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The Role of Fibroblast PPAR β / δ in Skin Homeostasis

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List of Abbreviations

α -SMA	α -smooth muscle actin
APC	Adenomatous polyposis coli
CAF	Cancer-associated fibroblast
CK6	Cytokeratin 6
CK10	Cytokeratin 10
Col1A2	Collagen type 1, alpha 2
ECM	Extracellular matrix
EGF	Epidermal growth factor
FIB	Normal fibroblast
FIB _{PPARβ/δ}	FIB with knockdown PPAR β / δ
FLAP	5-lipoxygenase activating protein
GPx1	Glutathione peroxidase 1
H ₂ O ₂	Hydrogen peroxide
HGF	Hepatocyte growth factor
HRE	Hormone response element
IL-1	Interleukin-1
LCM	Laser capture microdissection
LRG1	Leucine-rich alpha-2-glycoprotein 1
MMP	Matrix metalloproteinase
OTC	Organotypic co-culture
PCNA	Proliferating cell nuclear antigen

PDGF	Platelet-derived growth factor
PNF	Peri-normal fibroblast
PPAR	Peroxisome proliferator-activated receptor
PPRE	Peroxisome proliferator response element
qPCR	Quantitative real-time PCR
RAR	Retinoic acid receptor
ROS	Reactive oxygen species
Rpl27	Ribosomal protein L27
RXR	Retinoid X receptor
SF-OTC	Serum-free OTC
sIL-1ra	Secreted IL-1 receptor antagonist
siLrg1	Lrg1 gene knockdown by siRNA
siRNA	Small interfering ribonucleic acid
TBP	TATA box promoter
TGase1	Transglutaminase 1
TGF	Transforming growth factor

The Role of Fibroblast PPAR β/δ in Skin Homeostasis

Summary

The skin is the largest organ in the body and is made up of mainly the epidermis and dermis. The skin functions as a natural barrier against external stimuli, dehydration and injury. Peroxisome proliferator-activated receptor β/δ (PPAR β/δ) is one member of the PPAR family of nuclear receptors, and the predominant subtype found in the epidermis and dermis. Epidermal PPAR β/δ has well-established roles in maintaining skin homeostasis, such as during wound healing. However, in the mammalian system, skin homeostasis depends on a complex crosstalk between the epithelial and mesenchymal compartments, underscoring the importance for a tight regulation of the complex epiethelial-mesenchymal interaction.

Although epidermal PPAR β/δ in normal wound healing is well-studied, there has been no progress in clinical therapies for diabetic chronic wounds. This is also ensued with problems such as drug bioavailability and patients' compliance. In our study, we showed that pharmacological activation of fibroblasts PPAR β/δ activity using microencapsulated ligands can promote the healing of diabetic wounds via the modulation of ROS production.

The role of PPAR β/δ in other diseases, such as cancer, has also been controversial due to differences in experimental setups and genetic modifications of the PPAR β/δ gene. While epidermal PPAR β/δ has been widely investigated on in these diseases, the role of fibroblasts PPAR β/δ in skin homeostasis and disease has been neglected. Hence, we generated a novel mouse model with the PPAR β/δ gene deleted specifically in the fibroblasts. These animal exhibited skin abnormalities during development, such as

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dermal thickening and increased collagen production, recapitulating a morphea-like phenotype. In addition, the dermis manifest as a hotbed for inflammatory events. In our subsequent preliminary studies, we showed that cancer-associated fibroblasts from squamous cell carcinoma exhibit low levels of PPAR β/δ . Our novel mouse model will therefore serve as an appropriate model to study the role of fibroblasts PPAR β/δ in diseases, such as acute inflammation, wound healing and tumorigenesis in the future.

Chapter 1: Introduction

1.1 Tissue microenvironment

The tissue microenvironment resembles an ecological system that comprises various biotic and abiotic factors engaged in a complex interplay to maintain homeostasis and self-renewal. The biotic component of the tissue microenvironment is made up of different cell types, each having a specialized role to perform in synergy with others within the tissue. These cell types are constantly engaged in a tightly regulated crosstalk via autocrine and paracrine signaling, determining cell behavior and fate in a context-dependent manner. The abiotic component is made up of the extracellular matrix (ECM) components and structures, redox potential, pH, and mechanical forces. Taken together, these components work collaboratively, eliciting biological responses from cells to regulate cellular phenotypes and functions. Tissue epithelia are composed of continuous sheets of tightly connected cells. They constitute the surfaces, such as the skin epidermis, and the linings of various organs, such as the gastrointestinal tract of the body. In addition to providing a protective barrier against the external environment, the epithelia also regulate the absorption of water, nutrients, and glandular secretions. The epithelial tissues in different parts of the body share numerous molecular and cellular characteristics despite being categorized as either single-layered (simple) or multi-layered (stratified). In virtually all of these organs, the epithelial tissues interact with and are supported by the underlying mesenchymal compartment of the tissue. Such epithelial-mesenchymal interactions are essential as early as embryogenesis in an organism, allowing for normal organogenesis and cytodifferentiation to take place (Lim and Thiery, 2012). The architectural and functional dependence between the epithelium and mesenchyme persist

into the adult stages of growth and development, ensuring proper tissue homeostasis and coordinating responses towards external stimuli and injury (Kiesslich et al., 2013).

1.2 Skin

Skin is the largest organ in the human body. It has properties that function synergistically to act as both a barrier against external stimuli such as foreign organisms or ultraviolet radiation and as a protective layer to prevent dehydration or injury of the underlying tissues and organs. In addition, the skin helps regulate body temperature, detects stimuli by various sensory mechanisms, and provides immune surveillance against foreign intrusion of the body. The skin is made up of two primary layers – the outer epidermis and the inner dermis. The epidermal layer is largely composed of keratinocytes, with additional melanocytes and Langerhans cells. During skin development, keratinocytes undergo vectorial differentiation to form the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum layers (Figure 1.1). This vectorial differentiation involves the upward migration of undifferentiated basal cells towards the outer surface of the skin. Throughout this process, these basal cells undergo biochemical changes that result in the observed morphological modifications. These differentiating cells ultimately become dead, enucleated flake-like cells that can be shed from the skin surface (Segre, 2006). In contrast, the dermis is made up of the collagen-rich extracellular matrix (ECM), which provides necessary scaffold support and nutrients to the tissues and organs of the body. The principal cell type in the dermis is the fibroblast, but it also contains endothelial and immune cells. Soluble factors produced by these dermal cells play essential roles in regulating the proliferation and terminal differentiation of the

adjacent keratinocytes. Similarly, keratinocytes constantly produce signals to regulate the behavior of normal fibroblasts. Hence, epithelial-mesenchymal interactions are an integral part of maintaining skin homeostasis or repair in the human body (Maas-Szabowski et al., 1999).

Figure 1.1 Schematic diagram of the stratified layers of the skin. Keratinocytes that form the epidermis undergo a vectorial differentiation program. This begins with the basal layer, comprising cells that undergo active mitosis, to the spinous layer that comprises transcriptionally active cells. Keratinocytes then begin to lose their nuclei, forming the granular layer, and finally resulting in the differentiated squames of the stratum corneum. The stratum corneum, primarily made up of keratin macrofibrils and a cornified enveloped, surrounded by a lipid bilayer, provides the skin barrier. (“Republished with permission of The Journal of Clinical Investigation, from Epidermal barrier formation and recovery in skin disorders, Julia A. Segre, 116, 5, 2006; permission conveyed through Copyright Clearance Center, Inc.”).

1.3 Skin microenvironment

As mentioned in Section 1.2, the skin microenvironment is mainly composed of the epidermis and the dermis. The skin epidermis provides a protective shield against the external environment and regulates water and nutrient absorption. This epithelial tissue is supported by cells in the underlying mesenchymal tissue and constantly interacts with them to maintain skin homeostasis.

In the skin mesenchyme, fibroblasts are the most abundant mesenchymal cells. Fibroblasts manifest as spindle-shaped, elongated, and flat cells. Due to their abundance in the tissue microenvironment, fibroblasts exert strong influences on both the biotic and abiotic components of the tissue microenvironment. The ability of fibroblasts to secrete a plethora of mitogenic and chemotactic factors and chemicals enables them to regulate the proliferation, migration, and behavior of neighboring endothelial, immune and epithelial cells (Smith et al., 1997). Fibroblasts are also the main cell type responsible for the production of ECM molecules such as collagen, and matrix remodeling enzymes (Li and Wang, 2011; Kendall and Feghali-Bostwick, 2014). As a result, fibroblasts are the main architects of the scaffold that holds cells in place. Any dysregulated events in the homeostasis of ECM molecule production and matrix remodeling enzymes are therefore expected to result in undesirable consequences for the entire tissue.

The ability of fibroblasts to impact such a wide array of events highlights the importance of the microenvironmental cues provided by fibroblasts and the proper regulation of the epithelial-mesenchymal crosstalk processes in physiology and pathophysiology. However, the majority of current published studies studied the epithelia and mesenchyme as separate entities. Such experimental setups preclude the scrutiny of

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any microenvironmental effects. In recent years, various co-culture model systems have been designed in an attempt to investigate the cell-cell and cell-ECM communications. Although such *in vitro* co-culture models are relevant, they are unable to entirely recapitulate the complex intercellular communication and dynamic microenvironment that occurs in an *in vivo* environment, such as cancer and wound healing. Therefore, to have a better understanding of disease etiology, initiation and progression, appropriate animal models must be derived to complement physiologically relevant *in vitro* co-culture models.

1.4 Skin wounds

Skin wounds, also known as cutaneous wounds, can be categorized into two categories – acute and chronic cutaneous wounds. Acute wounds may occur anytime and anywhere on the body, ranging from superficial abrasions to deep wounds that may have injured blood vessels or muscles and surgical wounds. Acute wounds are able to heal within an expected duration, according to the physiological wound healing processes. In contrast, chronic wounds are acute wounds that fail to heal within an expected time frame or do not heal at all. Previous reports have indicated that almost 10 million wound cases were handled in the United States alone (Singer et al., 2006). In particular, almost 7 million patients were affected by chronic wounds (Sen et al., 2009), and these numbers are expected to rise annually due to an increasingly affluent society, especially in developed countries. The increasing incidences of lifestyle diseases such as diabetes and obesity, the increasing cost of healthcare, and an aging population are placing a huge burden on the ability of the health budgets of developed nations to treat chronic wounds. In addition, with current medical advancements many surgeries are performed on patients

for various medical interventions. Therefore, the need for post-surgical wound care is also on the rise. In wounds that do not heal as per the normal wound healing process, scarring of the skin occurs. Skin scarring contributes to an additional morbid manifestation that causes psychological impacts on patients, resulting in an estimated \$10 billion medical expenditure annually. So colossal is the impact of wound healing on a society and its economy that it warrants greater attention towards the understanding of the mechanisms behind skin wound complications.

1.5 Wound repair biology

Wound repair is a high priority survival response to skin injuries. Upon the onset of a cutaneous wound occurrence, the body system's primary goal is to re-construct an epidermal barrier that covers the wound site and to prevent tissue dehydration or opportunistic microbial infections (Tan et al., 2004). Cutaneous wound healing and closure involves a tightly regulated cascade of biological events that serve to harmoniously repair the damaged tissues and regenerate the epidermis through a procedure known as re-epithelialization. The normal wound healing process is made up of three major phases, known as the inflammatory, migratory (re-epithelialization), and ECM remodeling phases (Reinke and Sorg, 2012). These three phases of wound healing occur in an overlapping pattern via the production of cytokines and growth factors in a temporally and spatially regulated manner (Figure 1.2) (Tan et al., 2004).

Figure 1.2 Overlapping phases of wound healing. Wound healing involves a cascade of events that overlap with one another. Three phases can be identified: inflammation, re-epithelialisation (granular tissue formation), and remodelling. During inflammation, immune cells migrate to the wound site and clears up any debris while phagocytosing harmful bacteria. Various soluble factors are also released to attract cells that are involved in the re-epithelialisation phase. During this phase, events such as granular tissue formation, angiogenesis, epithelialisation, collagen deposition, and wound contraction are occurring. When wound re-epithelialisation approaches completion, excessive cells undergo apoptosis and collagen is remodelled and realigned to conclude the healing process. (Figure reproduced with permission from <http://www.camacs.ca/Directors.php>).

The inflammatory phase of wound healing begins immediately after the onset of injury. Injured blood vessels cause the release of signals to initiate localized blood clotting within seconds and the influx of inflammatory cells within minutes. Clotting is mainly the work of platelets, whose role is to activate various coagulation pathways by releasing chemotactic factors to attract more platelets, leukocytes and fibroblasts to the injured region (Jurk and Kehrel, 2005; Nurden et al., 2008). Leukocytes such as neutrophils and

macrophages sterilize the wound site through the phagocytosis of foreign microbes and the uptake of cellular debris. In addition, leukocytes produce several growth factors, including platelet-derived growth factor (PDGF), fibroblast-growth factor (FGF), transforming-growth factor β (TGF β), and TGF α . These growth factors stimulate the attraction and growth of fibroblasts. In turn, fibroblast activation initiates ECM protein production to modulate the matrix for proper wound closure. The inflammatory phase of wound healing ends when macrophages phagocytose the remaining neutrophils. Next, the migratory and proliferative phase begins.

The migratory phase comprises cellular proliferation and the migration of key resident cells, mainly fibroblasts, endothelial cells, and keratinocytes. This phase is driven by events such as hypoxia, which occurs due to the disruption of blood vessels upon injury. This decrease in oxygen levels within the wound environment is a crucial stimulus for the activation of fibroblasts and endothelial cells. Evidence pointing to such an event had been documented previously through the observation of growth factor synthesis in both cell types (Kourembanas et al., 1990; Falanga et al., 1991; Kourembanas et al., 1991; Shweiki et al., 1992). During this process, some fibroblasts undergo a morphological change, displaying characteristics of smooth muscle cells. These fibroblasts are known as myofibroblasts (Martin, 1997), which produce matrix proteins such as elastin, proteoglycans, glycosaminoglycans, and collagen. As a result of these events, new connective tissues are formed and aid in contracting the wound. Towards the end of the final stages of healing, the presence of myofibroblasts gradually decreases, likely due to the apoptosis of these cells (Rouabhia et al., 2013). On the other hand, keratinocytes are the main focus of the re-epithelialization event during this phase of wound healing. Re-

epithelialization involves a diverse yet interconnected range of transcriptional events (Schäfer and Werner, 2007). The changes in the transcriptional activities of the involved transcription factors modulate the restructuring of the cytoskeleton and keratin structures of keratinocytes, enabling them to proliferate and migrate from the wound edge towards the epidermal closure. For migration to occur, it is important that the migrating keratinocytes express certain enzymes to move through or along the matrix. Upregulation of tissue-type plasminogen activator (tPA) and the urokinase-type plasminogen activator (uPA) and its receptor by migrating keratinocytes was reported previously (Grøndahl-Hansen et al., 1988; Rømer et al., 1994). Matrix metalloproteinases (MMPs) have also been implicated in keratinocyte migration (Rohani and Parks, 2015). For example, MMP-1 (collagenase-1), MMP-9 (gelatinase), and MMP-10 (stromelysin-2) are expressed by keratinocytes at the migrating edge of the wound to facilitate its movement through the degradation of certain ECM proteins. In contrast, expression of MMP-28 (epilysin) was shown to only be upregulated proximal to the wound edge (Saarialho-Kere et al., 2002).

In the final phase of wound healing, the ECM undergoes a series of remodeling events. Unfortunately, this phase of wound healing has not been well-studied compared to the earlier two phases. In the earlier two phases of wound healing, exaggerated levels of immune responses, cellular proliferation, and ECM protein production ensure a proper wound closure. Hence, as the wound healing process ends, these excess materials must be removed. The final phase of wound healing downregulates the body's response to injury, bringing the body system back to its pre-wounded state. This normal wound healing process is vital to survival, as it ensures the well-being of the injured site and body homeostasis after wound closure. Unfortunately, this normal wound healing process is

impaired in certain situations, such as in diabetic patients, who experience chronic wounds.

1.6 Impaired wound healing in diabetic patients

In most scenarios, an acute wound should heal within its expected time frame. However, as our society becomes more affluent, increasing numbers of patients suffer from lifestyle diseases such as obesity and diabetes mellitus. In particular, diabetes affects millions worldwide. Diabetes mellitus, or diabetes for short, is a metabolic disorder that affects almost 200 million people worldwide, and this number is expected to double by 2030 (Wild et al., 2004). In Singapore alone, there are approximately 400,000 diabetic patients today, and this number is set to increase by another 200,000 by 2030 (SingHealth, 2015). Symptoms of diabetes include the frequent need to urinate and increased thirst and hunger, all of which are results of prolonged periods of high blood glucose levels.

There are two main types of diabetes. Type 1 diabetes is diagnosed when the pancreas has failed to produce sufficient insulin to remove glucose from the blood circulation. In contrast, type 2 diabetes occurs when cells or tissues initially fail to respond to insulin. As the disease progresses, insulin production may fail due to the insensitivity of cells and tissues towards insulin. Poor-healing diabetic wounds can be characterized by an accrual of devitalized tissues, increased inflammatory responses, and impaired angiogenesis (Falanga, 2005). The most obvious morbidity that diabetic patients face is the development of healing-impaired or non-healing ulceration of the lower extremities, in particular diabetic foot ulcers (DFU).

DFU is the main leading cause of limb amputations, and approximately 15% of diabetic patients are affected by DFU. Increased glucose levels in the body alongside altered protein and lipid metabolism causes the formation of stable products known as advanced glycation endproducts (AGEs). The formation of these products occurs on ECM proteins and alters the properties of ECM proteins such as collagen and laminin (Brownlee, 1995; Goldin et al., 2006; Singh et al., 2014).

The development of DFU can be attributed to several biological alterations. An impairment of nitric oxide (NO) synthesis was postulated to be a contributing event. Previous reports have suggested that diabetic ketoacidosis could reduce the production of NO synthases, or that high glucose-associated kidney dysfunction could promote the build-up of NO synthase inhibitors. The consequence of such event is that there is now reduced NO-associated fibroblast proliferation and, subsequently, reduced collagen production in the wound healing of diabetic patients (Goldin et al., 2006; Linden et al., 2008). Furthermore, NO was shown to be important for proper collagen crosslinking to ensure minimal scarring and the restoration of tensile strength to a healed wound (Obayashi et al., 2006).

MMPs have also been implicated in DFU. Due to their ability to degrade almost all ECM components, MMPs are involved in the migration of keratinocytes and fibroblasts during wound healing, as well as various aspects of tissue inflammation, reorganization and remodeling in healing wounds (Ravanti and Kähäri, 2000). However, in diabetic conditions the elevated levels of inflammatory molecules in the wound site together with decreased levels of tissue inhibitors of MMPs (TIMPs) resulted in an almost 30-fold increase in the activity of MMPs compared to that in acute wounds (Vaalamo et

al., 1999; Falanga, 2005; Muller et al., 2008). This resulted in an altered ratio between the levels of MMPs and TIMPs, thus causing an imbalanced proteolytic ratio and balance in diabetic wounds. The loss of this spatio-temporal regulation of MMP activity has also been touted as a major cause of excessive ECM breakdown and inhibition of crucial growth factor synthesis for tissue regeneration, thus leading to poor wound healing (Muller et al., 2008).

A low level of inflammation at the end of the inflammatory phase of wound healing is vital for faster wound healing coupled with minimal scarring (Ashcroft et al., 1999; Liechty et al., 2000; Martin et al., 2003). Unfortunately, recalcitrant diabetic wounds are imbued with vicious cycles of increased and prolonged inflammation, which in turn lead to delayed wound healing (Acosta et al., 2008). To worsen the situation, there is a possibility of neuropathy being coupled with DFU, in which the diabetic wound becomes a gateway for opportunistic infections that can result in dehiscence, sepsis, and death (Brem and Tomic-Canic, 2007). This warrants more understanding and resources in elucidating wound repair biology to identify novel pharmacological targets for wound healing in diabetic patients. To date, the only approved treatment for diabetic wounds is the administration of PDGF. Unfortunately, this sole treatment only targets a single aspect of the disease process and is obviously not an efficient therapeutic intervention for diabetic wound healing. Thus, it is imperative to identify more molecular targets through the better molecular understanding of chronic wounds to develop more therapeutic options for clinical use.

1.7 Reactive oxygen species (ROS) in wound healing

In addition to the complex interplay of secreted cytokines and growth factors, another important chemical mediator that coordinates the wound healing process is ROS. ROS is produced by key cellular metabolic processes, such as the electron transport chain in the inner mitochondria membrane. Due to its naturally destructive nature, ROS can damage a wide range of proteins and nucleic acids. Cells have therefore evolved to possess various systems to scavenge these ROS molecules and maintain a low concentration of ROS (Bienert et al., 2007). The tightly regulated production and degradation of ROS ensures proper redox control of the wound tissue during the various wound healing stages (Sen, 2003; Roy et al., 2006; Schreml et al., 2011). In normal wound healing, an intricate balance of various ROS molecules confers an antimicrobial effect and pro-mitogenic signals that are vital for proper wound healing (auf dem Keller et al., 2006; Bryan et al., 2012). During the first three days post-injury while the wound is still in the inflammatory phase, the large influx of inflammatory cells contributes to the production of large quantities of ROS, thus protecting the wound site against any invading pathogens (Mizuta et al., 2012). Subsequently, ROS levels decrease at the onset of the re-epithelialization phase.

In diabetic wounds, the poor healing rate is also often associated with a protracted inflammatory phase with spatiotemporally dysregulated ROS production in the diabetic wound microenvironment (Rasik and Shukla, 2000; Roy et al., 2006; Mizuta et al., 2012; Bryan et al., 2012). Such a prolonged and uncontrolled production of ROS had previously been linked to chronic hyperglycemia in diabetic rodents (Rasik and Shukla, 2000). Out of the wide range of ROS, hydrogen peroxide (H_2O_2) is the most stable ROS molecule due to

its relatively longer half-life (1 ms) compared to superoxide ions (1 μ s) (Bienert et al., 2006). Furthermore, due to its permanent dipole moment that is close to that of water, H_2O_2 can passively diffuse across lipid bilayer membranes (Bienert et al., 2006; Bienert et al., 2007; Bienert and Chaumont, 2014). Thus, H_2O_2 is a potential ROS target that is dysregulated within diabetic wounds, leading to impaired wound healing. H_2O_2 is removed by enzymes such as catalases and peroxidases. Should such detoxification be impaired, excess amounts of H_2O_2 diffuse and oxidize proteins and nucleic acids (Marinho et al., 2014). Thus, it is important to better understand the regulation of ROS and particularly H_2O_2 in diabetic wound healing.

1.8 Transcriptional regulation in wound healing

To better understand the molecular pathways involved in wound healing, it is important to understand what changes are occurring at the transcriptional level. Several transcription factors that are involved in key developmental pathways such as the TGF β , Wnt/ β -catenin, Notch, and Hedgehog pathways play essential roles at different stages of the wound healing process (Bielefeld et al., 2013). Studies performed on these key developmental signaling pathways may be useful, as mid-gestation fetal wounds heal without scars (Leung et al., 2012). Thus, results from studying these aspects of transcriptional regulation may one day yield results on how we can recapitulate the regenerative capabilities of fetal wound healing in chronic wound healing and particularly diabetic wound healing. In addition to the transcriptional regulation from these key developmental pathways, another group of transcription factors known as nuclear hormone receptors, or nuclear receptors, has also been implicated in modulating various

aspects of skin homeostasis in cutaneous physiology and pathology (Schmuth et al., 2007). This distinct set of transcription factors has been programmed to act as lipid sensors and thereby mediate lipid signaling via gene transcription modulation (Nagy et al., 2012). Based on their ability to modulate gene transcription activity through the detection of hormonal changes in their immediate vicinity, nuclear receptors have been targeted pharmacologically, and numerous metabolic therapies have been derived based on nuclear receptors (Xiao et al., 2013). This highlights the possibility of targeting this specialized group of transcription factors as potential therapeutic targets for diabetic wound healing.

1.9 Nuclear receptors

The members of the nuclear receptor superfamily can be classified into three categories: the classical endocrine receptors, the orphan receptors, and the adopted orphan receptors. The classical endocrine receptors were identified and characterized at a time when their endogenous ligands were already known. Orphan receptors are those whose ligands are yet to be identified. The adopted orphan receptors are receptors whose ligands have only been identified in recent years (Shi, 2007). There is an increasing interest in identifying endogenous and synthetic ligands for orphan nuclear receptors, due to the widely recognized fact that the classical endocrine receptors have critical roles in development, homeostasis, and disease (Giguère, 1999). Most nuclear receptors are usually found in the nucleus, regardless of whether their ligands are present. In the absence of ligands, most nuclear receptors are associated with co-repressor molecules and exert a repressive effect on their target genes. When their respective ligands are present, nuclear receptors interact with co-activators. Through a series of conformational changes,

the complex activates gene transcription in response to various hormones and metabolites (Giguère, 1999).

Most nuclear receptors are typically organized according to their structural and functional domains (Figure 1.3). The A/B region, or the amino-terminal region, encodes for activation function-1 (AF-1), which functions independently of any ligand. The C domain contains highly conserved α -helical DNA binding motifs together with two zinc finger-like structures. This structure is typical of various transcription factors and forms the DNA-binding domain (DBD) of the nuclear receptors. The D region encodes the hinge region, which is thought to provide flexibility for the independent movement of the ligand-binding domain (LBD). Last, the E/F region encodes the LBD, which contains a ligand-binding cavity. Upon ligand-binding, it induces a conformational change in the nuclear receptor, allowing it to be activated for DNA-binding and induce gene expression. This conformational change involves the transactivation domain activation function-2 (AF-2), which allows for the recruitment of co-activators and the release of co-repressors.

Figure 1.3 Schematic illustration of the structural organisation of nuclear receptors. The evolutionary divergent regions A/B and D, and the conserved regions of C and E/F are depicted here together with their known functions below the diagram.

Three main types of nuclear receptor-DNA binding patterns exist within mammals (Helsen et al., 2012). The first group of nuclear receptors is made up of the steroid receptors, including the glucocorticoid, mineralocorticoid, androgen, and estrogen receptors. They bind to their respective HREs as homodimers. The next group of nuclear receptors forms heterodimers with the retinoid X receptors (RXR α , β or γ). This group of nuclear receptors includes the retinoic acid receptor (RAR), vitamin D receptor (VDR), and the peroxisome proliferator-activated receptors (PPAR α , β or γ). The third group consists of nuclear receptors that preferentially bind to DNA as monomers. Examples include the steroidogenic factor (SF-1) and the estrogen-related receptor (ERR).

One subfamily of nuclear receptors, the PPARs, comprises members that are known to act as lipid sensors and that transcriptionally regulate nutrient metabolism and energy homeostasis (Berger et al., 2005). The three subtypes of PPAR, known as PPAR α , PPAR β/δ , and PPAR γ , can be distinguished by their tissue distribution patterns and developmental stage-specific expression (Boitier et al., 2003). Members of the PPAR family regulate lipid homeostasis in metabolically active tissues, inflammation, and cancer development and progression (Kersten et al., 2000; Tan et al., 2001; Röhrli et al., 2011). PPARs form obligate heterodimers with the retinoid X receptor (RXR), another nuclear receptor, and bind as a complex to peroxisome proliferator response elements (PPREs) located in the regulatory domains of their target genes. PPREs are cis-acting elements known as DR1 elements. These are direct TGACCT repeats separated by one nucleotide.

When ligand-binding is absent, PPAR-RXR heterodimers recruit co-repressors, histone deacetylases and chromatin-modifying factors. This forms a complex that actively

represses target genes. Similar to other nuclear receptors, ligand-binding induces a conformational change that permits the formation of a complex that enhances target gene expression. The promiscuity of the PPARs is shown by their ability to bind a diverse selection of amphipathic ligands and arachidonic acid-derived metabolites, such as fatty acids, triglycerides and prostacyclin (Seedorf and Aberle, 2007). This promiscuity is further augmented by the permissivity of binding to its heterodimeric partner, RXR.

RXR is an important nuclear receptor, and almost one-third of the 48 human nuclear receptors need to partner with it before they can carry out their biological actions (Tanaka and De Luca, 2009; Bushue and Wan, 2010; Dawson and Xia, 2012). The only known ligand for the RXRs is 9-cis retinoic acid (9-cis RA), although it cannot be detected in vitro or in vivo under normal circumstances (Bushue and Wan, 2010; Dawson and Xia, 2012). RXRs can homodimerize or heterodimerize with other nuclear receptors. The presence of different isoforms of RXR (α , β , and γ) further increases the pleiotropy of effects that this nuclear receptor can exert, depending on which partner nuclear receptor it interacts with (Lefebvre et al., 2010). The binding of RXR-containing heterodimers can be considered 'permissive' or 'non-permissive'. A non-permissive nuclear receptor can only be activated by its own agonist. In contrast, a permissive nuclear receptor can be transactivated by either the agonist of the nuclear receptor or that of RXR. The PPAR-RXR heterodimer is an example of a permissive heterodimer, and the presence of both ligands may give a concerted effort in inducing gene transcription (Bushue and Wan, 2010). Although RXRs have been implicated in cancer (Tanaka and De Luca, 2009; Bushue and Wan, 2010), the multiple activation pathways of RXRs resulting from its

ability to partner different nuclear receptors and its dimerization permissivity have made therapeutic studies targeting RXRs a highly complex task.

In contrast to RXR, the main physiological functions of PPARs include the mediation of lipid and glucose metabolism, cell differentiation, metabolic syndromes, and cancer (Bishop-Bailey and Bystrom, 2009). PPAR α is highly expressed in the liver and is one of the major regulators that promote fatty acid catabolism. It plays an essential role in mediating metabolic processes when the body is in a state of hypoglycemia. It is therefore a target of hypolipidemic drugs. On the other hand, PPAR γ is highly expressed in adipocytes and is necessary for adipocyte differentiation and lipid accumulation within the adipocytes. The role of PPAR γ in type 2 diabetes mellitus has also been demonstrated using insulin-sensitizing thiazolidinedione agents (Berger et al., 2005; Yessoufou and Wahli, 2010). In contrast, PPAR β/δ is ubiquitously expressed, albeit with varying levels of expression in different organs (Yessoufou and Wahli, 2010). Other than displaying a more widespread expression in specific tissues, PPAR β/δ also differs from the other two isoforms by its involvement in developmental processes and its emerging roles in muscle and heart fat metabolism (Seedorf and Aberle, 2007). In comparison with the PPAR α and PPAR γ agonists that are already in clinical use for treating hyperlipidemia and type 2 diabetes, the PPAR β/δ agonists have only recently been identified as novel approaches for possible therapeutics against metabolic diseases and the associated cardiovascular ailments. In particular, the ability of PPAR β/δ to regulate gene expression relating to epidermal differentiation and repair has underscored its potential as a therapeutic target for diabetic wound healing (Wahli, 2002; Billin, 2008).

1.10 PPAR β/δ in wound healing

PPAR β/δ is the dominant PPAR subtype in the human epidermis. Numerous reports have presented strong evidence for the involvement of PPAR β/δ in wound healing (Michalik et al., 2000; Michalik et al., 2001; Tan et al., 2002; Wahli, 2002; Di-Poï et al., 2002; Tan et al., 2003; Di-Poï et al., 2003; Tan et al., 2004; Tan et al., 2005; Di-Poï et al., 2005; Michalik and Wahli, 2006; Tan et al., 2007; Man et al., 2008; Billin, 2008; Chong et al., 2009). Upon skin injury, inflammatory stimuli such as the expression of pro-inflammatory tumor necrosis factor α (TNF α) were found to trigger the upregulation of PPAR β/δ expression via the activator protein-1 (AP-1) site in the promoter region. This increased PPAR β/δ expression upregulated the production of integrin-linked kinase and 3-phosphoinositide-dependent kinase-1, which subsequently phosphorylated and activated protein kinase B (Akt1). Akt1 activation suppressed the apoptosis of keratinocytes to ensure that the number of viable keratinocytes at the wound edge was sufficient for re-epithelialization to take place (Michalik et al., 2001; Tan et al., 2004). Furthermore, PPAR β/δ activation conferred an anti-apoptotic effect upon keratinocytes in the context of *in vivo* wound tissues (Di-Poï et al., 2002). Additional studies have also described the anti-inflammatory role of PPAR β/δ , which is important for the proper downregulation of the inflammatory phase (Man et al., 2008). In agreement with this report, other studies had suggested that PPAR β/δ agonists were able to suppress lipopolysaccharide (LPS)-induced pro-inflammatory genes such as iNOS and COX2 via NF- κ B (Welch et al., 2003; Kilgore and Billin, 2008).

In the context of a mammalian system, it is important to understand that wound repair is not dependent on the behavior of one cell type but involves the epithelial-

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mesenchymal interactions that occur within the tissue mediated by key central players such as growth factors. These soluble mediators then perform the necessary autocrine and paracrine activities, forming a complex and tightly regulated interplay between cellular components that culminate in appropriate cellular responses to maintain skin homeostasis and, more importantly, proper wound healing (Chong et al., 2009). Within the myriad of growth factors produced, interleukin-1 (IL-1) is a cytokine that exerts a strong influence on cutaneous wound repair and homeostasis (Schröder, 1995). Both IL-1 subtypes, IL-1 α and IL-1 β , bind to the same IL-1 receptor (IL-1r) with comparable affinities. IL-1r also exists in two distinct forms, IL-1r1 and IL-1r2. Of the two, IL-1r1 mediates most of the established IL-1 responses, whereas IL-1r2 is unable to activate any signaling cascades due to its short cytoplasmic tail. Interestingly, keratinocytes predominantly express IL-1r2 either constitutively or post-activation, indicating that keratinocytes do not receive any autocrine effect from self-derived IL-1 (Rauschmayr et al., 1997). On the other hand, dermal fibroblasts primarily express IL-1r1, rendering it highly receptive to keratinocyte-derived IL-1 through paracrine signaling. IL-1 signaling inhibition occurs when the IL-1r antagonist (IL-1ra) binds to IL-1r1. Both IL-1ra variants, secreted IL-1ra (sIL-1ra) and intracellular IL-1ra (icIL-1ra), are produced from the same gene via two different promoters and first exons. The affinity of IL-1ra to bind to IL-1r1 is much higher than IL-1r2, and it outcompetes IL-1 for receptor binding. Keratinocytes only constitutively produce the icIL-1ra variant, whereas fibroblasts produce both sIL-1ra and icIL-1ra when activated in the correct context (Arend et al., 1998).

In addition to studying keratinocytes alone in wound healing, our group also previously reported on a novel and critical role of PPAR β/δ in wound fibroblasts to

regulate and maintain skin homeostasis via paracrine signaling to the adjacent epithelial cells (Chong et al., 2009). Keratinocyte-derived IL-1 induced the expression of PPAR β/δ in the neighboring fibroblasts. Fibroblast PPAR β/δ subsequently stimulated the production of sIL-1ra to inhibit further IL-1-induced production of mitogenic factors from fibroblasts. This decrease in mitogenic factors aided in the regulation of keratinocyte proliferation during wound healing, ensuring a homeostatic control in the skin epidermis (Chong et al., 2009). Such a homeostasis event occurring in wound healing underscores the importance of tight regulation in such a complex epithelial-mesenchymal interaction. However, in diabetic wound healing the keratinocyte differentiation and migration as well as the epithelial-mesenchymal crosstalk mechanisms are defective (Stojadinovic et al., 2005; Brem et al., 2007). The administration of PPAR β/δ agonists may be a potential clinical solution (Tan et al., 2003; Tan et al., 2004).

1.11 PPAR β/δ in fibroblasts

PPAR β/δ is the predominant subtype in dermal fibroblasts and controls keratinocyte proliferation during wound healing (Di-Poï et al., 2002). In the previous chapter, we showed that ligand-activated PPAR β/δ accelerated diabetic wound healing. PPAR β/δ stimulated the expression of glutathione peroxidase 1 and catalase in fibroblasts, which prevented ROS-mediated modifications of ECM to facilitate keratinocyte migration (Wang et al., 2015). Similarly, a separate report highlighted the importance of fibroblast PPAR β/δ in attenuating UVB-induced MMP-2 secretion alongside increasing the elastin levels. Consequently, this may contribute to the maintenance of skin integrity by inhibiting ROS generation (Ham et al., 2014). In another study, the authors showed that

upon heat injury, the expression of PPAR β/δ in fibroblasts increased and activated mechanisms that led to the protection of cells from heat injury-induced morphological changes and increased proliferation (Zuo et al., 2012). In addition, activation of PPAR β/δ mediated the upregulation of Ski and subsequently promoted the proliferation of fibroblasts and wound repair (Li et al., 2012). Thus, existing studies suggested that PPAR β/δ positively influences wound healing. Despite the importance of epithelial-mesenchyme communication, little is known about the role of fibroblast PPAR β/δ in the architectural formation of the skin and its function in skin homeostasis. Therefore, it is crucial that a model organism be derived to allow for better *in vivo* studies to elucidate the important roles that fibroblast PPAR β/δ play in skin structure and homeostasis.

1.12 PPAR β/δ agonist and delivery

To date, less than ten PPAR β/δ selective agonists have been developed, with the most widely experimented and potent one being developed by GlaxoSmithKline and Ligand (Pelton, 2006; Chang et al., 2007). This agonist is 2-methyl-4-((4-methyl-2-(4-trifluoromethylphenyl)-1,3-thiazol-5-yl)-methylsulfanyl)phenoxy acetic acid, also known as GW501516 (GW). Several PPAR β/δ agonists are currently in different Phase II clinical trials on patients with dyslipidemia and metabolic syndrome (Chang et al., 2007). However, no pre-clinical studies have been performed to assess the pharmacological potential of GW or any other PPAR β/δ agonists as therapeutic interventions against diabetic wound healing, despite the prodigious amount of supportive results of the role of PPAR β/δ in skin homeostasis and wound repair. In addition, some issues must be overcome to bring such a compound from the bench to the bedside. PPAR β/δ agonists

such as GW are hydrophobic and lipophilic compounds. This renders the topical application of such compounds a major problem due to their bioavailability and pharmacokinetics. Furthermore, for suitable topical application of the compound, the material used for the wound dressing must be biocompatible. Of equal importance is the need to decipher a pharmacological dose that is clinically relevant for human patients.

Earlier studies have shown that an increased and subsequently prolonged expression of PPAR β/δ allowed for the accelerated closure of wounds in wild type and Smad3 null mice, compared to PPAR β/δ null or PPAR β/δ -Smad3 null mice (Tan et al., 2004; Tan et al., 2005). Hence, through our aim of studying the effect of GW on mice diabetic wound healing by microparticle delivery, we are likely the first group to extrapolate the results from basic bench research to diabetic mice wound healing specifically. Furthermore, this would be a novel development to derive a microencapsulated microparticle encompassing the appropriate dose of GW, thereby allowing the pharmacokinetics and pharmacodynamics of the drug to be controlled more effectively and selectively as compared to current clinical trials. Such a delivery method would confer a dual-release kinetic profile to increase and prolong the expression and activation of PPAR β/δ , and also resulting in a potential for nonsystemic exposure to the drug, and thereby establishing a more favourable safety profile of the drug.

1.13 Double-layered microparticles

Delivery of various drug compounds has been heavily studied due to problems such as the bioavailability and pharmacokinetics of these molecules. Over the years, a wide range of drug release systems ranging from coated tablets and gels to biodegradable

microspheres and osmotic pumps have been analyzed to understand their drug release profiles. Single-layered particles inherently produce a high initial release of drug particles near the surface (Lee et al., 2012). This abrupt release might be detrimental to patients due to a narrow pharmaceutical window or immense toxicity. Furthermore, hydrophilic drugs such as GW can only achieve low encapsulation efficiency in single-layered microparticles. To overcome these issues, a multiparticulate drug delivery system had to be employed, in which the outer layer could serve a rate-limiting membrane for drug diffusion. In recent decades, two polymeric compounds, poly(D,L-lactide-co-glycolide) (PLGA) and poly(L-lactide) (PLLA), have been the most attractive candidates in drug delivery and tissue engineering applications. Both polymers are biocompatible and biodegradable, exhibit a wide range of erosion times, and have tuneable mechanical properties. Most importantly, both polymers have attained FDA approval (Dong et al., 2008; Huang et al., 2008; Wenk et al., 2009; Makadia and Siegel, 2011; Gainza et al., 2013). Unfortunately, few reports studied the *in vivo* biological effects of controlled drug release with various different release kinetic profiles. In this chapter, the biological impact of varying controlled release of GW on diabetic wound healing and its mechanism of action will be examined. Formulations that aid in the controlled release of drug compounds can be utilized to reduce the necessary drug quantity to provide the desired therapeutic effect in patients. In addition, patient compliance is expected to improve with such formulations. Microencapsulation will improve the bioavailability and aqueous solubility for efficient uptake and subsequently offer diabetic patients a promising medical alternative for wound treatment.

1.14 Cre-loxP technology

To complement *in vitro* studies on the role of fibroblast PPAR β/δ , it was necessary to perform *in vivo* studies on an appropriate animal model. Using Cre-lox technology (Maddison and Clarke, 2005), we generated a novel mouse line with the *Pparb* gene deleted specifically in fibroblasts. Cre is a 38 kDa recombinase able to recognize 34 bp sequences defining the locus of crossover in P1 bacteriophage (*loxP*) sites that can be engineered into genes of interest. Cre is a member of the integrase family of site-specific recombinases and thus has characteristics that allow for optimal maintenance and gene manipulation activities within mammalian hosts (Buchholz et al., 1996). These properties include the conserved recombination activity, the ability to recognize 34 bp targets, the ability to function without any additional cofactors and accessory proteins, and an ideal efficiency at 37 °C. The *loxP* site contains two inverted 13 bp repeats separated by a nonpalindromic 8 bp spacer sequence. The *loxP* sites usually flank a particular portion of the gene of interest. Such a modified gene is commonly labeled “floxed” (fl). Endogenously, the Cre gene and *loxP* sites are absent in mice. Hence, both entities have to be introduced transgenically. The direction of the *loxP* sites is particularly crucial (Nagy, 2000). Opposing directions of *loxP* sites allow Cre to invert the floxed portion of the gene. With a trans arrangement of *loxP* sites on two different chromosomes, chromosomal translocation would occur in the presence of Cre. In this thesis, the *loxP* sites are arranged in the same direction on a particular segment of the chromosome, resulting in the Cre-mediated deletion of the floxed section of the gene of interest.

By employing the Cre-loxP technology, fibroblast-specific gene deletion can be achieved by the expression of Cre recombinase driven by the fibroblast-specific protein 1

(FSP) promoter. For the first time, it is possible to specifically delete a gene in fibroblasts and study its long-term functional and molecular impact in the tissue microenvironment and its consequences in development and homeostasis. One recent study employing such a gene manipulation strategy showed that the deletion of estrogen receptor α specifically in fibroblasts (FSPCre-*Era*) resulted in the prostates of these mice having significantly reduced branching morphogenesis, increased stromal apoptosis, and reduced expression of genes involved in normal prostate functions (Chen et al., 2012). This indicated the importance of fibroblasts in tissue development via paracrine interactions. Similarly, as the predominant cell type in the skin dermis, we hypothesized that the deletion of PPAR β/δ specifically in the fibroblasts could have a huge impact on skin development and homeostasis.

1.15 Gene knockout strategy

Herein, a novel mouse line will be generated using mice carrying the Cre transgene and mice with exon 4 of the *Pparb/d* gene flanked by loxP sites. With reference to the current controversies stemming from the use of different mouse knockout models, the method of genetic deletion employed would result in a different physiological impact on the mice. Two types of knockout models have currently been established, and controversial results have emerged due to the differences in the knockout mechanisms. Studies that illustrated the pro-tumorigenic role of PPAR β/δ reported their findings based on a mouse model that deleted exon 4 (PPAR $\beta/\delta^{\Delta\text{exon4}}$) of the *Pparb/d* gene. Exon 4 encodes the DBD of PPAR β/δ , and its deletion resulted in a truncated mRNA that underwent rapid nonsense-mediated mRNA decay, thus eliminating PPAR β/δ function.

The mutant protein, if present, lacks the DBD and would be unable to bind its cognate response element to transactivate gene expression. In contrast, other groups reported on the anti-tumorigenic role of PPAR β/δ in mice with exon 8 (PPAR $\beta/\delta^{\Delta\text{exon}8}$) of the *Pparb/d* gene deleted. It was previously proposed that this particular deletion strategy could result in a hypomorphic allele that produces a gene product with residual PPAR β/δ activity (Zuo et al., 2009). Exon 8 encodes helix 12 of the LBD of PPAR β/δ , and the resultant mutant protein retains its DNA-binding properties (Wang et al., 2014).

The novel mutant mice generated in this current study displayed profound morphological changes to the epithelia architecture of the skin. Microarray gene expression analysis and chromatin immunoprecipitation (ChIP) studies identified *Lrg1* as a novel target of PPAR β/δ in the fibroblasts. Our findings confirmed that the expression of PPAR β/δ in dermal fibroblasts plays important roles in the epithelial-mesenchymal interaction in the skin for normal morphogenesis, response to wounding and inflammatory stimuli.

1.16 Overview of cancer-associated fibroblasts (CAFs)

CAFs have attracted increasing attention as potential cancer drug targets due to their ability to stimulate tumor growth, invasion, angiogenesis and metastasis. Recent pre-clinical studies indicate that CAFs also mediate resistance to anti-angiogenic therapy and targeted therapy using tyrosine kinase inhibitors (Crawford et al., 2009; Wang et al., 2009). CAFs are large, spindle-shaped mesenchymal cells that can be identified based on a combination of markers such as α -smooth muscle actin (α -SMA), fibroblast activation protein, smooth muscle myosin, calponin, tenascin, vimentin, and desmin (Augsten, 2014).

In addition to the phenotypic alterations, an increasing number of *in vitro* and *in vivo* studies also suggested that the sustainability of CAFs arises in part due to possible genetic and epigenetic alterations to their DNA. Genetic alterations such as somatic p53 mutations detected within CAFs from many human cancers result in a higher inclination towards lymph node metastases. Parallel to this finding, p53^{+/-} fibroblasts lose the wild type allele upon exposure to cancer cells *in vivo* (Hill et al., 2005). Despite such findings, it was proposed that these genetically altered CAFs are minorities within a large heterogeneous fibroblastic population.

CAFs adopt a modified phenotype akin to wound fibroblasts present during normal wound healing. Fibroblasts found in normal, uninjured tissue are essential in maintaining a balanced level of ECM proteins and tissue homeostasis. Tissue injury initiates an early inflammatory response that is followed by an increase in fibroblasts and fibroblast-derived structural proteins at the injury site (Regan et al., 1991; Kalluri and Zeisberg, 2006). Fibroblasts present during the wound healing phase acquire an activated phenotype distinguished by a large oval euchromatic nucleus with one or two nucleoli, a rough endoplasmic reticulum, and a prominent Golgi apparatus (Kalluri and Zeisberg, 2006). This activation can be induced by growth factors derived from the injured epithelial cells and infiltrating monocytes and macrophages. These activated fibroblasts express cellular markers that are also observed in CAFs. Importantly, activated fibroblasts return to their quiescent state when wound healing is completed. In contrast, CAFs in tumors take on a phenotype that is constitutively active.

Previous studies comparing CAFs with normal fibroblasts showed that CAFs promote carcinogenesis in non-tumorigenic epithelial cells and support *in vivo* tumor

growth. This effect was only observed with CAFs but not with normal fibroblasts, suggesting an exclusive role of CAFs in promoting tumor growth. CAFs are crucial to supporting the entire tumor microenvironment and cancer progression via the heterotypic and homotypic communications that occur between the tumor cells and CAFs. Previous studies have also highlighted that CAFs contribute to the proliferation and growth of tumor cells in pancreatic, colon, breast, and skin cancers (Nakagawa et al., 2004; Hwang et al., 2008; Liao et al., 2009; Erez et al., 2010). In addition, CAFs dissected from human tumor biopsies revealed a contrasting microarray transcriptional profile from isolated cultured CAFs and normal fibroblasts (Orr et al., 2011). This indicates that cancer cells and CAFs should not be studied in isolation, given the indispensable reciprocal influence from their counterparts. Instead, it appears to be more appropriate to study the progression of cancer through investigations of how communication between tumor cells and the surrounding CAFs can modulate the growth and survival of cancer cells. Intriguingly, a major question that remains unanswered is whether CAFs co-evolved with cancer cells. Epithelial cells can be transformed through genetic alterations. At the same time, CAFs may also be generated through environmental cues that drive genetic instability towards transformation of normal fibroblasts. It remains debatable whether both entities co-evolved to drive tumor progression.

1.17 Tumor-CAF interactions

The tumor is a complex, heterogeneous tissue containing cancer cells that influence and respond to signals from the surrounding cells that constitute the tumor stroma. Epithelial-mesenchymal interaction initiates a reciprocal communication that

involves cytokines, growth factors, bioactive lipids, reactive oxygen and nitrogen species produced by either the cancer cells or the CAFs (Wang and Xie, 2010). Thus, the development and progression of neoplastic epithelia can be understood to arise from the deregulation of this dynamic interaction between normal epithelial and fibroblasts.

The co-existence of the cancer cells and the surrounding CAFs has prompted researchers to understand the signaling interactions that occur between the cell types. Cancer cells often secrete cytokines and growth factors such as transforming growth factor β (TGF- β) and platelet-derived growth factor (PDGF) that bind to their respective cognate receptors found on fibroblasts, altering the normal physiological functions of fibroblasts and thus affecting nearly every aspect of stroma remodeling and tumor growth (Kalluri and Zeisberg, 2006). CAFs are an established source of classical growth factors known to play tumor-promoting roles, including epidermal growth factor (EGF), TGF- β , hepatocyte growth factor (HGF) and other chemokines that bind to cognate receptors found on tumor cells (Kalluri and Zeisberg, 2006). As such, a more realistic representation of the *in vivo* conditions would require the analysis of the tumor-stroma interaction. Insights into the homotypic and heterotypic signaling pathways are required for a better understanding of how the tumor microenvironment supports the progression of cancer as a disease. The communication between CAFs and tumor cells via the various growth factors has been widely studied. However, despite the importance of CAFs, little is known about the transcription factors involved in the tumor-promoting functions of these cells or the oncogenic signals produced by these stromal fibroblasts for neoplastic transformation. A search for studies pertaining to the transcriptional regulation that

translates into the tumor-promoting effect of CAFs yielded a drought of information on this topic.

1.18 Transcriptional regulation in CAF

Transcription factors are an integral part of cellular behavior. Transcription factors are usually encoded by oncogenes and tumor-suppressor genes, with their functions being tightly regulated by transcriptional regulators. Numerous studies in tumor cells have consistently underscored the pivotal role of transcription factors in tumor development and progression. The aberrant activities of transcription factors such as p53, NF- κ B, STAT3, and Runx2 form the driving force for cancer progression (Pratap et al., 2006; Yu et al., 2007; Karin, 2009; Shostak and Chariot, 2011; Muller and Vousden, 2013; Lee et al., 2014). However, as we begin to understand the importance of epithelial-mesenchymal communications in dictating the progression of cancer, a better understanding of the transcriptional regulation of CAFs will be crucial for the development of more effective anti-cancer therapies. Despite the extensive studies of pro-tumorigenic growth factors shuttled between tumor cells and CAFs, there remains a knowledge gap with respect to transcriptional regulation in CAFs.

Transcriptional regulation plays an important role in the tumor-promoting function of CAFs, but little is known about the specific transcription factors that drive the transition from normal fibroblasts to CAFs or the role of transcription factors in the evolution of the reciprocal relationship between CAFs and tumor cells during growth. Despite being one of the largest known classes of transcription factors, the expression profiles of nuclear receptors in CAFs have not been defined and their functions in CAFs

remain unknown. This knowledge gap therefore warrants the need for a better understanding of the ways in which nuclear receptor signaling in CAFs contributes to cancer progression. The CAF-specific nuclear receptor expression pattern may offer the pharmaceutical industry a useful prognostic indicator and may also be used to identify specific nuclear receptors as potential pharmacological targets.

1.19 Current FSPCre *in vivo* studies on tumorigenesis

The use of animal models with genes deleted specifically in the fibroblasts to study tumorigenesis had been previously performed. One such study involved the fibroblast-specific ablation of *Pten* in the mouse via FSP-driven Cre recombinase (FSPCre-*Pten*) (Trimboli et al., 2009). Mammary glands of these FSPCre-*Pten* mice exhibited accelerated initiation, progression, and malignant transformation of mammary epithelial tumors. The tumorigenic event in FSPCre-*Pten* mice was accompanied by massive remodeling of the ECM, innate immune cell infiltration, and increased angiogenesis. Recently, reports on fibroblast-specific deletion of hypoxia-inducible factor 1 α (FSPCre-*Hif1a*) and androgen receptor (FSPCre-*Ar*) have also emerged (Kim et al., 2012; Yu et al., 2012). While FSPCre-*Hif1a* mice experienced accelerated tumorigenesis, FSPCre-*Ar* mice exhibited downregulated epithelial proliferation, increased apoptosis, and decreased collagen deposition. Importantly, these reports convincingly revealed that a modified transcriptomic profile of normal fibroblasts was sufficient to modulate the oncogenic potential of the adjacent epithelial layers. Hence, we hypothesized that the FSPCre-*Pparb*/ $\delta^{fl/fl}$ mice that we have generated will serve as a model to further our understanding of the role that PPAR β/δ plays in tumorigenesis.

Chapter 2: Materials and Methods

2.1 Cell culture

Human keratinocytes (Ker; ATCC, USA) were routinely cultured in Keratinocyte-SFM culture media (Invitrogen, USA). Adult human dermal fibroblasts were obtained via explants from human abdominal skin tissues as described previously (Huschtscha et al., 2012). The human abdominal skin tissues were provided by Dr. Marcus Wong from the Plastic, Reconstructive and Aesthetic Surgery department of Tan Tock Seng Hospital Singapore, approved by an ethical committee review board (NHG DSRB Ref: 2012/00071). The fibroblasts explants were routinely maintained in FibroGRO™ Complete Media (Millipore, USA). Human fibroblasts (FIB; ATCC, USA) were maintained in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific, USA) supplemented with 10% fetal bovin serum (FBS; Thermo Fisher Scientific, USA). Immortalized keratinocytes (HaCaT) and human embryonic kidney (HEK 293T) cells were cultured in DMEM supplemented with 10% FBS. Human monocyte cells (THP-1) was cultured in RPMI-1640 medium supplemented with 10% FBS. Overnight incubation of THP-1 with 50 ng/mL of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) allowed for differentiation into macrophages. Diabetic culture condition was achieved by using culture medium supplemented with 10% FBS containing 25 mmol/L glucose and 10 nmol/L insulin (Chong et al., 2014). All cultures were maintained in a humidified incubator at 37 °C with 5% CO₂.

2.2 Antibodies and chemicals

Primary antibodies used in this study: monoclonal anti-PPAR β/δ antibody (Millipore, USA), monoclonal anti- α -smooth muscle actin (α -SMA; Santa Cruz, USA), polyclonal

anti-PPAR β/δ , anti- β -tubulin, anti-transglutiminase 1 (TGase1), anti-FLAP, anti-collagen type 1 (Santa Cruz, USA), polyclonal anti-Ly6C/Ly-6G (BioLegend, USA), anti-keratin 10 (K10; BioLegend, USA), polyclonal anti-keratin 6 (K6; Thermo Scientific, USA), polyclonal anti-vimentin (Abcam, USA), anti-LRG1 (Sigma, USA) and polyclonal anti-Ki67 (Leica, USA). Secondary antibodies used included IRDye[®] 680LT goat anti-mouse IgG, IRDye[®] 680LT goat anti-rabbit IgG, IRDye[®] 680LT donkey anti-goat IgG (Li-Cor Biosciences, USA), DyLight[®] 488-conjugated goat anti-mouse IgG, DyLight[®] 488-conjugated goat anti-rabbit IgG, and biotinylated goat anti-rat IgG (Vector Laboratories, USA). GW501516 (IUPAC: {4-[(4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl)methyl)sulfonyl]-2-methylphenoxy}acetic acid; PPAR β/δ agonist; GW), GSK0660 (IUPAC: methyl 3-([2-(methoxy)-4 phenyl]amino)sulfonyl)-2-thiophenecarboxylate; PPAR β/δ antagonist; GSK), and compound 10h (IUPAC: methyl 3-(N-(4-(isopentylamino)-2-methoxyphenyl)sulfamoyl)-thiophene-2-carboxylate; PPAR β/δ inverse agonist; 10h) were dissolved in DMSO before use in culture. GW, GSK, and 10h were obtained from Associate Professor Chiba Shunsuke (School of Physical and Mathematical Sciences, Nanyang Technological University, Singapore).

2.3 Materials

PLGA (IV: 1.18; Bio Invigor, Taiwan), PLLA (intrinsic velocity (IV): 2.38; Bio Invigor, Taiwan), and poly(vinyl alcohol) (PVA) (Sigma-Aldrich, USA) were used as provided. Dichloromethane (DCM; Tedia Company Inc.) used was of high-performance liquid chromatography (HPLC) grade. Luciferase[®] Reporter Assay System and FuGENE[®] HD Transfection Reagent (Promega, USA). iScript[™] cDNA Synthesis Kit (Bio-Rad, USA).

SYBR[®] Green PCR Master Mix (Applied Biosystems, USA), and SuperFrost[™] Plus Microscopic Slides (Thermo Fisher Scientific, USA).

2.4 Microencapsulation of GW

The microencapsulation of GW into double-layered PLGA:PLLA (50:50) was performed by the oil-in-water (o/w) emulsion solvent evaporation procedure as described previously (Lee et al., 2010; Lee et al., 2010). Briefly, PLGA (67 mg), PLLA (33 mg), and GW (1 mg) were dissolved in 1.33 mL of DCM. The mixture was then added into 250 mL of aqueous PVA (0.5% w/v) and stirred at 2000 rpm with an overhead stirrer (Caframo BDC1850-220) at 25 °C. The evaporation of DCM caused a phase separation of PLGA and PLLA, giving rise to the double-layered microparticles loaded with GW in the hydrophilic core (PLGA:PLLA:GW). The formed microparticles were then centrifuged down and rinsed with deionised water, lyophilised and stored in a desiccator for subsequent characterization. Single layered GW-loaded particles (PLGA:GW and PLLA:GW) were prepared in the similar manner, where 100 mg of either polymer was used. Vehicle control microparticles were also prepared in the same way with no GW loaded (PLGA:PLLA:veh).

2.5 Determination of polymer distribution

In order to validate the distribution of the polymer within the microparticles, Raman mapping was performed. Microparticles were sectioned and viewed under a microscope objective at a laser power of approximately 20 mW. Raman point-by-point mapping measurements were obtained from both the x- and y-axes with an inVia Reflex Raman microscope (Renishaw, United Kingdom). The microscope was equipped with a near-

infrared enhanced deep depleted thermoelectrically Peltier-cooled CCD array detector (576 x 384 pixes) and a high-grade Leica microscope. Samples were irradiated with a 785 nm near-infrared diode laser, and objective lenses were used to harvest the backscattered light. A static 1800 groove/mm dispersive grating in a spectral window ranging 300 to 1900 cm^{-1} was used to collect the measurements. Acquisition time for each spectrum was approximately 35 s. Raman mapping data were subsequently analysed via the band target entropy minimization (BTEM) algorithm to reconstruct pure component spectral estimates.

2.6 Electron microscopy of microparticles

Surface and internal morphologies of the microparticles were studied using a JSM-6360 scanning electron microscope (SEM; JEOL, USA) at 5 kV. Prior to scanning, samples were mounted onto a metal stub and submerged in liquid nitrogen before being sectioned with a razor blade. Samples were subsequently coated with gold using a sputter coater (SPI-Module, USA).

2.7 Drug encapsulation efficiency

Encapsulation efficiency is defined as the ratio of actual to theoretical drug loading within the microparticles. For quantification of GW loading, 5 mg of microparticles were added into 1 mL of 1 M NaOH solution and incubated for 24 h at 37 °C. The mixture was then centrifuged and supernatant drawn. The concentration of GW was determined using a UV-Vis spectrophotometer (Shimadzu UV-2501, Japan) at 318 nm. All measurements were carried out in triplicates.

2.8 Transfection and luciferase assay

Drug release study was performed as previously described, except that serum-free culture medium was used (Lee et al., 2012). Cells were co-transfected with a PPAR response element (PPRE)-tk-luciferase reporter vector and pCDNA3.1-PPAR β/δ using Fugene[®] HD Transfection Reagent (Promega, USA). The renilla luciferase (RL) control reporter vector was used as a control for transfection efficiency. The next day, the culture medium was aspirated and replaced with conditioned medium, which were collected daily for luciferase assay for a period of 10 days. Luciferase assay was performed using Dual Luciferase[®] Reporter Assay System (Promega, USA) and the Glomax 20/20 Luminometer (Promega, USA) to monitor drug release kinetics and biological activity of released GW. TGF β activity in Ker keratinocytes was performed by transfecting Ker cells with TGF β -tk-luciferase reporter vector using the FuGENE[®] HD Transfection Reagent (Promega, USA). RL reporter vector was used as a control for transfection efficiency. The next day, cells were treated either with recombinant TGF β , LRG1, or both for 16 h. Cells were subsequently lysed and the luciferase assay was performed using the Dual Luciferase[®] Reporter Assay System (Promega, USA) and the Glomax 20/20 Luminometer (Promega, USA).

2.9 *In vitro* GW release kinetics assay

A rotating system was used to measure the *in vitro* GW release kinetics. 50 ng of GW-loaded microparticles were resuspended in 1 mL of saline and kept on the rotating system at 37 °C to allow the continuous release of GW from the microparticles. 10 μ L of the supernatant was taken daily to measure the concentrations of GW using a UV-Vis

spectrophotometer (Shimadzu UV-2501, Japan) at 318 nm. All measurements were done in triplicates. The assay was performed for 12 days.

2.10 Measurement of ROS

Extracellular hydrogen peroxide (H_2O_2) was measured using the Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Life Technologies, USA) according to the manufacturer's protocol. Briefly, 5×10^4 cells were incubated in assay reagent made up of 50 μM Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.1 U/mL horseradish peroxidase (HRP) in Krebs-Ringer phosphate buffer for 1 h at 37 °C. Next, cells were removed by centrifugation and resorufin fluorescence in the supernatant was measured at emission of 590 nm (excitation 485 nm) using Tecan SpectraFluor Plus plate reader (Tecan Systems, USA). Results were reported in arbitrary fluorescence units. For intracellular ROS measurement, 5×10^4 cells of interest were incubated with 10 μM 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester fluorophores (CM-H2DCFDA, Life Technologies) for 30 min at 37 °C. Next, cells were detached and analysed by the AccuriTM C6 flow cytometer (BD Biosciences, USA). ROS in wound fluid was measured using 20 μL of wound fluid in a 200 μL Amplex Red final reaction volume. Wound fluid was collected using a 5 mm x 5 mm polyvinyl alcohol sponge placed adjacent to the wound, and covered with a TegadermTM transparent film dressing (3M, USA). Sponges were collected at the respective timepoints and Amplex Red conversion to resorufin fluorescence was measured as described above.

2.11 *In vitro* catalase and GPx activities assays

10^6 cells for each assay were seeded and subsequently treated with indicated concentrations of GW for 6 h. Catalase and GPx activities were measured according to the Amplex Red Catalase Assay Kit (Molecular Probes, USA) and the Glutathione Peroxidase Assay Kit (Abcam, United Kingdom) manufacturers' protocols, respectively. Data was normalized against cell protein concentrations. Each experiment was repeated twice with triplicates.

2.12 Animal studies

All experimental animals were maintained in a specific pathogen-free facility with a 12 h/12 h light/dark cycle, and have *ad libitum* access to chow and water. Animal experiments were carried out in accordance to the guidelines of the University's Institutional Animal Care and Use Committee (IACUC, ARF-SBS/NIE-A0174AZ for drug encapsulation studies and ARF-SBS/NIE-A0216AZ for genetic knockout studies), Singapore.

2.13 Generation of fibroblast-specific PPAR β/δ -knockout mice

Pparb/d^{fl/fl} mice, having the exon 4 of their *Pparb/d* gene flanked by loxP sites (Iglesias et al., 2012), were mated with mice harbouring the *Cre* transgene under the control of the FSP promoter (Trimboli et al., 2008; Trimboli et al., 2009). FSPCre-*Pparb/d^{fl/+}* progenies were then bred with *Pparb/d^{fl/fl}* mice to obtain FSPCre-*Pparb/d^{fl/fl}* mice and the wild-type control mice. To sustain this genotype, we bred FSPCre-*Pparb/d^{fl/fl}* progenies with *Pparb/d^{fl/fl}* mice. FSPCre mice were always maintained as hemizygotes.

2.14 Genotyping of mice

Mice genotyping were performed using the Mouse Genotyping Kit HotStart according to the manufacturer’s protocol (KAPA Biosystems, USA). Briefly, genomic DNA was extracted from mouse ear clippings (~1 mm²) in a mixture containing 1X KAPA Express Extract Buffer and 2 units of KAPA Express Extract Enzyme per reaction. The extraction mixes were incubated in a thermocycler for 10 min at 75 °C, followed by protease deactivation for 5 min at 95 °C. PCR was immediately performed with KAPA2G Fast HotStart Genotyping Mix, primers (sequences are provided in Table 1.1) at a final concentration of 0.5 µM each, and 1 µL of supernatant from the respective KAPA Express Extract reaction. The PCR products were analysed in a 1.2% agarose gel via ethidium bromide staining.

Table 1.1 Oligonucleotide sequences of genotyping PCR primers.

<i>Genes</i>	Forward (5’ to 3’)	Reverse (5’ to 3’)
<i>PPARβ/δ Exons 4</i>	GCAGCTGCTCAGCTGCCTGC	CCGCCTCTCGCCATCCTTTCA GA
<i>PPARβ/δ Exon 5</i>	GCAGCTGCTCAGCTGCCTGC	CAGGTGCTGGGCGCCCTCTGA CAGAAT
<i>FSP1Cre Control</i>	CTAGGCCACAGAATTGAAAGA TC	GTAGGTGGAAATTCTAGCATC ATCC
<i>FSP1Cre Transgene</i>	GCGGTCTGGCAGTAAAACTA TC	GTGAAACAGCATTGCTGTCAC TT

2.15 Separation of epidermis and dermis from mouse skin biopsy punch

Mice in the telogen phase of their hair cycle were anaesthetized and depilated on their dorsal back before being subjected to a biopsy punch (~4 mm). The skin biopsy was then treated with 3.8% ammonium thiocyanate (Sigma, USA) in 1X PBS for 30 min at 37 °C. Subsequently, the epidermis was mechanically separated from the dermis with a pair of forceps. The dermis was then curetted with a 30-gauge needle. The dermis was washed

with 1X PBS before being subjected to the genotyping procedure, RNA extraction procedure for the real-time qPCR assay and Western blot.

2.16 *In vivo* wound healing experiments for drug encapsulation studies

Diabetic (db/db) and wild-type control (db/+) male mice of 6-8 weeks old were purchased from The Jackson Laboratory. Inclusion criteria were a blood glucose level lower than 200 mg/dL (control, db/+) or exceeding 300 mg/dL (diabetic, db/db). Two full-thickness excisional splint wounds were inflicted on the dorsal skin of each mouse as previously described (Tan and Wahli, 2013). GW (1 ng) or GW (1 ng)-loaded microparticles (PLGA:GW, PLLA:GW or PLGA:PLLA:GW) were re-suspended in saline and topically applied onto one wound. Control experiments were carried out on the other wound accordingly using vehicle DMSO alone, PLGA:veh, PLLA:veh or PLGA:PLLA:veh. Wounds were covered with 3M™ Tegaderm transparent film dressing (3M, USA) to prevent leakage of the microparticles or solution. Wound closure was measured daily in a double-blinded manner until end of experiment. Animals were sacrificed on days 3, 5, 7, 9 and 10 post wounding. Wound biopsies were harvested, centrally bisected and subjected to histology staining for measurement of the epithelial tongue length and wound epithelial area. At least 5 animals were used for each individual treatment at every single time point.

2.17 *In vivo* wound healing experiments for genetic knockout studies

Mice of ~8 weeks of age were shaved and full-thickness excisional wounds were inflicted on the dorsal skin of mice as previous described (Tan and Wahli, 2013). Wound surface areas and contraction rates were measured over the indicated duration (Tan et al., 2005; Tan and Wahli, 2013). Briefly, wound surface areas were measured from the length and

width of the wound surface on the mice dorsal backs. Wound contraction rate was measured from the changes in distance between the first hair follicles on the wound edge as a percentage of the distance from post-wounding day 0 sections.

2.18 Tissue sectioning and histological staining

Various tissues were harvested, fixed in 4% paraformaldehyde and were embedded in paraffin according to standard protocol (Canene-Adams, 2013). Briefly, tissues were dehydrated over an ethanol gradient and xylene, before being embedded in paraffin wax (Leica, USA). Tissue sections were rehydrated and stained with H&E, Van Gieson's or Masson's trichrome stains accordingly to manufacturer (Sigma-Aldrich, USA). Stained sections were dehydrated over an ethanol gradient and finally mounted with Eukitt[®] quick-hardening mounting medium (Sigma-Aldrich, USA). Images were taken using the Zeiss Axiovert 200M microscope using the Zeiss FLUAR 10x/0.5 NA objective and PALMRobo V4.3 software (Carl Zeiss, Germany).

2.19 Lucifer Yellow permeability assay

Lucifer Yellow dye (1 mg/mL; Sigma-Aldrich, USA) was topically applied on the epidermis for 1 h at 37 °C. Subsequently, excess Lucifer yellow was washed away with 1X PBS, and the skin tissues were cryofrozen with OCT tissue freezing medium (Leica, USA). Tissues were cryosectioned at 8 µm and mounted with Hoechst 33342 dye (Life Technologies, USA). Images were taken using the LSM 710 confocal microscope with the Zeiss EC Plan-NEOFLUAR 20x/0.5 NA objective. Analyses were performed with ZEN 2012 Light Edition software (Carl Zeiss, Germany).

2.20 Immunofluorescence staining

Tissue sections were fixed in 4% paraformaldehyde at room temperature for 10 min and washed in 1× PBS. Sections were subjected to heat antigen retrieval in citrate buffer (pH 6.0) and subsequently blocked with 5% normal goat serum (NGS) for 1 h at room temperature. Next, sections were then incubated with their respective primary antibodies overnight at 4°C, followed by incubation with the corresponding DyLight® Fluor-conjugated secondary antibodies for 1 h at room temperature in the dark. No primary antibody incubation served as negative controls. Sections were counterstained with Hoechst 33342 dye (Life Technologies, USA) and images were captured using the LSM 710 confocal microscope with the Zeiss EC Plan-NEOFLUAR 20×/0.5 NA objective. Analyses were performed with ZEN 2012 Light Edition software (Carl Zeiss, Germany).

2.21 Colorimetric staining

Sections were fixed, processed, and blocked with 5% NGS in PBS. Sections were incubated overnight at 4°C with the respective primary antibodies, followed by incubation with biotinylated secondary antibodies. After incubation with secondary antibodies, sections were incubated in avidin:biotinylated enzyme complex (Vector Laboratories, USA) for 30 min. Sections were developed with 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories, USA), which produced a brown stain. Slides were mounted with Fluka Eukitt® quick-hardening mounting medium (Sigma-Aldrich, USA). Negative controls did not include the primary antibody. Images were taken using the Zeiss Axiovert 200M microscope using the Zeiss FLUAR 10x/0.5 NA objective and PALMRobo V4.3 software (Carl Zeiss, Germany).

2.22 TPA-induced skin inflammation

Mice were shaven on their dorsal skin and topically treated with 6.5 nmol of TPA (Sigma-Aldrich, USA) in acetone for 24 h. The vehicle- and TPA-treated skins were then harvested and processed accordingly for histological studies and protein analysis.

2.23 Laser capture microdissection (LCM)

H&E stained tissues were subjected to LCM using the Arcturus[®] XT laser capture microdissection system according to the manufacturer's instructions (Life Technologies, USA). LCM tissues (dermal fibroblasts) were collected onto an adhesive CapSure[®] Macro LCM Caps (Life Technologies, USA). Samples were capped onto 500 μ L centrifuging tubes containing 15 μ L of lysis buffer and processed for RNA extraction and qPCR analysis.

2.24 Reverse transcription and real-time quantitative polymerase chain reaction (qPCR)

Samples (cultured cell pellets or skin wound tissues) were homogenized in TRIzol[®] Reagent (Life Technologies, USA) and total RNA was extracted according to the manufacturer's protocol. Reverse transcription was performed with iScript[™] cDNA Synthesis Kit (Bio-Rad, USA). The reverse transcription process was performed in a thermocycler for 5 min at 25 °C, 30 min at 42 °C, and 5 min at 85 °C. Real-time qPCR was subsequently performed with the respective primers at a final concentration of 0.2 μ M each, and KAPA SYBR[®] FAST Universal qPCR Kit (KAPA Biosystems, USA) in a CFX96 qPCR detection system (Bio-Rad, USA). Primer sequences of the specified genes are provided in Table 1.2.

Table 1.2 Oligonucleotide sequences of qPCR primers.

<i>Genes</i>	Forward (5' to 3')	Reverse (5' to 3')
<i>Gpx1</i>	AATGTCGCGTCTCTCTGAGG	TCCGAACTGATTGCACGGG
<i>Gpx2</i>	GAGCTGCAATGTCGCTTTCC	TGGGTAAGACTAAAGGTGGGC
<i>Sod1</i>	ATGGCGATGAAAGCGGTGT	CCTTGTGTATTGTCCCCATACTG
<i>Catalase</i>	CCCCTATTGCCGTTTCGATTCT	TTCAGGTGAGTCTGTGGGTTT
<i>sIL-1ra</i>	CTCAGATAGAAGGTCTTCTGG	CCGCAGTCACCTAATCACTCTCC T
<i>Nox1</i>	CTGCTTCCTGTGTGTCGCAA	AGGCAGATCATATAGGCCACC
<i>Nox2</i>	GAAGAAAGGCAAACACAACA CA	CCCCAGCCAAACCAGAAT
<i>Angptl4</i>	CCTCATGGTCTAGGTGCTTGT	GTCCACCGACCTCCCGTTA
<i>Mouse PPARα</i>	TCGGCGAACTATTCGGCTG	GCACTTGTGAAAACGGCAGT
<i>Mouse PPARβ/δ</i>	TTGAGCCCAAGTTCGAGTTTG	CGGTCTCCACACAGAATGATG
<i>Mouse PPARγ</i>	TGTGGGGATAAAGCATCAGG C	CCGGCAGTTAAGATCACACCTA T
<i>Human Lrg1</i>	CAGACAGCGACCAAAAAGC	GGAACACCTGGCAGTCTTTG
<i>Human Tgfbβ2</i>	CGTGGAGTCGTTCAAGCAGAC G	CCGCACCTTGAACCAAATGG
<i>Human Smad3</i>	GAACGTCAACACCAAGTGCA T	ACGCAGACCTCGTCCTTCT
<i>Human Col1A2</i>	GGTCTCGGTGGGAACTTTG	CCAGGTGGGCCTCTAGGT
<i>Rpl27</i>	AATGCCCACAAGGTACTCTGT	CTTGCGTTTAAGAGCAGGATCT
<i>18S</i>	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
<i>TBP</i>	GCTGGTTATCGGGAGTTGG	ACTGGCCTGGTGTCTAGAG

2.25 Microarray hybridization

cDNA synthesis was performed using the Applause WT-Amp ST System (NuGEN Technologies, USA) in accordance to the manufacturer’s protocol. The resulting cDNA was purified with MinElute Reaction Cleanup Kit (Qiagen, USA), followed by fragmentation and labeling using the Encore Biotin Module (NuGEN Technologies, USA). The fragmented and labeled cDNA were mixed with a hybridization master mix (Affymetrix, USA) and 90 μL of the resultant mixture were injected into the Genechip Mouse Gene 1.0 ST Array gene chips (Affymetrix, USA). Hybridization was allowed to take place for 18 h at 45 °C. Samples were then removed from the chips to allow for the

chips to be washed at a GCOS fluidics station (Affymetrix, USA). 100 µL of array holding buffer was then added into the chip for scanning. The scanned data was collected for analyses using the Partek software (Partek Inc., USA) and Ingenuity Pathway Analysis (Ingenuity Systems Inc., USA).

2.26 Chromatin immunoprecipitation (ChIP) assay

ChIP was performed with minor modifications from a previously described protocol (Chong et al., 2009). Briefly, fibroblasts with the specified treatments were crosslinked using 0.5-1% formaldehyde for 10-15 min at 37 °C and harvested for sonication in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) to obtain cross-linked DNA measuring 200-500 bp in length. Approximately 10% of the lysate was retained for input loading, while the remaining supernatant were subjected to ChIP with the monoclonal anti-PPARβ/δ antibody (4 µg; TransCruz Grade; Santa-Cruz, USA) or polyclonal anti-LRG1 antibody. Complexes that formed were then pulled down using Protein A/G (Santa Cruz, USA). DNA fragments were subsequently reverse crosslinked at 65 °C for 6 h. Pre-immune complex was used as negative control. The primer pairs used are shown in Table 1.3.

Table 1.3 Oligonucleotide sequences of ChIP primers.

Oligo name	Forward (5' to 3')	Reverse (5' to 3')
hcatalasePPRE1	TCCAAGCATGTGAATCCCAGT AG	CTGAGTCCTACTCAAGCCAT CTCC
hGpx1PPRE1,2	GGTCCAGGTTACCCTTCCAAG	GAGGGATCTAGGCTTCCGGT
mcatalasePPRE1	ACCACCCACAAATTAGTAGCA AACAAAG	GATAGGAAGAGACTGGGGA
mcatalasePPRE2 ,3	ACCCACAAATTAGTAGCAAA CAAAG	CCAATCCCGTCCTTTCTAGA GT
mGpx1PPRE1,2	TACCTGACCTGTTGCTCCCTT	AACTACTGTGTCTATTAAAC TT

<i>hLrg1</i>	CCAGGAATAGTGCCTTGCAAA C	CCAGTCCAGGCAGGTATAAG GC
<i>hLrg1 Negative</i>	CTCCTGGGTGCAAGCAATTCT C	GGGATTACAGGCGTGAGCC A
<i>mLrg1</i>	ACCTGCTTGGTTCCTTGTGGA AGAC	CAAACCTCAGTCCCTGGATCC TGGTG
<i>mLrg1 Negative</i>	TGTGCGTGTGTGCGACTCAG	CTTCGACCACCAGCTGACCA T

2.27 Protein extraction and Western blot

Total protein was extracted as previously described (Chong et al., 2014). Briefly, tissues were homogenized in ice-cold protein lysis buffer (20 mM Na₂H₂PO₄, 250 mM NaCl, 1% Triton-X, 0.1% SDS, 1 mM PMSF, and 200 μM sodium orthovanadate) and centrifuged at 12,000 xg for 20 min at 4 °C. The supernatant was retrieved for Western blot analysis. Equal amount of proteins were resolved in a SDS-PAGE and electrotransferred to a low-fluorescent PVDF membrane (IPFL00010; Merck Millipore, USA). Membrane was blocked for 1 h at room temperature with 0.5X Odyssey Blocking Buffer (LI-COR Biotechnology, USA) diluted with TBST (50 mM Tris HCl, pH7.6, 150 mM NaCl and 0.05% Tween-20). The membranes were then probed with the respective primary antibodies overnight at 4 °C in 1x Blocking Buffer with 0.1% Tween-20. Membrane was washed for 5 min thrice with TBST, and incubated with appropriate secondary antibody in 1X Blocking Buffer containing 0.1% Tween-20 and 0.01% SDS for 1 h at room temperature. After the excess secondary antibodies were washed off, the membrane was allowed to dry before being analyzed using the Odyssey CLx scanner and Image Studio V2.1 (LI-COR Biosciences, USA).

2.28 *In vitro* wound healing assay

Rat tail type I collagen (BD Biosciences, USA) was used to coat a 24-well-plate (Corning Life Sciences, USA) at a concentration of 50 $\mu\text{g}/\text{cm}^2$ for 1 h at 25 °C. PBS-diluted H_2O_2 with specified concentrations were used to treat the pre-coated plate for another 3 h at 25 °C. The wells were then washed thrice with PBS and dried. Individual silicone inserts (Ibidi, Germany), with a defined cell-free gap ($500 \mu\text{m} \pm 50 \mu\text{m}$), were attached into each well of the pre-treated plate. Subsequently, 10^4 keratinocytes were seeded into individual wells of each silicone insert. Cells were allowed to attach for 12 h before the inserts were carefully removed. Fresh culture media were provided to the cells and incubated in the Cell-IQ automated cell culture and analysis system (CM Technologies, Finland) for real-time imaging as the cells migrated and covered the cell-free gaps. Wound healing rates were assessed with the Cell-IQ analysis software (CM Technologies, Finland).

2.29 Protein array

Human inflammation antibody array 3 and Growth Factor antibody array membranes were processed according to manufacturer's instructions (RayBiotech, USA). Protein spots were developed using chemiluminescence. Signal intensities were quantified using ImageJ software, and normalized with the intensity of positive controls on each membrane.

2.30 Statistical analyses

Statistical analyses were performed with two-tailed Mann-Whitney U test using SPSS v.19 software (IBM Corporation, USA). A *P* value of <0.05 was considered significant.

Chapter 3: Results

EARLY CONTROLLED RELEASE OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR β/δ AGONIST GW501516 IMPROVES DIABETIC WOUND HEALING THROUGH REDOX MODULATION OF WOUND MICROENVIRONMENT

Publication:

Early controlled release of peroxisome proliferator-activated receptor β/δ agonist GW501516 improves diabetic wound healing through redox modulation of wound microenvironment

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Figures 2.1A, B, and 2.4A are not contributed by candidate.

3.1 Introduction

Diabetic wounds are imbued with early, excessive and protracted reactive oxygen species production. Despite the studies supporting PPAR β/δ as a valuable pharmacologic target in wound healing, the therapeutic potential of the PPAR β/δ agonist GW501516 (GW) as a wound-healing drug was never investigated. Using the topical application of polymer-encapsulated GW, we revealed that different drug release profiles significantly influenced the therapeutic efficacy of GW and, consequently, diabetic wound closure. We showed that double-layer encapsulated GW microparticles (PLGA:PLLA:GW) provided an earlier and sustained dose of GW to the wound and reduced the oxidative wound microenvironment to accelerate healing, in contrast to single-layered PLLA:GW microparticles. The underlying mechanism involved the early GW-mediated activation of PPAR β/δ that stimulated GPx1 and catalase expression in fibroblasts. GPx1 and catalase scavenged excessive H₂O₂ accumulation in diabetic wound beds, prevented H₂O₂-induced ECM modification and facilitated keratinocyte migration. The microparticles with an early and sustained rate of GW release had better therapeutic wound healing activity. The present study underscores the importance of drug release kinetics in the therapeutic efficacy of the drug and warrants further investigation to better appreciate the full potential of controlled drug release.

3.2 Results

3.2.1 Microencapsulation of GW, release and PPAR β / δ activation kinetics of PLGA:GW, PLLA:GW, and PLGA:PLLA:GW

To investigate the release kinetics of the polymers, GW was encapsulated in single-layered PLGA or PLLA and in double-layered PLGA:PLLA microparticles. Raman mapping was first performed on the double-layered encapsulated compound, and it confirmed the abundant distribution of PLGA and PLLA in the shell and core regions, respectively (Figure 2.1A). Cross-sectional analysis of the PLGA:PLLA:GW microparticles showed an even spread of the PLGA core all over PLLA:GW (Figure 2.1B). Both PLGA and PLLA have diameters that range from 75 – 125 μ m and are spherical with smooth surfaces (Figure 2.1B, left panels). Cross-sectional SEM imaging revealed the double- and single-layered structures of PLGA:PLLA:GW, PLLA:GW, and PLGA:GW (Figure 2.1B, right panels). The average encapsulated drug loadings were determined to be $0.2 \pm 0.014\%$, $0.065 \pm 0.005\%$, and $0.07 \pm 0.005\%$ for PLGA:PLLA:GW, PLLA:GW, and PLGA:GW, respectively.

Next, to determine the kinetics of the release of encapsulated GW from these microparticles, an *in vitro* release study was performed over a period of 10 days. The released GW content was measured using UV 318 nm absorbance. The results showed two distinct release profiles of GW between PLGA:PLLA:GW and PLGA:GW or PLLA:GW. In PLGA:PLLA:GW, GW was rapidly released and peaked within six days, with a sustained release until day 10 (Figure 2.1C). In contrast, PLGA:GW and PLLA:GW released GW at a slower rate for the first seven days, followed by a drastic increase to a peak by the 10th day (Figure 2.1C). To study the biological activity of

controlled release GW, a transient transactivation of HEK293T cells co-transfected with a PPAR β/δ expression vector and a luciferase reporter vector was performed. We first ensured that the respective drug-free vehicle microparticles did not result in any measurable transactivation activity even nine days post-transfection (Figure 2.1D). In contrast, the respective GW-encapsulated microparticles, resulted in two distinct PPAR β/δ activation profiles once again. PLGA:PLLA:GW rapidly increased the transactivation profile, reaching a sustained level by day 7 (Figure 2.1E). In contrast, PLGA:GW and PLLA:GW only weakly activated PPAR β/δ at earlier timepoints, with a rapid increase only at day 7 post-transfection (Figure 2.1E). There were no significant differences detected between the transactivation activity of PLGA:GW and PLLA:GW at a majority of the timepoints. However, this study ectopically expressed PPAR β/δ using a mammalian expression vector and did not elucidate the drug efficacy on endogenous PPAR β/δ at the cellular level. To do so, we studied the expression of the PPAR β/δ target genes angiopoietin-like protein 4 (ANGPTL4) and *sIL-1ra* in keratinocytes and fibroblasts, respectively (Pal et al., 2011; Chong et al., 2009). In agreement with the PPAR β/δ transactivation profiles obtained using HEK293T cells, PLGA:PLLA:GW, PLGA:GW, and PLLA:GW significantly increased the mRNA expressions of *Angptl4* in keratinocytes and *sIL-1ra* in fibroblasts (Figures 2.1F-G). The expression profiles exhibited a similar pattern to the drug release kinetics and PPAR β/δ transactivation profiles of PLGA:PLLA:GW, PLGA:GW, and PLLA:GW (Figures 2.1C, E-G). Taken together, double-layered GW-loaded microparticles (PLGA:PLLA:GW) resulted in an early PPAR β/δ activation profile, but single-layered GW-loaded microparticles (PLGA:GW and PLLA:GW) showed a delayed PPAR β/δ activation.

Figure 2.1 Properties of GW encapsulated microparticles. (A) Raman mapping of PLGA:PLLA:GW. Colour intensity scale indicates relative polymer concentration. (B) SEM graphs of microparticles. (C) In vitro drug release kinetics of microparticles measured in saline. (D) Drug release kinetics of microparticles measured in HEK 293T. Drug-free microparticles did not produce any transactivation activity. Relative luciferase activities were normalized to renilla luciferase activities. (E) Drug release kinetics of microparticles measured in HEK 293T. Relative luciferase activities were normalized to Renilla luciferase activities. (F, G) *ANGPTL4* (F) and *sIL1-ra* (G) mRNA expressions at indicated time points in keratinocyte and fibroblast, respectively. Rpl27 was used as the internal reference gene. (C, E-G) Data (means $\pm$ SD) from 3 independent experiments with at least triplicates of each sample. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to PLLA:GW at corresponding time point.

3.2.2 Topical application of GW-loaded microparticles accelerates diabetic wound closure

The effects of controlled release GW-loaded microparticles as pharmacological interventions for diabetic wounds were then tested on the re-epithelialization of full-thickness excisional splinting wounds on diabetic mice. Four random groups of mice were topically treated with either PLGA:PLLA:GW, PLGA:GW, PLLA:GW, or non-encapsulated GW along with their respective vehicle controls. In agreement with the pro-healing role of PPAR β/δ in wounds, wounds that were treated with non-encapsulated GW healed faster than wounds treated with DMSO vehicle seven days post-wounding. However, the observed healing rate was transiently slower compared to wounds that were topically treated with either the single- or double-layered microencapsulated GW (Figures 2.2A-B).

Although both the single- and double-layered microencapsulated treatments accelerated wound healing in diabetic mice, they executed their effects with different efficacies. Wounds treated with PLGA:PLLA:GW closed within seven days post-

wounding (Figures 2.2C-D). This closing rate was significantly faster than that of wounds treated with PLGA:PLLA:veh, PLGA:GW, PLLA:GW, or non-encapsulated GW (Figures 2.2C-D). Histomorphometric analyses of the wound biopsies were next performed. From day 3 post-wounding, PLGA:PLLA:GW-treated wound epithelia were significantly longer and thicker than wounds treated with PLGA:PLLA:veh (Figures 2.2E-F). On the other hand, wounds treated with either PLGA:GW or PLLA:GW closed only slightly faster than PLGA:veh or PLLA:veh, respectively (Figure 2.2C). This was further supported by histomorphometric analyses showing only marginally longer and thicker neo-epithelial layers covering the area between the wound site and the margin of the regenerating tissue (Figures 2.2E-F). These observations suggest that the two different release kinetics of encapsulated GW result in different diabetic wound healing acceleration efficacies.

Figure 2.2 Effects of GW-loaded microparticles on diabetic wound healing. (A) Representative macroscopic wounds (left) and H&E (right) sections of diabetic wounds from indicated test groups (n = 5) at day 7. Ruler units are in mm. Red arrows point to

epithelial wound edge. **(B)** Wounds closure kinetics of mice treated with indicated GW and vehicle. Data points and curves in grey color are retrieved from Figure 1.5D and re-plotted here for comparison. Wound surface area are plotted as percentage of day 0 (=100%). **(C)** Representative macroscopic wounds (left) and H&E (right) sections of diabetic wounds from indicated test groups ($n \geq 5$) at day 7. Ruler units are in mm. Red arrows point to epithelial wound edge. **(D)** Wounds closure kinetics of mice treated with indicated GW- and vehicle-loaded microparticles. Wound surface area is plotted as a percentage of day 0 (=100%). **(E, F)** Wound tongue length **(E)** and epithelial area **(F)** determined as previously described (Tan and Wahli, 2013). PLGA:PLLA:GW-treated wounds have completely re-epithelialized at day 7 post-wounding. Values (means $\pm$ SD) of 10 centrally dissected sections were from at least 5 mice. *P* values are based on comparisons between PLGA:PLLA:GW treatment and cognate PLGA:PLLA:veh.

3.2.3 PPAR β/δ activation modulates oxidative stress in diabetic wounds

Early controlled release of GW by PLGA:PLLA:GW significantly increased the rate of diabetic wound closure, whereas delayed GW release by either PLGA:GW or PLLA:GW only slightly improved re-epithelialization. To elucidate the mechanism behind this difference in efficacy, we performed microarray analyses on wound biopsies from three and five days post-wounding that were either treated with PLGA:PLLA:GW or PLGA:PLLA:veh. Comparative gene expression analyses between the samples revealed that several cellular oxidative stress-related genes were consistently altered (Figure 2.3A and Table 2.1). The expression of a cluster containing immune responsive genes was also altered (Figure 2.3B and Table 2.2). Wounds from wild type mice exhibited low and decreasing ROS levels, as expected of a non-diabetic wound (Figure 2.3C). This coincided with the resolution of inflammation and the beginning of the re-epithelialization phase of normal wound healing. In contrast, the ROS levels detected in diabetic wounds remained significantly higher than that in the wild type wounds over the entire experimental duration (Figure 2.3C). Notably, diabetic wounds that were treated with

PLGA:PLLA:GW showed a significant reduction in ROS levels, reaching a level that was closer to that seen in wild type wounds (Figure 2.3C). Subsequently, we further demonstrated using the Amplex Red assay that the H_2O_2 levels in wound fluids were significantly higher in diabetic mice compared to the wild type mice on days three and five post wounding (Figure 2.3D). These results implied that GW may accelerate the closure of diabetic wounds through the reduction of H_2O_2 levels in the diabetic wound environment.

Figure 2.3 PPAR β/δ activity modulates the redox status in diabetic wound. Graphs showed the expression of (A) ROS-related genes and (B) Immune response-related genes from gene expression microarray. Numbers beside the bar indicate number of genes. The gene list and fold change are in Tables 1.3 and 1.4. (C) Relative ROS levels of wound tissues as measured by DCF. (D) H₂O₂ levels measured by Amplex Red on wound fluids from indicated time points. Data (means $\pm$ SD) from 5 independent experiments with at least triplicates of each sample.

3.2.4 H₂O₂-modified collagen delayed keratinocyte migration

Wound healing involves the ECM, and therefore a corrupted or damaged ECM would undoubtedly impair cell-matrix communication, delaying cell migration and the wound healing process. Hence, we hypothesized that excessive H₂O₂ from the diabetic wound tissue could provisionally modify the ECM of the wound tissue. To this end, we investigated the direct effect of H₂O₂-treated collagen on cell migration using a modified *in vitro* wound assay. To first determine if H₂O₂ could modify collagen, we subjected H₂O₂-treated collagen to Fourier Transform Infrared (FTIR) spectroscopy analysis. FTIR data confirmed that a number of amide positions were modified by H₂O₂ compared to vehicle-treated collagen samples (Figure 2.4A).

Next, we performed an *in vitro* keratinocyte wound assay on an H₂O₂-treated collagen coated plate. Interestingly, we observed that the wound closure rate was significantly reduced on H₂O₂ (10 μ M and 100 μ M) pre-treated collagen compared with vehicle pre-treated collagen (Figure 2.4B-C). H₂O₂ treatment (10-100 μ M) showed little effect on keratinocyte apoptosis (Figure 2.4D-E) or proliferation (Figure 2.4F-G). These observations indicate that H₂O₂-modified collagen retards *in vitro* wound healing by impeding the keratinocyte migration rate.

Figure 2.4 H₂O₂ modulates collagen to retard *in vitro* wound closure. (A) FTIR spectra of collagen with indicated treatments. Spectral differences between the two treatments were highlighted in red circles (n=4). (B) *In vitro* scratch wound closure kinetic by keratinocytes. Wound closure as percentage of time 0 (width =100% at 500 μ m). (C) Representative time-lapsed images of scratch wounded cultures of keratinocytes. Culture plates were pre-coated with type I collagen and treated with or without indicated concentrations of H₂O₂ for 3 h before being used for the wound assay. Yellow dotted lines represent the wound gap at the time of wounding. Scale bar: 100 μ m. (D-G) Effects of H₂O₂ treatment on (D-E) cell survival (AV-/PI- population) and (F-G) proliferation (indicated as percentage of various cell cycle phases as measured by BrdU assay). Data (means $\pm$ SD) were obtained from 5 independent experiments with at least triplicates of each sample. n.s., not significant.

3.2.5 PPAR β/δ activation diminished H₂O₂ secretion from diabetic fibroblasts

The extracellular redox status of a wound microenvironment is partially determined by the intracellular redox state of the surrounding cells. Hence, to investigate how GW may directly modulate this extracellular redox status, we first determined the major producer of H₂O₂ in diabetic wounds. We measured both the intra- and extracellular H₂O₂ levels of the three major cell types found in skin cells, namely fibroblasts, keratinocytes, and macrophages, all maintained in diabetic culture conditions (Chong et al., 2014). We showed that fibroblasts produced significantly higher levels of intra- and extracellular H₂O₂ compared to keratinocytes and macrophages (Figures 2.5A and B). Notably, both the intra- and extracellular H₂O₂ levels produced by fibroblasts decreased upon treatment with GW in a dose-dependent manner (Figures 2.5A and B). A similar trend was observed in macrophages. However, the starting level of H₂O₂ production was much lower (Figures 2.5A and B). In contrast, GW had no apparent effect on keratinocyte-derived H₂O₂ (Figures 2.5A and B). To verify that the effect of GW was indeed mediated via PPAR β/δ , we also treated the cells with a PPAR β/δ antagonist (GSK)

and inverse agonist (Cpd) (Shearer et al., 2008; Naruhn et al., 2011). Both GSK and Cpd significantly annulled the effect of GW on H₂O₂ production by both fibroblasts and macrophages (Figures 2.5A and B), suggesting that GW exerted its effect on H₂O₂ production through PPAR β/δ activation.

Figure 2.5 GW inhibits H₂O₂ production in fibroblasts and macrophages. (A) Intracellular H₂O₂ and (B) extracellular ROS levels 6 h after the indicated treatments as

measured by CMH2DCFDA and Amplex Red reagents, respectively. Data (mean $\pm$ SD) from 3 independent experiments with at least triplicates. n.s., not significant.

3.2.6 GW-activated PPAR β/δ stimulated catalase and GPx1 expression

To understand the mechanism that links PPAR β/δ to H₂O₂ modulation, qPCR analyses of ROS-related genes were performed on fibroblasts obtained by LCM from wound biopsies. We showed that *catalase* and *GPx1* were the primary genes responsive to GW treatment (Figure 2.6A). Both genes regulate H₂O₂ production. The expression of both *catalase* and *GPx1* was significantly increased in PLGA:PLLA:GW-treated wounds compared to the PLGA:PLLA:veh-treated wounds (Figure 2.6B). Next, we performed a transient PPAR β/δ siRNA knockdown in fibroblasts. Knockdown efficiency was validated by qPCR (Figure 2.6B) alongside the expected reduction in expression of *siL-1ra*, a target gene of PPAR β/δ in fibroblasts. We noted that when PPAR β/δ was knocked down in fibroblasts, GW-induced *catalase* and *GPx1* mRNA expressions were abolished (Figure 2.6B). In addition, the activities of both catalase and GPx1 that were upregulated by GW were nullified in PPAR β/δ -deficient fibroblasts (Figures 2.6C-D). In agreement with the abolishment of catalase and GPx1 activities in PPAR β/δ -deficient fibroblasts, the loss of PPAR β/δ nullified the H₂O₂-reduction ability of GW in fibroblasts (Figure 2.6E), further supporting the observation that GW exerts its effect via PPAR β/δ . Finally, to investigate if the expression of catalase and GPx1 were regulated directly by PPAR β/δ , we performed *in vitro* ChIP on GW-treated diabetic fibroblasts. Our ChIP data illustrated that PPAR β/δ bounded specifically to the cognate PPAR response elements (PPREs) in the different promoter regions of both the human and mouse *catalase* and *GPx1* genes in the GW-

treated but not the vehicle-treated diabetic fibroblasts (Figure 2.6F). Neither immunoprecipitation nor amplification of any PCR products was observed with pre-immune IgG (p.i.) or with a control primer targeting a sequence upstream of PPRE regions (Figure 2.6F). Our data clearly indicated that both *catalase* and *GPx1* are direct target genes of PPAR β/δ . These observations suggest that GW released in an early and controlled manner could help diabetic wound healing via the reduction of excessive diabetic wound fibroblast- and macrophage-produced H₂O₂, thereby reducing the redox modifications of ECM.

Figure 2.6 PPAR β/δ activation by GW directly stimulates catalase and GPx1 expressions and activities to diminish fibroblast H₂O₂ production. (A) Relative mRNA expression of various ROS-related genes in wound fibroblasts extracted by LCM from PLGA:PLLA:veh- or PLGA:PLLA:GW-treated wound biopsies at indicated time points. **(B)** Relative mRNA expression of various genes and **(C–E)** catalase **(C)** and GPx **(D)** activities, and **(E)** extracellular H₂O₂ levels on *in vitro* cultured fibroblast or its PPAR β/δ counterpart upon indicated treatments. Data (mean $\pm$ SD), n $\geq$ 4. **(F)** Results of ChIP assay conducted using pre-immune IgG (p.i.) or antibody against PPAR β/δ in vehicle (veh) or GW-treated fibroblasts. Specific regions (PPREs, respective sequences shown at the right panels) spanning promoter binding sites of the GPx1 and catalase genes (from both human and mouse origins) were amplified using appropriate primers (Table 1.2). A control region served as a negative control.

Table 2.1 Expression of ROS related genes.

Gene Symbol	Gene ID	Fold Change of PLLA:PLGA:GW- vs. PLLA:PLGA:veh-treated biopsies		Gene Ontology Function
		Day 3	Day 5	
Cbr2	NM_007621	2.05862	1.31167	carbonyl reductase (NADPH) activity
Cbr3	NM_173047	-1.6893	-1.18594	
Dnajc10	NM_024181	7.5363	1.72642	cell redox homeostasis
Magt1	NM_001190409	6.05322	1.73061	
Aifm1	NM_012019	6.04561	1.36081	
Txn1	NM_011660	5.86263	1.17753	
Krit1	NR_033173	5.21196	1.25766	
P4hb	NM_011032	4.81143	2.04824	
Txndc9	NM_172054	4.2407	1.22302	
2810407C02Rik	NM_001040396	3.18237	1.41236	
Dld	NM_007861	2.5581	1.24805	
Glrx	NM_053108	2.48584	1.79347	
Tmx1	NM_028339	2.46694	1.62976	
Il6	NM_031168	2.2372	2.65185	
Sh3bgrl3	ENSMUST00000030651	2.14773	1.23318	
Qsox1	NM_001024945	1.8022	1.37962	
Pdia4	NM_009787	1.72525	1.03597	
Gsr	NM_010344	1.60079	2.01883	
Tusc3	NM_030254	1.23176	1.51385	
Glrx3	NM_023140	-1.58038	-1.79953	
Txndc8	NM_026132	-1.72146	-1.30604	
Grxcr1	NM_001018019	-2.0417	-1.07777	
Cbr4	NM_145595	4.57129	1.92942	NADPH binding
Decr1	NM_026172	2.77655	1.01894	NADPH regeneration
Pgd	NM_001081274	2.22418	1.0421	NADPH-hemoprotein reductase activity
Por	NM_008898	1.73584	1.43756	peroxidase activity
Mgst1	NM_019946	5.9749	1.3022	
Gpx8	NM_027127	5.46632	1.14281	
Cat	NM_009804	3.4342	2.02084	

Alox5ap	NM_009663	2.01433	3.36222	
Pxdn	NM_181395	1.76767	1.20692	
Mgst3	NM_025569	1.51937	1.07872	
Ptgs1	NM_008969	1.50134	1.33969	
Gpx1	NM_008160	2.12751	1.42129	
Prdx4	NM_016764	2.24803	1.64112	peroxiredoxin activity
Prdx6	NM_007453	2.23773	1.01909	
Prdx5	ENSMUST00000025904	-1.04655	1.55561	
Nfe2l2	NM_010902	13.4134	2.91583	regulation of superoxide metabolic process
Cd36	NM_001159557	9.14596	1.16784	
Il18	NM_008360	4.079	1.80827	
Egfr	NM_207655	2.33444	1.29242	
Aatf	NM_019816	1.81948	1.50574	
Agtr1a	NM_177322	1.57849	1.04589	
F2rl1	NM_007974	-1.7646	-1.7244	
Adh5	NM_007410	2.37215	1.29995	response to redox state
Cybb	NM_007807	5.34756	8.18011	superoxide metabolic process
Sh3pxd2a	NM_008018	4.97967	1.72867	
Cyb5r4	NM_024195	3.93283	1.82945	
Cyb5r4	NM_024195	3.57342	2.09008	
Atp7a	NM_001109757	1.82968	1.56075	
Edn1	NM_010104	-1.15968	1.50728	
Ncf2	NM_010877	-2.89628	-1.37019	superoxide-generating NADPH oxidase activity
Prdx1	NM_011034	4.49224	2.27324	thioredoxin peroxidase activity
Sep-15	NM_053102	3.88999	1.11073	
Prdx2	NM_011563	3.55543	1.48208	
Prdx1	NM_011034	3.2337	2.45724	
Prdx2	ENSMUST00000109734	2.55969	3.38465	
Pecr	NM_023523	-1.86497	-1.16975	trans-2-enoyl-CoA reductase (NADPH) activity

Table 2.2 Expression of immune response-related genes.

Gene Symbol	Gene ID	Fold Change of PLLA:PLGA:GW- vs. PLLA:PLGA:veh- treated biopsies		Gene Ontology Function
		Day 3	Day 5	
Nedd4	NM_010890	4.30301	1.70627	adaptive immune response
Ctsh	NM_007801	3.87012	1.96373	
Lilrb3	NM_011095	3.62063	2.01852	
Adam17	NM_009615	2.42499	2.28553	
Dnajc10	NM_024181	7.5363	1.72642	cell redox homeostasis
Magt1	NM_001190409	6.05322	1.73061	
Aifm1	NM_012019	6.04561	1.36081	
Txn1	NM_011660	5.86263	1.17753	
Krit1	NR_033173	5.21196	1.25766	
P4hb	NM_011032	4.81143	2.04824	
Txndc9	NM_172054	4.2407	1.22302	
2810407C02Rik	NM_001040396	3.18237	1.41236	
Dld	NM_007861	2.5581	1.24805	
GlrX	NM_053108	2.48584	1.79347	
Tmx1	NM_028339	2.46694	1.62976	
Sh3bgrl3	ENSMUST00000030651	2.14773	1.23318	
Qsox1	NM_001024945	1.8022	1.37962	
Pdia4	NM_009787	1.72525	1.03597	
Gsr	NM_010344	1.60079	2.01883	
Tusc3	NM_030254	1.23176	1.51385	
GlrX3	NM_023140	-1.58038	-1.79953	
Txndc8	NM_026132	-1.72146	-1.30604	
Grxcr1	NM_001018019	-2.0417	-1.07777	
Cfh	NM_009888	12.574	2.01242	humoral immune response
Cfb	NM_008198	5.66608	4.87115	
Serping1	NM_009776	4.82581	3.59873	
Cfp	NM_008823	2.02194	1.74565	
Clqb	NM_009777	1.69917	1.8	
Rbpj	NM_009035	1.67255	1.81147	
ClS	NM_144938	1.64529	1.71535	
Bmi1	NM_007552	1.58656	1.81977	
Cr11	NM_013499	-1.03976	-1.60131	
C8g	NM_027062	-1.70627	1.51436	
Ighm	BC053409	-1.69679	-1.62015	immune effector
Rnasel	NM_011882	15.5147	2.11269	
Bnip3l	NM_009761	7.2296	3.31257	

Ifitm1	NM_026820	2.8797	1.52035	process
Mll5	NM_026984	2.68176	1.65866	
Apobec1	NM_031159	2.17579	1.60903	
Ifitm3	NM_025378	2.02428	3.38953	
Eif2ak2	NM_011163	1.96352	2.04967	
Ifit3	NM_010501	1.91434	2.9002	
Ptx3	NM_008987	1.61419	2.23212	
Ifnar1	NM_010508	1.56055	1.90855	
Ifna2	NM_010503	1.53718	2.27848	
Oas2	NM_145227	1.38925	1.81569	
Ifitm2	NM_030694	1.08772	1.9322	
Gm13280	NM_206867	1.0143	3.02008	
Fut7	NM_013524	-1.23835	-1.66168	immune response
Prg4	NM_021400	34.1871	1.54689	
Ly96	NM_016923	17.6354	2.1893	
Ccl11	NM_011330	13.2333	1.76715	
Clec4e	NM_019948	4.75147	4.33999	
Enpp1	NM_008813	4.6931	1.57095	
Ccl6	NM_009139	4.25482	2.0765	
Ccl12	NM_011331	3.44691	1.88498	
Ccl9	NM_011338	3.40896	2.37272	
Lcn2	NM_008491	2.17977	5.95566	
Clec4a2	NM_001170333	2.15729	3.03311	
Clec4d	NM_010819	1.97196	2.7971	
Pf4	NM_019932	1.67868	3.10926	
Cxcl3	NM_203320	1.57223	3.68233	
Clec4n	NM_020001	1.50471	4.13891	
Csf1r	NM_001037859	1.46636	2.59322	
Ly86	NM_010745	1.36699	1.51563	
Naip2	NM_010872	1.07172	1.68188	
Naip5	NM_010870	1.04724	1.64674	
Hck	NM_010407	1.03704	1.55535	
Ube2n	NM_080560	7.59293	1.45007	immune response-activating cell surface receptor signaling pathway
Cd38	NM_007646	7.38302	6.42686	
Ube2n	NM_080560	7.36737	1.45427	
C3ar1	NM_009779	4.27815	6.63313	
Nckap1l	NM_153505	4.04978	2.27033	
Ptpn22	NM_008979	2.39281	1.08358	
Mapk1	NM_011949	1.72754	1.14953	
Plcg1	NM_021280	1.59743	1.09029	
Prkcb	NM_008855	1.5968	1.00956	
Lime1	NM_023684	-1.02061	-1.53681	
Cd19	NM_009844	-1.11141	-2.0396	

Gpld1	NM_008156	-1.3384	-1.76269	immune system development
Itk	NM_010583	-2.17581	-1.26318	
Spof2	NM_177123	-2.12649	-1.23153	
Spof2	HQ856050	-2.15837	-2.94716	
Ddx3x	NM_010028	19.7938	1.0257	immune system process
Spp1	NM_001204233	16.0907	1.72213	
Bnip3	NM_009760	15.2109	1.14843	
Fyb	ENSMUST00000090461	9.13032	7.69649	
Rps24	NM_011297	8.82003	1.51365	
Hprt	NM_013556	7.88289	1.5558	
Skap2	NM_018773	7.53205	1.61556	
Cd48	ENSMUST00000068584	6.60732	2.36939	
Rock1	ENSMUST00000067947	5.41465	1.21859	
Wwpl	NM_177327	4.85088	1.4686	
Rps24	NM_011297	4.83852	1.19876	
Jak2	NM_008413	4.63477	1.04391	
Dicer1	NM_148948	4.49698	1.40007	
Rac1	ENSMUST00000080537	4.10445	2.02798	
Ctsc	NM_009982	3.8931	1.325	
Chd7	NM_001081417	3.6337	1.80133	
Cd34	NM_001111059	3.47439	2.32254	
Herc6	NM_025992	3.16515	1.29776	
Iigp1	NM_001146275	3.16247	1.773	
Tshr	NM_011648	3.04609	1.08505	
Gon4l	NM_001242372	2.91174	1.02861	
Itgam	NM_001082960	2.86228	2.28844	
Abcc9	ENSMUST00000073173	2.80884	1.45056	
Rps17	NM_009092	2.78681	1.81688	
Ifitm1	NM_001112715	2.61809	1.15712	
Cxcr2	NM_009909	2.56447	1.8194	
Cxcl16	NM_023158	2.53035	1.48077	
Psmel	NM_011189	2.51217	1.2955	
Akirin2	NM_001007589	2.38811	1.35509	
Tnfrsf11a	NM_009399	2.3389	1.26773	
Itgav	NM_008402	2.33464	1.18542	
Ddx3x	NM_010028	2.18403	1.22372	
Ifnk	NM_199157	2.07431	1.01507	
Adam9	NM_007404	1.99581	1.9453	
Wnt4	NM_009523	1.98006	1.46732	
Cnpy3	NM_028065	1.92469	1.3705	
Polr3e	NM_025298	1.90496	1.04829	
Psmc2	ENSMUST00000104958	1.85701	1.87734	

Cd151	NM_009842	1.79999	1.30455
Rps17	NM_009092	1.76317	1.13243
Ireb2	NM_022655	1.75233	1.46103
Hfe	NM_010424	1.73469	1.49071
Trim25	NM_009546	1.72142	1.07456
Lcp2	ENSMUST00000169878	1.6707	5.26129
Ncor1	ENSMUST00000101066	1.65692	1.06599
Igbp1	NM_008784	1.65611	1.01096
Ptx3	NM_008987	1.61419	2.23212
Arhgef5	NM_133674	1.59546	1.01962
Ace	NM_207624	1.56921	1.43296
H60b	NM_001177775	1.56454	1.42547
Cklf	AF401531	1.5645	1.01826
Il18rap	NM_010553	1.5511	1.25655
Ifnab	NM_008336	1.53435	1.15731
Mpzl2	NM_007962	1.19224	1.53603
Dclrelc	NM_146114	1.16941	1.53843
Zbtb1	NM_178744	1.13292	1.50278
Trem12	NM_001033405	1.02233	1.9767
Axin1	NM_009733	-1.02653	-1.7137
Impdh2	NM_011830	-1.09053	-1.68852
Igbp1b	NM_015777	-1.50393	-1.00831
Nlrc3	NM_001081280	-1.5231	-1.18217
Ccr8	NM_007720	-1.53096	-1.09702
H2-Q10	ENSMUST00000174525	-1.54158	-1.29094
Wnt1	NM_021279	-1.54375	-1.24072
Ikbkg	NM_001136067	-1.55177	-1.10539
Tac4	NM_053093	-1.59519	-1.19084
Bag6	NM_057171	-1.62639	-1.19871
Prfl	NM_011073	-1.6397	-1.01514
Ifna7	NM_008334	-1.6449	-1.26737
Klrg1	NM_016970	-1.65945	-1.2494
Nlrx1	NM_178420	-1.67603	-1.23619
Ccr3	NM_009914	-1.68244	-1.23957
Ccnb2	NM_007630	-1.69335	-1.85622
Ccl25	ENSMUST00000024004	-1.69732	-1.04214
Mir18	NR_029736	-1.72055	-1.04069
Vav1	NM_011691	-1.7341	-1.09998
Fcamr	NM_001170632	-1.79761	-1.21435
Exosc5	NM_138586	-1.80247	-1.04595
Polr3h	NM_030229	-1.87398	-1.19855
Cxcr5	ENSMUST00000062215	-1.8786	-1.06505
Pfdn1	NM_026027	-1.96082	-1.15118

Tnfsf8	NM_009403	-2.08579	-1.32161	innate immune response- activating signal transduction
Ltb	NM_008518	-2.22741	-1.1425	
Mir20a	NR_029737	-2.29769	-1.06964	
Oas1b	NR_003507	-2.30545	-1.0265	
Hist1h2ba	NM_175663	-2.45403	-1.03217	
Nlrp3	NM_145827	-2.49225	-1.40218	
Aire	NM_009646	-2.59179	-1.15252	
Ifna12	NM_177361	-2.6592	-1.28773	
Il25	NM_080729	-2.70026	-1.12031	
Cd3d	NM_013487	-2.71109	-1.51753	
Sarm1	NM_001168521	-2.98593	-1.13337	
Osm	ENSMUST00000075221	-3.42782	-1.32258	
Rpl22	NM_009079	-3.49682	-1.23495	
Rps6ka3	NM_148945	10.5962	3.36536	
Tlr13	NM_205820	5.25851	2.80314	
Lsm14a	NM_025948	2.49267	2.44574	
Tlr1	NM_030682	2.24246	1.52399	
Tnip3	NM_001001495	1.31189	2.67867	
Cd86	NM_019388	1.18013	2.26398	
Mapkapk3	NM_178907	-1.32766	-1.64164	
Anxa3	NM_013470	3.74125	2.87499	
Ccl3	NM_011337	3.27917	9.60407	
Dock2	NM_033374	1.58217	4.92041	
Myo1f	NM_053214	1.0234	1.82183	
Lgals8	NM_001199043	5.63143	1.1508	
Lcp1	NM_008879	4.3217	5.17435	
Psen1	NM_008943	2.4286	2.30243	
Gpr183	NM_183031	1.56216	1.09701	
Il18r1	NM_008365	1.44407	1.79136	
Icam1	NM_010493	1.15219	1.67931	
F2rl1	NM_007974	-1.7646	-1.7244	lymphocyte activation involved in immune response
Ifna4	NM_010504	-1.81564	-1.19279	
Apod	NM_007470	66.7312	1.88079	
Trim30a	NM_009099	22.3429	2.48677	
Vsig4	NM_177789	13.6254	1.31432	
Socs6	NM_018821	8.0847	1.91529	
Itch	NM_008395	4.95765	1.21405	
Peli1	NM_023324	3.8309	1.24203	
Ptprj	NM_008982	3.38957	3.03045	
Thbs1	NM_011580	3.37505	3.49767	
Cblb	NM_001033238	2.7621	1.46177	
Col3a1	NM_009930	2.5606	1.43784	
Nr1d1	NM_145434	1.95936	1.59486	

Trim27	NM_009054	1.57489	1.48626	
Elf1	NM_007920	1.56552	1.5362	
Psmal	NM_011965	1.51369	1.06771	
Mertk	NM_008587	1.47299	3.09424	
Id2	NM_010496	1.45911	1.66252	
Grem1	ENSMUST00000099575	-1.28343	-1.8929	
Tspan32	ENSMUST00000075172	-1.65206	-1.14263	
Dhx58	NM_030150	-1.67372	-1.22971	
Btla	NM_001037719	-1.89467	-1.39153	
Sh2d1b2	NM_001033499	-2.11738	-1.1112	
Trim38	NM_001029935	-2.11976	-1.18899	
ErbB2	NM_001003817	-2.26486	-1.20993	
Casp3	NM_009810	-3.28036	-1.0473	
Mgst1	NM_019946	5.9749	1.3022	peroxidase activity
Gpx8	NM_027127	5.46632	1.14281	
Cat	NM_009804	3.4342	2.02084	
Alox5ap	NM_009663	2.01433	3.36222	
Pxdn	NM_181395	1.76767	1.20692	
Mgst3	NM_025569	1.51937	1.07872	

3.3 Conclusion

In this chapter, we studied the benefits of employing microparticles to deliver GW, an agonist of PPAR β/δ , to diabetic wounds via topical application. We demonstrated that single-layered PLGA:GW and PLLA:GW and double-layered PLGA:PLLA:GW accelerated diabetic wound healing, albeit with different efficiencies and drug release kinetic profiles. In comparison with PLGA:GW and PLLA:GW, double-layered PLGA:PLLA:GW exhibited an earlier and sustained controlled release of GW and significantly improved wound closure. We also revealed a novel role of PPAR β/δ in providing a protective effect on the ECM against the detrimental effects of ROS, in particular H₂O₂. In addition, we revealed that *catalase* and *GPx1* were directly regulated by PPAR β/δ . Increasing the expression of catalase and Gpx1 promoted the scavenging of excessive H₂O₂ found in diabetic wounds, reduced H₂O₂-induced modifications of the ECM and restored keratinocyte migration. These events helped to improve diabetic wound healing. Our findings highlight the importance of studying controlled drug release kinetics to improve pharmaceutical benefits and warrant more resources to enhance drug delivery systems to unleash the full potential of controlled drug release.

Chapter 4: Results

FIBROBLAST PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR β/δ IN SKIN HOMEOSTASIS, INFLAMMATION AND WOUNDING

4.1 Introduction

The skin is the largest organ in the human body. The epidermis of the skin provides a protective shield against the external environment and regulates water and nutrient absorption. On the other hand, the underlying dermis provides an architectural and functional support for the epidermis and the entire skin tissue. Largely made up of fibroblasts, the dermis is a huge source of mitogenic and chemotactic factors, which regulates tissue phenotype and functions. Working in synergy, the epidermis and dermis achieve skin homeostasis and regulate responses towards external stimuli and injury. The role of epidermal PPAR β/δ in skin homeostasis has been widely studied, particularly in inflammation and wound healing. However, little is known about the role of fibroblasts PPAR β/δ in regulating the cellular behavior and phenotype of the neighboring epidermis. Using the Cre-loxP technology, we generated a novel mouse model with *Pparb/d* deleted specifically in the fibroblasts. Herein, we revealed that the skin of these mice had enhanced inflammatory processes and increased wound contraction. Through chromatin immunoprecipitation, we found that *Lrg1* is a novel direct target of PPAR β/δ in the fibroblasts. Subsequently, this animal model can function as an appropriate model to study various pathologies, such as cancer.

4.2 Results

4.2.1 Mouse deleted of PPAR β/δ gene in fibroblasts is viable

To understand the role of fibroblast PPAR β/δ in epithelial-mesenchymal interaction, we generated a mouse line that had *Pparb/d* ablated specifically in the fibroblasts. We bred mouse that harbored a floxed *Pparb/d* (*Pparb/d^{f/f}*) (Iglesias et al., 2012) with transgenic mouse expressing the *Cre* transgene under the control of fibroblast-specific protein (FSPCre) (Trimboli et al., 2008). In order to verify the deletion of the floxed allele, PCR amplification was performed on the genomic DNA isolated from the deepithelized dermis. The excision of *Pparb/d^{f/f}* was identified by the amplification of the 600-bp DNA fragment in FSPCre-*Pparb/d^{f/f}* (lanes 3 and 4, Figure 3.1A) and FSPCre-*Pparb/d^{f/+}* mice (lanes 5 and 6, Figure 3.1A). The intact floxed *Pparb/d^{f/f}* gene was amplified as a 2.2kb band that appeared in the absence of the *Cre* transgene (lanes 1 and 2; Figure 3.1A) or in the presence of the wild-type allele (+) (lanes 5-8, Figure 3.1A). The mRNA and protein expression levels of PPAR β/δ in FSPCre-*Pparb/d^{f/f}* mice were undetectable in fibroblasts compared with *Pparb/d^{f/f}* mice, indicating an efficient deletion of the floxed allele (Figures 3.1B-C). The expression of the other two PPAR subtypes, *Ppara* and *Pparg* in fibroblasts were not altered by the genetic ablation of fibroblast *Pparb/d* (Figure 3.1B). The numbers of female and male progenies did not differ significantly in each litter (Figure 3.1D). Our observations on hair outgrowth between genotypes also showed that there were no differences in the first appearance of hair between the FSPCre-*Pparb/d^{f/f}* and *Pparb/d^{f/f}* mice (Figure 3.1E). The male FSPCre-*Pparb/d^{f/f}* mice were significantly smaller and lighter compared with its wild-type *Pparb/d^{f/f}* counterparts. This size difference was not apparent in female FSPCre-

Pparb/d^{fl/fl} mice (Figure 3.1F). These observations indicated that FSPCre-*Pparb/d^{fl/fl}* mouse is viable and that PPAR β/δ deletion in fibroblasts is dispensable for embryonic development.

Figure 3.1. *Pparb/d* ablation in fibroblast. (A) PCR analysis of mouse fibroblast DNA. The 2.2-kb band represents the non-deleted floxed allele (lanes 1 and 2) and wildtype allele (lanes 7 and 8). The 600-bp band represents the *Pparb/d*-deleted allele (lanes 3 and 4), confirming the excision of *Pparb/d* in FSPC*Cre-Pparb/d*^{f/f} mice. Heterozygous FSPC*Cre-Pparb/d*^{f/+} mice express both the wildtype and excised alleles (lanes 5 and 6). n.c., negative control (lane 9); 1kb DNA ladder (lane 10). (B) Relative mRNA expression of the three isoforms of PPAR (α , β/δ , γ) in the mouse fibroblast ($n = 3$). * $P < 0.01$; n.s., not significant. (C) Protein expression of PPAR β/δ in FSPC*Cre-Pparb/d*^{f/f} and *Pparb/d*^{f/f} mice fibroblasts. (D) Average number of male versus female pups in each litter. n.s., not significant. (E) No significant difference in first sighting of hair sprouting between FSPC*Cre-Pparb/d*^{f/f} and *Pparb/d*^{f/f} mice. (F) Male mutant mice were smaller in terms of body weight and length, as compared to their wildtype littermate during the weaning off period. This difference was not observed in female mice. * $P < 0.05$.

4.2.2 PPAR β/δ -deleted fibroblasts alter skin development pathways and epidermal inflammatory lipid metabolism

The FSPC*Cre-Pparb/d*^{f/f} mouse may exhibit other post-natal alterations, particularly skin development and functions. To determine what genes are affected by the ablation of PPAR β/δ in fibroblasts, we performed gene expression microarray analyses of epidermis and dermis isolated from FSPC*Cre-Pparb/d*^{f/f} and *Pparb/d*^{f/f} mice. Gene ontology (GO) analyses revealed 80 unique molecules that were differentially expressed in the FSPC*Cre-Pparb/d*^{f/f} epidermis, enriching the top 3 ontologies of lipid metabolism, molecular transport, and small molecule biochemistry (Figures 3.2A-B; Larger version of Figure 3.2B in Appendix A). Out of these, 68 molecules (37 upregulated, 31 downregulated) were lipid metabolism-related, and 60% (41 molecules) of this subset had overlapping functions in inflammatory responses. Further interrogation of these 41 molecules revealed the TGF β signaling pathway as a major signalling conduit, with known interactions with 61% (25 molecules) of the inflammation-related genes. This

observation indicated that the ablation of PPAR β/δ in fibroblasts primed the adjacent epidermis for an altered inflammatory effect via an altered TGF β signaling mechanism (Figure 3.2A).

Microarray analyses of the dermis highlighted a total of 78 unique molecules (49 upregulated, 29 downregulated) with altered expressions and known involvement in the gene ontologies of connective tissue development, tissue development, cellular growth and proliferation, immune cell trafficking and inflammatory response (Figures 3.2C-D; Larger version of Figure 3.2D in Appendix B). Similar to the epidermis, the deletion of *Pparb/d* in the fibroblasts resulted in altered TGF β signaling that directly or indirectly influenced the expression of 37% of the 78 identified molecules. In conjunction with the observation that the epidermis of FSPCre-*Pparb/d*^{f/f} mice was primed for inflammatory processes, the dermis of these mice also had 63% of the altered molecules associated with immune cell trafficking and inflammatory responses. This observation suggested that upon external stimuli or challenge, the skin of FSPCre-*Pparb/d*^{f/f} mice might be a hotbed for facilitated immune cell entry and an exacerbated local oxidative stress response with immune cell activation. In addition, 45% of the molecules that had altered expressions were associated with connective tissue development (Figure 3.2C). The predominant cell type in the dermis is the fibroblasts. It is well-established that fibroblasts are the main producers of ECM molecules, such as collagen. Thus, the deletion of *Pparb/d* in fibroblasts may have affected the skin architecture in these mutant mice, affecting biological processes, such as dermal wound healing.

B
(Please refer to Appendix A for larger picture)

D
(Please refer to Appendix B for larger picture)

Figure 3.2. Microarray analysis of FSPCre-*Pparb/d^{fl/fl}* epidermis and dermis. (A) Ingenuity Pathway Analysis (IPA) of genes differentially expressed by >1.5 fold in the FSPCre-*Pparb/d^{fl/fl}* epidermis, relative to that of the wildtype littermates. The top 5 gene ontologies (GO) ($P < 0.0341$) and their constituent number of molecules were identified. A lower P -value indicates a stronger association with the indicated GO. 80 unique molecules were further stratified into lipid- (turquoise), inflammation- (orange), and TGF β - (magenta) related sub-ontologies. Percentages indicate the proportion that each sub-ontology occupies with respect to the ontology above it. Bar chart represents the number of genes that are up- (red) or down- (green) regulated by >1.5 fold in the FSPCre-*Pparb/d^{fl/fl}* epidermis, as compared to that of the wildtype littermates. (B) Gene ontology (GO) of genes that were altered in the epidermis of FSPCre-*Pparb/d^{fl/fl}* mice. Genes that were altered were highly associated with lipid metabolism (80 genes). Out of these 80 genes approximately 60% (41 genes) were associated with inflammatory responses. One of the most upregulated genes was arachidonate 5-lipoxygenase activating protein (*Alox5ap*; circled in red), also known as 5-lipoxygenase activating protein (*Flap*), with an upregulation of approximately 4.6-folds. (C) IPA of genes differentially expressed by >1.5 fold in the FSPCre-*Pparb/d^{fl/fl}* dermis, relative to that of the wildtype littermates. The top 5 GO ($P < 0.0197$) and their constituent number of molecules were identified. A lower P -value indicates a stronger association with the indicated GO. 78 unique molecules were further stratified into connective tissue- (blue), migration/proliferation- (green), and TGF β - (magenta) related sub-ontologies. Percentages indicate the proportion that each sub-ontology occupies, with respect to the total number of unique molecules identified. Bar chart represents the number of genes that are up- (red) or down- (green) regulated by >1.5 fold in the FSPCre-*Pparb/d^{fl/fl}* dermis, as compared to that of the wildtype littermates. (D) GO of genes that were altered in the dermis of FSPCre-*Pparb/d^{fl/fl}* mice. Altered genes were highly associated with cellular function and maintenance, which could alter the behaviour and function of fibroblasts in the dermis. Leucine-rich α -2-glycoprotein 1 (*Lrg1*) was found to be one of the most downregulated genes detected upon deletion of *Pparb/d* in the fibroblasts. This downregulation was found to be approximately 4-folds.

4.2.3 Enhanced epidermal hyperplasia and inflammation in FSPCre-*Pparb/d^{fl/fl}* mice.

Microarray analysis revealed that the expression of a high number of molecules associated with cell death and survival was altered (Figure 3.2A). It was previously shown that fibroblasts with reduced PPAR β/δ promoted the proliferation of epidermal cells via

the modulation of IL-1 signaling (Chong et al., 2009). Thus, the deletion of PPAR β/δ will affect epithelial-mesenchymal interaction, leading to an altered morphogenesis of the organ. To this end, we characterized the skin of FSPCre-*Pparb/d^{fl/fl}* mice. We performed H&E analyses of skin tissues from both the FSPCre-*Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice. The FSPCre-*Pparb/d^{fl/fl}* mice exhibited a thicker epidermis, dermis, and hypodermis throughout weeks 1 to 8 of age (Figure 3.3A). Expectedly, the epidermal thickness decreased and, in contrast, the thickness of the dermis increased as the *Pparb/d^{fl/fl}* mice aged. Importantly, the FSPCre-*Pparb/d^{fl/fl}* mice consistently showed thicker epidermis and dermal layer compared with the wildtype mice over the study period (Figure 3.3B). To find out if modifications in the proliferation and differentiation of the skin could account for the thicker epidermis, we performed Western blot analyses for markers against cytokeratin 6 (CK6) and PCNA for proliferation, and CK10 and transglutaminase type 1 (TGase 1) for differentiation of skin samples from one-week old mice (Figure 3.3C). Immunohistochemistry analyses against PCNA (Figure 3.3D) and TGase1 (Figure 3.3E) supported the Western blot data. The FSPCre-*Pparb/d^{fl/fl}* mice had an elevated expression of proliferative and differentiation markers, suggesting that the increase in keratinocytes proceeded to differentiate, resulting in the observed thicker epidermis compared with their wildtype littermates. We did not detect any difference in the penetration of Lucifer Yellow into the epidermis of the FSPCre-*Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice (Figure 3.3F), suggesting that lipid and barrier function of the skin was not impaired.

Gene ontology analysis also suggested a dysregulated epidermal inflammatory response. To this end, we examined epidermal response to phorbol ester-induced inflammation. FSPCre-*Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice at week 4 of age were treated with

a single topical application of 6.5 nmol of TPA for 24 hours. Expectedly, TPA application on *Pparb/d^{fl/fl}* mice resulted in hyperplasia of the epidermis (Figures 3.4A-B). An enhanced epidermal hyperplasia was observed in *FSPCre-Pparb/d^{fl/fl}* when compared with *Pparb/d^{fl/fl}* mice (Figures 3.4A-B). The *FSPCre-Pparb/d^{fl/fl}* mice also exhibited more PCNA-positive proliferating keratinocytes than *Pparb/d^{fl/fl}* mice (Figures 3.4C-D). Consistently, the *FSPCre-Pparb/d^{fl/fl}* mice also exhibited intraepidermal microabscesses (Figure 3.4E). Immunoblot analysis of the skin tissues indicated a greater infiltration of Ly-6C/Ly-6G-positive neutrophils in *FSPCre-Pparb/d^{fl/fl}* when compared with *Pparb/d^{fl/fl}* mice (Figure 3.4F). This is further shown by immunohistochemical staining for Ly-6C/Ly-6G, confirming the infiltration of the neutrophils from the dermis into the epidermis (Figure 3.4G). Thus, PPAR β/δ ablation in the fibroblasts led to an aggravated response from adjacent epidermis to TPA.

Figure 3.3. Characterization of epidermis and dermis. (A) Morphological histology studies of the mice skin, illustrating the thicker epidermis and dermis in FSPCre-Pparb/d^{fl/fl} mice, as compared to Pparb/d^{fl/fl} mice at weeks 1, 4, and 8. Scale bar: 50 μm. (B) Quantitative measurements of the epidermis, dermis, hypodermis, and skeletal muscle

between both genotypes at weeks 1, 4, and 8 consistently showed thicker components in FSPCre-*Pparb*/*d^{fl/fl}* mice throughout skin development. * $P < 0.05$; ** $P < 0.01$; *** $P < 10^{-3}$. **(C)** Western blot analyses of epidermal markers for proliferation (CK6 and PCNA) and differentiation (CK10 and TGase1) illustrated that *Pparb*/*d* ablation in fibroblast led to enhanced proliferation and differentiation during skin development. **(D)** Representative colorimetric staining images of PCNA in skin tissues of the mice. Scale bar: 50 μm . **(E)** Representative colorimetric staining images of TGase1 in skin tissues of the mice. Scale bar: 50 μm . **(F)** Skin permeability was not altered by *Pparb*/*d* ablation in the fibroblast, as shown by the absence of Lucifer yellow leakages through the epidermis of both genotypes. Scale bar: 50 μm .

Figure 3.4. Characterization of epidermis and dermis. (A-B) Histological and quantitative analyses of epidermal thickening on mice dorsal skin treated with either vehicle (Veh) or 6.5 nmol of 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) for 24 hours. $*P < 0.001$; $**P < 10^{-7}$; $***P < 10^{-15}$. (C-D) Histological and quantitative analyses of PCNA-positive epidermal cells on mice dorsal skin treated with 6.5 nmol of TPA for 24 hours. $*P < 10^{-2}$; $**P < 10^{-7}$; $***P < 10^{-10}$. (E) Intraepidermal microabscesses were observed in FSPC*Cre-Pparb/d^{fl/fl}* mice upon TPA treatment. Scale bar: 200 μ m. (F) Western blot probing for neutrophil marker, Ly-6C/Ly-6G, in TPA-treated skin, indicated a more elevated neutrophil infiltration in the skin of FSPC*Cre-Pparb/d^{fl/fl}* mice, as compared to *Pparb/d^{fl/fl}* mice. (G) Colorimetric staining against neutrophil antigen, Ly-6C/Ly-6G, in FSPC*Cre-Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice. Scale bar: 50 μ m.

4.2.4 FSPC*Cre-Pparb/d^{fl/fl}* mice displayed dermal thickening

Dermal and hypodermal thickening were the most obvious phenotypic differences between FSPC*Cre-Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}*. Van Gieson's staining indicated that there was more collagen deposition in the dermis of the FSPC*Cre-Pparb/d^{fl/fl}* mice (Figure 3.5A). This was further supported by the hydroxyproline assay and Masson's trichrome staining performed on the biopsy punches and skin sections, respectively (Figures 3.5B-C). The hydroxyproline assay indicated a ~2-fold increase in collagen in the dermal region of the FSPC*Cre-Pparb/d^{fl/fl}* compared with *Pparb/d^{fl/fl}* (Figure 3.5B). Scanning electron microscopy analyses of the dermis region revealed larger mean cross-sectional area, but fewer number of the collagen fibrils in FSPC*Cre-Pparb/d^{fl/fl}* mice (Figures 3.5D-E). To investigate whether the increase in collagen production was due to an increase in number of fibroblasts in the dermis, immunofluorescence staining against vimentin was performed. Similar number of vimentin-positive fibroblasts was detected between wildtype and mutant mice (Figure 3.5F), that was further confirmed by our immunoblot data (Figure 3.5G). Immunofluorescence staining against α -smooth muscle actin (α -SMA) indicated an

increase in number of myofibroblasts in the dermis of FSPCre-*Pparb/d^{fl/fl}* (Figure 3.5F). Immunoblot analysis also showed a higher expression of α -SMA protein in FSPCre-*Pparb/d^{fl/fl}* mice compared with *Pparb/d^{fl/fl}* (Figure 3.5G). These observations suggested that PPAR β/δ deficiency in fibroblasts promotes the activation of fibroblasts to myofibroblasts, leading to an increase in secretion of collagen within the dermis.

To determine if the higher number of myofibroblasts and collagen content in the FSPCre-*Pparb/d^{fl/fl}* mice may play a role in dermal wound healing, we performed full-thickness excisional wounding on 8-weeks old mice from both genotypes. The surface areas of the wounds were measured for a period of 18 days and plotted as a percentage of the original wound surface area. Our data showed that the FSPCre-*Pparb/d^{fl/fl}* mice exhibited a more rapid wound closure than the *Pparb/d^{fl/fl}* mice (Figures 3.6A-B). Wound biopsies collected at days 0, 3, 5, 7, and 10 post-wounding were sectioned, stained with H&E, and wound contraction was measured (Tan and Wahli, 2013). Our analyses revealed that FSPCre-*Pparb/d^{fl/fl}* mice had a significantly higher rate of wound contraction than their wildtype littermate (Figures 3.6C-D). The higher number of myofibroblasts, increased collagen deposition with thicker collagen fibres contributed to the rapid wound contraction in FSPCre-*Pparb/d^{fl/fl}* mice. It is therefore crucial to understand the underlying mechanisms that contributed to these observations caused by PPAR β/δ ablation in the fibroblasts.

Figure 3.5. Collagen deposition differences in the dermis. (A) Van Gieson's staining against collagen in the dermis of mice skin showed that the dermis of FSPCre-*Pparb*/ $d^{fl/fl}$ mice had a more intense red staining, indicating higher collagen deposition, consistently throughout the period of study. Scale bar: 50 μ m. (B) Hydroxyproline content was higher in the dermis of the FSPCre-*Pparb*/ $d^{fl/fl}$ mice than in the *Pparb*/ $d^{fl/fl}$ mice. * $P < 0.01$. (C) Masson's trichrome staining against collagen in the dermis of mice skin confirmed higher collagen deposition in the dermis of FSPCre-*Pparb*/ $d^{fl/fl}$ mice. Scale bar: 50 μ m. (D) There were fewer collagen fibrils in the dermis of the FSPCre-*Pparb*/ $d^{fl/fl}$ mice, but they had larger mean cross-sectional areas, as compared to those of the *Pparb*/ $d^{fl/fl}$ mice. * $P < 0.05$; ** $P < 10^{-10}$. (E) Scanning electron microscopy images of skin tissues of the mice showed obvious number and cross-sectional area differences in the mice dermis. FSPCre-*Pparb*/ $d^{fl/fl}$ mice had fewer collagen fibrils, but each had a larger cross-sectional area than in the *Pparb*/ $d^{fl/fl}$ mice. Scale bars: 0.2 μ m (inset) and 1 μ m. (F) Representative immunofluorescence staining images against vimentin and α -SMA, illustrating similar number of fibroblasts (vimentin) in both genotypes, but an increase in fibroblasts activation (α -SMA) in FSPCre-*Pparb*/ $d^{fl/fl}$ mice. Scale bar: 50 μ m. (G) Protein analyses of vimentin and α -SMA in skin tissues of FSPCre-*Pparb*/ $d^{fl/fl}$ and *Pparb*/ $d^{fl/fl}$ mice.

Figure 3.6. Increased rate of wound contraction in FSPCre-Pparb/d^{fl/fl} mice. (A) Mice were subjected to full-thickness excisional wounding on their dorsal back, and were allowed to heal over a period of 18 days. Wound surface area was measured at indicated time points. Wound closure occurred faster in FSPCre-Pparb/d^{fl/fl} mice, as compared to Pparb/d^{fl/fl} mice. **P* < 0.05; ***P* < 0.01 (Mann-Whitney U test; n=7). (B) Wound closure was observed on mice subjected to full-thickness excisional wounding for a period of 18

days. The wounds on FSPCre-*Pparb/d^{fl/fl}* mice were observed to close at a faster rate, as compared to those on *Pparb/d^{fl/fl}* mice. **(C)** Rate of wound contraction was measured by the distance between the first hair follicles on the wound edges. A 5-mm full-thickness excisional wound was created on dorsal backs of mice. Wound biopsies were harvested at indicated days post-wounding and stained with H&E. The faster wound closure observed in FSPCre-*Pparb/d^{fl/fl}* mice occurred due to an enhanced contraction of the wound. $**P < 0.01$; $***P < 0.001$ (Mann-Whitney U test; $n=10$). **(D)** Representative images of the wound contraction over a period of 10 days in a 5-mm full-thickness excisional wound. Wound contraction is measured using the distance between the first hair follicles at the wound edges (red arrows). Scale bar: 500 μm .

4.2.5 FSPCre-*Pparb/d^{fl/fl}* mice exhibited a morphea-like skin phenotype

FSP1 is a key marker of a specific subset of macrophages in the liver during fibrosis and injury (Österreicher et al., 2011). The FSPCre-*Pparb/d^{fl/fl}* mice did not exhibit hepatomegaly or increased collagen deposition in the liver or gastrointestinal tract (Figures 3.7A-B). However, FSPCre-*Pparb/d^{fl/fl}* mice exhibited many pathological hallmarks of morphea, a localized form of scleroderma, including excessive collagen deposition leading to dermal thickening, increased myofibroblasts quantity, and dysregulated TGF β signaling. Previous studies concluded that localized scleroderma involves autoimmune abnormalities with elevated levels of anti-nucleosome antibodies (Sato et al., 2004; Takehara and Sato, 2005). We detected higher levels of anti-nucleosome antibodies in 69% of our FSPCre-*Pparb/d^{fl/fl}* mice (7 males, 22 females in total) (Figure 3.7C).

To further strengthen our findings, we compared microarray gene expression between skin biopsies from scleroderma patients and FSPCre-*Pparb/d^{fl/fl}* epidermis and dermis. Out of 372 genes, 74% from the FSPCre-*Pparb/d^{fl/fl}* mice had a similar profile as scleroderma patients. Of these genes, 32% were identically regulated among scleroderma

and both the dermis and epidermis from FSPCre-*Pparb*/*d^{fl/fl}* mice. GO analyses highlighted that these genes were closely associated with cellular behavior and function, indicating that the altered cellular functionalities were associated with the morphea-like features of the FSPCre-*Pparb*/*d^{fl/fl}* mice (Figure 3.7D). The remaining 68% of genes were regulated similarly in scleroderma patients and the dermis or epidermis (Figure 3.7E).

Although the best treatment for morphea is uncertain and current treatments often fail to help, benefits have been reported following topical imiquimod treatment. Imiquimod cream 5% (Aldara[®]), which induces interferon and subsequently inhibits TGF β , was previously utilized to treat patients with morphea (Man and Dytoc, 2004). FSPCre-*Pparb*/*d^{fl/fl}* mice topically treated with imiquimod exhibited a thinning of the thickened dermis (Figure 3.7F); the resultant dermal thickness was no longer significantly different from their wildtype littermates (Figure 3.7G).

Figure 3.7. Skin of FSPCre-*Pparb*/*d^{fl/fl}* mice exhibited a morphea-like phenotype. (A) There was no observed enlargement in the size of the FSPCre-*Pparb*/*d^{fl/fl}* mice liver, compared to that of *Pparb*/*d^{fl/fl}* mice. **(B)** Protein analyses of mature collagen type I in the gastrointestinal tract and liver showed the absence of increased collagen deposition in these organs. **(C)** Of the FSPCre-*Pparb*/*d^{fl/fl}* mice studied (7 males, 22 females), 69% were

positive for anti-nucleosome antibodies in their plasma. **(D)** Microarray expression in skin tissue from scleroderma patients compared to FSPCre-*Pparb/d^{fl/fl}* dermis and epidermis. Gene ontology for commonly regulated genes revealed a close association with cellular behavior and functional changes. **(E)** Out of a total of 372 genes, 74% (275 genes) from FSPCre-*Pparb/d^{fl/fl}* mice had a similar profile as scleroderma patients. Of these, 32% were identically regulated amongst FSPCre-*Pparb/d^{fl/fl}* epidermis, dermis and patients. The remaining 68% had either the FSPCre-*Pparb/d^{fl/fl}* epidermis or dermis regulated identically to patients. **(F)** H&E staining of mouse skin treated with imiquimod cream 4% daily for 4 days compared to vehicle-treated (Veh) mice showed that imiquimod application reverses the dermal thickening effect seen in FSPCre-*Pparb/d^{fl/fl}* mice. Scale bar = 50 μ m. **(G)** Measurement of the dermis revealed a thinning effect upon imiquimod treatment, and the difference in dermal thickness between FSPCre-*Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice was no longer significant.

4.2.6 Leucine-rich alpha-2-glycoprotein 1 (*Lrg1*) is a direct target gene of PPAR β/δ

Guided by our above observations and interrogation of our gene expression microarray data, we focused our investigation to the transforming growth factor- β (TGF β) signaling. TGF β plays central role in skin architectural formation and functions, including collagen synthesis and inflammation (Narayanan et al., 1989; Sanjabi et al., 2009). Out of the population of genes affected, we identified leucine-rich alpha-2-glycoprotein 1 (*Lrg1*) as one of the most downregulated gene with a 4-fold decrease in expression (Figure 3.2D). LRG1 is a relatively novel protein and had been implicated in the modulation of endothelial TGF β signaling to promote angiogenesis (Wang et al., 2013), as well as, in the suppression of metastatic events of hepatocellular carcinoma cells (Zhang et al., 2015).

We first performed Western blot in the dermis of FSPCre-*Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice. We revealed that dermal LRG1 expression was diminished in the skin section of the FSPCre-*Pparb/d^{fl/fl}* mice compared to the wildtype dermis (Figure 3.8A). Next we quantified the levels of mRNA and protein of LRG1 in human fibroblasts treated

with PPAR β/δ agonist, GW501516 (GW) in the absence or presence of either PPAR β/δ antagonist GSK0660 (GSK) or PPAR β/δ inverse agonist compound 10h (10h). The mRNA expression of *Lrg1* was upregulated by GW-activated PPAR β/δ as determined by qPCR. Importantly, this upregulation was attenuated in the presence of GSK or 10h (Figure 3.8B). The protein expression of LRG1 was also upregulated in the presence of GW, but not in the presence of GSK or cpd10 (Figure 3.8C). To further strengthen our finding, we performed chromatin immunoprecipitation (ChIP) on mouse- and human fibroblasts that were treated with either vehicle or 300 nM GW. *In silico* analysis of the intragenic regulatory region of mouse and human *Lrg1* genes revealed one and two PPAR response elements (PPREs), respectively (Figure 3.8D). The two human PPREs were found to be separated from each other by only 150 bp and these PPREs were found to resemble the consensus PPRE motif. Our results showed that in the presence of GW, PPAR β/δ was recruited to the PPRE of the *Lrg1* gene (Figure 3.8E). We identified *Lrg1* as a direct target gene of PPAR β/δ in fibroblasts, and a possible player in contributing to the phenotype of the FSPC*re-Pparb/d^{f/f}* mice.

Figure 3.8. *Lrg1* is a novel direct target of PPAR β/δ . (A) Representative immunoblot for LRG1 in skin dermis and epidermis of FSPCre-*Pparb/d^{fl/fl}* and *Pparb/d^{fl/fl}* mice. (B) Relative expression of mRNA levels in human fibroblasts treated with GW, and in combination with GSK0660 (GSK), a PPAR β/δ antagonist, and compound 10h (10h), a PPAR β/δ inverse agonist. **P* < 0.05; ***P* < 0.01. (C) Western blot analyses of LRG1 in human fibroblasts treated with GW, and in combination with GSK and 10h. (D) Identification of the PPRE sequences in mouse and human *Lrg1* genes (red boxes), and their respective NUBIScan scores. (E) Chromatin immunoprecipitation (ChIP) of *Lrg1* in mouse and human fibroblasts by PPAR β/δ in the presence of GW501516 (GW), a specific PPAR β/δ agonist.

4.2.7 Fibroblast PPAR β/δ regulates LRG1 to modulate homotypic and heterotypic TGF β signaling

Next, we set out to investigate how LRG1 affects the TGF β -SMAD3 pathway in fibroblasts for collagen production. We transiently knockdown *Lrg1* in fibroblasts using

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siRNA (Figures 3.9A-B). The knockdown of *Lrg1* led to an increase in the mRNA expressions of *Tgfb2*, *Smad3*, and *Colla2*, indicating that the reduction of LRG1 might activate fibroblasts to produce collagen through the TGF β -SMAD3 pathway (Figure 3.9C). Conversely, the treatment of *Lrg1*-deficient fibroblasts with recombinant LRG1 reduced the mRNA expressions of *Tgfb2* and *Smad3* (Figure 3.9C). The mRNA expression of *Colla2* was only significantly reduced at a dose of 100 ng/mL LRG1, indicating that the mRNA of *Colla2* maintained a certain level of stability upon production (Figure 3.9C). In addition, protein lysates from the dermis of FSPCre-*Pparb*/ $\delta^{\Delta/\Delta}$ and *Pparb*/ $\delta^{\Delta/\Delta}$ mice were probed for total TGF β RII and SMAD3, as well as, phospho-TGF β RII (p-TGF β RII) and phospho-SMAD3 (p-SMAD3). Our Western blot results showed that activation of TGF β RII, and subsequently, SMAD3, via phosphorylation were enhanced in the dermis of FSPCre-*Pparb*/ $\delta^{\Delta/\Delta}$ mice compared to the *Pparb*/ $\delta^{\Delta/\Delta}$ mice (Figure 3.9D). Furthermore, *in vitro* knockdown of *Lrg1* in fibroblasts led to an increase in the production of mature collagen type I (Figure 3.9D). However, this increase is not abrogated in the presence of recombinant LRG1, in agreement with the corresponding mRNA expression data (Figure 3.9C). These observations confirmed a role of fibroblast PPAR β/δ in homotypic regulation of TGF β -SMAD3 signaling in collagen production, and thus wound contraction, via the regulation of LRG1 production.

Our microarray analyses revealed that the epidermis of FSPCre-*Pparb*/ $\delta^{\Delta/\Delta}$ mice had upregulated expressions of genes related to lipid mediators of inflammation. We identified arachidonate 5-lipoxygenase-activating protein (*Alox5ap*), also known as 5-lipoxygenase activating protein (*Flap*), as one of the most upregulated molecule with a 4.6-fold increase in expression (Figure 3.2B). FLAP has a critical role in the mediation of

inflammatory responses towards external stimuli (Byrum et al., 1997). To confirm this observation, we analyzed the protein expression of FLAP in human keratinocytes treated with increasing doses of recombinant LRG1. In agreement with the microarray data, we observed a dose-dependent decrease in FLAP protein expression in LRG1-treated keratinocytes (Figure 3.9E). As a positive control, a TGF β luciferase reporter assay showed a dose-dependent increase in reporter activity when co-treated with LRG1, indicating that LRG1 exerted its effect on keratinocytes through the TGF β signaling pathway (Figure 3.9F). In addition, protein analyses of FLAP in the epidermis of FSPCre-*Pparb/d^{fl/fl}* mice skin showed an increase in FLAP expression, as compared with the wildtype littermates (Figure 3.9G). These results indicated that the expression of FLAP in the epidermis was regulated, in part, by paracrine production of LRG1 by the fibroblasts. The upregulation of *Flap* in the epidermis of FSPCre-*Pparb/d^{fl/fl}* mice contributed to the increase susceptibility of the epidermis to inflammatory insults. Taken together, our observations suggest that PPAR β/δ in the fibroblasts transcriptionally regulates LRG1, which acted in an autocrine and paracrine manner to augment cell-specific TGF β effect.

Figure 3.9. LRG1 mediates collagen production via TGFβ/SMAD3 signaling. (A) Knockdown efficiency of *Lrg1* in human fibroblasts using siRNA **P* < 10⁻⁷. (B) Protein analysis of LRG1 in human fibroblasts treated with either siRNA containing a scrambled sequence or siRNA targeting *Lrg1*. (C) Relative expressions of *Tgfr2*, *Smad3*, and *Col1a2* mRNAs in untreated human fibroblasts, *Lrg1*-knocked down human fibroblasts, and *Lrg1*-knocked down human fibroblasts treated with an increasing dose of recombinant LRG1. **P* < 0.05; ***P* < 0.01; ****P* < 10⁻⁴. (D) Knockdown of *Lrg1* (siLrg1) in fibroblasts resulted in the phosphorylation of TGFβRII and SMAD3, and the increased

production of collagen. This activation was reversed in a dose-dependent manner with exogenous recombinant LRG1 protein. **(E)** Western analyses of FLAP protein in Ker keratinocytes. LRG1 was able to downregulate FLAP expression in keratinocytes in a dose-dependent manner. **(F)** TGF β activity was measured in Ker keratinocytes in the presence of exogenous recombinant LRG1 protein. LRG1 increased the activity of TGF β in a dose-dependent manner, indicating that LRG1 could modulate the TGF β signaling pathway in keratinocytes. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **(G)** FSPCre-*Pparb*/ $d^{fl/fl}$ mice expressed more FLAP protein than its wildtype littermates.

Appendix A

Appendix B

4.3 Conclusion

In this chapter, we further elucidated the *in vivo* roles of fibroblast PPAR β/δ in skin architecture development as well as in mediating the skin response to injury or external chemical stimuli. We used a novel animal model in which *Pparb/d* is specifically deleted in fibroblasts. We demonstrated that these mutant mice exhibited thickening of the epidermis with increased proliferation and differentiation of the stratified layers and dermal thickening with a concomitant increase in collagen production. Due to the increased deposition of ECM products such as collagen, wounds closed faster in the mutant mice due to a faster rate of wound contraction. We also revealed that the deletion of fibroblast PPAR β/δ also rendered the skin tissue more susceptible to pro-inflammatory responses upon contact with external stimuli such as TPA, a known mouse skin irritant. These observations pointed to a scleroderma-like phenotype in the mutant mice. Furthermore, microarray analyses provided additional support that the epidermis had an upregulation of molecules associated with immunological and inflammatory processes, whereas the dermis displayed an increase in the expression of molecules related to an altered oxidative state, similar to observations in morphea, a localized form of scleroderma. This was supported by the high levels of anti-nucleosome antibodies in the serum of FSPC*Cre-Pparb/d^{fl/fl}* mice. Our findings highlight the importance of studying PPAR β/δ in the fibroblasts due to its significant impact on the development and homeostasis of the tissue as a whole in a paracrine fashion. Furthermore, this new animal model can be an excellent tool in clarifying the various controversies surrounding the role of PPAR β/δ in other diseases, especially cancer.

Chapter 5: Future Studies

ELUCIDATING THE ROLE OF FIBROBLAST PPAR β/δ IN TUMORIGENESIS

5.1 Introduction

Cancer is one of the leading causes of clinical death worldwide. Cancer cells acquire various hallmarks of cancer, including self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis, and evading apoptosis. Cancer cells bathe in a unique microenvironment that modulates tumor growth and progression. This tumor stroma is complex and dynamic, and its components change over the course of tumor development. Cancer-associated fibroblasts (CAFs) constitute a substantial volume of the tumor stroma and are known to promote carcinogenesis, unlike normal fibroblasts. Despite the importance of CAFs, little is known about the transcription factors involved in the tumor-promoting functions of CAFs and the oncogenic signals they produce for neoplastic transformation. Nuclear receptors form the second largest family of transcription factors, and many are currently targets of various therapeutic treatments. Recently, the nuclear receptor family member peroxisome proliferator-activated receptor β/δ (PPAR β/δ) has been the subject of controversy for its role in cancer. Using *in vitro* coculture models, we elucidated the profile of PPAR β/δ in CAFs and highlighted the importance of studying the role of fibroblast PPAR β/δ in tumorigenesis by using an appropriate animal model.

5.2 Preliminary Results

5.2.1 CAFs from squamous cell carcinoma (SCC) have reduced PPAR β/δ expression

To underscore the clinical relevance of PPAR β/δ signaling in CAFs, we examined the expression of PPAR β/δ in laser-microdissected CAFs from human SCC and the adjacent peri-normal tissues from 10 patients, courtesy of National Skin Centre, Singapore. In the microdissection procedure, we captured the stromal cells that were immediately associated with the tumor epithelial cells instead of stromal cells distal to the tumor cells. Lymphocytes and vascular structures were avoided and excluded from the capture (Figure 4.1A). All patients showed a reduced PPAR β/δ expression in CAFs compared to the peri-normal tissues (Figure 4.1B). Western blot analyses of fibroblasts isolated from freshly resected SCCs and distal normal tissues from four patients consistently showed decreased PPAR β/δ protein levels in CAFs. A representative Western blot from one patient is shown (Figure 4.1C). These observations suggested that reduced PPAR β/δ expression is common in CAFs and is associated with tumorigenic events.

Figure 4.1. CAF have downregulated expression of PPARβ/δ. (A) Laser-captured microdissection (LCM) of peri-normal fibroblasts (PNF) and cancer-associated fibroblasts (CAF) surrounding the epithelial cells. Scale bar: 1 cm. (B) Relative mRNA expression of PPARβ/δ in paired PNF and CAF from 10 sets of human squamous cell carcinomas. Ribosomal protein L27 (*Rpl27*) was used as the normalizing housekeeping gene. (C) Protein expression of PPARβ/δ between PNF and CAF. β-tubulin served as the loading control.

5.2.2 Increased expression of secreted pro-mitogenic factors in OTC with FIB_{PPARβ/δ}

CAFs are established sources of tumor-promoting growth factors (e.g., TGF-β and PDGF) as well as providers of pro-angiogenic factors (e.g., VEGF) that feed tumor

growth (Thijssen et al., 2004; Kalluri and Zeisberg, 2006). The paracrine effect of FIB_{PPAR β/δ} on epithelial tumor cell proliferation is likely mediated by changes in the expression of CAF factors. The serum-free defined OTC conditions that we have developed allowed us to detect changes in the protein levels of these factors without complications from serum. We performed an unbiased protein array comparing conditioned media from OTCs constructed using A-5RT3 tumor cells with either FIB or FIB_{PPAR β/δ} . A total of 76 distinct proteins were screened, and the results are shown in Table 3.1. Many well-known tumor-promoting and pro-angiogenic factors that also belong to the CAF-specific signature, such as GM-CSF, VEGF and PDGF, were highly expressed in A-5RT3 OTC with FIB_{PPAR β/δ} but not in A-5RT3 OTC with FIB. Notably, our preliminary analysis using A-5RT3 OTC with CAFs showed similar fold changes in several factors compared to A-5RT3 OTC with FIB_{PPAR β/δ} (Table 3.1, red). These results indicate that the reduced expression of PPAR β/δ in FIB_{PPAR β/δ} allows fibroblasts to exert a pronounced proliferative effect on tumor epithelial cells or to enhance the angiogenic responses from the tumor epithelial cells. More importantly, these findings underscore the important role of nuclear receptors as upstream mediators of CAF signals that promote tumor growth and metastasis and thus confirm nuclear receptors as potential anti-tumor targets.

Table 3.1 Relative fold change of protein expressions in A-5RT3 OTCs with FIB_{PPARβ/δ}, compared to FIB.

Cytokines/ Growth Factors	Fold Increase ^a
NT-3	3.92
IL-10	3.82
ICAM-1	3.53
NT-4	3.29
HB-EGF	2.88
TGF-β2	2.72
TGF-α	2.67
M-CSF	2.20
IL-7	2.08
PDGF-AA	1.99
GM-CSF	1.94
TGF-β3	1.89
PDGF-BB	1.80
VEGF	1.78
bFGF	1.74
IGF-11	1.74
VEGF-D	1.73
IL-8	1.52
IL-12p40	0.42
TGF-β1	0.37
FGF-4	0.26

^aSignal intensities were quantified by densitometry. Positive controls in the array blot, as provided by manufacturer (RayBiotech, USA), was used to normalize the results among the different membranes being compared. Values obtained from the A-5RT3/FIB OTC conditioned media were arbitrarily assigned a value of 1. Values represent fold increase as of conditioned media from A-5RT3/FIB_{PPARβ/δ} compared to that from A-5RT3/FIB.

We next investigated the changes in PPARβ/δ expression compared to the other two PPAR family members, PPARα and PPARγ. To this end, LCM of peri-normal fibroblasts (PNFs) and CAFs surrounding the tumor cells was performed. The PPARβ/δ level was significantly lower in CAFs than in PNFs (Figure 4.2), while the PPARα level remained unchanged and the PPARγ level was upregulated in CAFs. Recently, our group

also reported that in normal wound healing, IL-1-induced expression of PPAR β/δ in FIBs stimulated the production of sIL-1ra to regulate the production of fibroblast-secreted mitogenic factors via the IL-1 signaling pathways (Chong et al., 2009). To investigate if sIL-1ra plays a role in CAF-induced tumorigenicity, we also performed qPCR for sIL-1ra in both PNFs and CAFs. In accordance, the expression of sIL-1ra was downregulated significantly in CAFs (Figure 4.2), leading to a disruption in the inhibitory effect on the proliferative IL-1 signaling pathways. This rendered CAFs more pro-tumorigenic than FIBs.

Figure 4.2. Expressions of PPAR β/δ and sIL-1ra mRNA were altered in CAF. The mRNA expression of PPAR β/δ was found to be significantly downregulated in CAF. PPAR α and PPAR γ levels remain the same and upregulated, respectively, in CAF. The level of sIL-1ra mRNA was also found to have decreased significantly in CAF, with respect to PNF. ** $P<0.05$, *** $P<0.01$.

5.2.3 Reduced fibroblast PPAR β/δ contributes to increased growth of A-5RT3

To better understand the effects of stromal PPAR β/δ on epithelial tumor cells, OTC was set up with A-5RT3 cocultured with either FIBs or PPAR β/δ -knockdown FIBs

(FIB^{PPARβ/δ}). Knockdown efficiency was analyzed with qPCR and Western blot (Figure 4.3A). H&E staining indicated a thicker epithelial layer of A-5RT3 cells in the A-5RT3/FIB^{PPARβ/δ} coculture compared to that in the A-5RT3/FIB coculture (Figure 4.3B). Immunofluorescent staining against the proliferation marker Ki67 also suggested that the downregulation of PPARβ/δ in the underlying FIBs promoted the proliferation of the tumor cells (Figure 4.3B). Protein analyses of tumor cell lysate also highlighted the upregulation of the proliferative markers PCNA and cyclin D1 in the presence of FIB^{PPARβ/δ} (Figure 4.3C).

Figure 4.3. Reduced fibroblasts PPAR β/δ promotes epithelial tumor cells proliferation. (A) Knockdown efficiency of PPAR β/δ mRNA was determined by qPCR (left) and Western blot (right). (B) Organotypic coculture was set up with A-5RT3 cocultured with either FIB or FIB_{PPAR β/δ} . When PPAR β/δ level is reduced, the epithelial layer of A-5RT3 is thicker, as shown by H&E staining and increased Ki67 proliferation marker staining. (C) Western blot results indicate an increase in proliferation markers such as cyclin D1 and PCNA in tumor cells, when cocultured with FIB_{PPAR β/δ} .

5.3 Conclusion

In our preliminary studies, we showed that CAFs from SCCs expressed lower levels of PPAR β/δ as compared to normal fibroblasts. In order to study the paracrine effects that PPAR β/δ -knockdown fibroblasts had on the neighboring epithelial tumor cells, we constructed OTC models that utilized serum-free media conditions. Protein array analyses of the conditioned media from OTC models indicated that there was an upregulation in growth factors that enhanced tumor-promoting and pro-angiogenesis effects. We also showed by real-time qPCR that sIL-1ra was downregulated in CAFs, further confirming the loss in regulation of fibroblasts-derived mitogenic factors that play proliferative roles in the IL-1 signaling pathway (Chong et al., 2009). Finally, we performed histological studies on OTC constructs made up of A-5RT3 tumor cells and either normal or PPAR β/δ -knockdown fibroblasts. Our data showed that reduced fibroblasts PPAR β/δ promoted the proliferation of A-5RT3 tumor cells, alongside with increased Ki67, cyclin D1 and PCNA markers expressions. Collectively, our preliminary data demonstrated that reduced fibroblasts PPAR β/δ could play an important role within the tumor microenvironment in promoting tumor progression. Hence, our FSPCre-*Pparb/d^{f/f}* mouse can serve as an appropriate *in vivo* model to study the role of fibroblasts PPAR β/δ in tumorigesis.

Chapter 6: Discussion

In the first set of results, we demonstrated the use of microparticles to deliver an agonist of PPAR β/δ to diabetic wounds topically can be beneficial to patients. Wound healing is a complex, pro-survival response to injury. The process involves a milieu of overlapping biological events, including inflammation, re-epithelialization, and ECM remodeling. The tight regulation over the activation and withdrawal of these events serves the primary function of restoring the epidermal barrier of the skin and maintaining skin homeostasis. Throughout the entire process of wound closure, cellular communications with components of the ECM coordinate each phase of the healing process, providing a spatio-temporal regulatory effect over wound closure.

In the majority of diabetic patients, a skin injury would manifest into a chronic wound that does not heal properly. This impairment in wound healing is a major complication of the disease that results in extensive morbidity, loss in productivity, and healthcare expenses. Due to a decreased rate of blood flow, diabetic patients are commonly at an increased risk of developing complications from wounds. Diabetic wounds exhibit an accumulation of devitalized and injured tissues, poor local angiogenesis in the injured site, prolonged inflammation, and increased MMP activities that result in lower levels of crucial ECM components. These factors culminate in an impaired wound microenvironment that is unable to support or promote normal wound healing (Chong et al., 2014). Due to poor healing, diabetic wounds often result in ulcer formation as seen in DFU cases, subsequently leading to limb amputation. In addition to DFU, diabetic patients also tend to develop neuropathy, causing them to lose sensation in their hands or feet and preventing them from immediately noticing any injuries. Unfortunately, there are relatively few topical prescriptions available that are truly

effective towards ulcers. Therefore, there is currently still a high amputation rate among non-healing diabetic patients.

The role of PPAR β/δ in wound healing has been established over the decade (Michalik et al., 2001; Tan et al., 2004; Tan et al., 2005; Tan et al., 2007; Chong et al., 2009). PPAR β/δ was shown to prime keratinocytes to respond to chemotactic signals and to promote integrin recycling and remodeling of the cytoskeleton, thus promoting cell migration (Tan et al., 2007). In addition, PPAR β/δ plays a crucial role in balancing the inflammatory phase during wound healing (Tan et al., 2005), as well as contributing to the spatio-temporal control of keratinocyte proliferation to maintain a homeostatic control over proliferation and differentiation events (Chong et al., 2009). However, the use of PPAR β/δ agonists such as GW as clinical treatments is currently a challenge because of the low bioavailability of the compound and that suitable and economical drug delivery systems are unavailable. In addition, poor patient compliance makes it more difficult for the medication to exert its desired effects.

Several studies had been conducted to devise better drug delivery systems that are more efficient for wound healing. How different drug release kinetics affects the efficacy of a potential drug is still relatively unstudied. In this chapter, we explored the utilization of single-layered PLGA:GW and PLLA:GW and double-layered PLGA:PLLA:GW microparticles as potential formulations to promote better wound healing efficiency. The rationale behind employing such microencapsulation strategies is to enhance the delivery of a hydrophobic PPAR β/δ ligand (GW) via topical applications with various drug release profiles. At the same time, this allowed us to examine the therapeutic potential of using

GW to improve healing of diabetic wounds through topical application of encapsulated GW on full-thickness excisional splint wounds on diabetic mice.

Using the oil-in-water emulsion solvent evaporation technique, we created spherical microparticles comprised of PLGA:GW, PLLA:GW, and PLGA:PLLA:GW. These microparticles are of sizes ranging between 75 μm and 125 μm and differed in the average loading of GW amongst the various formulations (PLGA:GW 0.07%; PLLA:GW 0.065%; PLGA:PLLA:GW 0.2%). Hence, the efficiency of GW encapsulation was better than either of the single-layered formulations. This could be because PLGA was able to promote the shielding of the hydrophobic GW from dispersing into the PLLA from the outer aqueous phase during the fabrication of the encapsulated drug. Despite the difference in encapsulation efficiency, all three formulations retained the biological activity, overcame the difficulty in delivery a hydrophobic compound by topical application, and enhanced the *in vivo* biostability. In addition, compounding systemic influences and potential off-target side effects by GW can be countered to a large extent via encapsulated drug delivery. In this study, all three formulations displayed two distinct release kinetics of the drug. PLGA:PLLA:GW displayed a rapid release at the initial phase followed by a gradual and sustained drug release. On the other hand, the single-layered PLGA:GW and PLLA:GW initially released GW at a slow rate followed by a sudden increase to its peak by day 10. Upregulated expression of *sIL-1ra* and *ANGPTL4*, target genes of $\text{PPAR}\beta/\delta$ in fibroblasts and keratinocytes, respectively, proved that neither GW nor its biological activity were destroyed or altered during the fabrication process.

On the molecular level, diabetic wounds can be characterized by early, excessive, and protracted ROS production (Aktunc et al., 2010). This dysregulation in ROS levels

leads to aberrant wound healing in diabetic patients due to impaired cellular responsiveness to external signals (Eming et al., 2007; De Mattei et al., 2008). We detected an excessive amount of H_2O_2 in diabetic wound tissues and demonstrated that fibroblasts were the major contributors to this dysregulated production of H_2O_2 . Excessive H_2O_2 levels modified collagen, the major ECM protein of the wound bed, at numerous amide positions and subsequently caused abnormal keratinocyte migration along this modified ECM. An early dose of GW increased the expression and activity of catalase and GPx1, both of which reduce H_2O_2 to H_2O , thereby keeping the oxidative modification of collagen in check. The upregulation of *catalase* and *GPx1* were induced by the ligand activation of PPAR β/δ by GW. This was supported by several lines of evidence. First, in the presence of an antagonist (GSK) or inverse agonist (10h) of PPAR β/δ , levels of both intracellular and extracellular H_2O_2 were elevated. Second, GW was unable to quench extracellular H_2O_2 from fibroblasts when PPAR β/δ was transiently knocked down. Third, we identified a functional PPRE in the promoters of both *catalase* and *Gpx1* genes and a positive pull-down in our ChIP experiment in the presence of GW. Hence, diabetic wounds can be administered with an early and controlled timely release of GW dose to reduce the accumulation of H_2O_2 through the activities of catalase and GPx1. Contrastingly, the delayed release of GW and subsequently PPAR β/δ as seen in the release kinetic profiles of PLGA:GW and PLLA:GW would render the ECM susceptible to modification by H_2O_2 in the early phases of wound healing. This would cause retardation in keratinocytes migration in the later phases of wound closure. In view of the ability of GW to reduce H_2O_2 via the activation of PPAR β/δ , it could be speculated that GW may be a potential therapeutic drug for other diseases that are caused by detrimental

levels of H_2O_2 . Diabetic patients also commonly present with increased arterial wall stiffness (Chuang et al., 2014). Inflammatory cells at the arterial wall produce high level of superoxide radicals. The dismutation or reaction with nitric oxide would convert these radicals into H_2O_2 or other more reactive and damaging species, such as peroxynitrous acid, respectively. In addition, the hyperglycemic condition in diabetes can mediate ECM damage via glycoxidation or the formation of reactive aldehydes, which usually result in the formation of advanced glycation endproducts (AGE). Such overproduction of glycation products renders the matrix more difficult for normal turnover. Furthermore, diabetes-associated cardiovascular disease has also been attributed to ECM modifications that enhance the retention and accumulation of low-density lipoproteins through crosslinking with the ECM. Retention of these lipoproteins may enhance their modification by oxidation, giving rise to foam cells upon their uptake by monocytes and macrophages. Foam cells are capable of releasing a milieu of cytokines, MMPs, and other oxidants that could further damage the ECM. Such events lead to the subsequent dysfunction of the endothelial cells, increased smooth muscle cell infiltration and proliferation, and weakening the structure of the developing lesion, and thus increasing the risk of a rupture (Chuang et al., 2014). Another diabetes-related disease is diabetic nephropathy that leads to end-stage renal failure. As part of an inflammatory renal injury, alongside with aging, the ECM becomes oxidized, giving rise to microvascular complications in the kidney (Kashihara et al., 2010). Consequently, an oxidatively modified ECM perturbs interactions between cells and ECM, for example, disrupting the normal functions of mesangial cells, such as their cell adhesion capability and their proliferation, thus affecting the structural support and regulation of blood flow of the

glomerular capillaries (Pedchenko et al., 2005; Pozzi et al., 2009; Kolset et al., 2012). Interestingly, the use of GW against metabolic diseases, especially diabetes, is currently at phase II of clinical trials, although the finding is not publicly available yet. Hence, there is definitely a potential in the use of GW against ROS-mediated diseases, provided that further evidences surface to demonstrate the effectiveness of administering GW as an antioxidant in these diseases.

The current study using microencapsulated GW to treat diabetic wounds indicates that more attention should be given to drug release kinetic profiles, as these could impact the final outcome of any treatments. Using controlled release of GW on diabetic wounds helped us to comprehend how different drug release kinetics would lead to diverse healing outcomes. Importantly, our study provided additional insights into the currently known roles of PPAR β/δ in wound healing, which would be difficult to achieve had GW been applied directly on the wound. Although the wound-healing rate was faster with non-encapsulated GW treatment compared to vehicle treatment, the healing rate was still slower when compared to wounds treated with just single-layered encapsulated GW. This may be a consequence of poor stability, bioavailability, or the unwanted cytotoxicity of a single large bolus dose of GW, which might be difficult to predict *in vivo*. Hence, direct topical application may subsequently have a diminished effect on later stages of wound healing, such as angiogenesis and keratinocyte migration. The undesired consequences of directly applying GW topically highly diminished the pharmaceutical value of GW as a wound-healing drug for diabetic patients. This was improved through the microencapsulation of GW that allowed for a controlled release of the drug, enabling us to

better understand the potential of GW as a therapeutic intervention for diabetic wound healing.

Therefore, in the first set of results, we demonstrated the critical nature of being able to effectively deliver a drug to its target and carry out its full therapeutic effect in a controlled release manner. In addition to reducing the quantity of drug administered to a patient to achieve the desired therapeutic benefits, the use of such drug carriers allow for better patient compliance. Importantly, the fabrication process of encapsulating the drug preserved the integrity and biological activity of the drug compound and overcame hydrophobicity and stability problems to improve the delivery efficiency of the drug. Therefore, PLGA:PLLA:GW serves as a vital starting point for future development of clinical therapies such that affordability and effectiveness of diabetic wound healing are improved. In addition, our report justifies the importance of using an appropriate animal model for future studies of the role of PPAR β/δ in fibroblasts.

In order to further elucidate the role of fibroblasts PPAR β/δ in skin with an appropriate *in vivo* model, we generated a novel mouse line, with *Pparb/d* ablated specifically in the fibroblasts. Our study from the second set of results revealed a previously undescribed role of PPAR β/δ in fibroblasts associated with skin architectural formation, wound contraction and epidermal responses to inflammatory challenges. We showed that FSPCre-*Pparb/d*^{f/f} mice exhibited dermal thickening associated with excessive collagen production and greater numbers of myofibroblasts, which contributed to a greater wound contraction and faster healing compared with their wild type littermates. The epidermis of FSPCre-*Pparb/d*^{f/f} mice showed an aggravated response to TPA, suggesting that PPAR β/δ ablation in fibroblasts may render the mutant mice more

susceptible to developing inflammatory skin disorders. FSPCre-*Pparb*/ $d^{fl/fl}$ mice exhibited increased neutrophils migration upon acute TPA treatment, and our detailed analysis also revealed aggravated epidermal inflammation response. Future studies can investigate the response of the skin or skin repair upon skin bacterial infection, such as with *Staphylococcus aureus* or *Pseudomonas aeruginosa*. On one hand, we can speculate that the exposure of FSPCre-*Pparb*/ $d^{fl/fl}$ mice skin to pathogens may result in faster clearance of the bacterial invasion when compared to its wildtype littermates. On the other hand, the aggravated immune response to the pathogen may also lead to excessive tissue damage and manifest as chronic wounds. Further investigations will be necessary to understand the role of PPAR β/δ in infected wounds. The skin microbiome is an emerging field, and more studies have been conducted on human skin than on mouse skin. The cutaneous innate and adaptive immune responses can modulate the skin microbiota, but the microbiota also functions in educating the immune system. An enhanced understanding of the skin microbiome is necessary to gain insight into microbial involvement in human skin disorders and to enable novel promicrobial and antimicrobial therapeutic approaches for their treatment.

Mice with PPAR β/δ deleted in skin fibroblasts showed a more pronounced dermal thickness compared with wild type littermates. We did not observe any compensatory increased expression of PPAR α and PPAR γ . FSPCre-*Pparb*/ $d^{fl/fl}$ mice showed an enhanced rate of dermal wound closure concomitant with elevated collagen production and higher α -SMA-positive myofibroblasts. Notably, fibroblast-specific PPAR γ -deficient skin did not show significant alterations in skin thickness or matrix accumulation even four months after the PPAR γ gene was deleted, suggesting that PPAR β/δ and PPAR γ may

play different roles in skin architectural formation (Sha et al., 2012). Like FSPCre-*Pparb/d^{fl/fl}* mice, fibroblast-specific PPAR γ -deficient mice showed an enhanced rate of dermal wound closure associated with elevated phosphorylation of SMAD3, Akt and ERK and increased expression of PCNA, collagen and α -SMA (Sha et al., 2012). Wound contraction plays a limited role in human repair. However, if given enough time, even large open wounds of the human skull or posterior neck can completely close by contraction alone, usually leaving a small stellate area of neo-epithelialization (Ehrlich and Hunt, 2012). In addition to greater wound contraction, we also detected higher epidermal proliferation in FSPCre-*Pparb/d^{fl/fl}* mice that can contribute to faster re-epithelialization. This was consistent with a previous study using global PPAR β/δ knockout mice and *in vitro* 3D organotypic skin cultures that showed PPAR β/δ deficiency in fibroblasts led to increased epidermal proliferation (Chong et al., 2009). In response to interleukin-1 (IL-1) produced by keratinocytes, PPAR β/δ in the underlying fibroblasts stimulated the production of the secreted IL-1 receptor antagonist, resulting in an autocrine decrease in IL-1 signaling pathways, decreased production of secreted mitogenic factors by the fibroblasts and inhibited the mitotic activity of keratinocytes (Chong et al., 2009).

The skin of FSPCre-*Pparb/d^{fl/fl}* mice was characterized by increased collagen synthesis. Fibroblasts were recruited and activated to generate myofibroblasts. Through microarray analysis of the dermis of FSPCre-*Pparb/d^{fl/fl}* mice, we also detected dysregulated TGF- β signaling. Our results showed that in the dermis of FSPCre-*Pparb/d^{fl/fl}* mice, there was a higher expression level of phospho-TGF β RII and phospho-SMAD3 compared with *Pparb/d^{fl/fl}* mice. We further identified *Lrg1* as a direct

downstream target of PPAR β/δ , contributing to the observed phenotype. LRG1 is expressed in human tissues such as the breast, skin, and intestine, but little is known about its role in these tissues. Thus far, LRG1 has been reported to augment endothelial TGF β signaling and promote angiogenesis (Wang et al., 2013). Secreted LRG1 also acted in a paracrine manner to influence the epidermal TGF- β response to inflammatory challenge. A role for PPAR β/δ in lipid metabolism is well-recognized (Braissant et al., 1996; Kersten et al., 2000; Lee et al., 2003; Schmuth et al., 2004; Wagner and Wagner, 2010). Interestingly, our microarray analysis of the epidermis of FSPCre-*Pparb*/*d^{fl/fl}* mice also highlighted a significant number of lipid-associated genes involved in the immune response. A family of potent inflammatory lipid mediators known as leukotrienes is synthesized via the oxidation of arachidonic acid (AA). This process is facilitated by FLAP, which transports AA released from phospholipids lining the cell membranes to 5-lipoxygenase, the enzyme responsible for the oxidation of AA to produce leukotrienes. Thus, we showed that fibroblast PPAR β/δ can augment cell-specific TGF β responses via the transcriptional regulation of LRG1.

PPAR β/δ has been implicated in inflammatory skin diseases such as psoriasis, allergen-induced dermatitis and acne vulgaris (Michalik and Wahli, 2007; Man et al., 2008; Elmongy and Shaker, 2012). Interestingly, mice with PPAR β/δ deleted in skin fibroblasts displayed many sclerotic or morphoea-like pathological hallmarks. Morphoea, localized scleroderma, is a disorder characterized by excessive collagen deposition leading to the thickening of the dermis, subcutaneous tissues, or both. In addition, the pathophysiology of this disorder involves inflammation that culminates in fibrosis, a result of fibroblast activation and excessive ECM deposition (Gilbane et al., 2013). While

the etiology and the causes of fibrosis in scleroderma remain unclear, cytokines such as TGF- β and IL-4 that play crucial roles in the stimulation of collagen production have been implicated in the disease. Consistent with the findings of multiple studies of scleroderma-affected individuals, FSPCre-*Pparb*/ $d^{fl/fl}$ mice showed increased recruitment and activation of fibroblasts to myofibroblasts as well as dysregulated TGF- β signaling in these cells. These symptoms may be further aggravated by an enhanced epidermal inflammatory response in these mutant mice. This is similar to observations in clinical scleroderma, where fibroblast activation in the lesional skin tissues gave rise to an immune-inflammatory environment with abnormal repair of damaged vessels (Clark et al., 2015). In addition to a fibrotic and inflammatory environment, clinical scleroderma samples also exhibit increased ROS production in the dermis (Sfrent-Cornateanu et al., 2008; Piera-Velazquez and Jimenez, 2015; Piera-Velazquez et al., 2015; Spadoni et al., 2015). Although this study did not elucidate the changes in dermal ROS production in FSPCre-*Pparb*/ $d^{fl/fl}$ mice, our microarray analyses of the dermis showed the upregulation of numerous genes that would culminate in an increased ROS production in the PPAR β/δ -ablated dermis. Examples of these genes include BMP4 (Jo et al., 2006), CCL2 and CCL5 (Qiu et al., 2009; Li et al., 2014), CD52 (Elsner et al., 1996), and IL-18 (Yoon- et al., 2004). Furthermore, this observation is in agreement with the first set of data that the activation of PPAR β/δ in fibroblasts could upregulate the activities of catalase and glutathione peroxidase 1, which subsequently quenched excessive H₂O₂ in diabetic wound beds (Wang et al., 2015). Poor healing wounds, such as diabetic wounds, are usually characterized by impaired angiogenesis, prolonged inflammation and inflammation-associated scarring. In addition, many components of the ECM are destroyed or modified

by the increased macrophages infiltration, and thus the wound microenvironment does not support keratinocyte migration and wound re-epithelialization. In diabetic wounds, wound fibroblasts have a reduced expression and activity of PPAR β/δ compared to normal wound fibroblasts. Thus, in the diabetic wound study, we examined the effect of PPAR β/δ activation by exogenous ligand (GW) on healing of a splinted diabetic wound. A splint wound model allows us to study wound re-epithelialization, which is mainly contributed by keratinocyte migration, instead of by wound contraction. This wound healing model was also chosen in view of the clinical potential of GW as a wound healing drug. In human, wound healing is mainly by wound re-epithelialization. My finding revealed that and early PPAR β/δ activation upregulates the activities of catalase and GPx1 in the fibroblasts, reduces H₂O₂-mediated ECM modifications and aid keratinocytes migration (Wang et al., 2015). In addition, it would also be interesting to understand the role of fibroblast PPAR β/δ in diabetic wound healing using FSPCre-*Pparb/d^{fl/fl}* mice. My first study showed that extracellular matrix in diabetic wound is oxidatively modified by excessive H₂O₂ in the wound microenvironment. This modified matrix resulted in poor keratinocytes migration, thereby impeding proper wound closure. Furthermore, FSPCre-*Pparb/d^{fl/fl}* mice skin manifests as a hotbed for inflammatory events, which may exacerbate the inflammation event that is already prolonged in diabetic wounds. The increased proteolytic activity by the increased infiltration in macrophages may further corrupt the wound ECM. Using a splinted wound model, it can be speculated that wound healing would be impeded.

Taken together, our current work revealed new insights and understanding of the role of fibroblast PPAR β/δ in skin homeostasis, dermal wound healing and inflammatory

skin diseases. Furthermore, to fully understand the role of PPAR β/δ in tumorigenesis amidst the various controversies of its pro- and anti-tumorigenic roles, employing the use of FSPCre-*Pparb/d^{fl/fl}* mice in tumorigenesis studies will further our knowledge of the context-dependent role of PPAR β/δ in tumor growth and progression.

In the tumor microenvironment, there is a complex interplay between the tumor epithelial cells and the underlying CAFs. CAFs are an established source of growth factors known to possess tumor-promoting roles. In fact, the majority of the current research on CAFs has focused on their secretion of pro-tumorigenic signals (Kalluri and Zeisberg, 2006; Franco et al., 2010). Herein, our preliminary studies established that CAFs have decreased PPAR β/δ expression compared to normal fibroblasts. This downregulated fibroblast PPAR β/δ expression can affect the growth of tumor cells *in vitro*. Hence, with the generation of FSPCre-*Pparb/d^{fl/fl}* mice we can study how tumor progression in these mutant mice differs from that in their wild type littermates.

Cutaneous chemical carcinogenesis is a procedure involving the topical application of a mutagenic initiator known as 7,12-dimethylbenz[α]anthracene (DMBA) followed by repeated applications of the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). The use of a combination of DMBA and TPA to study cutaneous carcinogenesis is suitable because it recapitulates aspects of the multi-stage tumorigenesis process observed in human patients. Subsequently, tumor biopsies can be excised and analyzed. Following H&E staining, skin tumors will be graded according to the histopathological criteria defined by the Broder's classification (Kruszewski et al., 1987). To investigate if fibroblast PPAR β/δ ablation leads to increased tumor aggressiveness, we aim to study malignant progression susceptibility and signs of metastasis. Tumors will be

analyzed for mRNA and protein changes in the levels of the pro-oncogenic genes presented in Figure 2a. The expression of keratin-13, integrin β 1, N-cadherin and laminin-332 will also be used to identify various stages of skin tumor progression (Montagner et al., 2014). Upregulation of these markers is observed in the malignant progression of skin tumors. Laminin-332, a marker for basal lamina, is also an established candidate for the identification of premalignant progression. A clear lining of laminin-332 between the basal epithelial layer and the adjacent stroma indicates a non-malignant skin tumor stage. In contrast, advanced grade skin tumors show diffuse, punctate laminin-332 deposits. This will therefore allow us to fully elucidate the extent to which fibroblast PPAR β/δ ablation renders the FSPCre-*Pparb*/*d^{fl/fl}* mice more susceptible to tumorigenesis and cancer metastasis.

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Early controlled release of peroxisome proliferator-activated receptor β/δ agonist GW501516 improves diabetic wound healing through redox modulation of wound microenvironment

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ABSTRACT

Diabetic wounds are imbued with an early excessive and protracted reactive oxygen species production. Despite the studies supporting PPAR β/δ as a valuable pharmacologic wound-healing target, the therapeutic potential of PPAR β/δ agonist GW501516 (GW) as a wound healing drug was never investigated. Using topical application of polymer-encapsulated GW, we revealed that different drug release profiles can significantly influence the therapeutic efficacy of GW and consequently diabetic wound closure. We showed that double-layer encapsulated GW microparticles (PLLA:PLGA:GW) provided an earlier and sustained dose of GW to the wound and reduced the oxidative wound microenvironment to accelerate healing, in contrast to single-layered PLLA:GW microparticles. The underlying mechanism involved an early GW-mediated activation of PPAR β/δ that stimulated GPx1 and catalase expression in fibroblasts. GPx1 and catalase scavenged excessive H₂O₂ accumulation in diabetic wound beds, prevented H₂O₂-induced ECM modification and facilitated keratinocyte migration. The microparticles with early and sustained rate of GW release had better therapeutic wound healing activity. The present study underscores the importance of drug release kinetics on the therapeutic efficacy of the drug and warrants investigations to better appreciate the full potential of controlled drug release.

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1. Introduction

Diabetes is a complex metabolic disorder that may cause both acute and long-term complications, severely affecting ~200 million people worldwide [1]. One of the most debilitating complications in diabetes is refractory wound healing, resulting in severe complications that frequently cause morbidity and mortality [1]. About 15% of diabetic patients have an impaired, non-healing wound [2], among which 81%

has been attributed to diabetic amputations [3]. A better knowledge of diabetic wound pathology would lead to new therapeutic targets, more efficient wound management and eventually improved diabetic patient well-beings [4].

Normal wound healing involves intricate communications among different cell types, and their interactions with the wound microenvironment. These cells, through the release of numerous growth factors, cytokines and chemical mediators, regulate and coordinate the healing process via a series of events including inflammation, re-epithelialization and matrix remodelling [5]. An important chemical mediator is reactive oxygen species (ROS). A proper redox control of the wound microenvironment is essential for different stages of normal wound healing process [6–8]. Under normal conditions, a delicate balance of free radicals has antimicrobial and pro-mitogenic functions that are essential to wound healing [9,10]. Large amount of ROS produced by inflammatory cells during early phase of wound

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healing, until post-injury day 3, is essential to protect the wounded skin against invading pathogens [11]. Thereafter ROS level decreases and re-epithelialisation phase is initiated by migration of keratinocytes along the injured dermis. In contrast, poor healing diabetic wounds are often associated with a prolonged inflammatory stage and therefore a de-regulation on the time-dependent release of ROS in the wound microenvironment [7,9,11,12]. An excessive and protracted ROS production and decreased free radical scavenging enzyme activities are linked to chronic hyperglycaemia in diabetic rodents [12]. Therefore, ROS regulation in wound healing especially in diabetic conditions warrants further study.

Numerous studies have shown that peroxisome proliferator-activated receptor β/δ (PPAR β/δ), a ligand-activated transcription factor, plays pivotal roles in wound healing. The activation of PPAR β/δ conferred an anti-apoptotic effect on the keratinocytes *in vivo*, hence protecting them from cytokine-induced apoptosis during the inflammatory phase of wound repair, thereby maintaining a sufficient number of viable migratory keratinocytes for the re-epithelialization phase of the wound-healing process [13–15]. PPAR β/δ also induces the expression of pro-angiogenic angiopoietin-like 4 (ANGPTL4) and secreted interleukin-1 receptor antagonist (sIL-1ra) to influence wound angiogenesis, scar formation and tissue remodelling [16,17]. Despite the existing studies potentiating PPAR β/δ as a valuable pharmacologic wound healing target, affecting many events in all phases of wound healing [18,19], the roles of PPAR β/δ in regulating ROS in wound microenvironment and in diabetic wound healing remain unclear. Moreover, the clinical impact of a PPAR β/δ agonist as a pharmacological agent for the treatment of diabetic wounds has not been investigated.

In recent years, the study of controlled release of drugs and bioactive agents from polymeric materials and microparticulate drug carriers to a given target has been of great scientific interest. Controlled release formulations can be used to reduce the amount of drug necessary to provide the same therapeutic effect in patients. Another undoubted advantage is also improved patient compliance. Over the years of controlled release research, different systems, ranging from coated tablets, gels to biodegradable microspheres and osmotic systems have been explored to get predesigned release profiles. In the past two decades, poly(L-lactide) (PLLA) and poly(D,L-lactide-co-glycolide) (PLGA) have been among the most attractive polymeric candidates used to fabricate devices for drug delivery and tissue engineering applications. They are biocompatible and biodegradable, exhibit a wide range of erosion times, have tuneable mechanical properties and most importantly are FDA-approved polymers [20–24]. However, there is little literature examining the *in vivo* biological outcome of controlled delivery of drugs with different release kinetic profiles. In this study, we examine the biological impact of different controlled release of PPAR β/δ agonist GW501516 (IUPAC: [4-[[[4-methyl-2-[4-(trifluoromethyl)phenyl]-1,3-thiazol-5-yl)methyl]sulfanyl]-2-methylphenoxy]acetic acid, abbreviated as GW hereafter) on healing of diabetic wounds and study their mechanism of action.

2. Materials and methods

2.1. Materials

Poly(L-lactide) (PLLA) (intrinsic viscosity (IV): 2.38, Bio Invigor), poly(D,L-lactide-co-glycolide, 50:50) (PLGA) (IV: 1.18, Bio Invigor) and poly(vinyl alcohol) (PVA) (MW: 30–70 kDa, Sigma-Aldrich) were used without further purification. Dichloromethane (DCM) from Tedia Company Inc. was of high-performance liquid chromatography (HPLC) grade and used as received. Luciferase® Reporter Assay System and Eugene® HD Transfection Reagent (Promega, USA). SuperFrost Plus slides (Thermo Fisher Scientific). iScript™ cDNA Synthesis kit (Bio-Rad). SYBR Green PCR Master Mix (Applied Biosystem, CA). Monoclonal anti-PPAR β/δ antibody (Millipore).

2.2. Microencapsulation of GW

GW-loaded double-layered PLLA-PLGA (core-shell) microparticles were prepared using the oil-in-water (o/w) emulsion solvent evaporation method [25,26]. Briefly, the two polymers (67 mg of PLGA, 33 mg of PLLA) and GW501516 (1 mg) were dissolved in 1.33 mL DCM. This solution was then poured into the 250 mL of PVA aqueous solution (0.5% w/v). The emulsion was stirred at 2000 rpm using an overhead stirrer (Calframo BDC1850-220) at room temperature (25 °C). The evaporation of DCM gave rise to phase separation of PLGA and PLLA, yielding double-layered microparticles (designated as PLLA:PLGA:GW hereafter). Lastly, the particles were centrifuged, rinsed with deionised water, lyophilised and stored in a desiccator for further characterization. GW501516-loaded single-layered PLLA (designated as PLLA:GW hereafter) or PLGA microparticles (designated as PLGA:GW hereafter) were similarly prepared whereby 100 mg of polymer (PLLA or PLGA) was used. Vehicle control microparticles were made with similar process parameters as above whereby no GW was encapsulated.

2.3. Determination of polymer distribution

Raman mapping was utilized to verify the polymer distribution within the microparticles. Microparticles that had been pre-sectioned were placed under a microscope objective with a laser power of up to a ~20 mW. Raman point-by-point mapping measurements were performed in both x and y directions using a Raman microscope (InVia Reflex, Renishaw) equipped with a near-infrared enhanced deep depleted thermoelectrically Peltier-cooled CCD array detector (576 × 384 pixels) and a high-grade Leica microscope. The sample was irradiated with a 785 nm near-infrared diode laser, and an objective lens was used to collect the backscattered light. Measurement scans were collected using a static 1800-groove-per-mm dispersive grating in a spectral window from 300 to 1900 cm^{-1} , and the acquisition time for each spectrum was approximately 35 s. Raman mapping data were further analysed using the band target entropy minimization (BTEM) algorithm to reconstruct pure component spectral estimates.

2.4. Electron microscopy of the microparticles

The surface and internal morphologies of the microparticles were viewed using scanning electron microscopy (SEM, JSM-6360) at 5 kV. Before analysis, the samples were mounted onto a metal stub and submerged in liquid nitrogen before chopped with a razor blade. Samples were subsequently coated with gold using a sputter coater (SPI-Module).

2.5. Drug encapsulation efficiency

Encapsulation efficiency is defined as the ratio of actual to theoretical drug loading within the microparticles. For quantification of GW loading, 5 mg of microparticles were added into 1 mL of 1 M NaOH solution and incubated for 24 h at 37 °C. The mixture was then centrifuged and supernatant drawn. The concentration of GW501516 was determined using a UV-Vis spectrophotometer (Shimadzu UV-2501) at 318 nm. All measurements were done in triplicate.

2.6. *In vitro* GW release kinetic assay

In vitro GW release kinetic assay was performed using a rotating system. Briefly, 50 ng of GW-loaded microparticles were re-suspended in 1 mL of saline and kept in the rotating system for continuous release of GW from the microparticles at 37 °C. On a daily basis, 10 μL of the supernatant was used to measure the concentrations of GW on a UV-Vis spectrophotometer (Shimadzu UV-2501) at 318 nm. All measurements were done in triplicate. The assay was carried out up to 12 days.

2.7. Transfection and luciferase assay

Drug release study was performed as previously described, except that serum-free culture medium was used [27]. HEK293T cells were co-transfected with PPARE-tk-luciferase reporter vector and pCDNA3.1-PPAR β /6 using Fugene® HD Transfection Reagent (Promega, USA). RL Renilla luciferase control reporter vector was used as a control for transfection efficiency. The next day, medium was aspirated and replaced with conditioned medium were collected daily for luciferase assay for a period of 10 days. Luciferase assay was performed using Dual Luciferase® Reporter Assay System (Promega, USA) and the Glomax 20/20 Luminometer (Promega, USA) to monitor drug release kinetics and biological activity of released GW501516.

2.8. Measurement of reactive oxygen species (ROS)

Extracellular H₂O₂ was measured using Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Life Technologies) as described by manufacturer. Briefly, 5×10^4 fibroblasts, macrophages or keratinocytes were incubated with 50 μ M Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) and 0.1 U/ml horseradish peroxidase (HRP) in Krebs-Ringer phosphate buffer for 1 h at 37 °C. Next, cells were removed by centrifugation and resorufin fluorescence in the supernatant was measured at emission of 590 nm (excitation 485 nm) using Tecan SPECTRAFlour Plus (Tecan Systems) plate reader. Results were reported in arbitrary fluorescence units. For intracellular ROS measurement, 5×10^4 cells of interest were incubated with 10 μ M 5-(and 6-) chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester fluorophores (CM-H2DCFDA, Life Technologies) for 30 min at 37 °C. Next, cells were detached and analysed by Accuri C6 Flow cytometer (BD). ROS in wound fluid was measured using 20 μ l of wound fluid in a 200 μ l Amplex Red final reaction volume. Wound fluid was collected as previously described [14]. Amplex Red conversion to resorufin fluorescence was measured as described above.

2.9. In vitro catalase and GPx activities assays

Fibroblasts (10^6 per treatment) were seeded overnight and treated with indicated concentrations of GW501516 for 6 h. Catalase and GPx activities were measured according to the Amplex Red Catalase Assay Kit (Molecular Probes) and the Glutathione Peroxidase Assay Kit (Abcam) manufacturers' protocols, respectively. Cell protein concentrations were used for final normalization. Each experiment was repeated twice with triplicates.

2.10. In vivo wound healing experiments

Diabetic (db/db) and wild-type control (db/+) male mice of 6–8 weeks old were purchased from the Jackson Laboratory. Criteria for inclusion were a blood glucose level of less than 200 mg/dl (control, db/+) or over 300 mg/dl (diabetic, db/db). Two full-thickness excisional splint wounds were inflicted on the dorsal skin of each mouse as previously described [28]. GW (1 ng) or GW (1 ng)-loaded microparticles (PLGA:GW, PLLA:GW or PLLA:PLGA:GW) were re-suspended in saline and topically applied onto one wound. Control experiments were carried out accordingly using vehicle DMSO alone, PLGA:veh, PLLA:veh or PLLA:PLGA:veh on the other wound. Finally, the wounds were covered with 3M™ Tegaderm transparent film dressing to prevent leakage of the microparticles or solution. Wound closure was measured daily in a double-blinded manner until end of experiment. Animals were sacrificed on days 3, 5, 7, 9 and 10 post-wounding. Wound biopsies were harvested, centrally bisected and subjected to histology staining for measurement of the epithelial tongue length and wound epithelial area. At least 5 animals were used for each individual treatment at every single time point. All animals were maintained in specific pathogen-free

conditions. The animal studies were approved by the Institutional Animal Care and Use Committee (ARF-SBS/NIE- A0174AZ).

2.11. In vitro wound healing assay

Rat tail type I Collagen (BD Biosciences) was used to coat a 24-well plate (Corning Life Sciences) at a concentration of 50 μ g/cm² for 1 h at 25 °C. PBS-diluted hydrogen peroxide (H₂O₂) with varied concentrations was used to treat the pre-coated plate for 3 h at room temperature, and the plate was washed thrice with PBS and dried. Individual silicone insert (Ibidi Pte Ltd), with a defined cell-free gap (500 μ m $\pm$ 50 μ m), was placed and attached into each well of the pre-treated plate. Keratinocytes, 1×10^4 , were seed into individual well of each silicone insert. Cells were allowed to attach for 12 h before the insert was gently removed. Cells were replenished with fresh media and cultured in the Cell-IQ machine (CM technologies) for real-time photographing as the cells migrate and cover the cell-free gap. Wound healing rate was assessed using the Cell-IQ analysis software (CM Technologies).

2.12. Statistical analysis

Statistical analyses were performed using the Mann–Whitney test via SPSS v. 19 software (IBM Corporation, USA). A *p* value of <0.05 was considered significant.

3. Results

3.1. PPAR β /6 activation kinetics of PLLA:GW, PLGA:GW and PLLA:PLGA:GW

To achieve different release kinetics, PPAR β /6 agonist GW was encapsulated in single-layered PLLA and double-layered PLLA:PLGA microparticles. Raman mapping of PLLA:PLGA:GW showed that PLGA and PLLA abundantly distributed in shell and core regions, respectively (Fig. 1a). PLGA was spreaded evenly all over PLLA:GW as shown in the cross-sectional view of PLLA:PLGA:GW microparticles (Fig. 1b). Both types of microparticles, diameters ranging from 75 to 125 μ m, possessed spherical shapes with smooth surfaces (Fig. 1b, upper panels). Cross-sectional SEM micrographs revealed double- and single-layered structures of PLLA:PLGA:GW and PLLA:GW, respectively (Fig. 1b, lower panels). The average drug loading (weight) in the encapsulated microparticles was determined to be $0.07 \pm 0.005\%$, $0.065 \pm 0.005\%$ and $0.2 \pm 0.014\%$ for PLGA:GW, PLLA:GW and PLLA/PLGA:GW, respectively.

Next, to examine the release kinetics of encapsulated GW from these microparticles, we performed *in vitro* release study over a period of 10 days. The released GW was detected by UV 318 nm absorbance. Results showed two distinctive GW release profiles by PLLA:PLGA:GW, PLLA:GW and PLGA:GW. GW was released rapidly from PLLA:PLGA:GW, peaked within 6 days and thereafter sustained until day 10 (Fig. 1c). In contrast, GW was released slower from PLLA:GW and PLGA:GW for the first 7 days, followed by a drastic increase to its peak by day 10 (Fig. 1c). However, the *in vitro* acellular release study does not provide evidence about the biological activity of GW after encapsulation process. Thus, we performed a transient transactivation study using HEK 293T cells co-transfected with a PPAR β /6 expression vector and a luciferase reporter vector. We observed two PPAR β /6 activation profiles. PLLA:PLGA:GW rapidly increased the transactivation activity of PPAR β /6 when compared to PLLA:GW, reaching a sustained level by day 7 (Fig. 1d). PLLA:GW and PLGA:GW weakly activated PPAR β /6 at early time points and showed rapid increase at day 7 post-transfection (Fig. 1d). No significant difference was detected between PLLA:GW and PLGA:GW at most of the time points. The transient transfection study used ectopic expression of PPAR β /6 via a mammalian expression vector. To study the drug efficacy of the microparticle-released GW on endogenous cellular level of PPAR β /6, we examined the expression of two known PPAR β /6 target genes *ANGPTL4* and *sIL-1ra* in keratinocytes and fibroblasts, respectively [17,29]. Consistent with the characteristic

Fig. 1. Properties of GW encapsulated microparticles. (a) Raman mapping of PLLA:PLGA:GW. Color intensity scale indicates relative polymer concentration. (b) SEM graphs of microparticles. (c) *In vitro* drug release kinetics of microparticles measured in saline. (d) Drug release kinetics of microparticles measured in HEK 293T. Relative luciferase activities were normalized to Renilla luciferase activities. (e, f) *ANGPTL4* (e) and *sIL-1- α* (f) mRNA expressions at indicated time points in keratinocyte and fibroblast, respectively. L27 was used as the internal reference gene. (c–f) Data (means $\pm$ SD) from 3 independent experiments with at least triplicates of each sample. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to PLLA:GW at corresponding time point.

PPAR β/δ activation profiles obtained using HEK 293T, PLLA:PLGA:GW, PLLA:GW and PLGA:GW significantly increased ANGPTL4 expression in keratinocytes and siL-1ra expression in fibroblasts, respectively (Fig. 1e, f). Their expression profiles were found to be regulated in a pattern similar to the drug release kinetic and PPAR β/δ activation profiles of PLLA:PLGA:GW, PLLA:GW and PLGA:GW (Fig. 1c–f). Cognate control drug-free microparticles PLLA:PLGA:veh, PLLA:veh and PLGA:veh showed no measurable transactivation activity even after 9 days post-transfection (Fig. S1a). Taken together, the single-layered (PLLA:GW and PLGA:GW) and double-layered (PLLA:PLGA:GW) microparticles showed delayed and early PPAR β/δ activation profiles, respectively.

3.2. Topical application of GW-loaded microparticles accelerates diabetic wound closure

Next, we tested the effect of release-controlled microparticles as pharmacological agents for the treatment of diabetic wounds, on the re-epithelialization of full-thickness excisional splinted wounds in diabetic mouse. Mice were randomly divided into four groups and topically treated with either PLGA:GW, PLLA:GW, PLLA:PLGA:GW or non-encapsulated GW along with their cognate vehicle controls. Consistent with the known beneficial roles of PPAR β/δ in wound healing, mice treated with non-encapsulated GW accelerated wound healing. Wound healing rate was faster in non-encapsulated GW-treated wounds from day 5 post-wounding when compared to vehicle treatment. However, the healing rate was transiently slower when compared to wounds treated with single-layered microencapsulated GW (Fig. S1b, c). Non-encapsulated GW-treated wounds also closed slower than PLLA:PLGA:GW treatment.

Both GW-loaded microparticles accelerated wound healing in diabetic mice, albeit with different efficacies. Notably, wounds treated with PLLA:PLGA:GW closed within 7 days (Fig. 2a, b), which was significantly faster than PLLA:PLGA:veh, PLLA:GW, PLGA:GW and non-encapsulated GW (Fig. 2a, b). To gain further insight, we performed histomorphometric analyses of wound biopsies (Fig. 2c, d). From day 3 post-wounding, PLLA:PLGA:GW-treated wound epithelia were significantly longer and thicker than PLLA:PLGA:veh-treated wounds (Fig. 2c, d). In contrast, wounds treated with PLLA:GW or PLGA:GW closed only marginally faster than PLLA:veh (PLLA:GW vs PLLA:veh: 9 vs 10 days) and PLGA:veh (PLGA:GW vs PLGA:veh: 9 vs 10 days) (Fig. 2a). This was associated with a slightly longer and thicker neo-epithelial layer covering the area between the wound site and the margin of the regenerating tissue (Fig. 2c, d). Our observations suggest that the two different release kinetics of GW have different efficacies in accelerating wound healing.

3.3. PPAR β/δ activation modulates oxidative stress in diabetic wound

Early controlled release of GW via PLLA:PLGA:GW microparticles significantly accelerated diabetic wound closure, while delayed release of GW by PLLA:GW and PLGA:GW exhibited marginal improvement to the re-epithelialization. To understand the mechanism for the difference in efficacy, we chose day 3 and day 5 wound biopsies from PLLA:PLGA:GW- and PLLA:PLGA:veh-treated mice and subjected them to microarray analysis. Interestingly, comparative gene expression analysis between the samples revealed genes involved in cellular oxidative stresses to be consistently altered (Fig. S2a and Table S1). As expected, we also noticed changes in the expression of a cluster of immune responsive genes (Fig. S2b and Table S1). A study of wild-type wounds showed that ROS level in normal non-diabetic wounds decreased significantly (Fig. 2e), which coincided with the resolution of inflammation and the commencement of re-epithelialization. The ROS level in diabetic wounds remained significantly higher compared with wild-type wounds over the whole experimental period (Fig. 2e). Interestingly, wounds treated with PLLA:PLGA:GW showed significantly reduced ROS level, reaching a level closer to that of wild-type wounds (Fig. 2e). Next, we showed

that wound fluid levels of H₂O₂ were significantly higher in diabetic mouse as compared with wild-type counterparts on day 3 and 5 post-wounding as determined by a H₂O₂-sensitive Amplex Red assay (Fig. 2f). These observations suggest that GW may improve diabetic wound closure by reducing H₂O₂ level of the wound microenvironment.

3.4. H₂O₂-modified collagen delays keratinocyte migration

A corrupted or damaged extracellular matrix (ECM) directly impairs cell-matrix communication, delays cell migration and thus the healing process. Therefore, we proposed that excessive H₂O₂ might affect diabetic wound healing by acting as an oxidant that provisionally modifies wound ECM. To test this hypothesis, we examined the direct effect of H₂O₂-treated collagen on cell migration using a modified *in vitro* wound assay. Culture plate coated with a thin layer of rat tail type I collagen was treated for 3 h with various concentrations of H₂O₂. The residual H₂O₂ was thoroughly washed off with phosphate-buffered saline. To determine if H₂O₂ can modify collagen, we performed a Fourier transform infrared spectroscopy (FTIR) analysis on H₂O₂-treated collagen. FTIR results confirmed that several amide positions were modified by H₂O₂ when compared with vehicle treatment (Fig. 3a). Next, we performed *in vitro* keratinocyte wound assay on H₂O₂-treated collagen coated plate. Interestingly, we observed that wound closure rate was significantly reduced on H₂O₂ (10 μ M and 100 μ M) pre-treated collagen compared with vehicle pre-treated collagen (Figs. 3b and S3a). H₂O₂ treatment (10–100 μ M) showed little effect on keratinocyte apoptosis (Figs. 3c and S3b) or proliferation (Figs. 3d and S3c). These observations indicate that the modification of collagen by H₂O₂ retards *in vitro* wound healing through reducing keratinocyte migration rate.

3.5. PPAR β/δ activation diminishes H₂O₂ secretion from diabetic fibroblasts

The extracellular redox state of wound microenvironment is determined at least in part by the intracellular redox state in the surrounding cells. Thus, to understand how PPAR β/δ agonist GW may directly modulate the redox state of the wound microenvironment, we first determine the major producer of H₂O₂ in the diabetic wounds. We measured the intracellular and extracellular H₂O₂ levels of the three major skin cell types—fibroblast, keratinocyte and macrophage, maintained in diabetic culture conditions [30]. Fibroblasts produced significantly more intracellular and extracellular H₂O₂ as compared to the other two cell types (Fig. 4). Importantly, GW treatment dose-dependently decreased both intra- and extra-cellular H₂O₂ levels in fibroblasts (Fig. 4). As expected, macrophages also showed a similar trend upon GW treatment, albeit with a much lower starting H₂O₂ level (Fig. 4). GW has no significant effect on H₂O₂ production in keratinocyte (Fig. 4). Next, to determine whether the effect of GW was mediated by PPAR β/δ , we used PPAR β/δ antagonist GSK0660 (IUPAC: methyl 3-([2-(methoxy)-4 phenyl] amino)sulfonyl)-2-thiophenecarboxylate, abbreviated as GSK herein) and PPAR β/δ inverse agonist compound 10h (IUPAC: methyl 3-(N-(4-(isopentylamino)-2-methoxyphenyl)sulfamoyl)-thiophene-2-carboxylate, abbreviated as Cpd herein) [31,32]. Both GSK and Cpd significantly reversed the effect of GW on H₂O₂ level in both fibroblasts and macrophages (Fig. 4), indicating that the effect of GW on H₂O₂ level was mediated via PPAR β/δ .

3.6. GW-activated PPAR β/δ stimulates catalase and GPx1 expression

To understanding the underlying mechanism, we performed a qPCR screening of the ROS-related genes of fibroblasts that were obtained by laser microdissection from wound biopsies. Our results revealed that GPx1 and catalase were the major genes responsive to GW treatment (Fig. 5a), both of which are related to cellular H₂O₂ regulation. They were significantly increased in PLLA:PLGA:GW-treated wounds when compared with PLLA:PLGA:veh treatment (Fig. 5b). To strengthen our

Fig. 2. Effects of GW-loaded microparticles on diabetic wound healing. (a) Representative macroscopic wounds (left) and H&E (right) sections of diabetic wounds from indicated test groups ($n \geq 5$) at day 7. Ruler units are in millimeters. Red arrows point to epithelial wound edge. (b) Wounds closure kinetics of mice treated with indicated GW- and vehicle-loaded microparticles. Wound surface area is plotted as a percentage of day 0 (= 100%). (c, d) Wound tongue length (c) and epithelial area (d) determined as previously described [28]. PLLA:PLGA:GW-treated wounds have completely re-epithelialized at day 7 post-wounding. Values (means $\pm$ SD) of 10 centrally dissected sections were from at least 5 mice. p values are based on comparisons between PLLA:PLGA:GW treatment and cognate PLLA:PLGA:veh. (e) Relative ROS levels of wound tissues as measured by DCF. (f) H₂O₂ levels measured by Amplex Red on wound fluids from indicated time points. Data (means $\pm$ SD) from 5 independent experiments with at least triplicates of each sample.

finding, we performed a transient knockdown of PPAR β/δ in fibroblasts using siRNA. PPAR β/δ was successfully knockdown, which was further verified by the reduced expression of sIL-1ra, a target gene of PPAR β/δ , in fibroblasts, upon GW treatment (Fig. 5b). The up-regulation of GPx1 and catalase mRNA and activities by GW was nullified in PPAR β/δ -deficient fibroblasts (Fig. 5b–d). Consistently, PPAR β/δ -knockdown abolished H₂O₂-reducing effect by GW in fibroblasts (Fig. 5e), indicating that the effect of GW was PPAR β/δ -dependent. Finally, *in vitro* chromatin immunoprecipitation (ChIP) showed that PPAR β/δ specifically bound to the cognate responsive elements (PPREs) in different promoter regions of both human and mouse *catalase* and *GPx1* genes in GW-treated but not vehicle-treated diabetic fibroblasts (Fig. 5f). No immunoprecipitation or amplification was seen with pre-immune IgG (p.i.) or with a control sequence upstream of PPRE regions (Fig. 5f). These observations indicate that *catalase* and *GPx1* are direct target genes of PPAR β/δ . Our findings suggest early controlled release of GW improves diabetic wound closure by reducing redox modifications of ECM due to excessive H₂O₂ production from diabetic wound fibroblasts and macrophages.

4. Discussion

Numerous studies have been conducted to develop more efficient systems of drug delivery for wound healing. However, much remain to learn about how the efficacy of a potential drug on diabetic wound healing may be affected by different drug release kinetics. In the present study, we introduced single-layered PLLA:GW, PLGA:GW and double-layered PLLA:PLGA:GW microparticles as potential formulations for an efficient wound healing. The rationale behind the use of PLLA, PLGA and PLLA:PLGA is to deliver the encapsulated hydrophobic PPAR β/δ ligand GW501516 (GW) topically with delayed and early release profiles, respectively, and at the same time examine the therapeutic potential of GW on the healing rate of full-thickness excisional splint wounds of diabetic mice.

We have prepared PLLA:GW, PLGA:GW and PLLA:PLGA:GW by using the oil-in-water emulsion solvent evaporation technique that resulted in spherical microparticles of size ranging from 75 to 125 μ m with high GW encapsulation. There are differences in average drug

Fig. 3. H₂O₂ modulates collagen to retard *in vitro* wound closure. (a) FTIR spectra of collagen with indicated treatments. Spectral differences between the two treatments were highlighted in red circles (n = 4). (b) *In vitro* scratch wound closure kinetic by keratinocytes. Wound closure as percentage of time 0 width (= 100% is 500 μ m). Representative time-lapsed images as shown in Fig. S3a. Effects of H₂O₂ treatment on (c) cell survival (AV-/PI- population) and (d) proliferation (indicated as percentage of various cell cycle phases as measured by BrdU assay). Representative FAC plots are as shown in Fig. S3b and S3c. Data (means $\pm$ SD) from 5 independent experiments with at least triplicates of each sample. n.s., not significant.

Fig. 4. GW inhibits H_2O_2 production in fibroblasts and macrophages. (a) Intracellular H_2O_2 and (b) extracellular ROS levels 6 h after the indicated treatments as measured by CM-H2DCFDA and Amplex Red reagents, respectively. Data (mean $\pm$ SD) from 3 independent experiments with at least triplicates. n.s., not significant.

loading between microparticle formulations (PLLA:GW 0.065%, PLGA:GW 0.07% versus PLLA:PLGA:GW 0.2%). The encapsulation efficiency of PLLA:PLGA:GW particles was higher than that of PLLA and PLGA microparticles, possibly to the fact that PLGA helped to shield the drug disperse into PLLA from outer aqueous phase during fabrication. The PLLA:GW, PLGA:GW and PLLA:PLGA:GW retained the biological activity, enhanced the *in vivo* bio-stability and overcame the hydrophobicity problem of GW for topical application. This mode of delivery was adopted for drug administration to reduce the compounding systemic influences of GW and potential off-target side effects. The three formulations of encapsulation provided two distinctive release kinetics of GW. *In vitro* drug release of double-layered PLLA:PLGA:GW showed a rapid initial release followed by a gradual and sustained drug release. In contrast, the release of GW from PLLA:GW and PLGA:GW was initially slow, followed by a drastic increase to its peak by day 10. Moreover, the increased expression of PPAR β/δ target genes in fibroblasts and keratinocytes, two major cell types that constitutes the skin, confirmed that the preparation technique neither destroyed nor modified GW and its biological activity.

The physiological impact of non-encapsulated GW, PLLA:GW, PLGA:GW and PLLA:PLGA:GW on wound healing were evaluated in a splinted diabetic mouse full thickness excisional model. PPAR β/δ plays multiple roles in wound healing, such as angiogenesis, and keratinocytes viability and migration [18,30,33,34]. Indeed, we found that PPAR β/δ agonist GW accelerates diabetic wound closure. Interestingly, the drug release profile has a dramatic effect on the efficacy in wound healing. GW was released from PLLA:PLGA:GW in the early phase and remain sustained throughout the rest of the healing process. In contrast, GW was only released from PLLA:GW and PLGA:GW during the later stages of wound repair.

Diabetic wounds are characterised by an early excessive and protracted ROS production, which coincides with an aberrant inflammatory response [35]. Such dysregulated ROS level impairs multiple cellular processes and responsiveness to external signals, leading to

delayed wound healing [36,37]. Consistent with previous reports, we found an excessive H_2O_2 level in the diabetic wound microenvironment. We also revealed that fibroblasts and macrophages were major contributors of this oxidative microenvironment. Collagen, the major matrix protein in the wound bed, was modified by H_2O_2 at several amide positions. Furthermore, the migration of keratinocyte was impaired on this modified matrix. An early release of GW resulted in increased expressions and activities of GPx1 and catalase, which would facilitate the conversion of H_2O_2 to H_2O , consequently reining the oxidative modifications of ECM. The increased expressions of these two genes were mediated by ligand activation of PPAR β/δ . This was evidenced by the elevated levels of intracellular and extracellular H_2O_2 in the presence of PPAR β/δ antagonists GSK and Cpd, the inability of GW to quench extracellular H_2O_2 when PPAR β/δ was knocked-down in fibroblasts, the identification of a functional PPRE in the promoter of these two genes and our subsequent ChIP experiment in the presence of GW. As such, an earlier and timely release of GW dosage to the diabetic wound reduced accumulation of ROS, in particular, H_2O_2 in the diabetic wounds, via the continuous activation of GPx1 and catalase. Conceivably, a delayed release of GW, as in the case of PLLA:GW and PLGA:GW, would not be able to prevent this modification of the ECM during the early stages of wound repair, thereby retarding the migration of keratinocytes in the subsequent stage of wound closure.

The microencapsulation of GW in our study to target diabetic wounds underscored the importance of drug release kinetics on the outcome of a treatment. Microparticles to deliver GW to the diabetic wounds in a controlled release manner allowed us to understand how the release kinetics of a single drug from different microparticles can have different healing outcomes. Importantly, it provided novel insights into the roles of PPAR β/δ in wound healing, which would not have been possible if GW had been directly applied topically. Presumably, direct topical application, without any sustained release, may affect the early stages of wound healing but subsequently has a diminished impact on the rest of the cellular events, such as angiogenesis and keratinocyte migration. Wound healing rate was faster in non-encapsulated GW-treated wounds when compared to vehicle treatment. The healing rate was transiently slower when compared to wounds treated with single-layered microencapsulated GW, which may be due to the resultant effects from the stability, bioavailability and cytotoxicity by a single large bolus exposure to GW which could be difficult to predict *in vivo*. Further, such variations would likely diminish the pharmaceutical use of GW as a wound healing drug. Therefore, the microencapsulation of GW to allow for controlled drug release empowers us to better appreciate the therapeutic potential of GW as a wound healing drug.

Our study herein has demonstrated the importance of being able to deliver a drug to its target in a controlled release manner. Besides having better patient compliance, the use of such carriers can allow for reduced drug quantity having the same desired therapeutic effects in patients. We have also established that different release kinetics could result in different efficacies of a particular drug. Importantly, the encapsulation of the drug is able to maintain the integrity of the drug as well as to improve drug delivery efficacy by overcoming problems rendered by the hydrophobic nature and stability of GW. Thus, PLLA:PLGA:GW serves as an important starting tool towards further development of more affordable and effective clinical therapies for diabetic wound healing.

5. Conclusions

In this study, the efficacies of PPAR β/δ agonist GW-loaded microparticles on diabetic wound healing were investigated through topical application. We showed that microparticles, PLLA:GW, PLGA:GW and PLLA:PLGA:GW, accelerated diabetic wound closure, albeit with different release kinetics and efficacies. PLLA:PLGA:GW has an earlier controlled release of GW and significant improvement on the wound healing when compared with PLLA:GW and PLGA:GW. Our study also

Fig. 5. PPAR β/δ activation by GW directly stimulates catalase and GPx1 expressions and activities to diminish fibroblast H₂O₂ production. (a) Relative mRNA expression of various ROS-related genes in wound fibroblasts extracted by LCM from PLLA:PLGA:veh- or PLLA:PLGA:GW-treated wound biopsies at indicated time points. (b) Relative mRNA expression of various genes and (c–e) catalase (c) and Gpx (d) activities, and (e) extracellular H₂O₂ levels on *in vitro* cultured fibroblast or its PPAR β/δ counterpart upon indicated treatments. Data (mean $\pm$ SD), $n \geq 4$. (f) Results of ChIP assay conducted using pre-immune IgG (p.i.) or antibody against PPAR β/δ in vehicle (veh) or GW-treated fibroblasts. Specific regions (PPREs, respective sequences shown at the right panels) spanning promoter binding sites of the *GPx1* and *catalase* genes (from both human and mouse origins) were amplified using appropriate primers (Table S1). A control region served as a negative control.

revealed a novel protective role of PPAR β/δ on ECM against the detrimental ROS. We identified two novel PPAR β/δ target genes *GPx1* and *catalase*, their elevated expression scavenged excessive H₂O₂ in diabetic wounds, minimized H₂O₂-induced ECM modifications, restored keratinocyte migration and therefore diabetic wound healing. The present study underscores the important relationship between controlled drug release kinetics and therapeutic efficacy. Therefore, more investigations are warranted to better appreciate the full potential of controlled drug release.

Author contributions

XW, MKS and SF designed and performed the experiments and analysed the data, with intellectual inputs from PZ and NST. HCC, PZ and MBYT performed some studies and provided technical help. WLL, KWN and SCJL prepared and characterized the microparticles. BMKT and SC synthesized agonists and antagonists. MTCW provided the adult human skin tissues. MKS, PZ and NST were involved in manuscript preparation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jconrel.2014.11.001>.

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Src is activated by the nuclear receptor peroxisome proliferator-activated receptor β/δ in ultraviolet radiation-induced skin cancer

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Abstract

Although non-melanoma skin cancer (NMSC) is the most common human cancer and its incidence continues to rise worldwide, the mechanisms underlying its development remain incompletely understood. Here, we unveil a cascade of events involving peroxisome proliferator-activated receptor (PPAR) β/δ and the oncogene *Src*, which promotes the development of ultraviolet (UV)-induced skin cancer in mice. UV-induced PPAR β/δ activity, which directly stimulated *Src* expression, increased *Src* kinase activity and enhanced the EGFR/Erk1/2 signalling pathway, resulting in increased epithelial-to-mesenchymal transition (EMT) marker expression. Consistent with these observations, PPAR β/δ -null mice developed fewer and smaller skin tumours, and a PPAR β/δ antagonist prevented UV-dependent *Src* stimulation. Furthermore, the expression of PPAR β/δ positively correlated with the expression of *SRC* and EMT markers in human skin squamous cell carcinoma (SCC), and critically, linear models applied to several human epithelial cancers revealed an interaction between PPAR β/δ and *SRC* and TGF β 1 transcriptional levels. Taken together, these observations motivate the future evaluation of PPAR β/δ modulators to attenuate the development of several epithelial cancers.

Keywords keratinocyte; PPAR beta/delta; Skin cancer; *Src*; UV

Subject Categories Cancer; Skin

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Introduction

The incidence of non-melanoma skin cancer (NMSC) is increasing at a high rate (3–8% yearly), with over one million estimated new cases each year in the United States alone (Madan *et al*, 2010). Squamous cell carcinoma (SCC) and basal cell carcinoma, which both derive from keratinocytes, are the most common types of NMSC. Although mortality due to NMSC is low, its morbidity is high and is accompanied by heavy personal burden and enormous, rising costs for society. NMSCs are associated with excessive and/or chronic exposure to ultraviolet (UV) radiation and mainly occur on sun-exposed areas. In particular, UVB radiation (290–320 nm, sun-burn rays) predominantly affects keratinocytes because of its low skin penetration and is believed to act as a carcinogen (Ehrhart *et al*, 2003). Despite the prevalence of NMSCs, the mechanisms underlying their progression remain poorly defined.

Tumours have often been referred to as ‘wounds that never heal’. Gene expression profiling, which has revealed that a wound-response gene expression pattern predicts metastasis, survival likelihood and response to chemotherapy in cancer patients, supports this concept (Bissell & Radisky, 2001; Chang *et al*, 2004; Farmer *et al*, 2009; Nuyten & van de Vijver, 2006; Schafer & Werner, 2008). Apoptosis resistance, cell proliferation and migration, matrix remodelling, and angiogenesis are processes of life-saving importance after an injury but are also common features of tumour progression. The identification of molecular regulators of the mechanisms that acti-

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vate, sustain and eventually shut down these processes may advance our understanding of tumour development in general and of NMSC in particular.

Our previous work on skin wound healing in mice demonstrated the importance of the peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily, in tissue repair (Michalik *et al*, 2001). Injury strongly stimulates expression of PPAR α (NR1C1) and PPAR β/δ (NR1C2) in keratinocytes at the edges of skin wounds, and individual genetic deletion of these isotypes delays healing (Michalik *et al*, 2001). Both PPAR α and PPAR β/δ affect the early inflammatory reaction, but PPAR β/δ -null mice exhibit additional defects in healing resulting from impaired keratinocyte adhesion and migration, as well as increased apoptosis at wound edges (Di-Poi *et al*, 2002; Michalik *et al*, 2005; Tan *et al*, 2001; Tan *et al*, 2007). Thus, during wound healing, inflammation-induced PPAR β/δ controls processes that are also involved in carcinogenesis. Whether PPAR β/δ is pro- or anti-carcinogenic remains controversial, however, because both effects have been observed (Michalik *et al*, 2004; Muller-Brusselbach *et al*, 2007; Peters & Gonzalez, 2009; Schug *et al*, 2007; Schug *et al*, 2008). To address this question, we investigated PPAR β/δ function in keratinocyte-derived UV-induced skin tumours. Here, we unveil a previously unrecognized control by PPAR β/δ over a pro-oncogenic pathway, identifying PPAR β/δ as a key focus for further epithelial cancer studies.

Results

UV radiation stimulates PPAR β/δ expression and activity, enhancing skin tumour formation and progression *in vivo*

To test if PPAR β/δ is implicated in UV-dependent tumour development, we first investigated whether its expression responds to UV radiation. SKH-1 hairless mice were exposed to chronic low-dose UV radiation for up to 31 weeks to mimic repeated exposure to sunlight. Interestingly, PPAR β/δ expression increased in dorsal skin upon chronic UV exposure, PPAR α expression was reduced and PPAR γ expression was unchanged (Fig 1A, left) (supplementary

Fig S1). Additionally, the expression of *Tgfb1* and *Plin2*, two well-known PPAR β/δ target genes, was stimulated in wild-type but not in PPAR β/δ ^{-/-} mice, indicating activation of the receptor (Fig 1A). Importantly, when wild-type and PPAR β/δ ^{-/-}SKH-1 hairless mice were subjected to chronic UV radiation, tumour incidence and multiplicity were much higher in wild-type mice compared to PPAR β/δ ^{-/-} mice during the course of chronic UV exposure (Fig 1B). Wild-type mice fulfilled the criteria for withdrawal from the experiment (tumour larger than 9 mm in diameter) earlier than PPAR β/δ ^{-/-} mice (Fig 1C, D). Eventually, a 100% tumour incidence was reached in both genotypes after 22 weeks of UV exposure, which might have resulted from gene mutations arising from long-term UV exposure and associated modifications in gene expression in the PPAR β/δ ^{-/-} mice, which override the inhibitory effect of PPAR β/δ deficiency on tumorigenesis. Nevertheless, the growth rate of the tumours and their final size were higher in wild-type mice (Fig 1E).

To gain additional support for a PPAR β/δ -specific response to UV exposure, the SKH-1 mice were exposed to a single acute UV dose and sacrificed 24 h later. The UV treatment caused enhanced erythema in wild-type mice, characterized by an increased expression of inflammatory markers (supplementary Fig S2A). As with chronic irradiation, PPAR β/δ expression and activity were enhanced in the skin of wild-type animals upon acute UV exposure, especially in the epithelial compartment where carcinomas arise, as documented by the stimulation of two known target genes, *Tgfb1* and *Plin2* (Fig 1F) (supplementary Fig S2B). These observations suggest a major function for PPAR β/δ in the initiation and progression of skin tumours in response to UV irradiation.

Increased levels of activated PPAR β/δ enhance Src expression

Our previous gene expression analyses indicated a higher expression of the tyrosine kinase *Src*, a well-characterized proto-oncogene and enhancer of skin carcinoma (Guasch *et al*, 2007; Joseloff *et al*, 2002; Matsumoto *et al*, 2003; Matsumoto *et al*, 2004; Serrels *et al*, 2009; Yagi *et al*, 2007), in wild-type versus PPAR β/δ ^{-/-} primary keratinocytes (supplementary Fig S3A) (Montagner *et al*, 2011). In

Figure 1. PPAR β/δ is over-expressed and activated upon UV radiation and enhances skin tumour formation and growth.

- A** Quantification of PPAR β/δ (left), *Tgfb1* (middle) and *Plin2* (right) expression by RT-PCR in non-tumoural dorsal skin of chronically irradiated (Ch-UV, +) or non-irradiated (-) PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice. Values from non-irradiated dorsal skin of PPAR β/δ ^{+/+} mice (left) or from irradiated dorsal skin of PPAR β/δ ^{-/-} mice (right) were set to 1. Means $\pm$ SEM of 13 PPAR β/δ ^{+/+} and 9 PPAR β/δ ^{-/-} mice are presented. *p*-values are **p* = 0.035 for PPAR β/δ (left); **p* = 0.025 and ***p* = 0.008 for *Tgfb1*(middle); ***p* = 0.078 and ****p* = 0.0003 for *Plin2* (right) calculated by two-tailed Student's *t*-test.
- B,C** Percentage of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice bearing tumours (B) and average number of tumours per mouse (C) measured at the indicated time points of UV exposure. For (C), *p*-values are **p* = 0.025, ***p* = 0.008, ****p* = 0.0001 calculated by two-tailed Student's *t*-test. Data for 13 PPAR β/δ ^{+/+} and 9 PPAR β/δ ^{-/-} mice are presented.
- D** Percentage of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice involved in the experiment over time. Mice were withdrawn from the experiment based on criteria given in the Material and Methods section.
- E** Tumour growth rate (left) and final size (right) from PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice measured before sacrifice. Means $\pm$ SEM for 13 PPAR β/δ ^{+/+} and 9 PPAR β/δ ^{-/-} mice are presented. *p*-values are ***p* = 0.009 for tumour growth rate (left) and **p* = 0.045 for tumour size (right) calculated by two-tailed Student's *t*-test.
- F** Measurement of PPAR β/δ (left), *Tgfb1* (middle) and *Plin2* (right) expression by RT-PCR in dermis and epidermis compartments of dorsal skin of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice 24 h after acute UV (Ac-UV) irradiation. Values from dermis of non-irradiated dorsal skin of PPAR β/δ ^{+/+} mice (left) or from dermis of Ac-UV dorsal skin of PPAR β/δ ^{-/-} mice (middle and right) were set to 1. Means $\pm$ SEM for 12 PPAR β/δ ^{+/+} and 12 PPAR β/δ ^{-/-} mice are presented. Values are representative of three independent experiments; *p*-values are **p* = 0.025, ***p* = 0.007, ****p* = 0.002 for PPAR β/δ ; ****p* = 0.0003, **p* = 0.049, ***p* = 0.0093, ****p* = 0.0041, ****p* = 0.0004 from top to bottom for *Tgfb1*; ****p* = 0.0048, ****p* = 0.0013, ****p* = 0.0006, ****p* = 0.0009, ***p* = 0.0071, ****p* = 0.0035, ****p* = 0.0029 from top to bottom and from left to right for *Plin2* calculated by two-tailed Student's *t*-test; ns, not significant.

addition to Src, the Fyn and Yes tyrosine kinases, which also belong to the Src family kinases, were expressed in skin (Fig 2A) (supplementary Fig S3B). Interestingly, acute UV irradiation caused a

selective PPAR β/δ -dependent enhancement of Src mRNA and protein expression *in vivo* (Fig 2A, B) (supplementary Fig S3B). Similarly, Src mRNA and protein levels were consistently and strikingly

Figure 1.

higher *in vivo* in chronically irradiated wild-type skin versus *Pparβ/δ*^{-/-} skin (Fig 2C, D). This effect was selective since the expression of *Fyn* and *Yes* was not affected after chronic irradiation (supplementary Fig S3C).

The highest PPARβ/δ-dependent Src expression occurred in the epidermis, mainly in keratinocytes of the lower layers (Fig 2A, E) (supplementary Fig S4) where PPARβ/δ has been localized (Michalik *et al*, 2001). Consistent with these observations, human immor-

Figure 2.

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talized keratinocytes (HaCaT cells) stimulated with the PPAR β/δ agonist GW501516 exhibited higher Src mRNA and protein expression, similar to ANGPTL4, which was used as a positive control of PPAR β/δ activity (supplementary Fig S5A, B) (Kersten *et al*, 2000; Rieck *et al*, 2008). Characterization by 5' RACE and sequencing of the Src transcript induced in a PPAR β/δ -dependent manner by GW501516 in primary keratinocyte culture or by UV in mouse skin indicated its correspondence with the Src-001 transcript described in Ensembl (ENSMUST00000029175) (Fig 3A, B). *In silico* Src analysis revealed five peroxisome proliferator response elements (PPREs), which resemble the consensus PPRE motif, in the intragenic Src regulatory region (Fig 3A) (supplementary Fig S5C). These elements were potentially responsive to PPAR β/δ activation in a reporter assay (Fig 3C), indicating that Src is likely to be a PPAR β/δ target gene. This finding is in agreement with PPAR β/δ -induced SRC expression in the absence of *de novo* protein synthesis (supplementary Fig S5D).

To determine whether these PPREs serve as binding sites for PPAR β/δ , chromatin immunoprecipitation (ChIP) PPAR β/δ experiments followed by re-ChIP p300, a PPAR β/δ co-activator, were performed using lysates from control (wild-type) and PPAR β/δ -knockdown (KD) mouse keratinocytes (MKs, Fig 3D, E). The endogenous PPAR β/δ of KD cells was strongly downregulated by siRNA (supplementary Fig S5E). PPAR β/δ was constitutively associated with PPRE 3, 4 and 5 among the five PPREs identified *in silico* and tested in transactivation assays (Fig 3A, C), but p300 was recruited by PPAR β/δ only upon GW501516 activation. Thus, ligand activation appeared to be necessary for PPAR β/δ to regulate Src gene expression (Fig 3E), as for Tgfb1 (supplementary Fig S6).

Collectively, these observations demonstrated that UV induced PPAR β/δ expression and activity. This activity in turn directly increased Src gene and protein expression, results that define Src as a novel PPAR β/δ target gene.

PPAR β/δ enhances the Src-dependent EGFR/MAPK Erk1/2 signalling pathway in response to UV

UV irradiation activates the EGFR/MAPK Erk1/2 pathway via Src (Bode & Dong, 2003; Fritsche *et al*, 2007), a key mechanism in skin photocarcinogenesis in mice and humans (Rho *et al*, 2011). The possibility that PPAR β/δ activates not only Src but also the entire Src/EGFR/Erk1/2 downstream pathway was tested next in various PPAR β/δ and Src gain- and loss-of-function models. Src and Erk1/2

phosphorylation upon UV treatment was higher in wild-type than in PPAR $\beta/\delta^{-/-}$ primary keratinocytes (supplementary Fig S7), confirming the importance of PPAR β/δ activity for UV-dependent full activation of Src/Erk1/2. KD of PPAR β/δ in HaCaT cells consistently abolished UV-induced activation of the EGFR/Erk1/2 pathway (Fig 4A) (supplementary Fig S8), as did the absence of PPAR β/δ in skin *in vivo* (Fig 4B, C). Conversely, activation of PPAR β/δ by its agonist GW501516 enhanced the UV-induced phosphorylation of EGFR (Tyr845) and Erk1/2 in HaCaT cells (Fig 4A, D). Importantly, this action of PPAR β/δ completely depended on Src activity because Src loss of function, either by pharmacological inhibition using a PP2-specific Src family kinase inhibitor or Src KD by siRNA (supplementary Fig S8), prevented an increase in EGFR and Erk1/2 phosphorylation (Fig 4A, D). Taken together, these observations provide evidence in support of a PPAR β/δ -dependent UV stimulation of the entire Src/EGFR/Erk1/2 signalling pathway. Finally, *Ets1*, whose expression is stimulated by activated Erk1/2 (Fitsialis *et al*, 2007), was enhanced in the UV-irradiated dorsal skin of wild-type mice only (Fig 4E).

Wild-type and PPAR $\beta/\delta^{-/-}$ mice were then topically treated with the PPAR β/δ antagonist GSK0660 prior to acute UV irradiation. The UV-induced expression of two PPAR β/δ target genes, *Plin2* and *Tgfb1*, was prevented by GSK0660 (Fig 5A, B), which also abolished the increase in Src gene and protein expression (Fig 5C, D), resulting in both decreased phosphorylation levels of EGFR and Erk1/2 (Fig 5D) and stimulation of *Ets1* (Fig 5E). Interestingly, the drug did not affect expression of the pro-inflammatory markers *Ptgs2* (*Cox2*) and *Il6* (supplementary Fig S9A, B) or of *Ppar γ* and *Ppar α* (supplementary Fig S9C, D). However, UV-induced PPAR β/δ upregulation was abolished, suggesting a positive feedback loop in PPAR β/δ expression, possibly involving Src (Fig 5F). Taken together, these observations identified PPAR β/δ as a positive regulator of a major pathway in skin carcinogenesis via its effect on Src expression and activity.

PPAR β/δ coordinates a pro-tumoural gene program in advanced actinic keratosis

Skin tumour growth is associated with increased proliferation and migratory potential of keratinocytes, and in this process, Src promotes epithelial cell dedifferentiation during the epithelial-to-mesenchymal transition (EMT). The EMT might also be regulated by other

Figure 2. PPAR β/δ promotes Src expression in irradiated skin.

- A Src, Fyn and Yes mRNA expression by RT-PCR in acutely (Ac-UV, +) and non-irradiated (–) dermis and epidermis of PPAR $\beta/\delta^{+/+}$ and PPAR $\beta/\delta^{-/-}$ mice. The value from irradiated dermis of PPAR $\beta/\delta^{-/-}$ mice was set to 1. Means $\pm$ SEM are presented ($n = 6$ mice/genotype/group). Data are representative of three independent experiments; p -values are, from top to bottom and from left to right, *** $p = 0.0001$, *** $p = 0.0005$, ** $p = 0.01$, ** $p = 0.008$ for Src; *** $p = 0.0008$, * $p = 0.017$, *** $p = 0.0009$ for Fyn; *** $p = 0.0004$, *** $p = 0.0006$, *** $p = 0.0005$, *** $p = 0.0004$, *** $p = 0.0008$, *** $p = 0.0001$, *** $p = 0.001$ for Yes calculated by two-tailed Student's t -test.
- B Right, immunoblot of total Src in Ac-UV and non-irradiated skin of PPAR $\beta/\delta^{+/+}$ and PPAR $\beta/\delta^{-/-}$ mice. One of three independent experiments is shown ($n = 6$ mice/genotype/group). GAPDH was used as loading control; IB, immunoblot; left, quantification of the immunoblot (right) of total Src in Ac-UV and non-irradiated skin of PPAR $\beta/\delta^{+/+}$ and PPAR $\beta/\delta^{-/-}$ mice. Values are normalized to GAPDH, $n = 3$ mice/group; from left to right, ** $p = 0.010$, 0.009, t -test.
- C Src mRNA levels in non-tumoural skin of chronically irradiated (Ch-UV) PPAR $\beta/\delta^{+/+}$ mice and PPAR $\beta/\delta^{-/-}$ mice. Value from irradiated skin of PPAR $\beta/\delta^{-/-}$ was set to 1. Means $\pm$ SEM are given for 13 PPAR $\beta/\delta^{+/+}$ and 9 PPAR $\beta/\delta^{-/-}$ mice; * $p = 0.035$, t -test.
- D Left, quantification of Src protein from the immunoblot (right) normalized to GAPDH ($n = 5$); ** $p = 0.009$, t -test. Right, immunoblot of Src levels in Ch-UV non-tumourigenic skin of PPAR $\beta/\delta^{+/+}$ and PPAR $\beta/\delta^{-/-}$ mice ($n = 5$). One of three independent experiments is shown.
- E Representative pictures of Src immunohistochemistry in Ch-UV skin of 13 PPAR $\beta/\delta^{+/+}$ and 9 PPAR $\beta/\delta^{-/-}$ mice. Scale bars, 50 μ m.

Figure 3.

direct or indirect PPAR β/δ -modulated signalling pathways such as the Tgf β 1/SMAD pathway, which could act on *Src* expression (Glick, 2012; Han et al, 2005; Hoot et al, 2008; Martins et al, 2009; Nakamura & Tokura, 2011; Newkirk et al, 2007). Thus, we examined the ability of PPAR β/δ to enhance this transition by measuring expression of EMT markers in actinic keratosis with moderate atypia (grade II) of wild-type and *Ppar β/δ ^{-/-}* mice, including growth factors, transcription factors, cytoskeletal and cell surface markers, extracellular matrix proteins and regulators (Fig 6A). The expression of many of these markers was higher in wild-type mice than in *Ppar β/δ ^{-/-}* mice, suggesting that the wild-type advanced grade actinic keratosis presented a higher risk for premalignant progression. Results of basement membrane analysis using laminin 332 (α 3 β 3 γ 2) immunofluorescence supported this hypothesis (supplementary Fig S10A). The basement membrane between the basal keratinocyte layer and the adjacent stroma exhibited a clear lining of laminin 332 in *Ppar β/δ ^{-/-}* actinic keratosis with moderate atypia (grade II) whereas laminin 332 deposition was more diffuse and thicker in lesions of wild-type mice, reflecting perturbations of the basal layer. The proximity ligation assay (PLA) showed that these laminin 332 infiltrates in the basal and suprabasal epidermal layers in wild-type mice could bind β 4 integrin (Fig 6B, red positive signal quantified in Fig 6D) and transduce signal via Rac1 (Fig 6C, red positive signal quantified in Fig 6D). These observations were consistent with the increased expression compared to *Ppar β/δ ^{-/-}* mice of EMT markers in wild-type grade II actinic keratoses, which displayed a higher level of proliferating keratinocytes and more intense Keratin 13 staining, an early marker of malignant progression susceptibility (supplementary Fig S10B and S11). Moreover, histological grading of SCCs from wild-type and *Ppar β/δ ^{-/-}* mice according to the Broders' classification (Broders, 1921) showed that wild-type mice developed more advanced SCCs (Fig 6E) (supplementary Fig S12). However, because of the conditions imposed by the Veterinary Office (see Material and Methods for details), it was not possible to obtain more advanced UV-induced skin tumours (Grade IV according to Broders' classification) to observe metastatic SCCs. Interestingly, EMT markers were also expressed at higher levels in grade II SCCs from wild-type compared to *Ppar β/δ ^{-/-}* mice at the mRNA and protein levels. N-Cadherin staining confirmed this result (supplementary Fig S13).

Collectively, these observations suggested that keratinocytes of wild-type mice were more likely to invade the surrounding stroma. This characteristic represents a higher risk of malignant conversion in these mice compared to the *Ppar β/δ ^{-/-}* mice.

Correlation between PPAR β/δ and *Src* expression in various human carcinomas

We wished to investigate whether the proto-oncogene *Src* is also a PPAR β/δ target in human SCC. Interestingly, the levels of *SRC* and *PPARB/D* expression were highly and significantly correlated in these tumours (Fig 7A), as were the expression levels of *PPARB/D* and *TGFB1* and *MMP19*, respectively, and to a lesser extent, *MMP2*, *VEGFA*, *VIM* and *SNAI1* (Fig 7B, C) (supplementary Fig S14A–D). These data suggested that, as in UV-irradiated mouse skin, PPAR β/δ regulates *SRC* expression in human SCC, which is correlated with the expression of EMT markers.

To further examine the underpinnings of these observations, we investigated the relationships among *SRC*, *TGFB1* and *PPARB/D* expression levels in human carcinomas from various organs. More specifically, we tested whether the degree of linear dependency between *SRC* and *TGFB1* mRNA levels (degree of correlation) is influenced by the abundance of *PPARB/D* mRNA present in the tumour (the significance of the interaction term *TGFB1*: *PPARB/D* of the linear model *SRC* ~ *TGFB1* + *PPARB/D* + *TGFB1*: *PPARB/D*). This linear model was separately fitted to nine human tumour types, including skin SCC (Fig 7–D–F) (supplementary Fig S14E–I). The least square estimation of the model parameter revealed that the *TGFB1*: *PPARB/D* interaction coefficient term was significantly different from zero for lung (0.17 [0.06–0.29], 95% confidence interval in square brackets) and ovarian (0.15 [0.04–0.27]) carcinomas and oral squamous cell carcinoma (OSCC) (0.009 [0.003–0.014]). Combining these nine independent interaction coefficients into a random effects model, a method commonly used in statistical meta-analysis, produced a significant summarized interaction coefficient (0.081 [0.014–0.146]; Fig 7F). Results for SCC show that patients with a *PPARB/D* mRNA abundance lower than the median value had an absence of linear dependency between *SRC* and *TGFB1* (p = 0.75; Fig 7D) but that those with a *PPARB/D* abundance above or equal to the median of the dataset showed a significant linear relationship (p = 0.0336; Fig 7E). Results with the ovarian carcinoma

Figure 3. *Src* is a direct PPAR β/δ target gene.

- A Schematic structure of the murine *Src* gene. Untranslated and translated exons of *Src*-001 transcript and position of primers used for 5' RACE are depicted as indicated. GSP, gene-specific primer.
- B Identification of the *Src* transcript isoform expressed in wild-type primary keratinocyte cultures treated with GW501516 (100 nM) for 24 h (lane 1) and total skin of SKH-1 wild-type mice after acute irradiation (lane 2). In both samples, a major 650-bp product was obtained. Sequencing of the isolated DNA bands indicated that the transcripts corresponded to the *Src*-001 transcript (ENSMUST00000029175).
- C PPAR β/δ activity on wild-type or mutated PPRES in the *Src* promoter (supplementary Fig S5) via luciferase assays in NIH3T3 cells treated with GW501516 (300 nM) or vehicle (DMSO). Fold inductions were calculated as the ratio of firefly luciferase to Renilla luciferase activity using DMSO-treated NIH3T3 cells transfected with a TK-3PPRE construct designated as 1. Values are shown as the mean $\pm$ SD (n = 3). p -values are, from left to right, *** p = 0.0002, 0.0003, 0.0001, 0.0032, 0.0001, 0.0008 calculated by two-tailed Student's t -test. One of three independent experiments is shown.
- D,E Representative results of ChIP experiments using anti-PPAR β/δ antibody (D) followed by re-ChIP with anti-p300 (E) performed in mouse keratinocytes (MKs) downregulated for PPAR β/δ expression by siRNA [knockdown cells (KD)] or not (wild-type cells, WT) treated or not with GW501516. The results show a PCR amplification of the PPRES, 4 and 5 sites. Preimmune serum (p.i.) served as a control for ChIP. Primer sequences are given in supplementary Table S3. Data are representative of n = 3 independent experiments.

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Figure 4. PPAR β/δ -dependent upregulation of Src expression enhances EGFR/Erk1/2 signalling upon UV exposure *in vitro* and *in vivo*.

A Immunoblots of Src, pTyr845 EGFR, total EGFR, pErk1/2 and total Erk1/2 levels from whole-cell lysates of HaCaT cells transiently transfected with control (Ctrl), PPAR β/δ or Src siRNA, treated 24 h with GW501516 or DMSO, and then subjected to UVB radiation (40 mJ/cm²) before harvesting 30 min later. GAPDH was used as a loading control. Data are representative of three independent experiments.

B,C Immunoblot of pTyr845 EGFR, total EGFR, pErk1/2 and total Erk1/2 levels in protein extracts from chronically irradiated (Ch-UV) (B) or acutely irradiated (Ac-UV) (C) dorsal skin of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice. Data are representative of three independent experiments.

D Immunoblot of pTyr845 EGFR, total EGFR, pErk1/2 and total Erk1/2 levels from whole-cell lysates of HaCaT cells treated with the PPAR β/δ agonist GW501516 or DMSO for 24 h before UVB exposure (40 mJ/cm²) in the presence or absence of a Src family kinase inhibitor (PP2), and harvested 30 min later. Data are representative of three independent experiments.

E Real-time RT-PCR of Ets1 mRNA expression in non-irradiated and Ac-UV dorsal skin of PPAR β/δ ^{+/+} and PPAR β/δ ^{-/-} mice. Data are representative of three independent experiments for 12 PPAR β/δ ^{+/+} and 12 PPAR β/δ ^{-/-} mice; *p*-values are **p* = 0.032, ***p* = 0.009 calculated by two-tailed Student's *t*-test.

Figure 5. Pharmacological inhibition of PPARβ/δ prevents UV-induced Src expression and Src-dependent activation of EGFR/Erk1/2 signalling *in vivo*. Dorsal skin of *Pparβ/δ*^{+/+} and *Pparβ/δ*^{-/-} mice was topically treated with the PPARβ/δ antagonist GSK0660 or vehicle (Veh; 70% ethanol) prior to acute UV irradiation (Ac-UV); mice were sacrificed 24 h later.

A–C Quantification of *Plin2* (**A**), *Tgfβ1* (**B**) and *Src* (**C**) mRNA expression via real-time RT-PCR. Means ± SEM are presented (*n* = 6 mice/genotype/group). Data are representative of two independent experiments; *p*-values are **p* = 0.0190, ***p* = 0.0066, ****p* = 0.0004 for *Plin2*; **p* = 0.0421, 0.0153 (from left to right) and ***p* = 0.0098 for *Tgfβ1*; **p* = 0.0428, ***p* = 0.0098, ****p* = 0.0045 for *Src* calculated by two-tailed Student's *t*-test; ns, not significant.

D Immunoblot of total Src, EGFR, p-Tyr845 EGFR, Erk1/2 and p-Erk1/2 protein in extracts from dorsal skin of *Pparβ/δ*^{+/+} and *Pparβ/δ*^{-/-} mice. Data are representative of two independent experiments (*n* = 12 mice/genotype/experiment).

E Quantification of *Ets1* mRNA expression by real-time RT-PCR. Means ± SEM are given (*n* = 6); *p*-values are **p* = 0.0324 and ***p* = 0.0091, 0.082 (from left to right) calculated by two-tailed Student's *t*-test.

F Quantification of *Pparβ/δ* expression via real-time RT-PCR. Means ± SEM are presented (*n* = 6 mice/genotype/group). Data are representative of two independent experiments; *p*-values are ***p* = 0.0083 and ****p* = 0.0006 calculated by two-tailed Student's *t*-test; ns, not significant.

Figure 6.

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and OSCC datasets led to similar inferences (supplementary Fig S14F–I).

Thus, our statistical modelling provided evidence that the expression level of *PPARB/D* influences the degree of correlation between *SRC* and *TGFB1* levels across a wide variety of human carcinomas, an observation that supports our hypothesis of a direct influence of PPAR β/δ on *SRC* and *TGFB1* transcription. Taken together, our observations and analyses indicate that PPAR β/δ regulates *SRC* in human SCC, notably in skin SCC, and that this regulation also occurs in carcinomas of various tissue origins, thus revealing a broad pro-tumourigenic potential of PPAR β/δ via *SRC* upregulation.

Discussion

Our study demonstrates that PPAR β/δ promotes skin cancer by enhancing Src activity in response to UV radiation. UV is a skin carcinogen causing both DNA damage resulting in tumour initiation and activation of signalling cascades that promote tumour development (Devary *et al*, 1993; Matsumura & Ananthaswamy, 2004; Rho *et al*, 2011). The signalling pathway involving the tyrosine kinase Src, the tyrosine kinase receptor EGFR and the MAPK Erk1/2 cascade is activated during UV-induced skin inflammation and carcinogenesis, promoting initiation, progression and malignant conversion. This pathway is therefore a target for epithelial cancer prevention (Fritsche *et al*, 2007; Rho *et al*, 2011). Importantly, this pathway and its cellular effects may also be modulated by other PPAR β/δ -dependent or -independent signalling pathways, such as Tgf β 1/SMAD.

To date, the nuclear receptor PPAR β/δ has been less studied than PPAR α and PPAR γ , which are drug targets for the treatment of dyslipidemia and type 2 diabetes, respectively. Recently, the US Food and Drug Administration released safety information linking pioglitazone, which targets PPAR γ , to increased risk for bladder cancer. Although there is growing interest in developing PPAR β/δ agonists to treat dyslipidemia, no PPAR β/δ compound has been brought to market, and the role of this isotype in tumourigenesis has been debated (Michalik *et al*, 2004; Peters & Gonzalez, 2009; Peters *et al*, 2012).

Our study has yielded the first insights into how PPAR β/δ promotes UV-induced skin tumourigenesis (Fig 7G). *In vivo* UV

exposure increases PPAR β/δ expression at the transcriptional level and its activity. Once activated, PPAR β/δ upregulates Src expression and activity, which in turn, boosts EGFR/Erk1/2 signalling. Thus, in our mouse model, PPAR β/δ sensitizes keratinocytes to UV, promotes actinic keratosis development and progression, and stimulates the expression of EMT markers that affect basement membrane integrity. Importantly, *PPARB/D* expression and *SRC*, *MMP19* and *SNAIL* expression directly correlate in human skin SCC, which may indicate a direct effect of PPAR β/δ on EMT initiation. Furthermore, our meta-analysis of PPAR β/δ activity in various epithelial tumours, including human skin SCC, revealed a positive interaction between *PPARB/D* expression levels and those of two of its targets, *SRC* and *TGFB1*. Interestingly, the identification of this relationship in large independent datasets of various tumour types, particularly in lung, colon and ovarian cancers known for their high levels of Src expression and activity, strongly supports a regulatory role for PPAR β/δ in human carcinomas. We speculate that early in skin actinic keratosis formation, PPAR β/δ regulates Src and may act, in concert with other genetic change, to promote tumour progression by increasing keratinocyte migratory and proliferative properties. Our data help to define the pro-carcinogenic properties of PPAR β/δ in skin carcinoma and most likely in carcinomas from other tissue origins, as well, and identify Src as a direct PPAR β/δ target gene, at least in mouse.

In accordance with our observations, ErbB2 activation, in concert with ErbB3 and ErbB4, leads to the expression of *FABP5*, an intracellular lipid binding protein, which plays a crucial role in PPAR β/δ transcriptional activation in the MCF7 cell line, suggesting that the EGFR superfamily could also act upstream of PPAR β/δ to regulate its activity in a model of breast cancer (Kannan-Thulasiraman *et al*, 2010). Although its consequences remain unknown, the expression of PPAR β/δ has been reported to be increased in skin pathologies such as the lesional skin of psoriatic patients (Romanowska *et al*, 2010; Westergaard *et al*, 2003) and in human skin biopsies of pre-malignant and malignant skin carcinoma (Nijsten *et al*, 2005; Sertznig *et al*, 2008). In the latter, increased PPAR β/δ expression has been associated with a significant increase in microvessel density and strongly correlates with the expression of COX2, an enzyme implicated in skin carcinoma development (Trifan & Hla, 2003). Interestingly, our previous findings of PPAR β/δ participation in keratinocyte survival, proliferation and migration during skin wound healing (Tan *et al*, 2007) are somewhat analogous to the

Figure 6. PPAR β/δ upregulates pro-tumoural marker gene expression and laminin 332 deposition in actinic keratosis, enhancing SCC progression.

- mRNA expression of epithelial-to-mesenchymal transition (EMT) markers in 20 actinic keratoses with moderate atypia (grade II) of *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mice by real-time RT-PCR. Means $\pm$ SEM are presented; *p*-values are ***p* = 0.0077 (*Tgf β 1*), ****p* = 0.0009 (*Krt13*), **p* = 0.046 (*Vim*), **p* = 0.045 (*Snai1*), **p* = 0.032 (*Snai2*), **p* = 0.028 (*Gsc*), ***p* = 0.009 (*Itga5*), **p* = 0.025 (*Itga6*), **p* = 0.022 (*Itgb1*), **p* = 0.013 (*Itgb6*), **p* = 0.035 (*Cadhl2*), **p* = 0.020 (*Col7a1*), **p* = 0.035 (*Mmp19*), **p* = 0.025 (*Lam α 3*), **p* = 0.043 (*Mmp2*) calculated by two-tailed Student's *t*-test. The full gene names are given in supplementary Table S2.
- Representative immunofluorescence staining of PLA experiments for laminin 332/ β 4 integrin (red spots) in actinic keratosis with moderate atypia (grade II) sections from *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mice. DAPI, blue. Scale bars, 50 μ m.
- Representative immunofluorescence staining of PLA experiments for β 4 integrin/Rac1 (red spots) and laminin 332 in actinic keratosis with moderate atypia (grade II) sections from *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mice. DAPI, blue. Scale bars, 50 μ m.
- Quantification of PLA staining. Average number of PLA signals (red spots) per nucleus from *n* = 10 actinic keratoses with moderate atypia sections/genotype (**p* = 0.024, *t*-test).
- Actinic keratosis (AK) with grading of cellular atypia (mild, grade I; moderate, grade II; severe, grade III) and SCC distribution in 25 tumours collected from each *Ppar β/δ ^{+/+}* and *Ppar β/δ ^{-/-}* mouse graded histologically in a blinded manner according to Rowert-Huber *et al* and to the Broders' classification, respectively.

Figure 7.

tumour-development functions described in the current investigation, providing a further example of the molecular parallels between wound repair and cancer. It is intriguing to note that we and others have also described PPAR β/δ as a pro-differentiating factor in keratinocytes (Burdick *et al*, 2007; Peters & Gonzalez, 2009; Tan *et al*, 2001). Similarly, a pro-differentiation function has been attributed to the MAPK-Erk1/2 pathway in keratinocytes, a pathway that is clearly pro-carcinogenic in skin tumours (Rho *et al*, 2011; Seo *et al*, 2004). These apparently contradictory pro-differentiation and pro-carcinogenic roles may be due to different environmental stimuli and/or genetic contexts, which could lead to different kinetics and amplitude of Erk1/2 stimulation, switching the spectrum of their target proteins. This model could also accommodate a role for PPAR β/δ . Indeed, increasing evidence suggests that physiological or pathological contexts, including the genetic background, drive PPAR β/δ functions and might influence the extracellular signals, endogenous ligands produced, ligand-binding protein expression and post-translational modifications. These parameters are likely to affect the level and activity of PPAR β/δ and the expression pattern of its numerous target genes.

This hypothesized complexity could explain the between-study discrepancies regarding the role of PPAR β/δ in skin carcinogenesis. For example, Kim *et al* explored the anti-cancer properties of PPAR β/δ in skin using DMBA/TPA-induced carcinogenesis, a context in which tumour development was more severe in Ppar $\beta/\delta^{-/-}$ mice than in wild-type animals, suggesting that PPAR β/δ attenuates the development of chemically induced skin cancer (Kim *et al*, 2004). Inhibition of keratinocyte proliferation was thought to mediate this effect of PPAR β/δ mainly via the activity of protein kinase C α . However, in transgenic mice that over-express protein kinase C α , the susceptibility to DMBA/TPA-induced skin tumourigenesis was not affected (Cataisson *et al*, 2003; Wang & Smart, 1999). Moreover, induction of skin cancer by DMBA/TPA involves different molecular mechanisms from those associated with human skin cancer attributable to chronic UV exposure (Boukamp, 2005; Matsumura & Ananthaswamy, 2002). Notably, tumour initiation with a unique topical sub-carcinogenic dose of a genotoxic carcinogen (DMBA) primarily induces *Ha-Ras*-activating mutations, whose frequency reaches only 10–20% in human SCCs and basal cell carcinomas, reflecting a marginal contribution of this oncogene to these

carcinomas (Campbell *et al*, 1993; Lieu *et al*, 1991; Pierceall *et al*, 1991). In this context, our proposed model pathway could be masked by such *Ha-Ras*-activating mutations, rendering the MAPK Erk1/2 activation upstream independent of Src/EGFR stimulation.

Moreover, this apparent controversial function of PPAR β/δ in cancer development is also illustrated in other cancer types for which several studies have suggested an attenuation of carcinogenesis by PPAR β/δ , while others support a tumour-promoting role (reviewed in Michalik *et al*, 2004; Peters & Gonzalez, 2009; Peters *et al*, 2012). For instance, studies indicate that colon carcinogenesis is exacerbated in the absence of PPAR β/δ expression and/or that ligand activation of PPAR β/δ attenuates tumourigenesis (Harman *et al*, 2004; Hollingshead *et al*, 2008; Reed *et al*, 2004). In contrast, other studies have shown that genetic disruption of PPAR β/δ inhibits colorectal tumourigenesis and that ligand activation of PPAR β/δ promotes tumourigenesis (Gupta *et al*, 2004; Park *et al*, 2001; Wang *et al*, 2006; Zuo *et al*, 2009). Many of these studies are based on PPAR β/δ mRNA or protein expression levels, which do not necessarily reflect the ligand-dependent activity of the receptor. Interestingly, the expression of both PPAR β/δ and COX-2 in tissues of colorectal cancer patients leads to liver metastases, a condition associated with poor prognosis (Yoshinaga *et al*, 2011). PPAR β/δ gene expression is upregulated in patients with non-small cell lung cancer and correlated with the expression of *VEGFA* and *COX2*. Moreover, cell lines with relatively high PPAR β/δ activity (*e.g.* H441 and H358) exhibit proliferative and pro-survival responses, which are absent or minimal in cells with low levels of the receptor (*e.g.* A549) (Genini *et al*, 2012). In addition, PPAR β/δ , via its regulation of *ANGPTL4* gene expression, is suggested to be implicated in MDA-MB-231 cancer cell invasion into a three-dimensional matrix (Adhikary *et al*, 2012). These findings suggest a tumour-promoting function for PPAR β/δ , in accordance with our data on skin carcinoma, and this diversity of PPAR β/δ actions underscores the need for more work in mouse models and human tissue samples to decipher the complex PPAR β/δ functions in tumourigenesis. An illustration of this complexity is the importance of the isotype and expression levels of fatty acid-binding proteins in directing the same ligand towards RAR or PPAR β/δ . The latter promotes the pro-carcinogenic function of PPAR β/δ in breast tumour development (Kannan-Thulasiraman *et al*, 2010; Schug *et al*, 2007; Schug *et al*, 2008).

Figure 7. PPARB/D and SRC mRNA expression are correlated in human skin SCC and in other types of human carcinomas.

- A–C Correlation between PPARB/D and SRC (A), PPARB/D and *TGFB1* (B) and PPARB/D and *MMP19* (C) expression as assessed via real-time RT-PCR of RNA extracted from human SCC biopsies ($n = 9$). p -values are calculated by two-tailed Student's t -test.
- D,E Interaction between *TGFB1* and PPARB/D underlies SRC expression levels. All 42 SCCs were sorted by PPARB/D expression. Samples with either the highest ($n = 21$; top 50% of the samples) or lowest ($n = 21$, bottom 50% of the samples) PPARB/D levels were identified. (D) Least squares regression between *TGFB1* and SRC expression levels in samples with the lowest PPARB/D expression. The black line represents the least square fit. The observed linear regression coefficient (-0.23) was not significantly different from zero (Student p -value = 0.75). (E) Linear regression between *TGFB1* and SRC levels in the 21 tumour samples with the highest PPARB/D expression revealed a significant linear relationship.
- F Table presenting the interaction coefficient β_3 (estimate) and its 95% confidence interval for the linear model $SRC \sim \beta_0 + \beta_1TGFB1SRC + \beta_2PPARB/D + \beta_3TGFB1SRC:PPARB/D$. The summarized meta-analysis of the interaction coefficient was estimated using a random effects model.
- G Model of the molecular function of PPAR β/δ in UV-induced skin tumours. UV irradiation induces PPAR β/δ gene expression and activation. Once activated, PPAR β/δ drives the expression of Src, which correlates with an increased Src protein level and higher kinase activity. This activity leads to the activation of the Src-dependent EGFR/Erk1/2 signalling pathway, which drives the expression of genes involved in the epithelial-to-mesenchymal transition (EMT). In coordination with other PPAR β/δ -dependent or -independent mechanisms and/or genetic defects, this mechanism enhances skin tumour formation and progression upon UV exposure, identifying PPAR β/δ as a putative inducer of carcinoma.

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Src-centred investigations have thus far concentrated on the mechanisms of post-translational regulation of its kinase activity, but little is known regarding the factors regulating the expression of its gene. The observation that PPAR β/δ stimulates *Src* expression sheds new light on this powerful oncogene, and interest is growing in *Src* as a potential therapeutic target in skin carcinoma treatment (Serrels *et al*, 2009). Our study also raises fundamental biological questions related to cancer therapy. Inhibitors of *Src* are used in the treatment of many carcinomas but are associated with side effects (Kopetz *et al*, 2007). Because *Src* is a target of PPAR β/δ , specific antagonists of the receptor might be considered for the treatment of such cancers and/or prevention of malignant progression of actinic keratosis. Given that PPAR β/δ is a promising molecular target in the prevention and treatment of metabolic diseases such as dyslipidemia, obesity and diabetes, we suggest a careful evaluation of the use of high-affinity PPAR β/δ agonists such as GW501516, which is currently in phase IV clinical trials for the treatment of dyslipidemia (Ooi *et al*, 2011).

Materials and Methods

Animal model and UVB irradiation

SKH-1 hairless mice (Charles River) were crossed with SV129/BL6/*J* Ppar β/δ wild-type or Ppar β/δ ^{-/-} mice to obtain SKH-1/Ppar β/δ wild-type and SKH-1/Ppar β/δ ^{-/-} mouse strains. Mice were housed in quarters with a 12/12-h light/dark cycle and maintained with water and food *ad libitum*. For acute and chronic UV exposure, mice were UV irradiated on their backs with a GL40E 40W tube (SNEE), which emits most of its energy within the UVB range (90%; emission spectrum 280–370; 10% UVA). Doses of UVB (312 nm) and UVA (370 nm) were monitored using an appropriate radiometer.

For acute exposure, female SKH-1 wild-type and Ppar β/δ ^{-/-} mice ages 10–12 weeks were irradiated on their backs with the UV lamp. UVB radiation emission was controlled using a radiometer until a dose of 120 mJ/cm² was delivered. Non-irradiated age-matched mice were used as controls. Twenty-four hours after UV irradiation, animals were sacrificed using CO₂ gas, following by the acquisition of dorsal skin samples that were directly frozen in liquid nitrogen or prepared for histological analysis. For GSK0660 treatment, 200 μ l of GSK0660 (625 μ g/ μ l in 70% ethanol; Sigma, G5797) was applied topically on the back 1 h prior to UV exposure.

For chronic treatment, animals were irradiated on their backs three times per week with 70 mJ/cm² of UVB, which was monitored using a radiometer. The time of tumour appearance, number and size were monitored twice per week. Mice with one tumour reaching 9 mm in diameter were sacrificed using CO₂ gas in accordance with the requirements of the Veterinary Office of the Canton Vaud (Switzerland) and Federal Swiss Veterinary Office Guidelines. Non-irradiated age-matched mice were simultaneously used as controls and handled in the same fashion as the irradiated animals. Dorsal tumours or non-tumoural irradiated skin samples were then obtained and prepared as described above.

Tumour grading

Mouse skin tumours were paraformaldehyde-fixed and paraffin-embedded. Tissue sections (5 μ m) were stained with hematoxylin/eosin. Histological analysis of actinic keratosis and tumour

classification were performed in a blind manner by a pathologist. SCCs were classified according to the Broders' classification based on the degree of SCC keratinization and of keratinocyte differentiation (Broders, 1921). This classification is as follows: SCC Grade I: 75% keratinocytes are well differentiated; SCC Grade II: >50% keratinocytes are well differentiated; SCC Grade III: >25% keratinocytes are well differentiated and SCC Grade 4: <25% keratinocytes are well differentiated. Actinic keratoses were defined histologically and classified as grade I, II or III based on the degree of cytological atypia of epidermal keratinocytes and involvement of adnexal structures according to Rowert-Huber *et al* (Rowert-Huber *et al*, 2007). Representative pictures of actinic keratosis and SCCs are presented in supplementary Fig S12.

Human skin biopsies

Cutaneous SCC samples were obtained anonymously from the Department of Dermatology, University Hospital of Lausanne, Switzerland. Normal skin was from healthy adult volunteers or from the edges of skin tumours. SCC was diagnosed by experienced pathologists. Informed consent for research was obtained prior to routine diagnostic services. All samples include the dermis and the epidermis.

Preparation of expression data

We used expression microarrays from the Expression Project for Oncology Consortium (Affymetrix human genome HG-U133 plus 2.0 arrays) and from the Gene Expression Omnibus (accession code: GSE2109). Only the following tumour types were considered: breast ($n = 344$), lung ($n = 121$), liver ($n = 41$), pancreas ($n = 14$), colon ($n = 277$), ovarian ($n = 202$), prostate ($n = 67$) and kidney ($n = 259$). Other tumour types were excluded because of low sample numbers at the time of download.

Gene expression data were normalized with the robust multi-array method implemented in the library affymetrix. Three selection criteria were applied to probe sets: annotation to a defined Entrez gene, standard deviation of normalized expression greater than 0.1, and characterization as the most variable probe set for a given Entrez gene. The normalized, log-transformed expression measures were used for the analysis. For the OSCC dataset, because two datasets were available from the GEO database, optimal probe set selection among features mapped to TGFB1, SRC and PPARB/D was performed with the GSE30784 dataset and then applied in the GSE41613 (Chen *et al*, 2008; Lohavanichbutr *et al*, 2013). Only results obtained with the GSE41613 were considered in the main analysis. For the SSC dataset, the GSE32628 was taken using the normalized values provided by the contributor (Hameetman *et al*, 2013).

All data were analysed in the R programming environment (2.9.1). Linear regression models were used to assess the relationships between gene expression levels using additive and multiplicative models (interactions). Linear models were applied separately to each tumour type. A meta-analysis of the interaction coefficient estimates for the various tumour types was performed using a random effect model.

Src cDNA isolation, polyA-tailing and 5' RACE

To identify *Src* transcript isoform(s) induced by PPAR β/δ in skin and keratinocytes, *Src* mRNA was specifically reverse transcribed

with Superscript II (Invitrogen) using 50 ng of primer GSP1 (Gene Specific Primer 1, primer sequence given in supplementary Table S2) from 5 μ g of total RNA (extraction with TRIzol, Invitrogen) from a culture of wild-type primary keratinocytes treated for 24 h with GW501516 (100 nM) or total skin from wild-type SKH-1 mice acutely irradiated and sacrificed 24 h later. After treatment with RNase H (New England Biolabs) for 20 min at 37°C, first-strand Src cDNA was purified (Macherey-Nagel) before a polyA-tailing reaction in the presence of Tdt (Promega) and ATP. A first, PCR was performed using Frohmann and GSP2 (Gene Specific Primer 2) primers (Fig 3; sequences given in supplementary Table S2) using Herculanase II Fusion DNA Polymerase (Agilent Technologies) according to the manufacturer's instructions. A nested PCR was then performed with Q0 and GSP3 (Gene Specific Primer 3) primers (Fig 3; sequences given in supplementary Table S2) using Herculanase II Fusion DNA Polymerase (Agilent Technologies). PCR products were then purified (Macherey-Nagel) and loaded onto agarose gels for identification of the Src transcript(s). GSP1, GSP2 and GSP3 primers were designed to specifically amplify a region corresponding to Src-001, Src-007, Src-005, Src-006 or Src-201 transcripts described in Ensemble. The DNA bands revealed were then excised and purified before sequencing.

PPRE subcloning, mutagenesis and promoter activity assay

To analyse Src promoter activity, various regions of the promoter (annotated from the transcriptional start site in the mouse genome) were inserted into the plasmid vector pGL4 (Promega, E8421) to generate pGL4-PPRE1, pGL4-PPRE2, pGL4-PPRE3, pGL4-PPRE4 and pGL4-PPRE5. pGL4-PPRE mutant plasmids carried mutated PPRE sequences introduced with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Primers used for mutagenesis are given in supplementary Table S1. NIH3T3 cells were plated the day before transfection in 12-well plates. Either 0.8 μ g of wild-type or mutated pGL4-PPRE or 0.5 μ g of 3 $\times$ -PPRE-TK-Luc (gift from R. Evans) plasmids were introduced into the cells via Superfect (Qiagen), together with 0.5 μ g of pSG5-mouse-PPAR β/δ and 0.025 μ g of a Renilla luciferase construct. After overnight serum starvation, transfected cells were treated for 24 h with GW501516 (100 nM) and then lysed in 1 $\times$ passive lysis buffer and analysed for dual luciferase activity following the manufacturer's instructions (Promega). Relative luciferase activity was calculated as the ratio of firefly to Renilla luciferase activity. Data represent means $\pm$ standard deviations of relative luciferase activities for DMSO performed in triplicate.

Immunoblotting

Cells and tissue samples were lysed in TNE buffer containing 20 mM Tris [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% NP-40, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin and 1 mM Na₃VO₄. After quantification, proteins were separated by SDS-PAGE and subjected to immunoblotting. All primary antibodies were incubated overnight in 1 $\times$ Tris-buffered saline plus 0.1% Tween-20 and 5% bovine serum albumin at a dilution of 1:1000 except where indicated otherwise. Cell Signalling was the source for the following antibodies: Src (2123), phospho-Tyr845 EGFR (6963), EGFR (4267), phospho-Erk1/2 (4370), Erk1/2 (9102), β -Catenin (8480), phospho-Ser675 β -Catenin (4176), Slug (9585) and GAPDH (2118). Anti- β -tubulin and N-Cadherin were purchased from BD Biosciences (556321) and Millipore (04-1126; 1/50 000), respectively.

ChIP and re-ChIP

ChIP experiments were carried out with modifications of the experimental setup described by Tan *et al* (Tan *et al*, 2004). Briefly, chromatin was crosslinked using 0.5% formaldehyde for 10 min at 37°C and sonicated in SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1) to obtain crosslinked DNA that measured 200–500 bp in length. Approximately 10% of the supernatant was retained as input, while the remaining amount was processed with the ChIP using anti-PPAR β/δ antibody (4 μ g; SC-7197, TransCruz Grade; Santa Cruz), and complexes were pulled-down by Protein A/G (Santa Cruz, CA, USA). Re-ChIP was performed by subsequent probing with anti-p300 antibody (4 μ g; 05–257, Millipore); DNA fragments were reverse crosslinked at 65°C for 6 h. The pre-immune complex served as negative control. The ChIP primer sequences are listed in supplementary Table S3. Downregulation of PPAR β/δ in MK cells was performed using a siRNA construct against mouse *Ppar β/δ* (L-042751-01, Thermo Scientific), as recommended by the manufacturer (Dharmacon, Thermo Scientific). Briefly, MK cells were seeded in 10-cm dishes and transfected with 25 nM of siRNA in DharmaFECT1 transfection reagent (T-2001-03, Thermo Scientific). MK cells transfected with scrambled siRNA were used as controls. The efficiency of KD was tested by real-time PCR 48 h later (supplementary Fig S5E).

Immunohistochemistry and immunofluorescence

Src, Ki67 and Keratin 13 staining was performed on tissue sections (5 μ m) from paraformaldehyde-fixed, paraffin-embedded skin. Briefly, sections were quenched with 3% H₂O₂. Ki67 (Abcam, 15580) staining was performed as described by the manufacturer. For Src and Keratin 13 staining, the antigen-retrieval step was achieved in 0.1 M citrate buffer [pH 6] via microwave. After washing, sections were incubated in normal goat serum 1% for 30 min and probed with Src (Cell Signalling; 2109; 1:250) and Keratin 13 (Abcam; ab92551; 1:100) antibodies overnight at 4°C or at room temperature in blocking buffer, respectively. After washing, sections were incubated for 30 min with anti-rabbit horseradish peroxidase secondary antibody (EnVision+ rabbit-HRP; Dako; K4002). Visualization was performed with diaminobenzidine substrate (Vector Labs) for 14 min before being counterstained with Mayer hematoxylin and mounted. Keratin 10 and Keratin 14 co-staining and N-Cadherin staining were performed as described above with the following modifications. Tissue sections were probed with Keratin 10 (Progen; GP-K10; 1/200), Keratin 14 (Covance; PRB-155P; 1/1000) or N-Cadherin (Millipore; 04-1126; 1/50) overnight at 4°C, washed, and then incubated 30 min at room temperature with Goat anti-guinea-pig A488 (Molecular Probes; A11073; 1/200) and Goat anti-rabbit A568 (Molecular Probes; A21069; 1/1000), for Keratin 10/Keratin 14 co-staining or Goat anti-rabbit A568 (Molecular Probes; A21069; 1/1000) for N-Cadherin staining. After washing, counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI) for 5 min at RT.

Double staining for laminin 332 and pan-Cytokeratin was performed from OTC-embedded frozen skin samples. Briefly, sections were defrosted and incubated in acetone for 10 min, washed and blocked for 1 h in 1% normal goat serum/5% bovine serum albumin. Sections were probed overnight at 4°C with anti-laminin 332 (Abcam; 14509; 1:200) and anti-pan-Cytokeratin (BMA-T1341; 1:250) in blocking buffer. After being washed, sections were

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incubated for 30 min with anti-mouse STAV-Cy3 (Zymed; 43-4315; 1:1800) and anti-rabbit A488 (Molecular Probes; A111034, 1:2000) diluted in blocking buffer. Sections were then washed three times and incubated 5 min with DAPI (Sigma; D95542; 1:5000) and washed again before being mounted.

PLA and immunofluorescence

PLA experiments were performed in accordance with the manufacturer's protocol (Olink Biosciences, Sweden) using pairs of antibodies targeting the protein–protein interactions of interest. The antibodies were laminin 332 (1:20; Abcam, ab14509), active integrin $\beta 4$ (Abcam, ab29042; 1/50) and Rac1 (Abcam, ab97732; 1/20). PLA images were taken using the LSM 710 confocal microscope (Carl Zeiss, Germany) with the Plan-Apochromat 40 $\times$ /1.4 oil differential interference contrast objective. Analyses were performed with the ZEN 2009 Light Edition software (Carl Zeiss, Germany). Quantification of PLA signals was performed with BlobFinder version 3.2 (Allalou & Wahlby, 2009). Immunofluorescence for laminin 332 (1/250) was performed after PLA for integrin $\beta 4$ and Rac1 as previously described.

Epidermis and dermis separation for RNA extraction

Epidermis and dermis were prepared from whole dorsal skin of acutely irradiated mice according to the protocol developed by Clemmensen *et al* (Clemmensen *et al*, 2009), with some modifications. After dorsal skin harvest, adipose tissue was removed with a scalpel on ice, and the skin was cut into small pieces (1–2 mm) and immediately incubated for 15 min at RT in ammonium thiocyanate 3.8% in 1 $\times$ phosphate-buffered saline. Epidermis and dermis were then separated mechanically with forceps in TRIzol. After RNA extraction and quality control by Bioanalyser, RT-PCR and qPCR were performed as described below to generate cDNA. Specific markers of the epidermis (Keratins 10 and 14) and dermis (Collagen 4 $\alpha 1$) compartments were tested by qRT-PCR to check the proper separation of the two in each experiment (supplementary Fig S2D).

Statistics summary

Unless indicated otherwise, all data are presented as the means $\pm$ standard errors of the mean, and statistical differences were evaluated by two-tailed Student's *t*-tests. For all analyses, we considered *p* < 0.05 to be statistically significant.

Study approval

Collection of human skin biopsies was approved by the local (Centre Hospitalier Universitaire Vaudois) and cantonal (Canton de Vaud) research ethics committees. All experiments involving animals were approved by the Veterinary Office of the Canton Vaud (Switzerland) in accordance with the Federal Swiss Veterinary Office Guidelines. All experiments conform to European Commission Directive 86/609/EEC.

Supplementary information for this article is available online: <http://embomolmed.embopress.org>

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The paper explained

Problem

The incidence of non-melanoma skin cancer (NMSC) is increasing at a high rate, with over one million estimated new cases each year in the United States alone. Squamous cell carcinoma (SCC) and basal cell carcinoma, which both derive from keratinocytes, account for approximately 96% of all diagnosed skin cancers. Although mortality from NMSC is low, morbidity is high and is accompanied by a heavy personal burden and enormous rising costs for society. NMSCs are associated with excessive and/or chronic exposure to ultraviolet (UV) radiation, especially UVB, and mainly occur on sun-exposed areas. Despite the prevalence of NMSCs, the molecular mechanisms underlying their progression remain poorly defined. In skin, roles for the peroxisome proliferator-activated receptor beta/delta (PPAR β/δ), a member of the nuclear receptor superfamily, have been emphasized by its implication in keratinocyte adhesion, migration and survival during tissue repair. These processes also participate in skin cancer progression. However, whether PPAR β/δ is pro- or anti-carcinogenic remains controversial because both effects have been observed. We therefore investigated *in vivo* whether PPAR β/δ might be involved in UV-induced skin carcinoma, using SKH-1 hairless albino mice lacking PPAR β/δ activity, a relevant model for studying human UV-induced skin cancer.

Results

In vivo and *in cellulo*, we characterized a cascade of events involving PPAR β/δ as a regulator of oncogene Src transcription. We found that UV induces PPAR β/δ activity, which in turn stimulates Src gene expression, resulting in increased Src protein expression. We further demonstrated that UV exposure activates Src and enhances the EGFR/Erk1/2 signalling pathway, resulting in increased PPAR β/δ -dependent pro-tumoural gene expression. In line with this, PPAR β/δ -null mice developed fewer and less advanced tumours compared to wild-type mice. Moreover, topical application of a PPAR β/δ antagonist prevented UV-dependent stimulation of the Src/EGFR/Erk1/2 signalling pathway. In human SCCs, PPARB/D expression was also positively correlated with SRC expression. In a wide variety of human carcinomas from various tissue origins, we identified an interaction among PPARB/D, SRC and TGFBI at the transcriptional level, which suggests a broad pro-tumourigenic potential of PPAR β/δ via SRC upregulation.

Impact

Our present work motivates the future evaluation of PPAR β/δ antagonists as putative anti-cancer drugs that could be used to prevent skin carcinoma progression and, potentially, epithelial cancers from other tissue origin. Interestingly, Dasatinib (Sprycel), a dual BCR/ABL and Src family tyrosine kinase inhibitor previously used in patients with acute myeloid leukaemia, is currently being assessed in a phase II clinical trial for its ability to inhibit Src activity and treat SCC. The identification of PPAR β/δ as a regulator of Src gene expression will further help to refine this therapeutic strategy.

In addition, interest is increasing in PPAR β/δ agonists for treating patients with metabolic syndrome through a stimulatory effect on lipid oxidation in skeletal muscle. A PPAR β/δ agonist is currently being tested in a phase IV clinical trial with obese patients showing dyslipidemia. Our data underscore the necessity of a careful evaluation of the use of high-affinity PPAR β/δ agonists, especially in conditions necessitating long-term treatment.

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Author Contributions

AM and MBD designed and performed experiments and analysed the data. CTB, HM, JSKC, MKS, CM, GD, MA and MB performed experiments. TAL, YL, SW and NST provided material and were involved in manuscript preparation. DH and TAS screened and graded mouse tumours. Biostatistics analysis of human tumour mRNA expression profiles was conducted by PJF. LM and WW supervised the project, designed experiments and analysed the data. This manuscript was written by AM, LM and WW. All authors reviewed the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Review

Probing for protein-protein interactions during cell migration: limitations and challenges

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Summary. Cellular migration is a fundamental biological process occurring as early as embryogenesis to the pathological state of cancer metastasis. Nearly all cellular migrations involve an extracellular signal that is transduced internally by members of a signalling cascade. These signal transduction events are driven by protein-protein interactions (PPIs) that coordinate intracellular activities to enable a cell to migrate. Understanding these PPIs will provide valuable insight into how cellular migration can be modulated perhaps towards a therapeutic end. Histologically, not many techniques are available to demonstrate PPIs. Contrasting agents only demonstrate the presence of a particular protein, and perhaps its co-localisation with another protein. Yet, co-localisation need not necessarily indicate physical interaction between the two proteins. In this review, we highlight four commonly used methods that continue to expand our understanding of PPIs underlying cell migration: co-immunoprecipitation, bimolecular fluorescence complementation, proximity ligation assay and surface plasmon resonance. The methods discussed herein allow for the study of PPIs in a wide variety of biological samples, including cell lysates, live cells, fixed cells and tissues, enabling the quantification of endogenous PPIs and exploration of the nature of PPIs. We also include a rudimentary framework for researchers to decide which experimental method best suits their research goals.

Key words: Co-immunoprecipitation, BiFC, SPR, PLA

The importance of protein-protein interactions (PPIs) and cellular migration

The central dogma dictates that the genome is transcribed into mRNA and subsequently translated into protein (Crick, 1970). James Watson himself, in a keynote address at the Miami Nature Biotechnology Winter Symposium in 2003, referred to DNA as "the script" and proteins as "the actors" in the complex play of life. Although reverse transcription, epigenetic regulation, prions, catalytic and non-coding RNA highlight novel conduits by which genetic information flows, the central dogma still remains one of the most enduring tenets of molecular biology (Mattick, 2003; Shapiro, 2009). Intrinsically, proteins are the crucial workhorses that facilitate the manifestation of a phenotypic end point. The presence, absence, mutation or modification of a particular protein will define the line between normal and pathophysiology. For example, in sickle-cell disease, a single amino acid substitution (glutamic acid to valine) at position 6 in the beta-subunit of the haemoglobin protein promotes haemoglobin aggregation under hypoxic conditions, which leads to the characteristic sickle-shaped erythrocytes and anaemia (Kassim and DeBaun, 2013). However, not every event in biology can be easily attributed to a single protein. A large majority of proteins do not function in isolation but rather require interaction with other proteins (homomeric or heteromeric) to exert an effect on cellular behaviour (Gandhi et al., 2006). The study of PPIs thus becomes

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paramount to understanding the molecular underpinnings of health and disease. However, the detection and interpretation of these PPIs is challenging. The cell-type specific, time- and context-dependent expression of proteins and their binding partners, as well as their sub-cellular localisation, post-translational modifications and conformational changes before and after binding are all critical parameters that act in a combinatorial manner to allow for subtle fine-tuning of biological responses at all levels of physiology (Chautard et al., 2009; Zinzalla and Thurston, 2009).

The complexity of studying PPIs is further compounded by the individual nature of each PPI that occurs. PPIs are graded between the extremes of stable and transient interactions (Phizicky and Fields, 1995). Stable PPIs are those found among proteins belonging to a functional complex (e.g., haemoglobin, nucleoporins, integrin-ECM) and that form by default, i.e., the ideal complementary contacts among binding partners are intrinsically coded for by amino acid sequences, and thus, interactions occur with negligible extrinsic influences such as post-translational modifications. Correspondingly, stable PPIs are often retained over a longer time-course and are not easily disrupted. Such PPIs are less challenging to detect using conventional molecular techniques.

In contrast, transient PPIs typically require specific stimuli to encourage exposure of ideal complementary contacts before binding can occur. As implied, transient PPIs are not durable, because successful formation of the PPI results in a conformational change or interactions with other factors that undermine the precondition for binding (e.g. G-protein activation and deactivation, kinase phosphorylation and phosphatase dephosphorylation). Thus, transient PPIs play an important role as molecular switches that enable stringent and precise control of intracellular signal transduction. The temporary nature of such PPIs can pose tremendous difficulties in their detection. Nonetheless, a transient PPI can be detected with less difficulty in a particular cellular context if it occurs more frequently. Most biological events require the coordination of stable and transient PPIs for correct execution. Cellular migration, the focal biological event of our review, is no exception to this rule.

Nearly all cellular migrations involve an extracellular signal that is transduced internally by transient PPIs among members of a signalling cascade. These upstream initiators and effectors of migration are extremely diverse and remain the subjects of intense study and discovery. In contrast, downstream responses such as relinquishment of stable cell-ECM contacts and replacement by less stable (more transitory) cell-ECM contacts at the leading and withdrawing edges of the migrating cell appear more generic and are better understood (Palamidessi et al., 2013; Wehrle-Haller, 2012). Repeated iterations of these changes in PPIs eventually dictate when and where a cell should migrate to. During prenatal development, definition of the germ

layers of an early embryo already requires protein-directed cellular migration (Enders et al., 1978; Lawson and Pedersen, 1987; Poelmann, 1981). Rolling adhesion, subsequent extravasation and chemotactic movement of circulating immune cells to the site of infection within tissue also depends on appropriate PPIs between cells and the extracellular matrix (ECM) (O'Neill et al., 2000; Pribila and Shimizu, 2003; Yong and Khwaja, 1990). Wound repair and angiogenesis, which are key processes in the maintenance of organ integrity, can only occur correctly when keratinocytes and endothelial cells receive the appropriate signals for migration (Kim et al., 1994; Decline and Rousselle, 2001; Master et al., 2001). Such migration events during normal physiology also have parallels in pathology, and several diseases arise from or are exacerbated by deregulation of normal cell migration. Cancer, one of the most widely-diagnosed diseases of the 21st century that accounted for up to 13% of worldwide deaths in 2008 according to the World Health Organisation (WHO), sustains its nefarious growth by establishing its own network of blood vessels through de novo angiogenesis (Bergers and Benjamin, 2003; Chung et al., 2010). The most aggressive of cancers culminate their malignant progression in invasion and metastasis, which, in essence is a migratory incident (Hood and Cheres, 2002; Roussos et al., 2011; Sethi and Kang, 2011).

It becomes apparent that PPIs and their associated signalling networks largely control migration in varying contexts. Precisely probing for the PPIs that occur during cellular migration can thus reveal pertinent therapeutic interventions to promote or prevent migration. Although many techniques have been developed to test for PPIs, herein we feature the most commonly and easily used experimental methods, old and new, that have contributed to and are currently expanding our understanding of these interactions in cell migration. We broadly categorise these methods into probe-based and direct-labelling techniques and evaluate each technique's advantages, limitations and future potential in elucidating PPIs that are pertinent to cell migration.

Methods for probing PPIs

Co-Immunoprecipitation (Co-IP)

Classically, the study of PPIs has been probe-based and driven by antibody-dependent approaches such as Western blotting, pull-down assays and a combination of these two methods, co-IP. These methods are contingent upon the availability of a specific recognition domain on an antibody that is raised against a particular epitope, which even allows for the specific capture of a protein based on its status (e.g., its activation state or presence of any post-translational modifications). Known as the gold standard in its field of assays, co-IP remains a popular method for the detection of PPIs. The idea of co-IP stems from using a specific antibody to purify and

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detect antigens on a small-scale basis. The captured protein is immobilised onto a Protein A or G beaded support via the protein-specific antibody. From this stage, a co-IP procedure would then be able to pull-down any other protein-binding molecules from complex solutions through native interactions (see Figure 1). The power of co-IP comes from the modifiability of the assay to capture transient interactions that would otherwise be missed, or to map specific chains of events during the signalling cascade of interest.

The flexibility of co-IP was demonstrated by Master

et al in the characterisation of angiopoietin-1-dependent endothelial cell (EC) migration in angiogenesis (Master et al., 2001). Using antibodies that target phospho-proteins, in this case phospho-Dok-R, the authors demonstrated that the stimulation of EC migration by angiopoietin-1 is dependent on sequential PPIs between Dok-Nck-Pak that are involved in phosphorylation and activation of the indicated proteins. The versatility of co-IP was further extended to demonstrate that phosphorylation on a single tyrosine residue in Dok is necessary to activate this signalling axis to enhance EC

Fig. 1. Schematic depicting the underlying principle of co-immunoprecipitation (Co-IP). Cell lysates are incubated with bead-coupled antibodies specific to Protein A for immunoprecipitation. Protein B which physically interacts with Protein A remains bound while non-interacting proteins are washed off and the beads are pelleted by centrifugation. After elution, immunoblotting for Protein B is performed. A darker band appearing in the immunoblot for the migrating cells indicate that the protein interaction is enriched in cell migration.

migration. By mutating the tyrosine residue in Dok to remove the phosphorylation and activation of Dok, all sequential downstream PPIs were undetectable by co-IP, and the subsequent EC migration was impeded. This result highlights the ability of co-IP as a powerful tool to pinpoint PPIs to the resolution of a single amino acid. Co-IP is thus able to piece together crucial PPIs in a pair-wise manner to account for a migratory event through the use of specific antibodies that detect activated or other post-translationally modified members, within a protein complex. In addition, co-IP can highlight the domain architecture that is necessary for binding, whereas precise mutagenesis enables the detection of key single amino acid residues that anchor an entire protein complex. These versatile properties of a co-IP-based experiment can be particularly useful in peptide drug discovery which is aimed at mimicking native protein interactions that impact cellular migration.

Re-epithelialisation and migration of keratinocytes during wound healing is a complex process that requires the coordination of keratinocyte-ECM contacts and re-organisation of the cytoskeleton for directional motility. Sehgal et al used co-IP to highlight that integrin $\alpha 6 \beta 4$, once thought to be a minor player in laminin-332 patterning and deposition during keratinocyte migration, in fact plays a central role in enabling directional cell motility (Sehgal et al., 2006). Keratinocytes at the wound edge lay down the laminin-332 tracks on which they travel, and integrins $\alpha 3 \beta 1$ and $\alpha 6 \beta 4$ on keratinocyte plasma membranes are cognate binding partners of laminin-332 (Nguyen et al., 2000). Integrin $\alpha 3 \beta 1$ was first thought to be the major determinant of keratinocyte migration (deHart et al., 2003; Goldfinger et al., 1999). However, in the experimental context of dominant negative Rac-1 and Rac-1 inhibitors, a lack of Rac-1 activity caused circular laminin-332 deposition, which was an impediment to proper keratinocyte migration into the wound. Subsequently, a co-IP experiment clarified that integrin $\alpha 6 \beta 4$ is responsible for recruiting and activating Rac-1. In contrast, integrin $\alpha 3 \beta 1$ failed to co-precipitate with Rac-1, and Rac-1 recruitment by integrin $\alpha 6 \beta 4$ in turn activates cofilin to induce plectin/actin cytoskeletal rearrangements, which permitted integrin $\alpha 6 \beta 4$ to pattern laminin-332 deposition in a linear fashion for directional keratinocyte migration. In essence, co-IP has allowed the authors to link a less recognised integrin with Rac-1, and the latter's associated pathways, thus broadening the influence of integrin $\alpha 6 \beta 4$ in regulating the more subtle points of keratinocyte migration, for instance, the directionality of cellular movement.

Despite the utility and versatility of co-IP in (i) identifying PPIs, (ii) detailing the interfacing residues or unique characteristics of PPIs and (iii) associating PPIs with cellular migration in various contexts, co-IP does have limitations (Phizicky and Fields, 1995). The major limitation of co-IP is the inability to distinguish between direct PPIs or PPIs in a complex. For example, *in vivo* Protein A interacts with Protein B through protein C.

However, co-IP results will indicate that protein A interacts directly with B and would yield a false positive result (see Figure 1). Therefore, conclusions of direct PPI that are derived only from co-IP should be verified by another method that overcomes this limitation. Additionally, one crucial parameter is conspicuously missing from the co-IP experiments – the location within a cell where the PPI is occurring. This problem is inevitable because co-IP involves only proteins that are derived from cell lysates, therefore obviating any preservation of cellular structure and thus compartmentalisation of proteins. As such, non-physiological interactions may occur between proteins that are normally located in distinct cellular compartments. It is thus advisable that co-IP be performed in conjunction with immunofluorescence staining of the proteins involved in the PPI being probed to ascertain that the interacting proteins share the same intracellular compartment. In the Dok-R example, immunofluorescent staining followed by confocal imaging was performed to verify that Tek, Dok-R, Nck and Pak indeed co-localised and were found at the cell periphery of migrating ECs.

Given the myriad of proteins that are found in cell lysates, non-specific binding of unwanted proteins to the IP antibody is also possible. To minimize non-specific PPIs, one can enrich proteins in the pseudopodia protruding from the cell body compartment using polycarbonate microporous filters. Pseudopodia and cell bodies are then differentially scraped from the filter surface into lysis buffer for biochemical analysis. Using this method, it is possible to identify novel pseudopodia and cell body proteins, as well as study the spatiotemporal organization of signaling processes that regulate pseudopodium formation and cell polarity (Cho and Klemke, 2002; Tan et al., 2007; Wang and Klemke, 2007). In addition, thorough washing steps are typically involved to disrupt these weak interactions. Researchers can optimise the ionic strength of the buffers that are used in the assay, decrease the amount of target-specific antibody that is used to reduce signal noise, or pre-clear the samples by introducing non-specific antibodies to the cell lysate mixture prior to the actual IP steps.

Another consideration of a co-IP experiment is antibody contamination. The heavy and light chains of the antibody could be co-eluted with the complex of interest and will obscure the results on subsequent reducing SDS-PAGE runs. To circumvent this problem of antibody contamination, researchers may experiment with covalent immobilisation of the antibody to the beaded support. In this setup, beaded supports that are produced commercially can bind the primary amine groups on the antibodies, such that the antibody is permanently bound to the support. Another possibility for crosslinking the antibody to the Protein A/G-attached supports is through the use of crosslinkers, such as bisulfosuccinimidyl suberate (BS3) or its non-water soluble analogue, disuccinimidyl suberate (DSS). In this manner, covalent amide bonds are formed between the

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antibodies and the beaded support. These methods aim to reduce the occurrence of antibody contamination during the analysis of results.

Finally, researchers may face the difficulty of ensuring a stable physiological environment for a PPI of interest to occur. This issue is perhaps most apparent when multicolour immunofluorescence staining identifies strict co-localisation of proposed interacting partners that cannot be proven to physically interact via co-IP. The PPI that is formed is likely weak or transient and unable to withstand the mechanical and chemical stresses that are exerted on it during the co-IP assay; this weakness severely limits the sensitivity of co-IP. The incorporation of a fixation or cross-linking step may be necessary prior to cell lysis to stabilise such PPIs. The stringency of wash steps after immunoprecipitation may also need to be reduced. These modifications to a typical co-IP protocol would require optimisation to ensure that false positives do not surface with the reduction of wash stringency, and that epitopes are not masked by fixation or cross-linking.

The continued reliance on these antibody-based

methods in many current laboratories is a testament to their reliability, and many of the newer techniques that have been developed to probe PPIs still leverage on antibody specificity. The proximity ligation assay (PLA) is a prime example of one such novel antibody-based technique and will be discussed in greater detail later in this review.

Bimolecular Fluorescence Complementation (BiFC)

BiFC is an *in vivo* direct-labelling technique based on the principle of fluorescence complex formation upon the interaction of two protein molecules (see Figure 2). Two putative interacting partners are individually fused to a fragment of a fluorescent protein. Upon interaction, the fluorescent protein complex reassembles and fluoresces (Kerppola, 2008; Kodama and Hu, 2012). Unlike FRET, BiFC does not involve radiative transfer of energy and therefore the obtained signal is stronger and offers a directly measurable readout. Because PPIs occur within live cells, the compartmentalisation of the interacting protein partners is retained, and the sub-

Fig. 2. Schematic depicting the underlying principle of bimolecular fluorescence complementation (BiFC). Cells must first be transfected to exogenously express both proteins of the prospective interaction as fusion proteins. Each protein is fused with either the N- or C-terminus of a split reporter which refolds into a functional fluorophore when the PPI of interest occurs. Appearance of fluorescence signals at the leading edge of a migrating cell confirms the subcellular localisation where PPIs are formed. BiFC can be coupled with FRET to identify higher order interactions involving three or more proteins. Refolding of the fluorophore when two proteins interact results in fluorescence emission that excites a second fluorophore fused to a third interacting partner. The emission of the BiFC fluorophore is quenched while the emission of the FRET fluorophore is enhanced.

cellular localisations of the PPIs can be resolved by confocal microscopy. Thus, if an interaction is detected by co-IP but not observed in a BiFC experiment, it could be an indication that co-IP has picked up a non-native PPI between partners that share distinct sub-cellular compartments in an intact cell. Conversely, the two proteins could interact, but in a different orientation that does not allow the two fragments of the fluorescent protein to assemble into a functional protein.

A wide variety of split-fluorescent reporters are now commercially available, including split-CFP, YFP, GFP and mDsRed, as well as their respective modified variants with superior performance (brighter fluorescence, higher signal-to-noise ratio) (Kodama and Hu, 2012). Using split-fluorescence reporters with non-overlapping emission spectra enables the simultaneous monitoring of multiple complex formations in the same cell. More recently, the repertoire of split-fluorescent reporters has been further expanded with the development of the near-infrared BiFC reporter iSPLIT, which was modelled after the PAS and GAF domains of the bacterial phytochrome RpBphP2 and improved upon by directed evolution (Filonov and Verkhusha, 2013). iSPLIT widens the spectral range and thus the number of split-fluorescence reporters that can be used simultaneously, and it further enables imaging of PPIs in live animals because its excitation and emission spectra lie beyond the wavelengths of the autofluorescent compounds found in animal tissues.

In the context of cell migration, BiFC demonstrated the importance of the integrin-linked kinase (ILK) and p38 β interaction in promoting serum-induced bladder cancer cell migration. Yu et al. demonstrated that ILK and p38 β elevation in invasive bladder cancer tissues as opposed to that of normal bladder urothelium (Yu et al., 2014). Interestingly, the specific inhibition of ILK activity institutes a selective loss of p38 β in bladder cancer cells. ILK phosphorylation of p38 β at specific residues was later shown to confer protection against the proteosomal degradation of p38 β . Live-cell imaging of bladder cancer cells transfected with p38 β and ILK that were fused with the N- and C-termini of Venus, respectively, indicated that ILK specifically interacts with p38 β in the cytoplasm. The advantage of using BiFC is twofold. First, an overexpression of the interacting partners tends to accentuate a phenotypic readout, which leads to the ability of BiFC to prove a successful PPI and the subsequent cellular phenotype. Second, in contrast with co-IP, BiFC provides an insight into the sub-cellular localisation of PPIs, and rules out interactions between proteins that do not naturally share an intracellular space or organelle because the assay requires the use of intact, living cells by default. In addition, when a truncated or loss-of-function ILK mutant was fused to the C-terminus of Venus, and subjected to a BiFC assay with p38 β , it was definitively shown that the kinase domain is crucial for the interaction with p38 β . Similar to co-IP, the option of truncation and mutagenesis of proteins that are involved

in a PPI of interest is absolutely viable to identify the protein domains responsible for the PPI. Depletion of either ILK or p38 β resulted in a substantial decrease in serum-directed chemotaxis of the bladder cancer cells, thus confirming a causal relationship.

Despite its utility in identifying PPIs *in vivo* using live cells with sub-cellular localisation resolution, BiFC does have its limitations. The often irreversible reassembly of the fluorescence reporter protein stabilises transient, native PPIs, making it possible to visualise them. However, the drawback to this is that the dynamics of interactions are lost because permanent reassembly of the split-fluorescent probe unnaturally prolongs the very interaction being studied. Furthermore, the absence of a BiFC signal does not necessarily mean that PPIs did not occur. Steric hindrances could lead to a non-optimal orientation of the two fragments of the fluorescent protein to assemble into a functional fluorescent protein, even though the proteins-of-interest have interacted. To work around this, a flexible linker region is sometimes incorporated into the fusion construct, thus allowing sufficient range of molecular motion to drive refolding of the fluorescent reporter (Kerppola, 2008).

BiFC also assumes that expression of the split-fluorescent reporter that is fused to the putative interacting partners does not interfere with the native structures of the proteins of interest. Alterations in native protein structure can cause residues required for stabilising a PPI to be obscured, which would result in a false negative readout. A fusion protein with abnormal structure may also expose residues to allow for the formation of non-native PPIs that out-compete the native interaction or cause non-native interactions to be interpreted as legitimate. A BiFC experiment should be complemented with immunocytochemistry and co-IP to definitively prove the localisation and existence of a PPI between two proteins of interest.

Another issue to consider in a BiFC experiment is that the extent of expression of the fusion proteins cannot be strictly controlled even when expressed from an inducible promoter. This is a problem that is inherent to any experiment in which overexpression from an exogenous construct is involved and is not unique to the BiFC method. Non-physiological levels of fusion protein expression could strain the cell transcription and translation machinery, which could lead to cell death or the induction of an abnormal cellular state. Alternatively, high non-endogenous expression of fusion proteins may result in fluorescent reporter refolding that is independent of PPI formation, giving a false positive result (Kerppola, 2008). As with any experiment, optimisation of the transfection efficiency or induction of fusion protein expression should be ensured.

The principles behind FRET and BiFC allow both methods to be used concurrently to further increase the number of PPIs that can be observed simultaneously. Most PPI detection methods only detect interactions between two proteins at a time. Yet, some PPIs occur in

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ternary complexes and involve three or more proteins that are in physical contact with one another. An example of this situation is the formation of the pSmad2/3-TAP63-p53 complex in cholangiocarcinoma cells that promotes tumour cell migration, in addition to its survival (Lu et al., 2013). However, visualisation techniques for studying such ternary complex formations are lacking.

Recently, Shyu and colleagues established a BiFC-based FRET (BiFC-FRET) visualisation technique to study the formation of ternary complexes in living cells (Shyu et al., 2008). The BiFC-FRET technique encompasses the use of two mutant CFP and YFP proteins, known as Cerulean and Venus, respectively. In general, two proteins are conjugated to the two non-fluorescent fragments, as in the original BiFC technique. When an interaction successfully occurs, the two non-fluorescent fragments fuse to create the Venus fluorescent protein. In the presence of a third protein, which is fused to the Cerulean fluorescent protein, an interaction with the initially formed heterodimeric protein would bring Cerulean (FRET donor) into close proximity to Venus (FRET acceptor) and allow a successful FRET to occur. The visualisation of FRET in this case will be evident only upon the formation of a ternary complex. In their report, the team identified the formation of ternary complexes such as Jun-Fos-NFAT1 and Jun-Fos-p65 (Shyu et al., 2008).

An alternative method to detect and study ternary complex formation is known as the FRET-Fluorescence lifetime imaging microscopy (FLIM) assay (Kinoshita et al., 2007). This assay also relies on a FRET-based theory: two proteins that are part of a ternary complex are each conjugated to a chromophore. Changes in FRET signals in the presence of a third protein are then measured using the FLIM method. FLIM is designed to measure the fluorescence lifetimes of fluorescent proteins. When FLIM is applied as a downstream assay to FRET, protein-protein interactions and conformational changes of proteins in living cells can be spatially and temporally measured with high resolution. Whereas FRET occurrence is tracked by the decreasing lifetime of the donor chromophore in the presence of an acceptor chromophore, FRET-FLIM provides a certain advantage over traditional intensity-based FRET assays and has previously been discussed by Wallrabe and Periasamy (Wallrabe and Periasamy, 2005).

Because such techniques as BiFC-FRET and FRET-FLIM are still relatively novel, studies on ternary complex formations are still in their infancy stages. Future publications using such techniques will elucidate their role in cell migration.

Proximity Ligation Assay (PLA)

The need for a sensitive and specific assay to detect PPIs led to the development of the *in situ* PLA. PLA combines the robust and versatile properties of antibodies with a split-reporter assay to generate a

selective and specific method to detect PPIs. This method is similar to immunostaining (immunohisto- and immunofluorescence staining) in principle and allows for the detection of endogenous proteins down to single-cell resolution. This ability allows researchers to study PPIs in their most natural environment, within a cell, which is paramount in the context of cell migration.

In immunostaining, primary antibodies are used to target the proteins of interest, followed by the application of fluorophore-conjugated secondary antibodies to visualise the proteins of interest. This process provides information on the expression and co-localisation of the suspected interacting protein pairs. However, the limitation of microscopy resolution is a barrier to concluding whether the partners are indeed interacting or merely in close proximity. *In situ* PLA overcomes this limitation by replacing the secondary antibodies with a pair of proximity probes (antibodies conjugated to oligonucleotides) (see Figure 3). This pair of oligonucleotides acts as a template for the hybridisation and ligation of subsequently added connector oligonucleotides to form a circular DNA molecule. The hybridisation and ligation of the connector oligonucleotides is only possible if the proximal probes are close to each other (40 nm if employing secondary antibodies or 30 nm if primary antibodies are directly used), which underscores the most crucial innovation of the assay. The circular DNA acts as a template for infinite rolling circle amplification (RCA), a process performed by a DNA polymerase. This generates an extended single-stranded DNA molecule containing concatemeric copies of the original circular DNA sequence that remains attached to the proximal probes (Soderberg et al., 2006). These amplification products (AP) are then visualised using fluorescently labelled oligonucleotides that hybridise to a unique sequence on the AP. Because the AP contains numerous repeats of this sequence, the binding of the detection oligonucleotides generates a clear, localised fluorescence signal that is easily distinguishable from background readings. This feature greatly enhances the resolution of the assay. Because each PPI corresponds to one AP signal, the quantification of the number of interactions and the precise location of each interaction pair becomes possible, which provides further insights into cellular biology (Soderberg et al., 2006).

As mentioned in the BiFC section of this review, BiFC was used by Yu and colleagues to demonstrate the interaction between ILK and p38 β *in vivo* to substantiate its relevance to bladder cancer migration potential (Yu et al., 2014). Nonetheless, to validate their findings and ensure that endogenous ILK and p38 β indeed interacted in a serum-responsive manner, *in situ* PLA was their method of choice. In agreement with their BiFC observations, a sharp increase in the number of cytoplasmic PLA signals (each representative of an ILK-p38 β interaction) was observed when TSU-Pr1 cells were stimulated with 10% FBS. Treatment with the ILK inhibitor QLT-0267 prevented complex formation

between ILK and p38 β (as evidenced by the marked reduction of cytoplasmic PLA signals) and halved the serum-directed chemotaxis of serum-stimulated TSU-Pr1 cells (Yu et al., 2014).

In a study on wound-healing, PLA performed on primary human keratinocytes and mouse wound tissue sections also revealed the importance of ANGPTL4 in modulating interactions between keratinocytes and their ECM (Goh et al., 2010). ANGPTL4 is a secreted factor that is up-regulated by PPAR (Mandard et al., 2004) and hypoxia (Belanger et al., 2002) and has known implications in lipid and glucose metabolism (Oike et al., 2005) as well as breast cancer metastasis (Minn et al., 2005). It is cleaved post-translationally to give rise to N- and C-terminal fragments (nANGPTL4 and cANGPTL4) that are known to possess distinct functions. Goh and colleagues identified cANGPTL4-vitronectin and cANGPTL4-fibronectin interactions that occur in the wound bed and epithelium via PLA.

cANGPTL4 binding to vitronectin (or fibronectin) did not obviate cognate integrin $\alpha 5 \beta 1$ binding as visualised by triple PLA, wherein a PLA signal is only detected when all three members of the proposed tripartite complex interact. ANGPTL4 was subsequently found to stabilise vitronectin and fibronectin against matrix metalloprotease degradation, which in turn potentiates the activation of integrin-dependent intracellular signalling pathways that promote keratinocyte migration (Goh et al., 2010).

PLA offers researchers a convenient way to detect PPIs that occur between endogenous proteins of intact cells and tissue sections. This method preserves PPI localisation and ensures that only native interactions are highlighted, which positions PLA as a technique of high specificity. With the incorporation of an innovative amplification reaction that strengthens the signal output, PLA allows for detection sensitivity at the impressive resolution of a single PPI. PLA also allows for the

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detection of transient PPIs in cells and tissues, up to sub-cellular levels because fixation of the cells is involved. This technique provides a relative but objective quantification of PPIs, which is a characteristic that is not readily achieved when using other techniques. However, PLA also has its own limitations. Being an antibody-based assay means that the success of PLA is still dependent upon ensuring ideal antibody binding conditions and is vulnerable to the stresses of wash steps which may compromise the sensitivity towards transient PPIs. Hence, this requirement emphasises the need for positive and negative controls for each PPI being tested. For example, when probing for an interaction between cANGPTL4 and vitronectin with PLA, Goh et al also probed for vitronectin and integrin $\beta 5$, which is a well-established cognate interaction (Goh et al., 2010). As a negative control, one can consider probing for proteins that do not share the same cellular compartment under physiological conditions (e.g., integrin $\beta 5$ and nucleoporin).

Another limitation is that PLA has a low upper limit on the number of interactions that can be simultaneously detected. The triple PLA experiment for the identification of the cANGPTL4-integrin $\alpha 5 \beta 1$ -vitronectin ternary complex most likely required careful optimisation of antibody binding conditions and of the time that was allowed for amplification and fluorescent-labelled oligonucleotide binding. Increasing the number of PPIs that are being simultaneously probed could be complex and liable to failure. Additionally, permeabilisation of cells to allow for access by the primary antibodies means that PLA is used as an end-point experiment and is not amenable to live-cell imaging.

Surface Plasmon Resonance (SPR)

The concept of SPR has previously been described (Helmerhorst et al., 2012). Briefly, surface plasmon is an electromagnetic wave that propagates along the surface of a thin metal layer. To achieve SPR, a beam of

polarised light is shone through a prism at a precise angle where it undergoes total internal reflection at the prism/metal/medium interphase and results in the excitation of the surface plasmon to generate the SPR. The angle of the light is extremely sensitive to changes in the reflective indexes of the medium that is adjacent to the metal layer; therefore, valuable statistics on protein interactions can be obtained by directly measuring the intensity of the reflected light. Experimentally, the protein of interest (ligand) is bound to a sensor chip that normally has a gold polymer interface. The suspected protein (analyte) solution is then allowed to flow over the sensor chip. When binding of the ligand and analyte occurs, the reflective index of the medium changes, and this change is dependent upon the molecular weight and number of proteins that are bound, among others. The characteristics of the individual interactions, whether they are transient or stable, strong or weak, fast or slow directly impact the changes in intensity of the reflected light that is measured and yields real-time and highly sensitive information on protein interaction kinetics and equilibrium constants.

One recent article demonstrated the use of SPR to analyse the binding of plasminogen (PIg) to its postulated receptor, thrombomodulin (TM), in promoting endothelial migration and invasion (Chen et al., 2013). This experiment was performed by measuring the binding kinetics of PIg and TM using a Biacore sensor, and the readout was given in terms of the association and dissociation rate constants. High values for the association and dissociation rate constants indicated that the PIg-TM binding and dissociation events occurred rapidly.

One potential drawback of the SPR technique is the possibility of the protein-of-interest losing its native configuration upon its attachment onto the sensor chip surface, or that its orientation on the chip may confer steric hindrance that could prevent analyte binding. To address this problem, researchers may have to consider alternatives, such as switching the ligand to become the

Table 1. Summary of methods for probing PPIs during cell migration.

		Co-IP	BIFC/FRET/FLIM	PLA	SPR
Experimental requirement	Probe-based	✓		✓	✓
	Direct labelling		✓		
	Intact cell (PPI localisation retained)		✓	✓	
	Cell lysate (PPI localisation lost)	✓			✓
Nature of PPIs	Live-cell imaging		✓		
	Stable	✓	✓		✓
	Transient	✓*	✓	✓*	✓
	Post-translational modifications involved	✓		✓	✓
Readout	Semi-quantitative	✓	✓		
	Quantitative			✓	✓
	Affinity and kinetics				✓

*: denotes modification to protocol may be necessary.

analyte or optimising the attachment so that minimal binding heterogeneity to the sensor surface occurs. The presence of low-affinity or non-specific surface sites can also lead to an increase in non-specific binding effects at the surface of the sensor chip. Similarly, the pH and molarity of the buffers that are used will also influence the PPIs and questions remain as to whether these conditions reflect the endogenous context. Similar to co-IP, no subcellular localisation can be inferred from the interactions.

Despite these disadvantages, SPR has many advantages as well. In addition to it being a technique that is label-free, it provides rapid results and is highly sensitive; researchers are able to obtain real-time measurements of protein interactions and can determine the concentration of the analyte without the need for standard calibrations by applying calibration free concentration analysis (CFCA). CFCA provides an actual "active" concentration of the analyte that is compared to other techniques, such as ELISA or spectroscopic methods. This feature is critical, particularly in the pharmaceutical industries where the actual "active" concentration of an analyte is crucial to design an optimised production line. SPR is also the only technique that can provide data on the affinity constants (association and dissociation) of PPIs.

Conclusion

Cellular migration is a fundamental and important process that occurs during numerous biological activities, such as embryogenesis, angiogenesis, wound healing, inflammatory response and cancer metastases. Control of cellular migration is dependent upon PPIs that initiate and effect cytoskeletal remodelling alongside changes in cell-ECM contacts to enable a cell to move on its substratum in a directional manner. Given the multitude of proteins that coexist and interact within a cell at any point in time, it is often difficult to identify PPIs that are relevant to cellular migration. A reductionist approach to this problem is to demonstrate that a particular PPI associates strictly with a migratory event and that PPI formation is necessary and sufficient to promote cellular migration. To this end, probe-based and direct-labelling techniques have been developed to provide researchers a visual, semi- if not fully quantifiable readout of PPIs that drive the migratory events in cell biology. Each of these methods has its merits and shortcomings, but many serve complementary functions when applied in combination. Herein we have highlighted four methods, co-IP, BiFC (pure and hybrid versions), PLA and SPR which continue to enable researchers to gain insight into the molecular mechanisms that underlie cell migration. In summary, the decision to use one method over the other can be summarised through three main factors: (1) experimental requirement, (2) the nature of the PPIs being probed and (3) the preferred experimental readout

(see Table 1).

For example, if one chooses to employ co-IP for the identification of PPIs involved in migration, an antibody against proteins of interest must first be available, and the experimental setup, by default, forgoes the sub-cellular localisation of the PPI which could be important in some cases. Co-IP is more suitable for the detection of stable interactions; however, it is possible to stabilise transient interactions with the integration of a cross-linking step in its protocol. Although a researcher may choose to employ BiFC from the beginning because BiFC is directly capable of simultaneously capturing transient PPIs and the sub-cellular localisation where the PPI is occurring, the design of reporter-fusion protein constructs and optimisation of their *in vivo* expression may be costly and time-consuming. Hence, co-IP could be an efficient way for researchers to gain a foothold in demonstrating that the PPI indeed exists before committing to elucidating its localisation via BiFC.

Arguably, the best evidence for migration-related PPI formation is the observation of PPIs that form as migration occurs. Such observations can be made using BiFC and live-cell imaging in real time. Nonetheless, the readout of BiFC is only semi-quantitative because fluorescent labels only allow intensity-based inference of the frequency of PPI occurrence, and conclusions made from cells expressing exogenous, split-reporter-fused proteins may be viewed sceptically. These reservations can be overcome with PLA, which allows each PPI to be counted in non-migratory versus migrating cells which yields a precise quantitative result of whether a PPI is upregulated during cell migration. Moreover, probing is performed on endogenous proteins of unmodified cells, which alleviates any concerns over the non-native effects of the exogenous expression of split reporter-fused proteins.

As has been highlighted in this review, a greater depth into PPIs can be gained by truncating or mutating the proteins that are involved in a PPI of interest. Truncations or mutations that abolish PPI formation can reveal essential domains and residues that hold together entire protein complexes. Should further depth into the interaction be desired, SPR can be used to provide insight into the affinities and kinetics of binding.

To conclude, we have endeavoured to consolidate the advantages, limitations and considerations when using each of these methods to probe for PPIs in cell migration. The techniques discussed here are by no means exhaustive, but should suffice as a sound framework for researchers to decide on their preferred approach.

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Review

Emerging Roles of Angiopoietin-like 4 in Human Cancer

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Abstract

Angiopoietin-like 4 (ANGPTL4) is best known for its role as an adipokine involved in the regulation of lipid and glucose metabolism. The characterization of ANGPTL4 as an adipokine is largely due to our limited understanding of the interaction partners of ANGPTL4 and how ANGPTL4 initiates intracellular signaling. Recent findings have revealed a critical role for ANGPTL4 in cancer growth and progression, anoikis resistance, altered redox regulation, angiogenesis, and metastasis. Emerging evidence suggests that ANGPTL4 function may be drastically altered depending on the proteolytic processing and posttranslational modifications of ANGPTL4, which may clarify several conflicting roles of ANGPTL4 in different cancers. Although the *N*-terminal coiled-coil region of ANGPTL4 has been largely responsible for the endocrine regulatory role in lipid metabolism, insulin sensitivity, and glucose homeostasis, it has now emerged that the COOH-terminal fibrinogen-like domain of ANGPTL4 may be a key regulator in the multifaceted signaling during cancer development. New insights into the mechanistic action of this functional domain have opened a new chapter into the possible clinical application of ANGPTL4 as a promising candidate for clinical intervention in the fight against cancer. This review summarizes our current understanding of ANGPTL4 in cancer and highlights areas that warrant further investigation. A better understanding of the underlying cellular and molecular mechanisms of ANGPTL4 will reveal novel insights into other aspects of tumorigenesis and the potential therapeutic value of ANGPTL4. *Mol Cancer Res*; 10(6): 677–88. ©2012 AACR.

Introduction

Cancer is a disease in which a group of cells attains the ability to proliferate uncontrollably, invades surrounding tissues, and ultimately spreads to distal tissues and organs through the circulatory system to form metastatic tumors. Based on our current knowledge, the process of cancer development is somewhat perplexing and certainly multifactorial. Genetic instability fueled by DNA damage and errors generated by DNA replication machinery drive the initiation of tumorigenesis (1, 2). Extensive exposure to carcinogens or genotoxic chemicals is a prime factor that causes mutations and increases the risk of cancer. In addition, by-products of normal cellular metabolism, such as reactive oxygen species (ROS), also create a constant threat to DNA integrity (1). Genetic mutations or alterations of critical regulatory genes, such as tumor-suppressor genes and proto-oncogenes, cause uncontrolled proliferation and the deregulation of apoptosis further mediating tumor progression.

Tumors consist of heterogeneous cell types such as cancer-associated fibroblasts, endothelial cells, pericytes, immune inflammatory cells, and proliferating cancerous cells (3). This heterogeneity and interdependency sustain tumor growth and progression. Cancer-associated fibroblasts form the majority of the tumor stromal population and are capable of influencing cancer cell behavior via paracrine regulation. Endothelial cells that form the tumor vasculature act as a transportation route for nutrients not only to support tumor growth, but also to facilitate cancer metastasis. The pericytes provide paracrine support signals to stabilize tumor vascular integrity and its function. Immune inflammatory cells secrete proangiogenic effectors like angiogenic growth factor, VEGF, and proinvasive matrix metalloproteinases to promote tumor progression (3).

Reciprocal cell–cell and cell–matrix communications dictate nearly every aspect of tumor development, and they can drastically affect the efficacy of antitumor therapies. Such communication encourages the tumor cells of many human cancers to acquire several essential capabilities to sustain proliferative signals, evade growth suppressors, resist cell death, enable replicative immortality, induce angiogenesis, activate invasion and metastasis, reprogram their energy metabolism, and evade immune destruction (3). Research to identify critical mediators of these hallmarks will offer novel insights into the network of molecular mechanisms that are central to cancer development.

Ongoing cancer research focusing on understanding the interaction of cancer cells with their microenvironment has intensified over recent years, and a new class of proteins known as matricellular proteins have since been implicated

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(4–6). Matricellular proteins are a group of nonstructural, extracellular matrix (ECM)–associated glycoproteins secreted by cancer cells and neighboring stromal cells into the extracellular environment (4, 5). This large group of structurally and functionally diverse proteins modulates cell–matrix interactions and cell functions by interacting with membrane receptors, proteases, hormones, and other bioeffector molecules, as well as with structural matrix proteins such as collagen and vitronectin (7, 8). Recent studies have implicated several members of matricellular proteins, such as osteopontin, secreted protein acidic and rich in cysteine, and angiopoietin-like 4 (ANGPTL4) as important factors in tumor progression (4, 9–11). Of particular interest, ANGPTL4 is a novel matricellular protein that was reported to interact with specific ECM proteins and integrins to facilitate cell migration during wound healing (7, 8).

ANGPTL4 is a novel, glycosylated adipokine that belongs to a family of 7 ANGPTL proteins (Fig. 1; refs. 12,13). The 7 proteins, designated angiopoietin-like 1 to 7 (ANGPTL1–7), were identified in systemic circulation and share amino acid sequence similarity with the angiopoietin family (ANG). Despite being structurally similar to ANG, these ANGPTLs do not bind to the ANG receptors Tie 1 and Tie 2 to mediate their biological functions. The cognate receptors for ANGPTLs remain unknown; thus, ANGPTLs are considered orphan ligands (12, 14). ANGPTL3, 4, and 6 play critical roles in lipid and glucose metabolism (14, 15). Studies have also revealed that ANGPTL2, 3, 5, and 7 regulate hematopoietic stem cell activity (12). Interestingly, ANGPTL2 was recently reported to be a critical factor for carcinogenesis (16). ANGPTL2-associated chronic inflammation was also found to increase cancer susceptibility (16). ANGPTL2 was identified as a causative mediator of chronic

inflammation, and this was strongly correlated to the frequency of carcinogenesis *in vivo* using a chemically induced skin squamous cell carcinoma (SCC) mouse model. Furthermore, tumor-derived ANGPTL2 was shown to enhance metastasis and tumor angiogenesis (16). This finding suggests that other ANGPTL family proteins may have similar roles in cancer development.

Emerging evidence has identified a novel role for ANGPTL4 in cancer progression, although the other ANGPTL members have been mostly characterized as factors involved in angiogenesis and metabolism. The involvement of other ANGPTL family members in cancer remains unclear and warrants further investigation. In this review, we discuss the potentially diverse roles of ANGPTL4 in tumor development and discuss certain discrepancies with ANGPTL4 that require further clarification.

Functional Domains of ANGPTL4

ANGPTL4 is characterized by the presence of a highly hydrophobic signal peptide, an *N*-terminal coiled-coil domain, and a large ANG/fibrinogen-like COOH-terminal domain, which is well conserved in the ANG and ANGPTL families (Fig. 2; ref. 17). The native full-length ANGPTL4 (fANGPTL4) exists in the form of dimeric or tetrameric complexes that can undergo proteolytic processing to generate the *N*-terminal coiled-coil fragment (nANGPTL4) and the COOH-terminal fibrinogen-like domain (cANGPTL4; ref. 17). Although ANGPTL4s are known to be proteolytically processed, the mechanism of cleavage, the importance of the processing, and the roles of the various ANGPTL4 fragments are only beginning to be elucidated.

The proteolytic cleavage of ANGPTL4 is mediated by proprotein convertases (PC; Fig. 2; ref. 17). Because ANGPTL4 is proteolytically processed in the liver, the hepatocellular carcinoma cell line Huh7 has been used in studies investigating ANGPTL4 cleavage. Using Huh7 cells transfected with expression vectors encoding for the various convertases, Lei and colleagues showed that *in vitro* several PCs, including furin, PC5/6, paired basic amino acid–cleaving enzyme 4, and PC7, are able to cleave human ANGPTL4 at the -RRXR- consensus cleavage site (17). Although this study showed that ANGPTL4 is cleaved by PCs, the correlation between the expression of PCs and the truncated forms of ANGPTL4 remains unknown. The PCs have been shown to be enzymatically activated after passing through the late Golgi. Therefore, how ANGPTL4 is proteolytically processed remains to be determined (18). Importantly, the expression of these PCs in tumors and whether they are indeed responsible for the *in vivo* processing of fANGPTL4 is unknown.

The nANGPTL4 is responsible for the oligomeric assembly of ANGPTL4 and binds to lipoprotein lipases (LPL) to inhibit their activities (19). Disulfide bond formation facilitates the oligomeric formation of nANGPTL4 and fANGPTL4, which enhances its inhibitory effects on LPL activity. Mutations that prevent oligomerization severely compromise the capacity of ANGPTL4 to inhibit

Figure 1. Phylogenetic trees for the members of the ANG and ANGPTL families discovered in humans. There are 7 members in the ANGPTL family and 5 in the ANG family. Both families of proteins share structural similarities. However, ANGPTLs do not bind to ANG receptors Tie 1 and Tie 2 to mediate their biological functions. ANGPTLs are commonly considered to be orphan ligands.

Figure 2. Structure and biological functions of ANGPTL4. flANGPTL4 (~65 kDa) comprises a highly hydrophobic signal peptide (SP), an N-terminal coiled-coil domain (CCD), and a COOH-terminal fibrinogen-like domain (FLD). ANGPTL4 protein oligomerizes prior to secretion and cleavage. The N- and COOH-terminal domains are separated by a short linker that, after being secreted from the cell, can be cleaved into the nANGPTL4 (~26 kDa) and cANGPTL4 (~47 kDa) fragments. Cleavage is catalyzed by PCs at a conserved basic sequence (-RRKR-). The nANGPTL4 remains oligomeric, whereas cANGPTL4 will dissociate into monomers. Genetic variation in ANGPTL4 provides insights into protein processing and function. Oligomerized flANGPTL4 is able to bind to the extracellular matrix via the heparan sulfate proteoglycans and inhibit endothelial cell adhesion, migration, and tubule formation. Both flANGPTL4 and nANGPTL4 are involved in the modulation of triglyceride disposition via their inhibitory effect on LPL. However, this inhibitory effect on LPL seems to be weaker in the case of flANGPTL4 inhibition (dotted arrow). In contrast, cANGPTL4 binds to β1-integrin and activates 2 downstream processes. One process involves the stimulation of NADPH oxidase leading to the production of O₂⁻. A high ratio of O₂⁻ to H₂O₂ has been found to activate the Src/ERK/PKBα prosurvival pathway, thereby conferring anoikis resistance and promoting tumor growth. Alternatively, cANGPTL4-bound β1-integrin can activate the FAK/Src/PAK1 cascade to trigger the PKC-mediated signaling pathways that are crucial for effective wound healing. It is also interesting to note that cANGPTL4 has been found to be able to inhibit angiogenesis by suppressing the Raf/MEK/ERK signaling cascade.

LPL. The flANGPTL4 can also bind to heparan sulfate proteoglycans to participate in the inhibition of endothelial cell migration and tubule formation (20). However, it remains unknown whether flANGPTL4 and nANGPTL4 can directly stimulate intracellular signaling because neither a receptor nor a cell-surface interaction partner has been identified for ANGPTL4. The cANGPTL4 binds to and activates integrins β1 and β5 to regulate cell migration via the focal adhesion kinase (FAK)/p21-activated kinase (PAK)-signaling cascade (7). Recently, cANGPTL4 was found to trigger vascular disruption by binding to and dispersing vascular-endothelial cadherin (VE-Cad) and claudin-5 at endothelial junctions, via the activation of integrin α5β1 (21). Furthermore, cANGPTL4 can also

associate with specific matrix proteins and delay their proteolytic degradation by metalloproteinases. This interaction does not seem to interfere with integrin-matrix protein recognition (7, 8). Taken together, these studies suggest that different ANGPTL4 fragments exhibit tissue-dependent functions and that the different fragments of ANGPTL4 may have distinct roles in human cancers.

ANGPTL4 Expression and Its Transcriptional Regulation in Human Cancers

The expression of ANGPTL4 is regulated by the nuclear hormone receptors of the PPAR family, as well as by hypoxia and fasting (15, 22, 23). PPARs act as lipid biosensors and

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Table 1. Expression of ANGPTL4 protein in various human cancers

Type of cancer	Detection technique	Antibody used ^a	Author (reference)
Various cancers	Immunofluorescence, real-time PCR, and Western blot	nANGPTL4 and cANGPTL4	Zhu et al. (11)
CRC	Real-time PCR and Western blot	Full-length ANGPTL4	Nakayama et al. (47)
CRC	Real-time PCR and Western blot	Full-length ANGPTL4 and cANGPTL4	Kim et al. (25)
Breast cancer	Real-time PCR and Western blot	Full-length ANGPTL4	Zhang et al. (28)
Gastric cancer	Real-time PCR and Western blot	Full-length ANGPTL4	Nakayama et al. (48)
Esophageal cancer	Immunohistochemistry	Full-length ANGPTL4	Shibata et al. (49)
Kaposi sarcoma	Real-time PCR and Western blot	Full-length ANGPTL4	Ma et al. (43)

^aIndicates antibodies raised against full-length, N-terminal, and COOH-terminal ANGPTL4.

are crucial for lipid metabolism and homeostasis (15). Recently, PPARs have been implicated in the development of tumors (24). Studies have shown that ANGPTL4 is one of the PPAR target genes and contains several putative PPAR response element consensus sequences within its promoter (25). Reports have proposed that ANGPTL4 may be regulated by all 3 PPAR isoforms (PPAR α , PPAR β/δ , and PPAR γ), suggesting that ANGPTL4 may exert tissue-dependent effects governed by the differential expression of the PPAR isoforms (15). Furthermore, despite conflicting results, PPARs are known to be involved in tumorigenesis. This finding further implicates ANGPTL4 in cancer progression, although evidence that PPARs upregulate ANGPTL4 directly in tumor cells is still lacking. Girroir and colleagues suggested that PPAR β/δ modulates ANGPTL4 expression during tumorigenesis in human breast cancer and melanoma cell lines, although Zhu and colleagues reported no apparent correlation between the expression of PPARs and ANGPTL4 in SCC (11, 26). A recent study suggested that prostaglandin E2 (PGE₂) may transactivate PPAR β/δ to regulate the expression of ANGPTL4 under hypoxic conditions (25). Because of the aberrant growth of tumor cells and poor vascularization, the tumor microenvironment tends to become hypoxic. Several new studies have reported that hypoxia can induce the expression of ANGPTL4 and that the upregulation of ANGPTL4 is induced by the transcription factor hypoxia-inducible factor-1 α (HIF-1 α ; refs. 11, 25, 27). Indeed, Zhang and colleagues identified a functional HIF-1 α -binding site located 1.6 kb upstream of the human ANGPTL4 gene (28). This finding was further confirmed by another independent group using the hepatocellular carcinoma cell lines MHCC-97L and SMMC-7721 (27).

The tumor microenvironment is constantly under chronic inflammatory pressure (29). Recently, the potent regulator of inflammation TGF- β was found to regulate the expression of ANGPTL4 via a Smad3-signaling pathway (30). The upregulation of ANGPTL4 in cancer cells when they extravasate into the circulatory system could explain their inclination toward colonizing lung tissue. The rationale is based on the ability of ANGPTL4 to disrupt the integrity of vascular tight junctions, thereby increasing the permeability

of the capillaries in the lung to promote the intravasation into the lung tissue (30). Whether TGF- β can serve as a good therapeutic target still remains unclear because of its dual role in inhibiting and promoting tumor cell growth. However, this ambiguity shows the capacity of the primary tumor microenvironment to promote the progression of cancer via the production of cytokines (29).

Although early expression-profiling studies detected ANGPTL4 in a variety of organs or tissues, such as the skin, intestines, kidneys, adipose tissues, and the liver (14, 15, 22), little is known about the relative expression of the various ANGPTL4 fragments in these tissues. Recently, it was shown that adipocytes express the posttranslationally modified fANGPTL4 (~65 kDa), whereas the liver produces both cANGPTL4 (~47 kDa) and nANGPTL4 (~26 kDa; Fig. 2; refs. 17,31,32). The mechanism for such tissue-dependent expression of ANGPTL4 remains unclear.

Although information about the relative expression of the different ANGPTL4 fragments in normal tissues is emerging, much less is known about the expression of various ANGPTL4 fragments in various tumors (Table 1). To date, 4 studies have reported the elevated expression of cANGPTL4 in various tumors. Using an *in vitro* model in which they overexpressed ANGPTL4 cDNA in B16F0 mouse melanoma cells, Galaup and colleagues showed the presence of a small ANGPTL4 fragment whose molecular weight corresponded to the cANGPTL4 fragment (10). Using a monoclonal antibody against cANGPTL4 (mAb11F6C4), Zhu and colleagues showed that cANGPTL4, but not nANGPTL4, is highly expressed in major epithelial tumors such as SCC (11). Similarly, Huang and colleagues detected elevated cANGPTL4 from fine needle aspirates of breast tumors, basal cell carcinomas, melanomas, and in several cancer cell lines derived from breast, lung, and liver cancers (21). Consistent with the above findings, Kim and colleagues showed that colorectal cancer (CRC) cells only secrete cANGPTL4 proteins (25). A high ANGPTL4 expression in oral cancer correlates to an enhanced rate of tumorigenesis and a poor prognosis based on the tumor clinicopathology (Table 2; refs. 33, 34). An elevated ANGPTL4 in CRCs is also correlated with shorter disease-free survival rates (25). Furthermore, the

Table 2. Expression of ANGPTL4 in various human cancers and its implicated roles in tumor growth, metastasis, and angiogenesis

Type of cancer	Detection technique	Expression in tumor	Disease outcome	Author (reference)
Brain metastasizing melanoma	Real-time PCR	High expression in metastasized melanoma cells	Expression of ANGPTL4 correlated with metastasis of melanoma	Izraely et al. (46)
CRC	Real-time PCR and Western blot	High expression in CRC cells under synergistic effect of PGE ₂ and hypoxia	Increased cancer cell proliferation, survival, motility, and tumor-associated angiogenesis	Kim et al. (25)
Breast cancer	Real-time PCR and Western blot	High expression in breast cancer cells under hypoxia	Increased metastasis by promoting extravasation in the lungs	Zhang et al. (28)
CRC	Real-time PCR and Western blot	High expression in 100% of patients with metastatic tumor	Increased expression facilitates venous invasion and distant metastasis	Nakayama et al. (47)
Mammary carcinoma	Real-time PCR and bisulfite sequencing	Low expression in 12% of primary carcinoma	Methylation-silenced ANGPTL4 is present in mammary carcinoma	Hattori et al. (55)
Kaposi sarcoma	Real-time PCR and Western blot	High expression in 92% of oral Kaposi sarcoma	Enhances Kaposi sarcomagenesis	Hu et al. (33)
Various cancers	Immunofluorescence, real-time PCR, and Western blot	Elevated expression in tumors	Tumor-derived ANGPTL4 increases the O ₂ ⁻ to H ₂ O ₂ ratio, conferring anoikis resistance	Zhu et al. (11)
Endometrial cancer	DNA microarray, real-time PCR, and immunohistochemistry	Expressed in tumor		Mannelqvist et al. (56)
Prostate cancer	Global transcriptional profiling	High expression in tumors	ANGPTL4 promotes tumorigenesis	Feng et al. (35)
Esophageal SCC		High expression in metastatic tumor	ANGPTL4 promotes lymphovascular invasion	Shibata et al. (49)
Gastric cancer	Real-time PCR and Western blot	High expression of ANGPTL4 in 36.9% of adenocarcinomas	ANGPTL4 expression supports the progression of human gastric cancer	Nakayama et al. (48)
Kaposi sarcoma	Real-time PCR and Western blot	Upregulated by vGPCR	ANGPTL4 promotes angiogenesis and vessel permeability	Ma et al. (43)
Oral tongue SCC	Real-time PCR	High expression in tongue cancer	High expression is predictive of poor prognosis	Wang et al. (34)
Hepatoma		Increased expression in cancer	ANGPTL4 contributes to anoikis resistance	Zhang et al. (40)
Breast cancer	Microarray and real-time PCR	TGF-β enhances ANGPTL4 expression	ANGPTL4 facilitates metastasis to lungs	Padua et al. (30)
Gastric cancer	Oligonucleotide microarray	Lowered expression of ANGPTL4 in tumor	Role as a tumor biomarker	Arao et al. (57)
General cancer	Transgenic mice, corneal neovascularization, and Miles permeability assays	Low expression in tumor	Mice expressing Ap4 in the skin exhibited remarkable suppression of tumor growth	Ito et al. (37)
Gastric cancer		Low expression detected in tumor	ANGPTL4 gene is silenced by methylation, contributing to cancer development	Kaneda et al. (58)

downregulation of ANGPTL4 impairs tumor growth and metastasis (11, 35, 36). In contrast, studies have also shown that increased ANGPTL4 expression inhibits melanoma, lung, and colorectal tumor growth, metastasis, and angiogenesis (10, 37). High ANGPTL4 levels in mouse tumors also impaired tumor cell migration and invasiveness, thus inhibiting metastasis (10). The reason for this discrepancy is unclear. These results suggest that the expression and roles of ANGPTL4 may be context and tumor-type dependent. The expression of ANGPTL4 fragments in lymphatic and hematopoietic cancers, such as lymphoma and leukemia, also remains unclear. Despite the increasing emphasis on the differential roles of fANGPTL4, cANGPTL4, and nANGPTL4, most studies have not vigorously addressed the biological significance of this phenomenon in cancer. Emerging studies have also revealed that the posttranslational modification of ANGPTL4 can affect its biological functions, which suggests another level of complexity in understanding the role of ANGPTL4 in cancer. Thus, further investigation is necessary to better understand how tumor microenvironment influences the transcriptional regulation of ANGPTL4 in cancer.

ANGPTL4 and Tumor Growth

Increasing lines of evidence clearly support a tumor-promoting role of inflammation in the development and progression of cancer (38). The tumor microenvironment strongly resembles the wound environment, which contains numerous growth factors and proinflammatory lipid mediators such as prostanoids. However, instead of signaling for the removal of the abnormal cells, these proinflammatory mediators have been found to be coopted by the cancer cells to promote their growth, invasion, and metastasis (38).

COX-2 is an inducible enzyme that converts free arachidonic acid into prostanoids, including prostaglandins and thromboxanes (25). Under normal conditions, COX-2 expression is undetectable in most cells. During inflammation, COX-2 expression is upregulated in activated macrophages to increase the production of prostanoids that modulate the actions of the various immune cells (39). More recently, COX-2 has been shown to be upregulated in various carcinomas and seems to play a fundamental role in tumorigenesis. The expression of COX-2 is elevated in approximately 50% of colorectal adenomas and in up to 85% of adenocarcinomas; it is associated with poor survival among patients with CRC (25). Importantly, the tumor-promoting function of COX-2 is strongly linked to PGE₂, which is also the most abundant prostaglandin found in CRCs (25). A recent study showed a synergistic effect between PGE₂ and hypoxia in promoting CRC cell proliferation, and the authors ascribe tumor-promoting effects to ANGPTL4 (25). Specifically, hypoxia induced the expression of EP1, a PGE₂ receptor, in CRC. The elevated level of PGE₂ stimulated EP1 receptor signaling, which enhanced ANGPTL4 expression and cANGPTL4 secretion (Fig. 3). More importantly, the authors of this study went on to show that cANGPTL4, and not fANGPTL4 or nANGPTL4, was

responsible for the increased CRC cell proliferation and tumor growth *in vitro* and *in vivo* (25). Notably, the study further showed that cANGPTL4 regulates cancer cell proliferation and that STAT1 induction is dependent on an NADPH oxidase-mediated production of superoxide (O₂^{•−}), as well as the Src and mitogen-activated protein kinase (MAPK) pathways. This finding is consistent with the findings by Zhu and colleagues who showed that cANGPTL4 stimulates a redox-based mechanism to enhance tumor cell survival via the alteration of the O₂^{•−} to H₂O₂ ratio, leading to the activation of Src and extracellular signal-regulated kinase (ERK; ref. 11).

Given the distinct functions of the different ANGPTL4 fragments, this information provides a focal point for future ANGPTL4 research, highlighting the need to demarcate the different biological functions of cANGPTL4 and nANGPTL4 in cancer progression. More extensive studies will certainly be necessary to elucidate the underlying mechanisms by which ANGPTL4 modulates cancer proliferation. One important discovery will be the identification of the receptors that mediate the downstream effects of ANGPTL4.

ANGPTL4 and Anoikis Resistance

The abnormal growth pattern of tumor cells suggests that tumor cells grow under a myriad of physiologic stresses that should trigger apoptosis and halt cancer progression. However, tumor cells evade apoptosis using multiple dysregulated mechanisms, and anoikis resistance is a hallmark of cancer (3).

An early study showed that ANGPTL4 acts as a negative regulator of apoptosis, protecting endothelial cells from serum deprivation-induced apoptosis. Although the mechanism was unknown, it was postulated that ANGPTL4 could bind to a receptor to mediate its antiapoptotic effect. This suggests that cancer cells may employ a similar mechanism to resist stress-induced apoptosis (23). Indeed, ANGPTL4 has been shown to contribute to anoikis resistance in hepatoma cells grown in a detached state. These hepatoma cells tend to form a synokis-like multicellular aggregate that was resistant to apoptosis inducers (40). The suppression of ANGPTL4 by RNA interference enhanced detachment-induced apoptosis and sensitized the tumor cells to antitumor drug treatment (40). Recently, the anoikis-protective effect of ANGPTL4 was attributed to cANGPTL4. In this study, tumor-derived cANGPTL4 activates a redox-mediated survival mechanism that is used by tumor cells to facilitate cancer progression through the modulation of ROS (11). Tumor-derived cANGPTL4 interacts with integrins β1 and β5 to modulate O₂^{•−} levels using the NADPH oxidase-dependent machinery to maintain an elevated oncogenic O₂^{•−} to H₂O₂ ratio in tumors. This cANGPTL4–integrin interaction activates Src kinase by oxidation and consequently stimulates the prosurvival phosphoinositide 3-kinase (PI3K)/protein kinase B-α (PKBα) and mitogenic ERK pathways to sustain attachment-independent survival (Fig. 3). Importantly, the

Figure 3. Diverse roles of ANGPTL4 in human cancer. The roles of ANGPTL4 in human cancer are controversial. The flANGPTL4 (depicted by a rectangle and an oval) and cANGPTL4 (depicted by an oval) have been implicated in the various aspects of cancer. The tumor microenvironment is inflammatory and hypoxic in nature. In the event of promoting tumor growth, the expression of ANGPTL4 seems to be upregulated by proinflammatory TGF-β via the SMAD pathway, as well as by COX-2-induced PGE₂ signaling in carcinomas. ANGPTL4 subsequently activates NADPH oxidase (NOX) to promote tumor cell proliferation via the Src/ERK/STAT1 pathway. As the aberrant growth of tumor cells progresses, a mechanism to evade apoptosis via anoxia resistance ensures the survival of the tumor cells through ANGPTL4-mediated inhibition of proapoptotic Bcl-2 associated death promoter (BAD; see text). ANGPTL4 is also clearly involved in cancer metastasis. ANGPTL4 was found to increase tumor vascular permeability (see text). In addition, flANGPTL4 was shown to upregulate the expression of VCAM-1 on endothelial cells through an unknown mechanism facilitating the attachment of circulating metastatic cancer cells to endothelial cells, promoting their transendothelial extravasation and metastatic tumor formation. In contrast, antiangiogenic effects of flANGPTL4 have also been reported, whereby it inhibits endothelial cell proliferation, migration, and tubule formation via an unknown mechanism. In addition, N-glycosylated cANGPTL4 has been shown to impair bFGF and VEGF-induced angiogenesis by suppressing the Raf/MEK/ERK pathway, suggesting that other posttranslational modifications may also play a role in the modulation of the functions of ANGPTL4. One study has also shown the inhibitory effect that flANGPTL4 has on VEGF-induced vascular permeability; however, the mechanism remains unknown. Despite the establishment of an antiangiogenic effect, whether flANGPTL4 or either of its truncated fragments binds to a receptor to modulate these effects remains to be determined.

suppression of ANGPTL4 via RNA interference or immunosuppression by monoclonal antibody (mAb11F6C4) modulates intracellular ROS generation to attenuate tumor growth that is associated with enhanced apoptosis *in vitro* and *in vivo* (11). mAb11F6C4 binds to the human cANGPTL4 protein and has been shown to block the interactions between cANGPTL4 with integrin, VE-Cad, and claudin 5 to attenuate tumor growth and metastasis

(11, 21). Similar observations were also recently made in CRC, showing that cANGPTL4-mediated activation of ERK and Src kinase enhanced CRC cell proliferation under hypoxic conditions, further confirming the unique functions of cANGPTL4 in cancer (25). Despite the loss of cell–matrix attachment, the hijacking of integrin-mediated signaling by tumor-derived cANGPTL4 provides an alternative survival mechanism, which is important for cancer cells during

metastasis (11). This finding suggests that anticancer strategies focusing on anoikis or redox-based apoptosis in tumors are viable. However, more studies will be required to validate the role of cANGPTL4 in anoikis. Of particular interest would be studies on the effect of cANGPTL4 on other prosurvival pathways during anoikis and whether other ANGPTL4 fragments influence tumor survival.

ANGPTL4 and Angiogenesis

The oxygen consumption of rapidly growing tumor cells may outweigh an insufficient oxygen supply, leading to a pathologic condition known as hypoxia (41). This hypoxic environment triggers the onset of pathologic angiogenesis, which forms new blood vessels to supply oxygen and nutrients and remove metabolic waste products supporting tumor survival (41). Angiogenesis is a multistep process that is regulated by angiogenic factors like VEGF, ANG, and, more recently, ANGPTL4 (42). An *in vivo* hypoxic signature includes ANGPTL4 as a robust hypoxia-related gene. Previous studies have indicated that ANGPTL4 mRNA and protein levels become elevated in endothelial cells in response to hypoxia and exert a VEGF-independent proangiogenic effect (42). An increased expression of ANGPTL4 was also observed in the majority of human cancers, with the expression of cANGPTL4 highly correlated to the expression of HIF-1 α (see above, "ANGPTL4 Expression and Its Transcriptional Regulation in Human Cancers"). These observations indicate that ANGPTL4 could be a key modulator in tumor angiogenesis in a hypoxic tumor microenvironment.

Recent studies highlighted a prominent role for ANGPTL4 in tumor angiogenesis and vascular permeability. Using a transgenic mouse model expressing a viral G-protein coupled receptor (vGPCR), ANGPTL4 expression was upregulated by vGPCR in Kaposi sarcoma (43). This increase in ANGPTL4 expression enhanced endothelial cell migration and differentiation, both of which are important processes in angiogenesis, resulting in markedly enhanced neovascularization. The inhibition of ANGPTL4 using siRNA prevented the neovascularization process and reduced vascular permeability as well as vGPCR-induced tumorigenesis *in vivo* (43). In subsequent studies, Huang and colleagues revealed that ANGPTL4-knockdown allograft tumors express consistently lower levels of endothelial cell marker CD31 compared with wild-type allograft tumors, which is indicative of impaired angiogenesis, further confirming the proangiogenic role of ANGPTL4 in tumors (21). Although these findings suggest a proangiogenic role for ANGPTL4 in tumors, an antiangiogenic role has also been reported for ANGPTL4. Ito and colleagues found that ANGPTL4 acts as an antiangiogenic factor inhibiting VEGF-induced endothelial cell proliferation, chemotaxis, and tubule formation, which are important processes for vascularization (37). An *in vivo* corneal neovascularization assay revealed that ANGPTL4 strongly impairs VEGF-induced neovascularization and that ANGPTL4 alone does not influence neovascularization. These results seem to

suggest that in the context of VEGF-induced angiogenesis, ANGPTL4 exerts antiangiogenic effects. Furthermore, in a study investigating transgenic mice that overexpress ANGPTL4 in the basal epidermis, the authors showed that elevated levels of ANGPTL4 impaired neovascularization, resulting in a decreased amount of invading capillary vessels into the xenograft tumors, effectively limiting tumor growth (37). Interestingly, the same group also reported a proangiogenic effect for ANGPTL4, further highlighting the controversial roles of ANGPTL4 (10, 20, 42).

These findings suggest a role for ANGPTL4 in tumor vascularization, although the underlying mechanism by which ANGPTL4 modulates angiogenesis remains unresolved. The discrepancies between the findings suggest a context- and tissue-dependent effect of ANGPTL4, which is further complicated by posttranslational modification of ANGPTL4 in which *N*-glycosylated cANGPTL4 was found to suppress the Raf/MAP-ERK kinase (MEK)/ERK signaling cascade in endothelial cells, impairing basic fibroblast growth factor (bFGF) and VEGF-induced angiogenesis (32). To resolve these disagreements, future investigations should focus on understanding the roles of posttranslational modifications on ANGPTL4 and on the mechanisms that govern the pro- and antiangiogenic effect of hANGPTL4 and its truncated fragments.

ANGPTL4 in Tumor Invasion and Metastasis

Metastasis is the spread of malignant cells to distant organs and is frequently the final event in tumor progression (3). Metastasis is largely responsible for the majority of cancer deaths, as the metastatic cancer cells are typically highly aggressive and resistant to anticancer drugs. However, metastasis remains the most poorly understood aspect of cancer (3). Metastasis is a complex, multistep process in which tumor cells locally invade the surrounding tissue and then migrate across the vascular endothelium of the lymphatic and vascular systems (intravasation). While in circulation, tumor cells must evade immunodetection and resist anoikis before seeding at a distal site. These tumor cells leave the circulation through extravasation to establish a new secondary tumor (44). One of the fundamental mechanisms required during metastasis is the disruption of the vascular integrity, which facilitates the transmigration of the tumor cells across the endothelium (44).

Early studies have suggested that hANGPTL4 inhibits VEGF-induced angiogenesis and vascular permeability (37). In a different study, ANGPTL4 was shown to inhibit vascular leakiness, and this impaired the intravasation and extravasation of tumor cells *in vivo* (10). Notably, only a single form of ANGPTL4 was detected in sera *in vivo*. Because the molecular weight of the ANGPTL4 fragment size was not provided in the study, it was not possible to know whether the detected protein band corresponded to hANGPTL4, nANGPTL4, or cANGPTL4. Galaup and colleagues went further by using the overexpression of ANGPTL4 in melanoma to show that the expression of ANGPTL4 impairs the migration and invasion of melanoma

cells (10). Interestingly, sANGPTL4 in the presence of a smaller ANGPTL4 fragment, whose molecular weight corresponds to the cANGPTL4, was found to be produced by the melanoma cells (10). However, it is unclear which ANGPTL4 fragment was actually responsible for the inhibition of cell migration and invasion.

However, recent studies have shown that ANGPTL4 is highly expressed in metastatic cancers, suggesting that it may play a role in the metastatic processes. Furthermore, these studies have pinpointed ANGPTL4 as a critical mediator in the transmigration process (11, 21, 45). ANGPTL4 enhances vascular permeability and promotes the metastasis of breast tumor cells to the lung and the metastasis of melanoma cells to the brain (21, 28, 30, 46). TGF- β upregulates ANGPTL4 in breast tumor cells and disrupts the endothelial cell–cell junctions, leading to enhanced vascular leakiness and transendothelial migration of the tumor cells. Furthermore, clinical studies have correlated ANGPTL4 expression with venous and lymphatic invasion in human gastric and CRCs, which further emphasizes the role of ANGPTL4 in tumor metastasis (47–49). ANGPTL4 was found to promote transendothelial migration and metastasis of hepatocellular carcinoma cells through the upregulation of vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells and the activation of the VCAM-1/integrin β 1–signaling axis (27). In this mechanism, the increased VCAM-1 expression on endothelial cells facilitates the attachment of the metastatic cancer cells in circulation and the subsequent *trans*-endothelial extravasation to establish a metastatic tumor. ANGPTL4 also disrupts endothelial cell–endothelial cell interactions, leading to enhanced vascular leakiness, and promotes the metastasis of cancer cells to the lung (21). Another study pinpointed the vascular disruptive effect of ANGPTL4 to the cANGPTL4 fragment. In this study, the authors showed that cANGPTL4 interacts with integrin α 5 β 1 to activate the Rac/PAK–signaling axis, weakening the endothelial cell–endothelial cell contacts and facilitating cANGPTL4 interaction with VE-Cad and claudin-5 (Fig. 3; ref. 21). This interaction results in the internalization and declustering of the endothelial cell tight junction and adherens junction proteins, leading to the disruption of the vascular barrier. Clearly, more investigations are needed to clarify the prometastatic role of ANGPTL4 and its underlying mechanism of action. Taken together, these results suggest that ANGPTL4 may have both homotypic and heterotypic influences in the tumor microenvironment (Fig. 3).

The role of ANGPTL4 in vascular permeability and metastasis is controversial. The apparent discrepancy that was observed in previous studies seems to derive from the use of different experimental approaches and models in each study. Additionally, the functions of ANGPTL4 may be affected by the tumor microenvironment, suggesting a context- and tissue-specific activity. Presently, the lack of structural information on ANGPTL4 limits our ability to fully explain this phenomenon. Unfortunately, numerous studies that have attempted to decipher the role of ANGPTL4 in cancer have failed to address the posttrans-

lational modifications and proteolytic processing of ANGPTL4, which clearly have a significant influence on the functions of ANGPTL4 (10, 30, 37, 47–49). For example, the expression of either the nANGPTL4 or the cANGPTL4 truncation in transgenic mice remains unknown (43). The differential processing of ANGPTL4 has a major effect on the different biological functions of ANGPTL4 under various contexts and may have contributed to the observed discrepancies (11, 21, 50). Indeed, only cANGPTL4 can interact with integrin and stimulates intracellular signaling to promote tumor cell survival (7, 8, 11). In addition, it seems that the processing of ANGPTL4 in humans is tissue dependent, as different organs such as the liver and kidney have been found to use different forms of ANGPTL4 (50). One recent finding has implicated the glycosylated form of ANGPTL4 in augmenting the leakiness of the kidney glomerular epithelium, suggesting that this type of posttranslational modification of ANGPTL4 may direct its function in this case (50). Further *in vitro* and *in vivo* experiments are necessary to clarify the role of ANGPTL4 in metastasis.

Clinical Relevance of ANGPTL4

The endocrine regulatory role of ANGPTL4 in lipid metabolism, insulin sensitivity, and glucose homeostasis has been extensively studied (14, 15, 22). As a critical mediator in energy homeostasis, it is not surprising that ANGPTL4 has been suggested as a potential therapeutic target for metabolic and cardiovascular diseases. Antibodies against nANGPTL4 could potentially lower the levels of triglycerides and cholesterol in patients with heart disease (<http://www.lexpharma.com/news/2007-07-02-160145.html>). The targeted silencing of ANGPTL4 offers another potential therapeutic strategy in the treatment of atherosclerosis, dyslipidemia, and diabetic wounds (8, 14, 51, 52). These reports emphasize the clinical relevance of ANGPTL4 as an attractive therapeutic candidate.

ANGPTL4 as an antitumor and antimetastatic target remains debatable because the multifaceted roles of ANGPTL4 in tumorigenesis need further clarification. However, several research groups have shown promising results suggesting that ANGPTL4 could be a potential interventional therapeutic target in the battle against cancer (11, 21, 25, 30).

On one hand, it was shown that the immunosuppression of cANGPTL4 by a neutralizing antibody results in tumor regression, which was attributed to reduced cell proliferation and enhanced redox-based cell apoptosis of the regressed tumor (11). The suppression of cANGPTL4 by either a neutralizing antibody or RNA interference also reduces vascular disruption and, thus, metastasis (21). In addition, the inhibition of cANGPTL4 may confer chemosensitivity to cancer cells (25). On the other hand, an increased level of ANGPTL4 has been found to inhibit tumor angiogenesis, vascular permeability, and invasiveness of cancer cells (10, 20, 37, 42). Although discrepant in their results, these studies have used different cancer cell models, suggesting that potential inhibitory therapy may have a restricted therapeutic range or may be restricted to certain cancer types.

ANGPTL4 could also be a potential diagnostic biomarker in cancer. ANGPTL4 expression was observed to be elevated in numerous cancers at both the mRNA and protein level (Table 2). Given the implications that ANGPTL4 may have on cancer progression, a recent report suggested that ANGPTL4 mRNA could be used as a diagnostic marker to distinguish primary and metastatic clear cell renal cell carcinoma (53). Several other studies have also delineated the ANGPTL4 expression level in primary and invasive tumors, showing that high ANGPTL4 mRNA and protein expression in human tumor biopsies often correlates with a poor prognosis and a poor disease outcome (25, 33, 34). As a secreted protein, ANGPTL4 protein levels could also be detected in the serum (23). Zhu and colleagues have also shown that increased cANGPTL4 protein levels could be detected in the serum of tumor-bearing mice (11). cANGPTL4 was also found to be secreted by hypoxic cancer cells, lending further credence to the potential use of ANGPTL4 as a biomarker (25).

Conclusion and Future Directions

Recent work has suggested that ANGPTL4 has increasingly important roles in cancer development and progression. Therefore, many researchers have postulated that diverse and sometimes conflicting roles for ANGPTL4 exist in cancer. The collective data show that ANGPTL4 exerts a proangiogenic effect by promoting vascularization under hypoxic conditions in tumors (42). Moreover, ANGPTL4 promotes invasion and metastasis by enabling vascular permeability and conferring anoikis resistance to the tumor cells (11, 21, 28, 46). However, antitumorigenic roles of ANGPTL4 have also been established, particularly in the inhibition of tumor angiogenesis and metastasis (10, 37). The role of ANGPTL4 in human cancer could certainly be context dependent or tissue dependent. These seemingly controversial results underscore the need to address the function of fANGPTL4 and its truncated fragments in cancer and the posttranslational modifications that may clarify the context-dependent roles of the different fragments of ANGPTL4 in cancer.

The role of ANGPTL4 in systemic lipid and glucose metabolism has been established recently (15). However, it would be interesting to examine the impact that ANGPTL4 may have on tumor cell metabolism. Evidence has shown that AKT/PKB can regulate glucose consumption

and glycolysis during the metabolic reprogramming that occurs in cancer cells. Coupled with the ability of ANGPTL4 to regulate the function of AKT/PKB in conferring tumor cell resistance against anoikis, it remains to be observed if ANGPTL4 plays a role in the alteration of cancer cell metabolism as well (11, 54). Another area that remains to be elucidated is the biological function and the inherent signaling pathway of the different fragments of ANGPTL4 (Fig. 2). It is interesting to note that a "receptor" for the full-length and the *N*-terminal ANGPTL4 has yet to be identified (see above, "Functional Domains of ANGPTL4"). The cANGPTL4 has been reported to bind to several interacting partners, resulting in the activation of different pathways in a context- and tissue-dependent manner (11, 21, 25). However, it is unknown whether the COOH-terminal domain of the fANGPTL4 activates similar signaling pathways and biological functions.

New and promising findings have already earmarked ANGPTL4 as a diagnostic biomarker in various cancer types. Despite the reported conflicting roles of ANGPTL4, several exciting findings offer a novel perspective promoting the therapeutic use of cANGPTL4 antibody as a primary clinical drug. Clearly, the diverse roles of ANGPTL4 in cancer remain to be discovered. However, given the complex nature of cancer development and the many proteins involved, the likelihood of curing cancer by targeting a single protein is unlikely to achieve the desired outcome. Indeed, multimodal therapy is a new, yet extremely important, advance in cancer treatment. By building on the current mechanistic insights of ANGPTL4 function, elucidating the diversified roles of ANGPTL4 in human cancer promises to be a challenging yet provocative endeavor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Molecular Cancer Research

Emerging Roles of Angiopoietin-like 4 in Human Cancer

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ANGPTL4 modulates vascular junction integrity by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 clusters

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Vascular disruption induced by interactions between tumor-secreted permeability factors and adhesive proteins on endothelial cells facilitates metastasis. The role of tumor-secreted C-terminal fibrinogen-like domain of angiopoietin-like 4 (cANGPTL4) in vascular leakiness and metastasis is controversial because of the lack of understanding of how cANGPTL4 modulates vascular integrity. Here, we show that cANGPTL4 instigated the disruption of endothelial continuity by directly inter-

acting with 3 novel binding partners, integrin $\alpha 5\beta 1$, VE-cadherin, and claudin-5, in a temporally sequential manner, thus facilitating metastasis. We showed that cANGPTL4 binds and activates integrin $\alpha 5\beta 1$ -mediated Rac1/PAK signaling to weaken cell-cell contacts. cANGPTL4 subsequently associated with and declustered VE-cadherin and claudin-5, leading to endothelial disruption. Interfering with the formation of these cANGPTL4 complexes delayed vascular disruption. In vivo

vascular permeability and metastatic assays performed using ANGPTL4-knockout and wild-type mice injected with either control or ANGPTL4-knockdown tumors confirmed that cANGPTL4 induced vascular leakiness and facilitated lung metastasis in mice. Thus, our findings elucidate how cANGPTL4 induces endothelial disruption. Our findings have direct implications for targeting cANGPTL4 to treat cancer and other vascular pathologies. (Blood. 2011;118(14):3990-4002)

Introduction

Tumor metastasis is the main cause of mortality in cancer patients.¹ It is determined largely by vasculature leakiness and the critical steps of intravasation and extravasation, which involve the directional migration of tumor cells across the disrupted endothelium. The endothelium provides a semipermeable boundary between the bloodstream and tumor. The paracellular permeability of the endothelium is mediated primarily by transmembrane endothelial adherens junction (AJ) and tight junction (TJ) proteins that are linked to the actin cytoskeleton,² and connect adjacent endothelial cells (ECs).³ Thus, the disruption of major components of AJs and TJs, such as the intercellular VE-cadherin and claudin-5 clusters, leads to changes in the actin cytoskeleton, cell shape, and the activation of intracellular signaling pathways, which partly via β -catenin initiate gene transcription.⁴ Apart from AJs and TJs, integrins also strengthen EC-EC contacts and provide a scaffold for cytoskeletal reorganization during cell organization, proliferation, and migration.⁵ Specifically, the expression of integrin $\alpha 5\beta 1$ regulates endothelial monolayer integrity.⁶ Inherently, metastasis requires communication between tumor cells and ECs that culminates in the disruption of EC-EC contacts and the degradation of the vascular basement membrane. However, the molecular mechanisms that facilitate such communication are poorly understood.

In the tumor microenvironment, interactions among tumor cells, their secreted factors, and the endothelium induce vascular permeability to aid tumor metastasis.^{5,7} These factors bind to cognate

cellular receptors, setting off a cascade of downstream molecular signaling events that determine the outcome of malignancy.^{8,9} In this respect, angiopoietin-like 4 (ANGPTL4) protein has been implicated in cancer metastasis¹⁰; however, its precise role in vascular and cancer biology remains debatable. Earlier studies suggested that ANGPTL4 could prevent metastasis by inhibiting vascular leakiness.^{11,12} However, ANGPTL4 was identified as 1 of the most highly expressed genes in distant metastases¹³ and it is associated with breast cancer metastasis to the lungs.^{14,15} Our recent work revealed that tumors produced high levels of the C-terminal fibrinogen-like domain of ANGPTL4 (cANGPTL4). We also showed that cANGPTL4 stimulates integrin-mediated signaling to maintain an elevated, oncogenic $O_2^-:H_2O_2$ ratio to confer anoikis resistance to tumor cells via autocrine adhesion mimicry.¹⁶ Furthermore, we have demonstrated that through interaction with matrix proteins integrin $\beta 1$ and integrin $\beta 5$, cANGPTL4 facilitates cell migration and wound healing, both of which are processes highly reminiscent of metastasis.^{17,18} Despite the plethora of evidence implicating ANGPTL4 in cancer metastasis, the heterotypic role of ANGPTL4 in vascular integrity remains unclear.

Here, we show that tumor-derived cANGPTL4 instigated the disruption of endothelial continuity by directly interacting with integrin $\alpha 5\beta 1$, VE-cadherin, and claudin-5 in a temporally sequential manner. Tumor-derived cANGPTL4 and recombinant human (rh)-cANGPTL4 increased vascular permeability in vitro and in

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vivo. Using *ANGPTL4*-knockout and wild-type mice injected with either control or *ANGPTL4*-knockdown tumors confirmed that cANGPTL4 induced vascular leakiness and facilitated lung metastasis in mice. cANGPTL4 binds to and activates integrin $\alpha 5\beta 1$ -mediated PAK/Rac signaling that weakened EC-EC contacts and increased vascular leakiness. cANGPTL4 associated with the adhesive domains of VE-cadherin and claudin-5, resulting in their declustering and internalization, translocation of β -catenin to the nucleus, and a leaky endothelium. These results identify cANGPTL4 as a novel upstream mediator of endothelium permeability and a potential target in the treatment of cancer and other vascular-related pathologies.

Methods

Antibodies

Antibodies were obtained from the following sources: PAK1 and pPAK1 (Ser199/Ser204; Cell Signaling Technology); integrin $\beta 1$ (clone JB1A), $\alpha 5\beta 1$ (clone JBS5), $\beta 3$ (MAB2008), and c-Jun (all Millipore); CD29/activated integrin $\beta 1$ (HUTS-21; BD Biosciences); β -tubulin, β -catenin (12F7), epidermal growth factor receptor (1005; all Santa Cruz Biotechnology); VE-cadherin [BV9] and claudin-5 (Abcam); CD31 (Dako Denmark A/S); occludin (Invitrogen); zona occludens-1 (ZO-1; Zymed Laboratories); and Tie 1, Tie 2, junctional adhesion molecule (JAM)-C, β -tubulin, horseradish peroxidase (HRP)-conjugated goat anti-mouse, HRP-conjugated goat anti-rabbit, and HRP-conjugated donkey anti-goat (Santa Cruz Biotechnology). Mouse monoclonal anti-human cANGPTL4 mAb4A11H5 and rabbit polyclonal anti-human cANGPTL4 were produced in-house.¹⁶ Secondary antibodies Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexa Fluor 594-conjugated goat anti-mouse IgG also were used (Invitrogen).

Cell cultures

Primary human microvascular endothelial cells (HMVECs; Lonza) were cultured in EndoGRO-MV-VEGF medium (Millipore) in a humidified atmosphere of 5% CO₂ at 37°C. The culture surface was precoated with 0.1% gelatin in PBS. All other cell lines were cultured in DMEM supplemented with 10% FBS. Conditioned medium (CM) was obtained from 1×10^5 tumor cells grown in 1 mL of serum-free medium for 3 days.

Transient transfections assay

Transfections of HMVECs were carried out as per the manufacturer's protocol (Promega) with either constitutive-active Rac1 G12V or dominant-negative Rac1 T17N.

Expression and purification of recombinant cANGPTL4 proteins

The expression and purification of full-length ANGPTL4 and recombinant cANGPTL4 were performed as described previously.^{17,18}

In vivo tumorigenicity and miles vascular permeability assay

Six-week-old BALB/c athymic female nude mice (20–22 g) were injected either cANGPTL4 (6 μ g/mL), or 0.9% saline buffer was injected intradermally at adjacent locations on the back. To determine vascular permeability, tumor-bearing mice received an intravenous injection of Evans blue dye. Twenty minutes later, the mice were killed and Evans blue dye was extracted from the tumor and normal muscle as described in Miles vascular permeability assay.¹² The extravasated dye was extracted using formamide, and the amount was quantified by measuring absorbance at 610 nm. To determine the tumor vascular volume, 2MDa FITC-conjugated dextran (10 mg/kg) also was injected intravenously for 20 minutes. Fluorescence readings were obtained using 3300 fluorospectrometer (NanoDrop).

For another set of experiments, mice were injected subcutaneously at the interscapular region with either 2×10^6 or 8×10^6 cells (A-5RT3_{CTRL} or A-5RT3_{ΔANGPTL4}). The same procedures were carried out in C57BL/6J wild-type and ANGPTL4-knockout mice using B16F10_{CTRL} or B16F10_{ΔANGPTL4} cells. Injection sites were rotated to avoid site bias. The injected tumor cells were allowed to grow for 8 weeks.

In vivo metastasis assay

Wild-type and ANGPTL4-knockout C57BL/6J mice were injected intravenously with either 5×10^5 B16F10_{CTRL} or B16F10_{ΔANGPTL4} cells. For cANGPTL4 treatment, wild-type C57BL/6J mice intravenously injected with either 2×10^6 B16F10_{CTRL} or B16F10_{ΔANGPTL4} cells were treated with PBS or cANGPTL4 (3 mg/kg) thrice weekly. After 3 weeks, the mice were killed and the lungs were harvested for further analyses. Total metastatic burden were quantified by RT-PCR of melanin A. Total mRNA were extracted from lungs and reverse-transcribed as described previously.^{17,18} All animal studies were approved and carried out in compliance with the regulations of the Institutional Animal Care and Use Committee of Nanyang Technological University.

Proximity ligation assay

A proximity ligation assay (PLA) was carried out according to the manufacturer's protocol (Olink Bioscience) using indicated pairs of antibodies. Images were taken using an LSM 710 confocal microscope (Carl Zeiss) with a Plan-Apochromat 63 $\times$ /1.4 oil differential interference contrast objective and ZEN 2009 software (Carl Zeiss). PLA signals were quantified using BlobFinder software (Centre for Image Analysis, Uppsala University).¹⁹

TER measurement

Transendothelial electrical resistance (TER) was measured using an electrical cell-substrate impedance sensing system (Applied BioPhysics). HMVECs were seeded on sterile 8-well gold-plated electrode arrays precoated with fibronectin at 2×10^5 cells/well and allowed to adhere and spread for 4 hours at 37°C. Data from the electrical resistance experiments were obtained over the experimental time course at 5-minute intervals. Confluent HMVEC monolayers that had stable TERs for 1 hour preceding administration of indicated treatments were used. As cells adhered and spread over the microelectrode, TER increased, whereas cell retraction, rounding, or loss of adhesion was reflected by a decrease in TER. Resistance values for each microelectrode were normalized as the ratio of measured resistance to baseline resistance and are plotted as a function of time.

Internalization assay

Confluent HMVEC monolayers were treated with either PBS or cANGPTL4 (6 μ g/mL) for 3 hours. At indicated time point, an internalization assays was carried out as described previously.¹⁷

Immunofluorescence staining

Disrupted tight junctions were visualized by immunofluorescence staining for ZO-1. HMVECs were fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.2% Triton X-100 for 15 minutes, and blocked with 2% BSA containing 0.1% Triton X-100 in a humid chamber for 1 hour at room temperature. Cells were incubated overnight at 4°C with anti-human-ZO-1 antibodies (1:100) in 0.2% BSA. After 2 washes in PBS, cells were incubated for 1 hour at room temperature with Alexa 488-secondary antibodies (1:250). Cells were counterstained with Alexa 594-phalloidin for F-actin and 4,6-diamidino-2-phenylindole (DAPI) for nuclei. Immunostainings performed without primary antibodies served as negative controls. For β -catenin staining, cells were fixed with 4% paraformaldehyde containing 5% sucrose for 15 minutes, permeabilized with 0.5% Triton X-100 in PBS for 4 minutes, and blocked in 5% normal goat serum with 0.1% Triton X-100. Cells were incubated overnight at 4°C with anti-human β -catenin antibodies (1:200) in 3% normal goat serum, followed by Alexa 594-secondary antibodies. Images were acquired using an LSM710

confocal microscope (Carl Zeiss) with a 63×/1.40 oil objectives and AxioCan MRm camera (Carl Zeiss) and analyzed using ZEN 2009 software.

Surface plasmon resonance

Surface plasmon resonance (SPR) was carried out as described previously^{17,18} using a BIAcore 2000 system (BIAcore). Six concentrations (0.16, 0.32, 0.63, 1.25, 2.50, and 5.0 μ M) of recombinant integrin β 1, first extracellular domain of VE-cadherin (ECD1), extracellular repeat 1 domain of VE-cadherin (EC1), occludin, or JAM-A were used. Integrin was expressed in *Drosophila* S2 cells,¹⁷ and various extracellular domains of VE-cadherin and claudin-5 were purchased from Abnova. Global fitting of the SPR data were performed as described previously to determine the K_D value. Preinjection or preincubation with the indicated antibodies or preimmune IgG was performed to determine specific interactions. Each sensorgram was corrected by subtracting a sensorgram obtained from a reference flow cell with no immobilized protein. The R_{max} value was determined to be 283.1 resonance units using anti-cANGPTL4 antibodies against the immobilized cANGPTL4. Values are mean $\pm$ SD ($n = 3$).

Statistical analysis

Statistical analyses were performed using 2-tailed Mann-Whitney *U* tests with SPSS software (SPSS). All statistical tests were 2-sided. A *P* value of $\leq .05$ is considered significant.

Results

Elevated cANGPTL4 expression in human tumors

To examine the expression profile of cANGPTL4 in tumors, we performed immunofluorescence using anti-cANGPTL4 antibody on 2 human tumor tissue arrays that covered benign, malignant, and metastatic tumors originating from various anatomic sites. cANGPTL4 was elevated in all epithelial tumor samples compared with the corresponding normal tissue samples (supplemental Figure 1A-C, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Although fluorescence signals varied among different tumor types, they clearly showed that elevated cANGPTL4 was a common feature of tumors. Consistent with the observations in supplemental Figure 1A through C, cANGPTL4 protein level was elevated in all cancer cell lines compared with nontumorigenic lines (HaCaT and BJ; supplemental Figure 1D). Interestingly, we observed relatively higher cANGPTL4 levels in metastatic than in nonmetastatic cancer cell lines, suggesting a role of cANGPTL4 in tumor metastasis. To further corroborate the role of cANGPTL4 in metastasis clinically, we determined cANGPTL4 mRNA and protein levels in 6 human basal cell carcinoma (benign) and melanoma (metastatic) biopsies by quantitative real-time PCR and immunoblot analyses. We observed significant up-regulation of cANGPTL4 mRNA and protein levels in these epithelial cancers compared with their cognate peritumor normal samples. Notably, the melanomas expressed higher levels of cANGPTL4 mRNA and protein compared with benign basal cell carcinoma (Figure 1A-B). Similar trend was observed when comparing cANGPTL4 protein levels of fine-needle aspirates from 3 breast cancer patients to 4 human metastatic tumor lines (A-5RT3, MDA-MB231, MCF-7, and HT29) and 2 nontumorigenic lines (HaCaT and BJ). The mean cANGPTL4 protein concentrations were 12.4 μ g/mL (fine-needle aspirate), 5.9 μ g/mL (metastatic lines), and 0.8 μ g/mL (nontumorigenic lines; supplemental Figure 1E). These data suggest that epithelial tumors secrete elevated levels of cANGPTL4, which is

consistent with our recent report showing that tumor tissue expressed high levels of cANGPTL4 compared with basal level expression in the surrounding stroma.¹⁶ Altogether, our findings implicate a role for cANGPTL4 in cancer metastasis.

cANGPTL4 increased vascular leakiness in in vivo tumors

We next explored the role of cANGPTL4 in tumorigenesis. Metastatic human squamous cell carcinoma cell line A-5RT3 and murine melanoma B16F10 were transduced with either scrambled (control) small interfering (si)RNA (A-5RT3_{CTRL} or B16F10_{CTRL}) or siRNA against ANGPTL4 (A-5RT3_{ΔANGPTL4} or B16F10_{ΔANGPTL4}; supplemental Figure 2A) to suppress endogenous ANGPTL4, as described previously.^{16,18} The injection of A-5RT3_{CTRL} cells into immunodeficient mice induced large primary tumors at week 8, but A-5RT3_{ΔANGPTL4}-induced tumors displayed a 30% lower tumor volume (A-5RT3_{CTRL}, 1098 $\pm$ 422 mm³ vs A-5RT3_{ΔANGPTL4}, 551 $\pm$ 135 mm³; *P* < .05; $n = 6$; Figure 1C). Miles assays revealed decreased vascular leakiness in A-5RT3_{ΔANGPTL4}-induced tumors ($\sim$ 5-fold lower), as evidenced by decreased extravasation of Evans blue dye (Figure 1D; Table 1). Differences in vascular volume among the tumor types were accounted for when determining tumor vascular permeability (Table 1). This differential vascular permeability was independent of the tumor volume, as observed in A-5RT3_{CTRL}- and A-5RT3_{ΔANGPTL4}-induced tumors (Figure 1D). Furthermore, none of the mice injected with A-5RT3_{ΔANGPTL4} developed distant metastases to the lymph nodes, whereas 83% of mice injected with A-5RT3_{CTRL} showed metastases (supplemental Figure 2B). Notably, rh-cANGPTL4 was sufficient to induce vascular leakiness compared with the PBS control (rh-cANGPTL4, 0.084 $\pm$ 0.014 vs PBS, 0.04 $\pm$ 0.01; *P* < .05; $n = 3$; Figure 1E-F). Consistent to the increased vascular volume in A-5RT3_{CTRL}- and B16F10_{CTRL}-induced tumors, the expression of EC marker CD31 was elevated compared with their cognate ANGPTL4-deficient tumors (Figure 1G-H). Dual immunostaining for CD31 and cANGPTL4 also confirmed that epithelial tumors were the major producer of cANGPTL4, consistent with earlier findings using laser-captured microdissected samples (Figure 1H).¹⁶ Hence, these observations in vivo prompted us to investigate the molecular mechanism of cANGPTL4-induced endothelial permeability.

Confluent primary human microvascular ECs were cultured in 3-day-old CM from either A-5RT3_{CTRL} or A-5RT3_{ΔANGPTL4} cells. EC junction integrity was disrupted when the cells were cultured in A-5RT3_{CTRL} CM but not in A-5RT3_{ΔANGPTL4}CM, as indicated by ZO-1 staining. Junction integrity was maintained in the presence of anti-cANGPTL4 monoclonal antibody, suggesting that tumor-secreted cANGPTL4 plays a major role in endothelial junction disruption (Figure 1I). We found that EC-EC junction integrity was disrupted when the cells were treated with only rh-cANGPTL4 (6 μ g/mL) in the presence of either cycloheximide or actinomycin D, indicating that de novo protein synthesis and transcription were not required (supplemental Figure 2C). Treatment with cycloheximide, actinomycin D, or vehicle alone did not induce any vascular lesions (supplemental Figure 2C). This disruption was mirrored by a decrease in TER of the monolayer, suggesting a rapid and dose-dependent increase in paracellular permeability (Figure 1J). No change in TER was observed in the PBS control (Figure 1J). This effect was not because of apoptosis, even after 6 hours of rh-cANGPTL4 treatment (supplemental Figure 2D). Altogether, these results indicate that tumor-secreted cANGPTL4 disrupts endothelial integrity.

Figure 1. cANGPTL4 is elevated in metastatic tumors and disrupts endothelial junction integrity. (A-B) Expression levels of cANGPTL4 mRNA (A) and protein (B) in basal cell carcinoma (BCCs) and metastatic melanoma paired with peritumor normal tissues as determined by quantitative real-time PCR and immunoblot, respectively ($n = 3$). Data spots from same individual are linked by colored lines. Ribosomal protein L27 (L27) was used as a reference housekeeping gene. β -tubulin served as a loading and transfer control. (C-D) Evans blue dye extravasation induced by either A-5RT3 Δ CTRL- or A-5RT3 Δ ANGPTL4-induced tumors of different (C) or similar (D) tumor volume (A-5RT3 Δ CTRL, $1098 \pm 422 \text{ mm}^3$ vs A-5RT3 Δ ANGPTL4, $551 \pm 135 \text{ mm}^3$; $P < .05$; $n = 6$). (E-F) Evans blue dye extravasation induced by cANGPTL4 or PBS vehicle (G) and quantification (H) of extravasated Evans blue dye by measurement of absorbance at 610 nm. $*P < .05$. (G) Immunodetection of CD31 in A-5RT3 Δ CTRL- and A-5RT3 Δ ANGPTL4-induced tumors. (H) Immunofluorescence staining for CD31 and cANGPTL4 in B16F10 Δ CTRL- and B16F10 Δ ANGPTL4-induced tumors. Scale bar represents 100 μm . (I) Immunofluorescence staining for ZO-1 in a confluent HMVEC monolayer. Cells were treated with either CM from A-5RT3 Δ CTRL in the presence or absence of anti-cANGPTL4, from A-5RT3 Δ ANGPTL4 cells. HMVECs were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin stress fibers. Scale bar represents 40 μm . White arrows indicate endothelial junction lesions. (J) Transendothelial electrical resistance (TER) analysis of confluent monolayer HMVECs treated with the indicated amounts of cANGPTL4. PBS served as a vehicle control. Data (means $\pm$ SD) from 3 independent experiments.

Table 1. ANGPTL4 increases vascular permeability and vascular volume

Tumor (n = 15)	Vascular permeability, OD ₆₁₀ Evans blue/g,	Vascular volume, fluorescent unit (FITC)/g	Ratio VP/VV	Fold change
A-5RT3 _{CTRL}	0.76 ± 0.09*	0.82 ± 0.17†	0.927	5.332
A-5RT3 _{ΔANGPTL4}	0.073 ± 0.012	0.42 ± 0.09	0.174	1.000

Data represent the mean ± SD of 15 animals.
VP indicates vascular permeability; and VV, vacular volume.
*P < .001.
†P < .01.

cANGPTL4 interacts with Integrin α5β1, VE-cadherin, and claudin-5

VE-cadherin and claudin-5 contribute to the control of paracellular permeability, whereas integrin α5β1, which is localized to EC–EC junctions, also mediates endothelial leakiness.^{4,6} Hence, we hypothesized that tumor-derived cANGPTL4 induces vascular leakiness and ultimately disrupts the endothelium barrier via direct interactions with specific junction proteins. SPR analysis revealed that cANGPTL4 interacted with VE-cadherin ($K_D = 1.12 \times 10^{-7}$ M), claudin-5 ($K_D = 5.87 \times 10^{-8}$ M), and integrin α5β1 ($K_D = 1.26 \times 10^{-8}$ M) but not with occludin or JAM-A (Figure 2A). More significantly, cANGPTL4 targeted the VE-cadherin EC1 domain and claudin-5 ECD1 domain that are responsible for homophilic interactions between adjacent ECs, suggesting that the interactions of cANGPTL4 with VE-cadherin and claudin-5 could play an important role in regulating vascular permeability (Figure 2B).^{20,21} No interactions were observed between cANGPTL4 and integrin β3^{16,17} or other domains of VE-cadherin (EC3) and claudin-5 (second extracellular domain of claudin-5 [ECD2]), which also served as controls (Figure 2B). The observations of these interactions were corroborated by blocking with neutralizing antibodies against integrin α5β1 (Figure 2C), VE-cadherin (Figure 2D), claudin-5 (Figure 2E), and cANGPTL4 (supplemental Figure 3A–C). An *in situ* PLA detected complexes of integrin α5β1, claudin-5, or VE-cadherin with cANGPTL4 in both EC- (integrin, 22.50 ± 5.01 ; VE-cadherin, 16.43 ± 2.46 ; claudin-5, 12.35 ± 3.44 PLA spots/cell calculated from 250 cells) and A-5RT3_{CTRL}-induced tumor biopsies (Figure 2F; supplemental Figure 3D), confirming that these interactions also exists *in vivo*. No PLA signals, that is, no interactions were observed between CD31 and cANGPTL4, claudin-5, VE-cadherin, or integrin α5β1 (supplemental Figure 3E).

cANGPTL4 interacted directly with the EC1 and ECD1 adhesive domains of VE-cadherin and claudin-5, respectively, and may lead to disruption of intercellular VE-cadherin and claudin-5 cluster formation.²² To examine this possibility, membrane fractions of confluent ECs treated with cANGPTL4 were extracted, resolved under native conditions, and probed with either anti-VE-cadherin or anti-claudin-5. cANGPTL4 treatment resulted in smaller protein bands compared with PBS (Figure 2G). Treatment with EDTA, which disrupts intercellular adhesion, served as a positive control for declustered VE-cadherin and claudin-5. These findings confirm that cANGPTL4 interacts with integrin α5β1, VE-cadherin, and claudin-5 to disrupt intercellular adhesion.

cANGPTL4 modulates endothelial integrity in a temporally sequential mechanism

Next, we sought a mechanistic explanation for the effect of cANGPTL4 on the endothelium. We monitored the temporal interactions of cANGPTL4 and its 3 binding partners through kinetic PLA. A bimodal interaction profile of the cANGPTL4: integrin α5β1 complex was detected within 30–60 minutes, followed by cANGPTL4:VE-cadherin and cANGPTL4:claudin-5 at

120 minutes post-cANGPTL4 treatment (Figure 3A). *In vitro* coimmunoprecipitation of cANGPTL4 and its various interacting partners validated our PLA and SPR results (Figure 3B). Except for integrin α5β1, which showed elevated expression only 2 hours post-cANGPTL4 treatment, no change in protein level was observed for any other cANGPTL4-interacting partner, indicating that the bimodal cANGPTL4–protein interaction profiles did not result from increased protein expression (supplemental Figure 4A). We also showed that the initial cANGPTL4: integrin α5β1 interactions resulted in the complex being internalized into the cytoplasm at 40 minutes, followed by the internalization of the cANGPTL4: VE-cadherin and cANGPTL4:claudin-5 complexes between 120 and 150 minutes to result in the abrogation of the endothelial junctions (Figure 3C). This bimodal effect was not observed with the control treatment (supplemental Figure 4B). Notably, cANGPTL4: integrin α5β1 formation at 30–60 minutes coincided with a TER decrease, suggesting increased permeability (Figure 1K). Integrin β3, which does not interact with cANGPTL4, was internalized after 2 hours in both vehicle- and cANGPTL4-treated cells. Similar observations were reported in wild-type and ANGPTL4-knockdown keratinocytes,¹⁷ suggesting that this may be inherent to either integrin β3 or the experimental procedure. Thus, our results suggest that the initial interaction of cANGPTL4 with integrin α5β1 may be required to facilitate the subsequent interactions to mediate vascular disruption.

Inhibition of the cANGPTL4: Integrin α5β1 complex delays cANGPTL4 interactions with VE-cadherin and claudin-5

Antibody to integrin α5β1 increased endothelial leakiness but was insufficient to induce microscopic lesions at endothelial junctions.⁶ Thus, we sought to understand the impact of the upstream cANGPTL4: integrin α5β1 formation on the disruption of endothelial integrity. In the presence of monoclonal anti-integrin α5β1, cANGPTL4 required twice the length of time (6 hours) to induce lesions along the EC–EC contact compared with the cANGPTL4 alone (Figure 4A). Anti-integrin α5β1 blocked the interaction with cANGPTL4 (Figure 2E). ECs treated with anti-integrin α5β1 alone did not induce any lesions, even after 6 hours (Figure 4A). These results were corroborated by our TER data, which showed that the addition of anti-integrin α5β1 and cANGPTL4 (6 μg/mL) to ECs resulted in reduced endothelial permeability compared with ECs treated only with 6 μg/mL cANGPTL4 (Figure 4B). Accordingly, anti-integrin α5β1 caused a concomitant delay of ~ 90 minutes in the interaction profile of cANGPTL4:VE-cadherin and cANGPTL4: claudin-5 compared with control (compare Figures 3A and 4C). The mean fold change (0.4 ± 0.12) in the interactions between cANGPTL4 and these 2 proteins remained similar, albeit with a nonsignificant decrease of ~ 10% for VE-cadherin, suggesting that interactions with these 2 proteins are required for endothelial disruption (Figure 4C). Thus, we treated HMVECs with cANGPTL4 for 90 minutes, which allowed for cANGPTL4: integrin α5β1 interactions

Figure 2. cANGPTL4 interacts with integrin $\alpha 5 \beta 1$, VE-cadherin, and claudin-5. (A-E) Representative sensorgrams showing binding profiles between immobilized cANGPTL4 and integrin $\alpha 5 \beta 1$, VE-cadherin, claudin-5, occludin, and JAM-A (A); EC1, ECD1, VE-cadherin EC3, and claudin-5 ECD2 (B); integrin $\alpha 5 \beta 1$ (C), VE-cadherin (D), and claudin-5 (E) preblocked with either preimmune IgG or indicated cognate antibodies. (F) In situ PLA of cANGPTL4 and indicated binding partners. PLA signals are in red (integrin 22.50 ± 5.01 vs VE-cadherin 16.43 ± 2.46 vs claudin-5 12.35 ± 3.44 PLA spots/cell for 250 cells, $n = 3$). Cells were counterstained with Alexa 488-phalloidin for actin stress fibers (green) and Hoechst dye for nuclei (blue). Negative control was performed in the absence of anti-ANGPTL4. Scale bar represents 40 μm . (G) Immunodetection of VE-cadherin and claudin-5 from membrane extract of HMVECs treated with cANGPTL4 (3 or 6 $\mu\text{g}/\text{mL}$) for 3 hours. Vehicle (V) is PBS, and E indicates treatment with 2.5mM EDTA in PBS.

and initiated endothelial leakiness, followed by its removal. Immunostaining for ZO-1 revealed that cANGPTL4: integrin $\alpha 5 \beta 1$ interactions alone is not sufficient to induce endothelial disruption and that the later interactions are necessary for the observed disruption. (Figure 4D). Interestingly, we observed from our TER data that the removal of rh-cANGPTL4 promoted a “reversal effect” on endothelial permeability, conceivably because of the “re-zipping” of the EC-EC contacts, thus reinforcing the important of cANGPTL4: integrin $\alpha 5 \beta 1$ complex formation in the entire process of endothelial disruption (Figure 4E). Hence, our results

suggest that although integrin $\alpha 5 \beta 1$ is an essential player, its interaction with cANGPTL4 alone is insufficient to induce an endothelial disruption/lesion. The data suggest that the binding of cANGPTL4 to integrin $\alpha 5 \beta 1$ triggers internalization of the complex and “un-zipping” of the endothelial junctions to first promote endothelial leakiness. This facilitates the subsequent cANGPTL4 interactions with the adhesive domains of VE-cadherin and claudin-5 that weaken their homophilic contacts, resulting in their declustering and internalization and finally culminating in the disruption of EC-EC contacts.

Figure 3. cANGPTL4 interacts with integrin $\alpha 5 \beta 1$, VE-cadherin, and claudin-5 in a bimodal manner. (A) In situ kinetic PLA detection of various complexes between cANGPTL4 and indicated binding partners in HMVECs treated with 6 μ g/mL cANGPTL4. Values (means $\pm$ SD) represent mean fold change in the number of interactions compared with the zero time point, as determined from $n = 3$ independent experiments (600 HMVECs) using BlobFinder. Experiments were terminated when microscopic lesions were observed at 180 minutes after cANGPTL4 treatment. (B) Immunodetection of integrin $\alpha 5 \beta 1$, VE-cadherin, and claudin-5 in anti-cANGPTL4 immunoprecipitates from total protein lysates of HMVECs treated with 6 μ g/mL rh-cANGPTL4. (C) Immunodetection of integrins $\beta 1$, integrin $\alpha 5$, integrin $\beta 3$, VE-cadherin, and claudin-5 from total protein lysates versus internalized fraction of HMVECs treated with cANGPTL4. Protein lysates were collected every 10 minutes (0-180 minutes). Values below each band represent the mean fold change in protein expression level compared with the cognate zero time point ($n = 3$). * $P < .05$; ** $P < .01$.

cANGPTL4-induced disruption of the endothelium involves integrin-mediated PAK/Rac signaling

The pivotal role of integrin $\alpha 5 \beta 1$ in the process of EC-EC contact disruption lead us to examine the detailed molecular events

resulting from the cANGPTL4: integrin $\alpha 5 \beta 1$ complex formation. In vitro coimmunoprecipitation of cANGPTL4 with activated integrin $\beta 1$ (antibody clone Huts-21) indicated that integrin was activated on association with cANGPTL4 (Figure 5A-B). No

Figure 4. Inhibition of cANGPTL4:interacting partner complex formation delays endothelial disruption. (A,B,D,E) Immunofluorescence staining for ZO-1 (A,D) and TER measurement (B,E) in a confluent HMVEC monolayer. Cells were treated with either 6 μ g/mL cANGPTL4 in the presence of increasing anti-integrin $\beta 1$ concentrations (1:100 and 1:50; A-B) or 6 μ g/mL cANGPTL4 followed by removal of exogenous cANGPTL4 at 90 minutes (D,E). Treatments were for 3 or 6 hours. HMVECs were counterstained with DAPI (blue) for nuclei and phalloidin (red) for actin stress fibers. Scale bar represents 40 μ m. White arrows indicate endothelial junction lesions. Data (means $\pm$ SD) from 3 independent experiments. * $P < .05$; ** $P < .01$. (C) In situ kinetic PLA detection of cANGPTL4: indicated binding partner complexes in cANGPTL4-treated HMVECs in the presence of anti-integrin $\beta 1$. Values (means $\pm$ SD) represent mean fold change in the number of interactions compared with the zero time point, as determined from $n = 3$ independent experiments (~ 600 HMVECs) using BlobFinder. Experiments were terminated when microscopic lesions were observed (360 minutes after cANGPTL4 treatment).

interactions were observed between cANGPTL4 and Tie1, Tie2, integrin $\beta 3$, or the junction protein JAM-C (Figure 5B). Rac-GTP and PAK are downstream mediators of integrin signaling, whose roles in endothelial contractility and barrier function are well

recognized.^{23,24} Our coimmunoprecipitation results showed that ANGPTL4-activated integrin $\beta 1$ also stimulates the activation of Rac1 and phosphorylation of PAK, compared with PBS-treated ECs (Figure 5C). The increased cANGPTL4: integrin $\alpha 5 \beta 1$ interaction and

Figure 5. cANGPTL4-activated integrin β 1 mediates vascular permeability via activated Rac/PAK signaling axis. (A-B) Immunodetection of indicated proteins in anti-Huts-21 (A) and anti-cANGPTL4 (B) immunoprecipitates from total protein lysates of rh-cANGPTL4-treated HMVECs (6 μ g/mL). IgG immunoprecipitates serves as control. (C) Immunodetection of indicated proteins from anti-Huts-21 immunoprecipitates of HMVECs treated with either vehicle (PBS) or rh-cANGPTL4. Total integrin β 1, Rac1, and PAK served as controls. Protein lysates were collected every 20 minutes (0-180 minutes). Values below each band represent the mean fold change in protein expression level from 3 independent experiments compared with the cognate zero time point. * $P < .05$; ** $P < .01$. (D) In situ kinetic PLA detection of cANGPTL4: indicated binding partner complexes in cANGPTL4-treated HMVECs transfected with either constitutive-active Rac1 G12V or dominant-negative Rac1 T17N. Values (means $\pm$ SD) represent mean fold change in the number of interactions compared with the zero time point, as determined from $n = 3$ independent experiments (~ 600 HMVECs) using BlobFinder. * $P < .05$; ** $P < .01$.

activation of PAK/Rac1 signaling coincided with endothelial leakiness (Figure 1J), possibly by modulating integrin-mediated intercellular adhesion and contractility.^{23,25} To further underscore the role of Rac1, we transfected ECs with either constitutive active Rac1(G12V) or dominant-negative Rac1(T17N). Transfection of ECs with Rac1(G12V) accelerated the formation of cANGPTL4:VE-cadherin and cANGPTL4:

claudin-5 complexes, whereas the expression of Rac1(T17N) delayed the complex formation (compare Figures 3A and 5D). This suggests that cANGPTL4 regulates endothelial integrity via the integrin α 5 β 1-mediated Rac/PAK signaling axis that functions via signaling cross-talk to facilitate cANGPTL4 interactions with VE-cadherin and claudin-5, ultimately leading to vascular disruption.

cANGPTL4 modulates the nuclear translocation of β -catenin

The perturbations and disassembly of VE-cadherin result in the translocation of membrane β -catenin to the nucleus.^{26,27} Hence, we asked whether cANGPTL4-induced declustering of VE-cadherin and claudin-5 resulted in nuclear translocation of β -catenin. In PBS-treated ECs, β -catenin formed a continuous lining along the cell margin as indicated by the immunofluorescence staining and intensity plot (Figure 6A; supplemental Figure 5A), whereas treatment with either cANGPTL4 (6 μ g/mL for 3 hours) or 2.5mM EDTA (positive control) resulted in the disappearance of the β -catenin margin and accumulation in the nuclei, indicating cell-cell junction disruption (Figure 6A; supplemental Figure 5A). To better define the localization of β -catenin, we performed PLA that showed β -catenin in the nuclei at 180 minutes (β -catenin spots/nucleus increases from 1.2 ± 0.81 at 0 minutes to 17.5 ± 2.68 at 180 minutes, whereas those outside the nucleus decreases from 26.3 ± 3.29 minutes to 9.44 ± 3.98 minutes); this timing coincided with the declustering of VE-cadherin and claudin-5 (Figure 6B). Immunoblot analysis corroborated our PLA data (Figure 6C). Taken together, our data indicate that tumor-derived cANGPTL4 is a permeability-inducing factor capable of triggering endothelial disruption via sequential interactions with integrin $\alpha 5\beta 1$, VE-cadherin, and claudin-5, resulting in the accumulation of nuclear β -catenin (Figure 6D).

cANGPTL4 promotes metastasis to the lungs in vivo

To further investigate the role of cANGPTL4 in the metastatic process and to substantiate its biologic relevance in cancer, we examined its effect on lung metastasis using 2 different mice models. Wild-type C57BL/6J mice ($n = 12$ /group) were injected intravenously with either B16F10_{CTRL} or B16F10 Δ ANGPTL4 cells (2×10^6) and treated intravenously with either PBS or rh-cANGPTL4. Mice inoculated with B16F10 Δ ANGPTL4 had significantly fewer lung metastases compared with B16F10_{CTRL}-injected mice. Intravenous administration of rh-cANGPTL4 enhanced lung metastases in both cases, as evidenced by real-time PCR analysis for melanin A, a marker of melanoma (Figure 7A-B). Cross-section staining of the lung tissues corroborated our in vivo data (Figure 7C; supplemental Figure 5B), underscoring the biologic metastatic potential of cANGPTL4 in tumor cells (Figure 7A-B). In addition, we examined lung metastases using wild-type (ANGPTL4^{+/+}) and ANGPTL4-knockout (ANGPTL4^{-/-}) mice injected with either 0.5×10^5 B16F10_{CTRL} or B16F10 Δ ANGPTL4. There was a significantly reduced number of tumor nodules in the lungs of ANGPTL4^{-/-} compared with ANGPTL4^{+/+} mice, suggesting that metastasis of B16F10_{CTRL} was impaired (Figure 7D-E). Altogether, our data indicate cANGPTL4 is a prometastatic tumor-derived factor, and they provide new insight into the mechanistic action of this protein in cancer.

Discussion

Metastasis requires dynamic tumor-endothelium communication through tumor-secreted vascular permeability factors to breach the endothelial barrier, thereby allowing the migration and invasiveness of the tumor cells across the endothelium. Hence, a better understanding of the mechanism by which such vascular permeability factors disrupt endothelial integrity could offer new therapeutic strategies. Recent studies have implicated a role for ANGPTL4 in metastasis,¹⁰ yet the mechanism by which ANGPTL4 modulates

endothelial junction integrity remains unknown. Here, we provide insight into the heterotypic role of cANGPTL4 in vascular integrity. We show that cANGPTL4 disrupts intercellular adhesion via a novel integrin $\alpha 5\beta 1$ -mediated Rac/PAK signaling pathway and the declustering and internalization of VE-cadherin and claudin-5, resulting in nuclear accumulation of β -catenin. Using 2 different mouse models, our in vivo vascular permeability and metastatic assays confirmed that cANGPTL4 acts as a vascular permeability factor by inducing vascular leakiness and disruption. Interestingly, ANGPTL4-deficient tumors also showed decreased vascularization, suggesting that cANGPTL4 may facilitate metastasis through increased vascular permeability and volume.

EC-EC adhesion is mediated mainly by the $\alpha 5\beta 1$ integrin and EC-specific adhesion complexes containing VE-cadherin and claudin-5.^{6,28} Our work shows that cANGPTL4 interacts with these 3 adhesive complexes in a sequential manner to disrupt the endothelial junctions. A major route by which integrins regulate cell-cell contacts is the Rac/PAK pathway, leading to modulation of EC contractility.^{23,29,30} We show that the initial interaction between cANGPTL4 and integrin $\alpha 5\beta 1$ activates Rac and phosphorylates PAK, weakening cell-cell contacts and increasing endothelial leakiness. This facilitates cANGPTL4 access to the adhesive domains of VE-cadherin and claudin-5 to induce declustering of the adhesion molecules resulting in vascular disruption. We and others have shown that ANGPTL4 does not bind the angiopoietin receptor Tie1 or Tie2 to antagonize its action. Angiopoietin-1-mediated activation of Rac reduces vascular leak in the context of VEGF-induced vascular permeability. Thus, the role of Rac in vascular permeability is complex. The ligand and the cellular context that activate Rac will dictate its effect on vascular junction integrity.

The activation of Rac/PAK signaling trigger by cANGPTL4-bound integrin $\beta 1$ is necessary to facilitate cANGPTL4 access to VE-cadherin and claudin-5 and to trigger vascular disruption. By preventing the association of cANGPTL4 with integrin $\beta 1$ by anti-integrin $\beta 1$, we observed delayed cANGPTL4:VE-cadherin and cANGPTL4:claudin-5 complex formation and a concomitant decreased vascular permeability. A similar delay in cANGPTL4:VE-cadherin and cANGPTL4:claudin-5 complex formation was detected in ECs expressing dominant-negative Rac1, without affecting the cANGPTL4:integrin $\beta 1$ formation. This indicates that the Rac/PAK signaling acts downstream of integrin and facilitates cANGPTL4 interactions with VE-cadherin and claudin-5. Interestingly, by preventing the subsequent cANGPTL4 interaction with VE-cadherin and claudin-5, the initial increased in vascular permeability because of the activation of integrin-mediated Rac/PAK signaling did not propagate to observable vascular lesions. This confirms that Rac/PAK signaling is necessary to potentiate the vascular disruptive effect of cANGPTL4.

The interactions of cANGPTL4 with the VE-cadherin ED1 and claudin-5 EDC1 domains disrupt VE-cadherin and claudin-5 clustering. The EC intercellular clustering of VE-cadherin and claudin-5, mediated by their extracellular domains, is important for the maintenance of a semipermeable boundary between the bloodstream and the tumor.^{20,21,31} Our results also reveal a time-dependent cellular trafficking of β -catenin from the membrane to the nucleus in cANGPTL4-treated ECs, coinciding with endothelial disruption. The interactions between the cytoplasmic tail of VE-cadherin and the catenins are essential for the proper functioning of stable AJs.²⁷ The nuclear accumulation of β -catenin in leaky endothelium and release of β -catenin from AJ junctions into the nucleus is consistent with the declustering of AJs.^{26,27,32} Moreover,

Figure 6. cANGPTL4:interacting partner complex formation triggers the nuclear translocation of β-catenin. (A) Immunofluorescence staining for β-catenin (green) in an HMVEC monolayer treated with either 6 μg/mL cANGPTL4 or 2.5mM EDTA (as positive control) for 3 hours. HMVECs were counterstained with DAPI (blue) for nuclei. Scale bar represents 40 μm. White arrows indicate endothelial junction lesions associated with reduced β-catenin staining. Representative fluorescence intensity plot of β-catenin and DAPI, indicated by the white dotted line across the nuclei, were quantified using ZEN 2009 software (Carl Zeiss). (B) In situ PLA for β-catenin and quantification of the number of PLA spots per nuclei of cANGPTL4-treated HMVECs at the indicated time points. (0 minutes, 1.2 ± 0.81; 40 minutes, 3 ± 1.23; 90 minutes, 7.9 ± 1.93; 140 minutes, 13.3 ± 2.44; 180 minutes, 17.5 ± 2.68) PLA signals (red) revealed translocation of β-catenin into nuclei at 140 minutes after treatment (~250 HMVECs). Cells were counterstained with Hoechst dye for nuclei (blue). Scale bar represents 40 μm. *P < .05; **P < .01. (C) Immunodetection of β-catenin in membrane, cytosol, and nuclei fractions of HMVECs treated with either vehicle (PBS) or cANGPTL4. Protein lysates were collected at the indicated times. Values (means ± SD) below each band represent the mean fold change in protein expression level compared with the cognate zero time point (n = 3). *P < .05; **P < .01. (D) Schematic diagram of cANGPTL4-mediated disruption of endothelial junctions. (1) cANGPTL4: integrin α5β1 formation (30-50 minutes) coincides with (2) the activation of Rac-GTP and pPAK in ECs (40-60 minutes) and with vascular leakiness (3); the interaction between cANGPTL4 with VE-cadherin and claudin-5 (120-140 minutes) disrupts intercellular contact formation, and stimulates (4) nuclear translocation of β-catenin (180 minutes).

Figure 7. cANGPTL4 facilitates metastasis. (A-C) Representative macroscopic images of lungs (A); relative expression of melanin A (B); and representative eosin-stained images of lung sections (C). Black nodules in panel C indicate intravasated melanoma (n = 5). Micrographs of lung sections were taken with MIRAX MIDI (Carl Zeiss) with a Plan-Apochromatic 20×/0.8 objective and MIRAX Scan software. Scale bar: represents 100 μ m. (D-E) Number of nodules (D) and weights of lungs (E) from wild-type and ANGPTL4-knockout C57BL/6J mice intravenously injected with either 5×10^5 B16F10_{CTRL} or B16F10_{ΔANGPTL4} cells (n = 12). **P < .01; ***P < .001.

the membrane-liberated β -catenin induces gene transcription that contributes to angiogenic responses of ECs after episodes of vascular leakage that promotes tumor metastasis.^{26,27,33}

The role of ANGPTL4 in vascular leakiness, and thus metastasis, is controversial,^{10,12,34} and efforts to confirm it have been

further hampered by the lack of understanding of the mechanistic action of ANGPTL4. We show that cANGPTL4 behaves as a vascular permeability factor and disrupts endothelial integrity via integrin $\alpha 5 \beta 1$ -mediated Rac/PAK signaling and the declustering and internalization of VE-cadherin and claudin-5. The reason for

the apparent discrepancy is unclear, but it may be because of the position of fusion tags in recombinant ANGPTL4. Studies that suggest an inhibitory role of ANGPTL4 in vascular leakiness have used a C-terminal fusion ANGPTL4, whereas our work and that of others who have presented evidence that ANGPTL4 is a prometastatic agent that induces endothelial permeability has used either an N-terminal fusion ANGPTL4 or untagged ANGPTL4. A dysfunctional endothelium usually presents as a leaky or disrupted vasculature and is characteristic of many pathologic conditions, including inflammatory edema, diabetes, atherosclerosis, and cancer metastasis.^{32,35} The disruption of EC-EC contacts is a major route exploited by primary tumors to promote metastasis. Our findings suggest that targeting cANGPTL4 to treat metastasis and vascular-related diseases could be a viable option.

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Authorship

Contribution: R.-L.H. and Z.T. performed experiments, analyzed the results, and wrote the article; H.C.C., P.Z., M.J.T. C.K.T., C.R.I.L., and M.K.S. performed experiments; D.T.W.L. and S.M.T. performed experiments and contributed to discussion; S.K., J.L.D., and H.Y.L. analyzed the results, contributed to discussion, and edited the article; and N.S.T. analyzed the results, contributed to discussion, and reviewed and edited the article.

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ANGPTL4 modulates vascular junction integrity by integrin signaling and disruption of intercellular VE-cadherin and claudin-5 clusters

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