The role of Transforming Growth Factor (TGF) - Beta 1 Activated Kinase 1 (TAK1) in wound repair

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**THE ROLE OF TRANSFORMING GROWTH FACTOR (TGF)
- BETA 1 ACTIVATED KINASE 1 (TAK1) IN WOUND
REPAIR**

LAM CHEE REN IVAN

SCHOOL OF BIOLOGICAL SCIENCES

2013

**THE ROLE OF TRANSFORMING GROWTH FACTOR (TGF)
- BETA 1 ACTIVATED KINASE 1 (TAK1) IN WOUND
REPAIR**

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partial fulfilment of the requirement for the degree of Doctor of
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LIST OF ABBREVIATIONS

ng	Nanogram
µl	Microlitre
µm	Micrometre
µM	Micromolar
AKT1	see PKBα
ALS	Amyotrophic lateral sclerosis
AP-1	Activated protein-1
ATP	Adenosine-5'-triphosphate
BMP	Bone morphogenetic protein
BHLH	Basic helix-loop-helix
bp	Base pair
ca	Constitutively active
<i>C. elegans</i>	Caenorhabditis elegans
cDNA	Complementary deoxyribonucleic acid
c-FLIP	Cellular FLICE inhibitory protein
ChIP	Chromatin immunoprecipitation
cKit	c-Kit or tyrosine-protein kinase Kit
CM-H2DCFDA	Chloromethyl 2',7'-dichlorodihydrofluorescein diacetate
Col	Collagen
COX	Cyclooxygenase
Ctrl	Control
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DCF	Dichlorodihydrofluorescein
DD	Death domain
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
EMSA	Electrophoretic mobility shift assay
F	Fibroblast
FACS	Fluorescence activated cell sorter
FADD	Fas-associated death domain
FDA	Food drug administration
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1 beta-converting enzyme
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPX	glutathione peroxidase
GSH	Glutathione
GSK	Glycogen synthase kinase
h	Hour
HBSS	Hanks' balanced salt solution
HE	Haematoxylin and eosin staining
HGF	Hepatocyte growth factor
HMGA	High mobility group A2
HRP	Horse radish peroxidase
JNK	c-Jun N-terminal kinases
kDa	Kilo Dalton
kPa	Kilo pascal
K _{CTRL}	Human keratinocytes transduced with control scrambled siRNA
K _{TAK1}	Human keratinocytes transduced with TAK1 siRNA
I κ B α	Inhibitor of kappa B α (I κ B α)
IKK β	Inhibitor of I κ B kinase β

IKK γ	Inhibitor of I κ B kinase γ
IL-1	Interleukin 1
IL-1R	Interleukin 1 receptor
LOX	Lipoxygenase
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MAPKKK	MAPK kinase kinase
MEK	MAPK kinase
MEKK	MEK kinase
NAC	N-acetyl-L-cysteine
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF- κ B	Nuclear factor kappa B
NOX	NADPH oxidase complexes
NSC	Non-specific competitor
Oligo-dT	Oligo deoxy-thymine nucleotides
OTC	Organotypic co-culture
Pa	Pascal
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCNA	Proliferative cell nuclear antigen
PDGF	Platelet derived growth factor
pg	Picogram
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PKA	Protein Kinase A or cAMP-dependent Protein Kinase
PKB α	Protein kinase B α
PPAR β/γ	Peroxisome proliferator-activated receptor β/γ
pVHL	Von Hippel-Lindau
qPCR	Quantitative real-time polymerase chain reaction

ROS	Reactive oxygen species
RT-PCR	Reverse transcription PCR
SC	Specific competitor
SCF	Stem cell factor
S.D	Standard deviation
SEK-1	Stress/extracellular-regulated kinase-1
SGK	serum-and glucocorticoid-induced protein kinase
siRNA	Small interfering RNA
SMAD	Similar to Mother against Decapentaplegic drosophila
SOD	Superoxide dismutase
SODD	Silencer of death domain
TACE	TNF-converting enzyme
TAK1	Transforming growth factor- β activated kinase 1
TAM67	Dominant negative activator protein 1
TGF- β	Transforming growth factor- β
TLR	Toll-like receptor
TNF- α	Tumor necrosis factor- α
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
ZEB	zinc finger E-box-binding homeobox

CHAPTER 1

THE ROLE OF TRANSFORMING GROWTH FACTOR (TGF) - BETA 1 ACTIVATED KINASE 1 (TAK1) IN WOUND REPAIR

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All sections are contributed by the candidate except: Fig 1.5, Fig 1.7, Fig 1.8, Fig 1.10

ABSTRACT

The generation of reactive oxygen species (ROS) is deregulated in several human pathologies, including cancer and diabetes. During the inflammatory phase of normal wound repair, the epidermal ROS levels are increased by the infiltrating phagocytic immune cells and the native keratinocytes. This inflammation-induced ROS production is tightly controlled to contain the oxidative damage, the mechanisms of which remain unclear. Transforming growth factor β -activated kinase 1 (TAK1) is a pivotal inflammatory mediator, and its expression is transiently elevated during wound healing, corresponding to the epidermal ROS production profile in the wound. However, the implications of this relationship remain obscure. Herein, we show that TAK1 directly regulates the expression of stem cell factor (SCF), which activates the protein kinase B (PKB)- α pro-survival pathway in a cell-autonomous manner to protect keratinocytes from ROS-mediated cell death. TAK1 deficiency in keratinocytes led to increased apoptosis in response to anoikis and TNF- α treatment and was associated with elevated ROS levels as determined by fluorescence activated cell sorting (FACS). Using organotypic skin co-culture and comparative growth factor array analysis, we revealed a cell-autonomous mechanism that involved the SCF/c-Kit/PKB α signaling cascade. The ectopic expression of TAK1 or treatment with exogenous recombinant SCF restored the increased ROS production and apoptotic cell death in the TAK1-deficient keratinocytes. Conversely, the normal keratinocytes treated with various inhibitors targeting the SCF/c-Kit/PKB α pathway exhibited increased ROS production and TNF- α - and anoikis-induced apoptosis. Our study reveals a novel antiapoptotic and protective role for SCF in keratinocytes and identifies TAK1 as a novel player uniting inflammation and ROS regulation in skin redox biology.

1.1 INTRODUCTION

1.1.1) The importance of wound healing research

Health care for wound healing has been indispensable throughout human medical history¹. Even without the current medical literacy, ancient Egyptians were aware that closed wounds heal significantly faster than open ones, from which they conceptualized the technique of adhesive bandaging¹. Likewise, the long-held practice of stitching wounds or suturing was used by primitive Africans and South Americans to close an open wound and hence accelerate healing¹. In addition to improving the rate of wound recovery, the importance of wound care is best exemplified when its neglect almost certainly and entirely explains the high mortality rate of wounded or surgically treated individuals². Prior to the landmark implementation of antiseptic techniques in the late nineteenth century, the success of surgery was rare, and patients frequently died from complications of postoperative wound infections^{2,3}. Undoubtedly, the recovery and the success of surgical treatments are essentially conditional upon a positive state of wound healing. Although improved sanitation in recent times has greatly lowered the mortality rate of wound healing complications, the impact and implications of wounding nonetheless remain extensive. For example, in healthy individuals, wounding is still an important and regular cause of accidental morbidity and mortality. In fact, the incidence of superficial wounds remains substantially high; for example, more than 1.25 million people suffer from burns annually in the United States alone⁴. However, the need for wound care extends beyond that of inflicted injuries, as impaired wound healing commonly occurs when health is compromised by malnutrition, aging, immunosuppression, poor circulation, disease, among others⁵. Notably, wound healing is impaired as a result of diabetes⁶, which affects 170 million people worldwide and 20.8 million in the United States⁷. This impaired wound healing initially manifests as foot ulcers that can

potentially progress to limb-threatening infections that warrant amputation^{8,9}. Thus, even when wounding is not a terminal illness, its impact on disease morbidity is not insignificant. Hence, a great deal of research has been performed to better understand the molecular mechanisms of wound healing and how it can go awry in the context of diseases.

In addition to disrupted wound healing being a debilitating consequence of failing health (e.g., diabetes and ageing), it has been observed since the nineteenth century that wounding triggers several major pathologies. In patients with chronic inflammatory skin disease psoriasis, the wounding of uninvolved skin often leads to the subsequent formation of disease lesions at the wound boundaries. This observation, called Koebner's phenomenon, is similarly observed in many chronic inflammatory type skin diseases (e.g., lichen planus)¹⁰. Furthermore, cancer frequently results from chronic inflammatory disease, and chronic non-healing ulcers have also been observed to progress into malignant tumors^{11,12}. Moreover, cancers have been famously likened to wounds that do not heal because their inflammatory microenvironment is remarkably similar to that of a wound in the preliminary stages of healing¹². With the advent of molecular biology and the current improved understanding of wound healing, an interest in the classical relationship between wounding and disease has once again been revived and revisited¹². The immunopathological implications of wounding raise many important questions. What are the molecular factors in the wound microenvironment that can spontaneously trigger chronic inflammatory skin diseases or cancer? What are the biochemical similarities between wounding and disease? Answers to these questions will inspire novel treatment approaches for chronic inflammatory disease and cancer.

The skin is an excellent organ to study the effects of wound healing and inflammation due to its superficial location and position. In fact, most of the earliest observations of inflammation were

derived from the skin, as many inflammatory symptoms are first apparent in the skin without the need for dissection procedures. Moreover, the original word inflammation, derived from the Latin word ‘inflammare,’ which means ‘set on fire,’ was used to describe the localized redness, warmth, swelling and tenderness visible on the skin overlaying the site of tissue injury¹³. As such, a prerequisite understanding of skin structure and function will greatly aid the examination of wound healing and chronic inflammatory skin diseases.

1.1.2) The skin

1.1.2.1) Epidermis

The skin comprises of three connected layers: the innermost layer of subcutaneous tissue (i.e., fat tissue), the middle layer of dermis and the outermost layer of epidermis^{14,15} (Fig I).

Fig I. Anatomy of the skin. The skin consists of three main layers, namely the epidermis, dermis and the subcutaneous tissue. Interspersed within the three layers are accessory structures that support skin function, like blood vessels and sensory nerve endings, hair follicles, sweat glands and sebaceous glands.

As the most apical structure of the skin, the epidermis is constantly exposed to environmental insults, such as damaging sunrays, infection risks and the desiccating external environment¹⁶. To withstand these challenges, the epidermis must be capable of shielding the skin from UV, excluding foreign organisms and preventing evaporative water loss¹⁵. In fact, the surface of the skin is water-resistant, composed of insoluble flattened or squamous dead skin cell remnants that serve as both a physical and water barrier¹⁵. Understanding the epidermal structure (Fig II) and differentiation (Fig III) will help explain how this protective barrier is formed and maintained.

Fig II. Epidermal layers. A schematic drawing depicting the stratified epidermal layers. (Adapted from About.com(Dermatology))The epidermis is an epithelium composed mainly (95%) of keratinocytes, which are the most abundant epithelial cell type¹⁵. The epidermis is established on the basement membrane, which separates it from the dermis. A cross section of the skin reveals that the epidermis is stratified into five layers (Fig II), with each layer representing a particular phase of keratinocyte differentiation as the cells progress from the basement membrane to the skin surface.

Fig III. Keratinocyte differentiation in the epidermis. The outermost, protective epidermal layer is the cornified layer or *stratum corneum*. Here, cells are dead, flattened and squamous in shape, devoid of organelles and are metabolically inactive (Fig III)^{17,18}. These cells, or corneocytes, are enveloped by an insoluble layer of protein, thus contributing to the water-resistant character of the skin surface¹⁸. The corneocytes are shed daily through desquamation, yet the *stratum corneum* is not eroded because it is consistently replenished by freshly differentiated keratinocytes derived from ongoing mitotic divisions¹⁸. As such, the epidermis is not a static structure; rather, it is gradually turned over and renewed during cornification, which also fits its protective function¹⁸. This turnover process is termed epidermal homeostasis because it involves balancing keratinocyte proliferation and differentiation. Effectively, the terminal differentiation of keratinocytes during cornification mechanistically explains the underlying cellular dynamics of epidermal homeostasis and stratification¹⁷. Epidermal homeostasis progressively stratifies the epidermis in a manner that is gradual yet highly coordinated in time and space¹⁷.

1.1.2.2) Cornification

We shall now examine the changes at the tissue, cellular and molecular levels that underlie epidermal homeostasis and cornification. The innermost, single-cell epidermal layer attached to the basement membrane or basal lamina is called the *stratum basale*. The columnar shaped basal cells of the *stratum basale* are undifferentiated epidermal progenitors or keratinocyte stem cells (Fig III). $\beta 1$ -integrins mediate the attachment of the basal cells to the basement membrane. The interaction of the basal cell surface $\beta 1$ -integrins with the extracellular matrix of the basement membrane maintains the basal cells in an undifferentiated state¹⁹. The continuous growth and active proliferation of the basal cells replenishes the *stratum basale* with epidermal stem cells and adds keratinocytes to the suprabasal layers²⁰. When a basal cell divides, one of its daughter cells is committed to a stem cell fate, and the other moves into the suprabasal layers, subsequently detaching from the basement membrane²⁰. The keratinocytes destined for the suprabasal layers are termed the transit amplifying cells. The transit amplifying cells have lower $\beta 1$ -integrin expression than the basal stem cells; thus, they are more loosely adhered to the basement membrane and hence more easily detached from the basement membrane. As $\beta 1$ -integrins are unoccupied upon detachment, the transit amplifying cells are also triggered into terminal differentiation^{19,21,22}. Importantly, the transit amplifying cells retain a limited replicative potential, and they further divide during their transit to the superficial layers, thereby contributing to the multi-cell layered *stratum spinosum*. This proliferative process also pushes the existing keratinocytes into the upper layers^{18,23}.

The keratinocytes of the *stratum basale* and *stratum spinosum* are both metabolically and transcriptionally active^{15,24}. However, the basal cells are more mitotically active than the keratinocytes of the *stratum spinosum*, which are mostly post-mitotic and spent of their

replicative potential^{17,24}. Because terminal differentiation is initiated by cell detachment, the keratinocytes of the *stratum spinosum* also begin to use their transcriptional machinery for synthetic rather than mitotic purposes^{17,18,24}. A few key morphological, cellular and molecular changes occur during differentiation. Most visibly, the keratinocytes undergo cytoskeletal reorganization, which is evident when the columnar keratinocytes detach from the basement membrane and become polygonal shaped in the *stratum spinosum*²⁵. These keratinocytes then develop very well-defined desmosomal cell-cell adhesions, which appear as spines. Hence, the *stratum spinosum* is commonly referred to as the ‘spiny layer’. Importantly, these desmosomal connections contribute to the strength of the epidermis^{24,26,27}. Keratinocyte differentiation is further characterized by major changes in protein expression. The undifferentiated basal keratinocytes specifically express keratins 5 and 14, whereas the differentiating keratinocytes of the *stratum spinosum* express keratins 1 and 10^{24,28}.

In addition, the synthesis of structural proteins in preparation for cornification begins in the *stratum spinosum* and becomes histologically distinct when the synthesized proteins accumulate in the *stratum granulosum* or granular layer. When the keratinocytes reach the stratum granular layer, they stop dividing and largely increase their synthesis of structural proteins, keratin and keratohyalin^{23,24,29}. It is through this epidermal synthetic process that hair and nails are formed. Notably, the *stratum granulosum* is filled with dense cytoplasmic granules called keratohyalin granules²⁹. These proteins aid in the cross-linking of keratins to form the keratin filament network. Additionally, the keratinocytes from the *stratum spinosum* to the *stratum granulosum* become increasingly accumulated with intracellular lamellar bodies. These membranous bodies contain all the important differentiation-dependent structural and catalytic proteins, including involucrin, keratins, filaggrin and transglutaminase¹⁸. Involucrin, a soluble protein precursor of

the cornified envelope, is synthesized in the cells of the *stratum spinosum* and crosslinked in the *stratum granulosum* by transglutaminase, which is the enzyme used for the cornification of the envelope¹⁸. As the keratinocytes progress into the upper granular layer, they become increasingly permeable to calcium and other ions. This calcium influx activates transglutaminase, which cross links the amino groups of envelope and structural proteins²³. Because the cross linking usually involves the side-chain amino groups of the proteins, the protein networks thus formed are more resistant to protease degradation, thereby suiting the resistant role of the cornified layer against microbial invasion³⁰. In the keratinocytes of the *stratum granulosum*, the nucleus is disintegrated and its DNA content degraded; the cytoplasmic organelles are also destroyed, and the plasma membrane is progressively replaced by the cornified envelope and ceramide depositions from the lamellar bodies^{17,18,24}. The *stratum lucidum* is a three- to five-cell-thick transitional layer between the *stratum granulosum* and the *stratum corneum*, and it is only observed in thicker skin. The keratinocytes in the *stratum lucidum* continue the cornification process and become increasingly flattened as the keratinization or keratin fiber formation restructures and shapes the cell into thinner and flatter entities. The cornification process is effectively a programmed cell death process as the keratinocytes lose their cellular components and transform into lifeless structures^{18,23,24,31}.

1.1.3) The wound healing process

The aim of wound healing is to ultimately restore the disrupted epidermal barrier function. The general strategy involved in the repair of damaged skin is the heightened proliferation of epidermal and dermal cells neighboring the wound site followed by their subsequent migration to enclose the physical gap created by the wounding³². However, before wound enclosure, the wounded skin region has to be guarded against infection. Therefore, three key processes govern

wound healing: inflammation, re-epithelialization, and remodeling^{33,34}. The inflammatory phase begins immediately post-wounding and involves the gathering of immune cells at the wound site to combat the probable microbial invasion. Inflammation is strongly believed to aid in wound repair because the growth factors contributed by the immune cells also draw neighboring keratinocytes and fibroblasts to the wound site^{12,32,35}. These migrant cells then facilitate the replacement of the blood clot with granulation tissue, a connective tissue replacement for the damaged dermis that also serves as a favorable surface for re-epithelialization^{12,34,36,37}. This process promotes wound closure followed by the remodeling of the repaired cutaneous membrane. Given their interrelatedness and mutual dependence, these wound-healing processes operate in a manner that overlaps in time and space.

1.1.3.1) Inflammatory phase

When the functional epidermis and dermis are breached during wounding, the open wound is exposed to the non-sterile external environment and consequently subjected to infection. If pathogenic bacteria gain entry through a wound, the systemic spread of bacterial infection in the blood circulation (i.e., sepsis) is life threatening. As such, the natural wound-healing process involves the eradication of the microbes present at the wound site through immunomodulatory means. Hence, inflammation is necessarily the immediate response to wounding³⁵.

At the point of injury, the bleeding from the disrupted blood vessels leads to clotting (i.e., hemostasis), which beneficially blocks the wound opening from microbes. The extracellular matrix (ECM) of the blood clot, which is composed of cross-linked fibrin, fibronectin, vitronectin and thrombospondin, serves as a makeshift track for the navigation and migration of the cells involved in wound repair (i.e., the immune cells, keratinocytes and fibroblasts)^{12,36}. The

platelets in the clot also secrete molecular factors to support inflammation and wound healing. One key example is platelet-derived growth factor (PDGF), which serves as a chemoattractant to bring macrophages and fibroblasts to the wound site^{38,39}. In addition to PDGF, other vasoactive mediators and chemotactic factors are produced by the complement and coagulation cascades^{12,34,36,37}. These factors largely account for the recruitment of inflammatory leukocytes to the injury site. Neutrophils, which are the most abundant leukocyte type in the blood, are among the first immune cells to arrive at the site of injury within min⁴⁰. The infiltrating neutrophils scavenge the wound space and thoroughly ingest every trace of foreign particles, pathogens or parenchymal cellular debris^{38,40}. The pus commonly observed at the site of a wound or bacterial skin infection is mainly composed of the neutrophil masses infiltrating the inflamed site to perform this clearing function.

The neutrophils are followed by monocytes and lymphocytes^{38,39}. Monocytes, the second wave of immune cells converging on the wound site, are chemoattracted to the wound site by ECM fragments, transforming growth factor β (TGF β), and monocyte chemoattractant protein 1, where they further differentiate into activated macrophages, which are then capable of phagocytosis and priming the adaptive immune response^{35,36,39}. Furthermore, macrophages must adhere to the ECM at the wound site through integrin-mediated adhesions before they can secrete proinflammatory and growth-promoting cytokines. The macrophage-secreted cytokines include the proinflammatory cytokines tumor necrosis factor α (TNF α) and interleukin-1 (IL-1), PDGF, transforming growth factor- α (TGF- α) and insulin-like growth factor-1 (IGF-1)^{36,38,39,41}. All of these factors are also used as mitogenic influences to initiate the re-epithelization and growth of the keratinocytes at the wound edge during the wound closure effort. In addition to the phagocytic and cytokine-secreting roles at the wound site, the immune cell infiltrates (i.e., the

macrophages and neutrophils) also produce diverse forms of proteinases and reactive oxygen species (ROS) to combat the hostile microbial contaminants^{42,43}.

Inflammation is essentially a constitutive process that is inseparable from wound healing. However, inflammation must be executed in a timely and limited manner to positively promote wound recovery^{5,44}. In the event of deregulated and therefore chronic inflammation, wound healing would be prolonged, often times leading to a negative outcome of a chronic degenerative disease or even malignancy^{33,45}.

1.1.3.2) Reepithelialization and remodelling

Re-epithelialization refers to the mobilization of the epithelial cells or keratinocytes to the wound site and eventually the migration of the keratinocytes over the granulation tissue to seal the wound space. However, under the influence of the growth factors released by the immune cells and the blood clot, re-epithelialization occurs earlier than the establishment of the granulation tissue. In fact, a few hours post-injury, the epidermal cells originating from hair follicles rapidly proliferate and enter the wound space to remove the existing blood clots and stroma tissue debris³⁶. Although keratinocytes are abundantly present around a wound, they are rather stationary in their default epithelial state. To effectively modulate wound repair, the growth factors from the blood clot and immune cells induce many molecular changes to the keratinocytes neighboring the wound site to enhance their motility. Such changes include the retraction of intracellular tonofilaments, the dissolution of cell-cell desmosomal linkages and the formation of peripheral cytoplasmic actin filaments⁴⁶. In addition, the hemidesmosomal links between the epidermis and the basement membrane are also disrupted, allowing the lateral movement of the keratinocytes towards the wound site⁴⁷. Keratinocytes also express integrin

molecules on their surfaces that engage the ECM proteins (e.g., fibronectin and vitronectin) of the clot as they migrate⁴⁸. Because wound repair involves the removal of the clot, the epidermal cells also secrete matrix metalloproteinases to degrade the ECM; they break through the matrix-rich clot to separate it from the dermis⁴⁹⁻⁵¹.

Because the re-epithelialization process is initiated with the epidermal cells gaining motility and preparing for wound closure, the granulation tissue is concurrently formed by inflammatory cues. In the normal cutaneous structure, the epidermis is established on the dermis upon the basement membrane. During wounding, when the dermal layer of the skin is also damaged, re-epithelialization can only occur in the presence of an underlying dermal-like connective tissue. The granulation tissue is the precursor of the dermis replacement, and it also serves as a base on which the re-epithelializing keratinocytes can migrate and establish³⁶. The light pink or red granulation tissue that is moist in appearance and soft to the touch is a highly vascularized, fibrous connective tissue (Fig IV) that provides the highly metabolic wound site with an ample supply of nutrients and the drainage of waste³⁶. Because the connective tissues are synthesized by the fibroblasts, dermal repair must first occur with an assembly of fibroblasts. The fibroblasts are initiated and attracted to the wound site by several factors, including PDGF secreted by platelets and monocyte-derived activated macrophages of the inflammatory phase, TGF β 1 and other extracellular-matrix molecules present in the clot⁵²⁻⁵⁴. Like the epidermal cells, the activated fibroblasts also proliferate, express integrin receptors and secrete proteolytic enzymes for the migration through the clot^{50,55-57}. Once within the wound site, the fibroblasts begin synthesizing a provisional ECM, which is gradually replaced with a collagenous matrix^{58,59}. To form the perfused granulation tissue, this matrix must become a scaffold for angiogenesis. Many factors in the wound trigger this angiogenesis, including fibroblast growth factor (FGF), vascular

endothelial growth factor (VEGF), angiogenin, TGF- β , low oxygen tension and elevated lactic acid⁶⁰⁻⁶². Some of the angiogenic factors are secreted by the activated macrophages, reinforcing the importance of the preliminary inflammatory phase in the later phases of wound repair³⁸. The epidermal cells of the wound may also be activated to express these angiogenic factors⁴⁹. Like PDGF and TGF β , the cytokine factors that trigger granulation tissue formation also convert the granulation tissue fibroblasts to myofibroblasts, which have large cytoplasmic actin bundles and thus are highly contractile⁵⁸. Myofibroblast contraction is the basis for wound contraction, which effectively compacts the connective tissue and reduces the area of the wound space⁶³⁻⁶⁶. Finally, the epidermal cells at the wound edge can conveniently migrate across the granulation tissue to reepithelialize the wound space.

Fig IV. Granulation tissue. The granulation tissue is a well vascularised tissue with a moist appearance. Tissue vasculature supplies nutrients heavily demanded by the healing wound. This supports reepithelialisation efforts by neighbouring keratinocytes. (Adapted from the Angiogenesis Foundation)

1.1.3.3) Remodeling

Remodeling is the last stage of wound healing, and it is essentially the conversion of the granulation tissue to a mature scar. Most wounds result in scar formation and seldom revert back to the original state of the skin³⁶. The scar differs from the normal skin in that it lacks all appendages, such as hair follicles, sebaceous glands and sweat glands³⁴. Significantly, the matrix laid down by the fibroblasts during the formation of the granulation tissue is not static; rather, it is constantly turned over. Hence, remodeling involves constant collagen synthesis and catabolism by fibroblasts. Abnormalities and perturbations in this collagen turnover may lead to the two extremes of wound healing: an overhealing wound or a chronic, non-healing wound. If collagen synthesis is faster than catabolism, it is likely that thicker scar tissues will form, leading to hypertrophic and keloid scarring. The opposite is also true: if collagen catabolism is faster than synthesis, non-healing wounds will result⁶⁷.

Another key consensus regarding wound healing is that the earliest phase of inflammation usually concludes before the final remodeling phase can be affected. In chronic, non-healing wounds, such as diabetic wounds, inflammation is usually prolonged, and wound closure is therefore hindered⁵. Interestingly, cancer is often regarded as a wound that does not heal⁶⁸, so an accurate understanding of the science of wound inflammation is important, as it can potentially reveal important molecular or biochemical players with therapeutic implications in cancer.

1.1.4) Wounding and chronic inflammatory skin disease.

1.1.4.1) A classical case study: Psoriasis

Inflammation of the skin has important contributions towards wound healing. However, when inflammation fails to resolve appropriately, chronic inflammation occurs in the skin regions proximal to the wound, and healing is impeded⁵. Importantly, chronic skin inflammation is also the pathological manifestation of many lesional type skin diseases, including eczema, contact dermatitis and psoriasis⁶⁹⁻⁷².

In particular, psoriasis is an interesting disease case study because it is a chronic inflammatory skin disease with both epidermal and immune implications. With a high global incidence of 2%, psoriasis is one of the most prevalent skin diseases. Psoriasis is symptomized by the unsightly appearance of demarcated erythematous plaques covered with silvery white scales (Fig V) on the skin of predisposed body sites, including the elbows, knees, umbilicus and lumbar area^{71,73}. The entire skin surface is not usually lesional depending on the severity. The affected skin regions are usually referred to as the 'involved skin,' and the normal skin is termed 'uninvolved'.⁷⁴ The vasculature in the psoriatic dermis is morphologically distorted, taking the form of large, tortuous blood vessels that give the erythematous appearance or redness of the affected skin regions/psoriatic plaques⁷³. The psoriatic skin lesions are also significantly infiltrated by immune cells. This immune cell infiltrate has a characteristic distribution and is composed mainly of dendritic cells and macrophages in the dermis, neutrophils in the epidermis and T cells in both skin layers^{75,76}.

Fig V. Psoriatic lesions. Psoriatic lesions appear as demarcated erythematous plaques covered with silvery white scales. (Picture (top) is adapted from Psoriasis-Research.com) (Picture (bottom) is adapted from Skin Care Guide.CA)

Although much research has been performed to study psoriasis amid many challenges, the disease mechanism remains debatable. On the surface, psoriasis appears to be an inflammatory skin condition in which both epidermal and immune pathologies are involved⁷⁷. Theoretically,

either the epidermal or immune basis could equally contribute to the disease manifestation, complicating the dissection of the root cause of the disease^{77,78}. Another key difficulty comes with the fact that psoriasis does not naturally occur in animals, so most of the rodent psoriasis models that have been previously used are either existing spontaneous mice mutants or transgenic animals found to have psoriasiform-like features⁷⁹. Therefore, these models would never truly represent the actual human disease. In fact, because the psoriasiform features can be largely recapitulated in mice only with perturbations to their adaptive immune system, psoriasis has been termed and well regarded as a T cell-mediated autoimmune disease⁸⁰. In addition, a substantial number of psoriasis patients develop an extracutaneous psoriatic arthritic condition that is attributed to the systemic effects of the T cell-mediated responses⁸¹. Nevertheless, an epidermal trigger of the disease can be ruled out, and the question remains as to how the adaptive immune system is originally aggravated to induce psoriasis. Moreover, as clearly displayed by its cutaneous symptoms, psoriasis does indeed have very distinct epidermal changes. The psoriatic epidermis is approximately seven times thicker than the normal epidermis, and the basal and suprabasal keratinocytes of the diseased epidermis have been found to be hyperproliferative (i.e., epidermal hyperplasia)⁸². Hence, the *stratum spinosum* or spiny layer of the diseased skin is much thicker than that of the normal epidermis (i.e., acanthosis). The keratinocytes of the psoriatic skin are also prematurely differentiated, as reflected by the incomplete cornification of the *stratum corneum*, the retention of nuclei (i.e., parakeratosis) and the loss of the granular layer, subsequently resulting in an abnormally thickened *stratum corneum* (i.e., hyperkeratosis). Premature differentiation and incomplete cornification hinder desquamation. As a result, the psoriatic skin is scaly and flaky because the abnormal *stratum corneum* peels off in large sheets rather than by the single-cell shedding typical of normal desquamation⁸³. In addition, the nails of

most psoriasis patients are also affected with pitting and separation from the nail bed. Because nails are epidermal extensions derived from the epidermal expression of keratin, these changes reinforce the epidermal basis of psoriasis⁸⁴.

1.1.4.2) Koebner phenomenon

In 1877, German physician Heinrich Koebner observed psoriatic lesions at the wounded, uninvolved skin areas of a patient bitten by a horse. The psoriatic lesion that formed around the wound was uniquely shaped according to the outline of the bite wound. This classical observation is famously named after its discoverer as the Koebner phenomenon⁸⁵. With more clinical observations over time, the Koebner phenomenon expanded to more accurately and extensively include many other forms of skin injuries. These include insect bites, friction, pressure wounds, excoriation (e.g., shaved skin), burns, contact dermatitis, chemical irritation, infections, tattoos and photosensitivity (e.g., sunburns)⁸⁵. Indeed, psoriatic lesions have been frequently observed at the edges of the wounds on the uninvolved skin (Fig VI). These lesions often form 10-20 days post wounding, corresponding to the period of the wound-healing process⁸⁵. The onset, course and progression of psoriasis are unpredictable, leading to the important question of what triggers plaque formation in the previously normal-appearing, uninvolved skin. If the underlying cause of psoriasis is genetic or immune, then it is reasonable that the occurrence of lesions should be widespread because every skin cell would possess the mutation, or the systemic effects of the adaptive immune response would extensively damage the skin. However, the partial occurrence of the lesions more likely suggests that an additional trigger is needed to initiate the formation of lesions in the disease. Given that wounding

predictably triggers psoriasis plaque formation, as observed in the Koebner phenomenon, the trigger is most likely found in the wounding.

Fig VI. Koebner phenomenon. Scaly silvery psoriatic plaques forming around an incisional wound. (Picture adapted from Pediatrics Department, University of Wisconsin School of Medicine and Public Health)

Because wounding triggers psoriasis, much research has been focused on the identification of similarities between these two events. It was found that the wound microenvironment is largely similar to that of the psoriatic lesion⁸⁶. Both are highly inflamed, probably due to the exposure to the proinflammatory cytokines (e.g., TNF- α and IL-1) that are abundantly present.

Interestingly, the differentiation statuses of keratinocytes in the suprabasal layers of the wound epithelial and psoriatic epidermis are strikingly similar. Both show the up-regulated expression of keratin 6 and keratin 16 and the down-regulation of the normally expressed keratin 1 and keratin 10. Interestingly, keratin 6 and keratin 16 are found in both lesional and uninvolved psoriatic epidermis but are absent in the healthy epidermis. Furthermore, keratin 6 and keratin 16 are well known epidermal hyperproliferative markers and are relevant to the proliferative state of the wound epithelium and the hyperplasia of psoriatic epidermis^{87,88}. Wound healing studies have revealed that proinflammatory cytokines induce the up-regulation of the nuclear receptor PPAR β/δ in the wound keratinocytes. PPAR β/δ heterodimerizes with the retinoid X receptor

(RXR) and binds to the promoter region of its target genes. Upon endogenous ligand binding and activation of this heterodimer, the expression of target genes is hence initiated. It has also been shown that proinflammatory cytokines induce the up-regulation of the endogenous ligand of PPAR β/δ , hence enhancing the transcriptional activity of PPAR β/δ . This explains the unique differentiation pattern of the wound epithelium, as PPAR β/δ activity is responsible for the specific changes in the wound epidermis⁸⁹⁻⁹¹. The expression level of PPAR β/δ was also found to be similarly up-regulated in the psoriatic lesions, presumably explaining the biological similarity between the wounded and psoriatic skin tissues⁹².

These observations clearly show that the keratinocytes in the wounded and diseased tissues behave similarly in response to the same proinflammatory environment. It is thus important to evaluate the biological effects of inflammation on the keratinocytes to better evaluate the cause of psoriasis.

1.1.5) ROS

An important biological similarity between the wounded skin and psoriatic tissue is the elevated levels of ROS^{43,93}. ROS is the general term for molecules and free radicals (chemical species with one unpaired electron) derived from molecular oxygen. ROS result from the stepwise monovalent reduction of oxygen, usually produced from the leakage of electrons to molecular oxygen in the mitochondrial respiratory chain during ATP synthesis. In this manner, oxygen gains one electron and is reduced to the superoxide anion ($O_2^{\cdot-}$). *In vivo*, superoxide can be produced by enzymatic or non-enzymatic means. The enzymes producing superoxide are cell membrane-bound and include the reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and cytochrome P450-dependent oxygenase. Without enzymatic help, superoxide production can still occur with the direct transfer of a single electron to oxygen from

reduced coenzymes or prosthetic groups (e.g., flavins or iron sulfur clusters). Superoxide is the precursor of most ROS, and its subsequent reduction or dismutation leads to the production of hydrogen peroxide (H_2O_2). Dismutation can happen spontaneously or with the catalytic help of superoxide dismutase (SOD). H_2O_2 is then partially reduced to a hydroxyl radical ($\text{OH}\bullet$) or completely reduced to water. The hydroxyl radical is perhaps the strongest natural source of oxidants. In essence, $\text{O}_2^-\bullet$, H_2O_2 and $\text{OH}\bullet$ are the relatively stable intermediates from these monovalent reduction processes and are collectively termed ROS^{94,95}.

Given their highly reactive nature, ROS can capably damage all forms of biomolecules (i.e., lipids, nucleic acids and proteins). The ROS react with membrane phospholipids through lipid peroxidation reactions, damaging the membrane integrity. ROS damage to proteins results in even more drastic outcomes, including site-specific amino acid modifications, peptide chain fragmentation, cross-linking, altered isoelectric points and increased susceptibility to proteolysis. As such, ROS can hinder cellular function by severely disrupting protein function. DNA damage by ROS includes base degradation, single-strand breakage and the disruptive cross-linking to proteins⁹⁶.

1.1.5.1) ROS regulation through antioxidant defense systems

The highly reactive nature of ROS implies that their production needs be tightly controlled to prevent unnecessary damage to tissues through a network of regulatory pathways.⁹⁷ In addition, several antioxidant mechanisms also function in tissues to counter the oxidative stress arising from ROS. The two major strategies used are non-enzymatic or enzymatic. The non-enzymatic protection against oxidative stress involves small anti-oxidant molecules, such as vitamin E, vitamin C, glutathione (GSH), coenzyme Q and bilirubin, to quench ROS. During the enzymatic oxidative protection, the enzymes involved include the previously mentioned superoxide

dismutase for the removal of superoxide and glutathione peroxidase and catalase for the detoxification of hydrogen peroxide^{97,98}.

SODs belong to a family of metalloenzymes. Three types of SOD are involved in superoxide detoxification and are each found in a different cellular compartment. SOD1 is the variant in the cytoplasm and contains copper and zinc in its active site. An isozyme of SOD1 is also present in the intermembrane space of the mitochondria. SOD2 is found in the mitochondrial matrix and contains manganese at the active site. SOD3 is found in the extracellular space⁹⁷.

Hydrogen peroxide, the dismutation product of superoxide and the main precursor of the hydroxyl radical, is mainly degraded by the enzyme glutathione peroxidase, which is largely present and active in the liver. To a lesser extent, hydrogen peroxide is removed by the enzyme catalase, which is present in cytoplasmic peroxisomes⁹⁷.

1.1.5.2) Dysregulated ROS production and disease

Although natural anti-oxidant mechanisms are present in cells to counter oxidative stress, deregulated and elevated ROS production has been a widely accepted cause or promoter of malignancy and chronic inflammatory disease.

Most of the disease pathogeneses involving ROS are explained in terms of the destructive effects of ROS on biomolecules. In the case of the chronic inflammatory diseases leading to cancer, such as chronic hepatitis, cystic fibrosis and several autoimmune diseases, increased oxidative DNA damage has been observed. Similar forms of oxidative DNA damage are also found in cancerous tissues, which are well known to have up-regulated ROS levels. A typical example of oxidative DNA damage leading to mutations would be the guanine to thymidine (G to T) transversions. In fact, oxidative DNA damage is believed to be the cause of inflammatory breast cancer and hepatocellular carcinoma development because G to T transversions are known to

accumulate in these malignancies. In this manner, ROS acts as a carcinogen by generating mutations for malignant transformations⁹⁹⁻¹⁰¹.

Furthermore, oxidative stress is also implicated in a broad variety of clinical conditions, including neurodegenerative diseases, diabetes, atherosclerosis, viral infection and psoriasis^{93,102-106}. An example of a disease pathogenesis involving ROS would be that of neurodegenerative diseases. Neurodegenerative diseases result from the death and damage of specific brain regions or the central nervous system. The brain is a lipid-rich organ and thus particularly susceptible to the damaging effects of ROS because oxygen is significantly more soluble in lipids than in the aqueous phase. Furthermore, ROS production is easily elevated in the brain, which is also a highly metabolic environment^{107,108}. In fact, oxidative ROS damage has been reported in the areas of selective neurodegeneration in Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (ALS). These damages include protein oxidation in the hippocampus and neocortex of patients with Alzheimer's disease, in the Lewy bodies in Parkinson's disease and within the motor neurons in ALS¹⁰⁹⁻¹¹¹. Lipid peroxidation is also involved in the damage of the hippocampus and neocortex of patients with Alzheimer's disease, the substantia nigra of patients with Parkinson's disease and the spinal fluid of ALS patients¹⁰⁹⁻¹¹³. In addition to the degradative effects of ROS on biomolecules, brain damage can also occur through the induction of apoptosis and necrosis in astrocytes and neurons. An example would be the excitotoxic neuronal cell death induced through the calcium-related bursts of ROS production by the mitochondria¹¹⁴.

Chronic hepatitis is also an example of a ROS-linked disease pathogenesis. As an inflammatory liver disease, chronic hepatitis leads to liver fibrosis and hardening (i.e., cirrhosis). Essentially, liver transformation occurs during hepatitis and leads to a hardened fibrotic liver characterized

by massive collagen deposition. Chronic hepatitis, whether viral- (i.e., Hepatitis B and C) or alcohol-induced, has been associated with increased ROS levels in the liver¹¹⁵⁻¹¹⁸. Increased oxidative stress in the liver is gauged by the accumulation of cellular pro-oxidants, such as iron and nitric oxide^{119,120}. Viral infection or alcohol consumption are both known to disrupt the balance of the intra-hepatic pro- and anti-oxidant levels, mainly by increasing the hepatic iron content and inhibiting the synthesis of antioxidant enzymes (e.g., SODs)^{121,122}. Hepatic iron overload has been shown to be a trigger of liver fibrosis by *in vivo* experimental studies, thus reinforcing the contribution of the elevated hepatic oxidative stress to liver transformation¹²¹⁻¹²⁴. Furthermore, in addition to its ability to damage DNA, ROS is also believed to promote viral replication and activate oncogenic transcription factors, thereby leading to hepatocellular carcinoma, which is a common complication of viral hepatitis¹¹⁵.

Given that ROS is heavily implicated in many untreatable degenerative and chronic inflammatory diseases, it is thus important to investigate the cellular mechanisms of ROS regulation in the hope of treating these diseases.

1.1.5.3) Epidermal ROS production

Although cells and tissues have the antioxidant features to counter ROS-induced oxidative stress, it can be inferred through the prevalence of oxidative stress-induced diseases that these protective measures may not always be sufficiently up-regulated in the event of oxidative stress.

In the skin, ROS is usually elevated during wounding because the immune cells exploit the destructive property of ROS to defend against the invading pathogens. During the inflammatory phase of wound healing, neutrophils and macrophages accumulate at the wound site within hours of the injury, as mentioned previously. These phagocytic immune cells rapidly release high levels of superoxide radicals through the process of the “respiratory burst” via the NADPH

present in the plasma and phagosomal membranes of the phagocytic cells^{125,126}. Despite these high levels of ROS production, the wound tissue usually heals properly without much complication, presumably due to the optimally up-regulated anti-oxidant defenses in the keratinocytes of the wound epidermis. SOD mRNA and protein levels were found to be up-regulated particularly in the basal and suprabasal cells of the epidermis during wounding, especially in the early stages of wound repair when the oxidative burst occurs¹²⁷. This suggests that the keratinocytes have an exceptionally better defense against ROS. However, the underlying signaling regulation responsible for this ability of the keratinocytes remains unknown.

In addition to responding to immune cells, keratinocytes are also known to generate ROS in response to proinflammatory and stress triggers, such as UV induction. This is crucial for the protective role of the epidermis, as the superficially positioned keratinocytes are often subjected to UV damage¹²⁸. However, in normal circumstances, because keratinocytes are more efficient in their anti-oxidant response, ROS production is usually masked and suppressed. It was recently found that the knockout of TAK1 in mice leads to severe skin inflammation and animal death before postnatal day 7. This skin inflammation was characterized by massive keratinocyte death, which was later shown to result from TNF-induced ROS production, which was significantly elevated in the TAK1-knockout keratinocytes¹²⁹. It is likely that the protective role of keratinocytes against ROS is mediated through TAK1; however, little is known about the mechanism of this regulation.

1.1.6) TAK1

TAK1 is a signaling intermediate involved in immunological and developmental processes in many species. As suggested by its name, TAK1 is a kinase downstream of developmental morphogens, TGF- β and bone morphogenetic protein (BMP) in mammalian cells. However, TAK1 is regarded as a non-canonical downstream effector of TGF- β , which canonically signals through the Smad proteins. TAK1 was first discovered through a complementation screen of a murine cDNA library, in which it rescued a mitogen-activated protein kinase kinase kinase (MAPKKK)-deficient yeast strain, hence its classification as a serine/threonine kinase in the MAPKKK family^{130,131}. Subsequently, protein orthologs of TAK1 have also been identified in many other species, including *Xenopus*, zebrafish, *Drosophila*, *C. elegans* and humans. The developmental role of TAK1 is exemplified by the embryonic lethality arising from defects in vascularization, angiogenesis and smooth muscle formation in TAK1-knockout mice. This developmental role of TAK1 is not limited to mammalian systems, as TAK1 is also implicated in the processes of endoderm specification, axis formation and vasculature development in the classical model organisms *C. elegans*, *Xenopus* and zebrafish¹³¹.

1.1.6.1) TAK1 and inflammation

The immunological role of TAK1 is also applicable across different species. In *Drosophila*, TAK1 is involved in innate immune responses, in which it is required for the expression of antimicrobial peptides. The signaling role of TAK1 is unique in the sense that it does not fit into a clearly defined linear molecular pathway, but rather TAK1 responds to a wide variety of cytokine signals. Therefore, in both developmental and immunological signaling, TAK1 may be triggered by different growth factors or cytokines to evoke similar or different biological

outcomes. The inflamed wound microenvironment is a good example of the TAK1 signaling response to multiple distinct triggers¹³¹.

The microenvironment of a wound or any chronically inflamed tissue, including psoriatic skin lesions is concentrated with proinflammatory cytokines (e.g., TNF- α , IL-1) released by the assembled immune cell infiltrates. If the wound is infected, the presence of additional pathogenic factors, such as bacterial components (e.g., lipopolysaccharide (LPS), peptidoglycan and flagellin), also categorized as pathogen-associated molecular patterns (PAMPs), also initiate and promote inflammation. These proinflammatory molecules bind to their respective receptors on the membrane of the immune and tissue epithelial cells to invoke their respective innate immune responses, including inflammation, in mammalian systems¹³²⁻¹³⁴. For example, TNF- α binds to its respective TNF receptors 1 and 2, which dimerize upon ligand binding; IL-1 binds to the IL-1 receptor (IL-1R) to induce inflammation¹³⁵. Different PAMPs are recognized by exclusive members of the Toll-like receptor (TLR) superfamily¹³². Regardless of the proinflammatory signal type (TNF- α , IL-1, PAMPs), the resulting intracellular signaling response generally activates the transcription factors nuclear factor- κ B (NF- κ B) and AP-1, thereby leading to the expression of inflammation-related genes. Importantly, TNF- α is one of the gene products of NF- κ B and AP-1 transcriptional activity regardless of the proinflammatory trigger. Therefore, TNF- α can be positively up-regulated by itself or via other proinflammatory factors (e.g., IL-1, LPS) during inflammation¹³⁶.

1.1.6.2) TNF- α signaling in cell death

Human TNF- α is encoded by chromosome 6 and is a 17-kDa protein composed of 157 amino acids¹³⁷. In solution, TNF- α exists as a homotrimer¹³⁸. In addition to its main immune response-

based production by activated macrophages, T lymphocytes and natural killer cells, TNF- α also triggers signaling pathways leading to the activation of transcription factors, including nuclear factor κ B (NF κ B), AP-1 and CCAAT enhancer-binding protein β ^{139,140}. Physiologically, the homotrimeric TNF- α binds TNFR-1, which correspondingly leads to its trimerization. In the skin, a low level of TNF- α is constitutively expressed by the keratinocytes in the upper layer of the healthy epidermis. This keratinocyte TNF- α expression is greatly enhanced during proinflammatory events, such as injury, infection, UV irradiation and the application of contact sensitizers¹⁴¹. In cells, TNF- α is synthesized as a membrane-bound pro-form (26 kDa) that is activated and released upon the proteolytic cleavage by TNF-converting enzyme (TACE)^{142,143}. TNF- α acts through two distinct receptors, TNF receptor 1 (TNFR-1) and 2 (TNFR-2). The affinity of TNF- α for TNFR-2 is five times stronger than that of TNFR-1; however, most biological activities of TNF- α are mediated through TNFR-1. Furthermore, TNFR-1 is expressed in every cell type, whereas TNFR-2 is mostly restricted to immune cells, suggesting the different responses of immune cells and non-immune cells towards the same pro-inflammatory cytokine¹³⁵. The skin keratinocytes mainly express TNFR-1¹⁴¹. Another key difference between both receptors is that a death domain (DD) is present in TNFR-1 but not TNFR-2¹⁴⁴. Therefore, TNFR-1 belongs to the death receptor family whereby its signaling can lead to apoptotic cell death. However, TNFR-1 is reportedly a dual role receptor, implying it has the ability to induce both apoptotic death and survival¹⁴⁵. However, this life-or-death signaling regulation remains poorly understood. To modulate the diverse processes of cell growth and death, inflammation and stress responses, TNF- α binds to TNFR-1 and the silencer of death domain (SODD) protein, an inhibitor of the DD, is thereby released from TNFR-1. Then, TNFR-associated death domain (TRADD) binds to the DD of TNFR-1. This facilitates the recruitment of other adaptor proteins,

such as Fas-associated death domain (FADD), TNFR-associated factor-2 (TRAF-2) and TNFR-associated factor-6 (TRAF-6). These adaptor proteins in turn recruit key molecules responsible for the subsequent intracellular signaling. To promote apoptosis through the TNF- α binding to TNFR-1, FADD is recruited to the receptor complex, where it binds and activates pro-caspase-8. The activated caspase 8 then cleaves and activates pro-caspase-3, which in turn leads to downstream apoptotic events. Ultimately, apoptotic signaling also leads to the activation of endonucleases, which fragment the nuclear DNA, a main feature of apoptosis¹⁴⁶.

1.1.6.3) The prosurvival role of TAK1 in TNF- α signaling

The binding of TNF- α to TNFR-1 leads to diverging signaling outcomes. Whereas signaling through FADD leads to apoptosis, as mentioned above, parallel signaling through TRAF-2 and TRAF-6 leads to antiapoptotic, proliferative effects. A key downstream mediator that binds TRAF-2 and TRAF-6 to mediate this opposing effect is TAK1¹⁴⁷.

As a principal mediator of innate immunity, TAK1 serves as a key intermediate in the proinflammatory pathways initiated by TNF- α and interleukin (IL)-1.¹⁴⁸ As a member of the MAPK kinase kinase family, TAK1 can activate c-Jun N-terminal kinase (JNK) and p38 MAPK through the stimulation of its downstream MAPK cascade.¹⁴⁹ TAK1 also stimulates the degradation of I kappa B α (I κ B α), leading to the activation of NF- κ B. In the skin, these downstream mediators of TAK1 are important for the maintenance of epidermal homeostasis.^{150,151} Perturbations to the JNK pathway have been implicated in epidermal hyperplasia leading to cancer development, whereas reduced NF- κ B function is linked to epidermal squamous cell carcinoma. Blocking the NF- κ B pathway through inhibitor of I κ B kinase (IKK β or IKK γ) inactivation also leads to severe skin inflammation.^{152,153} Mice with a

keratinocyte-specific deletion of TAK1 develop severe skin inflammation, characterized by massive cellular apoptosis, and die at postnatal day 7.^{154,155} The histological examination of the TAK1-null mouse skin revealed a deregulated differentiation and increased keratinocyte proliferation, similar to that observed in TAK1-deficient human keratinocytes. Given the central role that TAK1 plays in inflammation, it has recently been implicated in ROS production. Indeed, the TAK1-deficient mouse keratinocytes are more susceptible to the TNF- α -mediated apoptosis associated with increased ROS production, which can be abrogated by a c-Jun-dependent mechanism.¹⁵⁶ Taken together, these observations suggest that TAK1 plays a homeostatic role in modulating ROS production in wound healing, but the precise underlying mechanisms remain unknown.

1.1.7) Aim of present study

TNF- α signaling has been implicated in chronic inflammation and malignancy, however its role is not exactly clear cut, often times demonstrating opposing functionality. While the diverging of TNF- α /TNFR1's downstream signaling pathways best explains the dual role nature of TNF- α /TNFR1 signaling, it also implies that the modulation of specific downstream players may determine the fate of TNF- α /TNFR1 signaling. The observation of epidermal TAK1 deficiency leading to chronic inflammation and massive keratinocyte death attributed to ROS up-regulation suggests an innate protective role of TAK1 against ROS induced oxidative stress which as previously mentioned is highly detrimental towards cell survival. We attempt to elucidate the mechanism of this protective role of TAK1 in human keratinocytes, which will shed light on inflammatory control and also reveal new perspectives for cancer research.

1.2 MATERIALS AND METHODS

1.2.1) Reagents

Antibodies purchased include TAK1 (Upstate Biotechnology) for immunofluorescence and TAK1, caspase-3, phospho(T183)-JNK, phospho(Y721)c-Kit, phospho(S473)PKB α , p(S9)GSK-3 β , total JNK, c-FLIP, c-Kit (Cell Signaling), involucrin (Novocastra), β -tubulin, PCNA, phospho-c-Jun, and secondary HRP-conjugated antibodies (Santa Cruz Biotechnology) for immunoblot. Neutralizing anti-SCF antibody and recombinant SCF were from Peprotech. Collagen Type I Rat Tail was from BD Biosciences. Human primary fibroblasts, keratinocytes and corresponding culture medium were purchased from Cascade Biologics. Transfection reagent ExGen 500 was purchased from Fermentas. Double-promoter lentivirus-based siRNA vector and pFIV packaging kit were acquired from System Biosciences. All restriction enzymes and DNA/RNA-modifying enzymes were obtained from Fermentas. Kinase inhibitors BAY 11-7082, SN50, 1,9-pyrazoloanthrone, [2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one] and PD98059 were acquired from Merck. PI3K inhibitor LY294002 was purchased from Cayman Chemical. All other chemicals were bought from Sigma-Aldrich.

1.2.2) Full-thickness wound induction, biopsy, staining

6 weeks old C57BL6J mice were anaesthetized prior to the shaving of dorsal hair. A 0.2 x 0.2 cm² area of dorsal skin was inflicted on both the left and right lower-dorsum of each mouse using surgical scissors and forceps. Wounded mouse was separately caged and induced wound was left exposed to air. At days 1, 3, 7 and 10 post wounding, 0.5 x 0.5 cm² area of wound biopsies sample were excised. Samples were frozen immediately in liquid nitrogen and stored in -80 °C for total RNA/protein extraction. For staining purposes, the wound biopsy was cut into

half and separately embedded in Tissue-Tek OCT compound medium (Sakura, Leica), frozen in liquid nitrogen and stored at -80 °C. Cryosections of 3 µm thickness were obtained using cryostat microtome (Leica CM 3050S) and slides were stored in -20 °C for subsequent analysis. The 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (CM-H₂DCFDA; herein referred to as DCF) (Invitrogen, USA) was used for ROS quantification. DCF is cell permeable and non-fluorescent until its oxidation by intracellular ROS. During dye loading, the dye diffuses freely into the cell and is trapped upon oxidation. This trapped fluorescent oxidized dye possesses absorption and emission spectra of 492-495 nm and 517-527nm respectively. For ROS detection staining, it is important that wound biopsies are not fixed prior to staining. Cryosections were washed with phosphate buffered saline (PBS) for 15 minutes prior to DCF staining. The sections were treated with 10 µM DCF dissolved in PBS and incubated at 37 °C for 30 minutes. The slides were washed with PBS and then mounted with coverslips. Images were taken using a Carl Zeiss LSM 710 META confocal laser scanning microscope and ZEN 2009 software. As for hematoxylin and eosin (H & E) staining, cryosections were fixed in 4 % paraformaldehyde for 10 min, following 1 x rinsing with PBS. Sections were immersed with hematoxylin solution modified according to Gill III (Merck, Germany) for 5 min and rinsed in 0.1 % HCl for 2 sec. Rinsing was continued with gentle running water for 5 sec. Finally slides were mounted with 90 % glycerol in 1 x PBS. Stained sections were viewed and images captured using Eclipse 90i microscope Nikon and Q-Capture Pro program respectively.

1.2.3) Cell culture conditions and organotypic skin culture (OTC).

Human dermal fibroblasts and keratinocytes were routinely cultured in a 37 °C, 5% CO₂, humidified incubator. Organotypic skin cultures were constructed as described in Fig VII.¹⁵¹ Briefly, a fibroblast density of 1 x 10⁵ cells/ml of collagen was used to reconstruct OTCs. The

air-exposed process was performed in a 37°C incubator with 5% CO₂ and 70% humidity. The OTC was cultured for another 14 days, changing the medium every 2 days. Inhibitors, agonists and antagonists were added into the OTC medium. Serum-free OTC medium was composed of DMEM:Ham's F12 in 3:1 volume ratio, 1 mg/ml fatty acid free albumin, 10 µM carnitine, 0.4 µg/ml hydrocortisone, 1 µM isoproterenol, 1 µM α-tocopherol, 5 µM linoleic acid, 7 µM arachidonic acid, 25 µM oleic acid, 50 µg/ml ascorbic acid, 10 µM serine, 100 nM adenine, 100 nM cholera toxin, 2 ng/ml epidermal growth factor (EGF), and 2 ng/ml TGF-α. OTCs were kept at 37°C, 5% CO₂ with a relative humidity of 70%.

Fig VII. Constructing the skin organotypic coculture (OTC). The OTC is an *in vitro* skin model where nourishment from the defined medium can only reach the epidermal layer via diffusion through the fibroblast/collagen matrix (white arrows), simulating the *in vivo* environment. **(A)** A mixture of 80% (by volume) of 3.89 mg/ml Collagen Type I, Rat Tail (BD Biosciences, USA) and 10% of 10X Hanks' balanced salt solution (HBSS) containing phenol red was maintained on ice and the acetic acid solubilized collagen was neutralized using 1 N NaOH through titration. Fibroblasts from culture flasks were constituted in 1X HBSS and added to the mixture, with constant stirring, to make up the remaining 10% of volume. A cell density of 5×10^4 per ml was achieved. **(B)** 3 ml of this mixture was added into each insert (Transwell® Permeable Supports [Corning, USA]) and incubated at 37 °C with 5% CO₂ for 1 h to achieve gelation. **(C)** Inserts were transferred to a 6-well Deep-Well Plate (BD BioCoat™, USA); glass rings were centrally placed onto the collagen matrices and the setup was incubated under the same conditions for 1 h. Following that, OTC medium was added to a level which submerged the collagen matrices, and the plate was incubated at 37 °C with 5% CO₂ for 24 h. **(D)** Medium was aspirated and keratinocytes were seeded into the centre of the glass ring (10^6 cells per insert). OTC medium was added to air-liquid interface and the setup was once again incubated at 37 °C with 5% CO₂ for 24 h to allow the keratinocytes to attach. **(E)** Rings were removed and the medium was replaced. **(F)** Medium was changed every 2 days for a total of 14 days.

1.2.4) Lentivirus siRNA constructs and transduction.

The sequences of TAK1 and control siRNA are shown in Table 1.1. The siRNAs were cloned into the pFIV-HI/U6 siRNA vector according to the manufacturer's protocol (System Biosciences). Positive clones were identified by PCR and by DNA sequencing. The pFIV/siRNA and packaging plasmids were transfected into HEK 293T cells using TransTL293. 10^5 cells were seeded into each well of a 6-well plate in complete medium. Medium was replaced for the seeded HEK 293T cells before transfection. 12 μ l of TransTL293 reagent was added to 200 μ l of serum free DMEM medium followed by incubation at room temperature for 20 minutes. 2 μ g of DNA comprising of equimolar amounts of pFIV/siRNA, pFIV-34N and pVSV-G respectively were added to this incubated mixture. The resultant mixture is then incubated at room temperature for 30 minutes and added drop wise to a single well of HEK 293T cells. The culture was then incubated for 48 h at conditions of 37 °C and 5% CO₂. The respective medium for each well was then collected and composed with 12 % polyethylene glycol 8000 (PEG) using working stocks dissolved in PBS. This mixture was incubated overnight in ice to allow for conjugation of pseudoviral particles to PEG. The PEG bound pseudoviral particles were then collected via a 4000 x g centrifugation and transduced into A-5RT3 cells in the presence of polybrene (10 μ g/ml). Successfully transduced cells were selected with 350 ng/ml puromycin for a week and further subjected to western blot and qPCR quantitation of knockdown efficiency.

Table 1.1- siRNA sequences

TAK knockdown forward primer	AAAGTGGCTTATCTTACACTGGA
TAK knockdown reverse primer	AAAATCCAGTGTAAGATAAGCCA
Control Knockdown forward sequence	GCAGCTTAGAATCCGTTGAATTA
Control Knockdown reverse sequence	GTACCAAGGAATAACGAATACAT

1.2.5) Total RNA isolation and real-time quantitative PCR (qPCR).

Total RNA was isolated from cells using a PureLink™ RNA kit (Invitrogen) following the supplier's protocol. Total RNA (2.5 µg) was reverse-transcribed with oligo-dT primers using RevertAid™ H Minus M-MuLV (Fermentas). Real-time PCR was performed with KAPA SYBR Fast qPCR (KAPABiosystem). Melt curve analysis was included to assure that only one PCR product was formed. Expression was normalized to the control gene ribosomal L27, which did not change under any of the experimental conditions studied. The sequences of primers are shown in Table 1.2.

Table 1.2- qPCR Primer sequences used in this study.

SCF qPCR primer 1	GGGATCTGCAGGAATCGTG
SCF qPCR primer 2	ACAATGCTGGCAATGCCATG
PDGF-AA qPCR primer 1	CGGATACCTCGCCCATGTTCT
PDGF-AA qPCR primer 2	TCCGGATTCAGGCTTGTGGTTCG
L27 qPCR primer 1	TGATGGCACCTCAGATCGC
L27 qPCR primer 2	AGAGTACCTTGTGGGCATTAGG

1.2.6) Transient transfection and transactivation assay.

TAK1 cDNA was subcloned into pCMV5 mammalian expression vector (Stratagene). A 983-bp (-853 to +130) promoter of the human SCF gene was PCR-amplified from human genomic DNA using *Pfu* polymerase.¹⁵⁷ The resulting fragment was subcloned into pGL-3 basic luciferase reporter vector (Promega). Site-directed mutagenesis of the AP-1 binding site at -573 was performed using QuikChange Site-Directed Mutagenesis kit (Stratagene). Primer sequences for PCR are given in Table 1.3. Keratinocytes were cotransfected with a luciferase reporter driven by the SCF promoter construct, cDNAs encoding for various kinases and pEF1-β-galactosidase

as a control for transfection efficiency. The constitutively active (ca) PKA, caMEK1 and caMEKK1 were from Clontech. The caMKK7 was from Cell BioLabs. The various TAK1, caSEK1, caJNK, caSGK and caIKK expression vectors were from E. Nishida (Kyoto University, Japan), R.J. Davis (University of Massachusetts Medical School, Worcester, MA), D.J. Templeton (University of Virginia Medical School, Charlottesville, VA) and D.V. Goeddel (Tularik, Inc). After transfection, cells were cultured for 48 h prior to lysis. Luciferase activity was measured using the Promega luciferase assay on a Microbeta Trilux (Perkin Elmer). β -galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl- β D-galactopyranoside as a substrate. The expression vector containing dominant-negative AP-1 (TAM67) was transfected in increasing doses into K_{CTRL}, and the expression level of SCF mRNA was determined by qPCR.

Table 1.3- PCR Primers used

phSCF promoter forward	AGATCTACACTAATAGATTTGATG
phSCF promoter reverse	TTTAGCTGTTCTGGAGCTCCAGCA

1.2.7) Electrophoretic mobility shift assay (EMSA).

Non-radioactive EMSA was performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). The AP-1 (-573) binding site of the human SCF promoter was examined.¹⁵⁷

1.2.8) Protein array analysis and enzyme-linked immunosorbent assay (ELISA).

A growth factor array membrane was processed according to the manufacturer's protocol (RayBiotech). Serum-free conditioned medium (1 ml) or 0.5 mg cell lysate proteins were used. Protein spots were detected by chemiluminescence. Signal intensities were quantified using ImageJ analysis software and were normalized to the mean intensity of the positive controls on

each membrane. The concentrations of growth factors were measured in culture supernatants using sandwich ELISA according to the manufacturer's instruction (R&D Systems).

1.2.9) Immunofluorescence and TUNEL assay.

Tissues were fixed with 4% paraformaldehyde in PBS for 2 h at room temperature, washed twice with PBS and embedded in Tissue-Tek OCT compound medium (Sakura, Leica). Eight-micrometer cryosections were processed for immunofluorescence as followed: 1 × PBS, 0.1% BSA/30 min/RT; primary antibodies in 1 × PBS buffer, 0.1% BSA/2 h/RT; washings in 1 × PBS buffer; FITC-conjugated IgG secondary antibody in 1 × PBS buffer, 0.1% BSA/1 h/RT. The slides were subsequently washed and mounted before microscopic observation. Apoptotic cells were detected using the TUNEL assay according to the manufacturer's protocol (Roche). As positive control for the TUNEL assay, the section was pre-treated with DNase I. The slides were counterstained with DAPI and mounted for microscopic observation. Images were taken using an LSM510 META confocal laser scanning microscope with a Plan-Apochromat 63x/1.40 Oil objective and ZEN 2009 software (Carl Zeiss).

1.2.10) Chromatin immunoprecipitation (ChIP).

In vivo ChIP was carried out as previously described, except anti-phospho-c-Jun antibody was used.¹⁵⁸ The putative AP-1 binding site in the human SCF promoter was previously described.¹⁵⁷

Primers used for ChIP are given in Table 1.4.

Table 1.4- ChIP primer sequences used

hSCF AP-1 chIP forward	AGACACAGTAACAAACCATTC
hSCF AP-1 chIP reverse	GAAATACCTCGCGCGCCAGA

1.2.11) Apoptosis treatments, ROS measurement and FACS analysis.

Keratinocytes were subjected to anoikis treatment as previously described.¹⁵⁹ Keratinocytes in serum-free medium were treated with 10 ng/ml TNF- α to induce apoptosis. Treated keratinocytes were incubated with 10 μ M CM-H2DCFDA (Invitrogen) for 30 min at 37°C, harvested, and analyzed by flow cytometry (LSR II from BD Biosciences). Apoptotic cells were detected using an Annexin V/propidium iodide staining kit (BD Biosciences) according to the manufacturer's protocol. For indicated experiments, cells were treated with either vehicle, 25 μ M LY294002, 200 ng/ml anti-SCF antibody, 50 ng/ml SCF or 1 mM NAC.

1.3 RESULTS

1.3.1) ROS production in wound epithelial coincides with TAK1 expression.

We have previously provided *in vivo* evidence that the expression of TAK1 is elevated in skin wound biopsies, which peaks at days 3–7 post-injury.¹⁵⁰ To establish a comparison between wound TAK1 expression and ROS production, we evaluated ROS production in the wound epithelial by staining wound biopsies with the fluorescent dye CM-H2DCFDA (DCF). We observed that ROS production peaked at 3–7 days post-injury (Fig 1.1A, B), coincident with the pattern of wound TAK1 expression shown previously (Fig 1.1C). As a negative control to prove that the observed fluorescence results entirely from ROS reaction, we performed DCF staining in the presence of an antioxidant N-acetyl-cysteine (NAC) which completely abolished the fluorescence signal (Fig 1.1D).

Fig 1.1. ROS production is elevated during wounding.

(A) Hematoxylin and eosin (H&E) (left panel) and dichlorodihydrofluorescein diacetate (DCF) staining of ROS (right panel) in murine skin wound biopsies at the indicated days post-wounding. Scale bar, 40 μ m. Dotted white line represents epidermal–dermal junction. Representative pictures of centrally dissected wound sections are shown.

(B) Relative DCF signal intensity in murine skin wound biopsies at indicated days post-wounding. Values (mean $\pm$ S.D.; n=5) represent mean fold changes in DCF signal intensity compared to the signal from Day-1 wound biopsies, as determined using ZEN 2009 (Carl Zeiss) software.

(C) Wound TAK1 protein expression profile as determined through immunoblotting. Tubulin expression was used as a loading control. Values shown are band intensities as determined using (Image J) software.

(D) Control for dichlorodihydrofluorescein diacetate (DCF) staining of mouse wound biopsy. DCF staining of a Day-7 post-wounding tissue section in the presence of vehicle (left) or an antioxidant, N-acetyl-cysteine (NAC) (right). NAC abolished all fluorescence signal, indicating the specificity of DCF staining for ROS. Scale bar, 40 μ m. Dotted white line represents epidermal–dermal junction.

1.3.2) Epidermis of organotypic skin co-cultures constructed from TAK1 knockdown human keratinocytes found with elevated ROS production and increased apoptosis associated with the JNK pathway.

As TAK1 levels were up-regulated during wounding in response to increased ROS production, we further examined the role of TAK1 in keratinocytes towards ROS production by constructing organotypic skin co-cultures (OTCs) (Fig 1.2A) using either control (K_{CTRL}) or TAK1-knockdown human keratinocytes (K_{TAK1}) with underlying human primary dermal fibroblasts. We observed that the overall epidermal thickness between K_{CTRL} and K_{TAK1} OTCs was similar. To reveal the suprabasal layers, we performed immunofluorescence staining against involucrin, a keratinocyte terminal differentiation marker expressed only in the suprabasal layers of the epidermis and observed that the suprabasal layer of K_{TAK1} epidermis was significantly thinner than that of K_{CTRL} OTC. Notably, ROS production was elevated in the K_{TAK1} -derived epithelia, as revealed by DCF staining (Fig 1.2B). Persistently elevated ROS is known to induce cellular apoptosis in normal cells. Indeed, K_{TAK1} OTCs showed more TUNEL-positive apoptotic cells than K_{CTRL} OTCs (32.6 ± 3.5 vs 12.7 ± 4.1 TUNEL-positive cells per microscopic field, respectively; Fig 1.2B). This was further confirmed by immunodetection of cleaved caspase-3, an apoptotic marker in the OTC epithelial (Fig 1.2C). Phosphorylation-mediated activation of JNK, the downstream target of TAK1 was also found via immunodetection to be decreased in the K_{TAK1} -derived OTC epithelial (Fig 1.2C). Taken together, these observations suggest that TAK1 plays a homeostatic role in modulating ROS level in keratinocytes during wound healing associated with its downstream JNK pathway.

Fig 1.2. ROS levels and cellular apoptosis are elevated in OTC epidermis formed by TAK1-deficient keratinocytes (K_{TAK1}).

(A) Schematic diagram of OTC.

(B) H&E staining (1st top panel), anti-involucrin immunofluorescence (2nd panel), DCF staining (3rd panel) and TUNEL analysis (bottom panel) of control (K_{CTRL})- and K_{TAK1}-derived organotypic coculture (OTC) sections. OTCs were constructed using either control (K_{CTRL}) or TAK1-knockdown human keratinocytes (K_{TAK1}) with underlying human primary fibroblasts (F). and harvested after 2 weeks. Sections used for involucrin staining and TUNEL assay were counterstained with DAPI for nuclei (blue). Scale bar, 40 μm. Dotted white line represents epidermis–dermal junction.

(C) Immunodetection of JNK, phosphorylated JNK (p(T183)JNK), intact and cleaved caspase-3 in K_{CTRL} and K_{TAK1} epithelial tissues from corresponding OTCs, constructed with underlying fibroblasts. β-tubulin was used as a loading and transfer control.

1.3.3) TAK1 confers anti-apoptotic properties in a cell-autonomous fashion.

Our above analysis revealed an increased number of apoptotic keratinocytes in K_{TAK1} epidermis. To investigate whether this observation was an autocrine or paracrine event, we examined cellular apoptosis in OTCs constructed using either K_{CTRL} or K_{TAK1} with only collagen as the OTC dermis, i.e., absence of underlying fibroblasts, denoted K_{CTRL}/Col and K_{TAK1}/Col, respectively. K_{TAK1}/Col OTCs showed a stronger DCF fluorescence signal than K_{CTRL}/Col (Fig 1.3A). Similar to K_{TAK1} OTCs constructed with fibroblasts (Fig 1.2B), higher number of apoptotic cells were observed in K_{TAK1}/Col when compared with K_{CTRL}/Col (45.3 ± 5.2 vs 17.9 ± 2.3 vs TUNEL-positive cells per microscopic field, respectively; Fig 1.3A). This was supported by immunoblot analysis of cleaved caspase-3 (Fig 1.3B).

Fig 1.3. TAK1 confers anti-apoptotic properties in a cell-autonomous fashion.

(A) H&E staining (1st panel), DCF staining (2nd panel), and *in situ* TUNEL analysis (bottom panel) of K_{CTRL} and K_{TAK1} OTC. OTCs were constructed on an underlying fibroblast-free collagen layer (Col). Sections were counterstained with DAPI for nuclei (blue). Scale bar, 40 μ m. Dotted white line represents epidermal–dermal junction. Representative pictures of centrally dissected OTC sections are shown.

(B) Immunoblot analysis of cleaved caspase-3 in K_{CTRL} and K_{TAK1} epidermis from corresponding OTCs constructed with underlying collagen. β -tubulin was used as a loading and transfer control.

1.3.4) TAK1 absence enhanced keratinocyte death through ROS overproduction in both anoikis and TNF- α -induced apoptosis.

Keratinocytes face two important death challenges in their normal development or wound repair role. During epidermal stratification, basal cells detach from the basement membrane into the suprabasal layers and are hence subjected to anoikis. However suprabasal cells remain viable, implying their anoikis resistance. During the inflammatory phase of wound healing where the wound epithelial is exposed to abundant levels of proinflammatory cytokines, keratinocytes are subjected to TNF- α -induced apoptosis.¹⁶⁰ To study the role of TAK1 with regards towards these cell death triggers, we subjected K_{CTRL} and K_{TAK1} to either anoikis or TNF- α treatment and examined their ROS and apoptotic levels by FACS. DCF labeling revealed increases in ROS production of more than 35% and 55% upon anoikis and TNF- α treatment, respectively (Fig 1.4). This increased intracellular oxidative state was associated with ~50% more apoptotic K_{TAK1} cells compared with K_{CTRL} under both challenges, as determined by Annexin V staining (Fig 1.4)

Fig 1.4. TAK1 deficiency increased ROS accumulation. Representative FACS-derived histograms showing increased ROS in TAK1-knockdown (K_{TAK1}) compared to control human keratinocytes (K_{CTRL}) during anoikis or 10ng/ml of TNF- α treatment. FACS analysis of K_{TAK1} and K_{CTRL} stained with DCF (10,000 events). No difference was observed with untreated control cells. Mean fluorescence intensities, indicated in brackets, were obtained from five independent experiments. Data were analyzed using CellQuest software (Becton Dickinson). The analyzer threshold was adjusted on the flow cytometer channel to exclude most of the subcellular debris to reduce the background noise using non-DCF stained cells.

Importantly, the co-treatment with NAC to quench the effect of ROS significantly reduced the apoptotic index in K_{TAK1} (Fig 1.5). Similarly, K_{TAK1}-derived OTCs treated with NAC showed reduced TUNEL-positive apoptotic epidermal cells, reaching a number that was comparable to K_{CTRL}-derived OTCs (Fig 1.6). No significant difference was observed in mean DCF signals and apoptotic index between untreated K_{CTRL} and K_{TAK1} (Fig 1.4, 1.5). Altogether, these results point towards a cell autonomous role for TAK1 in modulating ROS production and thus protecting keratinocytes against apoptosis and TNF- α -induced apoptosis.

Fig 1.5. TAK1 deficiency increased cell death. Percentage of apoptotic K_{CTRL} and K_{TAK1} after anoikis or 20ng/ml of TNF- α treatment, in the presence either vehicle or 1 mM of NAC, as analyzed by FACS (10,000 events). The sum of Annexin V⁺/PI⁻ (early apoptosis) and Annexin V⁺/PI⁺ cells (late apoptosis) were considered apoptotic. Values (mean $\pm$ S.D., bold) denote apoptotic cells (%) from five independent experiments.

Fig 1.6. TAK1 deficient epidermis found with increased keratinocyte death. TUNEL analysis of K_{CTRL} - and K_{TAK1} -derived organotypic coculture (OTC) sections. OTCs were constructed using either K_{CTRL} or K_{TAK1} with underlying human primary fibroblasts (F), cultured in the presence of either vehicle or 1 mM NAC and harvested after 2 weeks. Sections used for TUNEL assay were counterstained with DAPI for nuclei (blue). Scale bar, 40 μ m. Dotted white line represents epidermal–dermal junction.

1.3.5) Decreased SCF expression in TAK1 knockdown keratinocytes.

Our above findings showed that TAK1-deficient keratinocytes were hypersensitive to $TNF-\alpha$ -induced apoptosis associated with elevated ROS production and that the antiapoptotic effect of TAK1 was cell-autonomous. To understand the underlying mechanism for this TAK1 action, we conducted an unbiased proteomic analysis to screen for candidate growth factors and phosphoproteins. A growth factor array approach was used; conducting subtractive comparison of medium from OTCs constructed using either K_{TAK1} or K_{CTRL} with collagen, i.e., absence of underlying fibroblasts. A total of 41 growth factors were screened, and the results revealed a significant change only in the protein expression level of SCF. This was further confirmed by ELISA on the OTC culture medium (Fig 1.7A), whereas no detectable change was observed for PDGF-AA. The expression of PDGF-AA, which showed no difference between K_{TAK1} - and K_{CTRL} -derived OTC culture medium, served as control. Real-time PCR analysis of the K_{TAK1} - and K_{CTRL} -derived epidermis from OTCs showed corresponding changes at the mRNA level (Fig 1.7B). Furthermore, the re-introduction of an expression vector harboring TAK1 cDNA that

has a silent third-codon point mutation within the siRNA target region restored the expression of SCF (Fig 1.7C).

Fig 1.7. Decreased SCF expression in TAK1 knockdown keratinocytes.

(A–B) Expression level of stem cell factor (SCF) (A) protein and (B) mRNA in human keratinocytes transduced with either control (K_{CTRL}) or TAK1 siRNA (K_{TAK1}). SCF protein levels were determined from culture medium of K_{CTRL} and K_{TAK1} by ELISA. Platelet-derived growth factor (PDGF)-AA levels were also measured as a control. Real-time qPCR revealed the relative gene expression of SCF and PDGF-AA in K_{TAK1} and K_{CTRL}. Ribosomal L27 was used for normalization. Values (mean±S.D.) were from five independent experiments.

(C) Expression levels of SCF (upper panel) and TAK1 (lower panel) proteins in K_{CTRL} and K_{TAK1} transfected with either an empty vector or an expression vector encoding a TAK1 cDNA with a silent third-codon mutation in the siRNA targeting region (upper panel). Two independent transfections are shown. SCF and TAK1 proteins were measured by ELISA and immunoblot, respectively. β-tubulin was used as a loading and transfer control.

1.3.6) SCF activates c-Kit/ PI3K/ PKBα pathway to protect keratinocytes from ROS mediated death.

It is known that SCF binds and activates its cognate receptor c-Kit through phosphorylation.¹⁶¹

Corroborating our above finding, immunodetection indicated that the expression of phosphorylated c-Kit was downregulated in K_{TAK1}-derived epidermis, while total c-Kit protein remained unchanged (Fig 1.8A). The activation of c-Kit has been shown to trigger the phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB) α pro-survival pathway.¹⁶² Indeed,

immunoblot showed that the expression of phosphorylated PKB α and its downstream targets, like glycogen synthase kinase (GSK)-3 β were reduced in K_{TAK1} compared with K_{CTRL} (Fig 1.8A). The expression level of total PKB α remained unchanged (Fig 1.8A). Increased activity of PKB α i.e., higher phosphorylation, protects keratinocytes from apoptosis.^{159,160}

To further investigate the importance of SCF/c-Kit/PKB α , we cultured K_{CTRL} and K_{TAK1} OTCs in medium supplemented with recombinant SCF. We hypothesized that the increased apoptosis observed in K_{TAK1} could be rescued by exogenous SCF. Indeed, the addition of recombinant SCF lowered the number of TUNEL-positive apoptotic K_{CTRL} and K_{TAK1} OTCs compared to vehicle-treated OTCs (Fig 1.8B). Although SCF treatment appeared to induce a trend towards more Ki67-positive proliferating cells, this effect on proliferation was not significant (Fig 1.8B). This was also confirmed by immunodetection using anti-caspase-3 and anti-PCNA antibodies (Fig 1.8C). We have previously shown that epidermal TAK1 modulates a double paracrine PDGF/HGF signaling to regulate keratinocyte proliferation that is required for proper wound healing.¹⁵⁰ Specifically, K_{TAK1}-derived epidermis showed increased proliferation when compared with K_{CTRL}-derived epidermis as a result of the paracrine induction of dermal fibroblasts by keratinocyte-secreted PDGF-B. To rule out the probable influences exerted by this paracrine PDGF-mediated pathway, anti-PDGF antibodies were added as a control. K_{TAK1} OTCs treated with anti-PDGF antibody showed reduced epidermal proliferation (PCNA), but it has no effect on apoptosis (cleaved caspase-3) (Fig 1.8C). In contrast, the treatment with recombinant SCF reduced the number of apoptotic keratinocytes (Fig 1.8B-C). Importantly, it underscored the distinct anti-apoptotic role of TAK1-mediated SCF expression. Altogether, these results indicate that SCF/c-Kit/PKB α mediated autocrine signaling plays a pivotal role in the action of TAK1 in cell survival.

Fig 1.8. SCF triggers c-Kit/ PI3K/ PKB α pathway to protect keratinocytes from ROS mediated death.

(A) Immunoblot analysis of total and phosphorylated c-Kit and its downstream mediators of the PI3K/PKB α pathway in K_{CTRL} and K_{TAK1} epidermis from OTCs constructed with underlying collagen. The level of p(Y721)c-Kit, the activated form of the SCF receptor, was measured in K_{CTRL} and K_{TAK1} epidermis. Total c-Kit, PKB α and Coomassie-stained blots were used as loading and transfer controls.

(B) Quantification of Ki67 and TUNEL-positive K_{CTRL} and K_{TAK1} in OTCs supplemented with either vehicle (PBS) as a control or recombinant SCF (rec. SCF). Mean numbers of proliferating and apoptotic cells were numerated after detection by anti-Ki67 antibody or TUNEL, respectively. Values are means from five standardized microscopic fields per section, performed on seven sections from three independent OTCs.

(C) Immunodetection of cleaved caspase-3 (apoptotic marker) and PCNA (proliferation marker) in the epithelia from K_{CTRL}- and K_{TAK1}-derived OTCs treated with either PBS (-), anti-PDGF antibody or recombinant SCF (+). β -tubulin served as a transfer and loading control. Values below each band represent the mean fold differences (n=3) in expression level compared to K_{CTRL}, which was assigned the value of 1.

1.3.7) The human SCF gene is a direct target of TAK1/JNK/c-Jun signaling in keratinocytes.

Our above results showed that TAK1's absence increased apoptosis, likely due to a diminished SCF expression and thus the activation of the c-Kit/ PI3K/ PKB α pathway. However, the precise mechanism by which TAK1 regulates SCF level was still unclear. We first examined the expression of SCF mRNA and protein levels in K_{CTRL} treated with TNF- α in the presence of various kinase inhibitors. With the exception of the specific JNK inhibitor 1,9-pyrazoloanthrone,

all other inhibitors did not attenuate TNF- α -induced increase in SCF mRNA and protein compared to vehicle-treated K_{CTRL}, as determined by qPCR and ELISA, respectively (Fig 1.9A, B).

Fig 1.9. TAK1 regulates SCF expression via MKK7/JNK/c-Jun pathway.

Expression level of SCF (A) mRNA and (B) protein in K_{CTRL} treated with either DMSO (vehicle control) or specific inhibitors of the indicated kinases in the presence of TNF- α (10 ng/ml). The various kinase inhibitors were NF- κ B (I): BAY 11-7082; NF- κ B (II): SN50; JNK: 1,9-pyrazoloanthrone;p38:[2-(4-chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl]-1,2-dihydropyrazol-3-one]; and ERK1/2: PD98059. Ribosomal L27 was used for normalization in qPCR. Protein levels were determined by ELISA.

Next we studied the roles of different kinases in regulating the promoter of the human SCF gene. The human SCF promoter was isolated by PCR, and a proximal 853-bp fragment containing the transcription start site was subcloned into a luciferase reporter gene.¹⁵⁷ This SCF promoter reporter construct was co-transfected with expression vectors encoding constitutively active (ca) kinases into K_{CTRL}. The activation of TAK1 stimulates the activity of downstream mediators, including IKK α/β , MKK7 and JNK.¹⁴⁹ Transient transfection studies showed that TAK1, MKK7 and JNK stimulated SCF promoter activity (Fig 1.10), with no effect from IKK, PKA, MEK1, MEKK1 or SEK1. JNK activates transcription factor c-Jun, an obligate partner of AP-1. Previous

in silico analysis of the human SCF promoter has identified a putative AP-1 binding site.¹⁵⁷ Site-directed mutagenesis of the putative AP-1 binding site in the SCF promoter abolished the stimulating effect of TAK1, MKK7 and JNK (Fig 1.10).

Fig 1.10. TAK1, MKK7 and JNK stimulated SCF promoter activity. Transactivation assay in keratinocytes co-transfected with a luciferase reporter gene driven by the human SCF promoter (pGL-hSCFpro-luciferase), cDNA encoding for indicated constitutively active (ca) kinases and pEF1- β -galactosidase as control for transfection efficiency. Luciferase activity was measured, and normalized reporter activity is shown as fold induction compared to reporter construct-transfected K_{CTRL} (control). The SCF promoter reporter construct with a mutated AP-1 site is denoted pGL-mSCFpro-luciferase. Values (mean $\pm$ S.D.) are from three independent experiments.

A dominant-negative c-Jun (TAM67) blocks AP-1 activation by dimerizing with Jun or Fos family proteins. Transfection of an expression vector containing TAM67 induced a reduction in SCF mRNA expression in a dose-dependent fashion, as determined by qPCR (Fig 1.11), suggesting that human SCF is a direct target of c-Jun.

Fig 1.11. SCF is direct target of c-Jun. Relative SCF mRNA expression in K_{CTRL} transfected with increasing amount of dominant-negative c-Jun (TAM67). Ribosomal L27 was used for normalization.

To further determine if human SCF gene is a direct target gene of c-Jun, we performed an electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Specific protein–DNA complexes were detected by EMSA for the SCF AP-1 binding site, which was effectively competed by unlabeled consensus AP-1 site (specific competitor, SC), but not by a non-specific competitor (NSC) oligonucleotide (Fig 1.12A). Mutation to the SCF AP-1 binding sequence (SCF mAP-1) eliminated its interaction with K_{CTRL} nuclear extract. As positive control, the labeled consensus AP-1 (conAP-1) oligonucleotide was used as a probe, which was specifically competed by the SC (Fig 1.12A). ChIP was performed on K_{CTRL} and K_{TAK1} using c-Jun antibody. In K_{CTRL} , c-Jun bound to this AP-1 site, but it did not in K_{TAK1} cells (Fig 1.12B). Additionally, no immunoprecipitation was seen with pre-immune serum in K_{TAK1} cells, and no amplification was detected for a control sequence upstream of the AP-1 site in the promoter of SCF (Fig 1.12B). Taken together, these results suggest that TAK1/MKK7/JNK/c-Jun signaling is necessary for the induction of SCF expression in keratinocytes.

Fig 1.12. SCF is a direct target gene of transcription factor c-Jun. (A) EMSA of the human SCF AP-1 binding site. Biotin-labeled SCF AP-1 binding sequence was incubated with nuclear extract isolated from K_{CTRL}. NSC denotes non-specific competitor, a scrambled AP-1 binding sequence. SC denotes non-labeled consensus AP-1 sequence (conAP-1). As positive control, conAP-1 was used. Mutated SCF AP-1 site is denoted SCF mAP-1. (B) Human SCF is a direct AP-1 target gene. Chromatin immunoprecipitation was done in K_{CTRL} and K_{TAK1} using pre-immune IgG (pre) or antibody against phospho-c-Jun (Ab). The promoter region with the AP-1 binding site was immunoprecipitated and specifically amplified in K_{CTRL} using Ab. No amplified signal was obtained in K_{TAK1} or using pre-immune IgG (pre). A control region upstream of the AP-1 binding site served as a negative control. M: 100-bp DNA marker.

1.3.8 SCF stimulates c-Kit/ PI3K pathway to modulate ROS production and cellular apoptosis.

The above data showed that SCF is a direct target gene of c-Jun and that SCF binding to its receptor c-Kit subsequently stimulates PKB α activation. This novel link between TAK1 and PKB α via SCF functioned in an autocrine manner to regulate cell survival. However, it remained unclear if this signaling cascade plays a prominent role in ROS production and thus cellular apoptosis. To this end, we examined the effect of either neutralizing anti-SCF antibody, recombinant SCF or the PI3K inhibitor LY294002 on ROS production in K_{CTRL} and K_{TAK1} during anoikis and TNF- α exposure. K_{CTRL} incubated with LY294002 or neutralizing anti-SCF antibody produced more ROS upon anoikis treatment or TNF- α induction, as determined by DCF labeling followed by FACS analysis (Fig 1.13A, B).

Fig 1.13. FACS analysis of DCF staining (A–B) Representative FACS-derived histograms showing increased ROS level in K_{TAK1} and K_{CTRL} treated overnight with 25 μ M LY294002, 50 ng/ml recombinant SCF or 200 ng/ml neutralizing anti-SCF antibody, before DCF labeling and apoptosis induction by (A) anoikis or (B) TNF- α (10 ng/ml) treatments. Mean fluorescence intensities, indicated in brackets, were obtained from five independent experiments.

More importantly, this elevated level of ROS led to a higher percentage of apoptotic cells (Fig 1.14). K_{TAK1} treated with exogenous recombinant SCF showed diminished ROS level and reduced percentage of apoptotic cells compared to vehicle-treated K_{TAK1} (Fig 1.14).

Fig 1.14. FACS analysis of Annexin/PI staining Percentage of apoptotic K_{CTRL} and K_{TAK1} treated as described in (A–B) and analyzed by FACS (10,000 events). The totality of Annexin V⁺/PI⁻ (early apoptosis) and Annexin V⁺/PI⁺ cells (late apoptosis) were considered apoptotic. Values (mean $\pm$ S.D., bold) denote apoptotic cells (%) from five independent experiments.

Next, we cultured K_{TAK1} and K_{CTRL} OTCs in serum-free medium supplemented with the above inhibitors. The inhibition of the SCF/c-Kit pathway in K_{CTRL} resulted in elevated ROS production and increased keratinocyte apoptosis reminiscent of K_{TAK1} . The same phenotype in K_{TAK1} was likewise rescued by exogenous SCF (Fig 1.15A,B).

Fig 1.15. (A–B) DCF staining of (A) K_{CTRL} and (B) K_{TAK1} OTCs constructed on an underlying fibroblast-embedded collagen matrix, cultured in the presence of either neutralizing anti-SCF

antibody, the PI3K inhibitor LY294002 or exogenous recombinant SCF. H&E staining (top panel) reveals tissue structure and morphology. *In situ* TUNEL staining (bottom panel) identifies apoptotic cells. Cells were counterstained with DAPI (blue) for nuclei.

It has been reported that ROS accumulation activates JNK and enhances FLIP degradation, which might be responsible for the increased sensitivity to TNF- α -induced apoptosis in TAK1-knockout murine keratinocytes.¹⁷⁰ [missing]¹⁶³ Thus, we examined the effect of exogenous SCF or NAC on FLIP degradation. As expected, the treatment with TNF- α resulted in a rapid degradation of FLIP in K_{TAK1} cells. Importantly, immunoblot analysis showed that the degradation of FLIP was diminished in the presence of either SCF or NAC, supporting a pro-survival role of TAK1-mediated SCF pathway revealed herein (Fig 1.16). This was similarly observed for K_{TAK1} cells subjected to anoikis (Fig 1.16).

Fig 1.16. Immunodetection of FLIP protein from TNF- α - and anoikis-treated K_{CTRL} and K_{TAK1} cells in the absence (-) or presence (+) of either 50ng/ml of recombinant SCF or 1 mM of NAC. β -tubulin served as a transfer and loading control. Values below each band represent the mean fold differences (n=3) in expression level compared to vehicle-treated K_{CTRL}, which was assigned the value of 1.

Altogether, our results show that keratinocyte TAK1-mediated SCF expression, which stimulates PKB α activity, has a major role in modulating keratinocyte ROS level and apoptosis in an autocrine manner (Fig 1.17).

Fig 1.17. SCF stimulates PI3K/PKB α signaling to modulate ROS production and cellular apoptosis. TAK1 sustains keratinocyte survival by regulating ROS production. Epidermal TAK1 protects keratinocytes from ROS-mediated cell death by an autocrine mechanism through the release of SCF. Upon wounding, inflammatory signals, like $\text{TNF-}\alpha$, present at the wound site stimulate the production of ROS. Anoikis also induces ROS production, and anoikis resistance is essential for the survival of wound keratinocytes. Our study showed that the simultaneous activation of TAK1 allows for tightly controlled ROS generation via the autocrine action of SCF. TAK1 activates MKK7/JNK/c-Jun to stimulate the expression of SCF. c-Jun is an obligate partner of transcription factor AP-1. By binding to and activating its receptor c-Kit, SCF triggers the activation of PI3K/PKB α signaling to modulate ROS level and protect keratinocytes against ROS-mediated apoptosis. Thus, TAK1-deficient keratinocytes exhibit elevated ROS production and massive cellular apoptosis.

1.4 DISCUSSION

ROS have been implicated as critical cell signaling intermediates in several processes, including wound repair. In the aftermath of a superficial injury, constitutive inflammatory signals facilitate the repair at the wound site by inducing the temporal production of ROS, which signals through redox-sensitive signaling pathways and transcription factors to execute specific biological processes relevant for wound healing. Although ROS are necessary for diverse signaling roles, their deregulated overproduction for prolonged periods is associated with cancer, diabetes, inflammatory diseases, ischemia-related diseases, and neurodegeneration, and they are commonly thought to contribute to human pathologies and aging.¹⁷² [missing]^{164,165} This conclusion is explained by the highly reactive nature of ROS exerting their subsequent damaging influence on macromolecules. To prevent unnecessary damage to the intracellular biomolecular structures, it is crucial that the production of ROS be tightly controlled. In this study, we show that the keratinocyte-specific TAK1 protects the keratinocytes from the apoptosis induced by anoikis and TNF- α . This mode of protection is associated with diminished ROS and involves a novel cell-autonomous mechanism by which TAK1 mediates the expression of SCF to stimulate the activation of the antiapoptotic PIK3/PKB α pathway. Earlier studies have described a role of TAK1 in innate immunity and inflammation by serving as a downstream signaling effector of several proinflammatory factors (e.g., TNF- α , IL-1, LPS)¹⁶⁶. Our study further reveals a functional role for SCF in keratinocytes and identifies TAK1 as a novel player connecting inflammation and ROS regulation in skin redox biology.

During normal wound healing, we have consistently observed that the pattern of ROS production is correlated with the expression of TAK1 in mouse skin wound biopsies. As the skin is exposed to a surge in ROS levels during post-injury inflammation, which is attributed to the respiratory burst of phagocytic cells and native keratinocytes, it is likely that TAK1 expression is enhanced

to attenuate the heightened epidermal oxidative stress. Using RNA interference and organotypic skin cultures, we assessed the role of TAK1 in an epidermal environment void of immune influences and found that the suppression of TAK1 in human keratinocytes led to higher ROS levels accompanied by an increased susceptibility to anoikis and TNF- α -induced apoptosis. Together, these results suggest that TAK1 plays a homeostatic role in regulating ROS generation in the skin. In support of this hypothesis, an independent study showed that the keratinocyte-specific deletion of TAK1 hypersensitizes the mouse keratinocytes to the TNF- α -mediated apoptosis via elevated ROS, leading to massive cell death and chronic skin inflammation. In this particular study, a c-Jun-dependent mechanism was reported to inhibit the excessive ROS production and salvage the TAK1-deficient keratinocytes from the apoptotic fate; however, the precise underlying mechanism remains obscure.¹⁵⁶ Recently, TNF-related apoptosis-inducing ligand (TRAIL) was shown to induce the accumulation of ROS, enhance the activation of caspase-3 and increase apoptosis in the TAK1-null mouse keratinocytes. Further work showed that the TAK1-mediated prevention of TRAIL-induced cell death was mediated by a non-NF- κ B mechanism¹⁶⁷.

The role of SCF/c-Kit has been well documented to promote the survival, proliferation and differentiation of bone marrow-derived hematopoietic cells.¹⁶⁸ In the human skin, SCF/c-Kit signaling plays a pivotal role in the process of melanogenesis. Although the expression of SCF and its receptor c-Kit have been reported in keratinocytes¹⁶⁹⁻¹⁷¹, its homotypic role in keratinocyte biology remains elusive. However, SCF expression is interestingly found to be substantially up-regulated by three-fold in a human keratinocyte cell line (i.e., HaCaT) upon differentiation¹⁷². We showed that the TAK1/MKK7/JNK/c-Jun signaling pathway in human keratinocytes results in the increased expression of SCF, which induces its receptor c-Kit to

reduce ROS production and activates a PKB α -dependent mechanism, thereby inhibiting TNF- α -induced cell death. This novel cell-autonomous mechanism clarifies and supports our previous work, but more importantly, it underscores the homeostatic role of TAK1 and SCF/c-Kit signaling in the regulation of ROS production and demonstrates their novel antiapoptotic role in keratinocytes. A plausible explanation for the increased expression of SCF in the differentiated human keratinocytes could be that the differentiated keratinocytes *in vivo* are closer to the exterior surface of the skin, implying that they are more prone to pathogenic contact or oxidative stress that could arise from UV-induced inflammation. The up-regulated SCF levels in the differentiated keratinocytes could be a naturally inherent protective mechanism of the skin against imminent oxidative stress. These results further substantiate the highly detrimental impact of unattenuated oxidative stress and its significance in disease pathology.

Prolonged inflammation that induces oxidative stress has been implicated in dermatological conditions, including psoriasis.¹⁷³ Molecularly, psoriatic skin bears resemblance to the TAK1-deficient epidermis. Analysis of skin biopsies from patients with psoriasis has shown that c-Jun and Von Hippel–Lindau (pVHL) are down-regulated in psoriatic skin,^{174,175} coinciding with our previous findings and those of others who have investigated TAK1-deficient keratinocytes.¹⁵⁰ This reduced c-Jun activity in the TAK1-deficient keratinocytes results in a state of increased epidermal oxidative stress as likened to the psoriatic epidermis. Furthermore, mice with epidermal-deleted Jun protein are also used as a psoriasis animal model and also display the psoriatic arthritis complication.¹⁷⁶ Moreover, mice with an epidermal-specific deletion of TAK1 also developed a severe psoriasiform-like skin inflammation¹²⁹. In view of these data supporting a probable role of TAK1 signaling in the development of psoriasis, the next question would be

how the TAK1 signaling deficiency is involved in the epidermal redox regulation to initiate psoriasis in the uninvolved skin of patients.

Within the currently available knowledge, psoriasis is strongly believed to be an immune-related disease, coupled with the prominent appearance of chronically inflamed scaly skin on affected patients. In fact, as in the scenario of cancer, psoriasis also has a long classical link with wounding. It has been widely observed that in addition to the existing plaques on the involved skin of psoriatic patients, disease lesions frequently form at the boundaries of a wound inflicted on uninvolved skin. Such psoriatic lesions usually form within 10-20 days of the wounding event, coinciding with the duration of the wound-healing phase⁷³. This observation, famously known as the Koebner phenomenon, strongly suggests that although the psoriatic skin is predisposed to lesional plaque formation, the skin may still develop normally until it is substantially triggered by key epidermal perturbations (e.g., wounding)⁸⁵. With further observation and documentation, the Koebner phenomenon was later broadened to more accurately and extensively include the psoriasis arising from all possible forms of skin injuries. As such, a wider variety of wounds and other skin perturbations or traumas may include insect bites, friction, pressure wounds, excoriation (e.g., shaved skin), burns, contact dermatitis, chemical irritation, infections, tattoos, and photosensitivity (e.g., sunburns)¹⁰. If we were to extrapolate from the infinite causes of trauma, the most minute and microscopic forms of trauma would be the necrotic damage of keratinocytes. Furthermore, the necrotic form of cell death as characterized by cytoplasmic spillage also favors the onset of tissue inflammation¹⁷⁷. Given the inexhaustible means of initiating necrotic damage in keratinocytes, it is conceivable that some forms of wounds, perturbations and traumas to the skin are not as visibly obvious as others.

These subtle forms of epidermal injuries could lead to koebnerized psoriasis and still display the misleading outward appearance and impression of a spontaneous psoriatic outbreak.

The keratinocytes in the skin are naturally subjected to anoikis when they are developmentally detached from the basement membrane in the course of skin homeostasis.^{17,178} During normal skin development, the TAK1-deficient keratinocytes subjected to anoikis-induced ROS elevation are thus susceptible to psoriasis-triggering trauma because they cumulatively sustain ROS-induced oxidative damage, which can trigger both apoptotic and necrotic cell death through multiple mechanisms, including DNA fragmentation¹⁷⁹ and mitochondria cytochrome c release.¹⁸⁰ Whereas all the previously mentioned forms of skin trauma involve external perturbation to the epidermis, this mode of ROS-triggered necrotic cell death may represent the first endogenously originating trauma that could theoretically initialize koebnerized psoriasis. Such a ROS-triggered skin trauma could arise in apparently unwounded psoriatic skin due to the deregulation of the intracellular signaling and could help explain the spontaneous occurrence of psoriatic lesions.

If the relationship between wounding, inflammation and cancer could be illustrated, it would most likely be an overlapping Venn diagram. The closely related relationship of wounding, inflammation and cancer implies the sharing of many key molecular features and processes. As mentioned previously, wounds share many features with cancerous tumors, including their proinflammatory microenvironment. Wounds are also known to trigger cancer or chronic inflammatory diseases (e.g., koebnerization). Studies have suggested that for reasons yet unknown, the genes that are active during wound healing are also activated in the cancers with the worst prognoses.¹⁸¹ Like wound healing, inflammation is a critical component of tumor progression and has also been linked to a role in malignant transformation.¹⁸² Many cancers are

known to arise from sites of infection, chronic irritation and inflammation. Therefore, studies on inflammation are relevant in the search for a new treatment approach for cancer. It is now becoming clear that in response to stress in the tumor microenvironment, such as inflammation, tumor cells exploit various signaling molecules to sustain and promote their growth, invasion and metastasis.¹⁸² Recent studies have shown that tumors utilize a redox-based mechanism to prevent death by anoikis.¹⁸³ Anoikis resistance is an essential feature of cancer cells, yet its acquisition is a central mystery in cancer biology. Although not investigated in this study, our findings suggest, at least in part, a role for the TAK1-mediated SCF/c-Kit signaling in conferring anoikis resistance as a possible tumor response to inflammation. Our study offers new insights into anti-inflammatory- and anti-redox-based approaches as viable cancer treatment strategies.

1.5) FUTURE DIRECTIONS

In this study, we identified an up-regulation of ROS mediated oxidative stress in TAK1 deficient human keratinocytes. This ROS elevation was observed to enhance cell death in keratinocytes in the context of inflammatory type trigger (ie. TNF- α) or anoikis which is likened to keratinocytes in a differentiating epidermis subjected to detachment from the basement membrane. Given that TAK1, a key inflammatory signalling protein participated in this vital oxidative stress protective role of skin cells during wounding and inflammation, the implications of TAK1's redox modulatory role in skin tumor cells is thus worthy of a future study.

An excellent approach and comparative study to keratinocytes would be the usage of A5-RT3 cell line for the study of TAK1 deficiency in cancer. Being a high-grade metastatic human skin cancer cell line, hallmark tumorigenic processes such as the epithelial to mesenchymal transition, may be studied in A5-RT3 cells and correlated with the TAK1 activity and ROS production. As a preliminary characterization study attempt, lentiviral mediated siRNA knockdown of TAK1 may be effected on A5-RT3 cells to establish a stable cell line. Important research objectives inspired by our findings in chapter 1 would be to evaluate how TAK1 deficiency might possibly influence ROS mediated oxidative stress in skin tumor cells. Does TAK1 deficiency also up-regulate ROS in skin tumor cells as observed in their keratinocyte counterparts? If so, how would the increased oxidative stress contribute to the tumorigenic properties of skin tumor cells? How does oxidative stress differences, if any, pertaining to differential ROS production affect the survivability of skin tumor cells? Given that ROS increase is also observed in wounded epidermis and that keratinocytes at the leading wound edge are documented to possess the status of a partial EMT, does probable oxidative stress differences stemming from TAK1 deficiency have an influence on key tumorigenic features like EMT? Significantly, TAK1 is also a non-

canonical downstream signalling player of TGF- β 1, a well known inducer of EMT in the cancer context. How would TAK1 deficiency, a hindrance to the non-canonical downstream signalling of TGF- β 1 affect the tumorigenicity of skin tumor cells? As such, evaluation of TGF- β 1 induced EMT could be performed and the impact of TAK1 knockdown be investigated.

These questions and experimental requirements would be duly addressed in chapter 2, as we proceed with the preliminary characterization of TAK1 knockdown in skin tumor cells.

CHAPTER 2

PRELIMINARY CHARACTERIZATION OF TAK1 KNOCKDOWN IN SKIN TUMOR CELLS

All sections are contributed by the candidate except: Fig 2.5

ABSTRACT

Both wounding and chronic inflammatory disease have long been known through clinical observation for their cancer predisposition. Hence much research effort is ongoing to elucidate key connections between these pathological processes in a bid to define new chemotherapeutic strategies. A likely connection would be the elevation of reactive oxygen species (ROS), the main biological mediator of oxidative stress which has been implicated in all wounding, inflammation and cancer. Another link would be transforming growth factor β -activated kinase 1 (TAK1), a signaling protein which has key inflammatory signaling roles and is coincidentally downstream of TGF- β 1. In our previous study, we showed that TAK1 played vital oxidative stress protective roles in human keratinocytes by modulating ROS production. However the ROS regulatory role of TAK1 in cancer remains poorly defined.

In this present study, we showed that TAK1 deficiency elevated ROS levels in the high grade skin tumor cell line A-5RT3 and significantly enhanced their EMT. ROS elevated skin tumor cells manifested a more mesenchymal morphology characterized by increased focal adhesions, thickened stress fiber and also the up-regulated expression of cancer associated integrin α 5 β 1. Importantly, the inhibition of ROS levels with antioxidant N-acetyl-L-cysteine (NAC) also inhibited EMT of the TAK1 deficient skin tumor cells. This together suggests that ROS has an important role in facilitating cancer cell invasiveness, one which may involve the signaling implications of TAK1.

2.1 INTRODUCTION

2.1.1) The relationship between wounding, inflammation and cancer: The classical hypothesis.

The relationship between wounding, inflammation and cancer is one of the earliest and longest standing hypotheses concerning cancer. Famously postulated in 1863 by Rudolph Virchow, “the father of modern pathology”, this viewpoint arose rationally from the frequent clinical observations of cancer development at sites of chronic tissue inflammation and wounding^{12,184}. These observations suggest that similar molecular mechanisms underlie wounding, inflammation and cancer. However, the mechanistic basis for these occurrences was difficult to evaluate prior to the advent of molecular biology. With the current improved understanding of wounding and inflammation, this age-old cancer hypothesis is once again revisited. One of the most important research objectives would be to identify the critical events of inflammation and wounding that favor cancer progression.

2.1.2) Inflammatory stimuli are well-established cancer risk factors

2.1.2.1) Carcinogens as pro-inflammatory stimuli.

Most factors implicated in carcinogenesis are also known to trigger inflammation. For example, carcinogens used to induce experimental animal tumors such as the phorbol ester derivatives of Croton oil, are well-known chemical irritants¹⁸⁵ and inflammation inducers¹⁸⁶. Although oncogenic viruses are known to elevate cancer risk through the modification of host DNA¹⁸⁷, several observations have highlighted the conditional importance of wound-elicited inflammation in driving tumor formation. One classic experiment is the induction of tumors in chickens with the inoculation of Rous sarcoma virus¹⁸⁸⁻¹⁹⁰. Interestingly tumors only form at deliberately wounded sites in the infected chicken, including the initial site of virus injection.

Furthermore, administration of anti-inflammatory drugs to the infected chickens also successfully prevented tumor formation. These suggest that wound associated inflammation is critical for oncogenic transformation, tumor growth and progression.

2.1.2.2) Chronic inflammatory diseases affecting epithelial tissue enhances the risk of cancer development.

The continuous surface of the body contacting the external environment is composed of epithelial tissue, and key examples are the skin, lining of the oral cavity, gastrointestinal tract, lung alveoli, breast ductal glands, the ductal and parenchymal tissue of the liver. The epithelium is frequently the site of chronic inflammatory diseases and cancer, as it is most exposed to the damaging elements of the environment and the inflammatory aftermath¹⁹¹. In fact, cancer of the epithelial tissue (i.e. carcinoma) is the most common form of cancer. Many chronic inflammatory diseases have been validated as cancer risk factors¹¹. Notable examples include chronic hepatitis¹⁹², *Helicobacter pylori*-induced gastric inflammation^{193,194}, inflammatory bowel disease^{195,196} and epidermolysis bullosa^{197,198}.

Chronic liver inflammation or hepatitis can result from viral infection, alcohol or drug-induced liver damage¹⁹². The inflamed and subsequently damaged liver undergoes liver fibrosis or excessive scarring, resulting in liver hardening (ie. cirrhosis) and subsequently liver cancer in most patients¹⁹⁹. Similarly, prior infection by the bacterium *Helicobacter pylori* induces damage to the stomach epithelial lining^{200,201}, promoting ulceration of the stomach walls^{202,203} and thus setting the wounding inflammatory stage for the development of stomach cancer or gastric adenocarcinoma²⁰⁴. Ulcerative colitis, the chronic inflammation of the colon and a form of inflammatory bowel disease also increases the risk of colorectal cancer²⁰⁵⁻²⁰⁷. In the chronic

inflammatory skin condition epidermolysis bullosa, the diseased skin is vulnerable to blistering and susceptible to squamous cell carcinoma¹⁹⁷.

2.1.3) Similarities between the tumor, wound and inflammation.

The multiple events of wound healing were previously detailed in chapter 1. In essence, the wound is an active environment consisting of the signaling, growth, migration and differentiation of the cells needed to orchestrate healing³⁶. The regenerating wound tissue is also a significantly inflamed environment that is functionally adapted to prevent microbial invasion³⁵. Famously likened to a wound that does not heal⁶⁸, the tumor is known to be a highly inflamed environment^{208,209}. Conversely, chronic inflammation is also a hallmark feature of non-healing wounds³⁵. Superficially, skin cancers such as squamous cell and basal cell carcinomas typically appear as ulcerated outgrowths and resemble chronic wounds²¹⁰. Beyond these appearances, cancer and wounding, which overlap in terms of inflammation are indeed physiologically similar in several ways.

2.1.3.1) Blood clots are similar to the tumor matrix.

Similar to bleeding typified in wounds, intratumoral bleeding and microhemorrhages are common occurrences in cancer²¹¹⁻²¹³. In fact, advanced- stage cancer is usually detected as bleeding from the cancerous site, typified by the clinical observations of nose bleeding in nasal cancer patients²¹⁴ and the presence of blood in stools of colorectal cancer patients²¹⁵. The bleeding wound or tumor is foremost sealed by hemostasis, the clotting of blood. As earlier described in chapter 1, the blood clot is not biologically inert, rather it influences the wound or tumor through the release of factors including PDGF from its entrapped platelets, chemoattracting immune cells to elicit further inflammatory responses³⁶. Physically, the blood

clot is mainly composed of the ECM, which serves as a migratory path to direct the recruitment of immune cells and fibroblasts¹². As a result, the wound or tumor is subsequently infiltrated with an increasing number of immune cells, thus favoring inflammation.

Structurally, the blood clot typical of a wound is composed predominantly of cross-linked fibrin and extravasated fibronectin²¹⁶. Interestingly, non-bleeding tumors are also found with a matrix of cross-linked fibrin and fibronectin⁶⁸. This results from the hyperpermeability of tumor blood vessels, which permit the continuous accumulation of the plasma-derived fibrin and fibronectin⁶⁸. In the earlier phases of wounding, wound blood vessels under the temporal influence of vascular permeabilizing factors such as the inflammation- associated histamine, also become leaky with respect to the passage of plasma fibrin and fibronectin³⁷. Thus, the wound and tumor are largely exposed to a similar matrix.

2.1.3.2) Angiogenesis and lymphangiogenesis occurs in both wounds and tumors

The normal wound is a metabolically demanding environment, as cellular growth, proliferation and migration are needed in both the immune and cutaneous systems participating in repair²¹⁷. A rich blood circulation is needed to supply the wound site adequately with oxygen and nutrients, and to remove the accumulated metabolic waste²¹⁸. Angiogenesis, the sprouting of blood vessels from pre-existing vessels occurs to construct a well-perfused granulation tissue that then serves as the provisional dermis for reepithelialisation³⁴. Similar to the wound, the tumor is composed of hyperproliferative cells that likewise require sustainability in their metabolic demand²¹⁹. Angiogenesis is initiated in the tumor when it grows beyond a diameter of 2 mm and thus faces reduced oxygen levels and nutrient diffusibility^{220,221}. While this enhanced vascularization is

beneficial in wounds, it detrimentally encourages tumor metastasis as tumor cells are brought to a closer proximity to the blood circulation.

Another circulation-related tissue change occurring in both tumors and wounds is lymphangiogenesis, the formation of lymphatic tissues²²². The lymphatic tissue, or vasculature, is part of a circulatory system that directs the protein-rich blood plasma fluid drained out of the capillaries back into the blood²²³. The lymphatic vasculature plays an important immune role by directing blood-derived fluid to lymph nodes, whereby foreign antigens and pathogens are brought into contact with lymphocytes to prime an adaptive immune response²²³. When the lymphatic tissue is mechanically damaged during wounding, lymphangiogenesis is initiated to reconstruct the disrupted lymphatic vasculature²²². Lymphangiogenesis is likewise induced during cancer, where it facilitates metastasis similar to angiogenesis facilitates metastasis²²⁴.

Both angiogenesis and lymphangiogenesis in tumors and wounds are primarily triggered by the cytokine vascular endothelial growth factor (VEGF), which is up-regulated in both cancer and wound healing²²⁵. Other pro-angiogenic growth factors expressed in wounds and tumors include fibroblast growth factor-2 (FGF-2), hepatocyte growth factor (HGF) and granulocyte-macrophage colony stimulating factor^{34,219,226-229}. Anti-angiogenic treatments with endogenous or exogenous inhibitors delay wound healing and conversely hinder tumor growth²³⁰. Bevacizumab (ie. Avastin) is a Food Drug and Administration (FDA)-approved anti-angiogenic drug for the treatment of colorectal cancer^{231,232}. In line with the above-mentioned role of angiogenesis in wound and cancer, Avastin has the side effects of hindering wound recovery, which results in abdominal abscesses and intestinal hemorrhages^{233,234}.

2.1.3.3) Fibrotic tissue changes in both wounds and tumors

In addition to angiogenesis, another similar tissue change observed in tumors and wounds is the formation of fibrous tissues and scarring. Key cells responsible for fibrous tissue formation are the fibroblasts present in the stroma underlying the epithelial cell layer²³⁵. In the context of the skin, fibroblasts reside in the dermis, the skin stroma where they deposit large amounts of ECM²³⁶. A few days after skin wounding, a sub population of dermal fibroblasts differentiate into myofibroblasts that possess cytoplasmic contractile molecular elements (ie. myosin, smooth muscle actin, etc)²³⁷. These myofibroblasts function by contraction and aids the closing of the wound gap^{238,239}. Furthermore, stromal fibroblasts are mainly triggered by PDGF, which is released by platelets of the blood clot in a wound or excessively secreted by several tumor types³⁴. As such, fibroblasts in the wound and tumor are effectively subjected to the same molecular trigger that induces their proliferation. The populations of both fibroblasts and myofibroblasts are largely increased in carcinomas, especially during the advanced stages. Through matrix remodeling efforts, these fibroblasts cause the tumors to become more fibrotic or desmoplastic^{240,241}. A fibrotic tissue environment has significant implications in cancer formation as cancers are known to arise from the fibrotic tissues of chronic inflammatory diseases¹². In addition, the processes leading to fibrosis in tumors are highly similar to those in healing wounds. This has been supported by similarities of gene-expression signatures between skin wounds, malignant tumors and serum-treated fibroblasts^{242,243}. The natural wound healing response of fibrosis that leads to scarring also results from many chronic inflammatory diseases, preceding malignant transformation²⁴⁴. Fibrosis is characterized by a massive tissue ECM deposition, which explains the hardening of the liver in cirrhosis and the scarring of lung tissue in pulmonary fibrosis²⁴¹. Both chronic inflammatory conditions are widely known to progress

into cancer^{11,208,209}. In essence, many pathological changes in tissue characteristic of chronic inflammatory diseases, such as fibrosis and ulceration²⁴⁵, are actually features of impaired wound healing.

2.1.4) Inflammation-induced oxidative stress, a wound and cancer connection

Inflammation is a major common factor between wounds and tumors. Furthermore, persistently inflamed wounds have been known to progress into malignancy. Importantly, inflammatory mediators are potential drivers of tumorigenesis^{11,208,209}. What then are the tumor promoting aspects of inflammation?

The tumor and wound site are both infiltrated with abundant immune and inflammatory cells such as macrophages^{34,36,246}. As part of the effort to deter infection, oxidative stress is elevated during inflammation to eliminate pathogens. This can happen through various means. Primarily, macrophages in inflamed wounds and tumors secrete high levels of reactive oxygen species (ROS) through respiratory burst²⁴⁷⁻²⁴⁹. Stimulation with proinflammatory cytokines such as tumor necrosis factor- α (TNF α) also induces ROS production in the wound epidermis²⁵⁰. Moreover, the inflamed wound and tumor environment are regions of increased oxidative stress as they are inherently metabolic^{249,251-253}. Hence, cells and tissues of wounds and tumors are exposed to the influences of ROS.

2.1.4.1) ROS and cancer

A detailed description of ROS types and their generation has been introduced in chapter 1. As previously described, ROS are relatively stable intermediates resulting from the stepwise monovalent reduction of oxygen^{254,255}. This usually happens through the leakage of electrons to molecular oxygen in the mitochondrial respiratory chain during ATP synthesis. The major ROS

species are the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$). In comparison, $O_2^{\cdot-}$ and H_2O_2 are much less reactive than $OH^{\cdot}$, which is responsible for most of the ROS-induced damage to biomolecules. In addition to being toxic byproducts of respiration, several recent studies suggest that ROS have a functional role in membrane receptor signaling. In fact, ROS can be deliberately generated by the stimulus-induced activation of membrane-bound enzyme systems, such as NADPH oxidase complexes (NOX)²⁵⁵, xanthine oxidases²⁵⁶, lipoxygenases (LOX)²⁵⁷ and cyclooxygenases (COX)²⁵⁸. When activated, the membrane-bound systems result in a one-electron reduction of molecular oxygen to generate $O_2^{\cdot-}$, which is readily dismutated to H_2O_2 by the superoxide dismutases (SODs). $O_2^{\cdot-}$ and H_2O_2 may then interact to generate the highly destructive $OH^{\cdot}$ ^{254,255}. Thus, the presence of destructive ROS is delicately and tightly regulated in the cell to maintain normal physiological functioning. Under physiological conditions, the damaging potential of ROS and their intermediates is neutralized and counterbalanced by the cell's innate anti-oxidant defense, which consists of the $O_2^{\cdot-}$ scavenging enzyme SOD, the H_2O_2 -scavenging catalase and glutathione peroxidase (GPX)^{254,255}. Any perturbation to this balance favoring ROS up-regulation would cause the cell to be oxidatively stressed, resulting in disordered cell death (necrosis) or programmed cell death (apoptosis). Such occurrences have been blamed as the disease mechanism of various degenerative and terminal conditions including wound healing, aging²⁵¹, diabetes²⁵⁹, cardiovascular diseases²⁶⁰, fibrosis²⁶¹, neurodegenerative disease^{102,262} and significantly, cancer²⁴⁹.

Relative to normal cells, tumor cells have been observed to operate with higher levels of endogenous oxidative stress both in culture and *in vivo*²⁶³⁻²⁶⁶. Significantly, freshly isolated leukemia cells from patients with chronic lymphocytic leukemia were found to possess higher

ROS levels than normal lymphocytes²⁶⁷. Increased levels of oxidative damage products, such as oxidized DNA base (8OHdG), and lipid peroxidation products were also found in clinical tumor specimens, plasma and cancer cell lines^{266,268,269}. Protective endogenous anti-oxidant features in tumor cells such as the ROS-scavenging enzymes superoxide dismutase (SOD), glutathione peroxidase and peroxiredoxin are aberrantly regulated in cancer cells as well²⁶⁹. Elevated ROS levels in cancer cells appear to promote malignant initiation and progression²⁷⁰. Importantly, high ROS levels in cancer cells have been associated with the increased aggressiveness of tumors and poor prognosis of patients²⁷¹. Strong evidence suggests that the increased oxidative stress in tumor cells is a determinant to their acquisition of cancer hallmark features including immortalization and transformation, cell proliferation and mitogenic signalling, cell survival and disruption of cell death, epithelial-mesenchymal transition and metastasis, angiogenesis and chemoresistance²⁶⁶. Evidently, ROS-producing respiratory enzymes such as NOX have been found to promote tumor cell survival in pancreatic and lung cancers^{272,273}.

The current hypothesis is that ROS functions as double-edged sword in cancer progression, capable of promoting both tumor cell survival and death, in a context-dependent manner. The continuous pre-exposure of normal epithelial cells to low levels of exogenous oxidants adapts the cells to survive upon subsequent exposure to higher oxidative stress²⁷⁴. Thus, in regards to tumor cell survival, this consistently high ROS stress endured by tumor cells may trigger adaptive stress responses that effectively are survival factors²⁷⁵. In particular, hydrogen peroxide (H₂O₂) has also been shown to degrade IκBα, the inhibitory subunit of NF-κB, hence liberating NF-κB for its cell transformation, proliferation and angiogenic roles that ultimately support tumor growth. In addition to NF-κB, Nrf2, c-Jun and hypoxia-inducible factor 1 (HIF-1) are also known to function as redox-sensitive transcription factors responsible for the expression of ROS-

scavenging enzymes such as SOD, catalase, thioredoxin and glutathione antioxidant systems as well as cell-survival factors such as B-cell lymphoma 2 (BCL2) and myeloid cell leukemia 1 (MCL1). ROS induction also causes NF- κ B to transcriptionally repress cell death factors such as caspases²⁶⁶. Additionally, the increase in glutathione during ROS stress adaptation responses can enhance the export and inactivation of some anticancer drugs, rendering the tumor cell chemoresistant. While moderate increases in ROS may promote cell proliferation and survival, ROS levels reaching toxic threshold levels may exceed the cell's antioxidant capacity, leading to cell death. However, ROS-triggered cell death is also subjective. Excessive levels of ROS inflict substantial damage to biomolecules, including DNA. The tumor-initiating property of ROS is largely attributed to their DNA damaging effect^{266,276}. ROS induce a variety of DNA damage, including strand breaks, point mutations and aberrant DNA cross-linking²⁶⁹. These mutagenic effects may target proto-oncogenes and tumor-suppressor genes, encouraging neoplastic transformation in the subsequent tumor promotion stage²⁷⁷. In normal cells, the DNA damage would trigger p53 action, leading to DNA repair and the trigger of apoptosis. However, in tumor cells where p53 is defective, the mutations owing to DNA damage are left to accumulate²⁷⁸. These mutations may confer survival attributes to the tumor cells, favoring their natural selection from environmental stress challenges (ie. chemotherapeutic agents, radiation, etc.) hence leading to increased malignancy. Mutations may also equip tumor cells with increased invasive features, facilitating their spread and thus worsening cancer prognosis. Additionally, cancer cells may also lose their normal p53 function through ROS mediated damages and proceed into a state of genomic instability.

The latest findings on the redox biology of cancer also revealed that the outcome of tumor growth is conditional on the specific ROS composition produced, thus influencing ROS-

mediated tumor cell death or survival. It was proposed that the ratio between $O_2^{\cdot-}$ and H_2O_2 is especially important for the survival fate of a tumor cell. It is believed that $O_2^{\cdot-}$ predominance supports cell survival and drives tumor progression^{279,280}. Conversely, H_2O_2 predominance prevents carcinogenesis by facilitating cell death signalling. In fact, it has been shown that the angiopoietin-like 4 protein (ANGPTL4) interacts with integrins to stimulate the NADPH oxidase-dependent production of $O_2^{\cdot-}$. This increases the $O_2^{\cdot-}:H_2O_2$ ratio, which subsequently causes increased oxidation of Src, triggering the prosurvival phosphoinositide 3 kinase/ protein kinase B- α (PI3K/PKB α) and extracellular signal regulated kinase (ERK) pathways that confer anoikis resistance to the tumor cells²⁸¹. Suppression of ANGPTL4 expression led to reduced $O_2^{\cdot-}$ production and correspondingly a reduced $O_2^{\cdot-}:H_2O_2$ ratio. This resulted in increased tumor cell apoptosis that impaired tumor growth. Moreover, ANGPTL4's role in tumor survival is also evident in colorectal cancer, whereby hypoxic conditions is found to induce prostaglandin synthesizing enzyme COX2 expression which then abundantly synthesizes prostaglandin 2. ANGPTL4 expression is then induced by prostaglandin 2, leading to tumor survival outcomes²⁸².

Chronic inflammation is a source of oxidative stress that may promote malignancy in tumor cells. Augmenting the hypothesis that chronic inflammation-derived oxidative stress is a trigger of cancer, tumor promotion in animal models with chemical agents could be inhibited with the application of anti-inflammatory drugs such as steroids or anti-oxidants²⁸³. DNA bases are also found to have oxidative modifications when mouse skin is induced with phorbol ester-type chemical irritants²⁸⁴.

2.1.5) Epithelial mesenchymal transition (EMT).

Cancer results in death when the tumor disseminates via metastasis to several secondary sites, leading to widespread organ damage and failure²⁸⁵. Currently, more than 90% of cancer related deaths have been blamed on metastasis²⁸⁶. For this reason, the spread of cancer and its invasiveness inevitably becomes the paramount focus of cancer research, aimed at curbing the high cancer mortality rate.

The epithelial tissue consists of sheets of continuous and polarized cells along the apical-basal axis²⁸⁷. Cells in the epithelial tissue are closely associated to ensure mechanical integrity and to maintain a permeability barrier to segregate tissue in development²⁸⁸. As most cancers (i.e. carcinomas) arise from an epithelial tissue origin, it is widely believed that the eventual metastasis must necessarily involve the conversion of primary tumor epithelial cells to a migratory form, allowing their invasion into the surrounding stroma. How then is this biological feat accomplished?

In the context of the human body and mammalian tissue, there are several occasions whereby epithelial tissues temporally need to migrate. During embryogenesis, tightly interconnected epithelial cells that were rightfully polarized with basal and apical attributes lose their polarization and transit into a mesenchymal cell form. These mesenchymal cells being more motile with a changed shape and polarity, then delaminate and migrate to remote sites for the colonization of tissue where they eventually revert into the epithelial form²⁸⁹. A key example would be the epithelial cells of the neuroectoderm, which transform into individualized migratory neural crest cells before their dispersion and relocated to the right spatial location to form precursor cells of various tissues including those of the nervous, endocrine and skeletal muscle systems²⁹⁰. This conversion of epithelial cells is also observed in postnatal circumstances.

During wound healing, epidermal cells or keratinocytes at the leading edge of a wound would spontaneously migrate and close up the wound gap¹². These former epithelial cells must likewise adopt a mesenchymal status to fulfill the migratory role. These epithelial transforming processes are collectively termed the epithelial-mesenchymal transition (EMT). It is believed that epithelial tumor cells also pivotally undergo EMT to metastasize, losing their detachment from neighboring cells, followed by a local invasion into the surrounding tissue before tumor cells gain access to the blood or lymphatic circulation^{291,292}. Compelling data from early histological and recent imaging studies have pointed to the involvement of EMT in cancer spread²⁹³⁻²⁹⁵. The protein expression changes underlying EMT observed in embryogenesis are also significantly similar to those of cancer cells²⁹⁶. As such, EMT is extensively studied as the critical preceding event of metastasis.

2.1.5.1) Characteristic cellular changes during EMT

Epithelial cells are connected through intercellular junctions composed of desmosomes, tight junctions and adherens junctions. Several proteins serve stabilizing roles in tight junctions including claudins, connexions, occludins and zona occludens^{297,298}.

Epithelial-cadherin (E-cadherin) is the main molecule utilized by epithelial cells in adherens junctions. E-cadherin (i.e. encoded by CDH1) is a transmembrane glycoprotein of type I cadherin superfamily. The extracellular domain of E-cadherin consists of 5 repeats used for homodimer formation with the E-cadherin of a neighboring cell while its intracellular domain is associated with a protein complex comprised of β -, α - and p120-catenins that in turn interact with the tensile actin cytoskeletal network²⁹⁹. Through this molecular association and linkage between adjacent cells, all epithelial cells are thus held together securely to form an epithelial

sheet that can withstand the stress of mechanical forces³⁰⁰. Moreover, a signal communication pathway is established between cell-cell contact, cytoskeletal regulation and cell shape³⁰¹. It is noteworthy that the adherens junction comprising E-cadherin and its ancillary molecular associations are not static entities with only structural supporting roles, but rather, they govern important signal regulatory roles³⁰¹.

Even though malignant carcinoma cells are generally regarded as invasive and motile, as distinct from the normal epithelial tissue, the epithelial organization plan is intrinsic to the primary tumors of carcinomas. In tumours, well-organized sheets of epithelial cells are still present. During EMT, closely knit epithelial cells begin to lose their cell junction proteins (i.e. claudins, connexions, occludins and zona occludens), weakening the epithelial cell association. Resultantly, cells become more motile as they are less anchored upon one another. Epithelial tumor cells progressively lose their E-cadherin and gain the expression of neural-cadherin (N-cadherin).^{291,292,296} As such, mesenchymal tumor cells with N-cadherin expression migrate and associate themselves with stromal cells such as fibroblasts that also express N-cadherin and as a result invade the stromal region³⁰². As the stromal is well perfused with capillaries which are composed of endothelial cells which possess N-cadherin as one of its major cadherins, in addition to vascular endothelial-cadherin (VE-cadherin), invading mesenchymal tumour cells with elevated N-cadherin can associate with endothelial cells through homodimeric N-cadherin interactions, favoring the access of tumor cells into the circulation^{302,303}. For example, normal skin pigment cells, or melanocytes, express E-cadherin and form the appropriate firm interactions with keratinocytes³⁰⁴. However, when melanocytes transform into melanoma cells during malignancy, N-cadherin expression takes over as part of EMT and facilitates the invasion

of melanoma cells into the dermal stroma where they interact with fibroblasts and endothelial cells³⁰².

Moreover, the signaling activity of the adherens junction is affected as cell surface E-cadherin expression is lost and replaced with N-cadherin expression³⁰⁵. Most notably, β -catenin previously held to the cytoplasmic side of the cell membrane by E-cadherin cytoplasmic tails is now released into the cytoplasm where it translocates into the nucleus and serves as a transcription factor for the expression of other EMT-inducing transcription factors that further augments the EMT process³⁰⁶. When E-cadherin expression is suppressed, several EMT-associated morphological changes begin to occur in the cell. Some experiments have shown that the simple suppression of E-cadherin expression causes cells to acquire a mesenchymal morphology and enhanced motility³⁰⁵. In fact many forms of invasive carcinoma have been found with their E-cadherin gene, *CDH1*, repressed by promoter methylation or transcriptional repression or entirely inactivated by frameshift mutations. Such expressional loss of E-cadherin has been well documented in advanced carcinoma of the breast, colon, prostate, stomach, liver, esophagus, skin, kidney and lungs³⁰⁷⁻³¹³. Familial gastric cancer, which is hereditary, is found with mutant germline alleles of the *CDH1* gene. Conversely, re-expression of E-cadherin in invasive carcinoma cells has conversely been shown to suppress invasiveness and metastatic dissemination of these cancer cells as well, further supporting that E-cadherin mediated contacts could autonomously regulate EMT³¹⁴.

Apart from the above-mentioned changes in the membrane protein composition that leads to the dissolution of cell-cell contacts and interaction, EMT also involves several other changes that additively cause cells to increasingly attain an interaction with the ECM or substratum to gain traction for migration and invasion. Upon the induction of the EMT *in vitro*, regularly shaped

cobblestone-like epithelial cell colonies are transformed into dissociated, flattened, spindle or irregularly shaped mesenchymal cells. These cell shape changes are attributed to extensive cytoskeletal remodeling³¹⁵. Generally, cytokeratin-type intermediate filaments characteristic of epithelial cells are also replaced with vimentin expression in mesenchymal cells. Vimentin expression has been classically proposed as the critical marker for mesenchymal cells³¹⁶. However, this classification attempt is confounded by several observations that vimentin expression is not specific for mesenchymal cells. Vimentin has also been found in endothelial cells and carcinomas even in the absence of EMT³¹⁷. Other cytoskeletal-type mesenchymal markers found up-regulated in mesenchymal cells also include α -smooth muscle actin (α -SMA)³¹⁸. However, α -SMA expression is limited to a subset of activated fibroblast cells and hence may not be present during EMT^{319,320}. Importantly, the expression of mesenchymal markers in EMT may vary and be context dependent. Actin stress fiber formation is characteristic of EMT, as mesenchymal cells are found to possess thickened stress fibers in contrast to the cortical actin-type patterns typical of epithelial cells³²¹.

The increased stress fiber formation also occurs in conjunction with the increased interactions formed by mesenchymal cells with the ECM or substratum are are histologically visible as point-like contact anchorages. These cell-ECM contact points are specialized structures termed focal adhesions, where bundles of actin filaments originating from stress fibers are anchored to a transmembrane receptor of the integrin family through a multimeric protein complex. This multi-protein complex is composed of several anchor proteins including vinculin, filamin, talin and paxillin are associated with focal adhesion kinase (FAK), which helps mediate signaling upon integrin binding of the ECM³²².

2.1.5.1.1) Integrins

Integrins are major surface receptors for cell adhesion to the extracellular matrix or substratum. Structurally, integrins are noncovalent heterodimers of two glycoprotein subunits, namely the α and β subunits. The complete classification of human integrins consists of 9 types of β subunits and 24 types of α subunits. Each subunit is a single-pass membrane protein. The bulk of its amino acid residues (>1600 amino acids) reside in the extracellular space and it has two short cytoplasmic domains (20-50 amino acids). As receptors, integrins bind distinct types of matrix protein such as fibronectin, laminin and collagen through specific interactions with the arginine-glycine-aspartate (RGD) peptide sequence found in matrix proteins. Among the many integrin subtypes, the $\alpha 5 \beta 1$ integrin is found to be an EMT marker and specifically binds fibronectin, which is highly secreted by mesenchymal tumor cells²⁹¹. The binding of integrins to their respective ligands depends on extracellular divalent cations (.ie. Ca^{2+} or Mg^{2+}). The type of divalent cation may also influence the affinity and specificity of integrin's ligand binding³²³.

Integrins serve three primary functions between a cell and its physical environment. First, integrins physically link the actin cytoskeletal network of cells to the ECM as earlier described. This transmembrane mechanical link between the ECM or substratum to the actin cytoskeleton is critical for a robust adhesion of the cell³²⁴. When the actin cytoskeleton binding cytoplasmic domain of the β subunit was removed with DNA recombinant techniques, cells subsequently failed to bind the matrix or form focal adhesions³²⁴. All integrins bind to actin cytoskeleton, with the exception of $\alpha 6 \beta 4$ which binds to intermediate filaments instead³²⁵.

Second, being more than static adhesion-mediating structures, integrins further serve signal transducing roles, informing cells as to whether a successful attachment of an integrin receptor to

the ECM has been achieved³²⁶. When integrins cluster at sites of cell-matrix contact, FAK is recruited to focal adhesions by intracellular anchor proteins such as talin and paxillin. Clustered FAK molecules cross-phosphorylate one another on a specific tyrosine residue, creating a phosphotyrosine docking site for members of the Src family of cytoplasmic tyrosine kinases. These kinases then further phosphorylate FAK on other tyrosine sites and additionally introduce more docking sites for a variety of other signaling proteins³²⁷. These signalling arising from integrin binding or activation could result in gene expression changes or stimulate localized changes in the cytoplasmic region close to the cell-matrix contract. Such integrin-related signaling is also employed to convey cell survival signals and prevent apoptosis. This explains why and how cell attachment to the substratum necessarily maintains the survival of all adhesive cells³²⁸.

Third, integrin signaling is also bidirectional and thus, so an intracellular signalling may be transmitted to integrins, causing their extracellular portion to make and break contact with the ECM, effectively executing directed cellular movement. This inside-out signaling is more applicable for suspension-type cells like platelets and white blood cells, where integrins have to be pre-activated before capably mediating an adhesion^{329,330}. This is particularly useful for platelets, which constantly circulate in the blood but occasionally adhere to wound sites or damaged blood vessels during an incidental event of blood clotting. Integrin expression in the platelets need not be induced *de novo*, which is time consuming and inefficient for the vital process of blood clotting which needs to be instantaneous. Rather, integrins may be held in the less adhesive state until triggered by an intracellular signal, causing a switch of integrins to a strongly adhesive state to mediate their due function³³¹. It is believed that signal protein binding

to the cytoplasmic tail of integrins serves as the mechanistic basis leading to structural changes affecting integrin affinity^{329,330}.

2.1.5.1.2) Relevance to cell traction force

To migrate, cells must exert a traction force on their underlying substratum or ECM. This generated cell traction force (CTF) presents a major physical difference between a migrating mesenchymal cell and a relatively stationary epithelial cell^{332,333}. The physical changes that occur in a cell during EMT are responsible for this CTF difference. Cytoskeletal remodeling involving the gain of actin stress fibers as well as the expression of vimentin and smooth muscle actin all leads to the increased tensile strength of the cell³³². As cells lose their cell-cell interactions and gain cell-matrix interactions during EMT, they progressively become more attached to their physical environment through increased integrin-based focal contacts³²¹. Given that traction is defined by the maximum frictional force that may be produced between two contacting surfaces without slipping, a cell must first be attached to its underlying substratum or ECM in order to generate traction³³⁴. This is mediated by surface integrin binding of the ECM which is the basis for CTF generation (Fig VIII). A better connection of ECM-bound integrins to stress fibers also enhances this traction³³⁵. This is evident as the severing of the integrin and stress fiber contact tremendously weakens cell adhesion to the substratum or ECM. When the ECM is well connected to the enhanced cytoskeleton of the mesenchymal cell, CTF is then generated by actomyosin motors, which bridges actin fibers and induces a contractile force powered by ATP hydrolysis^{332,335}. The CTF can also be generated at the forward protruding leading edge of cell migration by actin polymerization³³⁶⁻³³⁸. In fact, a measured CTF map of a cell is predictive of its migration directionality. CTF studies evaluate the physical intent of cell and are increasingly

gaining interest³³². The importance and relevance of CTF studies are also substantiated by the fact that many physiological and pathological processes involve cell mobility, hence implicating CTF, with examples including wound healing, inflammation, embryogenesis, angiogenesis and metastasis³³⁹. In the case of EMT, the generation of traction force by cancer cells at the peripheral or leading front helps separate them from their neighboring cells³⁴⁰. The changes in traction force precede the disruptions of cell-cell contact are observable, thus these changes are likely to be one of the earliest measurable cellular changes necessary for epithelial cells to lose their epithelial cell-cell junctions^{337,338,341}. Currently, CTF is studied with cell traction force microscopy (CTFM), which allows for the visualization of the overall CTF field of an entire cell spreading on a two-dimensional (2D) substrate or ECM surface³³².

Fig VIII. Cell traction force (CTF) is generated by the migrating cell attached to the ECM substratum. (Adapted from Fig. 1 in J. H. Wang and J. S. Lin, 2007, *Biomech Model Mechanobiol* 6: 361-371)³³²

2.1.5.2) TGF- β 1 induced EMT

Members of the transforming growth factor- β (TGF- β) family of cytokines are the main and best-characterized EMT inducers. In fact, TGF- β 1 has been involved in many processes that involve mesenchymal cell functions, including embryonic development, wound healing, fibrotic

diseases and cancer pathogenesis. Importantly, TGF- β 1 is also highly expressed in the wound where it plays roles in wound healing and inflammatory responses³⁴². Released by degranulating platelets in the wound at early phases of wound repair, TGF- β 1 is one of the first cytokines to mediate the chemoattraction of proinflammatory cells to the wound site³⁴². In fact, TGF- β 1 elicits chemotactic and mitogenic cues to neutrophils, lymphocytes, monocytes, macrophages and fibroblasts. Many of these cells further secrete TGF- β 1 via positive feedback response³⁴². In addition, TGF- β 1 is well known for its role in almost every cancer hallmarks³⁴³, and is importantly a potent inducer of EMT in many cancer cell lines³⁴⁴. The abundant expression of TGF- β 1 in wounds or inflamed tissue environment paves a favorable ground for tumor and skin cells to undergo EMT. In regards to cancer, the presence of TGF- β 1 has been associated with a poor disease prognosis and implicated in most processes pertaining to malignancy, including tumor growth, angiogenesis and metastasis, among others. The tumor microenvironment is also found to be highly abundant with TGF β 1³⁴⁵⁻³⁵³.

TGF- β 1 induces EMT through multiple distinct mechanisms. Fundamentally, the induction of EMT-related proteins is achieved through the activation of TGF- β 1's canonical Smad signaling pathway. Herein, TGF- β 1 ligand binds to TGF- β 1 receptor 1 (TGFB1) and TGFB2. TGFB2 phosphorylates TGFB1, which subsequently phosphorylates and activates SMAD2 and SMAD3. Activated SMAD2 and SMAD3 then form a Smad complex with SMAD4 before their combined translocation into the nucleus. In the nucleus, the Smad complex then interacts with other DNA-binding transcription factors and co-activators and co-repressors, binds to the promoter regions of TGF- β 1 target genes and regulating their transcriptional expression^{344,350}.

Through the Smad signaling of the TGF- β 1-induced EMT, epithelial markers are lost and mesenchymal features are acquired. This happens through a transcription program involving

three families of transcription factors, namely the Snail, ZEB and bHLH families. These transcription factors repress epithelial marker gene expression and concomitantly induce mesenchymal gene expression^{344,354}.

2.1.5.3) Non-Smad signaling involvement in TGF- β 1-induced EMT

TGF- β 1 does not induce EMT exclusively through the Smad downstream pathway alone. In fact, TGF- β 1 may also induce EMT via the activation of effector pathways of other tyrosine kinase receptors, which are generally not affected by the attenuation of Smad signaling pathways³⁵⁵. The direct activation of non-Smad signaling pathways by TGF- β 1 can occur directly through TGF β 2 binding with or without TGF β 1³⁵⁵. This may also happen with the assistance of adaptor proteins. Some examples of non-Smad signaling responses of TGF- β 1-induced EMT include Erk MAP kinases, Rho GTPases and the PI3 kinase/Akt pathway³⁵⁵. Their activation by TGF- β 1 has been linked to the regulation of distinct processes such as cytoskeletal organization, cell growth, survival, migration and invasion^{355,356}. Chemical inhibition of one or several of these pathways has been shown to affect TGF- β 1-induced EMT, implying that these non-Smad signalling pathways complement Smad signaling³⁵⁶.

Ras and MAP kinase activation have been shown to regulate TGF- β 1-induced Smad signaling. Erk MAP kinases phosphorylate Smad proteins in their linker regions, inhibiting their nuclear translocation and thus functioning as a transcription factor³⁵⁷. Additionally, TGF- β 1 directly activates the MAP kinase signaling pathways through its cognate receptors. This activation is notably lower than that mediated through tyrosine kinase receptors. The direct activation of Erk MAP kinases by TGF- β 1 is initiated by Ras and leads to the activation of Raf and MEK1/2 kinases characteristic of a growth factor response³⁵⁵. Increased Ras-Erk MAP kinase signaling,

either attributing to the growth factor stimulation or due to the expression of mutant Ras, has been shown to enhance TGF- β 1-induced EMT^{358,359}. The chemical blockade of MEK1/2 kinase leading to the inactivation of Erk1/2 MAP kinases supports its inhibition of TGF- β 1-induced EMT³⁶⁰. Erk1/2 MAP kinases are known to target transcription factor expression; hence they may augment TGF- β 1-induced EMT via the down-regulation of epithelial markers and the up-regulation of mesenchymal markers³⁶¹. In a similar manner, TGF- β 1 induces activation of the p38 MAP kinase and JNK MAP kinase³⁶².

TGF- β 1 has been known to induce the activation of Rho, Rac and Cdc42 subfamilies of proteins, which are normally involved in the regulation of cytoskeletal organization, cell migration and gene regulation. This activation is found to be widespread in different cell systems. TGF- β 1's activation of RhoA leads to the activation of ROCK, which leads to the formation of actin stress fibers characteristic of EMT^{363,364}. Chemical inhibition of ROCK or downregulation of Rho expression results in the inhibition of TGF- β 1-induced actin reorganization and α -smooth muscle actin expression associated with EMT³⁶³⁻³⁶⁵. TGF- β 1 may also induce the disassembly of epithelial cell junctions through a Smad-independent mechanism. This involves direct regulation of RhoA activity by TGF- β 1 at the epithelial tight junctions. Partitioning-defective protein (Par6), a cell polarity protein responsible for asymmetric cell development is directly phosphorylated by TGFR2 upon TGF- β 1 binding. This leads to the enhanced ubiquitination and subsequent degradation of RhoA, thus resulting in the loss of basal-apical polarity and the dissolution of existing epithelial tight junctions^{365,366}. TGF- β has also been shown in diverse cell systems to rapidly activate phosphoinositide-3 kinase (PI3K), leading to the activation of the Akt kinase, which plays a major role in EMT. Inhibition of this pathway was shown to restrict the EMT phenotype³⁶⁷.

Apart from the above pathways which are affected by non-canonical signaling of TGF- β 1, there are signalling pathways that do not induce EMT by themselves, but rather they cooperate with TGF- β 1 signalling to modulate EMT in tissues. An example would be the canonical Wnt signalling pathway, which is mediated by β -catenin, an adherens junction protein physically linked to the cytoplasmic portion of E-cadherin. During the absence of Wnt signaling, β -catenin is targeted for ubiquitination and degradation via GSK-3 β phosphorylation. β -catenin is thus maintained at a low level in the cytoplasm. Activation of Wnt signaling inhibits GSK-3 β activity and rescues cytoplasmic levels of β -catenin, allowing for their sufficient translocation into the nucleus to mediate gene transcription. Thus, EMT-related genes are triggered indirectly by Wnt ligands^{368,369}.

2.1.6) Study Objective

The microenvironmental similarities between chronic inflammation, wounding and cancer are believed to be the key underlying drivers of cancer invasiveness. Both wounds and tumors are sites of heavy inflammation and high oxidative stress. Indeed, wounds and tumors are predisposed to EMT. To close the wound, keratinocytes at the leading wound edge undergoes partial EMT to migrate across the wound bed¹². We have previously shown that the healing wound is an oxidative stressful environment, whereby the expression and activity of TAK1 are up-regulated. We showed that TAK1 has a vital ROS protective effect on keratinocytes²⁵⁰. ROS-induced oxidative stress in tumors is also known to drive carcinogenesis. While the relationship between ROS and EMT could be inferred from the involvement of chronic inflammation in cancer progression, few studies establish a direct link between ROS and EMT. TAK1 resides at the crossroads of inflammation and cancer. TAK1 is a key inflammation signaling mediator of several proinflammatory cytokines (ie. IL-1, TNF- α , IFN- γ) and a non-canonical downstream

effector of TGF- β 1¹⁶⁶. Given its newly found role in oxidative stress response, the signaling role of TAK1 is likely a prime candidate to link chronic inflammation, wounding and cancer, especially the relationship between ROS and EMT.

In this study, we identified an up-regulation of ROS mediated oxidative stress in TAK1 deficient human keratinocytes. This ROS elevation was observed to enhance cell death in keratinocytes in the context of inflammatory type trigger (ie. TNF- α) or anoikis which is likened to keratinocytes in a differentiating epidermis subjected to detachment from the basement membrane. Given that TAK1, a key inflammatory signalling protein participated in this vital oxidative stress protective role of skin cells during wounding and inflammation, the implications of TAK1's redox modulatory role in skin tumor cells is thus worthy of a future study.

An excellent approach and comparative study to keratinocytes would be the usage of A5-RT3 cell line for the study of TAK1 deficiency in cancer. Being a high-grade metastatic human skin cancer cell line, hallmark tumorigenic processes such as the epithelial to mesenchymal transition, may be studied in A5-RT3 cells and correlated with the TAK1 activity and ROS production. As a preliminary characterization study attempt, lentiviral mediated siRNA knockdown of TAK1 may be effected on A5-RT3 cells to establish a stable cell line. Important research objectives inspired by our findings in chapter 1 would be to evaluate how TAK1 deficiency might possibly influence ROS mediated oxidative stress in skin tumor cells. Does TAK1 deficiency also up-regulate ROS in skin tumor cells as observed in their keratinocyte counterparts? If so, how would the increased oxidative stress contribute to the tumorigenic properties of skin tumor cells? How does oxidative stress differences, if any, pertaining to differential ROS production affect the survivability of skin tumor cells? Given that ROS increase is also observed in wounded epidermis and that keratinocytes at the leading wound edge are documented to possess the status

of a partial EMT, does probable oxidative stress differences stemming from TAK1 deficiency have an influence on key tumorigenic features like EMT? Significantly, TAK1 is also a non-canonical downstream signalling player of TGF- β 1, a well known inducer of EMT in the cancer context. How would TAK1 deficiency, a hindrance to the non-canonical downstream signalling of TGF- β 1 affect the tumorigenicity of skin tumor cells? As such, evaluation of TGF- β 1 induced EMT could be performed and the impact of TAK1 knockdown be investigated.

These questions and experimental requirements would be duly addressed in chapter 2, as we proceed with the preliminary characterization of TAK1 knockdown in skin tumor cells.

Our earlier study has uncovered a crucial ROS regulatory role of TAK1 in the epidermal context. If TAK1 modulates oxidative stress in skin tumor cells, a similarly induced TAK1 knockdown as in chapter 1 would likely bring forth ROS related influences in the context of a metastatic skin cancer. In our present study, we seek to understand the role of TAK1 in cancer during TGF- β 1-induced EMT. A-5RT3, a high grade metastatic human skin squamous carcinoma cell line was chosen because skin cells or their tumor cell derivatives could potentially be exposed to the chronic inflammatory influences characteristic of a wound. TAK1 signalling is thus relevant. A study of TAK1 deficiency in a skin tumor cell line would thus be most relevant in our understanding of cancer promotion in wound and inflammation.

2.2 MATERIALS AND METHODS

2.2.1) Reagents

Antibodies were purchased against integrin β 1, α 5 β 1, vimentin, SNAI2 (Millipore), integrin β 3, β 4, β 5, β 7, secondary NL493-conjugated anti-sheep IgG (R&D Systems), TAK1, NF κ B p65, phospho NF κ B p65, E-Cadherin (Cell Signaling), N-Cadherin, fibronectin, SNAI1, β -tubulin, and secondary HRP-conjugated antibodies (Santa Cruz Biotechnology) for immunoblotting or immunostaining. Transfection reagent ExGen 500 was purchased from Fermentas. Double-promoter lentivirus-based siRNA vector and pFIV packaging kit were acquired from System Biosciences. All restriction enzymes and DNA/RNA-modifying enzymes were obtained from Fermentas. TGF- β 1 and IL-1 were purchased from Peprotech. Otherwise, chemicals were purchased from Sigma-Aldrich. ROCK inhibitor Y27632 was purchased from Selleckchem.com.

2.2.2) Cell lines and culture conditions

Human embryonic kidney (HEK293-T) cells (ATCC CRL 11268) were purchased from American Type Culture Collection (ATCC). A-5RT3 cell line was kindly provided by Dr Petra Boukamp of German Cancer Research Center. A-5RT3 was derived from ras-transfected HaCaT through a series of *in vivo* passages to acquire metastatic traits³⁷⁰. Cell culture medium and supplements used include: Dulbecco's Modified Eagle's Medium (DMEM) and fetal bovine serum (FBS) (Hyclone), puromycin (A.G Scientific, Inc).

2.2.3) Lentivirus-mediated siRNA silencing of TAK-1 gene

2.2.3.1) Cloning of TAK-1 siRNA

Two pairs of sense and anti-sense siRNA sequences targeting human TAK-1 were synthesized (1st Base) (Table 2.1). TAK-1 siRNA set 1 sequence targets TAK-1's mRNA sequence position 757 to 778 while set 2 sequence targets position 1418 to 1438 respectively. These sense and anti-sense oligomers were annealed, phosphorylated and ligated to linearized pFIV-H1 / U6 double promoter vector (System Biosciences). The ligated product was subsequently transformed into *E.coli* Top 10 competent bacteria and selected for ampicillin resistance. Positive clones were identified by PCR using H1 and U6 primers followed by DNA sequencing. To generate control siRNA sequences, the TAK-1 siRNA sequences were scrambled and matched against all sequences in the human database to rule out homology. These scrambled sequences were likewise synthesized (1st Base) and cloned into the lentiviral vector as stated above.

Table 2.1 - Sequence of oligonucleotides used in the study

Name		Sequence
TAK-1 siRNA Set 1	Sense	5'AAAGAATGTGCTGCATGGTGCTGAA 3'
	Anti-sense	5'AAATTCAGCACCATGCAGCACATTC 3'
TAK-1 siRNA Set 2	Sense	5' AAAAATATGGAGCAAGTTCCTGCC3'
	Anti-sense	5' AAAGGCAGGAACTTGCTCCATATT3'
Scrambled siRNA Set 1	Sense	5'AAAGGCGTGTTAAACGGATGGTCAT3'
	Anti-sense	5'AAAATGACCATCCGTTTAACACGCC3'
Scrambled siRNA Set 2	Sense	5'AAAAGCAGCTTATCCGACTATCACA3'
	Anti-sense	5'AAAATGTGATAGTCGGATAAGCTGC3'
H1 primer	H1	5'CTGGGAAATCACCATAAACGTGAA 3'
U6 primer	U6	5'GCTTACCGTAACTTGAAAGTATTTTCG 3'

2.2.3.2) Transfection and transduction of TAK-1 siRNA

The pFIV/siRNA or pFIV/scrambled and packaging plasmids were respectively transfected into HEK 293T cells using TransIT-293 (Mirus). Cells were seeded into a 6-well multi-dish at a density of 10^5 cells per well under conditions of DMEM complete medium. Medium was replaced for the seeded HEK 293T cells before transfection. Twelve microliters of TransIT-293 reagent was added to 200 μ l of serum free DMEM medium followed by incubation at room temperature for 20 min. Two micrograms of DNA comprising of equimolar amounts of pFIV/siRNA, pFIV-34N and pVSV-G respectively were added to this mixture. The resultant mixture was then incubated at room temperature for 30 min and added dropwise to a single well of HEK 293T cells. The culture was then incubated for 48 h at 37°C and 5% CO₂. The medium was collected and reconstituted to a resulting composition of 12% polyethylene glycol 8000 (PEG) using PBS. This mixture was incubated overnight in ice to precipitate the pseudoviral particles. The PEG bound pseudoviral particles were then pelleted via a 4000 x g centrifugation. The pellet was subsequently resuspended with 500 μ l of DMEM complete medium containing 10 μ g/ml of polybrene and added to a single well of 1×10^5 A-5RT3 cells in a 6-well multi-dish. Cells were incubated overnight at culture conditions to facilitate transduction. Successfully transduced cells were selected with 500 ng/ml puromycin and further subjected to western blot and qPCR quantitation of knockdown efficiency.

2.2.4) Reverse-transcription (RT-PCR) and real-time quantitative PCR (qPCR)

Total RNA was isolated from cells or tissues using the RNeasy Mini Kit (Qiagen), following the supplier's protocol. One microgram of total RNA was reversed transcribed with 1 μ g of oligo-dT primers (1ST Base) using 200 units of M-MLV Reverse Transcriptase, RNase H Minus (Promega) in an overall reaction volume of 20 μ l. Resulting product cDNA were subjected to

real time PCR using SYBR-Green Master mix (KAPA Biosystem). Sequences of primers (1st Base) used in this study are listed in Table 2.2.

Table 2.2- qPCR Primer sequences used in this study.

TAK1 qPCR primer 1	GTTCCGTGTAAGGGCTTTGA
TAK1 qPCR primer 2	GAGCAGCTGCCACTTACCTTTACA
E-cadherin qPCR primer 1	GCCGAGAGCTACACGTTCA
E-cadherin qPCR primer 2	GACCGGTGCAATCTTCAAA
N-cadherin qPCR primer 1	TGCGGTACAGTGTAAGTGGG
N-cadherin qPCR primer 2	GAAACCGGGCTATCTGCTCG
Vimentin qPCR primer 1	GTTTCCCCTAAACCGCTAGG
Vimentin qPCR primer 2	AGCGAGAGTGGCAGAGGA
SNAIL qPCR primer 1	TCGGAAGCCTAACTACAGCGA
SNAIL qPCR primer 2	AGATGAGCATTGGCAGCGAG
L27 qPCR primer 1	TGATGGCACCTCAGATCGC
L27 qPCR primer 2	AGAGTACCTTGTGGGCATTAGG

2.2.5) Western blot analysis

Cells were seeded in 33-mm petri-dishes at a density of 5×10^5 cells in 2 ml of complete DMEM medium. Cells were subjected to 24 h of serum starvation prior to cell lysis in 100 μ l lysis buffer (50 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 250 mM NaCl, 1% Triton-X and 0.1% sodium dodecyl sulfate (SDS)). Lysates were incubated on ice for 10 min and centrifuged at $13,000 \times g$ for 10 min at 4°C. The supernatant was collected and protease inhibitor, phenylmethyl sulfonyl fluoride (PMSF; Sigma), was added to a final concentration of 1 mM. Protein concentrations were determined with Bradford assay using NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific Inc). Twenty microgram of proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-

PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore). Immunoblotting for β -tubulin was used for normalization. The primary antibodies were used at dilutions of 1:4000 (TAK-1), 1:2000 (NF κ B p65), 1:2000 (phospho NF κ B p65) and 1:5000 (β -tubulin). Secondary antibodies were used at 1:5000 (anti-rabbit IgG). All incubations were performed in 1 x TBS, pH 7.5, with 0.1% Tween-20 and either 5% fat-free milk (β -tubulin) or 5% bovine serum albumin (TAK-1). Washing was performed with the same buffer without milk or BSA.

2.2.6) Bromodeoxyuridine (BrdU) assay

Cell cycle and proliferation were measured with bromodeoxyuridine (BrdU) assay using reagents from BD Pharmingen™ FITC BrdU Flow Kit. Cells were grown to 50% confluency and incubated with 40 μ M BrdU in growth medium for 30 min at cell culturing conditions. BrdU pulsed cells were trypsinized and fixed with ice cold 70% ethanol for 5 min followed by DNA denaturation with 1.5 M HCl for 30 min. Cells were then treated with FITC conjugated anti-BrdU antibodies (1:50) for 1 h at 37°C in the dark. Cells were finally treated with propidium iodide (1:10) to stain overall DNA content. Between each of the above steps, cells were washed twice through resuspension in PBS. Treated cells were analyzed with the Becton Dickinson (BD™) Accuri C6 flow cytometer using the CFlow® Plus Software.

2.2.7) Immunostaining

A-5RT3_{CTRL} and A-5RT3_{TAK1} cells were fixed with 4% paraformaldehyde for 15 min before blocking and permeabilization in 3% bovine serum albumin (BSA) with 0.05% Triton-X for 1 h, both carried out at room temperature. Next, cells were incubated with anti-vinculin (1:100) or monoclonal anti-E-cadherin (1:25) or anti-N-cadherin (1:25) in 0.3% BSA overnight at 4°C.

Secondary goat anti-rabbit Alexa Fluor 488 antibodies (1:200) in 0.2% BSA were added with incubation for an h at room temperature in the dark. Cells were then incubated with 1:50 dilution of Alexa Fluor 594 phalloidin (Molecular Probes, USA) in 2% BS for 2 hours at room temperature in the dark and counterstained with DAPI (Vectashield). All of the above incubations were performed in a humid chamber to prevent evaporation. Between each step, slides were washed thrice with PBS. Immunofluorescent and phase contrast images were taken using Nikon 80I Eclipse microscope equipped with a Nikon DS-SI1 camera, using a (20x/0.5) DIC objective and the NIS element D3.0 software with constant exposure and gain.

2.2.8) Proximity Ligation Assay (PLA) and analysis

Proximity ligation assay (PLA) is an immunostaining based technology that surpasses conventional immunostaining methods, allowing precise detection of proteins or their interactions to the single molecule resolution, favoring *in situ* quantification³⁷¹. Proximity ligation strategy takes advantage of the fact that 2 DNA aptamers brought sufficiently close together in proximity enables the ligation of oligonucleotides linked to each aptamer affinity probe³⁷². The successful ligation of two such proximity probes brings forth an amplifiable DNA sequence reflective of the identity and quantity of a target. PLA exploits this principle for the detection of single targets, protein interactions etc. Two complementary PLA probes (ie. plus (+) and minus (-) respectively), which are essentially animal species-specific secondary antibodies each attached with a unique short DNA strand serve as the DNA aptamers for proximity ligation. For single target detection, complementary PLA probes both bind to the primary antibody bound to the protein of interest and are brought into proximity for ligation. For protein interaction detection, proximity is achieved when complementary PLA probes respectively bind to two different species primary antibodies each targeting an interacting protein partner. In either case,

two other circle-forming DNA oligonucleotides are then added to facilitate interaction of the DNA strands. DNA strands across the two probes can only cross interact when they are in close proximity ($< 30\text{ nm}$)³⁷¹. These oligonucleotides are enzyme ligated followed by their several-hundredfold replication via rolling circle amplification. Fluorescent labeled complementary oligonucleotide probes are finally added to highlight the amplification product. The resulting high concentration of fluorescence in each single-molecule amplification product is clearly visible as a distinct bright spot when viewed with a fluorescence microscope.

In our study, we employed PLA to detect, visualize and quantify single protein interactions. As a standard procedure for every PLA staining experiment, A-5RT3_{CTRL} and A-5RT3_{TAK1} cells were cultured on 22 x 22 mm coverslips (CellPath) in 6-well plates. Cells on coverslips were fixed with 4% paraformaldehyde for 15 min at room temperature before blocking in 3% Normal Goat Serum (NGS) with 0.05% Triton-X for 1 h at room temperature. Next, cells were incubated with either rabbit polyclonal anti-TAK1 (1:100) or mouse monoclonal anti- $\alpha 5\beta 1$ integrin (1:25) in 3% NGS overnight at 4 °C. Treated samples were washed with 3 changes of PBS of 5 min each, prior to treatment with the secondary PLA probes. Secondary PLA probe was added according to the mode of detection and animal origin of the primary antibodies used. For example in the single molecule detection of TAK1 using rabbit polyclonal primary antibodies, PLA probe anti-rabbit plus (+) and minus (-) probes were added. For the single molecule detection of $\alpha 5\beta 1$ integrin using mouse monoclonal primary antibodies, PLA probe anti-mouse plus (+) and minus (-) probes were added. All PLA probes were diluted 1:5 in 3 % BSA according to manufacturer's protocol and added to the PBS washed cells followed by incubation at 37 °C for 1 h. All probe treated cells were again washed with 3 changes of PBS prior to the performing of the detection protocol as per manufacturer protocol (Olink Bioscience, USA). 0.4 μm pore

membrane filtered PBS was used for all mentioned washing purposes throughout the PLA experiment to maintain the samples dust free. Autofluorescence of dust particles would appear as misleading signals. Images were taken using Carl Zeiss confocal microscope LSM 710 meta using a Plan-Apochromat 63x/1.4 oil DIC objective, and ZEN 2009 LE software with constant exposure and gain. The resulting fluorescent spots were quantified using the “Blobfinder”.³⁷³

2.2.9) Flow cytometry analysis of surface integrins

Cells were seeded and grown on 75 cm² culture flask (Corning) until 50% confluence. Cells were then washed with PBS and incubated with 25mM EDTA/PBS for 20 min at cell culturing conditions. EDTA suspended cells were then spun down at 700rpm and fixed with resuspension in 4% paraformaldehyde for 20 min at room temperature. Fixed cells were spun down at 700rpm and washed with PBS twice through resuspension. Washed cells were then treated with anti- β 1, β 3, β 4, β 5, β 7, α 5 β 1 integrin antibodies respectively at concentration (1:25) in 10% BSA and incubated at room temperature for 1 h. Cells were washed twice with PBS resuspension before incubation in secondary antibody conjugated with Alexa Fluor 488 at concentration (1:100) in 10% BSA at room temperature in the dark for 1 h. Stained cells were analyzed with Becton Dickinson (BDTM) LSRII flow cytometer. Analysis of flow cytometry results were performed with Flowjo software.

2.2.10) Apoptotic assay, ROS staining, imaging and flow cytometry analysis

Detection of apoptotic cells were performed using the Annexin V/propidium iodide staining kit (BD Biosciences) according to the manufacturer’s protocol. Cells were incubated with 10 μ M CM-H2DCFDA (Invitrogen) for 30 min at 37°C prior to trypsinization and analysis by flow

cytometry (LSR II from BD Biosciences). Results were analyzed by the Flowjo software. ROS was quenched with the addition of N-acetyl-L-cysteine (NAC) to a final concentration of 100 μ M. CM-H2DCFDA labeled cells were also imaged with the Nikon 80I Eclipse microscope equipped with a Nikon DS-SI1 camera, using a (20x/0.5) DIC objective and the NIS element D3.0 software with constant exposure and gain.

2.2.11) Cell traction force measurements

To measure cell contraction forces, cells were cultured on PDMS substrate embedded with fluorescent beads. Briefly, glass coverslips were cleaned by use of oxygen plasma before it was placed in a vacuum desiccator containing a small drop of 1H,1H,2H,2H-perfluorooctyltrichlorosilane (Sigma) for 30min. Following which, the surface treated coverslips were removed and 100 μ L of diluted yellow-green FluoSpheres® microspheres (0.2 μ m, carboxylate-modified, 0.1vol%, Molecular Probes) was added to the coverslips with the treated surface facing upwards. This step forms a monolayer of fluorescent beads on the coverslips. Thin film of PDMS were generated by placing 15 μ L of PDMS mixture (before curing, 1:60 w/w of crosslinker to base ratio) on the fluorescent microspheres coated glass coverslips. One layer of Scotch tapes was applied as a spacer before putting the other bare glass coverslip on the top of fluorescent microspheres coated glass coverslips. The PDMS droplet was flatten under the weight of the upper coverslip. The entire assembly was left undisturbed for 5 min and then placed in 37°C incubator overnight before carefully peeling off the upper bonded PDMS thin film with fluorescent microspheres on its top. Only samples with well-dispersed beads were used in the study. Substrates were UV sterilized for 30 min. As PDMS surface does not allow cells to adhere, the substrate was coated by incubation with human plasma derived fibronectin solution (50 μ g/mL in 0.02 M acetic acid for thin coating) overnight to promote cell attachment. Coated

substrates were rinsed with PBS (pH 7.4) solution, and stored in the dark at 4 °C prior to culture. 1×10^3 cells / inserts were seeded for 48h under cell culture conditions. Cells were subsequently treated with 10ng/ml TGF β 1 for 24h before traction force measurement. All experiments were triplicated.

Calculation of CTFs, was based on the elastic moduli of PA substrate and fluorescent beads displacements, which was similar to the technique described by Dembo and Wang (1999)³⁷⁴. Spatial displacement of the beads was tracked by taking images of the fluorescent beads before and after cell trypsinization. The cell traction mapping was based on the Differential Interference Contrast (DIC) image of hMSCs on PA substrate and the corresponding traction stress mapping in pseudocolor, which indicated regions of traction stresses (dark blue to light pink according to stresses from low to high). Briefly, the traction force at discrete point $\vec{f}_i$, located at the position (x_i, y_i) was calculated based on the following formulation: $\vec{u}_t(x, y) = \sum_{i=1}^n \vec{G}(x - x_i, y - y_i) \vec{f}_i$, where $\vec{G}$ denoted the Greens' tensor and $\vec{u}_t$ denoted the experimental displacements of fluorescent beads at position (x_i, y_i) . The overall force of the cell (F) is an integral of the traction field magnitude over the area, $F = \int \int \sqrt{T_x^2(x, y) + T_y^2(x, y)} dx dy$, where $T(x, y) = [T_x(x, y) + T_y(x, y)]$ is the continuous field of traction vectors defined at any spatial position (x,y) within the cell.

PDMS substrate preparation and CTF measurements were all performed by our collaborators, Chen Xiao Dong, Wu Yunlong and Cai Ping Qiang from the School of Material Science and Engineering. I assisted in the preliminary phases of substrate preparation, including the determination of optimal fluorescence bead density and also the bead coating of coverslips. I also determined the optimal PDMS substrate stiffness required for effective traction force measurement and subsequently performed all the required microscopic imaging.

2.3 RESULTS

2.3.1) Lentivirus mediated knockdown of TAK1 in A-5RT3 cells.

In this study, we investigated the effect of TAK1 knockdown in A-5RT3, a high grade malignant and metastatic human skin cancer cell line³⁷⁰. A-5RT3 cells were cotransduced with 2 concentrates of lentiviral particle, each carrying a unique TAK-1 targeting siRNA sequence to suppress endogenous TAK-1 levels through RNA interference. This TAK-1 knockdown cell line is denoted as A-5RT3_{TAK1}. As a control, A-5RT3 cells were similarly transduced with scrambled sequences, of which we will refer to as A-5RT3_{CTRL}. Quantitative real time PCR (qPCR) revealed about a 5-fold reduction in TAK-1 mRNA level (Fig 2.1) of A-5RT3_{TAK1} relative to A-5RT3_{CTRL}. Consistent with the qPCR results, western blot analysis showed TAK1 protein expression was reduced by about 79% in A-5RT3_{TAK1} (Fig 2.1).

Fig 2.1. Quantification of knockdown efficiency. Extracted mRNA from control and knockdown cells were reverse transcribed to cDNA for quantitative PCR reactions. TAK-1 mRNA levels as reflected by cDNA quantities reveal about a 5-fold decrease in the knockdown cells ($P < 0.0001$, $n = 5$). L27 was used as a normalization control. Cell lysates from serum starved control and knockdown cells were compared for TAK1 protein expression with western blotting. Representative blots were shown. TAK1 protein band intensity were measured and normalized with that of β -tubulin. Normalized values of A-5RT3_{CTRL} and A-5RT3_{TAK1} cells are respectively averaged ($n = 5$). Resultant values are divided by that of the control to derive the fold-change values for TAK1 protein levels as indicated above the respective blots. β -tubulin immunoblotting showed equal loading and transfer.

Fig 2.2. PLA verification of TAK1 knockdown. Proximity ligation assay (PLA) was performed on A-5RT3_{CTRL} and A-5RT3_{TAK1} cells to further verify TAK1 knockdown. Each PLA spot represent a TAK1 molecule detected by the primary antibody. PLA spots for 3 selected fields were quantified and averaged over total nuclei number in each field indicated by blue DAPI staining. Difference was found to be statistically significant through student's t-test ($p < 0.0001$). $n=3$

Fig 2.3. A-5RT3_{TAK1} showed reduced TAK1 downstream signaling. Western blot analysis of TAK1's phosphorylation of NF-κB p65 subunit. Cells were treated with TAK1 triggering inflammatory cytokine IL-1 at 20 ng/ml prior to cell lysis and immunoblotting.

The targeted knockdown was further verified with proximity ligation assay (PLA), using TAK1 targeting antibodies and A-5RT3_{TAK1} was found with lower TAK1 levels than A-5RT3_{CTRL} as reflected by a significantly lower count of PLA spots (Fig 2.2).

Immunoblotting of A-5RT3_{TAK1} cell lysates confirmed that TAK1 signalling is effectively diminished as reflected by suppressed nuclear factor kappa B (NFκB) phosphorylation upon treatment with TAK1 signalling inducing proinflammatory cytokine interleukin-1 (IL-1)¹⁶⁶ (Fig 2.3).

Mitogen-activated protein kinases (MAPK) have been known for their roles in modulating cellular processes like gene expression, differentiation, survival, apoptosis and cell proliferation in response to external influences like mitogens, osmotic stress and heat shock³⁷⁵. Being a MAP3K, TAK1 has been implicated in some previous studies to affect cell proliferation^{376,377}. It is thus important to evaluate if A-5RT3_{CTRL} and A-5RT3_{TAK1} cells possess significant differences in the aspects of cellular proliferation and death. Herein, we compared BrdU

incorporated A-5RT3_{CTRL} and A-5RT3_{TAK1} cells through flow cytometry and found no significant difference between their cellular proliferation, as reflected by the percentage of cells in the various cell cycle phases in A-5RT3_{CTRL} (S= 58.3%, G1=27.3%, G2/M=8.5%) vs A-5RT3_{TAK1} (S=59.7%, G1=25.8%, G2/M=8.2%) (Fig 2.4A). Apoptosis assay was also carried out with annexin and propidium iodide staining of A-5RT3_{CTRL} and A-5RT3_{TAK1} cells harvested from their normal culturing conditions (Fig 2.4B). The respectively stained cells were analyzed with flow cytometry and no significant difference was found between the percentage of live cells in A-5RT3_{CTRL} (99.21%) and A-5RT3_{TAK1} (99.05%). (Fig 2.4B)

A

B

Fig 2.4. Comparison of cellular proliferation and death between A-5RT3_{CTRL} and A-5RT3_{TAK1} cells. (A) A-5RT3_{CTRL} and A-5RT3_{TAK1} cells were assessed for proliferation with bromodeoxyuridine (BrdU) incorporation. Cells were pulsed with 40 μ M BrdU for 30 min. Incorporated BrdU were detected with FITC conjugated anti-BrdU antibodies staining. Cells were further stained with propidium iodide (P.I) as a measure of DNA content and subsequently analyzed by flow cytometry. Results were gated to show percentage of cell population in the various mitotic phases (ie. G1, S and G2/M). (B) A-5RT3_{CTRL} and A-5RT3_{TAK1} cells were briefly trypsinized and stained with FITC-conjugated annexin V (1 mg/ml) for 15 min at room temperature in the dark. Cells were further stained with propidium iodide (1 μ g/ml) prior to flow cytometry analysis. Quadrant percentages are indicated in the respective dot plots.

2.3.2) TAK1 deficiency enhanced TGF- β 1 induced EMT in A5-RT3 cells.

TGF- β 1 is a potent inducer of EMT in various cancer cell lines³¹⁴. We subjected A-5RT3_{CTRL} and A-5RT3_{TAK1} cells to TGF- β 1 treatment to investigate the role of TAK1 in the EMT process. Within 48 hours of TGF- β 1 treatment, both A-5RT3_{CTRL} and A-5RT3_{TAK1} cell colonies were observed to transform from previously rounded colonies to polarized colonies with lamellipodial protrusions. Furthermore, A-5RT3_{TAK1} cell colonies were observed to disperse into single cells, which are elongated and flattened as typical of a mesenchymal morphology. This cell dissociation behavior was especially prominent at the lamellipodial leading edges (Fig 2.5).

Fig 2.5. EMT was extensively induced by TGF- β 1 treatment in TAK1 deficient cells. TGF- β 1 treatment were performed for all the below at a concentration of 10 ng/ml for 48h. Representative phase contrast microscopy images of A-5RT3_{CTRL} and A-5RT3_{TAK1} cell colonies are shown. Scale bar = 150 μ m

Immunostaining comparisons of these cells further suggests that TAK1 deficiency led to TGF- β induced EMT, as substantiated by an apparently reduced E-cadherin staining in A-5RT3_{TAK1} post TGF- β 1 treatment. Conversely, N-cadherin staining was elevated in TGF- β 1 induced A-5RT3_{TAK1} (Fig 2.6).

Fig 2.6. Immunostaining of cell-cell adhesion proteins. Immunostaining of E-cadherin and N-cadherin (green) in A-5RT3_CTRL and A-5RT3_TAK1 cells under the indicated treatment respectively. Nuclei were counterstained with DAPI(blue). Scale bar = 150 μ m

Western blotting was performed on cell lysates of A-5RT3_CTRL and A-5RT3_TAK1 with and without 48 h of TGF- β 1 treatment to assess the protein expression of key EMT markers (Fig 2.7). This confirmed E-cadherin's down-regulation and N-cadherin's up-regulation in TGF- β 1-treated A-5RT3_TAK1 relative to A-5RT3_CTRL (Fig 2.7). Furthermore, the significant up-regulation of mesenchymal markers vimentin, fibronectin, SNAIL1 and SNAIL2 was also observed in TGF- β 1 treated A-5RT3_TAK1 (Fig 2.7).

Fig 2.7. Immunoblotting of EMT markers. Immunoblotting of whole cell lysates for EMT markers in A-5RT3_{CTRL} and A-5RT3_{TAK1} 48 h after TGFβ1 treatment. Representative western blots were shown. Samples were normalized with β-tubulin as a loading control. The densitometry values as indicated below respective lanes were normalised with respect to the control. n=3.

A real-time quantitative polymerase chain reaction (qPCR) analysis of EMT markers E-cadherin, N-cadherin, vimentin, fibronectin, SNAI1, SNAI2 and ZEB1³⁷⁸ further confirmed the EMT status of TGF-β1-treated A-5RT3_{TAK1} cells (Fig 2.8).

Fig 2.8. qPCR analysis of EMT markers. Real time qPCR of EMT markers in A-5RT3_{CTRL} and A-5RT3_{TAK1} 48 h after TGFβ1 treatment was performed. Samples were normalized with reference gene L27. Gene expression difference between A-5RT3_{CTRL} or A-5RT3_{TAK1} cells with and without TGF-β1 treatment was found to be statistically significant with the student's t-test ($p < 0.05$). Results shown were averaged from 3 independent experiments.

2.3.4) Increased focal adhesion formation in A-5RT3_{TAK1} cells corresponding to increased surface integrin levels.

Our above findings suggest that TAK1 deficiency significantly enhanced the mesenchymal status of A-5RT3, as evident in the increased EMT of TGF-β1 treated A-5RT3_{TAK1}. Interestingly, a routine focal adhesion staining revealed prominent vinculin staining in the cell-cell junctions and thickened actin stress fibers in A-5RT3_{TAK1} cells even prior to TGF-β1 treatment (Fig 2.9). These changes occur while the untreated A-5RT3_{TAK1} cells are still cobblestone-shaped and have yet to adopt the elongated and flattened mesenchymal morphology.

Fig 2.9. Focal adhesion formation is enhanced in TAK1 deficient tumor cells. Upper panel shows immunostaining of cells for vinculin (green/ 488 nm). Lower panel shows the same samples co-stained with rhodamine conjugated phalloidin (red/ 620 nm). TGF- β 1 was added at 10 ng/ml for 48hrs prior to staining. Representative images of 3 independent experiments were shown.

Functionally, focal adhesions are anchorage points that connect the intracellular stress bearing actin cytoskeleton to the ECM or an extracellular substratum. Hence, each focal adhesion is a macromolecular assembly of actin stress fibers coupled to a membrane integrin through adaptor proteins such as vinculin, talin, α -actinin and filamin, among others. Importantly, the membrane integrin mediates adhesion to the ECM. As we observed increased focal adhesions in A-5RT3_{TAK1}, it is likely that the expression of surface integrin of the TAK1-deficient cells is also increased. To identify the surface integrin changes in A-5RT3_{TAK1}, we conducted surface integrin staining for a randomized selection of integrin subunits β 1, β 3, β 4, β 5 and β 7 (Fig 2.10). Through subsequent flow cytometry analyses of these surface integrin-stained live tumor cells, we found that A-5RT3_{TAK1} had significantly increased surface levels of β 1 integrin and to a lesser extent, elevated levels of integrin β 3 (Fig 2.10).

Fig 2.10. FACS analysis of surface integrin. Tumor cells were incubated with the respective anti-integrin antibodies at 4°C for 30 min, washed and further incubated with FITC-conjugated secondary antibodies (green/ 488 nm). Cells were then harvested by suspension with 25 mM EDTA/PBS treatment. Stained tumor cells were analyzed by flow cytometry. The distribution of tumor cells with regard to their extent of integrin staining was presented in histogram plots with the identity of the stained integrin subunit indicated (top left). Mean fluorescence intensity values of integrin staining for A-5RT3_{CTRL} and A-5RT3_{TAK1} cells are stated above the histogram plots.

We further conducted staining of $\alpha 5\beta 1$ integrin and found that it is also significantly elevated in a manner reminiscent of integrin $\beta 1$ (Fig 2.11). Consistent with the flow cytometry results, PLA staining comparing A-5RT3_{CTRL} and A-5RT3_{TAK1} also showed that $\alpha 5\beta 1$ integrin levels were higher in TAK1-deficient cells (Fig 2.12). The PLA staining pattern also corresponded to that of the previously performed vinculin staining. A similar flow cytometry analysis of active integrin $\beta 1$ supportively showed significantly increased active integrin levels on the surface of A-5RT3_{TAK1} cells (Fig 2.11).

Fig 2.11. Surface integrin $\alpha 5 \beta 1$ and active integrin $\beta 1$ up-regulated in A-5RT3_{TAK1} cells. Surface integrin $\alpha 5 \beta 1$ and active integrin $\beta 1$ were analyzed as described in Fig 2.10. Mean fluorescence intensity values of integrin staining for A-5RT3_{CTRL} and A-5RT3_{TAK1} cells are stated above the histogram plots.

Fig 2.12 PLA staining for integrin $\alpha 5 \beta 1$. PLA was performed on tumor cells using antibody specific for integrin $\alpha 5 \beta 1$. Each red spot depicts an $\alpha 5 \beta 1$ integrin heterodimer. The fluorescent red spots representing integrin $\alpha 5 \beta 1$ staining were quantified for 3 selected microscopic fields and averaged over the total cell number reflected by DAPI (blue) nuclei staining. Results were

presented in a bar graph and the PLA spot difference between A-5RT3_{CTRL} and A-5RT3_{TAK1} cells was found to be statistically significant with the student's t-test ($p < 0.01$). $n=3$.

2.3.5) Cell traction force elevated in TAK1 deficient tumor cells

Epithelial tissue cells are mechanically coupled to one another as well as to the ECM of the basement membrane. Modulations in cell junction connectivity, cellular stress fibers and focal adhesion contact formation are known to effect differential cell traction force (CTF) generation, which are actively transmitted through the physical contacts of an epithelial cell. The eventual physical fate of the epithelial cell is essentially determined by the actual underlying counteracting traction force balance between that derived from cell-cell and cell-ECM contact. EMT is widely accepted as the key phenotypic alteration characteristic of tumor cells as they transit from the formerly static epithelial state to adopt a more motile and invasive cellular status. In fact, it is a hallmark EMT feature that epithelial cells increasingly lose their cell-cell interactions through cell junction dissolution and conversely gain cell-ECM interactions. Much reorganization also occurs to the tumor cell cytoskeleton and its associated molecular machineries to facilitate the EMT process. Together, these changes could result in traction force differences that represent early detectable changes in cells preceding the visual appearance of morphological changes and potentially serves as a useful prognostic indicator for cancer staging. CTF microscopy is the live-imaging technique used in our study to detect traction force changes in EMT. The advantage of CTF microscopy lies in its *in situ* detection which allows for a real time insight of physical force changes in live cells. We investigated the early EMT traction force changes arising in the control and TAK1-deficient tumor cells before cells were individualized from their former colonies, which coincided with a TGF- β 1 treatment period not exceeding 24 hrs. Cells were grown on a fibronectin-treated compliant PDMS substrate (10 kPa) embedding a

monolayer of fluorescence beads. The traction force is determined by measuring the displacement of the fluorescence beads in the x-y plane resulting from the contraction exerted by the cell. Strikingly, TAK1 deficient tumor cells generated a considerably different CTF profile from that of control tumor cells. While traction field was observed to be concentrated along the periphery of the A-5RT3_{CTRL}'s cell colony, intra-colony traction can be detected in the A-5RT3_{TAK1} group (Fig 2.13).

Fig 2.13. Cell traction force elevated in TAK1 deficient A-5RT3 cells. (A) Phase contrast images of cells grown on fibronectin coated PDMS substrates of 10 kPa stiffness and their traction force distribution per area (ie. traction stress (Pa)) indicated in colored areas. TGF- β 1 treatment was performed at a concentration of 10 ng/ml for 24 h. Color scale bar at bottom left of each image denotes traction stress (kPa). Distance scale bar at bottom right of image = 10 μ m. Representative images of 3 independent experiments were shown.

As the CTF exerted is also relative to the cell colony size and shape, measured CTF values are divided over the area covered by the cell colonies to derive traction stress values for comparison between cell colonies of differing sizes. Untreated A-5RT3_{CTRL} cells displayed a mean traction

stress value of 93.4 ± 14.8 Pa, while that of A-5RT3_{TAK1} displayed a mean CTF of 170.7 ± 20.2 Pa (Fig 2.14), indicating higher contractility in TAK1 deficient tumor cells. TGF- β 1 induction resulted in significant CTF increments for both A-5RT3_{CTRL} and A-5RT3_{TAK1} as reflected in their elevated traction stress values of 143.96 ± 31.7 Pa and 461.4 ± 113.22 Pa respectively (Fig 2.14). It is noteworthy that TGF- β 1 induced A-5RT3_{CTRL} cell colonies also adopted a CTF profile pattern comparable to that of untreated A-5RT3_{TAK1}.

Fig 2.14. Mean traction stress values obtained from CTF measurements. Values plotted are means $\pm$ standard deviation of 3 independent measurements. Mean traction stress values were compared between samples with the student's t-test and respective *p* values are indicated in the graph.

2.3.6) Rho inhibition hastened cell separation of TGF- β 1 induced TAK1 deficient tumor cell colonies.

Taken together, our observed CTF profiles corresponded well with our earlier observations of increased integrin expression, focal adhesion and stress fiber formation in untreated TAK1 deficient tumor cells. As stress fiber and focal adhesion formation are known to be regulated by

RhoA protein, a GTP-binding protein with key roles in cytoskeleton organization and cellular migration, we inhibited RhoA's activity to assess how EMT would be affected. We treated TGF- β 1 induced A-5RT3_{TAK1} cells with Y-27632, a selective inhibitor of Rho-associated protein kinase (p160ROCK), the downstream effector of RhoA. Interestingly, we observed that A-5RT3_{TAK1} cells under Rho inhibition dispersed significantly earlier upon TGF- β 1 treatment, within a 24h period as opposed to the usual 48h period (Fig 2.15). Rho inhibited cells were also found to stain weakly for E-cadherin (Fig 2.15).

Fig 2.15. RhoA activity inhibition led to cell separation and E-cadherin loss. A-5RT3_{TAK1} cells with and without TGF- β 1 induction were inhibited with Y27632 at concentration of 10 μ M for 24 h. Phase contrast images of treated cells (Top panel). E-cadherin (green/ 488 nm) immunostaining images (Bottom panel). Nucleus is stained with DAPI (blue). Scale bar = 150 μ m.

Rho inhibition dispersed A-5RT3_{TAK1} cells were immunoblotted for their EMT markers and mesenchymal genes were significantly found to be up-regulated through immunoblotting (Fig 2.16) and qPCR analysis (Fig. 2.17) respectively. We also found that Rho inhibition likewise hastened EMT like cell dispersion in A-5RT3_{CTRL} cells (Fig. 2.18) which was observable within 48 h of TGF- β 1 treatment.

Fig 2.16. Immunoblotting of EMT markers in RhoA inhibited A-5RT3_{TAK1}. Cell lysates of TGF-β1 induced A-5RT3_{TAK1} cells with and without Y27632 induced RhoA inhibition were immunoblotted for EMT markers. TGF-β1 treatment were performed at an concentration of 10 ng/ml for 24 h. Representative blots were shown. Samples were normalized with β-tubulin as a loading control. The densitometry values as indicated below respective lanes were normalised with respect to the control. n=3.

Fig 2.17. qPCR analysis of EMT markers in RhoA inhibited A-5RT3_{TAK1}. mRNA expression of EMT markers in RhoA inhibited A-5RT3_{TAK1} cells was assessed by qPCR. Samples were normalized with reference gene L27. Gene expression difference between TGF-β1 induced A-5RT3_{TAK1} cells with and without Y27632 treatment was found to be statistically significant with the student's t-test ($p < 0.01$). Results shown were averaged from 3 independent experiments

Fig 2.18. RhoA activity inhibition also hastened EMT in A-5RT3_{CTRL} cells. A-5RT3_{CTRL} cells with and without TGF-β1 induction were inhibited with Y27632 at concentration of 10 μM for 48 h. Representative phase contrast images of treated cells were shown. Scale bar = 150 μm.

2.3.7) Increased ROS production in TAK1 deficient A-5RT3 cells.

Our earlier results have clearly shown that TAK1 deficiency enhanced the mesenchymal state of A-5RT3 cells. Even though EMT is rightfully triggered through TGF-β1 induction, we have observed that TAK1 deficient cells were poised towards mesenchymal transformation even without TGF-β1 triggering. It is likely that TAK1 deficiency created a favorable cellular environment for A-5RT3 cells to adopt the mesenchymal form. We attempted to address the actual identity of this EMT-promoting cue.

In our earlier work, we found that TAK1 deficiency increased ROS production in human keratinocytes. We hypothesized that A-5RT3 cells may respond similarly towards TAK1 deficiency. We used CM-H₂DCFDA, a dye that reacts with intracellular ROS and is converted to fluorescent carboxy-H₂DCFDA to detect ROS changes in our tumor cells. Through flow cytometry analysis of A-5RT3_{TAK1} and control cells prestained with CM-H₂DCFDA, we found that ROS levels were likewise up-regulated in the TAK1-deficient skin cancer cells (Fig 2.19). Additionally we found that TGF-β1 treatment further up-regulated ROS levels in both A-5RT3_{CTRL} and A-5RT3_{TAK1} cells (Fig 2.19).

Fig 2.19. ROS production elevated in TAK1 deficient A-5RT3 cells and is implicated in TGF-β1 induced EMT process. ROS staining of live cells was conducted. A-5RT3_{CTRL} and A-5RT3_{TAK1} cells with the respectively indicated treatments (right panel) were incubated with 1 μM of CM-H2DCFDA for 30 min under cell culturing condition. Subsequently stained cells were trypsinized and analyzed by flow cytometry. Antioxidant NAC treated cells served as a negative control.

Notably, ROS levels of TGF-β1-treated A-5RT3_{CTRL} were raised to levels nearing that of untreated A-5RT3_{TAK1}. TGF-β1 treated A-5RT3_{CTRL} and A-5RT3_{TAK1} cells stained with CM-H2DCFDA were imaged with phase contrast microscopy. Mesenchymal TGF-β1 induced A-5RT3_{TAK1} cells showed significantly increased ROS levels relative to A-5RT3_{CTRL} (Fig 2.20).

Fig 2.20. Mesenchymal TAK1 knockdown cells have increased ROS. TGF-β1 treated cells were stained with 1 μM of CM-H2DCFDA for 30 min under cell culturing condition, and viewed with phase contrast and fluorescence microscopy. Representative images are shown. Scale bar = 100 μm.

Using antioxidant N-acetyl-L-cysteine (NAC), which quenches ROS production, we successfully inhibited the cell separation characteristic of TGF- β 1-induced EMT in A-5RT3_{TAK1} (Fig 2.21). These NAC treated A-5RT3_{TAK1} cells remained in colony form despite TGF- β 1 treatment and displayed positive E-cadherin immunostaining (Fig 2.22). Immunoblotting of EMT markers in these NAC treated A-5RT3_{TAK1} cells also showed significantly reduced mesenchymal gene expression despite TGF- β 1 induction (Fig. 2.23) which was further verified with qPCR analysis (Fig. 2.24). This substantially confirms a role of ROS in modulating EMT. While A-5RT3_{CTRL} cells usually require a significantly longer TGF- β 1 treatment time (ie. up to 72 h) than A-5RT3_{TAK1} cells to attain EMT, we found that NAC treatment could likewise prevent the occurrence of EMT in the control cells as well (Fig. 2.25).

Fig 2.21. NAC inhibition of TGF- β 1 induced EMT in A-5RT3_{TAK1} cells. Cells with and without TGF- β 1 treatment were treated with 100 μ M of NAC to quench ROS production and viewed with phase contrast microscopy. Scale bar = 150 μ m.

Fig 2.22. E-cadherin immunostaining of NAC treated cells. TGF-β1 treated A-5RT3_{TAK1} cells with and without NAC (100 μM) incubation were stained for E-cadherin. Scale Bar = 150 μm.

Fig 2.23. Immunoblotting of EMT markers in NAC treated A-5RT3_{TAK1}. Cell lysates of TGF-β1 induced A-5RT3_{TAK1} cells with and without NAC (100 μM) treatment were immunoblotted for EMT markers. TGF-β1 treatment were performed at a concentration of 10 ng/ml for 48 h. Representative blots were shown. Samples were normalized with β-tubulin as a loading control. The densitometry values as indicated below respective lanes were normalised with respect to the control. n=3.

Fig 2.24. qPCR analysis of EMT markers in NAC treated A-5RT3_{TAK1}. mRNA expression of EMT markers in NAC (100 μ M) treated A-5RT3_{TAK1} cells was assessed by qPCR. Samples were normalized with reference gene L27. All observed gene expression difference were found to be statistically significant with the student's t-test ($p < 0.01$). Results shown were averaged from 3 independent experiments.

Fig 2.25. Anti oxidant NAC treatment also inhibited EMT in A-5RT3_{CTRL} cells. TGF-β1 induced A-5RT3_{CTRL} cells were treated with NAC at concentration of 100 μ M. Representative phase contrast images of treated cells were shown. Scale bar = 150 μ m.

2.4) DISCUSSION

In this study, we knocked down TAK1 in metastatic human skin tumor cell line A-5RT3 and found that TGF- β 1 induced EMT was significantly hastened and enhanced as a result. This EMT change corresponded to significantly increased cell traction force changes, mesenchymal gene expression up-regulation, increased surface α 5 β 1 integrin expression etc. Similar to the response of human keratinocytes, ROS was also found to be up-regulated in TAK1 knockdown A-5RT3 through flow cytometry studies. Interestingly in TGF- β 1 induced A-5RT3_{TAK1} cell colonies that have partially undergone EMT, intense ROS staining was distinctively detected in the dispersed mesenchymal skin tumor cells as observed through phase contrast microscopy imaging. Moreover, we also found that this TGF- β 1 induced EMT could be inhibited by antioxidant treatment (ie. NAC) or hastened by chemical inhibition of RhoA.

Most cancers have also been linked to ROS, including cancer of the bladder, brain, breast, cervix, gastric, ovarian, liver and skin²⁴⁹. Cancer cells are known to have higher ROS levels than normal cells²⁶⁶, the dogma being that increased cellular ROS levels is associated with tumor aggressiveness and poor prognosis²⁶⁶. Moreover, the tumor cells of late-stage cancers are known to be further subjected to greater oxidative stress, suggesting an important role of ROS in facilitating the spread of cancer²⁶⁶. However, the exact mechanism underlying ROS involvement remains elusive.

In our current study, metastatic skin tumor cells with TAK1 deficiency were clearly observed to undergo TGF- β 1 induced EMT earlier. Uniquely, TAK1-deficient skin tumor cells were more motile with a greater display of focal adhesions and thick actin stress fibers, even in the absence of TGF- β 1 treatment. The increased focal adhesions observed in TAK1-deficient tumor cells are also associated with an increased surface expression of integrin α 5 β 1, which is a well known

mesenchymal marker in cancer. Integrin $\alpha 5\beta 1$ is the primary receptor for another EMT marker, namely fibronectin, an ECM protein highly abundant in cancer³⁷⁹. Thus, increased levels of tumor cell surface integrin $\alpha 5\beta 1$ may facilitate better cell migration over secreted fibronectin, enhancing EMT as observed in TAK1 deficient cells. We also found that these TAK1 deficient tumor cells with our previously described propensity towards TGF- $\beta 1$ induced EMT possess differences in their cell traction force profile patterns which represent early detectable *in situ* physical changes. In addition, RhoA inhibition of TAK1-deficient tumor cells significantly sped up cell separation consequent of TGF- $\beta 1$ induction. Furthermore, we also observed that TAK1 deficiency up-regulated ROS production in tumor cells, as in normal skin cells. Importantly, we found that an antioxidant inhibition of this elevated oxidative stress significantly suppressed EMT. This strongly suggests that TAK1 has a role to play in tumor EMT, with implications in the redox modulation of RhoA activity.

The Rho family of small GTP binding proteins play a central role in epithelial morphogenesis and dynamically control cell polarity. Within this family, Rho, Rac and Cdc42 are best characterized and are known to control many cellular processes in EMT³⁸⁰. Rho controls the formation of stress fibers and focal adhesion while Rac and Cdc42 regulate membrane ruffling and filopodium formation respectively³⁸⁰. The mesenchymal type motility of cells is known to be attributed to Rac1 activity which organizes actin polymerization and lamellipodium formation³⁸⁰. Hence Rac1 activity is enhanced at the leading edge of a migrating cell where the formation of actin-rich protrusions is most relevant for its forward movement³⁸⁰. RhoA activity on the other hand promotes cell edge retraction rather than protrusion, thus its activity is purposefully concentrated in the trailing edge of the cell. Befitting its role, RhoA activity leads to the rounding up of cell edges rather than their spreading on a substratum³⁸⁰. Hence RhoA activity needs to be

reduced at the cell front which is undergoing mesenchymal movement, to allow for the Rac1 induced extension of actin-rich protrusions. Mutual antagonism is known to exist between Rac and Rho GTPases function. This extracellular activation of Rac1 through the engagement of ECM by the integrin receptors of focal adhesion complexes directly enhances cellular ROS production which in turn leads to the redox inhibition of Low-molecular weight protein tyrosine phosphatase (LMW-PTP) activity by the oxidation of its catalytic cysteine residues responsible for intermediary phosphate group transfer^{380,381}. This results in the retained and increased phosphorylation of LMW-PTP's substrate p190RhoGAP which subsequently downregulates RhoA activity³⁸¹.

In our study, it is possible that ECM engagement through the increased focal adhesions and surface integrin expressed on TAK1 deficient tumor cells directly increased Rac1 activity and consequently the downstream ROS production. This elevated oxidative stress then inhibits RhoA activity through the redox modification of LMW-PTP. However, further studies are required to verify this signalling possibility. Importantly, RhoA function has also been implicated in the classical EMT of chick epiblast cells during gastrulation³⁸². Epithelial cells of the epiblast that do not undergo EMT were found with enriched RhoA localization and activity in their apical and basal cytoplasmic regions, presumably with epithelial cell polarity maintaining roles³⁸². In contrast, this RhoA presence in medial cells of the epiblast that were slated to undergo EMT was found to be absent³⁸². This again highlights the significance of RhoA inhibition and its involvement in EMT promotion.

The role of TAK1 in cancer has also been intensively studied by several other groups. In a separate study, skin tumors were generated in mice with a tumor initiator (i.e. DMBA) and promoter (i.e. TPA) treatment. These tumors were then subjected to epidermal-specific TAK1

knockout, whereby most tumor cells became apoptotic because of elevated ROS levels³⁸³. However, this epidermal TAK1 knockout only mildly caused apoptosis in the non-tumor regions of the adult mouse skin³⁸³. In contrast, TAK1-deficient tumor cells were not observed to be highly apoptotic in our present study even though TAK1 suppression similarly led to elevated ROS levels. This difference in cell survival status could be may be due to the influence of other surrounding cell types, such as cancer-associated fibroblasts, which may mask the effect of TAK1 deficiency or to differences between human and mouse tumor cells. .

Recently, TAK1 inhibition was importantly found to reverse chemoresistance in pancreatic cancer cells³⁸⁴. Pancreatic cancer is well known to be a long standing major treatment challenge. Being asymptomatic in early disease development, pancreatic cancer is usually diagnosed only at advanced stages whereby the efficacy of surgical resection is limited. This mainly leaves only chemotherapeutic treatment options which prospects are further worsened as pancreatic tumor cells are particularly known to resist most first line chemotherapeutic drugs^{385,386}. Much research has since been dedicated to further the understanding of chemoresistance typical of pancreatic cancer. In particular, TAK1 inhibition through knockdown and chemical means has been shown to augment the proapoptotic effect of chemotherapeutic drugs on pancreatic cancer cells³⁸⁴. This was related to the inhibition of the NFκB and activator protein-1 (AP-1) mediated prosurvival pathways which are downstream of TAK1³⁸⁴. However, the knockdown of TAK1 in pancreatic cancer cells in this study resulted in cells that were markedly different from control cells in terms of morphology, growing as highly clustered colonies that were less spread³⁸⁴. In other related studies, chemoresistant pancreatic cancer cells were found to be those undergoing EMT³⁸⁷⁻³⁸⁹. It is proposed that a more mesenchymal, and hence cancer stem-cell like state tumor cell is less proliferative and correspondingly less adversely affected by growth inhibitory drugs³⁹⁰. In fact, a

mesenchymal cell that has undergone a classical EMT is known to be unproliferative³⁹⁰. In these pancreatic cancer studies, ROS levels were not assessed either in the chemoresistant mesenchymal cells or TAK1-inhibited tumor cells. It is likely that knockdown or inhibition by a small molecule inhibitor (i.e. LYTAK1) led to a more thorough TAK1 suppression, hence spurring excessively high ROS levels, giving rise to oxidative stress challenged tumor cells that are death prone and susceptible to chemotherapy.

Tumor cells are consistently challenged by elevated oxidative stress, given their rapidly growing and metabolically demanding nature²⁵². As such, it is rational that tumor cells are better equipped with protective and survival features to counter the effects of excessive ROS²⁶⁶.

We also observed that TGF- β 1 induced ROS up-regulation in control skin tumor cells led to mesenchymal changes. Thus, it is likely that elevated ROS through TAK1 deficiency, TGF- β 1 induction or even chemotherapeutic treatments, might serve as a survival cue for tumor cells, driving their EMT. Given that anti-oxidant suppressed EMT through ROS inhibition and that EMT is characteristic of chemoresistance, it is likely that anti-oxidant treatment could serve as a strategy to diminish chemoresistance.

2.5 FUTURE DIRECTIONS

Having found that TAK1 deficiency led to elevated ROS levels in skin tumor cells, the next objective would be to uncover the mechanism underlying this dysregulation and how high ROS amounts trigger EMT. A large scale protein array could be conducted to assess the differences in cytokine expression of A-5RT3_{CTRL} and A-5RT3_{TAK1}. More studies may be performed to assess the status of ROS triggered pathways in A-5RT3_{TAK1} like the extent of AKT pathway's activation.

In our study, TAK1 deficient tumor cells are shown to possess higher surface expression of integrin β 1, β 3 and α 5 β 1, which may be implicated in up-regulating ROS production through Rac1 activation. This might then lead to the reciprocal control of Rac1 and RhoA. To evaluate this hypothesis, a function blocking anti- β 1 integrin antibody such as AIIB2³⁹¹ may be used to inhibit β 1 integrin mediated signalling to assess if Rac1 activation and ROS levels are hence affected. Rac1's role in inducing ROS production must also be verified. As a means to promote or diminish Rac1 activity, A-5RT3_{CTRL} and A-5RT3_{TAK1} cells could be transfected with plasmids encoding the constitutively active or the dominant negative Rac1 respectively. Transfected cells may be further analyzed with flow cytometry to evaluate oxidative stress levels. If Rac1 induced ROS production promotes EMT, TGF- β 1 induction of A-5RT3_{CTRL} cells transfected with constitutively active Rac1 should morphologically resemble that of A-5RT3_{TAK1}.

Importantly, TAK1 deficient tumor cells which possess higher ROS levels are hastened into EMT upon chemical based RhoA inhibition. Hence studies should also be conducted to assess if a redox inhibition of RhoA may be involved in promoting EMT. This could be achieved by assessing the oxidation status of LMW-PTP in A-5RT3_{TAK1} cells with and without anti-oxidant

treatment (ie. NAC), using proven antibody based detection methods. The higher ROS level in A-5RT3_{TAK1} should correspondingly increase its content of oxidized LMW-PTP, a feature that should also be rescued by NAC treatment. The sensitive cysteine-targeted biotin switch method to label oxidatively-modified proteins could be employed for the above purpose. The concurrent status of RhoA and Rac1 activity may be monitored with antibodies targeting their active or GTP bound forms. Sepharose beads with immobilised protein A may then be used to pull down and enrich these antibody bound GTP binding proteins for immunoblotting analysis.

It is also crucial to address how TAK1 knockdown skin tumor cells up-regulate their ROS levels. One probable link derived from our observation would be the increased surface display of integrin $\alpha 5 \beta 1$ on TAK1 deficient skin tumor cells. Given that integrin $\beta 1$ activation may activate Rac1 which subsequently activates NOX generate ROS³⁹². This rac/NOX/ros-dependent mechanism which interacts in a protein complex manner may be assessed by proximity ligation assay for dual targets using active integrin $\beta 1$, active Rac1 and Nox antibodies. To evaluate the relationship between Rac1 and ROS, plasmids expression vectors of constitutively active (G12V) and dominant negative Rac mutant (T17N), respectively could be transfected into A5RT3_{CTRL} and A5RT3_{TAK1}. The resultant cells could be DCF stained and subsequently analyzed with FACS for a comparative study of their ROS content. These transfected cells should also be assessed for their EMT potential upon TGF- $\beta 1$ induction.

Furthermore, the means of TGF- $\beta 1$ induced EMT ought to be evaluated. It is likely believed to be through SMAD signalling owing to TGF- $\beta 1$ involvement. SMAD inhibition should be performed, preferably with transient SMAD siRNA transfection mediated knockdown followed by the assessment of EMT status (ie. immunoblotting, qPCR) upon TGF- $\beta 1$ treatment.

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