STUDIES ON THE GENE EXPRESSION OF HUMAN HAIR FOLLICLE BULGE STEM CELLS AND HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS UNDER DIFFERENT CULTURE CONDITIONS

By

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ABSTRACT

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HO SHU CHEOW

In psychosocial communication, hair plays the role as a symbol of youth, fertility and sexual potency. Alopecia, hair loss is not a disease and it is not life-threatening, however it affects the personal physical attractiveness of a person. Hair loss often has an underestimated psychosocial impact on an individual's self-esteem, interpersonal relationships and positioning within a society. A great deal of research shows that whatever the cause of hair loss, be it genetic factors, environmental factors, food ingestion and/or hormonal disturbances, more and more individuals are affected by alopecia and they tend to start at an earlier age. Therefore, alopecia demands treatment. To date, medical treatment of alopecia includes drug therapy and hair transplantation, which both are not the most effective to cure alopecia. Stem cell therapy is emerging as a potential therapy in various diseases, thus it has been taken into consideration as hair loss treatment with the presence of stem cells in hair follicle. Various *in vitro* studies demonstrated the culture methods affecting the

gene expression and morphological changes of cells. This study aimed to identify the gene expressions changes of human hair follicle bulge stem cells and human adipose-derived mesenchymal stem cells under different culture conditions and explore the potential of the application in hair loss treatment. Human adipose-derived mesenchymal stem cells were induced to exhibit dermal papilla properties using hanging drop culture. Human hair follicle bulge stem cells and human adipose-derived mesenchymal stem cells were subjected to transwell co-culture and alginate bead culture respectively. Human adipose-derived mesenchymal stem cells demonstrated dermal papilla properties and the potential to be used in hair regeneration. The gene expression results obtained from transwell co-culture and alginate bead culture results showed inconsistency, elaborated the impact of culture conditions in altering the gene expression of cells. This study provides fundamental knowledge in culturing stem cells targeting hair loss problem.

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LIST OF ABBREVIATIONS

β-ME	β-mercaptoethanol
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
2D	two-dimensional
3D	three-dimensional
ALPL	alkaline phosphatase
BIO	6-bromoinirubin-3'-oxime
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CT	cycle threshold
CTNNB1	catenin beta 1
DAPI	4',6-diamidino-2-phenylindole
DHT	dihydrotestosterone
DMEM	Dulbecco's Modified Eagle Medium
DMEM/F12	Dulbecco's Modified Eagle Medium/ Nutrient Mixture F12
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FBS	fetal bovine serum
FDA	Food and Drug Administration

FGF-2	fibroblast growth factor
FGF-7	fibroblast growth factor 7
h	hour
HAD-MSC	human adipose-derived mesenchymal stem cell
HFDPC	human follicle dermal papilla cell
HGF	hepatocyte growth factor
HHFBSC	human hair follicle bulge stem cell
HHFK	human hair keratinocytes
IGF-1	insulin-like growth factor 1
K6hf	keratin 75
KM	keratinocyes medium
LEF1	lymphoid enhancer binding factor 1
M	molar
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger RNA
P0	Passage 0
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PLL	poly-L-lysine
RNA	ribonucleic acid
rpm	revolutions per minute

RT-qPCR	real-time reverse transcription polymerase chain reaction
s	second
SOX2	SRY-box 2
TCF3	transcription factor 3
VCAN	versican
WNT5A	wingless-type MMTV integration site family, member 5A
x*g*	time gravity

CHAPTER 1

INTRODUCTION

Hair cycle involves anagen (growth), catagen (regression), and telogen (rest) phase. Hair growth occur in anagen phase, follicle produce the entire hair from tip to root; catagen is transition period, where hair growth is reduced, prepared to enter telogen where there is no hair growth occur. Hair cycle will start again once receive signal through Wnt and Shh signalling pathway. Hair loss occurs when most of the follicles are in catagen and telogen. A great deal of research shows that whatever the cause of hair loss, be it genetic factors, environmental factors, food ingestion and/or hormonal disturbances, more and more individuals are affected by alopecia and they tend to start at an earlier age. In psychosocial communication, hair plays the role as a symbol of youth, fertility and sexual potency. Alopecia, hair loss is not a disease and it is not life-threatening, however it affects the personal physical attractiveness of a person. Hair loss often has an underestimated psychosocial impact on an individual's self-esteem, interpersonal relationships and positioning within a society.

Currently available treatments are medications and hair transplantation. Medications for instance minoxidil and finasteride are effective to certain type of alopecia, but they come with adverse effects. Hair transplantation is invasive and costly, hair is redistributed from the non-balding area to balding area to cover the balding. However, it does not promise new hair growth on the balding site. Thus, an effective and promising treatment is needed for alopecia.

Stem cell therapy is studied and developed in various diseases, with the stem cell technology, it has become potential in hair loss treatment.

Bulge area, a contiguous part of outer root sheath; consist of morphologically undifferentiated and slow-cycling cells under the normal conditions. The stem cells in bulge area have been proven to possess stem cell properties such as high proliferative capacity and multipotency to regenerate not only hair follicles but also sebaceous glands and epidermis. Dermal papilla cells are specialized mesenchymal cells which located at the base of the hair follicle. They maintain the hair growth of the hair shaft through interactions with hair matrix cells. Dermal papilla has been reported to play a role in induction of the formation of the hair follicle, maintenance of hair shaft growth, differentiation of stem cells in hair and control of the hair cyclic activity. Bulge stem cells and dermal papilla cells are known to be the crucial components in hair development, the epithelial-mesenchymal of the cells lead to the generation of hair. Mesenchymal stem cells are adult stem cells with self-renewing capability. They were successfully isolated from various tissues, including the bone marrow, adipose tissue, umbilical cord, skin, dental tissue and other tissues. They were well established from the aspect of isolation, biological properties and they showed potential in diverse disease models.

A range of cell culture methods were developed and incorporated in cell based studies. The purpose of cell culture not limited to expand the cells *in vitro*, it is used to study the cell nature, and involved in disease modelling, drug discovery and to develop cell based therapy. Nonetheless, different culture conditions

alter the cell gene expression. Thus, this study is aimed to study the impact of culture conditions on gene expression.

The objectives of this study are:

1. To determine the optimum *in vitro* culture media and coating for human hair follicle bulge stem cells.
2. To induce the human adipose-derived mesenchymal stem cells into dermal papilla cells.
3. To study the effect of transwell co-culture on the gene expression of human hair follicle bulge stem cells and adipose-derived mesenchymal stem cells.
4. To study the effect of alginate bead culture on the gene expression of human hair follicle bulge stem cells and adipose-derived mesenchymal stem cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Hair follicle

Hair follicles are complex tissues composed of the dermal papilla (DP), dermal sheath (DS), outer root sheath (ORS), inner root sheath (IRS) and hair shaft. Development of hair follicles are controlled by epidermal-mesenchymal interaction, which is a signalling cascade between epidermal and mesenchymal cell populations (Yoo et al., 2010; Stenn et al., 2007). Hair follicle, consisting of epithelial cylinders under control of a proximal lying mesenchymal papilla, reconstitutes themselves through the hair cycle, these regenerative properties suggesting the presence of intrinsic stem cells (Stenn et al., 2007; Ohyama, 2007). Bulge area, a contiguous part of outer root sheath; consist of morphologically undifferentiated and slow-cycling cells under the normal conditions. The bulge cells have been proven to possess stem cell properties such as high proliferative capacity and multipotency to regenerate not only hair follicles but also sebaceous glands and epidermis (Ohyama, 2007). Unlike mesenchymal stem cells, the stem cells in the bulge area were given different names by different groups of the researchers; however, the location of the stem cells and the marker expression indicated that they are the same type of cells. The name given includes bulge stem cells, epithelial stem cells, follicular stem cells, epithelial hair follicle stem cells, human hair follicle stem cells (Mitsiadis et al., 2007; Ohyama, 2007; Inoue et al., 2009; Kloepper et al., 2008; Oh et al., 2011). The surface markers expressions of the stem cells in bulge include CD

200^{+}, CK 15^{+}, and CD 34^{-} (Inoue et al., 2009; Oh et al., 2011). Oh and colleagues (2011) had successfully isolated the stem cells from hair follicle using positive marker CD 200 and negative marker CD 34. They considered these populations as the purest population of hair follicle stem cells.

The dermal (mesenchymal) portion of the hair follicle consists of dermal papilla (DP) and dermal sheath (DS). Dermal papilla is located at the base of the hair follicle where it maintains the hair growth of the hair shaft through interactions with hair matrix cells (Yoo et al., 2010). Dermal papilla has been reported to play a role in induction of the formation of the hair follicle, maintenance of hair shaft growth, differentiation of stem cells in hair and control of the hair cyclic activity (Elliott et al., 1999). Dermal papilla are specialized mesenchymal cells and they express their own distinct makers, which includes alkaline phosphatase, laminin, versican, type 4 collagen and α-smooth muscle actin (Yang et al., 2010; Yoo et al., 2010).

2.2 Hair cycle

Hair cycle is an synchronised event consisted of anagen, catagen and telogen, happens repeatedly in hair follicle. Anagen is growth phase, which last 2 to 6 years, cells proliferate in follicle and form inner root sheath and migrate upward to form hair shaft. Catagen is regression phase, is a transition period, usually lasts 1 to 2 weeks, where hair growth is reduced, dermal papilla starts to detach from hair follicle. Telogen is rest phase, lasts 2 to 4 months. There is no hair growth in this phase, dermal papilla is separated from hair follicle. Hair cycle will start again when received signals via Wnt and Shh signalling

pathway. Commonly, 85-90 % of follicles are in anagen phase, 1-2 % in catagen phase, and 10% in telogen phase. Hair loss occurs when anagen phase is shortened or telogen phase is prolonged (Paus and Foitzik, 2004; Krause and Foitzik, 2006; Wosicka and Cal, 2010; Banka et al., 2013).

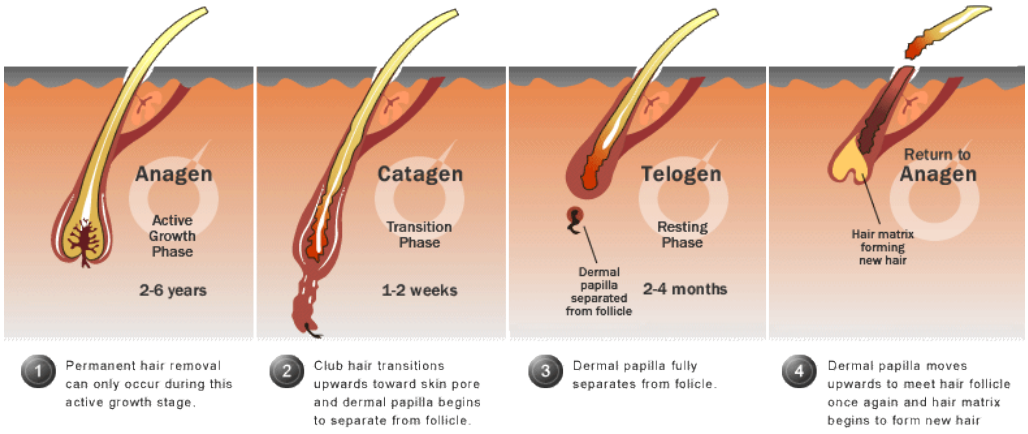

Figure 2.1 Hair Cycle

(Giselle, 2015)

Epithelial-mesenchymal interactions between the stem cells in hair follicle and dermal papilla cells are crucial for the hair development. During hair cycle, the stem cells in the hair follicle are stimulated to proliferate and differentiate as a response to the inductive signals from the underlying mesenchymal dermal papilla cells (Stenn et al., 2007; Roh et al., 2004). Wnt signalling is active during hair follicle morphogenesis; bulge stem cells express Tcf3, but not Lef1; the differentiation of the bulge stem cells leads to Tcf3 down-regulation and up-regulation of Lef1 (Mitsiadis et al., 2007). Therefore, Wnt mediated activation of Tcf3 and Lef1 appear to be important in determine the fate of the bulge stem cells. Besides, the proliferation markers Ki-67 and K10 is the

markers for the differentiated bulge stem cells (Zhang et al., 2006; Waters et al., 2007).

2.3 Hair loss

Hair loss or alopecia is common issue that bother both female and male. It refers to a condition where patient losing about 50% of the original hair (Zhao et al., 2008). There are a few classifications of hair loss, including female pattern hair loss, male pattern hair loss, alopecia areate, telogen effluvium, syphilitic alopecia and scarring alopecia (Jackson and Price, 2013).

Hair loss has psychological and social impact to patient even though it is not life-threatening disease. Hair loss patient regardless gender is reported loss of self-esteem, anxiety, obsessions, and distress (Banka et al., 2013; Brough and Torgerson, 2017).

The cause of hair loss can be hormonal change, stress, heredity, medication-induced and post-trauma. Despite the reason of hair loss, patients demand treatment. The currently available treatments are medication and hair transplantation. Minoxidil and finasteride are the FDA approved medications for male pattern hair loss; minoxidil is the FDA approved medication for female pattern hair loss (Banka et al., 2013; Adil and Godwin, 2017; Brough and Torgerson, 2017; Monsellise, 2017).

2.3.1 Current treatments and adverse effects

Minoxidil is approved by FDA at the 2 % and 5 % solution and 5 % foam, which are available over the counter. Minoxidil prolongs the anagen phase of hair follicles, and increases the diameter of existing hair (Banka et al., 2013; Nusbaum., 2013).

However, there are some adverse effects of minoxidil have been reported over the time. Allergic or irritant contact dermatitis is one of the most common side effects. Hypertrichosis is another adverse effect observed, with significantly increase incidence reported in women with 5 % minoxidil solution. Besides, tachycardia has been reported as one of the side effects of minoxidil. Patient with cardiovascular disease should be caution with minoxidil application. Other than that, Pregnant and breastfeeding women are not encouraged to use minoxidil (Banka et al., 2013; Nusbaum et al., 2013; Monselise et al., 2015).

Finasteride is a type II 5α-reductase inhibitor, reduce dihydrotestosterone (DHT) level in serum and scalp. It has been proven the most effective treatment for male pattern hair loss. Finasteride slows down the progression of male pattern hair loss, increase hair counts, hair diameter and growth rate (Ohyama, 2010; Banka et al., 2013; Nusbaum et al., 2013).

Despite the effectiveness of finasteride in treating male pattern hair loss, it causes loss of libido, ejaculatory dysfunction, gynecomastia, decreased ejaculate volume and depression. Discontinued of finasteride may resolve the

side effects, as the same time the effect of finasteride in treating hair loss also dissolve (Ohyama, 2010; Banka et al., 2013; Nusbaum et al., 2013).

Hair transplantation is surgical treatment for hair loss, which redistribute the hair on scalp, by removing the hair in a strip from non-bald area to the bald area. Hair transplantation does not promise hair growth on bald area; and it does not prevent the progression of hair loss. This procedure is costly and invasive, which come accompanied with risk of bleeding, infection, scarring and unnatural look after the transplantation (Jimenez-Acosta and Ponce, 2010; Ohyama, 2010; Vano-Galvan and Camacho, 2017).

Due the limitation and adverse effects of current treatments, hair loss requires effective treatment, which can solve the problem with minimal or no side effect.

2.4 Hair regeneration studies

Various *in* vitro studies have been carried out to generate hair. Based on concept of epithelial-mesenchymal interactions between keratinocytes stem cells and dermal papilla are crucial for hair follicle development, Roh et al. (2004) cultured keratinocytes stem cells and dermal papilla cells in transwell co-culture system for 5 days to study the gene expression of cells. The microarrays and protein level analysis suggested the stem cells were differentiate upon dermal papilla induction, however, they did not further report the cell morphology.

Ehama et al. (2007) cultured human keratinocytes derived from neonatal foreskin and murine dermal papilla cells in silicon chamber and co-grafting the cells into mice. Hair follicle-like structure growth was observed after 3 weeks. However, the hair follicle-like structure lack of regular hair structure indicated abnormal folliculogenesis.

Qiao et al. (2008) demonstrated the possibility to produce hair *in vitro*. They isolated follicular dermal and epidermal cells from embryonic mouse skin, forming aggregate using hanging drop method. The cellular aggregate form hair-like structure, termed "proto-hairs", was then implanted into mice, hair growth was observed after 2 weeks. However, they did not further report any studies employing human cells.

In 2012, Toyoshima and colleagues bioengineered a functional hair follicle through rearranging the stem cells and their niches in the bioengineered hair follicle. The study was conducted using mouse cells, epithelial cells and mesenchymal cells were isolated from mouse embryonic skin. Human stem cells and stem cells niche were suggested to be studied and optimised in order to produce bioengineered hair follicle.

Most of the studies were carried out using mouse model, and the results are fascinating and promising. However, further studies needed to be carried out using human cells in order to produce functional hair for clinical application.

2.5 Human adult stem cells

Stem cells are defined as undifferentiated cells that are able to self-renew, and differentiated into different lineage under appropriate conditions. Stem cells can be unipotent, multipotent, pluripotent, or totipotent, depending on the differentiation ability (Watt and Driskell, 2010; Chagastelles and Nardi, 2011; Sun et al., 2014).

Stem cells can be classified according to the source: embryonic stem cells and adult stem cells. Embryonic stem cells are isolated from embryo; adult stem cells are isolated from adult tissues (Schauwer et al., 2011; Sun et al., 2014).

Adult stem cells reside in stem cell niche in the body, they proliferate via symmetric division or asymmetric division. Symmetric division give rise to 2 stem cells, whereas asymmetric division give rise to 1 stem cells and 1 differentiated cell (Knoblich, 2008; Fuchs and Chen, 2012; Januschke and Nathke; 2014).

The source of adult stem cells include: bone marrow, adipose tissue, skin, skeletal muscle, heart, liver, amniotic fluid and blood (Chagastelles and Nardi, 2011; Dziadosz et al., 2016; Duelen and Sampaolesi, 2017).

2.5.1 Human mesenchymal stem cells

Human mesenchymal stem cells are adult stem cells with self-renewing ability. They are reported as multipotent stem cells, which are able to differentiate into multi lineage tissues. Human mesenchymal stem cells were first isolated from bone marrow in 1976 by Friedenstein and his colleagues. Subsequently

mesenchymal stem cells were isolated from various source of tissues including adipose tissue, umbilical cord, liver, brain, dental tissue, skeletal muscle, periosteum and amniotic fluid (Sakaguchi et al., 2005; Chen et al., 2008; Rastegar et al., 2010). Among the sources of mesenchymal stem cells, bone marrow, adipose tissue and umbilical cord-derived mesenchymal stem cells are more established with studies. Kern et al. (2006), "No significant differences concerning the morphology and immune phenotype of the MSCs derived from these sources were obvious".

International Society for Cellular Therapy has proposed 3 minimum criteria to define mesenchymal stem cells: (1) plastic adherent when maintained in standard culture conditions (2) express CD 105, CD 73 and CD 90; and lack of expression of CD 45, CD 34, CD 14, CD 19 and HLA-DR surface markers, (3) able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* (Dominici et al, 2006; Rastegar et al., 2010; Ullah et al., 2015).

The diverse distribution and ease of isolation of mesenchymal stem cells made them a potential candidate for therapeutic application. In addition, the immunoprivileged nature of mesenchymal stem cells permit allogenic transplantation without immunosuppression. The beneficial features of mesenchymal stem cells raise increasing interest, and hence they were studied in various disease model to explore the potential in clinical application, including orthopaedic application, cardiovascular disease, neurodegenerative disease, autoimmune disease, respiratory disorders (Chen et al., 2008; Meirelles et al., 2009; Ullah et al., 2015).

2.6 Three-dimensional cell culture

Mono layer culture method has been widely used in *in vitro* study to culture primary cells, however, mono layer culture cannot reflect *in vivo* condition, thus researchers have been looking for a culture method that manage to simulate *in vivo* condition. Three-dimensional (3D) culture has been adopted to replace mono layer culture in order to gather relevant *in vivo* like data in various study, especially disease modelling and tissue engineering. 3D cell culture can be with or without scaffold, cellular spheroids are simple 3D culture model without scaffold. Cellular spheroids can be produced from wide range of cells based on their aggregative tendency. The cellular spheroids produced are readily imaged by light and fluorescent microscope. Cellular spheroids are used in modelling solid tumour growth and metastasis studies, and in some therapeutic studies. Hanging drop method is one of the broadly used method to generate cellular spheroids, it is commonly used in formation of embryonic bodies in embryonic stem cell study (Haycock, 2011).

The emerging of cell-based therapies have stimulate the development 3D culture technologies, extracellular matrix, filter well insert, gel and microcarriers have been invented for 3D culture application. Filter well insert is one of the first technologies in 3D culture, allowing the interaction between cell populations. Transwell co-culture allow cell-cell interactions between population on upper compartment and population on lower compartment, with certain degree of separation between 2 populations in the culture. It is commonly used to study natural interactions in nature, to improve culturing

process and in engineering synthetic interactions between cell populations (Justice et al., 2009; Goers et al., 2014).

Alginate bead culture is a culture method that encapsulate the cell population in a scaffold. It allows the cells to proliferate, infiltrate and differentiate within the alginate bead. It is a common culture method to culture chondrocytes *in vitro.* Alginate is a naturally occurring anionic polymer that derived primarily from brown seaweed. It is biodegradable and non-animal origins; thus, it is widely used in tissue engineering, tissue regeneration and wound dressings. Human cells lack of receptors to alginate allow alginate to be used as scaffold in drug, growth factor and cell delivery (Lee and Mooney, 2012; Sun and Tan, 2013).

CHAPTER 3

MATERIALS AND METHOD

3.1 *In vitro* expansion of cells

Four different cells were used in this study; they are human hair follicular keratinocytes (HHFK), human hair follicle bulge stem cells (HHFBSCs), human adipose-derived mesenchymal stem cells (HAD-MSCs), and human follicle dermal papilla cells (HFDPC). The cells are acquired from different sources and manufacturers. They are *in vitro* expanded and cryopreserved upon receipt prior subjected to experiment.

3.1.1 Expansion of human hair follicular keratinocytes (HHFK)

Primary human hair keratinocytes (HHFK) are purchased from ScienCell (Carlsbad, CA, USA). The cells were isolated from human scalp, cryopreserved at P0 and delivered frozen as mentioned in data sheet (Appendix A). The cells were thawed and maintained in ScienCell's Keratinocyte Medium (KM) on poly-L-lysine coated culture vessel (2 μg/cm^2) at 37 ℃ in 5 % CO_2 humidified incubator. The medium was changed every 3 days and passaged at the 80% confluency using TrypLE Express™ at 37 ℃ for 3 to 5 min. TrypLE™ Express was removed by centrifugation at 300 x*g* for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in fresh medium. The cells were seeded in T75 flask pre-coated with poly-L-lysine at density of 5000 cells/cm^2. The cells were expanded and

cryopreserved in freezing medium (PromoCell, Heidelberg, Germany), at -196 ℃ in liquid nitrogen vapor phase until further experiment.

3.1.2 Expansion of human hair follicle bulge stem cells (HHFBSCs)

Primary human hair follicle stem cells (HHFSCs) were obtained from Celprogen (San Pedro, CA, USA). The cells were derived from donor's frontal region scalp, extracted from hair follicle bulge; the details were stated in data sheet (Appendix B) and certificate of analysis (Appendix C). The cells were received in frozen ampule, shipped with dry ice. The cells were thawed and maintained in Celprogen's Human Hair Follicle Complete Media with Serum on Celprogen's Human Hair Follicle Stem Cell Culture Extra-Cellular Matrix at 37 ℃ in 5 % CO_2 humidified incubator. The medium was changed every 2 days. The cells were passaged at the 80 % confluency using TrypLE™ Express (Gibco). The cells were trypsinised with TrypLE™ Express at 37 °C. The cells were seeded in T25 flask pre-coated with Celprogen's Human Hair Follicle Stem Cell Culture Extra-Cellular Matrix at density of 5000 cells/cm^2. The cells were expanded and cryopreserved in freezing medium (PromoCell), at -196 ℃ in liquid nitrogen vapor phase until further experiment.

3.1.3 Expansion of human follicle dermal papilla cells (HFDPCs)

Primary human follicle dermal papilla cells (HFDPCs) were obtained from PromoCell (Heidelberg, Germany). The cells were isolated from donor. The cells were received in frozen vial, shipped with dry ice. The cells were thawed upon receipt, and seeded in T25 flask, maintained in PromoCell Follicle Dermal Papilla Cell Growth Media at 37 ℃ in 5 % CO_2 humidified incubator.

The medium was changed every 2 days. The cells were passaged at the 80 % confluency using PromoCell DetachKit, followed manufacturer's protocol. The cells were seeded in T25 flask at density 8000 cells/cm^2. The cells were expanded and cryopreserved in freezing medium (PromoCell), at -196 ℃ in liquid nitrogen vapor phase until further experiment.

3.1.4 Expansion of human adipose-derived mesenchymal stem cells (HAD-MSCs)

Primary human adipose-derived mesenchymal stem cells (HAD-MSCs) were obtained from collaborator, CryoCord Sdn. Bhd. The cells were donated by liposuction patient to CryoCord Sdn. Bhd. The cells were maintained in Dulbecco's Modified Eagle Medium/ Nutrient Mixture F12 (DMEM/F12) supplemented with 10 % fetal bovine serum (FBS) and 1 % GlutaMAX™ (Table 3.1) at 37 ℃ in 5 % CO_2 humidified incubator. Five ng/mL epidermal growth factor (EGF) and 5 ng/mL fibroblast growth factor 2 (FGF-2) were added prior to feeding or passaging, to prevent absorption of growth factors to medium storage containers. The medium was changed every 2 days. The cells were passaged at the 80 % confluency using TrypLE™ Express. The cells were trypsinised with TrypLE™ Express at 37 °C for 3 to 5 min. TrypLE™ Express was removed by centrifugation at 300 x*g* for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in fresh medium. The cells were seeded in T75 flask at density of 5000 cells/cm^2. The cells were expanded and cryopreserved in freezing medium (PromoCell), at -196 ℃ in liquid nitrogen vapor phase until further experiment.

Table 3.1 HAD-MSCs media composition

Component	Concentration
DMEM/F12 basal medium	-
Fetal bovine serum (FBS)	10 %
GlutaMAX™	1 %
*Epidermal growth factor (EGF)	5 ng/ml
*Fibroblast growth factor 2 (FGF2)	5 ng/ml

*freshly added to cells during passaging or media changing.

3.2 Cell Culture Experiments

This study was divided into 4 parts:

Part 1: Optimisation of culture conditions for human hair follicle bulge stem cells.

Part 2: Induction of human adipose-derived msesenchymal stem cells into dermal papilla cells.

Part 3: Transwell co-culture of human hair follicle bulge stem cells and human adipose-derived mesenchymal stem cells using cell culture insert.

Part 4: 3D culture of bulge stem cells and adipose-derived mesenchymal stem cells using alginate bead culture.

3.2.1 Optimisation of culture conditions for human hair follicle bulge stem cells

Human hair follicle bulge stem cells were purchased from Celprogen, with cell culture media and pre-coated culture flasks. However, the culture media components and extracellular matrix in pre-coated culture flask information

were unknown, thus the below coating test and media test were carried out to find out the optimum culture conditions for further expansion the cells.

3.2.1.1 Coating Test

Human hair follicle bulge stem cells were subjected to 4 different coatings, and the pre-coated flask purchased from Celprogen to identify the optimum coating for expansion purpose. Pre-coated flask purchased from Celprogen served as the control, while the coatings selected for testing included normal cell culture dish, fibronectin, laminin and Matrigel. Human hair follicle bulge stem cells were seeded at the density of 5000 cells/cm^2, and maintained in the Celprogen's Human Hair Follicle Complete Media with Serum at 37 ℃ in 5 % CO_2 humidified incubator. The cultures were maintained for 4 days and sub-cultured when the cells reached 80 % confluency. Cell attachment and cell morphology were observed and imaged daily using camera attached inverted light microscope.

3.2.1.2 Media Test

Human hair follicle bulge stem cells were subjected to 4 culture media to find out the optimum culture media for expansion purpose. Culture media from Celprogen as control, 4 different culture media were prepared according to literature study. The formulation of culture media as in Table 3.2. Human hair follicle bulge stem cells were seeded at the density of 5000 cells/cm^2, and maintained in the Celprogen's culture media and 4 experimental culture media at 37 ℃ in 5 % CO_2 humidified incubator. The cultures were sub-cultured at 80 % confluency and maintained for 2 passages. Cell morphology was observed and imaged using camera attached inverted light microscope.

Table 3.2 Media composition for media testing for human hair follicle bulge stem cells.

Component	Control: Celprogen media	Media 1	Media 2	Media 3	Media 4
Basal media		William E	DMEM/F12 premix media (1:1)	Ham's F12	DMEM high glucose: Ham's F12 (3:1)
Fetal bovine serum (FBS)		-	5 %	5 %	5 %
Non-essential amino acid (NEAA)		-	1 %	1 %	1 %
GlutaMAX™		2 mM	2 mM	2 mM	2 mM
Minoxidil		200 ng/ml	200 ng/ml	200 ng/ml	200 ng/ml
Insulin	Celprogen's formula	10 µg/ml	10 µg/ml	10 µg/ml	10 µg/ml
Hydrocortisone		10 ng/ml	10 ng/ml	10 ng/ml	10 ng/ml
*β-mercaptoethanol (β-ME)		-	50 µM	50 µM	50 µM
*6-bromoinirubin-3'-oxime (BIO)		1 µM	1 µM	1 µM	-
*Hepatocyte growth factor (HGF)		20 ng/ml	20 ng/ml	20 ng/ml	-
*Insulin-like growth factor 1 (IGF-1)		20 ng/ml	20 ng/ml	20 ng/ml	-
*Fibroblast growth factor 7 (FGF-7)		10 ng/ml	10 ng/ml	10 ng/ml	-
*Epidermal growth factor (EGF)		-	-	-	10 ng/ml
*Fibroblast growth factor (FGF-2)		-	-	-	20 ng/ml

*freshly added to cells during passaging or media changing.

3.2.1.2 Media Test for Viability

This media test was carried out to study the viability of human hair follicle bulge stem cells in this in-house developed culture media. There were limited studies on this human hair follicle bulge stem cells, including the culture conditions, thus this media test is necessary. Human hair follicle bulge stem cells were seeded at the density of 5000 cells/cm^2, and maintained in the in-house developed culture media at 37 ℃ in 5 % CO_2 humidified incubator. The cultures were sub-cultured at 80 % confluency and maintained for 2 passages. Cell morphology was observed and imaged using camera attached inverted light microscope. The formula of the in-house developed culture media as shown in Table 3.3.

Table 3.3 Composition of in-house developed culture media

Component	Concentration
DMEM high glucose basal medium	3 parts
Ham's F12	1 part
Fetal bovine serum (FBS)	5%
Non-essential amino acid (NEAA)	1%
GlutaMAX™	1%
Minoxidil	200 ng/ml
Insulin	10 μg/ml
Hydrocortisone	10 ng/ml
*β-mercaptoethanol (β-ME)	50 μM
*6-bromoinirubin-3'-oxime (BIO)	1 μM
*Hepatocyte growth factor (HGF)	20 ng/ml
*Insulin-like growth factor 1 (IGF-1)	20 ng/ml
*Fibroblast growth factor 7 (FGF-7)	10 ng/ml

*freshly added to cells during passaging or media changing.

3.2.2 Induction of human adipose-derived msesenchymal stem cells into dermal papilla cells.

Human follicle dermal papilla cells and human adipose-derived mesenchymal stem cells were maintained in their culture media respectively until 80 % confluency. The cells were trypsinised using TrypLE™ Express at 37 ℃ for 3 to 5 min. TrypLE™ Express was removed by centrifugation at 300 x*g* for 5 min at room temperature. The supernatant was removed and the pellet was resuspended in fresh medium. Cell count was performed and the cell suspension was adjusted to 4 x 10^5 cells/ml. Five ml of phosphate buffered saline (PBS) was placed on the 60 mm tissue culture dish, to act as hydration chamber. The lid was inverted and 10 µl cell suspension was deposited onto the lid surface. The lid was then inverted onto the PBS-filled bottom chamber as shown in Figure 3.1 and incubated at 37 ℃ in 5 % CO_2 humidified incubator. The culture dish was observed after 24 h to assess aggregate formation. Aggregate formed were divided into 2 groups: a group of 4 to 5 aggregates were subjected to immunofluorescence staining; the rest of the aggregates were subjected to RNA isolation.

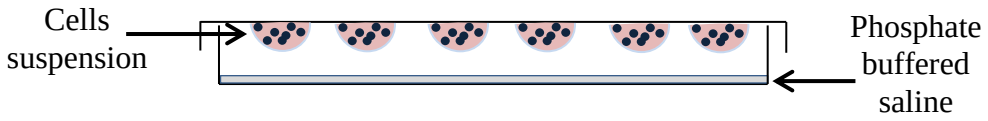

Figure 3.1 Hanging drop culture

3.2.2.1 Immunofluorescence staining

The aggregates were subjected to immunofluorescence staining with versican antibody (Bioss, USA). The aggregates were fixed in iced cold methanol for 7 min at room temperature. The aggregates were washed with phosphate buffered saline (PBS) for 4 min at room temperature twice to remove the methanol. The aggregates were then incubated with 1 % bovine serum albumin (BSA) for 1 h at room temperature to prevent nonspecific antibody binding. The aggregates were washed with PBS for 4 min at room temperature twice to remove the BSA. The aggregates were then incubated with rabbit anti-human versican antibody (1: 500) for 2 h at room temperature. The aggregates were washed with PBS for 4 min at room temperature twice to remove the antibody. Next, the aggregates were incubated with Texas Red conjugated goat anti-rabbit antibody (1:1000) for 2 h at room temperature in the dark. The aggregates were washed with PBS for 4 min at room temperature twice to remove the antibody. The aggregates were then counterstain with 4',6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature in the dark. The aggregates were then washed with PBS for 4 min at room temperature twice to remove DAPI. The aggregates were observed under fluorescence microscope.

3.2.2.2 RNA isolation

The aggregates formed were collected and trypsinised with TrypLE™ Express at 37 ℃ for on shaking incubator at 100 rpm for 10 min. The TrypLE™ Express was removed by centrifugation at 300 x*g* for 5 min at room temperature. The supernatant was removed and the pellet was subjected to RNA isolation using TRIzol (Invitrogen, Life Technologies, USA), according to manufacturer's instruction.

3.2.3 Transwell co-culture of human hair follicle bulge stem cells and human adipose-derived mesenchymal stem cells using cell culture insert.

Transwell co-culture system was used to study the interactions between two populations. Two different populations were cultured in the in-house developed media for 3 days. Six-well plate cell culture insert with 0.4 μm pore size was used to allow the diffusion of media components as well as the intercellular signaling between 2 cultured populations. The transwell co-culture system was illustrated in Figure 3.2 and the combination of transwell co-culture combinations was showed in Table 3.4. As control for the experiment, all 4 type cells were cultured individually in the in-house developed medium for 3 days.

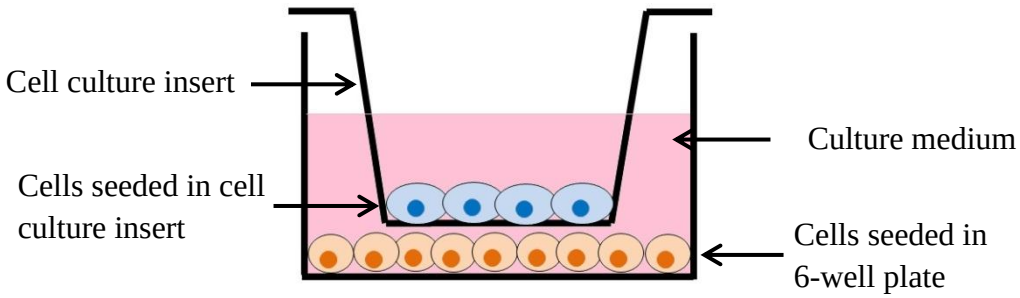

Figure 3.2 Transwell co-culture system

Table 3.4 Transwell co-culture combinations.

6-well plate	0.4 μm cell culture insert
HHFK	HAD-MSCs
HHFBSCs	HFDPCs
HHFBSCs	HAD-MSCs

3.2.3.1 Co-culture of human hair follicular keratinocytes (HHFK) and human adipose-derived mesenchymal stem cells (HAD-MSCs)

Six-well plate was pre-coated with poly-L-lysine (PLL), at coating density 2 μg/cm^2 by incubating at 37℃ for overnight. The plate was then rinse with sterile water twice to completely remove the coating solution. Human hair follicular keratinocytes (HHFK) were seeded in pre-coated 6-well plate at the density of 6000 cells/cm^2 and maintained in Keratinocyte Medium (KM) for 2 days. The media was then changed to 50% KM and 50% in-house developed media for another 2 days.

Human adipose-derived mesenchymal stem cells (HAD-MSCs) were plated in 0.4 μm cell culture insert at seeding density of 6000 cells/cm^2 and maintained in DMEM/F12 (Table 3.1), for 2 days. The media was then changed to 50% DMEM/F12 and 50% in-house developed media and maintained for another 2 days.

The 0.4 μm cell culture insert plated with HAD-MSCs was moved to the 6-well plate seeded with HHFK for co-culture. The media for both the insert (HAD-

MSCs) and 6-well plate (HHFK) were changed to 100% in-house developed media. The co-culture was maintained for 3 days.

3.2.3.2 Co-culture of human hair follicle bulge stem cells (HHFBSCs) and human follicle dermal papilla cells (HFDPCs)

Six-well plate was pre-coated with human fibronectin, at the density of 2 μg/cm^2. The plate was incubated at room temperature for at least 1 h, followed by washing with phosphate buffered- saline (PBS) twice to completely remove the coating solution. Human hair follicle bulge stem cells (HHFBSCs) were seeded in pre-coated 6-well plate at the density of 6000 cells/cm^2 and maintained in media 4 (result from media test in 3.2.1.2) for 2 days. The media was then changed to 50% HHFSCM and 50% in-house developed media for another 2 days.

Human follicle dermal papilla cells (HFDPCs) were plated in 0.4 μm cell culture insert at seeding density of 6000 cells/cm^2 and maintained in Follicle Dermal Papilla Cell Growth Media (FDPCGM) for 2 days. The media was then changed to 50% HFDPCs and 50% in-house developed media and maintained for another 2 days.

The 0.4 μm cell culture insert plated with HFDPCs was moved to the 6-well plate seeded with HHFSCs for co-culture. The media for both the insert (HFDPCs) and 6-well plate (HHFSCs) were changed to 100% in-house developed media. The co-culture was maintained for 3 days.

3.2.3.3 Co-culture of human hair follicle bulge stem cells (HHFBSCs) and human adipose-derived mesenchymal stem cells (HAD-MSCs)

Six-well plate was pre-coated with human fibronectin, at the density of 2 μg/cm^2. The plate was incubated at room temperature for at least 1 h, followed by washing with phosphate buffered saline (PBS) twice to completely remove the coating solution. Human hair follicle stem cells (HHFSCs) were seeded in pre-coated 6-well plate at the density of 6000 cells/cm^2 and maintained in media 4 (result from media test in 3.2.1.2) for 2 days. The media was then changed to 50% HHFSCM and 50% in-house developed media for another 2 days.

Human adipose-derived mesenchymal stem cells (HAD-MSCs) were plated in 0.4 μm cell culture insert at seeding density of 6000 cells/cm^2 and maintained in DMEM/F12 (Table 3.1), for 2 days. The media was then changed to 50% DMEM/F12 and 50% in-house developed media and maintained for another 2 days.

The 0.4 μm cell culture insert plated with HAD-MSCs was moved to the 6-well plate seeded with HHFSCs for co-culture. The media for both the insert (HAD-MSCs) and 6-well plate (HHFSCs) were changed to 100% in-house developed media. The co-culture was maintained for 3 days.

3.2.3.3 RNA isolation

All the cells involved in transwell co-culture were harvested for RNA isolation. The cells were trypsinised from cell culture inserts and 6-well plates respectively, using TrypLE™ Express at 37 ℃ for 3 to 5 min. TrypLE™ Express was removed by centrifugation at 300 x*g* for 5 min at room temperature. The supernatant was removed and the pellet was subjected to RNA isolation using TRIzol (Invitrogen, Life Technologies, USA), according to manufacturer's instruction.

3.2.4 3D culture of bulge stem cells and adipose-derived mesenchymal stem cells using alginate bead culture.

Alginate bead culture is a classic method to encapsulate and culture chondrocyte *in vitro*. This method was adopted in this study as three-dimensional (3D) culture to compare with transwell co-culture method (mentioned above). Alginate bead culture encapsulates cells in the 3D hydrogel enable maximum cell-cell interaction.

3.2.4.1 Preparation of alginate solution, calcium chloride solution and ethylenediaminetetraacetic acid (EDTA) solution

Alginate solution was prepared at the concentration 1.2%, by dissolving sodium alginate powder in distilled water, with 0.15 M of sodium chloride and 0.025 M HEPES buffer. The 1.2% alginate solution was filtered through a sterile 0.22 μm cellulose acetate filter, and stored at 4 ℃.

Calcium chloride solution was prepared at the concentration of 100 mM, by dissolving calcium chloride in distilled water and autoclaved at 121℃ for 15 min.

EDTA solution was prepared at the concentration of 5 % (w/v), pH 8.0 by dissolving EDTA pellet in pH 8.0 PBS. The solution was then filtered through 0.22 μm cellulose acetate filter, and stored at 4℃.

3.2.4.2 Encapsulation of cells in alginate bead

A mixture of epithelial and mesenchymal cells is encapsulated in the alginate bead, and mimics the *in vivo* hair follicle environment. The combinations of cells are showed in Table 3.5. The ratio of epithelial cell to mesenchymal cell is 2 : 1. Cell number in each bead is 9×10^4 cells.

Table 3.5 Alginate bead culture combinations

Combination	Epithelial Cell	Mesenchymal cells
1	-	HAD-MSCs
2	HHFBSCs	-
3	HHFBSCs	HAD-MSCs

Cells were trypsinised from the mono layer culture, and cell count was performed. Cell suspension of 9×10^6 cells/ml was prepared, and spun down. The cell pellet was re-suspended in 1.2 % alginate solution; the cell suspension was then aspirated into syringe capped with 22 gauge needles. The cell

suspension was released drop wise into 100 mM calcium chloride solution gently. The process of alginate bead production was illustrated in Figure 3.3. The alginate cell suspension polymerised instantly to form sphere bead (alginate bead) once they contacted with calcium chloride. The alginate beads were allowed to polymerise for another 15 min. Calcium chloride solution was then discarded, and the beads were washed with media twice. The beads were maintained in their expansion media for 2 days, then changed to 50% of expansion media and 50% of in-house developed media, maintained for another 2 days, then switched to 100% in-house developed media, culture for another 10 days.

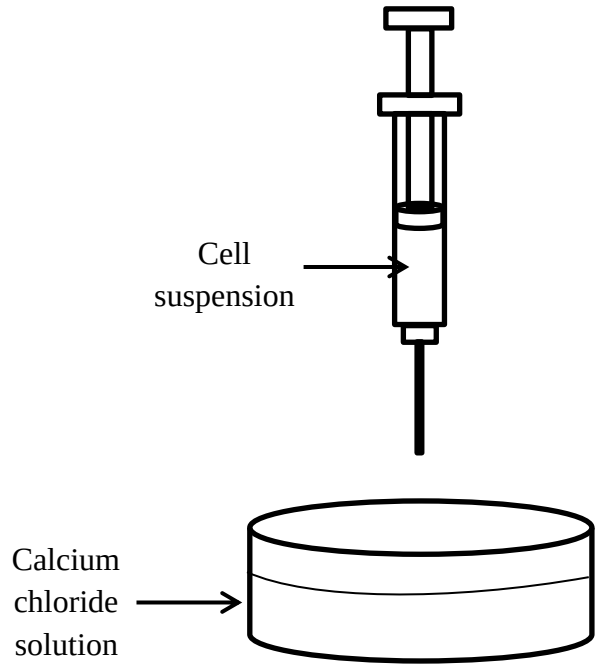

Figure 3. 3 Alginate bead production

3.2.4.3 RNA isolation

The alginate beads were dissolved using EDTA solution, at 37 ℃ for on shaking incubator at 100 rpm for 10 min. The EDTA was removed by centrifugation at 300 x*g* for 5 min at room temperature. The supernatant was removed and the pellet was subjected to RNA isolation using TRIzol (Invitrogen, Life Technologies, USA), according to manufacturer's instruction.

3.3 Real-time reverse transcription polymerase chain reaction (RT-qPCR)

Real-time reverse transcription polymerase chain reaction (RT-qPCR) was employed to study the gene expression of selected genes panel in the cultured samples.

Total RNA from the cultured cells was isolated from the cells using TRIzol (Invitrogen, Life Technologies, USA), according to the manufacturer's instructions. After total RNA isolation, the RNA was treated with DNase to remove DNA contamination. The concentration and purity of RNA was determined by Xpose reader (Trinean, US). The RNA was then kept at -80 ℃ with RNase inhibitor to minimize degradation.

Total RNA extracted from culture samples were converted into complementary DNA (cDNA) prior real-time polymerase chain reaction. The conversion was performed using Bio-Rad iScript™ reverse transcription supermix for RT-qPCR, followed manufacturer's instructions, the reaction setup and protocol as shown in Table 3.6 and Table 3.7.

Table 3.6 Reaction setup of reverse transcription

Components	Volume per Reaction, µl
5x iScript RT supermix	4
RNA template (1 µg total RNA)	Variable
Nuclease-free water	Variable
Total volume	20

Table 3.7 Reaction protocol of reverse transcription

Step	Temperature, ℃	Time, min
Priming	25	5
Reverse transcription	42	30
RT inactivation	85	5

Real-time PCR was performed using QuantiNova PCR Master Mix (Qiagen, Netherlands) and cDNA as template (obtained from reverse transcription), in Rotor Gene Q Cycler (Qiagen, Netherlands). The reaction setup and cycling conditions were shown in Table 3.8 and Table 3.9. Primers details are listed in Appendix D.

The mRNA level was normalised to housekeeping gene and the mRNA expression level was calculated using comparative CT method.

Table 3.8 Real-time PCR reaction setup

Components	Final concentration
2x SYBR Green PCR Master Mix	1x
Primer A	0.7 µM
Primer B	0.7 µM
Nuclease-free water	-
cDNA	25 ng

Table 3.9 Real-time PCR cycling conditions

Step	Temperature, ℃	Time	Cycle
PCR initial heat activation	95	2 min	1
Denaturation	95	15 s	40
Annealing	60	15 s	
Extension	72	10 s	

CHAPTER 4

RESULTS

Part 1: Optimisation of culture conditions for human hair follicle bulge stem cells

4.1 Expansion of human hair follicle bulge stem cells

Primary human hair follicle bulge stem cells were expanded in the manufacturer's culture media and coating. Human hair follicle bulge stem cells are cobblestone-shaped cells, with 95 % attached to the coating and 5 % in suspension.

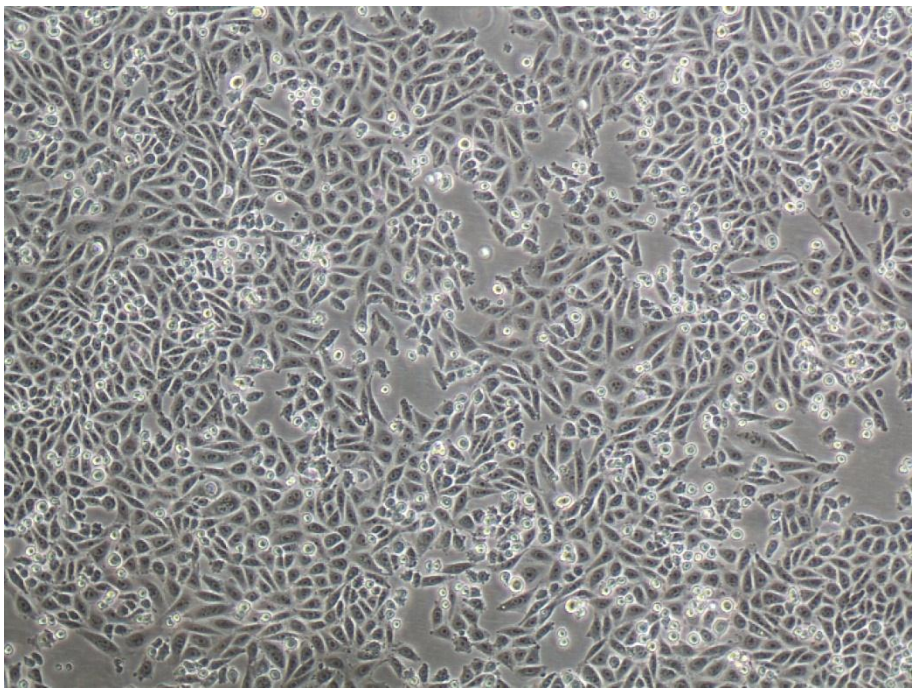

Figure 4.1 Morphology of human hair follicle bulge stem cells in manufacturer's culture media and coating. Magnification: 100 X

4.1.1 Coating test for human hair follicle bulge stem cells

Primary human hair follicle bulge stem cells were purchased from Celprogen Inc, which the coating information are classified. To achieve the therapeutic purpose of using human hair follicle bulge stem cells, the cells have to be cultured on the coating with known composition.

Primary human hair follicle bulge stem cells were subjected to 4 experimental coatings to identify the optimum coating to expand human hair follicle bulge stem cells. Human hair follicle bulge stem cells managed to survive in all experimental coatings, with different degree changes in morphology and attachment.

Figure 4.2 showed the morphology of human hair follicle bulge stem cells in the dish and 4 experimental coatings. Human hair follicle bulge stem cells exhibited cobblestone morphology with 95 % attached cells and 5 % suspension cells in the control dish. Cells on normal culture dish, which is the standard coating for cell culture dish, exhibited similar morphology, but the cells were less attached compare to the control cells, this was observed through the glowing of the edge of cells, the more glowing the edge, means it was less attached. Cells in laminin and Matrigel coated culture dish were loosely attached and did not exhibit the cobblestone morphology.

Cells in fibronectin coated culture dish, demonstrated cobblestone morphology and similar attachment as the cells in control dish. Therefore, fibronectin was chosen to expand human hair follicle bulge stem cells.

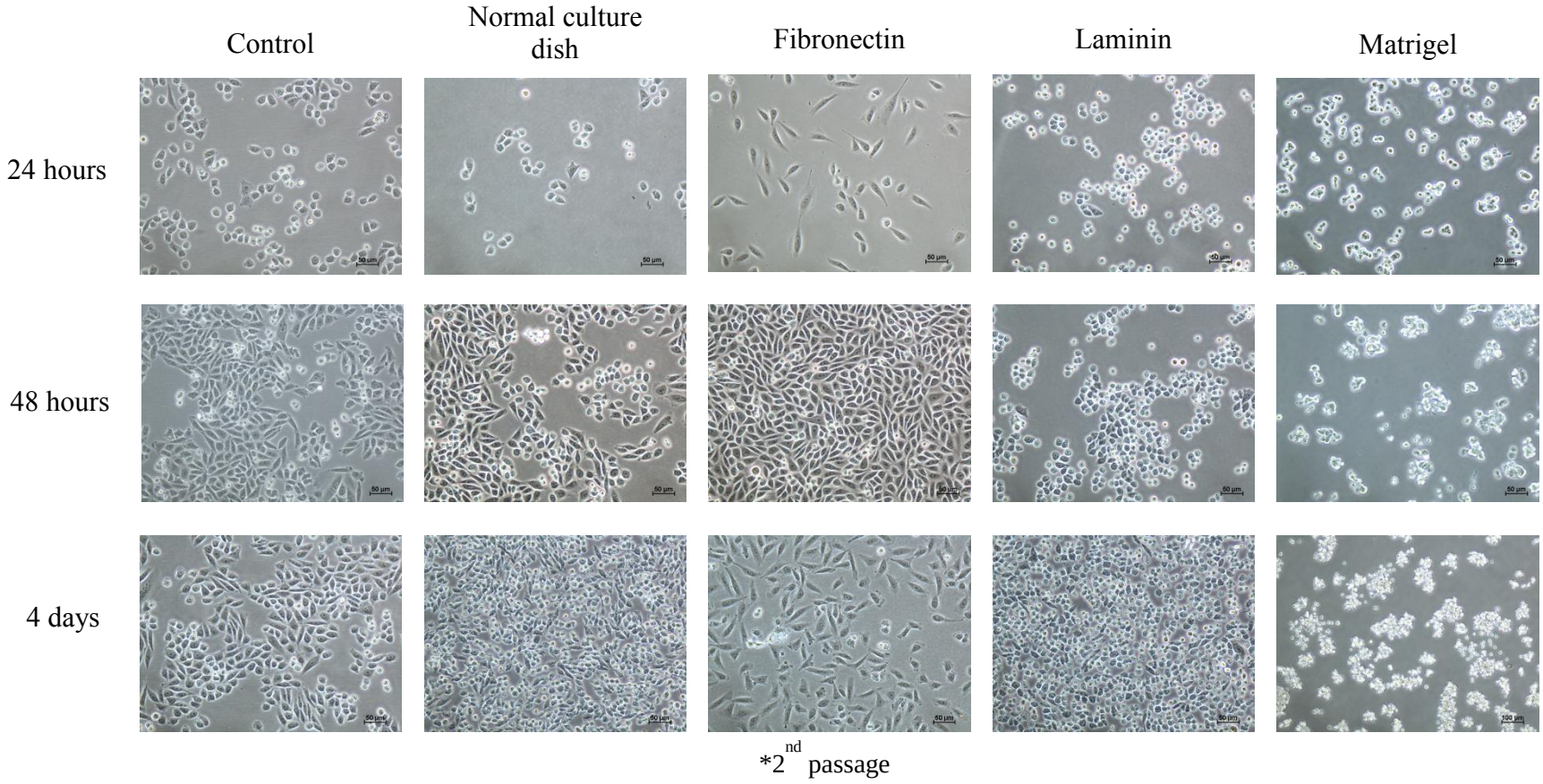

Figure 4.2 Morphology of human hair follicle bulge stem cells on different coatings. Magnification: 200 X

4.1.2 Media test for human hair follicle bulge stem cells

The formulation of culture media provided by manufacturer of human hair follicle bulge stem cells are classified information. To employ human hair follicle bulge stem cells in therapy, the composition of culture media must be studied and to minimise the possible adverse effects.

Primary human hair follicle bulge stem cells were subjected to 4 experimental culture media to find out the optimum culture media for expansion. At the same time, human hair follicle bulge stem cells were subjected to culture media 5, to learn the cells viability in the media. Media 5 was the in-house developed media, used in the following experiments.

Human hair follicle bulge stem cells managed to survive and proliferated in all the media with different degree of changes in morphology as showed in Figure 4.3. Cells in media 1 proliferated faster than the control cells, however, after one passage, the cells are merely attached and the morphology changed. Cells in media 2 and 3 were less attached to the culture dish. Cells with 2 different morphologies were observed; some cells were larger and flattened.

Cells in media 4 resembled the morphology like the cells in control cells, with uniform morphology and minimal differentiation, thus media 4 was selected to culture human hair follicle bulge stem cells for expansion purpose.

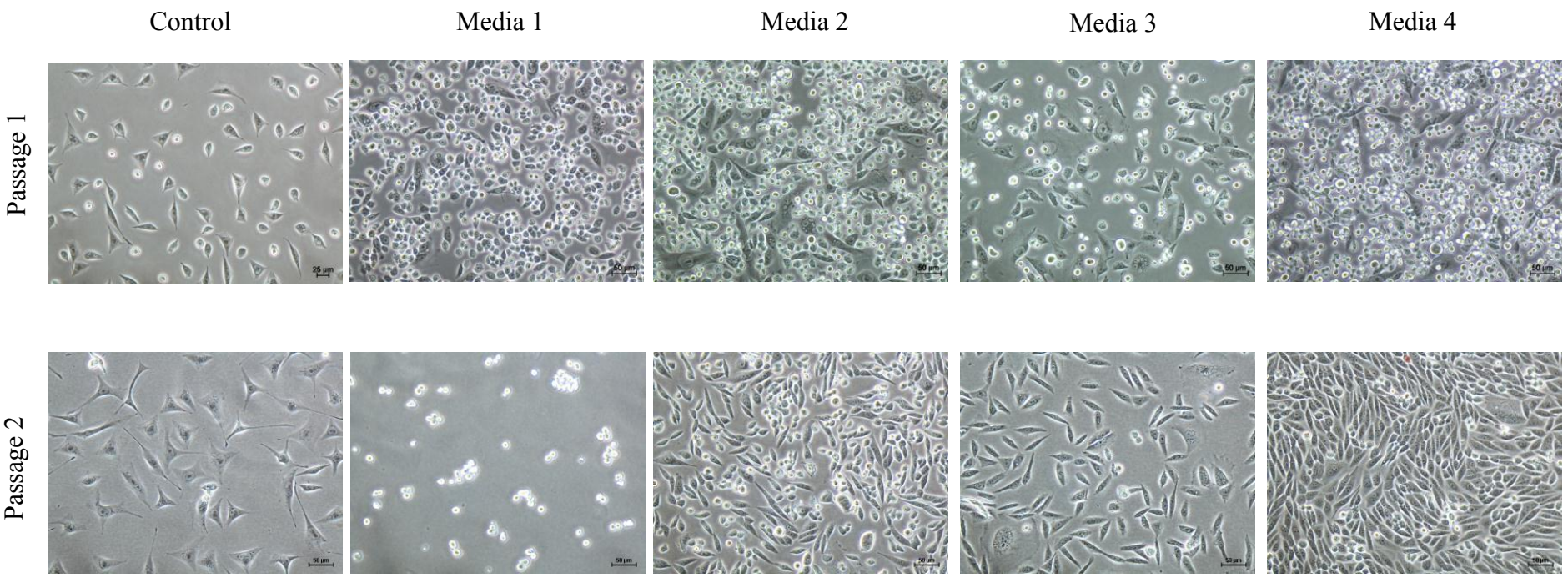

Figure 4.3: Morphology of cells in different cell culture media. Magnification: 200 X

4.1.3 Viability media test

Media 5 is the in-house developed media, to be used in the following co-culture experiments. Human hair follicle bulge stem cells were subjected to media 5 to test the viability prior co-culture.

Human hair follicle bulge stem cells were exhibiting two different morphologies in media 5 as showed in Figure 4.4. This test is mainly to test the viability of cells in the media, thus the morphological changes is not important in this test.

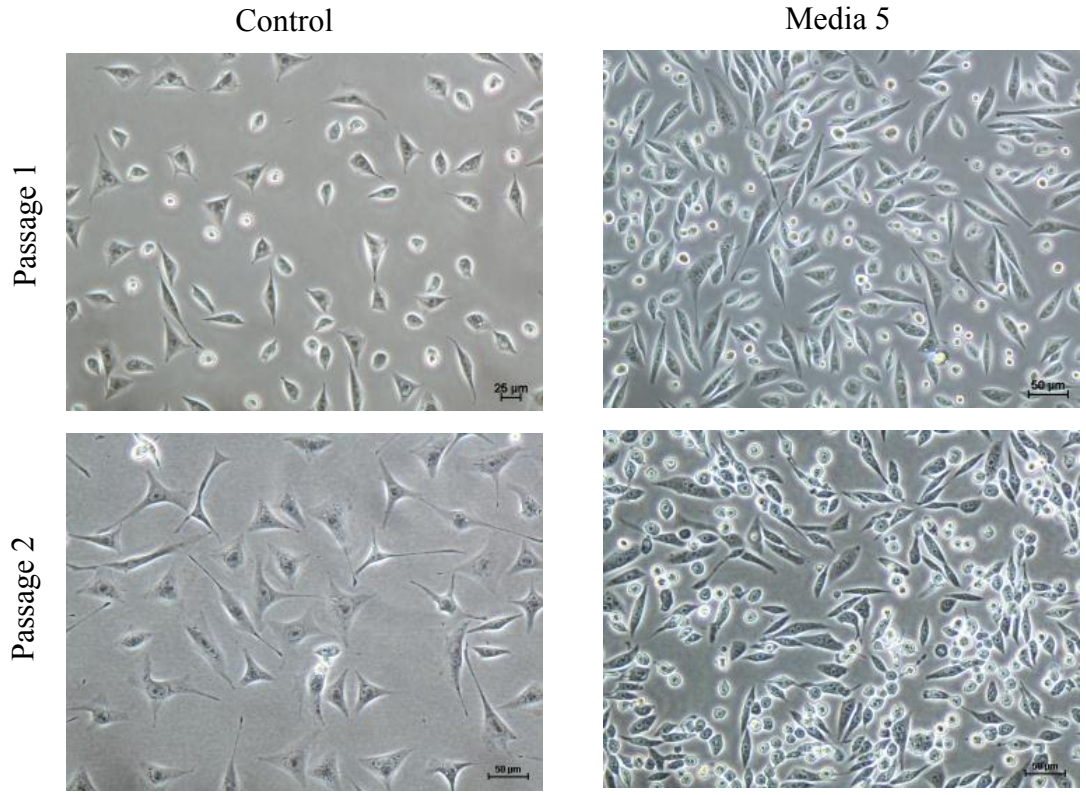

Figure 4.4 Morphology of human hair follicle bulge stem cells in control media and media 5. Magnification: 200 X

Part II: Induction of dermal papilla properties in human adipose-derived mesenchymal stem cells

4.2 Human dermal papilla cells and human adipose-derived mesenchymal stem cells in mono layer and hanging drop culture

Human dermal papilla cells and human adipose-derived mesenchymal stem cells were expanded in mono layer culture and then subjected to hanging drop culture. Both cells exhibited similar morphology in mono layer culture: bipolar fibroblast-shaped morphology as in Figure 4.5.

Both dermal papilla cells and human adipose-derived mesenchymal stem cells aggregated to form cellular spheroid in hanging drop culture as showed in Figure 4.5. Both the cellular spheroids were round with distinct edge.

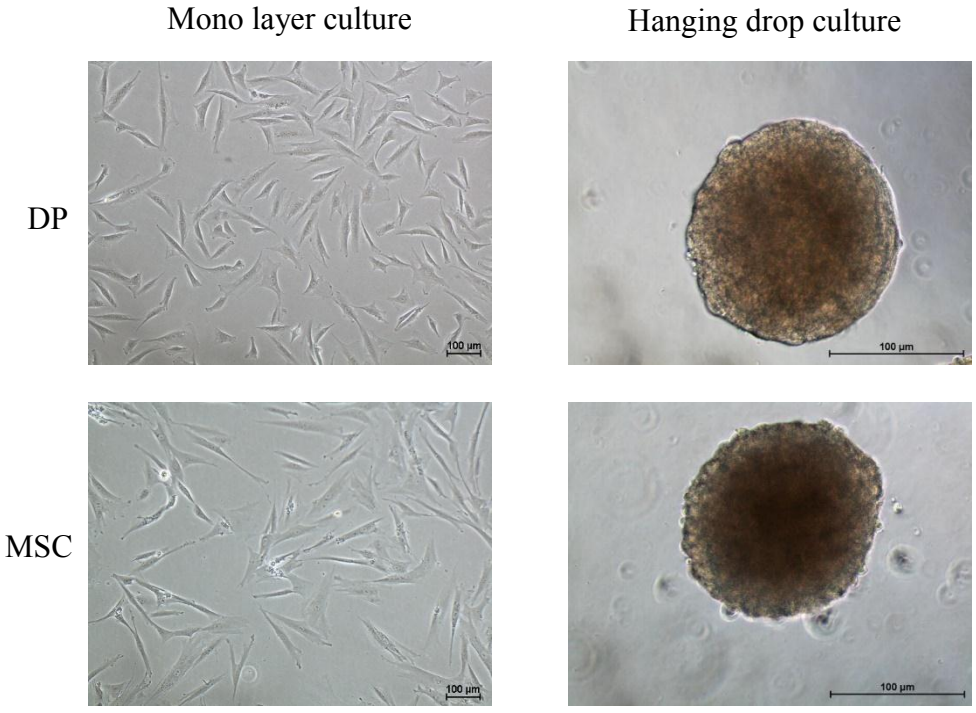

Figure 4.5 Morphology of human dermal papilla cells and human adipose-derived mesenchymal stem cells in mono layer and hanging drop culture. DP: dermal papilla cells; MSC: mesenchymal stem cells. Magnification: 100 X

4.3 Immunofluorescence staining of human dermal papilla cells and human adipose-derived mesenchymal stem cells in mono layer and hanging drop culture

Human dermal papilla cells and human adipose-derived mesenchymal stem cells in both mono layer and hanging drop culture were subjected to dermal papilla marker detection, using immunofluorescence staining technique. Versican is a dermal papilla marker, used as an indicator of the inductive capabilities of dermal papilla cells. Versican antibody with Texas Red fluorochrome was used to detect versican in cells, thus cells appeared red was positive for versican expression. Nuclei acid stain 4',6-diamidino-2-phenylindole (DAPI) was used to stain DNA, emitted blue fluorescence upon staining.

Figure 4.6 is the summary of the staining results, all the cells emitted blue fluorescence, confirmed the presence of cells. Versican was detected in mono layer culture of human dermal papilla cells and hanging drop culture of both dermal papilla cells and adipose-derived mesenchymal stem cells. Meanwhile, versican was not detected in mono layer of adipose-derived mesenchymal stem cells.

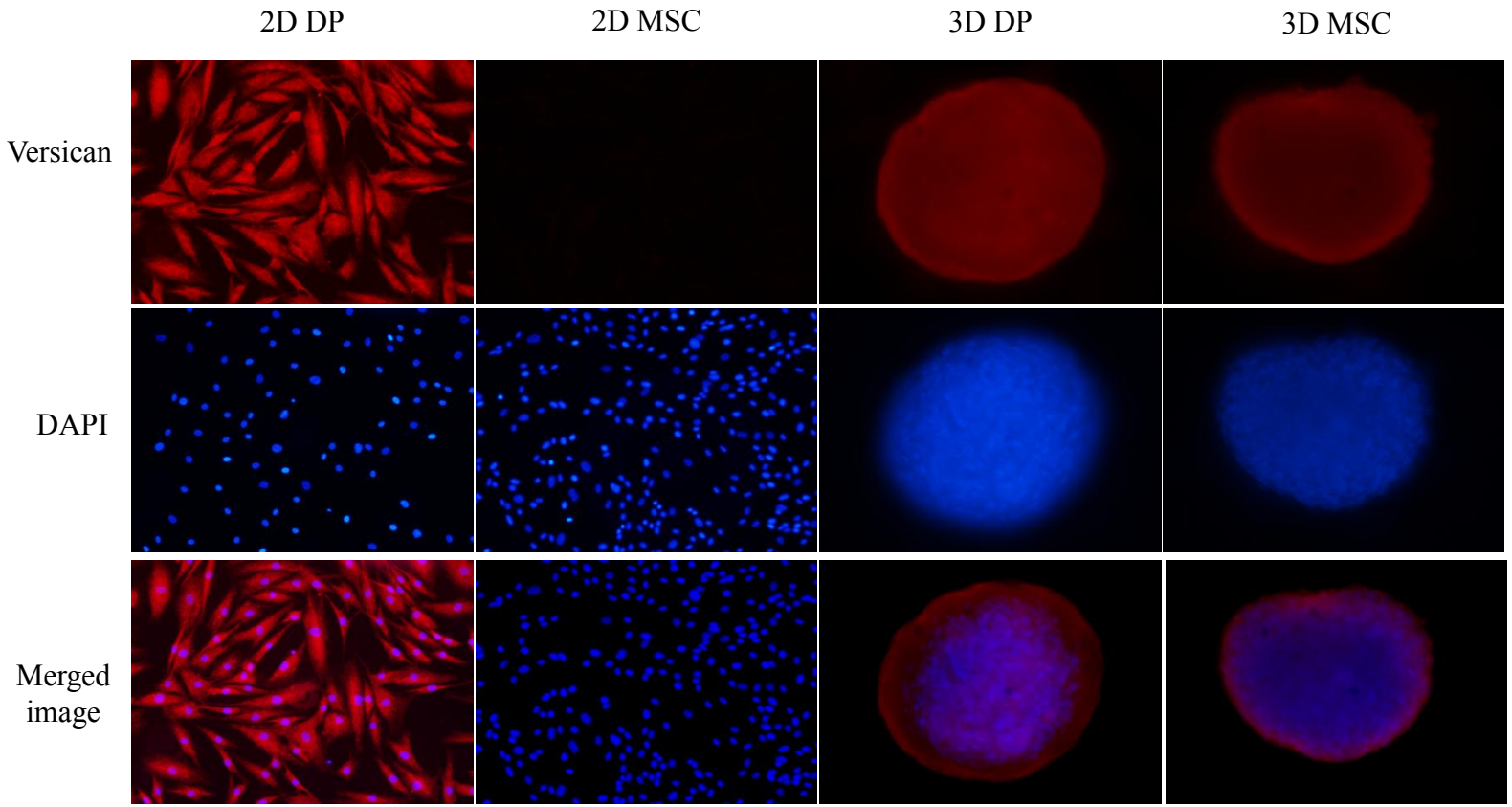

Figure 4.6 Immunofluorescence staining of dermal papilla marker, versican. 2D DP: dermal papilla cells in mono layer culture; 2D MSC: mesenchymal cells in mono layer culture; 3D DP: dermal papilla cells in hanging drop culture, 3D MSC: mesenchymal stem cells in hanging drop culture. Magnification: 100 X

4.4 RT-qPCR of dermal papilla related genes

RT-qPCR was performed to study the gene expression of human adipose-derived mesenchymal stem cells in mono layer and hanging drop culture, compared to the human dermal papilla cells in mono layer culture.

Dermal papilla related genes employed in this gene expression study included ALPL, WNT5A and CTNNB1. ALPL is alkaline phosphatase, a dermal papilla marker; WNT5A is Wnt Family member 5A, involves in maintenance of intrinsic properties in dermal papilla cells; CTNNB1 is Catenin Beta 1, expressed when there is cell to cell contact.

Figure 4.7 showed the mRNA expression level of the dermal papilla related genes in both mono layer culture and hanging drop culture. The expression of ALPL, WNT5A and CTNNB1 were upregulated in human adipose-derived mesenchymal stem cells in hanging drop culture. ALPL and WNT5A were down-regulated human adipose-derived mesenchymal stem cells in mono layer culture. CTNNB1 expression is slightly down-regulated in human adipose-derived mesenchymal stem cells in mono layer culture.

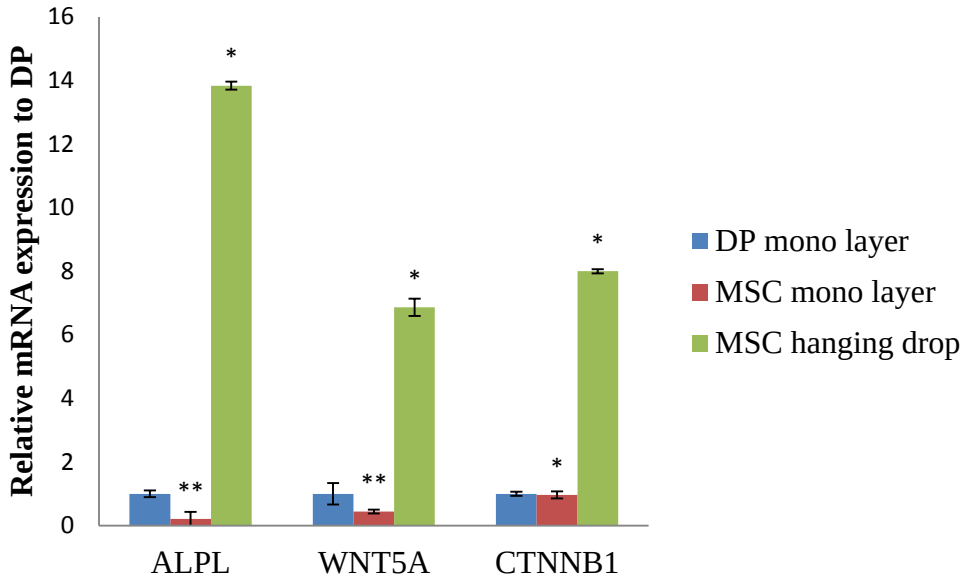

Figure 4.7 mRNA expression level of dermal papilla related genes.
DP: human dermal papilla cells; MSC: human adipose-derived mesenchymal stem cells in mono layer culture; MSC hanging drop: human adipose-derived mesenchymal stem cells in hanging drop culture. *p <0.05 and **p<0.01 were obtained in comparison to DP.

Part III: Transwell co-culture

4.5 RT-qPCR of dermal papilla related genes in transwell co-culture

Expression of dermal papilla related genes in human adipose-derived mesenchymal stem cells were studied. The genes selected were ALPL, CTNNB1, VCAN and WNT5A. The gene expression of human adipose-derived mesenchymal stem cells in co-culture with human hair follicle bulge stem cells and human hair keratinocytes were compared to the human adipose-derived mesenchymal stem cells culture in mono layer as in Figure 4.8.

Human adipose-derived mesenchymal stem cells demonstrated up-regulation in all the dermal papilla related genes when they were subjected to transwell co-culture with human hair follicle bulge stem cells.

Human adipose-derived mesenchymal stem cells showed inconsistency of expression when they were subjected to co-culture with human hair keratinocytes: ALPL, CTNBB1 and WNT5A were down-regulated meanwhile VCAN is the only gene upregulated.

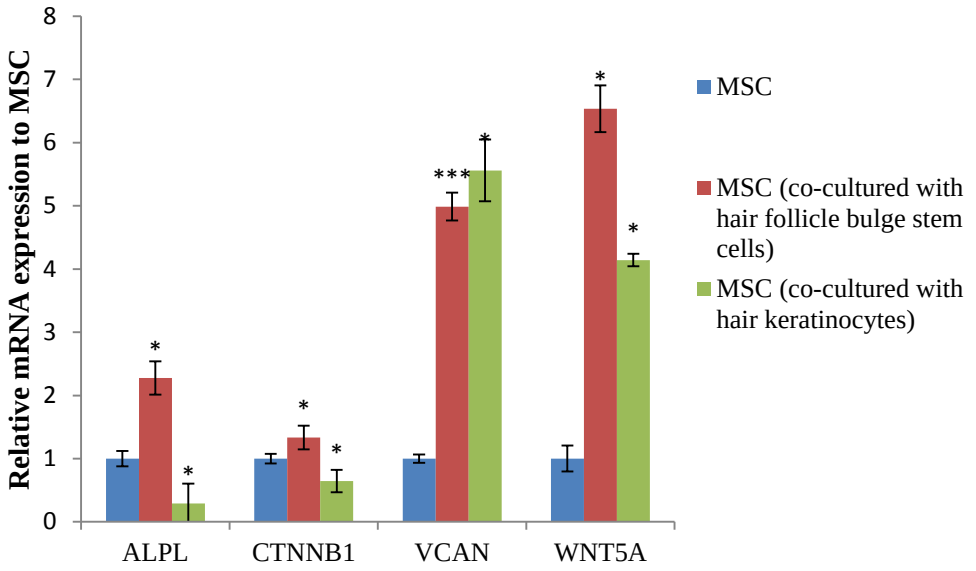

Figure 4.8 mRNA expression level of dermal papilla related genes in transwell co-culture. MSC: human adipose-derived mesenchymal stem cells in mono layer culture. *p <0.05 and ***p<0.001 were obtained in comparison to MSC (control).

4.6 RT-qPCR of bulge stem cells related genes in transwell co-culture

The gene expression changes of human hair follicle bulge stem cells in co-culture were studied through RT-qPCR, compared to the human hair follicle bulge stem cells in mono layer as in Figure 4.9.

Ki67 is a differentiation marker, while LEF1 is a bulge stem cells differentiation marker; both were not detected in all samples, including the human hair follicle bulge stem cells in mono layer. K6hf is a hair specific marker for dermal papilla-induced differentiation, it was down-regulated in human hair follicle bulge stem cells in both co-culture conditions: co-culture with mesenchymal stem cells and co-culture with dermal papilla cells. SOX2, a pluripotency marker, it was up-regulated in human hair follicle bulge stem cells in both co-culture conditions, as compared to the human hair follicle bulge stem cells in mono layer culture.

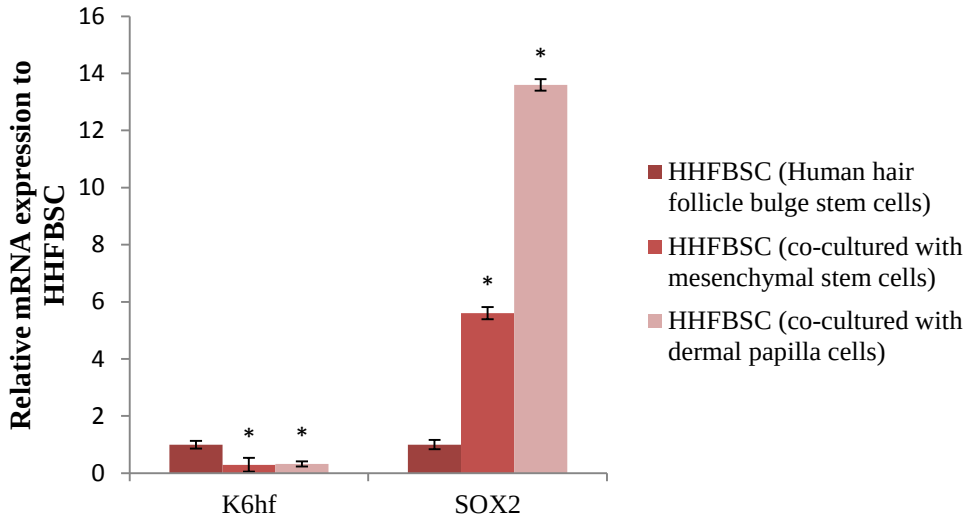

Figure 4.9 mRNA expression level of hair follicle bulge stem cells related genes in transwell co-culture. *p <0.05 was obtained in comparison to human hair follicle bulge stem cells.

Part IV: 3D Culture – Alginate bead culture

4.7 Morphological observation of alginate bead culture

Alginate bead culture method, a 3-dimensional culture method has been employed to culture human hair follicle bulge stem cells and human adipose-derived mesenchymal stem cells. The bead culture was maintained for 10 days in in-house developed culture media.

Cellular morphology was not able to be observed through microscope, due to the bead structure. But changes of the cells arrangement in the beads were captured through microscope, as in Figure 4.10. On day 1, cells were observed scattered in all bead cultures. On day 10, the control cells, which were human adipose-derived mesenchymal stem cells only bead culture and human hair follicle bulge stem cells only bead culture showed the same cells arrangement as day 1 in the bead; meanwhile the bead culture of human adipose-derived mesenchymal stem cells mixed with human hair follicle bulge stem cells showed tube-like arrangement in the bead. The cells appeared as tube-like, similar to hair structure in bead on day 10 culture, but there was nothing grown out from the bead, just the cells arrangement within the bead showed changes.

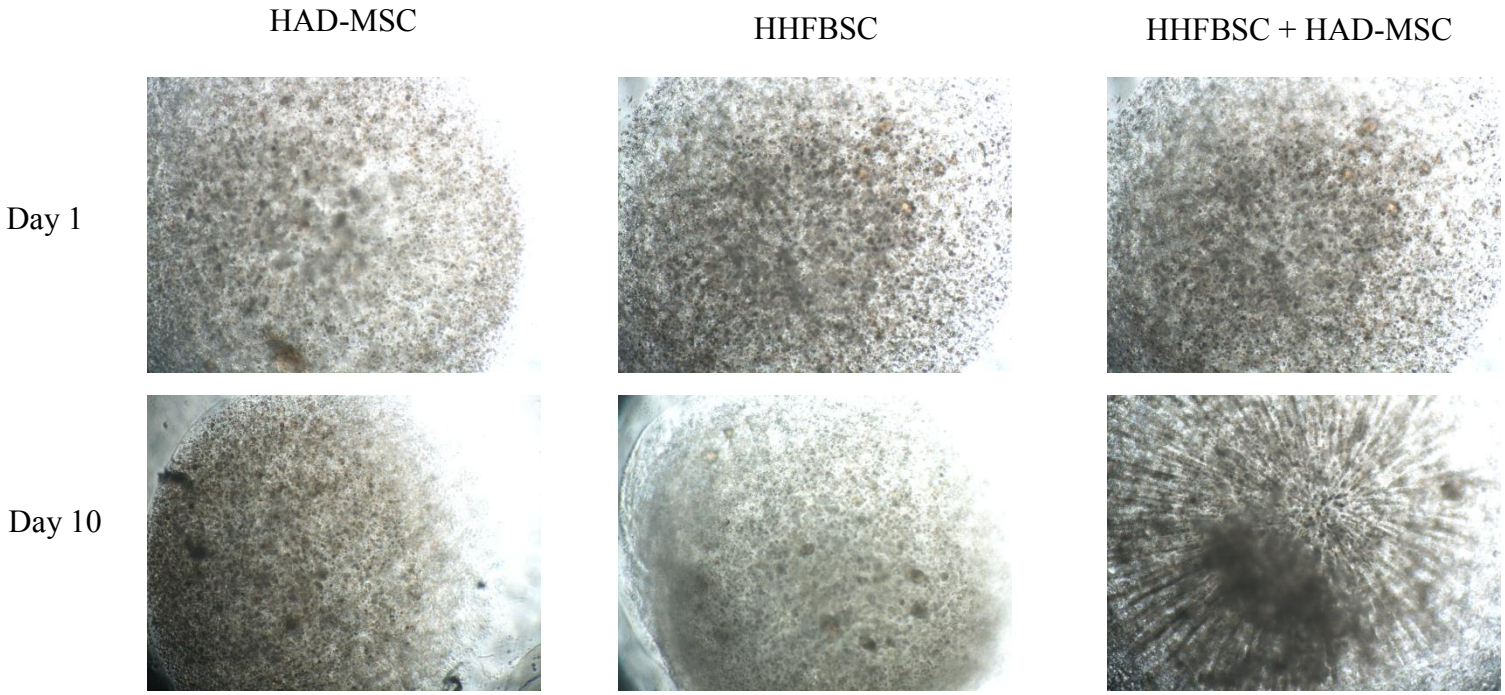

Figure 4.10 Changes of cells arrangement in bead culture. HAD-MSC: human adipose-derived mesenchymal stem cells; HHFBSC: human hair follicle bulge stem cells. Magnification: 40 X

4.8 qRT-PCR of cells in 3-dimensional alginate bead culture

The gene expression of cells involved in 3-dimensional bead culture was studied via qRT-PCR. Genes related to dermal papilla were compared to bead culture of human adipose-derived mesenchymal stem cells as in Figure 4.12; whereas genes related to human hair follicle bulge stem cells were compared to bead culture of human hair follicle bulge stem cells as in Figure 4.11.

The genes related to human hair follicle bulge stem cells were K6hf, TCF3, SOX2, Ki67 and LEF1. K6hf is a differentiation marker, specifically dermal papilla induced differentiation marker, it was slightly up-regulated in the bead culture of the mixture of human adipose-derived mesenchymal stem cells and human hair follicle bulge stem cells, compared to human hair follicle bulge stem cells bead culture. TCF3 is a differentiation marker, was remarkably up-regulated in the bead culture of the mixture of human adipose-derived mesenchymal stem cells and human hair follicle bulge stem cells. On the other hand, SOX2, Ki67 and LEF1 were not detected in all the samples.

The genes related to human adipose-derived mesenchymal stem cells were CTNNB1, VCAN, WNT5A, ALPL and LEF1, the expressions were compared to human adipose-derived mesenchymal stem cells bead culture. CTNNB1, VCAN, WNT5A were down-regulated in the bead culture of the mixture of human adipose-derived mesenchymal stem cells and human hair follicle bulge stem cells. ALPL and LEF1 were not detected in all samples.

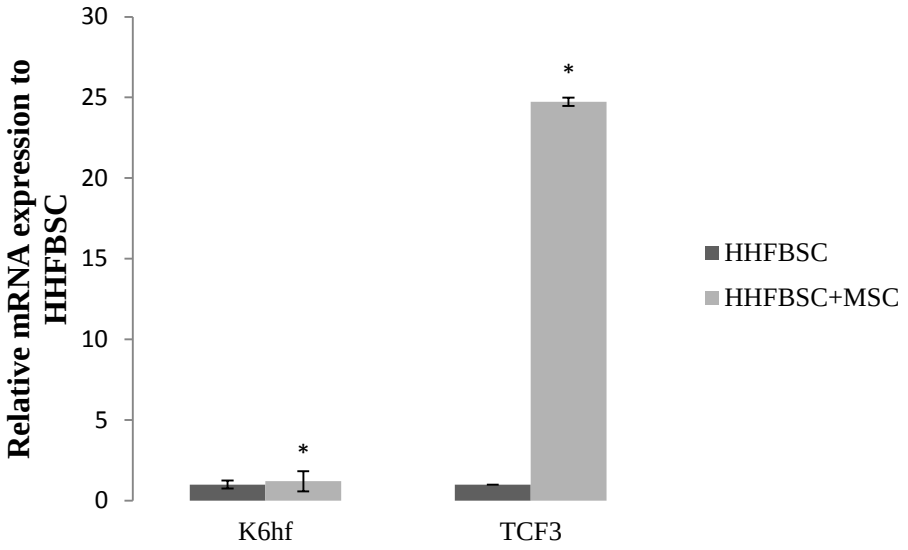

Figure 4.11 mRNA expression level of hair follicle bulge stem cells related genes in 3-dimensional alginate bead culture. *p <0.05 was obtained in comparison to human hair follicle bulge stem cells.

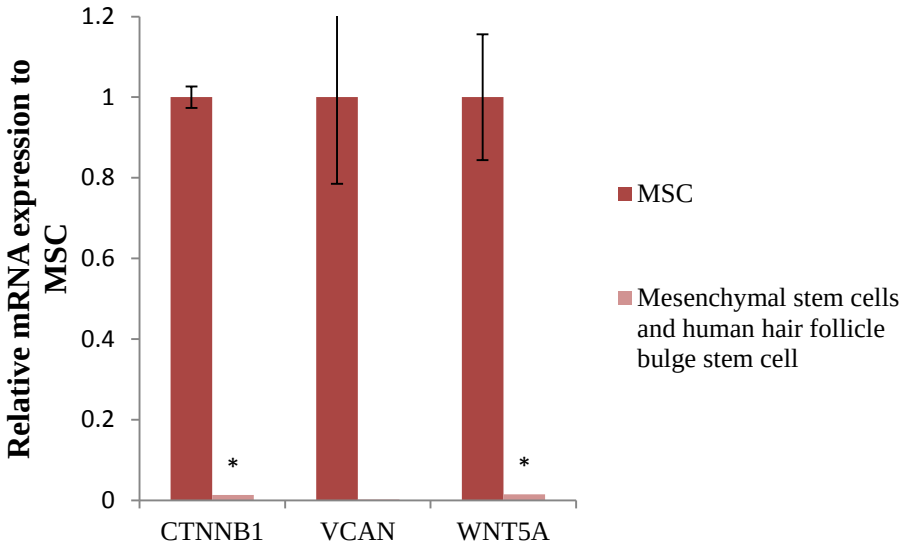

Figure 4.12 mRNA expression level of dermal papilla related genes in 3-dimensional alginate bead culture. MSC: human adipose-derived mesenchymal stem cells. *p <0.05 was obtained in comparison to human adipose-derived mesenchymal stem cells.

CHAPTER 5

DISCUSSION

The ultimate objective of this study was to identify the optimum culture conditions to generate hair *in vitro* for therapeutic purpose. To achieve the therapeutic purpose, human hair follicle bulge stem cells and dermal papilla cells should be isolated from hair loss patient. In this study, human hair follicle bulge stem cells were purchased from manufacturer due to the failure in maintaining the cells isolated from plucked hair (data not shown). Dermal papilla cells were purchased from manufacturer as there was no primary tissue available for isolation. The optimum culture conditions including the culture media and culture method, which possibly induce hair growth *in vitro.* However, in order in achieve the ultimate objectives, there are a few preliminary studies needed to be carried out. In this study, the effect of *in vitro* culture environments was studied through the gene expression of cells.

Human hair follicle bulge stem cells were purchased from manufacturer, considering that the media and coating from manufacturer were classified information, coating and media tests were carried out to find out alternatives in expanding the cells prior any down-stream experiments. Fibronectin was chosen as the coating for human hair follicle bulge stem cells based on the morphology of the human hair follicle bulge stem cells culture. Human hair follicle bulge stem cells demonstrated better attachment in 24 hours after

seeding and cells resembled cell morphology most similar to the control on fibronectin coating.

Human hair follicle bulge stem cells are 95 % adherent cells; thus, the attachment of cells is the first criteria in choosing coating. Media test was then carried out to identify the optimum media for human hair follicle bulge stem cells proliferation. Cells were cultured in 4 different media to compare the morphology of cells in control media. It was important to expand the cells prior subject to any further experiments; hence cells were cultured in the media test for 2 passages. Media 4 was chosen after observation throughout 2 passages of culture. Human hair follicle bulge stem cells resembled uniform cell morphology with minimal differentiation. Human hair follicle bulge stem cells were cultured in media 5 to test for cell viability. Media 5 was in-house developed cell culture media, based on literature studies, to promote stem cells differentiation *in vitro*. Human hair follicle bulge stem cells were subject to three-dimensional culture, which did not allow the observation of cellular morphology, thus the viability test was carried out beforehand. Human hair follicle bulge stem cells displayed morphology differ from control cells, suggested it may undergo certain differentiation in the media.

Human dermal papilla cells are well known for their inductive ability during hair development. Dermal papilla cells stimulate the proliferation and differentiation of bulge stem cells (Roh et al., 2004) and form hair bulb under favourable conditions (Matsuzaki & Yoshizato, 1998). However, human dermal papilla cells were reported to lose their hair inductive ability and intrinsic properties after prolong culture *in vitro* (Lu et al., 2006; Higgins et al.,

2010; Ohyama et al., 2012). Human dermal papilla is in aggregate form *in vivo*, but they are expanded in mono layer culture *in vitro*, explained the reason dermal papilla cells lost the inductive ability.

Human dermal papilla is mesenchyme-derived cells, thus human mesenchymal stem cells were proposed in this study. Human adipose-derived mesenchymal stem cells were employed in this study due to the consideration of autologous source of stem cells for therapeutic purpose. Adipose tissue can be obtained from patient through liposuction procedure, then the mesenchymal stem cells can be isolated from the adipose tissue. Yoo et al. (2010) demonstrated that human bone marrow-derived mesenchymal stem cells and human umbilical cord-derived mesenchymal stem cells abled to aggregate to form dermal papilla like tissue in their study. Qiao et al. (2008) used hanging drop method to produce cell aggregates from mouse embryonic skin keratinocytes and dermal cells. Human adipose-derived mesenchymal stem cells were proposed in to be induced into dermal papilla cells using hanging drop method. The immunofluorescence staining and RT-qPCR results demonstrated consistent result as reported by Higgins et al. (2010). Aggregative growth is one of the character of dermal papilla cells, human adipose-derived mesenchymal stem cells able to form cellular spheroid via hanging drop culture method, likewise the dermal papilla cells. Versican was detected when the human adipose-derived mesenchymal stem cells were cultured in hanging drop form, and not detected in mono layer culture, which is consistent with Yoo et al. (2010) and Yang et al. (2012), versican is correlated with the aggregative growth of dermal papilla. Gene expression analysis of dermal papilla genes further confirmed

the potential of human adipose-derived mesenchymal stem cells to be induced into dermal papilla cells using hanging drop culture. Alkaline phosphatase (ALPL), WNT5A and CTNNB1 were upregulated in human adipose-derived mesenchymal stem cells in hanging drop culture compared to human dermal papilla in mono layer culture. ALPL is a dermal papilla marker, associated with hair inductive capability of dermal papilla cells and indicator of healthy dermal papilla cells (Higgins et al., 2010, Yoo et al., 2010). The upregulation of ALPL was consistent with versican detection in immunofluorescence staining, indicated the human adipose-derived mesenchymal stem cells acquired hair inductive capability through hanging drop culture, further suggested it can replace dermal papilla in hair loss treatment. Wingless-type MMTV integration site family, member 5A (WNT5A) is a member of Wnt signalling, which involved in hair development. Wnt signalling plays vital role in hair follicle development and maintenance of dermal papilla intrinsic properties (Roh et al., 2004; Ohyama et al., 2012), was up-regulated in human adipose-derived mesenchymal stem cells hanging drop culture, indicated dermal papilla intrinsic properties was preserved in human adipose-derived mesenchymal stem cells when they were cultured in hanging drop culture. Lastly, CTNNB1 was up-regulated in human adipose-derived mesenchymal stem cells, indicated increased cell-cell contact in hanging drop culture, consistent with Higgins et al. (2010). CTNNB1 is catenin beta-1, involved in cell adhesion and expressed when there is cell-cell contact. The expression of CTNNB1 in mono layer culture of human adipose-derived mesenchymal stem cells is almost the same as the mono layer culture of dermal papilla cell, explained there was similar cell-cell contact for mono layer cultured cells.

Transwell co-culture was selected as one of the culture method to study the effect of culture conditions on the gene expression. Transwell co-culture is a culture method that allows cell interactions between 2 layers of cells. It is often used as an *in vitro* model to study epithelial-mesenchymal transition. Cell culture insert was regularly employed in transwell-co-culture. One type of cells was cultured on the cell culture insert, another type of cells was cultured on the surface of multi-well plate. The same culture media was used in transwell co-culture, which served as a media for the cell-cell signalling or cell-cell interactions. The signal can be transmitted through from cells in cell culture insert to multi-well culture plate or vice versa.

In this study, human hair follicle bulge stem cells required coating to proliferate, thus they were cultured on multi-well plate with fibronectin coating; human adipose-derived mesenchymal stem cells were cultured on the cell culture insert. Morphological changes of cells were observed daily, however, no photograph was taken due to the technical difficulties involved in photography, cell culture insert has to be removed from the culture well, to another empty well in order to take picture of the bottom culture. Hence, to minimise the changes of contamination, morphological changes of cells were not recorded through photography.

Human adipose-derived mesenchymal stem cells were co-cultured with human hair follicle bulge stem cells and hair keratinocytes, to compare the difference in gene expression, meanwhile human adipose-derived mesenchymal stem cells were cultured in the same culture media to serve as control. RNAs were

isolated from cell culture insert and multi-well plate for RT-qPCR respectively. Genes related to dermal papilla were selected: ALPL, CTNNB1, VCAN and WNT5A. Overall, ALPL, CTNNB1, VCAN and WNT5A were up-regulated in mesenchymal stem cells co-cultured with bulge stem cells, suggested human adipose-derived mesenchymal stem cells exhibited dermal papilla properties when they are allowed to interact with human hair follicle bulge stem cells. On the other hand, human adipose-derived mesenchymal stem cells showed fluctuating expression when they were co-cultured with human hair keratinocytes. VCAN and WNT5A were up-regulated, meanwhile ALPL and CTNNB1 were down-regulated compared to human adipose-derived mesenchymal stem cells. The data suggested the certain dermal papilla intrinsic properties were reserved, but further validation needed to be carried out.

Human hair follicle bulge stem cells were subjected to transwell co-culture with mesenchymal stem cells and human dermal papilla cells respectively, to study the difference in gene expression, compared to human hair follicle bulge stem cells mono layer culture. Differentiation markers and stem cells marker were selected, overall RT-qPCR results demonstrated human hair follicle bulge stem cells did not differentiated in neither co-culture with human adipose-derived mesenchymal stem cells nor co-culture with human dermal papilla cells. Ki67, a proliferation marker was down-regulated in both transwell co-cultures, suggested there were limited proliferation when human hair follicle bulge stem cells. K6hf, is a specific marker of dermal papilla induced differentiation, was down-regulated in both transwell co-cultures, coherent with undetectable LEF1 expression in all samples. LEF1 is an indicator of

bulge stem cells differentiation, which required Wnt signalling to control the stem cells differentiation. At the same time, SOX2, a pluripotency marker in stem cells, was up-regulated in both transwell co-cultures compared to human hair follicle bulge stem cells mono layer culture. These results proposed that human hair follicle stem cells maintained the stem cells stemness, did not undergo any differentiation in transwell co-culture.

Alginate bead culture method was employed in the study as another culture conditions to study the effect of culture condition on the gene expression. Alginate bead culture is a common method to culture chondrocytes, via encapsulating the chondrocytes in the semi-solid alginate bead. Alginate is studied widely due to its biodegradable properties and the potential in regenerative medicine (Lee and Mooney, 2012; Sun and Tan, 2013). Alginate bead culture is a relatively simple three-dimensional culture method, which allow mimicking *in vivo* condition *in vitro*, thus it was proposed in this study, to explore its potential in hair loss treatment.

Human adipose-derived mesenchymal stem cells and human hair follicle bulge stem cells were mixed in cell suspension and encapsulated in alginate bead culture, maintained in in-house developed culture media for 10 days. The morphological changes of bead cultures were observed. The mixture alginate bead displayed fascinating morphology at day 10. The cells aligned in tube like formation, similar to hair structure, which was contrast from the morphology observed in human adipose-derived mesenchymal stem cells control bead and human hair follicle bulge stem cells control bead. The cells in both control

beads were scattered in the beads, with no uniform alignment and no specific formation observed throughout 10 days incubation.

RNAs were isolated from the alginate bead then subjected to RT-qPCR. The expressions were compared to human adipose-derived mesenchymal stem cells control bead culture and human hair follicle bulge stem cells control bead culture respectively. As the bead culture involved 2 types of cells, the cell mixture was subjected to RNA isolation without cell sorting. Dermal papilla related genes were compared to mesenchymal stem cells while hair follicle bulge stem cells related genes were compared human hair follicle bulge stem cells.

Human hair follicle bulge stem cells required signal from dermal papilla to initiate differentiation, along with Wnt signalling (Roh et al., 2004). The data from RT-qPCR revealed human adipose-derived mesenchymal stem cells unable to be induced into dermal papilla through alginate bead culture. VCAN, WNT5A, and CTNNB1 were down-regulated compared to human adipose-derived mesenchymal stem cells control bead. Meanwhile ALPL was not detected in both control bead and mixture culture bead. The data suggested absence of dermal papilla inductive capability (ALPL and VCAN), intrinsic properties of dermal papilla was not displayed (WNT5A), limited cell to cell contact (CTNNB1) and absence of Wnt Signalling (WNT5A and CTNNB1).

On the other hand, the expression of human hair follicle bulge stem cells in accordance with the above data, suggested human hair follicle bulge stem cells were differentiated, but not induced by dermal papilla cells. TCF3 was up-regulated, indicated the differentiation of human hair follicle bulge cells, however, TCF3 acted independently of beta catenin, promoted differentiation of bulge stem cells other than epidermal terminal differentiation (Merrill et al., 2001). That SOX2 was not detected further suggested the human hair follicle bulge stem cells were no longer pluripotent in the culture. LEF1 was not detected in the culture, indicated there was no Wnt signalling; LEF1 promoted hair follicle bulge stem cells differentiation in response to Wnt signals (Merrill et al., 2001). The overall expression suggested that human hair follicle bulge stem cells underwent differentiation in the absence of Wnt signalling when they were cultured in alginate bead culture with human adipose-derived mesenchymal stem cells. Nevertheless, the differentiation was not induced by dermal papilla cells.

CHAPTER 6

CONCLUSION

6.1 Conclusion

Total of 4 different culture methods were employed in this study, which were mono layer culture, hanging drop culture, transwell co-culture and alginate bead culture. Transwell co-culture and alginate bead culture demonstrated that different culture conditions can cause changes in gene expressions although the same type of cells were involved in the culture. Both culture methods allow cell to cell interaction via signalling, however, different genes were expressed by the same type of cells when they were subjected to different culture conditions.

Human adipose-derived mesenchymal stem cells demonstrated aggregative behaviour as in dermal papilla cells, and exhibited the potential to be induced or remodel into dermal papilla via hanging drop culture. This finding suggested human adipose-derived mesenchymal stem cells can be a replacement for dermal papilla in hair loss therapy.

Dermal papilla induced-differentiation was not detected in both tranwell co-culture and alginate bead culture. At the same time Wnt signalling was absence in both culture conditions due to none detectable expression of Wnt signalling member. These data further confirmed the importance of Wnt signalling in hair follicle bulge stem cells differentiation.

6.2 Limitations of Present Study and Suggestion for Future Study

The in-house developed culture media used in present study can be further optimised via modifying the concentration of growth factors and perform comparison study. Growth factors in all culture media can be analysed using growth factor antibody array, to further understand the growth factor involved and improve the in-house culture media formula.

Alginate bead culture of human adipose-derived mesenchymal stem cells and human hair follicle bulge stem cells exhibited significant changes in the cells alignment in the bead. The culture period can be further extended to observed the changes. The bead culture can be subjected to immunofluorescence staining to study the expression of the marker and identify the cells distribution in the bead, for instance to find out the cells that aligned in tube-like formation. Besides, other three-dimensional culture method can be taken into consideration to perform co-culture.

Moreover, the signalling pathway can be further studied, and more genes along the pathway can be included in the study, to further understand the activation of the pathway in culture.

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Appendix A

Data sheet of human hair follicular keratinocytes

ScienCell™
Research Laboratories

Human Hair Follicular Keratinocytes (HHFK)
Catalog #2440

Cell Specification
Hair follicles are self-renewing structures that cycle and regenerate throughout life and also provide a critical niche for keratinocyte stem cells [1]. The interplay between keratinocytes and the underlying mesenchyme are critical for hair follicle morphogenesis and cycling. Several key developmental pathways are involved in determining follicular keratinocyte fate as well as the differentiation into several distinct cellular layers of the follicle during the growth phase of hair [2]. Studies indicate that follicular keratinocytes are distinct from epidermal keratinocytes in proliferative capacity and CD34 expression [3].

HHFK from ScienCell Research Laboratories are isolated from the human scalp. HHFK are cryopreserved at P0 and delivered frozen. Each vial contains >5 x 10^5 cells in 1 ml volume. HHFK are characterized by immunofluorescence with antibody specific to cytokeratin. HHFK are negative for HIV-1, HBV, HCV, mycoplasma, bacteria, yeast, and fungi. HHFK are guaranteed to further expand for 15 population doublings under the conditions provided by ScienCell Research Laboratories.

Recommended Medium
It is recommended to use Keratinocyte Medium (KM, Cat. #2101) for culturing HHFK *in vitro*.

Product Use
HHFK are for research use only. They are not approved for human or animal use, or for application in *in vitro* diagnostic procedures.

Storage
Upon receiving, directly and immediately transfer the cells from dry ice to liquid nitrogen and keep the cells in liquid nitrogen until they are needed for experiments.

Shipping
Dry ice.

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[1] Obyama M, Terunuma A, Tock CL, Radonovich MF, Pise-Masison CA, Hopping SB, Brady JN, Udey MC, Vogel JC. (2006) "Characterization and isolation of stem cell-enriched human hair follicle bulge cells." *J Clin Invest*. 116: 249-60.
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Appendix B

Data sheet of human hair follicle stem cells

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DATA SHEET

Human Hair Follicle Stem Cells – Frozen Vial

Catalog number: 36007-08

Description: Frozen Ampule (1.2 x 10^6 cells) of 1 x 10^6 viable cells upon thawing, shipped with dry-ice. Also available in T25, T75, T150, and T225 tissue culture flask with plated cells, shipped at room temperature. The Human Hair Follicle Stem Cells was derived from Human Frontal region Scalp extracted from Hair Follicle bulge. They were maintained in Celprogen's Human Hair Follicle Stem Cells Complete Growth Medium and sub-cultured every 24 to 48 hours on Human Hair Follicle Stem Cells Extracellular Matrix.

Source: Human Frontal region Scalp extracted from Hair Follicle bulge

Mycoplasma test: Negative-PCR and mycoplasma agar methods

Sterility: Negative for bacteria, yeast, and mold

Donors: All donors from which the cells were derived were pre-screened; donors tested negative for the usual blood donation infectious disease panel ABO/RH, Hepatitis B Surface Antigen, HIV1 and 2, Syphilis, hepatitis B Core, Human T Lymphocyte Virus 1 and 2, Hepatitis C Virus, Antibody Screen, Nucleic Amplification Test for HIV 1 HCV, West Nile Virus and Antibodies to Trypanosoma cruzi (the agent of Chagas disease).

Storage Conditions:

Frozen Vial: Liquid nitrogen vapor phase for frozen Ampule of Human Hair Follicle Stem Cells .

Plated Cells: For plated cells in tissue culture flask, upon receipt of the cells wipe the flask with 70% ethanol and transfer to sterile tissue culture hood. In the tissue culture hood remove the media from the cells and wash the cells with 1X PBS sterile solution, for 2-3 minutes, remove the 1X PBS solution and then Trypsinize. After Trypsinization of the Cells neutralize the Trypsin with equal volume of Human Hair Follicle Stem Cells Complete Growth Media with Serum and collect the Cell suspension in sterile conical centrifuge tube in the tissue culture hood. Centrifuge the cell suspension at 100g for 7 minutes in centrifuge. Plate cells 5x10^3 cells per pre-coated flasks with Human Hair Follicle Stem Cells Extra-cellular Matrix for Expansion in Human Hair Follicle Stem Cells Complete Growth Medium.

Thawing of Cryovials: It is vital to thaw cells correctly in order to maintain the viability of the culture and enable the culture to recover more quickly. Some cryoprotectants, such as DMSO, are toxic above 4°C therefore it is essential that cultures are thawed quickly (< 1 minute) and diluted in complete growth media with serum to minimize the toxic effects. Briefly, remove the cryovial containing the frozen cells from dry ice or from liquid nitrogen vapor phase, and immediately place in 37°C water bath or 37°C dry oven in a shaker. Quickly thaw the cell (< 1-2 minutes) by gently swirling the vial in the 37°C water bath or 37°C dry oven in a shaker, until there is just a small bit of ice left in the vial. Spray the vial with 70% alcohol and transfer the thawed vial in the tissue culture hood. [See protocol for detail]

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Positive Markers: b1-integrin, Keratin 15, Keratin 19, CD71, SA 200, S100, CK19+, CK14+ 10%, CD34, CD133, Nestin, Ki67, Sox 2, Neurogenin 3, Map 2, O 2A, S-100B & NF

Morphology & Proliferation: Mixed population of cells with approximately 95% attached cells and the other 5.0% in suspension, need to change cell culture media every day after 48 hours of initial cell culture or when the media starts changing color to slight yellow from pink. This is a fast growing cell culture (with a population doubling time of 24 hours). Change media with Celprogen's Human Hair Follicle Stem Cells Complete Growth Medium with the appropriate Human Hair Follicle Stem Cells Extra-cellular Matrix. Temperature 37°C in 5% CO_2 humidified incubator. Population doubling is 80-120 or up to 12 passages when cultured at 60% to 70% confluent in Celprogen culture system. The cells are shipped at passage 2.

Sub-culturing:

1. Thaw the vial with gentle agitation in a 37°C water bath or a dry 37°C shaking incubator. For water bath thawing keep the O-ring out of the water.
2. Remove the thawed vial and wipe with 70% ethanol. Then transfer to the tissue culture hood.
3. Transfer the vial contents to a sterile centrifuge tube, and gently add pre-warmed Human Hair Follicle Stem Cells Complete Growth Media to the centrifuge tube. Use additional Human Hair Follicle Stem Cells Complete Growth Media to rinse the vial and transfer the liquid to the centrifuge tube, repeat this once more to ensure you have all the cells transferred to the 15ml centrifuge tube. Centrifuge the cells at 100g for 7 minutes. Remove the supernatant and re-suspend the cell pellet in 500ul of Human Hair Follicle Stem Cells Complete Growth Medium.
4. Add the 500ul of cells to the appropriate size flask pre-coated with Human Hair Follicle Stem Cells Extra-cellular Matrix with 10ml of Human Hair Follicle Stem Cells Complete Growth Medium.
5. Incubate the cells in the appropriate size flask at 37°C in a 5% CO_2 humidified incubator. Perform 100% Media Change every 24 to 48 hours.
6. Medium renewal every other day or 2-3 days, sub-culturing ratio: 1:2 or 1:3 depending on the cell density.
7. Refer to protocols, flow diagrams and videos for more detail. http://celprogen.com/tech.htm

Seeding cells from Plated Tissue culture flasks:

For plated cells in tissue culture flask, upon receipt of the cells wipe the flask with 70% ethanol and transfer to sterile tissue culture hood. In the tissue culture hood remove the media from the cells and wash the cells with 1X PBS sterile solution, for 2-3 minutes, remove the 1X PBS solution and then Trypsinize. After Trypsinization of the Cells neutralize the Trypsin with equal volume of Human Hair Follicle Stem Cells Culture Complete Growth Media with Serum and collect the Cell suspension in sterile conical centrifuge tube in the tissue culture hood. Centrifuge the cell suspension at 100g for 7 minutes in centrifuge. Plate cells $5x10^5$ cells per pre-coated flasks with Human Hair Follicle Stem Cells Culture Extra-cellular Matrix for Expansion in Human Hair Follicle Stem Cells Culture Complete Growth Medium.

Freezing Medium: Available for purchase Cat# M36007-08FM

Trypsin: Available for purchase Cat# T1509-014

IX PBS: Available for Purchase Cat# P1408-013

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Storage temperature: Liquid nitrogen vapor phase

Product Orders: Before submitting an order you will be asked to read and accept the terms and conditions of Celprogen's Material Transfer Agreement (MTA).

Permits/Forms: In addition to the MTA mentioned above, other CELPROGEN and/or regulatory permits may be required for the transfer of this CELPROGEN material. Anyone purchasing CELPROGEN material is ultimately responsible for obtaining the permits.

Biosafety Level 1

Notices & Disclaimers: ***CELPROGEN products are intended for laboratory research purposes only. They are not intended for use in humans.*** The Product, Human Hair Follicle Stem Cells , is established and manufactured by CELPROGEN Inc., and is for Research Use Only. This product is not for re-sale or may not be transferred to a third party prior to written request and approval by CELPROGEN Inc.

Appendix C

Certificate of analysis of human hair follicle stem cells

Celprogen Inc
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CERTIFICATE OF ANALYSIS:

Product name: Human Hair Follicle Stem Cell Culture – Frozen Vial	**Lot #: 1316001-01**

Catalog Number:	36007-08
Description:	Frozen Ampule (1.2×10^6 cells) of 1×10^6 viable cells upon thawing, shipped with dry-ice. The Hair Follicle Stem Cell Culture was derived from Human Ovarian tissue. They have been maintained in Celprogen's Human Hair Follicle Stem Cell Complete Growth Medium and sub-cultured every 24 to 48 hours on Human Hair Follicle Stem Cell Extra-cellular matrix. Also available as plated cells in T25 flasks and T75 flasks.
Mycoplasma Test:	Negative – PCR and mycoplasma agar methods
Sterility:	Negative for bacteria, yeast, and mold
Storage Conditions:	Liquid nitrogen vapor phase for frozen vial of Human Hair Follicle Stem Cell Culture. Plated cells in Flasks are shipped at room temperature, upon arrival, remove 100% media and add 10mls of fresh media for T25 flasks and incubate at 37°C humidified tissue culture incubator for 2-3 hours. After incubation subculture the cells with 1X Trypsin EDTA and the subculture ratio 1:2 to 1:4
Tests Performed:	bl-integrin, Keratin 15, Keratin 19, CD71, SA 200, S100, CK19+, CK14+ 10%, CD34, CD133, Nestin, Ki67, Sox 2, Neurogenin 3, Map 2, O 2A, S-100B & NF
Morphology & Proliferation:	Mixed population of cells with approximately 95% attached cells and the other 5.0% in suspension, need to change cell culture media every day after 48 hours of initial cell culture or when the media starts changing color to slight yellow from pink. Fast growing cell culture, change media with Celprogen's Human Hair Follicle Stem Cell Complete Growth Medium with the appropriate Human Hair Follicle Stem Cell Extra-cellular Matrix. Temperature 37oC in 5% CO_2 humidified incubator.
CERTIFICATION:	*The product is Human Hair Follicle Stem cell culture established by CELPROGEN Inc., and is for Research Use Only. This product is for laboratory use only and is not for Human use. This product is manufactured and distributed by CELPROGEN Inc. Cells are only guaranteed with purchase of Media and Extra Cellular Matrix for appropriate cell culture for 30 days from the date of shipment.*

Certified by: Date: 9-13-13

NOT FOR RESALE OR TRANSFER TO THIRD PARTY WITHOUT PRIOR WRITTEN APPROVAL FROM CELPROGEN

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Appendix D

Data sheet of follicle dermal papilla cell

Follicle Dermal Papilla Cell

PromoCell

Instruction Manual

Product	Size	Catalog Number
Human Follicle Dermal Papilla Cells (HFDPC)	500,000 cryopreserved cells 500,000 proliferating cells	C-12071 C-12072

Product Description

Human follicle dermal papilla cells are located in the dermal papilla at the base of hair follicles. Embedded in a laminin and collagen IV rich extracellular matrix, they are essential for induction and maintenance of hair growth. Since these cells express androgen receptors, they represent a well-suited model system for androgen-related research. PromoCell offers Human Follicle Dermal Papilla Cells (HFDPC) produced at PromoCell's cell culture facility from normal human dermis of the lateral scalp. Information on donor hair and skin color is available for each lot.

Shortly after isolation, all PromoCell Human Follicle Dermal Papilla Cells are cryopreserved at passage 2 (P2) using PromoCell's proprietary, serum-free freezing medium, Cryo-SFM. Each cryo vial contains more than 500,000 viable cells after thawing. Proliferating cell cultures are made from 500,000 cryopreserved cells that have been thawed and cultured for three days at PromoCell.

Quality Control

Rigid quality control tests are performed for each lot of Follicle Dermal Papilla Cells.

They are tested for cell morphology, adherence rate, and cell viability. Immunohistochemical tests for significant markers, e.g. alkaline phosphatase, are carried out for each lot. Growth performance is tested through multiple passages up to 10 population doublings (PD) under culture conditions without antibiotics and antimycotics.

In addition, all cells have been tested for the absence of HIV-1, HIV-2, HBV, HCV, and microbial contaminants (fungi, bacteria, and mycoplasma).

A detailed certificate of analysis (CoA) for each lot can be downloaded at: www.promocell.com/coa

Intended Use

PromoCell Follicle Dermal Papilla Cells are for *in vitro* research use only and not for diagnostic or therapeutic procedures.

Warning

Although tested negative for HIV-1, HIV-2, HBV, and HCV, the cells - like all products of human origin - should be handled as potentially infectious. No test procedure can completely guarantee the absence of infectious agents.

Follow appropriate safety precautions!

After delivery, start immediately with the protocol for cryopreserved cells (see page 2) or the protocol for proliferating cells (see page 3).

Appendix E

Certificate of analysis of follicle dermal papilla cells

Certificate of Analysis

Human Follicle Dermal Papilla Cells (HFDPC)

PromoCell

author: CT created: 03. September 2013 page: 1/1

Description

Product Name:	HFDPC-c / HFDPC-p
Order Number	C-12071 / C-12072
Lot Number	3100203.2
Donor Age / Sex / Race	32 / male / caucasian
Tissue / Localization / Hair Color	skin / occipital / dark brown
Number of Viable Cells	700.000
Freezing Medium	Cryo-SFM (Order No.: C-29910)
QC Evaluation Medium	Follicle Dermal Papilla Cell Growth Medium (Order No.: C-26501)
Stage of Culture	HFDPC-c: thawing and seeding results in passage 2 (3rd culture) HFDPC-p: shipped in passage 2 (3rd culture)

Viability & Growth Characteristics

Parameter / Test method*	Result
Viability / ISO 13319	91 %
Population Doubling (PD) Time in Log Phase / PDL Test	27.7 h / PD
Population Doublings	> 10 PD*

Phenotypic Characterization (tested within the first two passages)

Parameter / Test method*	Result
Immunohistochemical staining	positive for alkaline phosphatase

Test for microbiological contaminants and infectious viruses

This lot is found to be nonreactive to following contaminations by validated test methods:

Parameter / Test method	Result
Bacteria, Fungi / Direct Plating	negative / negative
Mycoplasma Genus, M. Pulmonis / PCR	negative / negative
HIV-1, HIV-2 / PCR	negative / negative
HBV, HCV / PCR	negative / negative
HTLV-1, HTLV-2 / PCR	negative / negative

* Using PromoCell's standardized culture system and procedures. The stated values may vary under customer culture conditions.

The tissue used by PromoCell for the isolation of human cell cultures is derived from donors who have signed an informed consent form, which outlines in detail the purpose of the donation and the procedure for processing the tissue (www.promocell.com/ethics).

Dr. Irina Börcsök
Head of Quality Control

Date: Nov 20, 2013

FOR IN VITRO RESEARCH USE ONLY. NOT FOR DIAGNOSTIC OR THERAPEUTIC PROCEDURES.

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Version 3

Appendix F

Primers used in this study

Gene	Primer sequence
GAPDH	(F) 5’- GAAATCCCATCACCATCTTCCAGG -3’ (R) 5’- GAGCCCCAGCCTTCTCCATG -3’
ALPL	(F) 5’- GCTGGGAAATCTGTGGGCAT -3’ (R) 5’- AGCTGGTAGGCGATGTCCTT -3’
CTNNB1	(F) 5’- CGCCATTTTAAGCCTCTCGG -3’ (R) 5’- CTCCTCAGACCTTCCTCCGT -3’
K6hf	(F) 5’- GGCTATACCCATTCCCAGGC -3’ (R) 5’- GGAGAGAGCAGGCTTTGGTT -3’
Ki67	(F) 5’- CACTGCAAAAGAGGTGCGAG -3’ (R) 5’- CCGGGGATAGGTGAATTGGG -3’
LEF1	(F) 5’- AGAGCGAATGTCGTTGCTGA -3’ (R) 5’- TTTAGCCTGCTCTTCACGGG -3’
SOX2	(F) 5’- ATGTACAACATGATGGAGACG -3’ (R) 5’- GCGCTTGCTGATCTCCGAGT -3’
TCF3	(F) 5’- CCCATCTGTGTCCCATGTCC -3’ (R) 5’- TGGGAAGAGCTGGTTACCCT -3’
WNT5A	(F) 5’- GTTTCGGCTACAGACCCAGA -3’ (R) 5’- CCCCAGTTCATTCACACCACA -3’
VCAN	(F) 5’- TCAAGCTGCTGGCAAGTGAT -3’ (R) 5’- CCGCCCTGTAGTGAAACACA -3’