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**EFFECTS OF BMP SIGNALLING IN
REGULATION OF MELANOGENESIS,
PIGMENT TRANSFER AND
MELANOCYTE MIGRATION**

by

LIM WEE HAN JAMIEN

Lee Kong Chian School of Medicine

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by

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

2021

Statement of Originality

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A handwritten signature in black ink, appearing to read 'Carlos Clavel', written over a horizontal line.

Carlos Clavel, PhD

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- I prepared the manuscript draft.
- Joy assisted in the manuscript drafting and edited the drafts.
- The manuscript was revised by Dr Clavel.
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Lim Wee Han Jamien

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ABBREVIATIONS

ACTH	Adrenocorticotrophic Hormone
ActR	Activin receptor
APOE	Apolipoprotein E
ATP6V0B	V-type proton ATPase 21 kDa proteolipid subunit
α -MSH	Alpha Melanocyte-stimulating Hormone
BMP	Bone morphogenetic protein
BMP-R	Bone morphogenetic protein receptor
BMP4	Bone morphogenetic protein 4
BMP6	Bone morphogenetic protein 6
BP	Biological process
BPE	Bovine pituitary extract
BSA	Body surface area
CTHRC1	Collagen triple helix repeat containing 1
CTS	Connective tissue sheath
CTSL	Procathepsin L
CO-SMAD	Common-partner Smad
CP	Cellular process
CPM	Counts per million
CYBA	Cytochrome B-245 alpha chain
DAPI	4',6-diamidino-2-phenylindole
DEG	Differentially expressed genes
DP	Dermal papilla
DN	Double negative
DOPA	Dihydroxyphenylalanine
DOPACHrome	Dihydroxyphenylalanine-chrome
DQ	Dihydroxyphenylalanine –quinone
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
FACS	Fluorescence-activated cell sorting
FGF-5	Fibroblast Growth Factor-5
FM	Fontana Masson
GO	Gene ontology
GPX4	Glutathione peroxidase 4
H&E	Hematoxylin & Eosin
HF	Hair follicle
HGF	Hepatocyte Growth Factor
HMGS-2	Human melanocytes growth supplement-2

HR	Hairless
ID	DNA-binding protein inhibitor
IFE	Interfollicular epidermis
IGF1	Insulin-like Growth Factor 1
IRS	Inner root sheath
K14/ KRT14	Keratin 14
K15	Keratin 15
KC	Keratinocytes
Kitl	KIT ligand
KEGG	Kyoto encyclopedia of genes and genomes
KSFM	Keratinocyte serum free media
MAPK	Mitogen-activated protein kinase
MC1R	Melanocortin receptor-1
Me	Melanocytes
MeSC	Melanocyte stem cell
MITF	Microphthalmia-associated transcription factor
MyoX	Myosin X
NB-UVB	Narrow band-ultraviolet B
NCC	Neural crest cells
NSV	Nonsegmental vitiligo
N/TERT	Immortalised normal telomerase reverse transcriptase
ORS	Outer root sheath
OCT	Optimal cutting temperature
PBS	Phosphate-buffered saline
PCA	Principal component analysis
PI	Protease inhibitor
PMEL	Premelanosome protein
PRDX2	Peroxiredoxin 2
PTGDS	Prostaglandin-H2 D-isomerase
PUVA	Psoralen Ultraviolet A
QPCT	Glutaminy-peptide cyclotransferase
qRT-PCR	Real-time quantitative reverse transcription
R-SMAD	Receptor-regulated Smad
RAB38	Ras-related protein Rab-38
ROS	Reactive oxygen species
RPS3	Ribosomal protein S3
S100A1	S100 calcium-binding protein A1
SCF	Stem cell factor
SEM	Scanning electron microscope

SHH	Sonic Hedgehog
SLC24A5	Solute carrier family 24 member 5
SOSTDC1	Sclerostin domain containing 1
SV	Segmental vitiligo
TDT	Tdtomato
TGF- β	Transforming Growth Factor- β
TM4SF1	Transmembrane 4 L6 family member 1
TYR	Tyrosinase
TYRP1	Tyrosinase-related protein 1
TYRP2/ TRP2	Tyrosinase-related protein 2
MART-1	Melanoma-associated antigen recognized by T cells
MGST1	Microsomal glutathione S-transferase 1
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVR	Ultraviolet radiation
VAMP8	Vesicle-associated membrane protein 8

ABSTRACT

Vitiligo is a severe skin depigmentation disorder due to its visible nature and psychological burden. UV-induced migration of melanocytes can be achieved by phototherapy to trigger interfollicular re-pigmentation. However, this process is costly and requires repeated UVB exposure. To unravel the possibility in enhancing the treatment, we utilized our *in vitro* models to study the role of BMP signalling in melanocyte function. Here, we observed that BMP4 decreases both melanin production and migratory rate of melanocytes. To determine the functional role of BMP signalling in follicular re-pigmentation *in vivo*, we UV-induced melanocyte migration into the interfollicular epidermis (IFE) using a novel mouse model and subsequently quantified it with histological staining and melanin assay. We found that ablation of BMP4 results in the enhancement of melanocyte migration and managed to validate it by rescuing the phenotype. Finally, to elucidate the molecular mechanism, we isolated the MeSC from our mouse model and performed whole transcriptome analysis. Through the analysis, we identified candidates involved, with some candidates having direct transcription regulation by BMP4. Overall, we discovered the role of BMP signalling in regulating melanin concentration and melanocyte migration.

INTRODUCTION

Vitiligo: a skin pigmentation disorder

Vitiligo is one of the most common skin depigmentation disorders, with a prevalence of 0.5 – 2% of the population in both children and adults (Alikhan et al., 2011; Boisseau-Garsaud et al., 2000; Howitz et al., 1977; Krüger & Schallreuter, 2012). Moreover, vitiligo affects people of different ethnic groups, skin types, and genders without predilection (Alkhateeb et al., 2003; Ezzedine et al., 2015; Picardo et al., 2015). However, there are evidence of large geographic differences in terms of the prevalence rate of vitiligo. For example, the prevalence of vitiligo in Shaanxi Province of China is reported to be 0.093% (Lu et al., 2007), in contrast, certain parts of India are reported to have a high prevalence rate of 8.8% (Behl & Bhatia, 1972; Sehgal & Srivastava, 2007). The high prevalence may be due to the inclusion of cases where depigmentation is caused by chemicals and toxins (Sehgal & Srivastava, 2007). In addition, the high disparity in prevalence data may be due to lesions being more prominent in individuals with darker-skinned tones or higher reporting incidences in regions with extensive social and cultural stigma (Sehgal & Srivastava, 2007). Vitiligo is categorized into two major groups: segmental vitiligo (SV) and nonsegmental vitiligo (NSV). With regards to the classification, vitiligo is used to define all types of NSV, which includes mucosal, acrofacial, generalized, universal, mixed, and rare variants. On the other hand, SV includes unisegmental, bi-, or multisegmental and is differentiated from all other forms of vitiligo mainly because of its prognostic repercussions. NSV occurs throughout all ages, with higher occurrence between ages of 10 and 30 years. In contrast, SV occurs at a younger age as compared to NSV, with 41.3% of cases developed before the age of 10 years (Hann & Lee, 1996). Hann and Lee reported that the mean age of vitiligo onset was 15.6 years, with the earliest being immediately after birth and the latest was 54 years (Hann & Lee, 1996). Vitiligo is characterized by the appearance of white patches on the skin, which result from the loss of functional

epidermal melanocytes (Dell'anna & Picardo, 2006; Ezzedine et al., 2012; Le Poole et al., 1993; Rodrigues et al., 2017; Schallreuter et al., 2008). The exact main cause of the following disease remains to be elusive. However, multiple mechanisms were proposed for the destruction of melanocytes in vitiligo. These mechanisms include autoimmune-mediated responses and oxidative stress.

In the case of oxidative stress, it is suggested that the destruction of melanocytes is initiated by oxidative stress (Dell'Anna et al., 2001; Jimbow et al., 2001; Maresca et al., 1997; Speeckaert et al., 2018). Moreover, vitiligo patients displayed higher susceptibility to oxidative stress than unaffected individuals (Puri et al., 1987). In response to stress, reactive oxygen species (ROS) are released from melanocytes, resulting in an imbalance in the antioxidant system. This imbalance affects and increases the sensitivity of melanocytes to external pro-oxidant stimuli, subsequently resulting in the induction of a presenescent state (Kostyuk et al., 2010; Maresca et al., 1997; Morrone et al., 1992). In addition, the buildup of ROS leads to various detrimental cellular effects like DNA damage, protein oxidation, and fragmentation which results in the impairment of cellular function (Bickers & Athar, 2006; Dell'Anna et al., 2007). Moreover, melanin production consumes energy and generates a pro-oxidant state in the skin (Denat et al., 2014). The resulting oxidative stress causes tyrosinase-related protein 1 (TYRP1) to produce toxic melanin intermediates. In addition, oxidative stress causes modification of dihydropteridin reductase and results in the buildup of hydrogen peroxide and reduction in catalase levels, further contributing to cellular death (Hasse et al., 2004; Jimbow et al., 2001). Furthermore, vitiligo patients are found to have altered mitochondrial functionality. This leads to an increase in mitochondrial malate dehydrogenase activity and alteration in membrane lipid components (Dell'Anna et al., 2003, 2007; Jimbow et al., 2001). These constitute an impairment in the function of membrane lipids and cellular proteins and together with the promotion of calcium influx into the cell, facilitate apoptosis of melanocytes (Kang et al., 2018, p. 2).

With regards to autoimmune responses, both innate and adaptive aspects of the immune system appear to be involved (Bergqvist & Ezzedine, 2020), in which the epidermal melanocytes were destroyed which resulted in the loss of skin pigmentation (Ianella et al., 2016). In vitiligo, innate immunity connects the fissure between oxidative stress and adaptive immunity as it appears that innate immunity is activated early in vitiligo by sensing the stress signals released from melanocytes (Schallreuter et al., 2008; van den Boorn et al., 2011; Yu et al., 2012). This is evident from the genomic expression analysis of skin from vitiligo patients, as it emphasized an upregulated innate immunity in the local microenvironment of melanocytes.

In addition, natural killer cells were noticed to have infiltrated clinically normal skin of vitiligo patients, signifying the possible role of natural killer cells as early responders to the stressor signals from melanocytes (Yu et al., 2012). Both humoral and cell-mediated immune irregularities are involved in the development of vitiligo. This is evident as antibodies from the sera of vitiligo patients are found to induce the destruction of melanocytes via complement-mediated lysis and antibody-dependent cellular cytotoxicity (Naughton et al., 1983; Norris et al., 1988; Ongenae et al., 2003). Cytotoxic CD8⁺ T cells infiltrate the epidermis and dermis, subsequently targeting and destroying melanocytes. Moreover, past researches showed the correlation between the number of cytotoxic CD8⁺ T cells and vitiligo activity, further indicating the involvement of cytotoxic CD8⁺ T cells in the pathogenesis of vitiligo (Benzekri & Gauthier, 2017; Ogg et al., 1998; Wańkiewicz-Kalińska et al., 2003). On the other hand, depletion of cytotoxic CD8⁺ T cells leads to the inability to induce cytotoxicity and apoptosis, whereas the potency increases when CD8⁺ purified population is applied (van den Boorn et al., 2009). Destruction of melanocytes was observed to be related to the existence of cutaneous lymphocyte antigen-positive T cells and to date, the identified antigenic proteins of melanocytes in vitiligo include tyrosinase, tyrosinase-related proteins 1 and 2 (TYRP-1 & TYRP-2), gp100, and Melan-F/MART-1 (H. Xie et al., 2016). Vitiligo patients frequently harbour

melanocyte-specific antibodies and T-cells can be found surrounding the basal layer melanocytes, which eventually lead to the progressive destruction of melanocytes and thus impairing the pigmentation process (Richmond et al., 2013). In addition to autoimmune-mediated destruction, it was reported that certain chemical treatments and mechanical stress may also trigger the loss of epidermal melanocytes (Schallreuter et al., 1999; Schallreuter et al., 2008; Dammak et al., 2009). These external stressors that could lead to loss of epidermal melanocytes include sunburn and exposure to phenolic chemicals all of which leads to the induction of oxidative stress in melanocytes (Manga et al., 2016).

Currently available treatment options for vitiligo

In most cases, repigmentation of vitiligo is attainable with the assistance of several melanocytes stimulating treatment. Currently, several treatment options are clinically available to treat vitiligo and generally, this can be divided into two groups, non-surgical and surgical treatments (Felsten et al., 2011; Taieb et al., 2013). Non-surgical treatment options include topical treatments, systemic medications, and phototherapy. While on the other hand, surgical treatment options include a rather invasive approach such as skin grafting. The treatment options that are used to treat vitiligo will depend on the degree of severity as well as the percent of body surface area (BSA) that is affected by the lesions.

I. Topical treatments

Topical treatments are mainly used as the first-line treatment for minimizing disease progression and have been regarded as the most effective therapy producing favorable clinical outcomes (Felsten et al., 2011; Garg et al., 2010). Several agents are incorporated for topical treatment of vitiligo, these include the application of topical corticosteroids, calcineurin inhibitors, and calcipotriene which is a vitamin D₃ analog (Rodrigues et al., 2017). Among these topical

agents, the application of topical corticosteroids is shown to be the most effective in inducing repigmentation compared to the other topical agents. A retrospective study involving 101 children suffering from vitiligo treated with topical corticosteroid showed favorable outcomes, where 64% of the patients showed signs of repigmentation (Kwinter et al., 2007). However, despite the favorable outcomes, long-term applications of topical corticosteroids are still associated with several undesired side effects. It is reported that continuous application of topical corticosteroids may induce epidermal atrophy, telangiectasia, steroid folliculitis, and to a greater extent may lead to the occurrence of other side effects associated with system absorptions (Kwinter et al., 2007). Therefore, with that being said, this raises the issues for long-term use of topical corticosteroids for the treatment of vitiligo whether the related side effects will outweigh the outcome.

II. Phototherapy

Phototherapy is one of the most potent melanocyte stimulants for repigmentation of vitiligo, and this particular treatment involves direct exposure of either UVA or narrowband UVB (NBUVB) targeted directly towards affected areas (Mohammad et al., 2017; Osawa, 2008). This treatment is considered as the second line of therapy for vitiligo when treatment with the first line of therapy is unresponsive. In general, phototherapy induces repigmentation mainly by promoting melanocyte migration and proliferation, the melanin-producing cells (Wu et al., 2004; Wu et al., 2007). As mentioned previously, phototherapy can be done by either UVA where in most cases it is given in convergence with photosensitizer psoralen (PUVA), or by narrowband UVB (NBUVB) phototherapy (Bae et al., 2017).

Figure 1. Cellular events during vitiligo and skin repigmentation. Illustration of cellular events in vitiligo and phototherapy to repigment the skin. Phototherapy utilizes UV to stimulate the MeSCs to form pigment islands on the skin.

The former, PUVA, is the initiator of the phototherapy regimen for vitiligo and has been implemented as the mainstay since it was first introduced in 1948 (Bae et al., 2017). For years, PUVA phototherapy has shown promising results where patients receiving the following treatment showed a good prognosis indicated by faster repigmentation. In addition to that, the addition of psoralen to the regimen is also reported to significantly enhance the response rate as high as 78% in affected areas such as the head and neck (Felsten et al., 2011). However, despite the effectiveness of PUVA in inducing repigmentation, the application of PUVA therapy possesses several disastrous side effects that could be detrimental to the overall patient's health. For instance, continuous and long-term exposure to PUVA may lead to an increased risk of contracting skin cancer and other less extreme side effects such as nausea and phototoxicity (Pacifico &

Leone, 2011). Furthermore, PUVA phototherapy is not advised to be used for children and pregnant women due to the addition of psoralen.

Due to the following limitations of PUVA phototherapy, NBUVB has then slowly replaced the use of PUVA since it was introduced as another effective treatment for vitiligo in 1997 (Westerhof, 1997). The use of NBUVB is reported to be relatively safe in both children and adults when compared to PUVA phototherapy since NBUVB does not include the use of photosensitizer, psoralen, and thus emerged as the initial treatment of choice. Moreover, NBUVB is also given at a lower dose as compared to PUVA and thus this reduces the possible adverse effects. However, there are still several side effects associated with NBUVB, such as erythema, itching, and mild burning. Nonetheless, these effects that accompany NBUVB are considered to be less severe than that of PUVA and they are usually well-tolerated and are only present a few hours after the treatment (Bae et al., 2017).

However, even though phototherapy is considered the golden standard for the treatment of vitiligo, successfulness for repigmentation largely depends on the availability of functional melanocytes (Falabella, 2009). In addition to that, the process that it takes for repigmentation to occur via phototherapy is considered to be rather arduous and time-consuming. In this case, it requires the patient to be continuously exposed to phototherapy for a fixed period. A study conducted by Bae et al., reported that in order to achieve maximal treatment response, phototherapy must be conducted for at least one year (Bae et al., 2017). Furthermore, the limitation of phototherapy is amplified due to the variable clinical outcome among patients, further emphasizing the need to develop a novel therapeutic strategy for vitiligo.

III. Surgical treatment

Surgical treatment is perhaps considered as the last resort treatment option for treating vitiligo when cosmetically pleasing results are not achieved by non-surgical treatments. This particular treatment option however should only be reserved for individuals suffering from segmental vitiligo cases, where the lesions are more stable (Dillon et al., 2017). Several procedures fall under the surgical treatment options and these include, needling, skin grafting, and melanocyte suspension transplantation (Dillon et al., 2017; Felsten et al., 2011; Mulekar & Isedeh, 2013). Needling is considered to be the least invasive among the other surgical approaches. The following procedure involves the relocation of the remaining viable and functional melanocytes from the patient into depigmented areas of the skin. This relocation of melanocytes aims to serve as a reservoir for melanogenesis (Dillon et al., 2017). On the other hand, skin grafting involves a rather complex procedure where a small section of normal skin from the patient is transferred and transplanted to the targeted sites to facilitate repigmentation of the affected areas (Jill Feetham et al., 2012). Melanocyte suspension transplantation is much more complex and involves the harvesting of autologous tissue from a potential donor site, followed by releasing the cells into a suspension and then transplanting it to the targeted areas (Ramos et al., 2017; Sahni et al., 2011).

Even though surgical treatment appears to be the ultimate solution to treat vitiligo in cases where nonsurgical treatment appears to be ineffective, not all vitiligo patients are eligible to undertake this particular treatment. There are several limitations to the following treatment approach, for instance, surgical treatments are rather expensive, time-consuming, and require experienced staff to ensure a

successful outcome (Mulekar & Isadeh, 2013). Furthermore, the state and condition of vitiligo lesions also determine whether the patient is eligible for the procedure. Grafting procedure for instance is only limited to small-to-medium-sized lesions, usually from 250cm² or less, and therefore lesions larger than the following size are not eligible to undergo grafting procedure (Mulekar & Isadeh, 2013). Other criteria that determine whether a patient is suitable to undertake a surgical approach include the stable and constant size of lesions for at least six months, no appearance of new lesions during the following period, and lastly no history of scarring-related conditions such as keloids and koebnerization (Mulekar & Isadeh, 2013).

Skin not only acts as an organ of protection but also has a vital role in our interaction with the world. The visible nature of skin depigmentation disorders like vitiligo affects the psychosocial development of patients, largely due to the stigma created over these disorders (Elbuluk & Ezzedine, 2017; Ezzedine et al., 2015; Kostopoulou et al., 2009; Picardo et al., 2015; Porter et al., 1979). Since ancient times, people suffering from vitiligo were considered unsuitable for marriage, and the emergence of it can be considered as a defect in marriage and a reason for divorce. Vitiligo patients often have many psychological issues like anxiety, depression, and shame which are derived from social isolation and low self-esteem (Kostopoulou et al., 2009; Porter et al., 1979). Vitiligo is evident in impairing the quality of life in children with vitiligo and has a long-lasting impact on their self-esteem, and possibly accompanied by severe psychological trauma (Ingordo et al., 2012; Parsad et al., 2003). Overall, vitiligo is not simply a cosmetic disease that affects only the outlook of a person but also brings about psychosocial stress and various psychiatric comorbidities (Picardo et al., 2015). In addition, the significance of addressing vitiligo is further intensified as the

golden standard treatment of vitiligo, phototherapy, requires repeated exposure and has variable clinical outcomes among patients. Therefore, it is of paramount importance to understand more about the mechanism involved in repigmentation and develop novel therapeutic strategies.

The Skin

I. Anatomy of the skin

Skin is known as the largest organ of the body, where it covers the entire body's exterior. It is reported that the skin accounts for approximately 12% of the total body weight of a typical adult, earning its name as the largest organ of the body (Kolarsick et al., 2011). The skin itself is often considered as one of the body's most intricate organ, because it is made up of multiple layers of tissues that work in unity as a single structure to execute a variety of distinctive and critical functions. In general, the skin carried out many indispensable functions that are detrimental to a person's well-being, including acting as a protective barrier against external physical, chemical, and biological hazards, preventing excessive water loss from the body, and last but not least thermoregulation (Wickett & Visscher, 2006). Due to the following factors, skin along with its derivative structures is denoted as the integumentary system in humans protecting against various external stressors.

Generally, the skin is segregated into three distinct layers, the epidermis, dermis, and hypodermis, which is also often referred to as the subcutaneous tissue (Lai-Cheong & McGrath, 2017). The epidermis is known as the superficial or outermost layer of the skin that is predominantly populated by a specific cell type known as the keratinocytes, constituting almost 95% of the epidermal layer of the

skin (McGrath et al., 2004). Keratinocytes produce a long, threadlike protein referred to as keratins, which are a major structural protein of the epidermis. Apart from keratinocytes, the epidermis also harbours many miscellaneous cell populations, including melanocytes, Langerhans cells, and Merkel cells. Unlike keratinocytes, these other miscellaneous cell populations populate the epidermal layer in much lesser numbers, where they constitute the remaining 5% of the total cell population.

Primarily, the epidermis is divided into four distinct layers, and depending on the anatomical location of the body, sometimes it can be divided further into five discrete layers (Nafisi & Maibach, 2018). Skin, whose epidermal layer is divided into four distinct layers is referred to as “thin skin” or thin epidermis, usually covering areas such as the eyelids. Generally, most of the skin is classified as “thin skin”. The four layers of thin epidermis from deepest to the superficial layer are the basal cell layer (*stratum basale/germinativum*), the squamous cell layer (*stratum spinosum*), the granular cell layer (*stratum granulosum*), and the cornified or horny cell layer (*stratum corneum*) (Kolarsick et al., 2011). On the other hand, those with five layers are referred to as “thick skin” or thick epidermis, and these types of skin are only found in certain areas of the body such as palms and soles of the feet. The four layers that are found in the thin epidermis are also present in the thick epidermis, except that in the thick epidermis one additional cell layer is present between the *stratum corneum* and *stratum granulosum*. This additional cell layer is identified as *stratum lucidum* (Nafisi & Maibach, 2018).

The dermis, a layer located in between the epidermis and hypodermis, is regarded as the core of the integumentary system. Unlike the epidermis that is subdivided into four layers, the dermis is comprised of only two distinct layers, which are the papillary and reticular layer,

the former being the thinner layer and the latter being the thicker layer (Lai-Cheong & McGrath, 2017). The thin papillary dermis is the upper layer located just right below the dermal-epidermal junction, an interface between the epidermis and dermis, while the thick reticular dermis is the deeper layer. The dermis is predominantly populated by fibroblasts, a specialized cell type of connective tissue that synthesizes structural protein namely collagen. Collagen itself is considered as an eminent structural protein that is not only essential for the skin but also the entire body where it comprises almost approximately 70% of the skin's dry weight (McLafferty, Hendry, & Farley, 2012). In other words, collagen is regarded as a major stress-resistant constituent of the skin, providing robust strength to the overall skin structure preventing them from collapsing. Other than collagen, fibroblasts also produce other types of protein known as elastin. As its name suggests, elastin is a type of protein present in the skin that possesses elastic properties providing the skin with its elasticity and flexibility (Kolarsick et al., 2011). Other skin components that are found within the dermis include sweat glands, hair roots, blood and lymphatic vessels (Kolarsick et al., 2011).

The innermost layer of the skin, hypodermis, is mainly comprised of lipocytes or more commonly referred to as adipocytes. These fat cells mainly function to store energy in the form of fats. Besides acting as an energy reservoir, these fats at the same time also possess endocrine function that in turn aids in appetite regulation as well as metabolic energy control (Lai-Cheong & McGrath, 2017). Some components of the dermis also extend up to the hypodermis thereby fortifying the link between these two layers of the skin.

II. Melanocytes: the key player in melanogenesis

Skin pigmentation is a complex process that involves several different types of cells, such as keratinocytes and melanocytes. Keratinocytes are the most predominant cell type constituting almost 90% of the epidermal layer of the skin, providing skin structure and playing a role as the protective barrier against external stress (Costin & Hearing, 2007; McGrath et al., 2004). On the other hand, melanocytes are the pigment-producing cells that are responsible for providing the skin's color. In addition, melanocytes play a crucial role in protecting against harmful ultraviolet radiation (UVR), as they produce pigment which serves as a photoprotection for the keratinocytes (Andrzej Slominski et al., 2004). With that being said, melanocytes are considered the main cell type that is involved in pigmentation. Unlike keratinocytes, melanocytes are localized in the bottom layer of the skin's epidermis and they are also present in the hair follicles (HF). In addition, melanocytes are found in many other locations like the nervous system, inner ear, and heart (Brito & Kos, 2008; Tachibana, 1999). It is important to note that the development of melanocytes consists of numerous steps including lineage specification from melanoblasts, migration and proliferation of melanoblasts, followed by differentiation and maturation into melanocytes (Ernfors, 2010; O'Rahilly & Müller, 2007).

Melanocytes reside in the basal epidermal layer, where one melanocyte is associated with 30 – 40 keratinocytes, forming the epidermal melanin unit in the skin (Cichorek et al., 2013; Fitzpatrick & Breathnach, 1963; Mayer, 1973; Nishimura, 2011; Nishimura et al., 1999). In general, the ratio of melanocytes to keratinocytes in the epidermal basal layer is 1:10, whereas in the HF the ratio of melanocytes to keratinocytes is 1:5 (Cichorek et al., 2013; Slominski

et al., 2005). The size of the epidermal melanin unit is uniform in humans but varies between body parts. With that being said, melanocytic dendritic arrangement and intensity of melanin production are responsible for determining racial distinction (Miyamura et al., 2007). To date, the mechanisms involved in maintaining the organization remain elusive. However, it is suggested that melanocytes, keratinocytes, and fibroblasts converse via secreted factors and cell-cell interactions (Y. Yamaguchi et al., 2007). Through the system of cell adhesion molecules and paracrine growth factors, keratinocytes can control the growth and activity of melanocytes (Haass & Herlyn, 2005; A.-Y. Lee, 2012). This in turn regulates melanocyte proliferation, melanogenesis, and formation of melanocytic dendrites and in overall, plays a part in the maintenance of skin homeostasis (Sulaimon & Kitchell, 2003). Besides internal factors, external factors like UVR are known to influence the secretion of factors by keratinocytes which in turn affects the biological activities of melanocytes (Hirobe, 2011; H. Y. Park et al., 2009). The ability to perform melanin synthesis as well as their neural crest cells (NCC) origin is considered as the hallmark of melanocytes (Plonka et al., 2009). Melanocytes are molecularly identifiable by the presence of many melanocyte-specific proteins including tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and 2 (TYRP2), melanosomal matrix proteins (PMEL17, MART-1), as well as microphthalmia transcription factor (MITF) (Plonka et al., 2009).

In general, the pigmentation process is initiated with the production of melanin, in a process known as melanogenesis. Various enzymes play a role in the production of melanin, including TYR (Tyrosinase), TYRP1, and TYRP2, all of which play an implicating role throughout the whole melanogenesis process (D'Mello et al., 2016). Melanogenesis begins with the hydroxylation of tyrosine into L-3,4-

dihydroxyphenylalanine (DOPA) then followed by its subsequent oxidation into DOPA-quinone (DQ) which are all mediated by TYR. Depending on substrate availability, in this case, is cysteine, DQ can either be converted into DOPA-chrome or Cysteinyl-DOPA (Cichorek et al., 2013; Videira et al., 2013). In the presence of cysteine, DQ will be cleaved into Cysteinyl-DOPA and the resulting product will be pheomelanin – the lighter, yellow to red pigment. On the other hand, when cysteine is not present, DQ will undergo spontaneous cyclization to DOPA-chrome and subsequently the darker, brown to black pigment, eumelanin, is generated instead (Cichorek et al., 2013; Videira et al., 2013). The following conversion of DOPA-chrome into eumelanin is mediated by the two enzymes, namely TYRP1 and TYRP2. The ratio of eumelanin as compared to the total melanin determines the skin color, whereas the ratio between eumelanin and pheomelanin controls the hair color (Ito & Wakamatsu, 2011; Slominski et al., 2004). With regards to photoprotection, eumelanin has better resistance towards degradation and the ability to neutralize ROS, thereby improving the photoprotective properties of eumelanin as compared to pheomelanin (Abdel-Malek et al., 2010). Regulation of melanogenesis happens at multiple levels (subcellular, cellular, tissue, and environmental), where all the different levels play a part in affecting melanogenesis (Sulaimon & Kitchell, 2003). For proper synthesis and distribution of melanin to take place, several important events must first occur. (1) Development of melanoblasts and their migration from the neural crest to peripheral sites; (2) Differentiation of melanoblasts into melanocytes; (3) Survival and proliferation of melanocytes; (4) Production of melanosomes and melanin synthesis (Costin & Hearing, 2007). Once these four stages have occurred, melanogenesis can then start to take place. The process of melanogenesis itself takes place within the melanosome, a

melanin-containing organelle found in melanocytes (Ando et al., 2012). Melanosomes are created to separate the cytotoxic intermediate products derived from melanogenesis to protect the melanocyte. In addition, an increase in the amount of antiapoptotic protein Bcl-2 is produced to further protect the melanocyte from cellular death due to oxidative stress (Sulaimon & Kitchell, 2003). Melanosomes are suggested to originate from the endoplasmic reticulum of melanocytes and require TYR, tyrosinase-related proteins (TYRP1, TYRP2) for development. In melanosome development, it consists of four stages. Vesicles with an amorphous matrix are first formed (Stage I), before the formation of structured fibrillar matrix by glycoproteins (Stage II). Melanin production then begins (Stage III) and is settled on the internal fibrils. Lastly, melanin fills the melanosome (Stage IV) and is transported to surrounding keratinocytes (Cichorek et al., 2013; Kushimoto et al., 2001; Seiji et al., 1963).

III. Melanin transfer: an interplay between melanocytes and keratinocytes

The next event that occurs following melanogenesis is melanin transfer, a process that is characterized by the transference of melanin from one melanocyte to neighbouring keratinocytes. Unlike melanogenesis, where the whole process mainly only involves melanocytes, melanin transfer on the other hand is an orchestrated process that involves both melanocytes and keratinocytes. Synthesized melanin granules are packed and enclosed within the melanosomes. The exact mechanisms of melanin transfer have still yet to be unraveled, however, there have been various attempts and theories postulated to explain the following process. Early studies using electron microscopy reveal that melanin cores were enclosed by

one or two membranes, which resulted in various hypotheses with regards to the mechanism involved in melanin transfer: (1) phagocytosis of melanocyte filopodia by keratinocytes; (2) exocytosis of melanosome with subsequent internalization by keratinocytes; (3) membrane nanotubes-mediated melanosome transport; and/or (4) shedding of melanosome-enriched structures with subsequent phagocytosis of vesicles by keratinocytes (Ando et al., 2011; Delevoye, 2014). Among all the hypotheses, the most prominent one will be the transfer of melanosomes via membrane vesicle/shedding vesicle system (Van Den et al., 2006). The following process is initiated by the expulsion of melanosome containing melanin granules by the melanocyte via exocytosis. Exocytosis of melanosomes by melanocytes is then followed by endocytosis of the melanosome containing melanin granules by keratinocytes, where these melanin granules are released and deposited within the keratinocytes, giving off colors in the skin (Delevoye, 2014). Primarily, melanin functions to protect the skin cells from DNA damage due to aberrant UV exposure, and therefore melanin production is usually triggered by UV exposure (Ando et al., 2012). However, in the case of vitiligo, this pigmentation process is impaired due to the destruction of melanocytes by the immune cells and thus explains the lack of pigment.

The Hair Follicle

I. Anatomy of the HF unit

Hair is regarded as one of the most personifying characteristics of all mammals, including humans. The hair does not only function as an ornamentation structure, in fact, it also functions as an idiosyncratic miniorgan. For instance, in humans, hair carried out several cardinal roles, including acting as protection against external stressors,

controlling sebum and sweat production, as well as thermoregulation (Bologna et al., 2013; Buffoli et al., 2013). Other than performing the following functions that are critical to sustaining life, the hair also plays equally significant roles in one's social and sexual interaction (Bologna et al., 2013; Buffoli et al., 2013). The hair apparatus is distributed throughout the body, except on certain areas such as the palms of the hands, soles of the feet, mouth, and lips (Erdoğan, 2017). Primarily, the hair can be fractionalized into two disparate structures, comprising of the follicle – living part of the hair and hair shaft – non-living part of the hair (Millar, 2002).

The HF, or also denoted as the living part of the hair is defined as the layer of tissue that encloses the hair (Farvolden & Mahalingam, 2007). In general, the HF can be considered as the vital component of the hair structure that is essential for facilitating and maintaining proper growth of the overall hair. Structurally, the HF is divided into two parts, the upper part known as infundibulum and the lower part known as the isthmus. It is in the lower part, the isthmus where the HF stem cell, as well as other dermal stem cells, reside (Millar, 2002). Within the hair bulb, a group of mesenchymal cells known as the dermal papilla (DP) are also found and they are the master regulator of HF development and growth (Erdoğan, 2017). They are also thought to be the reservoir of various multipotent stem cells (Driskellet et al., 2011). Therefore, with that being said, the lower part of HF, the isthmus, undergoes continuous cycles of hair regeneration (Erdoğan, 2017). On the other hand, past research had shown that HF stem cells also reside in a niche called the bulge. The bulge region is located between the attachment site of the arrector pili muscle and the opening of the sebaceous gland (Joulai Veijouye et al., 2017). Moreover, it is important to note that melanocyte stem cells are most likely located

in the bulge region, thus simplifying the search and isolation of melanocytes (Lang et al., 2013; Nishimura et al., 2002).

Overall, the HF itself is a double-bounded two layers, an epithelial interior comprising of inner root sheath (IRS) and outer root sheaths (ORS), and connective tissue exterior namely the connective tissue sheath (CTS) (Hardy, 1992). CTS is the outermost layer covering the HF and is the layer that is in direct contact with the dermis. The middle layer, ORS, which is positioned in the outermost part of the hair infundibulum is a keratinized layer comprising of keratinocytes. The exterior of the ORS is connected to the CTS via the basal membrane, whereas the interior of the ORS is connected to the outermost layer of IRS, where the IRS itself is located inside of the ORS (Panteleyev, 2018).

II. The HF morphogenesis and hair growth cycle

The HF morphogenesis is considered an intricate process that involves various cell types and signalling pathways. In general, HF morphogenesis can be segregated into eight different stages. Each stage is characterized by the expression of different growth factors, receptors, intracellular signal transduction molecules as well as a variety of adhesion molecules present (Millar, 2002). Initiation of the HF morphogenesis is indicated by the release of signals from the dermis to the epidermis, which in turn results in the thickening of the epidermis and subsequently leads to the formation of hair placode (Duverger & Morasso, 2009; Erdoğan, 2017). Following the formation of hair placode is the development of hair germ. The hair placode will expand and at the same time continues sending signals to the underlying mesenchyme that eventually instigate condensation of the dermal cells adjacent to the placode. Thereafter, the hair germ continues to proliferate and subsequently penetrate the dermis,

forming the hair peg, ultimately leading to the formation of DP. Once the DP is formed, it will start to send signals towards adjacent epidermal cells which will, in turn, result in the differentiation of IRS and ORS (Duverger & Morasso, 2009; Erdoğan, 2017). IRS is the compartment where the destined hair shaft grows and develops.

The hair development process itself does not only ends with morphogenesis. In fact, following the initial morphogenesis, the HF goes through a cycle to replace old and dying HFs. The growth cycle is comprised of three different stages, which include anagen (growing phase), catagen (regressing phase), and telogen (resting phase) (Alonso & Fuchs, 2006). Anagen, often referred to as the active growth phase is denoted by the mitotic activity of secondary epithelial germ, which is located between the club hair and DP in a telogen HF. In this stage of the hair cycle, the HF went through an active growth phase whereby the follicle expands and begins to form into its original shape. The anagen is also the stage whereby the hair fiber, hair shaft synthesis, and pigmentation occur (Erdoğan, 2017). Tight coordination between mesenchymal and epithelial parts is the key in regulating hair follicle homeostasis (Stenn & Paus, 2001). Many molecular regulators like Transforming Growth Factor- β (TGF- β), Wnts, BMP family, and SHH not only play a role in morphogenesis but also aid in the regulation of the anagen phase (Foitzik et al., 1999, 2000; Schmidt-Ullrich & Paus, 2005). During the anagen phase, 8 different cell lines are formed from the epithelial stem cells, namely the ORS, companion layer, Henle's layer, Huxley's layer, cuticle of the IRS, hair shaft cuticle, cortex, and medulla (Reynolds & Jahoda, 1991). It was suggested that descending stem cells from the bulge area established the ORS layer, further indicating that derivatives of stem cells are responsible for matrix keratinocytes formation and thereby rebuilding the hair shaft (Panteleyev et al., 2001). Generally, almost

85%-90% of the scalp hairs are in this stage and this particular stage of the hair cycle may last up to 6 – 8 years (Erdoğan, 2017).

At the end of anagen, the HF then enters the next stage of the hair cycle, identified as catagen or also known as the regressing phase. This particular phase of the hair cycle is characterized by the diminished mitotic activity of the matrix cells. At the start of this phase, melanin production in the hair bulb is ceased. Next, the proximal hair shaft undergoes keratinization, whereas the distal portion of the follicle is convoluted by apoptosis (Oh et al., 2016). In contrast to ORS and hair matrix, DP is not programmed for apoptosis due to its expression of apoptosis suppressor bcl-2 (Lindner et al., 1997; Müller-Röver et al., 1999). Subsequently, the size of the lower HF is reduced hence causing the DP to be near the bulge stem cells (Botchkarev & Paus, 2003; Erdoğan, 2017; Millar, 2002). This movement is coordinated by the hairless gene (Hr), where its disruption is observed to prevent the DP from ascending and interacting with the bulge stem cells (Ahmad et al., 1998). Aside from decreased mitotic activity of the matrix cells and increased apoptotic activity in the follicle, melanogenesis in the hair bulb is also terminated (Paus & Foitzik, 2004). Many molecular regulators like Alpha Melanocyte-stimulating Hormone (α -MSH), melanocortins are involved in regulating pigmentation (Kausar et al., 2005; Slominski et al., 2004; Tobin & Paus, 2001). Typically, catagen may last up to 2 weeks in humans.

After catagen, the hair follicle will enter the last stage of the hair cycle, which is known as telogen. Telogen is defined as the resting phase of the hair cycle, in which relative quiescence is observed in both proliferative and biochemical activity. Until the hair germ begins to be responsive again to anagen initiating signals sent from the DP, the follicle will remain to stay in the telogen phase (Alonso & Fuchs,

2006). The telogen phase is characterized by the transformation of the hair shaft into club hair and eventually followed by shedding of the hair shaft itself (Greco et al., 2009). During this phase, BMP4 is produced by both DP fibroblasts and secondary germ keratinocytes and subsequently interacts with its respective receptor in the secondary germ. In this way, it inhibits the anagen development (Botchkarev et al., 2001). This is altered when Noggin antagonizes the interaction between BMP4 and its receptor, thereby allowing the activation of SHH signalling in the HF. SHH is important for HF morphogenesis and hair cycle initiation (Dlugosz, 1999). At the end of the late telogen phase, the follicle will once again start to display increased proliferative and transcriptional activity, which eventually leads to the initiation of anagen. The telogen phase of the hair cycle generally lasts up for 2 – 3 months until the initiation of the next anagen phase.

In contrast to the human hair cycle, the murine hair cycle is shorter in duration and begins in a wave, spreading from head to tail and down laterally to the sides of the mouse. All the HFs of the mouse go through the first and second hair cycles in a coordinated wave manner. The first cycle begins after hair morphogenesis at postnatal day 6 or P6, where the HF goes into the anagen phase. The HF then moves into the catagen phase at P14, which lasted for 3 – 4 days before moving into the telogen phase. The second hair cycle begins after the transition from telogen into anagen, where the 2nd anagen lasts for around 2 weeks before going into the catagen phase. The 2nd catagen phase then moves into the telogen phase after 1 week. Upon the end of the second hair cycle, the HFs then transit between phases in an uncoordinated pattern (Alonso & Fuchs, 2006; Müller-Röver et al., 2001).

Figure 2. Schematic diagram of the hair growth cycle. After hair morphogenesis, the hair growth cycle is primarily comprised of 3 series of different stages, which consist of anagen (growth), catagen (regression), and telogen (rest).

III. Hair bulge as the main reservoir of skin melanocytes

Skin pigmentation is the outcome of two processes: melanogenesis and melanin transfer from melanocytes to keratinocytes (Fitzpatrick & Szabo, 1959). Skin pigmentation is heterogeneous, occurring not only between different types of skin but also between different sites of the skin from the same individual. Regulation of gene expression also contributes to causing the heterogeneity of the skin by controlling the overall activity and expression of melanosomal proteins (Sturm et al., 1998).

In general, melanocytes that may participate in vitiligo repigmentation can be derived from three possible sources: the HF unit, unaffected melanocytes localized in areas of depigmented epidermis, and melanocytes that are distributed at the edge of vitiligo lesions (Falabella & Barona, 2009). Among these three possible sources, the HF unit is considered to be the main site of the

melanocyte reservoir, which participates in the skin pigmentation process. This notion is based on several pieces of evidence suggesting that these melanocytes participating in the skin pigmentation process originate from melanoblasts residing in the bulge (Osawa, 2009). In addition to that, other studies also demonstrated that repigmentation of vitiligo occurs in a perifollicular manner, providing further evidence of the follicular origin of the epidermal melanocytes (Falabella and Barona, 2009). Furthermore, a study involving histological analysis of the repigmentation process of vitiligo, conducted by Cui *et al.*, observed the upward migration of melanoblast from hair bulge to the interfollicular epidermis (IFE) (Cui et al., 1991). Moreover, Ortonne et al (Ortonne et al., 1980) discovered that the pigment islands on the repigmented skin after phototherapy had follicular cores, and the presence of melanocytes are found in the ORS of HFs. Taken all together, these observations provide plausible evidence that MeSC localized in the hair bulge is indeed the main melanocyte reservoir in which it does not only provide melanocyte responsible for hair pigmentation but also for skin pigmentation. This population of cells participated in the repopulation of the depleted melanocytes by migrating out of the HF into the IFE. Interestingly, phototherapies like narrow band ultraviolet B (NB-UVB) are most effective in areas where unpigmented patches hold normal pigmented HFs (Paus, 2013).

MeSCs are an unpigmented quiescence population of stem cells that plays a part in controlling hair pigmentation by synthesizing and transporting melanin to the matrix keratinocytes during the anagen (active growth) phase of the hair cycle (Schneider et al., 2009; Andrzej Slominski et al., 2005; Tobin, 2008). On the other hand, mature matrix melanocytes undergo apoptosis during catagen when the HF regresses (Tobin et al., 1998). Interestingly, the fates of

follicular melanocytes are in synchronization with hair cycle progression, suggesting that HF cycling plays a part in regulating follicular melanocytes regeneration (Nishimura et al., 2002; Slominski et al., 1994, 2005; Tobin et al., 1999).

IV. The Complexity of HF microenvironment

Besides producing hair shafts, HF is also the source of many molecular modulators (eg. Growth, pigment etc.) For instance, it can synthesize a large number of growth factors like TGF- β , Insulin-like Growth Factor 1 (IGF1), Hepatocyte Growth Factor (HGF) (Itami et al., 1995; Lindner et al., 2000). Moreover, it can metabolize proopiomelanocortin into Adrenocorticotrophic Hormone (ACTH), α -MSH, or β -endorphin (Stenn & Paus, 2001). These evidence suggested the autocrine and paracrine mechanisms involved in eliciting certain HF processes. Furthermore, the presence of diverse systemic extrafollicularly-generated molecular modulators complicates its delicate homeostasis more. Therefore, a minute change can steer the HF to undergo abnormal events.

It is accepted that HF undergoes repetitive cyclic activity, which confers the uniqueness it has as compared to other mammalian biological systems. This repetitive process is brought about by key regulators that confer gradients with competing stimulatory and inhibitory signals. With that, the HF experiences cycle of mitotic activity, then the apoptotic stage, and subsequently went into a 'dormant' phase before resuming into the next growth phase. According to postulated classical experiments in the past, 'chalcones' are responsible for the inhibition of epidermal proliferation, giving rise to the 'dormant' phase (Bullough, 1962). In recent years, many pieces of evidence had emerged and identified the role of the key compartments in HF and various signalling mechanisms in bringing

about its organ transformation. For instance, factors like the Wnt family, including its downstream activation of the β -catenin pathway, Noggin, and STAT3, are known to induce the anagen development (Botchkarev et al., 1999; Millar et al., 1999). In addition, factors like SHH, FGF7, and HGF are discovered as supporters of anagen and played a part in its development (Danilenko et al., 1995; Lindner et al., 2000; Sato et al., 1999). On the other hand, factors like the TGF- β , neurotrophins, Fibroblast Growth Factor-5 (FGF-5), and retinoids are catagen inducers (Botchkarev et al., 2004; Foitzik et al., 2005; Soma et al., 2002; Suzuki et al., 2000). After the HF enters the telogen phase, it will be influenced by BMP4 and 17 β -Estradiol to ensure its quiescent state (Botchkarev & Sharov, 2004; Conrad & Paus, 2004). Therefore, neutralization of BMP4 by Noggin and thereby upregulating SHH will be essential for the HF to transit into anagen phase once again (Botchkarev et al., 2001). This indicates the complexity involved in HF homeostasis where both stimulatory and inhibitory factors are expressed at the same time to instigate the transition between phases (Botchkarev & Sharov, 2004). To confuse the mechanism further, some HF modulators elicit different effects during different stages. For example, TGF- β 2 is known to expedite HF morphogenesis but induces catagen in mature anagen HF (Foitzik et al., 1999). It is important to note that even though some of the factors may have the potential to manipulate the HF homeostasis but still, none of these is the master regulator itself in directing the HF transformation.

Among all the key compartments, hair bulge is the only compartment that comprises diverse and complex stem cell repositories, where it is also regulated by the surrounding micro-environment. These stem cells interact with many different cell types such as fibroblast, muscle cells, adipocytes, and neurons, and even with their progeny that

migrates out of the niche during the anagen stage which will later return to the bulge during the telogen stage to generate a pro-quiescence environment (Solas & Benitah, 2013). Moreover, hair bulge is made up of various mininiches, in accordance with specific expression profiles (Woo & Oro, 2011). This cyclical activation of HF stem cells has been linked to the tandem pattern of inhibitor and activator signals. The two identified groups of signals were BMP 2/4 and Wnt/ β -catenin respectively. The BMP cycle was reported to be out of phase with the Wnt/ β -catenin cycle, and demonstrated the inhibitory effects of 'chalcones', presenting the complexity involved in hair bulge regulation (Fuchs, 2007; Houschyar et al., 2020; Jahoda & Christiano, 2011; Plikus et al., 2008). The balance between these two pathways will determine the bulge stem cells' quiescence or activation fates, leading to a new HF regeneration (Blanpain & Fuchs, 2009; Gat et al., 1998; Kandyba et al., 2013; J. Zhang et al., 2006). Inhibition of BMP will upregulate the signalling of Wnt (Perez-Moreno et al., 2003) and cause the hair state to change from quiescence to active (Greco et al., 2009). Moreover, BMP signalling plays an important role in regulating the fate of the MeSC, thus regulating BMP signalling will affect both melanogenesis and pigment transfer (Singh et al., 2012). These evidence, therefore, indicate the possible existence of a relationship between BMP signalling and skin pigmentation.

Bone morphogenetic protein (BMP)

In the superfamily of TGF- β , a group of signalling molecules, Bone Morphogenetic Proteins (BMPs), is known to play an important role in all organ systems. BMPs were first discovered for their ability to induce ectopic bone formation (Urist, 1965). Subsequently, more research was performed and confirmed the role of BMPs in bone and cartilage formation. Since then, more

discoveries were made which revealed that BMPs are also critical in many other processes like embryogenesis, development, and maintenance of tissue homeostasis (Hemmati-Brivanlou & Thomsen, 1995; Stewart et al., 2010; Zou & Niswander, 1996). Therefore, BMPs are often known as body morphogenetic proteins because of their diverse functions in all organ systems (Wagner et al., 2010). The effect of BMP is dependent on many factors like the type of BMP, type of receptors expressed by the target cell, the concentration of the tissue, and the development stage of the target tissue (Wang et al., 2014).

BMP signalling is mediated by two types of receptors, type 1 and 2. Type 1 receptor has two different forms: A and B. Binding of both receptors induces the phosphorylation of an intracellular domain in BMP-R1 by BMP-R2 and in turn activates the intracellular signal transduction (Yaar et al., 2006). There are seven type 1 receptors in total for the TGF- β family of ligands, with three of them binding for BMPs: type 1A BMP receptor (BMPR-1A), type 1B receptor (BMPR-1B), and type 1A activin receptor (ActR-1A) (Horbelt et al., 2012). On the other hand, there are four type 2 receptors, with three of them interacting with BMPs: type 2 BMP receptor (BMPR-2), type 2 activin receptor (ActR-2a), and type 2B receptor (ActR-2B). Due to the presence of preformed oligomeric complexes, ligands like BMP6 can signal either through the canonical or non-canonical pathway. It can either bind to and interact with type 2 receptor and recruit type 1 receptor or bind to a preformed oligomeric complex, both of which will activate a different pathway (de Caestecker, 2004; Nohe et al., 2002a). Moreover, it is important to note that the mechanism of the hetero-oligomeric complex formation can vary. For example, BMP6 and BMP7 interact with type 2 receptors and in turn recruit type 1 receptors. On the other hand, ligands like BMP2 and BMP4 will interact with type 1 receptors and recruit type 2 receptors (de Caestecker, 2004).

As mentioned before, BMP signalling can be achieved through the canonical or non-canonical pathway. In the non-canonical pathway of BMP signalling, ligands

like BMP6 bind to a type 2 receptor such as BMPR-2 and recruit a type 1 receptor to form a hetero-oligomeric complex which in turn leads to the activation of the p53-MAPK (Mitogen-activated protein kinase) pathway. Alternatively, the ligands like BMP6 can bind to a preformed hetero-oligomeric complex which consists of at least one type 1 and one type 2 receptor to activate the canonical BMP/SMAD signalling pathway (Nohe et al., 2002b). Besides MAPK signalling pathway, many other non-canonical, SMAD-independent signalling pathways for BMPs which are identified as BMPs have been found to affect TAK-1, PI3K/AKT, P/kc, Rho-GTPases, and many others (Derynck & Zhang, 2003; K. Yamaguchi et al., 1995; Y. E. Zhang, 2009).

In general, once BMPs are secreted, they will bind to the target cell expressing at least one specific type 1 and one specific type 2 receptor to activate the BMP signalling pathway. Depending on the type of receptor the BMPs bind to, the type of pathway it activates will be different as well. More than 15 unique BMPs are known structurally and are further categorized into subclasses based on amino acid or nucleotide similarity. These unique BMPs are categorized into BMP2/4, BMP5/6/7/8, BMP9/10, and BMP12/13/14 based on phylogenetic analysis (Mueller & Nickel, 2012).

Upon binding to a type 2 receptor, hetero-dimerization of type 1 and type 2 receptor is induced. This resulted in phosphorylation of receptor-regulated SMAD (R-SMAD) like SMAD1/5/8. In contrast, activin activates R-SMAD like SMAD2/3 (Heldin et al., 1997; Massagué & Wotton, 2000; L. Xu, 2006). Activated R-SMAD will then form a complex with Smad4, which is also known as Common-partner SMAD (Co-SMAD). This complex then translocates into the nucleus where regulation of specific gene transcription takes place. It is important to note that both Co-SMAD and R-SMAD contain two conserved regions known as MH1 (Mad homology 1) and MH2. MH1 domain is responsible for DNA binding, while MH2 domain manages the association with type 1 receptor, Co-Smad, SARA (SMAD-anchor for receptor activation), and p300/CBP (Heldin et al., 1997; Massagué & Wotton, 2000; L. Xu, 2006). Conversely, SMAD6/7 lack

the MH1 domain and therefore function as an inhibitory SMAD (I-SMAD), where it prevents the association between R-SMAD and type 1 receptor or Co-SMAD (Hata et al., 1998; Imamura et al., 1997; Nakao et al., 1997, p. 7). I-SMAD expression is induced by BMPs or TGF- β and therefore functions in a feedback manner, providing a mechanism to restrict and fine-tune BMP signaling and its control on cellular functions. Similar to TGF- β , BMPs impart their effects by motivating target gene transcription. With the presence of the β -hairpin loop in the MH1 domain, it allows R-SMAD and Co-SMAD to bind directly to the target genes, thereby achieving its transcriptional acts on the genes (Shi et al., 1998). Moreover, the complexity involved in the regulation of transcription by the SMAD pathway is further accentuated by other mechanisms. These mechanisms determine its specificities and modulate its binding affinity for genes. Firstly, SMAD was discovered to associate with tissue-specific transcription factors and thereby stimulate the expression of the target genes. Secondly, studies have suggested the presence of functional cooperation between SMAD and general transcription factors, in turn augmenting its transcriptional action on the specific genes. For example, SMAD3 cooperates with ATF-2 and C-JUN to bring about their transcriptional activity (Liberati et al., 1999; Sano et al., 1999, p. 2; Y. Zhang et al., 1998, p. 4). Thirdly, SMAD is capable of recruiting co-activators or co-repressors to support the activation or suppression of transcriptional activity respectively. In this way, biological effects can be appropriately modulated to ensure cellular processes are optimally conditioned (Liu et al., 1996; Wotton et al., 1999). Moreover, SMAD cross-talks with other signalling pathways to regulate cellular processes. For example, SMAD3 associates with the Wnt signalling pathway and participate in the formation of Spemann's organization (Crease et al., 1998; Nishita et al., 2000). Overall, SMAD demonstrates a complex and specific regulatory network, thereby coordinating signals in a precise manner.

As BMPs are stimulated to bring about regulation of multiple cellular processes, it is also important for its expression level to be restricted, to ensure signals are

well-coordinated. In this case, one of the most commonly known BMP antagonists is Noggin. Noggin is a 67-kD homodimeric glycoprotein and was also first identified as a dorsalizing factor. It resembles BMPs in a two-fold axis of symmetry and dimerizes through a core body, where 2 pairs of β -strands extend, preceding an N-terminal segment. It binds to many BMPs with different affinities, with a marked preference for BMP2 and BMP4 (Seemann et al., 2009, p. 5; Song et al., 2010; Zimmerman et al., 1996). Noggin snakes around BMP ligand, restricting the activity of BMPs and thereby preventing BMPs interaction with its respective receptors and thus inhibiting SMAD signalling (Krause et al., 2011). Similar to BMPs, Noggin can be found in various stages of embryonic development, working together in a coordinated manner to induce specific activities. For example, BMP2, 4, 7 are antagonized by Noggin during early gastrulation, leading to a BMP gradient directed dorsal-ventral patterning and subsequently developed into germ layer (Smith & Harland, 1992; Zimmerman et al., 1996). Moreover, expression of BMP4 is found all over ectoderm before neural induction and its expression level is crucial for the development of ectoderm derivatives. For example, inhibition of BMP4 expression in response to Noggin suppresses epidermal fate and gives rise to the neural plate by inducing the expression of neural-specific markers. Therefore, this indicates the importance of Noggin for neurogenesis and further emphasizes the interplay between BMPs and Noggin. (D. A. Lim et al., 2000; McMahon et al., 1998). In addition, the balance between BMP4 and Noggin, both localized in the dorsal neural tube, control the migration of neural crest progenitors from neuroepithelium. The neural crest is a distinct structure in the vertebrate embryos and its derivatives produce a wide variety of cell types. This list includes connective and adipose tissues, peripheral neurons, endocrine cells, melanocytes, tendon, and satellite cells to bones (Sela-Donenfeld & Kalcheim, 1999). Coordinated activity between BMP4 and Noggin is observed in the dorsal neural tube, which is essential for the delamination of SLUG-expressing neural crest cells (Sela-Donenfeld & Kalcheim, 1999). Several other studies also indicated

the importance of the synchronization between BMP4 and Noggin, where it controls the neural crest delamination, thus emphasizing the pivotal role and significance of the harmony between BMPs and their inhibitors in patterning crucial episodes during morphogenesis (Capdevila & Johnson, 1998; Dale et al., 1999; Fainsod et al., 1997; Mancilla & Mayor, 1996). This is also evident in the role of BMP6 during epidermal development, where Blessing et al. reported that high expression of BMP6 in the suprabasal epidermal layer inhibits proliferation and does not show a significant change in the distribution of differentiation markers. However, a moderate BMP6 expression is capable of inducing proliferation of basal epidermal keratinocytes, resulting in the atypical appearance of proliferating cells and keratins expressions in the suprabasal layer (Blessing et al., 1996). This harmony is further exemplified as several studies reported the involvement of BMP2 and BMP6 in inhibiting epidermal proliferation and stimulating their differentiation in postnatal skin (Drozdoff et al., 1994; D'Souza et al., 2001; McDonnell et al., 2001; G. T. Park & Morasso, 2002), suggesting the possible concentration-dependent effect of BMPs. Moreover, the complex interplay between BMPs and Noggin is also demonstrated in the development of tooth, hair follicles, and eyes (McMahon et al., 1998). For instance, BMP4 is discovered to be involved in hair follicle induction in embryonic skin organ cultures and Noggin is required to neutralize BMP4 expression for hair follicle development to take place (Botchkarev et al., 1999). Even in melanocytes of human newborn skin, BMP4 is observed to regulate proliferation, decrease melanin content through TYR expression (Yaar et al., 2006). Taken together, not only BMPs but also their interplay with respective antagonists are important components of the molecular network, which played crucial roles throughout the various developmental stages.

I. Bone morphogenetic protein 4 (BMP4) regulates melanogenesis

As mentioned previously, BMPs play an important role in the regulation of several processes. Moreover, it was reported that bone morphogenetic proteins (BMP) signalling plays a role in regulating the activities of MeSC in the HF compartment. BMP signalling in the MeSC compartment is responsible for maintaining the melanocyte stem cell localized in HF bulge quiescent and regulating melanogenesis (Park et al., 2009; Yaar et al., 2006).

Among the BMPs, two of the BMP family members originating from differing BMP subclasses have been shown to regulate pigmentation of the human skin cells. These members of the BMP include BMP 6 and BMP 4, where each one is shown to have contrasting effects on skin pigmentation (Singh et al., 2012). The former of the aforementioned BMP member, BMP 6 is reported to promote melanogenesis. Increased expression of BMP 6 is shown to positively affect skin pigmentation and this is observed by increased activity of melanosome transfer from the melanocytes to keratinocytes (Park et al., 2009; Singh et al., 2012). While on the other hand, the latter BMP 4 is reported to have an opposing effect on melanogenesis, where increased expression of BMP 4 is correlated with reduced melanin transfer (Singh et al. 2012). In addition to that, the following study conducted by Singh *et al.* demonstrated that both BMP 6 and BMP 4 along with their respective antagonists, Sclerostin and Noggin, as well as receptors are strongly expressed in normal adult human skin (Singh et al., 2012). Moreover, both BMPs were reported of their role in regulating morphogenesis of HF and epidermal layer. BMP4 is also reported to be responsible for ensuring the quiescent state of HF during the telogen phase.

Moreover, melanocytes treated with BMP4 *in vitro* are found to decrease microphthalmia-associated transcription factor (MITF) (H.-Y. Park et al., 2009) and TYR expression (Yaar et al., 2006). Furthermore, Noggin, an inhibitor of BMP4, was upregulated in transgenic mice. They exhibited darker hair and decreased Agouti expression, indicating a cross-talk between BMP4 and melanocortin receptor-1 (MC1R) that controls HF pigmentation (Sharov et al., 2005). BMP signalling has also been shown to inhibit keratinocyte migration in wound healing models (Lewis et al., 2014). All these evidence further indicated the role of BMP signalling in both melanogenesis and melanin transfer. Moreover, previous research by Clavel et al. discovered the effects of Sox2 in controlling hair growth and revealed the role of BMP signalling in regulating HF differentiation. In addition, Clavel et al. reported that Sox2 ablation led to an increased BMP expression, and fascinatingly, a pigment switch phenomenon was observed at the same time. This led to the speculation of possible involvement of BMP signalling in the regulation of melanocytes (Clavel et al., 2012; J. Lim et al., 2019). Therefore, this results in our hypothesis that downregulation of the BMP signalling will likely lead to the activation of the MeSC, thereby improving phototherapy treatment when used in conjunction with NBUVB. With that in mind, we aim to develop a novel strategy to enhance UV-induced follicular repigmentation in vitiligo by identifying and understanding the role of BMP signalling in melanogenesis, melanin transfer, and melanocyte migration.

Summary

Skin depigmentation disorder is among one of the most common dermatological disorders. One particular example of such disease is vitiligo, which affects about 0.5 – 2% of the world's population (Alikhan et al., 2011; Boisseau-Garsaud et al., 2000; Howitz et al., 1977; Krüger & Schallreuter, 2012), in both adults and children (Ezzedine et al., 2012). Vitiligo is characterized by the appearance of white patches in the skin, which arises from the lack of melanin (Dell'anna & Picardo, 2006; Ezzedine et al., 2012; Le Poole et al., 1993; Rodrigues et al., 2017; Schallreuter et al., 2008). Up until today, the exact cause that leads to development of vitiligo remains unclear. However, several theories have been developed as an attempt to explain the etiology of vitiligo and one of the most probable explanation is the autoimmune theory (Ianella et al., 2016). The autoimmune theory generally speculates that the lack of melanin in vitiligo patients arises as a result from progressive destruction of melanocytes by the immune cells (Ianella et al., 2016). In general, vitiligo can affect both men and women of different ethnic groups with similar prevalence (Alkhateeb et al., 2003; Ezzedine et al., 2015; Picardo et al., 2015), meaning that there is no particular pattern involved (Allam & Riad, 2013). As for the onset, vitiligo can develop at any age, however typically the disease arises before the age of 30 (Hann & Lee, 1996), which comprise 70% - 80% of vitiligo cases (Alikhan et al., 2011). Due to apparent and visible features of the disease, it is often that vitiligo patients also experience psychological stress like anxiety, depression and shame (Kostopoulou et al., 2009; Porter et al., 1979).

Currently there are several treatment options available to treat vitiligo. Phototherapy is one of the widely used treatment for vitiligo patients, which involves the exposure of narrowband UVB on affected areas of the skin to induce skin repigmentation (Bae et al., 2017; Mohammad et al., 2017). Results from clinical observations and research suggest this occurs due to UV-induced migration of melanocytes from the HF stem cell niche that trigger interfollicular

repigmentation (Tang et al., 2018; Wu et al., 2004). However, despite its efficacy, the process of phototherapy treatment itself may be considered as rather time consuming and the outcome varies between individuals. The reason being is that this particular treatment requires at least 48 exposures that are usually given three times a week in order for the result to be seen, with some individuals requiring more than 72 exposures to determine lack of response to phototherapy (Mohammad et al., 2017). Therefore, this study aims to enhance the UV-induced follicular repigmentation during vitiligo.

In the previous research performed by Clavel et al. involving the study on how Sox2 in the dermal papilla (DP) niche controls hair growth, the role of bone morphogenetic protein (BMP) signalling in regulating melanocyte function was first observed (Clavel et al., 2012; J. Lim et al., 2019). In the study, knocking out of Sox2 in the DP niche leads to increased BMP expressions, which was due to the reduced *Sostdc1* expression, one of the BMP antagonists. At the same time, Clavel et al. also discovered an interesting phenomenon whereby a pigment switch event was observed along with increased BMP expression. This then leads to the speculations that BMP signalling might played an implicating role in the regulation of melanocytes.

Furthermore, there are many recent researches that further confirm the existence of a relationship between BMPs and skin pigmentation. For instance, melanocytes treated with BMP4 *in vitro* were found to decrease microphthalmia-associated transcription factor (MITF) (H.-Y. Park et al., 2009) and TYR expression (Yaar et al., 2006). Furthermore, darker hairs and decreased Agouti expression were observed when Noggin, an antagonist of BMP4, was upregulated in mice, suggesting a possible relationship between BMP and HF pigmentation (Sharov et al., 2005). In addition, BMP signalling demonstrated an inhibition of keratinocyte migration in wound healing models (Lewis et al., 2014). Overall, all the evidences suggest the involvement of BMP signalling in regulating melanocyte functions. These findings lead to the idea of further scrutinizing the other implication of BMP signalling in regulating melanocyte

functions, whereby it can be used as a potential target to enhance skin repigmentation in vitiligo when used in conjunction with phototherapy.

Therefore, it is hypothesized that down regulation of the BMP signalling, will likely result in the activation of the MeSC. This will eventually result in the differentiation and migration of the melanocyte stem cell into the epidermis hence leading to skin repigmentation. Thus, the objective of this research is to identify the role of BMP signalling in melanogenesis, melanin transfer and melanocyte migration both *in vitro* and *in vivo*. Having said that, identifying and understanding the role of BMP signalling will be of great importance, since this could provide knowledge on understanding follicular repigmentation. The significance of this newly profound knowledge could then later on be implemented as a potential therapeutic strategy to treat vitiligo that could serve as a more efficient and reliable treatment option. Thereby relieving the psychological burden in vitiligo patients.

Figure 3. Schematic diagram of project hypothesis. The diagram depicts the cellular details during vitiligo, phototherapy, hypothesized novel therapy and enhanced repigmentation (aim of study). Reduction of BMP4 signalling in bulge region is proposed to achieve an enhanced repigmentation of the skin.

In the proposed study, several approaches will be implemented to justify the objectives of the following research. In general, the research is divided into two parts, *in vitro* and *in vivo*. The *in vitro* part of the research will mainly involve several cell culture methods such as 2D keratinocyte and melanocyte co-culture system to study melanogenesis and melanin transfer at the functional level by incorporating different cytokine treatments. In addition to 2D co-culture, melanocyte migration assay will also be integrated as part of the *in vitro* aspect. The *in vivo* part of the research will mainly involve mice work using genetically modified mouse models. Fluorescence-based analysis approaches, including immunofluorescence analysis using confocal microscopy and fluorescence-activated cell sorting (FACS) will also be incorporated. In addition to that, downstream experiments like melanin assay, qRT-PCR and RNA sequencing will also be included in the research to further study the effects of BMP signalling *in vivo*.

Objectives of the study

The main objective of the following project is to develop a novel strategy to enhance UV-induced follicular repigmentation in vitiligo. In order to test our hypothesis and achieve our objective, we are proposing the following specific aims:

Aim 1: To determine the functional role of BMP signalling in melanogenesis, melanin transfer and melanocyte migration *in vitro*

- A. Analyse the functional consequences of BMP signalling in melanogenesis, melanin transfer, and melanocyte migration *in vitro*
- B. Investigate the effects of UVR induction on BMP signalling and melanocyte migration

Aim 2: To determine the role of BMP signalling in follicular repigmentation *in vivo*

- A. Prospective isolation of the MeSC compartment from BMP4 mice model
- B. Analyse the functional consequences of BMP signalling in melanogenesis, melanin transfer and melanocyte migration in a BMP4 cKO mice model

Significance of study

Skin not only act as an organ of protection, but also has a vital role in our interaction with the world. The visible nature of skin depigmentation disorders like vitiligo affects the psychosocial development of patients. Vitiligo is not simply a cosmetic disease that affects only the outlook of a person, but also brings about psychosocial stress and various psychiatric comorbidities. In addition, the significance of addressing vitiligo is further intensified as the golden standard treatment of vitiligo, phototherapy, requires repeated exposure and has variable clinical outcome among patients. Therefore, it is of paramount importance to understand more about the mechanism involved in repigmentation and develop novel therapeutic strategies.

MATERIALS & METHODS

Melanocyte & Keratinocyte Cell lines

A primary melanocyte cell line (Thermo Fisher Scientific, Waltham, Massachusetts, United States) was used for this study. Medium 254 supplemented with PMA-free Human Melanocyte Growth Supplement-2 (HMGS-2) supplement from Thermo Fisher Scientific (Waltham, Massachusetts, USA) was used to culture the melanocyte cell lines. Media was changed every 48 to 72 hrs to ensure the growth of the melanocytes.

N/TERT cell line (Immortalised Normal Telomerase Reverse Transcriptase keratinocyte; loss of p16 expression) from Dr. Oliver Dreesen's laboratory was used for this study. Keratinocyte Serum-Free Media (KSFM) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) supplemented with Bovine Pituitary Extract (BPE), human recombinant Epidermal Growth Factor (EGF), and calcium chloride, was used to culture the N/TERT cell lines. Media was changed every 48 to 72 hrs to ensure the growth of the keratinocytes.

Epidermal Melanocyte-keratinocyte (Me-KC) 2D co-culture

To mimic the physiological ratio in the epidermis between the melanocytes and keratinocytes, the respective cells were seeded into a 6-well plate (9.6 cm² culture area) or 8-well chamber slide (1 cm² culture area) in a 1:10 ratio. Therefore, for the 8-well chamber slides, each well contains 4x10⁴ keratinocytes and 4x10³ melanocytes, whereas, for the 6-well plates, each well contains 3.8x10⁵ keratinocytes and 3.8x10⁴ melanocytes. The co-cultures were supplemented with HMGS-2 media and analyzed after a specific period based on the objective of the experiments conducted.

2D co-culture Melanin Assay

Co-cultures of N/TERT and primary melanocytes were first cultured in the appropriate ratio as discussed previously and supplemented with HMGS-2 media. These co-cultures were then treated with BMP4, in the presence and/or absence of its respective antagonist, Noggin. After 24 hrs of cytokine treatment, these co-cultures were subjected to 0.25% Trypsin-EDTA with phenol-red (Thermo Fisher Scientific, Waltham, Massachusetts, United States) to dislodge the cells from the plate. Thereafter, the melanin content in these samples was quantified with melanin assay.

Table 1

Cytokine	Concentration	Diluent used/Media for Co-culture
BMP4	200ng/ml	HMGS-2
Noggin	400ng/ml	

Table 1 shows the concentrations of cytokines added for co-culture treatments.

In vitro Migration assay

Co-cultures of N/TERT and primary melanocytes were performed and the conditioned media was harvested after 24 hrs for later use. On the other hand, primary melanocytes were cultured in Ibidi Culture-Insert 2 Well 24 (Martinsried, Planegg, Germany) with the harvested co-culture conditioned media, supplemented with 200ng/ml of recombinant human BMP4 and/or 400ng/ml of recombinant human Noggin (R&D Systems, Minneapolis, Minnesota, United States) and pre-treated overnight. The culture was then treated with Mitomycin-C (Sigma-Aldrich, St Louis, Missouri, United States) for 30mins before subjecting the culture to UVR exposure ($50\text{mJ}/\text{cm}^2$) if required. Then, insert was removed and fresh co-culture conditioned media supplemented with the respective cytokines was added into the wells. Subsequently, phase-contrast images of 6 non-overlapping fields of views in duplicated wells were taken every 4 hrs for 48 hrs with an IX83 microscope (Olympus, Shinjuku, Tokyo, Japan). Lastly, a macro script (Appendix A) was used in ImageJ to quantify the relative areas covered by migrating melanocytes.

Figure 4. Schematic diagram of the Ibidi Culture-Insert 2 well. The insert consists of a $500\mu\text{m} \pm 100\mu\text{m}$ cell-free gap between the two growth areas. 1.1×10^4 melanocytes were cultured in each growth area. After treatment of cells, inserts are removed before migration assay.

Sample Collection

Human

Ethical approval and consent were obtained for all skin and HF biopsies used in this study under IRB no. 2021-021.

Mice

BMP4^{fl/fl} and tdTomato (Tdt) transgenic line was obtained from Jackson Laboratories (Bar Harbor, Maine, United States). TyrCreER^{T2} transgenic line was previously described (Aktary et al., 2020; Bosenberg et al., 2006). Cross-breeding between the mice was performed to obtain a transgenic line of BMP4^{fl/fl} x TyrCreER^{T2} x tdTomato. To activate the Cre, mice were injected at P15 with 1mg/10g (weight of mouse) of Tamoxifen (Sigma-Aldrich, St Louis, Missouri, United States) for 3 days before subjecting the mice to UVR exposure (200mJ/cm²) for 5 days. Dorsal skin was then removed for further downstream processes. All animal experiments were performed in accordance with the guidelines from IACUC and Biological Resource Centre (BRC) at Agency for Science, Technology and Research (A*STAR).

Figure 5. Illustration on the timeline for Tamoxifen injection and UVR exposure. The mouse model is injected with Tamoxifen (1mg/10g of weight) for 3 days at P15 (Catagen Stage). Thereafter, the mouse will be subjected to UVR exposure at P20 (200mJ/cm²) for 5 days before sacrificing the mouse at P30.

After the removal of hair and subcutaneous fats from the skin tissue, it was first fixed in 4% paraformaldehyde (Sigma-Aldrich, St Louis, Missouri, United States) overnight before embedding in OCT for frozen sections or 48 hrs before further dehydrating it with ethanol and xylene and subsequently embedded in paraffin.

Table 2

Step	Reagent	Duration
1	4% Paraformaldehyde	48 hrs
2	70% Ethanol	30 mins
3	90% Ethanol	30 mins
4	95% Ethanol	30 mins
5	100% Ethanol	30 mins
6	100% Ethanol	30 mins
7	Xylene	15 mins
8	Xylene	30 mins
9	Xylene	30 mins
10	Paraffin	1 hr
11	Paraffin	1 hr

Table 2 shows the steps taken for the paraffinization process.

Dewaxing of paraffin-embedded sections was performed to remove the polyester wax surrounding the tissues, before subjecting the tissues to Dako Target Retrieval Solution (10x; pH 6; Dako, Brüsseler Straße, Germany) in the pressure cooker (121°C) for 30 mins to enable the antibodies to access the target protein.

Table 3

Step	Reagent	Duration
1	Incubate at 65°C	20 mins
2	Xylene	10 mins
3	Xylene	10 mins
4	100% Ethanol	2 mins
5	100% Ethanol	2 mins
6	95% Ethanol	2 mins
7	90% Ethanol	2 mins
8	70% Ethanol	2 mins
9	50% Ethanol	2 mins
10	Distilled water	2 mins
11	Antigen Retrieval at 121°C	30mins

Table 3 shows the steps taken to dewax paraffin-embedded sections.

Frozen sections were subjected to Hoechst 33342 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), followed by the counting of HF and IFE Tdt+ cells to quantify the number of events in the IFE and HF throughout the whole section.

Immunofluorescence (IF) Staining

Immunofluorescence staining was performed on paraffin sections to visualize the expression of various proteins in the skin. MOM basic kit (Vector Laboratories, Burlingame, California, United States) was used whenever applicable to prevent non-specific binding of mouse monoclonal antibodies. The list of antibodies and their respective dilutions use were: BMP4 (Rabbit, 1:50, Lifespan Biosciences, Seattle, Washington, United States), Phospho-SMAD1/5 (Rabbit 1:100, Cell Signalling Technology, Danvers, Massachusetts, United States), TRP2 (Mouse, 1:100, Santa Cruz Biotechnology, Dallas, Texas, United States), Sox10 (Goat, 1:100, Santa Cruz Biotechnology, Dallas, Texas, United States), K14 (Rabbit, 1:100, BioLegend, San Diego, California, United States) and NKI/GP100 (Mouse, 1:100, Abcam, Cambridge, United Kingdom). FITC, PE, and APC conjugated anti-mouse, -rabbit, -goat antibodies (1:1000, Abcam, Cambridge, United Kingdom) were used as secondary antibodies.

Table 4

Primary Antibody	Concentration	Secondary Antibody	Concentration
BMP4	1:50	FITC	1:1000
Phospho-SMAD 1/5	1:100	FITC	1:1000
TRP2	1:100	APC	1:1000
Sox10	1:100	PE	1:1000
K14	1:100	FITC	1:1000
NKI/GP100	1:100	PE	1:1000

Table 4 shows the concentrations of antibodies used in immunofluorescence staining.

Hematoxylin & Eosin (H&E) Stain

H&E staining was performed to view the morphological structure of the skin tissue, with the Hematoxylin (Sigma-Aldrich, St Louis, Missouri, United States) to stain nucleic acids by a complex and Eosin (Sigma-Aldrich, St Louis, Missouri, United States) to act as a counterstain as it stains proteins non-specifically. This staining was executed on paraffin-embedded sections as shown in **Table 5**. After dehydrating the H&E stain, it is sealed with Micromount medium (Leica Biosystems, Wetzlar, Germany) before imaging it under the BX51 microscope (Olympus, Shinjuku, Tokyo, Japan) and thereafter analyze with Image J.

Table 5

Step	Reagent	Duration
1	Hematoxylin	2 mins
2	Running water	2 mins
3	Scotts' blueing water	2 mins
4	Running water	2 mins
5	Eosin Red	20 secs
6	70% Ethanol	1 dip
7	80% Ethanol	20 secs
8	90% Ethanol	20 secs
9	100% Ethanol	3 mins
10	100% Ethanol	3 mins
11	Xylene	5 mins
12	Xylene	5 mins

Table 5 shows the steps taken for Hematoxylin & Eosin Staining.

Fontana Masson (FM) Stain

To visualize the melanin deposits in the skin tissue, Fontana Masson stain (Sigma-Aldrich, St Louis, Missouri, United States) was utilized to stain these deposits because melanin can bind to silver from a silver solution and reduce to visible metallic silver without a reducing agent. The staining is performed with steps as shown in **Table 6** after dehydrating the slides, it is sealed with Micromount medium (Leica Biosystems, Wetzlar, Germany) before imaging it under the BX51 microscope (Olympus, Shinjuku, Tokyo, Japan) and thereafter analyse with Image J.

Table 6

Step	Reagent	Duration
1	Warmed Ammoniacal Silver Solution (58°C to 60°C)	30 mins
2	Rinse with Distilled water	2 changes
3	Gold Chloride solution	30 secs
4	Rinse with Distilled water	2 changes
5	Sodium Thiosulfate solution	2 mins
6	Running water	2 mins
7	Rinse with Distilled water	2 changes
8	Nuclear Fast Red solution	5 mins
9	Running water	2 mins
10	Rinse with Distilled water	2 changes
11	70% Ethanol	1 dip
12	80% Ethanol	20 secs
13	90% Ethanol	20 secs
14	100% Ethanol	3 mins
15	100% Ethanol	3 mins
16	Xylene	5 mins
17	Xylene	5 mins

Table 6 shows the steps taken for Fontana Masson Staining.

BMP4 agarose bead preparation and implantation

Recombinant human BMP4 (R&D Systems, Minneapolis, Minnesota, United States) was reconstituted with 4mM of HCl to a final concentration of 10µg/ml. Thereafter, 200µl of Affi-Gel Blue beads (100-200 mesh, Bio-Rad, Hercules, California, United States) was added to 200µl of 10ug/ml BMP4 and incubated for 1 hr at 37°C. Controls were made by soaking the agarose beads in 4mM of HCl and was incubated for 1 hr at 37°C as well. Then, 150µl of the suspensions were injected into the dorsal skin of cKO with a 22-gauge needle, with 2 sites containing agarose beads coated with BMP4 and vice versa. Sites of implantation and the surrounding area of it were harvested after 7 days.

Figure 6. Illustration on the timeline for the beads implantation and sites of injections. Dermal injections will be performed on the cKO mouse where beads coated with/ without cytokines are injected into the dorsal skin of the mouse after treating it with Tamoxifen and UVR exposure.

Cell Isolation and FACs

Cell isolation from mice dorsal skin was carried out as described previously (Rendl et al., 2005). After shaving the mice, the dorsal skin is carefully removed from the mice. Then, subcutaneous fats will be removed before subjecting the skin tissue to 0.25% Trypsin-EDTA with phenol-red (Thermo Fisher Scientific, Waltham, Massachusetts, United States) overnight at 4°C. After overnight digestion of the skin tissue, the epidermis is cautiously detached from the dermis. To remove the collagen that is present in the dermis, it was first minced into pieces before exposing it to collagenase (Sigma-Aldrich, St Louis, Missouri, United States) for 45 mins to 1 hr at 37°C. DNase1(Roche, Basel, Switzerland) was added as well to get rid of any free-floating DNA which may induce cell apoptosis, in turn affecting the overall cell viability. Cold Hanks' Balanced Salt Solution (HBSS; with 10% FBS) was added into the digested mixture and successively centrifuged at 300g for 10mins to wash the mixture. The washing step was repeated a couple of times before subjecting the mixture to 0.25% Trypsin-EDTA with phenol-red for 5 mins at 37°C to separate the clumps into single cells. The mixture was then washed before adding DAPI into it and subsequently, cell sorted it with flow cytometry.

On the other hand, the epidermis that was removed earlier will be exposed to 0.25% Trypsin-EDTA with phenol-red for 5 mins at 37 °C immediately to separate it into single cells. It was then washed before adding DAPI and proceeded to isolate the cells with flow cytometry.

Cell sorting was performed on BD Influx™ Cell Sorter (BD Biosciences, Franklin Lakes, New Jersey, United States) to isolate the cells of interest. Cells were first gated using the Side Scattering (SSC) and Forward Scattering (FSC) parameters for live cells before drilling down into SSC Width-SSC and FSC Width-FSC parameters to exclude the clumped cells. The cells were further drilled down to isolate for viable cells with the help of DAPI and before drilling

down to further isolate the Tdt⁺ cells. Post-sorting analysis was subsequently performed to ensure the purity of the collected cells.

Figure 7. Diagram showing the FACS strategy utilized to obtain the final melanocyte (Tdt⁺) population. The cells were first sorted based on FSC-A and SSC-A to identify live cells and remove the debris. P1 is then drilled down through P2 and P3 to remove cell clumps and sorted based on DAPI to isolate the live cells. P4 is subsequently sorted based on PE-A to isolate the melanocytes

Melanin Assay

Cells were first lysed with the cOmplete™ Lysis-M EDTA-free buffer with Protease Inhibitor (PI) (Roche, Basel, Switzerland). Next, the samples were centrifuged twice at 14,000 rpm for 10 mins before dissolving the pellets in 1M NaOH and incubated at 80°C for 1 hr. Concurrently, serial dilution of synthetic melanin was performed and incubated at 80°C for 1 hr. The standard curve was built based on the absorbance level of the synthetic melanin (Sigma-Aldrich, St Louis, Missouri, United States). Both melanin standards and samples were plated into a clear, round-bottom 96-well plate (Greiner Bio-one, Kremsmünster, Austria) in duplicates. Absorbance values were obtained at 475nm using the SpectraMax M5 microplate reader (Molecular Devices, San Jose, California, United States). Subsequently, the melanin concentration of the samples was obtained by comparing the absorbance with the constructed standard curve.

Table 7

Standard	Melanin Concentration (µg/ml)	Volume of synthetic melanin (µl)	Volume of 1M NaOH (µl)
A	100	100 from stock	900
B	75	600 from A	200
C	50	400 from B	200
D	25	250 from C	250
E	5	60 from D	240
F	0	0	130

Table 7 shows the serial dilution of synthetic melanin for melanin assay standard curve.

Figure 8. Graph shows the standard curve for melanin assay. The standard curve is constructed with synthetic melanin serially diluted into various concentrations. Absorbance value is then obtained to construct the standard curve.

RNA isolation and RT-qPCR

Total RNAs were harvested and purified from FACs cells with Isolate II RNA Micro Kit (Bioline, London, United Kingdom) before assessing the quality and quantity by NanoDrop 8000 full Spectrum 8-Sample UV (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Next, 50ng of RNA was reverse transcribed with ReverTra AceTM qPCR RT Kit (Toyobo, Osaka, Japan) using oligo(dT) primers. Primers were designed (**Table 8**) and RT-qPCR was performed with LightCycler 480 (Roche, Basel, Switzerland) under these settings: 30 secs at 95°C for initial denaturing, followed by 50 cycles of 15 secs of 95°C of denaturation, 1 min of 60°C annealing and extension. iTaq Universal SYBR Green Supermix (Bio-Rad, Hercules, California, United States) is used and differences between the samples and controls were calculated with the $2^{-\Delta\Delta C_P}$ method. Ribosomal protein L13a is used as the housekeeping gene in RT-qPCR, where it standardizes the amount of expression between samples.

Table 8

S/N	Gene	Primer Sequence
1	<i>APOE</i> Forward	CTGGAGGCTAAGGACTTGTTTC
	<i>APOE</i> Reverse	CATCAGTGCCGTCAGTTCTT
2	<i>BMP4</i> Forward	TTATGGGCTGGCCATTGAGG
	<i>BMP4</i> Reverse	GCACAATGGCATGGTTGGTT
3	<i>CD63</i> Forward	GCCTGCAAGGAGAACTACTG
	<i>CD63</i> Reverse	CCCACAGCCCACAGTTATG
4	<i>CKIT</i> Forward	GCACCAGAGAGCATTTTCAGC
	<i>CKIT</i> Reverse	CCTGCTTGAATGTTGGCCTT
5	<i>CTHRC1</i> Forward	GGTCGGGATGGATTCAAAGG
	<i>CTHRC1</i> Reverse	GATGGCTTCGATGGGAAGAG
6	<i>CTSL</i> Forward	GGGTCTTGTTGGGCGTTTA
	<i>CTSL</i> Reverse	GCGAACTCGGCTCTGTATTT
7	<i>CYBA</i> Forward	CTCATCTGTCTGCTGGAGTATC
	<i>CYBA</i> Reverse	GCCGCTCCTTGGGTTTA
8	<i>GPX4</i> Forward	TTTACGAATCCTGGCCTTCC
	<i>GPX4</i> Reverse	CCACGCAGCCGTTCTTAT
9	<i>ID2</i> Forward	ACCCGATGAGTCTGCTCTAC
	<i>ID2</i> Reverse	GGATGCTGATGTCCGTGTTC
10	<i>MC1R</i> Forward	AGGGGAGGCTGTTGGCTTATC
	<i>MC1R</i> Reverse	CGGGACCGTGGTTTCCTAAAT

11	<i>MGST1</i> Forward	ACCGCATTCCAGAGGATAAC
	<i>MGST1</i> Reverse	GTAGATCCGAGCACCTACAAAG
12	<i>MITF</i> Forward	CACTGGGGAGAAGTTGATGTTGATA
	<i>MITF</i> Reverse	AGTGCTGCGGACCATAACAGAAA
13	<i>MLANA</i> Forward	ACCTCAAGGGGAAAGATGCTC
	<i>MLANA</i> Reverse	GGCGTCTACTCAGCCTTAAA
14	<i>PMEL</i> Forward	CAGCGGTCATAGATACCACAC
	<i>PMEL</i> Reverse	GCGAGGGAGAAAGAACCATATC
15	<i>PRDX2</i> Forward	CCACCTGGAATCTGGTGAATAG
	<i>PRDX2</i> Reverse	ATCCCTCCAGGCCTAGTAAT
16	<i>PTGDS</i> Forward	TCCTGGGTCTCTTGGGATT
	<i>PTGDS</i> Reverse	CATGATCTTGGTCTCACACTGG
17	<i>QPCT</i> Forward	GAGTAGGACTCCCTATGGCTATC
	<i>QPCT</i> Reverse	GTGAGAGATCTGGCTTGGAAC
18	<i>RAB38</i> Forward	GGGACACAGACAGCCCTAATA
	<i>RAB38</i> Reverse	GCACACGACTCTGAAGAGAAAC
19	<i>SELENOP</i> Forward	GAGCAAAGAGACAGGACGAAG
	<i>SELENOP</i> Reverse	GCCTGCAGAAGACACAAGTA
20	<i>SLC24A5</i> Forward	CAGGGACAAGCATACCAGATAC
	<i>SLC24A5</i> Reverse	CGGTCTAGTTTCCAGCCATT
21	<i>SMADI</i> Forward	CCGAGTAACTGCGTCACCAT
	<i>SMADI</i> Reverse	CATTTTGTCCCAGGTTGCGG
22	<i>SMAD5</i> Forward	GATTCTGCCTGGGATTGTTGTC
	<i>SMAD5</i> Reverse	CAGCTGCTGGGGATCTTACA
23	<i>SMAD9</i> Forward	TGGTACCAAGACACAGCGAG
	<i>SMAD9</i> Reverse	GCCGGAAGTCTGAGTGTTGA
24	<i>TM4SF1</i> Forward	CTGTGAGCATCGGCTTTCT
	<i>TM4SF1</i> Reverse	GTCCTCCGTAGCATACTTTGTC
25	<i>TRP2</i> Forward	CCCACCGGTGACTAATGAGG
	<i>TRP2</i> Reverse	CTCAAGATGAGCGACGGTGT
26	<i>TYR</i> Forward	GGGGGCTCTGAAATATGGAGG
	<i>TYR</i> Reverse	GGGTGACGACCTCCCAAGTA
27	<i>TYRP1</i> Forward	CAAGGCCACCACAAAGTCACAG
	<i>TYRP1</i> Reverse	CGCAGGCCTCTAAGATACGAGAA
28	<i>VAMP8</i> Forward	GACCACCTCCGAAACAAGAC
	<i>VAMP8</i> Reverse	GGCTCCTCTTGGCACATATTAG

Table 8 shows the sequences of the primers used in RT-qPCR.

RNA sequencing

Eluted RNA of samples were then sent to Novogene for RNA Sequencing, where preamplification, library preparation, and Illumina RNA sequencing were performed. Preamplification was achieved with SMART-Seq v4 Ultra Low RNA Kit (Clontech Laboratories, Mountain View, California, United States), where synthesis of the first strand took place before whole-length LD-PCR amplification. Next, NEB Next Ultra RNA Library Prep Kit for Illumina® was used to construct the library, where cDNA samples were sheared by the Covaris system, and subsequently end-paired, A-tailed, and ligated to sequencing adaptors. The samples were uniquely indexed with a barcode. Quantification of the final library size was performed with Qubit 2.0 fluorometer (Life Technologies, Carlsbad, California, United States) and then on an Agilent 2100 and qPCR. 125bp PE sequencing was done on Illumina NovaSeq6000 system to at least 45 million reads per sample.

Subsequently, analysis of RNA sequencing data was performed using Partek Flow Software, version 9. Data were first trimmed to remove adapter content contamination from the library preparation. With the help of the Cutadapt tool in Partek Flow and the list of adapter sequences provided (**Table 9**), trimming was performed on both 3' and 5' ends with some amendments to the default parameters: minimum overlap of 5 bp, error rate of 0.2 and a minimum post trim

Figure 9. Bar Chart showing the alignment breakdown of samples. >40M reads were detected with >85% aligned and obtained an average quality score of 36 for the reads.

size of 35bp. Post trim QC was performed to show that the total read count was not noticeably affected and all samples still had >40M reads (**Figure 9**). In addition, all samples were aligned successfully with >85% total alignment throughout.

Figure 10. Bar Chart showing the coverage breakdown of samples after annotation. >82% reads were aligned fully to exonic genomic regions with a small percentage lost to non-alignment and alignment to non-exonic regions.

Next, the data was aligned with TopHat2 (Kim et al., 2013) and annotated with RefSeq mm10 sequence, using the default parameters. All samples showed >82% reads aligned to fully exonic genomic regions (**Figure 10**). Before differential analysis, the samples were categorized into cKO and WT groups and were normalized using the counts per million (CPM) method.

Differential analysis was performed on the samples using DeSeq2 (Love et al., 2014) with default parameters to reveal the differentially expressed genes (DEGs) between samples and were filtered with the criteria of $FDR < 0.05$. To characterize the gene expression further, gene ontology (GO) analysis was performed on the DEGs with Panther version 15 (Thomas et al., 2003, 2006). Panther overrepresentation test (Released 20200407) was utilized, using Binomial test type and filtered with adjusted $p\text{-value} < 0.05$. KEGG pathway enrichment analysis was conducted with KOBAS version 3 (C. Xie et al., 2011) and pathways were filtered based on adjusted $p\text{-value} < 0.05$.

Table 9

3' Primer Adapters
CCCATGTACTCTGCGTTGATAACCACTGCTT
AAGCAGTGGTATCAACGCAGAGTACATGGG
AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTT TTTTTTTT
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGA TACCACTGCTT
CTGTCTCTTATACACATCTGACGCTGCCGACGACTAAGCCTGTGTA GATCTCGGTGGTCGCCGTATCATT
CTGTCTCTTATACACATCTCCGAGCCCACGAGACTAAGGCGAATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACCGTACTAGATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACAGGCAGAAATC TCGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACTCCTGAGCATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACGGACTCCTATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACTAGGCATGATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACCTCTCTACATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACCAGAGAGGATC TCGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACGCTACGCTATCT CGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACCGAGGCTGATCT CGTATGCCGTCTTCTGCTTG

CTGTCTCTTATACACATCTCCGAGCCCACGAGACAAGAGGCAATC TCGTATGCCGTCTTCTGCTTG
CTGTCTCTTATACACATCTCCGAGCCCACGAGACGTAGAGGAATC TCGTATGCCGTCTTCTGCTTG
5' Primer Adapters
CCCATGTACTCTGCGTTGATACCACTGCTT
AAGCAGTGGTATCAACGCAGAGTACATGGG
AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTT TTTTTTTT
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGTACTCTGCGTTGA TACCACTGCTT

Table 9 shows the list of adapter sequences.

Statistics

All data were presented as mean±s.d. with the number of replicates indicated. Parametric one-way ANOVA with Tukey post hoc test was used to calculate statistical significance when the comparison between multiple samples is required. In addition, our experiments setup only involves one variable, therefore explaining the use of one-way ANOVA with Tukey post hoc test.

Two-way ANOVA with Tukey post hoc test is used in the migration assay (**Figure 13B & 14B**) to calculate statistical significance. This allows appropriate comparison between multiple samples when the experimental setup involves two variables.

On the other hand, parametric one-tailed paired t-test was used to calculate the statistical significance in experiments that consist of only two conditions. Moreover, the experimental effect was expected to only move in one direction, therefore explaining the use of a one-tailed paired t-test. Statistical methods are indicated in the corresponding figures, with the use of GraphPad Prism 9. Statistical significance was considered if $p < 0.05$.

RESULTS

BMP signalling within the HF stem cell niche of human skin biopsies

The main goal of this thesis is to downregulate BMP signalling to enhance the melanocyte migration from the HF MeSC compartment into the IFE. Therefore, this project's foundation lays on the premise that BMP signalling is active within the human MeSCs. MeSCs are located in the bulge and share their niche with other HF stem cells for which several groups have reported the key role of BMP signalling in maintaining their quiescence (Infarinato et al., 2020). However, there is not enough evidence that undifferentiated melanocytes in the MeSC compartment maintain an active BMP signalling. As shown in **Supplementary Figure 1**, we have corroborated an active BMP signalling in the MeSC compartment of human HFs. Cryopreserved human follicular skin biopsies and histological sections were prepared for immunofluorescence analysis of BMP signalling reporters. To readily identify the basal layer melanocytes, MeSC, and bulb melanocytes, we co-stained our sections with the melanocyte-specific CKIT Ab. As it can be seen in **Supplementary Figure 1**, we corroborated that our preliminary analysis evidenced a strong BMP activity in the melanocytes of the epidermis and the HF. Specifically, the BMP signalling reporter pSmad1/5/8 was found in CKIT positive basal layer melanocytes (**Supplementary Figure 1C-Upper panel**), the MeSC compartment (K15+ in **Supplementary Figure 1D, E-Lower panel**), and the mature melanocytes found in the HF bulb (**Supplementary Figure 1C-Upper panel**). In addition, we have also positive detection of BMP4 and BMP6 in the melanocytes of the epidermal basal layer (**Supplementary Figure 1C-Lower panel** and **Supplementary Figure 1B-Upper panel**). Moreover, BMP6 co-stained with the mature bulb melanocytes in **Supplementary Figure 1B-Upper panel**. Thus, our preliminary data already confirms an active BMP signalling in the melanocytes of human skin.

Importantly, our data is also indicating that undifferentiated melanocytes of the HF MeSC are indeed maintaining high levels of BMP signalling.

Establishment of a 2D keratinocyte co-culture system to study the role of BMP signalling in skin pigmentation and migration

Skin melanocytes are our first line of defense against the harmful ultraviolet irradiation from the sun and are responsible for the production and maintenance of our skin pigmentation. To study melanogenesis, transfer, and migration at the functional level, a 2D keratinocyte and melanocyte co-culture was established. In our novel assay, melanin synthesis and transfer are measured by immune detection of melanin organelles by NKI/GP100 Ab and keratinocytes using the K14 Ab. As shown in **Figure 11**, we optimized cell culture conditions to co-culture human melanocytes and keratinocytes at physiologically relevant ratios (1:10-40). In addition, our assay allows the use of FACS to quantify pigment transfer from melanocytes to keratinocytes (by quantifying the percentage of cells that are both positive for K14 (keratinocyte marker) and NKI/GP100 (melanosome marker) (**Figure 11B**), as well as pigment production by measuring the median fluorescence intensity of NKI/GP100 in K14-negative melanocytes (**Figure 11C**).

A

B

C

Figure 11. 2D co-culture of human keratinocytes and melanocytes. Day 3 of Me:Kc (1:10 ratio) co-culture. **(A)** left panel: double K14, GP100 staining, middle panel: GP100 only and right panel: K14 only, middle and right panels. Note K14-positive keratinocytes that contain melanosomes (GP100) (left and middle panels). **(B)** FACS analysis. P4 gated population identifies K14-positive/GP100-positive keratinocytes as an example of pigment transfer. **(C)** Histogram of K14-negative/GP100-positive melanocytes to quantify pigment production. (Scale bar: 100 μ m)

BMP signalling regulates pigment production and transfer

As stated above during the introduction, it is known that BMP4 is a key regulator of skin homeostasis and interestingly, it has been also shown to be involved in the regulation of skin pigmentation. To validate the regulatory effect of BMP4 on pigmentation, we made use of our 2D keratinocyte and melanocyte co-culture system with various cytokine combinations. In this case, both BMP4 and BMP6/or their antagonist, Noggin and Sostdc1, were added into the co-cultures. Our 2D co-cultures were treated with 200 ng/ml of BMP4-6 and in to confirm that the treatment outcome is due to the effect of our BMP cytokines, we “rescued” our co-cultures by introducing Noggin (BMP4 inhibitor) or Sostdc1 (BMP6 inhibitor). Using our established 2D co-culture protocol, our preliminary data shown in **Supplementary Figure 2** shows the inhibition of melanogenesis and pigment transfer by BMP4. Interestingly, Noggin was able to partially rescue the BMP4 inhibition of melanogenesis (**Supplementary Figure 2**). Notably, no significant differences in pigment transfer (K14+/NKI+) were observed after treatment with either BMP4 or BMP6.

BMP4 signalling downregulates melanin production

To corroborate the BMP4 regulation of pigment production. We proceeded to quantify the total melanin content for the respective co-cultures. After treatment with BMP4, the total melanin amount was decreased as compared to the control (**Figure 12**). As expected, this reduction in melanin production was partially rescued when Noggin was added in the combination with BMP4. Interestingly, the addition of Noggin by itself was sufficient to significantly rescue the melanin levels (**Figure 12B, C**). Notably, these results were previously discussed by Singh et.al (Singh et al., 2012), where they saw the same outcomes for their experiments. Interestingly, when we added in Noggin only, an increase in melanin content as compared to control was observed. This is very important as it highlights the possibility of utilizing Noggin to further increase the overall skin pigmentation when coupled with phototherapy. Based on past research, it was known that BMP inhibited keratinocyte migration in wound healing models. Therefore, we wanted to investigate if BMP4 is capable of regulating the migratory functions of melanocytes.

Figure 12. Effects of BMP4 signaling in melanin production. (A) Phase Contrast and Immunofluorescence (IF) staining of co-cultures treating with different cytokines. Note that the 3rd row consists of IF staining for DAPI and NKI (stains for melanosomes). The red dots represent the melanosomes transferred to the keratinocytes. **(B)** Quantification of total melanin content in the co-cultures. Total melanin content was derived from the co-cultures and the different conditions were compared relative to the control. **(C)** The absolute amount of total melanin content in the co-cultures. One-way ANOVA is used for the statistical analysis of this experiment. (Scale bar for phase contrast: 200µm; Scale bar for IF: 100µm)

BMP signalling reduces migration in 2D co-cultures

In an effort to seek a possible BMP signalling effect on the migration of melanocytes. We first investigated the regulation of BMP4 and BMP6 on the migration of keratinocytes and melanocytes in our 2D co-cultures. For this purpose, we adapted the *in vitro* wound assay to our 2D keratinocytes-melanocytes co-cultures. We treated or co-cultures with BMP4 or BMP6 and measure overall wound closure and melanocyte migration. In addition, to the BMP4 and BMP6 treatment, we also rescued their effects using their inhibitors, Noggin and Sostdc1, respectively. After 2D co-cultures had reached sub-confluent densities, the culture was treated with mitomycin-C for only 2h. Then, we tracked the overall wound closure and melanocyte migration by digital imaging at 0,6,12,24,36, and 48 hours. As it can be seen in **Supplementary Figure 3**, our encouraging data shows that both BMP4 and BM6 significantly inhibited overall wound closure and melanocyte migration after 29 hours. It is also notable that BMP4 was a stronger inhibitor of migration than BMP6 and that both BMP4 and BMP6 inhibition could be rescued by Noggin and Sostdc1, respectively (**Supplementary Figure 3B, C**).

BMP4 signalling downregulates melanocyte migration

Cultured melanocytes do not migrate unless they are stimulated to do so. For example, in co-cultures, keratinocytes produce the major adhesion molecule E-Cadherin to establish a cell-cell adhesion migration stimulus to their neighboring melanocytes. Another known migration stimulus is UVR irradiation, as it has been used in skin wound models. To achieve this, we established an *in vitro* wound assay where melanocytes are induced to migrate using conditional media from our Kc:Me co-cultures (**Figure 13**). To determine the effect of BMP4 in melanocyte migration, we performed *in vitro* melanocyte migration assay with the use of Ibidi Culture-Insert 2 Well 24. To ensure the accuracy of this assay, the co-cultures were administered with mitomycin-C to prevent cell proliferation.

A macro script was used to quantify the area covered and with that, wound closure can be tracked with accordance to time (**Figure 13B**). To better monitor the migration behaviour, we utilized 2-point measurement method (with start point at 0% wound closure, and end point at 65% wound closure) as shown in **Figure 13C**. Moreover, to make the data comparable, we quantified the velocity of wound migration as illustrated in **Figure 13D**. Overall, we observed that melanocytes treated with BMP4 required a longer time to close the wound as compared to the controls.

This migration delay was ‘rescued’ when we added in the antagonist, Noggin, as observed in **Figure 13**. In contrast, when treated with Noggin only, it upregulated the migratory function of melanocytes as compared to the other conditions. Till this point, the result had indicated the potential of Noggin in enhancing the migratory function of melanocytes.

Figure 13. Melanocyte migration assay without UVR irradiation. (A) Phase Contrast images of the migration assay and magnified image of ‘migrating melanocytes’ (right), with lamellipodia pointed out. Migration assay of melanocytes was conducted for up to 48 hrs and the area covered by melanocytes was traced. A macro script was used to trace the area covered by melanocytes and a comparison was performed relative to the control. (B) % wound closure was tracked throughout and plotted against time. Two-way ANOVA is used for the statistical analysis of this experiment. (C) Quantification of the area covered by melanocytes based on 2-point measurement method. One-way ANOVA is used for the statistical analysis of this experiment. (D) Quantification of the wound closure rate between different conditions. One-way ANOVA is used for the statistical analysis of this experiment. (Scale bar: 500 μ m)

Figure 14. Melanocyte migration assay with UVR irradiation. (A) Phase Contrast images of the migration assay and magnified image of ‘migrating melanocytes’ (right), with lamellipodia pointed out. Migration assay of melanocytes was conducted for up to 48 hrs after UVR irradiation and area covered by melanocytes was traced. A macro script was used to trace the area covered by melanocytes and a comparison was performed relative to the control. (B) % wound closure was tracked throughout and plotted against time. Two-way ANOVA is used for the statistical analysis of this experiment. (C) Quantification of the area covered by melanocytes based on 2-point measurement method. One-way ANOVA is used for the statistical analysis of this experiment. (D) Quantification of the wound closure rate between different conditions. One-way ANOVA is used for the statistical analysis of this experiment. (Scale bar: 500 μ m)

However, little was known about its effect when used in conjunction with phototherapy, which was referred to as the golden standard in treating vitiligo. Moreover, UV was known to stimulate the migration of melanocytes out into the epidermal layer. Therefore, we proceeded to repeat the whole migration assay, with addition of UVR exposure ($50\text{mJ}/\text{cm}^2$) after the mitomycin-C treatment. Similarly, we observed that melanocytes treated with BMP4 required a longer time to close the wound whereas its counterpart required a shorter time to close the wound as shown in **Figure 14**.

Figure 15. Melanocyte Migration assay. Quantification of area covered by melanocytes of both migration assays. Macro script was used to trace the area covered by melanocytes and comparison was performed in relative to the non-UV control. One-way ANOVA is used for the statistical analysis of this experiment.

Fascinatingly, when these two graphs were conjoined together, Noggin was seen to have an additive effect in upregulating the migratory function when coupled with UV. Furthermore, it is important to note that the overall time wound closure time for the UV batch was shorter in contrast to the non-UV batch (**Figure 15**). This result further emphasized the potential of Noggin as a possible novel therapeutic component in vitiligo treatment.

In vivo HF MeSC-specific BMP4 ablation

Figure 16. *In vivo* BMP4 ablation in the MeSC compartment of the hair follicle. Schematic drawing of our BMP4 cKO strategy.

To further confirm the *in vitro* regulation of BMP signalling in melanocyte migration, we next wanted to validate our findings with an *in vivo* model. To specifically target the HF MeSC compartment, we first crossed Tamoxifen-inducible (TyrER^{T2}) mice lines with homozygous BMP4 floxed (BMP4^{fl/fl}) mice to conditionally knock out BMP4 specifically in Me of HFs. In addition, to report for the Cre activity and follow up our conditional cKO mice fate, we also crossed our lines with a Td-tomato reporter-generating our final BMP4^{fl/fl}/TyrCreER^{T2}/Td-tomato line. Then, to specifically target Me in the MeSC, we induced our Cre-lox system right at telogen. During the telogen phase of the HF, only melanocyte stem cells survive the catagen phase making this time ideal to target the MeSC. Therefore, at P15 when HFs enter telogen, mice were injected with tamoxifen (1mg/10g) for 3 consecutive days to induce Cre recombinase activity and conditionally ablate BMP4 at the MeSC compartment (**Figure 16**). Initial phenotypes were analyzed at the anagen phase around postnatal day 30 by harvesting dorsal skins and assessing efficient recombination and ablation of BMP4 by immunofluorescence. Before further downstream

processes were carried out, we first had to certify that BMP4 was indeed conditionally ablated at the MeSC niche of the HF. Here, we performed immunofluorescence staining using a cocktail of antibodies (BMP4, Sox10, and TRP2). As shown in **Figures 17I & 17II**, we demonstrated that BMP4 expression in the melanocytes was specifically ablated in the Me of cKO as compared to WT mice (both Guard and Awl/Auchene HF). These findings corroborated the TyrCre efficiency to target and ablate the BMP4 gene in melanocytes, which was validated with the help of the melanocyte-specific markers, Sox10 and TRP2. Moreover, it is important to note that the nuclear detection of Sox10 in cKO mice, suggests that the reduction in BMP4 expression was not due to the masking of signals by melanin but rather, ablation through Cre activity.

Even though we had successfully ablated BMP4 gene with this model, but we did not aim to completely abolish BMP signalling in the skin. This is because complete abolishment of BMP signalling will cause more harm than good as we knew that BMP signalling is a vital pathway for various cellular functions and processes. Therefore, we investigated the presence of BMP signalling by staining its downstream effectors, namely SMAD1/5/8 (**Figure 18I & 18II**).

Here, we validated the presence of the downstream effectors and in both cKO and WT. This result indicated that the ablation of BMP4 gene did not result in the complete abolishment of BMP signalling. With that being said, the proposed mouse model is established to be appropriate for our study.

Figure 17I. IF staining for BMP4, Sox10 and TRP2 expression in Guard HF. (A) IF Staining of guard hair in WT. BMP4 was stained to check for its expression, whereas Sox10 and TRP2 were stained to identify the melanocytes. **(B)** IF Staining of guard hair in cKO. BMP4 was stained to check for its expression, whereas Sox10 and TRP2 were stained to identify the melanocytes. Decreased BMP4 expression was observed in the cKO. (Scale bar: 100µm)

Figure 17II. IF staining for BMP4, Sox10 and TRP2 expression in Awl/Auchene HF. (A) IF Staining of awl/auchene hair in WT. BMP4 was stained to check for its expression, whereas Sox10 and TRP2 were stained to identify the melanocytes. **(B)** IF Staining of awl/auchene hair in cKO. BMP4 was stained to check for its expression, whereas Sox10 and TRP2 were stained to identify the melanocytes. Decreased BMP4 expression was observed in the cKO. (Scale bar: 100μm)

Figure 18I. IF staining for pSmad1/5, Sox10 and TRP2 expression in Guard HF. (A) IF Staining of guard hair in WT. pSmad1/5 was stained to check for its expression after BMP4 ablation, whereas Sox10 and TRP2 were stained to identify the melanocytes. **(B)** IF Staining of guard hair in cKO. pSmad1/5 was stained to check for its expression after BMP4 ablation, whereas Sox10 and TRP2 were stained to identify the melanocytes. No difference in expression is observed between cKO and WT. (Scale bar: 100µm)

BMP4 ablation in the HF MeSC upregulates interfollicular epidermal migration of melanocytes

After the establishment of our mouse model, we wanted to first determine the effect of BMP4 ablation on the migratory function of melanocytes. A noteworthy difference between the mouse and human epidermis is that most mice strains used in research do not contain melanocytes within the IFE. This lack of epidermal pigmentation is critical for developing pigmentation models more relevant to human melanocyte biology. As discussed during the introduction, UVR irradiation can be used to stimulate the migration of the MeSC from the bulge to the IFE. Notably, in WT mice, this migration into the epidermal layer after UVR exposure is limited but this phenomenon is more pronounced in the cKO when BMP4 is ablated.

Therefore, we utilized both Fontana Masson Stain, which stains for melanin, to investigate this by counting the number of melanin deposits that resided in the IFE and HF. In this experiment, more incidences of melanin were seen deposited in the IFE of the cKO as compared to the WT (**Figure 19B**). On the other hand, both conditions had almost an equal number of melanin deposits that resided in the HF. This observation indicated that cKO had more melanocytes migrating out into the IFE in contrast to the WT.

However, more validation is required to confirm this finding. With that in mind, we ensued to exploit the Td-tomato reporter gene in our mouse model to visualize and trace the movement of melanocytes. Correspondingly, a similar phenotype where more melanocytes migrated out into the IFE was seen after quantifying the number of melanocytes in both regions with the help of the reporter gene (**Figure 19D**). With that, it validated our previous observation with regards to the effects of BMP4 ablation in upregulating melanocyte migratory function. This finding was in conjunction with our *in vitro* data as well where we saw the co-cultures treated with Noggin displayed upregulated melanocyte migratory function.

Since BMP4 ablation had shown to upregulate the migratory function of melanocytes into the IFE, we wanted to further validate this phenomenon by performing a ‘rescue of phenotype’ experiment. In this experiment, we hoped to reverse the effects of BMP4 ablation by performing localized injection of BMP4 cytokines into the dorsal skin of our mouse model, and subsequently looking into these injected sites for signs of reversal. Implanted skin sites were harvested and quantification was performed based on the reporter gene of the mouse model (**Figure 20**). Here, we saw a significant reduction in the number of melanocytes migrating out into the IFE after the implantation (**Figure 20C**), suggesting that the previously observed phenotype was ‘rescued’ here, thus validating our findings. This is an important validation data for our mouse model as it not only demonstrated the effectiveness of our mouse model for this study but also greatly supported our hypothesis in controlling BMP4 signalling to enhance the migratory function of melanocytes.

Figure 19. Tracking of melanocyte migration incidences. (A) FM Stain of both cKO and WT mice to locate melanin deposits. (B) Comparison between cKO and WT on the number of deposits in IFE and HF. (C) Visualization of melanocytes through Tdt reporter gene. (D) Comparison between cKO and WT on number of melanocytes in IFE and HF. One-tail paired t-test is used for the statistical analysis of this experiment. (Scale bar: 200 μ m)

Figure 20. 'Rescue' assay of the proposed mouse model. (A) Visualization of melanocytes through Tdt reporter gene in both control and 'rescued' sites. Migration of melanocytes is still observed in control sites as compared to the sites injected with BMP4-coated beads. **(B)** H&E staining of both the control and 'rescued' sites. **(C)** Overall quantification between cKO, BMP4 cKO control, and BMP4 cKO 'rescued' sites. A final comparison is performed between all the different components in both HF and IFE. This is to visualize the impact of BMP4 dermal injection to 'rescue' the observed phenotype in cKO. One-way ANOVA is used for the statistical analysis of this experiment. (Scale bar for Tdt: 100µm; Scale bar for H&E: 200µm)

Prospective isolation and characterization of the HF MeSC niche during UVR-induced repigmentation

As previously reported by Singh et al. (Singh et al., 2012) and shown in our *in vitro* data, BMP4 was witnessed to play a role in downregulating melanogenesis and therefore, we went on to investigate this potential effect of BMP4 in our mouse model. For this, we first separated the epidermis and dermis of dorsal skin. Then, taking advantage of our genetic tool, we were able to isolate live melanocytes using FACSsort and select our Tdt reporter. As illustrated in **Figure 21**, Tdt⁺ cells were isolated using an appropriate gating strategy on FACs for both cKO and WT. Subsequently, we performed RT-qPCR on several known melanocytes signature genes to validate the identity of Tdt⁺ population. As shown in **Figure 21B**, the data obtained from RT-qPCR revealed the gene profile of melanocytes, thereby confirming the identity of Tdt⁺ population as melanocytes.

Figure 21. Prospective isolation strategy for melanocytes. (A) FACSsort strategy utilized to isolate melanocytes from dorsal skin of mouse. The dorsal skin of mouse is first processed into single cells before subjecting the cells through flow cytometry to filter out the melanocytes (Tdt⁺ cells). (B) Comparison of melanocyte signature genes between DN and Tdt⁺ populations. Various melanocyte signature genes were tested to determine the identity of the Tdt⁺ population. In this case, Tdt⁺ population demonstrated melanocytes profile based on the qPCR results. One-tail paired t-test is used for the statistical analysis of the comparison between DN and Tdt⁺ for each primer.

BMP4 ablation upregulates melanin production

Our live FACSsort strategy allowed us to study the first changes in overall melanin production. The sorted Tdt⁺ samples were normalized in the number of cells before undergoing melanin assay, where we compare the concentration of melanin in both cKO and WT. After quantification, the dermis of cKO had higher melanin concentration as compared to WT, further validating the role of BMP4 as reported previously (**Figure 22**). This was also in conjunction with the *in vitro* data previously discussed, where co-cultures treated with Noggin demonstrated a higher concentration of melanin content. On the other hand, the epidermis of both groups displayed almost the same amount of melanin concentration, although slightly upregulated in cKO epidermis, which could be because the number of melanocytes that had migrated out into the IFE was not large enough to elicit a difference in melanin concentration between the two samples. With all these results in mind, gene expression study was the next logical step to further understand the mechanisms involved in the regulation of melanocyte functions via BMP4 signalling.

Figure 22. Quantification of melanin content of Tdt⁺ population. (A) Melanin content of dermal Tdt⁺ population between WT and cKO. **(B)** Melanin content of total epidermis between WT and cKO. One-tail paired t-test is used for the statistical analysis of this experiment.

Gene Expression Analysis reveals distinct molecular pathways from BMP4 ablation

To answer the ultimate aim of this study, which is to understand the mechanisms involved, we executed RNA sequencing on the melanocytes population to gain insights into the various differential expressed genes between cKO and WT. Replicated samples originated from different litters of mice and a sufficient number of cells were collected to ensure a high-quality RNA collection. After obtaining the RNA sequencing data from Novogene, the samples were aligned using TopHat2 and annotated with RefSeq mm10 sequence. Next, DeSeq2 was used to normalize by CPM, resulting in various DEGs between cKO and WT to be identified. Two distinct cell populations were identified (cKO and WT), with clustered replicates as shown in the principal component analysis (PCA) (**Figure 23A**), which illustrated the gene expression difference between the two groups of the sample. Moreover, this clustering depicted the correlation involved between the sample replicates, thus assuring the accuracy of the data. These were further represented by the hierarchical heat map (**Figure 23B**) shown, where we observed the difference between the two groups of the sample as well as the correlation involved between the replicated samples. Next, we defined these signatures by filtering out transcripts with $FDR < 0.05$ and resulted in a list of differentially expressed genes, derived from this comparison.

Figure 23. Overview of samples used for RNA sequencing. (A) PCA plot of the samples used for RNA-seq. Two distinct categories were observed in the PCA plot, demonstrating the stark difference in gene expression between the two populations. **(B)** Hierarchical heatmap for the gene expressions between the two populations.

With this list, GO analysis was executed to uncover processes that were significantly regulated based on the list of DEGs. The result revealed various functions that suggested the impact of BMP4 ablation on melanocytes (**Figure 24**). As expected, GO analysis for biological processes showed that pigmentation was significantly regulated, which coincided with our previous findings where a difference in melanin concentration was observed between cKO and WT. Moreover, GO analysis for cellular processes revealed pigment granule and melanosome processes to be significantly regulated as well (**Figure 25**). These processes were associated with melanosome biogenesis, suggesting a relationship between BMP4 and melanosome biogenesis, in particular the trafficking process involved with melanosomes.

Figure 24. List of biological processes in Gene Ontology BP. Based on the RNA-seq data obtained, gene ontology analysis is performed to determine the biological processes involved to elicit the observed phenotype.

Figure 25. List of cellular processes in Gene Ontology CP. Based on the RNA-seq data obtained, gene ontology analysis is performed to determine the cellular processes involved to elicit the observed phenotype.

Figure 26. List of KEGG pathways involved. Based on the RNA-seq data obtained, KEGG pathways analysis is performed to identify the possible effect on various major pathways.

Next, we performed KEGG analysis to understand the high-level functions and utilities of the biological system (**Figure 26**). Here, the result displayed oxidative phosphorylation as one of the significantly regulated pathways, which might be caused by the increase in melanin production and thus leading to an increase in the overall oxidative phosphorylation pathway.

With that, we then looked into the functions of all filtered DEGs, and from the list of 85 DEGs, we filtered out the 24 melanocyte-related genes. Among these 24 genes, we discovered several pigmentation-related genes, and therefore further categorized them into the following groups; melanin biosynthesis and melanosome biogenesis. With regards to the melanin biosynthesis group, we saw genes like TYR being upregulated in the cKO, which was known to be involved in the synthesis of melanin. Moreover, it was also described previously that BMP4 played a role in inhibiting it (Singh et al., 2012). On the other hand, MLANA is a gene known to be involved in melanosome biogenesis as its presence is critical to the formation of stage II melanosomes. As a whole, it was suggested that in terms of pigmentation, BMP4 was not only involved in the regulation of melanin biosynthesis, but also melanosome biogenesis.

Besides pigmentation genes, it is important to note the presence of other melanocyte-related genes linked to differentiation, oxidative stress, migration, proliferation, and melanomas formation (**Figure 27**). This was expected especially because BMP4 was known to maintain cell quiescence. Furthermore, we had demonstrated previously the inhibitory role of BMP4 on melanocytes migration. Therefore, data from RNA sequencing is in conjunction with all other findings.

Figure 27. List of melanocyte-related genes identified in the DEGs list. The list of genes was categorized into pigmentation (orange), migration (green), proliferation (pink), differentiation (purple), oxidative stress response (blue), melanoma formation (grey). n=3 is achieved for all the listed primers. One-tail paired t-test is used for the statistical analysis of the comparison between WT and cKO for each primer.

Validation of Identified DEGs

To validate the findings from RNA sequencing, we next performed RT-qPCR on several signature melanocyte genes to determine the observed effect of BMP4. For this, we applied FACSsort to isolate the Tdt⁺ cells and thereby extract the total RNA before reverse transcribing it into cDNA as the starting material required in RT-qPCR. To ensure that the sorted cells were highly enriched in melanocytes, negative cells were isolated too for comparison by RT-qPCR for the expression of signature melanocyte genes. Furthermore, we verified the ablation of BMP4 and validated it by using the downstream effectors of the BMP/SMAD pathway, namely SMAD1/5/8 (**Figure 28C**).

From these data, ablation of BMP4 is validated in the cKO, as a significant downregulation in BMP4 and downstream effector genes (SMAD 1/5/8) was observed as shown in **Figure 28C**. This in turn confirms the presence and effectiveness of the Cre-lox recombinase system in creating a BMP4 cKO mouse model. With that in mind, we proceeded to investigate the gene expression of several melanocyte signature genes. Here, we saw that by ablating BMP4, signature melanocyte genes like CKIT, TYR, TRP2, and MC1R were upregulated, indicating the mechanisms involved for the upregulation of melanogenesis in cKO (**Figure 28B**). Putting all the data from both *in vitro* and *in vivo* together, we can conclude that not only did it support our hypothesis but also provided insights into the mechanisms involved.

With the list of the 24 selected DEGs that were known to be related to melanocytes, we sought to corroborate this data with RT-qPCR. Therefore, RT-qPCR was executed for these genes of interest. Correspondingly, we observed the same phenomenon as before for these genes, confirming the data we obtained through RNA sequencing (**Figure 28D**). With all the data from both *in vitro* and *in vivo* studies, we can conclude that not only did it support our hypothesis but also provided insights into the mechanisms involved.

Figure 28. Gene expression profile of both WT and cKO. (A) FACS sort strategy utilized to isolate melanocytes from dorsal skin of mouse. Dorsal skin of mouse is first processed into single cells before subjecting the cells through flow cytometry to filter out the melanocytes (Tdt+ cells). (B) qPCR results of melanocyte signature genes. Melanocyte signature genes were tested to determine the effect of BMP4 ablation. (C) qPCR results of BMP4 and its downstream effector genes. BMP4 and its downstream effector genes were tested to validate the ablation of BMP4. (D) Validation of identified melanocyte-related genes from the DEGs list. One-tail paired t-test is used for the statistical analysis of the comparison between WT and cKO for each primer.

DISCUSSION

In the case of vitiligo, it is known that the immune cells target and destroy the matured melanocytes present in the epidermal layer, thereby resulting in a lack of melanin and the appearance of white patches on the skin. However, these immune cells do not target the immature melanocytes that reside in the hair bulge compartment. With this in mind, we proposed to stimulate this group of immature melanocytes by conferring a change in its signalling pathway and thereby enhancing the rate of UV-induced follicular repigmentation. Among all the signalling pathways involved, two groups of signals, BMP2/4 and Wnt/ β -catenin, were identified to be linked to the tandem pattern of inhibitor and activator signals, which cause the cyclical activation of HF stem cells (Fuchs, 2007; Houshyar et al., 2020; Jahoda & Christiano, 2011; Plikus et al., 2008).

Before this study, previous research was performed by Clavel et al. on the effects of Sox2 in controlling hair growth and uncovered the role of BMP signalling in regulating HF differentiation. Clavel et al. reported that Sox2 ablation led to an increased BMP expression and interestingly, we have also recently linked it with a pigment switch phenomenon, which led to the speculation of possible involvement of BMP signalling in the regulation of melanocytes (Clavel et al., 2012; J. Lim et al., 2019). This speculation was further amplified when coupled with other research that confirmed the existence of a relationship between BMPs and skin pigmentation. With that, we hypothesized that downregulation of BMP4 signalling will result in the enhancement of the current phototherapy treatment for vitiligo, by upregulating both migratory and pigment-producing functions of melanocytes.

This study is divided into two parts, *in vitro*, and *in vivo*. To corroborate this study with the possibility to develop it as a novel treatment strategy for vitiligo patients, we first looked for the presence of BMP signalling in the human skin and hair follicles. As shown in **Supplementary Figure 1**, we detected active BMP signalling in the melanocytes of human skin. Notably, our data also

indicates that undifferentiated melanocytes of the HF MeSC are indeed maintaining high levels of BMP signalling. With that in mind, we utilized our established 2D co-culture system to aid us further in this study. With the help of FACS and our detection strategy, we can quantify both melanin synthesis and transfer, thereby allowing us to compare the different condition types. Based on past research, BMP4 is known to be a key regulator of skin homeostasis and interestingly, it has been also shown to be involved in regulation of skin pigmentation. Thus, to validate the regulatory effect of BMP4 on pigmentation, we made use of our 2D keratinocyte and melanocyte co-culture system with various cytokine combinations. In this case, both BMP4 and BMP6/or their antagonist, Noggin and Sostdc1, were added into the co-cultures. Our data detected the inhibitory role of BMP4 in melanin synthesis, in which Noggin was able to partially rescue the observed phenotype.

To validate our previous finding, 2D keratinocyte and melanocyte co-culture system is performed to mimic the physiological ratio of human skin, thereafter treated with cytokines and observed for changes in overall pigmentation via melanin assay. Our results demonstrated a decrease in melanin concentration when treated with BMP4 but are rescued if Noggin was added as well. In corroboration with other research, our findings have shown that BMP4 downregulates melanogenesis, further validating its effect in the regulation of melanogenesis as reported by Singh et al. (Singh et al., 2012), where they observed the same phenomenon after treating the cells with BMP4. Interestingly, melanin concentration increased when treated with Noggin and this is very important as it supports our hypothesis in which BMP4 is involved in the regulation of melanin content. Moreover, this finding brought us one step closer to understanding and deriving the possibility of Noggin to act as the novel therapeutic strategy in enhancing UV-induced follicular repigmentation. However, to fully achieve our aim, the migratory function of melanocytes is the key aspect to look into instead. With that in mind, we needed to investigate the capability of BMP4 in regulating the migratory function of melanocytes.

In an effort to seek for a possible BMP signalling effect on the migration of melanocytes, we investigated the regulation of BMP4 and BMP6 on the migration of keratinocytes and melanocytes in our 2D co-cultures. For this purpose, we adapted the *in vitro* wound assay to our 2D keratinocytes-melanocytes co-cultures. In our data, we observed the inhibitory role of both BMP4 and BMP6, with BMP4 as the stronger inhibitor of migration. These effects were rescued when we added in respective antagonists, Noggin and Sostdc1. This is an important step for us as it not only shows the possible inhibitory effect of BMP signalling on the migration of melanocytes but also the strength of BMP4 in affecting migratory functions. Moreover, it is important to note that our data also suggested an inhibitory role of both BMPs on the migration of keratinocytes as the wound closure is delayed in BMP-treated co-culture wells. This observation is consistent with other studies as well where BMP signalling is shown to inhibit keratinocyte migration in wound healing models (Lewis et al., 2014). However, we wanted to study the migration of melanocytes only, therefore we proceeded to treat it with the media from co-cultures. It is important to note that melanocytes require the factors released by keratinocytes to stimulate their migratory functions. Therefore, we aim to provide the melanocytes with such factors for our melanocyte migration assay. With that in mind, we went on to dive deeper into the functions of BMP4 signalling in the regulation of melanocytes.

Next, *in vitro* melanocyte migration assay was performed to look into the effects of BMP4 and Noggin in regulating the migratory functions of melanocytes. Moreover, melanocytes were pre-treated in accordance with our experimental layout (BMP4, BMP4 + Noggin, Noggin, Control), together with the conditioned media to first stimulate the melanocytes before ‘creating’ the wound. As hypothesized, BMP4 is observed to downregulate the migratory functions of melanocytes as it requires a longer time to close the wound as compared to control. Moreover, our observed phenotype was rescued when Noggin was added to counteract the effects of BMP4. Surprisingly, migration speed is enhanced

when only its antagonist is used to treat the culture, indicating a direct relationship between BMP4 and migratory function. Once again, Noggin is observed to possess the ability to enhance melanocyte functions. In the case of phototherapy, UV exposure is used to stimulate the migration of melanocytes out into the epidermal area. In this case, we wanted to mimic this scenario and therefore, subjected the melanocytes to UV exposure. Correspondingly, BMP4 is observed to reduce the migration speed of melanocytes as compared to control and is subsequently rescued when in the presence of Noggin. Notably, Noggin is witnessed to have an additive effect on the migratory functions of melanocytes, on top of the stimulation by UV exposure. This is an important finding for our study, as it not only supports the hypothesis but also demonstrated the possible role of Noggin to enhance phototherapy treatment. However, more data is required before concluding the potential of Noggin in the enhancement of phototherapy. Therefore, *in vivo* experiments are designed for the second part of the study to validate the previous findings. Moreover, *in vivo* model will be a better representation in terms of deducing the overall effect of BMP4 on the functions of melanocytes. In addition, the complex relationship between the different cell populations in the skin will also reveal the mechanisms involved between BMP4 and pigmentation.

With that in mind, an *in vivo* mouse model is created for our study, where BMP4 is conditionally knocked out in the immature melanocytes of the HF and labeled with an *in vivo* reporter gene, Td-tomato. In our model, we target the melanocytes using the genetic tool Cre driven by the melanocyte-specific TYR gene (Tyr^{Cre}). This model has its BMP4 gene specifically knocked out in melanocytes at the end of the catagen phase of the hair cycle when all the matured melanocytes are destroyed and leaving intact only the immature melanocytes that reside in the hair bulge. In this way, it ensures that only the immature melanocytes are targeted and when coupled with the help of UV exposure, it will then stimulate these melanocytes out into the epidermal layer. Therefore, when compared to WT, we will then be able to deduce the effects of BMP4 on melanocyte functions for our

in vivo model. To confirm the conditional knock-out of BMP4, we performed immunofluorescence staining to visualise the expression of BMP4 proteins in both cKO and WT mice. With the help of the two melanocyte markers, Sox2 and TRP2, we can locate the melanocytes in our tissue sections. Moreover, the role of Sox10 in this staining is also to prove that the staining of BMP4 is not being masked by the melanin in the melanocytes, as the existence of both proteins is found in the nucleus. Even though BMP4 is successfully knocked out in cKO, we still need to ensure that overall BMP signalling is still active. This is because a total shutdown of BMP signalling pathway will cause undesirable effects to the *in vivo* model as BMP is known to be involved in many processes (Hemmati-Brivanlou & Thomsen, 1995; Stewart et al., 2010; Zou & Niswander, 1996). In this case, we ensure the expression of pSmad 1/5 in the melanocytes as it is known to be the downstream effector genes of BMP4 through the canonical signalling pathway (Nohe et al., 2002b).

With the validated *in vivo* model, we delved deeper to investigate the relationship between BMP4 and melanocyte functions by conducting *in vivo* experiments to validate this finding. In conjunction with our *in vitro* data, we identify an increase in both the number of melanocytes migrating out into the IFE and the amount of melanin content produced in melanocytes. Although the upregulation of melanocyte migration is not statistically significant, it is interesting to note that even with partial reduction of BMP signalling an increase in the number of melanocytes migrating out into the IFE is observed, supporting our hypothesis for this study. To further validate the observed phenotype, we rescued the cKO by performing dermal injection of BMP4-coated beads into the cKO, thereby attempting to reverse the phenotype caused by the conditional knock-out of BMP4 gene. Through this novel approach, we once again identify that BMP4 is directly involved in regulating melanocyte migration. This is very important as it demonstrates the credibility of our *in vivo* model for this study, as it demonstrates that the migratory function of melanocytes can be controlled by the amount of BMP4 expression. Moreover, this result greatly supports our hypothesis for this

study. Besides studying the melanocyte migration *in vivo* model, we also wanted to investigate and validate the effects of BMP4 on pigmentation.

For this purpose, melanin assay was performed to obtain the melanin concentration and thereby revealing the effects of BMP4 on pigmentation. In this experiment, we observed an increase in the amount of melanin concentration in cKO as compared to WT. This result is in conjunction with the previous *in vitro* data, where a decrease in BMP4 expression leads to an overall increase in pigmentation. Once again, both the *in vitro* and *in vivo* data show similar effects elicited by BMP4 towards melanocyte functions, in response to our hypothesis for this study. Interestingly, a cross-talk between BMP4 and MC1R has been previously reported to control HF pigmentation (Sharov et al., 2005). However, more insights are required to understand the mechanisms involved between BMP4 and melanocyte functions. This is of paramount importance as more knowledge is needed to develop this study further into a novel therapeutic strategy for vitiligo. To achieve this, RNA sequencing is executed to unravel the mechanisms involved.

Our comprehensive molecular analysis with RNA sequencing in isolated melanocytes revealed multiple genes through BMP4 which caused the phenotype observed in this experiment. Through the PCA plot and hierarchical heat map, both cKO and WT displayed different properties which segregated them into two distinct groups. This is significant as it demonstrated that changes in BMP4 expression can constitute a distinct group from WT. After obtaining a list of differential genes with DeSeq2, GO analysis is first executed to reveal the processes that are significantly regulated based on this list of genes. As expected, the pigmentation process is listed as one of the significantly regulated processes. This finding is in conjunction with our previous data, where we observed that manipulation of BMP4 expression in melanocytes causes an effect on its melanin content. Interestingly, metabolic processes involving reactive oxygen species are shown in the list as well. This uncovers the possible reason for increased melanin content is due to an upregulation in melanogenesis, which is understandable as

an increase in melanin production will lead to a rise in oxidative stress, and therefore explains this list of significantly regulated processes. Notably, within the process of pigmentation, there are both melanosome biogenesis and melanin biosynthesis processes. Till now, the data has yet to answer the mechanism through which BMP4 undertakes to affect melanin content. With that in mind, GO analysis on cellular processes is performed to investigate in detail the pathway BMP4 took to affect melanin content. The result corroborated with the GO analysis on biological processes, where it reveals that the pigment granule process is one of the significantly regulated processes. This in turn clarified that BMP4 may have indeed regulated the melanin biosynthesis pathway, thereby increasing overall melanin content through this process. Surprisingly, the melanosome process is revealed to be significantly regulated as well. This points to the fact that BMP4 may also increase the overall melanin content by producing more melanosomes, which are vital to produce and contain the melanin.

After looking through the various processes through GO analysis, we wanted to dive deeper to investigate in detail the mechanisms involved with regards to BMP4 and pigmentation. Therefore, we went through the list of DEGs to identify genes that are known to be related to melanocytes, in the hope to unravel more about the mechanisms involved. Here, we identified 24 genes that are possibly related to the regulation of various melanocyte functions, such as pigmentation, migration, and differentiation. Moreover, transcription of some identified genes are directly regulated by the BMP/SMAD signalling pathway as illustrated in **Table 10**. In this way, it allows us to probe deeper into the possible pathways BMP4 undertakes to regulate the observed phenotypes.

Table 10

Tyrosinase (TYR)
<p>A known marker of melanocytes, where its role in melanocyte has been reported to be involved in the melanin biosynthesis process (Iozumi et al., 1993; SZABÓ, 1957). This copper-containing oxidase is an enzyme that catalyzes melanin production and other pigments from tyrosine by oxidation. Therefore, a change in the regulation of TYR will undoubtedly result in more melanin being produced as it catalyzes the initial and rate-limiting step in the melanin production process (Tsukamoto et al., 1992; Yamamoto et al., 1989).</p>
Melanoma antigen recognized by T-cells (MLANA/ MART-1)
<p>Known as melanoma antigen recognized by T-cells 1 (MART-1), is a surface protein of melanocytes. MLANA is a marker of melanocytes that are involved in the generation of melanosomes by safeguarding the stability of OA1 (Giordano et al., 2009). Moreover, MLANA plays an important role in the expression, stability, trafficking, and processing of PMEL, which is vital for the formation of melanosome structure and maturation, thus indicating the significant role of MLANA in regulating pigmentation (Hoashi et al., 2005).</p>
Premelanosome protein (PMEL)
<p>A transmembrane glycoprotein that is expressed in pigment cells like melanocytes. It targets the precursors of melanosome and is reported to participate in melanosome biogenesis. PMEL is required for the maturation of stage I melanosomes to stage II melanosomes, in which the vesicles are elongated from within the distinct fibrillar structures (Berson et al., 2001, p. 17; van Niel et al., 2011).</p>
CD63
<p>A cell-surface protein and member of the tetraspanin family is involved in multiple processes. For instance, CD63 participates in intracellular vesicular</p>

transport processes, in which its presence is vital for the formation and maturation of melanocytes (van Niel et al., 2011). In addition, CD63 is involved in the regulation of cell survival, cytoskeletal reorganization, cell adhesion, spreading, and migration through the pathway of AKT and FAK/PTK2 (S. Y. Lee et al., 2014).

Ras-related protein Rab-38 (RAB38)

Essential for TYRP1 trafficking, which affects the peripheral melanosomal distribution of TYRP1 in melanocytes (Tamura et al., 2011). Moreover, it is further reported that in cooperation with RAB32, RAB38 is identified to play a role in the trafficking of three melanogenic enzymes (TYR, TYRP1, TYRP2) to melanosomes in melanocytes. Overall, RAB38 is vital in both melanin production and melanosome biogenesis (Marubashi et al., 2016).

Apolipoprotein E (APOE)

A member of the fat-binding protein family participates in fat metabolism as it associates with lipid particles. Moreover, it is reported that APOE functions in lipoprotein-mediated lipid transport between organs (Arnon et al., 1991; Krimbou et al., 2004; Marcel et al., 1983). In addition, recent research had shown that APOE is associated with intraluminal vesicles within endosomes, making it vital in the ESCRT-independent sorting mechanism of PMEL onto intraluminal vesicles. Furthermore, APOE is found to be involved in the regulation of the endosomal formation of PMEL amyloid fibrils, thus indicating the role of APOE in the regulation of melanosome biogenesis (van Niel et al., 2015).

Vesicle-associated membrane protein 8 (VAMP8)

A type of soluble N-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) that is associated with the fusion of synaptic vesicles with

the presynaptic membrane. One of its known functions is that VAMP8 is involved with the autophagy process by controlling the fusion between autophagosome membrane and lysosome membrane (Diao et al., 2015, p. 14; Itakura et al., 2012). With regards to melanocytes, it is reported that VAMP8 cooperates with Syntaxin 7 in the regulation of fusion events within the late endosomal pathway, signifying its vital role in melanogenesis (Wade et al., 2001).

Solute carrier family 24 member 5 (SLC24A5)

A member of the potassium-dependent sodium/calcium exchanger family is found in the trans-Golgi network in melanocytes. This cation exchanger is reported to play a part in pigmentation, by facilitating the ion transport in melanosomes. SLC24A5 is the main sodium-calcium exchanger that exists in melanocytes, in which it exchanges 1 Ca²⁺ and 1 K⁺ to the melanosome with 4 cytoplasmic Na⁺ (Ginger et al., 2008, p. 5; Lamason et al., 2005, p. 5).

Microsomal glutathione S-transferase 1 (MGST1)

An enzyme involved in conjugating the reduced glutathione to exogenous and endogenous hydrophobic electrophiles. MSGT1 is found in the endoplasmic reticulum and outer mitochondrial membrane. Recent research had shown that MGST1 is essential in the conversion of dopaquinone to glutathionyl-DOPA, in which an absence of MGST1 leads to toxicities and reduced melanin content. Moreover, overexpression of MGST1 showed a concomitant increase in TYR protein level, indicating a possible relationship between the two proteins (J. Zhang et al., 2019).

Glutaminyl-peptide cyclotransferase (QPCT)
<p>An enzyme involved in the generation of pyroglutamyl peptides. QPCT is reported as a meta-gene to be involved in the regulation of pigmentation. Based on the report, QPCT is observed to have a higher expression level in hyperpigmented samples as compared to the less pigmented samples. Moreover, QPCT is found to colocalize with two melanocyte markers, MART-1 and MITF, in the same cell. In turn, this suggests QPCT as a melanocyte-specific gene and its role in pigmentation (Yin et al., 2015).</p>

V-type proton ATPase 21 kDa proteolipid subunit (ATP6V0B)
<p>An enzyme that regulates the acidification of intracellular compartments in eukaryotic cells. Interestingly, ATP6V0B is reported to be highly expressed in light-skinned individuals and is found to be responsible for upholding an acidic environment in melanosomes for melanin synthesis (Dooley et al., 2013, p. 2; López et al., 2015). Therefore, upregulation of ATP6V0B is expected as the mouse model is of Agouti background which supported the evolutionary role of ATPV60B in pigimentary phenotype.</p>
Collagen triple helix repeat containing 1 (CTHRC1)
<p>A secreted glycosylated protein is reported to play a role in the cellular response to arterial wall injury through vascular remodelling. Interestingly, CTHRC1 overexpression demonstrated enhanced migratory ability in fibroblasts and smooth muscle (Pyagay et al., 2005). Correspondingly, CTHRC1 is recognized to be an important regulator of migration and invasion in melanoma cells, further indicating the possibility of CTHRC1 being involved in the regulation of melanocyte migration (Eriksson et al., 2016).</p>
Prostaglandin-H2 D-isomerase (PTGDS)
<p>An enzyme that is responsible for the catalysis of prostaglandin H₂ to prostaglandin D₂ conversion. PTGDS is known to be involved in multiple</p>

processes such as smooth muscle contraction and relaxation, inhibition of platelet aggregation, and a variety of central nervous system functions (Zhou et al., 2010). Moreover, PTGDS is found to be a target of MITF and is involved in regulating melanocyte proliferation (Colombo et al., 2012). Furthermore, PTGDS is highly expressed as a marker in melanocytes and displayed an MC1R-dependent expression, indicating its role in eumelanogenesis as well (April & Barsh, 2006; Takeda et al., 2006).

Procathepsin L (CTSL)

A lysosomal endopeptidase enzyme is required for the initiation of protein degradation. In addition, CTSL is critical for many other processes like lysosome-mediated protein turnover, antigen processing, and bone remodelling (Joseph et al., 1988). Furthermore, CTSL is discovered to be essential for skin and HF homeostasis as it is associated with the differentiation of keratinocytes and melanocytes during HF morphogenesis and cycling, thus signifying the importance of CTSL in the role of pigmentation (Tobin et al., 2002).

Table 10 shows the information for some of the 24 melanocyte-related genes and possible transcription binding sites by SMAD1 for genes that are directly regulated. The list of genes was categorized into pigmentation (orange), migration (green), proliferation (pink), and differentiation (purple).

The remaining genes are associated with either response to oxidative stress (GPX4 and PRDX2) or melanoma formation (S100A1, CYBA, TM4SF1, ID2, ID3, RPS3). The rise in response to oxidative stress is expected as melanin production generates a pro-oxidant state in the cells (Denat et al., 2014). Therefore, an increase in melanin production via BMP4 ablation will eventually lead to an upregulation in cellular response towards oxidative stress. On the other hand, BMP signalling is required to maintain cell quiescence to maintain tissue homeostasis and BMP4 ablation will undoubtedly lead to an increased tendency towards melanoma formation. However, controlling the amount of BMP4 signalling will be the key towards finding the fine balance between enhancing phototherapy and causing dire effects to the patient.

Figure 29. Schematic illustration on the prospective mechanism of BMP4 signaling on the regulation of melanocytes.

Overall, we identified various melanocyte-related genes in our RNA sequencing data. Therefore, to affect various processes like pigmentation, migration, proliferation, and differentiation, BMP4 is suggested to achieve its regulation via multiple pathways. In addition, BMP signalling is known to be involved in many cellular processes and as such, modulation of BMP4 signalling will elicit changes in various processes. Moreover, a decrease in overall BMP signalling will undoubtedly increase overall Wnt signalling, in turn upregulating many different cellular processes like migration, proliferation, and differentiation (Fuchs, 2007; Houschyar et al., 2020; Jahoda & Christiano, 2011; Plikus et al., 2008).

After studying the RNA sequencing results, we went on ahead to validate our findings by first performing qPCR on BMP4 and its downstream effector genes to confirm the ablation of BMP4 via our mouse model. Our results indeed show the effectiveness of our mouse model in ablating BMP4 signalling, and thus validating that the observed phenotype is due to the decrease in BMP4 signalling. Next, the expression of various signature melanocyte genes is investigated and the qPCR data showcases the upregulation of genes like CKIT, TYR, TYRP2,

and MC1R. And these genes, corroborate our previous data of increased melanin production as they are involved in the melanin production pathway. Last but not least, we validated the results from RNA sequencing by carrying out qPCR on the list of melanocyte-related genes, and indeed, we observed a similar expression in our qPCR data as well.

On an interesting note, KEGG analysis reveals other processes where BMP4 ablation affects. Interestingly, the analysis reveals that Parkinson's disease is one of the processes affected. In Parkinson's disease, brain cells containing neuromelanin are selectively degenerated, particularly dopamine-producing cells in substantia nigra. The accumulation of neuromelanin with age in humans will lead to neuronal cell death and thereafter result in Parkinson's disease. However, in contrast to humans, mouse models lack neuromelanin and will therefore not be able to accumulate any neuromelanin to mimic Parkinson's disease (Barden & Levine, 1983; Zucca et al., 2017). With this in mind, the RNA sequencing result suggests the possibility of both BMP4 and TYR to be involved in other processes that may confer Parkinson's disease when affected, which may be an interesting mouse model for Parkinson's Disease. Next, we saw Alzheimer's disease listed in the KEGG analysis as well, and fascinatingly, BMP4 is reported to be involved in the modulation of neurogenesis in the adult hippocampus, which indicates a likely relationship between BMP4 and Alzheimer's disease (H. Xu et al., 2013, p. 4).

It is also important to note that in the case of vitiligo, matured melanocytes are targeted and destroyed by the immune cells. Even though the relationship between BMP4 and autoimmunity has yet to be discovered, but some evidences suggest a likely relationship between BMP4 and immune cells. Based on past research, BMP is known to participate in the regulation of T cell development within the thymus and human naive CD4⁺ T cell activation and homeostasis (Martínez et al., 2015; Tsai et al., 2003). However, more investigation is required to understand the implication of BMP4 in the subject of autoimmunity and vitiligo.

Future Work

Despite being successful in demonstrating the effects of BMP4 on melanocyte functions, it is still imperative for us to check the expression of BMP4 in the bulge region of human biopsies during vitiligo and after phototherapy. In this way, we can determine if BMP4 is indeed upregulated during vitiligo and down in phototherapy, thereby corroborating our current data and hypothesis.

Our *in vitro* and *in vivo* results suggest that BMP signalling inhibits melanogenesis, pigment transfer, and migration in melanocytes. In this aim, we will seek confirmation of the pivotal molecular pathways involved in the MeSCs migration, obtained from our *in vitro* and mouse *in vivo* results, in biopsies from vitiligo patients. The analysis will be focused on the MeSC niche of the HF and the epidermis. We can readily identify the MeSC of the HF using a combination of K15 and CKIT antibodies (K15+/CKIT+). Our histological analysis will start by identifying changes in the level of expression for BMP signalling reporters (pSmad1/5/8) within a particular set of biopsies. The main goal for this aim will be to corroborate the key pathways regulating melanocyte migration and maturation found in aims 1 and 2. We expect to detect less BMP signalling at the HF MeSC after the UVB treatment starts. In addition, using a combination of the migration cell surface marker MCAM, the proliferation marker ki67, and the melanocyte marker CKIT, we are expecting to detect migrating melanocytes leaving the HF MeSC compartment and entering the epidermis through the infundibulum. In addition, we are also expecting to see an increase in melanocyte differentiation markers (TYR, TRP1, NKI/GP100) as these migrating melanocytes start to repopulate the IFE. The presence of MC1R key pathway members (CKIT, TYRP2, TYR, PAX3, and Sox10) will be characterized by IF in WT and BMP4 cKO. In addition, the two major molecular pathways regulating the MC1R signalling pathway; Wnt and BMP will be analyzed by detecting nuclear B-catenin and pSmad 1/5, respectively. Finally, to further extrapolate the finding from our mouse model into our human biopsies, we will also study the

presence or absence of other molecular mechanistic changes. At the end of the histological analysis, we are anticipating that our established *in vitro* and *in vivo* results will be confirmed and that BMP signalling will be identified as a key regulator of melanocyte migration during UV-induced follicular re-pigmentation in vitiligo patients. Furthermore, Goldstein et al. reported a list of DEGs between biopsies obtained from untreated and NB-UVB-treated patients with vitiligo (Goldstein et al., 2021). Among the list of DEGs, BMP4 is found to be upregulated in the untreated group and subsequently downregulated after NB-UVB treatment. This finding greatly supports our hypothesis and further reinforced the need to investigate more to determine this phenomenon we observed.

In addition, we are proposing to further reinforce our mouse model data by establishing a vitiligo mouse model with a humanized epidermis. A noteworthy difference between the mouse and human epidermis is that most mice strains used in research do not contain melanocytes within the IFE. This lack of epidermal pigmentation is critical for developing pigmentation models more relevant to human melanocyte biology. In this way, we will attempt to induce a vitiligo-like phenotype, to generate a chemical-induced immune response within the epidermal melanocytes of our mouse model. The stem cell factor (SCF) is the kit ligand responsible for the postnatal maintenance of epidermal melanocytes in the skin. To achieve this goal, we will import a transgenic mouse where a human K14 promoter is driving the SCF expression KRT14-Kitl, these transgenic mice have epidermal melanocytes that persist into adulthood and cause hyperpigmentation. Then, KRT14-Kitl background is bearing human-like epidermal pigmentation. Our data has shown that we can upregulate the migration of HF melanocytes stem cells into the epidermis during UVB radiation by cKO BMP4. Now, generating this model into the KRT14-Kitl background will create an ideal model to study epidermal re-pigmentation. Finally, to induce a vitiligo-like epidermal depigmentation *in vivo*, we will chemically induce the loss of melanocytes by the administration of the skin-depigmenting agent

monobenzene. This chemical induces a stress-related systematic immune reaction in non-exposed areas and therefore, is thought to be responsible for inducing vitiligo-like phenotype. With this mouse model, we can once again apply our genetic tools to ablate BMP4 and investigate the effects of BMP signalling after UVR irradiation during epidermal repigmentation.

Here in this study, we have explored the different components of melanocytes and associated all the observed phenotypes with our RNA sequencing data. Our data is as hypothesized where modulation of BMP4 signalling will lead to the regulation of melanocyte functions.

In conclusion, our data have supported the hypothesis that BMP signalling is involved in the regulation of melanocytes. In this case, ablation of BMP4 results in higher melanin production and an enhanced migratory rate of melanocytes. Moreover, insights on the mechanism undertaken by BMP4 to achieve its regulation on melanocytes were explored in this study. With this, a better understanding of BMP's involvement in regulating melanocyte functions will help to achieve a more robust repigmentation strategy, to relieve the psychological burdens of vitiligo patients.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1. BMP signaling detection in human skin. Upper panel: Triple positive staining for pSmad1/5/8 (red), BMP6 (green) and CKIT (white) identifies the epidermal basal layer melanocytes and mature bulb melanocytes. (A-D left and right). Lower panel: BMP4 (green) and CKIT (red) staining of human epidermis. Note the co-stain with basal layer melanocytes (A-C). pSmad1/5/8 (green) and CKIT (red) double positive staining in melanocytes of the K15+MeSC compartment. Nuclei are counterstained with DAPI. (Scale bar: 100µm)

Supplementary Figure 2. Effects of BMP4 and BMP6 in Melanogenesis and Pigment transfer. 2D Keratinocytes and Melanocytes co-cultures were treated with 200ng/ml of BMP4, BMP4+Noggin, BMP6 or BMP6+Sostdc1 for 3 days. Double K14/GP100 staining and FACS analysis were performed to quantify MFI (Median Fluorescence Intensity) of single NKI+ cells (melanogenesis) and K14+/NKI+ cells (pigment transfer). **(A)** Upper panel: Brightfield and Lower panel: IF staining images of 2D co-cultures treated with various cytokines. **(B)** Quantification of MFI for NKI+ cells treated with BMP4, BMP4+Noggin, Noggin. **(C)** Quantification of MFI for K14+/NKI+ cells treated with BMP4, BMP4+Noggin, Noggin. **(D)** Quantification of MFI for NKI+ cells treated with BMP6, BMP6+Sostdc, Sostdc1. **(E)** Quantification of MFI for K14+/NKI+ cells treated with BMP6, BMP6+Sostdc, Sostdc1. (Scale bar for phase contrast: 400µm; Scale bar for IF: 100µm)

Supplementary Figure 3. In-vitro wound closure assay of Me:Kc co-cultures under BMP treatment. Me:Kc (1:10 ratio) co-culture where documented by digital imaging. **(A)** After 6 hrs of treatment the wound borders were recorded and used as a reference of closure at 29hrs. **(B)** Quantification of overall % wound closure. **(C)** Quantification of % of melanocyte migration within the wound. (Scale bar: 500µm)

APPENDIX

Macro Script for *In vitro* Migration Assay (Annex A)

```
//Parameters
run("Set Measurements...", "area redirect=None decimal=3");
min_area=50000;
slice=1;
mod=0.8; //adjust threshold
save_output=false;

//Reset
run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");
run("Select None");
run("Clear Results");
roiManager("reset");
roiManager("Associate", "true");
run("ROI Manager...");
run("Options...", "iterations=1 count=1 black edm=Overwrite");

setBatchMode(true);

name=getTitle();
id0=getImageID();
Stack.getDimensions(width, height, channels, slices, frames);
if(slices>1 && frames>1){
    exit("Reduce dimensional to XYT or XYZ");
}
if(frames>slices){
    slices=frames;
```

```
}  
path=getDirectory("image");  
  
bit=bitDepth();  
if(bit==24){  
    run("8-bit");  
}  
  
//run("Duplicate...", "title=Binary duplicate slices="+slice+" frames=1-  
"+frames); //For processing  
run("Duplicate...", "title=Binary duplicate range=1-"+slices); //For processing  
id1=getImageID();  
  
run("Find Edges", "stack");  
  
setAutoThreshold("Huang");  
getThreshold(t1,t2);  
setThreshold(t1,mod*t2);  
run("Convert to Mask", " black");  
run("Erode", "stack");  
run("Fill Holes", "stack");  
  
Count=newArray(slices);  
Total_Area=newArray(slices);  
  
//Initial ROI & measurement  
min_area2=min_area*10;  
setSlice(1);  
run("Analyze Particles...", "size="+min_area2+"-Infinity circularity=0.00-1.00  
show=Nothing clear display add slice");
```

```
if(roiManager("count")<1){
    exit("Error: No initial wound area was found, Adjust parameters");
}
rows=nResults;
if(rows>0){
    temp=newArray(rows);
    for(j=0;j<rows;j++){
        temp[j]=getResult("Area",j);
    }
    Total_Area[0]=Sum(temp);
    Count[0]=rows;
}
if(rows==0){
    Total_Area[0]=0;
    Count[0]=0;
}
selectImage(id1);
roiManager("Select", 0);
run("Enlarge...", "enlarge=25 pixel");
run("Clear Results");

s=0;
key=true;
for(i=2;i<=slices;i++){
    n1=roiManager("count");
    setSlice(i);
    run("Analyze Particles...", "size="+min_area+"-Infinity
circularity=0.00-1.00 show=Nothing display add slice");
    n2=roiManager("count");
```

```
rows=nResults;
if(rows>0){
    temp=newArray(rows);
    for(j=0;j<rows;j++){
        temp[j]=getResult("Area",j);
    }
    Total_Area[i-1]=Sum(temp);
    Count[i-1]=rows;
}
if(n2==n1){
    Total_Area[i-1]=0;
    Count[i-1]=0;
}
n2=roiManager("count");
n=n1+1;
if(n2==n && key==true){
    s=n2-1;
    roiManager("Select", s);
    run("Enlarge...", "enlarge=20 pixel");
}
else{
    key=false;
    run("Restore Selection");
}
run("Clear Results");
}

roiManager("Deselect");
selectImage(id1);
```

```
close();
selectImage(id0);
setBatchMode("exit and display");
//if(slices>1){
    //run("Duplicate...", "title=Wound_Healing duplicate slices="+slice+"
frames=1-"+frames);
//}
roiManager("Show All with labels");

Recovery_Ratio=newArray(slices);
for(i=0;i<slices;i++){
    setResult("Total_Area",i,Total_Area[i]);
    setResult("Count",i,Count[i]);
    Recovery_Ratio[i]=(Total_Area[0]-Total_Area[i])/Total_Area[0];
    setResult("Recovery",i,Recovery_Ratio[i]);
}

Plot.create("Wound Area", "Frame", "Area", Total_Area);
Plot.show();
Plot.create("Recovery", "Frames", "Recovery", Recovery_Ratio);
l=Recovery_Ratio.length;
Plot.setLimits(0, l, 0, 1);
Plot.show();

if(save_output==true){
    roiManager("Deselect");
    roiManager("Save", path+name+"RoiSet.zip");
    selectWindow("Results");
}
```

```
        saveAs("Results", path+name+"Results.txt");
        selectWindow("Recovery");
        saveAs("PNG", path+name+"Recovery");
        selectWindow("Wound Area");
        saveAs("PNG", path+name+"Wound_Area");
    }
```

```
function Sum(A){
    len=A.length;
    total=0;
    for(i=0;i<len;i++){
        total=total+A[i];
    }
    return total;
}
```