Understanding wound healing and cancer in the skin

Tan, Mark Wei Yi

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**NANYANG
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SINGAPORE

**UNDERSTANDING WOUND HEALING AND
CANCER IN THE SKIN**

TAN WEI YI, MARK

Interdisciplinary Graduate School

Nanyang Institute for Health Technologies

2020

UNDERSTANDING WOUND HEALING AND CANCER IN THE SKIN

TAN WEI YI, MARK

**Interdisciplinary Graduate School
Nanyang Institute for Health Technologies**

A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2020

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Assoc Prof Tan Nguan Soon

AUTHORSHIP

The thesis is written by Mr Tan Wei Yi, Mark. The research presented in the thesis are a combination of results obtained by himself, and that of the co-authors in the publications listed below.

15/1/2020

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Date

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TAN WEI YI, MARK

PUBLICATIONS

1. **Tan, W.Y.M.**, Kong Z.Q., Tan W.R., Toh, J.H., Wee W.K.J., Teo M.L.E., Wang X. and Tan N.S. High glucose restrains acetylcholine-induced keratinocyte EMT is mitigated by p38/STAT inhibition. (in revision)
2. **Tan, W.Y.M.***, Sng M.K.*, Cheng H.S., Low Z.S., Leong J.J.B., Chua D., Tan H.P.E., Chan S.K.J., Yip Y.S., Lee Y.H., Pal M., Wang X., Wahli W. and Tan N.S. Deficiency in fibroblast PPAR β/δ reduces non-melanoma skin cancers in mice. Cell Death Diff. (in press)

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LIST OF ABBREVIATIONS

β -catenin	Beta-catenin
Ach	Acetylcholine
AF	Activation function
Akt	RAC-alpha serine/threonine-protein kinase
AMPK	Adenosine monophosphate-activated kinase
ANGPTL4	Angiopoietin-like 4 protein
ANOVA	Analysis of variance
ARE	Antioxidant response element
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BCC	Basal cell carcinomas
B-Raf	B-Raf proto-oncogene, serine/threonine kinase
CAF	Cancer-associated fibroblast
CD44	Cluster of differentiation 44
cdc42	Cell division control protein 42 homolog
cDNA	Complementary DNA
CID	CtBP-interacting domain
CK	Cytokeratin
CSL	CBF1, Suppressor of Hairless, Lag-1
CtBP	C-terminal binding protein
Cul2	Cullin 2
DDR	Discordin domain receptor
DMBA	Dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E-box	Enhancer box
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial-mesenchymal transition
ErbB	Epidermal growth factor receptor
ERK	Extracellular signal–regulated kinases
EtOH	Ethanol
Fas	Tumor necrosis factor receptor superfamily 6
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FSP1	Fibroblast-specific protein 1
GPX1	Glutathione peroxidase 1
GSK-3 β	Glycogen synthase kinase 3 beta
H&E	Hematoxylin and eosin
HBS	Hank's buffered saline
HCl	Hydrochloric acid
HG	High glucose
Hras	Transforming protein p21
HRP	Horseradish peroxidase
IL	Interleukin
IPA	Ingenuity Pathway Analysis

K	Keratin
KGF	Keratinocyte growth factor
LCM	Laser capture microdissection
LG	Low glucose
LRG1	Leucine-rich alpha-2-glycoprotein 1
MAPK	Mitogen-activated protein kinase
MCP-1	Macrophage chemoattractant protein-1
MEK	Mitogen-activated protein kinase kinase
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
Myc	MYC, BHLH transcription factor
NAC	N-acetyl-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NCAD	N-cadherin
NEM	N-ethylmaleimide
NK	Natural killer
Nox4	NADPH oxidase 4 protein
NQO1	NAD(P)H Quinone Dehydrogenase 1
NR	Nuclear hormone receptor
NRF2	Nuclear factor erythroid 2-related factor 2
NSMC	Nonmelanoma skin cancer
OCLN	Occludin
OTC	Organotypic coculture
p53	Tumor protein 53

PAGE	Polyacrylamide gel electrophoresis
PB	Pyridostigmine bromide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
PPAR	Peroxisome proliferator-activated receptors
PTEN	Phosphatase and tensin homolog
qPCR	Quantitative polymerase chain reaction
Raf-1	RAF serine/threonine-protein kinase
RL	Renilla luciferase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-qPCR	Real time quantitative polymerase chain reaction
RTK	Receptor tyrosine kinases
SCC	Squamous cell carcinomas
SDS	Sodium dodecyl sulfate
siRNA	Small interfering RNA
SMAD	Mothers against decapentaplegic
SMAD3	Mothers against decapentaplegic homolog 3
SMT	Somatic mutation theory
SNAI	Snail family transcriptional repressor
Src	Proto-oncogene tyrosine-protein kinase Src
STZ	Streptozotocin

T2D	Type 2 diabetes
TGF- β	Transforming growth factor beta
TGF β R	Transforming growth factor beta receptor
TME	Tumor microenvironment
TNF α	Tumor necrosis factor alpha
TOFT	Tissue organization field theory
TWIST	Twist-related protein
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
VIM	Vimentin

Summary

The epidermis, being the outermost layer of the skin, is susceptible to wounds and cancer. In either of these events, the function of the skin as a barrier to external insults and regulation of several homeostatic processes is compromised. The dysregulation in chronic wounds and cancer need to be better understood in order for proper recovery of the epidermal barrier.

With globalization and lifestyle changes, diabetic wounds are becoming increasingly common. Although the re-epithelialization process leading up to wound closure in normal wound healing is well studied, not much progress has been made in the understanding of diabetic wounds, leading to the insufficiency observed in existing therapeutic methods. Diabetic neuropathy has been reported to be strongly correlated with diabetic wounds. In my study, I demonstrated that keratinocytes are sensitive to acetylcholine stimulation, leading to the activation of the Src-ERK signaling axis and the upregulation of TGF β R2, thus promoting epithelial-mesenchymal transition. Under hyperglycemic conditions, this upregulation is suppressed by p38 MAPK activation, rendering the keratinocytes resistant to acetylcholine. Inhibition of p38 MAPK restored acetylcholine signaling, and therefore, epithelial-mesenchymal transition, of diabetic keratinocytes.

Peroxisome proliferator-activated receptor β/δ is a lipid mediated nuclear receptor that exists as the predominant subtype in the epidermis and dermis. Although epidermal PPAR β/δ has been shown to be implicated in skin cancer, the role of PPAR β/δ in fibroblasts and its contribution to tumor progression remains obscure. Herein, I showed that deficiency of PPAR β/δ in fibroblasts led to changes in the LRG1-TGF β 1 signaling pathway that resulted in an increase

in Nox4 in keratinocytes. The resulting increase in oxidative stress led to activation of the B-Raf-MEK pathway and subsequent upregulation of NRF2, as well as its downstream antioxidant enzymatic targets. The antioxidative response delayed tumor initiation and progression.

Taken together, my studies conducted in this thesis provide valuable insight into the understanding of diabetic wounds and epithelial-mesenchymal crosstalk in skin cancer and highlight the importance of studying small non-proteinaceous biomolecules in these diseases.

Chapter 1: Introduction

1.1 Skin

The skin, covering the entire body, is not just the largest organ of the body but also serves as the first layer of protection against chemical and physical insults from the external environment as well as ultraviolet (UV) radiation and pathogens (Kubo and Amagai, 2019). In addition, the skin plays an important role in regulating homeostatic processes such as the modulation of body temperature and the release of water into the environment. For example, in the first instance, blood flow to the skin is increased via cutaneous vasodilation to dissipate excess heat in the body to the external environment (Petzelbauer et al., 2019). A study showed that the increase in blood flow can be as high as 500 % (Nagasaka et al., 1987). The release of water by the skin is also for temperature control. One of the main methods of water release is through the production of sweat by the sweat glands. The evaporation of water from the skin then helps to bring down the temperature of the skin. The dysregulation of water loss is observed in unhealthy or damaged skin (Blattner et al., 2014).

The three main layers that make up the skin are the hypodermis, dermis and epidermis, with the hypodermis being the lowermost layer of the skin and the epidermis being the uppermost layer in direct contact with the environment (Kubo and Amagai, 2019). Different cell types reside in each of these layers, conferring each of the layers with their own distinct functions in the biology of the skin (Lopez-Ojeda et al., 2019).

The hypodermis consists mainly of adipocytes, whose primary function is to provide insulation, in addition to acting as an energy storage site. Other structures related to the skin such as hair follicles, neurons and blood vessels, also exist in the hypodermis and extend to the upper layers of the skin.

The dermis, unlike the hypodermis, consists of a dense network of collagen and elastic fibers intertwined with a variety of glycosaminoglycans, such as hyaluronic acid. It also houses structures such as nerves, blood vessels, sweat glands and hair follicles. It therefore comes as no surprise that the dermis contains a variety of cells, including macrophages, mast cells, stem cells, Schwann cells, adipocytes and fibroblasts, with fibroblasts accounting for most of these cells (Prost-Squarcioni et al., 2008). The dermis can be further subdivided into the papillary layer and the reticular layer, where the reticular layer is the lower layer of the dermis, containing fewer cells and a larger and denser network of fibers, and the papillary layer is the uppermost layer of the dermis, containing more cells as well as a looser network of fibers. The main function of the dermis is to support the functions of the other layers of the skin, which is made possible by the variety of structures and cell types present in the dermis (Kubo and Amagai, 2019). For instance, the vasculature provides nutrients to the epidermis while at the same time acting as a point of entry for inflammatory cells during the event of an infection (Petzelbauer et al., 2019). In addition, the vasculature within the dermis acts together with the sweat glands in the regulation of the body's temperature. The nerves detect external stimuli and are therefore important in protecting the individual from harm (Brown and Krishnamurthy, 2019).

Finally, the epidermis is the uppermost layer of the skin and the layer that contributes most to the functionality of the skin as a protective barrier against the external environment. Similar to the dermis, the epidermis contains a variety of cell types, including keratinocytes, melanocytes, Langerhans cells and Merkel cells. In addition, the epidermis can be subdivided into multiple distinct layers

from bottom to top, such as the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. In contrast to the indistinct demarcation of the dermis's sublayers, the epidermis's sublayers are much more distinct. The stratum basale contains a single layer of cuboidal cells that directly adhere to the basement membrane via hemidesmosomes and are connected to adjoining cells via intermediate filaments. The majority of these cells are stem cells that proliferate constantly to produce keratinocytes that replace the upper layers of the epidermis, and they are therefore vital in ensuring that the thickness and functionality of the epidermis are maintained despite wear and tear on the upper layers (Lechler, 2019). Melanocytes are also present in the stratum basale, and their primary function is to produce melanin, which protects the cells from (UV) radiation. Melanin granules are taken up by surrounding keratinocytes through phagocytosis, and it is estimated that a single melanocyte produces melanin for 30-40 keratinocytes (Fitzpatrick and Breathnach, 1963; Ostrowski and Fisher, 2019). The last cell type found in the stratum basale is the Merkel cells, which are unique nerve cells that function as mechanoreceptors. The stratum spinosum contains the progenitor cells resulting from the most recent proliferative processes that have taken place in the stratum basale. These cells maintain the capacity to proliferate, but unlike the stratum basale, this ability is limited, and the shapes of these keratinocytes in the spinosum are irregular. In addition, they use desmosomes to maintain contact with their adjoining neighbors. Langerhans cells are also found in the stratum spinosum, which are specialized dendritic cells that are found in the skin and act as the skin's first line of defense. They are also important in the recruitment of the immune system in responding to infections via their capacity

to migrate to nearby lymph nodes and carry out antigen presentation (Gudjonsson and Modlin, 2019). The stratum granulosum, as the name suggests, contains keratinocytes that are heavily granulated. These granules contain the precursors of keratin, as well as glycolipids, both of which are vital in maintaining the barrier function of the epidermis as the cells migrate upwards towards the surface of the skin. The stratum lucidum is a layer that is usually present only in parts of the body with increased skin thickness, such as the soles of the feet and palms of the hands. In this layer, substances within granules are released, and products such as eleidin, are formed just before the cells migrate into the stratum corneum. The stratum corneum within the epidermis is composed of anucleate keratinocytes, also known as corneocytes, that are connected to each other through intermediate filaments known as cytokeratin, which are the mature products of the granules mentioned above (Kubo and Amagai, 2019). These filaments are bonded together by filaggrin. Coupled with the surrounding extracellular matrix (ECM), containing abundant fats, most of which are also products of the granules, the structure of the stratum corneum has been shown to be similar to that of a wall in which the corneocytes represent the bricks and the ECM, the cement. Extracellular structural proteins such as loricrin form a rigid casing that covers the cells, resulting in the development of a protein-lipid envelope surrounding the cells. The uppermost layer of the stratum corneum is covered by a thin layer of lipids, composed primarily of sebum, which is a product of the sebaceous glands, as well as lipids from the ECM, as mentioned above. Together, these lipids form a 3-dimensional structure that is vital for maintaining the integrity of the lipid layer. The composition of the lipids varies based on the characteristics of the individual

such as their genetics, age, and sex and environmental factors such as seasons, humidity, and ambient temperature (Kubo and Amagai, 2019). The abundance of corneocytes and the composition of the protein-lipid envelope dictate the ability of the skin to act as a barrier and to perform its homeostatic functions. Examples include the effects on the types of microorganisms that can colonize the surface of the skin, the proinflammatory and anti-inflammatory properties of the skin, skin moisturization, water transpiration, insulation, and antioxidative protection from UV radiation. The functional and structural integrity of the epidermal barrier is therefore very important for ensuring that the skin performs its functions properly, and its failure can result in the onset of skin pathologies and diseases (Yousef et al., 2019).

As the outermost layer of the skin, the epidermis is the layer of the skin that is most exposed to external insults and radiation damage, making it the layer of the skin that is most subjected to wounding and cancer. In the event of either, the epidermis is compromised, and most of the functional aspects of the skin with regard to acting as a barrier or contributing to homeostatic regulation are lost. As such, it is important that these two processes, including their potential dysregulation, are well studied and understood for effective recovery of the epidermal barrier.

National Cancer Institute

Figure 1. Anatomy of the skin. The skin comprises of three layers, the epidermis, dermis and hypodermis. Functionality of each layer is dependent on the structures and cell types that are present in the layer. The close-up image shows the differences in the morphology of the keratinocytes as they progress from the basal layer to the uppermost layer of the epidermis. Image is obtained from the website of the National Cancer Institute (<https://www.cancer.gov>) (Public Domain). Image by Don Bliss.

1.2 Wound Healing

1.2.1 The socioeconomic impact of poor wound healing

Poorly healing wounds, including chronic wounds, refer to wounds that fail to proceed through the normal reparative process, leading to slow and disordered healing, which results in impairment of the anatomic and functional integrity at the site of the injury (Lazarus et al., 1994). Because poorly healing wounds are usually comorbid, meaning that they coexist with other health conditions, such as obesity and diabetes, their significance has often been overshadowed by these coexisting diseases. This has led such wounds to become a so-called “silent epidemic” that poses a major threat to the economy and public health (Sen et al., 2009). For instance, in the United States alone, statistics from Medicare showed that wound healing and management costs ranges from \$28.1 billion to \$96.8 billion, with surgical and diabetic wounds being the two most expensive wounds to treat (Nussbaum et al., 2018). Singapore has one of the highest diabetes-related lower extremity amputations rates in the world, with about 180 lower extremity amputations performed in 2015 for every 100,000 adult Singaporeans with diabetes, as compared to the countries in Organization for Economic Co-operation and Development’s (OECD), whose average lies at 60 (Ministry of Health, Singapore).

Approximately 1 to 2 % of the population in developed countries is expected to suffer from a chronic wound within their lifetime (Gottrup, 2004). There is a heavy socioeconomic burden placed on both the patients and society because these poorly healing wounds frequently cause patients to have difficulty managing work commitments while complying with the medical management of their wounds. In some cases, these wounds cause permanent disability and

therefore impair job performance (O'Donnell et al., 1977; Phillips et al., 1994; Eberhardt and Raffetto, 2005). For example, venous ulcers alone have been reported to cause a loss of 2 million workdays per year, while at the same time forcing 12.5 % of the inflicted workers into early retirement (DaSilva et al., 1992). In addition, excessive scar formation is observed in most chronic wounds due to the prolonged inflammation associated with these wounds. These scars may then cause further disability by negatively impacting the patients' psychological, functional and cosmetic attributes (Wadman, 2005; Sen et al., 2009), as underlined by a psychological study which found that 27% of the people who have sustained facial trauma tend to suffer from posttraumatic stress (Bisson et al., 1997). Although no specific statistics have been collected on the number of scarring cases, it has been estimated that the market for efficient anti-scarring drugs for the skin is approximately \$12 billion in year 2008, and is expected to increase to \$35 billion dollars by the year 2023, indicating the large number of people inflicted with scars stemming from chronic wounds as well as the increasing occurrences of chronic wounds (Prescient Strategic Intelligence, 2018; The Times, 2008). However, to achieve successful advancements in the development of anti-scarring drugs, the biology behind chronic wounds leading up to scar formation has got to first be better understood.

Older adults face even greater biomedical and socioeconomic burdens associated with wound complications (Sen et al., 2009). They are more likely to have chronic wounds than the young, and the effect of chronic wounds on their quality of life is particularly profound because of the significantly lower frequency of wound closure in older patients (Holt et al., 1992). In Singapore, this poses an immediate problem since 11.2 % of the Singapore population was 65 years of

age or older in 2014, and the number of elderly citizens is expected to double, leading to about 1 million Singaporeans being above the age of 65 by 2030 (Singapore Ministry of Social and Family Development, 2015).

Unbeknownst to many, in addition to the profound effect this has on the quality of life of these individuals, there is also a high socioeconomic burden resulting from the disability of an individual (Ollendorf et al., 1998), ranging from \$10,000 to \$20,000 per year for each individual (Olsson et al., 2019). Thus, it is important for poorly healing wounds to be viewed as a serious public health concern alongside other comorbid diseases and given the same amount of attention as diseases such as obesity and diabetes. In this context, a better understanding of the underlying biological mechanisms at the site of the wound is required.

1.2.2 Obesity, diabetes and the aging population are risk factors for poorly healing wounds

With globalization, the Western diet has been introduced to many countries. Together with changes in lifestyle, behavior and the environment, this has led to a marked increase in the number of obese individuals around the world (James et al., 2001; Ng et al., 2014). Obesity increases the predisposition for several chronic diseases that have high mortality rates, such as heart diseases (23 %), type 2 diabetes (T2D) (3 %) and high blood pressure (1.3 %) – diseases that rank in the top 15 leading causes of death as reported in 2017 by the Centers for Disease Control and Prevention (Cordain et al., 2005; Centers for Disease Control and Prevention, 2017). However, while the risks of high blood pressure and heart disease have been estimated to be increased by

approximately 50 % in obese individuals compared to their counterparts, T2D is a disease that exists almost exclusively in overweight individuals, as evidenced in a study conducted by Public Health England in 2014, whereby 90 % of diabetic adults in England were overweight (BMI > 25) or obese (BMI > 30) (Public Health England, 2014). Unlike type 1 diabetes, which is caused by a genetic defect and therefore diagnosed at an early age, individuals are usually diagnosed with T2D when they are middle aged or older (American Diabetes Association, 2004). Although different in diagnosis, studies showed that unmanaged hyperglycemia results in a convergence of the two forms of diabetes. Type 1 diabetic patients become insulin resistant over time as a result of the production of “diabetogenic” hormones such as adrenaline and growth hormones, which are antagonists of insulin activity, and Type 2 diabetic patients become insulin deficient due to death of β -cells in the pancreas stemming from the stress of producing insulin due to continual stimulation by the hyperglycemic condition (Waldhäusl and Bratusch-Marrain, 1985; Naseem et al., 2012; Bjornstad et al., 2015). The resulting complications that arise from both types of diabetes, such as retinopathy, cardiomyopathy, and neuropathy, are the same, thus indicating hyperglycemia as the root cause of problem (Dabelea et al., 2017).

In 2010, approximately 150 to 220 million diabetic patients were estimated to exist worldwide, and this number is predicted to increase to 300 million by 2025 (Zimmet et al., 2001). In Singapore, 29.3 % of adults aged 18 and older were overweight in 2010, and 10.8% of these individuals are categorized as obese (Foo et al., 2013). At the same time, 11.3 % of our population suffers from diabetes, compared to ~6.4 % of the human population globally (Shaw et al.,

2010; Singapore Ministry of Health, 2010). As these numbers increase, so will the related health problems, including problems with wound healing. With the current trend of an aging population on a global scale, the number of T2D cases may further increase in the years to come (Lutz et al., 2008).

One of the most debilitating complications in diabetes is delayed wound healing, as it may lead to high morbidity complications as well as mortality (Brown et al., 1997). Changes in body systems that stem from obesity are also known to impede wound healing (Wilson and Clark, 2004). Approximately 15 % of diabetic patients exhibit a wound showing impaired healing or nonhealing (Palumbo et al., 1985), 81 % of which are a result of diabetic amputations (Pecoraro et al., 1990). In patients who manage to recover, there is a recurrence rate of 66 %, during which the rate of amputation increases to 12 % (Sen et al., 2009). Taking into consideration that the cost of healthcare per case of diabetic foot ulcer can be as high as \$20,000 and that each amputation procedure costs approximately \$38,000, an average of approximately 15 billion dollars is spent on healthcare for the treatment of diabetic foot ulcers each year in the U.S. alone (Shearer et al., 2003; Rogers et al., 2008). In Singapore's context, the cost of health care for diabetic foot ulcers is estimated to account for 15% to 25% of the healthcare costs for treating diabetes. It can range from \$1,000 to \$10,000 dollars per patient, depending on the type of care and surgery required, and a total of 1 million dollars is being spent on the treatment of diabetic foot ulcers each year (Tan et al., 2015). In a retrospective study of 214 patients, the total cost of treatment was estimated to be SGD 1.05 million per year (Tan et al., 2015).

Despite the enormous impact of these poorly healing wounds, there is a lack of effective therapies available to patients. To provide more effective management strategies and develop novel, efficient therapies that create an environment that is conducive for healing to occur, increased attention and allocation of resources toward understanding the underlying biological mechanisms involved in cutaneous wound complications is required (Witte and Barbul, 1997; Coulombe, 2003; Metz, 2003; Sen et al., 2009).

1.2.3 Phases of wound repair

Wound healing involves an orderly progression from injury to coagulation, inflammation, cell and matrix proliferation, cell migration, and tissue remodeling (Velnar et al., 2009; Alavi and Kirsner, 2019). The first phase of these processes is the coagulation and hemostatic phase. The main player in this phase is the platelets, which leak to the site of the injury from damaged blood vessels. Upon contact with the components of the ECM, these platelets are activated, resulting in the release of clotting factors. The end result is the formation of blood clots in damaged veins or arteries as well as at the site of the wound (Pool, 1977; Robson et al., 2001). This phase is important for preventing further loss of blood while simultaneously providing a matrix to ease the migration of cells in the later phases of wound repair (Jespersen, 1988; Lawrence, 1998).

The next overlapping phase is inflammation, which occurs almost immediately after clotting is completed. Cells that were damaged from the injury release pro-inflammatory molecules that cause a potent inflammatory response (Toy, 2005; Strecker-McGraw et al., 2007). Together with other inflammation-inducing molecules such as lipopolysaccharides from bacteria and interleukins

(IL) from platelets (Basset et al., 2003; Casoli et al., 2010), these molecules activate the complement cascade, which leads to the infiltration of a variety of immune cells at the wound site. One of the earliest immune cell types to reach the wound site is the neutrophils (Robson et al., 2001). These cells exit from leaky blood vessels at the wound site and phagocytose pathogens and foreign objects that are present (Flangan, 2000; Richardson, 2004). As compared to normal circulating neutrophils, gene expression microarray analysis of these neutrophils indicated that there is an upregulation of genes that promote the recruitment of even more immune cells, angiogenesis, and the proliferation of fibroblasts and keratinocytes (Theilgaard-Monch et al., 2004). Subsequently, these neutrophils either undergo apoptosis or are extruded via sloughing and are replaced by tissue macrophages. A study demonstrated that the ingestion of apoptotic neutrophils, by macrophages, is a critical step in wound healing because it inhibits the production of pro-inflammatory cytokines, without which wound reparation would not be able to proceed effectively (Fadok et al., 1998). Similar to neutrophils, macrophages help to remove pathogens and foreign objects via phagocytosis (Hess, 2000; Hart, 2002). However, unlike neutrophils, they play an additional role in activating other cell types, such as keratinocytes, endothelial cells and fibroblasts, which are vital in the third phase: the proliferative phase (Samuels and Tan, 1999; Ramasastry, 2005).

In the proliferative phase, wound re-epithelialization starts to take place, culminating in the formation of a neo-epidermis. Fibroblasts from nearby tissues are activated to become myofibroblasts, which then migrate to the wound site and start to form a new ECM (Witte and Barbul, 1997; Goldman, 2004). Matrix proteins such as collagen type 3 replace the previous matrix deposited by the

platelets. Myofibroblasts simultaneously contribute to wound contraction – a key step in wound repair (Goldman, 2004; Ramasastry, 2005). Concurrently, endothelial cells from nearby blood vessels undergo proliferation and growth, causing the sprouting of capillaries into the newly synthesized tissue (i.e., wound angiogenesis) (Folkman and Klagsbrun, 1987; Joseph-Silverstein and Rifkin, 1987). Both myofibroblasts and endothelial cells play important roles in the synthesis of granulation tissue. Another key player in this phase is the keratinocytes at the edges of the wound. These cells are required to undergo epithelial-mesenchymal transition (EMT), a trans-differentiation process that emphasizes the transient nature of these cells. The ability to transform into mesenchymal cells and back, either fully or partially, is an inherent characteristic of epithelial cells. During EMT, keratinocytes undergo changes such as cytoskeletal reorganization, cell junction remodeling, loss of cell polarity, morphological modifications and gene expression reprogramming. These processes culminate in a migratory phenotype that is important for the formation of the ECM and closure of wounds via re-epithelialization (Yan et al., 2010; Pastar et al., 2014).

The last phase is the remodeling phase, which involves architectural changes to the newly synthesized ECM after the completion of re-epithelialization. The initial deposits of fibronectin are cleared and replaced by collagen from the activated fibroblasts (Hunt, 1998). Eventually, the collagen build-up allows the injured region to regain up to 80 % of its original strength (Robson et al., 2001), and scar maturation occurs (Diegelmann and Evans, 2004). Irregularities in this phase may result in excessive scarring and the formation of hypertrophic and keloid scars (Tuan and Nichter, 1998). As healing

occurs, the fibroblasts and macrophages in the new ECM undergo apoptosis, and the growth of new capillaries slows and eventually stops (Clark, 1993; Greenhalgh, 1998).

Figure 2. Phases of wound repair. Wounds heal in 4 stages: hemostasis, inflammation, new tissue formation, and tissue remodeling. Hemostasis prevents excessive blood loss and inflammation occurs to clear the cellular debris and pathogens. In the third stage, new tissue formation occurs resulting in the proliferation and migration of keratinocytes and fibroblasts. Finally, in tissue remodeling, ECM reorganizes, degrades and is resynthesized. Eventually, these 4 steps culminate in proper wound closure. Image by Tan Wei Yi, Mark.

1.2.4 Chronic wounds and the re-epithelialization process

While acute and chronic wounds are different, all chronic wounds start as an acute wound (Alavi and Kirsner, 2019). In chronic wounds, the linear progression between the sequential phases of acute wound healing is lost (Demidova-Rice et al., 2012). The result of these impairments is a wound that does not heal and is constantly exposed to external insults such as bacterial infection, which can lead to a further reduction of healing (Bowler and Davies,

1999). To prevent this, it is fundamental that the epidermis, which is the outermost layer of the skin and functions as an airtight-waterproof barrier that protects us from the external environment, be reformed via re-epithelialization. In fact, re-epithelialization is regarded so highly that it is a common method of assessing wound healing in most clinical trials and has been recognized as the gold standard for wound closure assessment by the FDA (Gould and Li, 2019). It is therefore deemed imperative that research should be focused on the re-epithelialization process in an attempt to seal wounds from the external environment and facilitate better healing.

The key characteristic of re-epithelialization is the migration of keratinocytes at the edges of the wound across the wound bed. It has been speculated that the activation and mobilization of these cells are similar to the EMT that is frequently observed in cancer. Unlike the EMT process observed in cancer, keratinocytes do not achieve complete dissociation of cell-cell junctions or motility. Instead, keratinocytes undergo changes in their cytoskeleton and junctional complexes that result in intercellular gaps that ultimately confer the ability to migrate as a sheet but not as individual cells. This phenomenon has been referred to as “partial EMT” (Nieto et al., 2016). Previous studies have proven that transforming growth factor beta 1 (TGF- β 1) is a key player in the induction of this partial EMT both *in vivo* and *in vitro* and that TGF- β 1 activity in chronic wounds is decreased compared to that in normal wounds as a result of both TGF- β 1 production and decreased TGF- β Type II receptor (TGF β R2) expression (Kim et al., 2003). This strongly correlates with the longstanding hypothesis that the impairment of re-epithelialization observed in chronic wounds is due to a lack of EMT occurring in keratinocytes at the tip of the

epithelial tongue. However, studying this process has continually posed a technical challenge, as only a small number of keratinocytes undergo EMT, even under normal wound-healing conditions. Furthermore, *in vitro* studies of EMT have revealed heterogeneity between the cells within and at the edge of the colony, and because it is only a small number of cells at the edge of the colony that undergo EMT, studying EMT at the molecular level has also been very challenging (Mani et al., 2008). As such, the trigger for the activation of EMT in keratinocytes during re-epithelialization and how it is altered in chronic wounds remains unknown. Due to the many similarities that exist in both wounds and cancer, such as inflammation, ECM remodeling and the migration of epithelial cells, it has been suggested that knowledge obtained with respect to either wound healing and cancer could be cross examined with the other (Schafer and Werner, 2008; Antonio et al., 2015; Dvorak, 2015). In fact, cancer has been described as “a wound that does not heal,” implying that the cellular and biochemical processes associated with wound healing are similar to those involved in the growth and development of tumor stroma (Dvorak, 1986; Byun and Gardner, 2013).

1.2.5 EMT biomarkers

E-Cadherin

The transmembrane protein E-Cadherin contributes to epithelial cell polarity and cell-cell adhesiveness (Knust and Bossinger, 2002; Nelson, 2003; Perez-Moreno et al., 2003). This protein plays a crucial role in adherens junction formation and connects to the actin cytoskeleton, thereby allowing cell-to-cell binding within a tissue (Geiger and Ayalon, 1992; Lewis et al., 1994; Perez-

Moreno et al., 2003). For cell migration to occur, it is therefore necessary for these junctions to be lost; indeed, several studies have demonstrated that the loss of E-Cadherin in epithelial cells leads to the acquisition of a fibroblastoid phenotype together with migratory properties (Takeichi, 1995; Huber et al., 1996; De Wever and Marcel, 2003; Kalluri and Neilson, 2003; Huber et al., 2005).

The significance of E-Cadherin as a marker of the epithelial phenotype has been well established by many studies, particularly in cancer. First, the loss of E-Cadherin was reported to be positively correlated with the aggressiveness of tumors (Frixen et al., 1991; Vleminckx et al., 1991; Miyaki et al., 1995; Llorens et al., 1998). Second, E-Cadherin levels at the tumor invasion front have been shown to be lower than those in the central areas (Kirchner and Brabletz, 2000; Brabletz et al., 2001). Third, the repression of E-Cadherin has been shown to lead to the release of beta-catenin (β -catenin), which is anchored by E-Cadherin at adherens junctions, leading to the accumulation of nuclear β -catenin, which results in the induction of a tumorigenic phenotype (Hannigan et al., 2005). Thus, the downregulation of E-Cadherin is considered a hallmark of EMT (Kalluri and Neilson, 2003).

Epidermal growth factor receptor (ErbB) 3

Epidermal growth factor receptor 3 is one of the four members of the epidermal growth factor receptor (EGFR) family (Prigent et al., 1992; Carraway and Cantley, 1994; Normanno et al., 1994; Rajkumar and Gullick, 1994; Voldborg et al., 1997). Studies have shown that these receptors heterodimerize with each other and are involved in several cellular processes such as migration,

proliferation, differentiation and survival (Olayioye et al., 2000; Yarden and Sliwkowski, 2001; Mirschberger et al., 2013).

Unlike the other members of the EGFR family, ErbB3 has a non-functional intracellular tyrosine kinase domain (Guy et al., 1994; Sierke et al., 1997). As a result, it has been speculated that not only does ErbB3 require heterodimerization with another member of the EGFR family, but allosteric interactions between ErbB3 and its heterodimer partner are required for the partner's activation (Jackson-Fisher et al., 2004; Linggi and Carpenter, 2006). One of the most well studied heterodimerization is ErbB2/ErbB3, which is responsible for many biological processes such as apoptosis, maturation, angiogenesis and most importantly, cell proliferation and survival (Chang et al., 1997; Hermanto et al., 2001; Camenisch et al., 2002; Holbro et al., 2003; Kim et al., 2005). In the absence of its ligand, ErbB3 exists at low levels and self-oligomerizes to form an inactive dimer (Landgraf and Eisenberg, 2000). However, in the presence of neuregulin, the ligand for the ErbB3 receptor (Carraway et al., 1994; Sliwkowski et al., 1994; Garratt, 2006), ErbB3 oligomers detach from each other and conformational changes expose the interface required for interacting with ErbB2 (Kani et al., 2005).

A recent study has demonstrated that ErbB3 is involved in EMT and cell migration through activating Snail Family Transcription Repressor (SNAIL) 1, an EMT-inducing transcription factor (Kim et al., 2013). However, it has been found that ErbB3 expression exists specifically in epithelial cells (Prigent et al., 1992; Thomson et al., 2005; Argast et al., 2011; Thomson et al., 2011), thus making it a useful marker of the epithelial phenotype. This loss of ErbB3 after EMT has

been speculated to prevent further cell proliferation after the cell has taken on a mesenchymal-like phenotype (Salt et al., 2013).

Occludin (OCLN)

The occludins were the first tight junction proteins to be discovered (Furuse et al., 1993). Upon their initial discovery, they were thought to be a type of structural unit required for tight junction formation. However, a subsequent study showed that the cells of OCLN knockout mice could still form tight junctions, although histological changes were observed at the tissue level (Saitou et al., 2000). Occludins were later observed to be components within tight junctions that may play a role as sensors due to their colocalization with elements from the hippo pathway and may therefore be responsible for controlling cellular activities such as proliferation (Cravo et al., 2015). This possibility was further confirmed when a study proved that occludins had the ability to repress the proliferation and metastasis of cancer cells and has been demonstrated in other studies providing similar observations in multiple types of cancers, such as liver and breast carcinomas (Osanai et al., 2006; Orbán et al., 2008; Martin et al., 2010). While the ability of occludins to repress proliferation has been established to involve their inhibitory effects on RAF proto-oncogene serine/threonine-protein kinase (Raf-1), their ability to prevent metastasis has been inferred to be simply due to their function as proteins that promote adhesion (Wang et al., 2005; Lemieux et al., 2009). It therefore comes as no surprise that occludins have been found to be downregulated in most tumors, even more so when EMT is taking place (Vincent et al., 2009; Işeri et al., 2011)

Similar to what is observed in cancer, the induction of EMT through the expression of SNAI1 in normal mouse epithelial cells leads to a decrease in OCLN (Ikenouchi et al., 2003). This observation was further supported by another study in which the leading cells of the epithelial tongue were found to exhibit downregulated levels of OCLN compared to the rest of the epidermis in acute human wound samples, thus confirming that the loss or decreased expression of OCLN is a phenomenon that is required for EMT to take place, indicating that OCLN can be used as an epithelial marker (Volksdorf et al., 2017).

N-cadherin (NCAD)

Along with E-Cadherin, NCAD is a member of the cadherin family of proteins. However, unlike E-Cadherin, NCAD has been found to be positively associated with cancer cells that are undergoing EMT (Nakajima et al., 2004). In fact, the downregulation or loss of E-Cadherin expression and the concomitant increase in NCAD expression have been associated with an increase in the migratory and invasive capacity of cancer cells, causing this phenomenon to be described as a “cadherin switch” (Wheelock et al., 2008; Gheldof and Berx, 2013).

Interestingly, it has been found that downregulation of NCAD is sufficient to impair the migration capacity of prostate cancer cells, while its overexpression is sufficient to enhance their migration capacity (Wang et al., 2016). These phenomena were further highlighted by another group that found that upregulation of NCAD increases the invasiveness of cancer cells regardless of E-Cadherin expression (Hazan et al., 2004). In a study conducted on human neck cancer biopsies, NCAD was found to be increased in samples with metastasis compared to those without metastasis; this difference was strongly

correlated with survival probability, which was found to be decreased in patients with tumors showing higher NCAD expression (Zhu et al., 2018).

In the wound-healing context, NCAD has been found to be upregulated in keratinocytes with increased migratory potential (Terao et al., 2011). Similarly, keratocytes explanted from the wounds of zebrafish exhibit loss of E-Cadherin expression and an increase in NCAD (McDonald et al., 2013). These results were recapitulated in the wounds of horses, in which the loss of E-Cadherin and increase in NCAD were concentrated in the epithelium directly adjacent to the wound (Jørgensen et al., 2019).

The mechanisms whereby NCAD supports migration have also been studied. One of these mechanisms is the suppression of alpha (α) 5 integrins at the rear of the cell due to the formation of NCAD complexes with the following cells, leading to the promotion of Rac1 and Cell division control protein 42 homolog (cdc42) activity as well as the polarization of α 5 integrins at the leading edge of the cell and ultimately, collective cell migration (Ouyang et al., 2013). These observations were supported by another study that showed that NCAD inhibition had a converse effect on collective cell migration due to reduced polarization of α 5 integrins at the leading edge (Wasil and Shair, 2015). Other studies have revealed a second mechanism whereby NCAD is capable of binding to fibroblast growth factor receptor (FGFR) 1, leading to decreased internalization of the receptor when its ligand, fibroblast growth factor (FGF) 2, binds to it (Suyama et al., 2002; Rachagani et al., 2012). This in turn leads to the consistent activation of the MEK/ERK signaling pathway and production of migration-associated genes such as matrix metalloproteinase (MMP) 9 (Hazan et al., 2000; Suyama et al., 2002). Unlike the first mechanism, this mechanism

has been speculated to function in both individual and collective cell migrations (Kim et al., 2000; Sanchez-Heras et al., 2006).

Vimentin (VIM)

While keratin intermediate filaments have long been considered definitive of an epithelial phenotype, VIM is similarly definitive of the mesenchymal phenotype. During its initial discovery, VIM was thought to be involved only in maintaining the integrity and architecture of tissues. However, it was later discovered that VIM can interact with signaling molecules and form signaling complexes, thus suggesting that it is capable of orchestrating changes in cellular activities (Eriksson et al., 2009). One such activity is cell migration, which is supported by evidence that VIM is upregulated in many different types of cancers, and in the case of hepatic and lung cancer, VIM has been observed to positively correlate with the occurrence of metastasis (Hu et al., 2004; Helfand et al., 2011; Kidd et al., 2014). In fact, VIM is now being used to diagnose the progression of some forms of cancer from a noninvasive to an invasive state (Gilles et al., 1999; Geiger and Peeper, 2009). The importance of VIM was further highlighted in a comprehensive study using breast cancer epithelial cell lines and primary fibroblast cell lines. The study revealed that simply overexpressing VIM led the epithelial cells to take on a mesenchymal-like shape, internalize their desmosomes, and achieve increased motility, while the suppression of VIM in the fibroblasts yielded opposite results (Mendez et al., 2010).

In the context of EMT in wound healing, VIM-null mice have been found to exhibit decreased numbers of EMT-activated keratinocytes (Cheng et al., 2016).

Another study showed that VIM was only expressed in the keratinocytes at the leading edge of the epithelial tongue and colocalized with keratin (K) 14, while a mutated variant of VIM showed inability to interact with K14 led to a significant decline in the rate of re-epithelialization (Velez-delValle et al., 2016). Taken together, these studies indicate the importance of VIM in the migration of keratinocytes during wound healing. The mechanism that VIM uses to support the progression of EMT has been shown to involve the maintenance of phosphorylated SNAI2 via the activation of extracellular signal-regulated kinases (ERK) (Savagner, 2001; Shirley et al., 2010; Liu et al., 2015).

Twist-related protein (TWIST) 1

Twist-related protein 1 is a transcription factor that was first thought to be involved only in morphogenesis (Furlong et al., 2001), but many subsequent studies have shed light on its ability to promote EMT. The evidence of this ability includes the finding that TWIST1 is upregulated in various types of cancers, such as colorectal cancer and hepatic cancer (Lee et al., 2006; Celesti et al., 2013).

A clinical study on thyroid cancer also showed that TWIST1 was positively correlated with cancer metastasis (Lv et al., 2016), while another group identified TWIST1 upregulation as an indication of increased aggression and a lower survival probability in patients with prostate cancer (Kwok et al., 2005). Twist-related protein 1 has been shown to be so important in EMT that it is now considered an essential transcription factor for metastasis to occur (Yang et al., 2004). In addition to being a key regulator of EMT, studies have shown that TWIST1 has the potential to confer cancer cells with resistance to anti-cancer drugs such as tyrosine kinase inhibitors used against EGFR in lung cancer and Taxol, a drug that is commonly used to treat breast cancers (Wang et al., 2004; Yochum et al., 2019).

In a nontumorigenic setting, TWIST1 has been found to appear in human keratinocytes undergoing EMT induced by either tumor necrosis factor (TNF) α or TGF β 1 (Yan et al., 2010). Similar findings were obtained in mouse keratinocytes that were undergoing EMT during wound healing (Terao et al., 2011). Taken together, the evidence suggests that TWIST1 is an important regulator of EMT during the re-epithelialization process in wound healing.

The transcriptional activity of TWIST1, which contributes to its ability to induce EMT, has been studied, and various modes of action have been discovered. One of these mechanisms is the transcription of Cullin (Cul) 2, a circular ribonucleic acid (RNA) that binds to and inhibits micro ribonucleic acid (miRNAs) that target VIM, thus allowing an increase in VIM transcripts within the cell, leading to increased production of the protein (Meng et al., 2018). Another study demonstrated that TWIST1 is able to activate RAC- α serine/threonine-protein kinase (Akt)/ Glycogen synthase kinase 3 beta (GSK-

3 β)/ β -catenin, leading to the upregulation of cluster of differentiation 44 (CD44), a gene with pro-migratory relevance (Oh et al., 2016). E-Cadherin has also been found to be a target of TWIST1, in which TWIST1 binds to the enhancer box (E-box) sequence in the promoter region of the E-Cadherin gene and represses its expression (Onder et al., 2008). Finally, TWIST1 has been shown to upregulate the activation of the Akt/Protein kinase B (PKB) signaling pathway, which in turn positively mediates the activity of Phosphoinositide 3-kinase (PI3K)/Akt and TGF- β signaling, leading to the amplified induction of EMT; the Akt/PKB-mediated phosphorylation of TWIST1 promotes tumor metastasis by mediating the cross-talk between the PI3K/Akt and TGF- β signaling axes (Xue et al., 2012).

SNAI1

Snail family transcriptional repressor 1 has been identified as a transcription factor that functions as one of the master regulators of EMT due to its ability to directly suppress E-Cadherin, resulting in a loss of cell adhesion (Moll et al., 1993; Batlle et al., 2000; Cano et al., 2000; Come et al., 2006; Ansieau et al., 2008; Thiery et al., 2009), and it was shown to be expressed beginning in the early stages of EMT, whereas other transcriptional regulators of EMT are induced much later (Peinado et al., 2007). In addition to E-Cadherin, SNAI1 regulates many other genes that drive EMT (Moreno-Bueno et al., 2008), including genes involved in cell polarity, adherens junction formation, and cytoskeleton reorganization, as well as genes such as MMP2 and $\alpha\beta$ 3-integrin, which promote cell migration (Yokoyama et al., 2003; Miyoshi et al., 2005; Thiery and Sleeman, 2006; Sharma and Henderson, 2007; Haraguchi et al.,

2008; Yilmaz and Christofori, 2009). Collectively, SNAI1 expression results in the conversion of an epithelial phenotype to a fibroblastoid phenotype with an increased migratory ability (Cano et al., 2000; Guaita et al., 2002).

The ability of SNAI1 to recognize and regulate its target genes relies on 2 main domains – the zinc-finger domain in the C-terminus, which helps it to recognize target deoxyribonucleic (DNA) sequences such as the E-box within the promoter region of E-Cadherin (Batlle et al., 2000), and the SNAG domain in the N-terminus, which is responsible for the repressor activity of SNAI1 (Nieto, 2002; Peinado et al., 2004; Peinado et al., 2007). In addition to the SNAG domain, external factors such as corepressors and methyltransferases have been shown to aid in the repression of target genes by SNAI1 (Peinado et al., 2004; Dong et al., 2012; Dong et al., 2013).

The tight regulation of SNAI1 underscores its importance as a key inducer of EMT. Epithelial cells usually express low levels of SNAI1 in the cytoplasm – a mechanism that is controlled by the phosphorylation of SNAI1 by GSK-3 β , which results in the nuclear export, ubiquitination and degradation of the SNAI1 protein (Zhou et al., 2004). Microribonucleic acids such as miR-153 and miR-199a simultaneously target SNAI1 messenger RNA (mRNA) transcripts, leading to their degradation (Suzuki et al., 2014; Bai et al., 2015). The ectopic expression of SNAI1 alone is sufficient to initiate EMT (De Craene et al, 2005; Yook et al., 2005; Moreno-Bueno et al., 2006).

SNAI2

Like SNAI1, SNAI2 is part of the Snail protein family (Nieto, 2002). Similarly, SNAI2 has been discovered to be a potent repressor of E-Cadherin

and a key regulator of EMT whose presence positively correlates with the metastasis of cancer cells (Bolós et al., 2003; Gupta et al., 2005). These findings are supported by a study showing that breast cancers with higher levels of aggression presented higher SNAI2 activity compared to their less aggressive counterparts (Storci et al., 2008). Another study showed that SNAI2 is upregulated and suppresses epithelial markers during the induction of EMT in cancer cells by TWIST1; the cooperation of these transcription factors promotes the progression of EMT and, subsequently, metastasis in these cells (Casas et al., 2011). Surprisingly, similar findings were made in breast cancer stromal cells, leading to the hypothesis that these two transcription factors may not only support the EMT and, therefore, the metastasis of epithelial cancer cells, but their upregulation in nearby stromal cells may be essential for promoting these processes (Grzegorzolka et al., 2015). In addition to its potent EMT-inducing abilities, SNAI2 has been found to be responsible for the development of cisplatin resistance in ovarian cancers (Haslehurst et al., 2012).

In the context of wound healing, SNAI2 has been found to be expressed in migrating mouse keratinocytes both *in vivo* and *ex vivo* and in human keratinocytes *in vitro*. The same study showed the importance of SNAI2 in the wound-healing process, as observed from the compromised migration of keratinocytes that were explanted from SNAI2 knockout mice and the increase in the migratory phenotype of human keratinocytes overexpressing SNAI2 *in vitro* (Savagner et al., 2005). These findings were supported by another study showing that SNAI2 is important for maintaining the motility of migrating keratinocytes during re-epithelialization (Arnoux et al., 2008).

Similar to SNAI1, SNAI2 contains a zinc-finger domain at its C-terminus and a SNAG domain at its N-terminus, thus allowing it to bind to and repress its target genes and support the progression of EMT (Hemavathy et al., 2000).

Figure 3. Gene expression changes during EMT. Epithelial markers (left, pink box) are downregulated and mesenchymal markers and pro-EMT transcription factors (right, orange box) are upregulated during the EMT process. Image by Tan Wei Yi, Mark.

1.2.6 Crosstalk between keratinocytes and other cell types during wound healing

During the course of wound healing, the cells within the wound site are constantly interacting with each other either through autocrine or paracrine signaling (Ganapathy et al., 2012). This communication is vital, as it ensures the smooth and timely transition of the different phases during wound healing, thus facilitating successful wound closure. Much research has therefore been focused on identifying and piecing together the interactions between different cell types to better identify the irregularities in chronic wounds and develop therapies for resolving these irregularities. As a result, there is now a broad

understanding of the interactions between keratinocytes and some of the other cell types in the vicinity of re-epithelialization.

In the event of wounding, the sudden loss of cell-cell contacts results in the activation of keratinocytes at the edge of the wound (Wood et al., 1996). This sets off a series of changes in gene expression within these keratinocytes, causing them to produce signaling molecules that act on both keratinocytes and a variety of other cell types. These cells in turn produce signaling molecules that regulate keratinocyte activity. This cycle continues throughout the wound-healing process, with changes occurring in the type of signaling molecules that are produced as wound closure progresses.

Two of the earliest molecules that are released by keratinocytes are IL-1 and TNF α (Antoniades et al., 1993). Interleukin-1 acts on keratinocytes in an autocrine manner, increasing their capacity for migration and proliferation. Interleukin-1 also acts on fibroblasts, causing them to secrete keratinocyte growth factor (KGF), another molecule that aids in the migration and proliferation of keratinocytes (Maas-Szabowski et al., 1999). Similar to IL-1, TNF α stimulates keratinocyte migration. However, unlike IL-1, TNF α stimulates the production of FGFs in fibroblasts (Beer et al., 1997). Through autocrine signaling, these growth factors stimulate the migration of the fibroblasts and induce them to produce and deposit ECM components that are essential for promoting keratinocyte movement across the wound bed (Werner et al., 2007). In addition to the functions listed above, both IL-1 and TNF α are important initiators of the inflammatory cascade.

Chemokines produced by activated keratinocytes stimulate immune cell migration (mainly that of neutrophils) to the wound site. Interleukin-1 and TNF α

then activate these neutrophils and stimulate the production of proinflammatory molecules, leading to the beginning of the inflammatory phase of wound healing. One of these molecules is IL-6, which shows mitogenic and proliferative activities in keratinocytes (Mast and Schultz, 1996). Keratinocytes then produce macrophage chemoattractant protein-1 (MCP-1), which recruits macrophages to the wounded area (Mai et al., 2018). These macrophages are also capable of producing IL-6, leading to a positive feedback loop that promotes both macrophage recruitment and keratinocyte proliferation (Behm et al., 2012).

Endothelial cells also receive signals from keratinocytes, fibroblasts, immune cells and platelets in the form of proangiogenic growth factors, cytokines and cell-ECM interactions. This leads to their activation and migration to the wound site, where they proliferate and form a new vasculature. Notably, vascular endothelial growth factor (VEGF) is one of the earliest growth factors to accumulate at a wound, as it is released by platelets upon their activation and aggregation (Brouwers et al., 2013). This process is important, as the formation of new vasculature within the wound environment promotes the migration of immune cells to the site of the injury and allows the transport of nutrients and oxygen to the vicinity of the wound, which is a crucial step in maintaining an environment that supports the activities of the cells. Vascular endothelial growth factor also acts on keratinocytes to promote re-epithelialization (Behm et al., 2012).

Although much has already been discovered about the cross-talk between the abovementioned cell types and keratinocytes, little is currently known about the cross-talk between neural cells and keratinocytes. This shortfall exists despite the fact that the sensation of pain is an event that takes place

immediately upon wounding and persists throughout the wound-healing process until wound closure is achieved.

1.2.7 Summary and current gap of knowledge

The heavy psychological and socioeconomic impacts of chronic wounds call for better understanding of the chronic wound environment to facilitate the development of more effective therapeutic strategies. Diabetic wounds in particular, require more attention due to the rapidly increasing numbers of diabetics across the globe, in part due to globalization, leading to changes in diet and lifestyle, as well as an ageing population. Although these clinical realities have been known for decades, our understanding and ability to treat this disease remain unacceptably inadequate. Recombinant PDGF-BB (Becaplermin) gel remains the only pharmacological biologics approved by FDA for treatment of chronic diabetic wounds. However, the FDA has added a black box warning label about the risk of cancer associated with the use of three or more Becaplermin tubes. Experimental trials of growth factors and cytokines improve diabetic wound healing, however, these therapies often used doses that far exceed their endogenous levels, which increase their risk of side-effects (Okonkwo and DiPietro, 2017; Zubair and Ahmad, 2019). Thus, we are of the opinion that a better understanding of why diabetic wounds are less responsive to various experimental treatments, requiring high doses, will have the greatest need and potential effect on health.

In wounds that exhibit impaired wound healing, such as diabetic wounds, the re-epithelialization process has failed (Chen et al., 2016; Rousselle et al., 2018). It has been speculated that the initiation of re-epithelialization is similar

to the epithelial-mesenchymal transition (EMT) process observed during embryogenesis and cancer metastasis (Yan et al., 2010; Haensel and Dai, 2018). However, studying this process in wound healing has been technically challenging because only a small number of keratinocytes at the epithelial tongue underwent EMT even for normal healing wounds (Usui et al., 2008). Thus, molecular insights of EMT in keratinocytes during re-epithelialization, and how EMT affected diabetic keratinocytes remains unknown.

1.3 Skin Cancer

1.3.1 Cancer and the hallmarks of cancer

Cancer is a generic term that is used to describe the abnormal growth of cells in different parts of the body. Nomenclature for referring to the type of cells that have become cancerous has been developed. For example, cancers of epithelial origin are known as carcinomas, while cancers originating from the blood are known as leukemia, and cancers stemming from connective tissues are referred to as sarcomas.

Numerous studies have led to the identification of common traits that are found in cancers. These traits have come to be considered hallmarks of cancer. Eight hallmarks are currently considered to exist: sustained proliferation, evasion of growth suppressors, avoidance of immune surveillance, dysregulation and reprogramming of metabolism, resistance to cell death, induction of angiogenesis, increased metastatic potential, and immortality. In addition, inflammation and genomic instability are two characteristics that are now agreed to confer a predisposition to cancer formation and are considered enabling characteristics of cancer (Hanahan and Weinberg, 2011).

Figure 4. The 8 hallmarks and 2 enabling characteristics of cancer. Source: Hanahan and Weinberg's "Hallmarks of Cancer: the next generation" published in the journal, *Cell*, Volume 155, Issue 5, p646-674. Permission was obtained on 5th January 2020. The 8 hallmarks and 2 enabling characteristics of cancer are presented in the inner circle. Possible therapeutic strategies for each of these traits are listed in the outer circle.

The sustained proliferation of cancer cells can be a result of the excessive and/or continuous availability of growth factors in the microenvironment, the persistent expression of their receptors on the cancer cell surface, or both (Perona, 2006). An alternative method whereby cancer cells avail themselves of growth factors may involve the production of proteases that degrade the ECM, leading to the release of growth factors sequestered within (Egeblad and Werb,

2002). Mutations of the receptors and/or their downstream targets may also allow the constitutive activation of the mitogenic signaling pathway in the absence of the ligand. For instance, a study showed that mutations can lead to lowered thresholds of autophosphorylation in receptor tyrosine kinases (RTKs), thus allowing them to function in the absence of their ligands (Lemmon and Schlessinger, 2010).

Similar to sustained proliferation, there is a variety of different ways in which cancer cells evade growth suppressors. One such mechanism is the loss of cell-cell contact via the downregulation of adhesion molecules such as cadherins, which results in the loss of growth contact inhibition (Takai et al., 2008; Buda and Pignatelli, 2011; Li et al., 2012). Transcription regulators bound within these adhesion complexes, such as β -catenin, are then freed and begin to accumulate in the cytoplasm. These regulators subsequently translocate to the nucleus, leading to the transcription of proliferation-related genes (Jamieson et al., 2014). Next, the accumulation of mutations in growth-suppressing transcription factors leads to their inability to perform their functions. One common transcription factor that has been found to be mutated in many types of cancers is tumor protein 53 (p53), one of the key transcription factors that is required to stop the progression of the cell cycle (Manning and Dyson, 2012; Bieganski et al., 2014).

Resistance to programmed cell death, also known as apoptosis, is another key characteristic of cancer cells. Typically, normal cells activate their apoptotic pathways when events such as DNA damage occur. However, cancer cells use a variety of mechanisms, such as the upregulation and/or downregulation of anti-apoptotic and pro-apoptotic factors or the production of mutated copies of

pro-apoptotic factors, to prevent the activation of their apoptotic pathways (Cory and Adams, 2002; Adams and Cory, 2007; Cotter, 2009). In many tumors, the cells are under hypoxic and nutrient-depleted conditions, which would lead to the activation of the extrinsic apoptotic pathway and cell death under normal circumstances. However, the same signals that trigger apoptosis allow cells to undergo autophagy (Levine and Kroemer, 2008); thus, rather than undergoing apoptosis, cancer cells exhibit autophagy, saving them from starvation (Sinha and Levine, 2008).

Cell immortality refers to the ability of a cell to exhibit unlimited mitosis. Under normal circumstances, cells are only permitted to undergo mitosis a certain number of times before apoptosis is activated, which is dictated by the length of their telomeres, as each round of division leads to shortening of the telomeres (Mathon and Lloyd, 2001). Cancer cells have been found to express telomerase, the enzyme that is responsible for the extension of telomeres, conferring their ability to continually undergo cell division without the loss of genomic matter or apoptosis activation (Novak, 2003; Artandi and DePinho, 2010).

The induction of angiogenesis is a crucial characteristic of cancer cells, as continual growth naturally leads to a need for increased oxygen and nutrients for sustenance. By upregulating the production of growth factors, cancer cells recruit and activate endothelial cells either directly, as in the case of VEGF production, or indirectly, through the activation of surrounding mesenchymal cells via the production of FGF (Ferrara, 2009; Liang et al., 2013). In some cases, there may also be downregulation of the production of angiogenic inhibitors (Lawler and Lawler, 2012). More often than not, as in the case of proliferation,

pro-angiogenic factors are sequestered in the ECM, and cancer cells produce proteases such as MMPs as a means of releasing these factors, thus allowing angiogenesis to occur.

Cancer cells usually exhibit an increased metastatic capacity due to the induction of EMT by the transcription factors described above, such as SNAI1, TWIST1, and SNAI2 (Peinado et al., 2007; De Craene and Berx, 2013). Epithelial-mesenchymal transition causes cancer cells to lose their cell-cell attachments and adopt a more motile phenotype with transient cell-ECM attachments (Peinado et al., 2007). The upregulation of mesenchymal markers such as MMPs also aids in the degradation of the surrounding ECM and the basal lamina (Kessenbrock et al., 2010; Sutoh Yoneyama et al., 2014), which allows cancer cells to escape into the bloodstream and establish a secondary tumor in another part of the body, thus completing metastasis.

In an environment that is low in oxygen, cancer cells are more often than not unable to carry out the oxidative phosphorylation of glucose to obtain adenosine triphosphate (ATP) to meet their energy requirements. Instead, these cells are commonly observed to carry out glycolysis. As a result of selective pressure, most of the cancer cells that thrive meet their metabolic needs through glycolysis, which is now being described as the Warburg effect (Bensinger and Christofk, 2012; Upadhyay et al., 2013). Since the turnover rate of ATP during glycolysis is much lower, the demand for glucose by cancer cells is higher than in normal cells. In addition to environmental pressure, it has been proposed that many cancer cells exhibit dysfunctional mitochondria, thereby preventing them from carrying out the oxidative phosphorylation of glucose (Wallace, 2012).

Immune cells are responsible for inducing the death of abnormal cells such as cancer cells under normal physiological conditions. The recognition of abnormal cells is usually carried out through the expression of certain antigens on the surface of the abnormal cell. Cancer cells have been found to avoid immune surveillance through mutations in the tumor necrosis factor receptor superfamily member 6 (Fas) receptor, which results in a loss of function, leading the cells to become refractory to the Fas ligand expressed by cytotoxic T cells. In addition, some cancer cells have been observed to express decoy receptors that do not stimulate the activation of T cells (Fouque et al., 2014). The tumor microenvironment has also proven to be too acidic for effector T cells, leading to the suppression of their activity, which also leads to the recruitment of regulatory T cells, causing immunosuppression (Mougiakakos et al., 2010; Calcinotto et al., 2012). The production of TGF- β by cancer cells further impedes the activation of the immune system, as it impedes the activation of natural killer (NK) cells (Gigante et al., 2012).

1.3.2 Types of skin cancer and their associated risks

Despite the measures taken to reduce skin cancer risk, such as adequate protection from UV radiation emitted by the sun, the number of reported skin cancer cases has been increasing globally (Godar, 2011; Lomas et al., 2012). In most cases, skin cancer affects an individual's quality of life, while in more serious cases, it can lead to death or disfigurement (Lewis and Weinstock, 2004; Burdon-Jones et al., 2010). Similar to chronic wounds, skin cancer poses a burden on society and the economy. For instance, in the United States alone, an estimated \$ 8.1 billion is spent on the treatment of skin cancer annually

(Medical Expenditure Panel Survey. Rockville, MD: Agency for Healthcare Research and Quality, January 2014). Due to the impending epidemic that is predicted to occur in the near future, the Surgeon General of the United States has put forth a “Call to Action” to governments and healthcare groups, highlighting the importance of the development of new therapeutic techniques to address skin cancer (US Department of Health and Human Services. The Surgeon General's Call to Action to Prevent Skin Cancer. Washington (DC): Office of the Surgeon General (US); 2014. Skin Cancer as a Major Public Health Problem).

In general, skin cancer has been separated into two broad categories: melanoma and nonmelanoma skin cancer (NMSC), with NMSCs accounting for approximately 80 % of skin cancer cases (Medical Expenditure Panel Survey. Rockville, MD: Agency for Healthcare Research and Quality, January 2014). Statistics from Medicare revealed that within the years 2002-2006, the number of procedures used to treat NMSCs increased by 16 %, highlighting a sharp increase in NMSC incidence rates (Rogers et al., 2010). Although the presented figures are already large, these numbers are still not representative of the total number of people afflicted with skin cancer, as information on NMSCs is not routinely collected by cancer registries, and these data therefore only include data from medical claims and surveys. As a result, the predicted number of people suffering from NMSCs is expected to be larger than indicated above.

The two major types of NMSCs are basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs). BCCs are the most common form of skin cancer and occur mostly on the neck and head (Dai et al., 2005). The primary

cause of BCCs is UV light exposure, and individuals who work outdoors therefore present a higher risk of developing BCCs (Kamath et al, 2018). Similarly, geographic locations affect the risk of developing BCCs in the population, with locations at high and low latitudes presenting greater amount of exposure to UV radiation. The development of BCCs has been determined to stem from DNA damage, caused both directly and indirectly by UV radiation. Other factors such as age, sex, family history and race also affect the risk of BCCs, due to differences in genetic susceptibility or, in the case of age, immunodeficiency (McDaniel and Badri, 2018). In addition, people who have previously suffered from NMSC present a significantly increased risk of BCC development (Cameron et al., 2019). Despite the high incidence rates of BCCs, they grow slowly and show a low chance of metastasizing. Coupled with the facts that they are easily diagnosed and that the current methods of treatment result in high cure rates of 95 % or above, BCCs are relatively easy to manage and treat (Newlands et al., 2016; McDaniel and Badri, 2018).

Squamous cell carcinomas are the second most common type of skin cancer. Squamous cell carcinomas that arise in the skin largely exhibit similar causes and risk factors to BCCs (Howell and Ramsey, 2018). However, in contrast to BCCs, SCCs tend to be characterized by higher metastatic potential and therefore higher mortality rates (Clayman et al., 2005). In addition, SCCs commonly occur in areas of the body such as the head, neck, lungs and esophagus, making them the most common type of metastatic cancer in the world (Marinkovich, 2007). However, studies have shown that SCCs generally share many characteristics at both the phenotypic and molecular levels, and discoveries made in any form of SCC should therefore provide a deeper

understanding of SCCs as a whole (Travis, 2002; Famadieh et al., 2009). Although much work has been carried out in the study of SCCs, the advances in diagnostic techniques and treatment modes have not had a considerable impact on the survival rate of patients (Agada et al., 2009). This is largely due to the shortage of reliable biomarkers identified to date, which may in part stem from the lack of studies focused on the tumor microenvironment.

1.3.3 Field cancerization

In recent years, one of the leading hypotheses for tumor recurrence is the concept of field cancerization. The term “field cancerization” was first coined by a group that noticed that seemingly normal tissue at the borders of oral SCC had already started displaying malignancy, as indicated by the presence of hyperplasia and hyperkeratinization (Slaughter et al., 1953). This inference was supported by their observation that independent primary tumors presented a high tendency of being in close proximity to each other in each patient, suggesting that a particular region, or field, contains factors that support cancer growth and progression. Since then, similar observations have been made for other forms of cancer, such as lung and colon cancers (Torezan and Festa-Neto, 2013), and one study showed that the radius of the field can be as great as 7 cm from the site of the primary tumor (Tabor et al., 2002).

In support of this concept, multiple studies have shown that the cells within such fields show signs of premalignancy. For instance, a key tumor suppressor gene, p53, was found to contain mutations in cells obtained from the margins of neck and head tumors, despite the normal histological characteristics of these cells, and the recurrence rate of these tumors was positively correlated with the

mutations (Brennan et al., 1995). Similar observations have been made in pathologies that are known to present an increased risk of cancer development, such as the presence of p53 and Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-RAS) mutations in patients suffering from colitis and p53 mutations in actinic keratoses (Glogau, 2000; Quaedvlieg et al., 2006; Leedham et al., 2009). Since somatic mutations are rare and random events, the chance of different cells undergoing identical mutations is miniscule. As a result, field cancerization has been suggested to start from a single mutant cell that undergoes multiple rounds of cell division, leading to the entire “field” being littered with mutants born from a single origin (Torezan and Festa-Neto, 2013).

Although it was initially thought that this phenomenon may arise from the accumulation of mutations in epithelial cells after multiple rounds of cell division, recent studies have revealed that the mesenchyme can be a determining factor as well (Dotto, 2014). For example, it has been observed that exposure to UV radiation (UVA) causes changes in the ECM contents of the dermis as well as atrophy before any abnormalities are observed in the epidermis (Yaar and Gilchrist, 2007). This finding indicates that UV radiation primarily affects fibroblasts, resulting in the secretion of various factors by these cells and remodeling of the ECM, which in turn affects the behavior of the keratinocytes. This theory was supported by another study showing that the deletion of the CBF1, Suppressor of Hairless, Lag-1 (CSL) gene specifically in fibroblasts is sufficient to cause SCC. Similar observations were made when the same group transplanted CSL-deficient fibroblasts into immunodeficient mice (Hu et al., 2012). In contrast, other studies have reported that cancer cells implanted into regenerating salamander limbs display characteristics indicating that they have

reverted normal cell behavior (Seilern-Aspang and Kratochwil, 1962; Fior, 2014). Taken together, these studies have provided evidence that the microenvironment plays an important role in regulating the behavior of cells and is essential in determining whether they remain normal or become malignant, which has been designated the tissue organization field theory (TOFT).

Unlike the somatic mutation theory (SMT), which proposes that cancers are derived from cells that accumulate a variety of mutations over time, the TOFT follows the principle that the microenvironment affects intracellular communication, and when there is an imbalance in the microenvironment, cell-cell communication is also altered, leading to overall changes in cell behavior within the tissue (Tarin, 2011; Smythies, 2015). Understanding the defects in the field is therefore important because rectifying these defects could significantly reduce the likelihood of cancer relapse.

1.3.4 Cancer-associated fibroblasts (CAFs)

Cancer cells are never found in isolation in a tumor. Instead, they are usually surrounded by a variety of different cell types from different lineages, which together with the ECM, constitute the tumor stroma. It has been shown that the stroma not only plays a role in helping cancer cells avoid immune surveillance but is also responsible for maintaining the inflammatory state of the tumor tissue, which is an enabling characteristic of cancer (Hanahan and Weinburg, 2011). This role of the stroma is further evidenced by the fact that antagonizing the contributions of the stroma to the tumor can improve prognosis and survival, while increased stroma activity leads to opposite results (Tlsty and Hein, 2001).

As mentioned earlier, the dermis is the layer of the skin that is in contact with the epidermis, and its primary role is to provide support to the epidermal layer. The interactions between the keratinocytes and dermal cells are therefore extremely crucial for maintaining normal skin homeostasis; any dysregulation of these interactions could lead to detrimental changes to the epidermis, thus impairing its ability to effectively function as a barrier. Fibroblasts are the cells that are most abundant in the dermis. As a result, fibroblasts have a large impact on the microenvironment in which keratinocytes are present. The ability of fibroblasts to secrete numerous cytokines and chemokines that regulate the migration and proliferation of other cell types in the skin during wound healing (including keratinocytes), along with their main role as the producers of ECM components such as collagen as well as enzymes involved in ECM remodeling such as MMPs highlights their importance in maintaining the microenvironment within the skin and, therefore, their contribution to tissue cancerization (Witte and Barbul, 1997; Goldman, 2004).

Although CAFs are the main cell type present in the tumor stroma, it has been discovered that they may not only originate from fibroblasts. Instead, many different types of cells within the stroma have been found to be capable of differentiating into CAFs, such as bone marrow-derived mesenchymal stem cells, pericytes, endothelial cells, muscle cells, adipocytes, and even epithelial cells (Kalluri and Zeisberg, 2006; Anderberg and Pietras, 2009; Erez et al., 2010; Quail and Joyce, 2013). Regardless of their origin, CAFs are typically myofibroblast-like cells that are capable of promoting cancer progression. The differences between CAFs and normal fibroblasts have also been compared, and a study has shown that CAFs express many similar markers to activated

fibroblasts during wound healing (Augsten, 2014). This observation was supported by a study that showed that the transcriptional profiles of CAFs and normal fibroblasts were distinctly different (Orr et al., 2011). However, unlike fibroblasts involved in wound healing, CAFs do not return to a quiescent state and, thus, remain constitutively active.

In addition to the ability of CAFs to contribute to the growth of tumors in many different types of cancers (Nakagawa et al., 2004; Hwang et al., 2008; Liao et al., 2009; Erez et al., 2010), the extent of their capacity to contribute to tumor progression has been shown by their ability to convert nontumorigenic cells into tumorigenic cells and to support the growth of these cells *in vivo*. A study showed that the interaction between CAFs and cancer cells led to the production of many factors that contribute to cancer progression, such as growth factors, cytokines, reactive nitrogen species and reactive oxygen species (ROS), which may explain the observations made in the above studies (Erez et al., 2010). Growth factors are the most widely studied of these cancer progression-related factors, and CAFs have been established as the main producers of many growth factors involved in tumorigenesis, such as EGF and TGF- β (Kalluri and Zeisberg, 2006). In addition, these factors affect resident fibroblasts, leading to the remodeling of the ECM, which further promotes tumor growth. For these reasons, many studies have started to focus on CAFs for the development of anti-cancer drugs. For instance, it has been discovered that CAFs are able to confer resistance to cancer therapies such as tyrosine kinase inhibitors and anti-angiogenesis inhibitors upon tumor cells (Crawford et al., 2009; Wang et al., 2009). In addition, it has been proposed that CAFs arise due to mutations or epigenetic changes in DNA. This hypothesis was confirmed by the finding that

p53^{+/-} fibroblasts lost their wild-type allele of p53 when exposed to cancer cells *in vivo* (Hill et al., 2005). The results obtained from this study also helped to prove that crosstalk occurs between the cells, and this crosstalk may be essential for cancer progression.

Although much has been discovered with regard to the effects of the various factors on cancer progression and their downstream changes during the transcriptional regulation of cancer cells, the changes in transcriptional regulation within CAFs that lead to their existence or to their production of these factors remain unclear. Several different transcription factors have been identified as playing a crucial role in cancer, but one of the largest families of transcription factors, the nuclear receptors, has continually evaded scrutiny.

Figure 5. Tumor-CAF crosstalk leads to cancer progression. A schematic demonstrating the interactions between the cancer cells and CAFs resulting in a feedforward mechanism which promotes cancer progression and malignancy. Image by Tan Wei Yi, Mark.

1.3.5 Nuclear receptors and peroxisome proliferator-activated receptors (PPARs)

Unlike most transcription factors, nuclear receptors are generally found in the nucleus regardless of their transcriptional activation state. In their inactive

state, they bind to corepressors that prevent them from acting on their target genes. In the presence of their ligands, conformational changes lead to them bind to coactivators instead, causing gene transcription to take place (Giguere, 1999). The structures of most of these receptors are also fundamentally similar, and the different domains have been categorized as domains A-F in sequence starting from the N-terminus. First, the A/B domain contains activation function (AF) 1. The C domain is the DNA-binding domain and consists of a combination of α -helical DNA-binding regions with structures akin to zinc fingers. As the name suggests, this domain is critical for the nuclear receptor to recognize and bind to its target sequence. The D domain is known as the “hinge” and provides pivotal flexibility for conformational changes that take place when the ligand is present. Finally, the E/F domain contains the ligand-binding domain and AF-2. Activation function-2 is activated upon the conformational changes that are induced when the ligand binds, leading to the replacement of corepressors with coactivators.

Figure 6. Illustration of the domain organization of nuclear receptors. The different domains of a nuclear receptor, with the function of each domain being indicated below, respectively. Image by Tan Wei Yi, Mark.

In the past, the identified ligands of nuclear receptors have been related to hormones and the endocrine system, while a large group of nuclear receptors whose ligands remained obscure were referred to as “orphan” receptors. After the endocrine nuclear receptors were found to play an essential role in tissue

development, physiology, and pathology, interest in identifying the ligands for the orphan nuclear receptors increased (Giguere, 1999). One leading example is the PPARs, which have been demonstrated to be activated by lipids, leading to the transcriptional activation of genes involved in metabolism (Berger et al., 2005). The PPARs have been divided into three subtypes, PPAR α , PPAR β/δ , and PPAR γ , each of which is distinct in its distribution patterns in different developmental stages and types of tissues (Boitier et al., 2003). For instance, PPAR α is mainly expressed by the liver, where it is involved in the regulation of fatty acid metabolism and is therefore essential, particularly under hypoglycemic conditions. Peroxisome proliferator-activated receptor γ is also highly specific in terms of its localization in adipose tissue, where it is required for the differentiation of adipocytes as well as lipid accumulation (Berger et al., 2005). On the other hand, PPAR β/δ is expressed in almost all types of tissue, albeit at varying levels (Yessoufou and Wahli, 2010). Similar to its paralogs, PPAR β/δ regulates metabolism. However, it has also been found to be involved in tissue development.

Recent studies have shown that PPAR β/δ is the major subtype in the epidermis and plays a critical role in the epidermis during differentiation and wound repair (Tan et al., 2002; Tan et al., 2003; Tan et al., 2004; Tan et al., 2005; Tan et al., 2007; Chong et al., 2009). In addition, it has also been found that PPAR β/δ is the major subtype in dermal fibroblasts and is important for mediating cross-talk between the mesenchyme and the epidermis during wound healing. Examples include PPAR β/δ activation in fibroblasts leading to the proliferation of keratinocytes during re-epithelialization and the PPAR β/δ activation in fibroblasts facilitating keratinocyte migration via the reduction of

ECM oxidative stress and, therefore, ECM modifications through the production of catalase and glutathione peroxidase 1 (Di-Poi et al., 2002; Chong et al., 2009; Wang et al., 2015). Similar to wound healing, PPAR β/δ has been shown to play a role in the development of skin cancer. For instance, it has been revealed that UV light is capable of activating PPAR β/δ in epidermal cells, leading to the activation of Src and the expression of EMT markers, ultimately contributing to skin cancer progression (Montagner et al., 2014). However, there remains a gap in the knowledge of how PPAR β/δ in dermal fibroblasts can affect skin cancer progression, which could be extremely important in the development of new therapeutic strategies and interventions.

1.3.6 Summary and current gap of knowledge

Nuclear receptors are among the largest known classes of transcription factors that regulate gene expression in a ligand-dependent manner. Drugs targeting nuclear receptors have been used for various skin diseases (Lipid and Skin Health, Chapter 7). The importance of nuclear receptors in cancer has largely been evaluated within the tumor epithelium itself. Much less is known about the nuclear receptor status of the tumor microenvironment. This is in no small part due to the dynamism and heterogeneity of the various constituents of the tumor stroma. However, without a comprehensive understanding of the activities and functions of nuclear receptors within the tumor stroma it becomes difficult to access nuclear receptor-directed anticancer treatments that deprive cancer cells of the supportive structure that it provided by the tumor stroma.

In this aspect, CAFs are the dominant cell type in many cancers including cutaneous squamous cell carcinoma. Given the limited studies on nuclear

receptors in CAFs (Knower et al, 2013; Siletz et al 2013; Yu et al., 2014), no national or international statistics are available on the expression of any specific nuclear receptor in any cancer types. A pilot study comparing ten paired samples of CAFs and peri-tumoral normal fibroblasts from archived SCC biopsies revealed that the expression of PPARb/d was downregulated to the greatest extent in all the samples, followed by Vitamin D receptor and glucocorticoid receptor (Chan et al., 2018). *In vitro* co-culture system and gene expression microarray analysis indicate that fibroblast PPARb/d modulates cellular growth, migration and chemoresistance (Chan et al., 2018). However, the *in vivo* function of PPARb/d in skin tumorigenesis remains to be determined.

1.4 Hypotheses and aims

Aim 1: To determine the signaling pathway/s that initiate EMT in keratinocytes during re-epithelialization.

Aim 2: To elucidate how these signaling cascades are dysregulated by hyperglycemia.

In our first study, we hypothesized that hyperglycemia blocks signals that play a crucial role in the re-epithelialization process during wound healing and that the lack of these signals cause the impairment of the re-epithelialization process in diabetic wounds. To examine these hypotheses, we performed laser capture microdissection of immunohistologically stained samples to specifically capture migrating keratinocytes at the tip of the epithelial tongues using the downregulation of junctional E-Cadherin as a guide in the wounds of both diabetic and normal mice. Next, we conducted microarray analysis and gene ontology studies on the samples to observe changes that support our

hypothesis, specifically regarding proliferation, signaling, migration and transport, which would indicate the signals that are being impeded in the migration of diabetic keratinocytes. Using a combination of techniques including quantitative polymerase chain reaction (qPCR), Western blotting, immunostaining and flow cytometry analysis, we aimed to use the information provided by our microarray analysis to elucidate the mechanism that might be causing the impairment of re-epithelialization.

Aim 3: To determine the effect and the underlying mechanism by which PPAR β/δ in fibroblasts affect skin tumorigenesis.

In our second study, we hypothesized that PPAR β/δ in fibroblasts play a role in skin tumorigenesis. Using chemicals to induce skin carcinogenesis, we aimed to confirm our hypothesis by comparing the skin of mice in which PPAR β/δ was selectively deleted in the fibroblasts (FSPCre-*Pparb/d^{ex4}*) and that of their wild-type counterparts (*Pparb/d^{fl/fl}*). To ensure that we covered as many aspects of cancer progression as we can, we used a combination of proliferation and differentiation markers as well as hematoxylin and eosin staining, in addition to macroscopic observations, to check for differences in tumor progression. Guided by the observed differences, we used our previously obtained microarray data (GSE71419) from the epidermis and dermis of FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* mice to elucidate the possible mechanisms through which PPAR β/δ in fibroblasts might be using to modulate changes in the epidermis and, thus, cancer development and/or progression.

Chapter 2: Materials and Methods

2.1 Cell culture

Immortalized telomerase reverse transcriptase (TERT) human keratinocyte Ker-CT was from the American Type Culture Collection (ATCC, USA). HaCaT cells were from AddexBio (T0020001, USA). HaCaT cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v/v) fetal bovine serum (FBS) (Hyclone, USA), 4.0 mM L-glutamine, 11 mg/mL sodium pyruvate and 3.7 g/L sodium bicarbonate. Ker-CT cells were cultured in KGM-GOLD BulletKit medium (Lonza, Switzerland). Human primary fibroblasts were obtained from Asterand Bioscience in 2015 and maintained in FibroGro™-LS Complete Media Kit (Millipore, USA). Cells were used at low passage numbers (3–5 passages) and periodically tested to exclude mycoplasma contamination. Cells were maintained in a 37 °C, 5 % CO₂, humidified incubator. Cells were sub-cultured when they reached 70-80 % confluency. HaCaT cells were sub-cultured using 0.25 % trypsin in pH 7.4 Ethylenediaminetetraacetic acid (EDTA). Ker-CT and fibroblasts were sub-cultured using 0.05 % trypsin in pH 7.4 EDTA. For diabetic culture conditions, the respective culture medium was supplemented with 25 mmol/L glucose.

2.2 Glucose, TGFβ1, acetylcholine (ACh), choline and kinase inhibitor treatments

All cells were seeded at a density of 5×10^3 cells/mL. Cells were serum-starved overnight prior exposure to indicated treatments. Treatments: 25 mM glucose, 10 ng/mL TGFβ1 (Peprotech, USA), 5 mM acetylcholine chloride (Sigma, USA) 5 mM choline chloride (Sigma, USA), and inhibitors (at their IC₅₀) (Target

Molecule Corporation, USA). For exposure to multiple agents, the agents were added at 4 h intervals from each other.

2.3 Organotypic coculture (OTC)

Dermal equivalents for OTCs were prepared with native type I collagen extracted from rat tail tendons with 0.1 % acetic acid. The lyophilized collagen was dissolved with 0.1 % acetic acid to a final concentration of 4 mg/mL. Eight volumes of ice-cold collagen solution were mixed with 1 volume of 10 X Hank's buffered saline (HBS) followed by neutralization with 1 M NaOH. Fibroblasts were resuspended in 1 volume of 1 X HBS and added dropwise to the neutralized collagen to achieve a final cell density of 10^5 cells/mL of collagen. Three mL of this fibroblast/collagen mixture was dispensed into a 24 mm diameter Transwell culture insert (3 μ m pore size; BD). The culture insert was placed in a 6-well Deep-Well Plate (BD) and allowed to gel in a 37 °C, 5 % CO₂, 70 % humidity incubator (Thermo Fisher Scientific). Glass rings (outer diameter, 20 mm; thickness, 1.2 mm; height, 12 mm) were centrally placed onto the collagen. The glass rings serve to compress the collagen and delimit the area for the seeding of keratinocytes. The fibroblast-embedded collagen was submerged and cultured in serum-free OTC medium overnight. The serum-free OTC contains basal medium, 3:1 (vol/vol) DMEM (low glucose), Ham's F-12 nutrient mixture, basal supplements 5 μ g/mL insulin, 1 ng/mL epidermal growth factor, 0.4 μ g/mL hydrocortisone, 100 nM adenine, 10 μ M serine, 100 nM cholera toxin, 10 μ M carnitine, 1 mg/mL fatty acid-free albumin, lipid supplements 0.05 mM ethanolamine, 1 μ M isoproterenol, 1 μ M α -tocopherol, and 50 μ g/mL ascorbic acid. The following day, the medium was removed and

10⁶ keratinocytes were seeded into the center of the glass ring. Organotypic coculture medium was added, and the setup was once again incubated at 37 °C with 5 % CO₂ for 24 h to allow the keratinocytes to attach. The next day, the glass ring was removed, and culture was maintained at air liquid interface. Medium was changed every 3 days for a total of 2 weeks.

2.4 Cell transfection

Cells were seeded and left to incubate for 24 h at 37 °C with 5 % CO₂ before transfection. Fibroblasts were transfected with SMARTpool small interfering RNAs (siRNAs) targeting either *Pparb/d*, Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 4 protein (*Nox4*) or Leucine-rich alpha-2-glycoprotein 1 (*LRG1*) using DharmaFECT1 according to manufacturer's recommendation (Dharmacon, USA). Briefly, a transfection mixture containing 25 nM final concentration of siRNA was constituted from siRNA and DharmaFECT reagent and applied to cells in complete medium. For overexpression studies, plasmids encoding the selected genes were purchased from Missouri S&T cDNA Resource Center and Addgene (Addgene, USA). FugeneHD (Promega, USA) was used as the vector for plasmid delivery into the fibroblasts. Briefly, the fibroblasts were treated using a FugeneHD:plasmid ratio of 3:1 using 0.55 µg of plasmid cDNA. All knockdowns and overexpressions were validated by RT-qPCR before experimentation.

2.5 Animal wounding and wound fluid collection

For all experiments, male C57BL/6J mice of 8 weeks of age were used (Jackson Laboratory, ME, USA). All animals were caged individually and housed in a

temperature- controlled room (22 ± 1 °C) on a 12:12 light-dark cycle, fed with the standard mouse chow diet (60 % carbohydrate, 40 % protein), and water *ad libitum*. For microarray analysis, diabetes was induced by using high fat chow (60 % fat, 40 % protein) and 30 % fructose water over a period of 16 weeks. The mean daily unadjusted food intake was 4.4 ± 0.1 g/mouse. Subsequently diabetes was induced by injecting streptozotocin (STZ) either at low doses (40 mg/kg) over a span of one week, or one high dose (200 mg/kg). Mice were fasted overnight and blood glucose levels were monitored through tail vein sampling with a glucometer (Accu-Check Performa Glucometer, Roche, Germany). Fasted non-diabetic mice exhibited fasting blood glucose levels <5 mmol/L or 90 mg/dL glucose (normal 80-100 mg/dL glucose; Jackson Laboratory). STZ-induced diabetic mice have fasting blood glucose >15.1 mmol/L or 272 mg/dL glucose. Mice with diet-induced diabetes have mean fasting glucose of 152 mg/dL or 8.4 mmol/L glucose.

Full-thickness excisional wounds were inflicted on the mice by folding the skin of the mouse's back and punching through, creating two identical elliptical wounds, or by using a pair of surgical scissors to excise out all three layers of the skin. Wound fluids were collected using a 5 mm x 5 mm polyvinyl alcohol sponge placed adjacent to the wound, and covered with Tegaderm™ transparent film dressing (3M, USA) and collected at the specified times. In ACh-treated wounds, 10^{-9} M of acetylcholine chloride (Sigma-Aldrich, St. Louis, MO, USA) was added to the wounds immediately after wounding. Wounds were harvested at indicated intervals. All animal experiments were carried out in accordance with the guidelines of the institutional animal care and use

committee (ARF-SBS/NIE-A0112AZ, A0216AZ, A0324 and A0321, A0250, A19043 & A19032) of Nanyang Technological University, Singapore.

2.6 Separation of epidermis and dermis from mouse skin biopsy

Age matched mice in the telogen (resting) phase of their hair cycles were anaesthetized and depilated on their dorsal back before being subjected to a biopsy punch (~4 mm). The skin biopsy was treated with 3.8 % ammonium thiocyanate (Sigma, USA) in 1 X phosphate buffered saline (PBS) for 30 min at 37 °C. Subsequently, the epidermis was mechanically separated from the dermis using a pair of forceps. The dermis was then curetted with a 30-gauge needle and washed with 1 X PBS before being subjected to either genotyping, RNA extraction and RT-qPCR analysis, or Western blot analysis.

2.7 Genotyping of mice

Mice genotyping was performed using the mouse genotyping kit hotstart according to the manufacturer's protocol (KAPA Biosystems, USA). Briefly, genomic DNA was extracted from the mouse ear clippings (~1 mm²) in a mixture containing 1 X KAPA Express Extract Buffer and 2 units 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. Polymerase chain reaction was immediately performed with KAPA2G Fast Hotstart Genotyping Mix, 0.5 µM forward and reverse primer, and 1 µL of supernatant from the respective KAPA Express Extract reaction above. Polymerase chain reaction products were analysed in a 1.2 % agarose gel via ethidium bromide staining.

Table 1 Oligonucleotide sequences of genotyping PCR primers.

Genes	Forward (5' – 3')	Reverse (5' – 3')
PPAR β/δ Exon 4	GCAGCTGCTCAGCTGCCTGC	CCGCCTCTCGCCATCCTTTCAGA
PPAR β/δ Exon 5	GCAGCTGCTCAGCTGCCTGC	CAGGTGCTGGGCGCCCTCTGACAGAAT
Fibroblast-specific protein 1 (FSP1) Cre Control	CTAGGCCACAGAATTGAAAGATC	GTAGGTGGAAATTCTTAGCATCATCC
FSP1Cre Transgene	GCGGTCTGGCAGTAAAACTATC	GTGAAACAGCATTGCTGTCACTT

2.8 Chemical-induced skin carcinogenesis

Male FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* mice of C57BL/6 background were used for this study. Briefly, mice were shaved with surgical clippers two days before topical initiation of the back with 200 μ L of acetone containing 100 nmol of 7,12-dimethylbenz[a]anthracene (DMBA). One week after initiation, mice were treated thrice weekly with 30 mg benzoyl peroxide. The average number of dorsal skin tumors was recorded at 1-week intervals from 10th to 19th-week post-treatment. Skin tumors were harvested at 19 weeks. The tumor size was calculated using the modified ellipsoid formula: $\frac{1}{2}$ (length $\times$ width²). The greatest longitudinal diameter (length) and the greatest transverse diameter (width) of each tumor was determined using a digital caliper (Jensen et al., 2008). The size for each tumour was classified under 3 categories: <5 mm³ (small), 5-10 mm³ (medium) and >10 mm³ (large). Each tumor from FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* mice was measured and categorized accordingly.

2.9 Antioxidant response element (ARE) luciferase reporter assay

The activity of nuclear factor erythroid 2-related factor 2 (NRF2) in the cells was determined using ARE luciferase reporter vector (BPSBiosciences). This reporter contains a firefly luciferase gene under the control of multimerized ARE responsive elements located upstream of a minimal promoter. The renilla luciferase (RL) control reporter vector was used as a control for transfection efficiency. Cells were transfected using FugeneHD as described earlier (Promega). Reporter activity was measured using Dual-Glo Luciferase Assay System (Promega).

2.10 Extracellular hydrogen peroxide (H₂O₂) and intracellular ROS measurement

Extracellular H₂O₂ was measured using Amplex Red Hydrogen Peroxide/Peroxidase assay kit (Life Technologies, USA) according to manufacturer's protocol. Briefly, 5 x 10⁴ cells were incubated in assay reagent comprising of 50 µM Amplex Red reagent 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 x 10⁴ 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 using 10 µM pH 7.4 EDTA and analysed using the Accuri™ C6 flow cytometer (BD

Biosciences, USA). Reactive oxygen species in wound fluid was measured using 20 μ L of wound fluid in 200 μ L Amplex Red final reaction volume. Wound fluids were collected using a 5 mm x 5 mm polyvinyl alcohol sponge placed adjacent to the wound, and covered with Tegaderm™ transparent film dressing (3M, USA). Sponges were collected at the respective timepoints and Amplex Red conversion to resorufin fluorescence was measured as described above. The specificity of the assay for H_2O_2 was verified with catalase, and the degradation of H_2O_2 or inhibition of the assay system by the sample was analyzed by determining the recovery of exogenously added H_2O_2 .

2.11 Polyethylene glycol (PEG)-switch assay

Keratinocytes were lysed using M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, USA). Oxidized thiols were reduced using dithiothreitol (DTT) and alkylated using 2 mM PEG-maleimide with 0.5 % sodium dodecyl sulfate (SDS) for 2 h at room temperature. The alkylation reaction was quenched using 100 mM Tris–hydrochloric acid (HCl) buffer pH 6.8, 4 % SDS, 20 % glycerol, and 0.01 % bromophenol blue and 100 mM maleimide and the samples were resolved by SDS- polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblot. To analyse oxidatively modified phosphatase and tensin homolog (PTEN) and proto-oncogene tyrosine-protein kinase Src (Src), the cells were washed with oxygen-free ice-cold PBS containing 1 mM N-ethylmaleimide (NEM) and then lysed in lysis buffer (50 mM Tris, pH 6.8, 150 mM NaCl, 1 mM EDTA, 0.5 % Triton X-100 and 30 mM NEM). The lysates were further clarified using QIAshredder (Qiagen). Equal amounts of proteins were mixed with NEM loading dye (50

mM Tris, pH 6.8, 10 % glycerol, 2 % SDS, 20 mM NEM, and 0.02 % bromophenol blue) and resolved by non-reducing SDS-PAGE. As a control, the proteins were completely reduced by heating in SDS-loading dye containing DTT. The separated proteins were subjected to immunoblot analysis using antibodies against PTEN and Src.

2.12 H₂O₂ treatment of keratinocytes *in vitro*

Human primary keratinocytes were seeded at a density of 5×10^4 cells/mL and grown to 70 % confluency before starving overnight. Cells were then resuspended in PBS and treated with 100 nM H₂O₂ for 30 min before changing back to starvation medium. Cells were lysed for RNA extraction after incubating for 3.5 h post H₂O₂ treatment.

2.13 Tissue processing

Wound and tumor biopsies were harvested, fixed in 4 % paraformaldehyde overnight, and dehydrated stepwise in 70 %, 80 %, 90 % and 100 % ethanol (EtOH), followed by 50 % EtOH/50 % xylene and 100 % xylene at 1 h intervals before being embedded in paraffin. Sections of 5 μ m thickness were subjected to hematoxylin and eosin staining (H&E), immunostaining, or immunohistological staining.

2.14 Immunostaining and H&E staining

Five μ m thick paraffinized sections were deparaffinized in xylene for 20 min and hydrated through a graded alcohol series. The hydrated sections were washed with 1 X PBS buffer. Antigen retrieval was conducted using the Aptum Biologics

2100 Antigen Retriever. The sections were blocked with 3 % normal goat serum (NGS) for an hour. Respective primary antibody (**Table 2**) was added to the sections and incubated at 4 °C overnight. The following day, the sections were washed with PBS and incubated with cognate secondary antibodies (**Table 2**). Next, the sections were washed with PBS buffer, and the procedure was repeated with a second primary antibody (if any). Finally, the sections were washed, counterstained with Hoeschst 33342 dye (Life Technologies, USA) for nuclei and imaged using JuLI™ Stage Real-Time Cell History Recorder (NanoEnTek Inc., Korea) or Zeiss LSM 700 Microscope (Carl Zeiss, Germany). All immunostaining performed without primary antibodies served as negative controls. For H&E staining, the sections were washed in PBS buffer and stained with H&E as recommended by the manufacturer (Sigma, USA). The sections were dehydrated and mounted using the Eukitt® Quick-hardening Mounting Medium (Sigma, USA). Images of the sections were captured with the Zeiss Axio Scan.Z1 and analyzed with the Zen-Lite software (Carl Zeiss, Germany).

Table 2 Antibodies used for immunostaining.

Antibody	Species	Vendor	Catalog No.
SNAI1	Rabbit	Abnova	PAB27142
E-cadherin	Rabbit	Cell Signaling	3195S
Phospho-SMAD3	Rabbit	Abcam	ab52903
Alexa Fluor™ 488 Tyramide SuperBoost™ Kit, goat anti-rabbit IgG	Goat	Thermo Scientific	B40943

Alexa Fluor™ Plus 594	Goat	Thermo Scientific	A32740
Alexa Fluor™ 488	Goat	Thermo Scientific	A21042

2.15 Laser capture microdissection (LCM)

Laser capture microdissection was performed on immunohistologically stained sections of wounds obtained from wild-type and diabetic mice. Briefly, sections were incubated with E-cadherin antibody (3195S) (Cell Signaling, USA) overnight followed by incubation with biotinylated secondary antibodies. After incubation with the secondary antibodies, sections were incubated in avidin:biotinylated enzyme complex (Vector Laboratories, USA) for 30 min. Sections were then developed with 3,3'-diaminobenzidine (DAB) substrate (Vector Laboratories, USA), producing a brown stain. Using the Zeiss PALM Laser Capture Microdissection system (Carl Zeiss, Germany) according to manufacturer's instructions, LCM was carried out and the samples were collected using separate adhesive CapSure Macro LCM Caps (Life Technologies, Carlsbad, CA). 50 sections of each sample were accumulated within each tube. Images were taken using the Fluar 10x / 0.25 and Korr LD Plan-Neofluar 40x / 0.60 objectives alongside the AxioCam ICc1 color camera installed in the microscope.

2.16 Microarray gene analysis

RNA amplification and ss-DNA synthesis were carried out using GeneChip® WT Pico Kit (Affymetrix, Santa Clara, CA) on laser-captured samples. Samples were loaded onto GeneChip® Mouse Gene 1.0 ST Array as recommended by manufacturer (Affymetrix, USA) Hybridization was performed

using the GeneChip Hybridisation Oven 645 (Affymetrix, USA). Washing and staining was performed using GeneChip Fluidics Station 450 (Affymetrix, USA). Raw microarray data files (.cel) were then analysed using Partek Genomic Suite (v6.6). Analysis of variance (ANOVA) was performed for all samples. Diabetic mice were contrasted against the wild-type mice samples and gene ontology studies were carried out using a cutoff of 1.2-fold.

2.17 Ingenuity pathway analysis (IPA)

For low-glucose pathway, kinase list (in red) was derived from the unbiased kinase inhibitor array screen, which were then connected to 2 major nodes of acetylcholine and its receptor, and the TGF β RI, TGF β RII kinases and TGF β R nodes. Intermediate nodes (denoted in grey) are grown off by the curated pathway analysis algorithm. For high glucose pathway, previously derived low-glucose pathway was connected to p38- mitogen-activated protein kinase (MAPK) node (in blue) to denote the possible associations derived from previous experiment.

Identification of the Pparb/d, TGF β 1, Nox4 and NRF2 network connectivity was determined through the use of twelve molecules with significant fold change (>1.5 times) and confidence ($p < 0.05$) identified from a microarray previously carried out from our lab (GSE71419), using FSPCre-*Pparb/d*^{ex4} and *Pparb/d*^{fl/fl} epidermis and dermis.

2.18 Immunoblotting

Cells were lysed using ice-cold M-PER Mammalian Protein Extraction Reagent (Thermo Scientific, USA). Total protein lysates were resolved using

10 % SDS-PAGE and electrotransferred onto Immobilon-FL PVDF membrane (Merck Millipore, USA). Membranes were blocked with 1 X Odyssey Blocking buffer (LI-COR Biotechnology, USA) for 1 h at room temperature. The membrane was then incubated overnight at 4 °C with the indicated primary antibodies (**Table 3**) in 1 X Odyssey Blocking buffer containing 0.05 % Tween-20. Membranes were washed thrice with TBST (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05 % Tween-20), and incubated with appropriate IRDye®680- or 800-conjugated anti-IgG secondary antibodies (**Table 3**) in 1 X Odyssey Blocking buffer containing 0.05 % Tween-20 and 0.01 % SDS for 1 h at room temperature. Protein bands were revealed using the Odyssey®CLx Infrared Imaging System, and signals were quantified using ImageStudio Software (LI-COR Biotechnology, USA).

Table 3 Antibodies used for immunoblotting.

Antibody	Species	Vendor	Catalog No.
E-cadherin	Rabbit	Cell Signaling	3195S
Total SRC	Rabbit	Cell Signaling	2108S
Phospho-SRC	Rabbit	Cell Signaling	6943S
Total P38 MAPK	Rabbit	Cell Signaling	8690S
Phospho-P38 MAPK	Rabbit	Cell Signaling	4511S
Total MEK1/2	Rabbit	Cell Signaling	9122S
Phospho-MEK1/2	Rabbit	Cell Signaling	9121S
Total PTEN	Rabbit	Cell Signaling	9552S
Phospho-PTEN	Rabbit	Cell Signaling	9551S
Total-B-Raf	Rabbit	Cell Signaling	14814S
Phospho-B-Raf	Rabbit	Cell Signaling	2696S
NRF2	Rabbit	Cell Signaling	12721S
Total SMAD3	Rabbit	Abcam	ab40854

Phospho-SMAD3	Rabbit	Abcam	ab52903
LRG1	Rabbit	Abcam	ab178698
β -tubulin	Mouse	DHSB	E7
Total ERK1/2	Rabbit	Santa Cruz Biotechnology	sc-94
Phospho-ERK1/2	Rabbit	Santa Cruz Biotechnology	sc-16982
NOX4	Goat	Santa Cruz Biotechnology	sc-21860
PPAR β/δ	Goat	Santa Cruz Biotechnology	sc-1987
Snail	Rabbit	Abnova	PAB27142
IRDye® 680RD antiMouse IgG (H + L)	Goat	LI-COR	LIC-92568070
IRDye® 680RD antiRabbit IgG (H + L)	Goat	LI-COR	LIC-92568071
IRDye® 800CW antiMouse IgG (H + L)	Goat	LI-COR	LIC-92632210
IRDye® 800CW antiRabbit IgG (H + L)	Goat	LI-COR	LIC-92532211
IRDye® 800CW antiGoat IgG (H+L)	Donkey	LI-COR	LIC-92632214

IRDye® 680CW antiGoat IgG (H+L)	Donkey	LI-COR	LIC-92668074
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2.19 Live cell imaging and cell migration analysis

Live cell imaging was performed in 6-well plates at 1 h intervals over a period of 24 h using JuLI Stage real-time cell history recorder (NanoEnTek Inc., Korea) using a UPlanSApo 4X 0.16 numerical aperture objective lens (Olympus, Japan). Cell movement was tracked and measured using the Manual Tracking plugin on the ImageJ software (NIH, USA). Directionality plots were then made using the Chemotaxis and Migration Tool (Ibidi®, Germany).

2.20 Flow cytometry

Cells were rinsed with 1 X PBS before being treated with 10 mM of EDTA to dislodge cells from culture flasks. Cells were resuspended in 50 µL of blocking buffer (3 % BSA, 2 mM of EDTA in 1 X PBS) for 45 min before incubation with TGFβRII PE-conjugated antibody (REA903) for 1 h (1:50 dilution) (Miltenyi Biotec, USA). Flow cytometry analysis was carried out using BD Accuri™ C6 Plus (BD Biosciences, USA). Post analysis was performed using FlowJo v10.0.7 (FlowJo LLC, USA).

2.21 Acetylcholine/acetylcholinesterase assay

Acetylcholine amount was measured using the Amplex® Red ACh/acetylcholinesterase Assay Kit (Invitrogen, USA) according to manufacturer's protocol. Briefly, the biochemical principal of the assay measures total choline levels. Using acetylcholinesterase's inhibitor pyridostigmine bromide (PB) (Sigma-Aldrich, USA) to inhibit endogenous

acetylcholinesterase activity, we measured each sample twice, one with inhibitor and one without. Inhibited and uninhibited samples represented choline only and total choline concentrations, respectively. Samples were measured with a microplate reader (Infinite® 200, Tecan, Crailsheim, Germany) at Ex560 nm and Em590 nm. Standard curve was derived using exact choline concentrations. Concentration of ACh was obtained by subtracting the concentration of free choline from total choline.

2.22 RNA extraction, reverse transcription, and qPCR

Total RNA was extracted using TRIzol® Reagent followed by the PureLink™ Micro-to-Midi Total RNA Purification System according to the manufacturer's protocol (Invitrogen, USA). Total RNA was quantified based on the A260/A280 absorbance using the Nanodrop ND1000 (Thermo scientific, USA). Total RNA was reversed transcribed using iScript Reverse Transcription Supermix for RT-PCR (Bio-Rad, USA). Quantitative polymerase chain reaction was performed using the respective primers (**Table 4-6**).

Table 4 List of human qPCR Primers.

Gene	Forward (5' – 3')	Reverse (5' – 3')
OCLN	GACTTCAGGCAGCCTCGTTAC	GCCAGTTGTGTAGTCTGCTCA
CDH1	GCCGAGAGCTACACGTTCA	GACCGGTGCAATCTTCAAA
ERBB3	GACCCAGGTCTACGATGGGAA	GTGAGCTGAGTCAAGCGGAG
NCAD	AGCCAACCTTAACTGAGGAGT	GGCAAGTTGATTGGAGGGATG
VIM	AGTCCACTGAGTACCGGAGAC	CATTTCACGCATCTGGCGTTC
TWIST1	GTCCGCAGTCTTACGAGGAG	GCTTGAGGGTCTGAATCTTGCT
SNAI1	GCCTTCAACTGCAAATACTGC	CTTCTTGACATCTGAGTGGGTC

SNAI2	CGAACTGGACACACACACAGT	CTGAGGATCTCTGGTTTTGGT
TGFβ1	CAACAATTCCTGGCGATACCT	GCTAAGGCGAAAGCCCTCAAT
TGFβR2	CGTGGAGTCGTTCAAGCAGAC	CCGCACCTTGGAACCAAATGG
NOX4	TGCCTGCTCATTTGGCTGT	CCGGCACATAGGTAAAAGGATG
NRF2	ACACGGTCCACAGCTCATC	TGCCTCCAAAGTATGTCAATCA
SOD2	AAGTACCAGGAGGCGTTGG	TGAACTTCAGTGCAGGCTGA
SOD3	GGTGCAGCTCTCTTTTCAGG	AACACAGTGGCGCCAGCATGGCC
GPX1	TCTGTTGCTCGTAGCTGCTG	CTATGGATCACCGGGATTTTG
GPX4	TTTCCGTGTAACCAAGTTCG	TGCCTCCAAAGTATGTCAATCA
PRDX1	AATGCAAAAATTGGGTATCCTGC	CCGTGGGACACACAAAAGTAAA
NQO1	GAAGAGCACTGATCGTACTGGC	GGATACTGAAAGTTCGCAGGG

Table 5 List of mouse qPCR primers.

Gene	Forward (5' – 3')	Reverse (5' – 3')
NOX4	TGCCTGCTCATTTGGCTGT	CCGGCACATAGGTAAAAGGATG
NRF2	CCTAGTTCTCCGCTGCTGGG	CCATGTCCTGCTGGGACTGTA
SOD2	CAGACCTGCCTTACGACTATGG	CTCGGTGGCGTTGGATTGTT
SOD3	ACCGGCTTGGTTCTCTTCC	CTCCATCGGGTTGTAGTGCG
GPX1	AATGTCGCGTCTCTCTGAGG	TCCGAACTGATTGCACGGG
GPX4	GATGGAGCCCBTTCCTGAACC	CCCTGTAATTATCCAGGCAGA

Table 6 List of human and mouse housekeeping qPCR primers.

Gene	Forward (5' – 3')	Reverse (5' – 3')
18S	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG
RPL27	GCTGGTTATCGGGAGTTGG	ACTGGCCTGGTGTCTAGAG

2.23 Statistical analysis

Statistical differences were evaluated with two-tailed Mann–Whitney U-test or one-way ANOVA with SPSS software where appropriate. P-values <0.05 indicate statistical significance.

Chapter 3: Hyperglycemia dampens the sensitivity of keratinocytes to acetylcholine- induced epithelial-mesenchymal transition

TAN, MWY, KONG, ZQ, TAN, WR, TOH, JH, Wee, JWK, TEO, EML, WANG, XM and TAN, NS (2020). High glucose restrains acetylcholine-induced keratinocyte EMT is mitigated by p38 inhibition. (in revision)

3.1 Introduction

Peripheral diabetic neuropathy is a complication caused by chronically elevated blood sugar levels and diabetes (Boulton et al., 2004, Nickerson, 2014) that induces nerve damages in body extremities, resulting in clinical presentations of numbness and loss of sensation. Consequently, many diabetic patients who sustained trauma are often unaware of the injury and therefore do not receive timely, appropriate treatment. Although approximately 50 % of diabetics are expected to suffer from peripheral neuropathy within their lifetime (Kumar et al., 1994), there is no cure for peripheral neuropathy, and current standards of care are often inconsistent in symptom reliefs and possibly entail adverse effects.

Diabetic neuropathy can be life-threatening, as it tremendously increases the risk of developing foot ulcers and can lead to amputation, after which they are subjected to a 5-year survival rate of approximately 40 % (Tesfaye et al., 2010; Singh et al., 2014; Huang et al., 2018). Even if patients manage to recover, there is a high recurrence rate of 66 %, with a 12 % higher likelihood to result in amputation (Sen et al., 2009). In addition to the high medical and socioeconomic costs, it has a profound effect on the quality of life that diabetic patients incur from disability due to foot ulcers. Although these clinical realities have been appreciated for decades, our understanding and ability to treat diabetic wounds remain unacceptably deficient.

Wound re-epithelialization is a cooperative multifactorial process, dominated by cell migration, proliferation, and differentiation that restores the

intact epidermal barrier to prevent infection and excessive moisture loss. The cellular and molecular processes involved in the initiation, maintenance, and completion of re-epithelialization are essential for successful wound closure. However, in wounds that exhibit impaired wound healing, such as nonhealing diabetic wounds, the re-epithelialization process has failed (Medina et al., 2005; Chen et al., 2016; Rousselle et al., 2019). It has been speculated that the initiation of re-epithelialization is similar to the EMT process observed during embryogenesis and cancer metastasis (Yan et al., 2010; Haensel and Dai, 2018). However, studying this process in wound healing has been technically challenging because only a small number of keratinocytes at the epithelial tongue underwent EMT even for normal healing wounds (Usui et al., 2008). Thus, molecular insights of EMT in keratinocytes during re-epithelialization, and how EMT affected diabetic keratinocytes remains unknown.

It is well recognized that peripheral neuropathy is a major risk factor for poor wound healing; however, these two debilitating complications of diabetes were often studied independently. Numerous studies speculated that the alleviation of one tends to improve the other (Cameron and Cotter, 1997; Schratzberger et al., 2000). In support of this notion, it was documented that all phases of wound healing are impaired in denervated tissue (Barker et al., 2006). Acetylcholine is a well-established neurotransmitter in cholinergic neurotransmission. However, there is little emphasis on its non-neuronal functions which involve the activation of cholinergic signaling in non-neuronal cells. Emerging data have revealed a causal role for ACh in normal wound healing. Keratinocytes, the major cell type involved in re-epithelialization, have a functional cholinergic system which is suggestive of their responsiveness to

ACh (Hana et al., 2007; Kurzen et al., 2007). Several important functions in keratinocytes such as cell viability, proliferation, adhesion, migration, and differentiation, have been attributed to the non-neuronal cholinergic system, particularly muscarinic receptors (Beck et al., 2006; Uberti et al., 2017). Recently, a low dose of ACh was shown to accelerate healthy wound healing in mice (Uberti et al., 2018). However, the precise mechanism by which ACh modulates keratinocyte behavior and improves wound healing remains obscure. The effect of ACh on diabetic wounds is also unclear, emphasizing the need for a better understanding of the non-neuronal roles of ACh.

Taken together, the abovementioned studies suggest an association between the neurological system and wound healing at a molecular level which remains yet unknown. The emergence of non-neuronal roles of ACh on keratinocytes suggests the involvement of ACh as the bridge that links both diabetic neuropathy and diabetic ulcers. However, there is no studies so far that examined whether there is a dysregulation in ACh signaling in diabetic keratinocytes, or the detailed mechanism by which ACh regulates keratinocyte activity especially in the context of the EMT process during re-epithelialization.

In this study, we aim to determine the signaling pathway/s that initiate EMT in keratinocytes during re-epithelialization and to elucidate how these signaling cascades are dysregulated in diabetic wounds. Using gene expression microarray and gene ontological analyses of diabetic and healthy keratinocytes, we reveal that genes related to the neurological system/neurotransmitter activity are enriched, thus establishing a relationship between the neurological system and impaired keratinocyte migration. Amongst these genes were genes involved in ACh metabolism. We further show that ACh levels in healthy wounds

microenvironment gradually increases over time, but remains consistently high in diabetic wounds. Finally, we identified the mechanism by which ACh stimulates EMT and that a hyperglycemic environment dampens cellular responses to ACh, i.e. ACh resistance.

3.2 Results

3.2.1 Enrichment of neurotransmitter-related genes in keratinocytes undergoing EMT.

Epithelial-mesenchymal transition is a critical and early event in re-epithelialization, during which otherwise stationary keratinocytes migrate across the wound bed to restore the epidermal barrier. Re-epithelialization is activated at the earliest stages of wound healing, thus, to understand if delayed wound closure in diabetes can be attributed to differences in EMT, we examined high fat high fructose chow induced diabetic and healthy mice full-thickness excisional wounds at day 1-3 post-injury. Wound EMT, as indicated by a reduced E-cadherin and increased Snai1 staining, was observed as early as day 2 post wounding (**Fig 7A-B**). In contrast, wound EMT was significantly attenuated in diabetic wounds compared with normal wounds (**Fig 7A-B**). This observation suggests that impaired EMT of wound keratinocytes was associated with poor diabetic wound healing.

Figure 7. Impaired keratinocyte EMT in diabetic wounds. (A) Representative images of immunostaining for E-cadherin (green) and Snai1 (red) in the epithelial tongue of wild type and diabetic wounds at day 1-3 post injury. Hoechst 33342 was used as a nuclear counterstain. The cognate H&E images were also shown. (n = 9 mice for each group). Scale bars: 100 μ m. **(B)** Percentage of cells undergoing EMT as depicted by loss of E-cadherin staining (left panel) in wound tip (≤ 100 μ m) and wound surface area occupied by these cells (right panel) of wild type and diabetic wounds at day 1-3 post injury.

Data information: Data in **(B)** were expressed as means $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$ (Mann-Whitney test).

To gain further insight into the molecular mechanism of wound-associated EMT, we laser-microdissected keratinocytes undergoing EMT from wild type

and diabetic wounds, as guided by reduced E-cadherin staining, and performed gene expression microarray analysis (**Fig 8A**). Gene ontology for cellular functions and enrichment analysis of differentially expressed genes revealed that cellular processes involved in re-epithelialization such as cell proliferation, migration, cell-cell communication as well as receptor-mediated signaling were enriched in genes involved in neurotransmitter signaling (**Fig 8B**). Further interrogation revealed an enrichment of genes involved in ACh metabolism, indicating that dysregulated ACh activity could potentially be causing the impaired EMT observed in diabetic wounds.

A

B

Figure 8. Enrichment of neurotransmitter-related genes being differentially expressed in wild type and diabetic keratinocytes undergoing EMT. (A) Representative images of day 3 wounds obtained from

wild type and diabetic mice before and after LCM. Immunohistochemical staining of E-cadherin was identify keratinocytes undergoing EMT. (n = 9 mice for each group). Scale bars: 75 μ m. **(B)** Gene ontology analysis for differential expressed genes in laser-captured keratinocytes undergoing EMT from day 3 wild-type and diabetic wounds. Numbers in brackets denote the respective enrichment scores.

3.2.2 Topical ACh accelerates EMT in normal wounds but not diabetic wounds.

To examine if ACh can modulate EMT, we topically treated normal wild-type and diabetic wounds with exogenous ACh. As expected, vehicle-treated normal (wild-type) wounds were smaller than cognate diabetic wounds. Normal wounds treated with 2.5 nM of ACh showed the smallest wound area when compared with vehicle treatment (**Fig 9**). We observed more keratinocytes in ACh-treated wounds that underwent EMT compared to vehicle-treated wounds as revealed by immunostaining for E-cadherin and Snai1 (**Fig 10A-B**). Similarly, the total surface area of wound EMT was also greater than vehicle-treated control wounds (**Fig 10B**). Unexpectedly, exogenous ACh did not facilitate wound EMT in diabetic wounds when compared with vehicle-treated diabetic wounds (**Fig 9-10**).

Figure 9. ACh accelerates rate of wound closure in wild type but not diabetic wound. Representative macroscopic images of wild type and diabetic mice wounds before (Day 0) and after (Day 4) topical treatment of vehicle (PBS) or ACh. (n = 9 mice for each group). Graph showed percentage of wound closure with respect to Day 0 wounds.

Data information: Data were expressed as means $\pm$ SEM. *** $p < 0.001$ (Mann-Whitney test). n.s. denotes not significant.

Figure 10. ACh accelerates EMT in the wild type but not diabetic wound.

(A) Representative images of immunostaining for E-cadherin (green) and Snai1 (red) in the epithelial tongue of wild type and diabetic wounds at day 1-3 post injury with and without ACh treatment. Hoechst 33342 was used as a nuclear

counterstain. The cognate H&E images were also shown. Scale bars: 100 μ m. **(B)** Percentage of cells undergoing EMT as depicted by loss of E-cadherin staining (left panel) in wound tip (≤ 100 μ m) and wound surface area occupied by these cells (right panel) of vehicle- and ACh-treated wild type and diabetic wounds at day 1-3 post injury.

Data information: Data in **(B)** were expressed as means $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

Genes involved in ACh metabolism were enriched in our gene expression analysis. Thus, we hypothesize that the reduced response of diabetic keratinocytes to ACh may be due to the rapid degradation of ACh in diabetic wound milieu. We did not observe any difference in the mean acetylcholine esterase activity, a key enzyme in the catabolism of ACh, between wild type and diabetic wounds (**Fig 11A**). However, we detected a higher cumulative mean concentration of ACh in diabetic compared with wild type wound fluids 24 h after injury (**Fig 11B**). The level of ACh was elevated in diabetic wounds as early as 4 h post-injury and continued to increase over 24 h. On the other hand, the ACh level in normal wounds increased over the first 20 h of injury, albeit at a lower level (**Fig 11C**). To take the metabolic flux of ACh into account, we also determined the ratio of ACh and total choline in the wound fluid in the presence of an inhibitor of acetylcholine esterase. A higher ratio of ACh:total choline was detected in diabetic than in wild type wound fluids through the 24 h post-injury (**Fig 11D-E**), suggesting that the higher ACh in diabetic wound fluids was due to higher production of ACh. The high endogenous level of ACh in the diabetic wound was likely a compensatory response to the ACh resistance. Indeed, the ACh in wound fluids and ACh:total choline ratio were higher after STZ-induced diabetes than before STZ exposure (**Fig 11F-G**). Consistent with the diabetic

wound fluids, primary human keratinocytes (Ker-CT) conditioned HG (25 mM) media had a significantly higher level of ACh when compared with conditioned low glucose (LG) (5mM) media (**Fig 11H**). Our observation suggests that keratinocytes in normal wounds were responsive to ACh, whereas diabetic keratinocytes were refractory to either exogenous or high endogenous ACh, i.e. ACh resistance.

Figure 11. Levels of ACh and acetylcholine esterase activity in wound fluids of wild type and diabetic mice. (A) Mean endogenous acetylcholine esterase activity in wound fluids obtained 24 h post wounding. **(B)** Mean endogenous ACh levels in wound fluids obtained 24 h post wounding. **(C)** Mean endogenous ACh levels in wound fluids obtained at 4 h intervals over a period of 24 h. **(D)** Ratio of ACh:total choline in wound fluids obtained 24 h post wounding. **(E)** Ratio of ACh:total choline in wound fluids obtained at 4 h intervals over a period of 24 h. **(F)** Mean concentration of ACh in the wound fluids of mice before and after STZ-induced diabetes. **(G)** Ratio of ACh:total choline in the wound fluids of mice before and after STZ-induced diabetes. **(H)** Ratio of ACh:total choline in conditioned medium from Ker-CT cells cultured in normoglycaemic (LG 5 mM) and hyperglycaemic (HG 25 mM) conditions. Data information: n = 6 mice for each group. Data in **(A-H)** were expressed as means $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

3.2.3 ACh stimulates keratinocytes EMT in normoglycemic conditions.

Our above finding suggests that ACh stimulated EMT in normal wounds. To further confirm that ACh can induce EMT in keratinocytes, we used live-cell imaging to monitor the response of human keratinocytes (Ker-CT) and HaCaT cells cultured in normoglycemic conditions of 5 mM glucose (LG) to ACh. Vehicle and choline, a by-product of ACh breakdown, served as negative controls, whereas TGF β 1 served as a positive control of EMT. Regardless of Ker-CT or HaCaT cells, TGF β 1 treatment for 24 h upregulated and downregulated the expression of mesenchymal and epithelial markers of EMT, respectively, at the RNA and protein levels when compared to vehicle-treated control (**Fig 12A-B**). At LG condition, cells treated with ACh underwent incomplete EMT after 24 h (**Fig 12A-B**). An increase in cell migration and

forward cell movement were readily detected despite this partial response (**Fig 13A-B**). Choline-induced cells did not display any significant differences in EMT markers from the uninduced cells (**Fig 12C**).

Next, we observed that the effects by ACh and TGFβ1 on EMT markers and associated phenotypic changes were severely reduced in cells cultured in HG medium (**Fig 12A, 13A-B**). These observations suggest that ACh indirectly stimulates EMT and that HG condition attenuates the response of the cells to EMT stimuli, i.e. ACh resistance.

Figure 12. ACh and TGFβ1 induce EMT in keratinocytes under normoglycemic, but not hyperglycemic, conditions. (A) Heat map showing the upregulated (red) and downregulated (green) of EMT-related genes in HaCaT (left panel) and Ker-CT (right panel) cells after exposure to ACh or TGFβ1 for 24 h in normoglycemic (LG 5 mM) and hyperglycaemic (HG 25 mM) conditions as determined by qPCR. **(B)** Representative immunoblots of E-

cadherin and Snai1 from HaCaT and Ker-CT cells exposed to ACh or TGFβ1 in LG (5 mM) and HG (25 mM) conditions. β-tubulin serves as a loading control from the same samples. **(C)** Heat map showing the expression changes to EMT-related genes (right panel) in HaCaT and Ker-CT cells after exposure to choline for 24-48 h in normoglycemic (low glucose 5 mM) condition as determined by qPCR. Representative immunoblots of Snai1 (left panel) from HaCaT and Ker-CT cells exposed to choline in low glucose condition. β-tubulin serves as a loading control from the same samples.

Data information: Data in **(A-C)** were expressed as means ± SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

Figure 13. ACh and TGFβ1 increase the motility of keratinocytes under normoglycemic, but not hyperglycemic, conditions. (A) Graphs showing the cumulative distance travelled by HaCaT (left panel) and Ker-CT (right panel)

cells as quantified using live cell imaging over 24 h following exposure to either ACh (blue) or TGF β 1 (red) in low glucose (solid circles) and high glucose (open circles). (n = 15 cells for each group). **(B)** Cell tracking of HaCaT (left panel) and Ker-CT (right panel) cells using live cell imaging over 24 h after following exposure to conditions in (A) Displacement of each cell is indicated by the straight lines, and the radius of the circles indicate the average displacement of the cells of that treatment group. (n = 15 cells for each group).

Data information: Data in **(A)** were expressed as means $\pm$ SEM. ****** $p < 0.01$, ***** $p < 0.05$ (Mann-Whitney test).

3.2.4 ACh increases TGF β RII to potentiate EMT in keratinocytes.

TGF β 1 has the broadest spectrum of effects on wound healing and is also a potent EMT stimulus. We hypothesize that ACh indirectly stimulates EMT via the TGF β 1 pathway. To this end, we examined the effect of ACh on the expression TGF β 1 and its receptor TGF β RII. An increase in TGF β RII mRNA and higher surface expression of TGF β RII as confirmed by flow cytometry were detected in ACh-treated LG Ker-CT cells (**Fig 14A**). Despite the high endogenous or ectopic exogenous ACh, HG-treated keratinocytes did not respond to ACh as depicted by the lack of increase in TGF β RII mRNA and protein, consistent with the notion of ACh resistance (**Fig 14B**). Low glucose and HG culture condition per se did not alter the surface expression of TGF β RII (**Fig 14C**).

Figure 14. ACh upregulates TGFβRII. (A) Relative gene expression of TGFβ1, TGFβRII and surface expression of TGFβRII in vehicle- (blue) and ACh-treated (red) Ker-CT in low glucose (5 mM). (B) Relative gene expression of TGFβ1, TGFβRII and surface expression of TGFβRII in vehicle- (blue) and ACh-treated (red) Ker-CT in high glucose (25 mM). (C) Histogram showing the surface expression of TGFβRII in Ker-CT cells exposed to normoglycemic (blue) and hyperglycemic conditions (red).

Data information: Data in (A-C) were expressed as means ± SEM. *** $p < 0.001$ (Mann-Whitney test). n.s. denotes not significant.

Notably, Ker-CT cells exposed to ACh followed by TGF β 1 (ACh-TGF β 1) exhibited more robust changes in the expression of EMT markers and a significantly higher level of phosphorylated mothers against decapentaplegic homolog 3 (Smad3) compared with ACh or TGF β 1 alone, suggesting enhanced TGF β 1 signaling (**Fig 15A-B**). In concordance, cells treated with ACh-TGF β 1 showed more forward cell movement compared with treated ACh or TGF β 1 alone (**Fig 15E**). Consistent with the above findings, HG-treated keratinocytes have diminished TGF β 1 signaling, changes in the EMT marker expression profile, and motility (**Fig 15C-E**).

Figure 15. ACh upregulates TGFβRII to sensitize keratinocytes to TGFβ1-mediated EMT. (A) Relative fold change in mRNA of Snai1 and E-cadherin levels in Ker-CT, subjected to indicated treatments, cultured in low glucose conditions. **(B)** Relative fold change in protein levels of p-SMAD, Snai1 and E-cadherin in Ker-CT, subjected to indicated treatments, cultured in LG conditions. Representative immunoblots of pSMAD, total SMAD (t-SMAD), Snai1 and E-

cadherin are shown. β -tubulin serves as a loading control from the same samples. **(C)** Relative fold change in mRNA of Snai1 and E-cadherin levels in Ker-CT, subjected to indicated treatments, cultured in HG conditions. **(D)** Relative fold change in protein levels of p-SMAD, Snai1 and E-cadherin in Ker-CT, subjected to indicated treatments, cultured in high glucose conditions. Representative immunoblots of pSMAD, total SMAD (t-SMAD), Snai1 and E-cadherin are shown. β -tubulin serves as a loading control from the same samples. **(E)** Cell tracking of Ker-CT cells using live cell imaging over 24 h after following exposure to indicated conditions. Displacement of each cell is indicated by the straight lines, and the radius of the circles indicate the average displacement of the cells of that treatment group. (n = 15 cells for each group). Data information: Data in **(A-D)** were expressed as means $\pm$ SEM. $**p < 0.01$, $*p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

To verify the effect of ACh in vivo, we stained wound epithelial tongues of ACh- and vehicle-treated wild-type wounds, as well as diabetic wounds, for phospho-Smad3. We observed more keratinocytes at the tip of day 2 and 3 wounds were positive for phospho-Smad3 in ACh-treated wild-type wounds compared with vehicle-treated wild-type wounds (**Fig 16**). Despite the elevated endogenous ACh in diabetic wounds, phospho-Smad3 positive wound keratinocytes were only evident at day 3 post-injury, indicating delayed activation of TGF β 1 signaling.

Figure 16. Exogenous ACh promotes SMAD3 activation in wild type, but not diabetic, wounds. Representative images of immunostaining for pSMAD (green) in the epithelial tongue of wild type and diabetic wounds at day 1-3 post injury with and without ACh treatment. Hoechst 33342 was used as a nuclear counterstain. The cognate H&E images are also shown. hf indicates hair follicle. Dotted lines indicate the basal membrane. Scale bars: 100 μ m. (n = 6 mice for each group).

The above observations raise the question of how ACh increases the expression of TGF β RII to enhance TGF β 1-mediated EMT. We reasoned that kinase inhibitors that negated the ACh-mediated up-regulation of TGF β RII mRNA transcripts indicate the involvement of those specific kinases in the signaling cascades. Conversely, kinase inhibitors that did not attenuate the ACh-mediated up-regulation of TGF β RII would suggest that those kinases were not involved (**Fig 17A**). We subjected ACh-stimulated LG Ker-CT cells to an unbiased kinase inhibitor array screen to identify the signaling pathways triggered by ACh. TGF β RII mRNA was increased by ACh within 6 h in LG (**Fig 17B**). Our results showed that inhibitors against 13 targeted kinases attenuated the ACh-mediated increased in TGF β RII transcription (**Fig 17B**). Pathway analysis revealed Src and ERK as key signaling nodes that resulted in the activation of transcription factors, such as p53 and MYC proto-oncogene, BHLH transcription factor (Myc), required for the upregulation of TGF β RII by ACh (**Fig 17D**). Immunoblot analysis confirmed the enhanced phosphorylation of Src and ERK1 upon ACh treatment (**Fig 17B**).

A

Low glucose (5 mM)

B

Low glucose (5 mM)

C

Low glucose (5 mM)

Figure 17. ACh upregulates TGFβRII via the Src-Erk1/2 pathway. (A) A schematic diagram of the rationale behind the kinase inhibitor screen, which led to mapping the IPA pathways in Panel C. Possible outcomes of the kinase inhibitor screen of Ker-CT cultured in low glucose conditions during ACh-mediated upregulation of TGFβRII. **(B)** Relative fold change in TGFβRII mRNA in ACh-treated Ker-CT cells under low glucose conditions after the kinase inhibitor screen as determined by qPCR. Kinases involved in ACh-mediated upregulation of TGFβRII (red) are shown. Uninvolved kinases (black) and vehicle-treated (green) Ker-CT are also shown. Representative immunoblots of total and phosphorylated SRC and ERK1/2 in vehicle- and ACh-treated Ker-CT cells are shown. **(C)** Ingenuity pathway analysis showing the identified kinases (red) involved in the ACh-mediated upregulation of TGFβRII.

Data information: Data in **(B)** were expressed as means ± SEM. ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test).

In summary, we showed that ACh increases the expression of TGFβRII via Src-ERK mediated pathway in LG keratinocytes. These ACh-treated LG keratinocytes have enhanced TGFβ1 signaling and EMT markers. In contrast, TGFβRII expression was not increased by ACh in HG keratinocytes.

3.2.5 High glucose condition activates p38 pathway to attenuate cellular response to ACh.

We showed above that HG keratinocytes produced more ACh than LG keratinocytes (**Fig 11H**). Unlike LG keratinocytes, HG keratinocytes did not respond to ACh-mediated EMT. To decipher the mechanism underlying this ACh resistance, we performed another kinase inhibitors screen using HG Ker-CT. In this instance, kinase inhibitors that alleviate the suppressive effect of HG on ACh-mediated upregulation of TGF-βRII mRNA indicates that these kinases

are involved. Conversely, kinase inhibitors that did not diminish the impact of HG for the upregulation of TGF- β RII by ACh would suggest that those kinases were not involved (**Fig 18A**). Our results showed that inhibition of three kinases resulted in the upregulation of TGF β RII by ACh (**Fig 18B**). Pathway and immunoblot analyses revealed that HG activated the p38 MAPK pathway, which integrated with ACh-Src-ERK pathway, to suppress keratinocyte response to ACh (**Fig 18B-C**).

Figure 18. HG activates p38 MAPK. (A) A schematic diagram of the rationale behind the kinase inhibitor screen, which led to mapping the IPA pathways in Panel C. Possible outcomes of the kinase inhibitor screen of Ker-CT cultured in HG conditions during ACh-mediated upregulation of TGF β RII. **(B)** Relative fold

change in TGF β RII mRNA in ACh-treated Ker-CT cells under HG conditions after the kinase inhibitor screen as determined by qPCR. High glucose stimulates kinases involved in suppressing ACh-mediated upregulation of TGF β RII are indicated in blue. Uninvolved kinases (black) and vehicle-treated (green) Ker-CT are also shown. Representative immunoblots of total and phosphorylated p38 kinase in vehicle- and ACh-treated Ker-CT cells cultured in low (5 mM) and high (25 mM) glucose are shown. **(C)** Ingenuity pathway analysis showing the identified kinases (blue) involved in HG suppression of the ACh-mediated upregulation of TGF β RII. Blue lines indicate the possible mechanisms by which p38 MAPK exerts its repressive effects.

Data information: Data in **(B)** were expressed as means $\pm$ SEM. * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

We rationalize that ACh can activate Src-ERK pathway and increases TGF β RII in HG Ker-CT to facilitate EMT if p38 kinase was inhibited. Western blot analysis showed that HG Ker-CT treated with solamargine, a p38 kinase inhibitor, have higher pERK1/2, pMEK1/2 and increased surface expression of TGF β RII (**Fig 19A-B**). The cotreatment of solamargine with TGF β 1 facilitated EMT as evidenced by changes in the EMT markers, Snai1 and E-cadherin, and increased motility (**Fig 19C-D**). In summary, HG restrains keratinocytes to ACh-induced EMT which is mitigated by the inhibition of p38 kinase.

Figure 19. High glucose restrains ACh-induced EMT in keratinocytes via p38 MAPK. (A) Representative immunoblots of total and phosphorylated SRC, ERK1/2 and MEK1/2 in vehicle and p38 MAPK inhibitor (solamargine) treated Ker-CT cultured in high glucose (25 mM) conditions. **(B)** Relative gene expression of TGFβRII and surface expression of TGFβRII in vehicle- and p38 inhibitor-treated Ker-CT under hyperglycemic conditions. **(C)** Representative immunoblots of pSMAD, SMAD, Snai1 and E-cadherin in high glucose treated Ker-CT subjected to indicated treatments. **(D)** Cell tracking of Ker-CT cultured in high glucose cells using live cell imaging over 24 h after following exposure

to indicated treatments. Displacement of each cell is indicated by the straight lines, and the radius of the circles indicate the average displacement of the cells of that treatment group. (n = 15 cells for each group).

Data information: Data in **(A-C)** were expressed as means $\pm$ SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

3.3 Discussion

As the most abundant neurotransmitter, ACh is involved in almost all aspects of diabetes and its complications. For example, lower serum insulin level, along with reduced glucose tolerance, was detected in M3 muscarinic receptor knockout mice (Duttaroy et al., 2004). Similar findings were made in mice with a conditional deletion of M₃ muscarinic receptor gene in the pancreatic β -cells (Gautam et al., 2007). In the same study, the overexpression of M3 muscarinic receptors specifically in the pancreatic β -cells led to an increase in glucose tolerance and insulin production, underscoring the importance of the M3 muscarinic receptor in the secretion of insulin in β -cells. Acetylcholine also regulated the β -cell survival and mass through the $\alpha 7$ nicotinic ACh receptor (Gupta et al., 2018). Dysregulated ACh activity was also found to exist in the brain, heart and vasculature for diabetic rats (Akiyama et al., 1989; Welsh and Wecker, 1991; Ozansoy et al., 1993; McNally et al., 1994; Ebuehi and Dibia, 2015).

Diabetic neuropathic injuries represent a serious health care burden to patients and society. Although the management of chronic diabetic wounds has improved in recent years, it remains a frustrating problem for clinicians and caretakers. A couple of studies have shown that low doses of ACh can improve healthy wound healing through the activation of migration and proliferation of

keratinocytes *in vitro*, and an increased rate of wound closure *in vivo* (Uberti et al., 2017; Uberti et al., 2018). However, the precise mechanism and its role in diabetic wound remain unclear. These studies proposed a non-neuronal role for ACh on keratinocyte migration and proliferation (Słoniecka et al., 2015; Uberti et al., 2017). The finding that keratinocytes have a functional cholinergic system lends support to this hypothesis (Grando, 1997).

In our study, we used LCM to isolate keratinocytes undergoing EMT, followed by a combination of gene expression microarray and gene ontology analysis. We observed that there was an enrichment of neurologically related genes in the migration and signaling of these keratinocytes. These findings highlight a strong relationship between neuropathy and the migratory capacity of keratinocytes at the tip of the epithelial tongue during re-epithelialization. Our study highlights the high incidence rate of 85 % of diabetic ulcer cases that have underlying neuropathy (Boulton, 2008) and supports an impaired wound healing in denervated tissue (Barker et al., 2006). “Neurotransmitter” was the most commonly observed group to appear in most of the ontologies relevant to our analysis. Further interrogation into the group showed gene changes in AChR and AChE – the enzyme responsible for the degradation of ACh. Since prior studies conducted have revealed the capacity of ACh to instigate migration of keratinocytes both *in vivo* and *in vitro* (Uberti et al., 2017; Uberti et al., 2018) in non-diabetic condition, we first measured the innate levels of ACh in diabetic and non-diabetic wounds in the first 24 h period post-injury. In healthy wounds, the level of ACh in wound fluids begun to increase and plateau by 24 h. The early onset of ACh production post-wounding indicated a possible link between ACh and TGFβ1, which is a potent inducer of EMT and an early player of wound

healing. Fibroblasts isolated from chronic wounds and placed into culture are insensitive to TGF β 1 due to lowered levels of TGF β RII (Hasan et al., 1997). Thus, we investigate if the expression of TGF β RII expression can be modulated by ACh. In healthy wounds i.e. normoglycemic condition or LG, ACh upregulated the expression of TGF β RII in LG keratinocytes via the Src-ERK pathway. TGF β 1 activated Smad signaling to trigger EMT. The addition of ACh with exogenous TGF β 1 further enhanced the Smad3 activation, accompanied by significant changes in Snai1 and E-cadherin levels. There were also more keratinocytes at the tip of the wound tongue expressing p-Smad3. Taken together, we showed that ACh encourages EMT in keratinocytes *in vitro* and *in vivo*.

Our study showed that a hyperglycemic wound microenvironment could significantly attenuate the responsiveness of keratinocytes at the tip of diabetic wound tongues to ACh. We detected significantly higher ACh levels in diabetic wound fluids over 24 h post-injury when compared with wild-type wounds. Keratinocytes maintained in HG condition similarly produced more ACh into the medium. In contrast to LG keratinocytes, ACh was unable to increase TGF β RII in HG keratinocytes. The lower surface expression of TGF β RII led to reduced TGF β 1-mediated signaling and delayed EMT. Consistent with delayed EMT at the epithelial tongue of diabetic wounds, the response to TGF β 1 in diabetic wounds was muted as a result of reduced TGF β 1 and TGF β RII expression when compared with normal wounds (Bitar and Labbad, 1996; Jude et al., 2002; Kim et al., 2003; Al-Mulla et al., 2011). We observed fewer keratinocytes at the tip of the diabetic wound tongue expressing phosphorylated Smad3 than normal wounds treated, or not, with ACh. This observation again underlines ACh

resistance as a phenomenon in diabetic keratinocytes. Analogous to insulin resistance in diabetes, keratinocytes in a HG condition secreted more ACh, and yet they were unresponsive to ACh-mediated upregulation of TGF β RII. We further showed that HG stimulates the activation of p38 kinase pathway, which interferes with the Src-ERK cascade. Thus, this suppressive effect by HG must be resolved for ACh to participate in the activities important for wound re-epithelialization. Indeed, the inhibition of p38 kinase by solamargine sensitized HG keratinocytes to endogenous ACh and TGF β 1-mediated EMT. Our finding was in line with another group that showed the inhibition of p38 kinase led to improved wound healing at the macroscopic and histological level, although the group did not explore the mechanism behind their observation (Medicherla et al., 2009).

Recombinant PDGF-BB became the first and remained the only pharmacological biologics approved by FDA in 1997 for treatment of a chronic wound condition. Prior to approval, clinical trials using recombinant human PDGF-BB on diabetic foot ulcer and pressure ulcers, improved the median reduction in wound area by a significant 20 - 50 % more than those with placebo (Robson et al., 1992; Mustoe et al., 1994; Steed, 1995). In those studies, doses below 1 $\mu\text{g}/\text{cm}^2$ PDGF-BB was found to be ineffective in improving wound healing, which was two orders of magnitude higher than the physiological average of 500 pg/mL PDGF-BB detected in tears during corneal wound healing (Vesaluoma et al., 1997) or 13-21 pg/mL in skin wound exudate (Zhang et al., 2014). Numerous growth factors and cytokines have been shown to improve diabetic wound healing, however, their clinical adoption has generally been disappointing (Tsang et al., 2003; Okonkwo and DiPietro, 2017; Zubair and

Ahmad, 2019). It is conceivable that the elevated proteases in the wound microenvironment led to rapid degradation of these growth factors, or that cells downregulated the expression of their receptors. However, topical application of large concentrations of these growth factors inevitably increase the risk of cancer and other side effects (Yamakawa and Hayashida, 2019). It is tempting to speculate that hyperglycemic conditions exert similar suppressive impacts on other pro-healing growth factors. It is also conceivable that modulators that mitigate HG's effects can be potential adjunctive therapeutic agents. This finding has wide-ranging implications on the dose, duration, safety and efficacy of these growth factors to improve chronic diabetic wounds. In our study, adjunctive use of solamargine with TGF β 1 already accelerated wound healing, mitigating the side effects that accompanied high concentration of exogenous TGF β 1 required during diabetic wound therapies. Whether solamargine result in better clinical outcome in healthy non-diabetic wounds remains to be explored.

Chapter 4: PPAR β / δ deficiency in fibroblasts delays tumor initiation in keratinocytes by promoting antioxidative responses

TAN, MWY, SNG, MK, CHENG, HS, LOW, ZS, LEONG, BJJ, CHUA, D., TAN, EHP, CHAN, JSK, YIP, YS, LEE, YH, PAL, M, Wang, XM, and WAHLI, W and TAN, NS, (2020) Deficiency in fibroblast PPAR β/δ reduces non-melanoma skin cancers in mice. ***Cell Death & Diff.*** (in press)

4.1 Introduction

The incidence of NMSC, comprising of BCC and SCC, has been increasing worldwide (Leiter et al., 2004). Cutaneous squamous cell carcinoma (cSCC) is the second most common form of skin cancer, with an estimated 1 million cases treated in the United States each year (Rogers et al., 2010). Although most cSCCs are localized and can be easily treated, approximately 5 % of patients will experience local recurrence, 4 % will develop nodal metastases, and up to 2 % will die of the disease (Schmults et al., 2013). In addition to the small but significant number of deaths attributed to cSCC each year, the disease and its associated precancerous skin lesions contribute to a large financial burden of more than \$4.5 billion annually in the United States (Lim et al., 2017). Skin cancer is one of the top ten most frequent cancers in Singapore. There has been a general rising trend in the incidence of this cancer in both men and women over the last 40 years. According to the Trends in Cancer Incidence in Singapore 2011-2015 (Singapore Cancer Registry), there are 1,822 cases of skin cancer and 1,404 in Singapore men and women yearly respectively. Skin cancer ranks 6th in male cancers and 7th in female cancers in Singapore (Sng et al., 2009).

The development of NMSC is not a cell-autonomous process. Tumor-associated stromal cells in the reactive tumor microenvironment (TME) are now recognized as an important modulator and even a driver of tumorigenicity (Chan et al., 2018; Liao et al., 2018). Cancer-associated fibroblasts, which differ from normal fibroblasts, are the dominant stromal cells within the TME of many

tumors (Chan et al., 2017). These CAFs reinforce the pro-tumorigenic communication with adjacent tumor cells, exacerbating the hallmarks of cancer and accelerating tumor malignancy (Chan et al., 2018; Liao et al., 2018). Recent transcriptional profiling of the CAFs from clinical cutaneous SCCs revealed a unique signature profile of nuclear hormone receptors (NRs) (Chan et al., 2018; Cheng et al., 2019). The study showed that targeting specific NRs in CAFs with cognate ligands as concurrent therapy can inhibit the emergence of chemoresistant tumors (Chan et al., 2018). So far, most studies have focused on the CAFs in the TME of pre-existing tumors, and it has been found that CAFs can modulate important changes in the TME such as immunosuppression and angiogenesis, both of which are important factors for tumor progression (Ahmadzadeh and Rozenberg, 2005; Feig et al., 2013). One recent study further highlighted the importance of CAFs in cancer development by illustrating their ability of promoting metastasis (Kashima et al., 2019). However much less is known about the communication between normal fibroblasts and epithelia, whose disruption affects tumor initiation and the latency period in emergence of tumors. In fact, currently fibroblasts dysregulation, such as fibrosis, increases the risk of tumor formation in the area where the fibrosis is located, such as lungs, liver and breasts (Zhao et al., 2010; Uehara et al., 2013; Cox and Eler; 2014). Delineating the specific mechanism that mediates this epithelial-mesenchymal communication that contribute to NMSC could identify more effective targeted therapies to inhibit and/or prevent this disease.

Peroxisome proliferator-activated receptors are NRs that have attracted attention due to their crucial role in lipid homeostasis and tissue repair. Peroxisome proliferator-activated receptors are highly amenable for therapeutic

intervention because their activities can be directly modulated by agonists and antagonists (Grygiel-Gorniak, 2014). The PPAR β/δ isotype has been implicated in NMSC development, but its functions remain controversial as both oncogenic and tumor-suppressive roles have been reported (Kim et al., 2006; Montagner et al., 2014; Peters et al., 2015; Tan et al., 2016). Using chemically-induced skin carcinogenesis, Kim et al. showed that *Pparb/d^{ex8}* (deleted exon 8) knockout mice developed more and larger skin tumor (Kim et al., 2006). PPAR β/δ was also reported to promote Hras-induced senescence and tumor suppression by repressing AKT activation (Zhu et al., 2014). In contrast, *Pparb/d^{ex4}* (deleted exon 4) knockout mice developed fewer and smaller tumor when challenged with UV (Montagner et al., 2014). The study showed that UV induced PPAR β/δ activity, which increased Src kinase and EGFR/Erk1/2 activities, resulting in increased expression of EMT markers. This discrepancy has been attributed to several differences, including tumor initiation procedures, genetic disruption strategies of *Pparb/d* gene and the genetic background of the mouse (Peters et al., 2015; Tan et al., 2016). Importantly, these studies used whole-body *Pparb/d* knockout mouse, thus the contribution of fibroblast PPAR β/δ in NMSC was not investigated.

A role for fibroblast PPAR β/δ in skin physiology is emerging. Fibroblast PPAR β/δ has been shown to curb excessive epidermal proliferation during wound healing via the production of secreted IL-1 receptor antagonist (Chong et al., 2009). Wang et. al. showed that ligand-activated fibroblast PPAR β/δ reduced oxidative stress in diabetic wound microenvironment via the upregulation of Glutathione peroxidase 1 (Gpx1) and catalase, thus facilitating wound healing (Wang et al., 2015). Recently, Sng et al. showed that the skin of

FSPCre-*Pparb/d*^{ex4} mice, a fibroblast-selective deletion of *Pparb/d* gene, recapitulated various aspects of fibroproliferative II subtype of human scleroderma, which involves the upregulation of leucine-rich alpha-2-glycoprotein-1 (LRG1) by fibroblast PPARβ/δ that in turn acts in an autocrine and paracrine manner to modulate tissue-specific TGFβ1 responses (Sng et al., 2018). However, a role for fibroblast PPARβ/δ in skin carcinogenesis remains unknown. Given that the stromal fibroblasts have significant influence on skin cancer initiation and development, we investigate the role of fibroblasts PPARβ/δ in chemically-induced skin carcinogenesis on FSPCre-*Pparb/d*^{ex4} and wild-type *Pparb/d*^{fl/fl} mice. Our finding revealed a reduced activation of epidermal NRF2 by fibroblast PPARβ/δ resulted in increased skin tumor load.

4.2 Results

4.2.1 Fibroblast PPARβ/δ increased skin tumor load.

The FSPCre-*Pparb/d*^{ex4} and their wildtype *Pparb/d*^{fl/fl} mice were subjected to the two-stage chemical-induced skin carcinogenesis regime. The selective deletion of *Pparb/d* in the fibroblasts was confirmed by genotyping and western blot analysis (**Fig 20A-B**).

Figure 20. Fibroblast-specific PPARβ/δ knockout validation. (A)

Genotyping results of the fibroblasts from normal skin and tumors of *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{ex4}* mice (top panel) and the respective amplicon sizes and constructs (bottom panel). **(B)** Representative immunoblots of PPARβ/δ in the fibroblasts from normal skin and tumors of *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{ex4}* mice (top panel). β-tubulin served as housekeeping protein from the same samples. Densitometric quantification plots is (bottom panel).

Data information: Data in **(B)** were expressed as means ± S.D. n=15-18 mice.

****p*<0.001 (Mann-Whitney test).

The number and volume of tumors were monitored weekly throughout the regime. Palpable tumors were observed after 10-12 weeks of treatment in *Pparb/d^{fl/fl}* compared to 12-15 weeks in FSPC*Cre-Pparb/d^{ex4}* (**Fig 21A**). All *Pparb/d^{fl/fl}* mice developed tumor by week 10, whereas tumors were observed in all FSPC*Cre-Pparb/d^{ex4}* mice at week 13 (**Fig 21B**). We segregated the tumor numbers by volume, small (< 0.005 cm³), medium (0.005-0.01 cm³) and large (> 0.01 cm³) after 19 weeks of treatment. The FSPC*Cre-Pparb/d^{ex4}* mice developed fewer and smaller tumors compared with *Pparb/d^{fl/fl}* (**Fig 21C-D, and 22**).

Figure 21. Reduced skin tumor load on FSPC*Cre-Pparb/d^{ex4}*. (A) Graph showing the mean number of tumors in FSPC*Cre-Pparb/d^{ex4}* (open circle) and *Pparb/d^{fl/fl}* (solid circle) mice starting from week 10 post tumor initiation. **(B)** Graph showing the percentage of FSPC*Cre-Pparb/d^{ex4}* (open circle) and

Pparb/d^{fl/fl} (solid circle) mice that developed palpable tumors after chemical-induced skin carcinogenesis. The duration of tumor emergence in FSPCre-*Pparb/d^{ex4}* (median=12.5 weeks) and *Pparb/d^{fl/fl}* (median=9 weeks) mice were evaluated with log-rank test. *** $p<0.001$. **(C)** Box-and-whisker plot showing the distribution of tumor number and size in FSPCre-*Pparb/d^{ex4}* (white) and *Pparb/d^{fl/fl}* (grey) mice. Three categories were used for tumor volume classification: small (<5 mm³), medium (5-10 mm³), and large (>10 mm³). Outliers were identified by using the interquartile range and presented as black dots. Table below shows the weight of the tumors for each category. **(D)** Representative images of tumor-bearing *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{ex4}* mice at indicated weeks post DMBA/BP initiation.

Data information: Data in **(A, C)** were expressed as means $\pm$ S.D. n=15-18 mice. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ (Mann-Whitney test).

Figure 22. Smaller tumors in FSPCre-*Pparb/d^{ex4}* mice. Representative H&E images of normal, hyperplastic skins and tumors from *Pparb/d^{fl/fl}* (left panel) and FSPCre-*Pparb/d^{ex4}* (right panel) mice. Scale bar: 100µm.

Surprising, immunofluorescence staining for Ki67, a proliferation marker, revealed a higher number of Ki67-positive in FSPCre-*Pparb/d^{ex4}* than *Pparb/d^{fl/fl}*-derived tumors biopsies (**Fig 23A-B**). We utilized cytokeratin 1 (CK1) and 8 (CK8) to distinguish the stage of tumor differentiation. Cytokeratin 1 expression is elevated in the hyperplastic epidermis, and the loss of CK1 combined with an aberrant increase CK8 are characteristic of the progression of mouse skin papilloma to SCCs. As expected, CK1 was expressed in normal

and hyperplastic skin from both genotypes. Regardless of tumor size, no difference in CK8 expression between tumors from *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{ex4}* mice was observed, indicating that PPAR β/δ deficiency in fibroblasts does not affect tumor differentiation (**Fig 23A**). Taken together, our observation suggests an impaired tumor initiation, resulting in a longer latency period in emergence of tumors in FSPCre-*Pparb/d^{ex4}*.

Figure 23. Tumors in FSPCre-Pparb/dex4 mice have increased proliferation. (A)

Representative images of normal, hyperplastic skins, and tumours from FSPCre-Pparb/d^{ex4} and Pparb/d^{fl/fl} mice. Dual immunofluorescence staining was performed for CK1 (red) and CK8 (green). The nuclei were counterstained with DAPI (blue). Same sections were used for hematoxylin and eosin (H&E) stain. Proliferation marker Ki67 staining was performed with other sections from the same biopsies. Scale bar: 100 μ m. **(B)** Quantification of Ki-67 positive nuclei in normal, hyperplastic skins, and tumor sections from FSPCre-Pparb/d^{ex4} and Pparb/d^{fl/fl} mice.

Data information: Data in **(B)** were expressed as means $\pm$ S.D. n=15-18 mice. ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test).

4.2.2 PPAR β/δ deficiency in fibroblasts increased epidermal Nox4 and NRF2 expression.

To decipher the underlying mechanism for the delayed emergence of tumors in FSPC*Cre-Pparb/d^{ex4}*, we interrogated the intrinsic networks that underlie differentially regulated genes using the gene expression microarray data of FSPC*Cre-Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* epidermis and dermis (GSE71419). Using the IPA, a gene connectivity network has grouped diseases and functions into broad categories, with connective tissue development having the largest coverage, followed by organismal injury and abnormalities and cellular movement (**Fig 24A**). A core network linked dermal *Pparb/d* and *TGF- β 1* with epidermal *NRF2* and *Nox4* (NADPH oxidase-4) signalings (**Fig 24A-B**).

A

B

Category	Disease or function	p-value	Molecules involved
Tissue Morphology	Quantity of epithelial tissue	1.71E-07	PPARG, PRKCD, AKT1, TGFB1, NFE2L2
Cellular Movement, Connective Tissue Development	Cell movement of fibroblast cell lines	2.51E-07	PPARG, NOX4, PRKCD, AKT1, TGFB1
Inflammatory Response, Organismal Injury and Abnormalities	Inflammation of organ	3.51E-07	PPARG, PRKCD, TGFB1, IDI1, AKT1, HLA-DQB1, HLA-DRB5, APOA2, CCL27, NFE2L2
Connective Tissue Development and Function	Morphology of fibroblast cell lines	3.78E-07	NOX4, PRKCD, TGFB1, AKT1
Cellular Movement	Migration of dermal cells	4.03E-07	PPARG, TGFB1, AKT1, CCL27
Free Radical Scavenging	Generation of reactive oxygen species	5.94E-07	PPARG, NOX4, PRKCD, AKT1, TGFB1, NFE2L2
Hair and Skin Development and Function, Organ Development	Growth of skin	6.94E-07	PPARG, PRKCD, AKT1, TGFB1, NFE2L2
Cancer, Organismal Injury and Abnormalities	Actinic keratosis	3.64E-06	CCL27, PRKCD, NFE2L2
Cellular Development	Proliferation of keratinocytes	5.88E-06	PPARG, PRKCD, TGFB1, NFE2L2
Cellular Movement	Invasion of cells	1.09E-05	PPARG, PRKCD, TGFB1, AKT1, NOX4, SLC2A1, NFE2L2
Cellular Development, Connective Tissue Development	Differentiation of fibroblast cell lines	1.20E-05	PPARG, AKT1, TGFB1, NFE2L2
Organismal Injury and Abnormalities	Fibrosis of tissue	5.00E-05	TGFB1, AKT1, NOX4
Cancer, Organismal Injury and Abnormalities	Carcinoma	5.43E-05	TGFB1, AKT1, HLA-DQB1, NSD3, NOX4, SLC2A1, CCL27, HLA-DRB5, APOA2, ATF7, ALDH3A1, MRPS18B, PRKCD, IDI1, PPARG, NFE2L2
Connective Tissue Development and Function, Tissue Morphology	Quantity of connective tissue	8.62E-05	PPARG, AKT1, TGFB1, APOA2, NFE2L2
Free Radical Scavenging	Quantity of reactive oxygen species	1.34E-04	NOX4, AKT1, TGFB1, NFE2L2
Cellular Movement, Connective Tissue Development	Migration of fibroblast	1.41E-04	AKT1, TGFB1, PRKCD
Cellular Development, Hair and Skin Development	Differentiation of epithelial cell lines	1.90E-04	AKT1, TGFB1
Cancer, Organismal Injury and Abnormalities	Growth of tumor	1.96E-04	NOX4, PRKCD, SLC2A1, TGFB1, AKT1, NFE2L2
Cellular Development	Epithelial-mesenchymal transition of carcinoma	2.84E-04	TGFB1, AKT1

Figure 24. Pathway analysis and gene connectivity network. (A) A gene connectivity network links *Pparb/d*, *Nox4*, *NRF2* and *TGF-β1* signalings with indicated diseases and functions. Twelve molecules with significant fold change (>1.5 times) and confidence ($p < 0.05$) identified from the microarray were connected to these signaling networks. An activating (green), deactivating (red) and inconclusive (grey) effect on the various functions are indicated. Arcs surrounding the gene network group diseases and functions into broad categories that include connective tissue development, cancer and free radical scavenging. **(B)** Diseases and functions are tabulated and arranged into categories based on their *p-value*. The molecules responsible for each disease and function are also indicated.

Our analysis revealed a hitherto unknown connection linking *Pparb/d*, *NRF2* and *Nox4*, thus, we focused our investigation on this new network. We detected higher *Nox4* and *NRF2* in the epidermis of FSPCre-*Pparb/d^{ex4}* mice than *Pparb/d^{fl/fl}* (**Fig 25A**). We also detected higher extracellular H_2O_2 in conditioned medium from primary FSPCre-*Pparb/d^{ex4}* than *Pparb/d^{fl/fl}* keratinocyte cultures, which could be eliminated by exogenous catalase (**Fig 25B**). These changes were consistent with higher intracellular ROS as indicated by the higher mean CellRox fluorescence readings in keratin-positive cells from dorsal skins of FSPCre-*Pparb/d^{ex4}* than *Pparb/d^{fl/fl}* mice (**Fig 25C**). A higher basal *NRF2* activity, determined using an ARE-luciferase reporter transactivation assay, was also observed in keratinocytes co-cultured with primary fibroblasts from FSPCre-*Pparb/d^{ex4}* than that from *Pparb/d^{fl/fl}* (**Fig 25D**). We also detected higher mRNA levels of *NRF2*, along with *NRF2*-regulated antioxidant enzymes in the FSPCre-*Pparb/d^{ex4}* epidermis compared with *Pparb/d^{fl/fl}* (**Fig 25E**).

Figure 25. Elevated Nox4 stimulates NRF2-related responses to reduce epidermal ROS. (A) Representative immunoblots of Nox4 and NRF2 in epidermis from FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* mice. Densitometric quantification plots are shown (top panel). β -tubulin served as housekeeping protein from the same samples. (B) Extracellular H_2O_2 levels in FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* keratinocytes. Extracellular H_2O_2 was detected by Amplex Red Assay and normalized to cell number. (C) Histogram shows mean intracellular ROS (CellRox fluorescence readings) in cytokeratin-positive cells from skin of FSPCre-*Pparb/d^{ex4}* (red) and *Pparb/d^{fl/fl}* skin (grey). (D) Fold change in ARE-dependent luciferase activities in keratinocytes co-cultured with primary

fibroblasts from FSPCre-*Pparb/d^{ex4}* or *Pparb/d^{fl/fl}*. For reporter assay, firefly luciferase activity was normalized to Renilla luciferase. **(E)** Relative mRNA levels of indicated antioxidant genes in epidermis from FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* mice. Ribosomal protein L27 was used as housekeeping gene. Data information: Data in **(A-E)** were expressed as means $\pm$ S.D. ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test).

To understand how ROS, specifically H₂O₂, increased the mRNA level of *NRF2* mRNA, we subjected primary human keratinocytes to a kinase inhibitor screen and examined *NRF2* expression at 4 h post H₂O₂ stimulation. We observed that kinase inhibitors against B-Raf proto-oncogene, serine/threonine kinase (B-Raf) and Mitogen-activated protein kinase kinase (MEK) could attenuate H₂O₂-mediated upregulation of *NRF2*, suggesting that B-Raf-MEK pathway is involved in the transcription of *NRF2* (**Fig 26A**). The increase in *NRF2* mRNA was unlikely due to autoregulation as its increase occurred before the capacity of *NRF2* to upregulate its target genes such as NQO1 and GPX1 (**Fig 26B**). Western blot analyses confirmed the increase in the phosphorylation levels of B-Raf, MEK1/2 and ERK1/2 upon H₂O₂ exposure (**Fig 26C**).

Figure 26. B-Raf-MEK signaling axis regulates the transcription of *NRF2*.

(A) Relative mRNA level of *NRF2* in human primary keratinocytes treated with vehicle and kinase inhibitors against B-Raf and MEK1/2 at 4 h post H₂O₂ stimulation. Ribosomal protein L27 was used as housekeeping gene. **(B)** Relative fold change in mRNA of *NRF2* target genes, *GPX1* and NAD(P)H Quinone Dehydrogenase 1 (*NQO1*), in human primary keratinocytes after 4 h of H₂O₂ treatment. Ribosomal L27 gene serves as housekeeping gene. n=3 independent experiments. **(C)** Representative immunoblots of total and phosphorylated B-Raf, MEK1/2 and ERK1/2 in human keratinocytes treated with H₂O₂ (left panel). Densitometric quantification plot is shown (right panel). Total

B-Raf, MEK1/2 and ERK1/2 as cognate normalizing proteins were from the same samples.

Data information: Data in **(A-C)** were expressed as means $\pm$ S.D. $**p<0.01$, $*p<0.05$ (Mann-Whitney test). n.s. denotes not significant.

Consistent with the above observation, we observed a higher Nox4 and NRF2 expression in human keratinocytes (Ker) co-cultured with human dermal fibroblasts whose endogenous PPAR β/δ was suppressed by siRNA ($F_{siPparb/d}$) than with control fibroblasts ($F_{scrambled}$). The overexpression of PPAR β/δ in $F_{siPparb/d}$ fibroblasts reduced the Nox4 and NRF2 expression (**Fig 27A-C**). In summary, PPAR β/δ deficiency in fibroblasts increased Nox4 expression that triggers an enhanced antioxidant NRF2 response in the adjacent epidermis.

Figure 27. PPAR β/δ deficiency in fibroblasts increased epidermal Nox4 and NRF2 expression. **(A)** Representative immunoblots of Nox4 and NRF2 in human keratinocytes (Ker) cocultured with either dermal human fibroblasts knockdown of *Pparb/d* ($F_{siPparb/d}$), scrambled siRNA ($F_{scrambled}$) (bottom panel). β -tubulin served as housekeeping protein and were from the same samples. Densitometric quantification plot is shown (top panel). **(B)** Relative mRNA (top

panel) and protein (bottom panel) levels of PPAR β/δ in control (scrambled) and knockdown (*siPparb/d*) fibroblasts. β -tubulin served as housekeeping protein was from the same samples. n=5 independent experiments. **(C)** Representative immunoblots of Nox4 and NRF2 (top panel) in human keratinocytes (Ker) cocultured with either dermal human fibroblasts transfected with scrambled siRNA ($F_{\text{scrambled}}$), *siPparb/d* ($F_{\text{siPparb/d}}$) or *siPparb/d* and *Pparb/d* expression vector ($F_{\text{siPparb/d}} + Pparb/d$ vector). β -tubulin served as housekeeping protein were from the same samples. Relative mRNA level of PPAR β/δ (bottom panel) in control (scrambled), knockdown (*siPparb/d*) and rescued (*siPparb/d* + *Pparb/d* vector) human dermal fibroblasts. n=3 independent experiments. Data information: Data in **(A-C)** were expressed as means $\pm$ S.D. ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

4.2.3 TGF- β 1 elevates Nox4 expression in keratinocytes and was attenuated by LRG1.

The epithelial-mesenchymal communication between dermal *Pparb/d* and TGF- β 1 has been previously reported. PPAR β/δ in fibroblasts upregulates the expression of Lrg1 that attenuates epidermal and dermal responses to TGF β 1. We investigated the potential contribution of TGF- β 1 signaling, which was a key player in our IPA analysis, in the increase epidermal expression of Nox4 and NRF2. We showed that the expression of Nox4 (**Fig 28A**) and the activity of NRF2 (**Fig 28B**) were stimulated by TGF- β 1 in *Pparb/d^{fl/fl}* keratinocytes. The FSPC*re-Pparb/d^{ex4}* keratinocytes showed a higher basal ARE-dependent luciferase activity that was further increased by exogenous TGF- β 1 (**Fig 28B**). PPAR β/δ transcriptionally upregulates the expression of LRG1 in fibroblasts. LRG1 attenuates TGF- β 1 signaling in the skin and lung to suppress fibrosis. We observed that recombinant LRG1 dose-dependently attenuated the expression of Nox4 and the activity of the reporter luciferase. The suppression of

endogenous Nox4 (siNox4) or the exposure to the antioxidant N-acetyl-cysteine (NAC) attenuated ARE-dependent luciferase activity (**Fig 28B**).

Figure 28. TGF-β1 elevates Nox4 expression in keratinocytes and was attenuated by LRG1. (A) Relative mRNA (top panel) and protein (bottom panel) levels of Nox4 in *Pparb/d^{fl/fl}* keratinocytes treated with either TGFβ1 or LRG1 proteins. Representative immunoblot for Nox4 is shown. β-tubulin served as housekeeping protein was from the same samples. Densitometric quantification plot is shown. **(B)** Fold change in ARE-dependent luciferase activities in FSPC-Cre-*Pparb/d^{lox4}* and *Pparb/d^{fl/fl}* keratinocytes treated with either TGFβ1 (10 ng/mL) or LRG1 (50-100 ng/mL). Keratinocytes whose endogenous Nox4 was suppressed by siRNA were denoted by siNox4. Antioxidant NAC was used at 500 μM. For reporter assay, firefly luciferase activity was normalized to Renilla luciferase.

Data information: Data in **(A-B)** were expressed as means ± S.D. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

Human keratinocytes (Ker) co-cultured with *F_{siPparb/d}* or *F_{siLRG1}* and treated with exogenous LRG1 reduced the expression of Nox4 and NRF2 in Ker (**Fig 29A**). This observation suggests that LRG1-TGF-β1 signaling was involved in regulating Nox4 and NRF2 expression in keratinocytes. In human Ker cocultured with *F_{siPparb/d}*, the mRNA levels of several antioxidant enzymes were

also elevated (**Fig 29B**), but the intracellular ROS level remained higher than to those co-cultured with F_{scrambled} (**Fig 29C**). These results are consistent with our co-culture experiment of keratinocytes with primary fibroblasts from either *Pparb/d^{fl/fl}* or FSPCre-*Pparb/d^{ex4}* mice. Human keratinocytes alone treated with TGF- β 1 showed increased Nox4 and NRF2 expression accompanied by an increase in the expression of antioxidant enzymes (**Fig 29D**), indicating that NRF2 is a downstream mediator of TGF- β 1. The observed upregulation of Nox4 and NRF2 was attenuated by LRG1 (**Fig 29E**). Notably, the increase in NRF2, but not Nox4, was abolished by NAC suggesting that NRF2 expression was dependent on Nox4-derived H₂O₂ (**Fig 29E**). NADPH oxidase 4 protein increases oxidative stress by producing H₂O₂, whereas an increased NRF2 response elevates the expression of many antioxidant genes. To understand the relationship between Nox4 and NRF2, we examined the effect of wild-type Nox4 and various Nox4 mutants on NRF2 expression and activity. The ectopic expression of wild type Nox4 and H₂O₂-producing Nox4 Δ D-loop mutant increased NRF2 expression and activity (**Fig 29F-G**). In contrast, the overexpression of Nox4 Δ B-loop mutant, which is defective in H₂O₂ production neither stimulates NRF2 expression nor activity (**Fig 29F-G**). In summary, we showed that PPAR β/δ -deficient fibroblasts increased Nox4 expression in the adjacent keratinocytes which involved paracrine LRG1/TGF- β 1 signaling. The elevated epidermal Nox4 increased intracellular oxidative stress that triggered an enhanced NRF2 response as indicated by higher expression of antioxidant/detoxification genes in epidermis.

Figure 29. Fibroblast PPAR β / δ modulates epidermal Nox4 via LRG1-TGF β 1 signaling. **(A)** Relative mRNA (top-left panel) and protein (bottom-left panel) levels of LRG1 in control (scrambled) and knockdown (siLRG1) fibroblasts. β -tubulin served as housekeeping protein was from the same samples. n=5 independent experiments. Representative immunoblots of Nox4 and NRF2 in human keratinocytes (Ker) cocultured with either dermal human fibroblasts knockdown of *Pparb/d* ($F_{siPparb/d}$) or LRG1 (F_{siLRG1}) (bottom-right panel). β -tubulin served as housekeeping protein and was from the same samples. Densitometric quantification plot is shown (top-right panel). **(B)** Relative mRNA levels of indicated antioxidant genes in Ker co-cultured with $F_{siPparb/d}$ or $F_{scrambled}$. Ribosomal protein L27 was used as housekeeping gene. **(C)** Mean intracellular ROS level in Ker cocultured with either $F_{siPparb/d}$ (red) or $F_{scrambled}$ (grey). Histogram shows mean CellRox fluorescence readings in keratinocytes. **(D)** Relative fold change in mRNA of indicated NRF2 target genes in human primary keratinocytes after TGF- β 1 treatment. 18S gene serves as housekeeping gene. n=3 independent experiments. **(E)** Relative mRNA levels of Nox4 and NRF2 in Ker treated with either TGF β 1, LRG1 or NAC. Ribosomal 18S was used as housekeeping gene. **(F)** Representative immunoblots of Nox4 and NRF2 in primary wildtype mouse keratinocytes transfected with various Nox4 mutants. Densitometric quantification plots are shown (right panel). β -tubulin served as housekeeping protein from the same samples. **(G)** Fold change in ARE-dependent luciferase activities in mouse keratinocytes overexpressing wild-type, Nox4 Δ D-loop and Nox4 Δ B-loop Nox4 mutants. Empty vector served as control. For reporter assay, firefly luciferase activity was normalized to Renilla luciferase.

Data information: Data in **(A-G)** were expressed as means $\pm$ S.D. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test). n.s. denotes not significant.

4.2.4 Differential phosphorylation kinetics of B-Raf, MEK1/2, PTEN, Src and AKT prior to tumor formation.

The epidermis of FSPC*Cre-Pparb/d*^{ex4} has enhanced NRF2 response. The inhibition of either B-Raf and MEK1/2 kinases attenuated the mRNA

expression of *NRF2* by H_2O_2 . To gain further insight into mechanism *in vivo*, we examined the expression of B-Raf, MEK1/2 and *NRF2* in the epidermis of vehicle- or DMBA/BP-treated *Pparb/d^{fl/fl}* and FSPCre-*Pparb/d^{ex4}* mice between week 4 to 8, prior the emergence of palpable tumor at week 10 post-treatment in *Pparb/d^{fl/fl}* mice. The expression profiles of phospho-B-Raf and phospho-MEK1/2 were elevated in epidermis of vehicle-treated FSPCre-*Pparb/d^{ex4}* mice, concomitant with the higher expression of *NRF2*, when compared with cognate *Pparb/d^{fl/fl}* mice (**Fig 30A**). The expression of phospho-B-Raf, phospho-MEK1/2 and *NRF2* increased more rapidly in DMBA/BP-treated FSPCre-*Pparb/d^{ex4}* compared with *Pparb/d^{fl/fl}* (**Fig 30A**). Together with the *in vitro* data, the observation indicates that the upregulation of *NRF2* mRNA is mediated via B-Raf-MEK1/2 pathway.

The higher intracellular ROS in *Pparb/d^{fl/fl}* modifies the activities of tumor suppressors or oncogenes. Direct oxidative modification of products of tumor suppressors or oncogenes can have a direct impact on tumor formation. Previous studies showed that oxidation increases the autophosphorylation of PTEN and Src, which attenuates the tumor-suppressive catalytic activity of PTEN and triggers the oncogenic functions of Src. Phosphatase and tensin homolog and Src have been implicated in SCC formation. To this end, we examine the oxidative state of tumor suppressor PTEN and oncogenic Src in FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* mice. We used the PEG-switch assay which is a semi-quantitative method for measuring target reversible cysteine oxidation in complex protein mixtures derived from tissue or cultured cells. Quantification of pegylated PTEN and Src against the reduced form showed a significant increase in PTEN and Src oxidation, indicating reduced PTEN and

enhanced Src activities in *Pparb/d^{fl/fl}* compared with FSPCre-*Pparb/d^{ex4}* keratinocytes (**Fig 30B**).

We propose that difference between the phosphorylation levels of PTEN and Src between FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* also contributes to the delayed emergence of tumor in the former. There was no significant difference in the phosphorylation levels of PTEN and Src in the epidermis of vehicle-treated FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* (**Fig 30A**), suggesting that these pathways may not be responsible for the already elevated NRF2 in FSPCre-*Pparb/d^{ex4}* (**Fig 30A**). We observed a progressive increase in phosphorylated PTEN and Src in the epidermis of *Pparb/d^{fl/fl}* upon DMBA/BP treatment, indicating that altered activities of PTEN and Src preceded the emergence of tumor (**Fig 30A**). Phosphorylation of PTEN at Ser380 suppresses its activity by controlling its recruitment into the PTEN-associated complex as well as its stability. With a reduced PTEN activity, we observed higher level of phosphorylated AKT in the epidermis of *Pparb/d^{fl/fl}* than FSPCre-*Pparb/d^{ex4}*. This observation is consistent with the higher tumor burden in *Pparb/d^{fl/fl}* than FSPCre-*Pparb/d^{ex4}* (**Fig 30A**). In contrast, the increase in phosphorylated PTEN and Src in the epidermis of FSPCre-*Pparb/d^{ex4}* was muted and was only significant compared to cognate vehicle control from week 6 onwards (**Fig 30A**). Taken together, the late tumor emergence and reduced tumor load in FSPCre-*Pparb/d^{ex4}* compared with *Pparb/d^{fl/fl}* can be attributed in part to an altered NRF2, B-Raf, MEK1/2, PTEN, Src and AKT activation profiles during tumor initiation.

Figure 30. Protein expression profiles of NRF2, B-Raf, MEK1/2, PTEN, Src and AKT in epidermis of FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}*. (A) Representative immunoblots of NRF2, total and phosphorylated B-Raf, MEK1/2, PTEN, Src and AKT in the epidermis of vehicle control- and DMBA/BP-treated FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* between week 4 to 8 (top panel).

Densitometric quantification plots are shown (bottom panel). Total B-Raf, MEK1/2, PTEN, Src and AKT served as cognate normalizing controls were from the same samples. n=3 independent experiments. **(B)** Representative PEG-switch immunoblots for oxidized PTEN and Src proteins in FSPCre-*Pparb/d^{ex4}* and *Pparb/d^{fl/fl}* keratinocytes (bottom panel). Oxidized proteins become conjugated with PEG and have a higher molecular weight than their unoxidized counterparts. Quantification of oxidized/pegylated proteins against the reduced form of PTEN and Src (top panel).

Data information: Data in **(A-B)** were expressed as means $\pm$ S.D. ** $p < 0.01$, * $p < 0.05$ (Mann-Whitney test).

4.3 Discussion

Cancer-associated fibroblasts are the major cell population within the tumor stroma and are involved in cancer progression (Liao et al., 2018). Emerging studies have shown that concurrent therapies targeting CAFs increased the efficacy of conventional anticancer chemotherapeutics (Chan et al., 2018). Nuclear hormone receptors have surfaced as ideal drug targets because the binding of small lipophilic molecules precisely modulates their activities. In this context, PPAR β/δ is of great interest because it has been implicated in a variety of diseases, such as cardiovascular diseases and diabetes. However, the biological function of PPAR β/δ in NMSC remains controversial, due in part to a poor understanding of the role of fibroblast PPAR β/δ in NMSC (Tan et al., 2016). Furthermore, previous studies mainly focused on CAFs in pre-existing tumors (Cheng et al., 2019), rather than the epithelial-mesenchymal communication between normal fibroblasts and epithelia, whose disruption facilitates tumor development.

We observed a delayed emergence and reduced tumor burden in FSPCre-*Pparb/d^{ex4}* mice than with *Pparb/d^{fl/fl}* mice using a chemically induced

skin carcinogenesis model. However, FSPCre-*Pparb/d^{ex4}*-derived tumors showed increased proliferation, with no difference in differentiation compared to similarly-sized *Pparb/d^{fl/fl}*-derived tumors, suggesting a delayed tumour initiation. The underlying mechanism involves an enhanced epidermal *NRF2* and *Nox4* signaling which is in line with a previous study that concluded a protective effect of *NRF2* against UV-induced cutaneous squamous cell carcinomas (Knatko et al., 2016). In contrast, a tumor-promoting activity of *NRF2* in virus-induced skin tumorigenesis has been reported (Kitamura and Motohashi, 2018). The mixed evidence suggests a complex and context-dependent role of *NRF2* in tumor progression. In our study, the activation of *NRF2* and *Nox4* integrates into the PPAR β/δ and LRG1-TGF β 1 networks. Fibroblast PPAR β/δ transcriptionally upregulates the expression of LRG1 that inhibits TGF β 1 signaling in adjacent epidermis (Sng et al., 2018). We showed that TGF- β 1 stimulated the expression of *Nox4* in the epidermis and that PPAR β/δ -deficient fibroblasts also triggered an enhanced *NRF2*-mediated response, which was dependent on *Nox4*-derived H₂O₂, i.e. oxidative stress. We showed that H₂O₂ rapidly upregulated *NRF2* mRNA via the B-Raf-MEK1/2 pathway. The enhanced *NRF2*-related response altered the intracellular ROS that modulates the activities of tumor suppressor PTEN, oncogene Src and AKT. These findings supported a pro-tumorigenic role for fibroblast PPAR β/δ .

Although the genetic background, knockout mouse model and tumor induction protocol were different, our experimental outcome was consistent with the UV-induced skin carcinogenesis performed on full-body hairless SKH1/*Pparb/d^{ex4}* mice (Montagner et al., 2014). The induction of skin cancer by DMBA/BP involves different molecular mechanisms from those associated

with skin cancer attributable to chronic UV exposure (Matsumura and Ananthaswamy, 2002; Boukamp, 2005). These independent studies have underscored a pro-tumorigenic role for PPAR β/δ in skin cancers. Lending support, the long-term treatment of mice with PPAR β/δ agonist GW501516 caused rapid induction of cancers in several organs (Sahebkar et al., 2014). Our findings were at odd with cancer studies using whole-body *Pparb/d^{ex8}* mice, whose *Pparb/d* gene was inactivated by insertion of the phosphoribosyltransferase II gene at the last exon. It has been proposed that whole-body *Pparb/d^{ex8}* display a hypomorphic phenotype, i.e. a partial loss of function of PPAR β/δ (Bility et al., 2008; Peters et al., 2015; Tan et al., 2016).

Despite a delay in tumor initiation, FSPCre-*Pparb/d^{ex4}* mice had higher tumor proliferation compared to FSPCre-*Pparb/d^{fl/fl}* mice. The long-term effect of such enhanced tumor proliferation on cancer progression suggests a possible dual role in initiation and progression. Transforming growth factor beta and NRF2 signaling have been reported to play dual roles in tumorigenesis, differing in tumor initiation and progression (Huang and Blobe, 2016; Teo et al., 2017a). Given that fibroblast PPAR β/δ activates epidermal NRF2 via TGF β signaling, it is conceivable that fibroblast PPAR β/δ may similarly exhibit dichotomous roles. On one hand, PPAR β/δ deficiency in fibroblasts led to reduced oxidative stress in the epidermis, delayed tumour initiation and reduced tumor burden in FSPCre-*Pparb/d^{ex}*. On the other hand, tumors from FSPCre-*Pparb/d^{ex4}* have a higher number of proliferating Ki67-positive cells. Similarly, the proliferation of human skin cancer cells was reduced when cocultured with fibroblasts overexpressing PPAR β/δ (Chan et al., 2018). The mechanism underlying this dichotomy remains unclear, but it tempting to

speculate that it could be related to the transformation from normal fibroblasts to CAFs. It was shown that the acquisition of an oxidative, CAF-like state by normal fibroblasts could be achieved by the chronic exposure to oxidative stress and attenuated TGF- β signaling (Chan et al., 2017). Our study showed that a deficiency in PPAR β/δ in the fibroblasts disrupted the normal epithelial–mesenchymal communication and enhanced antioxidant/detoxification response to delay NMSC development.

Chapter 5: Discussion and future directions

5.1 Summary of findings

In the first study, we reveal a hitherto unknown role for neurotransmitter, ACh during the early stages of wound healing by promoting the capacity of keratinocytes to undergo EMT. ACh primes the keratinocytes to TGF β 1 induced-EMT via the upregulation of their cognate receptor, TGF β R2. Under normal circumstances, ACh activates the Src-ERK1/2 signaling axis to upregulate TGF β R2. However, in hyperglycemic condition, as is the case for diabetes, the high level of glucose activates p38 MAPK, which interferes with the Src-ERK1/2 activation. This cross-communication between the two pathways leads to the keratinocytes becoming resistant to ACh stimulation, and subsequently, a lack of TGF β R2 upregulation. The addition of solamargine, a p38 MAPK inhibitor, eliminates the inhibitory effect of p38 MAPK on ERK1/2, re-sensitizing the keratinocytes to ACh stimulation (**Fig 31**). Our data suggest that ACh is a novel regulator of EMT in keratinocytes. The ACh mediated signaling is impeded by a hyperglycemic environment, culminating in delayed re-epithelialization seen in diabetic wounds. The re-sensitization of keratinocytes to ACh signaling may be a crucial turning point in wound management and care for diabetic injuries. An enhanced local cellular response in a diabetic wound microenvironment may also be a useful strategy to increase the efficacy of therapeutic agents for the treatment of poor or non-healing diabetic wounds.

Figure 31. Schematic illustration of the mechanism in the first study. Under low glucose conditions, ACh upregulates TGFβRII through the Src-ERK1/2 axis. High glucose, as is observed in diabetes, activates p38 MAPK, which interferes with the Src-ERK1/2 signalling axis, rendering the cells resistant to ACh signalling. Inhibition of p38 MAPK rescues this phenotype.

In the second study, we show that the dysregulation of nuclear receptor PPARβ/δ in CAFs alters mesenchyme to epithelium communication and is instrumental in cancer initiation and progression. PPARβ/δ in fibroblasts modulates epithelial TGFβ1 response via the increased production of LRG1 into the microenvironment. An increase in TGFβ1 response upregulates the expression of Nox4. Nox4-derived H₂O₂ production triggers an NRF2 response in the keratinocytes. H₂O₂ upregulated NRF2 via the B-Raf-MEK1/2 pathway. This antioxidative response also acts as an “anti-cancer” protection by altering

the activities of PTEN, Src and AKT kinases (**Fig 32**). Indeed, using chemically induced skin carcinogenesis, FSPCre-*Pparb/d^{ex4}* mice, whose *Pparb/d* gene was selectively deleted in the fibroblasts, has delayed emergence and reduced tumor burden than control mice (*Pparb/d^{fl/fl}*). Our study demonstrates the importance of stromal cells in the microenvironment during cancer initiation and progression. It also reveals a dichotomous role for ROS as an essential signaling molecule that can conjure an anti-tumor mechanism (study herein), as well as for the propagation of field cancerization and in tumor progression (published papers from our lab).

Figure 32. Schematic illustration of the mechanism in the second study.

Fibroblast PPAR β/δ increases the expression of LRG1. The secreted LRG1 attenuates TGF- β 1 signaling, which is required for the upregulation of Nox 4 in the keratinocytes. Less Nox4-derived H_2O_2 production reduces the NRF2 expression and response, and consequently a lower expression of antioxidant/detoxification genes and higher intracellular ROS. More oxidatively modified

PTEN and Src were present in the epidermis of *Pparb/d^{fl/fl}* which contribute to earlier appearance of tumors compared to FSPCre-*Pparb/d^{ex4}*.

5.2 Small biomolecules in the microenvironment of chronic wounds and cancers.

Many studies have proposed that cancer shares many similarities with chronic wounds (Schäfer and Werner, 2008; Dvorak, 2015). Some prominent examples include exacerbated inflammation, enhanced proliferative capacity and a corrupted ECM that promote growth and migration, and immunosuppression (Balkwill and Mantovani, 2001; Schäfer and Werner, 2008; Colotta et al, 2009; Lu et al., 2012; Antonio et al., 2015). A study showed that cancer cells displayed accelerated tumor progression in mice if continually wounding occurred in proximity to the inoculation site (Stuelten et al., 2008). In another independent study, the continual wounding of mammary tumors in mice led to a higher rate of metastasis (Hobson et al., 2013). A similar finding was reported in zebrafish that were predisposed to melanoma (Antonio et al., 2015). Therefore, it is not surprising that the presence of a chronic wound leads to an increased risk of cancer development (Liddell, 1975; Ackroyd and Young, 1983). This relationship between chronic wounds and cancer was exemplified in Metformin, a widely prescribed drug for treating type 2 diabetes, has anti-cancer properties (Pernicova and Korbonits, 2014; Morales and Morris, 2015).

Metformin helps to lower blood glucose levels by reducing the amount of glucose produced and released by the liver and by increasing insulin sensitivity (Grzybowska et al, 2011). Metformin inhibits mTOR activity by activating ATM (ataxia telangiectasia mutated) and LKB1 (liver kinase B1) and then adenosine

monophosphate-activated kinase (AMPK), and thus prevents protein synthesis and cell growth (Inoki et al., 2003; Chiang and Abraham, 2007; Yoshida et al., 2011). Metformin can activate p53 by activating AMPK and thereby ultimately stop the cell cycle (Ben Sahra et al., 2011). Generally, Metformin can reduce the incidence and mortality from cancers, increase the response to radiotherapy and chemotherapy treatment, reduce malignancy, and the likelihood of relapse (Wu et al., 2014; Coyle et al., 2016). In contrast, Becaplermin, an approved drug for lower extremity, neuropathic, diabetic ulcers, is associated with an increased risk of cancer development. Becaplermin promotes cellular proliferation and angiogenesis; therefore, use is contraindicated in patients with known neoplastic disease at the site of application (Nagai and Embil, 2002; Barrientos et al., 2014). A study found that the rate ratio incidence for cancer development and the rate ratio for cancer mortality in becaplermin-exposed as compared to the unexposed comparator cohort to be 1.2 (95 % CI 0.7 to 1.9) and 1.8 (95 % CI, 0.7 to 4.9), respectively. However, among patients exposed to 3 or more tubes of becaplermin gel, the incidence of cancer mortality as compared to the unexposed cohort was 5.2 (95 % CI, 1.6 to 17.6) (Papanas and Maltezos, 2010). Despite the proclaimed similarities in the two pathologies, these findings indicate there are still differences between cancer and chronic wounds, and that a better understanding of the processes associated with each of them is required for the development and improvements in therapies for these two pathologies.

Although much research has been carried out on both pathologies, therapeutic interventions have not been making much headway. Most studies to date focus on the different cell types and the disarray cytokine landscape of

the diseases. While the presence of small non-proteinaceous biomolecules in the microenvironment, such as metabolites, hormones and phosphorylated nucleosides, has been well-recognized for many years, detailed investigations into their roles, the underlying mechanism, and their impact on clinical outcome are scarce. Our studies reveal the essential roles of two such molecules, ACh and H₂O₂ on the diabetic wound healing and cancer initiation, respectively.

To the best of our knowledge, our study is the first to explore the role of wound ACh in diabetic wounds. We found that ACh plays a role in wound healing through the upregulation of TGFβR2 on keratinocytes, therefore promoting TGFβ1 induction of the EMT process. Keratinocyte migration is a pivotal cellular process during the wound re-epithelialization. Unexpectedly, we found higher levels of ACh in diabetic wounds, and ACh signaling was attenuated in the keratinocytes by hyperglycemia, impairing the re-epithelialization process. Thus, while low doses of ACh accelerate healthy wound healing, our finding suggests that a similar topical ACh application is not a viable option to aid the healing of diabetic wounds. Our finding further suggests that adjunctive strategies that mitigate the suppressive effect by hyperglycemia should be considered.

Reactive oxygen species play a pivotal role in the orchestration of the normal wound healing response (Dunnill et al., 2017). Classically, ROS functions as the host's defense through phagocytes that induce a ROS burst onto the pathogens present in wounds, leading to their destruction. Excess ROS leakage into the surrounding environment has further bacteriostatic effects. They act as secondary messengers to many immunocytes and non-lymphoid cells, which are involved in the repair process, and appear to be important in

coordinating the recruitment of lymphoid cells to the wound site and effective tissue repair. ROS also possess the ability to regulate angiogenesis at the wound site and the optimal perfusion of blood into the wound healing area. Previous work from our lab showed that diabetic wounds are imbued with an early excessive and protracted ROS production, which coincides with an aberrant inflammatory response (Wang et al., 2015). Wound fibroblasts and macrophages are major contributors to this oxidative microenvironment. Such dysregulated extracellular ROS level impairs multiple cellular processes and responsiveness to external signals and also modifies the ECM. The chemotaxis and migration of keratinocytes are impeded on this modified matrix. Interestingly, the activation of PPAR β/δ in diabetic wound fibroblasts increases the expression and activities of GPX1 and catalase, reining the oxidative modifications of ECM required for active cell migration. It is clear that the precise balance between low versus high levels of ROS is critical in determining functional outcome. Methodologies or technologies that harness the potential benefits of low ROS by promoting a more normal (acute-type) wound-healing response, yet mitigating the detrimental consequence of high ROS, should be clinically tested. Their efficacy will provide a greater understanding of which wounds respond best to which treatment.

The formation of primary, secondary tumors or metastasis is greatly influenced by multifaceted tumor-stroma interactions, in which stromal components of the tumor microenvironment can affect the behavior of the cancer cells. Many studies have identified cytokines and growth factors as cell signaling molecules that aid cell to cell communication. The functional contribution of ROS and ACh as communication molecules are less understood.

Acetylcholine signaling is implicated in cancer development and progression. Unlike diabetic wounds, ACh signaling is heightened in cancer and supports proliferation, invasiveness, and angiogenesis (Ng et al., 2007; Catassi et al., 2008). The increase in ACh signaling has been attributed to different mechanisms, including elevated ACh receptors expression and the transactivation of growth factor receptors by ACh (Cheng et al., 2008; Huang et al., 2017; Yu et al., 2017). Various types of mesenchymal cells, such as the fibroblasts, endothelial cells, and immune cells, are known to express ACh receptors (Norel et al., 1996; Kawashima and Fujii, 2003; Pieper et al., 2007; Kolahian and Gosens, 2012). Although ACh signaling appears to be a potential anti-tumor target, many fundamental questions remain to be addressed - what are the changes in ACh level in the tumor microenvironment during tumor initiation and progression, which cell types are responsible for ACh production and what are the target cell type of ACh.

Numerous studies, including our previous work, agree that elevated rates of ROS are detected in almost all cancers, where they promote many aspects of tumor development and progression (Liou and Storz, 2010). This high level of ROS can result from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signaling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxygenases, and thymidine phosphorylase, or through crosstalk with infiltrating immune cells (Szatrowski and Nathan, 1991; Storz, 2005). As field cancerization and metastasis are the leading causes of cancer recurrence and mortality in cancer patients, our lab focuses on the role of ROS in these two events. We showed that tumor-derived angiopoietin-like 4 protein (ANGPTL4) is an important redox player in cancer

and a metastasis-related gene (Zhu et al., 2011; Teo et al., 2017b). We showed that ANGPTL4 interacts with integrins to stimulate the Nox1-dependent production of superoxide $O_2^{\cdot-}$. A high ratio of $O_2^{\cdot-}$: H_2O_2 activates oncogenic Src, triggering the PI3K/PKB α and ERK prosurvival pathways to confer anoikis resistance, thus promoting metastasis (Zhu et al., 2011). In another study, we showed cancer-associated fibroblasts (CAFs) enact field cancerization by promoting extratumoral oxidative stress (Chan et al., 2017). Our study showed that CAF-derived ROS, specifically H_2O_2 , induced oxidative stress in normal fibroblasts and triggered *de novo* CAF transformation *in vitro* and *in vivo*. The adoptive transfer of oxidatively transformed fibroblasts into malignant and non-tumorigenic epithelia xenografts resulted in faster-growing and more aggressive tumors, indicating exacerbated oncogenic transformation. This self-amplifying, field cancerization effect was due to impaired TGF β signalling. In a follow-up study using squamous cell carcinoma, we revealed CAFs have a distinct nuclear receptor profile compared with the normal fibroblasts (Chan et al., 2018). We further showed that targeting nuclear receptors in CAFs as concurrent therapy can inhibit the development of chemoresistant tumors. Our data therein revealed that PPAR β/δ expression was downregulated to the greatest extent in CAFs, followed by the vitamin D receptor (VDR) and the glucocorticoid receptor (GR).

In this study, we found that the deletion of the nuclear receptor PPAR β/δ in fibroblasts contributes to the upregulation of ROS, specifically H_2O_2 , in keratinocytes. The intracellular H_2O_2 stimulated a cellular anti-oxidative response that involved increased expression and activity of NRF2. This NRF2-mediated response delayed the transformation of healthy cells. Interestingly,

despite a delay in tumor initiation associated with PPAR β/δ -deficient fibroblasts, these tumors have higher proliferation compared to control. This observation again highlights the dichotomous roles of biomolecules in tumor initiation and progression.

Although we have studied ACh and ROS separately in different physiopathological contexts, we allude to a possible relationship between ACh and ROS. Thus far, this relationship has been demonstrated mainly in cardiomyocytes. On the one hand, ACh has been shown to protect against cardiac infarction and is dependent on generation of ROS. This ACh-induced production of ROS is dependent on PI3K and Src kinases activation and mitochondrial K(ATP) channel opening (Oldenburg et al., 2003). On the other hand, ACh has also been shown to modulate the cardiac redox environment and thereby suppress ROS generation during oxidative stress (Sun et al., 2014). ACh promotes ROS detoxification against hypoxia/reoxygenation-induced oxidative stress through Forkhead Box O3 (FoxO3a)/ Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) dependent superoxide dismutase. The reason for this discrepancy remains unclear. Whether this relationship exists in diabetic wounds and cancer is unknown.

5.3 Limitations of my studies

We recognize there are limitations to our current studies. While we are able to overcome some challenges using laser capture microdissection and microarray to study wound keratinocytes undergoing EMT, the minute amount of tissues and quality of RNA that can be obtained from a mouse remains a major technical hurdle. Consequently, we must pool laser-dissected samples from

multiple animals in order to obtain enough RNA for gene expression microarray analysis. Along the same line, we are unable to correlate the changes in the mRNA to protein level using western blot. To this end, we used HaCaT and Ker-CT cell lines as surrogates to investigate and decipher the mechanism by which ACh stimulates EMT. Clearly, these cells are unable to fully recapitulate the *in vivo* setting. It will be ideal to examine gene expression change using healthy and diabetic human wound biopsies, however, clinical settings and ethical challenges do not allow us to inflict a wound on healthy and diabetic patients, followed by the collection of biopsies to capture early events such as EMT. Majority of diabetic wound biopsies are already at different stages of wound healing. We monitored the change in ACh in wound exudate during 24 h post-injury. The drying of the wound and the formation of a scab over the wound prevented us from studying ACh levels beyond 24 h.

We also recognized specific limitations in our cancer study. We did not explore other tumor induction protocol such as UV irradiation or use FSPCre-*Pparb/d^{ex4}* mice from a different genetic background. The induction of skin cancer by chemicals (DMBA/TPA) involves different molecular mechanisms from those associated with human skin cancer attributable to chronic UV exposure (Matsumura and Ananthaswamy, 2002; Boukamp, 2005). The signalling pathways involving the tyrosine kinase Src, the tyrosine kinase receptor EGFR and the Erk1/2 MAPK cascade is activated during UV-induced skin inflammation and carcinogenesis, promoting initiation, progression and malignant conversion (Fritsche et al., 2007; Rho et al., 2011). In contrast, tumor initiation with 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 (Lieu et al., 1991; Pierceall et al., 1991; Campbell et al., 1993). It is conceivable that different mouse strains may exhibit different phenotypic severity or outcomes owing to differences in vulnerabilities to the oxidative stress and carcinogens. Fibroblast specific protein 1 is a key marker of a specific subset of macrophages in the liver during fibrosis and injury, although no report has described confounding issues in other organs (Osterreicher et al., 2011). Based on existing technology, it is not possible to achieve pure fibroblastic gene deletion nor any tissue-specific deletion with absolute certainty. Nevertheless, to the best of our knowledge, this is the first study to examine the role of fibroblast PPAR β/δ in NMSC.

5.4 Future directions

In the first study, we uncovered that ACh mediates EMT in keratinocytes during the early stages of wound healing through upregulating TGF β R2 through the Src-ERK signaling axis. P38 kinase is activated under hyperglycemic conditions and interferes with the activation of the Src-ERK axis, and inhibiting p38 brings about recovery of ACh signaling. In the short run, future studies should investigate whether other growth factors' activities are also primed through ACh, and if so, whether they are dysregulated in diabetic wounds. Also, since all the cells involved in wound healing possess ACh receptors, investigation into the roles and mechanisms of ACh in these cell types is warranted, both under the normal and diabetic wound contexts. Together, these studies may shed light on our limited understanding of diabetic wounds, and

allow for the development of better therapeutics. Acetylcholine-focused studies should also be carried out on other diabetic complications.

In our second study, we found that PPAR β/δ knockout in fibroblasts led to an increase in oxidative stress and subsequently, antioxidative responses in keratinocytes. The antioxidative responses acted as a form of anti-cancer protection, causing a delay in tumor initiation. To gain further insight into field cancerization, similar studies could be carried out on other transcription factors in fibroblasts and other stromal cells.

Non-healing diabetic wounds and cancer progression involve communication among multiple cell types. It is unlikely that the etiology of the two diseases resides in a particular cell type, but rather a miscommunication among the different cell types. It is evident that small biomolecules, growth factors, cytokines, among others, present in the microenvironment are critical mediators to effective autocrine and paracrine communications. As mentioned above, studies into small non-proteinaceous biomolecules are comparatively limited. In my work, I have examined the role of ACh, and ROS (H₂O₂) as signaling mediators in diabetic wounds and cancer, respectively. As mentioned above, the precise role ACh signaling in cancer development and progression warrant investigation. ACh has been reported to act as an autocrine growth factor for multiple human cancer types, such as lung, colon, and breast cancers. Recently, ACh was reported to induce stemness on diffuse gastric cancer cells (Nguyen et al., 2018).

Similarly, we should use technologies, such as ROS-responsive biomaterial scaffolds (Yao et al., 2019), to exploit the beneficial effect of ROS. ROS-responsive biomaterials have been identified as a type of promising

therapeutic substance to alleviate oxidative stress in tissue microenvironments due to their ability to interact with ROS molecules, leading to degradation or changes in solubility of the biomaterial.

Another small biomolecule that is of great interest is lipids. There are only a few studies that examine the composition and change in the lipidome of the wound and cancer microenvironment. Previous studies conducted on diabetic wounds showed that the type of lipids in the wound microenvironment could lead to the biosynthesis of either pro-inflammatory or pro-resolving lipid mediators, which then impacts the rate of wound closure through the modulation of inflammation (Tian et al., 2011a; Tian et al., 2011b). As tumor-promoting inflammation is an emerging hallmark of cancer, studying the lipid profile in the microenvironment of cancer could prove useful in the development of better therapies. For example, the ratio of phosphatidylinositol (4,5)-bisphosphate (PIP2) and phosphatidylinositol (3,4,5)-trisphosphate (PIP3) is essential for the activation of oncogenic PIK3 cascade, required for cell proliferation and directed migration in cancer and wound healing (Carnero and Paramio, 2014; Mkrtschjan et al., 2018). The introduction of specific fatty acids into the ECM has been shown to combat the effects of carcinogenic compounds (Gama et al., 2014). Furthermore, the transactivation activity of the nuclear receptor PPAR β/δ is modulated by fatty acids. Long-term future direction can be focused on studying the changes in the different types of biomolecules present in the microenvironments of both chronic wounds and cancer, particularly the inflammation response.

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