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THE ROLE OF ANGIOPOIETIN-LIKE 4 IN SKIN WOUND REPAIR

GOH YAN YIH

(M. Sc.)

School of Biological Sciences

A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirement for the degree of

Doctor of Philosophy

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This work is dedicated to my parents and my siblings for their understanding, support and love.

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LIST OF ABBREVIATIONS

°C	Degree celcius
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar
Ac5	Actin 5
Act-D	Actinomycin D
AE	Anion-exchange
Af	Affinity chromatography
ANGPTL	Angiopoietin-like protein
ANGPTL3	Angiopoietin-like 3
ANGPTL4	Angiopoietin-like 4
$ANGPTL4^{-/-}$	ANGPTL4-knockout mice
$ANGPTL4^{+/+}$	ANGPTL4 wild type mice
B	Bound
bp	Base pair
BSA	Bovine serum albumin
cANGPTL4	C-terminal fibrinogen-like domain of ANGPTL4
cDNA	Complementary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
CHX	Cycloheximide
CM	Conditioned medium
CMACs	Cell-matrix adhesion complexes
CO_2	Carbon dioxide
COL-1	Collagen-1
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
E	Epidermis
E. coli	Echerichia coli
ECM	Extracellular matirx
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-Linked Immunosorbent Assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FIAF	Fasting-induced adipose factor
FITC	Fluorescein isothiocyanate

FN	Fibronectin
GAG	Glycosaminoglycan
GF	Gel filtration
GM-CSF	Granulocyte-macrophage colony stimulating factor
GSK-3β	Glycogen synthase kinase-3β
GST	Glutathione-S-transferase
GST-PAK	GST coupled to Rac1/cdc42 binding domain of p21 activated kinase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
h	Hour
H&E	Haematoxylin & Eosin
HaCaT	Immortalized human keratinocytes
hf	Hair follicle
HFARP	Heatic fibrinogen/angiopoietin-related protein
HIF-1	Hypoxia inducible factor 1
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
IAA	Indole-3-acetic acid
IB	Immuno-blotting
IEF	Isoelectric focusing
IF	Immunofluorescence
IgG	Immunoglobulin G
IL-1	Interleukin-1
ILD	I-like domain
ILK	integrin-linked kinase
IPTG	isopropyl β-D-1-thiogalactopyranoside
JNK	c-Jun N-terminal kinase
K_{off}	Dissociation rate constant
K_{on}	Association rate constant
K_a	Association rate constant
$K_{ANGPTL4}$	Human keratinocytes transduced with ANGPTL4 sirna
K_{CTRL}	Human keratinocytes transduced with control scrambled sirna
K_d	Dissociation rate constant
K_D	Equilibriu dissociation constant
kDa	Kilodalton
KGF	Keratinocyte growth factor
KLC2	Kinesin light chain 2
LB	Luria broth
LCM	laser-capture microdissection
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LIMK1	LIM domain kinase 1
Ln	Laminin-5
M	Molar
mAB	Monoclonal antibody

MALDI	Matrix-assisted laser desorption/ionization
MAPK	Mitogen-activated protein kinase
MEK	MAPK kinase
MEK/ERK	Mitogen-activated protein kinase kinase/extracellular regulated kinase
MesNa	2-mercaptoethanesulfonate sodium
mg/L	Milligram per litre
min	Minutes
mit C	Mitomycin C
ml	Millilitre
mM	Millimolar
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Mass spectroscopy
NaCl	Sodium chloride
nANGPTL4	N-terminal coiled-coil domain of ANGPTL4
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHS	N-hydroxysulfosuccinimide
nm	Nanometre
OTC	Organotypic skin culture / Organotypic co-culture
pAB	Polyclonal antibody
PAI-1	Plasminogen activated inhibitor type-1
PAK	p21-activated kinase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF-B	Platelet-derived growth factor-B
PDK1	Phosphoinositide-dependent protein kinase 1
pFAK	Phosphorylated FAK
PI3K	Phosphotidylinositol 3-kinase
PIP3	Phosphotidylinositol 3,4,5- triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PKCα	Protein kinase Cα
PLA	Proximity ligation assay
PMSF	Phenylmethylsulphonyl fluoride
PPAR	Peroxisome proliferators-activated receptor
PPARα	Peroxisome-proliferator-activated receptor alpha
PPARβ/δ	Peroxisome-proliferator-activated receptor beta/delta
PPARγ	Peroxisome-proliferator-activated receptor gamma
PPRE	PPAR response element
PSI	Plexin semaphoring integrin
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time polymerase chain reaction

RACK1	Receptor for activated protein kinase C
RU	Response unit
s	Second
S.D.	Standard deviation
S2 cells	Schneider 2 cells
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
siRNA	Small interfering RNA
SPARC	Secreted protein acidic and rich in cysteine
SPR	Surface plasmon resonance
SPR-MS	Surface plasmon resonance coupled mass spectroscopy
SPRs	Small proline-rich proteins
TBS	Tris-buffered saline
TBST	Tris-buffered saline with Tween-20
TGase1	Transglutminase 1
TGF	Transforming growth factor
TGF-β	Transforming growth factor-beta
Tie-1	Tyrosine kinase-1
Tie-2	Tyrosine kinase-2
TNF-α	Tumor necrosis factor-alpha
tPA	Tissue-type plasminogen activator
TPA	Phorbol ester
TSP	Thrombospondin
TUNEL	Terminal deoxynucleotidyl transferase Biotin-dUTP nick end labeling
U	Unbound fractions
UI	Uninduced
uPA	urokinase-type plasminogen activator
VEGF	Vascular endothelial growth factor
Vn	Vitronection
vWF	von Willibrand factor
WF	Wound fluid/exudate

SUMMARY

A dynamic cell-matrix interaction is crucial for a rapid cellular response to changes in the environment. Using mice deficient in angiopoietin-like 4 (ANGPTL4) either by gene ablation or immuno-neutralization, we show that ANGPTL4 is important for the wound re-epithelialization. We demonstrate that ANGPTL4 produced by wound keratinocytes coordinates cell-matrix communication. ANGPTL4 interacts with vitronectin and fibronectin in the wound bed, delays their proteolytic degradation by metalloproteinases, and thereby regulates the availability of local extracellular matrix. Importantly, we identify integrins β1 and β5 as novel binding partners of ANGPTL4. These interactions activate the FAK-Src-PAK1 and 14-3-3-mediated signaling pathways to accelerate cell migration. Interestingly, ANGPTL4-bound integrins recruit protein kinase Cα to selectively internalized integrin that culminates in productive lamellipodia formation. The findings presented here reveal an unpredicted role of ANGPTL4. They provide insights into a novel control of keratinocyte behavior, which is mediated by ANGPTL4-mediated cell-matrix communication, and underscores the physiological importance of such modulation of integrin activity in angiogenesis and metastasis.

(160 words)

CHAPTER 1

ANGIOPOIETIN-LIKE 4 INTERACTS WITH INTEGRINS β1 AND β5 TO MODULATE KERATINOCYTE MIGRATION

Publication:

Angiopoietin-like 4 interacts with integrins beta1 and beta5 to modulate keratinocyte migration.

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1.1 ABSTRACT

Adipose tissue secretes adipocytokines for energy homeostasis, but recent evidence indicates that some adipocytokines also have a profound local impact on wound healing. Upon skin injury, keratinocytes use various signaling molecules to promote re-epithelialization for efficient wound closure. Here, we identify a novel function for adipocytokine angiopoietin-like 4 (ANGPTL4) in keratinocytes during wound healing via the control of integrin-mediated signaling and internalization. Using two different *in vivo* models based on topical immuno-neutralization of ANGPTL4 and ablation of the *ANGPTL4* gene, we show that ANGPTL4-deficient mice exhibit delayed wound re-epithelialization with impaired keratinocyte migration. Human keratinocytes, in which endogenous ANGPTL4 expression was suppressed by siRNA or by neutralizing antibody, showed impaired migration associated with diminished integrin-mediated signaling. Importantly, we identified integrins β1 and β5, but not β3, as novel binding partners of ANGPTL4. ANGPTL4-bound integrin β1 activated the FAK-Src-PAK1 signaling pathway which is important for cell migration. The findings presented here reveal an unexpected role for ANGPTL4 during wound healing and demonstrate how ANGPTL4 stimulates intracellular signaling to coordinate cellular behavior. Our findings provide insight into a novel cell migration control mechanism and underscore the physiological importance of the modulation of integrin activity in cancer metastasis.

1.2 INTRODUCTION

1.2.1 Human Skin

As the largest organ in the human body, the skin serves multiple functions, ranging from providing a barrier against external organisms and ultraviolet radiation through the pigmentation, to protecting the underlying tissues from dehydration and injury. Other functions of the skin include thermoregulation, sensory detection and immune surveillance. The skin is primarily made up of two layers – the thin outer epidermis and the thicker inner dermis. Keratinocytes, the main cell type of the epidermis, undergo a vectorial differentiation program to form different layers: the basal layer, the spinal layer, the granular layer and the corneal layer (stratum corneum) (Figure 1.1). The innermost, undifferentiated basal cells move upward to the outermost surface of the skin. During this migration, they undergo a series of morphological and biochemical changes that culminate in the production of dead, flattened, enucleated squames, which are sloughed off from the skin surface. The dermis, whose major cell type is the fibroblast, is made up of collagen rich connective tissue that provides nourishment and support, and regulates the proliferation and terminal differentiation of epidermal keratinocytes. Such communication between these two layers occurs via a double-paracrine mechanism (Maas-Szabowski et al., 1999), i.e., epithelial-mesenchymal interactions.

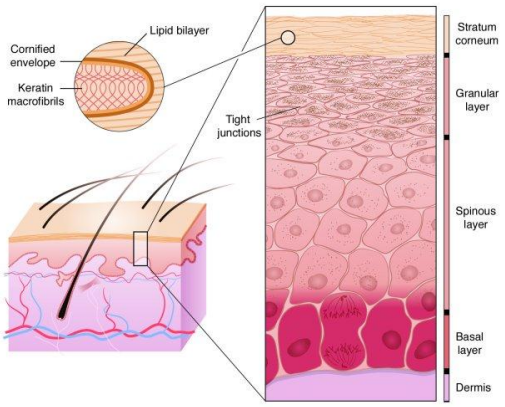

Figure 1.1 Schematic diagram of the stages of epidermal differentiation, resulting in a permeability barrier. Epidermal keratinocytes undergo a linear program of differentiation from mitotically active basal cells to transcriptionally active spinous cells, enucleated granular cells, resulting finally in differentiated squames in the stratum corneum. As shown in the inset, squames, which provide the primary barrier, are composed of keratin macrofibrils and cross-linked cornified envelopes encased in lipid bilayers. Tight junctions, located in the granular layer, also play an essential role in retaining the water content of the body. (Taken from (Segre, 2006)).

1.2.2 Wound Injury

Acute and chronic cutaneous wounds are very common. Traumatic lacerations of the skin are one of the most common problems seen and treated in emergency departments. An ongoing study conducted by the National Center for Health Statistics for the year 2004 has estimated that there were 10 million wounds cared for in United States emergency departments, including 2.5 million facial lacerations and 1.8 million extremity lacerations (Singer et al., 2006). In addition, chronic wounds affect 6.5 million patients. An estimated excess of US$25 billion is spent annually on the treatment of chronic wounds and the burden is rapidly growing due to increasing health care costs, an aging population and a sharp rise in the incidence of diabetes and obesity worldwide. The annual wound care products market is projected to reach $15.3 billion by 2010 (Sen et al., 2009). A total of 40 million inpatient surgical procedures were performed in the United States in 2000, followed closely by 31.5 million outpatient surgeries (Sen et al., 2009). Furthermore, the need for post-surgical wound care is sharply on the rise. An additional burden of wound healing is the problem of skin scarring, which is estimated to

cost $12 billion annually. The immense economic and social impact of wounds in our society calls for greater attention and resources to understand the biological mechanisms underlying cutaneous wound complications.

1.2.3 Biology of Wound Repair

Injury that results from skin exposure to external factors triggers a finely tuned pattern of integrated biological events to repair the damaged tissue and re-establish a new epithelial barrier. There are three recognized phases that characterized the cutaneous repair process: the inflammation, the proliferation or migratory phase (also known as the re-epithelialization phase), and the extracellular matrix (ECM) remodeling phase (Martin, 1997; Segre, 2006). Main players and events that characterized each phase will be discussed. However, it should be noted that separating the overall process of wound repair into these three phases only aim to aid explanation, in actual fact these phases overlap considerably (Figure 1.2).

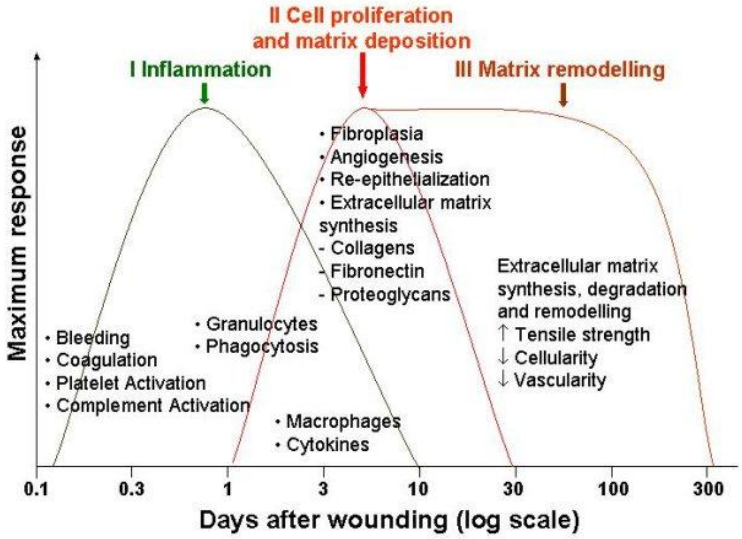

Figure 1.2 Phases of wound healing. Wound healing can be divided into three phases: inflammation, re-epithelialization (granulation tissue), and remodeling (wound contraction) phases. In the inflammatory phase, bacteria and debris are phagocytosed and removed, and factors are released that cause the migration and division of cells involved in the re-epithelialization phase. The re-epithelialization phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction. In angiogenesis, new blood vessels are formed by vascular endothelial cells. In fibroplasia and granulation tissue formation, fibroblasts grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin. Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and 'crawl' atop the wound bed, providing cover for the new tissue. In contraction, the wound is made smaller by the action of myofibroblasts, which establish a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. When the cells' roles are close to complete, unneeded cells undergo apoptosis. In the maturation and remodelling phase, collagen is remodelled and realigned along tension lines and cells that are no longer needed are removed by apoptosis.
(Taken from http://www.worldwidewounds.com/2004/august/Enoch/images/enochfig1.jpg)

1.2.3.1 Inflammation Phase (Phase 1)

In first phase of wound repair, inflammation begins immediately after an acute injury. The disruption of blood vessels leads to local release of blood cells and blood-borne elements resulting in clot formation. While the blood clot within the vessel lumen provides hemostasis, the clot within the injury site acts as a provisional matrix for cell migration (Clark et al., 1982b). The inflammation phase is dominated by the platelet, which directs clotting of the fresh wound by the intrinsic and extrinsic coagulation pathways. Platelets also release a number of chemotactic factors that attract other platelets, leukocytes, and fibroblasts to the site of injury. The inflammation phase

continues as leukocytes, specifically neutrophils and macrophages, enter the scene. Their initial role is to cleanse the wound by phagocytosing and killing bacteria and scavenging cellular debris. However, it should be recognized that neutrophils and macrophages also release growth factors and other important mediators during this period of time. This first phase of wound repair can be best understood by breaking it down into the following components: (a) platelet release and aggregation, (b) the processes of coagulation and inflammation, and (c) recruitment of leukocytes (Coulombe, 2003).

a) Platelet Release and Aggregation

Tissue injury leads to blood vessel damage, platelet release and activation of blood coagulation (Furie and Furie, 1988). Platelets first adhere to interstitial connective tissue and then aggregate, when they are exposed to thrombin and fibrillar collagen, which trigger their activation, adhesion and aggregation. During aggregation, platelets release many mediators, including fibrinogen, fibronectin, thrombospondin and von Willibrand factor VIII. Fibrinogen, fibronectin, and thrombospondin serve as ligands for platelets aggregation, whereas von Willibrand factor VIII facilitates platelet adhesion to fibrillar collagens, resulting in the formation of a platelet plug (Moncada et al., 1976). Thrombin polymerization of fibrinogen leads to fibrin, which amplifies the clot and will form part of the provisional extracellular matrix required for the migration of cells into the wound. Platelets also release a number of growth factors and cytokines. These factors participate in the initial inflammatory phase but also serve as signals for the migration of certain critical cells to the site of injury (Poppas et al., 1996). Among these factors are platelet-derived growth factors (PDGF), transforming growth factors β isoforms (TGF-βs) play a critical role in wound healing.

b) Coagulation and Inflammation

During the process of coagulation, plasma and other blood elements leak from injured blood vessels and contribute to the formation of a thrombus through the intrinsic and extrinsic pathways. Both pathways lead to the formation of thrombin, which cleaves fibrinopeptides A and B as well as other fragments from fibrinogen and ultimately leads to the polymerization of fibrinogen to fibrin (Furie and Furie, 1988). The stability of fibrin and its biological activity are depedent on its cross-linking. Blood clotting ends when the different stimuli for the activation of the coagulation cascade resolve.

Migration to the site of injury of a number of key cells, such as keratinocytes, fibroblasts, endothelial cells, and monocytes, is aided by their receptors for various components of the thrombus. The end result is increased vascular permeability, neutrophil and monocyte recruitment, and release of factors from mast cells (Weiss et al., 1998). Cleavage of fibrinogen leads to a number of active fragments, such as the fibrinopeptides, that are important in stimulating migration to the wound site of certain cells, including fibroblasts.

c) Recruitment of Leukocytes

There is a constant cascade of inflammatory molecules and recruitment of inflammatory cells during the early phases of wound healing (DiPietro et al., 2001). Neutrophils and monocytes arrive at the site of injury at about the same time. Initially, neutrophils are present in greater numbers because they constitute a larger fraction of peripheral white cells. Both neutrophils and monocytes are attracted to the wound by chemotactic factors such as kallikrein, fibrinopeptides released from fibrinogen, and fibrin degradation products. Neutrophils are important in tissue debridement and in bacterial killing, events that generate further products of complement activation with inflammation and chemotactic

properties. The action of neutrophils is enhanced by integrins, cell surface receptors that facilitate cell-matrix interaction (Grinnell et al., 1992).

As the inflammation process continues, within 24 – 48 h after injury, monocytes replace neutrophils and become the predominant leukocytes. Monocytes are attracted to the injury site by kallikrein, fibrinopeptides and fibrin degradation products, as well as fragments of collagen, fibronectin, elastin, and TGF-β1 (Doherty et al., 1987). Beside undergoing a phenotypic change to tissue macrophages that kill bacteria and scavenge tissue debris, monocytes are critical for the progression of wound healing because they release several growth factors, including PDGF, fibroblast growth factor (FGF), TGF-β, and TGF-α, thereby stimulating migration and proliferation of fibroblasts, as well as production and modulation of extracellular matrix (Newman et al., 1982).

A few days after tissue injury, the remaining neutrophils are phagocytosed by tissue macrophages, and the first phase of wound healing comes to an end, whereas the second phase of proliferation and tissue formation in under way.

1.2.3.2 Proliferation or Migratory (re-epithelialization) Phase (Phase 2)

In this phase of wound repair, one is dealing with both cellular proliferation and migration, processes that are aided by a number of events, such as hypoxia, as well as specific adhesion proteins and extracellular matrix components. The stages in the inflammation phase have served a number of functions that will now allow and facilitate the proliferation of key resident cells, such as fibroblasts, the migration of endothelial cells and the process of neovascularization, and keratinocyte migration. Keratinocytes undergo a remarkable change in morphology and function. They migrate to the wound bed, release a numbers of proteins and enzymes that help facilitate their migration and other cellular functions, and ultimately reconstitute the damaged epidermis and basement

membrane (Grose et al., 2002; Kirfel and Herzog, 2004). The later stages of the second phase of wound repair feature the formation of granulation tissue, the reconstitution of the dermal matrix (fibroplasia) and the development of new blood vessels (angiogenesis). Fibroblast and endothelial cells undergo activation, phenotypic alteration, and migration much like keratinocytes do. Another critical background component of this phase is hypoxia, which begins immediately after injury and severance of blood vessels and has a profound impact on the migration and proliferation of fibroblasts and endothelial cells as well as, keratinocytes migration.

a) Hypoxia

Immediately following acute injury, the wound becomes temporarily hypoxic due to severance of blood vessels. Evidence points to low oxygen tension as an important early stimulus for fibroblast and endothelial cells activation, including synthesis of a number of growth factors, TGF-β1 (Falanga et al., 1991), endothelin-1 (Kourembanas et al., 1991), PDGF B chain (Kourembanas et al., 1990), and VEGF in endothelial cells (Shweiki et al., 1992). It appears that the effect of hypoxic conditions is mediated by hypoxia inducible factor 1 (HIF-1).

b) Fibroplasia

Fibroplasia refers to formation of granulation tissue and reconstitution of the dermal matrix. It is generally accepted that fibroblasts migrate into the wound; produce large amounts of collagens, proteoglycans, elastin, and other matrix proteins; and participate in wound contraction. Fibroblasts, like keratinocytes, undergo phenotypic changes that modify their interactions with the extracellular matrix, allowing them to perform a number of functions (Clark et al., 1982b). Fibroblasts begin to migrate into the wound 48 h after injury. They move along the fibrin-fibronectin matrix deposited in the initial clot, and they themselves produce fibronectin, which can facilitate their movement. The Arg-Gly-Asp

(RGD) tripeptide, which is common to these and other extracellular matrix proteinsm is important in the binding of these molecules to cell surface integrin receptors (Herard et al., 1996a). Fibroblasts produce other extracellular matrix components, including type I and III collagen, elastin, glycosminoglycans, and proteoglycans. Synthesis of type III collagen becomes maximal 5 to 7 days after injury. As new connective tissue is formed, some fibroblasts undergo a further phenotypic change to actin-rich myofibroblasts. These cells display features characteristic of fibroblasts and smooth muscle cells. Myofibroblasts are largely responsible for wound contraction and are prominently present in granulation tissue (Martin, 1997). Unlike other cells involved in the wound healing process, myofibroblasts undergo organized arrangements along the lines of contraction (Grinnell, 1994).

c) Angiogenesis

As with other processes of wound repair, new vessel formation occurs in the context of the changing of extracellular matrix. The chief cell of angiogenesis is the endothelial cell, which must undergo specific changes in order to migrate to the wound bed, proliferate, and direct new vessel formation. Migration of endothelial cells into the wound depends on chemotactic signals supplied by the extracellular matrix and neighboring cells. Many factors such as fibronectin, heparin, fibroflast growth factor (FGF-2) appears to be critical to stimulate both endothelial proliferation and migration (Clark et al., 1982a). Low oxygen tension in the early wound environment appears to potentiate angiogenesis (Knighton et al., 1983). In addition, cell-to-cell and cell-to-matrix interactions play a key role in determining endothelial cell invasion, migration, and proliferation. During wound repair, a number of adhesive proteins are expressed in the basement membrane zone of blood vessels, including vWF, fibronectin,

and fibrinogen, Studies have shown that several integrin receptors, especially αvβ3 the receptor for vWF, fibrinogen and fibronectin, are upregulated on the surface of smooth muscle cells and endothelail cells during angiogenesis (Brooks et al., 1994a).

d) Keratinocyte Migration

The process of re-epithelialization begins several hours after tissue injury. The events regulating keratinocyte migration are high heterogenous and yet interrelated (Schafer and Werner, 2007). These events have to do with changes in the shape of keratinocytes, in restructuring of their cytoskeleton and keratin expression, and in expression of proteases. Phenotypic alteration of keratinocyte enables them to migrate both from the wound edge and from any adnexal structures remaining in the wound bed. One to two days after the injury, epidermal cells at the wound edge and within the wound begin to divide and proliferate, thereby contributing to the population of migrating cells. Basal keratinocytes undergo a morphologic change from their normal cuboidal shape to a flattened cell with extended lamelipodia that project into the wound bed (Martin, 1997). Hemidesmisomes are retracted from the plasma membrane, and the number of gap junctions increases. Integrin receptor expression changes to facilitate keratinocyte movement on collagens, and kerotinocytes begin to synthesize and release type I and type IV collagenases.

The expression of new integrins clearly aids keratinocyte migration. The keratinocytes at the edges of the wound upregulate proteases to allow their migration in the context of a fibrin clot. The migration of keratinocytes over extracellular matrix as well as the provisional matrix is aided by the expression of new integrins, mainly the α5β1 and αvβ6 fibronectin/tenascin receptors and the αvβ5 vitronectin receptors (Huang et al., 2000; Grose et al.,

2002). Movement over collagen requires the α2β1 integrin. Crawling over these surfaces by the keratinocytes is achieved through morphologic changes and lamellopodial movement (Grose et al., 2002). The cytoskeleton is critical to these changes and to cell migration. Thus contraction of the actinomyosin filaments inserting into adhesion complexes results in and facilitates cell crawling (Grose et al., 2002).

The process of reepithelialization could not occur without the appropriate expression and action of enzymes needed to dissolve substrates and matrix materials for keratinocytes to migrate. Ultimately, these enzymes also play a fundamental role in tissue remodeling. Migrating keratinocytes upregulate tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) and its receptor (Romer et al., 1994). These enzymes are critical for the movement of keratinocytes through the fibrin clot. Other proteases are also important. The matrix metalloproteinases (MMPs) enzyme family comprises more than 20 different members, some of which, during the process of cutaneous repair, are very tightly expressed and regulated. Collagenase-1 (MMP-1), stromelysin-2 (MMP-10), and the 92-kDa gelatinase (MMP-9) are expressed by keratinocytes at the edges of the wound. MMP-1 is needed for keratinocyte migrating on type I collagen and is upregulated in keratinocytes at the very edge of the wound, after the cells have freed themselves from the basal lamina, a process that is aided by MMP-9. MMP-10 degrades a numbers of extracellular matrix proteins other than collagen. Other MMPs are spatially upregulated just proximal to the wound's edge (Saarialho-Kere et al., 2002).

1.2.3.3 Extracellular Matrix (ECM) Remodeling Phase (Phase 3)

In the third and final phase of wound repair, the formed tissue is degraded and remodeled, and cells undergo apoptosis and other drastic changes, such as keratinocyte differentiation and myofibroblast transformation. Typically, this phase can last for several months (Martin, 1997). Although the functional outcome is not ideal, in that tissue regeneration has not occurred, the remodeling process allows the host to develop a stable scar that has approximately 70% of the original strength. In many ways, this phase of wound repair resembles many other unrelated physiological processes in which the initial outcome is exaggerated (Grose and Werner, 2003). Thus, during the process of wound repair, there is probably an overabundance of cellular migration and proliferation and even excessive deposition of many types of extracellular matrix components. This "exaggeration" of the repair response is important in ensuring a proper inflammatory reaction and clearing of bacteria, wound debridement, and removal of necrotic tissue. Fibroplasia and angiogenesis are critical to the development of the appropriate wound bed required for keratinocyte migration and reepithelialization. However, the abundance of cells and extracellular matrix must now be dealt with and a remodeling process is required for downregulating the response and returning to an approximation of the prewounded state. Apoptotic mechanisms and the enzymatic activity of MMPs and other proteases will be at work to achieve a balance within a newly reepithelialized wound.

Events of remodeling are closely linked to those which have allowed the deposition of certain extracellular matrix components and migration of cells to the wound site in the first place. The reason for this is that no remodeling can take place unless the primary stimulatory signals are turned off. However, the third phase of wound repair is not as well studied as the early two processes, and therefore many more questions remain. In addition, the remodeling phase does not occur homogeneously within the wound, either in location or in time. In the context of the remodeling phase of wound repair, the

following events and key extracellular matrix components will be discussed in turn: (a) fibronectin and associated components, (b) hyaluronic acid and proteoglycans, (c) collagen, and (d) myofibroblasts and contraction.

a) Fibronectin and Associated Components

Fibroblasts produce fibronectin as they enter the wound site, and at least initially, the concentrations of fibronectin are very high. By 4 to 5 days after injury, the fibronectin network is well established (Welch et al., 1990). Fibroblasts leave their collagen-rich environment to enter the wound site by modifying their behavior and characteristics, including become activated and modify their integrin repertoire, which will dictate what they bind to and where they will go. There is directionality to the deposition of fibronectin, which is important in the way other extracellular matrix proteins are laid down. Ultimately, during remodeling and contraction, myofibroblasts will make use of the fibronectin network to cause wound contraction. The deposition of ECM-associated moleculoes, such as tenascin, secreted protein acidic and rich in cysteine (SPARC), thrombospondin (TSP), and dermatan sulfate influences and modifies the overall ECM makeup (Bornstein, 2000). For example, SPARC upregulates fibroblast expression of MMPs, especially collagenases, whereas thrombospondin and tenescin have both adhesive and antiadhesive properties. Eventually, the fibronectin-rich environment of the initial wound is modified and remodeled by cell and plasma proteases. Over time, fibronectin is replaced by type III collagen and ultimately by type I collagen.

b) Hyaluronic Acid and Proteoglycans

The glycosaminoglycan (GAG) hyaluronic acid (hyaluronan) is also an abundant component of the provisional matrix and one whose deposition needs to be modified during remodeling process (Laurent and Fraser, 1992).

Fibroblasts from early granulation tissue produce large amounts of hyaluronic acid. Beside the issue of offering less resistance to cell movement, hyaluronic acid may stimulate cell motility by altering cell-matrix adhesion by weakening the adhesion of heparin sulfate and fibronectin. Eventually hyaluronic acid is degraded by hyaluronidase and replaced by sulfated proteoglycans, which contribute a stronger structural role in late granulation tissue formation and in scars while being less able to stimulate cellular movement (Lawrence, 1998).

c) Collagen

Three main classes of collagens are present normally in connective tissue: fibrillar collagen (types I, III and IV), basement membrane collagen (type IV), and other interstitial collagens (type VI, VII and VIII). During the initial phases of wound repair, it appears that the wound tends to recapitulate the processes involved in embryogenesis. Thus granulation tissue is initially comprised of large amount of type III collagen, which is a minor component of adult dermis and indeed is present in larger amounts in fetal wound repair (Singer and Clark, 1999). During the phase of remodeling, over a period of a year or more, type III collagen is gradually replaced by type I collagen, Type I collagen replacement is associated with increased tensile strength of the scar (Greenhalgh, 1998). However, the final tensile strength of a scar is only about 70% of that of preinjured skin. The process of converting the collagen content of the dermis from type III to type I collagen is controlled by interactions involving synthesis of new collagen with degradation of old collagen. Keys to this process of conversion are MMPs and specifically the collagenases.

d) Myofibroblasts and Contraction

During the earlier stages of wound repair, beginning around 4 days after injury, granulation tissue forms (Martin, 1997). Granulation tissue is so named based on

its granule-like appearance, which is rich in vascular structures. In the more mature wound and during remodeling, this granulation tissue has to be modified. One of the primary events is wound contraction. There are different degrees of contraction depending on the host, the location of the wound, and its depth. The utmost importance to the development and extent of contraction is wound depth. Partial-thickness wounds, down only to papillary dermis, heal with less scarring and contraction. Full-thickness wounds, which encompass the fat and subcutaneous tissue, tend to heal with much more scarring and rely on contraction for faster repair (Hinz et al., 2001). Nonetheless, the mass of tissue that has formed in the earlier phases of wound repair must now be made smaller, and contraction is an effective and dependable way of doing so. The myofibroblast plays a critical role in the contraction process (Grinnell, 1994).

Closure of the wound is of paramount important from a survival and evolutionary standpoint, and the outcome of repair reflects this priority and is a compromise between functional and structural needs. The barrier integrity of epithelial cells is restored after migrating cells have covered the denuded wound area. Aberrant wound healing results in chronic inflammatory diseases, tumor formation and metastasis (Prud'homme, 2007). Hence, understanding the precise molecular mechanism of wound repair regulation *in vivo*, especially in the cell migration, is important in developing effective therapeutic strategies and wound management.

1.2.4 Cell Migration

Re-epithelialization of the wound involves the proliferation and directed migration of keratinocytes from the wound edge, followed by their stratification and re-differentiation to form an intact epithelium. These pivotal events of wound repair through re-epithelialization of keratinocytes at the wound margin have been shown in various *in*

vivo and *in vitro* models (Herard et al., 1996b). Cell migration is a multistep process involving cell-substrate adhesion and changes in the cytoskeleton. There are two main theories as to how the cell stretches forward at the front edge, namely (A) the cytoskeletal model and (B) the membrane flow model (Figure 1.3). In the cytoskeletal model, soluble actin monomers undergo rapid polymerization to form filaments at the leading edge of the cells to produce the main motile force for advancing the cells (Pollard and Borisy, 2003). In the membrane flow model, studies have shown that the membrane on the front side of a cell is returned to the cell surface from internal membrane pools at the end of the endocytic cycle (Bretscher, 1996). This has led to the view that extension of the leading edge occurs due to exocytosis. Leading edge keratinocytes are guided towards the fibrin, fibronectin, and collagen bundles present beneath the desiccated blood clot, which they sense and adhere to via the display of specific integrin receptors on their surface (Coulombe, 2003). The actin filaments that form at the front might stabilize the extended membrane through an ECM-integrin-cytoskeleton connection, so that a structured extension, or lamella, is formed, rather than the cell directing membrane vesicles towards its anterior edge (Giannone and Sheetz, 2006).

The keratinocytes migrate as an epithelial sheet, where keratinocytes are released from cell-cell contact inhibition at the front of the wound while maintaining cell-cell contact at the rear. The mechanism by which epithelial and other strongly cell-cell adhesive cells migrate collectively as continuous sheets is not clear. However, emerging evidence suggest that the release of cell-cell contact inhibition marks the front of the migrating sheets. Further work also showed that the detection of chemoattractants at the migrating front results in an internal polarized response of accumulation of phosphatidylinositol-3,4,5-triphosphate (PIP3) at the pseudopodia of the migrating keratinocytes. In this respect, peroxisome proliferators-activated receptor β/δ (PPARβ/δ) has been shown to play important role in the directed migrating of the keratinocytes, by

regulating the intercellular communication at the organism level but participating in cell responses to a chemotactic signal through promoting integrin recycling and remodeling of the actin cytoskeleton (Nobes and Hall, 1999; Merlot and Firtel, 2003; Tan et al., 2007).

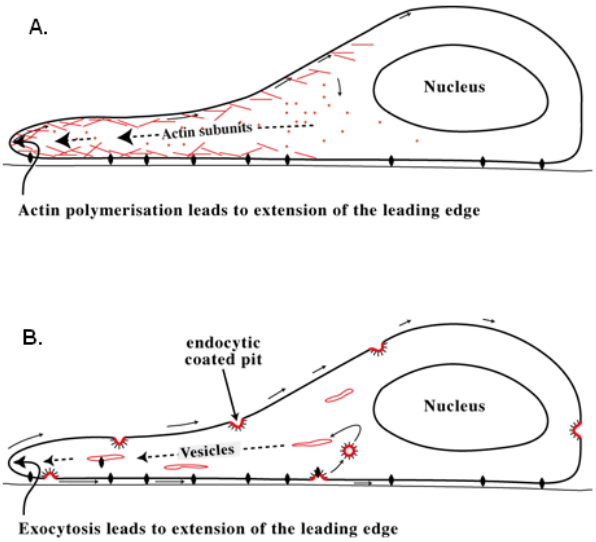

Figure 1.3 Two schemes for particle migration on motile cells. In both schemes, the cells are moving to the left. (A) Polarized actin cycle in which actin filaments polymerize at the leading edge, pushing the cell's front forward to extend it. Actin depolymerization ahead of the cell's nucleus generates actin subunits (g-actin) which diffuse to the cell's front for reuse. Filamentous actin, moving rearward, pulls tines (small arrows) and associated particles on the cell surface towards the cell's rear. Particles or patches often migrate with actin, although this is not always the case. (B) Polarized endocytic cycle in which exocytosis at the leading edge extends the cell surface, bringing a fresh source of substratum attachments with it. Endocytosis by coated pits occurs randomly on the cell surface, generating a lipid flow in the plasma membrane which decreases with increasing distance from the leading edge: this is indicated by the decreasing size of the arrows along the plasmalemma. This flow pushes on substratum-attached feet, providing a fluid drive to advance the cell. Particles or patches on the cell surface are swept towards the back of the cell, where rearward movement due to flow is balanced by random movement due to Brownian motion. (Taken from (Bretscher, 1996)).

1.2.5 Secreted Factors Affecting Cell Proliferation and Migration during Wound Repair

1.2.5.1 Growth Factors and Cytokines

The multitude of complex biological processes that occur during the different stages of wound repair is regulated spatio-temporally by the concerted actions of numerous growth factors and cytokines. Cell proliferation and migration are

indispensable during re-epithelialization for the rapid reconstitution of a cohesive epithelial structure. The variety of these signals and their effects on wound repair has been well studied (Werner and Grose, 2003). It is clear that epithelial-mesenchymal communication is essential for the proper production of these cytokines. For example, keratinocyte-derived interleukin-1 (IL-1) stimulates fibroblasts to produce growth factor keratinocyte growth factor KGF/fibroblast growth factor-7 (FGF-7), which in turn stimulates keratinocytes proliferation proliferation (Werner et al., 2007). On the other hand, growth factors like TGF (transforming growth factor) α and β exert opposing effects on cell proliferation and migration. Thus, KGF potentiates keratinocyte growth, while TGF-β exerts potent growth inhibitory action on epithelial cells. Studies have shown that crosstalk among different signaling cascades can result in the dominance of certain pathways culminates in appropriate cell responses (Tan et al., 2005).

Platelet-derived growth factors (PDGFs) and granulocyte-macrophage colony stimulating factor (GM-CSF), on the other hand, can stimulate cell migration of different cell types either by inducing chemokinesis, i.e., migratory reactions with random orientation, or chemotaxis, i.e., directional migrations following a gradient of growth factor concentrations through transmembraneous protein receptors on the cell surface (Nickoloff et al., 1988).

1.2.5.2 Extracellular Matrix

The ECM is a complex and dynamic structural network that serves as a stabilizing element for all multicellular organisms, to ensure their functional integrity and to mediate mechanical forces. The ECM is composed of structural proteins, proteoglycans, latent and active growth factors and matricellular proteins. The structural and functional diversity of different ECM proteins are predicted by site-specific biosynthesis of their structural components, such as vitronectin, fibronectin, collagen, laminin and elastin.

During the entire wound repair process, changes in the ECM composition, among others, would have a direct effect on cell-matrix communication and consequently on the behavior of the epithelial cells. Vitronectin is a glycoprotein with diverse distribution and functions. In the plasma, vitronectin has been found in complex with members of the complement cascade as well as the coagulation cascade (Preissner, 1991). In the tissues, vitronectin is found in the ECM, associated with angiogenesis (Brooks et al., 1994b) and tumor progression (Gladson and Cheresh, 1991), which suggests it has a role in directed cell migration. Fibronectin is a dimeric glycoprotein that interacts strongly with other components of the ECM and is involved in a number of biological processes, such as cellular adhesion, motility, differentiation and homeostasis. It has also been shown to play an important role in wound healing because it appears to be the stimulus for the migration of epidermal cells (Donaldson and Mahan, 1983; Takashima et al., 1986). Overall, the distribution and role of different ECM proteins in wound healing have been extensively studied, but the exact mechanism in wound healing is poorly known.

Within a single tissue, the ECM is constantly being remodeled as cells build and reshape the ECM by degrading and reassembling it; remodeling rates are particularly high during development and wound repair (Daley et al., 2008). The ECM can be remodeled in response to signals transmitted by ECM receptors – such as integrins, other laminin receptors and syndecans – or ECM-modifying proteins, such as matrix MMPs (Page-McCaw et al., 2007). For example, integrin-mediated assembly of the ECM glycoprotein fibronectin into fibrils is required for the deposition of other ECM proteins, including type I collagen and matricellular protein thrombospondin I (Sottile and Chandler, 2005). Proteolytic cleavage of ECM components represents another major mechanism by which the ECM is remodeled. Proteases, including those in the MMP, serine protease (e.g. plasmin, plasminogen activator and uPAR) and cysteine protease (e.g. cathepsins) families, influence matrix dynamics at multiple levels. For example, to

convert structural molecules to signaling molecules, proteases release small bioactive peptides, release growth factors stored within the ECM and degrade matrix proteins directly (Heissig et al., 2002). In addition, intact and proteolytic fragments of ECM may have different signaling properties and can affect cells differently. For instance, degraded collagen fragments lose the ability to maintain focal adhesion points, and promote the rapid disassembly of focal adhesion for cell migration (Carragher et al., 1999), while degradation of laminin 5 by matrix metalloproteinases generates fragments that promote cell migration (Giannelli et al., 1997).

1.2.5.3 Matricellular Proteins

Matricellular proteins, such as SPARC, TSP, tenascin and osteopontin, belong to a group of matrix-associated factors that modulate cell-matrix communications (Bornstein and Sage, 2002). Matricellular proteins do not serve a primarily structural role, in the sense that most collagens, laminins, and elastin are structural proteins. Rather, the complex nature of their function derives from their ability to interact with multiple cell-surface receptors, cytokines, growth factors, proteases, and structural proteins. The contextual nature of their function thus reflects the composition of the matrix, the availability of cytokines and proteases, and the expression of integrins and other receptors in a given cellular environment (Murphy-Ullrich, 2001). They are expressed when tissues undergo events that require tissue renewal, tissue remodeling or embryonic development. In contrast to the severe or lethal phenotype of mice null for structural matrix proteins, mice that have a targeted disruption of genes encoding matricellular proteins appear normal or have subtle phenotypes (Bornstein and Sage, 2002). In spite of the importance of matricellular proteins during wound repair, there is a lack of information about their roles and mechanism of action when compared to growth factors and ECM proteins (Werner and Grose, 2003; Midwood et al., 2004). The interaction of

integrin receptors with specific matricellular proteins appears to initiate a signal transduction pathway that involves activation of the intracellular protein Rho family of GTPase (Clark et al., 1998). Activation of Rho GTPases, including cdc42, Rac1 and RhoA, dramatically affects cell migration through cytoskeletal reorganization events.

1.2.5.4 Adipocytokines

Adipose tissue not only serves as a passive reservoir for energy storage, it also produces and secretes copious levels of a variety of bioactive molecules called adipocytokines, including leptin, tumor necrosis factor-α, resistin and plasminogen activator inhibitor type-1 (PAI-1) (Shimomura et al., 1996), that have both systemic and local effects on wound repair. The dysregulated production of adipocytokines is associated with the pathophysiology of obesity-related metabolic diseases (Hotamisligil et al., 1993). Recent works shows that some of these adipocytokines have a profound impact on wound healing. Leptin is a hypoxia-inducible pleiotropic adipocytokine known to participate in multiple cellular and physiological processes. Leptin is predominantly produced by white adipocytes and secreted into the bloodstream (Zhang et al., 1994). It is structurally related to cytokines and acts on receptors that have considerable homology to class I cytokine receptors (Tartaglia et al., 1995). The main effect of leptin in the human body lies in the regulation of energy homeostasis, especially under conditions of restricted energy availability. Circulating leptin is also involved in glucose and lipid metabolism, angiogenesis, blood pressure regulation, bone mass formation and wound healing processes. Leptin acts as a potent angiogenic and mitogenic stimulus to endothelial cells and keratinocytes during skin repair (Frank et al., 2000). Leptin is acutely upregulated in injured skin and it is proposed that local production of leptin serves a critical functional role as an autocrine/paracrine regulator of the normal wound healing process (Murad et al., 2003). Interestingly, the systemic and topical application

of leptin has been demonstrated to accelerate the wound healing process in wild type mice, suggesting a fundamental role during wound repair (Stallmeyer et al., 2001). However, its importance during wound healing under physiological circumstances and its mechanism of action remain unclear.

1.2.6 Integrins

Integrins are transmembrane receptors which promote both cell-cell and cell-ECM adhesion. They are heterodimers of non-covalently-bound α and β subunits that show a wide range of tissue distribution and play key roles in developmental, physiological and pathological processes. Their ligand specificity relies on a combination of both α/β subunits (18 different α integrins and 8 different β integrins). Ligand binding to the receptor extracellular domain induces a conformational change that is propagated to the cytoplasmic domain and initiates downstream signaling events related to cytoskeletal rearrangements. The major integrins in the epidermis are $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$ and $\alpha_v\beta_5$ (Kirfel and Herzog, 2004). Intracellular communication with the external environment can be triggered by receptor/ligand interaction. Receptor activation in response to ligand binding leads to changes in target gene expression that ultimately modulates cellular processes. Such cellular processes can also be influenced by intracellular signaling facilitated via various signaling networks. As a result, integrins are involved in many fundamental cellular functions, including proliferation, adhesion, motility, differentiation, survival, and apoptosis (Kirfel and Herzog, 2004).

1.2.7 Cell-Matrix Interaction

Cell-matrix interaction plays a crucial role throughout the entire wound repair process. After wounding, keratinocytes at the wound edge come into contact with dermal collagens I and III and with fibrin clot constituents fibrin, fibronectin, and vitronectin.

Keratinocytes migrate over the wound bed via an interaction between integrin and its cognate ECM. In the skin, the keratinocytes migrate via an interaction between cell surface receptor integrin and its cognate matrix proteins. The binding of integrin to matrix proteins induces a conformational change that is propagated to the cytoplasmic domain and initiates the activation of intracellular signaling pathways (Hynes, 2002). Simultaneously, the expression profile of integrins on the keratinocytes at the wound margin changes, characterized by the induction of specific integrins that bind proteins of the dermal matrix and specific components of the fibrin clot that act as a provisional matrix during wound repair (Grinnell, 1992).

During the entire wound repair process, changes in the ECM composition, among other changes, would have a direct effect on cell-matrix communication and consequently on the behavior of the epithelial cells. The ECM is composed of structural proteins, proteoglycans, latent and active growth factors, and matricellular proteins. Residing at the crossroads of cell-matrix communication, matricellular proteins serve a regulatory, instead of a structural, function.

1.2.8 Focal Adhesion Kinase (FAK) in Cell Migration

Engagement of integrin receptors with their cognate ECM ligands leads to the formation of well-defined structures linking the ECM and cytoplasmic actin cytoskeleton. In tissue culture cells, points of adhesion, the focal adhesions, are localized to the ends of actin stress fibers (Craig and Johnson, 1996). Sites of adhesion in motile cells, the focal complexes within filopodia and lamellipodia, are relatively small and transient. Collectively, they are called cell-matrix adhesion complexes (CMACs). Signal transduction through CMACs has been implicated in the regulation of a number of key cellular processes, including growth factor-induced mitogenic processes, cell survival and cell locomotion (Schwartz and Ingber, 1994). The dynamic regulation of cell

adhesion is of particular importance when cells move in response to a stimulus, such as cytokines, growth factors or wounding. Newly generated focal complexes formed at the leading edge of the cell provide new adhesion contacts and thus provide 'directionality' to cell movement; while existing focal adhesions at the rear dissociate, internalize and recycle to the leading edge to form new focal adhesions (Parsons et al., 2000). Coordinated regulation of the formation and turnover of adhesion complexes is central to how cells move in response to different signals.

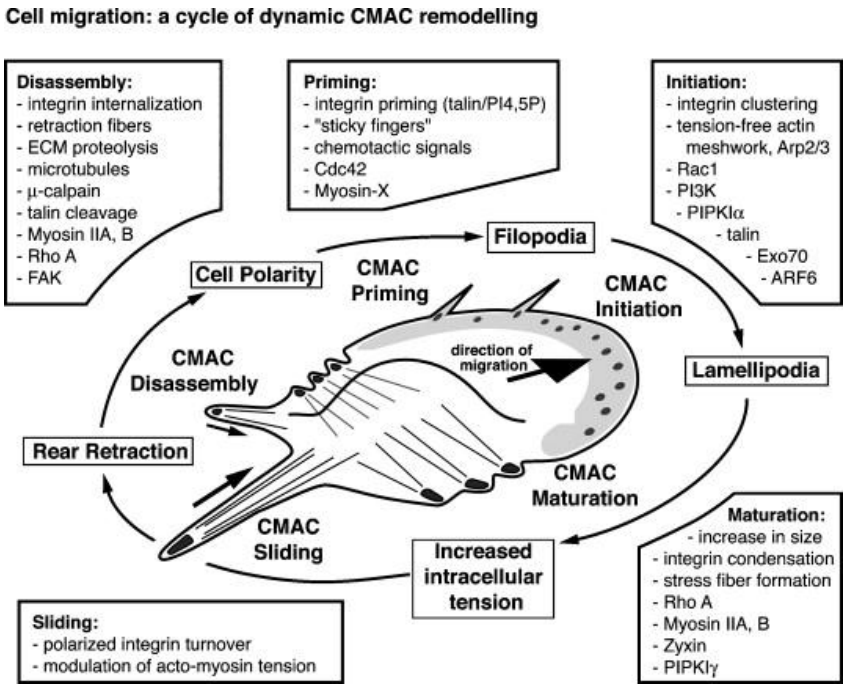

Figure 1.4 Cell migration — a cycle of dynamic CMAC remodeling. This graphical representation of a migrating cell highlights protruding and retracting features, such as filopodia, lamellipodia and retraction fibers, respectively. Different types and states of cell-matrix adhesion complexes (CMACs) are represented by dark dots or elongated shapes, either embedded within the lamellipodial actin meshwork (grey crescent) or attached to stress or retraction fibers (black lines). Each different CMAC state is described by an adjacent text box (refer to the text for more detailed descriptions). Please note that the different CMAC states are dynamically linked to morphological (filopodia, lamellipodia, rear retraction) or cellular features (increased intracellular tension, cell polarity) (Taken from (Lock et al., 2008)).

Clustering of integrins during the formation of new focal adhesions leads to the rapid recruitment and phosphorylation of focal adhesion kinase (FAK) (Parsons et al., 2000). FAK is a nonreceptor protein tyrosine kinase that is involved in signal transduction from integrin-enriched focal adhesion sites that mediate cell contact with the matrix proteins (Schlaepfer and Mitra, 2004). These "activated" FAKs further recruit Src family kinases to form a FAK-Src complex, which is the central "signaling hub" of downstream signaling pathways that control cell spreading, migration and survival (Hynes, 2002; Schlaepfer and Mitra, 2004). For example, PAK kinases appear to be important regulators of cytoskeleton rearrangements and lamellipodia formation (Sells et al., 1999), while PI3 kinase (PI3K) has been shown to be directly activated by FAK (Parsons et al., 2000). Furthermore, elevated FAK expression and activation have been correlated with progression to highly malignant and metastatic tumor phenotypes (Hecker and Gladson, 2003). Integrins can also cooperate with specific growth factor receptors to activate non-FAK-dependent pathways, such as the 14-3-3, phosphatidylinositol 3-kinase, mitogen-activated protein kinase and PKC-mediated pathways.

1.2.9 The Molecular Role of 14-3-3

Members of the 14-3-3 protein family form a group of highly conserved 30kDa acidic proteins expressed in a wide range of organisms and tissues. The five major isoforms in mammalian brains are $\alpha - \eta$, while α and δ are the phosphoforms of β and ζ, respectively. τ and σ are expressed in T cells and epithelial cells, respectively. 14-3-3 is now established as a family of dimeric proteins that can modulate interaction between proteins (including oncogene products of polyoma, Raf-1, AKT, and Bcr-Abl). They are involved in cell signaling, regulation of cell cycle progress intrecellular trafficking/targeting, cytoskeletal structure and transcription (Aitken, 2006). The

regulation of interaction usually involves phosphorylation of the interacting protein and in some cases the phosphorylation of 14-3-3 isoforms themselves may modulate interaction.

14-3-3 is expressed in all eukaryotic cells that bind to a plethora of client proteins, including kinases, phosphatases, and other signaling proteins, and transcription factors as ‘adapter proteins’ (Aitken, 2006; Porter et al., 2006). By mediating these effects in a diverse array of clients, 14-3-3 controls signaling transduction, intracellular trafficking/targeting, cell cycle progression, metabolism, cytoskeletal structure, oncogenesis, and apoptosis (Fu et al., 2000). Functioning as a phosphoserine/phosphothreonine binding protein, 14-3-3σ proteins involved in the regulation of apotosis through multiple interactions with proteins of the core mitochondrial machinery, pro-apoptotic transcription factors, and their upstream signaling pathways (Zha et al., 1996). 14-3-3 coordinates with survival kinases to inhibit multiple pro-apoptotic molecules (Tsuruta et al., 2004). one prominent mechanism for the suppression of apoptosis is through 14-3-3-mediated sequestration of pro-apoptotic client proteins (Samuel et al., 2001). On the other hand, cellular stresses appear to signal through the inhibition of 14-3-3 function to exert their pro-apototic effect. Global inhibition of 14-4-3/client protein interaction induces apoptosis. 14-3-3 also participates in phosphorylation-dependent protein-protein interactions that control progression through the cell cycle (Chan et al., 1999), initiation and maintenance of DNA damage checkpoints (Samuel et al., 2001), activation of the Ras-Raf-MAP signaling pathways (Clark et al., 1997), and coordination of integrin signaling and cytoskeletal dynamics (Fu et al., 2000).

The 14-3-3σ, whose original name was stratifin, is specifically expressed in the stratified epithelial cells, although other isoforms are also found in the keratinocytes (Kilani et al., 2008). The isolation and identification of 14-3-3σ as an intracellularcellular

keratinocyte-derived stimulator of protein kinase C-mediated phosphorylation as also been reported (Robinson et al., 1994; Dellambra et al., 1995; Van Der Hoeven et al., 2000). The secreted form of 14-3-3 from the keratinocytes targets fibloblast production of MMPs (Ghahary et al., 2004; Ghahary et al., 2005) and fibroblasts influence the expression profiles of 14-3-3 in keratinocytes (Medina et al., 2007; Carr et al., 2011). Recent work has demonstrated 14-3-3σ is a crucial regulator for skin proliferation and differentiation (Li et al., 2005), as well as interaction with an intermediate filament protein, keratin 17, to regulate keratinocyte cell growth during wound repair (Kim et al., 2006). In addition, 14-3-3 is shown to interaction with integrin β1 to regulate keratinocyte migration (Han et al., 2001; Santoro et al., 2003).

1.2.10 Signal Transduction through Integrin upon Cell-Matrix Interaction

Numerous effects of integrin ligation are mediated by the canonical focal adhesion kinase (FAK)-dependent pathway. Focal adhesion kinase is a nonreceptor protein tyrosine kinase involved in signal transduction from integrin-enriched focal adhesion sites that mediate cell contact with the ECM. The multiple protein-protein interaction sites allow FAK to associate with adaptor and structural proteins to modulate the activities of mitogen-activated protein (MAP) kinases, stress-activated protein kinases and small GTPases (Schlaepfer and Mitra, 2004). The FAK-enhanced signals have been shown to mediate the survival of anchorage-dependent cells and are critical for efficient cell migration in response to growth factor receptor and integrin stimulation (Schlaepfer et al., 2004). Integrins can also cooperate with specific growth factor receptors to activate non-FAK-dependent pathways such as the phosphatidylinositol 3-kinase, mitogen-activated protein kinase, 14-3-3 and PKC-mediated pathways (Shaw et al., 1997; Fu et al., 2000; Dellambra et al., 1995; Mercurio et al., 2001; Dans et al., 2001). Of these, 14-3-3σ–mediated pathway has one of the broadest effects (Fu et al., 2000). 14-3-3 proteins

act as adaptor proteins in many signaling cascades, mediate intracellular communication and regulate cellular homeostasis, protein synthesis, apoptosis and differentiation via effector proteins, such as protein kinases (Porter et al., 2006; Fu et al., 2000). The integration of the FAK-dependent or independent signaling pathways results in changes in target gene expression and culminates in the appropriate cellular responses, such as migration, proliferation and differentiation.

Keratinocytes migrate via an interaction between integrin and its cognate ECM. Intracellular communication with the external environment can be triggered by the receptor/ligand-binding activity. Receptor activation in response to ligand binding leads to changes in target gene expression which ultimately modulates cellular processes. Such cellular processes can also be influenced by intracellular communication facilitated via various signaling networks. It was recently shown that PPARβ/δ potentiates keratinocyte directional sensing and movement towards a cue such as the EGF, as well as the activity of AKT1/GSK-3β and Rac1/cdc42 pathways (Tan et al., 2007).

1.2.11 Integrin Internalization and Recycling during Cell Migration

Integrins on the cell surface are well suited to function as biosensors that constantly interrogate the wound environment and modulate cell responses accordingly. Cell adhesion, migration and the maintenance of cell polarity during cell migration are all processes that depend on the correct targeting of integrin and the dynamic remodeling of integrin-containing adhesion sites. The binding of an integrin to its cognate matrix protein activates intracellular signaling pathways to modulate a broad range of cellular processes, including cell migration (Caswell and Norman, 2008). The importance of the integrin endo/exocytic cycle as a key regulator of these functions is increasingly recognized. Ligand-activated integrins are continuously internalized from the plasma membrane into endosomal compartments and recycled back to the cell surface (Caswell

and Norman, 2006). Trafficking receptors for exocytosis at the advancing edge assists cell locomotion withunliganded adhesion receptors which are provided by internalized endocytosis at the retracting end of the cell (Caswell and Norman, 2006). It is well established that integrin recycling contributes to the motility of rapidly migrating cells, such as wound keratinocytes, and permits constant monitoring of the wound cellular environment. For example, the fibronectin receptor of CHO cells (integrin $\alpha5\beta1$) is constantly endocytosed and recycled, rather than degraded (Bretscher, 1989). It is also well known that integrins regulate matrix turnover by endocytosis (Jones et al., 2006). Integrin $\alpha v\beta5$ is internalized in an active, vitronectin-bound form, and recycled back to the membrane; the vitronectin however, is targeted for degradation (Panetti et al., 1995). Endocytosed $\beta1$ integrins have been shown to remain in active conformation and colocalize with fibronectin and collagen (Ng et al., 1999), which suggests that integrin traffic provides the cell with a constant supply of 'refreshed' receptors that can bind ligands. It is also evident that the recycling process is selective, with certain heterodimers being cycled rapidly, while others remained at the plasma membrane (Bretscher, 1992; Roberts et al., 2001). Effective directed cell migration is possible only through constant interaction with and modification of the microenvironment during wound healing. However, the extracellular factors and mechanisms that provide such selectivity remain unclear.

1.2.12 Angiopoietin-Like Protein Family

In recent years, a family of proteins found to be akin in structure to angiopoietins has been identified (Figure 1.5). This family was named angiopoietin-like (ANGPTL) proteins, and stand at seven members, ANGPTL1 to 7. The ANGPTL and angiopoietin families share common structural components, an N-terminal coiled-coil domain an a large fibrinogen-like C-terminal domain (Kim et al., 1999b; Kim et al., 1999a; Kersten et

al., 2000; Kim et al., 2000; Yoon et al., 2000; Camenisch et al., 2002; Ito et al., 2003; Oike et al., 2005a). Although these two protein families have structural similarities, ANGPTLs unlike angiopoietins, are not able to bind to the TIE1 or TIE2 receptor tyrosine kinases, most members show angiogenic effects. This binding property is dependent on the fibrinogen-like C-terminal domain, in which only four out of the six common cysteine residues have been conserved in ANGPTLs (Oike et al., 2004a; Oike et al., 2004b). This sequence disparity might account for their distinct inabilities to bind TIE1 and TIE2 receptors as well as to serve different functions from angiopoietins. Thus, ANGPTL4 are presently considered orphan ligands (Kim et al., 1999b; Kim et al., 1999a; Kersten et al., 2000; Kim et al., 2000; Camenisch et al., 2002; Shimizugawa et al., 2002; Ito et al., 2003; Le Jan et al., 2003b; Yoshida et al., 2004). ANGPTLs also contain a highly hydrophobic region at its N-terminal, which is similar to that of a signal sequence for protein secretion. In addition, it has been determined that ANGPTLs exists as glycoproteins.

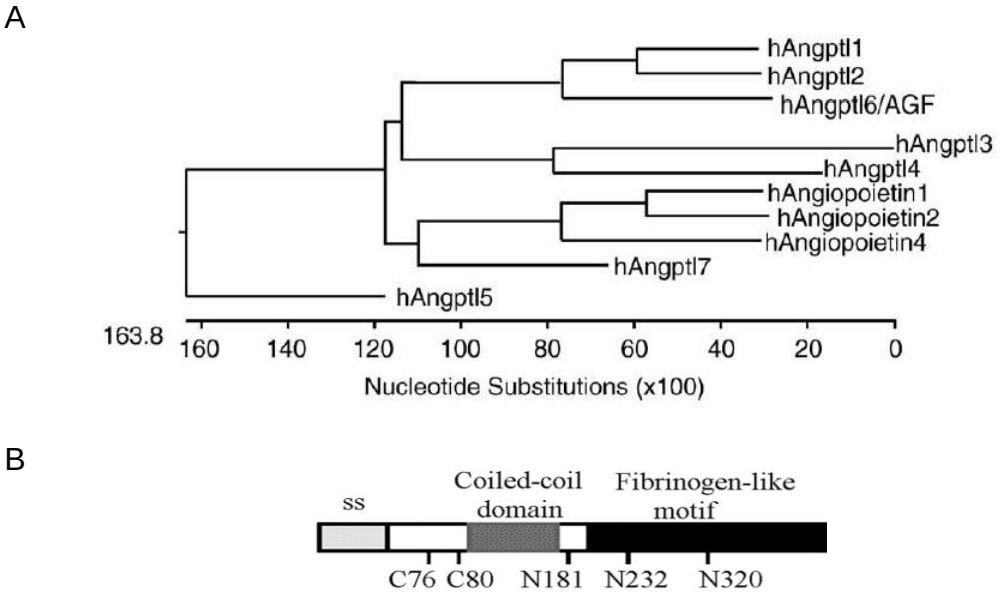

Figure 1.5 Phylogenetic of ANGPTLs and Angiopoietin and their general structure. (**A**) Phylogenetic clustering of human ANGPTLs and Angiopoietins. There are seven ANGPTLs and 4 Angiopoietin family members. Phylogenetic trees for ANGPTLs and angiopoietins. ANGPTLs have seven family members. ANGPTLs have been identified in both humans and mice, except for Angptl5, which is limited to humans. Angiopoietins (shown as Angs in the figure) contain four family members. Although Angiopoietin1 (Ang1) and Angiopoietin2 (Ang2) have been identified in both humans and mice, Angiopoietin3 (Ang3) and Angiopoietin4 (Ang4) have been identified only in mice and humans, respectively. (**B)** Schematic diagram of the structure of angiopoietin-like proteins, consisting of a signal sequence (ss), a coiled-coil and fibrinogen-like domain. (Taken from (Hato et al., 2008)).

Several lines of *in vivo* and *in vitro* evidence have pointed that ANGPTLs do function to modulate angiogenesis (Table 1.1). ANGPTL1 and ANGPTL2 have been Initially reported to exhibit significant but weak endothelial cell sprouting activities *in vitro* (Kim et al., 1999a; Kim et al., 1999b). Later, it was reported that ANGPTL1 inhibited VEGF-induced angiogenesis, and it was thus named "Angioarrestin" (Dhanabal et al., 2002). ANGPTL1 and ANGPTL2 have been shown to exhibit antiapoptotic activity (Kubota et al., 2005a; Kubota et al., 2005b). ANGPTL3 is shown to stimulate adhesion and migration of endothelial cells as well induces blood vessel formation (Camenisch et al., 2002). ANGPTL6 promotes angiogenesis (Oike et al., 2004a; Oike et al., 2004b; Zhang et al., 2006), while ANGPTL7 is shown to reduce aberrant blood

vessel formation by inducing massive fibrosis in a mouse xenograft model (Peek et al., 1998; Peek et al., 2002).

Table 1.1 Biological effects of ANGPTLs on angiogenesis and metabolism.

	Angiogenesis	**Vascular permeability**	**Hypoxia induction**	**Metabolism**
ANGPTL1	Pro/Anti	NR	No	NR
ANGPTL2	Pro	Enhance	Yes	NR
ANGPTL3	Pro	NR	No	Inhibit LPL activity and increase serum TG levels
ANGPTL4	Pro/Anti	Suppress	Yes	Inhibit LPL activity and increase serum TG levels
ANGPTL5	NR	NR	NR	NR
ANGPTL6	Pro	Enhance	Yes	Increase energy expenditure
ANGPTL7	Anti*	NR	NR	NR

NR : No record; Pro : proangiogenic; Anti : antiangiogenic; TG : triglyceride; LPL : Lipoprotein lipase.
* information from (Peek et al., 1998; Peek et al., 2002). (Taken from (Hato et al., 2008)).

ANGPTL-family members have also been found to be regulators of metabolism (Hato et al., 2008). At least four ANGPTLs (ANGPTL2, 3, 4 and 6) have been shown to have pronounced effects on energy metabolism. ANGPTL2 is a key mediator of chronic adipose tissue inflammation and obesity-related systemic insulin resistance (Tabata et al., 2009). It is primarily secreted by adipose tissue and its expression at both the mRNA and protein levels is increased by obesity and obesity related conditions, including hypoxia. Plasma ANGPTL2 levels are correlated to inflammation, adiposity and systemic insulin resistance. ANGPTL2 also activates an inflammatory cascade and induces chemotaxis of monocytes and macrophages in endothelial cells (Shoji et al., 2009).

The importance of ANGPTL3 for lipid metabolism was first indicated by the genetic analysis of a mutant strain of obese mice with low plasma lipid levels (Koishi et al., 2002). Administration of recombinant ANGPTL3 to ANGPTL3-deficient as well as wild type mice increased the plasma levels of triacylglycerol, non-esterified fatty acid and cholesterol (Koishi et al., 2002). ANGPTL3 decreases the clearance of very-low-

density-lipoprotein by inhibiting lipoprotein lipase (Shimizugawa et al., 2002), but able to activate lipolysis upon direct binding to adipocytes (Ono et al., 2003; Shimamura et al., 2003). ANGPTL3 also suppresses endothelial lipase thereby regulating high-density lipoprotein (Shimamura et al., 2007).

The effect of ANGPTL6 (angiopoietin-related growth factor; AGF) on metabolism has been revealed by studying ANGPTL6-deficient mice (Oike et al., 2005a). Surviving mice developed marked obesity, lipid metabolic disorders, and insulin resistance accompanied by reduced energy expenditure. In contrast, transgenic ANGPTL6-mice are lean and more insulin-sensitive despite their normal energy intake and serum leptin levels (Oike et al., 2005b). ANGPTL6 has been shown to suppress gluconeogenesis through an Akt/FocO1-dependent pathway (Kitazawa et al., 2007).

1.2.13 Angiopoietin-like 4 (ANGPTL4) – a Novel Adipocytokine: Implications for the Wound Healing Process

Angiopoietin-like 4 protein (ANGPTL4) was first described in mouse hepatocytes during a search for novel genes in hepatocytes during fasting and in adipocytes during adipogenesis (Kersten et al., 2000; Yoon et al., 2000). ANGPTL4 is a secreted glycoprotein of ~46kDa with a 25 amino acid long signal peptide at its N-terminus. It contains a coiled-coil like domain at its N-terminal region and a fibrinogen-like domain at its C-terminal region (Kersten et al., 2000). The ANGPTL4 protein exists as an oligomer containing intermolecular disulfide bonds. Oligomerized ANGPTL4 undergoes proteolysis to release its C-terminal fibrinogen-like domain, which circulates as a monomer (Kersten et al., 2000). The N-terminal coiled-coil domain mediates its oligomerization, which is sufficient to form a higher order oligomeric structure (Ge et al., 2004). It is known that ANGPTL4 binds to LPL and converts the enzyme from catalytically active dimers to inactive monomers via its N-terminal coiled-coil domain

(Sukonina et al., 2006), and thus regulates lipid metabolism. These findings demonstrate a novel property of ANGPTL and suggest that oligomerization and proteolytic processing of ANGPTL4 may regulate its biological activities *in vivo* (Ge et al., 2004).

ANGPTL4 is produced widely in the body, suggesting multiple functions for the protein. To date, its expression has been profiled in liver, adipose tissue, blood, pancreas, kidney, intestine, brain, placenta, and skin (Kersten et al., 2000; Yoon et al., 2000). It was first identified as a target gene of the nuclear hormone receptor PPAR, which directs lipid metabolism in the liver and white adipose tissue (Mandard et al., 2004b). The expression of ANGPTL4 is stimulated by hypolipidemic fibrate drugs and insulin-sensitizing thiazolidinediones (Mandard et al., 2004a). The expression level of this gene is up-regulated during fasting conditions in both hepatocytes and white adipose tissue, which are primary sites for triglyceride and lipid metabolism (Mandard et al., 2006; Koliwad et al., 2009; Legry et al., 2009). Its plasma abundance increases during fasting and decreases following chronic high-fat feeding (Lichtenstein, et al, 2010). Various studies have been carried out to understand the functions of this protein. Overexpression of ANGPTL4 severely impairs the clearance of plasma triglycerides and stimulates adipose tissue lipolysis, resulting in lipids being redirected from storage into circulation, and results in the uptake of fatty acids and cholesterol into tissues (Mandard et al., 2006; Lichtenstein et al., 2007). Subsequently, it has been shown to regulate triglyceride metabolism and adiposity (Mandard et al., 2006; Koster et al., 2005). The lipid-lowering effect of ANGPTL4 is also seen when anti-ANGPTL4 antibody and ANGPTL4-knockout mice were used (Desai et al., 2007). It is also reported that the down-regulation of ANGPTL4 in the intestine is essential for the increase in adipose mass induced by intestinal microbiota (Backhed et al., 2004).

ANGPTL4 protein is also involved in glucose metabolism. An earlier study showed that ANGPTL4 decreased blood glucose and improved glucose tolerance in mice

(Xu et al., 2005). In concordance with that study, serum levels of ANGPTL4 are found to be lower in patients with type 2 diabetes than in healthy subjects (Xu et al., 2005). Overexpression of ANGPTL4 in mice has altered mitochondrial activities and modulates the amino acid metabolic cycle in liver tissues (Wang et al., 2007). These data suggest a beneficial effect on glucose metabolism and may provide a therapy for complex metabolic disorders (Oike et al., 2005a). Apart from the physiological regulatory roles of ANGPTL4, mechanisms that initiate the intra- or intercellular signaling events still remain largely unknown.

The role of ANGPTL4 in angiogenesis was also examined. Recombinant ANGPTL4 protects endothelial cells from apoptosis and was reported as pro-angiogenic factor (Kim et al., 2000). Subsequently, ANGPTL4 was shown to be induced by hypoxia and resulted in a strong *in ovo* angiogenic response in the chicken chorioallantoic membrane (Belanger et al., 2002; Le Jan et al., 2003a). By contrast, Ito et al. showed that recombinant ANGPTL4 markedly inhibited proliferation, chemotaxis and tubule formation (Ito et al., 2003). It was also discovered that ECM-bound ANGPTL4, not soluble ANGPTL4, inhibited endothelial cell adhesion, migration and sprouting (Cazes et al., 2006). The study also indicated that the interaction with ECM is heparin/heparan-sulfate-proteoglycan dependent, and the matrix-associated and soluble forms of ANGPTL4 regulate its bioavailability (Cazes et al., 2006) through its coiled-coil N-terminal domain (Chomel et al., 2009). cANGPTL4 exists as a monomer, whose function is still relatively unclear, but it has been implicated in the maintenance of vascular endothelial integrity (Yang et al., 2008). As both pro- and anti-angiogenic effects of ANGPTL4 in endothelial cells have been observed, a clear conclusion cannot be drawn.

The role of ANGPTL4 in cancer metastasis was also studied. ANGPTL4 has also been identified as one of the most predictive genes that signal the metastasis of breast cancer to the lungs (Minn et al., 2005; Steeg, 2006). In addition, microarray data analyses

showed that ANGPTL4 is upregulated in cancer from various organs, especially the skin (Axelsen et al., 2007; Ghosh et al., 2007). Recent studies indicated that TGFβ primed cancer metastasis through ANGPTL4 by enhancing its anoikis resistance (Zhang et al., 2008) and endothelial permeability (Padua et al., 2008). It was reported that ANGPTL4 was involved in the functional partitioning of postnatal intestinal lymphatic and blood vessels (Backhed et al., 2007). On the other hand, inhibition of vascular permeability and tumor cell invasiveness through intravasation by ANGPTL4 was also reported (Ito et al., 2003; Galaup et al., 2006). As cancer metastasis involves both vascular permeability during intravasation and extravasation, as well as tumor cell motility and invasiveness, the role of ANGPTL4 in these processes requires thorough study. In addition, the molecular mechanisms in both angiogenesis and cancer metastasis are yet to be elucidated.

Despite the well-established role of ANGPTL4 in lipid and glucose homeostasis, no studies have been undertaken to elucidate the effect of ANGPTL4 on keratinocyte migration during wound healing. The significance of the different cleaved fragments of ANGPTL4 is only beginning to be understood. Importantly, the mechanism through which ANGPTL4 relays its action from the cell surface and initiates an intracellular signaling cascade remains unknown, which limits our understanding of the mechanisms by which ANGPTL4 contributes to wound healing and cancer metastasis. As a secretory protein, various forms of ANGPTL4 are likely to interact with specific membrane-bound receptors that initiate intracellular cascades leading to appropriate cellular responses, including cell migration. Thus, the goal of my present study is to examine the role of ANGPTL4 in keratinocytes and elucidate its intracellular signaling cascades.

Here we show that ANGPTL4 interacts with wound integrins β1 and β5. This interaction activates integrin-mediated intracellular signaling and allows for selective integrin recycling and enhances cell migration. ANGPTL4-deficient cells showed

impaired cell migration and diminished FAK-Src-PAK1 activation, which facilitate lamellipodia formation and selective integrin internalization during keratinocyte migration. This defect was observed *in vivo* as delayed re-epithelialization in *ANGPTL4*-knockout mice. Our results reveal a novel role for ANGPTL4 in modulating integrin-mediated signaling during wound healing. Considering the importance of cell migration to numerous pathophysiological processes, our findings fill crucial gaps in the understanding of integrin-mediated cell migration. The findings presented here reveal an unexpected role for ANGPTL4. They provide insights into the novel control of keratinocyte behavior that is mediated by ANGPTL4-mediated cell-matrix communication, and underscores the physiological importance of the modulation of integrin activity in angiogenesis and metastasis.

1.3 MATERIALS AND METHODS

1.3.1 Reagents: Glutathione sepharose 4B beads were purchased from Amersham Biosciences; purified vitronectin (Calbiochem); Pfu polymerase, transfection reagent and ExGen 500 (Fermentas); Amine coupling kit, Immobiline pK buffers, Ni-NTA resin, Sensor chip (CM5) (GE Healthcare); pAc5.1/V5-His A plasmid, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI), Platinum quantitative PCR superMix-UDG, transfection reagent, Cellfectin, Dulbecco's modified Eagle's medium (DMEM) and *Drosophila* medium for Schneider 2 (S2) insect cells (Invitrogen Life Technologies); Immobilin western chemiluminescent HRP substrate and PVDF membrane (Millipore); Seize X Protein G immunoprecipitation kit (Pierce Biotechnology); DNA purification, gel and PCR clean up kits (Promega); 10 kDa cut-off membrane (Sartorius); Otherwise stated all chemicals from Sigma-Aldrich.

Antibodies: p(Y397)-FAK, β-tubulin, His-tag, fibronectin and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology); vitronectin, integrins β1, β3, β5, α3, α5, αv, α5β1, αvβ3, αvβ5 (Chemicon); 14-3-3σ, 14-3-3β, PKCα, RACK1 (BD Biosciences); p(Y416)-Src, FAK, PAK1, p(T423)PAK1, ERK and EGF receptor (Cell Signaling); Rac1 (Cytoskeleton Inc.). Polyclonal antibodies against the C-terminal region human (186-406 amino acid) and mouse (190-410 amino acids) of ANGPTL4 were produced in-house.

1.3.2 Wounding Experiment

Wounding and treatment were performed as described (Chong et al., 2009). Wounds were topically treated daily with either 50 μg of pre-immune IgG, anti-cANGPTL4 antibodies or 10 μg recombinant ANGPTL4. The wounds were kept moist using occlusive Tegaderm (3M, USA) dressing. Treatments were rotated to avoid site bias. At the indicated days post-injury, wounds were excised for analysis. Pure bred *ANGPTL4*$^{+/+}$

and *ANGPTL4*$^{-/-}$ mice on a C57Bl/6 background were used (Koster et al., 2005). Animal experiments were approved by the University Institutional Animal Care and Use Committee (ARF-SBS/NIE-A-0093, -0078 and -004). Hematoxylin and eosin (H&E) stained images and histomorphometric measurements were taken using a MIRAX MIDI with Plan-Apochromatic 20x/0.8 objective and MIRAX Scan software (Carl Zeiss). Polyclonal antibodies against human (amino acids 186-406) and mouse (190-410) cANGPTL4 were produced in-house.

1.3.3 Knockdown of ANGPTL4 and Real-Time PCR

siRNA against human ANGPTL4 and a scrambled sequence as control were subcloned into the pFIV-H1/U6-puro pFIV/siRNA lentivirus system. Pseudovirus purification and transduction were performed as described (Chong et al., 2009). Endogenous *ANGPTL4* in human keratinocytes was transiently suppressed using either siGLO control or ON-TARGETplus SMARTpool *ANGPTL4* siRNA (Dharmacon; L-007807-00) by means of DharmaFECT1. The knockdown efficiency and relative expression level of indicated genes were determined by qPCR using the KAPA FAST qPCR kit (KAPA Biosystems). All oligonucleotides and TaqMan probe sequences are provided in Table 1.2. The Interferon Response Detection Kit was from System Biosciences.

Table 1.2 Oligonucleotide sequences of siRNA and real-time PCR primers used in this work.

siRNA		
ANGPTL4 siRNA	sense	5'- AAAGCTGCAAGATGACCTCAGATGGAGGCTG – 3'
	anti-sense	5'- AAAAGGCTTAAGAAGGGAATCTTCTGGAAGAC -3'
Control siRNA	sense	5'- AAAGCTGTCTTCAAGATTGATATCGAAGACTA -3'
	anti-sense	5'- AAAATAGTCTTCGATATCAAGCTTGAAGACA -3'
Real-time qPCR[a]		
Human ANGPTL4	forward	5'- CTCCCGTTAGCCCCTGAGAG -3'
	reverse	5'-AGGTGCTGCTTCTCCAGGTG -3'
	Taqman probe	5'-(6-FAM)ACCCTGAGGTCCTTCACAGCCTGC(TAMRA)-3'
Mouse ANGPTL4	Forward	5'- GCTTTGCATCCTGGGACGAG -3'
	Reverse	5'- CCCTGACAAGCGTTACCACAG -3'
	Taqman probe	5'-(6-FAM)ACTTGCTGGCTCACGGGCTGCTAC(TAMRA)-3'
L27	Forward	5'- CTGGTGGCTGGAATTGACCGCTA -3'
	Reverse	5'- CAAGGGGATATCCACAGAGTACCTTG -3'
	Taqman probe	5'-(HEX)CTGCCATGGGCAAGAAGAAGATCGCC(BHQ1)-3'

[a] Melting curve analysis was performed to assure that only one PCR product was formed. Primers were designed to generate a PCR amplification product of 100 to 250 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real-time PCR assays.

1.3.4 Recombinant ANGPTL4 Expression and Purification

The cDNAs encoding various domains of human ANGPTL4 were isolated by PCR, subcloned into pET30a vector and transformed into *E. coli* Rosetta-gami™ bacteria (Novagen). Protein expression was induced by 0.5 mM IPTG and purified either by affinity nickel-Sepharose, size-exclusion or anion-exchange chromatographies according to standard procedures. *Drosophila* S2 cells stably expressing either human integrin β1, β5 or β3 were maintained as previously described (Tan et al., 2000). S2 cells were routinely cultured in serum-free medium. Cell membranes were first isolated using the ProteoExtract Native Protein Extraction Kit (Calbiochem) and were enriched by step sucrose gradient ultracentrifugation (Tang, 2006).

1.3.5 *In vitro* Scratch-wound Assay

Scratch-wound assays were performed as described (Tan et al., 2009). Images were taken at 2-min intervals over 6 h using a temperature-controlled, 5% CO_2-chambered Axiovert 200M microscope (Carl Zeiss) with a Plan-Neofluar 10x/0.3 or 20x/0.5 objective, CoolSNAP HQ^2 camera (Photometrics) and MetaMorph software (Molecular Devices). Pre-immune IgG or anti-cANGPTL4 antibodies were used at 2 μg/ml, recombinant ANGPTL4 at 6 μg/ml.

1.3.6 Surface Plasmon Resonance (SPR)

Surface Plasmon resonance (SPR) was used to determine the dissociation constants of the interactions of integrins β1, β5 with ANGPTL4 immobilized onto CM5 chip using BIACORE 3000 (Biacore, Uppsala, Sweden). The surface plasmon resonance (SPR) phenomenon occurs when polarized light, under conditions of total internal reflection, strikes an electrically conducting gold layer at the interface between media of different refractive index: the glass of a sensor surface (high refractive index) and a buffer (low refractive index). A wedge of polarized light, covering a range of incident angles, is directed toward the glass face of the sensor surface. Reflected light is detected within a Biacore system. (More information available from http://www.biacore.com/lifesciences/technology/introduction/following_interaction/index.html).

SPR reflectivity measurements in the present application are used to detect molecular adsorption, such as polymers, DNA or proteins, etc. Technically, it is common, that the angle of the reflection minimum (absorption maximum) is measured. This angle changes in the order of 0.1° during thin (about nm thickness) film adsorption. In other cases the changes in the absorption wavelength is followed. The mechanism of detection is based on that the adsorbing molecules cause changes in the local index of refraction, changing the resonance conditions of the surface plasmon waves (Figure 1.6).

The change in SPR signal is recorded in Response Units (RU) where 1 RU is equivalent to a change in mass of 1 pg/mm^2 pf biomolecules at the surface layer. Real time measurements of the binding and dissociation of proteins in solutions to and from the immobilized molecules on the sensor chip can thus be detected by an increase and decrease in RU, respectively.

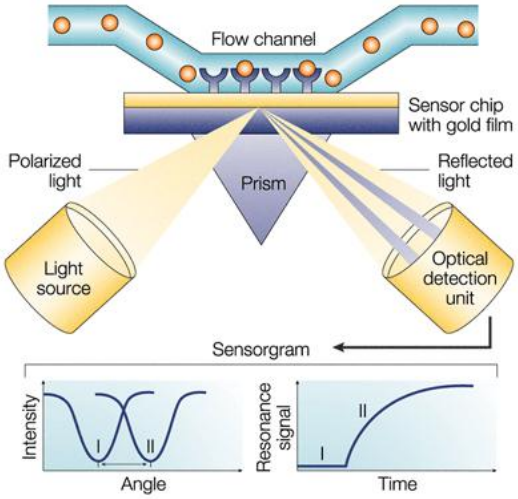

Figure 1.6 Basis of surface plasmon resonance (SPR). SPR detects changes in the refractive index in the immediate vicinity of the surface layer of a sensor chip. SPR is observed as a sharp shadow in the reflected light from the surface at an angle that is dependent on the mass of material at the surface. The SPR angle shifts (from I to II in the lower left-hand diagram) when biomolecules bind to the surface and change the mass of the surface layer. This change in resonant angle can be monitored non-invasively in real time as a plot of resonance signal (proportional to mass change) versus time. (Taken from Cooper, 2002 Nature Reviews Drug Discovery 1, 515-528)

Anti-cANGPTL4 antibodies against the immobilized ANGPTL4 determined the Rmax value of 251.8 resonance units (RU). Six concentrations (0.16, 0.32, 0.63, 1.25, 2.50 and 5.0 µM) of various integrins were used at a flow rate at 10 µl/ml. Details of interaction between ANGPTL4 and integrin β1 and β5 using SPR was performed by Mintu Pal, as reported in his Ph.D. thesis. Global fitting of the data to a Langmuir 1:1 model was used to determine the dissociation constant (K_D) using kinetic analysis calculated with the BiaEvaluation software (BIAcore, version 3.1). The experimental Rmax values of integrins β1 and β5 for ANGPTL4 were 261.1 RU and 229.3 RU,

respectively. Values are mean ± S.D. of five independent preparations of recombinant proteins. Various anti-integrin α5β1 and αvβ5 antibodies from R&D Systems and Abnova.

1.3.7 Affinity Co-precipitation Assay

In vivo coimmunoprecipitation was performed using indicated antibodies as previously described (IJpenberg et al., 2004). Briefly, cells at 2 x 10^6 cell/ml were rinsed twice with PBS before cross-linking using 1% formaldehyde in PBS for 10 min at 37 ℃, rinsed twice, scraped off, spinned down and resuspended in iced-cold lysis buffer (10mM EDTA, 50mM Tris–HCl, pH 8.0, 1% SDS, protease inhibitor cocktail (Roche Biochemicals, Mannheim, Germany) at 5x 10^6 cell/ml for 5 min. Five μl of 10 mg/ml RNase A were added and 3M NaCl was added to a final concentration of 150mM. The mixture was further incubated of 10 min on ice before spinned at 13,000 xg at 4℃ for 15 min. Co-immunoprecipitation was performed using immobilized with either anti-cANGPTL4, anti-integrin β1 or pre-immune IgG was first immobilized onto Protein A Sepharose resin at 4℃ for 4 h, and washed with ice-cold rinsing buffer eight times. Immunoprecipitates were released by Laemmli's buffer and probed with the indicated antibodies. For specificity of co-immunoprecipitation, immunodetection of cytoplasmic ERK, which does not interact directly with integrins β1 and β5 was performed.

1.3.8 Rho GTPase Assay

Active GTP-bound Rac1 was quantified as previously described (Tan et al., 2009). Briefly, 500 μg of cell lysates were incubated for 1 h at 4 ºC with GST-p21 binding domain of PAK coupled to glutathione Sepharose beads. Bound proteins were solubilized in Laemmli's buffer, resolved by SDS-PAGE, and immunoblotted using the

corresponding antibodies against Rac1. Total Rac1was detected using total cell lysate. Anti-Rac1 antibodies were from Cytoskeleton, Inc.

1.3.9 *In situ* Proximity Ligation Assay (PLA)

Wound biopsies were frozen in Tissue-Tek OCT compound medium (Sakura). Keratinocytes subcultured onto glass chamber slides (Lab-Tek), or wound sections were fixed with 4% paraformaldehyde for 15 min. The slides were washed twice with PBS, before DUOlink^{TM} *in situ* PLA was performed as recommended by the manufacturer (OLink Biosciences). Duolink *in situ* PLA Proximity Ligation Assay (PLA) enables detection, visualization and quantification of individual proteins, protein modifications and protein interactions in tissue and cell samples prepared for microscopy. The target is detected using one or two primary antibodies depending on the application. In the case that two primary antibodies are used, they must have been raised in different species.

The Duolink kits contain a pair of oligonucleotide labeled secondary antibodies (PLA probes) and a signal is generated only when the two PLA probes have bound in close proximity, either to the same primary antibody or two primary antibodies that have bound to the sample in close proximity. The signal from each detected pair of PLA probes is visualized as an individual fluorescent dot. These PLA signals can be quantified (counted) and assigned to a specific subcellular location based on microscopy images (Figure 1.7).

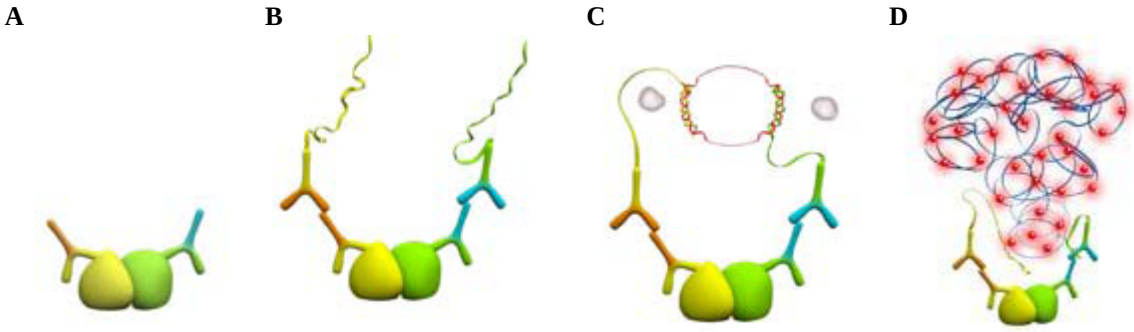

Figure 1.7 Principles of Proximity Ligation Assay (PLA). A. The samples are incubated with primary antibodies that bind to the protein(s) to be detected. B. Secondary antibodies conjugated with oligonucleotides (PLA probe MINUS and PLA probe PLUS) are added to the reaction and incubated. C. The Ligation solution, consisting of two oligonucleotides (illustrated as red bands) and Ligase, is added and the oligonucleotides will hybridize to the two PLA probes and join to a closed circle if they are in close proximity. D. The Amplification solution, consisting of nucleotides (not shown) and fluorescently labeled oligonucleotides, is added together with Polymerase. The oligonucleotide arm of one of the PLA probes acts as a primer for a rolling-circle amplification (RCA) reaction using the ligated circle as a template, generating a concatemeric (repeated sequence) product. The fluorescently labeled oligonucleotides will hybridize to the RCA product. The signal is easily visible as a distinct fluorescent spot and analyzed by fluorescence microscopy. (Information from http://www.bioscience.co.uk/userfiles/pdf/Duolink_II_Fluorescence_User_Manual.pdf).

Briefly, 40 µl Duolink blocking solution (2% BSA in PBS containing 0.1% Triton-X) was added to each slide and incubated in a pre-heated humidity chamber for 30 min at 37 ^{o}C. After tapping off the blocking solution, 20 µl of indicated antibody pairs at 1 mg/ml each was added onto the slides and incubated overnight at 4 ^{o}C. Primary antibodies were subsequently washed with PBST twice, before the two PLA probes (PLA probe MINUS and PLA probe PLUS) were added and incubated in a humidity chamber for 1 h at 37 ^{o}C. The negative control was performed without primary antibody. For detection, PLA probe solution was washed twice with PBST before 40 µl of hybridization solution was added and incubated for 10 min at 37 ^{o}C in a humidity chamber. After brief and gentle washing with PBST, ligation solution was added and the slides were incubated for another 15 min in a humidity chamber. After two gentle washing, 20 µl Duolink polymerase was subsequently added to the slides and incubated for 1 h at 37 ^{o}C. The slides were washed again twice with PBST, and subsequently

incubated with 20 µl of detection solution for 1h at 37 ^{o}C before final wash with SSC solutions, counter stained with Hoechst dye for nuclei and mounted on Duolink mounting medium. Images were taken using an LSM510 META confocal laser scanning microscope with a Plan-Apochromat 63x/1.40 Oil objective and ZEN 2008 software (Carl Zeiss).

1.3.10 Integrin Internalization

Surface labeling of membrane receptors was performed on adherent cells as described (Roberts et al., 2001), with minor modifications. Surface proteins were directly labelled at 4 °C with 0.2 mg/ml NHS-SS-biotin (Thermo) in PBS for 30 min. Labeled cells were washed twice with cold PBS and transferred to serum-free DMEM at 37 °C to eliminate exogenous ANGPTL4 and to permit internalization. After removing biotin from all remaining surface proteins using 20 mM MesNa for 15 min followed by 20 mM IAA for 10 min, cells were lysed. Supernatants were corrected to equivalent protein concentrations, and biotinylated proteins were captured overnight by NeutrAvidin agarose resins (Thermo) at 4 °C. Immobilized proteins were released using Laemmli's buffer and resolved by 10% SDS-PAGE, followed by immunoblot with the indicated anti-integrin antibodies. Cell surface expression of integrin at the indicated time was evaluated as previously described (Sterk et al., 2000).

1.3.11 Flow Cytometry (FACS)

Wound tissues were subjected to FACS analysis as previously described (Chen et al., 2008). Entire excised skin wounds were dispersed enzymatically into single cell suspensions. The tissue was incubated with dispase I (1 mg/ml) overnight at 4 ^{o}C, minced and incubated in digestion buffer containing hyaluronidase (1 mg/ml), collegenase D (1 mg/ml) and DNase (100 unit/ml) (Sigma-Aldrich) in a 37 ^{o}C shaking incubator for 2 h.

The dispase and hyaluronidase digests were pooled and filtered through a 70 μm Nylon cell strainer. Cells were washed, pelleted and resuspended in equal volume of PBS containing 3% FBS. For staining of surface marker, cells were first blocked with Mouse BD Fc Block and then incubated with either phyoerythrin (PE)- or FITC-conjugated monoclonal antibodies specific for F4/80 (macrophages) and CD31 (endothelial cells), or control isotype IgG on ice for 30 min. After washing with PBS, the samples were subjected to flow cytometry on a FACS Calibur system (Becton Dickinson). Data were analyzed using the 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.

1.3.12 Statistical Analysis

Statistical analysis was determined using the two-tailed Mann-Whitney test using SPSS software (IBM). $p<0.05$ was considered statistically significant.

1.4 RESULTS

1.4.1 Elevated ANGPTL4 Expression in Skin Wounds

We found that during the healing of a full-thickness excisional wound in mouse skin, *ANGPTL4* mRNA peaked at day 3-5 post-wounding, as shown by quantitative PCR (qPCR) and immunodetection (Figure 1.8A). Using polyclonal antibodies that recognize either the N- or C-terminal region of ANGPTL4, only the native ANGPTL4 and cANGPTL4 were detected in wound biopsies (Figure 1.8B). Immunoblot showed the specificity of anti-cANGPTL4 (Figure 1.8C). Dual immunofluorescence staining revealed that the expression of ANGPTL4 increased progressively in both the wound epithelia and wound bed, coinciding with an increase in Ki67-positive proliferating keratinocytes (Figure 1.9A). ANGPTL4 was detected only at basal levels in unwounded skin (Figure 1.9B). A retrospective examination of human skin ulcers, which reflects a situation of impaired healing, also revealed higher ANGPTL4 expression in ulcers compared with normal skin (Figure 1.10A and B). Although ulcers are different from acute wounds, their examination can provide clues on ANGPTL4 expression in human wounds, as it was not possible for us to obtain equivalent biopsies from healthy volunteers. These observations suggest an important role of ANGPTL4 during wound healing.

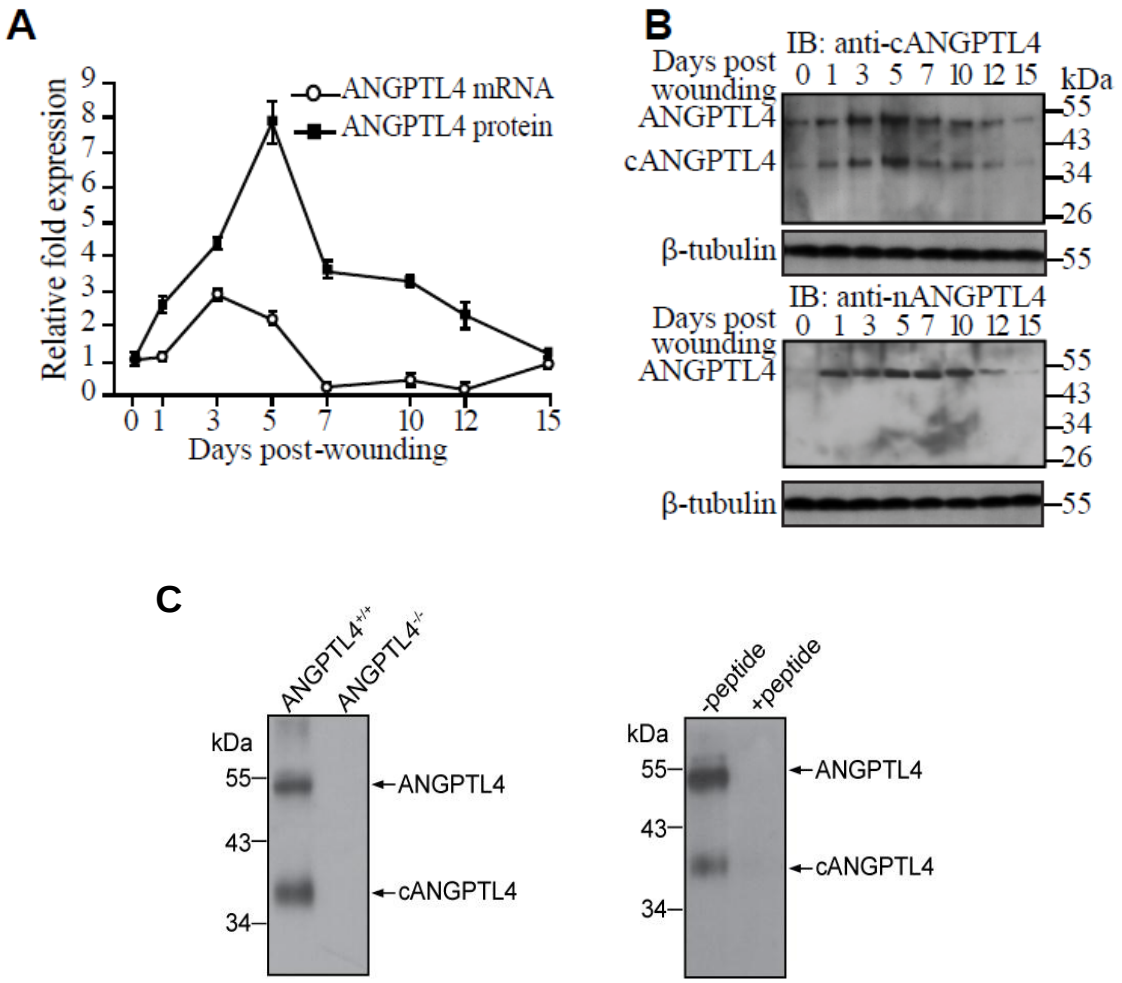

Figure 1.8 ANGPTL4 expression is induced during mice skin wounding. (A) ANGPTL4 mRNA and protein expression profiles during wound healing determined using qPCR and immunoblotting, respectively. Ribosomal protein L27 was used as a normalizing housekeeping gene.Values at each time point are mean±SEM of 15 mice. **(B)** Polyclonal antibodies that recognized the N- (anti-nANGPTL4) and C-terminal (anti-cANGPTL4) of ANGPTL4 were used. Coomassie-stained blot show equal loading and transfer. **(C)** Specificity of anti-nANGPTL4 was previously shown (Kersten et al., 2000) and anti-cANGPTL4 was verified by immunoblot analysis. Western blot analysis of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mouse skin lysate (left panel) or human plasma in the absence or presence of peptide epitope (right panel) using polyclonal anti-mouse or anti-human cANGPTL4 antibodies, respectively.

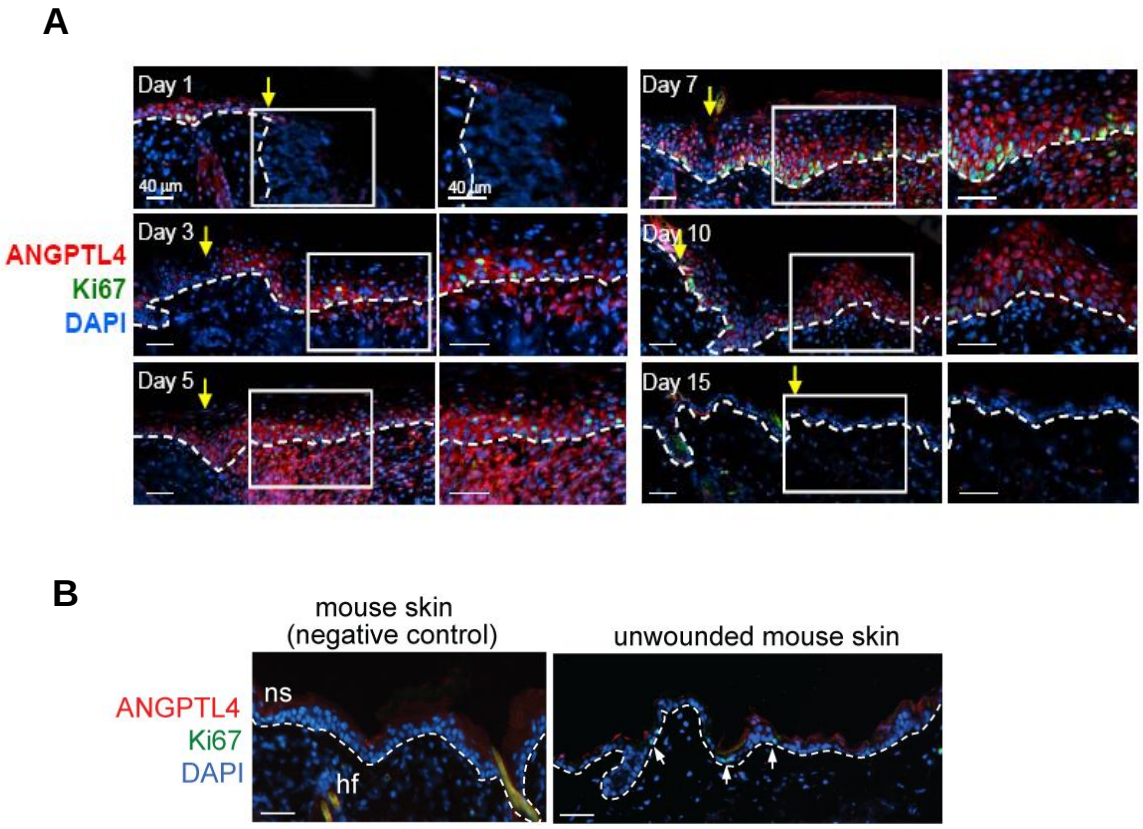

Figure 1.9 Immunofluorescence staining of ANGPTL4 in mice skin wound biopsies. (A) Mice skin wound biopsies at indicated days of post-wounding were cryosectioned and stained with antibodies against cANGPTL4 (red) and Ki67 (green), and counterstained with DAPI (blue). Representative pictures from wound edge and the adjacent wound bed are shown. The basal layer of keratinocytes and the underlying extracellular matrix are highlighted in the smaller windows next to the main figures. Higher magnifications are shown in boxes. Yellow arrow represents wound edge at day 0. **(B)** Immunofluorescence staining of ANGPTL4 in unwounded biopsy. Negative control of staining was performed without primary antibody. Dotted white line represents epidermal-dermal junction. Immunostaining were performed on five mice skin. Scale bar 40 µm.

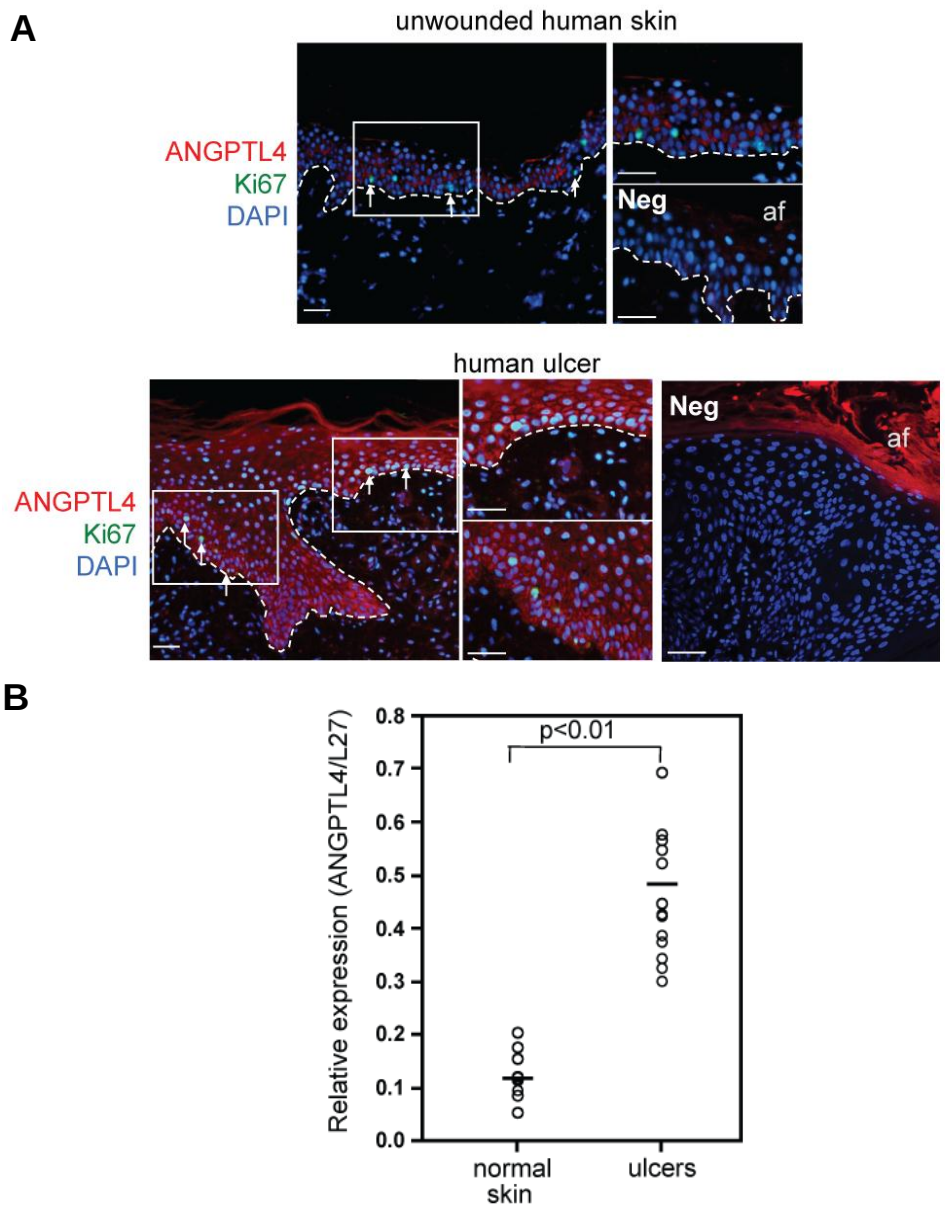

Figure 1.10 ANGPTL4 is expressed in unwounded human skin and ulcers. (A) Immunofluorescence staining of ANGPTL4 in unwounded and human ulcer biopsy. ANGPTL4 is expressed in unwounded human skin and ulcers. Higher magnifications are shown in boxes. Polyclonal antibodies against human cANGPTL4 were used. The anti-nANGPTL4 antibodies were not suitable for immunofluorescence staining. Negative (Neg) control performed without primary antibody showed autofluorescence (af) in the stratum corneum. White arrows indicate proliferating Ki67-positive keratinocytes (green). Scale bars 40 μm. Immunostaining were performed on eight paraffin-embedded human ulcer biopsies. Representative pictures are shown. Negative control of staining was performed without primary antibody. **(B)** Relative expression level of ANGPTL4 mRNA in normal human skin biopsies and ulcers as determined by qPCR. Ribosomal protein L27 was used as a normalizing housekeeping gene. Each point is mean±S.D. of 3 different paraffin sections from an individual sample. (p= 0.0016; two-tailed Mann-Whitney test).

1.4.2 ANGPTL4 Deficiency Delays Wound Re-epithelialization

We examined the healing of full-thickness skin wound in wild type (*ANGPTL4*$^{+/+}$) and ANGPTL4-knockout (*ANGPTL4*$^{-/-}$) mice. Histomorphometric analysis of day 3-10 wound biopsies showed delayed re-epithelialization of *ANGPTL4*$^{-/-}$ wounds when compared with *ANGPTL4*$^{+/+}$ wounds (Figure 1.11A). No difference in wound contraction was observed (Figure 1.11B). The length, thickness and area of the epithelial tongue were reduced in *ANGPTL4*$^{-/-}$ wounds at days 3-5 post-injury (Figures 1.11C-E). The *ANGPTL4*$^{+/+}$ wounds were completely re-epithelialized by day 7, in contrast to *ANGPTL4*$^{-/-}$ wounds (Figures 1.11A & F). The topical application of recombinant ANGPTL4 onto *ANGPTL4*$^{-/-}$ wounds resulted in complete re-epithelialization at day 7 post-application, in contrast to untreated *ANGPTL4*$^{-/-}$ wounds (Figure 1.11A). Recombinant full-length angiopeiotin-like 4 (ANGPTL4), coiled-coil N-terminal domain (nANGPTL4) and fibrinogen-like C-terminal domain (cANGPT4) were cloned using pET30a+, expressed using *E. coli Rosetta gami* and purified (Figures 1.12A & B). To eliminate a potential systemic effect of ANGPTL4 on wound closure, we examined the effect of topically applied anti-cANGPTL4 antibody on wound re-epithelialization. We reasoned that the antibody might interfere with the action of ANGPTL4, and thus recapitulate *ANGPTL4*$^{-/-}$ wounds. Our analysis revealed impaired re-epithelialization, reduced length and thickness of the epithelial tongue in wounds treated with anti-cANGPTL4 as compared to pre-immune IgG-treated wounds (Figures 1.13A & B). No significant difference in wound contraction was observed (Figure 1.13A). Images of serial sections encompassing complete wounds at days 3-10 post-injury showed that the impaired re-epithelialization of the epidermis was not a local random alteration; rather it was distributed over the entire healing wound edge. Altogether, these results indicate ANGPTL4 is important for efficient wound healing.

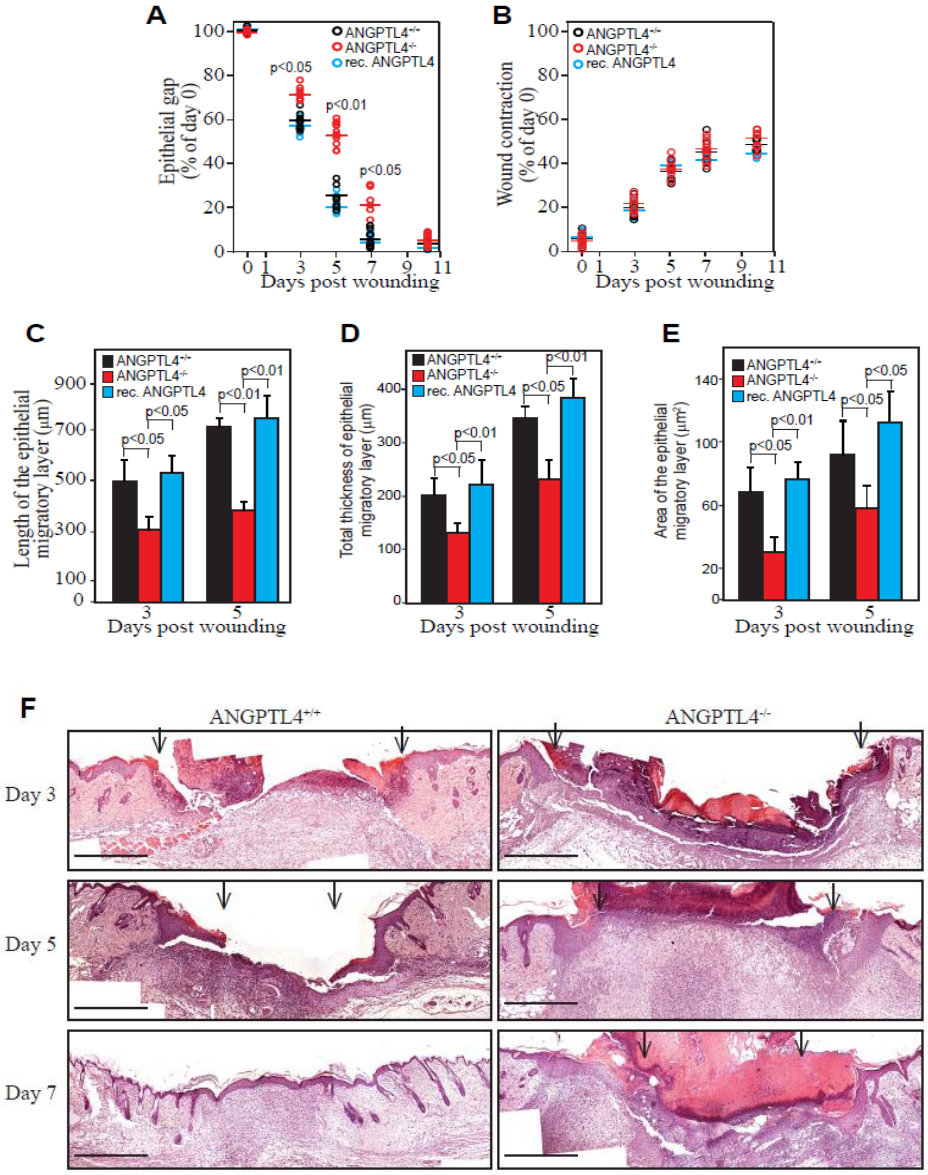

Figure 1.11 Deficiency in ANGPTL4 delays wound re-epithelialization. Quantification of the **(A)** epithelial gap, **(B)** wound contraction, **(C)** length, **(D)** thickness and **(E)** area of the migrating wound epidermis in ANGPTL4$^{+/+}$ (black), ANGPTL4$^{-/-}$(red) and ANGPTL4$^{-/-}$ mice topically treated with recombinant ANGPTL4 (rec. ANGPTL4, blue). At indicated day post-wounding, wounds were excised for analysis. Each circle shows the mean values of 10 centrally dissected sections obtained from individual mouse; horizontal bars show average values obtained for each genotype or treatment. Quantification of the thickness, area and length of the wound epithelia were the mean of left and right wound epithelial tongue measured using day 3 and 5 wound biopsies from 10 mice. Epithelial gap is defined as the distance between the advancing edges of clear, multiple layer neoepidermis. An epithelial gap of zero represents a completely re-epithelialized wound. Wound contraction is defined by the distance between the first hair follicle on both of the wound edge. The length of the wound epidermis measured from the first hair follicle to the tip of the wound epithelial tongue is used as an indicator of keratinocyte migration. Two-tailed Mann-Whitney statistical test was used. **(F)** Haematoxylin and eosin stained pictures of post-injury wound edges from ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice. Scale bar 500 μm. Arrows point to the epithelial wound edge. Representative pictures of a centrally dissected wound section are shown.

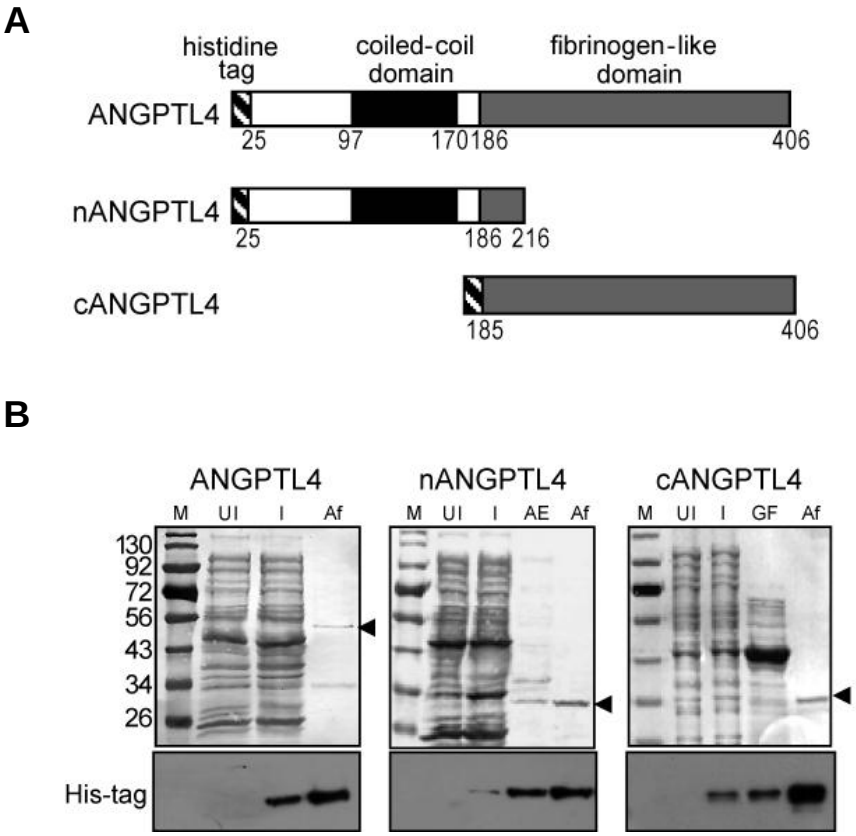

Figure 1.12 Expression and purification of ANGPTL4 recombinant proteins. (A) Schematic illustration of the subcloning of full-length ANGPTL4 (ANGPTL4), the coiled-coil N-terminal domain (nANGPTL4) and the fibrinogen-like C-terminal domain (cANGPTL4) into pET30a+ expression vector. The proteins were expressed in with *E. coli* Rosetta gami. Numbers indicated the amino acid position. **(B)** Purification of bacterially expressed N-terminal His-tagged ANGPTL4 proteins. M: molecular weight marker; UI and I denotes: un-induced and IPTG-induced bacterial lysates; Af: nickel-Sepharose affinity chromatography; AE: anion-exchange; GF: gel filtration. Coomassie stained gels (upper panel) and immunoblot analysis with anti-His antibody (lower panel).

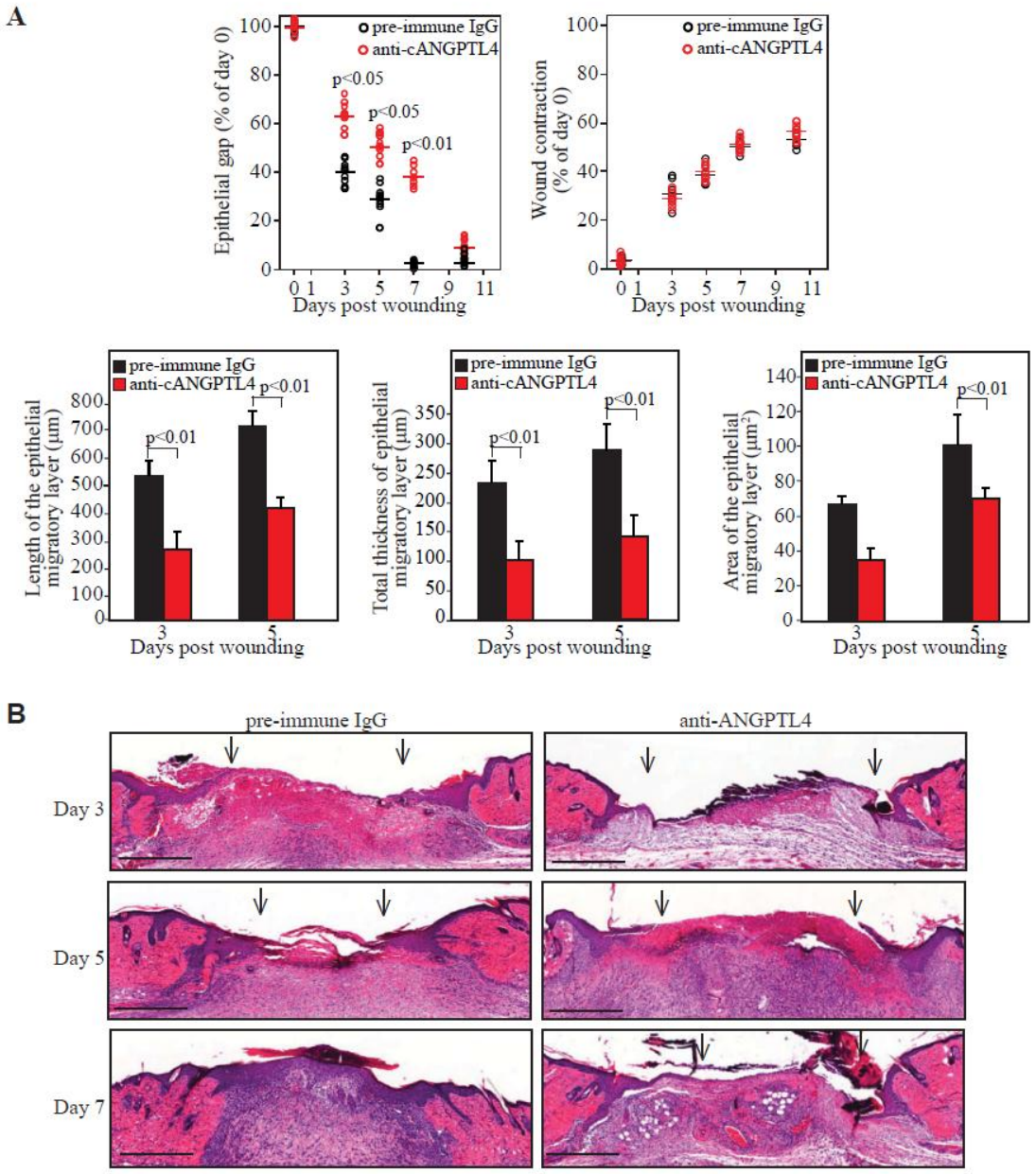

Figure 1.13 Neutralization of ANGPTL4 delays wound re-epithelialization. (A) Quantification of the epithelial gap, thickness, area and length of the migrating wound epidermis in pre-immune IgG (black) and anti-cANGPTL4 antibody (Ab)-treated (red) wounds. **(B)** Haematoxylin and eosin stained pictures of post-injury wound edges from pre-immune IgG and anti-cANGPTL4 antibody-treated wounds. At indicated day post-wounding, wounds were excised for analysis. Each circle shows the mean values of 10 centrally dissected sections obtained from individual mouse; horizontal bars show average values obtained for each genotype or treatment. Quantification of the thickness, area and length of the wound epithelia were the mean of left and right wound epithelial tongue measured using day 3 and 5 wound biopsies from 10 mice. Epithelial gap is defined as the distance between the advancing edges of clear, multiple layer neoepidermis. An epithelial gap of zero represents a completely re-epithelialized wound. Wound contraction is defined by the distance between the first hair follicle on both of the wound edge. The length of the wound epidermis measured from the first hair follicle to the tip of the wound epithelial tongue is used as an indicator of keratinocyte migration. Two-tailed Mann-Whitney statistical test was used.

1.4.3 ANGPTL4 Deficiency Impairs Cell Adhesion and Migration

To better understand the role of ANGPTL4, we examined the effect of ANGPTL4 on cell adhesion and migration using primary human keratinocytes. We suppressed endogenous *ANGPTL4* expression by RNA interference. Keratinocytes were either transduced with a lentivirus-mediated *ANGPTL4* siRNA (Figure 1.14A) or transiently transfected with ON-TARGETplus SMARTpool siRNAs (Figure 1.14B). Lentivirus-mediated control scrambled siRNA and siGLO siRNA served as corresponding controls. The *ANGPTL4* expression level in *ANGPTL4*-knockdown keratinocytes ($K_{ANGPTL4}$) was reduced by 90% compared with control-siRNA keratinocytes (K_{CTRL}) (Figure 1.14A). The induction of interferon responses has been reported as a challenge to the specificity of some RNA interference approaches (Bridge et al., 2003). Therefore, we measured the expression of key interferon response genes by qPCR, which showed no induction in $K_{ANGPTL4}$ when compared to either wild-type nontransduced cells or K_{CTRL} (Figure 1.14C), suggesting no off-target effect. $K_{ANGPTL4}$ did not undergo spontaneous apoptosis in standard growth conditions, as determined by FACS analysis (Figure 1.14D).

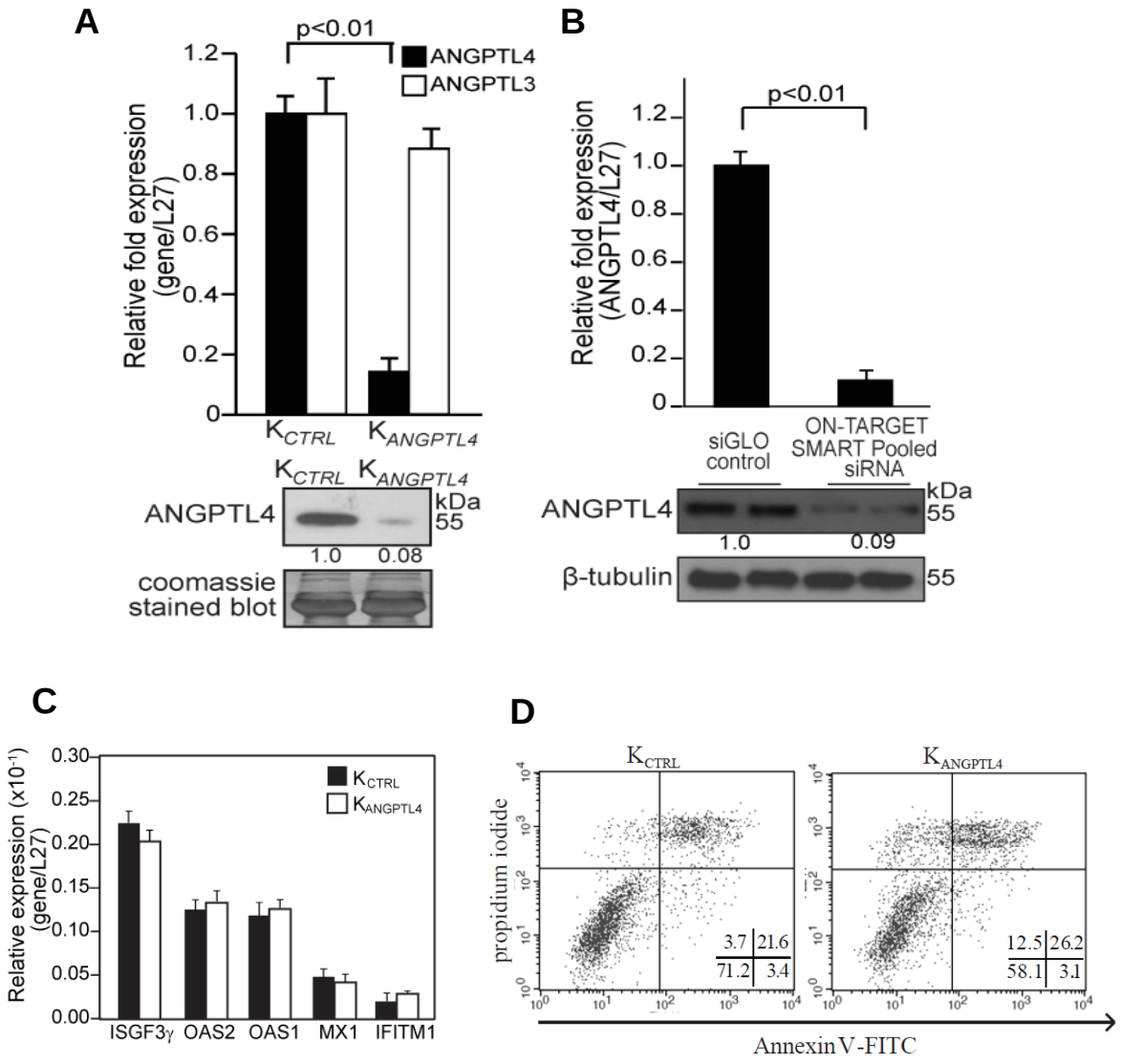

Figure 1.14 ANGPTL4 knockdown efficiency and specificity using lentivirus-mediated RNAi and transient siRNA transfection. Expression level of **(A)** ANGPTL4 and ANGPTL3 in keratinocytes transduced with either control (K_{CTRL}) or ANGPTL4 siRNA ($K_{ANGPTL4}$), **(B)** ANGPTL4 in keratinocytes transfected with either siGLO control or ON-TARGETplus SMARTpool ANGPTL4 siRNAs, **(C)** 2',5'-oligoadenylate synthetase isoforms 1 and 2 (OAS1, OAS2), interferon-induced myxovirus resistance 1 (MX1), interferon-inducible trans-membrane protein (IFITM) and interferon-stimulated transcription factor 3γ (ISGF3γ) mRNAs in K_{CTRL} and $K_{ANGPTL4}$ as determined by qPCR. Ribosomal protein L27 was used as a normalizing housekeeping gene. Values below each band represent the mean fold differences in protein expression level with respect to control from five independent experiments (n=5). Coomassie-stained blot or β-tubulin showed equal loading and transfer. **(D)** FACS analysis of K_{CTRL} and $K_{ANGPTL4}$ stained with annexin V-FITC/PI. The percentage of apoptotic cells (lower right quadrant) is indicated in bold.

Next, we performed a cell adhesion assay on K_{CTRL} using serum-free medium. The results showed that cells attached more rapidly onto ANGPTL4-coated surfaces compared to control uncoated surfaces. The attachment rate was delayed in the presence of anti-cANGPTL4 antibody compared with pre-immune IgG (Figure 1.15), suggesting that ANGPTL4 facilitated cell attachment. *ANGPTL4*$^{-/-}$ mouse primary keratinocytes adhered poorly to the culture surface and underwent apoptosis, so we were unable to culture sufficient cells for experiments.

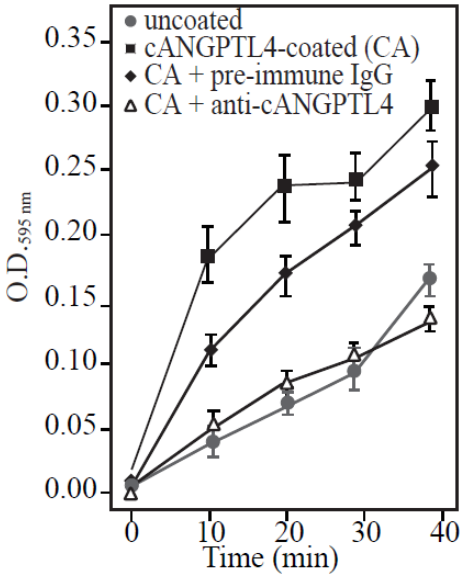

Figure 1.15 ANGPTL4 deficiency impairs keratinocyte adhesion. Cell adhesion onto cANGPTL4- was impaired in the presence of anti-cANGPTL4. Cells were detached with 5 mM EDTA in PBS and washed twice with serum-free medium. A total of 5 x 10^3 cells per well were pre-incubated with 2 μg/ml of anti-cANGPTL4 for 10 min. Cells were next seeded into cANGPTL4-coated wells. Adhered cells were identified by crystal violet staining at indicated time. Crystal violet was dissolved and absorbance at 595 nm was determined. Values are mean±S.D. of 3 replicate samples.

We examined the impact of ANGPTL4 on keratinocyte migration. In an *in vitro* scratch-wound assay, K_{CTRL} closed the wound by 6 h, whereas $K_{ANGPTL4}$ took 18 h, indicating impaired keratinocyte migration (K_{CTRL} vs. $K_{ANGPTL4}$: 11.92±0.31 vs. 6.66±0.12 μm/h, $P<0.05$) (Figure 1.16A). Similar observations were also made in transiently siRNA-transfected keratinocytes (5.87±0.15 μm/h), indicating that the impaired migration was not due to an adaptation to the reduced ANGPTL4 level (Figure 1.16A). Similar experiments in the presence of mitomycin C showed that K_{CTRL} closed the wound by 8 h, whereas $K_{ANGPLT4}$ and siRNA-transfected keratinocytes failed to close the wound even after 24 h (Figure 1.16A). Importantly, the application of recombinant ANGPTL4 rescued the impaired migration of $K_{ANGPTL4}$ (12.03±0.42 μm/h), regardless of mitomycin C treatment (9.24±0.37 μm/h) (Figure 1.16A). Conversely, the presence of anti-cANGPTL4 antibody delayed K_{CTRL} migration (preimmune vs anti-cANGPTL4: 13.09±0.23 vs 6.80±0.17μm/h) (Figure 1.17A). $K_{ANGPTL4}$ or anti-cANGPTL4-treated K_{CTRL} did not display pronounced lamellipodia at the leading edge of migrating cells (Figures 1.16B & 1.17B).

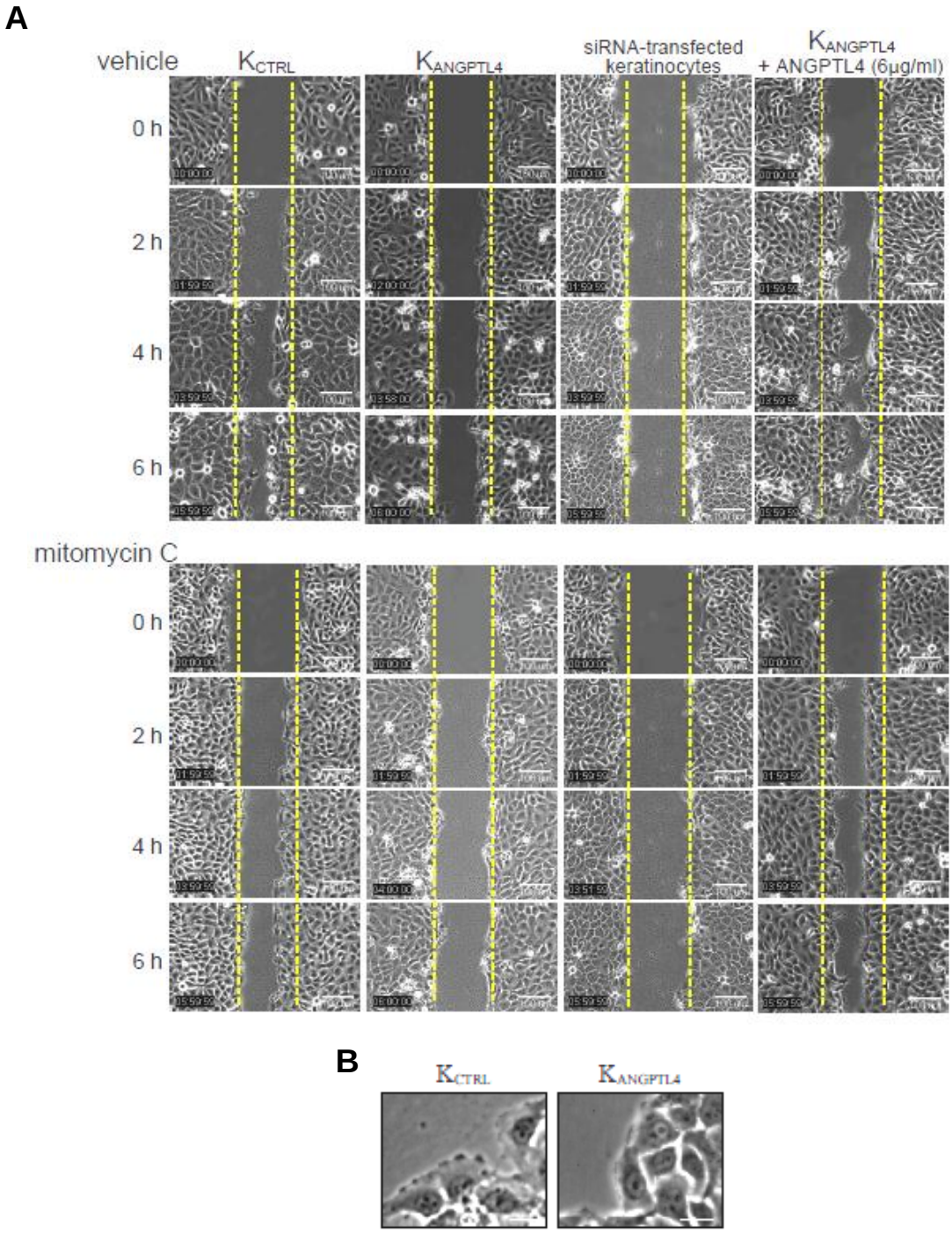

Figure 1.16 ANGPTL4 modulates cell migration. (A) Representative time-lapsed images of wounded cultures of K_{CTRL}, $K_{ANGPTL4}$, ANGPTL4 siRNA-transfected keratinocytes and with recombinant cANGPTL4 (rec. cANGPTL4)-treated $K_{ANGPTL4}$, in the presence (lower panel) or absence (upper panel) of mitomycin C (2 µg/ml). Yellow dotted lines represent the scratch gap at the time of wounding. Scale bar 100 µm. Five independent experiments were performed. **(B)** Phase contrast images showing lamellipodia of K_{CTRL} and $K_{ANGPTL4}$ during migration. Scale bar 20 µm. Five independent experiments were performed.

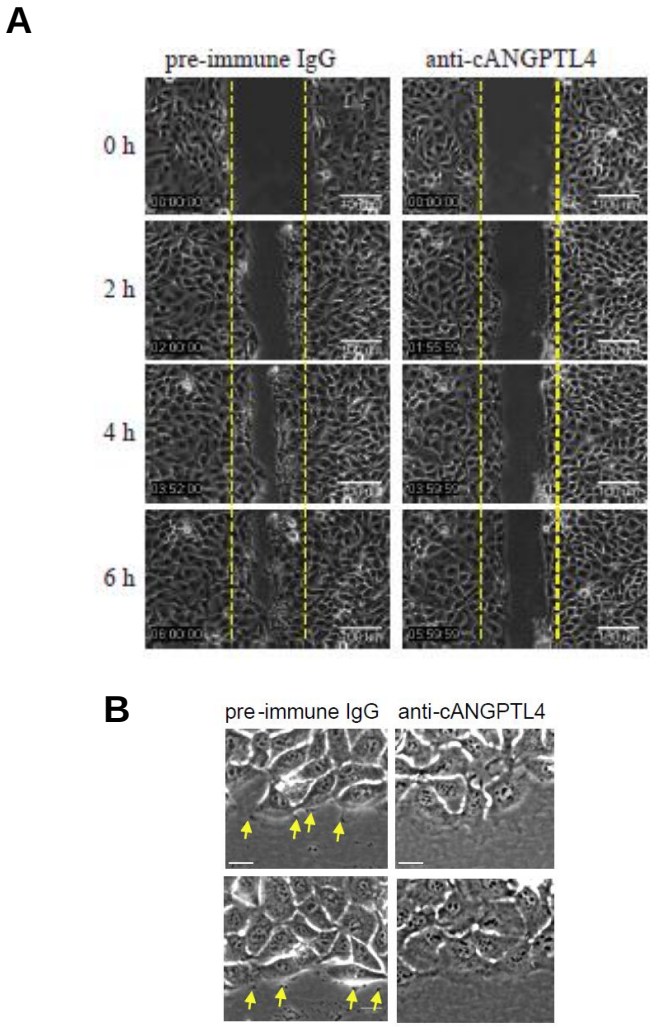

Figure 1.17 ANGPTL4 is important for cell migration. (A) Neutralization of ANGPTL4 with anti-cANGPTL4 antibodies in K_{CTRL} delays cell migration. Representative time-lapsed images of wounded cultures of K_{CTRL} treated with 2 μg/ml of either pre-immune IgG or anti-cANGPTL4 antibody (upper panel). Yellow dotted lines represent the scratch gap at the time of wounding. Scale bar 100 μm. **(B)** Phase contrast images showing lamellipodia of K_{CTRL} treated with 2 μg/ml of either pre-immune IgG or anti-cANGPTL4 antibody during migration. Arrows indicate the focal adhesion points in lamellipodia during migration. Scale bar 20 μm. Five independent experiments were performed.

1.4.4 ANGPTL4 Interacts with Integrin β1 and β5

(Details of interactions between ANGPTL4 and integrin β1 and β5 using SPR were performed by Mintu Pal as reported in his Ph.D.'s thesis and will not be described in detail here.)

How ANGPTL4 mediates its action remains a central question in our understanding of ANGPTL4 in cell migration. Cell migration is an integrin-dependent process, and ANGPTL3, a close relative of ANGPTL4, binds to integrin αvβ3 (Camenisch et al., 2002). This prompted us to inquire if ANGPTL4 interacts with integrins, particularly integrins β1 and β5 which are essential for keratinocyte migration and whose expression is increased during wound healing (Grose et al., 2002). We first bacterially expressed and purified the various domains of ANGPTL4. Next, we ectopically expressed and purified human integrins β1, β3 and β5 in *Drosophila* S2 cells cultured in serum-free medium (Figure 1.18A). Membrane extract enriched in either integrin β1, β3 or β5 was used for interaction studies with cANGPTL4 by surface plasmon resonance (SPR). Integrins β1 and β5, but not β3, interacted with cANGPTL4 with K_D of ~10^{-8} M (Figure 1.18B). The interaction between ANGPTL4 and integrin β1 or β5 was specific, as it was reciprocally blocked with neutralizing antibodies raised against either integrins (α5β1 or αvβ5), or cANGTPL4 (Figures 1.18C-E). Thus, ANGPTL4 can directly interact with specific integrins, in the absence of cognate matrix proteins. We further confirmed this interaction by ELISA (Figures 1.18F & G).

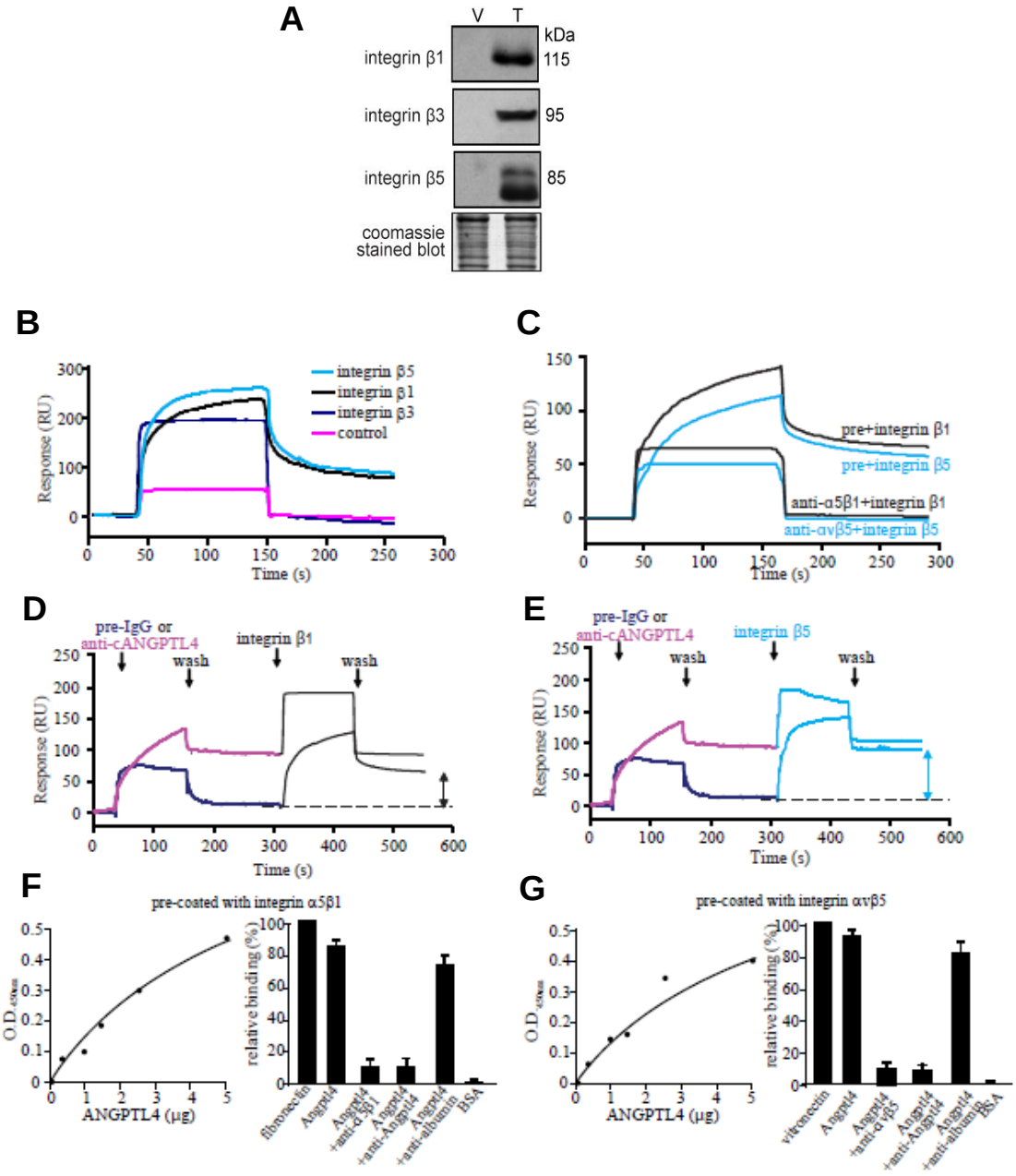

Figure 1.18 ANGPTL4 interacts with integrins β1 and β5. (A) Immunoblot analysis of enriched membrane extracts from Drosophila S2 cells transfected with either control empty expression vector (V) or vector encoding for either integrin β1, β3 or β5 (T). Coomassie-stained blot show equal loading and transfer. Representative sensorgrams showing binding profiles between immobilized-cANGPTL4 and **(B)** S2-membrane extracts containing either control, integrin β1, β3 or β5, or **(C)** integrin β1 or β5 pre-incubated with either pre-immune IgG (pre) or cognate anti-integrin antibody. **(D)** integrin β1 or **(E)** integrin β5 after pre-blocked with either pre-immune IgG or anti-cANGPTL4 antibody. Each sensorgram was corrected by subtracting a sensorgram obtained from a reference flow cell with no immobilized protein. Anti-cANGPTL4 antibodies against the immobilized cANGPTL4 determined the Rmax value to be 138.2 resonance units (RU). Five independent experiments were performed. Dose-dependent ANGPTL4 binding to immobilized **(F)** integrin α5β1 or **(G)** integrin αvβ5 which was specifically blocked by anti-cANGPTL4, as determined by ELISA.

In situ proximity ligation assays (PLA) performed using various antibody pairs on K_{CTRL} and day-5 wound sections confirmed that cANGPTL4 interacted with integrins β1 and β5 *in vivo* (Figure 1.19A). The PLA signal from each detected interacting protein pair is visualized as an individual red dot (Soderberg et al., 2008). Double immunostaining performed using anti-vinculin and anti-cANGPTL4 on K_{CTRL} of an "*in vitro*" scratch wound revealed strong ANGPTL4 expression near focal contact regions, which was further confirmed using PLA (ANGPTL4 & integrin β1) and immunofluorescence (vinculin), underscoring the role of ANGPTL4 in keratinocyte migration (Figure 1.19B).

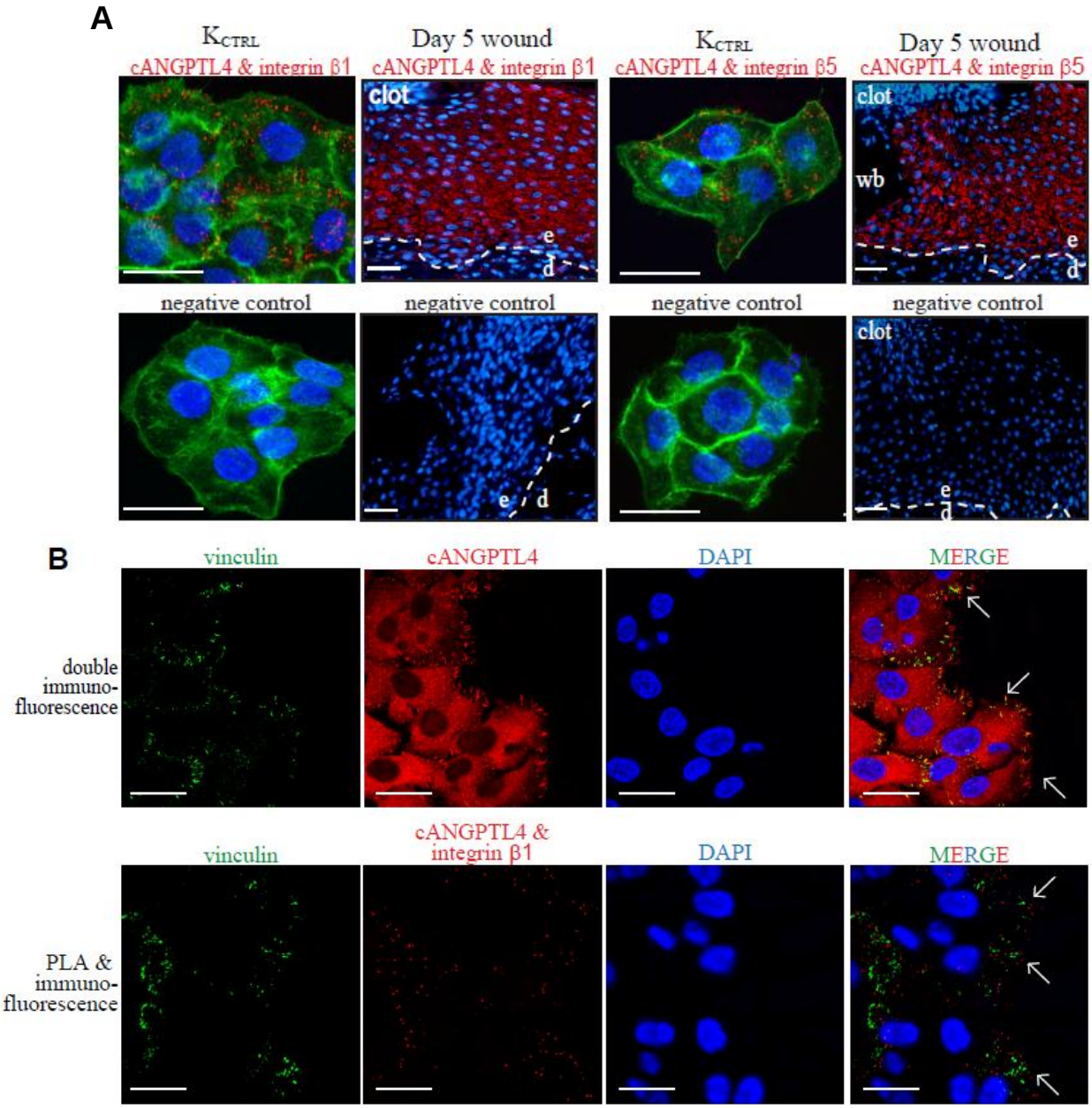

Figure 1.19 ANGPTL4 colocalizes with integrins β1 and β5 at focal contact regions. (A) Detection of the ANGPTL4-integrin β1 (left panel) and ANGPTL4-integrin β5 (right panel) complexes in K_{CTRL} and in day-5 *ANGPTL4*$^{+/+}$ wound biopsies using DUOlink™ PLA. PLA signals (red) and Hoechst dye for nuclei (blue). For K_{CTRL}, the cells were counterstained with Alexa 488-phallodin for actin stress fibers. The nuclear image was acquired in one z-plane using a LSM510 META confocal laser scanning microscope (Carl Zeiss). Dotted white line represents epidermal-dermal junction. Negative control was performed without primary antibodies Representative pictures from wound sections with epidermis (e), dermis (d), wound bed (wb) and K_{CTRL} from six independent experiments or sections from three mice are shown. **(B)** Double-immunofluorescence staining for ANGPTL4 (red), vinculin (green) (top panel) and PLA (ANGPTL4 and integrin β1; red) with single immunofluorescence staining for vinculin (green) (bottom panel) of K_{CTRL} cells at the migratory front of an '*in vitro*' scratch wound. Cells were counterstained with Hoechst dye for nuclei (blue). Scale bar 40 μm.

Immunoblot analysis of anti-cANGPTL4 and specific anti-integrin immunoprecipitates of *ANGPTL4*$^{+/+}$ and *ANGPTL4*$^{-/-}$ wound biopsy homogenates showed that the integrin β1 and β5 were present, as well as α3, α5 and αv subunits (Figures 1.20A & B). Interaction of integrin β1 and β5 with ANGPTL4 did not compete with the binding of integrins to their natural cognate ligands, but rather they appeared to strengthen the integrin-matrix interactions. Consistent with the above findings, integrin β3 was not detected in anti-cANGPTL4 immunoprecipitates. Next, we performed cell adhesion assays and *in vitro* wound assays on fibronectin- and vitronectin-coated surfaces. The results showed that $K_{ANGPTL4}$ adhered more slowly to both coated surfaces than K_{CTRL} (Figures 1.20C & D).

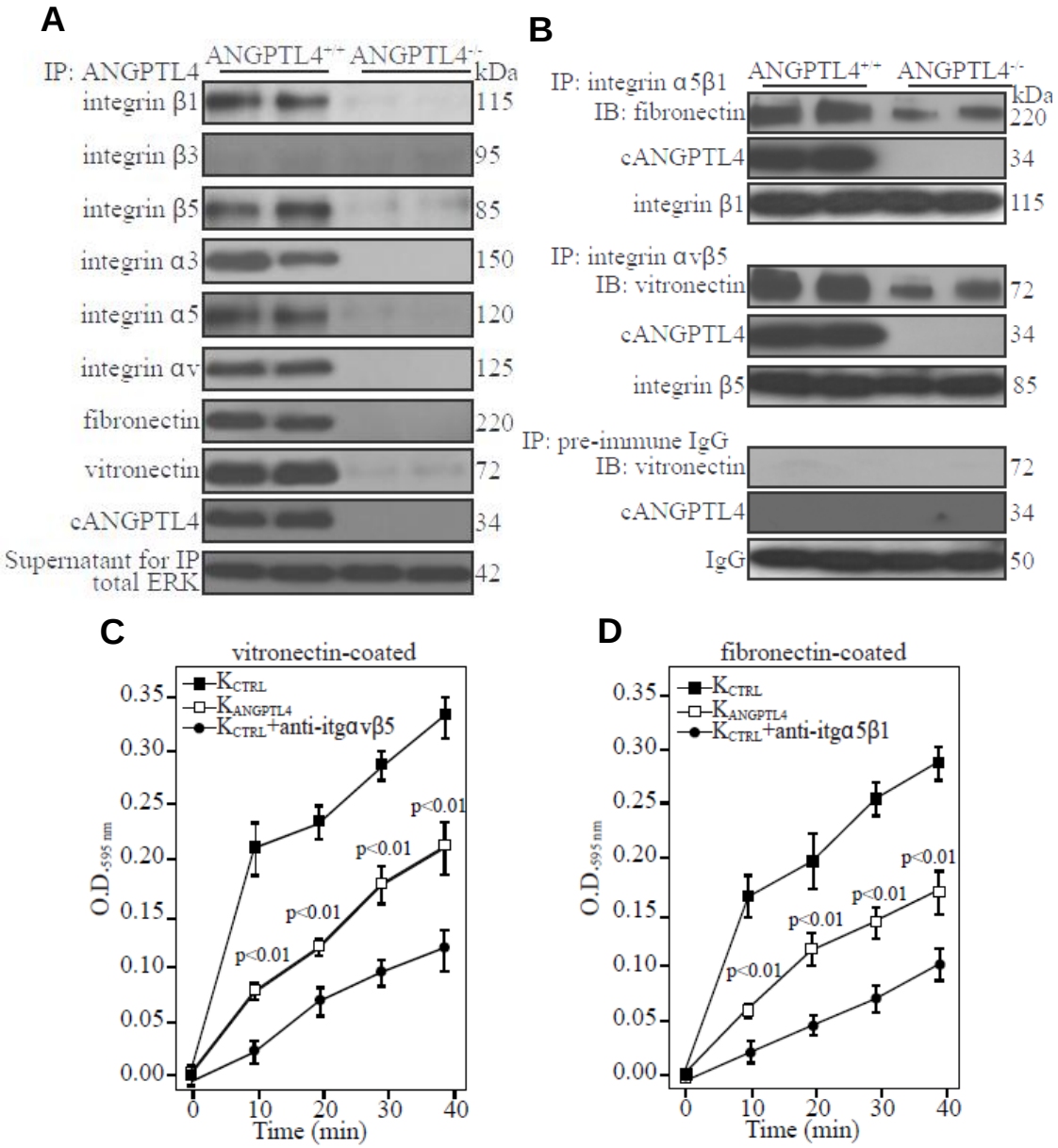

Figure 1.20 Interaction of integrin β1 and β5 with ANGPTL4 did not compete with the binding of integrins to their natural cognate ligands. (A) Immunodetection of indicated proteins from anti-cANGPTL4 immunoprecipitates of *ANGPTL4*$^{+/+}$ and *ANGPTL4*$^{-/-}$ wound biopsy homogenates. Total ERK from supernatant were used to verify equal loading. **(B)** Immunodetection of indicated proteins from anti-integrin α5β1, αvβ5 and pre-immune IgG immunoprecipitates of *ANGPTL4*$^{+/+}$ and *ANGPTL4*$^{-/-}$ wound biopsy homogenates. Integrin β subunits and IgG heavy chain were used to verify equal loading. Cell adhesion assays on **(C)** vitronectin- and **(D)** fibronectin-coated surfaces using K_{CTRL} and $K_{ANGPTL4}$. As controls, cognate anti-integrin antibodies were used.

Cell migration assays were also performed on coated surfaces using K_{CTRL} and $K_{ANGPTL4}$ treated with mitomycin C, to exclude any effects of proliferation (Figure 1.21). The re-population of the *in vitro* wound by K_{CTRL} and $K_{ANGPTL4}$ was faster on both matrix protein-coated surfaces compared with the cognate controls on uncoated surface (K_{CTRL} on coated vs. uncoated: 6 h vs. 8 h; $K_{ANGPTL4}$ on coated vs uncoated: 19 h vs. ≥24 h). Notably, the application of recombinant ANGPTL4 accelerated the migration and closure of the *in vitro* wound by $K_{ANGPTL4}$ (compare Figure 1.16A and Figure 1.21). Taken together, these data indicate that integrins β1 and β5, but not β3, are novel interacting protein partners of ANGPTL4. This interaction aided cell migration.

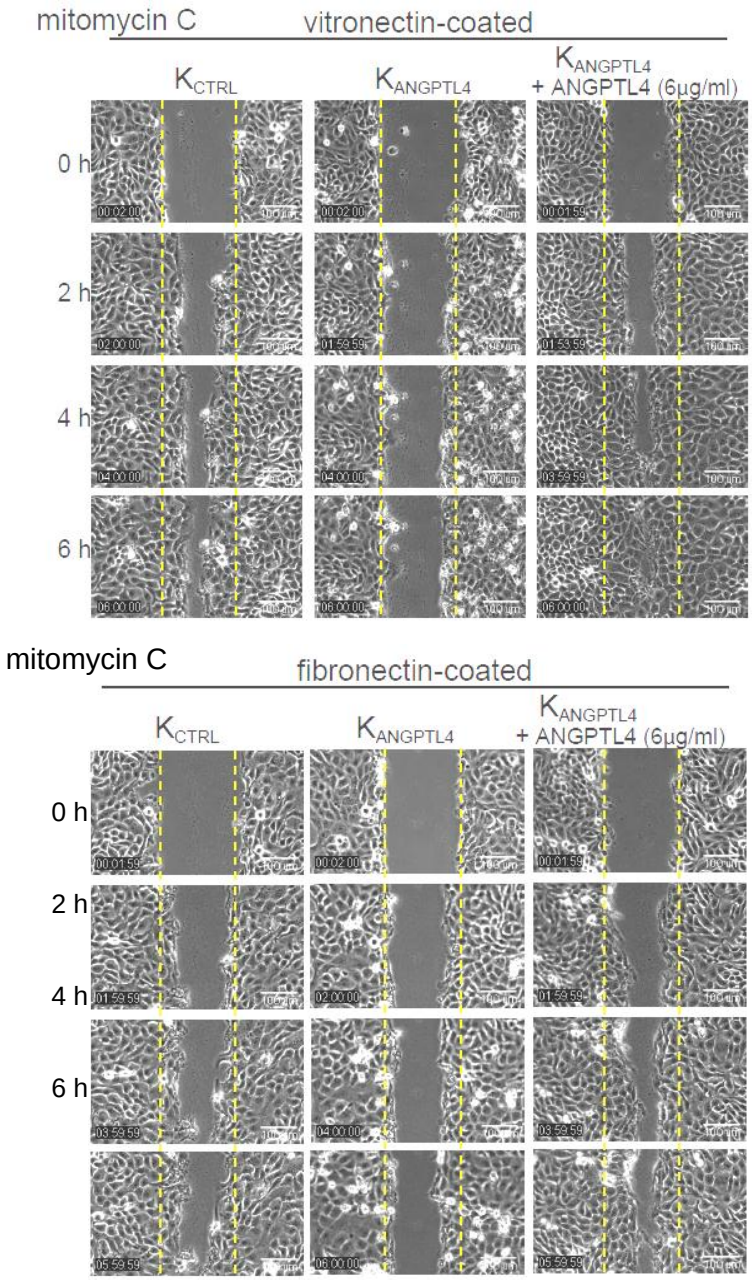

Figure 1.21 ANGPTL4 modulates cell migration on matrix-protein-coated plate. Representative time-lapsed images of wounded cultures of K_{CTRL} and $K_{ANGPTL4}$, and with recombinant cANGPTL4-treated $K_{ANGPTL4}$ in the presence of mitomycin C (2 µg/ml). Yellow dotted lines represent the scratch gap at the time of wounding. Scale bar 100 µm.

1.4.5 ANGPTL4 Modulates Integrin-Mediated Signaling and Internalization

To gain insight into the intracellular signaling pathway, we performed *in vivo* co-immunoprecipitation using either anti-cANGPTL4 or anti-integrin β1 antibodies, followed by immunodetection of specific mediators of integrin-mediated signaling. ANGPTL4 bound to integrin β1, recruited more FAK-Src complex with more active Rac1-GTP and phosphorylated PAK1 in the membrane fraction of *ANGPTL4*$^{+/+}$ and ANGPTL4-treated *ANGPTL4*$^{-/-}$ wounds than their cognate controls (Figures 1.22A & B). We detected cytoplasmic ERK, which does not interact directly with integrin, only in the supernatant of the immunoprecipitates, indicating the specificity of the affinity co-immunoprecipitation (Figures 1.22A & B). Neither integrin β1 nor ANGPTL4 was immunoprecipitated with pre-immune IgG (Figure 1.22C). Similar observations were also made in K_{CTRL} (Figure 1.22D & E), confirming that ANGPTL4 potentiates integrin-mediated signaling.

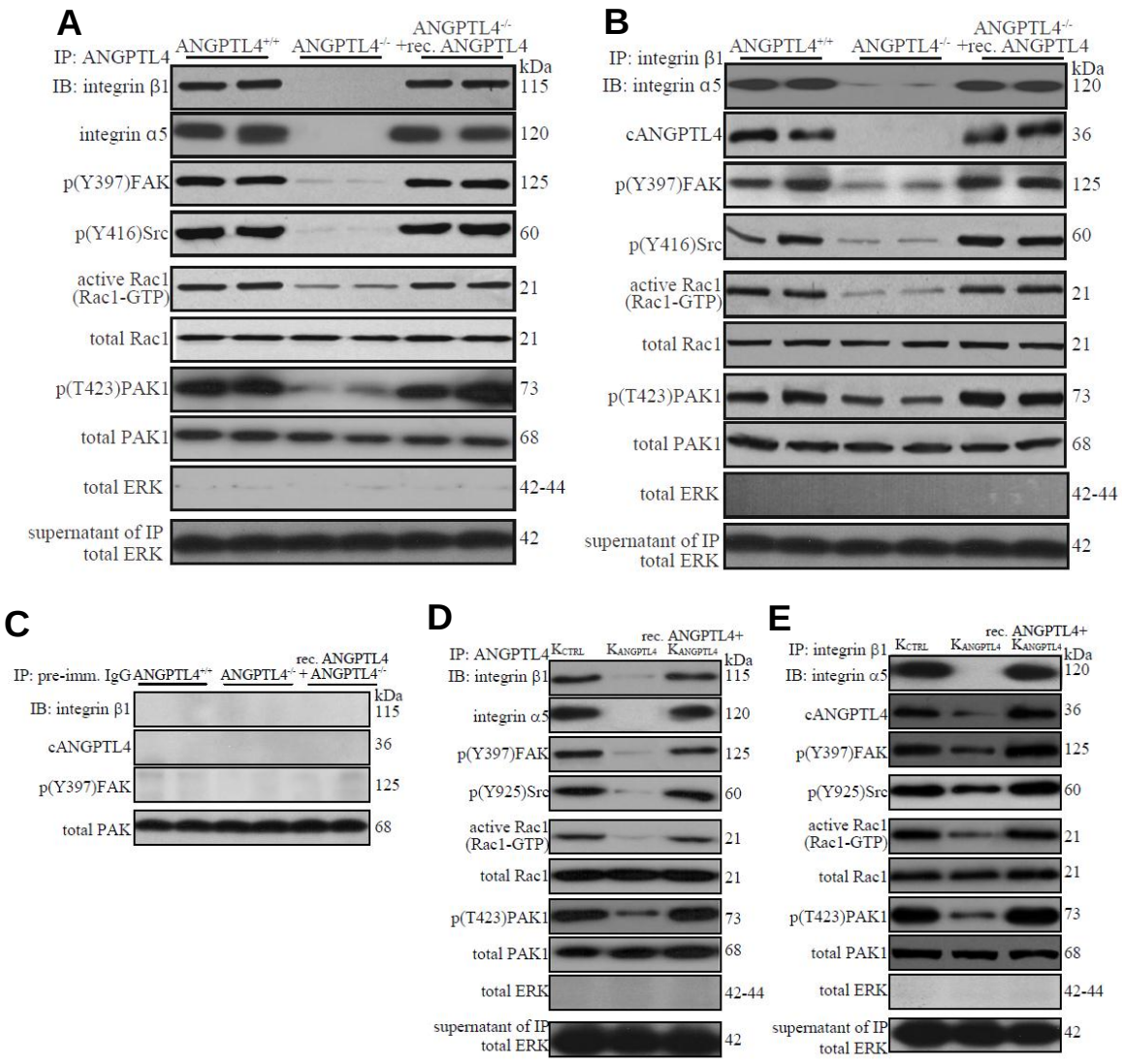

Figure 1.22 ANGPTL4 binds to integrin β1 to modulate FAK-dependent signaling. Immunodetection of indicated proteins in **(A)** anti-cANGPTL4 or **(B)** anti-integrin β1 immunoprecipitates (top panel); of Rac1-GTP and phosphorylated PAK1 (p(T423)PAK1) (lower panels) from membrane extract of indicated wound biopsies. Total PAK from total cell lysate were used to verify equal loading. Immunodetection of either integrin β1, integrin α5, ANGPTL4, phosphorylated focal adhesion kinase (p(Y397)FAK) or p(Y416)-Src in **(C)** pre-immune IgG immunoprecipitates of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$; **(D)** anti-cANGPTL4 or **(E)** anti-integrin β1 immunoprecipitates of K_{CTRL} and $K_{ANGPTL4}$ (top panel). Immunodetection of Rac1-GTP and phosphorylated PAK1 (p(T423)PAK1) (lower panels) from membrane extract of indicated cells or treatments. Recombinant ANGPTL4 (rec. ANGPTL4) was used at 6 µg/ml. Total PAK from total cell lysate were used to verify equal loading. Specificity of immunoprecipitation was verified by immunodetection of ERK in the immunoprecipitates and its supernatant.

Integrin recycling contributes to the motility of rapidly migrating cells and permits constant monitoring of the wound cellular environment (Jones et al., 2006). We observed a selective internalization of cell-surface biotin-labeled integrins β1 and β5, but not integrin β3. Importantly, the rapid internalization of integrins β1 and β5 was reduced in ANGPTL4 deficiency (Figure 1.23A). The internalization of integrin β3 was similar under all examined conditions (Figure 1.23A). These observations were further corroborated by FACS analysis of the cell-surface expression of integrins (Figures 1.23B & C). Similar results were also observed in K_{CTRL} treated with anti-cANGPTL4 antibody (Figure 1.23D).

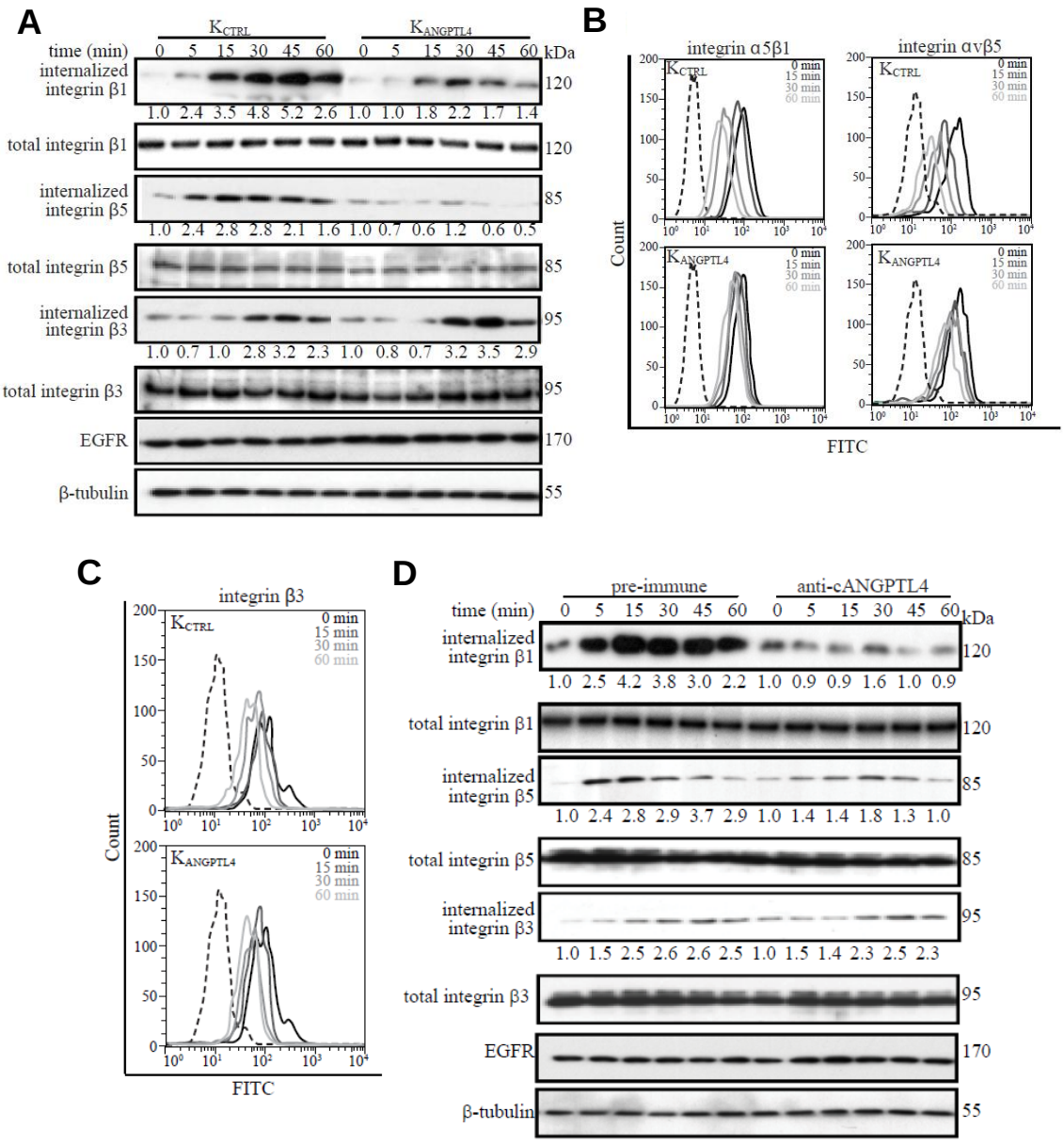

Figure 1.23 ANGPTL4 modulates integrin internalization. (A) Kinetics of integrin internalization. Internalized biotinylated-integrins were detected using corresponding antibodies after immunoprecipitation with NeutrAvidin agarose resins. The level of total integrins β1, β3 and β5 were determined using total cell lysate before immunoprecipitation. EGFR and β-tubulin from total cell lysate were used to verify equal loading. Values denote mean fold change of three independent experiments compared to K_{CTRL} at time zero. **(B)** Cell-surface expression of the integrins α5β1 and αvβ5 in K_{CTRL} and $K_{ANGPTL4}$ at the indicated time was determined by FACS. The negative control (only secondary antibody) is indicated by the dotted graph. **(C)** Cell-surface expression of the integrin β3 in K_{CTRL} and $K_{ANGPTL4}$ at the indicated time was determined by FACS. The negative control (only secondary antibody) is indicated by the dotted graph. **(D)** Kinetics of integrin internalization for keratinocytes treated with either pre-immune IgG or anti-cANGPTL4.

Integrins β1 and β5 are internalized with the aid of adaptor protein 14-3-3σ and protein kinase C alpha (PKCα), which binds directly to integrin cytoplasmic tails (Ng et al., 1999; Han et al., 2001). Prompted by our above observations, we examined the membrane expression of these proteins in ANGPTL4-deficient keratinocytes and wound biopsies. Immunodetection showed that the expression of 14-3-3σ, β and PKCα was significantly reduced in *ANGPTL4*$^{-/-}$ wound biopsies when compared with their cognate controls (Figure 1.24A). Besides reduced expression of 14-3-3σ, β and PKCα, the *ANGPTL4*$^{-/-}$ wounds also exhibited decreased expression of RACK1 (Panetti et al., 1995), indicating attenuated PKC-mediated signal transduction (Figure 1.21A). Similar findings were obtained in keratinocytes transiently transfected with *ANGPTL4*-siRNA, suggesting that the reduced levels of total signaling proteins observed is not an adaptation to the reduction in ANGPTL4 level (Figure 1.24B). To examine if ANGPTL4 has a direct effect on the expression of these signaling proteins, we examined their mRNA levels in $K_{ANGPTL4}$ treated with recombinant ANGPTL4 in the presence of either actinomycin D or cycloheximide. The increased mRNA levels of 14-3-3σ, β and PKCα induced by ANGPTL4 were abolished in actinomycin D- but not cycloheximide-treated cells, suggesting a transcriptional regulatory mechanism (Figure 1.24C). Thus, our results show that more activated FAK-Src complexes were formed when ANGPTL4 was bound to integrin β1, indicating that ANGPTL4 mediates its action at least partially via the FAK-Src-PAK1 axis. ANGPTL4 deficiency dysregulated 14-3-3σ and its effector PKCα expression, which would influence integrin internalization and thus keratinocyte migration. Altogether, our results reveal a novel function of ANGPTL4 in promoting keratinocyte migration during wound healing by activating integrin-mediated signaling and internalization.

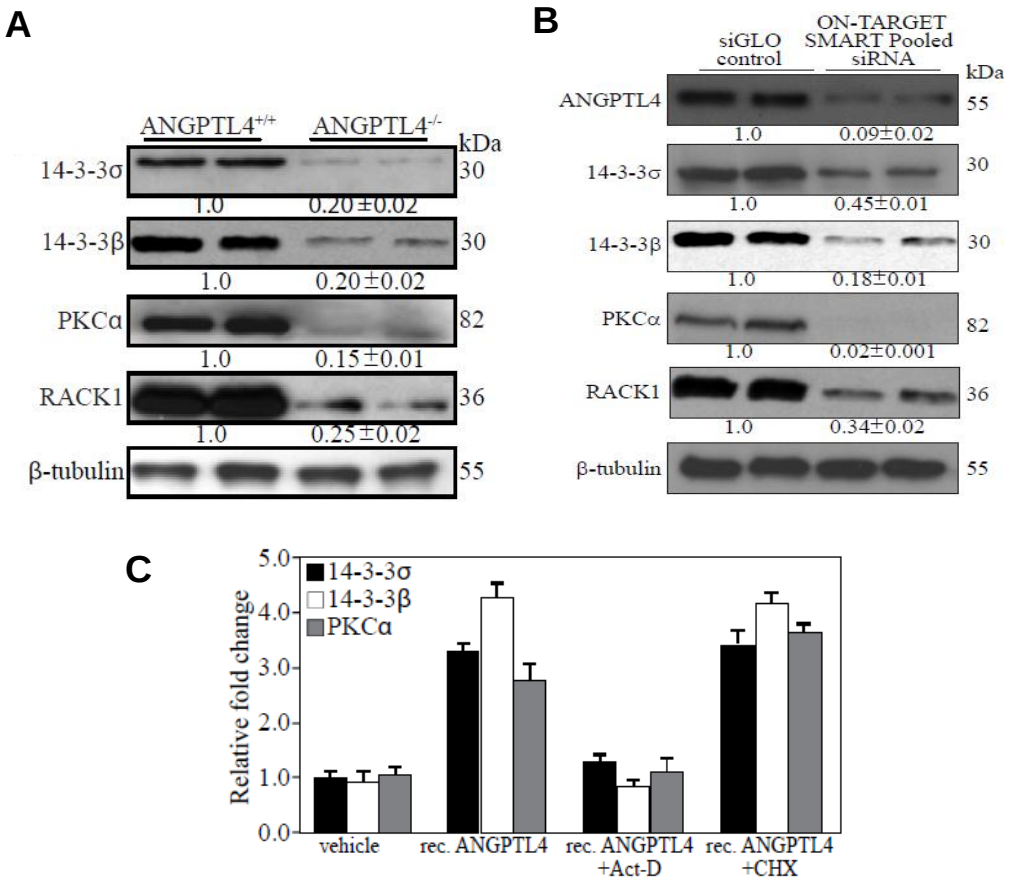

Figure 1.24 ANGPTL4 modulates transcriptional regulatory of 14-3-3 and PKC. (A) Immunoblot analysis of membrane extracts from day-5 *ANGPTL4*$^{+/+}$ and *ANGPTL4*$^{-/-}$ wound biopsies for indicated proteins. Values below the band represent the mean fold differences in protein expression levels relative to *ANGPTL4*$^{+/+}$ from eight wound biopsies for each genotype. β-tubulin was used as loading and transfer control. **(B)** Immunoblot analysis of membrane extracts from keratinocytes transiently transfected with either siGLO control siRNA or pooled ANGPTL4 siRNA. Values below the band represent the mean fold differences in protein expression levels relative to ANGPTL4$^{+/+}$ from three transfection studies. β-tubulin was used as loading and transfer control. **(C)** Relative fold change in mRNA levels of 14-3-3σ, β and PKCα in $K_{ANGPTL4}$ treated with either vehicle, recombinant ANGPTL4 (rec. ANGPTL4, 6 μg/ml) in the absence or presence of RNA synthesis (actinomycin D, Act-D) or protein synthesis (cycloheximide, CHX) inhibitors determined by qPCR. Act-D and CHX treatment alone did not affect the transcript level. Ribosomal protein L27 was used as a normalizing reference gene. Values are mean ± SEM of three independent experiments.

1.5 DISCUSSION

Wound healing is a complex process that involves a cascade of overlapping events, including inflammation, re-epithelialization and remodeling, all directed at the restoration of the epidermal barrier. Throughout the healing process, cellular interactions with ECM components coordinate the individual events, enabling temporal and spatial control. Re-epithelialization is accomplished by increased keratinocyte proliferation and guided migration of the keratinocytes over the wound ECM. This process requires orderly changes in keratinocyte behavior and phenotypes; in which integrin-mediated signaling plays a crucial role. We reveal a newly discovered role for ANGPTL4 in cell migration via direct interaction with integrin β1 and β5 to modulate integrin-mediated FAK-Src-PAK1 signaling and internalization (Figure 1.25).

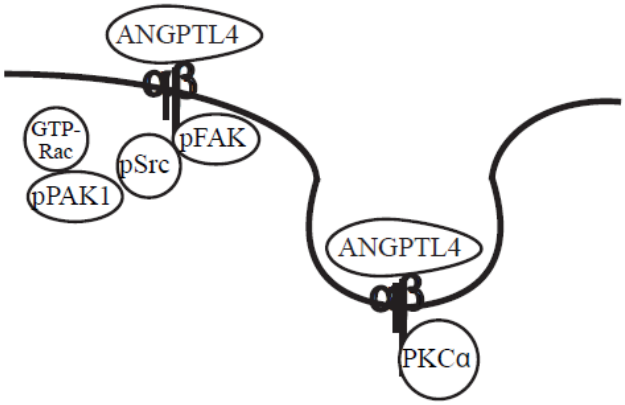

Figure 1.25 Schematic illustration showing ANGPTL4 interacting with integrin, activating FAK-Src-PAK1 signaling and facilitating integrin internalization, which involves PKCα and 14-3-3σ/β, to aid cell migration. PKCα is abundance in the cytoplasm, and hence it will be difficult to detect any translocation from cytoplasmic pool to the focal adhesion sites. It is also unlikely to have a distinct pool of PKCα for activation.

Adipocytokines secreted by adipose tissue play important roles in energy homeostasis (Bulcao et al., 2006). Emerging evidence points to additional non-metabolic roles, such as wound healing, of some adipocytokines. We show that the expression of the adipocytokine ANGPTL4, while only weakly detectable in normal intact skin, was markedly elevated during the re-epithelialization phase of wound healing. ANGPTL4 deficiency had a dramatic impact on cell migration *in vitro* and *in vivo*. Thus, in addition to its well-established role in energy homeostasis, we revealed an unsuspected role for ANGPTL4 as a matricellular protein in wound repair. Multiple functions of other matricellular proteins have been described, including SPARC, which is implicated in adipose tissue hyperplasia and adipogenesis (Bradshaw and Sage, 2001). ANGPTL4 undergoes proteolytic cleavage after secretion, to release the N-terminal coiled-coil domain (nANGPTL4) and a C-terminal fibrinogen-like domain (cANGPTL4). nANGPTL4 binds LPL and inhibits its activity (Sukonina et al., 2006), but little is known about the role of cANGPTL4. Therefore, how ANGPTL4 triggers intracellular signaling to propagate its effect remains a central question in relation to its functions. Although ANGPTL4 is related to angiopoietins, it does not bind to the Tie receptors (Kim et al., 2000). Using various methods, we identify integrin β1 and β5, but not β3, as novel interacting protein partners of ANGPTL4. We further show that the fibrinogen-like domain of ANGPTL4 associates with heterodimeric integrins, via the β1/β5 subunits and modulates integrin-mediated signaling, revealing crucial insight into its mechanism of action. This interaction modulates the FAK-Src-PAK1 signaling cascade, which is essential for keratinocyte migration (Schlaepfer and Mitra, 2004).

Integrins on the cell surface are well suited to function as biosensors to constantly monitor changes in the wound microenvironment. A few types of the variants (e.g. α2β1, α3β1 and α6β4) are found on normal keratinocytes and the variants changed during

wounding (e.g. α5β1, αvβ5, and α3β1) (Grinnell et al., 1992; Watt and Jones, 1993). Each αβ combination possesses specific binding and signaling properties. During wound healing, migrating keratinocytes enlarge their integrin repertoire concomitantly with changes in the extracellular matrix composition, suggesting a close interplay of these two groups of molecules during re-epithelialization. ANGPTL4 interacts with the β subunits of α5β1, αvβ5 and α3β1 to enhance integrin-mediated signaling, integrin internalization and keratinocyte migration. Consistent with the role of α5β1and αvβ5 in facilitating cell migration and adhesion, they showed increased expression in wound keratinocytes and their deficiencies have been associated with impaired cell migration, adhesion or wound healing (Bata-Csorgo et al., 1998; Huang et al., 2000; Grose et al., 2002). The role of integrin α3β1 in cell migration is controversial (Wen et al., 2010) depending on the complex of the various matrices present at the wound bed and, more importantly, on the context in which the intact matrix protein is presented to the cells. Small soluble matrix protein fragments generated by the action of proteases during re-epithelialization can compete with substrate-anchored matrix proteins for integrin and impair cell migration (Ginsberg et al., 2005; Giannone and Sheetz, 2006). We also showed that ANGPTL4 binding to specific integrins does not interfere with the association of integrins and their cognate matrix ligands. Although not studied here, it is tempting to speculate that ANGPTL4 may also interact with specific matrix proteins and form a ternary complex with its cognate integrin receptor to further fine-tune the cell-matrix communication that is crucial for cell migration.

During wound healing, migrating cells must display the appropriate cellular behavior in response to the changing wound environment to enable effective wound closure. Interestingly, deficiency in ANGPTL4 resulted in decreased expression of 14-3-3σ, β and PKCα, which also modulates cell migration via integrin internalization. The

underlying mechanism by which ANGPTL4 regulates their expression remains to be determined. Thus, ANGPTL4 is a novel matricellular protein that modulates keratinocyte migration on at least two fronts. First, ANGPTL4 potentiates integrin-mediated signaling to facilitate cell migration. ANGPTL4 binding to specific integrins does not interfere with the association of integrins and their cognate matrix ligands. Second, ANGPTL4-bound integrins provide a novel means by which selective integrin signaling cascades can be activated, depending on the local context of the ECM. ANGPTL4 regulates 14-3-3σ and its effector PKCα expression, which would influence integrin internalization. This allows migrating wound keratinocytes to better scrutinize the changes in the wound ECM and fine-tune their cellular behavior.

Tumour progression has been likened to the misregulation of wound healing processes. Immediately following acute injury, the wound becomes inflamed and temporarily hypoxic. The wound initiates a complex cascade of processes that recruit inflammatory cells, stimulate epithelial cell proliferation, cell migration, and induce angiogenesis to restore tissue integrity and function. These processes are temporally and spatially coordinated by cytokines, growth factors and extracellular matrix (ECM)-associated factors. Similarly, tumour cells are surrounded by an inflammatory and hypoxic milieu. Many of the repair events are constitutively active in the tumour and are critical for tumour growth, invasiveness and metastasis to distant organs. The present work on skin wound healing have identified ANGPTL4 as a novel matricellular protein with a beneficial effect on wound healing through the coordination of cellular behaviours during cell-matrix communications (Goh et al., 2010b). Transient inflammation-induced upregulation of ANGPTL4 promotes the interaction of ANGPTL4 with specific integrins and ECM proteins to modulate keratinocyte migration. Recent studies have revealed ANGPTL4 as a critical mediator in several hallmarks of cancer, which include resisting

cell death by anoikis and facilitating tumour metastasis, and possibly tumour cell metabolism (Minn et al., 2005; Minn et al., 2007; Padua et al., 2008).

Enhanced migration and proliferation of transformed epithelial cells is a principal characteristic of cancer metastasis, similarly observed during the re-epithelialization phase of wound healing. In wound healing and cancer, there is a consistent elevated level of cANGPTL, the C-terminus fibrinogen-like domain of ANGPTL4. We also showed that an interacting protein partner of cANGPTL4 is integrin β1. Indeed, recent studies have identified that β1 integrins are required for migration and proliferation for both wound keratinocytes and carcinoma cells (Grose et al., 2002; White et al., 2004). The chemokines, stromal-cell-derive factor 1 (SDF1), hepatocyte growth factor (HGF) and members of epidermal growth factor (EGF) family, are found upregulated in both wound and tumours. Their receptors and signal transducers (STAT3) are activated in both cases.

In contrast to the positive regulators of epithelial repair, transforming growth factor-β (TGF-β) and its downstream effector SMAD3 are negative regulators of wound re-epithelialization (Ashcroft et al., 1999; Wakefield and Roberts, 2002). This signaling pathway also inhibits proliferation of many carcinoma cells, at least in the early stages of tumorigenesis.

Divergent functions of growth factors in wound healing and cancer have also been reported. For example, the expression of a dominant-negative mutant of fibroblast growth factor (FGF) receptor-2-IIIb in the epidermis of transgenic mice caused impaired wound re-epithelialization (Grose et al., 2007). Unexpectedly, mice that lacked this receptor in the epidermis were more susceptible to chemically induced skin carcinogenesis, demonstrating a tumour-suppressive effect of this type of receptor. Therefore wound re-epithelialzation and growth of epithelial tumours are not always regulated by the same factors, and it will be important to identify the molecular mechanisms underlying these differences.

With respect to ANGPTL4, we noted that the expression of ANGPTL4 is transiently upregulated during re-epithelialization. It peaks at days 3-7 post injury, and thereafter maintained at low level until the remodeling phase (Figure 1.5). In contrast, cancer tissues exhibited sustained elevated level of ANGPTL4 (Zhu et al., 2011). Notably, metastatic tumor expressed high levels of ANGPTL4 to resist anoikis by stimulating NADPH oxidase-dependent production of $O2^{-}$, which activiate Src-dependent PI3K/PKBα and ERK prosurvival pathways.

Emerging evidence has suggested that ANGPTL4 function is drastically altered depending on the proteolytic processing and post-translational modifications of ANGPTL4. For example, in kidney podocytes (Clement et al., 2011) the hyposiaylation of ANGPTL4 has an impact on glomerulus leakiness. During lipid metabolism, the ability of ANGPTL4 to inhibit LPL is dependent on the processing of native ANGPTL4 (Lei et al., 2011). Although not examined in my studies, it is conceivable that processing of native ANGPTL4 may differ between wound and cancer.

Previous studies have reported that ANGPTL4 prevents metastasis by inhibiting vascular leakiness (Ito et al., 2003; Galaup et al., 2006). By contrast, recent work has revealed that one of the genes most highly associated with breast cancer metastasis to the lung is ANGPTL4 (Minn et al., 2005). Tumor-derived ANGPTL4 was proposed to disrupt endothelial cell-cell contacts that would aid the extravasation and metastasis of tumor cells (Padua et al., 2008). Therefore, whether ANGPTL4 promotes or inhibits vascular leakiness and thus cancer metastasis remains controversial. Although this question is not directly addressed in this study, our data clearly showed that ANGPTL4 binds to integrin β1, and it has been shown that neutralizing antibody against integrin α5β1 increases paracellular endothelial permeability (Lampugnani et al., 1991). Altogether, our findings that ANGPTL4 interacts with integrins β1 and β5 to modulate

integrin-FAK-Src-PAK1 signaling and integrin internalization provide valuable mechanistic insight into its roles in cancer metastasis.

1.6 CONCLUSION

It is known that ANGPTL4 binds to LPL and converts the enzyme from catalytically active dimers to inactive monomers via its coiled-coil N-terminal domain (Sukonina et al., 2006), however little is known about the role of the fibrinogen-like C-terminal domain of ANGPTL4, its binding partners, the precise mechanism and the intracellular signaling cascade mediated by ANGPTL4 (Li, 2006). We showed that the cANGPTL4 interacts with specific integrins and modulates integrin-mediated signaling; this has provided crucial insight into its molecular mechanism. Metastasis and wound repair share numerous characteristics during cell migration, thus it was not surprising that ANGPTL4 has been implicated in cancer metastasis (Steeg, 2006; Minn et al., 2005). It was reported that ANGPTL4 facilitated cancer metastasis by disrupting endothelial cell-cell contacts, but the mechanism remains unknown (Padua et al., 2008). Our findings provide a novel role for cANGPTL4 in wound healing through its interactions with and effects on integrins, and underscore its roles in metastasis. Indeed, recent work from other lab members showed that cANGPTL4 instigated the disruption of endothelial continuity by directly interacting with three novel binding partners, integrin α5β1, VE-cadherin and claudin-5, in a temporally sequential manner, thus facilitating metastasis (unpublished data). Interestingly, it also involved the activation of integrin α5β1–mediated Rac1/PAK signaling to weaken cell–cell contacts.

1.7 FUTURE STUDIES

1) The Role of ANGPTL4 in Wound Healing for Diabetic Patients

The role of ANGPTL4 in systemic glucose homeostasis is complex. The serum levels of ANGPTL4 were shown to be significantly lower in patients with type 2 diabetes than those in healthy subjects (Xu et al., 2005). However, the results are controversial because the antibodies used in the detection method may not be able to accurate determine the amount of oligomerized ANGPTL4 in the plasma (our unpublished data). Furthermore, the correlation between plasma ANGPTL4 and glucose may be complicated by the role of ANGPTL4 in lipid homeostasis which is mediated by the N-terminus coil-coiled fragment of ANGPTL4 (nANGPTL4).

Impaired wound healing in diabetic patients is a condition that predisposes a person to a high rate of medical complications. Dysregulation of growth factors and cytokines involved in inflammation, re-epithelialization, and angiogenesis is the underlying reason for impaired healing. To understand the interplay among these factors and discover possible therapeutic targets, *in vivo* data using mouse and human model can provide invaluable information. Microarray data from the skin of chronic (potentially diabetic) human wounds and normal skin has been analyzed. By analyzing the dynamics of gene expression during wound healing in wildtype (+/+) and diabetic (ob/ob) mice, we discovered that inflammatory mediators like TNFα, GM-CFS and IL-10, and angiogenic factors like VEGF and ANGPTL4 were aberrantly expressed. Validation of the data using realtime PCR and western blot will be performed. Administration of recombinant ANGPTL4 proteins, or manipulation of other factors to reverse the aberrant healing process and consequently, alleviate the impaired wound healing in diabetic mice may be tested.

2) The Role of ANGPTL4 in Tumor Development and Cancer Cell Migration.

The present study shows that ANGPTL4 facilitates cell migration through integrin-FAK-Src and 14-3-3-PKC signaling cascades. Since ANGPTL4 is one of the most predictive genes to signal the metastasis of breast cancer to the lung, it is important to examine the role of ANGPTL4 in cancer metastasis (Minn et al., 2005). First, the expression level of ANGPTL4 in various cancers from different organs can first be compared with the non-diseased stage. It is also not clear if the expression pattern of ANGPTL4 in different stages of tumor development is consistent. The ability of ANGPTL4 to facilitate tumor growth and metastasis can be determined by suppressing the expression of ANGPTL4 in various cancer cell lines, followed by injecting the cells into a mouse model. Since prior studies indicated that ANGPTL4 affected the angiogenesis (Kim et al., 2000; Cazes et al., 2006) and vascular permeability of endothelial cells (Galaup et al., 2006; Padua et al., 2008), further investigation of the role of ANGPTL4 in endothelial cells is required. Angiogenesis is inhibited when ANGPTL4 is bound to the ECM (Cazes et al., 2006). However, we hypothesize that when it is expressed in excess or when it is bound to the surface receptor of endothelial cells, the opposite effect may occur. This is highly possible during cancer cell migration, especially when ECM proteins are degraded (Giannelli et al., 1997).

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CHAPTER 2

ANGIOPOIETIN-LIKE 4 INTERACTS WITH MATRIX PROTEINS TO MODULATE WOUND HEALING

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Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing.

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2.1 ABSTRACT

A dynamic cell-matrix interaction is crucial for rapid cellular responses to changes in the environment. Appropriate cell behavior in response to the changing wound environment is required for efficient wound closure. However, the way in which wound keratinocytes modify the wound environment to coordinate with such cellular responses remains poorly understood. We demonstrated that ANGPTL4 produced by wound keratinocytes coordinates cell-matrix communication. ANGPTL4 interacts with vitronectin and fibronectin in the wound bed, delaying their proteolytic degradation by MMPs. This interaction does not interfere with integrin-matrix protein recognition, and directly affects cell-matrix communication by altering the availability of intact matrix proteins. These interactions stimulate integrin-FAK, 14-3-3 and PKC-mediated signaling pathways that are essential for effective wound healing. Deficiency of ANGPTL4 in mice delays wound re-epithelialization. Further analyses revealed that cell migration was impaired in the ANGPTL4-deficient keratinocytes. Altogether, the findings provide molecular insight into a novel control of wound healing via ANGPTL4-dependent regulation of cell-matrix communication. Given the known role of ANGPTL4 in glucose and lipid homeostasis, it is a prime therapeutic candidate for the treatment of diabetic wounds. It also underscores the importance of cell-matrix communication during angiogenesis and cancer metastasis.

2.2 INTRODUCTION

2.2.1 Wound Healing

Skin repair after an injury proceeds via a finely tuned pattern of integrated biological events aimed at the restoration of the epithelial barrier. The inflammatory stage of repair is followed by the proliferation and migration of keratinocytes, a process called re-epithelialization (Hynes, 2002). The barrier integrity of epithelial cells is restored after migrating cells have covered the denuded wound area. Understanding the precise molecular mechanism of wound repair regulation *in vivo* is important in developing effective therapeutic strategies and wound management.

Wound healing processes include inflammation, cell migration and proliferation (re-epithelialization), and extracellular matrix (ECM) remodelling (Martin, 1997; Segre, 2006). These events are regulated spatio-temporally by several classical growth factors and cytokines, the effects of which have been well-documented (Werner and Grose, 2003). Less studied are extracellular factors, such as matricellular proteins and adipocytokines, which have been shown to have a profound local impact during wound repair (Bornstein and Sage, 2002; Bulcao et al., 2006). The detail descriptions of the various events during wound repair are described in Chapter 1, section 1.2.3.

2.2.2 The Effect of Substrate–Anchored and Small Soluble Matrix Proteins on Cell Migration

Effective directed cell migration requires constant cellular interaction with the ECM in response to the changing wound environment. During the entire wound repair process, the ECM composition is constantly being remodeled as cells build and reshape the ECM by degrading and reassembling it (Daley et al., 2008). This has a direct effect on cell-matrix communication and consequently the behavior of epithelial cells. The ECM can be remodeled in response to signals transmitted by ECM receptors, such as

integrins, or ECM-modifying proteins, such as matrix metalloproteinases (MMPs) (Page-McCaw et al., 2007). Although the importance of cell-matrix communication in wound healing is well recognized, the mechanism that modifies the external wound microenvironment for coordinated keratinocyte behavior remains unclear.

Integrins on the cell surface often function as biosensors to constantly interrogate the wound environment and modulate cell responses accordingly. Binding of integrins to their cognate matrix proteins activates intracellular signaling pathways that modulate a broad range of cellular processes, including cell migration (Caswell and Norman, 2006). Integrin binding to the ECM induces the clustering and recruitment of scaffolding proteins that connect integrins to the actin cytoskeleton, activation of tyrosine kinase and phosphatase signaling, and the coupling of cell-generated forces with the ECM (Geiger et al., 2001). This interaction provides mechanical resistance that permits tensile forces to be generated via the acto-myosin system (Ginsberg et al., 2005). Forces applied to ECM-integrin-cytoskeleton connections, which can be generated by internal actin or external ECM motion, induce maturation of adhesion sites to focal adhesions, which are coupled to bundles of actin called stress fibers. In contrast, loss of force triggers the disassembly of stress fibers and adhesion sites (Balaban et al., 2001). In addition, small soluble matrix protein fragments generated by the action of proteases during re-epithelialization can compete with substrate-anchored matrix proteins for integrin binding and impair cell signalling (Choquet et al., 1997). Thus, productive integrin signaling depends on the context in which the intact matrix protein is presented to cells (Giannone and Sheetz, 2006). However, the way in which migrating wound keratinocytes coordinate the balance between substrate-anchored and small soluble matrix protein fragments by the specific induction of wound integrins requires further investigation.

2.2.3 Transcriptional Regulation of Cell-Matrix Interactions

Transcriptional regulation plays an important role in the control of keratinocyte behavior at different phases of wound repair (Schafer and Werner, 2007). for examples, the AP-1 transcription factors regulate the expression of MMPs, integrins, and growth factors involved in the re-epithelialization process (Angel et al., 2001). While Stat3 is a positive regulator of keratinocyte migration at the wound site (Sano et al., 1999), smad3 is a negative regulator during re-epithelialization (Flanders et al., 2003). However, little is known about the mechanism that modifies the wound microenvironment to coordinate with the changes in cellular behavior for cell-matrix communication. Effective cell-matrix communication is crucial for efficient wound healing.

Nuclear hormone receptors (NRs), one of the largest known classes of transcription factors, have been implicated in wound repair (Michalik et al., 2001; Tan et al., 2007). In humans, this superfamily comprises 48 ligand-dependent or "orphan" transcription factors. Agonist and antagonist drugs that target the NR family constitute one of the largest and most potent groups of pharmaceuticals currently in use, and thus hold great potential for use in improved wound care management (Grose et al., 2002b; Tan et al., 2003). Of interest, studies have shown that the nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR) β/δ is an early transcription factor that modulates the keratinocyte response to inflammation during wound healing (Tan et al., 2001; Tan et al., 2004a). Most studies have focused on intracellular signaling or the events mediated by PPARβ/δ that were important for keratinocyte survival and migration (Tan et al., 2001; Di Poi et al., 2002; Tan et al., 2007). PPARβ/δ gene expression results antiapoptotic effect mediated via integrin-linked kinase (ILK) and 3-phosphoinositide-dependent kinase 1 (PDK1) pathways (Tan et al., 2001; Tan et al., 2005), resulting in the activation of the antiapoptotic protein protein kinase B (PKB)/Akt1 (Di Poi et al., 2002). An interesting interaction between PPARβ/δ, AP-1, and Smad3 were identified, where

Smad3 inhibited the binding of the c-Jun-p300 complex on the PPARβ/δ promoter, resulting in the reduced expression of PPARβ/δ (Tan et al., 2004b). This example highlights the importance of transcriptional networks in the regulation of the wound healing process of PPARβ/δ. In addition, PPARβ/δ activity amplifies the response of keratinocytes to a chemotactic signal, promotes integrin recycling and remodeling of the actin cytoskeleton, and thereby favors cell migration (Tan et al., 2007). However, the mechanism by which PPARβ/δ alters the wound microenvironment for effective cell-matrix communication remains unknown. Conceivably, as an intracellular transcription factor, PPARβ/δ is likely to alter the wound microenvironment via extracellular factors.

2.2.4 Angiopoietin-Like 4 – a New Player in Cell-Matrix Communication

We showed that ANGPTL4 plays a novel role in keratinocyte migration during skin wound healing (see findings of Chapter 1). The full-length ANGPTL4 and the cANGPTL4 produced by wound keratinocytes during the inflammation, re-epithelialization phases of wound healing coordinate cell-matrix communication in several ways. ANGPTL4 specifically interacts with integrins β1 and β5 residing on the surface of the wound keratinocytes, which activate integrin-mediated intracellular signaling, allow for selective integrin recycling, enhances cell migration, and accelerates the wound healing process. Numerous intracellular signalling pathways, including FAK-, Rho GTPases and 14-3-3σ-dependent signalling cascades that emanate from integrins to modulate cell migration are activated upon activation by ANGPTL4. ANGPTL4-deficient mice exhibit delayed wound re-epithelialization with impaired keratinocyte migration.

Normal wound healing entails a continuum of events that includes inflammation, re-epithelialization and matrix remodelling, and involves a complex interplay among

connective tissue formation, cellular activity, and growth factor activation (Werner and Grose, 2003). Extracellular matrix (ECM) components integrate each phase during wound healing, communicating with cells and growth factors in a dynamic reciprocal manner that eventually results in proper wound closure. Our findings described in this chapter shows that ANGPTL4 is a PPARβ/δ target gene in keratinocytes and that its expression is elevated after injury. We show that ANGPTL4 produced by wound keratinocytes coordinates cell-matrix communication. Specifically, ANGPTL4 interacts with vitronectin and fibronectin in the wound bed, delaying their proteolytic degradation by metalloproteinases, thereby regulating the availability of the local ECM. This interaction does not interfere with the binding of a matrix protein to its cognate integrin receptor or with integrin-mediated signalling. Thus, ANGPTL4 is a novel matricellular protein. Our findings reveal a novel control of the wound environment by keratinocytes that coordinates the dynamic interactions between integrins and components of extracellular matrices.

2.3 MATERIALS AND METHODS

2.3.1 Reagents: Sensor CM5 chips, amine coupling kits, and immobiline pK buffers were from GE Healthcare; purified vitronectin, fibronectin and laminin were from Calbiochem; transfection reagent ExGen 500 was from Fermentas; real-time PCR KAPA SYBR Fast Master mix was from KAPABiosystem; DUOlink proximity ligation assay was from Olink Bioscience; *Drosophila* Schneider 2 (S2) expression vector harboring a proprietary secretory signal pSSAc5.1/V5-His A was as previously described (Tan et al., 2002); double promoter pFIV-U1/H6-Puro lentivirus-based siRNA vector (Cat. #SI110A-1) and pPACKF1 packaging plasmid kit were from System Biosciences; Purified matrix proteins were purchased from Sigma-Aldrich. All chemicals were from Sigma-Aldrich unless otherwise stated.

Antibodies: PAK, LIMK1, PKBα and their cognate phosphorylated forms were from Cell Signaling; Rac1 and cdc42 were from Cytoskeleton; β-tubulin, His-tag, laminin, fibronectin, MMPs and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology; vitronectin and integrin αvβ5 were from Chemicon; keratin 6 for wound keratinocytes and hair follicle, α-smooth muscle actin for myofibroblasts, F4/80 for macrophages and CD31 for endothelial cells were from BioLegend; anti-human PPARβ/δ monoclonal antibodies were from Perseus Proteomics Inc., Japan; rabbit polyclonal antibodies against the C-terminal region of human (186-406 amino acid) and mouse (190-410 amino acids) ANGPTL4 were produced in-house. Briefly, female rabbits (New Zealand White, 2-2.5kg) were injected intramuscularly with 300 µg of recombinant proteins homogenized with 500 µl of complete Freund's adjuvant solution. First and second booster immunization with the same immunization dose were performed three and six weeks after priming immunization using incomplete Freund's adjuvant, respectively. Final harvest was done by bleeding a whole blood volume and the rabbits were then culled with injection of euthanasia into the marginal ear vein. The carcasses

were disposed after confirming no heart beat and corneal reflex, pedal reflex reactions. Pre-immune blood sampling was collected as negative control. Antibodies were purified by Protein A affinity chromatography as recommended by manufacturer (GE Healthcare).

2.3.2 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 a 5% CO_2, 37 °C humidified incubator. This medium is a modification of the keratinocyte medium previously described (Rheinwald and Green, 1975; Rheinwald and Green, 1977). 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/cm^2) 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 $2.5x10^3$ cells/cm^2.

2.3.3 Chromatin Immunoprecipitation (ChIP)

chIP was performed according to the manufacturer's (Upstate Biotechnology) instructions with some modifications. Briefly, chIP assay was performed using the monoclonal PPARβ/δ antibody. Cells were treated with 1% formaldehyde at 37 ^oC for 15 min. Cross-linked DNA was sonicated to form fragments ranging from 200-500 bp in length. DNA fragments were reverse cross-linked at 65 ^oC for 6 h. The DNA was subsequently purified using Qiaquick column (Qiagen). DNA was amplified by PCR for 20-23 cycles. The chIP primers for the amplification of the PPAR-response element (PPRE) of the human ANGPTL4 gene were as previously described (Schug et al., 2007).

2.3.4 Skin Wounding Experiment

Wounding was performed as previously described (Chong et al., 2009; Tan et al., 2005). Briefly, the hair follicle cycle of each mouse was synchronized by shaving the back of the animal two weeks before the start of the experiment. After anesthetizing, the mice were shaven. A full thickness mid-dorsal wound (0.5-cm^2, square-shaped) was created by excising the skin and the underlying *panniculus carnosus*. Wound closure was measured daily in a double-blinded fashion until it was complete. At indicated days post-wounding, the entire wound, including a 5 mm margin, was excised. Wounds were dissected for immunohistochemistry, RNA and protein analyses (Chong et al., 2009; Tan et al., 2009). Six-week old PPARβ/$\delta^{+/+}$ and $^{-/-}$ (Tan et al., 2005) and pure bred wild type (ANGPTL4$^{+/+}$) and ANGPTL4-knockout (ANGPTL4$^{-/-}$) male mice were used (Koster et al., 2005). All mice used in this study had a C57BL/6 background and were individually caged, housed in a temperature-controlled room (23 ^{o}C) on a 10 h dark/ 14 h light cycle, and fed with the standard mouse chow diet. Animal experiments were approved by the University Institutional Animal Care and Use Committee (ARF-SBS/NIE-A-0093, ARF SBS/NIE-A-0078 and ARF SBS/NIE-A-004).

2.3.5 Western Blot and Immunofluorescence Assays

Cells or tissues were lysed in ice-cold lysis buffer (20 mM NaH_2PO_4, 250 mM NaCl, 1% Triton X-100, 0.1 % SDS) supplemented with complete protease inhibitors (Roche). Equal amounts of protein extracts were resolved by SDS-PAGE and electroblotted onto PVDF membranes for Western analysis. Membranes were processed according to standard protocol and developed using chemiluminescence (Millipore). Equal loading/ transfer was verified by Coomassie staining of gels or by immunodetection of β-tubulin. Wound biopsies were fixed with 4% paraformaldehyde in PBS for 2 h at 25 ^{o}C. The

fixed tissues were centrally bissected, washed twice with PBS and embedded in Tissue-Tek OCT compound medium (Sakura) overnight at 4 ^{o}C. The tissues were subsequently frozen at -70 ^{o}C for cryosectioning. Cryostat sections (8 μm) mounted on SuperFrost Plus slides were analyzed by immunofluorescence as previously described, except that anti-ANGPTL4 antibodies were used (Chong et al., 2009). The slides presenting the largest wound diameter was defined as the wound center. As a control for immunofluorescence staining, 10-fold more peptide antigen was pre-incubated with anti-ANGPTL4 at 4 ^{o}C for 1 h before use. Images were taken using a LSM710 confocal laser scanning microscope with a Plan-Apochromat 40x/1.40 oil objective and ZEN software (Carl Zeiss).

2.3.6 Laser-Capture Microdissection (LCM)

Paraffin-embedded sections of PPARβ/$\delta^{+/+}$ and $^{-/-}$ wounds (10 μm) were mounted onto MembraneSlides (Carl Zeiss). Hematoxylin and eosin (H&E) stained sections were then subjected to LCM using PALM MicroBeam according to the manufacturer's instructions (Carl Zeiss). LCM tissues were collected into microfuge tubes with opaque AdhesiveCaps (Carl Zeiss). RNA was extracted using Optimum™ FFPE RNA Isolation kit (Ambion) pooled from 8 LCM tissues. RNA was reverse transcribed using random primers and the resulting cDNA was used for real-time PCR.

2.3.7 Expression and Purification of Recombinant ANGPTL4 Proteins

The cDNA sequences encoding human full-length ANGPTL4, nANGPTL4 and cANGPTL4 were amplified by Pfu polymerase and subcloned into pSSAc5.1/V5-His-A (Tan et al., 2002). A histidine tag was introduced between the secretory signal and the ANGPTL4 cDNA. All ligated products were transformed into competent *E. coli* Top 10 bacteria and selected on Luria broth agar plates containing 80 μg/ml ampicillin. Positive clones were confirmed by DNA sequencing. Positive constructs were co-transfected with

hygromycin expression vector pCoHygro (Invitrogen) into S2 cells. Recombinant ANGPTL4 proteins were purified from the conditioned medium of stable ANGPTL4-expressing S2 cells by preparative isoelectric membrane electrophoresis as described (Tan et al., 2000).

2.3.8 Surface Plasmon Resonance (SPR) Coupled to Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Purified cANGPTL4 was immobilized onto a CM5 carboxylated dextran sensor chip by amine coupling using the Surface Prep Module of BIACORE 3000 as recommended by the manufacturer (BIAcore). Acute wound fluid/exudate (WF) was collected from two patients undergoing split-thickness skin grafting. The acute wound fluid was collected daily under sterile conditions, from beneath a vapor-permeable membrane applied to the donor site and changed every 24 h for 3 days postoperatively. WF was centrifuged, aliquoted, and frozen at -70 °C. WF buffered with 50 mM Tris, pH 8.0, was introduced into the cANGPTL4-conjugated CM5 chip at a flow rate of 5 μl/min for 10 min with running buffer (50 mM Tris, pH 8.0, 100 mM NaCl). After incubation for 45 s, the chamber was washed with the same buffer, and the bound molecules were subsequently eluted using 10 mM glycine (pH 6.0) and collected in a recovery vial. The CM5 chip was reused to pool more samples after washing with running buffer for 10 min at 20 μl/min. The recovered cANGPTL4-binding proteins were digested with trypsin, reduced, alkylated and then analyzed with a Finnigan Surveyor HPLC system coupled online to a LTQ-Orbitrap mass spectrometer (Thermo Electron) equipped with a nano-spray source. Proteins were identified using a Mascot search. SPR was used to determine the dissociation constant of the interaction between fibronectin and vitronectin with recombinant cANGPTL4 immobilized onto a CM5 chip. Six concentrations (0.16, 0.32, 0.63, 1.25, 2.50 and 5.0 μM) of various matrix proteins were used. Each sensorgram was

corrected by subtracting a sensorgram obtained from a reference flow cell with no immobilized protein. Anti-cANGPTL4 antibodies run against the immobilized cANGPTL4 determined the Rmax value to be 251.8 resonance units (RU). Global fitting of the SPR data to a Langmuir 1:1 model was used to determine the dissociation constant (K_D) with Scrubber 2 software. Values are given as the mean ± S.D. of 5 independent preparations of recombinant proteins.

2.3.9 Affinity Co-Precipitation Assay

Purified recombinant His-tagged ANGPTL4, nANGPTL4 or cANGPTL4 was immobilized onto Ni-NTA resin (GE Healthcare). The resin was washed with wash buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.1% TritonX-100) to remove excess ANGPTL4. An equal amount of ANGPTL4-bound resin was dispensed and incubated with 500 ng of purified matrix protein in PBS at 25 °C for 30 min. The resin was then thoroughly washed with wash buffer. The unbound fractions (U) were pooled and the bound (B) fractions were released by SDS-PAGE loading dye. Both fractions were analyzed by immunoblotting with their indicated antibodies. Resin treated with Tris-buffered saline was used as a control. *In vivo* co-immunoprecipitation was performed using corresponding antibodies as previously described (Tan et al., 2004c).

2.3.10 Sucrose Gradient Sedimentation Assay

Proteins (1 µg) were allowed to interact at 4 °C for 2 h in 150 µl of 50 mM Tris pH 8.0 and 100 mM NaCl. The protein mixture was size-fractionated by ultracentrifugation for 16 h at 132,000 x g at 18 °C through a 5 ml sucrose density gradient (25-40 %). Fractions of 300 µl were collected, chloroform/ethanol precipitated and analyzed by western blot using their respective antibodies.

2.3.11 *In Situ* Proximity Ligation Assay (PLA)

Keratinocytes subcultured overnight on glass chamber slides (Lab-Tek) or cryosections of wound biopsies were fixed with 4% paraformaldehyde for 15 min. The slides were washed twice with PBS, blocked for 1 h at room temperature with 2% BSA in PBS containing 0.1% Triton X-100 followed by incubation with indicated antibody pairs overnight at 4 °C. The slides were washed as described above. DUOlink^{TM} *in situ* PLA was performed as recommended by the manufacturer (OLink Biosciences) using a slide incubated without primary antibody as a negative control. Triple PLA was performed as previously described with minor modifications (Soderberg et al., 2006). Rabbit anti-cANGPTL4 (in house), mouse anti-integrin αvβ5 and goat anti-vitronectin antibodies (Chemicon) were used as proximity probes. DNA was ligated at 37 ^{o}C for 1 h. All probe sequences were as previously described (Soderberg et al., 2006) and were synthesized by Proligo (Sigma-Aldrich). As a negative control, rabbit anti-cANGPTL4 and mouse anti-integrin β5 proximity probes were omitted. Images were taken using a LSM710 confocal laser scanning microscope with a Plan-Apochromat 63x/1.40 oil objective and ZEN software (Carl Zeiss).

2.3.12 Knockdown of ANGPTL4 and Real-Time PCR

siRNA against human ANGPTL4 and a scrambled sequence control were subcloned into the pFIV-H1/U6-puro siRNA lentivirus system (System Biosciences). An equimolar ratio of sense and antisense oligonucleotide mixture was heated to 95 ^{o}C for 5 min and allowed to anneal in 20 mM Tris, pH 7.8, 100 mM NaCl and 0.2 mM EDTA by slow cooling to room temperature. The annealed oligonucleotide was phosphorylated using polynucleotide kinase prior to ligation with BbsI-linearized pFIV-H1/U6-puro siRNA vector. Ligated products were transformed into competent *E. coli* Top 10 bacteria and selected on Luria broth agar plates containing 80 μg/ml ampicillin. Positive clones were

confirmed by DNA sequencing. Positive constructs were co-transfected with pPACK packing plasmids into 293TN cells using ExGen 500. Supernatant was collected 48 h post-transfection and pseudovirus-containing precipitate was obtained by centrifugation at 50,000 x g for 90 min at 4 ^{o}C. Cells were transduced using polybrene according to the manufacturer's recommendation. Transduced cells were enriched by 350 µg/ml puromycin selection for 2 weeks. Knockdown efficiency of ANGPTL4 and relative expression level of indicated genes were determined by quantitative PCR (qPCR). All oligonucleotides and Taqman probes sequences were provided in Table 1.2. Control and ANGPTL4-knockdown keratinocytes were denoted as K_{CTRL} and $K_{ANGPTL4}$, respectively. The interferon response detection kit was from System Biosciences.

2.3.13 Matrix Protein Degradation Assay

Purified ECM proteins (200 ng) were first allowed to interact with various recombinant ANGPTL4 proteins (200 ng), prior to incubation at 37 ^{o}C with either WF or serum-free $K_{ANGPTL4}$ conditioned medium (CM). At the indicated time, aliquots of the reaction were stopped by the addition of SDS-PAGE loading dye. CM was prepared as follows: 3 x 10^6 $K_{ANGPTL4}$ cells were subcultured in a 10-cm dish the day before treatment. The next day, cells were treated with 50 µg/ml TNF-α in 3 ml of serum-free basal Quantum 153 medium for 12 h. CM was collected, sterile filtered and stored at -80 ^{o}C for use in assays. Three independent experiments from two WF samples were performed. Protease inhibition assays were performed using the protease inhibitors pepstatin A (8 µM), EDTA (8 mM) and PMSF (1 mM), either alone or in indicated combinations, in the CM. The matrix proteins were analyzed by Western blot using the corresponding antibodies.

2.4 RESULTS

2.4.1 ANGPTL4 Expression is Regulated by PPARβ/δ in the Keratinocytes

ANGPTL4 is a direct target gene of PPARβ/δ in HaCaT cells, a non-tumorigenic human keratinocyte cell line (Schug et al., 2007). However, the role, expression and regulation of ANGPTL4 in skin wound healing is unclear. To this end, we first examined the expression level of ANGPTL4 in human keratinocytes after ligand activation of specific PPAR isotypes. qPCR revealed that ANGPTL4 mRNA was up-regulated by all 3 PPAR isotypes (Figure 2.1A), with a ~8.5 fold induction with the specific PPARβ/δ ligand, GW501516. We found that serum, which contains undefined and complex mixture of lipid metabolites that can act as ligands for PPARs, also increased ANGPTL4 expression by ~5 fold (Figure 2.1A). Next, the PPRE of the ANGPTL4 (Schug et al., 2007) from human keratinocytes was analyzed by chIP using monoclonal anti-PPARβ/δ. Results showed that PPARβ/δ was bound to this site of the ANGPTL4 promoter region (Figure 2.1B), indicating that ANGPTL4 is a direct target of PPARβ/δ in keratinocytes.

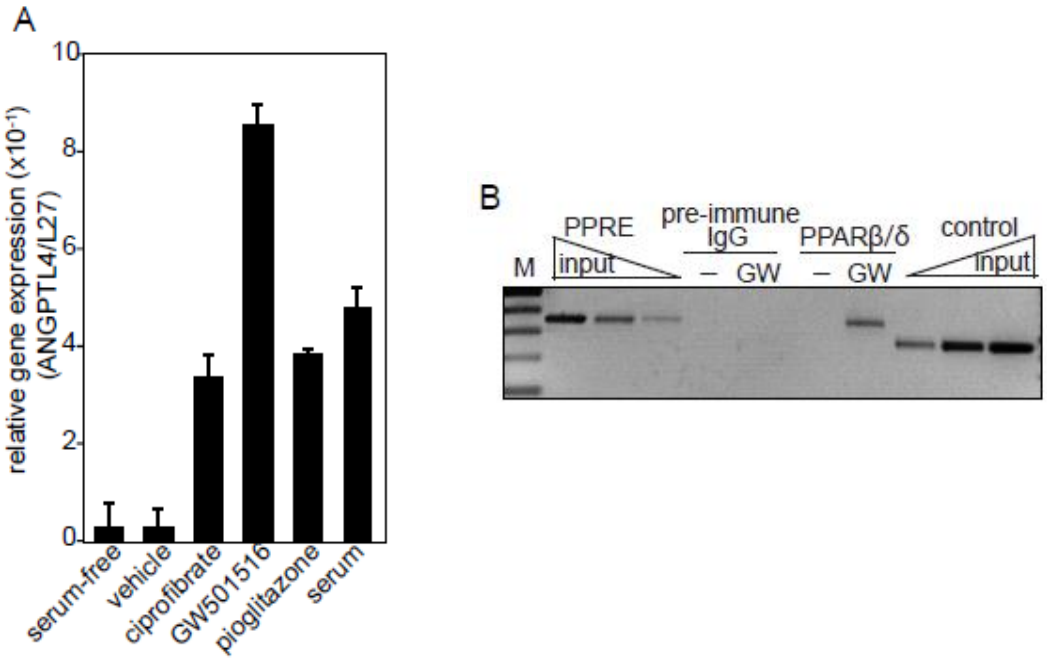

Figure 2.1 ANGPTL4 expression is induced by PPAR ligands. (A) Relative mRNA level of ANGPTL4 in human keratinocytes treated with different agonists selective for each PPAR isotype, ciprofibrate (30 μM, PPARα), GW501516 (2 nM, GW, PPARβ/δ) and pioglitazone (500 nM, PPARγ). Values are mean ± SEM of 4 independent studies. Ribosomal protein L27 used as a normalizing housekeeping gene. **(B)** chIP was done in keratinocytes using pre-immune IgG or monoclonal anti-PPARβ/δ. Regulatory region with the PPRE was immunoprecipitated with anti-PPARβ/δ and specifically amplified. No amplified signal was obtained with pre-immune IgG. A control region upstream of PPRE served as negative control. Aliquots of chromatin were analyzed before immunoprecipitation (input). M: 100 bp DNA marker.

Immunoblot analysis of Day-3 wound biopsies using different polyclonal anti-ANGPTL4 antibodies detected the native ANGPTL4 and cANGPTL4 in the PPARβ/$\delta^{+/+}$ mice, whereas the expression of ANGPTL4 was reduced in PPARβ/$\delta^{-/-}$ littermates (Figure 2.2A). The specificity of anti-cANGTPL4 is shown in Figure 2.2B and specificity of anti-nANGPTL4 was as previously reported (Mandard et al., 2004). Day-3 wound biopsies were used because PPARβ/δ expression peaked at day 3 post-wounding (Michalik et al., 2001). Immunofluorescence staining further confirmed that ANGPTL4 was highly expressed in both the wound epithelia and the wound bed in PPARβ/$\delta^{+/+}$ mice, whereas reduced expression was detected in their PPARβ/$\delta^{-/-}$ littermates (Figure 2.2C). qPCR analysis of the LCM wound epithelium, dermis and wound bed of Day-3 wound biopsies from PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ mice showed that the wound epithelium was the major producer of ANGPTL4 (Figures 2.3A & B). These results suggested that ANGPTL4 secreted by wound keratinocytes may play an important role during wound healing.

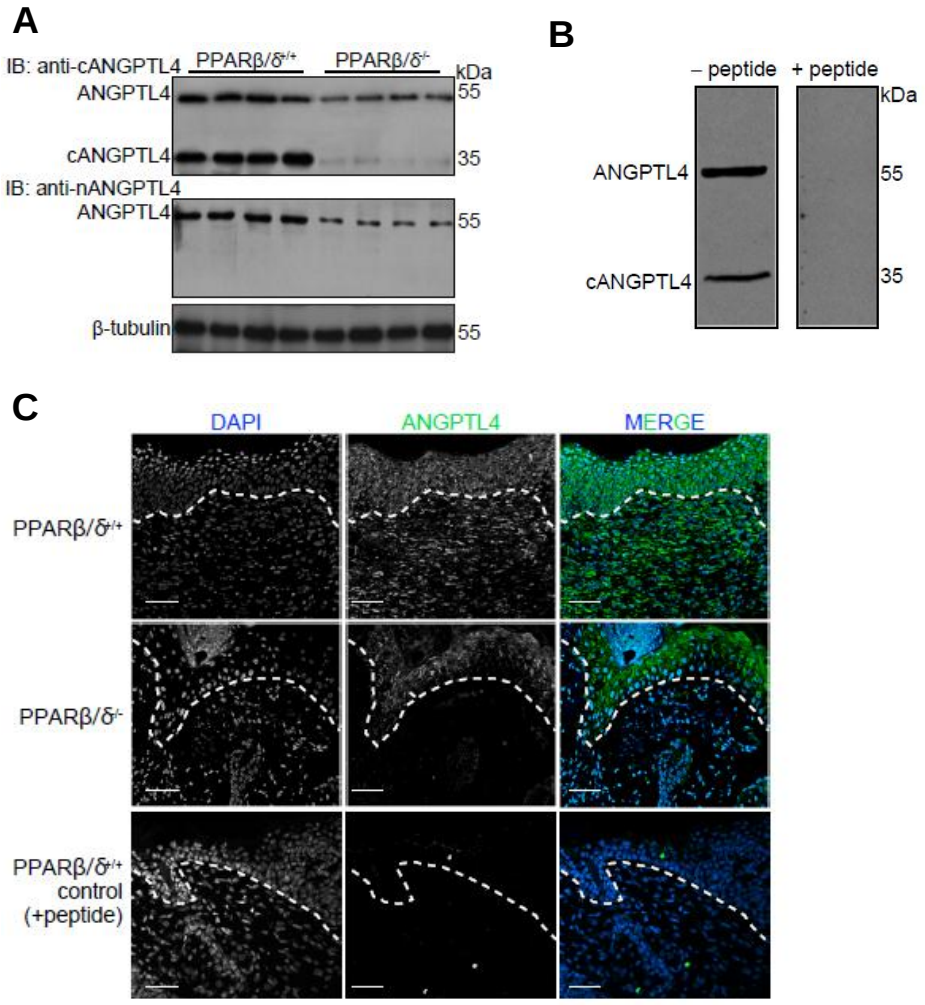

Figure 2.2 Reduced expression of ANGPLT4 in PPARβ/δ$^{-/-}$ mice wound. (A) Expression of ANGPTL4 protein in Day-3 post-wounding mice skin biopsies. Polyclonal antibodies that recognized the N- (anti-nANGPTL4) and C-terminal (anti-cANGPTL4) of ANGPTL4 were used. β-tubulin served as loading and transfer control. n=5. **(B)** Western blot of mouse wound biopsies using anti-cANGPTL4 antibody in the absence or presence of peptide epitope. Experiment was performed to test for specificity of the antibodies. **(C)** Immunofluorescence staining of ANGPTL4 in PPARβ/δ$^{+/+}$ and PPARβ/δ$^{-/-}$ Day-3 wound biopsies using anti-cANGPTL4. Sections were counterstained with DAPI. Negative control is performed with anti-cANGPTL4 pre-incubated with antigen peptide. Representative images from wound epithelia and wound bed were shown (n=5). Dotted white line denotes epidermal-dermal junction. Scale bar 40 μm.

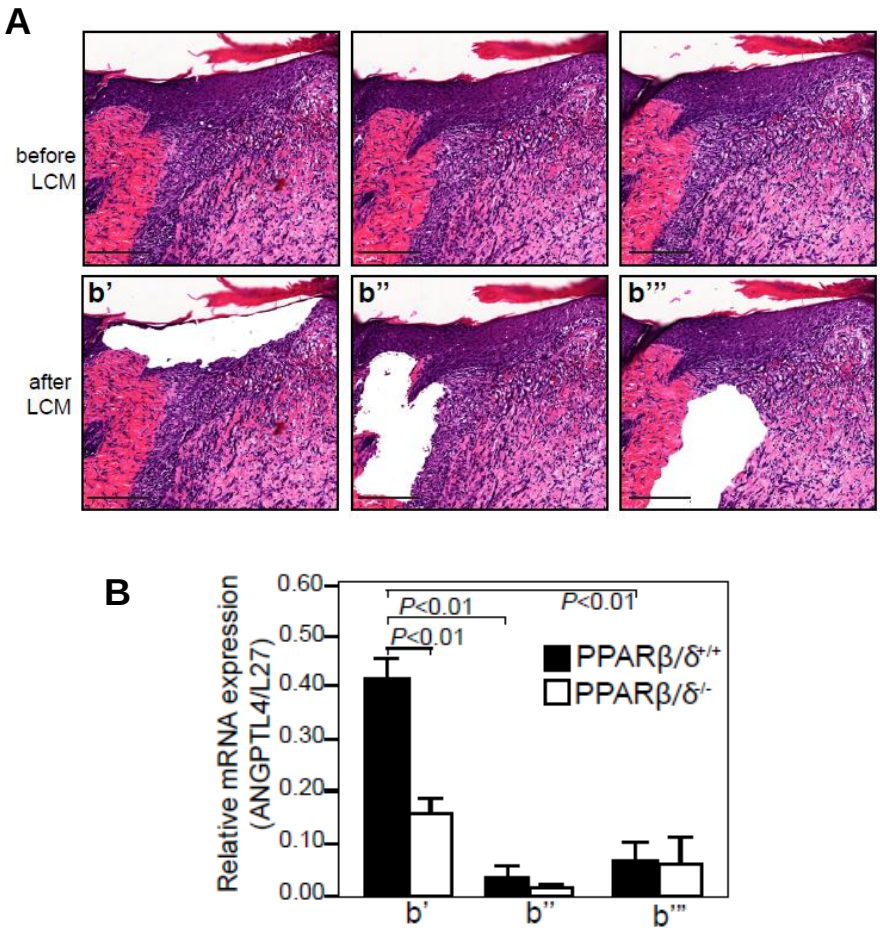

Figure 2.3 ANGPTL4 is mainly found in wound epithelium. (A) Laser capture microdissection of wound biopsies. Hematoxylin and eosin stained wound sections from PPARβ/$\delta^{+/+}$ and PPARβ/$\delta^{-/-}$ mice before and after laser capture microdissection using PALM Microbeam Axio Observer Z1 (Carl Zeiss) with a long distance Plan_Neofluar 20x/0.40 NA objective. The acquisition software used was PALM RoboSoftware 4.2 (Carl Zeiss). Images were taken with a charge-coupled device camera system (3-Chip; Hitachi), and representative H&E-stained sections are shown. Microdissected tissues were processed for real-time PCR analysis. (**B**) Relative mRNA level of ANGPTL4 in wound epithelium (b'), wound dermis (b'') and wound bed (b'''). Values are mean ± SEM of 4 independent studies. Ribosomal protein L27 used as a normalizing housekeeping gene.

2.4.2 cANGPTL4 Interacts with Specific Matrix Proteins

(Details of interactions between ANGPTL4 and matrix proteins using SPR were performed by Mintu Pal, as reported by his Ph.D's thesis and will not be discussed in detail here.)

To begin to understand the role of ANGPTL4 during wound healing, we sought to identify ANGPTL4-binding proteins using SPR-MS. Prompted by our initial observation (Figure 2.2C), we hypothesized that wound fluid (WF) may harbor ANGPTL4-interacting proteins. Using recombinant cANGPTL4 and WF as the bait and lysate, respectively, we identified the ECM proteins, vitronectin and fibronectin, as ANGPTL4-binding partners (Figure 2.4A). Recombinant cANGPTL4 was expressed and purified from *Drosophila* S2 culture medium (Figure 2.4B). Further analyses using SPR with purified vitronectin and fibronectin and ANGPTL4 revealed binding constants (K_D) of ~10^{-7} M (ANGPTL4 with fibronectin and vitronectin: 3.80±1.74 x 10^{-7} M and 3.04±1.33 x 10^{-7} M, respectively; cANGPTL4 with fibronectin and vitronectin: 3.52±1.41 x 10^{-7} M and 5.94±1.79, respectively) (Figures 2.5A & B). Specific anti-cANGPTL4 antibodies against immobilized cANGPTL4 determined the theoretical Rmax value to be 251.8 RU. The experimental Rmax values of fibronectin and vitronectin for cANGPTL4 were 238.6 RU and 218.5 RU, respectively, suggesting a 1:1 stoichiometry of binding. This interaction was specific, as the binding of anti-cANGTPL4 antibody, but not pre-immune IgG, to immobilized-ANGPTL4 blocked its interaction with vitronectin and fibronectin (Figures 2.5C & D). Specific interactions between cANGPTL4 with vitronectin and fibronectin were confirmed by *in vitro* affinity co-immunoprecipitation (Figure 2.5E). In addition, we also examined the formation of the cANGPTL4-matrix protein complex by sedimentation using sucrose gradient ultracentrifugation, which separates proteins and protein complexes according to their native molecular weight, with larger proteins/complexes sedimenting at a higher sucrose density. Consistent with our previous results, immunodetection after sucrose gradient ultracentrifugation showed that

cANGPTL4 associated with either vitronectin or fibronectin, which were detected in higher density fractions than the individual protein (Figure 2.5F). No interaction between nANGPTL4 with identified matrix proteins was observed by either SPR analysis or co-immunoprecipitation (Figure 2.5E).

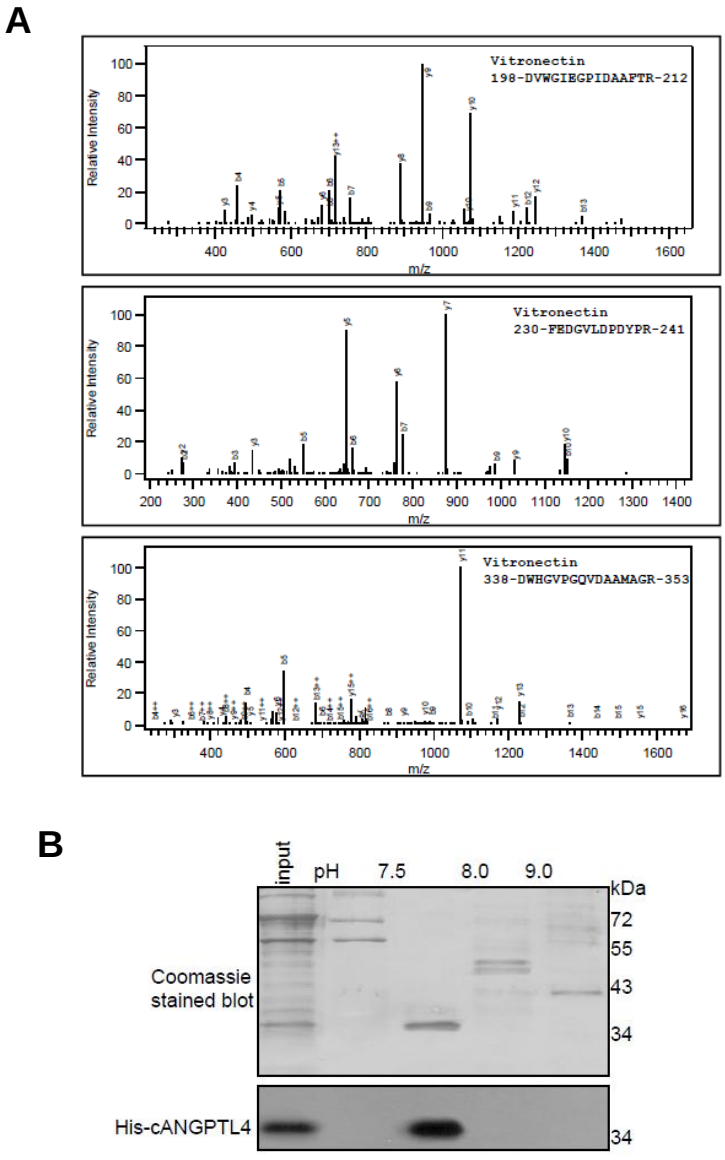

Figure 2.4 Wound ANGPTL4 interacts with vitronectin and fibronectin. (A) MS/MS spectra showing peptide sequences of trypsin-digested cANGPTL4-binding protein. The digested cANGPTL4-binding proteins from SPR-MS were analyzed with a HPLC system coupled to an LTQ-Orbitrap mass spectrometer equipped with a nano-spray source. MS/MS spectra were extracted from the raw data file by extract_msn packaged with Biowork 3.3 (TheromoFinnigan). Protein identification was achieved by searching the combined data against the IPI human protein database (downloaded on March 02, 2007, 67665 entries, 28462007 residues) via an in-house MASCOT server (version 2.2.1). Three representatives MS/MS spectra of tryptic peptides eluted from HPLC were identified as vitronectin. **(B)** Purification of cANGPTL4 from culture medium of S2 cells by preparative isoelectric membrane electrophoresis. The pI of cANGPTL4 was determined to be 7.8 at 4 ^{o}C. Immunodetection of cANGPTL4 was affected with anti-histidine antibody and visualized by chemiluminescence. Coomassie-stained blot showed that cANGPTL4 was purified to homogeneity.

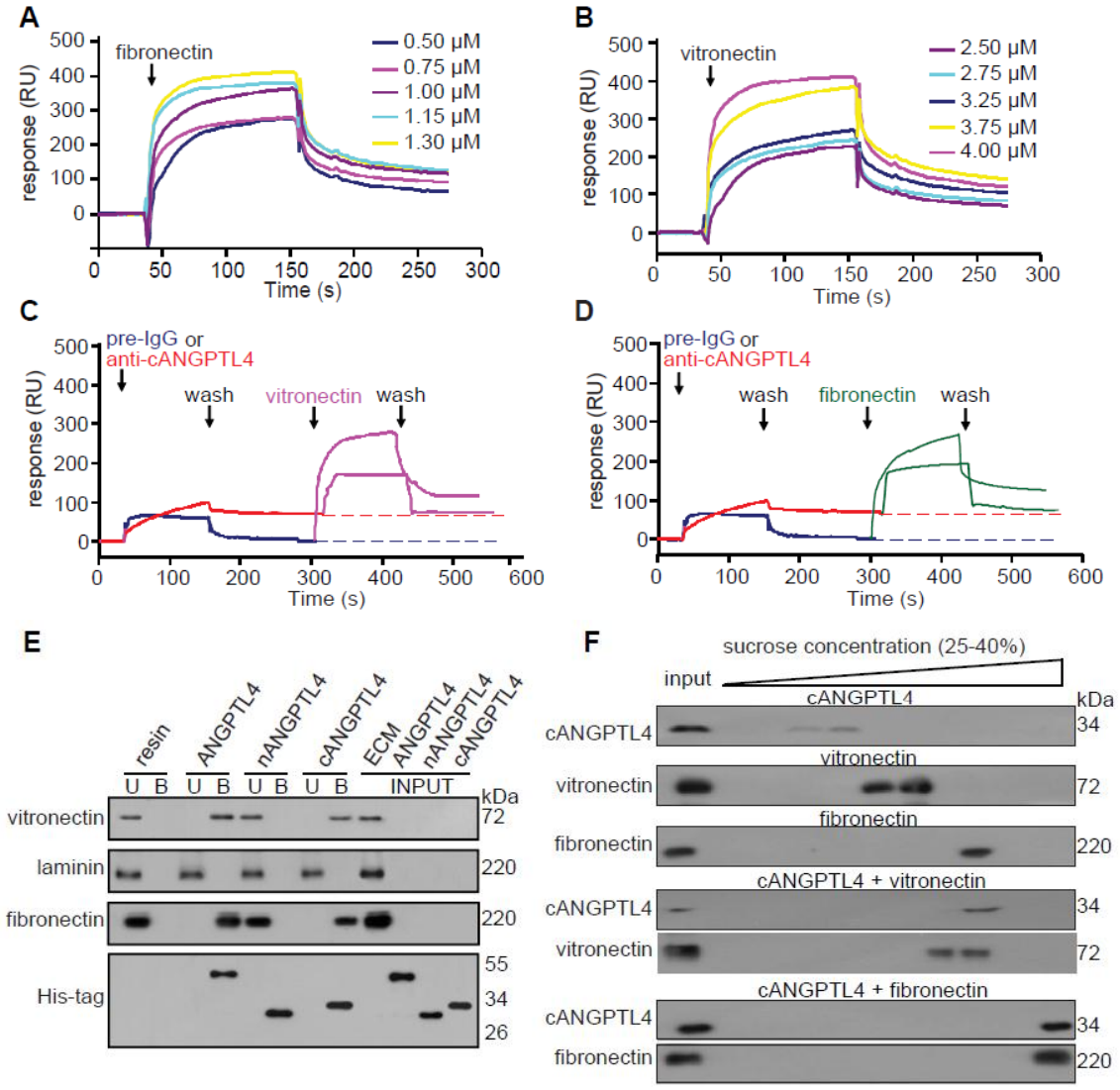

Figure 2.5 cANGPTL4 interacts with vitronectin and fibronectin. Sensograms showed binding profiles between immobilized-cANGPTL4 and indicated concentrations of either (**A**) fibronectin or (**B**) vitronectin. Representative sensorgram (n=5) showed binding profiles of (**C**) vitronectin (pink) and (**D**) fibronectin (green) with immobilized-cANGPTL4 CM5 chip after pre-blocking with either pre-immune IgG (blue) or anti-cANGPTL4 (red). **(E)** Co-immunoprecipitation assays (n=5) were carried out by using different forms of His-tagged ANGPTL4 proteins immobilized on nickel-Sepharose and incubated with indicated purified matrix molecules. Matrix proteins and ANGPTL4 were detected by immunodetected using corresponding antibodies and revealed by chemiluminescence. U and B denote the unbound/washed and bound fractions from the resin, respectively. **(F)** Immunodetection of indicated proteins from sucrose density gradient fractions. The proteins were allowed to interact in the indicated combinations prior separation by sucrose gradient ultracentrifugation. Blots showed increasing sucrose density from left to right. An aliquot of indicated protein, prior incubation and centrifugation, was also loaded (input) (n=5).

In situ PLA using indicated antibody pairs on primary human keratinocytes and Day-5 wound sections were subsequently used to examine whether these interactions occur *in vivo*. These experimental PLA signals, visualized as individual red dots, were observed in the wound bed, as well as in the wound epithelium (Figures 2.6A & B), confirming that cANGPTL4 interacts with vitronectin and fibronectin *in vivo*. As a positive control, vitronectin was shown to interact with integrin β5 (Figure 2.6C), whereas the negative control recommended by the manufacturer revealed negligible non-specific binding of PLA probes (Figures 2.6A-C). Taken together, these data indicate that ANGPTL4 directly interacts with specific matrix proteins in the wound bed during wound healing.

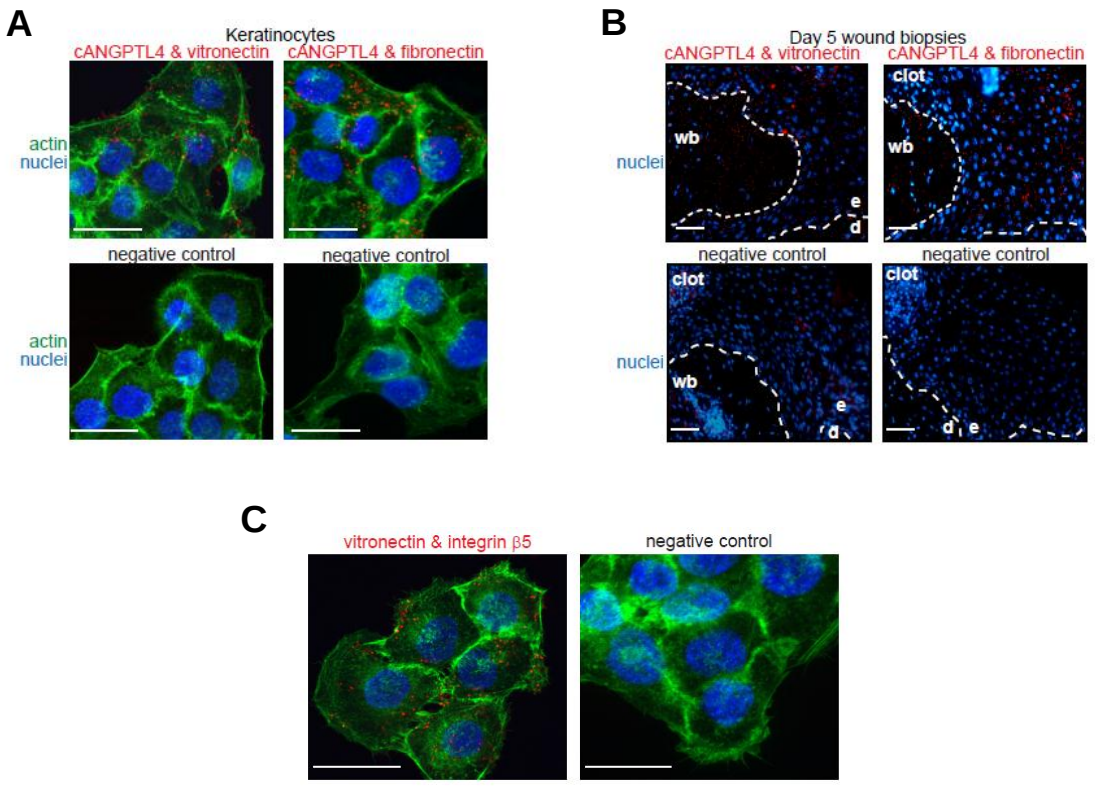

Figure 2.6 PLA co-localization of ANGPTL4 and matrix proteins. Detection of various complexes between ANGPTL4 and indicated binding partners **(A)** in K_{CTRL} and (**B)** in Day-5 wound biopsies using PLA. PLA signals (red), nuclei stained with Hoescht dye (blue) and actin stress fiber (green) by Alexa488-phalloidin. The nuclei-image has been acquired in one z-plane using LSM710 confocal microscope. Dotted white line represents epidermal-dermal junction. Negative control is performed without primary antibodies. Representative pictures from wound section with epidermis (e), dermis (d), the adjacent wound bed (wb) and K_{CTRL} from 6 independent experiments or sections from 3 mice are shown. **(C)** Positive control for DUOlink™ *in situ* proximity ligation assay (PLA). Detection of vitronectin and integrin β5 complexes in keratinocyte using PLA. PLA signals are shown in red, and cells were counterstained with Hoescht dye (blue) for nuclei and with Alexa488-phalloidin for actin stress fiber (green). The nuclei-image has been acquired in one z-plane using LSM510 META confocal laser scanning microscope (Carl Zeiss). Negative control was performed without primary antibodies. Scale bar 40 µm.

2.4.3 ANGPTL4-Bound Matrix Protein Can Interact with Integrins

Having shown that ANGPTL4 interacts with vitronectin and fibronectin, it was necessary to determine whether this interaction would interfere with integrin recognition of the matrix protein. To this end, we examined the ability of ANGPTL4-bound vitronectin to interact with the extracellular domain PSI-ILD of integrin β5. Purified recombinant cANGPTL4 with no histidine tag, histidine-tagged integrin β5 PSI-ILD and vitronectin were allowed to interact in solution and then sedimented using sucrose gradient ultracentrifugation. When the three proteins were present together, all three proteins were detected at a higher sucrose density, suggesting that ANGPTL4-bound matrix protein could still interact with PSI-ILD of integrin β5 (Figure 2.7A). *In vivo* co-immunoprecipitation and triple PLA were used to further confirm this observation. Co-immunoprecipitation from human keratinocyte lysate using antibodies against integrin αvβ5, vitronectin or cANGPTL4 followed by immunodetection showed that the corresponding two proteins were also found in the immunoprecipitates (Figure 2.7B). Finally, triple PLA further revealed the close proximity of ANGPTL4, integrin β5 and vitronectin at focal adhesions (Figure 2.7C). In retrospect, the PLA signals from ANGPTL4 and matrix proteins detected in wound epithelium (Figure 2.6B) represented ANGPTL4-bound matrix proteins that had interacted with their cognate integrins in keratinocytes. Taken together, our results suggest that the binding of ANGPTL4 to matrix proteins, such as vitronectin, does not prevent the matrix protein from associating with its cognate integrin.

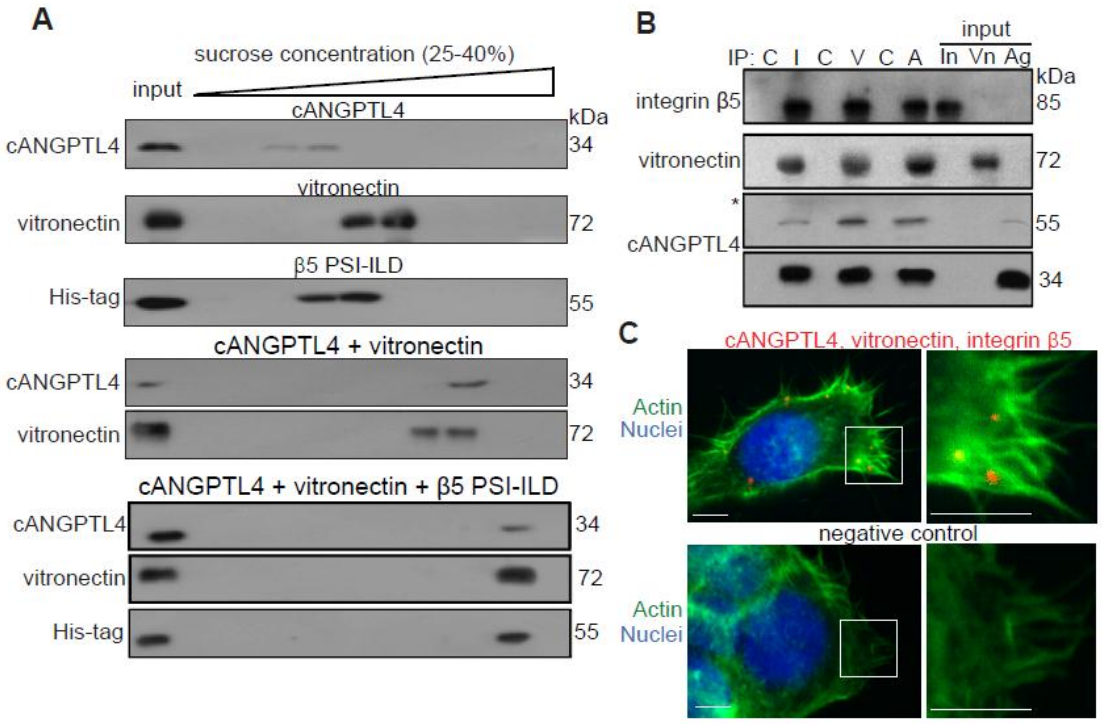

Figure 2.7 ANGPTL4-bound matrix proteins can interact with integrins. (A) Immunoblot of cANGPTL4, His-tagged integrin β5 PSI-ILD and vitronectin of sucrose density gradient fractions. The proteins were allowed to interact in the indicated combinations prior separation by sucrose density gradient ultracentrifugation. Blots showed increasing sucrose density from left to right. **(B)** *In vivo* co-immunoprecipitation (n=3) was performed by using antibodies against integrin β5 (I), vitronectin (V) and cANGPTL4 (A). As control, pre-immune IgG (C) was used. Antibodies were covalently crosslinked to Protein G on agarose beads were incubated with total keratinocyte cell lysate. Immunoprecipitates were detected by immunoblot using corresponding antibodies and revealed by chemiluminescence. Native ANGPTL4 of ~55 kDa was after longer exposure time as denoted by asterisk. Total cell lysate served as input. (In: integrin β5; Vn: vitronectin; Ag: ANGPTL4). **(C)** Triple PLA showed ternary complex in keratinocytes. Triple PLA signals (red), nuclei were stained with Hoescht dye (blue), and Alexa488-phallodin for actin fiber (green). Representative PLA images from 3 independent experiments are shown. Negative control is without anti-cANGPLT4 and anti-integrin proximity probes. Scale bar 40 μm.

2.4.4 ANGPTL4 Interacts with Matrix Proteins and Delays Their Degradation

Directed migration of wound keratinocytes over the provisional wound bed requires the controlled turnover of matrix proteins by proteases (Page-McCaw et al., 2007). We next examined the effect of the interaction between ANGPTL4 and matrix proteins on the turnover rate of matrix proteins. We pre-incubated purified ECM proteins with various recombinant ANGPTL4 proteins and subjected the mixture to WF. Our results revealed that the degradation of vitronectin and fibronectin was slower in the presence of ANGPTL4 and cANGPTL4 compared to either the vehicle control or nANGPTL4 (Figure 2.8A). As a control, laminin-5, which does not bind to cANGPTL4, was degraded at a similar rate regardless of the presence of ANGPTL4 (Figure 2.8A).

To eliminate the possible contribution of endogenous ANGPTL4 from WF, we performed a similar matrix degradation experiment with serum-free conditioned medium (CM). We initially suppressed endogenous ANGPTL4 expression by RNA interference in human keratinocytes. Keratinocytes were transduced with a lentivirus-mediated ANGPTL4 or control scrambled siRNA. The ANGPTL4 expression level in ANGPTL4-knockdown keratinocytes ($K_{ANGPTL4}$) was reduced by 90% compared to control siRNA keratinocytes (K_{CTRL}) (Figure 2.8B). The expression of β-tubulin remained unchanged, as did the transfer and loading control. The expression of ANGPTL3, a closely related member of the family, remained unchanged, indicating the specificity of the knockdown. The induction of interferon responses has been reported as a challenge to the specificity of some RNAi approaches (Bridge et al., 2003). Real-time PCR analysis of key interferon response genes OAS1, OAS2, MX1 and ISGF3γ revealed no significant difference between $K_{ANGPTL4}$ and either wild type non-transduced cells or K_{CTRL}, (Figure 2.8C). These data suggest that gene silencing is not associated with nonspecific interferon-response induction, namely, an off-target effect. Next, we stimulated the expression of proteases in $K_{ANGPTL4}$ by TNF-α treatment and used the resulting serum-

free CM for a matrix protein degradation assay. Consistent with our previous results, the degradation of vitronectin and fibronectin was slower in the presence of ANGPTL4 and cANGPTL4 (Figure 2.8D). Using different protease inhibitors, we further showed that ANGPTL4 mainly protected the degradation of vitronectin and fibronectin from matrix metalloproteinases (MMPs) (Figure 2.9A). SPR analysis failed to detect any interaction between recombinant MMP2 or MMP9 and cANGPTL4, arguing against a direct role of ANGPTL4 in the inhibition of MMPs (Figure 2.9B). Taken together, our results showed a physical interaction between ANGPTL4 with specific matrix proteins that resulted in the selective delay of the degradation of matrix proteins by MMPs during wound healing.

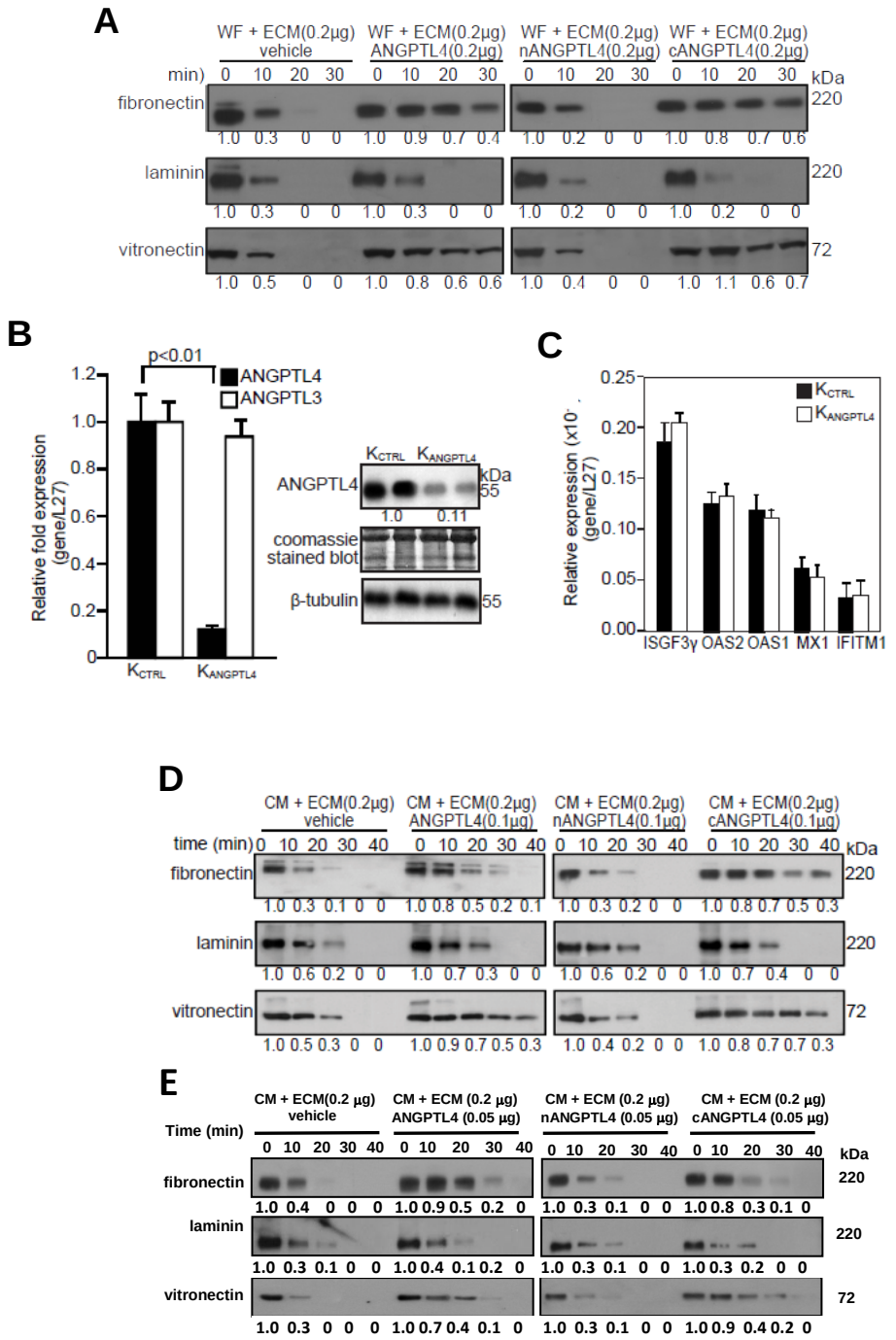

Figure 2.8 ANGPTL4 modulates matrix protein degradation. (A) Immunodetection of the matrix proteins, vitronectin and fibronectin, after incubation for indicated time with WF. Laminin which does not interacts with cANGPTL4 serves as control. Three independent experiments from two wound fluids were performed. Values below denote change in mean fold expression compared to input. **(B)** Expression level of ANGPTL4 and ANGPTL3 in keratinocytes transduced with either control (K_{CTRL}) or ANGPTL4 siRNA ($K_{ANGPTL4}$). Values below each band represent the mean fold differences in protein expression level compared to control from five independent experiments. Coomassie-stained blot and β-tubulin, which remained unchanged, showed equal loading. **(C)** qPCR of interferon response genes in $K_{ANGPTL4}$ compared with K_{CTRL}. 2',5'-Oligoadenylate synthetase isoforms 1 and 2 (OAS1, OAS2), interferon-induced myxovirus resistance 1 (MX1), interferon-inducible transmembrane protein (IFITM) and interferon-stimulated transcription factor 3γ (ISGF3γ). Ribosomal protein L27 was used as a normalizing reference gene. Dose-dependent degradation of the ECM. Immunodetection of the matrix proteins, vitronectin and fibronectin, after incubation for indicated time with TNF-α-treated $K_{ANGPTL4}$ conditioned medium (CM) using 0.1 µg **(D)** or 0.05 µg **(E)** recombinant proteins. Laminin which does not interact with cANGPTL4 served as control. Three independent experiments were performed. Values below denote mean fold expression change compared to input.

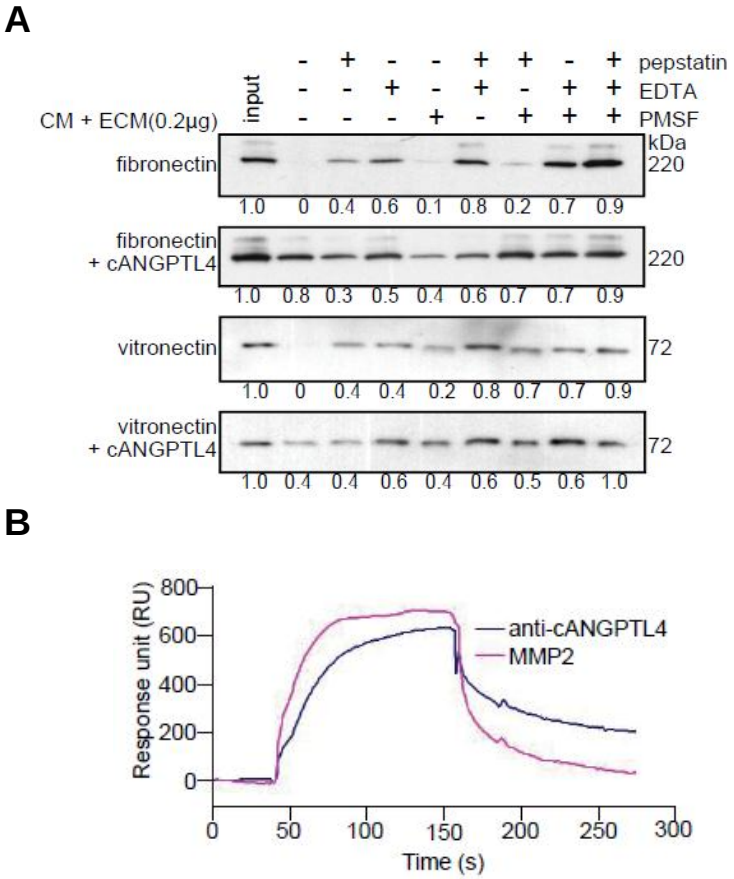

Figure 2.9 Interaction of ANGPTL4 with specific matrix proteins delays their degradation. (A) Immunodetection of the matrix proteins, vitronectin and fibronectin, after incubation for indicated time with TNF-α-treated $K_{ANGPTL4}$ CM in the presence of indicated protease inhibitors. Values below denote change in mean fold expression compared to input. **(B)** Representative sensorgrams showed binding profile between either anti-cANGPTL4 antibodies (blue) or recombinant MMP-2 (pink) on immobilized-cANGPTL4 CM5 chip. Each sensorgram was corrected by subtracting a sensorgram obtained from a reference flow cell not immobilized with protein. Anti-cANGPTL4 antibodies against the immobilized cANGPTL4 determined the Rmax value to be 138.2 resonance units (RU).

2.4.5 ANGPTL4 Deficiency Delays Wound Re-epithelialization

We showed that ANGPTL4 produced by keratinocytes interacts with vitronectin and fibronectin in the wound bed, and delays their proteolytic degradation by MMPs. To underscore the *in vivo* relevance of ANGPTL4 in the degradation of specific matrix proteins by MMPs, we examined the expression of MMPs and matrix proteins in ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ wound biopsies. Keratinocytes synthesize and secrete mainly MMP-1, 2, 9 and 10, and their expression is required to regenerate the injured tissue (Page-McCaw et al., 2007). Immunoblot analysis of Day-5 ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ wound biopsies showed that the protein levels of vitronectin and fibronectin, but not laminin, was reduced (Figure 2.10A). Our analysis did not reveal significant differences in the protein level of major MMPs (Figure 2.10A), indicating that the differential matrix protein level was a consequence of increased susceptibility of matrix proteins to proteolytic degradation.

We hypothesized that such actions would have a direct impact on wound healing. We first examined keratinocyte migration using *in vitro* scratch wound assays on surfaces coated with matrix proteins using K_{CTRL} and $K_{ANGPTL4}$ treated with mitomycin C to exclude any effects of proliferation. Our results showed that $K_{ANGPTL4}$ re-populated the *in vitro* wound significantly more slowly on fibronectin- and vitronectin-coated surfaces compared to K_{CTRL} (Figure 2.10B). No significant difference was observed on laminin-coated surfaces.

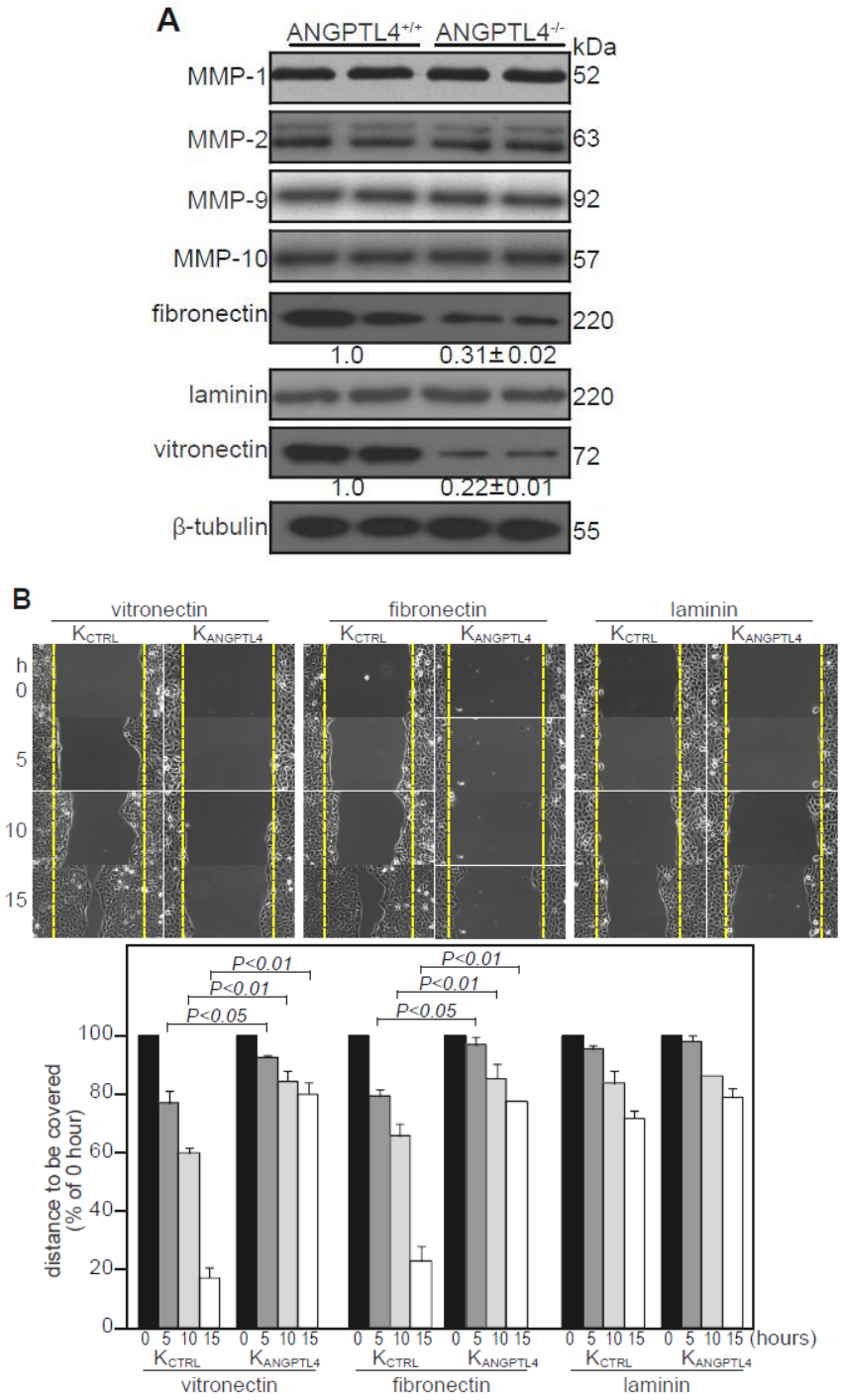

Figure 2.10 ANGPTL4 deficiency delays wound re-epithelialization. (A) Immunoblot analysis of MMP-1, -2, -9, -10, fibronectin, laminin and vitronectin from Day-5 wound biopsies of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice. Values below the band represent the mean fold differences in protein expression levels relative to ANGPTL4$^{+/+}$ from 8 wound biopsies for each genotype. β-tubulin served as loading and transfer control. **(B)** Wound closure kinetics of K_{CTRL} and $K_{ANGPTL4}$ treated with mitomycin C (2 µg/ml) on indicated matrix protein coated surfaces. Representative time-lapsed images of wounded cultures. Yellow dotted lines represent the scratch gap at the time of wounding. Graph shows distance to be covered by the migrating keratinocytes as percentage of zero hour (=100%) *in vitro* wound gap distance (± S.E.M., n=5, using Mann-Whitney test).

Next, we examined the healing of full-thickness skin wounds in ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice. Our analysis of the day 3-10 wound biopsies showed a delayed re-epithelialization of ANGPTL4$^{-/-}$ wounds compared to ANGPTL4$^{+/+}$ (Figure 2.11A). The length of the wound epidermis measured from the first hair follicle to the tip of the wound epithelial tongue is used as an indicator of keratinocyte migration, and this was also reduced in ANGPTL4$^{-/-}$ wounds (Figure 2.11B). No difference in wound contraction, defined by the distance between the first hair follicle on either side of the wound edge, was observed. Immunohistochemical staining of wound biopsies for keratin 6 identifies the wound epithelia and hair follicles, while α-smooth muscle actin reveals the myofibroblasts (Figure 2.11C & D). We harvested the entire wound along with a 5 mm perimeter of the surrounding tissue. Cell counts indicated cell number per wound at day 3 (ANGPTL4$^{+/+}$ 3.20±0.39x10^6; ANGPTL4$^{-/-}$ 3.01±0.44x10^6, n=4) and day 7 (ANGPTL4$^{+/+}$ 3.54±0.26x10^6; ANGPTL4$^{-/-}$ 3.33±0.54x10^6, n=4). The cell suspension was analyzed on FACS after staining with antibodies against F4/80 for macrophages showed no significant difference between ANGPTL4$^{-/-}$ and ANGPTL4$^{+/+}$ biopsies (Day 3: ANGPTL4$^{+/+}$ 15.1±1.2%; ANGPTL4$^{-/-}$ 14.1±2.1%; Day 7: ANGPTL4$^{+/+}$ 18.3±2.6%; ANGPTL4$^{-/-}$ 18.7±3.8% of cells in wound, n=3) (Figure 2.12A). We observed a consistently lower number of CD31$^+$ endothelial cells in ANGPTL4$^{-/-}$ compared to ANGPTL4$^{+/+}$ wounds (Day 3: ANGPTL4$^{+/+}$ 15.7±2.4%; ANGPTL4$^{-/-}$ 10.1±1.8%; Day 7: ANGPTL4$^{+/+}$ 19.7±3.6%; ANGPTL4$^{-/-}$ 14.7±2.8% of cells in wound, n=3) (Figure 2.12B). Our results showed that ANGPTL4 deficiency delays wound re-epithelialization associated, at least in part, with an increase in matrix protein degradation.

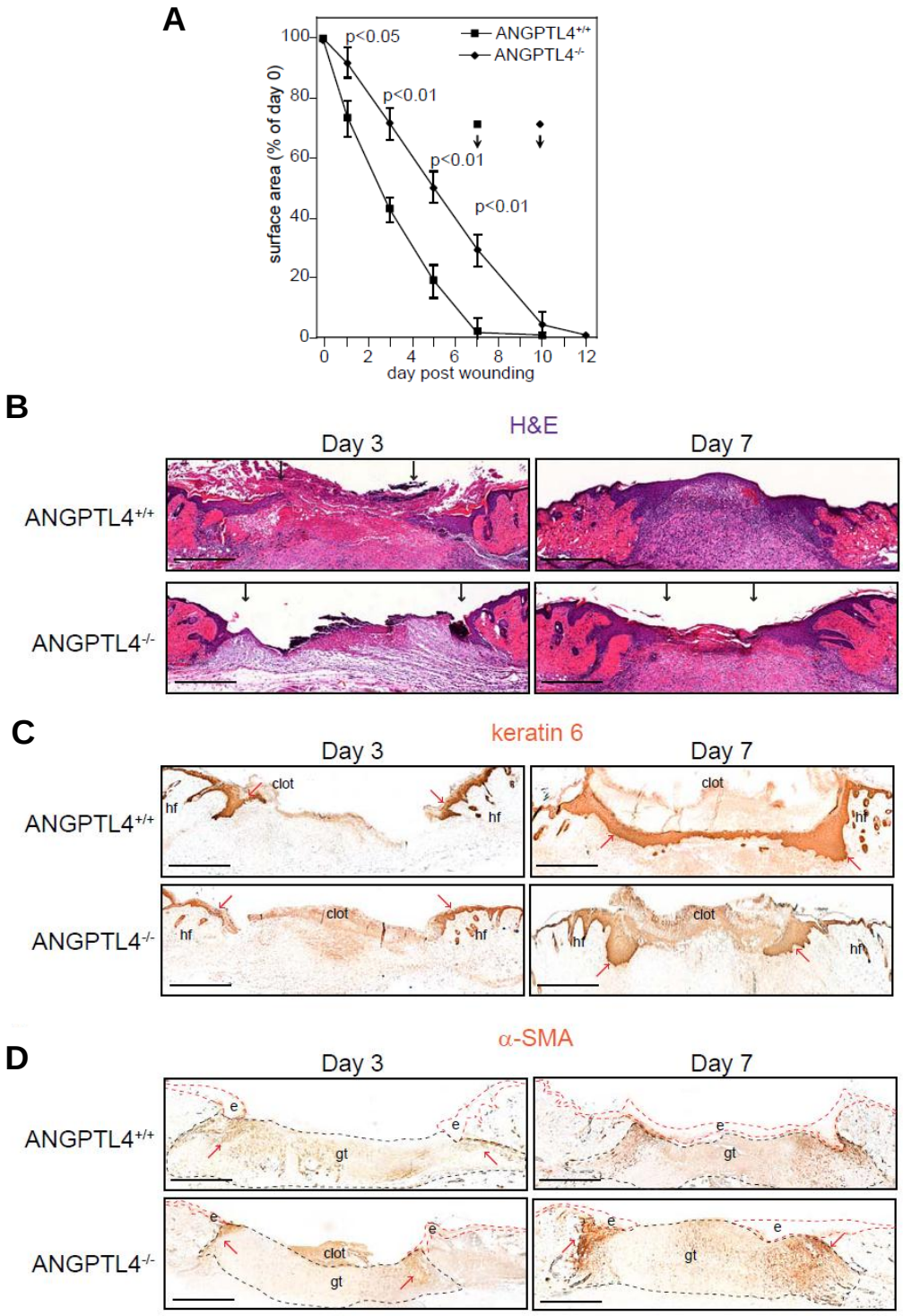

Figure 2.11 ANGPTL4-knockout mice displayed impaired wound re-epithelialization. (A) Wound closure kinetic of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice. Wound surface areas are plotted as percentage of day 0 (=100%) wound surface area (± S.E.M., n=10, using Mann-Whitney test). Arrows indicate the mean time for complete wound closure. **(B)** Haematoxylin and eosin stained skin biopsies taken from Day-3 and Day-7 post-wounding of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice. Black arrows point to the epithelial wound edge. Immunohistochemical staining of **(C)** keratin 6 (wound epidermis and hair follicles) and **(D)** α-smooth muscle actin (for myofibroblasts) in ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ Day-3 and Day-7 wound biopsies. Red arrows point to positively stained region. Dotted red and black lines demotes the wound epidermis (e) and granulation tissue (gt). hf: hair follicles. Proteins were revealed with VECTASTAIN® ABC kit (Vector Laboratories) using 3,3'-diaminobenzidine (DAB; brown) as substrate. Non-specific staining was observed in the clot region. Representative images of a centrally dissected wound sections are shown. Scale bar 500 μm.

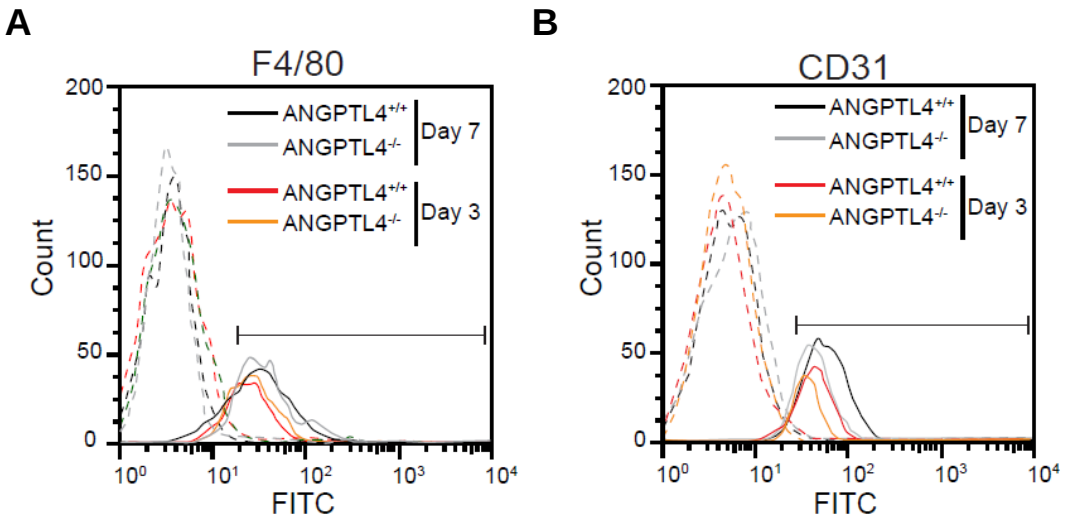

Figure 2.12 FACS analysis. FACS analysis of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ Day-3 and Day-7 wound biopsies-derived single cell suspensions (n=3-4) stained with **(A)** F4/80 (macrophages) or **(B)** CD31 (endothelial cells). The negative control (only secondary antibody) is indicated by the dotted graph, from which total cell number per wound was obtained.

2.4.6 ANGPTL4 Deficiency Affects FAK- and 14-3-3σ-dependent Signaling Pathways

Given that integrins are receptors for matrix proteins and having shown that ANGPTL4 deficiency affects matrix protein integrity and wound healing, it is conceivable that the underlying mechanism involves integrin-mediated signaling. Indeed, the expression or phosphorylation of downstream effectors like FAK- and 14-3-3-dependent signaling cascades was reduced in ANGPTL4$^{-/-}$ compared with ANGPTL4$^{+/+}$ wounds (Figure 2.13). 14-3-3 associates with integrins to modulate cell migration via a FAK-independent mechanism involving protein kinase C (PKC) (Dellambra et al., 1995). ANGPTL4$^{-/-}$ wounds also showed decreased expression of RACK1, indicating an attenuated PKC-mediated signal transduction (Figure 2.13) (Schechtman and Mochly-Rosen, 2001). A reduced activation of FAK is also known to converge with a decreased activation of the Raf-MEK-ERK signaling pathway (Porter et al., 2006).

The downstream mediators of the PI3K cascade such as PDK1, PKBα and GSK-3β were also altered (Figure 2.13). GSK3β is a target of PKBα known to phosphorylate kinesin light chain (KLC) and, thus, to negatively regulate kinesin-based motility and integrin recycling (Morfini et al., 2002; Roberts et al., 2004). We observed hyperphosphorylated KLC2 in ANGPTL4$^{-/-}$ wounds, which suggests that integrin recycling may be impaired in ANGPTL4 deficient keratinocytes (Figure 2.13). Small Rho GTPases are effectors of PI3K pathway. Among them, cdc42 and Rac1 are pivotal intracellular mediators for the formation of lamellipodia and cell migration. They activate downstream effectors such as PAK, which in turn activate LIM kinases (LIMKs) (Knaus and Bokoch, 1998). ANGPTL4 deficiency led to a reduction in the phosphorylation of PAK1 and LIMK1 (Figure 2.13). These would have a direct impact on lamellipodia formation and migration, consistent with our earlier observation that wound healing was delayed in $K_{ANGPTL4}$ (Figure 2.13).

Taken together, our results show that ANGPTL4 deficiency impairs the activation of numerous integrin-initiated downstream signaling cascades, including FAK and 14-3-3, to mediate gene expression involved in cell migration.

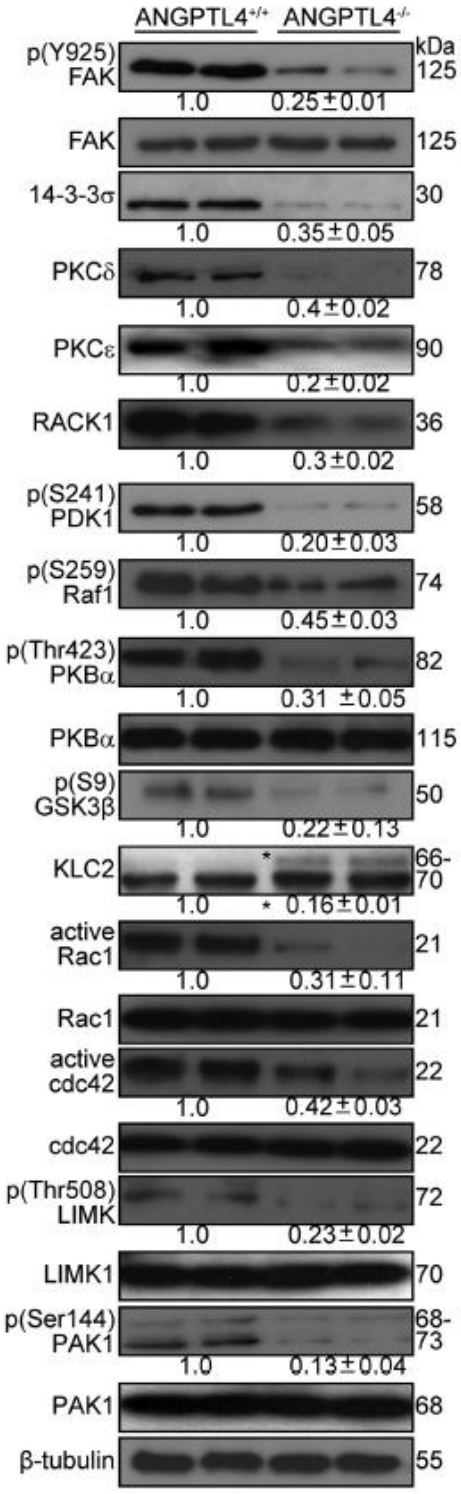

Figure 2.13 ANGPTL4 deficiency affects FAK- and 14-3-3σ–dependent signaling pathways. Immunoblot analyses of indicated proteins from ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice day 5 wound biopsies (n=5). Values below the bands represent the mean fold differences in protein expression levels when compared with ANGPTL4$^{+/+}$, which was assigned the value one. FAK: focal adhesion kinase; PKC: protein kinase C; RACK1: Receptor for activated protein kinase C; PKB: protein kinase B; GSK-3: glycogen synthase kinase-3; PDK1: 3-phosphoinositide-dependent kinase-1; KLC: kinesin-light chain; LIMK: LIM kianse; PAK: p21 activated kinase. β-tubulin served as loading and transfer control.

2.5 DISCUSSION

Wound healing is a complex process that involves a cascade of overlapping events, including inflammation, re-epithelialization and remodeling, all directed at the restoration of the epidermal barrier. Re-epithelialization is accomplished by increased keratinocyte proliferation and guided migration of the keratinocytes over the wound. Cellular interactions with ECM proteins, i.e., cell-matrix communication, among others, coordinate the individual events, enabling temporal and spatial control, as well as ordered changes in keratinocyte behavior and phenotype. We revealed a newly discovered cell-matrix communication role for ANGPTL4 in influencing wound re-epithelialization. Using SPR, PLA, sucrose gradient sedimentation and co-immunoprecipitation assays, we showed that cANGPTL4 associated with fibronectin and vitronectin. Further analysis also revealed that ANGPTL4 delays the degradation of fibronectin by MMPs. ANGPTL4 binds and delays the degradation of specific matrix proteins; they are thus available as intact components of the ECM to regulate cell-matrix communication. The precise mechanism is unknown, although it is tempting to speculate that ANGPTL4 may interfere with the accessibility of MMPs for the ECM protein.

An intact extracellular matrix (ECM) protein is essential for keratinocyte migration, where it allows the generation of traction force between the ECM and cells. The degradation of ECM protein into smaller soluble fragment prevent the generation of such traction force. Indeed, the addition of small soluble ECM peptide generally delays cell migration, unless these peptides are immobilized on an intact surface like the culture flask. To further understand this biophysical aspect, a collaborative study with researchers from School of Material Science Engineering has already started, where we examined the effect of matrix stiffness and nanopatterning on directed cell migration (unpublished data).

During wound healing, migrating cells must display appropriate cellular behavior in response to the changing wound environment to enable effective wound closure. Integrins on the cell surface function as biosensors to constantly monitor changes in the microenvironment. Simultaneously, the context in which the cognate matrix protein is presented to the cells dictates productive integrin activation. At a low protein ratio of soluble to substrate-anchored matrix, which is well below that required for blocking adhesion, one may observe accelerated turnover of integrin-matrix protein interactions (Legler et al., 2001). Deficiency in ANGPTL4 has a dramatic effect on wound closure. ANGPTL4 binds to specific matrix proteins via its C-terminal fibrinogen-like domain and delays their degradation by proteases. This association, however, does not interfere with integrin-matrix protein recognition. Instead, it directly affects integrin-mediated signaling by altering the balance between substrate-anchored matrix proteins and soluble matrix protein fragments, thereby modifying the availability of the local substrate-anchored ECM and consequently modulating cellular behavior.

Numerous studies have shown that inflammation-induced PPARβ/δ is crucial for wound repair. PPARβ/δ confers anti-apoptotic properties to keratinocytes, in part via transcriptional control of the PKBα signaling pathway, and by maintaining a sufficient number of viable wound keratinocytes at the wound edge (Tan et al., 2001; Di Poi et al., 2002). Recently, PPARβ/δ was shown to potentiate cell polarization and directed migration during re-epithelialization (Tan et al., 2007). Most of these studies focused on intracellular signaling or events mediated by PPARβ/δ that were important for cell survival and migration. Clearly, cell-matrix communication is needed for effective directed cell migration during wound healing. However, the mechanism by which PPARβ/δ modifies the wound microenvironment in order to coordinate cell-matrix communication remains unknown. Conceivably, as an intracellular transcription factor,

PPARβ/δ is likely to exert its effect via an extracellular factor. The expression of ANGPTL4, only weakly detectable in normal intact skin, was markedly elevated during the re-epithelialization phase of wound healing, as was similarly observed in PPARβ/δ-knockout mice (Michalik et al., 2001). We provide evidence that PPARβ/δ stimulates the expression of the adipocytokine ANGPTL4 in keratinocytes, which allows the migrating keratinocytes to modulate the wound microenvironment and to coordinate with cellular responses. Similar to its effect on integrin-matrix interactions, integrin-FAK-mediated signaling and key intracellular signaling cascades involved in actin polymerization and for the establishment of a leading lamellipodium in migrating cells are dependent on ANGPTL4. Besides regulating cell proliferation, PI3K/PKBα, PDK1 and GSK-3β are involved in the coordinated assembly and disassembly of actin filaments and integrin recycling, and contribute to the motility of rapidly migrating cells, such as wound keratinocytes (Enomoto et al., 2005).

The ANGPTL4-knockout mice did not have any obvious skin abnormalities, but displayed altered epidermal differentiation (Chapter 3). This suggests that a low level of ANGPTL4 may be required for normal skin homeostasis, and may play an important role during wound repair. The role of PPARβ/δ in epidermal differentiation and maintenance of a lipid barrier is well-recognized, although the underlying mechanism remains unclear (Burdick et al., 2006). Although not the focus of this study, we observed that the expression of 14-3-3, as assessed by immunoblot, was diminished in ANGPTL4$^{-/-}$ compared with ANGPTL4$^{+/+}$ mice, and this reduction may play a role in regulating epidermal differentiation. Studies have shown that 14-3-3 associates with PKC, which has a well-established role in epidermal differentiation (Denning, 2004).

Recent studies have shown that adipocytokines, such as leptin, have a profound local impact on wound healing (Frank et al., 2000). However, the mechanism for the

observed beneficial effect on wound repair is unclear and research efforts are currently being directed toward understanding the molecular regulation. We identified ANGPTL4, an adipocytokine, as having a beneficial effect on wound healing, in part due to its effect on the integrity of matrix proteins and cell-matrix communication. Given that ANGPTL4 is involved in lipid and glucose homeostasis, and that ANGPTL4 decreases blood glucose and improves glucose tolerance in mice (Xu et al., 2005), it is a prime therapeutic candidate for diseases such as diabetes, and for wound healing. A better understanding of its role in wound healing, especially in diabetic and chronic wounds would provide better wound management.

Non-insulin-dependent diabetes mellitus, also known as Type II diabetes, is a medical menace that affects ~200 million people and continues to be an increasing burden on healthcare resources worldwide with its morbidities of retinopathy, cardiovascular diseases, diabetic nephropathy and impaired wound repair (Brem and Tomic-Canic, 2007). Diabetic foot ulceration is a significant cause of morbidity and is the most common reason for hospital admission in diabetic patients. Approximately 15 % of diabetic patients will develop chronic ulcers during their lifetimes. In those who require lower-limb amputation, 70-90 % will be preceded by foot ulceration (Delmas, 2006). Furthermore, poor healing diabetic wound is an open portal for infections, often resulting in chronic inflammation, sepsis, dehiscence and death. To effectively manage these problems one must understand the healing process and to create a salubrious physical and biochemical environment conducive for healing.

Normal wound healing proceeds via a continuum of events that includes the acute inflammatory, proliferative and maturation phases (Coulombe, 2003; Werner and Grose, 2003) (Section 1.2.3). These events entail a complex interplay between connective tissue formation, cellular activity, and growth factor activation. All three of these physiologic processes are altered in the diabetic state. There is a cessation of epidermal growth and

migration over the wound surface which contributes to the poor healing of chronic diabetic ulcers (Falanga, 2005; Blakytny and Jude, 2009). Thus, diabetic ulcers are characterized by an accumulation of devitalized tissue, increased/prolonged inflammation, poor wound-related angiogenesis and reduced matrix deposition (Blakytny and Jude, 2009). Analysis of fluid from chronic wounds have showed elevated levels of MMPs directly resulting in increased proteolytic degradation of matrix protein and inactivation of growth factors that are necessary for proper wound healing (Falanga, 2005; Galkowska et al., 2006; Gallagher et al., 2007). Studies have investigated these alterations to better understand the wound healing abnormalities, and to target therapy specifically aimed at correcting these deficiencies. Recombinant PDGF-BB (becaplermin) is the only growth factor to date approved by the US Food and Drug Administration for the treatment of diabetic foot ulcers. PDGF-B is known to be a potent mitogen and chemotatic agent for stromal cells and may act to increase the wound vascularization by stimulating angiogenesis (Balfour and Noble, 1999).

Collective studies of wound healing in mice that lacks matricellular proteins indicate their ability to influence all phases of healing (Bornstein and Sage, 2002). Matricellular proteins, include SPARC, thrombospondin, tenascin, osteopontin and cysteine rich protein 61 belong to a group of matrix-associated factors that modulate cell-matrix communications (Bornstein, 2000; Yang et al., 2000; Puolakkainen et al., 2005). Matricellular proteins can associate with the diverse protein in extracellular matrix reservoir and bridged them with their cognate cell surface receptors. They act temporally and spatially to provide signals that influence cell activities such as migration, adhesion, inflammation and proliferation (Bornstein, 2000; Bornstein and Sage, 2002). Thus, matricellular proteins residing at the crossroads of cell-matrix communication can serve as a modulator for regulatory networks during wound healing. Presumably, the regulatory pathways consist of complex networks with many opportunities for

compensatory adjustments required for wound repair. Hence, targeting or replacing the necessary matricellular proteins may be more efficacious than individual cytokine-mediated candidates. Our findings from Chapter 1 and Chapter 2 justify the enrolment of ANGPTL4 as a new member of this family of protein. Despite the importance of ANGPTL4 in normal wound healing, its expression and roles in diabetic wound repair remains unclear. Preliminary investigation by other lab members showed that ANGPTL4-null mice had an increased inflammation and an impaired wound-related angiogenesis.

Altogether, ANGPTL4 modulates cell-matrix communications through its interactions with and effects on matrix proteins and integrins. We showed that ANGPTL4 deficiency delays wound re-epitehlialization and reduces matrix proteins expression. Importantly, it provides a novel means by which migrating wound keratinocytes can scrutinize the changes in the wound ECM and modulate their cell behavior.

2.6 CONCLUSION

ANGPTL4 interacts with matrix proteins and its cognate integrins, and exerts coordinated influences on cell-matrix communication, and thus on cellular behaviour (Figure 2.14). ANGPTL4 binds to specific matrix proteins via its C-terminal fibrinogen-like domain and delays their degradation by proteases. This directly affects integrin-mediated signaling by altering the balance between substrate-anchored matrix proteins and soluble matrix protein fragments, thereby modifying the availability of the local substrate-anchored ECM. Concordant with its effect on cell-matrix interaction, ANGPTL4 is a novel ligand of integrins β1 and β5. These interactions can activate the integrin-FAK-Src-PAK1 canonical signaling cascade that is needed for productive lamellipodia formation (Schlaepfer and Hunter, 1998; Schlaepfer and Mitra, 2004). Interestingly, the interaction of ANGPTL4 with specific integrins also recruits 14-3-3σ/β and PKCα, which also modulates cell migration via a FAK-independent mechanism (Fu et al., 2000; Han et al., 2001). Importantly, PKCα, a key regulator of the endo-exocytic machinery essential for integrin recycling, was also altered in ANGPTL4-deficient cells (Ng et al., 1999; Ivaska et al., 2005). Thus, ANGPTL4-bound integrins also provide a novel means by which selective integrin recycling can be achieved, depending on the local context of the ECM and also allows migrating wound keratinocytes to better scrutinize changes in the wound ECM and fine-tune their cellular behavior.

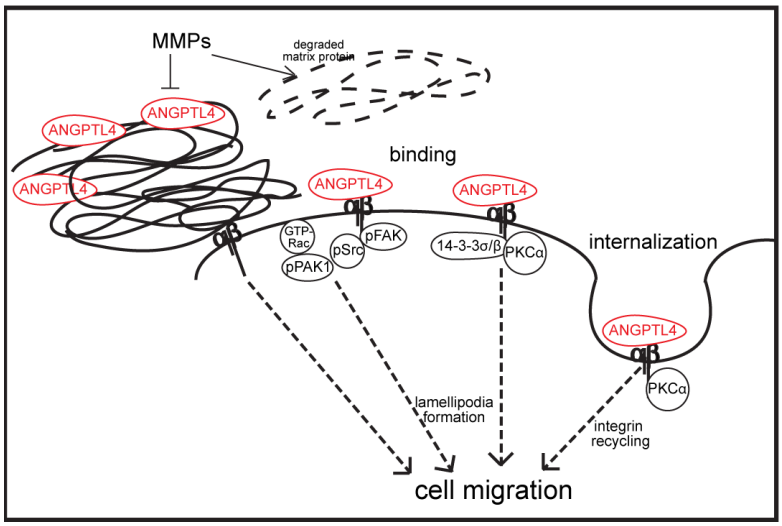

Figure 2.14 Schematic diagram of showing ANGPTL4 coordinating cell-matrix communication on two fronts. First, ANGPTL4 interacts with specific integrins to recruit and activate FAK-Src-PAK1-dependent and FAK-independent 14-3-3-PKCα pathways to facilitate productive lamellipodia formation and selective integrin internalization, which are important for rapid cell migration. Concomitantly, ANGPTL4 can also bind to specific matrix protein and delays their degradation by MMPs. This directly affects integrin-mediated signaling by altering the balance between substrate-anchored matrix proteins and soluble matrix protein fragments, thereby modifying the availability of the local substrate-anchored ECM.

2.7 FUTURE STUDIES

The Role of ANGPTL4 in Epithelial Keratinocyte Differentiation

The present study shows that ANGPTL4 affects keratinocytes cell migration during re-epithelialization. While re-epithelialization of the wound epithelium culminates in the formation of a new epidermis, it is important to further investigate the functional role of ANGPTL4 in the formation of a stratified epithelium. Previous studies have shown that lack of integrin β1 greatly impacts the balance of keratinocyte proliferation and differentiation, either through increased cellular apoptosis due to their detachment from the basal membrane or disrupted terminal differentiation (Dowling et al., 1996; Raghavan et al., 2000; Grose et al., 2002a). In addition, ANGPTL4 deficiency affects the expression of 14-3-3σ and protein kinase C (PKC), which are involved in the survival and differentiation of epithelium (Denning, 2004; Li et al., 2005). Organotypic skin culture (OTC) using human primary keratinocytes, which closely mimic epidermal regeneration during wound repair (Maas-Szabowski et al., 2001), may be used. In this system, keratinocytes can be seeded on a dermal fibroblast-embedded collagen matrix and cultured at an air-exposed interface to induce differentiation and stratification. Immunofluorescence and immunoblotting analyses using early, late and terminal differentiation markers can be performed to examine the degree of differentiation. Since keratinocyte differentiation requires detachment from the basal membrane, a balance of cells undergoing proliferation and differentiation become important. The number of proliferating and apoptotic cells can also be quantified using OTC. Pathways downstream of PKC signaling can also be determined.

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CHAPTER 3

PPARβ/δ REGULATES EPIDERMAL DIFERENTIATION THROUGH PROTEIN KINASE C AND Raf-MEK-ERK PATHWAYS MEDIATED BY SECRETION OF ANGIOPOIETIN-LIKE 4

Publications

(1)Regulation of epithelial-mesenchymal IL-1 signaling by PPARbeta/delta is essential for skin homeostasis and wound healing.
Chong HC, Tan MJ, Philippe V, Tan SH, Tan CK, Ku CW, **Goh YY**, Wahli W, Michalik L, Tan NS.
J Cell Biol. 2009 Mar 23;184(6):817-31.

(2)Angiopoietin-like 4 regulates epidermal differentiation by activation of AP-1.

Mintu Pal, Ming Jie Tan, Royston-Luke Huang, **Yan Yih Goh**, Mark Boon Yang Tang, and Nguan Soon Tan.

PLoS ONE 2011 Sep 22;6(9):e25377.

Figures 3.3, 3.4 and 3.5 of this chapter are included in publication (1).
Figures 3.6, 3.7, 3.8, 3.9 and table 3.4 of this chapter are included into the above manuscript (2).

3.1 ABSTRACT

Epithelium regeneration is crucial to re-establish a fully functional physical barrier after wounding. Using an organotypic culture model, we demonstrate that ANGPTL4 is essential for keratinocyte regeneration and differentiation. The ability to resist anoikis allows the keratinocytes to undergo terminal differentiation after detaching from the basal membrane, instead of undergoing apoptosis. Further observation of the interaction between ANGPTL4 and integrin in the activation of the FAK-Src-PAK1 and 14-3-3-mediated signaling pathways that results in the acceleration of cell migration reveals the involvement of integrin-mediated protein kinase C and raf-MEK-ERK signaling cascades. We showed that the deficiency of ANGPTL4 have diminished expression of various protein kinase C isotypes, which are well-established for their role in keratinocyte differentiation. Taken together, we showed that PPARβ/δ regulates epidermal maturation after wound closure via ANGPTL4-mediated signaling pathway.

3.2 INTRODUCTION

3.2.1 Epidermal Barrier Formation

Mammalian skin consists of the epidermis and dermis, separated by a basement membrane. The epidermis consists of a stratified squamous epithelium that is composed of several cell layers (Figure 3.1). Resting on the basement membrane is the basal layer (BL), consisting of proliferating transit-amplifying cells. The basal layer stratifies to give rise to differentiated cell layers of the spinous layer (SL), granular layer (GL) and the stratum corneum (SC). The stratified epithelium retains the ability to renew itself under both homeostatic and injurious conditions. The physical barrier resides within the exterior layers of the epidermis, which are sloughed off and replaced from the inner cells. The process of terminal differentiation begins when basal cells concomitantly withdraw from the cell cycle and lose their ability to adhere to the basement membrane (Fuchs and Raghavan, 2002). In the intermediate spinous layers, the cells reinforce a durable keratin filament framework to provide the mechanical strength necessary to resist physical injury. In the granular layers, lipids are produced, keratins are bundled into macrofibrils through their association with filaggrin, and a cornified envelope is assembled. As the cell loses its integrity towards the cornified layer, transglutaminase enzymes irreversibly cross-link the cornified envelope proteins, creating a tough, insoluble sac that surrounds the keratin fibers. Finally, lipids are extruded into the intercellular space onto the cornified envelope scaffold (Niemann and Watt, 2002). This process of differentiation from a mitotically active basal cell to a squame, or terminally differentiated squamous cell, is maintained throughout life as part of epidermal regeneration (Segre, 2006). The formation of mature

epidermis during embryogenesis, maintenance of skin homeostasis, and wound healing, involves keratinocyte proliferation, migration, differentiation and apoptosis. Different factors originating from mesenchymal and epithelial compartments, which act in paracrine and/or autocrine loops, are involved in regulating these events (Maas-Szabowski et al., 2001).

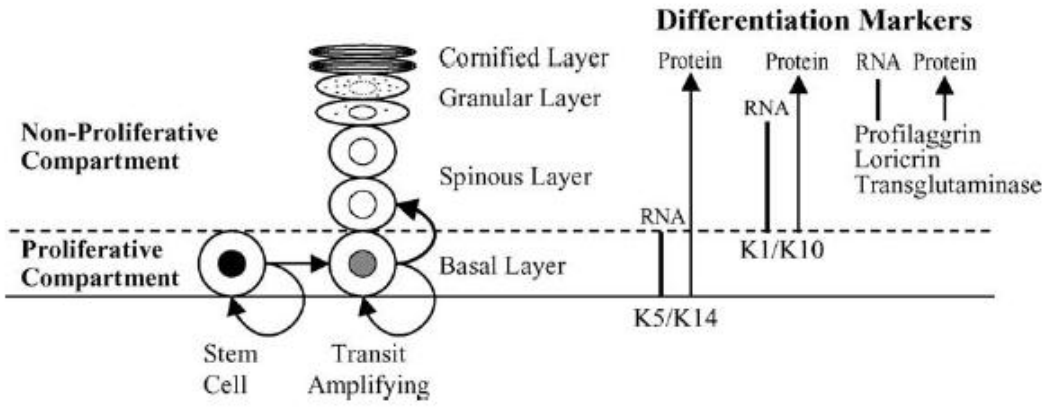

Figure 3.1 Compartmentalization of growth and differentiation in the epidermis. Epidermal stem cells residing in specific niches are self renewing, and give rise to transit amplifying that are responsible for the majority of the proliferative activity in the basal layer. Basal layer keratinocytes express keratin 5 and keratin 14, but as they detach from the basement membrane and migrate into the first suprabasal layer in the spinous layer, they irreversibly exit the cell cycle and switch to keratin 1 and keratin 10. Further up in the epidermis, the keratinocytes switch gene expression programs again and begin expressing the granular layer differentiation markers profilaggrin, loricrin and transglutaminase. The envelope precursors are then cross-linked by transglutaminase and the keratinocytes lose their nuclei and die as they become cornified envelopes. (Taken from Denning, 2004).

3.2.2 The Role of Protein Kinase C in Keratinocytes

PKC isoforms are regulated by multiple secondary messengers (calcium, diacylglycerol), lipids, auto- and trans-phosphorylation, protein-protein interactions, and proteolysis (Denning, 2004). Of the nine PKC genes in humans, most of them are expressed in keratinocytes. PKCα is the only classical PKC isoform expressed in keratinocytes and is activated by calcium and diacylglycerol. The novel isoforms, PKCδ, PKCε and PKCη, are calcium independent but are also activated by diacylglycerol. PKCζ is the only atypical PKC isoform in keratinocytes, and it is neither activated by calcium nor diacylglycerol. PKCμ/PKD-1 is activated by calcium or diacylglycerol in keratinocytes, but this kinase lacks significant homology to the PKC catalytic domains and can be classified in a distinct kinase subfamily. The diversity of PKC isoforms, and their central place in many signaling pathways makes them well suited to use for interpreting the multiple extracellular signals that must be sensed by the keratinocytes in order to perform its proper function in the epidermis (Figure 3.2) (Denning, 2004).

Several PKC knockout mice exhibiting impairment in a variety of cellular functions, especially in epidermal differentiation, have been reported (Denning, 2004). For example, PKCη knockout mice revealed the importance of inflammation in wound healing (Chida et al., 2003); PKCα is involved in the signaling response of desmosomal adhesion to wounding (Wallis et al., 2000); PKCδ is involved in transcriptional regulation, cell cycle progression and programmed cell death (Steinberg, 2004); PKCε, on the other hand, is involved in cell survival and proliferation (Griner and Kazanietz, 2007).

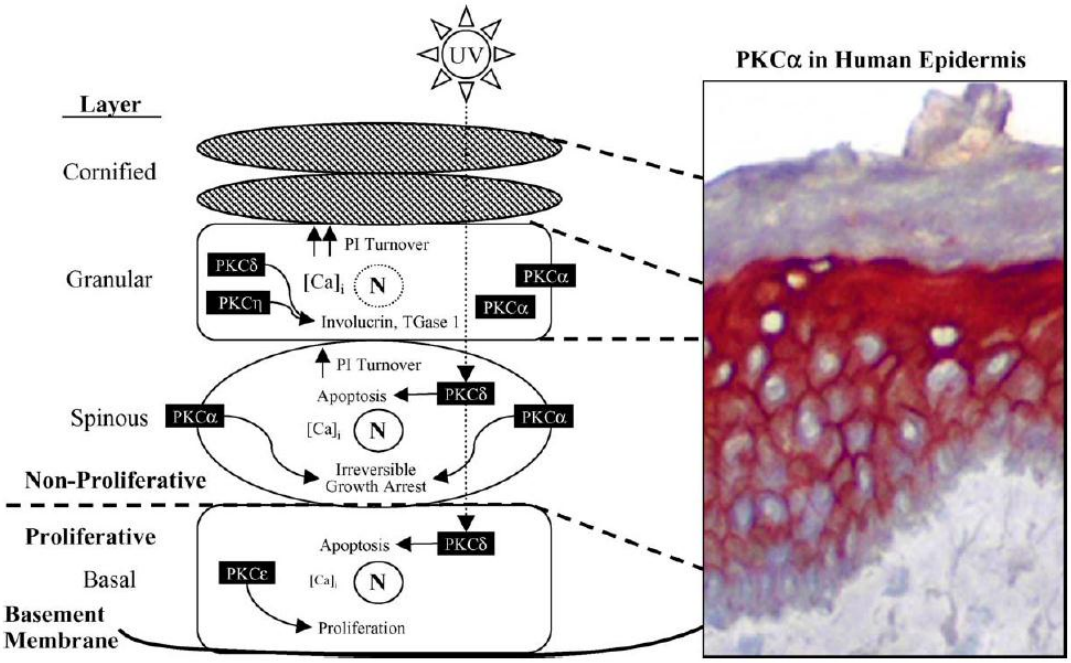

Figure 3.2 PKC isoforms in the epidermis. The schematic shows the expression profile and potential functions of several PKC isoforms in the epidermis as deduced from immunohistochemical staining *in vivo*, and *in vitro* studies. In the proliferative basal layer, high levels of PKCε are detected, suggesting that PKCε may help drive keratinocyte proliferation in the basal layer. As shown by immunohistochemical staining in the right panel (red-brown stain), PKCα is found in the membranes of suprabasal cells in the spinous and granular layers, highly indicative of activation in vivo. Activation of PKCα is associated with triggering irreversible growth arrest. PKCη is detected exclusively in the granular layer, and both PKCη and PKCδ are able to induce the differentiation markers involucrin and transglutaminase 1. PKCδ is detected at moderate levels throughout the epidermis, with slightly more in the basal/lower spinous layers. Activation of PKCδ by UV light is able to induce apoptosis and thus eliminate cells from the epidermis. The different localization of PKC isoforms in the epidermis reflects their different roles in keratinocyte proliferation, differentiation and death. (Taken from Denning, 2004).

3.2.3 Organotypic Skin Coculture – Advantages to Studying Epithelial-Mesenchymal Interactions

In order to study the molecular mechanisms of this regulation under defined *in vitro* conditions while maintaining the major principles of skin biology, three-dimensional organotypic co-cultures were developed, with keratinocytes growing on a matrix populated with fibroblasts (Maas-Szabowski et al., 1999).

By mimicking the natural tissue architecture, although in a simplified form, such conditions enable keratinocytes to recapitulate their tissue-specific growth and differentiation program *in vitro,* which is impossible to achieve using two dimensional culture methods (Maas-Szabowski et al., 2005). Organotypic co-cultures generate a normal epidermis, irrespective of the species and tissue origin of the fibroblasts. This facilitates the identification of the origin of compounds involved in epidermal tissue reconstitution and growth regulation (Maas-Szabowski et al., 2001). Moreover, the functional significance for the keratinocyte phenotype of genetically modified fibroblasts from transgenic or knockout mice, even those exhibiting an embryonic lethal phenotype, can be studied in such heterologous *in vitro* tissue equivalents.

3.2.4 Role of PPAR in Keratinocyte Differentiation

Several works have shown that ligand-activated PPARβ/δ can induce terminal differentiation of keratinocytes (Tan et al., 2001; Burdick et al., 2006). PPARβ/δ was first suggested to induce epidermal terminal differentiation when its expression was increased together with other differentiation markers in phorbol ester (TPA) treated

cultured human keratinocytes and mouse skin (Matsuura et al., 1999). Ligand activation of PPARβ/δ stimulated the expression of proteins required for terminal differentiation, including transglutaminase 1 (TGase 1), small proline-rich proteins (SPRs) and involucrin, but it is not found in similar treatments using PPARβ/δ-null mice (Schmuth et al., 2004; Kim et al., 2005). Despite convincing reports that PPARβ/δ can induce terminal differentiation of epidermal keratinocytes, it remains to be determined if PPARβ/δ directly regulates the expression of mRNAs encoding for differentiation genes, or if this effect is due to secondary events resulting from receptor activation (Burdick et al., 2006).

My findings in Chapter 1 and 2 showed that ANGPTL4 interacts with vitronectin and fibronectin in the wound bed, delays their proteolytic degradation by MMPs, and thereby regulates the availability of local extracellular matrix. In addition, we identified integrins β1 and β5 as novel binding partners of ANGPTL4. These interactions activate the FAK-Src-PAK1 and 14-3-3-mediated signaling pathways to accelerate cell migration (Chapters 1 & 2). Thus, ANGPTL4 is important for many biological events during the re-epithelialization phase of wound healing. We next questioned if ANGPTL4 also play a role in the formation of stratified epidermis upon wound closure. In this Chapter 3, I studied the effect of ANGPLT4 on keratinocyte differentiation during epithelium regeneration. We revealed a novel role of ANGPTL4 in keratinocyte differentiation through the activation of protein kinase C.

3.3 MATERIALS AND METHODS

3.3.1 Reagents: Rat tail collagen type I (BD Biosciences), GW501516 was obtained from Enzo Biochem, Inc. Primary neonatal human fibroblasts and keratinocytes were obtained from Invitrogen. All restriction enzymes and DNA/RNA modifying enzymes were obtained from Fermentas. Otherwise stated all chemicals from Sigma-Aldrich.

Antibodies: Ki67, keratin 5, keratin 10 and filaggrin (NovoCastra); Alexa488- or Alexa594-conjugated secondary antibodies (Molecular Probes); PPARα (Millipore), PPARβ/δ and PPARγ (Thermo Fisher Scientific); p(Y397)-FAK, β-tubulin, cyclin D1, PCNA, transglutaminase 1 (Tgase 1), ERK-1, p(T202/Y204)-ERK1/ERK2, laminin and HRP-conjugated secondary antibodies (Santa Cruz Biotechnology); 14-3-3σ, PKC isotypes, RACK1 (BD Biosciences); p(S241)PDK1, p(S9)GSK3β, FAK, p(S259)Raf1, caspase 3 and cleaved caspase 3 (Cell Signaling); polyclonal antibodies against the C-terminal region mouse (190-410 amino acids) of ANGPTL4 were produced in-house.

3.3.2 Lentivirus-Mediated Knockdown of PPARβ/δ

Two siRNAs targeting the human PPARβ/δ, 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 3.1. 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.

Table 3.1 Oligonucleotide sequences used for PPAR knockdown and RT-PCR.		
siRNA		Sequence (5'-3')
PPARβ/δ11	sense	AAAGGGAAGCAGTTGGTGAATGG
	antisense	AAAACCATTCACCAACTGCTTCC
PPARβ/δ9	sense	AAAGGAGCGCAGCTGCAAGATTC
	antisense	AAAAGAATCTTGCAGCTGCGCTC
Control siRNA	sense	AAAGCTGTCTTCAAGCTTGATATCGAAGACTA
	antisense	AAAATAGTCTTCGATATCAAGCTTGAAGACAG
Real-time qPCR		
PPARα	forward	ATGTCACACAACGCGATTC
	reverse	TCTTGGCCAGAGATTTGAGA
PPARβ/δ	forward	AGGAGCCATTCTGTGTGTGA
	reverse	TCCTGCCAGCAGAGAGTGAT
PPARγ	forward	AATGTGAAGCCCATTGAAGA
	reverse	AATGTGAAGCCCATTGAAGA
H1 sequencing primer		CTGGGAAATCACCATAAACGTGA

3.3.3 Organotypic Skin Culture (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 × HBSS containing phenol red was mixed with 8 vol 4 mg/ml rat tail type I collagen. The acetic acid was neutralized with 1 N NaOH on ice. Fibroblasts were resuspended in 1 vol 1 × HBSS and added dropwise to the neutralized collagen to achieve a final cell density of 10^5 cells/ml of collagen. 3 ml of this fibroblast/collagen mixture was dispensed into a 24-mm diameter Transwell culture insert (3-μm 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% CO_2, 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 μM carnitine, 1 mg/ml fatty acid – free albumin, lipid supplements 0.05 mM ethanolamine, 1 μM isoproterenol, 1 μM α-tocopherol, and 50 μg/ml ascorbic acid. The following day, the medium was removed and 10^6 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% CO_2 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.

3.3.4 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- μm cryostat tissue sections were mounted on SuperFrost Plus slides (Menzel-Glaser). 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 × /0.45 objective (Nikon), RetigaEXiFAST cooled mono 12-bit camera (QImaging), and Image-Pro Plus software (Media Cybernetics).

3.3.5 Chromatin Immunoprecipitation (ChIP)

ChIP was performed as described previously (Tan et al., 2004) with minor modifications. In brief, the keratinocytes were retrieved and crosslinked with 1% formaldehyde for 15 min at 37 °C prior to sonication in lysis buffer. Monoclonal anti-PPARβ/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 3.2.

Table 3.2 ChIP primers used in this work.

primer		Sequence (5'-3')
PPRE	forward	TTTACTCCTGGGGACATGTGCTGGTTTC
	reverse	GGCTGCCCCTGATTCAAATCCATGATT
Control	forward	GGCTGGGAGTGCAGGGCAGGAGC
	reverse	CACACAATGTGCAGAGCCTGTCTT

3.3.6 Knockdown of ANGPTL4 and ANGPTL4 Knockout Mice

siRNA against human ANGLPTL4 and scrambled sequence as control were subcloned into the pFIV-H1/U6-puro pFIV/siRNA lentivirus system. Pseudovirus were purified and transduced as described (Chong et al., 2009). Transient suppression of endogenous ANGPTL4 expression in human keratinocytes was performed using either siGLO control or ON-TARGETplus SMARTpool ANGPTL4 siRNA (Dharmacon; L-007807-00) by

means of DharmaFECT1 (Chapter 1). Pure bred ANGPTL4$^{+/+}$ and ANGPTL$^{-/-}$ mice on C57Bl/6 background were used (Koster et al., 2005).

3.3.7 Isolation of Total RNA, Real-Time PCR and Microarray

To obtain RNA of appropriate quality for chip analysis, fresh skin of pure bred ANGPTL4$^{+/+}$ and ANGPTL$^{-/-}$ mice was excised and snapped freeze in liquid nitrogen. RNA was first isolated using RNAeasy kit and Qiashredders (Qiagen) to homogenize cell extracts with centrifugation at 1,800 xg for 2 min and subjected to DNase I treatment. 5 μg 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 L27 (L27), which did not change under any of the experimental conditions studied. Oligonucleotides and Taqman probes sequences for determining knockdown efficiency of ANGPTL4 and relative expression level of indicated genes using are provided in Table 3.3. DNA microarray analysis was performed by Affymetrix platform on the isolated RNA.

Table 3.3. Oligonucleotide sequences used for RT-PCR.

L27 (universal)	forward	CTGGTGGCTGGAATTGACCGCTA
	reverse	CAAGGGGATATCCACAGAGTACCTTG
Human		
ANGPTL4	forward	CTCCCGTTAGCCCCTGAGAG
	reverse	AGGTGCTGCTTCTCCAGGTG
Mouse		
ANGPTL4	forward	GCTTTGCATCCTGGGACGAG
	reverse	CCCTGACAAGCGTTACCACAG
Itgb5	forward	GCTGCTGTCTGCAAGGAGAA
	reverse	AAGCAAGGCAAGCGATGGA
Sfn	forward	GTGTGTGCGACACCGTACT
	reverse	GCTATCTCGTAGTGGAAGACTGA
Ccnd1	forward	CAGAAGTGCGAAGAGGAGGTC
	reverse	TCATCTTAGAGGCCACGAACAT
Ctnnb1	forward	ACGCAGCAGCAGTTTGTGGAGG
	reverse	GTTGCCACGCCTTCATTCCTG
Timp1	forward	CTTGGTTCCCTGGCGTACTC
	reverse	ACCTGATCCGTCCACAAACAG
Plekhc1	forward	TGACCGAGTCTTCAAGGCTG
	reverse	GGCATGATAAGAGGTCCTTCCA
Pkcθ	forward	GGTCCTCCTGTACGAAATGCT
	reverse	AGTTGCTGTAGTCTGAAGGGG
Mmp13	forward	ACTCAAATGGTCCCAAACGAAC
	reverse	CAGGAGGAAAAGCGTGTGC

* All nucleotides and probes synthesized by Sigma-Aldrich. Melting curve analysis was performed to assure that only one PCR product was formed. Primers were designed to generate a PCR amplification product of 100 to 250 bp. Only primer pairs yielding unique amplification products without primer dimer formation were subsequently used for real-time PCR assays.

3.3.8 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 $Na_2H_2PO_4$, 250 mM NaCl, 1% Triton X-100, and 0.1% SDS). Equal amounts of protein extracts (50 μg) 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.

3.4 RESULTS

3.4.1 The Role of PPARβ/δ in Cutaneous Differentiation

Earlier studies have shown that ligand-activated PPARβ/δ induced the differentiation of human keratinocytes in monolayer cultures, while it has recently been shown that PPARβ/δ promotes corneal layer formation and epidermal permeability barrier development (Jiang et al., 2010). We examined the autocrine and paracrine consequences of PPARβ/δ deficiency during human epidermis formation using the organotypic skin culture (OTC) model. First, we examined the expression pattern of the three PPAR isotypes in fibroblasts and keratinocytes of the control OTC. Both quantitative PCR (qPCR) and ELISA analyses performed on mechanically separated dermis and epidermis equivalents of OTCs revealed that PPARβ/δ is the predominant isotype in the keratinocytes and dermal fibroblasts, whereas the lower levels of PPARα and PPARγ are comparable (Figure 3.3A). We next assessed the knockdown efficiency of PPARβ/δ expression in human keratinocytes and fibroblasts by lentivirus-mediated siRNAs using qPCR and immunoblot analyses. Analysis of the qPCR revealed a > 95% reduction of PPARβ/δ expression in cells transduced with the siRNA PPARβ/δ11 sequence. (Figure 3.3B, left). Immunoblot analyses also showed negligible levels of PPARβ/δ protein in the transduced cells. (Figure 3.3B, right). These cells were used for subsequent experiments.

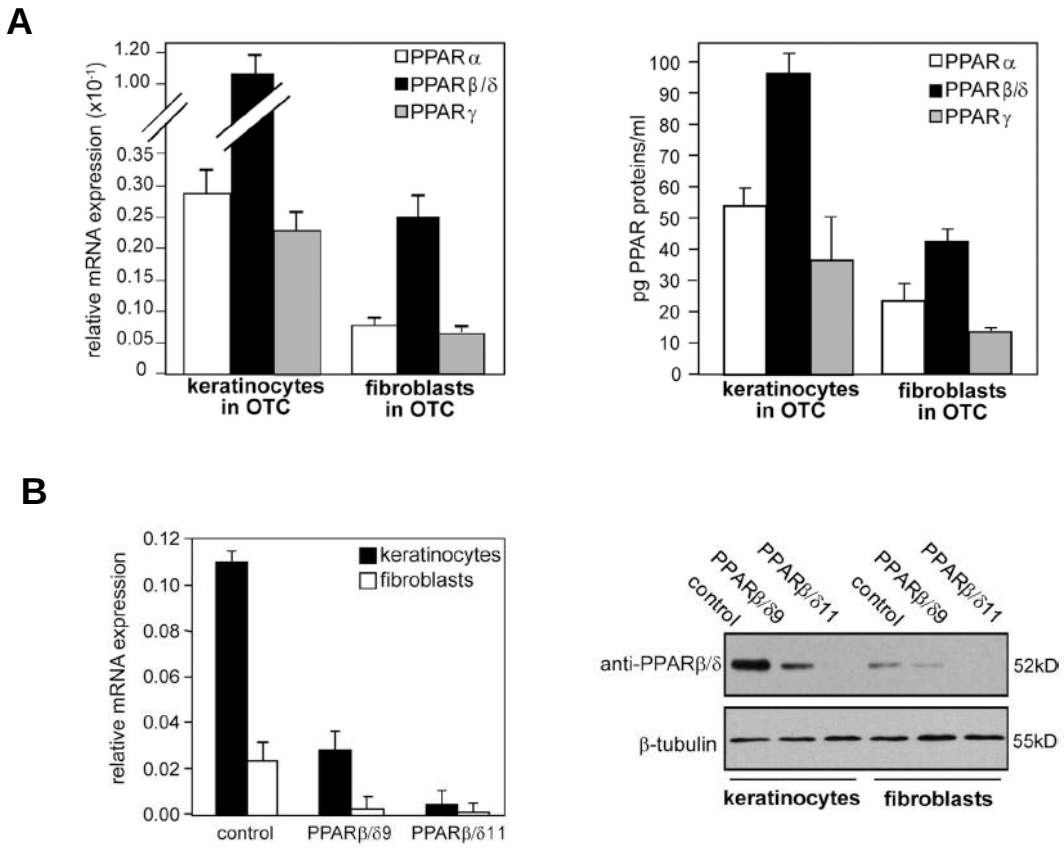

Figure 3.3 Expression of PPARβ/δ in keratinocytes. (A) Expression profile of PPARs in OTC keratinocytes and fibroblasts. Total RNA and protein were extracted from keratinocytes and fibroblasts in OTC. Expression levels of PPAR mRNA (left) and protein (right) were monitored by qPCR and PPAR transcription factor assay kit, respectively. PPARβ/δ mRNA was normalized with control ribosomal protein L27 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. Data are mean ± SD, n = 3.

Organotypic cultures were reconstructed using control keratinocytes (K_{CTRL}), PPARβ/δ knockdown keratinocytes ($K_{PPARβ/δ}$), control fibroblasts (F_{CTRL}), and PPARβ/δ knockdown fibroblasts ($F_{PPARβ/δ}$) in various combinations. Immunoblot analysis and immunofluorescence staining of the 2-week-old K_{CTRL}/F_{CTRL} OTCs showed the expected keratinocyte differentiation markers keratin 5, keratin 10, and involucrin (Figures 3.4 & 3.5). OTCs with $K_{PPARβ/δ}$ showed reduced expression of terminal markers, which is consistent with the known pro-differentiation role of PPARβ/δ (Figures 3.4 & 3.5; (Schmuth et al., 2004)). No difference in keratin 5 expression, localized to the basal layer of the epidermis, was observed among the various OTCs (Figure 3.4 and Figure 3.5). This provided evidence for the cell-autonomous action of PPARβ/δ in keratinocyte differentiation.

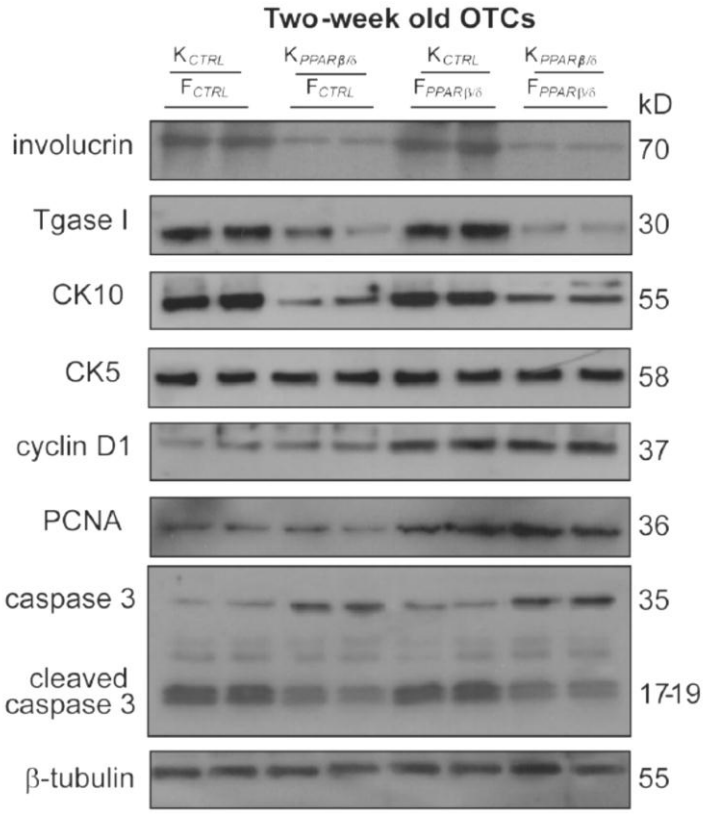

Figure 3.4 PPARβ/δ-deficient keratinocytes increase epidermal differentiation. Immunoblot analysis of epidermis from 2-wk-old OTCs constructed using K_{CTRL} or $K_{PPARβ/δ}$ and F_{CTRL} or $F_{PPARβ/δ}$. Involucrin and transglutaminase I (Tgase I) are terminal differentiation markers, and keratin 10 (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.

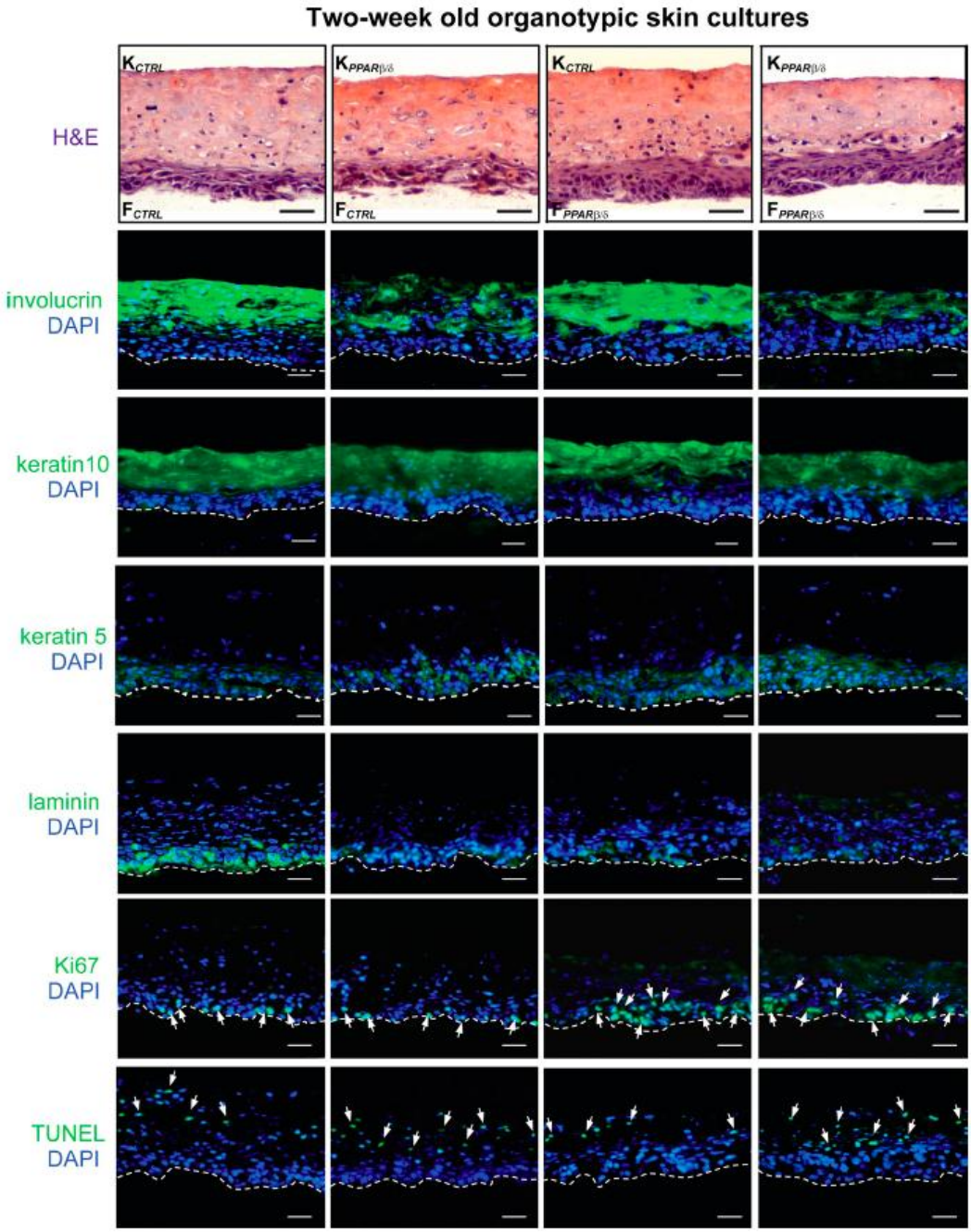

Figure 3.5 Immunofluorescence staining of 2-wk-old OTCs. OTCs constructed with control or PPARβ/δ knockdown keratinocytes (K_{CTRL} vs. $K_{PPARβ/δ}$) and fibroblasts (F_{CTRL} vs. $F_{PPARβ/δ}$). Pictures from representative immunostained sections are shown. $K_{PPARβ/δ}$ potentiates epithelial differentiation while $F_{PPARβ/δ}$ potentiates 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; $K_{PPARβ/δ}/F_{CTRL}$, 9 ± 1.8; K_{CTRL} / $F_{PPARβ/δ}$, 32.5 ± 2.11; $K_{PPARβ/δ}/F_{PPARβ/δ}$, 26.4 ± 3.3). H&E, hematoxylin and eosin staining. Dashed white lines show the epidermal–dermal junction. Bars: (H&E) 40 μm; (DAPI) 20 μm.

3.4.2 Impaired Epidermal Differentiation in ANGPTL4-Deficient Organotypic Culture

Although ligand activation of PPARβ/δ stimulates the expression of proteins required for terminal differentiation in epidermal keratinocytes in an autonomous manner, it remains unclear how the differentiation genes are modulated. To this end, we investigated the secondary events of PPARβ/δ. We examined the phenotypes in keratinocyte differentiation in organotypic cultures. If the impaired differentiation phenotypes of the PPARβ/δ-deficient cultures are mediated through the effect of secreted ANGPTL4, we should obtain a similar phenocopy when an ANGPTL4-deficient keratinocyte organotypic culture is performed. We first suppressed endogenous ANGPTL4 expression by RNA interference in primary human keratinocytes (refer to Chapter 1 for the generation of knockdown keratinocytes). In OTC, either K_{CTRL} or $K_{ANGPTL4}$ keratinocytes were seeded onto a dermal fibroblast-embedded collagen matrix and cultured in an air-exposed interface to induce stratification and differentiation. Consistent with the above findings in skin biopsies, hematoxylin and eosin staining revealed that the epidermis was thinner in $K_{ANGPTL4}$ than in K_{CTRL} ($K_{ANGPTL4}$ vs. K_{CTRL}: 248.7±25.1 vs. 328.9±27.4 μm, $p<0.001$) (Figure 3.6A). Immunofluorescence performed using the differentiation markers keratin 10 and filaggrin also showed that $K_{ANGPTL4}$ OTCs had impaired epidermal differentiation when compared with K_{CTRL} (Figure 3.6A). Immunoblot analyses using the differentiation markers keratin 10 and transglutaminase 1 further confirmed our findings (Figure 3.6B). $K_{ANGPTL4}$ OTCs also showed more apoptotic (TUNEL-positive) ($K_{ANGPTL4}$ vs. K_{CTRL}: 53±2.7 vs. 18±2.5 labeled cells per

microscopic field; $p<0.001$) and reduced Ki67-positive proliferating cells as compared to the control K_{CTRL} OTCs ($K_{ANGPTL4}$ vs. K_{CTRL}: 18±2.9 vs. 31±8.1; $p<0.001$) (Figure 3.6A). These data were further supported by immunoblotting with cyclin D1 and PCNA as proliferation markers, as well as with cleaved caspase 3 as an apoptotic marker (Figure 3.6B).

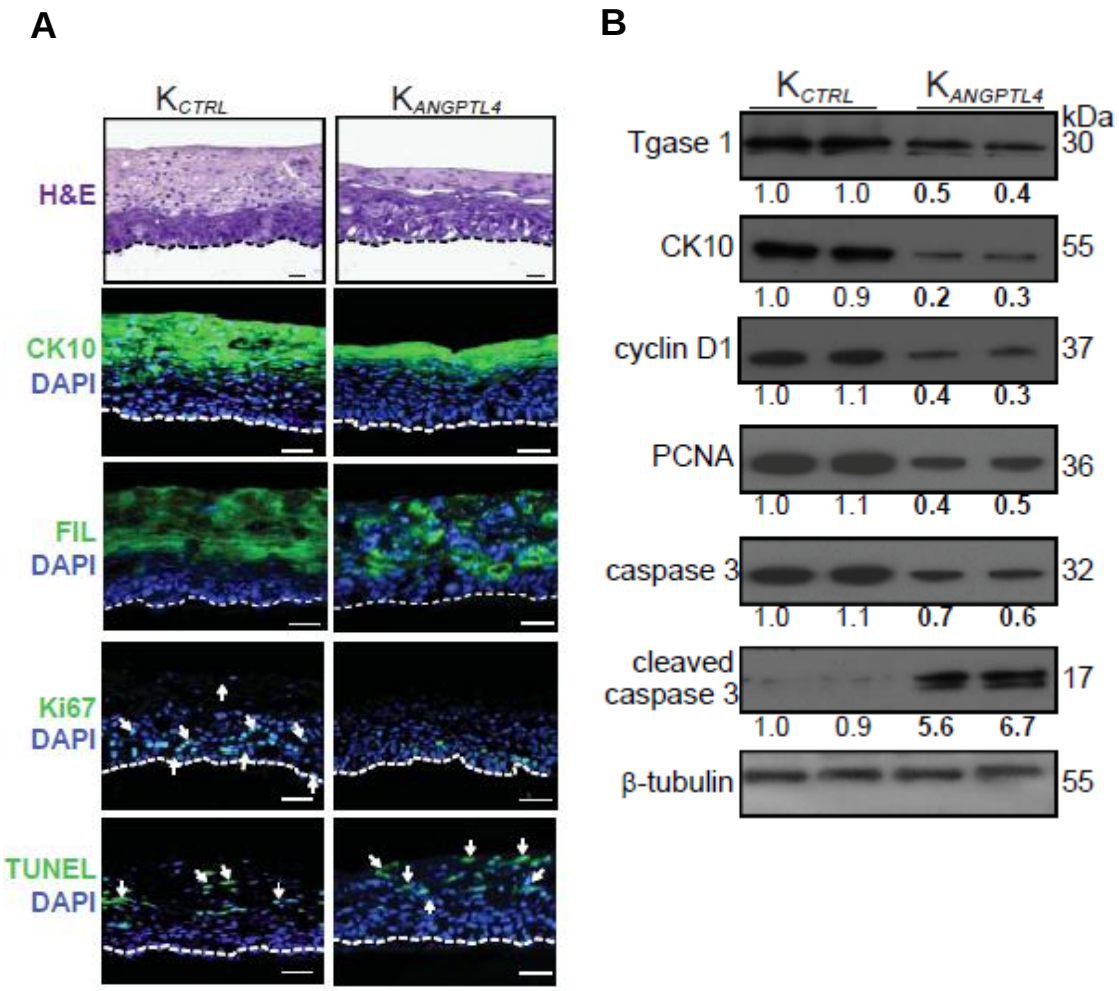

Figure 3.6 ANGPTL4 is crucial for epidermal differentiation. (A) Immunofluorescence staining of K_{CTRL} and $K_{ANGPTL4}$-dervied organotyipic skin culture (OTCs). Keratin 10 (CK10) and filaggrin (FIL) were late and terminal epidermal differentiation, respectively. Proliferating and apoptotic cells were identified using Ki67 antibody and TUNEL assay, respectively (white arrows). Sections were counterstained with DAPI (blue). Dotted white line represents epidermal-dermal junction. H&E: Haematoxylin and eosin stain Scale bar 40 μm. Mean proliferating and apoptotic cells were numerated from 3 standardized microscopic fields per section, performed on 3 sections from 4 OTCs (n=36). **(B)** Immunoblot analysis of K_{CTRL} and $K_{ANGPTL4}$ epidermis. Cyclin D1 and PCNA were used as proliferation markers. Cleaved caspase 3 was as apopotic marker. Values below the bands represent the mean fold differences in protein expression levels with respect to control K_{CTRL} or ANGPTL4$^{+/+}$, which was assigned the value one. Values in bold are significant with $p<0.05$ when compared to K_{CTRL} at corresponding time. Five independent experiments, animals or OTCs were used. Representative pictures of immunoblot were shown.

Re-epithelialization of the wounded epithelium culminates in the formation of a new epidermis. To gain insight into the underlying mechanisms, we first examined skin biopsies from ANGPTL4-null (ANGPTL4$^{-/-}$) and wildtype (ANTPTL4$^{+/+}$) mice (Figure 3.7A). Deficiency in ANGPTL4 resulted in a thinner epidermis (wildtype vs. ANGPTL4$^{-/-}$: 32.5±12.4 vs. 21.9±4.6 μm, $p<0.001$). Immunofluorescence performed using the differentiation markers keratin 10 and filaggrin showed that ANGPTL4$^{-/-}$ had impaired epidermal differentiation when compared with wildtype (Figure 3.7A). Immunoblot analyses using the differentiation markers keratin 10 and transglutaminase 1 further confirmed our findings (Figure 3.7B). ANGPTL4$^{-/-}$ also showed more apoptotic cells (TUNEL-positive) (ANGPTL4$^{-/-}$ vs. wildtype: 12 ± 3.4 vs. 4 ± 1.7 labeled cells per microscopic field; $p<0.001$ or ANGPTL4$^{-/-}$ vs. wildtype: 0.05% vs. 0.03%, $p<0.05$) and reduced Ki67-positive proliferating cells as compared to the control wildtype (ANGPTL4$^{-/-}$ vs. wildtype: 4 ± 0.7 vs. 5±1.1; $p<0.01$ or ANGPTL4$^{-/-}$ vs. wildtype: 0.02% vs. 0.04%; $p<0.05$) (Figure 3.7A). These data were further supported by immunoblotting with the proliferation markers cyclin D1 and PCNA, as well as with cleaved caspase 3 as an apoptotic marker (Figure 3.7B). Taken together, these data demonstrate that ANGPTL4 modulates epidermal differentiation.

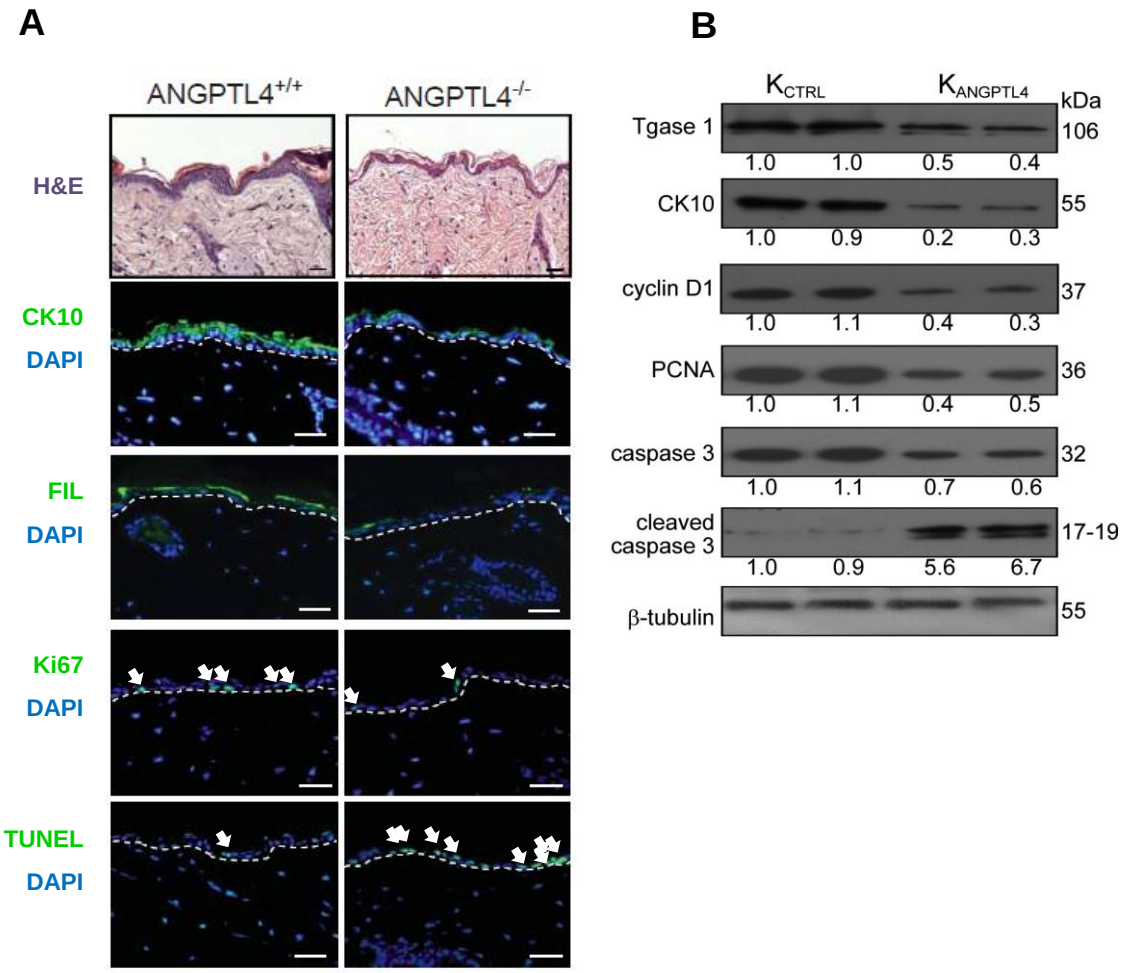

Figure 3.7 Deficiency in ANGPTL4 impairs epidermal differentiation. (A) Immunofluorescence staining of ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mouse skin. Keratin 10 (CK10) and filaggrin (FIL) were late and terminal epidermal differentiation, respectively. Proliferating and apoptotic cells were identified using Ki67 antibody and TUNEL assay, respectively (white arrows). Apoptotic cells (TUNEL-positive) (ANGPTL4$^{-/-}$ vs. wildtype: 12 ± 3.4 vs. 4 ± 1.7 labeled cells per microscopic field; $p<0.001$ or ANGPTL4$^{-/-}$ vs. wildtype: 0.05% vs. 0.03%, $p<0.05$) and Ki67-positive proliferating cells (ANGPTL4$^{-/-}$ vs. wildtype: 4 ± 0.7 vs. 5±1.1; $p<0.01$ or ANGPTL4$^{-/-}$ vs. wildtype: 0.02% vs. 0.04%; $p<0.05$). Sections were counterstained with DAPI (blue). Dotted white line represents epidermal-dermal junction. H&E: Haematoxylin and eosin stain Scale bar 40 μm. Mean proliferating and apoptotic cells were numerated from 3 standardized microscopic fields per section, performed on 3 sections from 4 mice (n=4). **(B)** Immunoblot analysis of wildtype (ANGPTL4$^{+/+}$) and knockout mice (ANGPTL4$^{-/-}$) skin. Cyclin D1 and PCNA were used as proliferation markers. Cleaved caspase 3 was as apopotic marker. Values below the bands represent the mean fold differences in protein expression levels with respect to control K_{CTRL} or ANGPTL4$^{+/+}$, which was assigned the value one. Values in bold are significant with $p<0.05$ when compared to K_{CTRL} at corresponding time. Five independent experiments, animals or OTCs were used. Representative pictures of immunoblot were shown.

Lending additional support, DNA microarray analysis comparing the skin from ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice (See chapter 1 for details of the knockout mice) confirmed the consistent down-regulation of numerous genes involved in differentiation, cytoskeletal dynamics, cell adhesion, and migration in the skin of the knockout mice (Figure 3.8A & Table 3.4). The mRNA expression levels of selected genes were further confirmed by qPCR (Figure 3.8B). Taken together, our results showed that ANGPTL4 is essential in mediating the gene expression involved in cell differentiation.

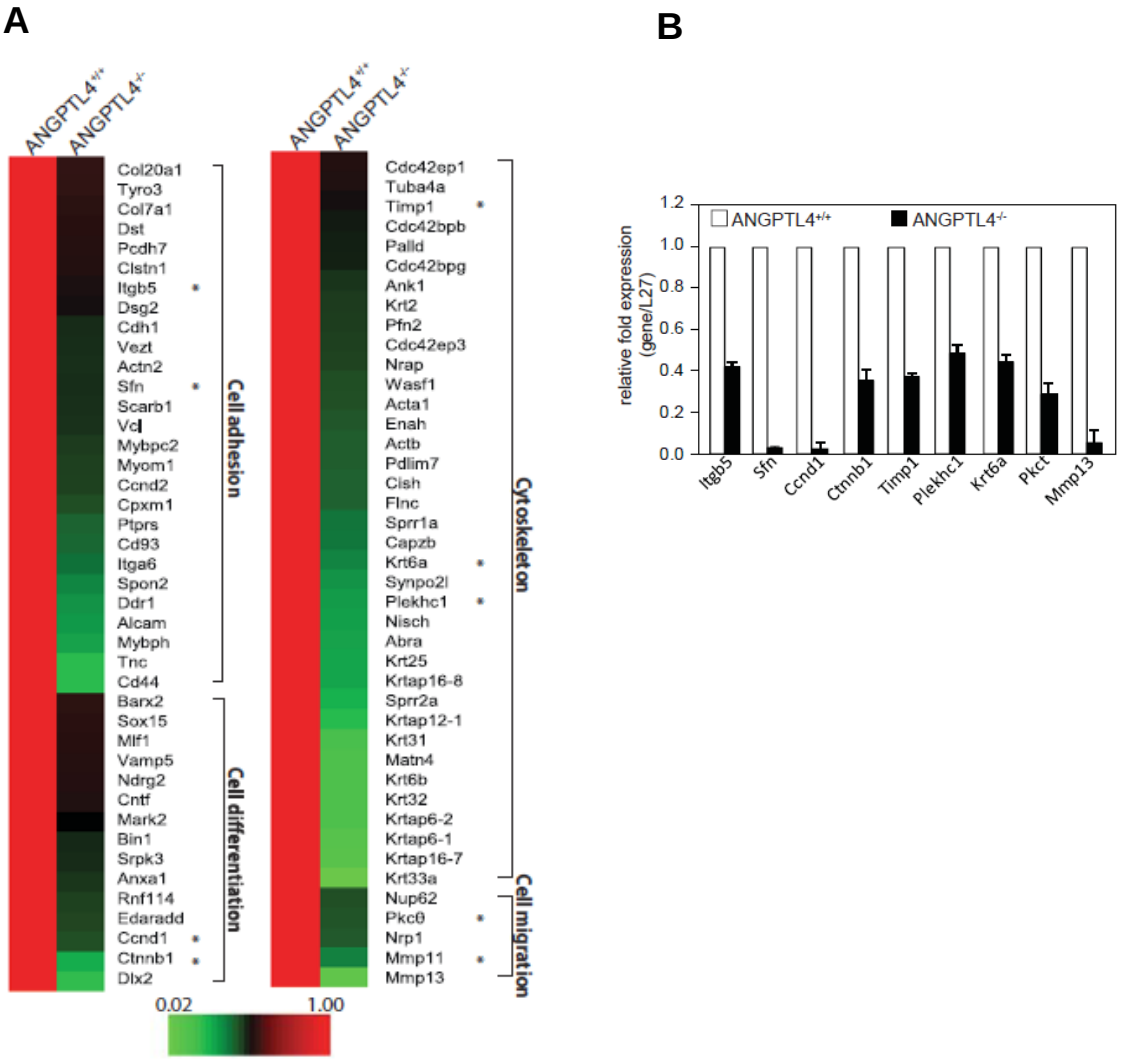

Figure 3.8 ANGPTL4 deficiency affects epidermal differentiation. (A) Heat map showing the genes down-regulated in skin of ANGPTL-null (ANGPTL4$^{-/-}$) and wild type (ANGPTL4$^{+/+}$) mice. DNA microarray analysis was performed by Affymetrix platform. Genes were classified into 4 functional clusters- cell adhesion, differentiation cytoskeleton and migration. The color scale is representative of the ratio of ANGPTL4$^{-/-}$ versus ANGPTL4$^{+/+}$. Results generated from three pairs of ANGPTL4$^{-/-}$ and ANGPTL4$^{+/+}$ mice. Detailed description of the genes and expression in fold changes is in Table 4.4. The expression of selected genes, whose expression was further confirmed by qPCR is indicated in asterisk. **(B)** Relative fold expression of mRNA expression level of indicated genes in ANGPTL4$^{-/-}$ with respect to control ANGPTL4$^{+/+}$ skin, which was assigned the value one. Ribosomal protein L27 was used as a normalizing housekeeping gene. Itgb5 : integrin beta 5; Sfn : Stratifin (14-3-3σ); Ccnd1 : Cyclin D1; Ctnnb1 : Catenin (cadherin-associated protein), beta 1, 88kDa; Timp1 : TIMP metallopeptidase inhibitor; Plekhc1 : Fermitin family homolog 2; Krt6a : Keratin 6A; Pkct : protein kinase C theta; Mmp13 : Matrix metallopeptidase 13.

Table 3.4 Genes down-regulated in mouse skin of ANGPTL4$^{-/-}$ (KO) as compared to ANGPTL4$^{+/+}$ (WT)

Access No.	*Symbol*	*Description*	[a]*Fold change*	*Functions*
XM_181390	Col20a1	Collagen, type XX, alpha 1	0.53	
NM_019392	Tyro3	TYRO3 protein tyrosine kinase	0.52	
NM_007738	Col7a1	Collagen, type VII, alpha 1	0.52	
NM_133833	Dst	Dystonin	0.51	
NM_018764	Pcdh7	Protocadherin 7	0.51	
NM_023051	Clstn1	Calsyntenin 1	0.50	
NM_010580	Itgb5	Integrin, beta 5	0.49	
NM_007883	Dsg2	Desmoglein 2	0.49	
NM_009864	Cdh1	Cadherin 1, type 1, E-cadherin (epithelial)	0.46	
NM_172538	Vezt	Vezatin, adherens junctions transmembrane protein	0.46	
NM_033268	Actn2	Actinin, alpha 2	0.45	
AF152893	Sfn	Stratifin	0.45	
AK080894	Scarb1	Scavenger receptor class B, member 1	0.45	
NM_009502	Vcl	Vinculin	0.45	Cell adhesion
NM_146189	Mybpc2	Myosin binding protein C, fast type	0.44	
NM_010867	Myom1	Myomesin 1, 185kDa	0.44	
NM_009829	Ccnd2	Cyclin D2	0.43	
NM_019696	Cpxm1	Carboxypeptidase X (M14 family), member 1	0.42	
AK032604	Ptprs	Protein tyrosine phosphatase, receptor type, S	0.38	
AK077882	Cd93	CD93 molecule	0.37	
NM_008397	Itga6	Integrin, alpha 6	0.36	
NM_133903	Spon2	Spondin 2, extracellular matrix protein	0.32	
NM_007584	Ddr1	Discoidin domain receptor tyrosine kinase 1	0.29	
NM_009655	Alcam	Activated leukocyte cell adhesion molecule	0.28	
NM_016749	Mybph	Myosin binding protein H	0.26	
NM_011607	Tnc	Tenascin C	0.18	
AK045226	Cd44	CD44 molecule (Indian blood group)	0.18	
NM_013800	Barx2	BARX homeobox 2	0.52	
NM_009235	Sox15	SRY (sex determining region Y)-box 15	0.51	
NM_010801	Mlf1	Myeloid leukemia factor 1	0.51	
NM_016872	Vamp5	Vesicle-associated membrane protein 5 (myobrevin)	0.51	
NM_013864	Ndrg2	NDRG family member 2	0.51	
NM_053007	Cntf	Ciliary neurotrophic factor	0.50	
NM_007928	Mark2	MAP/microtubule affinity-regulating kinase 2	0.49	
NM_009668	Bin1	Bridging integrator 1	0.46	Cell differentiation
NM_019684	Srpk3	SFRS protein kinase 3	0.46	
NM_010730	Anxa1	Annexin A1	0.45	
NM_030743	Rnf114	Ring finger protein 114	0.43	
NM_133643	Edaradd	EDAR-associated death domain	0.43	
NM_007631	Ccnd1	Cyclin D1	0.41	
AK020013	Ctnnb1	Catenin (cadherin-associated protein), beta 1, 88kDa	0.24	
NM_010054	Dlx2	Distal-less homeobox 2	0.16	
NM_027219	Cdc42ep1	CDC42 effector protein (Rho GTPase binding) 1	0.52	
NM_009447	Tuba4a	Tubulin, alpha 4a	0.52	
NM_003254	Timp1	TIMP metallopeptidase inhibitor 1	0.51	
NM_183016	Cdc42bpb	CDC42 binding protein kinase beta (DMPK-like)	0.50	
XM_204283	Palld	Palladin, cytoskeletal associated protein	0.50	
XM_140553	Cdc42bpg	CDC42 binding protein kinase gamma (DMPK-like)	0.49	
NM_031158	Ank1	Ankyrin 1, erythrocytic	0.47	
NM_010668	Krt2	Keratin 2	0.46	
NM_019410	Pfn2	Profilin 2	0.46	
NM_026514	Cdc42ep3	CDC42 effector protein (Rho GTPase binding) 3	0.46	
NM_198059	Nrap	Nebulin-related anchoring protein	0.45	
NM_031877	Wasf1	WAS protein family, member 1	0.44	
NM_009606	Acta1	Actin, alpha 1	0.44	
AK020248	Enah	Enabled homolog (Drosophila)	0.43	Cytoskeleton
NM_007393	Actb	Actin, beta	0.42	
NM_026131	Pdlim7	PDZ and LIM domain 7 (enigma)	0.42	
NM_009895	Cish	Cytokine inducible SH2-containing protein	0.41	
XM_284175	Flnc	Filamin C, gamma (actin binding protein 280)	0.41	
NM_009264	Sprr1a	Small proline-rich protein 1A	0.38	
U10406	Capzb	Capping protein (actin filament) muscle Z-line, beta	0.38	
NM_008476	Krt6a	Keratin 6A	0.35	
NM_175132	Synpo2l	Synaptopodin 2-like	0.32	
AK079588	Plekhc1	Fermitin family homolog 2 (Drosophila)	0.30	
NM_175456	Abra	Actin-binding Rho activating protein	0.29	
NM_133730	Krt25	Keratin 25	0.28	
NM_130856	Krtap16-8	Keratin associated protein 16-8	0.28	
NM_011468	Sprr2a	Small proline-rich protein 1B	0.25	
NM_010670	Krtap12-1	Keratin associated protein 12-1	0.22	
NM_010659	Krt31	Keratin 31	0.15	
NM_013592	Matn4	Matrilin 4	0.13	
NM_010669	Krt6b	Keratin 6B	0.13	
NM_010665	Krt32	Keratin 32	0.13	
NM_010673	Krtap6-2	Keratin associated protein 6-2	0.13	
NM_010672	Krtap6-1	Keratin associated protein 6-1	0.09	
NM_130875	Krtap16-7	Keratin associated protein 16-7	0.09	
NM_027983	Krt33a	Keratin 33A	0.01	
NM_053074	Nup62	Nucleoporin 62kDa	0.44	
NM_008859	Pkcθ	Protein kinase c theta	0.43	
NM_008737	Nrp1	Neuropilin 1	0.43	Cell migration
NM_008606	Mmp11	Matrix metallopeptidase 11 (stromelysin 3)	0.36	
NM_008607	Mmp13	Matrix metallopeptidase 13 (collagenase 3)	0.04	

[a] Two-tailed Mann-Whitney test was performed with $p < 0.05$. Access No. refers to the GeneBank accession.

3.4.3 ANGPTL4 Modulates Itegrin-Mediated FAK- and 14-3-3σ–Dependent Signaling Pathways

The activation of integrins initiate focal adhesion kinase (FAK)-dependent and independent signaling cascades to modulate cellular behavior, such as migration, growth and differentiation (Parsons et al., 2000). We showed earlier that ANGPTL4 interacts with the specific integrins β1 and β5, together with their cognitive ligands, vitronectin and fibronectin, to modulate integrin internalization and activate integrin-mediated signaling. To gain further insight into ANGPTL4-mediated signaling pathways, we performed immunoblot analyses of the intracellular mediators of integrin-induced signaling. Consistent with the notion of decreased integrin activation, the expression, i.e. phosphorylation, of numerous downstream effectors of FAK- and 14-3-3σ-dependent signaling cascades was reduced in $K_{ANGPTL4}$ as compared with K_{CTRL} (Figure 3.9A). In addition to reduced phosphorylated FAK, downstream mediators of the PI3K cascade, such as PDK1 and GSK-3β, were also altered (Figure 3.9A). Our immunoblot also showed attenuated expression of 14-3-3σ and of classical and novel PKC isoforms, namely PKCα, δ, ε and η in $K_{ANGPTL4}$, compared with K_{CTRL} (Figure 3.9A). The 14-3-3σ associates with integrins to modulate cell migration via an FAK-independent mechanism and with protein kinase C (PKC) (Dellambra et al., 1995; Fu et al., 2000) in the maintenance of proper epidermal differentiation (Li et al., 2005). The dysregulation of 14-3-3σ and its effector PKC would have a direct influence on keratinocyte differentiation (Tsuruta et al., 2004). We also detected diminished levels of phosphorylated ERK-1/2 and Raf1 in $K_{ANGPTL4}$, which has been shown to down-regulate

PKCδ (Schonwasser et al., 1998). Notably, reduced activation of FAK is also known to converge in decreased activation of the Raf-MEK-ERK signaling pathway (Porter et al., 2006). Besides reduced 14-3-3σ and PKC expression, $K_{ANGPTL4}$ also have decreased expression of RACK1, indicating attenuated PKC-mediated signal transduction (Figure 3.9A) (Schechtman and Mochly-Rosen, 2001). The results using transiently transfected keratinocytes using ANGPTL4-siRNA showed the similar reduction in the phosphorylation of PDK1, FAK, Raf1 and ERK1/2 and expression of signaling proteins when compared with lentivirus-transduced keratinocytes (Figure 3.9B). This suggests that the phosphorylation state of these proteins is likely due to ANGPTL4 deficiency, rather than an indirect consequence of the adaptation of the cells in response reduced ANGPTL4 levels.

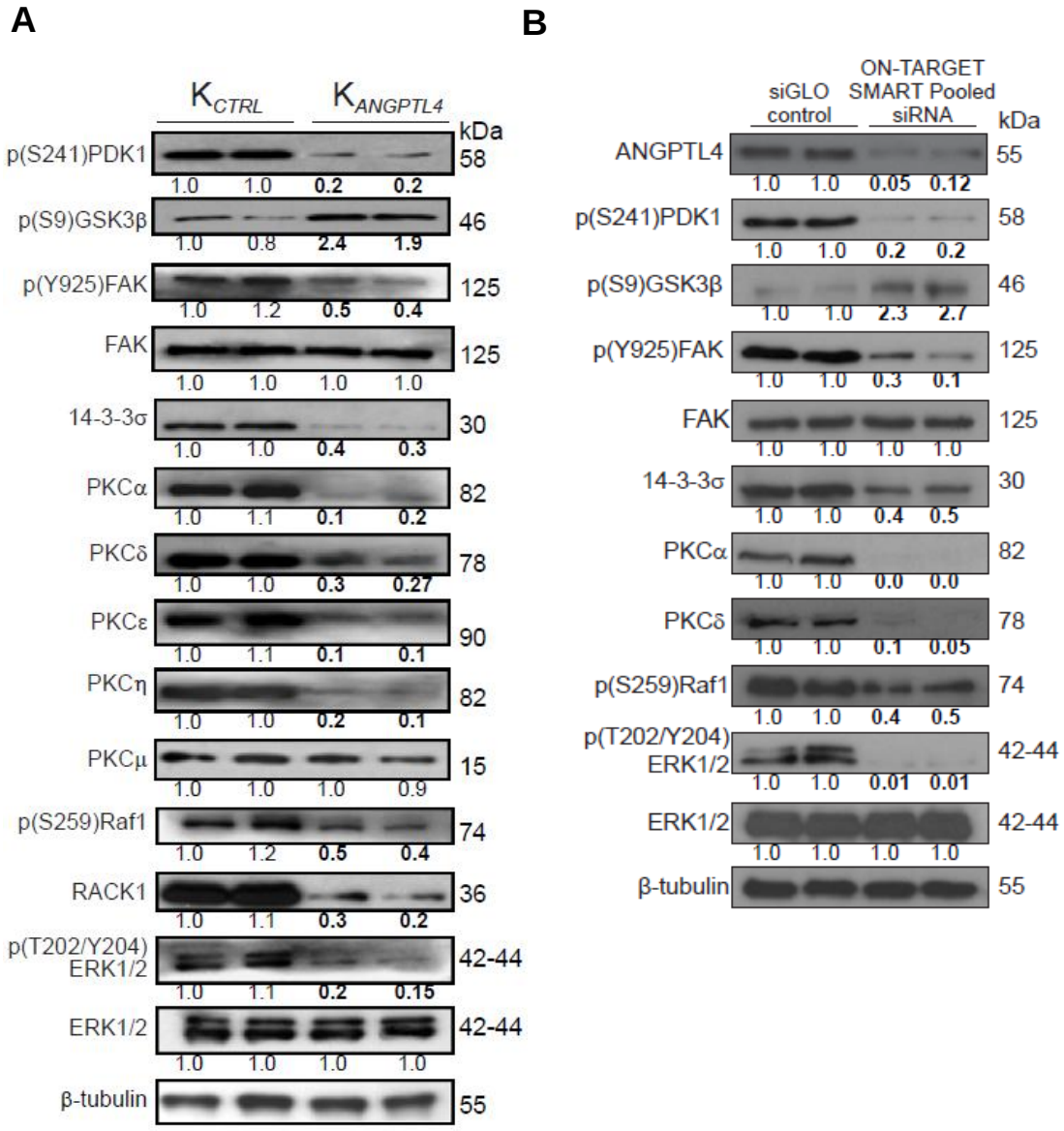

Figure 3.9 ANGPTL4 regulates integrin-mediated signalings and cellular differentiation. ANGPTL4 modulates FAK-dependent and 14-3-3σ mediated signalings. Immunoblot analysis of cytoplasmic proteins from **(A)** K_{CTRL} and $K_{ANGPTL4}$, and **(B)** siGLO control or ANGPTL4 siRNA-transfected keratinocytes. Values below the bands represent the mean fold differences in protein expression levels when compared with either vehicle (PBS) at time 0 or K_{CTRL}, which was assigned the value one. Values in bold are significant with $p<0.05$ when compared to K_{CTRL} at the corresponding time. Five independent OTCs were used. ERK1/2: p44/p42 MAPK; FAK: focal adhesion kinase; GSK-3: glycogen synthase kinase-3; PDK1: 3-phosphoinositide-dependent kinase-1; PKC: protein kinase C; RACK1: receptor for activate C kinase 1.

3.5 DISCUSSION

Following complete re-epithelialization of the damaged skin, reconstruction of the waterproof barrier is crucial to regain the protective function. Previous studies have shown that activation of PPARβ/δ stimulates the differentiation of keratinocytes in monolayer cultures (Westergaard et al., 2001) and promotes stratum corneum formation and epidermal permeability barrier development (Schmuth et al., 2004; Jiang et al., 2010). How PPARβ/δ, a nuclear transcription factor, exerts its effect on differentiation remains unclear. How PPARβ/δ, a nuclear transcription factor, exerts its effect on differentiation remains unclear. Although previous studies have greatly enhanced our understanding of wound healing, much remains to be elucidated on the molecular mechanism underlying epidermal maturation essential for the formation of a stratified neoepithelia. Here we provide a mechanism for the role of ANGPTL4, PPARβ/δ target gene, in epidermal differentiation via FAK- and 14-3-3σ- dependent signaling pathways.

Several studies have shown the importance of extracellular Ca^{2+} in the activation of protein kinase C (PKCs), Tyr and Fyn signaling in keratinocyte differentiation (Dotto, 1999; Denning et al., 2000) through transcription factors p300, SP-3 and AP-1 resulting in the expression of differentiation genes, like involucrin and transglutaminase (Yuspa et al., 1988; Denning et al., 2000). Recent studies also showed that PPARα and PPARγ interfere with NF-κB- or AP-1-mediated gene transactivation through direct protein-protein interactions in endothelial cells (Delerive et al., 1999; Wang et al., 2002). PPARα can also physically interact with c/EBPβ and inhibit target gene expression in hepatocytes (Mouthiers et al., 2005). More uniquely, PPARβ/δ may repress the

expression of PPARα and/or PPARγ-mediated target genes by binding to PPREs in association with co-repressors (Shi et al., 2002; Matsusue et al., 2004). We showed that deficiency of ANGPTL4, a target gene of PPARβ/δ, resulted in an impairment in epithelial differentiation. Analyses using microarray data and immunoblotting revealed that the autonomous regulation of epidermal differentiation by PPARβ/δ is mediated at least in part by ANGPTL4 and its intracellular modulation through integrin-mediated PKC and 14-3-3 signaling.

ANGPTL4-deficient epidermis displayed altered epidermal architecture with impaired keratinocyte differentiation, which is related to the impairment of FAK- and 14-3-3σ mediated signaling pathways. Studies have also shown that 14-3-3σ associates with PKC to regulate epidermal differentiation (Porter et al., 2006). Of the eleven isoforms of the protein kinase C (PKC) superfamily, the classical isoform PKCα and novel isoforms PKCη, PKCε and PKCδ found in keratinocytes have been clearly shown to activate raf-MEK-ERK pathways (Schonwasser et al., 1998). Together with RACK1 and MEK, PKC eventually phosphorylates the JNK-c-Jun/ AP-1 pathway (Lopez-Bergami et al., 2005), resulting in increased activation and the expression of transglutaminase I, a key marker of keratinocyte differentiation. Consistent with our finding that cANGPTL4 influences cellular behavior through cell-matrix communication via integrin and ECM, 14-3-3σ has been shown to associate with protein kinase C (PKC) (Fu et al., 2000) in the maintenance of proper epidermal differentiation (Li et al., 2005; Kim et al., 2006). Immunoblotting analyses showed a decrease in the expression of classical and novel PKC isoforms in $K_{ANGPTL4}$. Indeed, we detected reduced expression levels of phosphorylated ERK1/2 and

Raf1 in $K_{ANGPTL4}$. Consistent with the above observation, we also detected a ~3-fold decrease in the expression of RACK1, the receptor for activated C-kinase, in $K_{ANGPTL4,}$ which indicated attenuated PKC-mediated signal transduction (Schechtman and Mochly-Rosen, 2001).

Numerous intracellular signaling pathways, including FAK- and PI3K- dependent signaling cascades that emanate from integrins to modulate cell proliferation and differentiation (Schlaepfer and Mitra, 2004; Porter et al., 2006), were altered in ANGPTL4-deficient keratinocytes. Unlike angiopoietin 1, ANGPTL4 neither binds to Tie1 nor Tie2 receptors to initiate the phosphatidylinositol 3'-kinase (PI3K)/PKBα pathway (Kim et al., 2000). Integrin-mediated activation of FAK recruits SH2-containing proteins, including PI3K. In addition, FAK can either activate PKC (Yamamoto et al., 2003) or activate JNK via the Src/Pak1/MAPK pathway (Almeida et al., 2000). Consistent with a role for ANGPTL4 in enhancing integrin-mediated signaling (Chapter 1), its deficiency resulted in reduced phosphorylation (i.e. activation) of downstream mediators of the PI3K cascade, such as PDK1 and GSK-3β. In summary, our findings concerning the ANGPTL4-mediated signaling cascades via PKC and 14-3-3σ provide crucial insights into the molecular mechanism of PPARβ/δ-mediated differentiation.

Taken together, epidermal barrier development through keratinocyte differentiation is crucial to establish a physical barrier to protect the underlying tissues. The role of PPARβ/δ in epidermal differentiation remains to be elucidated. Although the present study does not fully answer this question, we herein show that the autonomous regulation

of epidermal differentiation by PPARβ/δ is mediated at least in part by ANGPTL4 expression.

3.6 CONCLUSION

In summary, our findings on the specific collaborative partnership between extracellular matrix proteins, integrins and ANGPTL4, provide crucial insights into the molecular mechanism of action of ANGPTL4, and has underscored its novel role in cell migration, differentiation and proliferation. Our data provide a novel facet to the regulation of the cell-matrix interactions and signaling during wound healing and cancer metastasis.

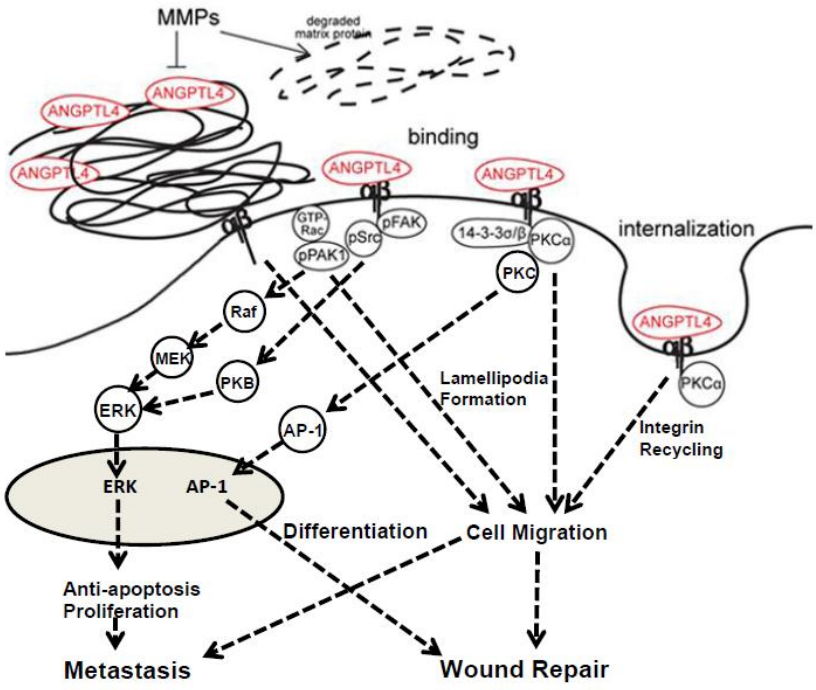

Figure 3.10 Schematic diagram showing ANGPTL4 coordinating cell-matrix communication on two fronts and intracellular signaling leading to keratinocyte migration and differentiation. ANGPTL4 interacts with integrin β1 and β5 to recruit and activate FAK-Src-PAK1-dependent and FAK-independent 14-3-3-PKCα pathways to facilitate lamellipodia formation and selective integrin internalization, which resulted in rapid cell migration at re-epithelialization phase during wound repair. Concomitantly, ANGPTL4 can also bind to vitronectin and fibronectin to delay their degradation by MMPs. During tissue remodeling phase, ANGPTL4 regulates the expression of differentiation markers, involucrin and transgutaminase type I genes through activation of JUNB and c-Jun transcriptional factor activator protein-1 (manuscript in revision, PLoS ONE). At the same time, ANGPTL4 confers anti-apoptotic effect and stimulate proliferation through Raf-MEK-ERK and PKB pathways, which is essential for metastasis as well (Zhu et al., 2011).

3.7 WORK IN PROGRESS AND FUTURE STUDIES

1) Investigate the anti-apoptotic role in ANGPTL4

ANGPTL4-deficient keratinocytes showed impaired epithelial differentiation. Cell differentiation is accompanied by detachment from the basement membrane and undergoes a series of differentiation programs, during which keratinocytes are required to confer resistance to anoikis. We have shown that $K_{ANGPTL4}$ OTCs have more apoptotic cells near the suprabasal layer, which was coincidental with the detachment of basal keratinocytes from the basal membrane. Thus $K_{ANGPTL4}$ may be more susceptible to detachment from the basal membrane and anoikis, which brought about the observed increase in apoptosis, altered epidermal architecture and differentiation. To examine the response of $K_{ANGPTL4}$ to anoikis, we performed anoikis assays and quantified the number of apoptotic cells by annexin staining (Figure 3.11A & B). ANGPTL4 confers anti-apoptotic effects on keratinocytes only when the cells were subjected to anoikis. When keratinocytes are attached on substratum, e.g. ECM, no difference in the percentage of cell death is observed (Zhu et al., 2011). During the formation of stratified epidermis, the basal proliferating keratinocytes must detached from the basal lamina and undergo a vectorial movement associated with cell differentiation. In this respect, the resistance to anoikis at this moment will be important for cell differentiation.

Interestingly, ANGPTL4 was suggested to act as an apoptosis survival factor for vascular endothelial cells (Kim et al., 2000), and contributed to anoikis resistance in hepatoma cells (Zhang et al., 2008). In both studies, the mechanism of

action remained unknown. ANGPTL4 also confers anti-apoptotic effects on keratinocytes, and this could be partially due to the elevated expression of 14-3-3σ that sequesters pro-apoptotic proteins like Bax from the mitochondria, inhibits cytochrome C release and thus prevent apoptosis (Samuel et al., 2001; Tsuruta et al., 2004). However, more experiments are required to determine the mechanistic details for the anti-apoptotic role of ANGPTL4.

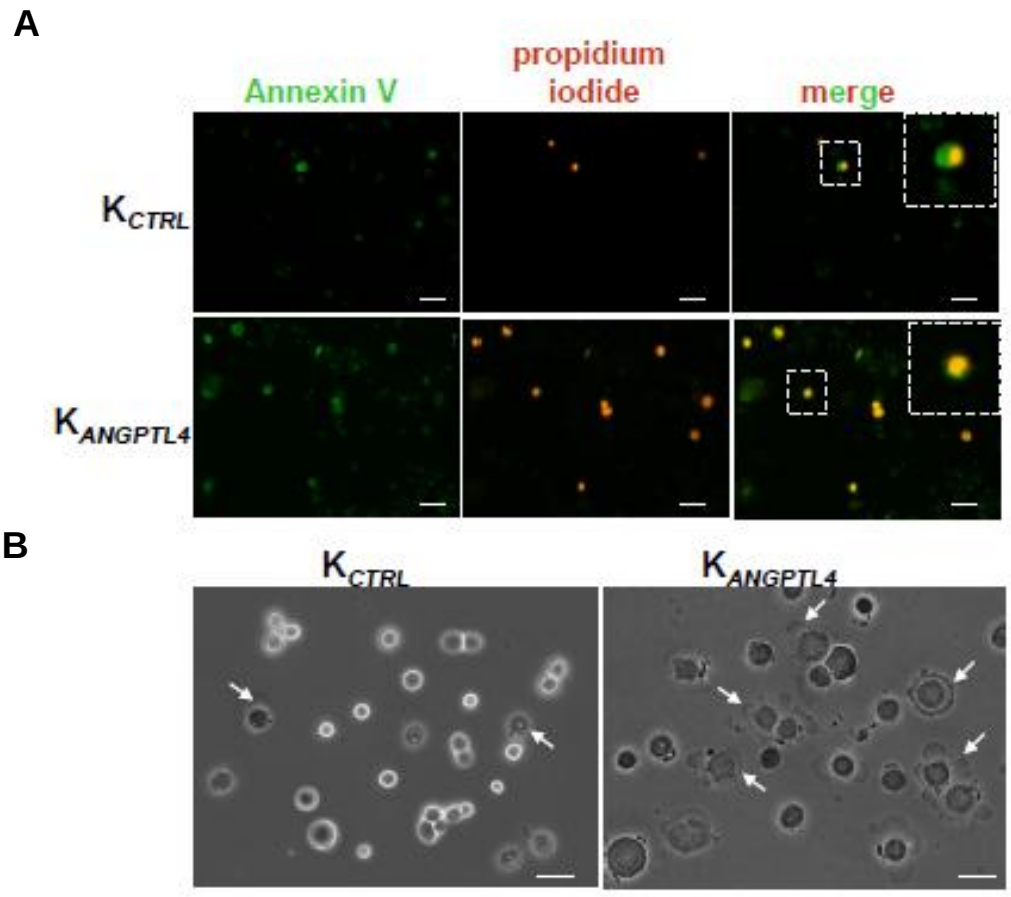

Figure 3.11 ANGPTL4-deficiency promotes anoikis. (A) $K_{ANGPTL4}$ showed higher number of positive cells for annexin V and propidium iodide, when compared to the control. Inset shows magnified picture of the cells in the dotted region. **(B)** Bright field microscopy showing the blebbing of cell membrane in $K_{ANGPTL4}$ after 2 h of forced detachment, when compared to similar treated K_{CTRL} cells. Arrow marks show the blebbing cells. Scale bars represent 40 μm in (**A**), and 20 μm in (**B**). Five independent experiments were performed. Representative pictures were shown.

We repeated the study for spontaneous apoptosis, anoikis assay and western blotting on PKC isoforms, Raf-MEK-ERK pathways using primary keratinocytes isolated from ANGPTL4$^{+/+}$ and ANGPTL4$^{-/-}$ mice. Our results revealed that the degradation of vitronectin and fibronectin was selectively degraded by MMPs in the absence of ANGPTL4 and cANGPTL4 (Chapter 2). We showed that cell adhesion is also affected in the absence of ANGPTL4 in ANGPTL4-knockdown keratinocytes (Chapter 2). In addition, our preliminary data have indicated the difficulties in obtaining primary keratinocytes from ANGPTL4$^{-/-}$ mice, judging by the difficulties in cell adhesion and the high number of apoptotic cells that were observed, resulting in extended cell doubling times during cell culture. Perhaps a coated surface with ECM and /or ANGPTL4 is required when culturing the primary keratinocytes, to isolate and maintain the cells before further experiments can be performed.

2) Investigate the role of transcriptional factor AP-1

The activation of PKC and various members of transcriptional factor AP-1 are important for the expression of different keratinocyte differentiation markers (Rutberg et al., 1996; Kamioka et al., 2010). Differentiation-promoting agents have been shown to regulate the expression of differentiation marker genes via activation of PKC-dependent signaling pathway that targets AP-1 proteins. ANGPTL4 interacts with specific integrins and their cognate ligands to activate integrin-mediated signaling (Goh et al., 2010a; Goh et al., 2010b; Zhu et al., 2011).

Hence the dysregulation of PKCs would have an influence on the activation of AP-1 proteins and subsequently keratinocyte differentiation (Rutberg et al., 1996; Angel et al., 2001).

To investigate the role of ANGPTL4 in differentiation through the activation / phosphorylation of AP-1 proteins, immunoblot analysis should be performed using K_{CTRL} vs $K_{ANGPTL4}$ and $ANGPTL4^{+/+}$ vs $ANGPTL4^{-/-}$. In addition, chromatin immunoprecipitation (ChIP) of differentiation genes, such as transglutaminase type 1 and involucrin, using AP-1 should be performed to study to differential expression of the genes in the absent of ANGPTL4. (Both experiments have been completed and the results are included in the manuscript to PLoS ONE.) (In revision, manuscript is attached at the end of thesis).

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