GENERATION AND CHARACTERISATION OF INDUCED PLURIPOTENT STEM CELLS DERIVED FROM HUMAN HAIR FOLLICLE KERATINOCYTES

By

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ABSTRACT

GENERATION AND CHARACTERISATION OF INDUCED PLURIPOTENT STEM CELLS DERIVED FROM HUMAN HAIR FOLLICLE KERATINOCYTES

Lim Sheng Jye

Induced pluripotent stem cells (iPSCs) are pluripotent cells formed through forced expression of transcriptional factors into somatic cells. iPSC resemble the human embryonic stem cell (hESC) in term of morphology, pluripotency marker expression, growth properties, and differentiation capability. In this study, the objectives were to generate iPSCs from human hair follicular keratinocytes using retrovirus method, to characterise the iPSCs generated from human hair follicular keratinocytes using immunofluorescence staining, flow cytometry, and reverse-transcription PCR (RT-PCR), and finally to differentiate derived-iPSCs into keratinocytes. The human hair follicular keratinocytes (HHFK) were transduced using retrovirus vectors to produce iPSCs. The iPSCs were picked and characterised for the expression of pluripotency markers using immunofluorescence staining, flow cytometry and RT-PCR. The iPSCs were further formed into embryoid bodies or monolayer cultures and differentiated into three germ layers in the presence of different differentiation growth factors. Lastly, the iPSCs were induced via directed differentiation into keratinocytes expressing cytokeratin 14. In this study,

iPSCs have been successfully generated from human hair follicular keratinocytes using the retrovirus carrying the four transcription factors, Oct4, Sox2, Klf4, and c-Myc. Using immunofluorescence staining and flow cytometry, the iPSCs generated expressed hESC-like surface and intracellular markers such as TRA-1-60, TRA-1-81, SSEA4, Oct4, Sox2 and Nanog. Using RT-PCR, the iPSCs generated expressed pluripotency markers such as Sox2, Oct4, Klf4, c-Myc, Nanog, Lefty, and Gabrb. The iPSCs were able to differentiate *in vitro* to form the embryonic three germ layers, namely, ectoderm, endoderm, and mesoderm. The iPSCs were directed differentiated into keratinocytes and the keratinocytes were expressing the cytokeratin 14 marker. This source of keratinocytes could be a valuable source for *in vitro* hair cloning or use to treat hair balding or for grafting in burn patients.

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FACULTY OF MEDICINE AND HEALTH SCIENCES

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It is hereby certified that **LIM SHENG JYE** (ID No: **11UMM07436**) has completed this final year project entitled "GENERATION AND CHARACTERISATION OF INDUCED PLURIPOTENT STEM CELLS DERIVED FROM HUMAN HAIR FOLLICLE KERATINOCYTES" under the supervisor of Associate Professor Dr. GAN SENG CHIEW (Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences, and Associate Professor Dr. ALAN ONG HAN KIAT (Co-Supervisor) from the Department of Pre-clinical Sciences, Faculty of Medicine and Health Sciences.

I hereby give permission to my supervisors to write and prepare manuscripts of these research findings for publishing in any form, if I do not prepare it within six (6) months from this date, provided that my name is included as one of the authors for this article. The arrangement of the name depends on my supervisor.

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This dissertation entitled **"GENERATION AND CHARACTERISATION OF INDUCED PLURIPOTENT STEM CELLS FROM HUMAN HAIR FOLLICLE KERATINOCYTES"** was prepared by LIM SHENG JYE and submitted as partial fulfilment of the requirements for the degree of Master of Medical Sciences at Universiti Tunku Abdul Rahman.

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(Lim Sheng Jye)

DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(Lim Sheng Jye)

Date: December 2015

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LIST OF ABBREVIATIONS

AMD	Age-related Macular Degeneration
AP	Alkaline phosphatase
BMP4	Bone Morphogenetic Protein 4
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CK15	Cytokeratin 15
CK19	Cytokeratin 19
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
DMEM/F12	Dulbecco's Modified Eagle's Medium/Ham's F-12
DMSO	Dimethyl Sulfoxide
EB	Embryoid Bodies
EGF	Epidermal Growth factor
EMT	Epithelial-to-mesenchymal Transition
FBS	Fetal Bovine Serum
FGF-2	Fibroblasts Growth Factor 2
FITC	Fluorescein Isothiocyanate
GFP	Green Fluorescent Protein
HDAC Inhibitor	Histone Deacetylase Inhibitor
HDF	Human Dermal Fibroblasts
hESC	Human Embryonic Stem Cell
hfK	Plucked Hair Follicle Keratinocytes
HGPS	Hutchinson-Gilford Progeria Syndrome
HHFK	Human Hair Follicular Keratinocytes
ICM	Inner Cell Mass
iPSC	Induced Pluripotent Stem Cell
KOSR	Knockout Serum Replacement
LB Broth	Luria-Bertani Broth
LIF	Leukemia Inhibitory Factor
LIF-STAT3	Leukemia Inhibitory Factor-Signal transducer and activator of transcription 3
MEF	Mouse Embryonic Fibroblasts
MET	Mesenchymal-to-epithelial Transition
miRNA	Micro RNA
NEAA	Non-essential Amino Acid
ORS	Outer Root Sheath
OSKM	Transcription Factors of Oct4, Sox2, Klf4, and c-Myc
OSNL	Transcription Factors of Oct4, Sox2, Nanog, and Lin28
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PVDF	Polyvinylidene Difluoride
RPE	Retinal Pigment Epithelium
RT-PCR	Reverse-transcription Polymerase Chain Reaction
RA	Retinoic Acid
SCID	Severe Combined Immunodeficiency
STO Fibroblast Feeder Layer	Sandos Inbred Mice Thioguanine/Ouabain-resistant mouse fibroblast feeder layer

TAE Buffer	Tris-Acetate-EDTA Buffer
TALEN	Transcription Activator-Like Effector Nuclease
VPA	Valproic Acid
ZFN	Zinc Finger Nucleases

CHAPTER 1

INTRODUCTION

Induced pluripotent stem cell (iPSC) was termed by Shinya Yamanaka and his team after reprogrammed mouse fibroblasts using four transcription factors in 2006 (Takahashi & Yamanaka, 2006). In 2007, Shinya Yamanaka reproduced the iPSCs generation on human fibroblasts (Takahashi, et al., 2007). This discovery has brought him to be awarded the Nobel Prize in 2012 along with Sir John Gurdon. iPSC is pluripotent cell formed through forced expression of transcriptional factors into somatic cells. iPSC resemble the human embryonic stem cell (hESC) in term of morphology, pluripotency markers expressions, growth properties, and differentiation capability (Takahashi & Yamanaka, 2006). A group of transcription factors which consists of Yamanaka's factor of Oct4, Sox2, Klf4, and c-Myc (Takahashi, et al., 2007) or Thomson's factor of Oct4, Sox2, Lin28, and Nanog (Yu, et al., 2007) can be used to reprogram most cell types back into the native state. However, most of reprogramming studies used the Yamanaka's factor for generation of iPSC compared to Thomson's factor (Planello, et al., 2014).

Around the same time in 2007, another group of scientists led by James Thomson successfully derived human iPSCs from human fibroblasts (Yu, et al., 2007). This group used different set of transcription factors consist of Oct4, Sox2, Lin28, and Nanog (Yu, et al., 2007). The difference was they used Lin28 and Nanog instead of Klf4 and c-Myc. The other difference was the virus

system used to infect the cells. This group used the lentiviral system to deliver the four transcription factors (Yu, et al., 2007) while Yamanaka used the retroviral delivery system (Takahashi, et al., 2007). The iPSCs derived using the Thomson's factor also showed the same characteristics as the iPSCs derived from Yamanaka's factor. The iPSCs showed similar morphology to typical hESC morphology (Yu, et al., 2007). It also showed similar surface antigens such as TRA-1-60, TRA-1-81, SSEA-3, and SSEA-4, (Yu, et al., 2007).

Apart from a set of four transcription factors, many studies also generated iPSCs using three and lesser transcription factors. IPSC has been generated with one factor from dermal papilla cells and neural stem cells. Both studies also generated iPSC using single factor, Oct4 (Kim, J. B. et al., 2009; Tsai, et al., 2011). Currently, various cell types have been reprogrammed into iPSCs such as keratinocytes (Aasen, et al., 2008), fibroblasts (Takahashi, et al., 2007), mature B lymphocytes (Hanna, et al., 2008), neural stem cells (Kim, J B et al., 2009), dermal papilla cells (Tsai, et al., 2011), pancreatic β cells (Stadtfeld, et al., 2008a) and bone marrow cells (Streckfuss-Bomeke, et al., 2013).

Apart from generation of iPSCs from normal cell types, iPSCs can also be generated from patient-specific or disease-specific cells (Takahashi, et al., 2007). The iPSCs generated may be used to elucidate the mechanisms involve in the disease and also to study the effect of drugs and cells or tissue for cell and tissue replacement therapies (Novak, et al., 2010). iPSCs have been

produced from patients with Parkinson disease, Huntington disease, and adenosine deaminase deficiency-related severe combined immunodeficiency (Park, et al., 2008).

Various methods have been employed for generation of iPSC. The first generated iPSC was based on retroviral delivery method (Takahashi & Yamanaka, 2006). Another viral reprogramming method which causes genomic integration is lentiviral based reprogramming (Yu, et al., 2007). Another two viral methods that do not cause genomic integration are adenovirus and sendai virus (Stadtfeld, et al., 2008b; Fusaki, et al., 2009). More recent iPSC generation, most study focused on using non-integrating method such as episomal vector, synthetic modified mRNA, miRNA, and protein-based reprogramming factors (Yu, et al., 2009; Kim, D., et al., 2009; Warren, et al., 2010; Miyoshi, et al., 2011). Another two integrating methods in which the inserts are removed from the cell genome later on are cre-mediated recombinase and Piggybac transposon (Woltjen, et al., 2009; Sommer, et al., 2010).

These transcription factors, Oct4, Sox2, Klf4, c-Myc, Lin28 and Nanog, are the common factors used in reprogramming, with each factor playing a different role but could co-operate to maintain pluripotency and prevent differentiation (Boyer, et al., 2005; Takahashi, et al., 2007; Yu, et al., 2007; Pan & Thomson, 2007). Oct4 together with Sox2 and Nanog are the main regulatory complex function in maintaining pluripotency and self-renewal of pluripotent stem cells (Boyer, et al., 2005). It has been revealed that these

factors occupy large number of promoter region at the same time (Boyer, et al., 2005). These genes co-operate in regulating large number of target genes in hESC which are involved in early hESC development (Boyer, et al., 2005).

As far as improvements are concern, however, the generation of iPSC still need to be fine-tuned to able to get a safe iPSC that can be used in clinical setting. Generally, reprogramming is a slow process which takes up at least one week and up to a month (Kiefer, 2011) and also an inefficient process with the first reported reprogramming efficiency of only 0.02% (Takahashi & Yamanaka, 2006). Nonetheless, the improvement in iPSC research area has led to the reduction in the generation time to eight day and increases the reprogramming up to 10% (Hong, et al., 2009; Chen, J., et al., 2011).

The first reprogramming experiment used OSKM as the transcription factors and the c-Myc was identified as oncogene (Kiefer, 2011). The inclusion of oncogene as reprogramming factor will introduce problem to the downstream applications especially for clinical use (Kiefer, 2011). The integrated oncogene will cause tumour formation when reactivated (Kiefer, 2011). This leads to current researchers to focus on eliminating the use of c-Myc as one of the reprogramming factors (Nagakawa, et al., 2008; Chen, J., et al., 2011).

The potential of iPSCs has driven many researchers to the studies of iPSCs. Research has been focused on finding the reprogramming method which involve non-viral delivery or without transgenes integrating method (Oh,

et al., 2012). The low efficiency of reprogramming is also a problem (Oh, et al., 2012). Many have come up with different methods to increase the efficiency such as with the use of chemicals or small molecules (Oh, et al., 2012) such as ERK, GSK TGFβ, histone deacetylase and DNA methyltransferase inhibitors (Kiefer, 2011). The continual advancement will promote further understanding of the mechanisms involve in reprogramming and the future implication in this field (Buganim, et al., 2013).

Human embryonic stem cells (hESCs) were isolated by James A. Thomson and his team in the year 1998. As indicated by their name hESC, the pluripotent cells were isolated from human embryos. They were the first to isolate hESCs from the inner cell mass of embryos (Thomson, et al., 1998). The hESCs have the following characteristics such as high ratio of nucleus to cytoplasm, round colony morphology, and prominent nucleoli (Thomson, et al., 1998). The hESCs expressed high levels of telomerase activity, SSEA3, SSEA4, alkaline phosphatase, TRA-1-60, and TRA-1-81. The hESCs also had normal XX and XY karyotypes (Thomson, et al., 1998). The hESCs were able to be differentiated *in vivo* to form the three germ layers (Thomson, et al., 1998).

The translation of pluripotent stem cells to clinical use have been performed for both hESCs and iPSCS (Alper, 2009). For hESCs, it was used to treat spinal cord injury in 2010 (Alper, 2009). But, the trial ended in 2011 due to financial problems even though there is no safety issues being reported (Sperling, 2013). The first ever clinical trial for iPSCs was for treating patient

with Age-Related Macular Degeneration (AMD) (Cyranoski, 2014b). According to Dr. Masayo Takahashi, the project leader for this trial, she mentioned that this procedure might not restore patient vision, but, any improvements or side effects are worth to know since it had attract interest from other researchers (Cyranoski, 2014b).

Hair follicles are considered as a mini-organ which is not only important in animals for protection from coldness but also important in humans (Marioka, 2006a). Although hair disorders are not life threatening, hair follicles still play a significant role for human apart from the role in social perspective (Randall & Botchkareva, 2008). Hair follicles are appendages which reside in the dermis and outgrowth through the epidermis to form the whole hair (Aasen & Belmonte, 2010). The visible hair which grows above the scalp is known as hair shaft (Aasen & Belmonte, 2010). The continual growth of hair happen in three phases, namely anagen phase, catagen phase, and telogen phase or called growing, regression and resting phase, respectively (Yoo, et al., 2007). The hair follicles are made up of outer root sheath, inner root sheath, dermal papilla, and hair shaft (Randall & Botchkareva, 2008).

Keratinocytes are the main cell type that made up the surface of skin, hair, and nails (Aasen, et al., 2008). The keratinocytes on the skin and appendages surface usually consist of terminally differentiated keratinocytes and it functions as protective barrier against external environment (Aasen, et al., 2008). Human hair follicle keratinocyte was first cultured on bovine eye lens capsules (Weterings, et al., 1981). Then, Limat and Noser (1986) were the

first to culture the primary human hair follicle keratinocytes on an irradiated 3T3 feeder layer. The feeder layer was able to sustain the growth of primary keratinocytes up to 3 passages (Limat & Noser, 1986). More recently, keratinocytes cultured in serum free medium was introduced with the addition of bovine pituitary extract and epidermal growth factor for serum free and feeder free culture (Yoo, et al., 2007).

Alopecia or hair loss has been affecting more people be it genetic or environment factors (Yoo, et al., 2010). The treatments for alopecia are drug therapy and hair transplantation. Drugs are only effective in mild cases of alopecia and it always brings side effects. Hair transplantation involves redistributing existing hairs to bald area. The procedure is very lengthy and bloody (Yoo, et al., 2010). If hair follicles can be formed *in vitro* from differentiated iPSCs, the hair follicles represent an alternative hair source for transplantation. Besides, burn patients always suffered from limited autografts donor sites due to extensive burns (Mcheik, et al., 2014). Although, methods to handle wounds are well-established, but, differentiated cells from pluripotent stem cells still represent a useful source (Mcheik, et al., 2014).

The objectives of this study are (i) to generate iPSCs from human hair follicular keratinocytes using retrovirus method, (ii) to characterise the iPSCs generated from human hair follicular keratinocytes using immunofluorescence staining, flow cytometry, and reverse-transcription PCR, and (iii) finally to differentiate the derived-iPSCs into keratinocytes.

CHAPTER 2

LITERATURE REVIEW

2.1 The Hair Systems

Hair not only has biological functions but, it plays an important psychosocial role in humans (Randall & Botchkareva, 2008). Every human, either man or woman, will spend some time to fix their hair. The hair follicle is one of mammalian main characteristic and is only present in mammals (Randall & Botchkareva, 2008). The hair follicle is form from several parts such as, inner root sheath, outer root sheath, dermal sheath, hair shaft and hair bulb, with keratinocytes as the main cell type present in the hair follicle apart from melanocytes, connective sheath cells or dermal papilla cells (Randall & Botchkareva, 2008).

2.1.1 Structures and Functions of Hair Follicles

The main structures of hair follicle consist of hair shaft, inner root sheath, outer root sheath, connective tissues sheath, and hair bulb (Figure 1.1). The medulla and cortex make up the hair shaft which is mainly consists of terminally differentiated keratinocytes (Randall & Botchkareva, 2008). The inner root sheath is made up of the cuticle, Huxley's layer, Henle's layer, and the companion layer. The cuticle layer of inner root sheath and hair shaft is partially overlapped, thus the hair shaft is supported by the hair follicle. The inner root sheath's major roles are to support the hair shaft and to guide its movement upward (Randall & Botchkareva, 2008).

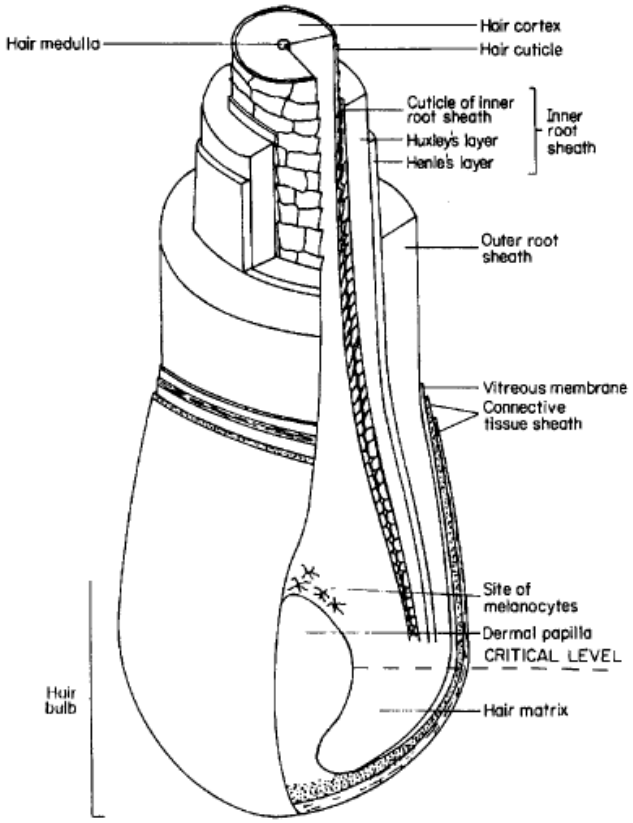

Figure 1.1 The structure of hair follicle. The diagram shows the structures of the hair follicle in 3-dimensional view. (Reproduced from Randall & Botchkareva, 2008)

The outer root sheath is a site with different cell population of keratinocytes, keratinocytes stem cells, melanocyte stem cells progeny, and differentiating melanocytes (Randall & Botchkareva, 2008). Below the area of sebaceous gland is the location of distinct bulge area, where the multi-potent stem cells are located. These multi-potent stem cells retained BrdU, expressed cytokeratin 15 (CK15), cytokeratin 19 (CK19) and CD200, and characterised by low proliferative rate and able to give rise to different cell types (Randall & Botchkareva, 2008). The multi-potent stem cells migrate to the hair bulb forming the cells that made up the inner root sheath and hair shaft. The stem

cells also reported to migrate to the skin surface whenever it is involved in wound repair (Marioka, 2006b).

The dermal papilla is located at the hair bulb area. This area is believed to be the main control area for hair growth, maintain vascular system for nutritional support and also involved in hair pigmentation (Kiyokazu, 2005). The actions of dermal papilla are driven by the presence of various growth factors such as noggin, keratinocyte growth factor, hepatocyte growth factor, and insulin-like growth factor-I to drive the proliferation and differentiation of the matrix cell (Randall & Botchkareva, 2008).

2.1.2 Keratinocytes

Keratinocytes are the main cell type that made up the surface of skin, hair follicle, and nails (Aasen & Belmonte, 2010). The keratinocytes on the skin surface and surfaces of appendages usually consist of terminally differentiated keratinocytes. It functions as a protective barrier against external environments such as pathogens, heat, and UV radiation, and prevents fluid loss (Baroni, et al., 2012).

Hair follicles are appendages which reside in the dermis and outgrowth through the epidermis to form the whole hair (Aasen & Belmonte, 2010). The visible hair which grows above the scalp is known as the hair shaft. The outer root sheath (ORS) provides a barrier to the hair follicle from the dermis that surrounding it and the ORS is connected to the epidermis (Aasen & Belmonte,

2010). The growth of hair happens in three phase, namely growing phase, regression phase, and resting phase (Aasen & Belmonte, 2010).

There are two ways of culturing keratinocytes, either feeder-based or feeder free. The feeder-based culture is where the keratinocytes are cultured on top of an inactivated fibroblasts cells such as 3T3 or human dermal fibroblasts (Limat & Hunziker, 1996; Aasen & Belmonte, 2010). The feeder free method usually uses a combination of coated flask with serum free medium (Aasen & Belmonte, 2010). Both culture methods have its own advantages and disadvantages and it is up to the preference of the user and dependent on the use of the cells.

Culturing of keratinocytes on feeder free condition is useful for primary cultures whereby it could eliminate the time required to prepare feeder cells and free from contaminating cells such as fibroblasts. Culture of feeder cells and inactivation of feeder cells usually takes several days to complete. Fibroblasts usually do not grow well in serum free conditions (Aasen & Belmonte, 2010). Culturing of keratinocytes on feeder cells is important for long term culture of keratinocytes. The feeder layer will provide certain growth factors that were needed for the long term maintenance of keratinocytes (Bisson, et al., 2013). This can mimic the growth condition *in vivo* whereby keratinocytes layer is on top of the fibroblasts.

2.1.3 Embryonic Hair Follicle Morphogenesis

Hair follicle morphogenesis involved the interaction between the neuroectoderm and mesoderm (Schmidt-Ullrich & Paus, 2005). Hair follicle development starts in embryonic stage. Hair follicle morphogenesis occurs in three stages, namely, induction, organogenesis, and cytodifferentiation (Rishikaysh, et al., 2014). The induction stage starts when the first signal arrived via the Wnt signalling pathway. The signal will direct the overlying epithelial cells to form thickening layers of cells called placode (Rishikaysh, et al., 2014). The organogenesis stage involves the interplay between the epithelial and dermal cells. The epithelial cells produced signals to dermal cells to proliferate and group under the placode, and thus forming the dermal condensate. The dermal condensate produced further signal to the epithelial cells to proliferate and grow downwards (Rishikaysh, et al., 2014). This placode downgrowth involved the dermal signals from Sonic Hedgehog (Shh) and Gli2 signalling pathway and ectodermal Wnt signals (Schmidt-Ullrich & Paus, 2005). Once the epithelial grow downwards in the cytodifferentiation stage, it will envelop the dermal condensate (Rishikaysh, et al., 2014). From this stage, the dermal condensate will be known as dermal papilla cells. The signals from this dermal papilla will direct the formation of the whole hair (Schmidt-Ullrich & Paus, 2005). Shh signal also involved in the maturation of the dermal papilla cells.

2.2 Induced Pluripotent Stem Cell

Induced pluripotent stem cell or in short called iPSC is somatic cell-derived pluripotent stem cell through the introduction of transcription factors.

The iPSC resembles hESC in terms of the pluripotency characteristics. The characteristics include morphology, unlimited self-renewal, pluripotency markers expression at the gene level and protein level, epigenetic status of pluripotent genes, teratoma formation and telomerase expression (Takahashi, et al., 2007).

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2.2.1 History of Induced Pluripotent Stem Cell

Induced pluripotent stem cell was generated and termed by Dr Shinya Yamanaka and Kazutoshi Takahashi in 2006 using the mouse embryonic fibroblasts (MEF) and adult mouse tail-tip fibroblasts. The fibroblasts were transduced using retroviral delivery system which carried four transcription factors (Oct4, Soc2, Klf4, and c-Myc). The iPSC generated showed the same morphology, growth properties, and pluripotency genes as embryonic stem cells. Tumours developed upon subcutaneous injection of iPSC into the nude mice and histological examination showed tissues of the three germs layers. The blastocysts were injected with iPSCs which then developed into embryos that showed green fluorescence protein (GFP)-positive due to the presence of iPSCs (Takahashi & Yamanaka, 2006). This showed that the injected iPSCs contributed to the development of the embryo.

In 2007, the same team lead by Dr. Shinya Yamanaka has generated the iPSCs from human dermal fibroblasts, primary human fibroblast-like synoviocytes, and BJ cells established from neonate fibroblasts. The fibroblasts were transduced using retroviral delivery system which carried four transcription factors (Oct4, Sox2, Klf4, and c-Myc). The authors successfully

generated iPSCs and proved that the iPSCs were identical to hESCs in term of proliferation rate, morphology, pluripotency markers expression, epigenetic status of pluripotency genes, and telomerase activity. The cells were able to be differentiated *in vitro* into three germ layers and were able to form *in vivo* teratoma formation (Takahashi, et al., 2007).

2.2.2 Methods for Reprogramming

There are various methods to derive iPSC. Reprogramming methods can be divided into genome integration method or genome integration-free method, viral based method or non-viral based methods, and nucleic acid based method based or non-nucleic acid based method. In this review, we reported the reprogramming method based on the genome integration and genome integration-free method. The genome integration methods include retrovirus, and lentivirus (Takahashi & Yamanaka, 2006; Yu, et al., 2007). For genome integration-free methods, the examples include adenoviral, episomal vector, sendai virus, synthetic modified mRNA, miRNA, protein-based transcription factors, cre-mediated recombinase, and Piggybac transposon (Stadtfeld, et al., 2008b; Fusaki, et al., 2009; Kim, et al., 2009; Woltjen, et al., 2009; Yu, et al., 2009; Mallanna & Rizzino, 2010; Sommer, et al., 2010; Mandal & Rossi, 2013).

2.2.2.1 Genome Integration Method

Retrovirus based reprogramming was the first method used for reprogramming by the Yamanaka group (Takahashi & Yamanaka, 2006). In that study, the authors used the pMXs-based retroviral vectors (Takahashi &

Yamanaka, 2006) to bring in the transcription genes into the target cells. Usually when using virus transduction method, a packaging cell is needed to produce the infectious virus particles and these virus particles are able to infect the target cells (Morita, et al., 2000). For retroviral vector, it can infect various types of actively dividing cells and the transgenes will be stably integrated into the host genome (Miller, et al., 1990; Okita & Yamanaka, 2010). Since, the transcription factors are actively expressed during the reprogramming process, thus, it will result in higher efficiency in comparison to the non-integrating method (Robinton & Daley, 2012).

The exogenous transgenes are usually silenced in iPSC but in some cases it could be expressed back (Okita & Yamanaka, 2010). Thus, it will disrupt the endogenous factors network and could prevent the downstream characterisation such as *in vitro* or *in vivo* differentiation (Okita & Yamanaka, 2010). There is another disadvantage of the integration method using viruses, which is when using for transplantation, it will increase the risk of tumour formation especially some of the transcription factors are potential oncogenes (Okita & Yamanaka, 2010).

Lentiviral vector was first used in the reprogramming by Thompson group in 2007 for human fibroblasts reprogramming using OSNL (Oct4, Sox2, Nanog, and Lin28) transcription factors (Yu, et al., 2007). One of the advantage of lentivirus compare to retrovirus is that lentivirus can infect any types of cells, both the dividing and non-dividing cells (Tanabe & Takahashi, 2011). Basically, the integrating method such as using lentivirus vector itself

will confer a higher efficiency in the reprogramming process. This could be due to the stable expression of the reprogramming factors (Tanabe & Takahashi, 2011).

Lentivirus vector would integrate the transgenes into the host genome and this event could lead to tumorigenesis (Tanabe & Takahashi, 2011). This is especially dangerous if the iPSC is for clinical use. The activity of lentiviral transgene is not silenced in the iPSC (Lois, et al., 2002; Maherali & Hochedlinger, 2008). It was shown that ngenomic integration was not helpful to promote reprogramming and genomic integration method could serve as a platform for optimization of reprogramming method (Stadtfeld, et al., 2008b). This is important so that reprogramming can be done in higher efficiency and more safe for clinical use (Maherali & Hochedlinger, 2008).

2.2.2.2 Genomic Integration-Free Method

Adenoviral vector is one of the non-integration methods for generation of iPSC. Adenoviral vector produce high level of transgenes expression and allow transient expression of the transgenes (Stadtfeld, et al., 2008b). This could explain the reason Zhou et al. (2009) performed two round of infection at Day 2 and Day 4. One advantage of using adenovirus compared to retrovirus and lentivirus is that the use of adenovirus vector did not cause genomic integration into the host (Stadtfeld, et al., 2008b). The efficiency of reprogramming using adenovirus was around 0.0005% to 0.0012% (Stadtfeld, et al., 2008b). The efficiency was extremely low compared to viruses integrating method, which was around 0.01% to 0.1% (Stadtfeld, et al., 2008b).

Yu et al. (2009) reported successful generation of iPSC using the episomal vectors without the integration of the transgenes and vector. The episomal vector was based on the oriP/EBNA1 vector which was derived from the Epstein-Barr virus (Yu, et al., 2009). This vector is suitable for reprogramming of human somatic cells because these plasmids do not need packaging cells to produce the virus particle and can be easily removed from the culture by stop drug selection (Yu, et al., 2009).

The oriP/EBNA1 vector is capable to replicating only once per cell cycle and will lost from cell population at around 5% per cell generation once selection is stop due to the defects in plasmid synthesis (Yates & Guan, 1991). The vectors are diluted out per cell division and thus, PCR analysis not able to detect any episomal vectors present in the cell genome (Yu, et al., 2009).

The drawback for episomal vector reprogramming is that the efficiency in generating iPSC is low. In the study of Yu et al. (2009), the authors were only able to derived 3 to 6 colonies per million of input cells (~0.0003 to ~0.0006%). In another episomal vector reprogramming by Okita et al. (2011), they generated iPSC with efficiency of 0.00039% which is roughly the same with Yu et al. (2009). The only difference between these two studies was the former group generated iPSC using seven factors and the latter group used 6 factors and substitute the c-Myc with L-Myc (Yu , et al., 2009; Okita , et al., 2011)

Another type of non-integrating method for generation of iPSC is by using sendai virus. Sendai virus is a type of single-stranded RNA virus (Fusaki, et al., 2009). Sendai virus able to infect a number of host cells and the most important is that it is non-pathogenic to human (MacArthur, et al., 2012). Sendai virus vectors will replicate in the cytoplasm of the cells and does not go through DNA phase. Thus, the transgenes do not integrate into the genome of the host (Fusaki & Ban , 2013). The virus vectors stably express the reprogramming factors that will continue to induce the cells into iPSC (Fusaki, et al., 2009).

The efficiency of reprogramming for using sendai virus varies from 0.005% to 1% depends on different conditions and factors (Ban, et al., 2011; Fusaki, et al., 2009). This proved that the efficiency for reprogramming using sendai virus could be matched with the use of integrating viruses such as lentivirus and retrovirus. But, this is also much depends on the number of transcription factors, type of cells and other factors being used (Ban, et al., 2011; Fusaki, et al., 2009; MacArthur, et al., 2012).

Induced pluripotent stem cell can also be generated from synthetic modified mRNA. In nature, RNA is unstable and the control of protein expression by modified mRNA will peak at 16 hours before its activity diminish after 36 hours (Mandal & Rossi, 2013). The whole process of reprogramming required at least 2 weeks and maximum at 4 weeks (Warren, et al., 2010). There is need to establish continuous reprogramming factors protein expression to support the conversion to pluripotent cells (Warren, et al., 2010).

The strategy involved repeated administration of modified mRNA into the culture (Warren, et al., 2010). The repeated administration was repeated for a total of 2 weeks and the continuous administration of the mRNA is the drawback of this method (Mandal & Rossi, 2013).

In the study by Warren et al. (2010), the authors were able to generate iPSC from various sources at the efficiency from 2 – 4.4%. This method produced the iPSC with the highest efficiency and without any genomic integration of the transgenes (Warren, et al., 2010). This method also allowed the control of expression of different transcription factors (Warren, et al., 2010). Further investigation to find out the expression of different factors at different time points could increase the understanding of the process and would lead to further improvement of the reprogramming efficiency (Warren, et al., 2010).

Micro RNA (miRNA) play a role in maintain ESC self-renewal, pluripotency, and differentiation (Mallanna & Rizzino, 2010). Micro RNA have been used to generate iPSC. A combination of miRNAs family was used for reprogramming and the combination consists of mir-200c, mir-302 s, and mir-369 s (Miyoshi, et al., 2011). Since the miRNA levels decreased drastically 72hours after transfection, four rounds of transfections were introduced at 48 hours intervals (Miyoshi, et al., 2011). The authors also reported a comparable efficiency to the reprogramming efficiency of that using retroviral based (Miyoshi, et al., 2011). This method do not cause integration of the transgene, thus it involved lower risk for mutation and tumourigenesis (Miyoshi, et al., 2011).

The use of protein-based transcription factors to induce reprogramming is among one of the safest method to generate iPSC (Kim, et al., 2009). The reprogramming factors proteins with the polyarginine peptide tags called the polyarginine cell penetrating peptide were used (Kim, et al., 2009). In a study by Kim et al. (2009), the starting cells were human fibroblasts. The authors successfully generated iPSCs using this method and showed characteristics of hESC in term of morphology, proliferation rate and expression of pluripotency markers, and the *in vitro* and *in vivo* three germ layers formation (Kim, et al., 2009). The authors further added that by using this method, the benefits were eliminating the risks of using viruses, DNA transfection and harmful chemicals and this method further provide a safe source of iPSCs for clinical use (Kim, et al., 2009).

The half-life of the reprogramming proteins is short thus the need for continuous supply of the reprogramming factors (Kim, et al., 2009). The developed the method that needed at least six cycles of transduction to successfully generate iPSCs (Kim, et al., 2009). Although the efficiency of this method was only 0.001% which is 10 fold lower than the virus method and also required longer time compared to the virus method, the two iPSC lines that they establishes were stable and can be maintained for more than thirty five passages (Kim, et al., 2009). The authors proved that the protein-mediated reprogramming is working and further improvements should be made such as the use of purified reprogramming proteins and further optimisation is needed (Kim, et al., 2009).

2.2.2.3 Excision-based Methods

IPSC is generated using the existing genomic integration method but later, the transgenes are removed from the host genome. The two methods that can be used for transgenes excision are cre-mediated recombinase and Piggybac transposon (Sommer, et al., 2010). Cre-mediated recombinase uses either Adeno-Cre mediated recombination or Cre-expression plasmids for the transient expression of Cre recombinase (Sommer, et al., 2010). This Cre recombinase will act as scissors to cut out the DNA sequence that present between the loxP sites (Sommer, et al., 2010).

This loxP is introduced into the viral vector flanking the both side of the reprogramming factors (Sommer, et al., 2010). However, the authors also emphasised on the viral LTR that left in the genome of the iPSC after excision of the transgenes (Sommer, et al., 2010). This 200bp DNA sequence was inactive and does not possessed promoter or enhancer activity (Sommer, et al., 2010). The integrated DNA from exogenous sources could always possess risk of insertional mutagenesis (Sommer, et al., 2010).

Piggybac transposon utilises plasmid to deliver the reprogramming factors into the cells (Woltjen, et al., 2009). Introduction of transposase to the cells will remove completely the Piggybac transposon which carrying the reprogramming factors (Woltjen, et al., 2009). The efficiency of the excision of the transgenes was more than 90% by analysis of the original insertion site of Piggybac transposon in the cells, which was reverted back to wild type (Woltjen, et al., 2009). The Piggybac transposon capable of delivery big size

DNA fragments up to 10 kb (Yusa, et al., 2009). The reported reprogramming efficiency was average of 2.5%, but the authors used a different way to calculate the overall reprogramming efficiency (Kaji, et al., 2009).

Apart from the reprogramming methods, there are ways to enhance the efficiency of reprogramming by using histone deacetylases (HDAC) inhibitors, histone methylases inhibitors, histone methyltransferases inhibitors (Oh, et al., 2012). The use of these small molecules involved in the modulation of chromatin modifications (Oh, et al., 2012). For example, in a study by Huangfu et al. (2008), the authors used valproic acid (VPA), an HDAC inhibitor coupled with the retroviral transduction (Huangfu, et al., 2008). It was reported that the reprogramming efficiency was improved by more than 100 fold (Huangfu, et al., 2008). The authors also reported that reprogramming efficiency with three reprogramming factors and VPA and in the absence of c-Myc was increased by 50 fold (Huangfu, et al., 2008).

2.2.3 Genes Involved In Reprogramming

IPSC can be generated through forced expression through Yamanaka factors (OSKM) or Thomson factors (OSNL). These factors involved in reprogramming included Oct4, Sox2, Klf4, c-Myc, Nanog, and Lin28 in both set of transcription factors.

2.2.3.1 Oct4

Oct4 (octamer-binding transcription factor 4) or Pou5f1 (POU domain, class 5, transcription factor 1) because Oct4 is encoded by the Pou5f1 gene.

POU domain of the Pou5f1 is highly conserved within the POU transcription factors superfamily. POU transcription factors are DNA-binding proteins which contain an octamer motif (of ATGCAAAT) within their promoter region (Pesce & Scholer, 2001).

Oct4 is an important regulator of pluripotency, regulator of the cells fate towards the germline cells, and Oct 4 is also the regulator factor that first present in the cells of the embryo (Niwa, et al., 2000). The Oct4 deficient embryos failed to form the mature inner cell mass (ICM) and will not formed extra-embryonic endoderm (Nichols, et al., 1998). When the Oct4 deficient embryos were put into *in vitro* culture, the supposing inner cell mass cells differentiate into the trophectoderm lineage (Pesce & Scholer, 2001).

The expression of Oct4 is tightly regulated. So, any changes to the amounts of Oct4 will lead to different fate for the embryonic stem cells. For example, an increase of 50% of Oct4 from normal level will caused differentiation into endoderm and mesoderm lineages. Meanwhile, a drop of 50% of Oct4 from normal expression level will cause the loss of pluripotency and differentiation into trophectoderm lineage (Niwa, et al., 2000).

Oct 4 is the most common transcription factors used in the production of iPSC. Oct 4 is working closely together with Sox2 and Nanog in regulating the pluripotency. Boyer et al. (2005) reported that all these three transcription factors bound to the same enhancer elements of genes that are involved in pluripotency and differentiation. Boyer et al. (2005) also showed that 352

genes are occupied by these three factors at the same time in human ESC. At the same time, Oct4, Sox2 and Nanog are also occupying their own promoter region and forming an interconnected autoregulation loop. These three factors will either positively or negatively regulate their own level of expression in the cells to maintain the pluripotency of ESC (Boyer, et al., 2005; Pan & Thomson, 2007).

2.2.3.2 Sox2

Sox2 or SRY (sex determining region Y)-box 2 is another important transcription factor being used frequently in the production of iPSC. Sox2 can only be substituted when there is endogenous expression of Sox2 in the target cells. The Sox family share the same highly conserved HMG box-DNA binding domains which make up of around 80 amino acids. Sox proteins have been studied extensively mainly related to mammalian cell development (Rizzino, 2009).

As explained above that the presence of Oct4 is required for the formation of ICM or else the Oct4 deficient inner cell mass (ICM) cells will differentiate into trophectoderm lineage. Avilion et al. (2002) presented that the Sox2 is also required for the ICM and epiblast formation, but, the level of Sox2 only detected after implantation. The presence of Sox2 protein in the pre-implantation stage could be of the maternal and the level of endogenous Sox2 spike up after implantation could be due to maternal Sox2 already used up due to the embryo rapid growth (Avilion, et al., 2002).

In the condition where Sox2 is absence, there is lack of rapidly proliferating cell and only the trophoblast giant cells and extraembryonic endoderm will survive. Thus, the presence of Sox2 is essential for epiblast cell formation, but, without Sox2, the cells will differentiate into other cell type (Avilion, et al., 2002).

Sox2 function in maintaining pluripotency and also in self-renewal in ESC. According to Avilion et al. (2002), the authors reported that ESC cannot be produced from the Oct4 deficient embryos. Sox2 together with Oct4 co-occupy the regions of genes involved in the pluripotency and also differentiation. This finding raised an interesting fact that apart from maintaining pluripotency which is seen as their main job. Both factors also play a role in differentiation process whereby during undifferentiated state, the differentiation factors are being suppressed and when in the differentiated state, both factors can readily activate the differentiation factors (Zhang & Cui, 2014).

A study by Masui et al. (2007) reported that Oct4 gene are not only bound by Sox 2 but also other Sox proteins such as Sox4, Sox11, and Sox15. Even though, all four Sox proteins are reported to be able to bind to the HMG/POU cassetes and activate the promoters. But, only Sox2 is involve in the maintainance of pluripotency and self-renewal of ESC. Furthermore, Matsui et al. (2007) reported that overexpression of Oct4 can rescue the knockdown effect of Sox2 in ESC by having both the self-renewal ability and differentiation ability back. This finding showed that Sox2 works in a way to

maintain the level of Oct4 in ESC. However, this finding also showed that Sox2 and Oct4 not necessarily have to worked together in maintaining pluripotency which is never been proved before (Ralston & Rossant, 2010).

2.2.3.3 Klf4

Klf4 or Kruppel-like factor 4 is another transcription factor used in reprogramming by the Takahashi group. Kruppel-like factors family consists of 17 members in which involves in various cellular processes such as cellular growth, differentiation, inhibit differentiation and induces pluripotency (El-Karim, et al., 2013). Klf4 consists of three zinc finger domains, which act as DNA binding site (Ralston & Rossant, 2010).

Klf4 is important for pluripotent stem cells self-renewal and maintaining pluripotency in the presence of other more important transcription factors, Oct4, Sox2 and c-Myc, but its presence is replaceable. Li et al. (2005) reported that overexpression of Klf4 was able to maintain pluripotency by inhibiting the differentiation process. The authors performed a secondary embryoid bodies (EB) assay and found out that the Klf4-tranduced EBs expressed 7 folds higher Oct4 level compared to empty vector or SOCS3-transduced EBs at day 6.

The presence of Klf4 alone is considered as not important for pluripotent cells self-renewal and pluripotency and can be replaced. In the study conducted by Nakagawa et al. (2008), Klf4 can be replaced by other kruppel-like factor protein. The authors used Klf1, Klf2, and Klf5 as

replacement for KLF4 and found out that Klf2 can be used for reprogramming with the same efficiency as Klf 4, and both Klf1 and Klf5 can replaced Klf4 as reprogramming factor but with lower efficiency.

Furthermore, other Krupple-like factors could play a role in the self-renewal process. Jiang et al. (2008) reported that by inhibiting the expression of single Klf (Klf2, 4, and 5) at a time by RNAi, the morphology of ES cells did not changed or it means no differentiation occurred. This is because in the absence one of the Klf proteins, the other Klf proteins can bind to the same gene to exert its functions. But, once three of the Klfs were knockdown, the ES cells differentiated and formed fibroblastic cells. In the same study by Jiang et al. (2008), the chromatin immunoprecipitation analysis revealed that the Klfs bounds to same target site of important pluripotency-related regulator genes such as Oct4, Sox2, Nanog, Essrb and others.

2.2.3.4 c-Myc

c-Myc belongs to the Myc family together with B-Myc, L-Myc, N-Myc, s-Myc. c-Myc is a proto-oncogene same as L-Myc and N-Myc, which involved in cellular processes such as cell growth, apoptosis, and metabolism. When dysregulated, these proto-oncogenes which involved in important cellular processes; will become oncogenes and will cause cancer. It was reported that c-Myc regulate self-renewal and pluripotency mainly through the blocking of differentiation by control of histone acetylation and specific micro RNAs (Lin, et al., 2009; Araki, et al., 2011).

c-Myc also found to be replaceable same to the Klf4 or even omitted. In a study by Nagakawa et al., (2008), they found out that other Myc protein such as N-Myc and L-Myc were able to replace c-Myc as reprogramming factor with almost equivalent efficiency.

In the case of iPSC generation without c-Myc, it is true that the authors (Nagakawa, et al., 2008; Araki, et al., 2011) omitted the c-Myc and reported successfully generated the iPSC. But, in study by Nagakawa et al. (2008), the authors reported that the MEFs also expressed endogenous c-Myc at the level of 20% of the mouse ESC. In Araki et al. (2011) study, the target cells used for reprogramming also MEFs. Thus, in the absence of exogenous c-Myc, the endogenous c-Myc could be recruited to participate in the reprogramming processes (Nagakawa, et al., 2008).

Furthermore, Thomson group (Yu, et al., 2007) also reported that their group was able to reprogramme neonatal fibroblasts without the conventional Yamanaka factors. Instead, this group used Oct4, Sox2, Nanog, and Lin28 for generation of iPSC. Lin28 could play a role in activating the endogenous Myc gene through activation of insulin-like growth factor 2 (Nagakawa, et al., 2008). Nagakawa et al. (2008) further reported that the reprogramming can proceed without c-Myc but the overall efficiency in generation of iPSC has decreased substantially.

2.2.3.5 Lin28

Lin28 is a gene first identified in nematodes, responsible for the self-renewal (Ralston & Rossant, 2010). Lin28 is a RNA-binding protein that was being used in reprogramming as one of the Thompson factor together with Oct4, Sox2, and Nanog (Yu, et al., 2007). It was reported that Lin28 can affect the frequency of the emergence of iPSC colonies and Lin28 presence was neither required for reprogramming nor for establishing the stable clones (Yu, et al., 2007).

Lin28 have been reported to be present in mouse and human ESC (Polesskaya, et al., 2007; Richards, et al., 2004) and its level will diminish after differentiation (Viswanathan, et al., 2008). Let-7 miRNA involved in the differentiation process by promoting differentiation upon maturation (Mallanna & Rizzino, 2010). It was reported that the expression of let-7 was regulated tightly and specifically by Lin28 (Viswanathan, et al., 2008). Lin28 completely blocked the processing of primary let-7 to precursor let-7 (Viswanathan, et al., 2008). Thus, Lin28 prevent the pri-let-7 from being transported out to the cytoplasm and prevent its maturation. This post-transcriptional control prevents the synthesis of mature let-7 in ESC and thus maintaining it in the undifferentiated condition (Viswanathan, et al., 2008).

In order to test for the specificity of Lin28, four primary miRNA, namely pri-let-7a, pri-let-7g, pri-miR-15a, and pri-miR-122 were transfected into 293T cells, which lack Lin28, in the presence or absence of Lin28 (Viswanathan, et al., 2008). The processing of pri-let-7a and pri-let-7g was

completely blocked but processing of pri-miR-15a, and pri-miR-122 was undisrupted (Viswanathan, et al., 2008). In ESC, since the maturation of pri-let-7 was blocked, the level of mature let-7 will decreased while the level of pri-let-7 was abundant (Viswanathan, et al., 2008). The promoters region of let-7 and Lin28 was pre-occupied by the Oct4/Sox2/Nanog/Tcf3 and this suggests that the transcription factors co-operate in regulating the let-7 and Lin28. This could be the reason that the Lin28 can be omitted from the reprogramming (Yu, et al., 2007).

2.2.3.6 Nanog

Nanog protein is made up of 305 amino acids and contains a conserved homeodomain region which is for DNA binding (Mitsui, et al., 2003). Nanog is one of the transcription factors in the Thompson factors, which was used in the reprogramming (Yu, et al., 2007). Nanog function in reprogramming is the same as Lin28 which mean that it is not required for initial reprogramming, but for cells self-renewal (Yu, et al., 2007). Nanog could be playing a role in ESC self-renewal through the inhibition of genes that are involved in differentiation (Mitsui, et al., 2003).

In the presence of Nanog when initial reprogramming, the survival rate of the reprogrammed cells increased (Yu, et al., 2007). Two events that explained the importance of Nanog when it is overexpressed were mouse ESC self-renewal can be maintained without the presence of leukemia inhibitory factor (LIF) bypassing the important pluripotent maintaining pathway, LIF-STAT3 pathway (Mitsui, et al., 2003; Chambers, et al., 2003) and the growth

of hESC can be maintained without the presence of feeder layer (Pan & Thomson, 2007).

Nanog-null mouse embryos failed to developed into blastocyst stage because without the presence of epiblasts (Mitsui, et al., 2003). This can explain the function of Nanog in maintaining the epiblasts. In both the mouse and human ESC, the absence of Nanog will promote differentiation of these cells into endoderm and trophectoderm lineages (Hyslop, et al., 2005).

Nanog was found to be regulated by the Oct4/Sox2 motif upstream of the promoter site (Kuroda, et al., 2005). However, the behaviour of both Nanog and Oct4 are totally different. Nanog downregulation in ESC will leads to endoderm lineage differentiation while Oct4-downregulation in ESC will cause trophectoderm differentiation (Hyslop, et al., 2005; Niwa, et al., 2000). Overexpression of Oct4 will leads to differentiation of ESC while overexpression of Nanog will leads to self-renewal (Pan & Thomson, 2007). These could explain the opposite function for Oct4 and Nanog (Mitsui, et al., 2003). Nanog not only prevents differentiation but also maintains pluripotency (Mitsui, et al., 2003).

Through a detailed molecular study, Chang et al. (2009) proposed that Nanog recruits either co-activators or co-repressors through the interaction with the C-terminus or N-terminus. This recruitment process will determine whether the interaction will activate or repress the promoter and thus, regulate the self-renewal activity in pluripotent stem cells (Chang, et al., 2009). The

examples of co-activators such as OCT4 and SALL4, while the examples of co-repressors such as HDAC2, polycomb, SWI/SNF, and NuRD complexes) (Chang, et al., 2009).

2.3 Embryonic Stem Cells

In 1998, embryonic stem cells were first isolated to be cultured *in vitro*. In that year, two groups successfully isolated ESCs from mouse embryos during the pre-implantation stage, which is at the blastocysts stage (Evans & Kaufman, 1981; Martin, 1981). Evans et al. (1981) reported the success of establishment of pluripotent cells from the mouse embryo's inner cell mass (ICM). The ICM cells were then plated on inactivated-STO fibroblasts feeder cells (Evans & Kaufman, 1981). The pluripotent cells were culture in Dulbecco's modified minimal essential medium with addition of 10% fetal calf serum and 10% newborn calf serum (Evans & Kaufman, 1981).

The authors called the isolated pluripotent cells from mouse embryos as EK cells (Evans & Kaufman, 1981). EK cells grow rapidly and were able to passage up to more than thirty passages *in vitro* (Evans & Kaufman, 1981). The EK cells formed embryoid bodies when cultured in suspension without the presence of feeder cells and formed outgrowth after being re-plated (Evans & Kaufman, 1981). When the EK cells were injected into the mice, all the mice developed tumours known as teratocarcinomas or teratoma (Evans & Kaufman, 1981).

Gail R. Martin was the first to use the term "embryonic stem cells" for the pluripotent cells isolated from the mouse embryo (Martin, 1981). The method of ESCs derivation was different for both papers. Evan's group isolated the ESCs from the large egg cylinder-like structures formed from the plated inner cell mass cells (Evans & Kaufman, 1981). The large egg cylinder-like structures were then dispersed and plated on feeders (Evans & Kaufman, 1981). While for the Martin's group, the ESCs were derived from the embryos ICM by immunosurgery (Martin, 1981). The inner cell mass cells were then plated on STO fibroblasts feeder layer (Martin, 1981).

The ESCs were cultured in conditioned medium from the embryonal carcinoma cell culture (Martin, 1981). The conditioned medium was mixed with fresh medium in 1:4 ratios with supplements of 10% calf serum and 0.1mM 2-mercaptoethanol (Martin, 1981). The ESCs isolated were also able to form tumour when injected into athymic mice (Martin, 1981). The teratocarcinoma formed contained the derivatives of the embryonic three germ layers (Martin, 1981). The authors further proved that each cells of the ESCs are pluripotent. A single cell was isolated and injected into athymic mice and tumour was developed (Martin, 1981). The ESCs also differentiated *in vitro* through EB formation. The migrating cells from the EBs were found belongs to the cells of three germ layers (Martin, 1981).

In 1998, Thompson's group reported the success of derivation of the human embryonic stem cells, seventeen years after the discovery of mouse ESC (Thomson, et al., 1998). Human ESC was derived from embryos

produced by *in vitro* fertilisation (Thomson, et al., 1998). The blastocyst stage embryos were used to isolate the ICM *via* immunosurgery (Thomson, et al., 1998). The ICM cells were plated on irradiated MEF with culture medium consists of Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 1mM glutamine, 0.1mM b-mercaptoethanol, and 1% nonessential amino acid (Thomson, et al., 1998).

The human embryonic stem cells possesses the following characteristics, such as the cells have prominent nucleoli with high ratio of nucleus to cytoplasm, retained normal karyotype even after *in vitro* culture for six months, possess high telomerase activity, and expressing SSEA3- SSEA-4, TRA-1-60, TRA-1-81, and alkaline phosphatase (Thomson, et al., 1998). When the ESCs were injected into immunodeficient mice, all mice formed tumour (Thomson, et al., 1998). The teratomas were cells belong to the three germ layers, such as gut epithelium (endoderm), bone, and neural epithelium. The hESCs were also differentiated *in vitro* without the presence of feeder layer (Thomson, et al., 1998). After 2 weeks, alpha-fetoprotein (endoderm) and human chorionic gonadotrophin (trophoblast) were detected in the culture medium (Thomson, et al., 1998).

2.4 Induced Pluripotent Stem Cells and Embryonic Stem Cells

IPSCs closely resemble ESCs but however iPSCs can be distinguished from ESCs. Numerous studies have reported that iPSCs and ESCs are indeed different subtype of pluripotent cells (Chin, et al., 2009; Ohi, et al., 2011) but there were other studies that proved it was difficult to differentiate between

iPSCs and ESCs (Bock, et al., 2011). These studies reported the differences based on gene expression, DNA methylation, miRNA expression, genomic stability, histone modification profiling, and by the ability to differentiate (Chin, et al., 2009; Ohi, et al., 2011; Bock, et al., 2011).

In 2009, Chin et al. (2009) reported that iPSCs are a unique subtype of pluripotent cell, separating it from ESC. The microarray expression study revealed that almost 860 genes differentially expressed between the late-passage human iPSCs and human ESCs (Chin, et al., 2009). The difference suggested that the gene expression pattern of original fibroblasts cells was not completely silenced and also the failure to activate the gene expression important for undifferentiated ESCs (Chin, et al., 2009).

The miRNA profiling also showed little difference between the miRNA expression for iPSCs and the miRNA expression for ESCs (Chin, et al., 2009). But, there were few miRNAs that expressed differently between iPSCs and ESCs in a consistent way. In histone modification profiling, the methylation pattern of histone H3K27 trimethylation for both iPSCs and ESCs showed almost 97% identical (Chin, et al., 2009). Lastly, the genomic stability analysis revealed a normal and stable genome for iPSCs. But, the authors reported the presence of some sub-karyotypic alterations in iPSCs compared to fibroblasts, which were not specific to each iPSC lines (Chin, et al., 2009).

Ohi et al. (2011) reported that although the hierarchical clustering classified the iPSC lines and ESC lines as clustered together, but there were

gene expression difference among both cell types due to incomplete silencing of somatic cell genes. The global pattern of differential gene expression showed that genes that commonly expressed in somatic cells in high level were repressed, but, in comparison to ESCs, the level expressed in iPSCs was still considered high (Ohi, et al., 2011).

Using a statistical method, Differential Expression *via* Distance Synthesis, to analyse the gene expression data, 50-60% of genes analysed showed significant difference expression between iPSCs and ESCs that represent a memory of the difference between the somatic cells and ESCs (Ohi, et al., 2011). This suggested that a significant number of genes expression were higher in iPSCs compared to ESCs was due to incomplete silencing of the somatic genes (Ohi, et al., 2011). On the other hand, a significant number of genes expression were lower in iPSCs compared to ESCs was due to incomplete reactivation of the pluripotency-related genes (Ohi, et al., 2011).

In the study by Bock et al. (2011), the authors compared 20 ESC lines and 12 iPSC lines by comparing DNA methylation, gene expression profiling, and quantitative differentiation assay. An ESC reference was based on the 20 ESC lines and was set up using the data obtained from DNA methylation and gene expression profiling assays (Bock, et al., 2011). The hierarchical clustering showed that all iPSC lines clustered together with the ESC lines, and not clustered to fibroblasts (Bock, et al., 2011).

The iPSC lines showed similar deviation as observed with ESC lines (Bock, et al., 2011). The majority of the genes showed similar deviation averages in all ESC and iPSC lines (Bock, et al., 2011). But, a small number of genes were seen to deviate from the ESC reference in the iPSC lines such as HTRA4, RLN1/2, and others (Bock, et al., 2011). By studying the HTRA4 genes methylation, this gene was hypermethylated in most iPSC lines compared to none in ESC lines (Bock, et al., 2011). This gene also hypermethylated in fibroblasts and this explained the reason of incomplete silencing as potential deviation of iPSC lines from the ESC reference (Bock, et al., 2011).

The authors developed a more accurate classifier using the data obtained to differentiate between an ESC lines and iPSC lines (Bock, et al., 2011). The authors concluded that mostly, but not all, iPSC lines expressed similar DNA methylation and gene expression profiles that can differentiate them from ESC lines (Bock, et al., 2011). In the study, the authors also developed deviation scorecard and lineage scorecard that able to predict the quality and the utility of the studied iPSCs and ESC lines (Bock, et al., 2011).

In a review paper by Yamanka (2012), there was an obvious difference in the studies conducted to support difference or similarity among iPSC and hESC. The studies that reported the difference between iPSC and hESC compared lower number of cell lines while the studies that reported the similarity among both lines compared more cell lines across multiple laboratories (Yamanaka, 2012)

These studies showed some degree of overlapping between iPSC and ESC. Although iPSC showed higher variation among clones and some showed variations from ESC expression, it should be noted as well that some iPSC clones are indistinguishable from ESC (Yamanaka, 2012). The author also added that differences between iPSC clones are largely attributed to factors such as factors combinations, reprogramming methods, culture conditions adapted, and stochastic events during reprogramming (Yamanaka, 2012).

2.5 Applications of Induced Pluripotent Stem Cells/Embryonic Stem Cells

The discovery of ESCs has bring several benefits such as to understand the underlying disease mechanisms, for drug screening by creating a disease modelling, or to treat patients through cell or tissue replacement therapy (Thomson, et al., 1998; Takahashi, et al., 2007). However, the use of ESCs itself brought a lot of problems regarding ethical issues surrounding the use of human embryos to derive the ESC lines or the immune rejection of ESCs derived cells or tissues for the use of transplantation (Thomson, et al., 1998). With the discovery of iPSCs, it avoids the same issues that surrounding the usage of ESCs.

2.5.1 Disease Modelling

iPSC is pluripotent stem cells which possess the ability of stem cells to indefinite self-renewal (Stadtfeld & Hochedlinger, 2010). iPSC also able to differentiate into three germ layers (Colman & Dreesen, 2009). Due to their abilities, it is possible to create a disease model that is able to recapitulate the

disease pathogenesis *in vitro* (Sterneckert, et al., 2014; Kanherkar, et al., 2014). This disease modelling enables the study of the mechanisms and pathways involved in the progression of the diseases (Kanherkar, et al., 2014). Apart from studying the mechanisms and pathways, it also enables the discovery of new biomarkers involved in the diseases and unlimited supply of affected cell sources for drug screening (Colman & Dreesen, 2009).

The generation of disease model from ESCs has been restricted and can only be generated through modification of available ESCs or to generate new ESCs with embryo carrying the diseases (Colman & Dreesen, 2009). To generate iPSC, any cell types from the patients suffering from any diseases can be extracted (Colman & Dreesen, 2009). Various studies had reported the generation of iPSC from patients suffering from diseases such as spinal muscular atrophy (Ebert, et al., 2009), Parkinson's disease (Soldner, et al., 2009), Hutchinson-Gilford Progeria Syndrome (Liu, et al., 2011), Down's syndrome (Hibaoui , et al., 2014), and others.

Hutchinson-Gliford Progeria Syndrome (HGPS) is a premature aging disease, with noted premature arteriosclerosis and vascular smooth muscle cells deterioration (Liu, et al., 2011). HGPS is cause by mutation to the LMNA gene, which responsible to produce laminin A (Liu, et al., 2011). The mutation will cause the production of progerin, a type of defective laminin A and the progerin build-up will eventually cause nuclear defects and finally the premature aging of the cells (Liu, et al., 2011).

iPSCs were generated from the HGPS patient fibroblasts. The iPSCs were found to express pluripotent genes, demethylation of Oct4 promoter, and able to be passaged for more than 50 passages (Liu, et al., 2011). The iPSCs did not produce any progerin and lack the nuclear defects and epigenetic alterations associated with HGPS conditions (Liu, et al., 2011). Upon differentiation into smooth muscle cells, progerin was detected and the cells showed premature senescence phenotypes (Liu, et al., 2011). This disease model for HGPS can use to study the pathogenesis of human aging-related vascular diseases (Liu, et al., 2011).

2.5.2 Cell/Tissue Replacement Therapy

Although the methods for reprogramming, and differentiation protocols are improving, but there are still a lot of issues to be addressed (Stadtfeld & Hochedlinger, 2010). Nevertheless, the use of iPSC in cell/tissue replacement therapy is very promising. The use of patient-specific iPSCs may prevent tissue rejection (Kanherkar, et al., 2014); thus, eliminating the use of immunosuppressive drugs (Stadtfeld & Hochedlinger, 2010). The replacement therapy works by reprogramming, genetic engineering by gene editing, and lastly cellular therapy by transplantation of the fixed cells back to the patient (Kanherkar, et al., 2014).

By using gene editing technology, genetic defects can be corrected and the corrected cells can be used to replace the damaged tissue and thus cure the genetic or degenerative diseases (Kanherkar, et al., 2014). This technique was easy to manipulate since the recent advancement and results in high precision

editing (Kanherkar, et al., 2014). The types of gene editing methods are zinc finger nucleases (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeats (CRISPR) (Kanherkar, et al., 2014).

In a study by Hanna et al. (2007), the authors showed that the humanized mouse model with sickle cell anemia was rescued after transplantation with genetically corrected-hematopoietic progenitor cells. The fibroblasts were obtained from the tail-tip of the mice (Hanna, et al., 2007). The fibroblasts were induced into iPSCs and the iPSCs were confirmed for its identity (Hanna, et al., 2007). The iPSCs were differentiated into hematopoietic progenitors (Hanna, et al., 2007). The human sickle hemoglobin allele of the hematopoietic progenitor was then corrected using homologous recombination and transplanted into the mice (Hanna, et al., 2007). The mice were shown to be rescued from the disease phenotype (Hanna, et al., 2007).

In a mice study for spinal cord injury, the mouse iPSCs were generated and were induced to differentiate through embryoid bodies formation with the presence of retinoic acid into neurospheres (Tsuji, et al., 2010). Before injected into the mice, the neurospheres were evaluated for its safety by transplantation into severe combined immunodeficiency (SCID) mice and no tumour was observed (Tsuji, et al., 2010). The neurospheres were then injected into mice with spinal cord injury (Tsuji, et al., 2010). The neurosphere differentiated into the neural lineages participate in the recovery of the serotonergic fibres thus promote movement recovery (Tsuji, et al., 2010).

2.6 Advancements in Induced Pluripotent Stem Cells Research

From the literature review above, we can see that iPSCs is a promising cell types for future personalised therapy. iPSCs can be used in cell or tissue replacement therapy or serve as disease modelling to know the underlying disease mechanism and the model can be used for testing and discovery of new drugs (Kanherkar, et al., 2014). Although, there are many more issues to be addressed, the advancement in certain areas such as safe, non-integrating method for reprogramming, culture conditions that is safe for clinical use, and discovery of more efficient methods for iPSCs differentiation.

The use of integration and viral methods has been shown to increased risk for tumourigenesis due to reactivation of the transgene or due to the viral insertion mutagenesis (Okano, et al., 2013). Now, the generation methods focus using the insertion-free method with the view of translating the iPSCs to clinical use (Okano, et al., 2013). As the literature review above have mentioned, there are various methods for generation of iPSCs without using virus and non-integrating such as modified mRNA (Warren, et al., 2010), episomal vectors (Yu, et al., 2009), plasmids, recombinant proteins, and microRNA (Miyoshi, et al., 2011).

Apart from that, the efficiency in generation of iPSCs also had been an issue. The use of chemical compounds or small molecules could improve both the quality of iPSC and efficiency of reprogramming (Okano, et al., 2013). Chemical compounds such as the inhibitors for histone deacetylase and protein kinases have been used in various studies (Okano, et al., 2013).

The culture of iPSCs with feeder layer and serum-containing medium was adapted from the culture of ESCs. The first generation of iPSCs was cultured on mouse embryonic fibroblasts feeder layer with serum containing-conditioned medium (Takahashi & Yamanaka, 2006). The culture condition of feeder-free and serum free has been used since before the discovery of iPSCs (Amit, et al., 2004). Although there are various commercial extracellular matrices such as Matrigel, Geltrex and CELLstart, but some contained animal products and only Matrigel is the most commonly used for feeder-free culture (Denham, et al., 2009).

Apart from the commercial extracellular matrices, various types of matrices are available; among those matrices are laminin and vitronectin. However, the price is too costly to do large scale culturing. Hence, most laboratories decided to produced own matrices (Chen, et al., 2011; Nagakawa, et al., 2013). A study by Chen et al (2011) used vitronectin variants, VTNNC and VTN-N, to culture iPSC and ESC. The authors found that the vitronectin variants support the growth of undifferentiated ESC and iPSC (Chen, et al., 2011). In another study, recombinant laminin-515 E8 fragment was used as matrices and the authors reported that laminin-515 was selected due to its ease to obtain and able to support the iPSCs for longer periods of time and the iPSCs were suitable for clinical use as well (Nagakawa, et al., 2013).

Apart from the matrices, the culture medium use for iPSCs is also important to determine its suitability for clinical use (Nagakawa, et al., 2013). The use of serum-containing medium will bring animal-related infectious

agents into the culture and when the iPSCs to be used in clinical, it could be transmit to human (Geraghty, et al., 2014). Commercial xeno-free media can be easily purchase such as TeSR2, NutriStem, StemFit, and Essential E8 medium (Nagakawa, et al., 2013). Proper use of animal free culture medium and matrices are very important to ensure safety to use iPSCs for clinical use (Nagakawa, et al., 2013).

2.7 Application in Clinical Setting

In this literature review, the use of first hESC for human clinical trial was reported to treat spinal cord injury and the use of first hiPSC for clinical trial to treat human was reported to treat age-related macular degeneration (AMD) (Cyranoski, 2012; Sperling, 2013). In October 2010, Geron, a biotechnology company announced that the company will conduct clinical trial to treat spinal cord injury using hESCs (Alper, 2009). United States Food and Drug Administration had approved the trials and Geron was allowed to proceed with the trials on patients with complete thoracic-level spinal cord injury (Alper, 2009).

The phase 1 multi-centre trial will involve ten patients with spinal cord injury and they will receive Geron's pioneering therapy known as "GRNOPC1 product" (Alper, 2009). "GRNOPC1 product" is the oligodendrocyte progenitor cells derived from hESCs which capable of remyelinating and stimulate nerve growth (Alper, 2009). Two patients were reported to have undergone the treatment and there was no serious safety issues reported, but, no conclusion can be made before the end of the trial (Alper, 2009). In

November 2011, Geron announced that the clinical trial will be dropped entirely due to reported financial problems (Sperling, 2013).

Since the first clinical study using the hESC ended due to reported financial problems, hence, we look at the current on-going clinical trial involving human iPSCs (Sperling, 2013). In 2013, the clinical trial was approved by the Japan's regulatory authorities to start collecting samples for the clinical-iPSC pilot study (Cyranoski, 2014a; Reisman & Adams, 2014). A patient in her seventies suffering from AMD was selected for the study (Cyranoski, 2014b). AMD was characterised by degeneration of the retinal pigment epithelium due to abnormal growth of blood vessel (Kamao, et al., 2014).

In the trial, skin cells were taken from the patient and the isolated skin cells were subjected to reprogramming (Cyranoski, 2014b). The reprogrammed cells or iPSCs were then differentiated into retinal pigment epithelium (RPE) cells (Cyranoski, 2014b). The RPE cells were cultured in a way to form a monolayer sheet with diameter size of around 1cm (Cyranoski, 2014b). A sheet of RPE cells of 1.3 by 3.0 millimetres was implanted into the eyes of the patient (Cyranoski, 2014b). Although this procedure might not restore the vision of the patient, instead, any improvement or side effects from the procedure that attract interest from other researchers around the world (Cyranoski, 2014b).

Before proceed with the transplantation, Dr. Masayo Takahashi, the project leader for this clinical pilot study, had done a lot of studies to test the safety of the RPE cells (Kamao, et al., 2014). In a study, Masayo and group had to optimise the hiPSC-derived RPE cell sheets that were suitable for clinical use in term of the quality, amount, consistency, and safety (Kamao, et al., 2014). The RPE cell sheets produced by her group were able to maintain its structure through tight junctions and basement-membrane-like structures (Kamao, et al., 2014). The RPE cell sheets expressed RPE markers such as RPE65, BEST1, MERTK, BEST1, and CRALBP (Kamao, et al., 2014).

Since only small size of RPE sheets was needed, $5x10^5$ cells were enough to produced RPE sheets of 1cm in diameter (Kamao, et al., 2014). The consistency of producing the RPE sheet was very stable (Kamao, et al., 2014). Hence, the major concern surrounding the trial is about safety (Kamao, et al., 2014). In another study, Dr Masayo and group tested RPE sheets through subcutaneous and subretinal transplantation into mouse model. The study concluded that the tumorigenicity of the RPE sheets were negligible (Kanemura, et al., 2014). The cost for the transplantation is very high, thus, there is a need to scale up the procedure and at the same time reduce the cost of manufacturing (Kamao, et al., 2014).

CHAPTER 3

MATERIALS & METHODS

3.1 Chemicals and Cell Culture Reagents

Refer to Appendix A.

3.2 Cell Culture & Maintenance

3.2.1 Culture of Keratinocytes Isolated from Hair Follicles (hfK)

hfK was cultured in keratinocytes medium (DMEM: DMEM/F-12 (3:1 ratio), supplemented with 10% fetal bovine serum, 1mM sodium pyruvate, 2mM L-glutamine, 1% antibiotic-antimycotic, 0.4ug/ml hydrocortisone, 2 x 10^{-4} M adenine, 5ug/ml insulin, 1 x 10^{-10}M cholera toxin, 5ug/ml transferrin, and 10ng/ml EGF.

3.2.2 Culture of Primary Human Hair Follicular Keratinocytes (HHFK)

HHFK was cultured in keratinocytes medium supplemented with keratinocyte growth supplement and 1% penicillin/streptomycin. HHFK was expanded in tissue culture flasks for the experiments throughout this study.

3.2.3 Culture of Phoenix Cells

Phoenix cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine. Phoenix cells were expanded in 100mm tissue culture dishes for the experiments throughout this study. Phoenix cells were kindly donated from Dr. Kenneth Raj (Health Protection

Agency, UK). The Phoenix cells were originally available from Professor Dr. Garry P. Nolan, Stanford University, Stanford, California.

3.2.4 Culture of Mouse Embryonic Fibroblasts (MEFs)

MEFs were cultured in DMEM supplemented with 10% FBS and 2mM L-glutamine. MEFs were expanded in 100mm tissue culture dishes for the experiments throughout this study.

3.2.5 Culture of Human Embryonic Stem Cell (hESC) and Human Induced Pluripotent Stem Cell (hiPSC)

hESC and hiPSC on feeder layer were cultured in ESC medium consists of 80% DMEM/F12, 20% Knockout Serum Replacement (KOSR) (Invitrogen, CA), 2mM L-glutamine, 0.1mM non-essential amino acid (NEAA), 0.1mM 2-mercapthoethanol, and 10ng/ml fibroblasts growth factor (FGF-2). FGF-2 was added fresh when changing of medium. hESC and hiPSC in feeder-free condition were cultured in mTeSR1 medium. The culture plates and dishes were coated with matrigel before being used for culture. hESC and hiPSC were expanded in multi-well plates, and 35mm tissue culture dishes for the experiments throughout this study.

3.2.6 Thawing of Cells

The thawing protocols were divided into three, firstly for thawing of primary HHFK cells, secondly for thawing of iPSCs or hESCs, and lastly for other cells such as Phoenix cells and MEFs.

3.2.6.1 Thawing of HHFK

For thawing primary keratinocytes, the flask was prepared one day before for the Poly-L-Lysine coating. The HHFK cells were thawed in 37^{o}C water bath after the vial was removed from storage in liquid nitrogen. Sufficient volume of medium was added to the flask and cells suspension was transferred into the culture medium drop by drop. The flask was incubated in 37^{o}C CO_2 incubator for overnight for at least 16 hours without moving. After 16 hours, the medium was changed with fresh medium. The medium was changed every three days until the cells are ready for passaging.

3.2.6.2 Thawing of iPSCs and hESCs

For thawing iPSCs or hESCs, the dish was prepared one day before for the Matrigel coating. The cells were thawed in 37^{o}C water bath after the vial was removed from storage in liquid nitrogen. Nine millilitres of medium was added into a 15ml centrifuge tube and the cells suspension was transferred to the tube drop by drop. The cell suspension was spun at 1500rpm for 5 minutes. The supernatant was carefully removed without disturbing the cell pellet. Medium was added to the cell pellet and the pellet was resuspended gently by tapping on the tube or by gentle flushing. The cell suspension was transferred to the culture dish. The dish was incubated in 37^{o}C CO_2 incubator. The medium was changed every day until the cells ready for passaging.

3.2.6.3 Thawing of Phoenix Cells and MEFs

The cells were thawed in a 37^{o}C water bath after the vial was removed from storage in liquid nitrogen. Nine millilitres of medium was added into a

15ml centrifuge tube and the cells suspension was transferred to the tube drop by drop. The cell suspension was spun at 2000rpm for 5 minutes. The supernatant was carefully removed without disturbing the cell pellet. Medium was added to the cell pellet and the pellet was resuspended by flushing. The cell suspension was transferred to the culture dish. The dish was incubated in 37^{o}C CO_2 incubator. The medium was changed every 2 or 3 days until the cells ready for passaging.

3.2.7 Passaging of Cultured Cells

Passaging protocols were divided into three protocols, one was for passaging HHFK, Phoenix cells, and MEF, and another two protocols for passaging iPSCs and hESCs.

3.2.7.1 Passaging of HHFK, Phoenix cells, and MEF

For passaging using the first protocol, the dissociation enzyme used was 0.05% trypsin-EDTA. The medium was removed from the flask and the cells were washed with phosphate buffered saline (PBS). The PBS was discarded and sufficient volume of 0.05% trypsin-EDTA was added according to the culture flask or dish size. The flask was incubated at 37^{o}C and the incubation time depends on the cell type. The Phoenix cells and MEF took around 5 minutes or less to detach from the flask. The HHFK took around 10 minutes to detach from the flask.

After completely detached from the flask, double volume of fresh medium was added to the cell suspension. The cell suspension was transferred to a 15ml centrifuge tube and the tube was spun at 2000 rpm for 5 minutes. The supernatant was carefully removed without disturbing the cell pellet. Medium was added to the cell pellet and the pellet was resuspended by flushing. The cell suspension was removed according to desire splitting ratio. The remaining cell suspension was transferred to the culture dish. The dish was incubated in 37^{o}C CO_2 incubator. The medium was changed every 2 or 3 days until the cells ready for passaging.

3.2.7.2 Passaging of iPSCs and hESCs

There were two protocols for passaging iPSC or hESC, either enzymatic using dispase or mechanical using blade and scapel. For enzymatic method, the medium was removed from the dish and the cells were washed with phosphate buffered saline (PBS) for once. The PBS was discarded and sufficient volume of 1mg/ml of dispase was added to the dish. The dish was incubated at 37^{o}C CO_2 incubator for around 5 minutes or until the border of the colonies start to fold up. After the border folded, the dish was removed from the incubator. Around 1ml of PBS was added to the dish and by using a 5ml pipette tip, the colonies were scrapped from the dish surface.

After all colonies had been lifted, transferred all the colonies to a 15ml centrifuge tube and spun at 1500 rpm for 5 minutes. The supernatant was removed and fresh medium was added to the cell pellet. The cell pellet was resuspended gently to avoid the cells dissociate into single cells. The cells were

split according to desired ratio and plated the cells back to the matrigel-coated dish. The dish was placed back to 37^{o}C CO_2 incubator. The medium was changed every day until the cells ready for passaging.

For mechanical passaging using scalpel and blade, a new blade and autoclaved scalpel were used for each time. The cells were removed from the incubator and each colony was cut in grid as shown in Figure 3.1. The amount of pieces can be cut is dependent on the size of the colony. The grid was cut on the undifferentiated colonies only. After each colony had been cut, each piece was lifted using the scalpel. After all the pieces have been lifted, the pieces were transferred to new dish.

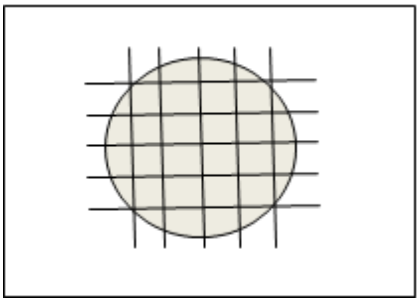

Figure 3.1 Mechanical method of cutting an iPSC or hESC colony. Each colony was cut in grid as shown in the picture above before being lifted up by using the scalpel.

3.2.8 Cryopreservation of Cells

Cells were typsinised or cut into small pieces according to the protocol described in Section 3.2.7 based on the cell types. After subcultured, the following protocol was adopted according to respective cell types.

3.2.8.1 Cryopreservation of MEF, Phoenix cells, and HHFK

After trypsinised, cell types such as MEF, Phoenix cells, and HHFK were transferred to a 15ml tube and spun at 2000rpm for 5 minutes to pellet the

cells. The supernatant was discarded from the tube. For MEF and Phoenix cells, 900ul of FBS was added to the tube and resuspended. The cell suspension was transferred to a cryovial and another 100µl of dimethyl sulfoxide (DMSO) was added to the cryovial. For HHFK, one millilitre of freezing medium (Promocell, Heidelberg, Germany) was added to the cells and the cell suspension was transferred to the cryovial. The cryovial was then stored in isopropanol cell storage (Heathrow Scientific, Nottingham, UK) and kept in -80^{o}C freezer for overnight. The vial was then transferred to liquid nitrogen storage tank the next day.

3.2.8.2 Cryopreservation of iPSC and hESC

For iPSC and hESC, the cells were transferred to a 15 ml tube after the mechanical or enzymatic passaging. The 15 ml tube was spun at 1500 rpm for 5 minutes. The supernatant was removed and 1ml of freezing medium was added into the tube. The cells were gently resuspended to lift the cells from the tube. The cells were then transferred to the cryovial. The cryovial was then stored in isopropanol cell storage and kept in -80^{o}C freezer for overnight. The vial was then transferred to liquid nitrogen storage tank the next day.

3.3 Primary Hair Keratinocytes Isolation

Complete whole hairs were plucked from healthy volunteers. After plucked, the hairs were immersed in PBS with 1% penicillin streptomycin and fungizone before transfer into the biological safety cabinet (BSC) for processing. The hair follicles were dipped in 70% ethanol for a few seconds before washed with PBS to remove the ethanol residue. The hair follicles were

transferred to another Petri dish and were washed with PBS for another few times.

For enzymatic method, the hair follicles were incubated in 0.05% trypsin/EDTA for 30 minutes with agitation. After 30 minutes, the cell suspension was collected and was centrifuged at 2000rpm for 5 minutes to pellet the cells. After centrifugation, the supernatant was decanted and the pellet was re-suspended with growth medium and plated on feeder layer.

For outgrowth method, the hair follicles were selected from dish and incubated into 0.05% trypsin-EDTA for 15 minutes in 37^{o}C CO_2 incubator. After 15 minutes, the 0.05% trypsin-EDTA was removed and the hair follicles were washed with PBS to remove excess 0.05% trypsin-EDTA. The enzymatic step is optional. The hair follicles were transferred to the feeder layer. Hair keratinocytes isolation protocols were adopted from Barlow & Pye, 1997 and Aasen & Belmonte, 2010.

3.3.1 Characterisation of Human Hair Follicular Keratinocytes

HHFK cells were characterised using immunofluorescence staining and reverse-transcription PCR.

3.3.1.1 Characterisation of Human Hair Follicular Keratinocytes using Immunofluorescence Staining

The HHFK were cultured in 24-well plate before used for staining. The cells were fixed with 4% paraformaldehyde for 10 minutes at room

temperature. After each step, the cells were washed with PBS for 3 times to remove the reagents leftover. Next, the cells were treated with 0.1% bovine serum albumin (BSA) in PBS for 15 minutes at room temperature. The cells were treated with BD Perm/Wash buffer (Becton Dickinson, New Jersey, USA) before proceed with antibody staining. Next, the cells were stained with fluorescent-conjugated antibody for 1 hour at room temperature or overnight at 4^{o}C. The cells were counter-stained with DAPI for 30 minutes at room temperature to stain the nucleus. The antibodies used for HHFK characterisation were cytokeratin 19 (Santa Cruz, Texas, USA) and cytokeratin 75 (Antibodies-online, Georgia, USA).

3.3.1.2 Characterisation of Human Hair Follicular Keratinocytes using RT-PCR

Total RNA was extracted from HHFK using the GeneAll Ribospin RNA extraction kit (GeneAll, Seoul, South Korea). The cDNA was synthesized from 500ng - 1µg of RNA depending on the RNA concentration using 5x iScript Reverse Transcription Supermix kit (Bio-Rad, California, USA). The conversion protocol and reaction setup were according to the given protocol by manufacturer. The reaction setup and conversion protocol as following:

Table 3.1 The components required in the conversion of RNA to cDNA.

Component	Volume per Reaction
5x iScript reverse transcription supermix	4µl
RNA template	Variable
Nuclease-free water	Variable
Total Volume	20µl

Table 3.2 The conversion conditions required for the conversion of RNA to cDNA.

Priming	5 min at 25^{o}C
Reverse Transcription	30 min at 42^{o}C
RT Inactivation	5 min at 85^{o}C

The PCR was performed using the reagents from MyTaq™ Red Mix (Bioline, London, UK). The primers used were CK19 and CK75. The following tables were the setup and cycling conditions for PCR:

Table 3.3 PCR reaction setup.

Template	200 ng
Primers (20 µM each)	1 µl
MyTaq HS Red Mix, 2x	25 µl
Water	Variable

Table 3.4 PCR cycling conditions.

Step	Temperature	Time	Cycles
Initial Denaturation	95^{o}C	1 min	1
Denaturation	95^{o}C	15 s	30
Annealing	Variable	15 s	
Extension	72^{o}C	5 min	

The protocols for PCR set-up and cycling conditions were modified from manufacturer's manual. Each PCR products were run in 1.5% agarose gel to check for its size.

3.3.1.2 Agarose Gel Electrophoresis

The 1.5% agarose gel was prepared in 1X TAE buffer. Ten microliters of sample were loaded into each well. The size was confirmed with the comparison with the 100bp DNA ladder loaded together with the samples. The agarose gel electrophoresis was run at 40 voltages for 70 minutes in running buffer, 1X TAE buffer. After run, the gel was stained with GelRed (Biotium Inc., Hayward, CA). Then, the stained gel was visualised and photographed under a UV transiluminator.

3.4 Preparation of OSKM Plasmid for Viral Transduction

Transformed *E. coli* encoding human pMX-GFP, pMXs-hOCT3/4, pMXs-hSOX2, pMXs-hKLF4, pMXs-hc-MYC plasmids were stored in glycerol stock. The transformed *E. coli* was kindly donated by Dr. Shigeki Sugii [A* STAR, Singapore Bioimaging Consortium (SBIC)] through Prof. Choo Kong Bung (Faculty of Medicine and Health Sciences, Universiti Tunku

Abdul Rahman). The transformed *E. coli* consists of ampicillin resistant gene and was used as a marker for selection.

3.4.1 Broth Culture of Competent Cells Carrying the OSKM gene

A single colony of bacteria was picked and dipped into the Luria-Bertani (LB) broth. The LB broth was cultured overnight at 37^{o}C with agitation. The broth was checked for bacterial growth the next day.

3.4.2 DNA Plasmid Extraction

Bacterial overnight culture was pelleted at 8000 rpm for three minutes at room temperature. Pelleted bacterial was re-suspended in 250 µl Buffer P1 and transferred to a micro centrifuge tube. Two hundred and fifty microliters Buffer P2 was added and mixed thoroughly by inverting the tube four to six times. Three hundred microliters Buffer N3 was added and mixed thoroughly by inverting the tube four to six times. The tube was centrifuged for 10 minutes at 13,000 rpm. The supernatant was transferred to the QIAprep spin column. The column was centrifuged for 1 minute and the flow-through was discarded. Five hundred microliters Buffer PB was added to wash the column. The column was centrifuged for one minute and the flow-through was discarded. Seven hundred microliters Buffer PE was added to wash the column. The column was centrifuged for 1 minute and the flow-through was discarded. The column was transferred to the collection tube. Then, the tube was centrifuged for one minute to remove residual wash buffer. The column was placed in a new 1.5ml micro centrifuge tube. Fifty microliters Buffer EB was added to the centre of the QIAprep spin column to elute DNA. The tube was let to stand for

one minute and centrifuged for one minute. The eluted DNA was measured for the concentration and purity. This protocol was followed as according to the QIAGEN's QIAprep Spin Midiprep Kit manual.

3.5 Generation of iPSCs from Human Hair Follicular Keratinocytes

3.5.1 Transfection of Phoenix Cells with Plasmid DNA

Phoenix cells were used as packaging cells to produce retroviral which carrying the OSKM gene. The retroviral supernatant was used to transduce the HHFK. Six point six micrograms of DNA plasmid was used to transfect into the Phoenix cells. The reagents were prepared as following:

Table 3.5: Transfection reagents.

Reagents	Volume/ Weight
Calcium Chloride	8.3 µl
Plasmid DNA	6.6µg
2X HBS	8.3µl
Distilled Water	Variable
Total Volume	83µl

The retroviral supernatant was collected at 72 hours and 96 hours for transduction. The retroviral supernatant was filtered with 0.45 µm polyvinylidene difluoride (PVDF) membrane and was used immediately after filtered.

3.5.2 Transduction of Human Hair Follicle Keratinocytes with Retroviral Supernatant

The primary keratinocytes were seeded at 5,000 cells per well in six well plate the day before transduction. The next day, the culture medium was removed from each well. The fresh filtered retroviral supernatant was added into each well. Eight microgram per millilitre (final concentration) of polybrene was added into all wells including GFP well and negative well. Negative well was added with supernatant with virus carrying empty vectors. The plate was transferred to a centrifuge and spinfection at 700 x*g* for 45 minutes at 32^{o}C. Next, the cells were left in the incubator for 20 minutes. The supernatant was removed and the cells were washed twice with PBS before replacing with serum free medium. After 24 hours, the cells were transduced for another time with the same protocol as above. One day after second infection, the cells were trypsinized and centrifuged at 1500 rpm for 5 minutes. The infected cells were resuspended in hESC medium and were plated in wells treated with 0.1% gelatin and pre-seeded with Mitomycin-C inactivated MEFs. After two days, the medium was changed daily until selection of potential colony around day 14 to 28.

3.5.3 Selection of Potential Reprogrammed Colonies for Expansion

The reprogramming process took around 14 – 28 days before selection of clones can be done. The selection of reprogrammed cells depends on cell types and clones. The large reprogrammed clones that grow fast can be picked, subcloned, cultured, and further characterised for identity confirmation.

3.6 Characterisation of Induced Pluripotent Stem Cells

3.6.1 Live Staining of iPSCs using Alkaline Phosphatase (AP) and SSEA4 Markers

For alkaline phosphatase (AP) live staining (Invitrogen, CA, USA), the growth medium was removed from the culture. The culture was washed with pre-warmed DMEM/F12 without supplements and serum for two to three minutes. The DMEM/F12 medium was removed and the same step was repeated for another time. The AP live staining working solution was prepared by adding 2µl of 500X stock of AP live staining in 1ml of DMEM/F12.

The AP Live Stain working solution was added onto the cell culture. The culture was incubated for 30 minutes in the CO_2 incubator. The AP Live Stain was removed from the culture and the culture was washed with DMEM/F12 for 5 minutes. The DMEM/F12 was removed and the washing step was repeated for another two times. Fresh DMEM/F12 was added to the culture before view under the fluorescent microscope using FITC filter. The images can be captured within 90 minutes after staining. After visualisation, the DMEM/F12 was removed and growth medium was added to the culture.

For SSEA4 live staining (Stemgent, Massachusetts, United States), the primary antibody was diluted to final concentration of 2.5µg/ml in DMEM/F12. The medium was removed from the culture. The diluted primary antibody was added to the culture. The culture was incubated for 30 minutes at 37^oC and 5% CO_2. The primary antibody was removed from the culture and the culture was washed with DMEM/F12 gently for two times. Fresh culture

medium was added to the culture and was examined under the fluorescent microscope using the phycoerythrin (PE) filter. After view, the culture was return to the incubator to continue culture at 37^{o}C and 5% CO_2.

3.6.2 Morphology

The morphology of iPSCs resembles the morphology of hESC in term of a compact colony with large nucleoli, large nucleus to cytoplasm ratio, distinct cell borders, and slightly rounder in shape.

3.6.3 Immunofluorescence Staining

The hESC-like colonies were cultured in 24-well plate before used for staining. The cells were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After each step, the cells were washed with PBS for 3 times to remove the reagents leftover. Next, the cells were treated with 0.1% BSA in PBS for 15 minutes at room temperature. For intracellular staining, the cells were treated with BD Perm/Wash buffer before proceed with antibody staining. Next, the cells were stained with fluorescent-conjugated antibody for 1 hour at room temperature or overnight at 4^{o}C. The cells were counter-stained with DAPI for 30 minutes at room temperature to stain the nucleus. The antibodies used were: SSEA4 (1:50), TRA-1-60 (1:50), TRA-1-81 (1:50), Nanog (1:50), Sox2 (1:50), and Oct4 (1:50). All antibodies were purchased from BD Bioscience.

3.6.4 Flow Cytometry Analysis

For cell surface marker staining, the iPSCs and hESCs were trypsinised using 0.05% Trypsin/EDTA to dissociate the cell into single cell. Cell count was performed to check for the cell number. Two hundred thousand cells per sample were aliquot into a 5 ml FACS tube. The cells were centrifuged at 300 *xg* for 5 minutes. While centrifuging, 100µl of antibody mix was prepared at optimal working dilution. After centrifuged, the supernatant was removed without disturbing the cell pellet and the cells were resuspended in the antibody mix. The tube was tapped gently to mix the antibody and the cells. The tubes were incubated for 60 minutes at room temperature in dark. One millilitre of 1% bovine serum albumin (BSA) in PBS was added to each tube, and was gently tapped at the base of the tube. The tubes were spun at 300 *xg* for 5 minutes. The supernatant was removed without disturbing the cell pellet and the cells were resuspended in 200µl of FASCFlow (Becton Dickinson, New Jersey, USA). The samples were kept on ice and in dark and were analysed by flow cytometry as soon as possible. The antibodies used were: SSEA4 (1:50), TRA-1-60 (1:50), and TRA-1-81 (1:50).All antibodies were purchased from BD Bioscience.

For intracellular staining, the iPSCs and hESCs were trypsinised using 0.05% Trypsin/EDTA to dissociate the cell into single cell. Cell count was performed to check for the cell number. Two hundred thousand cells per sample were aliquot into a 5ml FACS tube. The cells were centrifuged at 300 *xg* for 5 minutes. The cells were resuspended in 250 µl of 4% Paraformaldehyde and incubated for 10 minutes. One millilitre of 1% bovine

serum albumin (BSA) in PBS was added to each tube. The tubes were gently mixed and spun at 300 x*g* for 5 minutes. The supernatant was completely removed without disturbing the cell pellet and the cells were resuspended in 500 µl of 1X BD Perm/Wash buffer. The tubes were mixed by tapping and incubated at room temperature for 15 minutes. Then, the tubes were spun at 300 *xg* for 5 minutes. The 1X BD Perm/Wash buffer in 1% BSA/PBS was used as the diluent for the remaining of the procedure before analysis. While centrifuging, 100µl of antibody mix was prepared at optimal working dilution. After centrifuged, the supernatant was removed without disturbing the cell pellet and the cells were resuspended in the antibody mix. The tube was tapped gently to mix the antibody and the cells. The tubes were incubated for 60 minutes at room temperature in dark. One millilitre of 1% bovine serum albumin (BSA) in PBS was added to each tube, and were gently tapped at the base of the tube. The tubes were spun at 300 xg for 5 minutes. The supernatant was removed without disturbing the cell pellet and the cells were resuspended in 200 µl of FASCFlow. The samples were kept on ice and in dark and were analysed by flow cytometry as soon as possible. The antibodies used were: Nanog (1:50), Sox2 (1:50), and Oct4 (1:50). All antibodies were purchased from BD Bioscience.

3.6.5 Reverse-transcription Polymerase Chain Reaction (RT-PCR)

The RT-PCR protocol was according to the same protocol described in Part 3.3.1.2 - Characterisation of Human Hair Follicular Keratinocytes using RT-PCR. The primers used were Sox2, Oct4, Klf4, c-Myc, Nanog, Lefty, and Gabrb.

3.6.6 In vitro Differentiation for Three Germ Layers Formation

The iPSCs were plated for mesoderm differentiation on dishes coated with matrigel. The iPSCs were grown to 70% confluency before differentiation. For mesoderm differentiation, the culture medium was changed to mesoderm differentiation medium (R & D Systems, Minnesota, USA). The differentiation medium was changed again after 16 hours. The differentiated cells were harvested for immunofluorescence staining after 36 to 48 hours after the initial treatment.

The iPSCs were subcultured by either enzymatic or mechanical and the iPSCs were transferred to a well of non-treated cell culture 6-well plate. The medium used was hESC medium without FGF2. The iPSCs formed embryoid bodies the next day and cultured in the same medium for 5 days. The medium was changed every two days. After 5 days, the EBs were used for ectoderm and endoderm differentiation. For ectoderm and endoderm differentiation, the treatment was continued for 5 days with change of medium every 48 hours. For ectoderm differentiation, the growth factor used was noggin and noggin was added whenever changing of medium. For endoderm differentiation, activin A was added when changing of medium every 48 hours.

3.7 Directed Differentiation into Keratinocytes

The iPSCs were plated on dishes coated with matrigel. After the next day, the treatment started with addition of retinoic acid (RA) and bone morphogenic protein-4 (BMP-4) and the feeder free medium was changed to keratinocytes serum free medium. The medium with growth factors was

changed every 48 hours for 6 days. The differentiated cells were cultured for at least 15 days before harvested for immunofluorescence staining and RT-PCR.

3.7.1 Characterisation of Keratinocytes derived from iPSCs

Keratinocytes derived from iPSCs were characterised using immunofluorescence staining and reverse-transcription PCR.

3.7.1.1 Characterisation of Keratinocytes derived from iPSCs using Immunofluorescence Staining.

The immunofluorescence staining protocol was according to the same protocol described in Part 3.3.1.1 - Characterisation of Human Hair Follicular Keratinocytes using Immunofluorescence Staining. The antibody used for differentiated cells was cytokeratin 14 (abD Serotec, North Carolina, USA).

3.7.1.2 Characterisation of Keratinocytes derived from iPSCs using RT-PCR

The RT-PCR protocol was according to the same protocol described in Part 3.3.1.2 - Characterisation of Human Hair Follicular Keratinocytes using RT-PCR. The primer used was CK14.

CHAPTER 4

RESULTS

4.1 Keratinocytes Isolation from Human Hair Follicles

The criteria for hair follicle selection were the presence of outer root sheath (see arrow on Figure 4.1b) and the hair follicle must be in anagen phase (Figure 4.1b). The anagen phase hair follicle usually present in the occipital area of the scalp. The missing outer root sheath (Figure 4.1a) was due to incorrect plucking technique or due to the volunteer itself. Volunteers with softer scalp have yielded more complete plucked hair follicles with the presence of whole outer root sheath (Figure 4.1b). Volunteer with finer hair yielded more incomplete plucked hair follicles (Figure 4.1a).

Two methods were used to isolate the keratinocytes from the hair follicle. The two methods used were enzymatic based and outgrowth based methods. For the enzymatic method, agitation and 0.05% trypsin-EDTA were used to dissociate the keratinocytes from the hair follicles. The released keratinocytes usually appear as round shaped cells (Figure 4.2a) after being dissociated from the hair follicles and finally appear as normal cobblestone keratinocytes cell shape. On Day 3, the keratinocytes appeared to increase in number but the cells did not attach to the human dermal fibroblasts (Figure 4.2b). Apart from keratinocytes, a large number of transit-amplifying cells were isolated as well. These transit-amplifying cells increase in cell numbers after several days of culture and eventually stopped proliferating (Figure 4.2c).

For outgrowth method, the hair follicles were straightaway plated after processing. But, few problems arose such as some of the hair follicles were floating on the medium and did not attach to the feeder and for those attached hair follicles there were no observed outgrowth of cells after a few days. A single step was added to this outgrowth method and that was to trypsinise the hair follicles before plated on the feeder layer. Some keratinocytes were also released from the hair follicles and were seen floating on the medium and did not attach to the feeder (see figure 4.3a). Apart from the keratinocytes, some of the transit-amplifying cells were also released from the hair follicles (see figure 4.3b). Even with this additional step, there was no outgrowth of the hair follicles.

Since a sustainable primary keratinocytes were not able to be generated from the human plucked hair follicles, the primary keratinocytes were acquired from a commercial source. Human Hair Follicular Keratinocytes (HHFK) (Figure 4.4a & b) was acquired from Sciencell. The cells showed typical cobblestone shape (Figure 4.4b) which is the normal morphology for keratinocytes.

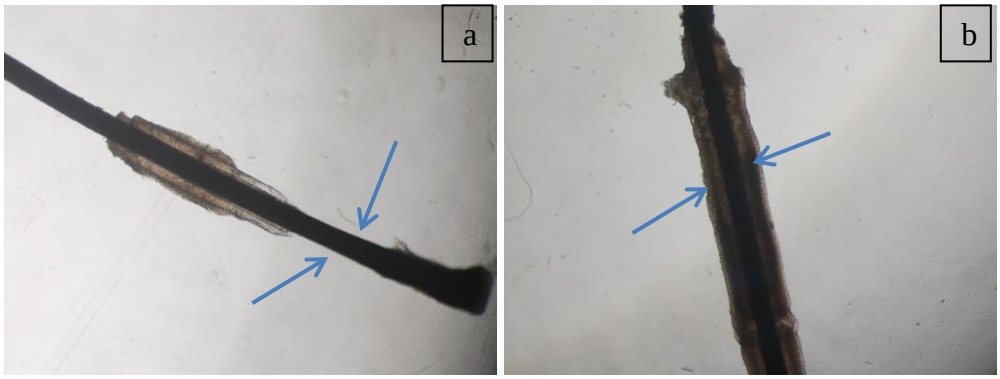

Figure 4.1 Morphology of plucked hair follicle. (a) Morphology of a plucked hair follicle with missing outer root sheath (see arrow) (b) Morphology of a complete plucked hair follicle with the presence of outer root sheath (see arrow) covering the hair shaft. Magnification: 40X.

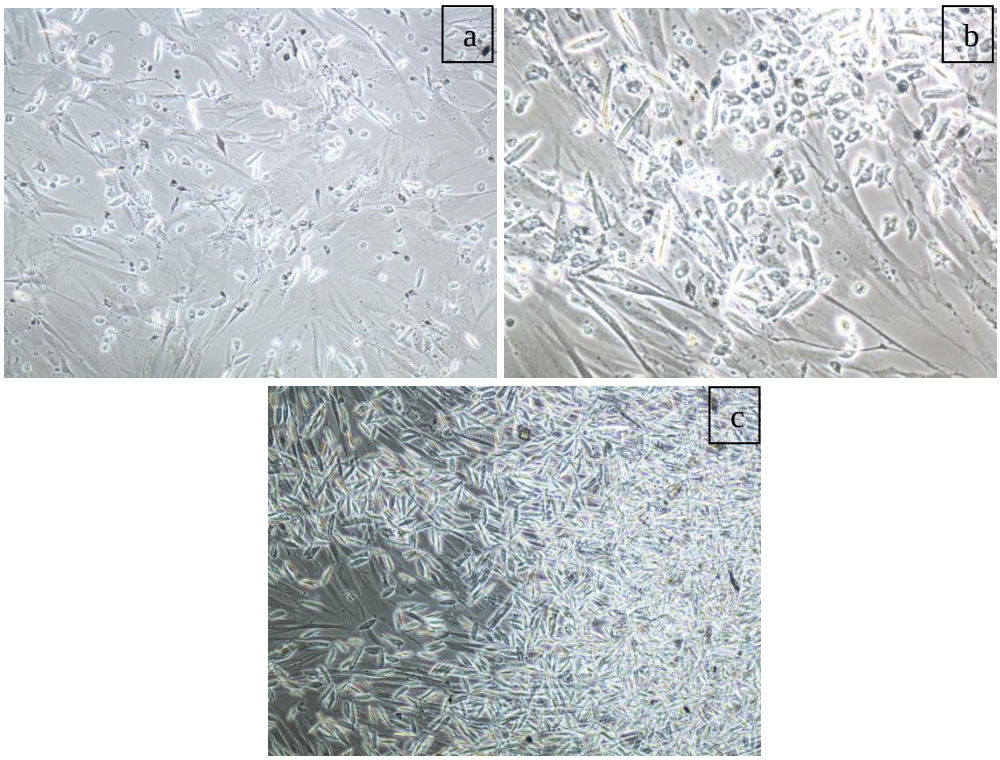

Figure 4.2 Isolation of keratinocytes from plucked hair follicles using enzymatic method. (a) Morphology of keratinocytes isolated from plucked hair follicles which appeared as round initially before forming the normal cobblestone keratinocytes cell shape. (b) The number of keratinocytes increased but it still does not attach to the feeder layer. (c) Transit amplying cells isolated from hair follicles using the trysinisation method. The cells proliferated for few passages before stop proliferating. Magnification: 40X (a, c) 100X (b).

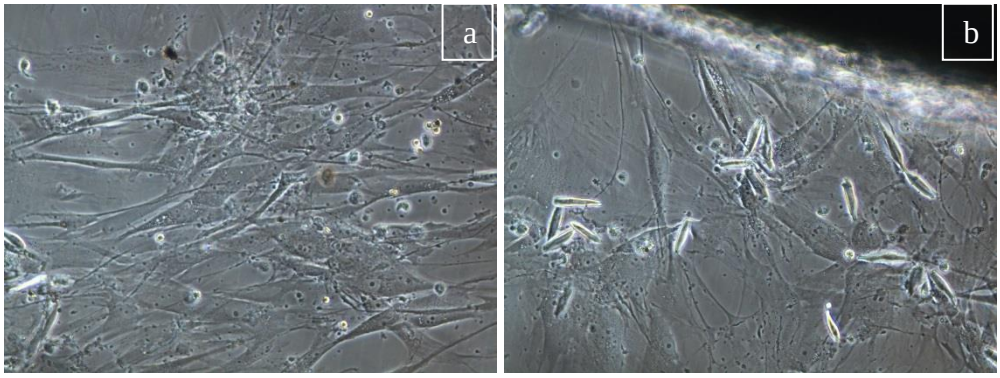

Figure 4.3 Isolation of keratinocytes from hair follicles using outgrowth method. (a) Keratinocytes can be seen floating on the medium but did not attach to the feeder (b). Transit-amplifying cells can be seen in the culture as well. Magnification: 100X.

4.2 Characterisation of Human Hair Follicular Keratinocytes

The HHFK was characterised against two cytokeratin antibodies, CK19 and CK75 using IF and RT-PCR. From IF and RT-PCR, both methods showed that HHFK expressed the CK19 and CK75 markers. CK19 is marker for simple epithelial keratin while CK75 is marker for hair follicle-specific epithelial keratins.

Using IF staining, HHFK showed green FITC signal for the expression of CK19 (Figure 4.5a) and CK75 (Figure 4.5b) markers. From RT-PCR, the band size for CK19 was 214 base pairs while the band size for CK75 was 434 base pairs. HHFK showed bands for both expressions of CK19 and CK75 (Figure 4.5c).

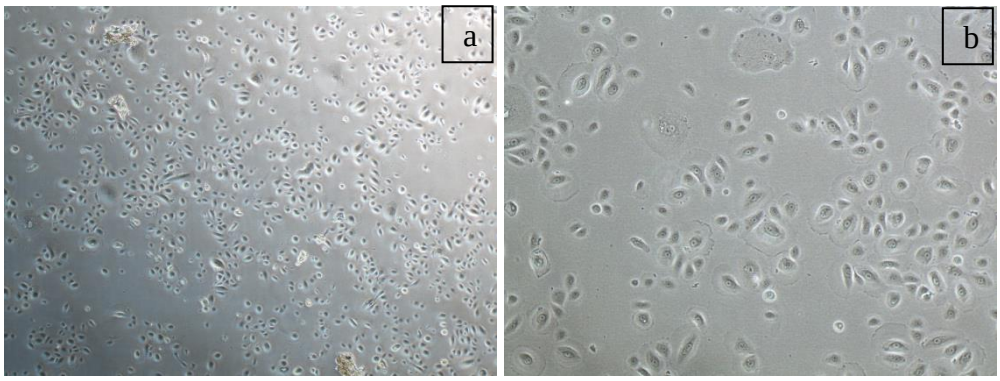

Figure 4.4: Human Hair Follicular Keratinocytes (HHFK). (a) Morphology of HHFK at passage 4 at low magnification. Magnification: 40X. (b) Morphology of HHFK at passage 4 at higher magnification. Magnification: 100X.

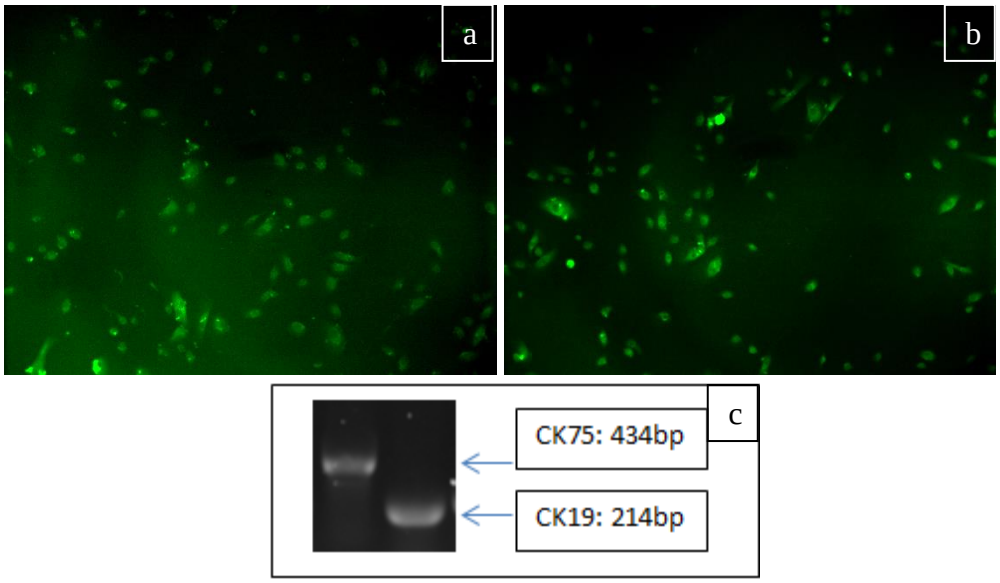

Figure 4.5 Characterisation of HHFK using immunofluorescence staining and RT-PCR. (a) Immunoflurescence image of HHFK stained with CK19 antibody. Magnification: 100X. (b) Immunofluorescene image of HHFK stained with CK75 antibody. Magnification: 100X. (c) Agarose gel image of cytokeratin-75 (434 base pairs) and cytokeratin-19 (214 base pairs).

4.3 Production of Retroviral Particles for Reprogramming

Phoenix cells were used as the packaging cells and they were able to produce the packaging proteins, gag and pol protein. The retrovirus was produced using the conventional calcium phosphate method to introduce the plasmids into the cells. The initial number of phoenix cells seeded was at around 70% to allow the cells to achieve optimum confluency for transfection.

The GFP signal was used as an indicator for the transfection efficiency. The GFP signal intensity was viewed under fluorescent microscope and determined under naked eyes. The time point at which the GFP signal was strongest would represent the optimum uptake of plasmids by the cells. It also represents the optimum time to collect the supernatant for reprogramming. The supernatants were collected at 72 hours (Figure 4.6c) and 96 hours (Figure 4.6d) where the GFP signals were at the strongest. The expression of GFP signals were more than 80% of the total cells at 72 hours and 96 hours.

4.4 Reprograming of HHFK and its Efficiency

iPSCs have been generated from HHFK using the retrovirus carrying four single-transcription factors. On a separate well, HHFK was transduced with retrovirus carrying the GFP gene (Figure 4.7). This well served as indicator well for the efficiency of the transduction in bringing in the plasmid DNA into the cells. The transduction efficiency was calculated based on number of cells expressing GFP to the total cell number. After 3 days, the transduction efficiency reached at least 60% of the total cells.

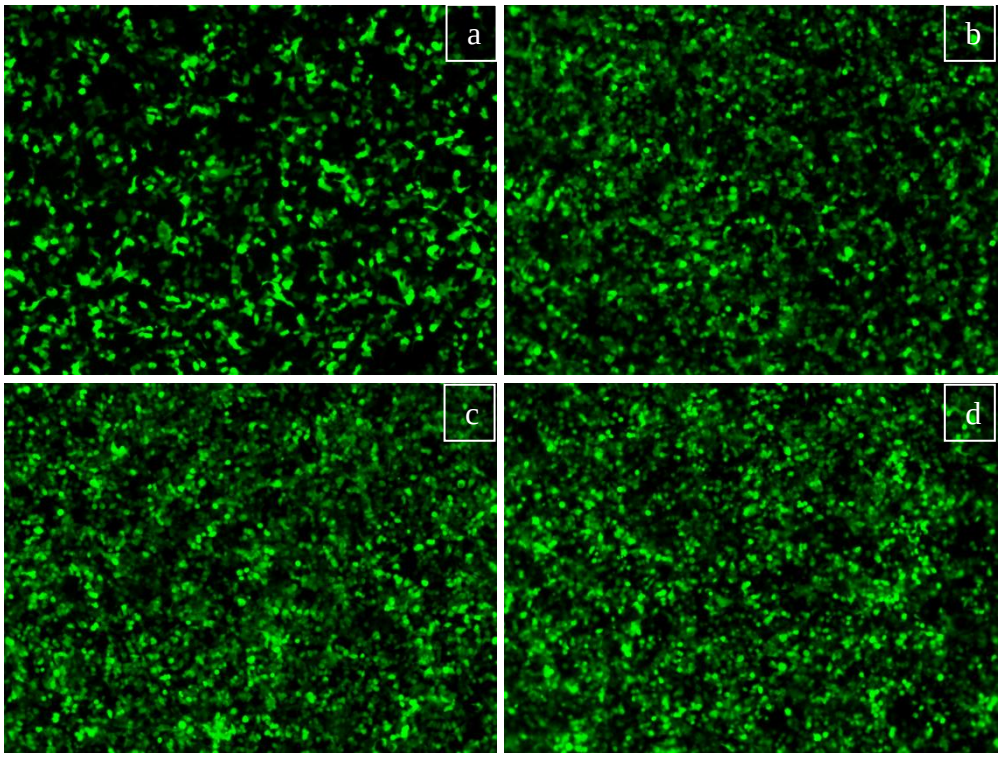

Figure 4.6 Green fluorescence protein (GFP) signal produced from Phoenix cells after transfected with GFP plasmid using calcium phosphate transfection method. The expressions of GFP at different time point of 24 hours (a), 48 hours (b), 72 hours (c), and 96 hours (d). Magnification: 100X.

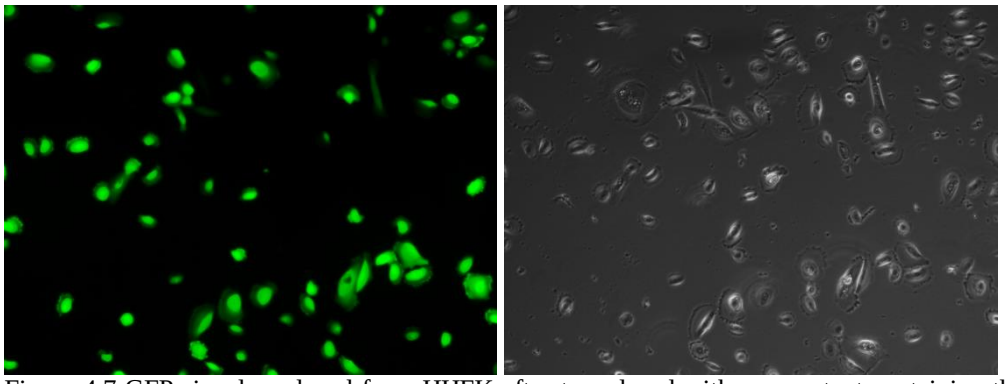

Figure 4.7 GFP signal produced from HHFK after transduced with supernatant containing the retrovirus. Magnification: 100X.

During the initial 10 days of reprogramming process (after replated on feeder layer), a small molecule, valproic acid (VPA), a histone deacetylase inhibitor, was added to the culture to enhance the overall reprogramming efficiency. The culture was placed in hypoxic condition, at 5 % oxygen level, to enhance the reprogramming efficiency.

The timing for selection of iPSC clones depends on cell types used and methods used. The range of time usually for cell picking is around 14 – 40 days. In this study, the iPSC clones were picked at around day 16 post initial-infection. The emergence of small iPSC-like colonies was at around day 6 after the first infection (Figure 4.8b). But, picking the colonies before day 14 will reduced the survival and viability of the clone.

After 16 days, the colonies which showed typical hESC morphology such as distinct border or compact colony were picked. From Figure 4.10a, the image showed one of the colonies that had been picked and expanded individually. This colony which was expanded individually will be considered as one clone. When few colonies are picked and expanded in the same well,

they are called mixed clone. So, mixed clone is not recommended since it is heterogeneous and could have different pluripotent marker expression.

The iPSCs were stained with live-staining antibodies to check for the initial pluripotent marker expression. The iPSCs were stained using the AP live staining kit (Invitrogen, CA, USA) and SSEA4 live staining kit (Miltenyi Biotec, Bergisch Gladbach, Germany). From Figure 4.9a, the image of the iPSC colonies showed green FITC signal for alkaline phosphatase. From Figure 4.9 c, the image showed that the iPSC colonies showed PE signal for SSEA4. These showed that the generated iPSC clones were expressing the AP and SSEA4. This live staining was performed before proceed to the next step of iPSC clones selection.

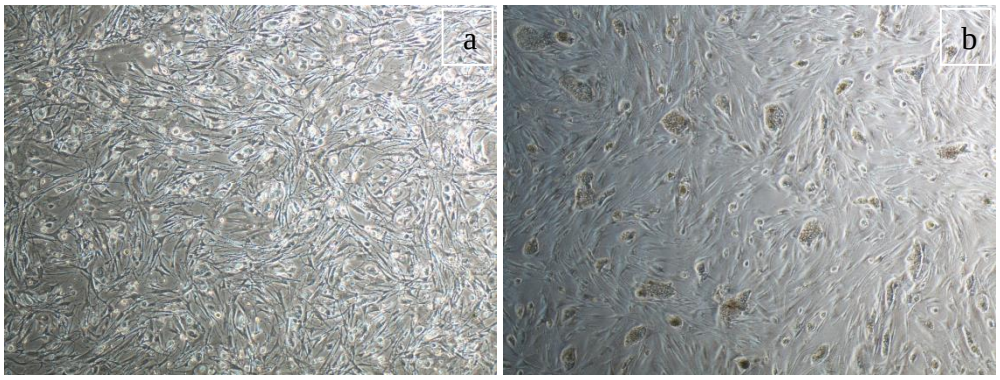

Figure 4.8 Reprogrammed HHFK after replated on MEF Feeder layer and under VPA treatment and hypoxic condition. The appearance of infected HHFK at Day 2 (a) and Day 8 (b) of VPA treatment. Magnification: 40X.

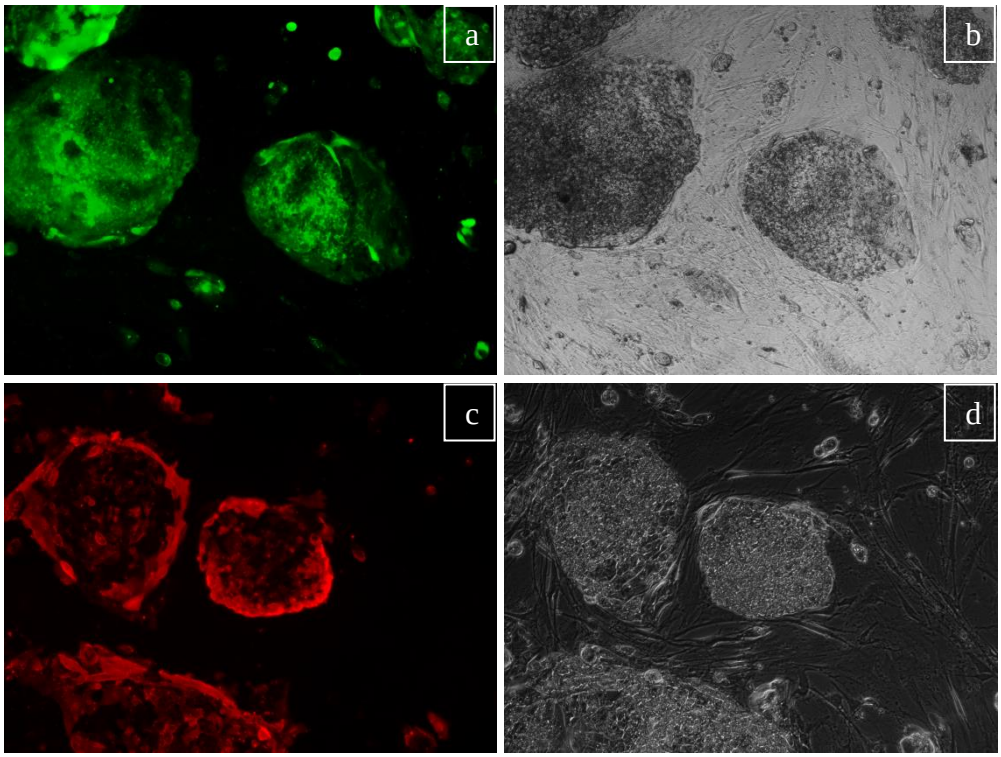

Figure 4.9 Alkaline phosphatase (AP) and SSEA-4 live staining. The green FITC signal of reprogrammed cells showed the expression of AP (a) and the same view of reprogrammed cells as (a) under brightfield imaging (b). The reprogrammed cells showed red PE signal for the expression of surface marker, SSEA-4 (c) and the same view of reprogrammed cells as (c) under brightfield imaging. Magnification: 100X.

In this study, about 34 clones were selected from three reprogramming experiments for further expansion. But, only three clones were selected for further characterisation and differentiation. During the expansion process, some clones were not able to survive while others survived for only a few passages. The selected iPSC clones were able to expand up to 47 passages in feeder free condition (Figure 4.10d). The clones were cultured on feeder layer up to 26 passages. The clones were switched to feeder-free culture system starting from as early as passage 9.

The images of representative iPSC clone were depicted at passage 2 in Figure 4.10b and passage 11 in Figure 4.10c and this clone was cultured on feeder layer. The image of iPSC clone after switching to feeder-free system at

passage 47 was depicted in Figure 4.10d. All the iPSC clones grew in colonies and showed typical hESC morphologies such as distinct border, and tightly packed cells (Figure 4.10 a, b, & c). The iPSCs can be cultured without any replicative crisis and the culture was terminated after completion of the differentiation step.

The reprogramming efficiency was calculated from the number of colonies formed to the total initial number of cells seeded. The colonies were counted based on morphology. We obtained around 218 to 372 iPSC colonies from three separate reprogramming experiments with the total infected HHFK of 50,000 cells. This represents reprogramming efficiency ranging from 0.44% to 0.74%.

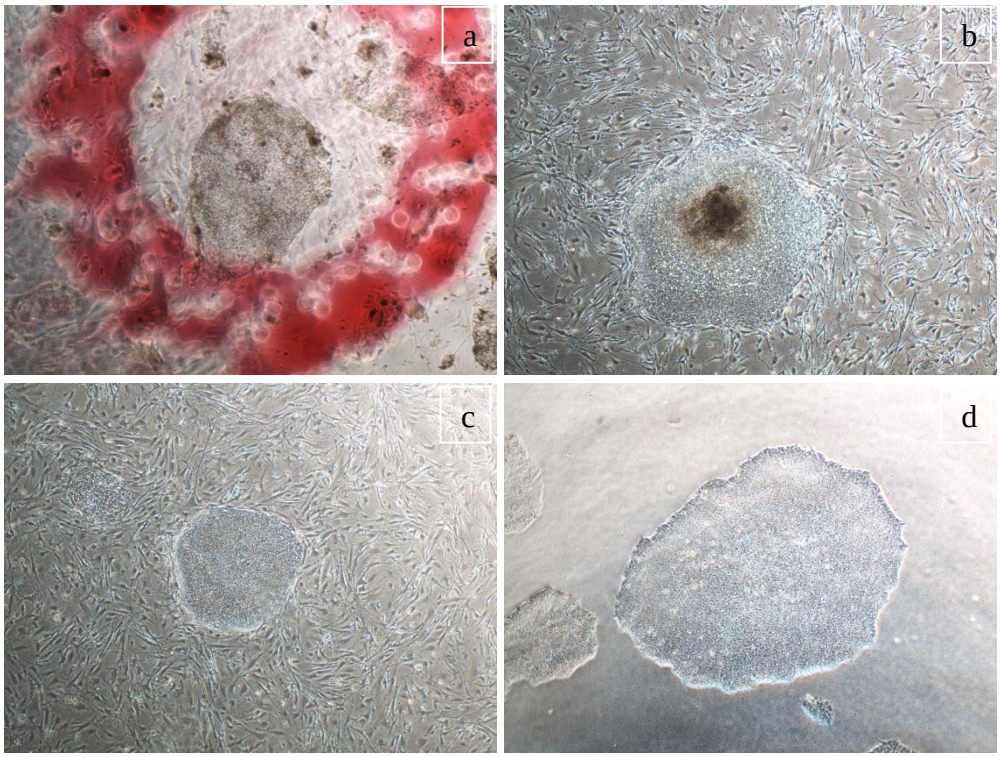

Figure 4.10 Morphology of representative iPSC clone. The image of iPSC colony circled in red (a) was captured before selection. The image of iPSC colony grew in feeder layer at passage 2 (b) and 11 (c). The image of iPSC colony grew in feeder-free system at passage 47. All the colonies showed typical hESC morphology. Magnification: 40X

4.5 Characterisation of iPSCs

The iPSCs were characterised using immunofluorescence and flow cytometry to detect the pluripotency-related protein, and RT-PCR to detect the pluripotency gene. Apart from IF, flow cytometry analysis, and RT-PCR, an *in vitro* differentiation was also performed for the characterisation of iPSC in order to know its ability to differentiate into three germ layers. Three germ layers formation is also one of the common ways to check for the identity of pluripotent cells.

4.5.1 Immunofluorescence Staining

After the selection and expansion of the iPSCs, the immunofluorescence staining was performed to check for the presence of pluripotent-related proteins. The generated iPSC clones were cultured up to at least passage 10 before characterisation for its pluripotency markers. iPSC clones were stained with antibodies against Oct4, Sox2, Nanog, SSEA4, TRA-1-60, and TRA-1-81 conjugated with different fluorochromes.

The immunofluorescence staining showed that the iPSC clones expressed intracellular pluripotency markers such as Oct4, Sox2, and Nanog (Figure 4.11) as well as the pluripotency surface markers such as stage specific embryonic antigen-4 (SSEA4), TRA-1-60, and TRA-1-81 (Figure 4.12). Figure 4.11 and Figure 4.12 showed the iPSCs expressed Oct4, Sox 2, and TRA-1-60 which were stained with PE-conjugated antibodies. While for Nanog, SSEA4, and TRA-1-81, the iPSCs were stained with FITC-conjugated antibodies. The hESC line, BGO1V was included for comparison.

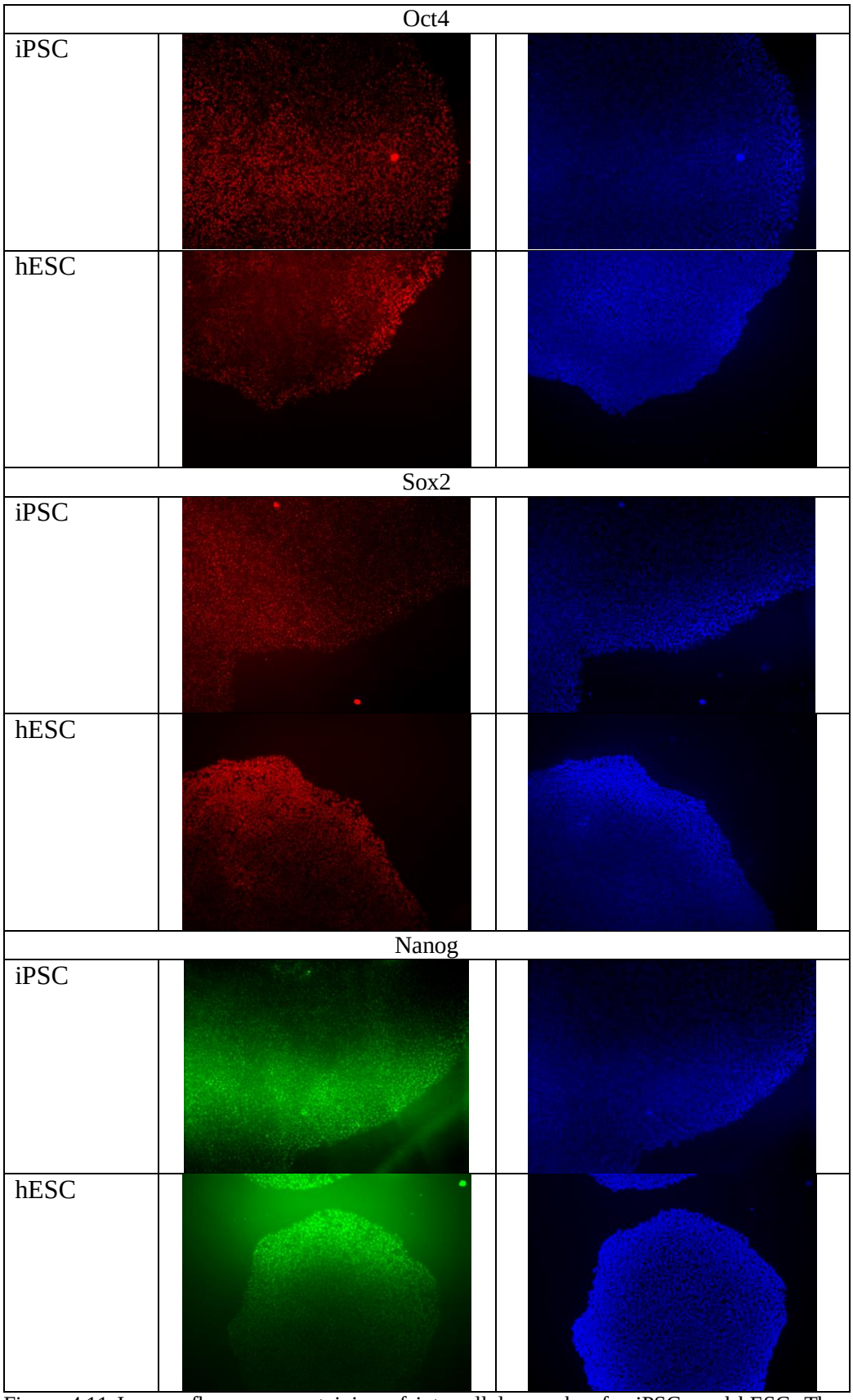

Figure 4.11 Immunofluorescence staining of intracellular marker for iPSCs and hESC. The mentioned intracellular markers include Oct4, Sox2, and Nanog. The images on the left panel were the fluorescence images stained for the pluripotent marker while the images on the right panel were the same colony that stained with DAPI. Magnification: 100X.

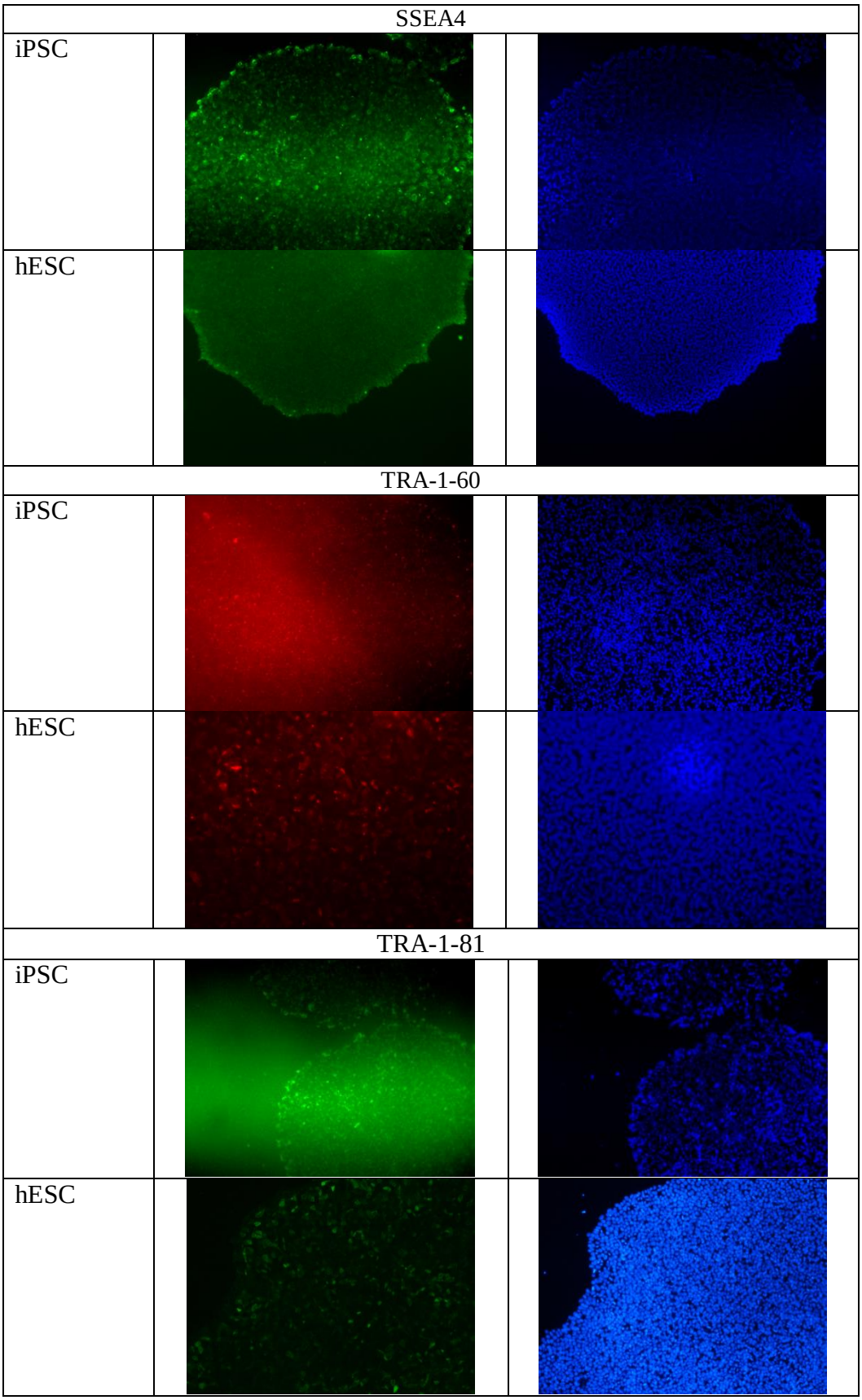

Figure 4.12 Immunofluorescence staining of surface marker for iPSCs and hESC. The mentioned cell surface markers include SSEA4, TRA-1-60, and TRA-1-81. The images on the left panel were the fluorescence images stained for the pluripotent marker while the images on the right panel were the same colony that stained with DAPI. Magnification: 100X.

4.5.2 Flow Cytometry Analysis

The iPSC clones were further characterised using flow cytometry to check for the percentage of the cells that express the pluripotent markers. From our flow results, we have generated the iPSC clones that showed the expression of pluripotent-related proteins such as Oct4, Sox2, Nanog, SSEA4, TRA-1-60, and TRA1-81. As a comparison, we have included the human embryonic stem cells line and parental cells, HHFK, for comparison. The hESC line is the BGO1V and the HHFK is the parental cells that we used for reprogramming, which are both available in our laboratory.

From the Figure 4.13, the dot plot showed the expression for Oct4, Sox2, and Nanog in iPSC, hESC, and HHFK. The expression of Oct4, Sox2 and Nanog by representative iPSC was 55.9%, 63.0% and 36.4%, respectively. For the hESC expression of pluripotency markers Oct4, Sox2, and Nanog, the percentages were 70.6%, 73.8%, and 37.4%, respectively. Even though, the level of expression was slightly lower in the iPSCs compared to the hESC, but the differences in the percentage were not more than 15%. This difference applies to all three generated iPSC clones. In the parental cells, the expression of Oct4, Sox2 and Nanog was 1.7%, 1.8%, and 0.5%, respectively. These results showed that the increase of the three markers in the HHFK to iPSCs was very significant.

For the expression of surface markers, the percentages of SSEA4, TRA-1-60, and TRA-1-81 in iPSC were 98.2%, 12.8% and 21.7%, respectively. On the other hand, the expression for hESC's SSEA4, TRA-1-60, and TRA-1-81

were 89.0%, 24.2%, and 15.2%, respectively. The level of SSEA4 expression in iPSCs was higher compared to the hESC. The level of TRA-1-60 and TRA-1-81 were just slightly lower, not more than 3% compared to hESC. The same trend applied to all generated iPSC clones. For the HHFK, the expression percentage for SSEA4, TRA-1-60, and TRA-1-81 were 0.5%, 1.7%, and 0.4% respectively. The same trend also showed in surface markers where the expression has increased significantly in iPSCs compared to the parental cells.

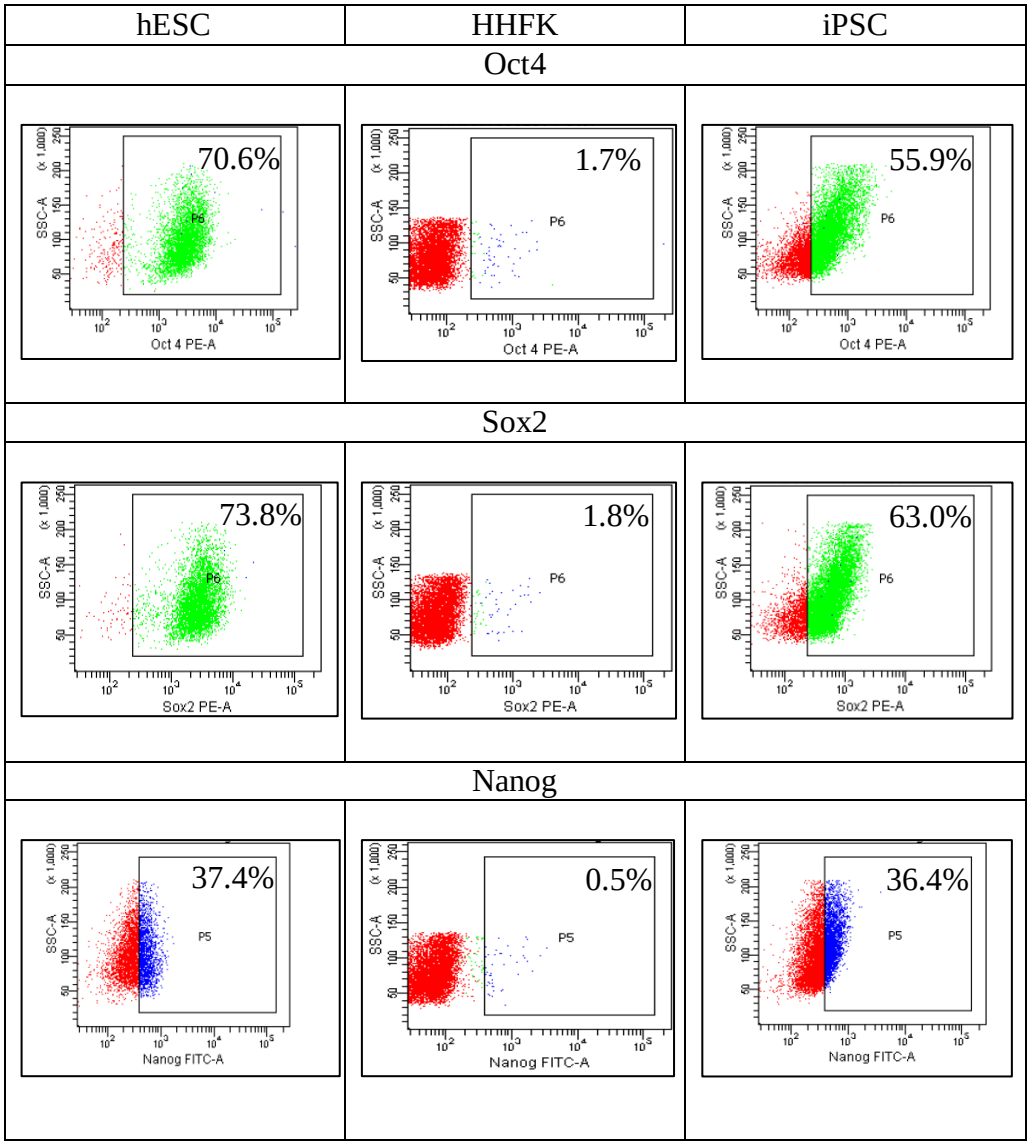

Figure 4.13 Characterisation of iPSCs using flow cytometry. The dot plot graph showed the flow cytometry analysis of the pluripotent markers expression for hESC, HHFK, and representative iPSC. The pluripotent markers involved were intracellular marker of Oct4, Sox2, and Nanog.

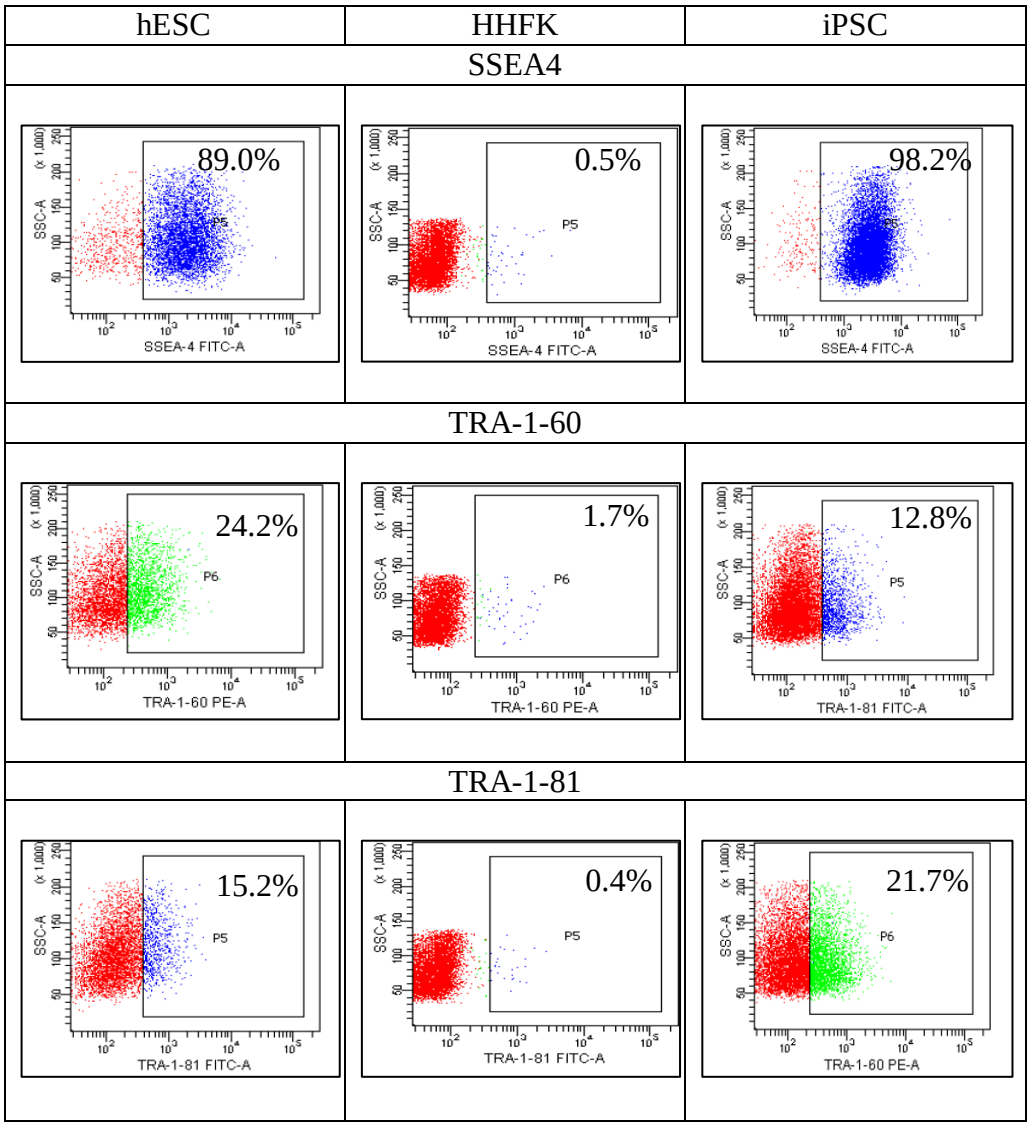

Figure 4.14 Characterisation of iPSCs using flow cytometry. The dot plot graph showed the flow cytometry analysis of the pluripotent markers expression for hESC, HHFK, and iPSC. The pluripotent markers involved were cell surface markers of SSEA4, TRA-1-60, and TRA-1-81.

4.5.3 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed to identify the expression of pluripotent marker in the iPSCs, hESC, and parental cells. A negative control lane was prepared on the most right hand side of each gel. A 100bp DNA ladder was prepared on the most left hand side of each gel. GAPDH was used as the housekeeping gene for control.

Using RT-PCR method, Sox2, Oct4, Klf4, c-Myc, Nanog, Lefty, and Gabrb were used as the pluripotent markers to check for its presence in iPSCs, hESC, and HHFK. From the gel image in Figure 4.15, all three clones (Lane 2 to Lane 5 for all images) showed the comparable expression of all above mentioned pluripotent markers. All three clones also showed comparable expression of all pluripotent markers to the positive control cells, hESC. For the parental cells, there was presence of certain pluripotent markers such as Klf4, c-Myc, Nanog, Lefty, and Gabrb, but, no Sox2 and Oct4 were detected.

4.5.4 *In vitro* Three Germ Layers Differentiation

Both the iPSCs and hESC were differentiated into mesoderm lineage using the monolayer method. The cells were plated on matrigel-coated plates before being induced to differentiate. The cells were cultured to 70% confluency before proceeding to differentiation. The cells took around 3 – 4 days to reach the expected confluency. Then, mesoderm differentiation medium was used to induce the iPSCs and hESC to differentiate into mesoderm lineage. The differentiation took about 2 days before harvested for staining. The differentiated iPSCs and hESC were stained with Brachyury, a

marker for mesoderm lineage. Figure 4.17a and Figure 4.17b showed the iPSC and hESC were able to form mesoderm lineage cells that expressed Brachyury.

For ectoderm and endoderm lineage differentiation, the iPSCs and hESC were formed EBs on uncoated multi-well plate for 5 days. The EBs (Figure 4.16) were transferred to a 0.1% gelatin-coated plate and were let to attach on it. The subcultured iPSCs or hESC colonies curled and formed round shaped bodies. These round shaped bodies were the embryoid bodies. At the same time, the differentiation growth factors were added. Activin A was added for endoderm lineage differentiation while Noggin was added for ectoderm lineage differentiation. After the EBs attached, the cells were grown out of the EBs. From Figure 4.17c and Figure 4.17d, the images showed the cells expressing the MAP2 marker, which is the ectoderm marker. Figure 4.17e and Figure 4.17f showed the iPSCs and hESC expressed Sox17, a marker for endoderm lineage.

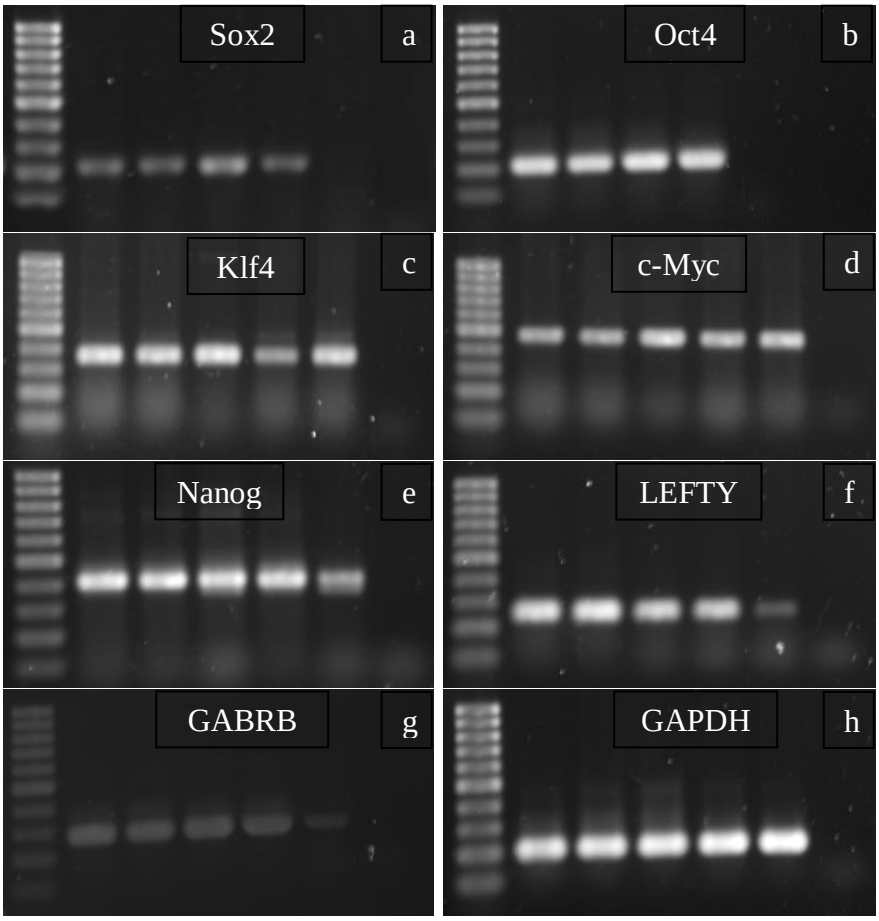

Figure 4.15 Characterisation of of iPSCs using RT-PCR. The pluripotent genes were being analysed such as Sox2 (a), Oct4 (b), Klf4 (c), c-Myc (d), Nanog (e), Lefty (f), and Gabrb (g) for the presence in iPSCs, hESC, and HHFK. The housekeeping gene being used was Gadph (h). The following lane arrangement was the same for all 8 images: (Lane 1)100bp DNA Ladder, (Lane 2) Clone 1, (Lane 3) Clone2, (Lane 4) Clone 3, (Lane 5) hESC, (Lane 6) HHFK, and (Lane 7) Negative.

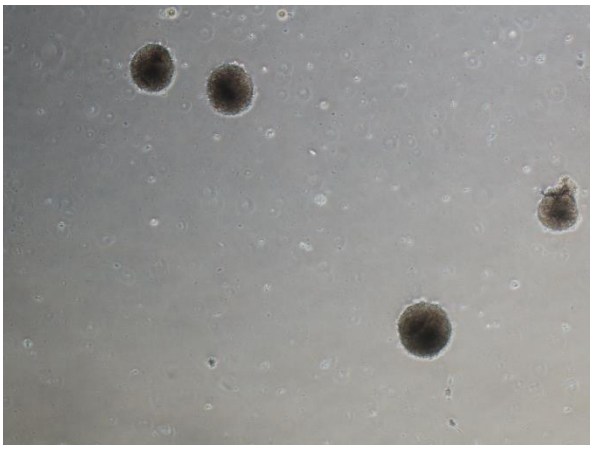

Figure 4.16 Appearance of embryoid bodies. The EBs formed round in shape after cultured in suspension overnight. The EBs were later plated on gelatin-coated plate to induce differentiation. Magnification: 40X.

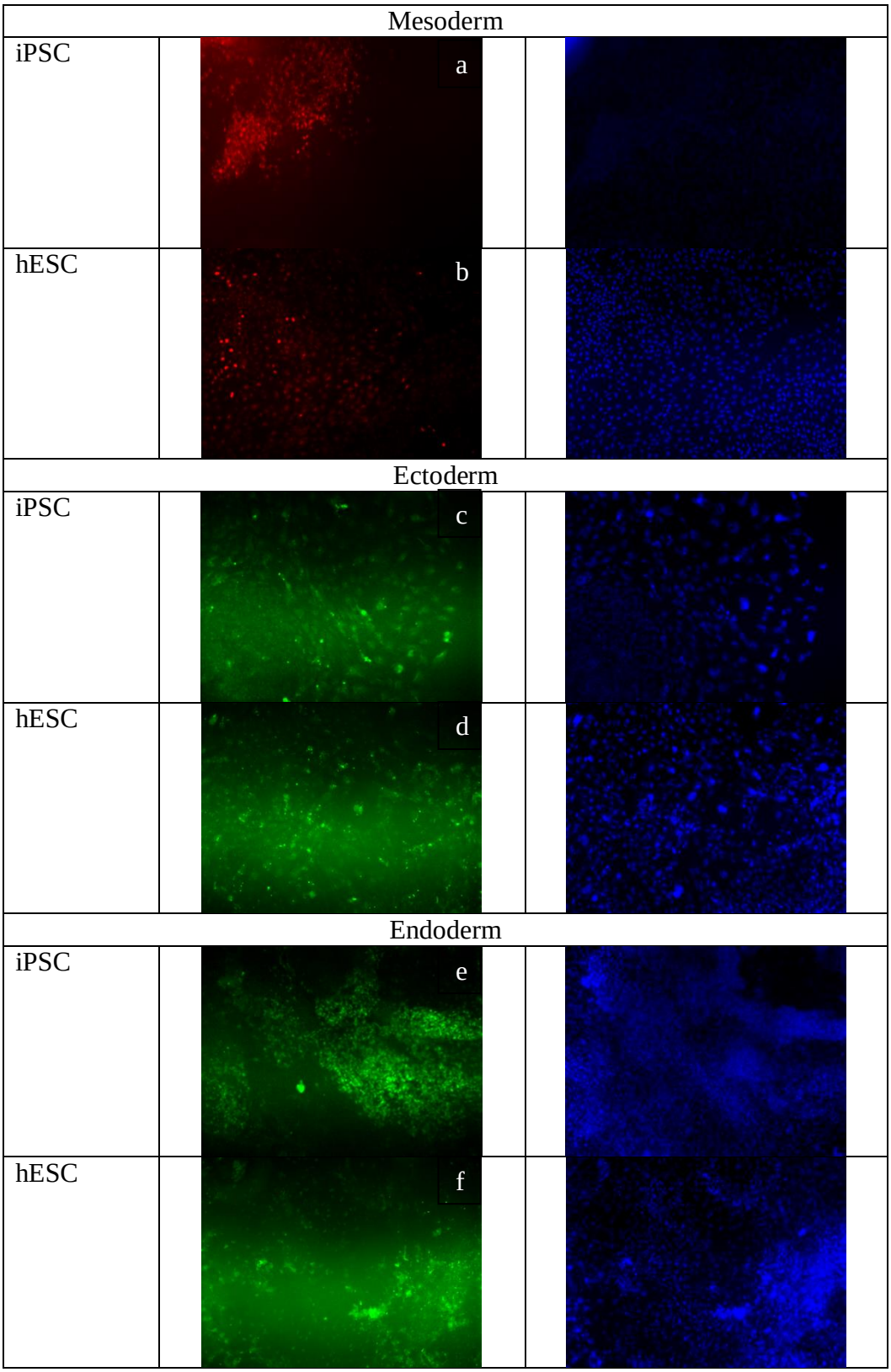

Figure 4.17 Differentiation of iPSC and hESC into three germ layers. The images showed fluorescence images of differentiated iPSC and hESC into mesoderm, ectoderm, and endoderm. The mesoderm lineage was stained against Brachyury (a, b), ectoderm was stained against MAP2 (c, d), and endoderm lineage was stained against Sox17 (e, f). The nucleic acids were stained with DAPI and the DAPI images were showed at the right side of the fluorescence images. Magnification: 100X.

4.6 Differentiation of iPSCs into Keratinocytes

After we successfully verified the identity of the iPSCs, the iPSCs were differentiated back into keratinocytes. The iPSCs and hESC were re-differentiated back into keratinocytes in the presence of bone morphogenetic-4 protein (BMP4) and retinoic acid (RA). The method of differentiation was monolayer method. After the cells were plated on matrigel-coated plate, the iPSCs were treated with BMP4 and RA the next day. The hESC was induced two days later after plated on matrigel-coated plate.

On the following day after treatment, the morphology for iPSC and hESC were changed and did not resemble the typical hESC morphology such as compact colony, distinct border, and larger nucleoli. After 15 days, the cells were harvested and subjected to immunofluorescence staining and reverse-transcription polymerase chain reaction to check for the cytokeratin 14 marker, which is a typical marker for basal keratinocytes.

4.6.1 Characterisation of iPSC-derived Keratinocytes using IF and RT-PCR

The harvested iPSC and hESC were stained for cytokeratin 14 marker. From Figure 4.19, both the differentiated cells from iPSC and hESC showed expression for CK14, a marker for basal keratinocytes. The differentiated cells from iPSC and hESC were also harvested and subjected to RNA isolation. The isolated RNA were used as a template to synthesise the cDNA. Then, the cDNA was used for conventional PCR to detect for CK14 gene. Figure 4.20

showed the three iPSC clones and hESC also expressed CK14 gene. The CK14 gene has a band size of 113base pairs.

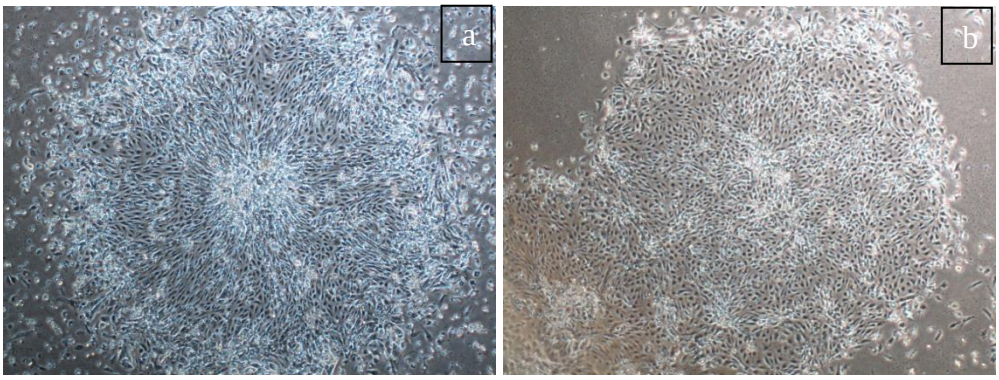

Figure 4.18 Morphology of differentiated iPSCs and hESC. The iPSC (a) and hESC (b) were plated and induced to differentiate with BMP4 and retinoic acid. The morphology no longer resembles of the typical hESC morphology such as compact colony, distinct border, and larger nucleoli. Magnification: 100X.

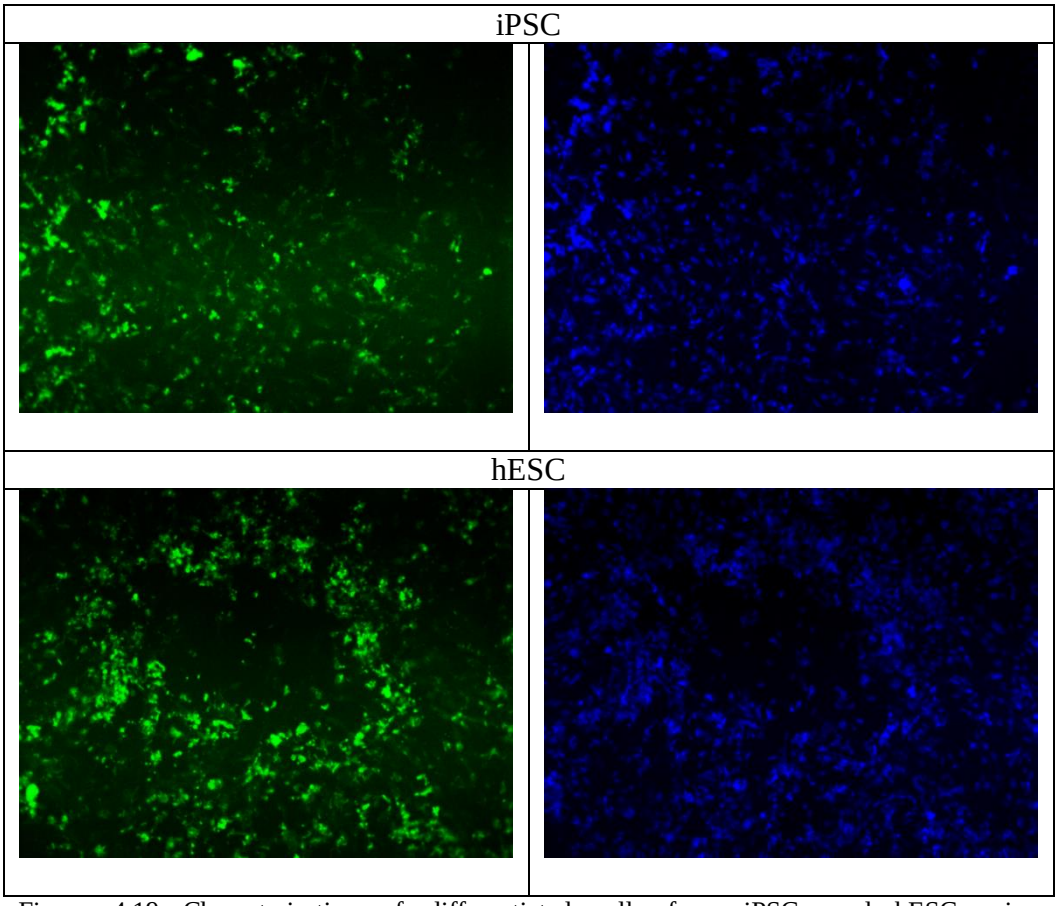

Figure 4.19 Characterisation of differentiated cells from iPSCs and hESC using immunofluorescence staining. Upon 15 days of BMP-4 and RA treatment, the differentiated cells were stained against cytokeratin 14 marker. The differentiated cells showed expression for CK14 marker. Magnification: 100X.

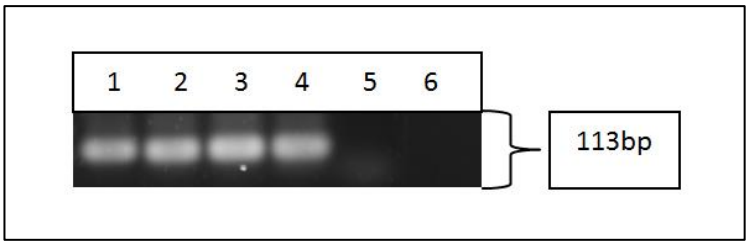

Figure 4.20 Characterisation of differentiated cells using RT-PCR. After 15 days, the differentiated cells were harvested and subjected to RNA extraction. The RNAs were used as template to synthesise the cDNA for the PCR. The PCR products were run on 1.5% agarose. The differentiated cells from iPSCs (Lane 1-3) and hESC (Lane 4) showed the expression of cytokeratin 14. (Lane 1) Clone 1, (Lane 2) Clone 2, (Lane 3) Clone 3, (Lane 4) hESC, (Lane 5) HHFK, and (Lane 6) Negative Control.

CHAPTER 5

DISCUSSION

Protocols for the isolation of keratinocytes from plucked human hair follicles were tested (Barlow & Pye, 1997; Aasen & Belmonte, 2010). But, a protocol for the isolation of keratinocytes and maintenance of the culture for at least few passages is not found. Hair cells were successfully isolated from the human hair follicles, but unable to establish the primary keratinocyte cell line. There could be specific factors that contribute to the unsuccessful establishment of the primary keratinocytes culture.

Human dermal fibroblasts (HDF) was subsequently used as feeder layer for keratinocytes culture isolated from plucked human hair follicles. According to Limat and Hunziker (1996), the use of HDF provides advantage compared to mouse 3T3 feeder layer. The advantage of using HDF is the reproducibility of the feeder layers, which can be cultured several weeks without loss of growth properties (Limat, et al., 1989; Limat & Hunziker, 1996). In reference to a study by Bisson et al. (2013), the authors compared the morphology and physiological properties between the mouse 3T3 feeder and HDF feeder. Both studies showed that there was no much difference in using either one as feeder cells except that there is an advantage for using human feeder layer because it is more suitable for clinical use (Bisson, et al., 2013).

The plucking of the hair follicles was very important. The correct plucking of hair follicles technique resulted in complete hair follicles with the outer root sheath. The hair need to be plucked fast and pulled properly from the base of the scalp (Limat & Hunziker, 1996). The hair follicles harvested from some donors yielded incomplete hair follicles or missing outer root sheath even with the correct plucking technique. This was because the scalp of the donor was too tough and some portion of the outer root sheath was retained (Limat & Hunziker, 1996).

The keratinocytes medium used for isolated keratinocytes from plucked human hair follicles was supplemented with hydrocortisone, adenine, insulin, cholera toxin, transferrin, and EGF. The medium compositions play an important role in promotes the keratinocytes growth (Limat & Noser, 1986). Each of the supplements played different roles in the keratinocytes culture. Hydrocortisone is a type of steroid hormone and its functions are to increase the growth rate and to maintain the stratified appearance of keratinocytes (Rheinwald & Green, 1975). Insulin induces keratinocytes proliferation through the mechanism that involves the activation of Na^+/K^+ pump (Shen, et al., 2001).

Transferrin is an iron carrier protein which function is to facilitate uptake of iron by cells (Young & Garner, 1990). All cell types need transferrin to grow hence it is an important supplement in culture medium (Barnes & Sato, 1980; Young & Garner, 1990). Transferrin is also present in foetal bovine serum but bovine transferrin binds poorly to human transferrin receptor (Young

& Garner, 1990). Adenine is one of the purine bases that make up the nucleic acid (Michal & Schomburg, 2012). Adenine is also part of the ATP, NAD, and FAD molecules that are involved in cellular metabolism (Michal & Schomburg, 2012). Thus, the function of adenine in culture medium enhances cellular metabolism (Michal & Schomburg, 2012).

The addition of cholera toxin to the culture medium will increase the keratinocytes growth rate by increasing the cAMP level (Okada et al, 1982). The cholera toxin only increases the keratinocytes proliferation when keratinocytes were seeded at low number and inhibit proliferation when the keratinocytes culture when fully confluent (Okada, et al., 1982). Cyclic AMP is involved in proliferation as regulator of cellular events (Okada, et al., 1982). EGF is one type of protein growth factor which increases the keratinocytes proliferation rate by 20 % (Karasek, 1983).

In this study, keratinocytes were isolated using both enzymatic and outgrowth method. For the enzymatic treatment, the trypsin concentration and enzyme treatment time were very important (Seo, et al., 2003). It was shown that with increase in concentration and time, the number of cells recovered increases, but the number of cells adhered was decreased and the cells tend to change in morphology (Seo, et al., 2003). According to Seo et al. (2003), the authors reasoned that the outgrowth method was not suitable for primary culture because of three reasons. The three reasons were the probability of cell outgrowth was low, the culture was contaminated with other cell types, and the lag phase was too long (Seo, et al., 2003).

In this study, iPSCs have been generated from the purchased human hair follicular keratinocytes (HHFK) using the retrovirus carrying the four transcription factors, Oct4, Sox2, Klf4, and c-Myc. The factors were delivered at the same time using a single factor vectors. The iPSCs generated were of true identity through the expression of hESC-like surface and intracellular markers such as TRA-1-60, TRA-1-81, SSEA4, Oct4, Sox2 and Nanog. The iPSCs were differentiated *in vitro* to form the embryonic three germ layers, namely, ectoderm, endoderm, and mesoderm. The characterisation methods used were those commonly used to check for the identity of true iPSC (Takahashi, et al., 2007).

It was the first successful generation of iPSCs from somatic cells reported in UTAR laboratory. Research in iPSC is a new area of research in Malaysia and no paper has yet been published on the successful generation of iPSCs from somatic cells. Retrovirus-based reprogramming method was chosen to reprogram HHFK. This method was among the first and most established method for generation of iPSCs as different type of cells (such as fibroblasts, neural stem cells, adipose cells, and amniotic cells) have been used to generate iPSCs successfully (Takahashi, et al., 2007; Robinton & Daley, 2012). This method also reported higher reprogramming efficiency compared to other viral methods such as adenoviral or Sendai virus (Oh, et al., 2012). Although it was a transgene~~s~~ integrating method, it is suitable for use as a platform to study the iPSCs generation and its pluripotency (Gonzalez, et al., 2011).

Three attempts of reprogramming experiments were performed on HHFK. From the three experiments, 34 clones had been selected for further passaging, but the number of clones that survived for at least 10 passages and above was seven. From the seven clones, three clones were selected for characterisation while the remaining clones were cryopreserved in liquid nitrogen as back-up. The selected three clones were from each of the three separate experiments. The biological replicate of three is usually sufficient for a study. Apart from that, the other reasons involved were due to time constraint, cost and work load. Due to the laborious work involved in maintenance of iPSC and the cost of the culture medium and supplements, the decision to focus on characterisation of only three clones was made.

In this study, the reprogramming efficiency of HHFK reported was in the range of 0.44% to 0.74%. This result represents at least 44-fold increase in reprogramming efficiency compared to fibroblasts reprogramming (Takahashi, et al., 2007). The cell source could be a factor in affecting the reprogramming efficiency. Even with the same cell source but different infection system, the reprogramming efficiency could be affected as well. For example, the reprogramming efficiency of retrovirus transduction in this study exceeded the reprogramming efficiency of keratinocytes using polycistronic lentivirus (Novak, et al., 2010).

Although, a study reported that only the polycistronic lentiviral vector can efficiently delivered the factors to keratinocytes and efficiently reprogrammed the keratinocytes (Novak, et al., 2010). Nevertheless,

keratinocyte reprogramming efficiency in this study was higher compared to the keratinocytes reprogrammed by using the polycistronic lentiviral vector. In another study, the single vector could minimize the integration site into the cells genome. But, this do not eliminate the risk of insertional mutagenesis, it only reduce the risk (Sommer, et al., 2009). The safest way would be totally eliminate the use of integrated system.

The inclusion of Oct4 and Sox2 in this study as the transcription factors for reprogramming is rather important. Both factors were also included in the Yamanaka factor and Thomson factor (Takahashi, et al., 2007; Yu, et al., 2007). Oct4 is the single most important factor among the four, but, it is not irreplaceable (Hochedlinger & Plath, 2009). This study showed that Oct4 is not irreplaceable as iPSCs were successfully generated by using Sox2, Klf4 and c-Myc and also a chemical inhibitor (Shi, et al., 2008; Hochedlinger & Plath, 2009). Furthermore, Oct4 will form heterodimer with Sox2 to regulate the ESC gene expression (Boyer, et al., 2005).

In this study, Nanog is not included as one of the reprogramming factor, but, was activated later after reprogramming. An Oct4/Sox2 motif was found located at the upstream of the transcription start site which was found to be important in the regulation of Nanog expression (Pan & Thomson, 2007). Oct4 and Sox2 will promote Nanog transcription in iPSCs (Pan & Thomson, 2007). Thus, the expression of Oct4, Sox2 and Nanog in iPSCs will promote the maintenance of pluripotency of iPSC apart from promoting cell self-renewal

and supress the genes involved in differentiation (Boyer, et al., 2005) (Pan & Thomson, 2007).

Apart from that, Nanog was detected in the parental cells, HHFK using the RT-PCR. A study by Novak et al. (2010) did not report any Nanog expression when studying human keratinocytes for inducing iPSC. Nevertheless, Nanog expression was reported in human keratinocytes cell line, HaCat, and keratinocytes stem cells derived from the bulge of the hair follicles (Yu, et al., 2006; Palla, et al., 2015). Even though, the mRNA of Nanog was detected in the parental cells, there is a possibility that the level is low in order to produce a detectable protein level. Various processes also involved in the regulation of protein abundance such as post-transcriptional modification and degradation of mRNAs and proteins (Vogel & Marcotte, 2012).

Although c-Myc can be omitted from the reprogramming, but, the inclusion of the c-Myc as one of the reprogramming factors will enhance the reprogramming efficiency (Nagakawa, et al., 2008). c-Myc acts on miR-21 and miR-29a by down-regulating the level of these two microRNAs (Yang, et al., 2011). The inhibition of these two mircoRNAs will inhibit the p53 and ERK1/2 pathways (Yang, et al., 2011). The inhibition of these two pathways leads to an increase in the reprogramming efficiency (Yang, et al., 2011). Recently, many reprogramming studies had eliminated c-Myc from their reprogramming cocktails due to c-Myc in the role as an oncogene (Aoki, et al., 2010; Okita & Yamanaka, 2010; Piao, et al., 2014).

Klf4 involved in suppression of early somatic genes such as Tgfb1, Pdgfra, and Col6a1 and followed by activation of pluripotency genes (Polo, et al., 2012). Klf4 binds to Oct4 and Sox2 via the C-terminus which contain the three zinc fingers (Wei, et al., 2009) and forming a complex. Klf4 and Oct4 binding site are also found to be present in the Nanog promoter (Wei, et al., 2009). When the Klf4, Oct4, and Sox2 complex being disrupted by the introduction of mutant Klf4, the reprogramming efficiently was further reduced (Wei, et al., 2009). Klf4, Oct4 and Sox2 complex activates Lefty expression which is highly expressed in embryonic stem cells (Nakatake, et al., 2006).

Among the surface markers that were commonly used to characterise iPSC are SSEA4, SSEA3, TRA-1-81, and TRA-1-60 (Abujarour, et al., 2013). Characterisation of iPSC using the surface markers SSEA4, TRA-1-81 and TRA-1-60 was performed at the protein level using the flow cytometry analysis and immunofluorescence. The results in this study showed comparable expression of the above mentioned surface markers for iPSC with the hESC. For TRA-1-60 and TRA-1-81, the percentage of expression was lower in iPSC (Figure 4.14) compared to hESC. But, the SSEA4 level was higher in iPSC (Figure 4.14) than hESC. There is no literature reporting on the differences in the lever of surface markers between iPSC and hESC and association between the level differences. SSEA4 is the carbohydrate epitopes of the globo-series glycolipids (Zhao, et al., 2012). SSEA4 are found in mesenchymal stem cells, neural progenitor cells and corneal epithelial cells (Truong, et al., 2011) apart from their presence in oocytes, zygotes and early cleavage-stage embryos (Zhao, et al., 2012). SSEA4 and SSEA3 usually express first before TRA-1-60

and TRA-1-81 (Chan, et al., 2009). SSEA4 was detected as early as days 2 compared to TRA-1-60 as early as days 6 (Chan, et al., 2009). Combination of surface markers and morphology identification is required to confirm the selection of true iPSC (Chan, et al., 2009).

Apart from common surface markers, alkaline phosphatase (AP) (or TRA-2-49) has also been used as pluripotency marker identification (Boulting, et al., 2011). AP is a type of enzymes that dephosphorylating molecules under alkaline conditions (Singh, et al., 2012). AP has been used as live staining for the identification of successfully reprogrammed iPSC clones and for calculation of reprogramming efficiency through number of AP positive colonies (Singh, et al., 2012). More recently, one negative cell surface marker was identified to be used with the positive surface markers for iPSC selection (Quintanilla, et al., 2014). The new negative surface marker is CD44 and CD44 is a type of surface glycoprotein that is expressed by a variety of cells including fibroblasts, epithelial cells, and lymphocytes (Quintanilla, et al., 2014).

Culture conditions, feeder cells and medium composition are the factors involved to regulate the reprogramming efficiency (Gonzalez, et al., 2011). During the reprogramming process, valproic acid was added during the initial period of reprogramming and the culture was stored in hypoxic conditions (5% oxygen level). In this study, no comparing of the reprogramming efficiency of using VPA and without VPA or incubate the cells in hypoxic or without hypoxic condition was performed as the focus of the study is not to study the reprogramming efficiency. Reprogramming under hypoxic condition in human

cells was observed to enhance the reprogramming efficiency by 4 fold (Gonzalez, et al., 2011). While, reprogramming under hypoxic condition and the presence of VPA for mouse cells, the efficiency was reported to be increased to 200 fold (Yoshida, et al., 2009).

Apart from increasing the efficiency using the VPA or hypoxic condition, the transduction efficiency can also be boosted by using centrifugation (or also known as spinfection method) and polybrene. Many studies had used both of these methods to increase the transduction efficiency (Aasen, et al., 2008; Hanna, et al., 2008). By applying centrifugation force of 700 x*g* for 45 minutes at 32^{o}C, it will bring the virus in the supernatant closer to the adherent cells on the dish surface (Damico & Bates, 2000). The close proximity between the viral glycoproteins and the cell's cell membrane will enhance the virus-cell fusion and thus facilitate the transfer of the viral genome to the cells (Damico & Bates, 2000). Polybrene is a cationic polymer and can be used to increase the infection efficiency of many retroviruses (Damico & Bates, 2000). Polybrene action is to reduce the repulsive charges difference on the surface of the virus and cell's membranes (Damico & Bates, 2000). Polybrene will promote the fusion of virus and cell during the attachment stage (Damico & Bates, 2000).

The mouse embryonic fibroblasts (MEFs) was selected as feeder layer as MEFs have been used as feeder layer since the first reported iPSCs (Takahashi & Yamanaka, 2006). The use of MEFs as feeder layer was a reliable choice as it will support the iPSCs growth and inhibit differentiation

(Dravid, et al., 2005; Gonzalez, et al., 2011). The use of hESC culture medium was selected which consists of DMEM/F12, knock-out serum replacement, L-glutamine, non-essential amino acids, b-mercaptoethanol, and basic fibroblasts growth factors. The same medium composition has been used in other studies for iPSCs culture (Yu, et al., 2007; Aasen , et al., 2008).

Keratinocytes was selected as the target cells from the human hair follicles due to the way the hair follicles can be obtained. The hair follicles can be obtained from the scalp using non-invasive method by plucking it. Thus, this cell type offers advantage over other cell types especially when the patient is not suitable for carrying out skin biopsy or bone marrow aspirate (Streckfuss-Bomeke, et al., 2013).

Apart than that, keratinocytes also offer advantage after the cells have been induced to iPSC. The non-reprogrammed-negative well-cells were attached to the feeder cells, but the cells did not proliferate, died off, and can be seen floating on the culture medium. The same event happened to the non-reprogrammed keratinocytes in the transduced wells. For keratinocytes that is not reprogrammed, the non-reprogrammed cells eventually died off and detached from the plate when the culture medium was switched to hESC culture medium (Aasen, et al., 2008; Streckfuss-Bomeke, et al., 2013). Thus, this leaves only the reprogrammed cells in the culture.

Epithelial-to-mesenchymal transition (EMT) is a process of cell fate determination, whereby the cells losing the epithelial characteristics to acquire

the mesenchymal characteristics (Li, et al., 2010). In iPSC generation, it is the reverse of EMT which is the mesenchymal-to-epithelial transition (MET) that occurs (Aasen, et al., 2008). In order to generate iPSC from fibroblasts, it is required to undergo the MET process thus requiring a bigger effort for the transition and resulted in lower efficiency (Aasen, et al., 2008). For keratinocytes, it skips the MET process and thus it is more favourable for reprogramming (Aasen, et al., 2008). This observation may be used to explain the reason that reprogramming efficiency in this study was much higher compared to those using fibroblasts (Takahashi, et al., 2007).

Proliferative capacity of target cells is also one of the important factors required for successful generation of iPSC. Keratinocytes used in this study is a type of highly proliferative epithelial cells although with limited doubling passage (Aasen & Belmonte, 2010). In a study by Chan et al. (2009), the authors reported that the isolated cell clusters expressed TRA-1-60+ SSEA-4+ but many did not form iPSC. The authors highlighted that this could be due to senescence or low proliferative capacity of the target cells (Chan, et al., 2009). In another study, it was reported that high proliferative capacity of target cells (MEFs) eventually lead to reduce reprogramming efficiency (Xu, et al., 2013). Nevertheless, proliferation of target cells is still needed when using retroviral infection system as retrovirus only infect dividing cells (Xu, et al., 2013).

HHFK was characterised for the presence of cytokeratin 19 (CK19) and cytokeratin 75 (CK75) expressions. CK19 is the secondary keratins of simple epithelial cells and usually appeared in simple ductal epithelial (Moll, et al.,

2008). But, CK19 is also one of the widely distributed simple-epithelial keratins (Moll, *et al*., 2008). CK19 also found to be present in lower and upper area of outer root sheath of anagen phase hair follicles (Yoo, et al., 2007). On the other hand, CK75 is one of the hair follicle-specific epithelial keratins (Moll, et al., 2008). The companion layer is the location where CK75 is specifically present within the hair follicle (Winter, et al., 1998; Moll, et al., 2008).

HHFK was purchased from Sciencell and the cells were characterised using the marker, CK19, which was used by the company to isolate the cells at primary culture and another marker, CK75, was found through detection of several cytokeratin markers that were available. The HHFK was checked for expression of CK19 and CK75 to confirm the cell source. Apart from that, the HHFK cells morphology matched with typical keratinocytes morphology which was typical cobblestone shape (Racila, et al., 2011).

CK19 was also identified as one of the putative stem cell marker present in the hair follicles (Michel, et al., 1996). The presence of these keratinocytes stem cells was thought to be the target of reprograming and thus increase the reprogramming efficiency (Aasen, et al., 2008).

In this study, the HHFK was identified to express endogenous Klf4 and c-Myc (Figure 4.15c & d). Although, endogenous Klf4 was reported to be highly expressed in keratinocyte, but, Klf4 is still needed for reprogramming (Aasen, et al., 2008). In fact, the endogenous expression of the reprogramming

factors in keratinocytes still contributed towards reprogramming (Aasen, et al., 2008). In another study, the dermal papilla cells isolated from hair follicles also expressed endogenous Sox2, Klf4, and c-Myc (Tsai, et al., 2011). Thus, the authors tried to reprogram the dermal papilla cells with the use of only one factor, Oct4 (Tsai, et al., 2011). The dermal papilla cells successfully generated iPSCs but with reduced efficiency of 0.088% and the colonies only appeared after 3 weeks (Tsai, et al., 2011).

In this study, the clones of iPSC were able to be differentiated into keratinocytes that expressed the cytokeratin 14 (Figure 4.20 and Figure 4.21), marker for basal keratinocytes of stratified squamous epithelia. CK14 expression in hair follicle keratinocytes has been reported in other studies (Gho, et al., 2004; Novak, et al., 2010). Two growth factors, retinoic acid (RA) and bone morphogenic protein-4 (BMP-4) were used to direct the differentiation into keratinocytes. RA direct iPSCs into epithelial fate while BMP-4 block differentiation into neural lineage (Metallo, et al., 2008). In addition, the RA-induced differentiation offered an efficient way to generate keratinocytes with higher proliferative capacity (Metallo, et al., 2008).

There is no published study on keratinocytes reprogramming using three and lesser factors. Thus, study to optimize the reprogramming method to facilitate the successful generation of keratinocytes using two factors is needed. This could be coupled with the use of small molecules to enhance the reprogramming efficiency (Oh, et al., 2012). With the high keratinocytes

reprogramming efficiency, keratinocytes can represent one of the non-invasive and easily accessible cell sources.

Since the iPSCs were successfully generated from human hair follicular keratinocytes, future work can focus on generating the HHFK derived iPSCs as a safer alternative and without integration into host genome. This safer cell source represents valuable cells for clinical use and most importantly patient-specific cells can be generated. The patient-specific cells can be used for the patient without the issue of rejection. In contrast, using hESC derived cell source for transplantation whereby rejection will most likely occur (Almeida, et al., 2013). With the improvement in the differentiation protocol, keratinocytes can be derived from the patient derived iPSCs. This keratinocytes source represents a valuable source for *in vitro* hair cloning or use to treat hair balding or for grafting of burn patients.

CHAPTER 6

CONCLUSION

In conclusion, induced pluripotent stem cells (iPSCs) have been successfully generated from human hair follicular keratinocytes (HHFK) using retroviral transduction method. The four transcription factors used were Oct4, Sox2, Klf4, and c-Myc. The iPSCs generated expressed hESC markers such as TRA-1-60, TRA-1-81, SSEA4, Oct4, Sox2, Nanog, Lefty, and Gabrb. The iPSCs were able to be differentiated *in vitro* to form the embryonic three germ layers, namely, ectoderm, endoderm, and mesoderm. For the HHFK-derived iPSCs, it was able to be differentiated into keratinocytes expressing CK14. This cell type could represent a new valuable source for *in vitro* hair cloning or use to treat hair balding or for grafting in burn patients.

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APPENDICES

APPENDIX A

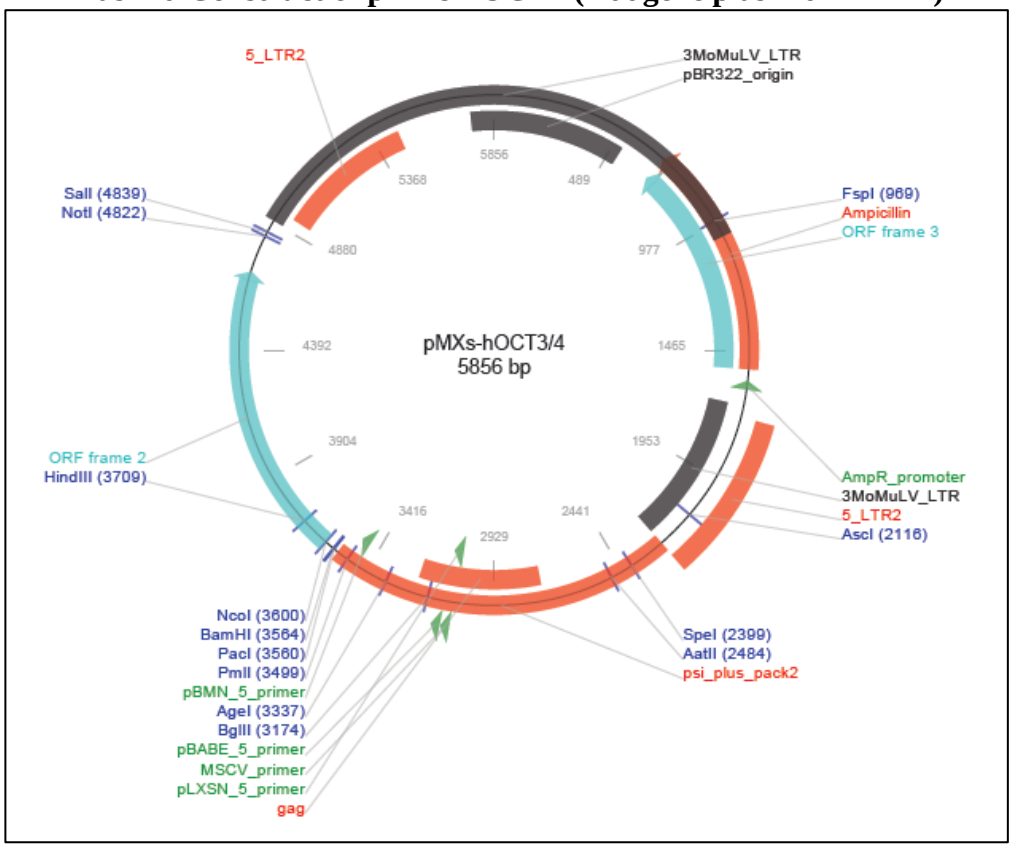
Chemicals and Cell Culture Reagents

Chemicals and Cell Culture Reagents	Brand	Country
0.05% trypsin-EDTA	Invitrogen	CA, USA
2-mercapthoethanol	MP Biomedicals	CA, USA
Adenine	Sigma	Missouri, USA
Bone morphogenetic protein 4	Invitrogen	CA, USA
Bovine serum albumin	Nacalai Tesque	Kyoto, Japan
Calcium Chloride	Calbiochem	Darmstadt, Germany
Cholera toxin	Sigma	Missouri, USA
DMEM/Ham's F-12 medium (DMEM/F12)	Invitrogen	CA, USA
Dulbecco's Modified Eagle's Medium (DMEM)	Invitrogen	CA, USA
Epidermal growth factor	Miltenyi Biotec	Bergisch Gladbach, Germany
Fetal bovine serum (FBS)	Invitrogen	CA, USA
Fibroblasts growth factor (FGF-2)	Miltenyi Biotec	Bergisch Gladbach, Germany
Gelatin	Sigma	Missouri, USA
Geneticin	Merck	Darmstadt, Germany
Keratinocytes serum free medium	ScienCell	CA, USA
Knockout serum replacement (KOSR)	Invitrogen	CA, USA
L-glutamine	Invitrogen	CA, USA
LB broth	Sigma	Missouri, USA
Matrigel	Becton Dickinson	New Jersey, USA
Mitomycin-C	Merck	Darmstadt, Germany
mTeSR1 medium	Stemcell Technologies	British Columbia, Canada
Non-essential amino acid (NEAA)	Invitrogen	CA, USA
Phosphate buffered saline (PBS)	MP Biomedicals	CA, USA
Poly-l-lysine	ScienCell	CA, USA
Retinoic acid	Merck	Darmstadt, Germany

Sodium pyruvate	Merck, Darmstadt	Germany
Triiodothyronine	Sigma	Missouri, USA
Transferrin	Sigma	Missouri, USA

APPENDIX B

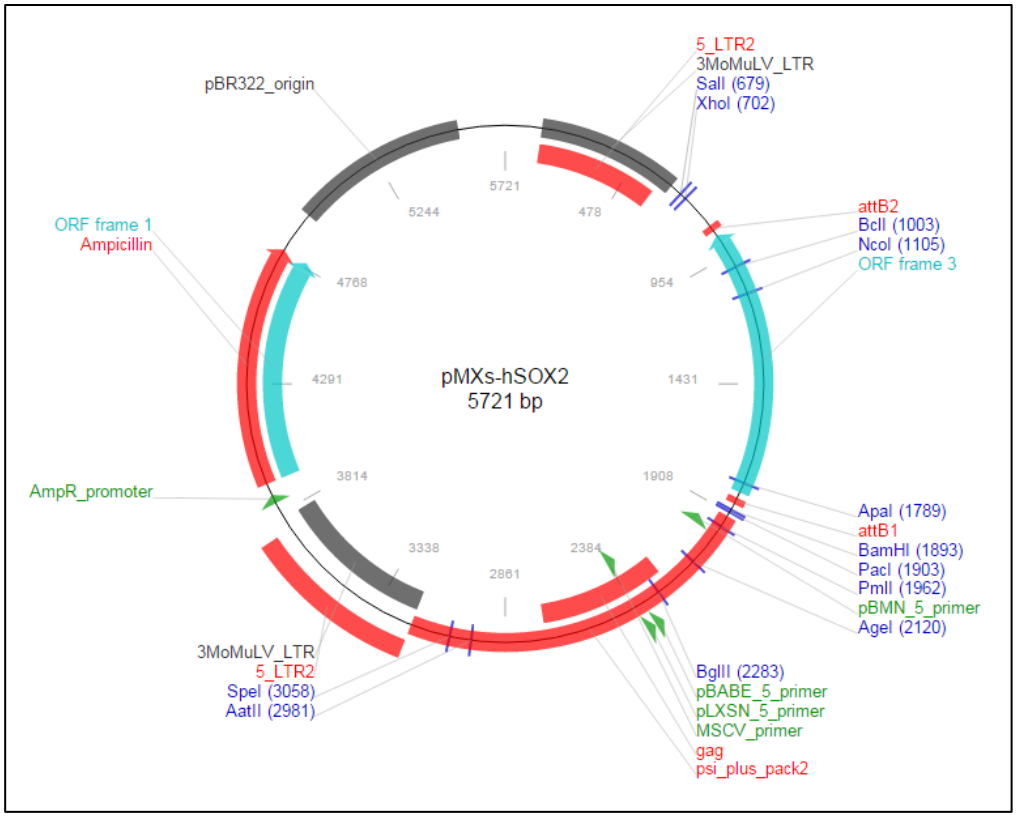
Plasmid Construct of pMXs-hOCT4 (Addgene plasmid # 17217)



Adapted from Takahashi, et al., 2007. At: https://www.addgene.org/17217/ [Accessed on 12th May 2015]

APPENDIX C

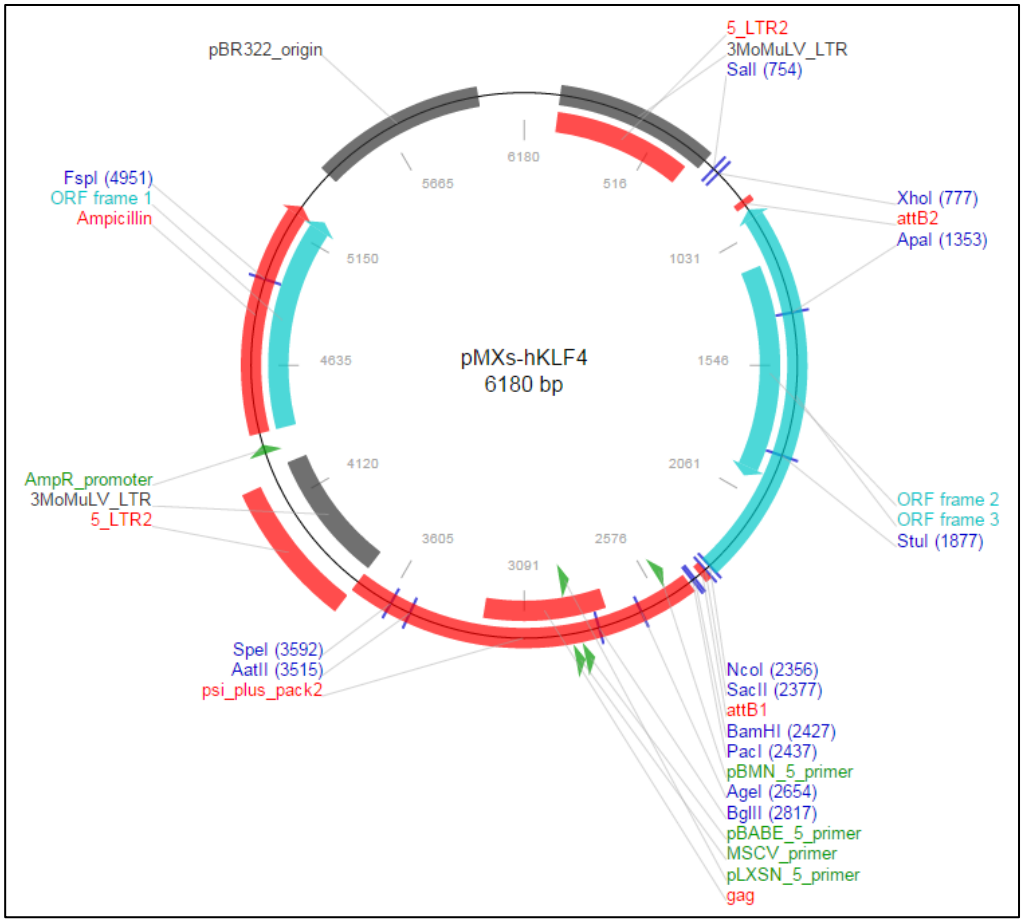
Plasmid Construct of pMXs-hSOX2 (Addgene plasmid # 17218)



Adapted from Takahashi, et al., 2007. At: https://www.addgene.org/17218/ [Accessed on 12th May 2015]

APPENDIX D

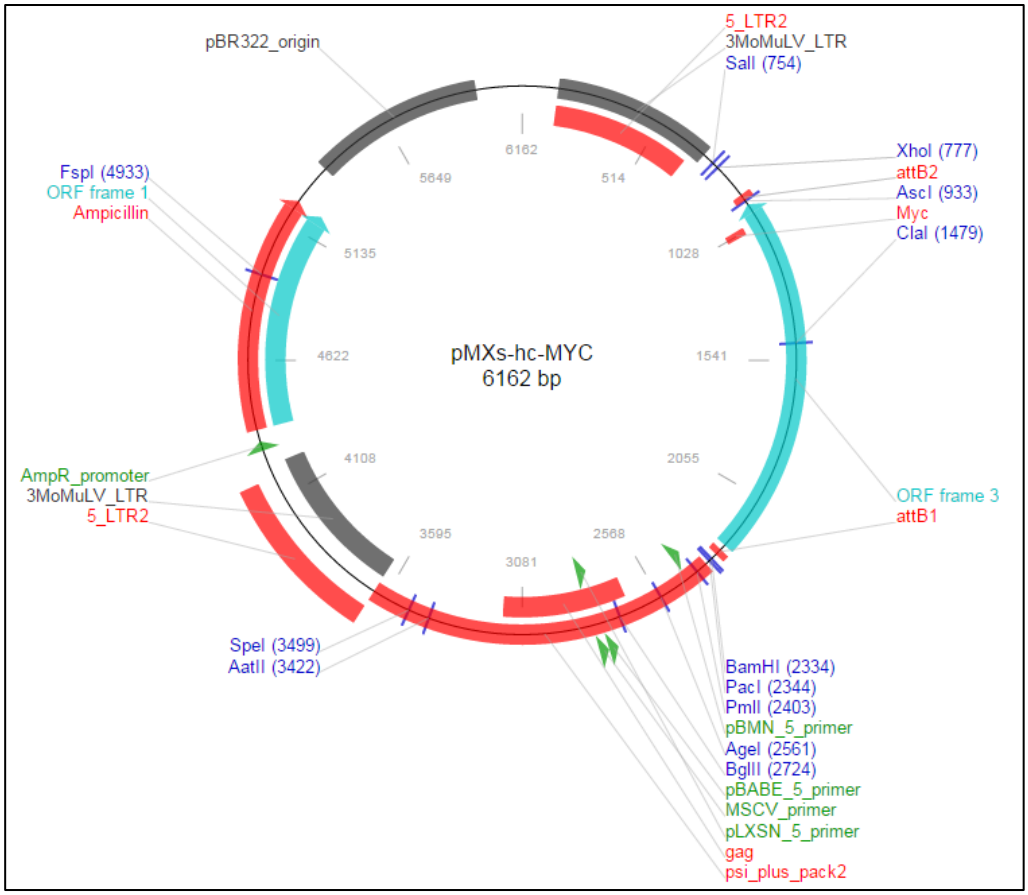
Plasmid Construct of pMXs-hKLF4 (Addgene plasmid # 13375)



Adapted from Takahashi, et al., 2007. At: https://www.addgene.org/13375/ [Accessed on 12th May 2015]

APPENDIX E

Plasmid Construct of pMXs-hc-MYC (Addgene plasmid # 17220)



(Adapted from Takahashi, et al., 2007. At: https://www.addgene.org/17220/ [Accessed on 12th May 2015]

APPENDIX F

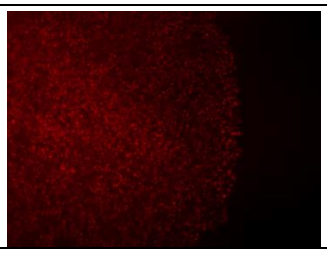
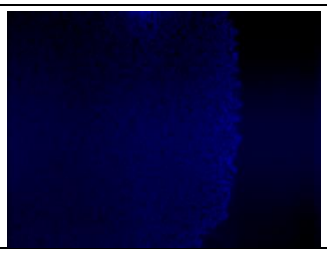
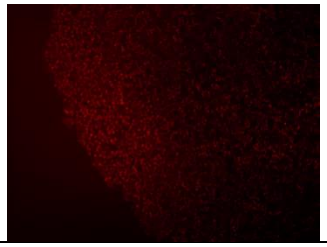
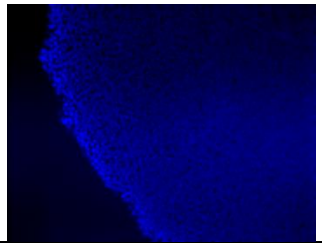
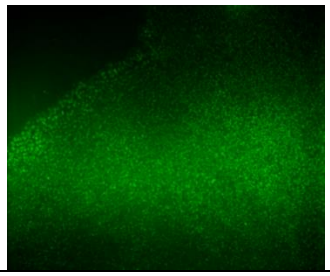
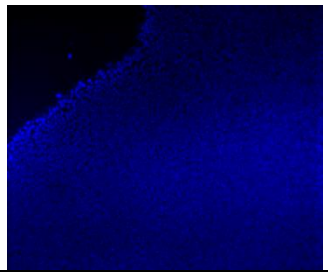
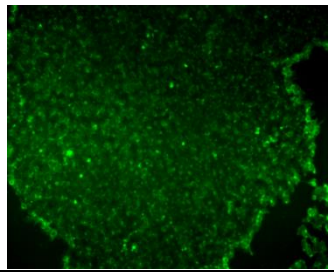
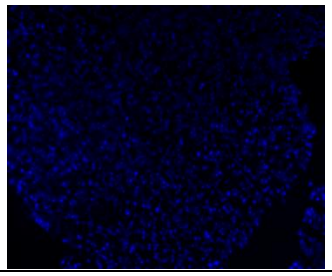
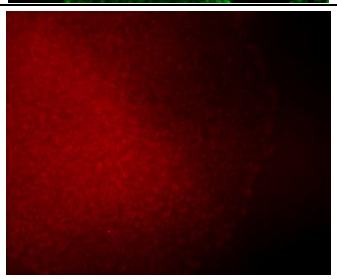
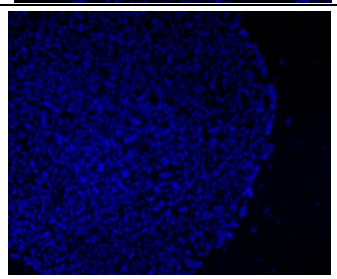
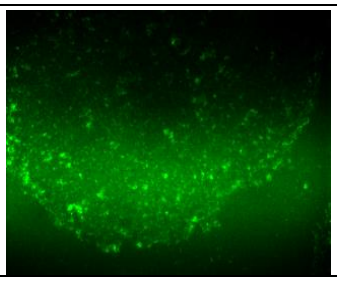
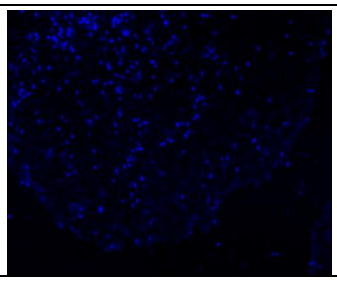
Primers Used in Reverse-transcription Polymerase Chain Reaction (RT-PCR) for Characterisation of iPSCs

Primers	Sequence	Product Size (bp)	Annealing Temperature (^{o}C)
CK14	Forward: 5'GACCATTGAGGA CCTGAGGA Reverse: 5'CATACTTGGTGC GGAAGTCA	113	58
CK19	Forward: 5'GCGGGACAA GATTCTTGGTG3' Reverse: 5'CTTCAGGCCTTC GATCTGCAT3'	214	58
CK75	Forward: 5'CCTTCCTCACCC ACCATGTCT3' Reverse: 5'GGGTCGATTTGC AGGTGAAG3'	434	60
Oct4	Forward: 5'GACAACAATGAA AATCTTCAGGAGA Reverse: 5'CTGGCGCCGGTT ACAGAACCA	195	58
Sox2	Forward: 5'ATGTACAACATG ATGGAGACG Reverse: 5'GCGCTTGCTGAT CTCCGAGT	220	58
Nanog	Forward: 5'ATGCCTGTGATT TGTGGGCC Reverse: 5'GCCAGTTGTTTT TCTGCCAC	403	58
Klf4	Forward: 5'CGATCAGATGCA GCCGCAAGTC Reverse: 5'TGTGTAAGGCGA GGTGGTCCGa	365	58
c-Myc	Forward: 5'TACCCTCTCAAC GACAGCAG Reverse: 5'TCTTGACATTCT CCTCGGTG	477	58
Lefty	Forward: 5'CTTGGGGACTATGG AGCTCAGGGCGAC Reverse: 5'CATGGGCAGCGAGT CAGTCTCCGAGG	255	67
GABRB	Forward: 5'CCTTGCCCAAAATC CCCTATGTCAAAGC Reverse: 5'GTATCGCCAATGCC GCCTGAGACCTC	277	64

GAPDH	Forward: 5’AGGGCTGCTTTT AACTCTGGT Reverse: 5’CCCCACTTGATT TTGGAGGGA	206	58

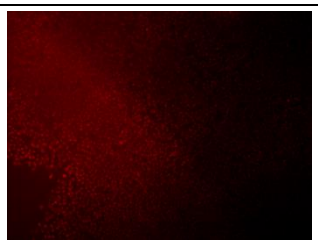
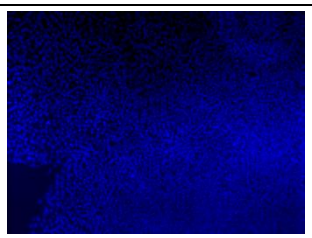
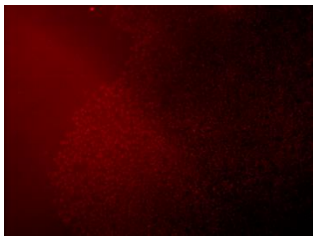
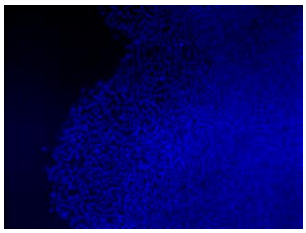
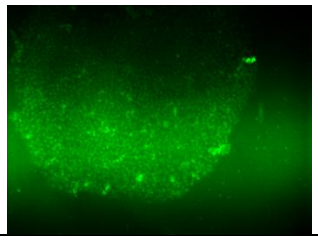
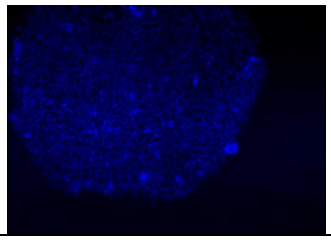
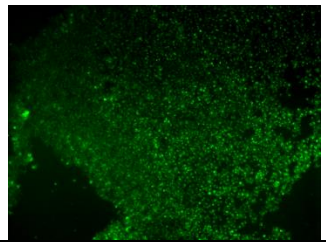
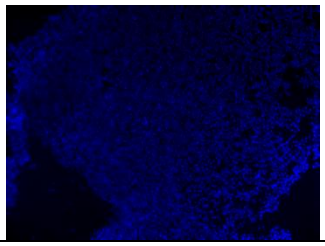
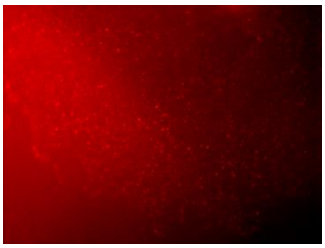
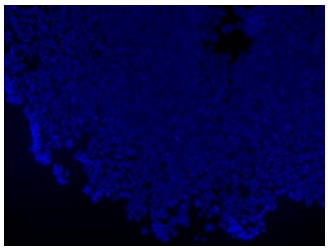
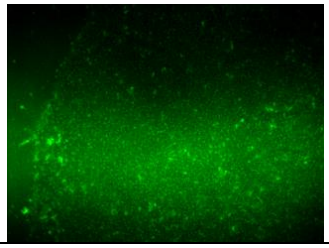
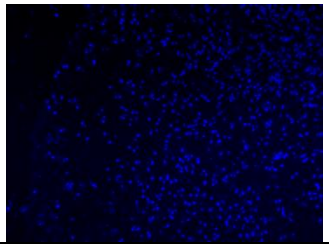
APPENDIX G

Immunofluorescence of Markers Expression by Keratinocytes-derived Induced Pluripotent Stem Cells (Clone 1)

Clone 1		
Oct4		
Sox2		
Nanog		
SSEA4		
TRA-1-60		
TRA-1-81		

APPENDIX H

Immunofluorescence of Markers Expression by Keratinocytes-derived Induced Pluripotent Stem Cells (Clone 2)

	Clone 2	
Oct4		
Sox2		
Nanog		
SSEA4		
TRA-1-60		
TRA-1-81		

APPENDIX I

Flow Cytometry Analysis of Markers Expression by Keratinocytes-derived Induced Pluripotent Stem Cells (Clone 1)

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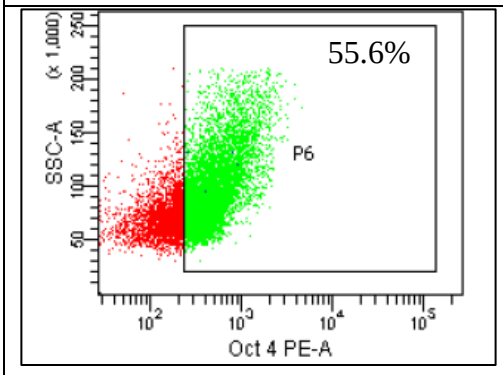
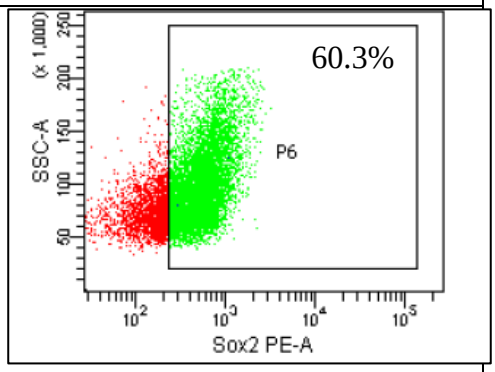
<tr><td> 
</td><td> 
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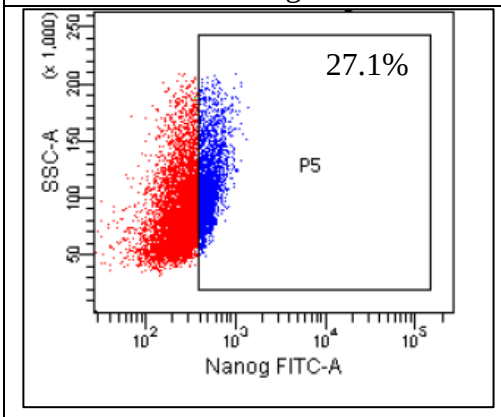
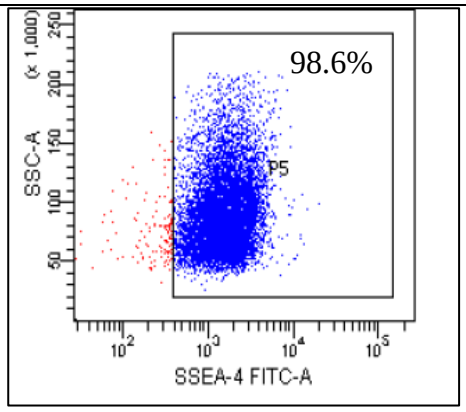
<tr><td> 
</td><td> 
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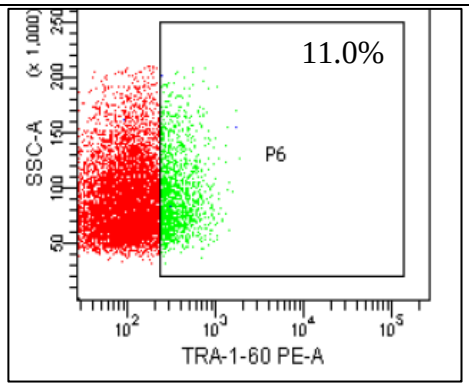
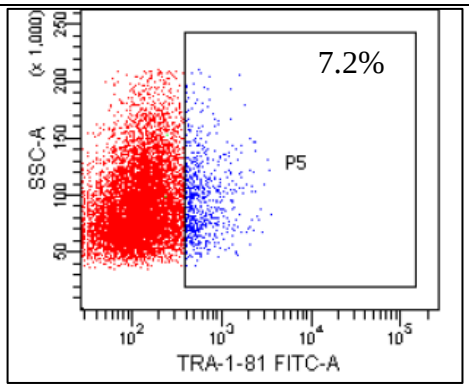
<tr><td> 
</td><td> 
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APPENDIX J

Flow Cytometry Analysis of Markers Expression by Keratinocytes-derived Induced Pluripotent Stem Cells (Clone 2)

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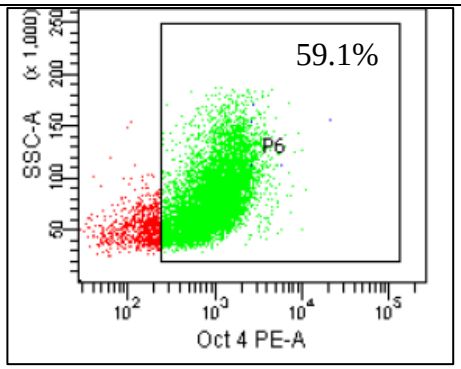
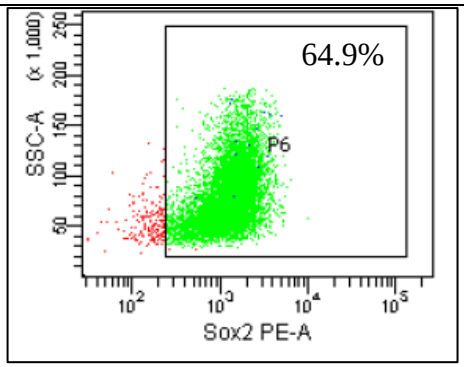
<tr><td> 
</td><td> 
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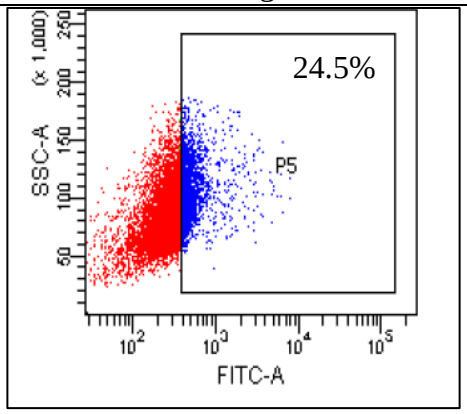
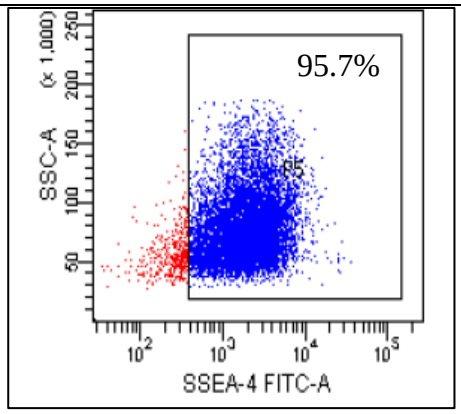
<tr><td> 
</td><td> 
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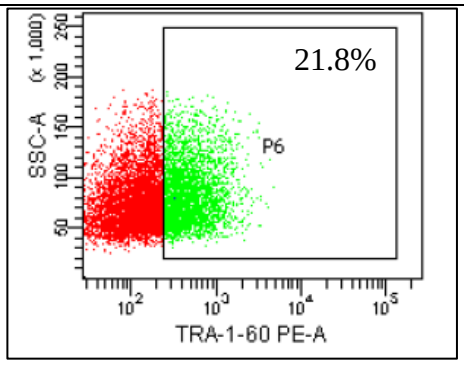
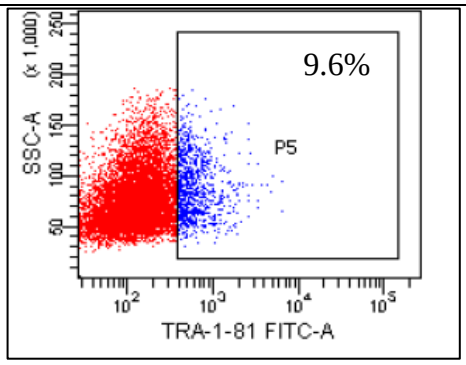
<tr><td> 
</td><td> 
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