert et al., 1997). The cells of the basal layer include the undifferentiated, mitotic keratinocyte stem cells and are important in maintaining the interfollicular epidermis.

Two types of proliferating basal keratinocytes exist: stem cells which have high proliferative potential and transit-ampliflying cells which are destined to undergo terminal differentiation. Stem cells are responsible for epidermal maintenance and repair. They give rise to daughter cells, which can either be stem cells themselves, or transit-amplifying cells. The transit-amplifying cells undergo a few cell divisions and are then committed to differentiate terminally (Jones et al., 1995). These committed cells detach from the basement membrane and migrate to the surface, undergoing a series of morphological and biochemical changes which can be identified at each layer of the epidermis (Fuchs, 1990).

Barandon and Green have identified three populations of cells in keratinocyte culture which have differences in proliferative potentiation: the holo-, para- and metaclones. The holocones have high proliferative potention and give rise to the largest colonies in clonal growth assays. Holoclones are rich in markers of

the epidermal basal layer, including β1 integrin. These cells correspond to the stem cell population. The paraclones differentiate after only limited proliferation and the meroclones have an intermediate proliferative capacity and morphology (Barrandon and Green, 1987; Eckert *et al.*, 2012). The paraclones correspond to the transient amplifying cells (Eckert et al., 2012).

The main structural proteins of the basal keratinocytes are keratins K5 and K14. They assemble into keratin intermediate filaments (KIF). Together with microtubules (tubulin) and microfilaments (actin), KIF's form the cytoskeleton of the keratinocyte. KIF's extend from the desmosomes towards the nuclear lamina bridging adjacent keratinocytes. Desmosomes scaffold keratinocytes into a three dimensional structure. They are composed of glycoproteins belonging to the cadherin family which are Ca^{2+} dependent cell-adhesion molecules. The cells of the basal layer are polarized with their basal surface attached to the basement membrane extracellular matrix components (including laminin and collagen IV secreted by keratinocytes). KIFs attach to hemidesmosomes via $α_6β_4$ integrins that act as heterodimeric transmembrane receptors adhering the basal keratinocyte to the basement membrane. The integrins are a family of cell surface receptors mediating cell-cell and cell-extracellular matrix adhesion. α6-integrin and β4-integrin are putative epidermal stem cell markers (Candi *et al.*, 2005; Radoja *et al.*, 2006; Eckert *et al.*, 2012).

Stratum spinosum: The keratinocytes in the suprabasal layers are post mitotic and produce a new set of structural keratins, K1 and K10, which form the cytoskeletal filaments that aggregate into tonofilaments(Fuchs, 1990; Candi *et al.*, 2005).   Other keratins are expressed in specific locations, such as K9 in the palms and soles(Candi et al., 2005). These suprabasal cells express involucrin (INV) and transglutaminase (TG) and also make envoplakin and periplakin(Steinert, 2000). Concurrently, lamellar granules (or Odland bodies) that synthesize and store lipids are also produced (Candi et al., 2005).

Stratum granulosum: As the spinous cells migrate to the granular layer further envelope proteins are expressed, namely loricrin and small proline-rich proteins

(SPRs) (Candi et al., 2005). The granular cells acquire characteristic cellular inclusions called keratohyalin granules (KHG) in cornifying epithelia and thus are found in the granular cells of the skin, cornea and hard palate but not in non-cornified squamous epithelia (McGrath et al., 2004). Human loricrin is deposited in the granular layer in KHGs and then intermixed with profilaggrin (Candi et al., 2005). Profilaggrin is a highly phosphorylated, insoluble protein consisting of an N-terminus and multiple filaggrin repeats joined by linker peptides. The filaggrin (FLG) repeats vary from 10-12 in humans (Pearton et al., 2002).

Stratum corneum and the formation of the cornified cell envelope: The formation of the cornified cell envelope is initiated by a rise in intracellular calcium ($[Ca^{2+}]_i$) levels which activates transglutaminase 1 (TG1) and triggers the expression of INV, envoplakin and periplakin, the latter two forming heterodimers. As $[Ca^{2+}]_i$ continues to rise TG1 crosslinks the plakins and INV by forming N^{ε}-(γ-glutamyl)lysine isopeptide bonds which form the scaffold of the cell envelope and provides it with its resistant and insoluble properties(Eckert et al., 2005).

Transglutaminases (TG) comprise a family of Ca^{2+} dependent enzymes that catalyse the formation of very stable isopeptide bonds (Eckert et al., 2005). Four of the nine mammalian TG's are expressed in the epidermis. TG1, TG3 and TG5 are expressed in the spinous and granular layers and are involved in CE assembly; TG2 is expressed under specific situations such as wound healing (Candi *et al.*, 2005; Eckert *et al.*, 2005).

Lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids, accumulate in the granular layer. At the interface of the granular and cornified layers, fusion of the lamellar body membranes' with the apical plasma membrane delivers their contents into the extracellular space (Kalinin et al, 2001). This enriches the plasma membrane with ω-hydroxy-ceramides. This coating of the CE contributes to epithelial elasticity and barrier function (Kalinin *et al.*, 2001; Candi *et al.*, 2005).

As cornification progresses the 400kD profilaggrin is dephosphorylated and proteolytically cleaved to release 10, 11 or 12 functional filaggrin subunits. Filaggrin (filament aggregating protein - FLG) binds to the cytoplasmic KIF to form tight macrofibril bundles (Pearton et al., 2002). This causes the KIF cytoskeleton to collapse forming the flattened cornified cells in which KIFs are aligned parallel to the outer surface of the epidermis. These events must occur rapidly because the half life of FLG is 6 hours and FLG is then degraded into free hygroscopic amino acids, 'natural moisturing factors', essential for water retention, osmolarity and also flexibility of the cornified layer (Candi *et al.*, 2005; S. J. Brown *et al.*, 2012).

Loricrin constitutes about 80% of the cornified cell envelope. Loricrin is complexed with various amounts of SPRs by TG3 and these oligomers are translocated to the cell periphery and crosslinked onto the pre-existing scaffold by TG1 (Kalinin et al., 2001). The ratio of SPRs to loricrin varies between different body sites. Both give mechanical strength to the CE but the varying amounts of SPRs may alter the physical properties of the skin at different anatomical sites (Candi et al., 2005).

The final anuclear, cornified cell consists mostly of bundled intermediate filaments of keratins (mainly 1, 2e, 10) embedded in a FLG matrix, covalently attached to envo- and periplakins and crosslinked to INV, loricrin and SPRs following degradation of organelles, microtubules, microfilaments and most desmosomal components. Several other proteins are also involved in epidermal differentiation such as S100 proteins, a family of Ca^{2+} binding proteins which are expressed in the basal and spinous layers, and later the late envelope proteins (LEPs). Corneocytes are surrounded by insoluble lipids and tightly attached to each other by corneodesmosomes (modified desmosomes), which are proteolytically cleaved in the uppermost layers of the stratum corneum to allow desquamation (Candi et al., 2005)(Candi et al, 2005).

Proteases: These are involved in three processes in epidermal differentiation: 1) proteolytic processing of some cornified-envelope precursors are required before cornified envelope formation can occur, 2) the loss of nuclei and

mitochondria requires proteolytic processing and 3) desquamation requires proteolysis of the corneodesmosomes. There are three classes of intracellular proteases: lysosomal proteases, calpains and caspases. The cathepsin family includes some lysosomal proteases. Cathepsin D is important in cell survival and in keratinocytes is involved in proteolytic processing of TG1 required for CE formation. The calpains may also be involved in TG1 processing. Caspases regulate apoptosis (human caspases -2, -3, -6, -7, -8, -9 and -10) and inflammation (human caspases -1, -4, -5). Caspase 14 is a member of the caspase family that is expressed and processed in association with keratinocyte differentiation at the transition from the granular to the cornified layer (Lippens *et al.*, 2000; Candi *et al.*, 2005).

1.1.3 The epidermal differentiation complex

A high proportion of the genes encoding the proteins important for terminal differentiation of the human epidermis are found in the epidermal differentiation complex (EDC) which maps to a 2.05-Mb region on chromosome 1q21 (Mischke et al., 1996). There are three families, related structurally, functionally and evolutionarily (Marenholz et al., 1996):

1. Structural proteins, including INV, loricrin and three classes of SPRs: two SPRR1 genes, eight SPRR2 genes and the SPRR3 gene.

2. The intermediate filament associated proteins profilaggrin and trichohyalin

3. 14 genes of the calcium-binding proteins S100 family (Eckert et al., 2004).

1.1.4 Epidermal gene regulation

The morphological changes occurring in the epidermis are the result of genes being turned on and off at specific times during the differentiation process. The most common mode of gene regulation is transcription regulation (i.e. rate of RNA synthesis). Every gene includes sequences designed to control RNA synthesis. These sequences can be divided into two classes. Firstly, sequences that control the rate of basal transcription and are common to most genes. They include the TATA box sequence and the INR (initiator) sequence. These sequences bind to ubiquitous proteins that are used by nearly all promoters to initiate and maintain RNA transcription and include the TATA

binding protein, RNA polymerase II (Pol II) and other transcription activation factors. This region of DNA ensures that RNA synthesis is initiated at the appropriate position along the DNA sequence and, in the absence of other transcriptional regulators, drive transcription at a low but physiologically relevant rate. The second class of sequences modulate basal transcription and mediate the response to specific stimuli (Eckert et al., 1997).

In keratinocytes, the expression of most genes is regulated at the transcription level by a class of proteins called transcription factors. Transcription factors are nuclear proteins that mediate the final part of the signal from the cell surface to the nucleus and the gene by binding to a specific DNA sequence that is usually located upstream and adjacent to the sequence that encodes the gene to control the rate of its transcription. This sequence is known as a silencer or enhancer and is called cis-acting as it is linked, on the same DNA strand, to the gene that it regulates. These sequences are often located 50-5000 bp upstream of the binding sites for the basal transcription proteins. Binding of the transcription factor to this site can either activate or suppress the gene (Eckert et al., 1997).

Transcription factors usually have several functional domains. All contain a DNA binding domain and a domain that regulates transcription, but also frequently contain a domain that regulates their activity via a phosphorylation site of the ligand binding site. Combinations of a transcription factor family can also assemble to regulate gene expression. Transcription factors can also integrate signals from the cell surface via signal transduction pathways. These pathways regulate keratinocyte proliferation and differentiation (Eckert et al., 1997):

- AP1 factors function as homo- and heterodimers of the jun (c-jun, junB, junD) and fos (Fra-1, Fra-2, c-fos, fosB) family members. AP1 sites have been shown to be functionally important as an activator of gene expression and that activation has been shown to be important for the transcription of keratins K1, K5, profilaggrin, filaggrin, Loricrin and TG1

as well as others. Different AP1 family members regulate expression of different genes (Eckert et al., 1997).

- AP2 is a transcription factor that has been shown to activate transcription of INV. In addition it has been shown to be important in activating transcription of K5,K14 and TG1 (Eckert et al., 1997).

- Sp1 is a transcription factor that has been identified as a regulator of TG3, INV and K5 (Eckert et al., 1997).

- NFκB activation results in protection against apoptosis by upregulating anti-apoptotic genes such as members of the Bcl-2 family and cellular inhibitors of apoptosis. In vitro and mouse data reveal that in epidermal differentiation the regulation of NFκb appears to be significant as the expression of NFκb dependent anti-apoptotic proteins is increased in differentiating keratinocytes. This may protect keratinocytes against apoptosis when cells undergo cornification (Lippens et al., 2005).

- NFAT is a family of transcription factors encompassing 5 proteins that are related to the Rel/ NFκb family. NFAT activates transcription of a large number of genes including the regulation of cell differentiation programs in a number of cell types (Hogan et al., 2003). The Reynolds lab has shown that endogenous NFAT translocates to the nucleus in human keratinocytes in response to differentiation stimuli following activation of calcineurin suggesting that the calcineurin/NFAT pathway may play a role in regulating keratinocyte differentiation (Al-Daraji et al., 2002).   This will be discussed in detail later (Chapter 3.1).

1.1.5 Molecular Controls of Epidermal Differentiation

These extracellular regulators control the balance between growth and terminal differentiation.

Growth stimulatory signals: Epidermal Growth Factor (EGF) stimulates cell proliferation and differentiation. Transforming growth factor α (TGF-α) is synthesized by keratinocytes and stimulates proliferation in an autocrine fashion. Retinoic acid (at concentrations of $10^{-7} – 10^{-10}$ M) promotes proliferation and these changes are mediated by nuclear retinoic acid receptors (RARs). Keratinocyte growth factor (KGF) and IL-6 are also growth stimulatory signals(Fuchs, 1990).

Growth inhibitory signals: Transforming growth factor β (TGF-β) inhibits DNA synthesis and cell division and inhibits growth of basal cells. TGF-βs are found predominantly in the differentiating layers and may be important in inflammation and repair regulation(Fuchs, 1990). TNF-α, IFN-α and IFN-γ have keratinocyte cytostatic effects.

1.1.6 Calcium

Calcium signalling: Calcium ions (Ca^{2+}) are important messengers carrying signals essential for the development and function of a cell from the beginning to the end of life.

Ca^{2+} signalling involves an intracellular increase in calcium ions. These signals usually involve a combination of release of Ca^{2+} from intracellular stores and influx of Ca^{2+} across the plasma membrane. Entry of extracellular Ca^{2+} maybe via voltage-operated channels (VOCs), receptor-operated channel (ROCs) or store operated channels (SOCs). SOC is also known as capacitative calcium entry (Berridge, 1997b; Putney, 2005).

The endoplasmic reticulum (ER), or a specialised component of it, provides an important store for Ca^{2+} providing a source for the intracellular release of Ca^{2+} to the cytoplasm in the course of Ca^{2+} - dependent signalling processes. The release of Ca^{2+} from intracellular stores is either via the messenger inositol trisphosphate (IP_3) and/or additionally via calcium-induced calcium release. The Ca^{2+} ATPase pump which transfers Ca^{2+} to the lumen of the ER or to the cytosol is known as SERCA. In most cell types, depletion of intra cellular Ca^{2+} stores signals the activation of capacitative calcium entry (Berridge, 1997a; Putney, 2005).

In order for Ca^{2+} to fulfil its role as a key second messenger depends largely on a number of heterogenous calcium binding proteins (CBP) such as calmodulin, S100 proteins and calcineurin. These CBP contribute to the intracellular control of Ca^{2+} concentration as well as acting as Ca^{2+} transporters across the cell membranes or as Ca^{2+} modulated sensors thus contributing to calcium homeostasis and signalling pathways (Yanez et al., 2012).

Calcium and Epidermal differentiation: Ca^{2+} is critical for controlling the balance of proliferation and differentiation. Elevation of extracellular Ca^{2+} ($[Ca^{2+}]_o$) to >1.0mM triggers an acute and then sustained increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and tightly controls epidermal differentiation and expression of EDC genes (Bikle et al., 1996).

In keratinocytes, $[Ca^{2+}]_o$ activates the phospholipase C (PLC) pathway resulting in a rise in inositol trisphosphate (IP_3) that coincides with the increase in $[Ca^{2+}]_i$. IP_3 may then mediate the rise in $[Ca^{2+}]_i$ by the result of calcium-activated PLC activity or by releasing calcium from intracellular stores. In addition, $[Ca^{2+}]_o$ stimulates transmembrane calcium flux by activating chloride channels, causing hyperpolarization of the membrane, and activating voltage independent non-specific cation channels permeable to Ca^{2+} (Bikle et al., 1996).

Specific functions of Ca^{2+} in keratinocyte differentiation are in transcription of EDC genes, activation of the TGs, desmosomal integrity, regulation of Ca^{2+}-dependent proteases and in protein deamination (Eckert et al., 1997).

1.1.6 Keratinocytes and cutaneous inflammation

Keratinocytes can themselves initiate inflammatory reactions. Many cytokines are produced by keratinocytes and include the pro-inflammatory cytokines IL-1, IL-3, IL-6, IL-8, Granulocyte colony-stimulating factor (G-CSF), Macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNF-α.
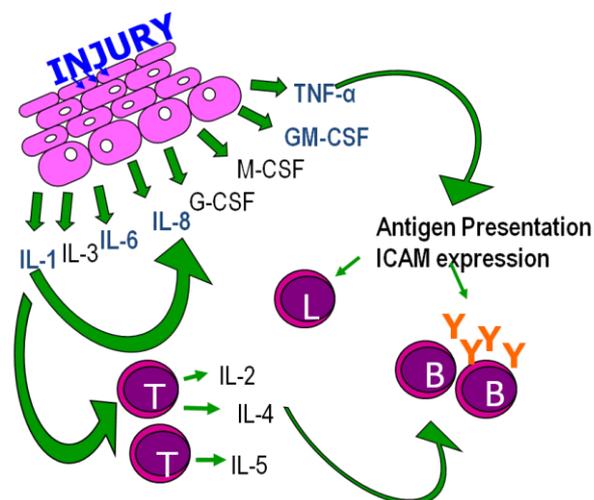
Figure 2. Rapid cascade of cytokines in response to keratinocyte injury, adapted from McKenzie and Sauder (McKenzie and Sauder, 1990)

In response to epidermal barrier disruption (Figure 2), keratinocytes can rapidly generate an inflammatory response via a cascade and network of cytokines.

The IL-1 family are essential proinflammatory cytokines. IL-1α and IL-1β both act via IL-1 receptor type I (IL-1R). IL-1α seems to exist at a constitutive level whereas IL-1β is only detectable upon stimulation (Contassot et al., 2012). Cytokines act in an autocrine fashion: IL-1 release stimulates further release of IL-1 and also stimulates the production of IL-8, IL-6, TNF-α, and GM-CSF from neighbouring keratinocytes. T-cells rapidly proliferate under the influence of IL-1. Activated T cells produce IL-2, IL-4 and IL-5 which stimulate B-cell proliferation and antibody production. TNF-α release from IL-1 stimulated keratinocytes enhances the inflammatory responses initiated by IL-1 and enhances MHC expression and antigen presentation to lymphocytes. IL-1 and TNF-α are not only chemotactic for leukocytes but also induce expression of intercellular adhesion molecules (ICAMS). Circulating leukocytes adhere to ICAM expressing cells at the site of injury and become activated by local cytokines. IL-1 stimulates keratinocyte chemotaxis, proliferation and differentiation (McKenzie and Sauder, 1990; Segre, 2006). High levels of cytokines IL-6 and IL-8 are found in psoriasis. Up-regulation of ICAM expression on keratinocytes is seen in atopic dermatitis and psoriasis (McKenzie and Sauder, 1990).

Chapter 2: Inflammatory Skin Disease

Atopic eczema and psoriasis are two of the most common inflammatory skin conditions. Immunological mechanisms have been primarily studied as the aetiology for atopic eczema and psoriasis. Histologically, both conditions are accompanied by a T-cell predominant inflammatory infiltrate of the dermis and epidermis. However, both are characterised by abnormal epidermal differentiation and impaired barrier function. There is increasing evidence to suggest that a primary epithelial barrier defect plays an important role in the pathogenesis of psoriasis and atopic eczema (Griffiths *et al.*, 2006; Nickoloff, 2006). In both, the degree of epidermal barrier disruption correlates with the severity of the condition (Segre, 2006).

Atopic eczema and atopic disease are increasing in frequency now affecting approximately 20% of the population in the developed world. Acute eczema is characterised by spongiosis (intercellular oedema) and is regularly accompanied by infiltration of leukocytes, mostly lymphocytes, into the epidermis and upper dermis where a switch from a Th1 to a Th2 cytokine profile (IL-4, IL-5, IL-10, IL-13) in lesional skin of AD is well established. As the disease becomes chronic, the infiltration of lymphocytes and spongiosis diminishes and acanthosis (thickening of the stratum spinosum), hyperkeratosis and parakeratosis, signifying abnormal keratinocyte differentiation, occur (Griffiths *et al.*, 2006; Howell *et al.*, 2007).

Psoriasis affects approximately 2% of the population in western countries. Psoriatic plaques are characterized by increased keratinocyte proliferation leading to epidermal hyperplasia and hyperkeratosis, abnormal keratinocyte differentiation (parakeratosis), infiltration of the epidermis and dermis by activated lymphocytes and neutrophils and tortuous and dilated upper dermal blood vessels. A T helper type 1 (Th1) profile of cytokines predominates (i.e. IFN-γ, IL-2, and IL-12) (Griffiths et al., 2006).

Both atopic eczema and psoriasis have complex genetic and environmental components contributing to their pathogenesis. Genetic linkage of both atopic

eczema and psoriasis to the EDC on chromosome 1q21 strongly suggests a role for barrier function and repair in these disorders (Sugiura *et al.*, 2005; Segre, 2006).   A large scale DNA microarray analysis of 23,000 genes in skin biopsies from 17 patients with atopic eczema revealed down-regulation of cornified envelope genes and up-regulation of the alternative keratinisation pathway – a mechanism in place which responds to conditions requiring rapid epidermal regeneration and protective gene expression which in atopic eczema may not be adequately regulated. The genes with the greatest difference in expression were FLG and loricrin which were down regulated and S100A8 and S100A7 which were upregulated (Sugiura et al., 2005). The S100 proteins are also markedly overexpressed in psoriasis (Eckert et al., 2004).

In support of this, mutations in the FLG gene have been shown to be implicated in the pathogenesis for atopic eczema implying impaired skin barrier function in the development of atopic disease. Reduced expression of FLG has been shown by immunofluorescence, in patients with atopic dermatitis (Palmer et al., 2006).

In psoriasis, an additional susceptibility locus has been mapped to PSORS4 on chromosome 1q21 within the EDC (Capon et al., 2001).

2.1 Atopic Eczema and Filaggrin
The world wide prevelance of atopic eczema has increased by approximately threefold since the 1960's with social and environmental factors influencing the expression for atopic eczema. Advances in understanding the pathogenesis of atopic eczema and atopic disease have been made possible following the identification that mutations in the FLG gene are associated with barrier defects (Eichenfield et al., 2012).

FLG was first identified in the late 1970s by Dale and its role in keratin filament aggregation established (Dale, 1977; Dale *et al.*, 1978). Sybert and colleagues, in 1985, identified that FLG abnormalities contribute to ichthyosis vulgaris (IV) (Smith *et al.*, 2006; Eichenfield *et al.*, 2012; McLean and Irvine, 2012). In 2006, Smith et al after overcoming the technical difficulty in sequencing FLG, showed

that null mutations in the FLG gene was the cause of IV which was inherited by a semi-dominant Mendelian inheritance (Smith, Irvine et al. 2006). IV is a common disorder, occurring in 1 in 250 individuals, characterized clinically by xerosis, scaling, hyperkeratosis, keratosis pilaris, palmar and plantar hyperlinearity, as well as a strong association with atopic disorders.

Loss-of-function mutations in the FLG gene are common with approximately 10% of individuals in European populations carrying at least one FLG mutation, which are associated with a 50% reduction in FLG protein production. The first two recurrent loss-of-function mutations identified were the R501X and 2282del4 mutations, but several others have been since identified with population-specific patterns being identified worldwide (Figure 3) (Sandilands *et al.*, 2007; Irvine *et al.*, 2011).
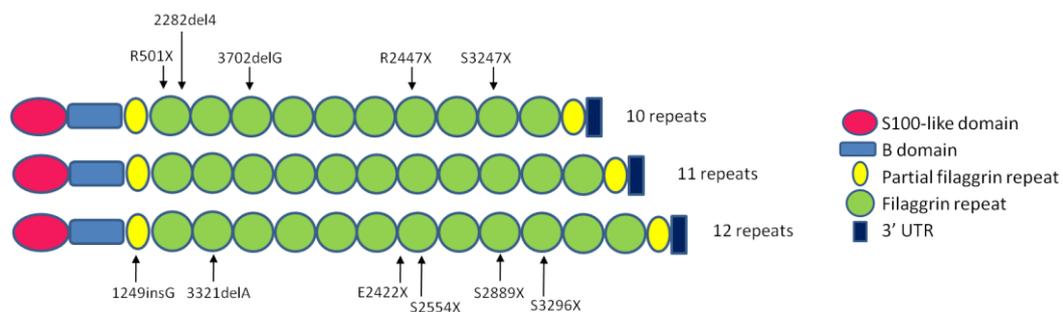
Figure 3. Schematic of the human FLG gene. The FLG gene is polymorphic with common allelic variants showing 10,11, or 12 almost identical tandem repeats in exon 3, demonstrating intragenic copy number variation. Each repeat at the DNA level encodes a complete copy of the functional 324 amino-acid filaggrin polypeptide product of the gene. The location of 11 known mutations are shown. Recurrent mutations can occur on the background of a 10-, 11- or 12- repeat allele; UTR – untranslated repeat (modified from O'Reagan et al and Brown et al (O'Regan et al., 2008), (S. J. Brown *et al.*, 2012)

The association of the loss-of-function FLG gene mutation and the development of atopic eczema has been widely established and replicated. In addition, patients with atopic eczema who have the FLG mutation have more severe and more persistant eczema and are more likely to develop asthma and multiple allergies including peanut allergies and are more at risk of acquiring herpes virus infections (S. J. Brown *et al.*, 2011; McLean and Irvine, 2012).

Interestingly, the intragenic copy number mutations are also important, as the total FLG repeat number (the FLG index) significantly predicts atopic eczema risk: an increase in FLG copy number leads to a reduced risk of developing atopic eczema (S. J. Brown *et al.*, 2012) .   Kesic et al have shown that FLG expression is downregulated in all cases of moderate-severe atopic eczema even in the absence of FLG-null alleles (Kezic *et al.*, 2011; McLean and Irvine, 2012).

2.2 Atopic eczema and antimicrobial peptides

Antimicrobial peptides (AMP) are innate defence proteins expressed in the skin. Two main groups of AMPs are the β-defensins and the cathelicidins. These innate defense proteins were first discovered for their antimicrobial actions but have since been found to play an important role in triggering the adaptive immune response. They have different regulatory pathways but both β-defensins and cathelicidins are released at high local concentrations when needed in response to infection or epidermal injury. The cathelicidins have been shown to protect against skin infections but many additional peptides have also been described to have antimicrobial properties. β-defensins are chemotactic for memory T cells and immature dendritic cells. Once stimulated they release a variety of cytokines (Figure 4) (Schauber and Gallo, 2007).
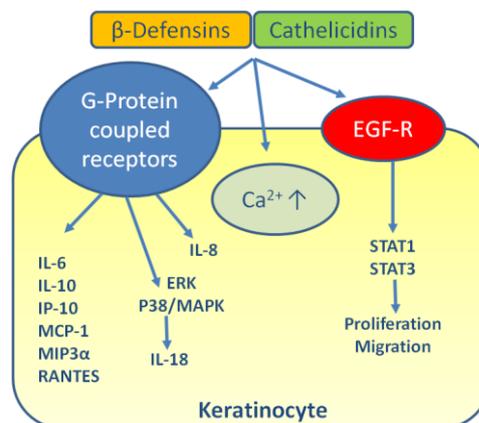
Figure 4. Schematic showing cytokine and chemokine realease from human kertinocytes following antibicrobial peptide stimulation, modified from Schauber and Gallo (Schauber and Gallo, 2007)

Patients with atopic eczema have been shown to have a relative deficiency of AMP compared to patients with other inflammatory skin diseases. This may be associated with staphylococcal colonization which is very common in atopic eczema, who are then at increased risk of developing infection (Eichenfield et al., 2012).

If a barrier defect allows secondary immunological changes that mediate the development of atopic eczema then early intervention may prevent its development and even halt the progression of the atopic march.

Chapter 3. CYCLOSPORINE A

3.1 Cyclosporine A, Calcineurin and NFAT

Cyclosporine (CsA) is a cyclic 11 amino-acid peptide produced by the fungus Tolypocladium inflatum and is an immunosuppressant agent which, along with FK506, revolutionised transplant surgery due to their ability to block the activation of T-lymphocytes and prevent graft rejection in organ and bone marrow transplantation (S. L. Schreiber and Crabtree, 1992).

CsA is known to act on the cells of the immune system with a preference for T cells. CsA blocks the activation and proliferation of T cells through inhibition of T cell receptor-mediated IL-2 production by preventing NFAT translocation to the nucleus (Buurman et al., 1986). CsA binds specifically to intracellular receptors called cyclophilin (CyP), a member of the immunophilin family. FK506 binds specifically to the immunophilin FKBP12 . The CsA-CyP complex then binds to and inhibits calcineurin, a Ca^{2+} and calmodulin dependent serine/threonine phosphatase. Inactivation of calcineurin prevents dephosphorylation of the nuclear localization sequence (NLS) of Nuclear Factor of Activated T cells (NFAT) (Buurman et al., 1986).

The NFAT family are a group of five transcription factors related to the Rel/NFκB family (NFAT 1-5). Normally, NFAT proteins are phosphorylated and reside in the cytoplasm in resting cells. NFAT 1-4 are regulated by Ca^{2+} and the Ca^{2+}/calmodulin-dependent serine phosphatase calcineurin; upon stimulation these NFAT proteins are dephosphorylated by calcineurin, exposing a NLS, and translocate to the nucleus. Here they become transcriptionally active, providing a direct link between intracellular Ca^{2+} signalling and gene expression (Figure 5) (Hogan et al., 2003).
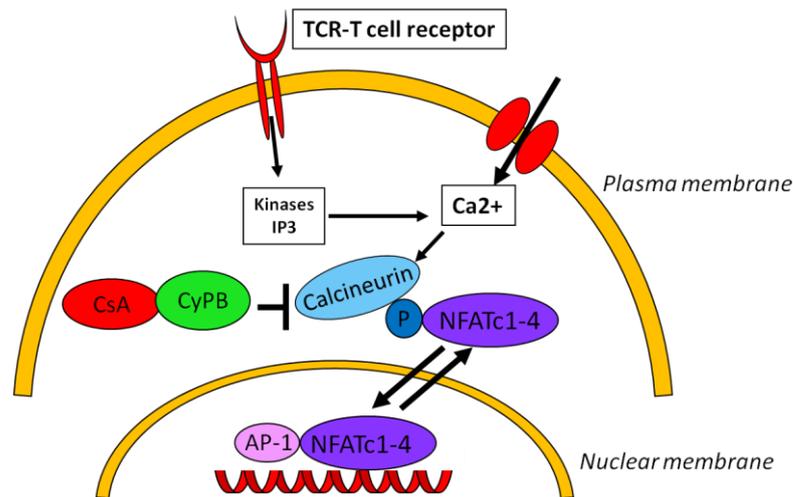
Figure 5. Mechanism of action of CsA. CsA binds to Cyclophilin (CypB). The CsA-CypB complex binds to regulatory subunit of calcineurin. Calcineurin is positively regulated by Ca^{2+} and negatively regulated by an endogenous inhibitor (CAIN). In immune cells, calcineurin dephosphorylates NFAT regulating expression of cytokines, their receptors and ion channels. NFAT complex is composed of NFAT proteins and AP-1 transcription factor activated via Ras/PKC/MAPK pathway. JNK (c-Jun N terminal MAP kinase) and GSK3β phosphorylate NFAT proteins counteract effects of calcineurin (modified from Kaminska et al, 2004 (Kaminska et al., 2004)).

NFAT acts synergistically with AP-1 but also with other transcription factors other than Fos and Jun (Hogan et al., 2003). NFAT activates transcription of a large number of genes during an effective immune response including IL-2, IL-3, GM-CSF, TNFα and Fas ligand (Rao et al., 1997). When Ca^{2+} entry is prevented or calcineurin activity is inhibited, NFAT is rephosphorylated by NFAT kinases and rapidly leaves the nucleus, terminating NFAT gene expression (Figure 5) (Hogan et al., 2003).

Thus, CsA inhibits calcineurin and prevents IL-2 production which is required for T cell activation.

3.2 Cyclosporine A in inflammatory skin disease

Since 1979, CsA has been used as effective treatment for psoriasis. Since then, it has been used effectively for atopic eczema (Figure 6) and for a number of other dermatoses such as pyoderma gangrenosum, lichen planus, Darier's disease, Hailey-Hailey's disease and autoimmune bullous disease (Griffiths et al., 2006).

(i)

(ii)
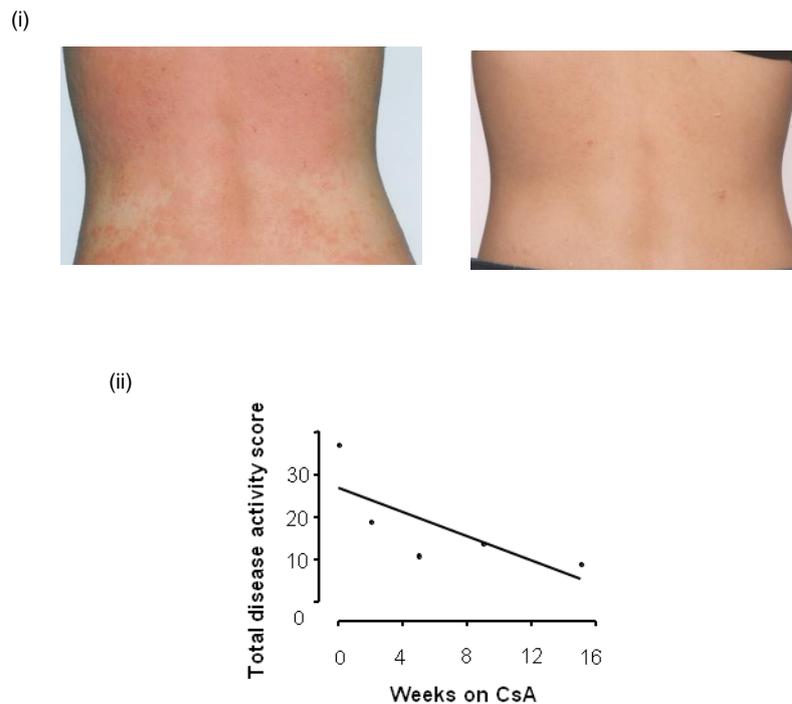
Figure 6. Clinical improvement of atopic eczema with CsA therapy. Example of (i) clinical improvement at 4 weeks and (ii) reduction in the total disease severity score (SASSAD) in response to CsA therapy (4mg/kg), with kind permission from NJ Reynolds.

However, its precise mechanism of action in skin and in particular psoriasis and atopic eczema is not completely understood. Both conditions are accompanied by a T-cell predominant inflammatory infiltrate. In psoriasis, a Th1 cytokine predominates within the plaques, thus cyclosporin may exert its effects here by inhibiting T cell activation via a reduction in IL-2 expression (Griffiths et al., 2006).

There is good evidence that suggests that CsA exerts effects in the skin that are independent of its action on T-cells. CsA has been shown to inhibit proliferation of cultured keratinocytes from psoriatic plaques at concentrations that are

therapeutically relevant (Fisher et al., 1988). CsA inhibits 12-O-tetradecanoyl-phorbol-13-acetate (TPA) induced epidermal hyperplasia, leukocyte infiltration and keratinocyte-derived TG activity and inhibits TPA induced inflammation in SCID mice in the absence of functioning T cells (Reynolds et al., 1998). CsA also induces hair growth indicating a direct epidermal effect (Taylor et al., 1993). CsA has also been shown to inhibit epidermal IL-1 and IL-8 expression in psoriatic skin before clinical improvement is seen and suggests that CsA also modulates keratinocyte cytokine expression (Prens et al., 1995).

Furthermore, the expression of the calcineurin/NFAT pathway has been shown in cultured human keratinocytes and in normal and psoriasis skin in vivo (Barrandon and Green, 1987; Al-Daraji *et al.*, 2002). CsA binding protein cyclophilin A and tacrolimus binding protein FKBP12, calcineurin and NFAT were all expressed predominantly in the keratinocytes of normal and psoriatic skin. Increased nuclear localization of NFAT1 in suprabasal keratinocytes of psoriatic epidermis was observed indicating calcineurin activation. In addition, calcineurin is functionally active by demonstration of nuclear translocation of endogenous NFAT in human keratinocytes which have been exposed to differentiation stimuli, specifically TPA, TPA plus ionomycin and raised $[Ca^{2+}]_o$ that is inhibited by CsA and tacrolimus in a dose dependent manner and at doses that are therapeutically relevant (Al-Daraji et al., 2002).

Activation of calcineurin/NFAT by agonists that induce keratinocyte differentiation suggest that this pathway may play a role in regulating keratinocyte differentiation. Regulation of transcription of mouse keratinocyte differentiation markers and the cyclin-dependent kinase inhibitor $p21^{WAF1}$ via calcineurin has been shown to occur through a mechanism involving an interaction between NFAT1/NFAT2 and the Sp1/Sp3 transcription factors (Santini et al., 2001).

These data indicate that inhibition of NFAT in keratinocytes by CsA may contribute to its therapeutic effects in psoriasis and that the calcineurin/NFAT pathway may play a role in epidermal differentiation.

Chapter 4. Cyclophilin B

4.1 The immunophilins

Three enzymes subfamilies, the cyclophilins, FK506 binding proteins (FKBPs) and parvulins, are collectively termed immunophilins on the basis of their affinity for immunosuppressive ligands. They are highly conserved and ubiquitous proteins, the majority of which possess the ability to catalyze the cis-trans isomerization of peptidyl-prolyl bonds. The cyclophilins were the first subfamily of immunophilins to be discovered in bovine thymocytes as ligands for the immunosuppressant drug CsA. FKBPs bind macrolides such as FK506 (tacrolimus) and rapamycin(sirolimus), that are structurally distinct from CsA. Parvulins bind Juglone (5-hydroxy-1,4-napthoquinone, an organic brown compound found in leaves, husk and barks of trees) (M. T. Ivery, 2000a; Galat and Bua, 2010).

A large number of isoforms of both cyclophilins and FKBPs have been isolated, varying primarily by their molecular weight and tissue distribution. All are characterised by their highly conserved ligand binding domain. Immunophilins are ubiquitous and occur in every compartment both as free cytosolic and membrane bound species. Their peptidyl-prolyl cis-trans isomerase (PPIase) activity is important for protein folding, assembly and trafficking. There is growing evidence to suggest that immunophilins perform other important functions within a range of organisms and cell types although their exact function remains to be elucidated (M. T. Ivery, 2000a).

Cyclophilins and FKBPs have structurally distinct PPIase domains that are unrelated in their amino acid sequence. The drugs bind to the catalytic pocket of the PPIase domain and inhibit their PPIase activity. In addition, the drug-immunophilin complexes, CyP-CsA and FKBP-FK506, inhibit calcineurin and subsequently block activation and proliferation of T-cells, the basis of immunosuppression. Although their drug-dependent functions which gave rise to their name have clinical relevance, this interaction does not appear, so far, to have any physiological relevance (McCaffrey *et al.*, 1993; Kumari *et al.*, 2012).

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4.2 The Cyclophilins

Cyclophilins are found in most cellular compartments in mammals, plants, insects, fungi and bacteria. There are 7 major cyclophilins in humans, CypA, CypB, CypC, CypD, CypE, Cyp40 and CypNK, with a total of 16 cyclophilin proteins identified so far. Their structure is conserved throughout evolution and all cyclophilins share a common central domain of approximately 10 amino acids, the cyclophilin core domain which includes the PPIase catalytic site and the CsA binding site (Wang and Heitman, 2005). In mammals, CypA and Cyp40 are cytosolic whereas CypB and CypC have amino-terminal signal sequences that target them to the ER protein secretory pathway. CypD has a signal sequence that directs it to the mitochondria; CypE has an amino-terminal RNA-binding domain and is localised in the nucleus, Cyp40 has a c-terminal tetratricopeptide repeat domain (TPR) and is located in the cytosol. Human CypNK is the largest cyclophilin, with a large, hydrophilic and positively charged carboxyl terminus, and is located in the cytosol (Wang and Heitman, 2005).

CypA and CypB are the most abundant isoforms of the cyclophilins (Wang and Heitman, 2005). The first characterized isoform of cyclophilin was cyclophilin A (CypA) and is an abundant cytosolic isoform recognised as the host cell receptor for CsA (Takahashi *et al.*, 1989; Edlich and Fischer, 2006). Cyclophilin B (CypB), a second CsA binding protein, was then characterised and was found to have a 10 times higher affinity for CsA than CypA and was distinguishable from CypA as an ER residential protein (Figure 7) (Hasel *et al.*, 1991; Spik *et al.*, 1991; Mikol *et al.*, 1994).
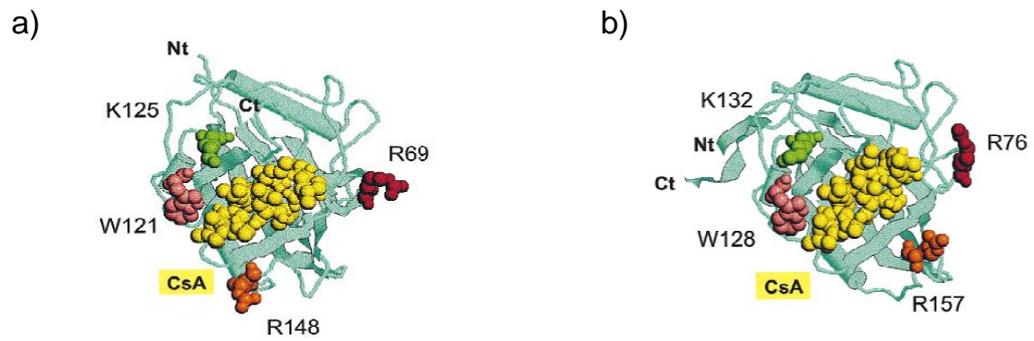
Figure 7. **Three-dimensional structures of (A) CypA and (B) CypB.** Visualized models with WinMGM program using the coordinate files (A) ICWA and (B) ICYN. CsA is represented with the yellow balls. The residue W121 is involved in the CsA binding site of CypA and W128A is involved in the CsA binding site of CypB. Taken from Carpentier el al, 2000 (Carpentier et al., 2000).

4.3 Characteristics of Cyclophilin B

CypB (also known as peptidylprolyl isomerise B or PPIB) is a 21kDa protein mapped to 15q21-q22.

Sequence alignment and comparison of human CypB (hCypB) to human CypA (hCypA), yeast CypB (yCypB) and rat cyclophilin-like protein (rCyLP), reveals domains with various degrees of conservation (E. R. Price *et al.*, 1991). Human CypB (Figure 8) contains 5 domains: Domain I, made up of 33 amino acids, at the N-terminal containing characteristic ER directed signal sequences and nuclear localisation sequence, absent from CypA; Domain II, III and IV (containing 61, 77 and 27 amino acids respectively) make up the CyP core domain; Domain V contains 10 amino acids which are not found in the CypA family (Figure 8b).
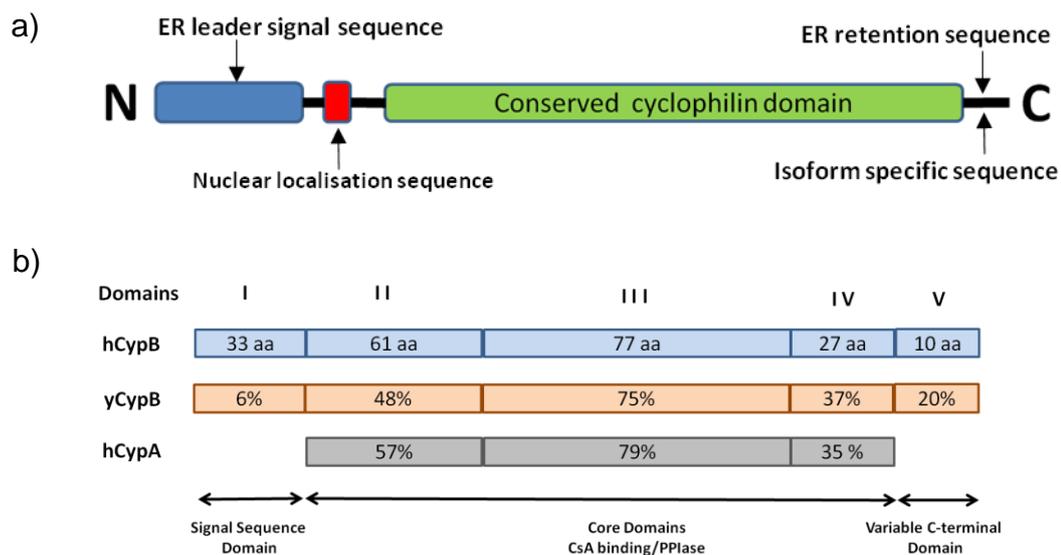
Figure 8. Schematic structure of CypB. a) Schematic showing domain structure of human CypB (modified from Price et al (E. R. Price *et al.*, 1991) and Fearon et al (Fearon et al., 2011) b) Schematic comparison of hCypB to yCypB and hCypA. For each domain the percentage of amino acid identity between hCypB and a particular homologue is displayed (modified from Price et al (E. R. Price *et al.*, 1991)).

CypB accumulates in the ER and in complexes on the cell surface. It has a cleavable N-terminal signal sequence that directs the protein to the ER (Schumacher et al., 1994). Regulation of protein expression during cell life and differentiation is reliant on the mechanisms operating in the ER and in the

downstream compartments of the secretory pathway (Ellgaard et al., 1999) (Ellgard et al, 1999). CypB lacks the C-terminal KDEL ER-retention signal found in most ER resident proteins but interacts with plasma membrane proteins in the ER via the VEKPFAIAKE motif at the C-terminus of CypB. The ER retention sequence (AIAKE) is proteolytically clipped in the ER enabling secretion of CypB into the extracellular environment (E. R. Price *et al.*, 1991; Arber *et al.*, 1992; E.R. Price *et al.*, 1994). The variable C-terminal domain is isoform specific. CsA causes a specific and rapid translocation of CypB from the ER to more distal portions of the secretory pathway (E.R. Price *et al.*, 1994). Within the N-terminus, CypB contains a nuclear translocation motif (DEKKKGPKV) (Figure 9) (E. R. Price *et al.*, 1991).

a)

```
   1  CGGGAACGCAACATGAAGGTGCTCCTTGCCGCCGCCCTCATCGCGGGGTCCGTCTTCTTC
                M   K   V   L   L   A   A   A   L   I   A   G   S   V   F   F

  61  CTGCTGCTGCCGGGACCTTCTGCGGCCGATGAGAAGAAGAAGGGGCCCAAAGTCACCGTC
           L   L   L   P   G   P   S   A   A   D   E   K   K   K   G   P   K   V   T   V

 121  AAGGTGTATTTTGACCTACGAATTGGAGATGAAGATGTAGGCCGGGTGATCTTTGGTCTC
           K   V   Y   F   D   L   R   I   G   D   E   D   V   G   R   V   I   F   G   L

 181  TTCGGAAAGACTGTTCCAAAAACAGTGGATAATTTTGTGGCCTTAGCTACAGGAGAGAAA
           F   G   K   T   V   P   K   T   V   D   N   F   V   A   L   A   T   G   E   K

 241  GGATTTGGCTACAAAAACAGCAAATTCCATCGTGTAATCAAGGACTTCATGATCCAGGGC
           G   F   G   Y   K   N   S   K   F   H   R   V   I   K   D   F   M   I   Q   G

 301  GGAGACTTCACCAGGGGAGATGGCACAGGAGGAAAGAGCATCTACGGTGAGCGCTTCCCC
           G   D   F   T   R   G   D   G   T   G   G   K   S   I   Y   G   E   R   F   P

 361  GATGAGAACTTCAAACTGAAGCACTACGGGCCTGGCTGGGTCAGCATGGCCAACGCAGGC
           D   E   N   F   K   L   K   H   Y   G   P   G   W   V   S   M   A   N   A   G

 421  AAAGACACCAACGGCTCCCAGTTCTTCATCACGACAGTCAAGACAGCCTGGCTAGATGGC
           K   D   T   N   G   S   Q   F   F   I   T   T   V   K   T   A   W   L   D   G

 481  AAGCATGTGGTGTTTGGCAAAGTTCTAGAGGGCATGGAGGTGGTGCGGAAGGTGGAGAGC
           K   H   V   V   F   G   K   V   L   E   G   M   E   V   V   R   K   V   E   S

 541  ACCAAGACAGACAGCCGGGATAAACCCCTGAAGGATGTGATCATCGCAGACTGCGGCAAG
           T   K   T   D   S   R   D   K   P   L   K   D   V   I   I   A   D   C   G   K

 601  ATCGAGGTGGAGAAGCCCTTTGCCATCGCCAAGGAGTAGGGCACAGGGACATCTTTCTTT
           I   E   V   E   K   P   F   A   I   A   K   E   @

 661  GAGTGACCGTCTGTGCAGGCCCTGTAGTCCGCCACAGGGCTCTGAGCTGCACTGGCCCCG

 721  GTGCTGGCATCTGGTGGAGCGGACCCACTCCCCTCACATTCCACAGGCCCATGGACTCAC

 781  TTTTGTAACAAACTCCTACCAACACTGACCAATAAAAAAAAAATGTGGGTTTTTTTTTTTT

 841  TTAATAAAAAA
```

b)

```
human CyPA: M.............................VNPTVFFDIAVDGEPLGRVSFELFADK
human CyPB: -kvllaaaliagsvfflllpgpsaadekkkgpk-TVK-Y---LRIGD-DV---I-G--GKT
yeast CyPB: -kfsglwcwlllflsvnviasdvgelidqddevITQK-----EHGE-KV--IVIG-YGKV
  rat CyLP: -kvlfaaalivgsvvflllpgpsvandkkkgpk-TVK-Y--FQIG-RTCRTSDLWTLWKD

human CyPA: VPKTAENFRALS.TGEKGFGYKGSCFHRIIPGFMCQGGDFTRHNGTGGKSIYGEKFEDEN
human CyPB: ----VD--V--A.---------N-K---V-KD--I-------GD----------R-P---
yeast CyPB: C----K--YK--t-TNSKK-FI--T---V--N--V------DGT-V-------DT-P---
  rat CyLP: CS--VD--V--A.---------N-K--HM-KD--I-------GD----------R-P---

human CyPA: FILKHTGPGILSMANAGPNTNGSQFFI.CTAKTEWLDGKHVVFGKVKEGMNIVEAME.RF
human CyPB: -K---Y---WV------KD--------.T-V-A-----------L---EV-RKV-sTK
yeast CyPB: -T---DRK-R-----R-KD--------tT-EEAS----------Q-VD--DV-NYIQ.HV
  rat CyLP: -K---Y---TV------KD--------.T-V--S-----------L---DV-RKV-nTK

human CyPA: G.SRNGKTSKKITIADCGQLE
human CyPB: T.DSRD-PL-DVI-----KI-VEKPFAIAKE
yeast CyPB: SrDA-D-PLEAVK--K--EWTPELSS
  rat CyLP: T.DSRD-PL-DVI-V---KI-VEKPFAIAKELREPRGPHPSKQLSVRVLSIPHR
```

Figure 9. Nucleotide and amino acid sequence of CypB. a) Nucleotide and amino acid sequence of hCypB and b): Amino acid sequence alignment showing homologies between human CypA (165 amino acid residues), human CypB (208 residues), yeast CypB (206 residues) and rat CyP-like protein (rCyLP) (231 residues) lower case letter indicate insertions relative to hCypA at the N-terminus (cleavable N-terminal sequence directing CypB to the ER), dots indicate deletions, and hyphens indicate identities, taken from Price et al (E. R. Price *et al.*, 1991).

4.4 Functions of Cyclophilin B

Increasing evidence reveals CypB to be involved in both intracellular and intercellular activity (Figure 10)
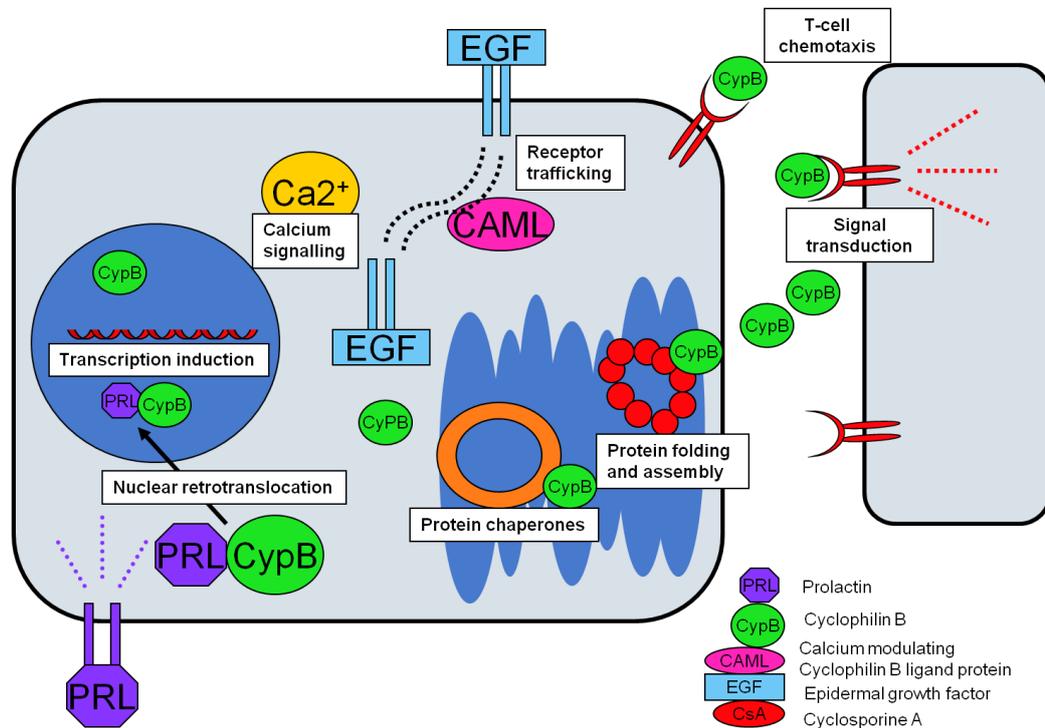
Figure 10. Schematic summarising intercellular and intracellular functions of CypB.

4.4.1 Intracellular functions of Cyclophilin B

PPIase activity and Protein folding and assembly

The PPIase activity of cyclophilins are important in protein folding and in the assembly of multidomain proteins. Peptide bonds can exist in two distinct isomeric forms – cis and trans. The trans peptide bond is a lower energy state with side chains at 180 degrees opposite to each other. These are sterically favoured and it is thought that the ribosome synthesizes peptide bonds in this form. In proline containing peptides, 10% of the peptidyl-prolyl bonds are in the unusual cis conformation where the side chains are adjacent to each other. CyPs stabilise the cis-trans transition state and accelerate isomerization (Figure 11).
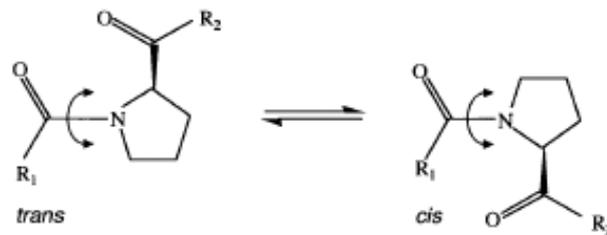
Figure 11. Cis-trans isomerization of a peptidyl-prolyl bond (Gothel and Marahiel, 1999).

This process is considered important not only in protein folding but also during the assembly of multidomain proteins (Gothel et al., 1996).

CsA blocks the PPIase activity of cyclophilins but the prolyl isomerase activity of these proteins is not essential for their immunosuppressive effects (Gothel and Marahiel, 1999).

Kim et al have shown that CypB expression is activated during ER stress via an ER stress response element and suppresses ER stress mediated apoptosis. Overexpression of CypB, but not the catalytically (PPIase) inactive mutant, protected cells against ER stress by preventing abnormal calcium leakage, ROS generation and mitochondrial membrane depolarization. Conversely, CypB knockdown increased the sensitivity of cells to ER stress. These studies show that CypB is required for cell survival in response to ER stress via its PPIase activity (J. Kim *et al.*, 2008).

Recently, mutations in the PPIB gene have been shown to contribute to a small percentage of individuals with oseogenesis imperfecta. It is thought that excessive post-translational modification of type1 procollagen results in phenotypes that range from lethal osteogenesis imperfecta in the perinatal period to severe deforming osteogenesis imperfecta and that normally the PPIase activity of CypB is required to produce effective type 1 procollagen (Pyott et al., 2011). However, a lack of CypB in osteogenesis imperfecta has also been associated with normal collagen folding but that there is a delay in folding due to the unavailability of CypB (Barnes et al., 2010).

Protein chaperones

An important role for CyPs is as chaperones for protein trafficking and macromolecular assembly. For example CypA and CypB assist viral replication. CypB interacts with the HCV RNA-dependent RNA polymerase (NS5B) required for viral replication. HCV replication is compromised if the CypB-NS5B interaction is competitively inhibited by CsA or mutation of NS5B which prevents its binding to CypB (Watashi et al, 2005). CypA promotes the formation and infectivity of HIV via its interaction with HIV-1 GAG (Galat, 1993; Stremlau et al, 2004). Non-immunosuppressive anolgues of CsA, namely Debio-025 and SCY-635 may alter certain replication steps of HCV (Fernandes *et al.*, 2010; Puyang *et al.*, 2010).

Calcium signalling

CypB has been implicated in Ca^{2+} signalling. Stimulation of T cells with CypB at varying concentrations induced Ca^{2+} mobilization which did not occur when CypB was preincubated in the presence of CsA (Denys et al., 1998a).

In the search for potential homologues of CsA, calcium modulating cyclophilin B ligand protein (CAML) was identified. Overexpression of CAML in Jurkat T cells constitutively activated an NFAT reporter in cells co-stimulated with phorbol ester (Bram and Crabtree, 1994; Holloway and Bram, 1998). NFAT requires a Ca^{2+} influx signal for activation which is usually provided following T cell receptor stimulation. This can be mimicked pharmacologically by treating cells with thapsigargin, a specific inhibitor of Ca^{2+}/APTase (SERCA) pumps (Hogan et al., 2003). CAML localizes to SERCA2-containing vesicles and overexpression of CAML markedly depleted internal Ca^{2+} stores and shifted the dose response of thapsigargin to lower concentrations.

Thus CAML may act by directly releasing intracellular Ca^{2+} and leading to consequent NFAT activation through the capacitative Ca^{2+} pathway (Holloway and Bram, 1998).

Receptor trafficking

CypB may be important in receptor recycling. CAML deficient mouse epithelioid cells had severely impaired proliferative responses to EGF. In these cells, EGF induced activation of signalling intermediates and internalization of the EGF receptor (EGFR) were normal, but recycling of the internalized receptor to the plasma membrane was defective leading to reduced surface accumulation. Enhanced recycling of surface receptors effectively leads to augmented ligand-induced proliferation by continuously replenishing surface receptor levels necessary for prolonged signalling (Tran et al., 2003).

Transcription induction

CypB has been shown to potentiate prolactin (PRL) driven proliferation, cell growth and nuclear retrotransport of PRL. PRL is necessary for mammary growth and differentiation and its pleotrophic actions are mediated by signalling through the PRL receptor. In addition to activation of signalling cascades, the PRL receptor is also associated with ligand internalisation via an endosomal like pathway across the ER and nuclear envelope known as nuclear retrotranslocation. The retrotranslocation of peptide hormones also appears to be widespread as several other peptide hormones such as EGF, growth hormone and insulin are also noted in the nucleus. CypB has been shown to potentiate PRL-driven proliferation and this was accompanied by a dramatic increase in nuclear retrotranslocation of PRL. Removal of the NLS in the N terminus of CypB abrogated the potentiation in PRL proliferation and the enhancement of PRL nuclear transport indicating a necessary role for CypB in these functions. Furthermore, PPIase activity of CypB is required for the potentiation of PRL-driven proliferation but not for PRL nuclear retrotransport (Rycyzyn et al., 2000). The PRL/CypB complex interacts directly with Stat5, a member of the signal transducers and activators of transcription (STATs) which comprise a family of several transcription factors that are activated by a variety of cytokines, hormones and growth factors. STATs are activated through tyrosine phosphorylation, mainly by JAK kinases, which lead to their dimerization, nuclear translocation and regulation of target genes expression (Rycyzyn and Clevenger, 2002; Valentino and Pierre, 2006). The interaction of the PRL/CypB complex with Stat5 results in the removal of the stat-repressor

protein inhibitor of activated Stat3 (PIAS3), enhancing Stat5 DNA-binding activity and PRL-induced Stat5 mediated gene expression (Rycyzyn and Clevenger, 2002).

CypB levels are increased in malignant breast epithelium and have been shown to play an important role in cancer progression, cell proliferation and cell growth. They are also implicated in the regulation of the above genes which are implicated in the pathogenesis of breast cancer (Fang et al., 2009). Knockdown of CypB decreases cell growth, proliferation and migration. Prolactin is increasingly appreciated as having a key role in the pathogenesis of breast cancer. CypB knockdown was shown to impair the patterns of gene expression induced by prolactin in breast cancer cells and that targeting both PRL-induced signals and CypB may have a synergistic potential in the treatment of breast cancer.

4.4.2 Intercellular functions of Cyclophilin B

CypB is the major component of the extracellular pool of cyclophilins. Appreciable quantities of CypB of up to 150ng/ml have been found in serum and breast milk (Allain *et al.*, 1995; Mariller *et al.*, 1996). CypB can also be released from chondrocytes by activated matrix metalloproteinases (MMPs) (De Ceuninck et al., 2003). In vitro, extracellular CypB interacts with two types of receptor at the surface of the T-lymphocytes.

The first type, known as Type I binding sites, were identified as CD147 (also known as Basigin or EMMPRIN) initially in T cells, but are now known to be expressed by epidermal keratinocytes, human skin mast cells and melanoma cells (Denys *et al.*, 1997; Ghannadan *et al.*, 1998; Kanekura *et al.*, 2002; Yurchenko *et al.*, 2010). Type I binding sites are involved in an endocytosis process and have been shown to be essential for cyclophilin-initiated signalling events (Denys et al., 1998a). The type I receptor-binding site region on CypB is highly conserved containing the CsA binding pocket– its interaction is inhibited by CsA. The PPIase activity of CypB is not required for receptor binding but are required for promoting cellular adhesion (Carpentier et al., 2002).

The second, Type II binding sites, are highly specific and correspond to glycosaminoglycans (GAGs) of the heparin family. The type II receptor-binding site is located in the N-terminal extension of CypB which is not present in CypA. In T-cells the interaction of CypB with GAGs does not only serve to increase the local concentration near the type I receptors but also affects the biological activity. The binding regions of CypB are located on opposite sides of the molecule (Denys *et al.*, 1998a; Yurchenko *et al.*, 2001; Carpentier *et al.*, 2002).

T-Cell chemotaxis and adhesion
CypB induces chemotaxis and integrin mediated adhesion of T cells to the ECM and appears to target memory CD4+ T cells predominantly in vitro which suggests that CypB may recruit specific populations of T cells into infected tissue in vivo thus playing a role in inflammation. CypB induced T-cell adhesion to the ECM in vitro was by its ability to bind to Type II GAGs receptors (Allain et al., 2002).

4.5 Cyclophilin B and inflammation
Accummulating data have implicated extracellular CypB (as well as CypA) as an intercellular mediator in inflammation. High levels of cyclophilins have been observed in inflammatory disorders such as sepsis, rheumatoid arthirits and atherosclerosis and systemic lupus erythematosus (Billich *et al.*, 1997; Tegeder *et al.*, 1997). CypB is constituitively secreted by tissue-resident cells (eg fibroblasts, chondrocytes and keratinocytes) and accumulates within the extracellular matrix (Gonzalez-Cuadrado *et al.*, 1996; De Ceuninck *et al.*, 2003; Fearon *et al.*, 2011). It is released intact by heparanase and MMPs suggesting it can participate in tissue inflammation. CypB contributes to leukocyte infiltration during the acute phase of inflammation. In addition it is able to trigger chemotaxis and integrin activation in T lymphocytes (Marcant et al., 2012).

4.6 Cyclophilin B and keratinocyte differentiation
SDS/PAGE immunoblot of cyclophilin and quanitifcation of cyclophilin mRNA in human epidermal cells revealed that CyP isoforms were consistently expressed throughout normal differentiating human keratinocytes but their function in the keratinocyte remains elusive (Chatellard-Gruaz *et al.*, 1994; Steele *et al.*, 2002).

CyP is found in normal human skin, lesional psoriatic skin and in non-differentiating and differentiating keratinocytes (Griffiths *et al.*, 1990; Chatellard-Gruaz *et al.*, 1994). The presence of two isoforms, a major and minor form of CyP were found in cultured human keratinocytes and in normal and psoriatic skin. The major CyP form was found in vivo and in vitro at similar levels in all samples but the minor form was expressed at significantly lower levels in psoriatic plaques as compared with normal skin. Inducing cultured keratinocytes to differentiate and stratify in the presence of 1.2mM $[Ca^{2+}]_o$ increased levels of the minor form of CyP by 5.8 fold (p<0.2) (Chatellard-Gruaz et al., 1994). These results strongly indicate that CyP may be an important factor in keratinocyte differentiation.

Chapter 5. Aims

As described above, CypB has been shown to be expressed and secreted by keratinocytes. However, the functional role of CypB in the skin has not yet been determined. In addition, CsA is an effective therapeutic option for patients with inflammatory skin disease. CypB is therefore an interesting protein to study in the skin and is a potential therapeutic target as:

a) Cyclophilins have been shown to increase in differentiating keratinoctyes.
b) CypB binds to CsA with 10 times greater efficiency than CypA.
c) CypB is secreted in response to CsA.
d) CypB has been shown to play a role in regulating the inflammatory response in a variety of other tissues/systems.

The work undertaken has been to investigate the hypothesis that a) CypB, a key intracellular receptor for CsA, regulates growth and differentiation of human keratinocytes and that b) CsA exerts a direct therapeutic effect on the epidermis in patients with atopic eczema through its effect on CypB.

The aims of this research were to:

1. Determine whether CypB influences growth, proliferation and differentiation in normal human epidermal keratinocytes and whether this effect is driven by intracellular or extracellular pathways.

2. Understand the functional and clinical significance of CypB and its role in mediating effects of CsA by studying a) skin equivalents overexpressing CypB and following CypB knockdown and b) skin biopsies obtained from patients with atopic eczema before and during the early phases of treatment with CsA.

Chapter 6. Materials and Methods

6.1 Human skin biopsies

Local ethical committee approval for the study was obtained (Ref: 2003/199). Patients were recruited having met the following criteria: 1) UK modification of Hanifin and Rajka's diagnostic criteria for atopic eczema (Williams et al., 1994), 2) Have been routinely prescribed CsA treatment, 3) 18 years old, 4) Over 18 years old, 4) Given informed consent to the study; 5) Have not used immune modifying drugs, topical calcineurin inhibitors, systemic eczema treatments, UVB, UVA, PUVA, sun-beds or significant exposure to sunlight for 3 months prior to inclusion; 6) Have not used very potent topical steroids for two weeks prior to inclusion.

5mm punch biopsies were taken under local anaesthesia from patients with atopic dermatitis before and during treatment with CsA. The severity of eczema was measured using the six area, six sign in atopic dermatitis (SASSAD) severity score for atopic eczema.

6.1.1 Preparation of skin biopsies

The tissue was embedded in OCT (Optimal Cutting Temperature compound) on a tissue chuck with the dermo-epidermal junction at 90° to the chuck and completely covered in OCT. The chuck, but not the tissue, was immersed in liquid nitrogen to freeze the tissue. The frozen OCT embedded tissue was then placed onto a cork base and stored at -80°C. Tissue sections were cut using the Leica CM 1900 rapid sectioning cryostat (Leica Microsystems, Milton Keynes, UK) and placed onto superfrost microscope slides coated with TESPA/APES (2% aminopropyltriethoxy-silane in dry acetone) or Supafrost Plus microscope (Thermo Scientific, UK).

6.1.2 Immunohistochemical analysis of skin biopsies

Five micron frozen sections of human skin biopsies were analysed for CypB FLG and CD147 expression. Rabbit polyclonal antibody to CypB (1:500, PA1-027A, ABR affinity BioReagents, USA), was used. This antibody is a synthetic

peptide corresponding to residues C(194) GKIEVEKPFAIAKE(208) of human CyPB. This sequence is almost completely conserved between human, mouse, rat, bovine, and chicken. This sequence is not found in CypA. Mouse monoclonal antibodies to FLG (1:100 Neomarkers, USA) and CD147 (1:750 BD Biosciences, USA) were used for FLG and CD147 expression. Substitution of the primary antibody with isotype-specific IgG and omission of the primary antibody served as negative controls. The secondary antibody used for CypB immuohistochemistry was Goat anti-Rabbit Oregon Green (1:300) and for FLG was Goat anti-Mouse Oregon Green (1:300). Tissue sections were defrosted at room temperature and fixed in acetone for 10 minutes. Sections were washed with Tween 20 (0.05%) in PBS (PBS/Tween). Each washing step was repeated 3 times with PBS/Tween. Cells were permeabilised with Triton X-100 (0.2%) for 10 minutes and then washed. Blocking buffer (NGS 1:60 in 1% BSA in PBS) was applied for 10 minutes, tipped off and the primary antibody applied for 1 hour at room temperature. Sections were washed and the secondary antibody applied for a further hour at room temperature. After washing, the nuclear stain toto-3 iodide was added (1:7000 in PBS) for 15 minutes and then washed for the last time. A coverslip was attached with Vectasheild ® (Vectalabs, UK) mounting medium, sealed with nail polish and stored at 4°C.

For CD147, labeled StreptAvidin Biotin (LSAB) method was used. Tissue sections were defrosted at room temperature and fixed in acetone for 10 mins. Sections were washed and permeabilised as above. Tissue sections were blocked with horse serum. Avidin D was applied for 15 minutes, slides washed and then Biotin block applied for 15 minutes. Following a further wash, the primary antibody was applied for 1 hour at room temperature, washed and biotinylated Horse anti-Mouse (1:300) applied for 1 hour. Following a further wash, Streptavidin Oregon Green (1:300) was applied for 30 minutes followed by the nuclear stain toto-3 iodide was added (1:7000 in PBS) for 15 minutes and then washed for the last time. A coverslip was attached with Vectasheild ® (Vectalabs, UK) mounting medium, sealed with nail polish and stored at 4°C.

Oregon Green 488 and Toto-3-iodide (642/660) were excited using the 488nm line supplied by the Argon ion laser and the 633 nm line supplied by the Helium

red neon laser, respectively. Images were captured using a Leica TCS SP II confocal laser microscope (Leica Microsystems, Milton Keynes, UK) and processed using Adobe Photoshop 6.0 and CS, USA.

6.2 Cell Culture

6.2.1 Cell culture of primary human keratinocytes

Primary human keraintocytes were cultured from skin samples obtained from urological and paediatric surgical procedures from the Royal Victoria Infirmary (RVI) following informed consent and ethical committee approval (Ref: 08/H0906/95). These tissue samples would have otherwise been discarded. Tissue samples were delivered in universal containers containing transport medium (Keratinocyte culture medium) and were stored at 4^0C and processed promptly.

Primary human keratinocytes were grown in Epilife^{TM} (Invitrogen, UK) supplemented with penicillin G (5 IU/ml) and streptomycin (5µg/ml) and Human keratinocyte Growth Supplement (Invitrogen, UK) and incubated at 37^0C with 5% CO_2 in a humidified incubator.

To prepare the skin the hypodermis was trimmed off with sterile scissors. The sample was then scored and placed in a universal container with phosphate buffered saline (PBS) and penicillin G (5 IU/ml), streptomycin (5µg/ml) and dispase (2mg/ml). Dispase, a protease, separates the dermis from the epidermis. The sample was stored at 4^0C. After 24 hours the epidermis was peeled off from the dermis with sterile forceps and incubated for 5 minutes at 37^0C with 5ml trypsin (0.05% trypsin and 0.02% EDTA, Lonza, UK) in a universal to separate the basal keratinocytes. The trypsin was neutralised with 2% FCS and the contents of the universal placed in a T75 or T175 flask with 10ml or 25mls respectively of Epilife^{TM} and incubated at 37^0C with 5% CO_2 in a humidified incubator overnight. The cells were washed with PBS and re-fed and incubated. Keratinocytes were fed every 2-3 days. Once 80% confluent the cells were either passaged or seeded for experiments. Primary keratinocytes were usually passaged up to 3 – 4 times.

6.2.2 Cell culture of cell lines

Cell lines used were the HaCaT, Retroviral Phoenix Amphoteric (RPA), 293T cells and 3T3 mouse fibroblast cell lines. All four cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Cambrex, UK), supplemented with 10% fetal calf serum (FCS) (Biosera, UK), penicillin G (5 IU/ml) and streptomycin (5µg/ml). The cells were incubated at 37^0C with 5% CO_2 in a humidified incubator.

The HaCaT cell line is an immortalised aneuploid human keratinocyte cell line which was a kind gift from Professor NE Rusenig [Heidelburg, Germany (Boukamp et al., 1988)]. The RPA cells are a retroviral packaging cell line and a kind gift from Dr G Nolan (Stanford, USA). The 293 cells are a lentiviral packaging cell line and were a kind gift from Dr O Heidenreich (NICR, Newcastle, UK). The 3T3 mouse fibroblasts were a kind gift from Dr J Reichart (ICM, Newcastle UK).

6.2.3 Passaging and seeding cells

The cells were washed with PBS and then detached with trypsin incubated at 37^0C with 5% CO_2. The cells were viewed regularly so that trypsin neutralisation can occur as soon as the cells have detached. DMEM was used for trypsin neutralisation. A 1 in 10 dilution of trypsin to neutralising medium was used. The medium containing the cells were transferred to a universal container and centrifuged at 1200rpm for 5 mins to obtain a cell pellet and supernatant discarded. For primary keratinocytes, the pellet was resuspended in 10mls Epilife^{TM} and for RPA cells, the cells were reseeded in complete DMEM.

For passaging, keratinocytes were reseeded into tissue culture flasks incubated and allowed to grow until 80-90% confluency. In order to seed keratinoctyes, 20µl was removed from the suspension in order to measure the concentration of cells using a haemocytometer. The required numbers of cells were then seeded into appropriate cell culture plates.

6.3 Retroviral Transduction

6.3.1 Retroviral Gene Transfer

Retroviral gene transfer allows for long-term, stable expression of introduced genetic elements. Retroviruses are able to integrate into the DNA of the host chromosome following which the retroviral promoter directs high-level, efficient expression of genes encoded within its genome.

The plasmid used as the retroviral vector was pLEGFP-N1 (Clontech, US). A second plasmid pLEGFP-C1 was also available (Figure 12). pLEGFP-N1 and pLEGFP-C1 facilitate the retroviral delivery and expression of enhanced green fluorescent protein (EGFP) or the amino-(N1) or carboxy-(C1) terminal fusions of EGFP protein of interest.

EGFP is a red-shifted variant of wild-type green fluorescent protein (GFP) of the jelly fish *Aequorea Victoria*. EGFP has a brighter fluorescence and higher expression in mammalian cells (excitation maximum = 488nm; emission maximum = 507nm).

pLEGFP derivatives expressing EGFP fusions to the gene of interest are stably transfected into a packaging cell line to allow the production of infectious viral particles. The packaging cell line used was the retroviral phoenix amphoteric (RPA) cell line, a generous gift from Dr G Nolan (Nolan's lab, Stanford University, USA). The RPA cells include the structural *gag, pol, env* genes to allow retroviral infection and delivery. pLEGFP transcripts produced in the packaging cell contain the RNA signal Ψ^+, the neomycin gene, transcription and processing elements and the gene of interest. EGFP or an EGFP fusion is expressed from the human cytomegalovirus (CMV) immediate early promoter (P_{CMV}). The vectors do not contain the structural *gag, pol and env* genes that are required for retroviral particle formation and replication, but are stably integrated in the packaging cell genome. In the cell, RNA from the vector is packaged into high-titer, infectious, replication-incompetent retroviral particals and thus can infect target cells and transmit the EGFP or EGFP-fusion gene, but cannot replicate within these cells since the cells lack the viral structural genes (BD Biosciences Clontech, US).
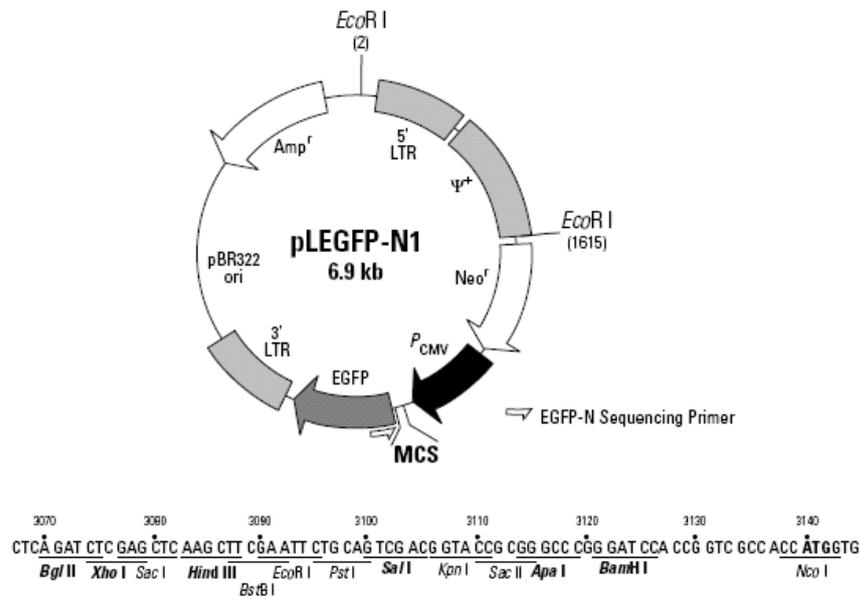
Figure 12. Restriction map and multiple cloning sites (MCS) of pLEGFP-N1 retroviral vector (Clontech, 2002).

6.3.2 Wild type CypB and W128A mutant CypB retroviral vectors (pLEGFP-CypBWT and pLEGFP-CypBW128A)

The wild type CypB (CypB$_{WT}$) and W128A mutant CypB (CypB$_{W128A}$) were a kind gift from Dr F Allain ([Lille, France;(Carpentier et al., 1999)]. CypB$_{WT}$ and CypB$_{W128A}$ had already been cloned into the pLEGFP retroviral vector and stably transfected into the RPA packaging cell line in the Reynolds lab by amplifying full length human CypB from keratinocyte complementary DNA and cloned into the pLEGFP-N1 vector between the *Hind*III and *BamH*I restriction sites to generate C-terminal fusion proteins forming the pLEGFP-CypB$_{WT}$ construct. The pLEGFP-CypB$_{W128A}$ was generated by subcloning a region containing the W128A mutation from the vector CypB$_{W128A}$ (Carpentier et al., 1999). The same primers used to generate full-length wild-type CypB was used to amplify the insert and the PCR product was cloned into pLEGFP-CypB$_{WT}$ between the Apa1 and BamH1 sites, replacing the wild-type CsA-binding site with the mutated one.

RPA cells transfected with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ were stored in cryovials in liquid nitrogen for long term storage.

6.3.3 Retroviral particle production and collection

RPA cells transfected with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ were defrosted and grown to 90-100% confluence in complete DMEM in T75 flasks at 37°C with 5% CO_2 in a humidified incubator. The medium was replaced gently with complete DMEM and the cells transferred to 32°C with 5% CO_2 in a humidified incubator. After 48 hours the medium containing the virus particles is harvested and stored in a sterile 15ml or 50ml Falcon conical tube at -80°C.

6.3.4 Retroviral transduction of keratinocytes

Retroviral transduction was performed in 6 well and 12 well plates. Primary human keratinocytes were seeded into 6 well and 24 well plates respectively. When the cells were at least 50-60% confluent, the medium was removed and they were transduced by adding 2mls per well of medium containing virus particles together with 10μl of hexadimethrine bromide (Polybrene, 5μg/ml) (Sigma, UK) and centrifuged at 1500rpm for 90 mins. Polybrene, a cationic polymer reduces the electrostatic repulsion between the negatively charged surfaces of the viruses and the cells or alternatively, may induce viruses to flocculate and form complexes, which due to their large size rapidly sediment onto the target cells, increasing the rate of gene transfer but can only be used at low concentrations (e.g., 2–20 μg/ml in most cases) due to their cytotoxicity (Davis *et al.*, 2004; Landazuri *et al.*, 2006).

After transduction they were washed with PBS and Epilife^{TM} (Invitrogen, UK) supplemented with penicillin G (5 IU/ml) and streptomycin (5μg/ml) and Human keratinocyte Growth Supplement (Invitrogen, UK) was added and the plates were incubated at 37^{0}C with 5% CO_2 in a humidified incubator.

6.3.5 Retroviral transduction efficiency.

Live cells were imaged on the Leica upright fluorescent microscope (Leica Microsystems, UK) to assess transduction efficiency. Alternatively, keratinocyte seeded onto coverslips and retrovirally transduced were fixed using 2ml GFP fixative per well for 15 minutes. The fixative is then aspirated off and the cells washed twice with 150nM Tris. The coverslips are mounted onto slides using Vectashield® (Vectorlabs, UK) and the edges sealed with nail varnish and

stored at 4°C until ready to visualise. GFP was excited using the 488nm line supplied by the Argon ion laser and images captured using a Leica TC SP II confocal laser microscope (Leica Microsystems, Milton Keynes, UK) and processed using Adobe Photoshop 6.0 or CS (Adobe, USA).

6.4 Lentiviral Transduction

6.4.1 Lentiviral Gene Transfers

Lentiviruses belong to the group, retroviruses, and are thus able to integrate into the host genome and direct highly efficient gene expression. Unlike retroviruses, which only infect dividing cells, lentiviruses can infect actively dividing as well as resting and differentiated cells and thus are more effective for long-term gene expression (Manjunath et al., 2009).

GIPZ Lentiviral shRNAmir viral vector (Figure 13) was used for shRNA knockdown. A cyclophilin B (PPIB) mouse GIPZ lentiviral shRNAmir clone was purchased in a glycerol stock (Open Biosystems, Thermo Scientific, UK). The DNA was extracted using the Qiagen Maxi Prep kit and used to transfect 293T cells for virus production. At the time of purchase, only one GIPZ lentiviral shRNAmir construct for CypB was available. This lentiviral system contains turbo GFP (tGFP) to mark cells expressing shRNAmir and was thus chosen. A non-silencing-GIPZ lentiviral shRNAmir control an pGIPZ lentiviral empty vector control were used (Open Biosystems, Thermo Scientific, UK).
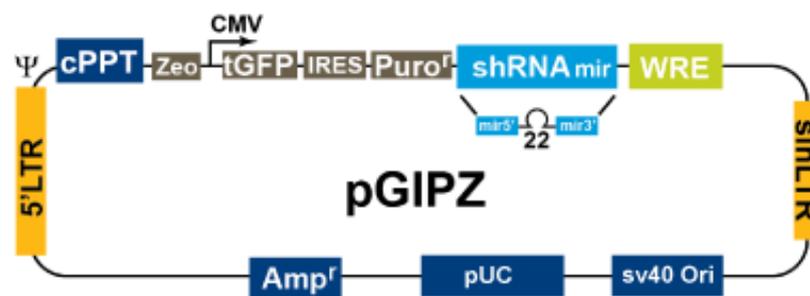
Figure 13. GIPZ Lentiviral shRNAmir vector (Open Biosystems, Thermo Scientific)

6.4.2 Co-transfection of 293T cells.

DNA transfections were performed using the Calcium phosphate coprecipitation technique (Graham and van der Eb, 1973). The day prior to co-transfection, 293T cell were seeded into 100mm tissue culture dishes in 10mls of complete DMEM for cells to be 50-60% confluent on the day of transfection. Prior to transfection the cells were re-fed with complete DMEM. On the day of transfection 20µg of the lentviral transfer vector is mixed with 5µg of envelope plasmid (*pMD2.G*) and 15µg of packaging plasmid (*pCMVdeltaR8.91*) in a sterile endotoxins-free epindorf. The envelope and packaging plasmids were kind gifts from Dr O Heidenreich (NICR, Newcastle, UK) and the protocol for co-transfection and particle collection, outlined below, developed by his laboratory. Special Water (125µl of 1M HEPES (ph7.3) to 50ml deionized water and solution sterilized using 0.22 µm filter and stored at 4^0C) was added to the plasmid mixture and the volume made up to 250µl. To this, 250µl of 0.5M $CaCl_2$ solution was added and mixed well. The plasmid/$CaCl_2$ mixture was added drop-wise, slowly onto 500µl HeBS while mixing by air bubbling. Prior to mixing, pre-warming of the special water, $CaCl_2$ solution and 2X HeBS to room temperature or in a water bath to 37^oC was important to standardise the precipitate characteristics. The mixture was allowed to stand for 30 minutes (maximum of 40mins) on the bench at room temperature, a critical time for the formation of a fine precipitate. The precipitate was slowly added, drop-wise, onto the cell monolayer while gently shaking the plate to ensure the precipitate was mixed well. The cells were incubated at 37^oC with 5% CO_2 in a humidified incubator. After 16-18 hours, a fine sandy precipitate was seen over the whole plate except on the cells and immediately around them. The reason for this was that DNA-calcium co-precipitate mixture formed strongly binds to the surface of the cell monolayer and enhances uptake of DNA by the cells (Graham and van der Eb, 1973). The medium was then carefully aspirated and the cells gently washed with warmed PBS. The cells were gently re-fed with complete DMEM and the dish incubated at 37^oC with 5% CO_2 in a humidified incubator for 3 days.

6.4.3 Lentiviral particle collection

Three days post co-transfection the lentiviral containing supernatant was
harvested by collecting the supernatant into a sterile 15ml – 50ml Falcon
conical tube. The collected supernatant was centrifuged at 3000 rpm for 14
mins to pellet cell debris. The viral supernatant was then filtered through a
0.45μm filter to remove any remaining cell debris. The viral particles were
stored in Falcon conical flasks at -80°C.

6.4.4 Lentiviral transduction of keratinocytes

Lentiviral transduction was performed in 6 plates. Keratincoytes were seeded
into 6 well plates respectively. When the cells were at least 50-60% confluent
they were transduced by adding 1mls per well of medium containing virus
particles and 1ml of complete DMEM together with 20μl of hexadimethrine
bromide (Polybrene, 5μg/ml) (Sigma, UK) and centrifuged at 1500rpm for 90
mins.

6.4.5 Lentiviral transduction effeciency.

Live cells were imaged on the Leica upright fluorescent microscope (Leica
Microsystems, UK) to assess transduction effeciency.

6.5 Cell Viability and proliferation assays
6.5.1 Sulforhodamine B (SRB) Assay

The SRB assay is widely used for cell proliferation and chemosensitivity testing.
SRB is a bright pink aminozanthene dye with two sulfonic groups. Under mildly
acidic conditions, SRB binds to protein basic amino acid residues in
trichloracetic acid (TCA) fixed cells to provide and index of cellular (cytoplasmic)
protein content. It does not stain cell debris. The relationship between
absorbance and cell number is linear and the colorimetric end-point is non-
destructive. SRB strongly fluoresces with laser excitation at 488nm and can be
measured quantitatively by static fluorescence cytometry (Skehan et al., 1990).

3×10^{3} HaCaT cells in 200μl medium were seeded per well into 96well plates.
Retroviral transduction with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and
pLEGFP-empty vector was performed the following day. The SRB assay was

performed on day1, day 5 and day 7 following retroviral transduction. Cells were fixed in 10% TCA for 1 hour at 4°C. Plates were washed by immersion in tap water, 5 times, and the excess water flicked off and the plates drained. The plates could be strored at 4°C until all time points were ready to assay. Fixed cells were stained with 0.4% SRB dissolved in 1% glacial acetic acid for 30 minutes at room temperature. At the end of the staining period the SRB was removed and the plates rinsed four times with 1% glacial acetic acid and the excess flicked off. The plates were then completely dried either overnight at room temperature or for 1 hour at 60°C. The bound dye was solubilisd with 200 µl 10mM unbuffered Tris base (ph10) for 5-10 minutes on a gyratory shaker. The optical density (OD) was measured using a spectrophotometer (SpectraMax 250, Molecular Devices, USA). Linear readings occurred with dye concentrations below 1.8 OD units. The most appropriate wavelength chosen was 490nm to ensure linear readings.

6.5.2 Colony forming Assays

Colony forming assays or clonogenic assys have been considered to be the optimal assay method for determining cell proliferation and survival following treatment of cells with various agents. It relies upon the ability of cells to form viable colonies derived from a single clone (Banasiak et al., 1999). Human epidermal cells in culture form three distinct types of colony (Barrandon and Green, 1987). The holoclone has the greatest reproductive capacity. Less than 5% of the colonies formed by cells of a holoclone terminally differentiate and thus form large rapidly growing colonies. These cells represent the epidermal stem cells. The paraclone consists of cells which have a short proliferative life span and stop dividing after a few cell divisions to differentiate, thereby producing small but uniform colonies. The meroclone, the third type of clone, contains a mixture of cells with different growth potential and represents a transitional stage between a holoclone and a paraclone (Barrandon and Green, 1987; Roh *et al.*, 2005).

The use of a non-proliferating feeder layer of mitomycin C-treated murine 3T3 fibroblasts was used to enhance the culturing of keratinocytes and formation of

colonies and was described by Rhinewald in 1975 (Rheinwald and Green, 1975).

Primary human keratinocytes were grown in 6 well plates and when at least 50-60% confluent were transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP-empty vector. Cells were grown until 80% confluent and visualised under a fluorescent microscope to ensure effective transduction efficiency. Keratinocytes were removed with trypsin-EDTA and neutralised as described in chapter 2.3. 200 cells were seeded onto mitomycin C treated mouse 3T3 fibroblasts in 60mm Petri dishes in quadruplet in 4mls Epilife^{TM} per dish.

Prior to use, the 3T3 mouse fibroblasts were grown in T75 tissue culture flasks to 70-80% confluency and then treated with Mitomycin C (10µl/ml) for 2 hours. The fibroblasts were then trypsinised as described in Chapter 6.2.3 and seeded into petri dishes and allowed to attach for 1 hour before adding the keratinocytes.

Once the keratinocytes were seeded onto the mitomycin C treated mouse 3T3 fibroblasts, the Petri dishes were incubated at 37°C with 5% CO$_2$ for 14 days. The medium was changed every 2 – 3 days. The Petri dishes were then fixed in Carnoy's fixative (75% methanol and 25% acetic acid) for 5 minutes, washed briefly with water and then stained using crystal violet (0.4% crystal violet in water). The plates were left to dry after rinsing off excess crystal violet with water. The numbers of colonies were counted by hand. A colony was defined as having at least 10 cells.

6.5.3 CellTitre-Glo® Assay Luminescent Cell Viability Assay (Promega®)
The CellTitre-Glo® assay Luminescent Cell Viability Assay is a homogeneous method which determines the number of viable cells in culture by quantifying the amount of the ATP present. ATP signals the presence of metabolically active cells. The procedure involves adding a single reagent (CellTiter-Glo® Reagent) directly to cells cultured in serum-supplemented medium.   This reagent results in cell lysis and generation of a luminescent signal proportional

to the amount of ATP present. The amount of ATP is directly proportional to the number of cells present in culture in. The CellTiter-Glo® Assay relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable "glow-type" luminescent signal.

5×10^3 primary human keratinocytes retrovirally transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP-empty vector for overexpression studies or with CypB GIPZ lentiviral shRNAmir, non-silencing-GIPZ lentiviral shRNAmir control or pGIPZ lentiviral empty vector shRNA knockdown studies, were seeded in 100µl Epilife^{TM} per well into a white 96 well plate with clear bottoms suitable for luminescence detection. After 24 hours, the cells were treated with either vehicle or Dithranol (5µM) as a negative control. Following a further 24 hours, the assay was performed according to the manufacturer's instructions: at the end of the experiment duration the plates were equilibrated at room temperature for 30 mins. 100µl of CellTitre-Glo® reagent, equilibrated to room temberature, was added to each well and mixed for 2 mins on an orbital shaker to induce cell lysis. After, a further 10 minute incubation period the luminescence was quantified using a luminometer (Microbeta® Trilux, PerkinElmer Precisely, Canada).

6.5.4   CytoTox Fluor^{TM} cytotoxicity assay (Promega®)

1×10^4 primary human keratinocytes were seeded into black 96 well plates with clear bottoms suitable for fluorescence detection and cultured in 100µl Epilife medium (Invitrogen, UK) supplemented with human keratinocyte growth serum (Invitrogen, UK) together with 5IU/ml penicillin G and 5µl/ml streptomycin at $37^{o}C$ with 5% CO_2. When the cells had reached 80-90% confluency, they were treated with either 0.5µM Ciclosporin (CsA) (Calbiochem, USA) or 1µM CsA for 24 hours. As a postitive control for cell death 5µM Dithranol was used. The Cytotox-Fluor® cytotoxicity assay (Promega, UK) was used to detect 'dead cell activity'. 20 µl of cytotoxicity reagent was added to each well and briefly mixed on an orbital shaker for 30 seconds. The plate was incubated for 1 hour at $37^{o}C$ prior. The assay measures a distinct protease activity released from cells that have lost membrane integrity using a fluorogenic peptide substrate (bis

AAF-R110). The bis-AAF-R110 substrate cannot cross the intact membrane of live cells and therefore gives no signal from live cells.

The signal intensity was measured using a Cary Eclipse fluorometer (Varian, Southampton, UK) using an excitation wavelength of 485nm and emission wavelengths of 520±2.5 nm (Promega, UK).

6.6 Differentiation Assays

6.6.1 Transglutaminase Luciferase assays

The 2.2 kb transglutaminase (TG) promoter-luciferase (TG-Luc) construct was a generous gift from Dr R Rice [California, USA (Mariniello et al., 1995)]. This construct, designated pTG-2.2LUC was made by generating a fragment including +70 to -2,210bp, counting upstream from the transcription start site in the 5'-flanking regions of the gene encoding TG. The construct was cloned into the pGL3-promoter vector (Promega, USA) and could be excised using *Kpn*I and *Bgl*II restriction enzymes. The backbone of the pGL3 Luciferase Reporter Vectors is designed for increased expression, and contains a modified coding region for firefly (*Photinus pyralis*) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. The pGL3-Promoter Vector (Figure 14) contains an SV40 promoter upstream of the luciferase gene (Promega, www.promega.com).
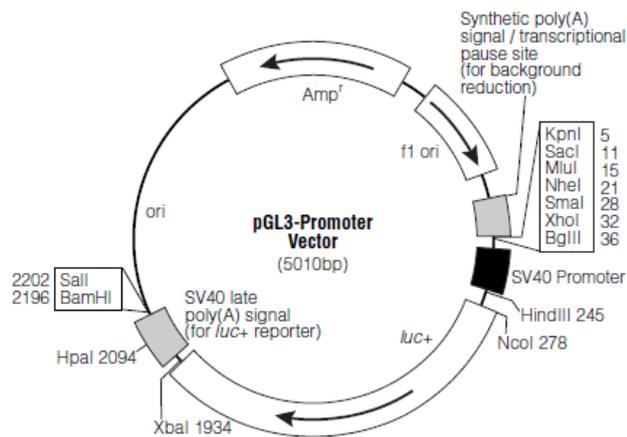
Figure 14. The pGL3-Promoter Vector circle map: luc+, cDNA encoding the modified firefly luciferase; Ampr, gene, conferring ampicillin resistance in E.coli; f1 ori,origin of replication derived from filamentous phage; ori, origin of replication in E.coli. Arrows within luc+ and the Ampr gene indicate the direction of transcription; the arrow in f1 ori indicates the direction of ssDNA strand synthesis (Promega).

Dual Luciferase® Reporter Assay system (DLR^{TM}, Promega)

The DLR^{TM} measures the activities of firefly (*Photinus pyralis*) and Renilla (*Renilla reniformis*, also known as sea pansy) sequentially from a single sample. The term 'dual reporter' refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. The 'experimental' reporter correlates with the effect of the specific experiment and the activity of the co-transfected Renilla 'control' reporter provides an internal control that serves as a baseline response. The activity of the experimental reporter is normalised to the activity of Renilla thus minimizing experimental variability caused by differences in cell viability or transfection efficiency (Promega, www.promega.com).

Transfection of keratinoctyes

Keratinocytes were transfected 1 hour following retroviral transduction at approximately 50-60% confluence per well in 24 well plates. Cells were transfected with 1µg total plasmid DNA (ie TG-Luc) and 0.05µg of Renilla as control, using Lipofectamine^{TM} and Plus^{TM} reagents, per well (Invitrogen, www.invitrogen.com). For each well, 1µg of plasmid DNA, 0.05µg of Renilla as control, 2µl Plus reagent and 100µl antibiotic and serum free Epilife^{TM} were placed in one falcon and in another 2 µl Lipofectamine and 100µl antibiotic and

serum free Epilife™ was added. Each falcon was mixed and incubated at room temperature for 15 minutes then both falcons were mixed together and incubated for a further 15 minutes at room temperature. The transfection mixture was added to each well and made up to 1ml with antibiotic and serum free medium and incubated at 37°C with 5% CO_2 for 4 hours. After this time point, the medium was aspirated and cells washed with PBS. 1ml of complete medium was added to each well and incubated. Medium was changed after 2 days. On day 4 the cells were lysed using 100ul 1X Passive Lysis Buffer per well and placed on a rocker and gently agitated for 20 minutes. The plates could be stored at -20°C until the DLR™ assay was ready to be performed.

DLR™ assay

In preparation of the DLR™ assay, the plates were defrosted whilst gently agitated on a rocker. 40µl of each sample was placed into a well of a 96-well solid bottomed white plate for reading luminescence. The firefly luciferase reporter was measured first by adding 65µl Luciferase Assay Reagent II (LARII). The firefly luminescence was quantified using a luminometer (Microbeta® Trilux, PerkinElmer Precisely, Canada). 65µl of the Stop & Glo Reagent was then added to the same well which quenches the firefly luciferase reaction and simultaneously initiates the *Renilla* luciferase reaction and the luminescence again quantified.

6.6.2 *Cadaverine incorporation technique*

Cadaverine is a polyamine, which can serve as an amine donor for transglutaminase reactions in place of lysine residues. Lysine residues are highly expressed in various protein differentiation markers such as involucrin and loricrin. Glutamine and lysine residue crosslinking is important in the formation of cornified envelopes during epithelial terminal differentiation and the enzymes responsible for carrying out this reaction are transglutaminase I and III (Candi et al., 2005). Cadaverine derivatives have been used experimentally as transglutaminase substrates and fluoroscein cadaverine has been shown to incorporate into the membrane proteins of epithelial cells and provide a quantitative and qualitative measurement of epidermal terminal differentiation (Gray et al., 1999).

In view of the presence of GFP labelled CypB used in our experiments, Texas Red® labelled cadaverine (Invitrogen) was used for measurement of epidermal differentiation studies.

Primary human keratinocytes were grown in 6 well plates and, when at least 50-60% confluent, were transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP-empty vector. Cells were grown until 80% confluent and visualised under a fluorescent microscope to ensure effective transduction efficiency. Keratinocytes were removed with trypsin-EDTA and neutralised as described in Chapter 6.2.3.

1-2 x 10^4 cells were seeded per well into black 96 well plates with clear bottoms, suitable for fluorescence detection in Epilife^{TM} medium.   1µM Texas Red® cadaverine (TRC) was added to the cells the following day and 1.2mM Calcium chloride added to control wells as a positive control for differentiation. Medium was changed every 2-3 days and after 6 days the assay performed. When the plate was ready the cells were washed twice with PBS and 100µl PBS added to each well. Signal intensity was measured using a Cary Eclipse fluorometer (Varian, Southampton, UK) using a excitation wavelengths of 550±10nm and emission wavelengths of 610±10nm. The cells were then fixed in 100µl fixative solution (acetic acid:ethanol:water - 1:49:50, by vol) and air dried. Cell protein content was determined using the kenacid blue method adapted from Knox et al. (1986): 150 µl kenacid blue stain (12mls acetic acid:88ml kenacid blue stock solution – 0.4g Coomassie Brillian Blue+250ml ethanol+ 630ml distilled water) added per well and plates shaken on a rotatest shaker for 2-3 hours. The stain was removed and cells washed x 2 for a minute each in 200µl washing solution (glacial acetic acid:ethanol:distilled water – 5:10:85 by volume). Cells were washed a third time and shaken for 20mins. The washing solution was then replaced with 150 µl desorb solution and agitated for 20 mins. The solution was diluted 1:5 in order to ensure a linear Optical Density (OD) reading within 0.4-1.2 range.   The OD value was used to represent the protein content and data presented as fluorescence/OD value.

6.7 Plasmid DNA Preparation

The bacterial vector containing the plasmid of interest was streaked to encourage growth of single colonies onto an agar plate containing ampicillin (100μg/ml) – the desired antibiotic for selection of the plasmids used. The plate is incubated upside down at 37°C overnight. A colony is picked and put into 200ml Luria-Bertani (LB) broth supplemented with ampicillin (100μg/ml) and grown overnight in an incubator shaker at 37°C for 14-16 hours.

Purified plasmid DNA was obtained with Qiagen's HiSpeed Maxi Kit (Qiagen) according to the manufacturers' instructions. Following bacterial culture, a bacterial pellet is obtained by centrifuging the LB cuture at 5000 x g for 15 mins at 4°C. The bacterial cells then undergo alkaline lysis and are poured into the barrel of a QIA filter Cartridge which are special filter units designed to completely remove SDS precipitates and clear bacterial lysates.   A plunger is inserted into the cartridge and the cell lysate is filtered into an equilibrated HiSpeed Tip. The HiSpeed tips contain an anion-exchange resin which allows for purification of nucleic acid. The HiSpeed tip is washed and the DNA within, eluted. The plasmid DNA is then precipitated by adding isopropanol to the eluted DNA, mixed and incubated at room temperature for 5 mins. The eluate/isopropranolol mixture is then filtered throught a QIA precipitator using constant pressure. The QIAprecipitator traps the precipitated DNA while the isopropanolol-buffer mixture flows through. The DNA is then eluted from the QIA precipitator into a microcentrifuge tube with TE buffer.

Restriction enzyme digest followed by agarose gel electrophoresis was performed to confirm the size of the plasmid and construct.

6.8 Formation of Human Epidermal Skin Equivalents

Human epidermal skin equivalents are formed from primary human keratinocytes cultured at high cell density in high calcium medium for 14 days on an inert polycarbonate filter (Millipore®) at the air-liquid interface (Poumay et al., 2004).

Primary human keratinocytes were grown in 6 well plates and when at least 50-60% confluent were transduced with pLEGFP-CypB$_{WT}$ or pLEGFP-CypB$_{W128A}$ or pLEGFP-empty vector for overexpression studies or with CypB GIPZ lentiviral shRNAmir, non-silencing-GIPZ lentiviral shRNAmir control or pGIPZ lentiviral empty vector shRNA knockdown studies. Cells were grown until 80% confluent and visualised under a fluorescent microscope to ensure effective transduction efficiency. Keratinocytes were then transferred to T75 flasks and allowed to proliferate to at least 80% confluency.

8.5×10^5 keratinocytes in Epilife™ were seeded onto the polycarbonate filter of an organotypic culture insert (Millipore®, Merck, Millipore, UK). Each insert was placed in a well of a 6 well plate. 2mls complete Epilife^{TM} was added to each well so that the cells were nourished from below the filter. The following day, the cells were switched to an air-liquid interface. The medium within the inserts was carefully aspirated so that the cells were dry and the medium below was replaced with 2ml Epilipfe ^{TM} containing 1.5mM Ca^{2+} and 5µl/ml vitamin C. The medium was changed in the wells every other day using freshly prepared Epilife^{TM} medium containing 1.5mM Ca^{2+} and 5µl/ml vitamin C. After 14 days the equivalents were differentiated.

The membrane was carefully removed from the insert using a scalpel blade and Each organotypic culture was divided into three: (i) snap frozen for lysate production and Western blotting, (ii) formalin fixed and paraffin embedded for H&E staining, (iii) OCT embedded and frozen for immumohistochemistry.

6.9 Preparation of organotypic culture for immunohistochemistry.

After the skin equivalent has been harvested, a third of the sample is fixed in OCT. The skin equivalent was embedded in OCT on a tissue chuck with the dermo-epidermal junction at 90° to the chuck and completely covered in OCT. The chuck, but not the tissue, was immersed in liquid nitrogen to freeze the tissue. The frozen OCT embedded tissue was then placed onto a cork base and stored at -80^oC. Tissue sections were cut using the Leica CM 1900 rapid sectioning cryostat (Leica Microsystems, Milton Keynes, UK) and placed onto superfrost microscope slides coated with TESPA/APES (2% aminopropyltriethoxy-silane in dry acetone) or Supafrost Plus microscope (Thermo Scientific, UK).

6.9.1 Immunohistochemical analysis of skin epidermal equivalents

5 micron frozen sections of the skin equivalents were analysed for CypB and FLG for skin equivalents formed from non transduced primary keratinocytes and for GFP in skin equivalents formed from retrovirally or lentivirally transduced skin equivalents. Mouse monoclonal antibody to FLG (1:100, Neomarkers, USA) were used. Substitution of the primary antibody with isotype-specific IgG and omission of the primary antibody served as negative controls. Secondary antibody used for FLG was Goat anti-Mouse Oregon Green (1:300). Tissue sections were defrosted at room temperature and fixed for 10 minutes in acetone for for FLG detection. For GFP detection, GFP fixative was used to fix the sections for 10 minutes. Sections were washed with Tween 20 (0.05%) in PBS (PBS/Tween). Each washing step was repeated 3 times with PBS/Tween. Cells were permeabilised with Triton X-100 (0.2%) for 10 minutes and then washed. For those sections in which CypB and FLG were assessed, blocking buffer (NGS 1:60 in 1% BSA in PBS) was applied for 10 minutes, tipped off and the primary antibody applied for 1 hour at room temperature. Sections were washed and the secondary antibody applied for a further hour at room temperature. After washing, the nuclear stain Toto-3 iodide (1:7000 in PBS) or ToPro (1:1000 in PBS) was added for 15 mins or 10mins respectively to all sections following 15 mins RNAase (1 in 1000) and then washed for the last time. A coverslip was attached with Vectasheild ® (Vectalabs, UK) mounting medium, sealed with nail polish and stored at 4^oC.

Oregon Green 488 and Toto-3-iodide (642/660) were excited using the 488nm line supplied by the Argon ion laser and the 633 nm line supplied by the Helium red neon laser, respectively. Images were captured using a Leica TCS SP II confocal laser microscope (Leica Microsystems, Milton Keynes, UK) and processed using Adobe Photoshop 6.0 and CS, USA.

6.9.2 Specimen processing for Haematoxylin and Eosin staining of Skin Equivalents

After the skin equivalent has been harvested, a third of the sample was fixed in formalin (10% formaldehyde in water). Using forceps, the sample was carefully wrapped in histology tissue (kindly donated by Histopathology Department, Royal Victoria Infirmary (RVI), Newcastle-upon-Tyne) and sandwiched between two biopsy foam pads. This was then transferred to a histology cassette and immersed in 70% ethanol ready for tissue processing. Tissue processing was performed by the histopathology laboratory at the RVI, Newcastle-upon-Tyne. Here the tissue was dehydrated by immersing the cassette, multiple times in progressively more concentrated ethanol baths. The tissue then undergoes clearing with xylene, a solvent which fully miscible with both ethanol and paraffin wax. Xylene replaces the ethanol so that the tissue will then be able to be infiltrated with paraffin wax. Hot paraffin wax then replaces the xylene in the tissue and once cooled to room temperature, solidifies.

Once processed the tissue was ready for embedding. The processed tissue is removed from the cassette and set in a paraffin block, using additional paraffin, and attached to the outside of the cassette on ice using the Leica EG1160 embedding centre (Leica, UK) courtesy of the RVI, histopathology department.

5 µm sections were cut from the paraffin block and placed into a water bath set at 37°C to smooth out any wrinkles. The sections were lifted out onto a glass microscope (Superfrost Plus) and placed into a rack and were dried at 60°C for 1hour. Once dried the slides were immersed successively, first in 90% ethanol and then 100% ethanol for 2 seconds each and then deparaffinised for 15 minutes in Histo-Clear^{TM}. They were re-immersed, first in 100% ethanol and

then in 90% ethanol for 2 seconds each. Following this the slides were stained with haematoxylin solution for 2 minutes and then immersed in tap water for 10 minutes, with a change of water midway. The slides were counterstained in 1% eosin for 10-15 seconds and rinsed in water and then consecutively immersed in 90% ethanol, then 100% ethanol each for 2 seconds, and cleared for 5 minutes in Histo-Clear^{TM}. The slides were mounted with DPX mounting medium and stored at room temperature.

The slides were imaged using 10x and 40x (0.75 NA) on a Zeiss AxioImager Z2 microscope (http://www.zeiss.com/) and images captured on the AxoVision 4.8 software, and processed in PowerPoint.

The thickness of the epidermal equivalent was measured from the image by hand using a ruler and taken from the top of the polycarbonate membrane to the base of the stratum corneum, and converted to micrometers.

6.9.3 Specimen preparation for Western Blotting

The final third of the skin equivalent was snap frozen in an eppendorf and stored at -80°C until analysis by Western blotting.   The samples were place on ice and the polycarbonate membrane was removed from the sample with forceps. 100µl RIPA buffer (see Chapter 6.10.3) was added to the eppendorf and the segment of the skin equivalent fully immersed. A glass homogeniser was cooled in Liquid nitrogen and then inserted into the eppendorf. The sample was homogenised, refrozen and the process repeated. The glass homogeniser was cleaned with ethanol between samples. Once homogenised each sample was sonicated at 8Hz for 4 seconds, 3 times. The samples were centrifuged at -2°C, at 23000rcf. The protein content was determined using the BCA assay and 10µg for protein prepared for Western blotting.

6.10 Western Blotting

Western blotting (or immunoblotting) is an important tool to study the presence, relative abundance, relative molecular mass, post translational modification and also specific interaction of proteins (MacPhee, 2010). Proteins, obtained from tissue or cell lysis are separated eletrophoretically on a polyacrylamide gel and transferred to a membrane – a Western blot, which represents a replica of the separated proteins. This method was developed by two laboratories (Towbin *et al.*, 1979; Burnette, 1981) and named Western blotting (Burnette, 1981; Kurien *et al.*, 2011) following Southern blotting, the process transferring nucleic acids from an agarose gel to a membrane, through capillary action and Northern blotting , the capillary transfer of RNA from an electrophoresis gel to a blotting membrane (Kurien et al., 2011). Following this, antibodies are directed against the membrane bound proteins and detected via chemiluminescence and captured on film or electronically (MacPhee, 2010).

6.10.1   Preparation of cell lysates and medium for Western blotting

$1x10^5$ primary human keratinocytes were seeded into each well of a 6 well plate and were transduced with either pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP-empty vector for overexpression studies or with CypB GIPZ lentiviral shRNAmir, non-silencing-GIPZ lentiviral shRNAmir control or pGIPZ lentiviral empty vector shRNA knockdown studies. Cells were grown for 24hrs, 48hrs, 72hrs and 96hrs. To determine the effect of CsA, cells were treated with 1μM CsA, equating to a therapeutic level.

6.10.2   Preparation of culture medium

At the end of the experiment, the culture medium was removed (approx 2mls) and concentrated using Amicon centrifugal filter units (Merck Millipore, UK). 10μl of sample was retained to measure protein concentration. The samples were stored at -20°C.

6.10.3   Preparation of cell lysates

The medium was removed and processed as described above. The cells were washed in 1ml ice cold PBS per well of 6 well plate. 100μl ice-cold modified radioimmunoprecipitation (RIPA) lysis buffer was prepared (50nM Tris-HCl

(pH7.5), 150mM NaCl, 0.25% (wt/v) sodium deoxycholate, 1% Nonidet P-40 (NP-40), 1mM NaF, 5mM β-Glycerophosphate, 1 tablet of Complete protease inhibitor cocktail tablet (Roche Applied Sciences,UK), made to a volume of 50ml ddH$_2$0 and stored at -20°C) and completed by adding 5µl sodium orthovanadate and 5µl of 200nM PMSF just prior to adding to the cells and incubated on ice for 5 minutes. The cells were scraped off the surface using a cell scraper and transferred to a 1.5ml eppendorf on ice. The samples were centrifuged for a maximum of 10 minutes to a speed of 10,000rpm. 10µl of sample was removed to measure protein concentration. The samples were stored at -20°C.

6.10.4 *Protein quantification using the Pierce® BCA protein assay kit*

The amount of protein in each sample was quantified using the Pierce® BCA protein assay kit (Thermo Scientific, UK). This assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) which allows for the colorimetric detection and quantification of total protein. The first step is the chelation of copper with protein in an alkaline medium which reduces Cu^{2+} to Cu$^+$ (the biuret reaction – based on the reaction between the organic compound biuret, NH$_2$-CO-NH-CO-NH$_2$ and the cupric ion to form a light blue tetradentate complex) The intensity of colour produced is proportional to the number of peptide bonds in the reaction. Single amino acids and dipeptides do not give the biuret reaction but tripeptides and larger polypeptides or proteins reat to produce a light blue – violet complex. The second step is the colorimetric detection of the cuprous cation (Cu$^+$) that was formed in step 1, using a reagent containing bicinchoninic acid. The chelation of two molecules of BCA with one cuprous ion forms a purple-coloured reaction product which is water soluble and exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000 µg/ml). The reaction that leads to BCA colour formation is also influenced by the presence of any of four amino acid residues (cysteine, cystine, tyrosine, tryptophan) in the amino acid sequence of the protein, and a single amino acid residue in the sample may result in the formation of a coloured BCA-Cu$^+$ chelate.

Protein quantification was determined using the Pierce® BCA protein assay kit according to the manufacturers guidelines. Protein samples were prepared in

duplicate and diluted 1 in 5 in H$_2$0 to 10µl.   The standard BCA Protein Assay detects protein concentrations from 20 to 2,000µg/mL and is provided with Reagent A (carbonate buffer containing BCA Reagent) and Reagent B (cupric sulphate solution). A working solution (WS) is prepared by mixing 50 parts of BCA Reagent A with 1 part of BCA Reagent B (50:1, Reagent A:B). The working solution is an apple green colour that turns purple after 30 minutes at 37°C in the presence of protein.   The plate was cooled to room temperature and the absorbance measured at 562nm on a spectrophotometer (SpectraMax 250, Molecular Devices, USA).

6.10.5   Sample preparation for Gel Ecectrophoresis

Samples were defrosted on ice and the volume for 10 µg of protein was mixed into an eppendorf with 2.5 µl NuPAGE ® LDS Sample Buffer (4x) (Invitrogen), 1 µl NuPAGE® Reducing Agent (10x), made up to 10 µl deionised H$_2$0 and heated to 70°C for 10 minutes. LDS (Lithium Dodecyl Sulfate) maintains polypeptides in denatured state following heating. The reducing agent contains 500 mM dithiothreitol (DTT) which breaks disulfide bonds to remove secondary and tertiary structure.

6.10.6   Gel Electrophoresis

Gel Electrophoresis was performed using the NuPAGE® (Invitrogen) 4-12% Bis-Tris gradient precast polyacrilamide gel cassettes were used to separate proteins. The comb from the gel cassette was removed and each well was flushed with deionised water. The gel cassette was inserted into the electrophoresis chamber of an XCell4*SureLock*^{TM} after peeling off the tape covering the slot on the back of the gel cassette.   The gel cassette was locked into place, the upper buffer chamber filled with 1X NuPAGE® MES SDS running buffer and 500 µl NuPAGE® Antioxidant and checked for leakage. The protein samples were loaded into the wells and a molecular weight marker (Novex® Sharp Protein Standard, Invitrogen) was also loaded to allow visualisation of the protein moleucar weight ranges and evaluation of western transfer efficiency. Two gel cassettes could be used in each XCell4*SureLock*^{TM} electrophoresis chamber. The Lower Buffer Chamber was filled and the lid placed on the so that the (-) and (+) electrodes are properly aligned. The electrode cords are

connected to the power supply and electrophoresis performed at 200V Constant for 40 minutes.

6.10.7 Protein Transfer to Nitrocellulose membrane

Following electrophoresis, the cassettes were removed and opened. The plates of the cassette were separated and the plate without the gel was discarded. A wet piece of blotting paper was layed over the gel and the gel carefully removed from the remaining plate using a gel knife to loosen the gel. The blotting paper, with the gel overlying it, was placed onto a wet transfer pad. The nitrocellulose membrane was cut and dampened and placed onto top of the gel. Another piece of blotting paper was placed over the nitrocellulose membrane. This and the gel were sandwiched between two pieces of blotting paper and two blotting pads and placed into a Novex transfer cassette. The cassette is placed into a Mini Trans-Blot Electrophoretic Transfer Cell (Bio Rad Laboratories) which can take two cassettes, thus transferring two gels together. The cell was filled with cold transfer buffer (1X NuPAGE transfer buffer, 10% methanol, 0.1% antioxidant at 4^0C) and an ice pack inserted. The lid was positioned and transfer performed at 100V Constant for 1 hour, ensuring that the protein runs from the gel to the nitrocellulose membrane from cathode to anode.

6.10.8 Immunodetection

Immunodetection is achieved in three steps: 1) blocking, to eliminate non-specific interactions of the antibody with the membrane, 2) probing, to detect the protein of interest using a specific primary antibody, following which a secondary antibody linked to the reporter enzyme, peroxidise is directed against the species-specific portion of the primary antibody, and 3) detection, using a chemiluminescent agent as a substrate that will luminesce when exposed to the peroxidise on the secondary antibody and captured digitally or using film. The nitrocellulose membrane is removed and washed in TBS-Tween 20 (TBS-T: 100mls 10X TBS (24.2g Tris Base, 80g NaCl made up to 1L deionised $H_2$0, pH 7.6), 1ml Tween 20, 899ml deionised H_2O). The membrane is placed in 5% non-fat milk powder in 1X TBS-Tween 20 to block non-specific binding sites, at room temperature 1 hour whilst gently agitated on a rotary shaker . The membrane was washed for 5 minutes in TBS-T. The membrane was placed in

a 50 ml Falcon and 4mls of the primary antibody, CypB 1:500 (ab16045, Abcam, UK - *a synthetic peptide conjugated to KLH (keyhole limpet haemocyanin) derived from within residues 150 to the C-terminus of human Cyclophilin B.*) in 5% non-fat milk powder in 1X TBS-T, was added. This was placed on a tube roller at room temperature for 1 hour. The membrane was washed 3 x 5 mins in TBS-T and then the secondary antibody, Anti-rabbit (Sigma) 1:2000 in 5% non-fat milk powder in 1X TBS-T added for 1 hour at room temperature. The membrane was washed 3 x 5 mins in TBS-T before detection. Chemiluminescence detection was performed using Amersham[TM] ECL[TM] Plus western blotting detection reagents (GE Healthcare, UK) according to the manufacturer's instructions. 40 parts of ECL Plus solution A to 1 part ECL Plus solution B (40:1 solution A: solution B) were mixed at room temperature for 15 minutes. After removing excess liquid, the membrane was laid on Saran wrap and the ECL Plus mixture pipetted equally over the membrane. This was covered from light for 10 minutes, after which the excess liquid was blotted off and placed on a piece of clean glass and covered with Saran wrap, taking care to remove any air bubbles. The membrane was then exposed on a phosphoimager. The ECL Plus detection reagents generate an intense long-lasting chemifluorescent signal which was detected on a phophorimager and digitally captured as a Tiff image using image analysis software . The membrane was washed in TBS-T and wrapped in Saran wrap and stored at -20°C. Anti β-actin anti-mouse (Sigma Aldrich) or anti GAPDH anti-mouse were used as loading controls in order to compare the band intensity of the target protein to this (www.gelifesciences.com, (Penna and Cahalan, 2007)).

6.11 TIRF imaging

Molecular events such as cell adhesion, secretion of neurotransmitters or cytokines often occur at the cell surface. If a protein bound to a fluorophore is excited, the resulting fluorescence bound to the surface is often overwhelmed by the background fluorescence. Total internal reflection fluorescence microscopy (TIRFM) was developed to overcome this problem. Total internal reflection is an optical phenomenon that occurs when a ray of light strikes a medium boundary at an angle larger than a particular critical angle with respect to the normal to the surface. If the refractive index is lower on the other side of the boundary, no light can pass through and all of the light is reflected. The critical angle is the angle of incidence above which the total internal reflection occurs (Figure 15).

$1x10^4$ primary human keratinocytes were seeded per well into a 6 well plate and transduced with either pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP-empty vector. Following transduction they were washed and trypsinised and seeded into glass bottom WillCo-dishes® for 24 hours. TIRF Images were acquired using an Evolve 512 EM CCD camera (Photometrics) mounted on a Nikon Eclipse TiE microscope. Samples were illuminated by 488 nm laser light (Argon) collected by a Plan Apochromat 60x/1.49 NA objective (Nikon). Images were acquired at a rate of 2 frames per second. Acquisition was controlled by Elements software (Nikon).
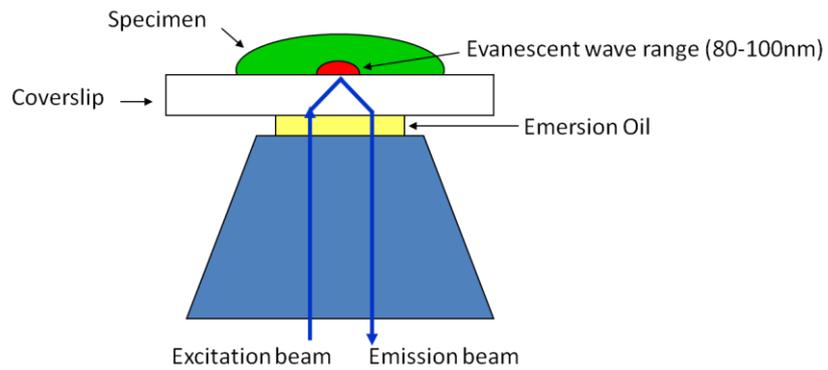
Figure 15. Total internal reflection fluorescence microscopy (TIRFM). When light strikes the interface between two materials, usually between a specimen and a glass coverslip or tissue culture container, at a sufficiently high angle, termed the critical angle, its refraction direction becomes parallel to the interface (90 degrees relative to the normal), and at larger angles it is reflected entirely back into the first medium. An induced evanescent wave or field is produced immediately adjacent to the interface between the two media having different refractive indices and this decays exponentially. As excitation of fluorophores in the bulk of the specimen is avoided, confining the secondary fluorescence emission to a very thin region, a much higher signal-to-noise ratio is achieved compared to conventional widefield epifluorescence illumination. This enhanced signal level makes it possible to detect single-molecule fluorescence by the TIRFM method (Ross et al.).

Chapter 7. Results

7.1 Expression of CypB in human keratinocytes and effect of CsA

CypA and CypB are the most abundantly found cyclophilins. Cyclophilins have been shown to be expressed by human keratinocytes via PAGE radiobinding assay, immunohistochemistry and PCR (Chatellard-Gruaz *et al.*, 1994; Al-Daraji *et al.*, 2002; Steele *et al.*, 2002) and so far CypA, CypB and CypD have been documented to be expressed in keratinocytes (Al-Daraji *et al.*, 2002; Fearon *et al.*, 2011; Ji *et al.*, 2012). CypA has been shown to be expressed in the cytoplasm of the basal layer of human epidermis by the Reynolds lab (Al-Daraji et al., 2002). The Reynolds lab have shown that CypB is secreted in response to CsA in normal human keratinocytes (Fearon et al., 2011) but the distribution of CypB in human epidermis has not, to our knowledge, been previously published. CD147 has been recognised as the Type 1 receptor for both CypA and CypB (Yurchenko *et al.*, 2001; Ji *et al.*, 2012). CD147 has been shown to be expressed in the basal and suprabasal layers of the epidermis (DeCastro et al., 1996). As CypB binds to CsA with 10 times greater efficiency than CypA, is secreted in response to CsA and has proinflammatory properties, we were interested to investigate its physiological role in human epidermis and its potential role in mediating the effects of CsA in atopic eczema (see section 7.6).

7.1.1 Immunohistochemical analysis of CypB and CD147 in normal skin.

To determine the expression of CypB and CD147 in normal skin, confocal images were taken of normal skin immunostained with CypB and CD147 (Type 1 receptor) antibodies. FLG was used to define the granular cell layer. Immunohistochemistry of FLG in normal skin was seen, as expected, in the granular layer of the differentiating epidermis in normal skin. CypB showed strong cytosolic expression within granular layer of the differentiating epidermis (Figure 16). There was an inverse gradient towards the basal layer where expression was much less than in the granular cell layer. Thus, the expression of CypB was highly distinct compared to CypA. CD147 was expressed in the cytoplasm and at the cell membrane of keratinocytes and expression was seen consistently from the basal layer of the epidermis throughout the suprabasal

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layers as previously described (DeCastro et al., 1996), but also to some extent, in the granular layer of the epidermis (Figure 16).
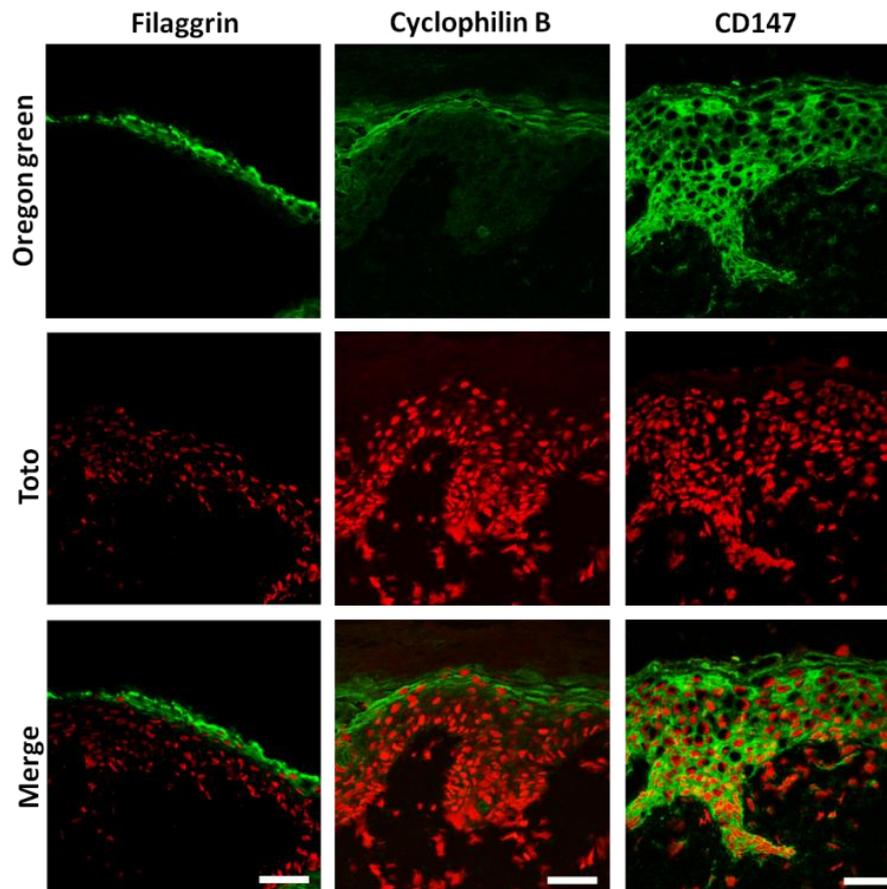
Figure 16: Localisation of CypB, CD147 and FLG in normal human skin.
Confocal images of frozen sections of normal epidermis immunostained with FLG, CypB and CD147 antibodies (Oregon green) and nuclear Toto-3 (red). Bar = 50µm.

FLG, CypB and CD147 expression in eczematous skin from patients with atopic eczema before and after two weeks of CsA treatment will be detailed in section 7.6.

7.1.2 Western blot analysis for CypB in normal human keratinocytes and in response to CsA

To investigate the physiological role of CypB, we utilised normal human keratinocytes cultured in monolayer and also studied 3-D epidermal equivalents (see section 7.5). Western blotting of normal human keratinocyte (NHEK) lysates detected a 21KDa band using a polyclonal antibody to CypB, as

previously established by the Reynolds lab (Fearon et al., 2011). Since our previous work, conditions for culturing human keratinocytes have been modified to include use of Epilife™ (Invitrogen, UK) keratinocyte culture medium. We therefore confirmed that CsA reproducibly induced secretion of CypB under these conditions: analysis of conditioned medium from NHEKs treated with and without CsA (1µm) was performed to detect any changes in CypB secretion in response to CsA. Primary human keratinocytes were treated for 24 and 48 hrs with CsA (Figure 17a).

Endogenous CypB was present in both vehicle and CsA treated keratinocyte whole cell lysates. Secretion of endogenous CypB into the medium increased in the presence of CsA which was present in a clinically relevant concentration (1 uM) (Fearon et al., 2011). In order to ensure that CsA was not inducing cell death, which could explain the appearance of CypB in the medium, a cytotoxicity assay was performed. NHEKs were treated with up to 1µm CsA for 24 hrs. This confirmed that no significant cell death occurred in the presence of CsA (Figure 17b).
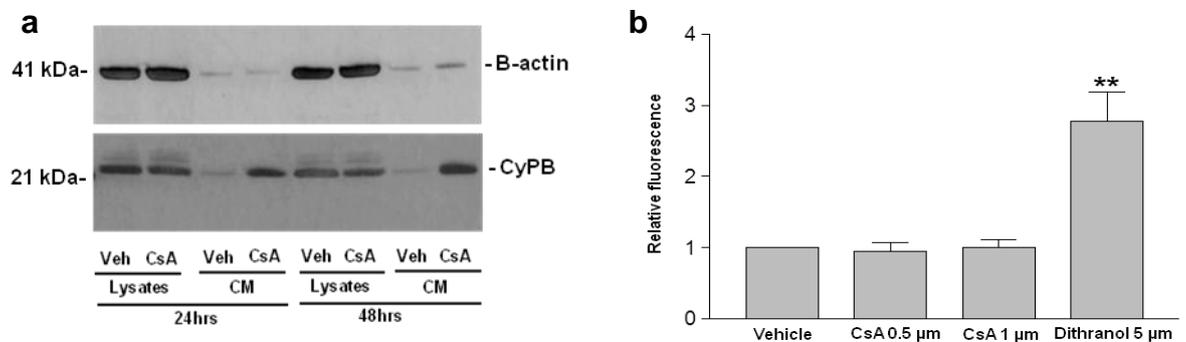
Figure 17: Secretion of CypB, an intracellular protein, increases in the presence of CsA. (a)Western blotting of keratinoyte whole cell lysates and concentrated medium (CM) collected from NHEKs, treated with 1µm CsA for 24 and 48 hrs (representative Western blot results shown from one of two donors). (b) Cytotoxicity was measured by detecting a fluorogenic protease released by dead cells. Bar chart showing no difference between the vehicle treated cells and those treated with CsA (n=9 wells from 3 independent experiments, **p<0.0001 compared with vehicle, one-way analysis of variance (ANOVA).

7.2 Retroviral transduction of normal human keratincytes with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$.

In order to investigate the effects of CypB on keratinocyte proliferation and differentiation, wild type CypB (CypB$_{WT}$) and mutant W128A CypB mutation (CypB$_{W128A}$) tagged at the C-terminus with green fluorescent protein (GFP) into the pLEGFP-N1 vector, already established in the Reynolds lab, were overexpressed in NHEKs by retroviral transduction. The CypB$_{W128A}$ mutation reduces the affinity of CypB for CsA by about 97%, with minimal effect on its PPIase activity (Carpentier et al., 2000), allowing us to establish functionality of the CsA binding region and CsA binding. The retroviral system was used to efficiently introduce stable expression of CypB and the expression of the enhanced green fluorescent protein allowed visualization of the protein within the cell.

7.2.1 Intracellular distribution and secretion of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in normal human keratinocytes following retroviral transduction and response to CsA

Previous work by the Reynolds lab (Fearon et al., 2011) had confirmed via Western blotting and confocal imaging, that pLEGFP-CypB$_{WT}$ was expressed in NHEKS, and that pLEGFP-CypB$_{W128A}$ was expressed in HaCaTs following retroviral transduction. In view of the changes in keratinocyte culture as described above in section 7.1.2, the intracellular distribution of GFP tagged CypB, and its response to CsA was confirmed in NHEKs following transduction with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$, treated with 1µm CsA and grown over 7 days. Live cell imaging and Western blot analysis of whole cell lysates were used to determine the intracellular distribution of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ compared to pLEGFP-empty vector control. Western blot analysis of conditioned medium was used to confirm if pLEGFP-CypB$_{WT}$ was secreted into the medium as seen with endogenous CypB and whether any differences were seen between pleGFP-CypB$_{WT}$ and pleGFP-CypB$_{W128A}$ compared to pleGFP-empty vector control.

Both live cell and confocal imaging of fixed cells confirmed the high efficiency of retroviral transduction (Figure 18a and b). pLEGFP-empty vector showed both

nuclear and cytoplasmic localisation compared to pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ which mainly show a cytoplasmic localisation on live cell imaging and when visualised using confocal microscopy following cell fixation (Figure 18a). pLEGFP-CypB$_{W128A}$ showed a reduced intracellular signal on live cell imaging compared to pLEGFP-CypB$_{WT}$, in the vehicle treated cells, suggesting less pLEGFP-CypB$_{W128A}$ present intracellular, however, western blot analysis revealed the presence of both pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in the lysates from 48 hrs to day 4. Both pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ were present in the medium at 48 hrs and day 4. At 48 hrs, western blot analysis revealed that more pLEGFP-CypB$_{W128A}$ was present in the medium compared to pLEGFP-CypB$_{WT}$. By day 4, intracellular levels of both pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ increased in vehicle treated NHEKs compared to 48hrs on western blot. The levels of intracellular pLEGFP-CypB$_{WT}$ shown on western blot appear to be higher than pLEGFP-CypB$_{W128A}$ in vehicle treated cells at both 48hrs and 4 days. Following CsA treatment, intracellular pLEGFP-CypB$_{WT}$ is lower compared to cells treated with vehicle after 48 hrs and also at day 4 on live cell imaging and this is confirmed on the western blot. After 4 days secretion of both pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ increase compared to 48hrs. This appears to be temporal and not just due to an increase in cell number. Together these data suggest that CsA induces secretion of CypB such that the intracellular concentration of CypB is decreased and that the rate of CypB production is insufficient to replenish levels and this can be seen to occur 24 hrs after treatment. Price et al demonstrated that CsA depleted CypB from the ER in HeLa cells and caused secretion of CypB (E.R. Price *et al.*, 1994). These data also indicate that CsA not only causes secretion of CypB into the medium, but that the CsA binding site is important in regulating the cellular distribution and secretion of CypB (Figure 18b and c).

CypB secretion was detected via western blot as this is a robust method of detecting the presence and abundance of proteins (MacPhee, 2010) and was used to validate the live cell images visualised using Confocal microscopy, determining the presence of CypB extracellularly in the medium. Measurement of CypB in the medium relied on the assumption that CypB is not protein bound to soluble receptors, carrier proteins or affected by inhibitors (Sullivan *et al.*,

2000). Although detecting immunofluorescence using live cell imaging indicated differences between pLEGFP-CypB$_{WT}$, pLEGFP-CypB$_{W128A}$ compared to pLEGFP-empty vector and each other, number of cells in each image was not known whereas, western blot allowed assessment of equal amounts of protein which was assessed by comparing the band intensity given using the loading control b-actin between samples and the band intensity of the target protein between the lysates and medium, following cell proliferation. B-actin bands show that loading for detection of CypB in lysates transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and treated with CsA was greater than for those treated with vehicle alone at 48hrs. Despite this we can still conclude that CsA secretion does increase over time as the western blots suggest less protein loaded in the day 4 samples but greater pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ secretion compared to 48 hrs. Techniques such as ELISA would allow quantification of the amount of CypB present in the samples. Furthermore, using FACS to count the number of cells in a sample would be helpful to accurately identify how much protein is being produced by a known number of cells.
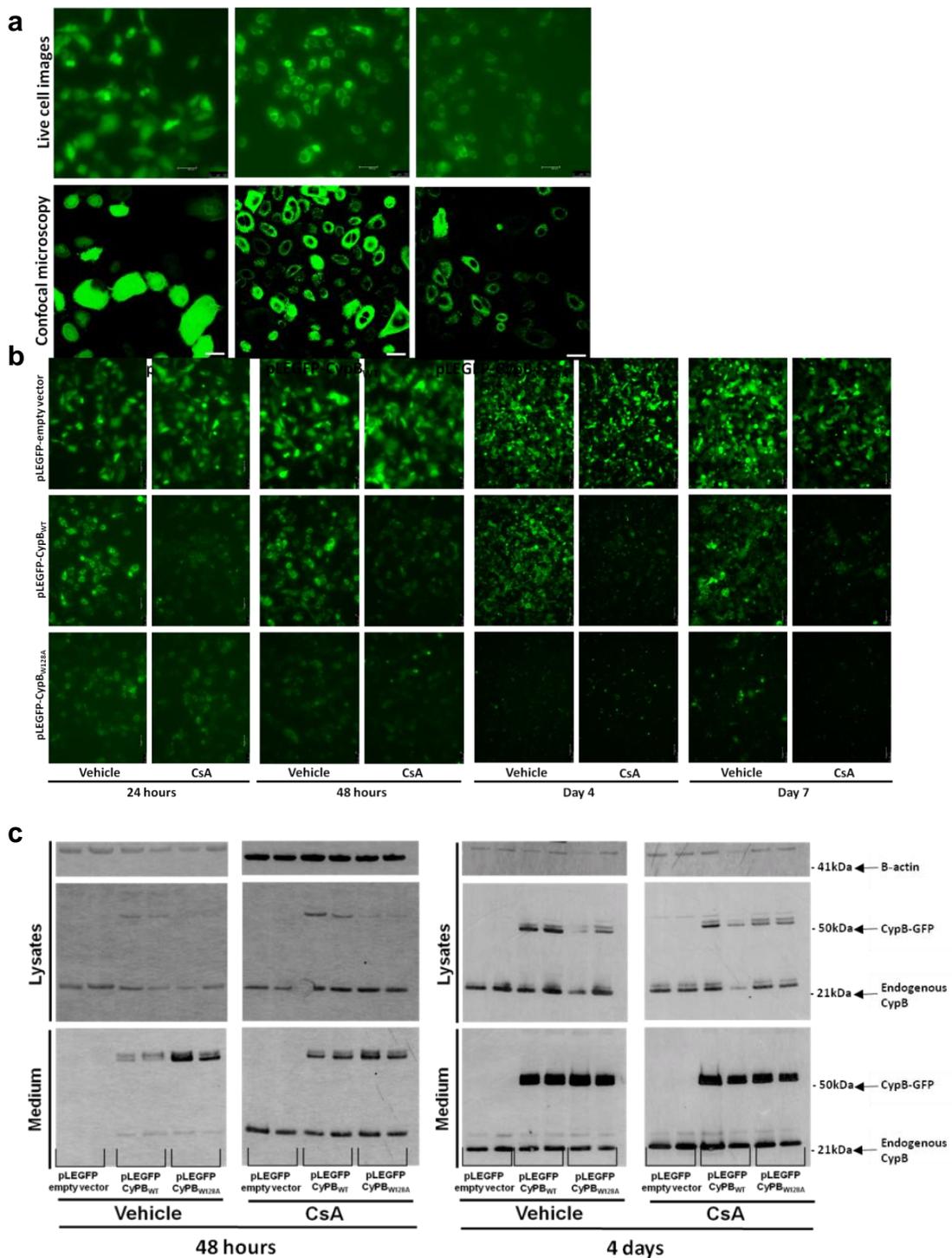
Figure 18. Localisation of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in NHEKs and response to CsA. (a) Live cell imaging and confocal images following cell fixation of NHEKs following retroviral transduction with pLEGFP-empty vector control, pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$. (b) Live cell imaging of primary human keratinocytes, transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and treated with CsA, grown over 7 days. d) Western blotting of cells and concentrated medium at 48 hours and day 4, β-actin acts as a cytoplasmic protein control.

7.2.2 TIRF Microscopy (TIRFM) of keratinocytes transduced with GFP tagged CypB

In order to study the process of secretion of CypB within NHEKs, primary human keratinocytes were transduced with pLEGPF-empty vector, pLEGFP-CypB$_{WT}$ and , pLEGFP-CypB$_{W128.}$ Live cell images were taken using TIRFM which allows viualisation of molecular events occurring at the cell surface (see enclosed CD of images).

A series of stills are shown below in Figures 19-21. In the keratinocyte shown transduced with pLEGFP-empty vector, keratinocyte pseudopodia, (as indicated by the white arrows) were seen and GFP was expressed homogenously throughout the cytoplasm and nucleus (Figure 19). Consistent with the ER localisation of CypB, a reticular pattern was observed in keratinocytes transduced with pLEGFP-CypB$_{WT}$ but also with pLEGFP-CypB$_{W128}$ (Figure 20 and 21). In the NHEKs transduced with pLEGFP-CypB$_{WT}$ dynamic activity occurring within the ER was seen in stills 1-3 (as indicated by the white arrows). In stills 4 and 5 a secretory vesicle (containing CypB, indicated by a white arrow) is shown moving from the cell to the extracellular space or over the glass interface. Towards the end of the series, in stills 8 and 9, new strands of ER containing CypB were seen to form. In NHEKs transduced with pLEGFP-CypB$_{W128A}$ (Figure 21) multiple secretory vesicles were seen moving rapidly within the keratinocyte (Figure 21a) and between the two keratinocytes shown (Figure 21b) which are not seen in the NHEK transduced with pLEGFP-CypB$_{WT}$. These vesicles correspond to secreted CypB. Visualisation of the dynamic changes are clearly seen in the movies in the DVD attached to the back cover of the thesis.
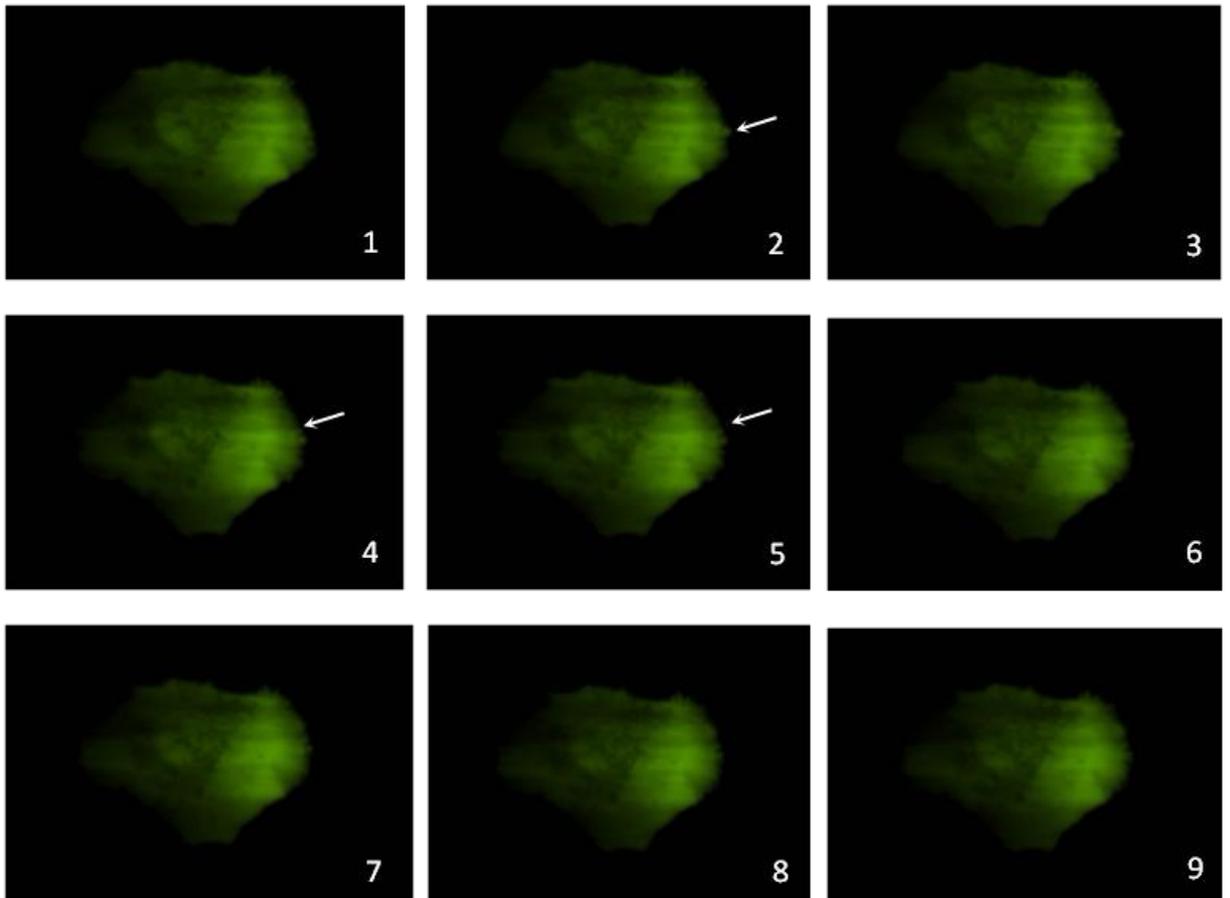
Figure 19. TIRFM stills of primary human keratinocytes overexpressing pLEGFP-empty vector. NHEKs transduced with pLEGFP- vector and imaged over 0.04 seconds shows diffuse pLEGFP empty vector expression. New keratinocyte pseudopodia are seen occurring in still 2 (not seen in still 1) and in still 5 (just seen to develop in still 4) marked with white arrows, Bar = 10μm.
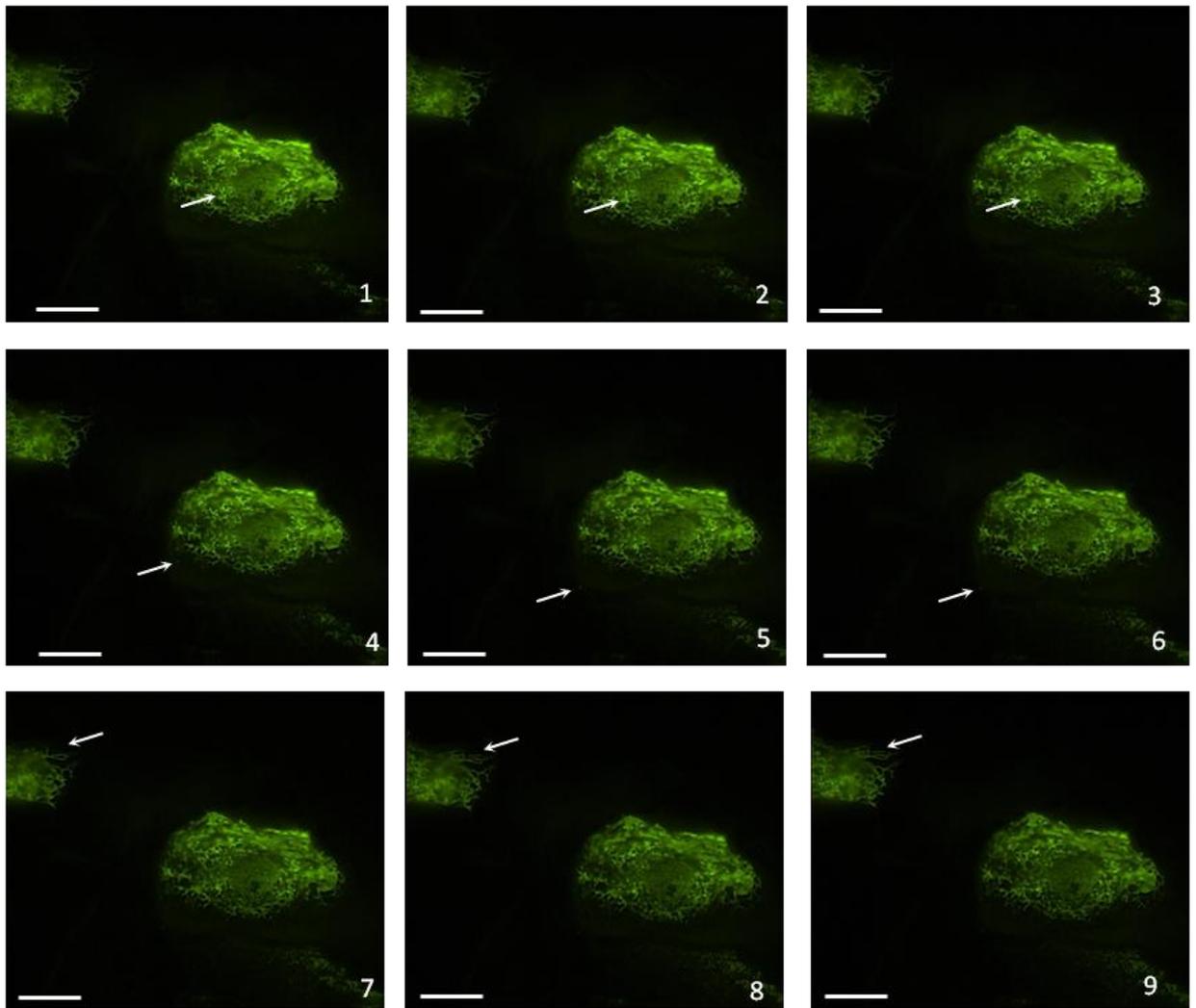
Figure 20. TIRFM stills of primary human keratinocytes overexpressing pLEGFP-CypB$_{WT}$. NHEKs transduced with pLEGFP-CypB$_{WT}$ and 9 stills taken from live cell imaging over 0.04 seconds. A reticular pattern indicates ER residence of pLEGFP-CypB$_{WT}$. White arrows in stills 1-3 identify change in pattern indicating dynamic activity of CypB on the ER. White arrow in stills 4 and 6 shows a secretory vesicle moving from the cell to the extracellular space or over the glass interface. In stills 8-9 there are new areas of ER formation not seen in 7, indicating CypB binding. Bar = 10μm.
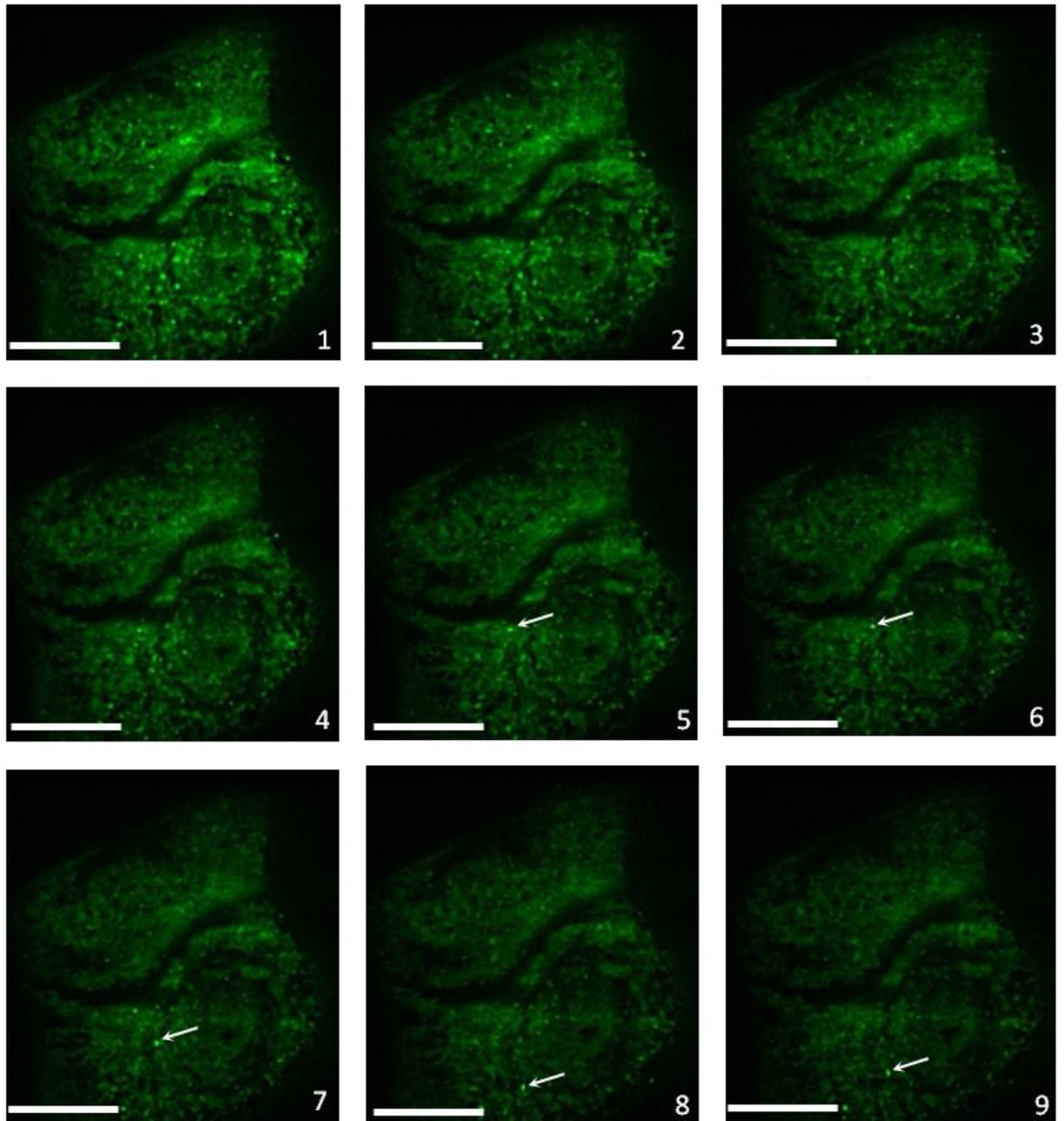
Figure 21 (a). TIRFM stills of primary human keratinocytes overexpressing pLEGFP-CypB$_{W128A}$. Nine stills taken every second of primary human keratinocytes transduced with pleGFP-CypB$_{W128A}$ and imaged over 9 seconds using TIRFM showing reticular distribution of pleGFP-CypB$_{W128A}$. Bleaching occurs over time. White arrows indicates movement of a CypB secretory vesicle within the cell that appears in 5 and moves down the cell through stills 6-9. There is a high degree of dynamic activity within the cells with GPF positive vesicles appearing and moving rapidly across the cells. This activity is clearly seen on the movie. Bar - 10µm

b
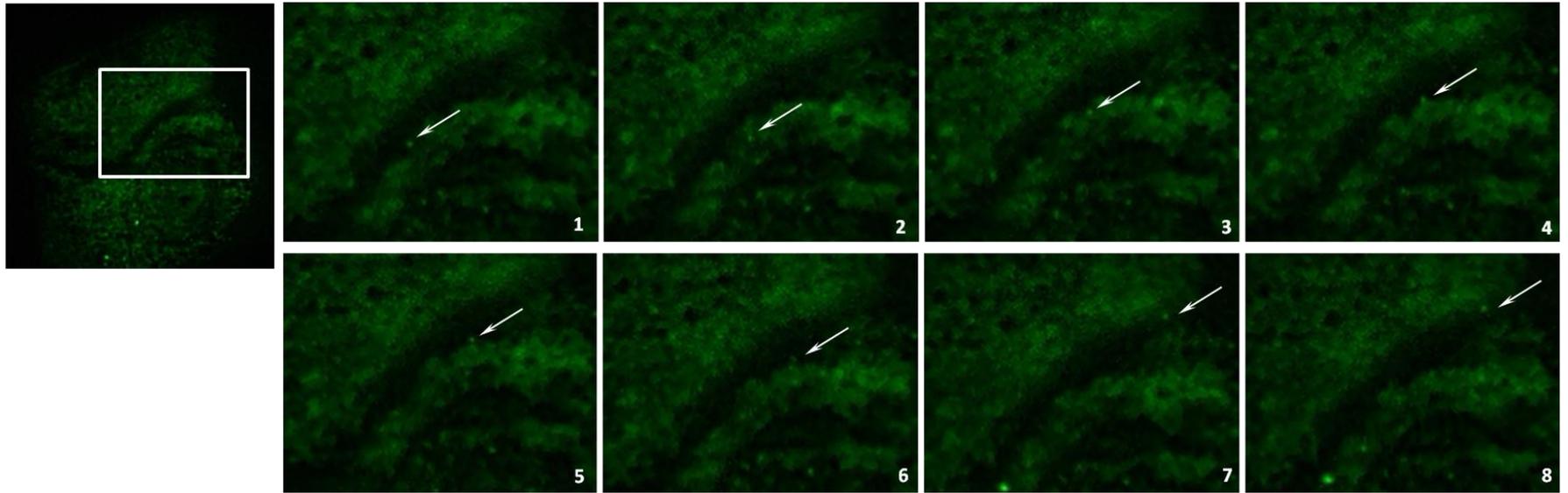
Figure 21(b) TIRFM stills of primary human keratinocytes overexpressing pLEGFP-CypB$_{W128A}$. Movement of secretory vesicle (as indicated by white arrows) from lower cell to cell above, over 0.02 seconds shown in stills 1-8. The secretory vesicle forms in still 1 and moves to the edge of the cell, shown in stills 4-5. It is released from the cell, shown in still 6 and taken up by the upper cell shown in stills 7-8. Bar = 5μm.

7.3 Overexpression of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and effect on keratinocyte proliferation.

To investigate whether CypB played a role in keratinocyte differentiation we overexpressed CypB in both HaCaT cells and NHEKs with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A,}$ by retroviral transduction, and assessed cell growth, proliferation and clonality. In addition, we assessed whether extracellular CypB was involved in cell growth and proliferation.

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7.3.1 Overexpression of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in HaCaT cells and effect on cell growth and proliferation.

In order to assess the influence of overexpressing CypB on keratinocyte proliferation we initially investigated the influence of CypB on HaCaT cells, an immortalized keratinocyte cell line. An SRB assay was performed which revealed that both CypB$_{WT}$ and CyPB$_{W128A}$ significantly influenced keratinocyte growth and proliferation (Figure 22).
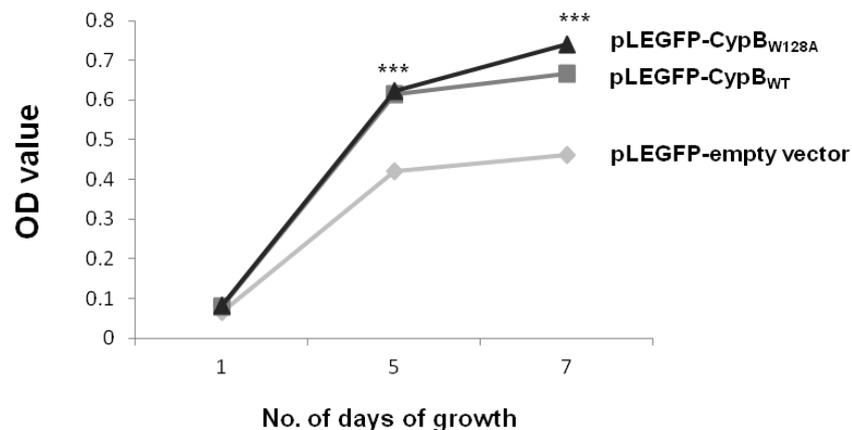
Figure 22. Overexpression of CypB in HaCaT cells significantly increases growth and proliferation. SRB in HaCaT keratinocytes growth over 7 days. By Day 5 and 7 HaCaT cells transduced with CyPB$_{WT}$ and CypB$_{W128A}$ showed significantly more growth compared to control, ***p<0.001, one way ANOVA, Neuman Keuls post test, n=36, results shown as the means ± SEM.

7.3.2 Overexpression of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in primary human keratinocytes and effect on colony forming assays

In order to investigate the effect of CypB in primary human keratinocytes, NHEKs were transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and seeded onto mitomycin C treated mouse 3T3 fibroblasts and grown for 14 days to allow colony formation[13] (Figure 23).

Significantly, more colonies were formed in those keratinocytes overexpressing CypB. In our clonogenic assays, keratinocytes transduced with pLEGFP-CypB$_{WT}$ grew more colonies than pLEGFP-CypB$_{W128A}$. Interestingly some donors showed more clonality than others and colonies were formed from some keratincytes passaged up to 4 times. Further, observations of the clones in one donor showed that colonies formed in those keratinoctyes overexpressing CypB were more likely to be holoclones (Figure 23c). In this donor (shown in the upper most panel in Figure 23b) the different colonies were easy to identify. In the other 3 donors (2 of which are shown in the lower panels of Figure 23b) colony size was smaller and therefore the different colonies were more difficult to distinguish, but this would suggest that the majority were, therefore, paraclones. These observations may suggest that CypB could influence the switch from keratinocytes proliferation to differentiation.

a

b

pLEGFP-empty vector   pLEGFP-CypB$_{WT}$ pLEGFP-CypB$_{W128A}$

c

Holoclones
Meroclones
Paraclones
Total

d

Meroclone

Paraclone

Holoclone

e

Age	Sex	Comordities	Skin used for keratinocyte culture taken from:
23	M	Nil	Foreskin
46	M	Varicose veins	Foreskin
81	M	Type 2 diabetes Duodenal ulcer	Foreskin
83	M	Unknown	Foreskin
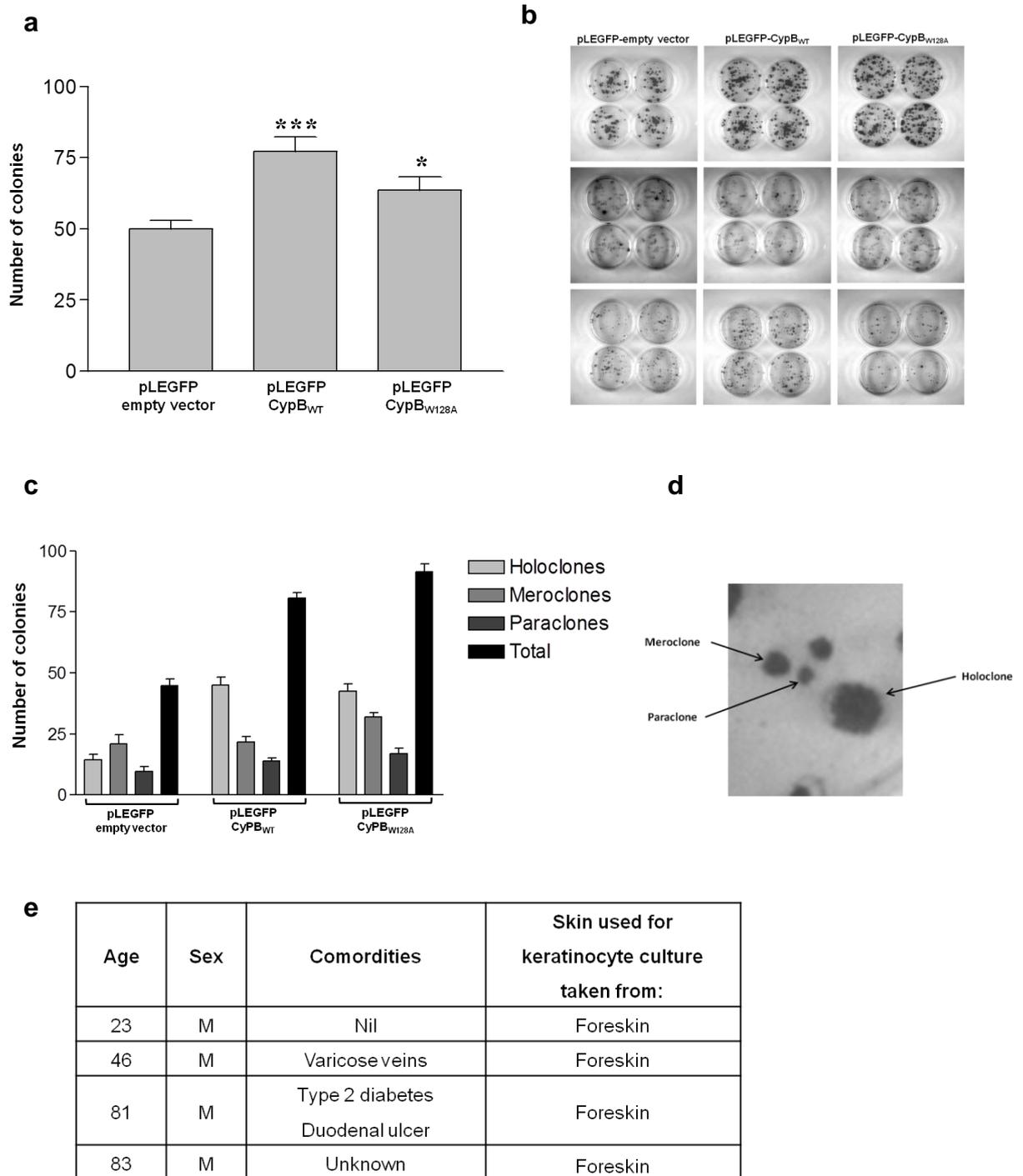
Figure 23. Overexpression of CypB in primary human keratinoctyes significantly increases colony formation.   (a) Retroviral transduction of NHEKs with pLEGFP-CyPB$_{WT}$ and pLEGFP-CyPB$_{W128A}$ significantly increased colony formation compared to pLEGFP empty vector (EV) by 1.4 fold and 1.15 fold respectively, results shown as the means ±SEM, **$p<0.001$, *$p<0.05$ n=16 in 4 independent experiments, from 4 different donors, one-way ANOVA, Neuman-Keuls post test analysis. (b) Representative images of colonies formed in 6mm petri dishes after 14 days of culture from 3 donors. (c) Bar chart showing the types of colonies formed in one donor, results shown as the means ±SEM.

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7.3.3 Overexpression of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in primary human keratinocytes and effect on cell growth and proliferation.

In order to further validate these observations, NHEKs were transduced with and grown in 96 well plates. In addition, CypB$_{WT}$ knockdown via lentiviral shRNA CypB$_{WT}$ transduction was achieved in primary human keratinocytes. Cell growth was measured after 48 hours using a cell proliferation assay as described in Chapter 6.5.3 (Figure 24).
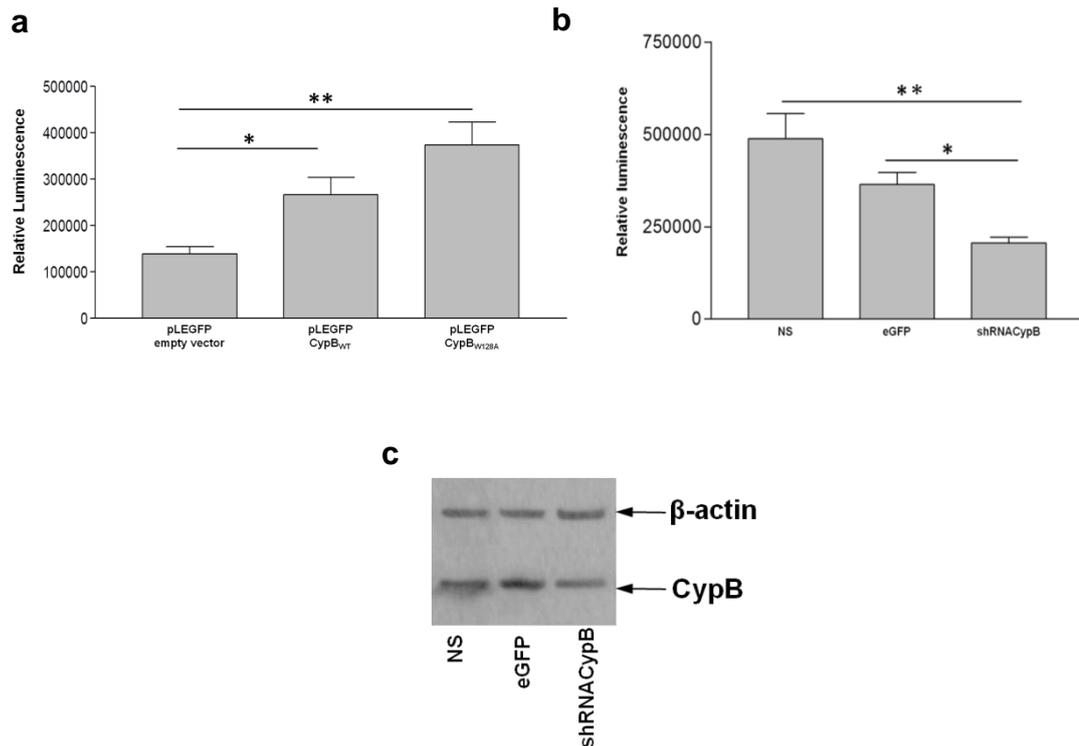
Figure 24. Overexpression of CypB in primary human keratinoctyes significantly increases cell growth and proliferation using the CellTitre-Glo® cell proliferation assay. (a) A significant increase in cell proliferation was seen in keratinocytes transduced with pLEGFP-CyPB$_{WT}$ by 1.9 fold and pLEGFP-CypB$_{W128A}$ by 2.7 fold when compared to pLEGFP empty vector, *p<0.05 and **p<0.001 respectively, n=20, in 4 independent experiments, one-way ANOVA, Neuman-Keuls post test. (b) Conversely, transduction of keratinocytes with shRNA CypB$_{WT}$ (shRNACyPB) resulted in reduced cell proliferation which was significant when compared with non-silencing (NS) and empty vector GFP control (eGFP), **p<0.001 and *p<0.05 respectively, n=36, in 4 independent experiments, one-way ANOVA. Results shown as the means ± SEM (c) Western blotting of primary human keratinocyte lysates shows shRNA CyPB$_{WT}$ knockdown.

Keratinocyte proliferation was seen to significantly increase in those keratinocytes overexpressing CypB. To further validate the role of CypB in

keratinocyte proliferation, we used shRNA to knockdown CyPB in keratinocytes. Western blotting confirmed CypB knockdown (Figure 24c). In addition this data showed that a significant reduction in keratinocyte proliferation occurred compared to normal controls when CypB knockdown was achieved.

Together, these experiments confirm that CypB influences keratinocyte growth and proliferation.

7.3.4 Effect of extracellular CypB induces keratinocyte growth and proliferation

In order to confirm whether intracellular or extracellular CypB is important for keratinocyte growth and proliferation, primary human keratinocytes, transduced with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in 6 well plates, were seeded into T75 flasks and expanded until 80% confluent the following day and the cells fed with complete Epilife® medium. Conditioned medium was collected after two further days of growth in T75 flasks because CypB accumulates in the medium, as shown previously. Conditioned medium was concentrated and 100μl added to naïve primary human keratinocytes (seeded the previous day) in 96 well plates and incubated for 48 hours. Cell growth in naïve NHEKs treated with concentrated conditioned medium was measured after 48 hours using a cell proliferation assay (Figure 25).
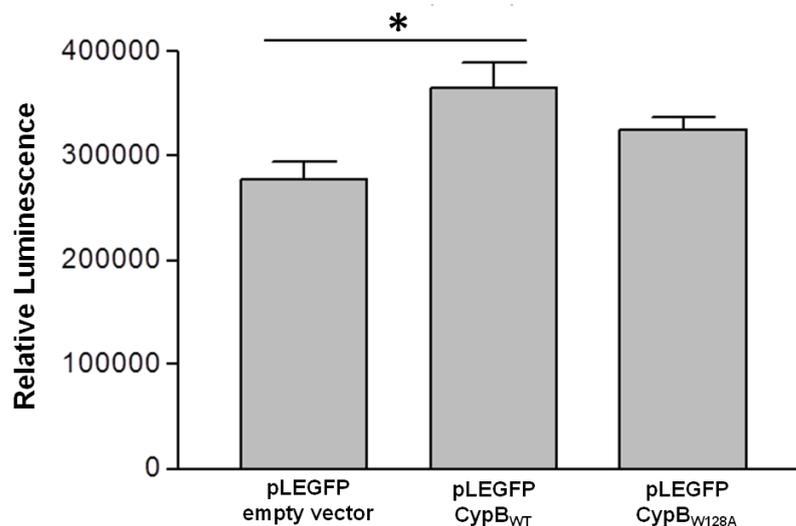
Figure 25: Extracellular CypB influences cell growth and proliferation using the CellTitre-Glo®proliferation assay. An increase in cell proliferation was seen in keratinocytes treated with conditioned medium from primary human keratinocytes transduced with pLEGFP-CyPB$_{WT}$ by 1.32 fold (*p<0.01), one-way ANOVA, and from cells transduced with pLEGFP-CypB$_{W128A}$ by 1.17 fold when compared to pleGFP empty vector control, n=36, in 3 independent experiments from 3 separate donors, results shown are the means ±SEM.

These experiments provide evidence that extracellular CypB influences growth and proliferation.

7.4 Overexpression of pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ in primary human keratinocytes and effect on keratinocyte differentiation.

As CypB is expressed in the granular layer of the epidermis we determined whether it played a role in regulating keratinocyte differentiation by assessing transglutaminase promoter luciferase activity and cadaverine incorporation in primary human keratinocytes transduced with CypB$_{WT}$ and CypB$_{W128A}$ (Figure 26).
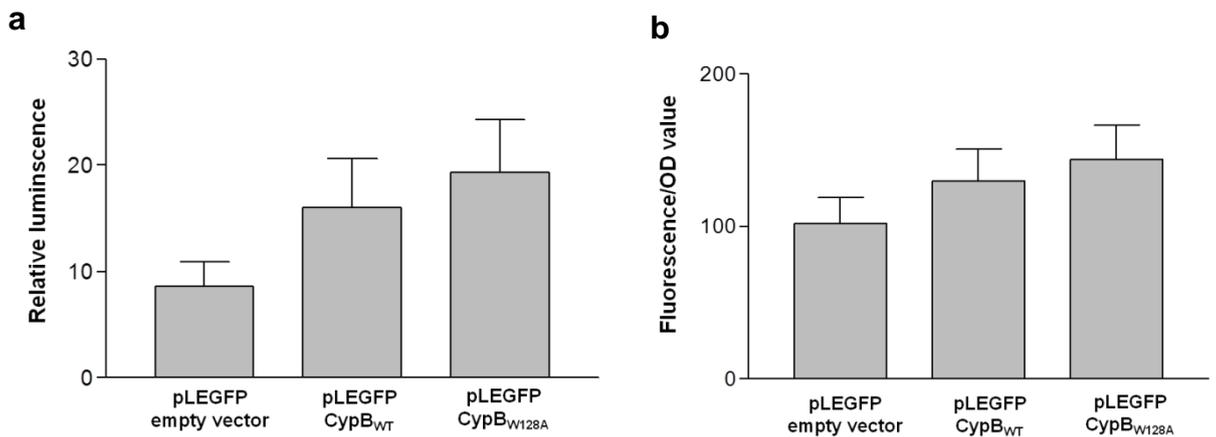
Figure 26. Overexpression of CypB regulates keratinocyte differentiation. (a) An increase in transgluatminase promoter activity was seen after 4 days in pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ when compared to pLEGFP-empty vector, n=34, 4 independent experiments (b) Increased transglutaminase activity measured by cadaverine incorporation (a substrate for transglutaminase) was seen after 6 days in pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ transduced keratinocytes when compared to pLEGFP-empty vector control, n=23, 3 independent experiments.

Using two different assays, keratinocytes overexpressing CypB showed an increase in transglutaminase activity which, although not statistically significant, is consistent with its presence in the differentiating layers of the epidermis. However, together these data suggest that CypB exerts a more profound effect on keratinocyte profileration and colony formation ability and that extracellular CypB may exert an important paracrine effect in this regard.

7.5 Organotypic cultures from primary human keratinocytes

To investigate the functional role of CypB we investigated the phenotype of organotypic cultures grown from NHEKs overexpressing CypB and following CypB knockdown. Skin equivalents were formed on a polycarbonate membrane allowing the formation of a fully differentiated cultured epidermis that is easily reproducible and therefore an important model for the study of human skin. The epidermal layers in these organotypic skin cultures display the same morphology as well as similar chemical and physical properties to normal human skin but are comprised of keratinocytes and do not contain melanocytes, Langerhan's cells or Merkel cells which are the other cells found in human epidermis (Poumay et al., 2004).

7.5.1 CypB andFLG expression in organotypic culture formed from primary human keratinocytes

Skin equivalents formed form NHEKs were confirmed to express CypB and FLG (Figure 27).
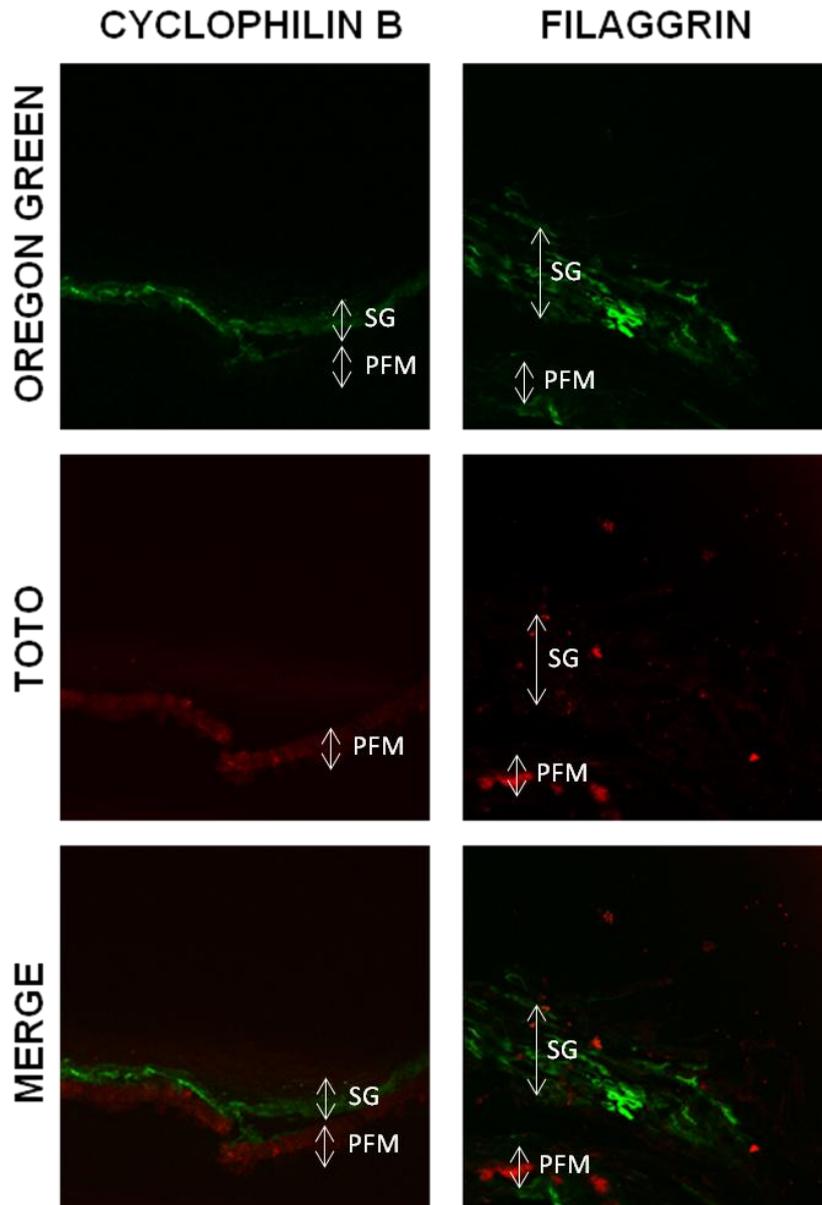
Figure 27. Localisation of CypB and FLG in skin equivalents. Confocal images of organotypic culture from one donor expressing CypB, FLG and nuclear Toto (red); SG = Straturm Granulosum , PFM= Polycarbonate filter membrane.

Organotypic cultures were observed to form basal, suprabasal, differentiating layers and a straturm corneum on light microscopy. Immunohistochemical analysis confirmed that these skin equivalents expressed FLG and CypB in the differentiating layer as expected.

7.5.2 Overexpression of CypB$_{WT}$ and CypB$_{W128A}$ and formation of epidermal organotypic cultures

We assessed the functional role of CypB by transducing primary human keratinocytes with pLEGFP-CypB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and using these keratinocytes to generate epidermal equivalents, as described in Chapter 6.8. Each organotypic culture was divided into three: (i) snap frozen for lysate production and Western blotting, (ii) formalin fixed and paraffin embedded for H&E staining, (iii) OCT embedded and frozen for immumohistochemistry (Figure 28).

The presence of GFP tagged CypB in addition to endogenous CypB in the epidermal equivalents was confirmed by western blotting and confocal imaging of the organotypic cultures. There was reduced expression of pLEGFP-CypB$_{W128A}$ compared to pLEGFP-CypB$_{WT}$, in keeping with lysates from monolayer experiments (Figure 28a). When examined using confocal microscopy, there was confirmation of GFP expression within the granular later of the epidermis in those organotypic cultures formed from keratinocytes overexpressing CypB (Figure 28c).

H&E sections of the organotypic cultures revealed the presence of a basal layer, supra-basal and differentiating layers together with the formation of a stratum corneum. Examination of the H&E sections suggest that in those epidermal equivalents formed from keratinocytes overexpressing CypB, the stratum corneum was more compacted in keeping with parakeratosis. Parakeratosis is often seen in psoriasis where there is overproliferation of the keratinocyte (Figure 28b and d).

The thickness of the epidermal equivalents were measured from the base of the basal layer to the top of the granular layer and revealed that those transduced with pleGFP-CyPB$_{WT}$ produced a thicker epidermis when compared to those organotypic cultures grown from keratinocytes transduced with empty vector control (Figure 29e), although this did not reach statistical significance.
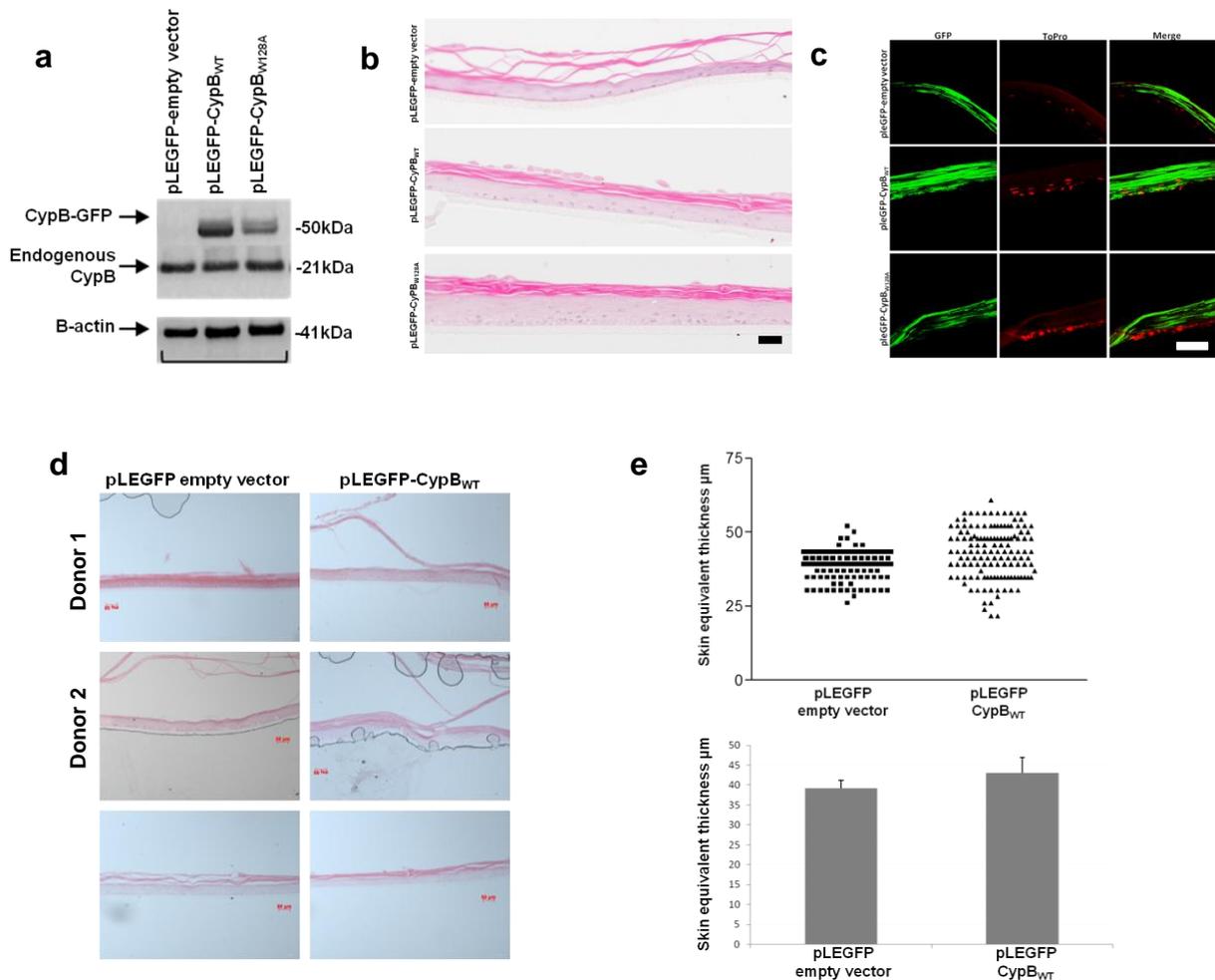
Figure 28. Organotypic cultures formed from NHEKs overexpressing pLEGFP-CyPB$_{WT}$ and pLEGFP-CypB$_{W128A}$. a) Western blotting of skin equivalents showed the presence of GFP tagged CypB in addition to endogenous CypB in equivalents formed from keratinocytes transduced with pLEGFP-CyPB$_{WT}$ and pLEGFP-CypB$_{W128A}$ whereas those equivalents formed from keratinocytes transduced with pLEGFP control showed presence of endogenous CypB alone. As in keeping with lysates from monolayer experiments, there was reduced expression of CypB$_{W128A}$ compared to CypB$_{WT}$. Western blot from one donor shown as a representative of experiments performed in triplicate. b) H&E sections of organotypic cultures formed from keratinocytes transduced with pLEGFP-CyPB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP control show basal layer of keratinocytes, supra-basal and differentiating layers and stratum corneum formation. c) Confocal images of organotypic cultures formed from keratinocytes transduced with pLEGFP-CyPB$_{WT}$ and pLEGFP-CypB$_{W128A}$ and pLEGFP control showing GFP expression in green and nuclear stain with ToPro in red. d) H&E sections of skin equivalents. Skin equivalents formed from keratinocytes from three different donors transduced with pLEGFP-empty vector and pLEGFP-CyPB$_{WT}$. e) Measurement of the thickness (measured from the base of the basal layer to the top of the granular layer) of each organotypic culture revealed those transduced with pLEGFP-CyPB$_{WT}$ produced a thicker epidermis when compared to those organotypic cultures grown from keratinocytes transduced with empty vector control (n=144, from 3 different donors - 48 measurements taken for each equivalent using 20x images shown in d) and measured manually); Bars=50μm.

7.5.3 Organotypic cultures from keratinocytes following lentiviral shRNA CypB$_{WT}$.

Primary keratinocytes were transduced with lentiviral shRNA CypB$_{WT}$ and allowed to proliferate in monolayer culture initially and then transferred into inserts and grown as epidermal equivalents before being processed as described above. Western blotting and H&E staining was performed (Figure 30).
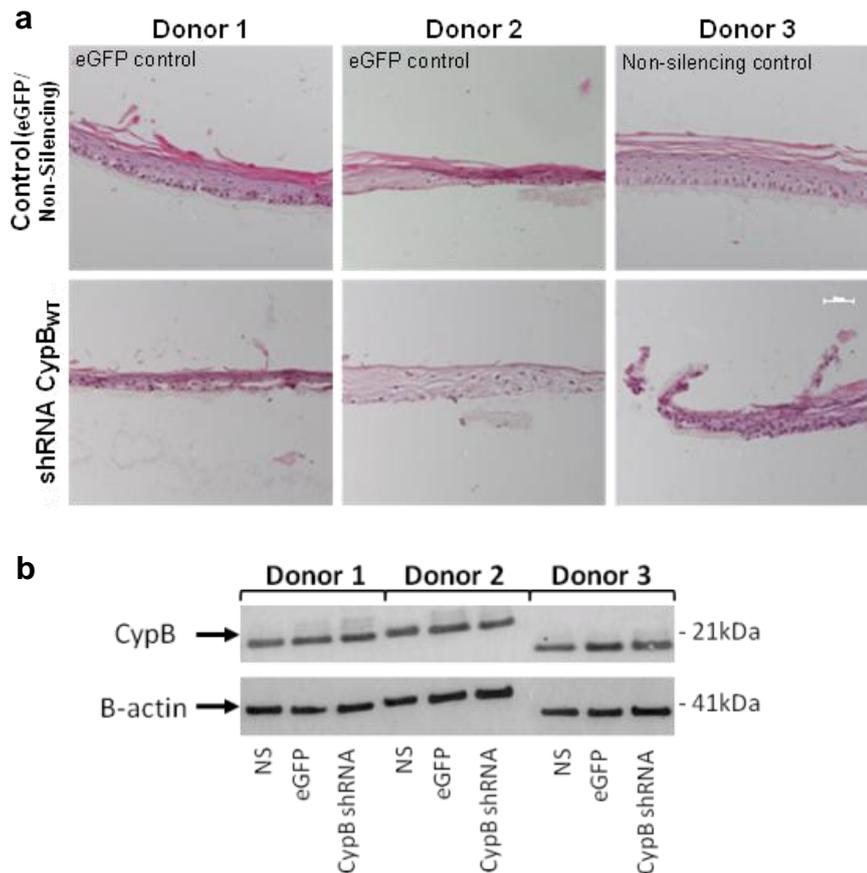
Figure 29. Organotypic cultures formed from NHEKs following shRNA CypB knockdown. (a) Skin equivalents from 3 different donors (labelled 1,2 and 3) following shRNA knockdown of CypB. (b) Western blotting of epidermal equivalents following shRNA knockdown, Bar=50µm.

Epidermal equivalents from keratinocytes from 3 different donors (labelled 1,2 and 3) and transduced with shRNA CypB$_{WT}$ did not show a normal skin phenotype. However although western blotting from monolayer cultures showed some reduction in CypB following shRNA knockdown, epidermal equivalents did not show a reduction in expression of CypB. This may reflect a lack of stable transfection or that those cells with continued CypB production were able to proliferate and produce an equivalent resembling an epidermis.

7.6 Immunohistochemical analysis of skin biopsies from patients with atopic eczema treated with Cyclosporine A (CsA)

In order to understand the clinical significance of CypB and its functional role in the epidermis we studied skin biopsies from eczematous skin of patients with atopic eczema before and after two weeks of CsA treatment at a dose of 4mg/kg. A summary of the patient details is shown in table 1 below. We reasoned that by studying changes in the epidermis of eczematous skin early during the treatment course, we were more likely to identify direct effects of CsA on atopic eczema rather than secondary changes that occur as a result of eczema clearance or as a result of barrier repair. Skin biopsies were taken and processed as described in chapter 6.1. We analysed the intensity of expression (intensity) of CypB, FLG and CD147 (Type I binding site (extracellular receptor) for CypB) in skin biopsies from patients with atopic eczema using confocal microscopy. The intensity for each protein was calculated by measuring its fluorescence intensity within the whole epidermis per 100µm of epidermis using image analysis software (Velocity, Perkin-Elmer, UK). Parameters were set to exclude background fluorescence.

Patient	Sex	Age	Hanifin and Rajka Diagnostic Criteria for Atopic Dermatitis	Biopsy site (pre CsA)	Biopsy site (post CsA)	Previous treatments	Co-morbidities
1	F	26	Y	buttock	buttock		
2	F	30	Y	lower back	lower back	UVB	iron deficiency, asthma, hayfever
3	M	54	Y	abdomen	abdomen		
4	M	NA	Y	NA	NA		
5	M	71	Y	arm	buttock		Photosensitive eczema
6	F	NA	Y	buttock	buttock		
7	F	42	Y	lower back	lower back		
8	F	NA	Y	lower back	lower back		
9	F	42	Y	lower back	lower back	topical tacrolimus, UVB	asthma
10	F	30	Y	buttock	buttock		
11	M	31	Y	lower/mid back	lower/ mid back	UVB	asthma, pneumonia
12	M	37	Y	lower back	lower back		

Table 1: 12 patients with atopic eczema donated skin biopsies for immunohistochemical analysis before and after treatment with CsA (M=male, F=female, Y=Yes, UVB=UVB phototherapy, NA= not available, CsA=ciclosporin)

7.6.1 CypB expression in skin biopsies from patients with atopic eczema treated with Cyclosporine A (CsA)

Immunohistochemical analysis of CypB undertaken in nine patients who had been treated with CsA at 4mg/kg for two weeks is shown in Figure 33. The fold change in the intensity of expression of CypB from pre treatment levels together with changes in the SASSAD scores for each patient pre-treatment and two weeks into CsA therapy is summarised below in Figure 30 and Table 2. Skin biopsies were taken as described in chapter 6.1. The normalised intensity measurements are shown in Figure 33. Any value less than 1 indicates that the intensity was reduced compared to the normal control for that image. The normal intensity was taken as the mean intensity measured from two normal controls (one representative image is shown). In the scatter plot (Figure 30), eight of the nine patients' data are shown. This is because in patient 3, who showed an increase in CypB intensity following CsA treatment in eczematous skin, had pre-treatment intensity levels calculated at zero due to the parameters set and thus the change in intensity could not be subsequently calculated.

Overall, there is no clear relationship between CypB expression within the epidermis and eczema severity. Seven out of nine patients showed a reduction in CypB expression in eczematous skin at baseline (prior to CsA treatment) compared to normal controls. In five of these patients, an increase in CypB expression was seen following 2 weeks of CsA treatment, but not to above that of the normal control. All patients showed an improvement in their SASSAD score. The remaining two of the nine patients had a higher CypB expression in their lesional skin compared to normal control but a decrease in expression was seen following CsA treatment. In patient 4, who showed a decrease in CypB expression following CsA treatment, diffuse CypB staining was seen throughout the epidermis pre CsA treatment, but following CsA treatment CypB expression was seen in the granular layer and present in similar levels to normal skin control.

Figure 30. Scatter plots to show changes in epidermal CypB expression in atopic eczema skin following 2 weeks of CsA treatment. 9 patients with atopic eczema were analysed for CypB expression. a) CypB epidermal intensity and SASSAD scores shown for 9 patients with atopic eczema prior to CsA therapy. b) CypB epidermal intensity and SASSAD scores shown for 9 patients with atopic eczema after two weeks of CsA therapy. c) 5 patients showed an increase in expression following CsA treatment from pre-treatment levels (as indicated by fold change values above 1, except for 1 patient (patient 3) where an increase in CypB expression was seen but pre-treatment intensity levels were calculated at zero due to the parameters set and thus the change in intensity levels is shown as zero). 4 patients showed a decrease in expression following CsA treatment from pre-treatment levels (as indicated by fold change values below 1), y-axis intersects x-axis at 1. All patients showed an improvement in atopic eczema as indicated by the % improvement in SASSAD score.

7.6.2 FLG expression in skin biopsies from patients with atopic eczema treated with Cyclosporine A (CsA)

Immunohistochemical analysis of skin biopsies from patients with atopic eczema who had been treated with CsA at 4mg/kg for two weeks to detect FLG expression was undertaken in 12 patients (Figure 34a and b). The normalised intensity measurements are shown in Figure 34 (any value less than 1 indicates that the intensity was reduced compared to the normal control for that image; where two images are shown, the intensity given is the mean of the two images). The normal intensity was taken as the mean intensity measured from two normal controls for all patients 1, 2, 4, 5, 7 and 10. For patients 3, 6, 8, 9, 11 and 12 the normal intensity was measured from one normal control. One representative image of the normal control is shown.

The fold change in intensity of expression following 2 weeks of CsA from pre treatment levels, together with changes seen in the SASSAD score are summarised below in Table 2 and in a scatter plot in Figure 31.

In 11 of the 12 atopic eczema patients, FLG expression was lower in lesional (baseline) skin compared to normal control except for patient 6 who had a higher expression of FLG in lesional atopic skin compared to the normal control. Following CsA treatment, FLG expression increased in 10 patients, all of whom had lower expression compared to the normal control, pre treatment. In seven of these 10 patients, the expression of FLG was higher following CsA treatment compared to normal control. In 9 of these 10 patients an improvement in SASSAD score was seen (SASSAD scores were unavailable for patients 10. FLG expression was lower following CsA treatment in the other two patients: patient 6 who had higher FLG expression compared to normal control pre treatment and an improvement in SASSAD, and patient 11 who had reduced FLG expression compared to normal skin pre CsA treatment and a decrease in FLG expression following 2 weeks of CsA treatment. However, the SASSAD score for patient 11 indicated particularly severe eczema which had minimally improved following 2 weeks of CsA treatment.
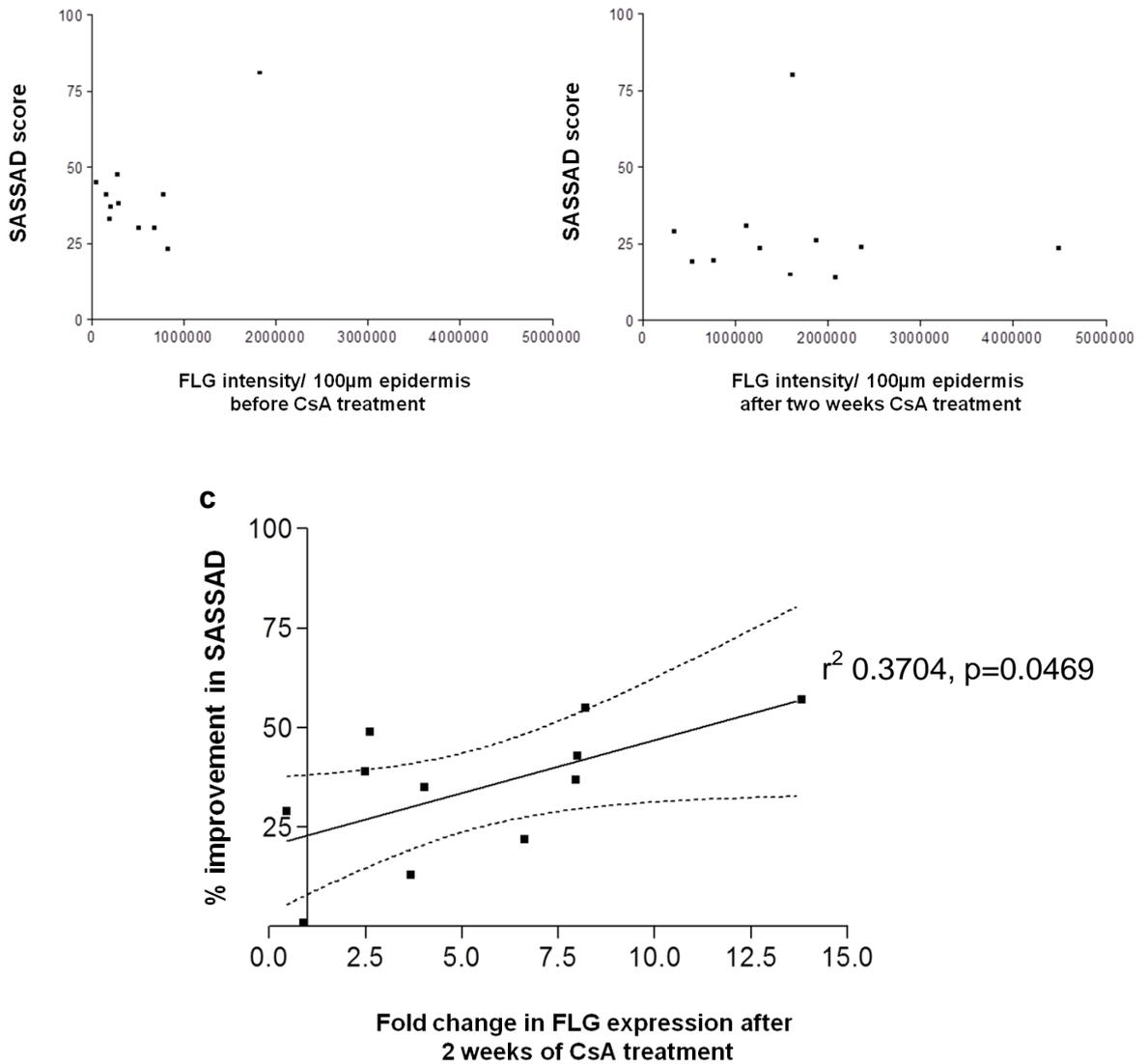
Figure 31. Scatter plots to show changes in epidermal FLG expression in atopic eczema skin following 2 weeks of CsA treatment. 12 patients with atopic eczema were analysed for FLG expression, 11 of the 12 patients analysed for FLG expression are shown above as SASSAD score for patient 10 not available. a) FLG epidermal intensity and SASSAD scores shown for 11 patients with atopic eczema prior to CsA therapy. b) FLG epidermal intensity and SASSAD scores shown for 11 patients with atopic eczema after two weeks of CsA therapy. c) 9 of the 11 patients shown showed an increase in expression following CsA treatment from pre-treatment levels (as indicated by fold change values above 1, r^2=0.3704, p+0.0469, linear regression analysis). 2 patients showed a decrease in expression following CsA treatment from pre-treatment levels (as indicated by fold change values below 1), y-axis intersects x-axis at 1. All patients showed an improvement in atopic eczema as indicated by the % improvement in SASSAD score. Solid line representing linear regression analysis with dotted lines representing 95% confidence intervals.

7.6.3 CD147 expression in human keratinocytes and atopic eczema.

CD147 has been identified as the Type I binding site for CypB and is known to be expressed on keratinocytes (Denys *et al.*, 1997; Ghannadan *et al.*, 1998; Kanekura *et al.*, 2002). We analysed CD147 expression in 6 patients with atopic eczema pre and post CsA treatment using confocal microscopy (Figure 35a and b) and analysed intensity of CD147 expression using velocity software (Perkin Elmer, UK). The normalised intensity measurements are shown in Figure 35 (any value less than 1 indicates that the intensity was reduced compared to the normal control for that image). The normal intensity was taken as the mean intensity measured from two normal controls (one representative image is shown). Table 2 and the scatter plot below (Figure 32) summarises the fold change seen in CD147 expression following 2 weeks of CsA therapy.

CD147 is expressed throughout the normal epidermis. The intensity of CD147 expression increased in four of the six patients investigated. A change in distribution of CD147 following treatment with CsA in patients with atopic eczema was seen (Figure 35). In patient 2 there was reduced expression of CD147 throughout the epidermis of atopic eczema in both pre and post CsA sections. In patients 4 and 7 the distrubtion of CD147 was found to change from being greatest at the basal layer, pre CsA treatment, to showing a greater intensity in the differentiating layers after two weeks of CsA therapy. In patient 6 the intensity of CD147 increases throughout the epidermis after two weeks of treatment. In patient 8 the intensity of CD147 expression increases in the basal layer of the epidermis.
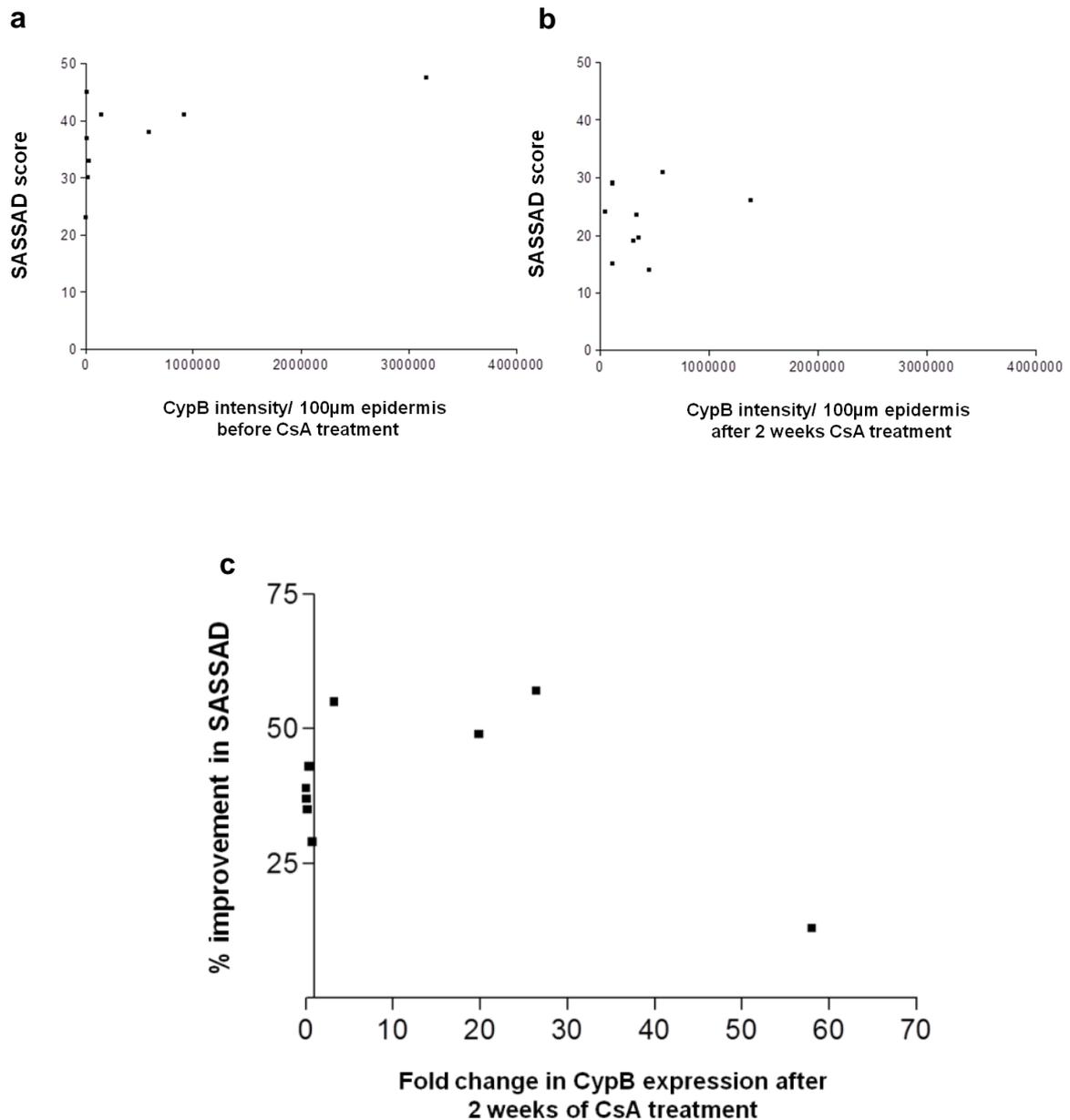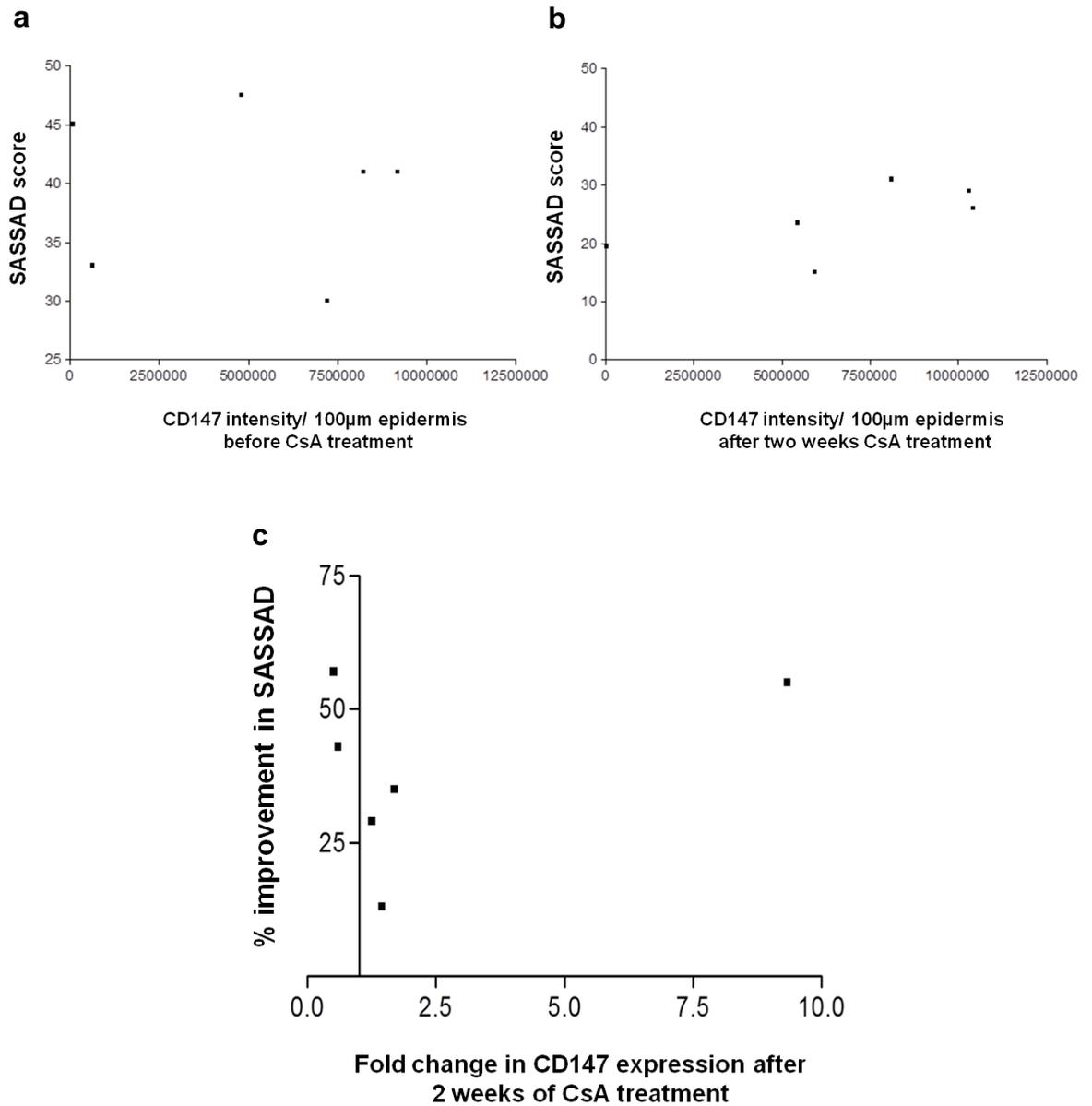
Figure 32. Scatter plots to show changes in epidermal CD147 expression in atopic eczema skin following 2 weeks of CsA treatment. 6 patients with atopic eczema were analysed for CD147 expression. a) CD147 epidermal intensity and SASSAD scores shown for 6 patients with atopic eczema prior to CsA therapy. b) CD147 epidermal intensity and SASSAD scores shown for 6 patients with atopic eczema after two weeks of CsA therapy. c) Of the 6 patients analysed, 4 patients showed an increase in expression following CsA treatment from pre-treatment levels as indicated by fold change values above 1). 2 patients showed a decrease in expression following CsA treatment from pre-treatment levels (as indicated by fold change values below 1), y-axis intersects x-axis at 1. All patients showed an improvement in atopic eczema as indicated by the % improvement in SASSAD score.
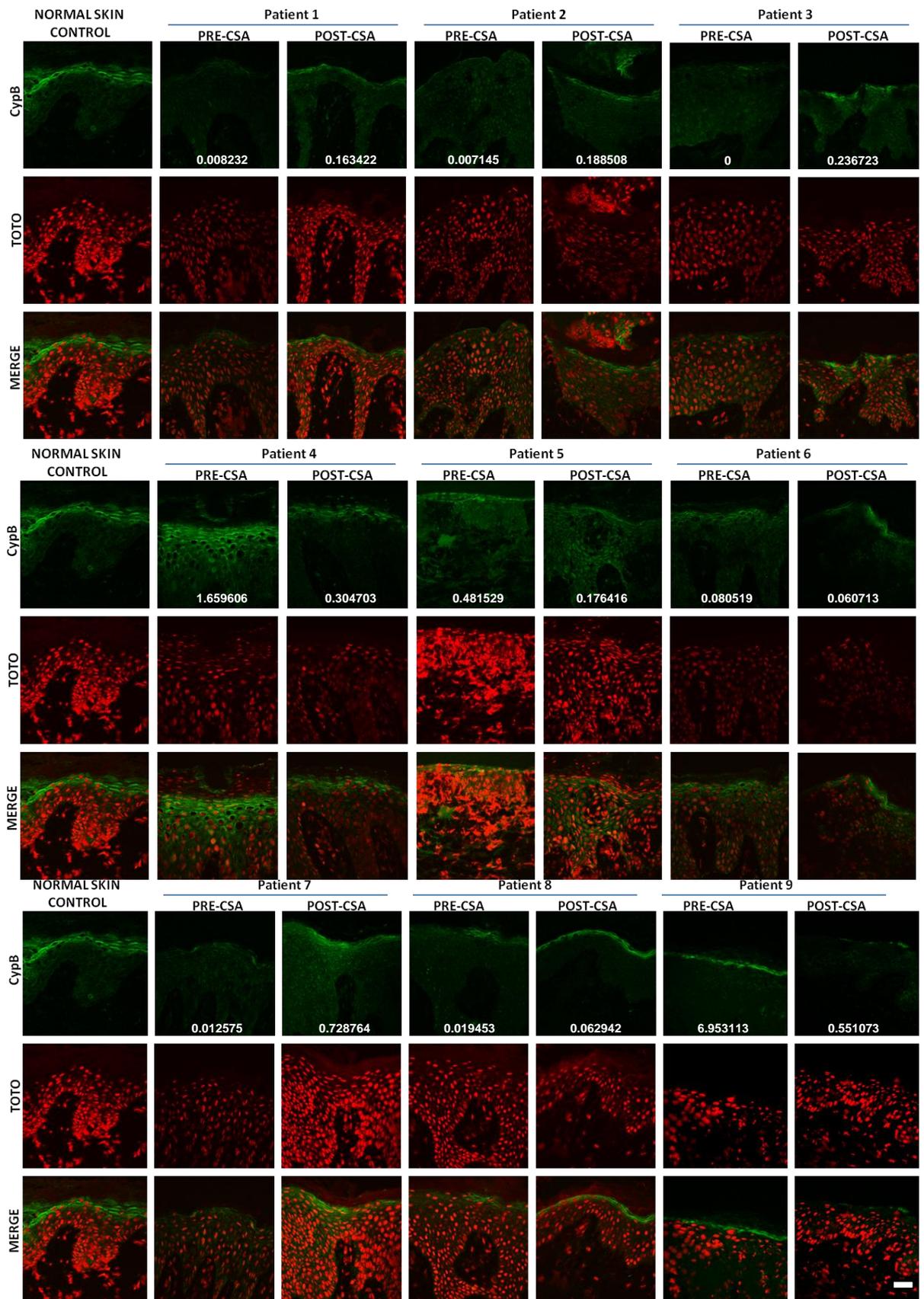
Figure 33. Localisation of CypB in frozen skin sections from patients with atopic eczema pre and post CsA treatment. Immunohistochemistry of eczematous skin revealed CypB expression throughout the epidermis, but mainly in the granular layer. For CypB expression one representative image is shown. The calculated normalised intensity is shown for each image. One representative image from two normal controls is shown. Bar = 100μm.

Figure 34 (a). Localisation of FLG in frozen skin sections from patients 1-6 with atopic eczema pre and post CsA treatment. Immunohistochemistry of frozen skin sections from patients 1-6 with atopic eczema pre and post 2 weeks of CsA treatment. FLG expression is seen in all patients but has a more patchy expression pre CsA treatment. Note FLG expression at baseline compared to normal control. An increase in FLG expression is seen in patients 1-5 following treatment. In patient 6 a decrease in FLG expression was seen pre CsA in atopic skin compared to normal and following 2 weeks of CsA therapy. Two representative images from skin sections for both pre and post CsA are shown except for patients 6 where one representative image is shown. Expression was quantified by measuring the intensity of green fluorescent signal measured per 100μm of epidermis, using velocity software (Perking Elmer) and the average intensity measured from the two images, except for patient 6. The calculated normalised intensity for each patent is shown. Where two representative images are shown, the calculated normalised intensity is the mean of the two images, Bar = 25μm.

Figure 34 (b). Localisation of FLG in frozen skin sections from patients 7-12 with atopic eczema pre and post CsA treatment.
Immunohistochemistry of frozen skin sections from patients 7-12 with atopic eczema pre and post CsA treatment. FLG expression is seen in all patients but has a more patchy expression pre CsA treatment. An increase in FLG expression is seen in patients 7, 8, 9, 10, 12 patients following treatment to above that of normal except in patients 9 and 10. Two representative images from skin sections for both pre and post CsA are shown except for patients 8 and 12 where one representative image is shown). Expression was quantified by measuring the intensity of green fluorescent signal measured per 100μm of epidermis, using velocity software (Perking Elmer) and the average intensity measured from the two images, except for patients 8 and 12. The calculated normalised intensity for each patent is shown. Where two representative images are shown, the calculated normalised intensity is the mean of the two images, Bar = 25μm.
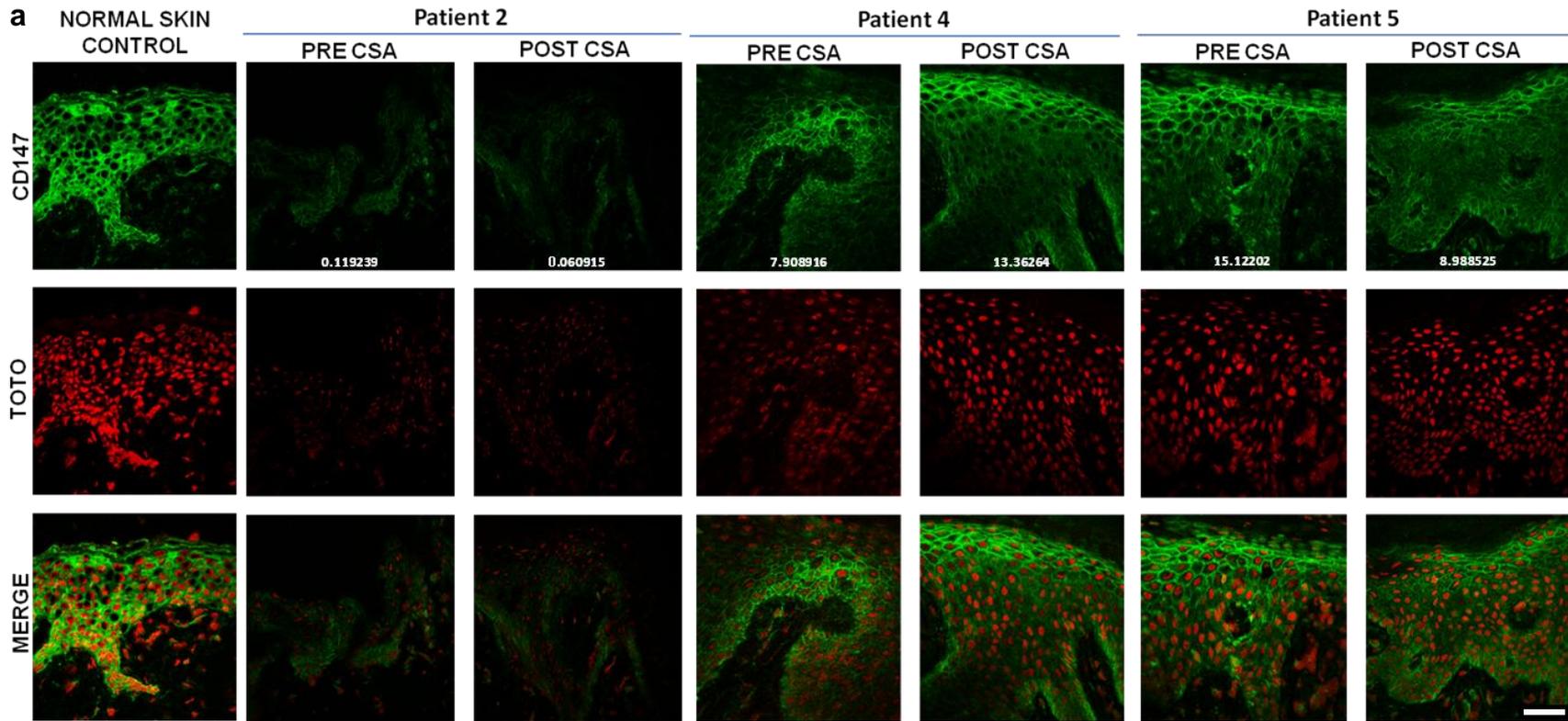
Figure 35 (a). Localisation of CD147 in frozen skin sections from patients 2,4,and 5 with atopic eczema. Immunohistochemistry of frozen skin sections from patients 2,4 and 5 with atopic eczema pre and post CsA treatment. CD147 expression is seen throughout normal epidermis. An increase in CD147 expression is seen in patient 4, following treatment. One representative image from skin sections for both pre and post CsA are shown. Expression was quantified by measuring the intensity of green fluorescent signal measured per 100µm of epidermis, using velocity software (Perking Elmer). Distribution of CD147 can be seen in the basal layer in patient 2, and is more apparent in both basal and differentiating layers for patients 4 and 5. The calculated normalised intensity is shown for each image. One representative image from two normal controls is shown, Bar = 50µm.
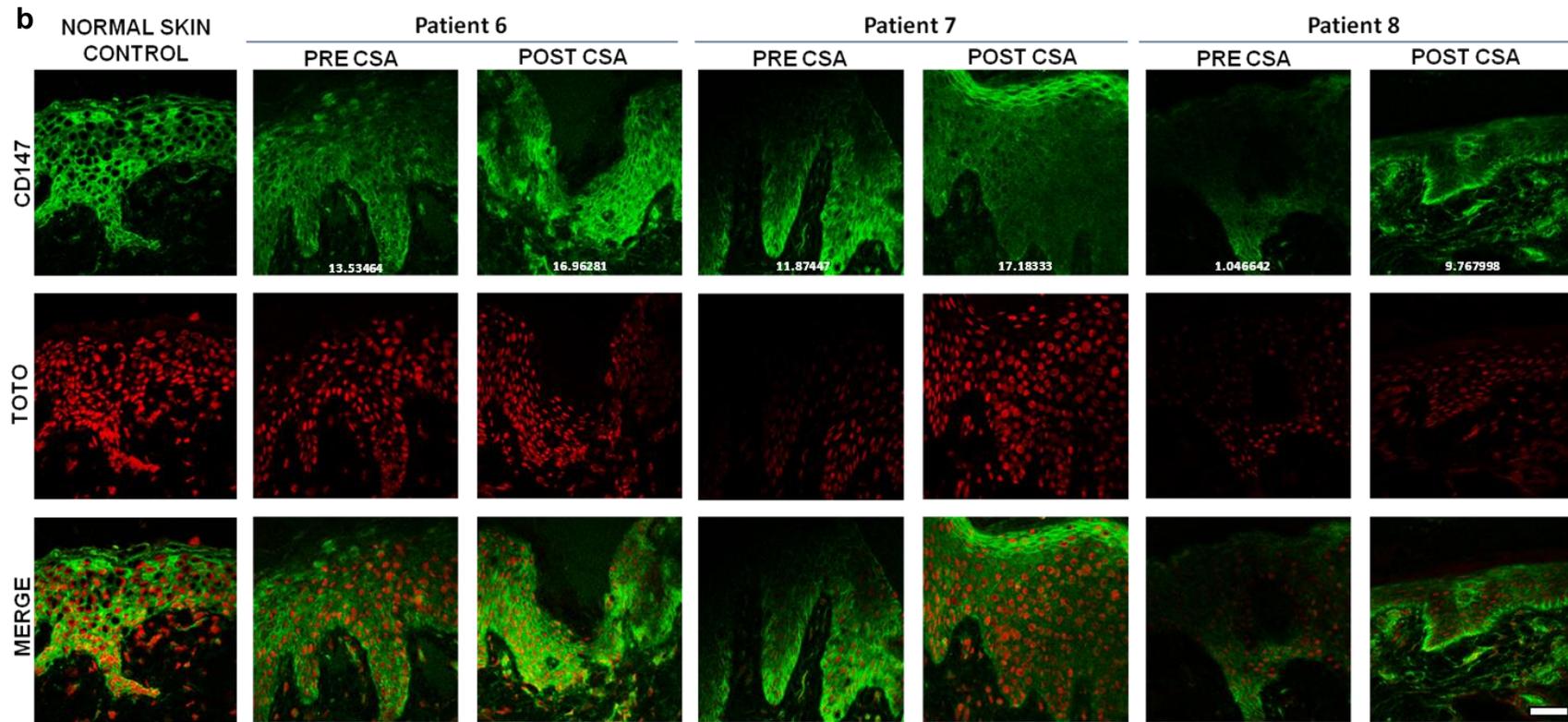
Figure 35 (b). Localisation of CD147 in frozen skin sections from patients 6,7 and 8 with atopic eczema. Immunohistochemistry of frozen skin sections from patients 6,7 and 8 with atopic eczema pre and post CsA treatment. An increase in CD147 expression is seen in patients 6,7 and 8, following treatment. One representative image from skin sections for both pre and post CsA are shown. Expression was quantified by measuring the intensity of green fluorescent signal measured per 100μm of epidermis, using velocity software (Perking Elmer). Distribution of CD147 is more apparent in both basal and differentiating layers for patients 6,7 and 8 (Bar = 50μm).

7.1.4 Summary of FLG, CypB and CD147 expression in skin biopsies from patients with atopic eczema treated with CsA

Patient	Fold change in CD147 expression following 2 weeks CsA treatment	Fold change in CypB expression following 2 weeks CsA treatment	Fold change in FLG expression following 2 weeks CsA treatment	SASSAD pre CsA	SASSAD post CsA	% improvement in SASSAD score
1	UNA	19.85	2.60	37	19	49
2	0.51	26.38	13.81	45	19.5	57
3	UNA	Increase*	2.49	23	14	39
4	1.69	0.18	4.03	47.5	31	35
5	0.59	0.37	7.98	41	23.5	43
6	1.25	0.75	0.45	41	29	29
7	1.45	57.96	3.67	30	26	13
8	9.33	3.24	8.20	33	15	55
9	UNA	0.08	7.96	38	24	37
10	UNA	UNA	1.64	UNA	UNA	UNA
11	UNA	UNA	0.89	81	80	1
12	UNA	UNA	6.62	30	23.5	22

Table 2. Summary of changes seen in expression of FLG, CypB and CD147 in patients with atopic dermatitis following CsA treatment. Values above 1 indicate an increase in expression (green cells); Values below 1 indicate a decrease in expression (orange cells) Improvement of eczema is indicated by % change in SASSAD score with green cells indicating an improvement in atopic eczema. **Increase*** - an increase in CypB expression was seen in patient 3 but pre-treatment intensity levels were calculated at zero due to the parameters set and thus the change in intensity could not be subsequently calculated.

In summary and notably, these results indicate that in all patients where an increase in CypB was seen following CsA treatment, an increase in FLG was also observed. However, of the four patients who had a decrease in CypB, three showed an increase in FLG expression and one showed a decrease in FLG expression. Further information may be gleaned if further biopsies were taken continually during treatment: in the patients where an increase in both CypB and FLG was seen post treatment, the SASSAD score fell to below 20 (except for patient 7), whereas they remained above 20 in those where there was a decrease in CypB following treatment, indicating that in these patients

improvement of eczema was not as marked. Changes in FLG expression may be seen earlier than in CypB expression during treatment. Further investigations are required to confirm this speculation. In addition, changes in CD147 expression are seen pre and post CsA treatment. CD147 expression is seen throughout the epidermis but an increase in basal and granular layer expression is seen.

FLG expression does increase in 10 out of 12 patients treated with CsA which suggest CsA may play a direct role in barrier repair. In all 5 patients with an increase in CypB expression following CsA treatment, FLG expression was also seen to increase. These observations and the changes in CD147 expression that were observed in the epidermis before and during CsA treatment need further investigation.

7.1.5 Reanalysis of FLG expression in skin biopsies from patients with atopic eczema treated with Cyclosporine A (CsA)

In order to validate the results seen previously, the FLG data was reanalysed so that the FLG intensity for each patient was normalised to internal nuclear (Toto-3) staining as the control, before and after CsA treatment. 12 patients with atopic eczema were reanalysed for FLG expression using Image J software. The average pixel intensity for each area of protein expression (FLG being expressed in the stratum granulosum and Toto-3 expressed between the stratum basale and stratum granulosum) was calculated/ 10000 pixels. FLG intensity was normalised to internal Toto-3. 11 out of 12 patients showed an increase in expression following CsA treatment from pre-treatment levels. Table 3 summarises the results. This data is shown as scatter plots in Figure 36.

Patient	Average pixel intensity per 10000 pixels pre CsA treatment	Average pixel intensity per 10000 pixels post CsA treatment	Fold change in FLG expression following 2 weeks CsA treatment	SASSAD pre CsA	SASSAD post CsA	% Improvement in SASSAD score
1	2.16	3.77	1.75	37	19	49
2	2.45	11.31	4.62	45	19.5	57
3	4.63	6.55	1.41	23	14	39
4	1.47	4.64	3.16	47.5	31	35
5	2.2	4.37	1.98	41	23.5	43
6	10.44	12.95	1.24	41	29	29
7	1.56	3.17	2.03	30	26	13
8	22.07	23.09	1.05	33	15	55
9	3.04	4.87	1.60	38	24	37
10	5.61	7.33	1.31	UNA	UNA	UNA
11	3.95	2.79	0.71	81	80	1
12	1.89	3	1.59	30	23.5	22

Table 3: Summary of changes seen in expression of FLG in patients with atopic dermatitis following CsA treatment following reanalysis. Values above 1 indicate an increase in expression (green cells); values below 1 indicate a decrease in expression (orange cells). Improvement of eczema is indicated by % change in SASSAD score with green cells indicating an improvement in atopic eczema. UNA=unavailable.

a

FLG intensity pre CsA/ SASSAD

b

FLG intensity post CsA/ SASSAD

c

r^2 0.1974, p=0.17
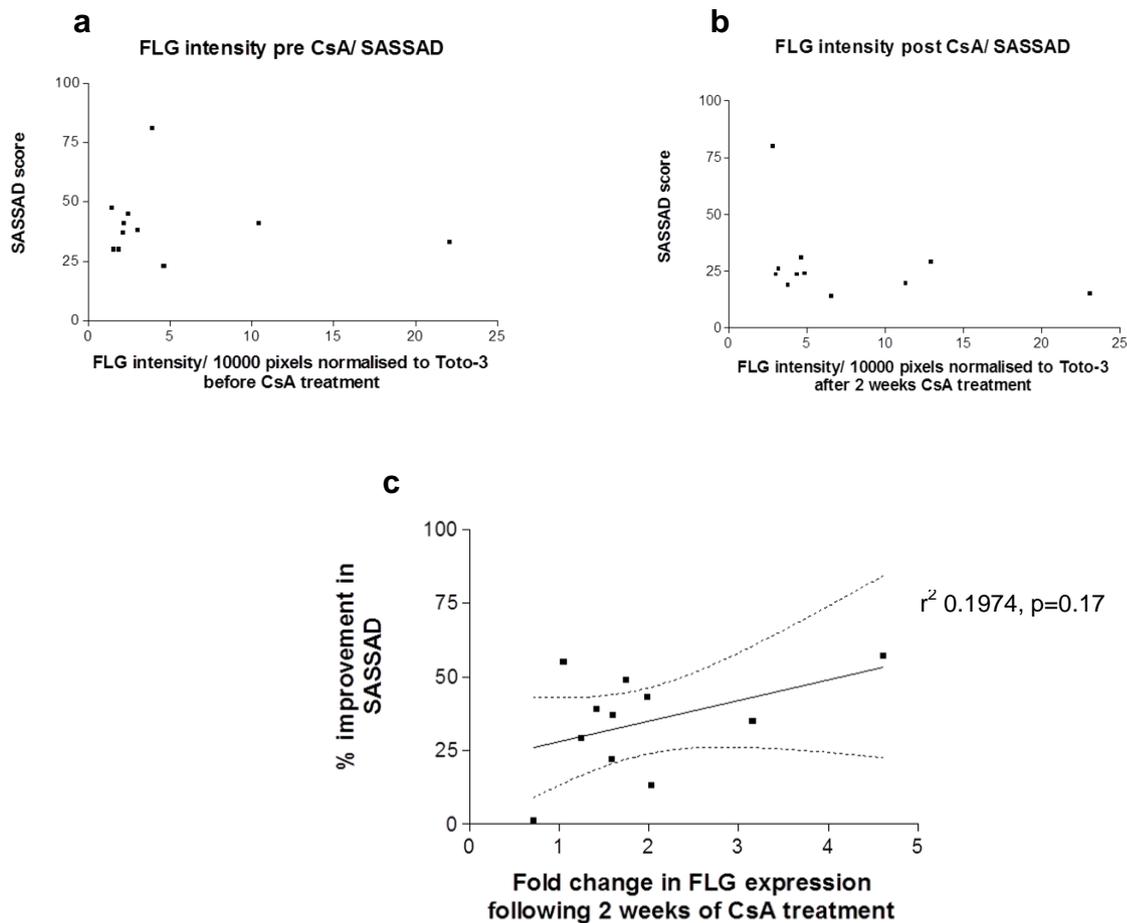
Figure 36: Reanalysis of FLG expression in skin biopsies from patients with atopic eczema treated with Cyclosporine A (CsA). Scatter plots to show changes in epidermal FLG expression in atopic eczema skin following 2 weeks of CsA treatment following reanalysis. 11 of the 12 patients analysed for FLG expression are shown above as SASSAD score for patient 10 was not available. a) FLG epidermal intensity and SASSAD scores shown for 11 patients with atopic eczema prior to CsA therapy. b) FLG epidermal intensity and SASSAD scores shown for 11 patients with atopic eczema after two weeks of CsA therapy. c) Chart to show fold change in FLG expression following CsA treatment in 11 patients reanalysed: 11 out of 12 patients showed an increase in expression following CsA treatment from pre-treatment levels – values above 1 indicate an increase in FLG expression and values below 1 indicate a decrease in expression. Patient 10 whose SASSAD score is not available and therefore not plotted showed an increase in FLG expression following CsA therapy All patients shown had an improvement in atopic eczema indicated by the % improvement in SASSAD score. Solid line representing linear regression analysis with dotted lines representing 95% confidence intervals.

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Chapter 8: Discussion

CsA has been recognised as an effective and rapidly acting drug for the treatment of atopic eczema for many years but its mechanism of action remains unclear in the skin. Although CsA binds to cyclophilin A and inhibits calcineurin in T cells, the relative contribution of this mechanism to the effects of CsA in inflammatory skin disease is incompletely understood. CsA has been shown to directly act on keratinocytes and moreover CsA inhibits inflammation and affects keratinocyte differentiation in SCID mice that lack functional T- cells (Fisher *et al.*, 1988; Reynolds *et al.*, 1998). In addition to the effect of the CsA-CypA complex on calcineurin, CsA interacts with other cyclophilins. CypB is highly expressed in T-cells and binds with high affinity to CsA (Hasel *et al.*, 1991; E. R. Price *et al.*, 1991; Spik *et al.*, 1991; Denys *et al.*, 1997; Denys *et al.*, 1998b). Moreover, over the last few years, CypB has been increasingly found to play an important role in a number of intracellular and extracellular functions in different cell types (Fang *et al.*, 2009; Galat and Bua, 2010; Kumari *et al.*, 2012). The studies reported in this thesis investigated the potential role of intracellular CypB, as well as CypB that is secreted by keratinocytes in response to CsA and their role in mediating the therapeutic effects of CsA in inflammatory skin disease. The studies show that CypB promotes keratinocyte proliferation and differentiation through both intra- and extracellular mechanisms and underscore the role of CypB in keratinocyte biology as well as the direct cutaneous effects of CsA that are independent of T cells.

Our studies confirm that CypB and its type I receptor CD147 are both expressed in human epidermis with the greatest expression of CypB found in the differentiating granular layer. CD147 has been recognised as the functional type 1 receptor for CypB which is involved in endocytosis and cyclophilin-initiated signalling in T-cells (Denys *et al.*, 1998a; Yurchenko *et al.*, 2001; Allain *et al.*, 2002). Positioned on the opposite side of CypB is its type II binding site and the location of type I and type II binding sites on opposing sides of the CypB molecule suggests that interactions with both types of binding can occur simultaneously (Carpentier et al., 1999). Type II binding of CypB serves to increase CypB concentration near the type I receptors as well as affecting the

biological activity of the protein which has been described in T-cells (Carpentier et al., 2002).

Our western blotting studies confirmed that CypB is secreted by human keratinocytes and that secretion is enhanced by CsA. In addition, using live cell imaging of GFP-tagged CypB constructs, we were able to visualise for the first time the actual secretion process of CypB secretion in keratinocytes and its uptake by adjacent keratinocytes in culture. We had previously shown that the addition of a GFP tag did not affect the distribution or behaviour of CypB in keratinocytes (Fearon et al., 2011). We verified in NHEKs that secretion of CypB in response to CsA was not due to CsA-induced cell death. Importantly, our studies have shown for the first time that CypB plays a role in regulating keratinocyte proliferation and differentiation. We established this by first overexpressing CypB-GFP in keratinocytes and then showing that CypB significantly promotes keratinocyte cell growth and proliferation using three different assays. Conversely, transduction with shRNA $CypB_{WT}$ resulting in knockdown of $CypB_{WT}$ reduced NHEK proliferation. Using conditioned media experiments we were able to provide evidence that extracellular CypB significantly promoted cell growth and proliferation in keratinocytes. Taken together, these results strongly suggest that CypB has an autocrine and/or paracrine role in promoting cell growth. In addition, we found that formation of skin equivalents produced from NHEKs overexpressing CypB formed a thicker epidermis supporting our observations that CypB plays a role in keratinocyte proliferation. Furthermore, using two different assays, overexpression of CypB in NHEKs positively increased transglutaminase activity, which is consistent with its expression in the differentiating layer of the epidermis.

Since CsA has been shown to exert a direct effect in keratinocytes we sought to investigate the relevance of the $CypB_{W128A}$ mutation (which substitutes alanine for tryptophan at residue 128 in the CsA-binding site) on keratinocyte proliferation and differentiation as this mutation reduces CsA binding by 97%, but does not have a significant effect on its PPIase activity (Carpentier et al., 1999). We found $CypB_{W128A}$ to be more readily secreted than $CypB_{WT}$ in primary keratinocytes, as shown via western blotting, and intracellular secretory

vesicles of CypB$_{W128A}$ were seen to be very prominent on TIRFM imaging in NHEKs. Previous work by the Reynolds lab has shown that the CsA-binding site of CypB is required for retaining CypB within the ER (Fearon et al., 2011): confocal imaging studies using the GFP-tagged CypB$_{WT}$ and CypB$_{W128A}$ revealed that CypB$_{W128A}$ showed significantly less colocalisation with an ER marker than CypB$_{WT}$ and that CsA reduced colocalisation of CypB$_{WT}$ but not CypB$_{W128A}$ with the ER (Fearon et al., 2011). In addition, an optical isomer of CsA lacking cyclophilin-binding activity (cyclosporin H) did not induce secretion of CypB which further supports evidence that the CsA site regulates CypB secretion. Furthermore, Fearon et al showed that the CsA binding site affects the speed and mobility of intracellular CypB. Following photobleaching in HaCaT cells, the completeness of recovery was significantly longer for CypB$_{W128A}$ compared to CypB$_{WT}$, and the addition of CsA also reduced the recovery of CypB$_{WT}$ but not CypB$_{W128A}$. This confirmed that the mutation in the CsA binding site reduced the speed and mobility of CypB suggesting that CypB had been directed out of the ER into secretory vesicles (Fearon et al., 2011). In our studies, using TIRFM live cell imaging, we were able to further study CypB localisation and motility within NHEKs.

Live cell imaging of primary human keratinocytes following transduction with GFP tagged CypB$_{WT}$ and GFP-tagged CypB$_{W128A}$ using TIRFM allowed us to visualise the differences between CypB$_{WT}$ and CypB$_{W128A}$. These images showed that both proteins localised to an interconnected network consistent with the ER. Moreover we observed the presence of much dynamic activity occurring within the ER. However, in the cells overexpressing wild type CypB, there was a much clearer reticular pattern seen than in those cells overexpressing GFP tagged CypB$_{W128A}$. This provided further evidence that CypB$_{WT}$ is able to bind to the ER, as expected. In addition, we were able to visualise formation of reticulate strands indicating active binding of CypB$_{WT}$ to the ER. Moreover, a secretory vesicle was seen to be released from the cell indicating secretion of CypB$_{WT}$. These secretory vesicles could be seen to a greater extent in the NHEKs overexpressing CypB$_{W128A}$. In these cells, a much greater number of secretory vesicles could be seen actively moving within the keratinocytes compared to CypB$_{WT}$. We were able to show the release of a

secretory vesicle from one cell and its uptake into the neighbouring cell. This further confirms that the presence of the CsA binding site mutation interferes with CypB binding to the ER and that a greater number of secretory vesicles containing CypB are therefore available within the keratinocyte. Taken together, these data suggest that under normal physiological conditions CypB may bind other proteins via the CsA-binding site to ER-binding partner(s) to retain CypB in the ER (Fearon et al., 2011) and/or that other cellular CypB-binding proteins interact with the CsA binding site to release CypB from the ER so that CypB may be biologically available. In the search for a potential homologue for CsA, CAML (calcium modulating cyclophilin B ligand protein) has been found to be involved in Ca^{2+} signalling, activates the calcium driven activation of NFAT and has been shown to be required for EGF receptor recycling to the cell surface (Bram and Crabtree, 1994; Holloway and Bram, 1998; Tran *et al.*, 2003). In preliminary results we have identified CAML RNA (via RT-PCR) from cultured human keratinocytes. It would be interesting to investigate if CAML plays a role in human keratinocytes as CAML has been shown to be associated with CypB and Ca^{2+} signalling and the ER provides an important store for calcium. In addition, CAML has been found to bind to the prolactin receptor during prolactin stimulation and participates in the receptor signalling and prolactin dependent proliferation of breast cells (Lim et al., 2011). As it is ubiquitously expressed, CAML may therefore play an interacting role in CypB signalling pathways but this is speculation and needs to be determined experimentally.

We hypothesised that CypB may play a role in keratinocyte proliferation and differentiation in keratinocytes. Although CypB protein was seen to be expressed throughout the human epidermis, we found the greatest expression in the granular layer. This observation suggests that CypB might regulate keratinocyte differentiation. On the other hand, the fact that CypB is known to be secreted (E.R. Price *et al.*, 1994; Mariller *et al.*, 1996; Billich *et al.*, 1997), and may regulate proliferation in other cell types (Rycyzyn *et al.*, 2000; Rycyzyn and Clevenger, 2002), raises the possibility that CypB secreted from keratinocytes in the granular cell layer might regulate the proliferation of stem and transit-amplifying keratinocytes in the basal and suprabasal layers. CD147

was previously shown to be expressed at the protein level within the basal and suprabasal layers by DeCastro et al (DeCastro et al., 1996), although it was not appreciated by these authors that CD147 may be the type 1 receptor for CypB. The work in this thesis extends these observations by demonstrating expression throughout human epidermis. This difference in expression may have been observed due to different antibodies used for detection. Moreover, these data provide a plausible biological mechanism and add further weight to the concept that CypB secreted in the granular cell layer may exert physiological effects on keratinocytes in basal and suprabasal layers of human epidermis.

We showed that overexpressing $CypB_{WT}$ and $CypB_{W128A}$ both significantly increased cell growth and proliferation in HaCaT and NHEK cells but also had a positive role in keratinocyte differentiation. For our cell growth and proliferation studies we performed an SRB assay on HaCaT cells which determines cell number and a cell viability assay in NHEKs which determines the number of viable cells in culture by quantifying the amount of ATP present. Both assays showed similar results – overexpression of both $CypB_{WT}$ and $CypB_{W128A}$ significantly expressed keratinocyte proliferation compared to normal control. In these experiments overexpression of $CypB_{W128A}$ was seen to influence keratinocyte proliferation to a greater extent compared to $CypB_{WT}$ (although this was only statistically significant in the cell growth and proliferation assay performed in NHEK cells). In addition, overexpression of both $CypB_{WT}$ and $CypB_{W128A}$ increased the number of colonies formed in NHEK culture suggesting that CypB regulates keratinocyte stem cell proliferation and that this effect is independent of the CsA binding site. In our colony forming experiments, overexpression of wild type CypB was seen to influence colony formation above that of $CypB_{W128A}$ overall. Secretion of CypB increases over time. If there is a depletion of intracellular CypB and the amount of extracellular CypB available is low (as would most likely be the case in colony forming assays as there are much fewer cells), there may be less $CypB_{W128A}$ overall compared to $CypB_{WT}$ which may explain why less colonies were seen to form in those keratinocytes overexpressing $CypB_{W128A}$ than in those overexpressing $CypB_{WT}$. Certainly, extracellular CypB does play a significant role in regulating NHEK proliferation as shown by our conditioned media experiments which

revealed that both extracellular $CypB_{W128A}$ and $CypB_{WT}$ influenced keratinocyte growth and proliferation compared to normal control. Here again, cells treated with conditioned medium derived from cells overexpressing $CypB_{WT}$ (containing $CypB_{WT}$ in the medium) significantly influenced cell growth and proliferation of NHEKs) whereas cells treated with conditioned medium derived from $CypB_{W128A}$ expressing cells showed less cell growth. These observations overall suggest that the W128A mutation does not modulate the ability of CypB to regulate keratinocyte proliferation per se but rather that the CsA binding site is important in regulating distribution and secretion of CypB and may affect the availability of CypB both intracellularly and extracellularly.

With regard to the colony forming assays, we observed that in one donor, which was able to produce the largest number of colonies and therefore cells overall, we were able to identify the different types of colonies produced and found that holoclones were the largest type of colonies formed by NHEKs overexpressing $CypB_{WT}$ and $CypB_{W128A}$. This supports our observations that CypB influences keratinocyte growth and proliferation as the holoclones represent proliferating stem cells (although this was an observation in one donor in the NHEK cells, we also observed this in preliminary HaCaT cell data, results not shown). Furthermore, our skin equivalent experiments showed that organotypic cultures formed from NHEKs overexpressing $CypB_{WT}$ formed a thicker epidermis compared to the normal control, again supporting our hypothesis that CypB influences keratinocyte growth and proliferation such as seen in conditions such as psoriasis, where increased epidermal proliferation occurs resulting in epidermal hyperplasia (thickening). Although our conditioned media studies provide persuasive data supporting that extracellular CypB plays an important role in keratinocyte cell growth and proliferation, due to lack of time we were unable to further validate this role. Specifically, in future studies, we will aim to confirm and validate these observations by performing immune-depletion studies and looking at the effects of blocking CypB binding via cell surface CypB binding sites (Type I and II binding with CsA and protamine, respectively). Carpentier et al have shown that the $CypB_{W128A}$ mutation interferes with type 1 binding (a 7-fold higher concentration of $CypB_{W128A}$ was required to displace 50% of radiolabeled ligand [125I]-CypB bound to the type I sites but that

CypB$_{W128A}$ was as efficient as CypB$_{WT}$ in inhibiting the binding of [^{125}I]-CypB to the type II binding sites) (Carpentier et al., 1999). T-cell adhesion to CypB occurs via complex mechanisms. Type II binding, via the N-terminal GAG clusters of CypB, is required for T cells to bind to CypB. However, when T-cell adhesion to the ECM component fibronectin is required, type I binding, type II binding and the PPIase site of CypB are necessary in order for biological activity to occur (Carpentier et al., 2002). Similar complex mechanisms may be involved in the role CypB plays in keratinocytes but the relative contribution of type I and type II binding sites in mediating the effects of extracellular CypB in keratinocytes requires further delineation and study.

Type II binding sites for CypB have been shown to be via GAGs, namely heparin sulphate proteoglycans (HSPG). Many biological functions mediated through cell surface proteoglycans are dependent on the interaction with extracellular mediators through their heparin sulphate moieties and the participation of their core proteins in signalling events (Allain et al., 2002). The syndecans are cell-surface HSPGs. Syndecan-1 has been shown to be associated with CD147 in CypB induced p44/p42 MAPK activation and consequent migration and adhesion of T-cells (Allain et al., 2002). Our results have shown that extracellular CypB$_{WT}$ significantly increased keratinocyte growth and proliferation but that there was also a trend towards positive regulation of keratinocyte growth and proliferation by extracellular CypB$_{W128A}$. These results indicate that Type I binding is probably not involved in keratinocyte proliferation.

In keratinocytes, GAGs have been shown to be synthesised and expressed at the level of the cell surface by proliferating human keratinocytes in culture (K. W. Brown and Parkinson, 1983; Roberts and Jenner, 1983). GAGs including the HSPGs have been shown to modulate the fate of several signalling molecules (growth factors and cytokines) implicated in epidermal homeostasis and repair (Ojeh et al., 2008). Syndecan-1 is strongly expressed in the suprabasal and differentiating layers with lower levels in the basal proliferating cells. However, its expression is upregulated in the wound edge keratinocytes upon wounding and has been shown to be an important modulator in mice of

epidermal cell proliferation with overexpression of syndcan-1 in basal keratinocytes promoting keratinocyte proliferation. (Elenius *et al.*, 2004; Bartlett *et al.*, 2007; Ojeh *et al.*, 2008). Syndecan-1 expression has been shown to be expressed in full thickness normal skin with the strongest reaction observed in the spinous and granular layer, whereas in psoriatic skin the strongest expression occurred in the basal and suprabasal layers and diminished through the spinous layer (Tomas et al., 2008). CypB may thus exert some of its biological functions in keratinocyte proliferation via type II binding or via another mechanism which may explain why we do see proliferation occurring in keratinocytes overexpressing both $CypB_{WT}$ and the $CypB_{W128A}$ mutation. However, we have no direct evidence for type II binding of CypB in keratinocytes, although further experiments blocking type II binding with Protamine or using CypB mutants corresponding to the protein(s) modified within its N-terminal that interacts with heparin and the type II binding sites (Carpentier et al., 1999), could be used to establish whether type II binding occurs in keratinocytes. Observing whether Syndecan-1 expression is present in lesional skin of patients with atopic dermatitis and whether there are any changes in its expression during treatment of CsA would be of interest.

Proliferation of cultured keratinocytes from psoriatic plaques has been shown to be inhibited by CsA at therapeutically relevant levels (Fisher et al., 1988). In addition, proliferation of cultured keratinocytes inhibited by CsA has been shown to correlate with blockade of the keratinocyte cell cycle at the G0/G1 phases (Karashima et al., 1996). Our results also confirmed that NHEKs treated with CsA at therapeutically relevant levels did not proliferate and in addition the lack of growth was confirmed not to be due to cell death. The action of CsA on keratinocytes is to block keratinocyte proliferation but a mutation in the CsA binding site does not inhibit keratinocyte proliferation. This may be because the actual CsA-CypB complex inactivates CypB but the mutation in the CsA binding site does not and the protein is still able to interact with other proteins. Further experiments to determine the effect of CsA on keratinocytes overexpressing $CypB_{WT}$ and $CypB_{W128A}$ may help to clarify these observations.

We used two different techniques to study the effect of CypB on transglutaminase activity and thus keratinocyte differentiation. Both confirmed that overexpression of CypB increased transglutaminase activity, with CypB $_{W128A}$ showing a greater trend than wild type CypB in influencing keratinocyte differentiation. This supports our observations that secreted CypB plays an important role in cellular activities but that the mutation in the CsA binding site does not inhibit keratinocyte differentiation. This may be due to the fact that a mutation in the CsA binding site increases secretion of CypB and allows for more intracellular bioavailability of CypB as well as an increase in extracellular CypB. Due to the complexities of these experiments, where NHEKs were subjected to retroviral transduction using spin transduction and then transfected for transglutaminase luciferase promoter activity assay or the addition of cadaverine post transduction and timing of experiments, the addition of CsA further complicated the assays. Due to lack of time we were unable to further pursue alternative methods for assessing differentiation, but the effects of CsA on keratinocyte differentiation and the effects of CypB on calcium influx and differentiation would be important areas to investigate. A rise in intracellular Ca^{2+} is a critical trigger for keratinocytes to switch from proliferation to differentiation. Yurchenko et al have also shown that CypB induced neutrophil chemotaxis and Ca^{2+} flux in CD147 expressing CHO cells (Yurchenko et al., 2001). As we have found CypB to positively regulate keratinocyte differentiation it maybe that it is involved in raising intracellular calcium levels via CAML or releasing Ca^{2+} from the ER, activating NFAT or interacting with CD147. It is possible that a naturally occurring homologue to CsA may bind to CypB, such as CAML, in order to regulate its activity.

Another possible mechanism of action by which CypB influences keratinocyte proliferation and differentiation might be via the calcineurin-NFAT pathway as evidence that CsA inhibits the expression of keratinocyte terminal differentiation markers and inhibits calcineurin and NFAT1 nuclear translocation has been shown in nude and depilated normal mice (Gafter-Gvili et al., 2003). In these mice, it was shown that the expression of p21$^{waf/cip1}$ and p27^{kip1}, two cyclin dependent kinase inhibitors usually induced with differentiation were blocked by CsA treatment (Gafter-Gvili et al., 2003). Santini et al showed that calcineurin

regulated the expression of differentiation markers and the activity of p21^{waf1} in cultured mouse keratinocytes in a pathway involving the interaction of NFAT1/NFAT2 and the Sp1/Sp3 transcription factors (Santini et al. 2001). Al-Daraji et al have confirmed that calcineurin/NFAT1 translocation to the nucleus in response to differentiation stimuli in normal human keratinocytes was inhibited by CsA (Al-Daraji et al, 2002). However, the CsA binding site is important in its interaction with calcineurin and the CypB$_{W128A}$ mutant does not significantly interact with calcineurin (Carpentier et al., 2000). In addition, Carpentier et al have found that in the absence of CsA there is no significant interaction between CypB and calcineurin (unpublished results (Carpentier et al., 2000)). In view of this, it seems unlikely that CypB$_{WT}$ and CypB$_{W128A}$ are exerting their effects on keratinocyte growth and differentiation via the calcineurin and suggests that CypB is promoting keratinocyte differentiation via an alternative pathway to the calcineurin-NFAT pathway.

If CypB does regulate keratinocyte proliferation and differentiation it must have some influence on transcription of genes involved in cell growth, proliferation and differentiation. Secreted factors and cell surface receptors can be internalised by endocytosis and translocated to the cytoplasm and nucleus (Planque, 2006) and we have confirmed that CypB is secreted by keratinocytes. By immunofluorescence and confocal studies, we found CypB was present in the nucleus of cultured NHEKs and in the nucleus of keratinocytes in skin biopsies of patients with atopic eczema treated with CsA. Overall, nuclear CypB was observed more in the basal and suprabasal layer compared to the differentiating layer in normal skin and atopic eczema skin, but this was not quantified. In breast cells, CypB has been shown to enhance prolactin (PRL) nuclear retrotranslocation: the PRL receptor is associated with ligand internalisation and the PRL/CypB interacts directly with STAT5 and thus potentiating PRL driven proliferation (Rycyzyn et al., 2000). Removal of the NLS in the N-terminus of CypB abrogated the potentiation of PRL driven proliferation and nuclear retrotransport. However, removal of the PPIase activity of CypB affected potentiation of PRL-driven proliferation but not nuclear retrotransport (Rycyzyn et al., 2000). STAT5a has been implicated to be involved in involucrin induction via PPARγ. The PPARs (proliferator-activated

receptors) bind to exogenous or endogenous ligands, form heterodimers with the retinoid X receptor (RXR), recruit a coactivator and facilitate transcription of target genes involved in many cellular functions including differentiation. All the PPAR members have been identified in keratinocytes and shown to play a physiological role in keratinocyte proliferation and differentiation (Dai et al., 2007). As CypB is found in the nucleus keratinocytes (Fearon et al., 2011) it may be involved in transcription of genes involved in cell growth and proliferation which then alters as keratinocytes differentiate.

As previously described, in healthy epidermis, keratinocytes undergo a carefully orchestrated programme of proliferation in the basal layer and differentiation in the suprabasal layers. However, once exposed to injury they are quickly activated to repair damaged tissue. The most common initiators are IL-1α and IL-1β. Once the keratinocyte is activated, further proinflammatory cytokines are released such as TNF-α, EGFR and ICAM-1 which in turn activate the MAPK/ERK signalling cascade pathway (Freedberg et al., 2001). CypB contributes to leukocyte infiltration during inflammation, triggers chemotaxis and integrin activation in T-cells and is released intact by heparanase and MMPs, all of which suggest it participates in tissue inflammation (Marcant et al., 2012). CypB has been shown to activate ERK signalling through CD147, together with increased ERK phosphorylation (Yurchenko *et al.*, 2001; K. Kim *et al.*, 2012). CypB is therefore a candidate as an additional pro-inflammatory cytokine, particularly as it is actively secreted and it would be interesting to investigate its potential role in wound healing.

During the early stages of CsA treatment in patients with atopic eczema, we found that the level and pattern of CypB expression within epidermis changed and this varied between patients. Individual variability to the immunosuppressive effects of CsA is well established (Klintmalm *et al.*, 1985; Sander *et al.*, 1986). Consistent with this, although all of our patients showed clinical improvement after 2 weeks of CsA therapy, we observed a wide range in % improvement in SASSAD scores. Denys et al showed that although there is individual variability in the clinical response to CsA, CypB potentiated the activity of CsA in blood mononuclear cells and that extracellular CypB modified

CsA distribution between plasma, erythrocytes and lymphocytes in a dose dependent manner by retaining the CypB-CsA complex extracellularly as well as promoting its accumulation intracellularly. Thus, the changes in CypB expression seen in our patients, may be related to individual variation but also due to dynamic changes occurring extra- and intracellularly.

Finally, we showed that FLG expression increased in 10 out of the 12 patients with atopic eczema after two weeks of CsA treatment and notably there was a significant positive correlation between the increase in FLG expression and clinical improvement following 2 weeks of therapy with CsA, suggesting that CsA may play a direct role in mediating barrier repair.

From our observations, it is not clear how CypB in atopic eczema skin treated with CsA is modulated by CsA therapy. Seven out of nine patients showed a reduction in CypB expression in eczematous skin at baseline (prior to CsA treatment) compared to normal controls. In five of these patients, an increase in CypB expression was seen following 2 weeks of CsA treatment, but not to above that of the normal control. The remaining 4 patients showed a decrease in CypB expression following CsA treatment despite all patients showing improvement in their eczema as indicated by the reduction in SASSAD scores. The presence of CypB in normal epidermis and its increased expression in the granular layer, changes seen in CypB expression in eczematous skin compared to normal skin and changes seen in CypB expression before and after CsA treatment all warrant further investigation. Our patient numbers are unfortunately small, but do illustrate that the expression of CypB is altered in atopic dermatitis and changes during treatment with CsA. Extracellular levels of CypB have correlated with an increased patient response to immunosuppression (Denys *et al.*, 1998b; Fearon *et al.*, 2011). In addition, the availability of CypB in the ECM may allow it to associate with its receptors, internalise and be directed to its intracellular functions. Secreted molecules such as growth factor and protein hormones, have been known to function by acting at the cell surface and then activating a cascade of intracellular second messengers. Recent evidence shows that the mode of action may be more complicated and that internalisation in endosomal vesicles may contribute to

ensuring they persist intracellularly in order to fulfil their biological role. Examples include fibroblast growth factors (FGF), EGF and PRL, with PRL requiring CypB to enhance its effects (Rycyzyn *et al.*, 2000; Tran *et al.*, 2003; Planque, 2006). Studying more patients and more time points correlated with clinical severity would be important to establish the effect of CsA on CypB in keratinocytes in vivo.

An interesting finding was that FLG gene expression changed in eczematous skin during CsA treatment. Since the discovery that mutations in the FLG gene contribute to atopic eczema (Palmer et al., 2006), the concept that an epidermal barrier defect plays a primary role in the development of inflammatory skin disease has strengthened. The intragenic copy number of FLG repeats is also known to be important: the greater the FLG copy number the less likely an individual is at risk of developing atopic eczema (S. J. Brown *et al.*, 2012). FLG expression has been shown to be downregulated in all cases of moderate-severe atopic eczema even in the absence of a FLG mutation (Kezic et al., 2011), probably as a result of inflammatory cytokines (Howell, Kim et al. 2007). Atopic eczema is characterised by the overexpression of T_H2 cytokines – IL-4 and IL-13, which are known to induce atopic disease and downregulate genes involved in the innate immune response (Howell et al., 2007). Howell et al showed that patients with atopic eczema acquire the FLG deficiency and subsequent barrier disruption as the result of the local inflammatory immune response which contributes to decreased FLG expression in patients with wild-type as well as null mutations in the FLG gene (Howell et al., 2007). They also showed that FLG staining in lesional and uninvolved skin from patients with atopic eczema was less intense than in skin from normal healthy subjects. FLG staining was also greater in uninvolved atopic eczema skin compared with lesional atopic eczema skin from the same subjects, suggesting that FLG mutations do not account for the decrease in filaggrin expression.

Approximately 8.8% of the general population carry one or more FLG null alleles, whereas 42% of patients with severe atopic eczema attending dermatology departments carry one or more of these FLG alleles (Barker et al., 2007). It would be important to establish the FLG mutation status of our

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patients in order to further understand the changes in FLG expression seen in eczematous skin of our patients as our results revealed that eleven out of twelve patients with atopic eczema showed a decrease in FLG expression in eczematous skin compared to the normal skin control prior to treatment with CsA. Following CsA treatment, ten of the patients with a reduction in FLG expression in eczematous skin showed an increase in FLG expression and of these, seven patients showed an increase in FLG expression to above the intensity seen in the normal control. These changes occurred early on during treatment, and in all patients an improvement in their eczema was seen as shown by their SASSAD score. Furthermore, we observed a positive correlation between disease activity and FLG expression where improvement in eczema severity correlated to an increase in FLG expression. Brown et al have shown that the copy number variation (CNV) within FLG significantly affects atopic eczema risk and this is independent of FLG null mutation although the overall effect size is smaller. The risk of atopic eczema decreases as the number of FLG repeats increases and a 5-10% increase in FLG 'dose' resulted in a significant reduction in the incidence of atopic eczema (S. J. Brown *et al.*, 2012). If CNV within FLG significantly affects atopic eczema, Brown et al suggest that treatments increasing FLG expression may have a therapeutic effect on prevention and/or treatment of atopic eczema (S. J. Brown *et al.*, 2012).

Our findings suggest that CsA does exert a direct effect on the epidermis, influencing FLG expression and thus potentially contributing to epidermal barrier repair. However, whether this effect is mediated through direct interaction with CypB, keratinocytes or through downregulation of inflammatory cytokines remains to be determined.   Human DNA microarrays used to identify gene expression profiles in skin biopsies taken from lesional atopic eczema skin revealed up-regulation of immune and inflammatory response genes, including cytokines, chemokines and cell surface antigens along with T-cells, dendritic cells and the complement pathway. Down-regulation of gene encoding enzymes involved in lipid homeostasis was also seen. In addition, transglutaminases were enhanced in atopic eczema skin (Saaf et al., 2008). Changes in gene expression in lesional atopic eczema skin have also been shown following

treatment. Jensen et al studied gene expression profiles in skin biopsies of patients with atopic eczema subjected to a within patient trial comparing topical 1% pimecrolimus cream on one upper limb and 0.1% betamethasone valerate on the other twice daily for 3 weeks. Both treatment regimens showed down regulation of genes involved in inflammation and the immune response which was more pronounced after treatment with betamethasone valerate than with pimecrolimus. Pimecrolimus specific effects were seen to be involved in cell proliferation and cell homeostasis. Both treatment modalities normalised the expression of FLG and loricrin (Jensen et al., 2012). Vahavihu et al showed that following narrowband UVB treatment a reduction in the initially increased expression of antimicrobial peptide human β-defensin, which acts as a proinflammatory mediator or alarmin in the skin, linking the adaptive and innate immune response, was seen in healing AD lesions (Vahavihu et al., 2010). Jensen et al have also shown that immunohistological staining of FLG in lesional skin of AE shows gaps that normalise during treatment with both pimecrolimus and betamethasone (Jensen et al., 2009). Our data also revealed patchy staining of FLG in lesional atopic eczema skin, which improved during treatment.

There is some overlap between the list of inflammatory conditions where there is an increase in cyclophilins and those conditions in which there is upregulation of CD147. These include lupus and rheumatoid arthritis (Yurchenko, Constant et al. 2010). Interestingly, in our experiments, changes in CypB expression and in CD147 expression were detected in the skin biopsies of patients with atopic eczema. Interestingly, the distribution of CD147 was seen to change following CsA treatment in eczematous skin. The changes were not consistent, as in one patient a reduction of CD147 was seen throughout the epidermis of atopic eczema in both pre and post CsA sections. In another two patients, the distribution of CD147 changed from increased expression in the basal layer, pre CsA treatment, to a greater expression in the differentiating layers during treatment. In another, the intensity of CD147 increases throughout the epidermis after two weeks of treatment and in a further patient the intensity of CD147 expression increases in the basal layer of the epidermis, during treatment. This may suggest that changes in expression of CD147 occur

depending on the extent of inflammation or in response to CsA therapy or represent the dynamic changes that may be occurring with regards to CypB secretion and uptake between proliferating and differentiating keratinoctyes. Further work in more patients would be required to further clarify these observations but the changes in CypB expression in eczematous skin following CsA treatment further suggests that CsA exerts a direct effect in the epidermis.

Two other areas of interest of CypB expression and function are in psoriasis and also in skin cancers. CypB positively influences keratinocyte growth and overproliferation of keratinocytes is a hallmark of psoriasis. Syndecan-1 is expressed throughout the basal and suprabasal layers of the epidermis in psoriasis which was felt to be associated with development of psoriasis (Tomas et al., 2008). Interestingly, a high expression of CD147 is seen in squamous cell carcinomas. A trend was observed in advanced SCCs expressing CD147 and reduced survival (Sweeny et al., 2012). This is highly interesting, not only because patients on CsA are at increased risk of SCC but also HPV infection, which in itself may be associated with non-melanoma skin cancer (Lajer and von Buchwald 2010). Certainly immunosuppressed patients with SCC are more likely to have HPV associated SCC than in immunocompetent groups (Harwood et al., 2000). With CypB playing a role in HCV and HIV viral replication, the role of CypB in HPV may be relevant. Recently, CypB has been shown to have an essential function in protecting hepatoma cells against ROS mediated apoptosis through binding to CD147 and regulating the ERK pathway. This protective effect is dependent on its PPIase activity (K. Kim *et al.*, 2012).

For a small protein, CypB has numerous functions and has been shown to be involved in a number of important intracellular and extracellular activities in a number of different cell types. In summary, these studies indicate that CypB stimulates keratinocyte differentiation consistent with its expression pattern but also significantly promotes proliferation through autocrine and/or paracrine effects and may be important in regulating epidermal homeostasis. Furthermore, we found that in eczematous skin treated with CsA, changes in expression of CypB and CD147 occur which need to be further explored. Moreover, the early changes in expression in FLG in eczematous skin during

treatment suggest that CsA acts by positively repairing impaired barrier function and further work is required to investigate this hypothesis.

These data suggest that CypB may have an autocrine or paracrine role in keratinocytes and that CsA may exert effects in inflammatory skin disease, at least in part, by regulating CypB. In addition to exploring if CypB works synergistically with other cytokines, growth factors or hormones in keratinocyte growth, proliferation and differentiation, further work investigating receptor binding/interactions with CypB and its signalling pathways and the role of PPIase activity in keratinocytes would also be important to shed further light on our findings. CypB could be a potential therapeutic target and further investigation into its functions in the skin is essential.

FINAL REFLECTION

The valued review of this data by the examiners has further helped to clarify some of the observations described above.

Review of the data regarding CypB secretion from keratinocytes transduced with wild type and mutant CypB compared to empty vector control, highlighted that more work in this area could help to validate our observations. Identification of CypB secretion from these cells was performed using western blot. Quantification of the amount of protein present can also be performed using western blot however, as there are no established secreted control proteins, this is difficult to perform. As CypB may be acting as a cytokine and/or synergistically with other proteins that are not yet identified, techniques to identify bound CypB would be important. As stated earlier, techniques such as ELISA would allow quantification of the amount of CypB present in the samples. Furthermore, using FACS to count the number of cells in a sample would be helpful to accurately identify how much protein is being produced by a known number of cells.

It is important to document information on the patient groups investigated. Providing details of the demographics of the patient groups from whom skin biopsies were taken for CypB, FLG and CD147 protein expression before and after CsA treatment, and also of the keratinocyte donors adds further information to the work, as does information regarding the site of biopsy and any co-morbidities. This allows clinicians to know whether study results are relevant to the patient populations they manage. The majority of our patients were between the ages of 25 – 45 and some had other atopic disease associated. Patients who have been referred as adults with atopic eczema to secondary and tertiary care often have a more severe, chronic eczema persisting from childhood into adult life. However there continues to be limited treatment and support available to patients (Skin, 2013).   Further work to establish each patient's FLG status (whether patients have an inherited FLG mutation, the number of FLG repeats they may have or whether they have acquired a FLG deficiency) would also be an important observation to establish

due to the association between reduced FLG protein and increased risk of atopic eczema and other atopic and allergic disease and to understand further the impact of treatment on FLG protein expression.

Re-examination and re-analysis of data is an important part of reassessing observations that have been found. Therefore, re-evaluation of the immunohistochemistry data and normalising FLG expression pre and post CsA to the Toto nuclear stain control was important to confirm the findings shown. The main reason to undertake this review was to reduce any errors introduced by pipetting technique and the observation that some of the images with the brightest protein expression also had the brightest toto-3 expression. In the skin biopsies identifying CypB localisation via immunohistochemistry, this was seen in one patient pre CsA treatment (patient 5) and one patient post CsA (patient 7). In the skin biopsies identifying FLG localisation this was seen in three patients (patients 5,7 and 9) post CsA treatment. In the skin biopsy identifying CD147 localisation, this was seen in one patient (patient 7) post CsA. Due to time restrictions, re-analysis was performed on the FLG samples only.

In the original analysis, the parameters set for detecting the signal were established using the normal control and the images subsequently captured for each sample pre and post CsA treatment were therefore controlled to the normal sample. Thus any brightness in signal was detected using the original parameters for detecting the signals on the normal control. Re-analysis was performed using Toto-3 nuclear stain as an internal control. Re-analysis showed similar results to the original, in that the majority of patients treated with CsA (11 out of 12) demonstrated an increase in FLG levels in the stratum granulosum following 2 weeks of CsA therapy. Patient 6 showed an increase in FLG expression following the re-analysis, whereas a decrease following CsA treatment was seen in the original analysis.

Using the normal skin control for comparison of the intensity of protein is an appropriate way of assessing changes in the protein levels seen in the skin. The sample of patients is small and further work looking at expression of FLG

before and after CsA treatment in more patients would help to consolidate our findings.

Whilst undertaking this research, there has been an expanse of knowledge regarding both CypB and FLG that has come to attention in the literature. Both have been shown to have important functions. Having investigated these proteins during this time has been both enjoyable and exciting. Further work to publish the data in this thesis will add to this knowledge.

I wish to extend my thanks to my examiners for their time and review of this work.

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