Functional roles of transforming growth factor activated Kinase-1 (TAK1) in skin wound healing

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**FUNCTIONAL ROLES OF
TRANSFORMING GROWTH FACTOR
ACTIVATED KINASE-1 (TAK1)
IN SKIN WOUND HEALING**

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ABSTRACT

Healing wound and maintaining new skin growth requires complex interactions of the epithelium and mesenchyme purportedly mediated by growth factors and cytokines. We show here that during wound healing, TAK1 activates von Hippel-Lindau tumor suppressor (pVHL) expression in keratinocytes which, in turn, represses the expression of PDGF-B and, integrins $\beta 1$ and $\beta 5$ via inhibition of the Sp1-mediated transcription. The reduced production of PDGF-B leads to a paracrine decrease in expression of hepatocyte growth factor (HGF) in the underlying fibroblasts, as well as reduced epidermal proliferation. This TAK1 regulation of the dual PDGF/HGF paracrine signaling system can regulate keratinocyte cell proliferation and is required for proper wound healing. Strikingly, TAK1 deficiency enhances cell migration. TAK1-deficient keratinocytes displayed lamellipodia formation with distinct microspikes protrusions, which was associated with an elevated expression of integrins $\beta 1$ and $\beta 5$, and sustained activation of cdc42, Rac1 and RhoA. Our findings provide evidence for a novel homeostatic control of keratinocyte proliferation and migration, mediated via TAK1 regulation of pVHL. Dysfunctional regulation of TAK1 may contribute to the pathology of chronic inflammatory wounds and psoriasis.

This thesis is based on the following original publications, with the main focus on publication (1). Some unpublished data are also included.

- (1) **Tan SH**, Pal M, Tan MJ, Wong MH, Tam FU, Teo JW, Chong HC, Tan CK, Goh YY, Tang MB, Cheung PC, Tan NS (2009) Regulation of cell proliferation and migration by TAK1 via transcriptional control of von Hippel-Lindau tumor suppressor. *J Biol Chem* 284: 18047-18058

- (2) Chong HC, Tan MJ, Philippe V, **Tan SH**, Tan CK, Ku CW, Goh YY, Wahli W, Michalik L, Tan NS (2009) Regulation of epithelial-mesenchymal IL-1 signaling by PPARbeta/delta is essential for skin homeostasis and wound healing. *J Cell Biol* 184: 817-831
- (3) Lam CR, **Tan SH**, Tan MJ, MBY Tang, Cheung, NS Tan (2011) TAK1 regulates SCF expression to modulate PKB-alpha activity that protects keratinocytes from ROS-induced apoptosis. *Cell Death Differ* doi:10.1038/cdd.2010.182

LIST OF ABBREVIATIONS

AKT1	see PKB α
AP-1	Activated protein-1
ATF	Activating transcription factor
BMP	Bone morphogenetic protein
ca	Constitutively active
ChIP	Chromatin immunoprecipitation
CK1	Cytokeratin 1
CK5	Cytokeratin 5
CK10	Cytokeratin 10
CK14	Cytokeratin 14
Col	Collagen
Ctrl	Control
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
EMT	Epithelial-mesenchymal transition
EphA	Receptors of class A ephrins proteins
EphB	Receptors of class A ephrins proteins
EMSA	Electrophoretic mobility shift assay
FAK	Focal adhesion kinase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GSK	Glycogen synthase kinase
HLA	Human leukocyte antigen
HE	Haematoxylin and eosin staining
HEK	Human embryonic kidney
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
Fgr	Gardner-Rasheed feline src viral oncogene homolog
IKK	NIK-I κ B kinase
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IL-1ra	Interleukin 1 receptor antagonist
IRS-1	Insulin receptor substrate-1
ISGF3 γ	Interferon-stimulated transcription factor 3 γ

JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K _{CTRL}	Control keratinocytes
K _{TAK1}	TAK1-knockdown keratinocytes
KGF	Keratinocyte growth factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MX1	Myxovirus resistance 1
NFκB	Nuclear factor kappa B
NGFR	Nerve growth factor receptor
NEDD8	Neural precursor cell expressed developmentally down-regulated protein 8
NIK	NFκB-inducing kinase
OAS1	2', 5'-oligoadenylate synthetase isoform 1
OAS2	2', 5'-oligoadenylate synthetase isoform 2
OTC	Organotypic skin culture
PAI-1	Plasminogen activator inhibitor-1
PBMC	Peripheral blood mononuclear cells
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PIGF	Placenta growth factor
PI3K	Phosphoinositide-3 kinase
PKBα	Protein kinase B alpha (also known as AKT1)
PLA	<i>In situ</i> proximity ligation assay
PPAR β/γ	Peroxisome proliferator-activated receptor β/γ
PTN	Pleiotrophin
pVHL	Von Hippel-Lindau tumor suppressor protein
qPCR	Quantitative real-time PCR
ROS	Reactive oxygen species
RT-PCR	Reverse-transcription PCR
SAPK	Stress-activated protein kinase
SDF-1	Stromal-derived factor 1
siRNA	Small interfering RNA
SMAD	Similar to Mother Against Decapentaplegic drosophila
SnoN	Ski-related novel protein
Src	Sarcoma

TAB	TAK1 binding proteins
TAK1	Transforming growth factor-beta activated kinase 1
TGF- β	Transforming growth factor-beta
TNF- α	Tumor necrosis factor-alpha
TNFRp55	TNF receptor p55
TRAF	TNF receptor-associated factor
TRAIL	TNF-related apoptosis-inducing ligand
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
pVHL	Von Hippel-Lindau tumor suppressor protein

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1 INTRODUCTION

1.1 The health importance of human skin in general and the epidermis in particular

Although researchers continue to develop various artificial substitutes for healthy, intact skin, there is currently no true replacement. Skin is the human body's largest and also one of the most complex organs. It provides many important functions in humans such as providing the body with a barrier against infection and helping to prevent dehydration. Healthy human skin can also serve to moderate body temperature by effecting changes in the blood flow and the evaporation of sweat. Moreover, as skin provides humans with the sense of touch, one of the five fundamental human senses, it therefore represents a vitally important sensory organ that enables humans to discern hot from cold, as well as and potentially harmful encounters with the environment.

Although the thickness of skin can vary as people age and location on the body, skin is remarkably thin, typically only 1 to 2 millimeters thick. Irrespective of its location or thickness, human skin consists of two discrete layers: (a) a thin outer epidermis and (b) a thicker dermis layer beneath which provides nourishment for the epidermis and is more sensitive than the underlying dermis as well (Edmondson et al, 2003).

Given the enormous amount of sensory information the outer epidermis must cope with throughout an individual's life, it is little wonder that this layer is less sensitive; if this were not so, one would be bombarded with an overload of tactile sensations. In fact, the top-most skin layer is comprised of rugged flat cells that are actually dead and are analogous to roofing shingles (Madison, 2003;

Nemes & Steinert, 1999). As they are worn away, these outermost skin cells are replaced with new cells from the epidermal layer below, which is composed of keratinocytes that divide rapidly and are thus able to furnish the body with these replacement cells as they are needed. Keratinocytes also manufacture a rugged protein known as keratin and they contain a type of vital fatty compound called sebum that serves to make skin waterproof (Madison, 2003; Nemes & Steinert, 1999). Sebum is made up of many types of lipids such as sapienic acid, squalene and wax esters. In addition, the epidermis contains melanocytes that provide skin with its color.

The most dominant cell type in the dermis is the fibroblast. However, the dermis also has lymph, blood vessels, as well as a series of nerves. Hair follicles and glands that produce sweat and oil glands are also situated deep in the dermis, which is composed primarily of connective tissue. The connective tissue embeds elastic fibers and collagen, which is the most commonly occurring protein in the human body, in the ground substance such as dermatan sulfate. Both collagen fibers and ground substance are bound to fibronectin, an adhesion protein, which serves to link them together. Collagen provides the skin with flexibility as well as structural support. The dermis is capable of keeping out unwanted pathogens and it also serves an important function during wound contraction and scarring (Ghahary & Ghaffari, 2007; Werner et al, 2007).

1.2 Epidermal structure and homeostasis

The epidermis is the outermost layer of the skin and it is primarily made up of keratinocytes. This epidermal layer of keratinocytes can be further differentiated into four striated layers of cells, which differ in their potential for both proliferation and differentiation (Fuchs & Raghavan, 2002). The four layers of keratinocytes are described further in Table 1 and illustrated in Figure 1.

Figure 1. A schematic drawing of the four striated layers of the epidermis with intercellular connections. *Adapted from (Fuchs & Raghavan, 2002)*

Table 1. The four striated layers of keratinocytes in the epidermal layer. (Menon, 2002)

Layer	Description
Basal layer	The basal layer is also known as stratum basale. It is adjacent to the cutaneous basement membrane. Proliferation principally occurs in this basal layer of epidermis. Cells located in the layers above the basal layer are terminally differentiated. As a result, they are least susceptible to damage that could produce a cancerous growth.
Spinous layer	The spinous layer is also known as stratum spinosum or “prickle cell” layer. From the basal layer, cells migrate into the spinous layer and are transformed from columnar to polygonal cells. Cells in this layer begin to synthesize keratin.
Granular layer	The granular layer is also known as stratum granulosum. This is the layer in which cells accumulate dense basophilic keratohyalin granules which contain lipids which, together with the desmosomal connections, function to assist in providing the waterproof barrier that serves to prevent fluid loss from the body.
Stratum corneum	This is the outermost, cornified layer also known as the keratinous or the "horny" layer.

The framework of normal skin development is based on its capacity to sustain control of epidermal proliferation, growth and cell death. The epidermis is a stratified squamous epithelium. It creates a barrier that prevents harmful pathogens from entering while concomitantly retaining body fluids (Lechler & Fuchs, 2005). In order to achieve these functions, proliferative basal cells contained in the innermost layer detach themselves from time to time from the underlying basement membrane of extracellular matrix, migrate outward and ultimately die. While they were at one point in the suprabasal layer, those cells subsequently cease their division and begin a differentiation program to create this aforementioned barrier. However, the mechanism by which stratification is achieved remains unclear (Lechler & Fuchs, 2005). *In vitro* research has suggested that stratification takes place through the delamination and subsequent movement of epidermal cells. However, the majority of culture conditions tend to support the notion that keratinocytes do not possess the polarity and cuboidal morphology of basal keratinocytes that are present in these tissues. These findings may be significant in terms of identifying an alternative mechanism; one possible explanation is that stratification takes place via asymmetric cell divisions, in which the mitotic spindle orients perpendicularly to the basement membrane (Lechler & Fuchs, 2005).

Currently, there are two paradigms of epidermal homeostasis that are widely accepted; these provide differing perspectives on how the mechanism of proliferation of basal keratinocytes (e.g. distribution of cellular contents and attachment of daughter cell to the underlying basement membrane) facilitates the differentiation and stratification of the epidermis (Figure 2).

Figure 2. Two potential models of how epidermal cells contribute to the dynamic maintenance of the epidermis. **(a)** Symmetric cell division or delamination model and **(b)** Asymmetric cell division. *Adapted from (Fuchs, 2007)*

The first paradigm supports symmetric cell division, in which daughter cells are initially attached to the basement membrane, and then tend to experience delamination following their receipt of differentiation signals (Fuchs, 2007; Mackenzie, 1970). In contrast, the second paradigm holds that asymmetric cell division is involved in which each division spawns a daughter stem cell that retains its attachment to the basement membrane as well as a detached suprabasal daughter cell (Lechler & Fuchs, 2005). The latter perspective also holds that the apical side will enter differentiation. Although these two paradigms differ in these important aspects, both of these systems most likely play a role in achieving and maintaining epidermal homeostasis.

In the process of epidermal homeostasis, proliferation and terminal differentiation are coordinated such that the former takes place primarily in the basal layer of epidermis. The epidermal cells will progressively engage in the differentiation program as they travel through the suprabasal layer from the dermal-epidermal boundary towards the apical side, thereby developing a well-organized stratified epithelium. Cytokeratin 5 (CK5) and 14 (CK14) are expressed

early in the differentiation program; in contrast, cytokeratin 1 (CK1) and 10 (CK10) are expressed during later phases of differentiation. Consequently, as the differentiation program is executed, the expression pattern of cytokeratins in the suprabasal keratinocytes changes (Byrne et al, 1994; Fuchs & Green, 1980). Terminally differentiated, keratinocytes express proteins such as involucrin, filaggrin and loricrin (Eckert & Green, 1986; Hohl et al, 1991; Rice & Green, 1977; Steven et al, 1990). The concentration of these proteins in keratinocytes, as well as the loss of nuclei and organelles, takes place during their commitment to terminally differentiate, resulting in the creation of stratum corneum. These biological events, among others, are orchestrated by a variety of growth factors and cytokines which simultaneously activate a network of signaling cascades that often have opposing effects (Maas-Szabowski et al, 1999).

The maintenance of epidermal homeostasis is not limited to keratinocyte regulatory signals. A few studies have demonstrated that maintenance of normal epidermal tissue requires a continuous exchange of signals with the underlying dermal environment, which is referred to as, epithelial-mesenchymal communication (Maas-Szabowski et al, 1999; Maas-Szabowski et al, 2000; Smola et al, 1993). Fibroblasts comprise a major cell type in the dermis, and their functions in the regulating extracellular matrix and the expression of growth factors determine the differentiation states of the epidermis.

1.3 Epithelial-mesenchymal communication during wound healing

Regardless of its specific mechanisms, the immediate objective of the skin in response to a wound is to repair itself, thereby re-establishing tissue integrity and homeostasis. The three phases of the healing process, inflammation, re-epithelialization and tissue remodeling, take place contemporaneously. The healing of wounds in the skin requires an enormously complex process that involves the interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating leukocytes. Re-epithelialization is achieved through an increase in keratinocyte proliferation and guided migration of the keratinocytes over the granulation tissue. Thus, the healing process requires ordered changes in keratinocyte behavior and phenotype, processes that are in turn mediated by epithelial-mesenchymal communication. This complex interplay requires the integration of diverse signals through a network of soluble factors, exerting autocrine and paracrine activity from the wound microenvironment, resulting in appropriate cellular response. However, when this signaling network fails to function properly, it has the potential to impair or enhance cell migration and proliferation, resulting in insufficient or excessive repair of future wounds and life-threatening consequences such as tumor growth and metastasis. Therefore, an attempt to understand the effect of single molecules on normal cellular function, studies into its role in this signaling network and how they culminate to an appropriate cell response have assumed increasing relevance and importance in recent years (Tan et al, 2009).

Although the importance of epithelial-mesenchymal communication in wound healing and epidermal homeostasis is well-recognized, few studies have aimed to identify these communication networks (Figure 3) (Angel & Szabowski,

2002; Chong et al, 2009; Maas-Szabowski et al, 1999; Maas-Szabowski et al, 2000).

Figure 3. A schematic diagram showing the epithelial-mesenchymal communication. Keratinocyte secretes interleukin 1(IL-1), which directly influences fibroblast to regulate the expression of c-Jun, JunB and nuclear factor kappa B (NFκB). Subsequently, the cytokines produced will be secreted to mediate proliferation and differentiation of keratinocyte. (Florin et al, 2005; Maas-Szabowski et al, 1999; Maas-Szabowski et al, 2000)

As demonstrated by Maas-Szabowski et al. (1999), proliferation of keratinocytes is enhanced when they are grown together with mouse fibroblasts (3T3 cells) or human fibroblasts (Limat et al, 1989). This mechanism involves a paracrine loop of soluble factors; keratinocytes secrete interleukin-1 (IL-1) that induces the production of keratinocyte growth factor (KGF) from fibroblasts, which in turn increases the proliferation of keratinocytes (Maas-Szabowski et al,

1999). However, the lack of c-Jun in fibroblasts decreases keratinocyte proliferation in cocultures. The importance of KGF for re-epithelialization during wound healing *in vivo* has been established previously (Werner et al, 1994). Another paracrine loop that drives the proliferation of keratinocytes has been discovered (Maas-Szabowski et al, 2000), which involves IL-1 from keratinocytes and granulocyte-macrophage colony-stimulating factor (GM-CSF) from fibroblasts. Modulated expression of several cytokines and cytokine receptors in fibroblasts, as well as keratinocytes, in co-cultures has been detected (Maas-Szabowski et al, 2000).

The activated protein-1(AP-1) family of transcription factors such as Jun, Fos and activating transcription factor (ATF) proteins has been shown to be of particular importance for the cross-talk between keratinocytes and fibroblasts (Angel & Szabowski, 2002). Two Jun-regulated cytokines, stromal-derived factor 1 (SDF-1) and pleiotrophin (PTN), are expressed *in vivo* in dermal fibroblasts and can stimulate in a paracrine manner *in vitro* proliferation of primary epidermal keratinocytes (Florin et al, 2005), thereby playing a role in epithelial-mesenchymal communication (Figure 3).

Recently, Chong et al. revealed another double-paracrine epithelial–mesenchymal network essential for efficient wound healing (Chong et al, 2009). The study showed that IL-1 produced by keratinocytes activates the peroxisome proliferator–activated receptor β/γ (PPAR β/γ) in the underlying fibroblasts, which serves to restrict the mitotic activity of keratinocytes through further constraints on the IL-1 signaling pathway (Chong et al, 2009). They showed that PPAR β/γ in the fibroblasts stimulates the production of the secreted IL-1 receptor antagonist (IL-1ra), which leads to an autocrine decrease in IL-1 signaling pathways and

consequently decreases production of secreted mitogenic factors by the fibroblasts (Figure 4). This fibroblast PPAR β/γ regulation of the IL-1 signaling is required for proper wound healing. Together, these findings provide evidence for a novel homeostatic control of keratinocyte proliferation and differentiation mediated via PPAR β/γ regulation in dermal fibroblasts of IL-1 signaling. Given the ubiquitous expression of PPAR β/γ , other epithelial-mesenchymal interactions may also be regulated in a similar manner (Chong et al, 2009).

Figure 4. The autonomous and non-autonomous actions of PPAR β/δ in maintaining epidermal homeostasis. Peroxisome proliferator-activated receptor β/γ (PPAR β/δ) confers anti-apoptotic properties to keratinocytes and potentiates their migration via AKT1 (or PKB α) and Rho GTPases in a cell-autonomous manner. PPAR β/δ regulates epidermal proliferation in a non-autonomous manner via a paracrine mechanism. The presence of PPAR β/δ in the fibroblasts also regulates an autocrine mechanism that influences epidermal proliferation. As such, the absence of PPAR β/δ in the fibroblasts will cause significant epidermal proliferation. Adapted from (Chong et al, 2009)

1.4 Transforming growth factor- β 1 (TGF- β 1)

Of all the secreted cytokines and growth factors present in the wound region, TGF- β 1 has the broadest impact affecting every aspect of the wound healing process. TGF- β 1 is the prototype for a conserved family of secreted polypeptides that includes BMP, activins, and several more distantly related factors (Chin et al, 2004). Through its effects on cell growth, differentiation, motility, adhesion, and apoptosis, TGF- β is an important regulator of cell fate determination and tissue morphogenesis. TGF- β signals through a heteromeric receptor complex of TGF β RI and TGF β RII serine/threonine kinase receptors that are assembled in response to ligand binding. Receptor activation mobilizes a complex network of signal transducers to produce pleiotropic responses. The canonical downstream TGF- β signaling cascade involves intracellular mediators from the Similar to Mother Against Decapentaplegic *Drosophila* (SMADs) family, specifically, SMAD2, SMAD3 and the common-mediator SMAD4. In response to TGF- β signals, SMAD2 and SMAD3 are phosphorylated at their C termini by the activated TGF β RI. These phosphorylated SMADs form stable complexes with Smad4 and translocate to the nucleus to act as transcriptional factors (Massague & Wotton, 2000). This mechanism is negatively regulated by the inhibitors SMAD6 and SMAD7. Endogenous inhibitor SMAD7 specifically prevents the access and subsequent phosphorylation of SMAD2 and SMAD3 to the activated TGF- β receptor complex (Nakao et al, 1997). Disruptions of the SMAD pathway or mutation of SMADs ablate both growth and transcriptional responses to TGF- β . Such disruptions or mutations are frequently observed in some human tumors, which also underscores their importance in TGF- β signaling (Engel et al, 1999).

Numerous lines of evidence indicate that SMADs cooperate with other transcription factors to modulate gene expression (Blume-Jensen et al, 1998; Byrne et al, 1994; Candi et al, 2006; Chen et al, 2006; Cheung et al, 2004; Eckert et al, 2002; Franke et al, 1997; Fuchs, 2007; Tan et al, 2007). Among these factors is AP-1, which is a heterodimer of c-Fos and c-Jun family members that binds specific sequences in target promoters (Fuchs & Raghavan, 2002). Whether the interaction between AP-1 and SMAD3 results in transcriptional activation or repression is promoter-dependent. For example, the plasminogen activator inhibitor-1 (PAI-1) promoter, where the TGF- β responsive regions and SMAD- and AP-1-binding elements are juxtaposed, expression was induced by TGF- β (Fuchs, 2007; Fuchs & Green, 1980). However, in gene promoters containing AP-1 binding elements, such as PPAR β/δ and monocyte chemoattractant protein-1 (MCP-1), the interaction between Smad3 and AP-1 leads to transcriptional inhibition.

The transcriptional factor Smad3 is a canonical intracellular mediator of TGF- β (Massague & Wotton, 2000). TGF- β plays a central role in fibrosis, contributing to the influx and activation of inflammatory cells, a process formally known as epithelial to mesenchymal transdifferentiation (EMT). TGF- β is also chemotactic for fibroblasts and their subsequent elaboration of extracellular matrix (ECM) (Clark & Coker, 1998; Clark et al, 1997). The use of Smad3-knockout (KO) mice shows that most of the pro-fibrotic activities of TGF- β are mediated by Smad3 (Ashcroft et al, 1999). These KO mice are resistant to radiation-induced cutaneous fibrosis, bleomycin-induced pulmonary fibrosis and carbon tetrachloride-induced hepatic fibrosis. In fibrotic conditions that are induced by EMT, such as proliferative vitreoretinopathy, ocular capsule injury and

glomerulosclerosis resulting from unilateral ureteral obstruction, KO mice also show an abrogated fibrotic response. Animal models of scleroderma, cystic fibrosis and cirrhosis implicate involvement of Smad3 in the observed fibrosis. Thus, small molecule inhibitors of Smad3 may have tremendous clinical potential in the treatment of pathological fibrotic diseases (Flanders, 2004).

1.5 Transforming growth factor β -activated kinase 1 (TAK1)

Being a multifunctional cytokine, TGF- β 1 also signals through other non-canonical pathways in which the TGF- β 1-activated kinase 1 (TAK1) occupies a pivotal role. TAK1 was identified from a mouse cDNA screen for TGF- β 1 responsive mitogen-activated protein kinase kinase kinase (MAPKKK) (Yamaguchi et al, 1995). TAK1 is a serine/threonine kinase that is rapidly activated by TGF- β 1 but the molecular mechanism of TAK1 activation remains incompletely understood (Kim et al, 2009). Studies suggest that under unstimulated conditions, endogenous TAK1 is stably associated with TGF β RI, whereas TGF- β 1 stimulation causes rapid dissociation from the receptor and induces TAK1 phosphorylation. In addition, they determined that TGF β RI-mediated TAK1 phosphorylation correlates with the degree of its association with TGF β RI and, requires kinase activity of TAK1, as well as TAK1 binding proteins (TAB). TAB1 does not interact with TGF- β receptors, but TAB1 is indispensable for TGF- β 1-induced TAK1 activation (Kim et al, 2009). The C-terminal 68 amino acids of TAB1 are sufficient for binding and activation of TAK1 in mammalian cells, while the N-terminal 418 amino acids act as a dominant-negative inhibitor of TGF β 1-induced gene expression. Taken together, studies indicate that TGF- β 1-induced interaction of TGF β RI and TGF β RII triggers dissociation of TAK1 from

TGF β RI, and that subsequently, TAK1 is phosphorylated through TAB1-mediated auto-phosphorylation and not by the receptor kinase activity of TGF β RI (Kim et al, 2009).

Interestingly, in addition to TGF- β (Yamaguchi et al, 1995), this serine/threonine kinase is also a primary intermediate in inflammatory cytokines tumor-necrosis factor α (TNF- α)- and IL-1-mediated signaling pathways (Ninomiya-Tsuji et al, 1999; Sato et al, 2005). Sakurai et al. showed that TAK1 could induce translocation to the nucleus of nuclear factor kappa B (NF κ B), composed of the p50/p65 heterodimer, accompanied by the degradation of I κ B α and I κ B β in a NF κ B-inducing kinase- (NIK-) independent manner (Sakurai et al, 1998). Activated TAK1 is also capable of stimulating a downstream MAPK cascade (Chen et al, 2006; Wang et al, 2001), which functions by activating c-Jun N-terminal kinase (JNK)/ AP-1 and p38 (Ninomiya-Tsuji et al, 1999; Takaesu et al, 2003; Yao et al, 2007). Specifically, TAK1 activates JNK through mitogen-activated protein kinase kinase 4 (MKK4) (Zhou et al, 1999), and p38 through MKK3 or MKK6 (Moriguchi, 1996). A deficiency in TAK1 will result in impaired TNF- α and IL-1 stimulated JNK activity and p38 phosphorylation (Shim et al, 2005; Singhirunnusorn et al, 2005; Takaesu et al, 2003). Consequently, expression of numerous AP-1 and NF κ B target genes are induced upon TAK1 activation. Importantly, NF κ B and members of the AP-1 transcription factor family are important mediators of inflammation, immune responses and, T and B cell activation, as well as epithelial cell survival, underscoring the role of TAK1 in these biological processes (Morioka et al, 2009; Ninomiya-Tsuji et al, 1999; Omori et al, 2008; Schuman et al, 2009).

In human cervical carcinoma HeLa cells, the specific knockdown of TAK1 using small interfering RNA (siRNA) not only abrogated the TNF-related apoptosis-inducing ligand (TRAIL)-induced activation of p65 and JNK, but also increased TRAIL-induced apoptotic signals such as caspase 3 (Choo et al, 2006). In addition, the binding of annexin V to the cell surface was synergistically increased by TRAIL in combination with the knockdown of TAK1. Choo *et. al* thus concluded that TAK1 mediates anti-apoptotic effects. Apart from its anti-apoptotic effect, TAK1 is also implicated in patterning and morphogenesis. Ventralization of *Xenopus* embryos is controlled by TGF- β /BMP signaling and experiments have shown that overexpression of TAK1 will block BMP-induced ventral patterning of *Xenopus* embryos (Shibuya et al, 1998). However in mice, targeted deletion of TAK1 causes lethality between embryonic day 10.5 and 12 (Jadrich et al, 2006; Shim et al, 2005). Altogether, TAK1 displays divergent roles in signaling pathways such as JNK, NF κ B and TGF- β /BMP.

1.6 Role of TAK1 in skin

Deletion of TAK1 gene in mice resulted in early embryonic death (Sato et al, 2005). The TAK1^{-/-} fibroblasts confirmed that TAK1 was required for IL-1 β - and TNF-induced NF κ B and JNK activation, as well as cytokine production. B cell-specific deletion of TAK1 led to impaired activation in response to Toll-like receptor ligands and B cell receptor stimulation. Thus, TAK1 has non-redundant functions in signaling pathways in inflammatory and immune responses (Sato et al, 2005). Furthermore, Liu et al. (2006) targeted TAK1 deletion in T cells in mice and found that TAK1 was essential for thymocyte development and activation (Liu et al, 2006). Deletion of TAK1 prevented maturation of single-positive thymocytes

displaying CD4 or CD8, leading to reduction of T cells in the peripheral tissues. Thymocytes that lacked TAK1 failed to activate NF κ B and JNK and were prone to apoptosis upon stimulation. Thus, TAK1 plays a central role in both innate and adaptive immunity.

Given the well-recognized role of NF κ B in regulating epidermal development, it is conceivable that upstream TAK1 could be essential for epidermal functions. To test this hypothesis, keratinocyte-specific TAK1-deficient mice were generated from Map3k7flox/flox mice and K5-Cre mice (Sayama et al, 2006). The keratinocyte-specific TAK1-deficient mice were macroscopically indistinguishable from their littermates until postnatal day 2 or 3, when the skin started to roughen and wrinkle. This phenotype progressed, and the mice died by postnatal day 7. Subsequently, a histological analysis determined that there was a thickening of the epidermis with foci of keratinocyte apoptosis and intra-epidermal micro-abscesses. In addition, immunohistochemical analysis revealed that the suprabasal keratinocytes of the TAK1-deficient epidermis expressed keratin 5 and keratin 14, which are normally confined to the basal layer. The expression of keratin 1, keratin 10, and loricrin, which are markers for the suprabasal and late phase differentiation of the epidermis, was absent from the TAK1-knockout epidermis (Sayama et al, 2006). However, in suspension culture, these TAK1-deficient keratinocytes can induce keratin 1 expression, indicating that these keratinocytes retain the ability to differentiate. The TAK1-deficient epidermis expressed keratin 16 and experienced an increased number of Ki67-positive proliferating cells. The deletion of TAK1 from cultured keratinocytes of Map3k7flox/flox mice resulted in apoptosis, an outcome that appeared at odds with the increase in *in vivo* cell proliferation. Nonetheless, these data suggest that TAK1

deficiency in keratinocytes results in abnormal differentiation, increased proliferation, and apoptosis in the epidermis.

1.7 Aim and conclusion of study

The studies to date that have employed keratinocyte-specific TAK1 knockout (TAK1-KO) mice have documented the role of TAK1 in skin inflammation. However, these mice died prematurely at postnatal day 7 and thus prevented long-term studies such as wound healing. Although, the TAK1-KO mice also exhibited abnormal epidermis with impaired differentiation and increased cellular proliferation, early *in vitro* studies have not identified any significant difference in proliferation index in *in vitro* culture of these phenotypically abnormal keratinocytes. The reason for this discrepancy is unclear, but these observations indicate an essential role of the underlying dermis in mitigating some effects of epidermal TAK1. Although the role of TAK1 in inflammatory response has been well documented, the precise role of TAK1 and its mechanism of action in keratinocyte proliferation and migration remains a mystery, which is the aim of this project.

Organotypic skin culture (OTC) offers an attractive technique in our study, which is aimed at unraveling TAK1 mechanistic pathway, because previous studies of TAK1 on animal models have caused lethality. Briefly, OTC is a construction of *in vitro* skin equivalent to mimic the *in vivo* development of human epidermis. The keratinocytes differentiate and stratify typically within two to three weeks into an epidermis, which closely resembles the native epidermal layers and the expression of epidermal differentiation markers. In addition, this *in vitro* epidermal model

allowed us to modulate the epidermal microenvironment such as supplementing exogenous growth factors in rescue studies.

Our work here has revealed that TAK1 in keratinocytes activates von Hippel-Lindau (VHL) tumor suppressor expression, which subsequently mediates cell migration. We have also showed an autocrine SCF/PKB α cascade that modulates cellular survival.

2 MATERIALS & METHODS

2.1 Reagents

Antibodies: anti-TAK1 for immunofluorescence (Upstate Biotechnology), anti-TAK1 for immunoblot (Cell Signaling); anti-cdc42-, anti-Rac1 and anti-RhoA (Cytoskeleton), anti-cytokeratin 10 (CK10), involucrin, Ki67 (Novacastra); neutralizing anti-PDGF-BB antibody (Peprotech); DAPI, Vectashield Mounting Medium (Vector Laboratories); AlexaFluor® 488 goat anti-mouse IgG (Molecular Probes, Invitrogen); goat anti-rabbit IgG HRP (Santa Cruz Biotechnology). Collagen Type I Rat Tail (BD Biosciences); Human primary fibroblasts, keratinocytes and corresponding culture medium (Cascade Biologics); Transfection reagent Fugene HD (Roche); Double-promoter lentivirus-based siRNA vector and pFIV Packaging kit were from System Biosciences. All restriction enzymes and DNA/RNA modifying enzymes were from Fermentas. Kinase inhibitors were from Merck. Otherwise, chemicals were from Sigma-Aldrich.

2.2 Keratinocyte culture

Primary human keratinocytes (Cascade Biologics) were cultured in Quantum 153 medium supplemented with insulin, transferrin, EGF, cholera toxin and 5% FBS (PAA Laboratories) in 5% CO₂, 37 °C humidified incubator. Medium was changed every 3 days. Cells were subcultured upon reaching 70% confluency. Briefly, medium was removed and the cells washed with PBS. Trypsin (0.25%)-EDTA (1-mM) in PBS was added to the culture (0.08 ml/cm²) and incubated at room temperature for 15 min. The flask was rapped gently to dislodge cells from the

surface of the flask. PBS containing 1% dialyzed FBS was added and the cells were collected by centrifugation. Cell pellet was resuspended with fresh medium and subcultured in new flask at 2.5×10^3 cells/cm².

2.3 Lentivirus siRNA constructs and transduction

The sequences of TAK1 and control siRNA are shown in Table 2. These phosphorylated siRNA templates were annealed and subcloned into the double promoter pFIV-HI/U6 siRNA vector. The ligated products were subsequently transformed into competent *E. coli* using heat-shock and the bacterial cells were grown on ampicillin-selective agar. Positive clones were identified by PCR and by DNA sequencing. Production of pseudoviral particles and transduction of cells were as described by the manufacturer. The lentiviral siRNA construct was transiently transfected into human embryonic kidney (HEK) 293T cells. Viral particles were produced with pFIV Packaging kit (System Biosciences). Following transduction, the cells were selected with 350 ng/ml puromycin for a week.

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

Total RNA was isolated from cells using Aurum total RNA kit (Bio-Rad) following the supplier's protocol. Total RNA (2.5 µg) was reverse-transcribed with oligo-dT primers using RevertAid™ H Minus M-MuLV. 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 sequence of primers is available in Table

Table 2. Oligonucleotide sequences

Primer pairs (S: sense; AS: anti-sense)	Sequence (5'-3')
Actin S	CTTCTCTCTAAGGAGAATGGCCCAG
Actin AS	CAACTGGTCTCAAGTCAGTGTACAGG
Actin RT1	ACGTGGACATCCGCAAAGAC
Actin RT2	CGCTCAGGAGGAGCAATGAT
HGF S	TCCCTTCTCGAGACTTGAAAGAT
HGF AS	CTGCATTTCTCATTTCCCATT
PDGFB S	TGAACATGACCCGCTCCCCTC
PDGFB AS	TGCCGTCTTGTCATGCGTGTG
PDGFA S	CGGATACCTCGCCCATGTTCTG
PDGFA AS	TCCGGATTCAGGCTTGTGGTCG
SCF S	GGGATCTGCAGGAATCGTGTG
SCF AS	ACAATGCTGGCAATGCCATG
TAK A1	<u>AAAGA</u> AAGAGGTTGTTGGAAGAGGAG
TAK A2	<u>AAAA</u> CTCCTCTTCCAACAACCTCTT
TAK B1	<u>AAAGT</u> GGCTTATCTTACACTGGA
TAK B2	<u>AAAA</u> TCCAGTGTAAGATAAGCCA
TAK1 RT1	GTTCCGTGTAAGGGCTTTGA
TAK1 RT2	GAGCAGCTGCCACTTACCTTTACA
U6	GCTTACCGTAACTTGAAAGTATTTTCG
H1	CTGGGAAATCACCATAAACGTGAA
pVHL promoter S	GATCCTCGAGCGTGATGATTGGGTGTTCCC
pVHL promoter AS	GATCAAGCTTCGACGCCTGCCTCCTCCGCG

Underlined nucleotides indicate 'sticky-ends' that facilitate ligation with the *Bbs*I-digested siRNA expression vector.

2. Interferon response detection was done as recommended by manufacturer (System Biosciences).

2.5 Transient transfection and transactivation assay

TAK1 cDNA was subcloned into pCMV5 mammalian expression vector (Stratagene). A ~732 bp proximal promoter of the human VHL was PCR amplified from human genomic DNA using Pfu polymerase (Zatyka et al, 2002). The resulting fragment was subcloned into pGL-3 basic luciferase reported vector (Promega). Site-directed mutagenesis was performed using QuikChange Site-Directed Mutagenesis kit (Stratagene). The sequence of primers is available in Table 3. Keratinocytes were cotransfected with a luciferase reporter driven by the VHL promoter construct, cDNAs encoding for various kinases and pEF1- β -galactosidase as a control of transfection efficiency (Tan et al, 2001). Briefly, one day before the transfection experiment, cells growing as monolayers were trypsinized and replated into six-well plates at 2.5×10^5 cells per well in 3 ml of medium. On the day of transfection, the cell density should be approximately 80% confluency. Fugene HD transfection reagent (Roche) was used to prepare 100 μ l transfection complex that contains 2 μ g DNA and 5 μ l transfection reagent. This transfection complex was incubated for 15 min at room temperature before adding dropwise to the cells. 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 constitutively active (ca) PKA, MEK1 and MEKK1 were from Clontech. The caMKK7 was from Cell BioLabs. The various caSEK1, caJNK, caSGK and caIKK

expression vectors were from E. Nishida (Kyoto University, Japan), R.J. Davis (University of Massachusetts Medical School, Worcester), D.J. Templeton (University of Virginia Medical School, Charlottesville) and D.V. Goeddel (Tularik, Inc). TAK1 was as previously described (Liu et al, 2008; Yamaguchi et al, 1995).

Table 3. Mutagenesis primer sequences

Primer pairs (S: sense; AS: anti-sense)	Sequence (5'-3')
VHL mutant S	GTGGATCGCGGAGGGAgatCtCCGGAGGGCGGAGAAC
VHL mutant AS	GTTCTCCGCCCTCCGGAGATCTCCCTCCGCGATCCAC

2.6 Organotypic skin culture (OTC)

Organotypic skin cultures were performed as previously described with some modifications (Maas-Szabowski et al, 1999). A fibroblast density of 1×10^5 cells/ml of collagen Type I rat tail (BD Biosciences) 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. Supplemented serum-free OTC medium was used (DMEM:Ham's F12 in 3:1 (v:v), 1 mg/ml fatty acid free albumin, 50 µg/ml ascorbic acid, 5 µg/ml transferrin, 0.4 µg/ml hydrocortisone, 25 µM oleic acid, 15 µM linoleic acid, 10 µM serine, 10 µM carnitine, 7 µM arachidonic acid, 1 µM isoproterenol, 1 µM α-tocopherol, 100 nM adenine and 100 nM cholera toxin).

2.7 Immunofluorescence and immunohistochemistry

OTCs 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- μ m cryosections were processed for immunofluorescence as described previously (Michalik et al, 2001). Apoptotic cells were detected using the TUNEL assay according to the manufacturer's protocol (Roche). As positive control for TUNEL assay, the section was pre-treated with DNase I. The slides were counterstained with DAPI and mounted for microscopic observation. Immunohistochemistry on the indicated wound section was performed with anti-TAK1 and anti-VHL antibodies (Upstate Biotechnology). The signal was amplified using the ABC-peroxidase method (Vector Laboratories) and revealed using 3,3'-diaminobenzidine (DAB, brown) for TAK1 or in the presence of nickel for pVHL (dark blue). Images were taken using MIRAX MIDI (Carl Zeiss) with a Plan-Apochromatic 20x/0.8 objective and MIRAX Scan software.

2.8 *In situ* proximity ligation assay (PLA)

K_{CTRL} and K_{TAK1-B} cells were subcultured on to glass chamber slides (Lan-Tek). The following day, the cells were fixed with methanol:acetone (1:1) at -20 °C for 10 min. the slides were washed twice with PBS. The slides were blocked with 1% BSA in PBS for 2 h at room temperature and were incubated with primary rabbit anti-pVHL and mouse anti-Sp1 antibodies overnight at 4 °C. The slides were washed as above. Duolink™ *in situ* PLA was performed as recommended by the manufacturer (OLink Biosciences). As negative control, no primary antibody was used. Images were taken using LSM510 META confocal laser scanning microscope (Carl Zeiss).

2.9 Protein array analysis

Phosphoproteins and Growth factors array membranes were processed according to the manufacturer's protocol (RayBiotech). The membrane-based RayBio® Human RTK Phosphorylation Antibody Array (Catalog #AAH-PRTK-1) contains 71 receptor tyrosine kinases and the array map is available online. The membrane-based RayBio® Human Cytokine Antibody Array 3 (Catalog #AAH-CYT-3) contains 42 cytokines for detection and the array map is also available online. Briefly, the array membranes were blocked with 1X Blocking Buffer and incubated at room temperature for 30 min. After which, the Growth factors membranes were incubated with 1 ml serum-free conditioned medium while the Phosphoproteins array membranes were incubated with protein samples containing 0.5 mg cell lysate proteins at 4 °C overnight. The membranes were then washed with 2 ml 1X Wash Buffer I and IX Wash Buffer II and incubated with 1 ml biotin-conjugated antibodies at room temperature at 4 °C overnight. Subsequently, the membranes were incubated with HRP-conjugated Streptavidin at room temperature for 2 h. Protein spots were detected by chemiluminescence and Kodak BioMax Light films were used for chemiluminescent imaging. Signal intensities were quantified using Image J analysis software and were normalized with the mean intensity of the positive controls on each membrane.

2.10 Enzyme-linked immunosorbent assay (ELISA) and integrin-mediated cell adhesion assay

The concentration of growth factors was measured using sandwich ELISA (R&D System). Briefly, 2×10^5 fibroblasts were cocultured with either K_{CTRL} or K_{TAK1-B} keratinocytes seeded in a culture insert (3 μ m pore size) of a 6-well multidish.

After 24 h, the media were harvested, filtered (0.2 μ m pore size) and treated by the addition of complete protease inhibitors (Roche). The level of growth factors was measured in culture supernatants according to the manufacturer's instruction. The integrin-mediated cell adhesion assay was performed as recommended by the manufacturer (Chemicon). Briefly, integrin-coated and uncoated control strips of 96-well microculture plates were rehydrated with PBS at room temperature for 10-min. 1×10^4 K_{CTRL} or K_{TAK1-B} cells in serum-free DMEM were added to each well and allowed to adhere in a 37 °C incubator with 5% CO₂ and 70% humidity for 1 h. Non-adherent cells were removed by gentle washing with PBS containing 1 mM CaCl₂ and 1 mM MgCl₂. Adherent cells were fixed with 1% glutaraldehyde in PBS for 15 min, stained with 0.1% crystal violet for 30 min and eluted by gentle rotation on shaker at room temperature for 10 min. Adhesion was quantified by measuring the absorbance of eluted stain at 562 nm on spectrophotometer.

2.11 Immunoblot analysis

Total protein was extracted from cells with ice-cold lysis buffer (20 mM Na₂H₂PO₄, 250 mM NaCl, 1% Triton-100, 0.1% SDS). Equal amount of protein extracts were resolved by SDS-PAGE and electrotransferred onto PVDF membranes. Membranes were processed according to standard procedure and proteins were detected by chemiluminescence. Coomassie blue-stained membrane was used to check for equal loading and transfer.

2.12 GTPases activation assay for cdc42, Rac1 and RhoA

GTPases activation assays were carried out as previously described (Tan et al, 2007). Purified recombinant glutathione S transferase (GST)-p21 binding domain (PBD) of PAK was used to measure active cdc42 and Rac1, while GST-Rho binding domain (RBD) of rhotekin was use to measure active RhoA.

2.13 *In vitro* cell migration assay

In vitro scratch wound assays were performed using K_{CTRL}, K_{TAK1-B} and TAK1-transfected K_{TAK1-B} keratinocytes as previously described (Michalik et al, 2001). Live cell images were captured at 2-min intervals during a 9 h experimental period using a temperature-controlled, 5 % CO₂-chambered microscope (Carl Zeiss). Similar experiments were also performed in the presence of 2 µg/ml of mitomycin C or 100 µM cobalt chloride-simulated hypoxia condition.

2.14 Anoikis assay

Anoikis was induced by forced suspension where 5.0×10^5 cells/ml K_{CTRL} and K_{TAK1-B} keratinocytes were seeded separately onto 0.9% agarose equilibrated with serum-free DMEM. The cells were harvested at indicated time and apoptotic cells were identified using ApoAlert Annexin V-FITC apoptosis kit (Clontech Laboratories, Inc.) and detected by fluorescence microscopy.

2.15 Protein-protein and chromatin immunoprecipitation (ChIP)

In vivo protein-protein crosslink and ChIP were carried out as previously described except anti-pVHL and anti-Sp1 antibodies were used (Ijpenberg et al, 2004). Primers used for ChIPs are available in Table 4. Briefly, chromatin was crosslinked using 1% formaldehyde for 10 min at 37°C and sonicated in lysis buffer to achieve crosslinked DNA of 200 – 600 bp in length. Crosslinking was stopped by adding glycine to a final concentration of 0.125M. Subsequently, samples were incubated at 25°C for 5 min, homogenized and pelleted by centrifugation. After centrifugation, 10% of the supernatant was used as input and the remaining amount was subjected to ChIP. The sheared chromatin sample, together with protease inhibitor cocktail, ChIP buffer and antibody, was mixed with protein G-coated magnetic beads and completely resuspended to a homogeneous slurry. This mixture was incubated overnight at 4°C. After which, the magnetic beads were isolated, washed and the chromatin on the magnetic beads was eluted. PCR was performed using 5-10 µl of the final ChIP eluate.

Table 4. Chromatin immunoprecipitation (ChIP) primer sequences

Primer pairs (S: sense; AS: anti-sense)	Sequence (5'-3')
ITGB1SP1_S	TCAGTGGACAAACGGGAGCGAG
ITGB1SP1_AS	CTCTCCCTCCGGAAACGCATTC
ITGB1control_S	TCGACATGCGTTCAGCCATTC
ITGB1control_AS	GGAGTGATAATGTGCTTGCCTG
ITGB3SP1_S	GGCGAGAGAGGAGCAATAG
ITGB3SP1_AS	GAGCCTCACTCACCTCCTAC
ITGB3control_S	TTGGAGCAGGATGAGGCTTTGC
ITGB3control_AS	CCCAGTACCAAAGAGGCCACTC
ITGB5SP1_S	CATTTCCCTCCCTCCTTC
ITGB5SP1_AS	TGTGAATTGAGTTTGTTC
ITGB5control_S	GAGAGACAGCCCAGCATCTCAG
ITGB5control_AS	CACGCACACTGTGGACTTCACC
PDGFBSP1_S	TCCGGGCCAGAAGAGGAAAG
PDGFBSP1_AS	CCCATCTTTGATCCTTCTG
PDGFBcontrol_S	GGTGAATCAGAGTGGAGTATG
PDGFBcontrol_AS	CTGTGGACTGTCCTGAGGAG

2.16 Wounding experiment

Wounding of the mice dorsal skin was initiated 2 weeks prior to carrying out excision by the removal of fur of the whole dorsal skin area using an electric razor. The mice were anesthetized, shaved and its back was swabbed with 70% alcohol. A full thickness middorsal wound (5 mm by 5 mm; square shaped) was created by excising the skin which removed the epidermal, dermal and subcutaneous layer including the panniculus carnosus. To examine TAK1 mRNA and protein expression at the site of injury, mice were sacrificed at day 1, 3, 7, 10, 12, 17 post-wounding to remove the wound biopsy samples. An area including the scab and a wound margin of about 5 mm was excised at each time point. For each mouse, a control of normal unwounded dorsal skin was taken at a distance away from the site of injury. Excised wound tissues were immediately frozen in liquid nitrogen and stored at -80 °C until further analysis.

2.17 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using the NucBuster protein extraction kit as described by the manufacturer (Novagen). VHL-NF κ B studies were done using 10- μ g cell nuclear extract and 32 P end-labeled oligonucleotides (20 000 cpm) in 20 μ l of binding buffer (10 mM HEPES [pH 7.5], 50 mM KCl, 0.1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 2.5 mM DTT, 0.05% NP40, and 500 μ g/ml poly(dI-dC). For competition, non-radiolled consensus NF κ B sequence was added at 10 - 100 molar excess. The reaction products were analyzed on a 6 % non-denaturing polyacrylamide gel in 0.5X TBE. The gel was dried and exposed to X-ray film

3 RESULTS

3.1 Elevated TAK1 expression in wound epithelia

Immediately after injury, an acute inflammation response ensures immune competence and orchestrates later wound healing events including re-epithelialization. The major goal of re-epithelialization, of which cell migration and proliferation are dominant cellular events, is the reconstitution of the epithelium as a functional barrier. While the role of TAK1 activation in inflammation is well-established, little is known about its role in cell migration, which is crucial to the re-establishment of the wounded area. In the first instance, we examine the expression profile of TAK1 during the healing of mouse skin full-thickness excisional wound. We found that during the healing of mouse skin full-thickness excisional wound, the expression profile of TAK1 mRNA and protein peaks at day 3-7 post-wounding, as shown by quantitative real-time PCR (qPCR) and immunoblot analysis (Figure 5). Immunohistochemistry staining with anti-TAK1 revealed that the expression of TAK1 was elevated in the wound epithelia (Figure 6A), compare to only basal level in unwounded skin. Immunostaining performed using pre-immune IgG did not display any staining (Figure 6B).

Figure 5. TAK1 mRNA and protein expression profiles during wound healing determined using qPCR (left panel) and immunoblotting (right panel). Ribosomal protein L27 was used as a normalizing housekeeping gene. β -tubulin showed equal loading and transfer. Data are mean $\pm$ SEM, day 0 unwounded (control) skin (n=7); n=4 of each post-wounding day.

To investigate whether the up-regulation of TAK1 was similarly observed in human skin, we performed a retrospective examination of human wounds by comparing the level of TAK1 mRNA. The human ulceric skin and normal skin biopsies differ in their morphology. The former shows hyperproliferative epidermis (i.e. presence of mitotically active cells beyond the basal layer) and parakeratosis epidermis (i.e. presence of nuclei in the cornified layer) indicate incomplete differentiation. However, mitotically active cells are only found in the basal layer of normal epidermis and it exhibits a well-defined cornified layer where parakeratosis is not detected. The TAK1 mRNA level was elevated in human ulceric skin biopsies compared to normal skin as determined by qPCR (Figure 7). These observations underscore an important role of TAK1 during re-epithelialization of skin wounds in rodents and humans.

Figure 6. Immunohistochemical analysis for TAK1 in skin wound biopsies. **(A)** Mice skin wound biopsies at indicated days of post-wounding were cryosectioned and stained with antibodies against TAK1. Representative pictures from wound epithelia were shown. **(B)** Negative control. Immunohistochemical staining of Day 5 mouse wound biopsies with pre-immune IgG as negative control. The section was processed as described in Figure 4(A). Representative picture from wound epithelium and the adjacent wound bed was shown. E: epidermis; WB: wound bed; hf: hair follicle. Scale bar 20 μ m.

Figure 7. Relative expression level of TAK1 mRNA in normal human skin (n=5) and chronically inflamed ulcer skin biopsies (n=7) as determined by qPCR. Cyclophilin was used as a normalizing housekeeping gene. Statistical analysis was determined using two-tailed Mann-Whitney test, *** denotes $p < 0.001$.

3.2 TAK1 deficiency enhances keratinocyte migration

Cell migration over the provisional wound bed is essential for re-epithelialization of injured tissues. As expression of TAK1 was increased during re-epithelialization of the wound, we questioned whether TAK1 deficiency affects keratinocyte migration. Thus, we generated human keratinocytes whose endogenous TAK1 expression was suppressed by lentivirus-mediated RNA interference. The efficiency of siRNA knockdown of TAK1 expression in human keratinocytes was assessed by PCR and immunoblot analysis. Keratinocytes transduced with either TAK1-A (K_{TAK1-A}) or TAK1-B (K_{TAK1-B}) sequences showed ~90% reduction in the expression of TAK1 mRNA when compared to unrelated control siRNA (K_{CTRL}) (Figure 8A). Consistent with the mRNA expression, immunoblot analysis showed negligible level of TAK1 protein in both K_{TAK1-A} and K_{TAK1-B} (Figure 8A). The induction of interferon responses has been reported as a challenge to the specificity of some RNAi approaches (Bridge et al, 2003).

A

B

Figure 8. Target-specific lentivirus-mediated knockdown of TAK1 in keratinocytes. **(A)** Lentivirus-mediated knockdown of TAK1 in keratinocytes. Human keratinocytes were transduced with a lentiviral vector harbouring a control or with two different TAK1 (TAK1-A and TAK1-B) siRNAs. Expression of TAK1 in either control (K_{CTRL}) or TAK1 siRNA (K_{TAK1-A} , K_{TAK1-B} or $K_{TAK1-AB}$) transduced keratinocytes was assessed by PCR and immunoblotting. $K_{TAK1-AB}$ were keratinocytes that were consecutively transduced with TAK1-A and TAK1-B siRNAs. Values below each band represent the mean fold differences in expression level with respect to control from five independent experiments ($n=5$). Keratinocytes were transduced with TAK1-B siRNA (K_{TAK1-B}) were used for subsequent studies. **(B)** Expression level of 2', 5'-oligoadenylated synthetase isoforms 1 and 2 (OAS1, OAS2), interferon-induced myxovirus resistance 1 (MX1) and interferon-stimulated transcription factor 3 γ (ISGF3 γ) mRNAs in K_{CTRL} and K_{TAK1-B} as determined by qPCR. Ribosomal protein L27 was used as a normalizing housekeeping gene.

To test whether gene silencing induced by the lentiviral-mediated RNA interference was associated with interferon-response induction, we measured the expression of some key interferon response genes by qPCR and the ability to rescue the K_{TAK1-B} phenotype by the reintroduction of an expression vector harboring TAK1 cDNA that has a silent third-codon point mutation within the target region. No induction of 2', 5'-oligoadenylate synthetase isoforms 1 and 2 (OAS1, OAS2), myxovirus resistance 1 (MX1) and interferon-stimulated transcription factor 3 γ (ISGF3 γ) was detected in the K_{TAK1-B} when compared with either wildtype untransduced cells or K_{CTRL} (Figure 8B). For subsequent analysis, K_{TAK1-B} keratinocytes were used. The expression of phosphorylated JNK/SAPK was downregulated in K_{TAK1-B} cells, consistent with the role of TAK1 in the activation of JNK/SAPK signaling pathway (Figure 9). There was no difference in phosphorylation of other MAPKs, such as ERK1/2 and ERK5 (Figure 9A). Similarly, the reintroduction of TAK1 into K_{TAK1-B} (TAK1-transfected K_{TAK1-B}) restored the expression of phosphorylated JNK and IKK α/β , but has no effect on

ERK1/2 (Figure 9B). Our results showed that gene silencing was not associated with non-specific interferon-response induction, i.e. off-target effect and that the deficiency of TAK1 attenuated the activation of JNK, p38 and IKK α/β .

Figure 9. TAK1 deficiency attenuated the activation of JNK/SAPK signaling pathway. **(A)** Expression of phosphorylated p(T183)JNK in K_{CTRL} and K_{TAK1-B}. One and five μ g of protein lysates were used. Reduced level of p(T183)JNK confirmed that endogenous TAK1 expression was suppressed. Values below each band represent the mean fold differences in expression level with respect to K_{CTRL}, which was assigned the value of one. **(B)** Expression of phosphorylated and total JNK, IKK α/β and ERK1/2 in K_{CTRL} and K_{TAK1-B} transfected with either expression vector or vector containing TAK1 cDNA. β -tubulin showed equal loading and transfer. Values below each band represent the mean fold differences in expression level with vector-transfected K_{CTRL}, which was assigned the value one.

Next, we investigated the effect of TAK1 deficiency in keratinocyte migration using *in vitro* scratch wound assay. Interestingly, our live-imaging results showed that K_{TAK1-B} keratinocytes closed the *in vitro* wound 3-4 h ahead of K_{CTRL} (Figure 10, videos 1 and 2). Cell proliferation and migration contributed to the closure of the *in vitro* wound. To more precisely determine the contribution of cell migration towards wound closure, we performed similar experiments in the presence of mitomycin C. Consistent with the above observation, K_{TAK1-B} closed the *in vitro* wound by 10 h in the presence of mitomycin C, a 2 h delay as compared to vehicle-treated K_{TAK1-B}. In contrast, K_{CTRL} failed to close the wound even by the end of the 12 h experimental period (Figure 11, videos 3 and 4). Close examination of the migratory front revealed that K_{TAK1-B} displayed more extended lamellipodia with numerous microspike-like features when compared to K_{CTRL} (Figure 10, videos 5 and 6). TAK1-transfected K_{TAK1-B} keratinocytes have a delayed migration when compared to K_{TAK1-B} and was similar to K_{CTRL} (Figure 10, video 7).

Figure 10. Time-lapsed images of wounded cultures of control keratinocytes (K_{CTRL}), TAK1-knockdown keratinocytes (K_{TAK1-B}) and TAK1-transfected K_{TAK1-B} (top five panels). Three independent experiments ($n=3$) were performed. Scale bar 100 μm . See videos 1, 2 and 7 respectively. Higher magnification images from video microscopy showing migratory front of K_{CTRL} , K_{TAK1-B} and TAK1-transfected K_{TAK1-B} (bottom two panels). Arrows indicate the focal adhesion points and microspike-like extensions in lamellipodia during migration. Scale bar 20 μm . Also see videos 5 and 6.

Figure 11. TAK1-deficient keratinocytes migrate faster, independent of cell proliferation. Time-lapsed images of wounded cultures of control (K_{CTRL}) and TAK1-knockdown (K_{TAK1-B}) treated with mitomycin C (2 μ g/ml). Three independent experiments (n=3) were performed. Scale bar 100 μ m. See videos 3 and 4. Higher magnification images from video microscopy showing migratory front of K_{CTRL} and K_{TAK1-B} (bottom two panels). Arrows indicate the focal adhesion points and microspike-like extensions in lamellipodia during migration. Scale bar 20 μ m.

Our earlier results demonstrated that TAK1-deficient cells migrated faster than the K_{CTRL} . The small Rho GTPases- cdc42, Rac1 and RhoA are pivotal intracellular mediators crucial for the formation of lamellipodia, cytoskeleton network and cell migration. Rac1 plays a role in the protrusion of lamellipodia and in forward movement, whereas cdc42 maintain cell polarity, including lamellipodia activity at the leading wound edge (Nobes & Hall, 1999). To gain further insight into the mechanism, we examined the activation of small Rho-GTPases, specifically cdc42, Rac1 and RhoA in K_{CTRL} and K_{TAK1-B} cells stimulated with serum. Consistent with the enhanced migration of K_{TAK1-B} cells, an elevated and sustained activation of cdc42, Rac1 and RhoA was observed (Figure 12). K_{CTRL} responded with a reduced activation of the three Rho GTPases. Notably, an elevated basal expression of active cdc42, Rac1 and RhoA was observed in K_{TAK1-B} when compared to K_{CTRL} before serum stimulation, thus we hypothesized that TAK1 likely exerted its action upstream of Rho GTPases activation. Integrin ligation and clustering triggers the subsequent activation of the GTPases- cdc42, Rac1 and RhoA. Immunoblot analysis revealed higher expression level of integrins $\beta 1$ and $\beta 5$, but not $\beta 3$ in K_{TAK1-B} when compared to K_{CTRL} (Figure 13A). Consistent with increased integrin ligation, we also detected enhanced activation i.e. phosphorylation of focal adhesion kinase (FAK) in K_{TAK1-B} (Figure 13A). The observation was further confirmed using integrin-mediated adhesion assay, which showed significant increase in integrins $\beta 1$ and $\beta 5$ expression in K_{TAK1-B} (Figure 13B). As expected, the expression of integrins $\beta 1$, $\beta 5$ and phosphorylated FAK in TAK1-transfected K_{TAK1-B} returned to similar level observed in K_{CTRL} (Figure 13C).

A

B

Figure 12. TAK1 deficiency activates small Rho-GTPases to enhance cell migration. **(A)** Graphs showed relative fold change of active cdc42 (top), Rac1 (bottom left) and RhoA (bottom right) in K_{CTRL} and K_{TAK1-B} exposed for the indicated time periods (min) with serum. Values represent the mean fold change in active cdc42, Rac1 and RhoA relative to level at zero minute of K_{CTRL} (mean $\pm$ SD; n=5). **(B)** Immunoblot analysis of active cdc42, Rac1 and RhoA in K_{CTRL} and K_{TAK1-B} exposed to serum for the indicated time periods (min). Values below the blots represent the mean fold change in active cdc42, Rac1 and RhoA relative to level at zero minute of K_{CTRL} (n=5). Representative pictures of immunoblot were shown.

Altogether, our results showed that the deficiency in TAK1 enhances keratinocyte migration associated with an elevated expression of integrins $\beta 1$, $\beta 5$ and exacerbated activation of cdc42, Rac1 and RhoA.

Figure 13. TAK1 deficiency upregulates the expression of integrin β1 and integrin β5. **(A)** TAK1 modulates the expression of integrins and activation of focal adhesion kinase (FAK). Immunoblot analyses of integrins β1, β3, β5, total FAK and phosphorylated FAK p(Y925)FAK in K_{CTRL} and K_{TAK1-B}. Coomassie-stained blot showed equal loading and transfer. **(B)** Integrin-mediated cell adhesion assay showed elevated expression of integrins β1 and αvβ5 in TAK1-deficient keratinocytes. Statistical analysis was determined using two-tailed Mann-Whitney test, denotes p<0.01. **(C)** Reintroduction of TAK1 into K_{TAK1-B} attenuated the expression of integrins and phosphorylation of FAK. Immunoblot analyses of integrins β1, β3, β5, total FAK and phosphorylated FAK p(Y925)FAK in vector- or TAK1-transfected K_{CTRL} and K_{TAK1-B} transfected with either expression vector or vector harboring TAK1 cDNA. β-tubulin showed equal loading and transfer.

3.3 TAK1-deficient epidermis showed impaired differentiation and increased proliferation

Besides cell migration, cell proliferation also plays an important role during re-epithelialization of the wound. We have shown that TAK1 deficiency enhances cell migration with increased expression of integrins $\beta 1$ and $\beta 5$ and active Rho GTPases. The epidermis of TAK1-KO mice displayed increased cell proliferation, but no significant difference was observed in *in vitro* culture of these mutant keratinocytes, thus suggesting the involvement of epithelial-mesenchymal communications (Omori et al, 2006; Sayama et al, 2006). To understand the role of TAK1 in cell proliferation, we constructed and examined organotypic skin cultures (OTCs) using either K_{CTRL} or K_{TAK1-B} keratinocytes with underlying human primary dermal fibroblasts. Our results showed that the overall epidermal thickness between K_{CTRL} and K_{TAK1-B} OTCs was similar, however, the suprabasal layer of the K_{TAK1-B} epidermis was significantly thinner and appeared as discontinuous patches when compared to K_{CTRL} OTC (K_{TAK1-B} vs K_{CTRL} : $38.3 \pm 0.7 \mu m$ vs $101.7 \pm 0.4 \mu m$) (Figure 14). Immunofluorescence performed using keratin 10 (CK 10) and involucrin, a late and a terminal differentiation marker, respectively, showed that K_{TAK1-B} OTCs had an impaired epidermal differentiation where the expression of differentiation markers were dysregulated when compared to K_{CTRL} OTCs (Figure 14).

Figure 14. TAK1-deficient epidermis showed impaired differentiation and increased proliferation. Immunofluorescence staining was performed on K_{CTRL} and K_{TAK1-B} -derived organotypic skin cultures (OTCs). Keratin 10 (CK 10) and involucrin (INV) were late and terminal epidermal differentiation, respectively. Proliferating and apoptotic cells were identified using Ki67 antibody and TUNEL assay, respectively (white arrows). OTC sections were counterstained with DAPI (blue). Dotted line represents epidermal-dermal junction. F denotes the underlying fibroblasts-embedded collagen. H&E: Haematoxylin and eosin staining. Scale bar 40 μ m. Mean proliferating and apoptotic cells were numerated from 3 standardized microscopic fields per section, performed on 3 sections from 4 independent OTC constructions (n=36).

This observation was further confirmed by immunoblot analysis using CK10 and transglutaminase 1, another terminal differentiation marker (Figure 15A). We next examined if TAK1 plays a role in the balance between keratinocyte apoptosis and proliferation, which is important for epidermal homeostasis. K_{TAK1-B} OTCs showed more Ki-67 positive proliferating cells (K_{TAK1-B} vs K_{CTRL} : 8.6 ± 2.8 vs 4.8 ± 1.2 labeled cells per microscopic field) as compared to the control K_{CTRL} OTCs (Figure 14). These were further corroborated by immunoblotting with cyclin D1 and proliferating cell nuclear antigen (PCNA) as proliferation markers (Figure 15A). To gain further insight, we examined cell proliferation in OTCs constructed using either K_{TAK1-B} or K_{CTRL} keratinocytes with collagen, i.e. absence of underlying fibroblasts denoted as K_{TAK1-B}/col and K_{CTRL}/col , respectively (Figure 15B). In contrast to K_{TAK1-B} OTCs constructed with fibroblasts, no significant difference in cell proliferation was observed in K_{TAK1-B}/col when compared to K_{CTRL}/col , which was further confirmed by immunoblot analysis using anti-PCNA antibody (compare Figures 15A with 15B, and Figures 14 with 15C). Our results point towards a homeostatic role of keratinocyte TAK1 that safeguard against epidermal hyperproliferation in a non-autonomous manner.

Figure 15. TAK1 plays homeostatic role in epidermal proliferation in a non-autonomous manner. Immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis from OTCs constructed either (A) normal fibroblasts or (B) collagen only. One and five μ g of protein lysates were used. Cytokeratin 10 (CK10) and transglutaminase I (TGase) were used as late and terminal differentiation markers, respectively. PCNA and cyclin D1 as proliferation markers; cleaved caspase 3 as an apoptotic marker. β -tubulin served as a loading control. Values below indicate the mean relative fold-change with respect to K_{CTRL} of 3 independent OTCs. (C) Immunofluorescence staining of K_{CTRL} and K_{TAK1-B}-derived OTCs cultured with only collagen (COL). In the absence of underlying fibroblasts, no difference in cell proliferation index was observed between K_{CTRL} and K_{TAK1-B} epidermis. Proliferating cells were identified using Ki67 antibody (white arrows). OTC sections were counterstained with DAPI (blue). White dotted lines indicate epidermal-dermal junction. Scale bar 40 μ m.

3.4 TAK1 regulates cell proliferation via double paracrine mechanism

Conceivably, this non-autonomous effect of TAK1 on cell proliferation is likely mediated by the changes in the production and secretion of mitogenic or anti-mitogenic factors by the fibroblasts upon stimulation by K_{TAK1} -derived factors. To understand the underlying mechanism of TAK1 deficiency in cell proliferation, an unbiased protein antibody array was done. Inflammatory and growth factor arrays were used to compare conditioned media from OTCs comprising of either K_{TAK1-B} or K_{CTRL} with normal primary fibroblasts. A total of 76 distinct proteins were screened and the results showed changes in the protein expression level of platelet-derived growth factor (PDGF)-BB and hepatocyte growth factor (HGF), which were further confirmed by ELISA measurement of the OTC culture medium (Figure 16A and 16B). No difference in PDGF-AA, IL-1 β and TGF- β 1 were detected (Figures 16A and 16B). Real-time PCR detected a 13.4-fold increase in the expression of PDGF-B mRNA in K_{TAK1-B} when compared to K_{CTRL} keratinocytes (Figure 16C). PDGF mRNA was not detected in the fibroblasts. An elevated HGF mRNA was detected in the fibroblasts extracted from K_{TAK1-B} OTCs, and was undetectable in the keratinocytes (Figure 16C). Notably, K_{TAK1-B} OTCs treated with anti-PDGF-BB neutralizing antibody showed reduced HGF production in the culture medium (Figure 16C) and in the fibroblasts from K_{CTRL} and K_{TAK1-B} OTCs. To further verify the above observation, we examined the activation status of HGF receptor, Met, in the epidermal keratinocytes. Immunoblot showed an increase in phosphorylation of Met in K_{TAK1-B} as compared to K_{CTRL} (Figure 16D). The increased proliferation seen in K_{TAK1-B} OTCs was not an effect of enhanced ERK activity, which remained unchanged in comparison to K_{CTRL} OTCs (Figure 16D).

A

B

Human Growth Factor Antibody Array I Map

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
2	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
3	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
5	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF R α	PDGF R β	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF R α	PDGF R β	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
7	SCF R	TGF-α	TGF-β	TGF-β 2	TGF-β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS
8	SCF R	TGF-α	TGF-β	TGF-β 2	TGF-β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS

* For use with serum, plasma, condition medium, urine and all other body fluids, cell lysates and certain tissue lysates samples

C

D

Figure 16. PDGF-B, but not PDGF-A, is over-expressed in TAK1-knockdown keratinocytes (K_{TAK1-B}). **(A)** Protein expression levels of platelet derived growth factor-AA and BB (PDGF-AA, PDGF-BB), hepatocyte growth factor (HGF) and interleukin-1 β (IL-1 β) as determined by ELISA. Conditioned serum-free OTC medium from K_{CTRL} and K_{TAK1-B} OTCs with fibroblasts, treated with either PBS (vehicle) or neutralizing anti-PDGF antibody, were used for ELISA measurement. Values are mean $\pm$ SD of three independent experiments (n=3). Statistical analysis was determined using two-tailed Mann-Whitney test, * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$. **(B)** Human Growth Factor Antibody Array I (RayBio®): Protein analysis of conditioned medium from two-week old K_{CTRL} and K_{TAK1-B} -derived OTCs. Spots corresponding to HGF, PDGF-BB and TGF- β were boxed. Representative pictures of array were shown. **(C)** mRNA expression levels of platelet derived growth factor-AA and BB (PDGF-AA, PDGF-BB), hepatocyte growth factor (HGF) and interleukin-1 β (IL-1 β) as determined by qPCR. Conditioned serum-free OTC medium from K_{CTRL} and K_{TAK1-B} OTCs with fibroblasts, treated with either PBS (vehicle) or neutralizing anti-PDGF antibody, were used for ELISA measurement. qPCR was performed using RNA extracted from epidermis and dermal fibroblasts of indicated OTCs. Epidermis was physically separated from OTC after a 20 min treatment with Dispase. Fibroblasts embedded in collagen were isolated after collagenase treatment. PDGF and HGF mRNAs were not detected in fibroblasts and keratinocytes, respectively. Values are mean $\pm$ SD of three independent experiments (n=3). Statistical analysis was determined using two-tailed Mann-Whitney test, * denotes $p<0.05$, ** $p<0.01$ and *** $p<0.001$. **(D)** Immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis from OTCs constructed with underlying fibroblasts. The expression of activated, i.e. phosphorylated HGF receptor, Met p(Y1349)Met was increased in K_{TAK1-B} epidermis. No difference was detected for total Met, total ERK1 and phosphorylated p(T202)ERK1. Values below each band represent the mean fold difference in expression level with K_{CTRL} , which was assigned the value of one.

To underscore the importance of PDGF/HGF signaling in the increased epidermal proliferation observed in TAK1-deficient epidermis. We cultured K_{CTRL} and K_{TAK1-B} OTCs in medium supplemented with anti-PDGF antibody. We hypothesized that if PDGF signaling played a major role in manifesting the phenotype of K_{TAK1-B} OTCs, the effect can be neutralized with anti-PDGF antibody. Indeed, the addition of neutralizing anti-PDGF antibody lowered the number of Ki67-positive proliferating cells in K_{CTRL} and K_{TAK1-B} OTCs when compared to vehicle-treated K_{CTRL} OTC (Figures 17A and 17B).

A

B

C

D

Figure 17. TAK1 mediates PDGF/HGF signaling to regulate cell proliferation. **(A)** K_{TAK1-B} OTCs with underlying fibroblasts were cultured in medium supplemented with anti-PDGF (200 nM) or vehicle (PBS). Mean numbers of proliferating and apoptotic cells were numerated after detection by anti-Ki67 antibody or TUNEL respectively. Values were mean from 5 standardized microscopic fields per section, performed on 7 sections from 3 independent OTCs. K_{CTRL} OTCs with underlying fibroblasts served as control. **(B)** Immunofluorescence staining of K_{CTRL} and K_{TAK1-B} -derived OTCs cultured with underlying fibroblasts (F) in the presence either vehicle (PBS) or neutralizing anti-PDGF antibody. Blocking the activity of PDGF negates the pro-mitogenic effect of TAK1 deficiency in keratinocytes. Dotted white line represents epidermal-dermal junction. H&E: Haematoxylin and eosin staining. Scale bar 40 μ m. mean proliferating cells was numerated from 3 standardized microscopic fields per section, performed on 3 sections from 3 independent OTC constructions (n=27). **(C)** Immunoblot analysis of K_{CTRL} and K_{TAK1} OTCs treated with either PBS (-) or anti-PDGF antibody (+) or **(D)** vector- and TAK1-transfected K_{CTRL} and K_{TAK1} OTCs. Cell proliferation marker (PCNA) was used. β -tubulin served as a loading control.

This was again confirmed by immunoblot analysis using anti-PCNA antibody (Figure 17C). Similarly, epidermis of TAK1-transfected K_{TAK1-B} OTCs showed a reduced expression of PCNA when compared to vector-transfected K_{TAK1-B} OTCs but was similar to K_{CTRL} OTCs (Figure 17D).

Altogether, our results indicate that the double paracrine PDGF/HGF signaling plays a pivotal role in the action of TAK1 in cell proliferation.

3.5 TAK1 suppresses Sp1 mediated signaling via increased expression of von Hippel-Lindau tumor suppressor

Our results showed that the absence of TAK1 enhances cell migration and proliferation associated with concomitant increased expressions of integrins β 1, β 5 and PDGF-B/HGF respectively. Based on these results, we ruled out the possibility that TAK1 has direct effect on the expression of PDGF and integrins. This is because if it were a direct effect of TAK1, TAK1-knockdown cells would have

shown a corresponding reduced expression of integrins and PDGF-B. Previously, we had also used IL-1 to stimulate TAK1 activation in actinomycin D- and cycloheximide-treated keratinocytes. Actinomycin D interferes with protein synthesis by its effect on transcription of RNA, while cycloheximide blocks translation. However, there was no effect on integrin and PDGF-B mRNA levels. This further concluded that TAK1 exerts its effect on integrins and PDGF-B via intermediate molecules. Next, we sought to investigate the mechanism by which TAK1 negatively regulate their expressions.

Earlier studies showed that von Hippel-Lindau tumor suppressor protein (pVHL)-Sp1 interaction suppresses PDGF-B expression (Rafty & Khachigian, 2002). We hypothesize that TAK1 directly increases the expression of pVHL, which sequesters Sp1 and consequently repressed PDGF-B expression. We first examined the expression level of pVHL mRNA and protein levels in K_{CTRL} treated with either IL-1 β or TNF- α in the presence of various kinase inhibitors. With the exception of two different NF κ B inhibitors, BAY 11-7082 and SN50, all the other inhibitors did not attenuate IL-1 β - or TNF- α -induced increase in pVHL mRNA and protein expression when compared to vehicle-treated K_{CTRL} (Figure 18).

A

B

Figure 18. NF κ B pathway is involved in the increased expression of pVHL. **(A)** pVHL mRNA and **(B)** protein expression in K_{CTRL} treated for 24 h with either DMSO (vehicle) or specific inhibitors of the indicated kinases in the presence of IL-1 β or TNF- α . 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], ERK1/2—PD98059. Ribosomal protein L27 was used as a normalizing housekeeping gene. β -tubulin showed equal loading and transfer.

Next, we studied the role of different kinases in regulating the promoter of the human VHL gene. The human VHL promoter was isolated by PCR and a proximal 732 bp fragment containing the transcription initiation site was subcloned into a luciferase reporter gene (Zatyka et al, 2002). This VHL 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 $IKK\alpha/\beta$, MKK7 and JNK (Adhikari et al, 2007; Delaney & Mlodzik, 2006). Transient transfection studies showed that $IKK\alpha$ and TAK1 stimulated the VHL promoter activity (Figure 19). Additional experiments ruled out the possible involvement of MKK7, JNK, PKA, MEK1, MEKK1 and SEK1 signalings in regulating human VHL expression. The human VHL gene promoter contains a putative NF κ B binding site (Zatyka et al, 2002). Site-directed mutagenesis of the putative NF κ B binding site in the VHL promoter abolished the stimulating effect of IKK and TAK1 (Figure 19).

Figure 19. $IKK\alpha$ and TAK1 stimulate the VHL promoter activity. Transactivation assay in keratinocytes co-transfected with a luciferase reporter gene driven by the human VHL promoter (pGL-VHLpro-luciferase), cDNA encoding for indicated constitutively active (ca) kinases and pEF1- β -galactosidase as control of transfection efficiency. Luciferase activity was measured and normalized reporter activity was shown as fold induction as compared to reporter construct transfected- K_{CTRL} (control). VHL promoter reporter construct, whose NF κ B site was mutated, was denoted as pGL-mVHLpro-luciferase. Data are mean $\pm$ SEM, n=3.

Next, we examined the protein expression level of pVHL, Sp1 and NFκB/p65 in K_{TAK1-B} and K_{CTRL} by immunoblot analysis. In concordance with the role of TAK1 in NFκB activation, the phosphorylation NFκB(p65) was reduced in TAK1-deficient keratinocyte. Interestingly, pVHL was barely detectable in the K_{TAK1-B} keratinocytes when compared to K_{CTRL} (Figure 20). No differences in total NFκB(p65) and Sp1 expression were observed (Figure 20). As expected, TAK1-transfected K_{TAK1-B} showed increased expression of pVHL and phosphorylated NFκB(p65) when compared to vector-transfected K_{TAK1-B} (Figure 20).

Figure 20. Immunoblot analysis of protein expression level of pVHL, Sp1 and NFκB/p65. Immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis (left panel) or vector- and TAK1-transfected K_{CTRL} and K_{TAK1-B} (right panel) using antibodies against pVHL, Sp1, total and phosphorylated NFκB(p65). β-tubulin served as a loading control. Values below each band represent the mean fold differences in expression level with respect to K_{CTRL} from five independent experiments (n=5).

To determine if human pVHL is a target gene of TAK1/NFκB-mediated signaling, we performed electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP). Specific protein-DNA complexes were detected in EMSA for VHL NFκB binding site, which was effectively competed by

unlabelled consensus NFκB (SC), but not by a non-specific competitor (NSC) oligonucleotide (Figure 21A). Mutation of the VHL NFκB binding sequence (VHL mNFκB) eliminated its interaction with nuclear extract from K_{CTRL}. As positive control, the labeled consensus NFκB (conNFκB) oligonucleotide was used as a probe, which was specifically competed by the SC (Figure 21A). Similarly, ChIP done on K_{CTRL} and K_{TAK1-B} using anti-NFκB(p65) antibody showed that NFκB(p65) specifically bound to this site in K_{CTRL} but not in K_{TAK1-B} (Figure 21B). No immunoprecipitation and amplification were seen with pre-immune IgG and with a control sequence upstream of the NFκB site on the promoter gene of VHL (Figure 21B). Taken together, we concluded that TAK1/ IKK/ NFκB signaling is necessary for the induction of pVHL expression.

A

B

Figure 21. The human pVHL gene expression is under the transcriptional regulation of TAK1/NF κ B signaling. **(A)** Electrophoretic mobility shift assay (EMSA) of human VHL NF κ B binding site. Radiolabelled VHL NF κ B binding sequence was incubated with nuclear extract isolated from K_{CTRL} . NSC denotes non-specific competitor, a scrambled NF κ B binding sequence. SC denotes non-radiolabelled consensus NF κ B sequence (conNF κ B). As positive control, conNF κ B was used. Mutated VHL NF κ B site is denoted by VHL mNF κ B. **(B)** pVHL is a direct NF κ B target gene. Chromatin immunoprecipitation (ChIP) were done in K_{CTRL} and K_{TAK1-B} using pre-immune IgG (pre) or antibody against p65 subunit of NF κ B (Ab). Promoter region with the NF κ B binding site was immunoprecipitated and specifically amplified in K_{CTRL} using Ab. No amplified signal was obtained in K_{TAK1-B} or using pre-immune IgG. A control region upstream of NF κ B binding site served as negative control. M: 100 bp DNA marker.

In silico analysis of the promoters of human PDGF-B, integrins $\beta 1$, $\beta 3$, $\beta 5$ genes revealed putative Sp1 binding elements (Cervella et al, 1993; Lai et al, 2000; Rafty & Khachigian, 2002; Villa-Garcia et al, 1994). In the first instance, we verified the interaction between pVHL and Sp1 in K_{TAK1-B} and K_{CTRL} by *in vivo* protein-protein crosslink and ChIP as described previously (IJpenberg et al, 2004). Our results showed that interaction between pVHL and Sp1 was only observed in K_{CTRL} (Figure 22A). To further strengthen this observation, *in situ* proximity ligation assay (PLA) was performed using anti-pVHL and anti-SP1 antibodies. PLA allows for the detection and quantification of interaction between two proteins. Our results showed a significant number of interacting pVHL-Sp1 pairs in the cytoplasm of the K_{CTRL} when compared to K_{TAK1-B} (Figure 22B).

A

B

Figure 22. TAK1 is required for the interaction between pVHL and Sp1. **(A)** *In vivo* co-immunoprecipitation of pVHL and Sp1. Immunoprecipitation (IP) was performed using Sp1 antibody, followed by immunoblot (IB) analysis with pVHL antibody and vice versa. **(B)** Detection of pVHL-Sp1 complex in K_{CTRL} and K_{TAK1-B} using DUOLink™ *in situ* proximity ligation assay (PLA) with anti-pVHL and anti-Sp1 antibodies. PLA-signals are shown in red and the nuclei in blue. The nuclei-image has been acquired in one z-plate. Negative control without primary antibody. Scale bar 50 μm.

Next, we performed ChIPs using anti-Sp1 antibody. Our results showed that Sp1 was bound to the Sp1 binding sites in integrins $\beta 1$, $\beta 5$ and PDGF-B, but not integrin $\beta 3$ (Figure 23). Conceivably, the reduced pVHL expression in K_{TAK1-B} allowed free Sp1 to bind and transactivate these genes. To strengthen these results, we performed ChIPs using anti-Sp1 antibody of these promoters in K_{TAK1-B} stably expressing pVHL (pVHL-transfected K_{TAK1-B}).

Figure 23. pVHL inhibits Sp1-mediated PDGF-B, integrins $\beta 1$, $\beta 5$, but not $\beta 3$, gene expression. Chromatin from K_{CTRL} and K_{TAK1-B} was immunoprecipitated with either a Sp1 antibody (Ab) or pre-immune IgG (pre). Enrichment of a DNA fragment encompassing the Sp1 binding sites was evaluated by PCR. Aliquots of the extracts were also used before immunoprecipitation (input). No amplified signal was obtained in using pre-immune IgG. A control region upstream of NF κ B binding site served as negative control.

The expression of pVHL in transfected K_{TAK1-B} was comparable to K_{CTRL} as determined by immunoblot analysis (Figure 24A). Consistent with the aforementioned results, these pVHL-transfected K_{TAK1-B} cells expressed comparable levels of integrins $\beta 1$, $\beta 5$ and PDGF-BB to that of the vector-transfected K_{CTRL} cells (Figure 24A). To further verify the results, we performed ChIP using anti-Sp1 antibody with these cells. The sequences spanning the Sp1 binding site were significantly reduced in the immunoprecipitates obtained from pVHL-transfected K_{TAK1-B} when compared with vector-transfected K_{TAK1-B} (Figure

24B). In addition, no signal was seen with pre-immune serum and no binding was detected to a control sequence.

A

B

Figure 24. Immunoblot analysis and chromatin immunoprecipitation (ChIP) analysis using pVHL-transfected K_{TAK1-B}. **(A)** Ectopic expression of pVHL in K_{TAK1-B} attenuates the expression of integrins $\beta 1$, $\beta 5$ and PDGF-B. Immunoblot analyses (left panel) and ELISA for PDGF-BB in conditioned medium (right panel) of K_{CTRL} and K_{TAK1-B} cells transfected with either empty expression vector or vector harboring cDNA of pVHL. **(B)** Ectopic expression of pVHL in K_{TAK1-B} inhibits Sp1 binding to the promoters of PDGF-B, integrins $\beta 1$ and $\beta 5$. Chromatin from vector- or pVHL-transfected K_{TAK1-B} was immunoprecipitated with either an anti-Sp1 antibody (Ab) or pre-immune IgG (pre). Enrichment of a DNA fragment encompassing the Sp1 binding sites was evaluated by PCR. Aliquots of the extracts were also used before immunoprecipitation (input). Two stable K_{TAK1-B} clones expressing pVHL were used. No amplified signal was obtained in using pre-immune IgG. A control region upstream of NF κ B binding site served as negative control.

The cellular role of pVHL can be hypoxia-inducible factor (HIF)-dependent or –independent manner. *In silico* analysis of the promoters of human PDGF-B, integrins $\beta 1$ and $\beta 5$ genes failed to identify putative HIF α binding site. Nevertheless, to eliminate this possibility, we first performed immunoblot analysis of HIF α using K_{CTRL} and K_{TAK1-B} epidermis. Our results showed no significant change in the expression of HIF α between K_{CTRL} and K_{TAK1-B} (Figure 25). Furthermore, cobalt chloride-simulated hypoxia condition, which increased HIF α expression, delayed K_{CTRL} migration, suggesting that HIF α was not responsible for the observed phenotype of K_{TAK1-B} (Figure 25; compare videos 1, 2 and 8).

Figure 25. pVHL suppresses migration and proliferation via a HIF α -independent fashion. Immunoblot analysis of HIF α on K_{CTRL} , K_{TAK1-B} and TAK1-transfected K_{TAK1-B} epidermis (top panel). β -tubulin showed equal loading and transfer. Time lapsed images of wounded cultures in cobalt chloride-simulated (50 mM) hypoxia condition (bottom panel). Three independent experiments (n=3) were performed. See video 8. Scale bar 100 μ m.

Finally, we examined the expression profile of pVHL mRNA and protein during wound healing. We found that during the healing of mouse skin full-thickness excisional wound, the expression profile of pVHL mRNA and protein was similar to that of TAK1, peaking on days 3-7 post-wounding, as shown by qPCR and immunoblot analysis (Figure 26A). Immunohistochemical analysis revealed that pVHL was strongly expressed in the wound epithelia, similar to TAK1 (Figure 26B). No primary anti-pVHL antibody served as negative control (Figure 26C).

Altogether, our results showed that TAK1/ NF κ B directly upregulate the expression of tumor suppressor pVHL which represses PDGF-B and selected integrins gene expression via Sp1 binding element in their cognate promoter during wound healing.

A

B

C

Figure 26. Expression profile of pVHL mRNA and protein during wound healing. **(A)** pVHL expression is induced during skin wounding. pVHL mRNA and protein expression profiles during wound healing determined using qPCR (left panel) and immunoblotting (right panel). Ribosomal protein L27 was used as a normalizing housekeeping gene. β -tubulin showed equal loading and transfer. **(B & C)** Immunohistochemical analysis for pVHL in skin wound biopsies. The section was processed as described in Figure 4A. DAB with nickel (dark blue) was used as substrate. Representative picture from wound epithelium and the adjacent wound bed was shown. E: epidermis; WB: wound bed; hf: hair follicle. Scale bar 20 μ m. **(B)** Mice skin wound biopsies at indicated days of post-wounding were cryosectioned and stained with antibodies against pVHL. **(C)** Immunohistochemical staining of Day 5 mouse wound biopsies with either pre-immune IgG as negative control (left panel) or with anti-pVHL antibody (right panel).

3.6 TAK1 modulates keratinocyte survival via an autocrine increase in protein kinase B alpha (PKB α) activity

Our above findings showed that the anti-apoptotic effect of TAK1 is cell-autonomous (Figures 15 and 27). The results of the anoikis assay and quantification of the number of apoptotic cells by annexin staining were consistent with our findings. The resistance to cell death due to lack of adhesion, i.e. anoikis is essential for proper epidermal differentiation (Gandarillas et al, 1999). The result revealed that there were more apoptotic K_{TAK1-B} when compared to K_{CTRL} cells over the 2 h experimental period (Figure 28).

Figure 27. Keratinocyte survival is regulated by an autocrine mechanism. Immunofluorescence staining of K_{CTRL} and K_{TAK1-B} -derived OTCs cultured with only collagen (COL). In the absence of underlying fibroblasts, there is increased apoptosis observed in K_{TAK1-B} epidermis. However, no difference in cell proliferation index was observed between K_{CTRL} and K_{TAK1-B} epidermis. Proliferating cells were identified using Ki67 antibody (white arrows). OTC sections were counterstained with DAPI (blue). White dotted lines indicate epidermal-dermal junction.

A

B

Figure 28. TAK1 promotes keratinocyte survival under adhesion-independent condition. **(A)** K_{CTRL} keratinocytes showed resistance to apoptosis but K_{TAK1-B} keratinocytes were more prone to apoptosis and showed significantly lower survival rate over the 2 h experimental period. Keratinocytes that were detected with annexin V fluorescent labeling were quantified. The values are mean $\pm$ SD of three independent experiments ($n = 3$). Approximately 300 cells were counted for each independent experiment. Statistically significant differences (* $P < 0.001$) were proven by student's t-test. **(B)** K_{TAK1-B} keratinocytes showed higher number of annexin V-positive and propidium iodide-positive cells. Annexin V-positive stain will indicate that a cell is in early apoptotic stage while a cell that is stained positive for both annexin V and propidium iodide is in the late apoptotic stage. However, a cell that is only stained positive for propidium iodide can be undergoing necrosis. Therefore, we only consider annexin V-positive cells as the apoptotic cells in our context. Representative pictures of K_{CTRL} and K_{TAK1-B} keratinocytes at the 1 h-time point of anoikis assay are shown. Arrows in the bright field microscopy image indicate apoptotic cells. More K_{TAK1-B} keratinocytes were detected to be in the early (arrows) and late (block arrows) apoptotic stages as compared to K_{CTRL} keratinocytes that have undergone the same treatment. BF: Bright field. Scale bars, 40 μ m.

To understand the underlying mechanism, additional unbiased proteomic analysis was done to screen for candidate growth factors and phosphoproteins. Using both growth factor array and phosphoprotein array, we conducted subtractive comparison of medium and intracellular proteins from K_{TAK1-B} and K_{CTRL} OTCs. Not only did the growth factor array reveal changes in the protein expression level of PDGF-BB and HGF (Figure 16C), it also revealed a change in the protein expression level of stem cell factor (SCF). This was further confirmed by ELISA measurement of the OTC culture medium (Figure 29A). Real-time PCR analysis of the keratinocytes and fibroblasts isolated from OTCs showed corresponding changes at the mRNA level (Figure 29B). Of particular interest, the growth factor array data collaborated with the phosphoprotein array data, which indicated that the expression of SCF and the activation (i.e. phosphorylation) of its receptor c-Kit, were downregulated in K_{TAK1-B} cells. Immunoblot analysis with phospho-c-Kit

antibody further confirmed our protein array findings (Figure 29C). The activation of c-Kit was shown to trigger the phosphoinositide-3 kinase (PI3K)/ protein kinase B alpha (PKB α) pro-survival pathway (Blume-Jensen, 1998). 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-B} as compared to K_{CTRL} cells (Figure 29C). The expression level of total PKB α remained unchanged (Figure 29C). An increase activity, i.e. higher phosphorylation of PKB α protects keratinocytes from apoptosis during their differentiation and epidermal stratification (Thrash, 2006).

The mechanism by which TAK1 regulates SCF abundance is still unclear. SCF is a direct target gene of c-JUN, an obligate partner of AP-1 transcription factor, in fibroblasts (Katiyar et al, 2007). *In silico* analysis of the human SCF promoter identified a putative AP-1 binding site (Taylor et al, 1996). To determine if c-JUN can binds to the identified AP-1, chromatin immunoprecipitation (ChIP) was performed on K_{CTRL} and K_{TAK1-B} using c-JUN antibody. In K_{CTRL}, c-JUN was bound to this AP-1 site compared to K_{TAK1-B} cells (Figure 29D). This effect was not observed in the K_{TAK1-B} and no immunoprecipitation was seen with pre-immune serum. In addition, no amplification was detected for a control sequence upstream of the AP-1 on the promoter gene of SCF (Figure 29D).

A

B

C

D

Figure 29. Anti-apoptotic role of TAK1 is mediated by stem cell factor (SCF) that stimulate protein kinase B alpha (PKB α). **(A)** Protein and **(B)** mRNA expression levels of SCF and interleukin-1 β (IL-1 β) as determined by ELISA and qPCR respectively. Conditioned serum-free OTC medium from K_{CTRL} and K_{TAK1-B} OTCs with fibroblasts were used for ELISA measurement. qPCR was performed using RNA extracted from OTC epidermis. Samples were prepared as described in Figures 14(A) and 14(B). **(C)** Immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis from OTCs constructed with fibroblasts. The expression of activated i.e. phosphorylated SCF receptor, c-KIT (p(Y721)c-KIT), PKB α (p(T308)PKB α and p(S473)PKA α) and its downstream effector, GSK-3 β (p(S9)GSK-3 β) were reduced in K_{TAK1-B} epidermis. Immunoblots were normalized to total PKB α and total c-KIT. Coomassie-stained blot served as transfer control. Values below indicate the mean relative fold-change with respect to K_{CTRL} of 3 independent OTCs. **(D)** SCF is a target gene of c-JUN. Chromatin immunoprecipitation (ChIP) assays were conducted in K_{CTRL} and K_{TAK1-B} using pre-immune IgG (pre) or antibody against c-JUN antibody. The promoter region containing AP-1 site of human SCF gene was specifically amplified in K_{CTRL} but not in K_{TAK1-B}. No immunoprecipitated product was amplified using pre-immune IgG (pre). A control region upstream of AP-1 on the promoter of SCF gene was used as negative control. M: 100 bp DNA marker.

Altogether, our data showed that SCF, c-Kit ligand, mediates the crosstalk between TAK1 and PKB α to regulate cellular survival in the human epidermis. TAK1 acts through the JNK pathway to activate c-JUN, which promotes the production of SCF. Secreted SCF binds and activates i.e. phosphorylate its receptor, c-Kit receptor and subsequently recruits p85 subunit of PI3K and progressively lead to PKB α activation (Lev et al, 1992).

3.7 Exogenous SCF rescue the phenotype of K_{TAK1-B} OTC

Our earlier results showed that SCF acted in an autocrine fashion to balance epidermal apoptosis. To underscore the importance of SCF signaling, we cultured K_{TAK1-B} OTC in medium supplemented with recombinant SCF. We hypothesized that if SCF signaling played a major role in manifesting the phenotype of K_{TAK1-B} OTCs, the effects can be negated using recombinant SCF. Similar to the earlier

results of exposing K_{TAK1-B} to neutralizing anti-PDGF antibody that negated the effect of TAK1 knockdown on proliferation in K_{TAK1-B} OTC (Figures 16A, 16B and 17A-C), the addition of exogenous SCF rescued the phenotype of K_{TAK1-B} OTC. The addition of exogenous SCF significantly lowered the number of apoptotic K_{TAK1-B} cells (Figure 31A). This was further confirmed by the reduced expression of cleaved caspase 3 (Figure 31B). Importantly, the exogenous SCF completely rescued the effect of TAK1 deficiency on cell survival indicating that SCF/c-Kit signaling is a major pathway. (Lam et al, Manuscript in preparation)

Figure 30. Exogenous stem cell factor (SCF) rescues the effect of TAK1 deficiency on cell survival. **(A)** K_{TAK1-B} OTCs cultured in medium supplemented with either recombinant SCF (50 nM), anti-PDGF (200 nM) or vehicle (PBS). Mean numbers of proliferating and apoptotic cells were numerated after detection by anti-Ki67 antibody or TUNEL respectively. Values were mean from 5 standardized microscopic fields per section, performed on 7 sections from 3 independent OTCs. **(B)** Immunoblot analysis of K_{CTRL} and K_{TAK1-B} OTCs supplemented with SCF or anti-PDGF antibody. Apoptotic markers (caspase 3 and cleaved caspase 3) and cell proliferation marker (PCNA) were used. β -tubulin served as a loading control. Values below indicate the mean relative fold-change with respect to K_{CTRL} for 3 independent OTCs.

4 VIDEO LEGENDS

Videos. *In vitro* scratch wound assay performed using **(Video 1)** control keratinocytes (K_{CTRL}) and **(Video 2)** TAK1-deficient keratinocytes (K_{TAK1-B}). Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

Videos. *In vitro* scratch wound assay performed using **(Video 3)** control keratinocytes (K_{CTRL}) and **(Video 4)** TAK1-deficient keratinocytes (K_{TAK1-B}) in the presence of 2 μ g/ml of mitomycin C. Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

Videos. Migratory front cell phenotype of **(Video 5)** K_{CTRL} and **(Video 6)** K_{TAK1-B} . Cell migration was recorded at 1 min interval for 2 h. Scale bar 20 μ m.

Video 7. *In vitro* scratch wound assay performed using TAK1-transfected K_{TAK1-B} . Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

Video 8. *In vitro* scratch wound assay performed using cobalt-simulated hypoxia condition on control keratinocytes (K_{CTRL}). Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

5 DISCUSSION

Following injury, the restoration of functional integrity is of utmost importance to the survival of the organism. The regeneration and maintenance of epithelium to close the wound is dictated by epithelial-mesenchymal interactions and purportedly mediated by the action of central players, such as chemokines and growth factors. This communication is crucial to preventing either insufficient or excessive wound repair. Previous works have shown that mice with a keratinocyte-specific deletion of TAK1 exhibit severe skin inflammation and displayed abnormal epidermis with impaired differentiation, increased cell proliferation and apoptosis (Omori et al, 2006; Sayama et al, 2006). In this study, we revealed that keratinocyte-specific TAK1 regulates epidermal proliferation via a double paracrine mechanism and characterized a novel unsuspected role of TAK1 in cell migration. We showed that pVHL, whose expression is regulated by TAK1/NF- κ B signaling, interacts and sequesters transcription factor Sp1, which is required for the expression of PDGF-B and integrins β 1 and β 5.

The formation of normal epidermal tissue requires a continuous exchange of signals with the underlying dermal fibroblasts (Cheng et al, 2005; Chong et al, 2009). Previous studies have indicated that gene expression changes in the dermal fibroblasts significantly influence epithelial cell fate (Bhowmick & Moses, 2005; Cheng et al, 2005). Keratinocyte-specific TAK1-knockout mice displayed enhanced epidermal proliferation that is not observed in monolayer culture, suggesting that dermal fibroblasts have a pivotal contribution. We revealed that a double paracrine PDGF/HGF signaling system plays an important role in TAK1-

mediated epidermal proliferation. For any cells to respond productively to PDGF, they must possess corresponding cell surface receptors, comprising either the α -PDGF or β -PDGF receptors. Although keratinocytes contribute to PDGF activity by their ample capacity to secrete PDGF, they do not express any transmembrane receptor recognizing any classical PDGF isoforms. Thus target cells for these ligands in skin are restricted to cells of mesenchymal origin e.g. dermal fibroblasts (Ansel et al, 1993; Reuterdaahl et al, 1993). We further showed that the expression of pVHL is regulated by NF- κ B, a downstream mediator of TAK1. The pVHL protein interacts and sequesters transcription factor Sp1, which is required for PDGF-B expression (Rafty & Khachigian, 2002). A similar mechanism was exploited by pVHL to repress VEGF gene expression (Mukhopadhyay et al, 1997). However, we cannot exclude the possibility that pVHL-Sp1 interaction is mediated by a decrease in the stability of Sp1. This is because the function of the pVHL tumor suppressor appears to be its ability to act as an E3 ubiquitin ligase, modulating protein stability by targeting proteins for ubiquitin-mediated degradation (Stebbins et al, 1999). Overall, our results showed that TAK1 deficiency resulted in reduced pVHL expression, allowing Sp1 to bind and activate the PDGF-B promoter. PDGF-B was previously reported to increase HGF production in fibroblasts to stimulate keratinocyte proliferation (Lederle et al, 2006). Indeed, PDGF-BB secreted by keratinocytes acts as a paracrine factor to induce HGF production by fibroblasts that in return, enhances epidermal proliferation (Figure 31). This study underscores the fact that in epidermal keratinocytes TAK1 fulfils a homeostatic role during epidermal formation, modulating cell proliferation in a double paracrine fashion, and involving the underlying dermal fibroblasts. The paracrine mechanism described herein

underscores the importance of epithelial-mesenchymal communication in the regulation of epidermal proliferation which was not observed in TAK1-knockout monolayer culture.

Figure 31. A schematic diagram showing how TAK1 plays a homeostatic role in regulating cell migration and proliferation. Epidermal TAK1 regulates keratinocyte proliferation by a double paracrine mechanism through the release of platelet-derived growth factor-BB (PDGF-BB) which induces hepatocyte growth factor (HGF) in the fibroblasts. TAK1 activates NF κ B to stimulate the expression of von Hippel-Lindau tumor suppressor (pVHL). This facilitates pVHL-Sp1 interaction and sequesters Sp1 from promoting PDGF-B expression. In response to PDGF, the fibroblasts increase the release of HGF that acts via its receptor, Met, to stimulate keratinocytes proliferation. Via a similar mechanism, TAK1 negatively regulate the expression of integrins $\beta 1$ and $\beta 5$ in a cell-autonomous manner.

Cell migration during re-epithelialization is equally crucial for efficient wound closure. In this study, we revealed a novel homeostatic role of TAK1 in the control of keratinocyte migration. When compared to their wildtype counterpart, TAK1-deficient keratinocytes migrated faster and were associated with enhanced expression of integrins $\beta 1$ and $\beta 5$, as well as activation of FAK and small Rho GTPases. Further quantitative evaluation to determine the level of cell surface expression of integrins $\beta 1$ and $\beta 5$ can be performed by cell-ELISA and flow cytometry. In fact, the former method will be suitable for studying cultured keratinocytes and is sufficient if median values for integrin densities are required, while the flow cytometry method should be useful for studying biopsies. The elevated expression profile of active Rho GTPases supports a faster migration rate and persistent lamellipodia formation. The functions of pVHL can be mediated via HIF α -dependent or -independent mechanisms. We consistently observed that the cobalt-chloride-treated cells adopt a different cellular morphology. This suggests that cobalt-chloride-mimicked hypoxia may have an effect on cytoskeleton organization. Indeed, hypoxia-induced cytoskeleton disruption has been observed in endothelial cells (Nishimura et al, 1998) and corneal epithelial cells (Kimura et al, 2010; Teranishi et al, 2008) among others. Our findings also suggest that hypoxia retarded keratinocyte migration. However, hypoxia-mediated migration is complex and context-dependent. It can be modulated by extracellular matrix, matricellular proteins, matrix metalloproteinases and other signaling pathways (O'Toole et al, 2008). It can also differ among keratinocytes isolated from donors of different ages (Xia et al, 2001). However, our findings suggest that the mechanism underlying the phenotype of TAK1-deficient keratinocytes is HIF α -independent. Interestingly, TAK1 employs a similar regulatory mechanism via

pVHL to modulate expression of integrins $\beta 1$ and $\beta 5$. In support of a role in cell migration for pVHL, it has been previously reported that highly aggressive breast cancer expressed either no pVHL or a low VHL level (Zia et al, 2007). High pVHL expression also resulted in a decrease of tubulin turnover indicating a role for pVHL in cellular processes such as migration, polarization, and cell-cell interactions (Lolkema et al, 2004).

The development, maintenance and regeneration of the epidermal integrity are likely to involve the concerted effort of numerous signaling pathways, including epithelial-mesenchymal communications. One of such communications is the IL-1/ KGF/ GM-CSF double paracrine mechanism (Maas-Szabowski et al, 1999). Given the pivotal role of TAK1 in propagating the effect of numerous inflammatory cytokines, such as TNF- α , the PDGF/HGF mechanism described herein, complements the IL-1/ KGF/ GM-CSF mechanism, particularly during wound repair. TAK1 may also modulate cell migration indirectly via HGF *in vivo*, when epithelial-mesenchymal interaction is evident. It was recently shown that HGF-mediated cell migration involves a PAK4-LIMK pathway (Ahmed et al, 2008). It is conceivable that both mechanisms are utilized for epidermal regeneration during wound healing.

We have found a novel link between TAK1 and PKB α via SCF, which acted in an autocrine manner to regulate cell survival (Figure 32).

Figure 32. A schematic diagram showing how TAK1 regulates SCF expression to modulate PKB α activity that promotes keratinocyte survival. TAK1 confers anti apoptotic properties to keratinocytes by increasing stem cell factor (SCF) expression that acts in an autocrine manner to stimulate PKB α activity. TAK1 directly increases the expression of SCF via the JNK/AP-1 pathway. SCF binds to its cognate receptor, c-KIT, and stimulates downstream PKB α survival pathway.

We showed that SCF is a direct target gene of c-JUN and SCF binds to its receptor c-KIT and subsequently stimulate PKB α activation, although the exact mechanism remains unknown (Lee et al, 2000). Sayama *et al.* (Sayama et al, 2006) reported that TAK1-KO mouse keratinocytes underwent spontaneous apoptosis, while another study revealed that these mutant cells were hypersensitive to TNF- α -induced apoptosis (Omori et al, 2006). It was recently reported that a NF κ B-independent pathway may be responsible for TAK1 to regulate reactive oxygen species and modulate cell death in keratinocytes (Omori et al, 2008). It was also shown that c-JUN likely played a role. Our recent unpublished data show that keratinocyte TAK1-mediated SCF expression, which PKB α activity, has a major role in modulating keratinocyte ROS level and apoptosis in an autocrine manner (Lam et al, Manuscript in preparation).

Taken together, our findings provide mechanistic insights into how the deregulation of epidermal TAK1 expression disrupts the equilibrium between keratinocyte proliferation and apoptosis. During epidermal differentiation, the basal keratinocytes detach from the basal lamina and undergo a vectorial upward movement that is usually accompanied by differentiation. These basal cells must be resistant to cell death induced by anoikis for epidermal differentiation to occur properly. Our observation that the suprabasal layer of K_{TAK1-B} OTC was thinner than K_{CTRL} suggests that TAK1-deficient keratinocytes is more susceptible to anoikis. Our results have also demonstrated that TAK1 inhibits both the migration and the proliferation of keratinocytes. Thus, it may appear difficult to reconcile with our observations that the inhibitory TAK1 is highly upregulated during the process of wound healing, when both migration and proliferation are necessary. During wound healing, stratification of wound keratinocytes occurs simultaneously that culminate to the formation of a neo-epidermis and anoikis resistance is also necessary for this to occur. The expression profile of TAK1 during wound healing can affirm our conclusion that TAK1 helps to keep a tight rein on wound healing. If TAK1 were to be downregulated during days 1 to 3 of post-wounding, excessive proliferation and migration without timely stratification of these keratinocytes would occur to render improper wound healing. Thus, anti-apoptotic, anti-mitogenic and migration signals are finely modulated by TAK1 in the epidermis so that the complex biochemical events during wound healing are regulated to reduce abnormal scarring.

Psoriasis is a non-contagious chronic inflammatory skin disease characterized by hyperproliferative epidermal growth, a phenotype that was similarly observed in mice with a keratinocyte-specific deletion of TAK1 (Omori

et al, 2006; Sayama et al, 2006). Recent report suggests that TAK1 deletion causes dysregulation of reactive oxygen species (ROS) in keratinocytes, which is causally associated with skin inflammation (Omori et al, 2006). ROS has been associated with psoriasis (Trouba et al, 2002; Young et al, 2008). Our study reveals the underlying mechanism by which TAK1 regulates epidermal proliferation via a double paracrine mechanism involving the underlying dermal fibroblasts. We showed that TAK1 in the keratinocytes directly up-regulated the expression of pVHL, reduced PDGF-B expression and consequently a diminished HGF/c-Met signaling. A deficiency in TAK1 resulted in reduced NF κ B activation and pVHL expression. Lending support, analysis of skin biopsies of patients with psoriasis showed that pVHL, a direct target of TAK1, was under-expressed in psoriatic skin and highly expressed in healthy skin (Tovar-Castillo et al, 2007). Furthermore, PDGF receptor expression was greatly elevated in psoriatic fibroblasts, rendering them highly responsive to PDGF and PDGF-induced HGF production (Krueger et al, 1990). Altogether, our findings herein and that of others, suggest that TAK1 deficiency may contribute to the etiology of psoriasis. The aberration in TAK1 signaling supports the development of inflammatory diseases, thus a better understanding of its mechanism of action paves new avenues in the development of innovative therapy for inflammatory diseases. For example, drugs or topical formulations can better target wound resolution for patients with sensitive skin and thus mitigate the risk of complications. Further insights of signaling pathway may also yield technological advances in tissue engineering of skin that mainly consists of activating regenerative abilities of the patient's keratinocytes that have aberrant functions and, if necessary, replace damaged skin tissues with skin tissue constructs.

6 FUTURE DIRECTIONS

The *vhl* susceptibility gene encodes a product that acts as a tumor suppressor, pVHL (Latif et al, 1993). It is well-established that VHL protein (pVHL) regulates the protein stability of the transcription factor HIF-1 α through proteolysis and in the regulation of fibronectin matrix assembly (Cockman et al, 2000; Kamura et al, 2000; Tanimoto et al, 2000). pVHL is commonly associated with tumorigenesis. Tumor cells that lack pVHL express high levels of HIF α (Maxwell et al, 1999). The results presented in this thesis made the first connection between pVHL and TAK1 and the cellular role of pVHL was shown to be HIF-independent. Therefore, identification of additional pVHL targets besides the HIF- α subunits is required in order to understand the cellular role of pVHL in the context of its novel link to TAK1.

The contribution of pVHL to the pathophysiology of cancer is well-documented (Kim & Kaelin, 2004; Stickle et al, 2004). The inactivation or otherwise silencing of this tumor suppressor gene was identified to be responsible for VHL disease or a concomitant incidence of kidney cancer. It was revealed that while no gene mutation was found tumors resulting from VHL disease, there was a biochemical change in the promoter of the tumor suppressor gene that had effectively led to it being silenced or inactivated. The latter process is now known to be important in various sporadic cancers i.e. those that occur without any prior family history (Kim & Kaelin, 2004). Furthermore, other studies had showed that an intact HIF pathway is insufficient to prevent VHL-associated tumorigenesis (Stickle et al, 2004). Ohh et al. (1998) showed a direct role of pVHL in fibronectin matrix assembly, important for tumor growth (Ohh et al, 1998). Our current

findings underscore the possible implications of TAK1 in tumourigenesis. Further studies of migration will be essential to establish our understanding of the molecular pathways that control the normal behavior of wound healing and metastasis of cancerous cell. The tumorigenic keratinocyte cell line, A5RT3, and TAK1 knockdown keratinocytes can be accessed for their migratory behavior using the *in vitro* transwell migration assay or invasion assay while *in vivo* studies can be conducted using Nude mice.

Changes in the epidermis such as abnormal differentiation and hyperproliferation are of importance in the pathogenesis of psoriasis. Thus far, this complex chronic skin disease has many questions remained to be answered regarding the underlying mechanism of disease development. The study by Tovar et al. (2007) reported on the differential expression of pVHL in psoriatic skin as compared to normal skin (Tovar-Castillo et al, 2007). The significance of TAK1 on this differential expression of pVHL can be further evaluated.

With recent reports that implicate TAK1 in reactive oxygen species (ROS) production (Morioka et al, 2009; Omori et al, 2008), it is of our interest to investigate the underlying mechanism in which TAK1 plays a homeostatic role in modulating ROS production during wound healing. While in the midst of working on this thesis, our lab has showed that the SCF-mediated activation of PKB α protects keratinocytes from TNF α -mediated apoptosis and anoikis. This mechanism modulates the level of ROS in keratinocytes, demonstrating a novel role of SCF in mediating the protective role of TAK1 against TNF-mediated killing of keratinocytes. Omori et al. (2008) suggested that FLIP degradation in TAK1^{-/-} keratinocytes could explain the increased sensitivity to death receptor-induced cell death. It is worthwhile to link our observations with further

investigation on the effects of SCF or ROS scavengers on FLIP degradation. Additional experiments such as blockade of ROS production by N-acetyl-L-cysteine (NAC), a reduced glutathione provider and direct scavenger of ROS, can be performed to affirm our previous data. By blocking the generation of ROS, the apoptotic phenotype in OTC will also be blocked. In contrast, there is other evidence that suggests that oxidative stress can trigger cell proliferation through the implication of ROS in mitogenic pathways (Liu et al, 2002). It may be interesting to extend our insights of TAK1 regulation to study the possible involvement of Rac1/Rho-GTPases in ROS production, which may contribute to mitogenic signaling. Hence, to test for Rac1's participation in ROS production, GTP-bound Rac1 pull-down assay can be carried out. Keratinocytes can also be transiently transfected with a dominant negative Rac1 mutant to be used for studies that investigate Rho GTPases' contribution to TAK1-mediated reduced cell proliferation.

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Regulation of Cell Proliferation and Migration by TAK1 via Transcriptional Control of von Hippel-Lindau Tumor Suppressor^{*S}

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Skin maintenance and healing after wounding requires complex epithelial-mesenchymal interactions purportedly mediated by growth factors and cytokines. We show here that, for wound healing, transforming growth factor- β -activated kinase 1 (TAK1) in keratinocytes activates von Hippel-Lindau tumor suppressor expression, which in turn represses the expression of platelet-derived growth factor-B (PDGF-B), integrin $\beta 1$, and integrin $\beta 5$ via inhibition of the Sp1-mediated signaling pathway in the keratinocytes. The reduced production of PDGF-B leads to a paracrine-decreased expression of hepatocyte growth factor in the underlying fibroblasts. This TAK1 regulation of the double paracrine PDGF/hepatocyte growth factor signaling can regulate keratinocyte cell proliferation and is required for proper wound healing. Strikingly, TAK1 deficiency enhances cell migration. TAK1-deficient keratinocytes displayed lamellipodia formation with distinct microspike protrusion, associated with an elevated expression of integrins $\beta 1$ and $\beta 5$ and sustained activation of cdc42, Rac1, and RhoA. Our findings provide evidence for a novel homeostatic control of keratinocyte proliferation and migration mediated via TAK1 regulation of von Hippel-Lindau tumor suppressor. Dysfunctional regulation of TAK1 may contribute to the pathology of non-healing chronic inflammatory wounds and psoriasis.

Wound healing is a highly dynamic process that involves complex interactions of extracellular matrix molecules, soluble mediators, various resident cells, and infiltrating leukocyte subtypes. The immediate goal in repair is to achieve tissue integrity and homeostasis. The healing process involves three phases that overlap in time and space, namely inflammation, re-epithelialization, and tissue remodeling. Re-epithelialization is accomplished by increased keratinocyte proliferation and guided migration of the keratinocytes over the granulation tissue. Such processes require ordered changes in keratinocyte

behavior and phenotype, which are dictated by the interplay of keratinocytes with dermal fibroblasts, *i.e.* epithelial-mesenchymal communication. This complex interplay demands the integration of diverse signals through a network of soluble factors exerting autocrine and paracrine activity from the wound microenvironment, culminating in appropriate cellular responses (1, 2). Aberrations to this signaling network may impair or enhance cell migration and proliferation, leading to insufficient or excessive wound repair and life-threatening consequences such as tumor growth and metastasis. Therefore, to understand the effect of any molecule in normal cellular function, studies into its role in this signaling network and how they culminate to an appropriate cell response become fundamental and necessary.

Transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) belongs to the MAPK kinase kinase family. This serine/threonine kinase is a key intermediate in inflammatory cytokines tumor necrosis factor- α (TNF- α) and interleukin 1 (IL-1) (3, 4) as well as TGF- β (5)-mediated signaling pathways. Activated TAK1 has the capacity to stimulate its downstream MAPK and NF- κ B-inducing kinase-I κ B kinase cascades (6). The former activates c-Jun N-terminal kinase (JNK) and p38 MAPK while the latter activates NF- κ B (3, 7, 8). A deficiency in TAK1 results in impaired TNF- α - and IL-1-stimulated JNK activity, p38 phosphorylation, and I κ B α degradation (7, 9). Studies of keratinocyte-specific TAK1 knock-out (TAK1-KO) mice confirmed the role of TAK1 in skin inflammation. These TAK1-KO mice died by postnatal day 7 and developed intra-epidermal micro-abscesses (10, 11). The TAK1-KO mice displayed abnor-

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⁴ The abbreviations used are: TGF- β , transforming growth factor- β ; ChIP, chromatin immunoprecipitation; CK10, cytokeratin 10; ELISA, enzyme-linked immunosorbent assay; HGF, hepatocyte growth factor; HIF α , hypoxia-inducible factor α ; IKK, I κ B kinase; IL-1, interleukin 1; JNK, c-Jun N-terminal kinase; siRNA, small interference RNA; K_{CTRL}, control siRNA keratinocytes; K_{TAK1-B}, TAK1 siRNA keratinocytes; KGF, keratinocyte growth factor; MAPK, mitogen-activated protein kinase; MEK1, MAPK kinase 1; MEK1, MAPK kinase 1; MKK7, MAPK kinase 7; OTC, organotypic skin culture; PDGF, platelet-derived growth factor; PLA, proximity ligation assay; pVHL, von Hippel-Lindau tumor suppressor; qPCR, real-time quantitative PCR; SEK1, MAPK kinase 4; SGK, serum and glucocorticoid-inducible kinase; TAK1, TGF- β -activated kinase 1; TNF- α , tumor necrosis factor- α ; KO, knock out; ca, constitutively active; PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase.

TAK1 Regulates Cell Proliferation and Migration

mal epidermis with impaired differentiation and increased cellular proliferation; however, no significant difference in proliferation index was observed in culture of these mutant keratinocytes *in vitro*. Nevertheless, the latter suggests a crucial role of the underlying dermis in mitigating some effects of epidermal TAK1. Although the role of TAK1 in inflammatory response is well established, the role of TAK1 and its mechanism of action in keratinocyte proliferation and migration remain unknown.

Herein, we show that the deficiency in TAK1 resulted in increased cell proliferation and migration. We provide evidence of a double paracrine mechanism that make a pivotal contribution to the enhanced cell proliferation in TAK1-deficient epidermis. This study also reveals a novel homeostatic role of TAK1 in controlling cell migration. These aberrant phenotypes, as a consequence of TAK1 deficiency, are mediated via the dysregulated expression of von Hippel-Lindau tumor suppressor.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—The reagents and antibodies were as follows: Anti-TAK1 and anti-pVHL for immunohistochemistry (Upstate Biotechnology); anti-TAK1 for immunoblot (Cell Signaling); anti-cdc42-, anti-Rac1, and anti-RhoA (Cytoskeleton), anti-cytokeratin 10 (CK10), involucrin, and Ki67 (Novacastra); neutralizing anti-PDGF-BB antibody (Peprotech); 4',6-diamidino-2-phenylindole and Vectashield mounting medium (Vector Laboratories); AlexaFluor® 488 goat anti-mouse IgG (Molecular Probes, Invitrogen); anti-Sp1 antibody and goat anti-rabbit IgG horseradish peroxidase (Santa Cruz Biotechnology); Collagen Type I Rat Tail (BD Biosciences); human primary fibroblasts, keratinocytes, and culture medium (Cascade Biologics); transfection reagent FuGENE HD (Roche Applied Science); and double-promoter lentivirus-based siRNA vector and pFIV Packaging kit were from System Biosciences. All restriction enzymes and DNA/RNA-modifying enzymes were from Fermentas. Kinase inhibitors were from Merck. Otherwise, chemicals were from Sigma-Aldrich.

Lentivirus siRNA Constructs and Transduction—The sequences of TAK1 and control siRNA were shown in supplemental Table S1. The siRNAs were into the pFIV-HI/U6 siRNA vector according to the manufacturer's protocol. Positive clones were identified by PCR and by DNA sequencing. Production of pseudoviral particles and transduction of cells were as described by the manufacturer. Following transduction, the cells were enriched with 350 ng/ml puromycin for a week.

Transient Transfection and Transactivation Assay—TAK1 cDNA was subcloned into pCMV5 mammalian expression vector (Stratagene). An ~732-bp proximal promoter of the human VHL was PCR-amplified from human genomic DNA using *Pfu* polymerase (12). The resulting fragment was subcloned into the pGL-3 Basic luciferase reporter vector (Promega, Madison, WI). Site-directed mutagenesis was performed using QuikChange site-directed mutagenesis kit (Stratagene). The sequence of primers is available in supplemental Table S1. Keratinocytes were cotransfected with a luciferase reporter driven by the VHL promoter construct, cDNAs encoding for various kinases and pEF1- β -galactosidase as a control of trans-

fection efficiency (13). The constitutively active (ca) protein kinase A, MEK1, and MEKK1 were from Clontech. The caMKK7 was from Cell BioLabs. The various caSEK1, caJNK, caSGK, and caKK expression vectors were from E. Nishida (Kyoto University, Japan), R. J. Davis (University of Massachusetts Medical School, Worcester), D. J. Templeton (University of Virginia Medical School, Charlottesville), and D. V. Goeddel (Tularik, Inc.). TAK1 was as previously described (5, 14). After transfection, cells were cultured for 48 h prior to lysis. Luciferase activity was measured using the Promega luciferase assay on a Microbeta Trilux (PerkinElmer Life Sciences). β -Galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl- β -D-galactopyranoside as a substrate.

Total RNA Isolation and Real-time qPCR—Total RNA was isolated from cells using an Aurum total RNA kit (Bio-Rad) following the supplier's protocol. Total RNA (2.5 μ g) was reverse-transcribed with oligo(dT) primers using RevertAid™ H Minus M-MuLV. 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 sequence of primers is available in supplemental Table S1. Interferon response detection was done as recommended by the manufacturer (System Biosciences).

Organotypic Skin Culture—Organotypic skin cultures (OTCs) were performed as previously described (15).

Immunofluorescence and Immunohistochemistry—OTCs 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-micron cryosections were processed for immunofluorescence as described previously (16). Apoptotic cells were detected using the TUNEL assay according to the manufacturer's protocol (Roche Applied Science). As positive control for TUNEL assay, the section was pre-treated with DNase I. The slides were counterstained with 4',6-diamidino-2-phenylindole and mounted for microscopic observation. Immunohistochemistry on the indicated wound section was performed with anti-TAK1 or anti-pVHL antibodies (Upstate Biotechnology). The signal was amplified using the ABC-peroxidase method (Vector Laboratories) and revealed using 3,3'-diaminobenzidine for TAK1 (brown) or in the presence of nickel for pVHL (dark blue). Images were taken using MIRAX MIDI (Carl Zeiss) with a Plan-Apochromatic 20 $\times$ /0.8 objective and MIRAX Scan software.

In Situ Proximity Ligation Assay—K_{CTRL} and K_{TAK1-B} cells were subcultured onto glass chamber slides (Lab-Tek). The following day, the cells were fixed with methanol:acetone (1:1) at -20 °C for 10 min. The slides were washed twice with PBS. The slides were blocked with 1% bovine serum albumin in PBS for 2 h at room temperature and were incubated with primary rabbit anti-pVHL and mouse anti-Sp1 antibodies overnight at 4 °C. The slides were washed as above. Duolink™ *in situ* PLA was performed as recommended by the manufacturer (OLink Biosciences). As negative control, no primary antibody was used. Images were taken using LSM510 META confocal laser scanning microscope (Carl Zeiss).

Protein Array Analysis—Phosphoprotein and growth factor array membranes were processed according to the manufacturer's protocol (RayBiotech). These arrays were used to compare conditioned media from 2-week-old OTCs reconstructed using K_{CTRL} and K_{TAK1-B} keratinocytes. Protein spots were detected by chemiluminescence. Signal intensities were quantified using ImageJ analysis software and were normalized with the mean intensity of the positive controls on each membrane.

ELISA and Integrin-mediated Cell Adhesion Assays—The concentration of growth factors was measured using sandwich ELISA (R&D Systems) according to the manufacturer's instruction. The integrin-mediated cell adhesion assay was performed as recommended by the manufacturer (Chemicon).

Immunoblot Analysis—Total protein was extracted from cells with ice-cold lysis buffer (20 mM $Na_2H_2PO_4$, 250 mM NaCl, 1% Triton X-100, 0.1% SDS). Equal amounts of protein extracts were resolved by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. Membranes were processed according to standard procedure, and proteins were detected by chemiluminescence. A Coomassie Blue-stained membrane was used to check for equal loading and transfer.

GTPases Activation Assay for cdc42, Rac1, and RhoA—GT-Pases activation assays were carried out as previously described (17). Purified recombinant GST-p21 binding domain of PAK was used to measure active cdc42 and Rac1, whereas GST-Rho binding domain of rhotekin was used to measure active RhoA.

Protein-Protein and ChIP—*In vivo* protein-protein cross-link and chromatin immunoprecipitation (ChIP) were carried out as previously described except anti-pVHL and anti-Sp1 antibodies were used (18). Primers used for ChIPs are available in supplemental Table S1.

RESULTS

Elevated TAK1 Expression in Wound Epithelia—Immediately after injury, an acute inflammation response ensures immune competence and orchestrates later wound healing events including re-epithelialization. The major goal of re-epithelialization, of which cell migration and proliferation are dominant cellular events, is the reconstitution of the epithelium as a functional barrier. Although the role of TAK1 activation in inflammation is well established, little is known about its role in cell migration, which is crucial to the re-establishment of the wounded area. In the first instance, we examined the expression profile of TAK1 during the healing of mouse skin full-thickness excisional wound. We found that, during this healing, the expression profile of TAK1 mRNA and protein peaked at day 3–7 post-wounding, as shown by quantitative real-time PCR (qPCR) and immunoblot analysis (Fig. 1A). Immunohistochemistry staining with anti-TAK1 revealed that the expression of TAK1 was elevated in the wound epithelia (Fig. 1B), compared with only a basal level in unwounded skin. Immunostaining performed using pre-immune IgG did not display any staining (supplemental Fig. S1A).

To investigate whether the up-regulation of TAK1 was similarly observed in human, we performed a retrospective examination of human wounds. The TAK1 mRNA level was elevated in human ulceric skin biopsies compared with normal skin as determined by qPCR (supplemental Fig. S1B).

These observations underscore an important role of TAK1 during re-epithelialization of rodents and human skin wounds.

TAK1 Deficiency Enhances Keratinocyte Migration—Cell migration over the provisional wound bed is essential for re-epithelialization of injured tissues. As expression of TAK1 was increased during re-epithelialization of the wound, we questioned whether TAK1 deficiency affects keratinocyte migration. Thus, we generated human keratinocytes whose endogenous TAK1 expression was suppressed by lentivirus-mediated RNA interference. The efficiency of siRNA knockdown of TAK1 expression in human keratinocytes was assessed by qPCR and immunoblot analysis. Keratinocytes transduced with either TAK1-A (K_{TAK1-A}) or TAK1-B (K_{TAK1-B}) sequences showed ~90% reduction in the expression of TAK1 mRNA when compared with unrelated control siRNA (K_{CTRL}) (Fig. 1C). Consistent with the mRNA expression, immunoblot analysis showed negligible level of TAK1 protein in both K_{TAK1-A} and K_{TAK1-B} (Fig. 1C). For subsequent analysis, K_{TAK1-B} keratinocytes were used. The expression of phosphorylated JNK, p38, and IKK α/β were down-regulated in K_{TAK1-B} cells, consistent with the role of TAK1 in the activation of these signaling pathways. There was no difference in phosphorylation of other MAPKs, such as ERK1/2 and ERK5 (Fig. 1D). The induction of interferon responses has been reported as a challenge to the specificity of some RNA interference approaches (19). To test whether gene silencing induced by the lentiviral-mediated RNA interference was associated with interferon-response induction, we measured the expression of some key interferon response genes by qPCR and the ability to rescue the K_{TAK1-B} phenotype by the re-introduction of an expression vector harboring TAK1 cDNA that has a silent third-codon point mutation within the target region. No induction of 2',5'-oligoadenylate synthetase isoforms 1 and 2, interferon-induced myxovirus resistance 1, and interferon-stimulated transcription factor 3 γ was detected in the K_{TAK1-B} when compared with either wild-type untransduced cells or K_{CTRL} (Fig. 1E). Similarly, the re-introduction of TAK1 into K_{TAK1-B} (TAK1-transfected K_{TAK1-B}) restored the expression of phosphorylated JNK and IKK α/β but had no effect on ERK1/2 (Fig. 1F). Our results showed that gene silencing was not associated with nonspecific interferon-response induction, *i.e.* off-target effect, and that the deficiency of TAK1 attenuated the activation of JNK, p38, and IKK α/β .

Next, we investigate the effect of TAK1 deficiency in keratinocyte migration using *in vitro* scratch wound assay. Interestingly, our live-imaging results showed that K_{TAK1-B} closed the *in vitro* wound 3–4 h ahead of K_{CTRL} (Fig. 2A and supplemental videos S1 and S2). Cell proliferation and migration contributed to the closure of the *in vitro* wound. To more precisely determine the contribution of cell migration toward wound closure, we performed similar experiments in the presence of mitomycin C. Consistent with the above observation, K_{TAK1-B} closed the *in vitro* wound by 10 h in the presence of mitomycin C, a 2-h delay as compared with vehicle-treated K_{TAK1-B} . In contrast, K_{CTRL} failed to close the wound even by the end of the 12-h experimental period (supplemental Fig. S2 and videos S3 and S4). Close examination of the migratory front revealed that K_{TAK1-B} displayed more extended lamellipodia with numerous

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microspike-like features when compared with K_{CTRL} (Fig. 2A and supplemental videos S5 and S6). TAK1-transfected K_{TAK1-B} cells have a delayed migration when compared with K_{TAK1-B} and were similar to K_{CTRL} (Fig. 2A and supplemental video S7).

Our earlier results demonstrated that TAK1-deficient cells migrated faster than the K_{CTRL} . The small Rho GTPases, cdc42, Rac1, and RhoA, are pivotal intracellular mediators crucial for the formation of lamellipodia, cytoskeleton network, and cell migration. Rac1 plays a role in the protrusion of lamellipodia and in forward movement, whereas cdc42 maintains cell polarity, including lamellipodia activity at the leading wound edge (20). To gain further insight into the mechanism, we examined the activation of small Rho-GTPases, specifically cdc42, Rac1, and RhoA in K_{CTRL} and K_{TAK1-B} cells stimulated with serum. Consistent with the enhanced migration of K_{TAK1-B} , an elevated and sustained activation of cdc42, Rac1, and RhoA was observed (Fig. 2B and supplemental Fig. S3). K_{CTRL} responded with a reduced activation of the three Rho GTPases. Notably, an elevated basal expression of active cdc42, Rac1, and RhoA was observed in K_{TAK1-B} when compared with K_{CTRL} before serum stimulation, thus we hypothesized that TAK1 likely exerted its action upstream of Rho GTPases activation. Integrin ligation and clustering triggers the subsequent activation of the GTPases: cdc42, Rac1, and RhoA. Immunoblot analysis revealed higher expression level of integrins $\beta 1$ and $\beta 5$, but not $\beta 3$, in K_{TAK1-B} when compared with K_{CTRL} (Fig. 2C). Consistent with increased integrin ligation, we also detected enhanced activation, *i.e.* phosphorylation of focal adhesion kinase (FAK) in K_{TAK1-B} (Fig. 2C). The observation was further confirmed using integrin-mediated adhesion assay, which showed significant increase in integrins $\beta 1$ and $\beta 5$ expression in K_{TAK1-B} (Fig. 2D). As expected, the expression of integrins $\beta 1$ and $\beta 5$ and phosphorylated FAK in TAK1-transfected K_{TAK1-B} returned to the similar level observed in K_{CTRL} (Fig. 2E). Altogether, our results showed that the deficiency in TAK1 enhances keratinocyte migration associated with an elevated expression of integrins $\beta 1$ and $\beta 5$ and exacerbated activation of cdc42, Rac1, and RhoA.

TAK1-deficient Epidermis Showed Impaired Differentiation and Increased Proliferation—Cell proliferation also plays important role during re-epithelialization of the wound. The epidermis of TAK1-KO mice displayed increased cell proliferation, but no significant difference was observed in *in vitro* culture of these mutant keratinocytes, thus suggesting the involve-

ment of epithelial-mesenchymal communications. To understand the role of TAK1 in cell proliferation, we constructed and examined OTCs using either K_{CTRL} or K_{TAK1-B} keratinocytes with underlying human primary dermal fibroblasts. Our results showed that the overall epidermal thickness between K_{CTRL} and K_{TAK1-B} OTCs was similar, however, the suprabasal layer of the K_{TAK1-B} epidermis was significantly thinner and appeared as discontinuous patches when compared with K_{CTRL} OTC (K_{TAK1-B} versus K_{CTRL} : $38.3 \pm 0.7 \mu m$ versus $101.7 \pm 0.4 \mu m$) (supplemental Fig. S4). Immunofluorescence performed using differentiation markers, keratin 10 (CK10) and involucrin, showed that K_{TAK1-B} OTCs had an impaired epidermal differentiation where the expression of differentiation markers were dysregulated when compared with K_{CTRL} OTCs (supplemental Fig. S4). This observation was further confirmed by immunoblot analysis using CK10 and transglutaminase 1, a terminal differentiation marker (Fig. 3A). K_{TAK1-B} OTCs showed more Ki67-positive proliferating cells (K_{TAK1-B} versus K_{CTRL} : 8.6 ± 2.8 versus 4.8 ± 1.2 labeled cells per microscopic field) as compared with the K_{CTRL} OTCs (supplemental Fig. S4). These were further corroborated by immunoblotting with cyclin D1 and PCNA as proliferation markers (Fig. 3A). To gain further insight, we examined cell proliferation in OTCs constructed using either K_{TAK1-B} or K_{CTRL} keratinocytes with collagen, *i.e.* absence of underlying fibroblasts denoted as K_{TAK1-B}/col and K_{CTRL}/col , respectively (Fig. 3B). In contrast to K_{TAK1-B} OTCs constructed with fibroblasts, no significant difference in cell proliferation was observed in K_{TAK1-B}/col when compared with K_{CTRL}/col , which was further confirmed by immunoblot analysis using anti-PCNA antibody (Fig. 3, compare A with B, and compare supplemental Fig. S4 with S5A). Our results point toward a homeostatic role of keratinocyte TAK1 that safeguard against epidermal hyperproliferation in a non autonomous manner.

TAK1 Regulates Cell Proliferation via Double Paracrine Mechanism—Conceivably, this non-autonomous effect of TAK1 on cell proliferation is likely mediated by the changes in the production and secretion of mitogenic or anti-mitogenic factors by the fibroblasts upon stimulation by K_{TAK1-B} -derived factors. To understand the underlying mechanism of TAK1 deficiency in cell proliferation, an unbiased protein antibody array was done. Inflammatory and growth factor arrays were used to compare conditioned media from OTCs composed of either K_{TAK1-B} or K_{CTRL} with normal primary fibroblasts. A total of 76 distinct proteins was screened, and the results

FIGURE 1. TAK1 expression is induced during skin wounding. A, TAK1 mRNA and protein expression profiles during wound healing determined using qPCR (left panel) and immunoblotting (right panel). Ribosomal protein L27 was used as a normalizing housekeeping gene. β -Tubulin showed equal loading and transfer. B, immunohistochemical analysis for TAK1 in skin wound biopsies. Mice skin wound biopsies at the indicated days of post-wounding were cryosectioned and stained with antibodies against TAK1. Representative pictures from wound epithelia were shown. E, epidermis; WB, wound bed; hf, hair follicle. Scale bar, 20 μm . C, lentivirus-mediated knockdown of TAK1 in keratinocytes. Human keratinocytes were transduced with a lentiviral vector harboring a control or with two different TAK1 (TAK1-A and TAK1-B) siRNAs. Expression of TAK1 in either control (K_{CTRL}) or TAK1 siRNA (K_{TAK1-A} or K_{TAK1-B}) transduced keratinocytes was assessed by qPCR and immunoblotting. Values below each band represent the mean $\pm$ fold differences in expression level with respect to control from five independent experiments ($n = 5$). Keratinocytes were transduced with TAK1-B siRNA (K_{TAK1-B}) were used for subsequent studies. D, expression of phosphorylated and total JNK, p38, ERK1/2, ERK5, and IKK α/β in K_{CTRL} and K_{TAK1-B} . Reduced levels of p(Thr183)JNK, p(Thr180/Tyr182)p38, and pIKK α/β confirmed that endogenous TAK1 expression was suppressed. Values below each band represent the mean $\pm$ fold differences in expression level with respect to K_{CTRL} , which was assigned the value of one. E, expression level of 2',5'-oligoadenylate synthetase isoforms 1 and 2 (OAS1 and OAS2), interferon-induced myxovirus resistance 1 (MX1), and interferon-stimulated transcription factor 3 γ (ISGF3 γ) mRNAs in K_{CTRL} and K_{TAK1-B} as determined by qPCR. Ribosomal protein L27 was used as a normalizing housekeeping gene. F, expression of phosphorylated and total JNK, IKK α/β , and ERK1/2 in K_{CTRL} and K_{TAK1-B} transfected with either expression vector or vector containing TAK1 cDNA. β -Tubulin showed equal loading and transfer. Values below each band represent the mean $\pm$ fold differences in expression level with vector-transfected K_{CTRL} , which was assigned the value of one.

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FIGURE 2. TAK1 deficiency enhances cell migration. *A*, time-lapsed images of wounded cultures of control (K_{CTRL}), TAK1-knockdown (K_{TAK1-B}), and TAK1-transfected K_{TAK1-B} (top five panels). Scale bar, 100 μ m. See supplemental videos S1, S2, and S7, respectively. Higher magnification images from video microscopy showing the migratory front of K_{CTRL} , K_{TAK1-B} , and TAK1-transfected K_{TAK1-B} (bottom two panels). Arrows indicate the focal adhesion points and microspike-like extensions in lamellipodia during migration. Scale bar, 20 μ m. Also see supplemental videos S5 and S6. *B*, graphs showed relative -fold change of active cdc42 (top), Rac1 (middle), and RhoA (bottom) in K_{CTRL} and K_{TAK1-B} exposed for the indicated time periods (minutes) with serum. Values represent the mean -fold change in active cdc42, Rac1, and RhoA relative to level at zero min of K_{CTRL} (mean $\pm$ S.D.; $n = 5$). *C*, TAK1 modulates the expression of integrins and activation of focal adhesion kinase (FAK). Immunoblot analyses of integrins $\beta 1$, $\beta 3$, and $\beta 5$, total FAK, and phosphorylated FAK p(Y925)FAK in K_{CTRL} and K_{TAK1-B} . Coomassie Blue-stained blot showed equal loading and transfer. *D*, integrin-mediated cell adhesion assay showed elevated expression of integrins $\beta 1$ and $\alpha v \beta 5$ in TAK1-deficient keratinocytes. Statistical analysis was determined using two-tailed Mann-Whitney test; *, $p < 0.01$. *E*, re-introduction of TAK1 into K_{TAK1-B} attenuated the expression of integrins and phosphorylation of FAK. Immunoblot analyses of integrins $\beta 1$, $\beta 3$, and $\beta 5$, total FAK, and phosphorylated FAK p(Y925)FAK in vector- or TAK1-transfected K_{CTRL} and K_{TAK1-B} transfected with either expression vector or vector harboring TAK1 cDNA. β -Tubulin showed equal loading and transfer.

showed changes in the protein expression level of platelet-derived growth factor (PDGF)-BB and hepatocyte growth factor (HGF), which were further confirmed by ELISA measurement

of the OTC culture medium (Figs. 3C and 55B). No difference in PDGF-AA, IL-1 β , and TGF- β 1 were detected (Figs. 3C and 55B). Real-time PCR detected a 13.4-fold increase in the

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FIGURE 3. TAK1 regulates keratinocyte proliferation via a double paracrine mechanism. Immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis from OTCs constructed either normal fibroblasts (A) or collagen only (B). 1 and 5 μ g of protein lysates were used. Cytokeratin 10 (CK10) and transglutaminase I (Tase) were used as a late and terminal differentiation makers, respectively. PCNA and cyclin D1 are proliferation markers; cleaved caspase 3 is an apoptotic marker. β -Tubulin served as a loading control. Values below indicate the mean relative fold change with respect to K_{CTRL} of three independent OTCs. Protein (C) and mRNA expression (D) levels of PDGF-AA and PDGF-BB, HGF, and IL-1 β as determined by ELISA and qPCR, respectively. Conditioned serum-free OTC medium from K_{CTRL} and K_{TAK1-B} OTCs with fibroblasts, treated with either PBS (vehicle) or neutralizing anti-PDGF antibody, were used for ELISA measurement. qPCR was performed using RNA extracted from epidermis and dermal fibroblasts of indicated OTCs. Epidermis was physically separated from OTC after a 20-min treatment with Dispase. Fibroblasts embedded in collagen were isolated after collagenase treatment. PDGF and HGF mRNAs were not detected in fibroblasts and keratinocytes, respectively. Values are mean $\pm$ S.D. of three independent experiments ($n = 3$). Statistical analysis was determined using two-tailed Mann-Whitney test; *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. E, immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis from OTCs constructed with underlying fibroblasts. The expression of activated, i.e. phosphorylated HGF receptor, Met p(Y1349)Met was increased in K_{TAK1-B} epidermis. No difference was detected for total Met, total ERK1, and phosphorylated p(T202)ERK1. F, K_{TAK1-B} OTCs with underlying fibroblasts were cultured in medium supplemented with anti-PDGF (200 nM) or vehicle (PBS). Mean numbers of proliferating and apoptotic cells were enumerated after detection by anti-Ki67 antibody or TUNEL, respectively. Values were mean from five standardized microscopic fields per section, performed on seven sections from three independent OTCs. K_{CTRL} OTCs with underlying fibroblasts served as control. Immunoblot analysis of epidermis from K_{CTRL} and K_{TAK1-B} OTCs treated with either PBS (-) or anti-PDGF antibody (+) (G) or vector- and TAK1-transfected K_{CTRL} and K_{TAK1-B} OTCs (H). A cell proliferation marker proliferating cell nuclear antigen (PCNA) was used. β -Tubulin served as a loading control. Values below each band represent the mean fold differences in expression level with K_{CTRL} , which was assigned the value of one.

expression of PDGF-B mRNA in K_{TAK1-B} when compared with K_{CTRL} (Fig. 3D). PDGF mRNA was not detected in the fibroblasts. An elevated HGF mRNA was detected in the fibroblasts extracted from K_{TAK1-B} OTCs and was undetectable in the keratinocytes (Fig. 3D). Notably, K_{TAK1-B} OTCs treated with anti-PDGF-BB-neutralizing antibody showed reduced HGF production in the culture medium (Fig. 3C) and in the fibroblasts

from K_{TAK1-B} OTCs (Fig. 3D), indicating a PDGF/HGF epithelial-mesenchymal communication. To further verify the above observation, we examined the activation status of HGF receptor, Met, in the epidermal keratinocytes. Immunoblot showed an increase in phosphorylation of Met in K_{TAK1-B} as compared with K_{CTRL} (Fig. 3E). The increased proliferation seen in K_{TAK1-B} OTCs was not an effect of enhanced ERK activity,

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which remained unchanged in comparison to K_{CTRL} OTCs (Fig. 3E).

To underscore the importance of PDGF/HGF signaling in the increased epidermal proliferation observed in TAK1-deficient epidermis. We cultured K_{CTRL} and K_{TAK1-B} OTCs in medium supplemented with anti-PDGF antibody. We hypothesized that, if PDGF signaling played a major role in manifesting the phenotype of K_{TAK1-B} OTCs, the effect can be neutralized with anti-PDGF antibody. Indeed, the addition of neutralizing anti-PDGF lowered the number of Ki67-positive proliferating cells in K_{CTRL} and K_{TAK1-B} OTCs when compared with vehicle-treated K_{CTRL} OTC (Figs. 3F and S5C). This was again confirmed by immunoblot analysis using anti-PCNA antibody (Fig. 3G). Similarly, epidermis of TAK1-transfected K_{TAK1-B} OTCs showed a reduced expression of PCNA when compared with vector-transfected K_{TAK1-B} OTCs but was similar to K_{CTRL} OTCs (Fig. 3H). Altogether, our results indicate that the double paracrine PDGF/HGF signaling plays a pivotal role in the action of TAK1 in cell proliferation.

TAK1 Suppresses Sp1-mediated Signaling via Increased Expression of von Hippel-Lindau Tumor Suppressor—Our results showed that the absence of TAK1 enhances cell migration and proliferation associated with concomitant increased expressions of integrins $\beta 1$ and $\beta 5$ and PDGF-B/HGF, respectively. Next, we sought to investigate the mechanism by which TAK1 negatively regulate their expressions.

Earlier studies showed that von Hippel-Lindau tumor suppressor protein (pVHL)-Sp1 interaction suppresses PDGF-B expression (21). We hypothesize that TAK1 directly increases the expression of pVHL, which sequesters Sp1 and consequently repressed PDGF-B expression. We first examined the expression of pVHL mRNA and protein levels in K_{CTRL} treated with either IL-1 β or TNF- α in the presence of various kinase inhibitors. With the exception of two different NF- κ B inhibitors, BAY 11-7082 and SN50, all other inhibitors did not attenuate IL-1 β - or TNF- α -induced increase in pVHL mRNA and protein expression when compared with vehicle-treated K_{CTRL} (Fig. 4A). Next, we studied the role of different kinases in regulating the promoter of the human VHL gene. The human VHL promoter was isolated by PCR, and a proximal 732-bp fragment containing the transcription initiation site was subcloned into a luciferase reporter gene (12). This VHL 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 (22, 23). Transient transfection studies showed that IKK α and TAK1 stimulated the VHL promoter activity (Fig. 4B). Additional experiments ruled out the possible involvement of MKK7, JNK, protein kinase A, MEK1, MEKK1, and SEK1 signals in regulating human VHL expression. The human VHL gene promoter contains a putative NF- κ B binding site (12). Site-directed mutagenesis of the putative NF- κ B binding site in the VHL promoter abolished the stimulating effect of IKK and TAK1 (Fig. 4B).

Next, we examined the protein expression level of pVHL, Sp1, and NF- κ B/p65 in K_{TAK1-B} and K_{CTRL} by immunoblot analysis. In concordance with the role of TAK1 in NF- κ B activation, the phosphorylation NF- κ B(p65) was reduced in TAK1-

deficient keratinocytes. Interestingly, pVHL was barely detectable in the K_{TAK1-B} keratinocytes when compared with K_{CTRL} (Fig. 4C). No difference in total NF- κ B(p65) and Sp1 expression was observed (Fig. 4C). As expected, TAK1-transfected K_{TAK1-B} showed increased expression of pVHL and phosphorylated NF- κ B(p65) when compared with vector-transfected K_{TAK1-B} (Fig. 4C).

To determine if human pVHL is a target gene of TAK1/NF- κ B, we performed electrophoretic mobility shift assay and ChIP. Specific protein-DNA complexes were detected in electrophoretic mobility shift assay for VHL NF- κ B binding site, which was effectively competed by unlabeled consensus NF- κ B but not by a nonspecific competitor oligonucleotide (Fig. 4D). Mutation to the VHL NF- κ B binding sequence (VHL mNF- κ B) eliminated its interaction with nuclear extract from K_{CTRL} . As positive control, the labeled consensus NF- κ B (conNF- κ B) oligonucleotide was used as a probe, which was specifically competed by the consensus NF- κ B (Fig. 4D). Similarly, ChIP done on K_{CTRL} and K_{TAK1-B} using anti-NF- κ B(p65) antibody showed that NF- κ B(p65) specifically bound to this site in K_{CTRL} but not in K_{TAK1-B} (Fig. 4E). No immunoprecipitation and amplification were seen with pre-immune IgG and with a control sequence upstream of the NF- κ B site on the promoter gene of VHL (Fig. 4E). Taken together, we concluded that TAK1/IKK/NF- κ B signaling is necessary for the induction of pVHL expression.

In silico analysis of the promoters of human PDGF-B and integrins $\beta 1$, $\beta 3$, and $\beta 5$ genes revealed putative Sp1 binding elements (21, 24–26). In the first instance, we verified the interaction between pVHL and Sp1 in K_{TAK1-B} and K_{CTRL} by *in vivo* protein-protein cross-link as previously described (18). Our result showed that interaction between pVHL and Sp1 was only observed in K_{CTRL} (Fig. 5A). To further strengthen this observation, *in situ* proximity ligation assay (PLA) was performed using anti-pVHL and anti-Sp1 antibodies. PLA allows for the detection and quantification of interacting proteins. Our results showed a significant number of interacting pVHL-Sp1 pairs in the cytoplasm of the K_{CTRL} when compared with K_{TAK1-B} (Fig. 5B).

Next, we performed ChIPs using anti-Sp1 antibody. Our results showed that Sp1 was bound to the Sp1 binding sites in integrins $\beta 1$ and $\beta 5$ and PDGF-B, but not integrin $\beta 3$ (Fig. 5C). Conceivably, the reduced pVHL expression in K_{TAK1-B} allowed free Sp1 to bind and transactivate these genes. To strengthen these results, we performed ChIPs using anti-Sp1 antibody of these promoters in K_{TAK1-B} stably expressing pVHL (pVHL-transfected K_{TAK1-B}). The expression of pVHL in transfected K_{TAK1-B} was comparable to K_{CTRL} as determined by immunoblot analysis (Fig. 5D). Consistent with the aforementioned results, these pVHL-transfected K_{TAK1-B} cells expressed a reduced expression of integrins $\beta 1$ and $\beta 5$ and PDGF-BB (Fig. 5D). To further verify the results, we performed ChIP using anti-Sp1 antibody with these cells. The sequences spanning the Sp1 binding site were significantly reduced in the immunoprecipitates obtained from pVHL-transfected K_{TAK1-B} when compared with vector-transfected K_{TAK1-B} (supplemental Fig. S6). In addition, no signal was seen with pre-immune serum, and no binding was detected in the control sequence.

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FIGURE 4. TAK1 regulates pVHL gene expression via NF-κB. *A*, pVHL mRNA (left panel) and protein (right panel) expression in K_{CTRL} treated for 24 h with either DMSO (vehicle) or specific inhibitors of the indicated kinases in the presence of with IL-1 β or TNF- α . The various kinase inhibitors were NF-κB(I), BAY 11-7082; NF-κB(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 protein L27 was used as a normalizing housekeeping gene. *B*, transactivation assay in keratinocytes co-transfected with a luciferase reporter gene driven by the human VHL promoter (pGL-VHLpro-luciferase), cDNA encoding for indicated constitutively active (*ca*) kinases and pEF1-β-galactosidase as control of transfection efficiency. Luciferase activity was measured, and normalized reporter activity is shown as -fold induction as compared with reporter construct transfected K_{CTRL} (control). VHL promoter reporter construct, whose NF-κB site was mutated, is denoted as pGL-mVHLpro-luciferase. Data are mean $\pm$ S.E., $n = 3$. *C*, immunoblot analysis of K_{CTRL} and K_{TAK1-B} epidermis (left panel) or vector- and TAK1-transfected K_{CTRL} and K_{TAK1-B} (right panel) using antibodies against pVHL, Sp1, total, and phosphorylated NF-κB(p65). β-Tubulin served as a loading control. Values below each band represent the mean fold differences in expression level with respect to K_{CTRL} from five independent experiments ($n = 5$). *D*, electrophoretic mobility shift assay of human VHL NF-κB binding site. Radiolabeled VHL NF-κB binding sequence was incubated with nuclear extract isolated from K_{CTRL} . NSC denotes nonspecific competitor, a scrambled NF-κB binding sequence. SC denotes non-radiolabeled consensus NF-κB sequence (conNF-κB). As positive control, conNF-κB was used. Mutated VHL NF-κB site is denoted by VHL mNF-κB. *E*, pVHL is a direct NF-κB target gene. Chromatin immunoprecipitation were done in K_{CTRL} and K_{TAK1-B} using pre-immune IgG (*pre*) or antibody against p65 subunit of NF-κB (*Ab*). Promoter region with the NF-κB binding site was immunoprecipitated and specifically amplified in K_{CTRL} using *Ab*. No amplified signal was obtained in K_{TAK1-B} or using pre-immune IgG. A control region upstream of NF-κB binding site served as negative control. *M*, 100-bp DNA marker.

The cellular role of pVHL can be hypoxia-inducible factor (HIF)-dependent or -independent manner. *In silico* analysis of the promoters of human PDGF-B and integrin β 1 and β 5 genes

failed to identify the putative HIF α binding site. Nonetheless, to eliminate this possibility, we first performed immunoblot analysis of HIF α using K_{CTRL} and K_{TAK1-B} epidermis. Our results

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FIGURE 6. TAK1 plays a homeostatic role in regulating cell migration and proliferation. Epidermal TAK1 regulates keratinocyte proliferation by a double paracrine mechanism through release of PDGF-B, which induces HGF in the fibroblasts. TAK1 activates NF- κ B to stimulate the expression of pVHL. This facilitates pVHL-Sp1 interaction and sequesters Sp1 from promoting PDGF-B expression. In response to PDGF, the fibroblasts increase the release of HGF, which acts via its receptor, Met, to stimulate keratinocytes proliferation. Via a similar mechanism, TAK1 negatively regulates the expression of integrins β 1 and β 5 in a cell-autonomous manner.

played enhanced epidermal proliferation, which was not observed in monolayer culture, suggesting that dermal fibroblasts have pivotal contribution. We reveal a double paracrine PDGF/HGF signaling that plays an important role in TAK1-mediated epidermal proliferation. For any cells to respond productively to PDGF, they must possess corresponding cell surface receptors, either the α -PDGF or β -PDGF receptors. Although keratinocytes contribute to PDGF activity by their ample capacity to secrete PDGF, they do not express any transmembrane receptor recognizing any classic PDGF isoforms; thus target cells for these ligands in skin are restricted to cells of mesenchymal origin, e.g. dermal fibroblasts (29, 30). We further show that NF- κ B, a downstream mediator of TAK1, regulates the expression of pVHL. The pVHL protein interacts and sequesters transcription factor Sp1, which is required for PDGF-B expression (21). A similar mechanism was also exploited by pVHL to repress VEGF gene expression (31). PDGF-B was previously reported to increase HGF production in the fibroblasts to stimulate keratinocyte proliferation (32). Indeed, PDGF-BB secreted by keratinocytes acts as a paracrine factor to induce HGF production by fibroblasts, which in return, enhance epidermal proliferation (Fig. 6). This study underscores the fact that TAK1 in the epidermal keratinocytes fulfills a homeostatic role during epidermal formation, modulating cell proliferation via a double paracrine fashion, involving the underlying dermal fibroblasts. The paracrine mechanism described herein emphasizes the importance of epithelial-mesenchymal communication in the regulation of epidermal proliferation, which was not observed in TAK1-knock-out monolayer culture.

Cell migration during re-epithelialization is equally crucial for efficient wound closure. In this study, we reveal a novel homeostatic role of TAK1 in the control of keratinocyte migration. TAK1-deficient keratinocytes migrate faster, associated with enhanced integrin β 1 and β 5 expression, activation of FAK, and small Rho GTPases when compared with its wild-type counterpart. The elevated active Rho GTPases expression profile supports a faster migration rate and persistent lamellipodia formation. The functions of pVHL can be mediated via HIF α -dependent or -independent mechanisms. Our findings suggested that hypoxia retarded keratinocyte migration. However, hypoxia-mediated migration is complex and context-dependent. It can be modulated by extracellular matrix, matricellular proteins, matrix metalloproteinases, and other signaling pathways (33). It can also differ among keratinocytes isolated from donors of different age (34). However, our findings suggest that the mechanism underlying the phenotype of TAK1-deficient keratinocytes is HIF α -independent. Interestingly, TAK1 employs similar regulatory mechanism, i.e. via pVHL to modulate integrin β 1 and β 5 expression. In support of a role in cell migration for pVHL, it was reported that highly aggressive breast cancer expressed either no pVHL or low pVHL level (35). High pVHL expression also resulted in a decrease of tubulin turnover indicating a role for pVHL in cellular processes such as migration, polarization, and cell-cell interactions (36).

The development, maintenance, and regeneration of the epidermal integrity are likely to involve the concerted effort of numerous signaling pathways, including epithelial-mesenchymal communications. One of such communications is the IL-1/keratinocyte growth factor/granulocyte macrophage-colony stimulating factor double paracrine mechanism (37). Given the pivotal role of TAK1 in propagating the effect of numerous inflammatory cytokines, such as TNF- α , the PDGF/HGF mechanism described herein complements the IL-1/keratinocyte growth factor/granulocyte macrophage-colony stimulating factor mechanism, particularly during wound repair. TAK1 may also modulate cell migration indirectly via HGF *in vivo*, when epithelial-mesenchymal interaction is evident. It was recently shown that HGF-mediated cell migration involves a PAK-LIMK pathway (38). It is conceivable that both mechanisms are utilized for epidermal regeneration during wound healing.

Psoriasis is a non-contagious chronic inflammatory skin disease characterized by hyperproliferative epidermal growth, a phenotype that was similarly observed in mice with a keratinocyte-specific deletion of TAK1 (10, 11). A recent report suggests that TAK1 deletion causes dysregulation of reactive oxygen species in keratinocytes, which is causally associated with skin inflammation (39). Reactive oxygen species has been associated with psoriasis (40, 41). Our study reveals the underlying mechanism by which TAK1 regulates epidermal proliferation via a double paracrine mechanism involving the underlying dermal fibroblasts. We showed that TAK1 in the keratinocytes directly up-regulated the expression of pVHL, reduced PDGF-B expression, and consequently diminished HGF/c-Met signaling. A deficiency in TAK1 resulted in reduced NF- κ B activation and pVHL expression. Lending support, analysis of skin biopsies of patients with psoriasis showed that pVHL, a

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direct target of TAK1, is underexpressed in psoriatic skin and highly expressed in healthy skin (42). Furthermore, PDGF receptor expression was greatly elevated in psoriatic fibroblasts, rendering them highly responsive to PDGF and PDGF-induced HGF production (27). Altogether, our findings herein and those of others suggest that TAK1 deficiency may contribute to the etiology of psoriasis.

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SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Immunohistochemical analysis of TAK1 skin wound biopsy. **(A)** Negative control. Immunohistochemical staining of Day 5 mouse wound biopsies with pre-immune IgG as negative control. The section was processed as described in Figure 1B. Representative picture from wound epithelium and the adjacent wound bed was shown. E: epidermis; WB: wound bed; Scale bar 20 μ m. **(B)** Relative expression level of TAK1 mRNA in normal human skin and biopsies from chronic non healing ulcers as determined by qPCR. Cyclophilin was used as a normalizing housekeeping gene. Statistical analysis was determined using two-tailed Mann-Whitney test, *** denotes $p < 0.001$.

Figure S2. TAK1-deficient keratinocytes migrate faster, independent of cell proliferation. **(A)** Time-lapsed images of wounded cultures of control (K_{CTRL}) and TAK1-knockdown (K_{TAK1-B}) treated with mitomycin C (2 μ g/ml). Scale bar 100 μ m. See videos S3-4. Higher magnification images from video microscopy showing migratory front of K_{CTRL} and K_{TAK1-B} (bottom two panels). Arrows indicate the focal adhesion points and microspike-like extensions in lamellipodia during migration. Scale bar 20 μ m.

Figure S3. TAK1 deficiency enhances cell migration. Immunoblot analysis of active cdc42, Rac1 and RhoA in K_{CTRL} and K_{TAK1-B} exposed to serum for the indicated time periods (min). Values below the blots represent the mean fold change in active cdc42, Rac1 and RhoA relative to level at zero min of K_{CTRL} (n=5). Representative pictures of immunoblot were shown.

Figure S4. TAK1 plays a homeostatic role in epidermal proliferation. Immunofluorescence staining of K_{CTRL} and K_{TAK1-B} -derived organotypic skin cultures (OTCs). Keratin 10 (CK 10) and involucrin (INV) were late and terminal epidermal differentiation, respectively. Proliferating and apoptotic cells were identified using Ki67 antibody and TUNEL assay, respectively (white arrows). OTC sections were counterstained with DAPI (blue). Dotted line represents epidermal-dermal junction. F denotes the underlying fibroblasts-embedded collagen. White dotted lines indicate epidermal-dermal junction. H&E: Haematoxylin and eosin staining. Scale bar 40 μ m. Mean proliferating and apoptotic cells were numerated from 3 standardized microscopic fields per section, performed on 3 sections from 4 independent OTC constructions (n=36).

Figure S5. TAK1 regulates epidermal proliferation via a double paracrine signaling involving PDGF-B. **(A)** Immunofluorescence staining of K_{CTRL} and K_{TAK1-B} -derived OTCs cultured with only collagen (COL). In the absence of underlying fibroblasts, no difference in cell proliferation index was observed between K_{CTRL} and K_{TAK1-B} epidermis. Proliferating cells were identified using Ki67 antibody (white arrows). OTC sections were counterstained with DAPI (blue). White dotted lines indicate epidermal-dermal junction. **(B)** Human Growth Factor Antibody Array I (RayBio®): Protein analysis of conditioned medium from two-weeks old K_{CTRL} and K_{TAK1-B} -derived OTCs. Spots corresponding to HGF, PDGF-BB and TGF- β were boxed. Representative pictures of array were shown. **(C)** Immunofluorescence staining of K_{CTRL} - and K_{TAK1-B} -derived OTCs cultured with underlying fibroblasts (F) in the presence either vehicle (PBS) or neutralizing anti-PDGF- antibody. Blocking the activity of PDGF negates the pro-mitogenic effect of TAK1 deficiency in keratinocytes. Dotted white line represents epidermal-dermal junction. H&E: Haematoxylin and eosin staining. Scale bar 40 μ m. Mean proliferating cells was numerated from 3 standardized microscopic fields per section, performed on 3 sections from 3 independent OTC constructions (n=27).

Figure S6. Ectopic expression of pVHL in K_{TAK1-B} inhibits Sp1 binding to the promoters of PDGF-B, integrins $\beta 1$ and $\beta 5$. Chromatin from vector- or pVHL-transfected K_{TAK1-B} was immunoprecipitated with either an anti-Sp1 antibody (Ab) or preimmune IgG (pre). Enrichment of a DNA fragment encompassing the Sp1 binding sites was evaluated by PCR. Aliquots of the extracts were

also used before immunoprecipitation (input). Two stable K_{TAK1-B} clones expressing pVHL were used. No amplified signal was obtained in using pre-immune IgG. A control region upstream of NF- κ B binding site served as negative control.

Figure S7. pVHL suppresses migration and proliferation via a HIF α -independent fashion. **(A)** Immunoblot analysis of HIF α on K_{CTRL} , K_{TAK1-B} and TAK1-transfected K_{TAK1-B} epidermis (left panel). β -tubulin showed equal loading and transfer. Time-lapsed images of wounded cultures in cobalt chloride-simulated (50 mM) hypoxia condition. See videos S8. Scale bar 100 μ m. **(B)** Immunohistochemical analysis of pVHL skin wound biopsy. Immunohistochemical staining of Day 5 mouse wound biopsies with either pre-immune IgG as negative control (left panel) or with anti-pVHL antibody (right panel). The section was processed as described in Figure 1B. DAB with nickel (dark blue) was used as substrate. Representative picture from wound epithelium and the adjacent wound bed was shown. Scale bar 20 μ m.

A

B

A

B

Human Growth Factor Antibody Array I Map

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
2	POS	POS	NEG	NEG	AR	bFGF	b-NGF	EGF	EGF R	FGF-4	FGF-6	FGF-7
3	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
4	GCSF	GDNF	GM-CSF	HB-EGF	HGF	IGFBP-1	IGFBP-2	IGFBP-3	IGFBP-4	IGFBP-6	IGF-I	IGF-I SR
5	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF R α	PDGF R β	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
6	IGF-II	M-CSF	M-CSF R	NT-3	NT-4	PDGF R α	PDGF R β	PDGF-AA	PDGF-AB	PDGF-BB	PIGF	SCF
7	SCF R	TGF- α	TGF- β	TGF- β 2	TGF- β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS
8	SCF R	TGF- α	TGF- β	TGF- β 2	TGF- β 3	VEGF	VEGF R2	VEGF R3	VEGF-D	BLANK	BLANK	POS

* For use with serum, plasma, condition medium, urine and all other body fluids, cell lysates and certain tissue lysates samples

C

SUPPLEMENTAL TABLE

Table S1. Oligonucleotide sequences

Primer pairs (S: sense; AS: anti-sense)	Sequence (5'-3')
Actin S	CTTCTCTCTAAGGAGAATGGCCCAG
Actin AS	CAACTGGTCTCAAGTCAGTGACAGG
Actin RT1	ACGTGGACATCCGCAAAGAC
Actin RT2	CGCTCAGGAGGAGCAATGAT
HGF S	TCCCTTCTCGAGACTTGAAAGAT
HGF AS	CTGCATTTCTCATTTCCCATT
PDGFB S	TGAACATGACCCGCTCCCACTC
PDGFB AS	TGCCGTCTTGTCATGCGTGTG
PDGFA S	CGGATACCTCGCCCATGTTCTG
PDGFA AS	TCCGGATTCAGGCTTGTGGTCG
TAK A1	<u>AAAG</u> AAGAGGTTGTTGGAAGAGGAG
TAK A2	<u>AAAA</u> CTCCTCTTCCAACAACCTCTT
TAK B1	<u>AAAG</u> TGGCTTATCTTACACTGGA
TAK B2	<u>AAAT</u> TCCAGTGTAAGATAAGCCA
TAK1 RT1	GTTCCGTGTAAGGGCTTTGA
TAK1 RT2	GAGCAGCTGCCACTTACCTTTACA
U6	GCTTACCGTAACTTGAAAGTATTTCTG
H1	CTGGGAAATCACCATAAACGTGAA
pVHL promoter S	GATCCTCGAGCGTGATGATTGGGTGTTCCC
pVHL promoter AS	GATCAAGCTTCGACGCCTGCCTCCTCCGCG
chIP primers (S, AS)	
ITGB1SP1_S	TCAGTGGACAAACGGGAGCGAG
ITGB1SP1_AS	CTCTCCCTCCGGAAACGCATTC
ITGB1control_S	TCGACATGCGTTCAGCCATTC

ITGB1control_AS	GGAGTGATAATGTGCTTGCCTG
ITGB3SP1_S	GGCGAGAGAGGAGCAATAG
ITGB3SP1_AS	GAGCCTCACTCACCTCCTAC
ITGB3control_S	TTGGAGCAGGATGAGGCTTTGC
ITGB3control_AS	CCCAGTACCAAAGAGGCCACTC
ITGB5SP1_S	CATTTCTTCCCTCCTTC
ITGB5SP1_AS	TGTGAATTGAGTTTGTTGC
ITGB5control_S	GAGAGACAGCCCAGCATCTCAG
ITGB5control_AS	CACGCACACTGTGGACTTCACC
PDGFBSP1_S	TCCGGGCCAGAAGAGGAAAG
PDGFBSP1_AS	CCCATCTTTGATCCTTCTG
PDGFBcontrol_S	GGTGAATCAGAGTGGAGTATG
PDGFBcontrol_AS	CTGTGGACTGTCCTGAGGAG
Mutagenesis primers	
VHL mutant S	GTGGATCGCGGAGGGAgatCtCCGGAGGGCGGAGAAC
VHL mutant AS	GTTCTCCGCCCTCCGGAGATCTCCCTCCGCGATCCAC

Underlined nucleotides indicate ‘sticky-ends’ that facilitate ligation with the *Bbs*I-digested siRNA expression vector.

SUPPLEMENTAL VIDEO LEGENDS

Videos. *In vitro* scratch wound assay performed using (S1) control keratinocytes (K_{CTRL}) and (S2) TAK1-deficient keratinocytes (K_{TAK1-B}). Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

Videos. *In vitro* scratch wound assay performed using (S3) control keratinocytes (K_{CTRL}) and (S4) TAK1-deficient keratinocytes (K_{TAK1-B}) in the presence of 2 μ g/ml of mitomycin C. Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

Videos. Migratory front cell phenotype of (S5) K_{CTRL} and (S6) K_{TAK1-B} . Cell migration was recorded at 1 min interval for 2 h. Scale bar 20 μ m.

Video S7. *In vitro* scratch wound assay performed using TAK1-transfected K_{TAK1-B} . Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

Video S8. *In vitro* scratch wound assay performed using cobalt chloride-simulated hypoxia condition on control keratinocytes (K_{CTRL}). Cell migration was recorded at 2 min interval for 9 h. Scale bar 100 μ m.

TAK1 regulates SCF expression to modulate PKB α activity that protects keratinocytes from ROS-induced apoptosis

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Dysregulated reactive oxygen species (ROS) generation contributes to many human pathologies, including cancer and diabetes. During normal wound repair, inflammation-induced ROS production must be tightly controlled, but the mechanisms reigning their generation remain unclear. Herein, we show that transforming growth factor β -activated kinase 1 (TAK1) directly regulates stem cell factor (SCF) expression, 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 is a pivotal inflammatory mediator whose expression was transiently elevated during wound healing, paralleling the ROS production profile. TAK1 deficiency in keratinocytes led to increased apoptosis in response to anoikis and TNF- α treatment and was associated with elevated ROS level as analyzed by 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. Ectopic expression of TAK1 or treatment with exogenous recombinant SCF restored the increased ROS production and apoptotic cell death in TAK1-deficient keratinocytes. Conversely, normal keratinocytes treated with various inhibitors targeting the SCF/c-Kit/PKB α pathway exhibited increased ROS production and TNF- α - or anoikis-induced apoptosis. Our study reveals a novel anti-apoptotic role for SCF in keratinocytes and identifies TAK1 as a novel player uniting inflammation and ROS regulation in skin redox biology.

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Wound healing comprises three progressive overlapping events: inflammation, re-epithelialization and tissue remodeling. Although wound healing is accompanied by inflammatory reactions, chronic inflammation impairs acute wound healing. Furthermore, prolonged inflammation has been the bane of chronic dermatological conditions like psoriasis¹ and wound healing complications like non-healing diabetic wounds.² It is thus of great importance to understand the underlying regulation of skin inflammation.

Reactive oxygen species (ROS) have profound effects on many biological processes, and thus it is crucial that ROS production be tightly controlled through a network of regulatory pathways.³ During normal wound healing, levels of the free radical superoxide peak at day 2 after injury in full-thickness excisional mouse wounds, as determined by redox-sensitive dye dihydroethidium and electron paramagnetic resonance.⁴ Low levels of and transient increases in ROS have antimicrobial action and operate through redox-sensitive signaling pathways and transcription factors to aid wound

repair.⁵ However, excessive or persistently elevated ROS within the cells can significantly damage macromolecules or alter cellular functions, eventually leading to irreversible injury and cell death. Furthermore, a chronically deregulated state in which ROS signaling can up-regulate patterns of gene expression and cell responses are believed to significantly contribute to and/or sustain the pathogenesis of major human diseases characterized by chronic inflammation, chronic wound healing and tissue fibrogenesis.⁶ Despite the importance of balanced ROS production in wound healing, the mechanisms underlying this regulation need further study.

Transforming growth factor (TGF)- β -activated kinase 1 (TAK1) has been strongly implicated in skin inflammation.⁷ As a principle mediator of innate immunity, TAK1 serves as a key intermediate in proinflammatory pathways initiated by tumor necrosis factor (TNF)- α and interleukin (IL)-1.⁸ As a member of the MAPK kinase family, TAK1 can activate c-Jun N-terminal kinase (JNK) and p38 MAPK through the stimulation of its downstream MAPK cascade.⁹ TAK1 also stimulates

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Abbreviations: AP-1, activating protein-1; Col, collagen; ChIP, chromatin immunoprecipitation; DAPI, 4',6-diamidino-2-phenylindole; DCF, dichlorodihydrofluorescein (CM-H2DCFDA); EMSA, electrophoretic mobility shift assay; FADD, Fas associated death domain; FLIP, FADD-like IL-1 β -converting enzyme-inhibitory protein; GSK, glycogen synthase kinase; H&E, hematoxylin and eosin staining; I κ B α , nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; IKK β , inhibitor of nuclear factor kappa B kinase beta; JNK, c-Jun N-terminal kinase; K_{CTRL}, keratinocyte control; K_{TAK1}, keratinocyte with TAK1 knockdown; NAC, N-acetylcysteine; OTC, organotypic skin co-culture; PCNA, proliferating cell nuclear antigen; PDGF, platelet derived growth factor; PI3K, phosphoinositide-3 kinase; PKB α , protein kinase B α ; ROS, reactive oxygen species; SCF, stem cell factor; siRNA, small interfering ribonucleic acid; TAK1, transforming growth factor β -activated kinase 1; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; TRAIL, TNF-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling

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the I κ B α kinase cascade, leading to the activation of NF- κ B.¹⁰ In the skin, these downstream mediators of TAK1 are important for the maintenance of epidermal homeostasis.^{10,11} Perturbations to the JNK pathway have been implicated in epidermal hyperplasia leading to cancer development, while reduced NF- κ B function is linked to epidermal squamous cell carcinoma. Blocking the NF- κ B pathway through IKK β or IKK γ inactivation also leads to severe skin inflammation.^{12,13} Mice with keratinocyte-specific deletion of TAK1 develop severe skin inflammation, characterized by massive cellular apoptosis, and die at postnatal day 7.^{7,14} Histological examination of TAK1-null mouse skin has revealed a dysregulated differentiation and increased keratinocyte proliferation, similarly observed in TAK1-deficient human keratinocytes. Given the central role that TAK1 has in inflammation, it has recently been implicated in ROS production. Indeed, TAK1-deficient mouse keratinocytes are more susceptible to TNF- α -mediated apoptosis associated with increased ROS production, which can be abrogated via c-Jun-dependent mechanism.¹⁵ Taken together, these observations suggest that TAK1 has a homeostatic role in modulating ROS production in wound healing, but the precise underlying mechanism remains unknown.

Herein, we provide mechanistic insight into the TAK1-regulated inhibition of TNF- α -mediated apoptosis in human keratinocytes. More importantly, we reveal that this protective role of TAK1 occurs in a cell-autonomous manner via the autocrine action of stem cell factor (SCF). The expression of SCF in keratinocytes is transcriptionally regulated via the TAK1/JNK/c-Jun signaling pathway. These findings identify a novel role for SCF in modulating the ROS level of keratinocytes.

Results

Elevated ROS production in wound epithelia. 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.¹⁰ To establish a causal relationship between TAK1 and ROS production profile in wound healing, we stained wound biopsies with the fluorescent dye CM-H2DCFDA (DCF). The staining revealed that the wound epithelia were a major site of ROS production that peaked at 3–7 days post-injury, coincident with elevated expression of TAK1 (Figures 1a and b). DCF staining in the presence of an antioxidant *N*-acetyl-cysteine (NAC) was

completely abolished, indicating that the fluorescence signal was specific (Supplementary Figure 1).

To further examine the role of TAK1 in ROS production during wound healing, we constructed and examined organotypic skin co-cultures (OTCs) using either control (K_{CTRL}) or TAK1-knockdown human keratinocytes (K_{TAK1}) with underlying human primary dermal fibroblasts. Our previous work has shown that this knockdown is specific and can be rescued by transfection with TAK1 cDNA.¹⁰ The overall epidermal thickness between K_{CTRL} and K_{TAK1} OTCs was similar, but the suprabasal layer of the K_{TAK1} epidermis was significantly thinner when compared with K_{CTRL} OTC, as evidenced by immunofluorescence staining for involucrin, a marker of keratinocyte terminal differentiation expressed only in the suprabasal layers of epidermis (Figure 1c).¹⁶ Notably, ROS production was elevated in the K_{TAK1}-derived epithelia, as revealed by DCF staining (Figure 1c). Persistently elevated ROS within the normal cells leads to cellular apoptosis. Indeed, K_{TAK1} OTCs showed more TUNEL-positive apoptotic cells rather than K_{CTRL} OTCs (32.6 $\pm$ 3.5 *versus* 12.7 $\pm$ 4.1 TUNEL-positive cells per microscopic field, respectively; Figure 1c). This was further confirmed by immunodetection of cleaved caspase-3, an apoptotic marker (Figure 1d). As expected, the phosphorylation of JNK, a downstream target of TAK1, was reduced in K_{TAK1} OTCs compared with K_{CTRL} OTCs (Figure 1d). Taken together, these observations suggest that TAK1 has a homeostatic role in modulating ROS level in keratinocytes during wound healing.

TAK1 confers anti-apoptotic properties in a cell-autonomous manner. 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 collagen, that is, 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 (Figure 1e). Similar to K_{TAK1} OTCs constructed with fibroblasts (Figure 1c), higher number of apoptotic cells were observed in K_{TAK1}/Col when compared with K_{CTRL}/Col (45.3 $\pm$ 5.2 *versus* 17.9 $\pm$ 2.3 *versus* TUNEL-positive cells per microscopic field, respectively; Figure 1e). This was corroborated by immunoblot analysis using cleaved caspase-3 (Figure 1f). Resistance to anoikis and to TNF- α -induced

Figure 1 ROS levels and cellular apoptosis are elevated in TAK1-deficient keratinocytes (K_{TAK1}). (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 with the signal from Day 1 wound biopsies, as determined using ZEN 2009 (Carl Zeiss) software. (c) H&E staining (first top panel), anti-involucrin immunofluorescence (second panel), DCF staining (third panel) and TUNEL analysis (bottom panel) of control (K_{CTRL})- and K_{TAK1}-derived 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 μ m. Dotted white line represents epidermis–dermal junction. (d) 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. (e) TAK1 confers anti-apoptotic properties in a cell-autonomous manner. H&E staining (first panel), DCF staining (second 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. (f) 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

apoptosis are needed during epidermal stratification and in response to inflammation during wound healing, respectively.¹⁷ Thus, we subjected K_{CTRL} and K_{TAK1} to either anoikis or TNF- α treatment and examined ROS levels and apoptosis by FACS. DCF labeling revealed increases in ROS production of more than 35 and 55% upon anoikis and

TNF- α treatment, respectively (Figure 2a). 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 (Figure 2b). Importantly, the co-treatment with NAC significantly reduced the apoptotic index in K_{TAK1}

Figure 2 TAK1 deficiency increased ROS accumulation. **(a)** Representative FACS-derived histograms showing increased ROS in TAK1-knockdown (K_{TAK1}) compared with control human keratinocytes (K_{CTRL}) during anoikis or 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. **(b)** Percentage of apoptotic K_{CTRL} and K_{TAK1} after anoikis or 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. **(c)** TUNEL analysis of K_{CTRL} - and K_{TAK1} -derived 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

(Figure 2b). 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 (Figure 2c). No significant difference was observed in mean DCF signals and apoptotic index between untreated K_{CTRL} and K_{TAK1} (Figures 2a and b). Altogether, these results point towards a role for TAK1 in modulating ROS production and protecting keratinocytes against apoptosis via an autocrine, cell-autonomous manner.

TAK1 modulates the SCF/c-Kit/PKB α cascade. Our above findings showed that TAK1-deficient keratinocytes were hypersensitive to TNF- α -induced apoptosis associated with elevated ROS production and that the anti-apoptotic effect of TAK1 was cell-autonomous. To understand the underlying mechanism for this TAK1 action, an unbiased proteomic analysis was done 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, that is, 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 enzyme-linked immunosorbent assay (ELISA) on the OTC culture medium (Figure 3a), 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 (Figure 3b). 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 (Figure 3c). SCF binds and phosphorylates, that is, activates, its cognate receptor c-Kit.¹⁸ 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 (Figure 3d). The activation of c-Kit has been shown to trigger the phosphoinositide-3 kinase (PI3K)/protein kinase B (PKB) α pro-survival pathway.¹⁹ 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} (Figure 3d). The expression level of total PKB α remained unchanged (Figure 3d). Increased activity of PKB α , that is, higher phosphorylation, protects keratinocytes from apoptosis.^{17,20}

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 with vehicle-treated OTCs (Figure 3e). Although SCF treatment appeared to induce a trend towards more Ki67-positive proliferating cells, this effect on proliferation was not significant (Figure 3e). This was also confirmed by immunodetection using anti-caspase-3 and anti-PCNA antibodies (Figure 3f). 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.¹⁰ 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) (Figure 3f). In contrast, the treatment with recombinant SCF reduced the number of apoptotic keratinocytes (Figures 3e and f). 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 has a pivotal role in the action of TAK1 in cell survival.

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

Our above results showed that the absence of TAK1 increased apoptosis due to diminished SCF expression and thus PKB α activation. 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 with vehicle-treated K_{CTRL} , as determined by quantitative PCR (qPCR) and ELISA, respectively (Figures 3g and h). 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.²¹ 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.⁹ Transient transfection studies showed that TAK1, MKK7 and JNK stimulated SCF promoter activity (Figure 3i), 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.²¹ Site-directed mutagenesis of the putative AP-1 binding site in the SCF promoter abolished the stimulating effect of TAK1, MKK7 and JNK (Figure 3i). 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 manner, as determined by qPCR (Figure 3j), suggesting that human SCF is a direct target of c-Jun. 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 (Figure 3k).

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 (Figure 3k). 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 bind in K_{TAK1} cells (Figure 3l). 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 (Figure 3l). Taken together, these results suggest that TAK1/MKK7/JNK/c-Jun signaling is necessary for the induction of SCF expression in keratinocytes.

SCF stimulates PKB α 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 has 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 (Figures 4a and b). More importantly, this elevated level of ROS led to a higher percentage of apoptotic cells (Figure 4c). K_{TAK1} treated with exogenous recombinant SCF showed diminished ROS level and reduced percentage of apoptotic cells compared with vehicle-treated K_{TAK1} (Figure 4c). Next, we cultured K_{TAK1} and K_{CTRL} OTCs in a serum-free medium supplemented with the above inhibitors. The inhibition of the SCF/c-Kit/PKB α 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 (Figure 4e).

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.^{22,23} 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 (Figure 4f). This was similarly observed for K_{TAK1} cells

subjected to anoikis (Figure 4f). 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 (Figure 4g).

Discussion

ROS are critical intermediates of cellular signaling pathways. Upon injury, inflammatory signals present at a wound site stimulate the transient production of ROS that operate through redox-sensitive signaling pathways and transcription factors to execute specific biological processes, aiding wound repair. However, the dysregulated production of ROS leading to persistently elevated levels is associated with cancer, diabetes, inflammatory diseases, ischemia-related diseases, and neurodegeneration and are commonly thought to contribute to human pathologies and aging.^{24–26} Thus, it is crucial that the production of ROS be tightly controlled. In this study, we show that keratinocyte-specific TAK1 protects keratinocytes from apoptosis induced by anoikis and TNF- α and is associated with the diminished ROS. This involves a novel cell-autonomous mechanism by which TAK1 mediates the expression of SCF to stimulate the activation of the anti-apoptotic PIK3/PKB α pathway. Our study reveals a functional role for SCF in keratinocytes and identifies TAK1 as a novel player linking inflammation and ROS regulation in skin redox biology.

During normal wound healing, we have consistently observed that increased ROS production is closely associated with the expression of TAK1 in mouse skin wound biopsies. Using RNA interference and OTCs, we previously found that the suppression of TAK1 in human keratinocytes leads to higher ROS levels accompanied by increased susceptibility to

Figure 3 TAK1 promotes cell survival through the SCF/c-Kit/PKB α pathway. (a and b) Expression level of 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 $\pm$ 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. (d) 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. (e) 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 enumerated 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. (f) 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 with K_{CTRL}, which was assigned the value of 1. (g and h) TAK1 regulates SCF expression via MKK7/JNK/c-Jun pathway. Expression level of SCF (g) mRNA and (h) 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. (i) 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 with 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. (j) Relative SCF mRNA expression in K_{CTRL} transfected with increasing amount of dominant-negative c-Jun (TAM67). Ribosomal L27 was used for normalization. (k) 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 as SCF mAP-1. (l) 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

Figure 4 SCF stimulates PI3K/PKB α signaling to modulate ROS production and cellular apoptosis. **(a and 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. **(c)** Percentage of apoptotic K_{CTRL} and K_{TAK1} treated as described in **(a and 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. **(d and e)** DCF staining of **(d)** K_{CTRL} and **(e)** 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. **(f)** Immunodetection of FLIP protein from TNF- α and anoikis-treated K_{CTRL} and K_{TAK1} cells in the absence (–) or presence (+) of either 50 ng/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 with vehicle-treated or anoikis-treated K_{CTRL}, which was assigned the value of 1. **(g)** 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 TNF- α , 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. Elevated ROS sensitize keratinocytes to TNF- α -induced cell death and anoikis by increasing FLIP degradation. Our study showed that the simultaneous activation of TAK1 allows for tightly controlled ROS generation via the autocrine action of SCF (bold). 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

anoikis and TNF- α -induced apoptosis, suggesting that TAK1 has a homeostatic role in regulating ROS generation in skin. Lending support to this hypothesis, keratinocyte-specific deletion of TAK1 hypersensitizes mouse keratinocytes to TNF- α -mediated apoptosis via an elevated ROS, leading to massive cell death and chronic skin inflammation. A c-Jun-dependent mechanism was reported to inhibit excessive ROS production and salvage TAK1-deficient keratinocytes from the apoptotic fate, but the precise underlying mechanism still

remains obscure.¹⁵ Recently, TNF-related apoptosis-inducing ligand (TRAIL) was shown to induce ROS accumulation, enhance the activation of caspase-3 and increase apoptosis in TAK1-null mouse keratinocytes.²⁷ Further work showed that TAK1-mediated prevention of TRAIL-induced cell death was mediated by a non-NF- κ B mechanism. The role of SCF/c-Kit has been well documented in promoting survival, proliferation and differentiation in bone marrow-derived hematopoietic cells.²⁸ In the human skin, SCF/c-Kit

signaling has a pivotal role in the process of melanogenesis. Although the expression of SCF and its receptor c-Kit has been reported in keratinocytes,^{29–31} its homotypic role in keratinocyte biology remains elusive. We showed that TAK1/MKK7/JNK/c-Jun signaling 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 inhibiting TNF- α -induced cell death and anoikis. This novel cell-autonomous mechanism clarifies and furthers our previous work, but importantly, it underscores the homeostatic role of TAK1 and SCF/c-Kit signaling in the regulation of ROS production and indicates their novel anti-apoptotic role in keratinocytes.

Prolonged inflammation that induces oxidative stress has been implicated in dermatological conditions like psoriasis.³² Our findings and those of others have shown that a deficiency in TAK1, and hence reduced c-Jun activity, results in a greater oxidative stress in keratinocytes. Our previous findings also show that TAK1-deficient keratinocytes have reduced NF- κ B activation and pVHL expression.¹⁰ Interestingly, analysis of skin biopsies from patients with psoriasis shows that c-Jun and pVHL are downregulated in psoriatic skin.^{33,34} Furthermore, a mouse model of psoriasis is also deficient in epidermal Jun proteins.³⁵ In view of these supporting evidence, it will be worth to examine the expression or activity of TAK1 in human psoriatic skin biopsies.

Wounds share many features with cancerous tumors. Studies have suggested that for reasons yet unknown, the genes active during wound healing are also activated in cancers with the worst outcomes.³⁶ Like wound healing, inflammation is a critical component of tumor progression.³⁷ Many cancers arise from sites of infection, chronic irritation and inflammation. 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.³⁷ Recent studies have shown that tumors utilize a redox-based mechanism to prevent death by anoikis.³⁸ Anoikis resistance is an essential feature of cancer cells, yet its acquisition is a central problem in cancer biology. Although not investigated in this study, our findings suggest, at least in part, a role for TAK1-mediated SCF/c-Kit signaling that confers 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.

Materials and Methods

Reagents. Antibodies were purchased against TAK1 (Upstate Biotechnology, Waltham, MA, USA) for immunofluorescence and against 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, Danvers, MA, USA), involucrin (Novocastra, Newcastle upon Tyne, UK), β -tubulin, PCNA, phospho-c-Jun and secondary HRP-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for immunoblot. Neutralizing anti-SCF antibody and recombinant SCF were from Peprotech (Rocky Hill, NJ, USA). Collagen Type I rat tail was obtained from BD Biosciences (Bedford, MA, USA). Human primary fibroblasts, keratinocytes and corresponding culture medium were purchased from Cascade Biologics (Portland, OR, USA). Transfection reagent ExGen 500 was purchased from Fermentas (Vilnius, Lithuania). Double-promoter lentivirus-based siRNA vector and pFIV packaging kit were acquired from System Biosciences (Mountain View, CA, USA). All restriction enzymes and DNA/RNA-modifying enzymes were obtained from

Fermentas. Kinase inhibitors were acquired from Merck (Darmstadt, Germany). PI3K inhibitor LY294002 was purchased from Cayman Chemical (Ann Arbor, MI, USA). Otherwise, chemicals were bought from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture conditions and organotypic skin co-culture. Human dermal fibroblasts and keratinocytes were routinely cultured in a 37°C, 5% CO₂, humidified incubator. OTCs were performed as previously described.¹¹ Briefly, a fibroblast density of 1×10^5 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 μ M carnitine, 0.4 μ g/ml hydrocortisone, 1 μ M isoproterenol, 1 μ M α -tocopherol, 5 μ M linoleic acid, 7 μ M arachidonic acid, 25 μ M oleic acid, 50 μ g/ml ascorbic acid, 10 μ M serine, 100 nM adenine, 100 nM cholera toxin, 2 ng/ml epidermal growth factor and 2 ng/ml TGF- α . OTCs were kept at 37°C, 5% CO₂ with a relative humidity of 70%.

Lentivirus siRNA constructs and transduction. The sequences of TAK1 and control siRNA are shown in Supplementary Table 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. Production of pseudoviral particles and transduction of cells were done as described by the manufacturer. Following transduction, the cells were enriched with 350 ng/ml puromycin for a week.

Total RNA isolation and real-time quantitative PCR. Total RNA was isolated from cells using a PureLink RNA kit (Invitrogen, Carlsbad, CA, USA) following the supplier's protocol. Total RNA (2.5 μ g) 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, Cape Town, South Africa). 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 Supplementary Table 1.

Transient transfection and transactivation assay. TAK1 cDNA was subcloned into pCMV5 mammalian expression vector (Stratagene, Santa Clara, CA, USA). A 983-bp (–853 to +130) promoter of the human SCF gene was PCR-amplified from human genomic DNA using *Pfu* polymerase.²¹ The resulting fragment was subcloned into pGL-3 basic luciferase reporter vector (Promega, Madison, WI, USA). 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 Supplementary Table 1. Keratinocytes were co-transfected 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 caPKA, caMEK1 and caMEKK1 were obtained from Clontech (Mountain View, CA, USA). The caMKK7 was obtained from Cell BioLabs (San Diego, CA, USA). The various TAK1, caSEK1, caJNK, caSGK and caLKK expression vectors were obtained from E Nishida (Kyoto University, Japan), RJ Davis (University of Massachusetts Medical School, Worcester, MA, USA), DJ Templeton (University of Virginia Medical School, Charlottesville, VA, USA) and DV Goeddel (Tularik, Inc., South San Francisco, CA, USA). After transfection, cells were cultured for 48 h before lysis. Luciferase activity was measured using the Promega luciferase assay on a Microbeta Trilux (Perkin-Elmer, Waltham, MA, USA). 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.

Electrophoretic mobility shift assay. Non-radioactive EMSA was performed using a LightShift Chemiluminescent EMSA kit (Thermo Scientific, Waltham, MA, USA). The AP-1 (–573)-binding site of the human SCF promoter was examined.²¹

Protein array analysis and enzyme-linked immunosorbent assay. A growth factor array membrane was processed according to the manufacturer's protocol (RayBiotech, Norcross, GA, USA). Serum-free conditioned medium (1 ml) or 0.5 mg cell lysate proteins was used. Protein spots were detected

by chemiluminescence. Signal intensities were quantified using ImageJ analysis software (National Institutes of Health, Bethesda, MD, USA), 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, Minnesota, MN, USA).

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, Minneapolis, MN, USA). Eight-micrometer cryosections were processed for immunofluorescence as described previously.¹¹ Apoptotic cells were detected using the TUNEL assay according to the manufacturer's protocol (Roche, Mannheim, Germany). 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 $\times 63/1.40$ oil objective and ZEN 2008 software (Carl Zeiss, Singapore, Singapore).

Chromatin immunoprecipitation. *In vivo* ChIP was carried out as previously described, except anti-phospho-c-Jun antibody was used.³⁹ The putative AP-1-binding site in the human SCF promoter was previously described.²¹ Primers used for ChIP are given in Supplementary Table 1.

Apoptosis treatments, ROS measurement and FACS analysis. Keratinocytes were subjected to anoikis treatment as previously described.²⁰ 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.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)

Lam et al, Supplemental Figure 1

Figure S1: 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.

Supplemental Table 1: Primer sequences used in this study.

TAK knockdown forward primer	<u>AAAGTGGCTTATCTT</u> ACACTGGA
TAK knockdown reverse primer	<u>AAAATCCAGTGT</u> AAGATAAGCCA
TAK1 qPCR primer 1	GTTCCGTGTAAGGGCTTTGA
TAK1 qPCR primer 2	GAGCAGCTGCCACTTACCTTTACA
SCF qPCR primer 1	GGGATCTGCAGGAATCGTGTG
SCF qPCR primer 2	ACAATGCTGGCAATGCCATG
U6	GCTTACCGTAACTTGAAAGTATTTCG
H1	CTGGGAAATCACCATAAACGTGAA
phSCF promoter forward	AGATCTACACTAATAGATTTGATG
phSCF promoter reverse	TTTAGCTGTTCTGGAGCTCCAGCA
hSCF AP-1 chIP forward	AGACACAGTAACAAACCATTCC
hSCF AP-1 chIP reverse	GAAATACCTCGCGCGCCAGA

Underlined nucleotides indicate ‘sticky-ends’ that facilitate ligation with the *Bbs*I-digested siRNA expression vector.

Regulation of epithelial–mesenchymal IL-1 signaling by PPAR β/δ is essential for skin homeostasis and wound healing

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Skin morphogenesis, maintenance, and healing after wounding require complex epithelial–mesenchymal interactions. In this study, we show that for skin homeostasis, interleukin-1 (IL-1) produced by keratinocytes activates peroxisome proliferator–activated receptor β/δ (PPAR β/δ) expression in underlying fibroblasts, which in turn inhibits the mitotic activity of keratinocytes via inhibition of the IL-1 signaling pathway. In fact, PPAR β/δ stimulates production of the secreted IL-1 receptor antagonist, which leads to an autocrine decrease in IL-1 signaling pathways and consequently decreases

production of secreted mitogenic factors by the fibroblasts. This fibroblast PPAR β/δ regulation of the IL-1 signaling is required for proper wound healing and can regulate tumor as well as normal human keratinocyte cell proliferation. Together, these findings provide evidence for a novel homeostatic control of keratinocyte proliferation and differentiation mediated via PPAR β/δ regulation in dermal fibroblasts of IL-1 signaling. Given the ubiquitous expression of PPAR β/δ , other epithelial–mesenchymal interactions may also be regulated in a similar manner.

Introduction

Adult epidermis is a stratified epithelium in which keratinocytes from the basal and suprabasal layers cease to divide concomitantly with their outward movement and terminal differentiation all through enucleation and keratinization. After injury, the restoration of its functional integrity is of utmost importance to the survival of the organism. The maintenance of epithelium and its regeneration to close the wound is orchestrated with the contribution of the underlying dermal tissue. This synchrony is key to preventing either insufficient or excess wound repair. The regulation of wound repair is dictated by epithelial–mesenchymal interactions and purportedly mediated by the action of central players such as growth factors. This complex

interplay demands the expression of soluble factors exerting autocrine and paracrine activities and, importantly, the integration of such diverse signals, which culminate in appropriate cellular responses (Fusenig, 1994). Although the importance of the epithelial–mesenchymal communication is well recognized, the mechanism underlying this process needs in-depth study.

Of the numerous cytokines produced by skin cells, interleukin-1 (IL-1) has a pronounced influence on skin homeostasis and wound repair, and there are multiple mechanisms to regulate IL-1 signaling (Schroder, 1995). Both human and mouse keratinocytes constitutively produce biologically active IL-1 α/β (Arend et al., 1998). The two distinct forms of IL-1, termed IL-1 α and IL-1 β , are capable of binding to the same receptor with similar affinities to trigger biological effects. For any cells to respond productively to IL-1 α/β , they must possess corresponding cell surface receptors. Two distinct IL-1 receptors (IL-1Rs) have been cloned and characterized. The type I IL-1R (IL-1R1) mediates all

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Abbreviations used in this paper: AP-1, activation protein-1; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; GM-CSF, granulocyte macrophage colony-stimulating factor; iIL-1ra, intracellular IL-1ra; IL, interleukin; IL-1R, IL-1 receptor; IL-1ra, IL-1R antagonist; KGF, keratinocyte growth factor; OTC, organotypic skin culture; PCNA, proliferating cell nuclear antigen; PlGF, placental growth factor; PPAR, peroxisome proliferator–activated receptor; PPRE, peroxisome proliferator response elements; qPCR, quantitative real-time PCR; SDF-1, stromal-derived growth factor-1; sIL-1ra, secreted IL-1ra; TAK1, TGF-activated kinase 1; WT, wild type.

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known responses of IL-1. In contrast, the type 2 IL-1R (IL-1R2) is incapable of participating in signal transduction by virtue of its short cytoplasmic tail and thus is thought to neutralize the action of the bound IL-1 α/β . Interestingly, the predominant IL-1R species expressed by keratinocytes, both constitutively and after activation of the cells, is the IL-1R2. Its production by keratinocytes would represent an efficient mean for these cells to escape autocrine IL-1 α/β signaling (Rauschmayr et al., 1997). In contrast, the dermal fibroblasts express mainly the IL-1R1 isotype and thus are very sensitive to IL-1 α/β signaling.

The effects of IL-1 α/β on target cells can also be inhibited via the binding of the IL-1R antagonist (IL-1ra) to IL-1R1. There are two structural variants of IL-1ra, secreted IL-1ra (sIL-1ra) and intracellular IL-1ra (icIL-1ra). Both isoforms are transcribed from the same gene via two different promoters and alternative first exons. IL-1ra binds to IL-1R1 with an affinity that is 100–500 times higher than for IL-1R2 and competitively inhibits IL-1 α/β binding to the receptor. Although icIL-1ra may be released from keratinocytes under some conditions to compete for receptor binding, it can inhibit IL-1 α/β -induced cytokine production intracellularly via the COP9 signalosome (Banda et al., 2005). Fibroblasts produce both IL-1ra isoforms when appropriately stimulated, whereas keratinocytes only constitutively produce the icIL-1ra (Arend et al., 1998).

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors involved in the control of chronic diseases such as diabetes and obesity (Kersten et al., 2000). The PPAR subgroup comprises three related members named PPAR α (NR1C1), PPAR β/δ (NR1C2), and PPAR γ (NR1C3; Nuclear Receptors Nomenclature Committee, 1999). Ligand-activated PPARs form obligate heterodimers with the retinoid X receptor and bind to defined peroxisome proliferator response elements (PPREs) in the regulatory regions of target genes. Several works have shown that ligand-activated PPAR β/δ can induce terminal differentiation of keratinocytes (Tan et al., 2001; Burdick et al., 2006). Other studies comparing PPAR β/δ -null (KO) with wild-type (WT) mice showed that inflammatory signals increase the expression and activity of PPAR β/δ , which confer a pronounced antiapoptotic capacity to the keratinocytes, mediated in part via the increased activity of the PKB α survival pathway (Tan et al., 2001; Di Poi et al., 2002). Nonetheless, it is paradoxical that in the KO mice, epidermal hyperproliferation was observed either in early wound repair, upon hair plucking, or in response to a topical challenge with the phorbol ester (TPA; Peters et al., 2000; Michalik et al., 2001). Thus, the role of PPAR β/δ on cell proliferation remains debatable, and its mechanism of action is not sufficiently known (Michalik et al., 2001; Kim et al., 2005; Man et al., 2008).

Herein, we provide evidence for a novel paracrine effect of PPAR β/δ on epithelial cell growth. We found that PPAR β/δ in the fibroblasts attenuates keratinocyte proliferation by directly increasing the expression of sIL-1ra, thereby repressing IL-1 signaling. Epithelial–mesenchymal communications and IL-1 signaling are responsible for a wide range of biological events such as wound repair and tumorigenesis. Our results underscore the paracrine role of PPAR β/δ in the control of cell proliferation involving epithelial–mesenchymal interactions.

Results

PPAR β/δ knockdown in dermal fibroblasts results in increased keratinocyte proliferation

Earlier studies have shown that ligand-activated PPAR β/δ induced the differentiation of human keratinocytes in monolayer cultures (Tan et al., 2001; Schmuth et al., 2004). Herein, we examined the autocrine and paracrine consequences of PPAR β/δ deficiency during human epidermis formation using the organotypic skin culture (OTC) model. We first examined the expression pattern of the three PPAR isotypes in fibroblasts and keratinocytes of control OTC. Both quantitative real-time PCR (qPCR) and ELISA analyses performed on mechanically separated dermis and epidermis equivalents of OTCs revealed that PPAR β/δ is the predominant isotype in the keratinocytes and dermal fibroblasts, whereas the lower levels of PPAR α and PPAR γ are comparable (Fig. 1 A). We next assessed the knockdown efficiency of PPAR β/δ expression in human keratinocytes and fibroblasts by lentivirus-mediated siRNAs using qPCR and immunoblot analyses. qPCR revealed a >95% reduction of PPAR β/δ expression in cells transduced with the siRNA PPAR β/δ 11 sequence (Fig. 1 B, left). Immunoblot analysis also showed negligible levels of PPAR β/δ protein in the transduced cells (Fig. 1 B, right). These cells were used for subsequent experiments.

Next, OTCs were reconstructed using control keratinocytes (K_{CTRL}), PPAR β/δ knockdown keratinocytes ($K_{PPAR\beta/\delta}$), control fibroblasts (F_{CTRL}), and PPAR β/δ knockdown fibroblasts ($F_{PPAR\beta/\delta}$) in various combinations. Immunoblot analysis and immunofluorescence staining of the 2-wk-old K_{CTRL}/F_{CTRL} OTCs showed the expected keratinocyte differentiation markers keratin 5, keratin 10, and involucrin (Fig. 1 C and Fig. S1). OTCs with $K_{PPAR\beta/\delta}$ showed a reduced expression of terminal markers, which is consistent with the known prodifferentiation role of PPAR β/δ (Fig. 1 C and Fig. S1; Schmuth et al., 2004). No difference in keratin 5 expression, localized to the basal layer of the epidermis, was observed among the various OTCs (Fig. 1 C and Fig. S1). This provided evidence for a cell-autonomous action of PPAR β/δ in keratinocyte differentiation. Interestingly, OTC with $F_{PPAR\beta/\delta}$ led to increased keratinocyte proliferation as indicated by an increase in expression of cyclin D1 and proliferating cell nuclear antigen (PCNA; Fig. 1 C) and in Ki67-positive cells with respect to the control OTC (Fig. S1). Notably, the $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ OTC similarly displayed this enhanced proliferation of keratinocytes regardless of the impaired differentiation of the $K_{PPAR\beta/\delta}$ (Fig. 1 C and Fig. S1). Furthermore, OTC with $K_{PPAR\beta/\delta}$ showed threefold more apoptotic cells (TUNEL positive) and a higher level of cleaved caspase 3 as compared with OTC with K_{CTRL} (Fig. 1 C and Fig. S1). A dynamic epithelial–mesenchymal interaction is essential for the proper formation of the basement membrane (Smola et al., 1998). The reduced laminin 5 staining in OTCs with either $K_{PPAR\beta/\delta}$ or $F_{PPAR\beta/\delta}$ suggested a dysregulated epithelial–mesenchymal communication (Fig. S1). Altogether, these results are consistent with the observations from KO mice (Peters et al., 2000; Michalik et al., 2001) and revealed a potent proliferation stimulatory effect of $F_{PPAR\beta/\delta}$.

Figure 1. PPARβ/δ-deficient fibroblasts increase epidermal proliferation. (A) Expression profile of PPARs in OTC keratinocytes and fibroblasts. Total RNA and protein were extracted from keratinocytes and fibroblasts in OTC. Expression levels of PPAR mRNA (left) and protein (right) were monitored by qPCR and PPAR transcription factor assay kit, respectively. PPARβ/δ mRNA was normalized with control ribosomal protein P0 mRNA. (B) Human keratinocytes or fibroblasts were transduced with a lentiviral vector harboring a control or two different PPARβ/δ (PPARβ/δ9 and PPARβ/δ11) siRNAs. (C) Immunoblot analysis of epidermis from 2-wk-old OTCs constructed using K_{CTRL} or K_{PPARβ/δ} and F_{CTRL} or F_{PPARβ/δ}. Involucrin and transglutaminase I (Tgase I) are terminal differentiation markers, and keratin 10 (CK10) is an early differentiation marker. Keratin 5 (CK5) identifies the basal keratinocytes. Cell proliferation was measured using PCNA and cyclin D1. Apoptosis was detected using caspase 3. β-Tubulin showed equal loading and transfer. Representative immunoblots of epidermis from two OTCs are shown. Data are mean ± SD, *n* = 3.

on cocultured keratinocytes, providing evidence for an important noncell autonomous PPAR β/δ -dependent mechanism regulating keratinocyte proliferation.

Organotypic cultures with F_{PPAR β/δ} show increased expression of mitogenic factors

The paracrine effect of F_{PPAR β/δ} on epidermal proliferation is likely mediated by changes in the secretion of mitogenic or antimitogenic factors by the fibroblasts. To understand the mechanism underlying the enhanced epidermal proliferation in OTC with F_{PPAR β/δ} , an unbiased protein array was performed. Inflammatory cytokine and growth factor arrays were used to compare conditioned media from OTCs reconstructed using WT keratinocytes with either F_{CTRL} or F_{PPAR β/δ} . A total of 76 distinct proteins were screened, and the results showed that the protein expression of several mitogenic factors and cytokines were increased in OTC with F_{PPAR β/δ} (Table I). Notably, most of the proteins whose expression was increased have a known mitogenic action on keratinocytes. The expression of TGF- β 1, which exerts an antiproliferative effect on keratinocytes, and the abundant IL-1 α , constitutively produced by keratinocytes, remained unchanged. The expression of two proangiogenic factors, namely VEGF and placental growth factor (PIGF), were reduced in OTC with F_{PPAR β/δ} when compared with F_{CTRL}.

To verify that a reduced PPAR β/δ expression leads to a transcriptional up-regulation of mitogenic factor expression, we performed qPCR on selected mitogenic genes from OTC F_{CTRL} and F_{PPAR β/δ} in the absence or presence of the PPAR β/δ agonist GW501516. Consistent with the protein array results, F_{CTRL} exposed to GW501516 for 12 h showed a decrease in the expression of these genes (Fig. 2 A). Importantly, F_{PPAR β/δ} expressed higher basal levels of these mitogenic factors and, as expected, ligand treatment had no effect. Altogether, fibroblasts deficient in PPAR β/δ have an increased expression of mitogenic factors (Fig. 2 A).

Table I. Relative fold change of protein expression in OTCs with F_{PPAR β/δ} compared with F_{CTRL}

Cytokines/growth factors	Fold increase ^a
KGF ^b	4.01 $\pm$ 0.11 ^c
IL-6 ^b	5.56 $\pm$ 0.02 ^c
IL-8 ^b	2.94 $\pm$ 0.14 ^d
IL-10	2.13 $\pm$ 0.07 ^c
IL-16	2.86 $\pm$ 0.11 ^d
GMCSF ^b	5.26 $\pm$ 0.10 ^c
I-309	3.33 $\pm$ 0.16 ^c
Eotaxin-2	3.03 $\pm$ 0.12 ^c
VEGF	0.25 $\pm$ 0.011 ^d
PIGF	0.33 $\pm$ 0.014 ^d

^aThe intensities of signals were quantified by densitometry. Positive control included in the array blot was used to normalize and compare the results from different membranes. Values obtained from the control K_{CTRL}/F_{CTRL} OTC were arbitrarily assigned a value of 1. Values represent the mean fold increase as compared with control OTC ($n = 3$).

^bmRNA expression levels of these genes were further verified by qPCR.

^c $P < 0.01$.

^d $P < 0.1$.

Increased expression of mitogenic factors by F_{PPAR β/δ} results from attenuated IL-1 β /TAK1/AP-1 activation

Next, we sought to identify the signaling cascade that culminates in the up-regulation of the mitogenic growth factors in F_{PPAR β/δ} . The expression of most of these genes is known to be regulated by the transcription factor activation protein-1 (AP-1; Szabowski et al., 2000; Florin et al., 2004). Although direct regulation of eotaxin-2 by AP-1 has not been reported, the expression of this gene is stimulated by IL-1 (Watanabe et al., 2002; Heiman et al., 2005). The increased expression of I-309 is likely the consequence of increased IL-8 level (Wiener et al., 2008). We first addressed the hypothesis that AP-1 belongs to the pathway through which PPAR β/δ regulates the expression of mitogenic factors in F_{PPAR β/δ} . We examined the levels of phosphorylated (activated) c-Jun in F_{PPAR β/δ} and F_{CTRL} fibroblasts extracted from K_{CTRL}/F_{PPAR β/δ} and K_{CTRL}/F_{CTRL} OTCs, respectively. Immunoblot analysis showed that F_{PPAR β/δ} isolated from the K_{CTRL}/F_{PPAR β/δ} OTCs had enhanced phosphorylated c-Jun, which is consistent with an increased activity of the AP-1 complex (Fig. 2 B) and increased production of growth factors.

The TGF-activated kinase 1 (TAK1) plays a pivotal role in the activation of many genes, including genes encoding mitogenic factors, via activation of AP-1 (Florin et al., 2004, 2005). The IL-1 α/β released by keratinocytes and TNF- α present in wound sites activate TAK1 (Shim et al., 2005). To investigate the effect of PPAR β/δ on TAK1 activation, we examined the expression of phosphorylated TAK1 in F_{CTRL} and F_{PPAR β/δ} after IL-1 β and TNF- α stimulations. F_{CTRL} and F_{PPAR β/δ} were treated with either vehicle (DMSO) or PPAR β/δ agonist (GW501516) for 24 h prior to stimulation by either IL-1 β or TNF- α . As expected, there was an increase in phospho-TAK1 in vehicle-treated F_{CTRL} exposed to either IL-1 β or TNF- α (Fig. 2 C). Cotreatment of these fibroblasts with IL-1 β and GW501516 significantly prevented the increase in phospho-TAK1 levels in a dose-dependent manner (Fig. 2 C and Fig. S2 A). In the F_{PPAR β/δ} , a more robust increase in phospho-TAK1 was observed, which was only marginally affected by GW501516, showing that the ligand effect in F_{CTRL} was PPAR β/δ specific (Fig. 2 C and Fig. S2 A). In contrast, neither ligand-activated PPAR β/δ nor PPAR β/δ deficiency had an effect on TNF- α -mediated TAK1 activation (Fig. 2 C and Fig. S2 A). To further this observation, we performed chromatin immunoprecipitation (ChIP) using phospho-c-Jun antibodies on the keratinocyte growth factor (KGF) and granulocyte macrophage colony-stimulating factor (GMCSF) gene promoters in fibroblasts from K_{CTRL}/F_{CTRL} and K_{CTRL}/F_{PPAR β/δ} OTCs (Fig. S2 B). There was more phospho-c-Jun immunoprecipitated chromatin from F_{PPAR β/δ} when compared with F_{CTRL}, pointing to enhanced AP-1 binding and activation of the KGF and GMCSF genes in F_{PPAR β/δ} (Fig. S2 B). Together, these results indicate that PPAR β/δ specifically attenuates IL-1 β -mediated TAK1 activation and thus AP-1 activity.

Neutralization of IL-1 α/β signaling abolishes the mitogenic action of F_{PPAR β/δ}

Our results showed that IL-1 β -mediated but not TNF- α -mediated TAK1 activation and AP-1-dependent expression of mitogenic

Figure 2. Reduced fibroblast $PPAR\beta/\delta$ expression increases IL-1 β activation of TAK1 and up-regulation of AP-1-controlled mitogenic target genes. (A) Expression of mitogenic factor mRNAs in 2-wk old OTC $F_{PPAR\beta/\delta}$ and F_{CTRL} treated with $PPAR\beta/\delta$ agonist (500 nM GW501516 for 24 h) or vehicle. The expression levels of the indicated mitogenic factors were analyzed by qPCR and normalized to control ribosomal protein P0. Results are represented in fold induction as compared with OTC F_{CTRL} . (B) Immunoblot analysis of phosphorylated c-Jun from $F_{PPAR\beta/\delta}$ and F_{CTRL} extracted from $K_{CTRL}/F_{PPAR\beta/\delta}$ and K_{CTRL}/F_{CTRL} OTCs ($n = 2$), respectively. Total c-Jun and TAK1 protein expression level, which remains unchanged, showed equal loading and transfer. (C) Immunoblot analysis of IL-1 β and TNF- α activation of TAK1 in F_{CTRL} or $F_{PPAR\beta/\delta}$. Cells were treated with either vehicle (DMSO) or 800 nM GW501516 for 24 h prior to exposure to 10 ng/ml IL-1 β (top) or TNF- α (bottom). At the indicated time points, total cell lysates were extracted. Equal amounts of total protein (50 μ g) were resolved, electrotransferred, and probed for phosphorylated TAK1 (Thr184/187), total TAK1, and β -tubulin. Values below each band represent the mean fold differences ($n = 3$) in expression level with respect to vehicle-treated F_{CTRL} at 5 min, which was assigned the value of one. Data are mean $\pm$ SEM, $n = 3$.

A IL-1 α and β neutralizing antibodies **B** KGF, GMCSF and IL-6 neutralizing antibodies **C** Pre-immune IgG control

Figure 3. **Neutralizing antibodies against IL-1 α / β or KGF, GMCSF, and IL-6 abolished the mitogenic effect of F_{PPAR β / δ} .** (A–C) Immunoblot analysis of epidermis from indicated OTCs treated with 400 ng/ml IL-1 α / β (A), KGF, GMCSF, and IL-6 (B) neutralizing antibodies (each at 400 ng/ml) or 400 ng/ml preimmune IgG (C). Antibodies were added to OTC

factors were increased in F_{PPAR β / δ} . This result suggested that the IL-1 α / β pathway is responsible for the mitogenic effect of PPAR β / δ knockdown in fibroblasts. Conceivably, negating the effect of IL-1 α / β with neutralizing antibodies against IL-1 α / β would counteract the effect of PPAR β / δ knockdown in F_{PPAR β / δ} . As anticipated, OTCs exposed to anti-IL-1 α / β antibodies displayed a reduction in Ki67-positive keratinocytes, and the mitogenic action of F_{PPAR β / δ} was completely abolished (Fig. S3, compare A with C), indicating that IL-1 α / β signaling is a major pathway in the control of keratinocyte proliferation. This was further confirmed by immunoblot analysis of cyclin D1 and PCNA (Fig. 3, compare A with C). Similarly, inhibiting the action of the mitogenic factors should replicate the neutralizing effect of anti-IL-1 α / β antibodies. To test this possibility, we treated the OTCs with neutralizing antibodies against KGF, GMCSF, and IL-6, which are the three most abundant mitogenic factors in our model. To avoid possible compensatory effects, we neutralized all three growth factors simultaneously. Consistent with the aforementioned results, all OTCs showed thinner epidermis and reduced keratinocyte proliferation when compared with the corresponding OTC treated with preimmune IgG (Fig. 3, B and C and Fig. S3, B and C). Altogether, our observations indicated that the mitogenic action of the F_{PPAR β / δ} was caused by an increase in IL-1 α / β -mediated expression of mitogenic factors via TAK1 and AP-1.

Human sIL-1ra gene is a direct target of PPAR β / δ in fibroblasts

In vivo, the release of IL-1 α / β by basal keratinocytes is sufficient to maintain tissue homeostasis and to initiate cutaneous inflammation (Groves et al., 1996; Maas-Szabowski et al., 2000). Although we did not detect a change in the expression of IL-1 α / β in the protein array (Table I), we clearly showed that PPAR β / δ specifically attenuated IL-1 β response, including the activation of TAK1. This suggested that the attenuation of IL-1 β signaling by PPAR β / δ occurs upstream of TAK1 activation. To examine whether PPAR β / δ has a regulatory effect in IL-1 α / β signaling cascade, we measured the mRNA levels of IL-1 α , IL-1 β , IL-1R1, IL-1R2, icIL-1ra, sIL-1ra, and IL-1R-associated kinase, which are all key players of IL-1 signaling, in OTC epidermal keratinocytes (K_{CTRL} and K_{PPAR β / δ}) and dermal fibroblasts (F_{CTRL} and F_{PPAR β / δ}). With the exception of a higher level of sIL-1ra in the OTC F_{CTRL}, we detected no change in the expression of the other mediators of IL-1 α / β signaling (Fig. 4 A and not depicted). Consistently, elevated sIL-1ra was detected in the medium of OTC with F_{CTRL} as compared with F_{PPAR β / δ} , but no difference was observed in icIL-1ra protein within the fibroblasts (Fig. 4 A).

To determine whether the sIL-1ra promoter is directly regulated by PPAR β / δ in the fibroblasts, a 4.4-kb fragment

medium at each change of medium. Cell proliferation was measured using cyclin D1 and PCNA. Values below each band represent the mean fold differences in expression level with respect to K_{CTRL} from K_{CTRL}/F_{CTRL} OTC, which was assigned the value of 1. β -Tubulin served as a loading control. Representative immunoblots of epidermis from two indicated OTCs are shown.

Figure 4. Human sIL-1ra is encoded in a direct PPARβ/δ target gene in fibroblasts. (A) Expression of sIL-1ra and icIL-1ra mRNA (left) and protein (right) in OTC keratinocytes (K_{CTRL} and K_{PPARβ/δ}) and fibroblasts (F_{CTRL} and F_{PPARβ/δ}). sIL-1ra and icIL-1ra mRNA were analyzed by qPCR and normalized to ribosomal protein P0. The sIL-1ra level was determined by ELISA from medium of K_{CTRL}/F_{CTRL} and K_{CTRL}/F_{PPARβ/δ} OTCs. The icIL-1ra levels were measured by ELISA from cell lysates. (B) PPARE1 and PPARE3 of the human sIL-1ra gene are functional. Transactivation assay in fibroblasts cotransfected with a luciferase (luc) promoter driven by the human sIL-1ra promoter and pEF1-β-galactosidase as control of transfection efficiency. Relative positions of the three putative PPAREs (PPARE 1–3) and their mutants (mPPARE 1–3) are represented in closed and open ovals, respectively. Cells were treated with either 500 nM GW501516 (GW) and/or 10 ng/ml IL-1β for 24 h. Luciferase activity was measured, and normalized reporter activity is shown as fold induction as compared with untreated fibroblasts. (C) EMSA of human sIL-1ra PPARE1 and PPARE3. Radiolabeled PPARE1 (left) and PPARE3 (middle) were incubated either with RXRα, PPARβ/δ, or both. NSC denotes nonspecific competitor, the nonfunctional MEd DR1 element in the malic enzyme promoter. SC denotes nonradiolabeled consensus PPARE (conPPARE). As positive control, conPPARE was used. Mutated consensus PPARE is denoted by mconPPARE. PPARβ/δ did not bind to PPARE2 and mutated PPARE probes (mconPPARE, mPPARE1, mPPARE2, and mPPARE3; right). (D) PPARβ/δ binds to PPARE1 and PPARE3 of the human sIL-1ra gene in fibroblasts. ChIP assays were conducted using preimmune IgG or antibodies against PPARβ/δ (AB) in F_{CTRL} (WT) and F_{PPARβ/δ} (kd) fibroblasts extracted from two independent OTCs (OTC1 and OTC2). The regions spanning PPARE1 and PPARE2 of the sIL-1ra gene were amplified using appropriate primers (Table S1). A control region between PPARE1 and PPARE2 served as negative control. *, P < 0.05; **, P < 0.01. Data are mean ± SEM, n = 4.

Figure 5. Reduced siL-1ra in fibroblasts potentiates epidermal keratinocyte proliferation. (A) Immunoblot analysis of epidermis from indicated OTCs treated with either vehicle (PBS) or 50 ng/ml exogenous IL-1ra. Cell proliferation was measured using PCNA and cyclin D1. Values below each band were derived as described in Fig. 3 C. (B) Specific knockdown of siL-1ra in human fibroblasts. (left) Knockdown efficiency was monitored by qPCR and normalized with control ribosomal protein P0. Specificity of knockdown was assessed by the relative expression level of icL-1ra. (right) Protein expression of siL-1ra and icL-1ra as determined by ELISA. (C) Reduced fibroblasts siL-1ra increase epidermal proliferation. OTCs were constructed using K_{CTRL} with either control (F_{CTRL}) or siL-1ra knockdown ($F_{siL-1ra}$) fibroblasts. H&E, hematoxylin and eosin staining. (top) Ki67, cell proliferation (white arrows); DAPI, nuclear staining. Bars, 40 μ m. (bottom) Bars, 20 μ m. (left) Mean numbers of proliferating cells were derived as described in Fig. S1. Broken lines denote epidermal–dermal junction. (right) Immunoblot analysis of keratinocytes and fibroblasts extracted from two independent indicated OTCs. Equal amounts of total protein (50 μ g) were resolved, electrotitrated, and probed for the indicated proteins. Values below each band represent the mean fold differences in expression level with respect to K_{CTRL} or F_{CTRL} extracted from K_{CTRL}/F_{CTRL} OTC. **, $P < 0.01$; ***, $P < 0.001$. Data are mean $\pm$ SD, $n = 3$.

of the promoter region was subcloned into a luciferase reporter vector and analyzed in transactivation assays. Three putative PPRE sequences were identified in the siL-1ra promoter

(GenBank no. X64532), PPRE1 at position $-1,038/-1,050$, PPRE2 at $-1,072/-1,084$, and PPRE3 at $-4,067/-4,079$, using NUBIScan (Fig. S4 A; Podvinec et al., 2002). Transfected

primary fibroblasts treated with the PPAR β/δ ligand GW501516 alone showed only a modest approximately threefold increase in reporter activity (Fig. 4 B). As expected, treatment with IL-1 β increased the reporter activity by ~ 20 -fold, which is consistent with an earlier study (Smith et al., 1994). Importantly, this IL-1 β -mediated transactivation was further enhanced by treatment with GW501516 (to ~ 50 -fold; Fig. 4 B). To identify the functional PPRES responsible for the PPAR-mediated up-regulation, site-directed mutagenesis of the PPRES was performed. Transactivation assays with the various mutated promoters clearly showed that the stimulatory effects of PPAR β/δ were mediated by two PPRES, i.e., PPRES1 and PPRES3. To investigate whether PPAR β/δ was truly bound to these identified PPRES, electrophoretic mobility shift assay (EMSA) and ChIP were performed. As expected, specific protein–DNA complexes were detected in EMSA for PPRES1 and PPRES3, which were effectively competed by a consensus PPRE oligonucleotide (Fig. 4 C). As a positive control, the consensus PPRE oligonucleotide was used as a probe, which was specifically competed by the unlabeled consensus PPRE but not by a nonspecific competitor containing a nonfunctional MED (malic enzyme distal PPRE) DR1 element from the malic enzyme promoter (Fig. S4 B; IJpenberg et al., 2004). Similarly, ChIP performed on OTC F_{CTRL} using a monoclonal anti-PPAR β/δ antibody showed the binding of PPAR β/δ to both PPRES1 and PPRES3 (Fig. 4 D). This effect was not observed in $F_{PPAR\beta/\delta}$, and no signal was seen with preimmune serum either. In addition, no binding was detected to a control sequence residing between PPRES1 and PPRES3. These data clearly showed that PPAR β/δ specifically binds to the identified functional PPRES in the human sIL-1ra promoter in OTC F_{CTRL} . They indicated that the human sIL-1ra promoter is a direct PPAR β/δ target in primary human dermal fibroblasts.

Altogether, we showed that IL-1 α/β secreted by the keratinocytes activates TAK1/AP-1 signaling, which up-regulates the expression of mitogenic factors in the fibroblasts and thus enhances epidermal proliferation. We also showed that PPAR β/δ in the fibroblasts plays a homeostatic role in which it stimulates the expression of the sIL-1ra, modulates IL-1 α/β -mediated mitogenic factors gene expression, and thus attenuates epidermal proliferation.

sIL-1ra knockdown fibroblasts increase keratinocyte proliferation

We showed that the sIL-1ra gene in fibroblasts is a direct target of PPAR β/δ and that accordingly, PPAR β/δ knockdown results in a reduced production of sIL-1ra. To test whether the reduced production of sIL-1ra by $F_{PPAR\beta/\delta}$ triggers keratinocyte proliferation, we examined the effect of exogenous IL-1ra supplemented into the medium of OTCs with $F_{PPAR\beta/\delta}$. Importantly, exogenous IL-1ra abolished the mitogenic effect of $F_{PPAR\beta/\delta}$ (Fig. S5 A). This was further confirmed by immunoblot analysis of cyclin D1 and PCNA on the epidermis from the various OTCs (Fig. 5 A). Thus, inhibition of IL-1 α/β signaling using IL-1ra can rescue the pro-proliferating effect that PPAR β/δ deficiency in the fibroblasts has on keratinocyte proliferation.

To strengthen these results, we examined the role of sIL-1ra on epidermal proliferation using RNA interference. An siRNA

Table II. Relative fold change of protein expression in OTCs with $F_{sIL-1ra}$ compared with F_{CTRL}

Cytokines/growth factors	Fold increase ^a
KGF ^b	5.47 $\pm$ 0.22 ^c
IL-6 ^b	6.11 $\pm$ 0.31 ^c
IL-8	3.47 $\pm$ 0.06 ^c
IL-10	3.88 $\pm$ 0.15 ^c
IL-16	3.78 $\pm$ 0.41 ^c
GMCSF ^b	7.74 $\pm$ 0.31 ^c
CXCL1/GRO- α	2.67 $\pm$ 0.16 ^d
SDF-1	2.33 $\pm$ 0.07 ^d
I-309	4.57 $\pm$ 0.20 ^c
Eotaxin-2	6.19 $\pm$ 0.62 ^c
VEGF	0.22 $\pm$ 0.030 ^d
PIGF	0.25 $\pm$ 0.021 ^d

GRO- α , growth-related oncogene- α .

^aThe intensities of signals were quantified by densitometry. Positive control included in the array blot was used to normalize and compare the results from different membranes. Values obtained from the control K_{CTRL}/F_{CTRL} OTC were arbitrarily assigned a value of 1. Values represent the mean fold increase as compared with control OTC ($n = 3$).

^bChIP was performed on these genes using phospho-c-Jun antibody.

^c $P < 0.01$.

^d $P < 0.1$.

designed to target sIL-1ra was introduced into the human primary fibroblasts. qPCR and ELISA analyses revealed $>95\%$ reduction in sIL-1ra mRNA and protein in the transduced fibroblasts (Fig. 5 B). The knockdown showed high specificity for the targeted sIL-1ra mRNA as the level of icIL-1ra mRNA was unchanged (Fig. 5 B, left). The sIL-1ra knockdown fibroblasts ($F_{sIL-1ra}$) were used in OTC. Consistent with the aforementioned results, reducing the sIL-1ra production in fibroblasts resulted in increased keratinocyte proliferation as indicated by the large increase in Ki67-positive cells and cyclin D1 and PCNA expression (Fig. 5 C, left and middle). This marked increase in epidermal cell proliferation is accompanied by enhanced activation of TAK1 and c-Jun in the $F_{sIL-1ra}$ when compared with F_{CTRL} (Fig. 5 C, right).

To provide further evidence that reduced sIL-1ra in fibroblasts results in the increased expression of mitogenic factors, protein arrays were used to compare conditioned media from OTCs using WT keratinocytes with either F_{CTRL} or $F_{sIL-1ra}$ (Table II). In addition to those listed in Table I, the expression of two additional factors, chemokine ligand 1 (CXCL1)/growth-related oncogene- α and stromal-derived growth factor-1 (SDF-1), were increased in OTC $F_{sIL-1ra}$ compared with F_{CTRL} . The expression of CXCL1 and SDF-1 is c-Jun dependent, and their expression increased keratinocyte proliferation (Steude et al., 2002; Florin et al., 2005). Similarly, when comparing OTCs with F_{CTRL} and $F_{PPAR\beta/\delta}$, there was a trend for higher CXCL1 and SDF-1 expression in OTCs $F_{PPAR\beta/\delta}$, although the fold increase was not significant (unpublished data).

Finally, we also performed ChIP using phospho-c-Jun antibodies on the KGF, GMCSF, and IL-6 gene promoters in fibroblasts extracted from K_{CTRL}/F_{CTRL} and $K_{CTRL}/F_{sIL-1ra}$ OTCs. The sequences spanning the AP-1-binding site were significantly enriched in the immunoprecipitates obtained from $F_{sIL-1ra}$

Figure 6. **Increased c-Jun binding to AP-1 site of human KGF, GMCSF, and IL-6 gene promoter in $F_{sIL-1ra}$.** ChIP of AP-1-binding site of human KGF (top), GMCSF (middle), and IL-6 (bottom) genes using phospho-c-Jun antibodies. The gene sequence spanning the AP-1 site and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of F_{CTRL} and $F_{sIL-1ra}$ fibroblasts extracted from K/ F_{CTRL} and K/ $F_{sIL-1ra}$ OTC, respectively. Preimmune serum was used as a control. qPCR was performed on immunoprecipitates of phospho-c-Jun antibodies and normalized to input (chromatin before immunoprecipitation). Results are represented in fold change as compared with F_{CTRL} extracted from K/ F_{CTRL} OTC. M, 100 bp DNA ladder.

compared with F_{CTRL} (Fig. 6, left). This was further confirmed by qPCR normalized to chromatin before immunoprecipitation, i.e., input (Fig. 6, right). Altogether, we concluded that reduced sIL-1ra production in the fibroblasts, as observed in $F_{PPAR\beta/\delta}$, had a pronounced mitogenic effect on the epidermis.

PPAR β/δ -KO mice show reduced sIL-1ra expression during early wound healing

Next, we evaluated the relevance of our findings during mouse skin wound healing, an *in vivo* model that involves the IL-1 α/β pathway. We first showed that activated PPAR β/δ increased the IL-1 β -induced sIL-1ra expression in WT but not in KO primary fibroblasts (Fig. 7, A and B). Next, we examined the expression of IL-1ra in skin wound biopsies collected during early wound healing. qPCR analysis of early wound biopsies (days 1–3) showed a significantly lower sIL-1ra expression in KO tissue

when compared with WT (Fig. 7 C), which is consistent with the enhanced cell proliferation previously reported during early wound healing (Tan et al., 2001). Notably, this difference in sIL-1ra expression was no longer detected in later wound biopsy specimens (Fig. 7 C, day 7). The analysis of wound fluids collected from day 2 and 3 wound beds showed reduced sIL-1ra protein in KO mice when compared with WT mice (Fig. 7 C). The expression of icIL-1ra was unchanged between WT and KO mice (unpublished data). Because keratinocytes only produced icIL-1ra, the change in sIL-1ra expression was attributed to the fibroblasts, a hypothesis that was verified by qPCR analysis of laser microdissected epithelium and dermis sections from day 4 wound biopsies of WT and KO mice (Fig. S5 B). Consistent with our aforementioned results, wound KO dermis had reduced sIL-1ra expression and concomitantly increased levels of IL-6, GMCSF, and KGF when compared with wound WT

Figure 7. Regulation of IL-1 β signaling via PPAR β/δ during wound repair in KO mice. (A and B) Expression level of sIL-1ra (A) and icIL-1ra (B) in WT and KO fibroblasts. WT and KO primary fibroblasts were treated with PPAR β/δ ligand (100 nM GW501516 [GW]) and/or 10 ng/ml IL-1 β . The mRNA (left) and protein (right) expression levels were measured by qPCR and ELISA, respectively. (C) Expression level of sIL-1ra in early wound biopsies of KO mice. Wound fluids and biopsies were collected at the indicated day post-wounding (days 1–3, $n = 7$; day 7, $n = 4$). Unwounded skin was used as control (ctrl; $n = 4$). sIL-1ra mRNA and protein levels were determined by qPCR and ELISA, respectively. (D) Expression of sIL-1ra, icIL-1ra, KGF, IL-6, and GMCSF mRNAs from laser capture microdissected (LCM) dermis of WT ($n = 3$) and KO ($n = 4$) wound biopsies. Indicated mitogenic factor mRNA levels were analyzed by qPCR and normalized to control ribosomal protein P0. (E) Expression levels of indicated factor mRNAs in vehicle (veh; carboxymethylcellulose)- and IL-1ra-treated ($3 \times 2.5 \mu\text{g}$) wounds of KO and WT mice as compared with corresponding unwounded (control) skin. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Data are mean $\pm$ SEM, $n = 4$.

dermis (Fig. 7 D). These results confirmed that the expression of sIL-1ra was also modulated by PPAR β/δ in mouse fibroblasts. We next examined the effect of topical application of exogenous IL-1ra on the expression of IL-6, GMCSF, and mouse chemokine ligand 1 (KC; also known as CXCL1) in early wound biopsies. As expected, exogenous IL-1ra reduced IL-6, GMCSF, and KC

expression in KO wounds (Fig. 7 E). In summary, in vivo wound biopsy and fluid analyses revealed lower sIL-1ra mRNA and protein in the KO mice. Our results showed that PPAR β/δ -deficient fibroblasts had reduced sIL-1ra expression compared with WT cells and that this reduced sIL-1ra production increased epidermal proliferation.

Figure 8. The autonomous and nonautonomous actions of PPAR β/δ . PPAR β/δ confers antiapoptotic properties to keratinocytes and potentiates their migration via AKT1 and Rho GTPases in a cell-autonomous manner (Di Poi et al., 2002; Tan et al., 2007). PPAR β/δ also influences keratinocyte differentiation in a yet unknown mechanism. PPAR β/δ regulates epidermal proliferation in a nonautonomous fashion via a paracrine mechanism. IL-1 constitutively produced by the adjacent keratinocytes activates c-Jun, an obligate partner of AP-1 transcription factor via TAK1, and consequently increases the production of several mitogenic factors. The expression of PPAR β/δ in the underlying fibroblasts attenuates this IL-1 signaling via the production of sIL-1ra. sIL-1ra has little affinity for IL-1R2, which is highly expressed in keratinocytes. Thus, sIL-1ra acts in an autocrine fashion onto the fibroblasts, which expressed the predominant functional IL-1R1. The binding of sIL-1ra to IL-1R1 modulates the IL-1-mediated signaling events and consequently decreases the production of several AP-1-mediated mitogenic factors. The mitogenic factors exert a reduced paracrine effect on the epithelial proliferation via their cognate receptors (R). Therefore, PPAR β/δ in the fibroblasts plays an important homeostatic role in maintaining epidermal homeostasis, the absence of PPAR β/δ resulting in significant epidermal proliferation.

Discussion

Wound healing is a complex process that consists of a cascade of overlapping events, including inflammation reepithelialization and remodeling, directed at the restoration of the epidermal barrier. Throughout the whole healing process, interaction between different cell types provides coordination of the individual events, allowing for a temporal and spatial control. Reepithelialization is accomplished by increased keratinocyte proliferation and guided migration over the granulation tissue. Such processes require ordered changes in keratinocyte behavior that is dictated by the interplay of keratinocytes with dermal fibroblasts, i.e., epithelial–mesenchymal communication. The results presented in this study characterize a novel unsuspected role of PPAR β/δ in fibroblasts, which ensure a balanced functional interaction between dermis and epidermis. We show that keratinocyte proliferation is controlled by the dermal fibroblasts that produce AP-1–dependent mitogenic factors under the control of the IL-1/TAK1 signaling cascade whose activity level is modulated by PPAR β/δ via a direct control sIL-1ra production (Fig. 8).

Epidermal homeostasis requires a continuous exchange of signals with the underlying dermal compartment. Fibroblasts are a major cell type in the dermis, and their function in regulating extracellular matrix deposition and growth factor expression determines the differentiation states of the epidermis. Previous studies have showed that gene expression changes in the dermal

fibroblasts significantly influence epithelial cell fate (Cheng et al., 2005). In the presence of IL-1–producing keratinocytes, PPAR β/δ was the predominant PPAR isotype in fibroblasts. Interestingly, PPAR β/δ directly increased the expression of sIL-1ra in the fibroblasts that acted in an autocrine manner to modulate the expression of several IL-1–dependent mitogenic factors (Fig. 8). This autocrine action is possible because IL-1R1 is the predominant IL-1R subtype in fibroblasts, whereas keratinocytes exhibit IL-1R2 that has very low affinity for sIL-1ra and protects them from an exacerbated autocrine action of the IL-1 α/β that they constitutively produce. This study underscores the fact that PPAR β/δ in the dermal fibroblasts fulfils a homeostatic role during epidermal formation, balancing cell proliferation via a control on IL-1 α/β signaling.

It was reported that the enhanced TPA-induced hyperplasia in KO mice was a result of reduced ubiquitin C, whose expression is regulated by PPAR β/δ (Kim et al., 2004). The impaired ubiquitin-dependent proteasome-mediated protein turnover in KO mice resulted in a higher PKC α level, increased Raf1, and MAPK/extracellular signal-regulated kinase activities, which led to an increase in cell proliferation (Kim et al., 2005). Other studies have shown that PKC α overexpression in two different models did not affect the proliferation of primary human keratinocytes and of PKC α transgenic mouse keratinocytes (Wang and Smart, 1999; Cataisson et al., 2003). In fact, PKC α transgenic mouse keratinocytes treated with TPA underwent apoptosis (Cataisson et al., 2003). This is in apparent

discrepancy with the delayed wound healing kinetics observed in KO mice. Enhanced keratinocyte proliferation was also observed in many situations not involving TPA exposure (Michalik et al., 2001; Tan et al., 2001). Furthermore, no difference was observed in phosphorylated MEK-1/2 and ERK-1/2 expression between untreated KO and WT mice (Kim et al., 2005). This raises the question of how epidermal hyperproliferation occurs at wound edges early after injury of KO mice (Michalik et al., 2001). These observations point to a complex role of PPAR β/δ in cell proliferation. Our results showing that PPAR β/δ deficiency in fibroblasts increases their mitogenic effect on epithelial cells unveil part of this complexity. The underlying mechanism involves reduced sIL-1ra production by the fibroblasts, allowing a stronger IL-1 signaling, resulting in increased TAK1 activity with a consequent rise in mitogenic growth factor production. The *in vivo* relevance of this mechanism was observed during early wound repair in KO mice with reduced sIL-1ra in early but not late wound biopsies. This pattern is consistent with the early epidermal hyperproliferation reported in KO mice during the inflammatory phase of wound healing (Tan et al., 2001).

This study and earlier studies suggest different roles of PPAR β/δ in the dermis and adjacent epidermis (Tan et al., 2001; Man et al., 2008). The activation of PPAR β/δ conferred antiapoptotic properties to keratinocytes, which are mediated in part by the increased activation of the PKB α pathway (Di Poi et al., 2002). This antiapoptotic role was similarly observed in mouse tubular renal epithelial cells (Letavernier et al., 2005) and in human keratinocyte HaCaT cells (Schug et al., 2007). Our data now clearly show that PPAR β/δ signaling in fibroblasts has a pronounced effect on the growth potential of adjacent epithelia. Such differential roles were previously observed for TGF- β 1 signaling in wound repair (Beanes et al., 2003). Interestingly, an *in vivo* cross talk between TGF- β 1/Smad3 and PPAR β/δ signaling in keratinocytes during wound repair has been reported (Tan et al., 2004). The development of a vascular supply is important for wound healing. Interestingly, we also observed a reduced expression of VEGF and PIGF in OTC with F_{PPAR β/δ} . Although it is tempting to speculate that both VEGF and PIGF may be target genes of PPAR β/δ , their regulation are complex and context dependent, involving numerous factors such as pVHL (von Hippel-Lindau tumor suppressor), Sp-1, NF- κ B (nuclear factor κ light chain enhancer of activated B cells), hypoxia-inducible factor α , and AP-1, among others (Mukhopadhyay et al., 1997; Green et al., 2001). Importantly, epithelial-mesenchymal interactions can also modulate VEGF expression (Ong et al., 2007). During wound repair, an impaired angiogenesis is in line with the delayed wound healing observed in KO mice (Michalik et al., 2001).

The tissue microenvironment plays a pivotal role in modulating gene expression and cellular behavior, including epithelial-mesenchymal communication. Likewise, the microenvironment milieu is constituted by factors produced and released by these various cell types as communication signals. Of interest, the activation of IL-1 signaling was reported to coerce the growth of tumors, whereas its repression by IL-1ra has an antitumor effect (Lewis et al., 2006). The role of PPAR β/δ

in tumorigenesis remains debatable because there is evidence that this PPAR isotype potentiates or attenuates epithelial cancers (Gupta et al., 2000; Barak et al., 2002; Harman et al., 2004). Although this question is not directly addressed in this study, our preliminary data showed that F_{PPAR β/δ} can increase the proliferation of human squamous carcinoma cells (unpublished data).

Materials and methods

Reagents

The following antibodies and suppliers were used: anti-Ki67, antikeratin 10, anti-involucrin, antikeratin 6 (Novocastra), anti-PPAR α , anti-PPAR β/δ , and anti-PPAR γ (Millipore and Thermo Fisher Scientific); all cytokines and neutralizing antibodies against IL-1 α , IL-1 β , KGF, IL-6, and GM-CSF (Pepro-Tech); anti-c-Jun, antiphospho(Ser63)-c-Jun, anti-TAK1, and antiphospho-TAK1 (Cell Signaling Technology); and Alexa Fluor 488-conjugated goat anti-mouse antibody (Invitrogen). Real-time reagent SYBR GreenER (Invitrogen), rat tail collagen type I (BD), and transfection reagent Superfect (QIAGEN) were used. All restriction enzymes and DNA/RNA modifying enzymes were obtained from Fermentas. Double-promoter lentivirus-based siRNA vector and pFIV Packaging kit (System Biosciences) was used. The PPAR α , β/δ , γ Complete Transcription Factor Assay kit was obtained from Cayman Chemical. Primary neonatal human fibroblasts and keratinocytes were obtained from Invitrogen. GW501516 was obtained from Enzo Biochem, Inc. Otherwise, chemicals were obtained from Sigma-Aldrich.

Total RNA isolation, reverse transcription PCR, and qPCR

Total RNA was extracted from fresh tissues using Aurum total RNA kit (Bio-Rad Laboratories) following the supplier's protocol. 5 μ g total RNA was reverse transcribed with oligo-dT primers using RevertAid H Minus M-MuLV (Fermentas). After reverse transcription, the RNAs were removed by RNase H digestion. qPCR was performed with platinum Taq polymerase and SYBR GreenER super mixes using a PCR machine (MiniOpticon; Bio-Rad Laboratories). Melt curve analysis was included to assure that only one PCR product was formed. Primers were designed to generate a PCR amplification product of 100–250 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real-time PCR assays. Expression was related to the control gene ribosomal protein P0 (RPLP0), which did not change under any of the experimental conditions studied. The sequence of primers is available in Table S1. For each wound biopsies, 10 8- μ m-thick tissues were microdissected and pooled. RNA was isolated from microdissected paraformaldehyde-fixed paraffin-embedded sections using RecoverAll Total Nucleic Acid Isolation kit (Applied Biosystems). 5 ng RNA was subjected to Full Spectrum Complete Transcriptome RNA Amplification kit (System Biosciences) prior to qPCR.

Lentivirus-mediated knockdown of PPAR β/δ and sIL-1ra

Two siRNAs targeting the human PPAR β/δ , one against sIL-1ra and one unrelated control siRNA, were subcloned into the lentiviral-based siRNA vector pFIV-H1/U6-puro. The correct pFIV siRNA constructs were verified by sequencing using H1 primer. The sequence of the siRNAs was as given in Table S1. Transduction-ready pseudoviral particles (System Biosciences) were produced and harvested as described by the manufacturer. Transduced cells were enriched by puromycin selection for 1 wk. Western blot analysis, ELISA, or qPCR were used to assess the efficiency of knockdown.

Transactivation assay

An $\sim$ 4.4-kb (–1572 to –5980) promoter of the human sIL-1ra was PCR amplified from human genomic DNA using Pfu polymerase. The primer pairs used are given in Table S1. The resulting fragment was used for a second PCR amplification step introducing SacI and BglII sites that were used for subcloning into the pGL3 Basic vector (Promega). Site-directed mutagenesis of the three putative PPREs (PPRE1 at –1038/–1050, PPRE2 at –1072/–1084, and PPRE3 at –4067/–4079) were achieved using QuikChange site-directed mutagenesis kit (Agilent Technologies). Human fibroblasts were routinely grown in medium 106 (Invitrogen). Fibroblasts were cotransfected with a luciferase reporter driven by the various sIL-1ra promoter constructs and pEF1- β -galactosidase as a control of transfection efficiency using Superfect. After transfection, cells were incubated in the

presence or absence of 500 nM PPAR β / δ ligand GW501516 and IL-1 β for 24 h before lysis. Transfections with the positive control reporter pGL-3xPPRE-tk-Luc were included (provided by R.M. Evans, The Salk Institute for Biological Studies, University of California, San Diego, La Jolla, CA). Luciferase activity was measured using the luciferase assay (Promega) on a Microbeta Trilux (PerkinElmer). β -Galactosidase activity was measured in the cell lysate by a standard assay using 2-nitrophenyl- β -D-galactopyranoside as a substrate.

ChIP

ChIP was performed as described previously (Tan et al., 2004) with minor modifications. In brief, epidermis from OTC was physically separated from the collagen-embedded human fibroblasts after 2.5 U/ml dispase treatment at 37°C for 20 min. The dermal equivalents were thoroughly washed with PBS. The collagen gel was cut into small pieces prior to digestion with 0.5% collagenase I at 37°C. The fibroblasts were retrieved and cross-linked with 1% formaldehyde for 15 min at 37°C prior to sonication in lysis buffer. Monoclonal anti-PPAR β / δ antibody was used. The immunoprecipitates were reverse cross-linked for PCR by heating at 65°C for 6 h. The primer pairs used are shown in Table S1.

OTC

Primary human keratinocytes and fibroblasts were routinely maintained in defined keratinocyte medium (EpiLife; Invitrogen) and medium 106, respectively, as described by the manufacturer. OTCs were performed as previously described (Maas-Szabowski et al., 2000) in serum-free OTC medium with some modifications. 1 vol 10 $\times$ HBSS containing phenol red was mixed with 8 vol 4 mg/ml rat tail type I collagen (BD). The acetic acid was neutralized with 1 N NaOH on ice. Fibroblasts were resuspended in 1 vol 1 $\times$ HBSS and added dropwise to the neutralized collagen to achieve a final cell density of 10⁵ cells/ml of collagen. 3 ml of this fibroblast/collagen mixture was dispensed into a 24-mm diameter Transwell culture insert (3- μ m pore size; BD). The culture insert was placed in a 6-well Deep-Well Plate (BD) and allowed to gel in a 37°C, 5% CO₂, 70% humidity incubator (Thermo Fisher Scientific). Glass rings (outer diameter, 20 mm; thickness, 1.2 mm; height, 12 mm) were centrally placed onto the collagen. The glass rings serve to compress the collagen and delimit the area for the seeding of keratinocytes. The fibroblast-embedded collagen was submerged and cultured in serum-free OTC medium overnight. The serum-free OTC contains basal medium, 3:1 (vol/vol) DME (low glucose), Ham's F-12 nutrient mixture, basal supplements 5 μ g/ml insulin, 1 ng/ml epidermal growth factor, 0.4 μ g/ml hydrocortisone, 100 nM adenine, 10 μ M serine, 100 nM cholera toxin, 10 μ M carnitine, 1 mg/ml fatty acid-free albumin, lipid supplements 0.05 mM ethanolamine, 1 μ M isotroterol, 1 μ M α -tocopherol, and 50 μ g/ml ascorbic acid. The following day, the medium was removed and 10⁶ keratinocytes were seeded into the center of the glass ring. OTC medium was added, and the setup was once again incubated at 37°C with 5% CO₂ for 24 h to allow the keratinocytes to attach. The next day, the glass ring was removed, and culture was maintained at air liquid interface. Medium was changed every 3 d for a total of 2 wk. Neutralizing antibodies (each at 400 ng/ml) were freshly supplemented at every change of medium throughout the 2-wk culture period.

Immunofluorescence

OTCs were fixed with 4% paraformaldehyde in PBS for 2 h at 25°C. The fixed OTCs were washed twice with PBS and embedded in Tissue-Tek OCT compound medium (Sakura) overnight at 4°C. The skin cultures were subsequently frozen at -70°C for cryosectioning. 10- μ m cryostat tissue sections were mounted on SuperFrost Plus slides (Menzel-Gläser). The sections were processed for immunofluorescence as described previously (Michalik et al., 2001) except that Alexa Fluor 488-conjugated goat anti-mouse secondary antibody was used. The apoptotic keratinocytes were detected using the TUNEL assay according to the manufacturer's protocol (Roche). As positive control for TUNEL assay, the section was pretreated with DNase I. The slides were mounted with antifade reagent (ProLong Gold; Invitrogen) with DAPI. Images were taken with an inverted microscope (ECLIPSE TE2000-U; Nikon) using a Plan Fluor 20 $\times$ /0.45 objective (Nikon), Retiga EXi FAST cooled mono 12-bit camera (QImaging), and Image-Pro Plus software (Media Cybernetics).

Wounding experiment

Wounding of the mice dorsal skin was performed as previously described (Michalik et al., 2001). Wound biopsies and fluids were isolated as previously described (Tan et al., 2004) and were immediately snap frozen in liquid nitrogen for further analysis. Topical application of exogenous IL-1 α treatment was performed as previously described (Tan et al., 2005) except that three applications of 2.5 μ g recombinant IL-1 α were added over 3 d.

Measurement of cytokines by ELISA

The concentration of sIL-1 α and iCL-1 α measured using sandwich ELISA (R&D Systems). In brief, 2 $\times$ 10⁵ cells were cultured in a 35-mm culture dish with 750 μ l of medium and subjected to the indicated treatments. After 24 h, the media were harvested and treated with the addition of Complete protease inhibitors (Roche). The level of sIL-1 α was measured in culture supernatants accordingly to the manufacturer's instruction. To measure iCL-1 α , the cells were washed thoroughly, harvested by treatment with trypsin, and collected by centrifugation in fresh serum-free culture medium. The cell pellet was resuspended in 750 μ l of medium, disrupted by sonication, and the debris was removed by centrifugation. The concentration of iCL-1 α in the supernatant was measured by ELISA.

Protein arrays

Human Inflammation Antibody Array 3 and Growth Factor Antibody Array membranes (RayBiotech) were processed according to the manufacturer's protocol. Protein spots were detected by chemiluminescence. Signal intensities were quantified using ImageJ (National Institutes of Health) analysis software and were normalized with the mean intensity of the positive controls on each membrane.

Western blot analysis

Epidermis was physically separated from OTC after a 20-min treatment with dispase. Fibroblasts embedded in collagen were isolated after collagenase treatment. For Western blotting, protein extracts were made in ice-cold lysis buffer (20 mM Na₂H₂PO₄, 250 mM NaCl, 1% Triton X-100, and 0.1% SDS). Equal amounts of protein extracts (50 μ g) were resolved by SDS-PAGE and electrotransferred onto PVDF membranes. Membranes were processed as described by the manufacturer of antibodies, and proteins were detected by chemiluminescence (Millipore). Coomassie blue-stained membrane or tubulin was used to check for equal loading and transfer.

Online supplemental material

Fig. S1 shows immunofluorescence staining of 2-wk old OTCs. Fig. S2 shows immunoblot analysis of IL-1 β and TNF- α activation of TAK1 in F_{CTRL} or F_{PPAR β / δ} fibroblasts and ChIP of AP-1-binding site of human KGF and GMCSF genes using phospho-c-Jun antibodies. Fig. S3 shows immunofluorescence staining of 2-wk-old OTCs treated with neutralizing antibodies against IL-1 α and β or against KGF, GMCSF, and IL-6. Fig. S4 shows the alignment of the two functional PPREs in human sIL-1 α promoter to consensus PPRE. Fig. S5 shows immunofluorescence staining of 2-wk old OTCs derived using PPAR β / δ -deficient fibroblasts (F_{PPAR β / δ}) treated with either vehicle or exogenous IL-1 α . Table S1 shows the sequence of primers used in this study. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200809028/DC1>.

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Two-week old organotypic skin cultures

Figure S1. **Immunofluorescence staining of 2-wk-old OTCs.** OTCs constructed with control or $PPAR\beta/\delta$ knockdown keratinocytes (K_{CTRL} vs. $K_{PPAR\beta/\delta}$) and fibroblasts (F_{CTRL} vs. $F_{PPAR\beta/\delta}$). Pictures from representative immunostained sections are shown. $F_{PPAR\beta/\delta}$ potentiate the adjacent epithelial proliferation. Ki67, cell proliferation (arrows); TUNEL, cellular apoptosis (arrows); DAPI (blue), nuclear staining. Mean numbers of proliferating and apoptotic cells were derived from five standardized microscopic fields per section performed on three sections from four independent OTC constructions for each combination. Mean Ki67-positive cells per microscopic field (K_{CTRL}/F_{CTRL} , 10 ± 1.1 ; $K_{PPAR\beta/\delta}/F_{CTRL}$, 9 ± 1.8 ; $K_{CTRL}/F_{PPAR\beta/\delta}$, 32.5 ± 2.11 ; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 26.4 ± 3.3). H&E, hematoxylin and eosin staining. Dashed white lines show the epidermal–dermal junction. Bars: (H&E) 40 μ m; (DAPI) 20 μ m.

Figure S2. **Reduced PPAR β/δ expression increases IL-1 β activation of TAK1 in the fibroblasts.** (A) Immunoblot analysis of IL-1 β and TNF- α activation of TAK1 in *F_{CTRL}* or *F_{PPAR β/δ}* fibroblasts. *F_{CTRL}* and *F_{PPAR β/δ}* were treated with either vehicle (DMSO) or PPAR β/δ ligand (GW501516; 500 nM) for 24 h prior to exposure to 10 ng/ml IL-1 β (top) or TNF- α (bottom). At indicated time points, total cell lysates were extracted. Equal amounts of total protein (50 μ g) were resolved, electrotransferred, and probed for phosphorylated TAK1 (Thr184/187), total TAK1, and β -tubulin. (B) ChIP of AP-1-binding site of human KGF (top) and GMCSF (bottom) genes using phospho-cJUN antibodies. The gene sequence spanning the AP-1 site and a random control sequence were analyzed by PCR in the immunoprecipitated chromatin of *F_{CTRL}* and *F_{PPAR β/δ}* fibroblasts extracted from K/*F_{CTRL}* and K/*F_{PPAR β/δ}* OTC, respectively. Pre-immune serum was used as a control. qPCR was performed on immunoprecipitates of phospho-cJUN antibodies and normalized to input (chromatin before immunoprecipitation). Results are represented in fold change as compared with *F_{CTRL}* fibroblasts from K/*F_{CTRL}* OTC. No immunoprecipitation was observed with preimmune serum, and no amplification was observed for a control sequence. M, DNA molecular weight ladder.

Figure S3. **Neutralizing antibodies against IL-1 α and - β or against KGF, GMCSF, and IL-6 abolished the mitogenic effect of $F_{PPAR\beta/\delta}$.** (A–C) Indicated OTCs were constructed and set at air interface for 3 d prior adding to IL-1 α and - β (A), KGF, GMCSF, and IL-6 neutralizing antibodies (B) or preimmune IgG control (C). Neutralizing antibodies or preimmune IgG, each at 400 ng/ml, were added to serum-free OTC medium at each change of medium. The epidermis thickness in OTCs treated with 10 μ g/ml neutralizing anti-IL-1 α / β antibodies was K_{CTRL}/F_{CTRL} , 89.84 ± 2.11 μ m; $K_{PPAR\beta/\delta}/F_{CTRL}$, 82.69 ± 1.06 μ m; $K_{CTRL}/F_{PPAR\beta/\delta}$, 90.86 ± 2.54 μ m; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 80.18 ± 2.77 μ m, whereas that in OTCs treated with anti-KGF, GMCSF, and IL-6 (0.8 μ g/ml each) was K_{CTRL}/F_{CTRL} , 112.42 ± 3.57 μ m; $K_{PPAR\beta/\delta}/F_{CTRL}$, 81.46 ± 2.03 μ m; $K_{CTRL}/F_{PPAR\beta/\delta}$, 88.94 ± 4.32 μ m; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 85.15 ± 1.69 μ m. All values lower (epidermis thinner) than those of the corresponding OTCs were treated with a preimmune IgG (K_{CTRL}/F_{CTRL} , 139.08 ± 3.39 μ m; $K_{PPAR\beta/\delta}/F_{CTRL}$, 113.69 ± 2.70 μ m; $K_{CTRL}/F_{PPAR\beta/\delta}$, 165.71 ± 3.14 μ m; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 114.86 ± 4.23 μ m). Data are mean $\pm$ SEM, $n = 4$. No difference in Ki67-stained (arrows) proliferating cells were observed among the different OTCs except for the OTCs $F_{PPAR\beta/\delta}$ treated with preimmune IgG in which the number of proliferating keratinocytes was higher, as expected. Mean Ki67-positive cells per microscopic field were as listed subsequently (neutralizing antibodies against IL-1 α / β : K_{CTRL}/F_{CTRL} , 6 ± 1.9 ; $K_{PPAR\beta/\delta}/F_{CTRL}$, 7 ± 1.3 ; $K_{CTRL}/F_{PPAR\beta/\delta}$, 8 ± 2.0 ; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 8 ± 2.3 ; neutralizing antibodies against KGF, GMCSF, and IL-6: K_{CTRL}/F_{CTRL} , 9 ± 3.4 ; $K_{PPAR\beta/\delta}/F_{CTRL}$, 6 ± 1.9 ; $K_{CTRL}/F_{PPAR\beta/\delta}$, 8 ± 2.7 ; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 7 ± 2.6 ; preimmune IgG control treatment: K_{CTRL}/F_{CTRL} , 10 ± 3.2 ; $K_{PPAR\beta/\delta}/F_{CTRL}$, 9 ± 1.7 ; $K_{CTRL}/F_{PPAR\beta/\delta}$, 34 ± 4.1 ; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 35 ± 2.2). Pictures from representative immunostained sections are shown. H&E, hematoxylin and eosin staining. Dashed white lines denote epidermal–dermal junction. Bars, 40 μ m.

Figure S4. **Human sIL-1ra promoter contains two functional PPRES.** (A) Alignment of the PPRES (hsIL-1ra PPRE 1–3) in the promoter of human sIL-1ra (hsIL-1ra) gene with the consensus PPRES. Site-directed mutations are indicated as underlined nucleotides (hsIL-1ra mPPRE 1–3). Electrophoretic mobility shift assays are shown. Radiolabeled human (B) consensus PPRES (conPPRE) was incubated either with RXR α , PPAR β/δ , or both. NSC denotes nonspecific competitor, which contains a nonfunctional MED DR1 element in the malic enzyme promoter (Upenberg, A., N.S. Tan, L. Gelman, S. Kersten, J. Seydoux, J. Xu, D. Metzger, L. Canaple, P. Chambon, W. Wahli, and B. Desvergne. 2004. *EMBO J.* 23:2083–2091.). SC denotes nonradiolabeled consensus PPRES. Increasing the amount of nonradiolabeled consensus PPRES, but not nonspecific competitor, effectively impaired the binding of PPAR to the consensus PPRES.

Figure S5. **Exogenous IL-1ra abolishes the increased keratinocyte proliferation caused by $F_{PPAR\beta/\delta}$** (A) Indicated OTCs were constructed as described in Material and methods and treated with either vehicle (PBS) or 10 $\mu\text{g/ml}$ IL-1ra from day 3 after air interface setting. Fresh medium containing either vehicle or IL-1ra were changed every 3 d for 2 wk. As control for comparison, vehicle-treated K_{CTRL}/F_{CTRL} was shown. Pictures from representative sections immunostained with the denoted markers are shown. Ki67, cell proliferation; DAPI, nuclear staining. Dashed white lines represent epidermal-dermal junction. Mean numbers of proliferating cells were determined as described in Fig. S1. Vehicle (PBS) treatment: K_{CTRL}/F_{CTRL} , 8.5 ± 1.7 ; $K_{CTRL}/F_{PPAR\beta/\delta}$, 30.5 ± 4.1 ; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 29 ± 3.6 ; exogenous IL-1ra treatment: $K_{CTRL}/F_{PPAR\beta/\delta}$, 8 ± 2.26 ; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 7 ± 2.04 . Bars: (top) 40 μm ; (bottom) 20 μm . (B) Laser capture microdissection of normal and wound biopsies. Hematoxylin and eosin (H&E) stained of normal skin and day 4 wound biopsies from wild-type and $PPAR\beta/\delta$ knockout mice before and after laser capture microdissection using PALM Microbeam Axio Observer Z1 (Carl Zeiss, Inc.) with a long distance Plan-Neofluar 20 $\times$ /0.40 NA objective. The acquisition software used was PALM RoboSoftware 4.2 (Carl Zeiss, Inc.). Pictures were taken with a charge-coupled device camera system (3-Chip; Hitachi), and representative hematoxylin and eosin-stained sections are shown. Microdissected tissues were subsequently processed for real-time PCR analysis. Bars, 20 μm .

Table S1. Sequences of primers

Real-time quantitative PCR	Sequence
Human	
PPAR α (forward)	5'-ATGTCACACAACGCGATTG-3'
PPAR α (reverse)	5'-TCTTGGCCAGAGATTGAGA-3'
PPAR δ (forward)	5'-AGGAGCCATTCTGTGTGA-3'
PPAR δ (reverse)	5'-TCCTGCCAGCAGAGAGTGAT-3'
PPAR γ (forward)	5'-AATGTGAAGCCCATTGAAGA-3'
PPAR γ (reverse)	5'-AATGTGAAGCCCATTGAAGA-3'
sIL-1ra (forward)	5'-CCGCAGTCACCTAATCACTCTCCT-3'
sIL-1ra (reverse)	5'-CTCAGATAGAAGGTCTTCTGG-3'
icIL-1ra (forward)	5'-GAAGACCTCAGAAGACCTCTGTCC-3'
icIL-1ra (reverse)	5'-CTCAGATAGAAGGTCTTCTGG-3'
RPLP0 (forward)	5'-AACTCTGCAITCTCGTTCC-3'
RPLP0 (reverse)	5'-TCGTTTGTACCCGTTGATGA-3'
KGF (forward)	5'-CATGACTCCAGAGCAAATGGC-3'
KGF (reverse)	5'-TCCTCAGGTACCACTGTGTTCC-3'
GMCSF (forward)	5'-AGCAGCACTGCCCTCCAAC-3'
GMCSF (reverse)	5'-CAGCAGTCAAAGGGGATGAC-3'
IL-6 (forward)	5'-CCCACACAGACAGCCACTCA-3'
IL-6 (reverse)	5'-CCAGTGCCTCTTTGCTGCTT-3'
IL-8 (forward)	5'-GAACTTAGATGTCAGTGCAT-3'
IL-8 (reverse)	5'-CCAGACAGAGCTCTCTCCATC-3'
IL-1 β (forward)	5'-CTGTACCTGTCTGCGTGT-3'
IL-1 β (reverse)	5'-GTGCTGATGTACCAAGTTGGG-3'
IL-1 α (forward)	5'-CAGTGCTGCTGAAGGAGATG-3'
IL-1 α (reverse)	5'-AGTTTGGATGGGCAACTGAT-3'
IL-1R1 (forward)	5'-GGTGGTTCACTGAAGAGCA-3'
IL-1R1 (reverse)	5'-ACTGTGGTCCCTGTGTAAGTC-3'
IL-1R2 (forward)	5'-CCACGCCAGGAATATTCAGA-3'
IL-1R2 (reverse)	5'-CCTTGACTGTGGTGCGTAGT-3'
Mouse	
sIL-1ra (forward)	5'-GCTCATTGCTGGGTAATTACAA-3'
sIL-1ra (reverse)	5'-CCAGACTTGGCACAAGACAGG-3'
icIL-1ra (forward)	5'-CAGTCCACCCTGGGAAGGT-3'
icIL-1ra (reverse)	5'-AGCCATGGGTGAGCTAAACAGGACA-3'
GMCSF (forward)	5'-TCGTCTTAACGAGTTCTCTT-3'
GMCSF (reverse)	5'-GCAGTATGTCTGGTAGTAGTGG-3'
IL-6 (forward)	5'-TAGTCTTCTACCCCAATTCC-3'
IL-6 (reverse)	5'-TTGGTCTTAGCCACTCCTC-3'
KC (forward)	5'-CTGGGATTCACCTCAAGAATC-3'
KC (reverse)	5'-CAGGGTCAAGGCAAGCCTC-3'
IL-10 (forward)	5'-GCTCTTACTGACTGGCATGAG-3'
IL-10 (reverse)	5'-CGCAGCTCTAGGAGCATGTG-3'
IL-16 (forward)	5'-AAGAGCCGGAAATCCACGAAA-3'
IL-16 (reverse)	5'-GTCTAAAAGGGTCAGGGTACT-3'
siRNA primers	
PPAR β /s11 (sense)	5'-AAAGGGAAGCAGTTGGTGAATGG-3'
PPAR β /s11 (antisense)	5'-AAAACCATTCACCAACTGCTCC-3'
PPAR β /s9 (sense)	5'-AAAGGAGCGCAGCTGCAAGATT-3'
PPAR β /s9 (antisense)	5'-AAAAGAATCTTGACGCTGCGCTC-3'
Control (sense)	5'-AAAGCTGTCTTCAAGCTTGATATCGAAGACTA-3'
Control (antisense)	5'-AAAATAGTCTTCGATATCAAGCTTGAAGACAG-3'
sIL-1ra (sense)	5'-AAAGATCACTCTCCTCCTCTCTG-3'
sIL-1ra (antisense)	5'-AAAACAGGAAGAGGAGGAGAGTGAT-3'
H1 sequencing primer	5'-CTGGGAAATCACCATAAACGTGAA-3'
Promoter primers	
Human sIL-1ra promoter (forward)	5'-GACCAACTCTCTGCTGAGAATAACTAG-3'
Human sIL-1ra promoter (reverse)	5'-CTGCCATTGCGGGCCCAGAGTTGTGG-3'
ChIP primers	
PPRE1 (forward)	5'-AGCCCGGGGCTGGAGGTGAGAAGACCT-3'
PPRE2 (reverse)	5'-AACCTCTGCCACTTGCCTTGTGCCACC-3'
PPRE3 (forward)	5'-TTTACTCTGGGGACATGTGCTGGTTTC-3'
PPRE3 (reverse)	5'-GGCTGCCCCCTGATCAAATCCATGATT-3'
Control sequence (forward)	5'-GGCTGGGAGTGACAGGGCAGGAGC-3'
Control sequence (reverse)	5'-CACACAATGTGCAGAGCCTGTCTT-3'
Human KGF AP-1 (forward)	5'-ATGACCATTTGAATATAACAAGCC-3'
Human KGF AP-1 (reverse)	5'-GTGCTTGTGTGTGTGTGA-3'
Human KGF control sequence (forward)	5'-TGCACCACCTGGTGGTAAAGTAG-3'
Human KGF control sequence (reverse)	5'-GCTATTTACTTTCCAGCATGAGTT-3'
Human GMCSF AP-1 (forward)	5'-GAAGTCCAACTGTGCCCTCAGAGG-3'
Human GMCSF AP-1 (reverse)	5'-GGGCTCACTGGCAAAAAGAGCTCTT-3'
Human GMCSF control sequence (forward)	5'-TCACCTTAGCCATGTGGACAGTG-3'
Human GMCSF control sequence (reverse)	5'-GAGCATCTACTAGGTTCCAGCAGG-3'
Human IL-6 AP-1 (forward)	5'-GCTTTACTCTTGAAGACATGC-3'
Human IL-6 AP-1 (reverse)	5'-CGTCATTGAGGCTAGCGCTAAGAA-3'
Human IL-6 control sequence (forward)	5'-CCTCTGCAAGAGACACCATCC-3'
Human IL-6 control sequence (reverse)	5'-TGTGGCATCTTGACAGAGTTGTGT-3'

Two-week old organotypic skin cultures

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Figure S3. Neutralizing antibodies against IL-1 α and - β or against KGF, GMCSF, and IL-6 abolished the mitogenic effect of $F_{PPAR\beta/\delta}$. (A–C) Indicated OTCs were constructed and set at air interface for 3 d prior adding to IL-1 α and - β (A), KGF, GMCSF, and IL-6 neutralizing antibodies (B) or preimmune IgG control (C). Neutralizing antibodies or preimmune IgG, each at 400 ng/ml, were added to serum-free OTC medium at each change of medium. The epidermis thickness in OTCs treated with 10 μ g/ml neutralizing anti-IL-1 α / β antibodies was K_{CTRL}/F_{CTRL} 89.84 ± 2.11 μ m; $K_{PPAR\beta/\delta}/F_{CTRL}$ 82.69 ± 1.06 μ m; $K_{CTRL}/F_{PPAR\beta/\delta}$ 90.86 ± 2.54 μ m; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ 80.18 ± 2.77 μ m, whereas that in OTCs treated with anti-KGF, GMCSF, and IL-6 (0.8 μ g/ml each) was K_{CTRL}/F_{CTRL} 112.42 ± 3.57 μ m; $K_{PPAR\beta/\delta}/F_{CTRL}$ 81.46 ± 2.03 μ m; $K_{CTRL}/F_{PPAR\beta/\delta}$ 88.94 ± 4.32 μ m; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ 85.15 ± 1.69 μ m. All values lower (epidermis thinner) than those of the corresponding OTCs were treated with a preimmune IgG (K_{CTRL}/F_{CTRL} 139.08 ± 3.39 μ m; $K_{PPAR\beta/\delta}/F_{CTRL}$ 113.69 ± 2.70 μ m; $K_{CTRL}/F_{PPAR\beta/\delta}$ 165.71 ± 3.14 μ m; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ 114.86 ± 4.23 μ m). Data are mean $\pm$ SEM, $n = 4$. No difference in Ki67-stained (arrows) proliferating cells were observed among the different OTCs except for the OTCs $F_{PPAR\beta/\delta}$ treated with preimmune IgG in which the number of proliferating keratinocytes was higher, as expected. Mean Ki67-positive cells per microscopic field were as listed subsequently (neutralizing antibodies against IL-1 α / β : K_{CTRL}/F_{CTRL} 6 ± 1.9 ; $K_{PPAR\beta/\delta}/F_{CTRL}$ 7 ± 1.3 ; $K_{CTRL}/F_{PPAR\beta/\delta}$ 8 ± 2.0 ; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ 8 ± 2.3 ; neutralizing antibodies against KGF, GMCSF, and IL-6: K_{CTRL}/F_{CTRL} 9 ± 3.4 ; $K_{PPAR\beta/\delta}/F_{CTRL}$ 6 ± 1.9 ; $K_{CTRL}/F_{PPAR\beta/\delta}$ 8 ± 2.7 ; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ 7 ± 2.6 ; preimmune IgG control treatment: K_{CTRL}/F_{CTRL} 10 ± 3.2 ; $K_{PPAR\beta/\delta}/F_{CTRL}$ 9 ± 1.7 ; $K_{CTRL}/F_{PPAR\beta/\delta}$ 34 ± 4.1 ; and $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$ 35 ± 2.2). Pictures from representative immunostained sections are shown. H&E, hematoxylin and eosin staining. Dashed white lines denote epidermal–dermal junction. Bars, 40 μ m.

Figure S4. **Human sIL-1ra promoter contains two functional PPAREs.** (A) Alignment of the PPAREs (hsIL-1ra PPARE 1–3) in the promoter of human sIL-1ra (hsIL-1ra) gene with the consensus PPARE. Site-directed mutations are indicated as underlined nucleotides (hsIL-1ra mPPARE 1–3). Electrophoretic mobility shift assays are shown. Radiolabeled human (B) consensus PPARE (conPPRE) was incubated either with RXR α , PPAR β/δ , or both. NSC denotes nonspecific competitor, which contains a nonfunctional MED DR1 element in the malic enzyme promoter (Upenberg, A., N.S. Tan, L. Gelman, S. Kersten, J. Seydoux, J. Xu, D. Metzger, L. Canaple, P. Chambon, W. Wahli, and B. Desvergne. 2004. *EMBO J.* 23:2083–2091.). SC denotes nonradiolabeled consensus PPARE. Increasing the amount of nonradiolabeled consensus PPARE, but not nonspecific competitor, effectively impaired the binding of PPAR to the consensus PPARE.

Figure S5. **Exogenous IL-1ra abolishes the increased keratinocyte proliferation caused by $F_{PPAR\beta/\delta}$** (A) Indicated OTCs were constructed as described in Material and methods and treated with either vehicle (PBS) or 10 $\mu\text{g/ml}$ IL-1ra from day 3 after air interface setting. Fresh medium containing either vehicle or IL-1ra were changed every 3 d for 2 wk. As control for comparison, vehicle-treated K_{CTRL}/F_{CTRL} was shown. Pictures from representative sections immunostained with the denoted markers are shown. Ki67, cell proliferation; DAPI, nuclear staining. Dashed white lines represent epidermal-dermal junction. Mean numbers of proliferating cells were determined as described in Fig. S1. Vehicle (PBS) treatment: K_{CTRL}/F_{CTRL} , 8.5 ± 1.7 ; $K_{CTRL}/F_{PPAR\beta/\delta}$, 30.5 ± 4.1 ; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 29 ± 3.6 ; exogenous IL-1ra treatment: $K_{CTRL}/F_{PPAR\beta/\delta}$, 8 ± 2.26 ; $K_{PPAR\beta/\delta}/F_{PPAR\beta/\delta}$, 7 ± 2.04 . Bars: (top) 40 μm ; (bottom) 20 μm . (B) Laser capture microdissection of normal and wound biopsies. Hematoxylin and eosin (H&E) stained of normal skin and day 4 wound biopsies from wild-type and $PPAR\beta/\delta$ knockout mice before and after laser capture microdissection using PALM Microbeam Axio Observer Z1 (Carl Zeiss, Inc.) with a long distance Plan-Neofluar 20 $\times$ /0.40 NA objective. The acquisition software used was PALM RoboSoftware 4.2 (Carl Zeiss, Inc.). Pictures were taken with a charge-coupled device camera system (3-Chip; Hitachi), and representative hematoxylin and eosin-stained sections are shown. Microdissected tissues were subsequently processed for real-time PCR analysis. Bars, 20 μm .

Table S1. Sequences of primers

Real-time quantitative PCR	Sequence
Human	
PPAR α (forward)	5'-ATGTCACACAACGCGATTG-3'
PPAR α (reverse)	5'-TCTTGGCCAGAGATTGAGA-3'
PPAR δ (forward)	5'-AGGAGCCATTCTGTGTGA-3'
PPAR δ (reverse)	5'-TCCTGCCAGCAGAGAGTGAT-3'
PPAR γ (forward)	5'-AATGTGAAGCCCATTGAAGA-3'
PPAR γ (reverse)	5'-AATGTGAAGCCCATTGAAGA-3'
sIL-1ra (forward)	5'-CCGCAGTCACCTAATCACTCTCCT-3'
sIL-1ra (reverse)	5'-CTCAGATAGAAGGTCTTCTGG-3'
icIL-1ra (forward)	5'-GAAGACCTCAGAAGACCTCTGTCC-3'
icIL-1ra (reverse)	5'-CTCAGATAGAAGGTCTTCTGG-3'
RPLP0 (forward)	5'-AACTCTGCAITCTCGTTCC-3'
RPLP0 (reverse)	5'-TCGTTTGATCCCGTTGATGA-3'
KGF (forward)	5'-CATGACTCCAGAGCAAATGGC-3'
KGF (reverse)	5'-TCCTCAGGTACCACTGTGTTCC-3'
GMCSF (forward)	5'-AGCAGCACTGCCCTCCAAC-3'
GMCSF (reverse)	5'-CAGCAGTCAAAGGGGATGAC-3'
IL-6 (forward)	5'-CCCACACAGACAGCCACTCA-3'
IL-6 (reverse)	5'-CCAGTGCCTCTTTGCTGCTT-3'
IL-8 (forward)	5'-GAACCTAGATGTCAGTGCAT-3'
IL-8 (reverse)	5'-CCAGACAGAGCTCTCTCCATC-3'
IL-1 β (forward)	5'-CTGTACCTGTCTGCGTGT-3'
IL-1 β (reverse)	5'-GTGCTGATGTACCAAGTTGGG-3'
IL-1 α (forward)	5'-CAGTGCTGCTGAAGGAGATG-3'
IL-1 α (reverse)	5'-AGTTTGGATGGGCAACTGAT-3'
IL-1R1 (forward)	5'-GGTGGTTCACTGAAGAGCA-3'
IL-1R1 (reverse)	5'-ACTGTGGTCCCTGTGTAAGTC-3'
IL-1RII (forward)	5'-CCACGCCAGGAATATTCAGA-3'
IL-1RII (reverse)	5'-CCTTGACTGTGGTGCGTAGT-3'
Mouse	
sIL-1ra (forward)	5'-GCTCATTGCTGGGTAATTACAA-3'
sIL-1ra (reverse)	5'-CCAGACTTGGCACAAGACAGG-3'
icIL-1ra (forward)	5'-CAGTCCACCCTGGGAAGGT-3'
icIL-1ra (reverse)	5'-AGCCATGGGTGAGCTAAACAGGACA-3'
GMCSF (forward)	5'-TCGTCTTAACGAGTTCTCCTT-3'
GMCSF (reverse)	5'-GCAGTATGTCTGGTAGTAGCTGG-3'
IL-6 (forward)	5'-TAGTCTTCTTACCCCAATTCC-3'
IL-6 (reverse)	5'-TTGGTCTTAGCCACTCCTTC-3'
KC (forward)	5'-CTGGGATTCACCTCAAGAATC-3'
KC (reverse)	5'-CAGGGTCAAGGCAAGCCTC-3'
IL-10 (forward)	5'-GCTCTTACTGACTGGCATGAG-3'
IL-10 (reverse)	5'-CGCAGCTCTAGGAGCATGTG-3'
IL-16 (forward)	5'-AAGAGCCGGAATCCACGAAA-3'
IL-16 (reverse)	5'-GTCTAAAAGGGTCAGGGTACT-3'
siRNA primers	
PPAR β /s11 (sense)	5'-AAAGGAAGCAGTTGGTGAATGG-3'
PPAR β /s11 (antisense)	5'-AAAACCATTCACCAACTGCTTCC-3'
PPAR β /s9 (sense)	5'-AAAGGAGCGCAGCTGCAAGATTG-3'
PPAR β /s9 (antisense)	5'-AAAAGAATCTTGACAGCTGCGCTC-3'
Control (sense)	5'-AAAGCTGTCTTCAAGCTTGATATCGAAGACTA-3'
Control (antisense)	5'-AAAATAGTCTTCGATATCAAGCTTGAAGACAG-3'
sIL-1ra (sense)	5'-AAAGATCACTCTCCTCCTCTTCTG-3'
sIL-1ra (antisense)	5'-AAAACAGGAAGAGGAGGAGAGTGAT-3'
H1 sequencing primer	5'-CTGGGAAATCACCATAAACGTGAA-3'
Promoter primers	
Human sIL-1ra promoter (forward)	5'-GACCAACTCTCTGCTGAGAATAACTAG-3'
Human sIL-1ra promoter (reverse)	5'-CTGCCATTGCGGGCCCAGAGTTGTGG-3'
ChIP primers	
PPRE1 (forward)	5'-AGCCCGGGGCTGGAGGTGAGAAGACCT-3'
PPRE2 (reverse)	5'-AACCTCTGCCACTTGCCTTGTGCCACC-3'
PPRE3 (forward)	5'-TTTACTCTGGGGACATGTGCTGGTTTC-3'
PPRE3 (reverse)	5'-GGCTGCCCCCTGATCAAATCCATGATT-3'
Control sequence (forward)	5'-GGCTGGGAGTGCAGGGCAGGAGC-3'
Control sequence (reverse)	5'-CACACAATGTGCAGAGCCTGTCTT-3'
Human KGF AP-1 (forward)	5'-ATGACCATGAACTATACAAGCC-3'
Human KGF AP-1 (reverse)	5'-GTGCTTGTGTGTGTGTGA-3'
Human KGF control sequence (forward)	5'-TGCACCACCTGGTGGTAAAGTAG-3'
Human KGF control sequence (reverse)	5'-GCTATTTACTTTCCAGCATGAGTT-3'
Human GMCSF AP-1 (forward)	5'-GAAGTCCAACTGTGCCCTCAGAGG-3'
Human GMCSF AP-1 (reverse)	5'-GGGCTCACTGGCAAAAAGAGCTCTT-3'
Human GMCSF control sequence (forward)	5'-TCACCTTAGCCATGTGGACAGTG-3'
Human GMCSF control sequence (reverse)	5'-GAGCATCTACTAGGTTCCAGCAGG-3'
Human IL-6 AP-1 (forward)	5'-GCTTTACTCTTGTCAAGACATGC-3'
Human IL-6 AP-1 (reverse)	5'-CGTCATTGAGGCTAGCGCTAAGAA-3'
Human IL-6 control sequence (forward)	5'-CCTCTGCAAGAGACACCATCC-3'
Human IL-6 control sequence (reverse)	5'-TGTGGCATCTTGACAGAGTTTGTGT-3'