CHARACTERIZATION OF HAIR FOLLICLE DERIVED STEM CELLS AND KERATINOCYTES INDUCTION FOLLOWING SEEDING ON CHITOSAN SCAFFOLD

By

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Thesis submitted in fulfillment of the requirements
for the degree of
Master of Science

UNIVERSITI SAINS MALAYSIA

April 2016

ACKNOWLEDGEMENTS

Bismillah,

In the name of Allah, The Most Gracious and The Most Merciful. Praise be to Allah S.W.T., Lord of the universe and selawat to Prophet Muhammad S.A.W., messenger of Allah. Thanks to Allah in the completion of this project. Alhamdulilah.

I would like to send my greatest appreciation to my understanding and beloved supervisor and co- supervisor; Professor Dr Ahmad Sukari Halim, Professor Zamzuri Idris and Dr Saidi Jaafar whom are very supportive and cooperative, guided me to achieve the good thesis and the advices, to that, I will always thankful. I am grateful to staffs at Tissue Engineering Laboratory (Unit Rawatan Kebakaran) and Operation Theater (OT) of Hospital Universiti Sains Malaysia (HUSM) and Advanced Materials Research Centre (AMREC)-SIRIM (Malaysia) for all the assistance. Due recognition is given to all the patients who willingly allowed and consented the use of their scalp samples during this project purposes.

I am indebted to many of my colleagues who have supported me directly and indirectly. I am forever grateful for all the personal guidance and assistance. I also wish to express my sincere gratitude to staff of Reconstructive Sciences Unit whom always been there to land hands throughout this period. I wish to express my deepest appreciation to my parents and family for their love and understanding. I would want to thank Ministry of Science and Technology Malaysia (MOSTI) for National Science

Fellowship (NSF) and Universiti Sains Malaysia (USM) for Research University Grant (1001/PPSP/812037) to support this research project.

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LIST OF SYMBOLS AND ABBREVIATIONS

AB	Alamar Blue
AMREC-SIRIM	Advanced Materials Research Centre
APC	Allophycocyanin
BrdU	bromodeoxyuridine bromodeoxyuridine
CeA	Culture epidermal Allograft
CK6	Cytokeratin 6
CnT	Cell-N-Tech

CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DP	Dermal Papilla
DPBS	Dulbecco's Phosphate Buffer Saline
ELISA	Enzyme Link Immunosorbent Assay
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
g	gravity
HFSCs	Hair Follicle Stem Cells
HRP	Horseradish Peroxidase
HUSM	Hospital Universiti Sains Malaysia
IHC	Immunohistochemistry
IRS	Inner Root Sheath
LRCs	Label Retaining Cells
MSC	Mesenchymal Stem Cells
OD	Optical Density
ORS	Outer Root Sheath
SIRIM	Standard and Industrial Research institute of Malaysia

SPSS	Statistical Package for the Social Sciences
SRT	Skin Regenerating Template
TBS	Tris Buffered Saline
USM	Universiti Sains Malaysia
3H-TdR	tritiated thymidine
Ca^{2+}	Calcium
ddH2O	Double distilled water
cm^2	Centimeter square
µg	Microgram
µL	Microliter
kGy	Kilogray
Kda	Kilo Dalton
mg	Milligram
mL	Milliliter
mm	Millimeter
mm^2	Millimeter square
nm	Nanometer

µM	Micromolar
mM	Millimolar
O_2	Oxygen
~	Approximately
%	Percentage
°C	Degree Celcius
v/v	volume/volume

PENCIRIAN SEL-SEL STEM DARIPADA FOLIKEL RAMBUT DAN MENGIDUKSIKAN SEL-SEL STEM KEPADA KERATINOSIT EPIDERMIS SETELAH DILETAKKAN KE ATAS SKAFOL/ACUAN KITOSAN

ABSTRAK

Folikel-folikel rambut yang berulangkali gugur akan kembali tumbuh. Ini menunjukkan terdapat sel stem intrinsik padanya. Sel stem dewasa diasingkan daripada folikel rambut mempunyai ciri unik iaitu berkebolehan untuk membeza kepada keratinosit epidermis. Kitosan templat regenerasi kulit (TRK) yang dihasilkan oleh AMREC-SIRIM telah berjaya digunakan sebagai skafol untuk kejuruteraan tisu kulit. Objektif kajian ini adalah untuk menyiasat kaedah pengasingan sel-sel stem folikel rambut (SSFR) daripada tisu kulit kepala dan pencirian sel-sel stem ini dilaksanakan. Kebolehan SSFR untuk melekat, pertumbuhan dan kebolehan untuk membeza kepada sel jenis lain di atas kitosan TRK juga telah dinilai. Pengasingan SSFR daripada kulit kepala manusia dijalankan menggunakan kaedah penceraian sel dan kemudiannya dikulturkan di dalam CnT-07 media pertumbuhan. SSFR yang berbentuk segi empat membentuk kumpulan-kumpulan sel yang bertumbuh baik di dalam media pertumbuhan CnT-07. Pencirian kultur SSFR dilakukan dengan menggunakan penanda sel stem iaitu K15 dan CD200. Kultur SSFR positif bagi kehadiran penanda K15 dan CD200. Pelekatan dan pertumbuhan SSFR di atas kitosan TRK dikaji menggunakan mikroskop imbasan elektron (MIE), ujian hidup/mati dan ujian Alamar Biru. Imej-imej MIE menunjukkan SSFR melekat dan bertumbuh di atas kitosan. Ujian hidup/mati menunjukkan populasi SSFR yang hidup di atas kitosan pada hari 1 adalah 216 ± 6 sementara itu SSFR yang mati adalah 99 ± 9 ($p=0.068$). Pada hari ke 2, populasi SSFR yang hidup di atas kitosan adalah 367 ± 18, manakala populasi SSFR yang mati adalah 213 ± 3 ($p=0.068$). Pada hari ke 3, populasi SSFR yang hidup adalah 452 ± 18 berbanding SSFR yang mati adalah 221 ± 9 ($p=0.068$). Populasi SSFR yang hidup dan mati di atas kitosan tidak menunjukkan perbezaan yang signifikan di antara pada hari 1, 2 dan 3. Ujian Alamar Blue juga menunjukkan OD dari hari 1 hingga hari 7 yang terus meningkat selari dengan

hari yang meningkat, menunjukkan SSFR dapat tumbuh dan berkembang pada kitosan. Min nilai OD SSFR yang berada di atas kitosan pada hari 7 adalah yang tertinggi berbanding dengan hari kultur pada 1 , 3, dan 5 (0,0207 ± 0,001 untuk hari 1 ; 0,0763 ± 0.003 untuk hari 3 ; 0,0746 ± 0,003 untuk hari 5; 0,1317 ± 0.020 untuk hari 7). Sel-sel stem ini juga diinduksi untuk membeza menjadi keratinosit epidermis menggunakan media pembezaan CnT-02. Pencirian epidermis keratinosit telah disahkan oleh kehadiran sel positif involucrin dan CK6. Dalam kajian ini, SSFR berjaya diasingkan dan bertumbuh di dalam media pertumbuhan CnT-07 dan mengekspresikan penanda sel stem K15 dan CD200. Keputusan kajian juga mengesahkan bahawa kitosan TRK sesuai bagi SSFR untuk melekat, bertumbuh dan juga menyokong pembezaan SSFR kepada epidermis keratinosit. Kajian ini memberikan pengetahuan tentang pengasingan, pertumbuhan dan kebolehan membeza SSRF di atas kitosan yang di masa hadapan boleh menjadi kaedah alternatif untuk merawat pesakit yang terbakar.

CHARACTERIZATION OF HAIR FOLLICLE DERIVED STEM CELLS AND KERATINOCYTES INDUCTION FOLLOWING SEEDING ON CHITOSAN SCAFFOLD

ABSTRACT

Hair follicles repeatedly regress and reconstitute themselves, suggesting the presence of intrinsic tissue stem cells. Adult stem cells isolated from hair follicle have a unique characteristic which is differentiating into keratinocytes. Chitosan skin regenerating template (SRT), produced by AMREC-SIRIM has been successfully used as a scaffold in skin tissue engineering. This study aims to investigate isolation of HFSCs from scalp tissues and the characterization of the stem cells was performed. The HFSCs attachment, growth and differentiation ability on chitosan SRTs were evaluated. HFSCs were isolated from human scalp tissues using cell dissociation method and then cultured in CnT-07 growth media. The squamous shaped HFSCs formed groups of cells and grown well in the CnT-07 growth media. The characterization of the cultured HFSCs was performed by using the stem cell marker of K15 and CD200. HFSCs culture were positive for the presence of K15 and CD200. Meanwhile, the attachment and growth of the HFSCs on the chitosan SRTs were evaluated using scanning electron microscope (SEM), Live/Dead assay and Alamar blue assay. The SEM images revealed that HFSCs were shown to attach and grown on chitosan. A live/dead assay shown that living HFSCs population on chitosan at day 1 was 216±6 meanwhile the dead HFSCs was 99±9 ($p=0.068$). At day 2, the population of viable HFSCs on the chitosan was 367±18, while the dead population of HFSCs was 213±3 ($p=0.068$). At day 3, the population of viable HFSCs was 452±18 compared with the dead HFSCs was 221±9 ($p=0.068$). The population of viable and dead HFSCs grown on chitosan showed no significant differences at day 1, day 2 and day 3. Alamar Blue assay also shown the OD from day 1 to day 7 continues to increase as the days increase, indicating HFSCs able to grow and proliferate on chitosan. The mean of the OD values of HFSCs grown on chitosan on the

day 7 was the highest compared to 1, 3, and 5 days of the culture (0.0207 ± 0.001 for day 1; 0.0763 ± 0.003 for day 3; 0.0746 ± 0.003 for day 5; 0.1317 ± 0.020 for day 7). These stem cells were also induced to differentiate into epidermal keratinocytes using CnT-02 differentiation media. The characterization of the epidermal keratinocytes was confirmed by the presence involucrin and K6 positive cells. In this present study, the HFSCs were successfully isolated, grown in CnT-07 growth media and expressing stem cell markers K15 and CD200. The study also proved that the chitosan SRT is suitable for HFSCs to attach, grow and also support the differentiation of HFSCs into epidermal keratinocytes. This study provides knowledge on HFSCs isolation and their growth and differentiation on chitosan that in future can be used as an alternative method in treating burn patients.

CHAPTER 1

INTRODUCTION

1.1 Research background

Hair follicles reconstitute themselves though the hair cycle, suggesting the presence of intrinsic stem cells. The stem cells were detected in the bulge area of hair follicles. The stem cells are morphologically undifferentiated and slow-cycling under the normal conditions. Studies demonstrated that stem cells possess stem cell properties such as high proliferative capacity and able to regenerate not only hair follicles but also sebaceous glands and epidermis confirming capacity to transform into different cell types (Cotsarelis *et al.*, 1990; Blanpain *et al.*, 2004; Cotsarelis, 2006; Ohyama, 2007). Hair follicle consists of epithelial sheaths, namely inner root sheath (IRS), hair shaft and outer root sheath (ORS) that is surrounded by a dermal sheath and connected to the dermal papilla (DP). These epithelial sheaths are located at the lower part of the follicles. The bulge region in the ORS is the reservoir for stem cells. Anagen (growing phase), catagen (regression phase) and telogen (resting phase) are the three phases required in the growth of hair follicle. The hair follicles are self-renewing structures due to the presence of stem cell (Paus and Cotsarelis, 1999; Ohyama, 2007).

A study has shown that, in case of epidermal injuries, stem cells from the bulge region migrated and proliferated to renew and repair wounded epidermal layer (Ito *et al.*, 2005). Apart from that, hair follicle stem cells generated all epithelial cell types of follicle involved in the formation of hair follicles (Morris *et al.*, 2004). The bulge region of the hair follicle comprises morphologically non-differentiated cells, providing the place of attachment for arrector pili muscle and are important to mark the lower most part of the hair follicle during the cell cycle (Cotsarelis *et al.*, 1990; Akiyama *et al.*, 1995). The advance in molecular and cellular biology paves way for researchers to study the various properties involving these stem cells (Ohyama *et al.*, 2006).

The location of stem cells was identified by taking advantage of the relative quiescence state of stem cells. Quiescence has been postulated to prevent stem cell exhaustion (Orford and Scadden, 2008) and is thought to help protect stem cells from acquiring mutations leading to putative cancer stem cells (Park and Gerson, 2005). Stem cells can be labeled by continuous administration of bromodeoxyuridine (BrdU) or tritiated thymidine (3H-TdR) for a prolonged period. During the subsequent chase period which the period of cells dilute their nuclear label through cell divisions. However, the cells that divide less frequently during the chase period retain the label. Hence they have been referred to as label-retaining cells. Label-retaining cells (LRCs) can be found in the bulge area using tritiated thymidine (3H-TdR) labeling technique. The LRCs are slow-cycling in nature (Cotsarelis *et al.*, 1990). The similar results were recorded when using 3H-TdR and BrdU double-labeling indicating LRCs are stem cells which were found in bulge region (Taylor *et al.*, 2000; Ito *et al.*, 2002).

These stem cells are self-renewable and can transform into hair follicles, epidermis and also sebaceous glands. The isolation of HFSC is necessary for future research and development in stem cell biology (Huang *et al.*, 2009). A study has shown success on regeneration of hair using hair follicle stem cells (HFSCs) via intracutaneous transplantation of engineered hair follicles germ cells (Toyoshima *et al.*, 2012). HFSCs have a high degree of plasticity and can be differentiated into a different cell phenotype when given appropriate stimuli. HFSCs can be transformed into neurons, melanocytes, smooth muscle cells in laboratory (Yu *et al.*, 2006), keratinocytes (Hilmi *et al.*, 2013b), corneal cells (Blazejewska *et al.*, 2009) and sweat gland cells (Wang *et al.*, 2012). Therefore, HFSCs have been a huge attraction as a cell source for tissue engineering (Mohd Hilmi *et al.*, 2013) and a target in gene therapy (Sugiyama-Nakagiri *et al.*, 2006).

In tissue engineering, cellular attachment, growth and their differentiation are among the important factors to be investigated. Tissue engineering is essential for the formation of biologically viable constructs to repair the damaged tissue. The main aspects of tissue engineering are cells and scaffold. Scaffold acts as template that guides cells to construct an organ (Lim and Halim, 2010). Naturally derived biomaterials have been attracting scientist's interest over the years. Natural based polymers offer the advantage of being similar to biological macromolecules, which the biological environment is prepared to recognize and deal with metabolically. Owing to their similarity with the extracellular matrix (ECM), natural polymers may also avoid the stimulation of chronic inflammation or immunological reactions and toxicity, often detected with synthetic polymers (Mano *et al.*, 2007).

Naturally derived biomaterial can be classified into classes of protein-based biomaterials (collagen) and polysaccharide-based biomaterials (chitin or chitosan). One of the widely used biomaterial is chitosan. Chitosan is the most abundant derivative of chitin. Chemical deacetylation of chitin produces commercial chitosan (Krajewska, 2005). Chitin can be found in the skeleton of marine and insects' invertebrates. Chitosan, structurally resembles with GAG polypeptide and consist of long chain, unbranched, repeating disaccharide units that is necessary in modulating cell morphology, differentiation and function (Lim and Halim, 2010). Chitosan have many other profitable features such as biocompatibility, non-toxicity (Lim *et al.*, 2011; Hilmi *et al.*, 2013a), antibacterial (Avadi *et al.*, 2004) and hemostatic (Periayah *et al.*, 2014). A chitosan SRT is a Malaysian made product which has been successfully used in skin tissue engineering (Lim *et al.*, 2011; Hilmi *et al.*, 2013a). This current study investigated the attachment, growth and differentiation ability of HFSCs on chitosan SRTs. These HFSCs have proven to be able to differentiate into various types of cells. Therefore, in future HFSCs can be used to produce a skin substitute with various functional features such as sensory and hairs.

1.2 The rationale of the study

As mentioned earlier the main objective of tissue engineering is to restore the function of lost or damaged tissues. Skin tissue engineering strategy involves combining isolated cells with suitable scaffolds. The scaffold acts as templates for tissue generation to guide the growth of a new tissue. A chitosan Skin Regenerating Template (SRT) is a template/scaffold produced by AMREC-SIRIM (Malaysia) which is suitable to be used in skin tissue engineering. Throughout the years, chitosan SRT has been investigated. Scientist has also developed ways to grow and culture adult stem cells. These stem cells have unique properties and are more suitable for skin tissue engineering as they can differentiate into different type of cells. However, investigation on the behavior of the HFSCs grown on the chitosan SRTs was none performed yet. This current study provides a report on the attachment, growth and differentiation potential of the HFSCs on chitosan SRTs. Besides that, this study also demonstrated on the isolation method of the HFSCs from scalps using a simple technique which is using collagenase type-I enzymatic digestion.

1.3 General objective

To isolate, culture and characterize hair follicle stem cells and its differentiation into epidermal keratinocytes on a chitosan Skin Regeneration Template (SRT).

1.4 Specific objectives

1) To isolate, culture and characterize hair follicle stem cells

2) To investigate attachment and growth of HFSCs on chitosan SRTs

3) To differentiate and analyze HFSCs into epidermal keratinocytes on chitosan SRTs

CHAPTER 2

LITERATURE REVIEW

2.1 Stem cells in general and hair follicles stem cells

Stem cells are known as undifferentiated cells that (1) self-renew for the long term (2) have the ability to differentiate into multiple cell types (3). Together, these properties will be referred to as “stemness” properties. Other cells in the body do not have these abilities. Three terms are frequently used to define the differentiation potential of stem cells: totipotent, pluripotent and multipotent. Cells from a fertilized oocyte, in the first few days after fertilization, are totipotent and can give rise to a fully functional organism. The pluripotent stem cells can give rise to every cell in the embryo

but will not give rise to the placenta or supporting tissues necessary for fetal development. While, the multipotent stem cells is capable to give rise to a subset of cell lineages (Watt and Hogan, 2000).

Stem cells can be classified as follows: embryonic and adult stem cells. Embryonic stem cells are found in the embryo, and are founder cells essential for tissue and organ development. Embryonic stem cells generally tend to self-renew better *in-vitro* and differentiate into a broader range of cells than adult stem cells. The therapeutic potential of embryonic stem cells has been experimentally demonstrated by many studies. However, the use of embryonic stem cells for tissue engineering is controversial from an ethical point of view (Thuret *et al.*, 2006).

Therefore, an alternative stem cell source is intensively studied for cell-based tissue engineering. One of the approaches is to isolate adult stem cells from tissues of patients (Moreau and Xu, 2009; Mohd Hilmi *et al.*, 2013). Adult stem cells are found in mature tissues and organs, and are responsible for the tissue maintenance. Adult stem cells are found not only in highly-regenerative tissues such as blood and hair follicles (Ohyama *et al.*, 2006), but also in less-regenerative organs such as the brain (Rietze *et al.*, 2001).

It has been known for several decades that the hair follicles contains a population of basal cells that exhibit the properties expected of somatic stem cells: slow cell cycle, high proliferative potential, location in a protected niche, capacity to maintain and repair the tissue in which they reside, and long life span (Lyle *et al.*, 1998; Morris and Potten,

1999; Cotsarelis, 2006; Ohyama, 2007). Slowly, cycling HFSCs have been identified by long-term nuclear retention of tritiated thymidine or bromodeoxyuridine label (Cotsarelis *et al.*, 1990; Lyle *et al.*, 1998). It was this technique, which showed that the bulge area hosts HFSCs (Cotsarelis *et al.*, 1990; Morris and Potten, 1999).

In vivo labelling and transplantation studies confirmed the ability of hair follicles stem cells to give rise to all cell lineages of the mature hair follicle (Taylor *et al.*, 2000; Morris *et al.*, 2004). In accordance with this hypothesis, major inflammatory damage of the stem cells can result in permanent alopecia (Mobini *et al.*, 2005). Unless the skin is injured, bulge stem cells exclusively contribute to hair follicle maintenance and regeneration (Ito *et al.*, 2005; Levy *et al.*, 2005). Apart from that, they are also capable of generating sebaceous glands and the interfollicular epidermis (Blanpain *et al.*, 2004; Morris *et al.*, 2004). Besides that, HFSCs able to transform into neurons and smooth muscle cells and many more (Yu *et al.*, 2006).

Therefore, HFSCs are isolated from mammalian adult hair follicles, and have been defined *in-vitro* as adherent cells that self-renew and have the capacity to clonally differentiate into multiple cell types. Because of these abilities, adult stem cells and their clinical applications have attracted many researchers for the past years. They may offer considerable opportunities for providing differentiated cells for gene therapy and tissue engineering. Stem cells could be harvested and transduced ex vivo and the corrected cells reintroduced into the host. Growth advantages of the corrected stem cells could offer new therapeutic approaches for genetic diseases. Besides that, HFSCs have been used for skin substitute, regeneration of cornea and bioengineered hair (Mavilio *et al.*, 2006; Meyer-Blazejewska *et al.*, 2011; Asakawa *et al.*, 2012; Mohd Hilmi *et al.*, 2013).

2.2 Anatomy of the human hair follicle

Hair follicles are involved in the production of hair and it is one of the defining characteristic of mammalian species. The presence of these skin appendages provides important insertion for microbial invasion into the mammalian organism, since they interrupt the integrity of the epidermal barrier. Hair has useful biological functions such as protection from the harmful external factors and dispersion of pheromones which are the excretion of the sweat-gland. Over five million hair follicles cover the human body, and about 100, 000 are found on the area of the scalp (Reithmayer, 2009). There are three important classes of hair follicles namely the lanugo, the vellus and the terminal hair follicle. The lanugo hair is very fine and normally shed *in utero* or during the first few weeks after the formation of the embryo. Vellus hairs are very short, non-pigmented, and normally not medullated and lack the presence of the arrector pili muscle. They are found all over the body surface with the highest density in the cheek and forehead where they display extremely large sebaceous glands. They undergo full hair cycle within a shorter period than terminal hair. Terminal hair is the long, pigmented and medullated hair which is predominantly found on the scalp. Apart from these differences, all hair follicles share the same functional construction (Paus *et al.*, 2008).

Mature hair follicles consist of eight epithelial layers; each of which came from terminal differentiation pathway. The epithelial layers are namely the medulla, cortex, and cuticle, which form the hair shaft (HS); another cuticle, the Huxley's and Henle's

layers (He), form the inner root sheath (IRS); the companion layer (Cl) and the outer root sheath (ORS) (Schneider *et al.*, 2009). ORS, IRS and HS are located in the bulge area, where the arrector pili muscle (APM) inserts into the ORS (Tiede *et al.*, 2007; Kloepper *et al.*, 2008). They originated from three main precursor cell populations descending from epithelial stem cells. The ORS which are produced from epithelial stem cells remain within the bulge area in later life, while the IRS and HS are postulated to be deposited in the secondary hair germ. The stem cells return to quiescent form after forming the new hair-generating cells. These stem cells rarely undergo the dividing phase (Paus and Cotsarelis, 1999; Reithmayer, 2009) (Figure 2.1).

During the dormant phase, the bulge cells exhibit stem-like characteristics which have long lifespan with their self-renewing capacity. This unique character inclines these cells into the formation of tumor. This is because longer lifespan of cells is prone to gain gene mutations due to environmental impacts like ultra violet rays or carcinogenic compounds (Morris *et al.*, 2004; Park and Gerson, 2005). The lower isthmus contains epithelial HFSCs in the bulge area. The bulge is the end of the permanent, non-cycling region. Bulge and anagen bulb (bulbar end of the hair follicle) are separated by a long stretch of suprabulbar hair-follicle epithelium (Cotsarelis *et al.*, 1990; Paus and Cotsarelis, 1999; Cotsarelis, 2006).

The hair cortex is equipped with associated proteins and intermediate filaments. The hair bulb has matrix cells that proliferates and produces hair shaft at the end. The melanocytes that are interspersed among the matrix cells produce pigment in the hair shaft. The DP which has specialized fibroblasts are found at the base of the follicle. They control the duration of anagen phase, the number of matrix cells, hair bulb size,

and hair shaft diameter and length. The interaction of the follicular epithelium with the adjacent mesenchymal dermal papilla plays an important role in determining the normal cycle and development of hair follicle (Schneider *et al.*, 2009).

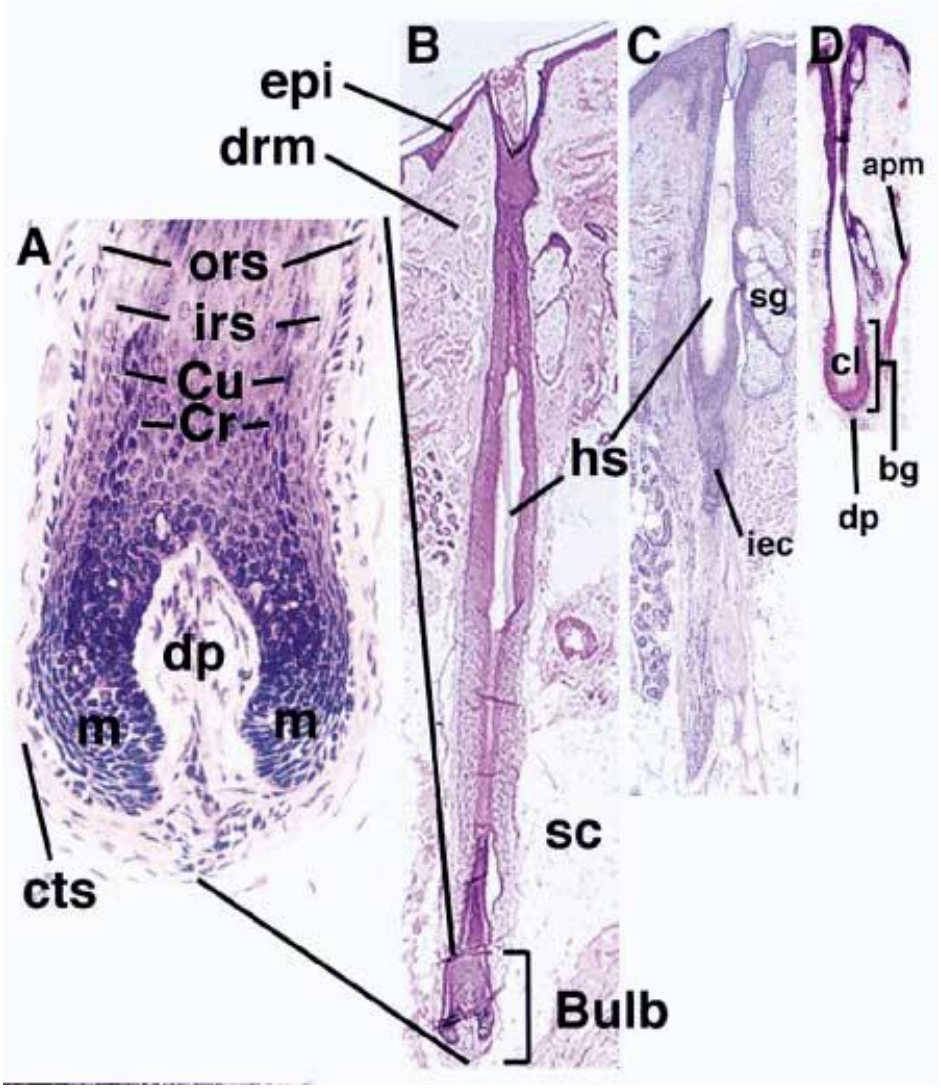

Figure 2.1: Scalp hair follicles structure during the Anagen, Catagen, and Telogen stages of hair cycling (Hematoxylin and Eosin). [A] (a high-magnification view of the portion of [B]). [A] Hair bulb during the anagen stage (100X magnification), [B] A hair follicle during the anagen stage (25X magnification), [C] A hair follicle during the catagen stage (40X magnifications). [D] A hair follicle during the telogen stage (25X magnification). (Notes: apm = arrector pili muscle, bg = bulge, cl=club hair, Cr=cortex, cts=connective-tissue sheath, Cu=cuticle, dp=dermal papilla, drm=dermis, epi=epidermis, hs=hair shaft, iec=involuting epithelial column, irs=inner-root sheath, m=matrix cells, ors=outer-root sheath, sc=subcutaneous fat, sg=sebaceous gland). Adapted from Paus and Cotsarelis (1999).

2.3 Location of hair follicle stem cell

Among the important biological features of stem cells is that they undergo a slow-cycling phase. This slow-cycling characteristic is particularly important as a way to maintain the proliferation potential of cells and reduce DNA replication-related errors (Morris *et al.*, 2004). Slow-cycling cells are also known as rarely cycling or Label Retaining Cells (LRCs) – (cells that retain labeled DNA for months) localize to a region of the outer root sheath (ORS) surrounding the hair shaft termed the "bulge." Accumulated evidence subsequently confirmed that the bulge is the repository of stem cells (Lyle *et al.*, 1998; Ohyama, 2007).

The LRCs experiment is a way to identify slow-cycling cells which represents keratinocyte stem cells (Taylor *et al.*, 2000; Ohyama *et al.*, 2006). The LRCs technique is performed by labeling all of the cells with a DNA precursor such as BrdU or 3H-TdR. This is followed by a washout procedure which results in labelling only slow-cycling stem cells. The labeled cells were found mostly at the bulge area which also known as HFSCs (Morris and Potten, 1999; Taylor *et al.*, 2000; Ito *et al.*, 2002; Ohyama *et al.*, 2006). Ohyama *et al.*, (2006) had done study using BrdU cells labelling to determine the localization or distribution of LRCs on the bulge region in order to isolate HFSCs which residing the bulge region. They also have reported on global gene expression profile of HFSCs that suitable to be used as markers for identification of isolated HFSCs. The HFSCs contribute to the cycling of the anagen part of the hair follicle. The HFSCs give rise to the follicle structures during each anagen phase (Stenn and Paus, 2001;

Cotsarelis, 2006). HFSCs appear to be programmed to able to regenerate epidermis under conditions of trauma, wounding, they can also completely regenerate a lost epidermis, e.g. after burns (Ito *et al.*, 2005). Thus, HFSCs have at least two different functions which are hair follicle remodeling in daily life and epidermal regeneration whenever skin integrity is severely compromised.

2.4 Hair Follicle cycle

Among all the organs of mammalian species, the hair follicle is the mammalian skin organ producing hair. The hair cycle is unique because of it is a long lasting cell cycling which is far outrunning the ovarian or endometrial cycle. Hair follicle morphogenesis begins since in embryonic development. Hair follicle morphogenesis starts from a signal of small epithelial budding in the epidermis right above a Dermal Papilla (DP) condensation and rapidly progresses to the generation of the mature hair follicle (pilosebaceous) unit. The maturation continues through the first postnatal week (Schmidt-Ullrich and Paus, 2005). At birth, the most mature hair follicles begin to break the skin surface. The first hair cycle starts when this mature hair follicle spontaneously enters the catagen phase. The hair cycle is divided into three main phases including anagen (the growing phase), catagen or also known as a regression phase and telogen (the resting phase). The exogen or also known as anagen onset phase is where the old hair shaft is shed out into environment (Cotsarelis, 2006) (Figure 2.2).

This growing phase of a hair follicle (anagen) is divided in 6 different stages (anagen I-VI), which can be distinguished using defined morphological criteria. In

average, an anagen scalp hair is growing 0.35mm a day and is not influenced by cutting or shaving. The duration of the growing phase defines the length of the hair follicle and it varies substantially from 2 to 6 years in terminal scalp hair to only 4 to 14 weeks in terminal *moustache* hair or even 6 to 12 weeks in vellus hair (Paus and Cotsarelis, 1999; Reithmayer, 2009). The catagen phase is divided into 8 stages (catagen I-VIII) and involves a rapid organ involution that driven by controlled apoptosis and terminal differentiation of a rather short time which is within 2 to 3 weeks.

While, in telogen (the resting phase) which last for about 3 months. During this resting period, the follicular melanin pigments production terminates, the hair shaft production stops and the hair shaft end is sealed off in a so called club. This club hair moves until it finds the non-cycling upper follicle. The DP shows no signs of apoptosis, but nevertheless condenses and also moves upwards (Stenn and Paus, 2001). After a telogen phase, a stimulus involving the DP signals the bulge stem cells or hair follicle stem cells at the base to initiate the regeneration of the lower follicle and produce a new hair shaft. Then, as the new hair shaft grows in, the old hair shaft is shed out into the environment. This is known as the exogen (shedding) phase (Figure 2). The only exit from this continuous cycling is the programmed organ deletion, in which selected, individual hair follicles are completely eliminated in the consequence of an inflammatory attack on the HFSCs. As this phenomenon occurs in otherwise healthy normal skin, it was postulated that this process is designed for clearing the skin from malfunctioning and undesired hair follicles (Cotsarelis, 2006).

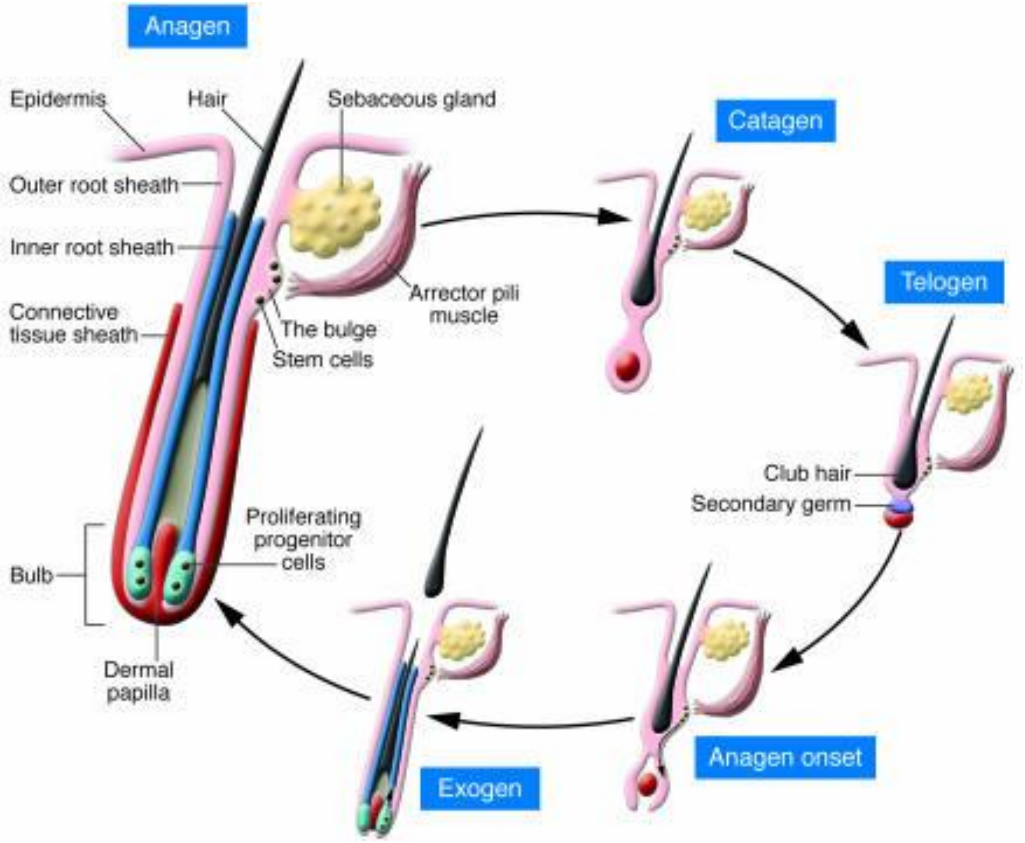

Figure 2.2: Hair follicle cycle. A hair follicle spontaneously enters the catagen phase and initiates the starts of the first hair cycle. The cycle is divided into catagen (regression phase), telogen (resting phase), anagen (growing phase) and exogen (shedding phase). Adapted from Cotsarelis (2006).

2.5 Multipotent (Differentiation ability) of HFSCs

Besides having a rarely dividing or slow cycling characteristic, HFSCs are known for their special property which is the ability to differentiate into various cell types or multipotent. HFSCs able to reproduce epidermal keratinocytes cells and hair matrix, inner and outer root sheath (IRS and ORS, respectively), companion layer, and hair shaft. This occurs during each new anagen phase under the control of an inductive, specialized mesenchyme (Cotsarelis *et al.*, 1990; Taylor *et al.*, 2000; Oshima *et al.*, 2001; Blanpain *et al.*, 2004; Cotsarelis, 2006). After isolation and cells expansion *in vitro*, HFSCs were shown to be able to transform into smooth muscle cells and sebaceous gland further confirming the multipotent capacity of the bulge cells (Sieber-Blum *et al.*, 2004; Yu *et al.*, 2006).

Ito *et al.*, have elucidated that HFSCs contribute to wound repair but do not contribute to normal epidermis homeostasis in non-wounded skin. Once the epidermis was damaged, the HFSCs and their progeny migrate out into epidermis to repair the wound. Thus, the HFSCs possess multipotent capacity associated with cutaneous epithelial stem cells but they predominantly reconstitute the hair follicles under normal conditions (Ito *et al.*, 2005). However, based on the clinical observation demonstrated that keratinocytes from hair follicles can repopulate wounds (Cotsarelis, 2006) or can be used to cure lesions like ulcers (Limat *et al.*, 2003).

Some cell populations (nestin-positive cells) isolated from the bulge area (the niche of HFSCs) able to transform into non-epithelial tissues such as neural cells. When implanted into the gap between ends of a severed sciatic nerve, these Nestin-positive cells transdifferentiated into predominantly Schwann cells, helping in the repair of the severed nerve (Amoh *et al.*, 2008; Amoh *et al.*, 2009). One of the major goals of various stem cell studies is to determine the ability of the stem cells to differentiate to another type of cells that can be used in tissue engineering. Therefore, in the current study, HFSCs differentiation ability were investigated. They were differentiated into epidermal keratinocytes which in future can be utilized to reform and re-establish the function of the damaged skin tissues.

2.6 Primary cell and tissue cultures

Cell culture refers to a culture derived from dispersed cells taken from original tissue of a primary culture or from an established cell line or cell strain using enzymatic and mechanical dissociation methods. The cells isolated from donors such as animal or human are known as primary cells (Schaffer, 1990). Cell culture or tissue culture is a process of harvesting cells from tissues and organs, culturing cells in appropriate growth media supplemented with growth factors and maintaining them under suitable cell culture conditions. The primary cell cultures are most commonly a combination of complex nutrient media, animal serum and also non-defined components. The culture process allows the cell to act as an independent single unit as seen in microorganisms like fungi and bacteria. The cells replicate by mitosis and this group of cells continues to

grow until reaching complete depletion of nutrients. The major advantage of cell culture is its physiochemical environment of cell culture such as osmotic pressure, pH, O_2, CO_2 and temperature can be controlled very precisely to allow consistency of the cells' growth. The original procedures or steps established for tissue culture involved maintenance of the tissue fragment on surfaces such as glass with supplied nutrients (Vunjak-Novakovic and Freshney, 2006).

However, the cells have a better growth performance when the tissues are separated out before culture. This can be done by cutting the tissue using scissors or blade which then further digested using proteolytic enzymes such as collagenase and trypsin. The proteolytic enzyme activity disaggregates the tissue to individual cells. The steps are commonly used in order to isolate cells, but some modifications if needed, are allowed to ensure a maximum yield of cells (Vunjak-Novakovic and Freshney, 2006). But the growth of cells could be hindered due to contamination of the culture by bacteria or fungi. The growth of the cells needs to be monitored closely as they could stop proliferating.

The development in cell culture techniques will be appreciated by various medical researchers. This is because tissue culture has also been used in many routine applications to the complicated medical treatments. As an example, epidermal keratinocytes cultures open possibilities in using individual's own cells in providing treatment to burn patients. This technique also known as *cultured epithelial autograft* (CEA). The patients' own epidermal keratinocytes isolated from a healthy skin grown in *in-vitro* condition and formed into sheets and later applied to the needed patient (Rheinwatd and Green, 1975; Auxenfans *et al.*, 2015). Another application of tissue

culture is to evaluate the effects or influence of various factors such as drugs and biomaterials. Tissue culture also could be used to test the toxicity of different types of products such as drugs and biomaterials *in vitro*. Cell culture techniques allow more specific investigation of the interested components and also produce rapid results by direct and indirect exposure to biomaterial. Besides that, it is cheaper than using a whole animal. Therefore, *in-vitro* toxicological testing has been used to test toxic components, drugs and biomaterials (Lim *et al.*, 2011). In addition, the issues of moral, ethical and legal questions when using animal could be avoided.

The major disadvantage of cell culture is loss of natural cell's microenvironment. The cells characteristic change after a particular time period and this hinders a long experimentation activity. This is due to depletion of nutrients and accumulation of waste products in the culture. The limitations in tissue culture are mainly involves the steps or the procedures used. Another disadvantage of tissue culture is that the culture techniques are needed to be done under strict aseptic conditions. This is to avoid cross and microorganism contamination. Besides that, the culture environment such as the incubation, pH, containment, workplace's aseptic and disposable of biohazards must be maintained regularly (Vunjak-Novakovic and Freshney, 2006).

2.7 HFSCs culture

Increase attentions in cell culture procedure have influenced the development of tissue culture techniques. Isolation of living bulge cells or HFSCs is important. It is much needed in clinical applications of regenerative medicine and to investigate the

HFSCs biology. Many experiments on isolation of HFSCs have been done. Previously, HFSCs were isolated from human scalp tissues by manual dissection under stereomicroscopy (Fu *et al.,* 2010). But the purity of the isolated cells was unclear. Therefore, Ohyama *et al.,* (2006) reported on scalp tissue dissection using an automatic navigated laser. The automatic navigated laser enables the team to guide and cut a better and precise HFSCs area for an accurate HFSCs isolation. They successfully isolated living HFSCs with *a* high proliferation rate. A different isolation technique for HFSCs has also developed for the isolation of HFSCs by flowcytometry-activated cell sorting (FACS). Tumbar and co-workers reported on a successful isolation of HFSCs via FACS. This method relied upon transgenic expressing fluorescent proteins that preferentially label HFSCs based on their slow-cycling nature (Tumbar *et al.,* 2004).

Yu and colleagues (2006) demonstrated a success in isolation of HFSCs from the scalp tissue via plucking out the hair follicles and cultured them in appropriate media (Yu *et al.,* 2006). Two times tissue enzymatic digestion using dispase solution followed by trypsin solution released HFSCs has also been reported (Yoo *et al.,* 2007). The current study demonstrated a simpler one type enzymatic tissue digestion using collagenase enzyme solution used to isolate HFSCs from human scalp tissue. The enzymatic digestion method to isolate HFSCs is feasible to yield healthy growing stem cells in a CnT-07 growth media (Noor *et al.*, 2012; Hilmi *et al.,* 2013b).

2.8 Markers for the characterization of HFSCs

One of the best ways to determine whether isolated HFSCs have been successfully cultured is by staining for surface markers. Many reports have described various biomarkers for identifying HFSCs. The most convincing evidence was provided in reports suggesting that K15 and CD200 are the efficient biomarkers for isolation of the HFSCs (Ohyama *et al.*, 2006; Inoue *et al.*, 2009). Keratins or cytokeratin are intermediate filament proteins responsible for structural integrity of epithelial cell. Lyle et al., (1998) reported that cytokeratin 15 (K15) is expressed in the location of the "Bulge". They demonstrated that the slowest-cycling keratinocytes, identified as LRCs, were dominant in the bulge area of the human hair follicle and also expressed K15 marker. Therefore, they are used as marker of relatively immature and undifferentiated keratinocytes in the bulge (Liu *et al.*, 2003; Yoo *et al.*, 2007; Hilmi *et al.*, 2013b). The higher expression of K15 protein was also observed during hyper proliferation in skin (Waseem *et al.*, 1999) or upon wounding (Porter *et al.*, 2000).

CD200 which was previously known as OX-2, is a type-1 transmembrane glycoprotein, expressed in different type of tissues including the thymus, nervous system, vascular endothelium, trophoblast, ovary, and various cells of the immune system (Barclay *et al.*, 2002; Clark *et al.*, 2003). When CD200 binds to its receptor CD200R, the signaling of CD200-CD200R attenuates the inflammatory cell activity surrounding hair follicles rescuing them from subsequent and highly damageable and autoimmune attack. The exact role of CD200 in bulge stem cell biology remains unclear. However, implanted skin grafts from $CD200^{-/-}$ mice into a wild-type mice shown a significant inflammation that eventually leading to the destruction of all hair follicles. Therefore, CD200 may play a role in the suppression of immune responses that help to

protect stem cells in the hair follicles from destruction by inflammation (Rosenblum *et al.*, 2004). Study by Inoue *et al.*, (2009) demonstrated that the expression of both K15 and CD200 were 33.3 % and 5.8 % respectively (Inoue *et al.*, 2009). CD200 protein is actively expressed in the basal layer of ORS which located within the human bulge area (Ohyama *et al.*, 2006). Reports done by Ohyama *et al* (2006) and Inoue *et al* (2009) have highlighted CD200 as among reliable marker to characterize HFSCs along with K15.

2.9 Marker for the characterization of keratinocytes

Keratinocytes is the major cell type (90% of epidermal cells) of the epidermis, which synthesizes keratin. The confirmation of the cells was performed using Keratin 6 or also known as Cytokeratin 6 (CK6) and involucrin markers. Cytokeratin 6 (CK6; 56 kDa) is one of the efficient markers for the characterisation of keratinocytes. CK6 is also well known for its strong induction in stratified epithelial cells that feature an enhanced cell proliferation rate or abnormal differentiation during wound healing in several disease such as psoriasis and cancers (Kaufmann *et al.*, 2001; Iizuka *et al.*, 2004). A study shown that using CK6 marker, approximately 80% of the isolated human skin epidermis keratinocytes and commercial normal human skin epidermal keratinocytes stained dark brown (Yusuf, 2012).

The skin provides an important barrier that protects the internal environment from outer influences. When the keratinocytes start dividing and migrates out from the

basal cell layer of epidermis to the outer surface of the skin, it reaches the terminal differentiation stage (Van Duijnhoven *et al.*, 1992). The synthesis of important structural, differentiation-dependent and catalytic proteins is initiated which consists of involucrin. Involucrin is a 96 kDa cell envelope protein that appears in the free form in the early stages of keratinocytes terminal differentiation (Eckert and Welter, 1996). Study done by Li and co-workers (2000) demonstrated that positive-involucrin cells were presence in the keratinocytes cultures (Li et al., 2000). Involucrin acts as a reliable marker for characterization of structural components of mature epithelial cells as it appears in the upper layer of the epidermis that involves in the differentiation of keratinocytes (Watt, 1983; Ivanova *et al.*, 2008). Involucrin marker was used in verification experiment of isolated human skin epidermis keratinocytes and commercial normal human skin epidermal keratinocytes. The involucrin shown approximately 80% brown staining in cytoplasm of the keratinocytes (Yusuf, 2012). Therefore, both CK6 and involucrin markers were used to characterize epidermal keratinocytes (Yusuf, 2012; Hilmi *et al.*, 2013b).

2.10 Chitin and Chitosan

Chitin (β-1,4-D-linked polymer of *N*-acetylglucosamine) is a natural polysaccharide. It is estimated that approximately one trillion tons of chitosan are produced every year making it one of the most abundant natural compounds on earth (Kurita, 2006). Chitin is visually characterized as a nitrogenous, inelastic, hard, white polysaccharide. Chitin is a common component of the exoskeleton of animals, especially