DEVELOPING METHODS OF MEASURING AND MANIPULATING MELANOCYTE/KERATINOCYTE RATIOS TO INFORM POTENTIAL TREATMENT OF VITILIGO VULGARIS

by

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ABSTRACT

Introduction

My doctoral work focuses on the disease vitiligo vulgaris. Several treatments already exist however, one totally successful remedy is unavailable. I have focused on cell-based technologies to refine the melanocyte–keratinocyte transplantation procedure (MKTP) that is currently in use. Treating this disease is important not just for its physical but its emotional effects.

Aim and objectives

The broad aim of my doctoral work was to establish techniques to enhance the MKTP vitiligo treatment by developing a method for isolating melanocyte and keratinocyte cells from skin, co-culturing in different ratios and manipulating this by different factors.

Methods

A co-culture system of melanocytes and keratinocytes at different ratios (1:3, 1:5 and 1:10) was established. Co-culture at different ratios was exposed to various concentrations of α-MSH (2.5 µg/ml, 5 µg/ml and 10 µg/ml) and UVB (10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2). Cells were evaluated for changes in morphology, melanin content, tyrosinase activity and the expression of selected markers (S100, TYRP1, TYR, pan-cytokeratin, cytokeratin 10 and filaggrin).

Results

In co-culture ratios 1:3 and 1.5 exhibited a higher population of melanocytes. Exposure of cells to various concentrations of α-MSH exhibited a dose dependent response and UVB caused a stimulated effect on melanin and its production.

Discussion & conclusion

As an *in vitro* study my doctoral work has provided a vital first step towards the development of melanocyte–keratinocyte co-cultured cell treatments for vitiligo vulgaris. The positive rate of melanocytes co-cultured with keratinocytes *in vitro* reached the maximum on the 12th day, and this co-culture model may be capable of being used in the treatment of vitiligo patients. Future applications of my work include informing the development of three-dimensional skin models and exosomes technology as an improvement to the co-culture method.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
ADF	Adult T cell leukemia-derived factor
bFGF	Basic fibroblast growth factor
BPE	Bovine pituitary extract
cAMP	Adenosine 3': 5'-cyclic monophosphate
CEA	Cultured epithelial autografts
CMV	Cytomegalovirus
CRE	CAMP response element
CT	Cholera toxin
DAF	Decay acceleration factor
DAG	1,2-Diacylglycerol
dbcAMP	Dibutyryl cAMP
DED	De-epidermised dermis
DHI	5,6-Dihydroxyindole
DHICA	Dihydroxyindole carboxylic acid
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethylsulphoxide
Dopa	3,4-Dihydroxyphenylalanine
ECM	Extracellular matrix
EDA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
EMEM	Eagle's modified essential medium
FBS	Fetal bovine serum
FCM	Fibroblast-conditioned medium
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward angle light scatter
cGMP	Guanosine 3': 5'-cyclic monophosphate
GM-CSF	Granulocyte/macrophage-colony stimulating factor

GS	Goat serum
H and E	Haematoxylin and Eosin
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
dH2O	Distilled water
HRP	Horseradish peroxidase
HAS	Human serum albumin
IBMX	3-Isobutyl-l-methylxanthine
ICAM-1	Intercellular adhesion molecule-1
IFN-y	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
IR	Immunoreactive
i3T3	Irradiated 3T3 Swiss mouse fibroblasts
KCM	Keratinocyte conditioned medium
LCM	Lymphocyte conditioned medium
LFA-3	Leukocyte function antigen-3
LT-C4/ D4	Leukotriene C4 or D4
MC1R	Melanocortin 1 receptor
MCP	Membrane cofactor protein
MBEH	Monobenzyl ether of hydroquinone
MHC	Major histocompatibility complex
MKTP	Melanocyte keratinocyte transplantation procedure
MOP	Methoxypsoralen
MSH	Melanocyte-stimulating hormone
MTT	[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide
NKCA	Natural killer cell activity
NCS	Newborn calf serum
NDP-MSH	Nle4DPhe7-alpha-melanocyte-stimulating hormone
NFKB	Nuclear factor kappa B
NGF	Nerve growth factor
NO	Nitric oxide

OAG	1-Oleoyl-2-acetyl-glycerol
O. D.	Optical density
P1 (2,3,4)	Passage 1 (2,3,4)
PBS	Phosphate buffered saline
PE R.	Phycoerythrin
PKA	Protein kinase A
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POMC	Proopiomelanocortin
PUVA	Psoralen plus ultraviolet A radiation
RER	Rough endoplasmic reticulum
Rpm	Revolutions per minute
SCF	Stem cell factor
SEM	Standard error of the mean
SSC	Side angle light scatter
TBS	Tris(hydroxymethyl)methylamine buffered saline
TNF-a	Tumour necrosis factor-alpha
TRP	Tyrosinase-related protein
Tween	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet
UVR	Ultraviolet irradiation
VKH	Vogt-Koyanagi-Harada syndrome
v/v	Volume to Volume

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melanocyte keratinocyte co-cultures (MK2 and MK4 cell lines) at 1:3 ratio after exposure to UVB at 30 mJ/cm^2

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CHAPTER ONE: INTRODUCTION TO THE STUDY

1.1 Introduction and rationale for the study

The purpose of my doctoral work was to focus on a disease called vitiligo vulgaris. There are several treatments already in place for this condition, however, at present there is still not one totally successful remedy. In this work, I have focussed on cell-based technologies to refine a treatment that is currently in use. As of the present date, cell and stem cell technologies are at the cutting edge of modern science and an understanding/grasp of how to use these is invaluable, not only in the field of cosmetic sciences but also in many other diseases in existence worldwide.

Developing treatments for the condition vitiligo vulgaris is important because whilst the skin is a primary target, other organs are also at risk, with its impact being far deeper than this. This condition can often have a devastating effect on the patient's emotional health with recent studies indicating that it might even have implications for the affected person's physical health too (Mishra *et al.*, 2014).

Normal healthy skin is a vital constituent of a person's psychological and physical welfare as well as being a crucial part of their sexual attractiveness, and their feelings of self-confidence and wellbeing (Allen, 2015). The skin is the largest and most visible organ of the human body which means that any skin discolourations are highly visible and thus profoundly affect that person with disease (Ahmed *et al.*, 2018).

Vitiligo is a condition where light white patches develop, most commonly affecting the skin, but it might also develop in the hair, the eyes or inside the mouth. It can be seen physically as

light-coloured or white patches in any of these areas while the amount of colour loss that occurs varies from patient to patient (Ghafourian *et al.,* 2014). This condition affects millions of people worldwide and it occurs in all skin types, affecting both men and women fairly equally. Approximately 50% of those affected receive a vitiligo diagnosis before the age of 20 with most patients having the disorder for the rest of their lives (Mahajan *et al.,* 2019). Although the physical symptoms are currently not thought to be so detrimental, it is widely reported that vitiligo can be a substantial contributing factor to emotional problems such as low self-esteem or depression (Ahmed *et al.,* 2018).

From a physical point of view, there appears to be a strong association of vitiligo with autoimmune disease comprising at least 20% of vitiligo patients being affected by both, and this linkage between both is known as comorbid condition (Gill *et al.,* 2016). This can be demonstrated with hyperthyroid disease and alopecia areata, where thyroid disease is 15 times more common in vitiligo patients and alopecia areata being 31 times more common (Saleem and Azim, 2016). Other conditions that show a heightened prevalence in vitiligo patients include lupus, inflammatory bowel disease, and Sjögren's syndrome, which presents clinically as dry eyes and a dry mouth. The prevalence of comorbid conditions means that everyone with vitiligo should be screened for them at least once a year (Gill *et al.,* 2016).

Vitiligo is a depigmentation disorder that affects 0.5-2% of the worlds' population (Anbar et al., 2019). It has been documented that historically vitiligo patients have suffered the same mental abuse as lepers with this going so far as sufferers being referred to as Sweta Kustha which means "White leprosy." It is devastating in all affected races but due to the strong contrast in dark skinned people, it is more of a problem for these people (Ezzedine *et al.,* 2019).

A study was documented in India which concluded that being afflicted with vitiligo is associated with suffering from serious social and psychological problems, a fact that has a heightened risk of occurrence in affected young women and children (Sarkar *et al.*, 2018). Due to India's status as being a very religious country where reincarnation is a popular theory, as described in ancient Hindu texts, many people still think that an individual suffers from vitiligo because they did wrong or "Guru Droh" in their previous life (Garg and Sarkar, 2014). Therefore, the social suffering from vitiligo of those affected is more prevalent in India than in other countries (Raman *et al.*, 2013). This is especially pronounced in young unmarried women due to the prevalence of arranged marriages with young women that have vitiligo having in some communities a minimal chance of being accepted for marriage. The lack of understanding is such that even if a woman develops vitiligo after their marriage they may have marital problems, which might even end up in divorce (Nguyen *et al.*, 2016).

For patients suffering from vitiligo, the economic and financial costs of this disease can be severe due to their need to take time off work so that they can attend hospital appointments for treatment, such as psoralen and ultraviolet A (PUVA). Psoralen (sor' a len) is a natural furocoumarin found in the seeds of Psoralea corylifolia and other botanicals and used for their photosensitizing activity in the therapy of psoriasis and vitiligo. Psoralen increases the skin's sensitivity to light. The theory behind their use is that they increase the amount of light the skin absorbs; this lets light into the skin. Psoralen is applied first to sensitise the skin, then UVA light is applied. The mode of action of psoralens is that it is actively taken up by epidermal cells and intercalates into DNA. Upon exposure to ultraviolet (UV) light, psoralen forms cross links between DNA causing cell injury and death (Madigan and Lim, 2016).

Another negative implication of suffering from this disease on a patient's employability is that visible lesions can also affect that person's chances of getting a job at an interview, thereby reducing their career choices (Lotti *et al.*, 2018). The psychological consequence of this disease is that a large proportion of patients feel distressed and stigmatized by their condition. Individuals that show visible signs of being affected by vitiligo often attract undue attention from the general public in the form of whispered comments, antagonism and ostracism (Vernwal, 2017). Of particular embarrassment are lesions over the face and parts of the hands and feet which can result in anger and disillusionment. This often leads to depression due to a marked reduction in the self-image of the affected person. Experiences such as these over a number of years can also reinforce the patient's negative feelings with regard to their illness (Chan *et al.*, 2013).

Many of the patients can report suffering from feelings such as embarrassment which can lead to a low self-esteem and social isolation. Mood disturbances, such as irritability and depression are particularly common in teenagers. Social withdrawal is a particularly common symptom as well as emotional distress, problems with employment, substance misuse and in extreme cases even severe depression leading to suicide attempts (Chan e*t al.*, 2013). Bullying related to this condition is associated with significant psychological trauma which can have long-lasting effects on the personal self-esteem of these children (Lotti et al., 2018).

Children with vitiligo lesions often avoid sports or restrict such activities and they might lose vital days from school. The extent of the psychological distress that these individuals suffer from can be dependent on the attitudes of those surrounding them, such as their parents, siblings, relatives, teachers, and friends. A consequence of this disease is depression and

frustration which can also manifest as problems in interpersonal relations and they can often feel that members of their family lack in understanding or support (Chan *et al.,* 2013).

Long term therapies for vitiligo including immunosuppressive treatments and PUVA/narrowband UVB therapy can carry a risk of side effects such as photo aging and carcinogenesis which has led to the need to find new safer alternatives (Shenoi and Prabhu, 2014). In one published report (Whitton *et al.,* 2015), 68% of all cases studied here reported adverse effects, especially so in topical treatments. The nature of these effects included itching, skin thinning, telangiectasia and atrophy. A few of the narrowband-UVB studies in this report (Whitton et al., 2015) caused a phototoxic reaction while PUVA treatment triggered the symptoms of dizziness and nausea (Whitton *et al.,* 2015). It is important that the psychological components of this disease are recognized and dealt with, so that those affected have an enhanced quality of life, and to improve response to treatment with counselling often helping to improve body image and self-esteem (Chan *et al.,* 2013) (Lotti *et al.,* 2018).

1.2 Currently available treatments for vitiligo vulgaris

Treatment of vitiligo vulgaris consists of three main options, namely medical, light based, surgical or a combination of all the aforementioned **(Table 1)**. After medical modalities, light-based therapies are the next treatment method but the limitations of these treatments are that stable colourless vitiligo patches that do not have any melanocytes left or do not have any inducible melanocyte precursors show no response to medical modalities (Nahhas *et al.,* 2019). An important note is that clear NICE (national institute for health and care excellence) guidelines are available for implementation of vitiligo treatment, the last date of revision of these was 2016 with a new review planned next in December 2021.

Table 1: Treatment algorithm for adults with vitiligo (Van Onselen, 2013)

Adult treatment algorithm	First-line	Second-line	Third-line
1. Diagnosis	Straightforward: Primary care diagnosis, including thyroid function test	Atypical presentations: referral and diagnosis by a dermatologist	
2. No treatment option	Sun screens	Cosmetic camouflage	
3. Topical treatment	Recent onset: potent or very potent topical steroid for two months	Ongoing treatment: topical pimecrolimus (alternative to topical steroid)	Severe vitiligo: De-pigmentation with p-(benzyloxy)phenol
4. Phototherapy	Narrow-band ultraviolet B	Psoralens and ultraviolet A phototherapy	
5. Systemic therapy	None		
6. Surgical treatments	Cosmetically sensitive site		

	only. Split skin grafts only.		
7. Psychological treatments	Should be offered to improve coping mechanisms in adults	Cognitive behavioural therapy	

The options for medical treatment of vitiligo involve using immune-modulating drugs on patients, such as vitamin supplements (especially vitamin B12 and folic acid), azathioprine, cyclophosphate, levamisole and systemic corticosteroids) (Nahhas *et al.*, 2019). Phototherapy is a medical procedure which involves exposing vitiligo lesions to fluorescent lamps which emit UV light. However, due to side effects, this should only be used when other treatments fail and UVB is emerging as the safer option. Heliotherapy is the therapeutic use of sunlight and its advantages are that it is cheap but unfortunately it does carry an increased risk of ageing skin, sunburn and skin cancer (Lotti *et al.*, 2018).

A product called Khellin is often used to enhance the treatment of vitiligo. Khellin is isolated from Khella, a Mediterranean plant and it acts as a photosensitizer which means that it acts directly on melanocytes to enhance proliferation and melanogenesis. For this treatment to be the most effective, it must be used in combination with PUVA or narrowband-UVB. Excellent repigmentation can occur within 2-4 months especially on the neck, face, upper limbs, abdomen and hands (Fenniche et al., 2017). Steroids have been known to be effective in the treatment of vitiligo, but they have been associated with serious side effects which have led to the search for alternative treatments (Lotti *et al.*, 2018).

The mode of action of steroids is to reduce both inflammation and the immune systems activity. Inflammation arises out of the body's white blood cells protecting it against infection and foreign substances such as bacteria and viruses. However, there are some diseases where the body's immune system does not act correctly. An outcome of this is inflammation working against the body's tissues thus causing unwanted damage. Signs of inflammation include: redness, warmth, swelling and pain. Steroids work by reducing the production of chemicals which can cause inflammation. The outcome of this is to help tissue damage to be kept as minimal as is possible. In summation steroids work by reducing the activity of the immune system by affecting the way white blood cells work (Grennan and Wang, 2019).

When the depigmentation covers more than 60% to 80% of the body, the easiest option is to depigment the entire skin rather than to try to repigment any remaining areas. Depigmentation occurs in the opposite way to pigmentation therapies by removing any pigment in the skin rather than regaining it. Before any adult is treated via the depigmentation process it is important that they are aware that the treatment involves the use of chemical depigmenting products for a long period of time. The patient must also be aware that this treatment will significantly change their physiognomy as well as creating the requirement for them to take special precautions, for the rest of their life, when exposing themselves to the sun. Established and potential depigmenting agents include: monomethyl ether of hydroquinone imiquimod, 88% phenol solution diphencyprone, laser catechol, cryotherapy hydroquinone bis (2-hydroxyethyl) ether, 4-methoxyphenol. The option of using depigmenting treatments should only be considered when more than 50% of the body is affected or when the patches are highly disfiguring (Grimes *et al.*, 2017).

A simple solution that does not require any sort of treatment is the use of camouflage creams/temporary makeup, such as creams and lotions (Kaliyadan and Kumar, 2012). Surgical treatments involve the blister technique, non-cultured and cultured autologous grafts, and epidermal/dermal grafts. For any individuals considering surgical treatment, it is important that they undergo a preoperative consultation to identify if they are suitable for it. The criteria for surgical treatment are that the candidates' disease should have been stable for at least 6 months and this is assessed by there being a lack of new or worsening lesions (Hossain *et al.,* 2016).

For surgical treatment, the site used as a skin donor (a site on the patient's own body) is usually positioned on the upper lateral thigh or the gluteal region and before being used the area is shaved to avoid any interference. The proximal portion of the lateral thigh is usually preferred as a donor site for grafting as it has a relatively more even plane and as far as cosmetic suitability is concerned, making it more preferable to patients (Nahhas *et al.,* 2019).

The blister technique (epidermal blister transplantation) is a surgical option, using the roofs of artificially created skin blisters as a source of melanocytes, to treat areas of vitiligo that are stable but refractory. There are three main stages to this technique which are: creation of the blisters, preparing the recipient site and transplanting the blisters to the recipient site. To create the skin blisters a special suction device is applied to the skin of the donor area. The recipient area is then prepared for receiving the donor skin by creation of superficial erosions, of 5-6mm in diameter, via ablation with an Erbium-YAG laser. The roofs of the created skin blisters are then removed with scissors and smooth forceps and transferred to the erosions that have been made in the recipient area. To protect these transplants silicone sheets are applied to them

before they were covered with a standard gauze and kept in place with an adhesive bandage to fix them in place and stop the transplant from moving (Dellatorre *et al.,* 2017).

One particularly novel technique for the treatment of vitiligo is the replenishment of melanocytes by autologous melanocytes. The advancement of culturing melanocytes means that it is now possible to treat large recipient areas with smaller skin samples. In this technique a superficial shave biopsy is taken, melanocytes are separated from the epidermis and cultured in M2 medium. For good cosmetic results, important factors to account for are the age of the patient, site of vitiligo and most importantly stability of vitiligo. In stable vitiligo, autologous melanocyte transfer is a simple and effective treatment to quickly produce homogeneous pigmentation. The disadvantage of cultured melanocytes over non-cultured melanocyte techniques are that they are time-consuming and labour intensive to prepare. The benefits of cultured melanocytes are that they require very little donor skin and that they can be used to cover an area that is over 100 times the donor area (Verma *et al.,* 2014). The limitations of autologous cultured melanocytes mean that non-cultured melanocyte transfer should always be the first choice of technique in not so widespread vitiligo. The use of autologous melanocytes when compared to cultured melanocytes produces a much better result with the non-cultured techniques since other techniques have various deficiencies, such as cobblestoning, pigment mismatch and patient discomfort (Verma *et al.,* 2014).

1.3 Significance of the study topic

As stated in the above sections, there are several treatment options for vitiligo that are already available which can broadly be defined as being either medical, light based, surgical or a combination of all of these (Nahhas *et al.,* 2019). The purpose of my doctoral work was to develop and modify a skin cell co-culturing technique with the aim of enhancing an already

existing surgical grafting technique entitled the melanocyte/keratinocyte transplantation procedure. Gauthier and Surleve-Bazeille were the first to introduce this technique in 1992 and since then, it has undergone many modifications. In recent years, the popularity of using the melanocyte/keratinocyte transplantation procedure (MKTP) for treating patients with vitiligo has shown a sharp rise. In this technique, a type of cell grafting, a suspension of cells is made from a graft of donor skin and it is applied to an appropriately prepared recipient site. The biggest advantage of this procedure is that a graft from a relatively small area can be used to treat a much larger area, usually at a size ratio of 1:10 (Altalhab *et al.*, 2019).

The aim of my doctoral work was to further improve the MKTP method that relies on the creation of a cellular suspension from a skin graft. The disadvantage of this method is that as of yet the constitution of the cellular suspension cannot be precisely defined and it was the aim of my doctoral work to improve upon this shortfall so that the treatment can be tailored appropriately to the patients' needs rather than being one general treatment to treat all cases of vitiligo, regardless of variance in disease presentations. (Bassiouny and Esmat, 2018).

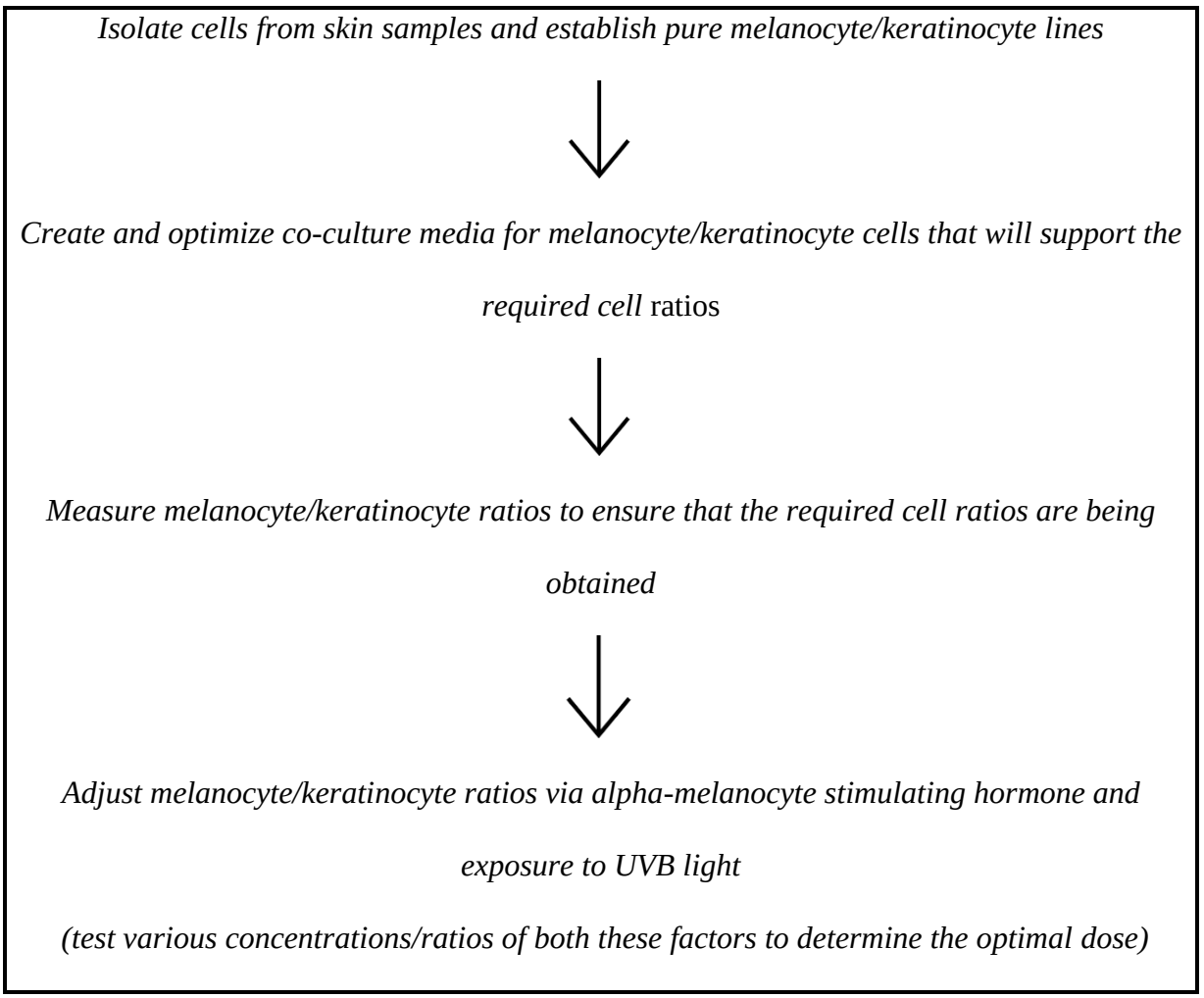
My work is novel since at present the exact ratio of melanocytes to keratinocytes in the cellular suspension is not controlled and developing a method to do this was one of my aims. Currently, a generic cell suspension is being used and as such the exact cellular suspension composition is not known, meaning it could consist of various cell types in any ratios. In my work, I have extracted not only melanocyte cells but also keratinocyte cells. Even if previous methods used a pure melanocyte suspension my work is an improvement of this since I have created a melanocyte/keratinocyte co-culture suspension. Not only was the melanocyte-keratinocyte ratio controlled but a method was developed to manipulate this ratio by the factors UV light and hormone (αMSH) to create the optimal ratio as required for the treatment of patients on an

individual case by case basis. The literature suggests that existing techniques prior to my study do not appear to do this (Tawfik *et al*., 2019).

To create a cell suspension of known composition, it was vital that I optimized a co-culture media to support the growth of both melanocyte and keratinocyte cell types. As of yet the availability of co-culture media is limited and they have been created for molecular biology rather than clinical use (Goers *et al.*, 2014). By creating my own co-culture media, I could be sure of its contents, meaning that I could also control it. Another disadvantage of commercially available media is that they are not cost-effective and are in fact very expensive to purchase (Kumar *et al.*, 2012). Once I have been able to optimise my co-culture media, it was important that I created a method to confirm that the required cells were present, this was done by flow cytometry and marker analysis. For clinical use, different melanocyte/keratinocyte ratios might be required for each patient depending on their needs, this was the driving force behind my creation of a technique to co-culture cells at different ratios, with my experimental work identifying that some ratios grow better in in vitro culture than others.

Further fine adjustments may be made to the cellular ratio in the composition by chemical/environment/physical stimulants. I chose to manipulate my cell proportions by exposing the cultures to the hormone alpha melanocyte-stimulating hormone (α-MSH) and UV light. I chose these two factors because they are widely and easily available, as well as this there is a reasonable amount of published literature on them (Nguyen and Fisher, 2019). The only way to measure the success of my melanocyte/cellular suspension for therapeutic work is via clinical trials, which is the next step after my basic scientific research work has been completed.

Figure 1: A flow diagram illustrating the steps that were undertaken for the experimental work of my study



1.4 Research questions

Aims and broad objectives

The broad aim of my doctoral work was to further develop the MKTP method by manipulating the melanocyte/keratinocyte ratios in the cellular suspension and before my study this had not been done. The longer-term aim as a result of my work is that the techniques developed in my doctoral work will help to inform the creation of patient specific treatments for the disease vitiligo vulgaris.

Specific objectives

1) To isolate and characterize melanocytes based on their cellular, biological and phenotypic properties.
2) To isolate and characterize keratinocytes based on their cellular and phenotypic features.
3) To establish and examine the co-culture methods for melanocytes and keratinocytes in different culture conditions.
4) To manipulate and assess the co-culture methods of melanocytes and keratinocytes by exposing to alpha melanocyte-stimulating hormone and UV light.

1.5 Structure of the thesis

In this chapter I have provided an introduction and background to the study. I have also provided a rationale for the study as well as an explanation of the significance of the study topic. Chapter two will provide a review of the current literature. Chapter three describes the methodology and outlines the methods that have been used. Chapter four will explain the results of the experiments that were conducted. Chapter five will critically discuss the results of the present study with already available literature on the study topic. Finally, the summary and conclusion of the study will be provided along with bibliography and appendices.

CHAPTER TWO: LITERATURE REVIEW

2.1 Introduction

In this chapter, I have built upon the introduction to the study in Chapter 1 with a more detailed background and context for this study. The first part of this chapter has identified the search strategy that was used. I will then provide an account of the anatomy and physiology of normal skin. After this I will focus on the topic of vitiligo which will involve a critical discussion of what the disorder is, its prevalence, as well as a summary of the different types of vitiligo and its histological/clinical presentation. After this I will then critically outline the currently available treatment methods.

2.1.1 Search strategy

The initial electronic searches that were used to identify relevant studies for my work consisted of searching selected online medical/scientific electronic databases and journals. The first search took place to assess how much relevant literature was available on my topic. Once a general search had been completed it was superseded by a more refined search and finally an inspection by eye of the indexes of a few chosen medical journals. Hand searching was carried out where it was possible to obtain the reference lists of relevant studies and these were searched to find appropriate further trials. Several commonly used health related electronic databases were identified and searched to allow for a wider range of articles to be captured as no one database is exhaustive.

The electronic databases that were searched were:

1. Web of science

This online citation indexing service gives the user admission to several databases referring to scientific research across a range of subjects.

2. Cinahl

A database that contains published research in healthcare but specifically for subjects related to the fields of nursing, allied health and biomedicine.

3. Cochrane library

A group of databases specialising in medicine and other specialties related to healthcare. It is essentially a database for systematic reviews and meta-analyses, summarising and interpreting medical research. This service aims to make the results of relevant healthcare trials readily available and it is an important tool for evidence-based medicine.

4. Google scholar

This is a web search facility which provides access to literature across a wide range of subjects.

5. Canterbury Christ Church University (CCCU)

This is the university's tool for accessing library resources, such as finding printed books, e-books and articles from journals. It can be used to find where printed books are located as well as identifying e-books and online journal articles.

6. Science direct

This website provides admission to over 12 million pieces of literature on research in science and medicine. This includes 34,000 e-books and 3,500 academic journals.

7. PubMed

This free search engine accesses literature on the topics of life sciences and biomedicine. It is particularly useful for identifying abstracts of relevant research topics.

The following search strings were used to conduct a preliminary search using the databases that are mentioned above:

1. co-culture AND melanocytes AND keratinocytes
2. vitiligo AND melanocytes AND keratinocytes
3. vitiligo treatment using cultured skin sheets

The following additional keywords/search strings that were not involved in the original proposal were used later to broaden the search and identify papers of more relevance. These were:

1. Treatment of vitiligo
2. Surgical treatment of vitiligo
3. Medical treatments for vitiligo
4. Autologous cultured melanocytes
5. Ultra thin epidermal sheets
6. Basal layer cell suspension
7. Micropigmentation
8. Image processing
9. Image J
10. Melanocyte stimulating hormone AND vitiligo
11. Basic fibroblast growth factor AND vitiligo
12. Interferon gamma AND vitiligo

13. Ultraviolet light AND vitiligo

14. Management/treatment of vitiligo in vitro

15. Measurement of cells in culture not suspension

These key words/ phrases were combined to produce various search strings using the “and” / “or” Boolean operators.

2.1.2 Primary search

The initial search used key words one at a time and then combined them into different phrases. Since a search such as the one described produced many hundreds of results it was decided to include parameters to narrow it down and make it easier to identify important journal papers. The date limit was set from 2013 to 2019. Papers before 2013 were discarded (unless the work was thought to be highly important or useful for explanatory purposes) as this field is very fast moving and any papers before 2013 are likely to be outdated or irrelevant. Papers over five years old were also not used because there is so much literature on the field that including any literature from a date earlier than this would identify so many papers it would be impossible to read them all but any later might miss key new discoveries in the field. Setting this time limit also allowed me to narrow down what was a very broad search in a manner that has reason and logic rather than it just being random.

2.1.3 Refined search

A secondary (refined) search using a more complex combination of the keywords/phrases depending on the number of hits produced by each key word was undertaken to narrow down and improve the accuracy/relevance of the results found. A literature search was performed to find abstracts and full text articles, and relevant articles were identified using a search by eye for the relevance of the article titles and abstracts to my study.

2.1.4 Appraisal

Every piece of literature that was identified as being relevant was subject to critical analysis before being accepted or rejected for inclusion as evidence to enable an answer to be obtained to the research study objectives. The critiquing of the research took a consistent approach and consistency in the way conclusions were made and how bias was removed in the appraisal process was achieved by the way in which the literature was analysed. In this review, I used several tools to critically appraise the literature.

The Preferred Reporting Items for Systematic Reviews and Meta-analyses (PRISMA) (Moher *et al.*, 2009) (www.prisma-statement.org) tool was used to assess the quality of published reviews. The primary function of this tool is to enhance how authors report systematic reviews and meta-analyses however; it can also enable the critical appraisal of qualitative studies (PRISMA, 2015). CASP (Critical Appraisal Skills Programme) is another useful tool. It was created with the aim of assisting health care workers to acquire research skills which fulfil the requirements for Evidence Based Medicine (CASP, 2015) (www.casp-uk.net). Currently eight critical appraisal tools are freely accessible from the CASP website.

Every specific tool is accompanied by a checklist that is unique to the literature being examined. When the questions are answered in their predetermined order to assess the relevant paper it can be decided whether the evidence in the paper is important and robust enough that it should be included or if it should be discarded. There are three steps in the approach to using CASP. Firstly, the study is valid if it is judged to be unbiased based upon evaluation of its methodology. After this, classification of the article is based around validity via a scale of levels of evidence.

When a study is presumed to be credible the next step is to analyse its results. The first aim of CASP is to examine if the results are of clinical importance. The second thing is to identify how certain the results are. This is achieved via the examination of statistical parameters for instance: p values, confidence intervals and the sensitivity analysis. CASP's third enquiry aims to find if the study results are useful and if they apply to the study questions. Each of the search terms provides a different classification of vitiligo, each of which will be discussed below. A criticism of using the CASP and PRISMA tools are that there are limitations to the number of keywords which means that the search must be repeated several times to make sure that all the required search query keywords have been included (Tursunbayeva et al., 2016). The next section of this review will examine what the disorder of vitiligo is and then classify it. The CASP and PRISMA tools are extremely useful as they provide a structured way to analyse the literature and determine its relevance.

2.2 Classification and incidence of vitiligo

The way that vitiligo is defined is influenced by a number of factors that include medical diagnosis, psychosocial impact, and whether recorded as prevalence or incidence. As described further in this section, several definitions are available for vitiligo. Vitiligo has been defined histopathologically as the "total absence of functioning melanocytes in the lesions, whilst the inflammatory cells most commonly found on the edges of the lesions are CD4+ and CD8+ T lymphocytes" (Faria *et al.*, 2014). One description of vitiligo is that it is: "an acquired chronic depigmenting disorder of the skin resulting from selective destruction of melanocytes" (Ezzedine *et al.*, 2015). According to another definition it is: "an acquired cutaneous skin and less frequently hair disease characterized by declining melanocyte function and depigmentation with an estimated prevalence of 0.5–1% in most populations." (Bellei *et al.*, 2013). Vitiligo was further defined as: "a common skin disorder causing depigmented macules that can impair

a patient's quality of life" (Komen *et al.,* 2015). Vitiligo is a condition where the skin's epidermis lacks melanin, which is due to the absence of melanocytes (Ghafourian *et al.*, 2014). Vitiligo as a disorder is reasonably common but as shown in **Table 2** below there are slightly different figures for the incidences of vitiligo ranging from 0.5% to 4%.

It has been reported that the large range that exists in the reported rate of vitiligo could be attributed to the fact that at present consensus is lacking with regards to defining and the methods of assessing this disorder making rates reported differ considerably (Al Ghamdi *et al.,* 2012). In both lesional and non-lesional skin of vitiligo patients, increased levels of reactive oxygen species (ROS) were observed, accompanied with reduced levels and activity of catalase (Li *et al.*, 2015).

One of the latest definitions of vitiligo taken from a nature article of 2018 is: Vitiligo is defined as a common depigmentary disease characterized by the destruction of melanocytes in the epidermis (Harris, 2018). Nature articles can be viewed as a robust body of evidence since publishing in high impact journals such as this one requires strong proof and noteworthy conclusions. Publishing an article in Nature is widely regarded as being highly prestigious and there are few journals that it is more difficult to publish in than this one.

The Ghafourian *et al.* (2014) definition gives a general description of vitiligo whilst other studies (Patel *et al.,* 2017) quote figures for the range of prevalence of vitiligo but sometimes the findings of different papers do not agree, for example they might quote a range of incidence of vitiligo that is much narrower than that reported in many other studies of a similar nature (Bellei *et al.,* 2013). The table below demonstrates the extent to which the reported incidence rate of vitiligo varies. As mentioned previously there are several reasons for this variance

which includes factors such as that when it comes to defining this condition there is a lack of agreement as to how this should be done.

Table 2: A table representing the range in vitiligo incidences reported amongst different studies

Vitiligo incidence rate	Paper	Author(s)	Year
0.5 % (average)	Vitiligo part 1	Tarle *et al.*	2013
0.5% to 2%	Vitiligo: symptoms, pathogenesis and treatment	Ghafourian *et al.*	2014
1% to 2%	Survey of dermatologists' phototherapy practices for vitiligo	AlGhamdi *et al.*	2012
1% to 3%	Advanced treatment modalities for vitiligo	Patel *et al.*	2012
0.5% to 1%	Concise review of recent studies in vitiligo	Allam and Riad	2013
1% to 4%	The efficacy of 308 nm UV excimer light as monotherapy and combination therapy with topical khellin 4% and/or tacrolimus 0.1% in the treatment of vitiligo	Bagherani	2015

The occurrence of a disease can be defined as its prevalence or as its incidence. The meaning of these two terms differs since prevalence is the proportion of cases at a given time in a population rather than how many new cases are occurring. The incidence gives us information as to how probable it is that a condition will occur during a specific period of time. In the papers that were referred to, the rate of incidence was used to report the occurrence of vitiligo in the

population. According to Tarle *et al.*, (2013), different countries report different figures for the prevalence of vitiligo for example: 0.093% in China, 0.34% on the island of Martinique, 0.38% in Denmark, 1% in U.S. and 0.5% to 1.13% in India.

Many cases of vitiligo involve children with approximately half of all affected individuals experiencing the start of their disease before the age of 20. Since childhood is such an important time in psychological and psychosocial development it is crucial that the full extent of the effect of this disease as well as the means of its alleviation is understood. As described in Cadmus *et al.* (2018), children affected with vitiligo definitely experience a decreased quality of life.

Cadmus *et al.*, (2018) explains that in children the psychological effect of this disease is greatest in those with dark skin as the patches are more striking and they have a greater disfiguring effect, but it is likely that this also extends to adult patients. It could be the fact that vitiligo is more visible in dark skin that has led to the different figures in Tarle *et al.* (2013) and Patel *et al.*, (2012). Differences in visibilities of this condition might mean that those with light skin but who still suffer from the condition are less likely to notice and report it in comparison to darker skin individuals which might lead to an interpretation of different occurrence rates in different countries.

Both men and women are affected equally by vitiligo with differences being apparent in the occurrence rate being down to the person's prototype and race. Previously it was believed that women are affected more than their male counterparts, but recent studies have found that bias does not exist with regard to sex (Steiner *et al.*, 2004; Tarle *et al.*, 2013). In 25% of cases the affected persons are children who show symptoms of the disease before they are 10 years old

with total variance in age being anything between 4 to 8 years. It has even been reported that very early onset, which can be as young as 3 months, has been previously acknowledged (Boniface *et al.*, 2017). However, the mean onset is at an age of approximately 20 years old (Tarle *et al.*, 2013).

To deal with there being a lacking consensus with regards to the classification and assessment of vitiligo, the Vitiligo European Task Force (VETFa) was formed in 2003 with three major goals, which are to: 1) propose a consensus definition of vitiligo 2) design biometric tools to assess the severity/stability and 3) creation of consensus scoring system (Taieb and Picardo, 2007; Akoglu *et al.*, 2018). The VETFa (Komen *et al.*, 2015) (Akoglu *et al.*, 2018) is a measurement tool that was established to evaluate the extent of existing depigmentation by using the rule of nine. It assesses the extent of pigmentation in four regions of the body which are: the head and neck, the trunk, the arms and the legs. Additionally, VETFa determines disease according to a scale of +1 to -1 (+1, progressive; 0, stable; -1, regressive).

It has been found that VETFa was a reliable assessment of depigmentation since both inter and intra observer reliability was found to be high (Komen *et al.*, 2015). To measure interobserver reliability in this study three experienced dermatologists/vitiligo physicians were recruited to independently assess the extent of depigmentation and compare their findings. In the case of intra observer reliability one physician repeated the measurement two weeks after the initial one. This was thought to be a reliable technique since it is unlikely that there will be a dramatic change in pigmentation over such a short period of time. Using just three observers was considered to be more than sufficient because the dermatologists are experts in their field so if two or more observers agree or disagree it will provide a majority opinion that is most likely to be totally reliable.

Komen *et al.* (2015) is the sole paper that describes the VETFa tool. Clinical scoring was performed according to the VASI score (Vitiligo area scoring index) which was originally described by Hamzavi *et al.* (2004) and later in Akoglu *et al.* (2018). The conclusion of this paper is that VASI and VETFa are reliable, but this is debatable. The number of patients sampled is small with only 31 patients being assessed for interobserver reliability and 27 patients being assessed for intraobserver reliability. The problem with a small study is that it reduces the reliability of the results (the statistical power) which means that it becomes more and more difficult to identify if a result is not a accurate representation of the actual correct conclusions of an entire study. With no other studies being available and such a small number of patients being studied further research must be done to come to a conclusion as to the reliability of this tool.

High prevalence rates should be considered with caution since it has been found that this tends to occur in some communities where inbreeding (close relatives having children together) is common, meaning that genetic factors are probably involved so this figure is indicative of that small community rather than the entire population at large. Another example of where prevalence rates are unreliable comes from a study, which arrived at a figure of 8% but was based at a skin institute in Delhi (Kruger and Schallreuter, 2012). Taking figures from a skin institute is an unreliable reflection of the actual prevalence rate because by its nature a skin institute will have a greater concentration of people presenting with skin disorders thus leading to higher figures of incidence reported. This fact is of relevance when considering the skin institute of interest was in India. By its very nature depigmented macules are more obvious in darker skinned individuals than their lighter skin counterparts meaning that more affected people will present themselves to a medical professional in a country such as India rather than in Europe.

2.3 Diversity in melanin production in human skin

It has been stated that one of the striking phenotypic differences that exists amongst humans is how they have different skin colours (Hudjashov *et al.*, 2013). This difference is thought to be because of natural selection with the geographical variation patterns being believed to correlate to the latitudinal differences that exist in the annual UV radiation level. It is thought that the optimal level of skin pigmentation is a balance between having skin that is dark enough to protect cells from UV radiation but light enough to allow vitamin D formation.

At a chemical level it is explained that the occurrence of such a range of pigmentation being present in the hair, skin and eyes of humans can be mostly a result of the diversity of pH in melanosomes (Wakamatsu *et al.*, 2017). For instance, an acidic pH is the factor that inhibits the production of melanin, particularly the process of eumelanogenesis. It has also been explained that the later part of eumelanogeneis after the dopachrome stage can be significantly suppressed by an acidic pH (Ito *et al.*, 2014). Pheomelanin production is thought to be the result of when tyrosine is oxidised by tyrosinase to form cysteinyldopa isomers, crucially cysteine is present. These isomers are oxidised further via benzothiazine intermediates.

The way in which these stages are being controlled by pH has not yet been identified. The outcomes of scientific analysis found that the presence of cysteine was required for pheomelanin production either from dopa or tyrosine by tyrosinase and this was greatest at pH values of 5.8-6.3, whilst eumelanin production was suppressed at pH 5.8. The inference that can be gained from these results is that mixed melanogenesis is chemically shifted to more pheomelanic states at a weakly acidic pH (Wakamatsu *et al.*, 2018).

2.4 Physical and emotional consequences of vitiligo

Vitiligo might not be life threatening but the appearance of chalky white lesions in various places and sizes can give rise to social stigma and leave sufferers vulnerable to psychological problems, in particular low self-esteem. This is particularly true of dark-skinned individuals where lesions stand out in stark contrast to "normal" healthy skin. Several studies on this topic arrive at the same conclusion: the psychological symptoms attributed to vitiligo and the impact that this has on the patient's quality of life is not to be taken lightly (Eves *et al.*, 2005) (Leeuw *et al.*, 2010). Vitiligo shows no preference for sex and it affects both adults and children of either gender in the same way (Kruger and Schallreuter, 2012), but women and girls tend to present more commonly due to greater prevailing social implications for them than for men (Ezzedine *et al.*, 2015).

The consequence of this disorder is that emotionally the patients may suffer with symptoms including stress, depression, low self-esteem and have a tendency towards suicidal thoughts (Paul, 2011). The psychosocial impact of this condition cannot be ignored, especially for those with darker/tanned skin where the disorder is more obvious. This condition is also particularly difficult for those individuals where highly visible areas such as the face or hands, and in children who might be teased or bullied when they reach school age (Whitton *et al*., 2015).

Quality of life studies have indicated that psychosocial problems that result from vitiligo are as significant as those found in patients with eczema and psoriasis (Bilgic *et al.*, 2010) (Hameed *et al.*, 2005). AlGhamdi and Kumar (2011) (this is an older paper but for the purposes of this study the results it presents are significant) have quantified the number of people affected by side effects of this disorder as depression (10%), suicidal thoughts (10%), sleep disturbances (20%) and anxiety (3.3%).

A study was presented where the relationship between bullying and skin disorders was observed (Magin, 2013). The term bullying is given to aggressive behaviour or/and intent to harm another, an action that might be carried out repeatedly over a period of time. A defining feature of bullying is that the victim finds it difficult to defend themselves due to the presence of an imbalance of power. One feature of bullying is that it is often appearance related which would therefore make it surprising if bullying was not associated with skin diseases. Studies have found that strangers often unintentionally caused offence via behaviours such as staring or comments that were not deliberate (Magin, 2013). It has been found that there is an association of mental health morbidity with bullying and teasing. Depression is one of the diseases that is most often associated with those found in the dermatologist's office (Tapia *et al.*, 2015) (Magin, 2013).

Since vitiligo is usually asymptomatic its effect on quality of life is usually attributed to psychological problems such as low self-confidence and unsuccessful social relationships. According to published literature many vitiligo sufferers experience shame and embarrassment as well as feelings of low self-confidence and social isolation (Hedayat *et al.*, 2016). In some developing countries, the situation is made worse by being associated with negative social beliefs, such as that the disease is a punishment from God for past sins. India is one such example of this where vitiligo is considered a form of white leprosy and is associated with significant stigma, even going as far as to being the grounds for divorce.

A study was carried out (25 patients as the pilot and another 173 patients as the main study group) to assess how having vitiligo affects the quality of life of people suffering from the diseases (Hedayat, 2016). In this paper a pilot study of 25 patients was first performed in order that essential modifications could be made to the questionnaire based on the opinions of the

research teams, so that the interviewers could adapt their technique accordingly. An example of such a modification was the change in the structure of complicated sentences to make them more comprehensible or to use words that were more familiar and made better sense. This study found that the best quality of life was in patients with disease duration of 5 years or less or 15-20 years. It is my conclusion that the reason for this is probably that it reflects either higher hopes for successful treatment in the early course of the disease or in the long term the patient getting used to it.

Moreover, apart from psychosocial impact, a lack of melanin pigmentation in lesions leaves this area vulnerable to UV damage from the sun resulting in an increased incidence of sunburn and cancer (Eves *et al.*, 2005) (Olsson and Juhlin, 2002) (Matsumura and Ananthaswamy, 2003) (Amaro-Ortiz, 2014) (Mujahid *et al.*, 2017). Such consequences of this disorder occur because if the person has white patches of skin it means that they have lost the solar protection that is usually offered to it by melanocytes (Olsson and Juhlin, 2002).

Table 3 is a presentation of the results of a study (Bonotis *et al.*, 2015) involving 216 adults who were diagnosed with vitiligo but no other skin disorder or major psychopathology. Unfortunately, a limitation of this is that the results of this study are difficult to interpret since nowhere in the paper is it mentioned which questionnaire each of these results come from. The table below uses several factors to compare how men and women affected by vitiligo react to it and it gives a p value to determine if there is any statistical significance to the differences between men and women.

Table 3: The effect of vitiligo on a study population in reference to quality of life indicators (Bonotis *et al*., 2015).

Variables	Men (n=101)		Women (n=115)		P value
	Mean	(S.D)	Mean	(S.D.)	
Age	35.81	12.37	35.65	10.41	0.917[1]
Self-esteem	21.95	4.10	20.69	4.51	0.03[1]
Neuroticism	11.85	4.19	14.58	4.24	<0.0001[1]
Extraversion	13.79	4.37	13.10	4.37	0.250[1]
Psychoticism	3.55	2.96	2.94	2.33	0.09[1]
Duration (months) median (10-90%)	48	5.2-225	72	7.8-228	0.07[2]
Extent of disease (percentage) median (10-90%)	5.02	1.5-18	7	2-15.4	0.06[2]
DLQI median (10-90%)	2	0-8	3	0-11	0.009[2]
GHQ-28 median (10-90%)	2	0-10.8	4	0-12.4	0.002[2]
		Abbr.: DLQI, Dermatological Quality of Life Index; GHQ-28, General Health Questionnaire 28, [1]t-test, [2]Mann-Whitney			

The p-value is an important aid to analysing the results because if there is a p value of less than/equal to 0.05 than the difference seen between men and women is statistically significant but if the p-value is higher than 0.05 then the difference is not statistically significant, thus indicating that evidence against the null hypothesis is weak. From the table above we can see there is now a significant difference in the age of the participants, extent and duration of their disease meaning that these factors should not influence the results. Self-esteem and neuroticism did exhibit significant p values meaning that men and women did differ in their

reaction with regard to these factors with women being significantly more affected in these areas.

2.5 Types of vitiligo

The two types of vitiligo are non-segmental vitiligo and segmental vitiligo. The most common is non-segmental vitiligo and it is characterized by lesions which are symmetrical and bilateral white patches. Symmetrical areas that these patches appear on are the: backs of hands, arms, skin around body openings (e.g. the eyes), knees, elbows and feet. The two types of vitiligo have been found to present different behaviours and responses to therapy (Picardo, 2011). There are several subtypes which are classified as generalized, acrofacial and universalis, each presenting with a bilateral distribution (affecting both sides of the body). Also, in occurrence are some unclassified and rare variants such as vitiligo punctate, vitiligo minor/hypochromic vitiligo and follicular vitiligo (Boniface *et al.*, 2017) which will be discussed in more detail further on.

The vitiligo society has also developed definitions for segmental and non-segmental vitiligo. According to this society segmental vitiligo is identified as a unilateral and localized spread of vitiligo patches. Van Geel (2013) states that the mechanisms behind vitiligo are an enigma. To develop a deeper understanding of this condition the distribution was compared amongst different skin conditions possessing mosaic or neurogenic backgrounds. The results of these observations found segmental vitiligo does not show a similar pattern of distribution to other skin conditions but there is more overlap between vitiligo and some mosaic skin disorders than others. The conclusion of Dr. Van Geel is that the classification of segmental

should be separate from other forms of vitiligo and the descriptor ‘vitiligo’ should be used as an umbrella term for all non-segmental forms of vitiligo.

It has been observed that non-segmental vitiligo can develop at any age but it is most common in young people between 10 and 30 years old. Segmental vitiligo characteristically appears in a unilateral distribution but this is less common and only happens in 5-16% of vitiligo cases. Non-segmental vitiligo is not limited to a set age range whilst segmental vitiligo is most common in the young, with 87% of cases appearing before 30 years and 41.3% before 10 years. The most basic diagnosis of both types of vitiligo is the loss of pigmentation in a patchy form from the skin, any overlying hair and in some cases the mucosa (Ezzedine *et al.*, 2015). The subset of vitiligo present in the patient must be defined so that treatments can be tailored accordingly. Often the reaction of the patient to any treatment is dependent upon the type of vitiligo that they are affected by.

2.5.1 Segmental vitiligo

Focal

This is where there are one or more colourless areas of skin but without a typical segmental distribution. Focal vitiligo is a rare subset of vitiligo with only 3.3% of patients out of the entire population identifying as being affected with this condition (Lommerts *et al.*, 2016).

Segmented

This is where there are one or more colourless patches of skin; this often follows dermatomal distribution and involves a unilateral body segment. Segmentary vitiligo presents onset at an

early age (between 5 and 30 years of age) and has no linkage to autoimmune diseases. Segmentary vitiligo has greater resistance to treatment than the non-segmentary type (Anbar et al., 2006) (Ezzedine *et al.*, 2011).

Mucosal

In this type only the mucous membranes are affected (Nagarajan *et al.*, 2014).

2.5.2 Non-segmental vitiligo

Research appears to suggest non-segmental vitiligo can be attributed to being an autoimmune condition where the effect of this is the destruction of melanocytes (pigment cells) (Whitton *et al.*, 2015).

Acrofacial

Typical lesions presenting in the toes or fingers (Zamanian *et al.*, 2014).

Vulgar

Colourless patches of skin showing a random distribution. There is no specific age for this and it is often linked to having an autoimmune disease, especially those of the thyroid (Schwartzmann-Solon, 1998) (Karelson *et al.*, 2011).

Mixed

A combination of different types such as acrofacial and vulgar or segmentary and acrofacial with/without vulgar (Kovacevic *et al.*, 2016).

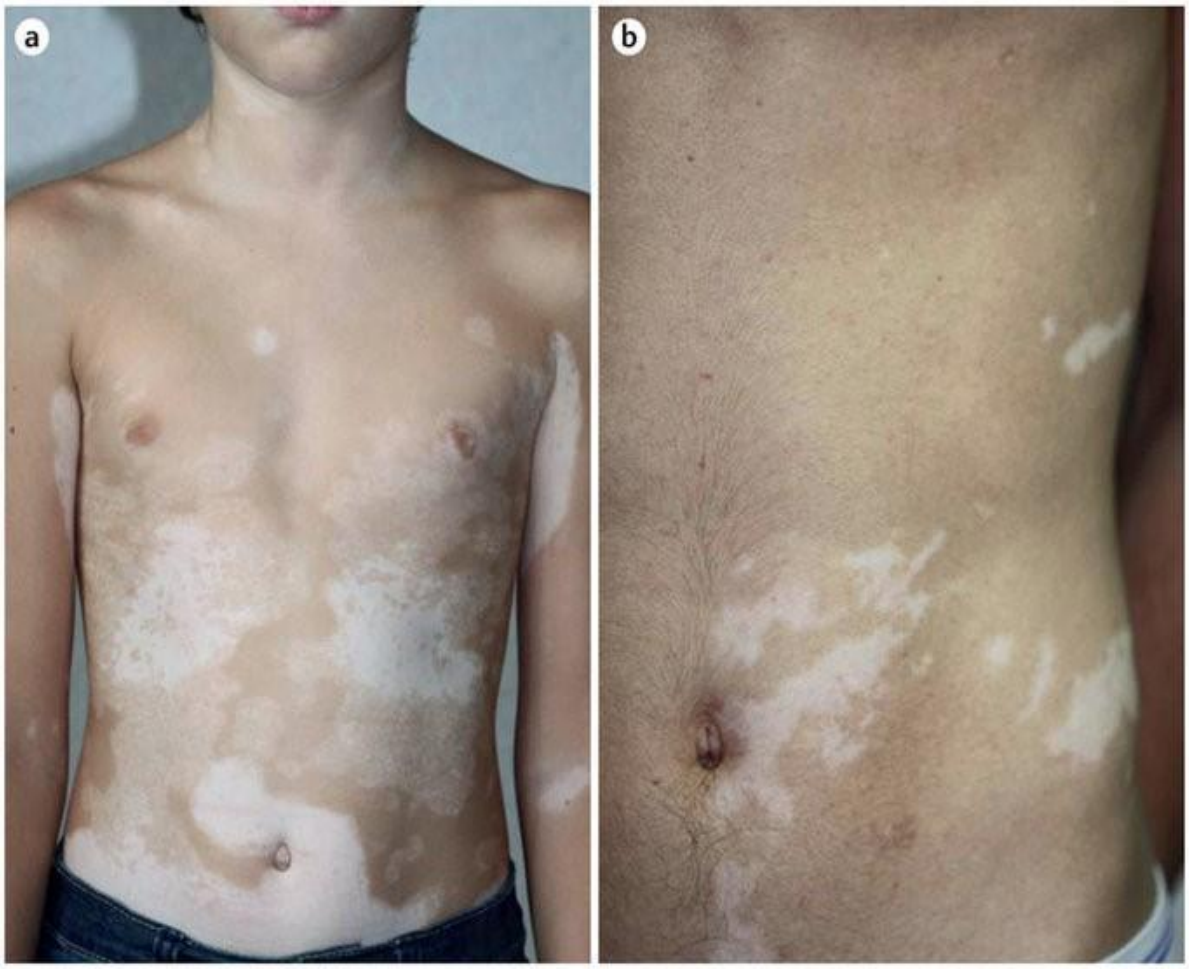
Universal vitiligo

A depigmentation of 50% or more of the skin area and/or mucous membranes (Al Ghamdi and Kumar, 2011) (Majid and Imran, 2017).

2.5.3 Clinical presentation

Clinical characteristics of this disorder are development of white macules of varying size, anything from a few millimetres to covering the entire body, which are attributed to the disappearance of functional melanocytes in the hair and/or skin (Ezzedine *et al.*, 2015) (**Figure 2)**. Characteristics of segmental vitiligo are that lesions are unilateral and segmental/band shaped. The lesions of non-segmental vitiligo show bilateral distribution in an acrofacial arrangement or they are most usually located over the entire body in a scattering of a symmetrical nature and show evolution over time (Ezzedine *et al.*, 2015) (Galvez *et al.*, 2018). It is possible for non-segmental vitiligo to initially present in a distribution that is acrofacial in nature and then progresses later to a generalised or universal form.

Figure 2: An illustration of how vitiligo lesions present themselves (Ezzedine *et al.*, 2015). Please note the stark white patches in contrast to normally pigmented skin. Histological examinations showed that in lesioned skin there is an absence of melanocytes, however in some cases an occasional melanocyte can be seen. The white areas in the above diagrams are the melanocyte absent vitiliginous regions.



2.5.4 Unclassified and rare variants

2.5.4.1 Vitiligo punctate

In this condition lesions appear as depigmented punctiform macules that are 1 to 1.5mm in size and have a sharp demarcation that can involve any body area. Guttate hypomelanosis is a common condition which must be distinguished histopathologically from vitiligo punctate and is where there is a lack in occurrence of melanocytes where they are found on chronically sun exposed sites, examples of which are the legs and forearms (Boniface *et al.*, 2017).

2.5.4.2 Vitiligo minor/ Hypochromic vitiligo

It has been stated that the only individuals that tend to be affected by this disease are those of darker skin (Boniface *et al.*, 2017). The 'Minor' part of the name of this condition can be attributed to the pigmentation defect being of a partial nature. This variety can be related to 'true' vitiligo via pathology and coexistence with more typical vitiligo macules. It is important to note that repeated biopsies are required to rule out cutaneous T cell lymphoma with molecular studies of clonality and long-term follow-up is needed before any diagnosis can be made (Sharma, 2015).

2.5.4.3 Follicular Vitiligo

This name is given in reference to a type that was described by (Boniface *et al.*, 2017). In this example a young black patient who had generalised vitiligo that mostly involved the reservoir of the follicular pigment had restricted skin involvement and contrasting marked generalized hair whitening. This contrasts with common vitiligo where hair depigmentation is followed by cutaneous depigmentation. This happens in skin with a clinically normal appearance as well as in vitiliginous areas (Gan *et al.*, 2016).

2.5.5 Diagnosis of vitiligo

The practice of diagnosis revolves around both normal and colourless skin patches existing in the same person. A woods lamp of 351 nm will cause affected skin to fluoresce blue white as because of 6-biopterine and 7-biopterine accumulating in these areas. This lamp is very useful for identifying lesions in which visualising by the naked eye is difficult (Allam and Riad, 2013) (Oiso *et al.*, 2013) (Plensdorf *et al.*, 2017).

2.5.6 Causes

The occurrence of vitiligo can be attributed to a dynamic interaction of environmental and genetic factors that initiate melanocytes in the skin being attacked by the autoimmune system (Rashighi and Harris, 2017). A variety of different theories have been presented for explaining the cause of vitiligo pathology and mechanisms that lead to functional epidermal melanocytes being lost (Bishnoi and Parsad, 2018). Important theories include the individual possessing a genetic disposition to vitiligo, the autoimmune destruction of melanocytes, melanocyte damage via free radicals or catecholamine's/neurotransmitters or heightened sympathetic response. It is the combination of all these theories that essentially explain the vitiligo pathogenesis (the combination theory) (Bishnoi and Parsad, 2018). The consequence of the autoimmune theory is that before any surgical transplantation treatment vitiligo lesions should exhibit stability (this will be described further later in this chapter).

The reason for melanocyte loss is subject to several theories, characteristically observed in those suffering with vitiligo. The theory of genetic causes is supported by the fact that in approximately 20% of cases the vitiligo patient has at least one close blood relative that is also affected by this condition. Genome wide association studies have managed to identify some loci that exhibit susceptibility for the development of generalised vitiligo, one locus is the gene

that encodes tyrosinase (TYR). Tyrosinase is an enzyme in melanocytes which catalyses the rate-limiting step in melanin biosynthesis. It may be stated that vitiligo should be classified as a polygenic disorder since its onset is known to involve at least three different allelic genes (Majumder *et al.*, 1993). This highlights the importance of genetic factors in developing vitiligo pathology.

A genetic predisposition for this disease means that certain environmental chemical substances can show selective toxicity to melanocytes. One such substance is hydroquinone, where hyper pigmented lesions are given topical treatment but for genetically susceptible individuals this has been found to be poisonous (Steiner *et al.*, 2004). Genetic factors are important even in typical vitiligo cases (Spritz and Anderson, 2017). In early clinical cases it was observed that probands relatives had a vitiligo frequency of 11% to 38%. Early clues to the heritability of vitiligo were in the fact that it happens more frequently in the immediate relatives of those patients with it. In fact, research mentions (Spritz and Anderson, 2017) that vitiligo heritability is 46% to 72%. For instance, in the general population it has been found that about 1% of individuals are affected by vitiligo but in a patient's sibling the risk of development is 6% whilst in a study of European-derived white monozygotic twins the concordance of vitiligo is 23%.

In a study of European derived monozygotic twins, it was found that in probands first-degree relatives the overall vitiligo frequency was 7% whilst the risk in probands parents and siblings was 7.8% and 6.1% respectively (Spitz and Anderson, 2017). These findings were consistent with polygenic, multifactorial inheritance and age-dependency of vitiligo onset. Another important outcome found by the study of vitiligo probands is that male and female subjects had an equal vitiligo frequency. This eliminates any female sex bias that is to be found in the

majority of vitiligo clinical case series. In studies (Spitz and Anderson, 2017), it is mentioned that in a study of families where multiple relatives were suffering from vitiligo there appeared to be an earlier age of onset as well as an increased skin surface involvement in comparison to singleton cases. The culture of melanocytes that are sourced from patients that have an active vitiligo have been found to have a reduced expression of c-Kit and stem cell factor. These receptors are important for melanocyte differentiation and any subsequent melanisation (Steiner *et al.*, 2004).

The cause of vitiligo is believed to be multifactorial as disease initiation and progression can be attributed to both genetic and environmental factors (Ezzedine *et al.*, 2015). In the case of between 10% and 76% of patients the onset of this disease is attributable to some sort of precipitating factor in those individuals that are genetically predisposed to the condition such as intensive stress, intense solar exposure or exposure to certain pesticides.

A progressive loss of melanocytes is often observed in cases of vitiligo, especially in non-segmental vitiligo. The full mechanism behind the development of vitiligo remains unknown but there are several key theories as to why this disorder occurs. Three popular theories behind the development of vitiligo are: the destruction of melanocytes (autoimmune, neural and impaired redox status), melanocytes inhibition or defective inhibition. At present, several different therapeutic modalities are available but therapeutic demands remain unmet. To bridge the gap over the last few years, new aetiology-based treatments have been developed (Lee *et al.*, 2012; Hameed *et al.*, 2005).

The melanocyte destruction theory is probably the most favoured for explaining the cause of vitiligo however this has never been clearly demonstrated. The destruction of melanocytes

occurs either by apoptosis or necrosis, and animal studies have suggested the most likely underlying pathway is apoptosis. In contrast to these animal studies, the apoptosis of human skin melanocytes has never been demonstrated. In addition, the intrinsic destruction of melanocytes via apoptosis is also reasonably higher than in healthy skin (Hameed *et al.*, 2005) (Yee *et al.*, 2000) (Qiao *et al.*, 2016).

The autoimmune theory is one of the leading hypotheses for the cause of vitiligo as it is based on the observation that there is often a co-morbidity between vitiligo and several other autoimmune disorders e.g. thyroiditis (Speeckaert *et al.*, 2014). Since one theory behind vitiligo is that it is an autoimmune disease, whose target is pigmentary cells, some treatments revolve around immune suppression. To suppress the immune reaction against melanocytes treatment methods include immunosuppressant's, such as topical steroids, cytotoxic drugs and PUVA. The strongest evidence for vitiligo being caused at least partly by immune reasons is that many patients have autoantibodies to melanocytes in their circulation giving even more support to the notion that vitiligo lesions must be stable before melanocyte transplantation is performed.

Studies (Spitz and Anderson, 2017) observed that there was a greater than normal frequency of autoimmune diseases in vitiligo subjects which suggests that in multiplex families of this type it is probably the case that genetic factors have a higher contribution to vitiligo risk than in singleton cases. This highlights the case that genetic factors are important as well as non-genetic contributors in the pathogenesis of vitiligo (Spritz and Anderson, 2017). In addition to this, patients with vitiligo and their relatives show a greater likelihood as far as the development of autoimmune diseases such as Addison disease, type 1 diabetes and pernicious anaemia (Rashighi and Harris, 2017).

The earliest evidence suggesting that vitiligo is of autoimmune origin was the original 1855 report of Addison disease (Spitz and Anderson, 2017). This report included a patient that had idiopathic adrenal anaemia, generalised vitiligo and idiopathic adrenal insufficiency and etiological factors were suggested by the co-occurrence of autoimmune disease. Since this case example various other investigators have noted that vitiligo often co-occurs with autoimmune disease. Therefore, for any surgical vitiligo treatment to be effective it is best if disease has been stable for at least 6 months signifying that the autoimmune component is at minimal activity. It is also important to note that these same autoimmune diseases happen more often in the first-degree relatives of vitiligo probands who themselves do not have vitiligo. This suggests that vitiligo shares at least some of its genetic foundations with autoimmune disease (Spitz and Anderson, 2017).

It is for this reason that a vitiligo patient must have disease stability for at least 6 months with lesions presenting no new expansion before they receive any interventional surgical treatment. The occurrence of stable disease patches minimizes the risk of the patient suffering from any active autoimmune component and maximizes the likelihood of successful treatment since it reduces the chances of the transplant failing due to being attacked by the person's immune system (Sahni and Parsad, 2013).

A study of 1098 patients (Gill *et al.*, 2016) found out that 217 (19.8%) of vitiligo patients were affected by at least one other comorbid autoimmune disease. The study originally consisted of 1873 patients who were seen between January 2002 and October 2002. 595 patients were excluded from the study for having less than 2 dermatology nodes and 180 for not having a diagnosis of vitiligo from a dermatologist which has led to the figure of 1098 patients as mentioned above. It was not found to be statistically significant but individuals with non-

segmental vitiligo were twice as likely as other vitiligo sufferers to have an associated autoimmune disease. An investigation (Saleh *et al.*, 2013) found that 83% of the 29 patients with vitiligo were found to have an autoimmune disease versus autoimmune disease being present in 7% of the healthy controls. Whilst this study might appear to be reasonably small (only 29 patients) it does give an indication that there is surely a link between suffering from autoimmune disease and vitiligo since vitiligo patients definitely have a greater incidence of autoimmune disease than normal healthy sufferers.

The most common conditions with an association to vitiligo are thyroid disease and alopecia areata. Both conditions present higher prevalence in these cases than in the general US population. In the general US population prevalence of thyroid disease is between 0.4% and 2.0% whilst Fricke and Mileva (2015) state that the prevalence of alopecia areata is 2%. Clinical thyroid disease showed a 15-fold increase in vitiligo patients compared to the general population and females had a 4-fold higher rate than males. The limitations to this study (Fricke and Mileva, 2015) were that a control group was lacking and this study only took place in one institution, this infers that there is a possibility for selection bias so the findings might not be an accurate representation of the overall vitiligo population. A control is needed so that the prevalence of autoimmune disease in the unaffected population can be gauged to get an understanding of how this compares to the affected population to be studied.

Relatives of vitiligo patients report an increased frequency of autoimmune disease suggesting that this disorder has a genetic component and is related to other auto-immune diseases. The findings of an epidemiological survey of the UK and North America were that in 19% of vitiligo sufferers over 20 years old there was a clinical history of autoimmune thyroid disease which was reported in comparison to 2 % in age matched controls from the general white

population. As of yet the association between vitiligo and autoimmune disease remains unexplained but valuable insights into this link have been provided by genetic data (Ezzedine *et al.*, 2015).

With regards to the theory of melanocyte destruction via an autoimmune method (Speeckaert *et al.*, 2014) (Bhardwaj *et al.*, 2017), autoimmune effector mechanisms direct cytotoxic T cells or autoantibodies to melanocyte surface antigens. The human immune system usually would not recognise its own antigens and this is known as self-tolerance. In autoimmune disorders, self-tolerance is lost and the immune systems recognise indigenous cells as a foreign body attacking melanocytes in the same way as a virus or bacterial cell. It has been reported that melanosomal antigens cause a response whereby there is an influx of Th1 lymphocytes in the epidermis. Th1 lymphocytes secrete factors that are lethal to melanocytes such as TNF-α and other cytokines. The presence of cytotoxic T cells has been described in the peripheral blood of vitiligo patients. It is the inference of an autoimmune component to vitiligo that has become the justification for using immunosuppressive drugs to treat it (Hameed *et al.*, 2005) (Li *et al.*, 2015).

An alternate suggestion for the cause of vitiligo is the neural theory where melanocytes can be lost either directly or indirectly because of unsuitable reactions to neuropeptides, catecholamine's or their metabolites or in some cases as a reaction to an overactive sympathetic nervous system. Support for this theory is via evidence that states that a common preceptor of vitiligo is stress. However vitiliginous skin does not have a high enough concentration of catecholamine's to kill melanocytes suggesting that other mechanisms are involved. It has been speculated that altered oxidative stress defences have a contribution to melanocyte loss

since generation of excess catecholamine's results in oxidative products that are toxic for the melanocytes (Hameed *et al.*, 2005) (Li *et al.*, 2015).

Melanocyte death in vitiligo may be the result of increased sensitivity to oxidative stress due to toxic intermediates made by melanin precursors or other sources. A low activity of the enzyme catalase in skin leads to accumulation of hydrogen peroxide in the epidermis which inhibits melanogenesis. Previous studies have suggested that catalase in non-segmental vitiligo patients can assist the establishment of melanocyte cultures and may have a role in re-establishing melanocyte function after exposure to hydrogen peroxide. Non-segmental vitiligo has been treated by oral or topical antioxidants (Hameed *et al.*, 2005).

Recent research (Rashighi and Harris, 2017) demonstrated that evidence for the theory of oxidative stress is increasing whereby melanocytes of vitiligo sufferers have a reduced capacity to manage cellular stress. For instance, an individual's epidermal cells which include their melanocytes have continual exposure to environmental stressors, for example various chemicals and ultraviolet radiation, causing an increase in the production of reactive oxygen species. In healthy melanocytes these stressors do not cause a problem but in the melanocytes of vitiligo patients they seem to be more vulnerable. Observations of the skin of vitiligo patients indicate that there is a high level of epidermal H_2O_2 and a reduced level of catalase, a vital enzyme for protecting cells from oxidative damage. Therefore when melanocytes are transferred onto affected areas it is essential to ensure that they are healthy (achieved by microscopic evaluation before transplant) enough that they can withstand the daily stressors that they will be exposed to reducing the likelihood of the transplant failing.

Another group of theories regarding the aetiology of vitiligo centre around it being attributed to the inhibition of melanocytes or a defect in their adhesion. Theory states that melanocyte death is not a must for depigmentation to occur in vitiligo but rather is due to an initial effect on melanogenesis inhibition or rather defective inhibition, which results in a disappearance of melanocytes. This theory is supported by several observations, such as that residual melanocytes in follicular reservoir leads to a vitiliginous area being repigmented after phototherapy (Patel *et al.*, 2017). Also, persistent melanocytes which show an ectopic distribution of pre-melanosomes in the keratinocytes of the suprabasal layers have been found within the lesioned skin of those suffering from long standing vitiligo (Hameed *et al.*, 2005).

It is thought that the development of vitiligo is a two-step process with the first stage consisting of a decrease in tyrosinase activity and the second involving the inhibition of melanisation which results in the death of the melanocytes (Ghafourian *et al.*, 2014). It is also thought that melanocyte loss in non-segmental vitiligo can be attributed to defective adhesion. In support of this theory is the finding that Tenacin-C, an extracellular matrix molecule, has been found in increased levels in the basal membrane and papillary dermis which suggests it inhibits melanocyte adhesion to fibronectin and therefore may add to melanocyte loss in vitiligo. This is also seen in Koebner phenomenon, which is where there is a detachment and loss of melanocytes after a minor trauma (Hameed *et al*., 2005) (Sagi and Trau, 2011).

Presently there are few studies to support the concept of melanocytorrhagy (Kumar and Parsad, 2012) (Gauthier *et al.*, 2003) with more research into it being needed, but the theory is that alteration of melanocytes response to friction and stress causes their weak attachment and therefore transepidermal loss. The dendrites of melanocytes are not only required for the transfer of melanosomes to the surrounding keratinocytes but they also have a function in

helping melanocytes adhere to the basal membrane. It has been observed that when vitiligo melanocytes are cultured their melanocytes appear to be 'stubby.' In addition to this when hydrogen peroxide is added to an established melanocyte culture a loss of dendricity is induced which in some cases leads to melanocyte detachment. The different phenotype of vitiligo melanocytes to normal cells means that they can be identified by confocal microscopy (Xiang *et al.*, 2015).

The increase in the levels of catecholamines might be a factor that aggravates non-segmental vitiligo. It has been suggested that in the case of adhesion deficient non-segmental vitiligo dendrites are lost as a response to oxygen species or due to an increase in the release of catecholamines, which exaggerates transepidermal loss (Hameed *et al.*, 2005) (Hirobe, 2014). Reactive oxygen species are chemically reactive and contain oxygen. The production of these is a natural by-product of normal oxygen metabolism but at times of stress their levels can have a dramatic increase resulting in significant damage to cell structures. Several abnormalities, in addition to defective adhesion and dendritic loss might cause the reduction of melanocytes frictional resistance in non-segmental vitiligo, which ultimately results in their detachment by chemical or mechanical injury.

The treatment of vitiligo should have either or both of two aims. The first involves halting the progression of active disease to reduce the expansion of the area that is involved in depigmentation whilst the second strategy involves the repigmentation of the depigmented area. In any treatment for vitiligo it should be tailored to suit the individual and it should be designed in such a manner that it prevents any relapse and it maintains the disease in a stable phase (Bishnoi and Parsad, 2018). This should be achieved by either of three distinct approaches which are to: reduce melanocyte stress, regulate the autoimmune response and

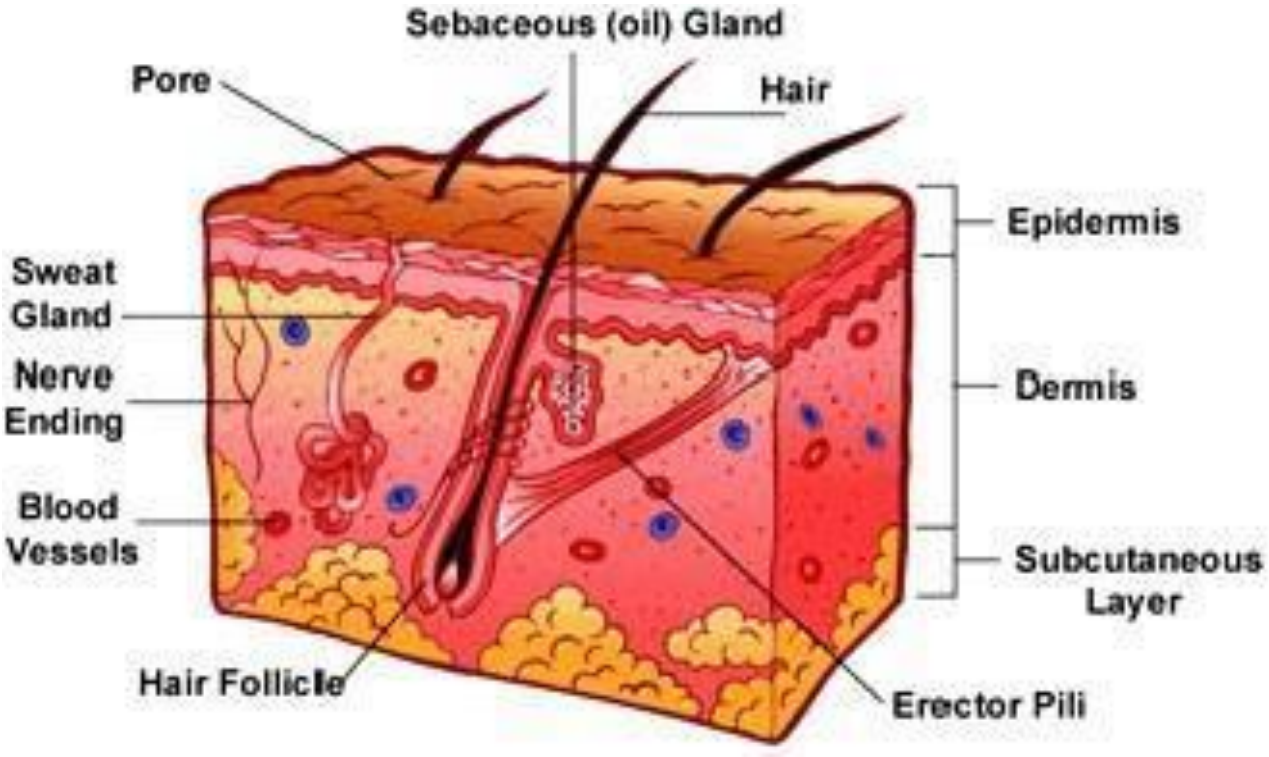
stimulate melanocyte regeneration. Treatments currently in existence partly address such requirements but in future any new therapies might do this in a more targeted way or they might utilize a combination of therapies to produce an overall better response (Rashighi and Harris, 2017).

In summary, the main theories regarding how vitiligo develops are that it might be attributed to melanocyte inhibition, a decrease in tyrosinase activity, inhibition of melanisation or the Koebner phenomenon. However, regardless of the theory as to how vitiligo develops the consequences of this disorder are the same.

2.6 Anatomy and cells of the skin

2.6.1 Structure of the skin

Figure 3: Structure of the skin (McLafferty E *et al.*, 2012).



This diagram illustrates the various layers of the skin as well as the location of several key features such as the sweat glands, nerve endings and blood vessels. Outermost epidermal layers comprise keratinocyte networks with interconnected melanocytes and a scattering of inflammatory cells. In the dermal layer there are fibroblasts and nerve endings, hair follicles, sweat glands, and blood vessels as well as other structures (Wang *et al.*, 2016).

Normal skin is the largest organ in the human body and is responsible for about 16% of total body weight. One of the roles of the skin is prevention of water loss from components of the body into the environment as well as to protect the human body from a range of environmental abrasions. The skin also has an important immune and sensory function as it has a role in the regulation of body temperature as well as the synthesis of vitamin D (Jablonski and Chaplin, 2018). The skin is composed of three main layers, as illustrated in **Figure 3**, which provide it with a strength and flexibility as well as it being involved in multiple functions. The skin also serves to protect the body from external stimuli, such as microbes (Kwiecien *et al.*, 2019).

The skin's top most layer is the epidermis and the function of this is to be a barrier to water loss from the body as well as microbial infection and the absorption of chemicals from the environment. The epidermis is composed of histologically stratified squamous epithelium, which needs to be constantly renewed throughout life from birth to death (Hirobe, 2014). The epidermis's barrier function occurs mostly in the upper stratum called the stratum corneum. The epidermis is made up of several layers starting with the basal layer that is found just above the dermis. This then proceeds upwards to the top layer through the prickle and granular layers.

In the epidermis, the most common cell type is the keratinocyte, which makes keratin and several other proteins. The antimicrobial peptides produced by the skin control microbial growth on its surface. The antimicrobial peptides have various roles in mediating inflammation and influence epithelial and inflammatory cells as well as influencing the proliferation of cells, wound healing cytokine/chemokine production and chemotaxis (Herman and Herman, 2019).

In the epidermis, there are two other important cell types **(Table 4)** which are melanocytes and Langerhans cells (Otsuka *et al.*, 2018). Melanocyte function is pigment production and these cells are found in the basal layer of the epidermis where they make pigment granules which contain melanin, these are called melanosomes. The source of melanosomes is the melanocytes from which they are transferred to epidermal keratinocytes. It is here that melanosomes provide the nucleus of the cell with some protection from ultraviolet light as well as giving skin its distinctive colour. The process of melanin synthesis is continuous, but it can be increased by skin exposure to UV light, seen physically as creation of the sun tan. There are two main melanin types called eumelanin and pheomelanin which will be described in much greater detail later in this literature review.

The Langerhans cell is important for creating an immune barrier for the epidermis (Jaitley and Saraswathi, 2012). Langerhans cells make up the skin and mucosa and are dendritic cells. These cells were first discovered in 1868 by Paul Langerhans and due to their dendritic nature were mistaken as being nerve cells of the epidermis. It is thought that these cells are important for combatting infection of the skin, since they pick up and process microbial antigens which lead to them being antigen presenting cells that are fully functional. Langerhans cells are important for induction of T-cell responses. Since Langerhans cells have an involvement in

the immune response against diseases they are potential targets for immunotherapy (Stoitzner, 2010). However, for the purposes of my study I will be focusing on only the melanocytes and keratinocytes because concentrating on the interactions of any more than two cell types will make my study too complex at this stage.

Table 4: Cells of the epidermis and their function (Yousef *et al.*, 2019).

Cell type	Function
Keratinocytes	Formation of epidermal water barrier by making and secreting lipids
Melanocytes	Melanin production
Langerhans' Cells	First line in skin defence and antigen presentation
Merkel cells	Sensory function as mechanoreceptors for light touch

The dermis and hypodermis are the two layers below the epidermis. Directly underneath the epidermis is the dermis which comprises tough connective tissue, hair follicles and sweat glands. The hypodermis is underneath and this is deeper subcutaneous tissue that is made from fat and connective tissue (Cheong and McGrath, 2013).

Another important constituent of the skin are the blood vessels which have the role of dilating or constricting respectively in response to heat or cold to maintain constant body temperatures. Sebum is secreted by the sebaceous glands, located at the base of hair follicles, preventing skin becoming excessively dry. Body temperature is also regulated by sweat glands

which produce sweat, in reaction to the body becoming excessively hot or stressed, or to cool it down. Sweat glands can be found all over the body but are of greatest abundance in palms, soles, forehead and underarms (Best *et al.*, 2019).

The base of hair follicles is in deeper dermal tissue from which they extend beyond the skins border. Arrector pili muscles and sebaceous glands anchor the hair follicle to the skin. These follicles possess many sensory fibres which provide sensory feedback by wrapping around their base and extending up the hair shaft. The base of the hair follicle is also innervated by autonomic hair fibres (Robertson *et al.*, 2011).

The most abundant protein in the dermis is collagen which makes up to 30% of the total human protein content and constitutes 70% of dry mass skin content. It is a vital part of the skin since collagen fibres make up the infrastructure for the protein Elastin. Elastin is found alongside collagen which is also responsible for giving the skin and organs structure, maintaining skin elasticity and for enabling hyaluronic acid to trap moisture. Over time the body's ability to produce collagen diminishes which is responsible for the appearance of wrinkles and fine lines. As well as collagen a reduction in the levels of these accessory proteins (e.g. elastin) will cause the skin to wrinkle and sag (Chong *et al.*, 2019).

Other skin components are keratinocytes and melanocytes, which will be discussed below. Different cells can be identified and distinguished by the presence of cell surface markers. Cell surface markers are proteins and carbohydrates that are attached to the cell membrane and are like a fingerprint for the cell. Each cell has its own unique set of cell markers (combinations of different proteins on the cell membrane). This will be discussed in more

detail in section 2.14 of this chapter but both melanocyte and keratinocyte cells have their own markers which can be used to confirm the presence or absence of these cells.

2.6.2 Keratinocytes

Keratinocytes have the important function of providing the body with protection from hostile forces as they are involved in expressing several surface antigens. These antigens are immunologically significant antigenic fragments and they are internalised, digested and expressed on the cell surface, for example the major histocompatibility complex class II (MHC class II) molecules making it easy for individual keratinocytes to recognise self from non-self (Weber, 2006).

Another function of keratinocytes is to secrete cytokines as well as being able to produce antimicrobial peptides. It is these natural antibiotics that enable a signal to be sent to the host immune system that an infectious agent has mounted a challenge (Weber, 2006). Therefore, the present study attempted to co-culture both cell types together as the addition of keratinocytes to the culture improves the functionality of melanocytes that are to be used in the treatment of vitiligo (Kumar *et al.*, 2012).

2.6.3 Melanocytes

There are several stages to the melanocyte life cycle **(Figure 4)** (Cichorek *et al.*, 2013). This view is confirmed by several other sources (Hirobe, 2014) such as the US national library of medicine and the national institutes of health websites (U.S. national library of medicine https://www.nlm.nih.gov/. accessed 3rd September, 2017).

Figure 4: Stages of the melanocyte life cycle

The specification of lineage from neural crest cells (melanoblasts)

The proliferation and migration of melanoblasts into melanocytes

The differentiation of melanocytes from melanoblasts.

The maturation of melanocytes (this involves the production of melanin in special organelles)

The transport of mature melanocytes to keratinocytes, which leads eventually to melanocyte cell death

Figure 5: Illustration of the relationship between keratinocytes and melanocytes (D'Mello *et al.*, 2016).

The basal layer of skin contains dendritic melanocytes which produce melanin. Melanosomes transfer melanin pigments to keratinocytes.

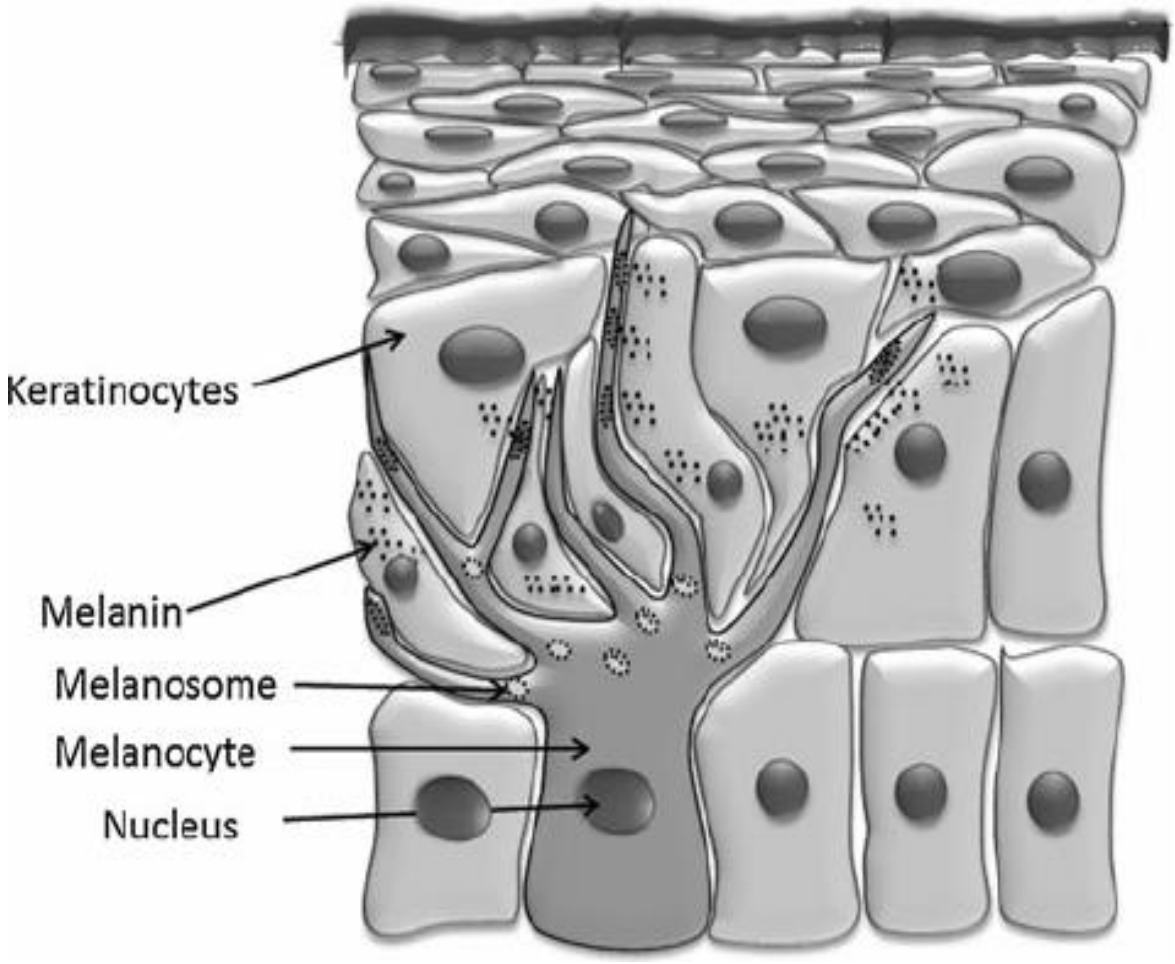


Figure 6: Production of melanin and its transfer to melanocytes (Hirobe, 2014).

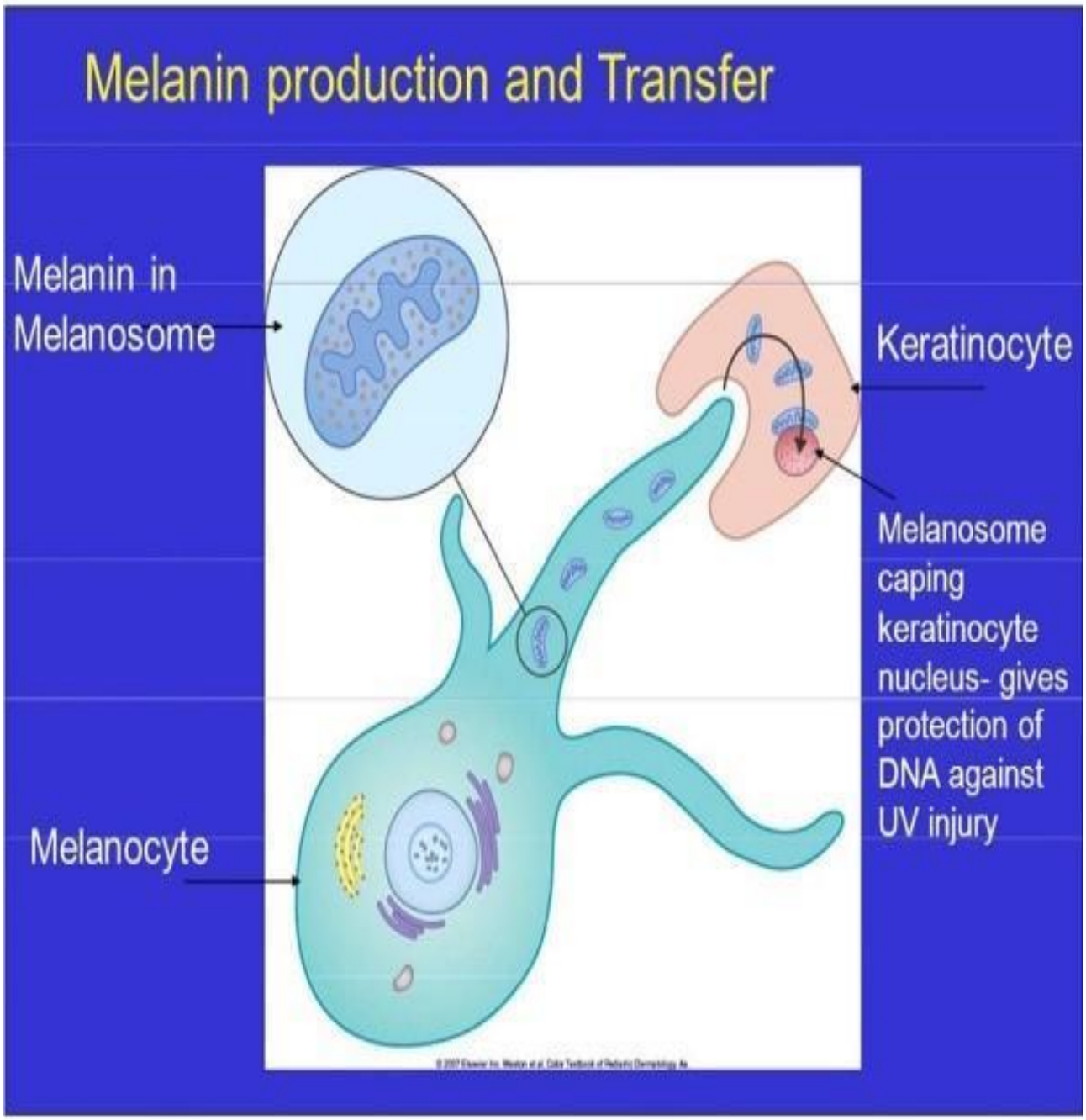
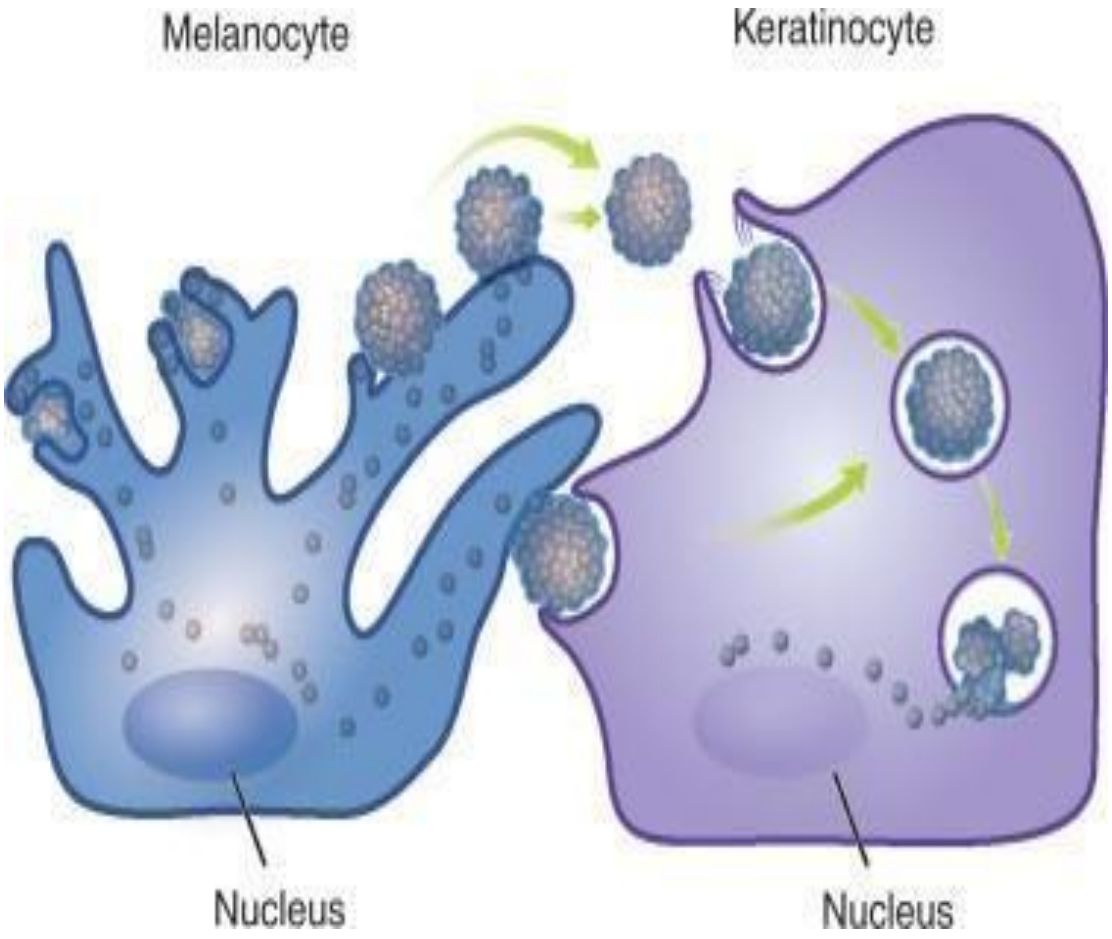


Figure 7: Melanosome transfer pathway (Ando *et al.*, 2011).

Globules of pigment that contain many melanosomes are released by the dendrites of melanocytes into extracellular space. Keratinocytes phagocytose these globules and the individual melanosomes are spread mostly around the keratinocyte's perinuclear region.



Fitzpatrik and Breathnach proposed in 1963 and cited in Thinges et al (2012) that the melanin unit comprises one melanocyte linking to about 36 keratinocytes (normally this is in the range of 30-40) **(Figure 5)**. It has been proposed (Thinges *et al.*, 2012) that keratinocytes are the major factor for determining the epidermal melanin unit's size. The epidermal melanin unit size is independent of race but does vary in different body areas. Difference in races occurs because of differences in the intensity of melanogenesis and the arrangement of melanocytic dendrites. Contact between keratinocytes and the dendrites of melanocytes **(Figure 6 & 7)** is required for melanin to be transferred into keratinocytes and for determination of skin colour (Cichorek *et al.*, 2013).

Melanocytes can be recognised molecularly by the identification of proteins that are specific to melanocytes which are tyrosinase (TYR), tyrosinase-related protein 1 and 2 (TYRP-1, TYRP-2/DCT), microphthalmia transcription factor (MITF) and melanosomal matrix proteins (Pmel17, MART-1) (Cichorek *et al.*, 2013). Various microscopical methods can be used to identify melanocytes. When the method of transmission electron microscopy is used sebaceous melanocytes can be identified as being of large shape which is either triangular or polygonal and a plasma membrane that does not have tonofilaments and desmosomes. Desmosomes are a cell structure that are specialised for cell cell adhesion. These cadherin-mediated junctions act via protein complexes to mechanically couple the intermediate filament network of neighbouring cells (Price *et al.*, 2018). Another structure specialised for cell cell adhesion is the hemidesmosome and although similar to desmosomes the difference is that these cells are found in keratinocytes where they attach adjacent cells to the extracellular matrix.

The nuclei could be observed as having a profile that is slightly irregular with shallow indentations. There is a difference between sebaceous melanocytes and those on the epidermal

basal surface with the former appearing to contain fewer granules containing melanin. Something to note is that melanosomes with an individual distribution in the suprabasal layer are much bigger than melanocytes of the epidermis and these melanin granules did not show migration to the surrounding sebocytes (Jang *et al.*, 2014). Microscopical methods are important to my project so that I can address my first research question which is to create a new method that is reliable and reproducible to measure the ratios of different cell types using image processing. If a picture of the cells in culture can be taken via a microscope then a method needs to be created whereby the different cell types can be identified and counted.

2.6.4 Melanocyte and keratinocyte interactions

Melanocytes and keratinocytes communicate with each other via cell-to-cell contact and secreted factors. Keratinocytes control melanocyte growth and activity via paracrine growth factors and cell adhesion molecules. Melanocytes and keratinocytes produce different hormones which are responsible for regulating melanocyte proliferation, melanogenesis and formation of melanocytic dendrites (please see below for further details regarding hormonal control of melanocyte proliferation) (Cichorek *et al.*, 2013).

As mentioned above the factors released by keratinocytes that influence melanocytes are hormones, cytokines and growth factors. Specifically, these are: $\boldsymbol{\alpha}$-melanocyte stimulating hormone, hepatocyte growth factor, basic fibroblast growth factor, leukaemia inhibitory factor, nerve growth factor, stem cell factor, endothelin's and granulocyte macrophage colony stimulating factor (Hirobe, 2014).

2.7 Melanogenesis

2.7.1 Description of melanogenesis

Melanogenesis is the name given to the process of melanin production and distribution by epidermal units in the skin. These epidermal units are an example of paracrine signalling consisting of a melanocyte that is surrounded by keratinocytes (Lei *et al.*, 2002). It is a complex process that consists of several different stages. Melanin is the primary determinant of the colour of the skin, hair and eyes. Melanin does not just define an important phenotypic trait in humans but it's vital for the body's photoprotection due to its ability to absorb ultraviolet radiation (UVR) (Videira *et al.*, 2013).

Melanocytes are very responsive cells that continuously sample their environment and can modulate their levels of melanogenesis or proliferation according to extrinsic signals, for example UV light, and other factors that originate from other cell types in the skin (Lei *et al.*, 2002) . The process of melanogenesis when disturbed can lead to a variety of pigmentation defects resulting in hypo or hyper pigmentation. These defects might occur both with and without an alteration in the number of melanocytes that are present (Videira *et al.*, 2013). Research into such defects is important because they have a detrimental impact upon quality of life and current treatments can be unsatisfactory. At present cosmetic and pharmaceutical companies are continuously looking for new treatments and solutions, a point which supports the value of this current study.

2.7.2 Melanosomes

Melanosomes are organelles that are found in all animal cells and they are where melanin is synthesised, stored and transported. Starting with the enzyme tyrosinase and then in association with other melanogenic enzymes (please see section 2.7.4), melanin granules that are made in

melanosome organelles are transported from melanocytes to keratinocytes. As the keratinocytes desquamate, so their melanin granule content is lost (Lei *et al*., 2002). Melanosomes can sometimes be seen to be arranged superficially to the keratinocyte nucleus, forming a physical barrier for keratinocyte DNA as well as having a scavenging role for free radicals induced through exposure of the skin to UV radiation.

Melanosomes are synthesised in several places which include the melanocytes of mammalian skin, choroidal melanocytes and in the retinal epithelial cells of the eye. The actual function and fate of melanosomes is dependent upon what type the cell is. For example, melanocytes in the epidermis supply their keratinocyte neighbours with melanosomes and this is responsible for pigmentation in the skin and hair, whilst in retinal epithelial cells of the eye pigment, granules are kept intracellularly (Wasmeier *et al.*, 2006).

Melanosomes are organelles of relatively large size (they have a diameter of 500 nm) which in combination with their dark pigment makes them easy to visualise with bright field microscopy. Because of this, melanosomes are very useful as a model organelle, in particular in the case of studies that are on the motility and biogenesis of organelles. Studies such as these have identified melanosomes as being lysosome related organelles. Study of melanosome biology has therefore provided crucial understanding of the biogenesis and transport of lysosome related organelles as well as how intercellular interactions happen in tissues that are complex (D'Alba and Shawkey, 2018).

2.7.3 Stages of melanogenesis

Melanosomes undergo four stages of development within the melanocyte, (**Figure 8**) which are:

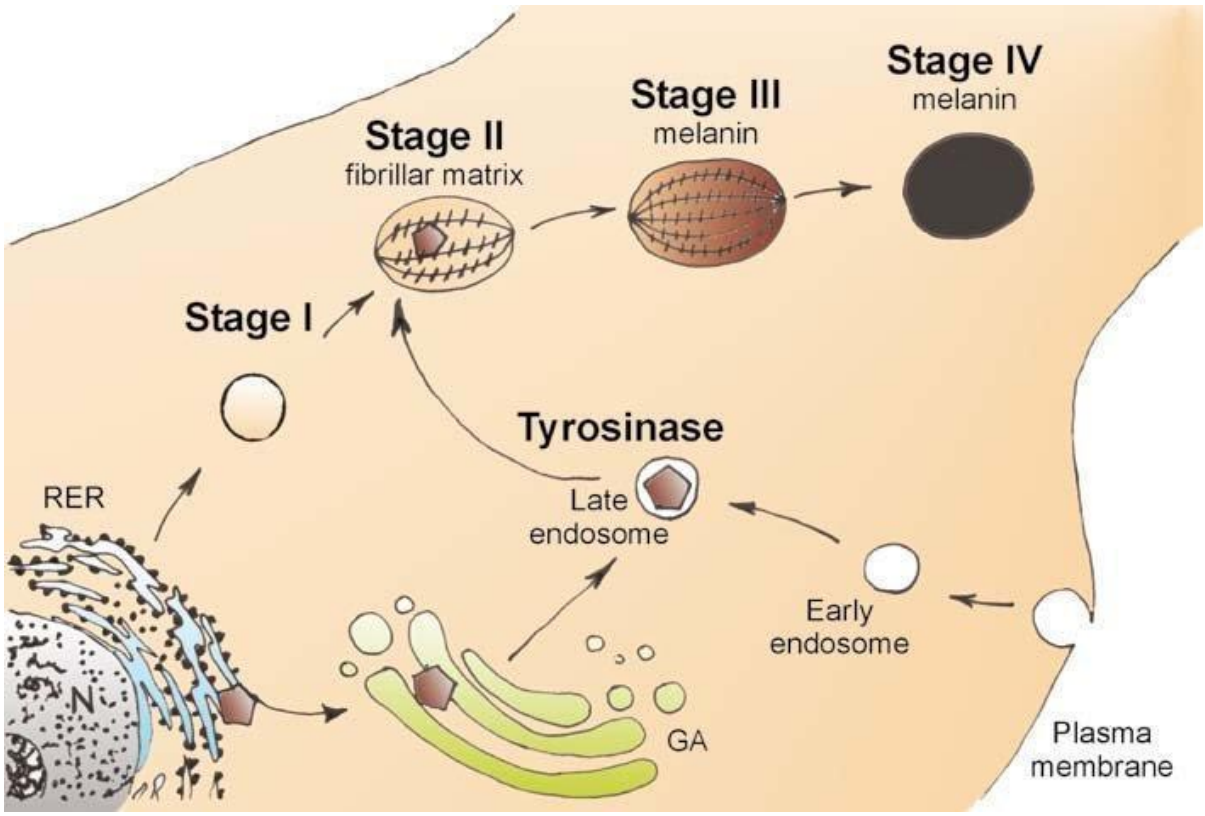
Stage 1: Premelanosomes – These are small round vesicles that have an amorphous matrix.

Stage 2: Melanosomes – These have a structured fibrillar matrix that is organised. There is tyrosine present but the synthesis of pigment has not yet occurred.

Stage 3: Beginning of melanin production

Stage 4: Pigment fills the entire melanosome (Hirobe, 2011).

Figure 8: An illustration of the stages of melanogenesis (Cichorek *et al.*, 2013).



2.7.4 Enzymes of Melanogenesis

2.7.4.1 Tyrosinase

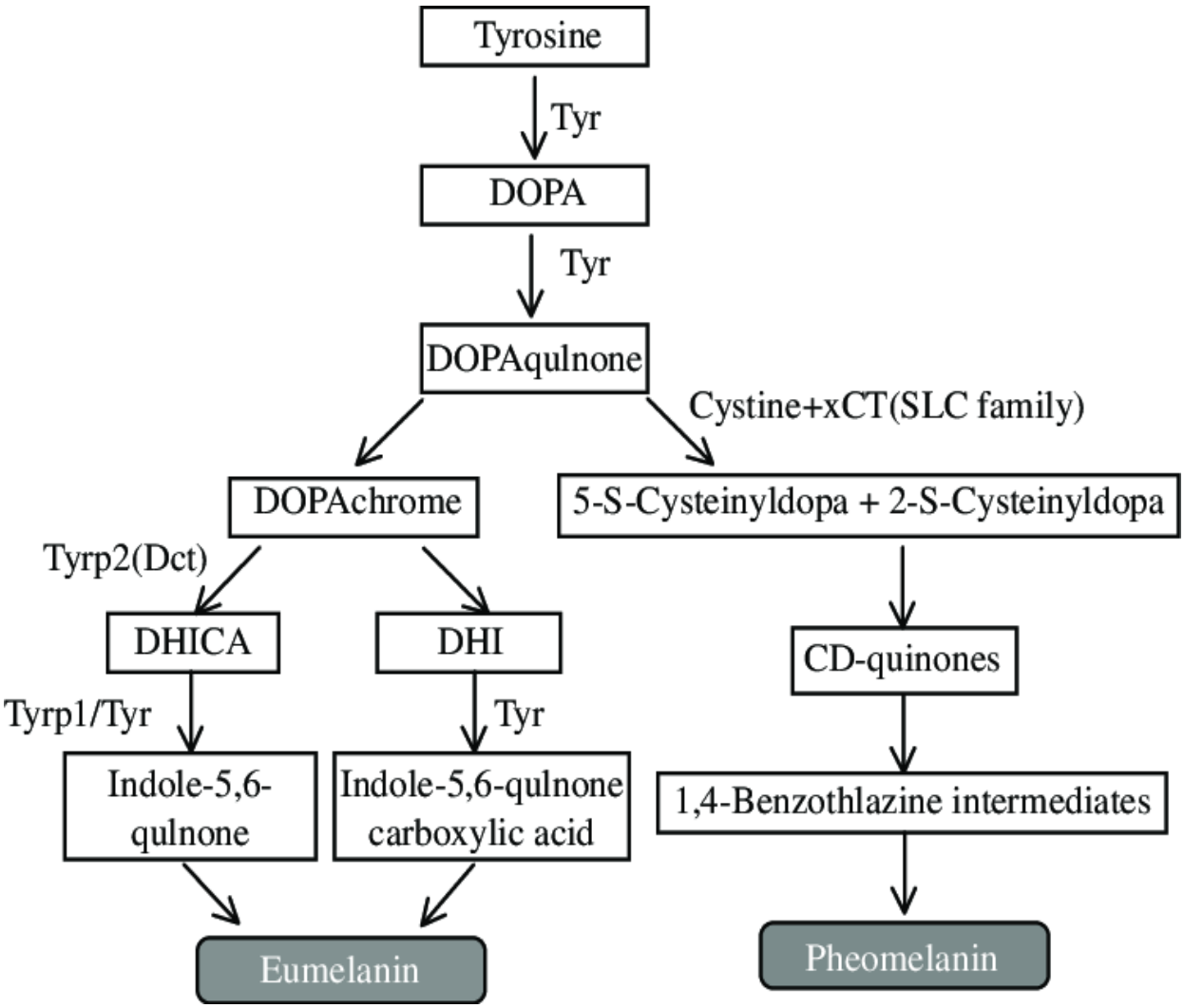
Tyrosinase is a glycoprotein that is found within the membrane of the melanosome. It has an internal, transmembrane and cytoplasmic domain. Its catalytic region (accounting for approximately 90% of the protein) is contained within an internal melanosomal domain and is where copper ions bind for reaction with histidine residues, these copper ions are needed for

its activity. This is since a copper dependent enzyme is responsible for the catalysis of the conversion of L-tyrosine into L-DOPA, which is the rate limiting step in melanin production (Gillbro and Olsson, 2011). Superoxide anions can be used as a substrate for melanogenesis, suggesting that tyrosinase may have a role in protecting melanocytes from reactive oxygen species (Videira *et al.*, 2013).

The transmembrane domain is short whilst the cytoplasmic domain is made up of around 30 amino acids. This enzyme's cytoplasmic domain aids in the enzymes transportation to melanosomes from the nucleus (Gillbro and Olsson, 2011). Once Dopaquinone production is complete the melanin synthesis pathway divides into producing red-yellow Pheomelanin or black-brown Eumelanin and there is an instantaneous conversion to Dopachrome and Leucodopachrome (Gillbro and Olsson, 2011) (**Figure 9**).

Figure 9: Illustration of how eumelanin and pheomelanin are produced from the same precursor because of the split of the pathway following the production of DOPAquinone (Zang *et al.*, 2018).

This pathway is conserved amongst species. Formation of eumelanin or pheomelanin depends upon the presence/ absence of cysteine (D'Mello, 2016).



The types of melanin formed and the amounts produced are determined genetically but they can be influenced by both extrinsic and intrinsic factors such as hormonal changes, age, inflammation and exposure to UV. All these stimuli exert their influence by affecting different melanogenic pathways (D'Mello, 2016).

The properties of these two variants differ since eumelanin can scavenge and extinguish both oxygen and carbon derived free radicals whilst eumelanin is lacking in these properties and can serve to be a source of free radicals when irradiated with UV (Gillbro and Olsson, 2011). It is the polymerisation of indoles and quinones that lead to the formation of eumelanin. The pheomelanin and eumelanin pathways diverge at the L-dopaquinone stage with the pheomelanin pathway being dependent upon cysteine being present, which is moved across the melanosomal membrane via active transport.

A characteristic of the eumelanin synthesis pathway is that dopachrome either undergoes spontaneous conversion to 5, 6-dihydroxyindole or enzymatic conversion to 5, 6-dihydroxyindole-2-carboxylic acid via tyrosine-related protein-2 (TRP-2). For pheomelanin synthesis cysteine needs to be present since it has a reaction with L-dopa producing cysteinyl-dopa. This then undergoes conversion into quinoneimine, alanine-hydroxyl, dihydrobenzothiazine and finally polymerisation into pheomelanin (Gillbro and Olsson, 2011).

A balance is made and maintained between the production of eumelanin and pheomelanin via redox conditions that are in the melanosomes. Levels of glutathione (GSH) directly determine which of these two substances are produced with them being at high levels for eumelanin and low levels for pheomelanin. The action of functional enzymes such as this (e.g. catalase, thioredoxin reductase, glutathione reductase and glutathione peroxidase) modify the melanogenic pathway (Gillbro and Olsson, 2011).

It has been shown that tyrosinase hydroxylase isoenzyme 1 (TH1) can indirectly activate tyrosinase. Its presence has been found in melanosomes and it can act as a catalyst for L-dopa synthesis with L-dopa then acting as a cofactor for tyrosinase (Gillbro and Olsson,

2011). Tyrosine hydroxylase isoform acts to catalyse L-tyrosine being converted into L-DOPA thus functioning as a promoter of tyrosinase activation. This enzyme is found in the melanosomal membrane adjacent to tyrosinase (Videira *et al.*, 2013).

Phenylalanine hydroxylase is found in the cytosol and it acts to catalyse L-phenylalanine being converted into L-tyrosine. This is the substrate for tyrosine, so it also acts as a promoter of its activation (Videira et *al.*, 2013). The three previously mentioned enzymes (tyrosinase, tyrosine hydroxylase and phenylalanine hydroxylase) are needed for the beginning of melanogenesis (Schallreuter *et al.*, 2008).

The melanosome membrane is also home to two proteins that exhibit similarity to tyrosinase (40% amino acid homology, suggesting a common ancestry). These proteins are a tyrosinase-related protein-1 (TRP-1) and tyrosinase-related protein-2 (TRP-2). The similarity of these proteins to tyrosinase is that they are found spanning the membrane of melanosomes. These proteins exact functions are still unknown but the suggestion is that TRP-1 is involved in: stabilising tyrosinase, the synthesis of melanosomes, increasing eumelanin to pheomelanin ratios and acting against oxidative stress, which can be attributed to its peroxidase effect (Videira *et al.*, 2013) (Gillbro and Olsson, 2011).

2.7.5 Regulation of melanogenesis in skin melanocytes

Skin pigmentation is regulated intrinsically by melanocytes, which produce several factors involved in inflammatory and immune responses that act via a paracrine or autocrine way on keratinocytes. Examples of this include leukotrienes, prostaglandins, nitric oxide, cytokines and POMC peptides (Videira *et al.*, 2013). UV irradiation is the primary extrinsic stimulus for the synthesis of melanin, this is particularly so in the case of black eumelanin. This stimulus

results in the generation of DNA photoproducts as well as release of paracrine and autocrine factors, most notably the secretion of α-melanocyte stimulating hormone (α-MSH) by keratinocytes. α-MSH activates the melanocortin 1 receptor in skin melanocytes plasma membrane. The effect of this is cAMP-dependent signalling and microphthalmia-associated transcription factor expression (MITF) being stimulated. A description for MITF is that it is a 'master regulator' for melanocyte function and the process of melanogenesis. MITF activity is important for causing expression of a multitude of genes needed for survival of melanocytes as well as their motility, differentiation, apoptosis and melanosome production (Wasmeier *et al.,* 2006).

2.7.6 Method of transfer of melanosomes to keratinocytes

Protease-activated receptor-2 (PAR-2) is a seven-transmembrane G-protein-coupled receptor expressed by keratinocytes that can regulate the transfer of melanosomes via the phagocytosis of keratinocyte (Ando *et al.,* 2012). It is known that the transfer of melanosomes is skin-colour dependent with the pattern of distribution of melanosomes that are transferred into keratinocytes being determined by the keratinocytes racial and ethnic origin. However, we still do not understand exactly how melanosomes are transferred from melanocytes to keratinocytes.

2.8 Main types of melanins

Different skin pigmentation phenotypes can generally be classified by the Fitzpatrik system. There are six phototypes (I-VI) which are classified by a person's reaction to exposure to UVR by grading erythema and acquired pigmentation (Ferreira *et al.*, 2012). A person's constitutive pigmentation reflects their level of melanin which is genetically determined. However, pigmentation can be altered by several regulatory factors, extrinsic or

intrinsic. Extrinsic factors include UVR or drugs whilst intrinsic factors may be released by fibroblasts, keratinocytes, endocrine, neuronal and inflammatory cells (Videira *et al.*, 2013).

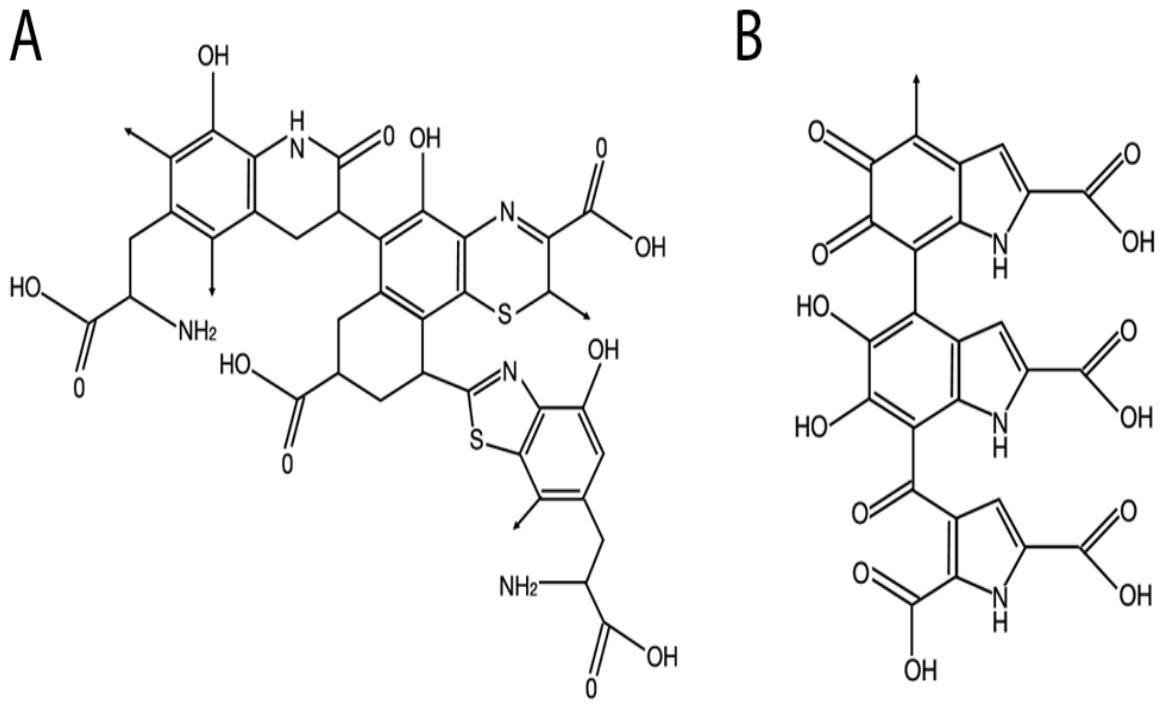
2.8.1 Difference in melanins of dark and light skinned individuals

Human skin colour is very variable across the globe with the variation in skin tones being a result of human migration and adaptation to tropical and non-tropical environments therefore, people don't have pure black, white, red or yellow skin. Melanin is the cause for skin colour and it is important because it protects individuals from environmental assaults and any potential subsequent cellular injury. It prevents DNA damage by absorbing any UV light that penetrates the skin and by acting as a scavenger of free radicals (Kumar *et al.*, 2012) (Lei *et al.*, 2002). A severe inverse correlation was found between the level of skin pigmentation and the occurrence of skin malignancies and UV photo damage (Lei *et al.*, 2002).

Differences in pigmentation phenotype are not attributable to there being any variance in the number of melanocytes since these numbers remain constant amongst the different ethnic groups but rather they are due to other causes such as how large the melanosomes are and how many are produced, the types and amounts of melanin that are produced and how melanin is distributed and transferred to the keratinocytes (Videira et *al.*, 2013). This is demonstrated by the fact that in dark skin the melanosomes are bigger, in greater quantities, and elongated meaning that their degradation in keratinocytes is delayed and there is an increase in pigmentation that is seen. These factors are determined at birth rather than by extrinsic factors (Videira *et al.*, 2013).

2.8.2 Division of the pigmentation pathway into eumelanin and pheomelanin

Figure 10: Molecular structure of pheomelanin (A) and eumelanin (B) (Ito & Wakamatsu, 2008).



The differences in skin pigmentation that are seen among different ethnic groups is dependent upon eumelanin content and the eumelanin to total melanin ratio is the determinant of the skin colour. It has been observed that eumelanins are better at protecting from the sun than their reddish-yellow pheomelanin counterparts which is why there is a 30-40 fold increased risk of skin cancer in lighter skin than in those with darker skin (**Figure 10**). Skin pigmentation is not correlated to the amount of pheomelanin as it is present in similar quantities in dark and light skin (Land and Riley, 2000).

Eumelanogenesis is the name of the process for creating eumelanin whilst that of phaeomelanin is called phaeomelanogenesis with both processes diverging at a fairly early stage in the

formation of pigment. This divergence occurs when dopaquinone, which is the first product of the oxidation of tyrosine, follows either one of two available paths for reaction, which are a reductive endo cyclisation where a Michael addition of the side-chain amino group occurs, or the production of cysteinyl dopa via the reductive addition of cysteine. For the first process the product is cyclo dopa which undergoes a redox exchange reaction with dopaquinone to produce dopachrome which is the precursor of the eumelanogenic pathway. In the case of the second mentioned reaction cysteinyl dopa leads to the formation of benzothiazoles which are known to be characteristic of pheomelanin. Cysteinyldopaquinone is the precursor molecule of the pheomelanin pathway (Land and Riley, 2000).

The biological benefits that are produced by pheomelanin are currently unknown so questions arise as to why its evolution has been promoted. One such hypothesis is that production of this pigment is required to remove cysteine since the presence of cysteine residues in proteins can make them susceptible to oxidative stress and any associated damage. It has been found in mammals that an excess of cysteine has associations with several conditions including rheumatoid arthritis, Parkinson's disease, Alzheimer's disease, systemic lupus erythematosus, an increase in the risk of cardiovascular disease and negative pregnancy outcomes (Galvan *et al.*, 2012).

Although not a recent study (Marmol *et al.*, 1996) explains that the mechanisms that govern the balance of pheomelanin to eumelanin are dependent on the three main factors of L-cysteine, glutathione and tyrosinase related protein-1 expression but it is the case that none of these factors alone is dominant in the direction of the production of one type of melanin over another. More recent descriptions are available (Wakamatsu *et al.*, 2017).

2.8.3 Eumelanogenesis

It is thought that the diversity that exists in the pigmentation of the skin, hair and eyes in humans can be down to the variation that exists in the pH of the melanosomes with acidic pH being responsible for the suppression of melanin production (Wakamatsu *et al.*, 2017). The enzyme tyrosinase shows optimum activity at a pH of 7.4 with this being greatly suppressed at lower pH levels (Ito *et al.*, 2013). In one study it was demonstrated that the action of depleting (Marmol *et al.*, 1996) extracellular l-cysteine significantly increased tyrosine hydroxylase activity and promoted eumelanogenesis. It must be acknowledged that this study is not relatively recent but a thorough literature search has not found any reason for these findings to be disputed.

2.8.4 Phaeomelanogenesis

The production of pheomelanin from either dopa or tyrosine by tyrosinase requires cysteine to be present and was maximised at the pH values of 5.8 to 6.3 whilst the production of eumelanin was suppressed at pH 5.8 (Wakamatsu *et al.*, 2017). This finding leads us to believe that at weakly acidic pH's mixed melanogenesis is chemically shifted to a more pheomelanic state.

2.8.5 Method of melanosome formation

The development and maturation of melanosomes can be categorised into four morphologically distinct stages by classical electron microscopy studies, which are stage I and II that includes unmelanized immature pre-melanosomes whilst stages III and IV contain melanised melanosomes (Hirobe, 2014).

Stage I pre-melanosomes are characterised as being non-pigmented vacuoles that originate from the endosomal system. In stage II these obtain characteristic internal striations whilst in

stage III the melanin pigment is placed upon these striations. The outcome of this is the creation of stage IV melanosomes that are mature and fully melanised. In addition to this, it appears that other cell types that are pigmented (such as retinal pigment epithelial cells) show similar progression in development (Wasmeier *et al.*, 2006).

2.8.6 Differences in melanosomes forming eumelanin and pheomelanin

The melanosomes that form eumelanin and pheomelanin are different and can be distinguished by the fact that those containing eumelanin (eumelanosomes) have an elliptical morphology with pigments being deposited longitudinally in intraluminal fibrils. This differs from melanosomes containing pheomelanin (pheomelanosomes) which are spherical and have pigments deposited granularly within multivesicular bodies (Hirobe, 2014).

The typical eumelansome is elongated (0.8-1 μm [800-1000 nm] long, and 200-400 nm wide) and has round ends (Raposa and Mark, 2007). Phaeomelanosomes on the other hand show much more variance in terms of size and are usually between 500nm and 700nm long (sometimes even up to 900nm) and have a width of 300nm to 600nm (Raposa and Mark, 2007).

2.9 Histological/clinical features of vitiligo

2.9.1 Structure of the skin

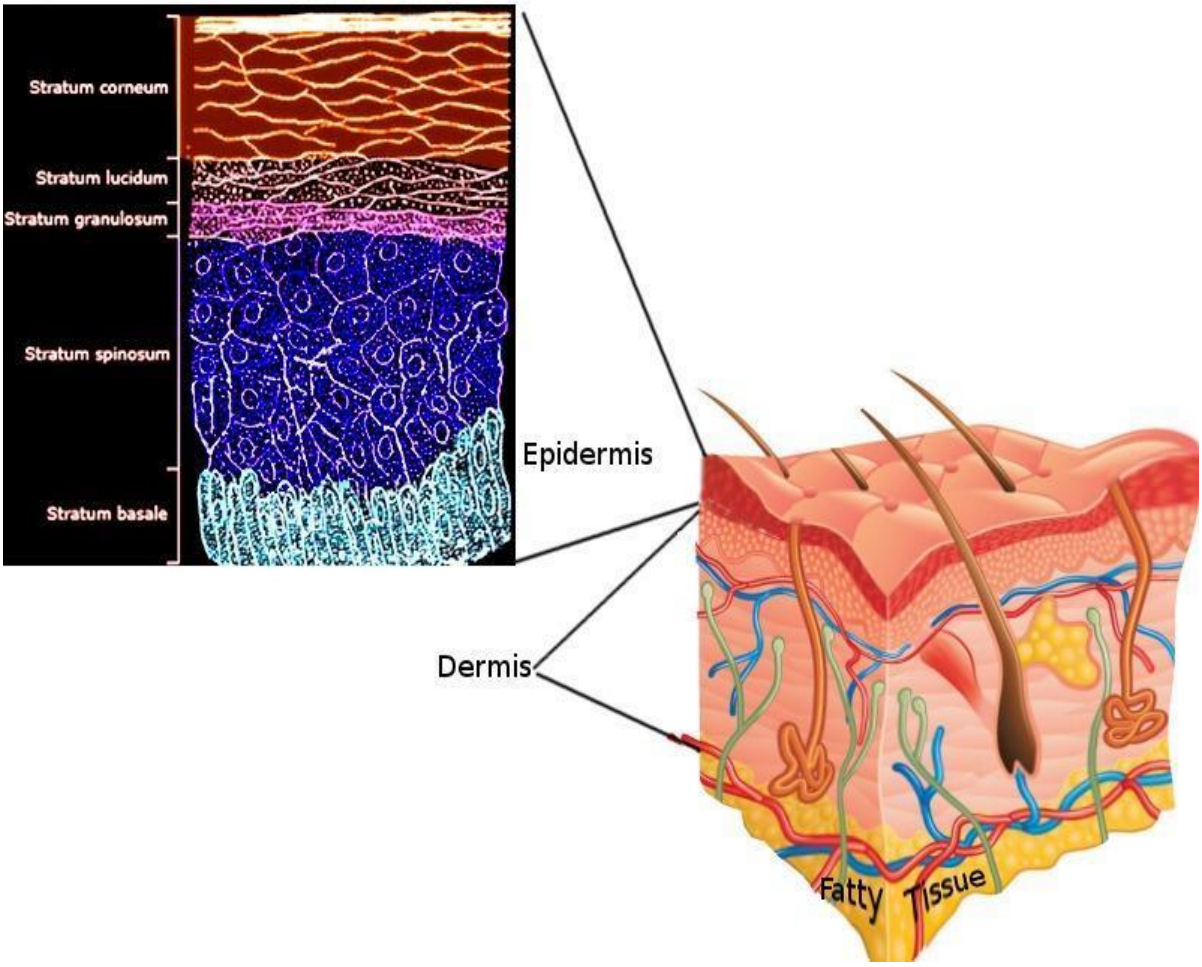
There are three main layers to the skin. The outermost layer of the skin is the epidermis which provides a waterproof layer. Below this is the dermis which comprises tough connective tissue, hair follicles and sweat glands. Under this is the hypodermis, this is a layer of subcutaneous tissue made from fat and connective tissue (Watkins, 2013). The epidermis can then be divided into five layers which are the stratum corneum, stratum lucidium, stratum granulosum, stratum spinosum and stratum germinativum (**Figure 11**). Stratum germinativum (also known as

stratum basale) is the layer at the base of the epidermis. It is a crucial layer since it has the only epidermal cells that have the capability for cell division. Cells essentially germinate here which has led to the word germinativum. It is in this layer that keratinocytes arise as a product of mitosis (Watkins, 2013).

Keratinocyte movement is from the stratum basale to the stratum spinosum. The stratum spinosum can lend such a characteristic name to the fact that its cells are of a spiny shape. The strength and flexibility of the skin can be in part attributed to this layer and keratinocytes move from here to the next layer which is the stratum granulosum, named as such because it contains many granules. It is in the granulosum that keratinocytes produce a large amount of keratin via keratinisation and the keratinocytes change to becoming flatter, more brittle and lose their nuclei (McGovern *et al.*, 2012). Once keratinocytes move out of the stratum granulosum they die and make the stratum lucidum. These cells die here because they are far from the rich blood supply which is below the stratum basale. Since these cells are lacking nutrients and oxygen the keratinocytes are pushed towards the skin's surface (McGovern *et al.*, 2012).

The stratum lucidum is named as such because its appearance is lucid (clear/transparent). This is found in the thickest areas of the skin such as the palms and soles of the feet. This is the only layer that is seen with the eyes and it is to where the keratinocytes move next from the stratum lucidum. It is here that the keratinocytes are called corneocytes and are absent of almost all water and a nucleus. These cells are basically dead and have a greater protein mass than an average cell because they are filled with the protein keratin. Corneocytes create a hard layer which protects against environmental trauma and they are eventually shed into the environment (Usui *et al.*, 2013). The entire keratinocyte life cycle from being produced in the stratum basale to being flaked off takes between 24 and 45 days (McGovern *et al.*, 2012).

Figure 11: Histological appearance of normal epidermis (Agarwal & Krishnamurthy, 2019).



2.9.2 Appearance of melanocytes and pigmented keratinocytes

Images from confocal scanning laser microscopy (CSLM) have enabled melanocytes and keratinocytes to be distinguished from each other. In these images keratinocytes are polygonal cohesive cells that have a variably bright granular cytoplasm. Melanocytes can be identified as bright cells that are round, dendritic, fusiform or oval (Busam *et al.*, 2001).

2.10 Description of rare forms of vitiligo

Fitzpatrick first coined the term trichrome vitiligo in 1964. It describes a condition where vitiligo lesions have an intermediate area of hypochromia, which is found between the principal unaffected skin and the achromic center. This is seen visibly as three shades of a colour (brown, tan and white) occurring in the same individual. After some time this trichome lesion will develop into a vitiligo macule that is normal. Trichrome vitiligo is only a transitory pigmentary state; however, it may exist for months or years without any change (Hann et al., 1999) (Lee *et al.*, 2011).

The description of 'chemical vitiligo' is given to the cases where vitiliginous processes are initially switched on by chemical exposure but then continue even after halting the use of these chemicals (Alam and Ghosh, 2015). Unfortunately, research on chemical vitiligo is very sparse, meaning that it is very difficult to gather any more information on this topic than what is presented here.

2.11 Treatment of vitiligo

A wide range of therapies have been developed to achieve repigmentation which range from the use of UV (e.g. narrowband UVB and PUVA), immunomodulatory therapies and laser to surgical procedures which include cultured epithelial autografts, cultured melanocyte suspensions, punch mini-grafting and suction epidermal grafting (Korobko, 2012). I will be

describing these treatments to give a background as to what therapies are currently available however; these treatments are not very effective, which is why my research is needed to improve upon them (Plensdorf *et al.*, 2017).

It is important to assess a person's psychological profile before treatment because as of yet no complete cure exists and the results of treatment vary depending on the individual, often proving to be unsatisfactory (Ezzedine *et al.*, 2015). The face shows the best response to vitiligo treatment whilst acral lesions show poor response. It must also be noted that treatment shows better results in lesions that have developed recently rather than older ones (Ezzedine *et al.*, 2015). The term 'Leucoderma' is used to give a name to disorders where the skin becomes white in a place which in some cases is the result of melanin not being present in epidermis (Olsson and Juhlin, 2002) (Navneet *et al*., 2012).

2.11.1 Phototherapies

One of the first line treatments for stimulating melanocyte regeneration in vitiligo is phototherapy; this is of special importance in patients with widespread disease (Anbar *et al.*, 2019). The exact mechanism of how this occurs is unknown but is probably because it induces both/either melanocyte stem cell differentiation and proliferation (Rashighi and Harris, 2017). One such key hormone that does this is alpha-melanocyte stimulating hormone. One drug that also serves this purpose is Afamelanotide which is a synthetic analogue of alpha-MSH. It is thought that Afamelanotide might mitigate photosensitivity and as such it might improve the outcomes of phototherapy in treating vitiligo (Fabrikant *et al.*, 2013). Clinical trials have found that combination therapy of NB-UVB and Afamelanotide led to better results in comparison to NB-UVB monotherapy and showed heightened response in darker skin types (Fitzpatrick skin

type IV to VI) however we are still unaware if Afamelanotide monotherapy (Rashighi and Harris, 2017) would produce favourable outcomes for vitiligo treatment.

2.11.2 Medical therapies and other treatment options

The options for medical treatment of vitiligo involve using immune-modulating drugs on patients such as vitamin supplements (especially vitamin B12 and folic acid), azathioprine, cyclophosphate, levamisole and systemic corticosteroids (Paul, 2011). The British Association of Dermatologists guidelines make recommendations for the management of vitiligo using narrow band ultraviolet B, tacrolimus and topical steroids (Kim *et al.*, 2012). The treatment options for vitiligo were reviewed with each of their advantages and disadvantages being clearly outlined (Steiner *et al.*, 2004) (Yazdani *et al.*, 2014). Below I will list each of the proposed therapies for adults and describe their advantages and disadvantages as set out in the British Association of Dermatology guidelines.

Topical potent or highly potent corticosteroids:

These can be used up to 2 months but it must be considered that a common side effect is skin atrophy

Depigmentation with p-(benzyloxy)phenol:

This is only suggested to be used where depigmentation is extensive and covers more than 50% of the body.

Systemic treatment:

Oral dexamethasone can arrest vitiligo progression but is not recommended as it is associated with a very high risk of causing side effects.

Phototherapy:

Due to side effects this should only be used when other traditional treatments fail.

Surgical treatments:

This should only be used in cosmetically sensitive sites when no lesions have appeared for at least 12 months.

In the treatment of vitiligo medical therapies should always be tried first and if this fails then the surgical option should be considered. In cases where vitiligo affects more than 20% of the body surface, UV light is thought to be the 'gold standard' for treating this. However, the limitations of this treatment are that the repigmenting process is slow and regular sessions of phototherapy are needed. This treatment usually displays good tolerance in children and adults however exposure to UV does carry with it an increased risk of developing cancer (Tippisetty *et al.*, 2013).

Medical treatments are much more acceptable to the patient than surgical therapies as they are much less invasive and they tend to be cheaper however when medical treatments fail there is not normally any other option. In my PhD project I have taken a commonly used surgical treatment, more specifically the autologous cell suspension transplantation technique, and attempted to modify it to improve its clinical outcomes. The use of autologous cells is preferable, as the body tends not to reject its own cells and I will be improving the technique by modifying the melanocyte/ keratinocyte ratio before replanting the cells back on the body rather than just extracting and transplanting the suspension without any modifications. The reason for modifying the melanocyte/keratinocyte ratio is that it has been found that melanocytes function much better in the presence of keratinocytes. This can be further improved if an optimal melanocyte/keratinocyte ratio can be found, which is the basis of my project.

A review that used narrow-band ultraviolet B (NB-UVB) in combination with other therapies (excluding surgical grafts and transplants) was published Yazdani *et al*. (2014). Several databases were searched with review and non-English articles being excluded. Included in the paper were the results of original investigations. In total 39 studies were identified for investigation in this paper. It is thought that there are two stages to repigmentation via NB-UVB. The first step is where inactive melanocyte stem cells found in hair follicles (specifically the outer root sheath) and perilesional skin have their proliferation and migration activated by light. It is important to note however that it is difficult to treat lesions on the distal extremities and bony prominences with this technique because hair follicles in these areas are sparse. The second step is where the release from keratinocytes of melanocyte growth factors, such as basic fibroblast growth factor and endothelin-1, induces the proliferation of melanocyte growth factors. This observation is supported by the fact that vitiliginous lesions have lower bFGF levels. Phosphorylated focal adhesion kinase and matrix metalloproteinase expression stimulate melanocyte migration.

Additionally, to this NB-UVB also serves to modulate the immune response in vitiligo since it works by inducing the production of interleukin-10 in the epidermis which then promotes differentiation of T regulatory cells and in turn suppresses the auto-reactive T cells. Direct T-lymphocyte apoptosis can be induced by NB-UVB. It is stated that the first-line treatments for vitiligo tend to involve topical treatments such as corticosteroids and calcineurin inhibitors. The areas that show the best response to this treatment are the face and extremities (Wong and Lin, 2013).

It is the case that there have been multiple investigations of the combination of tacrolimus 0.1% ointment and pimecrolimus 1% cream with NB- UVB (Yazdani *et al.*, 2014) (Wong and Lin,

2013). These studies were carried out for a duration of 3 to 12 months with the smallest study consisting of 3 participants and the largest 110. The results of one investigation (Yazdani *et al.*, 2014) show that there is clearly a better result seen when NB-UVB is combined with tacrolimus/pimecrolimus than when compared to a placebo. As much as 40% more subjects showed more than 50% repigmentation when NB-UVB pimecrolimus was used than with the control. Tacrolimus is an immunosuppressive drug that is usually used after allogeneic organ transplant to lower the risk of organ rejection. The fact that the use of tacrolimus has such an effect in improving the outcome of vitiligo treatment by NB-UVB suggests that the development of vitiligo is associated with an autoimmune reaction.

Limitations in conducting studies into the treatment of vitiligo include trying to establish an effective control arm. The two ways to do this are to utilise either an intra-individual or a parallel group comparison group design but both have challenges of their own. The limitations of intra-individual studies are that participants may find it difficult to meet the requirements such as administering topical therapies on only half of the body during the long months of treatment time. The improvement in quality of life is also difficult to assess and accurately identifying how much is due to combination therapy is challenging when only half of the body receives the treatment. On the contrary, parallel group comparisons may be difficult to accurately analyse because different patients do not share the same degree of melanocyte loss or they do not have the same prognostic factors for a treatment result. This worry can be dealt with by using large sample sizes and appropriate randomisation (Yazdani *et al.*, 2014).

One conclusion that has been reached (Yazdani *et al.*, 2014) is that combining NB-UVB with other treatments potentially shortens the length of time that is needed by patients for phototherapy and reduces their total radiation exposure. Much more investigation is needed to

find which combination regimes are best, to find out if clinical outcomes last and if there are any additional effects uncovered by long-term follow ups (Wong and Lin, 2013).

Topical corticosteroids have been found to be effective for use as monotherapy in vitiligo, especially in the areas of the head and neck but they do cause problems when used in these areas since the skin of these areas is amongst the thinnest of the body. Topical corticosteroids are commonly used for treating a variety of skin disorders, such as eczema and psoriasis, as they have anti-inflammatory properties. Fruitful use of corticosteroids as a monotherapy for vitiligo treatment once again adds more evidence to the theory that it is an autoimmune condition. Steroids cause skin atrophy so the use of calcineurin inhibitors is recommended as an alternative to this treatment as they do not cause this side effect. The efficacy of calcineurin inhibitors as a treatment for vitiligo was assessed and described (Wong and Lin, 2013) and in this, 23 studies concerning this treatment were reviewed. The number of studies assessed in this paper was substantial and had a participant number involvement ranging from eight to 110 people as well as a study duration of 8 weeks to 52 weeks. This review is also reliable since a double blind study was carried out where the use of the therapy was compared to a placebo and found to be superior (Leeuw *et al.*, 2010) (Wong and Lin, 2013).

Skin colour is also a factor in treatment success with vitiligo on the face of darker skin individuals showing superior treatment outcomes to their lighter skinned counterparts. The type of vitiligo being treated by steroids is also a consideration with better responses associated with patients with vulgar disease forms than those that have a localised or generalised illness. The final factor that must be considered is the duration of the disease with more recent lesions showing a better response.

The second line treatments involve phototherapy combined with steroid treatment (Haines et al., 2017). Light based treatments generally involve psoralen photochemotherapy (PUVAsol and PUVA) and NB-UVB, which are normally delivered to the whole body and a targeted phototherapy system which includes excimer laser and lamp (Paul, 2011) (Shenoi and Prabhu, 2014). There are three ways that Psoralens can be administered which are oral, topical or a combination of both. The best effects are witnessed with oral administration, with this also having the least side effects. In one study the therapeutic effect of combining mini-oral pulse steroids with Nb-UVB was compared to using the mini-oral pulse steroids on their own and Nb-UVB on their own as vitiligo treatments (El Mofty *et al.*, 2016). The study consisted of 45 patients who were randomly assigned into one of three groups.

The three groups were:

Group A: These subjects were treated with Nb-UVB in combination with mini-oral pulse (MOP) steroids

Group B: These subjects were treated with MOP steroids only

Group C: These patients were treated with only Nb-UVB

Allocating the patients randomly to each group is an attempt to remove any bias that might occur in the results (ElMofty *et al.*, 2016). Randomisation was done using the sealed envelope method. The patients had to satisfy the inclusion and exclusion criteria, these were:

1. Inclusion criteria:

- Age: over 15 years.
- Type of vitiligo: vulgaris.
- Stable disease with no new lesions for the past year.

- No systemic or topical treatment for vitiligo received for the past month.
- Extent: disease covers at least 10% of total body surface area.

2. Exclusion criteria:

- Contraindications to steroids.
- Pregnancy and lactation

3. Workup:

- Full personal and family history were taken

The study was conducted at the same dermatology and outpatient clinic and phototherapy unit at Kasr El-Aini hospital, Cairo University between September 2009 and February 2012. When the patients were divided into three groups, differences in age, sex, duration of disease, family history or relationship with stress showed no significance.

Table 5: A table showing a comparison of clinical data of patients during both the treatment and the follow-up periods in 3 different groups. This table illustrates that the results of group A and B are of a significant difference (El Mofty *et al.*, 2016).

		Group A (Nb-U.V.B and Steroids) n=15	Group B (Steroids only) n=15	Group C (Nb-U.V.B alone) n=15	p-value
VASI	Before therapy: Mean +SD	20.8+/-10.6	14 +/- 7.5	18.2 +/- 10.7	0.186
	After therapy: Mean +/- SD	8.2 +/- 6.5	13.6 +/- 7.6	15.3 +/- 8.9	0.041
Patients showing improvement	Number	15	5	15	<0.001
	%	100%	33.3%	100%	

Depigmentation of lesions	Number of patients	1	1	1	0.1
Appearance of new lesions	Number of patients	2	6	1	0.041

Patients were evaluated using VASI before and after three months of therapy. The VASI scores in the above table illustrates that those treated with Nb-UVB with steroids and Nb-UVB alone showed significant clinical improvement than those treated with steroids alone (El Mofty *et al.*, 2016) **(Table 5**).

There is no absolute definition of what the optimum duration of phototherapy should be with practice varying widely (Ezzedine *et al.*, 2015). Narrowband UVB phototherapy should only be suggested when patients couldn't be successfully managed with treatments that are more conservative or when the patient has widespread or localised vitiligo that impacts quality of life significantly (Leeuw *et al.*, 2010). When repigmentation does not happen within the first three treatment months irradiation is usually stopped however this is not to say that cases of repigmentation have not been recorded later on. An oral minipulse of betamethasone or dexamethasone in moderate dose can be used for 3-6 months to stop the disease progression when the vitiligo is of type that is fast spreading (Ezzedine *et al.*, 2015).

In patients who suffer with segmental vitiligo the lack of success of treatment with UV light and corticosteroids compared to those with generalised vitiligo (Steiner *et al*., 2004) (Van Geel *et al.*, 2012) can be explained by the fact that in this condition the hair follicles do not have any pigment cells and there is no involvement of an autoimmune component (Olsson and Juhlin, 2002) (Van Geel *et al.*, 2012). The success of this therapy is dependent on where on the body the lesion can be found with those on the face showing the best response and the distal areas

of the extremities and genitalia showing little response. An explanation for these areas not having successful treatment outcomes is that they do not have any hair follicles (Steiner *et al.*, 2004). For estimating the success of treatment factors that must be taken into consideration are the age and skin colour of the individual with better responses being in the young and those with dark skin.

Combination therapies tend to show better outcomes compared to monotherapy which is supported by a study search by Ezzedine. 29 studies that were similar in nature to Ezzedine where identified and all supported the same conclusion. These supporting studies did have their own limitations since the statistical power used was not adequate; only patients that have non-segmental or generalised vitiligo were included. These vitiligo types account for 85% of all vitiligo cases and they affect both body sides in adults (Ezzedine *et al.*, 2016).

Only 42% (40 out of 96) of studies that were referenced (Ezzedine *et al*, 2016) made use of an active control where the intervention was applied simultaneously on both sides of a specific body area in the same patient. It was therefore very difficult to identify which of the therapies used caused the adverse effect when combined therapies were used in the study. Another limitation is that none of the studies carried out an assessment of long-term repigmentation (e.g. follow up at two years) as a secondary outcome.

Current practice guidelines from the European Dermatology Forum (Taieb *et al.*, 2013) and the Vitiligo Japanese Task Force (Oiso *et al.*, 2013) support other findings (Ezzedine *et al.*, 2016). The recommendation of all these studies is that a combination of therapies is used with the primary treatment being light. The European Dermatology Forum recommends the use of narrowband UV-B excimer light in combination with topical steroids or with topical immunomodulators. The advice of the Japanese task force differs in that its recommendation

is for UV-B photochemotherapy in combination with UV-A and topical steroids or topical vitamin analogues. It can therefore be concluded that the recommendations of both organisations are in fact consistent with the findings of (Ezzedine et al., 2016). The use of combined therapies with narrowband light is a primary treatment and if this fails then the option of surgical treatment will be considered. My PhD study is an extension of this investigation. I will be studying a surgical intervention however; UV light will be used to modulate the melanocyte/ keratinocyte ratio in culture.

2.11.3 Heliotherapy

Heliotherapy can be described as being the use of natural sunlight to treat skin conditions such as vitiligo (Grzybowski *et al.*, 2016). The advantages of this treatment are that it is cheap and easily available. However, the disadvantages of this therapy are that there is a risk of sunburn, premature skin aging and skin cancer. Currently a thorough literature search has not identified any studies into the effects of heliotherapy when used as a treatment for vitiligo (Jarrett and Scragg, 2017).

2.11.4 Khellin

Khellin, a furano chrome, is extracted from the Mediterranean plant Khella. This extract has several properties such as that it is vasodilative, spasmolytic, can cause melanocyte proliferation and can inhibit the activity of fibroblasts. Most importantly in the context of my doctoral work Khellin has the ability to be a photosensitizer since its chemical structure is similar to that of the psoralen family. Khellin has the same photobiological and phototherapeutic effects as the psoralens but it doesn't have the same side effects of being phototoxic or carcinogenic (Beyazit et al., 2019). Such unwanted side effects are witnessed during treatment when ultraviolet A radiation (PUVA) is combined with psoralen.

Although as mentioned above Khellin is a good treatment for vitiligo its use should be done with caution. Research into the exact benefits of Khellin is still inconclusive and at times contradictions have been found (Leeuw *et al.*, 2011). Khellins mechanism of action is to directly enhance the proliferation and melanogenesis in isolated melanocytes. The first reported uses of Khellin in dermatology as a treatment for vitiligo were in 1982 when its use was combined with natural sunlight. Attempts to avoid systemic side effects meant that it was primarily used as an oral and then as a topical treatment (Fenniche *et al.*, 2018).

To improve Khellins action and diffusion different vehicles of delivery have been used such as: oil/water emulsions, microemulsions, cyclodextrin-containing gels, water/2-propanol/propylene glycol, and liposomes. Establishing the correct form to use is important because it can cause a substantial difference in the clinical results that are seen for vitiligo treatment (Fenniche *et al.*, 2018).

2.11.5 Topical, systemic and intralesional steroids

Steroids are an effective treatment with vitiligo but they have a major drawback in that they are associated with serious side effects which has resulted in a search for alternative therapies (Kumar *et al.*, 2004) (El Mofty *et al.*, 2016). Topical calcipotriol is one of several aetiology based treatments for vitiligo. It is a vitamin D derivative and acts in a similar manner to other derivatives of the same type in that it increases the intracellular calcium concentration via the hydrolysis of phosphatidyl inositol phosphate which results in the production of diacylglycerol and inositol triphosphate. The release of intracellular calcium stores that follows is essential because proliferation and differentiation of melanocytes, along with other cellular functions, are regulated by intracellular concentrations of calcium. The basis for the use of this treatment

is that a disturbed calcium homeostasis has been observed in vitiliginous skin. However, there are limitations to this treatment since studies have shown that for the treatment to be at optimal efficiency it must be used in conjunction with PUVA or narrow band UVB but if we are to assess its real efficiency then more research is needed. This treatment is not perfect since side effects have been observed in 10-34% of all cases but these are usually not very severe (Hameed *et al.*, 2005).

Ex vivo studies have identified that catalase enables the establishment of melanocyte cultures from non-segmental vitiligo cultures or to re-establish their function after exposure to hydrogen peroxide. This observation has served as the basis of the idea for using topical catalase in the treatment of vitiligo. A clinical study (Hameed *et al.*, 2005) made use of catalase over a period of 36 months with a sample of 33 patients. No explanation is given in the paper why 33 patients were chosen to be included in the study, we do not know if this is because only 33 patients were available or it was because a power calculation was conducted and this was the figure that was reached. Patients affected by both focal, segmental and vulgaris were included in the study and the results varied accordingly. In focal vitiligo all the cases studies exhibited a repigmentation in the range of 90-100%. Segmental vitiligo on the other hand did show a partial response but this happened slowly.

In vitiligo vulgaris the response was good to moderate but nothing was observed on the feet and toes. In Hameed et al. (2005) the participants applied a mixture of pseudocatalase and calcium chloride twice a day. Following this the entire body was exposed to erythemogenic UVB twice a week. Within 2-4 months it was found that excellent repigmentation occurred especially on the hands and feet. This study did identify that the success of the therapy was dependent upon the type of vitiligo since, for example, focal vitiligo showed 90-100%

repigmentation in all cases but the response in cases of segmental vitiligo was partial and extremely slow. The cases of vitiligo vulgaris showed moderate response but no repigmentation on the fingers and feet. Therefore, when we are considering what treatment to use it is important to consider the type of disease that is present in the observed patient.

A limitation to the study quoted (Hameed *et al.*, 2005) is that after 4 months the results of the treatment were no longer available. It must be emphasized that although the quoted (Hameed *et al.*) study is 12 years old and although this study is not recent at all it does identify the points that I am trying to make about how aetiology based treatments are useful.

2.11.6 Depigmentation

Camouflage treatments for vitiligo include temporary make up, such as creams and lotions whilst depigmentation therapy involves the removal of pigmented skin. Where the depigmentation covers more than 60% to 80% of the total body area (AlGhamdi and Kumar, 2011) it might in fact be more straightforward to remove pigment from all remaining skin rather than attempt to re-pigment the affected areas. Depigmentation occurs in the opposite way to pigmentation therapies by removing any remaining pigment in the skin rather than regaining it. The article states that there is a difference in opinion among practitioners as to what percentage of body coverage qualifies for depigmentation therapies. However (Steiner *et al.*, 2004) before any adult is treated via the depigmentation process, it is important that they are aware that the treatment involving chemical depigmenting products will be a long-term process. They must also be aware that this treatment will significantly change their physiognomy as well as creating requirements for extra precaution to solar exposure for the remainder of their lives. The choices that are available for depigmenting agents are listed in **Table 6**.

Table 6: A list of established and potential depigmenting agents (AlGhamdi and Kumar, 2011).

Established agents	Potential agents
Monobenzyl ether of hydroquinone	Imatinib
Monomethyl ether of hydroquinone	Imiquimod
88% phenol solution	Diphencyprone
Laser	Catechol
Cryotherapy	Hydroquinone bis (2-hydroxyethyl) ether
	4-Ethoxyphenol

The option of using depigmenting treatment should only be considered when more than 50% of that person's body is affected by vitiligo or when it is extremely disfiguring to the affected person. Bleaching of the skin can be achieved by several methods including: cryotherapy, laser treatment, monobenzone ethyl ester or 4-methoxyphenol (Ezzedine *et al.*, 2015). When medical treatments are not successful it is often the case that surgical therapies will be attempted as described below.

2.11.7 Surgical therapies

It has been seen from the literature that clinical therapies for vitiligo do not always work well which might occur for several reasons, for example this is not the right method for creating repigmentation or because there were not enough melanocytes in the lesion. This has led to the development of treatments that involve either a graft or melanocyte transplant (Razmi *et al.*, 2018). However, this form of treatment is only successful when the disease is stable. One judge of stability is whether the lesion has increased in size at all in the last two years. It is

preferred that patients recruited for therapy are emotionally stable adults who are in full knowledge of the risks of any surgical treatment such as scars or incomplete repigmentation (Steiner *et al.*, 2004).

There are only a few patients that would be considered to be suitable for surgical therapies as surgery is only considered an option for those with segmental vitiligo or with stable disease for at least one year after there has been a documented non-response by that patient to medical therapies (Ezzedine *et al.*, 2015). As mentioned previously, it is recommended that the disease has been inactive for a period of 6 months to 2 years (DeLeeuw *et al.*, 2011). When the vitiligo is stable (e.g. Segmental unilateral vitiligo) it is usually the case that the outcome of transplantation is very good and for some circumstances is the only treatment available that provides effective results. It is even possible for the same individual to have at the same time one area of skin with active vitiligo and another area that is inactive or regressive. As such, it is difficult to predict/assess the stability of the diseases in an individual (DeLeeuw *et al.*, 2011). Another difficulty in treatment of vitiligo is that (DeLeeuw *et al.*, 2011) no trustworthy tests are available to reliably assess and predict melanocyte transplantation outcomes in vitiligo patients.

The different treatments available for the treatment of vitiligo were evaluated (Olsson and Juhlin, 2002). This paper took several surgical treatments that will be critiqued later and followed up patients that had been treated by them after 1-7 years by questionnaires and clinical examinations. It found that the success of the treatment was dependent on the stability of the vitiligo beforehand. The limitation of this paper is that the follow up was performed on patients from Scandinavia between 1993 and 1999 meaning that differences between races and ethnicities are not accounted for and cannot be investigated further. The treatment was

determined to be a success depending upon the amount of re-pigmentation that occurred. For example, treatment was deemed successful when pigmentation levels of 65-100% were achieved whilst levels below 65% (0-64%) were judged to be a treatment failure. The study described in the aforementioned paper found that the age of onset of the disease was not important but the age of the patient when the treatment took place was important in determining the procedure's success.

The first stage of treatment of this disease usually involves the stimulation of pigment production in affected skin areas but after stimulation by ultraviolet light or dermabrasion they became active (Bagherani, 2016). The various treatment options proposed can be described as depigmentation, therapy, camouflage, surgical treatments, light-based treatment or medical treatment (Adotama *et al.*, 2015). However not all these treatments are effective for every person and success often depends on the area treated therefore this must be taken into consideration before the appropriate treatment is chosen.

2.11.8 Blister technique

The epidermal blister roof transplantation technique was created to repigment stable but refractory areas of vitiligo. In two studies (Iwanowski *et al.*, 2018) (DeLeeuw *et al.*, 2011) it was examined how combining the blister roof transplantation technique with UV light and khellin in liposomes improved outcomes for treating recalcitrant vitiligo by this method. After this it was important to gauge how satisfied patients were with the treatment (DeLeeuw *et al.*, 2011).

For the epidermal blister roof transplantation technique, the donor sites for melanocytes are the blister roofs themselves. There are three stages to the transplantation of blisters which are:

creation of the blisters, preparation of the recipient site and transplantation. Blisters were created by a suction device connected to a rectangular plastic block (Iwanowski *et al.*, 2018). The way the plastic block acts is to divide negative pressure across 28 holes, each of these have a diameter of 5 mm. This is then applied to a skin area where the pigmentation is normal (ideally the patient's upper arm). To obtain manageable blisters upon application of 300 mm mercury takes approximately one to one and a half hours but the procedure itself is painless and topical anaesthesia is not required (DeLeeuw *et al.*, 2011).

In the second stage which involves the preparation of the recipient area superficial erosions that have a diameter of 5-6 mm are made by ablation with an Erbium-YAG laser. The laser ablation is continued until pin-point haemorrhages appear as the appearance of these indicates that the ablation has reached the papillary dermis as required. The stage two procedures require topical anaesthesia with lidocaine 1-2% (in the absence of adrenalin) (DeLeeuw *et al.*, 2011).

The third stage involves the removal of the blister roofs with scissors and smooth forceps and the delivery of them to damaged sites in the recipient area (Dellatorre *et al.*, 2017). To protect transplants in the recipient area silicone sheeting was applied and this was covered with standard gauze which was fixed via an adhesive bandage to prevent it moving. Stitches were not used in this procedure because the blister roof grafts are too delicate for this to be possible. After a week the bandages were removed using water to enable the silicone sheets to be detached from the grafts as removal is far easier when the silicone sheets are wet. After this the grafts were protected for another week by a new bandage with paraffin gauze and standard gauze after which bandages were no longer used (DeLeeuw *et al.*, 2011).

The referenced (DeLeeuw *et al.*, 2011) study consisted of 19 patients. The study included 12 women and 7 men with ages ranging from 25-68 years. When analysing the results it should be remembered that that sex and age as well as area being treated (some areas of the body respond better to treatment than others) could have an impact on treatment outcomes however as described later in this section the inclusion/exclusion criteria do try to consider these conditions. It is also important to make sure that the recipient does not have any other co-morbid conditions, such as autoimmune disease, which could seriously hinder the success of the treatment. At the end of the procedure, the participants were interviewed and 75% of patients (12 out of 16) responded that they were satisfied with the results of the procedure and that its advantages outweigh its disadvantages whilst all the participants said that they would recommend it to other vitiligo patients. The reason that the participants' satisfaction was measured out of 16 patients rather than 19 was because 3 patients did not fill out the questionnaire at the end of the study. Since the study is so small the fact that three patients did not respond could be potentially significant. The loss of 3 patients from a total sample size of 19 is significant since this equates to a drop out of 16%. It might be that the inclusion and exclusion criteria for the (DeLeeuw *et al.*, 2011) study are so specific that only 19 patients were available in the time period that was studied.

For this study (DeLeeuw *et al.*, 2011) the inclusion criteria were that the vitiligo lesions should be stable and refractory despite having been treated with KLUV for a minimum of 1 year. The lesions that were studied were situated on: the hands (11 patients), the arms (four patients), the neck (three patients), the shoulders (one patient), the chest (one patient), the wrist (one patient), and the face (one patient). Only one patient with segmental vitiligo (on the neck) was included.

The exclusion criteria (DeLeeuw *et al.*, 2011) were pregnancy, lactation, history of allergy to sunlight, photo-induced diseases, and medication with drugs known to have potency for light

toxicity or light sensitization, phenylketonuria, impaired hepatic and renal functions, malignant skin diseases, a history of exposure to arsenates and ionizing radiation. This paper does not justify the reason for the study size that was used and there was no evidence of a power calculation or any statistical test being used.

2.11.9 Epidermal/dermal grafts

Cellular and tissue grafts are a type of surgical treatment for vitiligo but the advantages and disadvantages of these should be seriously considered before choosing this option. Tissue graft techniques include suction blister epidermal grafting (SBEG), punch grafting (PSG) and split thickness grafting (STG). STG has benefits including that large areas can be treated in a fairly short window of time but the limitations are colour mismatching, donor site scarring, milia and an unwanted appearance at the recipient area (Majid, 2013).

2.11.10 Non-cultured and cultured autologous cells

A novel technique for vitiligo treatment is the replenishment of melanocytes by autologous melanocytes. The advancement of culturing melanocytes means that there is now a possibility for large recipient areas to be treated with a relatively much smaller sample of skin. Various techniques are described in more detail below.

<u>Autologous cultured melanocytes</u>

Pigmented buttock skin is used as the source for a superficial shave biopsy (Verma *et al.*, 2014). Melanocytes are separated from the epidermis, cultured in M2 medium (PromoCell, Heidelberg, Germany) and transplanted. Czajkowski *et al.* (2008) state that a combination of autologous melanocyte transplantation with PUVA therapy resulted in repigmentation in 100% of recipient sites however it must be remembered that acral skin (skin of the extremities) is

extremely difficult to repigment. The strength of the paper (Pandya *et al.*, 2005) is that it gives a clear methodology for the preparation of autologous culture from melanocytes. The methodology is outlined systematically and gives an account of the reagents, equipment and processes required. This paper then goes on to describe the various factors that contribute to the success and failure of this technique such as body area being treated and the type of skin sample that cells are extracted from (e.g. punch biopsy versus superficial split thickness thin skin grafts).

For a satisfactory cosmetic result to be achieved correct patient selection is an important contributing factor (Majid, 2013). Other factors that must be considered are: patient age, vitiligo site, tendency for keloids along with crucially vitiligo stability. The term stable vitiligo refers to the disease process being inactive in the patient. When the vitiligo is stable it can be seen clinically as occurrence of no new lesions becoming present, with no spread of existing vitiligo lesions, and no Koebner's phenomenon. For stable vitiligo the transfer of autologous melanocytes is a simple and effective technique for quick production of homogeneous pigmentation. Cultured melanocytes are at a disadvantage over non-cultured melanocyte techniques since they are time consuming and labour intensive to create but the benefits of them are that they can be used to cover an area that is over 100 times the donor area making them suitable to cover large areas of body surfaces. An advantage of this technique is that it only requires very little donor skin (Verma *et al.*, 2014). Verma et al. (2014) is a study where melanocytes were harvested from a donor split thickness graft as an autologous melanocyte rich suspension. Cultured or non-cultured melanocytes were transplanted onto a recipient site that had been superficially debraded. 100 patches of vitiligo in patients were then allocated into any of these two groups to receive either of these interventions.

For dermatologists with the expertise and facilities to create autologous cultured melanocytes this offers a treatment for even the most difficult cases of vitiligo. However, the limitation of this technique is that most clinicians do not have the ability to maintain it (Czajkowski *et al.*, 2008). A critical analysis of this technique includes highlighting its limitations which are not only due to the lack of clinician skills but also its high cost and requirement of 3 weeks to obtain the cultured melanocytes (Fioramonti *et al.*, 2012). The limitations of using autologous cultured melanocytes mean that non-cultured melanocyte transfer should always be tried first when the vitiligo is not so widespread.

The effectiveness of using cultured versus non-cultured melanocytes in the treatment of vitiliginous areas was compared (Verma *et al.*, 2014). The paper found that using autologous melanocytes when compared to cultured melanocytes produced a much better result with the non-cultured technique showing an excellent response in 62.17% of cases and the culture technique showing an excellent response in 52% of cases. A limitation of this paper is that it does not give a definition of what an excellent response is or what it means. In total 100 patches of vitiligo were studied over a period of one year. This is a relatively large sample size and enough time was given for the results of the intervention to present themselves. To remove any anomalous results only patients who have had stable vitiligo for at least one year were included in the trial. This study's results are only three years old, meaning that they are current with the latest available techniques. To remove any bias the selection of whether patients should be recruited to the group for cultured or non-cultured melanocyte treatment was completely random. No blinding was done in this study however this should not have any impact on the results that are obtained. To remove any sex bias that might occur almost equal numbers of men and women were studied in each group.

The benefits of using autologous melanocytes over existing dermato-surgical procedures, such as punch grafting and split thickness skin grafting, are that the other techniques have various deficiencies such as cobblestoning, pigment mismatch and patient discomfort (Verma *et al.*, 2014). The (Verma *et al.*, 2014) paper is of relevance to my study because it discusses implanting both melanocytes and keratinocytes which then get entrapped within the healing site, multiply and repigment the depigmented site. However, what is not performed in the (Verma *et al.*, 2014) study is that the melanocyte/keratinocyte ratio is not manipulated prior to implantation; it is likely that doing so would improve the results seen although it might make the procedure more complicated. Keratinocytes secrete factors that improve melanocyte growth and function so implanting both cells types together rather than just melanocytes alone should improve the results that are seen.

2.11.11 Additional surgical therapies

Ultrathin epidermal sheets

A Zimmer – air dermatome (manufactured by Zimmer, Dover, OH, USA) was used to harvest ultrathin donor skin from normal gluteal skin that had been subject to local anaesthesia. This was then transplanted to the site where it is required (Olsson and Juhlin, 2002).

Basal layer cell suspension

Pigmented buttock skin was used as the source for a superficial shave biopsy (approximate size: 2 x 5cm^2). EMLA cream (a local anaesthetic cream) (Manufactured by Astra, Sodertalje, Sweden) and lignocaine (lidocaine) injection were used as local anaesthetic for the recipient area. The cell pellet underwent washing and centrifugation before its re-suspension in a small volume of s-minimal essential medium (S-MEM Life Technologies Ltd., Gaithersburg, MD, U.S.A). This was directly transferred to dermabraded skin from a test tube via a pipette before

Mepitel (silicon netting, moistened compresses) and Tegaderm semi-occlusive film were used to cover it.

In (Olsson and Juhlin, 2002), patients were given a questionnaire to complete as follow up as well as being offered a complimentary follow up consultation. In this study (Olsson and Juhlin, 2002) leucoderma patients that had been treated with autologous cultured melanocyte transplants, ultrathin epidermal sheets and basal cell layer suspension underwent long term follow up. Statistical methods were used to analyse this data which included Kruskal-Wallis test for group comparison and Spearman's rank correlation test. Re-pigmentation was assessed numerically with 65-100% pigmentation being judged successful and 0-64% as failure. This paper also gives a clear description of the methodology used to assess the effectiveness of the various techniques at follow up. The Olsson and Juhlin study was published in 2002 which means that it is at least 15 years old. It is possible that this paper is no longer entirely relevant as it is likely that since its publication new techniques or modifications to existing ones would have been developed. However, a literature search has confirmed that these techniques are still relevant in today's world (Pandya *et al.*, 2005).

Micropigmentation

Micropigmentation is a type of tattooing, a useful method for concealing vitiligo lesions, where small inert granules of pigment (approximately 6mm in size) are introduced into the superficial dermis (Hossain et al., 2016). These granules are made up of inert, nontoxic, insoluble chemicals which become permanently embedded into the dermis via mononuclear cells and collagen fibres when inserted into it. The pigment that is used for medical micropigmentation is mostly composed of iron oxide. When injected this has a light to dark brown colour in the skin and it is normally delivered via an electrical tattooing machine. This machine's active

component is an array of several 25-G needles with a penetration depth of about 1 to 2 mm and they can attain a speed of up to 9,500 strokes per minute. The correct colour dye is achieved by creating a paste or colloidal suspension mixing it before injection with various dyes, water, saline, or alcohol (Patel *et al.*, 2012).

The operator's skill can be a crucial determinant of the cosmetic outcome since a criterion for achieving a permanent uniform pigmentation is how deep the pigment granules are injected into the skin. Ideally insertion depth should be about 1.5mm and it should be done between the upper and mid-papillary dermis. When granules aren't injected to a sufficient depth, they tend to be expelled quickly after the procedure is performed but conversely if the injection is too deep then the pigment will be removed by macrophages (Patel *et al.*, 2012).

Other factors contributing to cosmetic appearance include: how thick the pigment paste is, how many needles are used in the tattooing machine, and how thick and elastic the recipient's skin is. Micropigmentation is normally adequately received by the body; the side effects are minimal. Side effects that can occur in the few days following micropigmentation procedure include: localized infection, oedema, bleeding, and crusting. Rare side effects that have been experienced are: granuloma formation, allergic reactions, keloid formation, and reactivation of herpes simplex virus (Patel *et al.*, 2012). Another technique that can be used is permanent dermal micropigmentation. This works in a similar way to eyelid tattooing. This technique shows few complications but there is a risk of infection by the herpes simplex virus in those individuals that are predisposed to it (Steiner *et al.*, 2004).

2.12 The use of therapies based on autologous co-cultured cells

Due to social and political pressure, the use of animals for research and clinical trials is now decreasing. Therefore, there is a necessity for versatile and translatable skin models to be developed so that the biology of pigmentation and the pathophysiology of the skin disease vitiligo can be studied. It is the case that many models have been developed however *in vitro* and *in vivo* models are often a poor representative of the clinical scenario. The use of *in vitro* models is at an advantage since they allow the analysis of environmental changes, substrate and dose response interactions in a standard fashion (Kumar *et al.*, 2012).

Artificial models of skin pigmentation are needed so that research can be performed on various pigment skin diseases and so that the regulation of pigmentation can be understood. If an artificial skin could be created this could be used to test chemicals and skin care products for any adverse effects. However, skin samples for chemical/cosmetic testing will not reveal all side effects as it's an artificially isolated part of a complex organism and as such may not mirror the biology as closely as is needed (Kumar *et al.*, 2012).

At present, the demand for artificial skin is not satisfied with experiments involving artificial skin being very expensive due to its limited availability. There have been many melanocyte/skin equivalent models used to assess the potential efficacy of melanogenic compounds in the regulation of pigmentation. Variation in such results can either be attributed to the use of different cell lines or due to monoculture of melanocytes/keratinocytes since in *vivo* melanocytes are usually present with keratinocytes (Kumar *et al.*, 2012).

The use of co-culture models is very important since they provide a better representative of the in vivo situation than a mono-culture or animal model does (Goers *et al.*, 2014) (Lei *et al.*,

2002) (Miki *et al.*, 2011). Experimenting with compounds on only melanocytes does not represent the conditions that are found in skin. Keratinocytes secrete many factors which act on melanocytes (Kumar *et al.*, 2012). Studies using models of in vivo systems have confirmed that human keratinocytes produce factors such as α-MSH, nerve growth factor and ACTH/ACTH fragments. The role of these factors is regulation of melanogenesis and/or dendritogenesis of human melanocytes in primary and/or serial cultures (Hirobe, 2014).

The use of co-culture techniques has an array of applications for achieving an understanding of the natural or synthetic interactions that happen between different populations of cells and they are vital in the quest to understanding any type of cell-cell interaction studies. In its most elementary form, there is some degree of contact between two or more different cell types when they are grown together (Goers *et al.*, 2014).

To develop an understanding of the mechanism by which keratinocytes regulate the growth of melanocytes using a co-culture model of these two cells proves to be invaluable. Processes where epidermal keratinocytes influence melanocyte growth include: proliferation, melanogenesis, differentiation and dendritogenesis of epidermal melanocytes. Using serum free media is preferred so that any unidentifiable factors can be eliminated as being a cause for influencing any of the processes being studied (Hirobe, 2014).

Many low complexity co-culture systems are in existence but higher complexity systems are much rarer and beyond certain levels of complexity they are non-existent. The problem with a high complexity system is that it inherently vastly complicates the experimental procedure. Therefore, most co-culture systems involve two populations with co-cultures of more than three distinct cell populations being very rare. However, (Goers *et al.*, 2014) it has

been suggested that co-culture studies with many populations might not actually be advantageous as it becomes more difficult to analyse the data/results. This outcome supports the main aim of synthetic biology which is to design a system that is as complex as necessary to reach the desired goal.

In any co-culture model the cell populations might exhibit similarities, e.g. the same strain with a minor difference in gene expression, or very different from each other e.g. different species. Common sense dictates that similarity of co-culture population's results in a more straightforward establishment of a stable system but many successful examples exist of co-cultures amongst contrasting species (Goers *et al.*, 2014).

In this study using a melanocyte/keratinocyte co-culture model is extremely relevant since it has been observed that both cell types influence each other in several ways. Melanocytes are extremely responsive cell types that modulate their levels of melanogenesis or proliferation in accordance to extrinsic signals and factors that are produced by keratinocytes (Kumar *et al.*, 2012). For example (Hirobe *et al.*, 2014) found that in human skins that have been irradiated with UVB or UVA, the keratinocyte derived factors Endothelin-1 and GMCSF regulate human melanocytes proliferation and melanogenesis/dendritogenesis. It was also found that human keratinocytes produced and released the factors IL-6 and IL-8 which have the action of stimulating dendritogenesis in cultured human melanocytes of the epidermis (Hirobe *et al.*, 2014).

The proliferation of melanocytes in humans has also been found to be regulated by bFGF as well as by stem cell factor being expressed by keratinocytes. When proteinase-activated receptor 2 is stimulated, prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) are released from

human keratinocytes. The effect of all these is the stimulation of dendritogenesis in cultured human epidermal melanocytes via prostaglandin EP1/EP3/FP receptors in a cAMP independent way (Hirobe, 2014).

In a co-culture the amount of contact that occurs between different cell types is dependent on the experimental design for example the different cell populations can either be thoroughly mixed or partially separated. The way in which the co-cultures are separated must be chosen and tailored to how the co-culture will ultimately be used. An example of this is that for some purposes different types of cells should be in direct contact; this is sometimes referred to as a 'mixed culture.' Direct contact such as this is occasionally required in mammalian tissues and some types of eukaryotic cell cultures so that physiological behaviours can be preserved e.g., the melanocyte/keratinocyte co-culture model. For mixed cell cultures different cell populations must be under symmetrical conditions. This is achievable when there are similar requirements amongst the two different cell types (Goers *et al.*, 2014).

When the populations are different but a mixed culture is desired it is necessary to optimise the growth medium so that the best medium for sustenance of the cell populations can be established. Before they are placed in co-culture both the melanocyte and keratinocyte cell populations are grown and established separately (Goers *et al.*, 2014). An additional aim of this study is the development of a co-culture medium that supports the growth of both melanocytes and keratinocytes in a mixed culture.

An important consideration that one must make when designing a co-culture model is that different cell types proliferate at different rates with one type being dominant over another. Therefore, in co-cultures it is often necessary that the population ratio is optimised so

that the culture is stable and one cell type does not eliminate another. In mammalian cultures, it is common for the population ratio to be set to a value that is in resemblance to the natural situation (Goers *et al.*, 2014).

2.12.1 Current methods available to manipulate and measure cell proportions in co-culture

Since keratinocytes produce a lot of growth factors for the proliferation and migration of melanocytes it is advisable that simultaneous transplantation of melanocytes and keratinocytes be attempted to improve the technique of cultured melanocyte transplantation (Lim *et al.*,2014).

Several preliminary experiments were performed where cells were seeded in various keratinocyte to melanocyte ratios of 1:1, 2:1, 5:1 or 10:1 in various medium formulations with differing levels of calcium (Lei *et al.*, 2002). A co-culture can be prepared with the ideal melanocyte to keratinocyte ratio being 1:5 (Kumar *et al.*, 2012). Investigations (Lei et al., 2002) go further to explain that optimum responses were obtained when low calcium was added to the co-culture. In (Kumar *et al.*, 2012) the co-culture could be maintained for up to 6 days. In the first step the keratinocytes were seeded in the culture plate and two days later melanocytes were added.

After one day the co-cultures underwent treatment with melanogenic compounds which were then replenished every two days. Lei *et al.* (2002) found that after day 6 the keratinocytes in the co-culture model were grown almost to confluence with melanocytes seeming to grow over them; phase contrast counting showed that by the end of the experiment the melanocyte to keratinocyte ratio had decreased from 5:1 to 2.5:1. My study is an extension to the other (Lei *et al.*, 2002) (Kumar *et al.*,2012) studies. In my study like in these studies a co-culture medium

was developed as part of research objective 1 and my cells were exposed to known amounts of melanogenic stimulators to find an optimal dose.

The effect of two melanogenic stimulator compounds, stem cell factor and latanoprost, on the co-culture system was examined (Kumar *et al.*, 2012). It was found that after treatment with melanogenic stimulators cellular melanin content increased considerably as well as cellular proliferation and tyrosinase activity. Skin pigmentation is attributed to tyrosinase-regulated melanin synthesis. The compounds MSH, DOPA and 8MOP were identified as being melanogenesis stimulators (Lei *et al*., 2002). MSH treatment in a dose dependent manner could stimulate tyrosinase activity and melanin content with a two to three times increase in comparison with untreated controls. The effects of a melanogenesis stimulator (Lei *et al.*, 2002) were much stronger in the case where melanocytes are co-cultured with keratinocytes. Findings such as this are an indication of the fact an important synergistic function is attributed to keratinocytes in the regulation of melanogenesis by MSH.

Since MSH is known to have an effect on melanocyte action and that is quite widely available it was decided that using controlled concentrations of this hormone could be used in the 2nd objective of my study to see how it can be used to manipulate melanocyte keratinocyte ratios in culture. Another environmental factor that is known to affect co-culture ratios is UV light. UV light is known to stimulate melanocyte action and this is also widely available. For example, dentists and dermatologists regularly use UV light sources in their practice so this equipment was easily available in KSHEMA. For this reason, to fulfil the second objective of my study, I also decided to investigate the effect of using UV light to manipulate melanocyte/keratinocyte ratios.

In recent studies (Lim *et al.*, 2014) the melanocyte keratinocyte co-culture model concept is taken one step further by the recommendation to use a co culture composed from melanocytes and adipose derived stem cells rather than melanocytes and keratinocytes. This suggestion is made since adipose cells are much easier to obtain than keratinocytes. It was found that although using adipose cells resulted in less proliferation than keratinocytes it was still significantly greater than melanocyte monocultures. Using co cultures of adipose stem cells and melanocytes could be an extension to and my PhD work in the future.

However, the Lim *et al.* (2014) study could be progressed further if the melanocyte adipose cell culture was performed on humans rather than mice as is currently the case. Mice are a useful model since they are mammals and the reaction can be indicative of a human result however if we want a more accurate assessment of the use of melanocyte/adipose co culture then the application of this to humans should be studied as well. Positive and negative controls were used and 4 weeks' time was given for any significant results to appear.

2.12.2 Methods of manipulating cell ratios in vitro co-culture

Melanocyte stimulating hormone (MSH)

Alpha melanocyte stimulating hormone, or α-MSH, is a peptide hormone that is made in the body. The differentiation of melanocytes is stimulated by melanocyte stimulating hormone (Hirobe, 2011). The origins of this hormone are in a precursor peptide called proopiomelanocortin (POMC) and as such the family of peptides that it belongs to are called melanocortins. α-MSH's amino acid sequence comprises 13 amino acids and it has a primary structure of: Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val. Hirobe (2011) gives details of the genetic factors involved in melanocyte differentiation. The limitations of this paper are that it uses mouse and not human models to assess how regulating different genes

affects melanocyte synthesis. As mentioned previously in section 2.12.1 mouse models are useful to give us an indication of how MSH will affect the co-culture but we will not know the definite effects of this in humans until the experiment has been performed in a human model. For the sake of safety, a mammalian model to test an experimental model is ideal but until this has really been translated into a human we cannot be sure of how it will really act in the human body.

Production of α-MSH occurs in epidermal cells of the skins, predominantly in the keratinocytes but this has also been observed occurring in melanocytes and Langerhans cells. α-MSH production happens as a defensive response against damage by UV radiation. It binds with the melanocytes MC1R receptor to stimulate melanin production. Following this granules of melanin are transferred to organelles called melanosomes via which they are transported to the dendrites which are found projecting from the ends of melanocytes. Melanin granules are released into neighbouring keratinocytes when the ends of melanocyte dendrites are engulfed by them. Once the melanin granules are in the keratinocyte, they form an arrangement as being a protective barrier over the nucleus of the keratinocytes. The effect of melanin in the cell nucleus is that it protects it from being damaged by UV light by absorbing, reflecting and refracting light (Bohm *et al.*, 2005).

Basic fibroblast growth factor

Basic fibroblast growth factor (FGF2) is a product of keratinocytes and the function of it is to prompt human melanocyte proliferation. FGF2 is potent because it can bind to all 4 FGF receptors and their subclasses. FGF2 is the most potent proliferation factor of all the 21 or so FGF molecules (Lee *et al.*, 2017). The presence of this cannot completely account for increase of melanocyte growth rate in vivo or in vitro, when it is thought that melanocyte proliferation

is potentially stimulated by co-culture keratinocytes. It is thought that the other factors that are responsible for the stimulation of the normal human melanocyte growth in monoculture or keratinocyte co-culture remains largely unknown (Luca *et al.*, 1993).

Alpha melanocyte stimulating hormone is thought to be one of the most obvious candidates for adenylate cyclase-dependent melanocyte growth stimulation. This has been shown to increase the intracellular levels of cAMP in target cells. Cholera toxin has also been found to increase cellular levels of cAMP. **α**-MSH (Luca *et al.*, 1993) does stimulate melanocyte proliferation in a dose dependent nature but for its mitogenic effect to occur it requires basic fibroblast growth factor (FGF2) and/or the activation of protein kinase C (Lee *et al.*, 2017).

A literature search revealed that the optimal cholera toxin and FGF2 concentrations for the induction of melanocyte growth were 10M and 1ng/ml respectively (Luca *et al.*, 1993). Maximum growth stimulation occurred upon the addition of FGF2 and alpha-MSH or Cholera toxin. The capability of cholera toxin to stimulate melanocyte growth has been confirmed (Kormos *et al.*, 2011). It was found that alpha-MSH was ineffective in its action when FGF2 was absent even at concentrations of up to 100ng/ml. 1ng/ml of bFGF was enough for the growth stimulatory effect of alpha-MSH at concentrations that were as low as 1ng/ml whilst maximal values were reached at 10ng/ml (Luca *et al.*, 1993).

<u>Interferon – Gamma</u>

Interferon-gamma, a type of cytokine, is expressed in the serum of those with vitiligo indicating that it could have a part to play in disease onset. In normal human melanocytes interferon-gamma causes cell cycle arrest and apoptosis. The effect of this can be seen physically since normal human melanocytes are pale and dendritic with small cell bodies whilst those

persistently exposed to interferon gamma treatment are larger and flatter with shorter and fewer dendrites.

UltraViolet light

Sunlight is made up of two main types of rays which are long wave ultraviolet (UVA) and short wave ultraviolet (UVB). These affect the skin differently with UVA penetrating deep into the thickest layer of the skin (the dermis) and UVB just burning the skin's uppermost layers.

It was shown that the normal melanocytes respond differently to UVA and UVB irradiation depending upon culture conditions and whether melanocytes were grown as a mono- culture or as a co-culture with keratinocytes. In the co-culture model the exposure to UVB irradiation at doses as low as $5mJ/cm^2$ led to the synthesis of melanin increasing whereas in monocultures of melanocytes it was found that UVB doses of up to $50mJ/cm^2$ had no melanogenic effect. UVA exposure differs from UVB in that it causes the same melanogenic effect in both mono and co cultures. When keratinocyte growth factors were removed from the culture medium the melanogenic response was abolished to UVB but not UVA exposure (Duval *et al.*, 2001) (this is a slightly older paper but its conclusions are still relevant). Tyrosinase is a vital enzyme of melanogenesis and it is activated by exposure of skin to UV radiation (Gillbro and Olsson, 2011).

Physical removal of cells

The dish containing the cells will be viewed under a microscope. Cells for removal will be identified and essentially ‘scratched out’ using the tip of a needle.

Melanogenic inhibitors

The effect of two melanogenic inhibitor compounds that are used in the treatment of hyper pigmentary disorders on the melanocyte/keratinocyte co-culture was examined (Kumar *et al.*, 2012). It was found that after treatment with Kojic acid and Hydroquinone there was a significant decrease in tyrosinase activity, melanin content and proliferation. Other melanogenic inhibitors include arbutin and niacinamide (Lei *et al*., 2002).

The rate limiting step of melanin synthesis is subject to the enzyme tyrosinase meaning the best way to control the formation of pigment is by inhibiting or stimulating the activity of tyrosinase. Four compounds are used by dermatologists to alter the appearance of disorders involving hyperpigmentation which are: hydroquinone, kojic acid, arbutin and niacinamide. Studies have found that when melanocytes and keratinocytes are co-cultured these components have an inhibitory effect that is more noticeable. This was seen by its effect on tyrosinase activity, melanin content and cellular proliferation. The assumption that can be reached from this observation is that keratinocytes have a fundamental role in modulating the function of melanocytes in situ (Lei *et al.*, 2002).

2.13 Vitiligo studies using animal models

It has been proposed that animal models be used to study the pathogenesis of vitiligo and to develop new treatments since before going directly to humans (Essien and Harris, 2014) a proof of concept is needed in a relevant animal model (Taieb, 2011). The limitations of using animal models for studying this disease are that its underlying pathogenesis is so complex that it is unlikely that just one animal model will be enough to give an accurate reflection of all the factors that have a role in disease initiation, progression and maintenance (Essien and Harris, 2014). It has been suggested that the best way to deal with the aforementioned problem is to

use multiple animal types since each has their own different strengths and weaknesses. However, when considering the use of animals, it must not be forgotten that doing this can be both costly and very time consuming. An example scenario of how an animal model differs from a human is that of melanocyte distribution in the skin. In mice these are found in hair follicle and are not present in the epidermis of hair bearing skin significantly different from humans where they are present in the follicles and epidermis of such skin (Singh *et al.*, 2016).

2.13.1 Mice

Mice have for a long time been viewed as a good model organism for studying the genetics of skin and hair pigmentation. Most mouse vitiligo models are carriers of transgenes leading to immune activation which means they can replicate autoimmune melanocyte destruction but not the other features of melanocyte loss (Singh *et al.*, 2016). Another setback to using murine models for vitiligo is that they do not have any interfollicular melanocytes (Manga and Orlow, 2012). A new mouse vitiligo model was made when melanocytes were kept by overexpressing a mutant form of the ligand kit (other names for this are stem cell factor, SCF or KITL). Transplanting melanocyte – targeting CD8+ T cells (CTLs) triggers melanocyte loss by immune factors (Harris *et al.*, 2012). The theory for destruction of melanocytes by immunological factors is that CTLs being targeted again certain proteins that are specific to melanocytes is a major part of this happening (Manga and Orlow, 2012).

The fact that mice are mammals means that their use is still preferable as animal models over other species and that within the mammal group they are relatively easy to maintain, for example, in contrast to chimpanzees and monkeys, but it is important to remember as

highlighted above that this is not a perfect system and that there are some disadvantages to it (Harris *et al.*, 2012).

2.13.2 Smyth line chicken

Smyth line chickens are a useful model organism for studying vitiligo because affected animals display a pathogenesis that has much in common with the human form. This means that there is a possibility that such an animal model can help us to understand how stress, defects in melanocytes, genetics and cellular immune responses interact with each other to cause this/the onset of disease. Sequencing of the chicken genome and the relative ease of obtaining parental control lines further adds to the advantages of using this model (Shi *et al.*, 2012).

Vitiligo in this chicken model presents itself as a depigmentation in its feathers. With regards to studying autoimmune mechanisms, 70-95% of hatchlings that are vaccinated against lymphoma develop depigmentation but when there is no vaccination these figures are much lower. It is hypothesised that viral infections can be a trigger for autoimmunity in vitiligo but this is yet to be definitively proven. The Smyth line chicken has a much greater incidence of vitiligo than their human counterparts (Essien and Harris, 2014).

Smyth line chickens exhibit a susceptibility to uveitis and autoimmune disorders that have an association with human vitiligo. Hypothyroidism and feather loss is viewed to have similarities with alopecia areata. The feathers of Smyth line chickens have increased levels of reactive oxygen species and their melanocytes can be seen to have a marked morphological abnormality. Similar to humans these defects alone are not enough for causing disease but rather also require the presence of immune responses. Similar to human vitiligo, $CD8^{+}$ T cells infiltrate the feathers of SL chickens with active disease and also IFN-γ and IFN-γ induced

genes are seen to be expressed in affected feathers. We can prove that melanocyte destruction might be caused by antibodies by observing the fact that performing Bursectomy procedure reduces disease incidence as well as this auto-antibodies have been isolated from vitiligo affected Smyth line chickens (Essien and Harris, 2014).

2.13.3 Dog breeds

There are breeds of dogs that have a genetic predisposition for developing vitiligo and these include: German Shorthaired Pointers, Dachshunds, Doberman Pinschers, Old English Sheepdogs, German Shepherds and Rottweilers. It has been observed that in animals with vitiligo their serum has autoantibodies for melanocyte antigens which are not present in the serum of healthy individuals. The use of dog breeds for genome wide association studies is especially powerful since they are genetically homogeneous meaning that fewer individuals are required for statistical power than in humans (Essien and Harris, 2014).

2.13.4 Sinclair swine

Swine are increasingly being used as a serious alternative to dogs and non-human primates in translational research. This can be attributed to the fact that swine have much in common with humans in terms of anatomy and physiology making them a much more suitable models for certain studies and procedures than other available species (Swindle *et al.*, 2012). Sinclair swine are particularly useful for modelling vitiligo since their disease state shows similar features to that of humans (Frisoli *et al.*, 2018).

Previously rats, mice and rabbits were used as models for experimental and toxicology studies of the skin but in recent times pigs are becoming the preferred choice for skin studies because there is a great deal of similarity in the skin of pigs and humans. In terms of structure pig and

human skin resemble each other because they have similar epidermal thickness as well as dermal-epidermal thickness ratio. The skin of these two species also has in common the fact that in both the skin is fairly hairless with a secure subcutaneous layer and hair follicles in the dermis. Pigs are a physically advantageous model because they respond to several growth factors, physically and molecularly, in a similar way to humans. Pigs also have a dermis with a similar content of dermal collagen and elasticity (Swindel *et al.*, 2012). Pig and human skin differ in the fact that pig skin is seen to be thicker on the neck dorsum as well as not being as vascular in all parts. Although like humans' pigs possess apocrine sweat glands and sebaceous glands all over their skin these have a negligible part to play in thermoregulation.

2.13.5 Horses

Horse breeds with the Gray coat hair allele (Andalusians, Arabians and Lipizzaners) have been observed to spontaneously develop vitiligo. This tends to present on body areas such as perigenital, perioral, perianal areas and the face. The mechanisms of vitiligo in horses are fairly unknown but horse sera of infected individuals have been found to have antibodies acting against surface melanocyte antigens. This has led to scientists theorizing that such horses are useful for studying the formation of vitiligo antibodies because quantities wise they can provide a large amount of serum for antibody purification (Essien and Harris, 2014).

2.14 Differentiation markers

Differentiation markers are most widely used to determine a cell's lineage and sub lineage. They are also known as cell surface antigens and function as monograms to enable the identification and classification of cells. In the majority of cases a cell marker is a molecule or antigen within a cell's plasma membrane with different cell types possessing specific combinations of markers or antigens. For example, T cells can be identified by the expression

of the CD3 marker. A mature T cell on the other hand might belong to the T4 subset in which case it will express the marker CD4. In such a manner there are markers for other cell populations and subpopulations (Zola, 2000).

Within a cell's lineage it is often helpful to be able to distinguish cells that are at different stages of differentiation and activation. It is particularly useful to use differentiation status in the diagnostic analysis of the lymphoid and myeloid malignancies as well as in research on the hematopoietic system. Examples of this include markers for the naïve or antigen experienced cells (especially for CD45 isoforms) and molecules such as CD9 which are to be found upon B-lineage precursors, including B lineage acute lymphoblastic leukaemia. The activation status is especially interesting in the studies of cell function. Markers of activation include CD25 (a growth factor receptor) and CD69 and CD98 (the cellular function of this is not yet fully understood).

It is markers such as these that have revolutionized aspects of pathology and research, and such is the case that the ease with which some cell populations can be identified has led to some unrealized, and perhaps unrealistic, expectations. It can be expected that the identification of T helper type 1 (TH1) and T helper type 2 (TH2) cells on the basis of a simple surface marker was frustrated by the lack of a single marker for all dendritic cells or for all NK cells, confused by the un-coordinated expression of different activation markers, or have an over-interpreted phenotype in some situations. Solutions to these problems can be examined by why the successful lineage markers work so well or by a reconsideration of our expectations (Zola, 2000). Several markers that are useful in the identification of melanocytes and keratinocytes have been identified and will be explained further below.

1α(OH)ase shows significant expression in keratinocytes. 1α(OH)ase-null mice do not display any particular phenotype but they do have a lowered expression of keratinocyte differentiation markers involucrin, profilaggrin and loricrin. In 1α(OH)ase-null mice one observation is they have a lowered capacity for recovering a normal functioning of the skin barrier once it has been disrupted (Hendy *et al.*, 2011).

It has been concluded that the local production of $1,25(OH)_2D$ is needed for epidermal cells to undergo normal differentiation. When the alleles for 1α(OH)ase were inactivated in a ras-transformed keratinocyte cell from nude mice, that make a squamous cell carcinoma, these tumours did not react to the inhibition of growth by 25(OH)D,that was administered locally, but they did react to $1,25(OH)_2D$ antiproliferative and differentiating effects. This makes us infer that the activity of keratinocyte 1α(OH)ase has a vital role in the autocrine regulation of keratinocyte differentiation and growth (Hendy *et al.*, 2011).

2.14.1 Filaggrin

Filaggrin (filament aggregating protein) is a matrix protein which exerts its function by binding to keratin fibres of the epithelial cells. Filaggrin is the product of a large profilaggrin precursor protein undergoing post translational hydrolysis to make ten to twelve filaggrin units when epithelial cells are terminally differentiating. Fillagrin is also important when the stratum corneum is forming because it becomes part of the physical barrier of the skin which has the responsibility of controlling the movement of molecules into and out of the skin. Further processing of Filaggrin happens in the upper stratum corneum where free amino acids are released which aids in water retention (Norreslet *et al.*, 2018).

A decrease in filaggrin expression or loss of function mutation are thought to present as causing an epidermal barrier defect that results in a heightened exposure to environmental allergens and greater susceptibility to bacterial and viral infections. This barrier defect also appears to result in an increased susceptibility to and exacerbation of asthma with a deficiency in Filaggrin being one of the top genome wide genetic determiners of asthma (Archer *et al.*, 2019). Another clinical presentation of filaggrin abnormality (truncation mutation in the gene) is that such individuals show a strong predisposition to severe dry skin. Almost 50% of all severe eczema cases present with this gene mutation (Brown and McLean, 2012).

When epidermal cells are terminally differentiating a huge (>400Da) profilaggrin precursor protein is post translationally hydrolysed into ten to twelve filaggrin units. The gene that codes for human profilaggrin is called FLG and it is a member of the fused type protein family S100. Filaggrin structures are found in several areas such as keratohyalin granules of the epidermis and they are a major constituent in cells of the stratum granulosum skin layer (Brown and McLean, 2012). Amidst the interface of the skin layers, the stratum granulosum and stratum corneum profilaggrin is reduced into filaggrin monomers by proteolytic processing which is assisted by enzymes that are calcium dependent. A characteristic of filaggrin is its high pH which can be attributed to the high presence of histidine in the primary structure and its relatively low constitution of methionine and cysteine (sulphur containing amino acids) (Brown and McLean, 2012) (Norreslet *et al.*, 2018).

2.14.2 Involucrin

In terms of terminal keratinocyte differentiation, Involucrin is a useful marker for this. It is coded for by the gene IVL and it is a soluble protein part of human skin. Involucrin is not

only present in humans but its size does differ in different species with it in dogs being of 285 residues whilst in orangutans it is of 835 residues in size. When it binds with the protein Loricrin it aids in the formation of the cross-linked envelope to which it is a precursor. The function of this envelope is to protect the skin's corneocytes. Involucrin is made once keratinocytes have departed from the basal layer and started to get bigger. However, this is some time before the envelope starts to cross-link and this only happens in the cells outermost layers (Watt, 1983).

Involucrins structure consists of a rod shaped α-helix and one of its functions is to be a substrate of the formation of covalent ε-(γ-glutamyl)lysine isopeptide bonds. This protein's N-terminal region is made up of approximately 75 amino acid residues which come before two segments containing glutamine-rich tandem repeats that are very variable in length. The function of tandem repeats is for the glutamine residues to act as a substrate for transglutaminase via a cross-linking reaction (Abadie *et al.*, 2019).

Involucrin is a soluble cytosolic protein when it is first made but when it terminally differentiates at the epithelial cell surface it is covalently cross linked via areas on the plasma membrane inner surface. It is in this region that Involucrin helps to stabilise the cells structure by it taking on the role of being a scaffold protein (Adhikary *et al.*, 2005). Since Involucrin is a type 1 transglutaminase it can be a catalyst for interprotein ε-(γ-glutamyl)lysine bond formation. This then serves to be a catalyst for interprotein covalent cross links being made by involucrin with other proteins (Adhikary *et al.*, 2005).

2.14.3 Loricrin

Loricrin is a structural protein, a characteristic of which is that it is terminally differentiating. It encompasses more than 70% of the cornified envelope and it aids in the function of the stratum corneum being a protective barrier. This protein is expressed in the stratified epithelia of all mammals in vivo. Its expression levels are particularly high in 'humid tissues' examples of which are: the new born epidermis, the epithelia of the oral cavity, anus, oesophagus, foreskin, vagina and epidermal parts of the sweat ducts. It is not expressed in the non-keratinizing epithelium and in actual fact its expression at these sites represents a defensive or protective mechanism of the body (Abadie *et al.*, 2019).

2.14.4 Keratin 10

This is a type I keratin that in combination with type II keratin 1 forms heteropolymeric structural proteins to produce the keratinocytes intermediate filament cytoskeleton in the skins spinous and granular layers. The function of the intermediate filaments in conjunction with microfilaments and microtubules is to build a network providing strength and stability to the epithelial cell's cytoskeleton (Frommherz *et al.*, 2019).

2.14.5 Keratin 5

Human Keratin 5 protein is coded for by the gene KRT5. Keratin 5 associates with the keratin 14 proteins in the human body to create an interaction that produces molecules which make up the cytoskeleton of basal epithelial cells; these are called the intermediate filaments. Failure of this protein has been attributed to being the cause of several diseases including epidermolysis bullosa simplex but also even breast and lung cancer (Abadie *et al.*, 2019).

Keratin 5 along with the case of other members of the keratin 5 acts as an intermediate filament protein. These polypeptides have characteristics such as a 310 residue central rod domain made from the four alpha helix segments (helix 1A, 1B, 2A and 2B) which are linked via the three short segments (L1, L1-2, and L2). It is observed that there is remarkable conservation amongst the extremities of the central rod domain (the helix initiation motif and the helix termination motif). These parts are needed to make the helix stable and to produce the heterodimer and filament. Variable, non-helical head and tail regions can be seen extending out from the intermediate filament surface and these make the differing IF polypeptides specific (Inaba *et al.*, 2018).

In the intermediate filament the central rods are made up of heptad repeats. These repeats are repeating patterns of seven residues which allow via hydrophobic interactions the two different IF proteins to intertwine with each other to form a coiled coil. These heterodimers are made by the specific pairing of type I (acidic) and type II keratin (basic). K5 is a type II keratin which can form a pair with type I keratin K14. In these coiled coil dimers, a stepwise assembly can happen when they connect in an antiparallel arrangement and cause interactions that are end to end with other coiled coils and then result in the formation of large 10nm intermediate filaments (Inaba *et al.*, 2018).

The majority of Keratin 5 (and K14) expression happens in the basal keratinocytes but they are also found in the epidermis (particularly the stratified epithelium that makes up the lining of the skin). The cytoskeletal scaffold that is found inside the epithelial cells is made by keratin intermediate filaments. The function of this scaffold is to support the architecture of the cells and to provide cells with the capability to endure both mechanical and non-mechanical stress. There is an extensive bundling of the K5/K14 keratin pairs as the non-helical tail of

K15 functions as a cross-linker at the surface of the intermediate filament. Via this bundling the intermediate filaments have an improved mechanical resilience and elasticity (Abadie *et al.*, 2019). The anchoring of basal cells intermediate filament desmosomes connects K5/K14 cells to neighbouring cells by way of desmoplakin and plakophilin-1. Association of plectin and BPAG1 with the transmembrane protein α6β4 integrin happens at the hemidesmosome. This adhesion molecule is how K5/K14 filaments found within basal cells are connected to the basal lamina (Inaba *et al.*, 2018).

2.14.6 Type IV collagen

The type IV collagens in mammals are all made from six α-chain polypeptides that are genetically distinct (α1–α6). The structures of the domains of the α-chains of these type IV collagens do have a similarity and at the amino acid level they demonstrate a homology of 50-70%. The α-chains mentioned here can be divided into three domains, these are: amino-terminal 7S domain, a middle triple-helical domain, and a carboxy-terminal, globular, non-collagenous (NC)-1 domain (Abreu-Velez and Howard, 2012).

The longest domain is the triple-helical domain which has a length of about 1,400 amino acids and approximately 22 interruptions in the collagen Gly-X-Y sequence motif. Every α-chain if the NCI domain has a length of 230 aa. Like other collagen types the importance of the NC1 domain is in aiding construction of the type IV collagen trimeric structure. A molecular interaction amongst the three α-chains is stimulated when a trimer beginning with the three NC1 domains is put together. The protomer trimerization continues from the carboxy-terminal end forming a protomer that has a flexibility enabling it to bend.

The next stage in the creation of this collagen is the forming of type IV collagen dimers. To do this two-type IV collagen protomers collaborate by way of their carboxy-terminal NC1 trimers to make a NC1 hexamer. Following this four protomers make tetramers via interactions at the glycosylated amino-terminal 7S region. The outcome of these interactions is creation of a nucleus for type IV collagen scaffold. Lateral associations and those of an end to end type amongst the type IV collagen protomers transform the scaffold into a type IV collagen superstructure (Wu and Ge, 2019).

At a genetic level COL4A3 codes for the alpha3(IV) chain of type IV collagen, this protein is flexible. In combination with two other types of alpha (IV) chains (the alpha4 and alpha5 chains) this makes a molecule of type IV collagen (Abreu-Velez and Howard, 2012). Type IV collagen molecules make complex protein networks by making bonds with each other. These protein networks are a large component of basement membrane zones (thin structures that are sheet-like) which act to separate and support the cells of various tissues. alpha3-4-5 networks of type IV collagen are important to the functioning of basement membrane zones in the eye, inner ear and the kidney (Wu and Ge, 2019).

Type IV collagen is composed of networks of Alphal(IV) and alpha2(IV) and in particular tissues it is the basement membranes principle skeletal macromolecule. Via carboxy-terminal NC1 domains these chains make heterotrimers which have a role in the collagenous domains folding into triple helices. By a few intermolecular interactions these helices have the ability to form networks. Associations between type IV collagen molecules make a non-fibrillar polygonal assembly which has the function of being a scaffold for other matrix glycoproteins being deposited in addition to cell attachment (Abreu-Velez and Howard, 2012).

2.14.7 Laminin V

Laminins are a collection of glycoproteins which have a contribution to basement membranes being formed and organized in nearly every tissue, their molecular weights are between 400 and 900kDA. The binding of these to cell surface receptors activates cell signalling cascades and interacts with ECM resulting in the production of scaffolds of migrating cells, ultimately this process/Laminins are active in causing cell adhesion (Hartwig *et al.*, 2006).

The structure of Laminins can be described as being a heterotrimeric molecule that has an alpha, beta and gamma chain all three of which are coded for by different genes. There have been identified five alpha, three beta and three gamma subunits that can arrange into at least 15 different types of laminins. There is the possibility to produce many more variations of these via processes such as proteolytic processing and alternative splicing (Armony *et al.*, 2016). Each of the chains has sections of approximately 600 amino acid residues at the C terminus which make coiled coils which possess other non-identical laminin subunits and stability is provided here via disulphide bonds. When the chains are assembled, they make a cross shaped trimer where the cross's short arms are made by the individual subunits N terminal parts (Armony *et al.*, 2016).

Laminin V is of particular interest, and it has also been known under any of the three names: Kalinin, Epiligrin and Nicein. This Laminin has also been named as Laminin 332 because its composition is one alpha 3, one beta 3 and one gamma 2 chain. This Laminin's biological role is to act as the major adhesive part of the epithelial basement membrane where it enables the stable attachment of keratinocytes which bind to α6β4 integrin via hemidesmosomes. For cells to be able to migrate in monolayers that are wounded interactions between laminin-5 and α3β1 are crucial. When laminin-5 is disrupted in the keratinocytes of mouse models the effect of

this is to cause a block and inhibition of keratinocyte migration caused by a response to EGF. Other functions of Laminin 5 include promoting gap junctional communication and cell cell interactions in keratinocyte cell culture (Hartwig e*t al.*, 2006).

Recent studies have identified laminin-5 is required for the keratinocytes polarized state and persistent linear migration. When the epidermis is subject to injury it can cause the activation of laminin-5 gene transcription resulting in keratinocytes and laminin-5 secretion into the basement membrane. Laminin 5 deposition over any exposed collagen has a fundamental part to play in re-establishing the integrity of the dermal epidermal junction. Laminin-5 has a pivotal part to play in the polarisation of cells that are at the leading edge. This can lead us to conclude that Laminin-5 is a crucial ligand for keratinocyte migration. Keratinocyte migrations are an essential requirement for wound healing to happen in the epidermis (Hartwig e*t al.*, 2006). When laminin V is missing it can present as the disorder epidermolysis bullosa which is a blistering disorder that is quite rare and recessively inherited (Matsui *et al.*, 1998).

2.14.8 Alpha 6 Beta 4-integrin

$\boldsymbol{\alpha}$6β4 is a protein whose function is to moderate basal keratinocytes interacting with laminin-332 these are found to be positioned between the dermal and epidermal skin layers. The importance of $\boldsymbol{\alpha}$6β4 is due to the fact that it is believed to have a function associated with skin maintenance, a theory which has been validated via knock-out mouse models and human studies. The effect of losing $\boldsymbol{\alpha}$6β4 in mouse models is seen physically as the epidermal skin layers separating from the dermal ones at the basement membrane zone whilst skin blistering and pot-natal lethality are other demonstrated effects. Mutations of the $\boldsymbol{\alpha}$6 or β4 integrin genes in humans presents clinically as a manifestation of the disorder junctional epidermolysis bullosa (JEB), symptoms of this are skin blistering and pyloric atresia (Klygis *et al.*, 2012).

α6β4integrins functions outside of being involved in stable adhesion are that they are up-regulated when wound healing and tumorigenesis are taking place where they have a key role in the determination of motile behaviour in skin cells. Various other integrin's that are improved epidermal basal cells include α3β1 integrin (receptor for laminin-332) and α2β1 integrin (receptor for collagen I), both are involved in regulating the healing of wounds in the skin and the migration of cells during tumorigenesis. α3β1 can improve proteolysis of the matrix during tumorigenesis including wound healing. Prevention of α3β1 action *in vitro* can prevent skin cell migration (Klygis *et al.,* 2012) (Stewart and O'Connor, 2015).

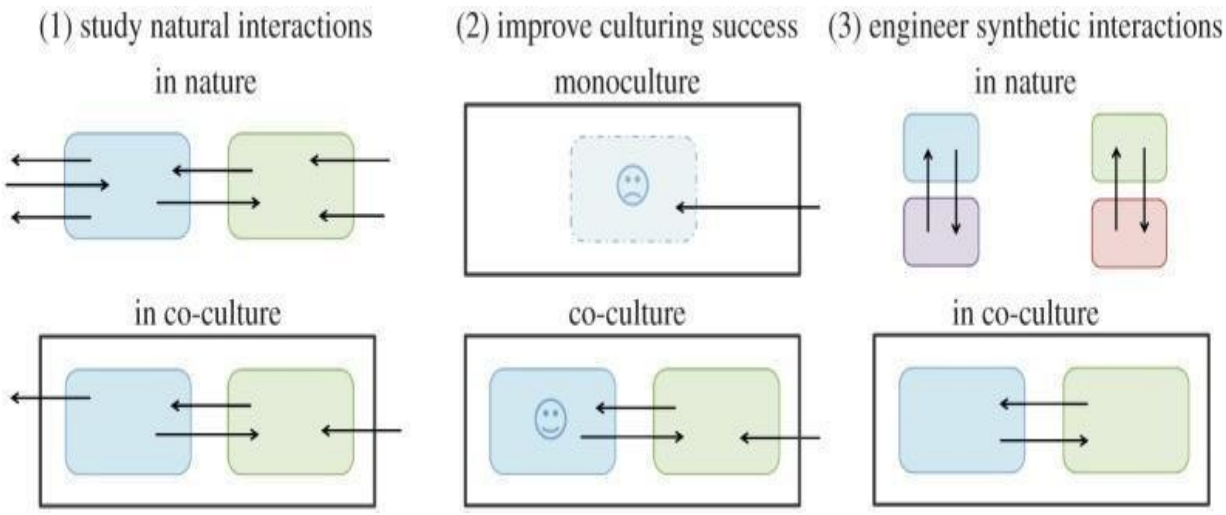
2.15 Benefits of the use of co-culture over mono-culture models

A co-culture at its most basic level is when two or more different cell populations are grown together with there being contact of some type between them. **Figure 12** demonstrates that there are three main reasons for using a co-culture setup (Goers *et al.*, 2014). One clear advantage of co-cultures over their monoculture counterparts is that they give a more accurate representation of the human in-vivo scenario. For instance, the body itself does not feature one cell type in isolation but rather many different cells interacting with each other (Kook *et al.*, 2017).

As is the case with my doctoral work sometimes a co-culture scenario is required because the growth of some cell types is improved by their interaction with a completely different population. Therefore, the presence of one population of cells might improve the success of culture or behaviour of another. A co-culture system can also enable us to understand how cells interact in nature (Miki *et al.*, 2012). Co-cultures have many favourable characteristics

over their monoculture counterparts such as that they are more robust, predictable, scalable and stable.

Figure 12: An illustration of the main reasons and benefits for conducting experiments with co-culture models



Synthetic biology is a branch of science that aims to benefit society via medical, environmental and industrial applications. Many synthetic systems can be benefitted by co-culture models as this is a method by which synthetic interactions can be modelled. For instance, before any synthetic systems are used in a medical, environmental or industrial context a co-culture model means that they can be tested for function and safety (Goers et *al.*, 2014). One of the most important reasons for using the co-culture technique is that cell-cell interactions of any type (natural or synthetic) can be studied or even so that new interactions can be designed (Kook *et al.*, 2017).

In designing a co-culture system, it must be remembered that the extracellular environment has a big influence on how cells interact and as such it might be important that the environment in

which the co-culture may finally be used must be replicated for any experiment. Ultimately a cell's phenotype is dependent upon the complex interactions of environment and genotype. There are many modifications of the basic co-culture model such as the number of separate populations being used, how much similarity there is between the populations, how the populations are separated and the extent to which this occurs, the amount of time over which the co-culture takes place and the differences in the local environments of the populations (Kook *et al.*, 2017).

Co-culture models do have limitations such as that many of the systems already in creation tend to be of low complexity with there being very few high complexity systems in comparison. A reason for the preferred simplicity is that studying any more than three populations interacting with each other at any one time can become very difficult to manage in terms of understanding molecular interactions and this often causes an element of instability and unpredictability (Goers *et al.*, 2014).

Therefore, as well as for how difficult it is to create a stable system when many more populations are involved, until recently most co-culture experiments have been limited to studying just two populations at any one time. These systems for a long time have been employed to study cell-cell interactions and as such their use is fundamental to doing this. Studies with more than three separate cell populations are relatively rare due to the increase in complexity that they require. However, this might not always be such a problem because sometimes in synthetic biology a system only needs to be as complex as to reach a desired goal (Bogdanowicz and Lu, 2013).

It is often concluded that the greater the similarity between populations, the easier it is for a stable system to be established but this does not mean that there are not successful co-cultures of populations from different species. Co-culture is probably the last step before creating artificial tissues and organs. By co-culturing different strains, we can simulate the natural environments and test evolutionary theories, it is also possible to study complex behaviours (Goers *et al.*, 2014).

2.16 Next steps

Now that I have provided a scientific background to my study and placed it in context the next chapter of my thesis will describe the methods used in my practical work in such a manner that any of these steps will be easy for the reader to follow and replicate. It clearly explains the workflow from extracting cells from the skin sample to manipulating cell ratios in co-culture. To support this chapter I have also included as appendices the plastic wares and list of equipment used. A further appendix lists the media, reagents and chemicals used along with the companies they were purchased from and catalogue number for ordering. The aim of these appendices is that they will further clarify the methods if there is any need for the reader to repeat them in the future.

CHAPTER THREE: MATERIALS, METHODOLOGY AND METHODS

3.1 Chapter introduction

This study was an *in vitro* investigative quantitative laboratory experiment which will form the basis of potential future quantitative studies targeted at informing the delivery of therapeutic clinical techniques in the treatment of vitiligo. Tissue was cultured, modulated and analysed entirely in the laboratory and the findings are qualitatively described. An *in vitro* approach was taken because it allows scientists to isolate the effects of compounds on two cell types without the distraction of looking at the whole organism. A co-culture of only two cell types was observed because studying a larger number of interacting populations of cells than this vastly complicates the experimental procedure. When there are more than three cell populations interacting with each other this can result in a level of complexity that is unmanageable in regard to the molecular interactions the consequence of this being unpredictability and instability.

The name quantitative research is given to a style that is used to quantify a problem by generating numerical data or data that can be changed into usable statistics. This style is used to quantify opinions, behaviours, attitudes and other defined variables. It can also be used to generalize results from a much larger sample population. Quantitative research works by using measurable data to formulate facts and identify patterns in research. Quantitative methods differ from the qualitative style in that they are much more structured. They include various types of survey such as systematic observations, online polls and face to face interviews (Bloomfield and Fisher, 2019).

A quantitative approach was used in this study since I had to determine the success of the various co-culture medium formulations by counting how many of each cell type grew in the medium over a period of time. I compared the number of cells that grew in several co-culture medium formulations to see which produced the best result. After this I determined the effect of the melanogenic stimulators alpha melanocyte stimulating hormone (α-MSH) and UV light by counting how this changed the number of each cell type, therefore taking another quantitative approach to measure outcomes.

3.2 Study settings

3.2.1 The two settings

The first setting was:

No.1a. This was the outpatient Plastic Surgery clinic at a private hospital in Kent where discarded skin from patients undergoing plastic surgery procedures was obtained with the patient's consent.

No. 1b. This was the Stem Cell Research and Advanced Bio-Engineering Laboratory (SCRABEL) at the Medway Campus of Canterbury Christ Church University (CCCU). Skin cells were cultured here.

The next setting was:

No. 2a. The Department of Plastic Surgery at Justice K. S. Hegde Charitable Hospital, Nitte University, Mangalore, Karnataka, India. This was the source of skin tissue samples for research.

No. 2b. The laboratory work was carried out in the Nitte University Centre for Stem Cell Research and Regenerative Medicine (NUCSReM), K. S. Hegde Medical Academy, Nitte University, Mangalore, Karnataka, India.

3.3 Ethics approval

3.3.1 Research ethics approval study setting one– England

An online research ethics approval application was completed and submitted through the IRAS (integrated research application system) to the London – City and East Research Ethics Committee. Approval was received in August 2016 **(Appendix 1)**.

3.3.2 Research ethics approval study setting two – India

An application was submitted to the Central Ethics Committee of Nitte University in India in September 2017 and was subsequently accepted and approved **(Appendix 2)**.

3.3.3 Confidentiality

Maintaining the anonymity and confidentiality of patients/participants is central to ethical practice in research. All of the work completed in this study complied with the Declaration of Helsinki (2000) which is a set of ethical guidelines with regard to human experimentation that was developed for the medical community by the World Medical Association (WMA). In order to maintain anonymity and confidentiality, each patient's consent form was given a number which was entered onto both the consent form and the tube holding the tissue. This made the patient's tissue anonymous but still enabled identification of the patient by the researcher's supervisor but not the researcher if the need had arisen for example, if a patient wanted to leave the study at any time. As the collection of skin samples was ultimately done in India, consent forms were kept in KSHEMA according to local procedures and guidelines.

3.4 Population

Study setting 1a) – Private hospital – Kent, UK

Participants were patients who were undergoing routine plastic surgery who met the later described inclusion and exclusion criteria. As mentioned previously after consideration no samples were eventually obtained from study setting 1.

Study setting 2a) – Nitte University, Mangalore, India

Collection of tissue samples: The Department of Plastic Surgery at Justice K. S. Hegde Charitable Hospital, Nitte University, Mangalore, Karnataka, India. Participants were patients who are undergoing routine Plastic Surgery who met the later described inclusion and exclusion criteria.

Laboratory experiments (*In vitro*): Nitte University Centre for Stem Cell Research and Regenerative Medicine (NUCSReM), K. S. Hegde Medical Academy (KSHEMA), Nitte University, Mangalore, Karnataka, India.

3.5 Recruitment and Consent

Two Consultant Plastic Surgeons who undertook surgical procedures at a private hospital in Kent agreed to take part in this study. The Consultants were sent a letter **(Appendix 3)** that explained the study. The researcher discussed the study with the Consultants as well as explaining the consent form **(Appendix 4)** and patient information leaflet **(Appendix 5)** to ensure that they fully understood the procedure and the ethical considerations. Patients attending the Consultant Plastic Surgeon's out-patient clinics, who met the study inclusion criteria (as described below) were recruited into the study by the Consultant Plastic Surgeon. The Consultants were asked to identify patients who attended their out-patient clinic that met

the inclusion/exclusion criteria and gave these patients a pack which consisted of three consent forms **(Appendix 6)**, a patient information leaflet and a pre-paid envelope. Patient consent was obtained for the use and storage of their tissue that would otherwise normally be discarded after routine surgery. The Consultant explained the study to the patient and invited them to take the information home with them. The patient was asked to sign each of the consent forms if they agreed to take part in the study and send the two signed forms back to the Consultant's secretary via a pre-paid envelope in the post. The patient retained the third signed consent form. The researcher ensured the consultant had information packs available to him for each of his clinics.

The person from whom the tissue sample was being collected from was not identifiable to the researcher. Participation in this study did not pose any additional risk to the patient since there was no change made to their surgical treatment. The sample/patient was allocated an identifying number and kept securely, with this number also being put in the patient's clinical notes for traceability. Consent forms were kept in patient's notes and the university in secure holdings in accordance with CCCU policies and the 1998 Data Protection Act. Transport of tissue followed (UN3373), storage of tissue (HTA guidelines/ www.hta.gov.uk) and disposal of tissue followed guidelines. They were put into a yellow bag and were collected by a waste disposal company to be incinerated.

The Spire Alexandra Hospital and Department of Plastic Surgery at Justice K. S. Hegde Charitable Hospital, Nitte University agreed to take part in the study subject to research study approval and were satisfied that protocols and procedures would be adhered to. To ensure that the patient consent process was unprejudiced it was important that I did not have any contact with the patients at any stage of this study. After transfer of the practical work to India, the

same procedure was followed for recruitment and consent and samples were still harvested from routine surgery by a Plastic Surgeon in India. As the work was carried out in India, the consent forms were kept in KSHEMA according to local procedures and guidelines.

3.5.1 Inclusion criteria

All patients who fulfilled the following criteria were eligible to take part in the study. These involved patients who:

1. underwent surgery which resulted in discarded skin that would normally be destroyed at the end of routine practice of procedure.
2. had agreed to take part and had capacity to consent.
3. were between 18 and 60 years old.
4. could provide a normal skin specimen that was not affected by vitiligo or any other disorder.
5. who were not taking any contravening medication or treatment that would affect the results.

3.5.2 Exclusion criteria

All patients who met the following criteria were ineligible to take part in the study. These were all patients who:

1. were aged less than 18 years old and who were ages above 60.
2. had not agreed to take part in the study.
3. did not have the capacity to clearly understand the study requirements and give informed consent.

3.6 Sample size

Previous similar studies such as Matsuzaki and Kumagai (2013) have included skin taken from 27 patients in order to enable quantitative analysis; however, this paper did not state how their power analysis was undertaken. The research statistician from a surgical research group at the Catholic University of South Korea (this group was initially providing scientific advice before my work was transferred to India), and the Canterbury Christ Church University statistician were consulted for advice. It is important that sufficient numbers of patients are recruited in order that data can be analysed to produce results of confidence (Biau *et al.*, 2008).

The results of this study were used to create a reverse power analysis to determine the minimum number of dishes required to accurately compare the rates of cell growth in our three conditions (control, exposure to UV and Hormone stimulation). Only three conditions were chosen at this stage since anymore becomes difficult to analyse at any one time. The conditions of UV light and hormone exposure were chosen for this study because they are both easily available and there is a vast amount of literature concerning these two factors. Obviously for any scientific experiment a control is needed so in this case the control will be a dish of cells being grown in an environment without any UV light or MSH hormone exposure. This study is only a pilot study to assess the number required by a complete analysis that would take more time than is available for the purposes of this study. There are four or five factors that are involved in a power analysis with the values for each being chosen before the analysis is done. The factors that must be considered are called: the effect size, alpha and beta/power (Nayak, 2010).

The effect size is the minimum deviation from the null hypothesis that is hoped to be detected. An example of this is if hens are treated with something that will change the sex ratio of their chicks. If it was decided by the experimental designer that the minimum change in the

proportion of sexes is 10% then you would set the effect size at 10%. The alpha value is the significance value of the test (the P value) and is the probability of rejecting the null hypothesis even if it is true (a false positive). It is usually the case that the alpha value is set at 0.05. The beta value is the probability of accepting the null hypothesis even though it is false (a false negative), when the real difference is equal to the minimum effect size. There is no clear consensus as to which value to use but the most common is a power of 80% (this is equivalent to a beta of 20%). The choice of power should be influenced by the cost of a false negative. It is absolutely necessary that the effect size is detected then a higher value for power (a lower value for beta) which will lead to a bigger sample size (Nayak, 2010).

The analysis was therefore performed for a one-way Analysis of variance (ANOVA) with a between subjects design and three levels for the one factor utilized. In this study the minimum power was set to beta=.80, the alpha level was set to .05, and because it was not possible to determine a suitable effect size from previous literature, given the innovative nature of this research, we performed three parallel analyses, one for each of three different effect sizes where f was set to large (g power suggesting a coefficient of .40), medium (with a suggested coefficient of .25) and small (with a suggested coefficient of .10). Analyses were performed using g-power version xxx. G-power is a very good free program that can be used by Mac and Windows, and it will do power analyses for a large variety of tests. The total sample sizes calculated were respectively 66; 159 and 969. This represents the total number of dishes necessary for each of the three different effect size coefficients. Considering that we are aiming to extract three tissue samples from each clinical participant, so that variability within each set of three conditions is reduced, we are aiming to obtain discarded tissues from 22, 53 and 323 different individuals. As mentioned previously, these numbers are so large it was not practical

to assess or obtain this many samples in my experimental work which should rather be referenced as a pilot rather than a full scale study (Mishra *et al.*, 2019).

There are two ways that a power analysis can be done which are either before (a priori) or after (post hoc) the data has been collected. The a priori power analysis is usually undertaken before the research study commences and is mostly used to estimate a sufficient sample size so that adequate power is achieved. A post hoc power analysis on the other hand is undertaken once the study has been completed. In a post hoc analysis the sample and effect size that is obtained is used to calculate the power in the study assuming that the sample effect size is equal to that in the population. However the use of retrospective techniques is controversial and as such in this study I used an a priori approach. It is thought that using statistical analysis of collected data to make an estimate of power will lead to uninformative and misleading values. In its simplest form the post hoc power has been shown to be a one to one function of the p value that is obtained. It has been shown that post hoc power analyses are susceptible to what is called the "power approach paradox" which is where a study with a null result shows more evidence that the null hypothesis is in fact true when the *p*-value is smaller, this occurs since the apparent power to detect an actual effect would be higher. In actuality a smaller *p*-value is properly understood to make the null hypothesis less likely to be true (Mishra *et al.*, 2019).

3.7 Chemicals, media, reagents and plastic wares

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All media and reagents were procured from Thermo Fisher Scientific (Gibco-Invitrogen, Life Technologies, Grand Island, NY, USA) **(Appendix 7)**, unless otherwise specified. All cell culture plastic wares, dishes and items were purchased from Thermo Fisher Scientific (Roskilde, Denmark) **(Appendix 8)**. A list of the laboratory equipment used is attached for reference **(Appendix 9)**.

3.8 Tissue handling procedure

The next few paragraphs will outline how the tissue will be handled starting with its collection from the hospital to how it will be stored in the laboratory prior to use.

3.8.1 Collection and transport of skin tissue from the hospital to the laboratory

Protocol for collecting skin tissue from the hospital

The laboratory technician collected the signed consent forms and samples from the hospital and filed these so that the researcher had no contact with the patient.

Protocol for receiving skin tissue at the lab

If the skin sample was to be processed straightaway, it was received in Dulbecco's phosphate buffer saline (DPBS) solution with 1% penicillin-streptomycin. It was found that the cells were prone to infection and two skin samples had to be discarded for this reason. It was therefore imperative that the transport medium that was used was freshly mixed with the antibiotic's penicillin streptomycin and fungizone (Amphotericin B) before the skin sample was placed within it.

If the sample was only to be processed after several hours then it was received in Dulbecco's modified eagle's medium (DMEM). The preference for DPBS is that it is a much cheaper alternative to DMEM. The antibiotics penicillin, streptomycin and fungizone were added to the transport solution (regardless of if it is DMEM or DPBS) to make sure that the sample did not become affected by any contamination. The advantage of DMEM over DPBS is that it contains additional growth factors such as vitamins and amino acids. If the sample is only to be stored for a short time then these factors are not required as they are not depleted from the

sample however if a longer time of storage is needed then these factors need to replace those lost from the original sample (Osmanovic *et al.*, 2018).

Protocol for storage of skin sample in laboratory

If the tissue was not to be processed immediately then it was stored in a 4^0C refrigerator until required. Tissue cannot be stored in DPBS for more than a few hours however it can be stored in DMEM overnight. This is because unlike DPBS, DMEM contains additional factors that support cell growth. DMEM is a modification of Basal Medium Eagle (BME) that contains a four-fold higher concentration of amino acids and vitamins, as well as additional supplementary components.

3.8.2 Extraction of cells from the skin sample (Ultimately all experimental work only occurred in KSHEMA, Mangalore, India, Study setting 2)

When cells were to be extracted from the skin sample, they were sequentially put into the enzymes, 0.25% trypsin-EDTA, for a time period of 2 to 4 hours at 37°C or overnight at 4°C, until the epidermis was seen to be peeling away easily from the dermis (shorter incubation tends to be at warmer temperatures than longer ones), and 0.1% collagenase (30 mins to 2 hours). There are several enzymes that might be used but a recently published study has found that digestion time appeared to affect the isolated cells more than the choice of enzyme (Skog *et al.*, 2019). It is preferable that an enzyme of non-mammalian origin is used in any human cell work to avoid any possibility of the occurrence of zoonotic diseases (Torgersona *et al.*, 2018). In the laboratory, for initial optimization of cell isolation procedures from skin samples, the primary preference for enzyme use was given to those that are more economical and easily available.

Skin source used for cell isolation

Since my work was carried out in a lab associated with a hospital that had a Plastic Surgery unit, it was decided to use cells from excess skin which was a surgical by-product as a matter of convenience. Best isolation results were obtained when the skin sample was placed in enzyme at 37°C for approximately 4 hours rather than the cells being left overnight in enzyme in the fridge (4°C). Different incubation times are required because the availability of the skin is dependent upon what time of day the surgeons operation is booked. If the operation happens in the morning, then it is possible for an incubation of 4 hours but if the operation is very late in the day then leaving the sample overnight might be the only option to avoid wasting it.

An important factor to take into consideration when comparing the experimental work in India and the UK was the differences in the skin used. It is much easier to isolate the epidermis from the dermis in India rather than the UK because in India the dermis in most cases is pigmented and appears brown. Therefore, it is easy to identify when the dermis has been removed from the dermis because the brown layer is removed leaving behind a whitish layer. In England since the population is mostly of lighter skin it is much more difficult to determine where the dermis begins and the epidermis is removed. When the skin sample was placed in enzymes to separate the epidermis from the dermis this was successful but initially the sample had to be closely monitored to ensure that the epidermis was not digested rather than just being separated from the dermis.

3.8.3 Monoculture of melanocyte and keratinocyte cells in the laboratory (Ultimately all experimental work only occurred in KSHEMA, Mangalore, India, Study setting 2)

1. All solutions and equipment coming into contact with living cells were sterile and aseptic techniques were used accordingly.

2. All (co) culture incubations were performed in a humidified 37°C, 5% CO_2

Protocol for mono-culture of melanocytes and keratinocytes

The study requires the culturing of melanocytes and keratinocytes, which have different proliferation potentials. In order to do this, established techniques for mono culture of these individual cell types were used, and these techniques were applied to co-culture.

Coating of cell culture dish with attachment factor

Attachment factors are used to coat the petri dish to be used for cell culture. They are structural proteins or protein-like substances that have adherent capabilities and serve to increase the cell-substrate interactions in a culture. It is the case that several glycoproteins have been identified that influence/promote the in vitro cell attachment to the surface or substratum of the culture vessel. Under normal circumstances the attachment and growth of a large number of cell types is dependent upon attachment factors and extracellular matrix components. Some cells are able to synthesize these components whilst others need an exogenous source, especially when grown in serum free culture. The glass or plastic culture flasks that are used as a substrate strongly influence the growth and differentiation of anchorage-dependent cells. To aid the attachment, cell spreading, and growth of cells, biological industries offer a wide line of attachment and matrix factors (Products and full product descriptions are available from the company 'Life technologies, India').

Method:

Petri dishes were coated with attachment factor by using 100 µL to 200 µL for 35 mm or 60 mm dishes or 500 µL for 100 mm dishes, and spread to form a thin layer. (Alternatively pipetted

1 mL of attachment factor into dishes to spread liquid and then pipetted back up). Then, the dishes were kept in sterile conditions to air dry with the lid of the dish at a loose angle.

Attachment factor is received as a stock solution of 1x strength. For use, it was diluted by adding 1 mL to 9 mL in DPBS to prepare a 0.1x solution.

Seeding density

The cell density that is used has a significant influence upon how cells grow *in vitro*. Using the correct seeding density for cell culture is important since cells growing in media tend to secrete a range of beneficial factors into it and as such they are able to condition the medium in which they are growing. The cell density has a direct effect upon the stimulation of neighbouring cells via the interaction of cell surface receptors and growth factors/cytokines that are secreted. An increase in the cell-cell contact can directly stimulate a variety of membrane-bound receptors. When considering what cell density to use it must be considered that as the cell density becomes greater there is an increase in the cell-cell interaction. (Xia *et al.*, 2011).

The recommended cell seeding density was: 3000 - 5000 cells per cm^2. Therefore depending on the size of the dish that is used for culture, the optimal seeding density is as follows:

35 mm dish = 0.05-0.1 million cells

60 mm dish = 0.1-0.5 million cells

100 mm dish = 0.5-1 million cells

These figures were calculated after discussion with a Senior Scientist based in the lab in India. This scientist has a lot of experience in cell culture work and as such these were the figures recommended by them.

After consulting the literature it was suggested to start with a 1:5 or 1:10 ratio of melanocytes to keratinocytes. Due to the highly specialised nature of this topic it was very difficult to find recent papers to consult when the experimental work commenced but one paper suggested that the initial seeding ratio of melanocytes to keratinocytes should be 1:5 (Kumar *et al.*, 2012) whilst another suggested a ratio of 1:10 (Liu *et al.*, 2008). After consultation with the Senior Scientist in India it was decided from their experience that a seeding density of 1:3 should also be investigated.

The titer experiments were performed to decide ratio for the experiments.

Passaging of cells

Cell passage (splitting) is necessary to maintain cells that are undergoing exponential growth (Liao *et al.*, 2014). Once cells have reached confluence in one dish they are removed from the dish using enzyme and split into several dishes to ensure that their growth continues. If the cells are not split into several dishes their growth will start to decrease because as the dish gets fuller and fuller they will start competing with each other for the same limited resources such as space and growth nutrients, once these nutrients are used up the cells will start to die.

Most cell types show the same patterns of behaviour which is that the ability to spread and migrate is best at early passages but that this does reduce after later passages, the exact number of passages before cell differentiation capacity reduces is dependent on cell type and culture conditions (Liao *et al.*, 2014).

3.8.4 Development of a co-culture medium that supports the growth of both melanocyte and keratinocyte cells at the same time (Ultimately all experimental work only occurred in KSHEMA, Mangalore, India, Study setting 2)

Protocol for co-culture of melanocytes and keratinocytes

There are several co-culture protocols available for melanocytes however at the time of writing this study was unique as there are no co-culture protocols for melanocytes and keratinocytes. Rather a literature search found that protocols are available for melanocytes and adipocytes and melanocytes with fibroblast cells (Bonifacino *et al.*, 2014; Shariati *et al.*, 2008).

To fulfill research question two, once a reliable protocol for the isolation of cells from skin had been created, a co-culture media and method for co-culture was developed. The co-cultured cells were exposed to specific intensities of UV light and concentrations of alpha-melanocyte stimulating hormone (α-MSH) following which the effects of these were observed.

A study that might be an extension of this project may be performed to observe the effects on cells of exposing them to both UV light and α-MSH at the same time. The rest of the protocols such as how to change media, passage cells, cryopreserve cells and revive cells are standard protocols that can be found within most molecular biology/cell biology textbooks.

Melanocyte and keratinocyte cells in co-culture

Initially the cells were isolated and grown individually in their specific medium. The cells were then seeded and grown together. The culture medium used for the co-culture was a mixture of both the melanocyte and keratinocytes mediums in the ratio to which the cells need to be seeded. For e.g. for 1:5 melanocytes to keratinocytes, the culture medium had a 1:5 mix

of melanocyte-keratinocyte medium. The purpose of this study is to develop optimized medium and culture conditions which support the growth of both cell types.

Developing a method of counting melanocyte and keratinocyte cells in culture

At present cell counting is carried out by a hemocytometer. The numbers of each cell type in a grid are counted by eye. It would be preferable if a computer program could be developed to do this automatically by identifying the different cell types and counting their number.

The current analysis of cell viability and counting requires taking cells out of culture and counting them in suspension. This interruption of the culture process may adversely affect the methods that are going to be used to modulate the cell culture. A way of addressing this is to see if image processing can allow us to derive the same data during the culture itself without disturbing the cells. Images captured may allow us to see the cell density, viability and phenotypic differentiation and allow us to better track the consequences of various methods of culture modification.

3.8.5 Investigation of whether ratios of cell populations in *in vitro* co-culture could be altered using environmental, biochemical or physical methods

Identifying factors to manipulate the cells

A literature search identified that Ultraviolet (UV)-light and α-MSH are common melanogenic stimulators. It is for this reason that the present study investigated the properties of these two factors on melanogenesis by adding factors to the culture medium or exposing the cells to them (Chung *et al.*, 2019).

UV-radiation serves as an environmental stress for human skin, and results in melanogenesis, with the pigment melanin having protective effects against UV induced damage. It is for this reason that I exposed the melanocyte/keratinocyte co-culture to UV light to observe the effects of exposure on cell proliferation (D'Mello *et al.*, 2016).

α-MSH is produced in the skin by epidermal skin cells (keratinocytes and less commonly melanocytes and Langerhans cells) as a protective response to any damage that is caused by ultraviolet radiation. α-MSH molecules work by binding to the MC1R on melanocytes and activating the production of melanin (this is done via a series of chemical reactions that are known as a signaling pathway). As such I exposed the cells in this experiment to α-MSH to explore the effect that this has on melanogenesis and cell proliferation (Chung *et al.*, 2019).

Identifying how the added factors manipulated cell ratios in co-culture

The cells in culture were observed over a period of time and the cells of each type counted frequently.

3.9 Protocols for experimental work

In the next sections of this chapter, I will be detailing the protocols used as well as the requirements for these such as materials and equipment/reagents required.

3.9.1 Isolation of epidermal cells

Normal skin was removed with sterilization toothless tweezers and washed in 10 mL DPBS and then they were disinfected in the 10-30 mL 75% medical alcohol for 30 sec. The disinfected skin was washed in 30 mL DPBS three times, and then put it into a petri dish and cut it into small squares in the size of 0.2×0.2 cm^2 with sterile scissors. The disinfected skin tissues

were put into a 50 mL aseptic mixing bottle, and 0.25% trypsin-EDTA and 0.1% collagenase were added sequentially, and incubated in 5% CO_2 at 37°C for 30 min to 2 hrs. After the digestion, the sample was filtered with nylon mesh, and DPBS was added to wash. Then the sample was centrifuged at 1500 g for 5 min. Supernatant was discarded with a pipette and washed with 20 mL DPBS centrifugally twice, and then used to resuspend cells.

3.9.2 Isolation and expansion of melanocytes

The isolated cells were then placed in the attachment factor coated culture dishes (35 mm dish, BD Falcon, Franklin Lakes, NJ, USA) along with complete media containing Minimum Essential Medium (MEM-α) supplemented with 10% fetal bovine serum (FBS), 1% Human Melanocyte Growth Supplement (HMGS) and 2 ng/ml epidermal growth factor (EGF, Biolegend, San Diego, CA, USA). Further, the plates were incubated in a 5% CO_2 humidified atmosphere at 37°C (Eppendorf Galaxy 170S CO_2 Incubator, Germany). The medium was firstly changed after 48 hrs and then twice a week with occasional wash with DPBS supplemented with antibiotics (Penicillin-Streptomycin) to ensure sterile cultures. Once the cells reach confluence of 90%, they are labeled as primary (P0) generation and passaged up to two times for further analysis using MEM-α supplemented with 10% FBS, 1% HMGS and 2 ng/mL EGF.

For expansion, the cells were suspended in Medium 254 (Gibco-Invitrogen) supplemented with fetal bovine serum (FBS), bovine pituitary extract, bovine insulin, hydrocortisone, bFGF, bovine transferrin, heparin, and phorbol 12-myristate 13-acetate. Taking FBS into account, the concentration was 0.5%. When the cells reached 80% confluence, they were detached from the flask and seeded into other culture flasks in preparation for an experimental procedure or to maintain and propagate the cell culture. Melanocyte cell lines were designated as MEL.

For further analysis, isolated and expanded melanocytes at early passages were cryopreserved using 80% FBS, 10% MEM-α and 10 dimethyl sulphoxide (DMSO) as a cryomedia at -80°C ultralow freezer or at -196°C in liquid nitrogen.

3.9.3 Isolation and expansion of keratinocytes

The isolated cells were placed in the attachment factor coated culture dishes (35 mm dish, BD Falcon) along with complete media containing MEM-α supplemented with 10% fetal bovine serum (FBS), 1% Human Keratinocyte Growth Supplement (HKGS), 2 ng/ml keratinocyte growth factor (KGF) (Peprotech, NJ, USA) and 2 ng/ml epidermal growth factor (EGF). Further, the plates were incubated in a 5% CO_2 humidified atmosphere at 37°C. The medium was firstly changed after 48 hrs and then twice a week with occasional wash with DPBS supplemented with antibiotics to ensure sterile cultures. Once the cells reach confluence of 90%, they were labeled as P0 generation and passaged up to two times for further analysis using MEM-α supplemented with 10% FBS, 1% HKGS, 2 ng/mL KGF and 2 ng/mL EGF.

For expansion of keratinocytes, instead of supplemented Medium 254, the cells were suspended in EpiLife Medium supplemented with Human Keratinocyte Growth Supplement (HKGS), human EGF and KGF. When the cells reached confluence, they were detached from the flask and seeded into other culture flasks. Keratinocyte cell lines were designated as KRT.

For further usage and analysis of keratinocytes, cells at early passages were cryopreserved using 80% FBS, 10% MEM-α and 10 dimethyl sulphoxide (DMSO) as a cryomedia at -80°C ultralow freezer or at -196°C in liquid nitrogen.

3.9.4 Co-culture of melanocytes and keratinocytes

Cultured keratinocytes were harvested by treatment with 0.25% trypsin/EDTA and seeded at 2.5×10^5 cells/well in six-well plates. After two days, cultured melanocytes (5×10^4) were collected by trypsin/EDTA and added to each well containing the keratinocytes. Co-cultures of melanocytes and keratinocytes were maintained in culture media (melanocyte media + keratinocyte media), the initial seeding ratio of melanocytes to keratinocytes being 1:3, 1:5 and 1:10. After one day of co-culture, fresh medium containing melanocyte inhibitors and stimulators were added. Again after two days fresh medium with fresh compounds at the appropriate concentrations were added; two days after that, cultures were photographed and then harvested with trypsin/EDTA. The cells were dislodged with trypsin/EDTA; media was added to inactivate the trypsin. The remaining cell suspension was centrifuged at 1500 g for 5 min, washed with DPBS, and assays were then conducted.

For co-culture experiments, the assays such as morphology, melanin content and tyrosinase activity by L-DOPA staining were performed.

3.9.5 Assessment of morphology and cell viability

Morphology of the cells was observed under phase contrast microscope (Olympus CKX41, Japan) and any change in cell structure was noted periodically at different passages. The viability of the cells at different passages was estimated by trypan blue exclusion method. Here, the cells are cultured for a period of 10-15 days or until confluence was reached. Then the cell suspension was obtained by detaching the cells with 0.25% trypsin-EDTA followed by treatment with 0.4% Trypan blue dye which traverses through membranes of dead cells only. 20 µL of cell suspension was treated with 20 µL of the dye and the number of live cells against dead cells was counted in 10 µL of the treated cell suspension under phase contrast microscope

using a hemocytometer chamber. The cell count was carried out in triplicates to obtain an accurate value.

3.9.6 Proliferation rate and population doubling time

To determine the proliferation rate of melanocytes and keratinocytes, 5000 cells were seeded in each well of a 12-well plate (Thermo Scientific, Roskilde, Denmark) and cultured for 12 days. Cells in every three wells were detached with 0.25% Trypsin-EDTA on days 3, 6, 9 and 12, and counted using a hemocytometer. The culture medium was changed every 3 days. Population doubling time (PDT) was calculated as, PDT = t (log2) / (log Nt-log No), where t represents culture time, and No and Nt are the cell numbers before and after seeding, respectively. PDT was then expressed as the average number of hours taken for the population of cells, undergoing exponential growth, to double its number.

3.9.7 Melanin content assay

Melanin content assay was carried out in melanocytes alone and also, after the culture of melanocytes with keratinocytes at different ratios. Melanin content was determined by following previously published protocol (Promden *et al.*, 2018). Cells were seeded in a 24-well plate at a density of 5×10^4 cells/well and cultured for 96 hours. Cells were lysed with 500 µL 2N NaOH containing 20% DMSO. Then, the cells were incubated at room temperature for 1 hour, and the extracted cells were centrifuged at 13000g for 5 minutes. A supernatant of 100 µL from each well was collected. Relative melanin content was determined by absorbance at 405 nm in a microplate reader.

3.9.8 Cytochemical staining of melanocytes by L-DOPA

To observe melanocytes more clearly, the cells were stained by L-DOPA (L-3, 4-dihydroxyphenylalanine) by following a previously published protocol (Zhang *et al.*, 2018) with minor modifications. Briefly, cultured melanocytes or melanocyte-keratinocyte co-cultures were washed with DPBS followed by fixation with 4% paraformaldehyde for 10 min at room temperature. The cells were washed with DPBS again for three times. Then the cells were stained with 5 mM L-DOPA and incubated for 2 hours at 37°C. Then the stained cells were washed with DPBS for three times. Control dishes were kept without adding L-DOPA. Finally, the cells were observed under a microscope (Olympus, Tokyo, Japan).

3.9.9 Tyrosinase assay

Tyrosinase activity in melanocytes was analyzed as DOPA oxidase activity using a protocol previously published by Tu *et al.*, (2012) with minor modifications. Cells were seeded in a 24-well plate at a density of 5×10^4 cells/well and cultured for 96 hours. The cells were washed with ice-cold 1x DPBS. 150 µL lysis buffer (1% Triton X-100 in 0.1 M phosphate buffer) was added to each 24-well plate. Then the plates were allowed to stand for 30 min at -20°C and immediately transferred to 37°C for 30 minutes. Cells were then scraped and transferred to a 1.5 ml Eppendorf tube, lysed through 3~5 times and centrifuged at 15000 rpm for 5 to 10 min at 4°C. The samples (300~500 µg /80 µL) were added to a new 96-well plate on the ice. 20 µL of 5 mM L-DOPA (L-3, 4- dihydroxyphenylalanine) was added to each well. The plates were then incubated at 37°C for 30 minutes, and measured absorbance at 475 nm (Tu *et al.*, 2012).

3.9.10 Flow cytometric analysis

To assess the purity of the melanocytes and the proportion of cells expressing specific internal markers (S100, TRP1 and TYR), fluorescence activated cell sorting (FACS) was performed on

cells at Passage 2 to 4 (FACSCalibur, BD Biosciences, Becton Dickinson, NJ, USA). After being harvested, the cells were detached by 0.25% Trypsin-EDTA, centrifuged and resuspended. Cells were fixed in 3.7% paraformaldehyde (Sigma-Aldrich) for 30 min and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 20 min. The cells were then incubated sequentially with unconjugated primary antibodies (mouse mAB, 1:100 dilution, Novus Biologicals, Centennial, CO, USA) prepared in cell staining buffer (Thermo Fisher or Biolegend) overnight at 4ºC and FITC-conjugated secondary antibody (Anti-mouse IgG FITC, 1:100 dilution, E-bioscience, Thermo Fisher, USA or Santa Cruz Biotechnology, San Diego, CA, USA) for 1 hour in the dark at room temperature. Further, the flow cytometric analysis of intracellular markers was performed by a BD FACSCalibur flow cytometer (BD FACSCalibur, Becton Dickinson, USA).

For phenotypic determination of keratinocytes, the specific markers were analysed by FACSCalibur (BD Biosciences, Becton Dickinson, NJ, USA) or PARTEC-flow cytometer (PARTEC-CY FLOW SPACE, Gloritz, Germany). Keratinocytes at the passage 2 to 4 were treated with 0.25% trypsin-EDTA and cells were harvested. Cells were fixed with 3.7% paraformaldehyde for 30 min. Unconjugated primary antibodies, pancytokeratin (K1, Novus Biologicals, CO, USA, 1:100), keratin 10 (K10, Novus Biologicals, 1:100) and filaggrin (Novus Biologicals, 1:100) prepared in cell staining buffer (Thermofisher or Bilegend) were incubated with cells for 1 hr at 37°C water bath. Further, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (E-bioscience, Thermo Fisher or Santa Cruz Biotechnology, San Diego, CA, USA, 1:100 dilution) as a secondary antibody and incubated at dark for 1 hr at room temperature. Isotype controls were kept in parallel and at least 10,000 events were acquired and analysed.

3.9.11 Co-culture of melanocytes and keratinocytes and α-MSH supplementation

The role of α-MSH in *in vitro* melanocytes-keratinocytes co-culture system was analyzed. The cells of passages 3 to 5 melanocytes and keratinocyte co-cultures at different ratios, such as 1:3, 1:5 and 1:10 were employed for the experiments on the supplementation of α-MSH (Sigma-Aldrich). After 48 hours of culture, the cells were exposed to α-MSH at different concentrations (2.5 µg/mL, 5 µg/mL and 10 µg/mL) and the changes in morphology were observed on day 3 and day 7 of culture. The exposed cell lines were further analyzed for morphological changes, melanin content assay and tyrosinase activity by L-DOPA assay.

3.9.12 Co-culture of melanocytes and keratinocytes and UVB irradiation

The cells of passages 3 to 5 were used for the following experiments. At 24 h after plating, the supernatant was replaced with a thin layer of DPBS with calcium and magnesium and exposed to UVB radiation after lids were removed. Immediately after irradiation, the PBS was removed and replaced with Medium 254 supplemented with HMGS and EpiLife supplemented with HKGS at the ratio of 1:3. Irradiation using various doses of UVB at 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2 (Schmid *et al.*, 2018) was performed using a UV irradiator thrice (24 hrs, 48 hrs and 72 hrs), and irradiated cells were cultivated for 2 weeks. Each cell line co-culture was irradiated three times with an interval of 24 hrs between treatments. Control cells were kept in the same culture conditions without UVB exposure. The exposed cell lines were further analyzed for morphological changes, melanin content assay, tyrosinase activity by L-DOPA assay and markers expression by flow cytometry.

In total 10 individual melanocyte (MEL1-MEL10) and keratinocyte (KRT1-KRT10) cell lines were established (10 patients) but for establishing co-culture and further analysis cell lines which showed the strongest growth were used (MK2, MK4, MK6).

Co-culture was established using cells from the same patient. Therefore MK2 means that in this co-culture the melanocyte and keratinocytes were both established from patient 2 samples.

Three melanocyte to keratinocyte cell culture medium ratios (1:3) (1:5) (1:10), three α-MSH concentrations (2.5 μg/ml) (5 μg/ml) (10 μg/ml) and three UVB intensities (10 mJ) (20 mJ) (30 mJ) were used as variables for this study.

3.9.13 Statistical analysis

All measurement data expressed as mean ± SD were collected and sorted in Excel (Microsoft). Statistical analyses were performed by analysis of variance (ANOVA) using Bonferroni's and Tukey's multiple comparison test with GraphPad Prism software programme, version 8.2 (GraphPad, San Diego, CA, USA). P value <0.05 was considered statistically significant for all tests. All the experiments were performed in a minimal of triplicates.

CHAPTER FOUR: RESULTS

The results of the study are presented under the four specific objectives as outlined below.

1. Isolation, and characterization of melanocytes and keratinocytes in terms cellular and phenotypic markers expression.

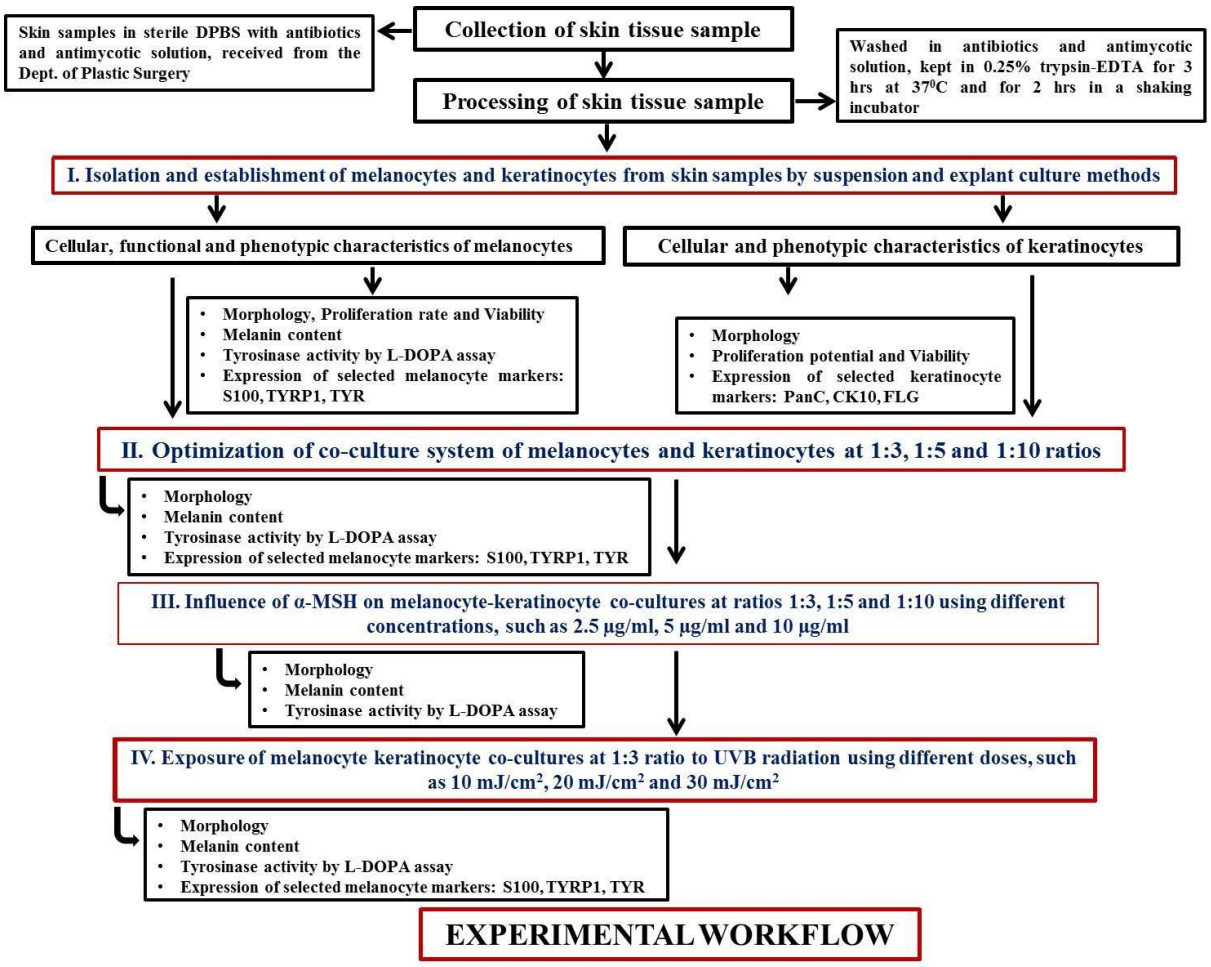
2. Establishment of a co-culture system of melanocytes and keratinocytes and their functional assessment.

3. Influence of α-MSH on melanocyte-keratinocyte co-culture systems.

4. Exposure of melanocyte-keratinocyte co-cultures to UVB radiation.

Figure 13 is a workflow schematic which explains how cells were derived from extraction of skin, to growth of monocultures and derivation of co-cultured cells. It is a summary of all the steps that were taken to enable completion of objectives 1 to 4.

Figure 13: Demonstration of the workflow schematic that was followed during my entire experimental procedure



Day 0 = Day of initial cell seeding for each assay performed with melanocytes and keratinocytes.

Total duration of lab work to complete all the assays from start to finish = Minimum 18 months

Objective 1: To isolate and characterize melanocytes based on their cellular, biological and phenotypic properties and Objective 2: To isolate and characterize keratinocytes based on their cellular and phenotypic features.

4.1 Observations of preliminary study

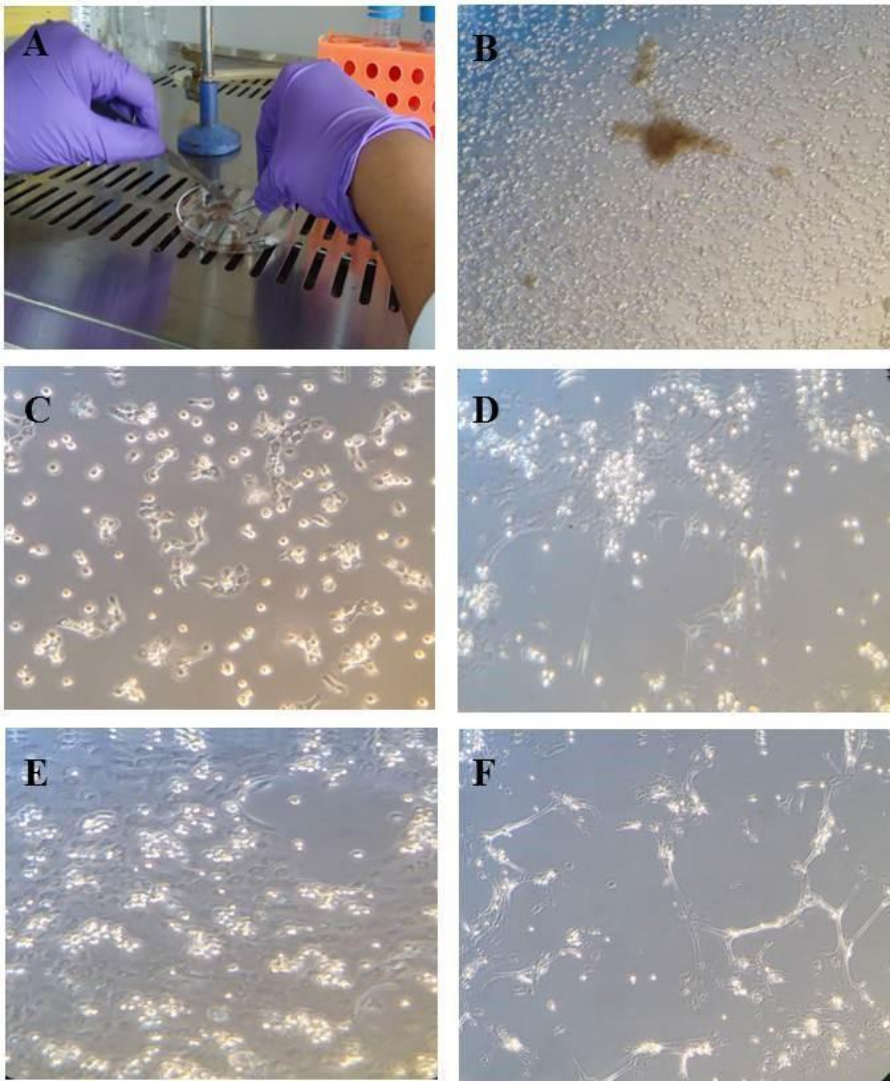
4.1.1 Isolation and establishment of melanocytes and keratinocytes from skin sample

The images of successful isolation and establishment of melanocytes and keratinocytes from skin samples during the preliminary study are presented in **Figure 14**. The skin specimens were collected from patients as a part of treatment procedures and brought to the laboratory for further processing. The samples were washed extensively in PBS and disinfected with medical grade alcohol as a part of the sterilization process. Later, the subcutaneous tissue and dermis, if any, were removed and the remaining epidermal tissue was cut into smaller pieces of 3-5 mm width and approximately 0.5 mm thickness. The tissue remnants were then placed in trypsin-EDTA solution at 37°C 30 min to 2 hrs. The digestion was terminated by the addition of serum and the cell suspension was obtained by filtering through strainers. The cells were seeded into culture dishes with seeding density of 10000 – 20000 per cm^2 and cultured at 37°C in a humidified atmosphere with 5% CO_2. On day 0, the round, single cells were observed after initial seeding. The culture medium was changed after 48 hrs. After two days of cells seeding, the initial adherence and growing of primary culture of skin cells were noticed. On day 5, the formation of small clusters by melanocytes and keratinocytes were clearly observed. Melanocytes were seen with dendritic features scattered among the epidermal cells and appeared round and small in culture. Representative image of primary cultured melanocytes with elongated bipolar or tripolar morphology is presented. However, the majority of the melanocytes had a spindle-shaped morphology. By day 7, primary cultures of keratinocytes

with round or polygonal shape were also present in culture dishes. The primary cultured cells were passaged after being grown for 2 to 3 weeks. Phase-contrast or inverted microscopy revealed that the primary culture had the combination of both melanocytes and keratinocytes in varied numbers or density. Subsequently, it was decided to continue the expansion in specific media for obtaining the pure population of melanocytes and keratinocytes with basic, distinguishable phenotypic characteristics.

When establishing and expanding melanocytes and keratinocytes from skin tissues, the presence of fibroblasts was observed and it was a common challenge to obtain the purity of desired cell population devoid of fibroblasts. However, the number of fibroblasts during the later stages of culture expansion gradually reduced, as the cells were maintained in specific media that supported the growth of either melanocytes or keratinocytes.

Figure 14: Isolation and establishment of melanocytes and keratinocytes from skin sample



A. The epidermal tissue was cut into smaller pieces of 4-5 mm width and placed in trypsin-EDTA solution.

B. Day 0. Suspension of single cells from epidermal tissue after initial seeding.

C. Day 2. Initial attachment and growing primary culture of skin cells.

D. Day 5. Formation of small clusters by melanocytes and keratinocytes.

E. Day 7. Representative image of primary cultured keratinocytes with round or polygonal shape.

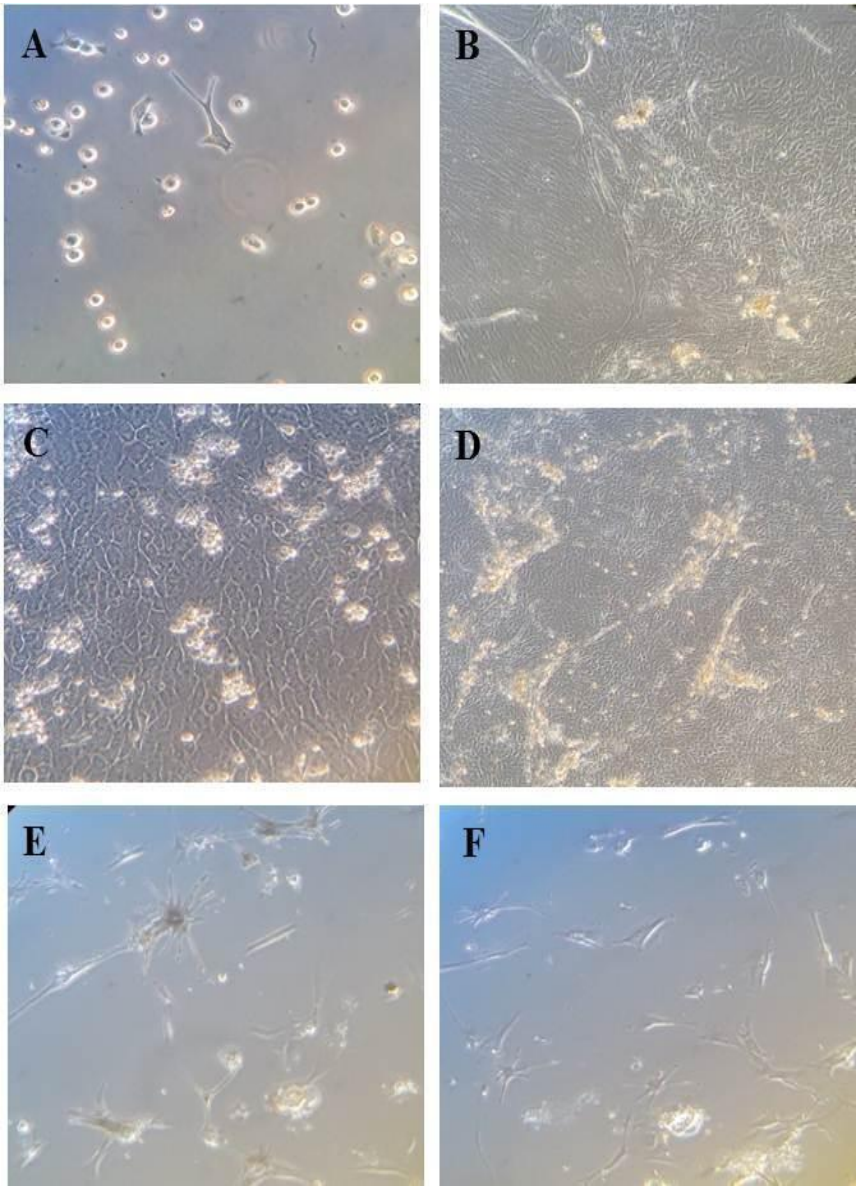
F. Day 7. Representative image of primary cultured melanocytes with elongated bipolar or tripolar morphology.

Magnification (B-F)- 10x

4.1.2 Culture and expansion of melanocytes and keratinocytes from skin sample

Established primary cultures of melanocytes and keratinocytes were expanded *in vitro*. The images of culture expansion of melanocytes and keratinocytes from skin samples are depicted in **Figure 15**. At passage 1, initial attachment and proliferation of skin cells in DMEM were observed by day 2. On day 6, a mixed population of cells representing melanocytes, keratinocytes and a smaller number of fibroblasts was noticed. Sub-cultured keratinocytes displayed round or polygonal shape, as their characteristic features. In addition, sub-cultured melanocytes in the M254 medium with human melanocyte growth supplement (HMGS) exhibited bipolar or multipolar morphology.

Figure 15: Culture and expansion of melanocytes and keratinocytes from skin sample Passage 1.



A. Day 2. Initial attachment and growing culture of skin cells in DMEM.

B. Day 6. Mixed population of cells representing melanocytes, keratinocytes and a smaller number of fibroblasts.

C. Day 6. Representative image of sub-cultured keratinocytes with clear round or polygonal shape.

D. Day 6. Representative image of subcultured keratinocytes and melanocytes growing as a mixed population of cells.

E & F. Representative images of subcultured melanocytes in M254 medium with human melanocyte growth supplement exhibiting bipolar or multipolar morphology.

Magnification (A-F)- 10x.

4.2 Processing of split skin sample

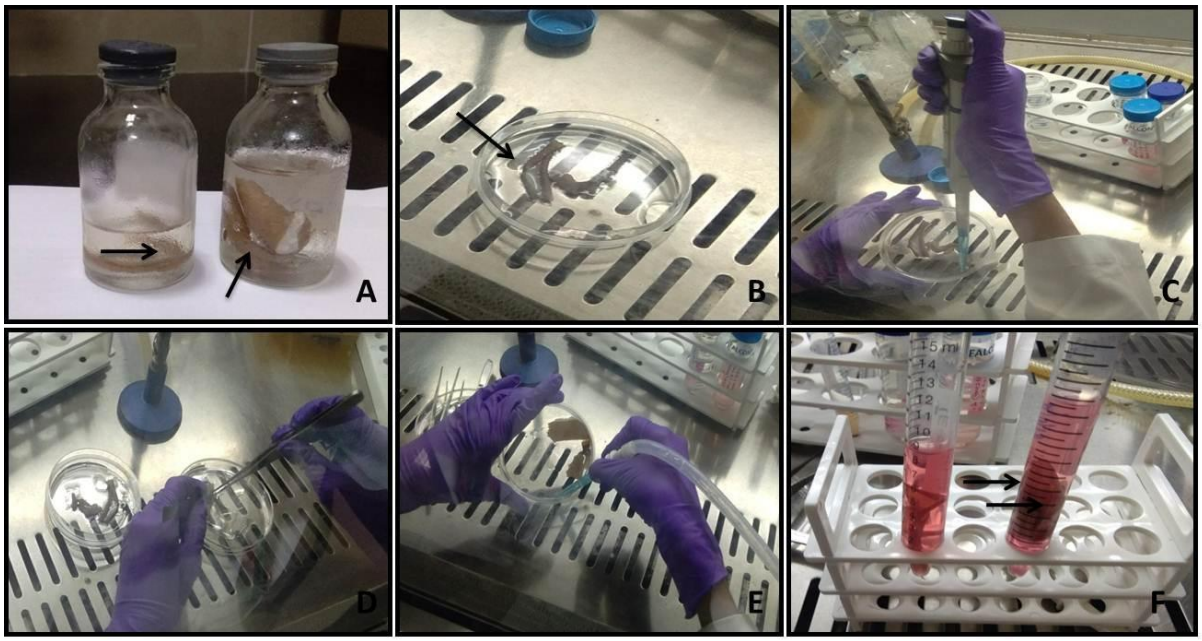
The split skin samples were obtained from normal skin of patients undergoing graft surgery, and the details of patients characteristics are presented in **Table 7**.

Table 7: Details of patients used for collection of skin samples and the *in vitro* establishment of melanocytes and keratinocytes

Sl. No.	Sample and cell lines ID	Age (years)	Gender	Condition
1	MEL1 & KRT1	57	Male	Normal skin was collected from the patients undergoing graft surgery.
2	MEL2 & KRT2	15	Male	
3	MEL3 & KRT3	20	Male	
4	MEL4 & KRT4	Not provided	Male	
5	MEL5 & KRT5	Not provided	Male	
6	MEL6 & KRT6	28	Male	
7	MEL7 & KRT7	30	Male	
8	MEL8 & KRT8	25	Male	
9	MEL9 & KRT9	58	Male	
10	MEL10 & KRT10	44	Male	

The steps involved in the processing of skin samples are presented in **Figure 16** and **Figure 17**.

Figure 16: Processing of split skin sample for the isolation of melanocytes

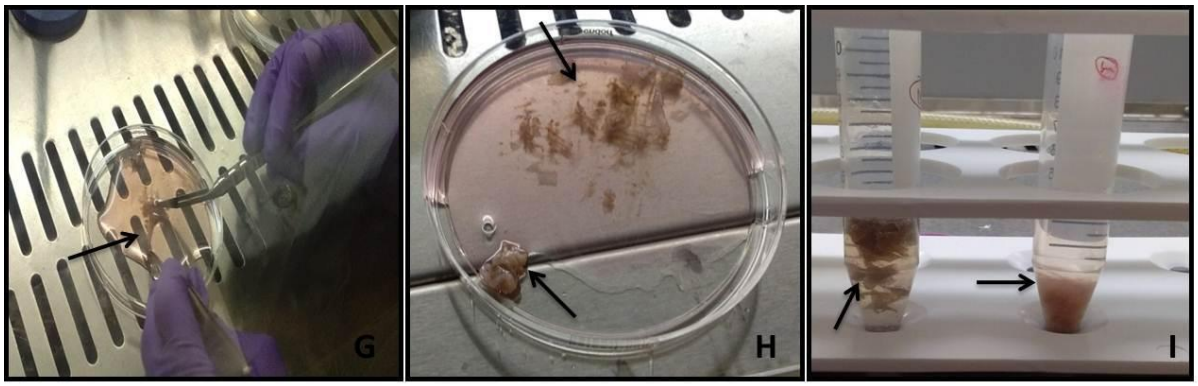


A. Split skin samples in sterile DPBS with antibiotics and antimycotic solution, received from the Dept. of Plastic Surgery, K. S. Hegde Charitable Hospital, Nitte (Deemed to be University) after signing informed consent from the donor.

B, C, D, and E. Skin sample was washed in DPBS with antibiotic and antimycotic solution twice then with 10 µg/ml gentamicin solution and dipped in iodine solution for 3 minutes and finally washed in 75% ethanol for 10 min. Each step was followed by DPBS wash. This sterilization procedure was followed strictly to avoid the sample-oriented contaminations.

F. Skin sample was kept in 0.25% trypsin-EDTA solution for 3 hrs at 37^0 C and for 2 hrs in a shaking incubator for the separation of epidermis from dermis.

Figure 17: Processing of split skin sample for the isolation of melanocytes (Contd.)



G. Collagenase activity was inhibited by washing the cells in DMEM medium with 10% foetal bovine serum (FBS). Later epidermis was separated from dermis using sterile surgical scalpel and blade.

H. Separated epidermis from dermis.

I. Dissociated cells after passing through 100 and 70 micron cell strainers.

4.3 Isolation, establishment and morphological characteristics of melanocytes from skin sample

Human melanocytes were isolated from skin tissue samples, by following enzymatic treatment that separates the dermis and epidermis, and incubation in trypsin-EDTA to obtain a single-cell suspension. In the present study, a total of 10 melanocyte cells lines were successfully established and designated as MEL1 to MEL10. Cell lines such as, MEL1 and MEL5 were also established by following an explant culture system. The processing of skin tissue, primary culture establishment, and morphological characteristics of melanocytes of MEL1 to MEL10 cell lines are presented. The melanocytes established by explant culture, such as MEL1, MEL5 and MEL8 are presented in **Figures 18, 19 and 20**, respectively. The remaining cell lines established by suspension culture are presented in **Figures 21 to 24**.

As explained in the methods section, isolated epidermal tissue remnants were pooled in a trypsin/EDTA solution and single-cell suspensions were prepared by filtering and pipetting. Separated cells were collected in serum to stop enzymatic action of trypsin. The pooled epidermal cell suspensions were centrifuged and resuspended in growth medium. Cell suspensions were placed in tissue culture dishes in growth medium containing HMGS. After 48 hrs of seeding cells, the medium was changed and unattached cells were removed. From day 3 onwards till the cells reached confluence, the attachment patterns and morphological features of melanocytes were observed regularly and the images were recorded.

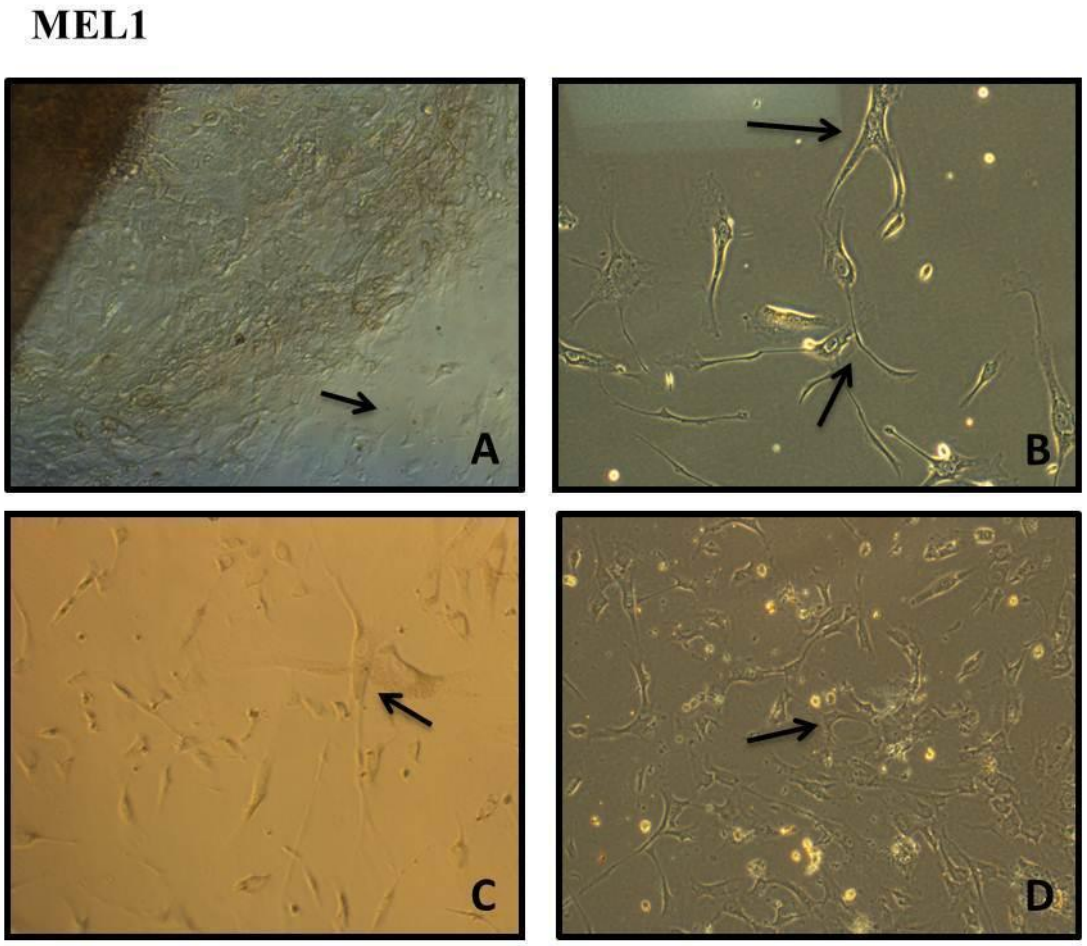
Melanocytes in tissue culture were easily distinguished from keratinocytes and fibroblasts by their morphological characteristics. Melanocytes appeared round, small, spindle-shaped during early stages of culture and later transformed into bipolar or polydendritic features.

Initially, the cultures had a fairly good number of fibroblasts showing the heterogeneity in cell population, but in later passages, their growth and number was reduced. In the primary culture, melanocytes readily attached onto the plastic culture dish/plates and proliferated best in the presence of attachment factor and the supplementation of HMGS.

During the initial culture period, a small number of melanocytes based on their characteristic smooth, bipolar shapes were observed. In some cultures, melanocytes commonly formed clusters of up to 10-20 cells and a close contact was observed between the two melanocyte populations, by extending cytoplasmic processes. In a couple of cases, melanocytes were observed to emerge from small fragments of tissues that attached to the bottom of culture dishes/plates. Primary cultures reached confluence after approximately 2- 3 weeks. At this point of time, the predominant cell type was melanocytes and a small portion of keratinocytes and fibroblasts were absent from cu1tures after the second or third passage. Melanocyte cultures were maintained up to at least 6 to 8 passages for further analysis.

Melanocytes were isolated and cultured in melanocyte growth media (M254) with melanocyte growth supplements. Cells were attached and continued to grow in this media having bipolar shape. In later passages, dendritic cells were more and the culture needed slightly more time to establish bipolar, proliferative, pure melanocyte culture. Another observed feature was, keratinocytes and fibroblasts could not attach and grow properly in this specific medium after 2-3 passages. Hence, the presence of keratinocytes and fibroblasts were less and could attain pure culture of melanocytes in minimum passages.

Figure 18: Isolation and morphology of melanocytes in explant culture (MEL1)



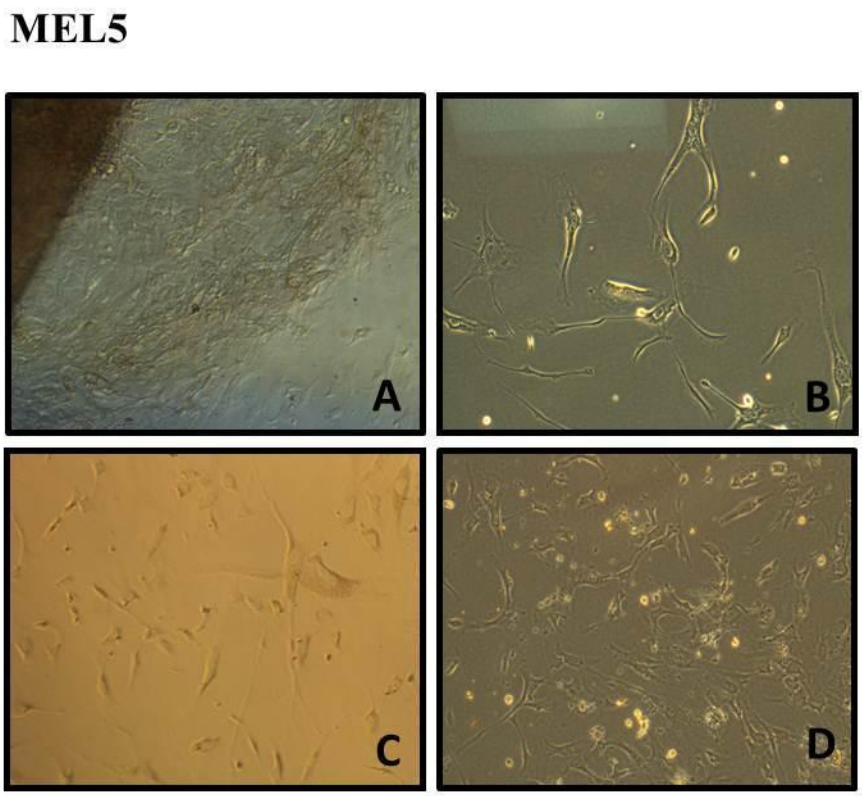
A. Cells are emerging from the epidermal explant at day 10.

B, C. Cells were attaining dendritic morphology on the 15th day of culture in basal MEM-alpha medium supplemented with HMGS (Human melanocyte growth supplements).

D. Human epidermal melanocytes after sub-passage.

Images magnification- 10x

Figure 19: Isolation and morphology of melanocytes in explant culture (MEL5)



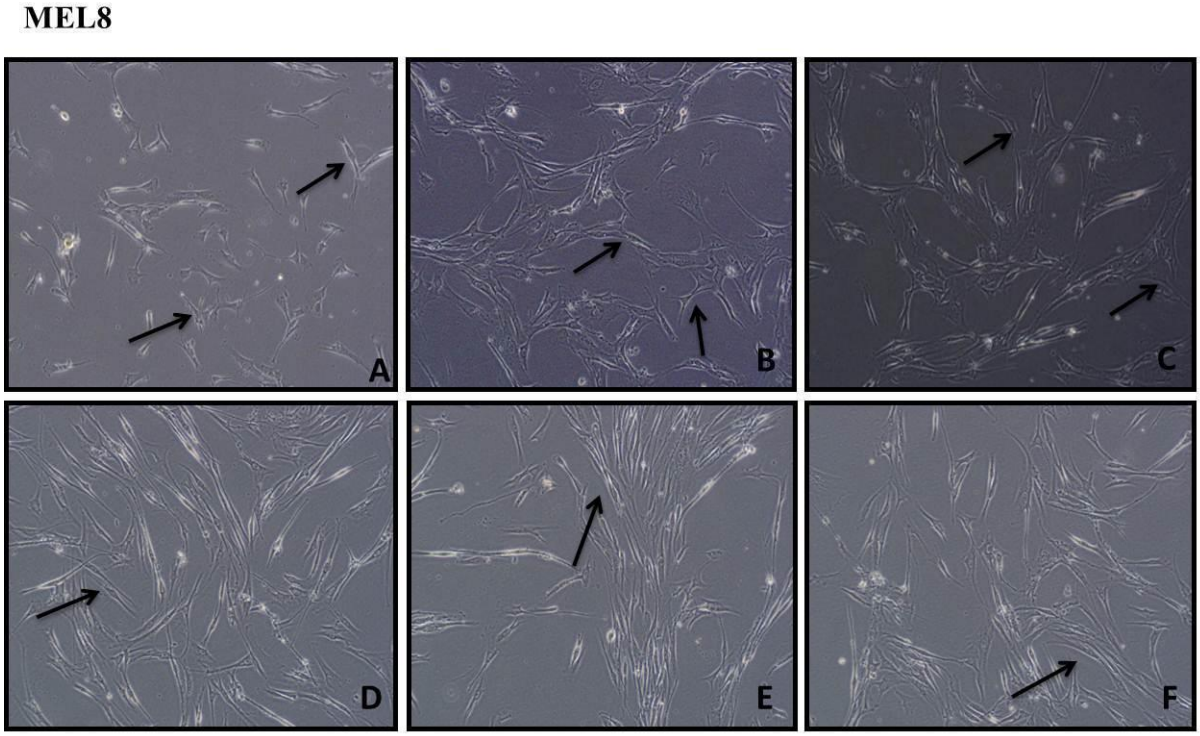
A. Cells emerged from the epidermal explant at day 10.

B, C. Cells were attaining dendritic morphology on the 15th day of culture in basal MEM-alpha medium supplemented with HMGS (Human melanocyte growth supplements).

D. Human epidermal melanocytes after sub-passage.

Images magnification- 10x

Figure 20: Isolation and morphology of melanocytes in explant culture (MEL8)

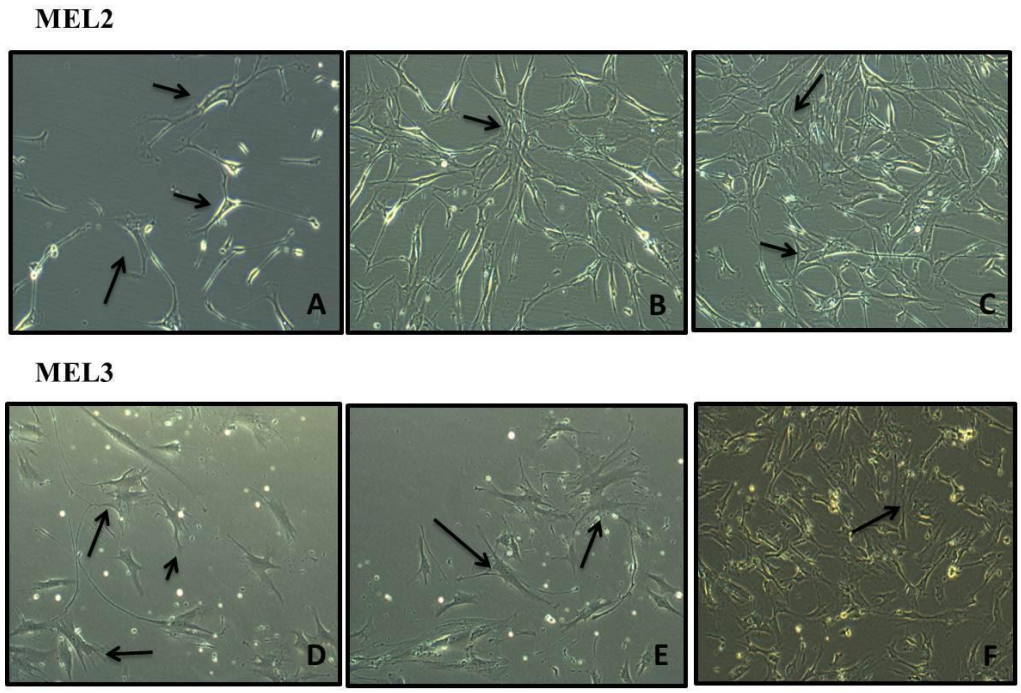


A, B, C: Cell line MEL8 suspension cultured cells showing attachment at P0.

D, E, F: Adhered human melanocytes showed cobblestone (thin arrow-like) structure along with long bipolar and tripolar morphology and a few cells are with dendritic arms in 15 days of culture and passage 1.

Images magnification- 10x

Figure 21: Isolation and morphology of melanocytes in suspension culture (MEL2 and MEL3)



A. Cell line MEL2 on the 3rd day of seeding.

B. A week after primary culture.

C. Cells at Passage 1.

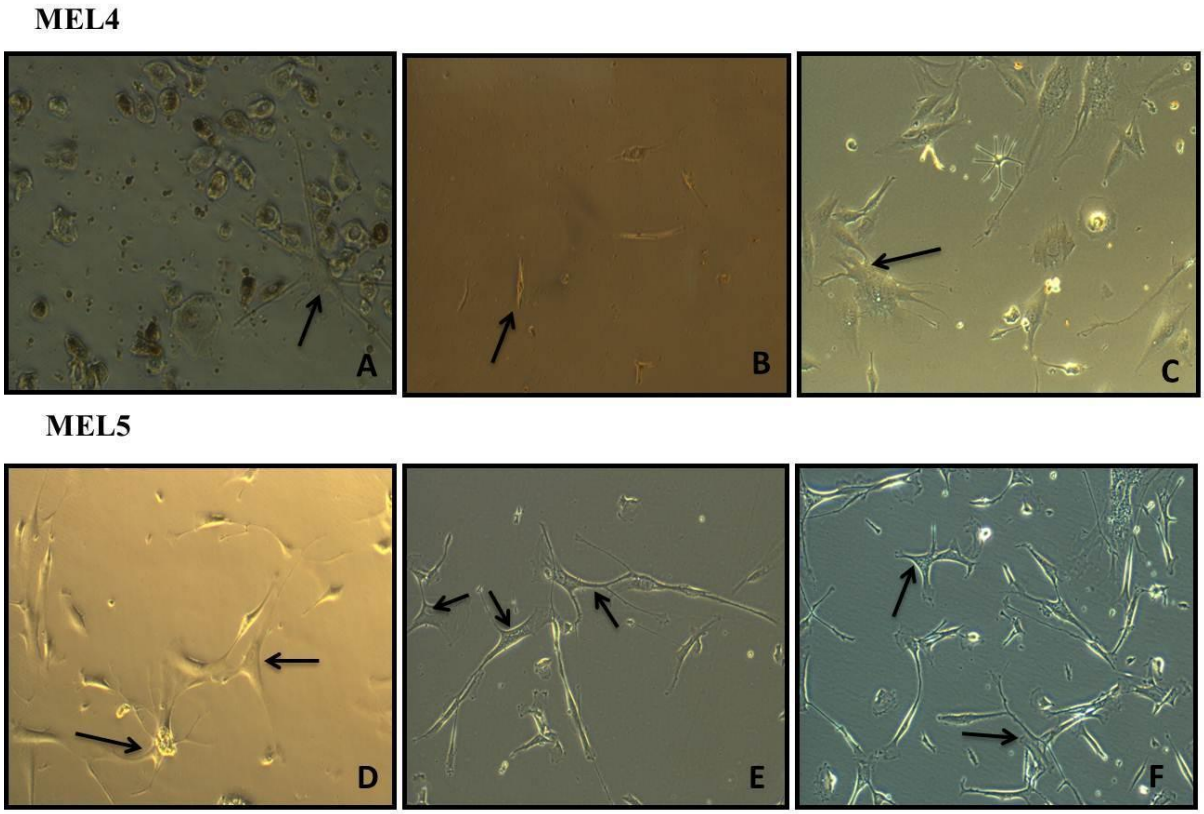
D. Cell line MEL3 on the 3rd day of seeding after given wash with DPBS.

E. Morphology of cells on day 7^{th} of culture.

F. At passage 1 reached 60% confluence. Melanocytes from both the samples showed spindle bipolar or multipolar dendritic processes.

Images magnification- 10x

Figure 22: Isolation and morphology of melanocytes in suspension culture (MEL4 and MEL5)

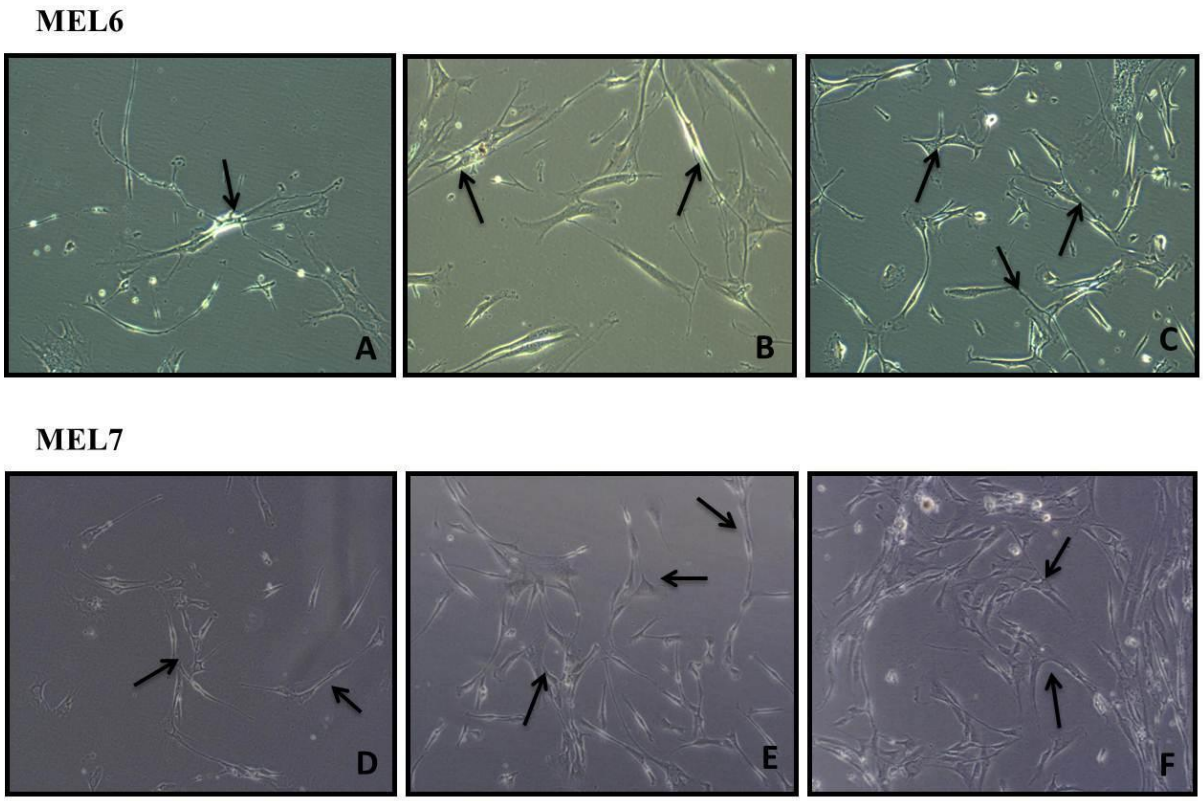


A, B, C: Cell line MEL4 suspension cultured cells showing attachment to the substratum coated with attachment factor at up to day 15 and there was a slow cell proliferation.

D, E, F: Cell line MEL5. Suspension cultured human melanocytes showed bi, tri or multipolar structures indicating the melanocyte morphology.

Images magnification- 10x

Figure 23: Isolation and morphology of melanocytes in suspension culture (MEL6 and MEL7)

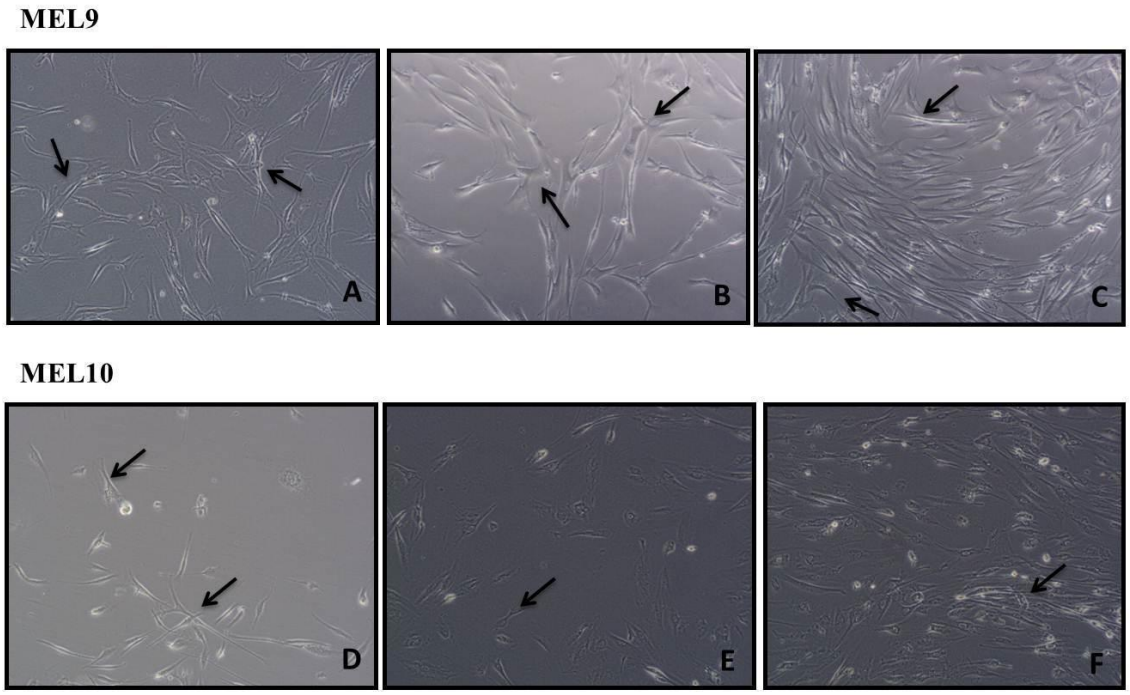


A, B, C: Cell line MEL6 suspension cultured cells showing attachment at P0.

D, E, F: Cell line MEL7. Adhered human melanocytes showed cobblestone (thin arrow-like) structure along with long bipolar and tripolar morphology. A few cells are with dendritic arms in 15 days of culture and at passage 1.

Images magnification- 10x

Figure 24: Isolation and morphology of melanocytes in suspension culture (MEL9 and MEL10).



A. Cell line MEL9 on 4th day of seeding.

B. A week after primary culture.

C. Cells at passage 1.

D. Cell line MEL10 on the 3rd day of seeding after given wash with DPBS.

E. On day 7th and F. At passage 1 reached 60% confluence. Melanocytes from both the samples showed spindle bipolar or multipolar dendritic processes.

Images magnification- 10x

4.4 Cellular, functional and phenotypic characteristics of melanocytes

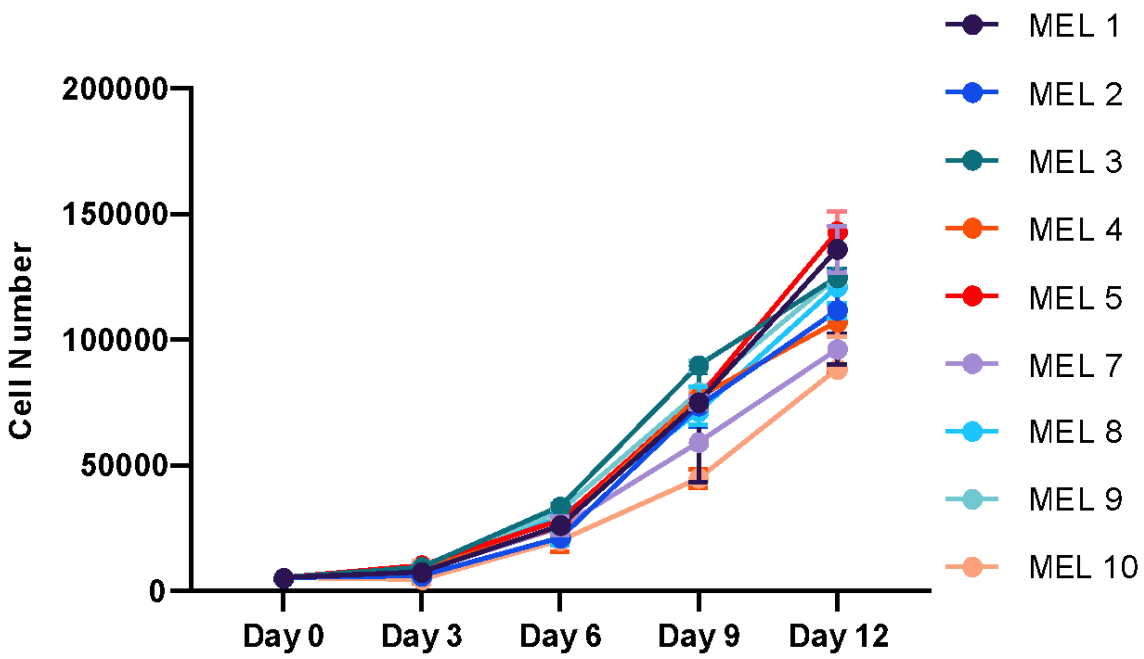
Established melanocytes were characterized based on their proliferation potential, viability, melanin content, tyrosinase activity and the expression of selected melanocyte markers. In the present study, it was aimed to clarify the growth characteristics of melanocytes *in vitro* and understand the mechanisms of culturing autologous melanocytes for the potential treatment of vitiligo.

4.4.1 Proliferation rate of melanocytes

The proliferation rate of melanocytes is presented in this section and the numbers are depicted in **Figure 25**. Melanocytes of passages 2 to 5 were employed for assessing their growth rate. Initially, the cells needed time for adherence to the plastic culture plates and hence the proliferation was slower. By day 6 in culture, the cells started proliferating at higher rates and reached the peak of growth phase in a linear manner by day 9 and day 12 of the culture period. All the established melanocyte cell lines showed higher proliferation potential and without significant differences in their growth behaviour. However, the division time varied slightly between the three different melanocyte cell lines, particularly during the early stages of cell culture.

In all the cell lines, cell number was significantly increased along with the time ($P<0.0001$). From day 9 to day 12 of culture, a significant difference in cell numbers was observed in all the cell lines.

Figure 25: Proliferation rate of melanocytes



Total number is represented in terms of an increase in cell number during the culture period of 12 days.

In all the cell lines, the cell number was significantly increased along with the time ($P<0.0001$) except from day 0 to day 3. From day 9 to day 12, a significant difference in cell numbers was observed in all the cell lines. The differences in terms of P value were varied between $P < 0.0026$ and $P< 0.0001$.

Error bars indicate standard deviation (SD) for mean values obtained from triplicates. Values on Y-axis denote cell number and x-axis denote the duration.

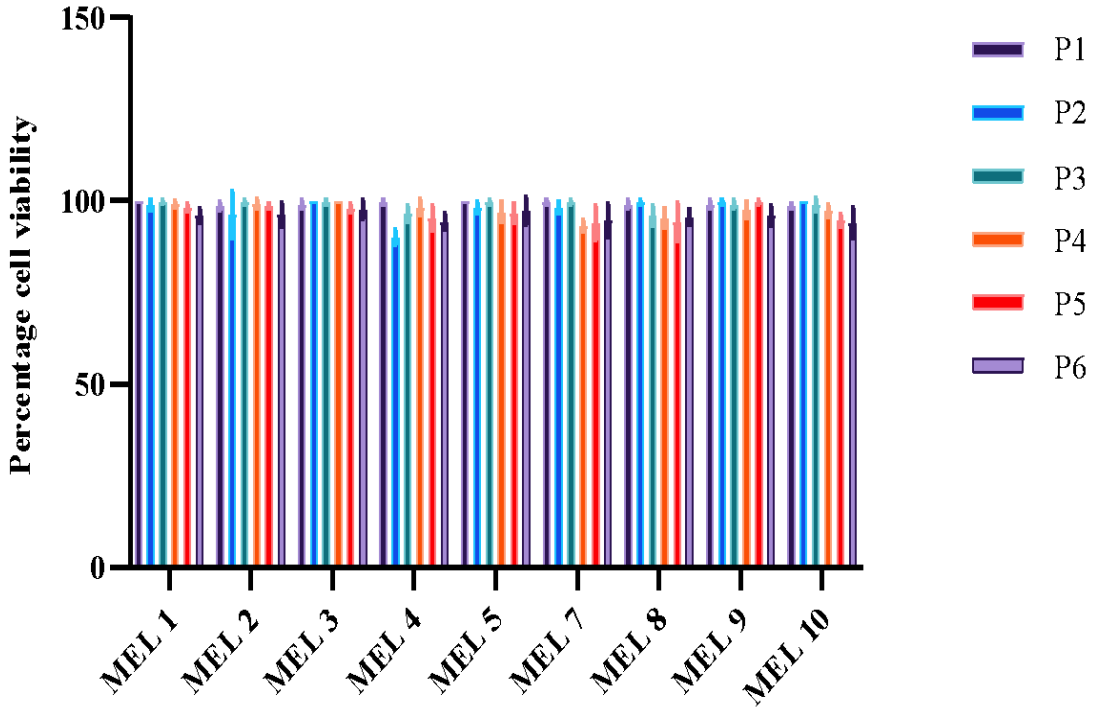
Proliferation assay was performed in triplicates.

4.4.2 Viability assay of melanocytes

Cell viability of cultured human melanocytes for a total of nine cell lines was assessed at different passages from one to six. More than 98% viability was noticed in every passage in all the cell lines when analysed using trypan blue dye exclusion assay method (**Figure 26, Figure 27 and Figure 28**). Melanocytes from all those passages had a proper morphology with dendritic structures and bipolar processes and remained proliferative with maintenance of high level of viability. The percentage of viable cells at all examined passages was greater than 90% and didn't show major differences between the cell lines.

All the cell lines showed more than 90% cell viability in every passage. Hence, there was no significant variation noticed in percentage cell viability except in MEL4, MEL7 and MEL10. In MEL 4, there was a significant difference in cell viability in P1 to P2 ($p<0.0001$), P1 to P5 ($p<0.0392$), P2 to P3, P4, P5 ($p<0.05$). In MEL, the differences were observed between P1 to P4 and P5 ($p<0.05$) and P3 to P4 and P5 ($p<0.05$). In MEL10, significant variation was noticed between P2 to P4 and P5 ($p<0.05$). Between the cell lines, there was no significant difference observed except in P2 for MEL1 vs MEL4 ($p<0.0001$).

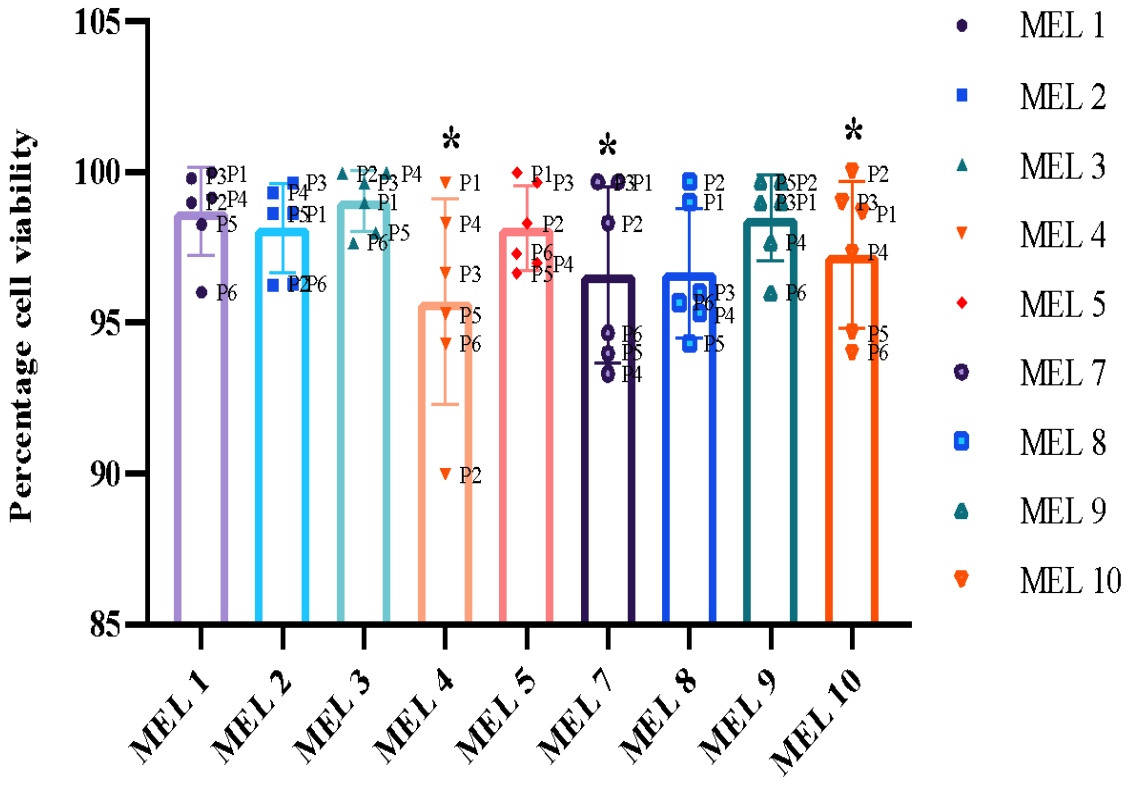
Figure 26: Cell viability of cultured human melanocytes (Total number=10) from passage 1 to passage 6



More than 98% viability was noticed in every passage in all the melanocyte cell lines when analysed using trypan-blue dye exclusion assay method.

Viability assay was performed with minimum of triplicate experiments.

Figure 27: Cell viability of cultured human melanocytes (Total number=10) from passage 1 to passage 6

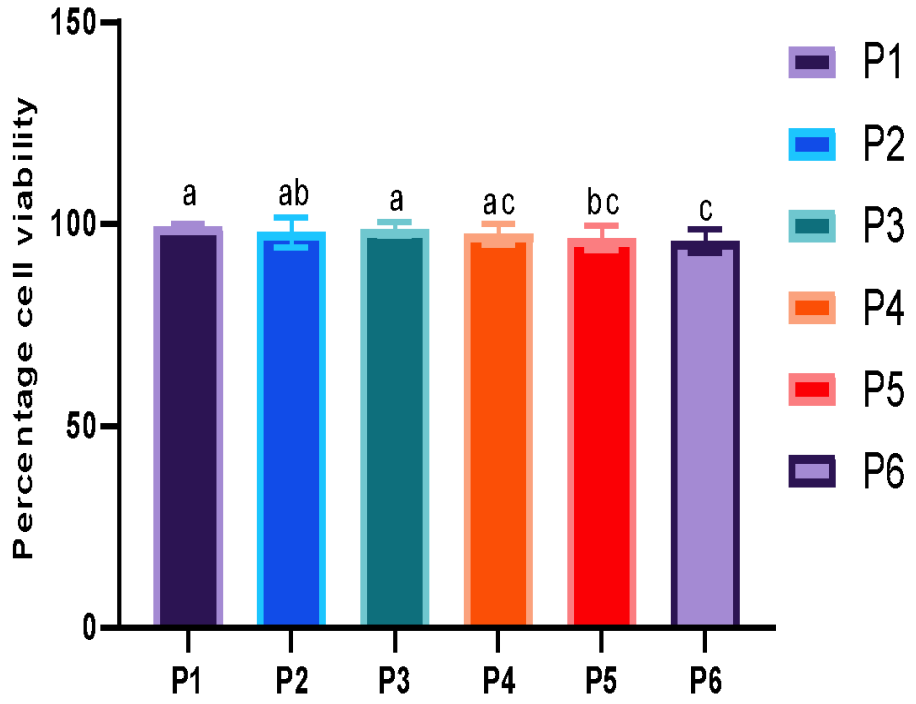


Percentage values of viability from passage 1 to passage 6 for every melanocyte cell line is presented in a single bar diagram.

* indicates a significant difference in values between the cell lines ($P<0.05$).

Viability assay was performed with minimum of triplicate experiments.

Figure 28: Average cell viability of cultured human melanocytes (n=10) from passage 1 to passage 6



Average percentage viability values from passage 1 to passage 6 for melanocyte cell lines (n=10) is presented.

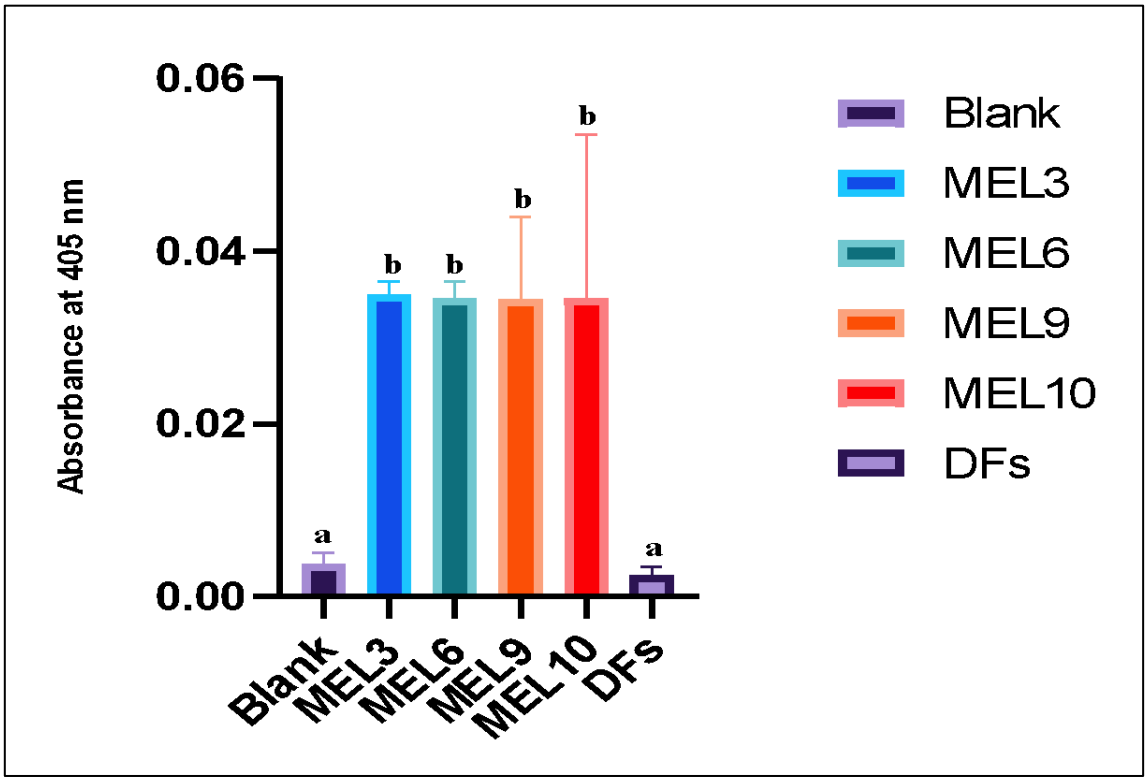
a, b, c indicates a significant difference in values between the passages ($P<0.05$).

Viability assay was performed with minimum of triplicate experiments.

4.4.3 Total melanin content assay

Total melanin content assay was performed to estimate the melanin levels in melanocytes and absorbance was taken in 405 nm in ELISA reader. A blank was taken without any cells and the dermal fibroblasts (DFs) were considered as a negative control for melanocytes. Every melanocyte cell line showed higher absorbance than the negative control which indicated the production of melanin during culture expansion period (**Figure 29**). A significant difference in absorbance values between the cell lines and blank or negative control (DFs) ($P<0.05$, $P<0.0001$) was observed. The melanin content was recorded at the absorbance at which both pheomelanin and eumelanin absorb light without the interference of pH from the cell culture medium.

Figure 29: Total melanin content assay



Total melanin content assay was performed to estimate the melanin content in melanocytes and absorbance was taken in 405 nm in ELISA reader. A blank was taken without any cells and dermal fibroblasts (DFs) were considered as a negative control for melanocytes. Every melanocyte cell line showed higher absorbance than the negative control which indicated the production of melanin during culture expansion period.

a & b indicate significant difference in values between the cell lines and blank or negative control (DFs) ($P<0.05$, $P<0.0001$).

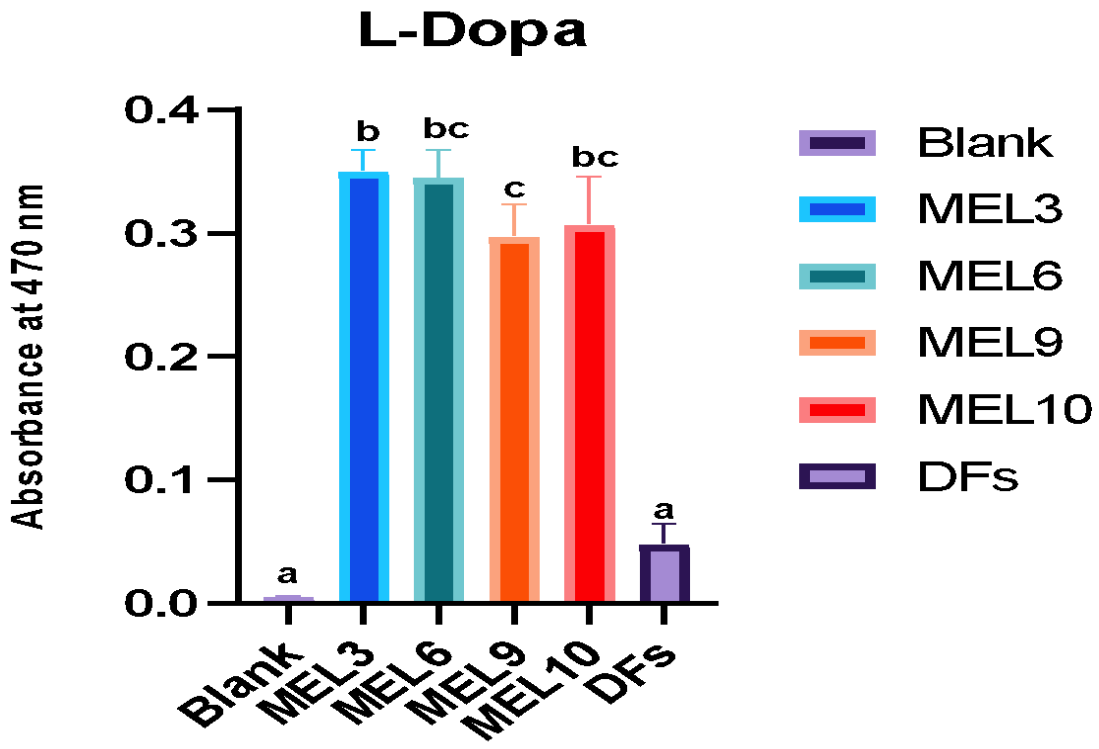
Melanin content assay was performed in triplicates.

4.4.4 Estimation of tyrosinase activity by L-DOPA assay

Tyrosinase activity in melanocytes was carried out by following the L-DOPA assay method. A blank value was recorded without any cells and dermal fibroblast cells were considered as a negative control for melanocytes. Tyrosinase activity was found to be highest in melanocyte cell lines as compared to dermal fibroblast cell lines (**Figure 30**). The results indicated that the established melanocytes were positive for the DOPA reaction.

L-Dopa activity was significantly higher in melanocytes when compared to dermal fibroblast as well as blank ($P<0.0001$). Between the melanocyte cell lines, there was no significant variation recorded except for MEL3 Vs MEL-9 ($p<0.0219$).

Figure 30: Estimation of tyrosinase activity by L-DOPA assay



Tyrosinase activity in melanocytes was carried out by following the L-dopa assay method. A blank was taken without any cells and dermal fibroblast (DFs) cells were considered as a negative control for melanocytes.

Tyrosinase activity was found to be highest in melanocyte cell lines as compared to dermal fibroblast (DFs) cell lines.

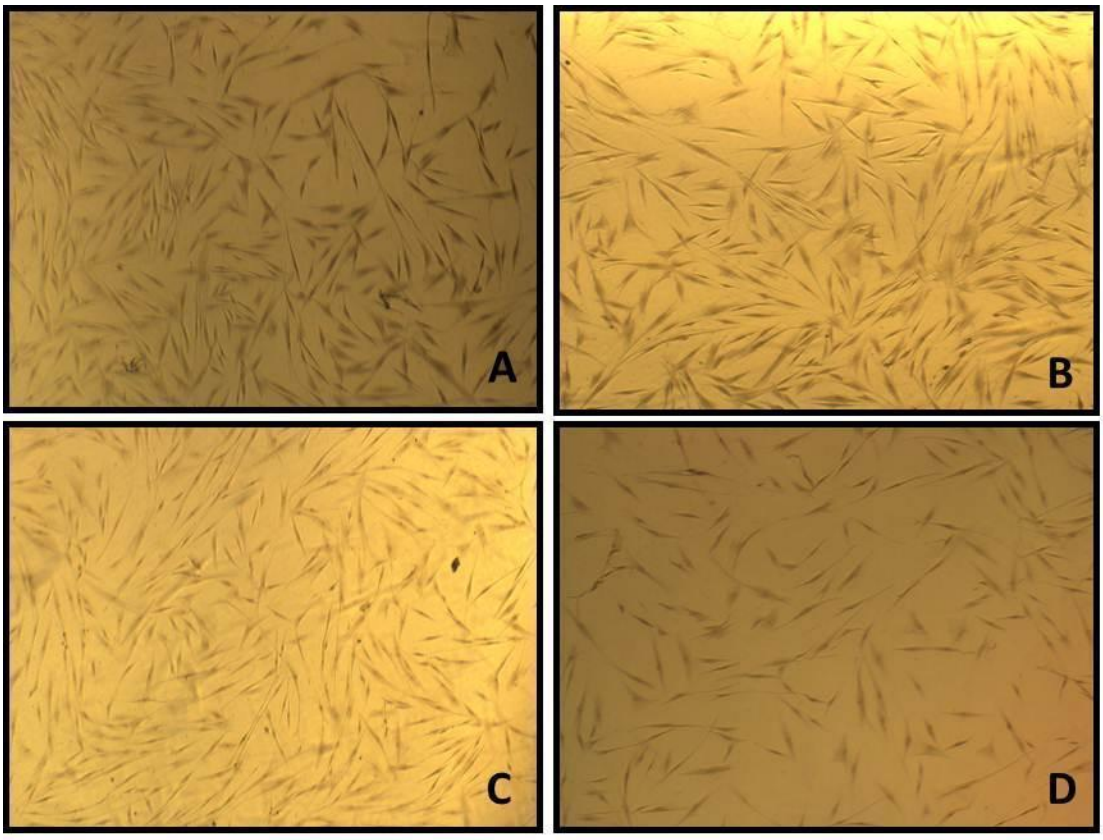
Between the melanocyte cell lines, there was no significant difference in L-DOPA recorded except for MEL3 Vs MEL-9 ($p<0.0219$).

a, b & c indicate significant difference in values between the cell lines and blank or negative control (DFs) ($P<0.05$). L-DOPA assay was performed in triplicates.

4.4.5 Staining of melanocytes by L-DOPA

The confirmation of melanocytes (MEL2, MEL3, MEL4 and MEL6 cell lines) by L-DOPA staining was examined by phase-contrast microscopy. Cultured cells were fixed in paraformaldehyde and stained in L-DOPA for 30 min at 37°C. Melanocytes were turned into dark brown after the staining procedures, and exhibited the positivity to the reaction (**Figure 31**). Melanocytes in the shape of dendritic structures were predominantly stained by DOPA and the nucleus was slightly smaller than dendritic features.

Figure 31: Confirmation of melanocytes by L-Dopa staining



A. MEL2 cell lines

B. MEL3 cell lines

C. MEL4 cell lines

D. MEL6 cell lines

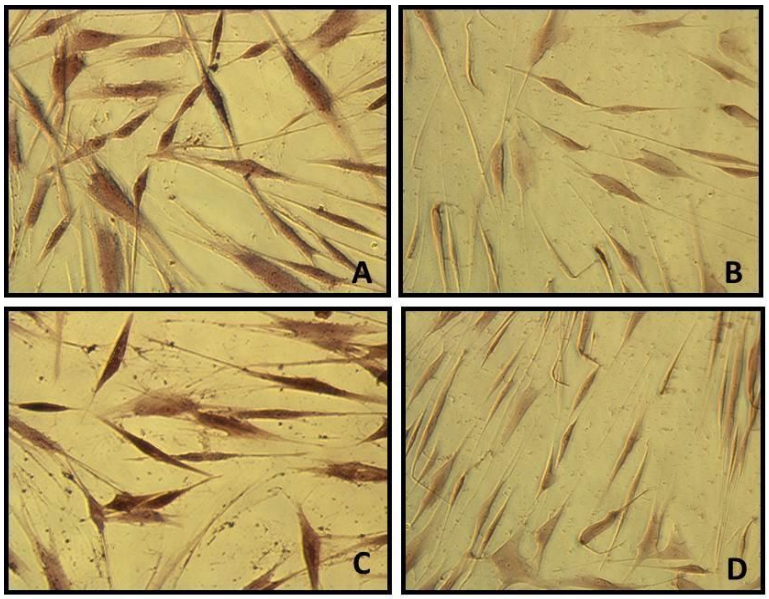
Cells were fixed in paraformaldehyde and stained in 1M L-DOPA for 30 minutes at 37^0C. Cells stained dark brown after staining.

Image magnification- 10x.

4.4.6 Melanin bleach assay

In addition to the above, melanin bleach assay was also performed using different cell lines. Melanocytes as a control without bleach, and the cells bleached with oxalic acid that removed the melanin pigmentation were clearly observed after the assays (**Figure 32**). These results confirmed that the cells isolated from the epidermal tissues were indeed melanocytes. With a starting material of a very small amount of tissue, a good number of human melanocytes were established with a pure population by the stage they reached passage 3 or 4. Although the melanocytes were isolated from various donors, significant differences in their morphological and proliferation rates were not observed. Further, the functional features of melanocytes, such as melanin synthesis and tyrosinase activity were clearly demonstrated by specific assays in the cultured cells.

Figure 32: Melanin bleach assay



A, B: Cell line MEL3. A is the control without bleach.

B is cells bleached with oxalic acid which removes the melanin pigmentation

C, D: Cell line MEL9. C is the control without bleach.

D is cells bleached with oxalic acid which removes the melanin pigmentation.

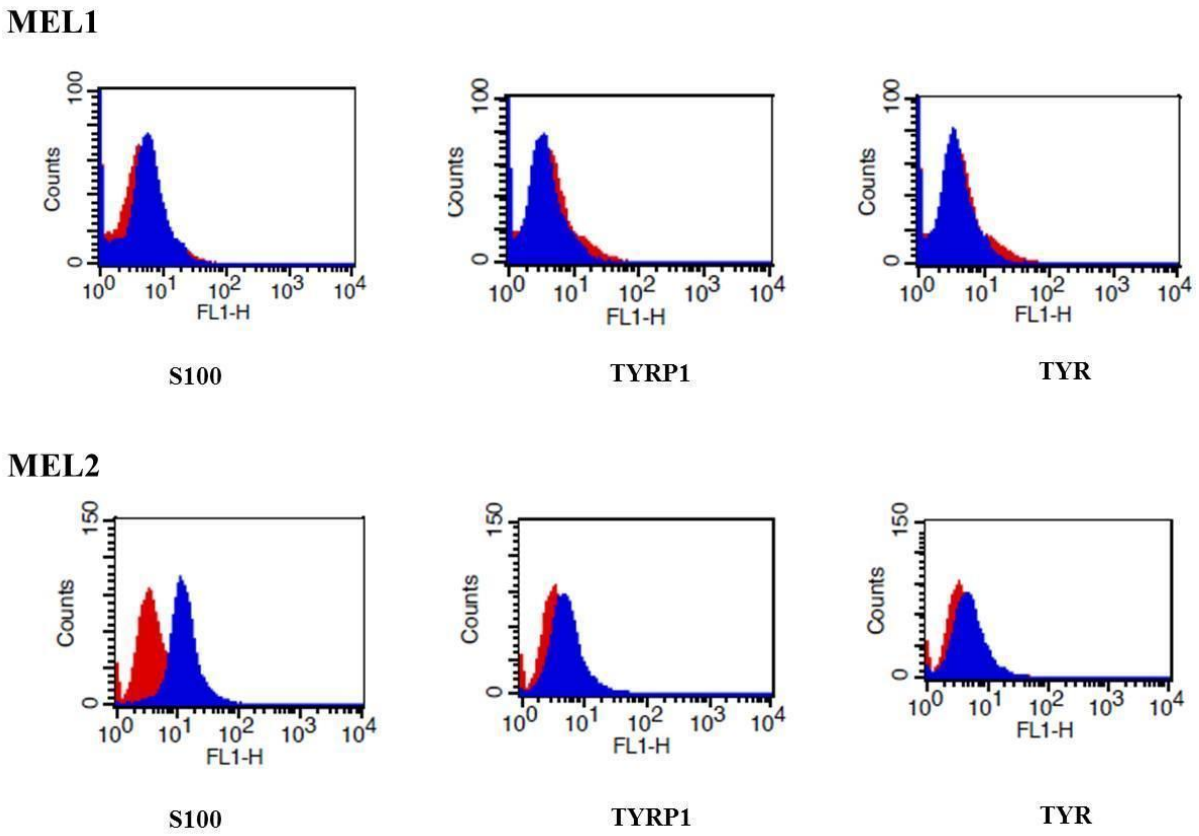
Image magnification- 10x.

4.4.7 Expression of melanocyte specific markers analysed by flow cytometry

The flow cytometry analysis of melanocytes (MEL1, MEL2, MEL3, MEL7 and MEL8 cell lines) was carried out to quantitatively examine the melanocyte-specific marker expression. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining (**Figure 33, Figure 34 and Figure 35**).

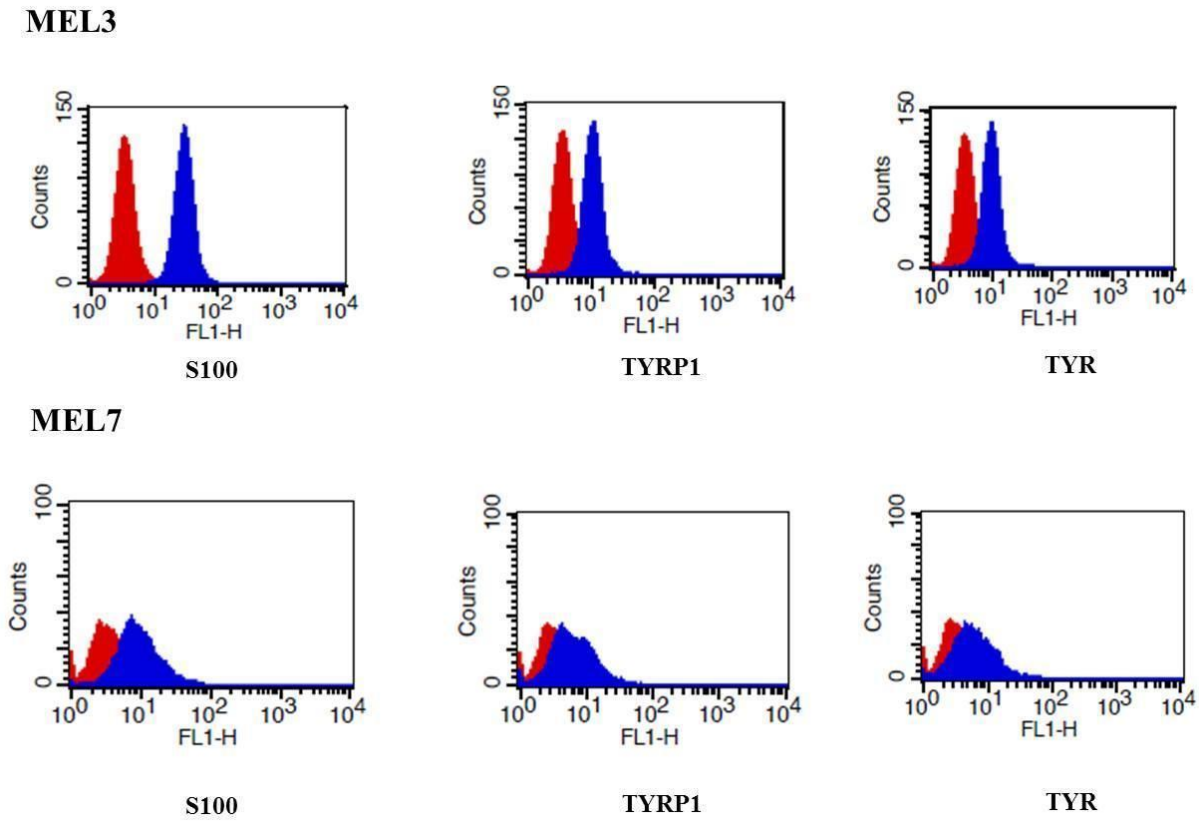
The expression of S100 was higher in all the cell lines examined. However, the expression of other melanocyte-specific markers, such as TYRP1 and TYR showed varied levels of positivity. Among the cell lines, MEL3 had a higher expression of these selected markers.

Figure 33: Representative results of flow cytometry analysis of melanocytes (MEL1 and MEL2 cell lines)



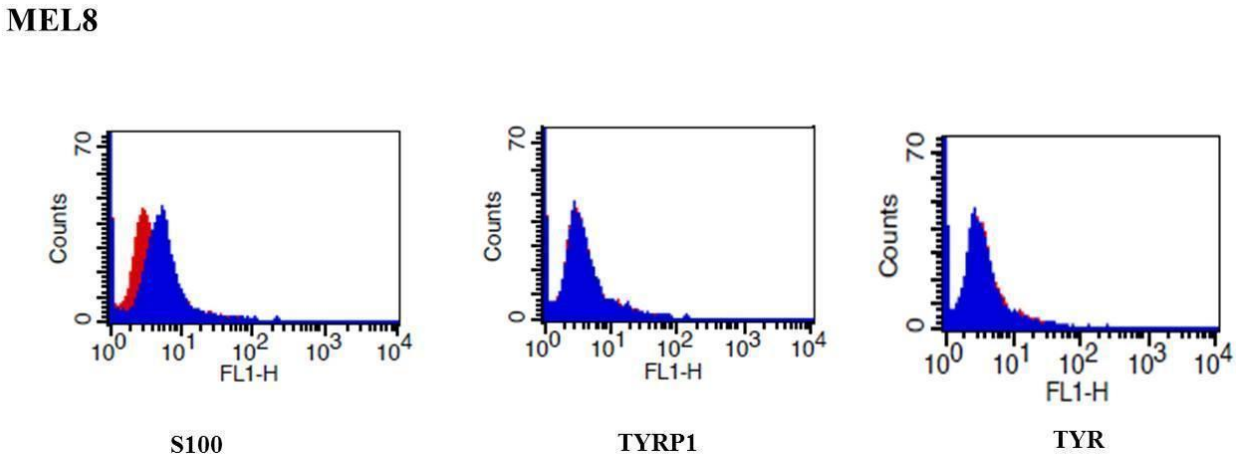
Melanocyte-specific marker expression was quantitatively examined. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody. Samples were analysed in duplicates.

Figure 34: Representative results of flow cytometry analysis of melanocytes (MEL3 and MEL7 cell lines)



Melanocyte-specific marker expression was quantitatively examined. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody. Samples were analysed in duplicates.

Figure 35: Representative results of flow cytometry analysis of melanocytes (MEL8 cell line)

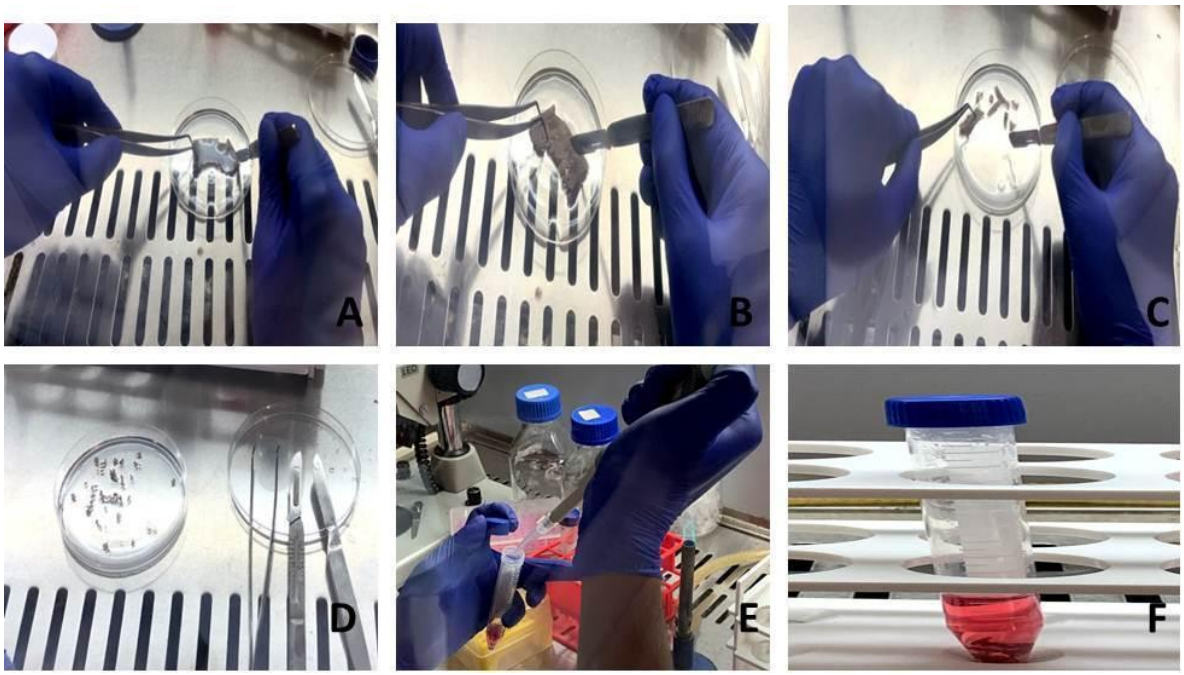


Melanocyte-specific marker expression was quantitatively examined. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody. Sample was analysed in duplicates.

4.5 Processing of skin for the isolation and establishment of keratinocytes

The steps involved in the processing of skin samples for the isolation of keratinocytes are presented in **Figure 36**.

Figure 36: Processing of split skin sample for the isolation of keratinocytes



A, B: Washing with sterile DPBS with 2X penicillin and streptomycin followed by 2 µg/ml fungizone and 10 µg/ml gentamicin solution. Further, the skin tissue surface was sterilized with 75% ethanol iodine solution.

C, D: Skin tissue was minced using a sterile surgical blade.

E: Minced tissue was incubated with 0.25% trypsin solution for 1.30 hrs.

F. Epithelial cells were scraped using a sterile scalpel and kept in culture medium.

4.6 Isolation, establishment and morphological features of keratinocytes from skin sample

An *in vitro* method followed for the establishment of primary culture of human keratinocytes was similar to that of melanocytes. Keratinocytes were isolated from skin tissue samples using trypsin-EDTA digestion for collecting the cells in suspension. In the present study, a total of 10 keratinocyte cell lines were successfully established and designated as KRT1 to KRT10. Further, an explant culture method was tried for establishing KRT5 cell line. The processing of skin tissue, primary culture establishment, and morphological characteristics of melanocytes of KRT1 to KRT10 cell lines are presented in this section.

The keratinocytes established by explant culture, such as KRT5, KRT8, KRT9 and KRT10 are presented in **Figures 37, 38** and **39**, respectively. The remaining cell lines established by suspension culture are presented in **Figure 40**, **Figure 41** and **42**.

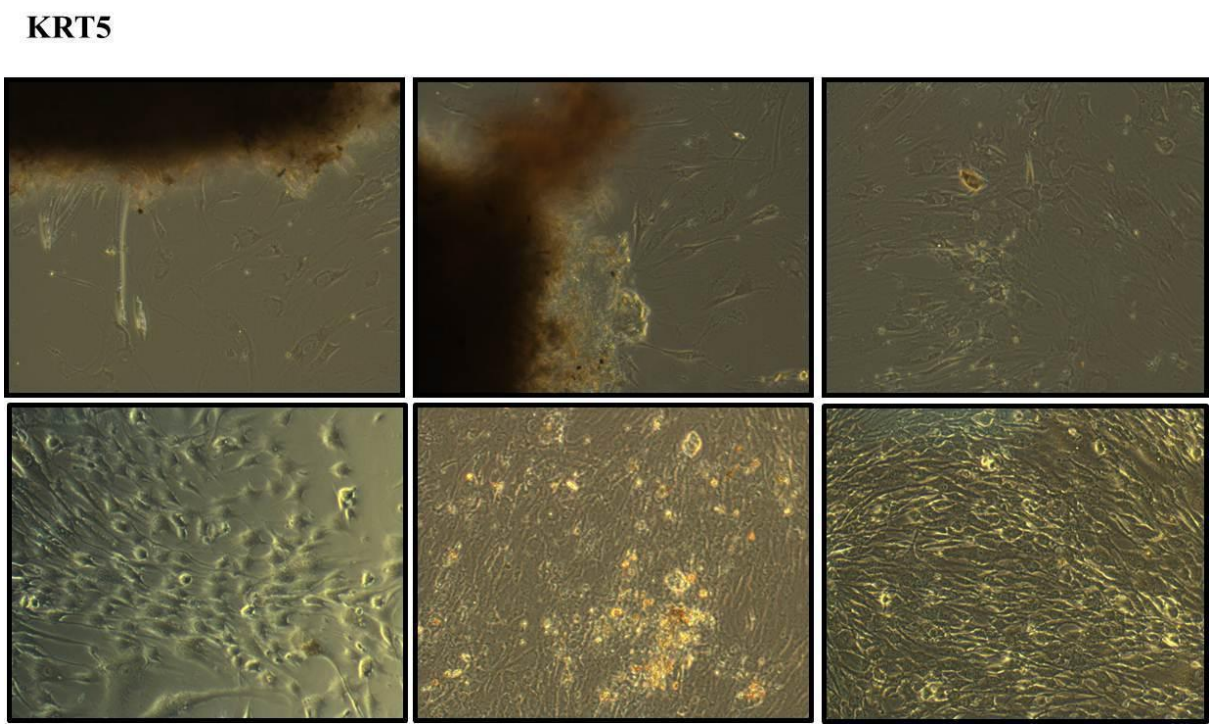
Keratinocytes were first observed growing out of the tissue explant or the attached cells in suspension culture. A monolayer of cells was formed between 48 and 96 hrs. By day 4, the outgrowth of cells surrounded the explants and over day 5 and 6, this outgrowth continued to expand. After 6 days in suspension culture, cells with spindle-shaped fibroblast or melanocyte morphology were also visible along with the keratinocyte ring. Sufficient numbers of cells for harvesting were attained in 2 to 3 weeks. When subcultured in Epilife medium supplemented with growth factors, these cells exhibited exemplary attachment and growth capabilities. After 2 to 3 days, cells were attached and were growing as a monolayer. Under these conditions, cells were more adhesive and formed tight colonies of keratinocytes. The cells showed epithelial morphology resembling a ‘pavement stone’. The presence of large and flat cells was

also noticed. Importantly, no fibroblast contamination was observed in the cultures of later passages. Confluent keratinocyte monolayers were generated in 2 weeks after passaging.

Established keratinocytes were highly proliferative around 2 weeks of seeding cells in suspension culture. Adhered keratinocytes had typical morphological characteristics and could be identified along with a small population of melanocytes and fibroblasts during the early stages of culture expansion. From a small skin tissue, it was possible to obtain confluent dishes within 3 to 4 weeks of culture. This was sufficient for sub-passaging and conducting the experiments at later passages. When seeding keratinocytes after trypsinization, more than 90% of cells were attached and initiated the proliferation within 24 to 48 hrs.

Direct outgrowth of keratinocytes from tissue explant was typically noticed within 3 to 4 days if successful. After 14 days in primary culture, a colony of cells from a single tissue piece was observed. For obtaining larger amounts of cells, subcultures were carried out as well as direct adaptation to EpiLife medium (at passage 0 or after). They typically reached confluence and were ready for sub-culturing in 2 to 3 weeks. After this, they were split 1:4 once with medium changes every 3 days.

Figure 37: Isolation and morphology of keratinocytes in explant culture (KRT5)



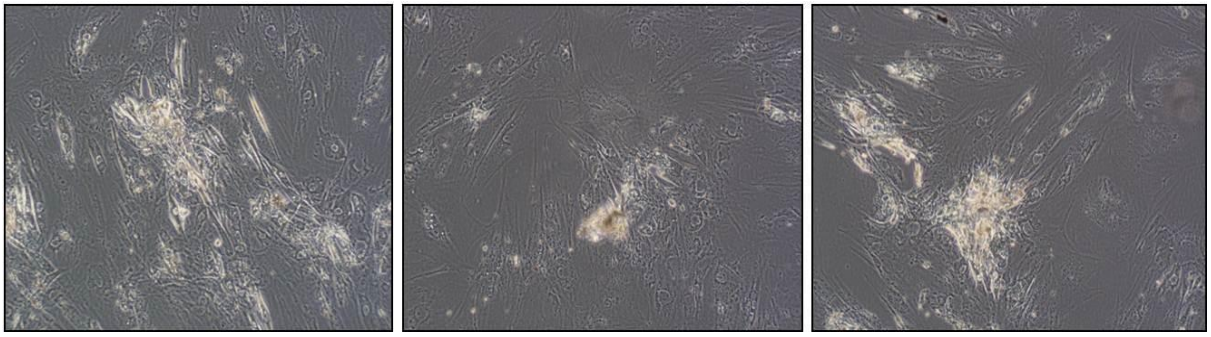
Under these conditions, cells were more adhesive and formed tight colonies of keratinocytes.

The presence of large and flat cells was also noticed.

Image magnification-10x

Figure 38: Isolation and morphology of keratinocytes in explant culture (KRT8)

KRT8

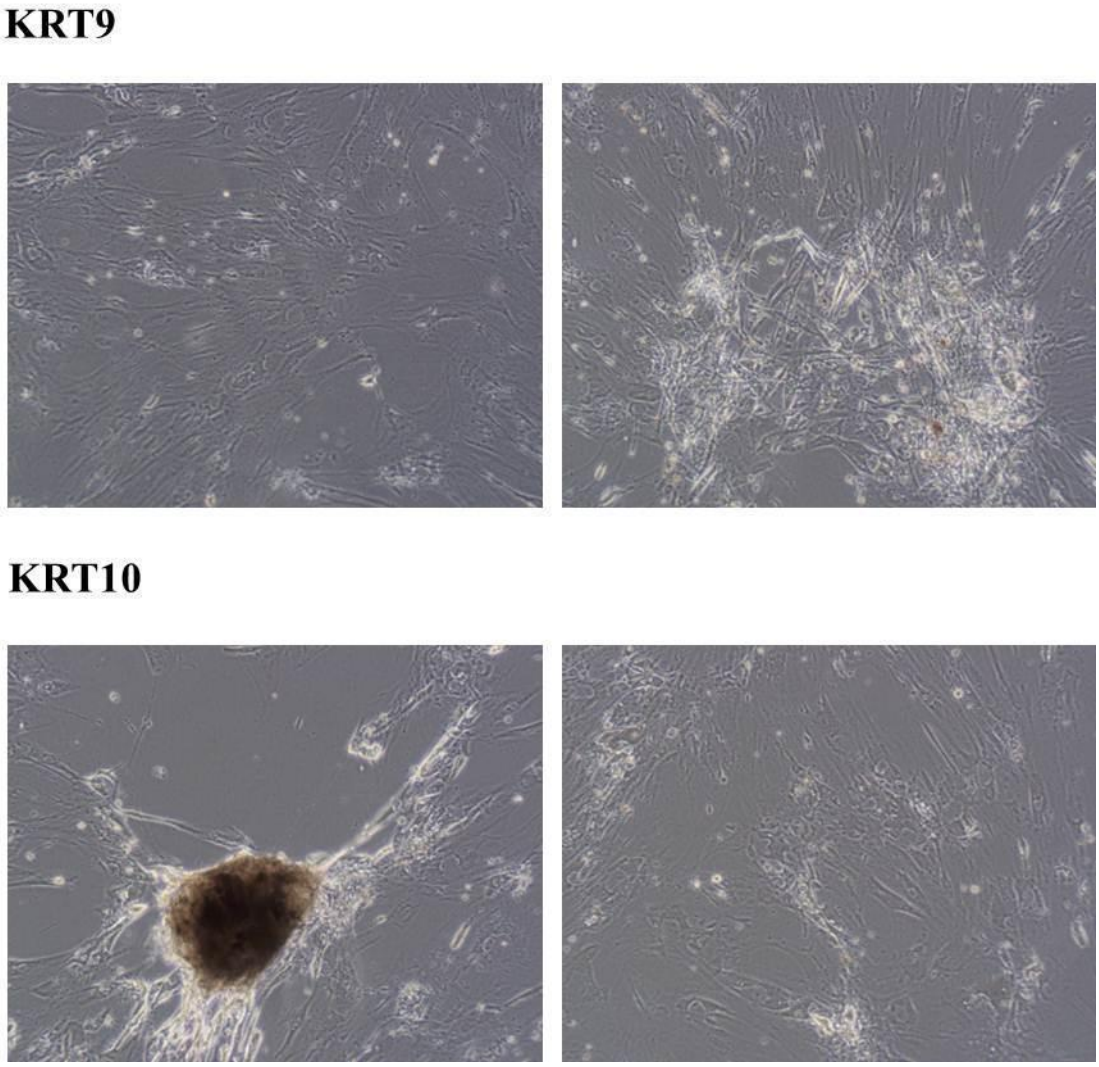


Under these conditions, cells were more adhesive and formed tight clusters or colonies of keratinocytes.

The presence of large and flat cells was also noticed.

Image magnification-10x

Figure 39: Isolation and morphology of keratinocytes in explant culture (KRT8 and KRT10)

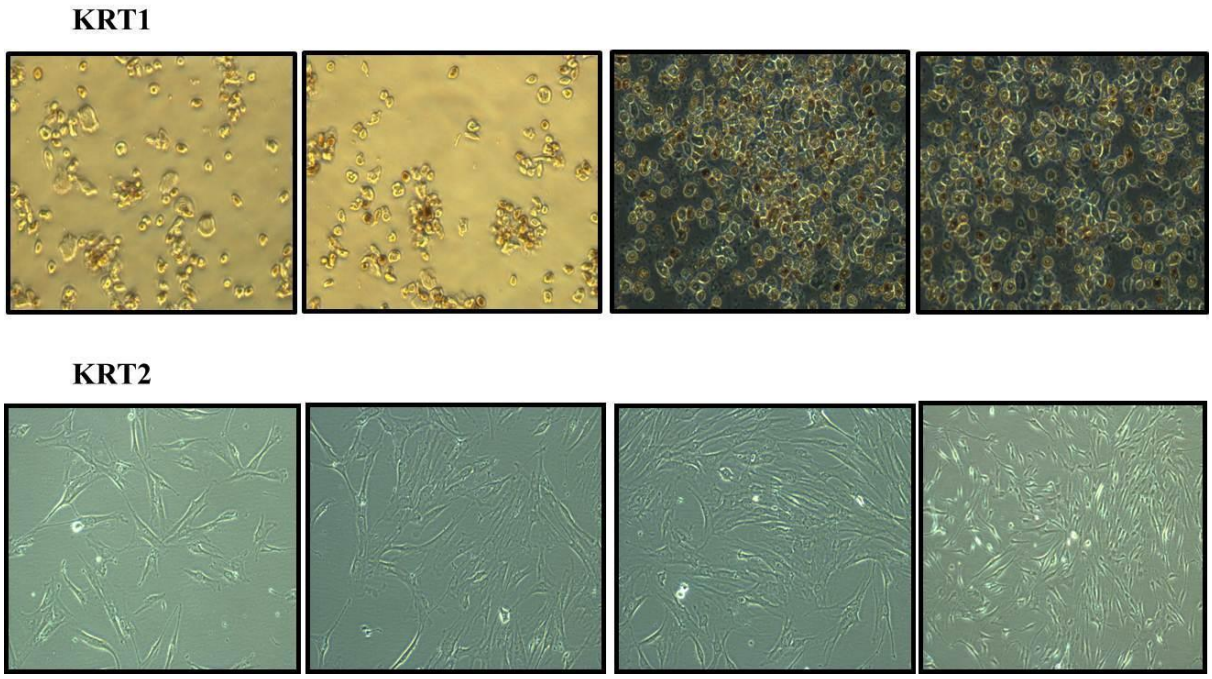


Under these conditions, cells were more adhesive and formed tight clusters or colonies of keratinocytes.

The presence of large and flat cells was also noticed.

Image magnification-10x

Figure 40: Isolation and morphology of keratinocytes in suspension culture (KRT1 and KRT2)

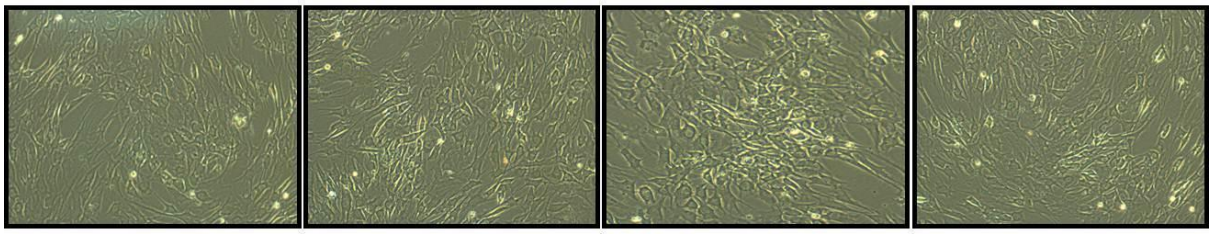


Phase-contrast microscopy revealed that the established keratinocytes formed a pavement-like monolayer under *in vitro* culture conditions.

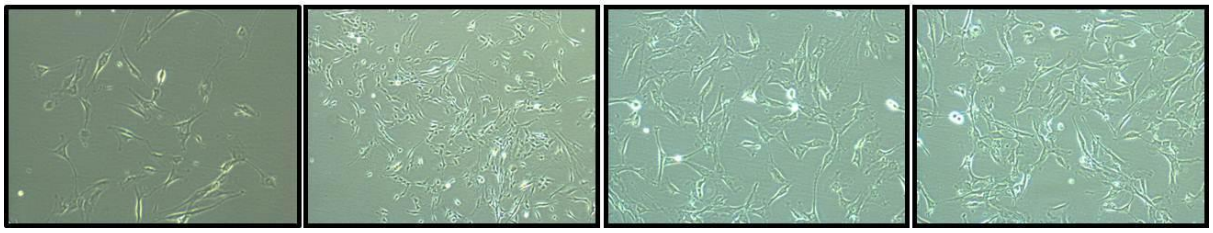
Image magnification-10x

Figure 41: Isolation and morphology of keratinocytes in suspension culture (KRT3 and KRT4)

KRT3



KRT4

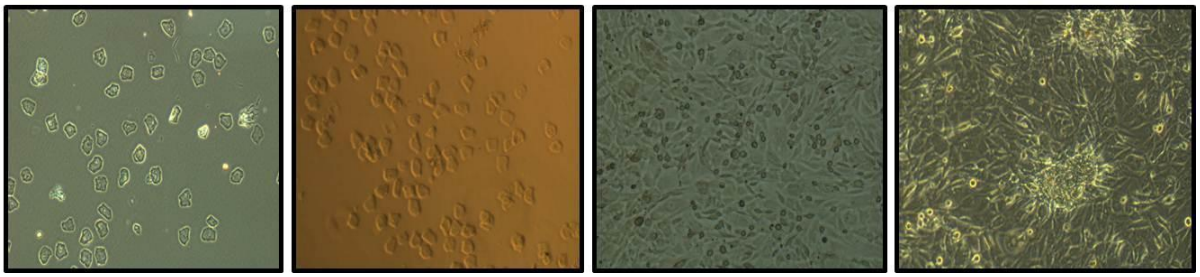


Phase-contrast microscopy revealed that the established keratinocytes formed a pavement-like monolayer under *in vitro* culture conditions.

Image magnification-10x

Figure 42: Isolation and morphology of keratinocytes in suspension culture (KRT6)

KRT6



Phase-contrast microscopy revealed that the established keratinocytes formed a pavement-like monolayer under *in vitro* culture conditions.

Image magnification-10x

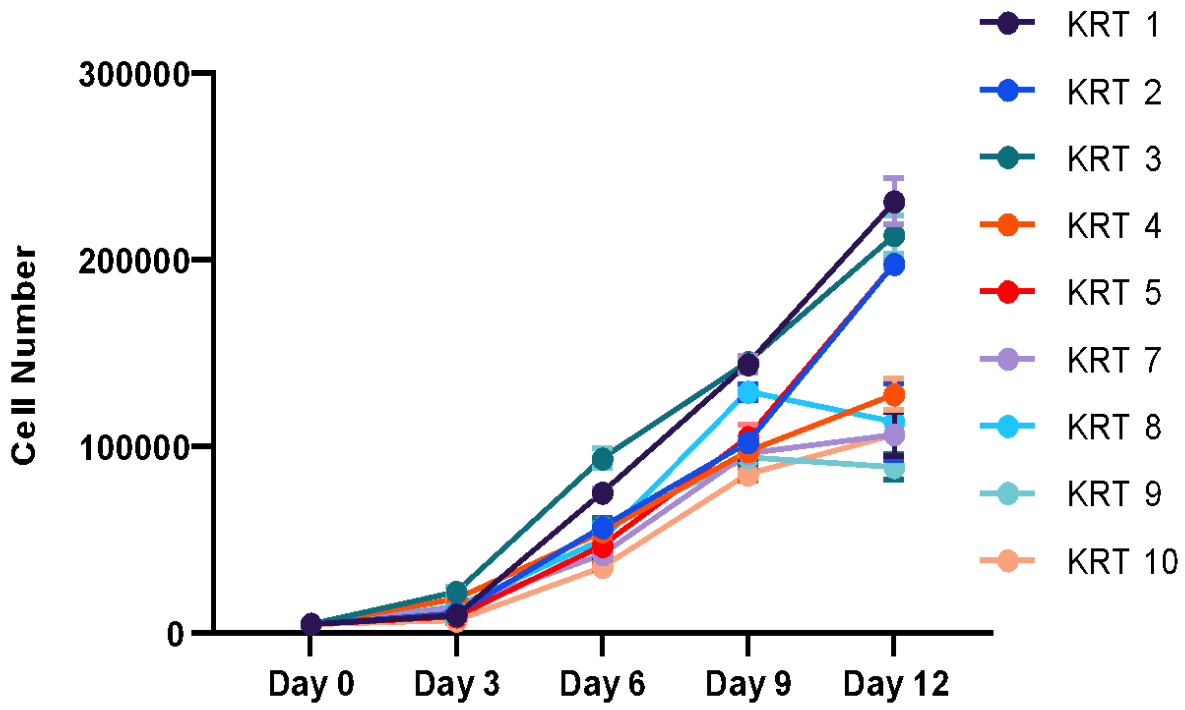
4.7 Cellular and phenotypic characteristics of keratinocytes

In vitro established keratinocytes were characterized based on their proliferation potential, viability, and the expression of selected keratinocyte-specific markers. In the present study, it was aimed to assess the growth characteristics of keratinocytes *in vitro* and understand the mechanisms of culturing autologous cells for establishing the optimal co-culture system with melanocytes.

4.7.1 Proliferation rate of keratinocytes

Proliferation rate of keratinocytes during the *in vitro* expansion for a period up to 12 days was examined. The cells were trypsinized and counted using a hemocytometer on every 3rd day. Initially, the cell growth rate was slightly slower. However, from day 6 onwards, there was a considerable increase in proliferation in all the cells cell lines was observed (**Figure 43**). Some of the cell lines showed greater proliferation even after day 9 in culture. Significant difference ($P<0.05$) in the cell number was observed between the time points of analysis.

Figure 43: Proliferation rate of keratinocytes during the *in vitro* expansion period up to 12 days



All keratinocyte cell lines showed significant increase in cell number as the time increased ($p<0.001$) except from day 0 to day 3.

From day 6 to day 12, a significant difference in cell numbers was observed in all the cell lines. The differences in terms of P value was <0.05.

Error bars indicate standard deviation (SD) for mean values obtained from triplicates.

Values on Y-axis denote cell number and x-axis denote the duration.

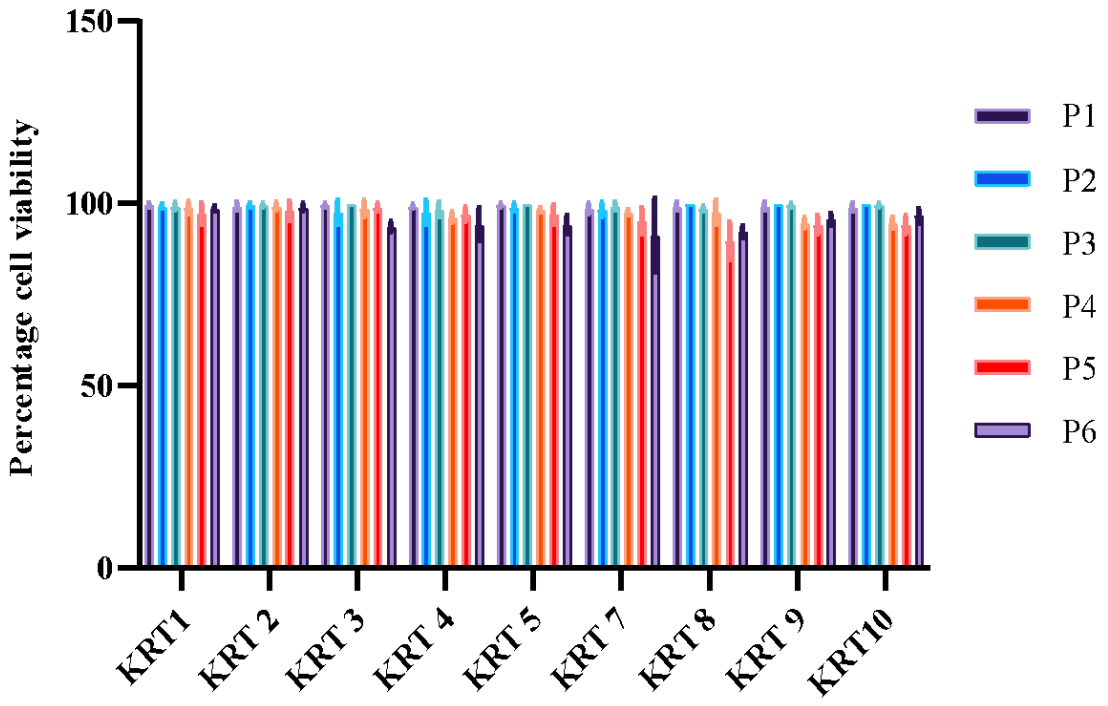
Proliferation assay was performed in triplicates.

4.7.2 Viability assay of keratinocytes

Viability of keratinocytes at different passages showed very high levels under *in vitro* culture conditions. Graphical representation of percentage values of viability of keratinocyte cell lines at different passages is made in **Figure 44**, **Figure 45** and **Figure 46**.

Amongst the keratinocyte cell lines, no significant difference was observed in percentage cell viability. But the KRT8 cell line showed significant variation in cell viability at (P1, 2, 3) vs P (5, 6) ($p<0.005$). Between the cell lines, few of the groups showed significant variation in percentage viability at P5 and P6. Those are at P5, KRT1 vs KRT8, KRT2 vs KRT8, KRT3 vs KRT7, KRT4 vs KRT8, KRT5 vs KRT8 and at P6, KRT1 vs KRT (7, 8) and KRT2 vs KRT (7, 8) at $p<0.05$. Further, slightly lower viability values were noticed in keratinocytes of later passages (Passage 5 and 6).

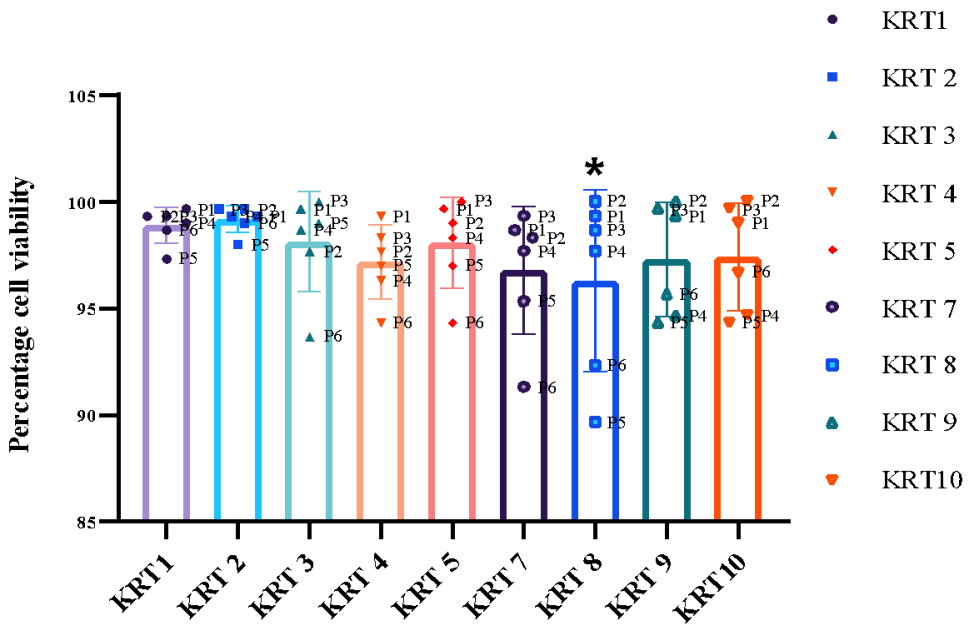
Figure 44: Cell viability of cultured human keratinocytes (Total number=10) from passage 1 to passage 6



More than 96% viability was noticed in every passage in all the keratinocyte cell lines when analysed using trypan-blue dye exclusion assay method.

Viability assay was performed with minimum of triplicate experiments.

Figure 45: Cell viability of cultured human keratinocytes (Total number=10) from passage 1 to passage 6

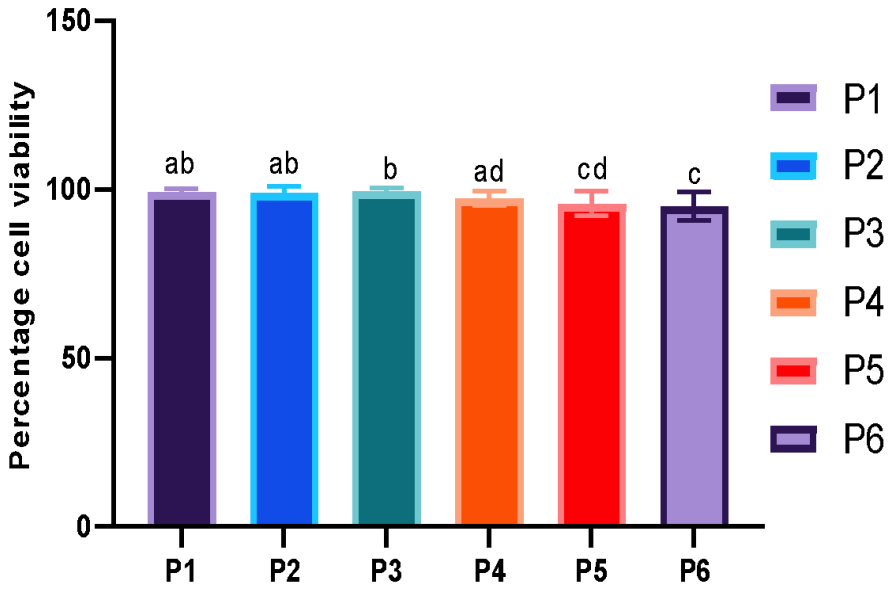


Percentage values of viability from passage 1 to passage 6 for every keratinocyte cell line is presented in a single bar diagram.

* indicates a significant difference in values between the cell lines ($P<0.05$).

Viability assay was performed with minimum of triplicate experiments.

Figure 46: Average cell viability of cultured human keratinocytes (n=10) from passage 1 to passage 6



Average percentage viability values from passage 1 to passage 6 for keratinocyte cell lines (n=10) is presented.

a, b, c & d indicate a significant difference in values between the passages ($P<0.05$).

Viability assay was performed with minimum of triplicate experiments.

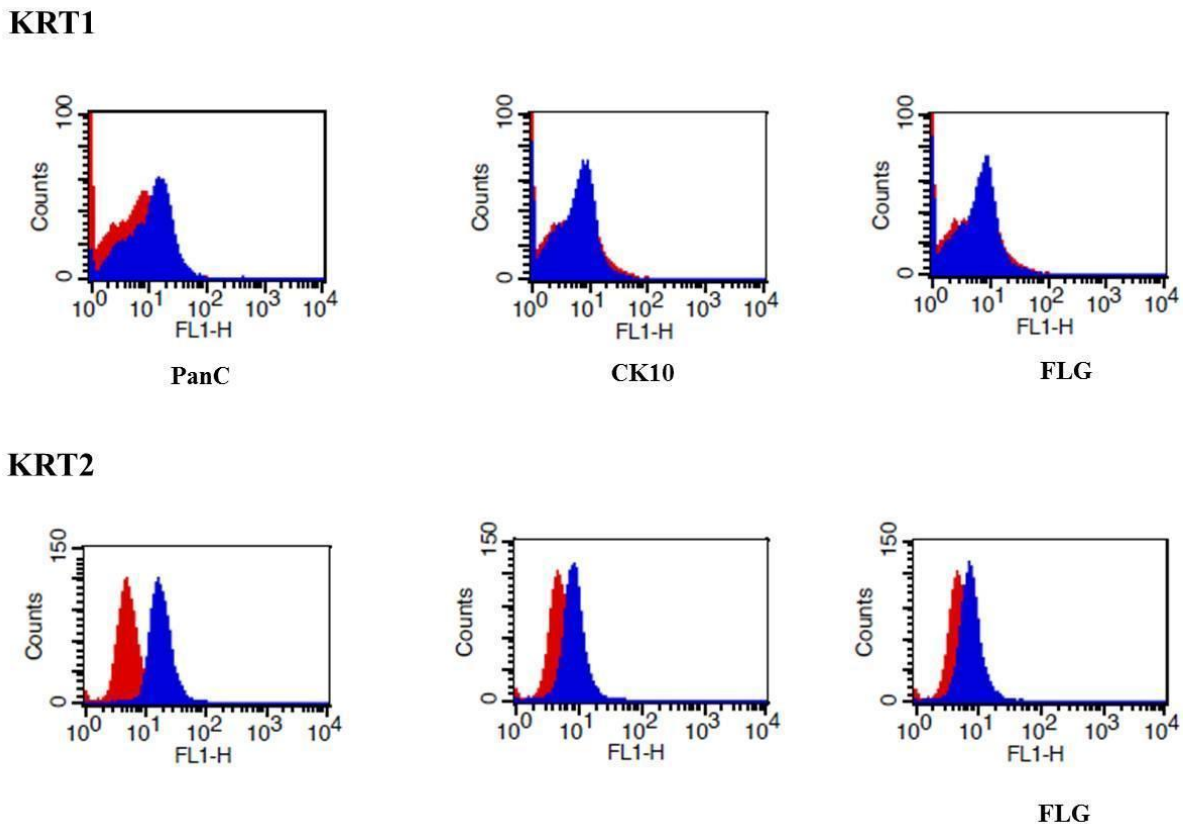
4.7.3 Expression of keratinocyte specific markers analysed by flow cytometry

Keratinocyte-specific marker expression was quantitatively examined by flow cytometry.

Assay demonstrated the expression of pan-cytokeratin (PanC), cytokeratin10 (CK10) and filaggrin (FLG) markers against FITC (Fluorescein isothiocyanate) staining (**Figure 47**, **Figure 48** and **Figure 49**).

The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody.

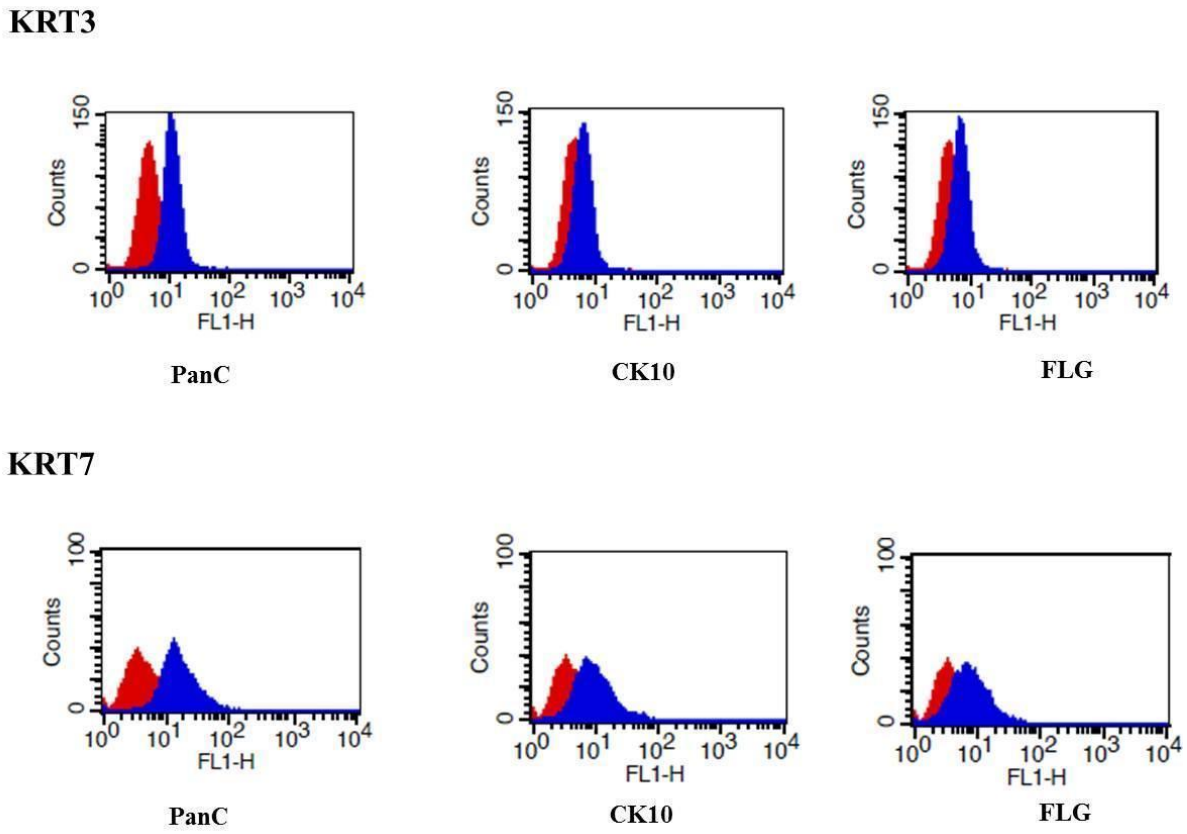
Figure 47: Representative results of flow cytometry analysis of keratinocytes (KRT1 and KRT2 cell lines)



Keratinocyte-specific marker expression was quantitatively examined. Assay demonstrated the expression of pan-cytokeratin (PanC), cytokeratin10 (CK10) and filaggrin (FLG) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody.

Samples were analysed in duplicates.

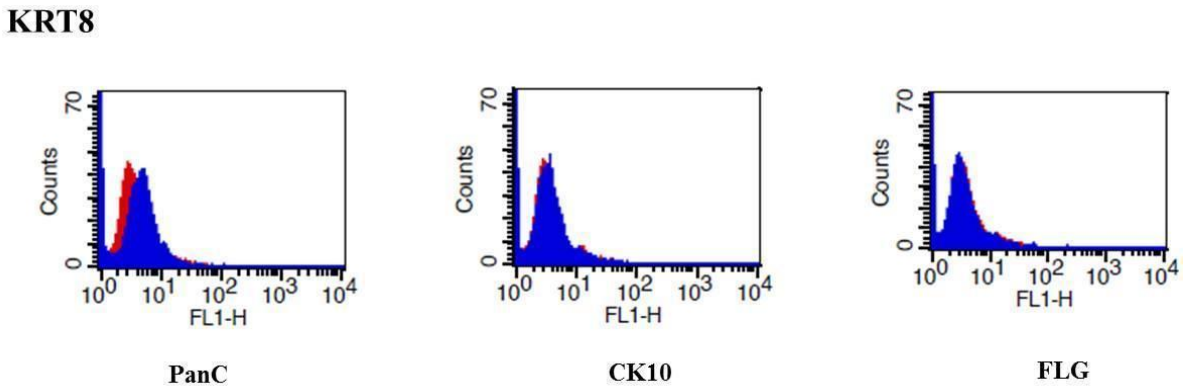
Figure 48: Representative results of flow cytometry analysis of keratinocytes (KRT3 and KRT7 cell lines)



Keratinocyte-specific marker expression was quantitatively examined. Assay demonstrated the expression of pan-cytokeratin (PanC), cytokeratin10 (CK10) and filaggrin (FLG) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody.

Samples were analysed in duplicates.

Figure 49: Representative results of flow cytometry analysis of keratinocyte (KRT8 cell line)



Keratinocyte-specific marker expression was quantitatively examined. Assay demonstrated the expression of pan-cytokeratin (PanC), cytokeratin10 (CK10) and filaggrin (FLG) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody.

Sample was analysed in duplicates.

Objective 3: To establish and examine the co-culture methods for melanocytes and keratinocytes in different culture conditions.

4.8 Co-culture of melanocytes and keratinocytes

For optimization of the co-culture system, *in vitro* established and characterized the population of melanocytes and keratinocytes were employed. The ratios of melanocytes to keratinocytes selected for optimization were 1:3, 1:5 and 1:10. The parameters, such as morphology, melanin content and the expression of phenotypic markers were analysed in co-culture systems. In **Table 8** the media composition that was used for the various co-culture ratios is listed.

Table 8: Composition of media used for *in vitro* culture

Culture Condition	Medium	FBS	Non-essential amino acids	Glutamax	β-mercaptoethanol	Pen-strep solution	Growth factors
Primary culture of keratinocytes	MEM-α	20%	1%	1%	900µL	1%	EGF 2 ng/mL + KGF 2 ng/mL + HKGS 1X
Primary culture of melanocytes	MEM-α	20%	1%	1%	900µL	1%	HMGS 1X
Maintenance of Keratinocytes	Epilife	--	--	--	--	1%	EGF 2 ng/mL + KGF 2 ng/mL + HKGS 1X
Maintenance of melanocytes	M254	--	--	--	--	1%	HMGS 1X
Co-culture of melanocytes and keratinocytes (1:3)	Above mentioned maintenance medium M254 and Epilife in the ratio 1:3						
Co-culture of melanocytes and keratinocytes (1:5)	Above mentioned maintenance medium M254 and Epilife in the ratio 1:5						
Co-culture of melanocytes and keratinocytes (1:10)	Above mentioned maintenance medium M254 and Epilife in the ratio 1:5						

HKGS contents:

Bovine pituitary extract (BPE): 0.2% v/v
Recombinant human insulin-like growth factor-I: 0.01 µg/mL
Hydrocortisone: 0.18 µg/mL
Bovine transferrin: 5 µg/mL
Human epidermal growth factor: 0.2 ng/mL

HMGS contents:

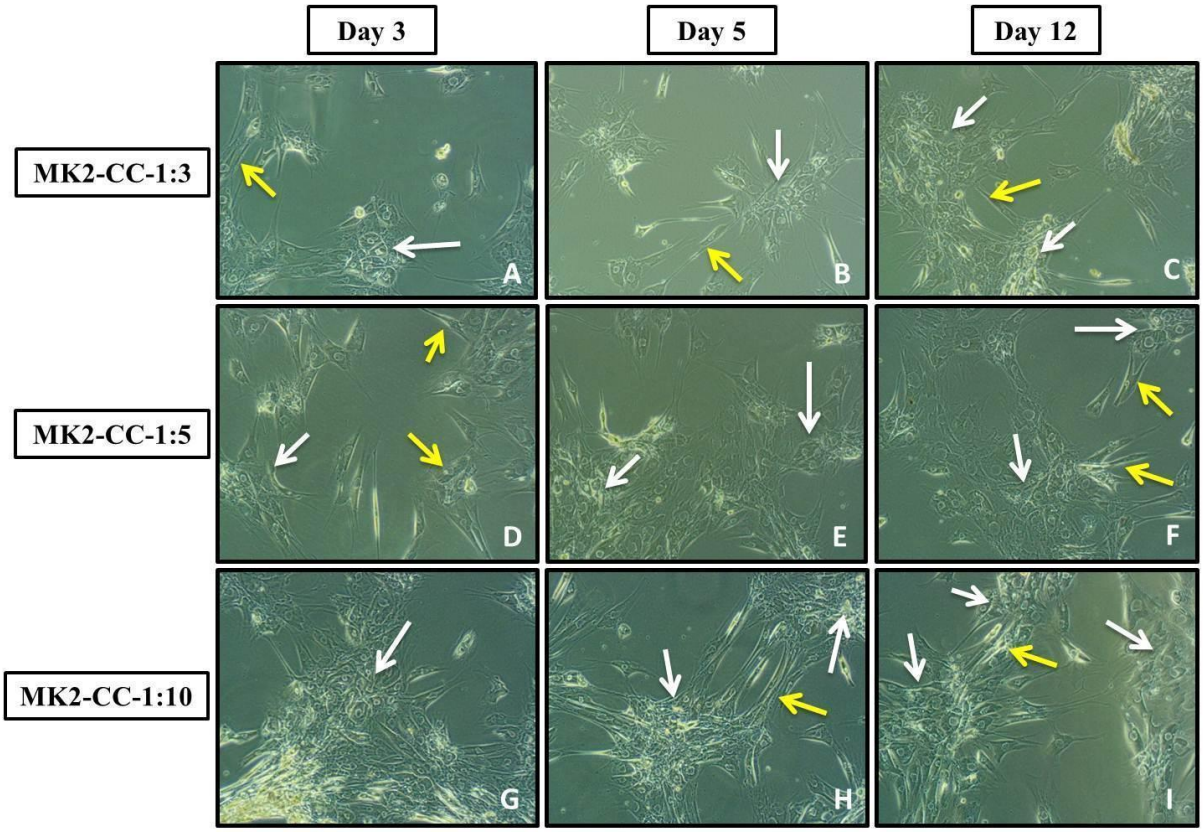
Bovine pituitary extract (BPE): 0.2% v/v
Fetal bovine serum: 0.5% v/v
Recombinant human insulin-like growth factor-I: 0.01 µg/mL
Bovine transferrin: 5 µg/mL
Basic fibroblast growth factor: 3 ng/mL
Hydrocortisone: 0.18 µg/mL
Heparin: 3 µg/mL
Phorbol 12-myristate 13-acetate (PMA): 10ng/mL

4.8.1 Morphological features in co-culture of melanocytes and keratinocytes

In this study, four cell lines of melanocytes and keratinocytes, such as MK2, MK3, MK4 and MK6 were employed for assessing the morphological changes at different ratios of 1:3, 1:5 and 1:10 co-culture systems on days 3, 5 and 10 of culture.

The differences in the morphological features were observed as per the ratios performed and the results are presented in **Figure 50** to **Figure 53**. Higher number of melanocytes was observed at ratios 1:3 and 1:5. Further, the presence of cells in co-cultures was confirmed by hematoxylin staining (**Figure 54**).

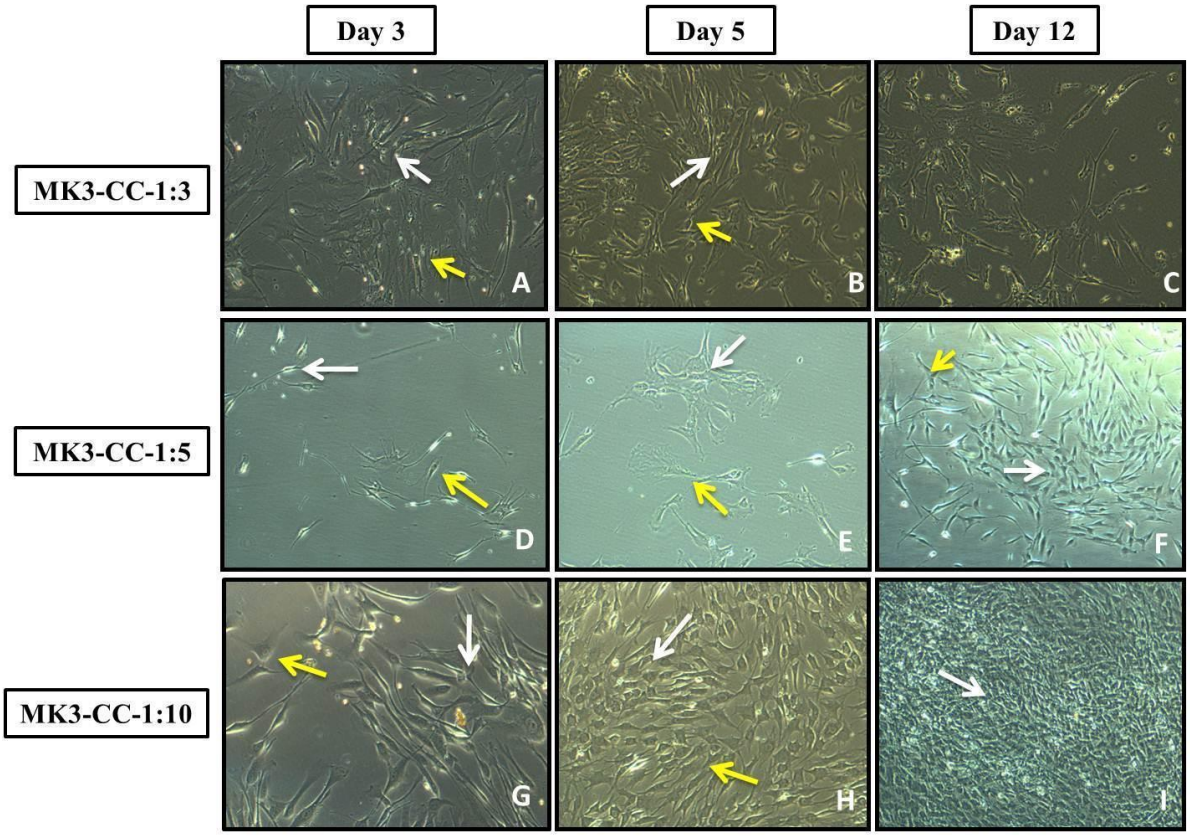
Figure 50: Representative images of morphologies of melanocyte-keratinocyte co-cultures (MK2-CC) at 1:3, 1:5 and 1:10 ratios observed on days 3, 5 and 12 (A to I)



Yellow arrows represent the melanocytes morphology and white arrows indicate keratinocyte cell clusters.

Image magnification-10x

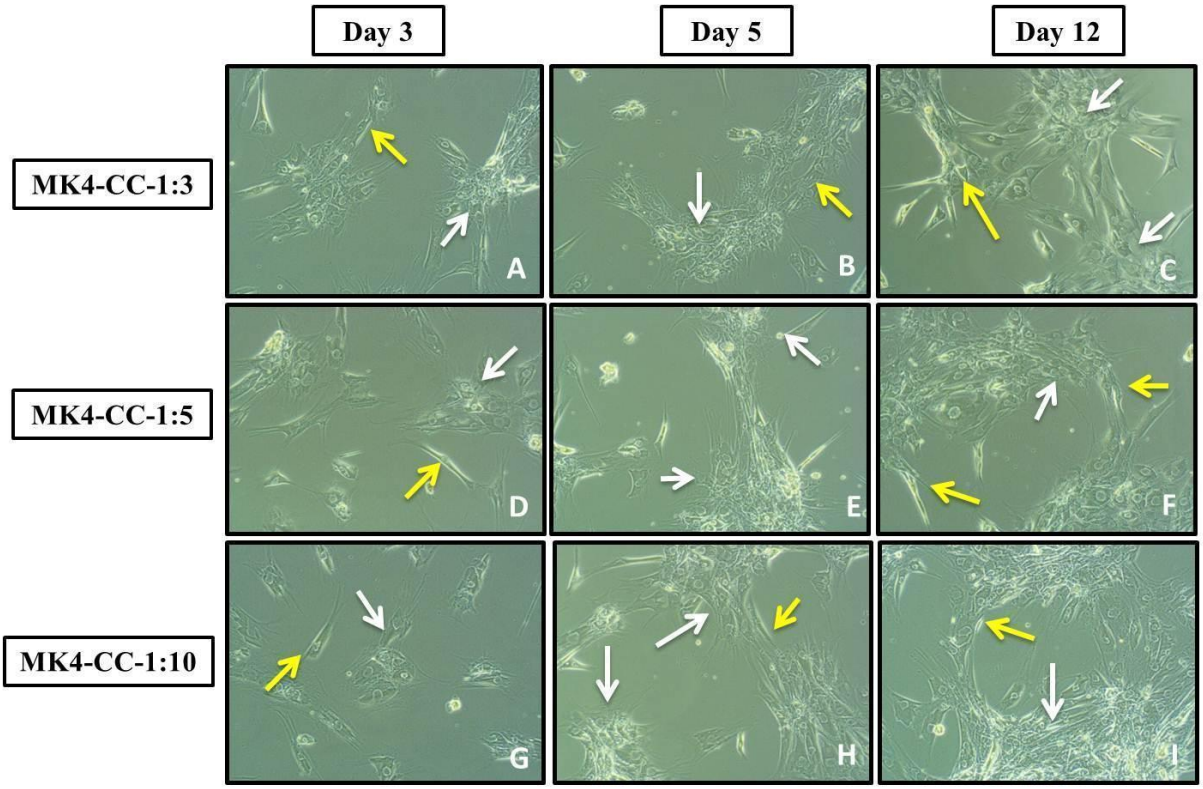
Figure 51: Representative images of morphologies of melanocyte-keratinocyte co-cultures (MK3-CC) at 1:3, 1:5 and 1:10 ratios observed on days 3, 5 and 12 (A to I)



Yellow arrows represent the melanocytes morphology and white arrows indicate keratinocyte cell clusters.

Image magnification-10x

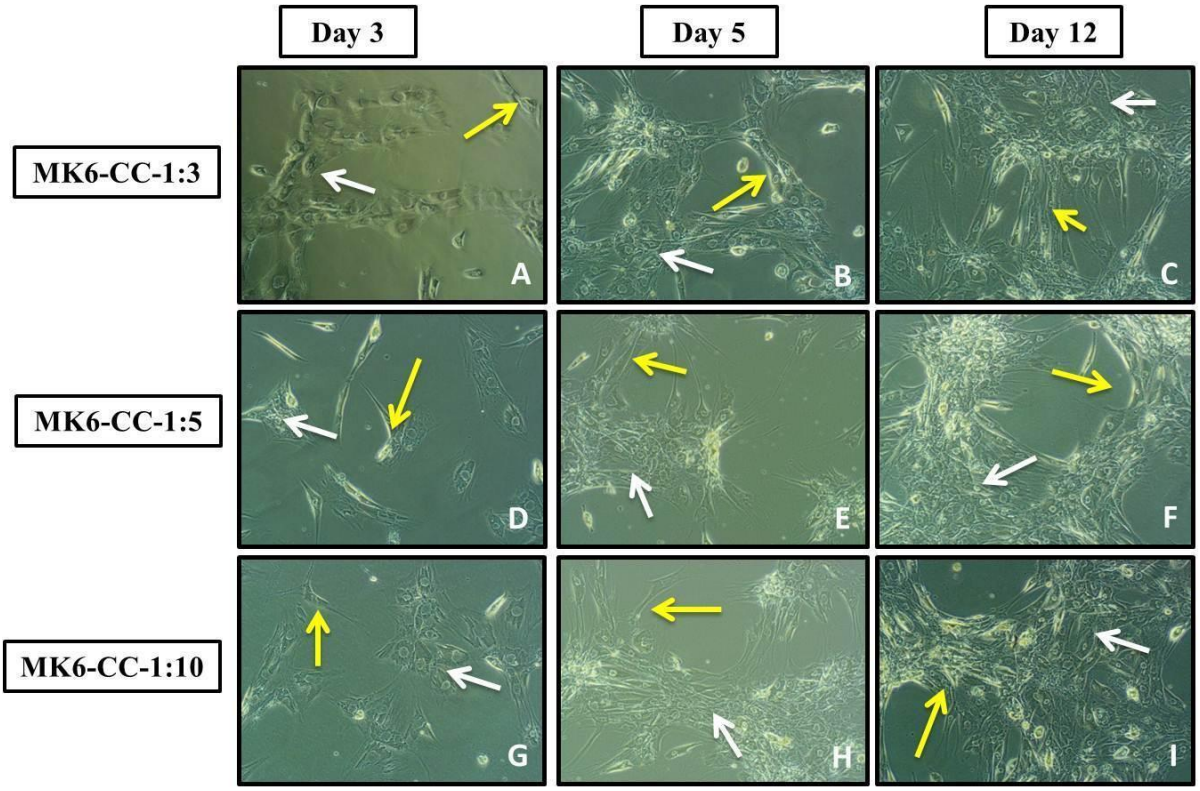
Figure 52: Representative images of morphologies of melanocyte-keratinocyte co-cultures (MK4-CC) at 1:3, 1:5 and 1:10 ratios observed on days 3, 5 and 12 (A to I)



Yellow arrows represent the melanocytes morphology and white arrows indicate keratinocyte cell clusters.

Image magnification-10x

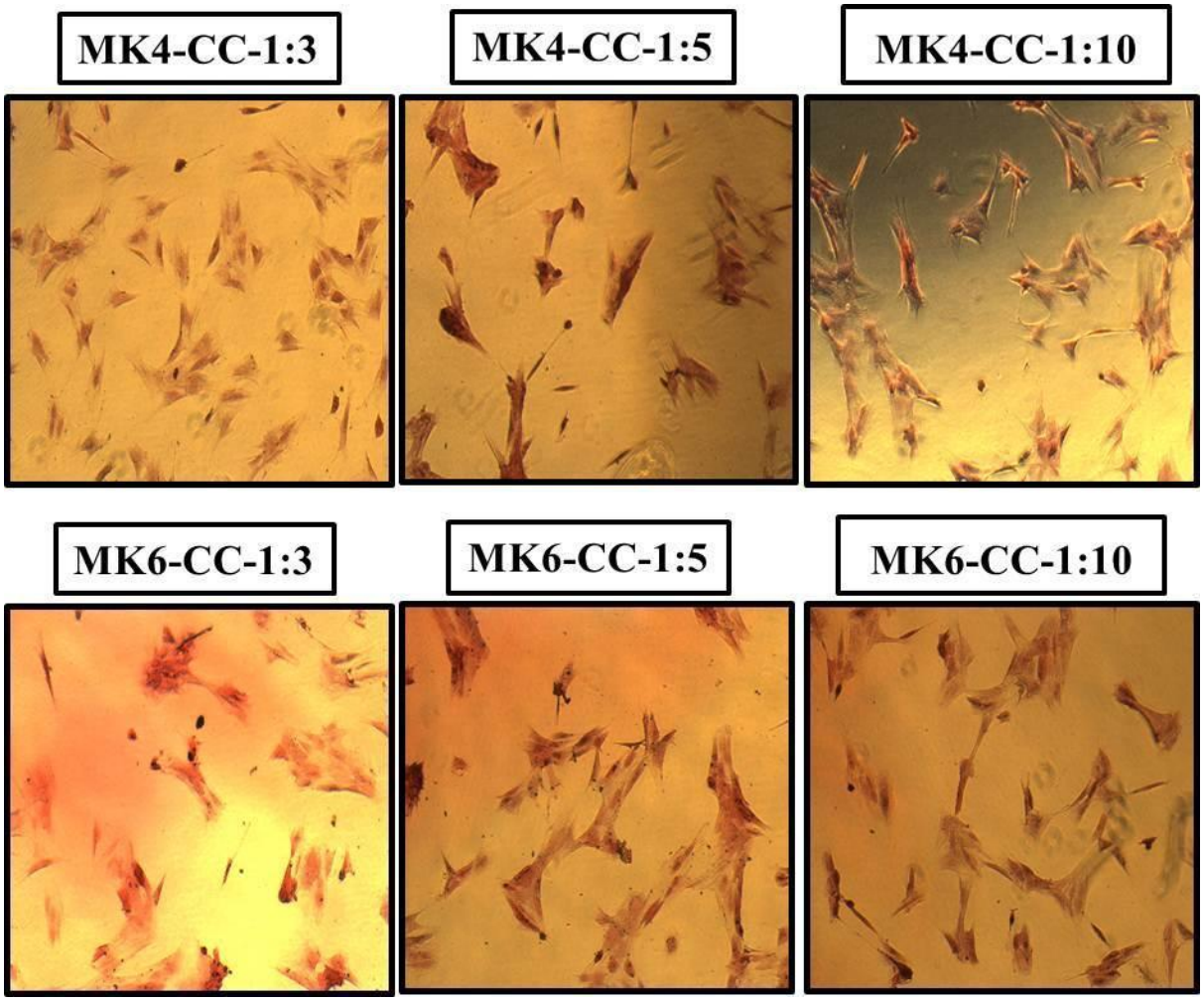
Figure 53: Representative images of morphologies of melanocyte-keratinocyte co-cultures (MK6-CC) at 1:3, 1:5 and 1:10 ratios observed on days 3, 5 and 12 (A to I)



Yellow arrows represent the melanocytes morphology and white arrows indicate keratinocyte cell clusters.

Image magnification-10x

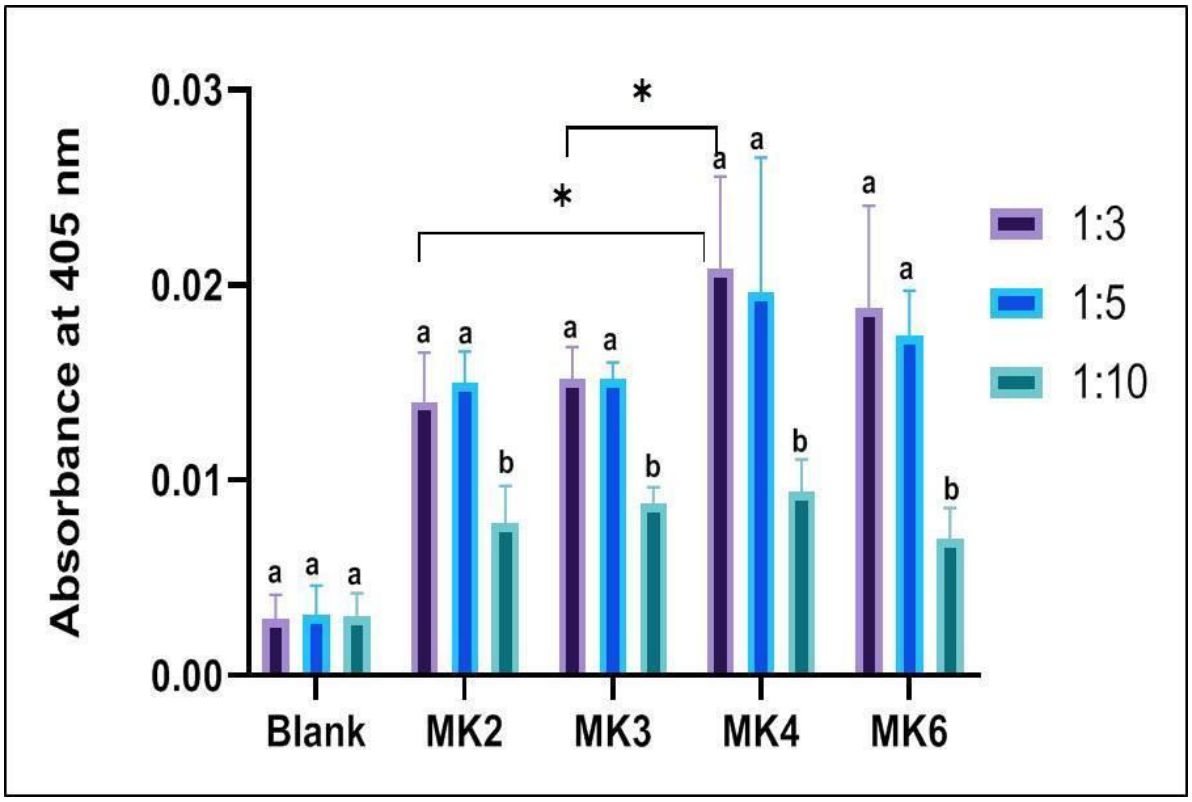
Figure 54: Representative images of melanocyte-keratinocyte co-cultures (MK4-CC and MK6-CC) at 1:3, 1:5 and 1:10 ratios stained by hematoxylin for confirmation



4.8.2 Estimation of melanin content in melanocyte keratinocyte co-cultures

Melanin content in the melanocyte-keratinocyte co-culture system at 1:3, 1:5 and 1:10 ratios was analysed in different cell lines. The results are presented in **Figure 55**.

Figure 55: Estimation of melanin content in melanocyte keratinocyte co-cultures (MK2, MK3, MK4 and MK6) cultured at 1:3, 1:5 and 1:10 ratios



Significantly higher secretion of melanin was observed in 1:3 and 1:5 ratio than 1:10 ratio ($p<0005$). Between the cell lines, MK4 cells showed significantly higher melanin content versus MK2 ($p=0.0058$) and MK3 ($p=0.0403$) cell lines in 1:3 ratio.

Melanin content was significantly higher in all the cell lines and all the ratios when compared to blank. In 1:3 ratio cultures, a significant difference in melanin content was observed between MEL2 vs MEL4 and MEL3 vs MEL4. In 1:5 and 1:10 ratios, there were no significant variations recorded in between the cell lines.

Between ratios 1:3 vs 1:5, co-cultures did not show any significant difference in melanin content. But, co-cultures in 1:3 vs 1:10 and 1:5 vs 1:10 showed highly significant variation in melanin content in all the cell lines.

*, a and b indicate a significant difference in values between the co-culture ratios and cell lines as indicated ($P<0.05$).

The samples were analysed in triplicates.

Values on Y-axis indicate absorbance units (absolute values)

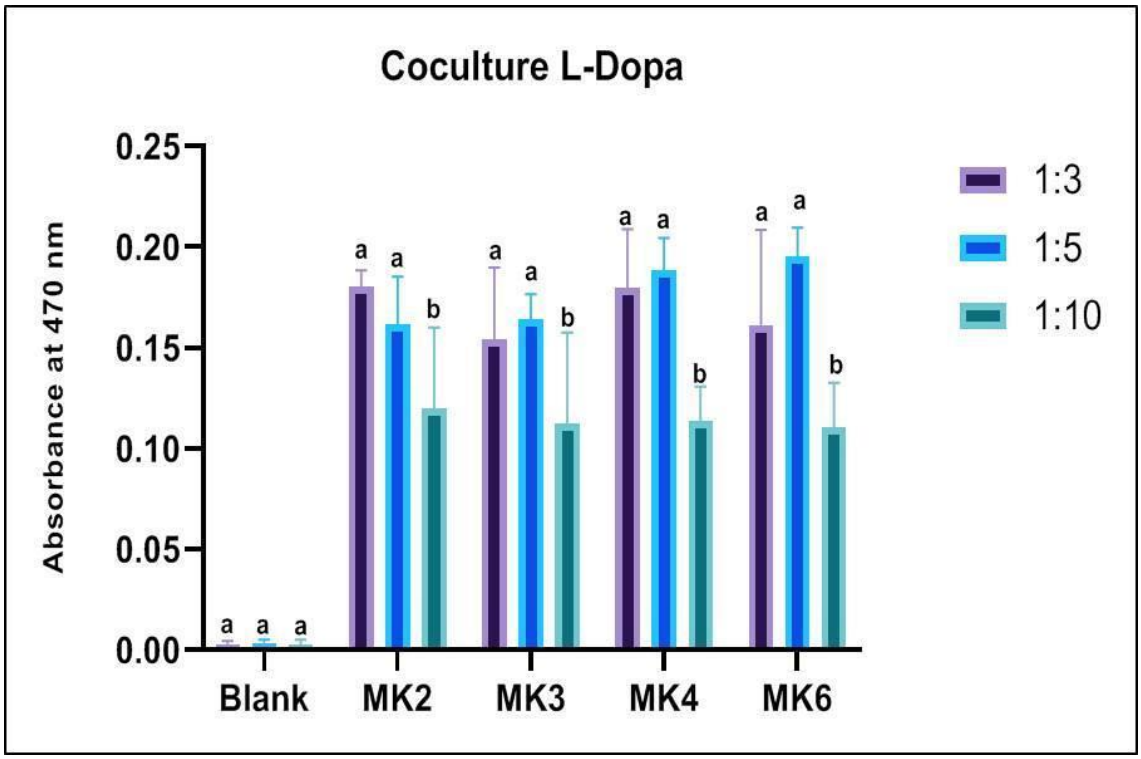
Dermal fibroblasts (DFs) were initially used as 'Negative control cell lines' in Total melanin content and L-DOPA assays (**Figure 29 & 30**, p224 & p226).

Later, for co-culture experiments, **e.g. Figure 55** (p261), **Figure 56** (p263) etc., only blanks were used. (Because DFs earlier showed very low melanin and tyrosinase, and the values were similar to blank values)

4.8.3 Analysis of tyrosinase activity in melanocyte keratinocyte co-cultures by L-DOPA

Analysis of tyrosinase activity in melanocyte keratinocyte co-cultures was performed by L-DOPA assay. The results are graphically presented in **Figure 56**.

Figure 56: Analysis of tyrosinase activity was performed in melanocyte keratinocyte co-cultures (MK2, MK3, MK4 and MK6) cultured at 1:3, 1:5 and 1:10 ratios



L-dopa activity was present in co-cultured cells and it was highly significant when compared with blank values ($p<0.0001$).

The increase in L-DOPA activity was not significant between the cells lines ($P>0.05$). But, between the different ratios of all the cell lines, the activity showed significant variation in 1:3 vs 1:10 and 1:5 vs 1:10. But, 1:3 vs 1:5 groups did not show any significant difference in all the cell lines ($P>0.05$).

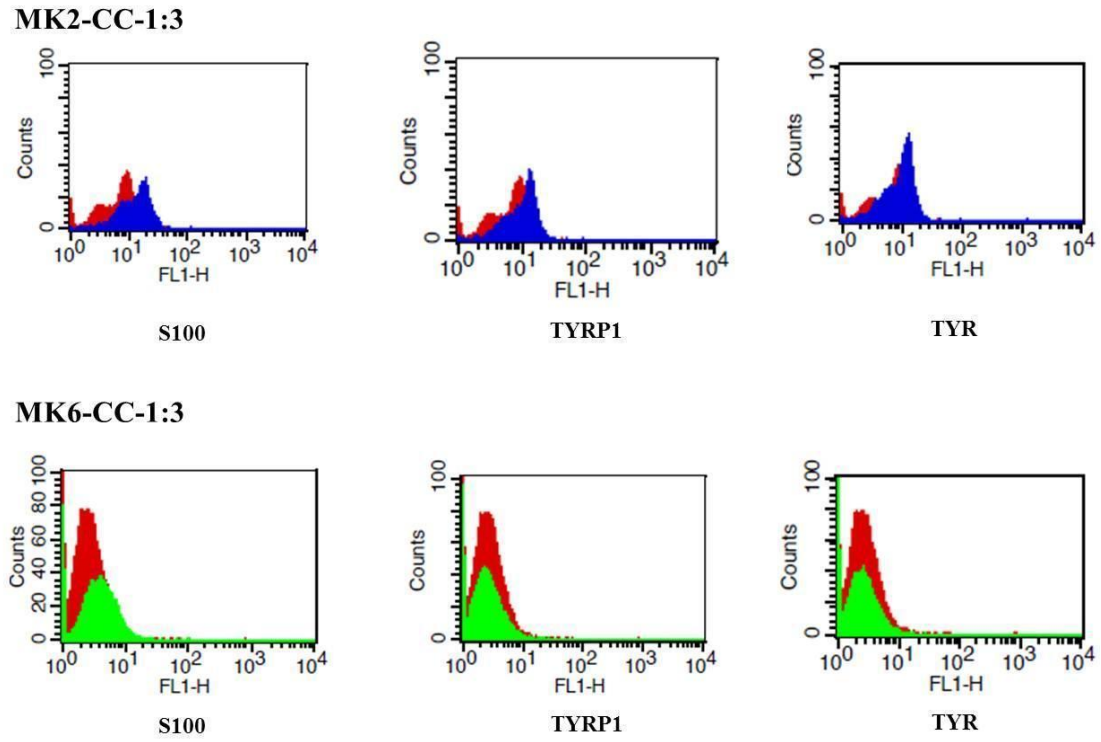
a and b indicate a significant difference in values between the co-culture ratios for each cell line as indicated ($P<0.05$).

4.8.4 Expression of phenotypic markers by flow cytometry analysis

Expression of selected melanocyte-specific markers was analysed in melanocyte-keratinocyte co-culture system using flow cytometer. The marker expression was quantitatively examined and the results are presented in **Figure 57**.

Analysis demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining.

Figure 57: Representative results of flow cytometry analysis of melanocytes and keratinocytes co-culture at 1:3 ratio (MK2 and MK6 cell lines)



The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody. The samples were analysed in duplicates for each marker as indicated.

Objective 4: To manipulate and assess the co-culture methods of melanocytes and keratinocytes by exposing to alpha melanocyte-stimulating hormone and UV light.

4.9 Influence of α-MSH on melanocyte-keratinocyte co-cultures

For further optimization of the co-culture system, the present study was aimed to assess the influence of α-MSH on melanocyte-keratinocyte at ratios 1:3, 1:5 and 1:10. Three cell lines were selected for these experiments and they were, MK2, MK4 and MK6. Further, α-MSH was tested at three different concentrations, such as 2.5 µg/ml, 5 µg/ml and 10 µg/ml. The cellular and functional parameters, including morphological characteristics, melanin content and tyrosinase activity by L-DOPA assay were performed.

4.9.1 Morphological features of melanocyte keratinocyte co-cultures at different ratios on days 3 and 7 after treatment with α-MSH at various concentrations

In this study, three cell lines of melanocytes and keratinocytes, such as MK2, MK4 and MK6 were employed for assessing the morphological changes at different ratios of 1:3, 1:5 and 1:10 co-culture systems on days 3 and 7 after their exposure to α-MSH at 2.5 µg/ml, 5 µg/ml and 10 µg/ml.

The differences in the morphological features were observed as per the concentrations of α-MSH used, and the results are presented for 1:3 ratio in **Figures 58** to **Figure 60**, 1:5 ratio in **Figures 61** to **Figures 63** and 1:10 ratio in **Figures 64** to **Figures 66**. In a dose dependent manner, the higher number of melanocytes was observed at ratios 1:3 and 1:5. Further, the day 0 images are also presented for ratios 1:3, 1:5 and 1:10 (**Figure 67**).

In co-cultures, melanocytes showed bipolar with dendritic morphology, whilst the cells with well-defined contours and round shapes were keratinocytes. Melanocytes appeared normal in the perinuclear region and also based on the dendrite shapes. Both cells showed proper adherence as observed on days 3 and 7 of culture.

Figure 58: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:3 ratio (MK2-CC-1:3) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

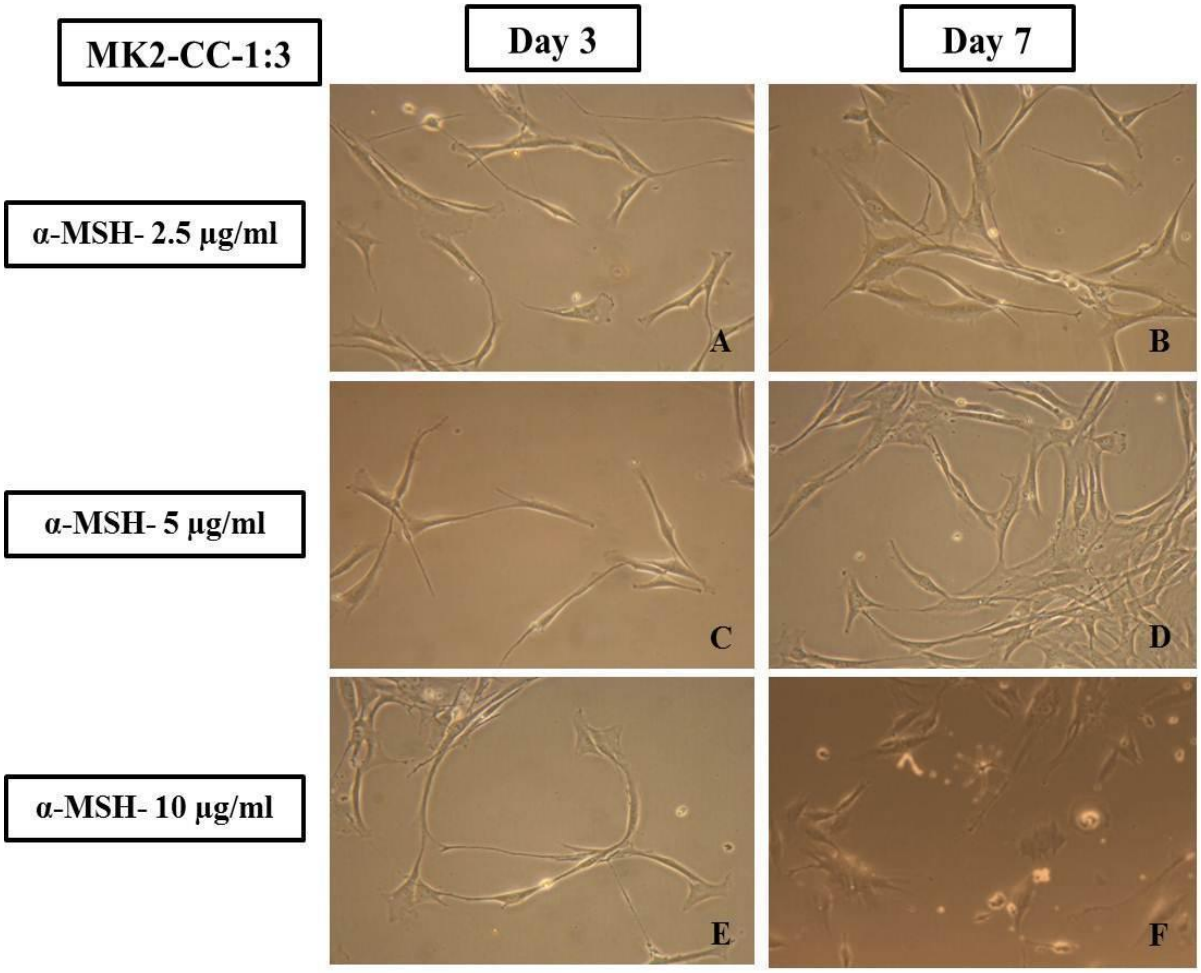


Image magnification-10x

Figure 59: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:3 ratio (MK4-CC-1:3) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

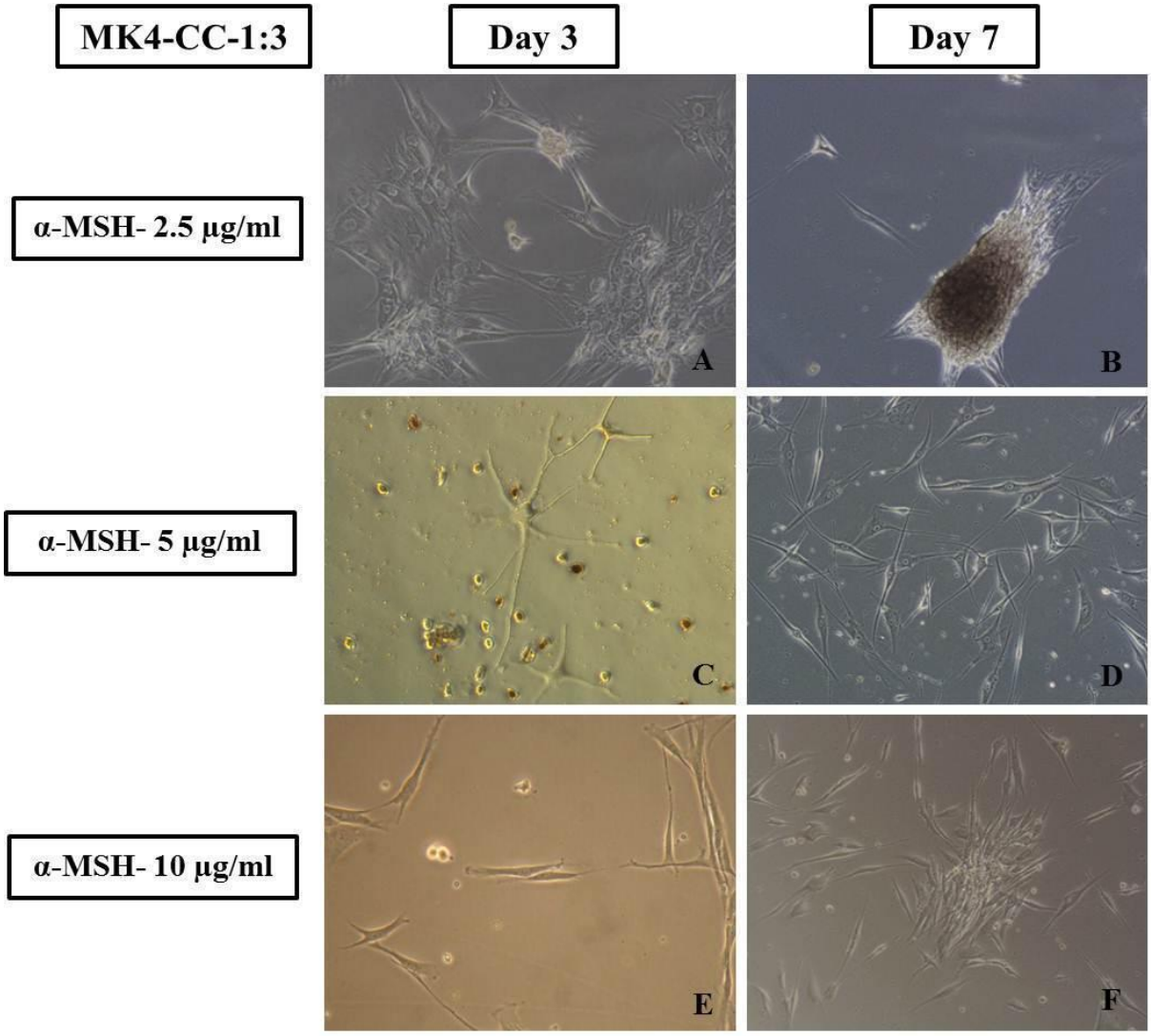


Image magnification-10x

Figure 60: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:3 ratio (MK6-CC-1:3) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

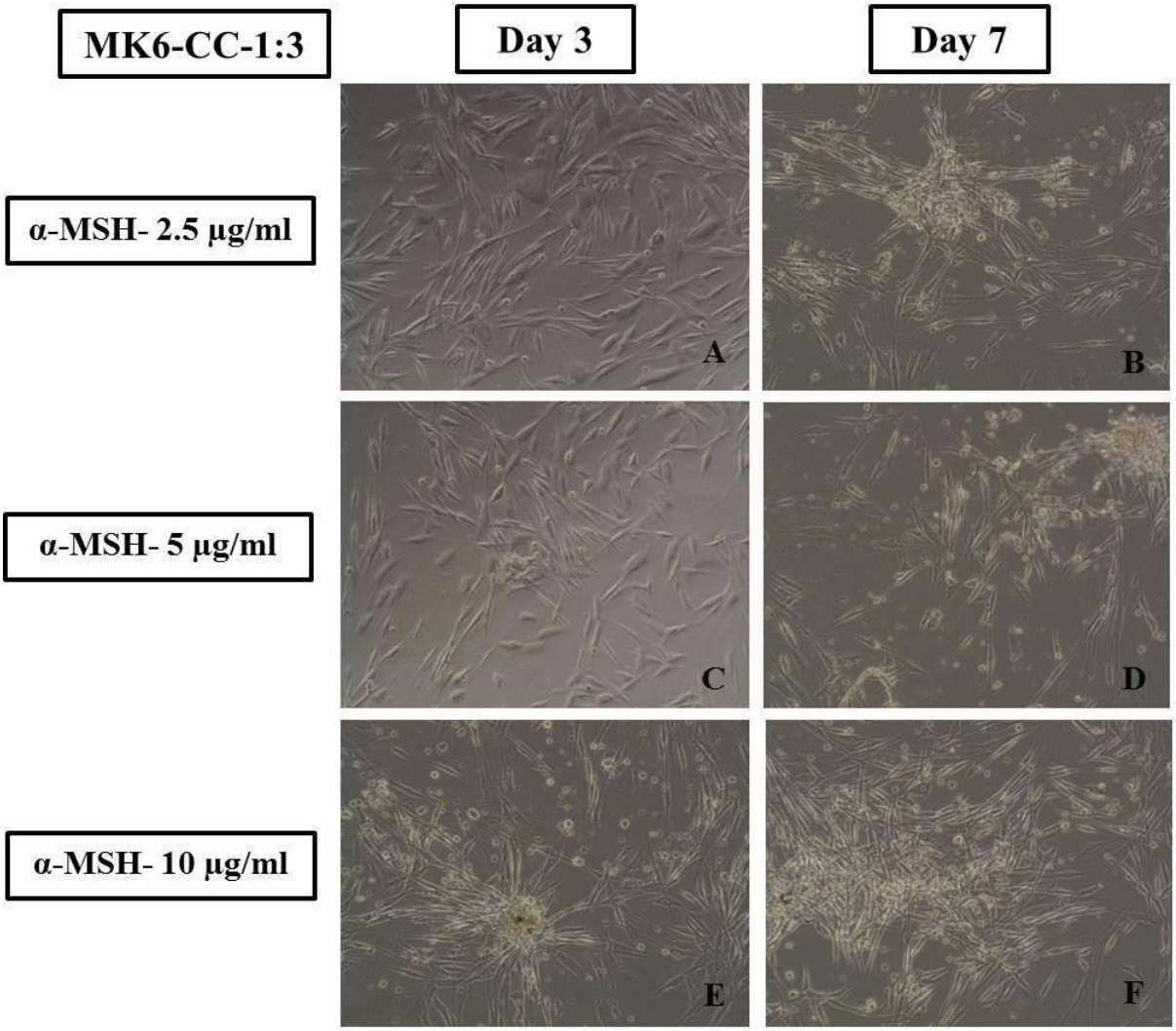


Image magnification-10x

Figure 61: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:5 ratio (MK2-CC-1:5) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

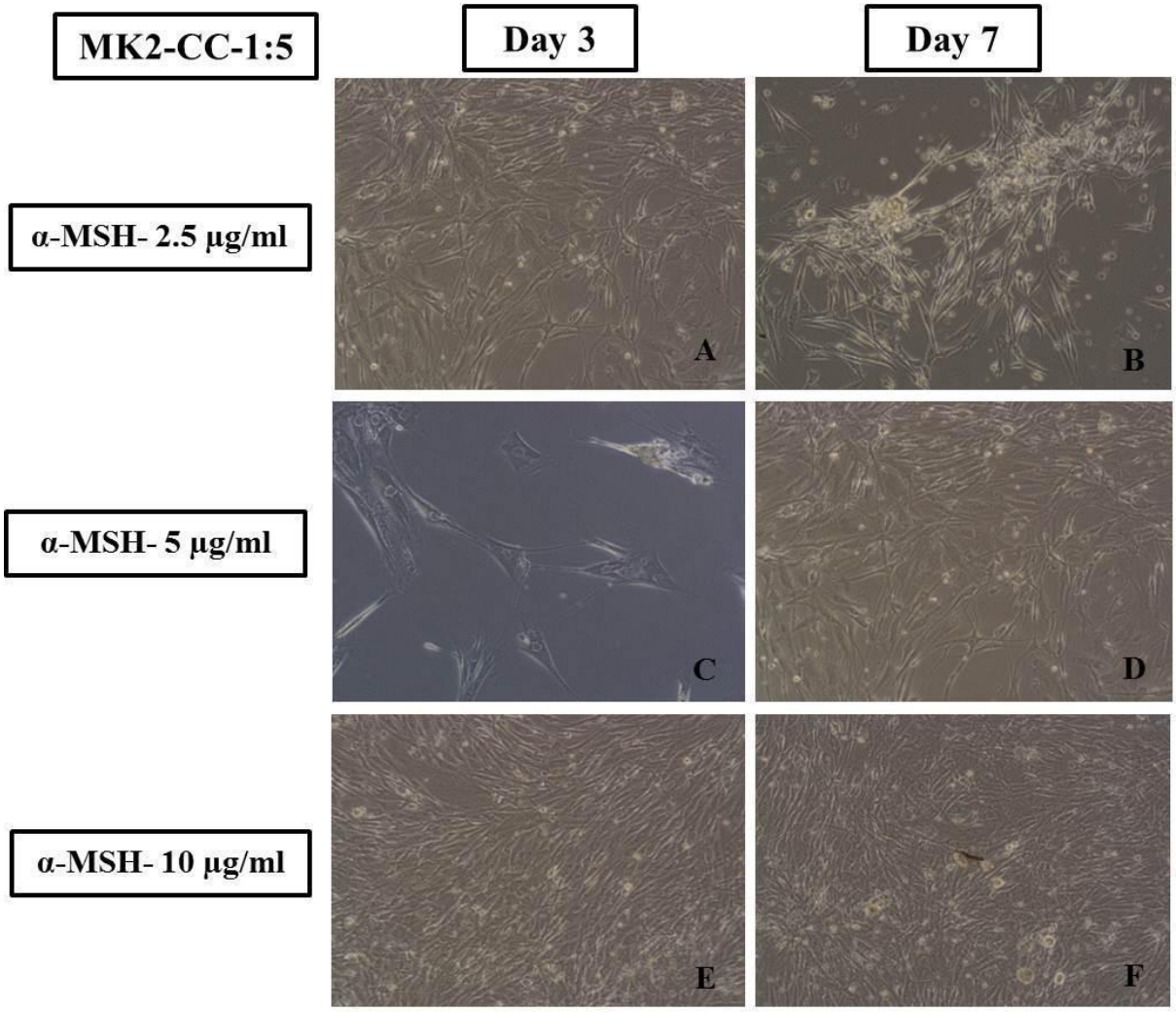


Image magnification-10x

Figure 62: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:5 ratio (MK4-CC-1:5) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

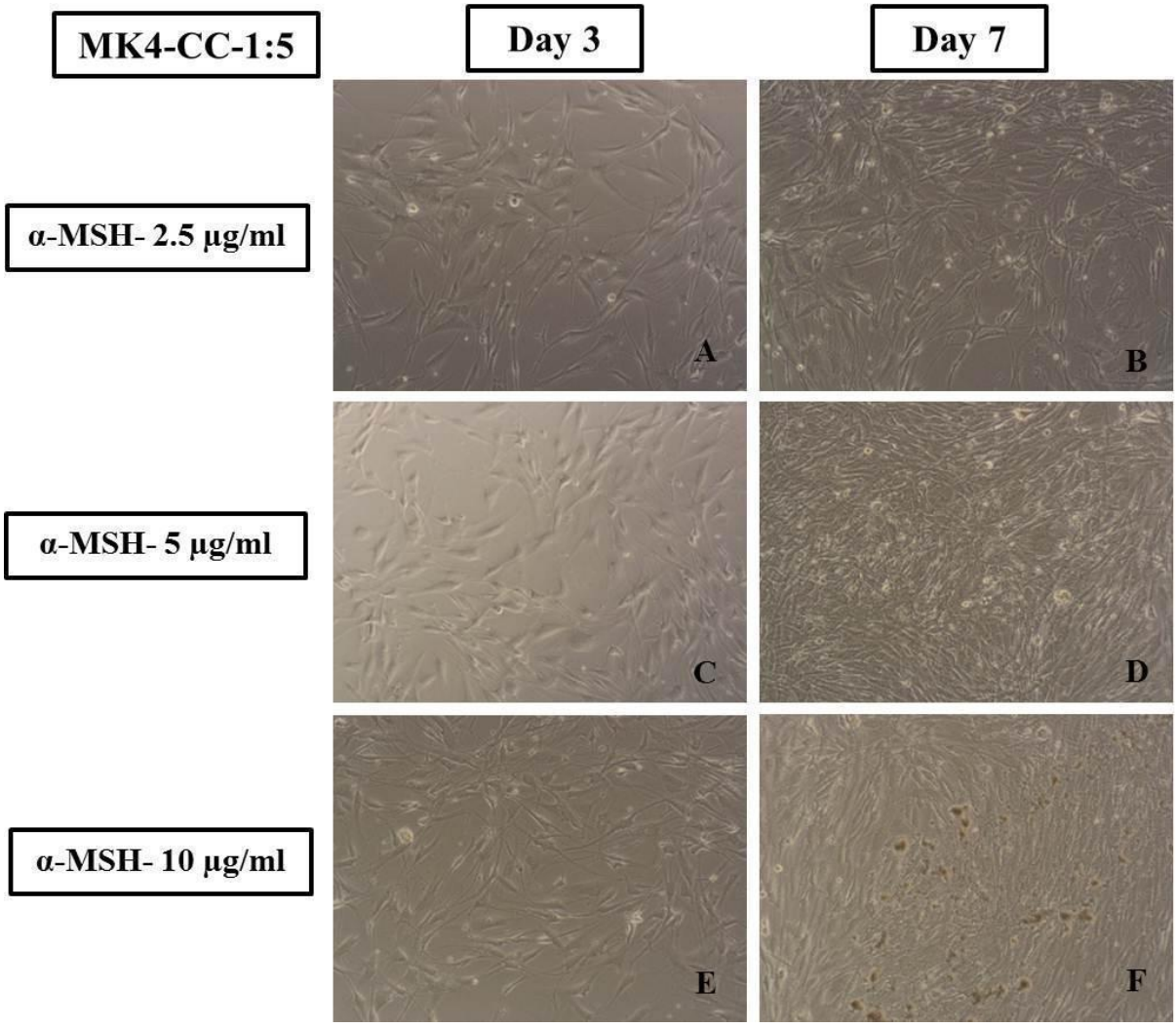


Image magnification-10x

Figure 63: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:5 ratio (MK6-CC-1:5) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

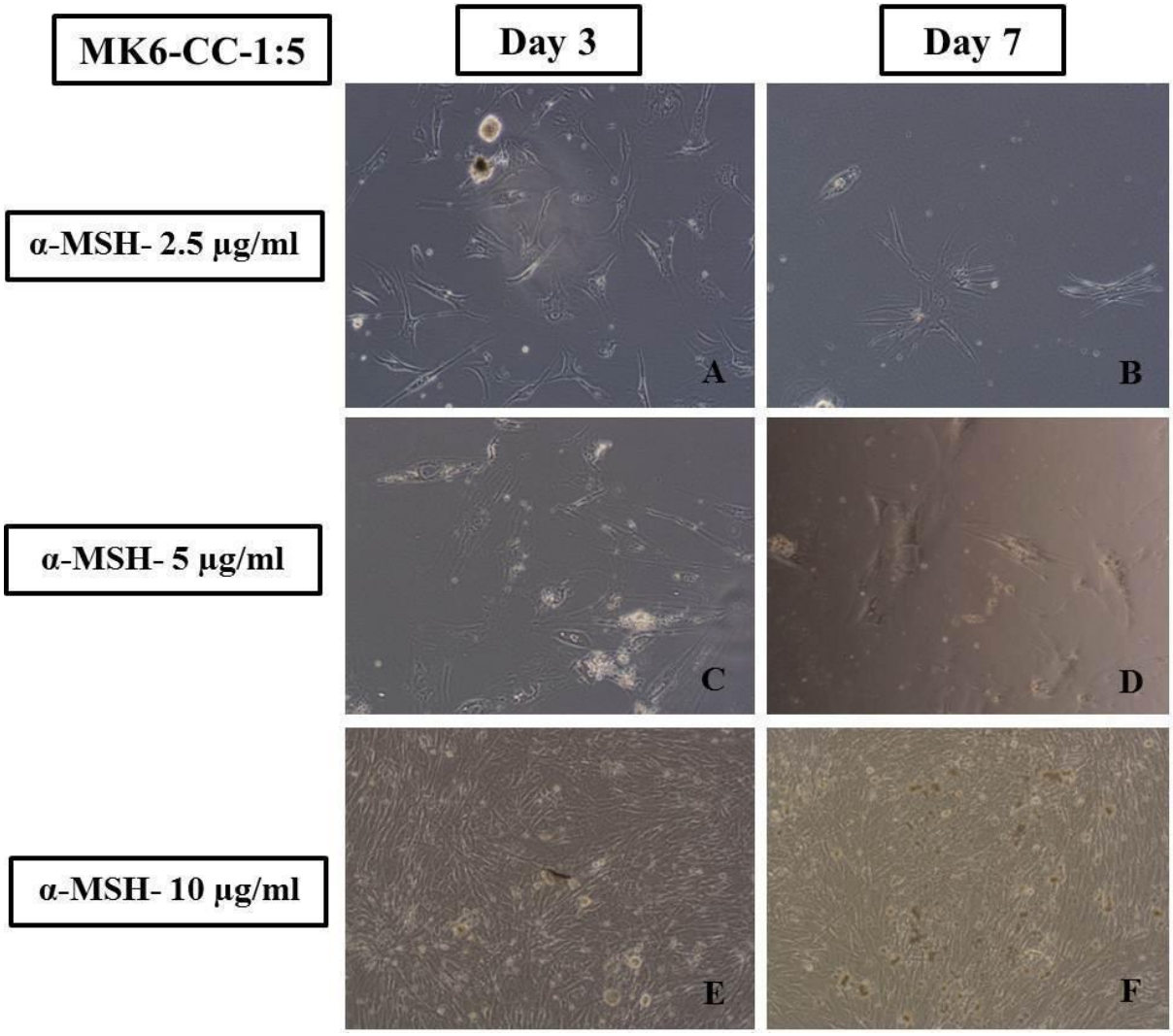


Image magnification-10x

Figure 64: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:10 ratio (MK2-CC-1:10) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

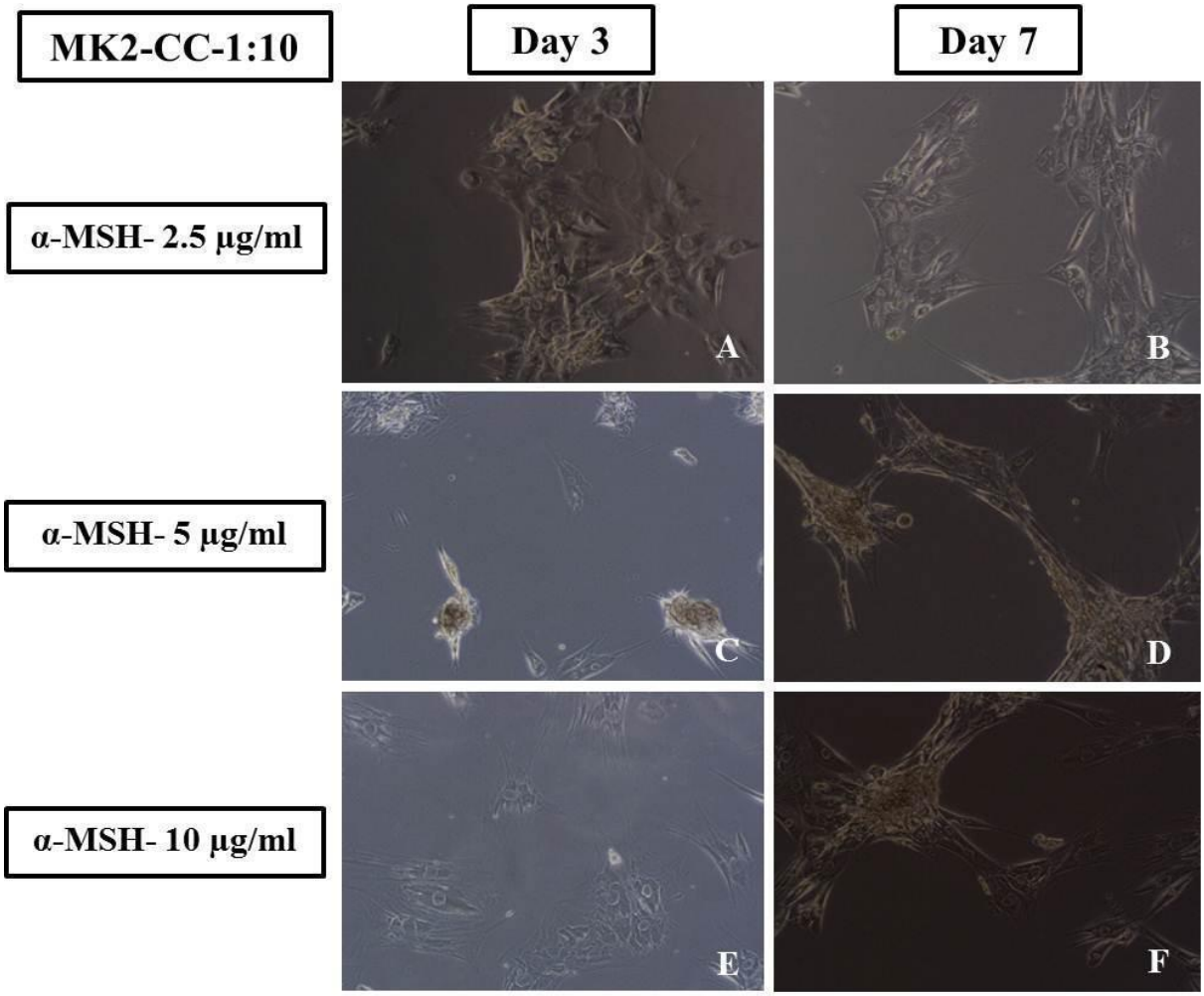


Image magnification-10x

Figure 65: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:10 ratio (MK4-CC-1:10) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

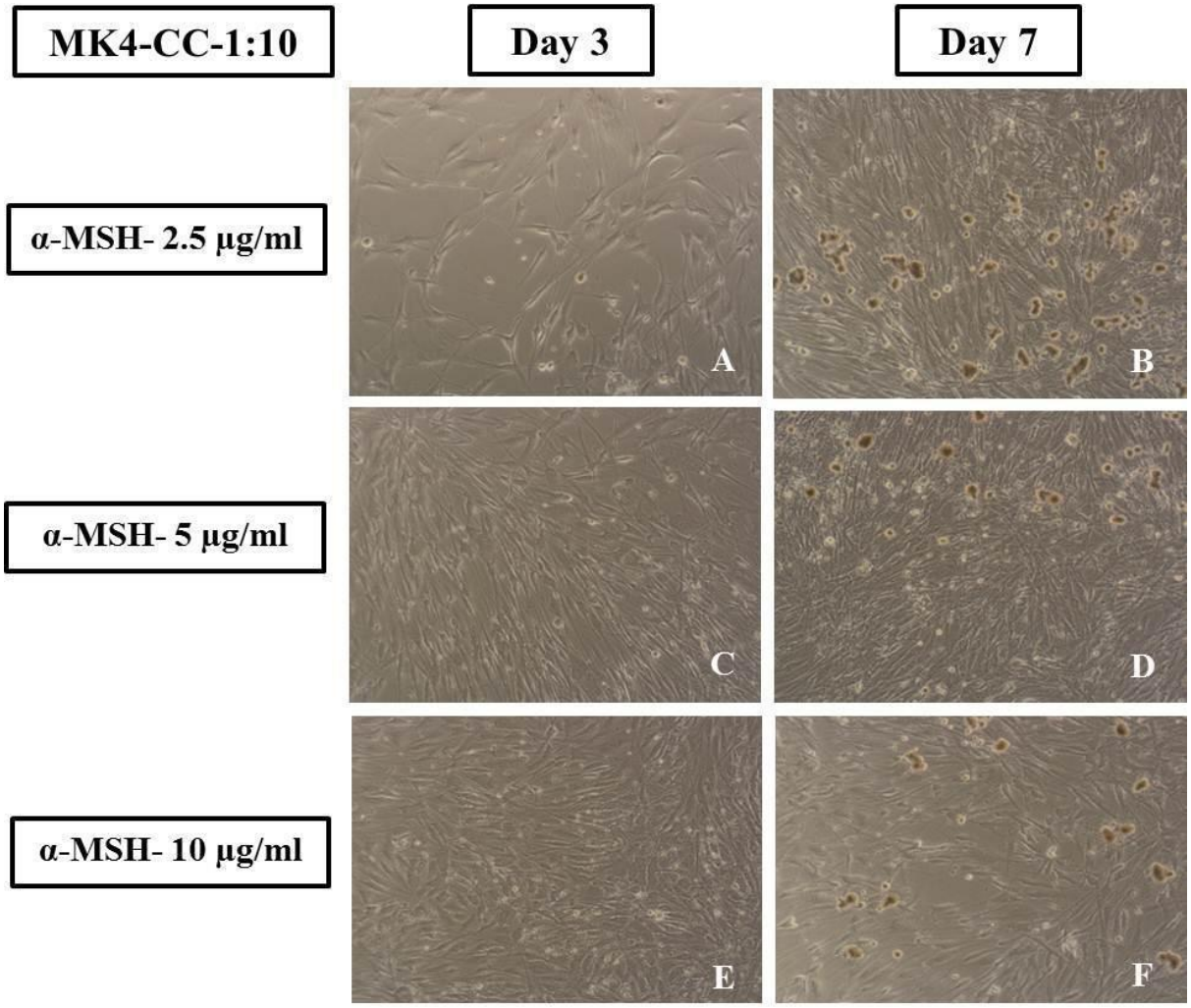


Image magnification-10x

Figure 66: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:10 ratio (MK6-CC-1:10) on days 3 and 7 after treatment with α-MSH at different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml)

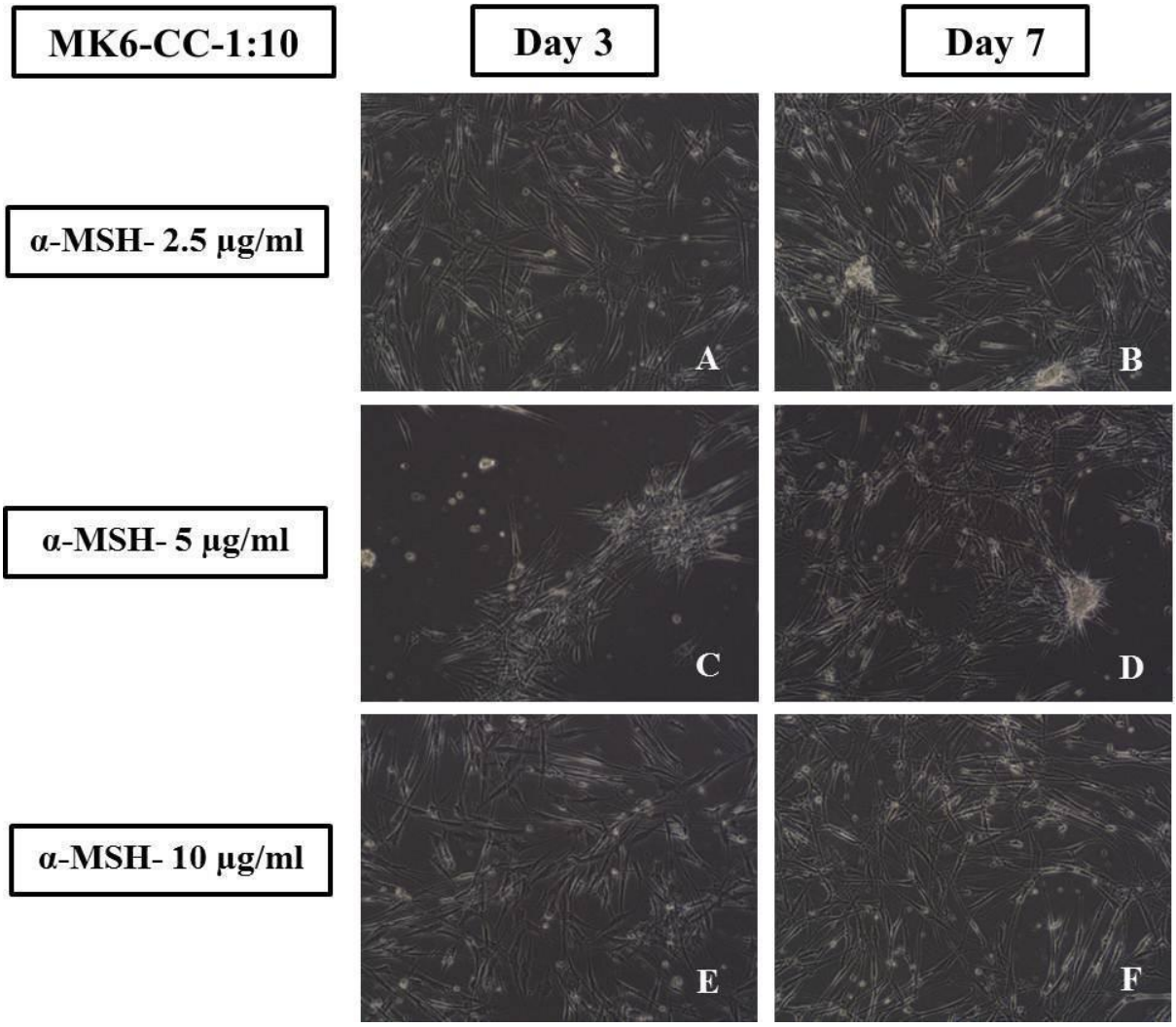


Image magnification-10x

Figure 67: Representative images of morphology of melanocyte keratinocyte co-cultures at 1:3, 1:5 and 1:10 ratio on day 0 after treatment with α-MSH at 2.5 µg/ml concentration

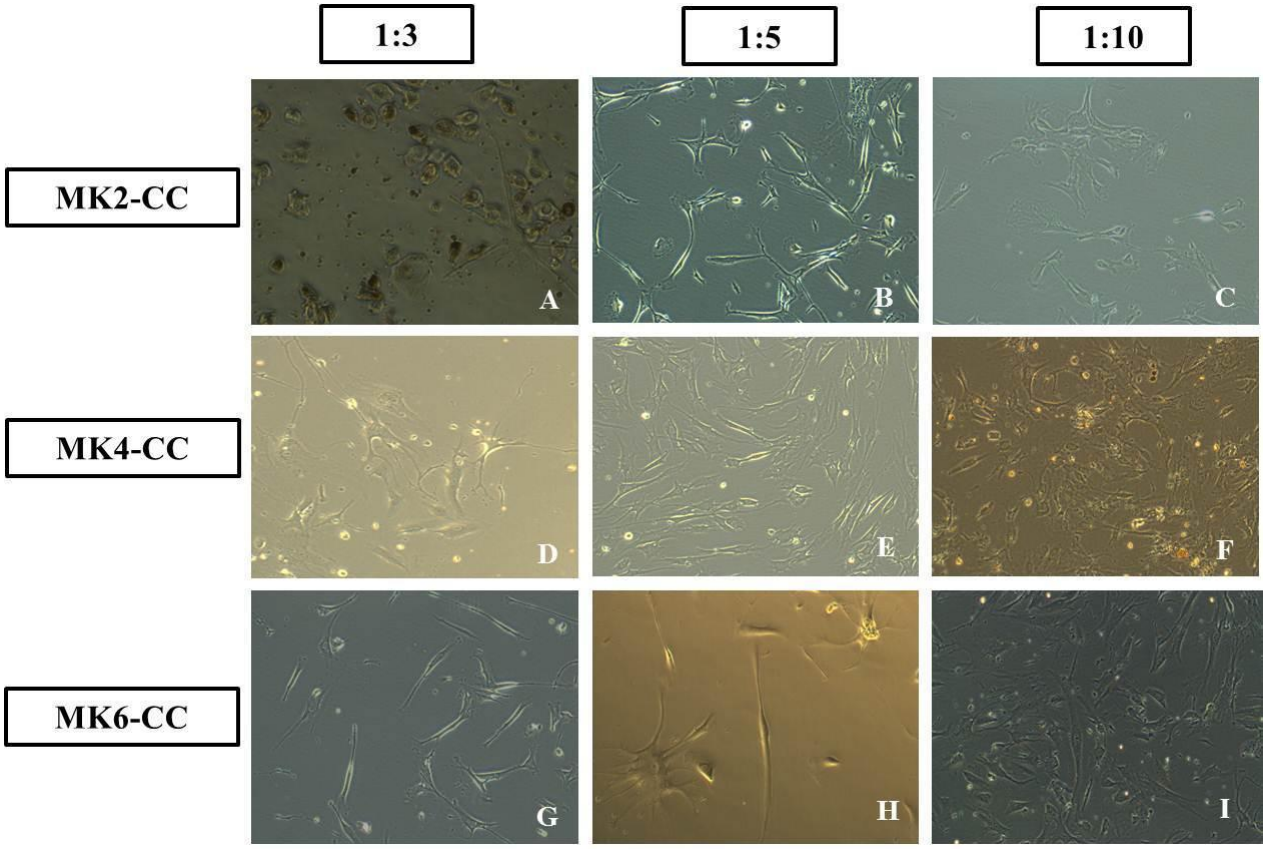
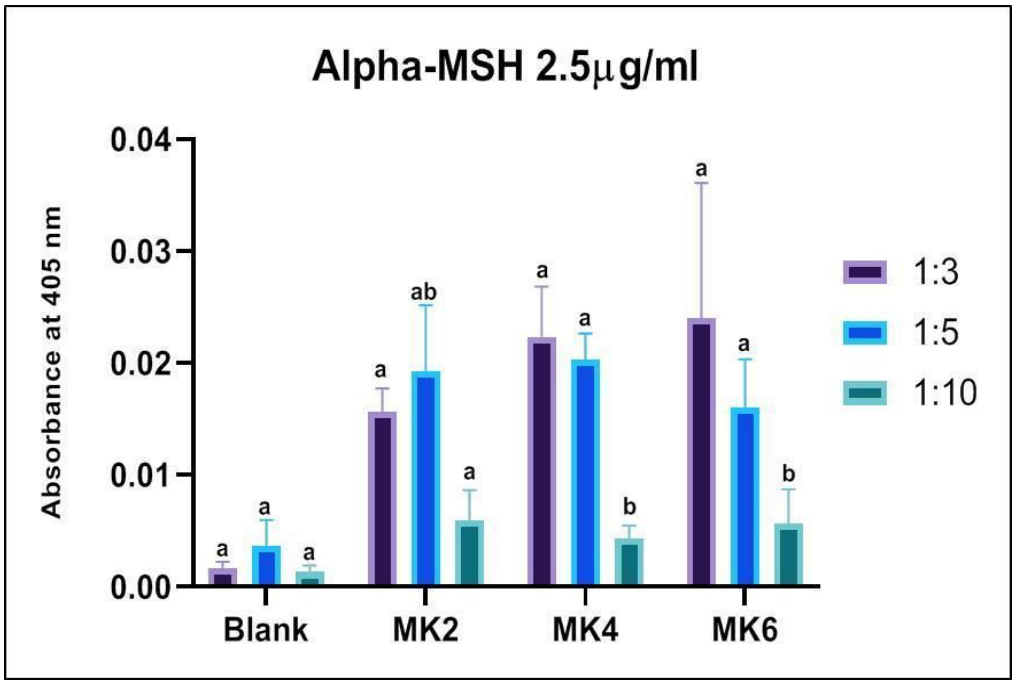


Image magnification-10x

4.9.2 Melanin content assay in melanocyte keratinocyte co-cultures at different ratios

Melanin content was analysed in melanocyte keratinocyte co-cultures at different ratios and exposed to α-MSH at various concentrations. All the three ratios of co-culture with 3 different concentrations of α-MSH (2.5, 5 and 10 µg/mL) showed significant increase in melanin content after treatment with α-MSH when compared to blank ($p<0.0001$) (**Figure 68**, **Figure 69** and **Figure 70**). Hence, after α-MSH treatment, all the cell lines showed increased secretion of melanin.

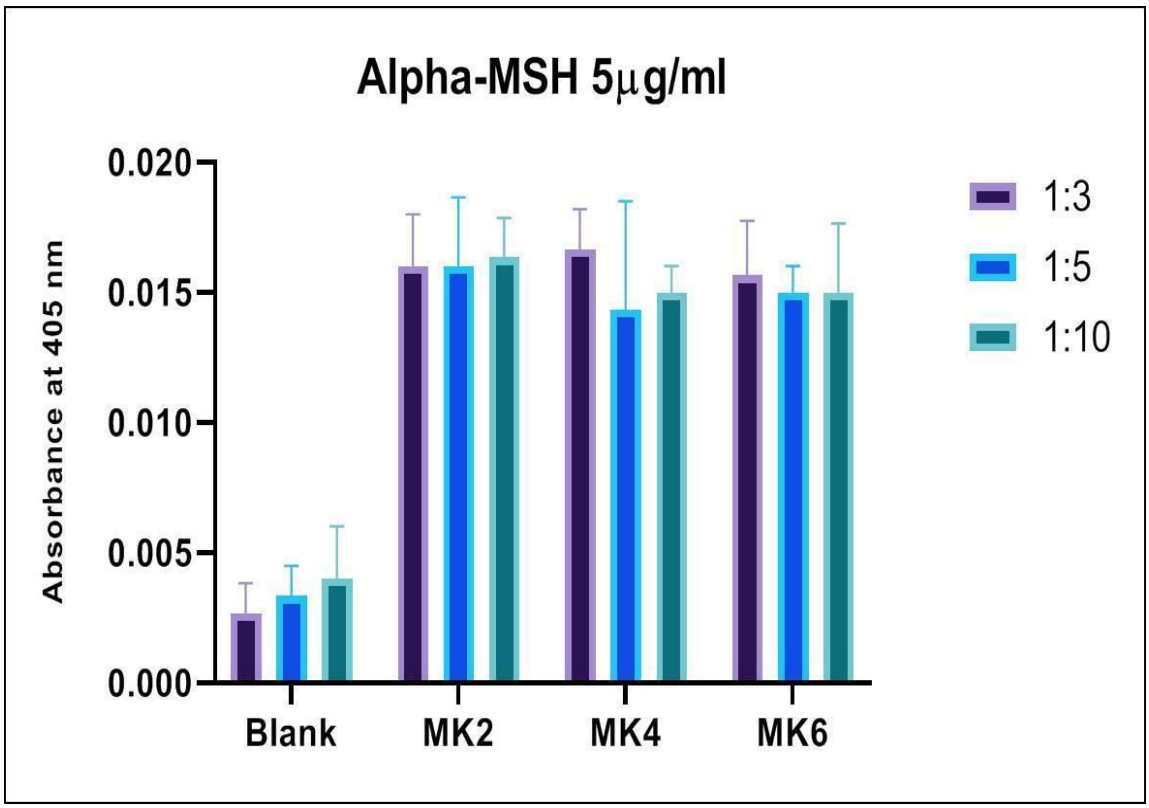
Figure 68: Melanin content assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines after treatment with α-MSH at 2.5 µg/ml concentration



There was a significantly increased melanin content in 1:3 and 1:5 ratios ($p<0.0334$).

The samples were analysed in triplicates. a and b indicate a significant difference in values between the co-culture ratios for each cell line as indicated ($P<0.05$).

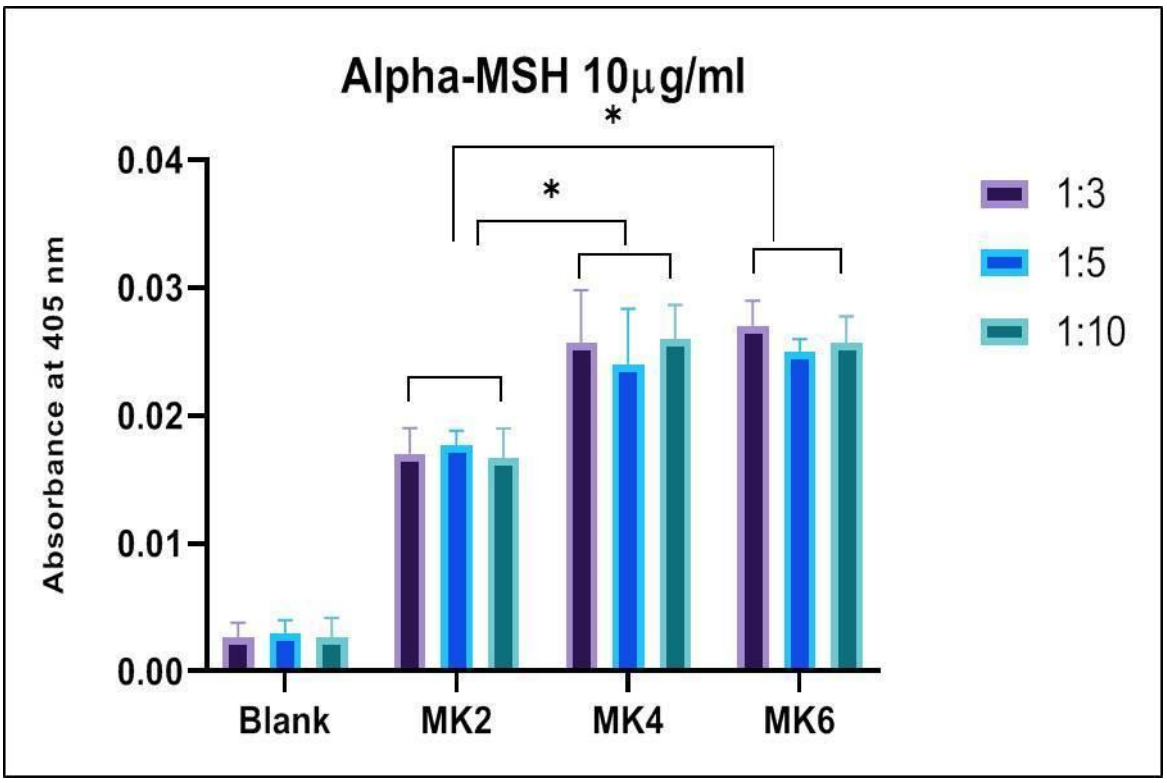
Figure 69: Melanin content assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines after treatment with α-MSH at 5 µg/ml concentration



No significant differences (P>0.05) in melanin content were recorded between the various ratios and the cell lines analysed.

The samples were analysed in triplicates.

Figure 70: Melanin content assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines after treatment with α-MSH at 10 µg/ml concentration



There was no significant difference (P>0.05) observed between the ratios of co-culture systems. But, between the cell lines, MK2 showed significantly lower secretion of melanin compared to MK4 and MK6 cell lines, when co-cultured cells were treated with 10 µg/mL α-MSH (P<0.02).

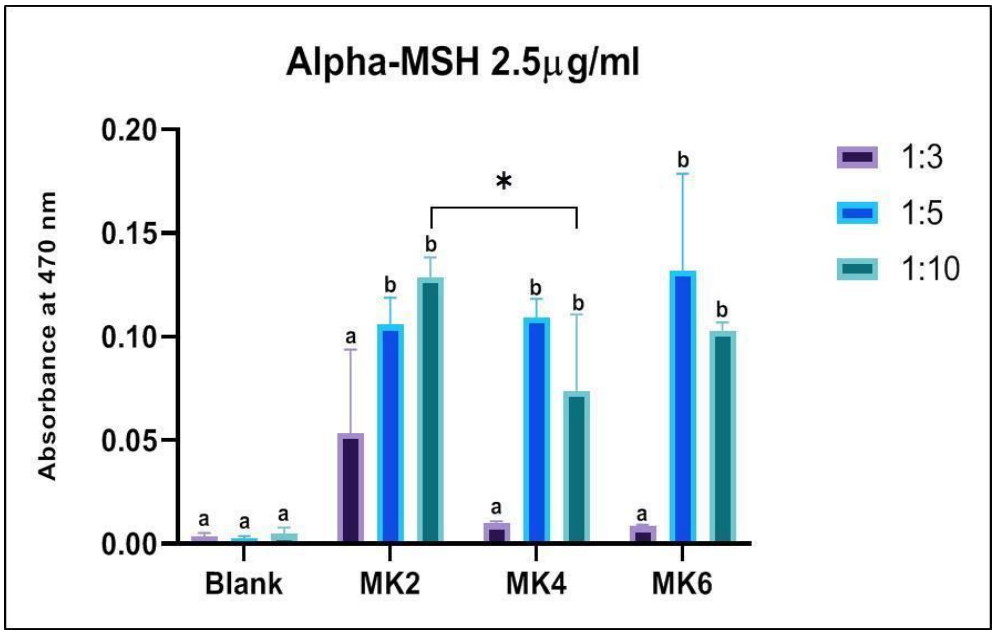
The samples were analysed in triplicates.

* indicates a significant difference in values between the cell lines as indicated (P<0.05).

4.9.3 Estimation of tyrosinase activity by L-DOPA assay in α-MSH (2.5, 5 and 10 µg/mL) treated co-cultured cell lines

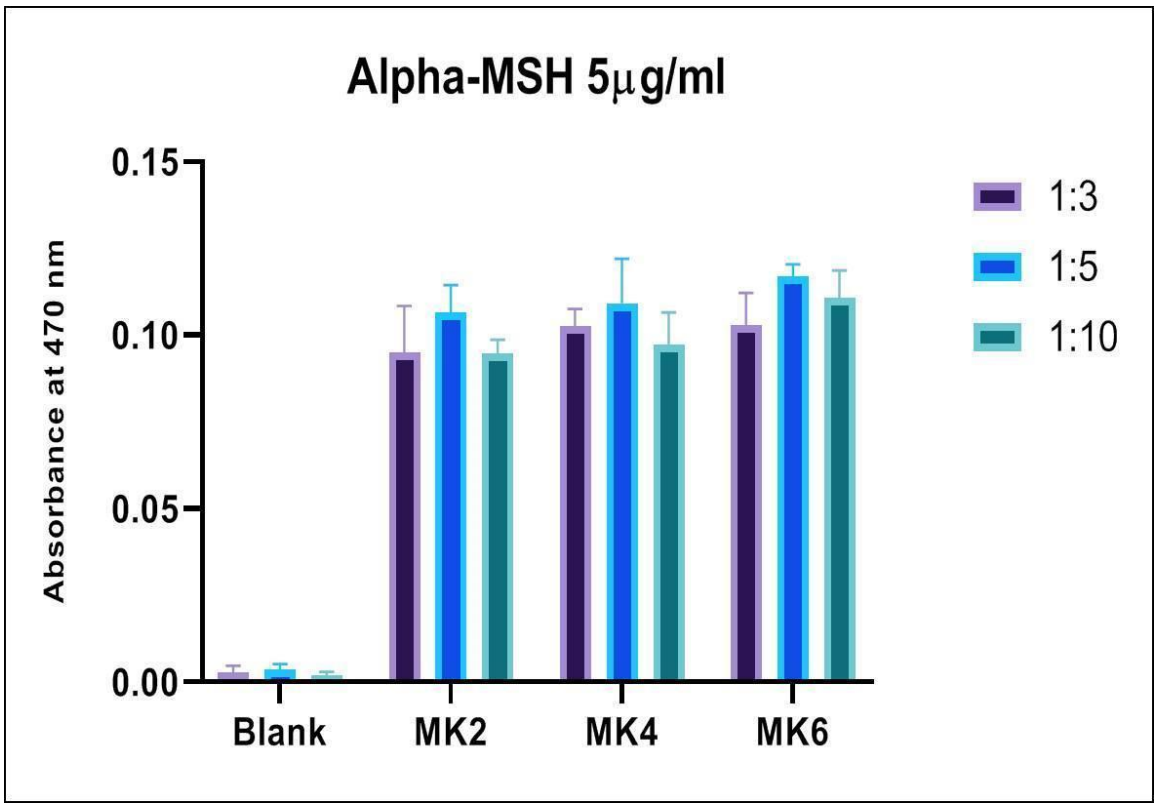
Tyrosinase activity by L-DOPA assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines was evaluated after treatment with α-MSH at 2.5, 5 and 10 µg/ml concentrations. The results are presented in **Figure 71**, **Figure 72** and **Figure 73**.

Figure 71: Tyrosinase activity by L-DOPA assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines evaluated after treatment with α-MSH at 2.5 µg/ml concentration



After α-MSH treatment all the cell lines showed increased tyrosinase activity. The tyrosinase activity was significantly high in 1:5 and 1:10 ratio in comparison with 1:3 ratio ($p < 0.05$). Between the cell lines, MK2 showed a significant increase in tyrosinase activity than MK4 in 1:10 ratio ($p=0.0273$). The samples were analysed in triplicates. *, a and b indicate a significant difference in values between the co-culture ratios for each cell line as indicated ($P<0.05$).

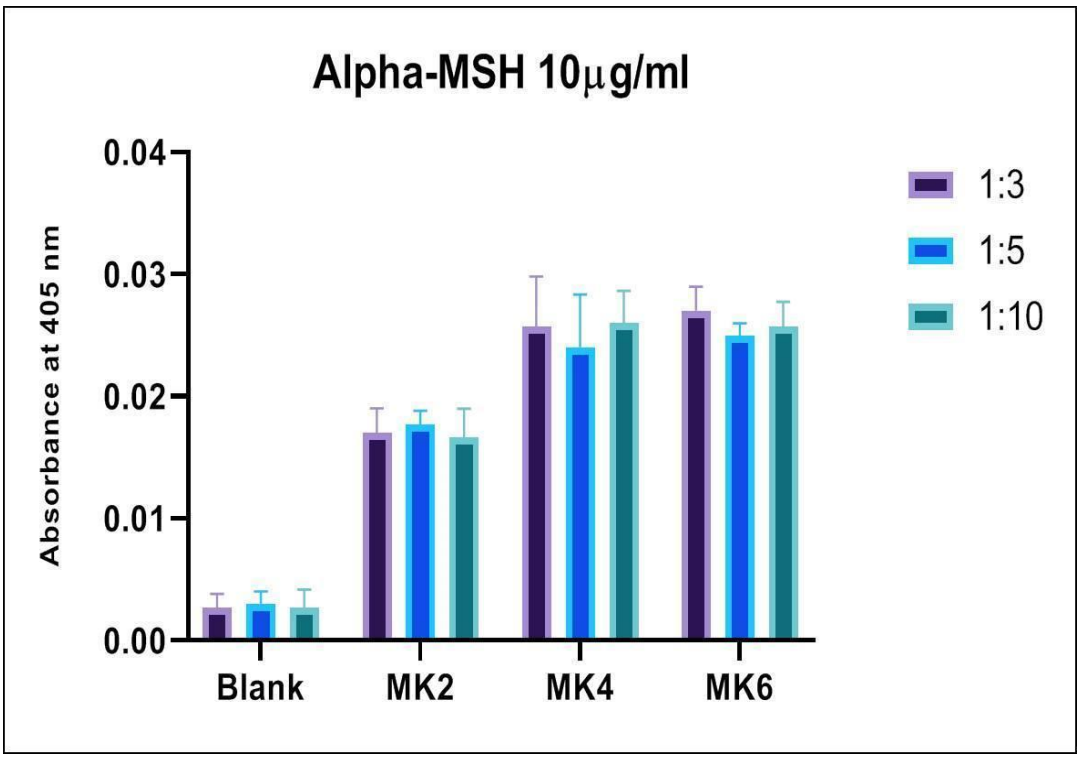
Figure 72: Tyrosinase activity by L-DOPA assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines after treatment with α-MSH at 5 µg/ml concentration



L- DOPA activity was recorded in all the cell lines. But, between the cell lines, its activity was not significantly different (P>0.05). Also, in between different ratios, the L-DOPA activity was not significant (P>0.05).

The samples were analysed in triplicates.

Figure 73: Tyrosinase activity by L-DOPA assay in melanocyte keratinocyte co-cultures at different ratios (1:3, 1:5 and 1:10) in MK2, MK4 and MK6 cell lines after treatment with α-MSH at 10 µg/ml concentration



L- DOPA activity was recorded in all the cell lines and was higher than blank. But, between the cell lines, its activity was not significantly different (P>0.05). Further, between different ratios also, the L-DOPA activity was not statistically significant (P>0.05).

The samples were analysed in triplicates.

4.10 Exposure of melanocyte keratinocyte co-cultures to UVB

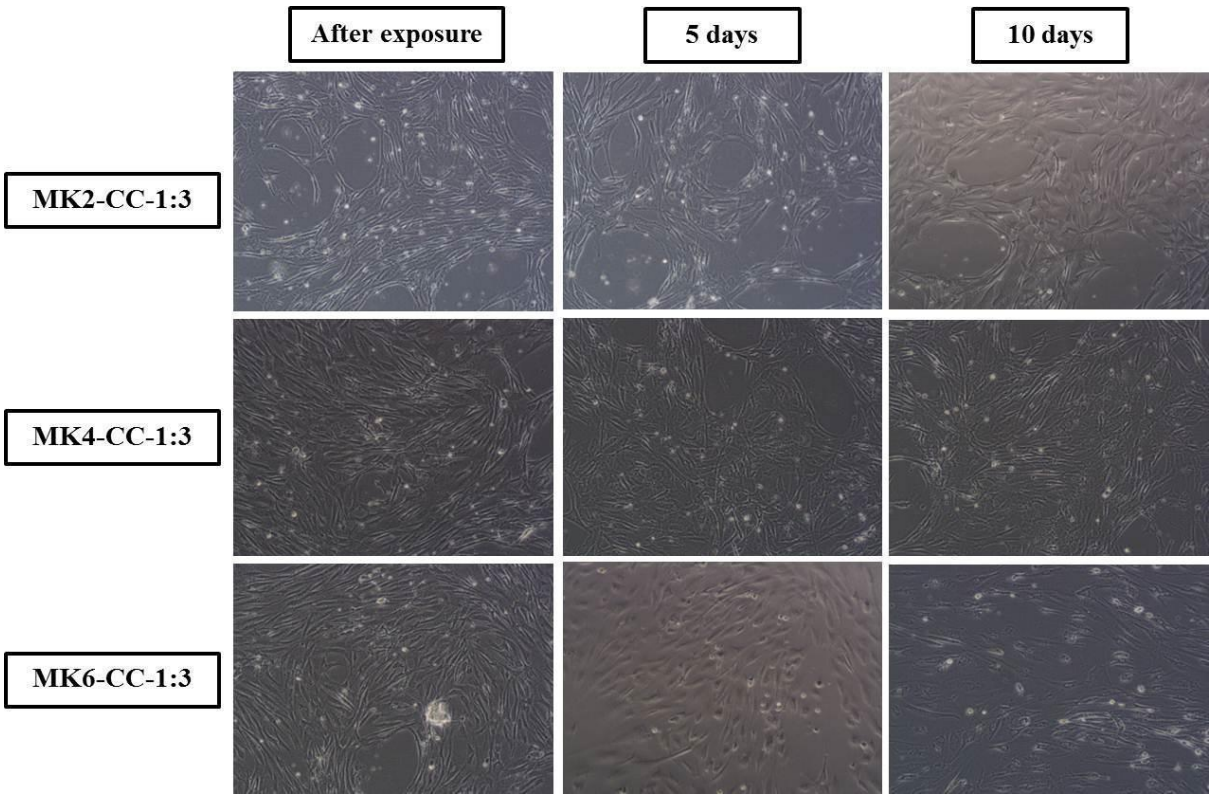
With an objective of further optimization of the co-culture system, the effect of UVB exposure on melanocyte-keratinocyte at ratios 1:3, 1:5 and 1:10 was also assessed. Three cell lines including, MK2, MK4 and MK6 were used for these experiments. Further, UVB exposure was tested at three different doses, such as 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2. The cellular and phenotypic characteristics of melanocytes and keratinocytes, such as morphology, melanin content, tyrosinase activity and the expression of selected markers were analysed in co-culture systems.

4.10.1 Morphological features of melanocyte keratinocyte co-cultures at 1:3 ratio exposed to UVB at different doses observed post-exposure, on day 5 and day 10

In this study, three cell lines of melanocytes and keratinocytes, such as MK2, MK4 and MK6 were employed for assessing the morphological changes at 1:3 ratio co-culture systems on days 5 and 10 after their exposure to UVB at 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2.

The differences in the morphological features were observed as per the doses of UVB used, and the results are presented for 1:3 ratio in **Figures 74** to **Figure 76**. In a dose dependent manner, the reduced number of cells with alterations in morphologies was observed in the co-culture system.

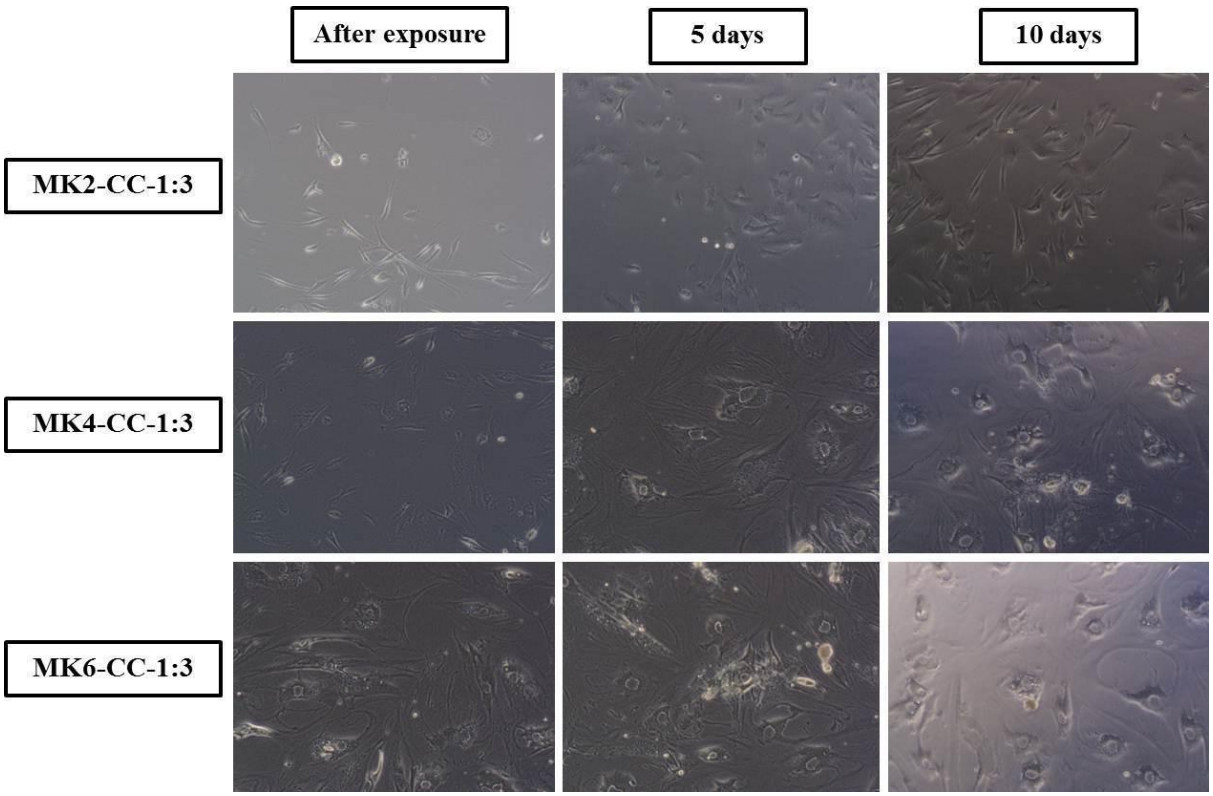
Figure 74: Representative images of morphologies of melanocyte keratinocyte co-cultures (MK2, MK4 and MK6 cell lines) at 1:3 ratio exposed to UVB at 10 mJ/cm^2 observed after exposure, on day 5 and day 10



Very moderate changes in morphologies were observed in melanocyte keratinocyte co-cultures after UVB exposure at 10 mJ/cm^2.

Image magnification-10x

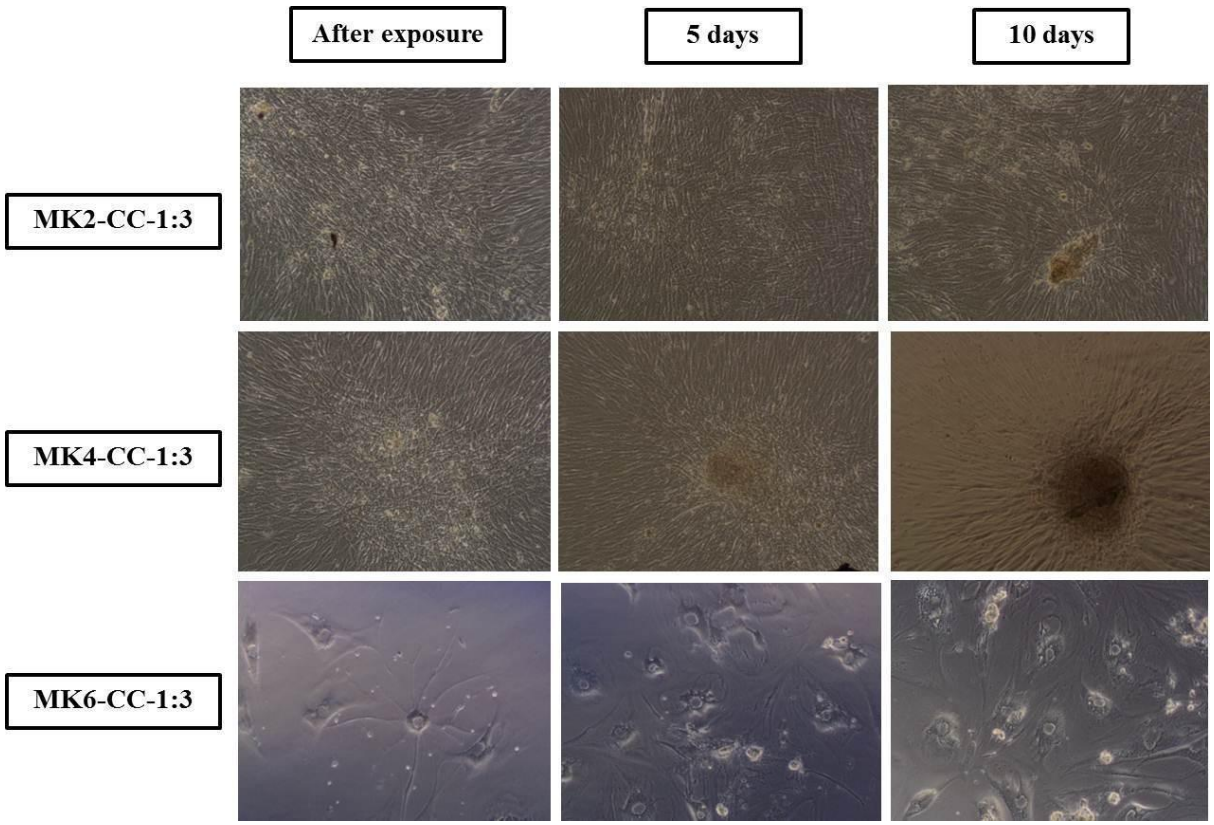
Figure 75: Representative images of morphologies of melanocyte keratinocyte co-cultures (MK2, MK4 and MK6 cell lines) at 1:3 ratio exposed to UVB at 20 mJ/cm^2 observed after exposure, on day 5 and day 10



Noticeable changes in morphologies were recorded in melanocyte keratinocyte co-cultures after UVB exposure at 20 mJ/cm^2. The cells formed clusters by day 5 and 10, and the number was reduced drastically in all the cell lines.

Image magnification-10x

Figure 76: Representative images of morphologies of melanocyte keratinocyte co-cultures (MK2, MK4 and MK6 cell lines) at 1:3 ratio exposed to UVB at 30 mJ/cm^2 observed after exposure, on day 5 and day 10



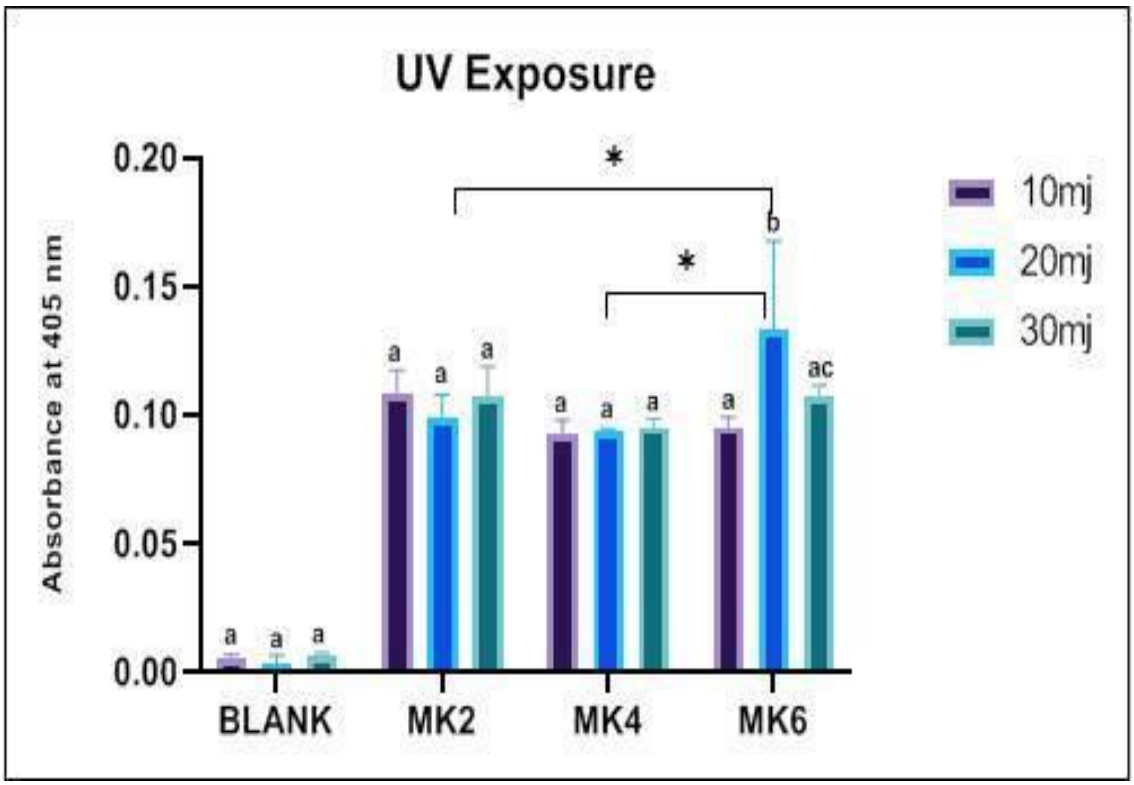
Drastic changes in morphologies were noticed in melanocyte keratinocyte co-cultures after UVB exposure at 30 mJ/cm^2. The cells formed clusters prominently by day 5 and 10, and the number of both melanocytes and keratinocytes was reduced considerably in all the cell lines.

Image magnification-10x

4.10.2 Melanin content assay in melanocyte keratinocyte co-cultures at 1:3 ratio exposed to UVB at different doses observed after exposure, on day 5 and day 10

Melanin content was analysed in melanocyte keratinocyte co-cultures at 1:3 ratio and exposed to UVB radiation at different doses. 1:3 ratio of co-culture with 3 different doses of UVB (10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2) showed significant increase in melanin content after exposure when compared to blank ($p<0.05$) (**Figure 77**). Hence, after UV radiation exposure, all the cell lines showed increased production of melanin.

Figure 77: Melanin content assay in melanocyte keratinocyte co-cultures (MK2, MK4 and MK6 cell lines) at 1:3 ratio exposed to UVB at 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2 observed after exposure, on day 5 and day 10



There was a significant increase in melanin content in all the three cell lines when compared with a blank. In 10 mJ/cm^2 and 30 mJ/cm^2, between the cell lines, there was no significant difference noticed. But, in 20 mJ/cm^2 significant difference in melanin content was observed in MK2 vs MK6 and MK4 vs MK6 ($p<0.009$). Statistical significant difference was observed only in MK6 cells between 10 mJ/cm^2 vs 20 mJ/cm^2 and 20 mJ/cm^2 vs 30 mJ/cm^2 ($p=0.0378$).

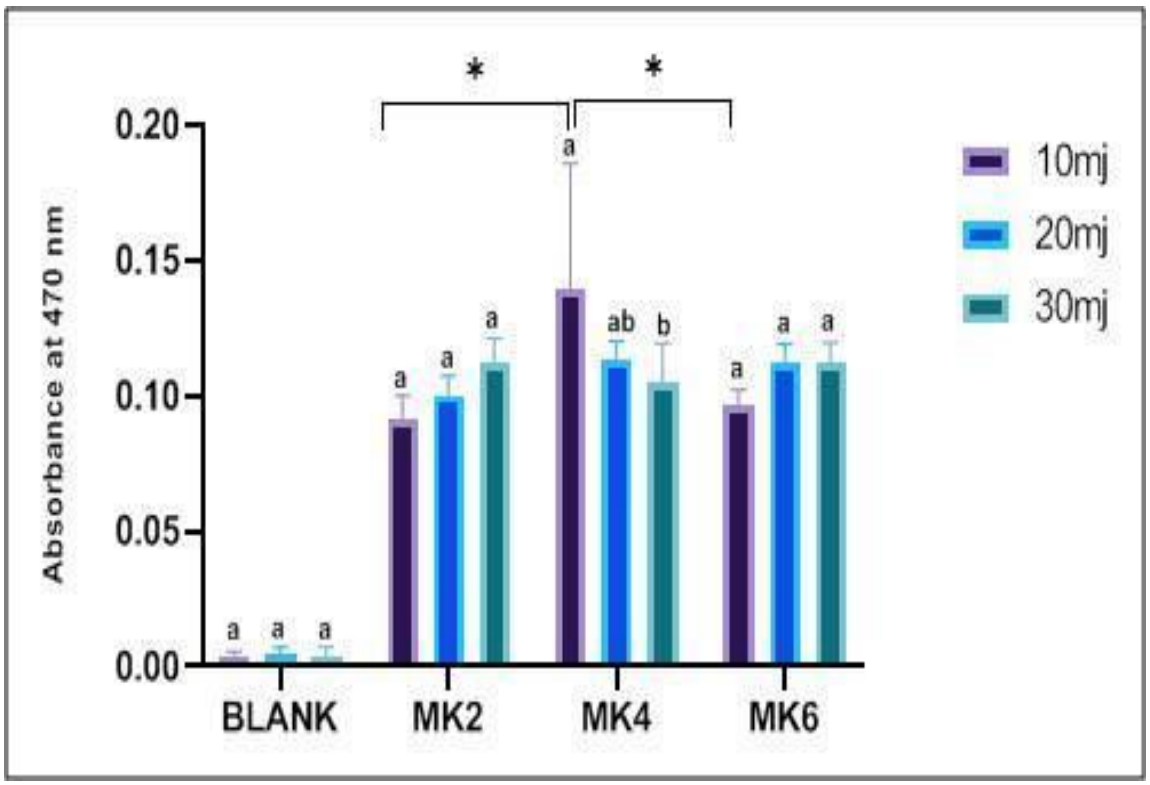
The samples were analysed in triplicates.

*, a, b and c indicate a significant difference in values between the UVB exposure doses for each cell line as indicated ($P<0.05$).

4.10.3 Tyrosinase activity by L-DOPA assay in melanocyte keratinocyte co-cultures (MK2, MK4 and MK6 cell lines) at 1:3 ratio exposed to UVB at 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2 observed after exposure, on day 5 and day 10

Tyrosinase activity by L-DOPA was analysed in melanocyte keratinocyte co-cultures at 1:3 ratio and exposed to UVB radiation at different doses. 1:3 ratio of co-culture with 3 different doses of UVB (10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2) showed significant increase in tyrosinase activity after exposure when compared to blank ($p<0.05$) (**Figure 78**).

Figure 78: Tyrosinase activity by L-DOPA assay in melanocyte keratinocyte co-cultures (MK2, MK4 and MK6 cell lines) at 1:3 ratio exposed to UVB at 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2 observed after exposure, on day 5 and day 10



Tyrosinase activity by L-DOPA assay was observed in all the cell lines exposed to various doses of UVB. However, a significant difference in L-DOPA activity was observed in MK4 cell line Vs MK6, MK2 ($p<0.0139$) in 20 mJ/cm^2. A significant difference was also noticed in the MK6 cell line between 10mj Vs 20mj ($p<0.0345$). Remaining exposure groups did not show any significant difference ($P>0.05$).

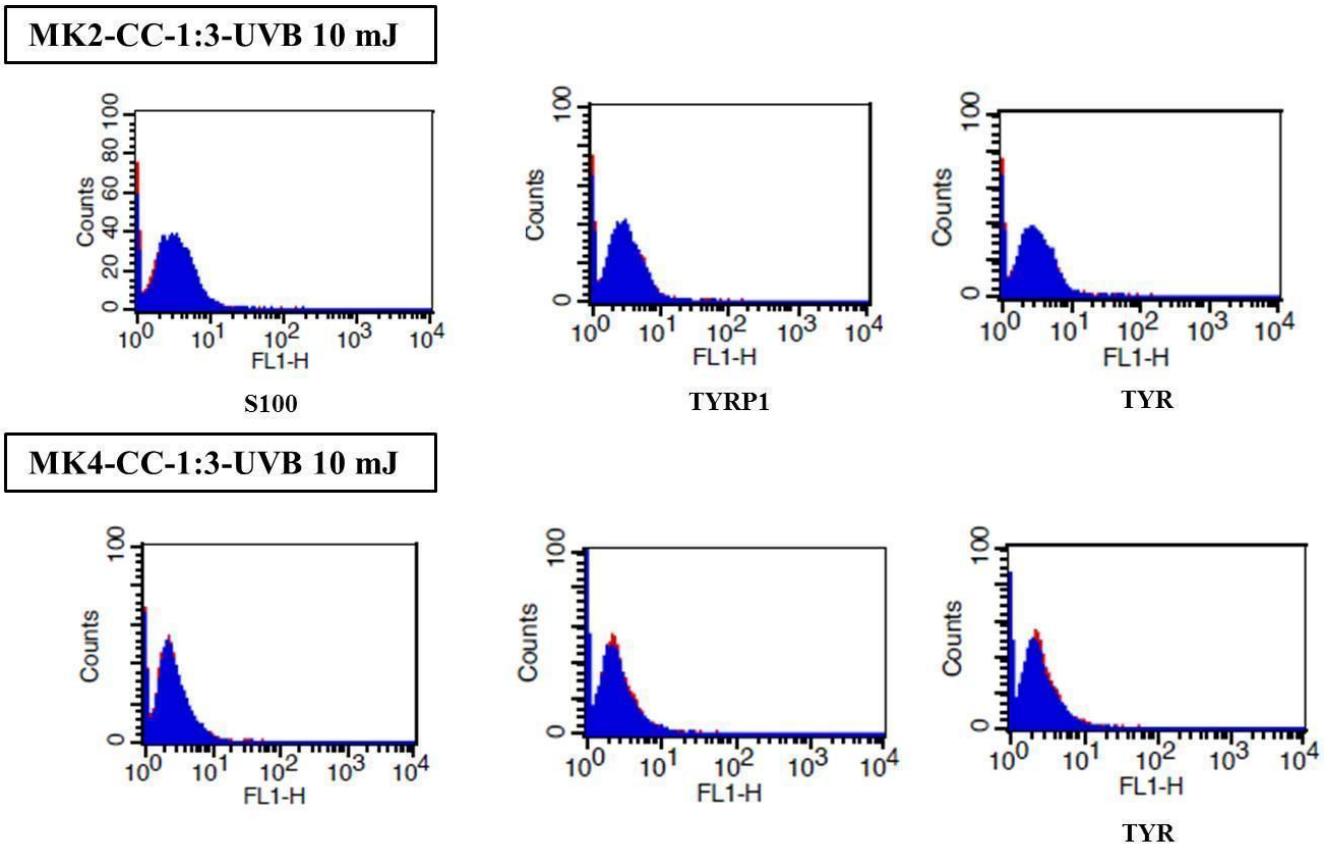
The samples were analysed in triplicates.

*, a and b indicate a significant difference in values between the UVB exposure doses for each cell line as indicated ($P<0.05$).

4.10.4 Phenotypic marker expression by flow cytometry analysis

Flow cytometric assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining in melanocyte keratinocyte co-cultures (MK2 and MK4 cell lines) at 1:3 ratio after exposure to UVB at 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2. Melanocyte-specific marker expression was quantitatively examined, and the results indicated that the exposure of UVB slightly enhanced the melanocyte markers expression (**Figure 79**, **Figure 80** and **Figure 81**). In addition, the expression of these selected markers was higher in MK2-CC-1:3 cells exposed at 20 mJ/cm^2.

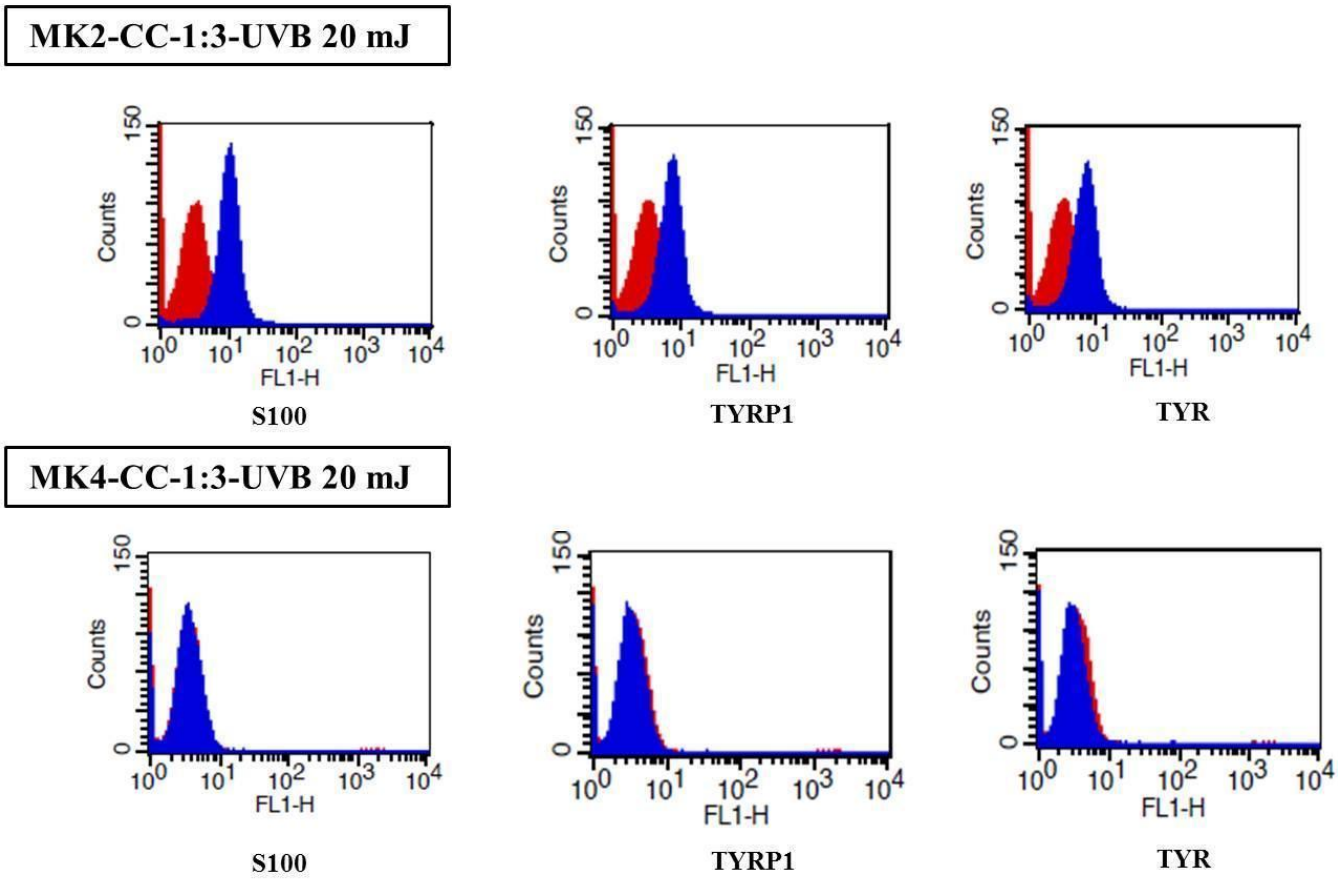
Figure 79: Representative results of flow cytometry analysis of melanocyte keratinocyte co-cultures (MK2 and MK4 cell lines) at 1:3 ratio after exposure to UVB at 10 mJ/cm^2



Melanocyte-specific marker expression was quantitatively examined by acquiring 10,000 cells per sample. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody.

The samples were analysed in duplicates.

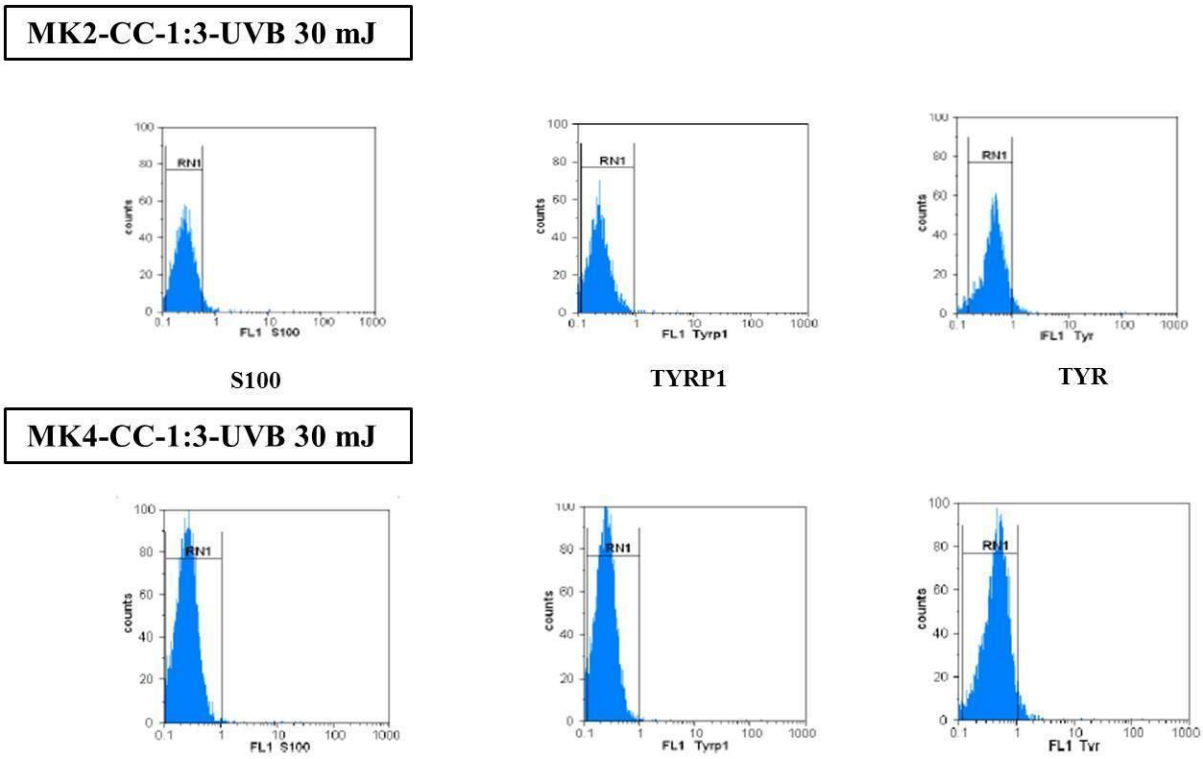
Figure 80: Representative results of flow cytometry analysis of melanocyte keratinocyte co-cultures (MK2 and MK4 cell lines) at 1:3 ratio after exposure to UVB at 20 mJ/cm^2



Melanocyte-specific marker expression was quantitatively examined by acquiring 10,000 cells per sample. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining. The blue histograms represent the specific staining, and the red histograms indicate background staining of an isotype-matched control antibody.

The samples were analysed in duplicates.

Figure 81: Representative results of flow cytometry (PARTEC) analysis of melanocyte keratinocyte co-cultures (MK2 and MK4 cell lines) at 1:3 ratio after exposure to UVB at 30 mJ/cm^2



Melanocyte-specific marker expression was quantitatively examined by acquiring 10,000 cells per sample. Assay demonstrated the expression of S100, tyrosinase related protein 1 (TYRP1) and tyrosinase (TYR) markers against FITC (Fluorescein isothiocyanate) staining.

The blue histograms represent the specific staining to the marker indicated.

The samples were analysed in duplicates.

4.11 Summary of results

Split-skin grafting (SSG) and full-thickness grafting (FTG) samples were obtained from subjects/patients undergoing routine graft surgery procedures with an informed consent. Digestion with collagenase type I and trypsin was employed to obtain epidermal cells by explant and suspension culture methods, respectively. Cell morphology, viability, proliferation and markers expression were assessed. Further, the co-culture system of melanocytes and keratinocytes at different rations (1:3, 1:5 and 1:10) was established and assessed various parameters, such as morphological changes at different days of culture duration, melanin content, tyrosinase activity and the expression of selected markers. Later, the same co-culture system of melanocytes and keratinocytes at different ratios were exposed to various concentrations of α-MSH (2.5 µg/ml, 5 µg/ml and 10 µg/ml) and examined for their morphological characteristics during culture duration along with melanin content assays and tyrosinase activity. Finally, the co-culture system of melanocytes and keratinocytes at different ratios were exposed UVB (10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2) and the cells were evaluated for changes in morphology, melanin content, tyrosinase activity and the expression of selected markers.

The primary cultures of melanocytes and keratinocytes from different human skin samples were successfully established (n=10) with highly proliferative features. Cells had a typical morphology with high viability and could be identified using specific markers, such as tyrosinase, S100, TYRP1, TYR, pan-cytokeratin, cytokeratin 10 and filaggrin. Further, the maintenance of cell cultures *in vitro* was carried out by employing cell specific media for further analyses.

After successfully establishing and culturing melanocytes and keratinocytes, the co-culture model was prepared at different ratios, such as 1:3, 1:5 and 1:10. These co-cultures were maintained up to 12 days. Morphological features were recorded at different timings and showed the higher population of melanocytes at ratios of 1:3 and 1:5. These results were supported by the higher synthesis of melanin content as well as the activity of tyrosinase in co-cultured cells.

The proliferation, melanin content and tyrosinase activity were increased in the co-culture model of melanocytes and keratinocytes when supplemented with different concentrations of α-MSH (2.5 µg/ml, 5 µg/ml and 10 µg/ml). The dose dependent response was observed in the cells with these parameters when compared to untreated controls.

The co-culture system of melanocytes and keratinocytes at different ratios when exposed to UVB at levels of 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2 resulted in stimulated effects on melanocytes and melanin production.

CHAPTER FIVE: DISCUSSION

There is a need for reliable techniques to isolate high quality melanocytes and keratinocytes, and expand them under *in vitro* culture systems to examine co-culture models for treating vitiligo subjects. Therefore, the present study was conducted to isolate and establish primary cultures of melanocytes and keratinocytes *in vitro* with a potential to prepare the appropriate skin co-culture model for replenishment.

The theory behind autologous transplantation is that melanocytes were harvested as an autologous melanocyte rich cell suspension from a donor split thickness graft. Melanocyte culture was performed in selected cases where the melanocyte cell count was insufficient to meet the requirement of the recipient area. These cells were then transplanted to the recipient area that had been superficially dermabraded.

A limitation of autologous transplantation is that it is unknown if the normal appearing non-lesional skin of vitiligo patients is in fact normal, therefore we cannot be sure if the melanocytes from non-lesional skin of vitiligo patients would in fact be impaired in their ability to produce melanin. Moretti et al. (2006) states that cultured melanocytes (showing an impaired growth) can only be obtained from pigmented (non lesional) skin of vitiligo patients and not from depigmented (lesional) vitiligo skin. One paper that investigated this further was Yu et al. (2012).

The conclusion of Yu et al. (2012) was that markers of heightened immune response were found to be upregulated in non-lesional as well as lesional skin of vitiligo patients. This suggests that melanocytes from non-lesional skin of vitiligo patients would in fact have an impaired functionality in comparison to those from a normal unaffected individual.

It should definitely be explored further if melanocytes from non lesional skin of vitiligo patients have an impaired ability to produce melanin. However, what must also be taken into consideration is the fact that the whole purpose of using autologous melanocytes as a treatment is that using cells from the same patient reduces the likelihood of rejection due to immune rejection. If we were to start using cells from a normal donor instead then this fact must be remembered.

Split-skin grafting (SSG) and full-thickness grafting (FTG) samples were obtained from patients undergoing routine graft surgery procedures with an informed consent. Digestion with collagenase type I and trypsin was employed to obtain epidermal cells by explant and suspension culture methods, respectively. The important factors required for establishing *in vitro* culture of melanocytes include the usage of appropriate enzymes and culture conditions. *In vitro* culture of adult human melanocytes was first successfully reported by Nielson and Don (1984). The improvement in the culture system was attempted by the supplementation of tumour promoting phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) (Eisinger and Marko, 1982). TPA was found to enhance the proliferation and attachment of melanocytes under *in vitro* culture. Moreover, the use of cyclic AMP (cAMP)-inducers, such as cholera toxin (CT) and isobutyl-methylxanthine (IBMX), observed to induce a mitogenic effect on

melanocytes, could be used to promote melanocyte cell proliferation in cell culture system (Eisinger and Marko 1982).

In the last two decades, the advancements in culture systems, especially by adding the selected growth factors in the medium have resulted in the enhanced yield of skin melanocytes. It has been demonstrated that basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and endothelin-1 (ET-1) enhance the melanocyte proliferation, tyrosinase activity and dendricity (Akio et al. 2004; Tang et al., 2013). Collectively, it is now established that either TPA or ET-1 is essential for the successful culture of melanocytes and hence, most of the commercially available melanocyte specific media consists of these supplements to support the culture of skin melanocytes.

In the present study, M254, a commercially available complete medium manufactured by GIBCO-INVITROGEN was employed for the culture of melanocytes after the initial usage of MEM-α. M254 medium contains essential and nonessential amino acids, vitamins, other organic substances, trace elements and salts to support the cell adherence and proliferation, but does not contain antibiotics, antifungal drugs, hormones, growth factors or other proteins. While establishing the culture, antibiotics (Penicillin-streptomycin) and antifungal (Amphotericin-B) were added to prevent the contamination. M254 medium also contains HEPES and bicarbonate buffers to maintain the optimal osmolality. After the initial primary culture establishment, supplementation of M254 medium with human melanocyte growth supplement (HMGS) is required for maintaining the long-term cultures of melanocytes. HMGS contains bovine pituitary extract (BPE), fetal bovine serum (FBS), bovine insulin, bovine transferrin, bFGF, hydrocortisone, heparin and phorbol esters, including phorbol 12-myristate

13-acetate (PMA), TPA, all of which are required for the growth of melanocytes under *in vitro* culture system (Swope et al. 1995; Tang et al., 2013).

As explained previously, isolated epidermal tissue remnants were pooled in a trypsin/EDTA solution and single-cell suspensions were prepared by filtering and pipetting. Separated cells were collected in serum to stop enzymatic action of trypsin. The pooled epidermal cell suspensions were centrifuged at low speed to harvest the cells and resuspended in growth medium. Cell suspensions were placed in tissue culture dishes in growth medium containing HMGS. After 48 hours of seeding cells, the medium was changed and unattached cells were removed. From day 3 onwards till the cells reached confluence, the attachment patterns and morphological features of melanocytes with dendritic features were observed regularly during their culture expansion. The cellular properties, such as proliferation ability and viability were analysed during their early culture system. Melanocytes were highly proliferative and did not show any noticeable differences in their cell number between the cell lines. Further, the viability values were also >98% at all cell passages (P1 to P6) analysed, and thus indicated their healthy status during the *in vitro* culture expansion.

Establishment of a pure population of melanocyte cultures facilitates the development of suitable co-culture models with keratinocytes and helps to study the factors controlling the pigmentation process. In this study, melanocytes derived from normal skin tissues showed bipolar structures with circular nucleus, and dendritic features. Similar morphological characteristics of melanocytes were observed in earlier studies (Tang et al., 2013; Zhang et al., 2018). However, Kumar et al. (2012) recorded some significant differences in the morphology of melanocytes between control skin and samples from unstable vitiligo patients. Melanocytes

from control samples were normal in perinuclear region and size. Whereas melanocytes from unstable vitiligo patients showed a bigger perinuclear region and the dendrites of perilesional unstable vitiligo patients were small with clubbed ends. In comparison, the results of the present study demonstrated that melanocytes from early passages (P1 to P6) exhibit key phenotypic characteristics of normal skin samples and possess relatively higher proliferative activity.

Apart from morphological and cellular characteristics, the conformation of melanocytes was carried out by melanin synthesis assay, L-DOPA staining and the expression of melanocyte specific markers, such as S100, TYRP1 and TYR. Established melanocytes were stained dark after L-DOPA incubation. L-DOPA positive staining confirms the presence of DOPA oxidase (tyrosinase) activity in the melanosomes as evidenced by previous reports (Munoz–Munoz et al. 2010; Tang eta l., 2013). This parameter is employed to positively identify melanocytes, and they are the only DOPA oxidase-positive cells in the cultures. L-DOPA functions as a second messenger subsequent to tyrosine hydroxylase activity, promoting the pigmentation through co-ordinated action with TYR (Stominski et al., 1988). Further, in melanocytes, L-DOPA activity enables the activation of TYR *in vitro* (Shi et al., 2016). In accordance with these observations, the results of this study showed strong reactivity in melanocytes, which corresponds with the expression of TYR marker in subsequent analysis.

The expressions of melanocyte specific markers, including S100, TYRP1 and TYR were analysed to validate that the cells possess functional features of normal melanocytes. S100 is a highly sensitive marker for melanocytes and is widely distributed in cells of neural crest origin and tumour cells. The observation of positive staining using both L-DOPA and S-100

proves that the cells established and cultured were indeed melanocytes (Petersson et al. 2009; Shi et al., 2016). TRP1 and TYR are related enzymes expressed in melanocytes and involved in melanin synthesis, which can also be employed to identify melanocytes (Tang et al., 2014). The expression of TRP1 has been closely related to melanin synthesis and its level of detection confirmed the purity and melanogenic capacity of melanocytes at different passages. Similarly, high expression of TYR is associated with increased melanin synthesis and its level of expression affects melanogenesis (Shi et al., 2016). Further, TYR catalyzes the rate of melanogenesis, and has been functionally correlated with the expression of a pigmented phenotype of melanocytes (Yamaguchi and Hearing, 2009). The results of the present study with the expression of these markers in established melanocyte cell lines showed that they have relatively higher melanogenic activity.

The present study attempted to establish and culture keratinocytes from normal skin tissue samples for developing suitable co-culture models with melanocytes. Previous reports have showed that keratinocytes can be cultured based on the traditional feeder-dependent method (Rheinwald and Green, 1975) or by other alternative methods using defined serum-free, low-calcium media, that do not require any feeder cells for cultivation (Tenchini et al., 1992). The current study succeeded in establishing keratinocyte subcultures without using feeder layers. Similar to melanocyte cell preparation, the cell suspension obtained after enzymatic digestion was filtered through a cell strainer and washed twice in DPBS. The harvested cells were resuspended in defined keratinocyte medium (EpiLife, GIBCO-INVITROGEN) supplemented with keratinocyte growth factor (KGF) and epidermal growth factor (EGF), and allowed for the establishment of primary culture with the medium being changed every third day. Microscopic observations revealed that the confluent keratinocytes formed a pavement-like monolayer and most importantly, no fibroblast contamination was observed in the cultures of

later passages. These cells could be sub-cultured to passage 6 without significant loss of morphological features. Established keratinocytes were highly proliferative around one week of seeding the cells and maintained their typical morphology. In agreement with these observations, isolation of primary keratinocytes was accomplished by enzymatic dissociation or with explant culture method using murine and human skin samples (Yano and Okochi, 2005; Aasen and Belmonte, 2010; Kumar et al., 2012; Zhang et al., 2017). In comparison with some cell types, such as fibroblasts, keratinocytes require more attention and issues such as apoptosis in low density and specifically, differentiation and senescence after reaching confluence have been observed as more prominent features (Aasen and Belmonte, 2010). However, in this study, these characteristics were not noticed, and obtained large numbers of keratinocytes for further analysis. Here, the role of EGF supplementation in culture media was evident as its positive effects on keratinocyte culture have been previously investigated (Yano and Okochi, 2005). Although the culture protocol followed in this study employed neither feeder layer nor any coating material, the supplementation of KGF and EGF was indispensable for the maintenance of keratinocyte cultures.

During the culture expansion of keratinocytes from skin tissues, fibroblast contamination poses a common challenge. Therefore, to determine the purity of the culture expanded keratinocytes, markers, such as pan-cytokeratin (PanC), cytokeratin10 (CK10) and filaggrin (FLG) expression was analysed by FACS method. Established keratinocytes at early passages were positive for the above markers indicating a high level of cell population in cultures. With this, the morphological analysis of keratinocytes was complemented by marker expression of basal cell markers (PanC and CK10) and the terminal differentiation marker, FLG. Earlier reports too demonstrated the expression levels of selected markers in keratinocytes using similar techniques (Yano and Okochi, 2005; Alkhilaiwi et al., 2018; Zhang et al., 2018). Collectively,

it is inferred that the keratinocyte cultures were established using a defined media and the supplementation of growth factors, such as KGF and EGF. A larger number of keratinocytes were obtained with normal morphology and proliferative features. Further, the purity of keratinocytes was ascertained by the expression of specific markers, which play a critical role in regulating the cellular and biological functions.

The melanocytes and keratinocytes would be a suitable co-culture model for different applications in vitiligo patients. Hence, the co-culture system of melanocytes and keratinocytes at different ratios (1:3, 1:5 and 1:10) was established and assessed on various parameters, such as morphological changes at different days of culture duration, melanin content, tyrosinase activity and the expression of selected markers. Later, the same co-culture system of melanocytes and keratinocytes at different ratios were exposed to various concentrations of α-MSH (2.5 µg/ml, 5 µg/ml and 10 µg/ml) and examined for their morphological characteristics during culture duration along with melanin content assays and tyrosinase activity. Finally, the co-culture system of melanocytes and keratinocytes at different ratios were exposed UVB (10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2) and the cells were evaluated for changes in morphology, melanin content, tyrosinase activity and the expression of selected markers.

It is known that melanocytes in epidermis exist in close contact with keratinocytes which are shown to affect melanocyte morphology and growth, and hence, the present study developed a co-culture system of melanocytes and keratinocytes at different ratios. In a co-culture system of different ratios (melanocytes: keratinocytes of 1:3, 1:5 and 1:10), melanocytes and keratinocytes from skin tissue samples were morphologically different, and the number in both types of cells varied at different time points of culture as observed on day 3, day 5 and day 12. Melanocytes are specialized epidermal cells, originated from the neural crest, with a

representation of 2-5% of the epidermal cells in the skin (Cooper and David, 2009). It has been showed that melanocytes, being pigment producing cells, transfer melanin pigment within their cytoplasmic melanosomes to surrounding keratinocytes. Further, their proliferation and melanogenic activities are stimulated by keratinocytes, fibroblasts and other regulatory factors (Liu et al., 2008; Zhang et al., 2018). This evidence indicates the existence of some complex interactions between melanocytes and keratinocytes through the secretion of a wide variety of molecules. To ascertain this, the melanin content and the tyrosinase activity by L-DOPA assays were performed in an established co-culture system. The results demonstrated that there was an increase in melanin secretion and also tyrosinase activity in melanocyte keratinocyte co-cultures at 1:3 and 1:5 ratios. An earlier study by Kumar et al. (2012), successfully cultured melanocytes and keratinocytes at 1:5 ratio from control and vitiligo patients and after that co-culture models were prepared. After treatment of a co-culture model with melanogenic stimulator, they recorded increased tyrosinase activity, cell proliferation and melanin content. In contrast, after treatment with melanogenic inhibitors, tyrosinase activity, cell proliferation and melanin content were decreased. Finally, the study concluded that the use of primary melanocytes and keratinocytes is more appropriate over the use of transformed cells for assessing the regulators of pigmentation. In a recent study, the number of melanocytes co-cultured with keratinocytes *in vitro* reached the maximum on the 20^{th} day of culture (Zhang et al., 2018). The authors opined that this co-cultured model might contribute to the growth of melanocytes which could be employed in the treatment of vitiligo. These collective facts demonstrate the existence of interactions and communicatory links between melanocytes and keratinocytes in co-cultures, resulting in modulated secretion of melanin and pigmentation process (Decean et al., 2013).

The increase in proliferation and migration of melanocytes is expected in mixed melanocyte and keratinocyte cultures (Duval et al., 2001; Kim et al., 2012; Zhang et al., 2018). With this interactive phenomenon, a study examined the effects of adipose-derived stem cells (ADSCs) on *in vitro* proliferation, differentiation, and migration of melanocytes, and compared with those of keratinocytes (Kim et al., 2012). The data showed that co-culturing with ADSCs stimulated both proliferation and migration of melanocytes although this was to a lesser degree compared with co-culturing with keratinocytes. In addition, ADSC co-culturing also increased the number of melanocyte precursor cells. These findings could be used to improve treatments of pigmentation disorders associated with the loss of melanocytes (Kim et al., 2012). Therefore, applications of co-cultured epidermis cells rather than just melanocytes to treat conditions, such as vitiligo have many advantages. This includes the regulation by keratinocytes on the growth and differentiation of the melanocytes (Zhang et al., 2018). Further, co-culturing will maintain the normal physiology characteristics of melanocytes, which mean melanocytes, could communicate with keratinocytes and dermal fibroblasts by secreted factors and cell contacts. Finally, culturing with keratinocytes is easier than melanocytes alone in a short time to obtain an autologous epidermis, and this method may not pose any serious adverse effects (Zhang et al., 2018).

Co-culturing of melanocytes with keratinocytes induced enhanced expression of melanocyte markers, such as S100, TYRP1 and TYR. The expression of TYRP1 showed a correlation with enhanced melanin content in a co-culture system. In a study by Kim et al. (2012), migration assay showed that co-culturing with ADSCs stimulated the melanocyte migration less than co-culturing with keratinocytes, although both cell types significantly stimulated migration compared with melanocyte monocultures. The report also demonstrated the expression of N-cadherin and E-cadherin proteins as they are transmembrane glycoproteins connected to the

actin cytoskeleton, and mediate haemophilic adhesion between neighbouring cells. In addition to this, the keratinocyte-derived growth factors, such as NGF, ET-1, and BFGF, promote melanocyte migration (Ma et al., 2006; Kim et al., 2012). However, additional studies are warranted to decipher the mechanisms by which mediators affect the melanocyte migration and melanogenic functions.

In the present research work, the effect of an important melanogenic stimulator compound, such as α-MSH was examined by supplementing different concentrations (2.5 µg/ml, 5 µg/ml and 10 µg/ml) on the melanocyte-keratinocyte co-culture system (1:3, 1:5 and 1:10). The results confirmed that proliferation, melanin content and tyrosinase activity based on L-DOPA assay increased in dose dependent manner in the established co-culture model. During the melanocyte culture, it has been observed that the cell-culture techniques yield heterogeneous cell populations and lead to eventual overgrowth by keratinocytes and fibroblasts (Zhang et al., 2018).

Thus, studies of *in vitro* characteristics of human melanocytes have been made difficult by the presence of contaminating populations of rapidly multiplying cells other than melanocytes. On the other hand, biosynthesis of melanin is initiated by multiple stimuli including hormonal signaling, UV irradiation and secretion of inflammatory cytokines (D'Mello et al., 2016). Particularly, α-MSH released from UV-exposed keratinocytes can stimulate melanin biosynthesis in epidermal melanocytes by activating the cAMP-PKA-CREB (cyclic adenosine monophosphate-protein kinase A-cAMP response element binding protein) axis (D'Mello et al., 2016). In the present study, it has been noticed that α-MSH directly influenced the melanin synthesis in a dose dependent manner, and this might have resulted in enhanced tyrosinase

activity that is independent of the transcriptional machinery associated with melanogenesis. These results suggest that α-MSH may be a potential component for the management of melanocytes and keratinocytes co-culture systems. This is supported by the fact that this neuropeptide was primarily known as melanogenic hormone. But, later its immunomodulatory role has been discovered and its anti-inflammatory effects have been implicated in the cure of many inflammatory conditions (Singh and Mukhopadhyay, 2014).

In the present study, the stimulatory effects of different concentrations of α-MSH were examined on the tyrosinase enzyme activity using cellular and cell-free tyrosinase activity assays and also based on L-DOPA assay. Based on the findings of melanocyte proliferation, melanin content and tyrosinase activity, the positive effect of α-MSH was demonstrated. However, the study did not look into a precise molecular mechanism as to how α-MSH enhances tyrosinase activity or melanin synthesis in melanocytes. This mechanism should be further explored to elucidate whether α-MSH directly interacts with tyrosinase and stimulates its oxidase activity or whether it has indirect effects that lead to increased tyrosinase activity.

In this study, the established co-culture model of melanocytes and keratinocytes was allowed to UVB exposure at different sub-cytotoxic doses, such as 10 mJ/cm^2, 20 mJ/cm^2 and 30 mJ/cm^2. Later, the exposed cells were cultured for 10 days and assessed for any changes in cellular and biological characteristics. The treatment resulted in features of enhanced number of melanocytes accompanied by hyperpigmentation as evidenced by enhanced melanin content. Additionally, increased tyrosinase activity was recorded by L-DOPA assay following UVB exposure. Further, the expression of markers, such as S100, TYRP1 and TYR was

considerably enhanced in melanocyte and keratinocyte co-culture of 1:3 ratio after UVB exposure to 20 mJ/cm^2.

It is well established that, upon exposure to UV-radiation, melanin produced by epidermal melanocytes is transferred to neighbouring keratinocytes and thus, permitting their absorption of UV-radiation to protect the cells from UVR-induced damage (Lin and Fisher, 2007). With this, it displays the role of photoprotection and thermoregulation activity. Under *in vitro* conditions, the epidermal melanin unit could be established as a co-culture system or as in tissue-engineered epidermis. These models are constructed to resemble the anatomical structure of human epidermis including the interaction between melanocytes and keratinocytes that ultimately results in macroscopically visible alterations. This pigmented reconstructed human epidermis may be employed as an alternative to animal model testing in cosmetic, pharmaceutical and medical research. However, no study has demonstrated yet a direct quantitative correlation between melanin content and the capacity to withstand natural UV-radiation under *in vitro* conditions. In a recent report by Choi et al. (2018), human epidermal melanocytes were exposed twice with 20 mJ/cm^2 UVB over a 24 hrs interval and subsequently cultivated for 2 weeks. The exposure induced melanocyte senescence, and senescent melanocytes were accompanied hyperpigmentation through the prolonged p53 expression. This study primarily examined the long-term effects of UV-radiation exposure in human epidermal melanocytes, the subsequent phenomena and key mediators of this process.

A distinct melanogenic response to UV exposure by human melanocytes in monoculture, in co-culture with keratinocytes and in reconstructed epidermis was documented (Duval et al., 2001). The study concluded that keratinocytes play an important role in mediating UVB-

induced pigmentation, but do not seem to be involved in the process of UVA stimulated pigmentation. This research further suggested that reconstructed pigmented epidermis is the most appropriate model to study UV-induced pigmentation under *in vitro* conditions. Thus, there is an increasing interest to study these processes in a standardized co-culture model of melanocytes and keratinocytes that mimics the *in vivo* situation as closely as possible. Moreover, melanin is detectable within the cytoplasm of keratinocytes and hence, a functional epidermal melanin unit, can be created in this model (Schmid et al., 2018).

In the current study, the effect of upper dose of UVB exposure at 30 mJ/cm^2 was noticeable in melanocytes and keratinocytes co-cultured at 1:3 ratios. Importantly, the reduction in the number of both the cells was observed at a dose of 30 mJ/cm^2. These observations indicate that doses of 10 mJ/cm^2 and 20 mJ/cm^2 might allow desirable cellular reactions in both melanocytes and keratinocytes, but higher UVB exposure might result in unfavourable changes, such as immediate cell death. These findings are in accordance with previous studies that indicated a connection between the functional properties of melanin unit and keratinocyte properties after UVB exposure (Duval et al., 2001; Choi et al. 2018; Schmid et al., 2018).

To summarize, the successful isolation and culture expansion of melanocytes and keratinocytes separately will enable trying out various ratios of these cells in a different medium as a co-culture method for potential therapy in vitiligo patients. Based on the findings, it is drawn the conclusion that the positive rate of melanocytes co-cultured with keratinocytes *in vitro* reached the maximum on the 12th day, and this co-culture model may be capable of being used in the treatment of vitiligo patients.

CHAPTER SIX: CONCLUSION

6.1 Scientific outcomes of my doctoral work

6.1.1 Developing a method to prepare skin tissue

A major revelation from my experimental work before the skin was processed in any way was just how susceptible the skin samples were to contamination. Although sorting this problem might seem relatively trivial it would actually be accurate to describe it as a rate limiting step and solving it completely was actually more complex than might originally be thought. When samples were initially received from the patient some were lost to contamination before any experimental work had begun and it seems that the location of the skin being on the surface of the body makes it particularly vulnerable. In summary it was found that the ideal way to reduce infection was to immediately place the sample, following extraction, into medium (DMEM or DPBS) containing penicillin, streptomycin and fungizone. Once the sample was received in the lab it was washed in 10ml of DPBS and disinfected with 10-30ml of 75% medical alcohol for 30 sec followed by washing with 30ml DPBS three times.

The effects of using different enzymes and digestion times to isolate cells from the skin sample was investigated and the best options were found to be to either perform a short incubation with enzyme at the warmer temperature of 37^0C for about 4 hours or a longer overnight incubation at 4^0C. The ideal isolation of cells from the skin sample occurred when a combination of enzymes was used. The preferred method to isolate cells from the skin sample

was found to be to first separate the epidermis from the dermis by placing the skin in 0.25% trypsin-EDTA followed by incubation in 0.1% collagenase to isolate the cells from this.

In conclusion as a result of my doctoral work an optimised procedure was developed for the isolation of melanocytes and keratinocytes from skin samples and the establishment of their subsequent monocultures. Cells isolated from skin are a mixed population of melanocytes, keratinocytes and fibroblasts and the population was encouraged to being of either a melanocyte or keratinocyte lineage by transferring the mixed culture, once established, to a cell specific medium that supported the growth of either melanocytes or keratinocytes.

6.1.2 Isolation and characterisation of melanocytes based on their cellular, biological and phenotypic properties.

Established melanocytes were characterized based on their proliferation potential, viability, melanin content, tyrosinase activity and the expression of selected melanocyte markers. All the melanocytes exhibited a proliferation potential that was initially slow but later occurred at higher rates whilst cell viability was consistently more than 90% amongst all cell lines. Melanocytes can be distinguished morphologically from other cells by their bipolar or polydendritic features. Melanin levels in the melanocyte cells were measured via ELISA reader at absorbance 405nm and compared to blank and dermal fibroblasts, used as controls, which confirmed the production of melanin during the culture expansion period. An L-DOPA

assay confirmed tyrosinase (enzyme of melanin synthesis) activity and these both gave a positive result in comparison to the controls at an absorbance of 470nm. The positive dark brown staining of cells by L-DOPA and their ability to be bleached in the melanin bleach assay also confirmed these cells were melanocytes. A positive result for expression of melanocyte specific markers S100, TYRP1 and TYR used via flow cytometry also identified that the cells in culture were melanocytes.

6.1.3 Isolation and characterisation of keratinocytes based on their cellular and phenotypic features

Morphologically keratinocytes in monoculture formed a pavement like monolayer/cells had a round polygonal shape. Established keratinocytes were highly proliferative and they exhibited high levels of viability in culture. Flow cytometry using the keratinocyte specific markers PanC, CK10 and FLG also confirmed keratinocyte presence.

6.1.4 Establishment and examination of co-culture methods for melanocytes and keratinocytes in different culture conditions

For the co-culture of melanocytes with keratinocytes the melanocyte to keratinocyte seeding densities of 1:3, 1:5 and 1:10 were used so that the growth of both cell types could be supported at the same time. Measuring absorbance in the co-cultures at 405nm using an ELISA reader showed that the best results for melanin content was at seeding ratios of 1:3 and 1:5, there was

not much difference in outcomes between them but both showed markedly superior results to culturing at 1:10. This result was confirmed by measuring L-DOPA via absorbance at 470nm. Presence of melanocytes was further confirmed using cell specific markers via flow cytometry in the 1:3 co-culture ratio.

6.1.5 Manipulation of culture conditions with alpha-melanocyte-stimulating hormone

An attempt was made to manipulate the melanin content in all three different co-culture ratios by exposing them to α-MSH at three different concentrations (2.5, 5 and 10 µg/mL). Proliferation, melanin content and tyrosinase activity were increased in co-culture upon supplementation with α-MSH. Exposing melanocytes to α-MSH in increasing concentrations stimulated melanin production in these cells in a dose dependent manner.

6.1.6 Manipulation of culture conditions with UV light

The effect of UV light as a stimulator of melanin production in melanocytes was observed and cells in all three co-culture ratios were exposed to UVB at 10mJ/cm^2, 20mJ/cm^2 and 30mJ/cm^2. The results of doing this confirmed that exposing the cells in co-culture to all three concentrations of UVB did have a stimulatory effect on melanocytes and melanin production in cells regardless of the seeding ratio.

6.2 Contribution of my work to future research

As an in vitro study my doctoral work has provided a vital first step towards the development of a treatment for vitiligo vulgaris. The aim of my doctoral study is that the outcomes of this serve to provide us with the ability to accurately estimate the actual number of participants required for a much larger full-scale study to obtain results that are statistically significant. Any scientific investigation requires that enough participants are recruited so that precise statistical analysis can be done. The aim of conducting a pilot study such as mine is if it can be used to design further work then significant results can be generated without the need for repetition of experiments and the consequent requirement for in this case the use of a more than necessary number of animals for in vivo work. Studies are usually undertaken in vitro first for ethical reasons since in vitro studies allow a potential treatment to be studied safely, without subjecting humans or animals to the possible side effects or toxicity of a new treatment. After the in vitro stage of development has been completed it is usually the case that this is followed by in vivo experimentation in animals (normally mammals such as mice) before any human clinical trials are performed. Non-animal techniques such as the one I developed in my doctoral work do obviously have inherent limitations however they are extremely useful for preliminary work before the necessary animal testing stage, of treatment development, however unfortunately one of the main disadvantages of using in vitro tests is that they can produce false or unreliable results with regards to applying these findings to an in vivo setting.

6.3 Future directions

6.3.1 The use of exosome technology as an improvement to the co-culture method

The use of co-culture systems is invaluable in molecular biology for investigating the interactions that occur between different cell populations. At its most simplistic level a co-

culture can be described as a setup where two or more different cell populations are grown with an element of contact between them. However, co-culture systems are not without their limitations and there is a requirement for the development of other methods to study cell interactions outside of the co-culture system. One major disadvantage of the co-culture system is that it is seriously limited with regards to system complexity. As far as high complexity systems are concerned these are often few and far between for reasons including that using multiple cell populations seriously complicates the procedure. This can be attributed to the fact that studying the interactions of any more than three populations at a time can lead to a complexity that is difficult to manage with regards to how they interact. Another disadvantage of using co-culture media is that creation of a media that is suitable for supporting the growth of multiple cell types at once can be extremely time consuming, requires a great deal of technical skill and can be very expensive.

An alternative to co-culture media that has been proposed is the development of a synthetic media that has a 'co-culture affect.' A suggestion for how this can be done is to completely step away from the concept of growing two cell types in the same media at the same time. Instead it has been suggested that a much simpler option would be to grow the two (or multiple) cell types in their own specific media. At its essence a co-culture media was thought to be important because when cells are grown together, they secrete factors that can impact the growth of neighbouring cell types. A possible useful extension to this project and alternative to co-culture could be to grow cells individually in their own specific media and then isolate the important influencing factors that they secrete during their growth (e.g. exosomes) directly from the media. Once these secreted factors have been isolated from the media they can be added to the specific media/culture for the cell type we want to investigate that it is believed to

be influencing, thus removing the entire need for developing a co-culture media and what could be a complex protocol for enabling the growth of multiple cells types at once.

The scientific theory behind developing a media with a 'co-culture' effect starts with the fact that extracellular vesicles, specifically exosomes, have a significant function with regards to enabling communication between various types of cells. The human body is obviously not composed of cells existing in isolation but rather it is made up of many different cell types existing and communicating with each other, all contributing to the in vivo system. Until recently it was thought that intercellular communication occurred via direct interactions and soluble molecules such as cytokines that are secreted by cells. However, more recent studies have identified that extracellular vesicles are important for communication between cells. Extracellular vesicles such as exosomes were once viewed as cellular waste but as of 2006/2007 it was discovered that exosomes contain microRNA (miRNA) and RNA highlighting that they have the capability for transmitting genetic information between cells (Shimasaki *et al.*, 2018).

The procedure behind a media enriched with exosomes would be loosely:

Step 1, Grow cell type A as normal in its specific media

Step 2, Expose cell type A to growth inhibiting or secreting factors e.g., UV light, MSH

Step 3, Extract exosomes from this media (the standard extraction method is ultracentrifugation of the media with exosomes existing in the resulting pellet)

Step 4, Administer the isolated exosomes to cell type B, growing in their specific media

Step 5, Analyse the effect of exosomes on the growth of cells growing in exosome enriched media.

A 2018 study (Shimasaki *et al.*, 2018) found that as of this date the number of papers published on the topic of exosomes and co-culture research totalled less than 180 but the number of publications has shown a dramatic increase in present times. Both exosome administration and co-culture analysis are techniques that can be used for studying cell interactions and the use of each has their own advantages and disadvantages. With exosome administration it is possible to study the effect of the exosomes on cells and the results are reasonably simple to interpret however cell interactions are not observed and the results might be artificial. With co-culture on the other hand it is possible to study the natural effect of cells on each other, there is no need to extract exosomes and the exchange, manufacture and secretion of exosomes can be evaluated by exosome surface marker with a fluorescent dye however the setback of this is that the results are not so simple to analyse.

6.3.2 Three dimensional skin models

However useful a two-dimensional melanocyte keratinocyte cell co-culture model might be, it is still limited in its ability to recapitulate the cellular complexity of human skin. Currently most cell-based studies are composed of the traditional set up of a 2D monolayer of cells that are grown on a substrate that is flat or rigid and whilst this was a useful arrangement for cell work the limitations of it are becoming increasingly acknowledged. In the in vivo situation cells are found surrounded by many other types and as such three-dimensional skin equivalents provide a much better representation of the complex architecture that is the human skin tissue. Current three-dimensional models consist of keratinocytes that are cultured on a dermal

substitute but with the work conducted with my melanocyte keratinocyte co-culture system there is a hope that there is much scope for the improvement of current skin models to make scientific systems that are a much more realistic representation of the system that exists in the human body. A result of this is that 3D skin models provide a much better scientific model than a two-dimensional cell culture model does and as such the creation of three-dimensional skin models would be a useful extension to my doctoral work. It can even be inferred that due to their lack of complexity the outcomes of tests using 2D cultures are not completely reliable and are a misleading representation of the in vivo response. (Edmondson *et al.*, 2014).

The two most commonly used types of 3D cell culture models are 3D spheroids and cells that are embedded into a 3D matrix. The principle behind cellular spheroids is that they are formed by aggregations of cell-cell adhesion when interactions at the cell-surface are interrupted. Spheroids can be a preferable alternative to artificial skin and animal models which can be comparatively more expensive and time consuming (Chung *et al.*, 2019). Multicellular spheroids have even been used in the treatment of vitiligo where they have been transplanted into the dermabraded skin lesions of vitiligo skin and repigmentation of the skin occurs when cells migrate from the transplanted spheroids. The use of spheroids is preferable over cell suspension for several reasons such as during the preparation of cell suspension melanocytes are easily damaged and they are subject to apoptosis when maintained in suspension medium (Hsiao and Young, 2019).

The creation of accurate three-dimensional models is so important for several reasons which include that the regulatory restrictions regarding the use of animal testing are getting increasingly stricter, with the use of animal testing for cosmetic being completely banned by

the European Union in 2013, whilst almost important to consider is that the availability of excised human skin is limited. Therefore, such models are crucial as a tool to reliably assess the safety and functionality of new cosmetic products with reliable skin models also being important in medical research and to the pharmaceutical industry where they can be used to evaluate drug formulations. These models give results that can be reproduced and are often translated to humans, reducing the reliance on animals whilst at the same time they give a fundamental insight into the cellular interactions that happen within tissue-like structures. Unfortunately at present the cost and technical requirements, such as a lack of standardisation of composition and construction, of multicellular human skin models are presenting as major limitations for their industrial use.

6.3.3 Further melanocyte/keratinocyte proliferation stimulating factors

A thorough literature search has identified basic fibroblast growth factor (bFGF) as being vital to the proliferation and survival of human melanocytes in pure culture. I did not investigate the use of this in my doctoral thesis however if my study was to be extended in the future the results of my literature search suggest that it would be useful to investigate how bFGF impacts melanocyte growth. A study published as early as 1988 (Halaban e*t al.*, 1998) identified that the presence of basic fibroblast growth factor is crucial to the survival and proliferation of human melanocytes and without it the cells die.

When melanocytes are cultured with keratinocytes without the addition of bFGF the cells can survive for several weeks suggesting that this growth factor is in fact produced by human keratinocytes. The release of bFGF is thought to be as a result of the stimulation of keratinocytes by UVB (Lee *et al.*, 2017) and since UVB was one of the growth factors that was

investigated in my doctoral work studying bFGF directly would be the next logical step. Other factors that are secreted by UV-B exposed keratinocytes and could be investigated in future studies include: nitric oxide, interleukin-1, interleukin-6, tumour necrosis factor, granulocyte macrophage colony stimulating factor (GM-CSF), endothelin-1, ***alpha- melanocyte stimulating hormone*** (this has already been investigated) and stem cell factor (Lee *et al.*, 2017). Agents that could be investigated for inhibiting the functions of melanocytes are: Arbutin, Kojic acid and Niacinamide.

6.3.4 Using different cell types for co-culture

A worthwhile future study would be to compare if alternative cell types to keratinocytes can be used in melanocyte co-culture to support their growth. Studies have already been conducted where adipose cells have been used in melanocyte co-culture instead of keratinocytes. An advantage of using adipose cells is that for experimental work the cells are easy to isolate and culture from adipose tissue that can be obtained by liposuction from a small incision. This is in comparison to using keratinocytes where there is a need to take a large amount of skin, the by-product of doing so is often scarring (Kim *et al.*, 2012). Therefore a balance must be obtained when considering cell types for co-culture between practicality and the quality of outcomes that are achieved.

6.4 Personal reflections on completing my PhD

The aim of my doctoral work was to successfully answer a research question and three research objectives which has been done. My work has raised several future research questions and this study has contributed towards future research. My study has also contributed new knowledge in the fields of cell co-culture, co-culture manipulation, in vitro cell technologies, vitiligo

research and aims to assist with the development of three-dimensional skin models for scientific testing in the future.

CHAPTER SEVEN: BIBLIOGRAPHY

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CHAPTER EIGHT: APPENDICES

Appendix 1 Research ethics approval – England

NHS
Health Research Authority

London - City & East Research Ethics Committee
Bristol Research Ethics
Committee Centre
WhitefriarsLevel 3,Block
B Lewins Mead

Bristol

BS1 2NT

Telephone: (020) 71048033

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval.

05 August 2016

Miss N Shetty

295 Hempstead Road

Hempstead

Kent ME73QJ

Dear Miss Shetty

Study title:	**Developing methods of measuring and manipulating melanocyte/keratinocyte ratios to inform potential treatment of vitiligo vulgaris.**
REC reference:	**16/LO/1395**
IRAS project ID:	**206446**

Thank you for your letter of 4 August 2016, responding to the Proportionate Review

Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mr Raj Khullar, nrescommittee.london-cityandeast@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Management permission must be obtained from each host organisation prior to the start of the

study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Covering letter on headed paper [IRAS Covering Letter Neha Shetty 12.07.2016]	1	12 July 2016
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [2016-17 PI TWIMC Letter Neha Shetty 12.07.2016]	1	12 July 2016
GP/consultant information sheets or letters [Consultant Information Letter Neha Shetty 12.07.2016]	1	12 July 2016
IRAS Application Form [IRAS_Form_04082016]		04 August 2016

IRAS Application Form XML file [IRAS_Form_04082016]		04 August 2016
IRAS Checklist XML [Checklist_04082016]		04 August 2016
Letter from sponsor [Sponsor Letter Neha Shetty]	1	12 July 2016
Other [Email from Neha Shetty]		12 July 2016
Other [RESEARCH STUDY PROTOCOL02.08.16final]	2	02 August 2016
Other [IRAS cover letter v2030816]	2	03 August 2016
Other [Patientinfosheetwithcomments]	1	04 August 2016
Other [patientconsentformtrackchanges]	1	04 August 2016
Participant consent form [Participant Consent Form Neha Shetty 02.08.2016final]	2	02 August 2016
Participant information sheet (PIS) [Participant Information Sheet Neha Shetty 12.07.2016]	2	02 August 2016
Referee's report or other scientific critique report [Referee Report Neha Shetty 12.07.2016]	1	12 July 2016
Research protocol or project proposal [Research Study Proposal FINAL Neha Shetty 12.07.2016]	1	12 July 2016
Summary CV for Chief Investigator (CI) [CV for Neha Shetty 12.07.2016]	1	12 July 2016

Summary CV for student [2nd Research Supervisor CV Prof Kim for Neha Shetty 12.07.2016]	2	03 August 2016
Summary CV for supervisor (student research) [Dr. S. Plummer CV 2016 re Neha Shetty]	1	12 July 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance

on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:

http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/LO/1395 Please quote this number on all correspondence

With the Committee's best wishes for the success of this project. Yours

sincerely

PP Dr John Keen

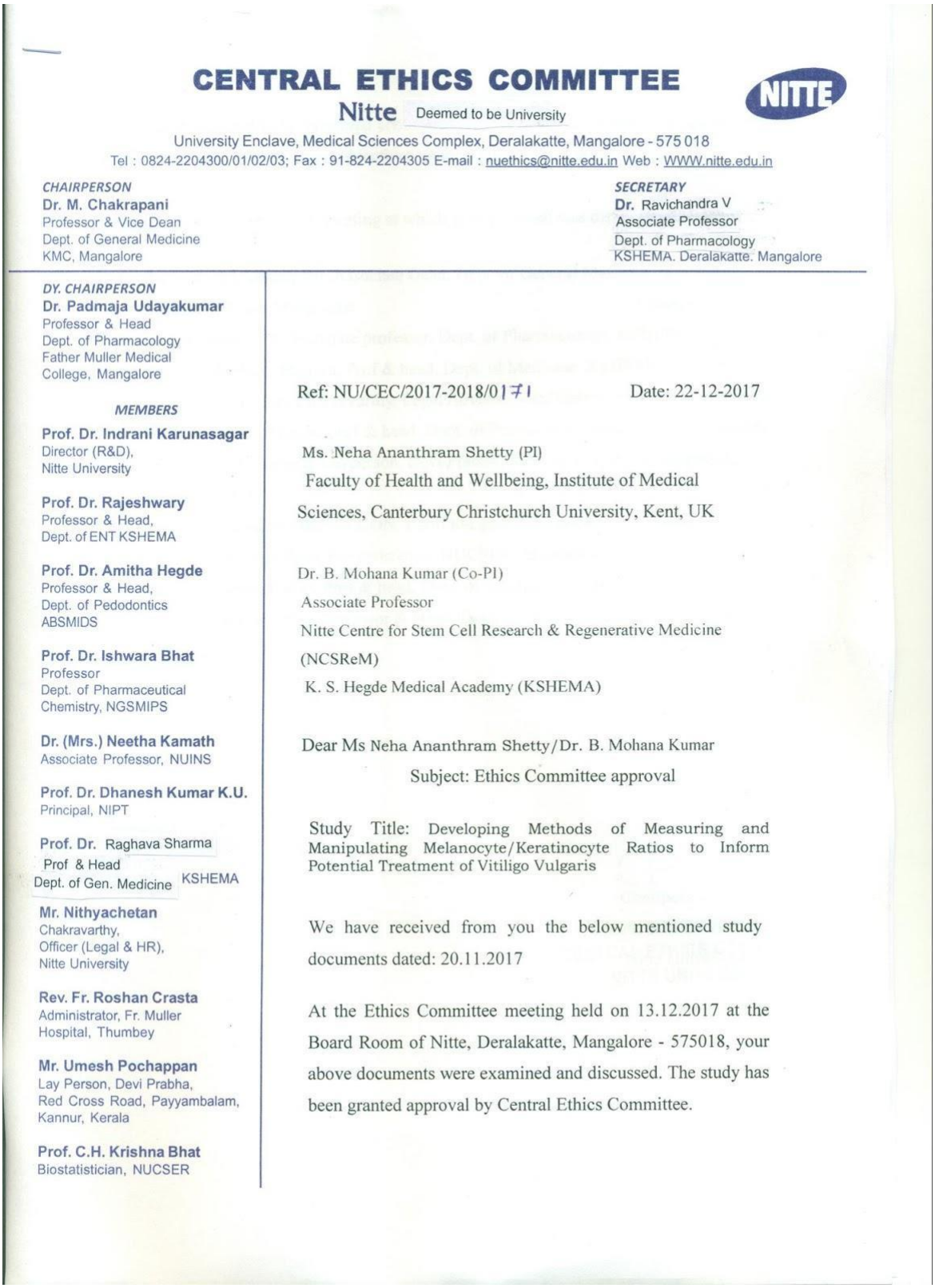
Chair

Email: nrescommittee.london-cityandeast@nhs.net

Enclosures: *"After ethical review – guidance for researchers"*

Copy to: Miss Neha Shetty

Appendix 2 Research ethics approval – India



CENTRAL ETHICS COMMITTEE

Nitte Deemed to be University

University Enclave, Medical Sciences Complex, Deralakatte, Mangalore - 575 018
Tel : 0824-2204300/01/02/03; Fax : 91-824-2204305 E-mail : nuethics@nitte.edu.in Web : WWW.nitte.edu.in

Ref: NU/CEC/2017-2018/0171 Date: 22-12-2017

Ms. Neha Ananthram Shetty (PI)
Faculty of Health and Wellbeing, Institute of Medical Sciences, Canterbury Christchurch University, Kent, UK

Dr. B. Mohana Kumar (Co-PI)
Associate Professor
Nitte Centre for Stem Cell Research & Regenerative Medicine (NCSReM)
K. S. Hegde Medical Academy (KSHEMA)

Dear Ms Neha Ananthram Shetty/Dr. B. Mohana Kumar

Subject: Ethics Committee approval

Study Title: Developing Methods of Measuring and Manipulating Melanocyte/Keratinocyte Ratios to Inform Potential Treatment of Vitiligo Vulgaris

We have received from you the below mentioned study documents dated: 20.11.2017

At the Ethics Committee meeting held on 13.12.2017 at the Board Room of Nitte, Deralakatte, Mangalore - 575018, your above documents were examined and discussed. The study has been granted approval by Central Ethics Committee.

Committee is expected to be informed about the progress of the study annually and also any changes in the title, methodology, objectives, guide of the study if any.

The members who attended the meeting at which your proposal was discussed are listed below:

1. Prof. Dr. M Chakarapani, Associate Dean, Dept. of General Medicine, Kasturba Medical College, Mangalore — Chairperson
2. Dr. Ravichandra V, Associate professor, Dept. of Pharmacology, KSHEMA, — Member Secretary
3. Prof. Dr. Raghava Sharma, Prof & head, Dept. of Medicine, KSHEMA, — Member
4. Mr. Nithya Chethan Chakravarthy, Legal Advisor, Nitte University — Member
5. Prof. Dr. Amitha Hegde, prof & head, Dept. of Pedodontics, ABSMIDS, — Member
6. Mr. Umesh Pochappan, Layperson, Devi Prabha, Red Cross road, Payyambalam, Kannur -Kerala. — Member
7. Shri Nama Nishtha Das, ISKCON, Kadri toll gate, Mangalore — Member
8. Prof. C.H Krishna Bhat, Biostatistician, NUCSER, Mangalore — Member
9. Prof. Dr. Satheesh Rao, Prof & head, Dept. of psychiatry, KSHEMA — Member
10. Prof. Dr. Ishwara Bhat, Professor & Head, Dept. of Pharmaceutical Chemistry, NGSMIPS — Member

Yours Truly

Chairperson
Central Ethics Committee
Nitte University

CHAIRPERSON
CENTRAL ETHICS COMMITTEE
NITTE UNIVERSITY

Appendix 3 Consultant information letter

Mr M. Jones

Consultant plastic surgeon

Spire Alexandra Hospital

Impton Lane

Walderslade

Kent

ME5 9PG

Dear Mr. Jones

Re: Research study

I am undertaking a research study for a PhD. The title of my study is: "Developing methods of measuring and manipulating melanocyte/keratinocyte ratios to inform potential treatments of Vitiligo Vulgaris." In this study I will be isolating cells from skin that is surplus to cosmetic procedures. This skin will be harvested and cells cultured. The purpose of my study is to culture cells from skin and manipulate them. The research questions that I will be attempting to address are:

1) Can a new method be created that will be reliable and reproducible for measuring ratios of different cell types during standard cell culture process using image processing.

2) Can ratios of cell populations in in vitro co-culture can be altered using environmental, biochemical or physical methods.

Patients who can be included in this study will be:

All patients who:

1. are undergoing surgery which will result in discarded skin that would normally be destroyed in routine practice of procedure
2. have agreed to take part - have capacity to give consent
3. are between 18-60 years' old

Patients who are excluded in this study will be:

All patients who:

1. are aged less than 18 years old and who are aged above 60
2. do not agree to take part in the study
3. do not have the capacity to give informed consent.
4. do not speak & understand fluent English

I would like to ask if you would agree to allow me to have surplus skin from the patients who consent to take part in this study so that I can use it as part of my research. If you do agree I would be extremely grateful.

When a patient who meets the above inclusion criteria attends your clinic would you please tell them that a study is being undertaken involving growing skin cells and that excess skin from their forthcoming surgery may be helpful in this study. Please could you give them an information pack which I will provide you with. This information pack consists of a patient information leaflet and three copies of a consent form. It is necessary that the patient takes the information pack home with them so that they have time to consider whether they wish to take part. It is important that no pressure is put on the patient to agree to take part. In the information pack, the patient is asked to return two signed copies of the consent form in the stamped addressed envelope that is also included in the pack. The envelope is addressed to you at the Spire Alexandra Hospital. Please would you put the two consent forms into the patient's notes. The patient will keep the third copy of the consent form for themselves.

I will provide you with transport medium and container. I will also provide your secretary with specimen form stickers to put into the patient's notes. Each sample will be given a unique reference number which will be written on the stickers. After the patient's surgery, whilst in theatre, please can you then complete the stickers with the information requested. Please put one sticker on the transport tube and one sticker on each of the two copies of the consent form. Please can you place these consent forms into the patient's notes. When I collect the skin sample I will also take one of the patient's consent forms with me in order that it can be kept in the study notes within locked cabinets in the University's secure laboratory. The Data Protection Act of 1998 will be followed.

It might be the case that the patient wants to ask more questions about the study. In this instance the patient has been asked to contact you. If you need any further information, please let me know.

Thank you for taking the time to read this letter and your cooperation would be highly appreciated.

Best wishes,

Neha Shetty

Appendix 4 Consent form – England

Unique Patient Identification No: ……..

Faculty of Health and Wellbeing

Institute of Medical Sciences

CONSENT FORM FOR PARTICIPATION IN RESEARCH STUDY : "DEVELOPING METHODS OF MEASURING AND MANIPULATING MELANOCYTE/KERATINOCYTE RATIOS TO INFORM POTENTIAL TREATMENT OF VITILIGO VULGARIS".

In the operation you will shortly have, some skin tissue will be removed. Normally this would be thrown away. Scientists and surgeons at Canterbury Christ Church University (CCCU) may be able to use this skin to develop new techniques which will be used to help us to understand how best to potentially treat a skin condition called Vitiligo Vulgaris.

1. We are asking for your permission to take a small sample – no more than 2 square centimetres (3.4 of an inch) of this surplus skin after your operation and use it for research purposes.

2. No additional skin will be taken during the operation other than that required as part of your routine surgery.

3. The skin will only be used for this research project which has been ethically approved by a Research Ethics Committee.

4. The skin will be destroyed after use in this study.

5. The skin will not be subject to any diagnostic testing.

6. The skin will not be used for the treatment of any patients. It is for laboratory based research only.

7. Your skin sample will be given a unique identification number to make it anonymous during this research

8. To comply with the Human Tissue Act (2004) to ensure all donated human tissues are used only for the purposes for which consent was given, a secure record will link your identity with the anonymous identification number. This will be made prior to the sample being used for research and will be identified thereafter by only the code number.

9. After being used, any remaining skin will be disposed of in a dignified manner in accordance with the University policy for the disposal of human tissue which is informed by the Human Tissue Act (2004)

10. If you do not wish to take part in this study and donate your surplus skin, this will not affect your current or future treatment in any way.

11. If you are willing to donate your surplus skin, please tick the "Agree" box below.

12. If you are not sure, please discuss with your Consultant.

Faculty of Health and Wellbeing

Institute of Medical Sciences

CONSENT FORM

Centre Number: Study Number:

Participant Identification Number for this trial:

Title of Project: "DEVELOPING METHODS OF MEASURING AND MANIPULATING MELANOCYTE/KERATINOCYTE RATIOS TO INFORM POTENTIAL TREATMENT OF VITILIGO VULGARIS".

Name of Researcher: Miss Neha Ananthram Shetty

Please initial

1. I confirm that I have read the information sheet dated.....02/08/16..... (version....3.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw without giving any reason, without my medical care or legal rights being affected.

3. I agree to take part in the above study.

Name of Participant	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature
_____	_____	_____

Please sign each of the three copies. Please keep one for yourself and return the other two to your Consultant in the stamped addressed envelope provided. Version 3 02.08.2016

Appendix 5 Patient information leaflet

Faculty of Health and Wellbeing

Institute of Medical Sciences

Can you help us in our skin research?

Patient Information Leaflet

Dear Sir/Madam,

We would like to invite you to take part in a research study that is being undertaken at Canterbury Christ Church University. This research study is an educational project for a PhD which is an advanced higher degree.

Purpose of study

The purpose of this study is to look at different ways of growing skin cells so that we can contribute to the current information about a skin disorder called Vitiligo Vulgaris. We hope that the results of this research study will help us and our colleagues around the world to have more understanding on how to potentially treat this distressing condition.

Vitiligo Vulgaris is a condition where pale white patches develop on the skin. It is due to a lack of colour (pigment) in the affected areas of skin. Vitiligo does not make someone feel ill, but the appearance of the white patches of skin can be very distressing and embarrassing. This is particularly so for darker-skinned people where white patches are noticeable.

Our Research

In order to study different ways of growing skin cells we need to have small samples of human skin so that we can remove the pigment cells and work on them in our laboratory. When someone has a routine cosmetic procedure that involves removing part of their skin, this excess skin is disposed of at the end of the operation by the hospital. With your permission, we would like to take a small part of this skin which will be no more than 2 square centimetres (3/4 of an inch). We would only take a sample from skin that would normally be disposed of.

Do I have to take part?

No, you don't have to take part. Participation is voluntary and if you choose not to take part this will not affect your current or future treatment and care in any way.

Why have I been chosen?

You have been approached for this study because you are going to have routine surgery that involves removing part of your skin. At the end of your operation, this excess skin would normally be discarded. Please be assured that you have not been chosen for this study because we are concerned that you might have vitiligo. By taking part in this study we are not confirming that you may or might have vitiligo. You have been selected purely randomly.

What do I have to do?

You do not have to do anything. Your operation would not be altered at all.

What are the potential benefits of taking part?

You will be providing us with skin cells which we can use to undertake scientific research on. Ultimately you will be contributing to research which will aid in potentially developing treatment of Vitiligo Vulgaris.

Will my participation in this study be kept confidential?

Yes. The only people that will know of your involvement are yourself and your Consultant. The skin sample that we take from you that would normally be discarded at the end of your operation will be coded and we will not know who it belongs to.

How will this affect my treatment?

There will be no change to your surgery and it will not affect your treatment at all.

What is the likelihood of discovering an illness or disease from my sample?

The sample we take from your excess skin will not be subject to diagnostic tests so any identification of illnesses or disease will not occur.

What risks are there in participation?

There are no risks to you in taking part in this study. It is important that we use only skin that would normally be discarded. We do not want any additional skin nor will any steps of the procedure be altered at all for the purpose of this research study.

How is my privacy protected?

Consent forms: One copy of your signed consent form will be kept in your medical notes in the Spire Alexandra Hospital. The only people who will access your medical notes will be your Consultant and the health care team at the hospital. A second copy of your signed consent form will be kept in a sealed envelope and stored in a locked cabinet in our secure laboratory at the Medway Campus of the University. Your skin sample will be given a unique identification number and this number will be put onto your consent forms. Your identity (your name) and your skin sample unique identification number will only be matched if you contact us having decided to withdraw from this study. This will enable us to remove your skin sample and any results we have on your skin. The only people who will have access to your consent form stored within the University will be the research study supervisor.

Skin sample: Your skin sample will be given a unique identification number. It will be stored and used within our secure laboratory at the Medway Campus of the University. Access to the laboratory is highly restricted. Results of our scientific work on your skin sample will be stored on our data base within the University's secure server. Access to these results are also highly restricted.

Will you sell to, or give other people my skin sample?

No, cells will only be used for this study.

How will the skin sample be disposed of?

Your skin sample will be disposed of in a dignified manner and within the strict laboratory regulations. No skin sample will be kept in the laboratory once the work on your cells is completed.

Can I withdraw from this study if I change my mind?

If, after you have signed and returned your consent forms, you decide that you no longer want to take part in the study please contact your Consultant - Mr. Martin Jones - who will let us know. Please be assured that if you decide to do this, it will not affect your current or future care in any way.

However, it will not be possible to withdraw you from the study after the point at which your skin cells have been used in our research and they have been analysed. This also means that results from your cells would be included in the overall analysis of all patients' skin cells and at that point would not be identifiable. The results of these analyses would be included in future studies.

Who has reviewed this study?

This study has been reviewed by the University, experts in skin cell culture and it has been approved by a Research Ethics Committee.

I am not sure whether I want to take part in this study. Where can I find more information?

If you are not sure, please contact your Consultant – Mr. Martin Jones – who will be able to give you some more information.

Will I be informed about the outcomes of the study?

Yes. At the end of this study, which we expect to take at least two years, you will be contacted by letter to inform you of the results of the study.

What do I do now?

If you would like to take part in this study, please sign each of the consent forms (three copies) that are enclosed in this pack. Please keep one copy for yourself. Then send the remaining two consent forms back to Mr. Martin Jones in the prepaid envelope that is also enclosed in this pack. When it is time for you to attend the hospital for your operation, Mr. Jones will know that you have agreed to take part in this study.

Thank you

Thank you for taking the time to read this information sheet, it is much appreciated.

If you have any complaints please contact Dr Susan Plummer at:

susan.plummer@canterbury.ac.uk

Research Team,

Institute of Medical Sciences

Canterbury Christ Church University

Version 3 020816

Appendix 6 Patient information sheet and consent form – India

01. Nature and purpose of study stating it as research	**DEVELOPING METHODS OF MEASURING AND MANIPULATING MELANOCYTE/KERATINOCYTE RATIOS TO INFORM POTENTIAL TREATMENT OF VITILIGO VULGARIS**
02. Duration of participation with number of participants:	**ated from a part of the skin sample which is collected for the treatment of patients who are undergoing skin treatment will only be used for this research study:.** **mples: 10**
03. Procedures to be followed:	**Collection, isolation, processing, in vitro expansion of cells, characterization, and storage of cells collected from the tissue**
04. Investigations, if any, to be performed	**Not Applicable**
05. Foreseeable risks and discomforts adequately described and whether project involves more than minimal risk	**NO**
06. Benefits to participant, community or medical profession as may be applicable	**NO direct benefit** **(Tissue and Cells will only be used for research studies)**
07. Policy on compensation	**No compensation is given**
08. Availability of medical treatment for such injuries or risk management	**Treatment facilities are available if any injuries or risks occur**

09. Alternative treatments if available	**Not applicable**
10. Steps taken for ensuring confidentiality	**Identity of patients, tissues and cells obtained from the harvested tissues from the patients will not be shared with third parties.**

11. No loss of benefits on withdrawal	**No**
12. Benefit sharing in the event of commercialization	**nmercialization as tissue and cells will be used only for research activity in this study.**
. Contact details of PI or local PI/Co-PI in multicentric studies for asking more information related to the research or in case of injury	**Ms. Neha Shetty/Dr. B. Mohana Kumar** **CSReM, 3rd Floor, K. S Hegde Medical Academy** **Email ID: mohanakumar@nitte.edu.in** **Mobile number: 9480504462**
4. Contact details of Chairman of the IEC for appeal against violation of rights	**Member Secretary/Chairperson** **Central Ethics Committee** **Nitte (Deemed to be University)** **Deralakatte, Mangaluru.** **Ph: 0824-2204300/01/02**
15. Voluntary participation	**will be collected from patients coming voluntarily for their skin treatment.**
IIf test for genetics and HIV is to be done, counseling for consent for testing must be given as per national guidelines	**Not applicable**
7. Storage period of biological sample and related data with choice offered to participant regarding future use of sample, refusal for storage and receipt of its results	**sfully cultured cells after being used for this research nly, and the remaining stock of cells if any left would be stored frozen until the study is completed.** **Maximum storage period is 5 years.**

Nitte (Deemed to be University)

JUSTICE K. S. HEGDE CHARITABLE HOSPITAL

(Unit of K. S. Hegde Medical Academy)

Medical Sciences Complex, Deralakatte, Mangaluru-575018, Karnataka

Ph.: 0824-2204471-76, Fax: 0824-2204016

Website: www.nitte.edu.in, E-mail: kshegde_hospital@nitte.edu.in

Patient Informed Consent Form

Study

Title_____

_

Study Number

_____

Subject's Full Name (with father's name) _____

Date of Birth/Age

_____

Address of subject

_____

_____

_____

Qualification of subject

_____

Name and address of nominee(s) and his relation to subject

_____
_____

_____
_____

1. I/We confirm that I have read and understood the information document dated _____for the above study and have had the opportunity to ask questions.

   OR I/We have been explained the nature of the study by the Investigator and had the opportunity to ask questions.

2. I/We understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason and without my medical care or legal rights being affected.

3. I/ We understand that no payment is made to me/us to carry out the above study.

4. I/We understand that the sponsor of the clinical trial/project, others working on the Sponsor's behalf, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the study. However, I/We understand that my Identity will not be revealed in any information released to third parties or published.

5. I/We agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

6. I/We agree to use collected/stored skin tissue sample to isolate keratinocytes and melanocytes, and grow them in laboratory to study the characteristics including DNA, RNA and protein analysis without any major modifications.

7. I/we agree that part of skin tissue collected for my treatment purpose will be used for above titled research.

8. I/we understand that my denial of participation in this study will not influence my treatment plan and outcome.

9. I/we understand that my health records and reports are stored for five years as per ICMR guidelines.

10. I /We agree to take part in the above study.

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative:

_____

Date_____

Signatory's Name_____

Date_____

Signature of the Investigator_____

Date_____

Study Investigator's Name _____

Signature of the Witness _____ Date_____

Name of the Witness _____

Appendix 7 Media, reagents and chemicals

LIST OF MEDIA, REAGENTS, CHEMICALS AND OTHER ITEMS

Item	Catalogue No.	Company
Dulbecco's phosphate buffer saline (DPBS)	21300-025	Gibco-Invitrogen, Thermo Fisher Scientific
Penicillin-streptomycin solution	15140-122	Gibco-Invitrogen, Thermo Fisher Scientific
Dulbecco's modified eagle's medium (DMEM)	11995-065	Gibco-Invitrogen, Thermo Fisher Scientific
Attachment factor protein	S-006-100	Gibco-Invitrogen, Thermo Fisher Scientific
Alpha-melanocyte stimulating hormone (α-MSH)	M4135-1 mg	Sigma-Aldrich
Ethyl alcohol (Ethanol)	F204325	Haymankimia
Collagenase, Type 1	17100-017	Gibco-Invitrogen, Thermo Fisher Scientific
Minimum Essential Medium-alpha (MEM-α)	12561-072	Gibco-Invitrogen, Thermo Fisher Scientific
Fetal bovine serum (FBS)	10270106	Gibco-Invitrogen, Thermo Fisher Scientific
0.4% Trypan blue	15250-061	Gibco-Invitrogen, Thermo Fisher Scientific
Epilife growth medium	MEPI500CA	Gibco-Invitrogen, Thermo Fisher Scientific
Epidermal growth factor (EGF)	585506	Biolegend
Medium 254	M254500	Gibco-Invitrogen, Thermo Fisher Scientific
Human melanocyte growth supplement (HMGS)	S-002-5	Gibco-Invitrogen, Thermo Fisher Scientific
Human keratinocyte growth supplement (HKGS)	S-001-5	Gibco-Invitrogen, Thermo Fisher Scientific

Keratinocyte growth factor (KGF)	100-19-02	Peprotech
0.25% trypsin/EDTA	25200-072	Gibco-Invitrogen, Thermo Fisher Scientific
Paraformaldehyde	P6148	Sigma-Aldrich
Sodium hydroxide (NaOH)	TCL002	Himedia
L-DOPA (L-3, 4-dihydroxyphenylalanine)	333786	Sigma-Aldrich
Triton X-100	X-100	Sigma-Aldrich
Fungizone (Amphotericin B)	15290-018	Gibco-Invitrogen, Thermo Fisher Scientific
Non-essential amino acid	11140-050	Gibco-Invitrogen, Thermo Fisher Scientific
S100	NBP2-45267SS	Novus Biologicals
TRP1	NBP2-32907	Novus Biologicals
TYR	NBP2-25238	Novus Biologicals
Cell staining buffer	420201	Biolegend
FITC-conjugated secondary antibody	11-4010-82	e-Bioscience, Thermo Fisher Scientific
FITC-conjugated secondary antibody	sc-516140	Santa Cruz Biotechnology
Normal mouse IgG	sc-3877	Santa Cruz Biotechnology
Filaggrin	NBP2-53245	Novus Biologicals
Pancytokeratin (K1)	NBP2-29429	Novus Biologicals
Keratin 10 (K10	NBP2-32962	Novus Biologicals
Glutamax (100X)	35050-061	Gibco-Invitrogen, Thermo Fisher Scientific
Phosphate buffered saline (PBS) solution	14190-144	Gibco-Invitrogen, Thermo Fisher Scientific
Fungizone (Amphotericin B)	15290-018	Gibco-Invitrogen, Thermo Fisher Scientific

Non-essential amino acid	11140-050	Gibco-Invitrogen, Thermo Fisher Scientific
2-Mercaptoethanol	21985-023	Gibco-Invitrogen, Thermo Fisher Scientific
Dimethyl sulphoxide (DMSO)	TC185	Himedia

Thermo Fisher Scientific, Gibco-Invitrogen, Life Technologies, Grand Island, NY, USA

Sigma-Aldrich, St. Louis, MO, USA

Biolegend, San Diego, CA, USA

Peprotech, NJ, USA

Novus Biologicals, Centennial, CO, USA

Himedia, Mumbai, India

Haymankimia, Chargen Life Sciences, Mumbai, India

Santa Cruz Biotechnology, San Diego, CA, USA

ITEMS	**COMPANY**	**CATALOGUE NUMBER**
MEDIA/REAGENTS		
DPBS Powder w/o calcium, magnesium	Gibco -Invitrogen	21300-025
Penicillin-streptomycin solution	Gibco -Invitrogen	15140-122
Fungizone (Amphotericin B)	Gibco -Invitrogen	15290-018
0.25% Trypsin-EDTA	Gibco -Invitrogen	25200-072
Collagenase Type 2, powder	Gibco -Invitrogen	17101-015
Gentamycin solution	Gibco -Invitrogen	15750060
Iodine solution	Himedia	R044
Ethanol	Hayman	F204325

Fetal bovine serum FBS (South America Origin)	Gibco -Invitrogen	10270106
MEM Alpha No nucleoside	Gibco -Invitrogen	12561072
Epilife growth medium	Gibco -Invitrogen	MEPI500CA
Human Keratinocyte growth supplement	Gibco -Invitrogen	S0015
Medium 254	Gibco -Invitrogen	M254500
Human melanocyte growth supplement (HMGS)	Gibco -Invitrogen	S-002-5
Keratinocyte growth factor (KGF)	Peprotech	100-19-2
EGF Recombinant human protein	Biolegend	585506
Attachment factor protein	Gibco -Invitrogen	S-006-100
Cell staining buffer	Gibco -Invitrogen	004222-26
Paraformaldehyde	Sigma-Aldrich	P6148
Glutamax (100X)	Gibco-Invitrogen	35050-061
MEM nonessential amino acids (100x)	Gibco-Invitrogen	11140-050
2-Mercaptoethanol	Gibco Invitrogen	21985-023
CHEMICALS/MARKERS		
L-DOPA-(Phenyl-D3)	Sigma-Aldrich	333786
a-Melanocyte stimulating harmone	Sigma-Aldrich	M4135
DMSO	Sigma-Aldrich	D-2650-100
Sodium hydroxide solution	Himedia	TCL002
Triton X-100	Sigma-Aldrich	X100-100ML
Tyrosinase Antibody	Novus Biologicals	NBP2-25238
Anti tyrp1	Novus Biologicals	NBP2-32907
Anti-S100B	Novus Biologicals	NBP2-45267SS
mIgkBP-FITC	SantaCruz	sc-516140
F(ab)2 Anti mouse IgG FITC	E-bioscience	11-4010-82

PAN Cytokeratin Antibody (K1)	Novus Biologicals	NBP2-29429
Cytokeratin 10 Antibody (k10)	Novus Biologicals	NBP2-32962
Filaggrin Antibody	Novus Biologicals	NBP2-53245

Appendix 8 Plastic wares, culture dishes and items

Company: Thermo Fisher Scientific, Roskilde, Denmark

Items	**Catalogue No.**
25 cm^2 flask (T25 flask	156367
75 cm^2 flask (T75 flask)	156499
Conical tube- 15 ml	LSCT15BS
Conical tube- 50 ml	LSCT50BS
6-well plate-TC treated	140675
12-well plate-TC treated	150628
24-well plate-TC treated	142475
48-well plate-TC treated	150687
96-well plate-TC treated	167008
35 mm culture dish	153066
60 mm culture dish	150288
100 mm culture dish	150350
Bacteriological Petri Dish- Non Treated sterile	157150
Serological pipette-5 mL	170355

Serological pipette-10 mL	170356
Cryo chill vial-1.5 mL	523182 (Tarsons)
Cell Strainer-Nylon-40 µm	22363547
Cell Strainer-Nylon-70 µm	22363548
Cell Strainer-Nylon-100 µm	22363549
Syringe Filter Unit, 0.22 µm (PVDF), individually packed	PVSY02225-100
Syringe Filter Unit, 0.45 µm (PVDF), individually packed	PVSY04525-100
Syringe Filter Unit, 0.88 µm (PVDF), individually packed	PVSY08825-100
Filter unit 250 mL PES membrane 50mm, 0.20 µm	568-0020
Filter unit 500 mL PES membrane 50mm, 0.20 µm	566-0020
Cell scraper	62407-141

ITEMS	**COMPANY**	**CATALOGUE NUMBER**
PLASTIC WARES		
35 mm dish	Thermoscientific	153066
60 mm dish	Thermoscientific	150288

6-well plate-TC treated	Thermoscientific	140675
12-well plate-TC treated	Thermoscientific	150628
24-well plate-TC treated	Thermoscientific	142475
25 cm2 flask (25T flask)	Thermoscientific	156367
Pipette tips- 1000µL	Tarsons	521020
Pipette tips- 200µL Yellow	Tarsons	521010Y
Pipette tips- 10µL	Tarsons	521000
Serological pipette-5ml	Thermoscientific	170355
Serological pipette-10ml	Thermoscientific	170356
Syringe Filter Unit, 0.22 µm (PVDF)	Thermoscientific	PVSY02225-100
Cell Strainer-Nylon-70µM	Thermoscientific	22363548
Cell Strainer-Nylon-100µM	Thermoscientific	22363549
Conical tube- 15 ml	Thermoscientific	LSCT15BS
Conical tube- 50 ml	Thermoscientific	LSCT50BS
Microcentrifuge tubes- 0.5 ml, colourless	Tarsons	500000
Microcentrifuge tubes- 1.5 ml, colourless	Tarsons	500010
Microcentrifuge tubes- 2.0 ml, colourless	Tarsons	500020

Appendix 9 List of equipment

K. S. HEGDE MEDICAL ACADEMY

Deralakatte, Mangaluru – 575 018

A constituent College of Nitte (Deemed to be University)

List of Equipments/Instruments for Research Purpose

Nitte University Centre for Stem Cell Research and Regenerative Medicine (NUCSReM)

Instrument	Centrifuge (incl. 15/50 ml adapters)	
Company	Eppendorf, 5810R	
Institute	KSHEMA	
Centre/Dept.	NUCSReM (Stem cell lab)	
Contact Person	Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	
Applications	1) Remove cellular elements from blood or body fluids 2) Concentration of cellular elements for microscopy 3) Isolation of macro- and micromolecules, such as DNA, RNA, proteins or lipids 4) Applicable to 15ml/50ml conical tube	

Instrument	Centrifuge (Refrigerated 24 tubes)	
Company	Eppendorf, 5418R	
Institute	KSHEMA	
Centre/Dept.	NUCSReM (Stem cell lab)	
Contact Person	Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	
Applications	1) Remove cellular elements from blood or body fluids 2) Concentration of cellular elements for microscopy 3) Isolation of macro- and micromolecules, such as DNA, RNA, proteins or lipids 4) Applicable to 1.5ml/2ml micro centrifuge tube	
Instrument	Centrifuge	
Company	Eppendorf, 5427R	
Institute	KSHEMA	
Centre/Dept.	NUCSReM (Stem cell lab)	
Contact Person	Dr. B. Mohan Kumar M:9480504462 E: mohanakumar@nitte.edu.in	
Applications	1) Remove cellular elements from blood or body fluids 2) Concentration of cellular elements for microscopy 3) Isolation of macro- and micromolecules, such as DNA, RNA, proteins or lipids	

	4) Applicable to 1.5ml/2ml micro centrifuge tube	
Instrument Company Institute Centre/Dept. Contact Person Applications	Centrifuge, Minispin plus Eppendorf, 5453 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Isolation of macro- and micromolecules, such as DNA, RNA, proteins or lipids 2) Applicable to 1.5ml/2ml micro centrifuge tube	
Instrument Company Institute Centre/Dept. Contact Person 	CO_2 Incubator Eppendorf, Galaxy 170S KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	

Applications	1) Used in scientific research to grow and maintain cell lines 2) High temperature disinfection system	
Instrument Company Institute Centre/Dept. Contact Person Applications	CO_2 Incubator Eppendorf, Galaxy 170R KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Used in scientific research to grow and maintain cell lines 2) Option of oxygen control for hypoxia experiments 3) Availability of tri-gas monitoring option 4) High temperature disinfection system	
Instrument Company Institute Centre/Dept. Contact	-80°C Ultra freezer Eppendorf U570, New Brunswick KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	

Applications	1) Cryostorage of tissue/cell samples at -80°C for long term storage 2) Shelves for keeping samples in cryoboxes	
Instrument Company Institute Centre/Dept. Contact Person Applications	Vertical autoclave Panasonic, MLS-3751 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Provides a safe, reliable high pressure steam sterilization environment 2) Applicable in culture media preparation, labware sterilization, etc.	
Instrument Company Institute Centre/Dept. Contact Person Applications	Cryogenic Storage Vessel Thermoscientific,8246 Locator 4w/monitor KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Cryogenic storage vessel for storage of tissue samples or cell lines in liquid nitrogen	

	2) Cryopreservation accessories to meet the rigorous storage requirements of cryogenic samples	
Instrument Company Institute Centre/Dept. Contact Person Applications	Electric Oven Panasonic, MOV-112-PK KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Sterilization and drying of glass wares, microtips, surgical items etc.	
Instrument Company Institute Centre/Dept. Contact Person Applications	Pharmaceutical refrigerator with freezer (-30ºC) Panasonic, MPR-414F-PE KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	

	1) It provides exact and strict storage temperature for keeping cell culture essentials, such as media, reagents etc. 2) Storage of materials at 4°C and -30°C	
Instrument Company Institute Centre/Dept. Contact Person Applications	-30ºC Deep freezer Panasonic, MDF-U537D-PK KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) It provides exact and strict storage temperature for keeping cell culture essentials, such as media, reagents etc. and other biological materials	
Instrument Company Institute Centre/Dept. Contact Person Applications	Pharma refrigerator (4ºC) Thermo Scientific, 288R AEV-TS KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Storage of cell culture essentials, such as media, reagents etc. and other biological materials	

	2) Storage of research consumables	
Instrument Company Institute Centre/Dept. Contact Person Applications	Biosafety cabinet with base stand, Class II Type A2 Labconco, No. 302681170, 6 ft width KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Workplace for safe handling and working with materials requiring a defined biosafety level 2) With high efficiency particulate air (HEPA) filters, cabinets are designed to protect against exposure to particulates, including biological agents	
Instrument Company Institute Centre/Dept. Contact Person Applications	Liquid nitrogen container and accessories Taylor Wharton, LS6000 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	

	1) Cryogenic vessel for storage of cell/tissue samples in liquid nitrogen at -196°C 2) Cryopreservation accessories are designed to meet the rigorous storage requirements of cryogenic samples	
Instrument Company Institute Centre/Dept. Contact Person Applications	Liquid nitrogen withdrawal device Taylor Wharton, LD35 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Provides convenient pressure transfers of liquid nitrogen from LD35	
Instrument Company Institute Centre/Dept. Contact Person Applications	Countess- automated cell counter Life Technologies, Thermo Scientific, AMQAF1000 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Performs cell count and viability measurement by using trypan blue stain	

	2) Eliminate the subjectivity of manual cell counting and user-to-user variability 3) Operated using an intuitive user interface	
Instrument Company Institute Centre/Dept. Contact Person Applications	Water bath Grant, JBN5 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Incubate samples in water at a constant temperature over certain period of time 2) Also suitable for use with heat transfer beads	
Instrument Company Institute Centre/Dept. Contact Person	pH meter EUTECH, ECION27004GS KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in	

Applications	1) To measure pH values in media and buffer solutions employed in cell culture activities 2) This is a micro-processor based instrument	
Instrument Company Institute Centre/Dept. Contact Person Applications	Electronic balance, 220G, 0.0001G Sartorious, BSA224S-CW KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) To measure weight with accuracy	
Instrument Company Institute Centre/Dept. Contact Person Applications	Trinocular inverted research microscope Nikon, Japan, TS100LED-F-MV KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) To observe and investigate small objects and their structure	

Instrument Company Institute Centre/Dept. Contact Person Applications	Haemocytometer complete set Neubauer KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Used for manually counting the cells under microscope	
Instrument Company Institute Centre/Dept. Contact Person Applications	MilliQ Direct 8 ultrapure water purification System Merck Millipore, Direct 8 Reservoir(TANKPE030) KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Water purification system providing lab with pure and ultrapure water directly from tap water with RO facility	

Instrument Company Institute Centre/Dept. Contact Person Applications	Cryo 1ºC freezing containers, System Thermo Scientific, Nalgene KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) To store cells at the rate of cooling-1ºC/minute prior to cryopreservation at ultralow temperatures	
Instrument Company Institute Centre/Dept. Contact Person Applications	Autoclave- vertical type Rotek, RAV-04 KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Used in culture media preparation, lab wares and dissection items sterilization etc.	

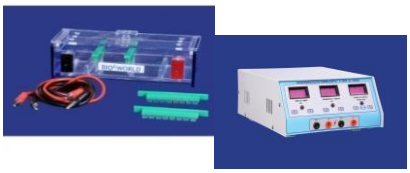
Instrument Company Institute Centre/Dept. Contact Person Applications	Magnetic stirrer Remi, 5MLH KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Employs a rotating magnetic field to cause stir bar spin quickly to dissolve chemicals or particulates	
Instrument Company Institute Centre/Dept. Contact Person Applications	Vortex mixture Remi, CM101 KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Used in proper mixing of liquids in small vials	
Instrument Company Institute Centre/Dept. Contact Person 	Vortex mixture Labnet, Z5030327 KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355	

	E: shamarao.88@nitte.edu.in	
Applications	1) Used in proper mixing of liquids in small vials	
Instrument	Hot air oven	
Company	Rotek, RHOM-18HSP	
Institute	KSHEMA	
Centre/Dept.	NUCSReM (Stem cell lab)	
Contact Person	Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in	
Applications	1) Drying of glass wares, microtips, surgical items etc.	
Instrument	Water bath	
Company	Rotek, RPTB-02SP	
Institute	KSHEMA	
Centre/Dept.	NUCSReM (Stem cell lab)	
Contact Person	Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in	
Applications	1) Used to incubate sample in water at a constant temperature over certain period of time	

Instrument Company Institute Centre/Dept. Contact Person Applications	Cryocan- for liquid nitrogen IOC, TA-55 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) For safe transportation of liquid nitrogen	
Instrument Company Institute Centre/Dept. Contact Person Applications	Centrifuge (incl. 15/50 ml adapters) Eppendorf, 5804R KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Remove cellular elements from blood or body fluids 2) Concentration of cellular elements for microscopy	

	3) Isolation of macro- and micromolecules, such as DNA, RNA, proteins or lipids 4) Applicable to 15ml/50ml conical tube	
Instrument Company Institute Centre/Dept. Contact Person Applications	Freezer- High efficiency -86°C Eppendorf, New-Brunswick, U101 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Cryostorage of tissue/cell samples at -80°C for long term storage 2) Shelves for keeping samples in cryoboxes	
Instrument Company Institute Centre/Dept. Contact Person	Microbiological incubator Bio-Bee KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355	

	E: shamarao.88@nitte.edu.in	
Applications	1) Microbial culture incubation for sterility testing 2) Detection of bacterial and fungal contamination in cell culture media	
Instrument Company Institute Centre/Dept. Contact Person Applications	Biosafety cabinet with base stand, class II Type A2 Thermo Scientific, 1300 series KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Workplace for safe handling and working with materials requiring a defined biosafety level 2) With high efficiency particulate air (HEPA) filters, cabinets are designed to protect against exposure to particulates, including biological agents	
Instrument Company Institute Centre/Dept. Contact Person	Deep freezer -20°C, ES series lab freezer Thermo Scientific, 232F-AEV-TS KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in	

Applications	1) For general-purpose lab storage and routine sample protection	
Instrument Company Institute Centre/Dept. Contact Person Applications	Electrophoresis system- Midi sub system Bioworld-03-02 KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) To separate charged molecules, such as DNA, RNA etc on electrophoretic gels	
Instrument Company Institute Centre/Dept. Contact Person Applications	Heat block with digital temperature ADJ Single block, D1100-230V KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Holds individual containers, such as test tubes, microtubes, or PCR tubes, for constant heating	

Instrument Company Institute Centre/Dept. Contact Person Applications	Olympus-Trinocular inverted microscope with Q imaging Olympus, CKX41 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Suitable for regular cell observations 2) Software with digital camera is connected for efficient documentation tasks and archiving in a computer	
Instrument Company Institute Centre/Dept. Contact Person Applications	Mixmate with 3 tube holders (0.5ml/1.5ml/2.0ml) Eppendorf, 5353000.014 KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in	

	1) High speed instrument provides complete mixing of samples in seconds 2) Applicable to 0.5ml/1.5ml/2.0ml tubes	
Instrument Company Institute Centre/Dept. Contact Person Applications	Master cycler Pro S, 230v (6325000.510) Eppendorf, PRO-S KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) PCR thermocycler is commonly used in molecular biology for amplifying single copy or few copy segments of DNA to generate thousand to millions of copies 2) Applications in the field of cell/stem cell biology, genetic research, medicine, forensic science etc. 3) Heated lid with integrated Thermal Sample Protection (TSP) technology	

Instrument Company Institute Centre/Dept. Contact Person Applications	Mini centrifuge 10000rpm including accessories FBS-MINI-10K KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Concentrate and remove cellular elements from biological fluids 2) Applicable to 1.5ml/2ml micro centrifuge tube	
Instrument Company Institute Centre/Dept. Contact Person Applications	Liquid nitrogen container, Locator 4 with level monitor Thermo Scientific, CY509107 Locator 4 KSHEMA NUCSReM (Stem cell lab) Dr. B. Mohana Kumar M: 9480504462 E: mohanakumar@nitte.edu.in 1) Cryogenic vessel for storage of cell/tissue samples in liquid nitrogen at -196°C 2) Cryopreservation accessories are designed to meet the rigorous storage requirements of cryogenic samples	

Instrument Company Institute Centre/Dept. Contact Person Applications	Vacuum pressure pump kit Millipore, Vacuum pump kit KSHEMA NUCSReM (Stem cell lab) Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Cell culture media aspiration 2) Filter sterilization of medium and other cell culture solutions	
Instrument Company Institute Centre/Dept. Contact Person Applications	Incubator shaker Bio-Bee KSHEMA NUCSReM Dr. Shama Rao M: 8095239355 E: shamarao.88@nitte.edu.in 1) Equipment is used to mix, blend and agitate substance in a tube or flask 2) It is mainly used in the field of chemistry and biology	

Appendix 10 List of conferences/meetings participated as a DELEGATE

1. The 2nd Biennial National Conference on "Advances in Stem Cell Research and Cell Based Therapy with Emphasis on Orthopedic, Dermatological and Dental Applications" held at K. S. Hegde Medical Academy (KSHEMA) from 22nd to 23rd December, 2017. This conference was jointly organized by Nitte University Centre for Stem Cell Research & Regenerative Medicine (NUCSReM) and Department of Orthopaedics, KSHEMA.

2. National Symposium on "Regenerative and Cancer Stem Cells" organized by Stem Cell and Regenerative Medicine Division of Yenepoya Research centre, Yenepoya (Deemed to be University) at Mangaluru on 1st February 2018.

3. Two days lecture series in 'Molecular Genetics' organized by K. S. Hegde Medical Academy (KSHEMA) in association with Karnataka Science and Technology Academy (KSTA) at Deralakatte, Mangaluru on 24th and 25th August 2018.

4. PIKNIKH Series XXVIII Indo-Japan symposium on 'Molecular Medicine' organized by School of Life Sciences (SLS), Manipal Academy of Higher Education (MAHE), Manipal on 19th September 2018.

5. International Conference on Radiation Biology (ICRB-2018) and 14th biennial meeting of Indian Society of Radiation Biology (ISRB) organized by K. S. Hegde Medical Academy (KSHEMA) from 4th to 6th October 2018 at Deralakatte, Mangaluru.

6. Presentation of part of PhD work at 87th Annual Conference of Society of Biological Chemists (India) with a theme on "Genome Biology in Health and Diseases" organized by School of Life Sciences (SLS), Manipal Academy of Higher Education (MAHE), Manipal from 25th to 27th November 2018.

Poster abstract presented and published in proceedings:

Title: DERIVATION AND CULTIVATION OF HUMAN MELANOCYTES AND KERATINOCYTES IN VITRO

Authors: Neha A. Shetty, Shama Rao, Vinutha E, Nikhil S. Shetty, Veena Shetty A, Jayaprakasha Shetty K, Mohana Kumar B.

Appendix 11 Departmental seminars, journal clubs presented during period

1. Regularly participated in the weekly meetings/seminars at Nitte University Centre for Stem Cell Research & Regenerative Medicine (NUCSReM) and Journal Club meetings of KSHEMA-CRL, Genetics and NUCSReM.
2. Presented the research articles related to PhD dissertation as a part of meetings/seminar.
3. Attended the guest/invited lectures organized at K. S. Hegde Medical Academy (KSHEMA).