CHAPTER I: INTRODUCTION AND LITERATURE REVIEW

1.1. Acne

1.1.1. Introduction

Acne is characterized by chronic inflammation of the skin and comedones, which are cystic sebaceous glands.[1] Comedones are thought to arise from abnormally differentiated keratinocytes from the infundibulum, the distalmost area of the hair follicle which is connected to the sebaceous gland through a duct. It was also observed that the sebaceous gland undergoes atrophy in acne, yet sebum production is increased.[2] Mild acne is usually self-limiting. However, severe acne would leave scars behind. In general, scarring is caused by abnormal collagen remodeling and reorganization.[3]

Acne is ubiquitous in adolescence, with a prevalence up to 100% depending on the population studied. Acne usually resolves spontaneously after the age of 25. However, it could persist into adulthood in 1% of men and 5% of women. It is thought that 95% of acne patients would scar to some extent.[4,5]

1.1.2. Physical, Psychological and Economical Burden of Acne

Acne lesions are pruritic, painful, and cosmetically disfiguring.[6–9] The morbidity of severe acne inflicts a psychological burden.[10] Acne could bring about anxiety, self-consciousness, and depression, which in turn could lead to suicide ideation. This might be associated with the expectations of the 'flawless' skin portrayed by the media.[11] The quality of life associated with acne is comparable or even worse of that of patients suffering from coronary disease, diabetes, or epilepsy.[12]

In the United States, expenditure related to acne exceeds $2.5 billion annually.[11,13] An American study found that unemployment rates within acne patients (between 18 and 30 years old) are high. Furthermore, low performance in work/school has been associated with acne.[11] The most recent report by the American Academy of Dermatology reported $398 million is lost every day in

opportunity cost because of acne in the United States.[14] Money used for prescription drugs covers $1,740 million with the addition of $329 million on over-the-counter drugs annually.[15]

1.1.3. Acne Pathogenesis

Four factors classically implied in acne pathogenesis are keratinocyte hyperproliferation, hyperseborrhoea, inflammation, and *Cutibacterium acnes* (previously known as *Propionibacterium acnes*) colonization.[1,13,16] The comedo switch hypothesis postulates that the junctional cells (the cells that generate the infundibulum and sebaceous gland, located in the area where the sebaceous gland meets the hair follicle) favor increased proliferation of the infundibular keratinocytes over the sebocyte proliferation upon comedogenic stimuli such as *C. acnes* metabolites.[2,17] Sebum, the oily substance secreted by the sebaceous gland, is thought to protect the skin against friction and increases its resistance to moisture.[18] Receptors expressed in the sebaceous gland (such as androgen receptor) are thought to induce sebum production.[17] The inflammation occurring in acne is still a highly elusive field. It is noted that inflammation occurs either with or without the presence of *C. acnes*. However, which pathways activated are yet to be elucidated.[9] Furthermore, the metabolites of *C. acnes* are known to induce keratinocyte proliferation into microcomedones.[17]

Many factors and hypotheses have been suggested to create a picture of acne's elusive pathogenesis. However, there are still many puzzle pieces that are not found to create the big picture of acne's pathogenesis. The precise pathogenesis of acne and acne scarring has yet to be elucidated.[3]

1.2. Current Acne and Acne Scar Therapeutics

1.2.1. Acne Treatment

Mild acne could be effectively treated with topical agents, such as retinoids and/or benzoyl peroxide.[1,8] Topical antibiotics (such as tetracycline and erythromycin) that have inherent anti-inflammatory activity are used in acne for their elusive anti-inflammatory property, and thus bacterial

resistance would not affect the efficacy of the treatment. Antibiotics used in acne include the 30s ribosome inhibiting tetracyclines and the 50s ribosome inhibiting erythromycin and azithromycin.[8,10] However, usage of antibiotics, regardless, will risk antibiotic resistance. Most antibiotic treatments are coupled with topical benzoyl peroxide, a bactericidal agent, to prevent resistance. However, benzoyl peroxidase is known to cause skin irritation and is often inconvenient as is bleaches fabric.[8,19]

Moderate and severe acne should be treated with systemic drugs, especially if the acne lesions are not only limited to the face.[8] Combination of oral antibiotics and topical retinoids could be used for moderate to severe acne. If the acne is severe, as different compounds of different mechanism of action could help alleviate acne. Furthermore, it is recommended to avoid the use of oral antibiotic as monotherapy as it could cause bacterial resistance.[1]

Isotretinoin (13-cis-retinoic acid) has been a mainstay in acne treatment since its approval by the Food and Drug Administration in 1982. Retinoids treat acne by inhibiting the four known factors in acne pathogenesis: they reduce inflammation, *C. acnes* levels in the ducts of the sebaceous gland, comedogenesis, and sebum production.[20] It was found that higher cumulative doses are more effective in decreasing acne relapse risks.[21] However, despite its efficacy, isotretinoin treatment has serious side effects such as dry lips, xerosis, facial erythema, nose bleeds, cheilitis, and myalgia due to rhabdomyolysis. Furthermore, the use of isotretinoin in pregnancy is associated with teratogenicity.[22,23] Previously, depression and suicide ideation were tied with the use of isotretinoin; however, the issue has been controversial. Depression and suicide ideation side effect by isotretinoin is rare and idiopathic, though the close evaluation of patients performing isotretinoin treatment is still recommended.[21,22] A small study found that the efficacy of low dosage of retinoic acid was comparable with the normal dosage group. Although fewer side effects were observed in the low dosage group compared to those at a normal dosage, however, the study was performed in a small study, and thus a study in larger cohorts should be performed to further confirm the efficacy of low dose treatment.[24]

1.2.2. Acne Scar Treatment

Prevention of acne scarring by readily treating severe acne is preferred, mainly by mitigating the inflammation. However, most literature focuses on treating acne or acne scarring instead of scar prevention. Only 3 topical treatments have been tested: 0.1% retinaldehyde/6% glycolic acid combination cream, 0.025% retinoic acid/12% glycolic acid combination and 0.1% adapalene/2.5% benzoyl peroxide combination. These three topical are claimed to improve acne scarring.[25] However, it seems the improvement primarily encompasses erythema and hyperpigmentation, while the scars do not completely disappear.

1.2.3. A Need for New Drugs

Overall, no acne drug has a desired risk/benefit tradeoff due to severe side effects that it incurs. Furthermore, the literature on drugs preventing acne scars is limited.[25] This phenomenon illustrates that there is a need for new acne drugs. However, up to today, there are no *in vitro* or *in silico* assays for medium/high throughput screening for acne drug development; thus lack of assays leaves an unmet demand for the development of new preclinical models for acne and acne scarring to test new/existing pharmaceuticals for acne treatment.

1.3. New Scope in Studying Acne: Monogenetic Syndromes

1.3.1. Monogenetic Genodermatoses: A Puzzle Piece of the Whole Image

One way of unraveling the pathogenesis of acne is to piece together smaller fragments. As such, studying human monogenetic genodermatoses allows for the elucidation of the role of specific genes in the pathogenesis of more common disorders. This is best illustrated by insights gained in the pathophysiology of atopic dermatitis from studying the genodermatosis ichthyosis vulgaris (IV; OMIM #146700). IV is an autosomal dominant disease caused by a mutation in *filaggrin*. IV presents with dry and scaling skin.[26] Previously, the exact cause of IV was unknown; it was only until a decade ago that

a mutation in *filaggrin* was identified to be the cause of IV. This discovery has allowed the elucidation of filaggrin and its importance in epidermal barrier function.[27] Studying such monogenetic disease in a small population has allowed the observation of the defective gene's property and postulation of its effects in the general population. Not only did the filaggrin study elucidate filaggrin's physiological function, but it was also used to study other diseases associated with skin barrier abnormalities. It was then found that *filaggrin* mutations are also associated with atopic dermatitis.[26]

1.3.2. Monogenetic Diseases Associated with Acne

There are monogenetic acne-related diseases, such as Apert syndrome (OMIM #101200) and pyogenic arthritis, pyoderma gangrenosum, and acne syndrome (OMIM #604416). Although rare, these disorders are still more common than Winchester syndrome, a disorder of particular interest to the ASGP. Winchester syndrome (WS, OMIM #277950) is a rare genetic disease that affects the skin, heart, and bone. Two twins clinically diagnosed in 2007 had coarse a face with broad a forehead and prominent jaw, mitral valve prolapse, kyphosis, a hypertrophic surgical scar on the chest (following valvuloplasty) with comedones, and acne in the face, auricle and on the upper thorax.[28] Previously, the cause of WS was still elusive. However, genetic analysis revealed that WS patients are homozygous for a mutation in *MMP14*.[29]

Matrix metalloproteinases (MMPs) are the main enzymes that function to breakdown the extracellular matrix (ECM). There are 24 MMPs which collectively degrade the different molecules in the ECM.[3,30] The ECM contains proteins that contribute towards the flexibility, structure, cell organization, and regulation of tissues. Collagen is the most abundant protein in the ECM. There are 28 collagens in vertebrates. Fibrillar collagens I, III and V are expressed in the dermis; notably, 80% of all collagen expressed in the skin is collagen I. Aside from the dermis, collagens are also primarily found in the basement membrane. During wound healing, the loose ECM during remodeling shifts towards a dense ECM.[3] In humans, collagen deposition following wound healing is tightly aligned while collagen in an unwounded dermis has a random orthogonal pattern.[3,31] Furthermore, collagen I to

collagen III ratio may play a role in scar formation as it was observed that a ratio higher than 5:1 is observed in scars.[3]

1.4. Zebrafish

1.4.1. Zebrafish Use in Modelling Human Diseases

One way of studying gene function is through knocking genes out in animal models through genetic modifications.[32] Mice have been highly used as the classically most-used animal model for studying human diseases. However, there has been a demand for a replacement in mammalian models towards other alternative models.[33] Despite the evolutionary distance between humans and laboratory animals, varieties of genes and cellular processes are conserved. This phenomenon has been used to create model organisms for human diseases in organisms such as *Caenorhabditis elegans* (nematode) and *Drosophila melanogaster* (fruit fly). Such lower-level organisms could model diseases at a molecular level but not at a higher level.[34,35] However, the presence of organ systems (which are present in *C. elegans* and *D. melanogaster*, although only present as simple counterparts of some human organs) during modeling is crucial as most human diseases are tied to pathogenesis within organs.[35] Note that the skin in worms and flies only consist of simple epithelial cells with an outer cuticle layer.[36]

Another alternative model, *Danio rerio* (zebrafish), a vertebrate, has been used in the laboratory setting for the past decade.[33] Unlike *C. elegans* and *D. melanogaster*, zebrafish possess organs that are more functionally similar to mammalian organs, except for lungs and mammary glands.[37] Internal organs develop rapidly within the first two days of life.[34] Furthermore, human and fish skin architecture is rather similar. Similar to human skin, zebrafish skin consists of the outer epidermis and the underlying dermis and hypodermis; note that the zebrafish epidermis consists of multiple layers that are equivalent to the layers of the human epidermis.[38] Moreover, fish skin act as a physical and chemical barrier and temperature regulator as similar to human skin.[39,40] In humans,

the outermost layer of the skin (stratum corneum) consists of a keratinized layer of terminally differentiated cells. Zebrafish skin lacks a keratinizing epidermis but instead is covered by a mucous layer.[41] Furthermore, zebrafish do not have pilosebaceous units but instead have other specialized units such as mucous cells, alarm cells, and scleroblasts that creates the dermal scales.[39,41] These factors limit the studies of terminal epidermis differentiation (as zebrafish lack genes regulating epidermis differentiation) and processes associated with the sebaceous gland in zebrafish. However, melanocytes are present in fish, thus allowing pigmentation research in zebrafish.[41]

The zebrafish is a well-documented model for metabolic disease, inflammation, infection, and cancer (as summarized in Patton & Tobin, 2019).[42] There has been a 909% increased use of overall alternative *in vivo* (including zebrafish) and *in silico* models from 1990 until 2015.[33] The zebrafish is a highly favored organism as it has a cheap maintenance cost, transparent embryos that develop outside of the mother's body, and it consistently generates more offspring compared to mice. Transparency allows evaluation of biological processes occurring during early development.[34] Moreover, mice have a longer life span.[43]

It is calculated that 71.4% of human genes have zebrafish counterparts. Of the 3,176 genes associated with disease recorded in the Online Mendelian Inheritance in Man (OMIM), 82% has a zebrafish ortholog, making zebrafish a tractable model for forward and reverse genetic screening.[44] One thing to note is that, duplicative genes is a feature of the zebrafish genome as they undergo several genome duplications throughout its evolution. Most duplicated genes are either lost or silenced, but some duplicated genes are functional and may function differently or have different expression distributed thought the organism.[45]

During drug discovery, usage of animal models allows the evaluation of signaling pathways and crosstalk between tissues that are not assessable with cell-based evaluations. Currently, we could only rely on animal models to evaluate the effects of drugs with a high degree of application towards humans.[46] There are two approaches, screening the phenotype asserted due to the drug library or screening the ability of chemical libraries to reverse a disease phenotype.[34]

1.4.2. The Past and Present of Zebrafish Use in Genetics

Early forward genetic screening in zebrafish utilized random mutagenesis (e.g., with N-ethyl-N-nitrosourea), after which individuals with aberrant phenotypes were genetically characterized to identify the causative gene mutation. However, with random mutagenesis, there is a need to seek the altered gene, which is time and cost consuming, and thus, a more targeted approach was sought out.[47,48]

Techniques for reverse genetics, causing a targeted mutation to generate a model with hypothesized the disease phenotype, were subsequently developed.[48] Morpholino oligonucleotides (MOs) were used to specifically knock-down a specific gene. However, it was shown that MOs cause p53-mediated neurotoxicity. The use of MOs would result in phenotypes that are unrelated to the targeted gene and mask the actual result. Although there are many ways to avoid such side effects (as discussed in Eisen & Smith, 2008),[49] MOs are only active until ~50 hours post-fertilization, and thus most research with MOs is limited to early stages of development.[50]

The rise of targeted nuclease technologies such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and CRISPR has led to the targeted editing of the zebrafish genome. CRISPR is preferred over ZFNs, and TALENs as the engineering of ZFNs and TALENs is intricate compared to the easily programmable CRISPR.[47] CRISPR was shown to be highly specific with an off-target rate of around 1-3%. Furthermore, off-target mutations could be segregated through breeding.[51]

1.4.3. Modelling Winchester Syndrome in Zebrafish

MMP14 is conserved between humans and zebrafish. Thus it was possible to create a zebrafish *mmp14* knockout (KO) model to study WS. It was found that *mmp14* KO fish generated by CRISPR (clustered regularly interspaced short palindromic repeats) primarily recapitulate the skeletal phenotypes of the WS patients. Zebrafish with mutated *mmp14* had a shorter body length and head,

exophthalmia, thoracic kyphosis, reduced bone mineral density, and a shorter life span when compared to wild type fish. Furthermore, abnormal collagen remodeling was found in several areas of the fish, including the bone, thus reflecting the abnormal collagen deposition seen in human patients. However, no remarkable cutaneous or cardiac phenotype was observed in the zebrafish model.[5]

1.4.4. The Pretzel Zebrafish Model

After the WS zebrafish model, the Acne and Sebaceous Gland Programme (ASGP) from the Skin Research Institute Singapore (SRIS) have created additional zebrafish models, that are deficient of proteins functionally linked with *MMP14*, using the CRISPR/Cas9 system. One particular zebrafish that has an astounding skin phenotype is the zebrafish canonically deficient of Przl. The gene that causes such phenotype will be called *pretzel* (*przl*) hereafter due to confidentiality. The term "pretzel" was chosen because the skeletal phenotype present in the mutant zebrafish involves folding of the body axis. Patients with such mutation were reported to have skeletal malformations, contractures, and severe acne. The zebrafish in which *przl* was canonically knocked out will be termed the "Pretzel model" (unpublished data).

Initial observation of the Pretzel model included the severe body axis bending accompanied by an abnormal swimming pattern at adult age (Supplemental fig. 1). The observed abnormal swimming pattern was the result of the limited range of motion in the pectoral and dorsal fins. On close inspection, it was revealed that the fins, most notably in the anterior base of the dorsal fin, has a white spot (Supplemental fig. 2). The opaque white spot was suspected to be fibrotic (unpublished data).

1.4.5. The Pretzel Model as a Preclinical Model for Acne Scarring Drug Discovery

It is previously mentioned that zebrafish do not have sebaceous glands and thus are unable to model acne. Furthermore, unlike humans, zebrafish typically do not form scars, even when

wounded. Collagen deposition in wounded zebrafish would develop to resemble an unwounded zebrafish skin as it heals.[52] Typically, collagen fibers in the dermis are arranged in an orthogonal pattern.[31] However, the Pretzel model undergoes spontaneous fibrosis, whereas no literature has described such phenomenon in zebrafish. The occurrence of the abnormal collagen deposition in the Pretzel model brought about the hypothesis that the *przl* mutant could be leveraged as a new acne scarring drug discovery model. Abnormal collagen deposition, as seen in WS, is thought to have cause acne and scarring in WS patients.[5] Since the process intended to be observed is not acne per se but the process in which collagen abnormally deposits, the Pretzel model is hypothetically usable as a preclinical model for acne scarring. Moreover, the aim of the Pretzel model is not to create a model that could reverse the preexisting scar, but rather screen therapeutics that could prevent fibrosis.

It is important to assess whether the scarring is inducible at an earlier stage by wounding. If the collagen deposition post-wounding were measurable in larvae, it would render a cheaper assay as smaller volumes of drugs would be needed to treat larvae kept in petri dishes, as compared to treatment of adult zebrafish in larger tanks. Furthermore, analyzing inducibility of scarring after tail fin wounding was tested as tail wounding is a common procedure done towards zebrafish and is previously well-documented.[53,54] Inducibility is an important factor to explore, as the exact cause and rate to which the fish spontaneously undergo fibrosis is unknown and thus drug treatment would need to be commenced at an early stage up until the 5-month time point. This will result in an expensive and time-consuming assay. If the scar is inducible at the adult phase, treatment will commence only after wounding and fish at 3-4 weeks post-wounding, thus rendering a cheaper and less labor-intense assay.

1.5. Aims and Objectives

The aim of the research is (i) to formally assess the phenotype present in the pretzel model and (ii) to characterize the potential of the pretzel model as a drug discovery assay through validating whether the fibrosis could be induced by wounding at an earlier stage (as the spontaneous fibrosis

only occurs at a late stage), either at the larval stage (10 days post-fertilization) or the adults stage (5 months post-fertilization).