Investigating the Role of iASPP in Skin Homeostasis and Tumourigenesis

Ho Ki Kathryn Chung
St. John's College
University of Oxford

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Abstract

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Candidate: Ho Ki Kathryn Chung
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The epidermis of skin is an essential barrier that protects the body from external stresses and retains internal body fluids. The fine balance between cell proliferation, differentiation and apoptosis as well as a well-coordinated immune response is vital in maintaining a healthy skin barrier. The iASPP protein is inhibitor of p53-mediated apoptosis and can potentially function as a proto-oncogene in tumourigenesis. Recent research has also demonstrated the regulatory role of iASPP on the transcriptional activity of p63 in mediating proper epidermal stratification. Furthermore, early data has suggested that iASPP could inhibit the DNA binding activity of the NF-κB transcription factor p65 which is critical in controlling inflammatory gene expression. The aim of this project was to investigate the autonomous role of epidermal iASPP in epidermal development and homeostasis using an *in vivo* transgenic mouse model system. Moreover, the role of epidermal iASPP in chemically induced skin carcinogenesis, and the possible interaction between iASPP and p65 in keratinocytes were examined.

Transgenic mice with epidermal specific iASPP deletion (K14-iASPP-/-) exhibited wavy coat and open eyelid phenotype similar to that observed in the total knockout model. K14-iASPP-/- mice showed focal epidermal thickenings, with signs of immune cell infiltrates in the dermis. Interestingly K14-iASPP-deficient mice were more susceptible to DMBA/TPA induced skin carcinogenesis, and had significantly higher papilloma burden with early onset of papilloma development compared to the wild type. Primary keratinocytes expressed higher levels of inflammatory mediators in the absence of iASPP, and abnormal expression of such mediators was detected in the K14-iASPP-/- mouse epidermis.

These results suggest that epidermal iASPP deficiency provided an inflammatory microenvironment that supports the development of papillomas. Therefore epidermal iASPP plays a key role in maintaining normal skin immunohomeostasis and offering protection against chemically induced skin carcinogenesis. Further research is required to decipher the molecular mechanisms by which iASPP modulates inflammatory signalling pathways.

Declaration

This thesis is submitted to the University of Oxford in support of my application for the degree of Doctor of Philosophy in Clinical Medicine. It has been composed by myself and has not been submitted in any previous application for any degree. I have carried out all of the work presented in this thesis, except for the RNA sequencing experiment which was performed in collaboration with Dr. Jo Zhou at the Radboud University in the Netherlands.

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Table of Contents

Abbreviations

4OHT	4-hydroxytamoxifen
AMP	Anti-microbial peptide
ANK	Ankyrin
AP-1	Activator Protein-1
APC	Antigen presenting cell
APS	Ammonium persulphate
ASPP	Ankyrin repeat, SH3 domain, and proline-rich containing protein
BAX	Bcl-2-associated X protein
BCC	Basal cell carcinoma
Bcl-2	B-cell lymphoma 2
BCR	B cell receptor
BM	Basement membrane
BrdU	5-bromo-2-deoxyuridine
BSA	Bovine serum albumin
CBP	CREB-binding protein
C/EBP	CCAAT/enhancer binding protein
CXCL	Chemokine (C-X-C motif) ligand
DAB	3, 3'- diaminobenzidine
DAMP	Danger-associated molecular pattern
DBD	DNA binding domain
DC	Dendritic cells
DETC	Dendritic epidermal T cells
DMBA	*7, 12*-Dimethylbenzanthracene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DP	Dermal papillae
EDTA	Ethylenediaminetetraacetic acid
EPB	Epidermal permeability barrier assay
ERK	Extracellular signal-regulated protein kinase
EtBr	Ethidium Bromide

FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FcR	Fragment crystallisable receptor
GRR	Glycine rich region
H&E	Haematoxylin and Eosin
HF	Hair follicle
iASPP	Inhibitor of apoptosis-stimulating protein of p53
iASPP CreER	iASPP*loxP*/*loxP* Cre+ERT
IBD	Inflammatory bowel disease
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IF	Immunofluorescence
IFE	Interfollicular epidermis
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRS	Inner root sheath
JNK	c-Jun N-terminal kinase
K14	Keratin 14
KGF	Keratinocyte growth factor
LC	Langerhans cell
LPS	Lipopolysaccharide
MAPK	Mitogen associated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MEF	Mouse embryonic fibroblast
MHC	Major histocompatibility complex
MK	MAPK-activated protein kinase
MPO	Myeloperoxidase
MSK	Mitogen- and stress-activated kinase
NDS	Normal donkey serum

NEMO	NF-κB essential modulator
NGS	Normal goat serum
NF-κB	Nuclear factor-κB
NK	Natural killer cell
NMSC	Non-melanoma skin carcinoma
OCHRe	Oxford Centre for Histopathology Research
OD	Oligomerisation domain
ORS	Outer root sheath
pAb	Polyclonal antibody
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PIG3	p53-induced gene 3
PKA	Protein kinase A
PRR	Pattern recognition receptor
PUMA	p53 upregulated modulator of apoptosis
RA	Rheumatoid arthritis
RaDAR	RanGDP/ankyrin repeat
RAI	RelA-associated inhibitor
RFLP	Restriction fragment length polymorphism
RHD	Rel-homology domain
RSK	Ribosomal-subunit kinase
qPCR	Quantitative PCR
SAM	Sterile α-motif
SCC	Squamous cell carcinoma
SDS-PAGE	SDS- Polyacrylamide gel electrophoresis
SEM	Standard error of mean
TA	Transactivation domain
TAE	Tris-acetate EDTA
TAM	Tumour-associated macrophage
TBE	Tris-borate EDTA

TBS-T	Tris buffered saline tween
TCR	T cell receptor
TGF-β	Tumour growth factor β
T_H	T helper cell
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TID	Transcription inhibition domain
TLR	Toll-like receptor
TPA	*12-O*-tetradecanoyl-13-phorbol acetate
TSS	Transcription start site
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WB	Western blot

List of Figures

List of Tables

Chapter 1 Introduction

1.1 Inflammation and Cancer

Progress in cancer research has shed light on the intrinsic capabilities that malignant cells acquire during multistage cancer development. Such distinctive fundamental properties of cancer include: self-sufficiency in growth signals, insensitivity to anti-proliferative cues, evasion of apoptosis, unlimited replicative potential, sustained angiogenesis and metastasis (Hanahan *et al*., 2011). In addition, an inflammatory tumour microenvironment is increasingly recognised as an important component in carcinogenesis, and has been referred to as the "seventh hallmark of cancer" (Vesely *et al*., 2011). Cancer-related inflammation can be attributed to the intrinsic properties of transformed cells promoting pro-inflammatory signalling due to genetic alterations, or extrinsic inflammation caused by environmental factors, inflammatory diseases and tissue responses towards abnormal cells (Colotta *et al*., 2009; Trinchieri, 2012).

The conceptual link between inflammation and cancer dates back to the 1860s, when Virchow proposed that cancers originate from sites of chronic inflammation (Coussens *et al*., 2002; Aggarwal *et al*., 2009). Various epidemiological and clinical studies have since provided evidence illustrating their close interrelationship. Known environmental factors increasing the risk of tumourigenesis, such as obesity and smoking, have been associated with persistent

inflammation (Aggarwal *et al*., 2009; Grivennikov *et al*., 2010; Trinchieri, 2012). Inflammation associated with chronic infection may also contribute to cancer development. *Helicobacter pylori* bacterial infection of the gastrointestinal tract increases the risk of gastric carcinogenesis, while hepatitis B and C viral infection predisposes to liver cancer development (Uemura *et al*., 2001; Bosch *et al*., 2004). Epidemiological data has also indicated a link between certain chronic inflammatory diseases and an increased susceptibility to neoplasms. For example patients with rheumatoid arthritis (RA) are more likely to develop lymphoma and lung cancer, and those suffering from persistent inflammatory bowel disease (IBD) are at a higher risk of colorectal carcinoma (Smitten *et al*., 2008; Triantafillidis *et al*., 2009). Long-term intake of non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin reduces the incidence of colorectal cancer, and may possibly affect incidence of other solid cancers such as head and neck cancer, illustrating the role of inflammation in favouring tumourigenesis (Rothwell *et al*., 2012; Trinchieri, 2012).

1.1.1 Pro-tumour Immunity

The pro-tumourigenic effects of inflammation are thought to be facilitated by the promotion of genetic instability, angiogenesis, tumour cell growth/survival and metastasis. Reactive oxygen and nitrogen species produced by inflammatory cells such as macrophages and

neutrophils might induce DNA damage and generate mutations favouring tumour development. Such oxidative species and inflammatory cytokines could also inhibit DNA repair through the down-regulation of mismatch repair proteins, further enhancing genetic instability (Colotta *et al*., 2009; Grivennikov *et al*., 2010). Pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) could trigger the expression of hypoxia induced factor 1α (HIF-1α) in malignant cells. HIF-1α signalling and the hypoxic environment of solid tumours in turn induces the expression of vascular endothelial growth factor (VEGF) in cancer and immune cells, mediating angiogenesis in support of tumour growth (Grivennikov *et al*., 2010). Immune cells infiltrating the tumour mass release cytokines, growth factors and metalloproteinases to promote cell proliferation, survival and metastasis of malignant cells. For example, inflammatory signalling pathways could induce the expression of anti-apoptotic factors such as Bcl-2 to mediate tumour cell survival (Colotta *et al*., 2009). The epithelial-mesenchymal transition (EMT) of tumour epithelial cells is facilitated by the down-regulation of cell adhesion protein E-cadherin upon TNF-α signalling, and the action of tumour growth factor β (TGF-β) cytokine produced by cancer and immune cells. These processes, along with the action of metalloproteinases in modifying the extracellular matrix, enable neoplastic cells to penetrate epithelial linings and metastasise (Colotta *et al*., 2009; Grivennikov *et al*., 2010).

1.1.2 Anti-tumour Immunity

On the other hand, studies have indicated the anti-tumourigenic role of the immune system in hampering cancer development. Inflammatory cells are responsible for the suppression and elimination of infectious agents such as viruses and bacteria, some of which impose higher risk to developing malignancies with infectious aetiologies. This is supported by the higher rates of certain neoplasms observed in patients who have received solid organ transplants or who suffer with the autoimmune deficiency syndrome. These immunosuppressed patients show higher incidence rates in Hodgkin's lymphomas, liver cancer and stomach cancer which are associated with Epstein-Barr virus, hepatitis viruses and *Helicobacter pylori* respectively (Grulich *et al*., 2007; Vesely *et al*., 2011). Moreover, renal transplant recipients also showed increased prevalence of skin cancers such as squamous cell carcinoma (Hardie, 1995).

Another aspect of anti-tumour immunity is immunosurveillance for abnormal malignant cells. The expression of tumour antigens such as mutated peptides or overexpressed products could allow the immune system to differentiate abnormal cells from normal ones. Various immunodeficient mouse models such as B cell and T cell deficient Rag2-/- mice are prone to spontaneous intestinal or lung adenocarcinomas (Shankaran *et al*., 2001; Swann *et al*., 2007). A long-term follow-up study on healthy individuals in Japan showed that higher cytotoxic activity

of blood lymphocytes is associated with reduced general cancer risk (Imai *et al*., 2000). Research has further demonstrated that cancer cells can generate an immunosuppressive microenvironment by producing TGF-β to promote the generation of regulatory T cells and escape elimination by the immune system (Massagué, 2008; Mellman *et al*., 2011). Indeed the avoidance of immunosurveillance by malignant cells is also being referred to as an element of the "seventh hallmark of cancer" (Zitvogel *et al*., 2006). Continual research on cancer immunotherapy is underway in an attempt to establish and activate the endogenous immune response towards malignant cells, such as antibodies against the inhibitory receptor programmed death 1 (PD1) expressed on tumour-specific T cells to reactivate T cell response towards cancer cells (Mellman *et al*., 2011; Ribas, 2012).

1.2 Pathological Conditions of the Skin

A fine balance between keratinocyte proliferation, differentiation and apoptosis is vital to maintain a healthy skin barrier during tissue homeostasis and in response to trauma. Likewise, precise control of pro-inflammatory and anti-inflammatory signalling between keratinocytes and immune cells is also essential in terms of tissue immunohomeostasis. Disruption of these tightly regulated biological systems leads to the development of various skin diseases.

A recent analysis looking at the prevalence of skin diseases at a global level has demonstrated that skin disorders are amongst the most common diseases worldwide, occupying 4th place amongst all non-fatal diseases (Hay *et al*., 2013). Eight skin conditions including eczema and acne vulgaris were ranked amongst the top 50 most prevalent diseases, which illustrate the contribution of skin diseases towards the global health burden. While skin diseases have a negative impact on public health in terms of disfigurement and fatality, the long-term psychological distress that patients suffer due to de-valued body image should not be underestimated.

1.2.1 Eczema/Dermatitis

Eczema, also referred to as dermatitis, is an inflammatory skin condition that reflects a reaction towards exogenous or endogenous stimuli, many of which are still unknown. Affected skin displays redness, scaling and itchiness, and the current clinical classification is inconsistent (Gawkrodger *et al*., 2012).

'Atopic' eczema/dermatitis refers to individuals with a genetic predisposition to developing such allergies, with high immunoglobulin IgE levels circulating in their system. These individuals tend to have a defective innate response and significant adaptive response with

CD4+ T cell infiltration. Patients exhibit abnormal skin barrier function and reduced recruitment of innate immune cells such as neutrophils and dendritic cells (DC). Decreased AMP production and mutations in the toll-like receptor 2 (TLR2) can also be detected. The disruption of the skin's innate immunity contributes to the susceptibility of atopic eczema patients to infections and hypersensitivity. Moreover, a polarised T_H2 response with increased interleukin-4 (IL-4), IL-5 and IL-13 production in atopic eczema can influence the innate immune system. These T_H2 cytokines have been demonstrated to bring about the down-regulation of filaggrin and anti-microbial peptides (AMP) expression in keratinocytes for barrier function, and increased IgE production by B cells (De Benedetto *et al*., 2009; Werfel 2009).

1.2.2 Psoriasis

Psoriasis is a chronic inflammatory skin lesion characterised by demarcated erythematous plaques covered with silvery scales, and can be associated with the development of arthritis. Basal and suprabasal keratinocytes are hyper-proliferative in the diseased skin, and abnormal infiltration of neutrophils, macrophages and T cells can be observed (Gawkrodger *et al*., 2012).

Studies have indicated that various factors such as T cells, DCs and keratinocytes are involved in the pathogenesis of this autoimmune disease. Stressed keratinocytes signal dermal DCs via IL-1, IL-6 and TNF-α to stimulate the differentiation of naïve T cells into T helper (T_H) cellsT_H1 and T_H17. Keratinocyte-derived chemokines such as CCL20 and CXCL10 attract an influx of activated T_H1/T_H17 cells and result in the development of psoriatic plaques. The T cells in turn induce keratinocytes to produce AMPs like defensins and S100 proteins, along with chemokines like CXCL1/GRO-α and CXCL8 for neutrophil recruitment (Schön *et al*., 2005; Nestle *et al*., 2009). Unlike eczema patients, those with psoriatic plaques are not prone to skin infections, which could be due to the protection offered by increased AMP expression.

1.2.3 Non-Melanoma Skin Carcinoma

Non-melanoma skin carcinoma (NMSC) has become increasingly prevalent worldwide, and is the most common type of cancer within white populations (Lomas *et al*., 2012). The most common types of NMSC are basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). BCC refers to tumours that derive from basal keratinocytes, and is locally invasive but rarely metastasises. SCC is more likely to metastasise and is thought to arise from keratinocytes of the epidermis and hair follicle (HF), due to the usual features of squamous cell differentiation SCC retains (Bagheri *et al*., 2001).

Ultraviolet (UV) radiation exposure has been identified as a major epidemiological risk factor for skin cancer development. Mutations in the p53 tumour suppressor gene have been detected in roughly 50% of all skin cancers, mostly associated with UV-specific p53 mutations (Boukamp, 2005). While p53-deficient transgenic mice are prone to the spontaneous development of lymphomas and sarcomas, the animals do not often develop epithelial carcinomas (Donehower *et al*., 1992; Harvey *et al*., 1993). Moreover, p53-deficient mice in DMBA/TPA induced two stage skin carcinogenesis assays did not have a higher burden of benign papillomas. Instead, papillomas on p53-/- animals progressed to malignant SCC much more quickly, suggesting that p53 mutation might be important for malignant conversion, but not the initiation of tumour development (Kemp *et al*., 1993). Another gene mutation found in around 10-20% of NMSC cases is the Ras proto-oncogene (Owens *et al*., 2003; Boukamp, 2005; Pylayeva-Gupta *et al*., 2011). Ras activation in the epidermis has a pro-proliferative role and inhibits differentiation, but seems to require additional gene mutations for the development of malignancies (Dajee *et al*., 2002; Dajee *et al*., 2003).

1.2.4 Inflammation and Non-Melanoma Skin Carcinoma

Despite the various studies mentioned earlier that have demonstrated that certain chronic inflammatory diseases predispose individuals to neoplasia, such association in cutaneous

inflammatory diseases and skin carcinoma development has yet to be established. Early studies of chemically induced skin carcinogenesis on athymic and TNF-α-/- murine models suggested an essential role for immune components in promoting papilloma formation, as wild type mice were more susceptible to tumourigenesis (Gershwin *et al*., 1978; Moore *et al*., 1999). However, the epidemiological data currently available has yet to establish a conclusive link between eczema and NMSC (Schmitt *et al*., 2011). Similarly psoriasis did not confer an enhanced risk of developing NMSC, although significantly increased risk was observed in psoriatic patients that had received the psoralen plus UV light A (PUVA) treatment (Nijsten *et al*., 2003; Nickoloff *et al*., 2005; Brauchli *et al*., 2009). It has been speculated that the skin might not be regularly exposed to environmental and dietary carcinogens like the liver and stomach, which could account for the inconclusive link between inflammatory skin disease and NMSC (Grivennikov *et al*., 2010). The pro-tumourigenic effect of PUVA might be due to DNA damage and gene mutations in Ras and p53 induced by such treatment (Nataraj *et al*., 1996; Kreimer-Erlacher *et al*., 2007). Moreover, a modest association with increased risk of pancreatic cancer and lymphoma was detected in patients with psoriasis (Brauchli *et al*., 2009). The precise relationship between inflammation and the development of NMSC remains ambiguous. To better understand such interaction in the skin, it is essential to first

comprehend the biology of the skin as well as the major signalling pathways involved in dictating its development and homeostasis.

1.3 The Epidermis of Skin

The skin is one of the largest organs of the body and is made of three different layers: the epidermis, the dermis and the hypodermis. The outermost layer, the epidermis, is a stratified squamous epithelium that protects the organism against mechanical trauma and pathological insults from microorganisms present in the external environment. The epithelial layer is also responsible for retaining body fluids and preventing desiccation due to its relative impermeability to water.

1.3.1 Embryonic Epidermal Development

The mouse epidermis originates from a single layer of ectodermal cells specified to the epidermal lineage, on receiving signals from the dermal mesenchymal cells at around E8.5 during embryogenesis (Green *et al*, 2003; Koster *et al*., 2004; Koster *et al*., 2007; Blanpain *et al*., 2009). The stratification of mouse embryonic skin during embryogenesis begins at E12.5 when the ectodermal markers K8/K18 are replaced by epidermal K5/K14 expression. Epidermal stratification completes at E17.5 when the skin barrier is fully established *in utero*.

1.3.2 The Anatomy of the Epidermis

The epidermis is composed of four different layers, reflecting the different stages of epidermal differentiation (Fig 1.1). The basal layer (*stratum basale*) consists of proliferative keratinocytes adhering to a scaffold of extracellular matrix known as the basement membrane (BM) via hemidesmosome structures. The BM, which is composed of collagens, proteoglycans and adhesive glycoproteins supports the proliferative potential of basal cells and separates the epidermis from the dermis (Yurchenco, *et al*., 2011). Progenitors of the basal keratinocytes can stratify and differentiate upwards to give the suprabasal spinous layer (*stratum spinosum*). This is accompanied by a switch in cytokeratin expression from K5/K14 in basal cells to K1/K10 in spinous cells. The newly synthesized K1 and K10 cytokeratins are organised into intermediate filaments, and are anchored at intercellular junctions known as the desmosomes. These adjoin adjacent spinous keratinocytes to provide the mechanical strength required in the epidermis.

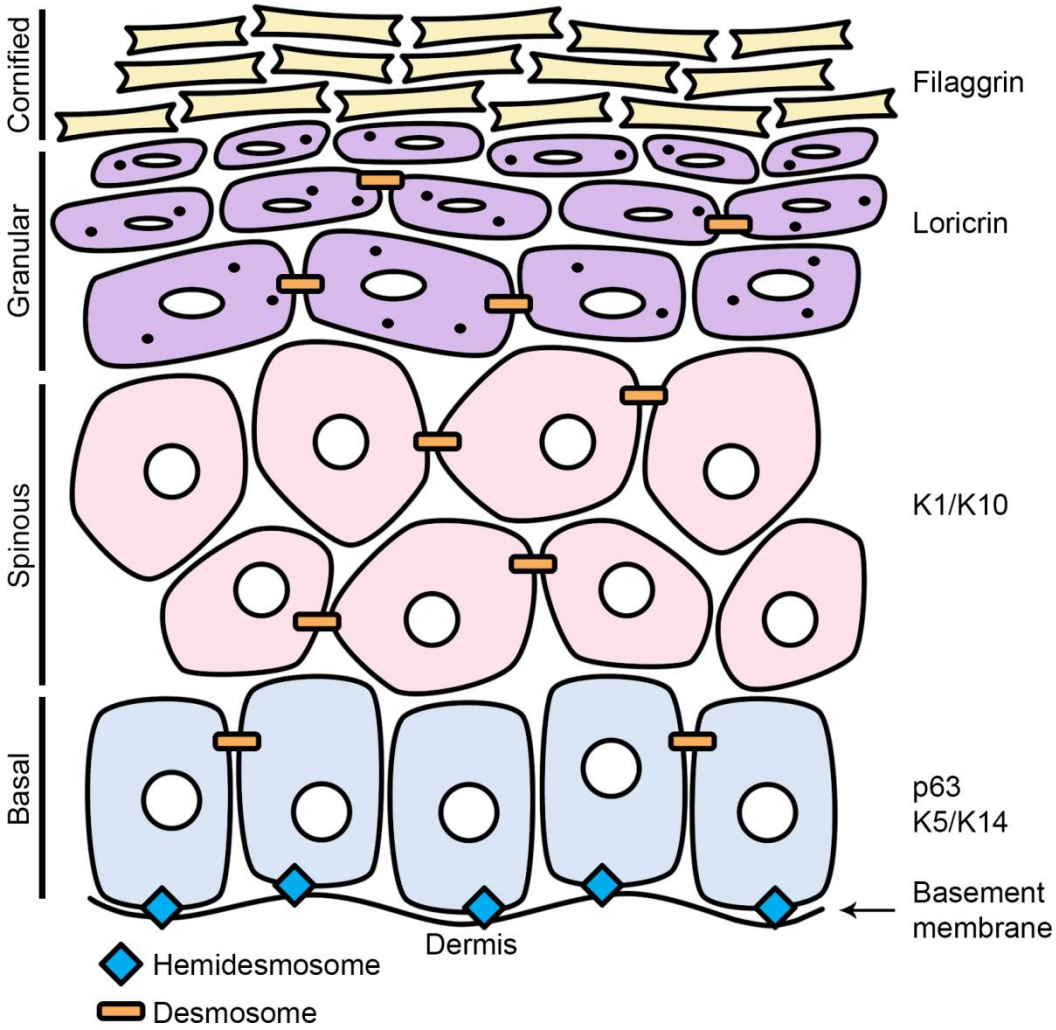


Fig 1.1 Schematic diagram of the stratified epidermal structure.

The epidermis contains a proliferative basal layer that produces progenitor cells that stratify and give rise to the differentiated upper layers. The basal keratinocytes are anchored to the BM via hemidesmosomes, and keratinocytes within the epidermis adhere to each other through desmosomes. The differentiated layers above the basal layer include the spinous, granular and cornified layers, and each layer expresses specific differentiation markers as shown in the diagram.

Spinous cells continue to differentiate upwards to form the granular layer (*stratum granulosum*), in which cells deposit cornified envelope proteins under the plasma membrane

to be cross-linked by transglutaminase. Granular cells also produce keratohyalin granules containing pro-filaggrin, which is processed into filaggrin present in the outermost cornified layer (*stratum corneum*). Terminal differentiation occurs in the cornified layer through destruction of cytoplasmic organelles to form enucleated cornified cells. The lipid content derived from lamellar granules is expelled into the intercellular spaces between corneocytes to facilitate skin barrier function. The dead corneocytes are gradually shed from the skin surface, as keratinocytes differentiate progressively from the basal layer to replace the upper layers (Candi *et al*., 2005; Fuchs *et al*., 2007; Koster *et al*., 2007, Blanpain *et al*., 2009).

1.3.3 Stem Cell Populations in the Epidermis

Lineage tracing experiments of the mouse skin have identified the presence of epidermal stem cells in the basal layer of the interfollicular epidermis (IFE). New born mice were given repeated injections of nucleotide analogue such as 5-bromo-2-deoxyuridine (BrdU) and ^{3}H-thymidine, which would be incorporated into the DNA of keratinocytes in the proliferative neonatal epidermis (Braun et al., 2004). Such labels were then chased into adulthood to identify label retaining cells, which would represent epidermal stem cell populations that rarely divide.

Two mechanisms have been proposed to explain how IFE stem cells contribute to skin homeostasis. The first model describes the existence of epidermal proliferative units which consist of one quiescent stem cell surrounded by several proliferative transit-amplifying cells, responsible for maintaining the continual replenishment of suprabasal epithelial cells. A more recent model suggests that the basal IFE does not necessarily contain transit-amplifying cells, but that basal cells can polarise regulatory cellular components and divide asymmetrically to give two distinct daughter cells for stem cell renewal and epidermal differentiation (Clayton *et al*., 2007; Lechler *et al*., 2007; Sotiropoulou *et al*., 2012). Further investigation is required to determine which model best describes the biology of IFE stem cells, although both models might not be mutually exclusive as the dynamics of stem cell maintenance and IFE homeostasis might differ under different physiological conditions.

Label-retaining experiments have also identified stem cell populations in the hair follicle (HF), an epidermal appendage which would be described in the section below. HF stem cell was shown to reside in the bulge present between the sebaceous gland and arrector pili muscle, and within the K5/K14-positive ORS epidermal cells. These HF stem cells have been demonstrated to sustain matrix cell proliferation for hair growth, and can be mobilised to

contribute to IFE homeostasis upon wounding (Botchkarev *et al*., 2003; Fuchs *et al*., 2007; Schneider *et al*., 2009).

1.4 Epidermal Appendages

Other than serving as a barrier to protect the body from external stresses and to maintain internal integrity, the epidermis contains a number of different epidermal appendages that confer additional functions of the skin. These include: the pilo-sebaceous unit composed of HF, sebaceous glands and arrector pili muscle; the sweat glands; and the touch domes. The HFs and sweat glands are important in maintaining body temperature, and the production of hair shafts is involved in the display of animal social behaviour. The sebaceous glands within the pilo-sebaceous unit are responsible for secreting sebum that lubricates the skin and possesses an anti-bacterial component, while touch dome helps in sensing the external environment (Schneider *et al*., 2009; Sotiropoulou *et al*., 2012).

1.4.1 The Hair Follicle

Amongst the different epidermal appendages present on skin, the HF attracts great interest due to its ability to undergo cyclic regeneration throughout mammalian adulthood and the presence of another epidermal stem cell population within the mini-organ.

1.4.2 Hair Follicle Morphogenesis and Cycling

HF morphogenesis during embryogenesis and perinatal development, as well as the cycling of mature HF all depend on the close interaction between epithelial and mesenchymal cells (Schneider *et al*., 2009; Sotiropoulou *et al*., 2012). The initial cue for signalling epidermal cells to undergo HF-specific differentiation during embryogenesis comes from the mesenchymal cells, instructing them to give regularly-spaced hair placodes. The hair placodes in turn signal mesenchymal cells underneath to form a condensate structure known as the dermal papillae, which promotes the proliferation and differentiation of HF cells to produce the mature follicle.

The post-natal mouse follicle enters its first HF cycle about 16 days after birth and undergoes HF involution (Müller-Röver *et al*., 2001). The cyclic progression of HF development can be divided into three stages: the regressive catagen phase, the resting telogen phase and the proliferative anagen phase (Fig 1.2). During catagen, follicular cell proliferation and differentiation ceases, and the lower two-thirds of the HF undergo apoptosis-mediated degeneration. HF involution in catagen is followed by the relatively quiescent telogen phase, during which the hair shaft matures into club hair and proliferation is minimal. Initiation of the anagen phase involves the activation of HF stem cells present in the telogen hair bulge and secondary hair germ for cell proliferation and differentiation to support hair growth.

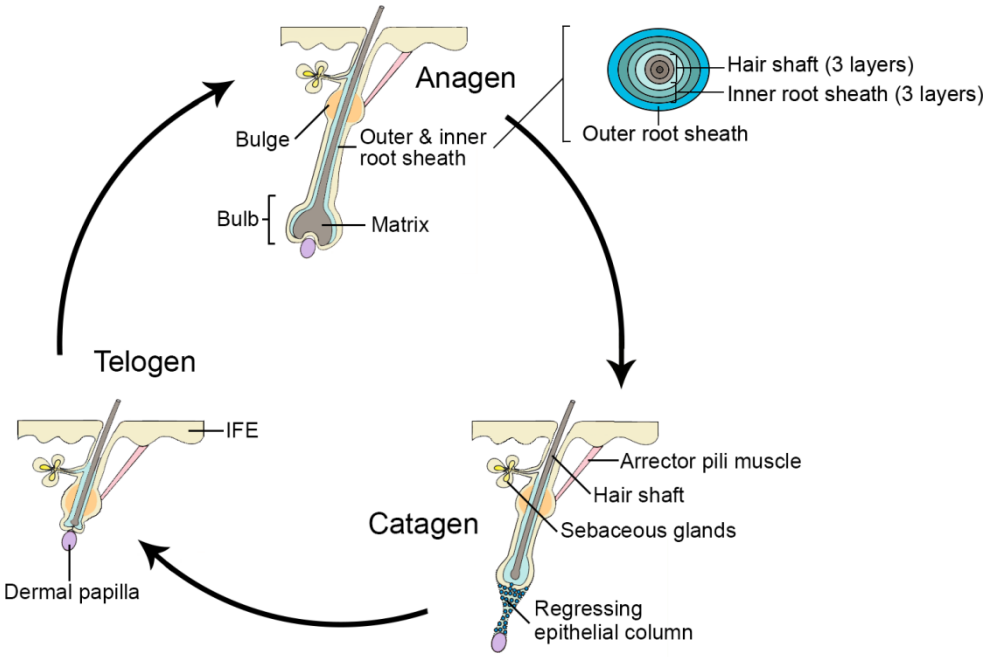


Fig 1.2 Schematic representation of the hair cycle and hair follicle structure.

The HF undergoes cyclic regeneration, starting from the anagen phase as cells in the HF matrix proliferate to give progenitors that form the hair shaft. The HF then progresses to the destructive catagen phase, during which the lower two-thirds of the HF undergo apoptosis and regress. This is followed by the resting telogen phase as the DP resides underneath the HF bulge, until the HF stem cells are activated again for re-entry into the anagen phase. The HF, sebaceous glands and arrector pili muscle form the pilo-sebaceous unit. The hair shaft (3 layers: medulla, hair cortex and cuticle) is surrounded by the 3 layers of inner root sheath (cuticle, Huxley's, Henley's layer). A companion layer is present that separates the inner and outer root sheath. The outer root sheath is continuous with the IFE basal layer.

1.4.3 The Structure of Hair Follicles

The mature anagen HF is organized as seven concentric layers of cells that arise from transiently amplifying progenitor cells present in the matrix of mature HF. These progenitors follow lineage-specific differentiation according to their positions relative to the DP to give rise

to differentiated structures such as the inner root sheath (IRS) and hair shaft. The DP mesenchymal condensate is enclosed within the bulb and is involved in epidermal-mesenchymal signalling during HF development. The outer root sheath (ORS) is continuous with the basal IFE.

1.4.4 Hair Shaft Structure

The anagen HFs are responsible for producing four different hair types found in the mouse coat: guard, awl, auchene and zigzag hairs (excluding specialised hairs such as the sensory vibrissae and tail hair). Different hair types are induced on embryonic skin successively at different timings during HF morphogenesis, and differ in hair shaft structure and distribution on the skin (Müller-Röver *et al*., 2001). These differences are illustrated in Table1.1 below:

Hair Types:	Guard	Awl	Auchene	Zigzag
Size (relative to guard hair)	1 (~1 cm)	1/2 to 2/3		1/2 to 2/3
Number of medulla columns	2	2–4		1
Number of bends	0	0	1	3-4
Frequency	1–3%	30%	0.1%	65-70%

Table 1.1 Characteristics of different hair types found on murine skin.

1.5 The Transcription Factor p63

The transcription factor p63 is the most evolutionarily conserved member of the p53 gene family. It has been observed that ectodermal commitment to epidermal fate is preceded by

the expression of the transcription factor p63 in the development of embryonic mouse skin (Koster *et al*., 2004, Laurikkala *et al*., 2006). In contrast to the tumour suppressor p53 that regulates gene expression in causing cell cycle arrest or apoptosis upon stress signals, p63 plays a fundamental role in epidermal development and homeostasis (Crum *et al*., 2010; Dȍtsch *et al*., 2010). p63-deficient mice exhibited severe defects in epidermal morphogenesis, and were born with the absence of normal stratified epithelia leading to perinatal death caused by dehydration (Mills *et al*., 1999; Yang *et al*., 1999). These p63-/- animals were also devoid of ectodermal appendages, limb and craniofacial development, which required close interaction between the epithelial and mesenchymal compartments (Mills *et al*., 1999; Yang *et al*., 1999).

1.5.1 The p63 Isoforms

Transcription of the gene p63 can be initiated at two different promoters to give the TAp63 and the ΔNp63 isoforms. The first promoter (P1) produces TAp63, which consists of the transactivation (TA) domain, the DNA-binding domain (DBD) and the oligomerisation (OD) domain (Fig 1.3). It has been shown that TAp63 can transactivate the p53-reporter due to its homology with the tumour suppressor p53 (Yang *et al*., 1998). The second promoter (P2) gives the truncated ΔNp63 isoform, which lacks the transactivation domain and is found to act as a

dominant negative inhibitor of p53 and TAp63 transactivation activities (Yang, *et al*., 1998). However, further studies have revealed the ability of ΔNp63 to activate transcription with an additional transactivation domain at the N terminus (King *et al*., 2003; Helton *et al*., 2006).

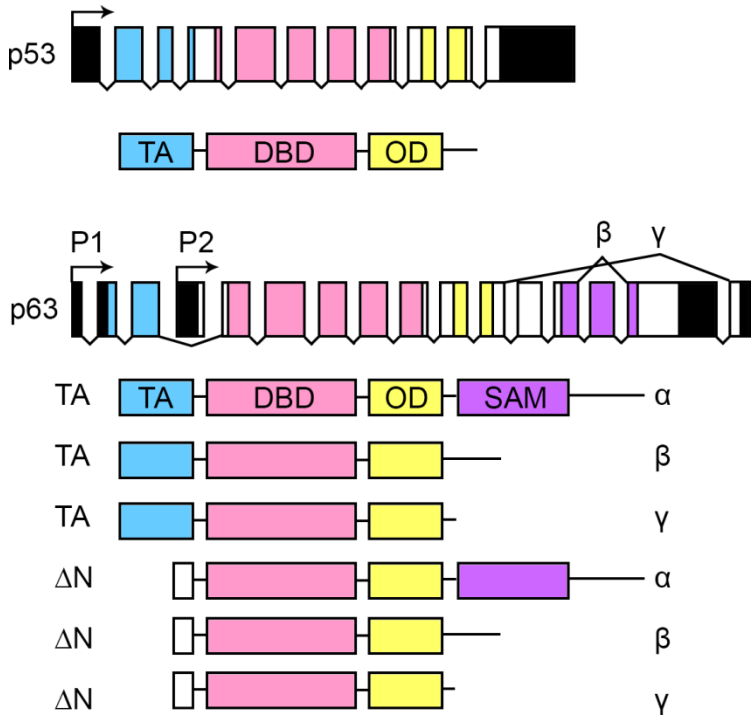


Fig 1.3 Schematics of p53 and p63 gene structures, with their protein isoforms.

The p53 tumour suppressor protein contains transactivation domain (TA), DNA binding domain (DBD) and oligomerisation domain (OD). p63 gene expression can be initiated at alternative promoters (P1 and P2) to give the TA and ΔN p63 isoforms. Transcripts can undergo alternative splicing to give six different p63 isoforms (TAp63α/β/γ and ΔNp63α/β/γ). TAp63α resembles p53 by having homologous TA, DBD and OD motifs along with the sterile α-motif (SAM).

Both TAp63 and ΔNp63 transcripts undergo alternative splicing at the 3'-end to give at least 6 different isoforms that differ at their C-termini (α, β, γ). The p63α isoform contains the sterile

α-motif (SAM) and transcription inhibition domain (TID), which are responsible for protein-protein interaction and hampering transcriptional activity respectively (Serber *et al*. 2002; Straub *et al*., 2010). The ΔNp63 isoform is predominantly expressed in mature epidermis over TAp63, whereas TAp63 is the predominant isoform in female oocytes to protect the integrity of the female germline (Yang *et al*., 1998; Liefer *et al*., 2000; Suh *et al*., 2006).

1.5.2 The Role of p63 in the Epidermis

Two models have emerged to explain for the function of p63 in stratified epithelial development: p63 could be involved in maintaining the proliferative potential of epidermal stem cells, or in signalling to ectodermal cells to commit to epidermal cell fate (Mills *et al*., 1999; Yang *et al*., 1999). Various studies have been performed to test these two hypotheses and to address the contribution of the different p63 isoforms to epidermal development since the generation of p63-deficient transgenic mice.

It remains uncertain whether TAp63 or ΔNp63 provide signals for epithelial lineage commitment, as both have been reported as being the initial predominant p63 isoform expressed in the ectoderm (Koster *et al*., 2004; Laurikkala *et al*., 2006). Furthermore, some have provided evidence illustrating that p63 is required to maintain epithelial stem cells but is

not involved in lineage commitment and differentiation in the skin and thymic epithelia (Yang *et al*., 1999; Senoo *et al*., 2007).

The role of ΔNp63 in supporting the proliferative potential of the basal epithelium has been demonstrated by p63-deficient mice complemented with TAp63 or ΔNp63 isoforms as well as human epidermal organotypic cultures in the absence of specific isoforms (Candi *et al*., 2006; Truong *et al*., 2006). Mice complemented with basal expression of ΔNp63α in a p63-null background exhibited larger regions of basal IFE development when compared to the rare patches of poorly differentiated epidermis in p63-deficient mice. These studies have also suggested that TAp63 may be involved in the late differentiation process, as TAp63 was found to induce transcription of differentiation genes such as K1 and involucrin. However, overexpression of TAp63α in mice with a p63+/- background inhibited terminal epithelial differentiation and resulted in progeny with fragile skin (Koster *et al*., 2004). While p63 is absolutely essential in stratified epithelial biology, perhaps it is the fine balance between the TAp63 and ΔNp63 isoforms that dictates the outcome of tissue development and homeostasis.

1.6 The Skin Immune Network

As the first line of defence against the external environment, the skin needs to initiate rapid and effective immune responses towards injuries and pathogens to restore normal skin

homeostasis. The concept of skin as an immunological organ has been proposed in the literature multiple times since 1983, in which the cellular components involved in maintaining cutaneous immunohomeostasis were described (Streilein, 1983; Bos, *et al*., 1987; Nickoloff, 1993). Indeed a number of immune cells can be found residing in the epidermis and dermis under normal physiological conditions, and additional immune cell populations are recruited to the organ during cutaneous inflammation.

Other than keratinocytes which compose the majority of the epidermis, Langerhans cells (LC) and dendritic epidermal T cells (DETC) can be also be found residing in the epithelium (Fig 1.4). The collagen-rich dermis is constituted by dermal fibroblasts, networks of the blood and lymphatic vasculatures, and scattered with different leukocytes such as neutrophils, macrophages, mast cells, dermal dendritic cells, natural killer (NK) cells, B cells and T cells. These cellular components interact and influence each other for the efficient sensing of danger signals, and to induce the appropriate immune response in the skin.

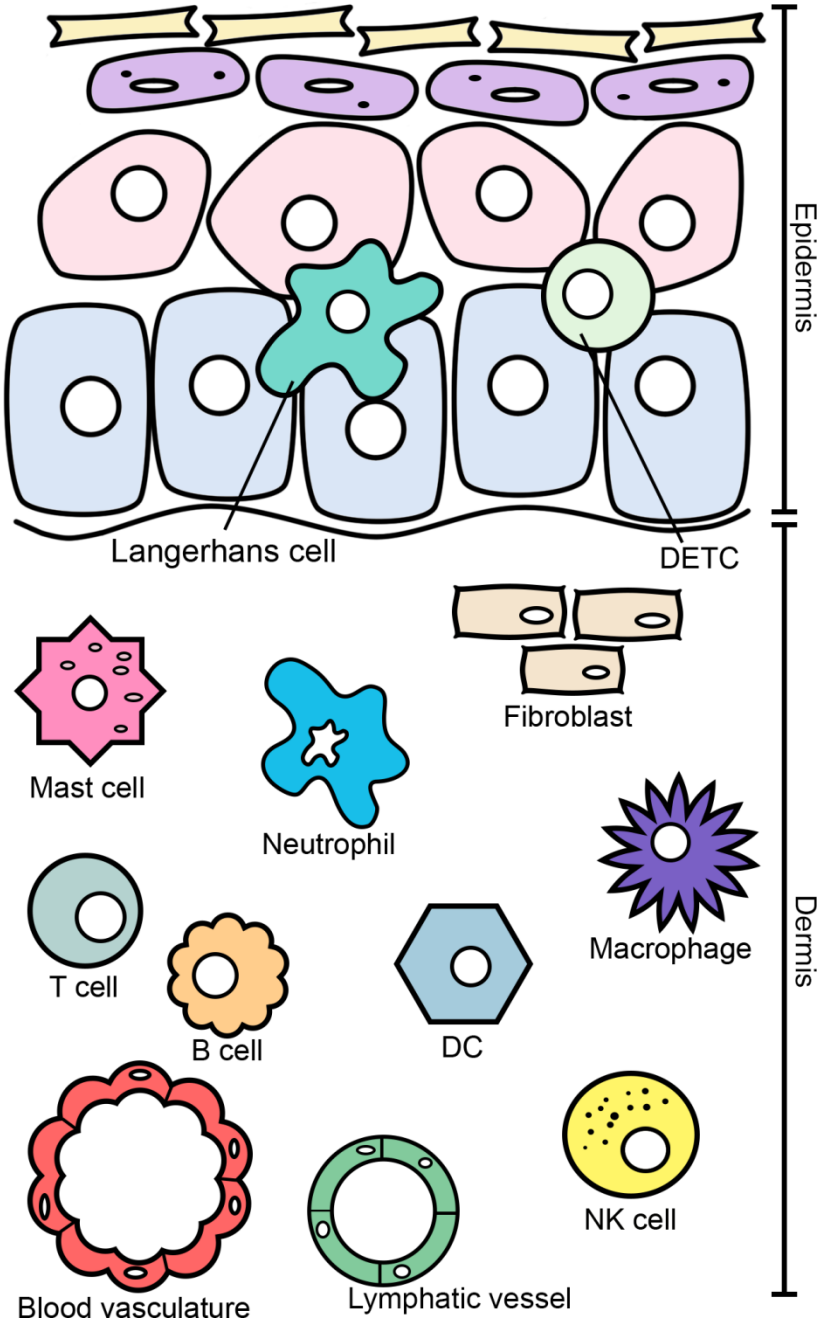


Fig 1.4 Diagram illustrating the different components present that comprise the skin immune system.

Other than keratinocytes which can function as initiators of skin inflammation, LCs (and DETCs in mouse skin) can be found in the epidermis patrolling for antigens that have breached the skin barrier. The dermis contains a variety of immune cells including: mast cells, neutrophils, macrophages, NK cells, DCs, T cells and B cells. These cells interact with each other to form the innate and adaptive skin immune network. Blood and lymphatic vasculatures present in the

dermis facilitate the trafficking of immune cells and chemokine signalling to generate an efficient inflammatory response. Dermal fibroblasts are also involved by secreting factors that modulate keratinocyte proliferation and differentiation.

1.7 The Role of Keratinocytes in Cutaneous Immunology

In addition to their protective role as the mechanical barrier against external stresses, epidermal keratinocytes have been demonstrated to actively participate in skin immune responses.

1.7.1 Keratinocytes Sensing Danger Signals

Keratinocytes are capable of sensing danger signals by recognising pathogen-associated molecular patterns (PAMPs) or danger-associated molecular pattern (DAMPs), which refer to the conserved structures shared by microbial pathogens and toxins respectively. The recognition of PAMP/DAMP occurs via binding to TLR present on the surface of keratinocytes (TLR1, 2, 4-6) or in endosomes (TLR3 & 9) within the cell (Nestle *et al*., 2009; Di Meglio *et al*., 2011). The binding of TLR ligands to the receptor leads to the activation of downstream signalling pathways and the production of AMP, cytokines and chemokines to mediate innate and adaptive cutaneous immunity.

1.7.2 AMP Production by Keratinocytes

AMPs such as cathelicidins, β-defensins and S100 proteins are cationic peptides that protect epithelial surfaces from microbial infection. Their positive charge allows them to lyse anionic bacteria walls by forming pore complexes. AMP production can be enhanced in keratinocytes upon the stimulation of TLRs by pro-inflammatory cytokines such as IL-1, TNF-α, IL-17A and IL-22 (Nestle *et al*., 2009; Di Meglio *et al*., 2011).

1.7.3 Cytokines from Keratinocytes

Cytokines are polypeptides or glycoproteins that function in modulating inflammation through their pro-inflammatory or anti-inflammatory properties. Keratinocytes produce a wide range of cytokines constitutively or in response to inflammatory signals. Unstimulated keratinocytes constitutively express certain pro-inflammatory (e.g. IL-1, IL-6 and TNF-α) and anti-inflammatory cytokines (e.g. TGF-β). Expression of these cytokines along with additional inflammatory components (e.g. CXCL1, IL-10 and GM-SCF) is further enhanced in activated keratinocytes. These keratinocyte-derived cytokines mediate the close crosstalk between epidermal cells and other cellular components of the skin to regulate cutaneous immunohomeostasis (Nestle *et al*., 2009; Di Meglio *et al*., 2011).

1.7.4 Interleukin-1 in the Epidermis

Amongst the many cytokines present in the skin, IL-1 was the first to be identified and exhibits various pleiotropic biological functions. It is involved in promoting B cell proliferation and maturation, T helper cell and DC activation, and up-regulation of adhesion molecules on endothelial cells (Luger *et al*., 1982; Pike *et al*., 1985; Nestle, *et al*., 2009; Gabay *et al*., 2010, Sims *et al*., 2010). Keratinocytes express members of the IL-1 family consisting of three ligands (IL-1α, IL-1β and IL-1Ra) along with two receptors (IL-1RI and IL-1RII).

Both IL-1α and IL-1β are constitutively synthesised as 31kDa precursors (pro-IL1α, pro-IL1β) in keratinocytes. The pro-IL1α precursor is biologically active, but has been shown to be processed by calcium-dependent calpain at a low frequency to give mature IL-1α in macrophages (Carruth *et al*., 1991). In contrast, pro-IL1β is inert and requires caspase 1-mediated cleavage in the inflammasome to release the mature functional 17kDa IL-1β. Of the two, IL-1α is the predominant form produced in keratinocytes, which could be explained by the limited caspase 1 activity detected in these cells. However, the reverse is observed in immune cells such as macrophages, LC and DC in which IL-1β dominates. The IL-1α and IL-1β ligands have similar biological activities, although IL-1α acts locally being tethered to the cell's plasma membrane while IL-1β is secreted and exerts its effect systematically (Sims *et al*., 2010).

The IL-1Ra ligand is an IL-1RI antagonist, as it competes with IL-1 for the receptor but does not induce any downstream signalling. The biologically inactive receptor IL-1RII takes on a similar inhibitory role in IL-1 signalling. This is achieved by it being released into the extracellular environment to sequester ligands or to act as a decoy receptor in competition with IL-1RI for ligands (Gabay *et al*., 2010, Sims *et al*., 2010).

IL-1 binding to the IL-1RI receptor is followed by the engagement of accessory protein IL-1RAcP. The complex formed triggers further recruitment of the adapter proteins MyD88, IRAK and TRAF6, which leads to the subsequent activation of signalling pathways such as nuclear factor-κB (NF-κB), mitogen-associated protein kinases (MAPK) p38, extracellular signal-regulated protein kinases (Erk1/2), activator protein-1 (AP-1) and c-Jun N-terminal kinase (JNK) (Saklatvala *et al*., 1993; Dunne *et al*., 2003; Gabay *et al*., 2010).

1.7.5 Keratinocyte-Derived Chemokines and Growth Factors

Keratinocytes produce a number of chemokines that have chemotactic properties and regulate the dynamics of immune cell infiltration to the skin. Other than IL-1 which acts as a chemo-attractant for neutrophils and macrophages, examples of chemokines produced by activated keratinocytes include CXCL1, CCL5/RANTES and CCL20/MIP-3α. CXCL1 is chemotactic

for neutrophils and supports epithelial proliferation during wound healing (Werner *et al*., 2003). CCL5/RANTES attracts the infiltration of monocytes, T cells and eosinophils into the skin, whereas CCL20 is a potent chemokine for recruiting LCs and T cells (Schall., *et al*., 1990; Dieu-Nosjean, *et al*., 2000). Moreover, activated keratinocytes can secrete growth factors such as GM-CSF, stimulating the survival and activation of macrophages and neutrophils, as well as promoting angiogenesis (Hamilton, 2008; Mascia *et al*., 2010).

The vast collection of cytokines and growth factors derived from keratinocytes play important roles in interacting with other immuno-competent cells and together modulate the progression of cutaneous inflammation. A quick overview of the immune cells that participate in this process is outlined in the following section. Although not listed below, it is important to keep in mind the contribution of the blood and lymphatic vasculatures in transducing inflammatory signals and recruiting immune cells. Epithelial-fibroblast interaction in the skin has also been recognised to contribute to skin homeostasis, as keratinocytes can stimulate fibroblasts by secreting factors like IL-1. Stimulated fibroblasts then signal back in a double paracrine manner through factors such as keratinocyte growth factor (KGF), heparin binding-epidermal growth factor (HB-EGF) and IL-6 to promote keratinocyte proliferation and differentiation (Werner *et al*., 2007).

1.8 Innate Skin Immunity

Innate immunity provides a rapid pre-existing defence response against pathogens by directly discriminating between self and non-self-patterns (Fig 1.5). Such recognition is mostly mediated by pattern recognition receptors (PRRs) present on immune cells binding to PAMP/DAMPs. Leukocytes such as neutrophils, macrophages, mast cells and NK cells form part of the skin's innate immunity along with keratinocytes.

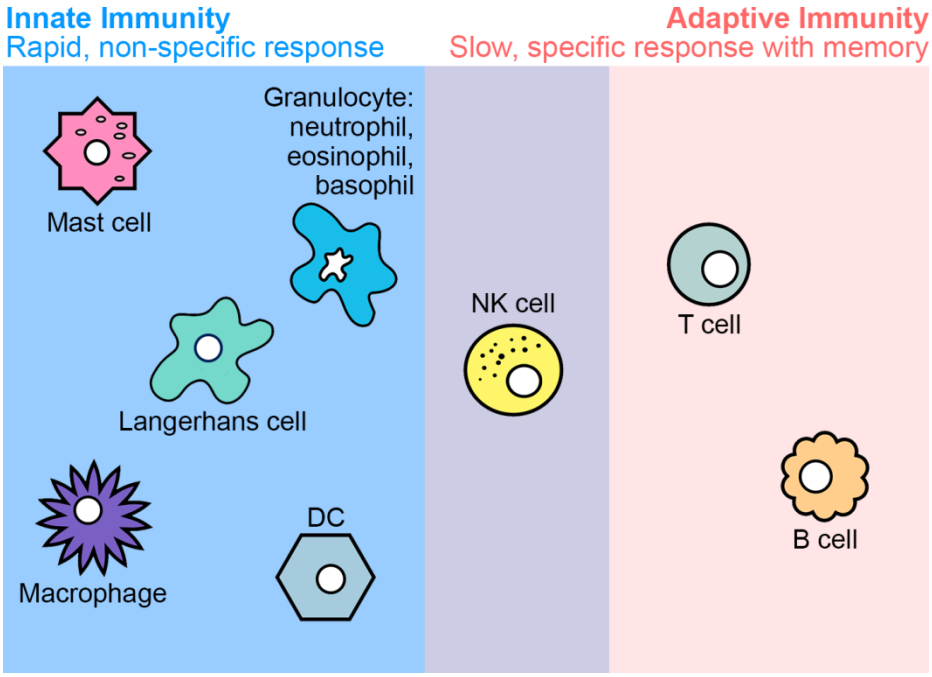


Fig 1.5 Diagram illustrating the types of immune cells involved in innate and adaptive immunity.

The innate immunity provides first-line rapid non-specific response against pathogens. In addition to soluble factors such as complement proteins, cellular components of the innate immunity include: mast cells, macrophages, LCs, granulocytes (neutrophils, eosinophils and basophils) and DCs. NK cells participate in innate immunity, but have been shown to be involved in adaptive immunity as well. The adaptive immunity provides slower antigen-specific response against pathogens, and creates immunological memory. T cells and B cells, as well as the antigen-specific antibodies produced by B cells are involved in adaptive immunity.

1.8.1 Neutrophils

Three types of granulocyte are present in the immune system: eosinophils, basophils and neutrophils, the latter being the most abundant in steady state. Neutrophils and eosinophils can phagocytose and destroy microorganisms, a process mediated by the presence of granules in their cytoplasm. These granules are composed of various anti-microbial cytotoxic components, proteinases and hydrolases, which are then responsible for degrading ingested material in the phagolysosome. Basophils on the other hand have histamine granules that cause a local inflammatory response and dilation of the vasculature when released. Neutrophils can respond to a number of chemotactic stimuli such as complement component C5a, IL-8 and CXCL1, and are the predominant leukocytes present during the early stages of acute inflammation. Neutrophils are short-lived and are quickly replaced by infiltrating macrophages as inflammation progresses (Gurtner *et al*., 2008).

1.8.2 Macrophages

Upon chemotactic signals, macrophage precursors known as monocytes migrate out of circulation and differentiate into macrophages to reach inflamed tissues. Macrophages patrol the tissues and participate in innate immunity by phagocytosis of antigenic materials. Macrophages that have taken up antigens can serve as antigen presenting cells (APC) for

lymphocytes in the lymph node to stimulate the activation of naïve T cells. These phagocytic cells can be classified into three categories based on their function: classically activated M1, alternatively activated M2 and regulatory macrophages (Murray *et al*., 2011; Pasparakis *et al*., 2014). M1 macrophages are activated by TLR ligands and IFN-γ, and produce pro-inflammatory cytokines. Research has shown that these phagocytic M1 macrophages could be responsible for acute and chronic skin inflammation. On the contrary, M2 macrophages are immune suppressive and pro-angiogenic due to the production of IL-10, VEGF and TGF-β. Regulatory macrophages also have anti-inflammatory functions by secreting IL-10 when their FcR cell surface receptors are bound to the Fc region of antibody molecules. It has been demonstrated that macrophages are plastic in nature and can respond differently according to the microenvironment they are exposed to. For example, M1 macrophages can differentiate into the M2 subtype upon sensing IL-4 secreted from basophils.

1.8.3 Dendritic Cells

APCs are capable of capturing, processing and presenting phagocytosed antigens on their cell surface to stimulate naïve T cells, providing a link between innate and adaptive immunity. Other than macrophages, DCs are known to serve as professional APCs and can cross-present exogenous antigens to CD8+ T cells via their major histocompatibility complex (MHC) Class I

receptors. The skin harbours two populations of DCs, the epidermal LCs and the dermal DCs, which are located in distinct regions of the skin (Nestle *et al*., 2009; Di Meglio *et al*., 2011; Pasparakis *et al*., 2014). LCs reside in the suprabasal layer of the epidermis to capture pathogens that have passed the cornified layer. Studies have demonstrated that LCs can present antigen epitopes to CD4+ T cells and induce IL-22 production by T cells. However, the exact role of LCs in skin immunity is still unclear, as opposing evidence has also been presented where LCs caused T cell anergy i.e. immune unresponsiveness. Dermal DCs can activate T cells to generate either the pro-inflammatory T_H1/T_H17 or the anti-inflammatory T_H2 response depending on the DC subset involved (Heath *et al*., 2013; Pasparakis *et al*., 2014).

1.8.4 Mast Cells

Upon the binding of antigen IgE onto their cell surface receptor FcR, mast cells are activated to release preformed cytosolic granules which contain a cocktail of pro-inflammatory substances such as histamine, tryptase, VEGF and TNF-α (Urb *et al*., 2012). The released inflammatory mediators can cause an increase in vasculature permeability, vasodilation and modulate responses of other immune cells such as recruiting neutrophils and T_H2 cells (Bischoff *et al*., 2007; Urb *et al*., 2012; Pasparakis *et al*., 2014).

1.8.5 Natural Killer Cells

NK cells are cytotoxic lymphocytes that are accountable for destroying virus-infected cells. The lower expression levels of MHC class I proteins on virally infected cells, compared to healthy cells allows for their selective recognition by NK cells. Furthermore, NK cells are capable of producing cytokines such as IFN-γ to facilitate cytokine crosstalk with other leukocytes (Cooper *et al.*, 2001). Other than the role of NK cell in innate response, further investigation is required for its possible involvement in adaptive immunity as demonstrated by recent studies on memory-like NK cells that could mediate robust contact hypersensitivity response to repeated haptens exposure (O'Leary *et al.*, 2006).

1.9 Adaptive Skin Immunity

Adaptive immunity can be stimulated with the help of the innate immune system, and provides an antigen-specific immune response. Immunological memory is generated in the process to enable a rapid and enhanced secondary response if the organism is subsequently exposed to the same antigen. The main effectors of adaptive immunity are B and T cells, which are stimulated by APCs such as macrophages and DCs. The adaptive response can be broadly divided into two classes: the cell-mediated response delivered by T cells, and the humoral response mediated by antibodies from B cells.

1.9.1 T Cells

T cells are present in the basal and suprabasal epidermis, and also in the dermis clustering around the epidermal-dermal junction and blood vasculatures (Nestle *et al*., 2009; Di Meglio *et al*., 2011; Pasparakis *et al*., 2014). Cell-mediated adaptive immunity is established by the biological activities of T cells.

CD4+ and CD8+ T Cells

Two main classes of T cells have been identified: CD4+ helper T cells are responsible for regulating the immune responses of other cells such as B cells, macrophages and CD8+ cytotoxic T cells; whereas CD8+ cytotoxic T cells kill infected host cells via receptor/ligand mediated or granule exocytosis-mediated cell lysis. Both types of T cells have T cell receptors (TCR) that recognise antigens presented on MHC molecules, and their interaction is facilitated by the surface co-receptors CD4 and CD8. TCR can exist as either the αβ heterodimer or γδ heterodimer, with the majority of peripheral T cells expressing the αβ-TCR. CD4 on helper T cells binds to class II MHC proteins, while CD8 on cytotoxic T cells stabilises binding to class I MHCs. This directs different T cell types to interact with specific target cells, as class I MHCs are present in all nucleated host cells but class II can only be found on certain cells such as APCs and B cells (Nestle *et al*., 2009; Di Meglio *et al*., 2011; Pasparakis *et al*., 2014).

Subclasses of Effector T Cells – T_H1, T_H2, T_C1, T_C2, DETC

CD4+ and CD8+ T cells, activated through antigen presentation, can differentiate into further subtypes depending on the mixture of cytokines present in the microenvironment (Woodland *et al*., 2003). CD4+ T cells can differentiate into either T_H1 or T_H2 effector cells with different cytokine production profiles. The development of T_H1 can be promoted by cytokines like IL-12, and results in the secretion of TNF-α and IFN-γ to activate macrophages and cytotoxic T cells. T_H2 cells, prompted by cytokines like IL-2, produce different cytokines such as IL-4 and IL-10 to stimulate B cell antibody production and the activation of mast cells and eosinophils. Therefore, the pro-inflammatory environment created by the T_H1 response is ideal to tackle intracellular pathogens, while the relatively anti-inflammatory T_H2 response is suitable when facing extracellular pathogens. Likewise CD8+ cytotoxic T cell differentiation into the Tc1 and Tc2 subtypes has been demonstrated, which showed different homing specificities into tissues without affecting their cytotoxic activity (Woodland *et al*., 2003).

A unique population of T cells has been identified in the mouse skin epidermis and is referred as DETCs. DETCs express the invariant γδ-TCR that recognizes antigens on stressed keratinocytes. They produce cytokines such as IFN-γ and IL-2, and therefore do not fit into the T_H1/T_H2 classification. These skin-resident DETCs seem to play important roles in regulating

keratinocyte proliferation as well as maintaining the anti-microbial skin barrier (Sharp *et al*., 2005; MacLeod *et al*., 2013).

1.9.2 B Cells

Antibodies produced by B cells mediate humoral adaptive immunity by inactivating antigens and marking them to aid the recruitment of phagocytes in eliminating pathogens. Analogous to T cell activation, naïve B cells are stimulated as B cell receptors (BCR) bind to processed antigens presented on MHC Class II proteins. Each B cell clone makes antibodies with unique antigen binding sites, and undergoes clonal expansion when activated. The B cell progenitors then differentiate to give either antibody producing effector cells or memory B cells. The presence of memory B cells allows for a rapid humoral response when the body is challenged by the same antigen again (Avalos *et al*., 2014).

Unlike T cells, which are present in both the epidermis and dermis, B cells are not present as extensively as T cells in normal healthy skin. B cells actively infiltrate into the skin from the skin lymphatic network in response to CCL20 signals during cutaneous inflammation. Infiltrating B cells express high levels of MHC Class II, CD80/86 and CD1, and have been suggested to be responsible for activating T cells present in the inflamed skin (Geherin *et al*., 2012). Further

research on the contribution of B cells in skin inflammation is required, as B cells were previously thought to play only a minor role in maintaining skin homeostasis.

1.10 The NF-κB Signalling Pathway

The controlled coordination of the immune system is an important part of the maintenance of tissue homeostasis. The nuclear factor-κB (NF-κB) signalling pathway is well established as having a central role in transactivating genes involved in the development and function of immunity such as pro-inflammatory cytokines IL-1 and TNF-α, adhesion molecules ICAM-1, and immunoreceptor MHC class I (Oeckinghaus *et al*., 2009). Moreover, NF-κB can also modulate the expression of genes such as growth factors and apoptotic mediators in controlling cell proliferation and survival.

The mammalian NF-κB family of transcription factors consists of five different subunits: p65/RelA, p50/p105 (NF-κB1), p52/p100 (NF-κB2), RelB and c-Rel which exist as homodimers or heterodimers (Li *et al*., 2002; Hayden *et al*., 2008; Pasparakis *et al*., 2009). All members contain the N-terminal Rel-homology domain (RHD) required for subunit dimerisation, nuclear localisation, and binding to NF-κB consensus DNA sequences (Fig 1.6). The p65, RelB and c-Rel subunits have transactivation domains (TA) at their C-termini to activate the transcription of

target genes. The p50 and p52 members are generated by proteolytic processing of their precursors p105 and p100 respectively. The precursor proteins p105 and p100 have C-terminal ankyrin (ANK) repeats that are absent from p50 and p52. The lack of TA domain on p50 and p52 means that they are transcriptionally inactive on their own, but their abilities to bind DNA and to dimerise with TA domain-containing subunits allow them to participate in gene regulation.

In unstimulated cells, NF-κB dimers are present in the cytoplasm, and are kept inactive by associating with inhibitory IκB proteins (Li *et al*., 2002; Hayden *et al*., 2008; Pasparakis *et al*., 2009). The IκB family is characterised by the presence of ANK repeats at the C-terminus, and consists of IκBα, IκBβ, IκBγ, IκBε and the atypical members (Bcl-3, IκBζ, IκBNS). The precursors p105 and p100 also act in a similar way as the inhibitory IκB with similar C-terminal ANK repeats to conceal the nuclear localisation sequence (NLS) present on the RHD.

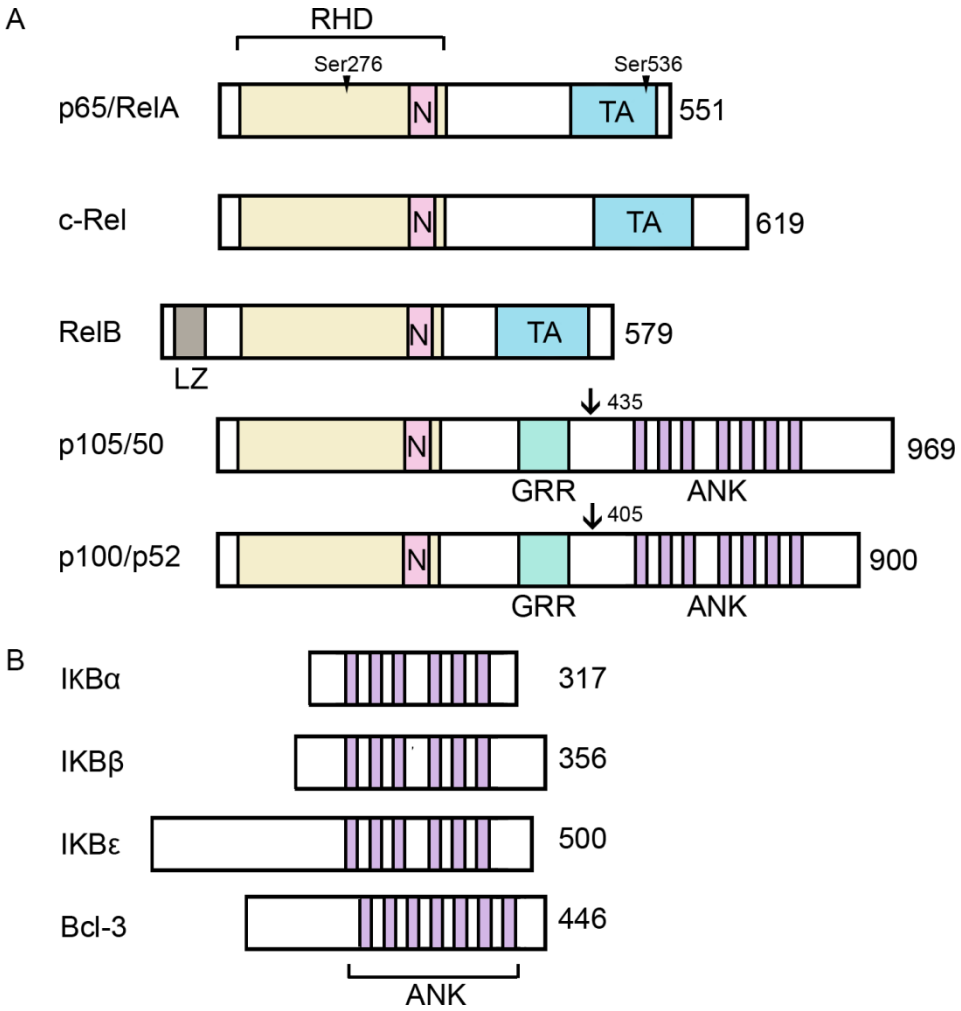


Fig 1.6 Members of the Mammalian NF-κB Family.

(A) Schematic representation of the NF-κB family members, all of which contain the Rel-homology domain (RHD) where the nuclear localisation signal (N) resides. RelB has an additional leucine-zipper domain at the N-terminal end. Examples of serine residues acting as phosphor-acceptor sites on p65 are indicated in the diagram. p65, RelB and c-Rel have a transactivation domain (TA) for activating target gene transcription but not p105/50 and p100/52. Instead p105 and p100 contain the glycine rich region (GRR) and ankyrin repeats (ANK) at the C-termini. The arrows indicate where proteolytic cleavage occurs to generate p50 and p52 respectively.

(B) Schematic representation of IκB family members, all containing the ankyrin repeats for protein-protein interaction.

1.10.1 Canonical and Non-Canonical NF-κB Pathways

The NF-κB signalling pathway can be activated in response to a wide range of stimuli, and two distinct courses of activation have been described: the canonical/classical pathway and the non-canonical/alternative pathway (Li *et al*., 2002; Hayden *et al*., 2008; Pasparakis *et al*., 2009). Both pathways require the phosphorylation activities of the IKK complex which is comprised of two catalytic subunits IKKα/IKK1 and IKKβ/IKK2, and the regulatory subunit NF-κB essential modulator (NEMO).

The canonical pathway can be induced by most physiological stimuli such as pro-inflammatory cytokines and activated TLRs (Fig 1.7). Adapter proteins are recruited to the stimulated receptors for intracellular signalling cascades, which subsequently results in the activation of the IKK complex. IKKβ is mainly responsible for the phosphorylation of IκBα at two specific serine residues in this classical pathway. Phosphorylated IκBα is marked for polyubiquitination and is degraded by the 26S proteasome. This releases the sequestered NF-κB dimers, predominantly the p65/p50 heterodimer, to translocate into the nucleus for gene activation (Li *et al*., 2002; Pasparakis *et al*., 2009).

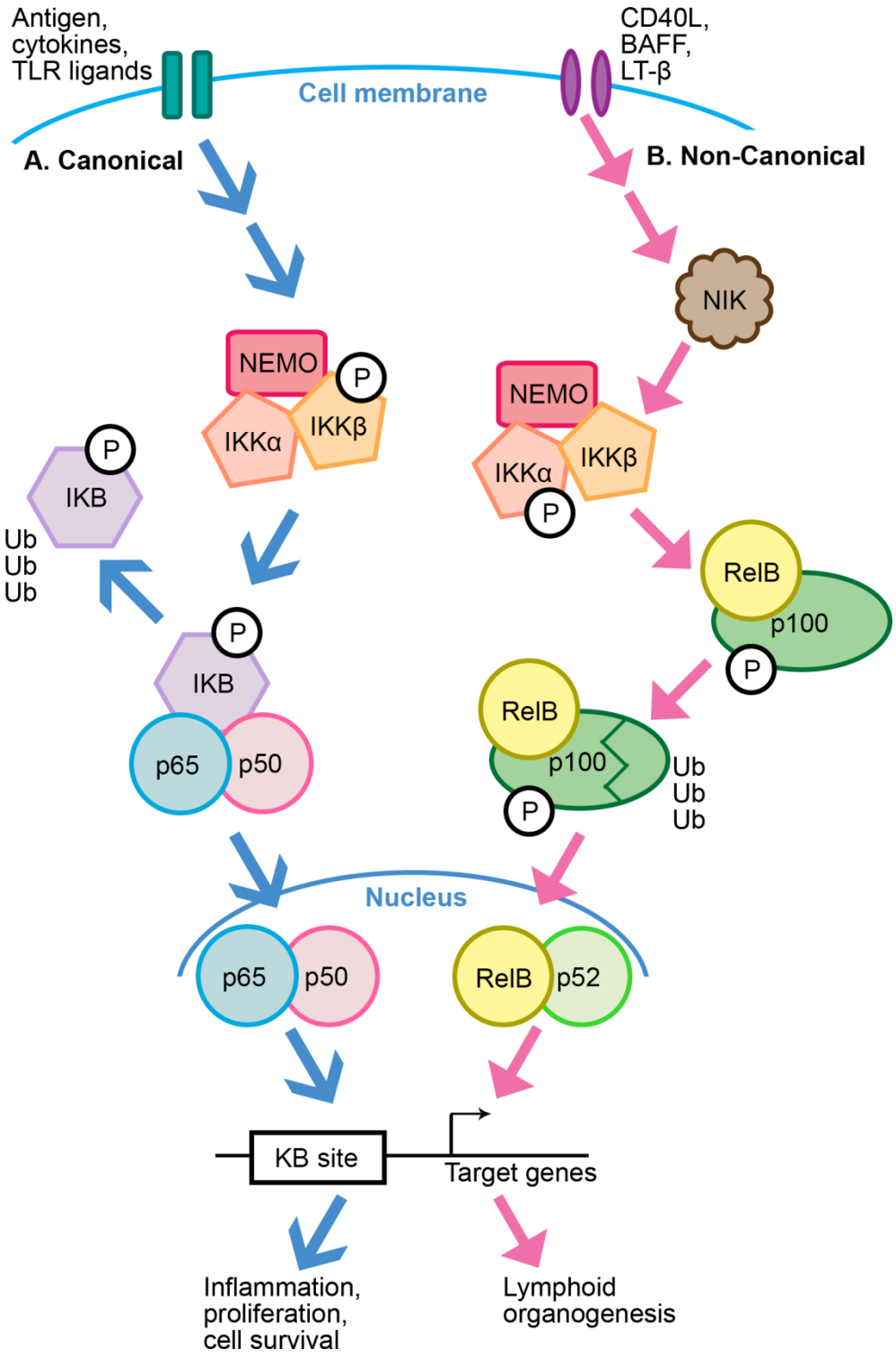


Fig 1.7 Canonical and non-canonical NF-κB signalling pathways.

(A) The canonical NF-κB pathway can be triggered by various signals, which leads to downstream signalling and phosphorylation of IKKβ within the IKK complex. Activated IKK phosphorylates IKB, marking it for polyubiquitination and proteosomal degradation. This

releases the p65/p50 heterodimer to translocate into the nucleus and transactivate target genes responsible for controlling cell survival, proliferation and inflammation.
(B) The non-canonical pathway can be triggered by a limited subset of ligands. The IKK complex is activated upon phosphorylation of IKKα, which then phosphorylates p100. Phosphorylated p100 undergoes proteolytic processing to give the mature p52 subunit. The RelB/p52 heterodimer then enters the nucleus for gene transactivation.

The non-canonical pathway responds only to certain TNF family cytokines such as CD40L, BAFF and lymphotoxin-β (LT-β), and is mainly involved in lymphoid organogenesis and lymphocyte development (Pasparakis *et al*., 2009; Oeckinghaus *et al*., 2011). Activation of the alternative pathway is dependent on IKKα instead to phosphorylate p100 for the generation of p52 to give the transcriptionally active p52/RelB dimer.

1.10.2 Activation of p65 by Phosphorylation

In addition to the nuclear translocation of released NF-κB dimers, NF-κB complexes can undergo post-translational modifications such as phosphorylation and acetylation to provide further control of their transcriptional activities. p65 is known to be a major target for phosphorylation by a number of kinases at its serine phosphor-acceptor sites (Schitmz *et al*., 2001; Chen *et al*., 2004). The Ser276 residue within the RHD is phosphorylated by the catalytic subunit of protein kinase A (PKA) and mitogen- and stress-activated kinase-1 (MSK1) upon lipopolysaccharide (LPS) and TNF-α stimulation respectively. The Ser536 residue within the TA

domain can be phosphorylated by TNF-α/ LPS-activated IKKs, or by the ribosomal-subunit kinase-1 (RSK1) in response to IL-1 or p53 activation (Chen *et al*., 2004; Roux *et al*., 2004). Phosphorylation of p65 promotes its binding with the transcription co-activator CREB-binding protein (CBP)/p300 and thus enhances the transactivation ability of p65 (Schitmz *et al*., 2001).

1.10.3 NF-κB in Cutaneous Inflammation

Expression of NF-κB components such as p65, p50, p52 and IκBα has been reported in the basal and suprabasal epidermis of mouse skin under normal physiological conditions (Seitz *et al*., 1998; Budunova *et al*., 1999; Mulero *et al*., 2013). The role of NF-κB in skin inflammation has also been investigated using transgenic mouse models lacking or overexpressing the NF-κB pathway.

1.10.4 Skin Inflammation upon NF-κB Activation

Transgenic mice overexpressing IKKβ in the epidermis (K5-IKKβ) develop cutaneous inflammation 2 weeks after birth, and have bald hyperplastic skin with abnormal epidermal appendages (Page *et al*., 2010). Increased NF-κB activity in the epidermis is associated with dermal infiltration of macrophages and T cells. Up-regulation of the production of cytokines such as IL-1α, IL-6, TNF-α and GM-CSF could also be detected in keratinocytes overexpressing

IKKβ. K5-IKKβ skin transplantation onto immunodeficient NOD/SCID mice that lack functional T and B cells did not impede the development of such skin lesions, hence adaptive immune cells do not seem to play a role in this model of skin inflammation.

Mice deficient in IκBα suffered postnatal multi-organ inflammation and died shortly after birth. The inflamed skin from these mice showed an increase in keratinocyte proliferation and infiltration of B and T cells (Beg *et al*., 1995; Klement *et al*., 1996; Rebholz *et al*., 2007; Pasparakis *et al*., 2009). The inflammatory skin phenotype in IκBα-/- mice could be rescued by either crossing into the Rag2-/- background to remove T and B lymphocytes, or by knocking out p65 expression in the epidermis. Moreover, IκBα-/- skin only showed signs of hyperplasia, and the ablation of IκBα expression in both keratinocytes and T cells was required to induce skin inflammation. The studies suggest that increased NF-κB activation in both keratinocytes and T cells can induce skin inflammation, which may be dependent on p65.

A recent study using skin-specific p65 knockout mice (K14-p65^{EKO}) illustrated that these mice were less susceptible towards the skin inflammatory and hyperplastic response induced by *12-O*-tetradecanoyl-13-phorbol acetate (TPA) treatment (Kim *et al*., 2014). Reduced infiltration of macrophages and granulocytes was observed in TPA-treated K14-p65^{EKO} skin, and

p65 deficiency inhibited the expression of pro-inflammatory cytokines and chemokines by keratinocytes. This finding was complemented by a previous study which showed that knock-in mice with constitutively active p65 (p65 S276D) displayed systemic hyper-inflammation in organs such as the skin, lungs and liver (Dong *et al*., 2010).

1.10.5 Skin Inflammation upon NF-κB Inhibition

Although increased NF-κB signalling in keratinocytes can disrupt the cutaneous immune system, other studies have demonstrated that a dampening of NF-κB activity can also cause inflammatory skin lesions. While IKKβ overexpression in the mouse epidermis (K5-IKKβ) caused skin inflammation, a transgenic mouse model with cutaneous deletion of IKKβ (K14-IKKβ^{EKO}) exhibited epidermal hyperplasia and inflammatory skin lesions less than a week after birth (Pasparakis *et al*., 2002). IKKβ deficiency in keratinocytes had impaired NF-κB activation but did not affect cell proliferation, differentiation and apoptosis. This suggests that epidermal hyperplasia was secondary to skin inflammation.

Another mouse model with epidermal expression of the super-repressor IκBα that is resistant to proteolytic degradation (K5-IκBαSR) also developed skin inflammation and epidermal hyperplasia (van Hogerlinden *et al*., 1999). Spontaneous SCC development was observed in

these animals later in life. Both cutaneous inflammation and tumour development were prevented when mice were crossed into a TNFR1-deficient background but not when crossed into an IL-1R1 background, illustrating the requirement for TNF signalling in mediating such a skin phenotype (Lind *et al*., 2004).

All these studies illustrate the importance of NF-κB in regulating skin immunohomeostasis and the involvement of keratinocytes as initiators of skin pathogenesis. They also emphasised the need for a better understanding of how NF-κB signalling can be modulated to give opposing effects on skin immunity.

1.10.6 NF-κB-Independent Function of IKKα in Skin

Recent studies have illustrated the NF-κB independent functions of IKK components, which were once thought to be specific for IκB proteins (Oeckinghaus *et al*., 2009). In skin, IKKα functions to promote terminal differentiation as IKKα-/- mice exhibit epidermal thickening but do not have the outermost cornified layer. IKKα-/- keratinocytes undergo hyperproliferation in the absence of terminal differentiation, which can be reversed by re-introducing IKKα lacking kinase activity and in the absence of NEMO binding (Hu *et al*., 2001; Sil *et al*., 2004).

1.11 The Biological Significance of iASPP

In view of the importance of transcription factors p63 and p65 in regulating epidermal development and homeostasis, it is important to gain a better understanding of the regulators involved in controlling their transcriptional activities. One such regulator is the inhibitor of apoptosis-stimulating protein of p53 (iASPP) protein, which serves as a binding partner of both p63 and p65 and could potentially regulate their activities in skin. Moreover, iASPP is known to inhibit p53-mediated cell death and might contribute in the pathogenesis of skin carcinomas (Yang *et al*., 1999; Bergamaschi *et al*., 2003).

iASPP is the most evolutionarily conserved member of the ASPP protein family (Bergamaschi *et al*., 2003). The nomenclature of the ASPP family reflects both the structural signature ankyrin repeat, SH3 domain, and proline-rich containing protein (ASPP) domains shared among members, as well as their function as apoptosis-stimulating protein of p53. Members of the ASPP family: ASPP1, ASPP2 and iASPP resemble each other at their C-terminal ends where the ASPP signature sequences are located (Fig 1.8A). The ASPP proteins are most well known for their regulatory roles in facilitating cell apoptosis mediated by the p53 family.

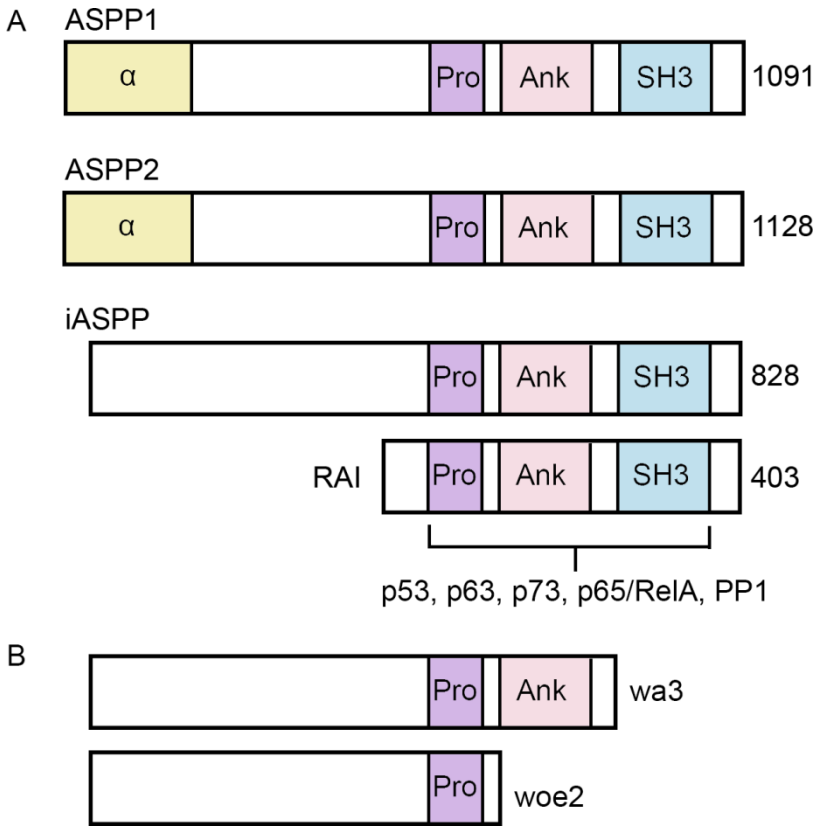


Fig 1.8 The ASPP Protein Family.

(A) Representation of the ASPP1, ASPP2 and iASPP/RAI proteins. All members contain the ASPP signature domain: proline-rich (Pro), ankyrin repeats (ANK) and SH3 motifs at the C-terminal ends. The C-terminal end is responsible for interacting with protein partners such as PP1 and the p53 family. ASPP1 and ASPP2 have an additional α-helical domain (α) at the N-termini.
(B) Representation of the iASPP truncation mutations detected in spontaneous mutant mice wa3 and woe2. Expression of iASPP is disrupted by the wa3 mutation, and the woe2 mutation is likely to have resulted in a complete loss of iASPP function.

The iASPP protein was initially identified as the RelA-associated inhibitor (RAI) protein due to its ability to interact with p65 in a yeast two-hybrid assay and to inhibit the DNA binding activity of p65 when co-transfected in human embryonic kidney cells (Yang *et al.*, 1999; Takada *et al.*, 2002). Subsequently it was discovered that the 403 amino acid RAI represents a shorter

homologue of the 828 amino acid full-length iASPP protein, with which it shares similarities at the C-terminal end and is expressed predominantly. The C-terminal region of iASPP provides the interacting site for proteins such as PP1 (Llanos *et al*., 2011), p65 (Yang *et al*., 1999), and the p53 family consisting of the p53, p63 and p73 members (Bergamaschi *et al*., 2003). The N-terminus of iASPP is required for the cytoplasmic localisation of the protein, which could explain the nuclear localisation of RAI on account of it missing the N-terminus (Yang *et al*., 1999; Slee *et al*., 2004). Moreover, a recent publication has demonstrated that ANK repeat-containing proteins, such as those of the ASPP protein family, can translocate into the nucleus via the importin-independent RanGDP/ankyrin repeat (RaDAR) nuclear import pathway through the interaction between RanGDP and ANK repeats (Lu et al., 2014).

The biological significance of iASPP in skin and other organs is demonstrated by spontaneous mutant mice that were found to harbour recessive mutations in the PPP1R13L gene encoding for iASPP (Fig 1.8B). Two mutant mouse models, known as wa3 and woe2, exhibited open eyelids at birth, abnormal wavy coat and dilated cardiomyopathy (Herron *et al*., 2005; Toonen *et al*, 2012). The wa3 mutation has disrupted RNA and protein expression of iASPP, while the woe2 mutation is likely to have resulted in a complete loss of iASPP function. Histological analysis showed that the open eyelid phenotype was caused by a lack of normal embryonic

eyelid closure. The eyes of adult mice did not have meibomian glands and developed severe corneal opacities with abnormalities in the anterior segment (Toonen *et al*., 2012). Aberrant HF orientation and abnormal hair shafts were associated with the abnormal fur texture of these mice (Herron *et al*., 2005). However, the development of other appendages such as teeth, salivary, mammary and sebaceous glands did not seem to be affected (Toonen *et al*., 2012). Heart from homozygous mutant mice had focal lesions on the ventricles with signs of myofiber degeneration, necrosis and mineralisation (Herron *et al*., 2005).

1.11.1 The p53 Gene Family

The p53 family members p53, p63 and p73 share homology between their N-terminal TA domain, central DBD and C-terminal OD domains (Fig 1.3). The transcription factor p53 is a tumour suppressor that is responsible for regulating gene expression to induce cell cycle arrest or cell death in response to stress (Dötsch *et al*., 2010; Beckerman *et al*., 2010). Under stressed conditions, phosphorylated p53 is released from the E3 ubiquitin ligase MDM2, which normally suppresses p53 activity and targets it for proteasomal degradation. The p53 tetramer is then freed to transactivate pro-apoptotic effectors (e.g. BAX, PUMA and PIG3) or those that result in cell cycle arrest (e.g. p21, 14-3-3σ). This allows the stressed cells to repair damaged DNA during cell cycle arrest, or to undergo permanent cell senescence or apoptosis if the damage is beyond repair (Donehower *et al*., 2009; Beckerman *et al*., 2010).

The ability of p53 to suppress proliferation of damaged cells accounts for the prevalence of p53 mutation observed in a wide range of malignancies such as oesophageal and head and neck SCC (Olivier *et al*., 2010). Patients with Li-Fraumeni syndrome are predisposed to cancer development due to the inheritance of germ-line p53 mutation, while p53-deficient transgenic mice showed higher rates of malignant conversion in chemically induced skin carcinogenesis assays (Kemp *et al*., 1993; Olivier *et al*., 2010). Moreover, p53 mutations have been detected in chronic inflammatory diseases such as RA and IBD, which could be attributed to the DNA damage caused by oxidative stress present in the inflammatory environment (Yamanishi *et al*., 2002).

Although the p63 and p73 homologues have been demonstrated to be able to transactivate certain p53 target genes in inducing cell senescence and apoptosis, they appear to play more important roles in development and differentiation (Yang *et al*., 2002; Dötsch *et al*., 2010). As mentioned previously, the p63 transcription factor is essential in the development and maintenance of stratified epithelia. On the other hand, p73 is fundamental in the development of signalling networks as p73-deficient mice exhibit defects in neurogenesis, pheromone-based social interactions and cerebral spinal fluid homeostasis (Dötsch *et al*., 2010). Their

developmental roles are also supported by the fact that p63 and p73 mutations in human tumours are rarely observed relative to the frequent mutation rate of p53.

1.11.2 The ASPP Protein Family Members

The ASPP proteins are capable of interacting with the p53 family members through their conserved C-termini. ASPP1 and ASPP2 proteins can enhance the transactivation activity of p53 towards pro-apoptotic genes such as BAX, PUMA and PIG3 specifically (Fig 1.9), but do not affect genes related to cell cycle arrest (Samuels-Lev *et al*., 2001; Bergamaschi *et al*., 2006). Moreover, expression of ASPP1 and ASPP2 in rat embryonic fibroblasts can inhibit the transforming activities of oncogenes Ras and E1A (Bergamaschi *et al*., 2003). Therefore ASPP1 and ASPP2 may serve as tumour suppressors by facilitating p53-mediated apoptosis. This is supported by an *in vivo* study of ASPP2 transgenic mice, which illustrated that ASPP2+/- mice had higher tumour incidence over their lifespan compared to the wild type (Vives *et al*., 2006). Furthermore, ASPP2+/- p53+/- double heterozygous mice had advanced onset of tumour development relative to ASPP2+/- single heterozygotes.

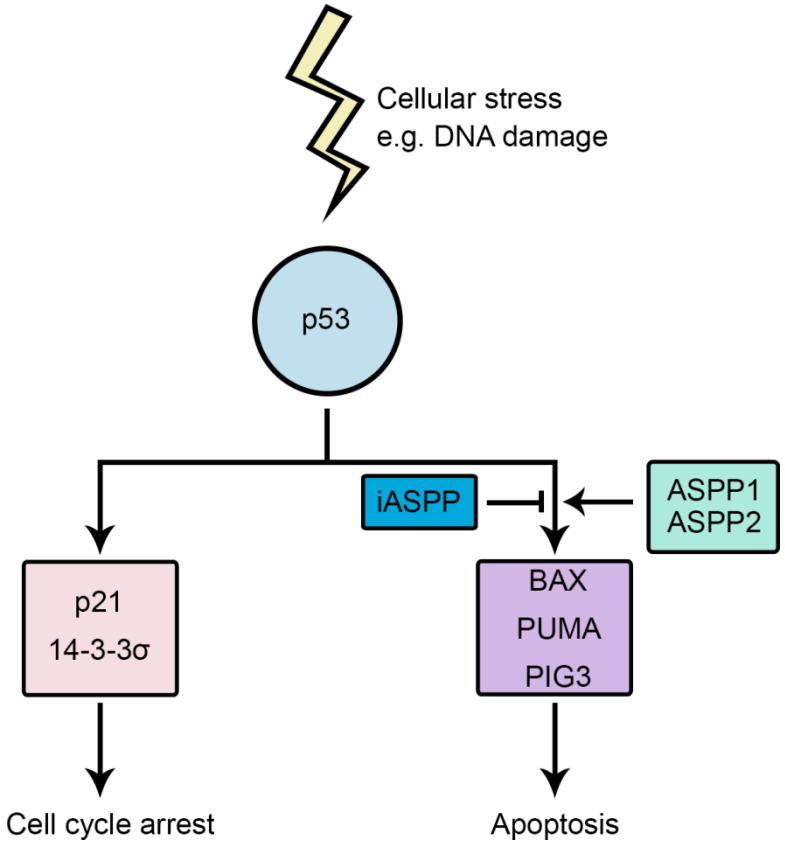


Fig 1.9 Regulation of p53-mediated apoptosis by the ASPP proteins.

p53 is activated in stressed cells to stimulate gene expression that results in either cell cycle arrest at G1 (p21, 14-3-3 σ) or apoptosis (BAX, PUMA, PIG3). The ASPP protein family specifically modulates gene expression associated with p53-mediated apoptosis, but not cell cycle arrest. ASPP1 and ASPP2 positively regulate apoptosis by enhancing p53 activity towards BAX, PUMA and PIG3, whereas iASPP acts as an inhibitor of such activity.

To the contrary, iASPP serves to inhibit p53-mediated apoptosis by specifically suppressing p53 activation of pro-apoptotic genes (Samuels-Lev *et al*., 2001; Bergamaschi *et al*., 2006). Several lines of evidence have suggested the potential role of iASPP as a proto-oncogene. iASPP expression enhances cell transformation abilities of Ras and E1A in rat embryonic fibroblasts (Bergamaschi *et al*., 2003). Overexpression of iASPP in human osteosarcoma cells and breast

cancer cells also offer protection against apoptosis induced by UV radiation and cisplatin treatment. Moreover, an up-regulation of iASPP expression has been reported in human tumour samples such as breast carcinoma and non-small cell lung cancer (Bergamaschi *et al*., 2003; Chen *et al*., 2010).

In addition to the role of the ASPPs in modulating cell apoptosis, increasing evidence has established their involvement in other aspects of biology. ASPP1 takes part in regulating lymphatic vessel development, as ASPP1 knockout mice show abnormalities in the formation and patterning of lymphatic vessels during embryogenesis that recover in adulthood (Hirashima *et al*., 2008). ASPP2 is important in maintaining cell polarity and the integrity of tight junctions through its ability to bind Par3 and to control its apical/junctional localisation (Sottocornola *et al*., 2010; Cong *et al*., 2010). This is backed up by the abnormal development of the central nervous system in ASPP2-deficient mice, which suffer from the dysregulated proliferation of neural progenitor cells and a disorganised neuroepithelium (Sottocornola *et al*., 2010). Our lab has also identified that iASPP localises at the desmosomes of cardiomyocytes in the heart and is important in maintaining the integrity of intercalated discs. This could explain the development of cardiomyopathy, with the abnormal dilation of the right heart ventricle observed in iASPP-deficient mice (Notari, 2012).

1.11.3 iASPP in Epidermal Biology

Besides its interaction with p53, the C-terminal end of iASPP has been demonstrated to bind the DBD of p63 with stronger affinity compared to that of ASPP2 (Robinson *et al*., 2008). In the epidermis, iASPP is expressed in the basal layer where it partially co-localises with nuclear p63 (Chikh *et al*., 2011; Notari *et al*., 2011). Increased levels of loricrin and involucrin mRNA can be detected in iASPP-deficient keratinocytes, which suggests that iASPP is a negative regulator of keratinocyte differentiation (Notari *et al*., 2011). Moderate epidermal thickening of the K1-positive spinous layer and the loricrin-positive granular layer was observed in iASPP-deficient mice. Moreover, signs of early entrance into cellular senescence are observed in iASPP-deficient mouse embryonic fibroblasts (MEFs). Therefore, iASPP plays a role in sustaining normal epidermal homeostasis by regulating gene expression associated with epidermal differentiation and can act to prevent premature senescence. Furthermore, early data has suggested the inhibitory function of iASPP on the NF-κB transcription factor p65 (Yang *et al*., 1999; Takada *et al*., 2002). While the interruption of normal NF-κB signalling is demonstrated to influence skin inflammation and tumour growth, epidermal expression of p65 seems to be essential in mediating cutaneous inflammation and cancer development (van Hogerlindden *et al*., 1999; Lind *et al*., 2004, Kim *et al*., 2014). Although the interaction between iASPP and p65 is unlikely to contribute to epithelial stratification (Notari *et al*., 2011), their

possible involvement in maintaining proper skin immune system and perhaps cancer development has yet to be examined.

1.11.4 The role of p53, p63 and p65 in Skin Carcinogenesis

The speculation of iASPP possibly acting as a proto-oncogene is due to its inhibitory function in suppressing p53 activation of pro-apoptotic genes (Samuels-Lev *et al.,* 2001; Bergamaschi *et al.,* 2006). The involvement of p53 in skin cancer development has been investigated using p53-deficent transgenic mice in various experimental carcinogenesis protocols (Table1.2). p53-deficiency reduced the latency in spontaneous cancer development of predominantly thymic T cell lymphomas and soft-tissue sarcomas (Donehower *et al.,* 1992). Exposure to ionizing radiation further accelerated cancer development in p53-null mice, but did not induce skin tumours (Kemp *et al.,* 1994). Mice deficient of p53 showed no difference in the formation of benign papillomas as compared to wild type animals in DMBA/TPA skin carcinogenesis assay, but exhibited accelerated malignant conversion into SCC (Kemp *et al.,* 1993). However, p53-null mice displayed enhanced susceptibility to UV-induced SCC development, which were associated with premalignant lesions resembling actinic keratoses but not papillomas (Jiang *et al.,* 1999). These models indicated the inhibitory role of p53 in epidermal carcinogenesis, and

also reflected the differences in the biology of cancers developed in DMBA/TPA and UV-mediated tumour induction assays.

The possible involvement of p63 in tumourigenesis remains a controversial topic (Table1.2). The fact that TAp63 shares extensive DBD homology with the tumour suppressor p53 led to speculation that it can act as a tumour suppressor by transactivating p53 target genes. Transgenic p63+/- mice and double mutant p63+/- p53 +/- mice have been utilised to test their susceptibility towards spontaneous tumourigenesis until 24 months of age. One group reported that p63+/- mice in the C57BL/6×129/SvJae background are prone to neoplastic development such as sarcomas and carcinomas compared to wild type. Moreover, p63+/- p53 +/- mice had higher tumour burden compared to p53+/- heterozygous mice (Flores *et al*., 2005). However, the other group that has developed their mouse model in the C57BL/6J×129S5 background failed to observe such a difference in spontaneous tumourigenesis, even in the settings where mice were treated with two-stage DMBA/TPA carcinogenesis protocol (Keyes *et al*., 2006).

Mouse Model	Method	Phenotype	Reference
p53-/-	Spontaneous cancer development	• Significantly reduced latency in spontaneous tumour development • Primarily lymphomas and sarcomas	Donehower *et al.,* 1992
	Ionising radiation	• Further acceleration in carcinogenesis, with similar tumour types observed in untreated p53-/- mice	Kemp *et al.*, 1994
	DMBA/TPA	• No difference in benign papilloma development compared to p53+/+ mice • Accelerated malignant conversion of papillomas	Kemp *et al.*, 1993
	UVB radiation	• Increased susceptibility to SCC development, associated with actinic keratose-like lesions	Jiang *et al.,* 1999
p63+/-	Spontaneous cancer development	• p63+/- mice more susceptible to developing sarcomas and carcinomas • p63+/- p53 +/- mice had higher tumour burden compared to p53+/- mice	Flores *et al*., 2005;
		• No difference in tumour susceptibility between p63+/- and p63+/+ mice	Keyes *et al*., 2006
	DMBA/TPA	• No difference in tumour susceptibility between p63+/- and p63+/+ mice	Keyes *et al*., 2006
Er/+ p63+/−	DMBA/TPA	• Less prone to skin carcinogenesis compared to Er/+ p63+/+ mice	Li *et al*., 2011
TNF-α-/-	DMBA/TPA	• Less prone to developing skin tumours compared to wild type • Ras-transformed IL-1R−/− and p65-/- keratinocytes failed to up-regulate pro-inflammatory gene expression	Moore *et al.,* 1999
IL-1R-/-			Cataisson *et al*., 2012
K14-p65^{EKO}			Kim *et al.,* 2014

Table 1.2 Mouse models used in examining skin carcinogenesis.

While p53 is a common target for deleterious and inactivating mutations in human cancers, such mutations are rarely spotted in the p63 gene (Moll *et al*., 2004). However, the chromosome region harbouring p63 has been found to be amplified in cases of SCC, and p63 overexpression has been detected in head and neck SCC and squamous cell lung carcinomas (DeYoung *et al*., 2007; Dȍtsch *et al*., 2010). As ΔNp63 is the predominant isoform expressed in SCC, it may possibly serve as a proto-oncogene, through its inhibitory influence on the transactivation activity of p53 (DeYoung *et al*., 2007; Dȍtsch *et al*., 2010). A recent study presented data that supported the tumour promoting activity of p63, using the Er/+ mouse model harbouring dominant-negative mutation in the tumour suppressor gene 14-3-3σ (Li *et al*., 2011). The group showed that Er/+ p63+/− mice has increased resistance against DMBA/TPA-induced carcinogenesis when compared with Er/+ p63+/+ mice, and 14-3-3σ deficient keratinocytes had strong expression of the ΔNp63 isoform. This might suggest that ΔNp63 could be involved in cancer development, but in conjunction with other gene mutations to facilitate the process. Further research is required to better understand the biological role of different p63 isoforms in skin cancer development.

Susceptibility towards chemically induced skin carcinogenesis has also been shown to be influenced by alterations in skin immunologic activities (Table1.2). Transgenic mice deficient of

the pro-inflammatory cytokine TNF-α were less prone to developing benign papillomas and malignant SCC (Moore *et al*., 1999). Moreover, IL-1R knockout mice also displayed increased resistance to the DMBA/TPA protocol and Ras-transformed IL-1R−/− keratinocytes could not up-regulate pro-inflammatory gene expression (Cataisson *et al*., 2012). Epidermal-specific p65 knockout mice K14-p65^{EKO}, with reduced expression of pro-inflammatory cytokines and chemokines by keratinocytes, showed increased resistance to chemically induced skin carcinogenesis compared to the wild type (Kim *et al*., 2014). Therefore, epidermal p65 expression is required in mediating skin cancer development by enhancing immunologic activities in the skin and supporting tumour cell growth.

1.12 The Aim of Research

iASPP has been demonstrated to be a binding partner the p53 protein family and can regulate their transactivation activities towards target genes (Bergamaschi *et al.,* 2003). The presence of iASPP expression is observed in both embryonic and postnatal murine skin, and the protein is predominantly expressed in the K14/p63-positive basal layer of the epidermis (Chikh *et al*., 2011; Notari *et al*., 2011). The display of a wavy hair phenotype in iASPP knockout mice emphasises the significance of the iASPP protein in regulating skin homeostasis, and initial evidence has pointed towards a role for it in preventing premature keratinocyte differentiation.

Mild thickening of the suprabasal differentiated epidermal layers was observed in iASPP-deficient mice, and iASPP-deficient keratinocytes showed increased levels of loricrin and involucrin expression (Notari *et al*., 2011).

This research project attempted to investigate whether epidermal iASPP expression is required in the embryogenic epidermal development. Moreover, the project also intended to examine the autonomous role of epidermal iASPP in maintaining normal postnatal skin biology. This was achieved by utilising the K14-iASPP-deficient mouse model with specific iASPP knockout in the K14-positive epidermis, rather than the previously developed complete iASPP-knockout mice. *In vitro* evidence from various studies has suggested that iASPP may act as a proto-oncogene, due to its inhibitory role against p53-mediated apoptosis and its overexpression profiles in certain human cancers. Therefore, another objective of this research project was to address the biological function of iASPP in skin carcinogenesis, and to assess its capacity to behave as a proto-oncogene using the DMBA/TPA chemically induced tumourigenesis protocol on the K14-iASPP-deficient *in vivo* mouse model. Moreover, iASPP was shown to be an inhibitor of p65, which is a NF-κB transcription factor essential in modulating inflammatory responses (Yang *et al*., 1999; Takada *et al*., 2002). Further

examination was made to test for the possible influence of iASPP on p65 in keratinocytes, and whether such interaction would contribute to the phenotypes observed in K14-iASPP-/-skin.

Therefore, this research project aimed to address the following aspects of epidermal iASPP function in skin:

1. The possible involvement of epidermal iASPP in embryonic skin development
2. The autonomous function of epidermal iASPP in postnatal skin biology
3. The biological role of epidermal iASPP in chemically induced skin carcinogenesis
4. The possible interaction between iASPP and p65 in keratinocytes

Chapter 2 Materials and Methods

This chapter provides an overview of the materials and methods used during the course of this doctoral degree. Specific details are included in the relevant results chapters where appropriate. Chemicals used were from Sigma-Aldrich unless otherwise stated.

2.1 Antibodies

For immunocytochemistry/immunofluorescence (ICC/IF) on cell cultures and immunohistochemistry on paraffin sections (IHC-P), antibodies were diluted in either 5% (v/v) normal goat serum (NGS) or normal donkey serum (NDS) in PBS. For western blot (WB) analysis, antibodies were diluted in 5% (w/v) milk in PBS, except for Cell Signalling antibodies which were diluted in 5% (w/v) bovine serum albumin (BSA) instead.

Antigen	Clone	Host	Source	Applications
BrdU	BU1/75	Rat mAb	Abcam (ab6326)	ICC/IF, IHC-P (1:250)
CD3	N/A	Rabbit pAb	Abcam (ab5690)	IHC-P (1:250)
CD31	N/A	Rabbit pAb	Abcam (ab28364)	IHC-P (1:300)
CD45R	RA3-6B2	Rat mAb	BD Biosciences (550286)	IHC-P (1:250)
CD8	N/A	Rabbit pAb	Stratech (bs-0648R-BSS)	IHC-P (1:250)
c-Rel	N/A	Rabbit pAb	Santa Cruz (sc71)	WB (1:500)
Erk1/2	N/A	Rabbit pAb	Cell Signalling (9102)	WB (1:1000)
F4/80	Cl:A3-1	Rat mAb	AbD Serotec (MCA497R)	IHC-P (1:300)

iASPP	LX49.3	Mouse pAb	Ascite, Lu Lab	WB (1:1000)
iASPP	LX49.3	Mouse mAb	Purified ascite, Lu Lab	ICC/IF, IHC-P (1:250)
IKBα	C-21	Rabbit pAb	Santa Cruz (sc371)	WB (1:500)
Keratin-1	AF87	Rabbit pAb	Covance (PRB-149P)	IHC-P (1:1000)
Keratin-6	N/A	Rabbit pAb	Covance (PRB-169P)	IHC-P (1:1000)
Keratin-13	EPR3671	Rabbit mAb	Abcam (ab92551)	IHC-P (1:200)
Keratin-14	AF64	Rabbit pAb	Covance (PRB-155P)	IHC-P (1:1000)
Loricrin	AF62	Rabbit pAb	Covance (PRB-145P)	IHC-P (1:1000)
MPO	E15	Goat pAb	Santa Cruz (sc-34159)	IHC-P (1:300)
p38	N/A	Rabbit pAb	Cell Signalling (9212)	WB (1:1000)
p-p38 (Thr180/Tyr182)	N/A	Rabbit pAb	Cell Signalling (9211)	WB (1:1000)
p53	CM5	Rabbit pAb	Leica (P53-CM5P)	IHC-P (1:250), WB (1:2000)
p63	4A4	Mouse pAb	Santa Cruz (sc8431)	IHC-P (1:500), WB (1:1000)
p65	N/A	Rabbit pAb	Santa Cruz (sc109)	ICC/IF (1:250), WB (1:1000)
p-p65 (Ser536)	93H1	Rabbit pAb	Cell Signalling (3033)	WB (1:1000)
p-Erk1/2 (Thr202/Tyr204)	N/A	Rabbit pAb	Cell Signalling (9101)	WB (1:1000)
PP1	E-9	Mouse mAb	Santa Cruz (sc-7482)	WB (1:1000)
Rel B	C-19	Rabbit pAb	Santa Cruz (sc226)	WB (1:500)
S100a8	N/A	Goat pAb	R&D (AF3059)	IHC-P (1:750)
S100a9	N/A	Goat pAb	R&D (AF2065)	IHC-P (1:750), WB (1:1000)
β-tubulin	TUB2.1	Mouse mAb	Abcam (ab11308)	WB (1:2000)

Table 2.1 List of primary antibodies used during the course of research.

mAb and pAb are abbreviations for monoclonal and polyclonal antibodies respectively.

Secondary Antibodies	Host	Source	Applications
Alexa Fluor® 488 Anti-Mouse IgG (H+L)	Goat	Invitrogen	ICC/IF, IHC-P (1:400)
Alexa Fluor® 488 Anti-Rabbit IgG (H+L)	Goat	Invitrogen	ICC/IF, IHC-P (1:400)
Alexa Fluor® 546 Anti-Mouse IgG (H+L)	Goat	Invitrogen	ICC/IF, IHC-P (1:400)
Alexa Fluor® 546 Anti-Rabbit IgG (H+L)	Goat	Invitrogen	ICC/IF, IHC-P (1:400)
Alexa Fluor® 488 Anti-Rat IgG (H+L)	Goat	Invitrogen	ICC/IF, IHC-P (1:400)
Biotinylated Anti-Mouse antibody	Goat	Vector Labs	IHC-P (1:400)
Biotinylated Anti-Rabbit antibody	Goat	Vector Labs	IHC-P (1:400)
Biotinylated Anti-Goat antibody	Horse	Vector Labs	IHC-P (1:400)
Anti-Mouse Immunoglobulins/HRP	Rabbit	Dako	WB (1:2000)
Anti-Rabbit Immunoglobulins/HRP	Swine	Dako	WB (1:2000)
Anti-Goat Immunoglobulins/HRP	Rabbit	Dako	WB (1:2000)

Table 2.2 Secondary antibodies used during the course of research.

2.2 Animal Studies

Materials:

4% Paraformaldehyde (PFA) Solution

4g paraformaldehyde was dissolved in 1L PBS, and dissolving aided by gentle heating to give 4% PFA. Solution was stored in aliquots at -20°C.

GNT Buffer for Genotyping

GNT buffer was prepared in distilled water with the following components:

50mM KCl

1.5mM $MgCl_2$

10mM Tris HCl pH 8.5

0.01% gelatin

0.45% NP-40

0.45% Tween-20

GNT buffer was autoclaved and stored at 4°C.

Methods:

Intraperitoneal Injection of BrdU

15mg/ml 5-bromo-2-deoxyuridine (BrdU) solution was prepared by dissolving BrdU powder (Sigma-Aldrich) into PBS. The solution was sterilised through a 0.22µm filter (Startorius Ltd.) and stored in aliquots at -80°C. BrdU solution was warmed up before intraperitoneal injections and 1mg/kg BrdU was injected 1 hour prior to sacrificing the animals.

2.3 Mouse Colonies

All animal procedures were approved by local ethical review and licensed by the U.K. Home Office (PIL: 30/9354, PPL: 30/2862). Animals were kept in individually ventilated cages (IVCs) at the Wellcome Trust Centre for Human Genetics, Oxford.

iASPP*loxP*/*loxP* Cre+ERT mouse colonies with 4-hydroxytamoxifen (4OHT) inducible recombinase expression were generated by crossing iASPP *loxP*/*loxP* mice with R26Cre+ERT mice in a mixed genetic background of C57BL/6 and 129/Sv, as described previously (Notari *et al*., 2011). K14Cre-iASPP-/- mutant mice were generated by crossing C57BL/6 transgenic mice containing *loxP* sites flanking iASPP exon8 and mice with the K14-Cre transgene expressing recombinase under the human K14 promoter (The Jackson Laboratory, U.S.) in a C57BL/6 background. The resulting mutant would have specific deletion of iASPP exon8 in K14-positive basal epithelial cells only. Genotyping of K14Cre iASPP-/- mutants and iASPP*loxP/loxP* Cre+ERT mouse colonies was performed using the primers listed in the table below.

Transgene genotyped	Primers used	Bands expected
iASPP exon8	FLP2: 5'-CCGAATTGGAGAAGTGAAGC-3 I8-2: 5'-CCGAATTGGAGAAGTGAAGC-3' E8-2: 5'-AGAGCAGCCTCAGAGCATGG-3'	iASPP+/+: 600bp iASPPΔ8/+:600/ 700bp iASPPΔ8/ Δ8:700bp
loxP	FLP2: 5'-CCGAATTGGAGAAGTGAAGC-3 FRANT9:5'-GGGTAGGAAAAAGGGCTGAG-3'	Wild type: 285bp *loxP*: 400bp
K14-Cre and Actin control	Cre-R: 5'-ATTCTCCCACCGTCAGTACG-3' K14-1: 5'-GCTCTCTGTCACCCTGGCTA-3' Actin-F: 5'-GGTGTCATGGTAGGTATGGGT-3' Actin-B: 5'-CGCACAATCTCACGTTCAG-3'	K14-Cre: 900bp Actin: 780bp
Cre+ERT	Cre-F: 5'-CATTTGGGCCAGCTAAACAT-3' Cre-B: 5'-ATTCTCCCACCGTCAGTACG-3'	Cre: 308bp

Table 2.3 List of primers used to genotype mouse colonies

GoTaq® Green Master Mix (Promega) was used for genotyping PCR reactions. The thermal cycles used for nested PCRs were as follows:

94°C for 5min

30cycles of 94°C for 40s, 60°C for 40s, 72°C for 40s

72°C for 5min

DNA Extraction from Mouse Ear and Tail Biopsies

Ear biopsies of mice were taken for mouse identification and genotyping. Ear tissue was digested in 100µl GNT buffer with 7.8mg/ml proteinase K (Qiagen) at 55°C overnight. Digested tissue was boiled at 97°C for 5minutes and 0.5µl was used for genotyping PCR analysis. The same extraction protocol was used for tail tissue taken from embryos.

DMBA/TPA Two-Stage Skin Carcinogenesis

8-10 week old C57BL/6 female mice at telogen phase were recruited to examine the susceptibility of K14Cre iASPP mice to chemically-induced tumours in the skin. Dorsal hair was carefully removed with a shaver one day before the application of chemicals. A single dose of 25µg *7,12*-Dimethylbenzanthracene (DMBA) in 200µl acetone, or acetone for the *12-O*-tetradecanoyl-13-phorbol acetate (TPA) control group, was applied on shaved dorsal skin. This was followed by twice weekly application of 4µg TPA in 200µl acetone for 15weeks. Animals were monitored closely throughout the assay in terms of weight, activity and tumour

development. Mice could be observed for up to 1 year or when endpoints were reached requiring immediate termination by the Schedule 1 method as listed on the project license.

Epidermal Permeability Barrier (EPB) Assay

Tail tissues from mouse embryos were first taken for genotyping PCR analysis. Unfixed, untreated mouse embryos at E16.5 and E17.5 were transferred up and down a methanol gradient (25%, 50%, 75% and 100% methanol in distilled water) for 1 minute per step at room temperature. Embryos were rehydrated in PBS for 1minute and incubated in 0.1% (w/v) toluidine blue O in water for 10minutes. Embryos were then washed thoroughly with PBS to visualise the EPB staining pattern.

Mouse Hair Samples for Hair Shaft Analysis

Hair samples were gently plucked out of the dorsal skin of sacrificed animals using a pair of forceps. Hair samples were placed on microscope slides with mounting medium (Vectamount, Vector Labs) and mounted with coverslips.

Mouse Skin Tissue for Paraffin Sections

Shaved dorsal skin and papilloma tissues obtained were fixed in 4% PFA overnight at 4°C. Dorsal skin was spread out on grade 1 Whatman filter paper during fixation to keep the section flat. Fixed tissues were processed by routine paraffin embedding using automated devices and serial 4µm sections were cut.

Mouse Skin Tissue for DNA/RNA/Protein Extraction

Mouse tissues collected upon scarification of animals were frozen immediately in liquid nitrogen. Samples were stored at -80°C.

Short-Term TPA Treatment for Acute Skin Inflammation

8-10week old C57BL/6 female mice at telogen phase were recruited to examine the susceptibility of K14Cre iASPP mice to TPA-induced inflammation in the skin. Dorsal hair was carefully removed with a shaver one day before the application of chemicals. 4μg TPA in 200μl acetone, or acetone only for negative control, was applied on days 0 and 4. Mice were sacrificed 24hours after the last TPA application, and injected with BrdU one hour prior to Schedule 1.

2.4 Bacterial Culture

Materials:

Ampicillin Stock Solution

1000x ampicillin stock of 50mg/ml was prepared by dissolving 0.5g ampicillin in 10ml sterile distilled water. Stock was stored in aliquots at -20°C.

Luria-Broth (LB) Agar Plates and LB medium

LB Agar plates with 100µg/ml ampicillin and autoclaved LB media were prepared by the Ludwig Institute for Cancer Research technical services.

Methods:

Sub-cloning Plasmids

Chemically competent Escherichia coli of the alpha-select™ bronze efficiency (Bioline) strain were used as bacterial host for heat shock transformation of plasmids. Competent cells were thawed on ice and 50ng of plasmid DNA was added and incubated for 10 minutes. The mixture was placed in a 42°C water bath for 45 seconds and placed on ice again for 2 minutes. 1ml of LB (without antibiotics) was added and incubated for 1hour at 37°C. 100µl of bacterial culture was spread on LB plates with 100µg/ml ampicillin and incubated at 37°C overnight. Single colonies of bacteria were picked 12-16 hours later and cultured in LB media containing 100µg/ml ampicillin in flasks shaking at 37°C for 4 hours. The bacterial suspensions obtained were used to inoculate 250ml of LB with 100µg/ml ampicillin in a shaker at 37°C overnight. Plasmid DNA was extracted from the bacterial culture with the Qiafilter Maxi DNA kit (Qiagen) according to the manufacturer's protocols.

2.5 Cell Culture

Materials:

Cell Lines

Cell Line	Host	Source
HaCaT	Human, spontaneously transformed keratinocyte	ATCC
HaCaT II-4	Human, malignant variant of HaCaT transfected with H-Ras oncogene	Prof. P. Boukamp, German Cancer Research Centre, Germany
H1299	Human, non-small cell lung epithelial cell	ATCC

Table 2.4 Immortal cell lines used throughout the course of research

Immortal cell lines HaCaT and H1299 used were from Prof. Xin Lu's cryopreserved stock in the lab. All cell lines were tested to be mycoplasma-free before usage, but did not undergo cell line authentication during this research project through single nucleotide polymorphism (SNP) sequencing or next generation sequencing (NGS) analysis.

Chelation of Fetal Bovine Serum (FBS)

80g of Chelex100 resin (Bio-Rad) was added into 1L of distilled deionized water, and the mixture adjusted to pH7.5 with HCl. The resin was filtered through grade 1 Whatman filter paper (GE Healthcare). The filtered resin was added to 500ml of heat inactivated FBS (PAA Laboratories) and mixed at room temperature for a minimum of 2hours to remove divalent cations such as Ca^{2+}. Resin was separated from the FBS with 1MM Whatman filter paper. The

chelated FBS was sterilised using a 0.45µm filter followed by a 0.22µm filter (Startorius Ltd.) under the laminar flow hood. Sterilised chelated FBS was stored in aliquots at -20°C.

FAD Medium for Primary Mouse Keratinocytes

Mouse epidermal keratinocytes were isolated from new-born pups of age P0-P2 and cultured in FAD Medium, which consisted of calcium-free, high glucose, no glutamine Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with the following components:

1.8×10^{-4}M adenine

10% (v/v) chelated FBS

0.5µg/ml hydrocortisone

5µg/ml insulin

10^{-10}M cholera toxin

10ng/ml epidermal growth factor (EGF; Peprotech EC)

100units/ml penicillin, 100µg/ml streptomycin and 250ng/ml amphotericin B

2mM L-glutamine (Gibco, Life Technologies)

$CaCl_2$ was added to a final concentration of 0.05mM for low-calcium FAD medium. FAD medium was stored at 4°C and used within 1 week.

Freezing Medium for Cells

90% (v/v) FBS (PAA Laboratories)

10% (v/v) DMSO

Methods:

Maintenance of HaCaT Human Keratinocytes

Cells were grown in calcium-free, high glucose, no glutamine DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2mM L-Glutamine (Gibco, Life Technologies) and 10% (v/v) chelated FBS. Low-calcium DMEM was obtained by adding $CaCl_2$ to a final concentration of 0.05mM. Cells were maintained in a Heraeus incubator at 37°C in the presence of 5% CO_2.

Maintenance of HaCaT II-4 Human Keratinocytes

DMEM (Gibco, Life Technologies) was supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 2mM L-Glutamine (Gibco, Life Technologies) and 10% (v/v) FBS (PAA Laboratories). Cells were maintained in a Heraeus incubator at 37°C in the presence of 5% CO_2.

Maintenance of H1299 Non-Small Cell Lung Epithelial Cells

H1299 cells were cultured in RPMI-1640 (Gibco, Life Technologies) with 100units/ml penicillin, 100µg/ml streptomycin, 2mM L-Glutamine (Gibco, Life Technologies) and 10% (v/v) FBS (PAA Laboratories). Cultures were maintained in a Heraeus incubator at 37°C with 5% CO_2.

Primary Mouse Keratinocyte Isolation

Primary mouse keratinocytes were prepared from new-born iASPP*loxP/loxP* Cre+ERT mice by floating skin on 0.25% trypsin-EDTA (Gibco, Life Technologies) at 4°C overnight. Detailed protocols for extracting primary keratinocytes have been described previously (Lichti *et al.*, 2008). Keratinocytes were plated on rat collagen type I (BD Bioscience) coated plastic dishes in

low-calcium FAD medium. Culture medium was replaced the next day and iASPP was deleted by incubating cells in 1μM 4OHT for 4 days. Primary mouse keratinocytes were maintained in a Heraeus incubator at 35°C in the presence of 7% CO_2. Keratinocyte cultures were washed with warm PBS twice and incubated in serum-free low calcium DMEM for 16hours before cytokine treatment in the cell-based assays described.

2.6 Cell-Based Assay

Dual-Luciferase Transcription Studies

The dual-luciferase assay involves the sequential quantification of two luminescent reporters from a single sample, with the pGL3 plasmid construct for firefly luciferase and pRL construct for Renilla luciferase. Promoter sequence of the gene of interest is inserted into the pGL3 reporter and the luminescence reading of firefly reflects the activity of such promoter when transfected into cells. Signal from the firefly luciferase is then quenched, along with the activation of renilla luciferase activity which is used as a control reporter to normalise for differences in transfection efficiency and cell viability.

For HaCaT cells, siRNA-mediated gene knockdown was performed 2 days before luciferase plasmid transfection. HaCaT were at ~80% confluency in 24-well plates on the day of transfection. 400ng of S100a9-pGL3 was transfected using Fugene6 (Promega) according to

the manufacturer's protocol. In the case of H1299, cells were seeded at ~70% confluence in 24-well plates and transfected with expression plasmids and luciferase reporters the next day with Fugene6 (Promega). The Dual-Luciferase Reporter Assay System (Promega) was used according to the manufacturer's standard instructions 48hours after transfection. Luciferase activities were measured on an automated luminometer (ClarioStar, BMG Labtech).

Immunocytochemistry (ICC/IF)

HaCaT and HaCaT II-4 cells were cultured on plastic µ-Slide 8 well (Ibidi) slides to ~80% confluence. Cells were fixed in 4% PFA for 15 min at room temperature and washed with PBS three times. Cells were incubated in 0.25% triton-X100 in PBS for 10minutes at room temperature for cell permeabilisation. Samples were washed three times with PBS for 5minutes, and blocked in 5% NGS in PBS for 1hour at room temperature. Cells were then incubated with primary antibodies diluted in 5% NGS for an hour at room temperature in a humidified chamber. Cells were washed three times with PBS, and incubated with secondary antibody in 5% NGS for 1hour at room temperature in the dark. Cells were washed again with PBS three times, and covered with Fluoromount-G mounting medium (Southern Biotech) with 1µg/ml DAPI.

Plasmids for Cell Transfection

Plasmid	Description	Source
pGL3	Luciferase plasmid backbone	Prof. Xin Lu, LICR Oxford

S100a9-pGL3	Luciferase reporter containing the Human S100a9 promoter (-786bp to +39bp)	Prof. Zhihua Liu, Chinese Academy of Medical Sciences (Li *et al*., 2009)
pcDNA3	Control plasmid	Prof. Xin Lu, LICR Oxford
pcDNA3-p65	Expression plasmid for Human p65	Prof. Xin Lu, LICR Oxford
pRL-TK	*Renilla* luciferase plasmid	Promega

Table 2.5 Plasmids used in luciferase reporter assays.

Plasmids were sent to Source BioScience Sequencing to verify plasmid constructs through sequencing.

Stimulation with Cytokines

Human and mouse TNF-α, IL-1α and IL-1β (Peprotech) cytokines were used at a final concentration of 10ng/ml in cell cultures. Cells were washed with warmed PBS twice and incubated in serum-free medium for 16 hours before cytokine treatment.

siRNA Transfection

siRNA oligonucleotides (Set of 4 Upgrade: ON-TARGETplus) against human iASPP and p65, and negative control siGENOME RISC-free were purchased from Dharmacon, GE Healthcare. siRNA at a final concentration of 25nM (p65 siRNA) or 50nM (iASPP siRNA) was transfected into cells using Dharmafect 1 reagent (Dharmacon, GE Healthcare) according to the manufacturer's protocol. Cells were examined 4days after transfection.

siRNA	Catalogue no.	Sequence
Human iASPP	LU-003815-00-0002	AGUAAAGUCUAGCAGGAUA GCACGGGUGUUGGCGGAAA GCAGACGUCGAGCAGAGUA UCGAGAAGUGCGACCCUUA

Human p65	LU-003533-00-0002	GAUGAGAUCUUCCUACUGU CAAGAUCAAUGGCUACACA GGAUUGAGGAGAAACGUAA CTCAAGAUCUGCCGAGUGA
RISC-free	D-001220-01-05	Sequence is a property of Dharmacon

Table 2.6 Sequences of siRNA probes used.

2.7 DNA and RNA Techniques

Materials:

Ammonium Persulphate (APS)

10% (w/v) APS stock solution was dissolved in distilled water and prepared fresh for each experiment.

EDTA Solution

0.5M EDTA stock solution was prepared by dissolving 186g of EDTA in 700ml distilled water. The solution was adjusted to pH 8 with NaOH and the volume topped up to 1L.

Ethidium Bromide (EtBr)

0.2g EtBr was dissolved in 20ml of distilled water for a 50000x stock solution of 10mg/ml, which was stored at 4°C in the dark.

50x Tris-Acetate EDTA (TAE) Solution

242g Tris base

57.1ml Glacial acetic acid

100ml 0.5M EDTA pH 8

Solution adjusted to ~pH 8.5

10x Tris-Borate EDTA (TBE) Solution

108g Tris base

55g boric acid

40ml 0.5 M EDTA pH 8

Adjusted solution to ~pH 8.3

Methods:

Agarose Gel Electrophoresis

A 1% agarose gel to resolve PCR products of size 0.5-10kb was prepared by dissolving agarose powder (Gibco, Life Technologies) in 1xTAE solution with a microwave. EtBr was added at a final concentration of 20µg/ml to the dissolved gel before pouring into a casting tray with a well comb in place. 10µl of PCR product was loaded onto the gel and a 1kb ladder (New England Biolabs) was used as a size marker. DNA was electrophoresed in 1xTAE buffer at 100V for approximately 30min. A BioDoc-it benchtop trans-illuminator (UVP) was used to visualise the PCR products.

cDNA Conversion of RNA

500ng of total RNA sample was used for cDNA conversion with the SuperScript II First Strand Synthesis System (Invitrogen). Oligo(dT) primers were used for cDNA synthesis according to the manufacturer's protocols.

DNA Extraction from Mouse Skin/Papilloma Tissue

DNA was extracted from mouse skin and papilloma tissues with a RNeasy Lipid Tissue Mini Kit (Qiagen) according to the manufacturer's protocol, which is suitable for DNA extraction from fibrous skin tissues.

Detection of Ras Mutation in Tissue Samples from the DMBA/TPA Mouse Cohort

The application of the carcinogen DMBA on mouse skin could cause an activating mutation on the 61st codon of H-Ras from the wild-type CAA allele to a CTA mutant allele. Nested PCR and Restriction Fragment Length Polymorphism (RFLP) analysis were used to characterise the presence of activated H-Ras mutation in DNA samples extracted from mouse tissues as described previously (Finch *et al*., 1996). The H-Ras 61st codon was amplified using PCR1_F and PCR1_R primers on 500ng of the extracted DNA sample to give a 267bp fragment. Nested PCR was carried out on the PCR product with primers PCR2_F and PCR2_R to give a 176bp product.

Primers	Sequence
PCR1_F	5'- CTGTGA ATTCTCTGGTCTGAGGAG-3'
PCR1_R	5'- TAGGTGGCTCACCTGTACTG-3'

PCR2_F	5'-CTA AGCCTGTTGTTTTGCAGGAC-3'
PCR2_R	5'-GGA ACTTGGTGTTGTTGATGGC-3'

Table 2.7 Primers used in nested PCR for Ras mutation.

GoTaq® Green Master Mix (Promega) was used for PCR reactions, and the thermal cycles used for nested PCR were as follows:

94°C for 5min

30cycles of 94°C for 40s, 60°C for 40s, 72°C for 40s

72°C for 5min

10µl of the nested PCR product was digested with 10units of XbaI (New England Biolabs) in a total volume of 50µl according to the manufacturer's protocol at 37°C overnight. Restriction enzymes were heat inactivated at 65°C for 20minutes and the digested products analysed on a 8% polyacrylamide gel.

Polyacrylamide DNA Gel Electrophoresis (for Short DNA Fragments)

Recipe for 10ml of 8% polyacrylamide gel:

1ml 10x TBE solution

70µl APS (10%w/v)

2ml 40% 29:1 acrylamide solution

6.26ml water

4µl TEMED

To resolve PCR products of size 60-400bp, a 8% polyacrylamide gel was prepared according to the above recipe and left to set at room temperature with a well comb in place. 10µl of PCR

product was loaded onto the gel and a 25bp ladder (New England Biolabs) was used as a size marker. DNA was electrophoresed in 1xTBE buffer at 80V for approximately 60min. The gel was soaked in 1:10^5 SyBr Green I stock (Invitrogen) diluted in 1xTBE for 40min in the dark at room temperature, and visualised with a BioDoc-it benchtop transilluminator (UVP).

Quantitative Polymerase Chain Reaction (qPCR)

Real time qPCR was performed on cDNA samples using the QuantiTect SyBr Green PCR Kit (Qiagen) on the 7500 real time PCR system (Applied Biosystem). Each qPCR reaction was carried out in duplicates using 2.5µl of 1:5 diluted cDNA samples in a 25µl reaction volume. The thermal cycle: 10 minutes at 95°C followed by 40 cycles of 95°C for 30s, 58°C for 40s, 72°C for 40s, and 72°C for 5 minutes elongation was used for all samples. The expression level of target genes was analysed using the comparative Ct method ($\Delta\Delta$Ct) with GAPDH as the internal control (Yuan *et al*., 2006). The experiment was repeated on three independent cultures of primary mouse keratinocytes.

Gene	**qPCR Primer Sequence**
CXCL1	F: 5’- CCAGAGCTTGAAGGTGTTGC-3’ R: 5’- TCTCCGTTACTTGGGGACAC-3’
GAPDH	F: 5'-CAGCAAGGACACTGAGCAAGA-3' R: 5'-GGCCCCTCCTGTTATTATGG-3'
iASPP	F: 5’-AGACTCCAGCACCTCAACAGA-3’ R: 5’-GACCGTGCCTTCATCTGC-3’

IL-1α	F: 5’-ATCAGCAACGTCAAGCAACG-3’ R: 5’- AGGTGCTGATCTGGGTTGGA-3’
IL-1β	F: 5’-CATCTCGGAGCCTGTAGTGC-3’ R: 5’-CGTGGACCTTCCAGGATGAG-3’
IL-6	F: 5’- AGAAGGAGTGGCTAAGGACCA-3’ R: 5’-AACGCACTAGGTTTGCCGA-3’
IL-1f6	F: 5'-CCCACTCATTCTGACCCAAG-3' R: 5'-GAGAGAGTGCCACAGAGCAA-3'
IL-1f8	F: 5'- TGCCTGCTGTCATAACTTCG-3' R: 5'-CAAGATTGTGGAGGGAAAGC-3'
S100a8	F: 5'-CCTTTGTCAGCTCCGTCTTC-3' R: 5'-TAGAGGGCATGGTGATTTCC-3'
S100a9	F: 5’- GCTGCATGAGAACAACCCAC-3’ R: 5’- TCCCTTTAGACTTGGTTGGGC-3’
TNF-α	F: 5’-GGCAGGTCTACTTTGGAGTCATTGC-3’ R: 5’-ACATTCGAGGCTCCAGTGAATTCGG-3’

Table 2.8 Primers for qPCR on cDNA samples from mouse keratinocytes

RNA Extraction from Cells

RNA was extracted from cells with the RNeasy Mini Kit (Qiagen) following the manufacturer’s protocols. On-column DNase I digestion was performed during RNA extraction.

2.8 Histology Techniques

Materials:

Sodium Citrate Buffer

10mM sodium citrate solution was prepared in water and adjusted to pH 6.

DAPI Staining Solution

DAPI powder was dissolved in distilled water at 1mg/ml and stored in aliquots at -20°C.

Toluidine Blue Stock Solution

1g Toluidine Blue O

100ml 70% alcohol

Stock solution to be used within 6 months.

Methods:

Haematoxylin and Eosin (H&E) Staining

Paraffin sections were deparaffinised in 2 changes of histoclear, rehydrated in a gradient of ethanol (100%, 90%, 70%, 30%) and washed in water. Slides were incubated in Harris haematoxylin for 3 minutes. Slides were rinsed in running tap water to remove excess stain, and differentiated in 1% acidic alcohol for 1s. Slides were washed again in tap water and immersed in Scott's water for 30s. Slides were rinsed in tap water and stained in eosin for 4 minutes. Slides were then washed in water to remove excess eosin and dehydrated with an increasing gradient of ethanol solutions. Slides were cleared via 2 changes of 5 minutes in histoclear, and mounted with non-aqueous mounting medium (Vectamount, Vector Labs) and coverslips.

Immunohistochemistry (IHC) on Paraffin Sections

Paraffin sections of mouse tissues at a thickness of 4µm were deparaffinised and hydrated. For 3, 3'-diaminobenzidine (DAB) staining, endogenous peroxidases were inactivated by incubating in 3% (v/v) H_2O_2 in methanol for 10minutes at room temperature. This step was not performed for IF staining. Heat-induced antigen retrieval was performed in sodium citrate buffer at 100°C for 4 minutes with a pressure cooker. Slides were left in the buffer to cool, and samples were blocked with 5% NGS/ NDS in PBS for 1 hour at room temperature. Sections were incubated in primary antibody diluted in 5% NGS/ NDS overnight at 4°C inside a humidified chamber. Slides were washed 3 times in PBS for 15 minutes, and secondary antibodies diluted in 5% NGS/ NDS were added for 1 hour in the dark at room temperature.

For IF staining, 1µg/ml DAPI was added along with the appropriate fluorophore conjugated secondary antibodies in the blocking solution. Slides were washed with PBS three times and mounted with fluoromount-G mounting media (Southern Biotech) and coverslips.

For DAB staining, biotinylated secondary antibodies were used instead and slides were washed in PBS 3 times for 15 minutes after incubation. Sections were then incubated in avidin-biotin peroxidase solution (VECTASTAIN Elite ABC Reagent, Vector Labs) for 15 minutes at room temperature. Slides were washed in PBS, 3 times for 5 minutes each. Sections were then incubated in HRP substrate solution (DAB substrate kit, Vector Labs) for 10 minutes at room temperature. Slides were rinsed in water, counterstained in haematoxylin for 5 seconds and

washed again in water to remove excess staining. The slides were dehydrated through an increasing gradient of ethanol, cleared in 2 changes of histoclear for 5 minutes and mounted with mounting medium (Vectamount, Vector Labs) and coverslips.

Toluidine Blue Staining for Mast Cells

Working toluidine blue solution was prepared fresh by diluting toluidine blue stock solution 1:10 in 1% (w/v) sodium chloride aqueous solution. Paraffin sections were deparaffinised and rehydrated as in H&E staining. Slides were incubated in working toluidine blue solution for 1-2 minutes and were washed 3 times in water for 5 minutes each. Slides were then dehydrated in ethanol gradients and cleared in histoclear as described in the H&E staining protocol.

Histological Analysis of Mouse Skin Tissue and Papillomas

Histological analyses of H&E stained mouse skin and papilloma tissue sections were conducted in collaboration with Dr. R. Asher, consultant dermatopathologist at the Oxford John Radcliffe Hospital.

2.9 Human Patient Samples

Paraffin sections of Human skin samples from normal skin, psoriasis and eczema patients (9 cases each) were obtained from the Oxford Centre for Histopathology Research (OCHRe)

under ethical approval (NRES approval: 09/H0606/78) and in collaboration with Dr. R. Asher, consultant dermatopathologist at the Oxford John Radcliffe Hospital.

2.10 Protein Analysis

Materials:

COmplete™ Protease Inhibitor Cocktail

One COmplete™ protease inhibitor tablet (Boehringer Mannheim, Germany) was dissolved in 2ml of sterile distilled water to form a 25x stock solution.

NETN/NP-40 Buffer

50mM Tris pH 8

150mM NaCl

1mM EDTA

1% (v/v) NP-40

Buffer stored at 4°C. Protease and phosphatase inhibitors were added before use.

Phosphatase Inhibitor

One PhosSTOP phosphatase inhibitor tablet (Roche) was dissolved into 10ml of cold NETN/NP-40 or RIPA lysis buffer before use.

RIPA Buffer

150mM NaCl

1% (v/v) NP-40

0.1% (w/v) SDS

50mM Tris base

Buffer stored at 4°C. Protease and phosphatase inhibitors were added before use.

5x SDS-PAGE Loading Dye

250mM Tris HCl

10% (w/v) SDS

50% (v/v) Glycerol

12.5% (v/v) β-Mercaptoethanol

0.5% (w/v) Bromophenol blue

10x SDS-PAGE Running Buffer

720g Glycine

150g Tris

50g SDS

Adjusted to 5L with distilled water

10x SDS-PAGE Transfer Buffer

725g Glycine

145g Tris

Adjusted to 5L with distilled water for 10x stock. 1x buffer was prepared in 20% ethanol.

Stripping Buffer

62.5mM Tris HCl pH 6.7

100mM β-mercaptoethanol

2% SDS

Adjusted to 250ml in distilled water. Buffer was prepared fresh and used on membranes, rocking at 55°C for 30 minutes.

10x Tris Buffered Saline Tween (TBS-T)

121g Tris base

36.53g NaCl

250ml Tween-20

10x solution adjusted to pH 7.6 with HCl with a total volume of 5L.

UREA Lysis Buffer

8M Urea

1M Thiourea

0.5% (w/v) CHAPS

50mM Dithiothreitol (DTT)

24mM Spermine

Methods:

Determining Protein Concentration

Protein concentrations of cell lysates were determined using the Bio-Rad Protein Assay (Bio-Rad). 1µl of cell lysate was added into 200µl 1x Bio-Rad Assay Reagent on a 96-well plate in duplicates, and measurements were taken at 595nm with a spectrophotometer (Anthos Labtech). Readings from increasing known concentrations of BSA (Sigma-Aldrich) in the Bio-Rad Assay Reagent were taken to generate a standard curve.

Immunoprecipitation (IP) Assay

Protein G sepharose beads (Pharmacia Biotech) stored in 20% ethanol at 4°C were washed with cold PBS three times and suspended in a volume of cold PBS before use. Cell cultures were washed with PBS three times and lysed in cold NETN/NP-40 or RIPA lysis buffer. Cell lysate was obtained by scraping with a sterile disposable cell scraper (Greiner) and left on ice for 30 minutes. Cell lysate was centrifuged at 15000g for 30 minutes at 4°C for the supernatant. Protein concentration in the supernatant was measured with the Bio-Rad Assay System (Bio-Rad). 1mg of cell lysate was pre-cleared with 50% of slurry protein sepharose beads in PBS for 30-60 minutes rotating at 4°C. The lysate was then centrifuged at 2000g for 2 minutes and the supernatant transferred into a fresh tube. Around 2µg of purified antibody or 2-5µl of pAb antiserum/serum was added to the 1mg pre-cleared lysate and left on a rotator at 4°C overnight.

Immuno-complexes were obtained by centrifugation at 2000g for 3 minutes and the supernatant was discarded. The beads were washed with cold NETN/NP-40 or RIPA lysis buffer three times. After removing the residual supernatant, the IP beads were mixed with 30µl of 5xSDS-PAGE sample buffer and boiled at 95°C for 5 minutes. The mixture was centrifuged briefly at 15000g and the supernatant loaded onto a SDS-PAGE gel for protein analysis.

Protein Sample Preparation from Cells

Cell cultures were washed twice with PBS, and lysed in the appropriate lysis buffer for specific assays. Cells were scraped with a sterile disposable cell scraper (Greiner) and transferred to an autoclaved Eppendorf tube. Protein lysate was sonicated briefly with 3 pulses of 10s each to shear DNA, and sample was left on ice for 30 minutes with occasional vortexing. The lysate was cleared by centrifugation at 15000g for 20 minutes at 4°C to remove insoluble cell debris and the resulting supernatant was used for protein analysis.

SDS-Polyacrylamide (SDS-PAGE) Gels

SDS-PAGE gels were made using the Mini-PROTEAN® Tetra Handcast Systems (Bio-Rad) according to the recipe in Table 2.9. The resolving gel was overlaid with isopropanol and left to polymerise, after which the isopropanol was removed before the placement of the stacking gel and well comb.

For 10ml total volume:	Resolving SDS-PAGE gel					Stacking
Acrylamide concentration/%	6	8	10	12	15	4
Separation Range/kDa	50-200	25-200	15-100	10-70	12-45	N/A
30% Acryl/Bis 37.5:1 Acrylamide/ml	2	2.7	3.3	4	5	1.3
1.5M Tris HCl pH 8.8/ml	2.5					N/A
1M Tris HCl pH6.8/ml	N/A					2.5
10% SDS/µl	100					100
10% APS/µl	100					100
TEMED/µl	10	8	5	4	4	10
Distilled H_2O/ml	5.3	4.6	4	3.3	2.3	6.1

Table 2.9 Compositions of SDS-PAGE gels

Preparation of Protein Lysates for SDS-PAGE

5x SDS-PAGE sample loading buffer was added to protein lysates boiled at 95°C for 5 minutes. Samples were collected by brief centrifugation and loaded onto SDS-PAGE gels along with a broad-range pre-stained protein marker (New England Biolabs). Gels were run in 1x SDS-PAGE running buffer with the Mini-PROTEAN Tetra cell (Bio-Rad) at a constant voltage of 100-135V.

Immunoblotting

Proteins separated in SDS-PAGE gels were transferred to nitrocellulose membrane (Whatman) by wet blotting in a Hoefer Transphor Electrophoresis unit. Protein transfer was carried out at 4°C for 2.5 hours at 85V, or overnight at 30V. The membrane was quickly stained with Ponceau S solution (Sigma-Aldrich) to confirm the successful transfer of proteins. The membrane was washed in distilled water and blocked in 5% (w/v) fat-free milk (Marvel) in 1x TBS-T solution for 1hour at room temperature with rocking. Primary antibody diluted in 5% (w/v) milk or 5% (w/v) BSA was added to the membrane for overnight incubation at 4°C or 3 hours at room temperature with rocking. The membrane was washed with 1xTBS-T three times for 5 minutes each, and incubated with secondary HRP-conjugated antibody at room temperature for 1 hour. The membrane was washed again three times with 1xTBS-T for 15 minutes, and incubated in enhanced chemiluminescence (ECL) western blot detection reagents (Amersham Pharmacia

Biotech) for 1 minute at room temperature. Proteins were visualised with the film developer in the dark room using Fujifilm autoradiography films (Fisher Scientific).

2.11 Data Analysis from Images

All autoradiographs from western blots were scanned using an Epson perfection 1660 photo scanner. Adobe Photoshop 7.0 and ImageJ software were used to manipulate images only as a whole for size, brightness and contrast. No signal was modified in relation to the raw image otherwise.

For cell counting from DAB/immunofluorescence stained tissue sections, averaged data for each tissue sample was collected from three different tissue sections at least 40µm apart. Data was obtained from at least three different fields of view under the microscope for each section. Positive staining was quantified by automated counting using ImageJ analysis software, with technical assistance from Mark Shipman. Positively stained immune cells were quantitatively analysed using the publically available Yen thresholding algorithm on ImageJ, with settings of circularity 0.25-1 and size 100-10000 on all samples. The numbers of positive stained immune cells were then divided by the area of skin section to obtain the density of immune cell population within each sample.

2.12 Statistics

Statistical analysis was carried out using Microsoft Excel, GraphPad Prism and IBM SPSS. Differences were considered significant at a p-value of $p \leq 0.05$. All error bars shown in graphs represented standard errors of the mean (SEM).

The log-rank (Mantel-Cox) test was performed to determine the statistical significance of tumour-free survival of K14-iASPP mice in the DMBA/TPA cohort (Bland and Altman, 2004). Mixed-design/split plot ANOVA for repeated measure generalised linear model was used in testing data on papilloma number per mouse. One-way ANOVA was utilised to analyse human patient sample immunofluorescence data. Two-tailed paired t tests were carried out for qPCR analysis, and unpaired t tests for measurements from immunohistochemistry staining on mouse tissues. The assumption of normal data distribution was taken, and parametric tests were selected as rank based methods could not be used for the small sample sizes in this research project (Bland and Altman, 2009). I have noted that the small sample size was insufficient to examine the normal distribution shape of data points, and a larger sample size would be required to confirm such assumption.

Chapter 3 iASPP Deficiency in K14-Positive Basal Epidermis Resulted in Wavy Coats, Open Eyelids at Birth and Abnormal Skin Immunohomeostasis

3.1 Introduction

The expression of iASPP in skin is detected predominantly in the K14-positive basal epidermal layer (Chikh *et al*., 2011; Notari *et al*., 2011). These proliferative basal keratinocytes also express the transcription factor p63, upon which iASPP exerts a negative regulatory effect over p63-mediated activation of epidermal differentiation. The epidermal differentiation programme was impaired in iASPP-deficient keratinocytes, with enhanced expression of the loricrin and involucrin genes. In addition, iASPP deficiency in MEFs induced premature senescence as shown by a SA-β-gal assay performed on primary cell cultures (Notari *et al*., 2011). Moreover, the presence of NF-κB transcription factor p65 in the basal epidermis has been reported previously (Budunova *et al*., 1999; Kim *et al*., 2014). Early studies have shown the possible suppressive role of iASPP on p65 transcriptional activity (Yang *et al*., 1999; Takada *et al*., 2002), and further investigation is needed to see if iASPP and p65 interact in the basal epidermal layer.

Our lab has previously generated a transgenic mouse model with complete ablation of iASPP expression (iASPP-/-) in a mixed C57BL/6x129Sv background (Notari *et al*., 2011). This was done by crossing mice carrying constitutive Cre recombinase expression with those that have *loxP* sites flanking exon 8 of iASPP. Deletion of iASPP exon 8 led to the loss of iASPP protein expression in the mutants. These iASPP-/- animals exhibited a wavy coat and open eyelids at birth phenotype with complete penetrance (Notari, 2012). Adult iASPP-deficient mice had cataracts in the eyes, which could be secondary to the open eyelids phenotype leaving the eyes unprotected during development. Modest epidermal thickening of the K1-positive spinous layer and the loricrin-positive granular layer was also detected in these iASPP-deficient animals, corresponding to the defective epidermal differentiation detected in iASPP-/- keratinocytes (Notari *et al*., 2011). Moreover, iASPP-deficient mice exhibited dilated cardiomyopathy with focal opaque white plaques covering the heart surface (Notari, 2012). The iASPP-/- mutants were susceptible to sudden death from 10 weeks of age due to impaired cardiac function. These abnormalities were highly similar to the phenotypes of the two spontaneous mutant mouse models wa3 and woe2, which had previously been reported to carry truncation mutations in the iASPP gene (Herron *et al*., 2005; Toonen *et al*, 2012).

The cardiac defects of iASPP-deficient mice impede any long-term animal study, for example to monitor possible spontaneous tumour development later in life. In order to study the biological role of iASPP in skin carcinogenesis and to circumvent the early deaths of iASPP-deficient mice, a new transgenic mouse model with specific iASPP deletion in the K14-positive basal epidermis was generated in this project. Moreover, it has been demonstrated that other cell types such as MEFs express iASPP, and that a lack of iASPP could lead to the premature senescence of these cells (Notari *et al*., 2011). The expression of iASPP transcripts was also detected in dermal fibroblasts of the murine skin. Such K14-iASPP-deficient mouse models would facilitate research into the autonomous role of iASPP specifically in the basal epidermal layer. The interaction between epidermal and dermal components is of particular importance in the development of epidermal appendages in the skin (Schneider *et al*., 2009; Sotiropoulou *et al*., 2012). The development of K14-iASPP-/- mice would enable the investigation of whether the skin phenotype observed in total iASPP knockout animals was dependent on the biological activity of iASPP in basal keratinocytes, where iASPP could possibly influence the transcriptional activities of its binding partners p63 and p65.

3.2 Results

3.2.1 K14-iASPP-Deficient Mice Displayed Wavy Coat and Open Eyelids at Birth, but not Dilated Cardiomyopathy

Basal epidermis-specific iASPP knockout mice were generated by crossing mice that carried the Cre recombinase gene driven by an epidermis-specific promoter, with mice carrying the floxed iASPP exon8 gene construct (Fig 3.1A). Our lab purchased K14-Cre transgenic mice with a keratin 14 promoter directing the expression of Cre recombinase. Stratification of the mouse embryonic skin and the expression of K5/K14 keratins commences at E12.5, during which expression of Cre recombinase under the K14 promoter should be induced (Koster et al., 2004, Laurikkala et al., 2006). The loss of iASPP signal in K14-iASPP-/- embryonic skin demonstrated successful knockout of iASPP by E16.5. This enabled deletion of iASPP exon8 and the ablation of iASPP protein expression in tissues expressing keratin 14, such as the skin, eyes and tongue.

The resulting K14-iASPP-/- mutant in a pure C57BL/6 background presented the sparse wavy coat phenotype with full penetrance (K14-iASPP+/+ n=35, -/- n=57). This indicated the significance of iASPP expression in keratinocytes with regard to enabling normal skin homeostasis and HF development. About half of the K14-iASPP-deficient adults also had eye cataracts, but none of the mutants had opaque plaques on the heart (Fig 3.1B, C). K14-iASPP-/-

mutants were left to age to over a year old, and none of the animals were lost due to sudden death. The abnormal coat texture and eye defects remained in aged K14-iASPP-/- animals, which showed no signs of spontaneous skin tumour development.

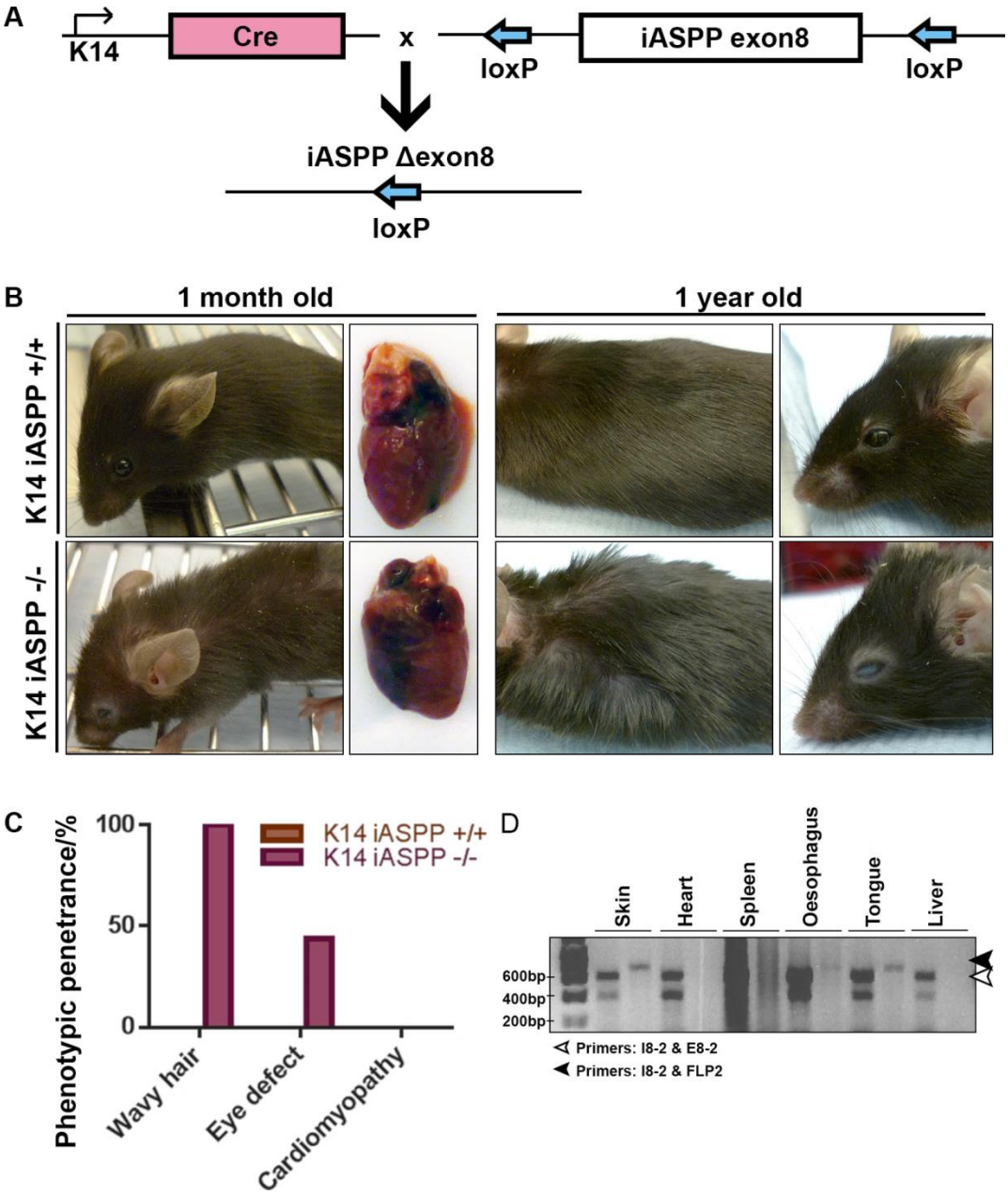


Fig 3.1 Mice with specific iASPP deletion in K14-positive epidermis displayed wavy hair and eye defect phenotypes, but not cardiomyopathy.

(A) Schematic illustrating the generation of iASPP conditional knockout mice K14-iASPP-/- by

crossing mice with Cre recombinase expression under the K14-promoter and those with *loxP* flanking exon 8 of the iASPP gene.
(B) K14-iASPP-/- mice had abnormal sparse wavy coat and eye defects similar to those observed in complete iASPP knockout mice, but did not show any heart abnormalities.
(C) The wavy hair phenotype showed complete penetrance in K14-iASPP-/- mice, but only 44% of K14-iASPP-/- postnatal mice had eye defects (K14-iASPP+/+ n=35, -/- n=57).
(D) PCR analysis of the iASPP gene performed on DNA samples extracted from different organs of the K14-iASPP-/- mouse. The white arrow refers to the 600bp band generated from the PCR reaction using primers I8-2 and E8-2, which indicates the presence of wild type iASPP exon 8. The black arrow points to the 700bp band derived from the PCR reaction using primers I8-2 and FLP2, which indicates the deletion of iASPP exon8.

DNA samples were extracted from various organs of K14-iASPP-/- mice and examined for iASPP exon 8 deletion by PCR analysis (Fig 3.1D). Tissues that consist of stratified squamous epithelium such as the skin, tongue and oesophagus are known to express K14 and hence the Cre recombinase would be activated to facilitate iASPP ablation. The presence of a 700bp band from PCR reactions on these samples indicated that *loxP* recombination of iASPP exon8 had taken place (Fig 3.1D). Such a band was not present for other tissue samples such as the heart, spleen and liver. Result from the PCR analysis confirmed the specificity of iASPP exon 8 deletion, mediated by the activity of K14-Cre recombinase.

3.2.2 Epidermal iASPP Deficiency did not Impair Embryonic Skin Development

Embryonic skin samples were taken from K14-iASPP+/+ and -/- embryos at E16.5-E17.5 to identify any developmental defects in the skin. Histological analysis through H&E staining

showed no clear difference in the epidermal structure between wild type and K14-iASPP-deficient mice (Fig 3.2A).

The expression profile of iASPP during embryonic skin development was analysed using immunofluorescence. The loss of any iASPP signal in K14-iASPP-/- embryonic skin demonstrated successful knockout of iASPP by E16.5. iASPP present in embryonic mouse skin was predominantly cytoplasmic in the K14-positve basal epidermis (Fig 3.2B). Unlike the adult murine skin in which iASPP was predominantly basal (Notari *et al*., 2011), cytoplasmic and junctional iASPP expression was also detected in the K14-negative suprabasal layers of embryonic skin.

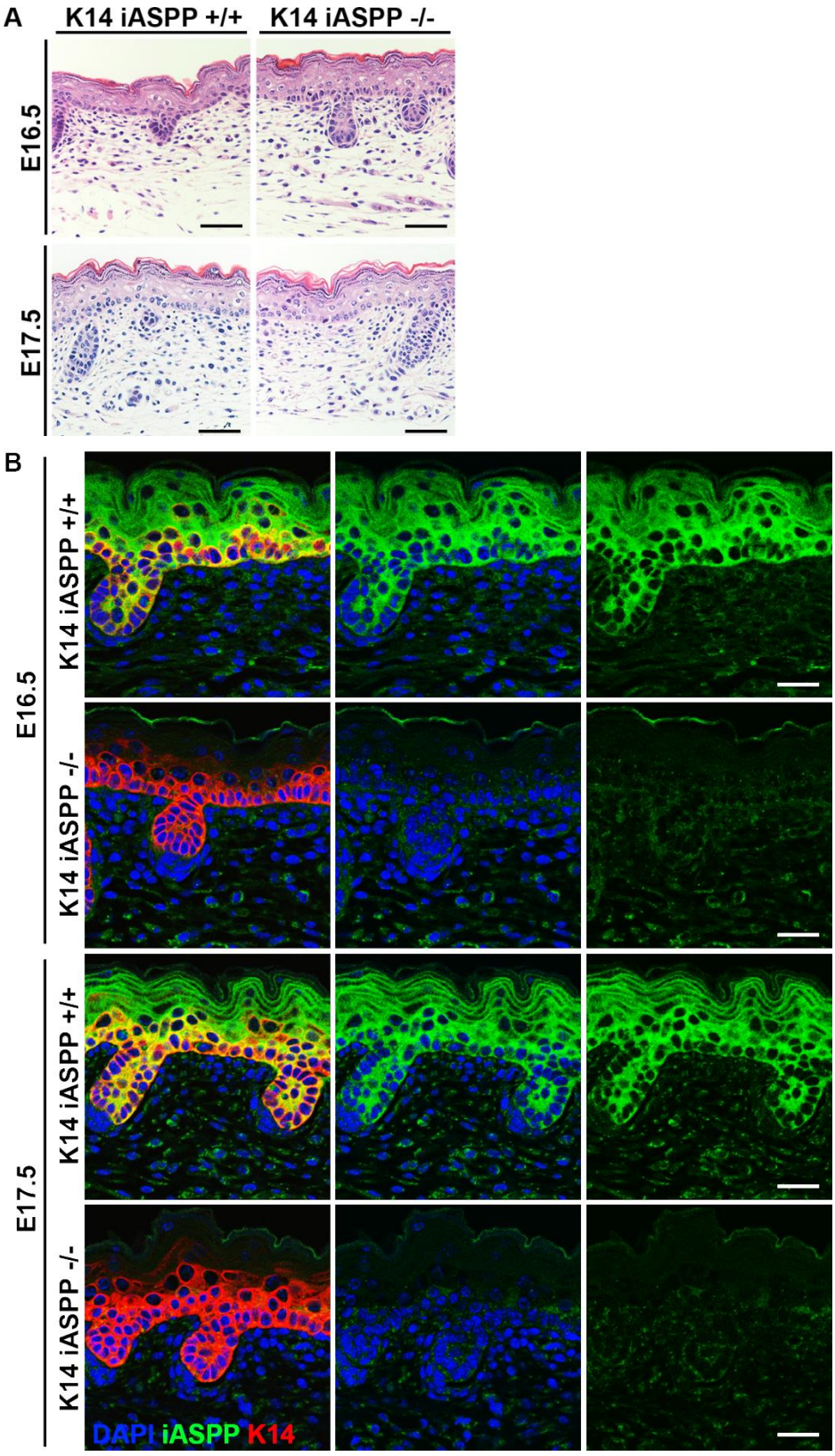


Fig 3.2 iASPP is expressed throughout E16.5-17.5 embryonic mouse epidermis

(A) H&E staining of K14-iASPP+/+ and K14-iASPP-/- embryonic skin sections did not reveal any

developmental skin defects (scale bar=50μm).
(B) iASPP immunofluorescence staining of embryonic skin sections showed predominantly cytoplasmic localisation in the basal layer and junctional iASPP in the suprabasal layer (scale bar=25μm).

The localisation of epidermal differentiation markers in K14-iASPP+/+ and -/- embryonic skin was also assessed. Skin samples were stained for basal markers K14 and p63, the spinous marker K1 and the granular marker loricrin (Fig 3.3A). iASPP deficiency in the embryonic epidermis did not affect the expression profiles of these differentiation markers.

Furthermore, K14-iASPP+/+ and -/- embryos were subjected to toluidine blue skin permeability assay to examine the skin barrier function of K14-iASPP-deficient embryonic skin. It has been demonstrated that the formation of the mouse skin barrier is initiated late in embryogenesis at E16 and completes by E17 (Hardman *et al*., 1998). Blue staining of the skin implies epidermal penetration of the toluidine blue dye where skin barrier function has yet to be established. Results of the assay showed that iASPP deficiency in the epidermis did not impair the acquisition of the skin barrier by E17.5 (Fig 3.3B).

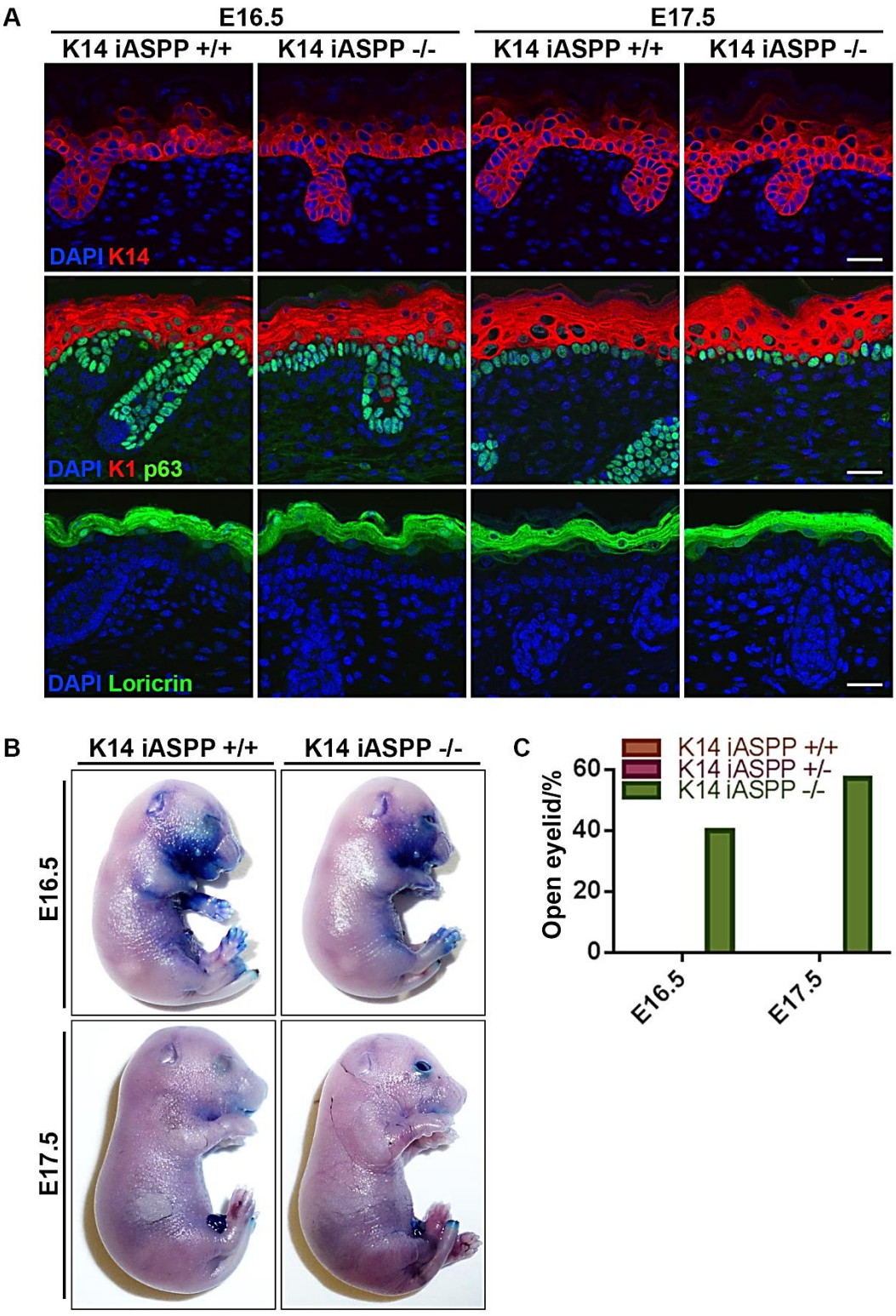


Fig 3.3 The absence of iASPP expression in embryonic skin did not affect embryonic skin development and barrier formation.

(A) Immunofluorescence staining of epidermal differentiation markers K14, K1, loricrin and p63

on E16.5-E17.5 skin from K14-iASPP+/+ and K14-iASPP-/- embryos. Expression patterns of these markers were not affected in the absence of iASPP in embryonic skin (scale bar=25μm).
(B) Skin permeability assay on K14-iASPP+/+ and K14-iASPP-/- embryos showed no impairment of skin barrier formation.
(C) Impaired eyelid closure was observed in 50% of K14-iASPP-/- embryos at E16.5-17.5 (E16.5: K14-iASPP+/+ n=4, -/- n=5; E17.5: K14-iASPP+/+ n=10, -/- n=7).

Open eyelid defects were observed in roughly 50% of K14-iASPP-/- embryos at E16.5-17.5, whereas all of the wild type embryos had closed eyelids (Fig 3.3C). Eyelid closure in mice begins at E14.5, when the epithelial leading edges extend from the eyelid root on both sides. The leading edges meet and fuse to form an epithelial bridge at E16.5, followed by mesenchymal extension at E17.5 (Tao *et al.*, 2005). K14-iASPP-deficient embryos could potentially have eyelid developmental defects before E16.5, leading to the display of open eyelids at E16.5-17.5.

As epidermal iASPP deficiency did not lead to overt abnormalities in the highly proliferative embryonic skin which might be indicative of alteration in the skin stem cell population, BrdU label retaining studies were not performed to quantify stem cell numbers present. However, it would be interesting to see if the dynamics of various stem cell populations, such as those in the HF bulge, would be influenced in postnatal K14-iASPP-/- skin.

3.2.3 Epidermal iASPP is Required to Maintain Normal HF Polarity, Hair Shaft Production and Proper Timing of the Hair Cycle in Adult Skin

iASPP expression in the adult skin of K14-iASPP+/+ and -/- mice at postnatal day 35 (P35) was examined by immunofluorescence staining. Predominantly cytoplasmic iASPP was detected in the IFE (Fig 3.4A). Moreover, strong iASPP expression could be detected in anagen HF, particularly in the hair bulb where the proliferative matrix cells reside as well as the ORS which is continuous with the IFE (Fig 3.4B). Such an iASPP signal was absent in skin samples from K14-iASPP-/- mice, which confirmed the specificity of the iASPP antibody (Fig 3.4C, D).

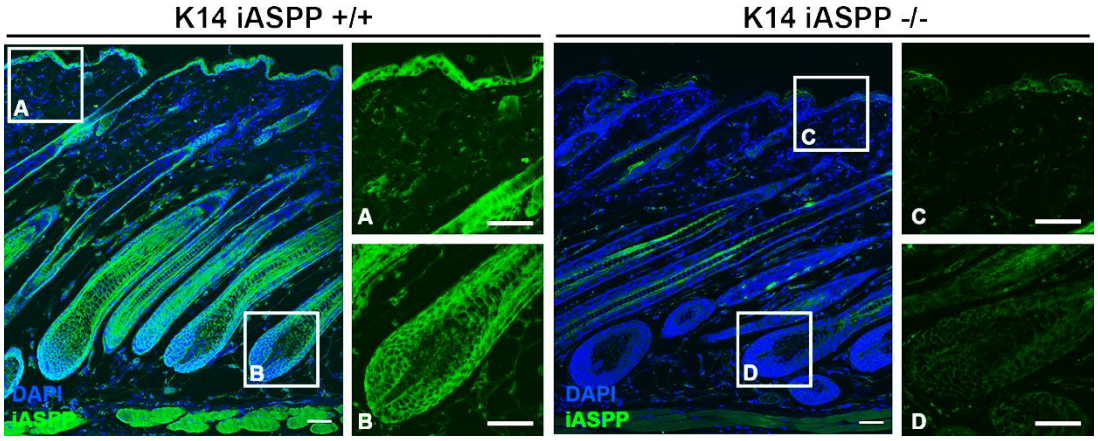


Fig 3.4 iASPP is expressed in the epidermis and hair follicles of adult mouse skin.
(A&C) Cytoplasmic expression of iASPP was detected on the epidermis of mouse skin at P35 by immunofluorescence staining.
(B&D) iASPP was detected in the anagen hair bulb (scale bar=25μm).

Histological analysis of adult mouse skin was performed by H&E staining of sections obtained from K14-iASPP wild type and knockout mice. Skin samples were collected from age and sex

matched littermates at P35 and P56 to examine the anagen and telogen phases of the hair cycle respectively. This was facilitated by the synchronised and therefore predictable hair growth cycles of mice during the first few months after birth (Müller-Röver *et al*., 2001).

A note of caution when conducting dermatological research on mouse skin is that the synchronised hair cycles in mice can lead to substantial changes in skin architecture (Müller-Röver *et al*., 2001; Stenn *et al*., 2001). Mouse skin with HFs at the proliferative anagen phase is known to have a thicker epidermis and dermis, along with an increase in dermal vascularity and stromal content. It is therefore vital that skin sections are taken at the same HF phase if comparable meaningful results are to be generated, especially when examining the thickness of mouse skin.

The H&E stained K14-iASPP+/+ and -/- skin sections did not reveal any obvious difference in the general thickness of the IFE, associated with anagen or telogen HFs (K14-iASPP+/+ n=3, -/- n=3 per group). However, unlike the wild type anagen HFs growing at an angle to the skin, misalignment of HFs could be observed in K14-iASPP-deficient skin that pointed downwards to the muscle layer (white arrowhead in Fig 3.5A). Interestingly, HF misalignment could only be detected at the anagen phase and not in the telogen phase of the hair cycle. The lack of

epidermal iASPP disrupted anagen HF polarity, but did not seem to affect the distribution of HFs in the skin (Fig 3.5B).

The timing of the hair cycle was also disrupted in the absence of epidermal iASPP expression. C57BL/6 female mice are expected to have a synchronous postnatal hair cycle, entering their first anagen phase at P28, catagen at P42 and telogen at P49 (Müller-Röver *et al*., 2001). The skin pigmentation of C57BL/6 mice aids the identification of which hair stage the skin is in, with anagen phase associated with black pigmented skin and telogen phase with pink skin. However, about half of the K14-iASPP-deficient female mice had anagen hair growth with black pigmented skin at 7-8weeks old whereas all of the wild type littermates had pink skin (Fig 3.5C). These K14-iASPP-deficient females eventually entered the telogen phase around 9-10weeks after birth. This could be due to a prolonged anagen phase, or accelerated initiation of the second anagen growth phase (K14-iASPP+/+ n=13, -/- n=13).

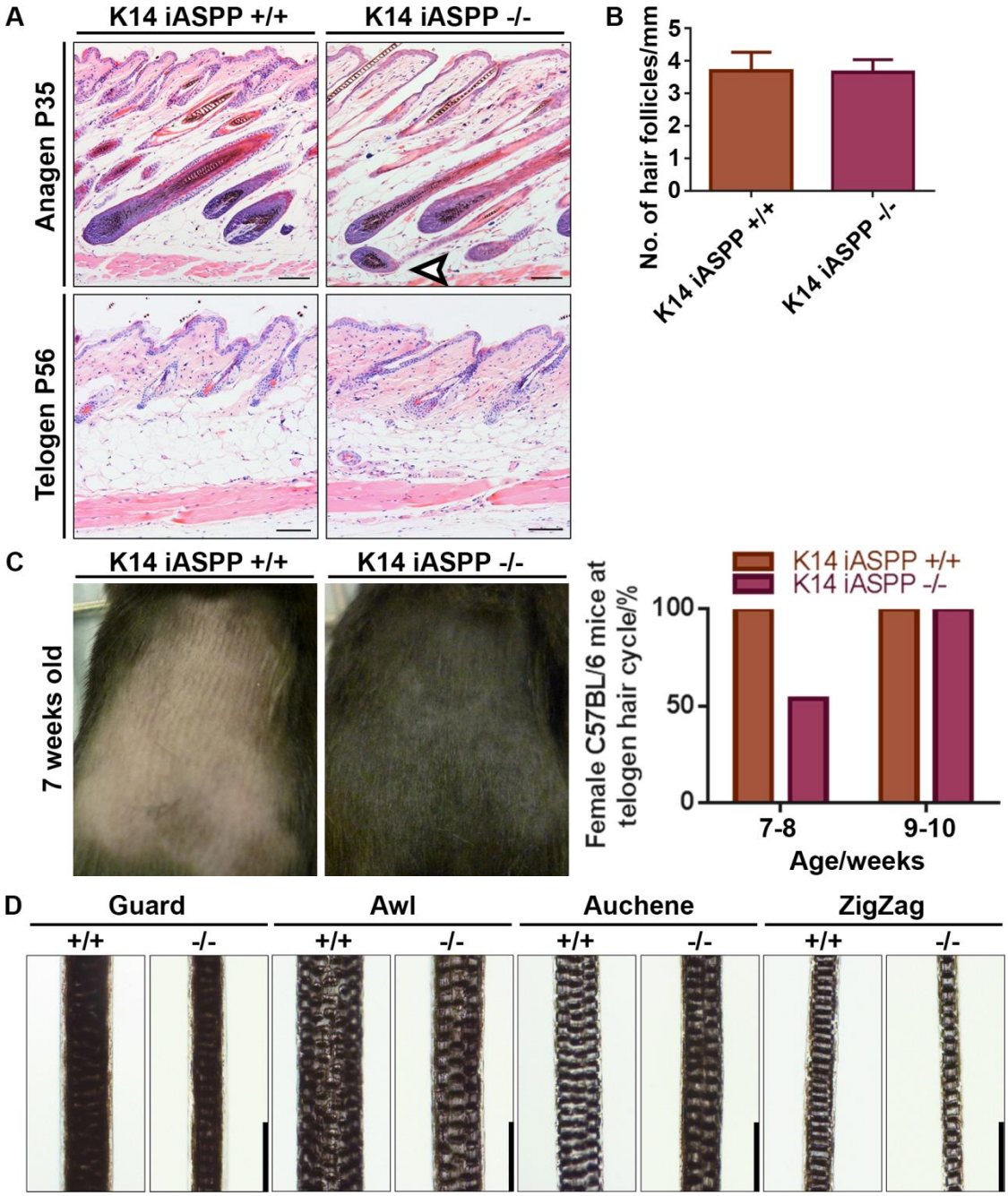


Fig 3.5 The lack of epidermal iASPP expression in postnatal skin led to misalignment of HFs at anagen, disrupting normal hair cycling and production.

(A) Histological analysis of K14-iASPP+/+ and K14-iASPP-/- murine skin at postnatal day 35 (P35) and day 56 (P56). White arrowhead indicates a misaligned anagen HF found in K14-iASPP-/- skin. HF alignment at telogen was not affected in the absence of iASPP. (Scale bar=100µm).

(B) No significant difference in the number of HFs per millimetre length of skin was observed between K14-iASPP+/+ and K14-iASPP-/- skin at P56.

(C) Pictures showing differences in skin pigmentation of K14-iASPP+/+ and K14-iASPP-/- C57Bl/6 females at 7 weeks old. The pale pink colour indicates the resting telogen phase of the hair cycle, and black indicates the active hair growth anagen phase. Deregulated timing of the hair cycle was observed in 50% of K14-iASPP-/- females when compared to the wild type (K14-iASPP+/+ n=13, -/- n=13).
(D) K14-iASPP-/- mice produced hairs with abnormal hair shaft structures compared to the wild type (K14-iASPP+/+ n=5, -/-n=7; scale bar=50μm).

Lastly, K14-iASPP-/- HFs produced hair shafts with altered structure for all hair types found on the murine coat (Fig 3.5D). This observation corresponded with the abnormal hair shaft production previously reported in the spontaneous mutant wa3 mouse (Herron *et al*., 2005). It would be interesting to further examine whether epidermal iASPP deficiency would significantly affect the normal distribution of different hair types found in mouse coat (Table1.1). These results suggested that a lack of iASPP in the epidermis is sufficient to disrupt HF biology in terms of HF polarity, cycling and hair shaft production, and could together contribute to the abnormal coat texture of K14-iASPP-/- mice.

3.2.4 The Lack of iASPP in Epidermis Disrupted Epithelial Differentiation and Resulted in Abnormal Focal Epidermal Thickenings

To investigate whether iASPP deficiency in the epidermis would impair its stratification, skin sections from K14-iASPP+/+ and -/- mice at P35 were stained for epidermal differentiation

markers. Although the general expression profiles of K14 and p63, which mark the basal layer, were comparable between wild type and iASPP knockout mice, mild focal thickening of the K14/p63-positive basal layer could be observed in K14-iASPP-deficient skin (Fig 3.6A, B). A similar observation was made for the spinous marker K1, as moderate focal thickening of the K1-positive spinous epidermal layer was also detected (Fig 3.6C).

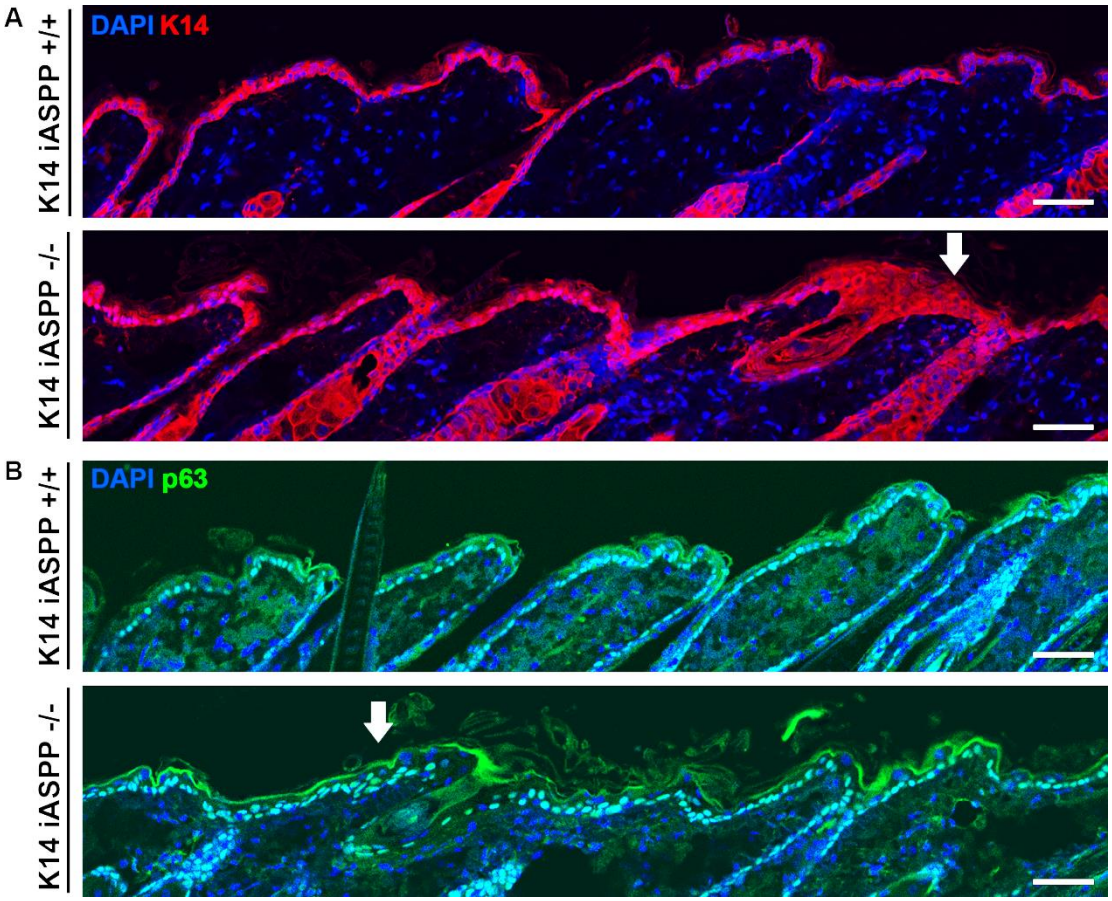


Fig 3.6 Focal thickening of the epidermis was observed in K14-iASPP-/- skin at P35 compared to wild type littermates.

Expression of basal epithelial markers was examined by immunofluorescence staining.

(A) K14 expression was examined. The white arrow indicates mild focal thickening of the

K14-positive basal layer in K14-iASPP-/- skin compared with wild type littermates.

(B) p63-positive cells were present as a single-cell layer in general in both K14-iASPP+/+ and K14-iASPP-/- skin, with the white arrow indicating a mild thickening of the p63-positive layer in K14-iASPP-/- mice (K14-iASPP+/+ n=3, -/- n=3).

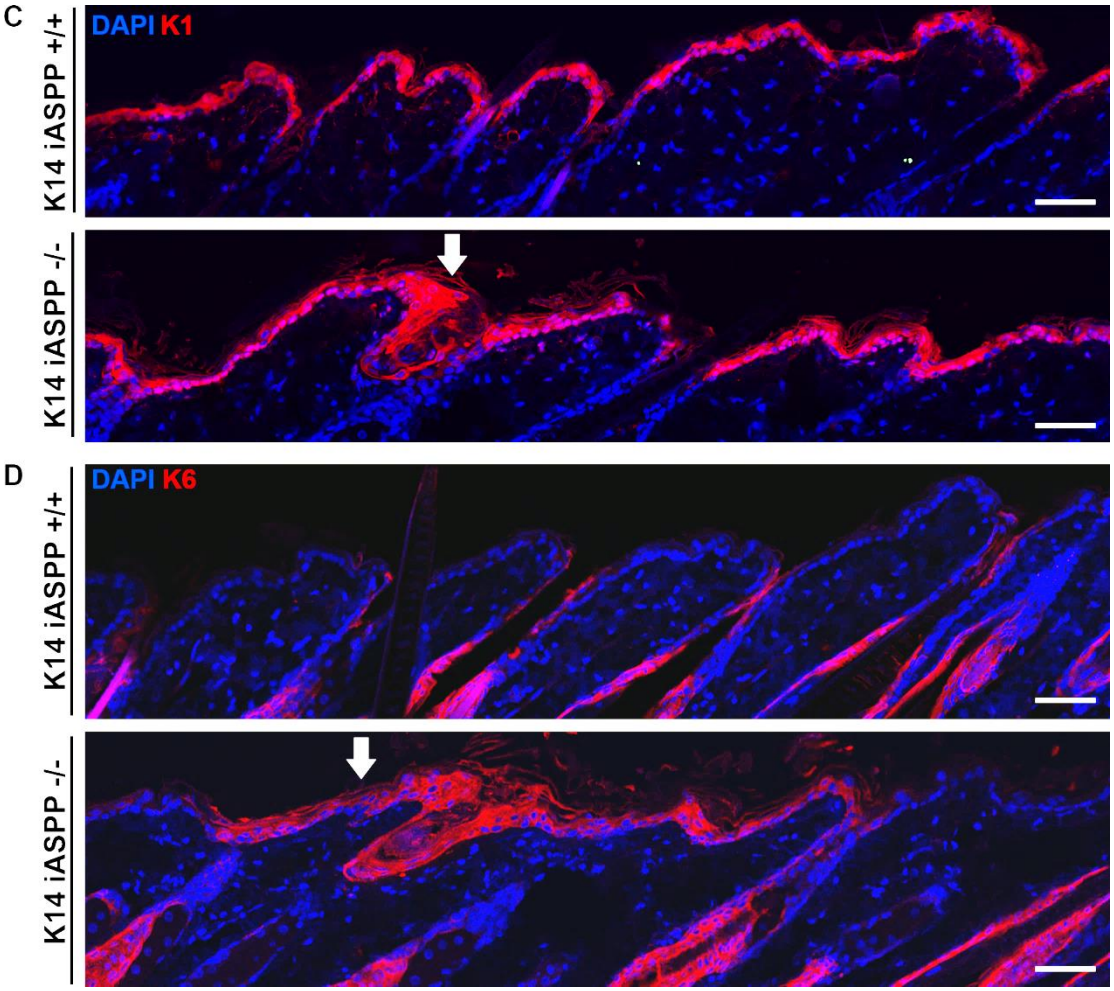


Fig 3.6 Focal thickening of the epidermis was observed in K14-iASPP-/- skin at P35 when compared to wild type littermates.

(C) Suprabasal epithelial marker K1 was detected by immunofluorescence staining. White arrow indicates mild focal thickening of the K1-positive suprabasal layer in K14-iASPP-/- skin compared to wild type littermates.

(D) The abnormal expression of hyperproliferative keratin marker K6 was detected in K14-iASPP-/- IFE but not in wild type mice (K14-iASPP+/+ n=3, -/-n=3).

These skin sections were then stained for the hyperproliferative marker K6 (Fig 3.6D). K6 is normally absent in healthy IFE, but is expressed as a structural component in the ORS of HF (Rothnagel *et al*., 1999; Komine *et al*., 2001). K6 expression is induced in IFE in response to stimuli such as wounding and exposure to phorbol esters, or in pathological conditions such as psoriasis and cancer. The focal expression of K6 on K14-iASPP-/- IFE indicated perturbed normal keratinocyte function in the absence of iASPP expression.

3.2.5 Abnormal Infiltration of Immune Cells in K14-iASPP-Deficient Skin

K14-iASPP-deficient mice and their wild type littermates were left to age to over 1year old, in order to monitor for any spontaneous development of skin pathologies in the absence of epidermal iASPP expression. While skin tumour development was not observed in these aged animals, the abnormal focal epidermal thickenings seen in P35 K14-iASPP-/- mice were detected on aged K14-iASPP-deficient skin as well (Fig3.7A). The focal epidermal thickenings were positively stained with K6, illustrating a disrupted epithelial differentiation programme within these regions (Fig 3.7B). Such lesions appeared to be more frequently observed in aged K14-iASPP-/- skin when compared to that of young animals, but formal quantification would be required to confirm that.

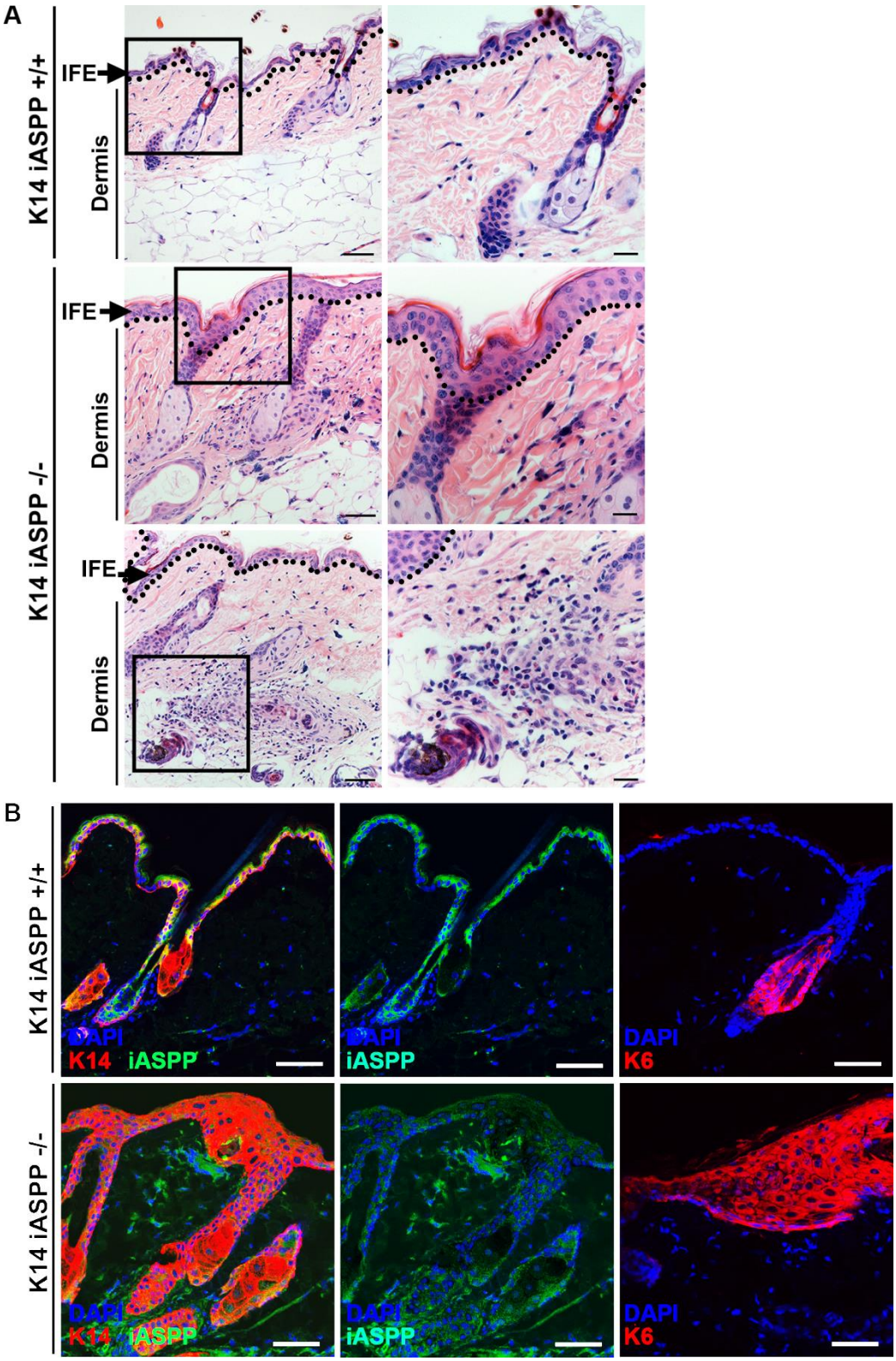


Fig 3.7 Immune infiltrates and focal epidermal thickenings were present in 1 year old K14-iASPP-/- mouse skin when compared to wild type littermates.

(A) Histological analysis of H&E-stained murine skin sections from 1 year old K14-iASPP+/+ and

K14-iASPP-/- females (K14-iASPP+/+ n=4, -/- n=7) showed the presence of dermal immune cell infiltrates and IFE thickening in the knockouts. Dotted line represents the border between the IFE and the dermis. (Left panel scale bar=50μm, right panel zoom-in scale bar=20μm).
(B) Immunofluorescence detection of iASPP, K14 and K6 demonstrated the expansion of the K14-positive basal layer and abnormal K6 expression in K14-iASPP-/- skin focal thickenings (scale bar=50μm).

Moreover immune cell infiltrates were found in the dermis of K14-iASPP-/- skin, some of which were associated with distorted HFs (Fig 3.7A). These deformed HF structures could possibly contribute to the abnormal coat developed by K14-iASPP-deficient mutants. All these abnormalities could not be detected in the skin of wild type mice (K14-iASPP+/+ n=4, -/- n=7).

To determine the identity of the immune cells observed in K14-iASPP-deficient murine skin, sections were stained with specific markers for different immune cell types by immunohistochemistry. The immune cell infiltrates appeared to predominantly consist of T cells, which were stained positively with the pan-T cell marker CD3 (Fig 3.8A). Unfortunately, because of technical issues, the staining for CD4 which marks T helper cells was unsuccessful. However, only a few immune cells that were stained positive for the T killer cell marker CD8 could be detected (Fig 3.8A). This led to speculation that the rest of the CD3+ CD8- T cell population could potentially be CD4+ T helper cells, although this needs experimental

verification. Moreover, a number of CD45R-positive B cells were detected within the immune cell cluster (Fig 3.8A).

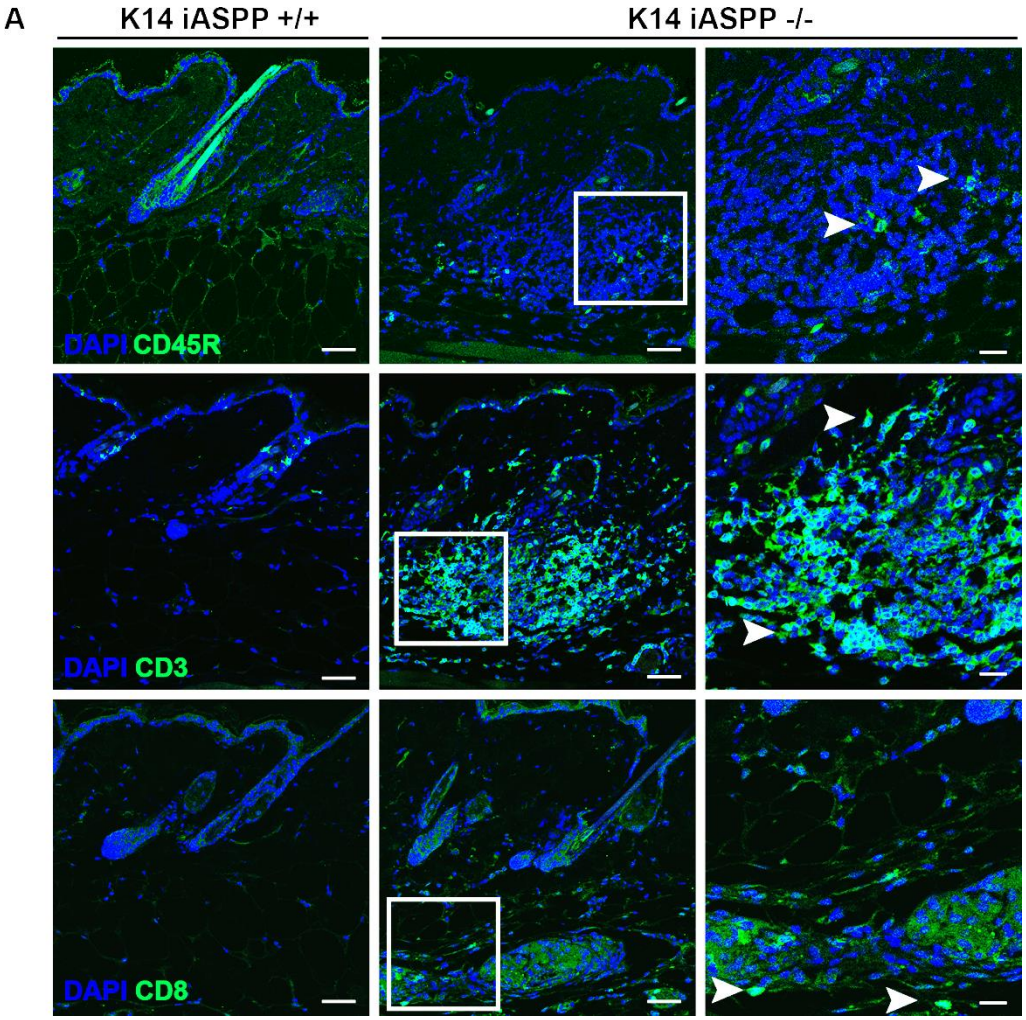


Fig 3.8 Immune cell infiltrates observed in K14-iASPP-/- skin consisted mainly of T cells and macrophages, with the presence of some B cells and neutrophils.

(A) Immunofluorescence detection of B cell marker CD45R, T cell marker CD3 and T killer cell marker CD8 on 1 year old K14-iASPP+/+ and K14-iASPP-/- skin. Staining predominantly demonstrated the presence of T cells, with some B cells in the immune infiltrates as indicated by the white arrows (scale bar=50μm, right panel zoom-in scale bar=20μm).

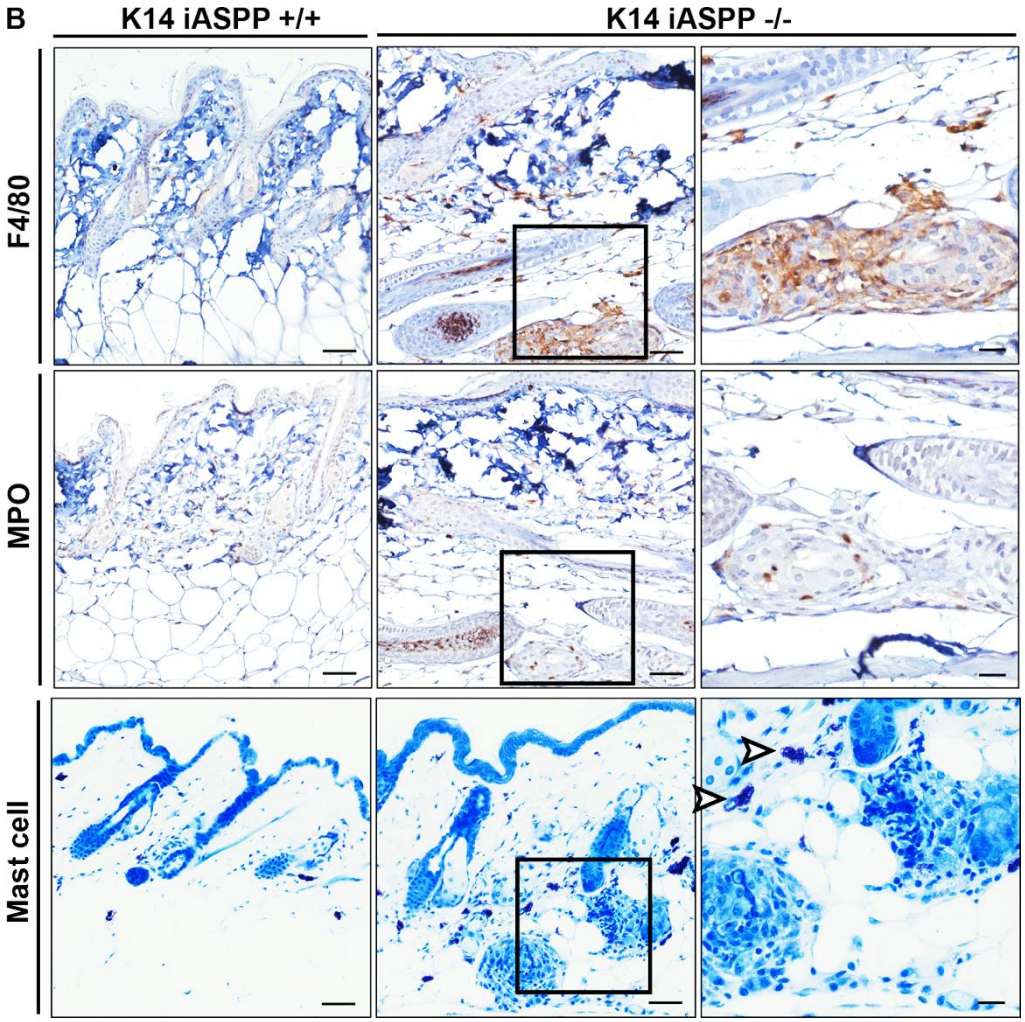


Fig 3.8 Immune cell infiltrates observed in K14-iASPP-/- skin consisted mainly of T cells and macrophages, with the presence of B cells and neutrophils.

(B) DAB staining for macrophage marker F4/80 and neutrophil marker MPO, as well as toluidine blue staining for mast cells on 1 year old mouse skin. Immune cell infiltrates found in K14-iASPP-/- skin consisted of F4/80-positive macrophages and MPO-positive neutrophils but not the metachromatically stained mast cells indicated with white arrowheads (scale bar=50μm, right panel zoom-in scale bar=20μm).

DAB staining was carried out to test for the presence of any innate immune cells in K14-iASPP-/- skin. F4/80-positive macrophages appeared to be the predominant infiltrating

innate cell type, along with a few MPO-positive neutrophils within such clusters (Fig 3.8B). Toluidine blue staining was performed to identify mast cells that would be metachromatically stained, appearing with a characteristic red-purple colour in contrast to blue orthochromatic background staining (Fig 3.8B). The results of the staining suggested that mast cells did not seem to contribute to the composition of cellular infiltrates.

3.3 Discussion

The presentation of abnormal coat and open eyelids at birth defects in K14-iASPP-/- mutants mirrored the phenotypes displayed by iASPP complete knockout mice. This provided strong *in vivo* evidence regarding the essential nature of iASPP expression in the basal epidermis to maintain proper skin homeostasis and eyelid development. The absence of any heart defects in K14-iASPP-/- mice confirmed the specificity of iASPP gene ablation in K14-expressing epithelial tissues. The integrity of the iASPP gene in K14-negative tissues was further proved by genetic analysis on DNA samples extracted from such tissues, in comparison to the deletion of iASPP exon 8 detected in K14-expressing tissues.

Interestingly the open eyelid defect showed incomplete penetrance in K14-iASPP-deficient mice in the C57BL/6 background. This was in contrast to the full phenotypic penetrance observed in iASPP complete knockout mice in the C57BL/6x129Sv background. Several possibilities could explain such difference in phenotypic display. It is possible that the pure C57BL/6 genetic background contains certain modifier genes that partially rescue the embryonic eyelid developmental defect, which might be diluted in the mixed C57BL/6x129Sv background. Secondly, the loss of iASPP expression in the eyelids of K14-iASPP-deficient mutants has not been tested. It is therefore plausible that mutants with normal eyelid closure

still retain iASPP protein expression, as embryonic eyelid closure commences at E14.5 and the absence of iASPP has only been verified in E16.5 embryonic skin. Thirdly, epithelial-mesenchymal interaction is important during eyelid morphogenesis. It is still unknown whether, and if so how much, mesenchymal iASPP contributes to this developmental process relative to iASPP expression in the epithelial compartment.

Although iASPP deficiency did not have a drastic effect on embryonic skin development, focal hyperproliferative epidermal thickenings were detected on K14-iASPP-deficient adult skin. Furthermore, abnormal infiltration of immune cells could be observed in the mutant dermis. Such cellular infiltrates appeared to consist of predominantly T cells and macrophages, and to a lesser extent B cells and neutrophils. This observations suggest that the lack of iASPP expression in keratinocytes could somehow lead to the aberrant recruitment of immune cells into the skin. Previous studies have indicated that iASPP has a regulatory role on epidermal differentiation via the modulation of p63 transcriptional activities (Chikh *et al*., 2011; Notari *et al*., 2011). It would be interesting to find out whether the infiltration of immune cells observed here is a secondary defensive response towards abnormal epidermal differentiation in the skin. Another possibility is that iASPP-deficient keratinocytes might be involved in the active

recruitment of immune cells, as keratinocytes are known to be capable of acting as inflammatory initiators.

Chapter 4 K14-iASPP-Deficient Mice were More Sensitive towards Chemically Induced Skin Carcinogenesis and Phorbol Ester Induced Skin Inflammation

4.1 Introduction

The most well-known function of the ASPP protein family is their ability to specifically regulate the apoptotic but not the cell cycle arrest function of p53 (Samuels-Lev *et al*., 2001). ASPP1 and ASPP2 proteins enhance the transactivation activity of p53 towards pro-apoptotic genes such as BAX, PUMA and PIG3, while iASPP inhibits such activities. Previous studies have demonstrated that iASPP could enhance the transforming activities of Ras and E1A in rat embryonic fibroblasts, and was overexpressed in a number of human tumours (Bergamaschi *et al*., 2003; Chen *et al*., 2010). In view of the prominent role of iASPP in skin biology and the need for *in vivo* data to determine whether iASPP can act as a proto-oncogene in supporting tumour growth, the DMBA/TPA two-stage chemical carcinogenesis experiment was performed on the K14-iASPP mouse cohort.

The chemically induced skin carcinogenesis protocol is a very well-established system that is used to study multistage cancer development and progression (Wu *et al*., 2001; Filler *et al*.,

2007). It involves three main stages of tumour development: initiation, promotion and progression. Epidermal cells are first initiated with a single topical application of the carcinogen *7, 12*-dimethyl-benzanthracene (DMBA) on mouse skin. DMBA is known to cause specific irreversible activating mutations in the H-Ras proto-oncogene, frequently an A-to-T transversion of the second nucleotide in codon 61 (Finch *et al*., 1996; Filler *et al*, 2007). The promotion phase consists of repeated topical application of the phorbol ester TPA to induce skin inflammation. This promotes the proliferation of initiated cells, leading to the development of benign papillomas. These benign papillomas may spontaneously progress into malignant squamous carcinomas, in cases where additional gene mutations are acquired, or when the transgenic animals carry genetic alterations that cause them to be more tumour-prone.

As mentioned above, iASPP has been shown to inhibit p53-mediated apoptotic gene transcription and can enhance Ras transforming activity in rat embryonic fibroblasts (Bergamaschi *et al*., 2003). Transgenic p53-deficient mice in DMBA/TPA studies exhibited more rapid malignant conversion of the papillomas formed, but developed a similar number of benign papillomas when compared with the wild type (Kemp *et al*., 1993). This illustrated that p53 is required to prevent malignant transformation but might not be involved in the initiation

of cancer development. The lack of iASPP expression in K14-positive basal epidermis might allow the activation of p53-mediated apoptotic gene transcription, as well as hampering the proliferation of Ras-activated keratinocytes upon DMBA treatment. Therefore one might expect that K14-iASPP-deficient mice would have reduced sensitivity towards the DMBA/TPA assay. This could be evidenced through reduced number of papillomas formed due to decreased proliferative potential of Ras-transformed keratinocytes, or reduced malignant conversion of papillomas as p53 is no longer inhibited by iASPP in the basal epidermis.

In addition, a recent study has demonstrated the negative regulatory function of iASPP over p63-mediated gene expression in preventing premature keratinocyte differentiation (Notari *et al*., 2011). Studies of spontaneous cancer development in p53+/- p63+/- transgenic mice did not give conclusive results on whether p63 would be involved in tumourigenesis (Flores *et al*., 2005; Keyes *et al*., 2006). However, considering the role of iASPP in inhibiting p63-mediated loricrin and involucrin expression, iASPP-deficient basal epidermis would be less prone to DMBA/TPA-induced skin carcinogenesis as iASPP-/- keratinocytes would undergo premature differentiation.

Moreover, iASPP has been previously reported to be able to bind to and inhibit the DNA binding activity of the NF-κB transcription factor p65 (Yang *et al*., 1999; Takada *et al*., 2002), although such interaction has yet been tested in keratinocytes. The K14-p65-deficient mice displayed decreased susceptibility towards TPA-induced cutaneous inflammation as well as DMBA/TPA skin tumourigenesis compared to the wild type (Kim *et al*., 2014). If the inhibitory effects of iASPP on p65 exist in the basal epithelium and predominates during DMBA/TPA treatment, one might speculate that the absence of iASPP in the basal layer would result in increased papilloma formation on K14-iASPP-/- mice.

Therefore, the aim of this chapter was to examine whether epidermis-specific deletion of iASPP would influence susceptibility towards the DMBA/TPA-induced skin carcinogenesis protocol. This would provide an indication on the role of iASPP in cancer development, and perhaps reveal hints on the possible interaction of iASPP with its binding partners p53, p63 and p65 during neoplastic progression.

4.2 Results

4.2.1 K14-iASPP-Deficient Mice Showed Earlier Onset of Benign Papilloma Development and had Higher Papilloma Burden than Wild Type Mice

Several components of murine skin biology must be taken into consideration when performing the DMBA/TPA induced skin carcinogenesis assay. The synchronous HF cycling of mouse skin can influence different aspects of skin biology at a particular hair phase. Besides the thickness of the skin, studies have demonstrated changes in blood vasculature and the skin immune system associated with HF cycling. The anagen HF phase is correlated with an increase in angiogenesis and production of anti-inflammatory mediators such as IL-10 (Mecklenburg *et al*., 2000; Müller-Röver *et al*., 2001; Stenn *et al*., 2001). Moreover, some studies have suggested that the topical application of DMBA to murine skin at the proliferative anagen phase could generate more papillomas when compared to skin in the telogen phase (Miller *et al*., 1993). Therefore, age-matched mice with HFs at telogen phase should be recruited for DMBA/TPA treatment. Female mice should also be used for this assay instead of males, as males tend to fight and potentially cause wounds on the skin and introduce bias to the assay.

Another factor to be considered when utilising the two-stage skin carcinogenesis protocol is the marked differences in response of treatment according to the mouse strain used (Naito *et*

al., 1988; Imamoto *et al*., 1993; Woodworth *et al*., 2004; Filler *et al*., 2007). Different mouse strain shows different susceptibilities towards TPA-induced hyperplasia and the malignant conversion of papillomas formed. Although the C57BL/6 genetic background of the K14-iASPP mouse cohort is ideal for studying HFs due to its obvious skin pigmentation, this strain is particularly resistant to DMBA/TPA tumourigenesis when compared to the more susceptible strains such as SENCAR.

Female mice with wild type, heterozygous and homozygous iASPP deletion in the K14-positive epidermis were enrolled into the DMBA/TPA treatment cohort (K14-iASPP+/+ n=14, +/- n=9, -/- n=11). A single dose of DMBA was applied topically to the dorsal skin that had been shaved on the previous day, and was followed by 15 weeks of twice-weekly TPA applications (Fig 4.1A). A small cohort of mice were allocated into the TPA-only treatment group (K14-iASPP+/+ n=5, -/- n=4) to test whether papilloma development was dependent on DMBA-mediated Ras gene activation, or could result from inflammation alone. Mice were then observed for up to 1 year, or when endpoints were reached requiring immediate termination as determined by the project license.

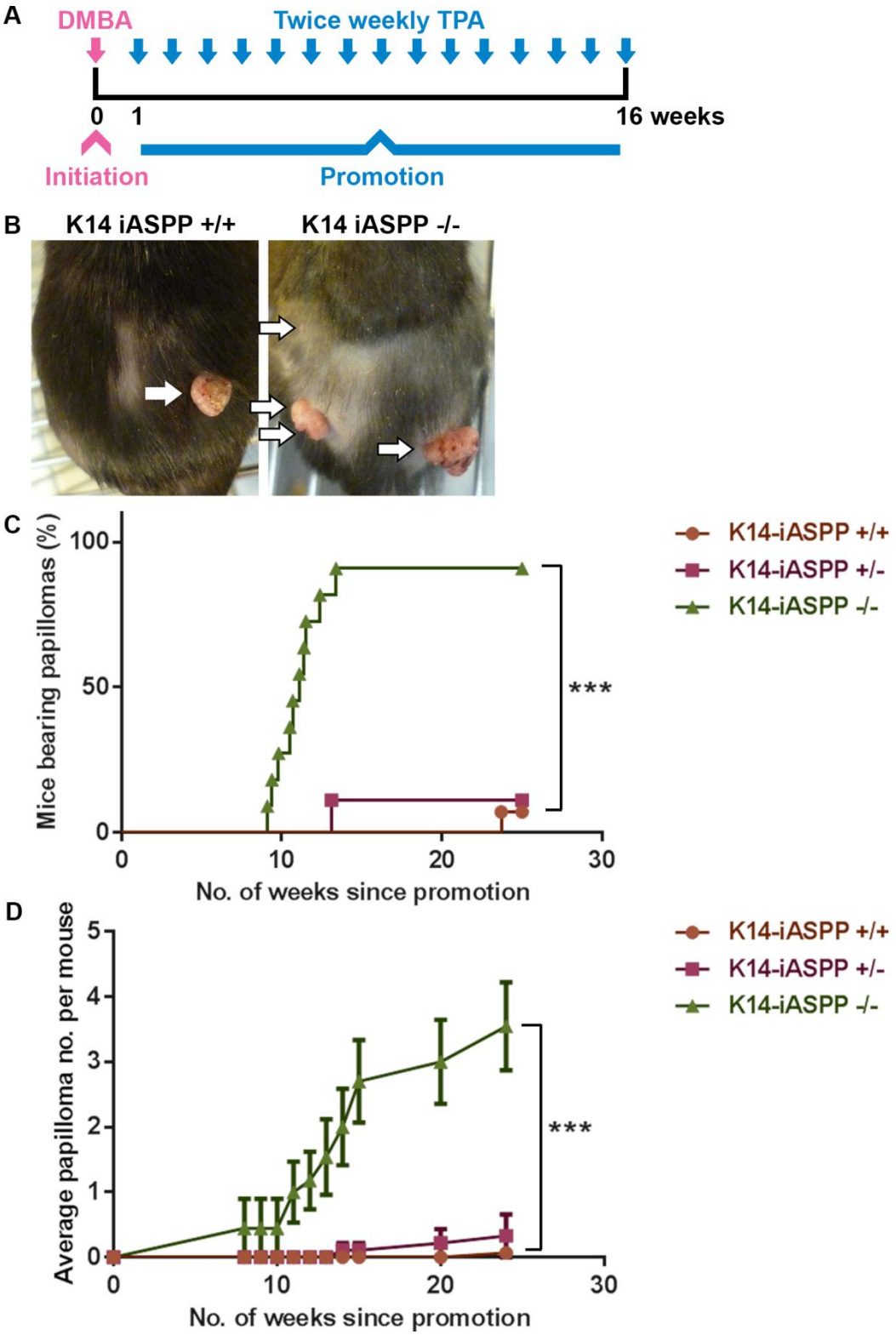


Fig 4.1 K14-iASPP-/- mice were more susceptible to DMBA/TPA-induced skin carcinogenesis compared to K14-iASPP+/+ mice.

(A) Schematic illustrating the DMBA/TPA two-stage tumour induction protocol.

(B) Dorsal view of papillomas developed on K14-iASPP+/+ and K14-iASPP-/- mice.
(C) Papilloma incidence represented as a percentage of mice bearing papillomas (K14-iASPP+/+ n=14, +/- n=9, -/- n=11).
(D) Formation of papillomas observed in different mouse cohorts (***P<0.001, error bar represents SEM).

Curiously, the K14-iASPP-/- mice were significantly more susceptible to the DMBA/TPA tumour induction protocol used. Almost all of the K14-iASPP-deficient mice in the DMBA/TPA cohort developed exophytic papillomas, in contrast to low frequencies of papilloma incidence in both wild type and K14-iASPP+/- animals (K14-iASPP+/+=0.07%, +/-=0.11%, -/-=91%). K14-iASPP-deficient mice also showed a much earlier onset of papilloma growth when compared to iASPP wild type and heterozygous mice (Fig 4.1C). Moreover, the average number of papillomas formed per mouse was significantly higher on K14-iASPP-deficient dorsal skin (Fig 4.1D). None of the mice exhibited tumour growth in the TPA-only cohort, irrespective of their genotype. Macroscopically, none of the papillomas showed signs of malignant conversion such as endophytic growth and ulceration during the course of the DMBA/TPA treatment and monitoring periods (Fig 4.1B).

A previous study has investigated the skin tumour inducing activity of DMBA/TPA treatment on the C57BL/6 mouse strain, and demonstrated its high resistance towards such skin

carcinogenesis assays. The group used 25nmol/6.5µg DMBA for initiation, followed by 23 weeks of twice-weekly 6.8nmol/4µg TPA application to give around 0.08 papillomas per mouse (Imamoto *et al*., 1993). The protocol utilised in this research project involved the application of 25µg DMBA, but only 15 weeks of twice weekly 4µg TPA. This led to the development of about 0.07 papillomas per wild type C57BL/6 mouse, a low frequency of papilloma formation comparable to what has been previously reported. On the other hand, an average of 3.55 papillomas per K14-iASPP-deficient mouse was obtained 24 weeks after the beginning of the DMBA/TPA treatment.

4.2.2 Papillomas Developed on Mice Harboured DMBA-induced H-Ras Activating Mutation

DNA samples were extracted from papillomas formed on the animals to identify the presence of H-Ras mutation induced by DMBA. Nested PCR reactions were performed to amplify exon 2 of the H-Ras gene where codon 61 resides (Fig 4.2A). The conversion of wild type CAA allele to CTA in codon 61 produces an XbaI restriction enzyme cleavage site. This can be utilised for the diagnostic detection of H-Ras point mutations, referred as restriction fragment length polymorphisms (RFLP).

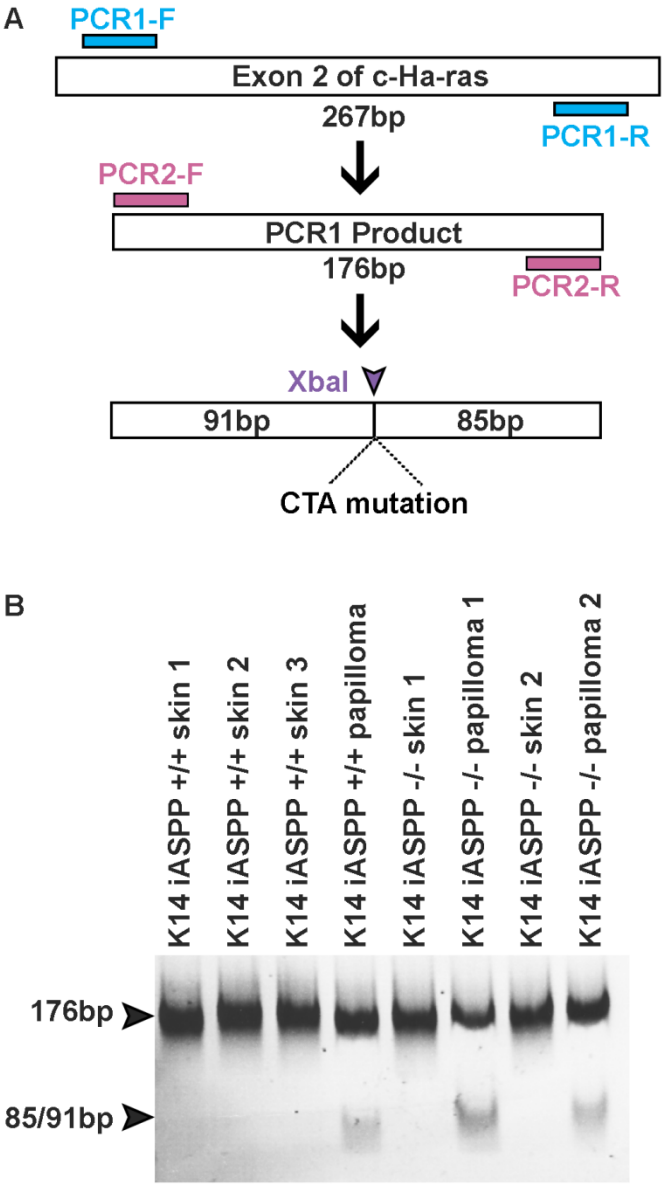


Fig 4.2 Presence of Ras mutation in papillomas formed on both K14-iASPP+/+ and K14-iASPP-/- murine dorsal skin.

(A) Schematic diagram showing the detection of CAA → CTA H-Ras mutation through nested PCR and RFLP.

(B) Nested PCR was performed on DNA extracted from papillomas and tumour-free skin samples from mice in the DMBA/TPA cohort. The presence of H-Ras mutation would have introduced the XbaI restriction site, causing the production of shorter fragments (85/91bp) from the unmutated PCR product (176bp). Papillomas from both K14-iASPP+/+ and K14-iASPP-/- mice contained H-Ras mutations, whereas tumour-free skin samples did not.

Digested PCR products were run on a polyacrylamide gel to visualise the shorter 89/91bp bands, which indicates the presence of CAA→CTA H-Ras mutation (Fig 4.2B). Such shorter bands were only found in reaction samples from papillomas and not those from tumour-free skin. This indicated that papillomas developed on K14-iASPP+/+ and K14-iASPP-/- mice harboured DMBA-induced Ras mutation, and that tumour formation on these mice was dependent on Ras activation as the TPA-only cohort did not develop any papillomas.

4.2.3 Papillomas Observed in the DMBA/TPA Treated Mouse Cohort were Well-Differentiated and Benign

Samples were taken from mouse skin and papillomas, and sections were stained by H&E to examine tissue morphology (Fig 4.3). Histological analysis was carried out with the guidance of dermatologist Dr. Ruth Asher at the John Radcliffe Hospital. Papillomas from both K14-iASPP+/+ and K14-iASPP-/- animals exhibited exophytic growth, and were well-differentiated, covered with extensive layers of keratin (Fig 4.3A). All of the papillomas examined were benign in nature as no signs of epidermal invasion into the dermis and underlying muscle layers were observed (Fig 4.3B). Epidermal spongiosis was detected in the papillomas, indicating the presence of intercellular oedema between keratinocytes (black arrowheads in Fig 4.3C). Parakeratosis, the retention of nuclei in the cornified layer, was also noted indicating a disruption of normal epidermal differentiation (white arrowheads in Fig

4.3C). Infiltration of immune cells such as macrophages, neutrophils, T cells and B cells was observed in the papillomas developed (Fig 4.4).

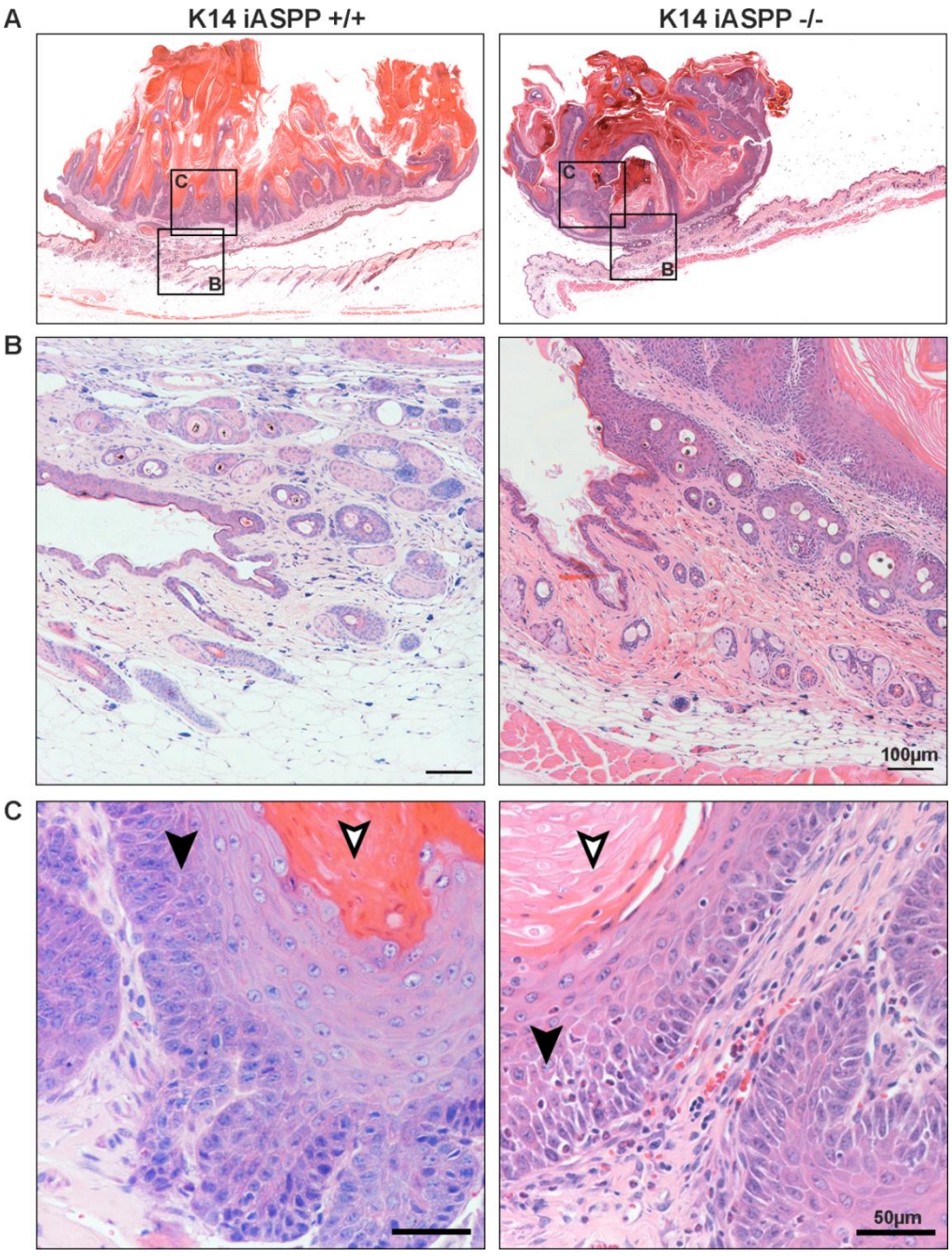


Fig 4.3 Outgrowth of benign, highly differentiated papillomas on K14-iASPP+/+ and K14-iASPP-/- mice in the DMBA/TPA cohort.

(A) Histology of papillomas developed through H&E analysis.

(B) No signs of tumour invasion into the dermis and the underlying muscles were observed in

papillomas from iASPP wild type and knockout animals (Scale bar=100µm).

(C) Signs of parakeratosis (white arrowheads) and spongiosis (black arrowheads) were observed in papillomas from both K14-iASPP+/+ and -/- mice (Scale bar=50µm).

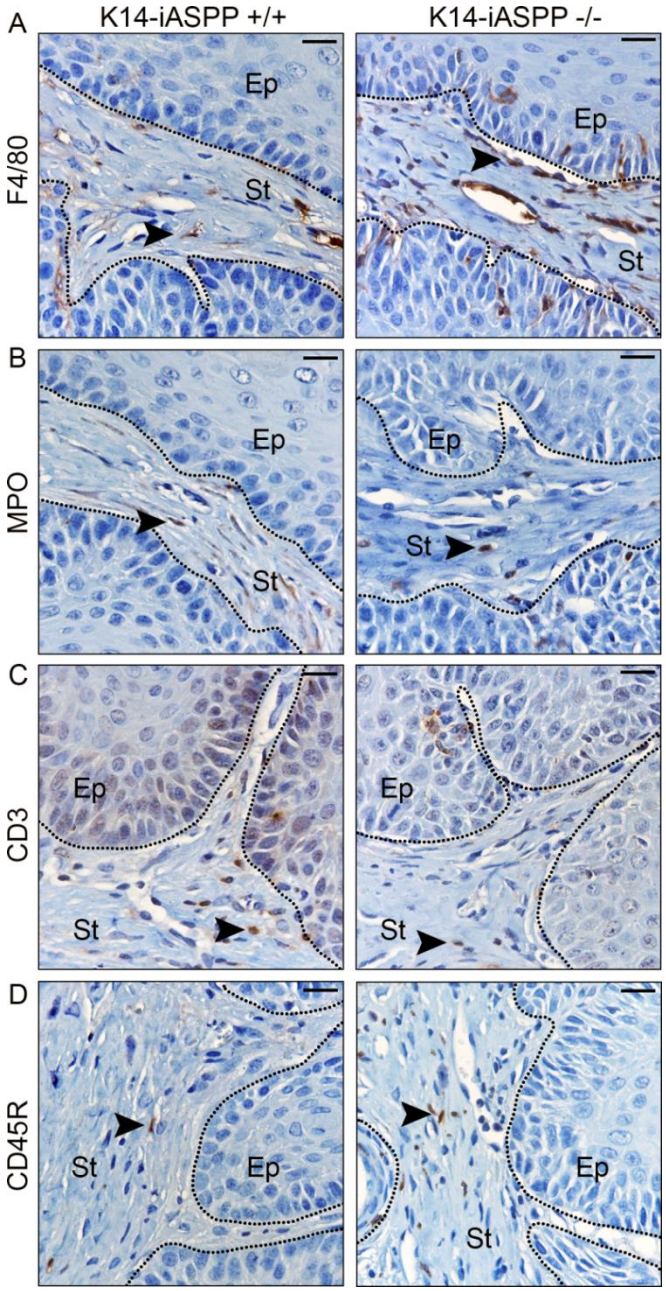


Fig 4.4 Immune cells present in papillomas developed on dorsal mouse skin.

DAB staining of papilloma sections for (A) macrophage marker F4/80, (B) MPO for neutrophils,

(C) CD3 labelling T cells and (D) CD45R for B cells (Scale bar=20μm, dashed line represents the border between the epidermis Ep and stromal region St).

Paraffin sections of papillomas and the flanking tumour-free skin were stained for iASPP by immunofluorescence staining (Fig 4.5A). The absence of iASPP staining demonstrated the ablation of iASPP expression in both K14-iASPP-deficient skin and papilloma tissues. Nuclear iASPP localisation could be detected in keratinocytes of K14-iASPP+/+ papilloma (white arrowheads, Fig 4.5B) although the majority showed cytoplasmic iASPP expression. In comparison, iASPP was predominantly cytoplasmic in the surrounding tumour-free epidermis, with nuclear iASPP localisation being less frequently seen (Fig 4.5C).

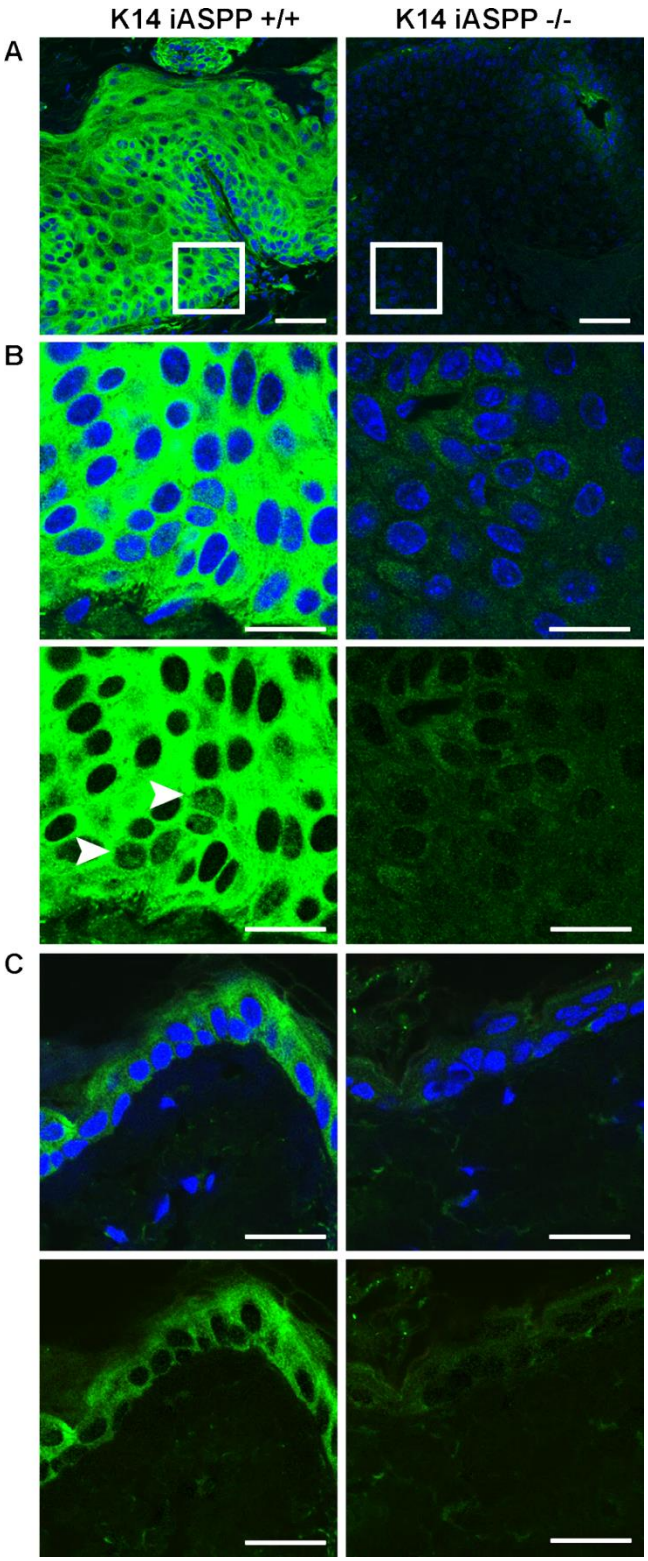


Fig 4.5 Nuclear iASPP observed in keratinocytes of K14-iASPP+/+ papillomas.

(A) Absence of iASPP immunofluorescence staining in papillomas on K14-iASPP-/- mice.

(B) Nuclear iASPP staining observed (white arrowheads) in keratinocytes of papilloma from

wild type mice.
(C) iASPP was predominantly cytoplasmic in tumour-free skin of K14-iASPP+/+ mice (Scale bar=50μm).

K14-iASPP+/+ and -/- papillomas were then stained for various differentiation markers to examine keratinocyte differentiation within such outgrowths. Marked expansion of K14 expression domains were detected in K14-iASPP+/+ and -/- papillomas (Fig 4.6A). A similar expression profile was observed for the transcription factor p63, which was no longer restricted to a single cell layer in the basal epithelia of normal skin (Fig 4.6B). However, K1 staining appeared to be weak, reflecting the disruption of the epidermal differentiation programme in the benign tumour (Fig 4.6B). Loricrin expression was present in the papillomas, consistent with the well-differentiated phenotype observed in histological studies (Fig 4.6C). As expected, the papillomas extensively expressed the marker K6 due to their hyper-proliferative states (Fig 4.6D). Both K14-iASPP+/+ and -/- papillomas expressed K13 (Fig 4.6E), a type of keratin previously shown to be a pre-malignant progression marker (Gimenez-Conti *et al*., 1990).

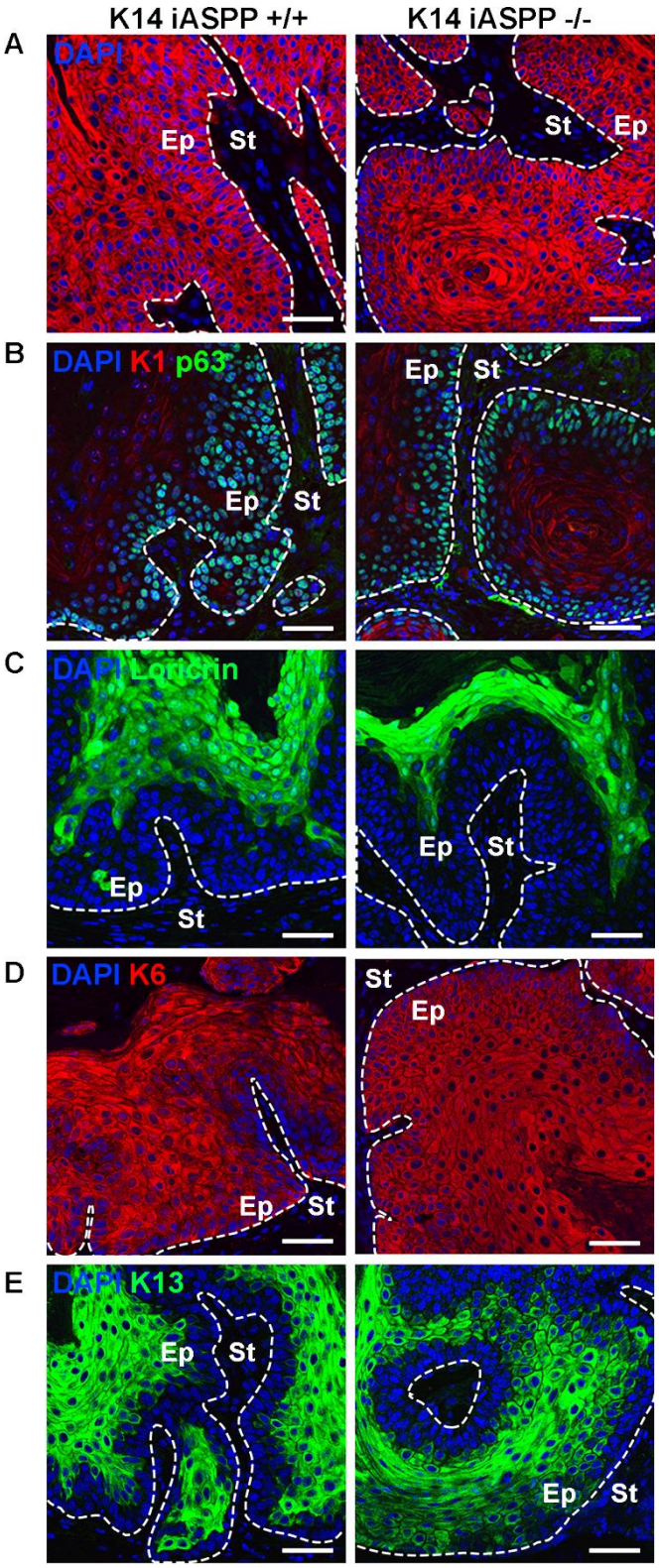


Fig 4.6 Characterisation of benign papillomas from K14-iASPP+/+ and K14-iASPP-/- mice by immunofluorescence.

(A) Extensive K14 expression in papillomas and (B) the thickened p63-positive basal layer with

reduced K1 expression observed.

(C) Positive loricrin staining in papillomas.

(D) Expression of hyperproliferative inflammatory keratin K6 and (E) pre-malignant marker K13 (Scale bar=50µm, dashed line represents the border between the epidermis Ep and stromal region St).

Immunofluorescence staining of papillomas sections showed the nuclear expression of the p53 tumour suppressor in both K14-iASPP+/+ and K14-iASPP-/- papillomas (Fig 4.7A). Nuclear localisation of p65 was also detected in both wild type and K14-iASPP-/- papillomas, and co-localisation of nuclear p65 and iASPP could be observed (Fig 4.7B).

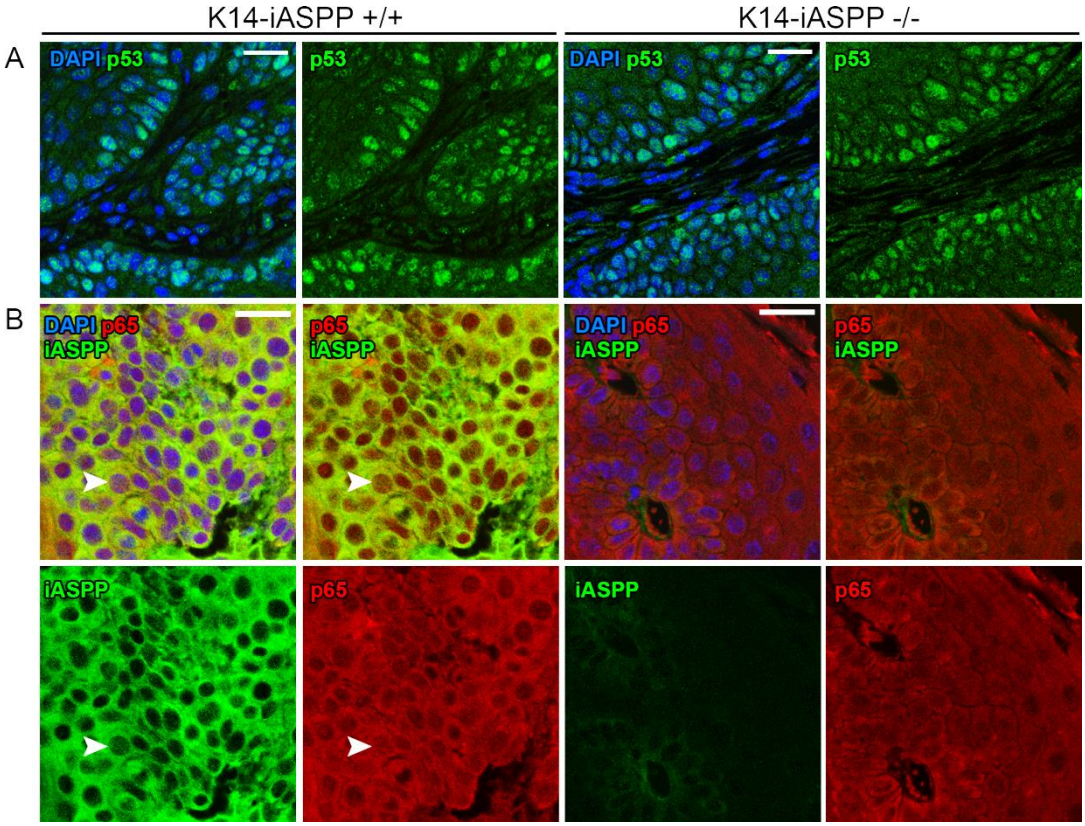


Fig 4.7 Nuclear localisation of p53 and p65 transcription factors in papillomas on K14-iASPP+/+ and -/- mice.

(A) Papillomas from both K14-iASPP wild type and knockout mice were positive for nuclear p53

expression detected by immunofluorescence staining.
(B) Nuclear p65 localisation present in keratinocytes with nuclear iASPP expression within papillomas from K14-iASPP+/+ mice (Scale bar=25μm).

The nuclear localisation of iASPP in keratinocytes of the papillomas would enable the protein to interact with transcription factors present in the nuclei. The C-terminal end of iASPP has been demonstrated to interact with p53 family members, p65 and PP1 proteins (Yang *et al*., 1999; Bergamaschi *et al*., 2003; Llanos *et al*., 2011). As K14-iASPP+/+ mice had a significantly lower papilloma burden compared to the knockout, it might be unlikely that the inhibition of p53/TAp63-mediated apoptosis by nuclear iASPP would contribute to the difference in papilloma incidence between wild type and knockout mice. It would be interesting to investigate whether nuclear iASPP could be acting to suppress p65 transcriptional activities, and down-regulate the expression of inflammatory mediators that could promote tumour development.

4.2.4 Short-Term Topical TPA Treatment of the K14-iASPP Mouse Cohort to Induce Acute Cutaneous Inflammation

The unanticipated increase in the sensitivity of K14-iASPP-deficient mutants towards DMBA/TPA-mediated tumourigenesis and the abnormal immune cell infiltrates present in their skin, led to speculation that a deficiency of iASPP in the epidermis could affect the cutaneous immune system. The abnormal immunohomeostasis of K14-iASPP-deficient skin could potentially provide a microenvironment that supports the proliferation of initiated keratinocytes to form papillomas in the context of the DMBA/TPA experiment. To test this revised hypothesis, K14-iASPP+/+ and K14-iASPP-/- female mice were placed in either a short-term TPA treatment cohort (K14-iASPP+/+ n=4, -/- n=3) or an acetone control group (K14-iASPP+/+ n=3, -/- n=4).

As already mentioned, the HF phase of mouse skin influences the skin immune system. Caution was therefore taken to recruit age-matched female mice in the telogen hair phase, particularly when iASPP deficiency in the epidermis disrupted HF cycling. Dorsal skin of female mice was gently shaved and given its first topical TPA treatment the next day (Fig 4.8A). Mice received intraperitoneal injections of BrdU and were sacrificed the day following the second TPA treatment.

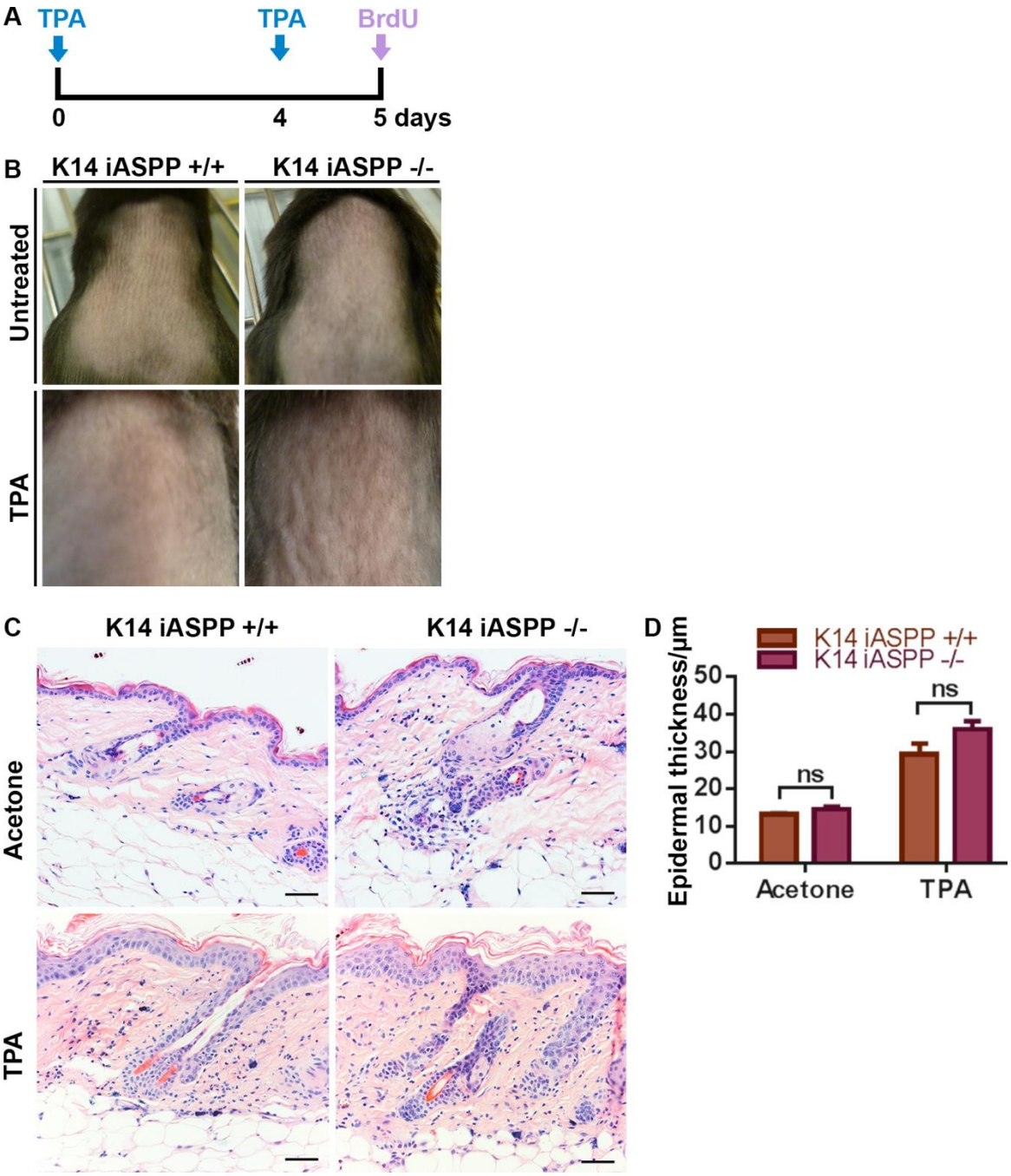


Fig 4.8 No significant differences were observed in epidermal thickness between K14-iASPP+/+ and K14-iASPP-/- murine dorsal skin samples.

(A) Schematic of short-term TPA treatment (2 doses of 4µg TPA in acetone) on murine dorsal skin to induce acute inflammation.

(B) Representative view of murine dorsal skin after acetone control or TPA treatment.

(C) H&E analysis of skin showed a thickened epidermis and increased dermal cellularity in TPA-treated skin when compared with acetone controls.

(D) Epidermal thickness between K14-iASPP+/+ and K14-iASPP-/- mice in both the acetone (K14-iASPP+/+ n=3, -/- n=4) and TPA cohorts (K14-iASPP+/+ n=4, -/- n=3) was not significantly different (scale bar=50μm, error bar represents SEM).

As shown in Fig 4.8B, no macroscopic differences could be observed between K14-iASPP+/+ and K14-iASPP-/- skin before or after topical TPA applications. Skin sections from these animals were H&E stained and analysed histologically. Epidermal hyperplasia could be observed in both wild type and mutant mice in the TPA treatment cohort, which was absent in skin from mice of the acetone control group (Fig 4.8C). An increase in dermal cellularity could also be seen in TPA-treated skin, indicating the induction of cutaneous inflammation upon TPA application. No significant difference between IFE thickness of K14-iASPP+/+ and -/- skin could be detected in the acetone-treated control cohort (Fig 4.8D, K14-iASPP+/+ mean=13.3μm vs. K14-iASPP-/- mean=14.7μm, p=0.162). The slightly thicker IFE of TPA-treated K14-iASPP-deficient mutants was not significantly greater than that of TPA-treated wild type mice (K14-iASPP+/+ mean=29.3μm vs. K14-iASPP-/- mean=36.0μm, p=0.145).

Immunofluorescence staining of BrdU on skin sections was carried out to examine cell proliferation in the IFE. Intraperitoneal injection of BrdU enabled the labelling of cells that had entered the S phase of the cell cycle, and had incorporated the thymidine analogue during

active DNA synthesis (Fig 4.9A). The numbers of BrdU-positive cells per length of the K14-postive IFE (number/mm) were comparable between wild type and mutant mice in the acetone control group (K14-iASPP+/+mean=2.93 vs. K14-iASPP-/- mean=3.15, *p*=0.72). An increase in BrdU incorporation within the IFE could be detected upon TPA treatment. TPA-treated K14-iASPP-deficient IFE showed a slightly higher frequency of BrdU-positive cells per length of basal IFE than the wild type, but has yet to reach statistical significance of *p*=0.05 (K14-iASPP+/+ mean=33.3 vs. K14-iASPP-/- mean=45.7, *p*=0.092).

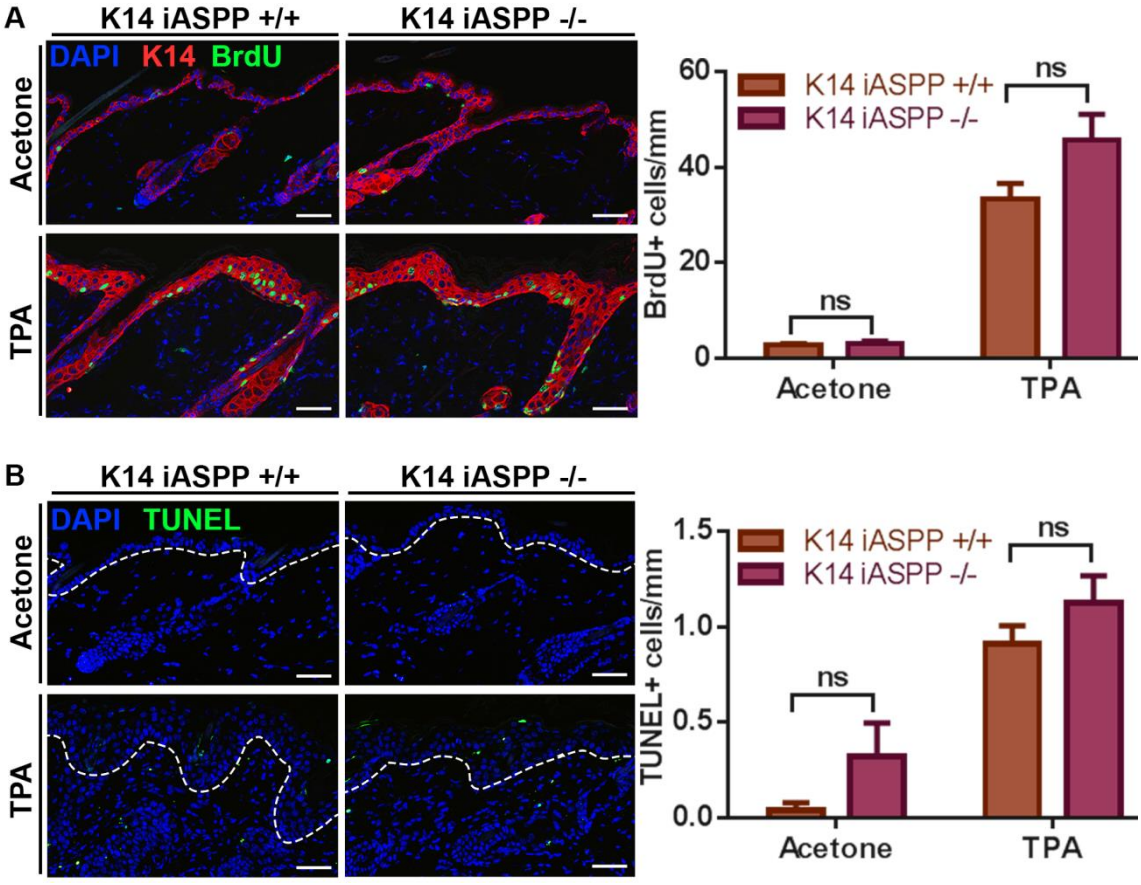


Fig 4.9 No significant difference was observed between cellular proliferation and apoptosis in the interfollicular epidermis of K14-iASPP+/+ and K14-iASPP-/- dorsal skin.

Immunofluorescence staining of BrdU to detect proliferative cells, and TUNEL assays to detect

apoptotic cells in mouse skin.
(A) Similar numbers of BrdU+ cells in K14+ IFE were observed in K14-iASPP+/+ and K14-iASPP-/- dorsal skin from both the acetone control and TPA-treated cohorts.
(B) No significant difference in the number of TUNEL+ cells was observed between K14-iASPP+/+ and K14-iASPP-/- IFE. (Scale bars= 50μm, error bar represents SEM, dashed lines indicate the epidermal-dermal junctions)

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) was performed to detect apoptotic events on these skin sections (Fig 4.9B). The assay marked the ends of DNA fragments produced in the last phase of cell apoptosis. The number of apoptotic event detected per length of IFE (number/mm) in wild type and mutant epidermis did not differ significantly in either the acetone group (K14-iASPP+/+ mean=0.041 vs. K14-iASPP-/- mean=0.32, p=0.23) or in the TPA cohort (K14-iASPP+/+ mean=0.91 vs. K14-iASPP-/- mean=1.12, p=0.25).

4.2.5 iASPP Expression in the Mouse Epidermis upon TPA-Induced Acute Cutaneous Inflammation

Positive K6 staining on TPA-treated mouse skin confirmed the hyperproliferative response of IFE towards phorbol ester treatment (Fig 4.10B). Such staining was absent in the negative control group as acetone could not induce epidermal hyperplasia and inflammation.

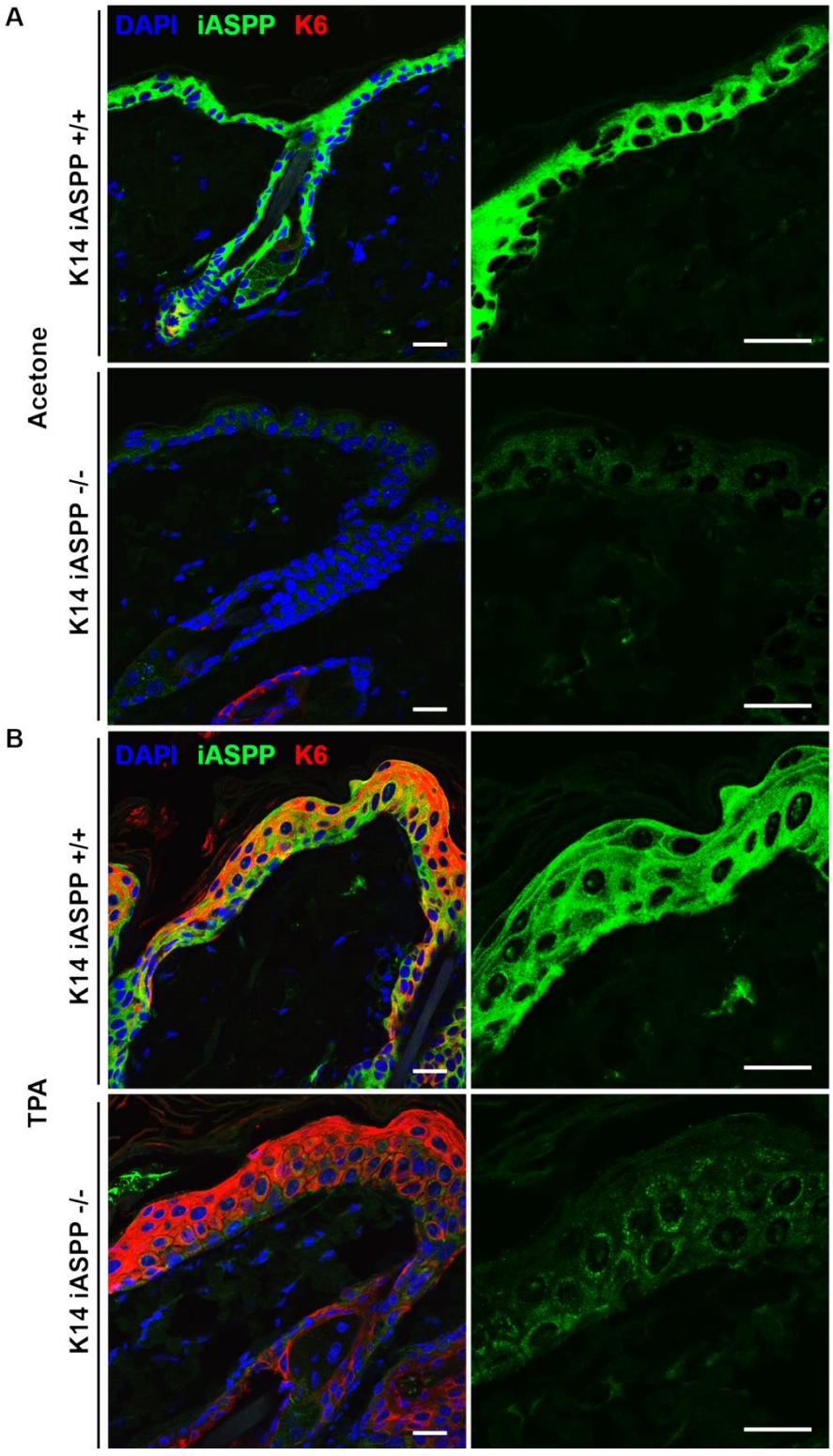


Fig 4.10 Predominantly cytoplasmic iASPP localisation in both acetone control and TPA-treated mouse skin.

(A) Cytoplasmic iASPP expression and an absence of K6 in IFE of acetone-treated skin observed

through immunofluorescence staining.
(B) iASPP remained predominantly cytoplasmic, with junctional localisation in the suprabasal layer in TPA-treated dorsal skin (scale bar=20μm).

Localisation of iASPP protein in the epidermis did not seem to be altered upon TPA-induced acute skin inflammation, as iASPP remained predominantly cytoplasmic in both the acetone and TPA treatment groups (Fig 4.10). However, junctional iASPP present in the suprabasal layers became more pronounced in the thickened hyperplastic epidermis, similar to that observed in embryonic mouse skin (Fig 4.10B).

4.2.6 Increased Macrophage Infiltration in K14-iASPP-/- Skin within Acetone Control and TPA-Treated Cohort

Mouse skin sections were next stained with a panel of immune cell markers to investigate whether epidermal iASPP deficiency could affect the populations of infiltrating immune cells during chemically induced acute skin inflammation. Antibodies against F4/80 and MPO were used to mark macrophages and neutrophils respectively (Fig 4.11A, B). Toluidine blue staining was performed to identify mast cells, while blood vasculatures were labelled with CD31 antibodies (Fig 4.11C, D). The numbers of infiltrated innate immune cells as well as CD31-positive structures per area of skin (number/mm^2) were counted in skin samples from the acetone and TPA-treated groups. Short term TPA treatment on C57BL/6 mouse dorsal skin

clearly induced an infiltration of macrophages and neutrophils, along with a mild increase in the number of mast cells and blood vasculatures.

The lack of iASPP expression in the epidermis led to an increase in macrophage number per skin area (number/mm^2) in the negative control group (Fig 4.11A, K14-iASPP+/+ mean=727 vs. K14-iASPP-/- mean=842, p=0.029). A similarly significant increase in macrophage number was also detected amongst the TPA cohort (K14-iASPP+/+ mean=1437 vs. K14-iASPP-/- mean=1896, p=0.044). However, neutrophil infiltration between K14-iASPP wild type and knockout skin was similar in unstimulated acetone-treated (Fig 4.11B, K14-iASPP+/+ mean=801 vs. K14-iASPP-/- mean=772, p=0.55) and stimulated TPA-treated skin (K14-iASPP+/+ mean=1744 vs. K14-iASPP-/- mean=1686, p=0.83). Comparable numbers were also obtained with mast cells within the control group (Fig 4.12A, K14-iASPP+/+ mean=47 vs. K14-iASPP-/- mean=48, p=0.90) and after TPA treatment (K14-iASPP+/+ mean=56 vs. K14-iASPP-/- mean=54, p=0.64). Epidermal iASPP deficiency did not significantly affect the distribution of blood vasculature present in acetone-treated (Fig 4.12B, K14-iASPP+/+ mean=120 vs. K14-iASPP-/- mean=158, p=0.08) and TPA-treated skin dermis (K14-iASPP+/+ mean=195 vs. K14-iASPP-/- mean=188, p=0.78) in terms of the number of CD31-positive structures per skin area.

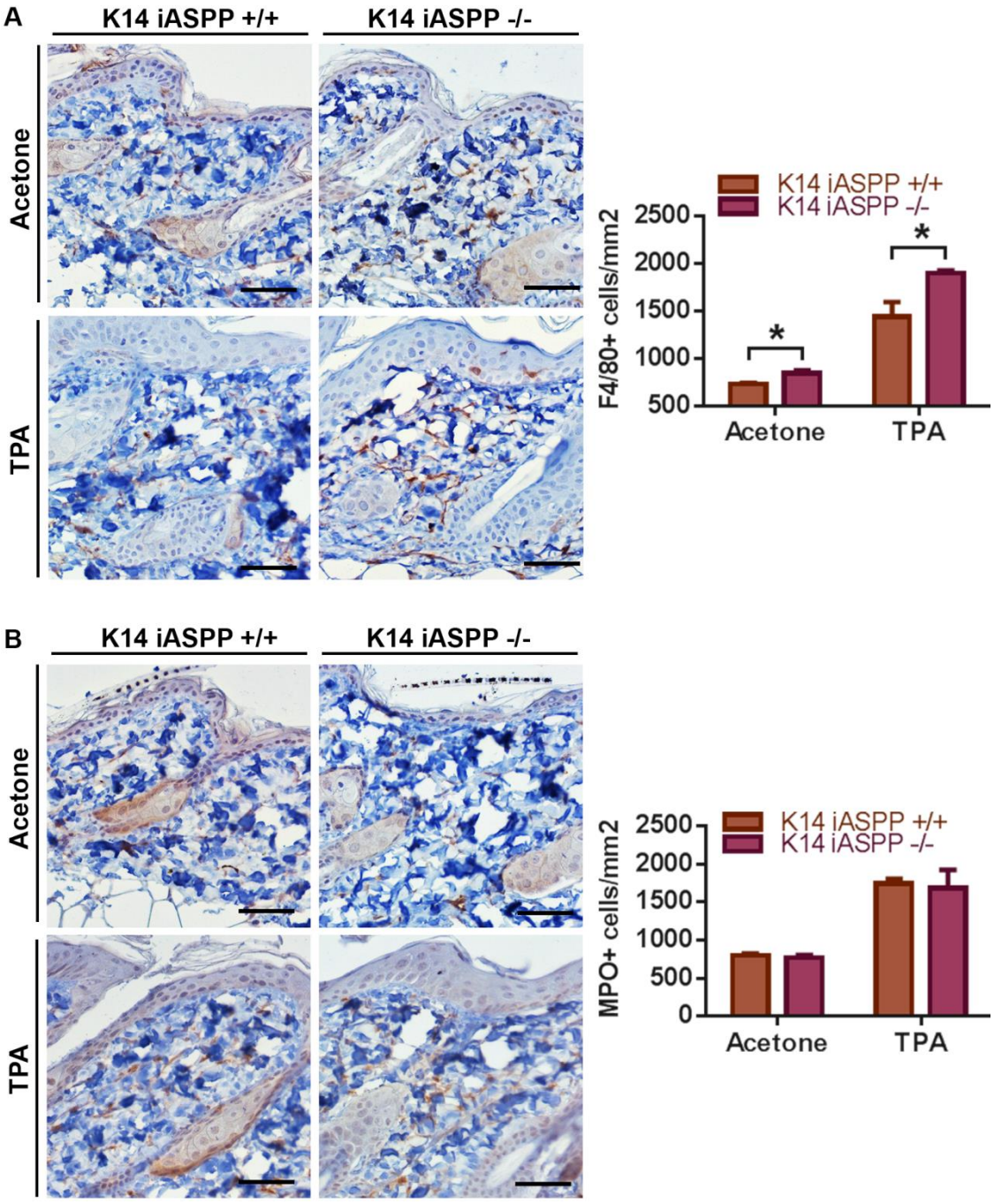


Fig 4.11 Increased macrophage, but not neutrophil infiltration in K14-iASPP-/- mouse skin compared to the wild type.

DAB staining to detect (A) F4/80+ macrophages and (B) MPO+ neutrophils in mouse skin from the acetone and TPA cohorts. Infiltration of macrophages and neutrophils could be detected upon TPA treatment. A significant increase in macrophage infiltration was found in K14-iASPP-/- skin in both acetone and TPA settings (*p<0.05, scale bar=50μm).

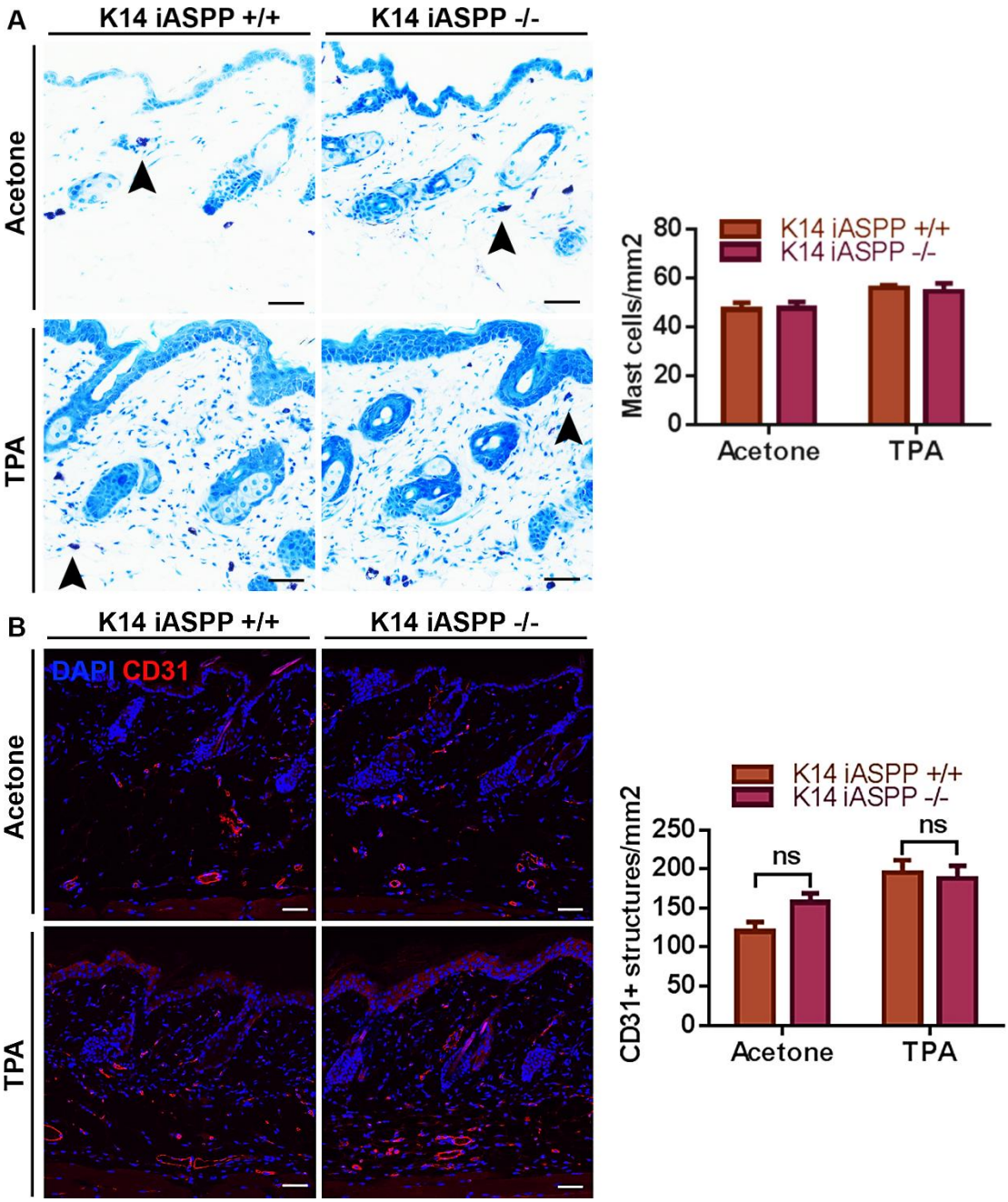
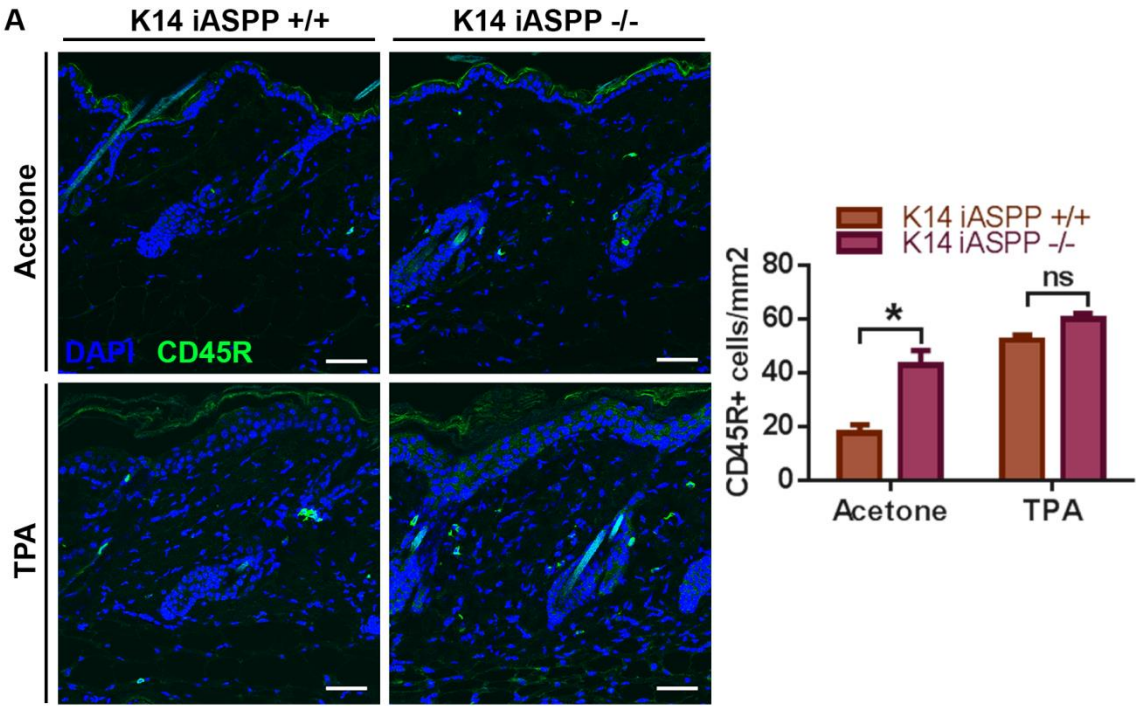


Fig 4.12 Mast cell infiltration and blood vasculature in K14-iASPP+/+ and K14-iASPP-/- mouse skin did not differ significantly.

(A) Toluidine blue staining to detect mast cells, and (B) CD31 immunofluorescence staining for blood vasculature, was performed on mouse skin from the acetone and TPA treatment cohorts. No significant differences in the number of infiltrated mast cells and blood vasculatures could be observed between K14-iASPP+/+ and -/- mice (scale bar=50µm).

4.2.7 Increased B Cell Numbers were Present in K14-iASPP-/- Skin in Acetone-Treated Control Cohort

The infiltration of adaptive immune cell populations such as T and B cells was examined through immunofluorescence staining. K14-iASPP-deficient skin contained an increased number of CD45R+ B cells per skin area (number/mm^2) when compared to wild type skin (Fig 4.13A, K14-iASPP+/+ mean=18 vs. K14-iASPP-/- mean=43, p=0.016). Such a difference, though relatively reduced, was maintained upon TPA-mediated skin inflammation and was close to being statistically significant (K14-iASPP+/+ mean=52 vs. K14-iASPP-/- mean=60, p=0.058).



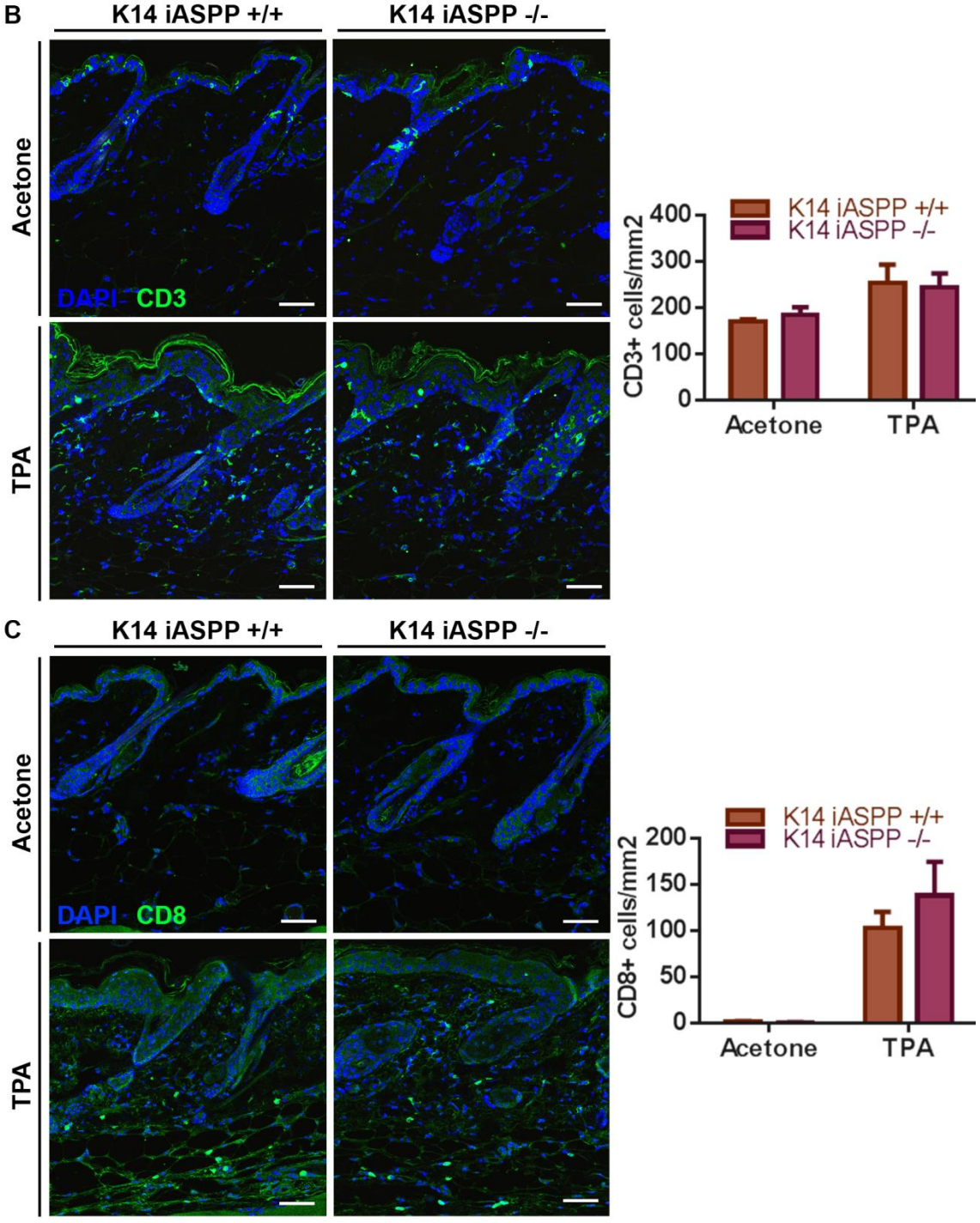


Fig 4.13 Increased B cell infiltration in acetone-treated K14-iASPP-/- mouse skin when compared to wild type skin from the control cohort.

(A) Immunofluorescence staining of B cell marker CD45R on control and TPA-treated mouse skin revealed an increase in B cell infiltration upon TPA treatment. A significant increase in B cell infiltration in control K14-iASPP-/- skin was detected in comparison to the wild type

(*p<0.05, scale bar=50μm).
(B) Immunofluorescence staining of T cell marker CD3 on control and TPA treated mouse skin demonstrated an increase in T cell infiltration upon TPA treatment. No significant difference in T cell infiltration was observed between wild type and knockout mice.
(C) Immunofluorescence staining of T killer cell marker CD8 on control and TPA treated mouse skin showed an increase in T killer cell infiltration upon TPA treatment, but no significant difference was detected between K14-iASPP+/+ and -/- mice.

The numbers of infiltrating CD3+ T cells per skin area were similar in acetone-treated wild type and K14-iASPP-deficient skin (Fig 4.13B, K14-iASPP+/+ mean=169 vs. K14-iASPP-/- mean=184, p=0.51). Skin CD3+ T cell numbers went up in response to TPA treatment, but no significant differences were observed between wild type and knockout (K14-iASPP+/+ mean=253 vs. K14-iASPP-/- mean=244, p=0.87). Similarly, no difference was detected in CD8+ T killer cell distribution in the acetone cohort (Fig 4.13C, K14-iASPP+/+ mean=1.07 vs. K14-iASPP-/- mean=0.45, p=0.36) and TPA cohorts (K14-iASPP+/+ mean=103 vs. K14-iASPP=138, p=0.38).

Although previous studies suggested that acetone treatment did not influence the distribution of immune cells in dorsal mouse skin (Wang et al., 1999; Rashel et al., 2014), it would be useful to examine the number of macrophages and B cells present in untreated K14-iASPP+/+ and K14-iASPP-/- skin to ensure that such difference observed was not caused by acetone exposure.

4.3 Discussion

The negative regulatory function of iASPP towards p53-mediated apoptosis and keratinocyte differentiation, together with reports of iASPP overexpression in human cancers, led to the hypothesis that iASPP could be oncogenic. Such theory is being challenged by the unforeseen increased susceptibility of K14-iASPP-deficient mice towards chemically induced skin carcinogenesis demonstrated in this study. K14-iASPP-deficient mice exhibited earlier onset of papilloma development, alongside significantly increased incidence of papilloma and average papilloma number per animal relative to the wild type.

The absence of spontaneous tumour development in aged K14-iASPP-/- mice and in the TPA control cohort within the DMBA/TPA assay indicated that iASPP deficiency alone was insufficient to initiate tumour growth. Papilloma development was dependent on the activation of Ras oncogene, and a lack of epidermal iASPP seemed to provide a growth advantage to these initiated cells resulting in the higher papilloma burden of knockout mice. However, the possible involvement of epidermal iASPP in the malignant conversion of papillomas could not be addressed due to the absence of such events in the two-stage skin tumour induction assay performed on the C57Bl/6 mouse strain. It was demonstrated previously that p53-deficient mice showed no difference in the formation of benign papillomas

in DMBA/TPA skin carcinogenesis assay, but exhibited accelerated malignant conversion as compared to wild type (Kemp *et al.,* 1993). Thus, the presence of p53 mutations in papillomas from K14-iASPP+/+ and K14-iASPP-/- mice has not been determined. However, it would be interesting to see if the absence of epidermal iASPP could provide selective pressure on the possible p53 mutations preset in papillomas.

Considering the presence of abnormal immune cell infiltration in K14-iASPP-deficient skin and the interaction between iASPP and p65 reported in literature, it was hypothesized that epidermal iASPP deficiency could bring about an inflammatory microenvironment in skin to support the proliferation of initiated keratinocytes. Thus, a short-term TPA treatment assay was performed to explore whether K14-iASPP-/- skin would respond differently towards TPA-induced skin inflammation.

K14-iASPP-deficient epidermis showed a trend of increased cell proliferation upon TPA stimulation. Moreover, there was also a tendency of K14-iASPP-deficient epidermis to have increased cell apoptotic events. A larger sample size is required to see whether such trends indicate a significant but mild change in cell proliferation and apoptosis in the absence of epidermal iASPP expression. Interestingly, analysis of data collected from the short-term TPA

treatment assay indicated an increase in macrophages and B cell infiltration present in acetone-treated negative control K14-iASPP-deficient skin when compared to the wild type.

According to these *in vivo* studies on the K14-iASPP mouse cohort, epidermal iASPP seems to be playing an important role in maintaining normal skin immunohomeostasis. The lack of iASPP expression in the acetone-treated epidermis could lead to an abnormal infiltration of leukocytes such as macrophages and B cells. It would be interesting if such differences in immune cell numbers could be replicated in untreated K14-iASPP+/+ and K14-iASPP-/- skin. In the absence of epidermal iASPP expression, the deregulated skin immune system could potentially contribute to papilloma development in the context of chemically induced skin tumourigenesis on mouse skin.

Chapter 5 A Lack of iASPP led to Increased Epidermal Expression of Inflammatory Mediators *in vitro* and *in vivo*

5.1 Introduction

Studies performed on K14-iASPP transgenic mice indicated that deprivation of epidermal iASPP expression led to the deregulation of skin immunity and increased susceptibility towards chemically induced skin carcinogenesis. This demonstrated that the iASPP protein is vital in regulating the biological activities of keratinocytes in such a way that it enables epithelial cells to interact with their surrounding neighbours, thus maintaining normal skin immune homeostasis as a whole.

Preceding studies on iASPP have indicated the possible interaction between iASPP and the transcription factor p65. Binding between the shorter iASPP homologue RAI and p65 was detected in the yeast two-hybrid assay, and the DNA binding activity of p65 to κB motifs was inhibited by RAI in rat embryonic fibroblasts (Yang *et al*., 1999; Takada *et al*., 2002). The transcription factor p65 is involved in mediating the canonical NF-κB signalling pathway, which is critical in regulating the innate and adaptive immune responses (Oeckinghaus *et al*., 2009). The participation of p65 in regulating cutaneous immunohomeostasis was evidenced by the systemic hyper-inflammation affecting the skin, lung and liver in transgenic knock-in mice with

constitutively active p65 (Dong *et al*., 2010). Transgenic mice deficient of p65 expression in the K14-positive basal epidermis showed resistance towards the TPA-induced skin inflammatory response and DMBA/TPA-induced tumourigenesis (Kim *et al*., 2014). The aberrant infiltration of immune cells and increased susceptibility towards chemically induced papilloma development in K14-iASPP-/- mice might be an indication of the inhibitory influence of iASPP on p65 transcriptional activity in basal keratinocytes. Enhanced p65 activity on inflammatory gene expression in iASPP-deficient keratinocytes might disrupt the normal homeostasis of the cutaneous immune system.

As discussed earlier, an increasing amount of evidence has emphasised the ability of keratinocytes to participate in immune responses through the production of AMPs, cytokines and chemokines. To better understand the role of iASPP in the biology of keratinocytes, notably its influence on inflammatory signalling pathways, primary mouse keratinocytes and the non-tumorigenic human keratinocyte cell line HaCaT were utilised as *in vitro* models for such studies (Boukamp *et al*., 1988). Primary mouse keratinocytes were prepared from iASPP*loxP*/*loxP* Cre+ERT (iASPP CreER) new-born pups, a transgenic mouse cohort established in the Lu Lab that enables conditional iASPP deletion upon the addition of tamoxifen (Notari, *et al*., 2011). HaCaT cells are spontaneously immortalised human keratinocytes that are widely

used in the study of epidermal biology due to their conserved epidermal differentiation capacity (Schoop *et al*., 1999). RNA and protein analyses were performed on these cell cultures, with attempts made to translate such findings into the *in vivo* K14-iASPP-deficient mouse model and human patient samples.

5.2 Results

5.2.1 Increased mRNA Levels of Inflammatory Mediators Detected in iASPP-Deficient Keratinocytes

In order to investigate how iASPP deficiency could affect inflammatory signalling in keratinocytes, RNA samples were extracted from iASPP+/+ and -/- primary mouse keratinocyte cultures to measure gene expression levels. Keratinocytes were generated from iASPP CreER pups to give tamoxifen-treated iASPP-/- cells and negative control ethanol-treated iASPP+/+ keratinocytes. Cells were also administered with TNF-α, a potent pro-inflammatory cytokine, for the indicated time periods to detect any differential response of iASPP-deficient cells towards such stimuli.

A panel of inflammatory mediators known to be expressed by keratinocytes was selected to be tested for the amount of mRNA present through qPCR analysis. Successful knockout of iASPP gene expression in iASPP CreER mouse keratinocytes was achieved after 4 days of tamoxifen treatment (Fig 5.1A).

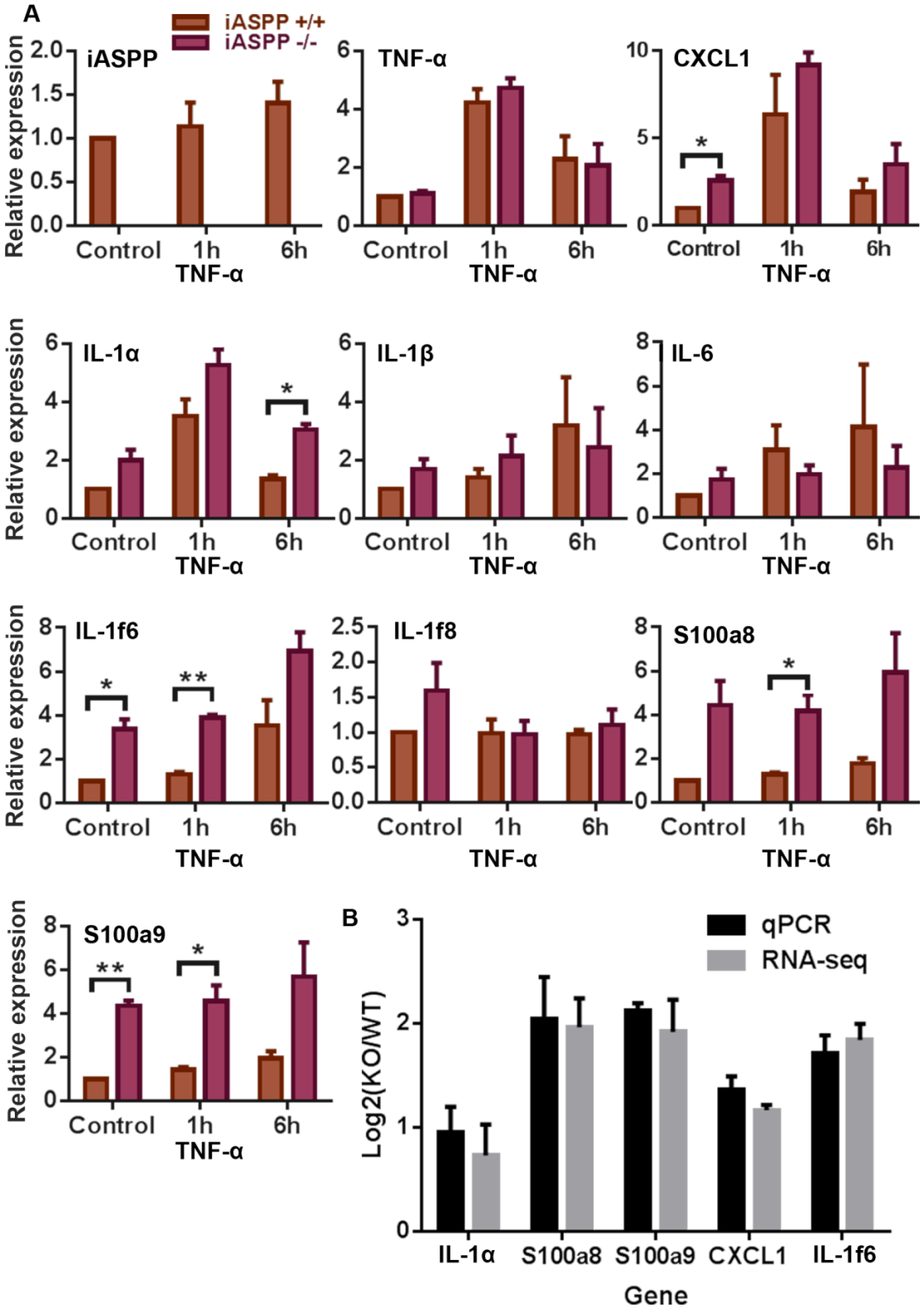


Fig 5.1 Up-regulated expression of genes associated with inflammation in iASPP-/- primary mouse keratinocytes relative to iASPP+/+ cells.

RNA samples were extracted from iASPP CreER primary mouse keratinocytes treated with

ethanol (control) or tamoxifen to induce iASPP deletion. iASPP+/+ and iASPP-/- keratinocytes were treated with or without 10ng/ml TNF-α for the indicated time periods.
(A) Fold change in mRNA was calculated relative to that of untreated wild type keratinocytes and normalised to GAPDH (n=3, *p<0.05, **p<0.01).
(B) Comparable fold changes in gene expression level between data from qPCR and RNA-seq experiments on iASPP-/- samples relative to iASPP+/+ samples.

Expression levels of target genes were analysed using the ΔΔCt method with GAPDH as the internal control, and presented as fold-change relative to the value obtained in untreated iASPP+/+ keratinocytes (Fig 5.1A). No significant alteration was detected in iASPP mRNA levels upon TNF-α treatment in wild type keratinocytes (Fold change for: Unstimulated=1 vs. 1hTNF-α=1.14, p=0.66; 6hTNF-α=1.41, p=0.52). Significant down-regulation of iASPP mRNA was observed in iASPP-/- keratinocytes upon tamoxifen treatment (Fold change: iASPP+/+=1 vs. iASPP-/-=0.0003, p=2.81x10^{-8}).

TNF-α positively regulated its own gene expression in keratinocytes as reported previously (Banno *et al*., 2004). However, a lack of iASPP did not affect relative TNF-α mRNA levels in the conditions tested (Fold change for: Unstimulated: iASPP+/+=1 vs. iASPP-/-=1.11, p=0.31; 1hTNF-α: iASPP+/+=4.23 vs. iASPP-/-=4.73, p=0.41). CXCL1, another known TNF-α-regulated target with chemotactic effects on neutrophils, exhibited increased expression upon TNF-α treatment (Banno *et al*., 2004), and showed a trend of increased expression without iASPP

(Fold change for: Unstimulated: iASPP+/+=1 vs. iASPP-/-=2.59, *p*=0.023; 6hTNF-α: iASPP+/+=1.91 vs. iASPP-/-=3.51, *p*=0.074). No effect was observed on the mRNA levels of IL-6 (Fold change for: Untreated: iASPP+/+=1 vs. iASPP-/-=1.74, *p*=0.28; 1hTNF-α: iASPP+/+=3.08 vs. iASPP-/-=2.00, *p*=0.29), a pleiotropic cytokine involved in wound healing and skin barrier function (Wang *et al*., 2004).

The mRNA levels of IL-1α, the pre-dominant IL-1 ligand produced by keratinocytes, were significantly enhanced in iASPP-deficient keratinocytes when compared to wild type cells in response to TNF-α (Fold change for 6hTNF-α: iASPP+/+=1.34 vs. iASPP-/-=3.05, *p*=0.020). However no difference was seen in the relative expression level of IL-1β, the less abundant IL-1 ligand in keratinocytes (Sims and Smith, 2010).

IL-1f6/IL-36α and IL-1f8/IL-36β are members of the IL-1 family due to their homology to the IL-1 ligands, and have full biological function as full-length proteins without proteolytic processing (Sims and Smith, 2010). Overexpression of the IL-36 cytokines has been reported in human psoriasis skin lesions (Carrier *et al*., 2011). IL-1f6/IL-36α expression was significantly increased in iASPP-deficient keratinocytes relative to that of iASPP+/+ cells (Fold change for: Unstimulated: iASPP+/+=1 vs. iASPP-/-=3.39, *p*=0.032; 1hTNF-α: iASPP+/+=1.32 vs.

iASPP-/-=3.91, *p*=0.0070; 6hTNF-α: iASPP+/+=3.54 vs. iASPP-/-=6.93, *p*=0.060). No significant gene expression induction was observed for IL-1f8/IL-36β upon TNF-α (Fold change for: Unstimulated: iASPP+/+=1 vs. iASPP-/-=1.60, *p*=0.27; 6hTNF-α: iASPP+/+=0.98 vs. iASPP-/-=1.10, *p*=0.54), which could be explained by the specificity of its induction in response to IFN-γ only (Carrier *et al*., 2011).

S100a8 (MRP-8/calgranulin A) and S100a9 (MRP-14/calgranulin B) are members of the S100 cytoplasmic calcium-binding protein family, and preferentially form the S100a8/S100a9 heterodimer referred as calprotectin (Eckert *et al*., 2004; Kerkhoff *et al*., 2012). Constitutive expression of S100a8 and S100a9 is observed in myeloid cells such as monocytes and neutrophils, and the heterodimer is involved in promoting phagocyte migration via the regulation of tubulin dynamics in a calcium-dependent manner (Vogl *et al*., 2004). S100a8 and S100a9 have anti-microbial properties and show chemotactic activities towards neutrophils, as they can be released by neutrophils and macrophages to the extracellular environment (Rykman *et al*., 2003; Kolls *et al*., 2008). Expression of these S100 members is minimal in healthy epidermis, but is induced upon stresses such as wounding, TPA-treatment and exposure to bacterial flagellin (Gebhardt *et al*., 2002; Eckert *et al*., 2006; Abtin *et al*., 2010), or in pathological conditions such as psoriasis and cancer (Eckert *et al*., 2004; Kerkhoff *et al*.,

2012). In unstimulated iASPP-deficient keratinocytes, the expression of S100a9 (Fold change: iASPP+/+=1 vs. iASPP-/-=4.37, *p*=0.0043) was induced when compared to wild type cells. This enhanced expression in iASPP knockout keratinocytes was also observed in TNF-α stimulated conditions. After 1 hour of TNF-α treatment, the S100a8 mRNA level was roughly three times higher in iASPP-deficient cells relative to that in the wild type (Fold change: iASPP+/+=1.29 vs. iASPP-/-=4.19, *p*=0.044) and a similar fold change was observed for S100a9 (Fold change: iASPP+/+=1.43 vs. iASPP-/-=4.59, *p*=0.036).

A recent collaboration has been established with Dr. Jo Zhou's lab at the Radboud University to study the transcriptome of iASPP+/+ and iASPP-/- keratinocytes through RNA-seq deep sequencing. RNA samples from ethanol-control and tamoxifen-treated iASPP CreER primary mouse keratinocytes were extracted and sent to the Netherlands for RNA-seq analysis. Initial gene ontology analysis showed enrichment in up-regulated genes associated with keratinisation and the stress response in iASPP-/- keratinocytes when compared to wild type cells (Table 5.1).

Genes involved in keratinisation were up-regulated in iASPP-deficient cells, consistent with previous studies which suggested that iASPP could act as a negative regulator of keratinocyte

differentiation (Chikh *et al*., 2011; Notari *et al*., 2011). Moreover, genes involved in stress response such as S100a7a, IL-1f9 and CCL20 were also over-represented in iASPP knockout cells. The list of genes included inflammatory mediators that have been found to show enhanced expression through qPCR experiments (Fig 5.1A). Moreover, expression fold-change of such genes was comparable between qPCR data and results from RNA-seq (Fig 5.1B).

Gene Ontology Term	Examples (FDR<0.05)
Keratinisation	Cnfn, IL-1α, Sprr2d, Sprr2e, Sprr2g, Sprr2h, Tgm3, Ptgs1, Upk1b, Wnt16
Stress Response	IL-1α, S100a7a,S100a8, S100a9, IL-1f6, IL-1f9, Ccl20, Cxcl1, Cryab, Defb14, Glul, Hmox1, ler3, Nupr1, Plk3, Prdx5, Ptgs1, Trex2, Upk3bl

Table 5.1 List of genes up-regulated in primary mouse keratinocytes in the absence of iASPP. RNA samples were extracted from iASPP CreER primary mouse keratinocytes treated with ethanol (control) or tamoxifen for conditional iASPP deletion. Samples were sent to Dr. Jo Zhou's lab (Radboud University, Netherlands) for RNA-seq analysis (n=3).

5.2.2 iASPP and p65 Interaction Not Detected in HaCaT Human Keratinocytes under Normal Physiological Conditions

The NF-κB signalling pathway is central to the control of biological processes that are involved in mediating immune and inflammatory responses. The list of stress response components that were up-regulated in iASPP-deficient keratinocytes included genes that have been previously associated with NF-κB signalling such as IL-1α (Mori *et al*., 1996), CXCL1 (Anisowicz

et al, 1991), CCL20 (Battaglia *et al*., 2008), S100a8/a9 (Németh *et al*., 2009), Plk3 (Li *et al*., 2005), HMOX1/HO-1(Lin *et al*., 2007) and Ier3/IEX-1L/DIF2 (Wu *et al*., 1998; Witcher *et al*., 2008). Considering a previous report on the interaction of iASPP with the NF-κB transcription factor p65 (Yang *et al*., 1999), the possibility of binding occurring between the two proteins was investigated in HaCaT keratinocytes (Fig 5.2A).

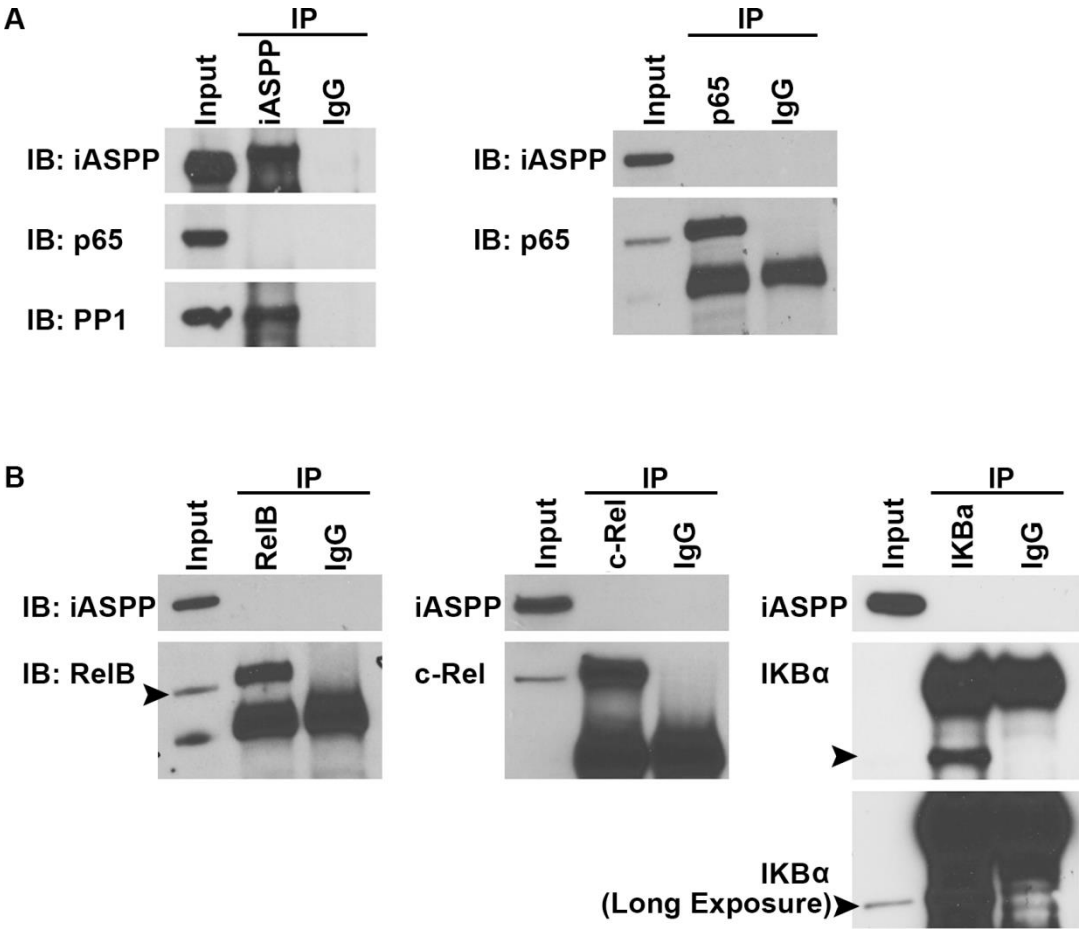


Fig 5.2 Binding between endogenous iASPP and p65 was not detected in HaCaT human keratinocytes under normal physiological conditions.

Interaction between endogenous iASPP and NF-κB components was tested by co-immunoprecipitation assays in HaCaT cells.

(A) HaCaT protein lysate was immunoprecipitated with polyclonal anti-iASPP LX49.3 antibody

and control IgG, and pull-down was blotted for p65 and PP1. iASPP co-immunoprecipitated with PP1, but not p65. Immunoprecipitation with p65 and immunoblotting for iASPP was also unable to detect binding between the two proteins.
(B) HaCaT protein lysate was immunoprecipitated with RelB, c-Rel and IκBα and blotted for iASPP on western blots. No binding could be observed between iASPP and these NF-κB components.

HaCaT cells were lysed with either RIPA or NETN/NP-40 cold lysis buffer, and immunoprecipitation (IP) assays were performed with the appropriate antibodies. The presence of a PP1 signal on the western blot for iASPP immunoprecipitation indicated successful iASPP pull-down in the IP reaction, as binding between iASPP and PP1 has been demonstrated previously (Llanos *et al*., 2011). However, no binding between endogenous iASPP and p65 could be detected in HaCaT cells under normal physiological conditions (Fig 5.2A). Other NF-κB components such as RelB, c-Rel and IκBα were also tested for binding with endogenous iASPP but protein-protein interaction could not be detected in the IP assays (Fig 5.2B).

5.2.3 Cytokine Treatment did not Affect iASPP Cellular Localisation

Although iASPP did not seem to interact with p65 under normal cellular conditions, protein-protein interaction might occur under stimulatory or stressed conditions. This speculation came from the observation that nuclear iASPP co-localised with nuclear p65 in

papillomas developed during the DMBA/TPA study (Fig 4.7). In an attempt to mimic the inflammatory conditions present in papillomas that might contribute to the nuclear translocation of p65, HaCaT cells were treated with different inflammatory cytokines and immunofluorescently stained for iASPP and p65 (Fig 5.3).

The cytokines TNF-α, IL-1α and IL-1β were selected due to their known involvement in keratinocyte biology, and also based on the qPCR and RNA-seq results in which several IL-1 family members showed up as being differentially expressed in the absence of iASPP. iASPP expression was silenced in HaCaT cells using short interfering RNA (siRNA) mediated gene knockdown to confirm staining specificity and to detect any potential impact on p65 localisation in keratinocytes. HaCaT cells were treated with PBS control or with TNF-α, IL-1α and IL-1β for the indicated time periods. TNF-α acted as a potent pro-inflammatory cytokine to induce the nuclear translocation of p65 shortly after treatment, but did not alter the cellular localisation of iASPP that remained predominantly cytoplasmic in HaCaT cells (Fig 5.3A). In comparison, IL-1α and IL-1β had moderate effects on p65 nuclear translocation detectable 1 hour after application (Fig 5.3B-C). However, the IL-1 ligands had no effects on the iASPP cytoplasmic expression profile in HaCaT cells.

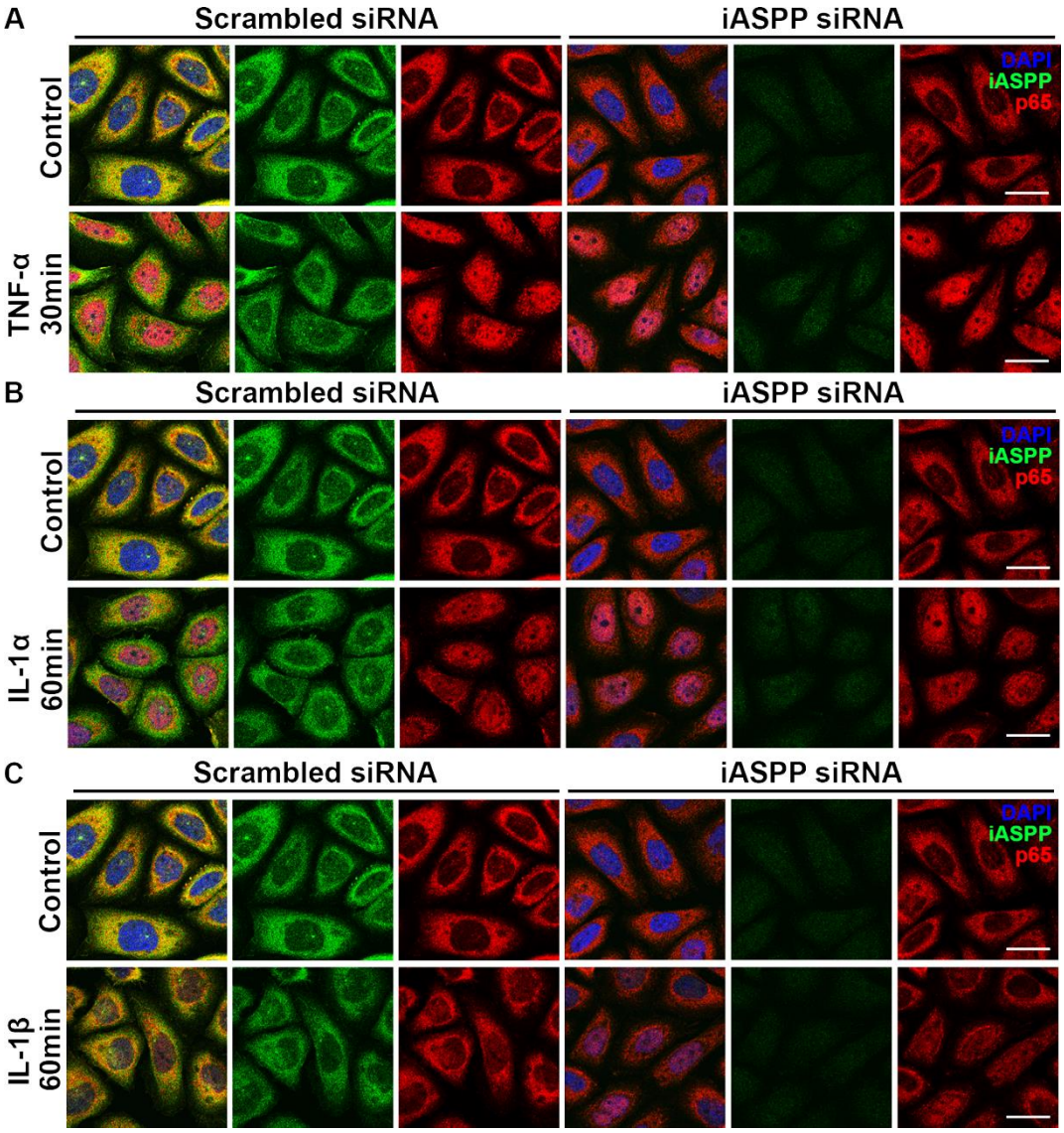


Fig 5.3 iASPP localisation in human keratinocytes HaCaT was not affected upon TNF-α, IL-1α or IL-1β treatment.

HaCaT cells were treated with scrambled siRNA or siRNA against iASPP for 4 days. HaCaT cells were exposed to PBS control or 10ng/ml of (A) TNF-α (B) IL-1α or (C) IL-1β for the indicated time periods (scale bar=20μm). Cells were fixed in PFA and the localisation of iASPP and p65 detected by immunofluorescence staining. iASPP localisation upon cytokine treatment and p65 nuclear translocation in the absence of iASPP were not affected.

To validate that the nuclear localisation of iASPP observed in papillomas was not a consequence of activated Ras oncogene being present in these initiated keratinocytes (Fig 4.5), the malignant variant of HaCaT cells, referred as HaCaT II-4, was obtained from Prof. Boukamp at the German Cancer Research Centre in Germany. HaCaT-II4 cells were generated by transfecting HaCaT cells with the H-Ras oncogene, and can induce the development of highly differentiated SCC upon subcutaneous injection into nude mice (Boukamp *et al*., 1990).

iASPP in transformed HaCaTII-4 cells was predominantly cytoplasmic under normal conditions, indicating that activated Ras activity was unlikely to have contributed to the nuclear iASPP detected in papilloma samples (Fig 5.4A). Again, the addition of cytokines TNF-α, IL-1α and IL-1β into HaCaTII-4 cultures did not change the localisation of iASPP protein (Fig 5.4). It would be interesting to investigate under what stimulatory conditions iASPP would translocate into the nuclei of keratinocytes, and perhaps then an interaction between endogenous iASPP and p65 would be observed in immunoprecipitation assays.

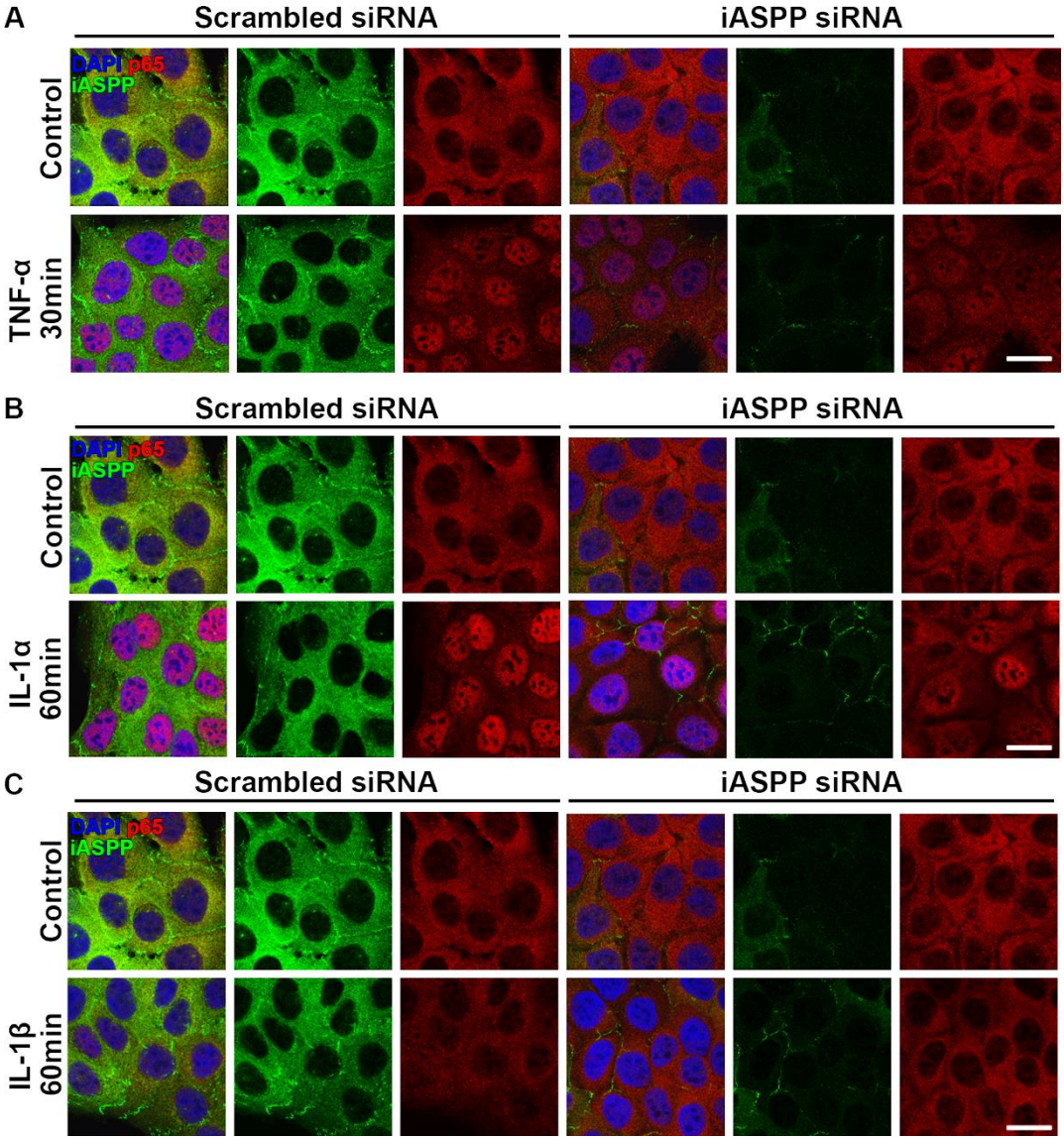


Fig 5.4 iASPP remained predominantly cytoplasmic in Ras transformed human keratinocytes HaCaT II-4.

HaCaT II-4 cells were treated with scrambled siRNA or siRNA against iASPP for 4days. PBS control or 10ng/ml of (A) TNF-α (B) IL-1α or (C) IL-1β was added into cell culture for the indicated time period (scale bar=20μm). iASPP and p65 localisation was detected by immunofluorescence staining. Cytoplasmic and junctional iASPP could be observed in Ras transformed HaCaT II-4 cells. iASPP localisation upon cytokine treatment, and p65 nuclear translocation in the absence of iASPP were not affected.

5.2.4 Aberrant MAPK Phosphorylation in iASPP-Deficient Keratinocytes

In consideration of the enhanced expression of a number of IL-1 family members in iASPP-deficient keratinocytes and the autocrine activity of IL-1α in up-regulating its own expression as well as other IL-1 family members (Yano *et al*., 2008; Sims *et al*., 2010), the cellular responses of iASPP+/+ and iASPP-/- keratinocyte cultures towards IL-1α stimulation were examined and how downstream signalling pathways could be affected.

External stimuli sensed by cells can be translated into intracellular responses through the MAPK signalling cascade. The MAPK cascade involves the sequential serine/threonine phosphorylation activities of MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). The stimulation of membrane receptors leads to the phosphorylation and activation of MAPKKK, which in turn phosphorylates MAPKK downstream (Zhang *et al*., 2002; Roux *et al*., 2004). Activated MAPKK then phosphorylates MAPK, allowing it to mediate biological responses by phosphorylating various substrates such as transcription factors, phospholipases, cytoskeletal proteins and MAPK-activated protein kinases (MK).

The p38 MAPK cascade is known to be activated by inflammatory cytokines such as IL-1 and TNF-α as well as stress signals like UV irradiation (Zhang *et al*., 2002; Roux *et al*., 2004; Zarubin

et al., 2005). Downstream substrates activated by p38 MAPK include the transcription factors p53 and NF-κB, and MKs such as MSK1/2 (Roux *et al*., 2004; Zarubin *et al*., 2005). It is therefore not surprising that activation of the p38 signalling cascade is associated with inflammatory diseases such as RA and IBD (Zarubin *et al*., 2005).

The Erk1/2 MAPK, also referred as p44/p42, is an important component of another MAPK signalling cascade that predominantly responds to mitogenic stimuli and to a lesser extent inflammatory and stress signals (Zhang *et al*., 2002; Roux *et al*., 2004). Upon cell surface receptor perceiving the presence of mitogenic signals, a number of adapter proteins are recruited to the stimulated receptor to activate the small GTP-binding protein Ras. Activated Ras then triggers the activity of MAPKKK Raf, which relays the signal to MAPKK Mek followed by Erk1/2 MAPK activation. Phosphorylated Erk1/2 MAPK translocates into the nucleus to induce downstream signalling that is largely involved in controlling cell proliferation, differentiation and developmental processes (Chang *et al*., 2003, Roux *et al*., 2004). The Erk1/2 signalling pathway has also been implicated in keratinocyte chemokine expression and cutaneous inflammation (Pastore *et al*., 2005). Examples of Erk1/2 MAPK substrates include the Stat1/3 transcription factors and MSK1/2 (Chang *et al*., 2003, Roux *et al*., 2004).

The phosphorylation states of p38 and Erk1/2 MAPKs were examined in iASPP-deficient keratinocytes relative to wild type cells due to their recognised function in signalling inflammatory gene expression. Moreover, it would be interesting to look into Erk1/2 phosphorylation due to the apparent dependence of K14-iASPP-deficient papilloma development on activated Ras and the involvement of Ras in the Ras/Raf/Mek/Erk cascade. Loss of iASPP expression could possibly facilitate the activation of the Erk1/2 MAPK signalling pathway.

Primary mouse keratinocytes extracted from iASPP CreER pups were cultured with the addition of ethanol (control) or tamoxifen for iASPP conditional knockout. Three days after ethanol/tamoxifen treatment, keratinocytes were serum-starved overnight and treated with either PBS control or IL-1α for 30 minutes or 16 hours. Keratinocytes incubated with tamoxifen showed a considerable reduction in iASPP expression. Moreover, iASPP protein level was not altered upon IL-1α treatment (Fig 5.5). Beta-tubulin was used as a loading control to confirm that similar amounts of protein were loaded between samples subjected to western blot analysis.

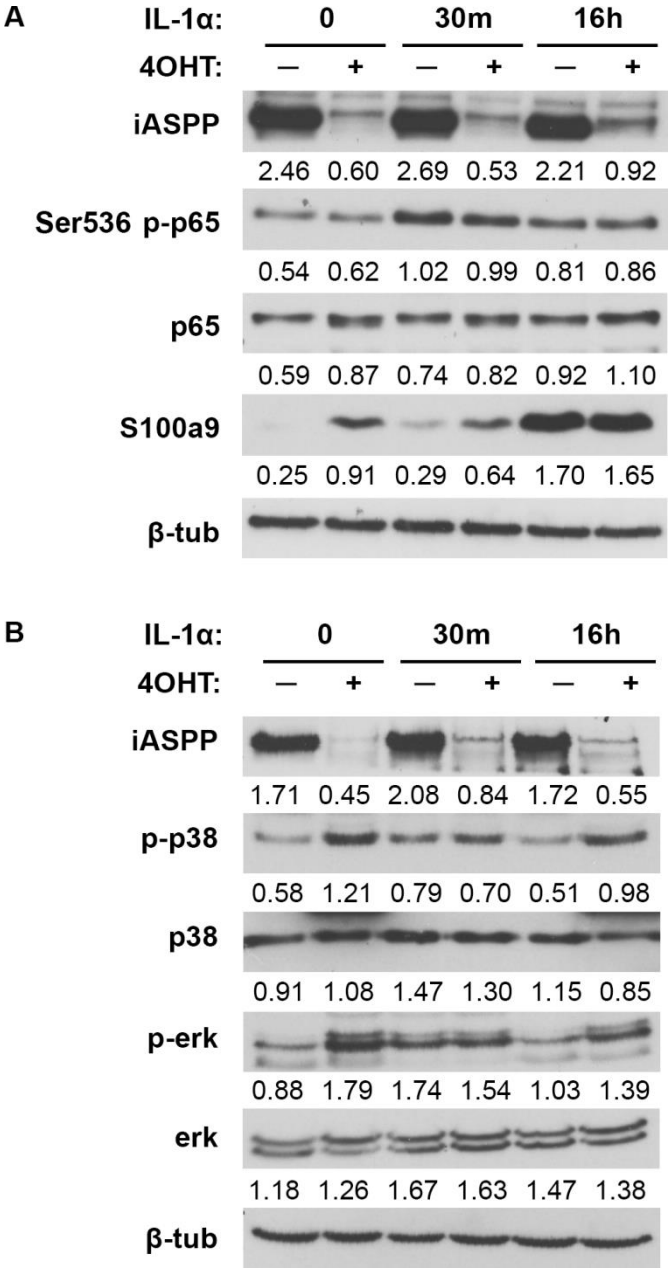


Fig 5.5 Increased phosphorylation of Erk1/2 and p38 was observed in iASPP-/- primary mouse keratinocytes.

iASPP CreER primary mouse keratinocytes were treated with ethanol control or tamoxifen to induce iASPP deletion. Cells were treated with PBS or 10ng/ml IL-1α for the indicated time period. Total protein lysate was extracted using UREA lysis buffer. Experiment was repeated twice. Number under each band represents the relative amount of protein normalised to that of β-tubulin.

(A) iASPP+/+ and iASPP-/- keratinocytes had similar p65 phosphorylation levels to PBS controls

and upon IL-1α treatment. iASPP-/- keratinocytes expressed increased levels of S100a9 proteins which could be induced upon IL-1α treatment in wild type cells.
(B) Increased phosphorylation levels of Erk1/2 and p38 were detected in iASPP-/- keratinocytes, which could be induced upon IL-1α treatment for 30 minutes. Levels of p-Erk1/2 and p-p38 decreased in iASPP+/+ keratinocytes after 16 hours of IL-1α treatment, but not in iASPP-/- keratinocytes.

The phosphorylation status of the NF-κB transcription factor p65 was investigated which reflected the activation of the NF-κB pathway and p65 transcriptional activation. IL-1α treatment of keratinocytes induced an increase in p65 Ser536 phosphorylation, but the phosphorylation levels between wild type and iASPP-deficient keratinocytes were comparable in all treatment conditions tested (Fig 5.5A). IL-1α stimulation also enhanced the production of S100a9 proteins in wild type keratinocytes, consistent with previous reports demonstrating IL-1α modulation of S100a9 expression (Bando *et al*., 2010; Abtin *et al*., 2010). S100a9 protein was absent in non-treated wild type keratinocyte lysates, as cells should not be expressing S100a9 under normal physiological conditions (Fig 5.5A). However, iASPP-deficient keratinocytes expressed higher levels of S100a9 proteins, which agreed with the earlier observation in this study that the S100a9 mRNA level was significantly greater in iASPP-deficient cells (Fig 5.1). The increase in S100a9 protein level in iASPP-/- keratinocytes seemed to be independent of Ser536 p65 phosphorylation.

Phosphorylation of the MAPKs p38 and Erk1/2 was also examined by immunoblotting (Fig 5.5B). Ablation of iASPP expression in keratinocytes did not affect the protein levels of MAPK p38 and Erk1/2. Treatment of IL-1α induced the phosphorylation of both p38 and Erk1/2 MAPKs 30 minutes after application, which came down to non-stimulated basal levels after 16 hours in iASPP+/+ keratinocytes. Phosphorylation levels of p38 and Erk1/2 in iASPP-/- keratinocytes, however, were abnormally induced even under normal conditions, and were maintained at the different time points of IL-1α incubation. Further investigation is required to see whether the aberrant MAPK phosphorylation levels observed in iASPP-deficient keratinocytes contribute to the up-regulation of S100a9, as well as other inflammatory mediators.

5.2.5 Enhanced S100a9 Promoter Activity in iASPP-Deficient HaCaT Cells

To further investigate whether iASPP could modulate the transactivation activity of p65, the possible influence of iASPP on S100a9 promoter activity was studied using the dual-luciferase assay. S100a9 was selected due to its significant response towards an absence of iASPP in terms of its mRNA and protein levels even under physiological condition. Moreover it has been previously reported to be a NF-κB target gene (Németh *et al*., 2009), with the presence of a NF-κB consensus motif GGGAATTTCAC upstream of the human S100a9 gene transcription start

site (TSS). This binding site resides -567bp upstream of the TSS, and was confirmed by sequence analysis on the human genome browser (Fig 5.6A). The binding motif also matched with the consensus sequence available on the JASPER transcription factor binding site database (JASPER, 2014).

The S100a9-pGL3 luciferase plasmid was a generous gift from Prof. Zhihua Liu at the Chinese Academy of Medical Sciences in China (Li *et al*., 2009). The reporter contains the -786bp to +39bp region of the human S100a9 gene promoter cloned into a basic pGL3 plasmid backbone. A quick dual-luciferase assay on the reporter plasmid was carried out to confirm the induction of S100a9 expression upon ectopic p65 expression (Németh *et al*., 2009). Human non-small cell lung carcinoma H1299 cells were transfected with either the pGL3 vector or S100a9-pGL3 reporter. Cells were also transfected with either pcDNA3 as a negative control or pcDNA3-p65, as well as the pRL *Renilla* plasmid to correct for transfection efficiency. Consistent with previous report, p65 transfection increased the relative luciferase activity of the S100a9-pGL3 reporter by roughly two-fold (Fig 5.6B). Western blot analysis on H1299 cell lysates showed the increase in p65 protein levels in pcDNA3-p65 transfected samples, with no effects on iASPP protein levels. The expression of endogenous p65 in H1299 cells could contribute to the S100a9-pGL3 luciferase activity observed in cells transfected with pcDNA3 control plasmid.

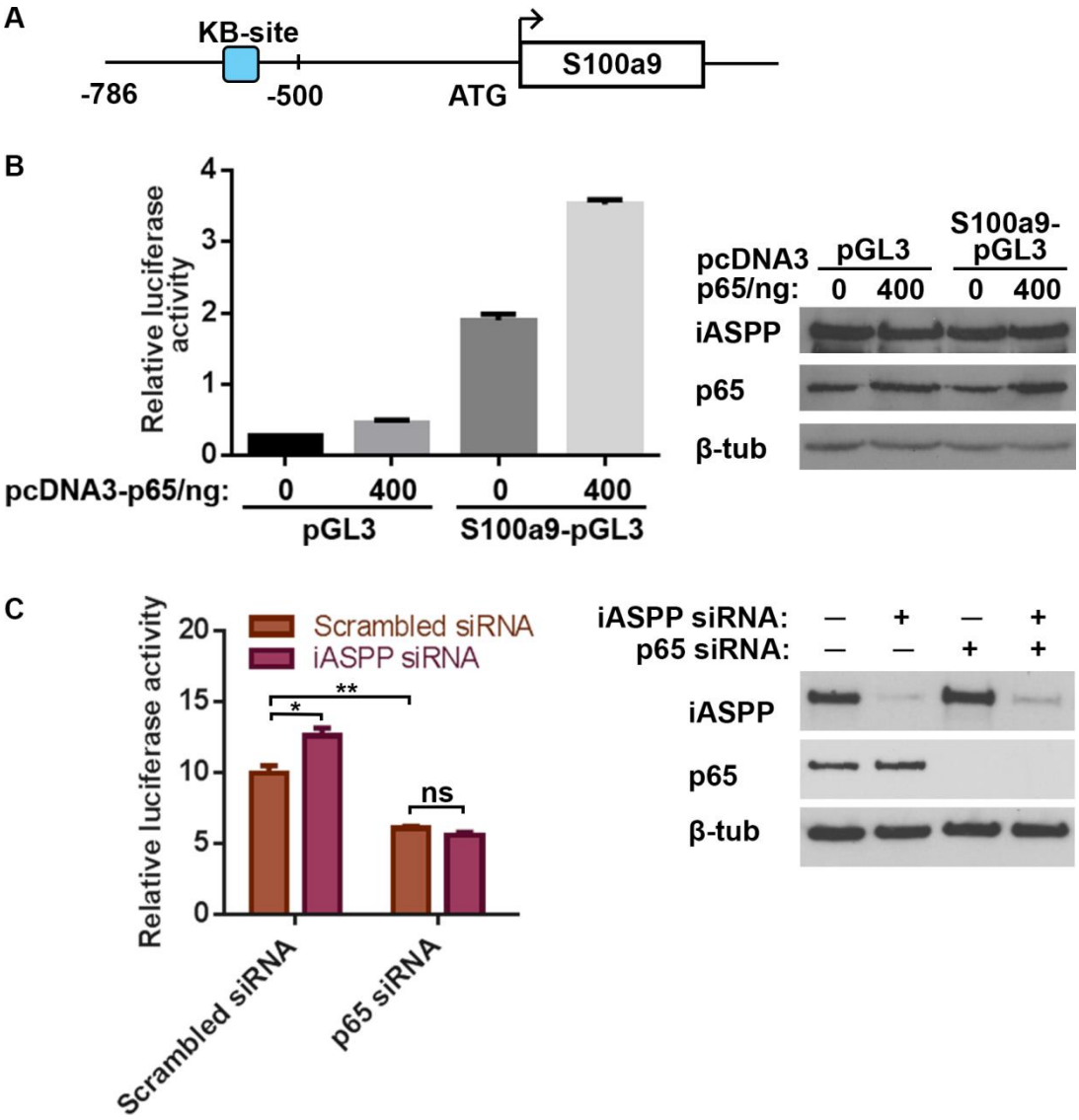


Fig 5.6 Luciferase activity of the human S100a9 promoter reporter in HaCaT was increased in the absence of iASPP.

(A) Schematic representation of the KB-site (GGGAATTTCAC) present -567bp upstream of the transcription start site (TSS) of the human S100a9 promoter.

(B) p65 induction of S100a9 expression reported in the literature (Németh *et al*., 2009) was confirmed in H1299 cells by transfection with pcDNA3 control or 400ng of pcDNA3-p65 (n=2, error bar represents SEM). Relative luciferase activity was obtained by normalising with *Renilla* luciferase readings. Protein analysis of cell lysates collected from H1299 luciferase assay showing enhanced p65 protein level upon pcDNA3-p65 transfection.

(C) HaCaT cells were treated with scrambled siRNA, siRNA against either iASPP or p65, or siRNA against both genes. Luciferase reporter containing the human S100a9 promoter (-786bp to

+39bp) was transfected into HaCaT cells for 48 hours. Luciferase readings were taken and normalised against *Renilla* internal control. Increased relative luciferase activity was detected in HaCaT with the lack of iASPP. Relative luciferase activity was reduced in the absence of p65, upon which iASPP deletion had no effect (n=4, *p<0.05, **p<0.01, error bar represents SEM). Protein analysis of cell lysates collected from the HaCaT luciferase assay demonstrating siRNA down-regulation of iASPP and p65.

The S100a9-pGL3 reporter was then tested in human keratinocytes HaCaT with siRNA mediated knockdown of iASPP and p65 expression. Successful silencing of iASPP and p65 expression was confirmed by the immunoblotting of cell lysates (Fig 5.6C). The lack of iASPP expression did not affect p65 protein levels in HaCaT, and *vice versa*. The relationship between iASPP and p65 in keratinocytes seemed to differ from that in hepatocellular carcinoma cells, which was notable as a previous study has suggested that iASPP is a transcriptional target of NF-κB (Lu *et al*., 2010).

Relative luciferase activity of the S100a9-pGL3 reporter was enhanced in HaCaT cells with iASPP siRNA knockdown (Fig 5.6C, scrambled siRNA=10 vs. iASPP siRNA=12.6, p=0.013). Silencing of p65 expression led to a significant reduction in the relative luciferase signal of S100a9-pGL3 (scrambled siRNA=10 vs. p65 siRNA=6.08, p=0.003). Reporter activity in HaCaT cells with both iASPP and p65 siRNA knockdown was similar to that detected in cells with p65 silencing only (p65 siRNA=6.08 vs. iASPP and p65 siRNA=5.56, p=0.12). Deletion of iASPP

expression did not influence the transcriptional activity of the S100a9 promoter in p65-deficient cells. This makes it difficult to completely rule out the possibility of interaction between iASPP and p65 in controlling S100a9 expression, as p65 could lie downstream of iASPP on the same signalling pathway.

5.2.6 Abnormal Expression of Inflammatory Mediators on K14-iASPP-Deificient Mouse Epidermis

Considering the significant effects of iASPP deletion on S100a9 transcription and protein levels in keratinocytes, further investigation was done to see whether such *in vitro* evidence could be translated into the *in vivo* K14-iASPP-deficient mouse model. Skin sections from K14-iASPP+/+ and -/- mice were stained for the S100a8 and S100a9 proteins by DAB immunohistochemistry.

S100a8 and S100a9 expression is not detected in healthy epidermis, but is induced in stressed or pathological conditions (Gebhardt *et al*., 2002; Eckert *et al*., 2004; Abtin *et al*., 2010; Kerkhoff *et al*., 2012). As expected, the K14-iASPP+/+ mouse epidermis was negative for both S100a8 and S100a9 staining (Fig 5.7A). However, focal expression of S100a8 and S100a9 was detected on K14-iASPP-/- epidermis. S100a8 and S100a9 positive staining was present in the same epidermal region on adjacent skin sections, suggesting the possible formation of

heterodimeric S100a8/a9 calprotectin in K14-iASPP-/- skin. All sections from TPA-treated murine skin and papilloma samples exhibited positive S100a8/9 staining irrespective of the mouse genotype, corresponding with previous reports in the literature (Fig 5.7B).

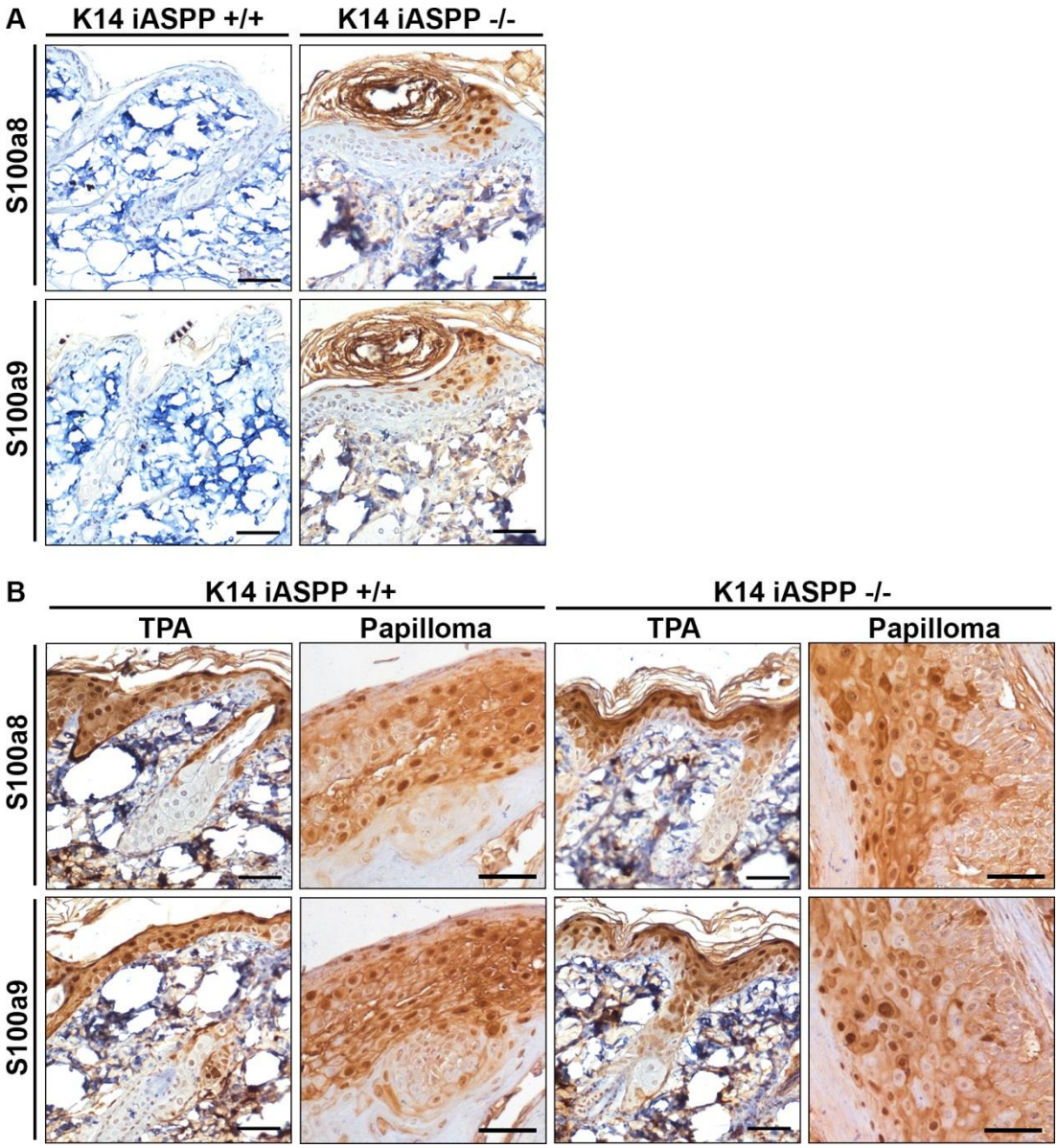


Fig 5.7 Abnormal expression of S100a8 and S100a9 was detected on 1 year old K14-iASPP-/- mouse skin, but not K14-iASPP+/+.

(A) Expression of S100a8 and S100a9 was detected at focal thickenings observed in K14-iASPP-/- epidermis through DAB staining analysis, but not on K14-iASPP+/+ epidermis.

(B) Expression of S100a8 and S100a9 was detected in TPA-treated skin and papilloma sections from K14-iASPP+/+ and K14-iASPP-/- mice (scale bar=50µm)

5.2.7 iASPP Expression in Human Inflammatory Skin Disorders

Having seen the contribution of epidermal iASPP in maintaining proper immunohomeostasis in mouse skin, whether or not ASPP is involved in the development of human inflammatory skin diseases such as eczema and psoriasis was investigated. Paraffin sections of normal human epidermis and chronic inflammatory skin lesions were obtained from the OCHRe biobank with the assistance of dermatologist Dr. R. Asher. Epidermal iASPP localisation was examined on human skin sections of normal epidermis, eczema and psoriasis samples (n=9 per group) through DAB staining.

Expression of iASPP was detected in the epidermis of all samples from both normal human epidermis and chronically inflamed skin lesions (Fig 5.8). Nuclear and cytoplasmic iASPP staining was observed in normal human epidermis, agreeing with the findings of previous report (Notari *et al*., 2011). Abundant epidermal iASPP expression was also detected in eczema and psoriasis samples, but cells with low or undetectable nuclear iASPP were identified in the epidermis more frequently relative to normal epidermis (Fig 5.8B-C).

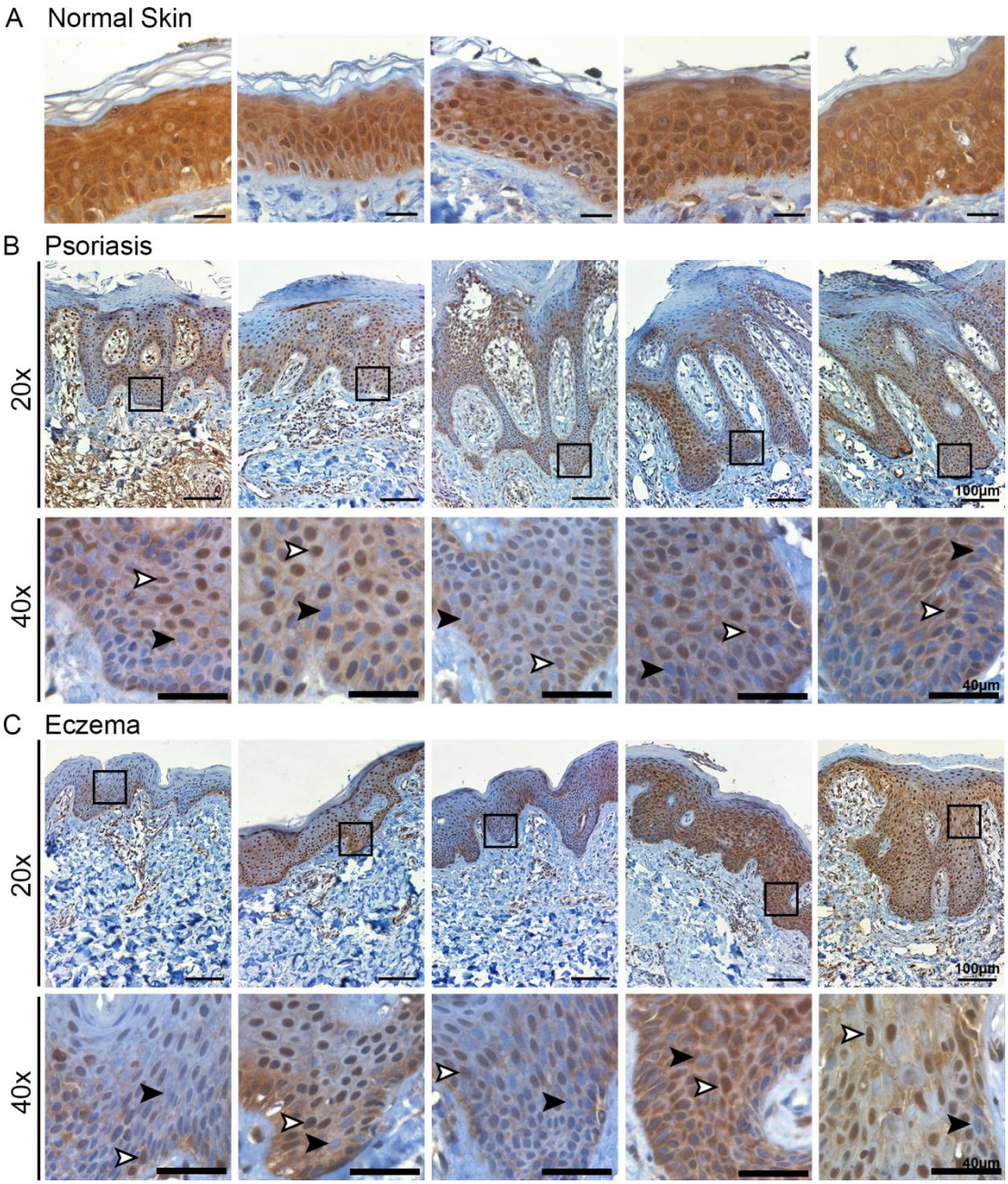


Fig 5.8 iASPP expression in human chronic inflammatory skin diseases.

iASPP localisation was examined by DAB staining on paraffin sections from human normal epidermis and inflammatory skin lesions.

(A) Cytoplasmic and nuclear iASPP staining was detected in normal human epidermis (scale bar=20μm).

(B) Epidermal iASPP expression detected in human psoriasis lesions. Epidermal cells with nuclear iASPP (white arrowheads) and those with low/undetectable nuclear iASPP (black arrowheads) could be detected (scale bar=40μm)

(C) Eczema lesions showed epidermal iASPP staining. Figures were labelled with white arrowheads indicating cells with nuclear iASPP and black arrowheads for cells with low/undetectable nuclear iASPP (scale bar=40μm)

In order to examine whether the epidermis of eczema and psoriasis lesions contained less nuclear iASPP staining quantitatively, samples were immunofluorescently co-stained with basal marker K14 and iASPP (Fig 5.9A). The immunofluorescent signal of iASPP present in K14-positive epidermis within normal skin, eczema and psoriasis samples was measured and analysed (Fig 5.9B-C).

Nuclear iASPP signals as fractions of total iASPP staining measured in the K14-positive epidermis were represented in percentages (normal=44.6% vs. eczema=39.4% vs. psoriasis=36.7%, p=0.093). The amount of iASPP signal per cell in the basal layer was obtained, dividing the value of total iASPP signal by the number of nuclei present (normal=1312x10^3 vs. eczema=1222x10^3 vs. psoriasis=1151x10^3, p=0.72). Similar iASPP immunofluorescence per cell was obtained from normal epidermis, eczema and psoriasis samples, but a weak trend showing a relative reduction in nuclear iASPP staining in chronically inflamed epidermis was observed. The trend did not reach statistical significance owing to the small sample size available for this study, and the large biological variation between human skin tissue donors.

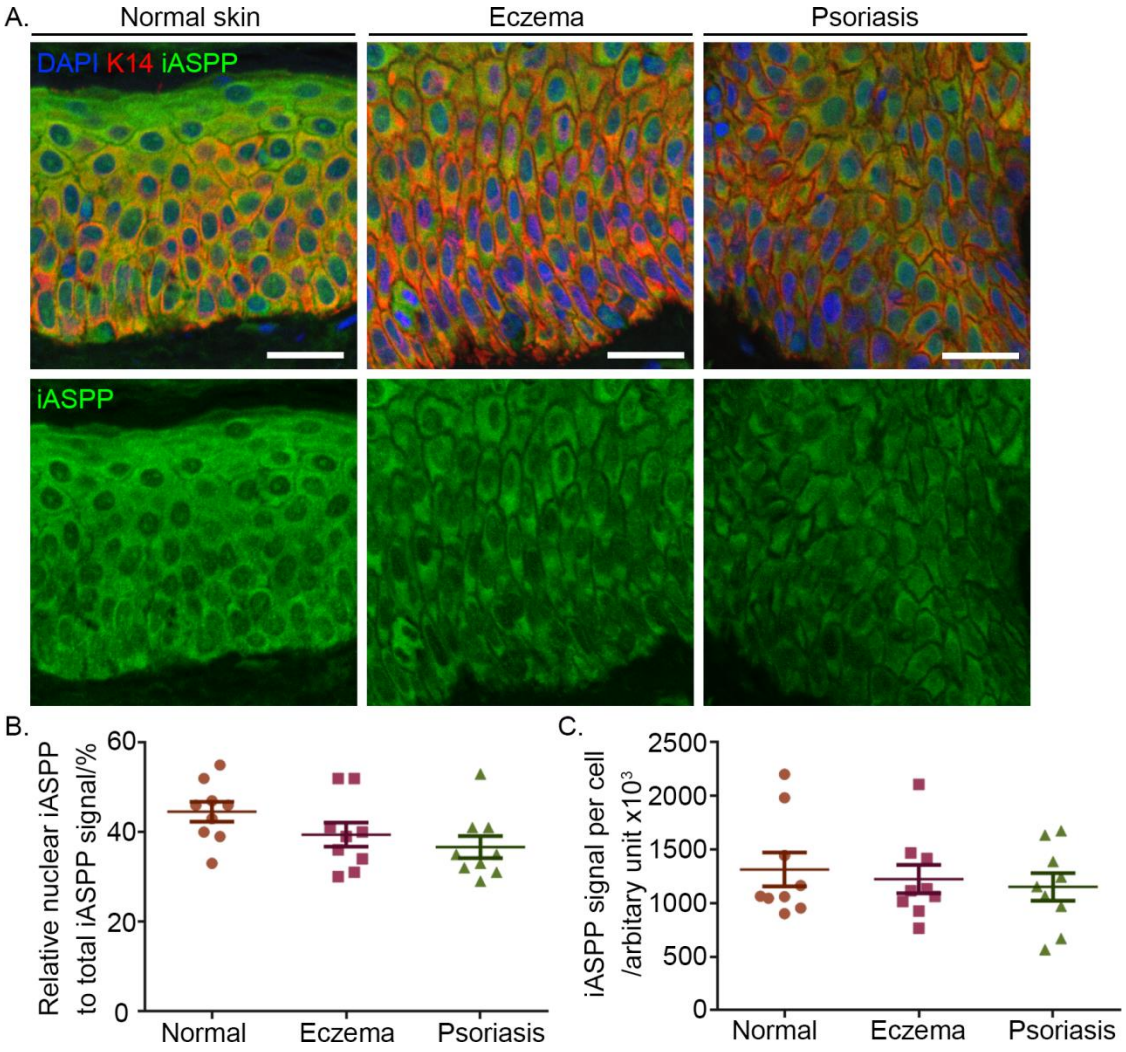


Fig 5.9 Co-staining of iASPP and K14 on normal human epidermis and samples from eczema and psoriasis lesions.

(A) iASPP localisation in K14-positive epidermis was investigated by immunofluorescence staining of human normal epidermis, eczema and psoriasis sections (scale bar=25μm).

(B) Percentage of nuclear iASPP signal relative to the total iASPP immunofluorescence signal measured in the K14-positive basal epidermis (n=9 per group, p=0.093)

(C) iASPP immunofluorescence signal per cell within the K14-positive epidermis was measured on stained human skin sections from normal epidermis, eczema and psoriasis lesions (n=9 per group, p=0.72).

5.3 Discussion

The ablation of iASPP expression in primary mouse keratinocytes resulted in the up-regulated expression of inflammatory genes such as IL-1α, S100a8/9, IL-1f6/9, CXCL1 and CCL20. This observation could offer an explanation for the abnormal presence of immune cell infiltrates in K14-iASPP-deficient skin, in which iASPP-/- keratinocytes were promoting an inflammatory skin microenvironment. Aberrant expression of the inflammatory mediators S100a8 and S100a9 on K14-iASPP-deficieint mouse skin was detected, in accordance with the *in vitro* data obtained from keratinocyte cultures. Furthermore, the intimate link between inflammation and cancer progression has been recognised in the literature (Hanahan *et al*., 2011). The increased incidence of papillomas on K14-iASPP-deficient mice subjected to the DMBA/TPA assay relative to the wild type could be the result of an inflammatory microenvironment provided by iASPP-/- keratinocytes.

It is puzzling that the interaction between iASPP and p65 previously reported (Yang *et al*., 1999) could not be reproduced in human keratinocyte HaCaT cells, considering the high endogenous level of both proteins in this cell line. Perhaps the binding between iASPP and p65 in HaCaT was too weak to be detectable with endogenous proteins. It Is also probable that the interaction between iASPP and p65 is indirect, and is mediated by an adapter protein.

Repeating the conditions used in the previous study by transfecting human embryonic kidney cells 293 with iASPP and p65 expression plasmids for immunoprecipitation experiments would be an attractive way to re-examine such a binding event. There is also the possibility that such protein-protein interaction only occurs transiently under certain biological conditions, which do not include normal physiological cell culturing. This speculation comes from the observation that full-length iASPP is predominantly cytoplasmic whereas the short variant RAI used in the reported iASPP-p65 binding assay is predominantly nuclear (Yang *et al*., 1999; Slee *et al*., 2004). It would be interesting to investigate whether binding between iASPP and p65 could be achieved in HaCaT cells, under conditions in which iASPP is predominantly nuclear. Unfortunately, the addition of cytokines TNF-α, IL-1α and IL-1β into HaCaT cultures did not induce such nuclear translocation. It would be intriguing to discover the conditions that can replicate the relatively pronounced nuclear iASPP staining observed in mouse papillomas.

The link between iASPP and p65 was made more perplexing with the similar phosphorylation levels of Ser536 p65 detected in iASPP+/+ and iASPP-/- keratinocytes, in both basal and IL-1α stimulated conditions. As reviewed previously, p65 phosphorylation at serine phosphor-acceptor sites reflects its enhanced transactivation activity and the activation of the NF-κB pathway (Schitmz *et al*., 2001; Chen *et al*., 2004). Although no difference was observed

in the phosphorylation status of Ser536 p65 in iASPP wild type and knockout keratinocytes, that of the p65 Ser276 residue requires further examination. Moreover, the phosphorylation of MAPKs Erk1/2 and p38 was abnormally induced in iASPP-deficient keratinocytes in basal conditions, suggesting the dysregulation of MAPK signalling pathways without iASPP expression.

In addition, the dual-luciferase assay on S100a9-pGL3 reporter activity indicated that both p65 and iASPP are involved in regulating the transcriptional activity of the human S100a9 promoter. Reporter activity was reduced in the absence of p65 expression, confirming that S100a9 is an NF-κB target gene. Without endogenous iASPP expression, the S100a9-pGL3 reporter showed increased activity illustrating the repressive role of iASPP on the S100a9 promoter. However, deletion of iASPP expression in p65-deficient HaCaT cells did not influence luciferase readings relative to those of p65-deficient cells. The results of the dual-luciferase assay could suggest that p65 is downstream of iASPP in regulating S100a9 promoter activity, and hence iASPP deficiency failed to rescue S100a9 promoter activation in p65-deficient cells. On the other hand, it is possible that S100a9 expression is controlled by iASPP and p65 on different pathways, and that the effects of p65 overpower those of iASPP on S100a9 promoter activity in normal cellular conditions. Overexpression of p65 in cells, along with the transfection of

increasing amounts of iASPP expression plasmid would provide a more direct indication of whether iASPP does indeed inhibit p65 transactivational activity on the S100a9 promoter.

Altogether, the evidence presented here illustrates the function of iASPP in regulating the gene expression of inflammatory regulators in keratinocytes to maintain proper skin immunity. A trend of reduced nuclear iASPP expression in human inflammatory skin lesions, such as eczema and psoriasis, was detected relative to normal human epidermis. This suggests that nuclear iASPP is required to regulate inflammatory gene expression, and that the down-regulation of iASPP could be associated with the pathogenesis of such skin disorders. However, the data reported here did not reach statistical significance due to the limited sample size and large variations present between the human samples. Ideally, a human skin tissue microarray containing hundreds of samples from diseased skin and normal controls should be used, but such samples were unfortunately unavailable at the OCHRe biobank. It would be interesting to obtain such skin tissue arrays from a different human tissue biorepository, or to increase the sample size substantially in future studies.

Chapter 6 Final Discussion and Perspectives

6.1 Overview

The aim of this research project was to assess the oncogenic potential of iASPP, a gene which has been described to inhibit p53-mediated apoptosis and suppress keratinocyte differentiation in the epidermis. The findings presented in this thesis did not support the notion of iASPP being a proto-oncogene, and instead revealed a novel role for epidermal iASPP in the regulation of skin immunohomeostasis and the protection it offered against chemically induced skin carcinogenesis.

The importance of iASPP in epidermal biology was highlighted by the development of abnormal coats and eyelid closure defects in K14-iASPP-/- mice with specific iASPP deletion in the epidermis. K14-iASPP-deficient skin exhibited signs of abnormal immune cell infiltration, focal epidermal thickenings and increased susceptibility to DMBA/TPA induced carcinogenesis when compared to the wild type. Furthermore, increased numbers of macrophages and B cells were present in K14-iASPP-/- skin.

Gene expression analysis of primary mouse keratinocytes showed significant up-regulation of inflammatory gene transcription in the absence of iASPP. A corresponding increase in cellular

protein level and aberrant expression on K14-iASPP-/- epidermis was detected for S100a8/9, one of the inflammatory mediators found to be enhanced in iASPP-/- keratinocytes. Analysis of protein lysates from wild type and iASPP-deficient keratinocytes indicated aberrant phosphorylation of Erk1/2 and p38 MAPKs in iASPP-/- keratinocytes at the basal level, which was absent in iASPP+/+ cells. This illustrated a dysregulation of MAPK signalling pathways in the absence of iASPP expression, possibly contributing to the abnormal expression of inflammatory genes. Further studies will be required to unravel the possible interaction between iASPP and p65 in the regulation of inflammatory signalling pathways, as results obtained during this project were not sufficient to make firm conclusions.

The interesting results obtained in the DMBA/TPA-mediated skin carcinogenesis assay demonstrated that iASPP expression in the epidermis prevented the development of benign papillomas. This indicated a tumour suppressor function of iASPP in the epidermis, despite previous publications suggesting its potential role as a proto-oncogene by protecting cells against apoptosis and enhancing cell transformation (Bergamaschi *et al*., 2003; Chen *et al*., 2010). It would be intriguing to investigate if the absence of epidermal iASPP could provide growth advantages to keratinocytes by facilitating an inflammatory microenvironment that supports tumour development. Furthermore, it would be interesting to test the involvement of

epidermal iASPP in other skin carcinogenesis models such as UVB-irradiation mediating p53 mutations, and to further understand its role in other tumour induction assays and not only in the context of DMBA/TPA assay that is dependent on the activation of Ras proto-oncogene.

Lastly, iASPP expression in human normal epidermis and inflammatory skin lesions from eczema and psoriasis patients was examined. More samples are required to test whether the correlation observed here between reduced nuclear iASPP expression and chronically inflamed epidermis is statistically significant and clinically relevant.

6.2 The Possible Role of Macrophages and B Cells in K14-iASPP-Deficient Skin

In K14-iASPP-/- mouse skin within the acetone-treated control cohort, greater numbers of macrophages and B cells were found to be present relative to those of K14-iASPP+/+. Although further investigation is required to dissect how these leukocyte populations might be responsible for the phenotypes seen in K14-iASPP-deficient mice, several lines of evidence have indicated their participation in the pathogenesis of inflammatory skin lesions and cutaneous malignancies.

Macrophages are professional phagocytes that patrol tissues for the removal of pathogenic materials and present antigens to T cells to mediate the cellular immune response. These highly plastic cells have been associated with cancer development and inflammatory diseases (Murray *et al*., 2011). The pro-inflammatory M1 macrophages have been described as participating in chronic inflammatory and autoimmune conditions such as Crohn's disease and psoriasiform dermatitis (Murray *et al*., 2011; Pasparakis *et al*., 2014). However in tumour development, M1 macrophages seem to offer a protective role with their tumour-killing function and amplify the anti-tumour T_H1 response. In contrast, most tumour-associated macrophages (TAM) have similar properties to the regulatory M2 macrophages that promote tissue repair and dampen inflammation (Murray *et al*., 2011; Pasparakis *et al*., 2014). It would be tempting to explore the expression profiles of the macrophages present in K14-iASPP-/- skin to examine their M1/M2 subtype polarisation. The depletion of macrophage in mice through treatment with clodronate encapsulated in liposomes (clodrolip) might also be utilised at a later stage to investigate their contribution to papilloma development (Nasser *et al*., 2012).

The involvement of B cells in cutaneous homeostasis has been underappreciated compared to the amount of research done on T cells. B cells were once considered to be absent or present in insignificant number within healthy skin, but have now been discovered to traffic through

and reside in non-inflamed skin (Geherin *et al*., 2012). Auto-antibodies produced by self-reactive B cells have been found to mediate the pathogenesis of autoimmune diseases such as RA, which result in tissue damage due to the functional inhibition of target self-proteins and the induction of complement activation (Martin *et al*., 2004). The role of B cells in carcinogenesis has been revealed by recent studies which utilised the K14-HPV16 transgenic mouse model to mimic multistage SCC development (de Visser *et al*., 2005; Andreu *et al*., 2010). Their work demonstrated that B cell-deficient mice (K14-HPV16 JH-/-) had reduced tumour incidence and that immune complexes produced by B cells stimulated the pro-angiogenic and pro-tumourigenic properties of mast cells and macrophages. Moreover, macrophages in B cell-deficient K14-HPV16 JH-/- animals exhibited polarisation towards the pro-inflammatory M1 function but not the M2 like profile associated with TAMs. This suggests that B cell-mediated humoral immunity can promote skin carcinogenesis by activating FcR on myeloid cells and modulating the bioeffector functions of macrophages (de Visser *et al*., 2005; Andreu *et al*., 2010). It would be compelling to test whether the incidence of papillomas on K14-iASPP-deficient mice can be affected by B cell depletion, or if such depletion can influence macrophage activity in mutant skin. Moreover, aged K14-iASPP-/- mice could be tested for any possible deposition of immunoglobulin IgG and IgM in skin, similar to what has been observed in K14-HPV16 mice as a sign of chronic inflammation (de Visser *et al*., 2005).

Lastly, it would be interesting to study the distribution of LCs and dermal DCs in K14-iASPP-deficient skin, cutaneous immune cell populations that were not explored in this project. LCs are epidermal antigen presenting DCs that are capable of stimulating T cell subsets, and keratinocytes have been shown to support LC development (Merad *et al*., 2008; Chu *et al*., 2011). Studies have begun to suggest the association of activated LCs, and perhaps dermal DCs in the development of contact hypersensitivity and autoimmune disease (Merad *et al*., 2008).

6.3 Possible Interaction between iASPP-Deficient Keratinocyte and its Surroundings

A lack of iASPP in keratinocytes resulted in the elevated expression of inflammatory genes such as IL-1α, IL-1f6/9, S100a7a, S100a8/9 and CCL20 (Fig 6.1). Questions over how these inflammatory mediators might contribute to the many phenotypic displays observed in K14-iASPP-/- mice remain to be addressed. How do iASPP-deficient keratinocytes interact with their surroundings through these mediators? Which cell type receives such signals and what are the biological consequences? What influence do these activated cells impose on keratinocytes?

6.3.1 IL-1 Inflammatory Signalling in Skin

With the increased expression of IL-1α in iASPP-deficient keratinocytes, larger amounts of this pleiotropic cytokine would be released into the extracellular environment upon cellular stress and inflammatory stimulation compared to wild type cells. The IL-1 cytokine can exert its pro-inflammatory influence on various cell types present in K14-iASPP-/- skin. Firstly, IL-1α can induce its own expression in keratinocyte in an autocrine manner, activating the downstream IL-1 signalling pathway to stimulate keratinocyte proliferation and migration (Lee *et al*., 1991; Chen *et al*., 1995; Di Meglio *et al*., 2011). These activated keratinocytes would also produce increased amounts of MHC class II surface receptors along with AMPs, cytokines, chemokines and growth factors upon receipt of the IL-1 signals (Yano *et al*., 2008; Sims *et al*., 2010). Keratinocyte derived IL-1 could also act on dermal fibroblasts to induce the expression of IL-1, further amplifying the pro-inflammatory response in the skin (Maas-Szabowski *et al*., 1996). The expression of growth factors and cytokines such as KGF, HGF and IL-8 would be induced in the activated fibroblasts, supporting the proliferation of keratinocytes. Moreover, IL-1 could trigger the expression of adhesion molecule ICAM-1 on endothelial cells and could mediate the recruitment of infiltrating leukocytes during inflammation (Detmar *et al*., 1992).

IL-1 can also signal to the various immune cell populations present in the skin. The pro-inflammatory mediator can activate mast cell production of cytokines such as TNF and IL-6 (Sims *et al*., 2010). DCs are known to respond to IL-1 stimulation with increased expression of CD40, OX40L and IL-12, and enhanced stimulatory activity towards T cells. IL-1 also has a positive influence over the stimulatory ability of LCs in activating T cells (Heufler *et al*., 1988). Some evidence has further indicated that IL-1 is capable of recruiting neutrophils and macrophages (Rider *et al*., 2011). Adaptive immune cells are also responsive towards IL-1 signalling. IL-1 supports the proliferation and survival of naïve T cells, as well as the development of T_H17 cells (Sims *et al*., 2010). B cell proliferation induced by immunoglobulin crosslinking or CD40 binding on the cell surface is further enhanced in the presence of IL-1 (Falkoff *et al*., 1983; Rousset *et al*., 1991; Sims *et al*., 2010).

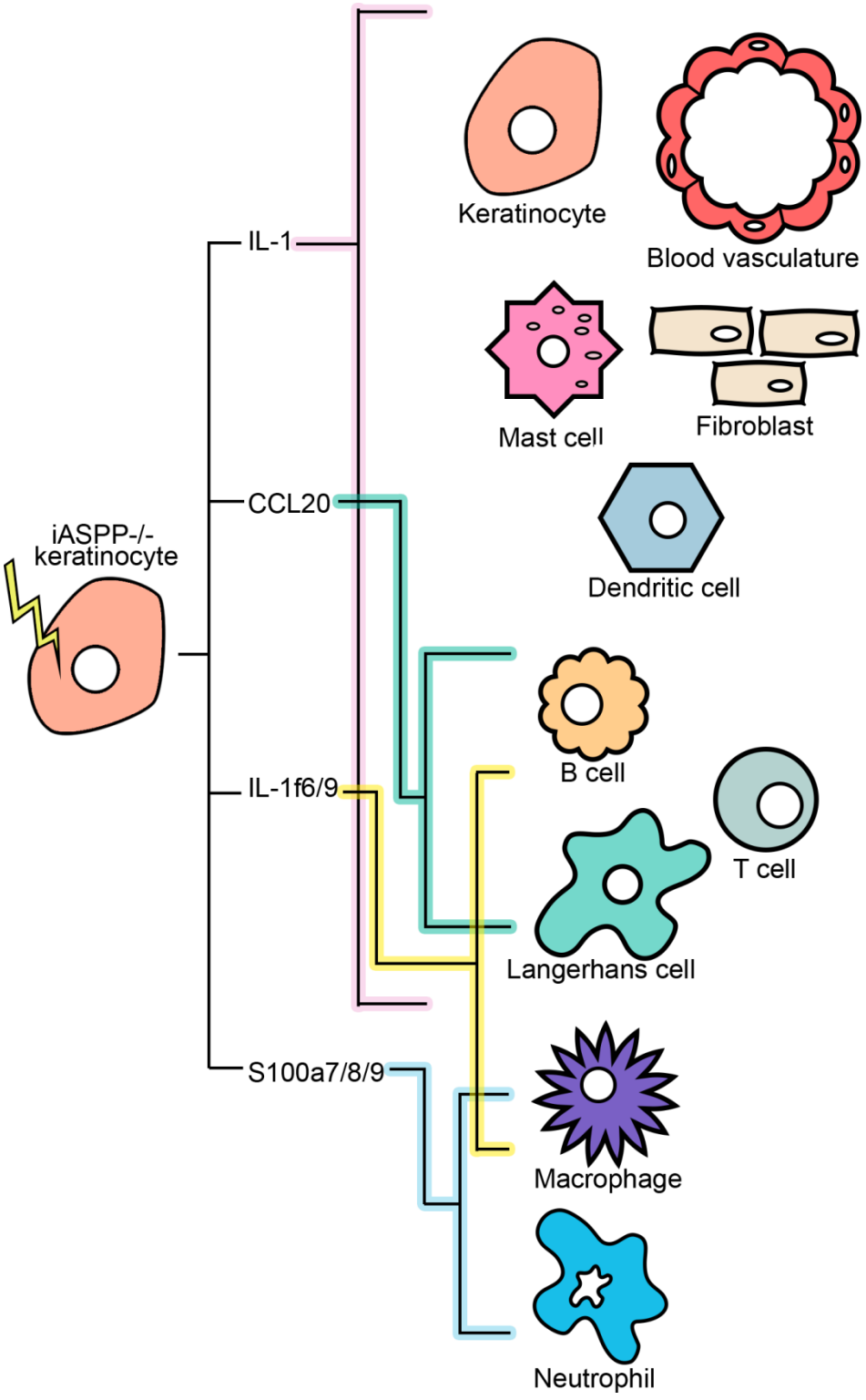


Fig 6.1 Possible influences of iASPP-/- keratinocytes on cellular components of the skin due to the up-regulated expression of inflammatory mediators.

Enhanced expression of inflammatory mediators such as IL-1α, CCL20, IL-1f6/9 and S100a7/8/9 in iASPP-deficient keratinocytes could mediate interaction with other cell populations in the

skin. This could disturb normal cutaneous immunohomeostasis, and contribute to the abnormal immune cell infiltrates, epidermal thickenings and increased papilloma susceptibility observed in K14-iASPP-/- mouse skin.

6.3.2 IL-1f6 and IL-1f9 Influencing Macrophage, LC and T cell Infiltration

IL-1f6 and IL-1f9, also known as IL-36α and IL-36γ, are members of the IL-1 family that are implicated in the pathogenesis of psoriasis (Carrier *et al*., 2011). While the expression of IL-36 ligands seems to be restricted to epithelial cells, the IL-1RL2 receptor that binds such ligands can be found on immune cells such as macrophages, B cells and DCs (Sims *et al*., 2010; Carrier *et al*., 2011). The overexpression of IL-1f6 in the basal epithelium results in the abnormal infiltration of macrophages, LCs and T cells into skin (Blumberg *et al*., 2007). This is accompanied by an increase in the levels of cytokines and chemokines like GM-CSF and MIP-2. This leads to speculation about whether the increased expression of IL-1f6 and IL-1f9 in iASPP-deficient keratinocytes could have similar effects on skin biology.

6.3.3 Overexpression of S100 Proteins in Skin Inflammation

The S100a7/psoriasin, S100a8 and S100a9 proteins have elicited recent interest in dermatological research due to their overexpression profiles in wound healing, inflammatory skin lesions and neoplasia (Eckert *et al*., 2006; Kerkhoff *et al*., 2012). Transgenic mice

overexpressing S100a7 and S100a15 in mammary epithelial cells had increased cell proliferation and production of cytokines such as IL-1, IL-11 and CXCL1 (Nasser *et al*., 2012). Increased infiltration of macrophages with a biased M2 phenotype was also observed, and the depletion of these macrophages suppressed tumour growth (Nasser *et al*., 2012). S100a8/9 has been demonstrated to induce neutrophil chemotaxis in a murine air pouch model (Ryckman *et al*., 2003). Moreover, S100a8/9 overexpression in HaCaT keratinocytes led to increased expression of the differentiation markers involucrin and filaggrin (Voss *et al*., 2011). Cells also showed enhanced NADPH oxidase and NF-κB activities, which could be involved in generating reactive oxygen species and the expression of inflammatory genes to further amplify the cutaneous inflammatory response (Benedyk *et al*., 2007). The overexpression of S100a7, S100a8 and S100a9 in iASPP-/- keratinocytes might therefore contribute to the presence of focal epidermal thickenings and abnormal macrophage infiltration.

6.3.4 CCL20 and B Cells in K14-iASPP-Deficient Skin

Increased CCL20 expression is associated with chronic inflammatory skin lesions like atopic dermatitis and psoriasis (Schutyser *et al*., 2003). CCL20 expressed in the epidermis was recently shown to be responsible for signalling B cells to migrate into the skin (Geherin *et al*., 2012). The enhanced CCL20 expression observed in this study in iASPP-deficient keratinocytes

could partly explain the increased infiltration of B cells observed in K14-iASPP-/- skin. However, CCL20 has also been demonstrated to have chemotactic properties for LCs and skin-homing T cells expressing the CCL20 receptor CCR6 (Schutyser *et al*., 2003). The chemokine CXCL1, which can be derived from macrophages and mast cells, is known to be chemotactic for neutrophils in inflamed tissues (De Filippo *et al*., 2013). How the up-regulated CXCL1 expression in iASPP-deficient keratinocytes could be involved in the K14-iASPP-/- skin phenotype remains an open question.

Immunohistochemical staining of immune cell markers was performed during this study to investigate the population of immune cells present in mouse skin. Other experimental techniques such as fluorescence-activated cell sorting (FACS) and enzyme-linked immunosorbent assay (ELISA) could be used to give a more systemic understanding of K14-iASPP-/- skin with better throughput. FACS analysis of skin tissue lysates from wild type and K14-iASPP-deficient skin could offer a more informative and clearer picture of the immune cell populations present. The technique could be utilised to expose the precise identities of immune cells, which could be followed by cell profiling to characterise the types of cytokines being produced. ELISA or qPCR analysis could then be performed to recognise any changes in the cytokine profiles of immune cells under the influence of iASPP-deficient keratinocytes. However, one would need to overcome technical difficulties in isolating cells from fibrous skin

tissues through enzymatic digestion while ensuring good sample quality with intact cell surface antigens.

6.4 Deregulated Inflammatory Signalling in the Absence of Epidermal iASPP Expression

It is perplexing how the previously reported protein-protein interaction between iASPP and p65 could not be replicated during this project, and yet the connection cannot be entirely disregarded based on the results from the dual-luciferase assay on S100a9 transcriptional control. Possible explanations for these observations have been explored in the discussion section of chapter 5. While phosphorylation of the Ser536 residue on p65 was not affected in the absence of iASPP expression, the phosphor-acceptor site Ser276 was not investigated. The p65 Ser276 residue seems an interesting target to study considering its involvement in inflammatory signalling (Schitmz *et al*., 2001; Chen *et al*., 2004) and the increased phosphorylation levels of MAPKs p38 and Erk1/2 seen in iASPP-/- keratinocytes. Both p38 and Erk1/2 have been illustrated to activate MSK1, which can mediate the phosphorylation of p65 Ser276 (Vermeulen *et al*., 2003). Abnormal MSK1 activation has been illustrated in human psoriatic skin, involved in regulating the expression of pro-inflammatory cytokines (Funding *et al*., 2006). It would be intriguing to investigate whether aberrant p65 Ser276 phosphorylation occurs in keratinocytes deficient of iASPP expression (Fig 6.2). Furthermore, a luciferase assay

with the revised experimental set-up discussed in the previous chapter would have to be carried out to clarify the link between iASPP and p65 in keratinocytes.

It is possible that the enhanced phosphorylation of MAPKs p38 and Erk1/2 in iASPP-deficient keratinocytes could be activating downstream transcription factors other than p65, in modulating inflammatory gene expression. An increase in p38 and Erk1/2 phosphorylation but not JNK1/2 has been reported in human psoriatic lesions, suggesting their involvement in skin inflammation (Johansen *et al*., 2005). Another transcription factor that may be worthy of attention is the basic leucine zipper transcription factor CCAAT/enhancer binding protein (C/EBP), a target of the MAPKs p38 and Erk1/2 (Nakajima *et al*., 1993; Zarubin *et al*., 2005). Epidermal C/EBPβ (also known as NF-1L6) was required for tumour development in DMBA/TPA induced skin carcinogenesis, in which Ras stimulated C/EBPβ activity through Erk1/2-mediated phosphorylation of C/EBPβ (Zhu *et al*., 2002; Sterneck *et al*., 2006). While the interaction between C/EBPβ and cyclin D1 has been implicated in promoting cell proliferation of epithelial cancers, the transcription factor is also responsible for regulating the gene expression of cytokines (Poli *et al*., 1998; Kracht *et al*., 2002; Nerlov *et al*., 2007).

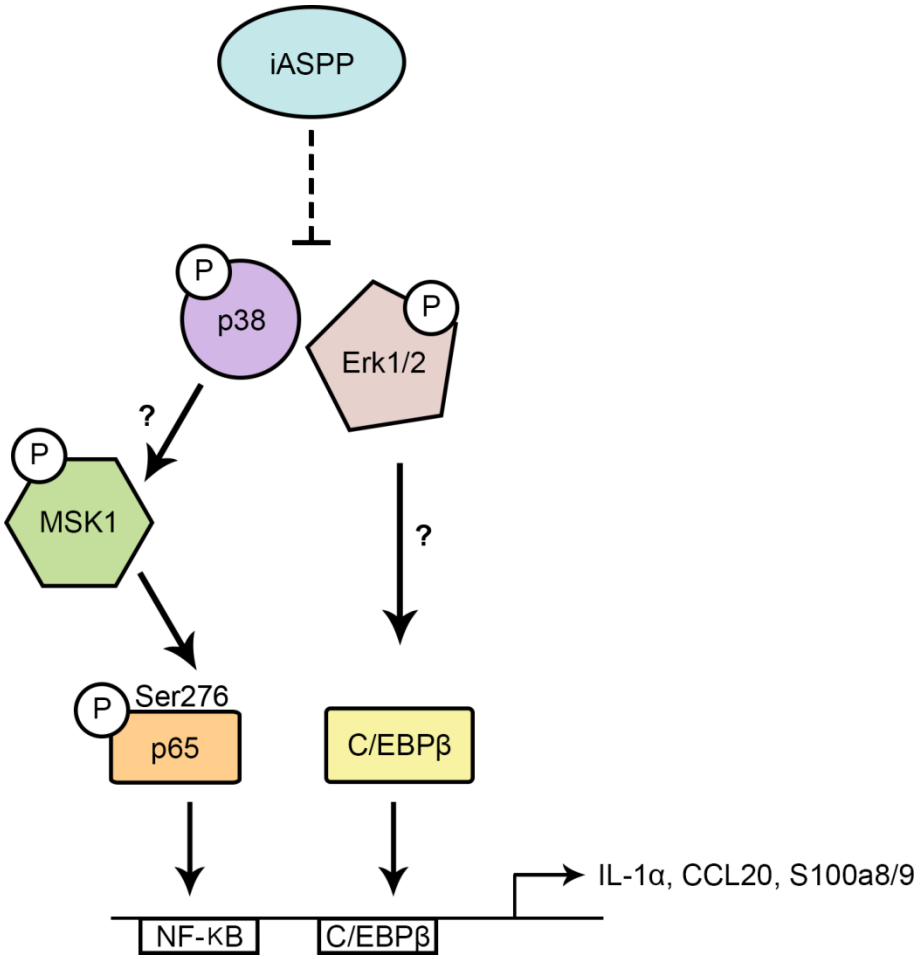


Fig 6.2 Proposed model illustrating the contribution of iASPP in regulating inflammatory gene expression.

In the absence of iASPP expression in keratinocytes, MAPK signalling pathways are disrupted. MAPKs such as p38 and Erk1/2 are phosphorylated aberrantly, which could lead to p65 Ser276 phosphorylation mediated by MSK1 or transcriptional activation of C/EBPβ. This results in enhanced gene expression of inflammatory mediators such as IL-1α, CCL20 and S100a8/9.

The presence of NF-κB consensus sites in the promoter regions of genes found to be differentially modulated in the absence of iASPP suggests a link between iASPP and NF-κB. Additionally C/EBPβ binding sites are also present upstream of the TSS and can regulate the transcription of genes such as IL-1α, CCL20, S100a8 and S100a9 (Stylianou *et al*., 1998;

Kuruto-Niwa *et al*., 1998; Schutyser *et al*., 2003; Miao *et al*., 2012; Sperling *et al*., 2012; Bando *et al*., 2013). Studies have also indicated crosstalk between the NF-κB and C/EBPβ signalling pathways, which might differ depending on the cellular context (Zwergal *et al*., 2006; Cappello *et al*., 2009). It could be interesting to examine the possible effects of iASPP on C/EBP, along with NF-κB transcriptional activity (Fig 6.2).

6.5 A Note on the Differences between Mouse and Human Skin

Mouse skin represents one of the best biological models for us to understand the biology of human skin, especially since the development of transgenic mouse models for investigating the pathogenic processes of various skin disorders. However, we should always be aware of the differences between mouse and human skin in terms of their anatomy and physiology when studying the organ and in applying data from murine models to human diseases.

The human skin epidermis is much thicker with a slower turnover rate compared to that of the mouse, and has epidermal projections into the dermis between DPs known as the rete ridges (Koster *et al*., 2010; Di Meglio *et al*., 2011; Alcolea *et al*., 2014). Melanocytes are found in the basal IFE of human skin, but are mostly restricted in the HFs of mouse skin. Moreover, mouse skin does not have sweat glands like the human skin.

While human skin has a much sparser HF distribution than the thick coat developed on mice, it also contains hair types that differ from the rodent hairs described previously. Humans only have two types of hair: unpigmented vellus hairs that do not penetrate into the dermis, and pigmented terminal hairs that penetrate into the hypodermis (Porter *et al*., 2003). Furthermore, human HFs cycle independently of each other while synchronised HF cycling can be observed in mouse skin during the first few weeks after birth (Müller-Röver *et al*., 2001). Lastly the presence of the panniculosus carnosus muscle layer in mouse skin allows for rapid wound contraction for scarless healing, which is distinct from human wounds that heal by re-epithelialisation and granulation tissue formation (Gudjonsson *et al*., 2007; Di Meglio *et al*., 2011).

Differences in skin immunology between mice and humans have also been demonstrated, which again remind us of the caution needed when extrapolating data yielded in mouse models to the human system (Mestas *et al*., 2004; Di Meglio *et al*., 2011). Firstly, the human homologue of IL-8, a cytokine that is secreted by human keratinocytes in response to IFN-γ and TNF-α, is not detected in mouse. However, it has been suggested that CXCL1 could serve as a functional IL-8 mouse homologue (Barker *et al*., 1991; Bozic *et al*., 1994; Mestas *et al*., 2004). Although γδ-TCR bearing DETCs are the predominant T cell population found in mouse skin, an

equivalent cell population has yet to be identified in human skin, in which the αβ-T cells predominate instead. Moreover, DCs equivalent to mouse langerin+ CD103+ DCs have yet to be discovered in human skin.

6.6 Final Remarks

This project has demonstrated the contribution of autonomous iASPP function in keratinocytes to skin immunohomeostasis and tumourigenesis. Basal epidermal iASPP expression is required to maintain normal cutaneous immune system, and the deletion of iASPP results in enhanced expression of inflammatory mediators and abnormal immune cell infiltration. This led to the speculation that iASPP-deficient keratinocytes provided an inflammatory tissue environment that supports the development of papillomas on K14-iASPP-/- mice. Therefore, despite several lines of evidence indicating the possibility of p53-inhibitor iASPP to act as a proto-oncogene, caution needs to be taken when considering iASPP as a cancer drug target in the context of skin cancers. The absence of iASPP expression in basal epithelium could potentiate keratinocyte proliferation and might impose a risk to neoplastic development. Further studies are needed to establish whether iASPP is involved in the pathogenesis of chronic inflammatory skin diseases in human patients, and whether iASPP could be utilised as a prognostic marker or a therapeutic target for these skin disorders.

It would be intriguing to investigate whether iASPP is also involved in the transcriptional regulation of inflammatory mediators in other epithelial tissues such as the stomach and lungs, where iASPP expression has been previously detected (Notari, 2012). The protective role of iASPP in skin basal epithelium against the development of papillomas could also differ in different tissue types. As spontaneous tumour development was not detected in K14-iASPP-deficient mice even in the skin, it might be necessary to utilise tumour induction assays or other stimulatory conditions to explore the biological role of iASPP in inflammation and carcinogenesis in these organs.

Chapter 7 References

Abtin, A., Eckhart, L., Glaser, R., Gmeiner, R., Mildner, M., & Tschachler, E. (2010). The antimicrobial heterodimer S100A8/S100A9 (calprotectin) is upregulated by bacterial flagellin in human epidermal keratinocytes. *J Invest Dermatol, 130*(10), 2423-2430. doi: 10.1038/jid.2010.158

Aggarwal, B. B., Vijayalekshmi, R. V., & Sung, B. (2009). Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. *Clin Cancer Res, 15*(2), 425-430. doi: 10.1158/1078-0432.CCR-08-0149

Alcolea, M. P., & Jones, P. H. (2014). Lineage analysis of epidermal stem cells. *Cold Spring Harb Perspect Med, 4*(1), a015206. doi: 10.1101/cshperspect.a015206

Andreu, P., Johansson, M., Affara, N. I., Pucci, F., Tan, T., Junankar, S., . . . Coussens, L. M. (2010). FcRgamma activation regulates inflammation-associated squamous carcinogenesis. *Cancer Cell, 17*(2), 121-134. doi: 10.1016/j.ccr.2009.12.019

Anisowicz, A., Messineo, M., Lee, S. W., & Sager, R. (1991). An NF-kappa B-like transcription factor mediates IL-1/TNF-alpha induction of gro in human fibroblasts. *J Immunol, 147*(2), 520-527.

Avalos, A. M., & Ploegh, H. L. (2014). Early BCR Events and Antigen Capture, Processing, and Loading on MHC Class II on B Cells. *Front Immunol, 5*, 92. doi: 10.3389/fimmu.2014.00092

Bagheri, M. M., & Safai, B. (2001). Cutaneous malignancies of keratinocytic origin. *Clin Dermatol, 19*(3), 244-252.

Bando, M., Hiroshima, Y., Kataoka, M., Herzberg, M. C., Ross, K. F., Shinohara, Y., . . . Kido, J. (2010). Modulation of calprotectin in human keratinocytes by keratinocyte growth factor and interleukin-1alpha. *Immunol Cell Biol, 88*(3), 328-333. doi: 10.1038/icb.2009.104

Bando, M., Zou, X., Hiroshima, Y., Kataoka, M., Ross, K. F., Shinohara, Y., . . . Kido, J. (2013). Mechanism of interleukin-1alpha transcriptional regulation of S100A9 in a human epidermal keratinocyte cell line. *Biochim Biophys Acta, 1829*(9), 954-962. doi: 10.1016/j.bbagrm.2013.03.010

Banno, T., Gazel, A., & Blumenberg, M. (2004). Effects of tumor necrosis factor-alpha (TNF alpha) in epidermal keratinocytes revealed using global transcriptional profiling. *J Biol Chem, 279*(31), 32633-32642. doi: 10.1074/jbc.M400642200

Barker, J. N., Jones, M. L., Mitra, R. S., Crockett-Torabe, E., Fantone, J. C., Kunkel, S. L., . . . Nickoloff, B. J. (1991). Modulation of keratinocyte-derived interleukin-8 which is chemotactic for neutrophils and T lymphocytes. *Am J Pathol, 139*(4), 869-876.

Battaglia, F., Delfino, S., Merello, E., Puppo, M., Piva, R., Varesio, L., & Bosco, M. C. (2008). Hypoxia transcriptionally induces macrophage-inflammatory protein-3alpha/CCL-20 in primary human mononuclear phagocytes through nuclear factor (NF)-kappaB. *J Leukoc Biol, 83*(3), 648-662. doi: 10.1189/jlb.0607349

Beckerman, R., & Prives, C. (2010). Transcriptional regulation by p53. *Cold Spring Harb Perspect Biol, 2*(8), a000935. doi: 10.1101/cshperspect.a000935

Beg, A. A., Sha, W. C., Bronson, R. T., & Baltimore, D. (1995). Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. *Genes Dev, 9*(22), 2736-2746.

Benedyk, M., Sopalla, C., Nacken, W., Bode, G., Melkonyan, H., Banfi, B., & Kerkhoff, C. (2007). HaCaT keratinocytes overexpressing the S100 proteins S100A8 and S100A9 show increased NADPH oxidase and NF-kappaB activities. *J Invest Dermatol, 127*(8), 2001-2011. doi: 10.1038/sj.jid.5700820

Bergamaschi, D., Samuels, Y., O'Neil, N. J., Trigiante, G., Crook, T., Hsieh, J. K., . . . Lu, X. (2003). iASPP oncoprotein is a key inhibitor of p53 conserved from worm to human. *Nat Genet, 33*(2), 162-167. doi: 10.1038/ng1070

Bergamaschi, D., Samuels, Y., Sullivan, A., Zvelebil, M., Breyssens, H., Bisso, A., . . . Lu, X. (2006). iASPP preferentially binds p53 proline-rich region and modulates apoptotic function of codon 72-polymorphic p53. *Nat Genet, 38*(10), 1133-1141. doi: 10.1038/ng1879

Bischoff, S. C. (2007). Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat Rev Immunol, 7*(2), 93-104. doi: 10.1038/nri2018

Bland, J. M., & Altman, D. G. (2004). The logrank test. *BMJ, 328*(7447), 1073. doi: 10.1136/bmj.328.7447.1073

Bland, J. M., & Altman, D. G. (2009). Analysis of continuous data from small samples. *BMJ, 338*, a3166. doi: 10.1136/bmj.a3166

Blanpain, C., & Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. *Nat Rev Mol Cell Biol, 10*(3), 207-217. doi: 10.1038/nrm2636

Blumberg, H., Dinh, H., Trueblood, E. S., Pretorius, J., Kugler, D., Weng, N., . . . Peschon, J. J. (2007). Opposing activities of two novel members of the IL-1 ligand family regulate skin inflammation. *J Exp Med, 204*(11), 2603-2614. doi: 10.1084/jem.20070157

Bos, J. D., Zonneveld, I., Das, P. K., Krieg, S. R., van der Loos, C. M., & Kapsenberg, M. L. (1987). The skin immune system (SIS): distribution and immunophenotype of lymphocyte subpopulations in normal human skin. *J Invest Dermatol, 88*(5), 569-573.

Bosch, F. X., Ribes, J., Diaz, M., & Cleries, R. (2004). Primary liver cancer: worldwide incidence and trends. *Gastroenterology, 127*(5 Suppl 1), S5-S16.

Botchkarev, V. A., & Paus, R. (2003). Molecular biology of hair morphogenesis: development and cycling. *J Exp Zool B Mol Dev Evol, 298*(1), 164-180. doi: 10.1002/jez.b.33

Boukamp, P. (2005). Non-melanoma skin cancer: what drives tumor development and progression? *Carcinogenesis, 26*(10), 1657-1667. doi: 10.1093/carcin/bgi123

Boukamp, P., Petrussevska, R. T., Breitkreutz, D., Hornung, J., Markham, A., & Fusenig, N. E. (1988). Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol, 106*(3), 761-771.

Boukamp, P., Stanbridge, E. J., Foo, D. Y., Cerutti, P. A., & Fusenig, N. E. (1990). c-Ha-ras oncogene expression in immortalized human keratinocytes (HaCaT) alters growth potential in vivo but lacks correlation with malignancy. *Cancer Res, 50*(9), 2840-2847.

Bozic, C. R., Gerard, N. P., von Uexkull-Guldenband, C., Kolakowski, L. F., Jr., Conklyn, M. J., Breslow, R., . . . Gerard, C. (1994). The murine interleukin 8 type B receptor homologue and its ligands. Expression and biological characterization. *J Biol Chem, 269*(47), 29355-29358.

Budunova, I. V., Perez, P., Vaden, V. R., Spiegelman, V. S., Slaga, T. J., & Jorcano, J. L. (1999). Increased expression of p50-NF-kappaB and constitutive activation of NF-kappaB transcription factors during mouse skin carcinogenesis. *Oncogene, 18*(52), 7423-7431. doi: 10.1038/sj.onc.1203104

Cai, Y., Qiu, S., Gao, X., Gu, S. Z., & Liu, Z. J. (2012). iASPP inhibits p53-independent apoptosis by inhibiting transcriptional activity of p63/p73 on promoters of proapoptotic genes. *Apoptosis, 17*(8), 777-783. doi: 10.1007/s10495-012-0728-z

Candi, E., Rufini, A., Terrinoni, A., Dinsdale, D., Ranalli, M., Paradisi, A., . . . Melino, G. (2006). Differential roles of p63 isoforms in epidermal development: selective genetic complementation in p63 null mice. *Cell Death Differ, 13*(6), 1037-1047. doi: 10.1038/sj.cdd.4401926

Candi, E., Schmidt, R., & Melino, G. (2005). The cornified envelope: a model of cell death in the skin. *Nat Rev Mol Cell Biol, 6*(4), 328-340. doi: 10.1038/nrm1619

Cappello, C., Zwergal, A., Kanclerski, S., Haas, S. C., Kandemir, J. D., Huber, R., . . . Brand, K. (2009). C/EBPbeta enhances NF-kappaB-associated signalling by reducing the level of IkappaB-alpha. *Cell Signal, 21*(12), 1918-1924. doi: 10.1016/j.cellsig.2009.08.009

Carrier, Y., Ma, H. L., Ramon, H. E., Napierata, L., Small, C., O'Toole, M., . . . Medley, Q. G. (2011). Inter-regulation of Th17 cytokines and the IL-36 cytokines in vitro and in vivo: implications in psoriasis pathogenesis. *J Invest Dermatol, 131*(12), 2428-2437. doi: 10.1038/jid.2011.234

Carruth, L. M., Demczuk, S., & Mizel, S. B. (1991). Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor. *J Biol Chem, 266*(19), 12162-7.

Cataisson, C., Salcedo, R., Hakim, S., Moffitt, B. A., Wright, L., Yi, M., . . . Trinchieri, G. (2012). IL-1R-MyD88 signaling in keratinocyte transformation and carcinogenesis. *J Exp Med, 209*(9), 1689-1702. doi: 10.1084/jem.20101355

Chang, F., Steelman, L. S., Shelton, J. G., Lee, J. T., Navolanic, P. M., Blalock, W. L., . . . McCubrey, J. A. (2003). Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway (Review). *Int J Oncol, 22*(3), 469-480.

Chen, J. D., Lapiere, J. C., Sauder, D. N., Peavey, C., & Woodley, D. T. (1995). Interleukin-1 alpha stimulates keratinocyte migration through an epidermal growth factor/transforming growth factor-alpha-independent pathway. *J Invest Dermatol, 104*(5), 729-733.

Chen, J., Xie, F., Zhang, L., & Jiang, W. G. (2010). iASPP is over-expressed in human non-small cell lung cancer and regulates the proliferation of lung cancer cells through a p53 associated pathway. *BMC Cancer, 10*, 694. doi: 10.1186/1471-2407-10-694

Chen, L. F., & Greene, W. C. (2004). Shaping the nuclear action of NF-kappaB. *Nat Rev Mol Cell Biol, 5*(5), 392-401. doi: 10.1038/nrm1368

Chikh, A., Matin, R. N., Senatore, V., Hufbauer, M., Lavery, D., Raimondi, C., . . . Bergamaschi, D. (2011). iASPP/p63 autoregulatory feedback loop is required for the homeostasis of stratified epithelia. *EMBO J, 30*(20), 4261-4273. doi: 10.1038/emboj.2011.302

Chu, C. C., Di Meglio, P., & Nestle, F. O. (2011). Harnessing dendritic cells in inflammatory skin diseases. *Semin Immunol, 23*(1), 28-41. doi: 10.1016/j.smim.2011.01.006

Clayton, E., Doupe, D. P., Klein, A. M., Winton, D. J., Simons, B. D., & Jones, P. H. (2007). A single type of progenitor cell maintains normal epidermis. *Nature, 446*(7132), 185-189. doi: 10.1038/nature05574

Colotta, F., Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability. *Carcinogenesis, 30*(7), 1073-1081. doi: 10.1093/carcin/bgp127

Cong, W., Hirose, T., Harita, Y., Yamashita, A., Mizuno, K., Hirano, H., & Ohno, S. (2010). ASPP2 regulates epithelial cell polarity through the PAR complex. *Curr Biol, 20*(15), 1408-1414. doi: 10.1016/j.cub.2010.06.024

Cooper, M. A., Fehniger, T. A., & Caligiuri, M. A. (2001). The biology of human natural killer-cell subsets. *Trends Immunol, 22*(11), 633-640.

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Nature, 420*(6917), 860-867. doi: 10.1038/nature01322

Crum, C. P., & McKeon, F. D. (2010). p63 in epithelial survival, germ cell surveillance, and neoplasia. *Annu Rev Pathol, 5*, 349-371. doi: 10.1146/annurev-pathol-121808-102117

Dajee, M., Lazarov, M., Zhang, J. Y., Cai, T., Green, C. L., Russell, A. J., . . . Khavari, P. A. (2003). NF-kappaB blockade and oncogenic Ras trigger invasive human epidermal neoplasia. *Nature, 421*(6923), 639-643. doi: 10.1038/nature01283

Dajee, M., Tarutani, M., Deng, H., Cai, T., & Khavari, P. A. (2002). Epidermal Ras blockade demonstrates spatially localized Ras promotion of proliferation and inhibition of differentiation. *Oncogene, 21*(10), 1527-1538. doi: 10.1038/sj.onc.1205287

De Benedetto, A., Agnihothri, R., McGirt, L. Y., Bankova, L. G., & Beck, L. A. (2009). Atopic dermatitis: a disease caused by innate immune defects? *J Invest Dermatol, 129*(1), 14-30. doi: 10.1038/jid.2008.259

De Filippo, K., Dudeck, A., Hasenberg, M., Nye, E., van Rooijen, N., Hartmann, K., . . . Hogg, N. (2013). Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. *Blood, 121*(24), 4930-4937. doi: 10.1182/blood-2013-02-486217

de Visser, K. E., Korets, L. V., & Coussens, L. M. (2005). De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell, 7*(5), 411-423. doi: 10.1016/j.ccr.2005.04.014

Detmar, M., Tenorio, S., Hettmannsperger, U., Ruszczak, Z., & Orfanos, C. E. (1992). Cytokine regulation of proliferation and ICAM-1 expression of human dermal microvascular endothelial cells in vitro. *J Invest Dermatol, 98*(2), 147-153.

Deyoung, M. P., & Ellisen, L. W. (2007). p63 and p73 in human cancer: defining the network. *Oncogene, 26*(36), 5169-5183. doi: 10.1038/sj.onc.1210337

Di Meglio, P., Perera, G. K., & Nestle, F. O. (2011). The multitasking organ: recent insights into skin immune function. *Immunity, 35*(6), 857-869. doi: 10.1016/j.immuni.2011.12.003

Dieu-Nosjean, M. C., Massacrier, C., Homey, B., Vanbervliet, B., Pin, J. J., Vicari, A., . . . Caux, C. (2000). Macrophage inflammatory protein 3alpha is expressed at inflamed epithelial surfaces and is the most potent chemokine known in attracting Langerhans cell precursors. *J Exp Med, 192*(5), 705-718.

Dinarello, C., Arend, W., Sims, J., Smith, D., Blumberg, H., O'Neill, L., . . . Gabel, C. (2010). IL-1 family nomenclature. *Nat Immunol, 11*(11), 973. doi: 10.1038/ni1110-973

Donehower, L. A. (2009). Using mice to examine p53 functions in cancer, aging, and longevity. *Cold Spring Harb Perspect Biol, 1*(6), a001081. doi: 10.1101/cshperspect.a001081

Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., & Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature, 356*(6366), 215-221. doi: 10.1038/356215a0

Dong, J., Jimi, E., Zeiss, C., Hayden, M. S., & Ghosh, S. (2010). Constitutively active NF-kappaB triggers systemic TNFalpha-dependent inflammation and localized TNFalpha-independent inflammatory disease. *Genes Dev, 24*(16), 1709-1717. doi: 10.1101/gad.1958410

Dotsch, V., Bernassola, F., Coutandin, D., Candi, E., & Melino, G. (2010). p63 and p73, the ancestors of p53. *Cold Spring Harb Perspect Biol, 2*(9), a004887. doi: 10.1101/cshperspect.a004887

Dunne, A., & O'Neill, L. A. (2003). The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE, 2003*(171), re3. doi: 10.1126/stke.2003.171.re3

Eckert, R. L., Broome, A. M., Ruse, M., Robinson, N., Ryan, D., & Lee, K. (2004). S100 proteins in the epidermis. *J Invest Dermatol, 123*(1), 23-33. doi: 10.1111/j.0022-202X.2004.22719.x

Eckert, R. L., & Lee, K. C. (2006). S100A7 (Psoriasin): a story of mice and men. *J Invest Dermatol, 126*(7), 1442-1444. doi: 10.1038/sj.jid.5700265

Falkoff, R. J., Muraguchi, A., Hong, J. X., Butler, J. L., Dinarello, C. A., & Fauci, A. S. (1983). The effects of interleukin 1 on human B cell activation and proliferation. *J Immunol, 131*(2), 801-805.

Filler, R. B., Roberts, S. J., & Girardi, M. (2007). Cutaneous two-stage chemical carcinogenesis. *CSH Protoc, 2007*, pdb prot4837. doi: 10.1101/pdb.prot4837

Finch, J. S., Albino, H. E., & Bowden, G. T. (1996). Quantitation of early clonal expansion of two mutant 61st codon c-Ha-ras alleles in DMBA/TPA treated mouse skin by nested PCR/RFLP. *Carcinogenesis, 17*(12), 2551-2557.

Flores, E. R., Sengupta, S., Miller, J. B., Newman, J. J., Bronson, R., Crowley, D., . . . Jacks, T. (2005). Tumor predisposition in mice mutant for p63 and p73: evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell, 7*(4), 363-373. doi: 10.1016/j.ccr.2005.02.019

Fuchs, E. (2007). Scratching the surface of skin development. *Nature, 445*(7130), 834-842. doi: 10.1038/nature05659

Funding, A. T., Johansen, C., Kragballe, K., Otkjaer, K., Jensen, U. B., Madsen, M. W., . . . Iversen, L. (2006). Mitogen- and stress-activated protein kinase 1 is activated in lesional psoriatic epidermis and regulates the expression of pro-inflammatory cytokines. *J Invest Dermatol, 126*(8), 1784-1791. doi: 10.1038/sj.jid.5700252

Gabay, C., Lamacchia, C., & Palmer, G. (2010). IL-1 pathways in inflammation and human diseases. *Nat Rev Rheumatol, 6*(4), 232-241. doi: 10.1038/nrrheum.2010.4

Gebhardt, C., Breitenbach, U., Tuckermann, J. P., Dittrich, B. T., Richter, K. H., & Angel, P. (2002). Calgranulins S100A8 and S100A9 are negatively regulated by glucocorticoids in a c-Fos-dependent manner and overexpressed throughout skin carcinogenesis. *Oncogene, 21*(27), 4266-4276. doi: 10.1038/sj.onc.1205521

Geherin, S. A., Fintushel, S. R., Lee, M. H., Wilson, R. P., Patel, R. T., Alt, C., . . . Debes, G. F. (2012). The skin, a novel niche for recirculating B cells. *J Immunol, 188*(12), 6027-6035. doi: 10.4049/jimmunol.1102639

Gillotin, S. (2009). iASPP, a potential drug target in cancer therapy. *Leuk Res, 33*(9), 1175-1177. doi: 10.1016/j.leukres.2009.04.037

Gimenez-Conti, I., Aldaz, C. M., Bianchi, A. B., Roop, D. R., Slaga, T. J., & Conti, C. J. (1990). Early expression of type I K13 keratin in the progression of mouse skin papillomas. *Carcinogenesis, 11*(11), 1995-1999.

Green, H., Easley, K., & Iuchi, S. (2003). Marker succession during the development of keratinocytes from cultured human embryonic stem cells. *Proc Natl Acad Sci U S A, 100*(26), 15625-15630. doi: 10.1073/pnas.0307226100

Grivennikov, S. I., Greten, F. R., & Karin, M. (2010). Immunity, inflammation, and cancer. *Cell, 140*(6), 883-899. doi: 10.1016/j.cell.2010.01.025

Grulich, A. E., van Leeuwen, M. T., Falster, M. O., & Vajdic, C. M. (2007). Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis. *Lancet, 370*(9581), 59-67. doi: 10.1016/S0140-6736(07)61050-2

Gudjonsson, J. E., Johnston, A., Dyson, M., Valdimarsson, H., & Elder, J. T. (2007). Mouse models of psoriasis. *J Invest Dermatol, 127*(6), 1292-1308. doi: 10.1038/sj.jid.5700807

Gurtner, G. C., Werner, S., Barrandon, Y., & Longaker, M. T. (2008). Wound repair and regeneration. *Nature, 453*(7193), 314-321. doi: 10.1038/nature07039

Hamilton, J. A. (2008). Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol, 8*(7), 533-544. doi: 10.1038/nri2356

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell, 144*(5), 646-674. doi: 10.1016/j.cell.2011.02.013

Hardman, M. J., Sisi, P., Banbury, D. N., & Byrne, C. (1998). Patterned acquisition of skin barrier function during development. *Development, 125*(8), 1541-1552.

Harvey, M., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., Bradley, A., & Donehower, L. A. (1993). Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat Genet, 5*(3), 225-229. doi: 10.1038/ng1193-225

Hay, R. J., Johns, N. E., Williams, H. C., Bolliger, I. W., Dellavalle, R. P., Margolis, D. J., . . . Naghavi, M. (2014). The global burden of skin disease in 2010: an analysis of the

prevalence and impact of skin conditions. *J Invest Dermatol, 134*(6), 1527-1534. doi: 10.1038/jid.2013.446

Hayden, M. S., & Ghosh, S. (2008). Shared principles in NF-kappaB signaling. *Cell, 132*(3), 344-362. doi: 10.1016/j.cell.2008.01.020

Heath, W. R., & Carbone, F. R. (2013). The skin-resident and migratory immune system in steady state and memory: innate lymphocytes, dendritic cells and T cells. *Nat Immunol, 14*(10), 978-985. doi: 10.1038/ni.2680

Helton, E. S., Zhu, J., & Chen, X. (2006). The unique NH2-terminally deleted (DeltaN) residues, the PXXP motif, and the PPXY motif are required for the transcriptional activity of the DeltaN variant of p63. *J Biol Chem, 281*(5), 2533-2542. doi: 10.1074/jbc.M507964200

Herron, B. J., Rao, C., Liu, S., Laprade, L., Richardson, J. A., Olivieri, E., . . . Beier, D. R. (2005). A mutation in NFkB interacting protein 1 results in cardiomyopathy and abnormal skin development in wa3 mice. *Hum Mol Genet, 14*(5), 667-677. doi: 10.1093/hmg/ddi063

Heufler, C., Koch, F., & Schuler, G. (1988). Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J Exp Med, 167*(2), 700-705.

Hirashima, M., Sano, K., Morisada, T., Murakami, K., Rossant, J., & Suda, T. (2008). Lymphatic vessel assembly is impaired in Aspp1-deficient mouse embryos. *Dev Biol, 316*(1), 149-159. doi: 10.1016/j.ydbio.2008.01.023

Hu, Y., Baud, V., Oga, T., Kim, K. I., Yoshida, K., & Karin, M. (2001). IKKalpha controls formation of the epidermis independently of NF-kappaB. *Nature, 410*(6829), 710-714. doi: 10.1038/35070605

Imai, K., Matsuyama, S., Miyake, S., Suga, K., & Nakachi, K. (2000). Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. *Lancet, 356*(9244), 1795-1799. doi: 10.1016/S0140-6736(00)03231-1

Imamoto, A., Wang, X. J., Fujiki, H., Walker, S. E., Beltran, L. M., & DiGiovanni, J. (1993). Comparison of 12-O-tetradecanoylphorbol-13-acetate and teleocidin for induction of epidermal hyperplasia, activation of epidermal PKC isozymes and skin tumor promotion in SENCAR and C57BL/6 mice. *Carcinogenesis, 14*(4), 719-724.

JASPER core database ver 5.0 alpha. (13 May 2014). Available from: http://jaspar.genereg.net.

Jiang, W., Ananthaswamy, H. N., Muller, H. K., & Kripke, M. L. (1999). p53 protects against skin cancer induction by UV-B radiation. *Oncogene, 18*(29), 4247-4253. doi: 10.1038/sj.onc.1202789

Johansen, C., Kragballe, K., Westergaard, M., Henningsen, J., Kristiansen, K., & Iversen, L. (2005). The mitogen-activated protein kinases p38 and ERK1/2 are increased in

lesional psoriatic skin. *Br J Dermatol, 152*(1), 37-42. doi: 10.1111/j.1365-2133.2004.06304.x

Kemp, C. J., Donehower, L. A., Bradley, A., & Balmain, A. (1993). Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. *Cell, 74*(5), 813-822.

Kemp, C. J., Wheldon, T., & Balmain, A. (1994). p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nat Genet, 8*(1), 66-69. doi: 10.1038/ng0994-66

Kerkhoff, C., Voss, A., Scholzen, T. E., Averill, M. M., Zanker, K. S., & Bornfeldt, K. E. (2012). Novel insights into the role of S100A8/A9 in skin biology. *Exp Dermatol, 21*(11), 822-826. doi: 10.1111/j.1600-0625.2012.01571.x

Keyes, W. M., Vogel, H., Koster, M. I., Guo, X., Qi, Y., Petherbridge, K. M., . . . Mills, A. A. (2006). p63 heterozygous mutant mice are not prone to spontaneous or chemically induced tumors. *Proc Natl Acad Sci U S A, 103*(22), 8435-8440. doi: 10.1073/pnas.0602477103

Kim, C., & Pasparakis, M. (2014). Epidermal p65/NF-kappaB signalling is essential for skin carcinogenesis. *EMBO Mol Med, 6*(7), 970-983. doi: 10.15252/emmm.201303541

King, K. E., Ponnamperuma, R. M., Yamashita, T., Tokino, T., Lee, L. A., Young, M. F., & Weinberg, W. C. (2003). deltaNp63alpha functions as both a positive and a negative transcriptional regulator and blocks in vitro differentiation of murine keratinocytes. *Oncogene, 22*(23), 3635-3644. doi: 10.1038/sj.onc.1206536

Klement, J. F., Rice, N. R., Car, B. D., Abbondanzo, S. J., Powers, G. D., Bhatt, P. H., . . . Stewart, C. L. (1996). IkappaBalpha deficiency results in a sustained NF-kappaB response and severe widespread dermatitis in mice. *Mol Cell Biol, 16*(5), 2341-2349.

Komine, M., Rao, L. S., Freedberg, I. M., Simon, M., Milisavljevic, V., & Blumenberg, M. (2001). Interleukin-1 induces transcription of keratin K6 in human epidermal keratinocytes. *J Invest Dermatol, 116*(2), 330-338. doi: 10.1046/j.1523-1747.2001.01249.x

Koster, M. I. (2010). p63 in skin development and ectodermal dysplasias. *J Invest Dermatol, 130*(10), 2352-2358. doi: 10.1038/jid.2010.119

Koster, M. I., Dai, D., Marinari, B., Sano, Y., Costanzo, A., Karin, M., & Roop, D. R. (2007). p63 induces key target genes required for epidermal morphogenesis. *Proc Natl Acad Sci U S A, 104*(9), 3255-3260. doi: 10.1073/pnas.0611376104

Koster, M. I., Kim, S., Mills, A. A., DeMayo, F. J., & Roop, D. R. (2004). p63 is the molecular switch for initiation of an epithelial stratification program. *Genes Dev, 18*(2), 126-131. doi: 10.1101/gad.1165104

Kracht, M., & Saklatvala, J. (2002). Transcriptional and post-transcriptional control of gene expression in inflammation. *Cytokine, 20*(3), 91-106.

Kuruto-Niwa, R., Nakamura, M., Takeishi, K., & Nozawa, R. (1998). Transcriptional regulation by C/EBP alpha and -beta in the expression of the gene for the MRP14 myeloid calcium binding protein. *Cell Struct Funct, 23*(3), 109-118.

Laurikkala, J., Mikkola, M. L., James, M., Tummers, M., Mills, A. A., & Thesleff, I. (2006). p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. *Development, 133*(8), 1553-1563. doi: 10.1242/dev.02325

Lechler, T., & Fuchs, E. (2007). Desmoplakin: an unexpected regulator of microtubule organization in the epidermis. *J Cell Biol, 176*(2), 147-154. doi: 10.1083/jcb.200609109

Lee, S. W., Morhenn, V. B., Ilnicka, M., Eugui, E. M., & Allison, A. C. (1991). Autocrine stimulation of interleukin-1 alpha and transforming growth factor alpha production in human keratinocytes and its antagonism by glucocorticoids. *J Invest Dermatol, 97*(1), 106-110.

Li, C., Chen, H., Ding, F., Zhang, Y., Luo, A., Wang, M., & Liu, Z. (2009). A novel p53 target gene, S100A9, induces p53-dependent cellular apoptosis and mediates the p53 apoptosis pathway. *Biochem J, 422*(2), 363-372. doi: 10.1042/BJ20090465

Li, Q., Sambandam, S. A., Lu, H. J., Thomson, A., Kim, S. H., Lu, H., . . . Lu, Q. (2011). 14-3-3sigma and p63 play opposing roles in epidermal tumorigenesis. *Carcinogenesis, 32*(12), 1782-1788. doi: 10.1093/carcin/bgr207

Li, Q., & Verma, I. M. (2002). NF-kappaB regulation in the immune system. *Nat Rev Immunol, 2*(10), 725-734. doi: 10.1038/nri910

Li, Z., Niu, J., Uwagawa, T., Peng, B., & Chiao, P. J. (2005). Function of polo-like kinase 3 in NF-kappaB-mediated proapoptotic response. *J Biol Chem, 280*(17), 16843-16850. doi: 10.1074/jbc.M410119200

Liefer, K. M., Koster, M. I., Wang, X. J., Yang, A., McKeon, F., & Roop, D. R. (2000). Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res, 60*(15), 4016-4020.

Lin, C. C., Chiang, L. L., Lin, C. H., Shih, C. H., Liao, Y. T., Hsu, M. J., & Chen, B. C. (2007). Transforming growth factor-beta1 stimulates heme oxygenase-1 expression via the PI3K/Akt and NF-kappaB pathways in human lung epithelial cells. *Eur J Pharmacol, 560*(2-3), 101-109. doi: 10.1016/j.ejphar.2007.01.025

Lind, M. H., Rozell, B., Wallin, R. P., van Hogerlinden, M., Ljunggren, H. G., Toftgard, R., & Sur, I. (2004). Tumor necrosis factor receptor 1-mediated signaling is required for skin cancer development induced by NF-kappaB inhibition. *Proc Natl Acad Sci U S A, 101*(14), 4972-4977. doi: 10.1073/pnas.0307106101

Liu, Z., Zhang, X., Huang, D., Liu, Y., Zhang, X., Liu, L., . . . Tian, Y. (2012). Elevated expression of iASPP in head and neck squamous cell carcinoma and its clinical significance. *Med Oncol, 29*(5), 3381-3388. doi: 10.1007/s12032-012-0306-9

Llanos, S., Royer, C., Lu, M., Bergamaschi, D., Lee, W. H., & Lu, X. (2011). Inhibitory member of the apoptosis-stimulating proteins of the p53 family (iASPP) interacts with protein phosphatase 1 via a noncanonical binding motif. *J Biol Chem, 286*(50), 43039-43044. doi: 10.1074/jbc.M111.270751

Lomas, A., Leonardi-Bee, J., & Bath-Hextall, F. (2012). A systematic review of worldwide incidence of nonmelanoma skin cancer. *Br J Dermatol, 166*(5), 1069-1080. doi: 10.1111/j.1365-2133.2012.10830.x

Lu, B., Guo, H., Zhao, J., Wang, C., Wu, G., Pang, M., . . . Guo, Y. (2010). Increased expression of iASPP, regulated by hepatitis B virus X protein-mediated NF-kappaB activation, in hepatocellular carcinoma. *Gastroenterology, 139*(6), 2183-2194 e2185. doi: 10.1053/j.gastro.2010.06.049

Lu, M., Zak, J., Chen, S., Sanchez-Pulido, L., Severson, D. T., Endicott, J., . . . Lu, X. (2014). A code for RanGDP binding in ankyrin repeats defines a nuclear import pathway. *Cell, 157*(5), 1130-1145. doi: 10.1016/j.cell.2014.05.006

Luger, T. A., Stadler, B. M., Luger, B. M., Mathieson, B. J., Mage, M., Schmidt, J. A., & Oppenheim, J. J. (1982). Murine epidermal cell-derived thymocyte-activating factor resembles murine interleukin 1. *J Immunol, 128*(5), 2147-2152.

Maas-Szabowski, N., & Fusenig, N. E. (1996). Interleukin-1-induced growth factor expression in postmitotic and resting fibroblasts. *J Invest Dermatol, 107*(6), 849-855.

MacLeod, A. S., Hemmers, S., Garijo, O., Chabod, M., Mowen, K., Witherden, D. A., & Havran, W. L. (2013). Dendritic epidermal T cells regulate skin antimicrobial barrier function. *J Clin Invest, 123*(10), 4364-4374. doi: 10.1172/JCI70064

Martin, F., & Chan, A. C. (2004). Pathogenic roles of B cells in human autoimmunity; insights from the clinic. *Immunity, 20*(5), 517-527.

Mascia, F., Cataisson, C., Lee, T. C., Threadgill, D., Mariani, V., Amerio, P., . . . Pastore, S. (2010). EGFR regulates the expression of keratinocyte-derived granulocyte/macrophage colony-stimulating factor in vitro and in vivo. *J Invest Dermatol, 130*(3), 682-693. doi: 10.1038/jid.2009.336

Massague, J. (2008). TGFbeta in Cancer. *Cell, 134*(2), 215-230. doi: 10.1016/j.cell.2008.07.001

Mecklenburg, L., Tobin, D. J., Muller-Rover, S., Handjiski, B., Wendt, G., Peters, E. M., . . . Paus, R. (2000). Active hair growth (anagen) is associated with angiogenesis. *J Invest Dermatol, 114*(5), 909-916. doi: 10.1046/j.1523-1747.2000.00954.x

Mellman, I., Coukos, G., & Dranoff, G. (2011). Cancer immunotherapy comes of age. *Nature, 480*(7378), 480-489. doi: 10.1038/nature10673

Merad, M., Ginhoux, F., & Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol, 8*(12), 935-947. doi: 10.1038/nri2455

Mestas, J., & Hughes, C. C. (2004). Of mice and not men: differences between mouse and human immunology. *J Immunol, 172*(5), 2731-2738.

Miao, L., Grebhardt, S., Shi, J., Peipe, I., Zhang, J., & Mayer, D. (2012). Prostaglandin E2 stimulates S100A8 expression by activating protein kinase A and CCAAT/enhancer-binding-protein-beta in prostate cancer cells. *Int J Biochem Cell Biol, 44*(11), 1919-1928. doi: 10.1016/j.biocel.2012.06.015

Miller, S. J., Wei, Z. G., Wilson, C., Dzubow, L., Sun, T. T., & Lavker, R. M. (1993). Mouse skin is particularly susceptible to tumor initiation during early anagen of the hair cycle: possible involvement of hair follicle stem cells. *J Invest Dermatol, 101*(4), 591-594.

Mills, A. A., Zheng, B., Wang, X. J., Vogel, H., Roop, D. R., & Bradley, A. (1999). p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature, 398*(6729), 708-713. doi: 10.1038/19531

Moll, U. M., & Slade, N. (2004). p63 and p73: roles in development and tumor formation. *Mol Cancer Res, 2*(7), 371-386.

Moore, R. J., Owens, D. M., Stamp, G., Arnott, C., Burke, F., East, N., . . . Balkwill, F. (1999). Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med, 5*(7), 828-831. doi: 10.1038/10552

Mori, N., & Prager, D. (1996). Transactivation of the interleukin-1alpha promoter by human T-cell leukemia virus type I and type II Tax proteins. *Blood, 87*(8), 3410-3417.

Mulero, M. C., Ferres-Marco, D., Islam, A., Margalef, P., Pecoraro, M., Toll, A., . . . Espinosa, L. (2013). Chromatin-bound IkappaBalpha regulates a subset of polycomb target genes in differentiation and cancer. *Cancer Cell, 24*(2), 151-166. doi: 10.1016/j.ccr.2013.06.003

Muller-Rover, S., Handjiski, B., van der Veen, C., Eichmuller, S., Foitzik, K., McKay, I. A., . . . Paus, R. (2001). A comprehensive guide for the accurate classification of murine hair follicles in distinct hair cycle stages. *J Invest Dermatol, 117*(1), 3-15. doi: 10.1046/j.0022-202x.2001.01377.x

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. *Nat Rev Immunol, 11*(11), 723-737. doi: 10.1038/nri3073

Naito, M., Chenicek, K. J., Naito, Y., & DiGiovanni, J. (1988). Susceptibility to phorbol ester skin tumor promotion in (C57BL/6 x DBA/2) F1 mice is inherited as an incomplete dominant trait: evidence for multi-locus involvement. *Carcinogenesis, 9*(4), 639-645.

Nakajima, T., Kinoshita, S., Sasagawa, T., Sasaki, K., Naruto, M., Kishimoto, T., & Akira, S. (1993). Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein kinase cascade is essential for transcription factor NF-IL6. *Proc Natl Acad Sci U S A, 90*(6), 2207-2211.

Nasser, M. W., Qamri, Z., Deol, Y. S., Ravi, J., Powell, C. A., Trikha, P., . . . Ganju, R. K. (2012). S100A7 enhances mammary tumorigenesis through upregulation of inflammatory pathways. *Cancer Res, 72*(3), 604-615. doi: 10.1158/0008-5472.CAN-11-0669

Nemeth, J., Stein, I., Haag, D., Riehl, A., Longerich, T., Horwitz, E., . . . Hess, J. (2009). S100A8 and S100A9 are novel nuclear factor kappa B target genes during malignant progression of murine and human liver carcinogenesis. *Hepatology, 50*(4), 1251-1262. doi: 10.1002/hep.23099

Nerlov, C. (2007). The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends Cell Biol, 17*(7), 318-324. doi: 10.1016/j.tcb.2007.07.004

Nestle, F. O., Di Meglio, P., Qin, J. Z., & Nickoloff, B. J. (2009). Skin immune sentinels in health and disease. *Nat Rev Immunol, 9*(10), 679-691. doi: 10.1038/nri2622

Nickoloff, B. J., & Turka, L. A. (1993). Keratinocytes: key immunocytes of the integument. *Am J Pathol, 143*(2), 325-331.

Notari, M., Hu, Y., Koch, S., Lu, M., Ratnayaka, I., Zhong, S., . . . Lu, X. (2011). Inhibitor of apoptosis-stimulating protein of p53 (iASPP) prevents senescence and is required for epithelial stratification. *Proc Natl Acad Sci U S A, 108*(40), 16645-16650. doi: 10.1073/pnas.1102292108

Notari, M. (2012). Investigation of the biological role of iASPP in vivo. DPhil thesis. *University of Oxford, U.K.*

O'Leary, J. G., Goodarzi, M., Drayton, D. L., & von Andrian, U. H. (2006). T cell- and B cell-independent adaptive immunity mediated by natural killer cells. *Nat Immunol, 7*(5), 507-516. doi: 10.1038/ni1332

Oeckinghaus, A., & Ghosh, S. (2009). The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol, 1*(4), a000034. doi: 10.1101/cshperspect.a000034

Oeckinghaus, A., Hayden, M. S., & Ghosh, S. (2011). Crosstalk in NF-kappaB signaling pathways. *Nat Immunol, 12*(8), 695-708. doi: 10.1038/ni.2065

Olivier, M., Hollstein, M., & Hainaut, P. (2010). TP53 mutations in human cancers: origins, consequences, and clinical use. *Cold Spring Harb Perspect Biol, 2*(1), a001008. doi: 10.1101/cshperspect.a001008

Owens, D. M., & Watt, F. M. (2003). Contribution of stem cells and differentiated cells to epidermal tumours. *Nat Rev Cancer, 3*(6), 444-451. doi: 10.1038/nrc1096

Page, A., Navarro, M., Garin, M., Perez, P., Casanova, M. L., Moreno, R., . . . Ramirez, A. (2010). IKKbeta leads to an inflammatory skin disease resembling interface dermatitis. *J Invest Dermatol, 130*(6), 1598-1610. doi: 10.1038/jid.2010.28

Pasparakis, M. (2009). Regulation of tissue homeostasis by NF-kappaB signalling: implications for inflammatory diseases. *Nat Rev Immunol, 9*(11), 778-788. doi: 10.1038/nri2655

Pasparakis, M., Courtois, G., Hafner, M., Schmidt-Supprian, M., Nenci, A., Toksoy, A., . . . Haase, I. (2002). TNF-mediated inflammatory skin disease in mice with epidermis-specific deletion of IKK2. *Nature, 417*(6891), 861-866. doi: 10.1038/nature00820

Pasparakis, M., Haase, I., & Nestle, F. O. (2014). Mechanisms regulating skin immunity and inflammation. *Nat Rev Immunol, 14*(5), 289-301. doi: 10.1038/nri3646

Pastore, S., Mascia, F., Mariotti, F., Dattilo, C., Mariani, V., & Girolomoni, G. (2005). ERK1/2 regulates epidermal chemokine expression and skin inflammation. *J Immunol, 174*(8), 5047-5056.

Pike, B. L., & Nossal, G. J. (1985). Interleukin 1 can act as a B-cell growth and differentiation factor. *Proc Natl Acad Sci U S A, 82*(23), 8153-8157.

Poli, V. (1998). The role of C/EBP isoforms in the control of inflammatory and native immunity functions. *J Biol Chem, 273*(45), 29279-29282.

Porter, R. M. (2003). Mouse models for human hair loss disorders. *J Anat, 202*(1), 125-131.

Pylayeva-Gupta, Y., Grabocka, E., & Bar-Sagi, D. (2011). RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer, 11*(11), 761-774. doi: 10.1038/nrc3106

Rashel, M., Alston, N., & Ghazizadeh, S. (2014). Protein kinase D1 has a key role in wound healing and skin carcinogenesis. *J Invest Dermatol, 134*(4), 902-909. doi: 10.1038/jid.2013.474

Rebholz, B., Haase, I., Eckelt, B., Paxian, S., Flaig, M. J., Ghoreschi, K., . . . Rupec, R. A. (2007). Crosstalk between keratinocytes and adaptive immune cells in an IkappaBalpha protein-mediated inflammatory disease of the skin. *Immunity, 27*(2), 296-307. doi: 10.1016/j.immuni.2007.05.024

Rider, P., Carmi, Y., Guttman, O., Braiman, A., Cohen, I., Voronov, E., . . . Apte, R. N. (2011). IL-1alpha and IL-1beta recruit different myeloid cells and promote different stages of sterile inflammation. *J Immunol, 187*(9), 4835-4843. doi: 10.4049/jimmunol.1102048

Robinson, R. A., Lu, X., Jones, E. Y., & Siebold, C. (2008). Biochemical and structural studies of ASPP proteins reveal differential binding to p53, p63, and p73. *Structure, 16*(2), 259-268. doi: 10.1016/j.str.2007.11.012

Rothnagel, J. A., Seki, T., Ogo, M., Longley, M. A., Wojcik, S. M., Bundman, D. S., . . . Roop, D. R. (1999). The mouse keratin 6 isoforms are differentially expressed in the hair follicle, footpad, tongue and activated epidermis. *Differentiation, 65*(2), 119-130. doi: 10.1046/j.1432-0436.1999.6520119.x

Rothwell, P. M., Wilson, M., Price, J. F., Belch, J. F., Meade, T. W., & Mehta, Z. (2012). Effect of daily aspirin on risk of cancer metastasis: a study of incident cancers during randomised controlled trials. *Lancet, 379*(9826), 1591-1601. doi: 10.1016/S0140-6736(12)60209-8

Rousset, F., Garcia, E., & Banchereau, J. (1991). Cytokine-induced proliferation and immunoglobulin production of human B lymphocytes triggered through their CD40 antigen. *J Exp Med, 173*(3), 705-710.

Roux, P. P., & Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. *Microbiol Mol Biol Rev, 68*(2), 320-344. doi: 10.1128/MMBR.68.2.320-344.2004

Ryckman, C., Vandal, K., Rouleau, P., Talbot, M., & Tessier, P. A. (2003). Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. *J Immunol, 170*(6), 3233-3242.

Saklatvala, J., Rawlinson, L. M., Marshall, C. J., & Kracht, M. (1993). Interleukin 1 and tumour necrosis factor activate the mitogen-activated protein (MAP) kinase kinase in cultured cells. *FEBS Lett, 334*(2), 189-192.

Samuels-Lev, Y., O'Connor, D. J., Bergamaschi, D., Trigiante, G., Hsieh, J. K., Zhong, S., . . . Lu, X. (2001). ASPP proteins specifically stimulate the apoptotic function of p53. *Mol Cell, 8*(4), 781-794.

Schall, T. J., Bacon, K., Toy, K. J., & Goeddel, D. V. (1990). Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature, 347*(6294), 669-671. doi: 10.1038/347669a0

Schmitz, M. L., Bacher, S., & Kracht, M. (2001). I kappa B-independent control of NF-kappa B activity by modulatory phosphorylations. *Trends Biochem Sci, 26*(3), 186-190.

Schneider, M. R., Schmidt-Ullrich, R., & Paus, R. (2009). The hair follicle as a dynamic miniorgan. *Curr Biol, 19*(3), R132-142. doi: 10.1016/j.cub.2008.12.005

Schon, M. P., & Boehncke, W. H. (2005). Psoriasis. *N Engl J Med, 352*(18), 1899-1912. doi: 10.1056/NEJMra041320

Schoop, V. M., Mirancea, N., & Fusenig, N. E. (1999). Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. *J Invest Dermatol, 112*(3), 343-353. doi: 10.1046/j.1523-1747.1999.00524.x

Schutyser, E., Struyf, S., & Van Damme, J. (2003). The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev, 14*(5), 409-426.

Seitz, C. S., Lin, Q., Deng, H., & Khavari, P. A. (1998). Alterations in NF-kappaB function in transgenic epithelial tissue demonstrate a growth inhibitory role for NF-kappaB. *Proc Natl Acad Sci U S A, 95*(5), 2307-2312.

Senoo, M., Pinto, F., Crum, C. P., & McKeon, F. (2007). p63 Is essential for the proliferative potential of stem cells in stratified epithelia. *Cell, 129*(3), 523-536. doi: 10.1016/j.cell.2007.02.045

Serber, Z., Lai, H. C., Yang, A., Ou, H. D., Sigal, M. S., Kelly, A. E., . . . Dotsch, V. (2002). A C-terminal inhibitory domain controls the activity of p63 by an intramolecular mechanism. *Mol Cell Biol, 22*(24), 8601-8611.

Shankaran, V., Ikeda, H., Bruce, A. T., White, J. M., Swanson, P. E., Old, L. J., & Schreiber, R. D. (2001). IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature, 410*(6832), 1107-1111. doi: 10.1038/35074122

Sharp, L. L., Jameson, J. M., Cauvi, G., & Havran, W. L. (2005). Dendritic epidermal T cells regulate skin homeostasis through local production of insulin-like growth factor 1. *Nat Immunol, 6*(1), 73-79. doi: 10.1038/ni1152

Sil, A. K., Maeda, S., Sano, Y., Roop, D. R., & Karin, M. (2004). IkappaB kinase-alpha acts in the epidermis to control skeletal and craniofacial morphogenesis. *Nature, 428*(6983), 660-664. doi: 10.1038/nature02421

Sims, J. E., & Smith, D. E. (2010). The IL-1 family: regulators of immunity. *Nat Rev Immunol, 10*(2), 89-102. doi: 10.1038/nri2691

Slee, E. A., Gillotin, S., Bergamaschi, D., Royer, C., Llanos, S., Ali, S., . . . Lu, X. (2004). The N-terminus of a novel isoform of human iASPP is required for its cytoplasmic localization. *Oncogene, 23*(56), 9007-9016. doi: 10.1038/sj.onc.1208088

Smitten, A. L., Simon, T. A., Hochberg, M. C., & Suissa, S. (2008). A meta-analysis of the incidence of malignancy in adult patients with rheumatoid arthritis. *Arthritis Res Ther, 10*(2), R45. doi: 10.1186/ar2404

Sotiropoulou, P. A., & Blanpain, C. (2012). Development and homeostasis of the skin epidermis. *Cold Spring Harb Perspect Biol, 4*(7), a008383. doi: 10.1101/cshperspect.a008383

Sottocornola, R., Royer, C., Vives, V., Tordella, L., Zhong, S., Wang, Y., . . . Lu, X. (2010). ASPP2 binds Par-3 and controls the polarity and proliferation of neural progenitors during CNS development. *Dev Cell, 19*(1), 126-137. doi: 10.1016/j.devcel.2010.06.003

Sperling, T., Oldak, M., Walch-Ruckheim, B., Wickenhauser, C., Doorbar, J., Pfister, H., . . . Smola, S. (2012). Human papillomavirus type 8 interferes with a novel C/EBPbeta-mediated mechanism of keratinocyte CCL20 chemokine expression and

Langerhans cell migration. *PLoS Pathog, 8*(7), e1002833. doi: 10.1371/journal.ppat.1002833

Stenn, K. S., & Paus, R. (2001). Controls of hair follicle cycling. *Physiol Rev, 81*(1), 449-494.

Sterneck, E., Zhu, S., Ramirez, A., Jorcano, J. L., & Smart, R. C. (2006). Conditional ablation of C/EBP beta demonstrates its keratinocyte-specific requirement for cell survival and mouse skin tumorigenesis. *Oncogene, 25*(8), 1272-1276. doi: 10.1038/sj.onc.1209144

Straub, W. E., Weber, T. A., Schafer, B., Candi, E., Durst, F., Ou, H. D., . . . Dotsch, V. (2010). The C-terminus of p63 contains multiple regulatory elements with different functions. *Cell Death Dis, 1*, e5. doi: 10.1038/cddis.2009.1

Streilein, J. W. (1983). Skin-associated lymphoid tissues (SALT): origins and functions. *J Invest Dermatol, 80 Suppl*, 12s-16s.

Stylianou, E., & Saklatvala, J. (1998). Interleukin-1. *Int J Biochem Cell Biol, 30*(10), 1075-1079.

Suh, E. K., Yang, A., Kettenbach, A., Bamberger, C., Michaelis, A. H., Zhu, Z., . . . McKeon, F. (2006). p63 protects the female germ line during meiotic arrest. *Nature, 444*(7119), 624-628. doi: 10.1038/nature05337

Swann, J. B., & Smyth, M. J. (2007). Immune surveillance of tumors. *J Clin Invest, 117*(5), 1137-1146. doi: 10.1172/JCI31405

Takada, N., Sanda, T., Okamoto, H., Yang, J. P., Asamitsu, K., Sarol, L., . . . Okamoto, T. (2002). RelA-associated inhibitor blocks transcription of human immunodeficiency virus type 1 by inhibiting NF-kappaB and Sp1 actions. *J Virol, 76*(16), 8019-8030.

Tao, H., Shimizu, M., Kusumoto, R., Ono, K., Noji, S., & Ohuchi, H. (2005). A dual role of FGF10 in proliferation and coordinated migration of epithelial leading edge cells during mouse eyelid development. *Development, 132*(14), 3217-3230. doi: 10.1242/dev.01892

Toonen, J., Liang, L., & Sidjanin, D. J. (2012). Waved with open eyelids 2 (woe2) is a novel spontaneous mouse mutation in the protein phosphatase 1, regulatory (inhibitor) subunit 13 like (Ppp1r13l) gene. *BMC Genet, 13*, 76. doi: 10.1186/1471-2156-13-76

Triantafillidis, J. K., Nasioulas, G., & Kosmidis, P. A. (2009). Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. *Anticancer Res, 29*(7), 2727-2737.

Trinchieri, G. (2012). Cancer and inflammation: an old intuition with rapidly evolving new concepts. *Annu Rev Immunol, 30*, 677-706. doi: 10.1146/annurev-immunol-020711-075008

Truong, A. B., Kretz, M., Ridky, T. W., Kimmel, R., & Khavari, P. A. (2006). p63 regulates proliferation and differentiation of developmentally mature keratinocytes. *Genes Dev, 20*(22), 3185-3197. doi: 10.1101/gad.1463206

Uemura, N., Okamoto, S., Yamamoto, S., Matsumura, N., Yamaguchi, S., Yamakido, M., . . . Schlemper, R. J. (2001). Helicobacter pylori infection and the development of gastric cancer. *N Engl J Med, 345*(11), 784-789. doi: 10.1056/NEJMoa001999

Urb, M., & Sheppard, D. C. (2012). The role of mast cells in the defence against pathogens. *PLoS Pathog, 8*(4), e1002619. doi: 10.1371/journal.ppat.1002619

van Hogerlinden, M., Rozell, B. L., Ahrlund-Richter, L., & Toftgard, R. (1999). Squamous cell carcinomas and increased apoptosis in skin with inhibited Rel/nuclear factor-kappaB signaling. *Cancer Res, 59*(14), 3299-3303.

Vermeulen, L., De Wilde, G., Van Damme, P., Vanden Berghe, W., & Haegeman, G. (2003). Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO J, 22*(6), 1313-1324. doi: 10.1093/emboj/cdg139

Vesely, M. D., Kershaw, M. H., Schreiber, R. D., & Smyth, M. J. (2011). Natural innate and adaptive immunity to cancer. *Annu Rev Immunol, 29*, 235-271. doi: 10.1146/annurev-immunol-031210-101324

Vives, V., Su, J., Zhong, S., Ratnayaka, I., Slee, E., Goldin, R., & Lu, X. (2006). ASPP2 is a haploinsufficient tumor suppressor that cooperates with p53 to suppress tumor growth. *Genes Dev, 20*(10), 1262-1267. doi: 10.1101/gad.374006

Vogl, T., Ludwig, S., Goebeler, M., Strey, A., Thorey, I. S., Reichelt, R., . . . Roth, J. (2004). MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. *Blood, 104*(13), 4260-4268. doi: 10.1182/blood-2004-02-0446

Voss, A., Bode, G., Sopalla, C., Benedyk, M., Varga, G., Bohm, M., . . . Kerkhoff, C. (2011). Expression of S100A8/A9 in HaCaT keratinocytes alters the rate of cell proliferation and differentiation. *FEBS Lett, 585*(2), 440-446. doi: 10.1016/j.febslet.2010.12.037

Wang, H. Q., & Smart, R. C. (1999). Overexpression of protein kinase C-alpha in the epidermis of transgenic mice results in striking alterations in phorbol ester-induced inflammation and COX-2, MIP-2 and TNF-alpha expression but not tumor promotion. *J Cell Sci, 112 (Pt 20)*, 3497-3506.

Wang, X. P., Schunck, M., Kallen, K. J., Neumann, C., Trautwein, C., Rose-John, S., & Proksch, E. (2004). The interleukin-6 cytokine system regulates epidermal permeability barrier homeostasis. *J Invest Dermatol, 123*(1), 124-131. doi: 10.1111/j.0022-202X.2004.22736.x

Werfel, T. (2009). The role of leukocytes, keratinocytes, and allergen-specific IgE in the development of atopic dermatitis. *J Invest Dermatol, 129*(8), 1878-1891. doi: 10.1038/jid.2009.71

Werner, S., & Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiol Rev, 83*(3), 835-870. doi: 10.1152/physrev.00031.2002

Werner, S., Krieg, T., & Smola, H. (2007). Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol, 127*(5), 998-1008. doi: 10.1038/sj.jid.5700786

Witcher, M., Pettersson, F., Dupere-Richer, D., Padovani, A., Summers-Deluca, L., Baldwin, A. S., & Miller, W. H., Jr. (2008). Retinoic acid modulates chromatin to potentiate tumor necrosis factor alpha signaling on the DIF2 promoter. *Nucleic Acids Res, 36*(2), 435-443. doi: 10.1093/nar/gkm1058

Woodland, D. L., & Dutton, R. W. (2003). Heterogeneity of CD4(+) and CD8(+) T cells. *Curr Opin Immunol, 15*(3), 336-342.

Woodworth, C. D., Michael, E., Smith, L., Vijayachandra, K., Glick, A., Hennings, H., & Yuspa, S. H. (2004). Strain-dependent differences in malignant conversion of mouse skin tumors is an inherent property of the epidermal keratinocyte. *Carcinogenesis, 25*(9), 1771-1778. doi: 10.1093/carcin/bgh170

Wu, M. X., Ao, Z., Prasad, K. V., Wu, R., & Schlossman, S. F. (1998). IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. *Science, 281*(5379), 998-1001.

Wu, X., & Pandolfi, P. P. (2001). Mouse models for multistep tumorigenesis. *Trends Cell Biol, 11*(11), S2-9.

Yamanishi, Y., Boyle, D. L., Rosengren, S., Green, D. R., Zvaifler, N. J., & Firestein, G. S. (2002). Regional analysis of p53 mutations in rheumatoid arthritis synovium. *Proc Natl Acad Sci U S A, 99*(15), 10025-10030. doi: 10.1073/pnas.152333199

Yang, A., Kaghad, M., Caput, D., & McKeon, F. (2002). On the shoulders of giants: p63, p73 and the rise of p53. *Trends Genet, 18*(2), 90-95.

Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., . . . McKeon, F. (1998). p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell, 2*(3), 305-316.

Yang, A., Schweitzer, R., Sun, D., Kaghad, M., Walker, N., Bronson, R. T., . . . McKeon, F. (1999). p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature, 398*(6729), 714-718. doi: 10.1038/19539

Yang, J. P., Hori, M., Sanda, T., & Okamoto, T. (1999). Identification of a novel inhibitor of nuclear factor-kappaB, RelA-associated inhibitor. *J Biol Chem, 274*(22), 15662-15670.

Yano, S., Banno, T., Walsh, R., & Blumenberg, M. (2008). Transcriptional responses of human epidermal keratinocytes to cytokine interleukin-1. *J Cell Physiol, 214*(1), 1-13. doi: 10.1002/jcp.21300

Yuan, J. S., Reed, A., Chen, F., & Stewart, C. N., Jr. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics, 7*, 85. doi: 10.1186/1471-2105-7-85

Yurchenco, P. D. (2011). Basement membranes: cell scaffoldings and signaling platforms. *Cold Spring Harb Perspect Biol, 3*(2). doi: 10.1101/cshperspect.a004911

Zarubin, T., & Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Res, 15*(1), 11-18. doi: 10.1038/sj.cr.7290257

Zhang, W., & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res, 12*(1), 9-18. doi: 10.1038/sj.cr.7290105

Zhu, S., Yoon, K., Sterneck, E., Johnson, P. F., & Smart, R. C. (2002). CCAAT/enhancer binding protein-beta is a mediator of keratinocyte survival and skin tumorigenesis involving oncogenic Ras signaling. *Proc Natl Acad Sci U S A, 99*(1), 207-212. doi: 10.1073/pnas.012437299

Zitvogel, L., Tesniere, A., & Kroemer, G. (2006). Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol, 6*(10), 715-727. doi: 10.1038/nri1936

Zwergal, A., Quirling, M., Saugel, B., Huth, K. C., Sydlik, C., Poli, V., . . . Brand, K. (2006). C/EBP beta blocks p65 phosphorylation and thereby NF-kappa B-mediated transcription in TNF-tolerant cells. *J Immunol, 177*(1), 665-672.