The Use of *In vitro* 2D Co-culture Models to Determine the Optimal Keratinocyte: Melanocyte Ratio to be used in the Development of Pigmented 3D Skin Model

MARIBANYANA LEBEKO

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Department of Human Biology
Faculty of Health Sciences
UNIVERSITY OF CAPE TOWN

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DECLARATION

I, **Maribanyana Lebeko**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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Signature:

Date: **July 2015**

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

α	Alpha
β	Beta
γ	Gamma
μg	microgram
μl	microliter
%	Percent
^{0} C	Degrees Celsius
ANOVA	Analysis of variance
APC	Allophyco-cyanine
ddH2O	double distilled water
bFGF	basic fibroblast growth factor
BCA	Bradford Calorimetric Assay
BSA	Bovine serum albumin
PBE	Bovine pituitary extract
CAMs	Cellular adhesion molecules
cm	centimetre
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle medium
DNA	Deoxyribonucleic acid
DOPA	dihydroxyphenylalanine
EB	Embryoid bodies
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMU	Epidermal melanin unit
ET-1	Endothelin-1
ECM	Extracellular matrix
FACS	Fluorescence-activated cell sorting
Fb	Fibroblasts
FCS	Fetal calf serum
FETI	Hams **F**10 medium, bFGF, **E**T-1, **T**PA and **I**BMX
FITC	Flourescein isothiocyanate
g	grams
GAGs	Glucosaminoglycans
GREENS	Original keratinocyte growth medium
HBSS	Hanks Buffered Saline Solution
h	hour(s)
HCl	Hydrochloric acid
HGF	Hepatocyte growth factor
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HFb-CM	Human fibroblast conditioned medium
IBMX	3-isobutyl-1-methyl xanthine
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IL	Interleukins
IFN-γ	Interferon gamma
Kc	keratinocytes
KGF	Keratinocyte growth factor
KSFM	Keratinocyte specific medium
L	litre
LIF	Leukemia inhibitory factor
M	Molarity
MART-1	Melanoma antigen recognised by T-cells 1
MMPs	Matrix metalloproteinases
Mc	melanocytes
MSH	Melanocortin stimulating hormone
ml	millilitre
mg	milligram
min	minutes
mm	millimetre
n	number
ng	nanogram
nm	nanometre
nM	nanomolar
O_2	Oxygen
PBS	Phosphate buffered saline
PE	Phycoerythrin
P/S	Penicillin/streptomycin
PerCP	Peridinin chlorophyll
PDGF	Platelet derived growth factor
PI	Proliferation index
qs	Quantity sufficient
RIPA	Radio-immunoprecipitation assay
rpm	Revolutions per minute
SDF-1β	Stromal derived factor-1 beta
SDS	Sodium dodecyl sulphate
SEM	Standard error mean
t	Time
TBSA	Total body surface area
TE	Trypsin-EDTA
TIPMs	Tissue inhibitors of metalloproteinases
TPA	12-O-tetradecanoylphorbol-13-acetate
TGF- β	transforming growth factor beta

TRP1	Tyrosinase-related protein-1
T/T3	3, 3', 5-Trioiodo-L-thyronine sodium salt
TRP2	Tyrosinase-related protein-2/ dopachrome tautomerase
TNF-α	Tumour necrosis factor alpha
U	units
WHO	World Health Organisation

ABSTRACT

Burn injuries are among the most devastating of all injuries and a major global public health crisis, with fire related burns accounting for approximately 265 000 deaths annually. The African continent, most especially Sub-Saharan Africa, has the second highest mortality rates (15% of global mortality rates). In South Africa, 3.2 % of the total population sustains burn injuries, with 50 % of these cases as children under the age of 20 years. Studies have also shown that most of these incidences are prevalent within the age groups of 0-5 years, and account for the 3rd most common cause of mortality in children under the age of 15 years.

In depth knowledge and understanding of cellular facets of wound healing has allowed for a greater stance in the interventions aimed at circumventing problems associated with development of effective wound defects treatment regimen. Burn treatment options are largely dependent on the degree and extensiveness of burns. A wide body of literature exists with regards to traditional as well as current treatment options. These include, for instance the use of various forms of skin auto-grafts. Despite such great success with all kinds of innovative ideas surrounding the use of autologous skin grafting, lack of available donor sites for skin grafts still remains a problem, more so in cases where patients suffer burns spanning more than 70% TBSA. This therefore has inspired the design and use of bioengineered skin substitutes as well as cultured/non-cultured autologous epidermal cells.

Unfortunately, to date, no tissue engineering technique has fully been able to recapitulate the anatomy and physiology of the skin, or has attained the biological stability as well as achieving the aesthetic outcome. Several hurdles are yet to be overcome to achieve this. Amongst many, inclusion of melanocytes, other skin appendages as well as potential progenitor cells is some of the attributes of an ideal 3D skin equivalent. Therefore pigmented 3D skin constructs are of great interest as they address not only the issues of complete wound healing, but also the aesthetic outcomes. In light of this, correct keratinocyte to melanocyte ratios are also of great importance in designing such pigmented 3D constructs. Therefore the major aim of this study was to isolate skin melanocytes and keratinocytes, and co-culture them at different ratios in order to attain optimal pigment production and/or consequent improved wound healing outcome.

To determine the best keratinocyte to melanocyte ratio to use in developing pigmented 3D skin constructs, the following co-culture ratios were used: 5:1, 10:1 and 20:1. Proliferation assays were employed to further elucidate the growth dynamics of both human skin melanocytes and keratinocytes in either mono- or co-culture system. Secondly, FACS was used to develop a reliable technique to be used to separate the two cell types from a co-culture system in order to perform downstream analyses. Thirdly, to establish the roles of the co-cultured cells in wound healing (with regards to proliferation and migration), scratch wound healing assays were employed. Lastly, FACS was used to infer the effect of such ratios on pigment production.

Our results demonstrated that keratinocytes, compared to melanocytes mono-cultures have higher proliferation capacity. On the contrary melanocyte's proliferation is up-regulated by the presence of keratinocytes in a co-culture, whereas higher numbers of melanocytes in co-culture with keratinocytes resulted in less proliferative keratinocyte phenotype. The FACS separation technique worked excellently in identifying keratinocyte population from melanocytes, with an almost 100% accuracy. This is shown by melanocytes being sorted as 93% of MART-1^{+} cells in a mono-culture, followed by an approximately 5:1 separation of keratinocytes from melanocytes (77% Kc and 17% Mc). In vitro scratch assays demonstrated that none of the co-culture ratios was significantly superior with regards to wound healing capacities and pigment production, in the absence of fibroblast-conditioned medium.

In conclusion, the 5:1 co-culture ratio seemed to yield a non-significant, yet best outcome with regards to wound healing capacity (only in the presence of fibroblast-derived factors), thus conferring it as a potential optimal ratio of keratinocytes to melanocytes, to be used in development of our pigmented 3D constructs.

1.1. Introduction

1.1.1. Burn statistics

Burn injuries are among the most devastating of all injuries and a major global public health crisis, with fire related burns accounting for approximately 265 000 deaths annually (1). Burn injuries have been rated as the sixth most common type of trauma in children aged 5-14 years (1, 2, 3). Approximately 95% of fatal fire related burns occur in low to middle income countries, as they generally lack the necessary infrastructure to reduce the incidence and severity of burns (1, 2). Followed by South East Asia (Figure 1.1), the African continent, most especially Sub-Saharan Africa, has the second highest mortality rates worldwide (15% of global mortality rates) (1).

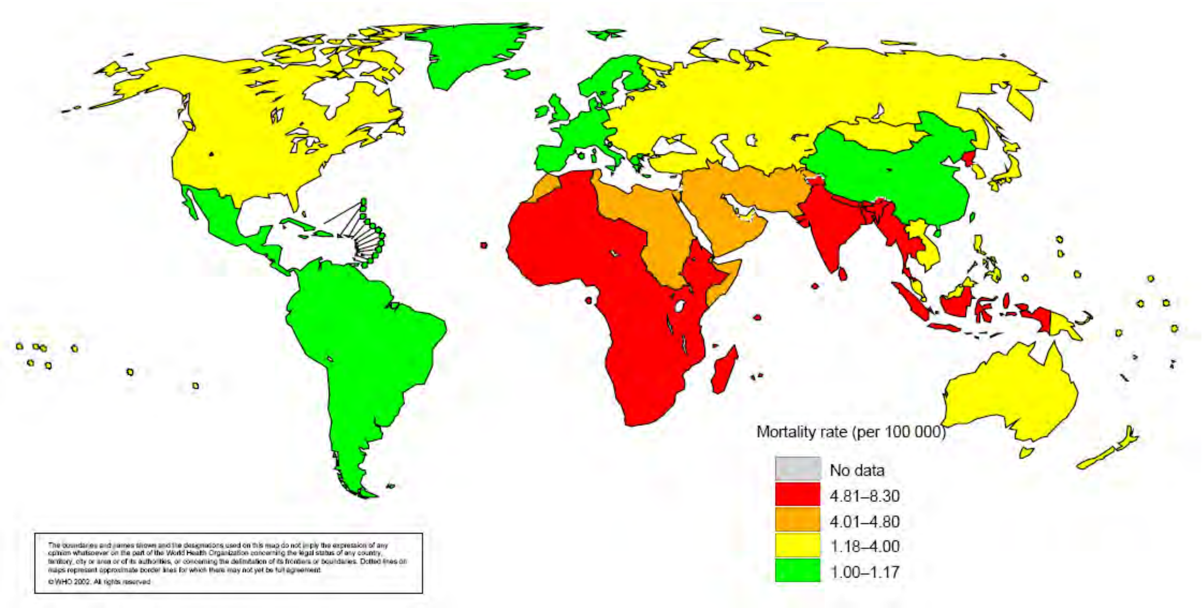


Figure 1.1: Global fire-burn related mortality rates in 2000 (4).

Several factors have been implicated in the increased burn injuries incidences in the Sub-Saharan Africa; these include poverty, mass illiteracy as well as urban migration, thus the development of informal settlements (5).

1.1.2. Epidemiology of burns in SA

In South Africa, 3.2 % of the total population sustains burn injuries, with 50 % of these cases as children under the age of 20 years (5,6).

Incidence and prevalence data are particularly lacking in Africa as most reported causes of burn injuries are from hospitals, compounded by a further lack of statistics because of community-based medicine. Studies have also shown that most of these incidences are prevalent within the age groups of 0-5 years, and account for the 3rd most common cause of mortality in children under the age of 15 years (6-8). Adolescent females sustain household based injuries (7). In older age groups, young males are at increased risk. Toddlers sustain burns mostly on the upper body parts due to their exploring nature; they touch, pull and grab even hot objects. Burns affecting the perineum, axilla and buttocks are difficult to keep clean and frequently lead to infections, with associated increased morbidity (9).

1.2. The skin

Skin, being the largest continuous organ in the human body, comprises cells which make up various layers, including keratinocytes (Kc), melanocytes (Mc), Langerhans cells (LC), Merkel cells, and "resident" T-cells (dendritic epidermal T-cells) (Figure 1.2). Keratinocytes represent the most abundant cell type and make up the bulk of the epidermis (10). Keratinocytes originate from a single layer of ectoderm during gestation. These cells then divide and form multiple layers, which will eventually form distinctive spinous and granular layers characteristic of the mature epidermis and express a variety of differentiation-dependant keratins (11). Keratinocytes exist in varying differentiation states, ranging from the undifferentiated, highly proliferative state (basal keratinocytes), to terminally differentiated, granular, upper layers of skin (12). Keratin expression is then stopped as the cells become cornified, which then forms the uppermost layer of skin, which is in constant contact with the environment.

Resting on the basement membrane are melanocytes (pigment producing cells). Melanocyte precursors (melanoblasts) originate from the neural crest cells (13,14) and migrate dorso-laterally along the neural tube in order to localise and differentiate into mature melanocytes at epidermal layer of skin (15). These cells are intercalated in between keratinocytes, to form a functional epidermal melanin unit (EMU) (16) . In this unit, 1 Mc interacts with 36 Kc, However, the ratio in the basal layer of the epidermis has been suggested to be approximately 1 Mc: 5 Kc (17,18). These melanocytes are maintained in a less differentiated state (controlled pigmentation and proliferation, which mark their differentiated state) and reside in the basement membrane of the epidermis under the tight control of TFG-β signalling via keratinocytes ((19-21). The involvement of other key growth factors includes the keratinocyte-derived factors such as endothelins (ETs) and fibroblast growth factors (bFGFs) have been shown to have a profound role in both the regulation of pigmentation and melanocyte proliferation (17).

The dermal layer is composed of cells of mesenchymal origin; fibroblasts (22), fundamentally responsible for replenishment and maintenance of extracellular matrix (ECM) (23,24). These cells make up the dermal layer of skin and form two distinct regions; the upper dermal layer (dermal papilla), which comprises papillary fibroblasts and the deeper dermal layer (reticular dermis) that is made up of less densely populated reticular fibroblasts (25). Fibroblasts are not only useful in maintaining the structural integrity of the vast majority of vertebrate tissue, but are also the major role players in bioengineered skin constructs as they perform the structural role or maintain the skin substitute integrity. These cells perform critical roles through their interaction with keratinocytes. Studies have implicated this cross talk during wound healing, where fibroblasts secrete keratinocyte mitogens to allow for proliferation (26,27), and in turn keratinocytes activate fibroblasts (via interleukins) to allow for increased proliferation, collagen deposition and ECM reorganization (28).

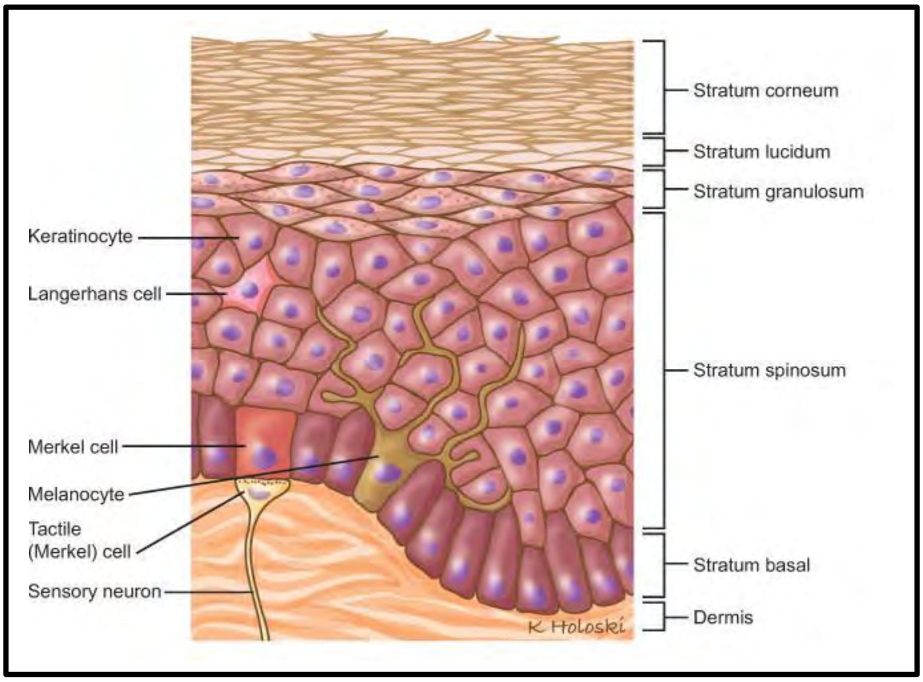


Figure 1.2: schematic depiction of the skin, showing mainly the epidermis with various cells types, as well as part of the dermis (29).

1.3. Biology of wound healing

The primary goals of burn treatment options are a rapidly closed wound and regaining of functionality of the affected area or body part. However, current treatment modalities aim to achieve both minimal as well as aesthetically presentable scarring. This wound care has been greatly impacted by our broadened understanding of biological processes involved in wound healing. Wound healing is an orchestrated phenomenon involving an overlap between inflammation, new tissue formation (re-epithelisation) and tissue remodelling (Figure 1.3).

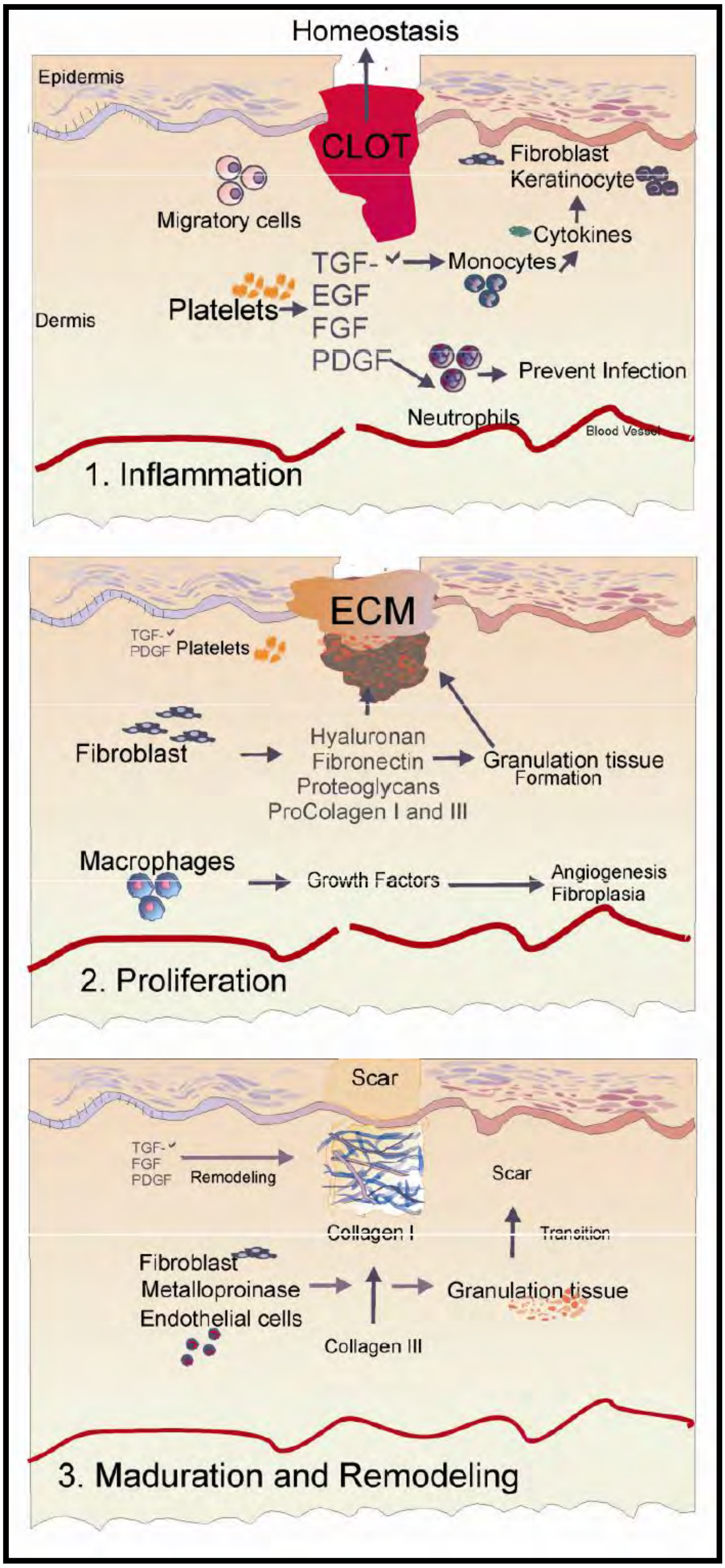


Figure 1.3: Schematic representation of stages of normal wound healing: inflammation, proliferation, maturation and remodelling (30).

1.3.1. Inflammation phase

This phase of wound healing is initiated within 24 hours post wounding (Figure 1.4) and may last for up to 2 weeks during normal wound healing (31). Mast cells produce vast amounts of mediators such as vaso-active amines as well as histamine rich granules, which result in the leakiness of blood vessels (32), thus allowing for efficient neutrophil movement and their subsequent recruitment to the site of injury (33). At this stage, the wound is therefore characterized by redness, swelling, heat and pain.

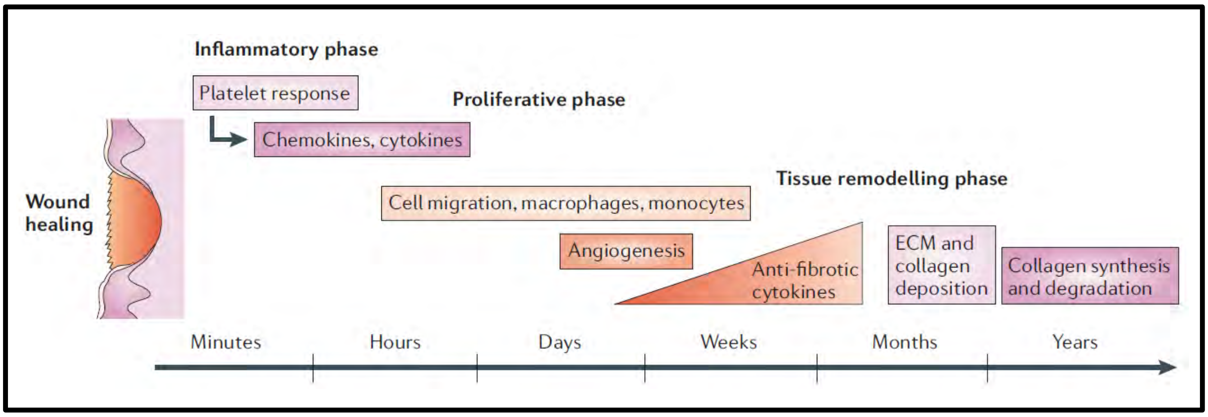


Figure 1.4: Phases of normal wound healing over time (31).

The major cellular role players in this phase are neutrophils and macrophages that are recruited by surrounding endothelial cells. Endothelial cells express cellular adhesion molecules (CAM) such as selectins, which in turn recruit neutrophils to the site of injury. This recruitment allows for neutrophils to provide the first line of defense by phagocytosis of micro-organisms as well as debris (33). In addition, tissue necrosis factor alpha (TNF-α) and interleukin 1 (IL-1) (34,35) are also secreted by activated macrophages and neutrophils to aid in initiation of new tissue formation (36).

1.3.2. New tissue formation (proliferation phase)

This phase is initiated approximately four days post-wounding and is mainly characterized by the loss of contact between epidermal and dermal cells, thereby allowing for migration of such cells from their various niches such as the hair follicle. Endothelial cells are activated by TNF-α and bFGF to initiate angiogenesis, thus allowing for increased blood flow and nutrient circulation. On the other hand, macrophages and platelets secrete PDGF and TGF-β (37), which allow for fibroblast migration (38) and proliferation (28). These cells in turn release matrix metalloproteinase (MMP's) that facilitate their movement through the matrix and subsequently express collagen and proteoglycans (39). This ultimately results in granulation tissue formation, thus forming a scar characterized by a network of blood vessels and capillaries, macrophages as well as a population of fibroblasts with randomly organized collagen fibres (40). Finally, epidermal stem cells from the hair follicle migrate to the wound edges and start to proliferate and differentiate into keratinocytes in order to form the protective outer layer (stratum corneum), a process known as re-epithelisation (41,42).

1.3.3. Tissue remodelling (maturation phase)

During this phase, a scar matures to form granulation tissue, and may last for up to two years post wounding. The type, density and organization of collagen fibres play a pivotal role in tensile strength of the healed wound. Mainly collagen type III is deposited, which is replaced by type I collagen (43). As extracellular matrix re-organization occurs, there is an ongoing collagen synthesis and degradation with the aim of achieving normal skin phenotype (a highly organized structure). This process is mainly driven by MMP's (44). However, the result is often a scar with only 80% tensile strength compared to normal tissue (45).

As mentioned earlier that keratinocytes are the major role players in the regaining of skin's physical barrier, it would be important to discuss some of the molecular facets that drive that particular re-epithelisation process. These cellular processes include keratinocyte migration and subsequent proliferation following injury, alongside the dynamic keratin expression profile, which largely drives their phenotypic changes.

1.4. Keratinocyte migration and proliferation, as well as cytokeratin expression profiling in response to wound healing

One of the vital facets of wound phases is re-epithelialization, which is orchestrated by epidermal keratinocytes, wherein upon wounding, these cells (in close proximity to wound edge) are activated to a migratory phenotype, followed by a proliferative burst that occurs just behind the migrating tongue (46,47). The migratory and proliferation role as well as phenotypic changes (at the level of keratin expression profiles; Figure 1.4) of keratinocytes is further dealt with in the following section.

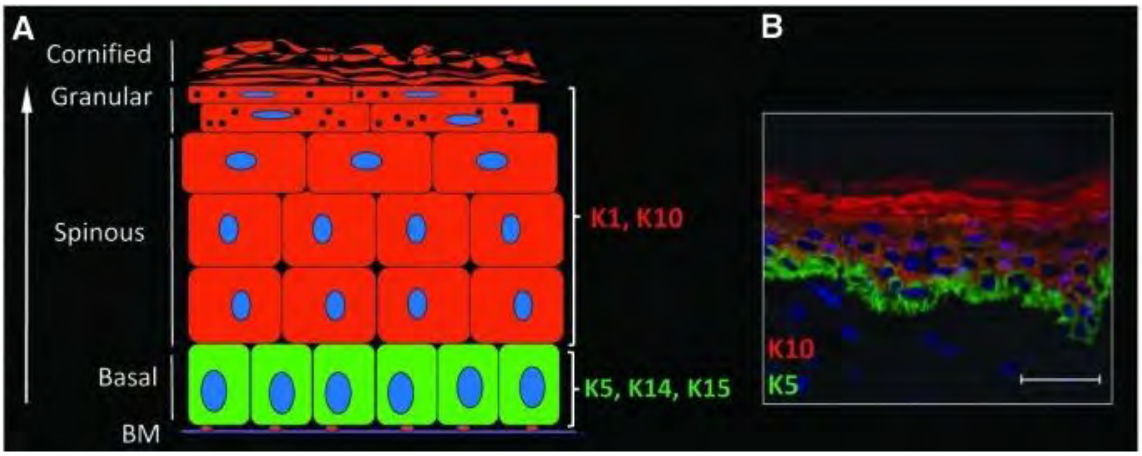


Figure 1.5: Structure of the epidermis. **(A)** Schematic illustration of keratinocytes in different layers of epidermis, alongside keratins expressed. Mitotically active basal layer keratinocytes are characterized by keratins 5, 14, and 15. As they differentiate, keratinocytes form suprabasal layers and express K1 and K10. **(B)** Immunolocalization of K5 (green) and K10 (red) in human epidermis. Nuclei are visualized with DAPI (blue). Basement membrane is depicted as BM. Adapted from (48)

1.4.1. Migration

In response to acute wound injury, suprabasal keratinocytes at the wound margin begin to change their keratin (K) expression profiles. These cells normally express a pair of keratins 1 and 10. However, ~8–24 hr after wounding, keratins 6 and 16 are expressed (47) and depict keratinocytes with a more activated phenotype (46). This keratin expression profile changes allow for the downregulation of cell-cell and cell-substrate attachment molecules; desmosomes and hemi-desmosomes respectively. This then allows for keratinocyte migration at the wound edge towards the denuded area (49,50).

The keratin expression profiles are accompanied by the integrin expression profiles of keratinocytes during migration, wherein these cells switch the expression from $\alpha 6\beta 4$ to $\alpha 3\beta 1$ (51). Keratinocytes' migration further induces of K6, K16, and K17 expression, which in turn is postulated to increase viscoelastic properties of migrating cells (52). Accompanied with keratin and integrin expression is the up-regulation of epidermal growth factors (EGF) and fibroblast growth factors (FGF-2), leading to further keratinocyte migration (53,54).

IL-1, IL-6, and TGF-α, are amongst some cytokines that can also modulate migratory phenotypes of keratinocytes. For instance, during wound healing, IL-1 has been shown to augment secretion of FGF-7 (55) whereas IL-6 allows keratinocytes to respond to mitogenic factors, thereby subsequently stimulating migration through the STAT3-dependent pathway (26). Finally, TGF-β1 can also promote keratinocyte migration by stimulating MMPs such as MMP-1, which is expressed abundantly at the wound edges, and is thought to sustain keratinocyte migration on type 1 collagen (56,57). Thereafter proliferation occurs.

1.4.2. Proliferation

The regulation of keratinocyte proliferation is largely dependent on the availability of various stimuli, such as that of growth factors, degree of cell differentiation, as well cell attachment to the substrate. Studies have shown that the basal keratinocytes have the ability to proliferate, while the keratinocytes in the suprabasal layer are of migratory phenotype (reviewed by (58,59).

Similar to migration, growth factor modulation plays a major role in the proliferative process, wherein growth factors such as HB-EGF, TGF-α, KGF and IGF-1 aid in stimulating keratinocyte proliferation (60). Finally, Imai et al., (1997) (61) has shown that MMPs, through their proteolytic activity, not only release growth factors from the wound matrix, but could possibly activate such growth factors. After being activated to repair an injury, keratinocytes lose the proliferation signals and return to their normal differentiation pathway, where the stratification process begins.

A deeper understanding of how wound healing is orchestrated, and how various cell types drive the different stages of wound healing has allowed for scientists to delineate burn injuries into distinctive types, or to varying degrees. The next section deals with the classification of burns as well as their clinical manifestations.

1.5. Types of burns

Burn injuries can be classified into various degrees, depending on how deep they occur into the skin. Such burns range from first degree (I, superficial), to fourth degree (IV, full thickness) burns (Figure 1.5). Literature had delved greatly into such clinical manifestations, therefore for the purposes of this review, we will not go into details on some the anatomical correlates and clinical aspects of various degrees of burn injuries. Figure 1.5 gives a broad overview of these aspects.

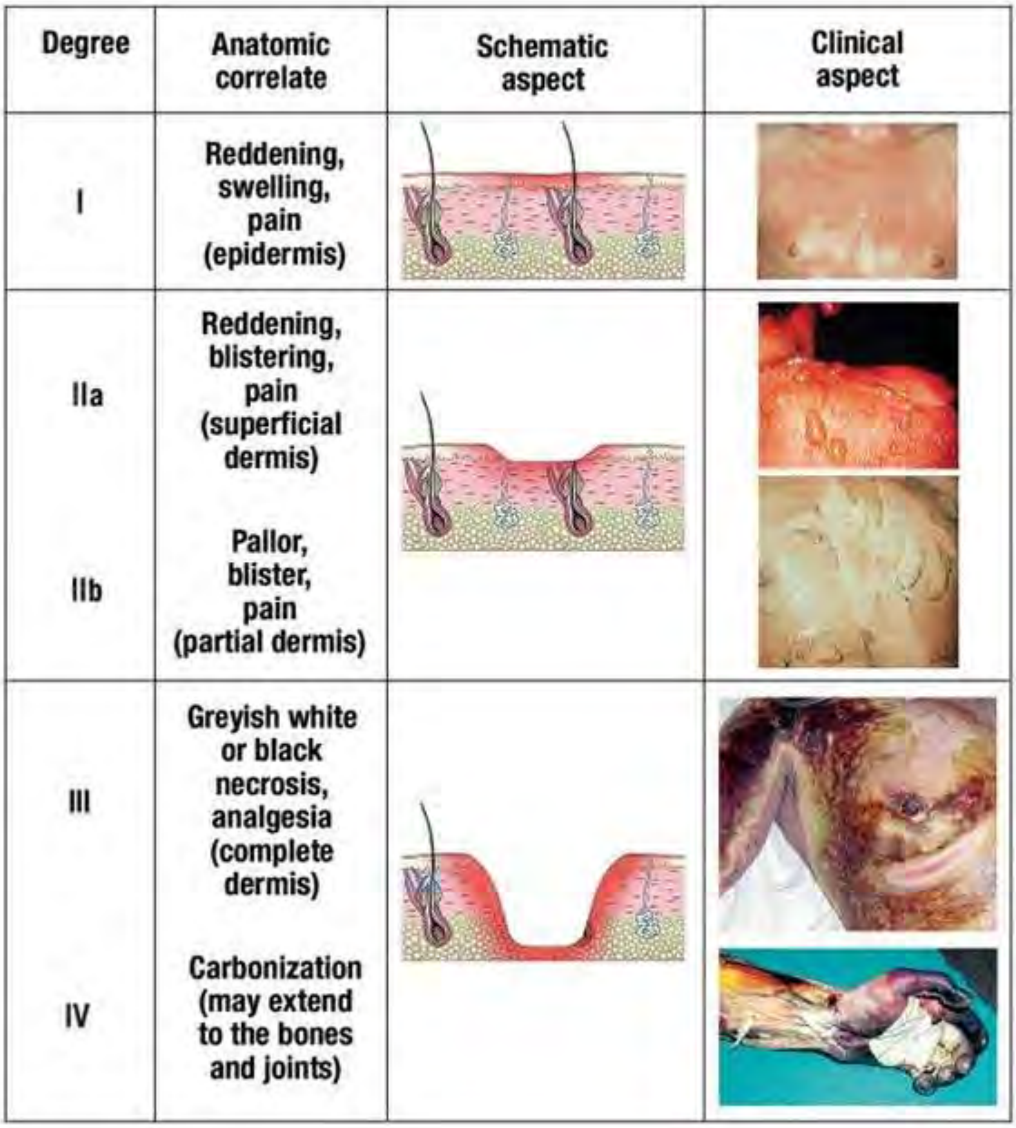


Figure 1.6: Degree of burns (62)

In-depth knowledge and understanding of cellular facets of wound healing has allowed for a greater stance in the interventions aimed at circumventing problems associated with development of effective wound defects treatment regimen. A large body of literature exist about various treatment modalities, therefore the next part of this literature deals with only some of the current burn injury treatment regimes.

1.6. Current clinical treatment options

Burn treatment options are largely dependent on the degree and extensiveness of burns. A wide body of literature exists with regards to traditional as well as current treatment options. These include, for instance the use of various forms of skin auto-grafts (63,64), use of cultured epithelial sheets (65), use of bio-engineered skin substitutes (64,66,67) as well as potential use of stem cell therapy (68,69). For the purpose of this study, only some of the major current treatment advances will be reviewed.

1.6.1. Autologous skin grafting

Skin grafting is a technique that has been used for treatment of severe skin defects, more so third degree burn injuries as well as in reconstructive surgery (70,71). This technique has also played a significant role in treatment of non-healing diabetic foot or leg ulcers (72,73). Skin grafting originates in ancient India, where it was used for treatment of mutilations of ear, nose and lips (74).

This technique was performed in various ways, including "slapping the skin of the buttock with a wooden paddle until it was quite congested and then, with a leaf cut to proper shape as a pattern, cutting out a piece of skin with its subcutaneous fat, transplanting it and sewing it into place, uniting it to the freshened edges of the defect" (…as cited in Chick, 1988 (74)). Since the 19th century, skin grafts have been harvested by means of a device developed by Padgett and Hood in the 1940's (Padgett-Hood dermatome), which yields a piece of skin with a defined thickness, thereby resulting in either full thickness skin graft (FTSG) or split thickness skin graft (STSG) (75). Furthermore, these techniques have been further optimised to cater for the well-known problem of lack of donor sites following extensive burn injuries. Such techniques include amongst many; meshed skin grafts as well as micro-skin grafts. Fabrication, clinical application as well as advantages and disadvantages of these techniques are tabulated below (Table 1.1).

Table 1.1: Various types of skin grafts, their development, subsequent pros and cons, as well as their clinical applications.

Graft type/name	1st developed by	Technique	Advantages/disadvantages and clinical applications
Full thickness skin graft (FTSG)	Well-developed technique, in use since 2500BC	Full epidermis and dermal layers of skin harvested from donor sites	Has all major skin appendages, less susceptible to trauma and normal pigmentation is regained (72). However, there's increased risk of graft "take", prolonged donor site healing times as well as decreased donor site aesthetics (74,76).
Split thickness skin graft (STSG)	Technique modified from FTSG	Full epidermis and partial dermal layer of skin harvested from donor sites	Shorter donor site healing time and increased aesthetics (74,76**.** There are increased contraction risks and hyperpigmentation (74,77)**.**(78)
Meshed skin graft (MSG)	Gabarro et al., 1945; Meek et al., 1958	Small pieces of skin cut into uniform square pieces (~3mmx3mm)	Works efficiently on extensive burns 9~70% TBSA) and spacing in between the uniformly layers skin pieces allows for uniform re-epithelialisation ((78).
Micro-skin graft (Micro-SG)	Zhang et al., 1989	Minute skin pieces (~1mm^2), meshed by use of conventional food processors, thereafter placed onto a fenestrated wound dressing	Excellent graft "take" and higher expansion ratios (20:1). Associated with increased contracture as well as increased donor site pain (79-81).

Despite the widespread use of both FTSG and STSG, and accompanying shortcomings, split thickness skin grafts remain the gold standard for treatment of various skin defects. A vast body of literature has delved into use of such techniques in treatment of severe burns and the interesting case studies are highlighted below.

Zhao et al., (2012) (64) Reported on a case of a female (32 years), with extensive facial burns scars. The group grafted a full face, whole full-thickness skin graft. Ten days post-operatively, there was a 100% graft "acceptance/take", accompanied by its survival even 4 years later. The patient regained her natural smile, normal sensation, as well regaining a fully operational mouth and eyes. Most importantly, the technique was accompanied by patient satisfaction, alongside a boost in self-confidence. This technique therefore highlights the holistic nature of the therapy as well as an optimal clinical outcome (64).

Due to the well-known problem of lack of availability of donor skin following burn injuries covering more than 50% TBSA, several approaches have been introduced. Between 1998 and 2008, Zheng et al., (2010) (82) embarked on a technique where they harvested split thickness skin graft (STSG) from scar tissue. Scar tissue grafts were 20cm x 20cm x 0.8cm in length, width and thickness. Three reconstructive operations were performed on various areas of burn scars at 6-monthly intervals. Most importantly, stamp-sized skin was also grafted onto the donor areas. The patients were followed up for 4 years, during which time a 100% "take" rate was observed. This was accompanied by complete donor skin healing exhibiting normal tensile strength and pressure resistance of the recipient area up to two years later. This case report highlights the significance of using scar tissue STSG, which exhibited similar post-operative outcomes such as satisfactory functional recovery and improved appearance, compared to normal STSG (82).

Despite such great success with all kinds of innovative ideas surrounding the use of autologous skin grafting, lack of available donor sites for skin grafts still remains a problem, more so in cases where patients suffer burns spanning more than 70% TBSA. This therefore has inspired the design and use of bioengineered skin substitutes as well as cultured/non-cultured autologous epidermal cells.

1.6.2. Bioengineered skin substitutes

Tissue engineering is an interdisciplinary (incorporating material science, regenerative biology, as well as proteomics and genomics) approach, where the ultimate objective is to develop bio-compatible materials that can be used in repair of damaged tissue. A technique of this nature not only requires the use and clear knowledge of support structures (bio-materials that mimic the ECM), but also the sources and characteristics of cells to be incorporated (77,83).

To date, no tissue engineering technique has fully been able to recapitulate the anatomy and physiology of the skin, attained the biological stability as well as achieving the aesthetic outcome. Therefore, current tissue engineering techniques should meet most of the following criteria: easy handling and application onto wound site, appropriate physical and biological properties, retaining skin barrier function, while maintaining appropriate porosity to allow for cell colonisation and fluid exchange (84,85). Furthermore, these techniques should possess adherent cues, controlled degradation capacity and advanced sterility (non-immunogenic or non-toxic), alongside decreased inflammatory response. They should also facilitate angiogenesis (reviewed by (84,85)).

Bio-materials

One of the requirements for tissue engineered skin is the use of bio-materials, which can either be naturally occurring (collagen, fibronectin, GAGs, or chitosan) or of synthetic origin (various forms of bio-polymers, such as poly-tetra flour ethylene). These biologically active matrix scaffolds have been widely used in the development of tissue engineered skin substitutes, which have been used to treat several skin defects.

Due to the limited properties of some of these bio-materials, there have been advances in the development of "new age" bio-polymers such as matrigel (collagen, fibrin, laminin or elastin) and polyethylene glycol (PEG). As a result of its natural resemblance to the ECM, its ability to provide certain signalling cues and to form three-dimensional structure, matrigel has gained a lot of momentum in its use in tissue engineering. On the other hand, PEG, a molecularly engineered synthetic hydrogel serves as an inert structural platform due to its hydrophilic nature (86). One of the major drawbacks of synthetic bio-materials is their inability to possess cell signalling cues; thus the requirement of cell adhesion peptides. Therefore, this synthetic polymer allows for incorporation of peptides (87), thus making it an ideal ECM mimic with cell adhesion and proteolytic degradation properties (88).

Cell sources

Alongside the requirement of biologically active bio-materials, cells are of equal importance as they serve various functions ranging from regaining the physical barrier to reconstituting the structural integrity of skin following injury. Reliable cell sourcing and routine isolation methods are pivotal for large scale production of cell for tissue engineering (89). Such cells can be sourced from various platforms, such as local sites (where fibroblasts, keratinocytes, melanocytes and adipocytes can be harvested), systemic (blood or circulating bone marrow cells) as well as from progenitor cell populations (MSCs or bone marrow derived stem cells). Various skin cells are used for the development of varying types of tissue engineered skin substitutes, ranging from epidermal to dermo-epidermal substitutes, as reviewed in this section. This section deals with the various tissue engineered platforms, the clinical outcomes as well as their shortcomings.

1.6.2.1. Epidermal substitutes

1.6.2.1.1. Cultured epidermal sheets

Cultured epithelial autografts (CEA) are defined as thin sheets (3-5 cell layers) of epidermal keratinocytes (Figure 1.7), that can either be cultured on synthetic materials such as petrolatum gauze (Epicel®) or silicone membrane (Epidex®) to confluence or sub-confluence (90). Culturing process takes approximately 3-5 weeks post harvesting, which is subsequently followed by their transplantation as epidermal sheets to treat various skin defects (Figure 1.6) (Table 1.2). Recently this technique has evolved to use of stratified cultured epidermal sheets, as a result of pioneering work by Bernstam et al., (1986) (91) and Prunieras et al., (1983) (92), who developed a method of in vitro stratification of keratinocytes by exposing them to air-liquid interface. This allows for spontaneous stratification.

Figure 1.7: Structure of CEA. Adapted from Wood, (2006) (93). Keynote presentation at SCAR Meeting in Montpellier, France.

Since their development by Green et al., (1979) (89), CEA have been extensively used as early as 1980's in treatment of severe burns (where patients have suffered burns of more than 50% total body surface area). This large surface area coverage of CEA has allowed them a platform to circumvent problems associated with lack of donor sites in the burn treatment modality arena (90,94,95). For instance, early studies by Gobet et al., (1997) (90) demonstrated potential use of this technique on pediatric burn injuries. It was also shown that the grafts persisted for over 2.5 years post transplantation, thereby highlighting the importance of CEA's ability to "take" and persist. Similarly, Cirodde et al., (2011) (95) used CEA in the treatment of 68 severely (80% TBSA, with 70% of burns as full thickness) burned patients. The group showed a 60% "take" success rate, which was attributed to age of patients.

Despite their desired use in the treatment of large skin defects and their persistence at injury sites, CEA are not without any shortcomings. This technique has been shown to be expensive, with complex application. Due to their nature (thin epidermal sheets), CEA are very fragile, and are susceptible to infections (65).

Table 1.2: The clinical use of (and product description) of various commercially available CEA.

Technology	Company	FDA approval	Brief product summary
Epicel®	Genzyme Bio surgery (Cambridge, MA)	2007	Autologous keratinocytes cultured from patient skin biopsy, transplanted as epidermal sheet using petrolatum gauze support. Permanent wound closure in patients with burn with greater than 30% TBSA injury and in patients with congenital nevus (90,94).
Epidex®	Modex Therapeutiques, (Lausanne, Switzerland)	Clinical trials	Autologous keratinocytes isolated from outer root sheath of scalp hair follicles; Supplied as epidermal sheet discs with a silicone membrane support. Treatment of chronic leg ulcers (96).
CellSpray	Clinical Cell Culture (C3 Perth, Australia)	2006	Autologous undifferentiated keratinocytes cultured from patient skin biopsy to sub-confluence and transplanted as a suspension. Treatment of deep and extensive wounds (97).

1.6.2.1.2. Cultured/non-cultured epidermal cells in suspension

Due to the prolonged waiting period of culturing partially differentiated autologous epidermal sheets, variability in product quality (cell numbers and yield), their fragile nature and difficulty to apply, associated poor "take" in shear prone areas as well as high costs (98), several studies have explored the use of (spraying) highly proliferative, undifferentiated sub-confluent epidermal keratinocyte cells in a suspension (97,99-102).

Since the 80's, use of non-cultured isolated keratinocytes in fibrin gel, known as keratinocyte-fibrin gel suspension (KFGS) onto patients with full-thickness burns and chronic wounds inspired the much appreciated use of non-/cultured CEA in suspension. Since the work done by Horch as well as Wood in the early 90's, the use of cultured sub-confluent KFGS for treatment of various skin defects became a much appraised technique (100,101,103,104).

For instance, Stoner and Wood, (1999) (97) demonstrated that CEA in suspension can be incorporated and persist in the recipient skin for about 3 weeks, thereby implying that wound healing was not a result of release of growth factors, rather of the direct incorporation of donor cells. Similarly Hartmann et al., (2007) (99) sprayed cultured keratinocytes onto prepared wound beds of deep dermal burn (face and neck) victims. They showed that the technique offered an excellent cosmetic outcome, with no adverse effects.

Such cultured epithelial autografts have numerous advantages: they are rapidly available (after 10 days of culture), can be harvested from same patient (autologous), with the advantages of being site matched, they offer reliable wound adherence with minimal donor site morbidity. Such grafts are clinically manageable, affordable and have been implicated in improved quality of scar (review by Horch et al., (2005) (98)).

However, the lack of dermal support has rendered use of CEA (as sheets or in suspension) undesirable for treatment of major full thickness burns, thereby highlighting the importance of use of dermal components, to provide support and restore mechanical strength of transplants.

1.6.2.2. Dermal substitutes

Dermal substitutes offer mechanical support and allow for cell and vascular invasion. They have been shown to retain moisture and increase surface adherence of cells, as well their superiority in biodegradability capacity (105). This therefore renders tissue engineered dermal substitutes, great candidates for temporary wound closure and mechanical support as the neo-dermis is formed.

In the early 80's Burke et al., (1981) (106) pioneered the development of dermal substitutes. Since then, such substitutes gained a lot of momentum in tissue engineering and regenerative medicine (Table 1.3). Some tissue engineered dermal substitutes are made of cultured human fibroblasts (in a 3D environment; polymer scaffold), thereby secreting growth factors and ECM components, which remain active following cryopreservation (107). On the other hand, synthetic dermal substitutes are made of a bilayer of bio-degradable ECM components such as collagen and chondroitin (Integra®) or on synthetic scaffolds (TransCyte®/ Dermagraft®), and serve as temporary dermal support until an autograft is ready (108,109). Other dermal substitutes are made of a structurally intact de-cellularised (by freeze-drying) human dermis (109).

Table 1.3: The clinical use of (and product description) of various commercially available dermal substitutes.

Technology	Company	FDA approval	Brief product summary
Integra	Integra Life Sciences, (Plainsboro, NJ)	1996	Bilayer structure; biodegradable dermal layer made of porous bovine collagen-chondroitin-6-sulfate matrix; temporary epidermal layer made of synthetic silicone polymer. Grafting of deep partial- or full-thickness burns; epidermal layer removed when donor sites available for autografting (110,111).
Alloderm	LifeCell Corporation, (Branchburg, NJ)	2010	Structurally intact allogeneic acellular dermis; freeze-dried after cells were removed with detergent treatment; rehydrated before grafting. Dermal template for grafting to burns and other wounds; repair of soft tissue defects (112,113).
TransCyte	Smith and Nephew, (Largo, FL)	1997	Allogeneic neonatal foreskin fibroblasts seeded on nylon mesh; freeze-dried to kill cells and preserve dermal matrix. Temporary covering for excised deep partial- and full-thickness burns before autografting (109,114).
Dermagraft	Smith and Nephew, (Largo, FL)	2001	Cryopreserved allogeneic neonatal foreskin fibroblasts seeded on bioabsorbable polyglactin mesh scaffold; cells are metabolically active at grafting. Treatment of full-thickness chronic diabetic foot ulcers (107).

Extensive clinical trials have been undertaken to validate the safety and efficacy aspects of now commercially available dermal substitutes. Heimbach et al., (1988) (108) embarked on a clinical trial where they enrolled 149 patients who suffered burns spanning approximately 50% of TBSA. Twenty six of these patients were followed up for at least one year post treatment. The group showed that recipients of Integra® exhibited 80% "take" rate, and that these patients preferred this treatment modality as compared to meshed autografts and normal wound dressings. However, infection rates seemed to significantly lower the "take" rates, therefore a multi-centre post approval clinical trial had to be put in place. This is where Heimbach et al., (2003) (110) enrolled a larger cohort of 216 patients, and they showed host dermal regeneration 2-3 weeks post transplantation. They further demonstrated a "take" rate of 70%, accompanied by lower infection rates, thereby postulating (along with other authors) that dermal substitutes could be used in treatment of severe pediatric and adult burns (106,115).

Despite the great success of dermal substitutes as scaffolds used in treatment of full thickness burn wounds, there are some considerable problems associated with this technique. Firstly, most of the FDA approved dermal substitutes serve only as temporary scaffolds (excluding Integra). Secondly, these substitutes cannot be exclusively used as they lack the epidermal (physical barrier) component, thereby highlighting their dependence on subsequent use of epidermal autografts (115,116).

1.6.2.3. Dermo-epidermal substitutes

Due to their fragility and susceptibility to infections (65), CEA need dermal supports as advanced tissue engineered modalities for treatment of skin defects. On the other hand, dermal substitutes due to lack of epidermal layer have been shown to be useful as temporary scaffolds (116). This reciprocity has therefore allowed for the sequential application of dermal application followed by epidermal replacement. However, several problems arise with such a technique, whereby studies have shown delayed CEA propagation on dermal substitutes as well as increased complexity of transferring fragile CEA sheets onto dermal scaffolds autografts (115,116). For these reasons, tissue engineering techniques as well as regenerative medicine has allowed for the development of dermo-epidermal substitutes such as the FDA approved Apligraf® and OrCell®, as well as many other techniques (TissueTech Autograft System and Stratagraft) that are still in clinical trials (Table 1.4). Figure 1.8 illustrates an overview of epidermal-dermal substitute fabrication.

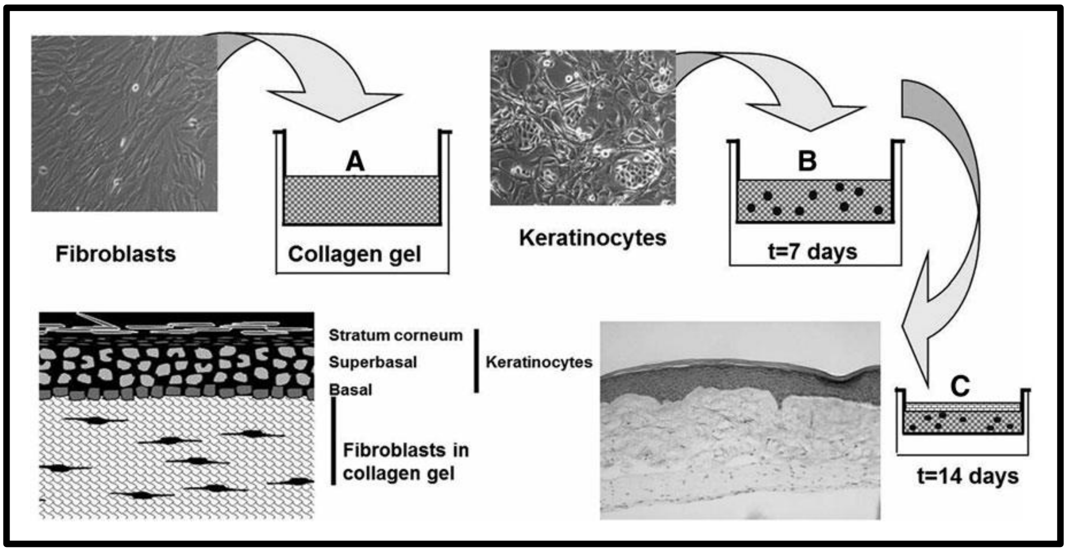


Figure 1.8: Fabrication of human skin-equivalent (HSE) tissues. Assembly of HSE involves preparation of cellular collagen layer, which serves as a dermal equivalent containing fibroblasts (**A**). After 7 days, keratinocytes are seeded on top of this contracted cellular layer (**B**). Developing tissues are maintained submerged in media for 7 days, and then lifted to an air-liquid interface to allow full stratification (**C**) (117).

Many of these dermo-epidermal substitutes have widely been used in treatment of acute surgical wounds (118), venous ulcers (119) and other skin diseases (66), but not in the treatment of burn wounds.

Table 1.4: The clinical use of (and product description) of various commercially available epidermal-dermal substitutes.

Technology	Company	FDA approval	Brief product summary
Apligraf®	Organogenesis (Massachusetts, USA)	1998	Bilayered human skin directly applied to a wound (burns and ulcers). Made of allogeneic neonatal foreskin fibroblasts and keratinocytes in bovine collagen gel. Treatment of leg ulcers and acute surgical wounds (66,119).
OrCel®	Ortec International (New York, USA)	2001/2008	Bilayer; allogeneic neonatal foreskin fibroblasts and keratinocytes cultured in bovine collagen sponge. Treatment of split-thickness donor sites in patients with burn and surgical wounds in EB (120,121).
The TissueTech® Autograft System	FIDIA Advanced Biopolymers, (Abano Terme, Italy)	Clinical trials	Comprises an autologous dermal substitute made of a three-dimensional matrix onto which autologous fibroblasts are seeded and expanded, and a three-dimensional matrix containing preconfluent autologous keratinocytes (122).
Stratagraft	StrataTech (Madison, WI)	Clinical trials	Dermal equivalent containing human dermal fibroblasts and a fully-stratified, biologically active epidermis derived from NIKS cells (a near-diploid human keratinocytes cell line). Treatment of severe burns and other skin defects (118,123).

1.7. Study rationale

Despite the success and clinical benefits of application of cultured 3D skin substitutes in treating burn wounds, these skin substitutes are devoid of many of the structures and functions of uninjured skin. Such include sebaceous glands, hair follicles, basement membrane, endothelial cells, nerve cells; immune cells and most importantly melanocytes (for re-pigmentation) (see review by Supp, (2012) (124)). Lately, to clinicians the best clinical outcome to burn victims is indeed not only a rapid healed wound, but also a satisfactory aesthetic outcome. Therefore the implementation of 3D skin constructs (pigmented skin equivalents - PSE) that are made of both epidermal cells (melanocytes and keratinocytes) and indeed dermal (fibroblasts) cells is an interesting design to explore. For this reason, the mechanism of epidermal cells' interactions with respect to wound healing and repigmentation (due to the presence of melanocytes) is pertinent to explore. In light of this, correct keratinocyte to melanocyte ratios are also of great importance in designing such pigmented 3D skin equivalents.

1.8. AIM

The overall aim of this study was to, through the use of primary isolated skin melanocytes and keratinocytes develop an in vitro co-culture system to obtain optimal pigment production in vitro and its applicability to an improved wound healing outcome.

1.9. Objectives

To address the above aim, the following objectives were formulated:

- To understand the growth dynamics (by means of proliferation assays) of both human skin melanocytes and keratinocytes in either a mono- or co-culture system
- To develop a reliable technique to separate the two cell types from a co-culture system in order to perform downstream intercellular analyses
- To develop a co-culture system at different ratios of epidermal cells and to analyse the effect such ratios have on pigment production
- To analyse the effect the co-cultured cells have on characteristics associated with wound healing such as cell proliferation and migration

2.1. Standard operating procedures and materials

2.1.1. Primary skin cells and cell lines used in this study

Primary human skin melanocytes and fibroblasts as well as immortalised human keratinocytes (HaCaTs; which will be referred to as keratinocytes) (125) were used in this study. Melanocytes and fibroblasts were harvested from infant foreskins using a modified method (126). See Figure 2.1, schematic overview of isolation and culturing protocol below. Please note that all cells and tissues obtained for this study was ethically approved with consent (REC REF 493/2011).

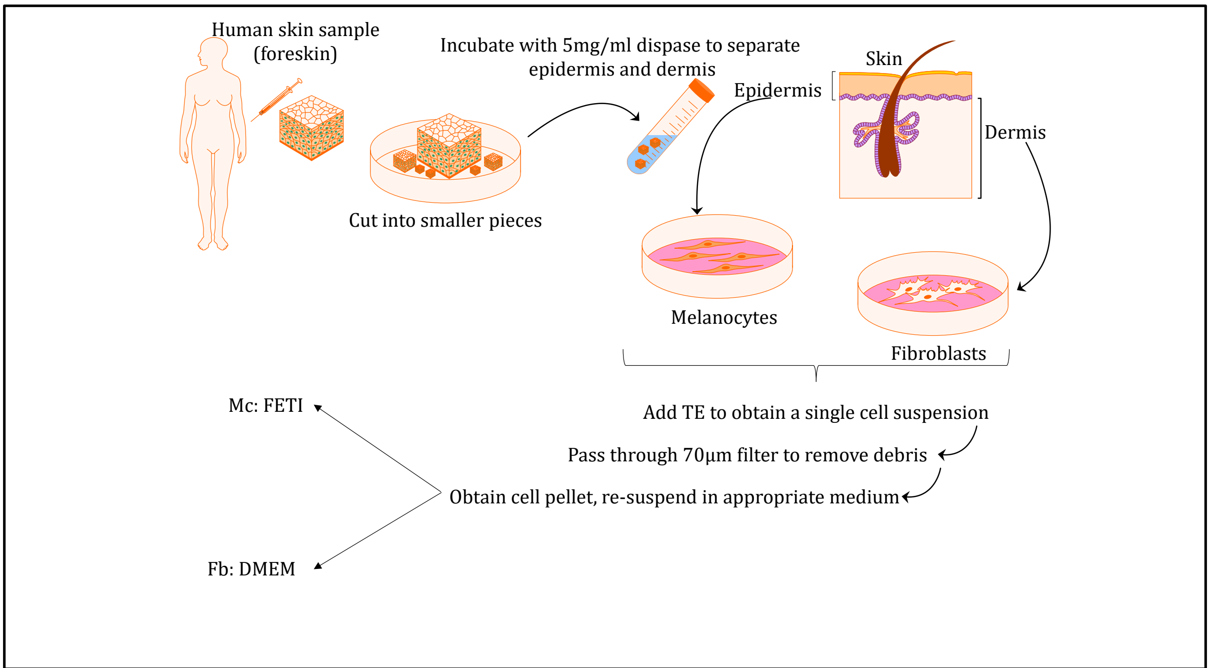


Figure 2.1: A schematic overview of the isolation and culturing protocol of human foreskin primary keratinocytes, melanocytes and fibroblasts.

2.1.1.1. Isolation of human foreskin melanocytes

Briefly, neonatal foreskins were transported in 10ml Dulbecco's Modified Eagle's Medium (Highveld Biological, SA; Appendix A) supplemented with 100U/ml Penicillin/ 100µg/ml Streptomycin (Sigma, SA; Appendix C) upon collection from the hospital. Aseptically, excess adipose tissue was removed from the foreskins and they were cut into thin, long strips (2mm x 5mm) and incubated in a dispase solution (5 mg/ml in HBSS, Appendix C) at 4°C overnight to allow for dissociation of the epidermis from the dermis. The following day, the thin pieces were placed in PBS (Appendix B) supplemented with antibiotic and the epidermis was gently separated from dermis with two fine forceps (KIMIX, RSA). Using a rounded scalpel blade (KIMIX, RSA), the epidermis (for melanocytes) was then chopped into further small pieces and transferred into 5ml of 0.1% trypsin with 0.02% EDTA (TE, Appendix C) to obtain a single cell suspension. The suspension was incubated at 37°C in a water bath (Memmert, Germany) for 15mins with gentle agitation every 5mins (the trypsin became cloudy as single cells were released from tissue). The tissue suspension was then triturated and transferred into a 15ml tube (Greiner Bio-One, Germany). Trypsin was inactivated with 1/10 volume of FCS (Appendix C), with further trituration. To remove debris, the cell suspension was filtered through a 70µm filter (BD Biosciences, USA), followed by centrifugation at 1000g for 10 minutes (MSE LTD, London). The pellet was re-suspended with melanocyte specific medium; FETI (Appendix A) and the cells seeded into 6cm culture dishes (Greiner Bio-One, Germany) and incubated (5% CO_2/95% O_2, Sanyo, Japan) at 37°C for two days to allow for cell attachment. No melanocyte cultures used in this project exceeded 11 passages.

2.1.1.2. Isolation of human foreskin fibroblasts

Using the same preparation procedure (section 2.1.1.1), primary human foreskin fibroblasts were harvested. Following collection and dispase treatment overnight and cutting of tissue into small pieces, 2ml of complete medium was added, while holding the cover slip down with a 1ml pipette tip (Greiner Bio-One, Germany). The small pieces of tissue under cover slips were incubated at 37°C. Fibroblast outgrowths were cultured until confluence with a change of medium every second day. Cells were expanded at confluence and frozen until needed. No fibroblasts used in this study exceeded 11 passages.

2.1.2. Tissue culture conditions

Melanocytes were cultured in specialised medium (FETI; Appendix A), while keratinocytes were cultured in DMEM supplemented with 10% v/v heat inactivated fetal bovine serum (Highveld Biological, SA; Appendix C) and 100U/ml Penicillin/ 100µg/ml Streptomycin. The co-culture medium (GREENS; Appendix A) was used when the melanocytes and keratinocytes were cultured together for different assays. All cells were cultured in a tissue culture incubator at 37°C with humidified 5% CO_2/95% O_2. Medium was changed every 3rd day and cells were passaged by means of trypsinization with trypsin/EDTA at confluence. These cells were routinely checked (every month) for mycoplasma using the nuclear stain, Hoechst 33342 (Appendix C).

2.1.3. Growth dynamics

One of the simple methods of inferring in vitro growth dynamics of cells is by means of growth curves. This method allows for counting of cells over a specified time period. In this study, monoculture growth curves were completed. Briefly, the cells ($2x10^4$) were seeded in 35mm dishes (Nest Biotechnology, China) and cultured for a period of 7 days. At days 1, 2, 3, 5 and 7, the cells were trypsinised off the dishes by standard methods and counted using a 0.002mm^2 Neubauer counting chamber (Superior Marienfeld, Germany). All experiments were conducted for a minimum of 3 times (n=3) containing duplicates within each experiment. The growth curve results were plotted as mean ± SEM values.

2.1.4. Co-culture models and culture conditions

Keratinocytes and melanocytes were co-cultured in GREENS medium as indicated in Section 2.1.2. **Table** E1-E4 (Appendix E) indicates the numbers of cells used for various co-culture experiments.

2.1.5. Scratch assays

A scratch assay is a simple in vitro wound healing simulation model used to observe and measure migration and/or proliferation of a monolayer of cells into a denuded area (127). In this study, in vitro scratch assays were performed through combining keratinocytes and melanocytes at various ratios, as indicated in Table E1 (Appendix E). Cells were pre-mixed in 1.5ml microfuge tubes and seeded onto 35mm glass coverslips (Superior Marienfeld, Germany) in 35mm tissue culture dishes and cultured for 24 h to 70-80% confluence prior to wounding.

A scratch "wound" was made with a 1ml micropipette tip, using a ruler as a guide to obtain consistency across the cover slip (Figure 2.2). Debris and floating cells were removed by washing with medium, followed by addition of 2ml fresh medium. Three images (top, centre and bottom of scratch, as indicated in boxes, Figure 2.2) per tissue culture dish were taken immediately and were used as control images (denoted as time zero). Thereafter, images were taken at specified time periods (denoted as times “n”).

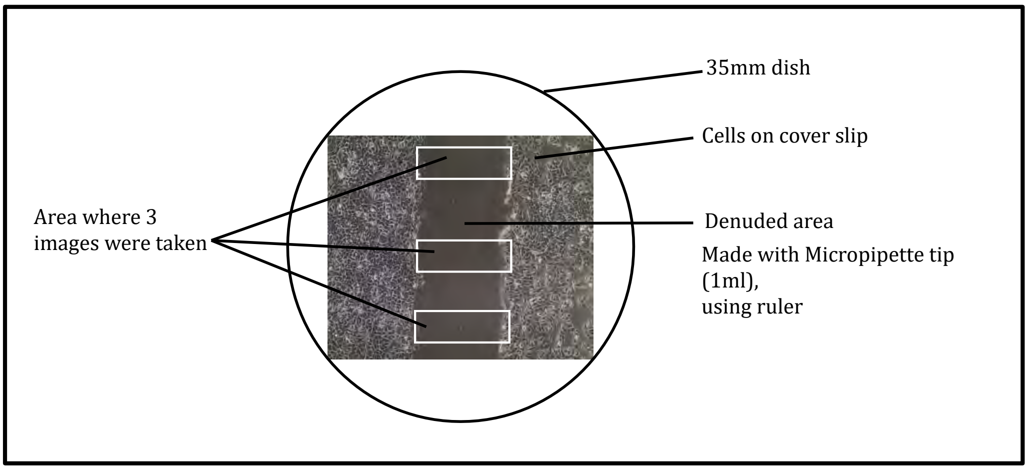


Figure 2.2: A schematic depicting a scratch assay made by the use of a 1ml micropipette tip, aided by a ruler.

The scratched co-cultures were cultured for a period ranging from 48 hours to 8 days at 37^{o}C. Camera images (EVOS, life Technologies, SA) were captured at various time points during wound healing (three images at each time-point). Wound closure was calculated using Image J software (version 1.43u; National Institutes of Health, USA). Briefly the denuded area on the scratch was measured for each of the three images at each time point (Figure 2.3). Percentage wound closure was subsequently calculated as per the equation below (where “t” is time, “n” is time post wounding):

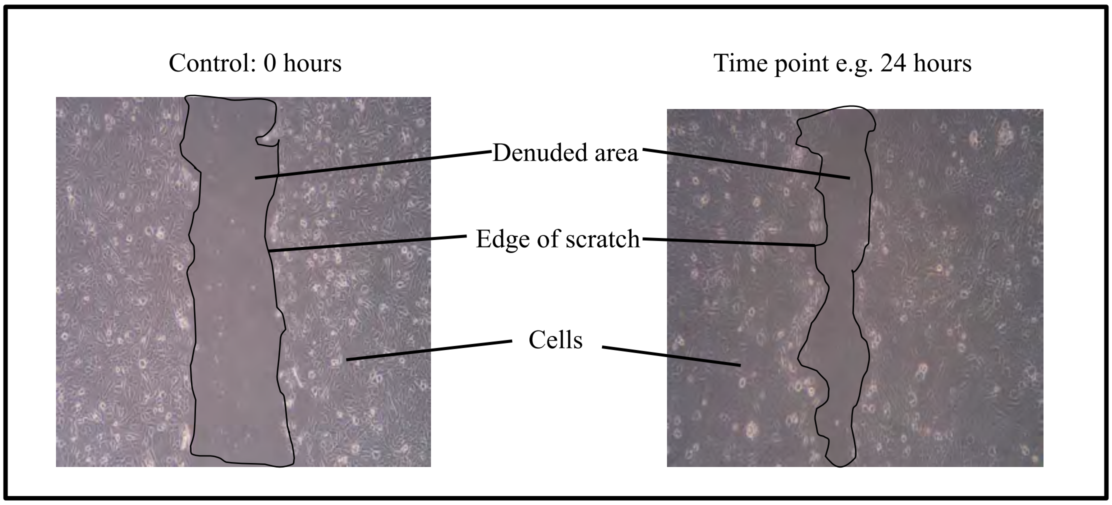


Figure 2.3: Image showing how a scratch is measured to depict percentage wound closure at various time points.

$$\% \text{ wound closure} = \frac{[\text{wound area } (t_0) - \text{wound area } (t_n)]}{\text{Wound area } (t_0)} \times 100$$

2.1.6. Fluorescent activated cell sorting (FACS)

Fluorescent activated cell sorting (FACS) is a technique used to sort cells according to size and granularity. Cells can be tagged with fluorescent dyes and/or antibodies and then sorted. In this study we aimed at separating keratinocytes and melanocytes (in a ratio of 5:1, 10:1 and 20:1) from a co-culture in order to perform further analyses. Briefly, cells were cultured as either mono or co-cultures in specific medium for 24 hours. Cells were then trypsinised and centrifuged for 5 minutes at 2 500 rpm. The resultant pellet was washed twice in PBS, with centrifugation for 1 minute at 2 500 rpm between each wash. The cells were re-suspended and fixed in 2ml of cold 4% paraformaldehyde (Appendix C) for 15 minutes at ambient temperature and washed twice in PBS. Cells were then permeabilized for 15 minutes using a perm/wash buffer (BD Biosciences, USA) following manufacturer's instructions. Cells were re-suspended in FACS buffer (R & D Systems, USA) and transferred to FACS tubes (BD Falcon, USA). Finally a specific fluorescent antibody (Section 2.2) was added to the cell suspension and tubes were covered with foil to prevent light exposure. The cells were incubated for 1 hour at ambient temperature on a shaker (The Belly Dancer, Stoval Life Sciences. USA) and washed with "perm/wash" buffer. The cells were then re-suspended in FACS buffer and immediately acquired (10 000 events or total cell population) on a flow cytometer (FACS Aria; BD Biosciences, USA).

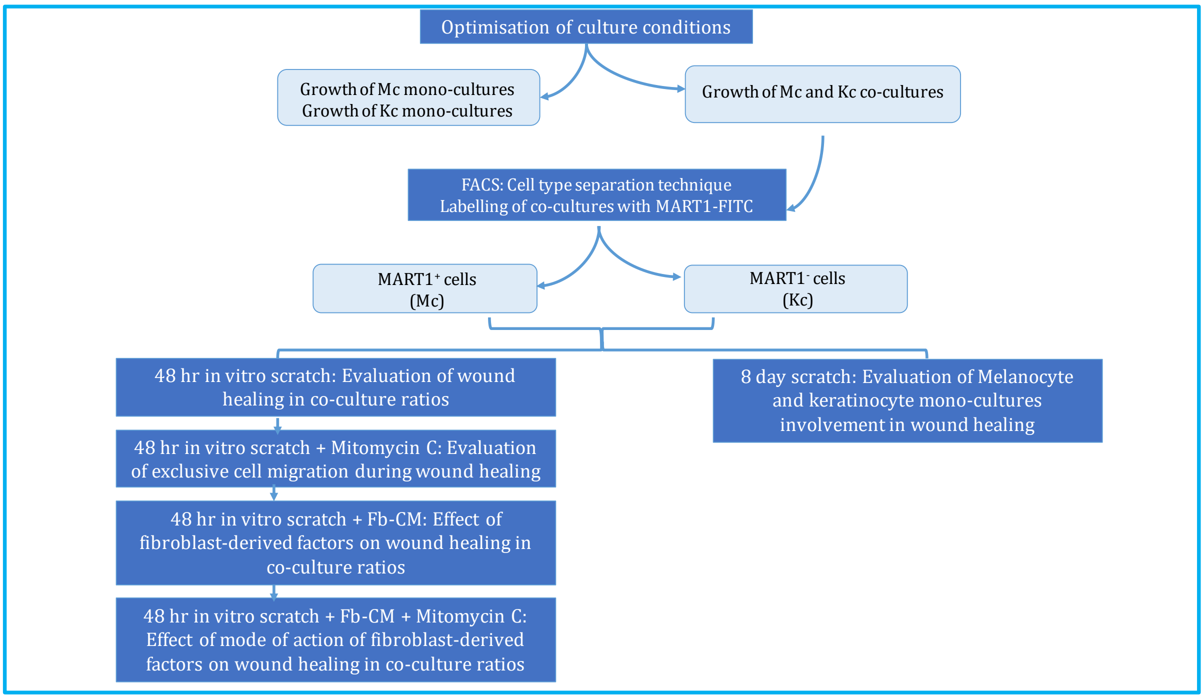
2.1.7. Harvesting of fibroblast conditioned medium

Primary human foreskin derived fibroblasts at passages 5-7 were used in this study to harvest fibroblast conditioned medium, which was subsequently used to infer the effect of fibroblast-derived factors on our in vitro co-culture models. Firstly these primary cells were isolated and cultured as mentioned in Section 2.1.1. Fibroblasts were seeded into 10cm dishes in complete DMEM to reach 70-80% confluence and cultured for a further 24hrs to enrich the medium. The resultant medium was collected into 15ml tubes, centrifuged at 2 000rpm for 2 minutes to remove all debris and medium was transferred into new tubes and further filtered through a 0.25μm filter (Sartorius Stedim

Biotechnology, Germany) to remove any finer debris. The resultant conditioned medium was then stored at 4^{o}C or -20^{o}C for short and long term storage, respectively.

2.2. Experimental setup

To address the aims and objectives of this study, the following specific experimental procedures were undertaken. Figure 2.4 below depicts the schematic workflow.



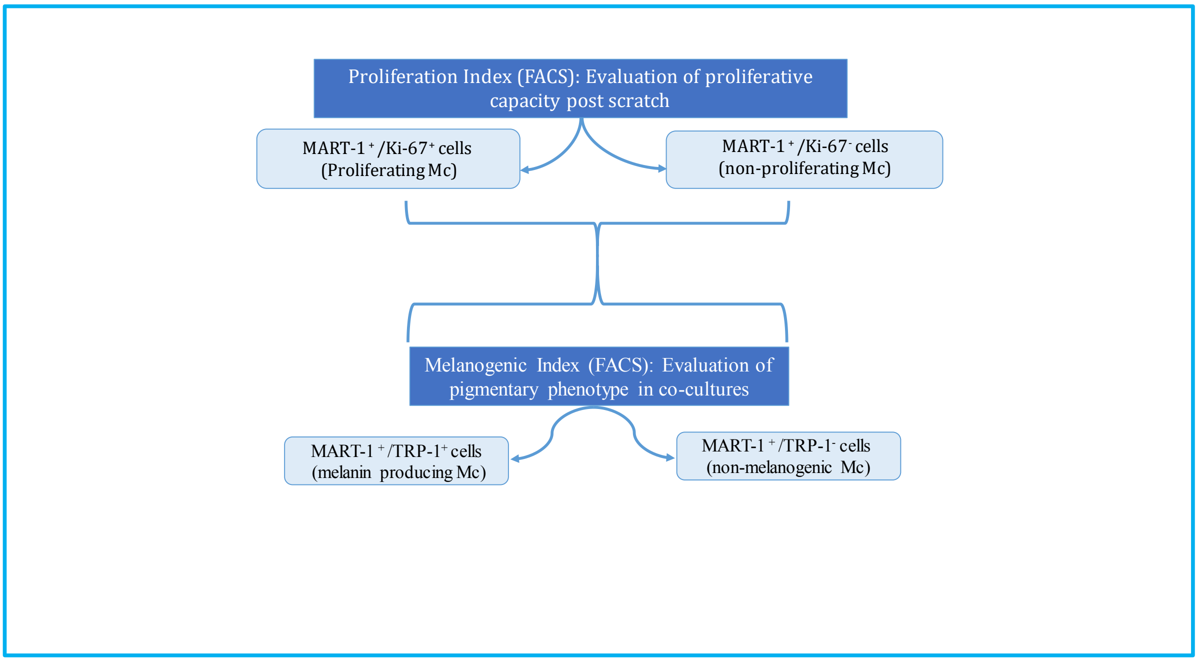


Figure 2.4: Schematic/overview of experimental setup followed in this study.

2.2.1. Co-culture growth dynamics

Three ratios of co-cultures of keratinocytes to melanocytes were used. These included 5:1, 10:1 and 20:1 keratinocytes to melanocytes, respectively.

In this study we used FACS to separate melanocytes and keratinocytes from a co-culture. This dynamic approach allowed us to count melanocytes (as a Mart-1-FITC positive population) and keratinocytes (as a FITC negative population). Therefore, co-culture growth dynamics were evaluated by means of growth curves, wherein FACS was used to separate the cells. Briefly, cells were seeded (as indicated in Table 2, Appendix E) and cultured for 7 days. At days 1, 3, 5 and 7, cells were trypsinised, and then washed with PBS. Thereafter, they were fixed and permeabilised (Section 2.1.6). The cell suspension was then labelled with 1µg of melanoma antigen recognised by T-cells (MART-1) conjugated to FITC (Santa Cruz Biotechnology, SA), followed by acquisition by means of FACS (wherein a total number of both melanocytes and keratinocytes in co-culture was established). The Mart-1^{+} cells were melanocytes, while Mart-1^{-} cells were acquired as a negative population.

2.2.2. Evaluation of melanogenesis in co-cultures

2.2.2.1. TRP-1 expression analyses (melanogenesis)

Using the FACS approach, we evaluated the melanogenic capacity of melanocytes in various co-cultures. We used TRP-1, which is a melanocyte-specific enzyme involved in melanin synthesis. It catalyses the last step in the melanogenic pathway, resulting in formation of eumelanin.

For this, cells were seeded (as indicated in Table 3, Appendix E) and cultured for 3 days. At days 0 and 3, cells were trypsinised, fixed and permeabilized as before (Section 2.1.6). The cell suspension was then double-labelled with 1µg of MART-1 (FITC) (Santa Cruz Biotechnology, SA) and TRP-1 (Alexa 647) (Santa Cruz Biotechnology, SA). Cells were acquired by means of FACS (wherein a total number of MART-1^{+}/TRP-1^{+} melanocytes in co-culture was established as a melanogenic population).

2.2.3. Evaluation of in vitro wound healing using various co-culture ratios (48hr scratch)

In order to determine the ratio of cells in a co-culture, which yields an optimal wound healing phenotype, both keratinocytes and primary foreskin melanocytes were seeded at various ratios (see Table E1, Appendix E) on cover slips (section 2.1.5), for 24 hours. Co-cultures were then scratched and images were taken at 0, 12, 16, 20, 24 and 36 hours post-scratch (Figure 2.5). Image J software was used to calculate the denuded area over time and the percentage wound closure was calculated.

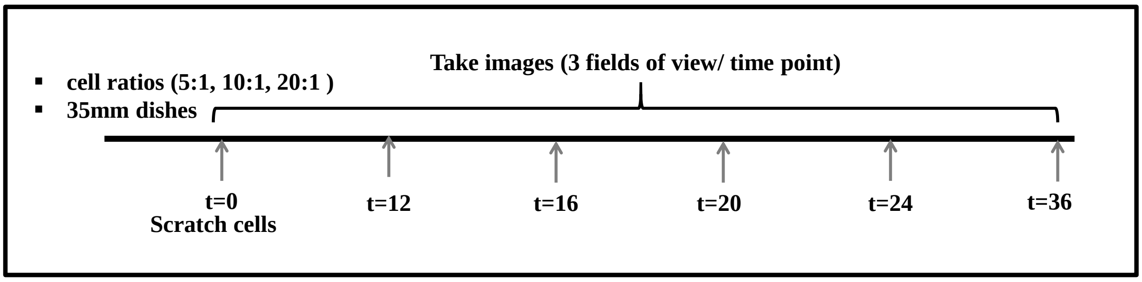


Figure 2.5: The in vitro wound healing protocol of various co-culture models (5:1, 10:1 and 20:1), over a 36 hour period post scratch.

2.2.3.1. Evaluation of cell migration during wound healing (Mitomycin C treatment)

Cells have been shown to close the wound via two exclusively mutual events; migration and proliferation. For this study, it was of great interest to infer how cells in a co-culture close the in vitro wounds. As previously mentioned, various ratios (5:1, 10:1 and 20:1) of co-culture models were seeded for 24 hours onto coverslips in 35mm dishes, followed by treatment with 0.01 µg/µl of mitomycin C (a mitogen inhibitor) for 2.5 hours. The medium with treatment was then removed and cells were washed twice with complete medium, followed by addition of fresh medium. The cells were then scratched and images were taken at 0, 12, 24, 36 and 48 hours post-wounding (Figure 2.6). Percentage wound closure was calculated as before.

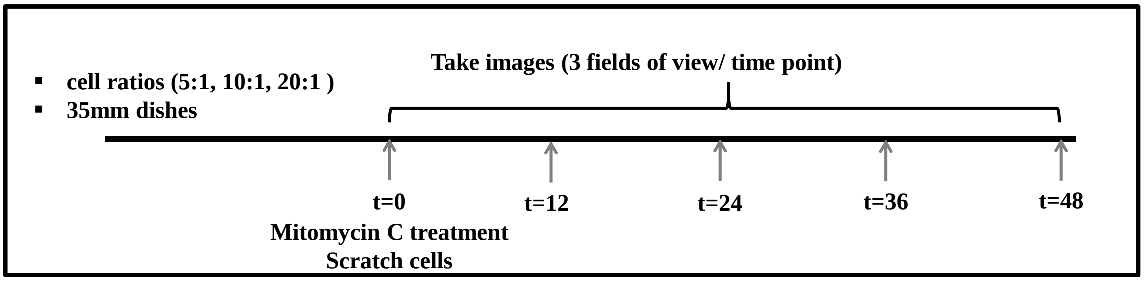


Figure 2.6: The in vitro wound healing protocol of various co-culture models (5:1, 10:1 and 20:1) over a 48 hour period, following addition of mitomycin C.

2.2.3.2. Determination of Proliferation index in various co-culture ratios, during wound healing

FACS was utilised to measure the proliferation index (PI) of co-culture ratios (5:1, 10:1 and 20:1). Briefly, cells were cultured for 24 hours in co-culture. The cells were scratched as before and at the following time points (24 and 48 hours) post-scratch, cells in suspension were fixed, permeabilised (as outlined in section 2.1.6) and dually labelled with 1µg and 2µg of MART-1 (FITC) and Ki-67 (PE) (Santa Cruz Biotechnology, USA), respectively. At each time point, the index (PI) was calculated using the following formula:

$$PI = \frac{\text{Number of Ki-67}^{+}\text{ cells}}{\text{Total number of cells}} \times 100$$

Equation depicting calculation of proliferative index (PI)

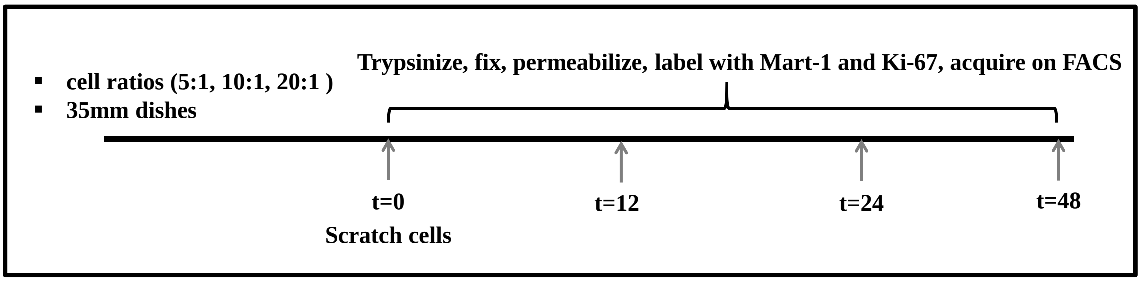


Figure 2.7: The schematic, representing the proliferative index (PI) protocol.

2.2.4. Effect of human fibroblast growth factors on wound healing (Fb-conditioned medium)

In order to evaluate the effect of fibroblast-derived factors on in vitro wound healing, we harvested fibroblast-conditioned medium, which was then added to our co-cultures. Briefly, the various cell ratios were seeded for 24 hours (section 2.1.5). Human fibroblast conditioned medium (harvested as outlined in section 2.1.7) was added to the cells in a 1:1 dilution (growth medium: conditioned medium). The cells cultured in a 1:1 ratio of DMEM (fibroblast growth medium) to respective growth medium were used as the control. The scratches were introduced onto these cell mono-layers and images were taken. Images were taken at times 0, 12, 24, 36 and 48 hours post-wounding (Figure 2.8).

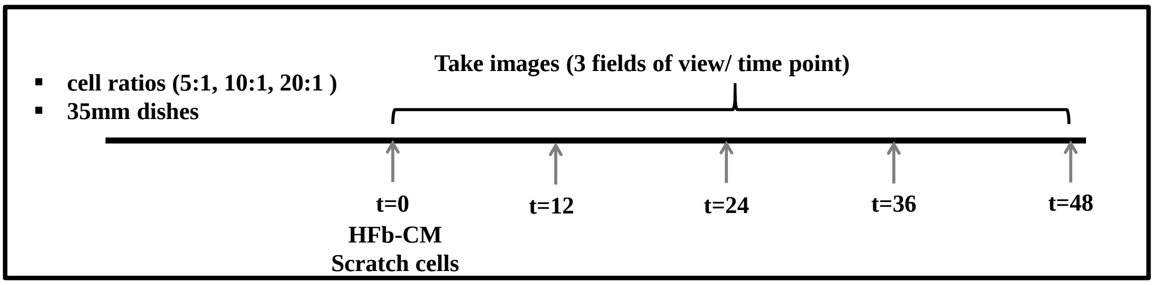


Figure 2.8: Protocol followed in determining the effect of human fibroblast growth factors on wound healing.

2.2.4.1. Evaluation of mode of action of fibroblast growth factors in wound healing (Fb-conditioned medium and Mitomycin C treatment)

In order to investigate how the fibroblast conditioned medium influences in vitro wound healing, ratios (5:1, 10:1 and 20:1) of co-culture models were seeded for 24 hours onto cover slips in 35mm dishes, followed by treatment with 0.01 µg/µl of mitomycin C (a mitogen inhibitor) for 2.5 hours (Section 2.2.4). Cells were scratched; images were taken at 0, 12, 24, 36 and 48 hours post-wounding (Figure 2.9) and percentage wound closure was calculated.

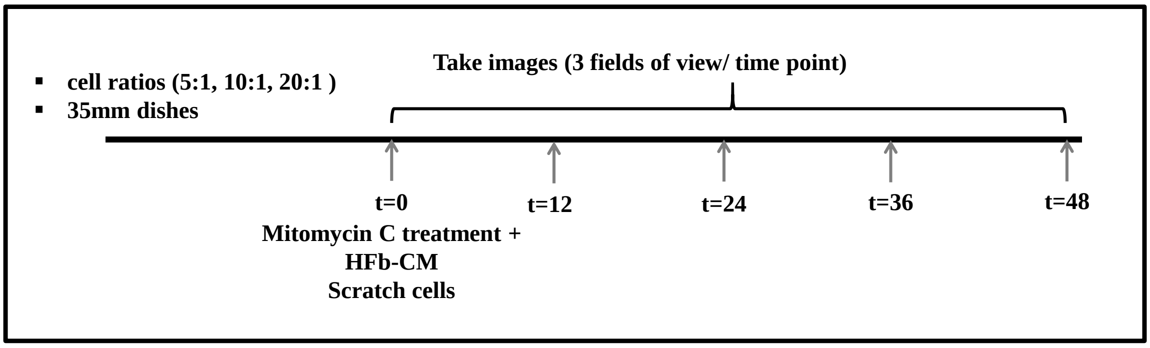


Figure 2.9: Evaluation of mode of action of fibroblast growth factors in wound healing protocol.

2.3. Statistical analyses

All data were presented as mean ± SEM. One way analysis of variance (ANOVA) with Bonferroni *post hoc* test was used to determine statistical significance. A value of $P<0.05$ was considered significant. All the graphs and statistics were performed using GraphPad Prism (Version 5.01, USA).

Despite the promising success and clinical benefits of using cultured 3D skin substitutes in treating burn wounds, their main disadvantage is the fact that they are devoid of many of the structures and functions of uninjured skin. These include amongst many, hair follicles and melanocytes (124). Furthermore, to clinicians, the best clinical outcome for burn victims is indeed not only a rapidly healed wound, but also a satisfactory aesthetic outcome. The inclusion therefore of pigment producing cells (melanocytes) in 3D skin constructs is of great importance. In light of the melanocytes and keratinocytes forming a functional epidermal melanin unit, correct keratinocyte to melanocyte ratios are of great importance in designing pigmented 3D constructs.

This study was aimed at isolating skin melanocytes from human skin samples, followed by co-culturing of these cells together with keratinocyte cell line at different ratios in order to determine the optimal keratinocyte: melanocyte ratio which would ultimately yield the best pigment production and/or consequent improved wound healing outcome. Clinically, this data would be useful in the future design of pigmented 3D skin constructs.

One of the main objectives of this study was to use *in vitro* co-culture models to evaluate the cellular mechanisms of wound healing. To meet this objective, various ratios of human keratinocytes (HaCaTs) to melanocytes (5:1, 10:1 and 20:1) were used to infer how such cells migrate and/or proliferate into *in vitro* scratch assays. Since the culture system involved two different epidermal cell types, their growth dynamics had to be evaluated. We therefore cultured melanocytes and keratinocytes as either mono- or co-cultures and evaluated their growth rates over a 7 day period.

3.1. Mono-culture growth dynamics

Keratinocytes, in a mono-culture exhibited a much faster growth compared to melanocytes (Figure 3.1), with a population doubling time of 0.6 days. This corroborated early work by Boukamp et al., (1988) (125) where they reported a doubling time of 1 day. Both keratinocytes and melanocytes in culture exhibited a classical growth dynamic, characterised by a lag phase (~ 2 days in both cell types), followed by an exponential phase. During the latter phase, cells exhibited marked differences in growth dynamics, with keratinocytes proliferating much faster, from day 5 to 7. By the end of the growth period (day 7), there were approximately 9-fold more keratinocytes (9 x 10^5) than melanocytes (1 x 10^5), highlighting the differences in growth dynamics between these epidermal cells. The estimated doubling times were 1.63 and 3.39 days for keratinocytes and melanocytes, respectively. The doubling time for melanocytes corroborated work by De Luca et al., (1988) (128) who showed the primary melanocyte doubling rate of 4 -10 days.

Such difference in growth patterns of melanocytes and keratinocytes was expected as studies have observed that melanocytes are largely dependent on surrounding keratinocytes *in vitro* and *in vivo* for survival and maintenance (17,129,130). Therefore in mono-culture, melanocyte growth is largely hampered due to the absence of contributing paracrine growth factors and mitogens secreted by surrounding keratinocytes *in vivo* or in co-culture.

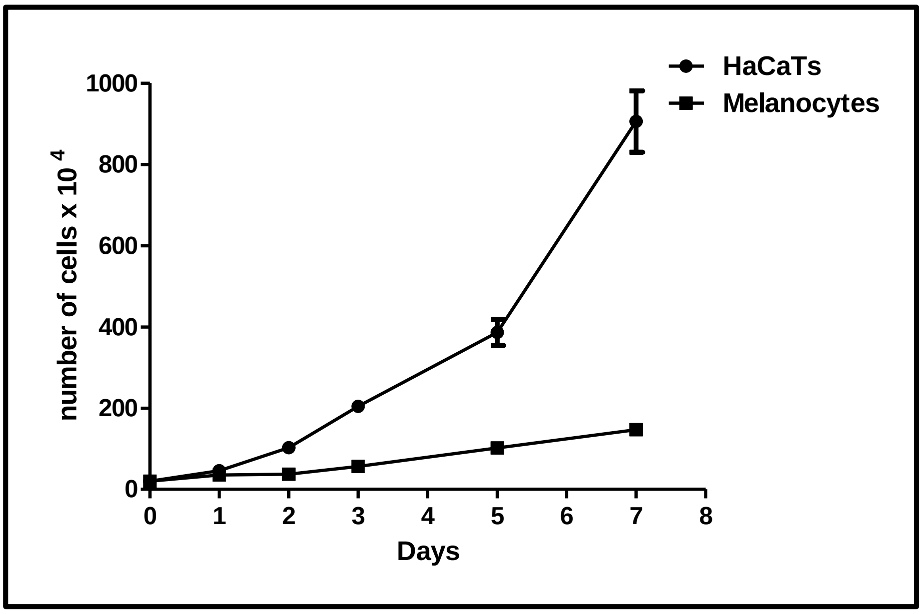


Figure 3.1: The growth curves of immortalised human keratinocytes and primary human melanocytes cultured in DMEM and FETI respectively, for a maximum of 7 days. At days 1, 2, 3, 5 and 7, cells were lifted and counted. Cell numbers are presented as mean ± SEM values. n=3

3.2. Co-culture growth conditions

Having confirmed the growth differences of keratinocytes and melanocytes in a mono-culture, it was pertinent to explore how such differences may be altered in a co-culture model. To do this, keratinocytes and melanocytes were co-cultured at ratios of 5:1, 10:1

and 20:1 respectively, over a 7 day period. We observed proliferation over time using cell separation by FACS and counting the number of events on each day (Figure 3.2). This is a unique model that allows for separation of melanocytes from keratinocytes based on the positive identification of melanocytes (MART-1$^+$) following labelling of co-cultured cells with MART-1 (melanocytes specific antibody). Keratinocytes in turn are sorted as a MART-1 negative (MART-1$^-$) population by FACS (detailed procedure and optimisation data are shown in Appendix F and G).

In a co-culture model, these cells displayed a different growth dynamic, which was largely dependent on the co-culture ratio.

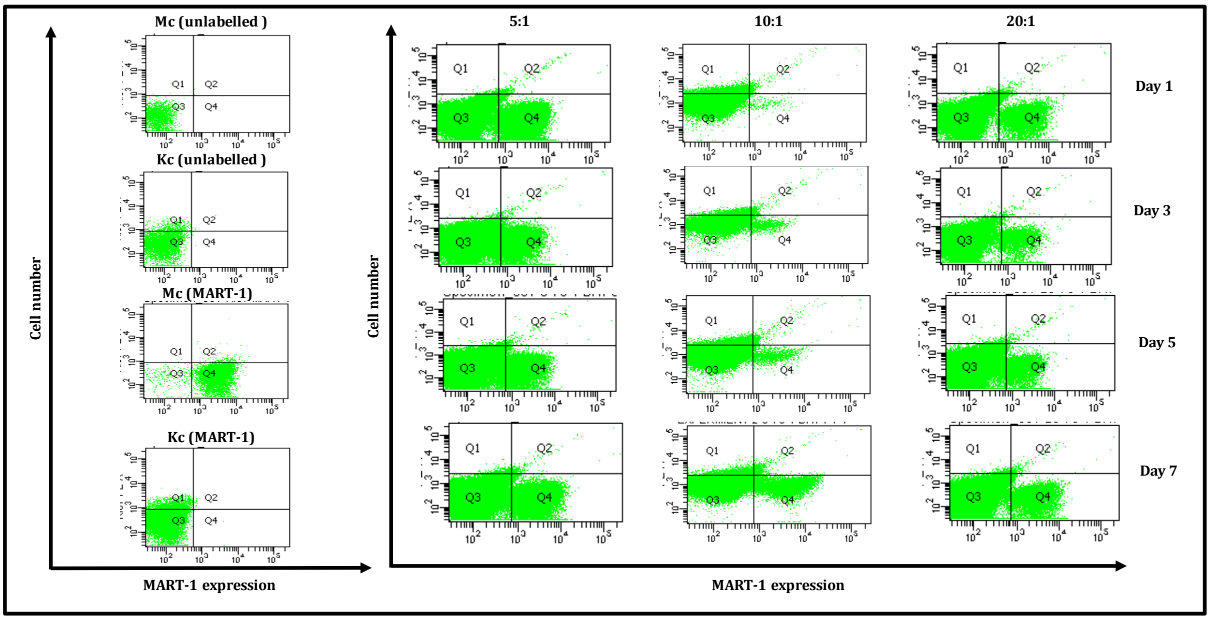


Figure 3.2: Proliferation of keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1). Cells were cultured for a maximum of 7 days in Greens. At days 1, 3, 5 and 7, cells were lifted, fixed, permeabilised, stained with the melanocyte specific antibody (MART1-1) and acquired by FACS.

Though keratinocyte numbers increased with time, melanocytes numbers stayed relatively the same (figure 3.3), implying that despite the proliferative capacity of keratinocytes (which could bring about competitive growth advantage); melanocytes were able to survive in a co-culture for 7 days. This is also in accordance with the study by Abercrombie et al., (1964) (45) who also showed that despite the paracrine signalling exerted by keratinocytes, melanocyte numbers are not affected in an *in vitro* co-culture model.

In culture, Swope et al., (2002) (131) reported that melanocytes lacked adequate expression of adhesion molecules and exhibited low proliferative capacities, thus rendering them unable to sustain themselves in prolonged cultures. Furthermore, keratinocytes at high densities are capable of hindering melanocyte growth in culture (132). Despite these known limitations, we observed melanocyte survival over a 7 day period, amidst the high proliferative activity of keratinocytes in our co-culture models.

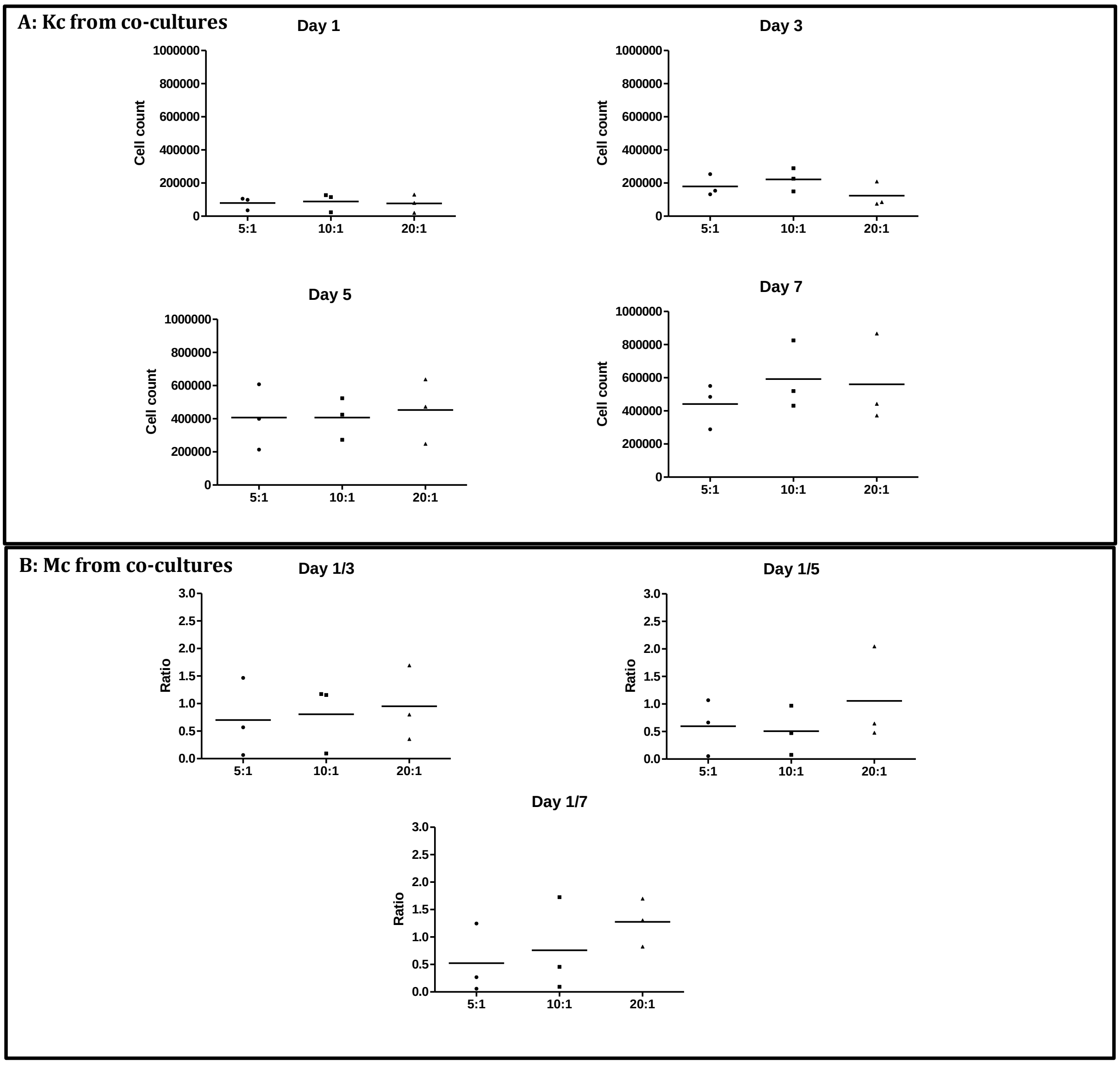


Figure 3.3: The growth curves of keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1). Cells were cultured for a maximum of 7 days in Greens. At days 1, 3, 5 and 7, cells were lifted, fixed, permeabilised and acquired by FACS. **A**: Kc represented as MART1$^-$ cells as cell counts, while **B**: Mc were sorted as MART1$^+$ cells and presented as a ratio of day 1. Cell numbers are represented as mean values. n=3

Though there were no significant differences in growth dynamics between all ratios of both keratinocytes and melanocytes in co-culture (Figure 3.3A and B), there seemed to be marked differences in their population doubling times (PD), wherein we observed a vast decline in PD of keratinocytes from a mono-culture into a co-culture (0.6 days in mono-culture to about 1.63 days in co-culture). This PD was largely influenced by the co-culture ratio, where we saw that keratinocytes in a 20: 1 ratio to melanocytes exhibited the highest proliferative capacity (PD of 1.29 days compared to 1.76 and 1.37 days in 5:1 and 10:1 ratios, respectively) (Table 3.1). Moreover, the population doubling for keratinocytes was highest in a 5:1 ratio, implying that in this ratio, keratinocyte proliferation is slowed.

Table 3.1: The population doubling times of keratinocyte to melanocytes co-cultures

Ratios	**Population doubling times (days) of cells in a co-culture**	
	Keratinocytes	Melanocytes
5:1	**1.76**	**1.71**
10:1	1.37	2.04
20:1	1.29	2.14

Similar to the keratinocytes, melanocytes exhibited marked ratio-dependant differences in growth dynamics. The melanocytes showed the highest proliferative capacity in a 5:1 ratio, with a PD of 1.76 days, compared to 2.04 and 2.14 days in 10:1 and 20:1 ratios, respectively. Furthermore, the melanocytes in a co-culture had a far greater proliferation capacity compared to mono-cultures. However, compared to keratinocytes, melanocytes still exhibited a slightly slower growth (average PD of more than 2 days for melanocytes and PD of less than 2 days for keratinocytes). The slower melanocyte proliferation is in accordance with studies that have implicated the tight control of melanocyte growth and differentiation in co-culture with keratinocytes (19-21). For instance, it has been shown that TGF-β signaling cascade maintains melanocytes in an undifferentiated, non-proliferative state. This is achieved by the phosphorylation of Smad 2/3 by TGF-β, which leads to down regulation of MITF, the master transcriptional regulator of melanocyte differentiation, and its downstream melanogenic genes (20).

Interestingly we observed an inverse correlation of PD within both keratinocytes and melanocytes in co-culture. Where keratinocytes' proliferation was greatly slowed in a 5:1 ratio, melanocytes exhibited the highest proliferation rates. This further emphasises the reciprocal influence of both cell types on each other in co-culture.

The results thus suggest that the 5:1 (keratinocyte: melanocyte) ratio may be the best co-culture ratio as we observed the greatest proliferation of melanocytes (lowest PD of 1.71 days) and most importantly, this ratio seems to be optimal for synchronisation of population doubling times (or growth rates of both keratinocytes and melanocytes) (column in bold; Table 3.1).

The synchronisation of population doubling times of both melanocytes and keratinocytes is a vital phenomenon as it implies that for clinical purposes, we would be able to co-culture these cells in vitro without any delays in the growth of any other cell type. Therefore this addresses the known fact that melanocytes grow much slower than keratinocytes in mono-culture.

3.3. Determination of melanocyte and keratinocyte involvement in an *in vitro* wound healing model

Having confirmed the growth dynamics of the epidermal cells in either a mono- or co-culture system, we set out to determine the co-culture ratio of melanocytes to keratinocytes that would yield the best quantifiable wound healing outcome. To do this, an *in vitro* migratory (scratch) assay was employed. This is a simple technique that determines the rate of wound closure following an induced scratch on a mono-layer of cells (127,133).

3.3.1. Involvement of melanocytes and keratinocyte mono-cultures

Firstly, we evaluated the wound healing capacities of each cell type in a mono-culture. The cells were seeded as outlined in materials and methods (Section 2.1.5). A consistent scratch (using a 1ml pipette tip cut at the edge, to create a larger scratch) was made on the mono-layer of cells (primary keratinocytes or melanocytes) and the cells were monitored daily over a 7-day period.

As shown in Figure 3.4, significant differences in percentage wound closure at days 3, 4 and 5 post wounding (3 and 4 days: $p < 0.001$, while 5 days: $p < 0.05$) were observed between keratinocytes and melanocytes. Keratinocytes exhibited a 96.8% wound closure compared by day 3. Melanocytes in contrast, exhibited a 64.5% wound closure. By day 4 post wounding, keratinocytes had fully (100% wound closure) closed the scratch, while melanocytes showed a 73.6% wound closure.

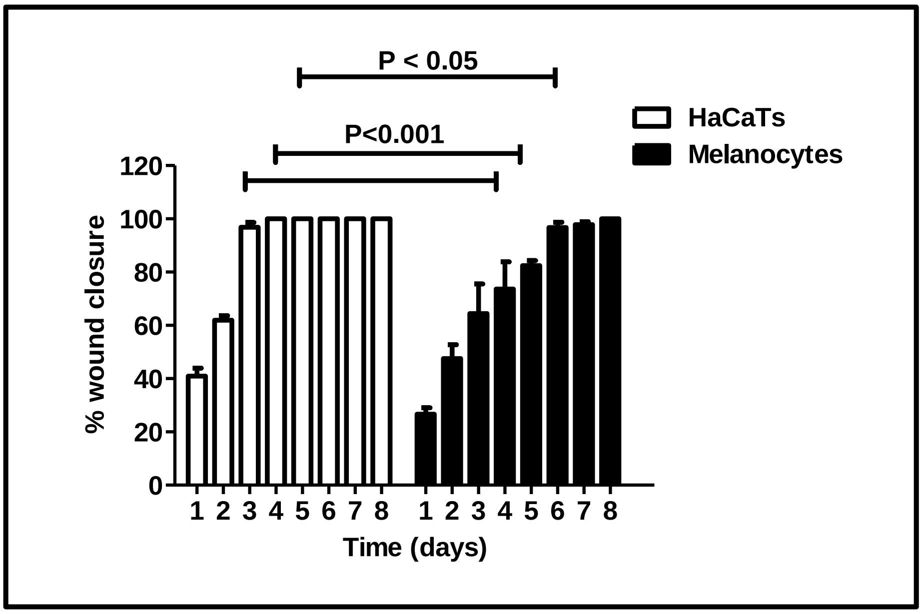


Figure 3.4: Evaluation of wound closure in melanocyte or keratinocyte mono-cultures. A scratch was made 24 hours post co-culturing and wound closure was monitored for 8 days. Three images per field of view were randomly taken daily and Image J software was then used to calculate percentage (%) wound closure. The % wound closure is represented as mean ± SEM, n=3.

Such significant differences in wound closure by keratinocyte and melanocyte individual monocultures may be attributed to the fact that melanocytes have been shown to have a low proliferative activity when in mono-culture (131). Moreover, their apparent lack of accelerated growth in the absence of surrounding keratinocytes re-emphasises their dependence on paracrine factors secreted by surrounding keratinocytes for optimal growth.

3.4. Evaluation of wound closure capacities by co-cultures

Studies have shown that both keratinocytes and melanocytes secrete paracrine and autocrine factors (such as bFGF, ET's, HGF and α-MSH) that are pivotal to their survival and maintenance in vivo (17,128,134,135). Such factors have also been implicated in their response to cellular stresses such as UV, where melanocytes are stimulated by keratinocytes to synthesise melanin (128,136,137).

To our knowledge, no studies have implicated these paracrine signals in wound healing. Therefore in our study, we were interested in determining what effect, if any, these cells have on the overall wound healing progression and how they may interact to promote this effect at the level of their proliferative potential and migratory phenotypes. To understand this, keratinocytes and melanocytes were co-cultured at various ratios (5:1, 10:1 and 20:1) and accompanying cellular processes (proliferation and migration) were analysed via the scratch assay (Figure 3.5).

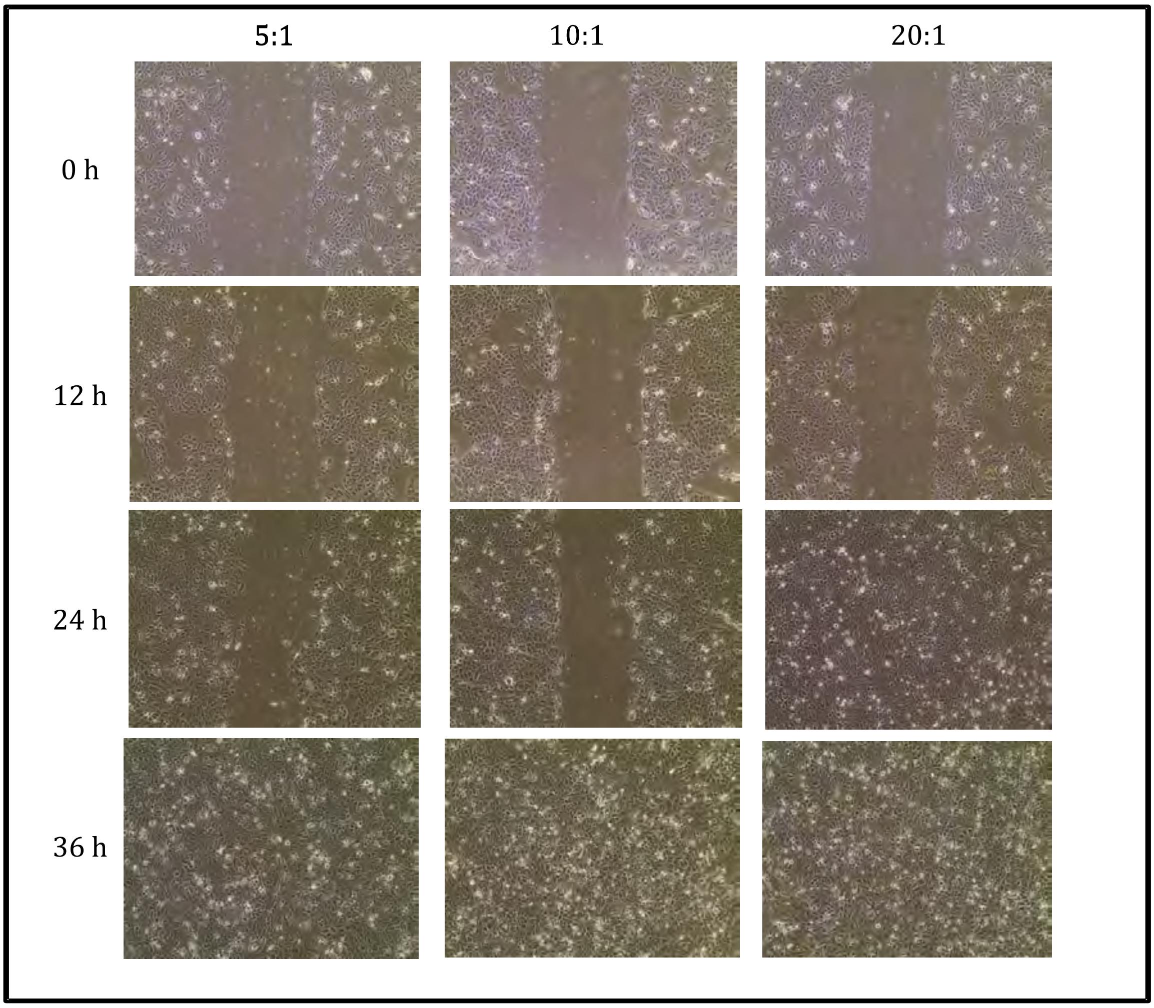


Figure 3.5: Scratch assay images showing the wound closure of the keratinocyte to melanocyte ratios (5:1, 10:1 and 20:1), 0, 12, 24 and 36 hours post wounding. Magnification, 100 X.

During the first 12 hours post wounding, co-cultures exhibited no significant differences in percentage wound closure (Figure 3.6). However, significant differences were evident at the 20 hour time point with the 20:1 ratio yielding a significantly (97%, p<0.001) higher percentage wound closure compared to 5:1 (58%) and 10:1 (60%) ratios. By 24 hours, this had progressed to even more disparate wound closure differences with the 20:1 displaying a 100% closure as opposed to 80% and 60% for the 10:1 and 5:1, respectively. Thirty six hours after wounding, 100% wound closure was evident for all the ratios.

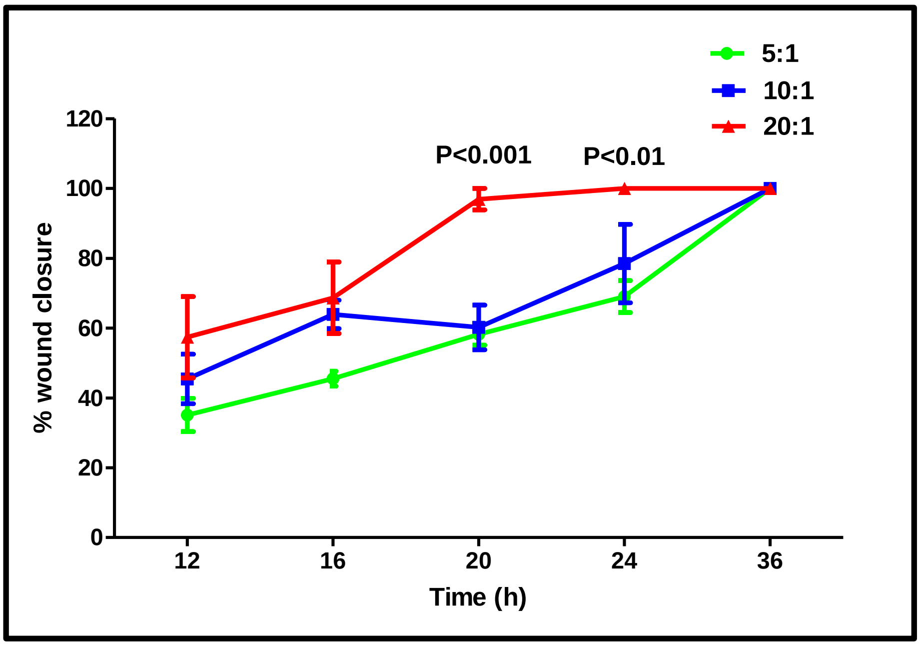


Figure 3.6: Determination of wound closure in various ratios of keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1). A scratch was made 24 hours post co-culturing. At 0, 12, 16, 20, 24, and 36 hours post scratching, three images per field of view were randomly taken using a camera and Image J software was then used to calculate % wound closure. The %wound closure is represented as mean ± SEM, n=3

These differences in wound closure may be attributed to several factors. Firstly, keratinocyte proliferation has been shown to be negatively impacted by the physical presence of melanocytes (138). In their study, Deveci et al., (2001) (138) reported that cell-cell contact of melanocytes and keratinocytes exerted a negative proliferative effect. In contrast, addition of melanocyte-conditioned medium to keratinocyte cultures enhanced proliferation. It therefore seems plausible that in the higher keratinocyte to melanocyte ratios (10:1 and 20:1), less melanocytes exist to exert this negative impact and thus faster closure ensues (Figure 3.6).

Based on these results the 20:1 ratio of keratinocytes to melanocytes may potentially yield a better wound healing outcome (Figure 3.6). In addition, a significant difference in wound healing occurs at around the 20 hour time point (Figure 3.6). Moreover, this corroborates our previous finding (Table 3.1), where the keratinocyte numbers increased in the 20:1 ratio. However, it does not support the previous observation where the 5:1 ratio enhanced the population doubling times of melanocytes in co-culture and clearly a different mechanism may be at play in that instance.

3.5. Evaluation of migratory and proliferative phenotypes of co-cultures during *in vitro* wound healing

One of the essential phases of wound healing is re-epithelialization, which is orchestrated by epidermal keratinocytes. This phase, upon wounding, involves the keratinocytes (those with close proximity to the wound edge) to be activated to a migratory cellular phenotype, followed by a proliferative burst that occurs just behind the migrating tongue (46,47) Studies have implicated mutual exclusiveness of these cellular processes (139,140), and thus it was pertinent to explore how and which of these processes are mostly attributable to the phenotypic differences in wound closure capacities of keratinocytes and melanocytes in a co-culture. The co-cultured cells at different ratios were subjected to mitomycin treatment (to inhibit proliferation) and wound closure over a 48 hour period was evaluated as a consequence of exclusive cellular migration.

3.5.1. Evaluation of migration capacity (inhibition of proliferation by mitomycin treatment) of *in vitro* co-cultures

To determine whether wound closure was due to migration of cells, we exposed the keratinocytes and melanocytes co-cultures to a mitogen inhibitor (mitomycin C) before inducing a scratch wound. To determine the effectiveness of the mitogen inhibitor, keratinocyte mono-cultures were treated with 10µg/µl of mitomycin C for 2.5 hours. Proliferation was halted without adversely affecting cellular morphology as early as three days of exposure (Figure 3.7).

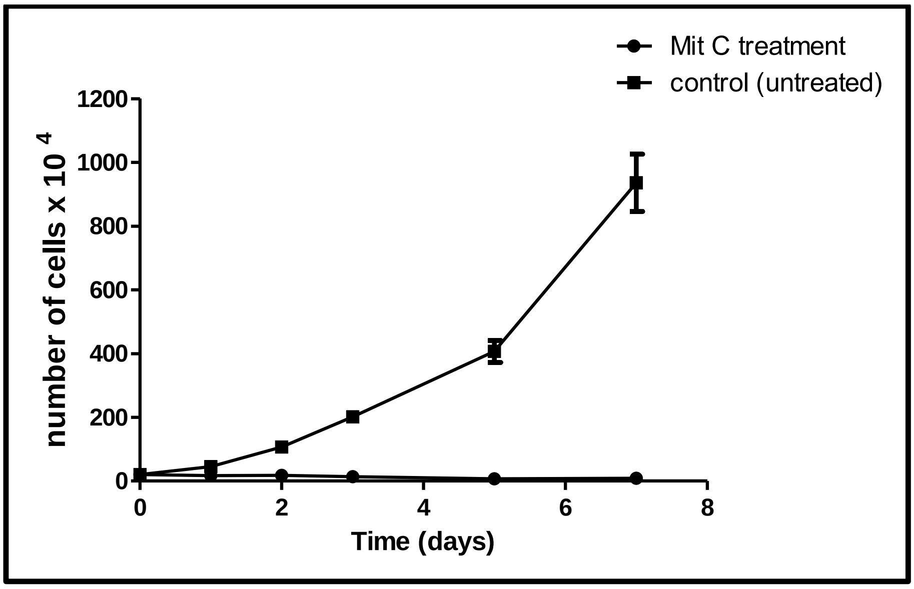


Figure 3.7: Evaluation of the effect of mitomycin C on proliferation of kerationocytes by means of growth curves. Cells were treated with 10μg/μl mitomycin C for 2.5 hours, and then cultured in DMEM for a maximum of 7 days. At days 1, 2, 3, 5 and 7, cells were lifted and counted. The graph represents the mean ± SEM values. n=3

Following the addition of proliferation inhibitor to the co-culture models, we saw no significant differences in the migratory pattern of cells in co-cultures, despite the 20:1 ratio showing a slightly superior, albeit non-significant, percentage wound closure at all-time points post-scratch (Figure 3.8).

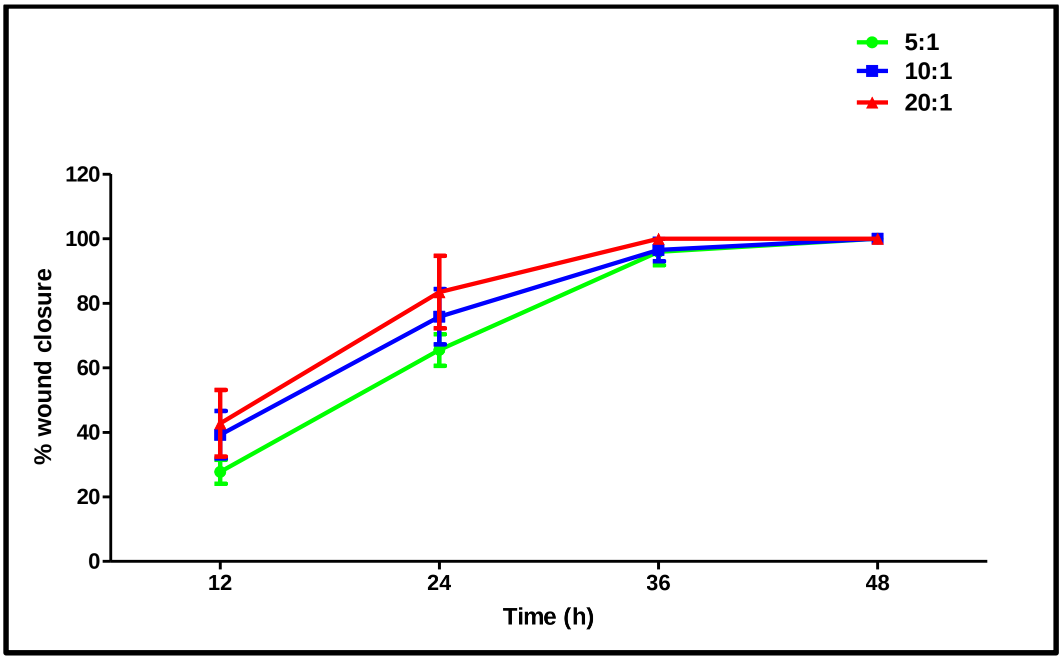


Figure 3.8: Evaluation of migratory pattern in various ratios of keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1). Cells were treated with 10µg/µl mitomycin C for 2.5 hours. A scratch was made 24 hours post co-culturing. At 0, 12, 24, and 48 hours post scratching, three images per field of view were randomly taken using a camera and Image J software was then used to calculate % wound closure. The % wound closure is represented as mean ± SEM. n=3

This result compared to the previous result of the ratios without the mitogen inhibitor (Figure 3.6) points to the fact that the wound closure was not due to migration, but probably due to different rates of cellular proliferation at the wound site. Taken together, this led us to postulate that in a 20:1 ratio of keratinocytes to melanocytes, the cells may mainly proliferate and in so doing, close the wound.

3.5.2. Evaluation of proliferative capacity of keratinocytes and melanocytes *in vitro* co-cultures

With the hypothesis that the wound healing phenotype was due to the proliferative activity of co-cultured cells into the wound, we were interested in determining which of the two cell types (either or both keratinocytes and melanocytes) in various co-culture ratios were chiefly responsible for the proliferative wound healing phenotype. To realise this objective, co-cultures of keratinocytes to melanocytes (5:1, 10:1 and 20:1) were prepared, scratched and cultured for 48 hours post-wounding followed by the calculation of a proliferation index as detailed in materials and methods (Section 2.2.5).

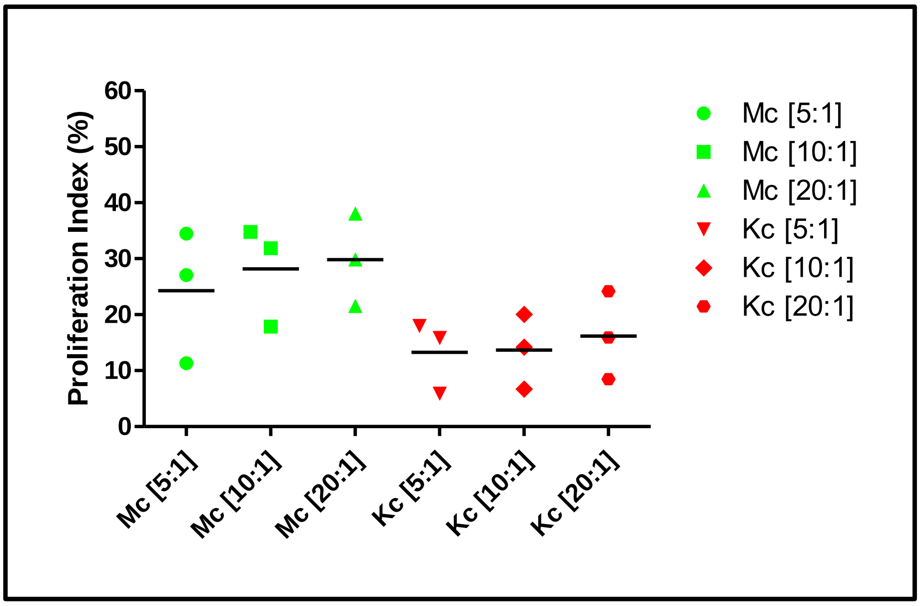


Figure 3.9: Determination of proliferation index (PI) in various ratios of keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1). A scratch was made 24 hours post co-culturing. At 0, 24, and 48 hours post scratching, cells were harvested, lifted and labelled with MART-1 and Ki-67. Ten thousand events were acquired through FACS followed by calculation of PI (as percentage Ki-67$^+$ cells). Values represented as means, n = 3.

There were no significant differences in the PI of both keratinocytes and melanocytes from the co-cultures; however there was a trend towards melanocytes being slightly more proliferative when compared to the keratinocytes (Figure 3.9). To further investigate the proliferative capacity of these cells, we compared the PI of mono-cultures to co-cultures (Figure 3.10). There were significant ($p<0.05$) differences at 24 hours post wounding in the PI of melanocytes when co-cultured, as compared to mono-cultures. On the contrary, keratinocytes in either mono-/co-cultures showed no significant differences in proliferation index.

This finding is fascinating in light of the fact that the identical numbers of melanocytes are seeded and only the keratinocyte numbers change. Despite the melanocyte numbers not changing significantly between the different co-culture ratios (Figure 3.3), significant differences in proliferative index exist between the monoculture and co-cultured melanocytes (Figure 3.10A). This result further emphasises the influence of keratinocytes on melanocyte proliferation through paracrine mechanisms and corroborates the earlier works of (17,128,134,135).

Despite this being previously reported in different systems, such as in determination of melanin transfer from melanocytes to surrounding keratinocytes (130,134), this possible paracrine influence of melanocyte proliferation by keratinocytes has not been shown in primary human cultures with respect to wound healing.

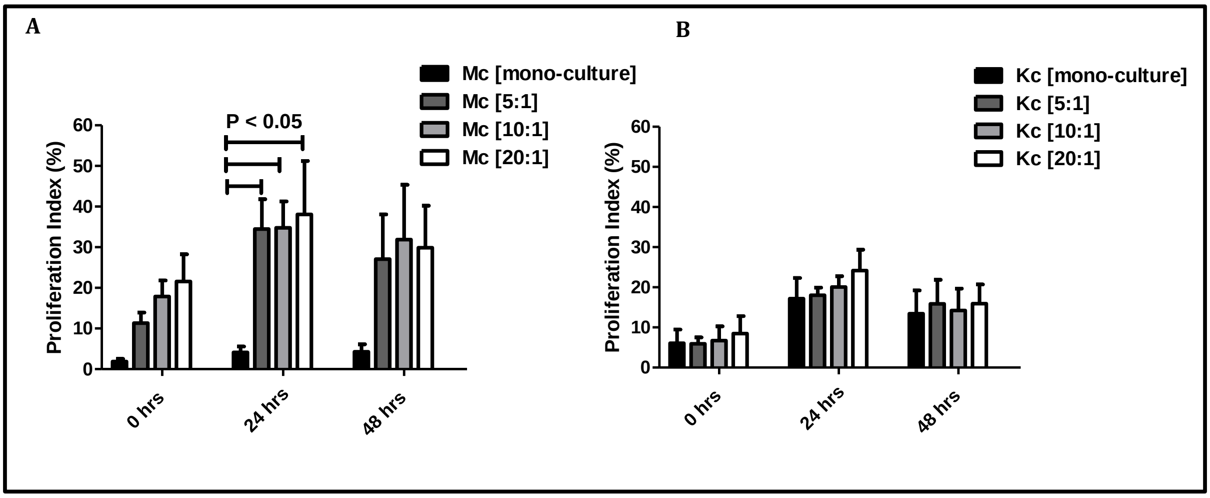


Figure 3.10: Comparison of proliferation index (PI) between keratinocyte and melanocytes mono- and co-cultures. **A:** Comparison of proliferation index (PI) of keratinocytes mono-cultures with keratinocytes sorted from co-cultures (5:1, 10:1 and 20:1). **B:** Comparison of proliferation index (PI) of melanocytes mono-cultures with melanocytes sorted from co-cultures (5:1, 10:1 and 20:1). A scratch was made 24 hours post co-culturing. At 0, 24, and 48 hours post scratching, cells were harvested, lifted and labelled with MART-1 and Ki-67. Ten thousand events were acquired through FACS followed by calculation of PI (as percentage Ki-67+ cells). Values represented ± SEM values. n=3

Taken together, the data so far implicates both keratinocyte and melanocyte proliferation, not migration as the major driving forces in wound healing post scratch.

3.6. Effect of human fibroblast-derived factors on wound closure

As wound closure involves both the epidermal and dermal cellular components of the skin, it was important in our *in vitro* model, to assess the wound healing influence of the dermal cell component i.e. fibroblasts. To investigate this, we harvested fibroblast-conditioned medium, as opposed to culturing fibroblasts alongside the keratinocytes and melanocytes (which would result in the complexity of determining the optimal culture conditions). The fibroblast-conditioned medium was then added to the co-cultures at a dilution of 1:1 (co-culture medium to fibroblast conditioned medium) after which wound closure was monitored over a 48 hour period (Figure 3.11).

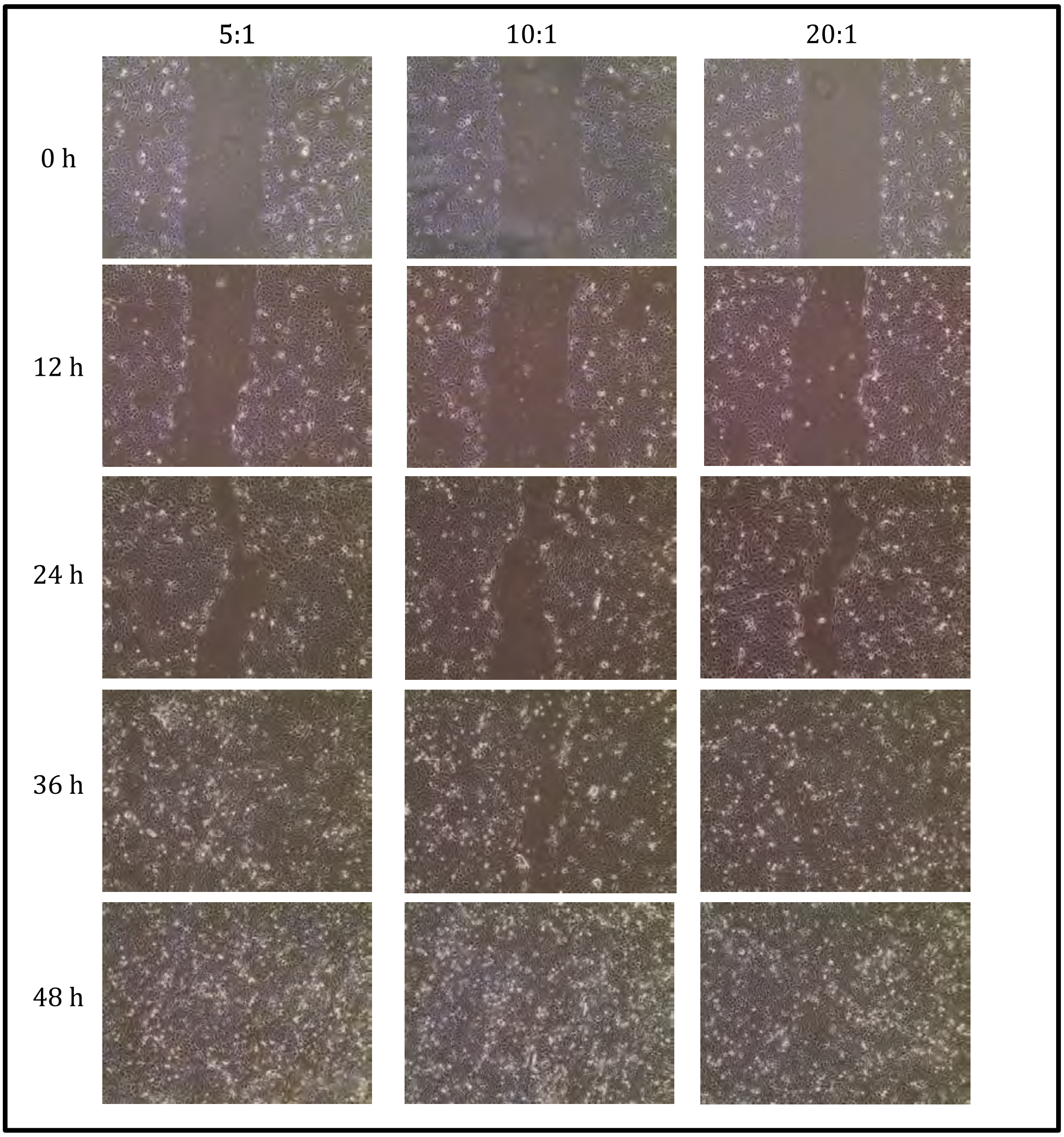


Figure 3.11: Scratch assay images showing the wound closure of the keratinocyte to melanocyte ratios, following addition of fibroblast-conditioned medium. Magnification: 100 X.

Our data showed no significant differences in wound healing profiles for any of the ratios in co-culture. However, the 5:1 ratio showed a trend towards a slightly higher percentage (10% higher than all other ratios at each time point) wound closure (Figure 3.12). This is in contrast with the previous result (Figure 3.6), where the 20:1 ratio (without fibroblast conditioned medium) yielded a better wound healing outcome. As the 10: 1 ratio was not affected by the inhibition of proliferation, this ratio was omitted in subsequent experiments.

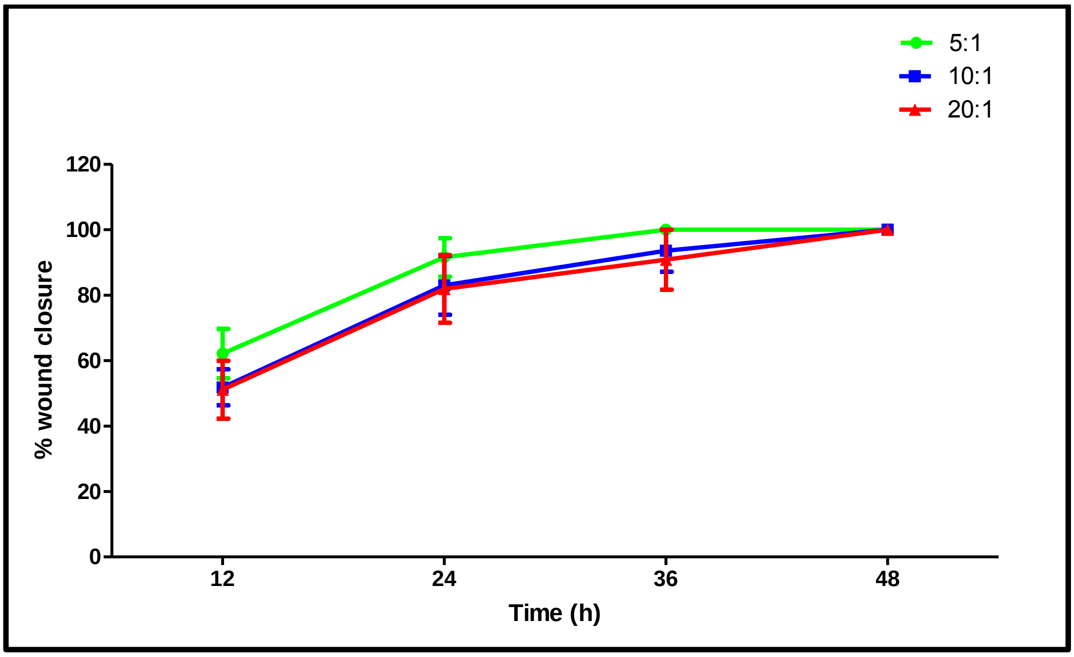


Figure 3.12: Effect of human fibroblast-derived factors on wound closure. Keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1) were scratched 24 hours post co-culturing. At 0, 24, and 48 hours post scratching, three images per field of view were randomly taken using a camera and Image J software was then used to calculate % wound closure. The %wound closure is represented as mean ± SEM. n=3

To further investigate such subtle variations in wound closure capacities of our co-culture models (5:1 and 20:1 only), we graphed the percentage wound closure with or without addition of conditioned medium (Figure 3.13).

The 5:1 ratio displayed significant differences ($p < 0.001$) in wound closure upon addition of fibroblast-conditioned medium at both the 12 and 24 hour time points (Figure 3.13A). In contrast, no significant differences were shown over the entire 48 hour time period for both the 10:1 (data not shown) and 20:1 ratios (Figure 3.13B), Despite the wound closing at the same time point (36 hours) in the 5:1 ratio, the result implies that fibroblast-conditioned medium i.e. fibroblast-derived factors, elicit a faster rate of closure over the first 24 hours. This result supports the observations of Imokawa et al., (1998) (141) who showed that fibroblast-conditioned medium was capable of activating mitogen-activated protein (MAP) kinase of human melanocytes, causing them to proliferate in the presence of conditioned medium. More recently, Cario-Andre et al., (2006) (142) also showed that fibroblast secretion significantly influences melanocyte proliferation and melanin distribution/degradation. Although the exact nature of the fibroblast-derived influence was difficult to isolate in our system, we can hypothesize that because there are more melanocytes to keratinocytes in the 5:1 ratio, that the melanocytes may be the key players in the acceleration of wound closure in the presence of fibroblast-derived paracrine factors.

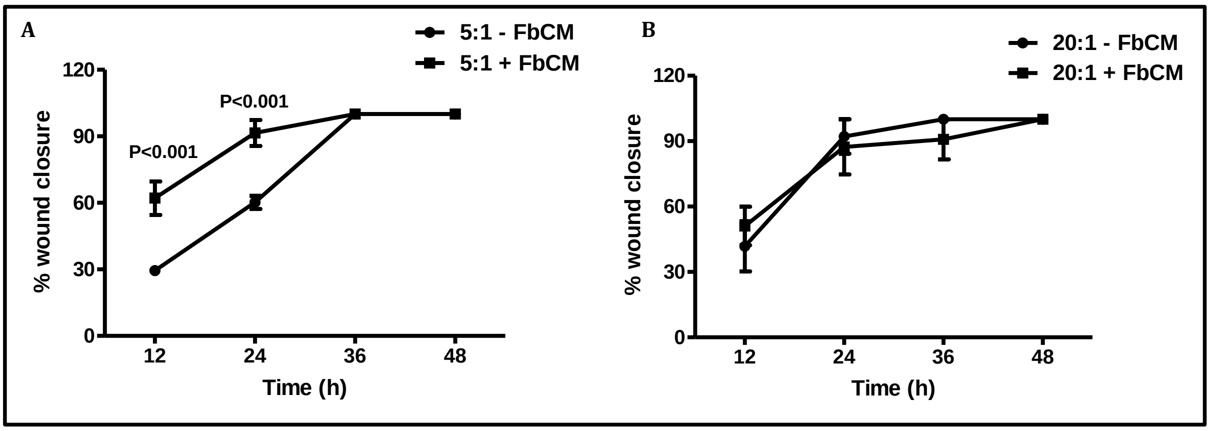


Figure 3.13: Comparison of the effect of human fibroblast-derived factors on wound closure between 5:1 and 20:1 ratios. Keratinocytes and melanocytes co-cultures (5:1 and 20:1) were scratched 24 hours post co-culturing as before. The %wound closure is represented as mean ± SEM. n=3

Overall these data shows that fibroblast-derived factors have a positive effect on the wound healing capacity of cells in 5:1 ratio, whereas such factors may not be as beneficial with a higher ratio of keratinocytes to melanocytes (20:1). The possibility further exists that inhibitory factors or factors that cause rapid degradation of the fibroblast-derived mitogens, may be released into the milieu from the increased number of keratinocytes in the 10:1 and 20:1 ratios. This will have to be further investigated in the future. For these reasons, we wanted to determine how such variations were manifested and therefore treated our co-cultures with mitomycin C, followed by addition of conditioned medium as before, with monitoring of wound closure over 48 hours (Figure 3.14).

3.6.1. Evaluation of migratory phenotype of co-cultures following addition of fibroblast conditioned medium

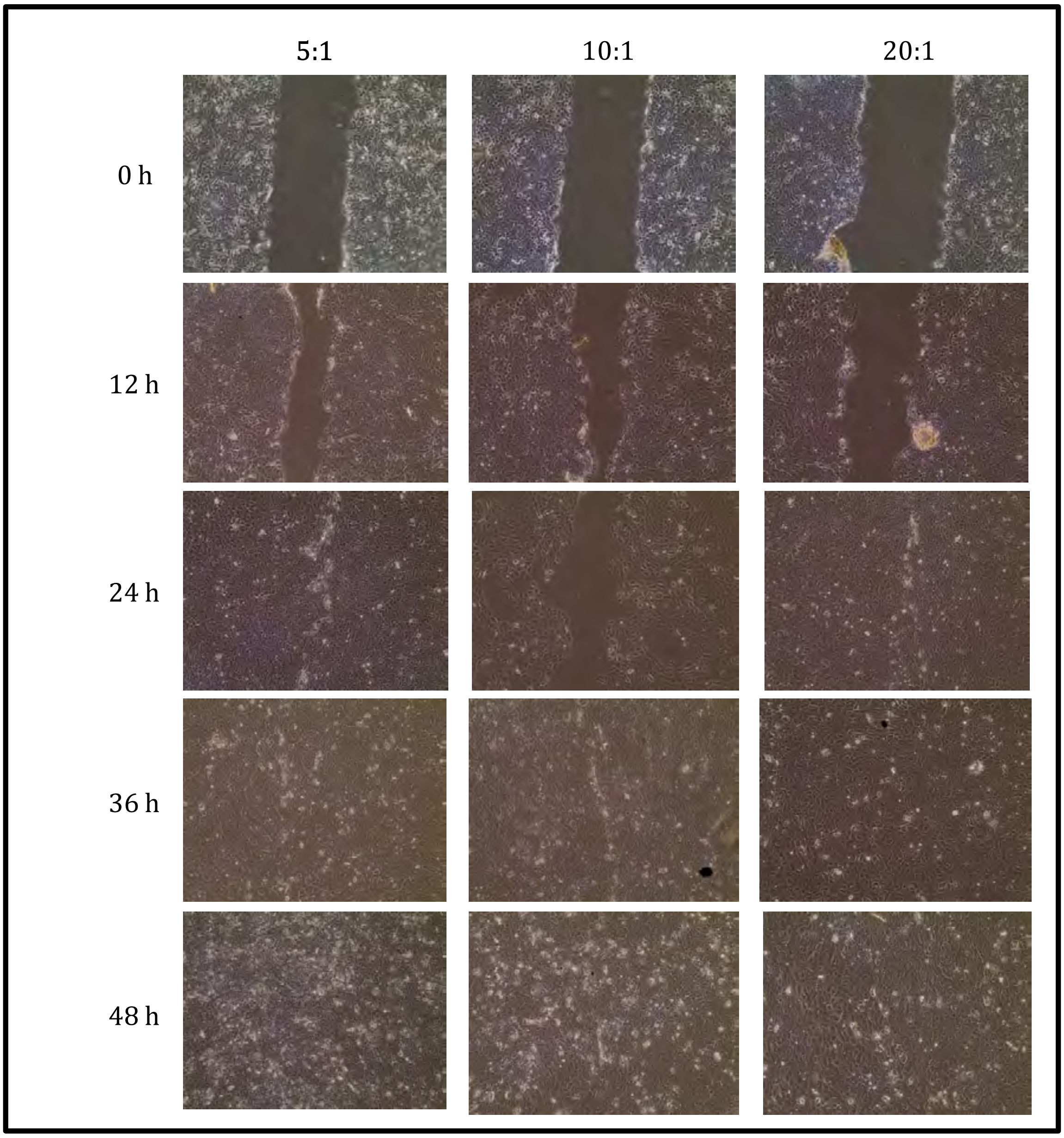


Figure 3.14: Scratch assay images showing the wound closure of the ratios, following addition of mitogen inhibitor (mitomycin C) and fibroblast conditioned medium. Magnification: 100 X.

Following the treatment of co-cultures with mitomycin C, no significant differences in percentage wound closure was observed in any of the ratios (Figure 3.15). Despite this, the 5:1 ratio fascinatingly displayed an increase in the rate of migration during the early wound healing phase (12-24h), followed by a non-significant drop in the migration rate at 48 hours post scratch (Figure 3.16), while the 20:1 ratio exhibited a reduction in migration rates over the same time period. Furthermore, 20:1 ratio displayed significant ($p<0.05$) reduction in the rate of migration, whereas the 10:1 ratio showed a uniform steady-state reduction in its migratory rate over time. Specifically, there was an inverse correlation in migratory potential of these two keratinocyte to melanocyte ratios - in the 5:1 ratio, the highest migratory rate was evident 24-36 hours post scratch (36 078µm/h), while the 20:1 ratio, displayed the fastest rate of migration (52 089µm/h) in the earlier (12-24 hours) phase of wound healing (Figure 3.16).

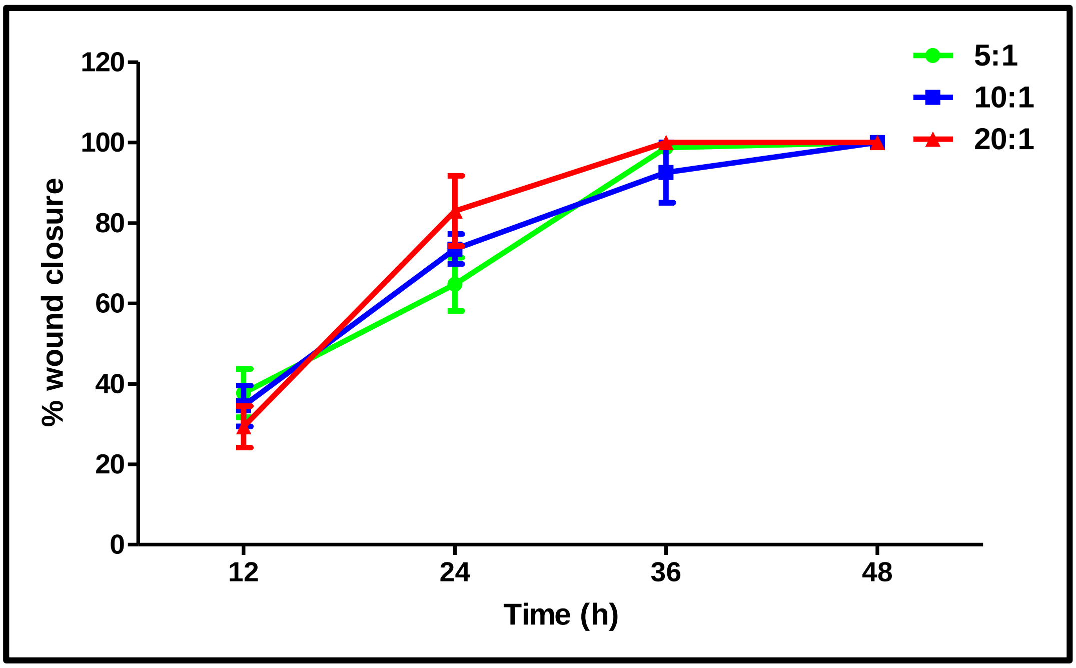


Figure 3.15: Effect of human fibroblast-derived factors on cell migration. Keratinocytes and melanocytes co-cultures (5:1, 10:1 and 20:1) were treated with 10µg/µl mitomycin C for 2.5 hours and scratched at 24 hours post co-culturing. At 0, 24, 36 and 48 hours post scratching, three images per field of view were randomly taken using a camera and Image J software was then used to calculate percentage wound closure. The percentage wound closure is represented as mean ± SEM. n=3

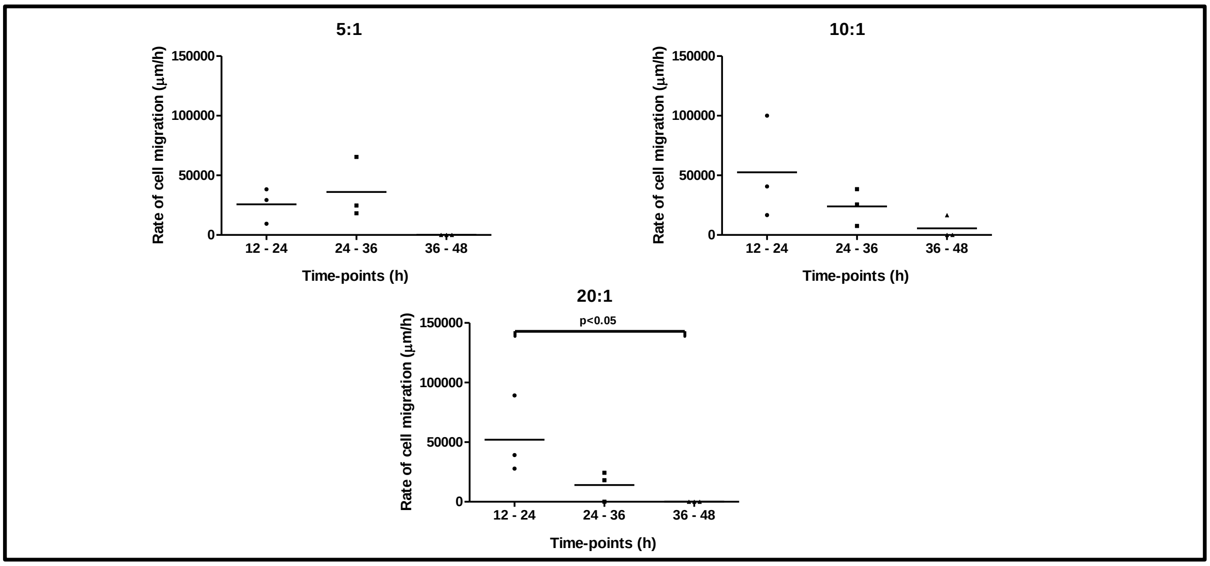


Figure 3.16: Determination of rate of migration in various co-culture ratios (5:1, 10:1 and 20:1). The rate was calculated as μm/h and data represented as mean ± SEM. n=3

Such variations in co-culture ratio-dependant wound healing capacities are important considerations in designing pigmented 3D skin constructs. A study by (131) showed that melanocyte titration is pivotal in the control of pigmentation in skin constructs. They showed, expectedly, that higher number of melanocytes yielded a better pigmentary skin phenotype. Another study conducted by (143) convincingly showed that addition of melanocytes onto 3D skin constructs yielded the similar colour to the donor skin. Therefore, this may also hold true for proliferation and migration, which are both important facets of epithelial wound healing. With this in mind, we sought to infer if any of these differences were existent, and if so how would they impact on the wound healing capacities of either melanocytes or keratinocytes in co-culture.

3.7. Evaluation of migratory and/or proliferation phenotype in co-cultures, with or without addition of fibroblast conditioned medium (Fb-CM)

Such differences in migratory rate (i.e. the 5:1 ratio displaying a late peak migratory rate and the 20:1 ratio showing an earlier peak migration rate) may be correlated to the proliferation capacities of cells in co-culture. Since it is well documented that proliferation and migration are mutually exclusive events in cellular dynamics (139,140), we observed an early significant increase in proliferation capacity of cells in a 5:1 ratio (Figure 3.13) 12-24 hours post-scratch upon addition of Fb-CM, which was accompanied by a late non-significant increase in migratory rate (Figure 3.16) that occurred 36 hours post-scratch.

This early proliferation capacity of cells in the 5:1 ratio was deduced from the observation that there was a significant increase in percentage wound closure upon the addition of Fb-CM (red line to green line in Figure 3.17). In contrast, this increase was abrogated upon the addition of a proliferation inhibitor (Green to blue lines) (Figure 3.17).

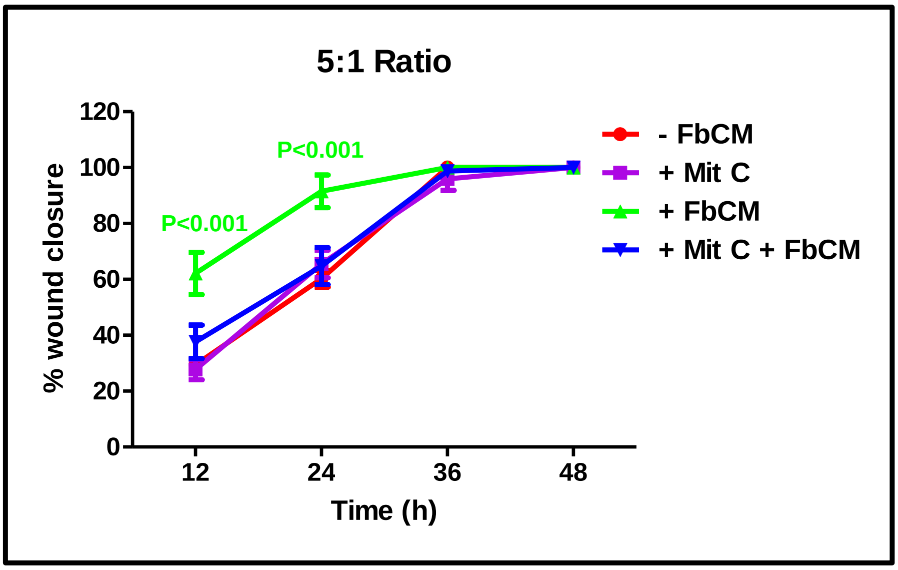


Figure 3.17: Evaluation of migratory and/or proliferation phenotype in 5:1 co-culture ratio, with/without addition of fibroblast conditioned medium. Cells were co-cultured; thereafter Fb-CM and/or mitomycin C were added post scratch. Scratches were monitored for 48 hours. Data is represented as mean ± SEM. n=3

3.8. Evaluation of migratory and/or proliferation phenotype in mono-cultures, with/without addition of fibroblast conditioned medium

Having observed such variations in wound closure capacities of cells in various co-culture models, we set out to determine the role of each cell type in effecting these changes. To do this, keratinocyte and melanocyte mono-cultures were scratched as before, followed by the addition of Fb-CM and/or mitogen inhibitor.

Interestingly, addition of Fb-CM to keratinocyte mono-cultures led to a significant ($p<0.05$) delay in wound closure at 36 hours post-scratch (Figure 3.18). The melanocytes in contrast, only exhibited a significant difference ($p<0.01$) in wound closure 48 hours after being wounded (Figure 3.18). Addition of the proliferation inhibitor, mitomycin C, resulted in a rescuing of the wound closure capacity of keratinocytes, which supports the hypothesis that keratinocytes' migratory phenotype is favoured upon addition of Fb-CM. On the other hand, melanocytes showed no differences in percentage wound closure following addition of the fibroblast conditioned medium and mitomycin C (Figure 3.18). In support of a number of reports (53,54,141), this difference between the two cell types' responses may be due to fibroblast derived factors such as bFGF and ET-1. It is suspected that in this system, these factors are responsible for the increased proliferation in melanocyte mono-cultures, whereas some of these factors seem to slow down keratinocyte proliferation, in favour of their migration (139,140).

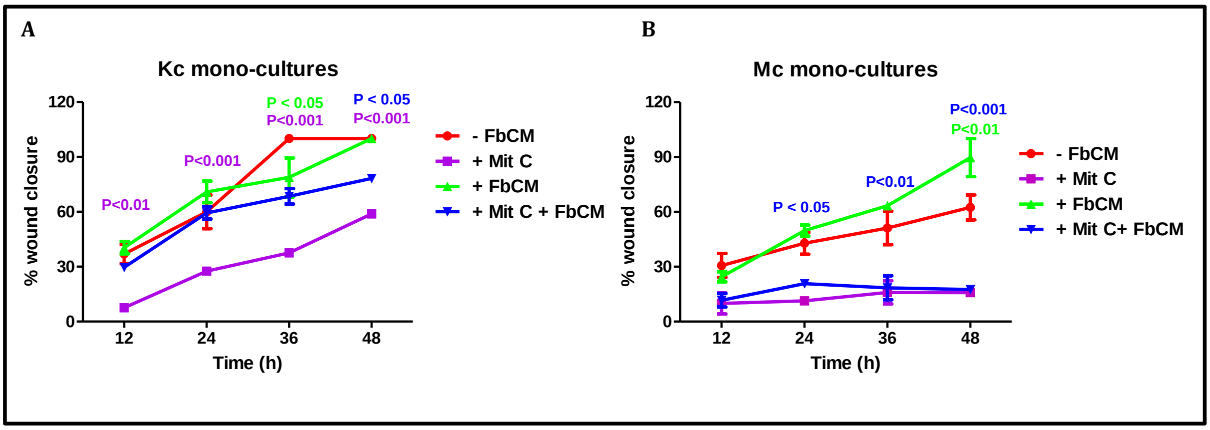


Figure 3.18: Evaluation of migratory and/or proliferation phenotype in mono-cultures, with/without addition of fibroblast conditioned medium. Cells were cultured; thereafter Fb-CM and/or mitomycin C were added post scratch. Scratches were monitored for 48 hours. Data is represented as mean ± SEM. n=3

Taken together, such Fb-CM studies have highlighted the importance of partially inhibiting proliferation in keratinocytes, while allowing for subsequent melanocyte proliferation. Such growth dynamic variations seem to be ideal in enhancing wound closure capacities in our co-culture models (where we see increased percentage wound closure in a 5:1 ratio, upon addition of Fb-CM) and have a direct application to the clinical response of wound healing.

3.9. Pigmentary phenotypes of co-cultures

Tyrosinase and TRP-1 are two of the best-characterised melanocyte differentiation proteins associated with melanin synthesis (144). Our question was therefore to evaluate whether the presence of keratinocytes in co-cultures with the melanocytes, affect their level of pigmentation. The challenge was to analyse this aspect after co-cultures. This was overcome by labelling the cells with a melanocyte-specific antibody (MART-1) and then quantifying the number of TRP-1-positive cells as a marker of differentiated, pigmented melanocytes using fluorescent activated cell sorting (FACS). While we observed a significant ($p<0.0001$) reduction in pigmented "melanogenic-competent"/TRP-1-positive melanocytes in the co-cultures compared to mono-cultures, no significant differences in TRP-1 positivity existed between the individual ratios (Figure 3.19). A number of reasons could be postulated for this seemingly lack of "melanogenesis-competent" cells in co-culture. Firstly, (131) showed that a decrease in pigmentation of their cultured skin constructs was due to the dilution of slow growing melanocytes by the fast growing keratinocytes. Corroborating this was a study by (132) who observed not only a decrease in melanocyte numbers, but an associated decrease in pigmentary function (as evidenced by a lack of TRP-1 expression). They postulated this to be a possible factor for subsequent melanocyte loss.

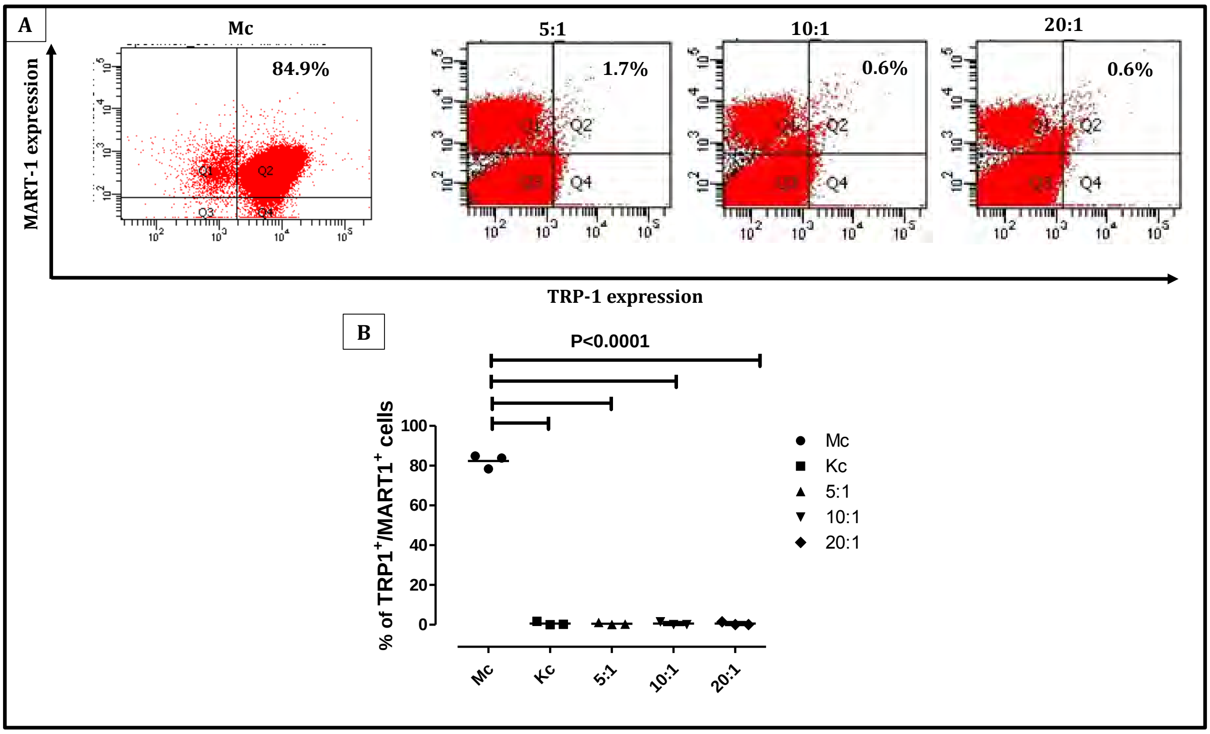


Figure 3.19: Evaluation of pigmentary phenotype of melanocytes in mono-/co-cultures with keratinocytes (5:1, 10:1 and 20:1Cells were co-cultured for 3 days, thereafter lifted, permeabilised and labelled with MART-1 and TRP-1. **A**: FACS was used to acquire 10 000 events. **B**: Melanogenic melanocytes represented as MART-1$^+$/TRP-1$^+$ cells. n=3

Despite the lack of pigmentation in our co-cultures, we hypothesise that when recapitulating pigmented skin equivalents (PSE), the non-pigmentary melanocytes would subsequently re-pigment due to the presence of fibroblasts in the prospective PSE models. Although the testing of this hypothesis falls beyond the scope of this study, a study by (145) using a 3D construct showed that dermal fibroblasts do indeed impact the degree of skin pigmentation.

CHAPTER 4: CONCLUSIONS AND FUTURE PERSPERCTIVES

The overall aim of this study was to, through the use of primary isolated skin melanocytes and keratinocytes develop an *in vitro* co-culture system to obtain optimal pigment production *in vitro* and to evaluate its applicability to an improved wound healing outcome. Specifically, the study was set out to analyse the effect the co-cultured epidermal cells (at various ratios) may have on characteristics associated with wound healing such as cell proliferation and migration. Furthermore, we were interested in determining the pigmentary phenotype of such co-cultures. Therefore keratinocytes and melanocytes were co-cultured at 5:1, 10:1 and 20:1 ratios respectively.

Firstly the proliferation capacities of both keratinocytes and melanocytes in co-culture were evaluated. Melanocyte's proliferation capacity increased with their subsequent co-culture with keratinocytes in all ratios, with the highest proliferation in the 5:1 ratio. On the contrary, keratinocytes proliferated better with less of melanocytes (20:1) in co-culture. These implied that melanocytes are hugely dependant on keratinocyte derived factors for proliferation, whereas keratinocytes are negatively impacted by greater melanocyte numbers.

Secondly, wound healing phenotypes of co-cultured keratinocytes and melanocytes were evaluated. The significantly better wound healing capacity was much more evident with the 20:1 ratio, where such was attributed to the increased proliferative capacity of cells into the denuded area. This significantly better wound healing outcome was abrogated by the subsequent presence of fibroblast derived factors, which in turn enhanced the proliferative capacity of melanocytes, while increasing the migratory pattern of keratinocytes in co-culture. This resulted in a non-significantly superior wound healing outcome in the 5:1 ratio of keratinocytes to melanocytes. It is in this ratio where we also observed a synchronised population doubling time (approximately 48 hours) of both epidermal cells in co-culture.

Thirdly, the pigmentary phenotypes of the three ratios of co-cultures was evaluated, where we observed a significant reduction in number of TRP-1$^+$ melanocytes when co-cultured with keratinocytes compared to melanocytes mono-cultures. Alongside this observation, all co-culture ratios exhibited similar low numbers of "melanogenic-competent" melanocytes. This further highlights the tight regulation of keratinocytes in preventing melanocytes from being fully differentiated, thus not being capacitated to pigment.

Therefore, we finally postulate that a 5:1 ratio would be a better ratio to incorporate into our prospective PSE (pigmented skin equivalents), as in this co-culture ratio, melanocytes and keratinocytes growth rates were similar, and also because such ratio yielded a better wound healing outcome in the presence of fibroblast derived factors. Furthermore, the presence of the highest number of melanocytes may result in a greater pigmentation outcome in the presence of fibroblast derived factors, though this will have to be tested.

However, few limitations exist in this study. Firstly immortalised keratinocytes; HaCaTs, instead of primary keratinocytes were used throughout this study. One could imagine that such spontaneously transformed cells may be tumorigenic or may exhibit abnormal genotypic and phenotypic characteristics. However, a study by Boukamp et al., 1988 (125) has shown that HaCaTs, similar to normal keratinocytes form an orderly structure, with normal differentiation patterns when transplanted onto nude mice. Pivotal differentiation markers such as K1, K10, involucrin and filaggrin are also expressed and regularly located. Furthermore, these transformed cell line possesses the normal DNA fingerprint pattern, which remains unaffected during pro-longed cultivation in vitro. The use of primary human keratinocytes seems an obvious choice. However, in our lab, we have experienced short life-span of these cells in vitro. The cells cannot be cultured beyond passage 4, therefore the use of immortalised cell line seemed the best option for this undertaking. We are however optimising the growth conditions of primary keratinocytes as these cells will have to be used in the development of PSE models.

Another limitation is the use of infant foreskin melanocytes in place of normal skin melanocytes. Foreskin melanocytes due to their juvenile nature grow faster than adult skin melanocytes. Therefore the use of the latter cells would imply slow cell expansion during development of PSE models. However, it has been mentioned (paragraph below) that the addition of skin fibroblasts and correct keratinocyte cells numbers could potentially increase melanocytes’ proliferative capacity.

In future, as previously mentioned, further studies to evaluate the pigmentation competence of melanocytes in co-culture with keratinocytes in the presence of fibroblast derived factors is of great importance. This postulation is driven by the observation that melanocyte proliferation is enhanced by such growth factors, which may in turn increase the pigmentation. Furthermore to enhance their melanogenic capacity, we could subject melanocytes to low dose UV, or co-culture them with less differentiated keratinocytes as these have been shown to release a greater magnitude of paracrine factors essential for melanocyte survival, proliferation and pigmentation.

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APPENDICES

Appendix A: Media used in the study

Quantity sufficient (qs) is defined as enough liquid (e.g. ddH2O) that is used to make up a certain final volume, given in litres or millilitres.

DMEM (pH 7.4, 1L)

DMEM powder	27.06g
$NaHCO_3$	7.4g
Autoclaved ddH_20	qs
Adjust to pH 7.4, sterilize through a 0.2µm filter and store in aliquots at 4°C	

Complete culture medium (DMEM/10%FCS/1xP/S, 200ml)

FCS (100%)	20ml
P/S (100x)	2ml
DMEM (pH7.4)	qs
Store in aliquots at 4°C	

FETI (1L)

Component	Concentration	Volumes
FCS	2%	10ml
Ultroser G	1%	5ml
hFGF-2	2ng/ml	100µl
ET-1	2ng/ml	100µl
TPA	16nM	200µl
IBMX	0.05mM	5ml
P/S	100U/ml; 100µg/ml	5ml
Hams F10		qs
Filter sterilize through a 0.2µm filter and store in aliquots at -20°C		

GREENS (0.8L)

Component	Concentration	Volumes
DMEM/F12	1:1	qs
Insulin	5µg/ml	2ml
Hydrocortisone	0.4µg/ml	1.6ml
Cholera toxin	0.1nM	800µl
T/T3	2nM	800µl
EGF*	10ng/ml	800µl
P/S	100U/ml; 100µg/ml	8ml
Gentamycin	0.2mg/ml	16ml
Sodium bicarbonate	0.075%	8ml
HEPES buffer**	20mM	16ml
FCS	10%	80ml
Filter sterilize through a 0.2µm filter, *add EGF after re-filtering **Make HEPES (1M) fresh Store in aliquots at -20°C		

KSFM complete medium (1L)

EGF (2.5µg)	**0.5ml**
BPE (25µg)	2.5ml
P/S (1x)	5ml
KSFM	500ml
Store in aliquots at -20°C	

Appendix B: Buffers

Hanks Buffered Saline Solution (HBSS, 1L)

HBSS powder	**9.5g**
$NaHCO_3$	0.35g
Autoclaved ddH_20	qs

HEPES buffer (1M, pH 7.3, 10ml)

HEPES	**2.383g**
ddH_20	qs
Make up fresh for Greens medium	

Phosphate Buffered Saline (1xPBS, 0.14M NaCl, 8.8M Na_2HPO_4, 2.7M KCl, 1.47M $KHPO_4$, pH7.4, 1L)

NaCl	**8g**
Na_2HPO_4	1.26g
KCl	0.2g
$KHPO_4$	0.2g
ddH_20	qs
Autoclave and store in aliquots at 4^oC	

Appendix C: Solutions

Dispase (5mg/ml)

Dispase 11	**500mg**
Hanks Balanced Salt Solution	100ml

EDTA (0.05%)

EDTA (disodium salt)	**0.25g**
PBS	500ml
Sterilize through a 0.2μm filter and store in aliquots at -20°C	

Endothelin-1 (ET-1, 10μg/ml)

ET-1	**0.1mg**
PBS	1ml
ET-1 (solution, 10μg/ml)	
ET-1 (10μg/ml)	100μl
PBS	400μl
Aliquot at store at -20°C	

Fetal Calf Serum (FCS, 500ml)

FCS	**500ml**
Heat inactivate by 20 minutes incubation in a 56°C water bath	
Allow to cool and store in aliquots at -20°C	

Hoechst (5μg/ml)

Hoechst (1000 μg/ml)	**20 μl**
autoclaved ddH2O	900 μl
Store in aliquots at -20°C	

IBMX (isobutyl-1-methyl xanthine; 20ml, 5mM)

IBMX (to make 10mM)	**0.022g**
ddH_2O	10ml
Autoclave, then allow to cool	
IBMX (10mM)	10ml
Hams F10	10ml
Store the resultant 5mM IBMX at room temperature	

L-Glutamine (200mM)

L-Glutamine	**2.92g**
ddH_2O	100ml
Sterilize through a 0.2µm filter and store in aliquots at -20°C	

L-DOPA (0.25 mM, 10 ml)

L-DOPA	**0.0005 g**
PBS	10 ml
Make fresh	

Mitomycin C (0.01µg/µl, 2ml)

Mitomycin C	**2mg**
SABAX H_20	2ml
Filter through a 0.2µm filter, wrap in foil and store in aliquots at -20°C	

Mowviol

Mowiol	**2.4g**
Glycerol	6g
Tris buffer (0.2 M) pH 8.5	12ml
ddH2O	6ml
Stir for a few hours at 50°C, centrifuge at 12000 rpm for 20 min. Store supernatant in 1 ml aliquots at -20°C	

Mowviol with anti-fade

Mowiol	**1 ml**
n-Propyl gallate (Sigma-Aldrich, USA)	tip of a small spatula
Vortex and heat at 50°C for 1 h, mixing occasionally. Centrifuge at 13000 rpm for 15-20 min. Transfer supernatant to clean tube and store at -20°C.	

Penicillin (1000U/ml)/ Streptomycin (100µg/ml) (100x P/S, 1L)

Penicillin (1662 U/mg)	**6g**
Streptomycin (750 U/mg)	10g
ddH_20	qs
Sterilize through a 0.2µm filter and store in aliquots at -20°C	

PFA (4%, 100ml)

Paraformaldehyde	**4g**
PBS	qs
Heat at 50°C to dissolve and store in aliquots at -20°C	

Sodium phosphate buffer (1 M, 200 ml)

$NaH_2PO_4 \cdot 2H_2O$	**15.6 g**
$Na_2HPO_4 \cdot 12H_2O$	35.8 g
ddH_2O	qs
Adjust to pH 7-7.4 and store at room temperature	

Sodium Phosphate Buffer (0.1 M, pH 7.2, 100 ml)

Sodium phosphate buffer (1 M)	**10 ml**
ddH^2O	qs
Adjust to pH 7.2 and store at room temperature	

TPA (12-O-tetradecanoylphorbol-13-acetate, 2mM stock)

TPA (powder)	**10mg**
Absolute ethanol	1ml
Transfer to a 1ml tube	
TPA (solution)	1ml
Absolute ethanol	7.11ml
Aliquot and cover with foil, store at -20^oC	

Triton X-100 (1%, 100ml)

Triton X-100	**1ml**
ddH_20	99ml
Allow to dissolve at room temperature overnight.	

Trypsin/EDTA (0.25% T/0.05% E, 100ml)

Trypsin	**0.25g**
EDTA	0.005g
PBS	qs
Adjust to pH7.5 and sterilize through a 0.2μm filter and store in aliquots at -20^oC	

Appendix D: Reagents

Name	Product number	Supplier
Acetic acid (glacial) 100%	SAAR1021020LC	Merck
Albumin, from bovine serum	A7906	Sigma-Aldrich
BCA Protein Assay Kit (Roche)	23225	Thermo Scientific Pierce
Cholera toxin	C8052	Sigma-Aldrich
DMEM	N.P02	Highveld Biological
DMEM/F12	31600-83	GIBCO, Life Technologies
DOPA	D9628	Sigma-Aldrich
EDTA	E9884	Sigma-Aldrich
EGF	E5036	Sigma-Aldrich
ET-1	E7764	Sigma-Aldrich
Ethanol absolute	1.00983.2500	Merck
Fetal Calf Serum	No.306	GIBCO, Life Technologies/ Highveld Biological
hFGF-2	130-104-924	Miltenyi Biotec
FITC Anti-Human IL-2	340448	BD Biosciences
Fungizone	No. 228	Highveld Biological
Gentamycin	G1272	Sigma-Aldrich
L-Glutamine	21051-016	GIBCO, Life Technologies
Glycerol	2676500LC	Merck
Hams F10	No. P08	Highveld Biological
HCl (37%)	SAAR3063054LCA	Merck
HBSS	No. P206	Highveld Biological
HEPES	H3375	Sigma-Aldrich
Hoechst	33342 H1399	Merck
Hydrocortisone	H0888	Sigma-Aldrich
IBMX	I5879	Sigma-Aldrich
Insulin	Reg: W/22.1/288	Novo Nordisk (Pty) Ltd
Isopropanol	SAAR5075040LC	Merck
KCl (potassium chloride)	SAAR5042000EM	Merck
KH_2PO_4 (potassium dihydrogen orthophosphate)	10203	BDH
KSFM	17005-034	GIBCO, Life Technologies
Methanol	SAAR4164060LP	Merck
Microscope cover glasses 22x22mm	101050	Marienfeld
Microscope slides	1001412	Marienfeld
Mitomycin C	M4287	Sigma Aldrich
Mowiol	P3130	Sigma Aldrich
NaCl (sodium chloride)	SAAR5822320EM	Merck

Na_2HPO_4 (di-sodium hydrogen phosphate)	1.06586.0500	Merck
PFA (paraformaldehyde)	S55065-016	Merck
Penicillin G sodium salt	P3032	Sigma-Aldrich
Transferrin	T0665	Sigma-Aldrich
Streptomycin	S9137	Sigma-Aldrich
Supplements for KSFM	37000-015	Sigma Aldrich
TPA	P1585	Sigma-Aldrich
Triton X-100	T9284	Sigma-Aldrich
Trypsin, from porcine pancreas	T4799	Sigma-Aldrich
T/T3	T6397	Sigma-Aldrich
Tween-20	P9416	Sigma-Aldrich
Ultroser G	15950-017	PALL Life Sciences

Appendix E: Tables

Table E.1: Number of cells used for a scratch assay

Ratio	Keratinocytes	Melanocytes
5:1	4.5×10^5	9×10^4
10:1	4.5×10^5	4.5×10^4
20:1	4.5×10^5	2.25×10^4
Keratinocyte control	4.5×10^5	-
Melanocyte control	-	5×10^5

Table E.2: Number of cells used for co-culture proliferation (growth curves) assays

Ratio	Keratinocytes	Melanocytes
5:1	4.5×10^5	9×10^4
10:1	4.5×10^5	4.5×10^4
20:1	4.5×10^5	2.25×10^4
Keratinocytes		
Melanocytes		

Table E.3: Number of cells used for co-culture proliferation Index (PI)

Ratio	Keratinocytes	Melanocytes
5:1	9×10^5	1.8×10^5
10:1	9×10^5	0.9×10^5
20:1	9×10^5	0.45×10^5
Keratinocytes	9×10^5	-
Melanocytes	-	7.5×10^5

Table E.4: Number of cells used for TRP-1 staining (for FACS)

Ratio	Keratinocytes	Melanocytes
5:1	1×10^5	2×10^4
10:1	2×10^5	2×10^4
20:1	4×10^5	2×10^4
Keratinocyte control	5×10^5	-
Melanocyte control	-	5×10^5

Table E.5: Antibodies used in this study.

Antibody	Concentration	Supplier
MART-1 (FITC)	1µg	Santa Cruz Biotechnology
Ki-67 (PE)	2µg	Santa Cruz Biotechnology
TRP-1 (Alexa Flour 647)	2µg	R & D Systems

Appendix F: supplementary protocols

Mycoplasma test – Hoechst

To test for the presence of mycoplasma in the cultures, cells were grown in their respective medium on coverslips for 7 days without antibiotics. The growth medium was then removed and cover slips (with cells) were washed with 1x PBS. Cells were fixed with 1 ml glacial acetic acid:methanol (1:3) for 6 minutes at room temperature, thereafter, fixative was removed and cover slips were allowed to air-dry. Cells were subsequently stained cells with 5µg/ml Hoechst for 6minutes. Hoechst was removed and cells were washed a few times with 1x PBS. Finally the coverslips were mounted onto microscope glass slides, after which cells were viewed on a fluorescent microscope.

Optimisation of co-culture growth conditions (GREENS/FETI)

One of the fundamentals of Co-culturing is the determination of optimal growth conditions for both cell types. Such includes mainly the growth medium conducive for both cells in a co-culture. Typically, a mono-culture of HaCaT cell lines is grown in DMEM, while the optimal growth medium for melanocytes is FETI. Therefore in this study, the optimal growth medium for HaCaT cell lines and melanocytes in a co-culture was determined as follows:

Primary skin melanocytes ($2x10^4$ cells) were seeded in 35mm dishes in duplicate and cultured for a period of 7 days. The cells were grown in either FETI or GREENS, during which at days 1, 2, 3, 5 and 7, cells were lifted off the dishes by means of trypsin/EDTA and counted as mentioned in Section 2.1.3. Similarly, HaCaT cells ($2x10^4$ cells) were seeded, cultured in either GREENS or FETI and counted at aforementioned days. For all experiments, 3 biological repeats were done.

Optimisation of FACS analyses for co-cultures (MART-1/Ki-67 and MART-1/TRP-1)

In order to separate the two cell types, FACS was used to sort such cells following labelling with specific antibodies. In the same manner, we used FACS analyses to decipher the various cell populations with different phenotypic changes. For instance, we used MART-1/Ki-67 and MART-1/TRP-1 antibody combinations to infer the proliferation and melanogenic capacities respectively, of melanocytes from a co-culture.

In order to optimise the separation technique, briefly cells were cultured as indicated in Table E2 for 24 hours, followed by trypsinisation as before. The cells were re-suspended and fixed in 2ml of cold 4% paraformaldehyde for 15 minutes at ambient temperature and washed twice in PBS. Cells were then permeabilized for 15 minutes using a perm/wash buffer following manufacturer's instructions. Cells were re-suspended in FACS buffer and transferred to FACS tubes. Finally a melanocyte specific antibody; 1µg of MART-1 conjugated to FITC was added to the cell suspension and tubes were covered in foil to prevent light exposure. The cells were incubated for 1 hour at ambient temperature on a shaker and washed with perm/wash buffer. The cells were then re-suspended in FACS buffer and immediately acquired (10 000 events or total cell population) on a flow cytometer. Table indicates the antibody combinations used for various assays.

Table F.1: The antibody concentrations used for specific assays.

Antibody	**Concentration**	**Assay**
MART-1 (FITC) only	1µg	Growth curves
MART-1 (FITC)/Ki-67 (PE)	1µg/2µg	Proliferation index
MART-1 (FITC)/TRP-1 (Alexa 647)	1µg/2µg	Melanogenesis

Appendix G: supplementary data

Optimisation of co-culture growth conditions

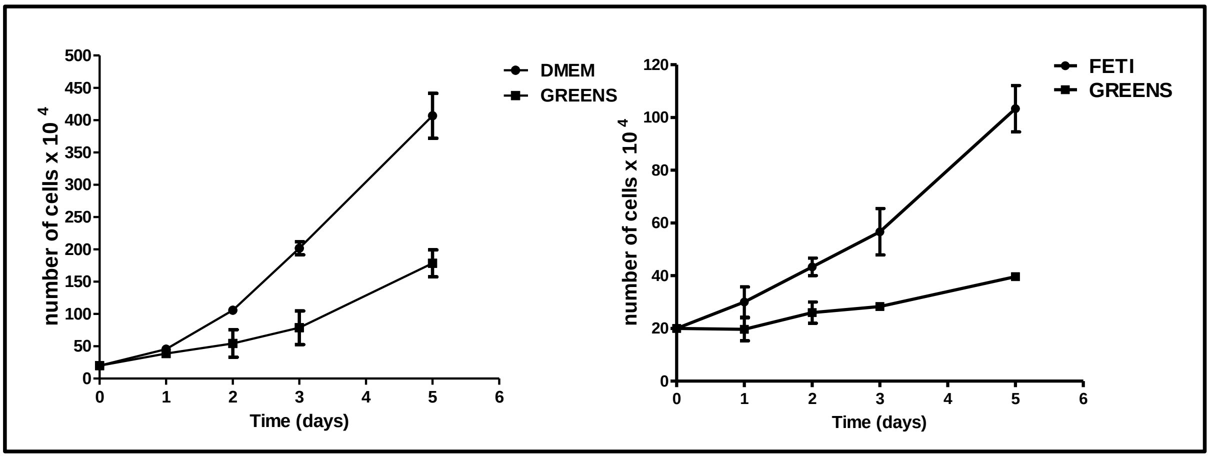

Figure F.1: The growth curves of immortalised human keratinocytes cultured in either DMEM or GREENS, and that of primary skin melanocytes cultured in either FETI of GREENS for a maximum of 7 days. At days 1, 2, 3, 5 and 7, cells were trypsinised and counted. Cell numbers are presented as mean ± SEM values. n=3.

Mono-cultures in their native media exhibit a much better growth as compared to when cells are cultured in GREENS (Figure F.1). However, when both keratinocytes and melanocytes are grown in a co-culture, there seems to be a stable maintenance of melanocyte numbers in a co-culture, with keratinocytes depicting much better growth rates in a co-culture (Figure F1; Section 2.2). Overall, these data let us to choose an optimal growth medium as GREENS due to that it relatively supports growth of these cells in a co-culture setting. Therefore all subsequent co-culture experiments were done in GREENS.

Optimisation of FACS analyses for co-cultures

The FACS separation technique worked excellently in deciphering keratinocyte population from melanocytes, with an almost 100% accuracy. This is shown by melanocytes being sorted as 93% of MART-1+ cells in a mono-culture, followed by an approximately 5:1 separation of keratinocytes from melanocytes (77% Kc and 17% Mc), as shown in Figure F.2. We therefore used this separation technique for all subsequent co-culture experiments.

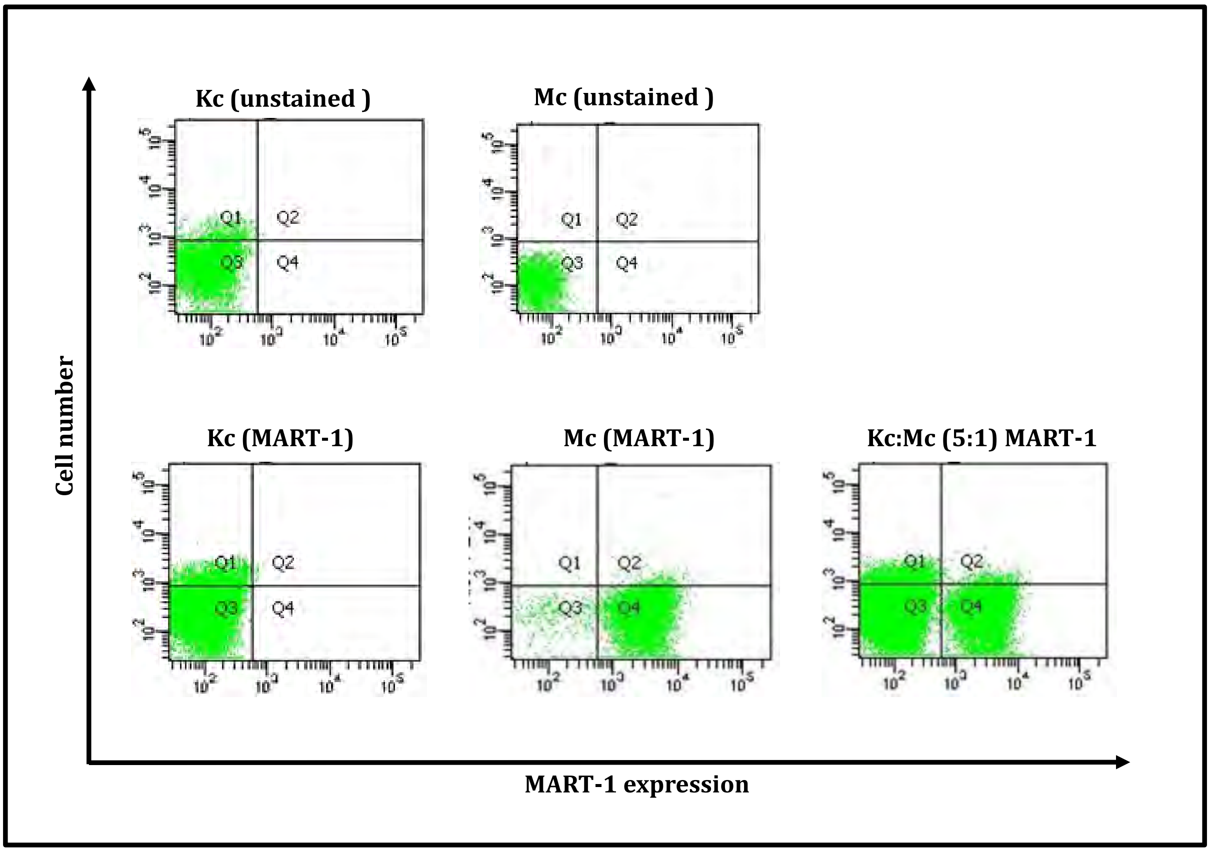

Figure F.2: Optimisation of the separation technique of keratinocytes and melanocyte co-cultures. Cells were culture overnight as either mono-/co-cultures, and then cells were trypsinised, fixed, permeabilised and acquired by FACS. Kc represented as MART1^{-} cells, while Mc were sorted as MART1^{+} cells. Cell numbers are represented as mean ± SEM values. n=3