STIMULATION OF HAIR GROWTH USING CONDITIONED MEDIA DERIVED FROM DENTAL PULP STEM CELLS EXPANDED IN SERUM FREE MEDIA

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FACULTY OF DENTISTRY
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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT

Introduction: Alopecia is a clinical condition caused by the hair loss and may result in baldness. The current treatment methods for this condition involve the use of drugs or natural substances. However, hair loss may be accelerated upon the discontinuation of medication. Alternatively, alopecia surgery could also be carried out though it has its limitations in the number of surgeries and the number of hair strands transplanted. Stem cell therapy, a recently emerged approach for alopecia treatment involves tedious procedures, complicated safety and quality management, low survival rate of transplanted cells and potential adverse immunological responses for the recipients. As an alternative, use of paracrine factors secreted by the stem cells showed promise in therapeutic applications. Paracrine factors involved in regulation of hair growth have been observed to be secreted by stem cells, in the culture media, termed as conditioned media (CM). **Objectives:** In this study we first verified the mesenchymal stem cell (MSC) like properties of human exfoliated or extracted deciduous dental pulp (SHED) and hair follicle stem cells (HFSCs) cultured in different concentrations of serum supplemented in DMEM-KO and in chemically defined media namely, STK2 (TwoCELLs, Japan). Secondly, hair growth regulatory paracrine factor profiles of CM prepared through culture of SHED and HFSCs in the respective media were ascertained. Lastly, the potential of SHED- and HFSC-CM to stimulate hair growth under *in vitro* and *in vivo* conditions were evaluated. **Methods:** SHED (n=3) and HFSCs (n=3) were cultured in, STK2 and other media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS and profiled for the presence of positive hair regulatory factors namely SDF-1, VEGF, HGF, PDGF-B and negative hair regulatory factors namely TGF-β, bFGF, TNF-α, IL-1 and BDNF. The potential of the prepared CM to stimulate hair growth was evaluated based on the paracrine profile and the observed *in vitro* hair growth dynamics. The administration of the CM media via

subcutaneous injection to telogen-staged synchronized 7-week old C3H/HeN female mice was carried out to further study the potential of the CM to stimulate hair growth *in vivo*. **Results:** SHED and HFSCs maintained their MSC characteristics in all media combinations. Cells cultured in STK2 based media showed better population doubling time, higher viability and better maintenance of MSC characteristics in comparison to cells cultured in DMEM-KO based media. SHED and HFSCs showed higher growth kinetics at passages 3 and 4. Cells cultured in DMEM-KO based media showed a higher expression of positive hair regulatory factors; SDF-1, VEGF, HGF, PDGF-B than cells cultured in STK2 based media. However, the difference was not statistically significant. Moreover, STK2 based CM contained only two negative hair regulatory factors, TNF-α, IL-1 while DMEM-KO based CM media contained all negative hair regulatory factors that were tested. The *in vitro* studies confirmed that treatment with CM of passage 3 cells prepared in STK2 based media resulted in a significantly higher number of anagen-staged hair follicles ($p < 0.05$) and a significantly lower number of telogen-staged hair follicles ($p < 0.05$). Administration of SHED-CM prepared in STK2 to C3H/HeN mice resulted in a significantly faster stimulation of hair growth in comparison to the HFSC-CM prepared in STK2 ($p < 0.05$). **Conclusions:** Within the limitations of the study, STK2 is a better media for the expansion of both SHED and HFSCs and the maintenance of stem cell characteristics in comparison to DMEM-KO based media. SHED-CM prepared using STK2, significantly enhanced the stimulation of hair growth compared to HFSC-CM.

ABSTRAK

Pengenalan: *Alopecia* adalah suatu keadaan klinikal yang disebabkan oleh keguguran rambut dan boleh menyebabkan seseorang menjadi botak. Kaedah rawatan sedia ada melibatkan penggunaan ubat-ubatan berasaskan bahan kimia atau bahan semula jadi. Walaubagaimanapun, keguguran rambut boleh berlaku dengan lebih cepat sekiranya pengambilan ubat dihentikan. Sebagai alternatif, pembedahan *alopecia* boleh dilakukan walaupun ada kekangan terhadap jumlah pembedahan dan jumlah rambut pemindahan rambut yang boleh dilaksanakan. Terapi sel stem adalah pendekatan baru yang melibatkan pelbagai prosedur, isu keselamatan dan pengurusan kualiti yang rumit, kadar kemandirian sel yang dipindahkan adalah rendah dan boleh mencetuskan tindak balas imunologi kepada penerima. Sebagai alternatif, faktor parakrin yang dirembes oleh sel-sel stem di dalam medium kultur, yang diistilahkan sebagai *conditioned media* (CM) telah menunjukkan keputusan yang memberangsangkan. **Objektif:** Objektif pertama adalah pencirian dan pengekalan status sel stem bagi sel dari pulpa gigi desidus manusia yang terlupas (exfoliated) atau dicabut (SHED) dan sel-sel folikel rambut (HFSCs) apabila dikultur dalam medium DMEM-KO dan STK2 dengan penambahan serum yang pelbagai kepekatan. STK2 (TwoCELLs, Jepun), suatu medium kultur yang komposisi kimianya telah ditakrifkan. Kedua, faktor parakrin yang bertanggungjawab bagi pengawalaturan pertumbuhan rambut di dalam CM yang disediakan melalui pengkulturan SHED dan HFSCs juga ditentukan. Akhir sekali, potensi SHED- dan HFSC-CM dalam merangsang pertumbuhan rambut di dalam keadaan *in vitro* dan *in vivo* dinilai. **Kaedah:** SHED (n=3) dan HFSCs (n=3) dikulturkan dalam STK2 dan pelbagai gabungan medium lain; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS dan menunjukkan profil faktor positif bagi pengawalaturan rambut; SDF-1, VEGF, HGF, PDGF-B dan faktor negatif bagi pengawalaturan rambut; TGF-β, bFGF, TNF-α, IL-1 dan BDNF. Penilaian potensi CM yang disediakan untuk

merangsang pertumbuhan rambut dijalankan berdasarkan profil parakrin dan kajian *in vitro* pertumbuhan rambut. Suntikan CM secara subkutaneus kepada tikus betina C3H/HeN yang berumur 7 minggu dimana fasa Telogennya telah disegerakkan bagi mengkaji potensi CM untuk merangsang pertumbuhan rambut *in vivo*. **Keputusan:** SHED dan HFSCs mampu mempertahankan ciri-ciri MSCs dalam semua kombinasi medium. Sel-sel yang dikultur dalam medium STK2 menunjukkan masa penggandaan populasi, kebolehhidupan dan pengekalan status MSCs yang lebih baik berbanding dengan sel-sel yang dikultur dalam DMEM-KO. SHED dan HFSCs menunjukkan kinetik pertumbuhan yang lebih baik dalam *passage* 3 dan 4. Sel dikultur dalam medium berasaskan DMEM-KO menunjukkan ekspresi faktor positif bagi pengawalaturan rambut yang lebih tinggi; SDF-1, VEGF, HGF, PDGF-B berbanding sel yang dikultur dalam medium yang berasaskan STK2. Walau bagaimanapun, perbezaan ini didapati tidak signifikan secara statistik. Tambahan pula, medium berasaskan STK2 hanya mengekspresikan dua faktor negatif bagi pengawalaturan rambut, TNF-α, IL-1 manakala CM dari medium berasaskan DMEM-KO mengekspresikan semua faktor negatif bagi pengawalaturan rambut. Kajian *in vitro* jelas menunjukkan bahawa CM dari sel-sel dalam *passage* 3 yang dikultur dalam STK2 menunjukkan peningkatan yang ketara dalam bilangan folikel rambut di fasa anagen ($p<0.05$) dan penurunan yang ketara dalam bilangan folikel rambut di fasa telogen ($p<0.05$). Suntikan CM yang berasaskan STK2 kepada tikus betina C3H/HeN, SHED-CM menunjukkan rangsangan pertumbuhan rambut yang lebih cepat berbanding HFSC-CM ($p<0.05$). **Kesimpulan:** Berdasarkan pengehadan kajian, STK2 adalah medium yang lebih baik bagi meningkatkan bilang kedua-dua jenis sel SHED dan HFSCs dan pengekalan status sel stem berbanding medium yang berasaskan DMEM-KO. SHED-CM yang berasaskan STK2, meningkatkan rangsangan pertumbuhan rambut yang signifikan berbanding HFSC-CM.

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LIST OF SYMBOLS AND ABBREVIATIONS

ADSC	: Adipose Derived Stem Cells
aFGF	: Acidic Fibroblast Growth Factor
Ang-1	: Angiopoietin-1
APC	: Allopycocyanin
BDNF	: Brain Derived Neurotrophic Factor
bFGF	: Basic Fibroblast Growth Factor
BMSCs	: Bone Marrow Stem Cells
CD	: Cluster of Differentiation
CNTF	: Ciliary Neurotrophic Factor
CM	: Conditioned Media
DHT	: Dihydroxy Testosterone
DMEM-KO	: Dulbecco's Modified Eagle's Medium- Knock Out
DPBS	: Dulbecco's Phosphate Buffered Saline
DPSCs	: Dental Pulp Stem Cells
EDTA	: Ethylenediaminetetraacetic Acid
EGF	: Epidermal Growth Factor
EPC	: Endothelial Progenitor Cells
FBS	: Feotal Bovine Serum
FDA	: Food and Drug Administration
FITC	: Fluorescein Isothiocynate
GCSF	: Granulocyte Colony Stimulating Factor
GDNF	: Glial Cell Derived Neurotrophic Factor
GMCSF	: Granulocyte Macrophage Colony Stimulating Factor
GRO-α	: Growth Regulated Protein Alpha
H&E	: Hematoxyline and Eosin

HEK : Human Epithelial Keratinocytes

hESC : Human Embryonic Stem Cells

HF : Hair Follicle

HFDPC : Hair Follicle Dermal Papilla Cells

HFSCs : Hair Follicle Stem Cells

HGF : Hepatocyte Growth Factor

HLA-DR : Human Leukocyte Antigen- Antigen D Related

HLA-G : Human Leukocyte Antigen- G

ICAM-1 : Intercellular Adhesion Molecule 1

ICR : Imprinting Control Region

IFN-g : Interferon Gamma

IGF : Insulin Like Growth Factor

IGFBP-7 : Insulin Like Growth Factor Binding Protein 7

IL : Interleukin

IP : Intraperitoneal

KGF : Keratinocyte Growth Factor

KSFM : Keratinocyte Serum Free Media

LIF : Leukaemia Inhibitory Factor

MCOA : Middle Cerebral Artery Occlusion Surgery

MCP-1 : Monocyte Chemoattractant Protein 1

MCSF : Macrophage Colony Stimulating Factor

microRNA : micro Ribo Nucleic Acid

MSCs : Mesenchymal Stem Cells

NCF-1 : Neutrophil Cytosolic Factor 1

Nd : Not detected

NGF : Nerve Growth Factor

P	: Passage
PDGF	: Platelet- Derived Growth Factor
PDLSCs	: Periodontal Ligament Stem Cells
PDT	: Population Doubling Time
PE	: Phycoerythrin
Pen-Strep	: Penicillin-Streptomycin
PerCP	: Periclinin Chlorophyll Protein Complex
PBS	: Phosphate Buffer Saline
PIGF	: Placental Growth Factor
PTHrP	: Parathyroid Hormone Related Peptide
RANKL	: Receptor Activator of Nuclear Factor Kappa-B Ligand
RANTES	: Regulated on Activation, Normal T cell Expressed and Secreted
SAPE	: Streptavidin-R-Phycoerythrin Conjugate
SCF	: Stem Cell Factor
SDF-1	: Stromal Derived Factor 1
SF	: Scatter Factor
SHED	: Stem Cells from Human Exfoliated Dental Pulp
SHH	: Sonic Hedge Hog
SPARC	: Secreted Protein, Acidic and Rich in Cysteine
Strp2	: Striatin Interacting Protein 2
TGF	: Transforming Growth Factor
TNF-α	:Tumor Necrosis Factor-alpha
TRKC	: Tyrosine Kinase Receptor
UV	: Ultraviolet
VCAM-1	: Vascular Cell Adhesion Molecule 1
VEGF	: Vascular Endothelial Growth Factor

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CHAPTER 1:INTRODUCTION

1.1 Overview

Hair-loss has a major impact on the social interactions and psychological wellbeing of an individual (Cotsarelis & Millar 2001), as hair plays a critical role in non-verbal communication (Stenn & Cotsarelis, 2005). The condition of hair loss from the head or body in clinical terms is referred to as ~~–a~~lopecia", which may eventually result in baldness (Yoo et al., 2010). Androgenetic alopecia is a common form of alopecia which generally affects approximately 73% of the female and male population in Asia (Lee & Lee, 2012), 55% of the females who suffer from hair loss are prone for depression, while 78% of men suffer from anxiety or 22% of the males has been reported to be aggressive (Levy & Emer, 2013).

The current treatment modalities for alopecia involves the use of drugs such as Finastride (Kaufmann et al., 1998) and Minoxidil (Price, 1987) or natural substances such as *Hibiscus rosa-sinensis* (Adhirajan, Kumar, Shanmugasundaram & Babu, 1994). Although proven to be effective, discontinuation of these treatment modalities showed the risk of accelerating hair loss. Alternatively, alopecia surgery may also be performed. However, it has several limitations including the number of surgeries one could undergo; firstly, the surgery can only be performed on an individual for a maximum of 3 times, and secondly, the number of hair strands that could be transplanted during each surgical procedure is limited to a maximum of 2000 (Yoo et al., 2010). Thus, effective treatment strategies are yet to be developed, in order to overcome the issues that are faced by current treatment strategies. A clear understanding of the process of hair growth in the human body and the underlying mechanisms of alopecia is also essential in order to progress further.

The hair regeneration is a cyclic process categorized in to four main stages; anagen (active phase), catagen (regression phase), telogen (resting phase) and exogen. In all four major forms of alopecia; androgenic alopecia, telogen effluvium, chemotherapy induced alopecia and alopecia areata, the hair follicles at the anagen stage enter the catagen or telogen stages simultaneously resulting in early shedding of hair. However, it is pertinent to note that the latter two forms of alopecia are generally reversible conditions since the hair follicle stem cells (HFSCs) are not affected. HFSCs are the native stem cell source that is responsible for the regeneration of hair, owing to their differentiation capacities (Cotsarelis & Millar 2001). In terms of the developmental biology, HFSCs are well defined and they are located in discrete compartments or niches making them one of the best models to study the regenerative potential of adult stem cells (Walters, Richardson, & Jahoda, 2007). HFSCs are present in a specialized structure that is known as the bulge region, located within the outer root sheath (Costsarelis, Sun & Lavker, 1990) of a hair follicle in an unspecialized stage. The main role of HFSCs is to generate the hair follicle and the shaft (Tiede et al., 2007). During skin injuries, they also contribute to the regeneration of the sebaceous glands (Cotsarelis, 2006), epidermis (Blanpain, Lowry, Geoghegan, Polak & Fuchs, 2004) and neurons (Amoh et al., 2009). These phenomena suggest that stem cells could be a potential resource for regulating and restoring the hair growth cycle, thus providing an effective approach for treating alopecia.

Currently, HFSCs obtained from scalp biopsies have been expanded under *in vitro* culture conditions and injected into the bald scalp regions for the stimulation of hair growth (Adams, 2011). The Repli-Cel™ is currently undergoing a phase II clinical trial whereby autologous dermal cells are injected in to the scalp in order to create new hair follicles (RepliCel Life Sciences, 2016). Adipose derived stem cells (ADSCs) have also been recently explored for the treatment of androgenic alopecia and are currently under

phase II clinical trials (Kerastem Technologies, 2016). Cell-based therapies for regeneration possess a number of issues that need to be addressed as discussed in Table 2.1 (Cho et al., 2012; Ide et al., 2010; Osugi et al., 2012; Mamidi et al 2012; Di Santo et al., 2009; Yang, Di Santo, & Kalka, 2010). To minimize these negative issues, the regeneration capacity of stem cells is capitalized through novel approaches such as replacement of cell transplantation by administering paracrine factors secreted by these cells.

Conditioned media (CM) is a rich source of paracrine factors (Osugi et al., 2012) that are regarded as the main driving force behind stem cells' therapeutic action (Jones, Estirado, Redondo, Bueno & Martinez, 2012). Thus, cell free CM could be an alternative to cell-based therapies. An *in vivo* study involving an animal model has been carried out using ADSC-CM to stimulate hair growth (Park et al., 2008). AAPE™ (now known as NGAL™), an advanced CM prepared from ADCSs is also being used in combination with microneedling for alopecia treatment in human (Fukuoka & Suga, 2015; Shin, Ryu, Kwon, Park, & Jo, 2015).

Even though dental pulp stem cells (DPSCs) have been shown to have a regenerative potential for the generation of hair follicles (Reynolds & Jahoda, 2004), further studies are required to warrant its application to treat alopecia. DPSCs were first isolated by Gronthos and co-workers from the impacted third molars in the year 2000 (Gronthos, Mankani, Brahim, Robey, & Shi, 2000), following which the same group isolated them from exfoliated deciduous teeth (Miura et al., 2003). In comparison to the current stem cell sources used for hair growth (Park et al., 2010; Shin et al., 2015; Zhao et al., 2008) DPSCs can be collected easily from exfoliated or extracted teeth. Since these stem cells are isolated from extracted or exfoliated teeth which could be considered as a waste product it acts as a non-controversial source, thus drawing less ethical concerns

(Nakashima, Iohara, & Murakami, 2013). It also has advantages with respect to its safety, minimal invasiveness and discomfort caused to the donors during the process of harvesting of stem cells from the source. Furthermore, DPSCs from extracted or exfoliated teeth can be obtained from young donors and stored for future usage (Huang, Chen, Lin, Shieh, & Chan, 2008). Compared to many other sources dental pulp tissues also yield a relatively higher number of stem cells (Kanafi, Pal, & Gupta, 2013). These cells has also been shown to possess a high proliferative capacity (Huang, Gronthos, & Shi, 2009) and a better immunosuppressive activity (Pierdomenico et al., 2005) in comparison to the gold standard, bone marrow derived stem cells (BMSCs). Additionally, DPSCs have been demonstrated to possess the ability to differentiate into hair follicles, neural cells, elastic cartilage cells, skeletal and/or smooth muscle cells, endothelial cells, adipocytes, osteoblasts, dentine producing odontoblasts under *in vitro* and *in vivo* conditions (Karaoz et al., 2010; Nakashima et al., 2013). The paracrine factor profiles of these cells indicated the presence of many positive paracrine factors known to support hair growth and only TGF-β as the negative hair growth regulatory factor (Nakashima et al., 2013). Based on the capability of DPSCs to regenerate hair follicles, and their paracrine secretion profiles, we aimed to investigate the hair growth potential of CM of dental pulp stem cells obtained from human deciduous extracted or exfoliated teeth (SHED) in comparison to the CM obtained from HFSCs.

To achieve this aim, careful consideration was taken when choosing the suitable media for culturing stem cells and the production of CM. The media should be able to facilitate the secretion of the desired paracrine factors in sufficient concentration and quantity. The conventional media used in culturing stem cells include feotal bovine serum (FBS) as a supplement due its high content of growth factors and attachment factors (Selvaggi, Walker & Fleisher, 1997). However, supplementing media with FBS pose some risks. FBS holds the possibility of transmitting viruses, bacteria, yeasts,

prions and other endotoxins, which may be potentially infectious to the recipient (Heiskanen et al., 2007; Shahdadfar, Fronsdal, Haug, Reinholt & Brinchmann, 2005; Spees et al., 2004). The quality of FBS also varies on the batch that FBS is extracted, which may affect the proliferation and differentiation potential of the cells. High concentration of serum is also known to lead to spontaneous differentiation (Gou et al., 2010; Tseng, Chem, Sheu, Yu, & Huang, 2007) and malignant transformation (Karbanova et al., 2011) of the cultured cells. Studies have been conducted using alternatives to FBS such as allogeneic and autologous human serum (Tateishi et al., 2008), and platelet-derived supplements (Schallmoser & Strunk, 2009). However, they too exhibit the potential of production lot-to-lot variation (Parker, Shang, Khurgel, & Katz, 2007) and are often only available in a limited amount. Chemically defined xeno-free culture media has been reported to be safe and efficient in expanding stem cells for therapeutic purposes (Wang et al., 2015).

STK2 is a chemically defined media developed by Two CELLs Inc. Co, Japan. The media is formulated with FGF, Platelet- Derived Growth Factor (PDGF), Hepatocyte Growth Factor (HGF), Transforming Growth Factor beta (TGF-β) and epidermal growth factor (EGF) (Koichiro, 2016). However, the exact composition of the media is not disclosed. Under serum free conditions, STK2 could eliminate the negative effects caused by the addition of serum, thus providing a safer approach for cell culture and CM collection for clinical applications. The STK media series has been used to expand cells for clinical trials in Japan (Uematsu, Negata, Kawase, Suzuki, & Takagi, 2013). This media is currently being used to culture synovial mesenchymal stem cells, ADSCs, human gingivial fibroblasts and dental derived stem cells. STK2 media in comparison to DMEM-KO media has minimal effect on the gene expression and morphology of cultured cells (Takeda-Kawaguchi et al., 2014; Tsugeno, Sato, Muragaki, & Kato,

2014). To the best of our knowledge, no previous studies have been reported using this media to culture HFSCs.

The overarching goal of the current study is to use CM of SHED to stimulate hair growth. The treatment strategy for hair loss involves an approach of prolonging the anagen phase of the hair cycle and to reduce the number of hair follicles that are in the telogen stage using paracrine factors derived from stem cells. Thus, in this study we propose to utilize the aforementioned approach by employing a cell-free strategy to stimulate the hair growth and the overview of the study framework is illustrated in Figure 1.1.

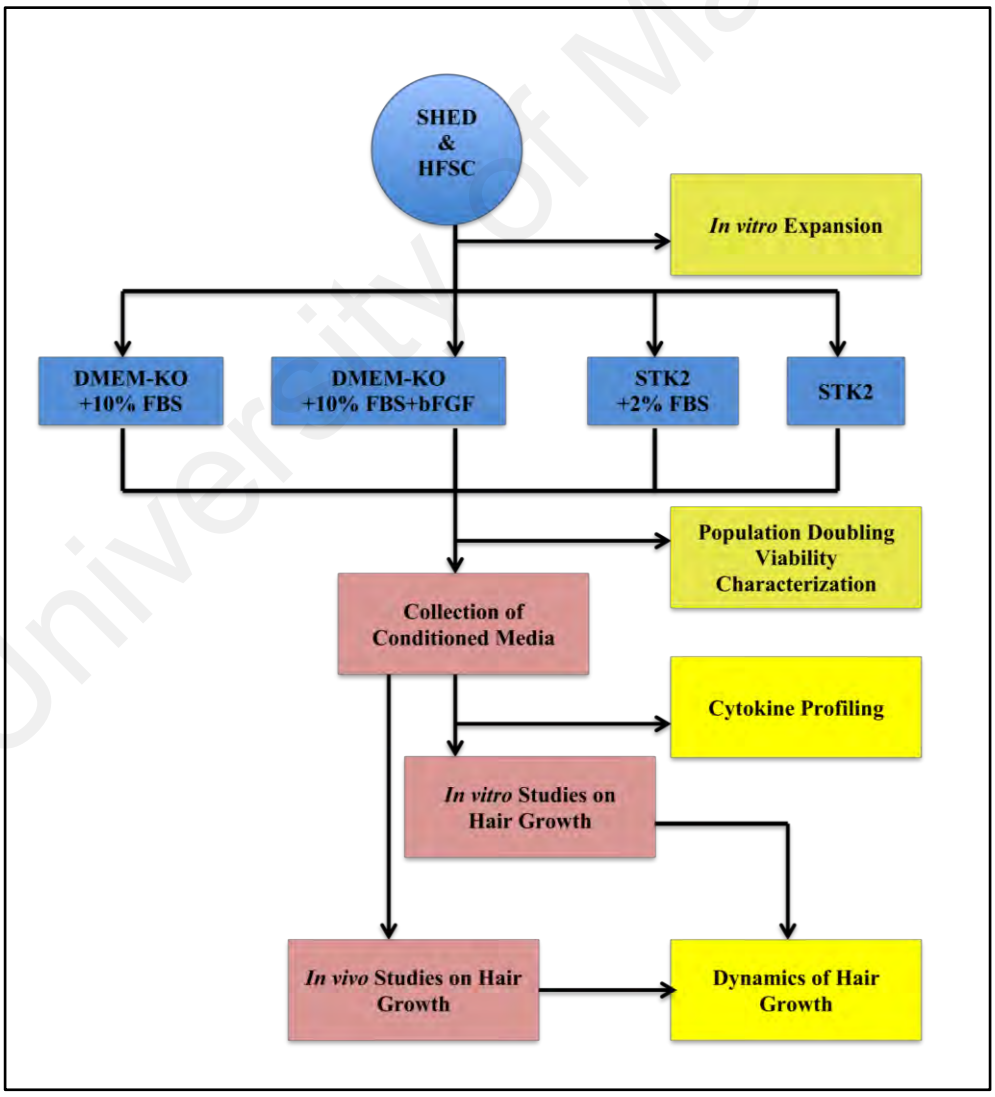


Figure 1.1: Overview of the experimental design

This study focuses on the following research questions-

I. Is there any effect of the various formulations of culture media on the growth characteristics and stem cell properties of SHED and HFSCs?
II. What is the optimal media composition to stimulate SHED and HFSCs for the secretion of positive hair regulatory factors?
III. Is SHED-CM as effective as HFSCs in inducing the anagen stage?
IV. Does the stem cell CM collected from different donors have an effect on hair growth?

1.2 Aim and objectives

1.2.1 Aim

The aim of this study is to stimulate hair growth using conditioned media derived from human extracted deciduous dental pulp stem cells.

1.2.2 General and specific objectives

1) To characterize the properties of stem cells of human exfoliated deciduous teeth (SHED) and hair follicle stem cells (HFSCs) cultured in various culture media. The experimental work was carried out to derive the following specific objectives;

a) To compare the differences in the *in vitro* population doubling time and viability of SHED and HFSCs with different combinations of serum supplement in either DMEM-KO or STK2.
b) To ascertain the influence of DMEM-KO and STK2 with different composition of serum supplementation on the stemness of SHED and HFSCs during *in vitro* culture.

2) To profile hair growth regulatory paracrine factors in *in vitro* SHED and HFSCs cultures. The following specific objectives were addressed under this objective;

a) To determine the paracrine factors secreted by SHED and HFSCs under different media combinations.
b) To study the effect of passage in the paracrine secretion profile of stem cells.

3) To evaluate the potential of STK2 based conditioned media (CM) using SHED (SHED-CM) and HFSCs (HFSC-CM) for hair growth in *in vitro* and *in vivo* models. The experiments were carried out to achieve the following specific objectives;

a) To compare the potential of the different media in promoting the anagen stage of hair follicles *in vitro.*
b) To determine the most suitable passage for the collection of CM in stimulating the anagen stage of hair follicles *in vitro.*
c) To determine and compare the time point for the appearance of the anagen stage in an animal model when treated with SHED-CM and HFSC-CM.
d) To ascertain if CM prepared from stem cells of different donors has an effect on the stimulation of hair growth in an animal model.

CHAPTER 2: LITERATURE REVIEW

2.1 Development of the hair follicle and hair growth

Understanding the normal hair follicle morphogenesis and growth would enable the development of better treatment approaches in stimulation of hair growth. The hair follicle development of an individual begins towards the end of the first trimester during pregnancy. This process is controlled by epidermal-mesenchymal interactions through a cascade of signals and cues between the basal epidermal layers and mesenchymal cell populations of the embryo during around 80 days of the estimated gestational age (Holbrook & Minami, 1991; Sennett & Rendl, 2012; Wang & Zhang, 2012; Yoo et al., 2010). Even though no new hair follicles are formed after birth, unlike other organs, the hair follicles regenerate themselves in a cyclical manner by the interaction of epithelial stem cells in the bulge region (Figure 2.1) with the adjacent mesenchymal derived dermal papilla cells (Stenn & Cotsarelis, 2005). The progression of hair growth consists of a unique cycle, which can be divided in to four stages namely, the anagen, catagen, telogen and exogen stages (Figure 2.2).

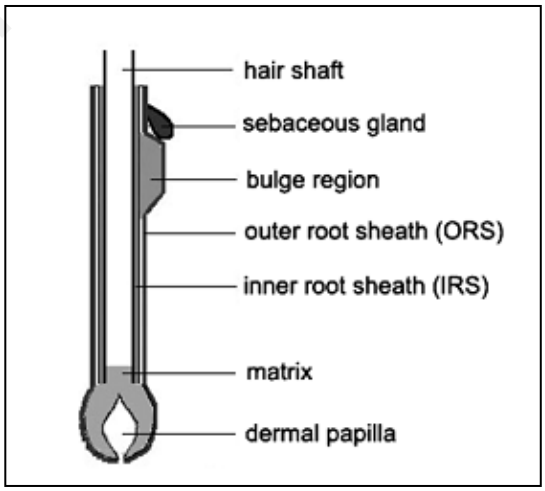


Figure 2.1: Structure of the hair follicle. The different parts of the hair follicle denoting the bulge region which carries the hair follicle stem cells. *Adapted from (Zhao et al., 2008)

During the anagen stage (growth period), the matrix cells at the epithelium base proliferate rapidly through mitotic division. They differentiate to produce hair fibres and other layers that are present within the hair root sheath. When the mitotic activity of these cells reduce, the hair follicle enters the catagen stage (regression period) where the cells present in the lower, transient, section of the follicle undergo apoptosis and the formation of a club fibre can be observed. The dermal papilla reaches to close proximity with the bulge region that consists of the stem cells which is followed by the telogen stage (resting/quiescence period). Following the hair shaft shedding (exogen stage), a new hair growth cycle begins (Waters et al., 2007). Thus, in adults, the hair follicle re-forms itself by the interaction between the stem cells within the bulge region and the adjacent mesenchymal derived dermal papilla cells (Stenn & Cotsarelis, 2005). An average hair cycle of a human being ranges for about 6-7 years of which the anagen phase lasts for approximately 2-6 years and consists of approximately 90-95% of the total hair. The catagen stage lasts for approximately 2-3 weeks and about 1% of the hairs are considered to be in this stage at a given time. The telogen stage lasts for about 2-3 months while 5% of the hair follicles are considered to be in this stage (Tully, Schwartzenberg, & Studdiford, 2010).

Each of these growth stages are regulated by different signalling cues such as Wingless-Type MMTV site Integration family member (Wnt), bone marrow morphogenetic protein (BMP), Notch and Sonic Hedge Hog (SHH) and activates pathways to activate different cellular processes. These cellular processes which are regulated in hair growth are modulated by some of the key transcription factors namely; distal –less homeobox 3 (Dlx3) , runt related transcription factor (Runx1), transcription factor 3/4 (Tcf3/4), nuclear factor of activated T cells 1 (NFATC1), LIM homeobox 2 (Lhx2), SRY-related HMG-box 9 (Sox9), T-box 1 (Tbx1), p63, lymphoid enhancer binding factor (Lef1), MX dynamin like GTPase 2/ Forkhead Box N1 (Mx2/Foxn1),

Gli, GATA binding protein 3 (GATA3). These factors are also responsible in regulating the hair follicle stem cell activation, self-renewal and differentiation (Lee & Tumbar, 2012).

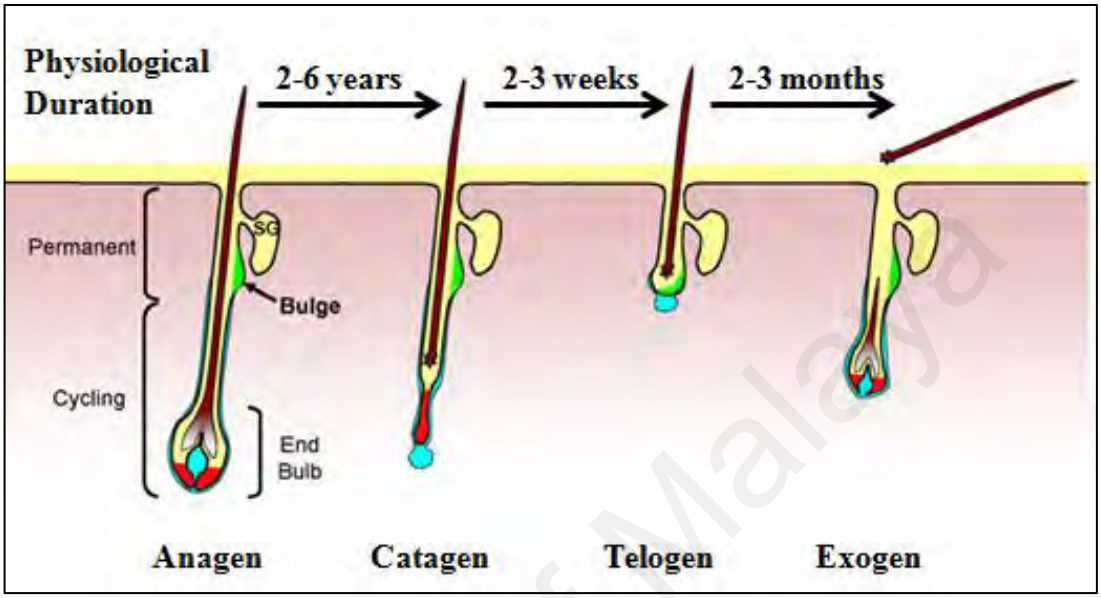


Figure 2.2: The events and physiological duration of the different stages of the human hair cycle. The schematic representation of the duration for the hair cycle stages: anagen, catagen, and telogen. The increase and decrease of the hair follicle length in relation to the position of the hair bulb is indicated. *Adapted from (Waters et al., 2007) SG-Sebaceous Gland

2.2 Signaling pathways regulating hair growth

I. Anagen stage

The onset of the anagen stage from the telogen-staged hair follicles is initiated by SHH proteins (Sato, Leopold & Crystal, 1999). It is also reported that this signal cue is required for the proliferation of epithelial cells later during the anagen stage but not for the transition from telogen stage in to the new cycle of hair follicle growth (Wang et al., 2000). During the anagen stage the dermal papilla secretes signaling ligands such as Wnt10a and Wnt10b required for proliferation to the follicular epithelium (Hardy, 1992; Lim & Nusse, 2013). The mitogenic effect of fibroblast growth factor 7 (FGF-7) also

plays a role during this stage for proliferative activity of the cells (Cotsarelis & Milar, 2001). The daughter cells of the proliferative matrix adopt the layers present in the inner root sheath or hair shaft. The cells in the hair shaft later differentiate into highly tensile structures following the process of keratinization. The duration of anagen phase eventually determines the length of the hair, which in turn is dependent on the proliferation of the matrix cells at the base of the hair follicle. The transition from anagen to catagen stage depends on the presence of many factors; fibroblast growth factor 5 (FGF-5), EGF, brain derived neurotrophic factor (BDNF), p75 neurotrophin receptor and TGF-β (Alonso & Fuchs, 2006).

II. Catagen stage

This stage is known for its massive programmed death. However, the molecular level understanding of this stage is not very well understood. The epithelial cells in the bulb and the outer root sheath undergo apoptosis. The differentiation of hair sheath retards and generates a rounded structure, called the club, which later moves upwards and remains at the same position during the telogen stage (Alonso & Fuchs, 2006). TGF-β and p75 neurotrophin receptors have been shown to play a role during this stage (Botchkarev et al., 2000, Stenn & Paus, 2003). TGF-β is considered to be responsible in the onset of the catagen stage (Botchkarev & Paus, 2003) while the p75 neurotrophin receptors are responsible in the induction of keratinocyte apoptosis during this stage (Botchkarev et al., 2000).

III. Telogen stage

The hair follicles remain dormant during this stage and thus this phase is also known as a dormant or resting phase. The transition of the stage from telogen to anagen begins when the hair follicle stem cells are activated to synthesize a new follicle. Its formation

occurs next to the club hair, which was formed previously. Upon shedding of the club hair, the bulge region is then exposed and stem cell activation leads the transition into the next stage, the anagen stage (Alonso & Fuchs, 2006).

2.3 Alopecia

The generic term for hair loss is "alopecia", is a phenomenon of lessening of visible hair. Loss of hair leads to a stressful state, which may lead to severe emotional impacts on the affected individual. This problem lowers the confidence level, self-image and esteem of the individual (Tully, Schwartzenberger, & Studdiford, 2010). Studies conducted by Wells et al. (1995) have reported that 75% of the men who undergo hair loss are less confident, especially during interaction with the opposite sex (Wells, Willmoth, & Russell, 1995). It is also reported that younger men are more prone to be mentally affected than elderly men (Cash, 1990). Thus, even though alopecia is not a life-threatening condition, the problem is needed to be addressed and treated to improve the socio-physiological wellbeing of the affected individuals.

Alopecia has been divided in to four major forms: androgenic alopecia, telogen effluvium, chemotherapy induced alopecia and alopecia areata (Cotsarelis & Millar, 2001). Androgenic alopecia affects nearly 50% of the men by the age of 50 (Price, 1999) and is a condition implicated at a genetic level manifested by the shortening of anagen phase. Meanwhile, telogen effluvium involves an excessive hair shedding, owing to the synchronous entry of hair follicles into the exogen stage. The treatment approaches for telogen effluvium includes the reduction of the exogen-staged hair follicles and the increase of hair follicles in the anagen stage. A similar treatment strategy is used in treating androgenic alopecia, to prolong the anagen stage of hair follicles.

Hair fall is one of the major side effects observed in cancer patients who undergo chemotherapy. Chemotherapy results in the disruption of the rapidly proliferating hair bulb, which later is responsible in the production of the hair shaft. Thus the hair follicles in the anagen stage enter the catagen stage. The hair loss observed is due to the removal of the hair shafts. However, this is a reversible hair loss condition, since the HFSCs remain unaffected by chemotherapy. In the condition alopecia areata, which is an autoimmune disorder, it is observed that the hair bulb is attacked by the lymphocytes. The hair falls off similarly to the conditions observed in chemotherapy induced alopecia (Cotsarelis & Millar, 2001). The common phenomenon observed in all forms of alopecia is the shortening of the anagen stage followed by the synchronous entry to the catagen and telogen stages, finally resulting in the shedding of hair. Thus, the treatment approaches for alopecia rationalize to prolong the anagen stage of the hair growth (Yoo et al., 2010).

Minoxidil and Finastride are the two drugs that have been approved by the Food and Drug Administration (FDA) for treating individuals with alopecia conditions (Lin et al., 2015). While Finastride is administered at a dose of 1 mg orally, the Minoxidil is the first tropically applied treatment for alopecia (Mysore & Shashikumar, 2016). For men suffering from alopecia, 5% of Minoxidil is currently suggested by the FDA (Kelly, Blanco, & Tosti, 2016).

Despite not clearly understanding the mode of action for Minoxidil, it is postulated to be mediated through the opening of potassium channels, which leads to an increased cutaneous blood flow, subsequently increasing the exposure to a higher concentration of Vascular Endothelial Growth Factor (VEGF). These eventually promote the hair growth of the dermal papilla (Rossi et al., 2012). It is also proposed that the increased production of prostaglandin E2 by the stimulation of prostaglandin endoperoxide

synthase-1 (Michelet, Commo, Billoni, Mahe, & Bernard, 1997) and Minoxidil sulphate, a metabolite that is required for the stimulation of hair growth is present in Minoxidil (Buhl, Waldon, Baker & Johnson, 1990) is responsible in aiding the therapeutic action of Minoxidil. Despite having the beneficial effect of facilitating hair growth, the use of Minoxidil is reported to cause adverse effects such as dryness of skin, scaling, localised irritation and dermatitis (Gelfuso, Gratieri, Delgado-Charro, & Vianna Lopez, 2013; Tarlow et al., 1994), which limits its therapeutic outcomes.

Finastride is a type 2 5α-reductase inhibitor which is responsible in decreasing the testosterone conversion to dihydroxy testosterone (DHT). DHT is responsible in the miniaturisation of the hair follicles, which is observed in male androgenetic alopecia. Finasteride also has its after effects causing sexually adverse effects due to the long-term suppression of DHT levels of the individual (Kelly et al., 2016).

Due to the limitations and adverse effects reported with respect to the currently available drugs in the market, other treatment options such as alopecia surgery have been explored. Surgical treatment approaches have its advantage on the distribution of hair, which can be predicted easily with relatively faster recovery time. However, there is an occurrence of spotty appearance on the scalp as the individual continues to shed the natural hair surrounding the transplanted area. There are also negative effects such as appearance of scar in the donor region, the limited number of successful transplanted hair and the high expense involved for the procedures.

It is noteworthy, that in case of chemotherapy induced alopecia and alopecia areata, the conditions are generally reversible since the HFSCs are not affected (Cotsarelis & Millar, 2001). Hence strategies that could modulate the therapeutic activities of the native or transplanted HFSCs can be used as an effective treatment approach for alopecia.

2.4 Stem cells and its therapeutic applications

Upon tissues damage and loss attributed due to diseases, aging or injury our body has the capability to regenerate the tissue to replace damaged tissues and accelerate the healing. A unique population of cells, referred as stem cells, largely executes this phenomenon. In the event where by the damage is beyond the repair capacity of the body, the concept of regenerative therapy using stem cells has been proposed to aid tissue regeneration.

Stem cells, are a population of unspecialized cells having two unique properties (i) self-renewal capacity above the Hayflick's limit (being able to proliferate over 50 population doublings) (Hayflick 1965; Suchanek et al., 2007) and (ii) differentiation potential to specific lineages under suitable conditions or cues making them successful candidates in regenerative medicine (Majumdar, Thiede, Haynesworth, Bruder, & Gerson, 2000b; Yu et al., 2007). They are also very attractive cellular resources owing to the characteristics of (i) ability to collect and harvest using minimal invasive procedures, (ii) ability to differentiate to multiple cell lineage pathways, and (iii) ability to transplant to an autologous or allogenic host (Bunnell, Flaat, Gagliardi, Patel, & Ripoll, 2008). Stem cells are widely used for therapeutic applications for treating myriad degenerative diseases and injuries including myocardial infarction (Orlic, Hill, & Arai, 2002), brain and spinal cord injury, stroke (Kim & Vellis, 2009), diabetes (Hussain & Theise, 2004), cartilage and bone injury (Bruder, Fink, & Caplan, 1994), Crohn's disease (Dalal, Gandy, & Domen, 2012) and graft versus host disease (Ringden & Le Blanc, 2011).

2.5 Stem cells applications for hair growth

Owing to their therapeutic values, stem cells have been explored for their potential application in the stimulation of hair growth. As discussed previously, stem cells play an important role in the generation of the hair follicle and maintaining the hair cycle. Studies conducted by Yoo et.al have shown that the BMSCs and umbilical cord stem cells have the capability to differentiate into dermal papilla-like tissues and outer root sheath cells of the hair follicle (Yoo et al., 2010) suggesting the possibility of using mesenchymal stem cells (MSCs) from various sources for reconstruction of the hair structure to eventually treat alopecia.

HFSCs in combination with dermal papilla cells have been explored in hair multiplication by injecting *in vitro* cultivated cells and bio-artificial dermal papilla tissues onto the skin. However, the utilisation of HFSCs in combination with MSCs as a treatment source for hair growth is rarely explored (Zhao et al., 2008). Despite the therapeutic advantage of stem cells, their application for regenerative therapy holds a number of concerns as listed in Table 2.1, indicating the necessity to explore different approaches in using stem cells as a treatment source to minimize its undesirable impacts in tissue regeneration. Thus, understanding the underlying mechanism of their therapeutic actions in accelerating tissue regeneration is a pre-requisite for successful therapies for degenerative diseases or disorders.

The mechanisms related to the therapeutic activity of stem cells upon transplantation is attributed to many actions including the ability of the transplanted cells to engraft at the site of disease, release trophic factors, exhibit immunomodulatory activity, differentiate into cell types required for the replacement or to activate the host somatic and progenitor cells (Jones et al., 2012). It is reported that the driving potency of the stem cells to confer these therapeutic activities are the paracrine factors that are secreted

by these cells (Cheng & Yau, 2008; Fedak et al., 2005). Of these mechanisms it is suggested that the paracrine factors secreted by stem cells play a predominant role in tissue regeneration, which is clearly demonstrated by many studies. This is particularly enlightening that acute tissue degeneration can be treated using the paracrine effect of stem cells without having the risk of tissue rejection upon stem cell transplantation.

Table 2.1: Stem cells therapy for tissue regeneration and the challenges faced

Challenges in stem cell therapy	References
Low survival rate of transplanted cells due to low oxygen and glucose and altered pH conditions	Cho et al., 2012; Ide at al., 2010; Osugui et al., 2012; Mamidi et al., 2012
Immunological responses in the event of post-administration and due to the apoptosis undergone by the transplanted cells	Di Santo et al., 2009; Yang, Di Santo & Kalka, 2010; Chuang et al., 2012
Reduced regenerative potential (proliferation and differentiation) of transplanted cells	Vandervelde, Van Luyn, Tio, & Harmsen, 2005
Lower treatment efficiency due to the presence of heterogeneous population	Ratajczak et al., 2012
Increased oncogenic potential of the genetically manipulated cultured stem cells	Baglio, Pegtel, & Baldini, 2012
Acquiring a cancer cell like behaviour when administered to normal tissues	Cho et al., 2012; Clarke & Fuller, 2006

CM and the paracrine factors secreted by stem cells have been shown to be therapeutically valuable in many instances. Studies suggest that approximately 50% or lesser number of the transplanted BMSCs formed new capillaries during the process of vascularization (Iba et al., 2002; Wang, Shum-Tim, Chedrawy, & Chiu, 2001).

Administration of CM of MSC in a mouse model for hind limb ischemia has demonstrated the effect of arteriogenic paracrine factors such as VEGF and bFGF secreted by the BMSCs to have a combined effect, increased collateral growth compared to the incorporation of the transplanted stem cells in the affected area (Kinnaird et al., 2004a). Reports suggest that the transplanted stem cells reached nearly 80% of their therapeutic potential due to the paracrine-mediated events exhibited by the cytokines and growth factors (Jones et al., 2012).

Even though positive effects observed upon the transplantation of stem cells for cardiac diseases were initially attributed towards the regeneration of cardiomyocytes by the transplanted cells, currently it is believed that paracrine factors are the key therapeutic avenue that could be responsible for cardiomyocyte regeneration and vasculogenesis (Cheng & Yau, 2008; Fedak et al., 2005; Gnecchi, Zhang, Ni, & Dzau, 2008; Tang et al., 2005). Reports also suggest that in the case of postnatal neovascularisation by endothelial progenitor cells, the secretion of paracrine factors play a dominant role (Di Santo et al., 2009). Enhancing of neovascularisation has shown that there was no clear dose response following the increasing number of administered endothelial progenitor cells beyond the plateau, suggesting that the tissue regeneration process is not fully cell dependent (Karp & Leng Teo, 2009). This suggests that the paracrine factors secreted by the stem cells played a pre-dominant role in the tissue regeneration process (Gnecchi et al., 2005; Yang, Di Santo & Kalka, 2010). An acute improvement of organ function has been observed within a period of 72 h following stem cell transplantation. This duration does not support the possibility of the transplanted stem cells to graft and differentiate to replace the damaged cells in the damaged organ. Instead, it was suggested that the action of paracrine factors secreted by these cells to mediate the observed regenerative effects (Crisostomo, Markel, Wang, &

Meldrum, 2008). Thus, using CM comprising of paracrine factors secreted by stem cells could potentially provide a therapeutic advantage for tissue regeneration.

2.6 Application of stem cell conditioned media as a therapeutic strategy

The culture media which comprises of the secretome from the cultured cell is referred to as CM (Pawitan, 2014) and is a known source carrying a pool of paracrine factors (Osugi et al., 2012). The stem cell secretome consists of various therapeutic molecules such as proteins, microRNAs, growth factors, anti-oxidants, receptor, proteasomes and exosomes (Maguire, 2013). It has been reported that the stem cells are known to secrete some common paracrine factors such as the VEGF, stem cell factor (SCF), HGF, insulin-like growth factor 1 (IGF-1), insulin-like growth factor 2 (IGF-2) and stromal derived factor (SDF) during *in vitro* cell culture (Ratajczak et al., 2012). These paracrine factors contribute to various biological and cellular activities and can serve in triggering anti-apoptotic events, increasing the metabolic activity of a damaged organ, increase the blood supply of the diseased area, strengthen the damaged area, thus preventing further damage to the diseased organ and accelerate the healing process (Crisostomo et al., 2008; Gnecchi et al., 2006; Ratajczak et al., 2012; Yang et al., 2010).

Stem cell CM has also been shown to be efficient in curing diseased conditions such as stroke (Egashira et al., 2012), wound healing (Walter, Wright, Fuller, MacNeil, & Johnson, 2010), myocardial infarction (Timmers et al., 2007; Iso et al., 2007), tissue ischemia (Kinnaird et al., 2004a), bone marrow regeneration (Osugi et al., 2012), including hair growth studies as summarized in Table 2.2.

The paracrine factors secreted by stem cells have been reported to promote neovascularisation, reduce leakage in vessels and provide trophic support to the cells

that are affected in conditions of stroke. Stem cells transplanted during the treatment has shown to have over expressed growth factors VEGF, BDNF, glial cell derived neurotrophic factor (GDNF), SDF-1, IGF-1 and HGF indicating the therapeutic efficiency of these factors for treatment (Paul & Anisimov, 2013). VEGF aids in the formation of immature vessels (Carmeliet & Collen, 1997). BNDF and GDNF cause the reduction of the size of the lesions speeding up the recovery process of patients suffering from stroke (Kocsis & Honmou, 2012). SDF-1 promotes the homing of stem cells and integrating the cells in to the ischemic area (Hill et al., 2004) enhancing the process of regeneration. The studies conducted in adult rats have shown that IGF-1 and BDNF are responsible in enhancing the cell proliferation (Dempsey, Sailor, Bowen, Tureyen, & Vemuganti, 2003).

Paracrine factors are reported to facilitate the migration, differentiation and survival of endogenous cells, reduce oxidative stress and increase angiogenesis under Huntington's disease conditions (Kerkis, Haddad, Valverde, & Glosman, 2015). Paracrine factors such as BDNF, Ciliary Neurotrophic Factor (CNTF), FGF-2 (bFGF), FGF-8, FGF-20, GDNF, IGF-1, Interleukin 6 (IL-6), Nerve Growth Factor (NGF), SDF-1α, TGF-β1, Tumor Necrosis Factor-alpha (TNF-α), Tyrosine Kinase Receptor (TRKC) and VEGF (Paul & Anisimov, 2013) have shown to be responsible for neurotrophic, neuroprotective and immunomodulatory effects in augmenting the recovery of Parkinson's disease in animal models (Kang et al., 2013, Wang et. al., 2013). All these factors and their biological roles will in return accelerate the healing and regeneration of tissue.

Table 2.2: Impact of paracrine factors in stem cell- CM on various diseased conditions

Condition	Cell source	Culture Media	Experimental model	Method of administration	Paracrine factors responsible	Impact on the disease
Hair loss	hADSC (Park et al. 2010)	DMEM/F12	7 week old C3H/NeH mice CCK-8 kit measurement if HFDPCs and HEKs	Subcutaneous infusion of CM at 3 day intervals	VEGF, PDGF, IGF-I, IGFBP	Induction of anagen hair follicle stage Increase of proliferation of hair follicle dermal papilla and human epidermal keratinocytes through mesenchymal-epithelial interactions Activation of hair follicle dermal papilla cells
Skin rejuvenation	hESC-derived EPC (Lee et al., 2014)	Commercially available CM of hESC-EPC	25 human subjects (mean 51.6 years)	Microneedling plus hESC-EPC CM, 5 treatments at 2 week intervals	EGF, FGF2, fractalkine, GM-CSF, IL-6, PDGF-AA, VEGF	Clinical improvement of pigmentation, erythema and wrinkles

Table 2.2: Impact of paracrine secretions in stem cell- CM on various disease conditions

Condition	Cell source	Culture Media	Experimental model	Method of infusion	Paracrine factors responsible	Impact on the disease
Wound healing	hBMSC (Walter et al., 2010)	DMEM/F12	Single cell and co-culture assays of murine origin L929 fibroblasts and human origin HaCaT keratinocytes	*In vitro* scratch assays	TGF-β1, IL-6, IL-8, MCP-1, RANTES, Collagen Type I, Fibronectin, SPARC, IGFBP-7	Enhancement of wound closure rate by acceleration of mainly fibroblast and keratinocyte cell migration
	Balb/C mice BMSC (Walter et al., 2010)	DMEM αMEM	Balb/C mice	Subcutaneous injection and tropical application	VEGF-α, IGF-1, PDGF-BB, Ang-1	Stimulation of macrophage and endothelial migration Increased vascularisation
Myocardial infarction	MSCs derived from hESCs (Timmers et al, 2007)	Chemically defined serum free media	Porcine	Intravenous infusion 5min before and intracoronary infusion after reperfusion	-	Reduction of myocardial nuclear oxidative stress Reduction of phosphor SMAD2 K active caspase 3 Reduction of systolic and diastolic function

Table 2.2: Impact of paracrine secretions in stem cell- CM on various disease conditions

Condition	Cell source	Culture Media	Experimental model	Method of infusion	Paracrine factors responsible	Impact on the disease
Myocardial injury	MSC (Mirotsou et al., 2007)	αMEM	Sprague Dawley rats	Injection at five different sites of the border zone of the infarcted area	Strp2	Decrease of caspase 3 activity Induction of dose-dependent increase in nuclear and total cellular B-catenin levels of cardiomyocytes Up regulation of antiapoptotic gene Birc1b
Myocardial fibrosis	Rat BMSC and Cardiac fibroblast (Ohnishi, Sumiyoshi, Kitamura, & Nagaya, 2007)	αMEM	Rat cardiac fibroblasts	-	-	Attenuation of cardio fibroblast proliferation Down regulation of Type I and III collagenase synthesis
Acute Myocarditis	MSC (Ohnishi, Yanagawa et al., 2007)	DMEM	10 week old male Lewis rats	Intravenous injection following 7days of induced myocarditis	VEGF, HGF	Attenuation increase of CD68 positive cells and MCP-I

Table 2.2: Impact of paracrine secretions in stem cell- CM on various disease conditions

4/6

Condition	Cell source	Culture Media	Experimental model	Method of infusion	Paracrine factors responsible	Impact on the disease
Friedreich's Ataxia (FA)	hADSC (Jones et al., 2012)	Animal origin free MSC medium (StemCells Technologies)	FA cells submitted to oxidative stress	-	BDNF	Increase of cell survival rate in response to oxidative stress by reduction of active caspase 3 levels
Tissue ischemia	MSC (Kinnaird et al., 2004a)	DMEM	12 week old Balb/C mice	Injection to adductor muscle 24hrs following surgical insult of unilateral hind limb ischemia	VEGF, bFGF, PIGF, MCP-1	Augmentation of collateral remodelling
Stroke	hADSC and Murine ADSC (Egashira et al., 2012)	DMEM	Ischemic stroke mice SH-SY5Y human neuroblastoma cells	ICV infusion of murine ADSC-CM one hour prior to MCOA or Immediately following MCOA	VEGF, IGF-1, HGF, TIMP-1, Progrnulin	Reduction of infract volume and brain swelling Reduction of glutamate induced excitotoxicity
	hADSC (Cho et	αMEM	8 Week old Sprague	Continuous infusion of CM to lateral and	VEGF, TGF-β1	Increase of endothelial cell proliferation

	al., 2012)		Dawley rats	ventricle following 8 days of MCOA		Reduction of neural cell apoptosis Mild astrogliosis

Table 2.2: Impact of paracrine secretions in stem cell- CM on various disease conditions

Condition	**Cell source**	**Culture Media**	**Experimental model**	**Method of infusion**	**Paracrine factors responsible**	**Impact on the disease**
Hind limb ischemia	Endothelial progenitor cells (Di Santo et al., 2009)	Growth factor free endothelial basal medium-2 (EBM-2)	Rat	Intra-muscular injection	Angiogenin, HGF, IL-8, PDGF, SDF-1 and VEGF	Increase of blood flow in the presence of neovascularization, vascular maturation and muscle function
Bone marrow regeneration	hBMSC (Osugi et al., 2012)	DMEM	10 week old male Wistar/SD rats	Implantation	IGF-1, VEGF	Increase of migration and proliferation of bone marrow cells
Distraction osteogenesis	hBMSC (Ando et al., 2014)	DMEM	8-10 week old ICR mice	Injection translucently to the zone of distraction on days 3,5 and 7.	MCP-1, MCP-3, IL-3, IL-6, RANTES, VEGF-C, IL-22	Acceleration of endogenous murine BMSC recruitment and blood vessel formation Recruitment of stem/progenitor cells and promotion of osteogenic differentiation

Table 2.2: Impact of paracrine secretions in stem cell- CM on various disease conditions

Condition	**Cell source**	**Culture Media**	**Experimental model**	**Method of infusion**	**Paracrine factors responsible**	**Impact on the disease**
Liver regeneration	ADSC (Lee, Lee, & Kim 2015)	DMEM low glucose	8 week old male Balb/c mice	Intravenous injection via tail vein within 03 hours of partial hepatectomy	-	Phosphorilation of Akt, STAT3 and Erk 1/2 Higher expression levels of mouse albumin
	hADSC (Lee, Lee, & Kim, 2015)	DMEM low glucose, stimulated with LPS	8 week old male Balb/c mice	Intravenous injection	HGF, VEGF	-

Abbreviations- Hair Follicle Dermal Papilla Cells (HFDPC), Human Epithelial Keratinocytes (HEK), Hunman Embryonic Stem Cells (hESC), Endothelial Progenitor Cells (EPC), Middle Cerebral Artery Occlusion Surgery (MCOA), Ppatelet-Derived Growth Factor (PDGF), Insulin Like Growth Factor Binding Protein (IGFBP), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Interleukin 8(IL-8), Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), Secreted Protein, Acidic and Rich in Cysteine (SPARC), Insulin Like Growth Factor Binding Protein 7 (IGFBP-7), Leukaemia Inhibitory Factor (LIF), Striatin Interacting Protein 2 (Strp 2), Placental Growth Factor (PIGF), Monocyte Chemoattractant Protein 1 (MCP-1), TIMP metallopeptidase 1 (TIMP-1), Monocyte Chemoattractant Protein 3(MCP-3)

2.7 Stem cell conditioned media for hair growth

Paracrine factors play an important role in the stimulation of molecular and cellular processes that govern hair growth. The terminal differentiation of the follicular matrix cells are highly dependent on the regulatory molecules that are expressed in the epithelial cells, which are influenced by the mesenchymal cell population in the hair follicle (Tsuboi, 1997). It was reported that various paracrine factors are present in the hair follicle, moreover recent studies have demonstrated that paracrine factors EGF, TGF-β, IGF-1, HGF, VEGF (Wang & Zhang, 2012) and FGF-10 are responsible in the regulation of human hair growth. Additionally, TGF-α, aFGF, bFGF, FGF-5 and PTHrP (Table 2.3) are known to regulate the hair growth in animal models such as sheep and mice (Danilenko, Ring, & Pierce, 1996; Tsuboi, 1997).

Growth factors such as FGF-1, bFGF, FGF-7, FGF-10, IGF-1, IGF-2 and EGF are responsible in promoting the cell cycle and cell proliferation. Thus these growth factors have potential in promoting the hair cycle and stimulating the hair growth under *in vitro* and *in vivo* conditions. FGF-1 and bFGF has been shown to influence in the suppression of the hair growth cycle (du Cros, 1993a, 1993b). Studies also suggest that bFGF promoted the proliferation of the dermal papilla and resulting in the increase size of the hair follicles in mice (Lin et al., 2015; Zine & de Ribaupierre, 1998). EGF and TGF-α are involved in the cell proliferation and regeneration of the avian utricles (Zine & de Ribaupierre 1998). bFGF and IGF-1 have also shown to be involved in the regeneration of hair cells in rat utricle (Zheng, Helbig, & Gao, 1997). Keratinocyte growth factor (KGF) is a growth factor that plays a role in protecting the hair follicles from UV irradiation and chemotherapy induced cell death (Braun et al., 2006).

Table 2.3: Paracrine factors and their roles in hair growth

Paracrine Factor	Role
Positive hair regulatory factors	
Insulin-like growth factor (IGF-1)	Prevents entry of catagen phase and maintenance of anagen phase
Hepatocyte growth factor (HGF)	Stimulates follicular proliferation
Vascular endothelial growth factor (VEGF)	Stimulates dermal papilla (uncertain) Modulates angiogenesis
Keratinocyte growth factor (KGF)	Stimulates follicular proliferation and induces normal differentiation and keratinization of hair
Stromal cell-derived growth factor 1 (SDF-1)	Migration of CXCR4+ stem cells
Platelet derived growth factor bb (PDGF-bb)	Induces and maintains anagen phase
Negative hair regulatory factors	
Transforming growth factor-alpha (TGF-α)	Necessary for normal follicular position, retards hair growth
acidic- Fibroblast growth factor (aFGF)	Delays follicular development. Retards hair growth and cycling
basic Fibroblast growth factor (bFGF)	Delays follicular development. Retards hair growth and cycling
Fibroblast Growth Factor 5 (FGF-5)	Induces transition of anagen hair cycle to catagen phase
Epidermal growth factor (EGF)	Induces transition of anagen hair cycle to catagen phase
Brain derived neurotrophic growth factor (BDNF)	Induces transition of anagen hair cycle to catagen phase
Transforming growth factor-beta (TGF-β)	Inhibits follicular development and follicular proliferation
Interleukine 1 (IL-1)	Inhibits follicular proliferation

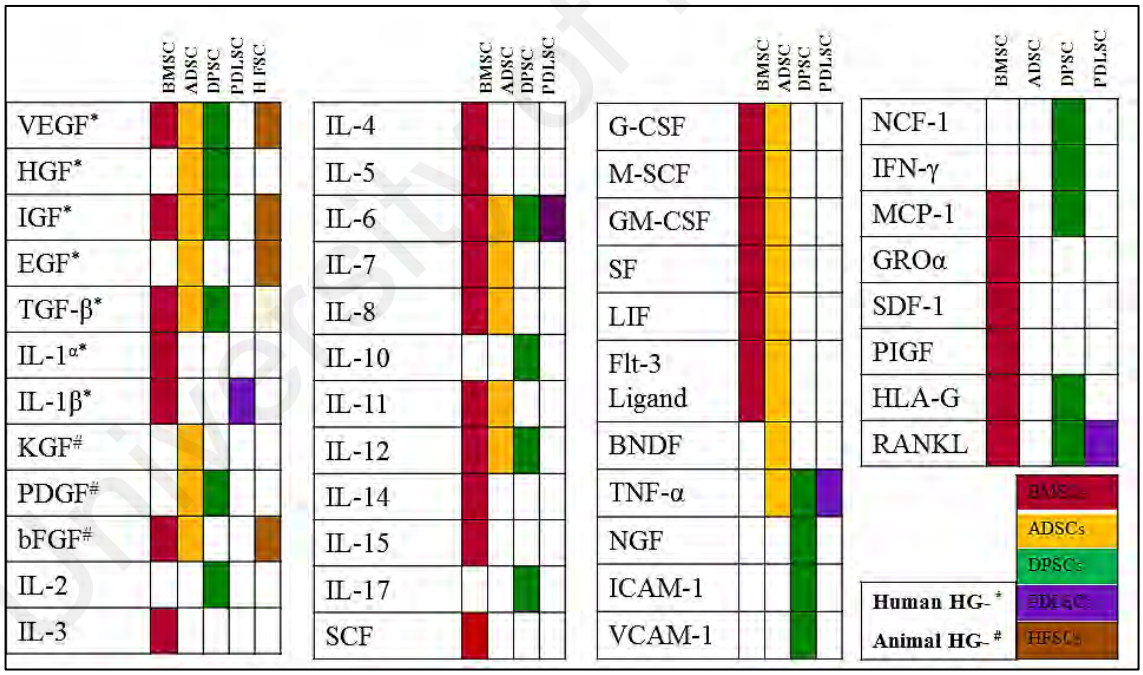
Currently, studies have been conducted using human ADSC-CM and keratinocyte stem cell-CM as a treatment source for alopecia in mice models (Park et al., 2010; Won et al., 2015). ADSC derived paracrine factors such as VEGF, PDGF, IGF-1, HGF and KGF have been reported as important factors that are involved in hair growth regulation (Jindo, Tsuboi, Takamori, & Ogawa, 1998; Limat et al., 1993; Lindner et al., 2000; Park et al., 2008; Su, Hickford, Bickerstaffe, & Palmer, 1999; Weger & Schlake, 2005). These factors are thought to be responsible in stimulating hair growth by modulating the cell cycle to stimulate the proliferation of dermal papilla cells and epithelial cells and also by activating the anagen stage of hair growth (Won et al., 2010). VEGF controls the hair follicles cycle through the modulation of angiogenesis then aids in the maintenance of the follicular size (Yano, Brown, & Detmar 2001). IGF-1 has been reported to be required for the maintenance of the anagen stage of hair growth (Ahn, Pi, Hwang, & Lee, 2012). TGF-β mediates both positive and negative effects on hair growth. It acts as an anagen stage inducer and also known to have an inhibitory effect on the hair growth (Bernard, 2016). TGF-β1, FGF5, EGF and BDNF are responsible in the transition of anagen stage hair follicles to the catagen stage (Alonso & Fuchs, 2006). Murine models have demonstrated the importance of PDGF in the maintenance of the anagen stage in the hair cycle (Tomita, Akiyama, & Shimizu, 2006).

In comparison to using stem cells as a treatment option, CM in regenerative therapy would render opportunities to overcome challenges such as immune-compatibility and requirement of matching donor-recipients which are required to be fulfilled when using cell based treatment approaches. Also, during the treatment procedure, CM application does not require sterile facilities, unlike the stem cell transplantation conditions making the application process of the therapy more convenient (Pawitan, 2014). Utilizing stem cell CM can also be considered as a cost-efficient treatment approach since the waiting time for cell expansion is reduced (Chuang et al., 2012).

Paracrine profiles listed in Table 2.4 indicate the secretion of various hair regulatory factors by different MSCs sources (Demircan et al., 2011; Gandia et al., 2008; Kilroy et al., 2007; Kim et al., 2005; Kinnaird et al., 2004b; Majumdar, Thiede, Haynesworth, Bruder, & Gerson, 2000a, Shu et al., 2008).

The presence of different factors and their concentrations is stem cell source dependent. Thus studying the paracrine secretion profiles of different stem cell sources would provide the possibility of choosing the most appropriate stem cell source for CM preparation to stimulate hair growth.

Table 2.4: The paracrine secretion profile of stem cells. The paracrine factors secreted by stem cell from bone marrow (BMSC), adipose (ADSC), dental pulp (DPSC), periodontal ligament (PDLSC) and hair follicles (HFSC) that are involved in human and animal hair growth (HG).



DPSCs secrete mainly the positive hair regulatory factors in comparison to other stem cell sources such as ADSCs. Thus, we propose to evaluate the potential of SHED-CM, for its ability to stimulate hair growth by conducting the characterisation of their paracrine secretion profiles followed by *in vitro* and *in vivo* experiments for hair growth.

2.8 Challenges and research gaps in conditioned media based studies for hair growth and treatment of hair loss

Based on the literature, the key research gap identified in using CM as an efficient therapeutic tool is the variation in paracrine factors detected attributed to the use of different culture media, stem cells source and the protocols involved in the collection of CM.

Culture media plays an important role in the *in vitro* expansion of stem cells to ensure the stem cells propagation, viability, stemness and their paracrine profile during culture conditions (Kanafi et al., 2013). DMEM-KO is reported to be one of the most suitable basal media for culturing MSCs including SHED (Dimarakis & Levicar, 2006; Gottipamula, Sharma, Krishnamurthy, Majumdar, & Seetharam 2012; Govindasamy et al., 2010; Nekanti et al., 2010; Pal, Hanwate, Jan, & Totey, 2009) when supplemented with serum. Serum provides growth factors, hormones and other elements that are required for the *in vitro* cell cultures (Selvaggi et al., 1997). However, using serum pose a number of risks such as spontaneous differentiation (Gou et al., 2010; Tseng et al., 2007) and malignant transformation (Karbanova et al., 2011). The batch-to-batch variation of serum also affects the cellular properties of the cultured cells, there by affecting the paracrine secretion profiles, which is one of the major concerns when preparing CM. Hence, the possibilities of culturing these cells in xeno free media has been explored for the preparation of CM (Timmers et al., 2007).

Based on the literature, StemPro® (Gibco Invitrogen, Carlsbad, CA, USA) and STK2 media (TwoCELLS, Japan) has been shown to support proliferation of MSCs (Agata et al., 2009; Chase, Lakshmipathy, Solchaga, Rao, & Vemuri., 2010; Gottipamula et al., 2013; Ishikawa et al., 2009; Ng et al., 2008). Both StemPro® and STK2 xeno free media

have been previously used for culturing dental derived stem cells (Gottipamula et al., 2013, Khanna-Jain et al., 2013; Takeda-Kawaguchi et al., 2014). However, evidence that these media support HFSCs growth is still lacking.

Whilst the composition of StemPro® is not disclosed, STK2 media comprised of a combination of mitogenic and morphogenic growth factors namely FGF, PDGF, HGF, TGF-β and EGF (Koichiro, et al., 2016) and approximately 70 other elements including fat compounds and rare elements (Kato & Shao, 2009), however detailed composition and the concentrations of these supplements were not revealed. It has been reported that STK2 media is also used for clinical trials in Japan (Uematsu et al., 2013).

Sawada et al. (2010) studied the differences in gene expression profile of MSCs when cultured in STK2. They concluded that STK2, a growth factor supplemented media caused cells to exhibit different gene expression patterns cell cycle, proliferation, and cell growth (Sawada, Yamada, Tsuchiya & Matsuoka, 2010), which in turn could influence the secretion of paracrine factors. This necessitates the exploration of different media combinations, if CM is to be used as a therapeutic reagent for tissue regeneration.

The choice of the stem cell source plays an important role during stem cell therapeutic approaches. However, the choice of stem cell source to prepare CM for potential therapeutic purposes has been largely unspecific (Table 2.2). It was reported that cells with high commonalities such as cellular characteristics, differentiation potential and genetic resources may produce a similar paracrine secretion profiles even though they may not be identical. This has been shown in the studies conducted by Park et al. using umbilical cord blood and bone marrow derived stem cells (Park et al., 2009). Thus the selection of an optimal stem cell source is of prime importance. Currently, the preparation of CM for hair growth involves the utilization of ADSCs. Even though adipose tissues such as craniofacial fat is considered to be of ectodermal origin (Le

Lievre & Le Douarin, 1975) the widely used white adipose tissue for the extraction of stem cells is of mesodermal origin. The DPSCs on the other hand are of ectodermal origin which is similar to the origin of the HFSCs. Therefore, using cells from a similar origin to generate CM will be explored during the current study.

The secretome of cells may also vary depending on the age of the primary source (Maguire, 2013) and the type of culture medium used for cell culture (Gheisari et al., 2011; Van Koppen et al., 2012). There has been no published standardized protocol with respect to the donor age of the primary source during the preparation of CM. This may have an effect for the consistency of the therapeutic effects that are exhibited by CM generated in various studies. During the currently proposed study, we have limited the sample collection to SHED which gives a clear demarcation for the primary source below the age of 12.

The currently available protocols for the preparation of ADSC-CM for hair growth utilizes serum supplemented media for the expansion of the cells (Park et al., 2008; Park et al., 2010; Won et al., 2010). The use of serum in media could alter the stem cell behavior, which in turn would alter their paracrine secretion profiles. The behavioural alteration could be minimized when stem cells are expanded and the collection of CM is conducted under serum free conditions. Thus, there is a possibility that this behavioural alteration could be minimized when stem cells are expanded and CM is collected under serum free conditions.

2.9 Animal models for hair growth

Even though the most relevant model to study human hair loss disorders is humans, alternative models have been proposed due to the ethical concern. The most commonly used whole animal systems for hair growth study include; mice (Chase, 1954; Sundberg et al., 2013), rats (Orasan, Bolfa, Coneac, Muresan, & Mihu, 2016), sheep (Hynd, Schlink, Phillips, & Scobie, 1986) and monkeys (Uno, 1991). Even though following human models, the macaque is regarded as the second best model for hair growth studies, due to the rarity, high expenditure, difficulty in handling and housing, these animals are rarely used. Thus, the laboratory mice are chosen as the animal models of choice for hair growth in many studies (Stenn & Paus, 2001; Sundberg, Beamer, Uno, Van Neste, & King, 1999).

2.9.1 Hair growth cycle in mouse model

The hair growth cycle in mouse is not completely similar to that of humans. In comparison to the human scalp hair, they have an added advantage of having the first two hair cycle stages synchronized and possessing a shorter hair cycle duration, which lasts for about a period of one month (Hashimoto et al., 2000; Porter, 2003). For this, they serve as a suitable model for the study of hair growth with an additional advantage of having the hair growth cycle stages, anagen, catagen and telogen. The key parameters to be considered in order to classify the hair growth stages in mice have been described earlier (Chase, Rauch, & Smith, 1951; Chase, 1954). A more detailed classification of the hair growth cycle in mouse based on the parameters set by Chase has been refined by Muller-Rover et al. (Muller-Rover et al., 2001).

Even though, factors such as sex, genetic background, environment and nutrition influence the murine hair cycle, the time frame for the first post-natal hair cycle is known to follow a specific time scale as illustrated in Figure 2.3a (Muller-Rover et al., 2001). The most commonly used mouse strains for hair growth study include C57BL/6 and C3H strains (Table 2.5). At 7 weeks, the hair growth cycle in these mice strain is synchronized to the telogen stage, indicated by the pink skin colouration following shaving. The activation of pigment production by the follicular melanocytes during anagen phase, causes changes in the skin colour. This provides an easy visual identification of hair growth stimulation (Stenn & Paus, 2001). The development of the anagen stage is demonstrated by the skin pigmentation as illustrated in Figure 2.3b (Muller- Rover et al., 2001; Park et al., 2010; Won et al., 2010). A major drawback for this animal model is that following the second wave of hair growth, the hair growth stages will be desynchronized and scattered dark patches can be seen upon shaving (Ranndall, Sundberg, & Philpott, 2003). Therefore, careful selection of mice must be made to ensure that their hair growth cycle is within the synchronized stage.

Table 2.5: Varied hair growth stimulants tested in commonly used mouse models

Hair growth stimulant	Mouse model	Observation of hair growth
ADSC-CM (Park et al. 2010)	C3H/HeN	8 weeks (appearance of dark patches) 12 weeks (almost complete coverage of hair growth)
Single growth factor-FGF-2, FGF-5, FGF-7 (Lin et al. 2015)	C57/BL6	2 weeks (appearance of dark patches)
Ketoconazole (Jiang et al. 2005)	C3H/HeN	3 weeks (appearance of dark patches)

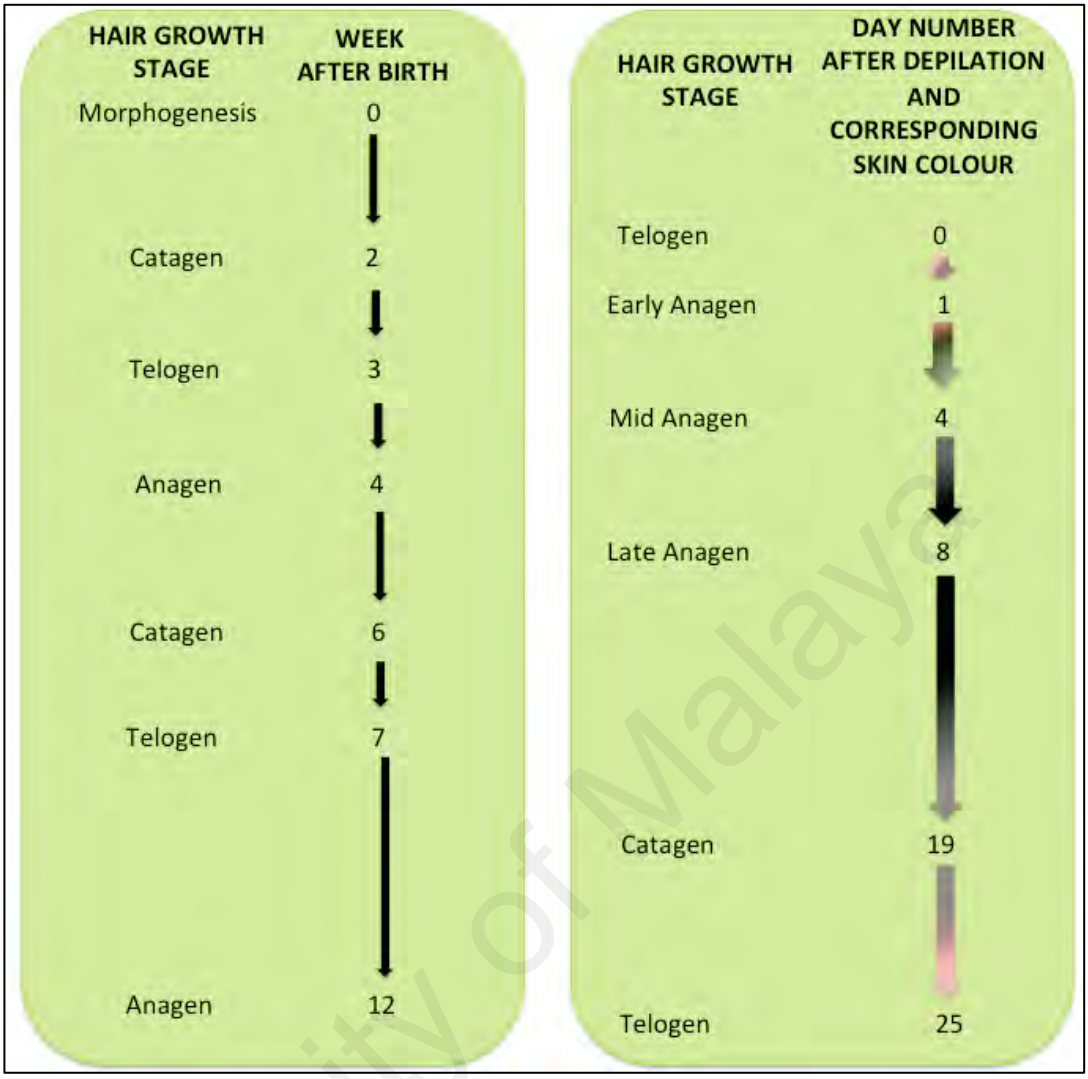


Figure 2.3: Time frame for hair growth in mice. (a) The hair cycle time frame for the first 14 weeks of birth for mice models (b) The time frame reported for mice hair growth stages, following depilation with the respective change of skin pigmentation in correspondence to hair growth stage.

CHAPTER 3: MEDIA FOR CULTURE AND PREPARATION OF CONDITIONED MEDIA- PILOT STUDY

3.1 Introduction

The self-renewal and regenerative capacity of stem cells make them a suitable therapeutic tool for tissue regeneration. One of the key mechanisms that foster the therapeutic benefits of stem cells is mainly attributed to the paracrine factors they secrete. In order to obtain the paracrine factors of interest in a sufficient quantity, it is necessary to expand the cells in optimal culture conditions while maintaining their stem cell characteristics and viability. Thus, culture media plays a vital role in providing such microniche in maintaining or enhancing their therapeutic values.

Most commonly used supplement in media to culture stem cells is FBS. FBS facilitates the attachment of cells to the culture plasticware surfaces and provides signaling cues and growth factors required for the survival, growth and proliferation of cells. However, the addition of FBS to cell culture media provokes ethical concerns over the procedures employed during harvesting, carries the risk of transmitting diseases to individuals, creates a high variability in terms of the production batch which results in the production of heterogeneous stem cell populations (Gottipamula, Muttigi, Kolkundkar, & Seetharam, 2013). These factors necessitate more attention to be drawn towards the development of serum free culture media in order to support stem cell culture and their potential applications in clinical settings. Hence, this pilot study was aimed to determine the suitable culture conditions for SHED and HFSCs.

Based on the literature we initially cultured SHED isolated from the primary source and the HFSCs obtained commercially as the control stem cell source in a wide range of media combinations to understand the culture media combinations that would best support the proliferation and viability of cells before further experiments were

conducted ; (i) DMEM-KO+10% FBS (ii) DMEM-KO+10% FBS+bFGF (iii) DMEM-KO+10% FBS+20% STK2 (iv) STK2+2% FBS (v) STK2 (vi) StemPro®

3.2 Aim and objectives

3.2.1 Aim

To screen for suitable media combinations that support culturing of stem cells from human extracted deciduous teeth (SHED) and hair follicle stem cells (HFSCs) at an optimal level.

3.2.2 Objectives

1. To determine the capability of expanding, and maintaining the morphology of SHED and HFSCs cultured under different media combinations.
2. To compare the effect of serum supplemented media, xeno free media and their combinations on the population doubling times and viability of SHED and HFSCs.
3. To compare the effect of serum supplementation in culture media on the population doubling times and viability of SHED and HFSCs.
4. To determine the effect of different CM collection methods on the hair growth related paracrine profiles.

3.3 Material and methods

3.3.1 Materials

All materials used during the pilot study are listed in Appendix E with the respective manufacturer details.

3.3.2 Methods

3.3.2.1 Isolation of SHED from extracted deciduous teeth

A molar deciduous tooth was collected from a 6 year old child who underwent surgical extraction at the Department of Pediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya. The collection protocol was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (DFCO1107/0066[L]) with informed consent from the respective parents. The deciduous teeth were transported in Dulbecco's phosphate buffered saline (DPBS) transport media containing 0.2% (*v/v*) Penicillin-Streptomycin (Pen-Strep) (10,000 units/ml Penicillin and 10,000 µg/ml Streptomycin), for the isolation of the pulp. Pulp extirpation was carried out by cutting the tooth in cross sectional slices using a high speed diamond disc on a turbine hand piece under copious irrigation. The pulp was then carefully transferred into a 1.5 ml eppendorf tube containing 0.5 ml complete media (Dulbeccos's minimal essential media- knock out + 10% Foetal Bovine Serum (DMEM-KO+10% FBS)) and was processed immediately for isolation of SHED.

The pulp tissues were subjected to three washes in DPBS and pen-strep (1:1 ratio) and were transferred to 500 µl of freshly prepared 1% (*v/v*) collagenase type I and minced to minute pieces using sterilized scissors. The minced pulp was incubated at 37°C for 30 min. Three hundred microliters of the incubated solution was pipetted out and substituted with an equal volume of 1% collagenase type I. The samples were further incubated for 15 min at 37 °C. The digested sample was transferred to 5 ml

DMEM-KO basal media for neutralization and was centrifuged at 310 g for 7 min. The supernatant was discarded without disturbing the pellet. The pellet was re-suspended in 1 ml of DMEM-KO+10% FBS complete media and the samples were seeded in T-25 flasks and incubated at 37 °C, 5% CO_2 until distinct colonies were obtained. These colonies were further sub-cultured as stated in the following section 3.3.2.3.

3.3.2.2 Revival of HFSCs

Passage 1 HFSCs ($0.5x10^6$ cells/vial) were purchased (ICELLTIS, Labege, France) and was immediately stored in liquid nitrogen for further use upon receiving. To revive the cells, the vials were thawed in a 37 °C water bath until a single frozen liquid droplet was observed. One milliliter of complete media (DMEM-KO+10% FBS) was added to the cryovial drop-wise and mixed gently by pipetting. The contents of the cryovial were transferred to a centrifuge tube containing 5 ml complete media and centrifuged at 310 g for 6 min. The supernatant was discarded and the pellet was dissolved in 1 ml complete media. Cell counting was carried out using Countess® Cell Counter to determine the cell number and viability of cells. The cells were seeded in 6-well plates at a seeding density of 5000 cells/cm^2 under different media combinations as follows, and media change was carried out every three days;

(a) DMEM-KO+10% FBS+1% 4mM Glutamax+0.5% pen-strep,

(b) DMEM-KO+10% FBS+10 ng bFGF+ 1% 4 mM Glutamax+ 0.5% pen-strep,

(c) DMEM-KO+10% FBS+20% STK2 +1% 4 mM Glutamax+0.5% pen-strep,

(d) STK2 + 2%FBS + 1% 4 mM Glutamax+0.5% pen-strep,

(e) STK2 + 1% 4 mM Glutamax+ 0.5% pen-strep,

(f) StemPro®+1% 4 mM Glutamax+ 0.5% pen-strep.

3.3.2.3 Sub-culturing and expansion of SHED and HFSCs

Sub-culturing of passage 1 HFSCs and SHED to passages 2-5 was carried out upon 80% confluency (Figure 3.1). The cells were subjected to two washes with 4 ml DPBS (without calcium/magnesium). One milliliter of Versene was added to the HFSCs sample and incubated for 2 min after which the solution was pipetted out and transferred into a 15 ml centrifuge tube containing 3 ml of respective complete media and kept at room temperature (24 ± 1°C). Then, 0.5 ml TrypLE Express was added to detach the cells. The flasks were incubated for 2 min following the addition of an equal volume of complete media. The flasks were washed by using complete media until the surface of the culture flask was cell free when viewed under phase contrast microscope at 4× magnification. The solution containing HFSCs was transferred into the earlier kept 15 ml centrifuge tube containing Versene solution. These suspended cell solutions were subjected to centrifugation at 310 g for 6 min. The supernatant was discarded without disturbing the pellet and the cell pellet was suspended in 1 ml complete media. The number of cells were counted using Countess® Cell Counting Chamber slides and the cells were seeded under the different media combinations at a seeding density of 5000 cells/cm^2 for further passaging. The culture media was changed at every three days.

Sub-culturing of SHED was also carried out as described above. However, since the HFSCs were more tightly attached to the culture flask, an additional chelating step was carried out by using Versene, which acts as an EDTA chelating agent. The addition of Versene was not required for SHED as they could be easily detached from the culture flask only by the addition of TrypLE Express, which comprises of combination of cell dissociating enzymes.

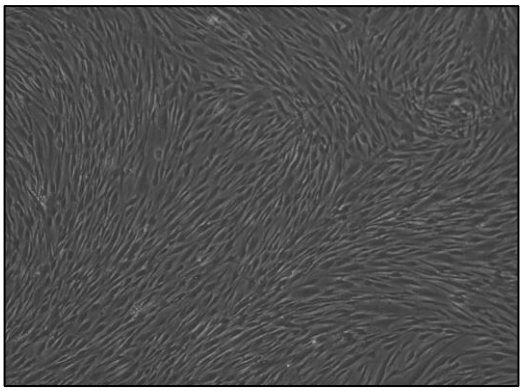


Figure 3.1 - Representative image for 80% confluency. The 80% confluency of cells at 4× magnification during which the supplementation of serum free basal media was carried out for the collection of CM.

3.3.2.4 Determination of growth kinetics for SHED and HFSCs in different media combinations

Following trypsinisation as stated in 3.3.2.3, the SHED and HFSCs viability assessment for each passage was carried out using Countess® Automated cell counter. Ten microliters of the re-suspended cells was added to 10 µl Tryphan blue dye and mixed well by pipetting up and down. Ten microliters of this mixture was loaded to the Countess® cell chamber slide and the slide was inserted to the cell counter. The cell images were focused and the count cell command was entered, in order to obtain the reading for the total, live and dead cell count, and the viable cell percentage.

The population doubling time (PDT) for each sample was measured using the following formula (Korzynska & Zychowicz, 2008).

$$PDT = \frac{\Delta t}{\log_2 \left\{ \left(\frac{\Delta N}{N0} \right) + 1 \right\}}$$

PDT = population doubling time

Δt =time taken for 80% confluency

ΔN =difference in cell number

N_0 = total cell number seeded

3.3.2.5 Selection of suitable culture media for the preparation of conditioned media (CM) collection

Low PDT and high cell viability obtained from the growth kinetic study (Section 3.3.2.4) was the determining factors for the selection of suitable culture media to expand the cells. Upon selection of the culture media, and when expanded cells reached 80% confluency, cells were washed with 5ml of DBPS followed by supplementation with serum free DMEM-KO and STK2 and incubated for 24 hrs. This process constitutes the serum free conditions for the CM preparation.

In order to determine if STK2, a growth factor rich media would have an effect on the paracrine secretions of the cells, conditioned media was also collected using DMEM-KO. SHED (n=1) and HFSCs (n=1) were supplemented with STK2 media, while the other set of samples were supplemented with DMEM-KO and incubated for 24 h. The CM was collected in pre-chilled tubes followed by centrifugation at 310 g for 6 min at 4 °C. The media was filtered using 2 μm filters and collected in pre-chilled 1.5 ml eppendorf tubes and stored at -80 °C until further use.

When conducting the Luminex assay, the CM stored at -80 °C was thawed and 500 μl of each sample was added to the U bottomed 96-well plate. The preparation of antigen standards was carried out by centrifugation of the antigen standard vial at 200 g for 10 sec followed by the addition of 250 μl fresh culture media. The vial was vortexed gently for 30 sec and incubated in ice for 5 min. A four-fold dilution of the reconstituted standards was carried out.

The Luminex based multiplex assay to profile the paracrine factors SDF-1, HGF, VEGF-A, PDGF-BB, IL-1β, TGF-β, bFGF, TNF-α and BDNF was carried out by vortexing the antibody magnetic beads for 30 sec. Fifty microliters of antibody magnetic beads were added to each well. The procata 96-well flat bottomed plate was

inserted to the hand held magnetic plate washer and was left for 2 min to allow the antibody magnetic beads to accumulate at the bottom of each well. The liquid in the wells was removed by inverting the hand held magnetic plate washer and 96-well plate assembly. One hundred and fifty microliters of 1× washing buffer was added to each well and left for 30 sec to allow the antibody magnetic beads to settle at the bottom of each well. Fifty microliters of standard or 50 µl sample was added to the respective wells while complete culture media was added to the wells marked as blank. The 96-well plate was sealed and removed from the hand-held magnetic plate washer and wrapped with aluminum foil. The plates were shaken well at 700 rpm for 1 h at room temperature (24 ± 1°C). The 96-well plate was inserted to the hand held magnetic plate washer and left for 2 min. The plates were carefully removed and the supernatant in the well was removed quickly by inverting the hand held magnetic plate washer. One hundred and fifty microliters of 1× wash buffer was added to each well and left for 30 min for the antibody magnetic beads to accumulate on the bottom of the wells. The supernatants were removed quickly by inverting the 96-well plates and the procedure of washing with 150 µl 1× washing buffer was repeated 3 times. Twenty-five microliters of detection antibodies were added to each well and the plate was sealed well. The 96-well plate was removed from the hand held magnetic plate washer and wrapped with an aluminum foil and was shaken at 700 rpm for 30 min at room temperature (24 ± 1°C). The washing of the wells using 150 µl 1× washing buffer was repeated for four times. The SAPE solution vial was vortexed for 20 sec. Fifty microliters of SAPE was added to each well and the plates were sealed. The 96-well plate was removed from the hand held magnetic plate washer and wrapped with an aluminum foil. The 96-well plate was shaken at 700 rpm for 30 min at room temperature (24 ± 1°C) followed by washing the wells four times using 150 µl 1× buffer. One hundred and twenty microliters of reading buffer was added to each well and the plate was sealed. The 96-well plate was removed

from the hand held magnetic plate washer and wrapped with an aluminum foil and shaken for 5 min at room temperature (24 ± 1°C) at a speed of 7000 rpm. The plate seal was removed prior to reading the 96-well plate by the Luminex instrument.

3.4 Results

3.4.1 Morphology of SHED and HFSCs

SHED and HFSCs culturing was supported by all serum supplemented media combinations, at a varying degree from passages 2 to 5. The cells maintained spindle-shaped morphology, typical for the adult stem cell characteristics in these media (Figure 3.2). SHED demonstrated a higher cell density in comparison to the HFSCs in all media combinations. Of the serum-based media, DMEM-KO+10% FBS+20% STK2 media combination showed the highest visually observed cell density at a selected time point for SHED in all passages. However, the morphological visual assessment indicated the STK2+2% FBS supports HFSCs at the most optimal level in comparison to the other media.

SHED and HFSCs were also cultured in the two commercially available serum-free media; STK2 and StemPro®. The STK2 supported the culturing of both SHED and HFSCs, and the cells maintained the spindled shaped morphology throughout the passages, while StemPro® media supported the culturing of SHED, but not HFSCs (Figure 3.3). It was also observed that STK2 under serum free conditions conferred better support for culturing SHED as the passaging increases in comparison to StemPro®.

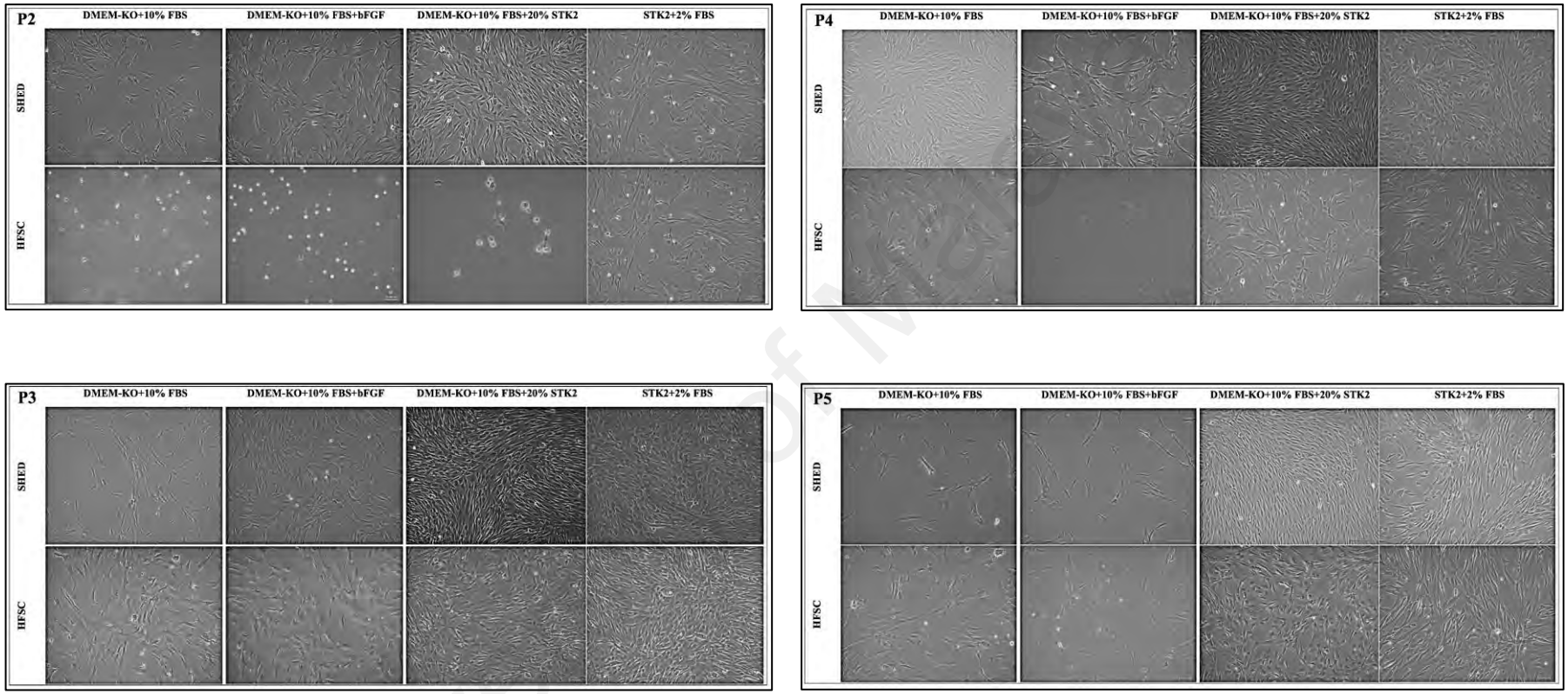


Figure 3.2: Morphology of SHED and HFSCs cultured under serum supplemented media combinations. The morphology of SHED and HFSCs when cultured in different combinations of serum supplement in DMEM-KO and STK2: DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, DMEM-KO+10% FBS+20% STK2, STK2+2% FBS at Day 3 of each passage, observed under 10× magnification**.** *P-Passage

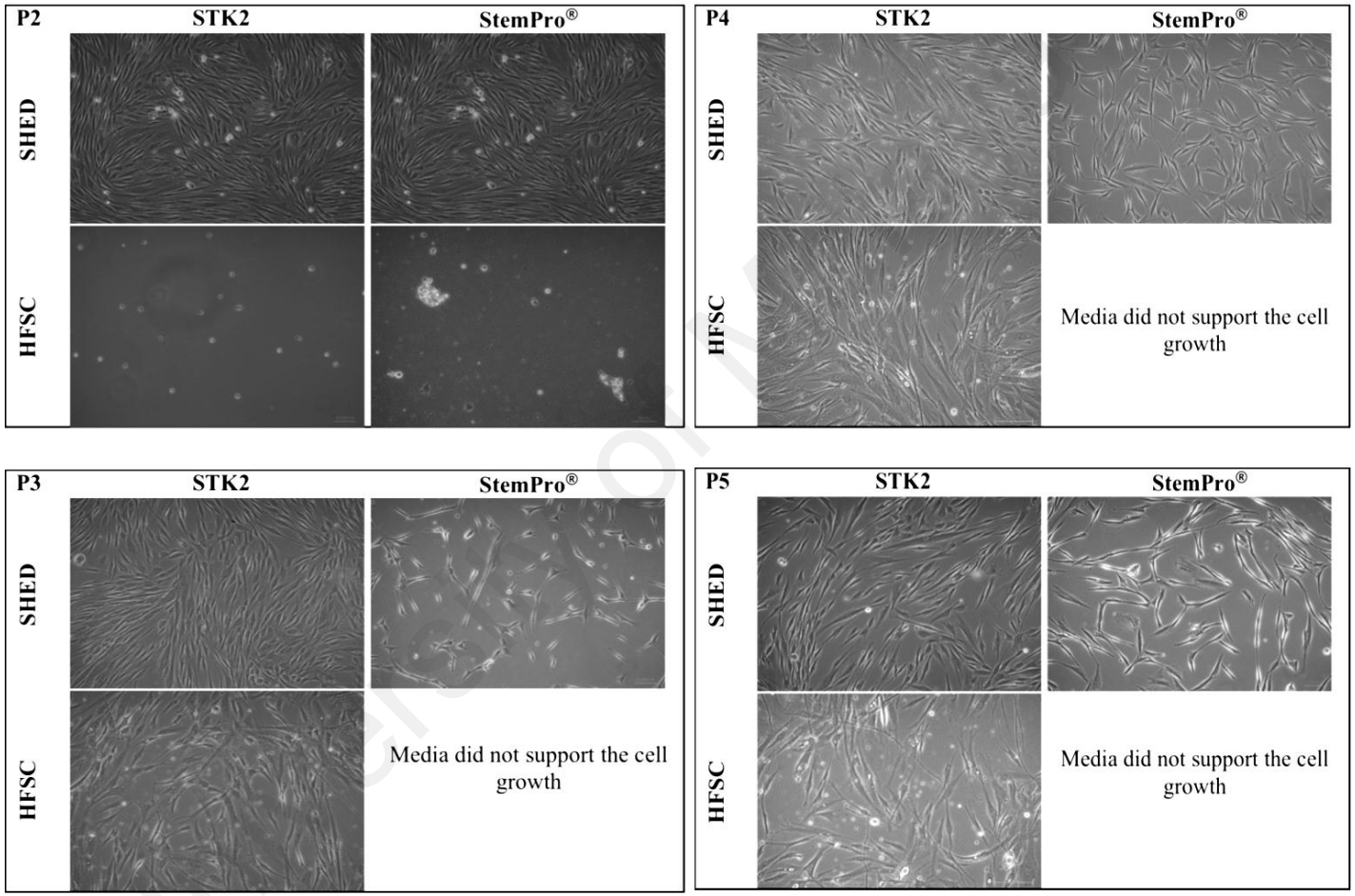


Figure 3.3: Morphology of SHED and HFSCs cultured under serum free media combinations. The morphology of SHED and HFSCs cultured in serum free media combinations STK2 and StemPro® at Day 3 of each passage, observed under 10× magnification. *P-Passage

3.4.2 Population doubling time and viability of SHED and HFSCs

PDT was calculated based on the yield of cells obtained at different passages for SHED and HFSCs. SHED exhibited a lower PDT compared to HFSCs throughout the different passages in different media combinations, indicating higher proliferative capacity (Table 3.1). The lowest PDTs were recorded for the cells cultured in the media combinations (i) DMEM-KO+10% FBS+20% STK2 and (ii) STK2+2% FBS for both SHED and HFSCs, which was prominently evident in passages 3 and 4. The PDT was lowest in the STK2 based media, while it was highest in the DMEM-KO, as recorded in all passages. It is also apparent that the addition of 20% STK2 and 2% FBS to DMEM-KO and STK2 respectively showed a better PDT, suggesting that STK2 and FBS as critical components that may influence the PDT and proliferative characteristics of stem cells.

The viability of cells cultured in different media combinations from passage 2-5 was also evaluated during this pilot study (Figure 3.4). In general, SHED showed a higher percentage of viability in comparison to HFSCs. The viability of SHED ranges between 90-99% in all assessed passages, in both DMEM-KO and STK2 based media, except for cells in DMEM-KO+10% FBS+bFGF at passage 4. However, SHED has shown a lower viability in StemPro® at passages 2 and 5, in comparison to the other media combinations.

Lower cell viability was observed when HFSCs was cultured in xeno free STK2 media at passages 2 and 3, however the viability increased at subsequent two passages. In addition, the viability of HFSCs cultured in DMEM-KO based media remained at a constant level from passage 3 to 5, ranging between 90-94% in the absence of STK2.

Supplementation of bFGF to DMEM-KO based media showed an increase in the percentage of viable cells.

Table 3.1 The population doubling times of SHED and HFSCs from passage 2 to 5 when cultured in different media combinations

Media Compositions	Population Doubling Time (hours) ± SE							
	P2		P3		P4		P5	
	SHED	HFSC	SHED	HFSC	SHED	HFSC	SHED	HFSC
DMEM-KO+10% FBS	34±2.2	135±37.8	38±2.8	47±3.8	27±3.0	63±2.1	56±4.0	71±2.4
DMEM-KO+10% FBS+ bFGF	31±2.4	157±15.1	24±0.5	33±4.8	35±1.6	62±1.0	68±3.4	71±3.4
DMEM-KO+10%FBS+20%STK2	22±0.7	68±1.3	15±0.4	16±0.2	18±0.3	17±0.1	19±0.2	23±0.6
STK2+2% FBS	23±0.6	68±5.2	13±0.7	20±2.7	13±0.3	20±0.8	22±2.4	22±1.3
STK2	22±0.5	164±7.7	21±2.1	25±1.3	23±0.2	31±2.6	25±0.7	58±2.8
StemPro®	25±0.2	DC	43±1.6	NC	38±2.8	NC	42±1.0	NC

P = passage number, DC= detached cells, NC= not cultured

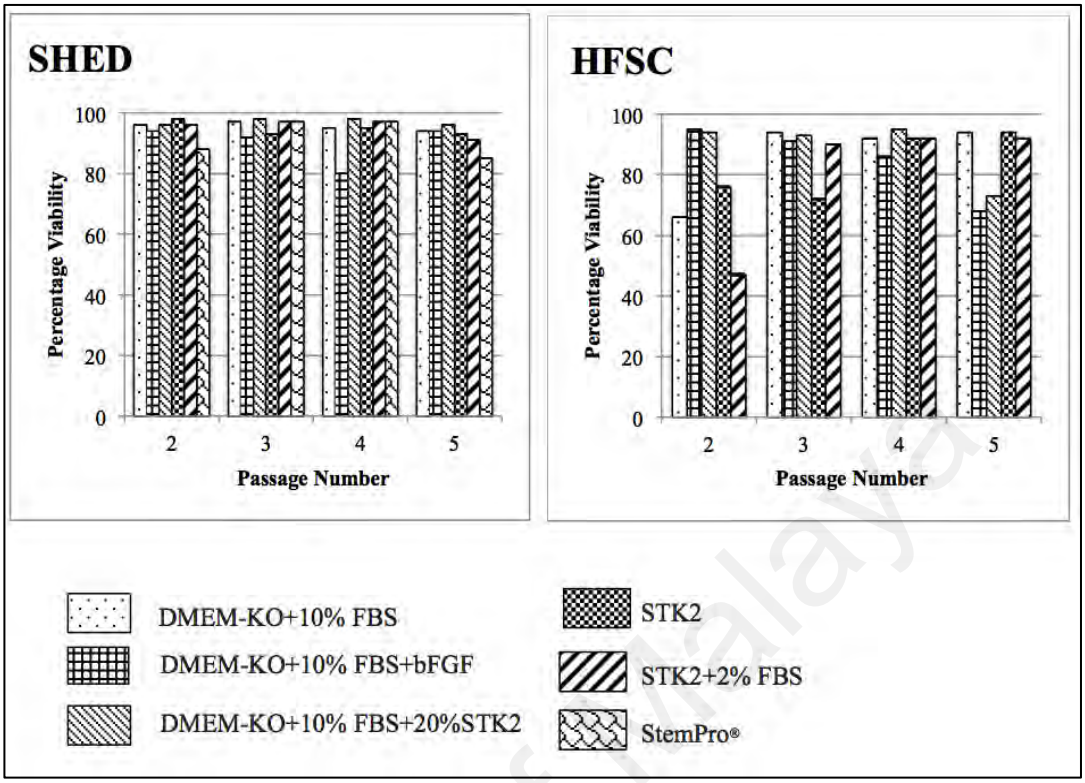


Figure 3.4: Viability of SHED and HFSCs. The viability of SHED and HFSCs when cultured in media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, DMEM-KO+10% FBS+20% STK2, STK2, STK2+2% FBS and StemPro® from passages 2 to 5. No growth was observed when HFSCs was cultured in StemPro® media.

3.4.3 Effect of DMEM-KO and STK2 on hair growth paracrine factor secretion in conditioned media

The CM was collected by incubating the cells in both STK2 and DMEM-KO basal media upon the cells reaching 80% confluency, for STK2 cultured SHED (n=1) and HFSCs (n=1). It was observed that the positive hair regulatory factors SDF-1 and PDGF-BB was secreted only in the CM prepared by DMEM-KO (Table 3.2). However, the negative hair growth regulatory factor secretion has also been facilitated when the cells were cultured in DMEM-KO based media.

Table 3.2: The hair regulatory paracrine profiles of the CM collected in STK2 and DMEM-KO for SHED and HFSCs.

(a) Paracrine factor concentration (ng/10^6 cells) for SHED

Paracrine Factor	Expansion media (STK2+2%FBS)				Expansion media (STK2)			
	P3		P4		P3		P4	
	STK2"	DMEM-KO"	STK2"	DMEM-KO"	STK2"	DMEM-KO"	STK2"	DMEM-KO"
SDF-1*	nd	3.01	nd	2.79	nd	3.67	nd	2.89
HGF*	5.24	4.82	0.16	3.82	8.11	2.69	0.18	2.19
VEGF-A*	174	54.80	32.20	52.30	101.00	15.10	13.6	8.19
PDGF-BB*	nd	0.25	nd	0.24	nd	3.33	nd	40.10
IL-1β#	nd	nd	nd	0.06	nd	0.08	nd	0.06
TGF-β#	nd	0.33	nd	0.32	nd	0.31	nd	0.30
bFGF#	nd	0.64	nd	0.61	nd	0.79	nd	0.67
TNF-α#	nd	0.64	nd	0.61	nd	0.87	nd	0.77
BDNF#	0.02	0.41	nd	0.03	nd	0.05	nd	0.05

***Positive hair regulatory factors, # Negative hair regulatory factors, " Media used for collection of CM (serum free condition), P Passage number**

(b) Paracrine factor concentration ng/10^6cells) for HFSCs

Paracrine Factor	Expansion media (STK2+2%FBS)				Expansion media (STK2)			
	P3		P4		P3		P4	
	STK2"	DMEM-KO"	STK2"	DMEM-KO"	STK2"	DMEM-KO"	STK2"	DMEM-KO"
SDF-1*	nd	1.41	nd	3.95	nd	1.47	nd	11.10
HGF*	59.30	0.51	119.00	1.60	nd	0.29	81.1	4.85
VEGF-A*	96.50	16.00	165.00	35.80	4.10	8.01	237.0	120.00
PDGF-BB*	nd	0.17	nd	0.51	nd	0.19	nd	1.94
IL-1β#	nd	0.04	nd	nd	0.89	0.04	nd	0.29
TGF-β#	nd	0.19	nd	0.81	0.18	0.20	nd	1.73
bFGF#	nd	0.29	nd	1.00	nd	0.46	nd	3.41
TNF-α#	nd	0.43	nd	0.65	nd	0.50	nd	3.12
BDNF#	nd	nd	nd	0.11	nd	0.03	nd	nd

*** Positive hair regulatory factors, # Negative hair regulatory factors, " Media used for collection of CM (serum free condition), P Passage number**

The profiling of the paracrine factor concentrations were carried out following the normalization against STK2 and DMEM-KO accordingly *nd- not detected

3.5 Discussion and conclusion

The main aim of the pilot study was to screen the most suitable media combinations that would support culturing of SHED and HFSCs at an optimal level which was assessed by the ability of the different media combinations in supporting the culturing of these cells in terms of the cell morphology, proliferation, PDT and viability.

The average PDT for SHED when cultured in DMEM-KO+10% FBS, which is one of the most commonly used media combinations in culturing SHED (Govindasamy et al., 2010) was 38.8±3 h. Our results for SHED concurred with the average PDT reported in literature, 38.5±13.4 h (Yu et al., 2014). However to date, the PDT for HFSCs cultured in DMEM-KO+10%FBS has not been reported in the literature as the widely used protocol involve the addition of bFGF or other growth factor combinations, in addition to FBS (Yu et al., 2006).

In this study, the addition of bFGF to DMEM-KO+10% FBS lowered the PDT for HFSCs. Interestingly, the addition of bFGF when culturing SHED caused a shorter PDT for passage 2 and 3 cells, however an increase in PDT was observed for passage 4 and 5 cells. The addition of bFGF increases the rate of cellular proliferation through MAPK/ERK and PI3K/AKT pathways which in turn activate their downstream processes which govern the survival, growth, protein synthesis in the cells (Gottipamula et al., 2013).

In this current study, StemPro® did not support HFSCs culturing. This may be due to the reason that the growth factor composition/ osmolality of the media being less supportive for the proliferation of the HFSCs. Furthermore, StemPro® also showed a higher PDT in comparison to the xeno free STK2 media when culturing of SHED. It has

been reported that StemPro® and STK2 media support better growth kinetics for SHED than the conventional media (Harada et al., 2015; Khanna-Jain et al., 2013). However, no studies reported the culture of HFSCs in STK2 or StemPro® media. STK2 also supported a better PDT (25±1.3 h) for HFSCs in comparison to the previously used Keratinocyte serum free media (KSFM), which reports a PDT of 30.73±0.75 h (Hilmi et al., 2013). Due to this reason STK2 media was chosen as the xeno free media for our subsequent experimental work.

We also used reduced serum culture conditions (2% FBS) when preparing the STK2 culture media to explore the possibility of using minimal FBS in culture conditions in combination to STK2. Since there is no previous study reporting the use of this media to culture HFSCs, the addition of 2% FBS was thought to provide the necessary anchoring properties for the attachment of SHED and HFSCs. The STK2+2% FBS media combination showed a shorter PDT for HFSCs and SHED compared to STK2 media under serum free conditions (Table 3.1).

When HFSCs were cultured in DMEM-KO+10% FBS+20% STK2 the PDT decreased by 4 h and 3 h for passages 3 and 4 cells respectively, in comparison to STK2+2% FBS. This observation could be attributed to better cell attachment in the presence of 10% compared to 2% FBS and the growth factors provided by both 10% FBS and 20% STK2. However, a 2 h and 5 hr increase in the PDT was observed for SHED when cultured in DMEM-KO+10% FBS+ 20% STK2 than in STK2+2% FBS at passages 3 and 4 respectively (Table 3.1).

In conclusion, after consideration on the feasibility of the media combinations to support the cell growth, the morphology of the cells, density, PDT and viability shown in the different media combinations, and in the efforts of minimizing the use of FBS, the following media combinations were chosen for the next stage of the study;

(a) DMEM-KO+10% FBS (control media),

(b) DMEM-KO+10% FBS+bFGF (control media),

(c) STK2+2% FBS

(d) STK2 media

StemPro® was eliminated for further studies since it did not support the proliferation of HFSCs. DMEM-KO+10% FBS+20% STK2 was also eliminated since it showed intermediate growth kinetics between STK2+2% FBS and DMEM-KO+10% FBS, and it has a higher percentage of serum supplementation.

Since the better growth kinetics observed in the cells cultured in STK2 based media can be an indication of a growth factor rich composition in STK2, it is possible that the cells integrate a negative feedback mechanism during the production and excretion of paracrine factors to the culture media. Thus the collection of CM using STK2 for the cells cultured primarily in STK2 based media was carried out in two ways. Upon reaching 80% confluency, one batch of cells were supplemented with STK2 media while the other batch of cells were supplemented in DMEM-KO based media and incubated for 24 h for the CM collection.

The CM prepared using STK2 showed a higher positive hair regulatory factor VEGF-A secretion than the CM prepared using DMEM-KO (Table 3.2). However, when supplemented with DMEM-KO, the secretion of positive hair regulatory factors SDF-1 and PDGF-BB were observed. It was also observed that the secretion of negative hair regulatory factors was also stimulated upon the collection of CM using DMEM-KO. Thus the CM collection in the subsequent experiments were carried out using

STK2 media for the cells cultured in STK2 based media and DMEM-KO for the cells cultured in the DMEM-KO based media.

CHAPTER 4: CONDITIONED MEDIA FOR STIMULATION OF HAIR GROWTH- MATERIALS AND METHODS

Upon identification of suitable culture media and establishment of the conditioned media collection procedure, the potential of conditioned media to stimulate hair growth from SHED and HFSC were further investigated. The following experiments were conducted to address the objectives outlined in Chapter 1.

4.1 Materials

All materials and instrumentation used in this section are listed in the Appendix D and E with the respective manufacturer details.

4.2 Methods

4.2.1 Culture expansion and growth kinetics of SHED and HFSCs

Passage 1 SHED (n=3) and HFSCs (n=3) were purchased and stored in the liquid nitrogen tank until further use. The cells were cultured and expanded in DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS and STK2 media and the growth kinetics were studied as described in Chapter 3, Section 3.3.2.4.

4.2.2 Mesenchymal stem cell characterization of SHED and HFSCs by Flow cytometry analysis

The sample preparation for the flow cytometry analysis was conducted as follows; At 80% confluency, passage 3 SHED and HFSCs were trypsinised and the cell numbers were determined using the Countess® chamber slides. Two aliquots of $0.5x10^6$ cells/ml were prepared in 1.5 ml eppendorf tubes for each cell source cultured in the different media combinations. The cells were centrifuged at 300 g for 10 min. The supernatant

was aspirated completely. The pellets obtained were re-suspended in 100 µl phosphate buffer saline (PBS) pH=7.2 containing 0.5% bovine serum albumin and 2 mM EDTA. One of the aliquots was mixed with 10 µl MSC phenotyping cocktail, while the other aliquot was mixed with 10 µl isotype control cocktail. The contents were mixed well and incubated in dark for 10 min at 4 °C, followed by the addition of 1ml PBS buffer (pH=7.2). These aliquots were centrifuged at 300 g for 10 min. The supernatant was aspirated completely. The cells were re-suspended in 1 ml PBS buffer (pH=7.2) and re-centrifuged at 300 g for 10 min. The supernatant was discarded. One milliliter of 1% cold para-formaldehyde dissolved in 0.85% saline was added to the pellets and the tubes were flipped well several times and mixed by pipetting. The samples were transferred to the flow cytometry tubes and were stored in dark at 4 °C until further use.

The samples for compensation were prepared as described below. Following trypsinisation of the 80% confluent passage 3 SHED and HFSCs, the cell number was determined using the Countess® chamber slides. Five aliquots of $0.5x10^6$ cells/ml were prepared for each type of sample grown in the respective media. The cell suspensions were centrifuged at 300 g for 10 min. The supernatant was aspirated completely. The cell pellets were re-suspended in fluorochromes; Periclinin Chlorophyll Protein Complex (PerCP), Phycoerythrin (PE), Allopycocyanin (APC) and Fluorescein isothiocynate (FITC) in 100 µl buffer respectively. The aliquot blank was re-suspended in 500 µl buffer and was preceded directly for compensation. Ten microliter of CD73-Biotin was added to the PerCP aliquot, 10 µl CD105-PE to the PE aliquot, 10 µl CD73-APC to the APC aliquot and 10 µl CD90-FITC to the FITC aliquot . Each aliquot was mixed well and incubated for 10 min in the dark at 4 °C. Following the incubation, 1 ml PBS buffer (pH=7.2) was added to each aliquot and centrifuged at 300 g for 10 min. The supernatant was aspirated completely. Ten microliter of Anti-Biotin-PerCP was added to aliquot PerCP and mixed well. The aliquots were incubated for 10 min in the

dark at 4 °C. The cells were washed by adding 1 ml PBS buffer (pH=7.2) and centrifuged at 300 g for 10 min. The supernatant was aspirated completely and the pellet was re-suspended in 500 µl PBS buffer (pH=7.2).

4.2.3 Mesenchymal stem cell characterization of SHED and HFSCs by Tri-lineage differentiation

Passage 3 SHED and HFSCs were seeded in 4 wells of a 6-well plate at a density of 5000 cells/cm^2, for each media combination. Upon 80% confluency, 2 ml each of adipogenic, chondrogenic, osteogenic differentiation media and the corresponding culture media was added to each well and media change for the corresponding differentiation lineage was carried out every 3 days. Staining was conducted for adipogenic lineage at day 14 and osteogenic lineage at day 22 and chondrogenic lineage at day 28 as follows:

(a) Adipogenic differentiation

The cells were washed with 2 ml 10% formaline and incubated for 40 min at 37 °C and washed twice with 2 ml distilled water. Two milliliters 60% iso-proponol was added to the cells and incubated at 37 °C for 5 min followed by the addition of Red Oil O and incubated for 1 h. The cells were washed four times using 2 ml distilled water per each wash. The cells were observed under Olympus CKX41 inverted microscope at 4×, 10× and 20× objectives.

(b) Chondrogenic differentiation

The cells were washed with 2 ml 10% formaline and incubated for 20 min at 37 °C, followed by two washes of 2 ml PBS. Two milliliters 1% acetic acid was added and the cells were incubated at 37 °C for 15 s followed by the addition of 2 ml safranin for 15

min. The cells were washed well four times using 2 ml PBS per wash and observed under Olympus CKX41 inverted microscope at 4×, 10× and 20× objectives.

(c) Osteogenic differentiation

The cells were washed with 2 ml 10% formaline and incubated for 30 min at 37 °C. Following the incubation the cells were washed twice with 2 ml distilled water per wash. Two milliliters of Alzarin Red was added to the cells and incubated at 37 °C for 1 h followed by four washes using 2 ml PBS. The cells were observed under Olympus CKX41 inverted microscope at 4×, 10× and 20× objectives.

4.2.4 Collection of conditioned media and profiling of paracrine factors

Changing of media was carried out every three days. Upon observation of 80% confluency in passage 3 and 4 cultures, the media was completely removed and supplemented with the respective basal media (DMEM-KO or STK2). The basal media supplemented cultures were incubated for 24 h, following which the media was collected in pre-cooled 15 ml centrifuged tubes. The media was centrifuged at 310 g for 6 min at 4 °C, followed by filtering using 2 μm filters. The filtered media was aliquot in pre-chilled 1.5 ml eppendorf tubes and stored at -80 °C until further use.

Profiling the paracrine factors secreted by SHED and HFSCs was carried out by employing the Luminex assay as described in section 3.3.2.5 in the pilot study.

4.2.5 Analysis of *in vitro* hair growth stimulation by conditioned media

An in *vitro study* was carried out to determine the most suitable CM for the stimulation of hair growth. Seven week old female Imprinting Control Region (ICR) mice were purchased from the Animal Experimental Unit, Faculty of Medicine, University of Malaya. All animal experimental protocols were carried out following the ethics approval obtained from the Animal Ethics Committee of University Malaya

(2015-180407/RESTD/R/NHAK). The ICR mice were euthanized in CO_2 euthinisation chamber for rodents. The dorsal region of the mice was shaved with clippers, and 0.5 cm x 0.5 cm skin samples from the shaved area were cut till the subcutaneous region. The skin samples were transported to the laboratory in cold PBS.

The CM was thawed following which 300 µl of the thawed media was added to each well of a 24-well plate. Three cut of skin pieces measuring approximately 0.5 cm x 0.5 cm were placed in each of the well and incubated in the CM at 37 °C for 72 h. At every 24 h time point one skin sample from each well was processed for histological analysis by fixing in 10% of formaline in a dark room for 48 h.

In order to conduct the histological analysis of the skin samples for the determination of hair growth stages the skin tissues were processed overnight in the tissue processor, Leica TP1020. Within the processor, the tissues were sequentially exposed to 10% formaline for 1 h, 70% alcohol for 1 h, 95% alcohol I for 1 h, 95% alcohol II for 1 h, absolute alcohol I for 1 h, absolute alcohol II for 1 h, absolute alcohol III for 1 h, xylene I for 1 h, xylene II for 1 h, xylene III for 1.5 h, paraffin wax I (2 h) and paraffin wax II for 3.5 h.

The skin tissues were positioned vertically and embedded in paraffin wax. The tissues were sectioned using the Leica Microtome longitudinally to obtain 5 µm sections. Each tissue section was transferred to a 40 °C water bath and was fished to a snowcoat slide. These slides were incubated at 60 °C for 15 min followed by heamatoxylin and eosin (H&E) staining.

During H&E staining the slides were processed in the chemicals as follows; Xylene I for 5 min, Xylene II for 4 min, 100% alcohol for 3 min, 95% alcohol for 3 min, 70% alcohol of 3 min, running water for 3 min, heamatoxylin stain for 5 min, running water

for 3 min, 0.5% acidic alcohol for a count of 1-10, running water for 3 min, 80% alcohol for 1 min, Eosin for 3 min, 95% alcohol 4 dips, 100% alcohol I for 2 min, 100% alcohol II for 2 min, xylene I for 3 min, xylene II for 3 min and xylene III for 3 min. Following mounting, the slides were scanned using the digital scanning system model-Pannoramic Desk and the slide images were read using the Pannoramic viewer.

The stage of murine hair growth (Figure 4.1) were identified using a scoring chart (Table 4.1) prepared as per guidelines set by Muller-Rover et. al (Muller-Rover et al., 2001). The scoring was conducted blinded and the inter-reliability was calibrated against an oral pathologist. The percentage of hair follicles present in the anagen, telogen and catagen stages were tabulate. Students paired t-test (SPSS Version 22) was carried out to ascertain the differences between the four CM in supporting the transition of hair growth to the different stages, at the confidence level of 95%.

Table 4.1: Score chart for the determination of murine hair growth stage adapted from (Muller Rover et al. 2001)

	Position of Hair Follicle					Consistency of Dermal Papilla		Shape of Dermal Papilla			Inner Root Sheath				
											Present			Absent	
	Papillary Dermis	Reticular Dermis	Border of Dermis and Subcutaneous	Mid Sub-Cutaneous	Deep SC above Panniculus Carnosus	Consistent	Diffused	Ball	Onion	Narrow	Cone	Bellow Sebaceous Gland	At Sebaceous Gland	Above Germ Cell	Bellow Germ Cell
Early Anagen															
Mid Anagen															
Late Anagen															
Catagen															
Telogen															

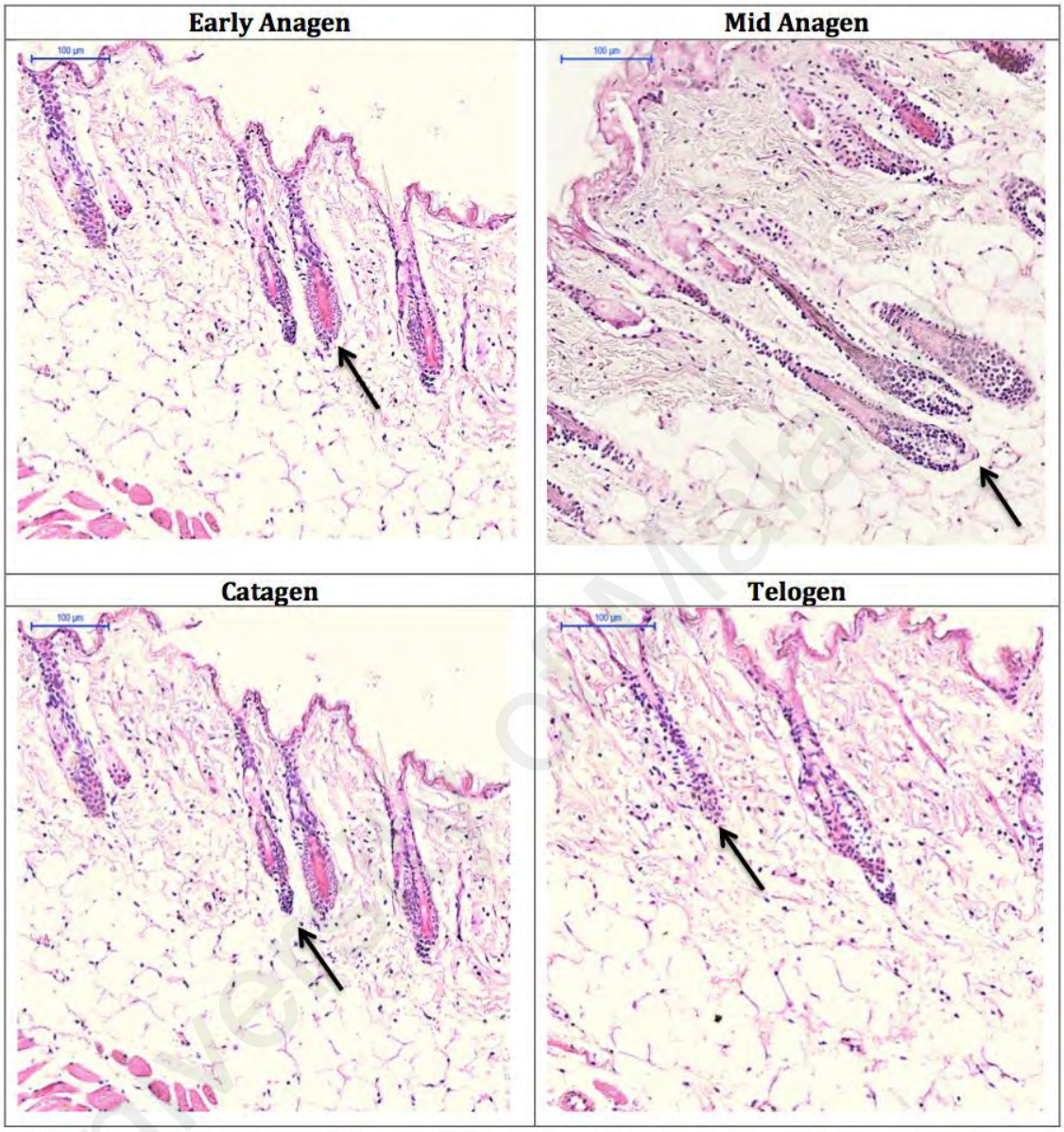


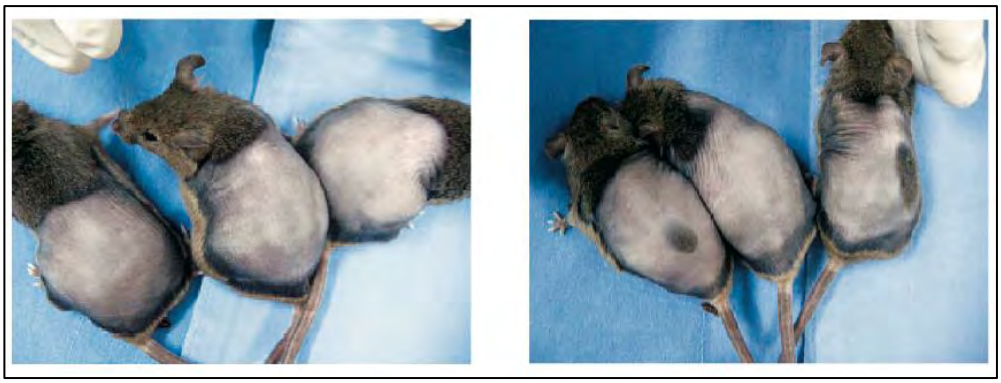
Figure 4.1: Representative slide images to indicate the different hair growth stages. The representative images of the hair follicle indicating the different hair growth stages, identified based on the position of the hair follicle, consistency and shape of the dermal papilla and the presence and absence of the inner root sheath.

4.2.6 Analysis of *in vivo* hair growth stimulation by conditioned media

Five week old C3H/HeN female mice (n=20) was purchased from M-Clea Bioresources Co.,Ltd., Thailand. The mice were acclimatized under controlled environmental conditions (room temperature 24 ± 1°C, humidity 65-75%) for 14 days. The mice were fed on an altromin diet, comprised of crude protein (18.5% per kg), fat (6.2% per kg), fiber (5.6% per kg), ash (7.2% per kg), moisture (11.3% per kg) and nitrogen free extract (51% per kg).

Upon seven weeks of age the mice were anesthetized using ketamine, xylasine intraperitoneal (IP) and the dorsal region was shaved with clippers. The pink coloured skin observed upon shaving, indicating that the hair growth stage is now synchronized to telogen stage (Muller-Rover et al., 2001). The mice were divided into four groups; Group 1- mice administered with SHED-CM (n=9), group 2- mice administered with HFSC-CM (n=9), group 3- mice administered with STK2 media (n=3), group 4- mice with no treatment (n=2), where in groups 3 and 4 are the control groups.

Each mouse in groups 1, 2 and 3 were administered with 3 sub-cutaneous injections of 100 µl of the selected media at the dorsal loose skin region of the neck at 3 day intervals and the darkening of skin was monitored pictorially on alternate days to observe the hair growth progression (Figure 4.2) (Park et al., 2010). The mice were euthanized using CO_2 gas in the euthanisation chamber for rodents when almost complete hair growth was observed in the shaved area. The Students paired t-test was chosen to determine the differences between all groups in stimulating the anagen stage of hair growth at the confidence level of 95%.



(a)Telogen stage pink skin **(b) Anagen stage dark patches**

Figure 4.2: Progression of mice hair growth in different stages (Park et al, 2010). (a) Pink skin of mice upon synchronizing the hair growth stages to telogen stage (b) The appearance of dark patches indicating the transition of hair follicles to the anagen stage.

CHAPTER 5: CONDITIONED MEDIA FOR STIMULATION OF HAIR GROWTH- RESULTS

5.1 Morphology and growth kinetics of SHED and HFSCs cultured in different media combinations

Based on the findings from the pilot study, the four media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+10 ng bFGF, STK2+2% FBS and STK2 were selected to further assess the effect of media on SHED and HFSCs to secrete hair growth related paracrine factors. The stem cells were cultured from passage 2 to 5. The Figure 5.1 illustrates the representatives of the phase contrast images of SHED and HFSCs at passage 3 on day 3 post seeding. The cells exhibit a spindle shaped morphology when cultured in all media combinations. However, cells cultured in DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF and STK2 media were more elongated in comparison to those cultured in STK2+2% FBS. Short, rounded cell morphology was observed for cells cultured in STK2+2% FBS media during the initial days of culture, following which the cells became elongated upon an increased confluency of approximately 80%.

Based on the morphological assessment, the highest cell density was in STK2+2% FBS media, while the cell density was similar in DMEM-KO+10% FBS+bFGF, to the serum free STK2. The commonly used standard culture media, DMEM-KO+10% FBS produced the least density in comparison to the other media combinations at a given period (Figure 5.1).

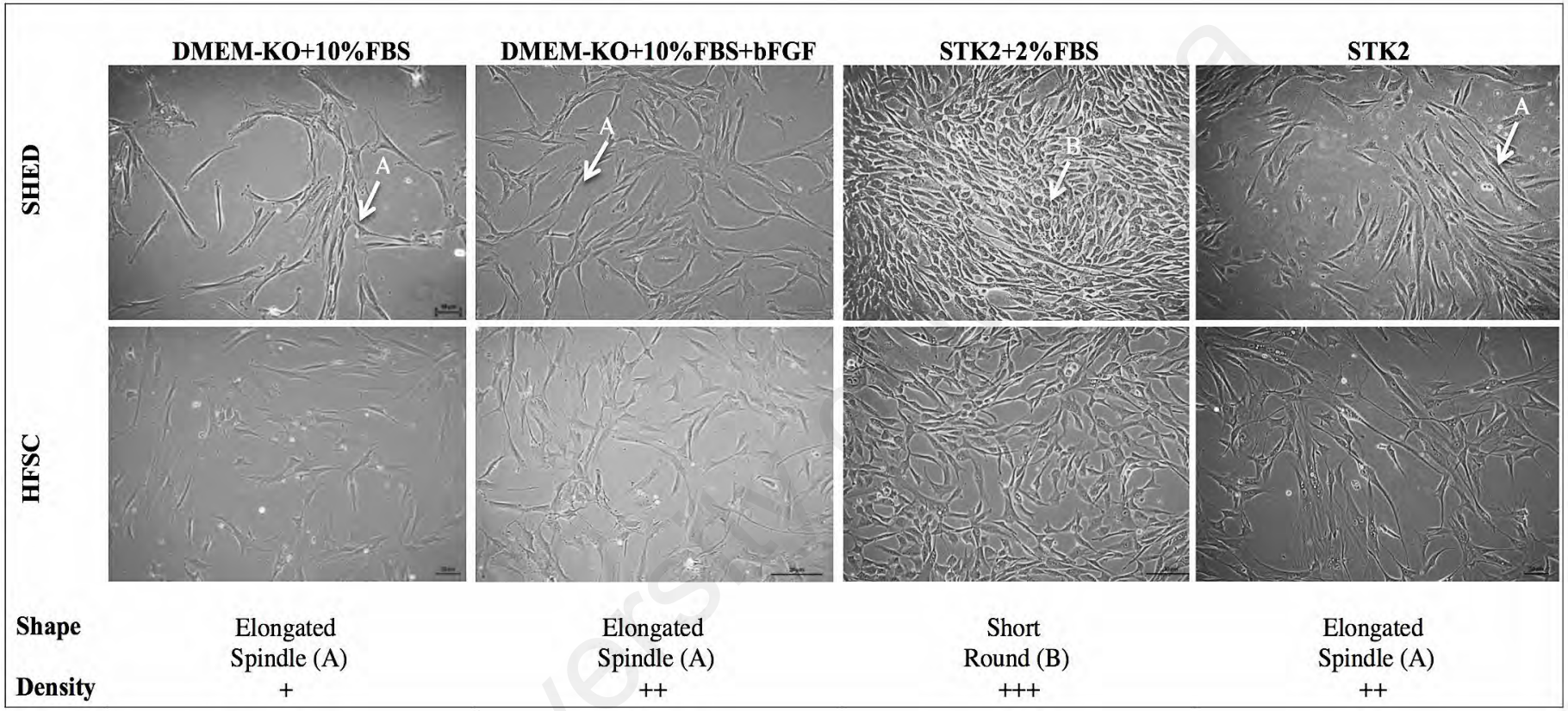


Figure 5.1: Morphology and cell density of SHED and HFSCs cultured in different media combinations. The cell morphology of SHED and HFSCs cultured in the media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS+STK2 at Day 3 of passage 3, observed under 10× magnification. *A- elongated spindle shape, B-short round shape, +-low cell density, ++- moderate cell density, +++- high cell density

The assessment of PDT indicated that SHED has a lower PDT than HFSCs (Table 5.1), suggesting its higher proliferation capacity. A lower PDT is observed in both stem cell sources at passage 3 and 4 in comparison to the later passages indicating their proliferative capacity reduces along the passages. The studies also showed that cells cultured in STK2 based media with or without supplementation of serum has a lower PDT in comparison to DMEM-KO based media combinations.

SHED cultured in STK2+2% FBS at passage 4, showed the lowest population doubling of 18±5 h. Statistical analysis conducted using Kruskal-Wallis indicated that SHED cultured in STK2 based media showed significantly ($p<0.05$) lower PDT than DMEM-KO based media at passages 2 and 5.

HFSCs showed the lowest PDT when cultured in STK2+2% FBS media in both passages 3 (23±3 h). The HFSCs cultured in the STK2 based media for all passages showed significantly ($p< 0.05$) lower PDT than DMEM-KO based media.

Table 5.1: The population doubling times of SHED and HFSCs cultured in the different media combinations from passage 2 to passage 5.

Media	Population Doubling Time (h) ±SE							
	Passage 2		Passage 3		Passage 4		Passage 5	
	SHED	HFSC	SHED	HFSC	SHED	HFSC	SHED	HFSC
DMEM-KO+10% FBS	30±5[a]	105±30	43±5	44±3[h]	46±18	58±5[k]	58±3[c]	110±39[m]
DMEM-KO+10% FBS+ bFGF	29±2[b]	120±38[f,g]	41±4	52±19[i,j]	32±3	85±23[l]	61±7[d,e]	105±34[n]
STK2+2% FBS	23±1[a,b]	63±6[f]	19±7	23±3[h,i]	18±5	24±4[k,l]	33±12[c,d]	26±4[m,n]
STK2	24±1	120±8[g]	27±6	29±4[j]	26±2	37±6	39±14[e]	53±6

*The same alphabet indicate a significant difference ($p<0.05$). There was no statistically significant difference between the population doubling times between SHED and HFSC.

In the assessment of the status of viability of the cells in these different culture media, it was observed that the percentage of viability was higher in passages 3 and 4 in comparison to the later passages SHED and HFSCs (Figure 5.2). Cells cultured in STK2 based media showed a higher viability in all passages in comparison to the DMEM-KO based media. The highest viability was observed in passage 3 for SHED cultured in STK2+2% FBS (98%±0.001), and at passage 4 for HFSCs when cultured in STK2 (98%±0.007). However, Kruskal-Wallis statistical analysis did not indicate any significant difference ($p>0.05$) for the viability between SHED and HFSCs or between the media combinations for each source.

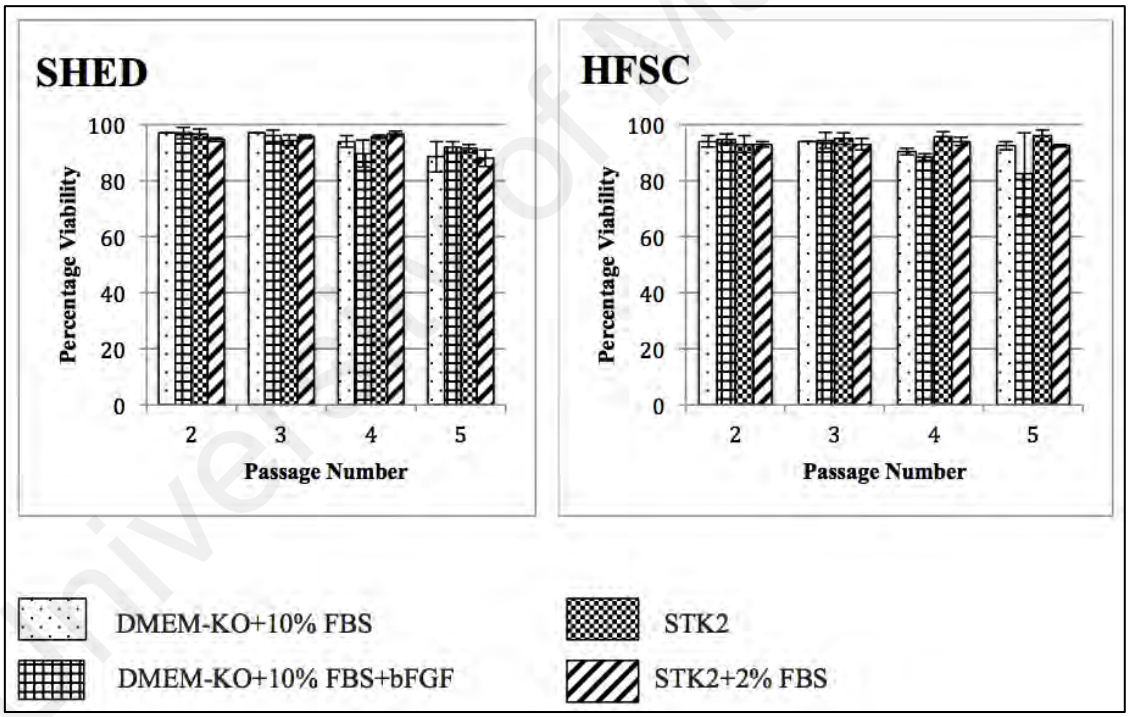


Figure 5.2: Viability of SHED and HFSCs. The viability of SHED and HFSCs when cultured in media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS and from passages 2 to 5.

5.2 Characterization of SHED and HFSCs for their mesenchymal stem cell properties

5.2.1 Determination of the mesenchymal stem cell marker expression

The morphological assessment of cells cultured in different culture media was of supportive evidence that these media promote the maintenance of SHED and HFSCs in the culture. However, there was a need for a validation of their MSC properties, as per the guidelines specified by the International Society for Cellular Therapy. Hence, flow-cytometry analysis was conducted to determine the expression of positive markers CD90, CD105 and CD73, and negative markers CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR (Horwitz et al., 2005) (Figure 5.3). SHED cultured in STK2 based media, the positive CD90 marker was expressed 99% while in DMEM-KO based media it was 94%.

However, in HFSCs CD90 was expressed 95% (Figure 5.4) in STK2 based media and ranged 69%-73% in DMEM-KO based media. The positive markers CD105 and CD73 expression levels were between 95-100% in all media combinations for both SHED and HFSCs. Thus all four media supported the expression of positive markers in SHED, even though a better positive marker expression level for HFSCs was observed in STK2 based media. The results also indicated that a higher homogeneity of the HFSCs was better maintained in the STK2 based media in comparison to the DMEM-KO based media.

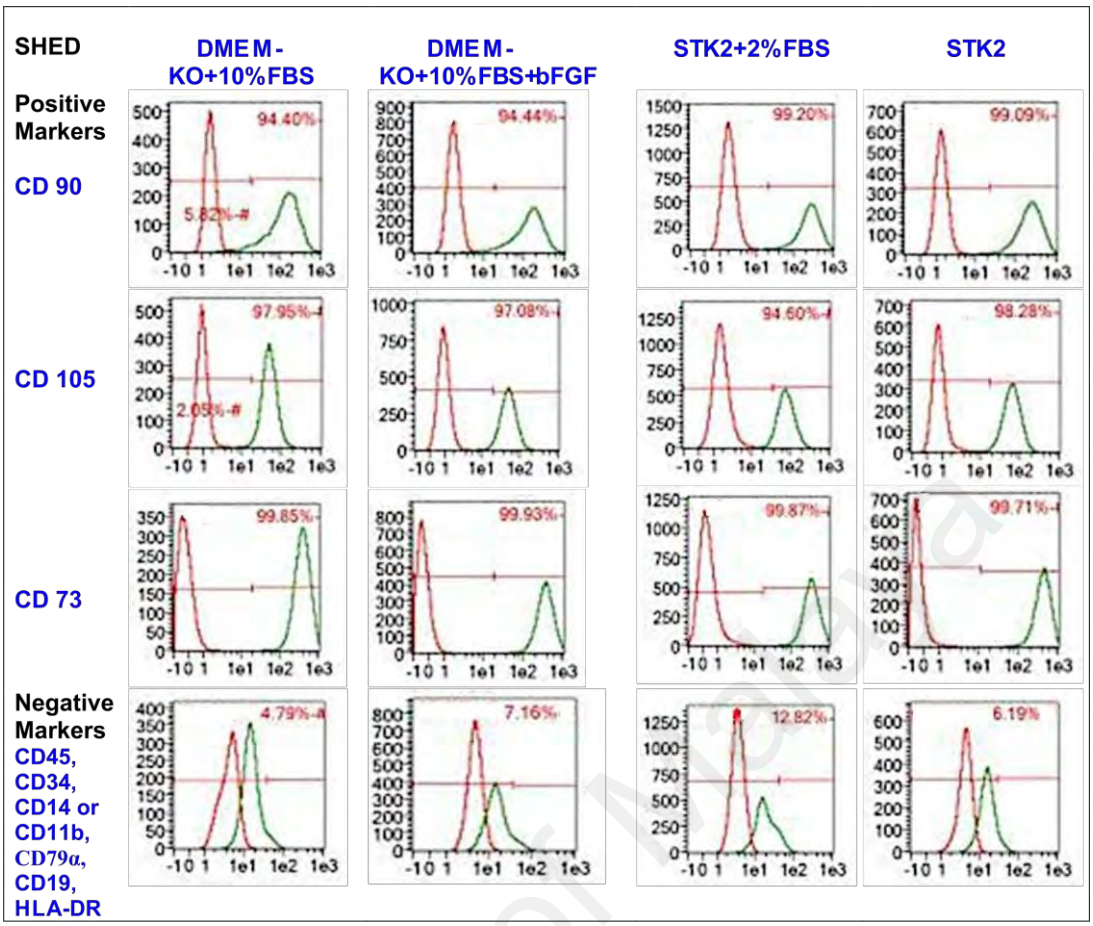


Figure 5.3: CD marker expression of SHED. The positive and negative MSC marker expression of SHED when cultured in media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS and STK2. The analysis was carried out for the cells at passage 3 upon 80% confluency.

SHED in STK2+2% FBS media showed negative marker expression level of 13% while HFSCs in DMEM-KO+10%FBS+bFGF media it was 25%. For all other media combinations the negative marker expression was less than 7% for both sources.

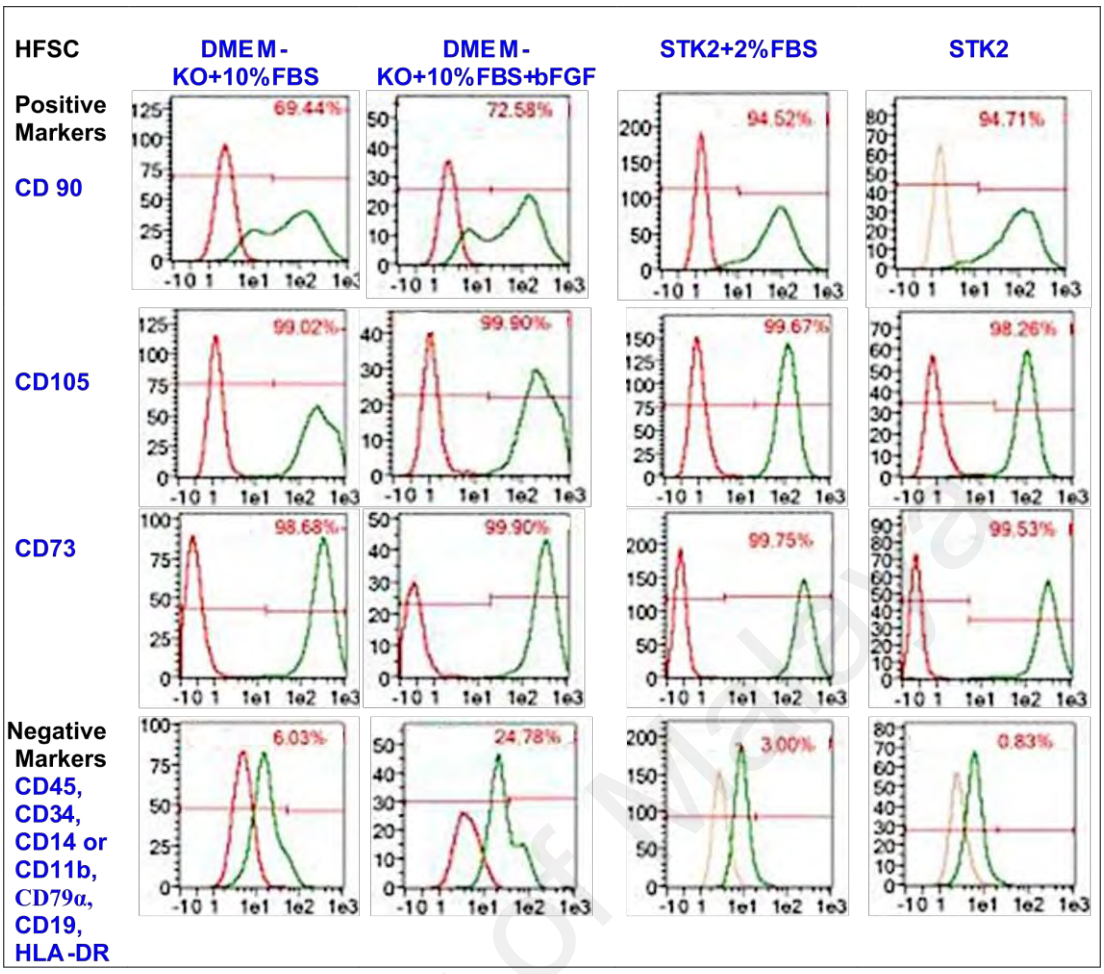
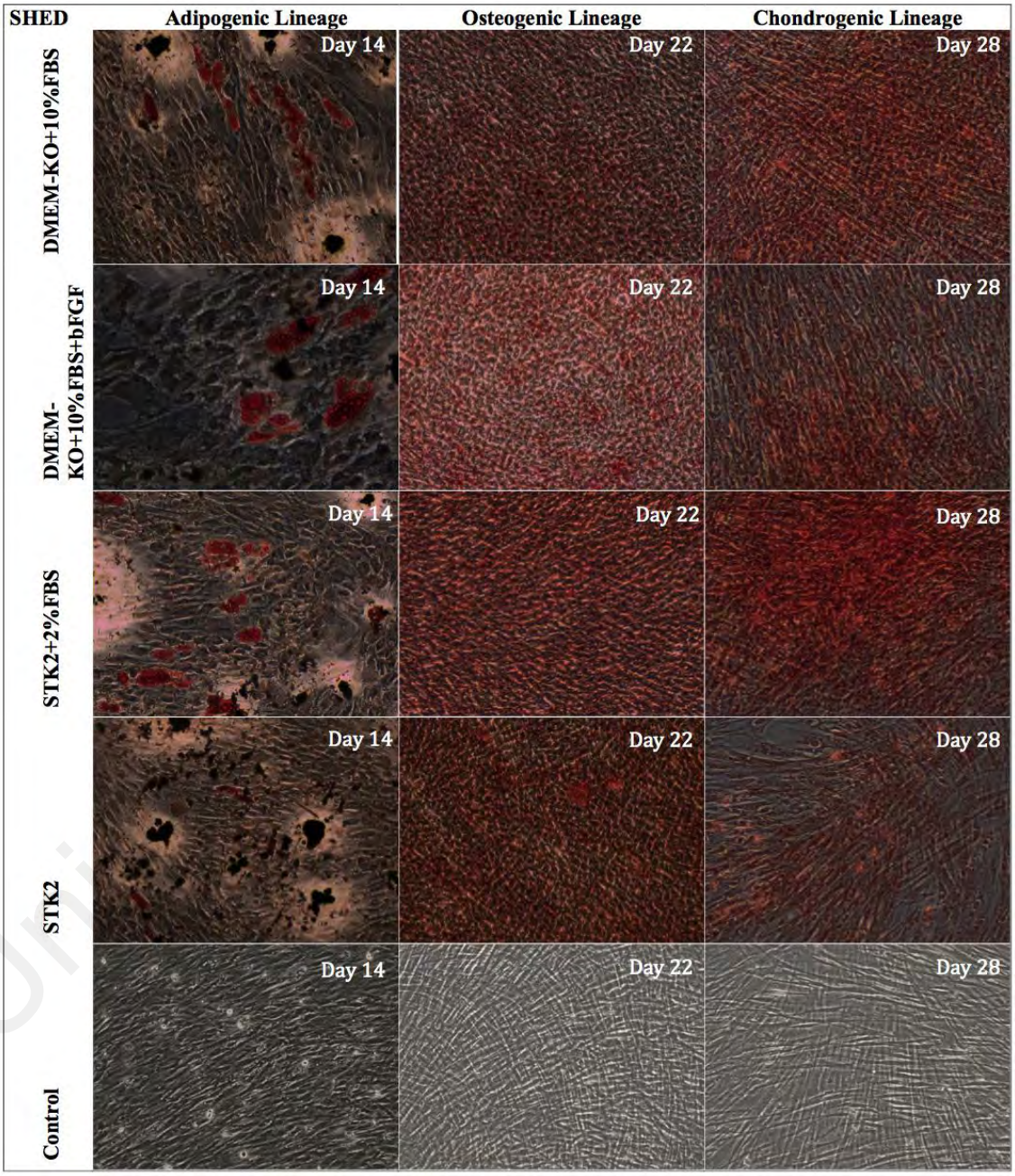


Figure 5.4: CD marker expression of HFSCs. The positive and negative MSC marker expression of HFSCs when cultured in media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS and STK2. The analysis was carried out for the cells at passage 3 upon 80% confluency.

5.2.2 Maintenance of the lineage differentiation potential of SHED and HFSCs

While the SHED and HFSCs maintained their CD marker expression profile the cells were subjected to the differentiation assay to verify their potential to differentiate to chondrogenic, osteogenic and adipogenic lineages under differentiation specific conditions. The adipogenic differentiation was detected by staining the intra-cytoplasmic lipid droplets with Oil Red O. The staining of proteoglycans with Safranin confirmed the chondrogenic differentiation while the differentiation of the cells to osteogenic lineage was confirmed by the staining of extracellular calcium deposits by Alzarin red. This is to confirm the maintenance of their differentiation capability as for

the guidelines specified by the International Society for Cellular Therapy (Horwitz et al., 2005) (Figure 5.5). The result from the differentiation studies confirmed that all media combinations supported the maintenance of their stem cell characteristics in which these cells were able to commit to the respective mesenchymal lineages.



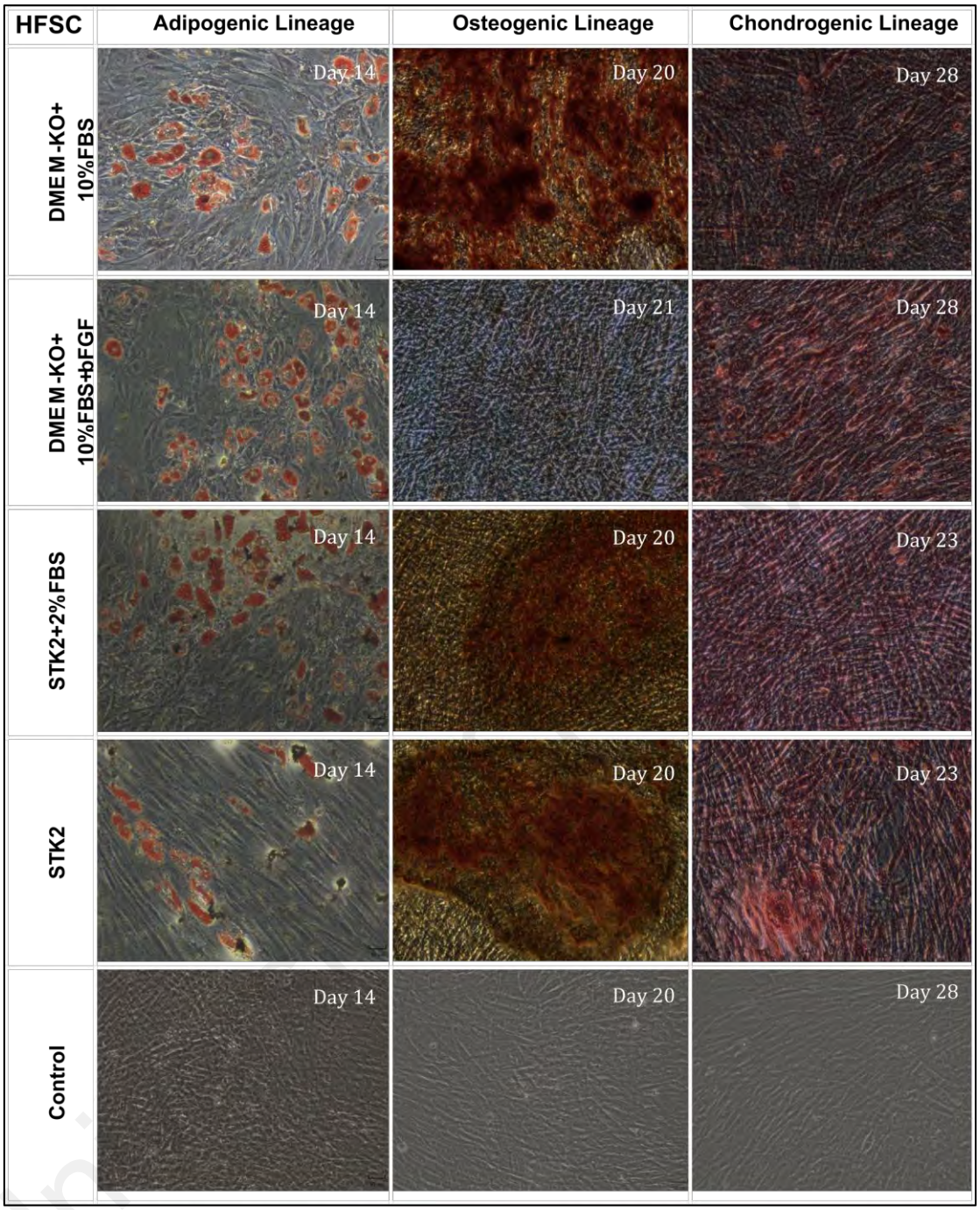


Figure 5.5: Tri-lineage differentiation of SHED and HFSCs. The trilineage differentiation studies conducted to study the maintenance of MSC lineages; adipogenic, chondrogenic and osteogenic for SHED and HFSCs when cultured in media combinations; DMEM-KO+10% FBS, DMEM-KO+10% FBS+bFGF, STK2+2% FBS and STK2.The representative images of cells cultured in DMEM-KO+10% FBS as the control media. The study was carried out for the cells at passage 3 upon 80% confluency.

5.3 Profiling of paracrine secretion of SHED and HFSCs

5.3.1 Hair growth related paracrine secretion profile of SHED and HFSCs

The profiling of paracrine secretion was carried out for three donors for SHED and HFSCs indicated that paracrine factor secretion levels are highly donor dependent (Table 5.2) and is also dependent on the culture media combinations used when culturing these cells.

All media combinations indicate the expression of positive regulatory factors for hair growth; VEGF-A and HGF. SHED and HFSCs cultured in STK2 based media secretes only negative hair regulatory factor BDNF and IL-1. It is also seen that the secretion of negative hair regulatory factors is comparatively high in SHED and HFSCs, when cultured in DMEM-KO+10% FBS. The SHED and HFSCs paracrine profiles indicated that STK2 media has not facilitated the secretion of SDF-1, while STK2+2% FBS has not facilitated the secretion of PDGF-BB. It is also observed that SHED secreted a higher amount of positive hair regulatory factors than HFSCs, with an exception of VEGF-A, while the negative hair regulatory factors are secreted higher in HFSCs.

Table 5.2: The mean (±SE) hair regulatory paracrine factor concentrations in ng/10^6cells for SHED and HFSCs cultured under the different culture media combinations; DMEM-KO+10% FBS, STK2+2% FBS and STK2 at passage 3 and passage 4

(a) The hair regulatory paracrine factor concentration (ng/10^6 cells) for SHED

Paracrine Factor	DMEM-KO+10% FBS		STK2+2%FBS		STK2	
	P3	P4	P3	P4	P3	P4
SDF-1*	11.80± 4.52	9.96±1.63	0.55± 0.42	0.74± 0.74	nd	nd
HGF*	13.20± 6.57	11.30±5.77	2.42± 1.52	0.08± 0.05	2.85± 2.63	0.13±0.07
VEGF-A*	44.10±20.70	39.80 ±2.33	104.00 ±49.40	27.90±9.67	39.80±30.60	9.36±2.78
PDGF-BB*	0.45± 0.30	0.24±0.01	nd	nd	0.59± 0.59	0.39±0.39
IL-1α#	0.11± 0.08	0.04±0.03	0.02± 0.02	nd	0.004±0.004	0.007±0.007
IL-1β#	0.14± 0.08	0.07±0.04	nd	nd	nd	nd
TGF-β#	0.84 ± 0.45	0.53±0.28	nd	nd	nd	nd
bFGF#	1.36 ± 0.85	0.73±0.37	nd	nd	nd	nd
TNF-α#	1.29 ± 0.77	0.85±0.43	nd	nd	nd	nd
BDNF#	0.94 ± 0.67	1.40 ±1.25	0.61± 0.59	1.01±0.99	0.008±0.008	0.007±0.007

*** Positive hair regulatory factors, # Negative hair regulatory factors, P Passage number**

(b) The hair regulatory paracrine factor concentration (ng/10^6 cells) for HFSC

Paracrine Factor	DMEM-KO+10% FBS		STK2+2%FBS		STK2	
	P3	P4	P3	P4	P3	P4
SDF-1*	34.70±10.20	35.40± 22.80	2.86± 2.86	2.42± 2.42	nd	nd
HGF*	57.54± 3.46	2.81± 2.60	39.50±17.20	66.10±33.50	9.41± 8.58	28.10±26.50
VEGF-A*	175.00±85.70	159.00±136.00	81.30±32.50	106.00±47.50	273.0±20.90	90.60±73.40
PDGF-BB*	0.79± 0.04	1.31± 1.31	nd	nd	1.28± 1.28	2.31± 2.31
IL-1α#	0.21± 0.08	0.22± 0.15	0.05±0.02	nd	0.05± 0.05	0.10± 0.10
IL-1β#	0.32± 0.18	0.32± 0.32	nd	nd	0.01± 0.01	0.01± 0.01
TGF-β#	1.05± 0.53	1.83± 1.83	nd	nd	nd	nd
bFGF#	2.23± 1.23	1.87± 1.87	nd	nd	nd	nd
TNF-α#	3.72± 2.21	3.39± 3.39	nd	nd	nd	nd
BDNF#	1.41± 1.05	1.97± 1.97	0.19±0.19	nd	0.02±0.02	0.03±0.03
*** Positive hair regulatory factors, # Negative hair regulatory factors, P Passage number**						

*nd- not detected

In addition to the hair growth regulatory factors, the profiling was carried out for paracrine factors that were commonly secreted by other stem cell sources such as ADSCs and BMSCs (Table 5.3). The profiling studies indicated that SHED and HFSCs both secreted the paracrine factors SCF, G-CSF, IL-6, IL-8, LIF, VCAM-1, MCP-1 and sICAM-1 in all media combinations. The secretion of all the profiled paracrine factors can be observed when the both cell sources are cultured in DMEM-KO+10% FBS media, except for M-CSF secretion by HFSCs at passage 4. The secretion of paracrine factors in STK2+2% FBS is observed in a higher trend than for the STK2 media without serum supplementation. It is also seen that HFSCs secrete more amount of paracrine factors, in comparison to SHED.

Table 5.3: The mean (±SE) paracrine factor concentrations in ng/10^6 cells for SHED and HFSCs cultured under the different media combinations; DMEM-KO+10% FBS, STK2+2% FBS and STK2 at passage 3 and passage 4
a) The paracrine factor concentration (ng/10^6 cells) for SHED

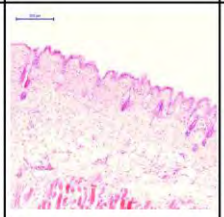
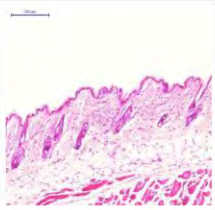
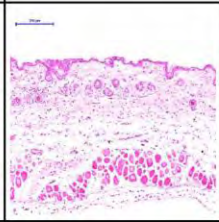
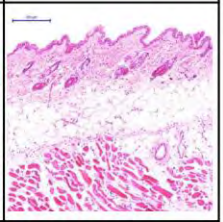
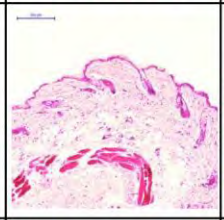
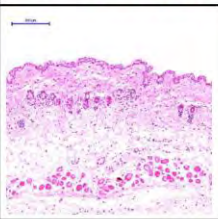
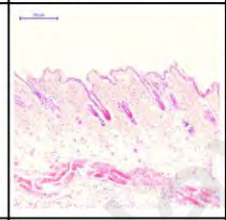
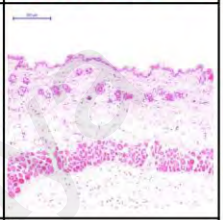
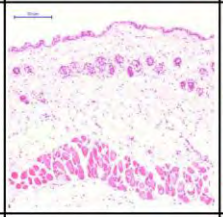
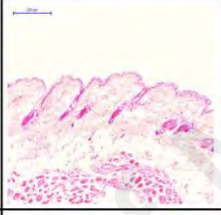
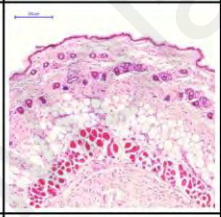
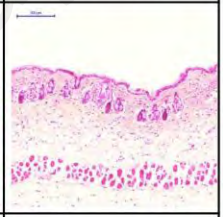
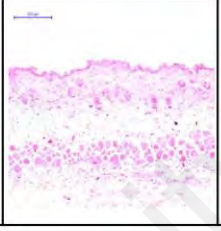
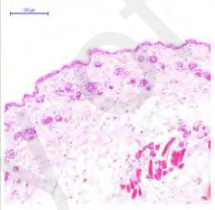
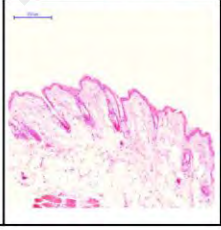
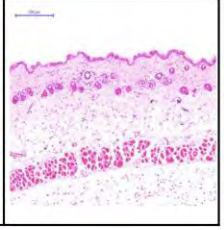
Paracrine Factor	DMEM-KO+10% FBS		STK2+2%FBS		STK2	
	Passage 3	Passage 4	Passage 3	Passage 4	Passage 3	Passage 4
SCF	0.51±0.23	0.49±0.10	0.12±0.06	0.01±0.01	0.02±0.02	0.01±0.01
GCSF	22.10±13.90	13.10±6.59	nd	nd	0.06±0.06	0.02±0.02
GM-CSF	1.06± 0.53	0.71±0.39	nd	nd	0.01±0.01	0.01±0.01
IL-2	3.77± 2.45	2.01±1.01	nd	nd	nd	nd
IL-4	0.99± 0.64	0.55±0.26	nd	nd	nd	nd
IL-6	4.54± 1.55	3.95±0.92	0.78±0.09	0.58±0.29	0.10±0.10	0.31±0.25
IL-7	0.12± 0.08	0.06±0.05	nd	nd	0.04±0.04	0.03±2.70
IL-8	2.24± 0.61	0.92±0.22	3.14±1.55	2.21±0.84	1.27±1.11	0.46±0.30
IL-10	0.21± 0.13	0.12±0.06	nd	nd	nd	nd
IL-15	1.86± 1.09	1.16±0.61	nd	nd	nd	nd
IL-17a	0.15± 0.09	0.07±0.03	nd	nd	nd	nd
IFN-g	0.26± 0.18	0.12±0.06	nd	nd	nd	nd
LIF	5.38± 1.02	3.62±1.33	2.49±0.99	1.23±0.48	1.33±1.09	0.68±0.11
M-CSF	0.99± 0.63	0.28±0.27	nd	nd	0.02±0.02	0.02±0.02
bNGF	0.80± 0.47	0.54±0.27	nd	nd	nd	nd
VCAM-1	5.69± 2.89	3.13±1.64	3.19±1.61	0.82±0.51	2.04±2.02	1.04 ±0.54
MCP-1	3.78± 0.87	2.01±1.08	2.42±1.23	1.78±1.29	1.40±1.26	0.81±0.40
sICAM-1	22.30±13.30	1.21±6.08	5.15±2.64	1.93±0.98	6.13±6.13	nd

(b) The paracrine factor concentration (ng/10^6 cells) for HFSCs

Paracrine Factor	DMEM-KO+10% FBS		STK2+2%FBS		STK2	
	Passage 3	Passage 4	Passage 3	Passage 4	Passage 3	Passage 4
SCF	1.93± 0.26	3.02± 1.29	0.56±0.14	0.59±0.06	0.18±0.10	0.24± 0.14
GCSF	41.40±23.30	72.60±72.60	6.91±2.24	9.72±3.64	0.83±0.41	0.81± 0.41
GM-CSF	2.86± 1.76	4.08± 4.08	nd	0.10±0.05	0.03±2.90	0.05± 0.05
IL-2	8.08± 4.46	8.23± 8.23	nd	nd	0.0007±0.0007	0.02± 0.02
IL-4	2.24± 1.07	3.16± 2.77	nd	nd	0.02±0.02	5.10± 5.10
IL-6	80.90±29.90	110.00± 7.08	5.58±2.00	4.33 ±1.08	1.81±1.54	3.03± 2.76
IL-7	0.34± 0.10	0.49± 0.38	nd	nd	0.17±0.17	0.25± 0.25
IL-8	13.10± 5.48	19.30±10.80	21.8±9.25	33.54±0.14	4.66±3.29	13.30± 5.33
IL-10	0.49± 0.25	0.44± 0.38	nd	nd	0.003±0.003	0.01± 0.01
IL-15	3.46± 1.73	7.41± 7.41	nd	nd	0.126±0.126	0.17± 0.17
IL-17a	0.38 ± 0.22	0.36± 0.36	0.08±0.08.	0.01±0.01	nd	nd
IFN-g	0.57± 0.30	0.69± 0.69	nd	nd	0.004±0.004	0.01± 0.01
LIF	8.65± 2.46	7.85 ± 0.19	3.11±0.24	3.44 ±0.53	3.35±1.71	6.87± 2.17
M-CSF	3.92± 1.31	nd	0.84±0.84	0.75 ±0.35	0.27±0.27	0.46± 0.46
bNGF	2.37± 1.41	2.99 ± 2.99	nd	nd	nd	nd
VCAM-1	1.19± 8.46	28.50±28.50	0.45±0.19	0.10 ± 0.10	0.58 ± 0.43	53.20 ±52.90
MCP-1	113.00± 5.90	48.10±29.40	17.30±5.88	27.00±11.90	10.00± 6.63	30.60±16.00
sICAM-1	112.00±67.4	69.8±69.60	29.90±16.90	48.80±24.40	11.70±10.90	63.10±31.70

5.4 Determination of the most suitable conditioned media to stimulate hair growth *in vitro*

Following the paracrine profiling studies, *in vitro* hair growth stimulation for ICR mice skin was observed using SHED- and HFSC-CM to identify the most suitable CM combinations to stimulate hair growth. The histology slides scoring was carried out in two independent researchers by an oral pathologist and the researcher. The kappa score for the inter-reliability tests was 0.8. During scoring the number of hair follicles in early, mid and late anagen, catagen and telogen stages were counted based on the images obtained as represented in Figure 5.6, and the percentage of the hair follicles at each hair growth stage was tabulated to compare the transition of telogen hair follicles to the successive hair growth stages within three days of *in vitro* testing (Figure 5.7).

SHED	Passage 3		Passage 4	
	Day 1	Day 3	Day 1	Day 3
DMEM-KO +10% FBS				
DMEM-KO +10% FBS +bFGF				
STK2 +2% FBS				
STK2				

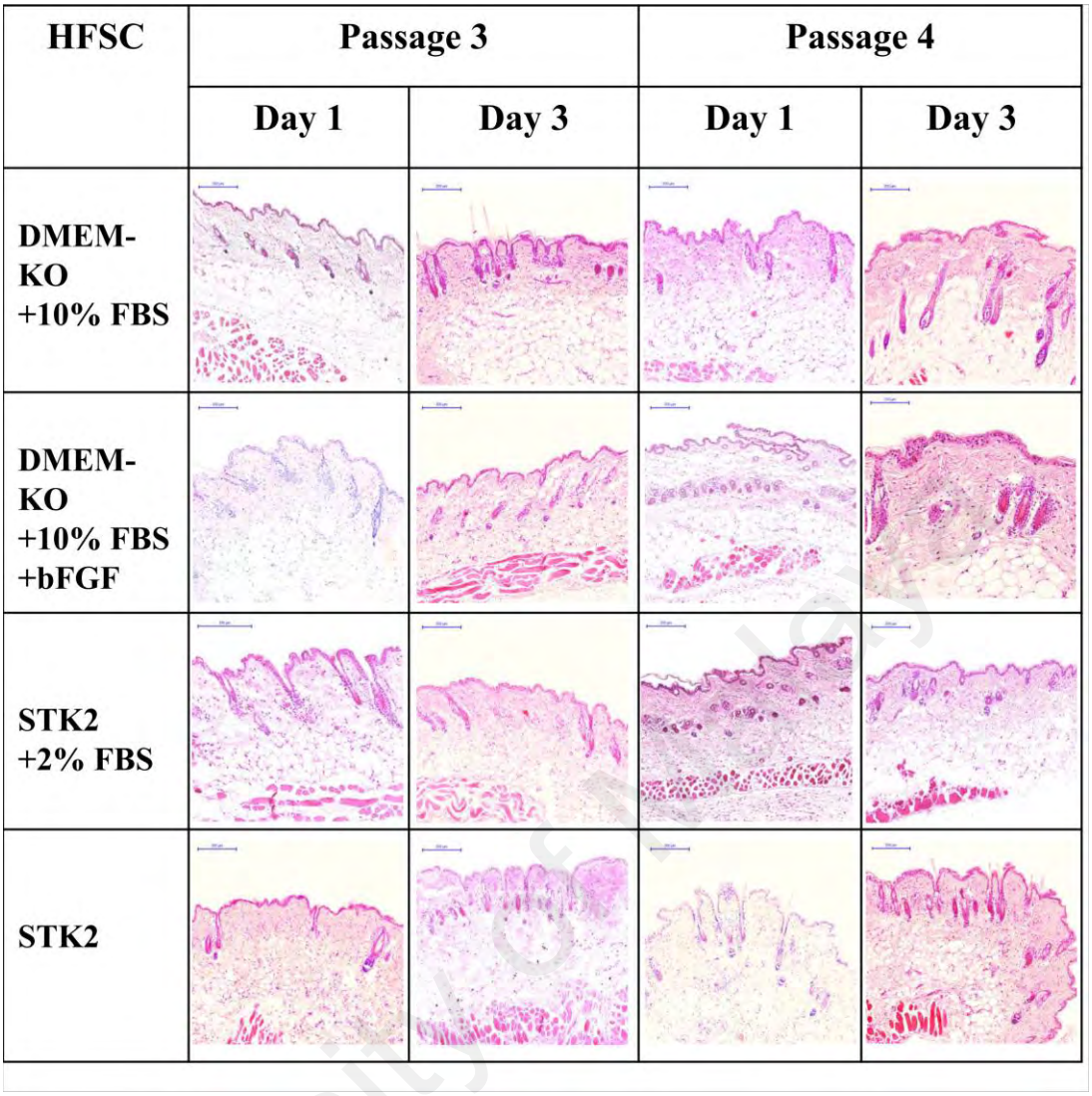


Figure 5.6: Representative H&E images of ICR mouse skin samples. The representative images of the ICR mouse skin samples treated with SHED-CM and HFSC-CM at 24 h and 72 h indicating the transition of telogen-staged hair follicles to the subsequent stages of hair growth following the CM treatment.

The *in vitro* study indicated the transition of hair follicles to subsequent hair growth stages following the synchronization of the hair follicles to telogen stage. Students paired t-test indicated that all the tissues treated with CM showed a significant increase ($p<0.05$) in the early anagen stage hair follicles by day 3 in comparison to day 1, except for the CM obtained from HFSCs and SHED cultured in STK2 media at passage 4 (Figure 5.7). The mid anagen hair follicle number significantly increased ($p<0.05$) in HFSC-CM collected in cells expanded in DMEM-KO+10% FBS at passage 4 and STK2+2%FBS at passage 3 and 4. The number of catagen hair follicles were

significantly increased ($p<0.05$) in HFSC-CM prepared by the expansion of cells in STK2+2% FBS at passage 3, SHED-CM prepared by expansion of cells in DMEM-KO+10% FBS+ bFGF in passage 3, DMEM-KO+10% FBS passage 4, STK2+2% FBS in passage 4 making the media not suitable for the preparation for CM to stimulate hair growth. The number of hair follicles in telogen stage was significantly increased ($p<0.05$) by day 3 in SHED-CM and HFSC-CM collected in STK2+2% FBS at passage 3 and DMEM-KO+10% FBS in passage 4. This was also observed in SHED-CM collected by expansion of cells in STK2 and STK2+2% FBS in passage 4. Thus due to the undesirable increase in the hair follicle numbers in telogen stage, we eliminated these media for further *in vivo* studies.

However, a significant decrease ($p<0.05$) of the catagen hair follicle number was observed in SHED-CM in STK2 media at passage 3 and DMEM-KO+10% FBS+bFGF in passage 4. There was no significant increase of the number of hair follicles by day 3 in the control media. DMEM-KO+10% FBS, STK2, STK2+2%FBS media showed a significant increase ($p<0.05$) in the number of mid anagen-staged hair follicles. An increase in the catagen stage hair follicle number was observed in DMEM-KO+10% FBS+bFGF. This media also showed a decrease in the number of hair follicles in the telogen stage. STK2+2% FBS also showed a significant decrease in the number of telogen hair follicles in day 3.

Specimens treated with STK2 media at passage 3 indicated a significant increase ($p<0.05$) in anagen stage hair follicles and a significant decrease ($p<0.05$) in the catagen and telogen stage hair follicles with respect to SHED-CM, while the same media at passage 3 showed a significant increase ($p<0.05$) in the anagen stage hair follicles and a significant decrease ($p<0.05$) in the telogen stage hair follicles by day 3 for HFSC-CM.

Based on the *in vitro* study results the STK2 media at passage 3 was chosen for subsequent *in vivo* studies.

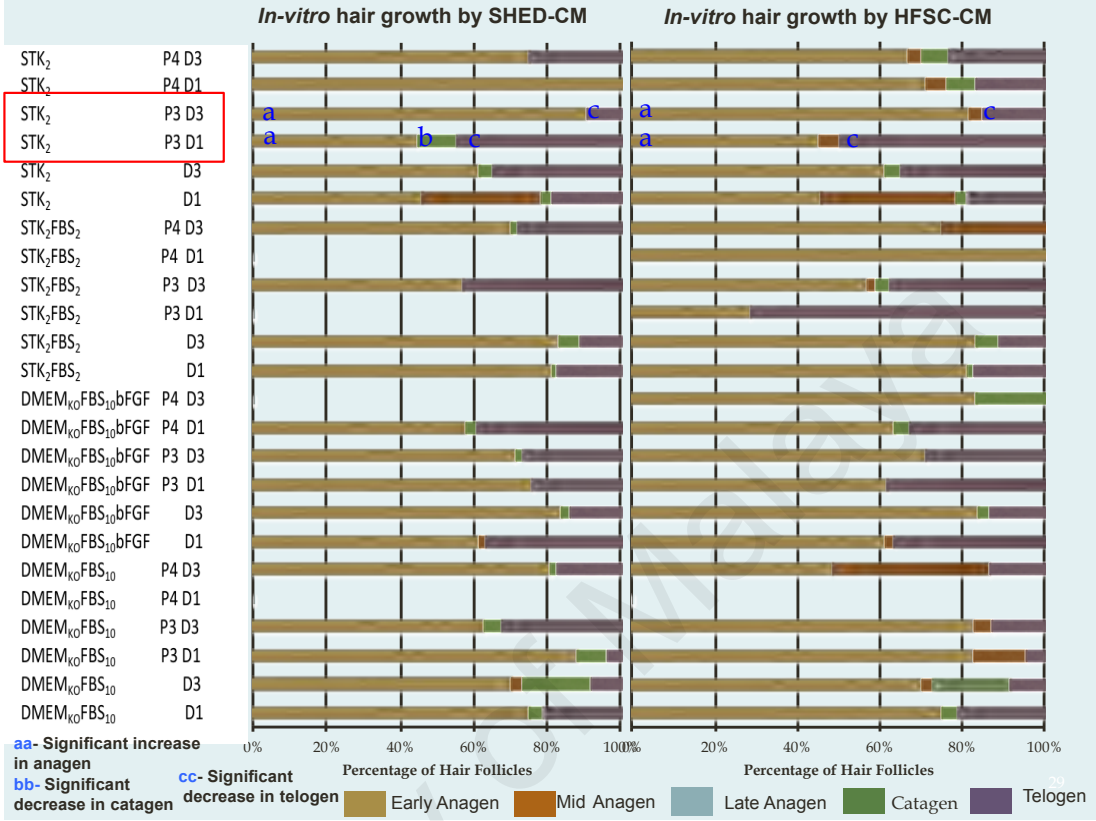


Figure 5.7: Percentage of hair follicles at each hair growth stage on day 1 and day 3, post CM treatment under *in vitro* conditions. The percentage of hair follicles at each hair growth stage at day 1 and 3 post CM treatment indicating the transition of telogen-staged hair follicles to the subsequent stages of hair growth following the CM treatment. *The same alphabet indicates a significant difference ($p<0.05$).D=Day P= Passage

5.5 Determination of the potential of conditioned media to stimulate hair growth under *in vivo* conditions

The *in vivo* study was conducted by injecting 3 sub-cutaneous injections of 100 µl of CM to telogen synchronized C3H/HeN mice at three-day intervals. Pictorial recording was made for each mouse at alternate days for the observation of the appearance of dark patches and until almost complete hair coverage of the skin was observed (Figure 5.8). The visual observance for the appearance of dark patches indicating the transition of

from telogen to anagen stage by SHED-CM ranged from 8 to 12 days while HFSC-CM ranged from 12 to 15 days (Figure 5.9) post-administration of CM. This demonstrates that in SHED-CM, the transition of telogen stage to anagen stage has occurred comparatively faster. However, the almost complete hair growth of the shaved skin area in SHED-CM ranges between 31 to 51 days, while HFSC-CM treatment has taken only 26 to 44 days.

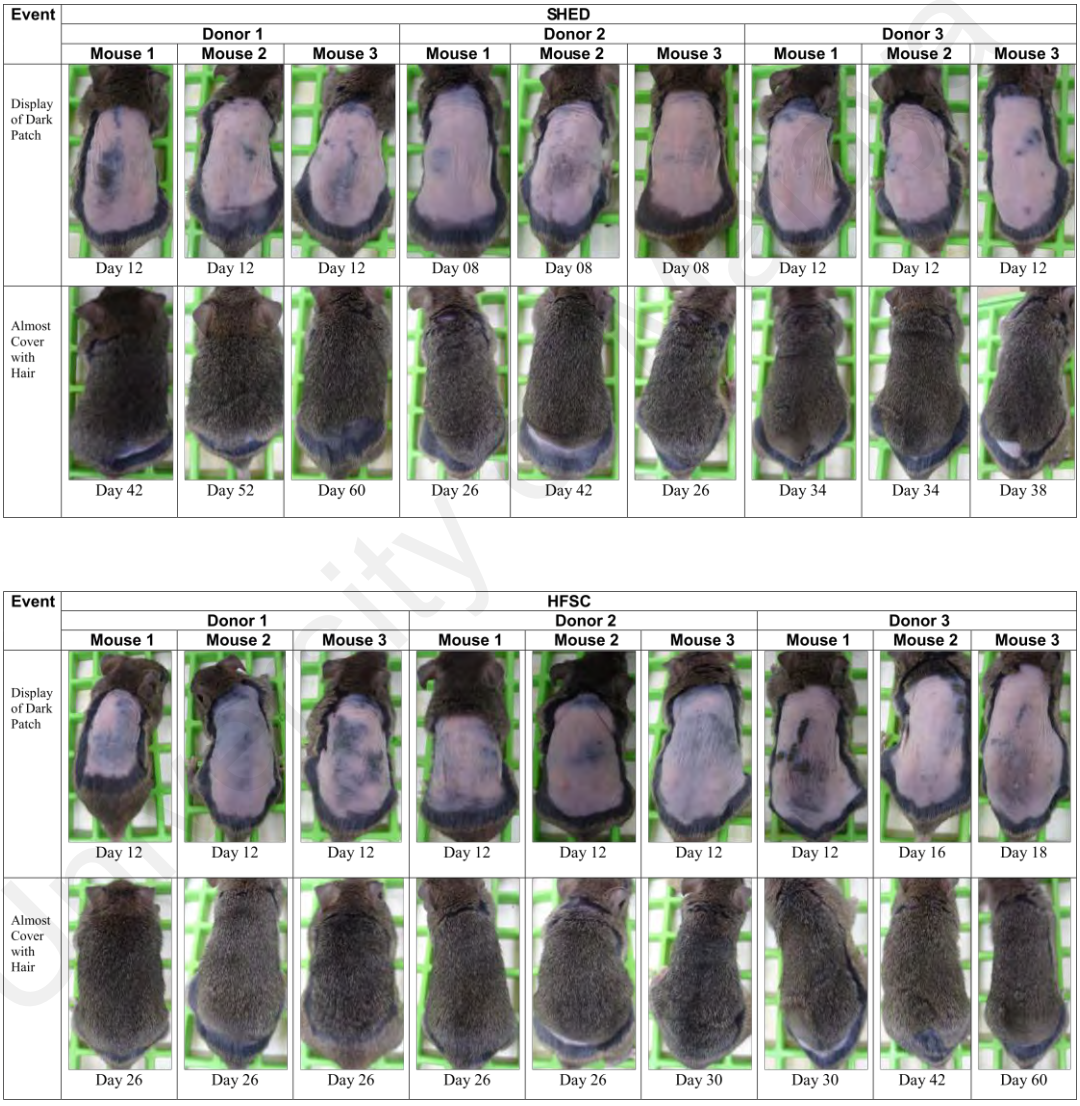
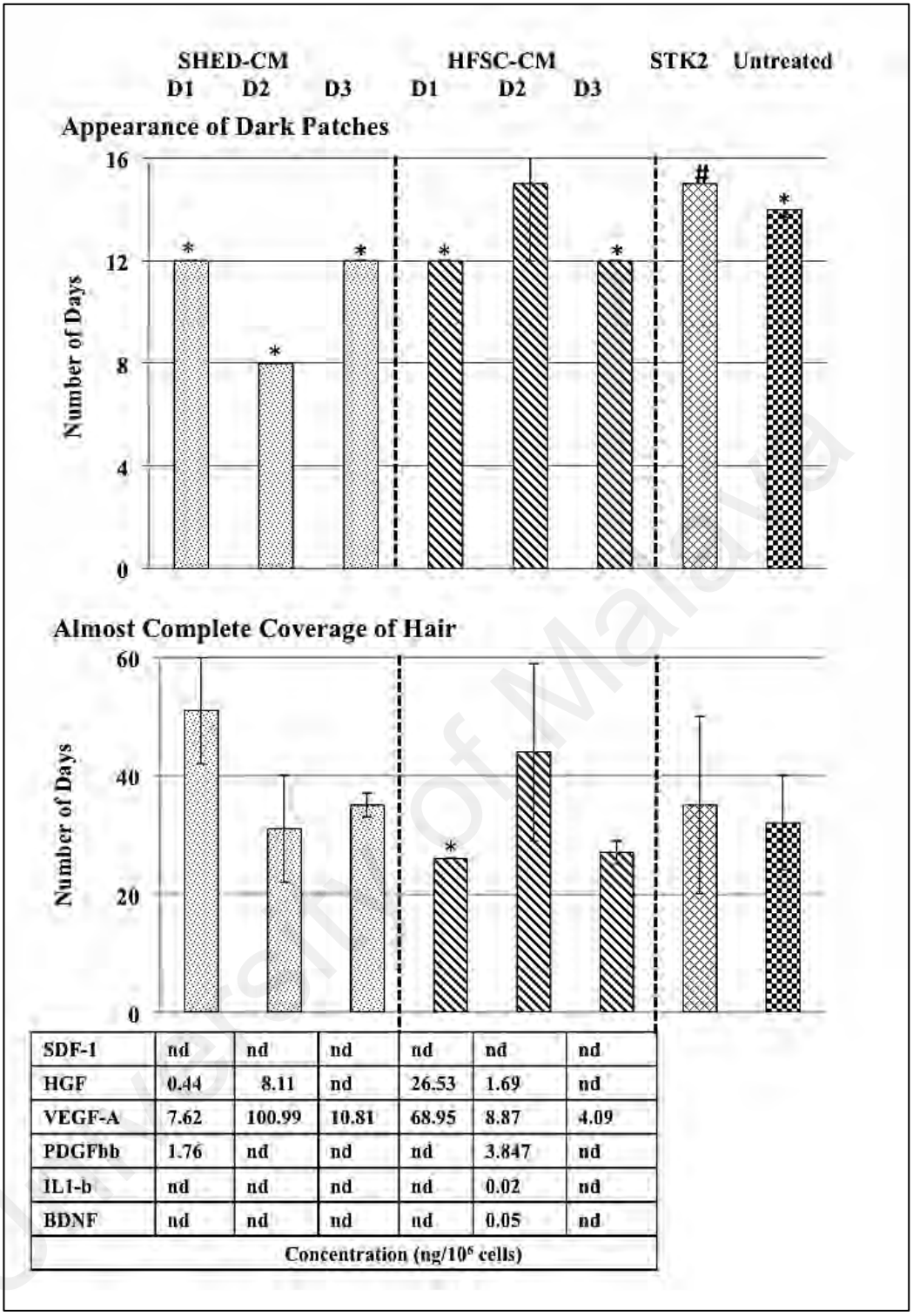


Figure 5.8: Pictorial representation for the appearance of dark patches and almost complete coverage with newly grown hair. The images of the telogen synchronized 7 week old female C3H/HeN mice following the subcutaneous injection of 100µl of SHED-CM (n=9) and HFSC-CM (n=9) administered at three day intervals for three days, for the observation of dark patches and almost complete coverage with newly grown hair.

The mouse treated with STK2 showed the appearance of a single dark patch on day 15. This is longer than the average number of days taken for the appearance of dark patches, for SHED-CM and HFSC-CM treatments. For the untreated mouse, dark patches were observed on day 14, higher than the mean number of days taken for SHED-CM and HFSC-CM. The paracrine profiling carried out for the individual donors demonstrate that the CM which contained a higher VEGF–A and HGF level have shown a quicker transition of the telogen stage to anagen stage (SHED-CM- Donor 2, HFSC-CM- Donor 1).



SDF-1	nd	nd	nd	nd	nd	nd
HGF	0.44	8.11	nd	26.53	1.69	nd
VEGF-A	7.62	100.99	10.81	68.95	8.87	4.09
PDGFbb	1.76	nd	nd	nd	3.847	nd
IL1-b	nd	nd	nd	nd	0.02	nd
BDNF	nd	nd	nd	nd	0.05	nd
Concentration (ng/10^6 cells)						

Figure 5.9: Number of days taken for the appearance of dark patches and almost complete hair coverage with the corresponding paracrine factor profiling of the CM prepared in STK2-serum free media. The number of days taken for the appearance of dark patches and almost complete hair coverage in C3H/HeN mice when treated with three subcutaneous injections of 100µl of SHED-CM (n=3), HFSC-CM (n=3) and STK2 (n=3) at three day intervals and the untreated C3H/HeN mice (n=2). The corresponding cytokine profiling for the donor from which the CM was prepared in also indicated. * Standard error = 0, # Standard error =1 and D =Donor

In order to compare the effect of the stem cell source in hair growth stimulation, statistical analysis was conducted using students paired t-test, combining the results for the SHED-CM and HFSC-CM (n=9 per stem cell source). The appearance of dark patches was significantly faster ($p<0.05$) in SHED-CM in comparison to the HFSC-CM (Figure 5.10 a). The almost complete hair coverage was observed earlier when treated by HFSC-CM (Figure 5.10 b). However, there was no significant difference between SHED-CM and HFSC-CM with respect to the observation made related to the effect of the two sources of CM in reaching an almost complete hair coverage.

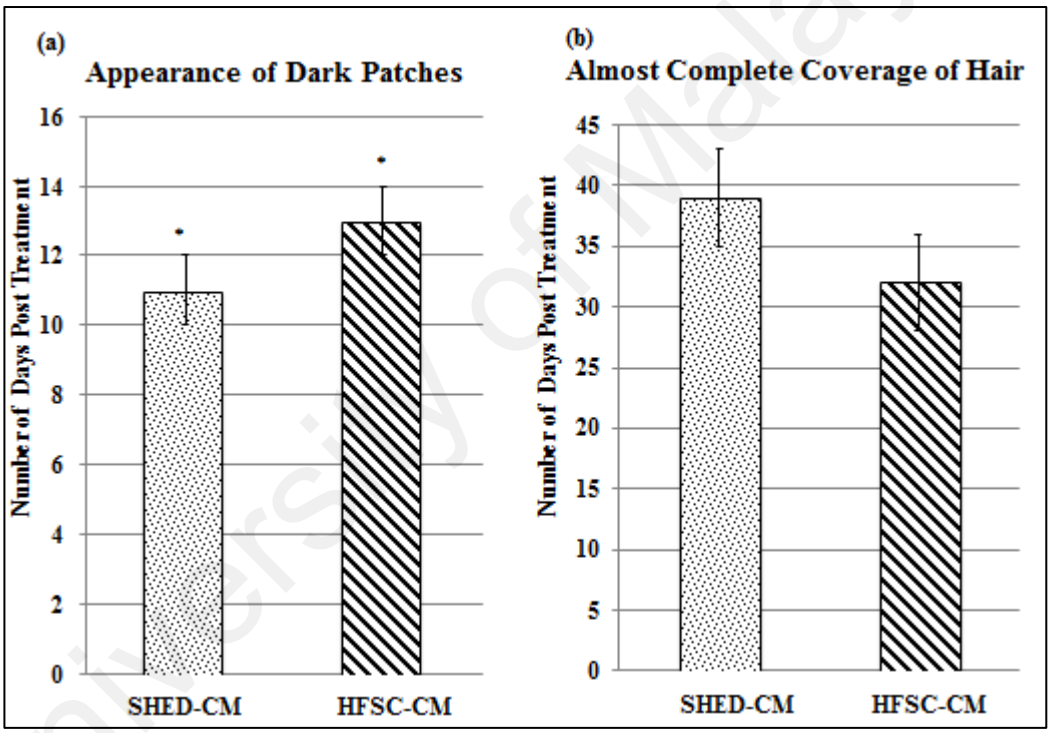


Figure 5.10: Time duration taken for the appearance of dark patches and almost complete coverage of hair upon treatment with SHED-CM and HFSC-CM. (a) The number of days taken for the appearance of dark patches in C3H/HeN mice (n=9) when treated with three subcutaneous injections of 100 µl of SHED-CM and HFSC-CM at three day intervals (b) The number of days taken for almost complete hair coverage of the C3H/HeN mice (n=9) when treated with three subcutaneous injections of 100µl of SHED-CM and HFSC-CM at three day intervals. * indicates a significant difference ($p<0.05$)

The percentage of hair growth observed between day 7-14 (Figure 5.11) also indicated that the stimulation of hair growth is faster in SHED-CM in comparison to the HFSC-CM. The HFSC-CM and the control groups (STK2 and untreated samples) only showed hair growth stimulation after day 12. It was observed that following 2 weeks of the CM administration, the HFSC-CM indicated the highest hair growth stimulation followed by SHED-CM, STK2 control media and untreated mice. However, 3 weeks post-treatment, hair growth in the untreated mice increased than the SHED-CM treated mice. The activity of HFSC-CM indicted to be declining following the 3rd week in comparison to the SHED-CM. The STK2 media shows a higher percentage of hair growth stimulation than the untreated mice until week 3. However, at week 4 post–treatment the untreated mice groups showed the highest percentage of hair growth.

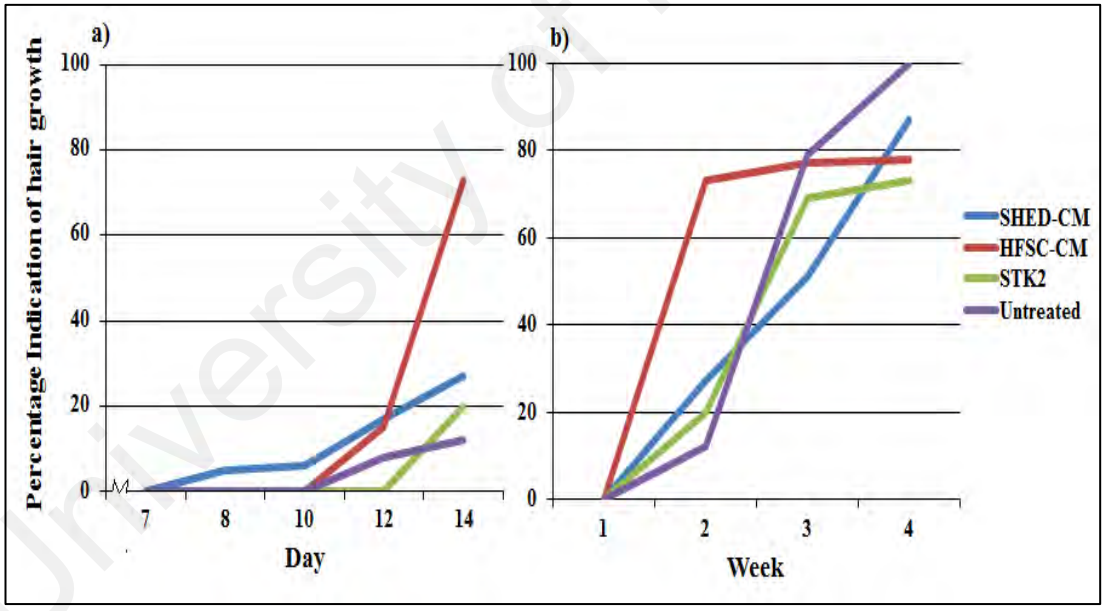


Figure 5.11: Percentage indication of hair growth. (a) The percentage of hair growth from Day 7- Day 14, following three subcutaneous injections of 100 µl of SHED-CM (n=9), HFSC-CM (n=9), STK2 (n=3) at three-day intervals to the C3H/HeN mice and the percentage indication of hair growth for the untreated C3H/HeN mice (n=2) (b)Weekly progress of the percentage of hair growth following three subcutaneous injections of 100 µl of SHED-CM (n=9), HFSC-CM (n=9), STK2 (n=3) at three-day intervals to the C3H/HeN mice and the percentage of hair growth for the untreated C3H/HeN mice (n=2)

CHAPTER 6: CONDITIONED MEDIA FOR STIMULATION OF HAIR GROWTH- DISCUSSION

The two hallmark properties of stem cells; to self-renew and differentiate to mature cell lineages have made them attractive sources in tissue regeneration. Following the isolation of stem cells, they need to be expanded under *in vitro* culture conditions to generate numbers that are therapeutically sufficient. It is necessary for the safety and efficacy of these cells to be maintained during the process of expansion, to cater for their high demand in clinical applications. In the human body, stem cells are provided with the necessary growth factor and environment to maintain their stemness (Shen et al., 2008). However, during *in vitro* culturing stem cells often lose their characteristics by entering cell senescence phase (Bonab et al., 2006). Thus it is necessary to retain the stemness and regenerative capacity of these cells by providing optimal the culture conditions.

The selection of basal media for culturing cells plays an important role in the maintenance of the therapeutic properties of MSC (Kanafi et al., 2013). BMSCs and DPSCs have been previously cultured in different media formulations such as DMEM-KO, DMEM-Low glucose, DMEM-F12 and α-MEM. However, Pal et al. (2009) has shown that the BMSC are better isolated and cultured in DMEM-KO and DMEM/F12 media (Pal, Hanwate, Jan, & Totey, 2009) while Govindasamy et al. has shown that DPSCs are better supported by α-MEM and DMEM-KO (Govindasamy et al., 2010). DMEM-KO has also been used in culturing HFSCs (Meyer- Blazejewska et al., 2011; Yu et al., 2006). Taken these studies to consideration it shows that the proliferation and maintenance of the therapeutic potential of MSCs from different sources are best supported by different media formulation. Thus during this current study, we have used different media combinations, to identify the media that would be the most suitable to

expand SHED and HFSCs. We have used DMEM-KO supplemented with 10% FBS as an expansion media. However, since Xeno free media has always been considered as an alternative to FBS supplemented media for cell-based therapy, serum free STK2 media was also chosen for the expansion of cells. STK2 media comprises of a combination of growth factors FGF, PDGF, HGF, TGF-β and EGF (Yasufumi et al., 2016). This media has been used to culture DPSCs, BMSCs, ADSCs and Synovial MSCs (Fukuta et al., 2014: Kato & Shao, 2009; Takeda-Kawaguchi et al., 2014). However, to the best of our knowledge no attempt has been reported to culture HFSCs in this media. Thus, the cells were also cultured in STK2 with minimal serum supplementation (2% FBS), in order to provide better attachment for cell growth.

The cells cultured in the different media combinations were assessed for their cell density, morphology, PDT and viability. The cell density observed in STK2 based media is comparatively higher than the DMEM-KO based. The cells showed fibroblast like, spindled-shaped morphology as earlier described by Gronthos et al. for SHED (Miura et al., 2003) and as described by Jahoda et al. for the HFSCs (Jahoda et al., 2003). However, for both SHED and HFSCs cultured in DMEM-KO based media, the cells changed to a more flattened shape as confluency increased. This may be an indication of aging of stem cells during culture (Wagner et al., 2008). Whilst, the cells cultured in the STK2 based media were short and rounded in shape signifying that the cells to be in a more actively dividing stage displaying a better homogeneity in comparison to the cells cultured in DMEM-KO based media.

The lowest PDT and highest viability for SHED was observed when cultured in STK2+2% FBS at passage 4, while for HFSCs, in passages 3 and 4. STK2 media in passage 4 showed the highest viability for HFSCs. The PDT of SHED was significantly lower ($p<0.05$) for STK2 +2% FBS at passage 2 in comparison to the DMEM-KO

based media and in STK2 based media at passage 5, in comparison to the DMEM-KO based media. HFSCs cultured in STK2 based media shows a significantly lower ($p<0.05$) PDT than in DMEM-KO+10% FBS+bFGF in passage 2 while in passage 3 the STK2 based media shows a significantly lower ($p<0.05$) PDT than the DMEM-KO based media. HFSCs at passages 4 and 5, when cultured in STK2+2% FBS media shows a significantly lower ($p<0.05$) PDT in comparison to the DMEM-KO based cultures.

Even though DMEM-KO is supplemented with FBS to increase the cell attachment and supply the necessary nutrients to the cells, it also contains cell growth inhibitors. The addition of high concentration of FBS (10% or more) could affect cell proliferation capacity negatively (Tekkatte et al. 2011). In the STK2 media supplemented with 2% FBS which is considered a low serum culture condition, the negative effects of serum may be minimized. The cells would be enriched with more growth factors in addition to the STK2 media alone that was reflected in the decrease in PDT for both SHED and HFSCs. The high proliferation rate of cells cultured in STK2 media is likely to be due to the formulation of STK2 which consist of growth factors, lipids, nutrients, fatty acids and phospholipids (Ishikawa et al. 2009). Thus, STK2 based media has the added advantage as it comprises only of cell growth-promoting factors.

The addition of bFGF to DMEM-KO is expected to promote the proliferation and in turn foster the maintenance of stemness of the cells (Coutu & Galipeau, 2011; Sotiropoulou et al. 2006). Although the addition of bFGF showed a decrease in the PDT for both SHED and HFSCs, the PDT were not significantly lower than STK2 based media. This observation may be due to the low stability of bFGF in the media. It has been reported that following the addition of bFGF, 50% of its initial concentration declines within a period of 4 h (Lotz et al., 2013). Furthermore, the 3-day cycle for

media change could also be a contributing factor. It is also reported that the activity of bFGF is better exhibited at a seeding density of 1000 cells/cm^2, in comparison to 5000 cells/cm^2 (Tsutsumi et al., 2001). However in this study the choice of seeding density was made based on the requirement to maintain a consistent protocol throughout the study as HFSCs is generally cultured at 5000 cells/cm^2. Although one would have thought of not including this media combination, in the following hair growth stimulation *in vitro* study, we wanted to ascertain if bFGF could affect hair growth stimulation since it is a negative hair regulatory factor. The findings will be further elaborated during the discussion of the *in vitro* study.

The PDT between the two stem cell sources in the given culture conditions was not significantly different. However, SHED showed a better proliferative capacity in comparison to the HFSCs. This may be also due to the donors' age; HFSCs was obtained from donors between 38-52 years old as claimed by the supplier. Although the supplier did not disclosed the age of the donors for SHED, we could assumed that SHED was from young donors since they were derived from deciduous teeth and the last deciduous tooth usually exfoliate at the age of 12. Interestingly, in lower passages (P3 to P5), the difference in the PDT between SHED and HFSCs is not highly variant when cultured in STK2 based media. This may suggest that supplying suitable media conditions could help overcome the differences in the proliferation capacity of cells originated from different sources and the impact caused by the age of donors. This hypothesis is further substantiated by Ding et al. (2013), where they reported that the donors' age (30-60 years old) does not have an effect on the growth kinetics of ADSCs cultured in low calcium and low serum supplemented with hormone and antioxidant supplements keratinocyte serum free medium (Ding et al., 2013). Thus considering the morphology and growth kinetics of cells, STK2 based media could be the media of choice for SHED and HFSCs compared to DMEM-KO based media.

In addition to the increase in cell number, the culture conditions should also be able to maintain the homogeneity of cells without inducing spontaneous differentiation. The media should support the maintenance of stemness for cells to differentiate into the required lineages. Thus, we conducted flow cytometry analysis and the trilineage differentiation studies to determine the maintenance of the MSC characteristic, as per the guidelines stated by the International Society for Cellular Therapy (Horwitz et al., 2005).

The flow cytometry analysis was conducted in order to determine the expression of the positive markers for MSC, CD 90, CD 105 and CD 73 and the negative markers CD 45, CD 34, CD 14 or CD 11b, CD 79α, CD 19 and HLA-DR, known markers for hematopoietic and endothelial cells. The positive and the negative markers expressed by SHED indicated that their MSC status was maintained in all media combinations, with the exception of STK2+2% FBS where the negative marker expression was high (13%). Such observations have also been reported by Yu et al. 2010, in ADSCs reporting that the negative marker level expressions ranged from 15.5%-12% for CD44 and CD45 markers respectively (Yu et al., 2010).

Expression level for the positive markers in HFSCs was between 94-100% except for the DMEM-KO+10% FBS and DMEM-KO+10% FBS+bFGF that shows a value of 69% and 73% for the CD 90 expression respectively. In HFSCs, the negative marker expression level is between 0-3% in all media combinations, except for DMEM-KO+10% FBS+bFGF which shows an expression level of 25%. These results may suggest that the maintenance of HFSCs for its stemness is poor in the DMEM-KO based media..

Trilineage differentiation studies confirmed that both SHED and HFSCs cultured in all media combinations retained their differentiation potential towards all three lineages

(Figure 5.5). The accumulation of the lipid vacuoles in the cytoplasm, stained by Oil Red O verified that both SHED and HFSCs committed to the adipogenic lineage. The osteogenic lineage commitment was shown to be more intense in the HFSCs cultured in DMEM-KO+10% FBS, STK2+2% FBS and STK2 media in comparison to SHED or HFSCs cultured in DMEM-KO+10% FBS+bFGF. From the flow cytometry and trilineage differentiation studies it can be observed that the MSC properties of the cells were maintained in all media combinations, even though the degree of the maintenance of these properties were varied among the different media and source. The maintenance of the stem cell properties is an indication that the cells function better due to which the possibility of these cells secreting inflammatory paracrine factors is lesser, which in turn would confer the negative cellular effects in the CM prepared.

The paracrine secretion profiles of SHED and HFSCs were studied following the conformation of the maintenance of their stem cell characteristics (Table 5.2). This would help in identifying the most suitable media for SHED and HFSCs for the secretion of hair regulatory growth factors. The development of the hair follicles commences during the first trimester of pregnancy (Yoo et al., 2010). The development of hair follicles undergo cyclic events and is divided in to four main phases; growth, resting, regression and shedding termed as anagen, catagen, telogen and exogen respectively (Cotsarelis & Millar 2001). The process of hair cycle is maintained by a series of cascade events controlled by paracrine factors thus, during the current study we have profiled the main positive hair regulatory factors SDF-1, HGF, VEGF-A and PDGF-BB and the main negative hair regulatory growth factors IL-1β, TGF-β, bFGF, TNF-α and BDNF. In addition to the hair regulatory paracrine factors, we have also profiled the expression of the paracrine factors LIF, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-15, IL-17a, SCF, G-CSF, M-CSF, GM-CSF, IFN-g, MCP-1, bNGF, GRO-α, sICAM-1 and VCAM-1 by SHED and HFSCs when cultured in the different

media combinations as they are involved in other cellular processes which could indirectly affect hair stimulation (Table 5.3).

The secretion of the growth factor, HGF was observed in all combinations of CM (Table 5.2). Murine vibrissal organ culture systems have shown that HGF is responsible in increasing the hair follicle length (Jindo et al., 1994) while in *in vitro* cultured human hair bulb derived keratinocytes HGF shown to stimulate the DNA synthesis to enhance the hair growth (Shimaoka et al., 1995). Upon administering HGF to new born mice in the second anagen stage of the hair cycle, it has been indicated that the transition of anagen stage to the catagen stage was delayed (Lindner et al., 2000; Tsuboi, 1997). HGF is also shown to be responsible in the formation of blood vessels around the hair follicles (Fushimi et al., 2011; Kikkawa et al., 2009; Xu et al., 2011) and in the proliferation, differentiation and apoptosis of the keratinocyte cells (Kikkawa et al., 2009). It has been reported that the HGF also facilitates the protein β-catenin to enter the nucleus of the stem cells (Qi et al., 2016) which in turn would differentiate in to follicular keratinocytes (Huelsken et al., 2001). Administration of ADSC-CM to individuals has shown that HGF is responsible in the increased hair numbers that were observed during the study (Fukuoka & Suga, 2015). Similarly in the current study secretion of HGF by SHED and HFSCs was facilitated in all media combinations. However, HGF was highly secreted in HFSCs when cultured in STK2+2% FBS and STK2 media at passage 4 in comparison to all media combinations, and in comparison to SHED.

VEGF leads to the increase in hair follicular size by increasing the perifollicular angiogenesis (Yano, Brown, & Detmar, 2001). Alteration of vasculature is observed in several hair growth disorders. However, it was observed that VEGF was highly secreted by both sources in comparison to all other paracrine factors. However, VEGF-A was

highly secreted in HFSC cultured in DMEM-KO+10% FBS at passages 3 and 4, followed by SHED cultured STK2 at passage 3.

IL-1, is known to be involved in the pathogenesis of alopecia areata through the recruitment of inflammatory cells including T cells (Dudda-Subramanya, Alexis, Siu, & Sinha, 2007), Also, IL-1α, IL-1β (Tarlow et al., 1994) and TNF-α are identified as potential paracrine factors in deteriorating the hair growth under *in vitro* conditions (Philpott, Sanders, Bowen, & Kealey, 1996). TGF-β is a growth factor that is responsible in initiating the catagen phase of hair growth (Oshimori & Fuchs, 2012). During the profiling studies carried out it was observed that the DMEM-KO based media seem to promote the secretion of the negative hair regulatory factors, TGF-β and IL-1β, bFGF, TNFα and BDNF while STK2 based media only influenced in the secretion of the negative hair regulatory factor BDNF.

Studies conducted using sheep and mouse models have reported that bFGF has a negative effect on hair growth by delaying the follicular development and retarding the hair growth (du Cros, 1993b; Philpott, Green, & Kelly, 1990). However, during the current profiling studies conducted the bFGF secretion was in undetectable amounts. Apart from the possibility of the low secretion levels of bFGF it is also possible that the non-detection may be due to its low stability (Lin et al., 2015) owing to the short half-life of 7.6 h (Beenken & Mohammadi, 2009).

Although our observations showed that a higher amount of paracrine factors are been secreted by HFSCs compared to SHED in terms of per million number of cells, the number of cells obtained from SHED (STK2- $9.6x10^6$, STK2+2% FBS- $5.7x10^6$, DMEM-KO+10%FBS-$4.2x10^6$) is higher than HFSCs (STK2- $9.1x10^6$, STK2+2% FBS- $6.1x10^6$, DMEM-KO+10% FBS- $1.9x10^6$). Therefore, we postulate that SHED would secrete equal or even higher amounts of paracrine factors in the prepared CM.

This could mean that CM prepared from SHED may potentially be as good as HFSC in the stimulation of hair growth.

Considering the results obtained from the population doubling, viability, flow cytometry and trilineage differentiation studies it was evident that the STK2 based media was more suitable for the expansion of cells. This is further the media of choice to prepare the CM, since the paracrine factor profiling studies also indicated that STK2 based media do not promote the secretion of the negative hair regulatory paracrine factors in comparison to DMEM-KO based media.

The transition of the hair follicle to subsequent stages of the hair growth cycle depends on a network of activators, inhibitors, morphogens and the presence of growth factors (Murray et al.,2012). An imbalance of the transition between the hair follicle stages is regarded as a main underlying cause for hair loss. A short anagen stage followed by a synchronized entry of the hair follicles to the telogen stage is mainly observed in all forms of hair related diseases. Thus, in order to evaluate the potential of the CM to stimulate the hair growth, the capability of the CM to stimulate the hair follicles in telogen stage to enter the anagen stage followed by the maintenance of the anagen stage, an *in vitro* study was conducted. Muller Rover (2001) reported that the anagen stage transition begins following 24 h of telogen stage synchronization (Muller-Rover et al., 2001). This was also observed in the *in vitro* study, where most of the hair follicles in the telogen stage entered the anagen stage within the 24 h period. However, no significant difference in the number of hair follicles entering the anagen stage was observed for tissues immersed in culture media that was not conditioned by the stem cells even after an incubation period of 72 h.

An early onset of the catagen stage was observed in the unconditioned STK2 and DMEM-KO media, SHED-CM prepared in STK2 at passage 3, STK2+2% FBS at

passage 4 and DMEM-KO+10% FBS at passages 3 and 4, and HFSC-CM prepared in STK2 at passage 4 and STK2+2% FBS at passage 3. The early appearance of the catagen stage observed under the *in vitro* conditions may be due to the absence of insulin in the CM that had been collected in the absence of FBS. Insulin is responsible in preventing the entry of hair follicles from anagen to catagen stage under normal physiological conditions (Philpott, Sanders, & Kealey, 1994). It is also seen that with respect to STK2 based media, the hair follicles entered the catagen more rapidly i.e. by day 3 for tissues supplemented with SHED-CM from passage 4 compared to passage 3. The reverse was observed for HFSCs cultured in STK2 media. This may be due to the lower amount of positive hair regulatory factors secreted during the culturing of SHED and HFSCs in passages 3 and 4 respectively. Positive hair regulatory factors are required to support the maintenance of hair follicles in the anagen stage.

In SHED, STK2 media in passage 3 was the only STK2 based media that secreted no negative hair regulatory factors and the least amount of negative hair regulatory factor BDNF. The *in vitro* studies confirmed that STK2 media in passage 3 as most suitable in the stimulation of hair growth. This media significantly ($p<0.05$) increased the number of hair follicles in the anagen stage and significantly decreased ($p<0.05$) the number of hair follicles in the catagen and telogen stages.

One of the major concerns while conducting the *in vitro* study is that CM was exposed to 37 ± 1°C for a period of 72 hours, during which the stability of the growth factors could have reduced, resulting in their lower mitogenic activity. The fast early catagen stage onset may also be due to environmental stress experienced by the hair follicles under *in vitro* conditions (Philpott, Green, & Kealey, 1992). Examination of the entire hair follicle was also a major challenge faced during the histological analysis. This difficulty could be due to the presence of hair follicles in random directions instead

of them being aligned in a longitudinal direction and the shrinkage of the dermal tissues under *in vitro* conditions that has been reported (Hashimoto et al., 2000).

Since the *in vitro* study only accounts for a partial sequence of events that occurred within a biological system a preliminary *in vivo* study was conducted using CM from STK2 when SHED and HFSCs were cultured at passage 3. Muller-Rover (2001), Park (2010) and Won (2010) have reported that the transition of telogen-staged hair follicles to the anagen stage is depicted by the appearance of dark patches (Muller-Rover et al., 2001; Park et al., 2008; Won et al., 2010). The *in vivo* study using SHED-CM demonstrates the appearance of dark patches within 11±2 days post-treatment while the HFSC-CM demonstrated the appearance of dark patches with 13±2 days. We hypothesize that this observation could be attributed to the change in the paracrine gradient created following the administration of CM. This resulted in an increased paracrine factor gradient in the mouse physiological system thus stimulating hair growth. It has been reported that CM stimulate the migration of the endogenous MSCs resulting in mitotic expansion and differentiation of the MSCs (Caplan & Bruder, 2001). Further, it may also induce cellular proliferation and increase vascularization, thus promoting an increased metabolic activity and oxygen supply (Ratajczak et al., 2012). Therefore, it is very likely that the increase in paracrine gradient caused the HFSCs in the niche to proliferate and differentiate, and increase vascularization in the treated area. This phenomenon may hold the same in case for the patients suffering from hair loss when treated with CM, as described in Figure 6.1.

Our results showed that the appearance of dark patches indicating the onset of the anagen stage was between day 11 to day 15, where the earliest was visualized in the group treated with SHED-CM, followed by HFSC-CM, untreated and STK2 group (Figure 5.9). Our results concurred with Muller Rover (2010) who showed that the

anagen to telogen stage of hair transition occurs within 25 days (Figure 2.3). However, the exact stage of hair cycle in a mouse model could only be determined histologically.

All mice groups showed almost complete hair coverage between days 31 to 39 in average. Although the mice in SHED-CM group showed the longest duration to reach almost complete hair coverage, the difference was not significant ($p>0.05$) to HFSC-CM. A stable paracrine factor gradient is required in order to maintain the aforementioned physiological activities (De Donatis, Ranaldi, & Cirri, 2010). It is important to note that the degradation of paracrine factors occurs in several distinct pathways such as proteolysis, oxidation and denaturation (Lee, Silva & Mooney, 2011) within the biological system. For example VEGF when administered intravenously shows a half-life of 30 min (Eppler et al., 2002). This results in the reduction of paracrine gradient with time (Shen et al., 2007) which in turn reduces the efficiency of the administered CM to exhibit biological activities (Peled et al., 1999). Thus, it is likely that the mice would have regained their normal physiological activities. Therefore, following two weeks post-administration of CM, no difference in the hair growth percentages was observed between the different groups of mice. Further studies should be conducted to develop suitable delivery systems that would maintain the stability of the pool of paracrine factors that are present in the CM (Lin et al., 2015). Furthermore the increase of the frequency of CM application, optimization of an efficient dosage and establishment of suitable delivery methods (Lee et al., 2011) should be explored to improve the long-term outcome.

The time taken for the stimulation of anagen stage by CM prepared by both sources were faster compared to the observation of Park et al (2010) when the same mice strain (C3H/HeN) were treated with ADSC-CM. They reported the appearance of dark patches only after 8 weeks post-treatment. It took 12 weeks for the hair to be almost re-

grown (Park et al. 2010). Studies conducted by Won et al. have reported that telogen matched C3H/HeN male mice treated with ADSCs intradermal injection and tropical application of ADSC-CM, both groups showed enhanced hair growth after 12 weeks. In the same study an increase number of hair follicles was reported by the 4th week of the application of ADSC-CM (Won et al., 2010). Although, there is a difference of almost 6 weeks between these two studies, our observation is within the normal time range for the telogen transition to anagen stage in the murine hair cycle reported by Muller-Rover (2001), Jiang (2005) and Lin (2015) (Muller-Rover et al., 2001; Jiang et al., 2005; Lin et al., 2015).

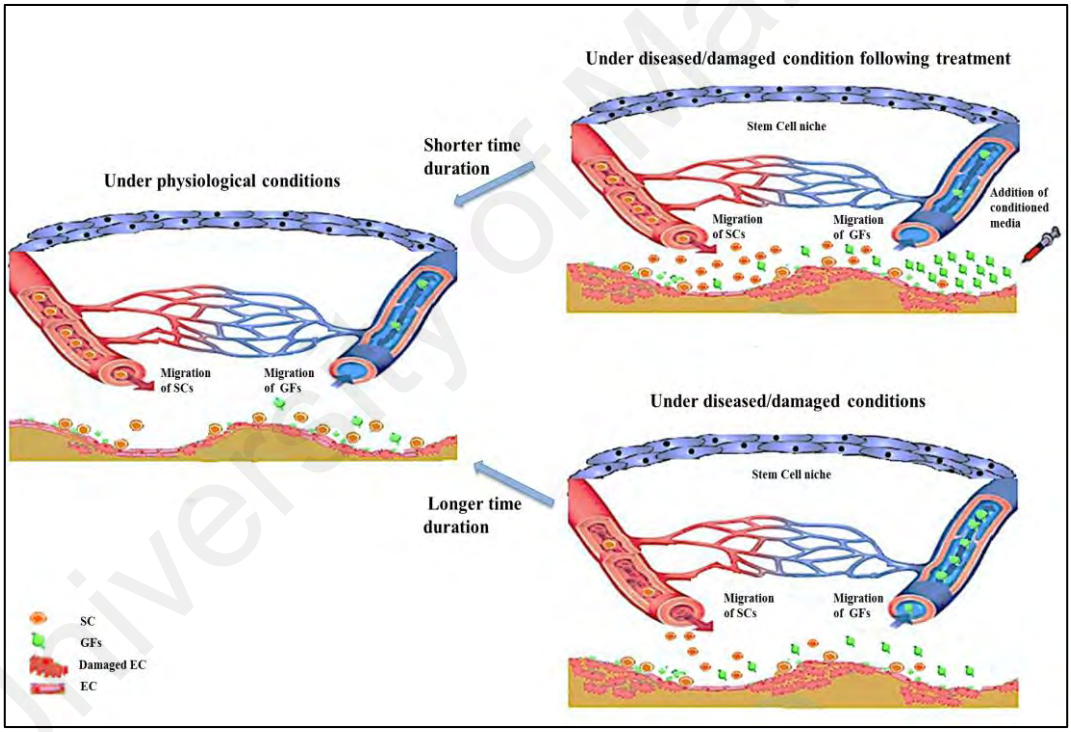


Figure 6.1: Potential role of stem cell-CM in the process of hair stimulation. The application of paracrine factor rich CM enhances the paracrine factor gradient within a specific area, thus increasing the migration of the stem cells towards the site of application. The migrated stem cells will differentiate to the organ specific cell type. These factors would also be responsible in activating and proliferating the cells at the site of application while inhibiting further apoptosis and depleting further inflammation of the area compared to the rate that occurring under normal physiological conditions.

In another study, Won et al (2015) administered keratinocyte stem cell (KSC)-CM through three subcutaneous injections to seven week old C3H/HeN female mice at a two day interval and the darkening of skin was observed after 10 days post-treatment (Won et al., 2015). The results of this study concurred with our observation when the same mice strain was treated with SHED-CM.

In addition to stem cell CM, chemical compounds have also been studied for its potential in stimulating hair growth. Ketoconazole shampoo improves hair growth in patients suffering from androgenetic alopecia by clearing the scalp from micro-flora such as the lipophilic microorganisms *Malassezia* spp. (Pierard, Pierard-Franchimont, Nikkels-Tassoudji, Nikkels, & Leger, 1996). In six week old male C3H/HeN mice model, it was reported that the dark patches was observed on day 21 post-treatment with tropical application of 2% ketoconazole (Jiang, Tsuboi, Kojima, & Ogawa, 2005). Quercetin is a natural compound that results in the down regulation of NF-κB (Nam, 2006), a factor that is responsible in enhancing the levels of TNF- α and IL-1 during the pathogenesis of alopecia areata (Dudda-Subramanya et al., 2007; Gregoriou et al., 2010). Daily subcutaneous injections of 100µl quercetin for eight days in alopecia areata mouse model (C3H/HeJ) stimulated hair regrowth within a period of 6 weeks (Wickramanayake et al., 2012). It is noteworthy that certain growth factors could affect the production of factors such as NF- κB. Thus, it would be interesting to explore the effect of SHED-CM in this regard. Early appearance of dark patches (14 days of post-treatment) has been observed when three different groups of C57BL/6 mice were injected with bFGF, FGF-5 and FGF-7 (Lin et al., 2015). Although the application of specific growth factors seem to be a possible approach for stimulation of hair growth the evidence is still lacking in the literature. Di Santo (2009) stated that a pool of paracrine factors is necessary to stimulate biological events efficiently rather than the application of specific growth factor (Di Santo et al., 2009).

The aforementioned *in vivo* studies postulated different mechanisms related to the stimulation of hair growth. It has been suggested that ADSC-CM stimulate hair growth through epithelial- mesenchymal interactions. Park et al. (2010) demonstrated this mechanism by supplementing co-cultures of epithelium derived human epidermal keratinocytes and mesenchymal derived hair follicle dermal papilla cells with ADSC-CM which resulted in a significant increase in the proliferation of the keratinocytes. It was also reported that the ADSC-CM activates the hair follicle dermal papilla by up-regulating the β-catenine signaling pathway (Park et al. 2010). Interestingly, passage 3 SHED-CM prepared using STK2, showed a similar paracrine profile to the Adipose Derived stem cell Protein Extract (AAPE™) developed by Prostemics Co. Ltd. (Korea) currently marketed for hair growth stimulation. The presence of similar paracrine factor combination to AAPE™, may suggest that the stimulation of the anagen stage onset by SHED-CM may also follow the similar sequence of cellular process of ADSC-CM. APPE comprises of both positive and negative hair regulatory factors, namely PDGF (44.41±2.56pg/ml), KGF (86.28±20.33 pg/ml), HGF (670±86.92pg/ml), VEGF (809±95.98pg/ml) and bFGF (131.35±30.31pg/ml) and TGF β1 (103.33±1.70pg/ml) respectively. It also contains collagen (921.47±46.65pg/ml), fibronectin (1466.48±460.21pg/ml), and superoxide dismutase on 5ml saline solution (Hirotaro Fukuoka, Suga, Narita, Watanabe, & Shintani, 2012). Whilst the paracrine profile of SHED-CM used in the *in vivo* study revealed the presence of PDGF (334 pg/ml), HGF (1,299 pg/ml), VEGF (11,963 pg/ml) while HFSC-CM showed a composition of PDGF (616 pg/ml), HGF (4,075 pg/ml), VEGF (8,391 pg/ml). However, bFGF and TGF-β1 were not detected in both SHED-CM and HFSC-CM. It is worthy to note that the CM prepared in the current *in vivo* study showed a higher positive hair regulatory composition and the negative hair regulatory factors were in an undetectable amount.

This, could be the reason why the induction of anagen stage by the SHED-CM and HFSC-CM were more efficient than ADSC-CM.

Won et al. (2015) postulated that KSC-CM stimulated hair growth through phosphorylation of AKT and ERK1/2 molecules. This mechanism results in an increased proliferation of the dermal papilla cells and outer root sheath of the hair follicle. They reported that paracrine factors involved were AREG, IGFBP2, IGFBP5, GM-CSF, PDGF and VEGF and the suggested minimum concentration required in accelerating hair growth is 1ng/ml (Won et al., 2015). The results obtained during the current study indicated that even though VEGF was within the concentration indicated, PDGF and GM-CSF was not within the suggested limits. However, the dark patches in our *in vivo* study appeared within the duration similar to the aforementioned study, suggesting that the SHED-CM may possess additional growth factors to KSC-CM that are required for the onset of anagen.

CM holds a promising approach in tissue regeneration. This treatment modality can also be used as a potential therapeutic option for alopecia. The results obtained from the current study indicated that SHED-CM appeared to be better in hair growth stimulation than HFSC-CM and the other CM sources that have been reported in the literature. Thus this preliminary *in vivo* study formed a strong foundation to necessitate the exploration of SHED-CM as a potential therapeutic tool for alopecia.

CHAPTER 7: CONDITIONED MEDIA FOR STIMULATION OF HAIR GROWTH- CONCLUSION

7.1 Conclusions

The expansion of SHED and HFSCs were carried out in media combinations of DMEM-KO, STK2 and StemPro®. HFSCs were expanded successfully in STK2 based media for the first time. The expansion of SHED and HFSCs was better in STK2 based media. The PDT and viability of cells in passages 3 and 4 were better. Thus, these passages were chosen for all the experiments and further studies. The paracrine profile showed a lower concentration of positive hair regulatory factors when cells were cultured in STK2 media. However, in comparison to DMEM-KO based media BDNF was the only negative hair regulatory factor secreted by the SHED, while only BDNF and IL-1 were secreted by HFSCs cultured in STK2 media. It was evident that passage 3 SHED-CM prepared in STK2 was the most suitable CM to stimulate hair growth *in vitro*. It showed a significant ($p<0.05$) increase in the stimulation of anagen-staged hair follicles and a significant decrease ($p<0.05$) in the catagen and telogen-staged hair follicles. It was also found that in comparison to HFSC-CM, SHED-CM demonstrates a significantly ($p<0.05$) higher potential to stimulate hair growth under *in vivo* conditions. However, upon the observation of almost complete coverage of hair no significant difference ($p<0.05$) was observed between the two sources.

Thus within the limitations of the current study, the following conclusions are drawn:

1. STK2 media supported the expansion of HFSCs.
2. The cell morphology PDT, viability of SHED and HFSCs are better at passages 3 and 4.
3. Both STK2 and DMEM-KO based media supported the stem cell characteristics for both SHED and HFSCs.
4. STK2 based media promoted better growth kinetics and stem cell characteristics in comparison to DMEM-KO based media.
5. The donor, source of stem cells and media used for culturing these cells effect paracrine profiles.
6. Stem cells cultured in STK2 based media showed a lower concentration of positive hair regulatory factors while the only negative hair regulatory factors expressed were BDNF and IL-1.
7. *In vitro* studies indicated that passage 3 SHED-CM cultured in STK2 based media showed a significant increase in the anagen-staged hair follicles ($p<0.05$) and a significant decrease ($p<0.05$) in the catagen and telogen-staged hair follicles.
8. *In vivo* studies confirmed that SHED-CM is significantly better ($p<0.05$) than HFSC-CM in the stimulation of hair growth.

7.2 Limitations of the study

1. The time duration in which the *in vitro* study can be conducted, acts as a limiting factor, since the skin tissues cannot be maintained for a longer duration. Further, the exposure of CM at 37° C, throughout the immersion period (72 h) would decrease the activity of the paracrine factors in the CM.
2. Even though the C3H mouse strain was considered to be a highly homogenous model to study hair growth patterns, a synchronized telogen stage was not observed in approximately 20 mice that were supplied at 7 weeks of age. These mice displayed a dark patchy appearance upon shaving, which may be due to the biological variance of the mice.
3. Most studies reported shaving of the dorsal region of mice during the hair growth studies. However, there is no standardization of the surface area to be shaved. Thus, it was difficult to conduct a direct comparison between each of these studies. It was also observed that the intensity and the area of the dark patch indicating the anagen stage onset were different in each mouse. Thus, the appearance of the dark patches cannot be standardized as it was done by visual observation. The difference of the area and intensity may denote various degrees of stimulation of hair growth by CM. A colorimetric analysis could be an option, however, the accuracy of data and sample preparation may be challenging.
4. Even though we have studied the effect of positive and negative hair regulatory paracrine factors, CM is a composition of the cell secretome, which comprises of proteins, microRNA, growth factors, anti-oxidants and proteasomes. These molecules can be freely available or present as exosomes/extracellular vesicles (EVS) which may have an effect in the

stimulation of hair growth. The effect of these molecules can be investigated in the future studies at address their role on the hair growth stimulation.

7.3 Future recommendations

The difficulty in collection and preparation of CM remains a significant challenge due to the low concentration and low dilution of paracrine factors present in the CM. Expression vectors, complement cascade cleavage fragments/ inductive agents or stimulating growth factors can be used to induce the targeted paracrine factors (Ratajczak et al., 2012). Ultra centrifugation steps during the preparation step of CM can also be carried out to overcome this issue during the future studies.

Studying the effect of different culture conditions such as hypoxic and 3D culture conditions which can express a higher level of arteriogenic paracrine factors (Cheng & Yau, 2008; Cho et al., 2012; Di Santo et al., 2009; Uemura, Xu, Ahmad, & Ashraf, 2006) can be further explored.

MSCs can be polarized into two phenotypes depending upon the toll like receptor signaling provided. They can be polarised towards a pro-inflammatory phenotype upon stimulation of toll like receptor 4 while toll like receptor 3 polarizes them to an immunosuppressive phenotype. Upon the type of stimulation received the cells tend to secrete paracrine factors related to the respective phenotype. It has been reported that preconditioning of ADSCs using lipopolysaccharide enhances the secretion of IL-6, TNF-α, HGF and VEGF (Lee et al., 2015). Thus stimulating and preconditioning SHED to obtain a better paracrine secretion profile can be conducted.

Since the half-life of each paracrine factor varies, further studies should be conducted to study the stability of the paracrine factors present in the CM. During the current

study, the application of the CM was carried out under a cold temperature, to preserve the stability of the paracrine factors. However, during a clinical application, this procedure may be not feasible. Thus methods such as freeze-drying of the CM can be considered to increase the shelf life of the paracrine factors.

In addition to profiling the paracrine factors that exhibit an effect on the hair growth, the components of the secretome can be profiled, and studied. This may provide render an opportunity to more effectively utilize CM in the stimulation of hair growth.

Further studies can be carried out to explore the signaling pathways that were stimulated by the application of CM. A time point based study on the signaling pathway activation would give an insight on how the CM acts, and the stability of the CM within the *in vivo* systems. It would also provide an opportunity to determine the frequency of which the CM can be applied for maintaining its regenerative capacity for hair growth application.

Furthermore, comparative studies can be conducted with the currently FDA approved drugs Finastride and Minoxidil and AAPE™, to study the efficacy of SHED-CM to the other treatment strategies that are currently available. Additionally, the application of CM can be studied in combination of other alopecia treatment methods such as micro-needling and hair plucking, which would increase its efficiency.

Since the application of the CM is carried out at the sub-cutaneous layer, the amount of CM entering the blood vessels is negligible. The mice reported no signs of adverse reactions. However, it is recommended that separate toxicology studies to be carried out to confirm this statement.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

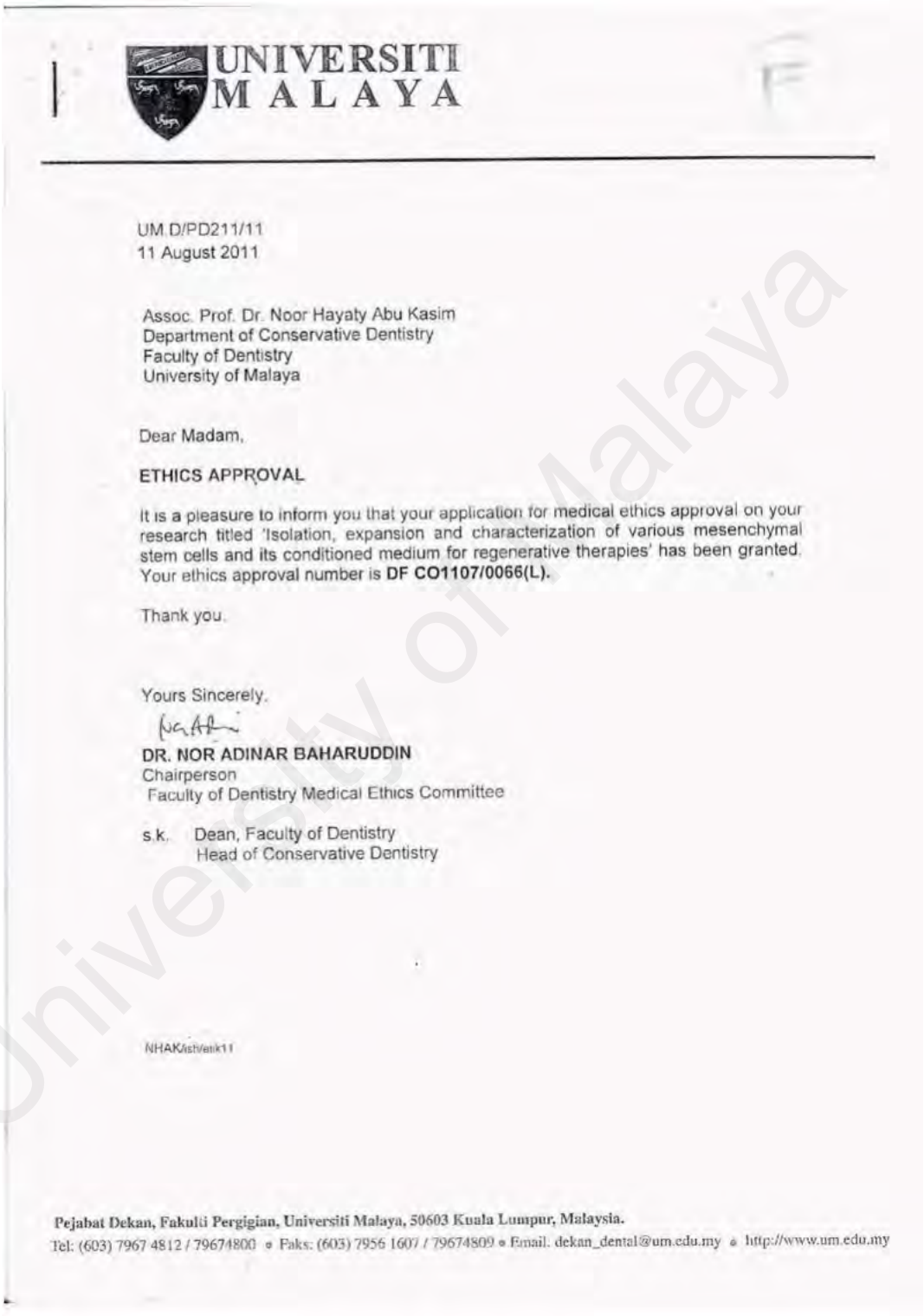
CONFERENCES

- Poster Presentation at the 9th World Conference for Hair Research in Florida, USA- Release of Hair Growth Regulatory Factors by Hair Follicle Follicle Stem Cells When Cultured in Four Different Media: A Preliminary Study. Tharini N. Gunawardena, Selvee T. Ramasamy, Mohammad T. Rahman, Hayaty N. Abu Kasim

MANUSCRIPTS

- Stem Cell Conditioned Media: the next generation of regenerative therapy (Submitted to International Journal of Biological Sciences)
- Effect of different culture media on growth kinetics of stem cells and comparison of cytokine profiles of mesenchymal stem cells (In preparation)
- Evaluation of the effect of mesenchymal stem cell conditioned media on hair growth (In preparation)

APPENDIX A: ETHICAL APPROVAL FROM THE FACULTY OF DENTISTRY, UNIVERSITY OF MALAYA, MEDICAL ETHICS COMMITTEE



UNIVERSITI MALAYA

UM.D/PD211/11
11 August 2011

Assoc. Prof. Dr. Noor Hayaty Abu Kasim
Department of Conservative Dentistry
Faculty of Dentistry
University of Malaya

Dear Madam,

ETHICS APPROVAL

It is a pleasure to inform you that your application for medical ethics approval on your research titled 'Isolation, expansion and characterization of various mesenchymal stem cells and its conditioned medium for regenerative therapies' has been granted. Your ethics approval number is **DF CO1107/0066(L).**

Thank you.

Yours Sincerely.

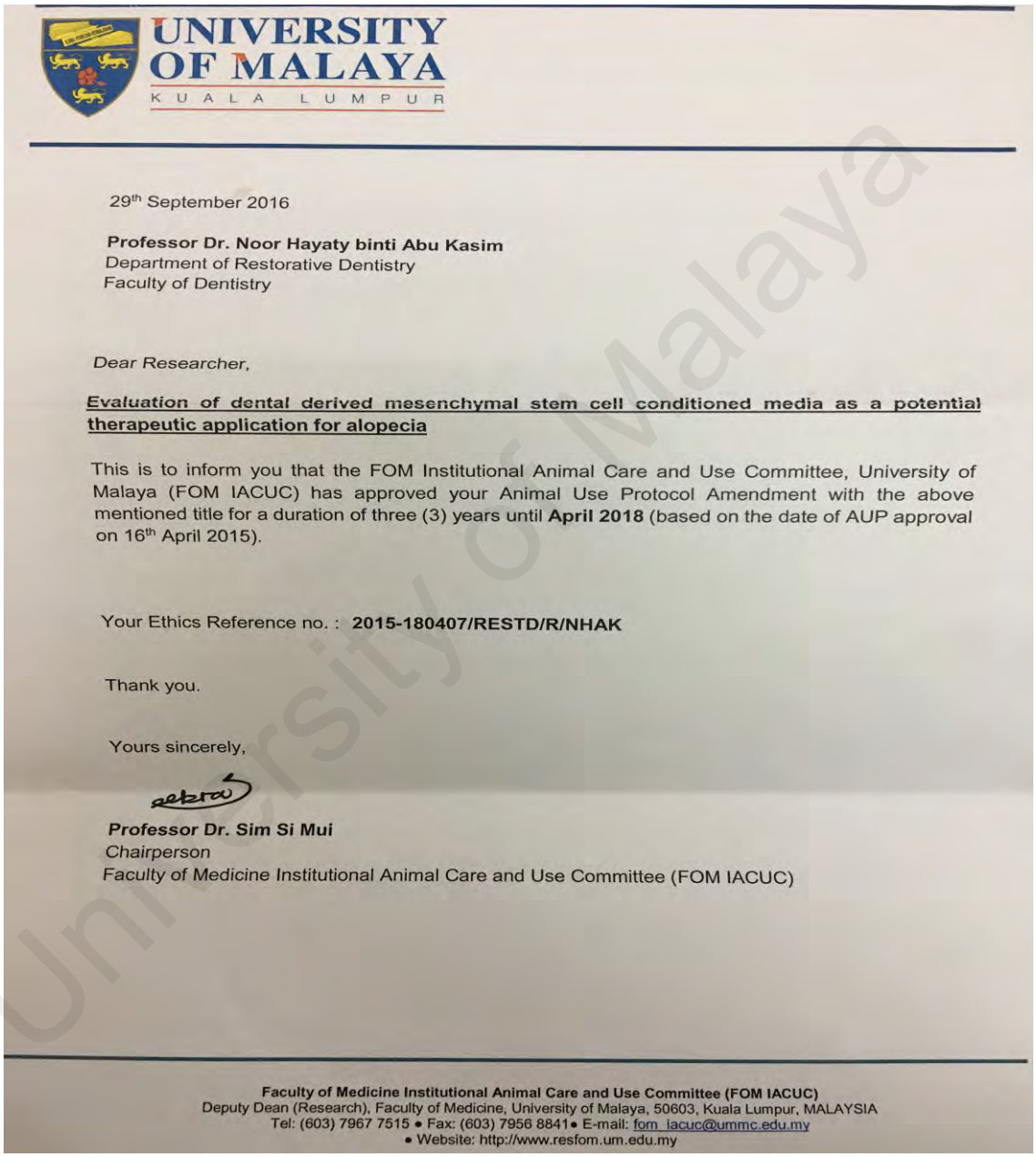
DR. NOR ADINAR BAHARUDDIN
Chairperson
Faculty of Dentistry Medical Ethics Committee

s.k. Dean, Faculty of Dentistry
Head of Conservative Dentistry

NHAK/ishvenk11

Pejabat Dekan, Fakulti Pergigian, Universiti Malaya, 50603 Kuala Lumpur, Malaysia.
Tel: (603) 7967 4812 / 79674800 • Faks: (603) 7956 1607 / 79674809 • Email. dekan_dental@um.edu.my • http://www.um.edu.my

APPENDIX B: ETHICAL APPROVAL FROM THE FACULTY OF MEDICINE, UNIVERSITY OF MALAYA, ANIMAL CARE AND USE COMMITTEE



UNIVERSITY OF MALAYA
KUALA LUMPUR

29th September 2016

Professor Dr. Noor Hayaty binti Abu Kasim
Department of Restorative Dentistry
Faculty of Dentistry

Dear Researcher,

Evaluation of dental derived mesenchymal stem cell conditioned media as a potential therapeutic application for alopecia

This is to inform you that the FOM Institutional Animal Care and Use Committee, University of Malaya (FOM IACUC) has approved your Animal Use Protocol Amendment with the above mentioned title for a duration of three (3) years until **April 2018** (based on the date of AUP approval on 16th April 2015).

Your Ethics Reference no. : **2015-180407/RESTD/R/NHAK**

Thank you.

Yours sincerely,

Professor Dr. Sim Si Mui
Chairperson
Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC)

Faculty of Medicine Institutional Animal Care and Use Committee (FOM IACUC)
Deputy Dean (Research), Faculty of Medicine, University of Malaya, 50603, Kuala Lumpur, MALAYSIA
Tel: (603) 7967 7515 • Fax: (603) 7956 8841• E-mail: fom_iacuc@ummc.edu.my
• Website: http://www.resfom.um.edu.my

APPENDIX C: DATA SHEETS FOR CELLS PURCHASED

ALLCELLS
1301 Harbor Bay Parkway, Suite 200, Alameda, CA 94502
Phone: 510-521-2600; Fax: 510-521-7600
Email: info@allcells.com; Web: www.allcells.com

CERTIFICATE OF ANALYSIS

Catalog Number: DP004F

Product Description: Dental Pulp Stem Cells, Deciduous Teeth

Lot Number: SHED092211-01

Donor Status: (single/pooled) **Single**

Passage Number: Passage-2

Donor Information:

Age	*Weight*	*Height*	*Sex*	*Race*	*Blood Type*	*Smoker*	*Taken Allergy Med.<1 wk?*
N/A	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**	**N/A**

DONOR VIRUS TESTING:	***HIV***	***Negative***
	HBV	***Negative***
	HCV	***Negative***

QA/QC Tests:	**Specification**	**Result**
1. Number of viable cells/vial:	**1x10^6**	**Approved**
2. Viability by trypan blue exclusion:	**90%**	**Approved**
3. Growth Curve:	**See attached**	**Approved**
4. FACS Analysis	**See attached**	**Approved**
5. Bacteria, Yeast, Other Fungi (Direct culture test)	**Negative**	**Approved**
6. Date and Serum Type of Cryopreservation:	**10/17/2011**	**with serum**

All donor tissue/cells were procured through an IRB approved donor program.

***Processing and QA/QC testing for this product were performed by AllCells' Supplier.**

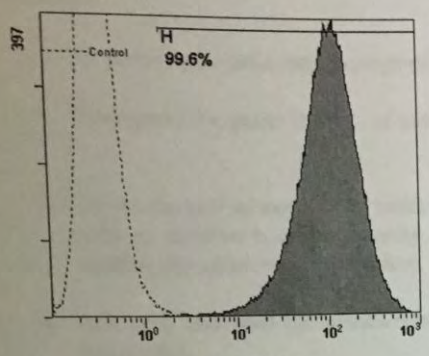
Recommended Storage Condition: Please use products immediately upon arrival.

These cells are for research purposes only and are not intended for human use. By your acceptance of these cells you are acknowledging that these products are potentially a biohazardous material and should be handled as such, even if available serological reports are negative. These cells should be handled by establishing or following an appropriate safety control procedures to ensure safety when using these products.

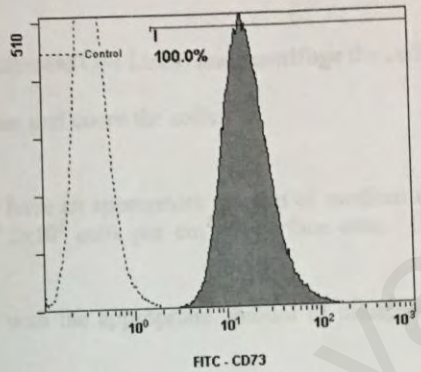
Signature:

Email: info@allcells.com ***website: www.allcells.com***

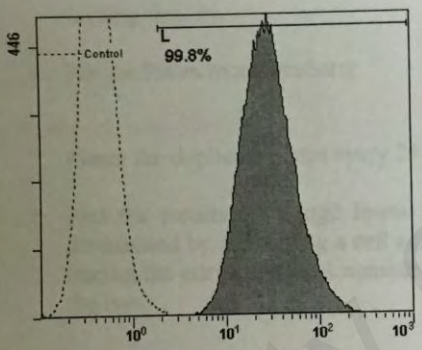
ALLCELLS
1301 Harbor Bay Parkway, Suite 200, Alameda, CA 94502
Phone: 510-521-2600; Fax: 510-521-7600
Email: info@allcells.com; Web: www.allcells.com



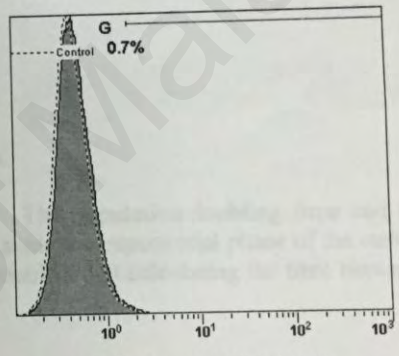
FITC - CD90



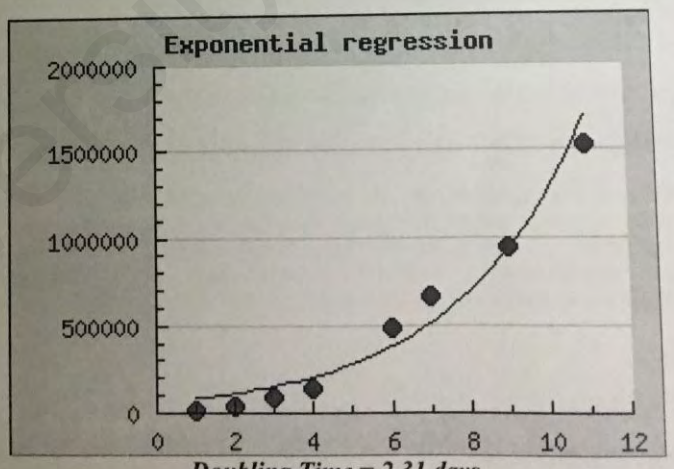
FITC - CD73



PE - CD105



PE - CD14, CD19, CD34, CD45, HLA-DR



Doubling Time = 2.31 days

ICELLTIS

Hair follicle mesenchymal stem cells
Batch HFMSC

Technical data sheet : cryopreserved cells

For in vitro use only

CHARACTERISTICS

- Human donors, batch : 14001
  - Tissue from hair origin
  - Safety of material : absence of hepatitis B, C, HIV-1, HIV-2
  - Test for presence of mycoplasma : negative

Notice: although controls were performed, human material has to be considered as potentially dangerous. Take maximum care in order to protect yourself and your colleagues.

- Cell isolation
  - According to the methods of VOGEL J.C.. M.Ohyama, A.Terunuma, C.L. Tock, M. F. Radonovich, C. A. Pise-Masison, S.B. Hopping, J.N. Brady, M. C. Udey, J.C. Vogel. "Characterization and isolation of stem-cell-enriched human hair bulge cells." J. Clin. Invest. 116 (1),249-260 (2006).

- Cell thawing
  - Number of viable cells per vial after thawing : ≥ 500 000
  - Cell viability after thawing (trypan blue exclusion test) : # 50 %

CONTROLS

- Cell morphology
- Cell density

STORAGE

In liquid nitrogen

USE

The conditions of differentiation suggested must be followed to differentiate into adipocytes, chondrocytes or osteoblasts.

VISA FOR BATCH RELEASE

Name	function	qualification	signature	date
Magdeleine	Product Manager	Biologist		22.1.15

ICELLTIS

SPECIFICATIONS

HUMAN MESENCHYMAL STEM CELLS FROM HAIR FOLLICLE

Reference : HFMSC-C05, batch 14001

Cells are isolated from normal human hair follicle and screened for hepatitis B and C and HIV-1 and HIV-2.

HFMSC-C05 are tested for their ability to differentiate *in vitro* into adipocytes, chondrocytes and osteoblasts. Cells are available cryopreserved or proliferating.

Biologic material

Origin sample: human adult hair follicle

Safety data:

Serological screening	Expected results
Hepatitis B (HBs antigen, anti HBc antibody)	negative
Hepatitis C (anti HCV antibody)	negative
HIV-1 and HIV-2 (anti HIV-1 and anti HIV-2 antibodies)	negative

Cell controls

Functional parameters:

Controls	Methods	Expected results
Viability	Trypan blue dye exclusion test	>85%

Sterility control:

Controls	Methods	Expected results
Mycoplasma	Biochemical test	negative
Bacteria, yeast, fungi	5 days culture without antibiotics, with a daily observation under microscope	No contamination

Packaging and delivery

Cryopreserved cells: 0,5 x 10^6 cells/cryotube, delivery in Carbon ice or dry nitrogen according to the customer

APPENDIX D : LIST OF CHEMICALS FOR FLOW CYTOMETRY ANALYSIS AND TRI LINEAGE DIFFERENTIATION

Flow-cytometry:

BUFFER (Storage at 4°C)

Phosphate buffer –saline	pH7.2
Bovine serum albumine	0.5%
EDTA	2mM

500µl MSC PHENOTYPING COCKTAIL, HUMAN (MACS, Miltenyi Biotech)
Cocktail of flurochrome-conjugated monoclonal antibodies
CD14-PerCP (clone TÜK4, iso type; mouse IgG2a)
CD20-PerCP (clone LT20.B4, isotype; mouse IgG1)
CD34-PerCP (clone AC136, isotype; mouse IgG2a)
CD45-PerCP (clone 5B1, isotype; mouse IgG2a)
CD73-APC (clone AD2, isotype; mouse IgG1)
CD90-FITC (clone DG3, isotype; mouse IgG1)
CD105-PE (clone 43A4E1, isotype; mouse IgG1)

500µl ISOTYPE COCKTAIL, HUMAN (MACS, Miltenyi Biotech)
Cocktail of flurochrome-conjugated monoclonal antibodies;
Mouse IgG1-FITC (clone; IS5-21F5)
Mouse IgG1-PE (clone; IS5-21F5)
Mouse IgG1-APC (clone; IS5-21F5)
Mouse IgG1-PerCP (clone; IS5-21F5)
Mouse IgG2a-PerCP (clone; S43.10)
50µl CD90-FITC human (clone DG3, isotype; mouse IgG1)
50µl CD105-PE human (clone 43A4E1, isotype; mouse IgG1)
50µl CD73-APC human (clone AD2, isotype; mouse IgG1)
50µl CD73-Biotin human (clone AD2, isotype; mouse IgG1)
50µl Anti-Biotin-PerCP (clone Bio3-18E7 isotype:mouse IgG1)

Tri-lineage differentiation:

PBS BUFFER- pH7.2
01 pellet of PBS dissolved in 200ml of distilled water

RED OIL O
2 parts water : 3 parts Red Oil O

ADIPOGENESIS DIFFERENTIATION MEDIA (ThermoFisher Scientific)

StemPro® Adipocyte Differentiation Basal Medium	90ml
StemPro® Adipocyte Supplement	10ml
Gentamycin Reagent (10mg/ml)	50µl

CHONDROGENESIS DIFFERENTIATION MEDIA (ThermoFisher Scientific)

StemPro® Osteocyte/Chondrocyte Differentiation Basal Medium	90ml
StemPro® Chondrogenesis Supplement	10ml
Gentamycin Reagent (10mg/ml)	50µl

OSTEOGENESIS DIFFERENTIATION MEDIA (ThermoFisher Scientific)

StemPro® Osteocyte/Chondrocyte Differentiation Basal Medium	90ml
StemPro® Osteogenesis Supplement	10ml
Gentamycin Reagent (10mg/ml)	50µl

APPENDIX E: OTHER MATERIALS, REAGENTS AND INSTRUMENTS USED FOR THE STUDY

Cell Sources and Animals	Company
SHED	AllCELLS, Alameda, Canada
HFSCs	ICELLTIS, Labege, France
C3H/HeN Mice	M-Clea Bioresources Co., Ltd., Thailand

Instruments	Company
Hand Held Magnetic Plate Washer	Affymetrix, Ebioscience, USA
Olympus CKX41	Olympus, Tokyo, Japan
Pannoramic Desk	3DHISTECH, Budapest, Hungary

Chemicals and Reagents	Company
4mM Glutamax	Gibco Invitrogen, Carlsbad, CA, USA
Absolute Alcohol	Thermo Fisher Scientific, Massachusettes, USA
Adipogenic, Chondrogenic and Osteogenic Differentiation Media	Sigma-Aldrich, Missouri, USA
Alzarin Red	Sigma-Aldrich, Missouri, USA
bFGF	Gibco Invitrogen, Carlsbad, CA, USA
Bovine Serum Albumin	Merk
Collagenase Type I	Gibco Invitrogen, Carlsbad, CA, USA
DMEM-KO	Gibco Invitrogen, Carlsbad, CA, USA
DPBS	Gibco Invitrogen, Carlsbad, CA, USA
EDTA	Sigma-Aldrich, Missouri, USA
FBS	Gibco Invitrogen, Carlsbad, CA, USA
Iso-propanol	Sigma-Aldrich, Missouri, USA
Luminex Assay	Affymetrix, Ebioscience, USA
Mesenchymal Stem Cell Phenotyping Cocktail	Meltenyi Biotech Inc., Bergisch Gladbach, Germany
Para-formaldehyde	Sigma-Aldrich, Missouri, USA
Pen-Strep	Gibco Invitrogen, Carlsbad, CA, USA
Phosphate Buffer Saline	Gibco Invitrogen, Carlsbad, CA, USA
Red Oil O	Sigma-Aldrich, Missouri, USA
Safranin	Sigma-Aldrich, Missouri, USA
StemPro®	Gibco Invitrogen, Carlsbad, CA, USA
STK2	TwoCELLS, Japan
TrypLE Express	Gibco Invitrogen, Carlsbad, CA, USA
Versene	Gibco Invitrogen, Carlsbad, CA, USA

Plasticware	Company
15 ml Centrifuge tube	BD Pharmingen, San Diego, CA, USA
2 μm Syringe Filters	Thermo Fisher Scientific, Massachusettes, USA
6- well Plates	BD Pharmingen, San Diego, CA, USA
96-well Flat Bottomed Plate	BD Pharmingen, San Diego, CA, USA
Countess® Cell Counting Chamber slides	Gibco Invitrogen, Carlsbad, CA, USA
Cryovial	Corning, New York, USA
Eppendorf Tube	Eppendorf, Hauppauge, NY,USA
Snowcoat Slide	Leica, Wetzlar, Germany
T-25 Flasks	BD Pharmingen, San Diego, CA, USA
U Bottomed 96-Well Plate	BD Pharmingen, San Diego, CA, USA