COMPARATIVE MOLECULAR SIGNATURES OF HUMAN MESENCHYMAL STROMAL CELLS DURING *IN VITRO* PASSAGING

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COMPARATIVE MOLECULAR SIGNATURES OF HUMAN MESENCHYMAL STROMAL CELLS DURING *IN VITRO* PASSAGING

ABSTRACT

Significant progress has been witnessed in the last decade documenting the use of human mesenchymal stem/stromal cells (hMSCs) as an alternative resource for cell replacement therapy owing to their intrinsic features of self-renewal and differentiation capacity into therapeutically valuable cell types. However, the yield of isolated hMSCs from donor tissues is usually inadequate, suggesting the need for the expansion of hMSCs in culture to meet the clinical need. However, hMSCs undergo cellular ageing, thus limiting their proliferation and differentiation potential in long term-culture. Cellular ageing is the manifestation of a complex interplay of molecular pathways between the gene expression and microniche which is govern by many processes including miRNA dysregulation. In this study, we aimed to determine molecular signatures of primary hMSCs isolated from deciduous pulp (SHED) and Wharton's Jelly (WJSCs) associated with cellular ageing during *in vitro* passaging. We found phenotypic changes of primary hMSCs isolated from SHED and WJSCs by passaging in culture. Subsequently, molecular profiling showed a set of diverse miRNA and mRNAs that were deregulated in SHED and WJSCs. With this platform, an overlap of up-regulated and down-regulated miRNAs between SHED and WJSCs was observed. Notably, the hsa-miR-22, hsa-miR-485-5p and hsa-miR-let-7a have been identified as the cellular senescence inducers were show highly up-regulation. Whereas, hsa-miR-302a, hsa-miR-373 and hsa-miR-520e were found commonly down-regulation in both cell types. These miRNAs are known as signature miRNAs in stem cells involved in maintaining stemness biological process. Moreover, the predicted miRNA targets of both samples that captured in this study are show involved in modulate cellular activity by regulating many cellular processes including cell cycle, senescence, proliferative pathways, cell death and survival as well as metabolism related functions. Importantly, the integration analysis of miRNA/mRNA revealed that differentially expressed genes were associated with inflammatory signalling and cell cycle G2/M DNA damage regulation. The data presented here is in agreement with the basic characteristic studies

which demonstrate the physiological changes of SHED and WJSCs during *in vitro* passaging. Taken together, it is intended that our study will contribute to the understanding of these miRNA/mRNA driving the biological process of cellular ageing in hMSCs. These will not only improve our fundamental knowledge of the ageing process but will also advance the development of a more effective and affordable targeted intervention approach for generating therapeutically valuable cell resources for treating many degenerative diseases.

Keywords: Molecular signatures, SHED, WJSCs, cellular ageing, *in vitro* passaging

ABSTRAK

Kemajuan yang ketara telah didokumenkan pada dekad yang lalu bagi penggunaan sel mesenchymal/stromal manusia (hMSCs) sebagai sumber alternatif untuk terapi disebabkan oleh ciri-ciri intrinsik mereka yang mempunyai keupayaan pembaharuan dan pembezaan kepada pelbagai jenis sel yang mempunyai nilai terapeutik yang tinggi. Walaubagaimanapun, penghasilan hMSC yang diasingkan daripada tisu penderma biasanya tidak mencukupi, lalu penjanaan pengembangan hMSC di dalam kultur telah disarankan bagi memenuhi keperluan klinikal. Namun demikian, hMSC menjalani penuaan selular di mana proses ini akan mengehadkan potensi pengembangan/ pertambahan dan pembezaan mereka di dalam kultur untuk jangka masa panjang. Penuaan selular adalah manifestasi hasil dari interaksi molekular yang kompleks yang melibatkan ekspresi gen dan *microniche* yang dipengaruhi oleh pelbagai proses termasuk nyahkawalselia (dysregulation) miRNA. Matlamat kajian ini adalah untuk menentukan petanda molekular yang berhubungkait dengan penuaan selular sepanjang proses penjanaan sel secara *in vitro* di dalam hMSC primer yang diasingkan dari pulpa gigi desidus (SHED) dan Jelly Wharton (WJSCs). Kami mendapati hMSC primer yang diasingkan dari SHED dan WJSCs mengalami perubahan fenotip apabila melalui proses penjanaan di dalam kultur. Seterusnya, dapatan profil molekular juga menunjukkan terdapat satu set pelbagai miRNA dan mRNA yang dinyahkawalselia dalam SHED dan WJSCs. Dari platform ini, kami mendapati ada pertindihan regulasi miRNA sama ada yang meningkat dan menurun bagi SHED dan WJSCs. Pemerhatian yang paling ketara ialah kadar ekspresi hsa-miR-22, hsa-miR-485-5p dan hsa-miR-let-7a yang meningkat, miRNA-miRNA ini adalah antara miRNA telah dikenalpasti sebagai pencetus kesenesensan. Manakala, hsa-miR-302a, hsa-miR-373 dan hsa-miR-520e didapati menurun sepertimana kebiasaanya di dalam kedua-dua jenis sel. MiRNA-miRNA ini dikenali sebagai petanda miRNA dalam sel stem yang terlibat dalam mengekalkan proses biologi yang mengawal sifat-sifat stem sel. Selain itu, sasaran miRNA yang diramalkan dalam kedua-dua sampel menunjukkan penglibatnya dalam proses memodulasikan aktiviti selular dengan mengawal selia pelbagai proses selular termasuk kitaran sel, senesen, jalur proliferatif, kematian sel dan kemandirian sel serta fungsi yang berkaitan dengan metabolisma.

Tambahan lagi, analisis integrasi miRNA/mRNA menunjukkan bahawa gen yang diekspres secara berbeza berkaitrapat dengan isyarat inflamasi dan kitaran sel di dalam fasa G2 / M yang terlibat dalam kawalan kerosakan DNA. Data yang didapati di sini adalah selaras dengan kajian ciri-ciri asas yang menunjukkan perubahan fisiologi SHED dan WJSCs semasa proses penjanaan sel secara *in vitro*. Justeru, diharapkan kajian ini akan menyumbang dalam memahami miRNA / mRNA yang memacu proses biologi penuaan sel dalam hMSCs. Ini bukan sahaja akan meningkatkan pengetahuan asas tentang proses penuaan sel tetapi juga akan menjadi pemangkin kepada pembangunan pendekatan intervensi bersasar yang lebih berkesan dengan kos yang berpatutan untuk menjana sumber sel yang yang mempunyai nilai terapeutik untuk merawat pelbagai penyakit degeneratif.

Katakunci: Petanda molekular, SHED, WJSCs, Penuaan selular, Penjanaan sel *in vitro*

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3’ UTR	: 3’ un-translated regions
A	: absorbance
anti-anti	: antibiotic-antimycotic
APC	: allophycocyanin
APE 1	: apurinic/apyrimidinic endonuclease 1
bp	: base pair
β	: beta
bFGF	: basic fibroblast growth factor
BSA	: bovine serum albumin
CD	: cluster of differentiation
Cdk4	: cyclin-dependent kinase 4
Cdk6	: cyclin-dependent kinase 6
CKS1	: cyclin-dependent kinases regulatory subunit 1
CO_2	: carbon dioxide
$CaCl_2$	: calcium chloride
cells/cm^2	: cells per centimeter square
cm	: centimeter
cDNA	: complementary deoxyribonucleic acid
cRNA	: complementary ribonucleic acid
Ct	: cycle threshold
Δ	: delta
ddH_2O	: sterile distilled water
DMEM-KO	: Dulbecco’s modified Eagle Medium knockout
DPBS--	: Dulbecco’s phosphate buffer saline without calcium and magnesium
°C	: degree Celsius
DNase	: deoxyribonucleas
DNA	: deoxyribonucleic acid
dNTP	: deoxynucleoside triphosphate
dUTP	: deoxyuridine triphosphate
DMSO	: dimethyl sulfoxide
DTT	: dithiotreitol
EDTA	: ethylenediamine tetraacetic acid
ESCs	: embryonic stem cells

FBS : fetal bovine serum

FITC : fluorescein isothiocyanate

g : gram

g : gravity

h : hour

hMSCs : human mesenchymal stromal cells

iPSCs : induced pluripotent stem cells

IgG : Immunoglobulin G

kg : kilogram

Mg : magnesium

mg : milligram

$MgCl_2$: magnesium chloride

miRNA : micro ribonucleic acid

mRNA : messenger ribonucleic acid

MSCs : mesenchymal stem cells

µg/µL : microgram per microlitre

µg/mL : microgram per milliliter

µL : microlitre

µm : micrometer

µM : micromolar

mM : millimolar

m : minute

MAP3K : mitogen-activated protein 3 kinases

M : molar

ng : nanogram

ng/µL : nanogram per microlitre

nM : nanomolar

nm : nanometers

nucleotides: : nucleotides- Adenine, thymine, cytosine and guanine A, T, C, G

Oct 4 : octamer-binding transcription factor 4

OD : optical density

pen-step : penicillin-streptomycin

PerCp : peridinin chlorphyll protein

% : percentage

PE : phycoerythrobilin

PBS : phosphate buffered saline
pg : picogram
pM : picomolar
PCR : polymerase chain reaction
PD : population doubling
PDT : population doubling time
RT : reverse transcriptase
RT-PCR : reverse transcriptase polymerase chain reaction
rpm : revolutions per minute
RNase : ribonuclease
RNA : ribonucleic acid
SD : standard deviation
s : second
SHED : stem cell from human extracted deciduous tooth
Sox2 : SRY (sex determining region Y)-box 2
NaCl : sodium chloride
TNFR2 : tumor necrosis factor receptor 2
U : unit
U/µL : unit per microlitre
UDG : uracil-DNA glycosylase
vol : volume
vol/vol : volume per volume
H_2O : water
WJSCs : Wharton's jelly stem cells
wt : weight
wt/vol : weight per volume
wt/wt : weight per weight

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 General introduction

Cell-based therapy is one of most promising and cutting-edge therapeutic approach in medical field. The principle behind the therapeutic strategy is that healthy and functioning cells from exogenous resources are engrafted to facilitate the repair and replacement of damaged tissues or organs (Buzhor *et al*., 2014). Whilst significant progress has been made in this innovative technology, obtaining sufficient number of cells and tissues with such regeneration function for transplantation purpose still remains as a major challenge in this field (Calne, 2005). For the past few decades, research has demonstrated the potential of using stem cells from various sources including embryonic stem cells (ESCs), mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) as an alternative cellular resource for cell-based therapy owing to their therapeutics features; i) self-renewal and ii) differentiation into functional cell types (Tewarie *et al*., 2009; Wang *et al*., 2010; Carlos Sepúlveda *et al*., 2014; Stuckey and Shah, 2014).

Notably, MSCs have attracted extensive attentions in a wide variety of biomedical disciplines due to their advantages for a promising clinical outcome (Wei *et al*., 2013). To date, many research groups have shown that MSCs are obtainable from various parts of the human body such as bone marrow, peripheral blood, and adipose tissue (Kern *et al*., 2006; Bourin *et al*., 2013; Wei *et al*., 2013; Byrne *et al*., 2016). Hence, the human body offers an abundant variety of stem cell sources for therapeutic purposes. From immunological and transplantation medicine perspectives, autologous stem cells are preferred for cell-based therapy in comparison to ESCs as they help to resolve ethical concerns and circumvent the immune rejection in patients. Furthermore, MSCs that could be isolated from dental tissue, amniotic fluid, umbilical cord and cord blood

which is consider as biological clinical waste have been shown to possess potential in cell-based therapies (Gronthos *et al*., 2000; Da Sacco *et al*., 2010; Ilic *et al*., 2011; Batsali *et al*., 2013). Thus, no additional surgical procedures are required.

Studies in cell-based therapy have been particularly instructive in revealing that it is imperative to use high cellular doses for clinical applications, in which several millions cells/ patient body weight in kilogram is required (Forbes *et al*., 2014; Heathman *et al*., 2015; Liu *et al*., 2016). In this regard, stem cells from any given sources need to be expanded *in vitro* to meet the number of cells for their application in transplantation or to further induced to differentiate into therapeutically valuable cells types. Although MSCs are easily isolatable and accessible in comparison to ESCs, they can only be cultured and expanded for limited passages in which they lost their proliferative and therapeutic potential along the culture course. This phenomenon is, in large part, due to MSCs are prone to undergo replicative stress and senescence state. A prerequisite mechanism underlying cellular ageing is that they do not proliferate yet remains metabolically active after several passages (Hayflick, 1965; von Zglinicki and Martin-Ruiz, 2005), suggesting the cells may undergo deregulation of those processes involved in proliferation, but not the survival / apoptosis pathway. The fact that MSCs could lose the ability to proliferate and differentiation in the passage-dependent manner could greatly hinder the further application of MSCs in cell-based therapies (Wagner *et al*., 2008; Wagner *et al*., 2010b; Carlos Sepúlveda *et al*., 2014). Therefore, it would be timely to explore the biology underlying the stem cell ageing and development of strategies towards enhancing the quality of later-passage stem cells for cell-based therapies.

The molecular mechanisms underlying the regulation of therapeutic characteristics; self-renewal, stemness, migration, secretion of paracrine factors in stem cells from various

sources have been comprehensively explored. Numerous studies have showed that the transcription factors form the signaling networks that crosstalk to modulate stemness potential (Chen and Daley, 2008; Greenow and Clarke, 2012; Dalton, 2013). For example, Oct4, Nanog, Sox2, c-Myc and Klf4 are critical factors in establishing the pluripotency state as well as suppress from the cell fate in differentiating into lineage (Stefanovic *et al*., 2009; Wang et al., 2012; Pan *et al*., 2016). Extensive research have also described that the signaling network that control the phenotype of stem cells is tightly coordinated by many sophisticated cellular and molecular mechanisms, which microRNAs (miRNAs) regulation is of one major mechanism (Wang *et al*., 2008; van Rooij 2011; Mathieu *et al*., 2013).

miRNAs are the non-protein coding ribonucleic acids, which are approximately 22 nucleotides in length (Bartel *et al*., 2004). Expression of these evolutionarily conserved, small RNAs is responded to the changes in cellular environment and mediated suppression of gene expression at post-transcriptional level (Bartel *et al*., 2004; Shomron and Levy, 2009). When expression of miRNAs are induced, they irreversibly induce messenger ribonucleic acids (mRNAs) silencing by binding to the 3' un-translated regions (3' UTR) of cognate mRNAs, resulting in either the degradation of the target mRNA or inhibition of protein translation (Bartel *et al*., 2004; Lee *et al*., 2007). Notwithstanding the apparent complexity, it is becoming clear that miRNAs have significant impact on cellular senescence (Dhahbi *et al*., 2011; Benhamed *et al*, 2012; Liu *et al*., 2012). Thus, to study the regulation of miRNA in regulating stem cell behavior especially the *in vitro* senescence would be of utmost importance. By delineating the miRNA deregulation in cellular ageing of MSCs, we will able uncover the critical mechanisms that responsible for maintenance of stem cell characteristics. This may pave a path for the development of strategies to delay or reverse the ageing of

MSCs during the expansion, hence produces higher quality cellular resources for clinical applications.

1.2 Research questions

In order to address the above issue, this study therefore focuses on the following research questions;

1. How do the stem cells characteristics differ between the early passage and the later passage in human mesenchymal stromal cells (hMSCs) isolated from primary sources especially those considered as biological waste?
2. What are the changes in miRNA signature of hMSCs undergoing ageing caused by *in vitro* passaging?
3. What are the changes in mRNA signature of hMSCs undergoing ageing caused by *in vitro* passaging?
4. How does the miRNA and mRNA integration works in response to cellular ageing caused by *in vitro* passaging?

1.3 Research hypothesis

In this study, we proposed that the isolated hMSCs from pulp of extracted deciduous teeth (SHED) and Wharton's jelly stem cells (WJSCs) are more likely to lose their stem cells characteristics at later passages during *in vitro* passaging. We also speculated that there are differences in mRNAs and miRNAs expression profile of hMSCs at early and later passages. It is worthy to explore the relationship of mRNA as well as the miRNAs and its putative-targeted mRNAs to identify the determinants of replicative senescence

and cellular ageing in stem cell population. In addition, we posited that the self-renewal and stemness of hMSCs could be partially rejuvenated at later passage by inhibiting the expression of miRNA, which is associated with pluripotency and self-renewal.

1.4 Aim and objectives

Increasing stem cell numbers that are clinically efficient to meet the needs of cell-based therapy via cell expansion and passaging forms the aim of this research which will be divided into four sections as follows;

Section 1: The effect of passaging on cellular ageing of human mesenchymal stromal cells isolated from the pulp of extracted deciduous teeth and Wharton's jelly derived from primary sources.

Objectives;

1. To isolate and passage hMSCs from pulp of extracted deciduous teeth and Wharton's jelly obtained from primary sources.
2. To confirm the MSC status of isolated SHED and WJSCs.
3. To determine the cell phenotypic changes related to cellular ageing of SHED and WJSCs at early and late passages.

Section 2: Comparative microRNA expression of human mesenchymal stromal cells isolated from the pulp of extracted deciduous teeth and Wharton's jelly during *in vitro* passaging

Objectives;

1. To ascertain the differentially expressed miRNAs in SHED and WJSCs at selected late passage (P6) compared to early passage (P3).
2. To determine the characteristics of differentially expressed miRNA profiles through their functional clustering based on their putative miRNA-targets.
3. To conduct quantitative validation study of selected differentially expressed miRNAs involved in cellular ageing by qPCR analysis.

Section 3: Comparative mRNA expression of human mesenchymal stromal cells isolated from the pulp of extracted deciduous teeth and Wharton's jelly during *in vitro* passaging

Objectives;

1. To determine differentially expressed mRNAs of SHED and WJSCs at selected late passage (P6) compared to early passage (P3).
2. To assess the characteristics of differentially expressed mRNA profiles through their functional clustering.
3. To conduct quantitative validation study of selected differentially expressed mRNAs involved in cellular ageing by qPCR analysis.

Section 4: miRNA and mRNA interaction on cellular ageing due to *in vitro* passaging of human mesenchymal stromal cells isolated from the pulp of extracted deciduous teeth and Wharton's Jelly

Objectives;

1. To correlate cellular ageing related miRNAs and mRNAs.
2. To validate the selected miRNA-mRNA interactions by negative linear regression analysis.
3. To model the highly differentially expressed miRNAs which are involved in the regulation of cellular ageing.

1.5 Research framework

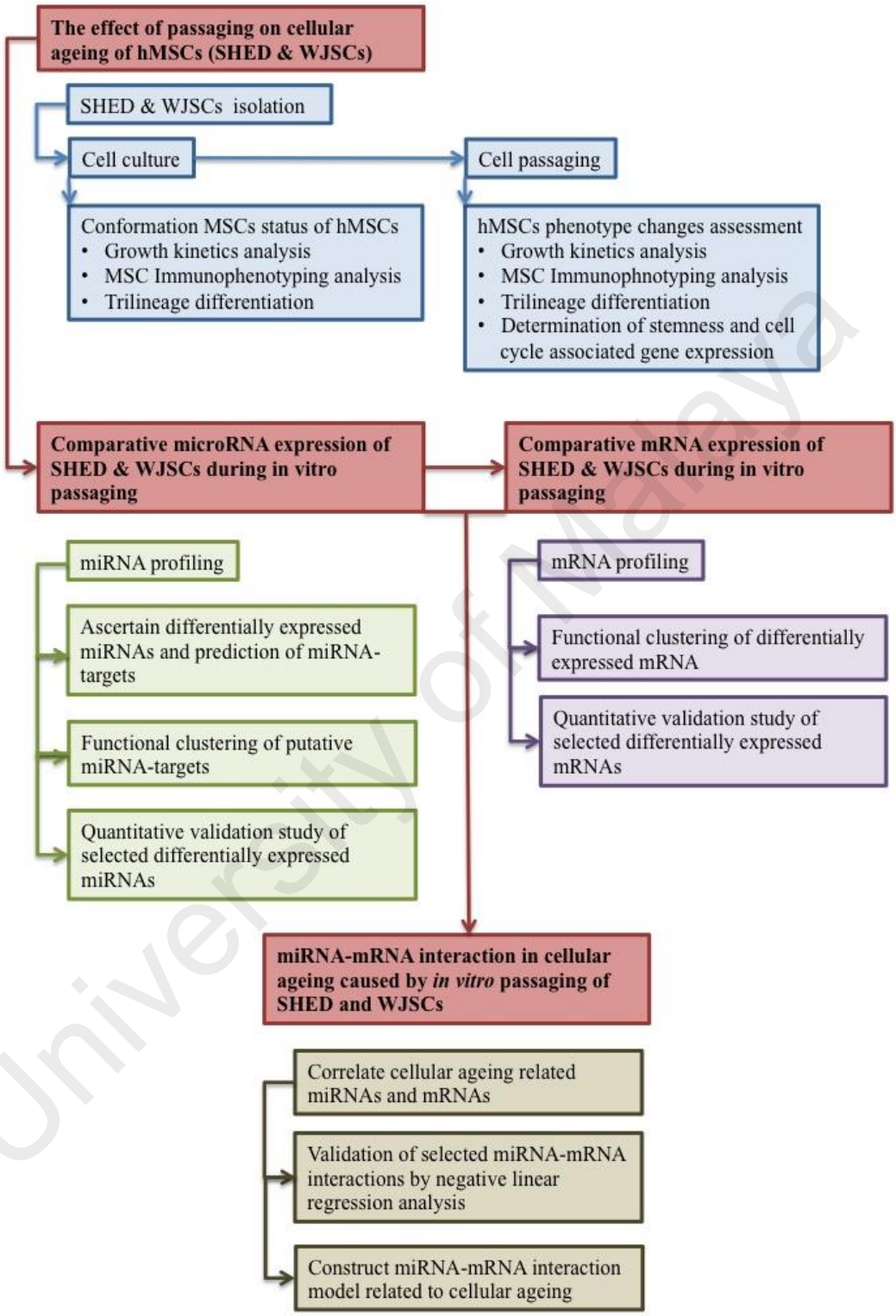


Figure 1.1 Flowchart of this study to determine the molecular signatures of SHED and WJSCs during *in vitro* passaging
(SHED, hMSCs from the pulp of extracted deciduous teeth; WJSCs, hMSCs from Wharton's jelly)

CHAPTER 2: LITERATURE REVIEW

2.1 Cell based therapy

The advances in biological sciences have shaped much of our understanding of the human cells and the pathogenesis of some diseases, which enhance the capability of early diagnosis and constructive intervention that help in treatment of diseases. Conventionally, treatment has been focused on the drug therapy, however many patients fail to respond to this therapy due to drug resistance. On the other hand, organ transplant is often the only the therapeutic option for patients with terminal diseases, yet organ shortage is the main hindrance to the progress of this treatment. In recent years, cell-based therapy has arisen to be a new therapeutic tool where healthy and functioning cells are transplanted at the site of injury in order to facilitate and improve their functions. Despite this technology has shown promising potential in treating incurable and irreversible condition caused by injury or disease, this technology remains challenging given the difficulty of obtaining donor cells. Hence, cell based therapy needs to be optimized in several manner in order to achieve the desired outcomes of this treatment modality.

For the past few decades, research has gradually explored the use for stem cells as an alternative source for cell replacement. Both embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) hold great promises in this therapeutic application owing to its self-renewal and pluripotency characteristics. The first successful replacement therapy was a human bone marrow transplantation carried out in 1968 (Gatti *et al*., 1968). This was exciting for the field of stem cell research and the knowledge continues to grow since then. Later, more promising results were obtained from other studies where *in vitro* differentiation of stem cells into other cell types including neurons (Ambasudhan *et al*., 2011), pancreatic insulin producing cells

(D'Amour *et al*., 2006), hepatic-like cells (Agarwal *et al*., 2008), osteoblast (Granero-Molto *et al*., 2008) and others was found possible.

The ethical concern in the isolation procedure of ESCs has advocated the development of induced pluripotent stem cells (iPSCs). Yamanaka's group generated the first iPSCs in 2006, where pluripotent cells were generated from somatic cells via cellular reprogramming approach (Takahashi and Yamanaka, 2006). However, even with similar properties as the ESCs, iPSCs might seriously compromise their efficacy in clinical application due to their potential in teratoma formation (Ben-David and Benvenisty, 2011). For this reason, MSCs may offer potential in clinical advantage for autologous stem cells donation. Such fascinating features of MSCs have drawn the attention of researchers from diverse fields for the success of regenerative medicine. Previous and emerging data have supported the idea that various stem cells therapies may have achieved advanced clinical trial phase to be employed in clinical setting in the near future (Heathman *et al*., 2015; Squillaro *et al*., 2016). For example, clinical study using bone-marrow derived progenitor cells in acute myocardial infarction exhibited a promising therapeutic significance in which recovery and improvement of cardiac function as well as reduction of the occurrence of adverse cardiovascular events (Assmus *et al*., 2010).

2.2 Stem cells and their therapeutic characteristics

By definition, stem cells are referring as unspecialized cell that can maintain their differentiation potential while repopulating in a long-term clonal growth. Stem cells can be classified into five categories based on their differentiation potential namely, totipotent stem cells (zygote formed from fertilized egg, cells that can differentiate into

embryonic cell types), pluripotent stem cells (ability to differentiate into almost all cell types of endoderm, mesoderm and ectoderm germ layers); multipotent stem cells (ability to differentiate into a closely related family of cells); oligopotent stem cells (ability to differentiate into few cell types), unipotent stem cells (ability to differentiate into single cell type).

In the modern era of stem cell research, the term stemness characteristic is commonly used to identify stem cell population. Stem cells markers (Oct4, Sox2, c-Myc and Klf4) are used to isolate and identification of stem cells and functional assays (such as *in vitro* functional differentiation) are used to determine therapeutic purposes of stem cells. This overview was demonstrated in the pioneer study of stem cell research by Till & McCulloch in 1961, shown that hematopoietic cells with multi-lineage differentiation potential and self-renewal capability. Along with the discovery of stem cell, the first ESCs were successfully isolated from mouse embryos and grew in culture by Evans & Kaufman (1981). Generally, ESCs are referred as "gold standard" of stem cells sources. They are derived from inner cell mass (ICM) at the blastocyst stage which are self-renewal and show pluripotency characteristics (Thomson *et al*., 1998). Stem cells can either propagate by cell division or inhibit their self-renewal molecular programs and prompt into differentiation stage.

2.2.1 Self-renewal of stem cell

An important feature that distinguishes stem cells from mature cells is their capability to self-renewal. Stem cell undergoes both asymmetric and symmetric cell division which is important for their identity maintenance and cell propagation (Shahriyari and Komarova, 2013). The asymmetric cell division is normal employed by stem cells or

progenitor cells in different tissues and the generated daughter cells have different cell fate (Gómex-López *et al*., 2014). Only one of the daughter cell inherit the same phenotype as the mother cells and retain the stemness property in next generation cells, whereas the other daughter cell is programmed to differentiate into specific cells along the lineage (Morrison and Kimble, 2006). On the other hand, symmetric cell division gives rise to identical daughter cells for the purpose to expand cell population after injury or during development (Morrison and Kimble, 2006).

The importance of cell cycle is known historically because of effects on the embryonic growth (White and Dalton, 2005) and later development (Branzei and Foiani, 2008; Pietras *et al*., 2011; Hindley and Philpott, 2012; Cheung and Rando, 2013). Previous studies indicated that cell division requires a series of molecular signaling and biochemistry events in precise order to ensure the daughter cells receive a complete set of molecules or materials required for survival (Nurse, 2000). Defects in the regulatory mechanism of cell cycle event would resulted in unregulated cell division, which may lead to cell proliferation arrest or cancer (Sherr, 1996; Massague, 2004). Based on previous and emerging data, stem cells exhibits an unique mechanism of cell cycle structure and regulation, where more than half of the cycle is dedicated to S phase and a shorter duration in G1 and G2 phases (Li *et al*., 2012; Ahuja *et al*., 2016).

2.2.2 Core pluripotency factors of stem cell

Up to date, determinants such as transcriptional factors including Oct4, Sox2, c-Myc and Klf4 are reported as core contributors that govern stem cell pluripotency and their competency in regeneration therapies (Niwa *et al*., 2000; Avilion *et al*., 2003; Chambers *et al*., 2003; Cartwright *et al*., 2005; Li *et al*., 2005; Loh *et al*., 2006; Zhao *et al*., 2012).

Indeed, these pluripotent factors are known to direct the formation of ICM during embryogenesis (Niwa, 2007). For instance, early report showed that the epiblast cells failed to form during gastrulation in the absence of Oct4 (Nichols *et al*., 1998). Like ESCs, MSCs also found to express Oct4, Sox2, c-Myc and Klf4; however, the expression level is lower than in ESCs, hence having lesser capacity to differentiate into other cell types (Fong *et al*., 2011). As such, this implicated that the determination of stem cells potency is reflected through the expression level of pluripotent factors (Figure 2.1). It has been shown that activation of these key pluripotency factors are frequently modulated by a combination of intrinsic and extrinsic signaling cascades (Chen *et al*., 2008; Orkin and Hochedlinger, 2011).

Figure 2.1. Schematic representation of pluripotent factors controlling stem cell states

2.2.3 Stemness-associated molecular signaling pathway

For the past few decades, great afford are made to study the signaling pathways in stem cells to understand how they function as a whole to sustain a long-term proliferation in undifferentiated state. Cell signaling refers to a communication system that directs basic cellular activities in response to its microenvironment. Early study suggested that transforming growth factor beta (TGF-β), phosphatidylinositol 3-kinase (PI3K), fibroblast growth factor (FGF), Wnt and other signaling pathways (Figure 2.2)

orchestrate in regulating stem cells phenotype (Bieberich and Wang, 2013; Hoffman and Benoit, 2017). Dynamic changes in any of the signaling cascade may influence cell fate decisions which inhibit stem cells proliferation and direct stem cells into differentiation stage (Dalton, 2013). In the rest of this chapter, we will review the key signaling pathways that are important in regulating stem cell characteristics.

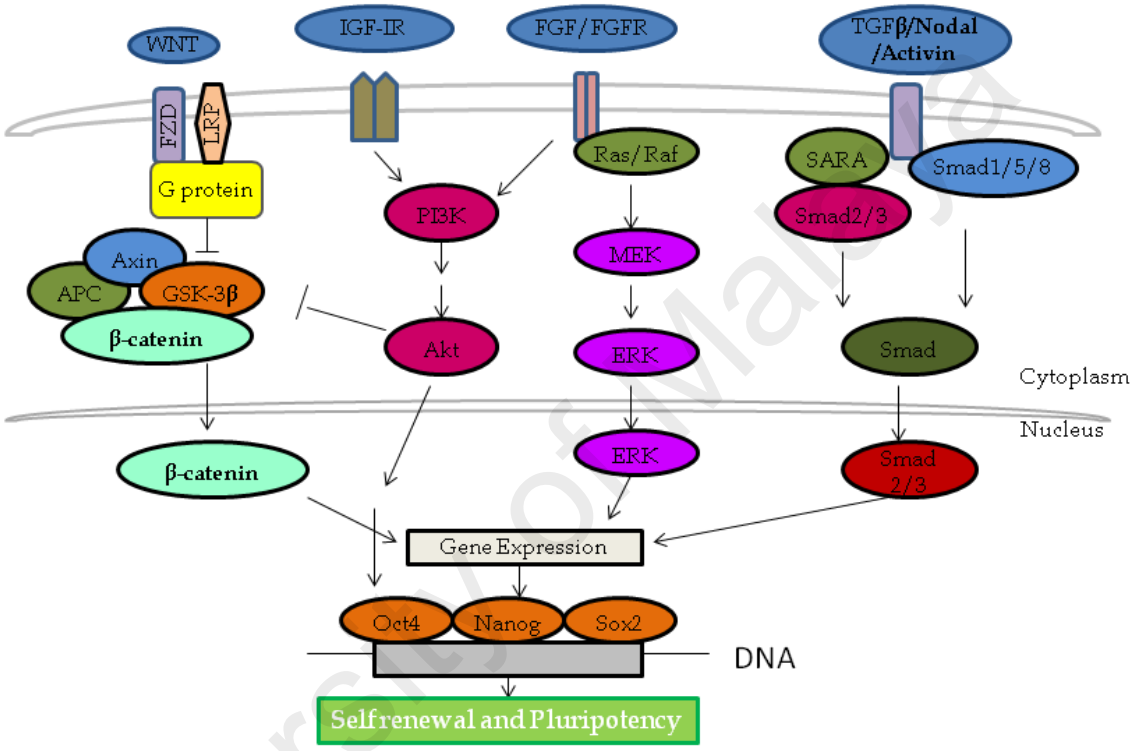


Figure 2.2. Schematic representation of signaling pathway regulating pluripotency of stem cell as discussed in text (source from Bieberich and Wang, 2013)

2.2.3.1 Transforming growth factor beta (TGF-β) signaling pathway

There are two main branches of TGF-β signaling pathway including canonical Smad pathway (Shi *et al*., 2003) and non-Smad pathway (Moustakas and Heldin, 2005). The signal transduction of Smad/TGF-β signaling pathway begins with the binding of TGF-β ligand; Activin, Nodal, bone morphogenetic protein (BMP) or TGF-β to the TGF-β type II receptor, which subsequently phosphorylate type I receptor followed by the

activation of Smad proteins (Lebrun *et al*., 2012). The phosphorylated Smads 2/3 and Smads 1/5/8 proteins then form a complex with Smad 4 (common mediator Smad; co-SMAD) and translocate into the nucleus. Eventually the Smad complex interacts with different transcriptional promoter and lead to the transcription of mRNAs that regulate stem cell stemness (Padua and Massagué, 2009; Gaarenstroom and Hill, 2014; Ghosh *et al*., 2017). It has been reported that, TGF-β signals at different cellular contexts express diverse set of Smad-interacting transcriptional promoters could determine specific gene expression and lead to elicit different biological response (Heldin *et al*., 2009; Wang *et al*., 2012).

On the other hand, the TGF-β can activate the non-Smad/TGF-β signaling pathways including the mitogen activated protein kinase (MAPK) (Davies *et al*., 2005; Lee *et al*., 2007), stress-activated kinase (p38/JNK) (Bakin *et al*., 2002; De Guise *et al*., 2006), RhoGTPas (Bhowmick *et al*., 2001; Edlund *et al*., 2002), PI3K (Chen *et al*., 1998) which shown to elicit stress response, migration and other biological effects. It is clear that these two major branches of TGF-β signaling pathway operate differently to regulate stem cells' stemness. Study done by Vallier *et al*., (2005) showed that the activation of TGF-β signaling via Smad 2/3 supports stem cells in maintaining its pluripotency. On the contrary, BMP signaling was reported associated with stem cells differentiation (Conley *et al*., 2007; Beederman *et al*., 2013), and inhibition of BMP signaling with Noggin is able to sustain ESCs and MSCs in undifferentiated state (Xu *et al*., 2002; Xu *et al*., 2005; Fan *et al*., 2013).

2.2.3.2 Phosphatidylinositol 3-kinase (PI3K) signaling pathway

PI3K pathway has been related to the cellular survival and growth that is activated by the binding of a range of extracellular signals including the growth factors and

hormones to the receptor tyrosine kinase (RTK) of cell membrane (Takahashi *et al*., 2005; Castellano and Downward, 2011). The binding of extracellular ligands to RTK causes the receptor dimerization and phosphorylation of tyrosine residues in the intracellular domains. Subsequently, the regulatory subunits p85 of PI3K will bind to the phosphorylated tyrosine residues and recruits p110, another catalytic subunits of PI3K to form an active PI3K. The activated PI3K then phosphorylates the inositol ring of phosphatidylinositol (PI) to produce phosphoinositides including PI(3)P, $PI(3,4)P_2$ and $PI(3,4,5)P_3$ that can anchor to the plasma membrane. Following that, the inactivated Akt translocates from the cytosol to the plasma membrane and binds to the $PI(3,4,5)P_3$ leading to conformational change upon exposure of phosphorylation sites threonine 308 and serine 473 for its activating kinases phosphoinositide dependent kinase 1 and 2 respectively for activation.

It has been reported that activation of Akt regulates various gene targets that involved in both cell survival and apoptosis (Chen *et al*., 2013). This pathway is crucial in regulating the biological homeostasis in order to maintain cellular function. As defective apoptosis processes lead to unlimited proliferation which may promotes tumour development (Hanahan and Weinberg, 2000), whereas uncontrolled cell apoptosis is associated with neurodegenerative disorder that has been observed in Alzheimer's disease (Mattson, 2000).

2.2.3.3 Fibroblast growth factor (FGF) signaling pathway

Recent emerging evidence supports the importance of FGF signaling to regulate cellular pluripotency and differentiation in stem cells (Kunath *et al*., 2007; Lanner and Rossant, 2010; Lai *et al*., 2011; Cai *et al*., 2013). Indeed, fibroblast growth factor 2 (FGF2) is one of the commonly use component for stem cells culturing (Ahn *et al*., 2009, Lotz *et*

al., 2013). The heparin sulfate proteoglycans (HSPGs) on the cell surface as cofactor that facilitates the binding of FGFs to the FGF receptors (FGFRs) (Ornitz and Itoh, 2015). The activated FGFR is further relayed in signaling cascades through four different signaling pathway including MAPKs, signal transducer and activator of transcription (STAT), phosphatidylinositol 3-kinase (PI3K) and phosphoinositide phospholipase C (PLCγ) signaling pathway.

Upon FGFR activation, the fibroblast growth factor receptor substrate 2 (Frs2α) which is associated with the FGFR is then phosphorylated by the FGFR kinase to form a complex with Src homology region 2 domain containing phosphatase 2 (Shp2) and growth factor receptor-bound protein 2 (Grb2). The FGFR- Frs2α- Grb2 complex recruits the son of sevenless homology (SOS) and then activates MAPK signaling pathway. MAPK signaling pathway was reported involved in regulation of diverse genes expression via E26 transformation-specific (ETS) transcription factors. In contrast to the MAPK pathway, PI3K is activated by FGFR- Frs2α- Grb2 complex via the Grb2-associated binding protein 1 (Gab1), which then phosphorylates the AKT. The phosphorylated AKT in the PI3K signaling activates the mTOR complex 1 through inhibition of tuberous sclerosis complex 2 (TSC2) and then leads to cell growth and proliferation (Manning and Cantley, 2007). In addition to that, phosphorylated AKT inhibits the activity of forkhead box class transcription factor (FOXO1), a pro-apoptosis effector to exit from nucleus for cell survival (Manning and Cantley, 2007; Liu and Deng, 2017).

2.2.3.4 Wnt signaling pathway

Beta-catenin (β-catenin) acts as a co-activator for transcriptional factors TCF/LEF to mediate transcriptional induction of target gene that plays crucial role in stem cell

maintenance (Nusse *et al*., 2008; Zhang *et al*., 2011). Generally, the existence and stability of β-catenin molecules is maintained via Wnt signaling pathway (Nelson and Nusse, 2004; MacDonald *et al*., 2009). In the absence of Wnt molecules, the β-catenin is phosphorylated by destruction complex (Axin, adenomatosis polyposis coli- APC, glycogen synthase kinase 3- GSK3 and casein kinase 1- CK1 α) by targeting it for ubiquitination and eventually degradation by proteasome. As result, the no free β-catenin enters the nucleus to bind to a transcriptional factor and activates transcription of protein such as TCF/LEF.

In the presence of Wnt molecules, it binds to the Frizzled (Fz) receptor and lipoprotein receptor related protein (LRP) co-receptor that lead to the recruit of CK1α and GSK3 to the membrane and phosphorylates LRP. This allows the translocation of Axin from cytosol to plasma membrane and associate with the phosphorylated LRP. Hence, the stability of beta-catenin is maintained, as Axin of the destruction complex is preoccupied. Previous studies have showed that Wnt signaling pathway has a prominent role in regulating various biological process of stem cell including proliferation and differentiation (Ring *et al*., 2014; Kim *et al*., 2015). It has been proposed that the effects of Wnt signaling on stem cell homeostasis are not simply reliant on "on and off" principle, but are relatively dependent on the intensity of signals (Luis *et al*., 2011; Ring *et al*., 2014).

2.3 Biology of human mesenchymal stem cells

Like ESCs, MSCs possess self-renewal and differentiate capability to facilitate the repair and regeneration of damaged or injured tissue. Normally, multipotent MSCs have limited differentiation potential with a significant role in tissue repair (Boquest *et al*., 2006). As fetus developed, the adult stem cells or MSCs will reside in the stem cell

niche and maintain in a quiescent stage (Morrison and Spradling, 2008). MSCs will be activated by the signal from their micro-environment upon tissue or cell injury (Hsu and Fuchs, 2012). Stem cell niche provides the homeostasis of MSCs, regulate stem cell proliferation and differentiation activity (Giai Via *et al*., 2012). Pervious research has shown that the primary hMSCs are obtainable from various tissues such as bone marrow, peripheral blood, adipose tissue, and others (Kern *et al*., 2006; Bourin *et al*., 2013; Wei *et al*., 2013; Byrne *et al*., 2016). Hence, hMSCs offers abundant variety of stem cell sources for therapeutic purposes. Research on stem cell biology in the past decade has demonstrated the potential of hMSCs to be used as a therapy resource for many diseases such as heart failure (Koninckx *et al*., 2010; Bockeria *et al*., 2013), bone and cartilage repair (Granerp-Molto *et al*., 2008), neurodegenerative diseases (Tanna and Sachan, 2014), diabetes (Bouwens *et al*., 2013; Rekittke *et al*., 2016) and others. Collectively, the implications of stem cells' characteristic and behavior in different tissue niches support the respective organs and tissues development.

The ability to self-renew and differentiate into other cells makes hMSCs valuable cellular resources for research and clinical applications. Although recent studies have greatly advanced our understanding of hMSCs and its potential efficacy, protocol for isolation, expansion and characterisation of the cells varies among research groups. Thus it is difficult to compare study outcomes, which hampers progress in the field. To address this issue, the International Society for Cellular Therapy (ISCT) proposed minimal criterial to define hMSCs which include: 1) adherence to plastic in standard culture conditions; 2) expression of surface molecules CD73, CD90, CD015 and absence of CD11b or CD14, CD19 or CD79a, CD34, CD45, HLA-DR; 3) capacity to differentiate into adipocytes, chondroblasts and osteoblasts *in vitro*. The minimal set of standard criteria to characterise hMSCs will facilitate investigators from multi disciplinary field to exchange data and reduce inconsistencies in the research conduct.

2.4 Challenge with MSCs in regeneration therapy

Over the past few decades, there has been a breakthrough in our understanding on the potential of stem cells in regeneration therapies. The emerging evidence linking the stem cells' stemness to the regeneration efficacy suggests that stem cells with proper pluripotent characteristics might be an effective candidate to differentiate into the desire cell types (Robinton and Daley, 2012). These have laid the foundation for the stem cells studies in regenerative medicine that followed. Although MSCs can be easily obtained from several tissues or self-donated, they are usually insufficient to meet the demand of clinically relevant number where a minimum of hundred of million cells are required (Introna *et al*., 2014, Heathman *et al*., 2015, Zhao *et al*., 2016, Wang *et al*., 2017). For this reason, the isolated MSCs need to expand *in vitro* before implantation (Figure 2.3).

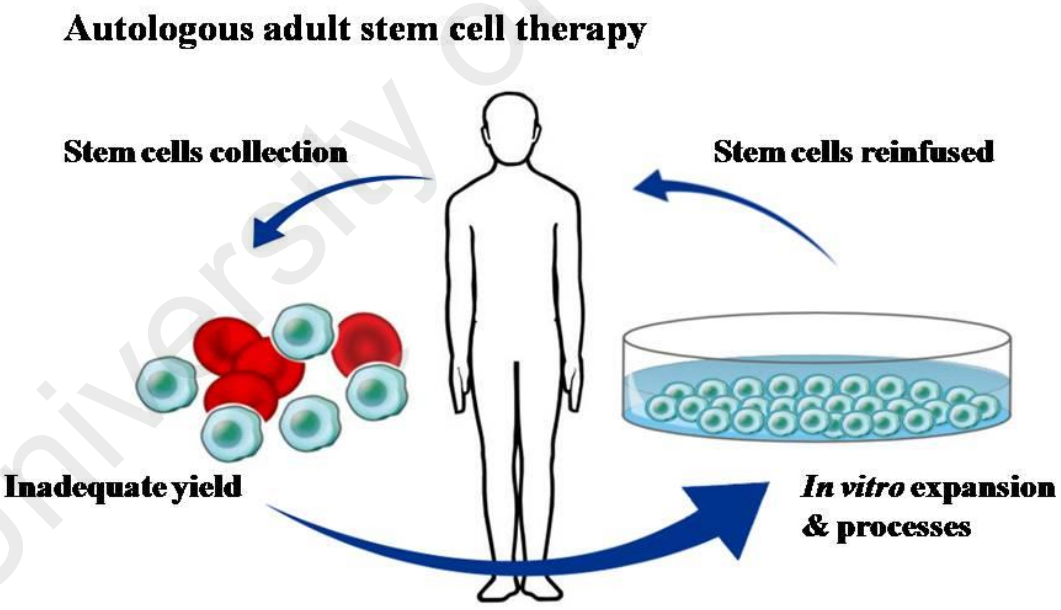


Figure 2.3. Autologous adult stem cells therapy
Patient uses their own stem cells for disease treatment purposes, in which collected stem cells required to expand *in vitro* due to insufficient to meet demand for clinical application.

Nevertheless, there are many technical challenges need to be overcome prior to the clinical use including the quantity and quality of the cells, heterogeneity, integration of

the cells at the site-specific manner and other. It has been reported that the therapeutic potential of MSCs for cell-based therapy is greatly influenced by the *in vitro* culture condition including the medium composition and the culture conditions (Placzek *et al*., 2009; Heathman *et al*., 2015). Therefore, it is necessary to develop a stem cell bioprocessing procedure for cell expansion and differentiation in order to generate reproducible and high quality cells in accordance with good manufacturing practice grade (Panchalingam *et al*., 2015). The current MSCs culture procedure is normally carried out in the presence of supplement from the xenogenic origin (such as bovine serum) and thus limits their clinical application. This is because the compositions in the serum are poorly defined and may vary from batch-to batch. This has led to an increased demand for defined serum-free medium to substitute conventional serum-containing medium.

Aside from this, MSCs isolated from primary sources may produce inconsistent outcomes that have been attributed to the heterogeneity population within primary cell cultures (Muraglia *et al*., 2000; Torensma *et al*., 2013; Whitfield *et al*., 2013). In order to increase the confidence that the cells employed are homogeneity, MSCs isolated from primary source need to be purified through antibody-based cell sorting approach (Amos *et al*., 2011). This idea is supported by early study from Cai *et al*., (2010), who demonstrated that an increase efficiency in generate a homogeneous pancreatic progenitors culture with PDX1 expression from the purified CXCR4 positive endodermal cells.

Another important aspect for the therapeutic efficacy of cell-based therapy is the administration method to ensure integration, survival and highest regenerative benefit of the introduced cells. The optimum cell delivery method will depend upon the functions of the exploited MSCs. There are two types of MSCs administration routes, namely

local delivery (e.g. intraperitoneal or intramuscular injection) and systemic delivery (vascular route either venous or arterial). The most common and convenient method of MSCs transplantation is by systemic delivery, however patients might encounter cell lost during relocation (Kurtz, 2008; Kean *et al*., 2013). In comparison with systemic delivery, the local delivery may advantageous as cells can be directly introduced at the site of injury (Kim *et al*., 2014).

Additionally, MSCs characteristics such as proliferation and stemness changes mimicking ageing like phenotype during expansion were commonly observed in many studies, where MSCs exhibit reduced proliferation rate and ultimately lead to premature growth arrest (Hayflick 1965, von Zglinicki and Martin-Ruiz, 2005; Wagner *et al*., 2009; Wagner *et al*., 2010b), affecting their clinical application potential in regeneration medicine. The basic premise of this view is that replication senescence during *in vitro* culture refers to biological phenomenon in which cells undergoing irreversible growth arrest. The initial study of cellular senescence was described by Hayflick in 1965 (Hayflick, 1965). Subsequent findings also demonstrated that other exogenous and endogenous factors which contribute to the cellular senescence including telomerase shortening, DNA damage, oxidation stress, and many others (Bodnar *et al*., 1998; Chen *et al*., 2007; Pole *et al*., 2016).

2.5 Cellular and molecular processes during *in vitro* passaging

The advances in molecular biology have paved a way to interpret the balanced interplay of molecular processes involving transcriptional factors, microRNAs (miRNAs), epigenetics and others for proper stem cell maintenance (Chen and Daley, 2008; Choi *et al*., 2013) (Figure 2.4). By delineating the related and predicted molecular components

of stemness influence machineries, we will be able to develop strategies to delay the aspects of cellular ageing in culture and thus help to minimize the impact of replication senescence in long-term culture. In the following of this chapter, we will review the key signaling pathways that are important in regulating stem cell ageing.

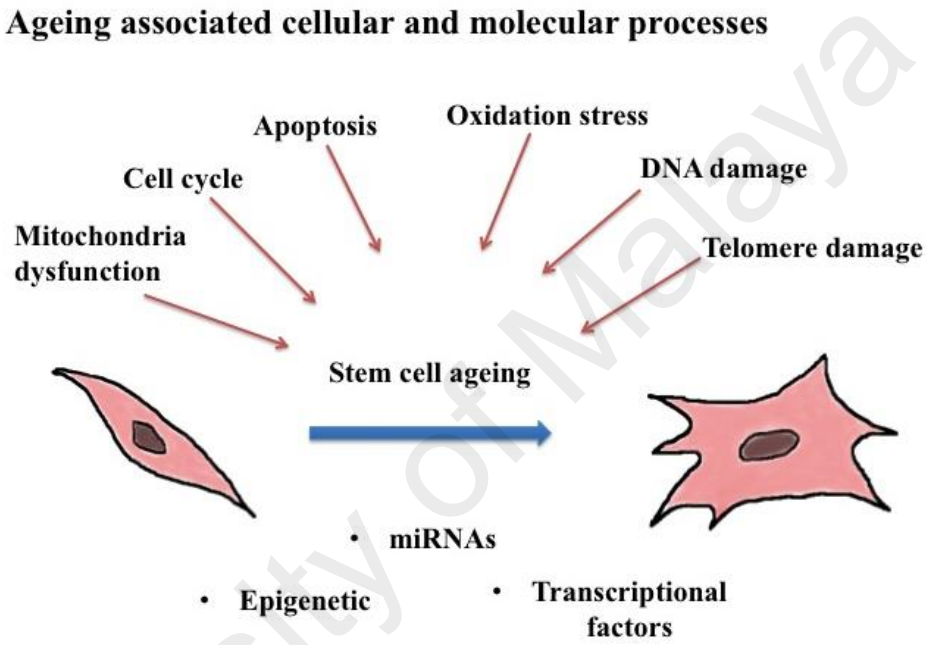


Figure 2.4. Stem cell ageing is affected by cellular and molecular process
Stem cell is able to react to exogenous and endogenous factors with a repertoire of molecular signaling pathways. Cell cycle arrest, apoptosis, DNA damage, telomere damage, oxidation stress, mitochondria dysfunction influence stem cell maintenance negatively.

2.5.1 Telomere shortening

Telomere is the end structure of chromosome that composed of 5'-TTAGGG-3' repeats and terminates with 3' single-stranded overhang (Meyne *et al.*, 1989). This single-stranded TTAGGG overhang forms a hairpin loop and serves as a cap to protect DNA from damage (Griffith *et al.*, 1999a; Griffith *et al.*, 1999b). Maser and DePinho (2004)

reported that telomeres may be completely lost after several rounds of cell division and affect the gene near to the chromosome end, subsequently leading to cease in cell proliferation. The telomere length is normally maintained by the telomerase, a DNA repair enzyme that is highly expressed in MSCs and ESCs (Armstrong *et al*., 2000; Serakinci *et la*., 2008). Telomerase bind to the 3' overhang of the lagging strand of the telomere which is complementary to the telomerase RNA (Sarek *et al*., 2015).

Following that, the 3' overhang is elongated by adding nucleotides by using telomerase RNA as template. Then, telomerase relocates to the 3' terminus of the lagging strand allows to repeat the second cycle. From this, DNA polymerase is able to complement the lagging strand. It has been reported that the telomerase facilitate the replication feature of ESCs without compromising the quality of the replicated products (Armstrong *et al*., 2000; Serakinci *et la*., 2008). Thus, analysis of telomere length and the expression level of telomerase have been frequently used as biomarker to measure cellular senescence.

2.5.2 Cell cycle regulation and DNA damage

Cell division cycle takes place in four stages, it begins in G1 phase where cells started to grow in size and synthesize RNA as well as proteins that required for DNA synthesis (Figure 2.5). In G1 phase, the elevation of Cyclin D form a complex with CDK4 and CDK6 then followed by phosphorylation of transcriptional repressor retinoblastoma (Rb), p017, and p130. Phosphorylation of these transcriptional repressors lead to release of its bind targets, the E2F including E2F1-3, E2F4, and E2F5 which are the transcriptional factors important in promoting transcription of genes that are essential for DNA synthesis. These free E2F proteins translocate from nucleus to cytoplasm and

activate down-stream targets including Cyclin E, Cyclin A, DNA polymerase, and other proteins found to be essential in cell cycle progression. Cyclin E plays critical roles in G1 phase, where it forms a complex with Cdk-2 and continues to phosphorylate Rb in order to prevent it from binding with E2F transcriptional factor. Aside from this, Cyclin E-Cdk2 complex promotes expression of genes that is important in directing G1-S transition.

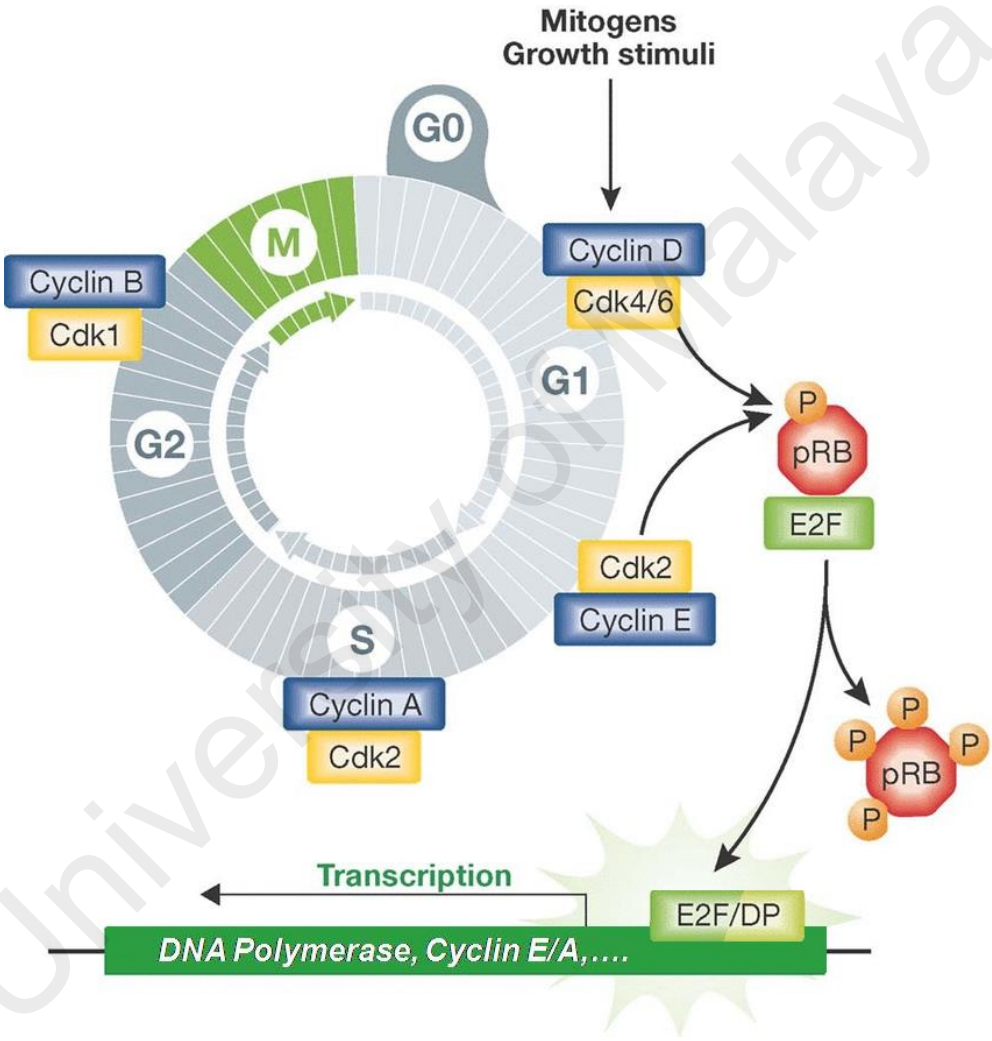


Figure 2.5: Schematic representation of the molecular regulation of cell cycle discussed in the text (modified from Aquilar and Fajas, 2010).

When cell cycle event proceed from G1 phase into S phase, E2F induces the expression of Cyclin A to substitute Cyclin E and form complex with Cdk-2 followed by initiation DNA synthesis. As a result, cell started to replicate its own DNA content to produce identical DNA copies (each chromosome has 2 sister chromatids) for daughter cells. As

cell move into late S phase, Cyclin A started to associates with Cdk-1 until it reaches G2 late phase where it activates Cyclin B. In the G2 phase, new proteins are synthesized and preparation for mitosis. When the expression level of Cyclin B-Cdk 1 complex reaches the threshold of mitosis promoting factor at late G2 phase, cells enter mitosis phase whereby duplicated DNA content is divided equally into two daughter cells. The division of nucleus is followed by cytokinesis, a division of cytoplasm. There are two checkpoints in cell cycle system including G1/S and G2/M, which known as the surveillance system to monitor the cell cycle progression (Bartek and Lukas, 2001; Gorgoulis *et al*., 2005). These checkpoints function to introduce cell cycle arrest in response to DNA damage in order for the DNA repair to take place before entering to the next phase (Giglia-Mari *et al*., 2011).

When a cell detects DNA damage, cell cycle checkpoint (G1/S and G2/M checkpoint) is activated to allow cellular repair before continuing the cell cycle events (Löbrich and Jeggo, 2007; Yu and Kang, 2013; Shaltiel *et al*., 2015). DNA damage detected by protein kinases such as Ataxia telangiectasia mutated (ATM) or Ataxia Telangiectasia and Rad3 related (ATR) at G1 phase will trigger a series of biochemical cascades leading to the inactivation of Cyclin E- CDK2, delaying and ceasing the cell cycle progression (Abraham, 2001; Giglia-Mari *et al*., 2011). The ATM is auto-phosphorylated upon DNA damage and activates Chk1 and Chk2 by phosphorylation, which in turn phosphorylates Cdc25A for degradation (Brown *et al*., 1999; Bartek and Lukas, 2001; Falck *et al*., 2001). In the absence of Cdc25A, inhibitory phosphates remains bound with CDK2 and hamper the activity of Cyclin E- CDK2 (Sandhu *et al*., 2000; Lavecchia *et al*., 2009).

Also, DNA damage signals induced activation of p53, which lead to the synthesis of p21 (Cardaci *et al*., 2008). In the presence of p21, Rb molecules remains

unphosphorylated and continue bound to E2F protein, blocking its activities which resulting in cell cycle arrested at G1 phase (Sherr *et al*., 1999; Narita *et al*., 2003; Benson *et al*., 2014). On the other hand, G2/M checkpoint control cells arrest in G2 phase before entering mitosis phase in response to DNA damage via inhibitory regulation of CDK. Previous studies showed that CDK1 is inhibited by three transcriptional targets of p53, namely p21, Gadd45, and 14-3-3σ (Spitkovsky*et al*., 1997; Löhr *et al*., 2003).

2.5.3 Oxidative stress

Oxidation stress reflects a disturbance in the balance of reactive oxygen species (ROS) and antioxidant in the cells that lead to cell proliferation and DNA damage that compromise cell survival (Coso *et al*., 2012). ROS are unstable molecules such as superoxide anions (O_2^-), hydroxyl radicals (OH), as well as hydrogen peroxide (H_2O_2) that derived from the reaction of molecular oxygen with free moving electrons generated mainly from mitochondria (Nathan and Ding, 2010; Ozougwu, 2016). Under normal physiological condition, cells synthesize enzymatic antioxidant molecules such as catalase (Sies, 2015), superoxide dismutases (Antonenkov *et al*., 2010), glutathione peroxidase (Molavian *et al*., 2015) to prevent development and accumulation of ROS. It has been reported that MSCs decrease in proliferation due to the high level of ROS stresses (Ko *et al*., 2012). Also, high levels of ROS were frequently observed in differentiating cell, as there is an increase in mitochondria mass (Nesti *et al*., 2007).

Indeed, activation of ROS has been demonstrated to affect several signaling pathways. For instance, ROS activates the NF-*κ*B signaling pathway that regulates the cell senescence, apoptosis, and inflammatory response by inhibiting the phosphorylation of

IκBα (Basak and Hoffmann, 2008; Gloire and Piette, 2009). Apart from this, high levels of ROS activates c-Jun N-terminal kinases (JNK) pathway which is critical signaling in cell differentiation, survival and apoptosis through disruption of specific phosphateses that normally inhibit the activity of JNK (Matsukawa *et al*., 2004, Castro-Caldas *et al*., 2012). The accumulation of cellular ROS will trigger expression of Nrf2 to modulate the expression of antioxidant genes as a negative feedback inhibition to balance the oxidation stress (Kasper *et al*., 2009).

2.5.4 p53 mediate apoptosis cascade

Tumor suppressor p53 is known as transcriptional factor that activates gene transcription that play key role in apoptosis, cell cycle arrest or growth suppressing activity (Haupt *et al*., 2003; Boregowda *et al*., 2018). There are two distinct signaling pathways (intrinsic and extrinsic) where p53 promotes apoptosis (Moll *et al*., 2005; Chowdhury *et al*., 2006). Generally, p53 initiates the extrinsic apoptosis pathway by inducing transcription of genes that encodetrans-membrane receptor of tumor necrosis factor receptor (TNF-R) family including Fas and DR5 (Nagata and Golstein, 1995; Ashkenazi and Dixit, 1998). The activation of Fas (Müller *et al*., 1998) and DR5 (Wu *et al*., 1997) by the binding of FasL and TNF-related apoptosis-inducing ligand (TRAIL) respectively and leading to the formation of death-induced signaling complex composed of apoptosis-inducing receptor, FADD, caspase-8 (Peter and Krammer, 2003; Feltham *et al*., 2017). Subsequently, this death-induced signaling complex triggers the cascade signaling including caspase-3 and caspase-7 that promote cell apoptosis (Hirata *et al*., 1998; Porter and Jänicke, 1999; Brentnall *et al*., 2013; Feltham *et al*., 2017).

On the other hand, the intrinsic apoptosis pathway is activated when the cytochrom c is released from the depolarized mitochondrial's inner membrane into the cytosol of cells in response to DNA damage (Gogvadze *et al*., 2006; Wang and Youle, 2009). The release of cytochrom c is controlled by the Bcl-2 family genes (Adams and Cory, 2002; Kuwana *et al*., 2002) including *Bax*, *Noxa*, *PUMA* and *Bid* which are targets of p53 (Haupt *et al*., 2003). The presence of cytochrome c in turn forms an apoptosome complex together with apoptotic protease-activating factor 1 (ApaF1) and procaspase-9 in order to activate caspase-9 (Adams and Cory, 2002). Subsequently, caspase-9 leads to the activation of caspase-3, caspase-6 and caspase-7 which promotes cell apoptosis (Maclachlan *et al*., 2002). Intriguingly, it was reported that p53 not only triggers the apoptosis pathways, it also plays a critical role in promoting crosstalk of both intrinsic and extrinsic pathways through Bid regulation (Haupt *et al*., 2003). Activated caspase-8 from the extrinsic pathway cleave the cytoplasmic Bid to induce conformational changes followed by translocation of Bid into the membrane of mitochondria to activate BAX and leading to apoptosome formation (Haupt *et al*., 2003).

2.6 Role of miRNAs in regulating gene expression

As previously mentioned, genes expression and its related signaling pathways have significant implications in the control of key stem cell programs including channeling cell differentiation, self-renewal, cell fate decisions, senescence and apoptosis. Recent evidence suggests that the genes expression is highly regulated through various mechanisms (Glubb and Innocenti, 2011). Among these, microRNAs (miRNAs) are reported as dynamic regulators that modulating the levels of gene expression by post-transcriptional mechanism (Maroney *et al*., 2006; Thomson *et al*., 2006; Filipowicz *et al*., 2008; Heinrich and Dimmeler, 2012; Zhao *et al*., 2014; Ba *et al*., 2016). The

binding of miRNA to the targeted mRNA obstructs the translation of target transcripts leading to inactivation or down-regulation of protein expression. Study by Friedman *et al*., (2009) suggested that more than 60% of human protein-coding genes are targets of miRNAs. With this, expression of miRNAs can therefore affect different signaling pathway of many cellular processes (Kim, 2005; Ason *et al*., 2006; Liu and Olson, 2010; Pritchard *et al*., 2012; Danger *et al*., 2014; Berrien-Elliott *et al*., 2016; Hoye *et al*., 2017).

2.6.1 Biogenesis of miRNA

miRNAs are referred as a class of non-coding RNAs which are 18 to 25 nucleotides in size (Ambros *et al*., 2003) that control the translation of mRNAs (Ambros, 2004; Valencia-Sanchez et al., 2006; Ha and Kim, 2014) . The coding sequences of miRNAs are located either in intergenic or intragenic region of DNA (Saini *et al*., 2007). The hairpin-shaped primary-miRNAs (pri-miRNAs) that transcribed by polymerase II (Lee *et al*., 2004) in nucleus are recognized and cleaved by microprocessor complex which composed of RNAse III polymerase (Drosha) and RNA binding protein DiGeorge syndrome Critical Region Gene 8 (DGCR 8) (Figure 2.6).

This microprocessor complex trimmed the pri-miRNAs and generates an approximately 70-nucleotide precursor-miRNAs (pre-miRNAs). The pre-miRNAs are subsequently processed after transported from nucleus into cytoplasm by Exportin-5. A heterotrimeric complex DICER (another type of RNAase III) which composed of double–stranded RNA-binding protein TRBP and Argonaute protein (Ago) crops the pre-miRNAs (at the approximately 22 nucleotides from the Drosha cleavage site) to remove the hairpin-loop and produce mature miRNA duplex with 20-24 nucleotides in size. DICER further

separates the mature miRNA duplex, in which one of the RNA is remains integrate with Argo molecule and another RNA strand is subjected to degradation.

mRNA regulation process by miRNA is triggered when miRNA–inducing silencing complex miRISC (containing miRNA strand with Ago molecule) recognizes the mRNA target (Maroney *et al*., 2006). During which the complimentary seed motif located at nucleotide 2-8 of mature miRNAs get to bind to the mRNA target (Nielsen *et al*., 2007, Friedman *et al*., 2009). This resulting in post-transcriptional gene silencing including mRNA target degradation or reduction in mRNA stability (Valencia-Sanchez *et al*., 2006; Ha and Kim, 2014). Hundreds of different mRNA can be recognized by miRNA and may be simultaneously targeted by multiple different miRNAs (Lewis *et al*., 2005, Friedman *et al*., 2009).

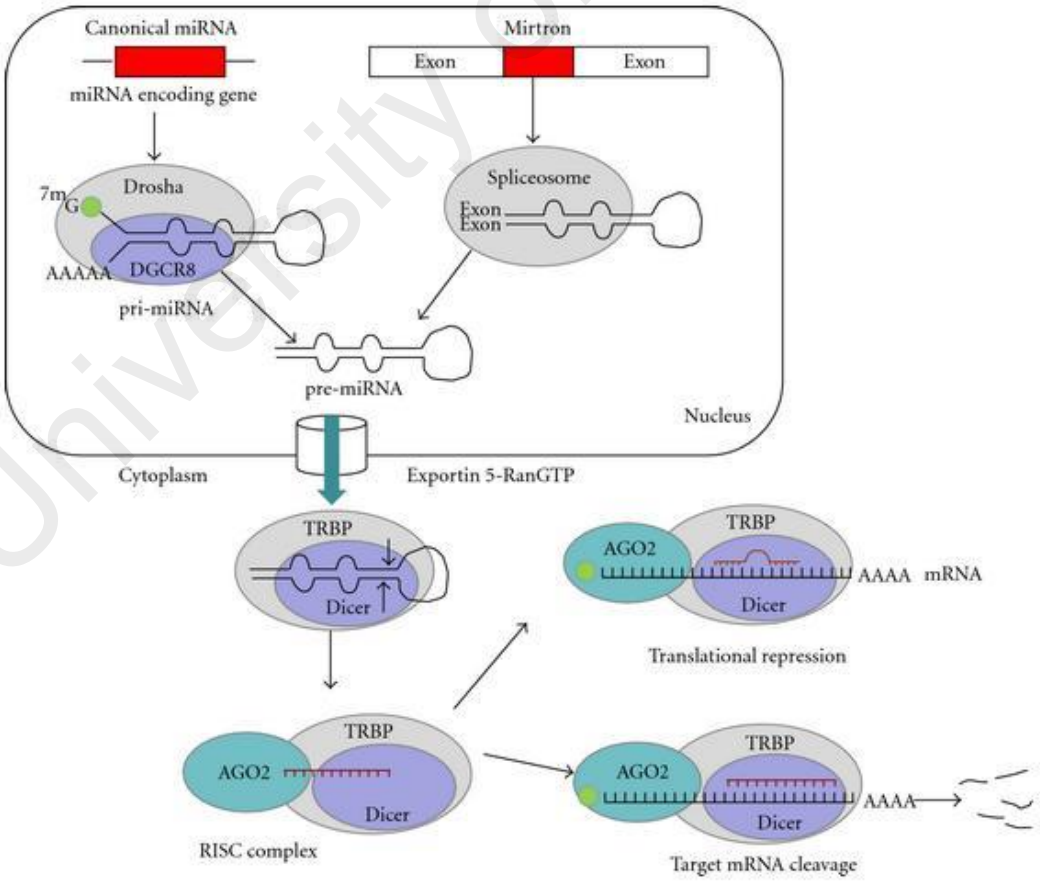


Figure 2.6. Schematic representation of miRNA biogenesis discussed in text (source from Feliciano *et al*., 2011)

2.6.2 miRNAs expression in regulating senescence related genes

It has been reported that p53 acts as a core factor that modulates diversity of senescence-related cellular processes in response to stress signals via exert transcription regulation of its target genes and miRNAs (Spike and Wahl, 2011; Rosa and Brivanlou, 2013). As a transcriptional factor, p53 directly induces the expression of cyclin-dependent kinases inhibitors (CDKIs) p21 (Figure 2.7), which is proven to be involved in modulating the cell cycle arrest (Itahana *et al*., 2001; Fridman and Lowe, 2003). On the other hand, p53 binds to the promoter region of miR-34 (Kato *et al*., 2009) and initiates the transcription of miR-34a and miR-34 cluster (miR-34b and miR-34c) which negatively regulates the progression of cell cycle by down-regulating the Cyclin D, Cyclin E, CDK4, CDK6, and E2F (He *et al*., 2007; Tazawa *et al*., 2007; Sun *et al*., 2008). Along with miR-34 cluster, other miRNAs factors such as miR-let-7 (Schultz *et al*., 2008) and miR-15 (Bonci *et al*., 2008) family were reported as transcriptional repressor of Cyclins and CDKs.

In addition of that, few studies have reported that Cyclins (Cyclin D and E) are direct targets of miR-26a (Kota *et al*., 2009), whereas CDKs are targeted by miR-24 (Lal *et al*., 2009). C-MYC is a transcriptional factor in genes regulation involved in cellular proliferation, differentiation and apoptosis. A study by Sachdeva *et al*., (2009) found that p53 represses the expression of c-MYC by inducing the expression of miR-145 leading to inhibition of tumor cell growth. Apart from this, c-MYC was down-regulated by other miRNAs including miR-let-7 family and miR-24 (Sampson *et al*., 2007; Lal *et al*., 2009).

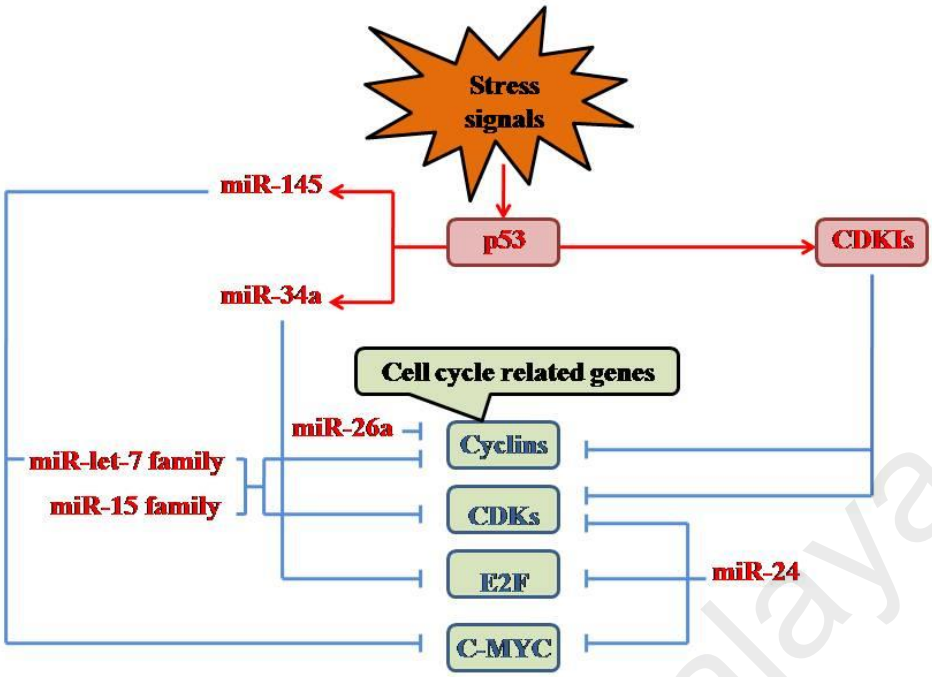


Figure 2.7. Schematic representation of stress mediated miRNAs expression in regulating cell cycle related genes discussed in text
Red arrow, miRNAs and genes indicates increased expression, whereas blue line and miRNAs indicates decreased expression.

Furthermore, the importance of miRNAs act as the biomarkers of cellular senescence has been shown in other studies. An example of miRNA that plays a role in cell senescence is miR-22 (Lee *et al*., 2015; Lee *et al*., 2017). miR-22 is known to direct target mediator of DNA damage checkpoint protein (MDC)1 when triggered by DNA damage response (DDR). It has been shown that DDR is an important signaling network critical for DNA repair to maintain genomic stability (Giglia-Mari *et al*., 2011). In addition, Zhang *et al*., (2017) reported that overexpression of miR-22 lead to reduction of proliferation and promoted cell apoptosis in breast cancer cells by inhibiting DNA damage regulator sirtuni 1 (SIRT1).

2.7 The road ahead

Over the past several decades, the fascinating features of stem cell have drawn the attention of researchers from various fields as it hold great promise for applications in regenerative medicine. However, MSCs were found undergoing cellular ageing after multiple passaging *in vitro* and ultimately leading to growth arrest, hence hampering its clinical application (Hayflick 1965, von Zglinicki and Martin-Ruiz, 2005; Wagner *et al*., 2009; Wagner *et al*., 2010b). Advances in molecular biology research have paved the way for understanding cell signaling system, suggesting that miRNAs profoundly influence the mRNA translation machinery and affect cellular phenotypes directly. Despite the apparent complexity, these early studies agree with the idea of multiple-to-multiple relationship between miRNAs and their targeted genes. The implication of such distinct physiological role with diversified regulatory effects of miRNAs might be valuable information in regarding the maintenance of cellular homeostasis.

This idea has sparked an interest in linking miRNAs as one of the core determinants that influence stem cells' stemness. Although many efforts have been made to acquire insights into how miRNA affect stem cells characteristics by altering their targeted genes expression. It is yet inadequate to fully explain the role of miRNAs in modulating stem cell ageing during *in vitro* expansion especially for MSCs derived from primary sources. Thus, it is crucial to extend our knowledge of miRNAs in regulating cellular ageing. This may facilitate the development and design of new strategies to restore cell integrity by specific interference with the activity of certain miRNA expression for research and clinical purpose.

CHAPTER 3: THE EFFECT OF PASSAGING ON CELLULAR AGEING OF HUMAN MESENCHYMAL STROMAL CELLS ISOLATED FROM THE PULP OF EXTRACTED DECIDUOUS TEETH AND WHARTON'S JELLY DERIVED FROM PRIMARY SOURCES

3.1 Introduction

Mesenchymal stem cells (MSCs) represent the multipotent adult stem cells that reside in the stem cells niches of different tissue throughout the body. Like embryonic stem cells (ESCs), these MSCs possess self-renewal and differentiate capability to facilitate the repair and regeneration of damaged or injured tissue. They are maintained in a quiescent stage upon activation by intrinsic or extrinsic signals during tissue injury. Research on stem cell biology in the past decade has demonstrated the potential of MSCs to be used as a therapeutic agent for many diseases such as heart failure, bone and cartilage repair, neurodegenerative diseases, diabetes and others (Granero-Molto *et al*., 2008; Koninckx *et al*., 2011; Bockeria *et al*., 2013; Tanna and Sachan, 2014).

In spite of that, the important factor for the success of stem cell therapies is the efficacy of the cells that are used, this including cellular homogeneity, integrity, proliferation capacity and differentiation propensity and others. In general there is consensus that difference in donors, tissue source, isolation methodology and culture conditions of MSCs will produce different population of cell types with potentially different potency. Indeed, early evidence showed the existence of heterogeneity of cell types within the adult stem cell sources, for example, at least two distinct stem cell populations were found in bone marrow; hematopoietic stem cells and MSCs (Méndez-Ferrer *et al*., 2010). Thus, the recognition of the cellular heterogeneity and defining the characteristics of MSCs culture that isolated from primary tissues is essential prior to further research. In fact, using a well-defined homogenous stem cell population in research would help to reduce variation in the experimental design and conduct.

Although MSCs have shown potential application in regenerative medicine, isolated MSCs from donor tissues may not be sufficient to meet the clinical needs during transplantation (Heathman *et al*, 2015). Hence it is essential to increase the number of MSCs in culture while retaining their therapeutic potential prior to clinical application. There are still many hurdles to be overcome in order to employ MSCs for clinical application. One of which is reduced cell proliferation and differentiation of MSCs when maintained in long-term culture. Unlike ESCs, which can undergo unlimited proliferation while retaining their pluripotency potential, MSCs can only be cultured for a certain period of time and ultimately halts in proliferation (Hayflick, 1965; von Zglinicki and Martin-Ruiz, 2005). It still remains unclear what causes the premature *in vitro* ageing in MSCs culture, hence this warrants the further investigation.

The aim of this study was to investigate the effect of passaging on cellular ageing of human mesenchymal stromal stem cells (hMSCs) from two primary sources; pulp of extracted deciduous teeth (SHED) and Wharton's jelly (WJSCs). The research objectives of this study are as follows;

1. To isolate and passage hMSCs from the pulp of extracted deciduous teeth and Wharton's jelly obtained from primary sources.
2. To confirm the MSC status of isolated SHED and WJSCs.
3. To determine the cell phenotypic changes related to cellular ageing of SHED and WJSCs at early and late passages.

3.2 Materials and methods

3.2.1 Isolation of hMSCs from extracted deciduous pulp

Dental pulp tissues used in this study were obtained from extracted deciduous teeth (n = 3). The teeth were indicated for extraction by the attending paediatric dentist at the Department of Paediatric Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya and prior to the tooth extraction informed consent was obtained from the parents of the donors, aged between four to twelve (Medical ethics approval: DF RD1503/0013 [L]). All chemicals were purchased from Gibco (USA) unless stated otherwise. First, the collected pulp tissues were rinsed three times with washing buffer containing 1× Dulbecco's phosphate buffer saline without calcium and magnesium (DPBS--), 1% (vol/vol) antibiotic-antimycotic (anti-anti), and 1% (vol/vol) penicillin-streptomycin (pen-step) to remove the blood cells and debris. The washed tissues were then minced into small fragments and digested with 3 mg/mL collagenase type I (Invitrogen, USA) in Dulbecco's modified Eagle's medium knockout (DMEM-KO) at 37°C for 40 min.

The digested mixture were then neutralized with an equal volume of culture media containing DMEM-KO, 10% (vol/vol) fetal bovine serum (FBS), 1% (vol/vol) Glutamax and 0.5% (vol/vol) anti-anti and followed by centrifugation at 1250 rpm for 5 min. The collected cell pellet was re-suspended with culture media supplemented with 2 ng/mL of basic fibroblast growth factor (bFGF) before seeding in culture flask and incubated in 5% CO_2 incubator at 37°C. Once cells attachment was observed under inverted light microscope the culture media was periodically changed every three days.

3.2.2 Isolation of hMSCs from Wharton's jelly

Umbilical cords used in this study were obtained from the Department of Obstetrics and Gynaecology, Faculty of Medicine, University of Malaya with ethical approval (Medical ethics approval: DF RD1503/0013 [L]) and informed consent was obtained the donors, aged from 25 to 35. All chemicals were purchased from Gibco unless stated otherwise. The cords were obtained after vaginal delivery or caesarean section were kept at 4°C in transport/washing buffer comprising of 1× DPBS--, 1% (vol/vol) anti-anti, and 1% (vol/vol) pen-step. Each cord sample was processed within 24 h upon collection. First, the umbilical cord was cut into 3 cm fragments and the blood contaminants were removed by rinsing with 1× DPBS-- or sterile distilled water (ddH_2O). Following that, the embedded umbilical arteries and veins in the Wharton's jelly were removed. The cord tissues were rinsed three times with washing buffer, followed by 30 s soaking in 70% ethanol and a quick rinse with ddH_2O immediately. Then, antibiotics treatment was carried out by incubating the cord tissues in 1× DPBS--, 10% (vol/vol) anti-anti and 10% (vol/vol) pen-strep at 37°C for 1 to 2 h.

The cord tissues were then digested with 0.1% (wt/vol) collagenase type I in DMEM-KO at 37°C for 9 h. Digested mixture was neutralized with an equal volume of culture media. The undigested debris was filtered using sterile 100 µm nylon filters (BD falcon, USA) and the filtrate was subjected to centrifugation at 1250 rmp for 6 min. Cells pellet was re-suspended with culture media supplemented with bFGF (2 ng/mL) before seeding into culture flask (BD falcon, USA) and incubation in CO_2 incubator at 37°C. Once cells attachment was observed under inverted light microscope the culture media was periodically changed every three days.

3.2.3 Cell culture, expansion and cryo-preservation

Upon reaching confluency of 80% to 90%, culture media were discarded from the culture flask followed by rinsing the adherent cells with 1× DPBS-- twice to remove excess culture medium. Adherent cells were dissociated using 1 to 2 mL of TrypLE Express (Gibco, USA) at 37°C for 3 min. Detached cells mixture was then neutralized with double volume of culture media. Cells suspension was harvested by centrifugation at 1250 rmp for 6 min. After the supernatant was removed, undisturbed pellet was re-suspended with culture media and was seeded at a density of 5000 cells/cm^2 into a fresh culture flask or plate (BD falcon). On the other hand, approximate 1 million of cells were stored by cryo-preservation with addition of 1 mL freezing medium containing 45% (vol/vol) of culture medium, 45% (vol/vol) of FBS and 10% (vol/vol) of dimethylsulfoxide (Sigma Aldrich, USA) in cryogenic vial (Corning, USA). Once frozen, vials were transferred into liquid nitrogen container in the vapor phase for long-term storage to ensure maximum viability of cells.

3.2.4 Growth kinetics analysis

The proliferation rate of culture was determined by plating cells with density of 5000 cells/cm^2 into a 6-well culture dish. Three replicates were prepared for each passage. Cell cultures were washed by DPBS (-)(-) twice to remove floaters (floating cells in medium are sign of cellular death). After 96 h of cultivation, cell culture was dissociated from the culture plate using TrypLE Express and cell count was conducted. Cell number (including live and dead cell) was determined using Trypan Blue exclusion method prior to cryopreservation and sub-cultures. 10 μL of cells suspension were diluted in Trypan Blue dye (Gibco, USA) in 1: 1 dilution factor and cell count was

performed using a cell counter (Invitrogen, USA). Growth kinetics was determined by calculating the population doubling time (PDT) and population doubling (PD) using the following equations;

PDT = D log(2) /(log(NH) – log(NI)) and PD = (log(NH) – log(NI))/log(2),

where NI is the inoculum cell number, NH is the cell harvest number, D is the duration of the culture in hours.

3.2.5 MSCs Immuno-phenotyping

A florescence compensation control was conducted before running the actual samples in order to correct the overlapped emission spectra. This process is to ensure the detected florescence is obtained from the fluorochrome that is being measured. In brief, four sets of 0.5 $\times 10^6$ hMSCs suspension aliquots in 100 µL of sample buffer containing 1× phosphate buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 2 mM methylenediaminetetraacetic acid (EDTA) and one set of blank aliquot in 500 µL of sample buffer were prepared. Approximately 10 µL of fluorochrome conjugated antibodies CD73-Biotin, CD105-PE, CD73-APC and CD90-FITC were added to each 100 µL cell suspension respectively and the mixture was mixed by pipetting up and down. After 10 min of incubation in a dark environment at 4ºC, the aliquots was washed by adding 1 to 2 mL of sample buffer followed by centrifugation at 300 ×*g* for 10 min, except for the aliquot CD-73-Biotin. After removing the supernatant, cell pellet was re-suspended in 500 µL sample buffer. Simultaneously, 10 µL of anti-biotin-PerCP was added to the CD-73-Biotin aliquot and incubated for another 10 min the dark. Then the mixture was washed by adding 1 to 2 mL of sample buffer followed by centrifugation at 300 ×*g* for 10 min. After removing the supernatant, CD73-PerCP conjugated cell pellet

was re-suspended with 500 µL of sample buffer. All five sets of fluorochrome-conjugated aliquot were then subjected to compensation analysis following the manufacturer instructions.

Cells were obtained by dissociation using TrypLE Express and were then collected after centrifugation at 300 ×*g* for 10 min. After determination of cell number, approximately 1×10^6 of cells was re-suspended with 100 µL sample buffer. Approximately10 µL of each MSC phenotyping cocktail (containing positive markers: FITC-CD90, PE-CD105, APC-CD73; negative markers: PerCP-CD34/CD45/CD14/CD20) and isotype control cocktail (IgG1-FITC, IgG1-PE, IgG1-APC, IgG1-PerCp, IgG2a-PerCP) (Miltenyi Biotec, Germany) was added into the cell suspension respectively. The cell suspension was then mixed and incubated for 10 min in dark refrigerator (2 to 8°C). Next, the cells were washed with 1 mL sample buffer and collected by centrifugation 300 ×*g* for 10 min. Cells pellet was then re-suspended with 0.5 mL of sample buffer for flow cytometric analysis using MACS Quant® Analyzer (Miltenyi Biotec, Germany) where at least 10,000 events were collected for each cocktail to determine percentage of cells expressing the respective markers.

3.2.6 *In vitro* trilineage differentiation studies

An *in vitro* trilineage differentiation study was performed to determine the multi-lineage capacity of the mesenchymal stem cells. Cell cultures at 80% confluency were subjected to *in vitro* differentiation into adipocytes, chondrocytes and osteoblasts lineages using StemPro® adipogenesis, chondrogenesis and osteogenesis differentiation kits (Gibco, USA) respectively. The cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2 and were re-fed at three days intervals. The

differentiation phenotypes were then confirmed by staining method after 21 days of differentiation period. After fixation with 10% formalin solution, cultures were rinsed with PBS for three times. Then, 0.3% of Oil Red O solution was used to stain the oil droplet red in the differentiated adipocytes and 1% of Safranin O solution will stain glucosaminoglycan in the differentiated chondrocytes orange under the inverted light microscope. The calcium deposits in the differentiated osteocytes culture were stained bright orange-red with 2% of Alizarin Red solution.

3.2.7 Cell passaging

In continuation of this study, SHED and WJSCs culture were expanded for another three consecutive passages after P3. Growth kinetic, MSCs immune-phenotyping and trilineage differentiation analysis were performed according to the protocol as described earlier in sections 3.2.4, 3.2.6 and 3.2.6 respectively. In addition, the cumulative population doubling level (cPDL) for both cell types was also calculated by adding the PD from P2 to P6.

3.2.8 RNA extraction

Cell cultured were washed with 1× DPBS (--) twice and adherent cells were directly lysed with 1 mL of TRIzol reagent (Ambion, USA) by pipetting the mixture up and down several times. TRIzol-lysate was then collected into 1.5 mL micro-centrifuge tube and 200 µL of chloroform was added. The mixture was thoroughly mixed by inverting for five times and incubated for 3 min at room temperature. The mixture was then subjected to centrifugation at 12000 rpm for 15 min at 4°C and was allowed to separate

into three liquid phases, represented by a colourless, white and pink layers in an ascending order. The top colourless aqueous layer was then transferred into a sterile 1.5 mL micro-centrifuge tube and mixed with 0.5 mL of isopropanol alcohol. RNA was allowed to precipitate out of the solution after incubation at room temperature for 10 min prior to a 10 min centrifugation at 12000 rpm. The RNA pellet was washed twice with 75% of ethanol and then was left to air-dry before dissolving in 30 µL of RNase and DNase free water. All RNA samples were stored at -30°C. The total RNA yield and its purity were determined by using spectrophotometer with absorbance measurement at 260 nm and 280 nm. RNA purity of samples was assessed by A_{260}/A_{280} ratio, with expected value of 1.8 to 2.1.

3.2.9 Quantitative polymerase chain reaction (qPCR)

The extracted RNA samples were subjected to DNase treatment to remove the residual DNA by mixing with 2 units of DNase I with 1× DNase buffer (Ambion, USA) and incubation at 37°C for 30 min, followed by another incubation period at 75°C for 5 min to deactivate the DNase I. Subsequently, 1 µg of total RNA was reverse-transcribed using TaqMan® Reverse Transcription Reagent (Applied Biosystems, USA). The reaction mixture consists of 1× RT buffer, 1.75 mM $MgCl_2$, 0.5 mM each of dNTP mix, 2.5 µM of random hexamers, 1.0 U/µL of RNase Inhibitor, and 2.5 U/µL of MultiScribe™ Reverse Transcriptase. cDNA was generated using the recommended three cycling conditions as follows; 25°C for 10 min, then 37°C for 30 min and 95°C for 5 min. Following that, qPCR analysis was performed with 7500 Fast system (Applied Biosystems, USA) in a total reaction mixture of 20 µL containing 100 ng of cDNA, 1× of TaqMan® Fast Advanced Master Mix, 1 µL of 20× TaqMan® Gene Expression Assay (Cdk4: Hs00262861, Cyclin D: Hs00176481. Cyclin E: Hs01026536,

p53: Hs01034249, p21: Hs00355782, Nanog: Hs02387400, Oct4: Hs04260367, gapdh: Hs02786624). The thermal cycling condition was initiated with 20 s polymerase activation at 95ºC followed by forty repeated cycles of denature at 95ºC for 3 s and annealing at 60ºC for 30 s.

Quantification of gene expression with cycle threshold (C_T) of 35 or less was accessed by DeltaDeltaC_T ($\Delta\Delta C_T$) data analysis method. C_T value of gene of interest (GOI) was normalized by the C_T of GADPH (as house keeping gene-HKG): $2^{-\Delta CT} = 2^{-[CT(GOI) - CT(HKG)]}$. Furthermore, the fold-change of gene of interest were calculated as $2^{-(\Delta\Delta CT)}$ in which the normalized expression of the GOI in the experimental sample (P6) is divided by the normalized expression of the same GOI in the control sample (P3):

$2^{-\Delta\Delta CT} = 2^{-[\Delta CT\ (Experiment) - \Delta CT(Control)]}$.

3.2.10 Statistical analysis

Statistical significance was determined using *t-test* and ANOVA, Statistical Package for the Social Sciences (SPSS version 20.0). Data was presented as mean ± SD and $p<0.05$ was considered as statistically significant.

3.3 Results

3.3.1 hMSCs isolation from extracted deciduous pulp and Wharton's jelly

In this study, hMSCs from extracted deciduous pulp (SHED) and Wharton's jelly (WJSCs) were successfully isolated, where each sample types was obtained from three different donors. Colonies formations were observed under an inverted light microscope at two to four days post-seeding. Both cultures at initial passage (P0) were maintained in the culture media supplemented with 2 µg/mL of bFGF until confluency of 80% to 90% followed by cryo-preservation and sub-culture for further experiments.

3.3.2 Confirmation MSCs status of SHED and WJSCs obtained from primary source

3.3.2.1 Cell morphology examination

Both polyclonal population of SHED and WJSCs were successfully expanded to a seeding density of 5000 cells/cm^2. Both samples were maintained in an undifferentiated culture up to three consecutive passages (P1 to P3) and required an average of four to six days to attain 90% confluency. Monolayer adherent fibroblastic spindle-like shape morphology was been observed in all cultures (Figure 3.1).

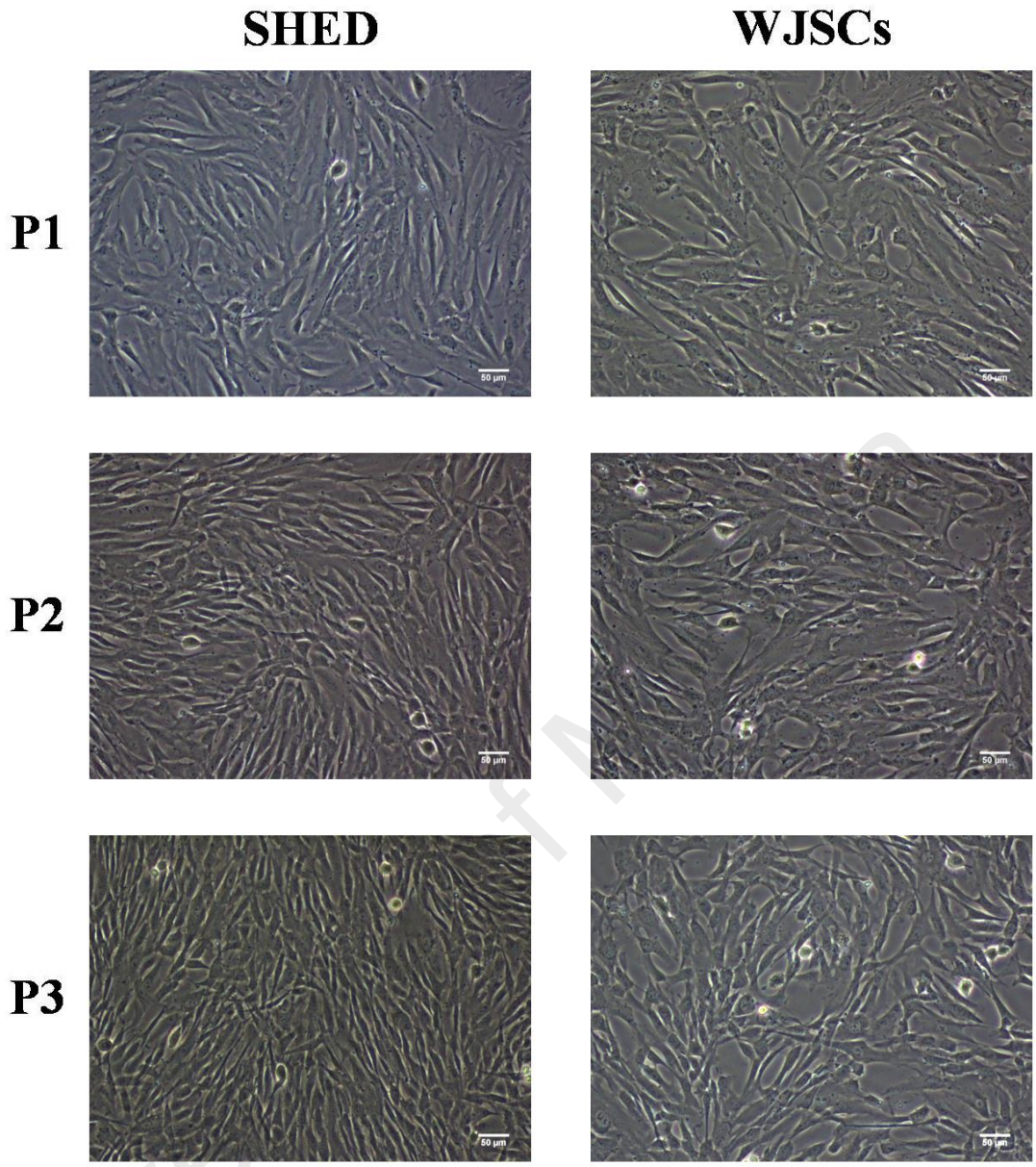
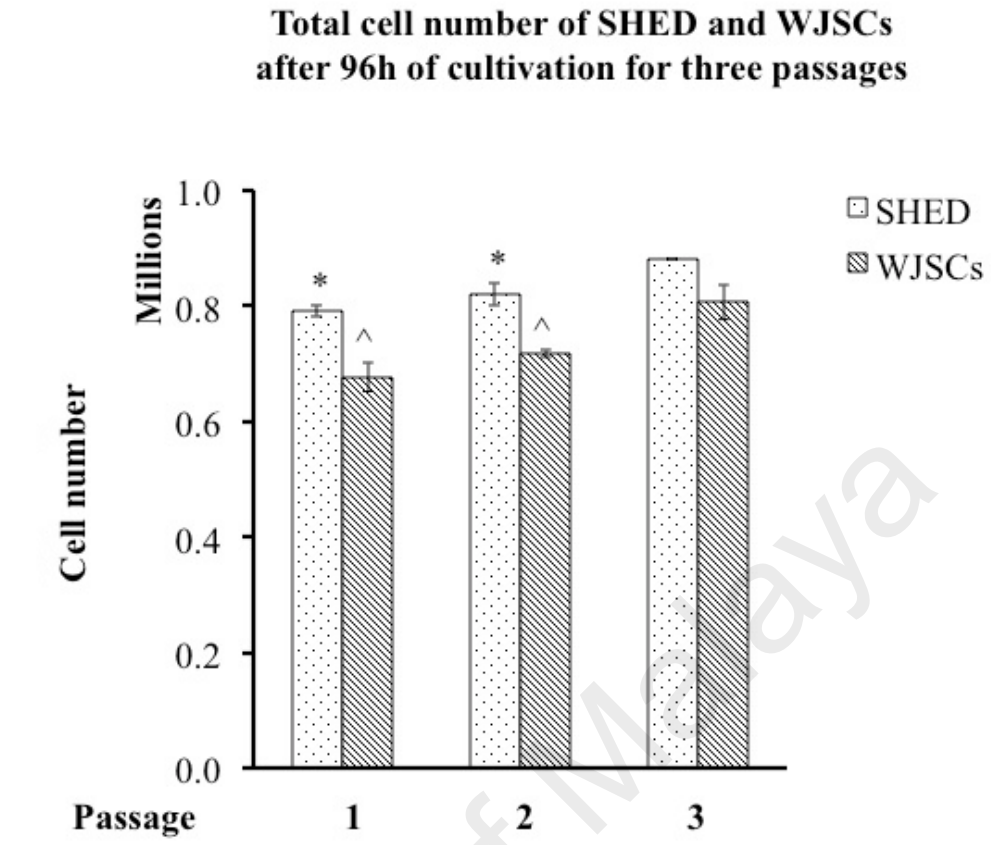


Figure 3.1. Morphology of SHED and WJSCs during *in vitro* culture for three passages (P1 to P3)

Bright-field images (total of 100× magnification; 10× ocular, 10× objective) of SHED and WJSCs at P1 to P3 taken after 96 h of cultivation. Fibroblastic spindle-like cell morphology was observed in both SHED and WJSCs culture.

3.3.2.2 Growth kinetics

In this study, the growth kinetics of isolated SHED and WJSCs including the number of live and dead cells were for three consecutive passages (P1 to P3) were determined. Triplicate cultures of SHED and WJSCs at seeding density of 50000 cells/well were plated in 6-well culture plates. Cell count and population-doubling time (PDT) was determined at each passage after 96 h of cultivation. Under the culture conditions, both SHED and WJSCs demonstrated an increase in proliferation. As shown in Figure 3.2, both cell types exhibited similar growth kinetic trends; where the cell count peaked at P3. The PDT results showed varied values across passages in both SHED and WJSCs cultures, where the shortest average PDT for SHED and WJSCs was 23.20 h and 23.94 ±0.32 h respectively at P3. P3 exhibited a significant lower PDT compare to those at P1 and P2 ($p< 0.05$) (Figure 3.2).



PDT				
	SHED	24.11 ±0.11 *	23.79 ±0.21 *	23.20 ±0.00
	WJSCs	25.55 ±0.37 ^	24.99 ±0.08 ^	23.94 ±0.32

Figure 3.2. Growth kinetics of SHED and WJSCs during *in vitro* culture for three passages (P1 to P3)

Total cell number and population doubling time (PDT) in all 3 passages for SHED and WJSCs after 96 h of cultivation. Data represent mean ± standard deviation of n=3 biological replicates. The results of cell count and PDT analysis show there were statistically significant differences between each samples and across each passages ($p > 0.05$), unless stated otherwise, same notation (^*) indicate no significant difference.

3.3.2.3 MSCs immuno-phenotyping

Based on the minimal criteria proposed by the International Society for Cellular Therapy (ISCT), both cell types at P3 were further characterized with regards to their morphologic homogeneity of MSC phenotype via assessing their cell surface antigen profile by immune-phenotyping technique. Number of cells in population expressing cluster of differentiation (CD)105, CD90, and CD73 antigen (markers of human MSCs) and CD45, CD34, CD20, as well as CD14 (hematopoietic markers) were determined by flow cytometry. The flow cytometric analysis revealed that more than 90% of both cells population were positive for expression of human MSC markers. In contrast, less than 1% of both cells population expressed heamatopoictic makers (Figure 3.3).

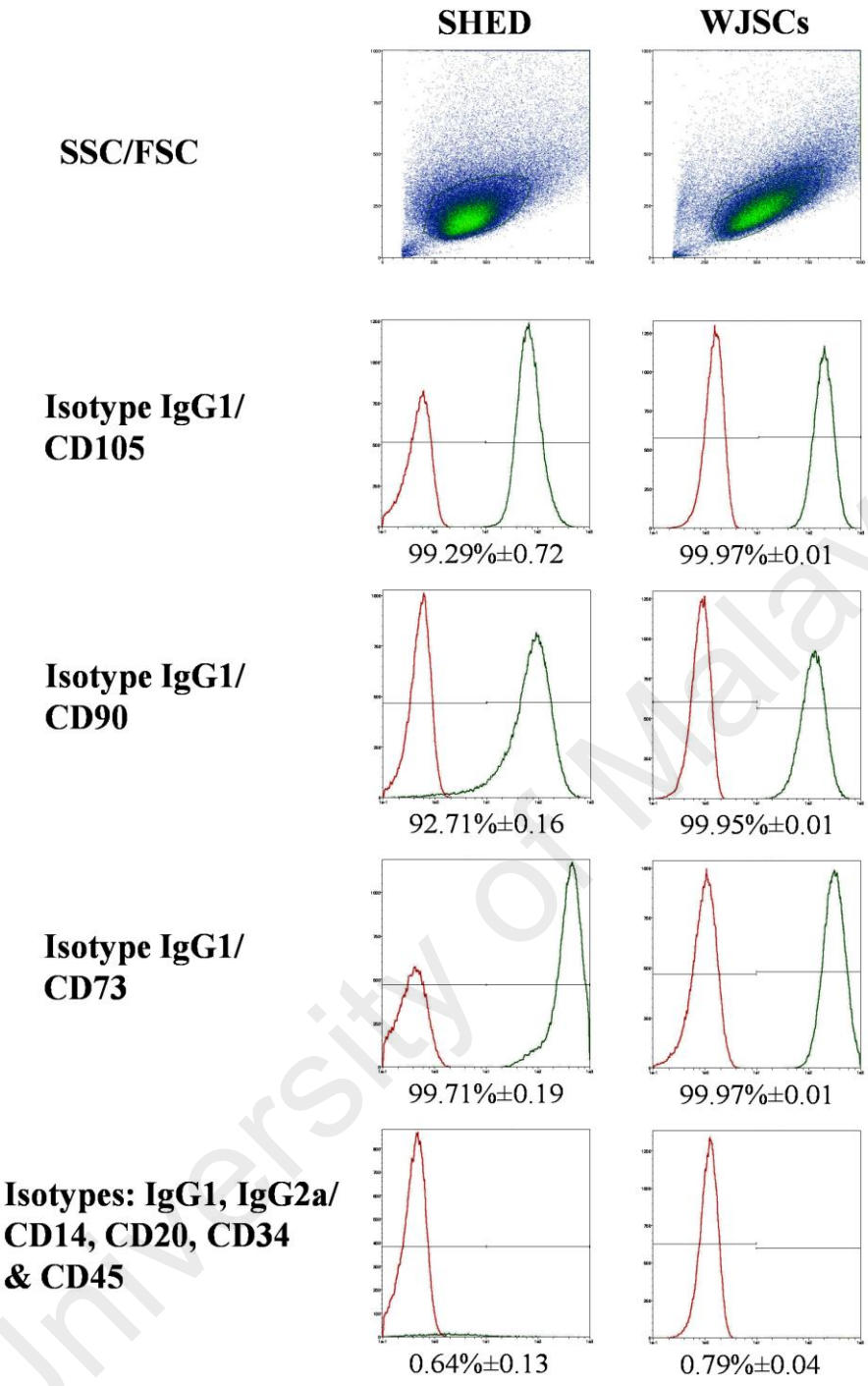


Figure 3.3. MSCs immune-phenotyping of SHED and WJSCs at P3
Flow cytometry analysis histograms demonstrating percentage of SHED and WJSCs at P3 stained for human MSCs surface markers (CD105, CD90 and CD73) and hematopoietic markers (CD45, CD34, CD20, and CD14). Data represent mean ± standard deviation of n=3 biological replicates; side scatter (SSC); forward scatter (FSC); red colour histograms are for control immunoglobulins and green colour histograms are for specific markers. Both SHED and WJSCs were showed positive for the expression of human MSCs markers and negative for the expression of hematopoietic markers.

3.3.2.4 Trilineage differentiation

Cell cultures of both SEHD and WJSCs at P3 were subjected for *in vitro* differentiation into adipocytes, chondrocytes and osteocytes in order to determine their multi-potent mesenchymal differentiation capacity. After 21 days of adipogenic differentiation, cells with oil droplets deposited in cytoplasm were observed in all cultures when stained with Oil Red O (Figure 3.4). Staining results showed that WJSCs exhibited better adipogenic differentiation capability than SHED. On the other hand, the expression of proteoglycan extracellular matrix in the chondrogenic-differentiated cell for both SHED and WJSCs cultures were confirmed by Safranin O staining. Additionally, differentiation of SHED and WJSCs into osteoblast was verified by Alizarin Red staining in which calcium precipitate was detected.

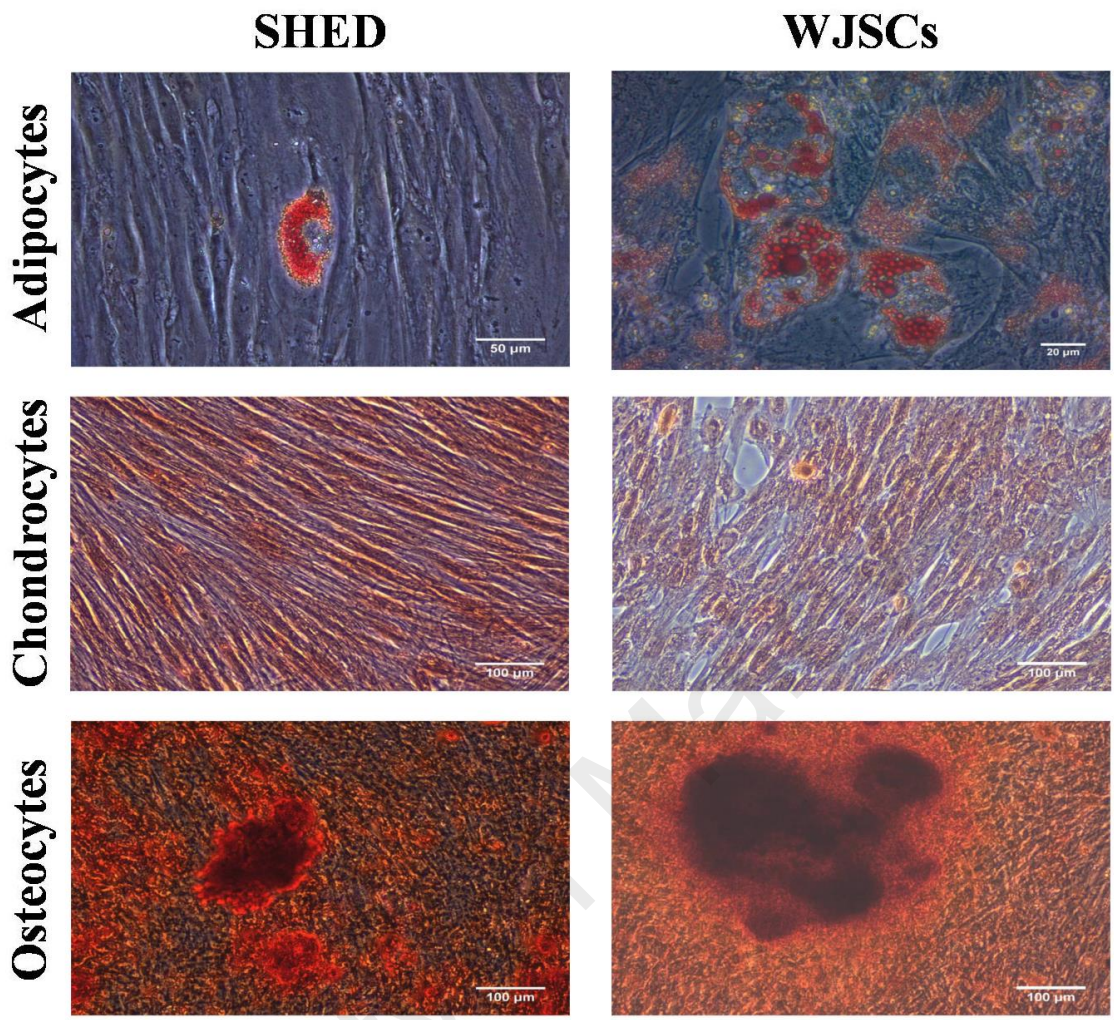


Figure 3.4. Trilineage differentiation capacity of SHED and WJSCs at P3
Phase contrast images of adipogenic (total of 200× magnification; 10× ocular, 20× objective), chondrogenic (total of 100× magnification; 10× ocular, 10× objective) and osteogenic (total of 100× magnification; 10× ocular, 10× objective) differentiation potential of SHED and WJSCs at P6 for 21 days of induction period. Oil Red O staining of intracellular lipid droplets in red confirmed adipogenesis, Safranin O staining of extracellular deposition of glycosaminoglycans in orange confirmed chondrogenesis and Alizarin red staining of calcium deposits in bright orange-red confirmed osteogenesis.

3.3.3 Phenotypic changes of SHED and WJSCs at early and late passages

3.3.3.1 Cell morphology examination

Morphology characterization has been widely reported as an important method to determine stem cells phenotypic status during cultivation (Wagner *et al*., 2008). In this study, plastic-adherent fibroblast-like cells were observed in both SHED and WJSCs from the first 3 early passages of (P1 to P3) cultivation (Figure 3.1). At the later passages (P4 to P6), some cells exhibited flattened and enlarged morphology (Figure 3.5).

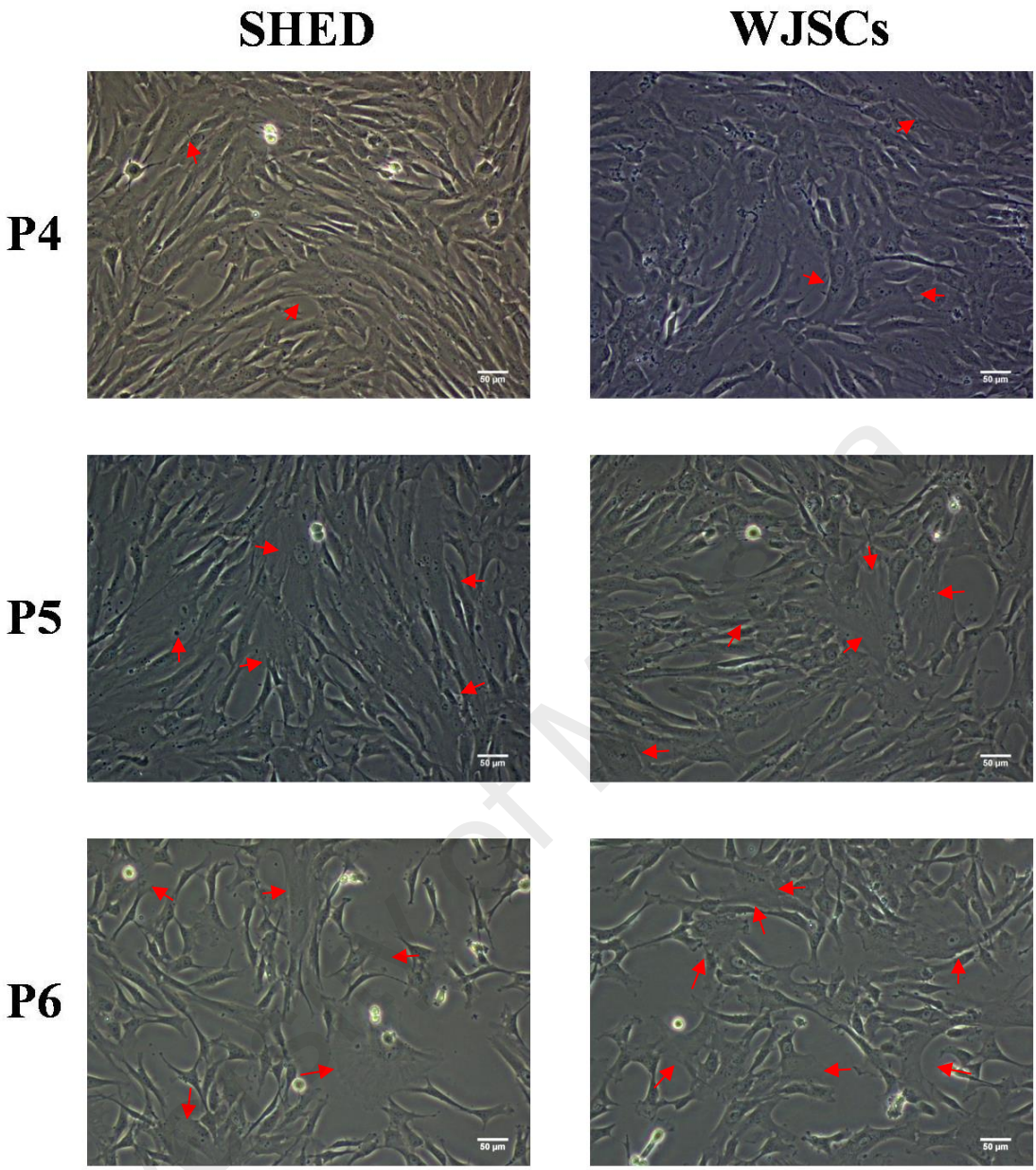


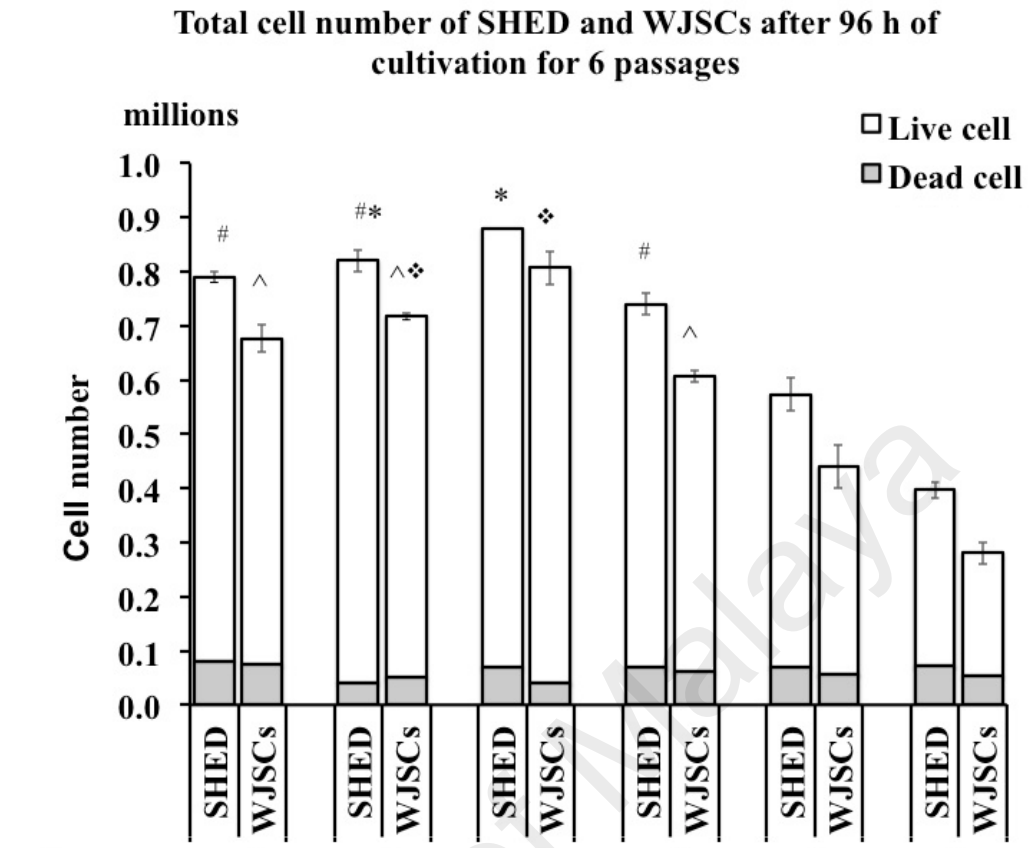
Figure 3.5. Morphology alteration of SHED and WJSCs during late passages (P4 to P6) of *in vitro* culture

Bright-field images (total of 100× magnification; 10× ocular, 10× objective) of SHED and WJSCs after 96 h of cultivation at P4 to P6, where some cells showed enlarged and flattened morphology (red arrow).

3.3.3.2 Growth kinetics

In addition to cell morphology examination, the growth kinetics of isolated hMSCs for another three consecutive passages after P3 were determined. As shown in Figure 3.6, a substantial decrease in cell proliferation rate of SHED and WJSCs were observed after P3. It is worth noting that there was an approximate 50% reduction in cell number from P3 to P6 for both cell types. Less proportion of dead cells were observed at P2 and P3 in both cell types which is less the 10%.

In addition, the PDT reflecting the cell count were evaluated, where the shortest PDT was found at P3 in both cell types at 23 h and lengthen to 37 h at P6. There was a significant increase of PDT in WJSCs after P3 in comparison to SHED, indicating that WJSCs underwent proliferation arrest earlier than SHED. These preliminary growth kinetics observations indicated that SHED and WJSCs cultures used in this study entered senescent after P3. It was also observed that SHED was able to undergo 22.55 PD during 6 passages culture while WJSCs could only accumulate a total numbers of 20.83 PD during the same period of time.



		Passage 1	Passage 2	Passage 3	Passage 4	Passage 5	Passage 6
PDT	SHED	24.11 ±0.11	23.79 ±0.21	23.20 ±0.00	24.70 ±0.25	27.30 ±0.61	32.14 ±0.61
	WJSCs	25.55 ±0.37	24.99 ±0.08	23.94 ±0.32	26.66 ±0.20	30.67 ±1.29	38.71 ±1.61
cPDL	SHED	3.96 ±0.02	8.02 ±0.05	12.15 ±0.05	16.04 ±0.09	19.56 ±0.17	22.55 ±0.23
	WJSCs	3.76 ±0.05	7.60 ±0.07	11.61 ±0.12	15.21 ±0.15	18.34 ±0.28	20.83 ±0.38

Figure 3.6. Growth kinetics of SHED and WJSCs during *in vitro* culture for six passages (P1 to P6)

Total cell number (live and dead cell), PDT and cPDL for each of six passages in SHED and WJSCs after 96 h of cultivation to assess their *in vitro* proliferation capability. Data represent mean ± standard deviation of n=3 biological replicates. The results of cell count and PDT analysis show there were statistically significant differences between each samples and across each passages ($p > 0.05$), unless stated otherwise, same notation ($^{\wedge * \diamond \#}$) indicate no significant difference. Growth kinetics of SHED and WJSCs showed approximately 50% reduction in cell number and increases in PDT reflects their lowering proliferative capacity along the passaging.

3.3.3.3 MSCs immune-phenotyping

Subsequently, the MSC surface antigen expression of SHED and WJSCs at P6 were examined. As showed in Figure 3.7 and Table 3.1, both SHED and WJSCs at P6 co-expressed MSC markers including CD105, CD90, and CD73 but lacking expression for CD45, CD34, CD20, and CD14. The results were found to be relatively comparable to that at P3 (Table 3.1). Overall results implied that a homogenous of SHED and WJSCs population resembled their MSCs identity in P3 and P6.

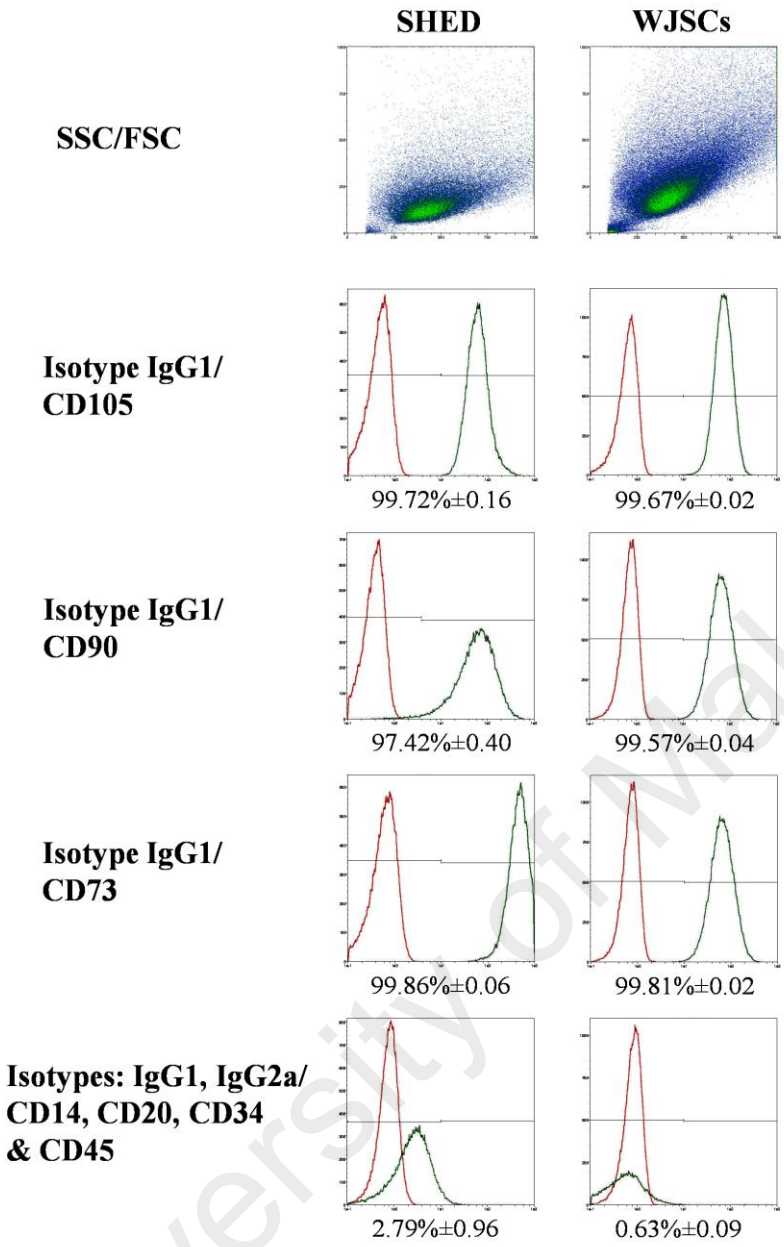


Figure 3.7. MSCs immune-phenotyping of SHED and WJSCs at P6
Flow cytometry analysis histograms demonstrating percentage of SHED and WJSCs at P6 stained for hMSCs surface markers (CD105, CD90 and CD73) and hematopoietic markers (CD45, CD34, CD20, andCD14). Data represent mean ± standard deviation of n=3 biological replicates; side scatter (SSC); forward scatter (FSC); red colour histograms are for control immunoglobulins and green colour histograms are for specific markers. Both SHED and WJSCs were showed positive for the expression of human MSCs markers and negative for the expression of hematopoietic markers.

Table 3.1. Immuno-phenotyping analysis of SHED and WJSCs at P3 and P6

Passage	Sample	MSC markers			Hematopoietic markers
		CD 105	CD 90	CD 73	CD45, CD34, CD20, and CD14
3	SHED	99.29% ± 0.72	92.71% ± 0.16	99.71% ± 0.19	0.64% ± 0.13
	WJSCs	99.97% ± 0.01	99.95% ± 0.01	99.97% ± 0.01	0.79% ± 0.04
6	SHED	99.72% ± 0.16	97.42% ± 0.40	99.86% ± 0.06	2.79% ± 0.96
	WJSCs	99.67% ± 0.02	99.57% ± 0.04	99.81% ± 0.02	0.63% ± 0.09

3.3.3.4 Trilineage differentiation

SHED and WJSCs at P6 were analyzed for trilineage differentiation. The staining evaluation on trilineage inductive cultures showed their capability to differentiate into adipocytes, chondrocytes, and osteocytes (Figure 3.8). Similarly to P3, WJSCs exhibited better adipogenic differentiation capabilities than SHED were observed.

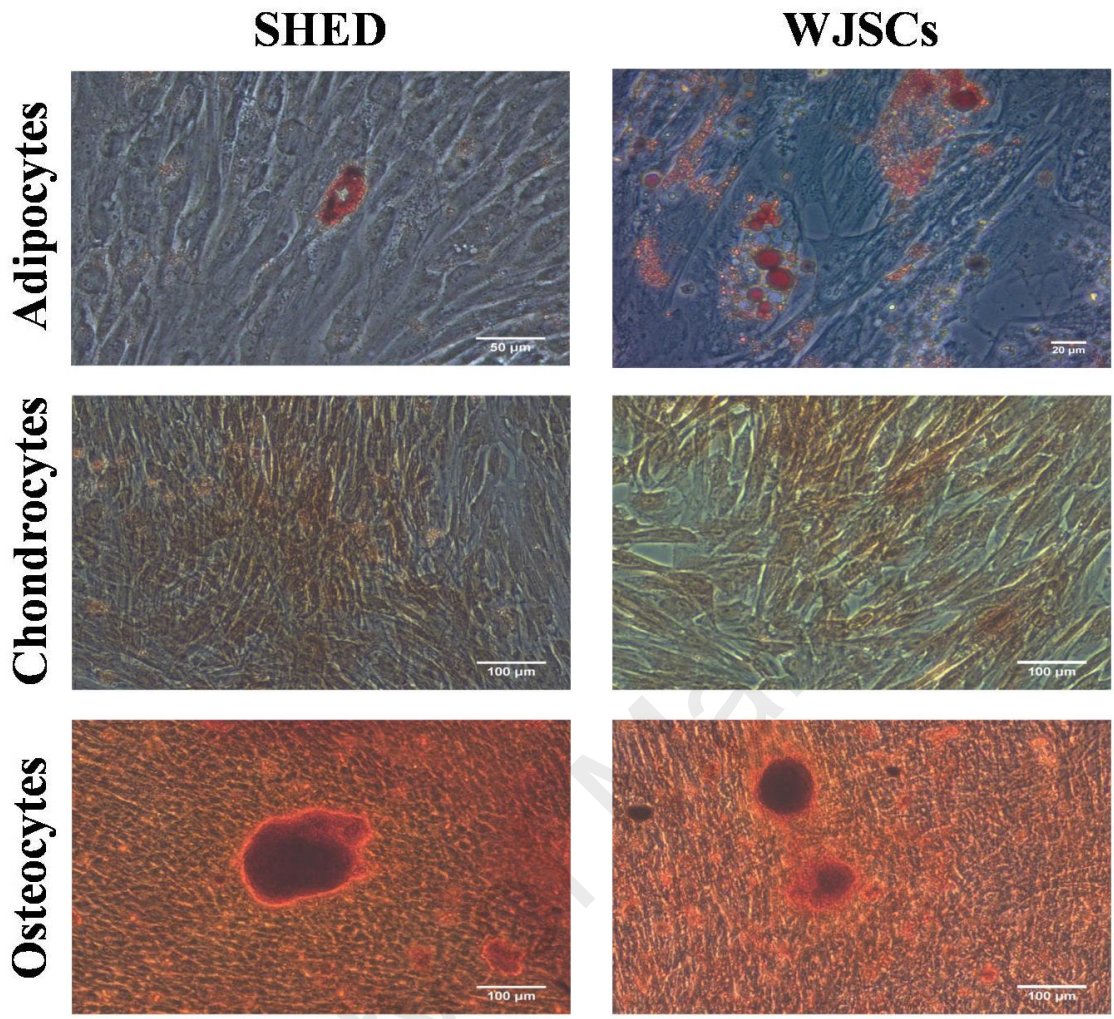


Figure 3.8. Trilineage differentiation capacity of SHED and WJSCs at P6
Representative microscopic images of adipogenic (total of 200× magnification; 10× ocular, 20× objective), chondrogenic (total of 100× magnification; 10× ocular, 10× objective) and osteogenic (total of 100× magnification; 10× ocular, 10× objective) differentiation potential of SHED and WJSCs at P6 for 21 days of induction period. Oil Red O staining of intracellular lipid droplets in red confirmed adipogenesis, Safranin O staining of extracellular deposition of glycosaminoglycans in orange confirmed chondrogenesis and Alizarin red staining of calcium deposits in bright orange-red confirmed osteogenesis.

3.3.3.5 Reduction of stemness and cell cycle associated gene expression in hMSCs during *in vitro* passaging.

In order to evaluate the effect of ageing caused by *in vitro* passaging on gene expression, RNA sample were extracted from SHED and WJSCs at both P3 and P6 for qPCR analysis. Figure 3.9 showed that the cell cycle-related genes such as *Cyclin-D*, *Cyclin-E* and *CDK4* declined significantly as passage number increases. To further assess the risk of cellular ageing upon passaging, the gene expression level of cyclin-dependent kinases inhibitor were examined. The qPCR results showed that *p53* and *p21* transcript level elevated from P3 to P6 in WJSCs, whilst the opposite patterns were observed in SHED (Figure 3.9). The results suggested that the changes of *p53* and *p21* levels in WJSCs might due to DNA damage recognition upon cellular senescence. Additionally, transcriptional factors such as *Oct4* and *Nanog* that are essential in maintaining plutipotency of stem cells phenotype reduced significantly from P3 to P6 in both cell types. Collectively, these results revealed that there were significant differences in gene expressions showing cell senescence-associated signature in P3 and P6 for both cell types.

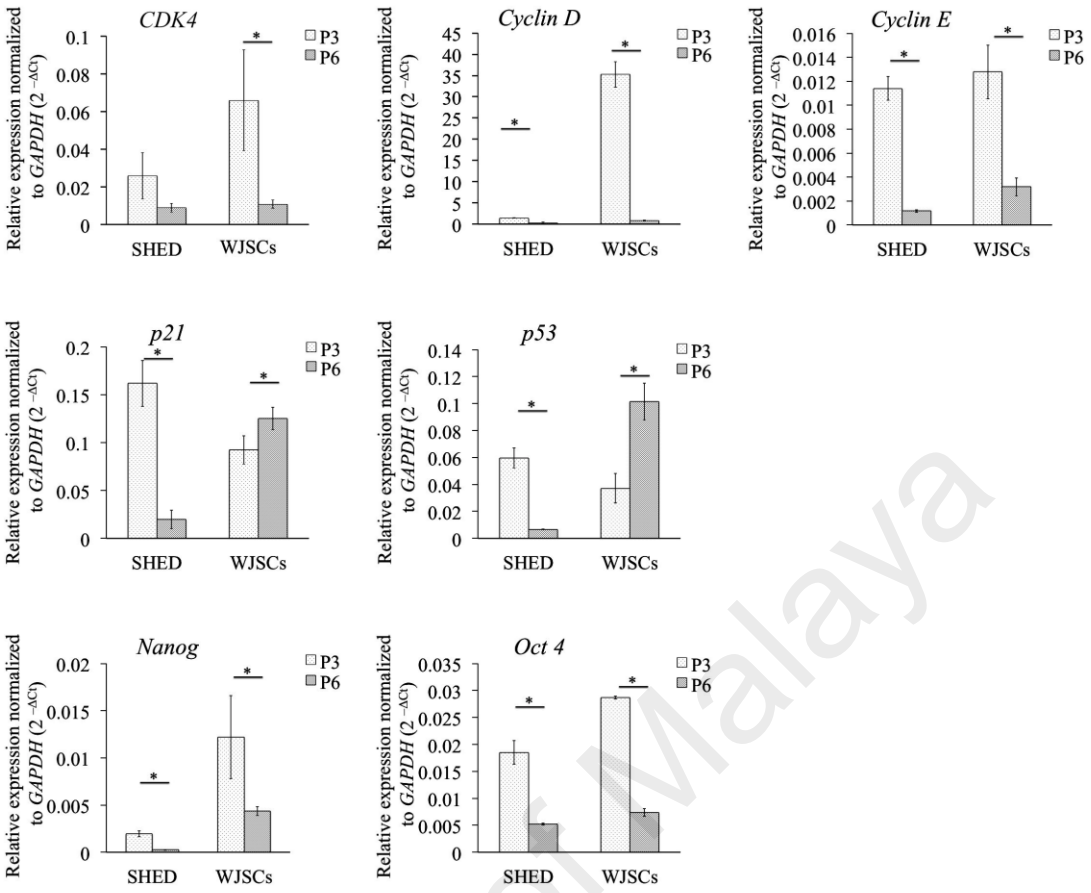


Figure 3.9. Changes in gene expression for *in vitro* SHED and WJSCs culture
qPCR assay using RNA extracted from SHED and WJSCs at P3 and P6 was employed to analyze transcript levels for genes associated with cell cycle (*CDK 4*, *Cyclin D* and *Cyclin E*), stress markers *(p21* and *p53*) and pluripotency markers (*Nanog* and *Oct4*). Data represent mean ± standard deviation of n=3 biological replicates. Same notation * indicate significant difference (*p*>.05).

3.4 Discussion

Recent development in regenerative medicine suggested that MSCs have excellent prospects in cell-based therapies due to their features of self-renewal, pluripotency and differentiation (Tewarie *et al*., 2009; Wang *et al*., 2010; Stuckey and Shah, 2014). They possess great potential in supporting the growing demands of tissues or organs transplantation as tissue donation from patients has its own limitation (Fossett and Khan, 2012). Ideally, MSCs are preferably isolated from patient via procedures with minimal invasion, followed by cell expansion in culture to recover abundance of quality cells before returning to the same patient for cell-based treatment (Heathman *et al*., 2015). The need for *in vitro* expansion of these stem cells has been heightened by the increasing importance of generating enough numbers of cells that are required clinically for cell based therapies purposes. However, it has been extensively reported that *in vitro* passaging of hMSCs often resulted in culture-related changes at the cellular and molecular level (Sethe *et al*., 2006; Wagner *et al*., 2010a; Wagner *et al*., 2010b). Hence, the use of MSCs in clinical applications is under intensive debate as MSCs undergo cellular senescence during passaging and thus limits their expansion and therapeutic applications (Pacini, 2009; Turinetto *et al*., 2016).

In this study, polyclonal populations of hMSCs from deciduous pulp and Wharton's jelly were successfully isolated and expanded for several passages. Concurrently, cellular morphology examination was imposed to determine population characteristics. Morphology alteration which is similar to senescence-like was observed in both SHED and WJSCs culture as passage number increases. Cell count was also conducted in order to assess the proliferation potential of SHED and WJSCs. Interestingly, the growth kinetic pattern between SHED and WJSCs cultures were found nearly identical. The cell count analysis showed that the SHED and WJSCs culture reduced in passage-

dependent manner and there was approximately 50% reduction in cell number yield from P3 to P6. These results in agreement with many other studies including by Jin *et al*., (2013) who reported a decline in the population doubling of MSCs after P2 or P3 and evidences of cellular senescence stage at P6.

The ageing process in cultured MSCs has been extensively described in previous studies, where cells expanded for a limited number of passages before entering cellular senescence stage and ultimately termination in proliferation (Hayflick, 1965; von Zglinicki and Martin-Ruiz, 2005; Wagner *et al*., 2008). These "Hayflick limit" phenomenon of *in vitro* MSCs culture is commonly reported in the cPDL (Koch *et al*., 2013; Brown *et al*., 2014). In this study, both SHED and WJSCs showed approximately of 20 cPDL during 24 days of six passages culture with reseeded cell density of 5000 cell/cm^2. The value was half of the *in vitro* lifespan of hMSCs derived from bone marrow as reported by Koch *et al*., (2013), in which were capable to expand to 35 cPDL during 80 days of culture until they entered growth arrest. This result indicated that the *in vitro* growth capacity of hMSCs might vary among different tissue origin although the culture conditions are very similar (Wegmeyer *et al*., 2013; Brown *et al*., 2014). Overall, the significant changes in morphology and growth kinetics comparing cultures showed signs of SHED and WJSCs entering early stage of cellular senescence after P3. Therefore we designated cell cultures that undergo rapid cell division as early passages, whereas those reduced proliferation as later passages.

There is increasing evidence that the self-renewal and differentiation capability of adult stem cell declines with age (Stenderup et al., 2003; Stolzing et al., 2008). One of the contribution factors is telomere length; as it progressively decreases with advanced age (Sahin & DePinho, 2010). Telomeres are essential to maintain the genomes stability during cell division, and therefore decrease in telomere length can lead to accumulation

of DNA damage (Maser & DePinho, 2004). In this regard, neonatal MSCs such as WJSCs with shorter prenatal lifespan should have retained their telomeres at a longer length compared to adult stem cells. Thus, adult stem cells are more susceptible to undergo senescence in comparison to neonatal MSCs. In contrast to previous reports, we do not observe a similar proliferation trend in SHED and WJSCs. Our results showed that SHED have higher proliferation rate than WJSCs, which implicates that decrease of proliferation capacity of both cell types may not be influenced by age. These results have prompted further investigation in regards to the impact of in vitro passaging on early stage cellular senescence at P6 in reference to P3 of SHED and WJSCs.

Next, the MSCs surface antigen expression analysis and trilineage differentiation was conducted to investigate the identity of SHED and WJSCs. From the flow cytometer analysis, cultures at P3 and P6 possessed MSCs marker combinations CD73, CD90, CD105 and low level express of hematopoietic markers CD14, CD20, CD34 as well as CD45. On the other hand, both samples were capable of differentiating into adipocytes, chondrocytes and osteocytes at P3 and P6 respectively. Adipogenic potential seems to be enhanced in WJSCs as compared to SHED, which require a further study to quantify their potential. The overall results suggested that despite the loss in propagation capacity during *in vitro* passaging, the MSC cell surface marker phenotypes of SHED and WJSCs were retained at a later stage of cultivation. Collectively, the above studies demonstrated that the SHED and WJSCs isolated from primary sources met the minimal criterion to define MSC as recommended by ISCT including adherence to plastic, expression of MSC-related surface antigen and capacity for trilineage mesenchymal differentiation. Hence, with great anticipation these primary SHED and WJSCs would have the potential in regenerative research.

Recent studies suggested that the cellular ageing or senescence is dynamically controlled by sophisticated signaling network (Chen *et al*., 2007; Chen and Daley, 2008; Oh *et al*., 2014; Pole *et al*., 2016); particularly, the cell cycle mechanism that associated with the maintenance of rapid proliferation and differentiation of the stem cells (Pauklin and Vallier, 2013). It has been reported that the stem cells have a distinctively shorter cell cycle period compared to somatic cells due to shortening G1 phase (Neganova and Lako, 2008; Coronada *et al*., 2013; Ahuja *et al*., 2016). The formation of cyclin and cyclin-dependent kinases (CDKs) complexes in the G1 phase is thus considered as a key molecule that modulates the progression of cell cycle (Becker *et al*., 2006; Li and Kirschner, 2014). They phosphorylate retinoblastoma susceptibility protein (Rb) to dissociate transcriptional factor E2F from E2F/DP1/Rb complex, allowing E2F to initiate the G1 /S phase transition (Helin *et al*., 1993; Bertoli *et al*., 2013). In this study, the expression of *cyclin D, cyclin E* and *CDK 4* were found significantly decreased in P6 in comparison with P3 coincides with decreased proliferation, which is consistent with the notion that reduction in the expression of cell cycle associated genes would lead to cell division arrest.

An underlying hallmark of cell senescence is cell cease to divide, which is directly associated with accumulation of DNA damage in the nucleus. This lead to the activation of DNA damage responses such as the checkpoints in cell cycle to allow the damaged cell to conduct necessary DNA restoration (Jain *et al*., 2012). It has been reported that p53, tumour suppressor / guardian of genome is one of the crucial regulator produced upon DNA damage, cellular senescence or stress (Solozobova and Blattner, 2011). As a result of the p53 activation in damaged cells, other down-stream genes such as *p21*, CDK inhibitor, as well as post-transcriptional inhibitor (microRNA) will be triggered to inhibit the CDKs at G1/S transition and hold the cell cycle progression (Liu and Lozano, 2005; Chang *et al*., 2007). In reference to this study, the elevated level of *p53*

and *p21* expression was analysed in WJSCs culture at passage 6 but this phenomenon was not detected in SHED. This may imply that the WJSCs could possess the ability to recover from senescence via the DNA damage repair or an alternative pathway(s) may be triggered in comparison to SHED. However, this hypothesis need to be further investigated.

It is well-reported that the Oct4, Nanog and Sox2 are the key transcriptional factors that form regulation networks to activate genes responsible to maintain stem cells in undifferentiated stage and also to suppress development-related genes (Niwa, 2007; Hammachi *et al*., 2012; Wang *et al*., 2012). Generation of induced pluripotent stem cells (iPSCs) that mimic the expression mode of the endogenous gene in embryonic stem cells (ESCs) was once achieved by introducing these factors directly into the somatic cells (Takahashi and Yamanaka, 2006). Recently, Radzisheuskaya *et al*., (2013) reported that suppression or knockdown of pluripotent-associated genes could promote the exits of stem cells from undifferentiated stage and progress to differentiation program. Here, in this study showed that the expression of pluripotent related genes of both SHED and WJSCs were lowered during *in vitro* passaging. It is speculated that the ageing of hMSCs may accompany by the lowering in stemness network. It has been reported that reprogramming efficiency of iPSCs generation was affected by the activation of p53 (Hong *et al*., 2009; Kawamura *et al*., 2009). However, the mechanisms underlying this phenomenon remain to be explored.

In summary, this study demonstrated polyclonal/ heterogeneous populations of SHED and WJSCs culture from primary source undergo ageing-like phenotypye alteration caused by *in vitro* passaging as early as after p3 with approximately 50% cell number reduction at P6. It was hypothesized that both intrinsic signal and extrinsic factors from microenvironment governed the process of *in vitro* ageing in stem cells. In this study,

bFGF was used as cell culture supplement to promote cell proliferation. We speculated that rapid cell proliferation with deficiency of DNA repair might accelerate the accumulation of DNA damage may lead to replication senescence. Our observations are in line with previous studies showing cells are likely to accumulate DNA damage as they continuously re-populate (Mandal *et al*., 2011; Gu *et al*., 2016; Simara *et al*., 2017). Cells activate senescence program as a naturally occurring event in order to protect themselves from genetic defects in the next generations (Solozobavo and Blattner, 2014). One of the possible reasons may due to the gene expression level associated in cell cycle events such as DNA damage repair, mitosis and DNA replication are declined at later passages (Wagner *et al*., 2010a; Wagner *et al*., 2010b). Consequently, these errors lead to DNA damage and mutation.

It is important to recognize that the population heterogeneity of hMSCs isolated from primary source in this study may be one of the contributing factors to the *in vitro* ageing. Study by Muraglia *et al*., (2000) reported that bone marrow derived MSCs for cell-based therapies produced inconsistent results which was attributed to the population heterogeneity in the primary cultures. Cells at dissimilar stages of maturation commonly seen in culture, could hamper the research and clinical application (Whitfield *et al*., 2013). Heterogeneity can be observed within the clonal population of MSCs upon further expansion, where mixture of fibroblast-like and larger, flatten epithelial-like morphology within the MSCs culture. Interestlingly, few studies have described that the small, fibroblast-like cells as high proliferative MSCs, whereas flat and large cell as low proliferative and mature committed MSCs (Colter et al., 2001; Katsube et al, 2008). Therefore, cell sorting has been recommended as an essential step for the hMSCs isolated from primary source prior to research and clinical application in order to obtain homogeneity cell population. Recent study provides evidence that bone marrow derived hMSCs with average cell size of 17 to 21μm shows significantly higher

proliferation rate and chondrocytes differentiation capacity in comparison to cells with average cell size of 23 to 25 μm (Yin *et al*., 2018). Also, these larger size hMSCs expresses higher level of senescence-associated β-galactosidase. While the literature indicated the advantages of obtaining homogeneity, however at this stage of our study we aim to observe the behavior of cells at different passages. Futher work is required to ascertain if there are differences between hetergenous and homogenous cell population.

Collectively, these distinct phenotypic changes in SHED and WJSCs during passaging have provide an important evidence of cells entering lower proliferative state reflected by the deregulation of cell cycle progression and arrest associated markers after P3. In this regards, it was designated that SHED and WJSCs cultures with high-quality stemness characteristics at P1 to P3 as the early passages, whereas cultures that showed replicative senescence phenotypes at passages after P3 as late passage. Based on the present results, it is suggested that optimum passage numbers of hMSCs culture for research or clinical application should be on early passages. This observation is consistent with the study by von Bahr *et al*., (2012), who demonstrated that patients treated with MSCs generated at early passage (1 to 2) for acute graft-versus-host disease could be correlated to better response and survival compared to passage 3 to 4.

However, many questions remained with regards to the regulation of *in vitro* ageing of MSCs. Hence, further research would be necessary in order to unravel the molecular mechanisms underlying this occurrence, and thus provide us an in-depth understanding to develop a better strategy to expand and enhance the qualities of hMSCs produced for research and related regenerative medicine. This remains as a key step towards the goal of utilizing hMSCs obtained from various primary sources for cell-based therapies for future therapeutic benefits.

3.5 Conclusion

1. hMSCs were successfully isolated and expanded *in vitro* from deciduous dental pulp and Wharton's jelly obtained from primary tissue source.
2. Isolated SHED and WJSCs met the minimal criterion for MSCs defined by ISCT including adherence to plastic, expression of MSCs related surface antigen and capacity for trilineage mesenchymal differentiation.
3. SHED and WJSCs culture showed ageing associated phenotype during *in vitro* passaging including morphology alteration and reduced in growth kinetics as passage number increases.
4. Mesenchymal stem cell phenotypes and trilineage differentiation capacity of SHED and WJSCs were retained at different stage of cultivation namely, passage 3 and passage 6.
5. hMSCs from passage 6 showed significantly higher ($p< 0.05$) senescence markers and lower ($p< 0.05$) cell cycle and stemness associated genes compared to those from passage 3 for both SHED and WJSCs.

CHAPTER 4: COMPARATIVE MICRORNA EXPRESSION OF MESENCHYMAL STROMAL CELLS ISOLATED FROM THE PULP OF EXTRACTED DECIDUOUS TEETH AND WHARTON'S JELLY DURING *IN VITRO* PASSAGING

4.1 Introduction

The key mechanism that governs stem cell phenotype and function has proven to be predominantly associated with the molecular regulatory network and gene expression (Song *et al*., 2006; Bieberich and Wang, 2013; Dalton, 2013). Therefore, alteration in the gene expression dictates cellular activities as well as cell fate specification in stem cells. Evidence from previous studies showed that the unique feature of microRNAs (miRNAs) can impact the translational machinery of messenger ribonucleic acid (mRNA) via degradation of their mRNA target or reduction in the stability of mRNA, thereby contributing to differential or modulated gene expression (Maroney *et al*., 2006; Thomson *et al*., 2006; Filipowicz *et al*., 2008; Heinrich and Dimmeler, 2012; Zhao *et al*., 2014; Ba *et al*., 2016).

It is important to note that a single miRNA can have hundreds of different mRNA targets (Lewis *et al*., 2005; Friedman *et al*., 2009), in which they can be bound to the 3' un-translated regions (3'UTR) of cognate mRNAs, resulting in either the degradation of the target mRNA and resulting in inhibition of protein translation (Bartel *et al*., 2004, Lee *et al*., 2007). This suggests that deregulation of some miRNAs could lead to aberrant changes in cellular processes regulated by their target mRNAs/proteins. For instance, up-regulation of miR-34a changes the mRNA and proteins expression of cyclin-dependent kinase (CDK) 4, *CDK6*, MYC proto-oncogene (c-Myc) and E2F those plays a critical role in cell cycle (Kaller *et al*., 2011; Marzi *et al*., 2012) as well as the anti-apoptotic gene sirtuni 1 (SIRT1) (Hermeking, 2010; Zhang *et al*., 2015).

Hence, over the past decades, much progress on understanding of molecular mechanism underlying development, disease, establishment and maintenance of cellular homeostasis was made through studies focusing on miRNAs expression and regulation (Ason *et al*., 2006; Liu and Olson, 2010; Pritchard *et al*., 2012; Danger *et al*., 2014; Berrien-Elliott *et al*., 2016; Hoye *et al*., 2017). Not only in the above mentioned fields, miRNAs expression analysis is indeed another important means of understanding the regulation of different stem cell fates or phenotypes as miRNA alter the expression of their target genes thereby regulation signaling pathways that affect their stemness homeostasis. For example, miR-145 has been shown to regulate the expression of Oct4, Sox2, Klf4 and c-Myc, whereby up-regulation of miR-145 resulting in the impairment of self-renewal and reprogramming capacity in stem cells (Xu *et al*., 2009; Barta *et al*., 2016). The knowledge of miRNAs expression states within stem cells can be used to define benchmark of stem cell fates and phenotypes, however information is still lacking regarding the role of miRNAs of human mesenchyaml stromal cells (hMSCs) during *in vitro* passaging.

In the previous chapter reported that the hMSCs cultures from the pulp of extracted deciduous teeth (SHED) and Wharton's jelly (WJSCs) exhibited significant changes morphology, growth kinetics and gene expression related to cell cycle, stemness as well as stress during *in vitro* passaging. Given the importance of miRNAs in affecting stem cell fate and their therapeutic potential, here in this study, we aim to determine the differential miRNAs expression in SHED and WJSCs during *in vitro* passaging, which may explain the lower regenerative potential of these cells in the later passage in comparison to those from early passage. We hypothesized that the alteration of cellular phenotype in hMSCs during *in vitro* passaging may be associated by deregulation expression of miRNAs. This study highlighted the impact of miRNAs expression in signaling networks and directing cellular phenotype which provides valuable insight

into the functional information towards the differential regulation of miRNAs in the regulatory networks underlying cellular senescence, stress induction in proliferative capacity caused by *in vitro* passaging. This may provide important clues for the findings from previous chapter regarding how the reduction of stemness capacity in SHED and WJSCs occurs during *in vitro* passaging. Thus, these studies would shed light in the way to develop an approach to restore stemness and self-renewal capacity or rejuvenate hMSCs at later passages for research and clinical purposes. The research objectives of this study are as follows;

1. To ascertain the differentially expressed miRNAs in SHED and WJSCs at selected late passage (P6) compared to early passage (P3).
2. To determine the characteristics of differentially expressed miRNA profiles through their functional clustering based on their putative miRNA-targets.
3. To conduct quantitative validation study of selected differentially expressed miRNAs by qPCR analysis.

4.2 Materials and methods

4.2.1 miRNA extraction

miRNAs were isolated from SHED and WJSCs (two biological samples for each) at passage 3 and passage 6 respectively after 96 h of cultivation using *mir*Vana™ miRNA extraction kit (Ambion, USA). First, culture media were discarded and cells were rinsed twice with Dulbecco's phosphate buffer saline without calcium and magnesium. 600 µL of Lysis/Binding solution were added directly into the culture flask to lyse the cells. The lysate was then collected and transferred into a fresh 1.5 mL micro-centrifuge tube and mixed with 1/10 volume (to the total lysate volume) of Homogenate Additive. The mixture was then mixed by inverting the tubes several times followed by incubation on ice for 10 min. Total ribonucleic acid (RNA) was extracted from the lysate by adding a volume of Acid-Phenol:Chloroform (equal to lysate volume) and vortexed for 60 s. The solution was centrifuged for 5 min at 10,000 ×*g* at room temperature to separate the aqueous and organic phase. The desired RNA located at aqueous phase (colourless upper layer) was transferred into a fresh tube and mixed with 1.25 volume (to the aqueous phase volume) of 100% ethanol. Following that, the total RNA was extracted by passing the aqueous phase/ethanol mixture through a filter cartridge with the aid of centrifugal force at 10,000 ×*g* for 15 s. Total RNA that retained in the glass fiber filter was washed once 700 µL of miRNA wash solution 1 once, the twice by 500 µL of wash solution 2/3. After the washing procedure, the filter cartridge was spun at 10,000 ×*g* for 1 min to remove the remaining washing buffer. Finally, the total RNA was eluded out from the filter by 50 µL of preheated (95ºC) nuclease-free water with the aid of centrifugal force at maximum speed for 30 s and stored at -20ºC or colder. The concentration as well as the purity of isolated RNA was measured using spectrophotometer and the expected ratio of $A_{260/280}$ is 1.8 to 2.1

4.2.2 miRNA gene expression analysis

A total of 1 µg of extracted RNA was reverse-transcribed into complementary Taqman® microRNA using Human Megaplex RT Primers pool A and pool B (Applied Biosystems, USA) with Taqman® MicroRNA Reverse Transcription Kit (Applied Biosystems, USA) according to manufacturer's instruction. Complementary deoxyribonucleic acid (cDNA) conversion was performed in 7.5 µL reaction mixtures which consist of reagents as listed in Table 4.1. Reaction mixtures were incubated on an Applied Biosystems Veriti Thermal Cycler (Applied Biosystems, USA) and the following thermal-cycling conditions were performed: 40 cycles at 16ºC for 2 min, 42ºC for 1 min and 50ºC for 1 min, followed by 85ºC for 5 min.

Table 4.1. Components of reverse transcription reaction mixture for Taqman® microRNA array

Components	Volume for one reaction (µL)
Megaplex RT primer A or B (10×)	0.80
dNTPs (100mM)	0.20
MultiScribe Reverse Transcriptase (50 U/µL)	1.50
RT Buffer (10×)	0.80
$MgCl_2$ (25 mM)	0.90
RNase Inhibitor (20 U/µL)	0.10
Nuclease-free water	0.20
Total miRNA (1000 ng in 3 µL)	3.00
Total	7.50

Next, the reverse-transcribed miRNA were then run on TaqMan® MicroRNA Array Cards A and B (Applied Biosystems, USA) respectively which consist a total of 754 human miRNAs sequence-specific primers and probe that aligned with Sanger miRBase v20 (including three endogenous controls and one negative control assay). Approximately, 6 µL of cDNA product was mixed with 450 µL Taqman® Universal PCR Master Mix (no UNG) (Applied Biosystems, USA), and 444 µL of nuclease-free water followed by brief mixing and centrifugation. Next, 100 µL of prepared reagent

was loaded into each port of the array card then proceeded to be centrifuged and sealed. The existence of targets in the sample were amplified using Quantstudio 12K Real Time PCR (Applied Biosystems, USA) which was performed with Taqman® low density array default thermal cycling conditions as followed: 45ºC for 10 min, 94ºC for 10 min and 40 cycles at 94ºC for 30 s followed by 60ºC for 1 min.

4.2.3 Data analysis

Figure 4.1 illustrates the work flow for the analysis of miRNA profiling data. In brief, the cycle threshold (C_T) values that were generated from qPCR were employed to determine relative expression levels of target miRNA by using $2^{-\Delta\Delta CT}$ algorithm, where RNU48 was the designated endogenous control. In order identify the putative predicted miRNA-targets, miRNA expression levels with 2 fold up-regulation and 2 fold down-regulation were used as inputs and analysed using the ingenuity pathway analysis (IPA) software. The predicted miRNA-mRNA relationship was based on the experimentally validated interactions from TargetScan, TarBase and miRecords database. Subsequently, the predicted miRNA-targets were subjected to gene ontology analysis to identify its related molecular functions, network function and canonical pathway via miRNA-target filter based on the interested biological criteria. Right-tailed Fisher's exact test was used to calculate a p-value determining the significant of the association between the genes in the dataset and the biological function or pathway in the Ingenuity Knowledge Base. The p-value was computed in IPA to determine the probability that the association between the genes in the observed values and the biological function is explained by chance alone using right-tailed Fisher's exact test.

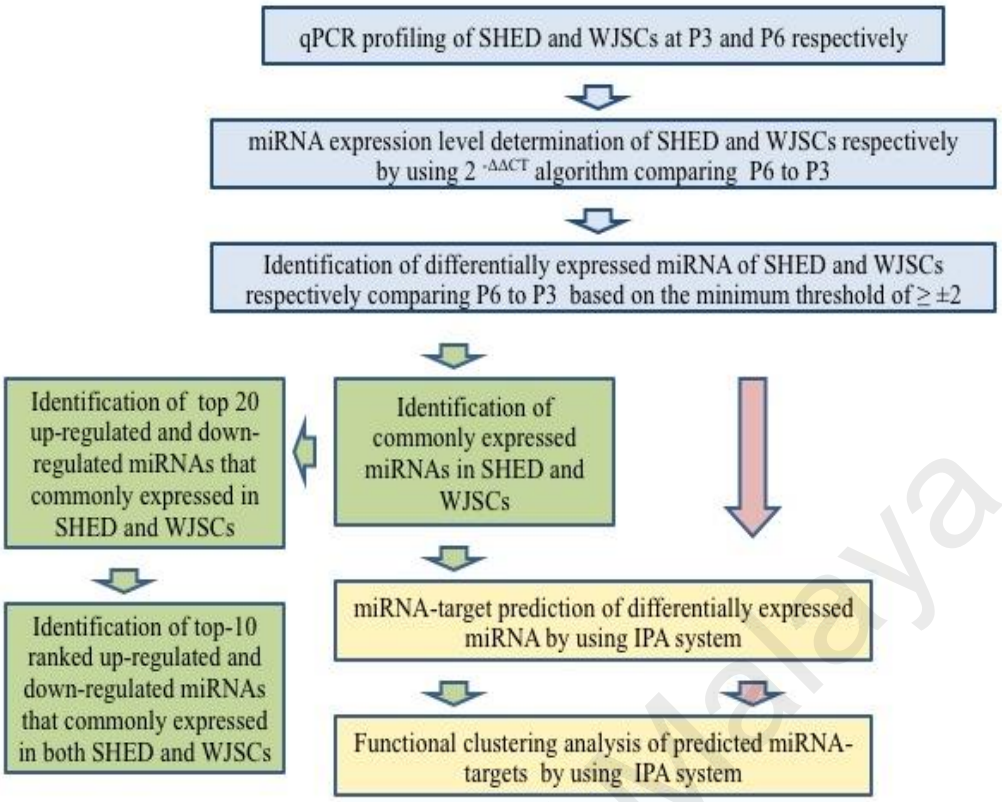


Figure 4.1. Flowchart for the analysis of miRNA profiling data
SHED, hMSCs from the pulp of extracted deciduous teeth; WJSCs, hMSCs from Wharton's jelly; IPA, Ingenuity Pathway Analysis. The highlighted boxes in blue represent procedure to determine relative expression levels of miRNAs, green represent procedure to identify commonly expressed miRNAs between SHED and WJSCs, yellow represent procedure of IPA.

4.2.4 miRNA validation by qPCR analysis

After initial screening, the differential expression of six selected miRNAs was quantitatively validated using qPCR technique. These miRNAs were selected based on features observed previously in experiment; 1) commonly expressed in both SHED and WJSCs; 2) fall into the top 10 ranked differentially expressed; 3) target to genes associated with the cell cycle, proliferation or stem cells' stemness in referring to published data as mentioned in the earlier discussion. First, the extracted miRNA from SHED and WJSCs at P3 and P6 (three biological samples for each) were reverse transcribed into cDNA using small RNA-specific reverse transcription (RT) primer

(hsa-miR-22-000398, hsa-let-7a-000377, hsa-miR-485-5p-001036, hsa-miR-508-5p-002092, hsa-miR-520e-001119, hsa-miR-373-000561, and RNU48-001006) with TaqMan® MicroRNA Reverse Transcription System according to manufacturer's instruction. The cDNA conversion was performed in 15 µL reaction mixtures which consist of reagents as listed in Table 4.2. Reaction mixtures were incubated on an Applied Biosystems Veriti Thermal Cycler and the following thermal-cycling conditions were performed: 16ºC for 30 min, 42ºC for 30 min followed by 85ºC for 5 min.

Quantification of miRNA expression with cycle threshold (C_T) of 35 or less was accessed by DeltaDeltaC_T ($\Delta\Delta C_T$) data analysis method. C_T value of miRNAs of interest (MOI) was normalized by the C_T of RNU48 (as house keeping gene-HKG): $2^{-\Delta CT} = 2^{-[CT(MOI) - CT(HKG)]}$. Furthermore, the fold-change of gene of interest were calculated as $2^{-(\Delta\Delta CT)}$ in which the normalized expression of the MOI in the experimental sample (P6) is divided by the normalized expression of the same MOI in the calibrator sample (P3): $2^{-\Delta\Delta CT} = 2^{-[\Delta CT\ (Experiment) - \Delta CT(Control)]}$.

Table 4.2. Components of reverse transcription reaction mixture for Taqman® microRNA single assay

Components	Volume for one reaction (µL)
Small RNA-specific RT primer (5×)	3.00
dNTPs (100mM)	0.15
MultiScribe Reverse Transcriptase (50U/µL)	1.00
RT Buffer (10×)	1.50
RNase Inhibitor (20 U/µL)	0.19
Nuclease-free water	4.16
Total miRNA(10 ng in 5 µL)	5.00
Total	7.00

Next, qPCR were performed in a 20 µL reaction mixture which consist of 1.33 µL of reverse-transcribed product, 10 µL of 2× TaqMan® Universal PCR Master Mix (no

UNG), 1µL of 20× TaqMan® small RNA assay (Applied Biosystems, USA), and 7.67 µL of nuclease-free water. The existence of targets in the sample were amplified using 7500 Fast Real-Time PCR system (Applied Biosystems, USA) which was performed with Taqman® low density array default thermal cycling conditions as followed: 95ºC for 10 min and 40 cycles at 94ºC for 15 s followed by 60ºC for 1 min. The cycle threshold (C_T) values that generated from qPCR were employed to determine relative expression levels of target miRNA by using $2^{-\Delta\Delta CT}$ algorithm, where RNU48 was the designated endogenous control.

4.3 Results

4.3.1 Differential expression of miRNAs in SHED and WJSCs at P6 compared to P3

In order to determine the differentially expressed miRNAs during *in vitro* passaging, miRNA expression profiles of SHED and WJSCs at P6 were assessed in comparison to P3 using Taqman® array miRNA cards (coverage a total of 768 miRNAs including twelve housekeeping miRNAs). Both samples exhibited different miRNA expression, in which more miRNAs were detected at P6 in WJSCs compared to SHED and vice versa have been observed at P3. Counts of detected miRNAs were tabulated in Table 4.3.

Table 4.3. Counts of detected miRNAs in SHED and WJSCs at P6 and P3 respectively

Passage	Total number of detected miRNA	
	SHED	WJSCs
6	295	336
3	441	280

4.3.2 Analysis of miRNAs expression profiles of SHED and WJSCs at P6 in compared to P3

Next, analysis of relative expression level of miRNAs was performed using $2^{-\Delta\Delta CT}$ algorithm normalization to identify differentially expressed miRNAs comparing cells at P6 to P3. The performed analysis was tabulated in Table 4.4. Out of 756 examined miRNAs only 7.53% and 29.23% of the miRNAs in SHED and WJSCs respectively were up-regulated by ≥ 2 fold change. In contrast, 28.57% and 10.71% of miRNAs were down-regulated by ≥ 2 fold change in SHED and WJSCs respectively. The list of

differentially expressed miRNAs in both SHED and WJSCs can be found in Appendix A and B respectively.

Table 4.4. Differentially expressed miRNAs in P6 compared to P3 of SHED and WJSCs

Level of miRNA expression in P6 compared to P3	Number of differentially expressed miRNA over the total of 756 miRNAs	
	SHED	WJSCs
Up-regulation	57	221
Down-regulation	216	81

Using the normalized data, list of the top 20 up-regulated and down-regulated miRNAs in SHED and WJSCs at P6 relative to P3 were identified and compared (Figure 4.2). Among the up-regulated miRNAs, miR-22 and miR-194-3p were found to be at the highest in SHED and WJSCs respectively with more than 7 $\log_2$(fold change). In contrary, miR-1243 and miR-146b-3p were found most down-regulated miRNAs in both SHED and WJSCs respectively with more than -15 $\log_2$(fold change).

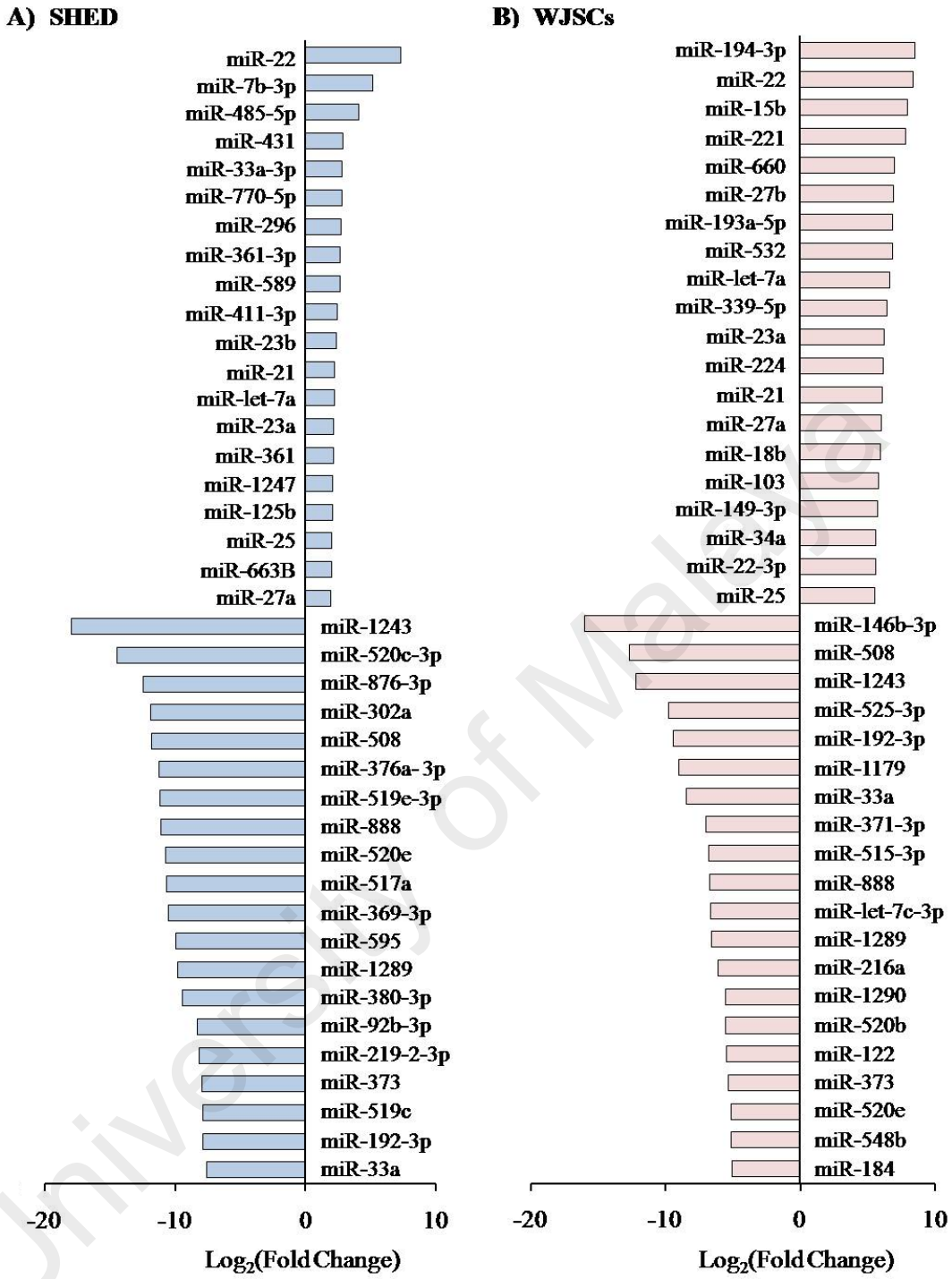


Figure 4.2. Top 20 differentially up-regulated and down-regulated miRNAs in SHED and WJSCs at P6 relative to P3

Data were expressed in $\log_2$(fold change), bars represent average reading from two miRNA arrays of two biological replicates (n=2) for each SHED (blue) and WJSCs (red).

In addition, a total of 49 miRNAs were commonly up-regulated, meanwhile 59 that were down-regulated in both SHED and WJSCs (Figure 4.3). Of the common miRNAs, the top 20 up-regulated and down-regulated miRNAs profiles in SHED and WJSCs were presented in Figure 4.4. Subsequently, the identified top 20 commonly up-regulated and down-regulated miRNAs in both SHED and WJSCs were ranked according to their average of the differential expression level as shown in Figure 4.5.

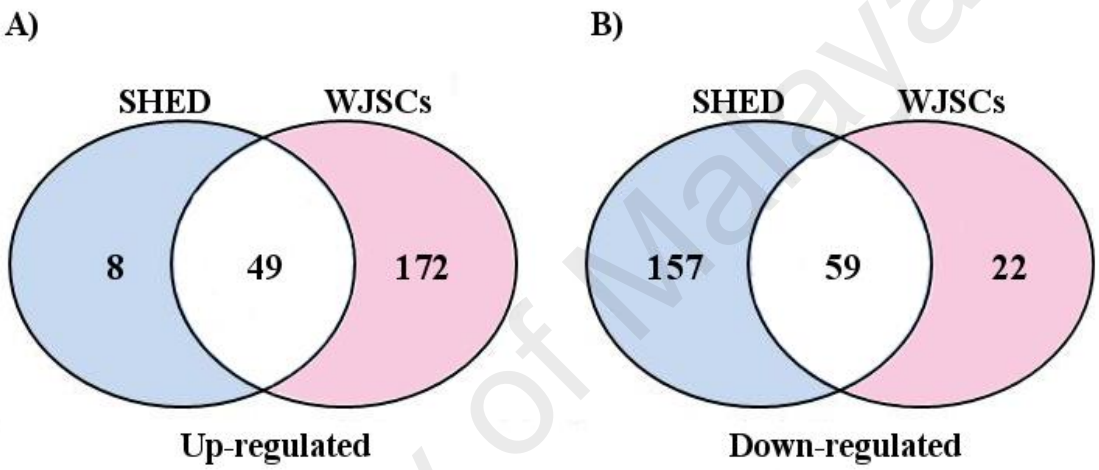


Figure 4.3. Comparison of differentially expressed miRNAs between SHED (blue) and WJSCs (red) at P6 compared to P3
Venn diagrams show number of up-regulated miRNAs (A), down-regulated (B), and the common between SHED and WJSCs (white).

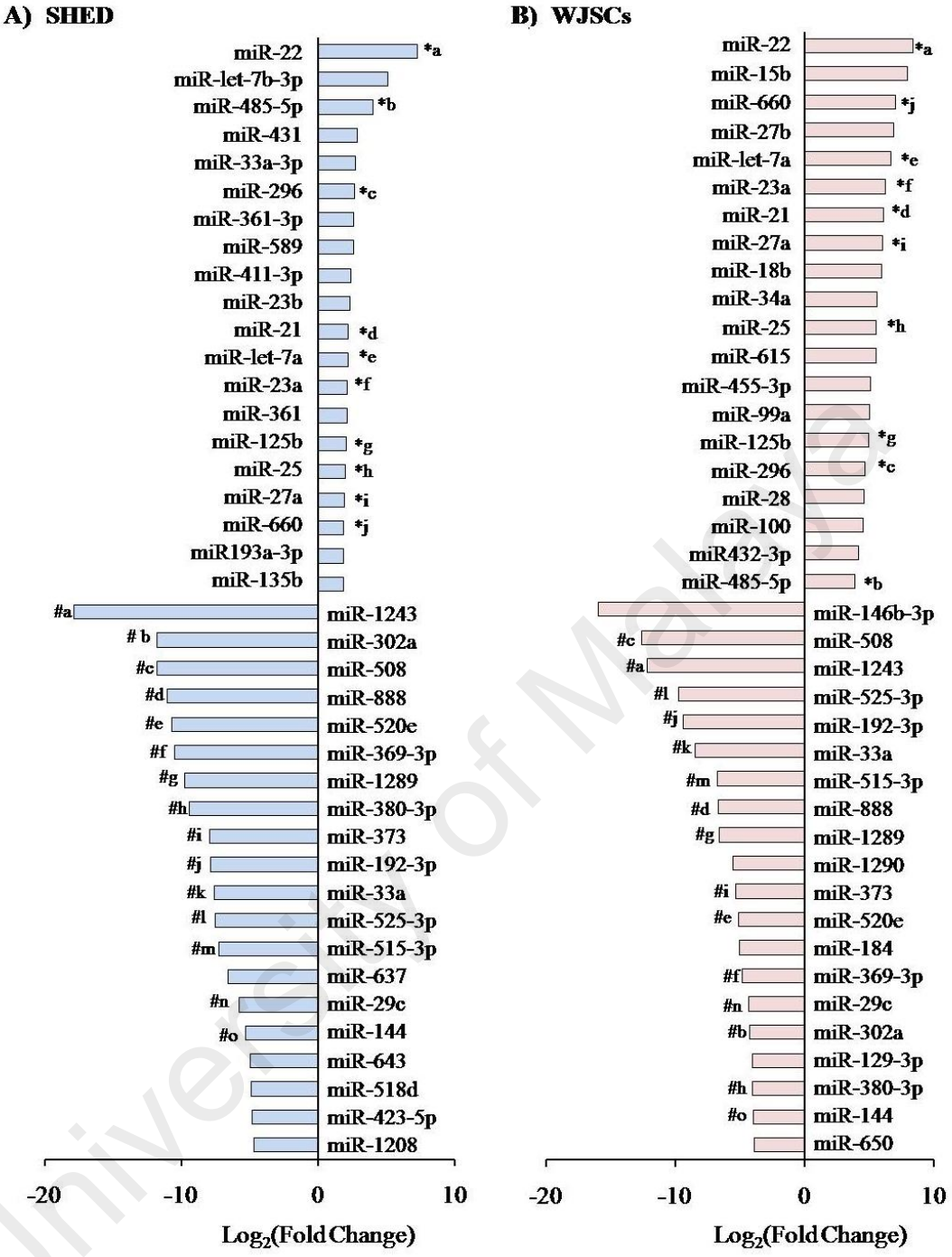


Figure 4.4. The top 20 up-regulated and down-regulated miRNAs profiles that commonly expressed in SHED (A) and WJSCs (B)

Data were expressed in $\log_2$(fold change), bars represent average datasets from two miRNA arrays of two biological replicates (n=2) for each cell types, bars that designated with same symbol and letter indicates common miRNAs that expressed between SHED and WJSCs which is fall within the top 20.

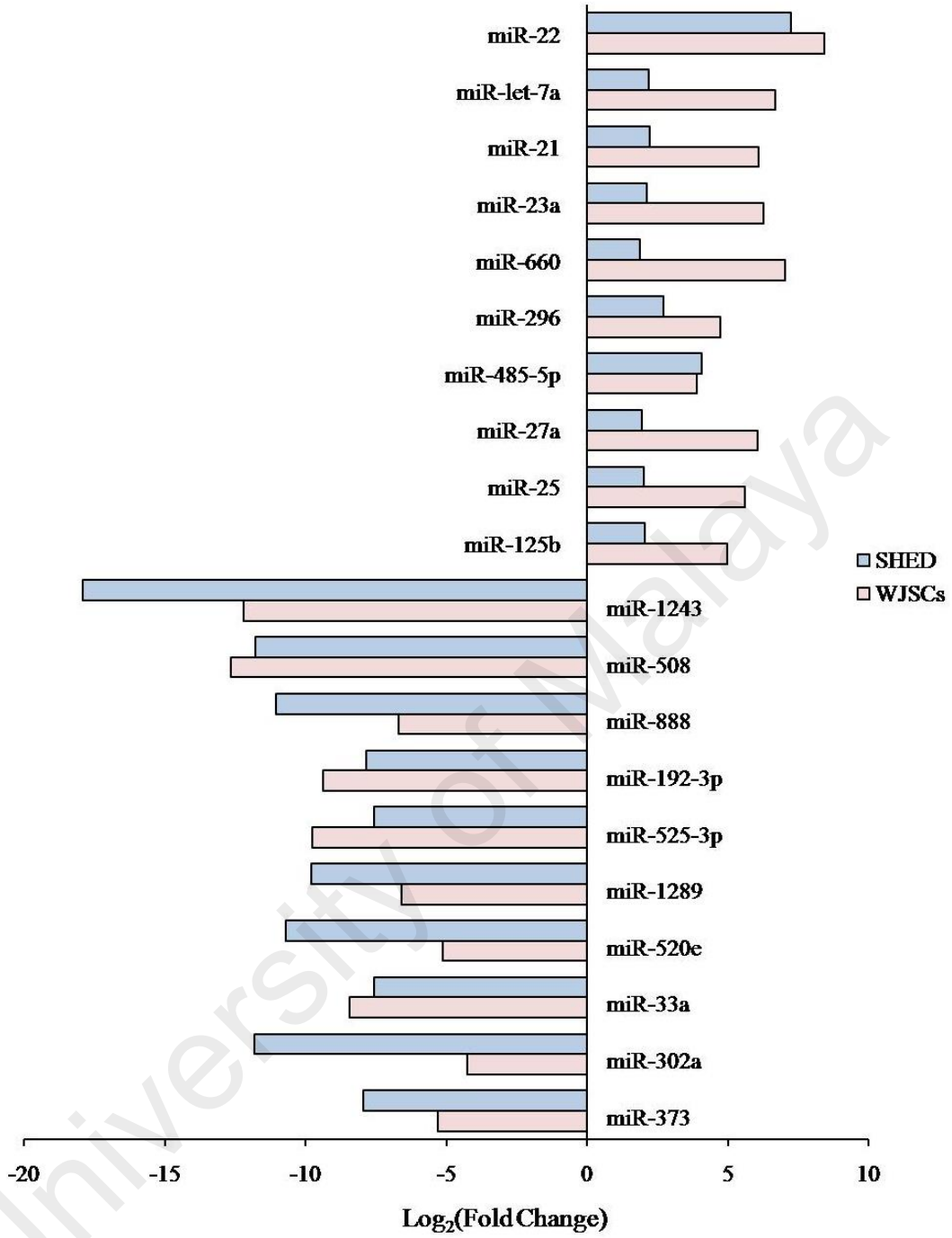


Figure 4.5. The top 10 ranked up-regulated and down-regulated miRNAs profiles that commonly expressed in both SHED and WJSCs

Data were expressed in $\log_2$(fold change) of two miRNA arrays datasets of two biological replicates (n=2) for each cell types.

4.3.3 Functional clustering analysis of differentially expressed miRNA in SHED and WJSCs

In order to determine the biological functions that were impinged by the differentially expressed miRNAs in SHED and WJSCs, putative miRNA-target, were first predicted using ingenuity pathway analysis (IPA) system. Only those miRNAs whose expression level with cut of value of $p< 0.05$ with fold change ≥ 2 of either up-regulation and down-regulation were subjected for miRNA-target prediction analysis via "microRNA Target filter" based on the six selected signaling pathway categories including i) apoptosis, ii) cell cycle, iii) growth and proliferation, iv) stress and injury, v) growth factors as well as vi) transcriptional factor. IPA analysis identified a total of 21644 and 25013 miRNA-target genes in SHED and WJSCs respectively.

These putative predicted miRNA-targets were then subjected for cellular function analysis by using IPA system and the top ten ranked cellular functions were tabulated in Table 4.5. Interestingly, those predicted miRNA-targets in both cell types were grouped similarly in terms of cellular functions despite insignificant difference in their ranking. The analysis showed that the predicted genes were clustered into cellular function including cell proliferation, cell death, necrosis, cell injury, apoptosis and others. This implicate that the differentially expressed miRNA during *in vitro* passaging have great impact in deregulation of genes that associated with cellular growth, survival and cell death.

Table 4.5. Top 10 cellular functions of putative predicted miRNA-targets on six selected signaling pathway categories generated from IPA software

SHED			Rank	WJSCs		
Categories	Cellular functions	p-value, # Target		p-value, # Target	Cellular functions	Categories
Cellular Growth and Proliferation	Cellular proliferation	2.21×10^{-255}, 1027	1	3.63×10^{-255}, 1033	Cellular proliferation	Cellular Growth and Proliferation
Cell Death and Survival	Cell death	2.57×10^{-252}, 960	2	2.60×10^{-247}, 959	Cell death	Cell Death and Survival
Cell Death and Survival	Necrosis	1.04×10^{-251}, 902	3	1.05×10^{-247}, 902	Necrosis	Cell Death and Survival
Cell Death and Survival	Apoptosis	4.20×10^{-248}, 814	4	2.69×10^{-245}, 815	Apoptosis	Cell Death and Survival
Cell Death and Survival	Cell death of tumor cells	1.27×10^{-213}, 763	5	1.02×10^{-224}, 1284	Cancer	Cell Injury and Abnormalities
Cell Injury and Abnormalities	Cancer	1.48×10^{-211}, 1254	6	2.95×10^{-210}, 777	Cell proliferation of tumor cell lines	Cellular Development,
Cellular Development	Cell proliferation of tumor cell lines	1.34×10^{-207}, 769	7	7.23×10^{-209}, 761	Cell death of tumor cell lines	Cell Death and Survival
Cell Death and Survival	Apoptosis of tumor cell lines	1.58×10^{-202}, 652	8	6.19×10^{-203}, 656	Apoptosis of tumor cell lines	Cell Death and Survival
Cell Injury and Abnormalities	Malignant solid tumor	1.43×10^{-184}, 1154	9	1.11×10^{-195}, 1181	Malignant solid tumor	Cell Injury and Abnormalities
Cell Death and Survival	Cell survival	5.58×10^{-170}, 546	10	3.08×10^{-166}, 544	Cell survival	Cell Death and Survival

p-value = probability that the association between the genes in the observed values and the biological function is explained by chance alone using right-tailed Fisher's exact test.

This finding was further examined by canonical pathway analysis. The canonical pathways were grouped similarly in between SHED and WJSCs despite insignificant difference in their ranking (Appendix C and D). The IPA identified canonical pathway and regulatory network including integrin, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), phosphatase and tensin homolog (PTEN), high mobility group box 1 (HMGB1), mechanistic target of rapamycin (mTOR), 5' adenosine monophosphate-activated protein kinase (AMPK) and other signaling pathways as the candidate of the putatively predicted miRNA-targets in both cell types (Table 4.6).

Table 4.6. Selected canonical pathways of putative predicted miRNA-targets that common between SHED and WJSCs

Functional categories	Selected canonical pathway
Apoptosis	14-3-3-mediated signaling
	NF-κB signaling
	PTEN signaling
Cell cycle	Integrin signaling
	CXCR4 signaling
	ERK/MAPK signaling
Growth and proliferation	AMPK signaling
	mTOR signaling
Stress and injury	NRF2-mediated oxidative stress response
	HMGB1 signaling
	p38 MAPK signaling

To assess the functional roles and pathways that mediated by the miRNAs that are common between SHED and WJSCs during *in vitro* passaging, functional clustering and canonical pathway analysis on the predicted miRNA-targets of commonly expressed miRNAs between SHED and WJSCs were performed. The analysis revealed that a majority of the putative candidate genes have been found to be highly associated with the regulation of cell death, necrosis, apoptosis, cell proliferation as well as cellular movement (Table 4.7). On the other hand, the canonical pathways as depicted in Table 4.8 including integrin, NF-κB, PTEN, mTOR, AMPK, and other signaling pathway suggested to have involved in changing the stem cells competence of SHED and WJSCs during *in vitro* passaging.

Table 4.7. Top 10 cellular functions involving putative predicted miRNA-targets of the commonly expressed miRNAs in both SHED and WJSCs on six selected signaling pathway categories generated from IPA software

Categories	Diseases or functions annotation	p-Value, #Target
Cell Death and Survival	cell death	3.53×10^{-263}, 989
Cell Death and Survival	necrosis	3.67×10^{-258}, 925
Cell Death and Survival	apoptosis	6.9×10^{-254}, 830
Cellular Development, Cellular Growth and Proliferation	cell proliferation of tumor cell lines	1.78×10^{-221}, 810
Cell Death and Survival	cell death of tumor cell lines	9.49×10^{-217}, 777
Cancer, Organismal Injury and Abnormalities	cancer	6.77×10^{-212}, 1335
Cell Death and Survival	apoptosis of tumor cell lines	1.95×10^{-208}, 668
Cancer, Organismal Injury and Abnormalities	solid tumor	1.71×10^{-194}, 1316
Cellular Movement	cell movement	7.81×10^{-194}, 663
Cellular Movement	migration of cells	1.27×10^{-183}, 605

p-value = probability that the association between the genes in the observed values and the canonical pathway is explained by chance alone using right-tailed Fisher's exact test

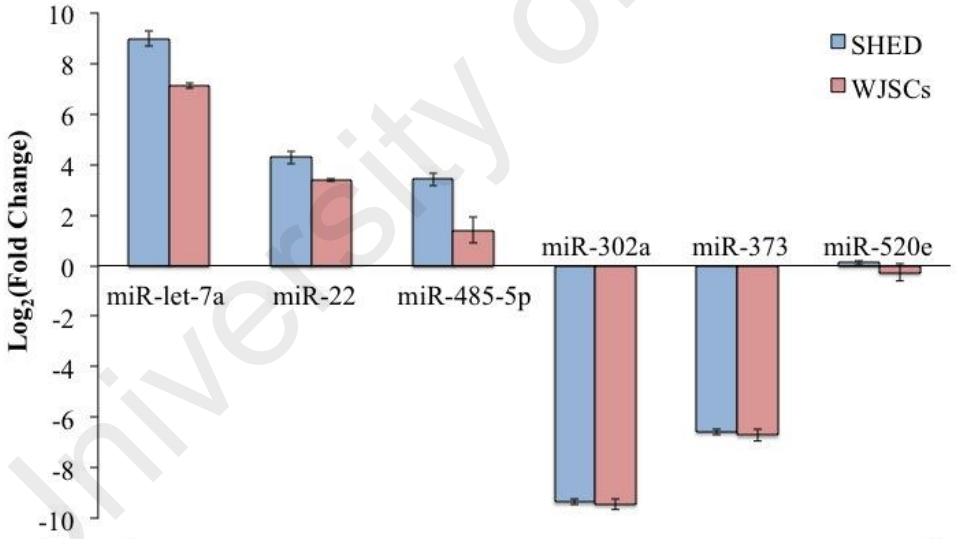
Table 4.8. Top 10 canonical pathways of putative predicted miRNA-targets of the commonly expressed miRNAs in both SHED and WJSCs on six selected signaling pathway categories generated from IPA software

Apoptosis	-log(p-value)	Cell cycle	-log(p-value)	Growth factor	-log(p-value)
Tight Junction Signaling	191	Integrin Signaling	252	Relaxin Signaling	189
Aryl Hydrocarbon Receptor Signaling	152	Tight Junction Signaling	191	Molecular Mechanisms of Cancer	168
NF-κB Signaling	152	Molecular Mechanisms of Cancer	178	HGF Signaling	153
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	149	Aryl Hydrocarbon Receptor Signaling	152	Cardiac Hypertrophy Signaling	152
14-3-3-mediated Signaling	144	Breast Cancer Regulation by Stathmin1	146	Renin-Angiotensin Signaling	150
Molecular Mechanisms of Cancer	137	14-3-3-mediated Signaling	141	Role of NFAT in Cardiac Hypertrophy	141
PTEN Signaling	134	ILK Signaling	133	IGF-1 Signaling	141
Role of NFAT in Cardiac Hypertrophy	132	CXCR4 Signaling	133	NGF Signaling	141
Sertoli Cell-Sertoli Cell Junction Signaling	131	Thrombin Signaling	130	B Cell Receptor Signaling	139
B Cell Receptor Signaling	127	ERK/MAPK Signaling	127	GNRH Signaling	132
Growth and proliferation	**–log(p-value)**	**Stress and injury**	**-log(p-value)**	**Transcriptional factor**	**-log(p-value)**
Molecular Mechanisms of Cancer	226	NRF2-mediated Oxidative Stress Response	189	Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	178
Integrin Signaling	192	EIF2 Signaling	149	Molecular Mechanisms of Cancer	148
Cardiac Hypertrophy Signaling	181	mTOR Signaling	144	Sumoylation Pathway	147
Axonal Guidance Signaling	179	HMGB1 Signaling	138	GlioblastomaMultiforme Signaling	128
ILK Signaling	160	p70S6K Signaling	130	Mouse Embryonic Stem Cell Pluripotency	124
CREB Signaling in Neurons	157	HIF1α Signaling	124	Colorectal Cancer Metastasis Signaling	122
AMPK Signaling	154	Regulation of eIF4 and p70S6K Signaling	124	Human Embryonic Stem Cell Pluripotency	121
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	153	Type II Diabetes Mellitus Signaling	124	Glucocorticoid Receptor Signaling	106
Th1 and Th2 Activation Pathway	149	Glucocorticoid Receptor Signaling	122	STAT3 Pathway	105
Colorectal Cancer Metastasis Signaling	147	p38 MAPK Signaling	122	Regulation of the Epithelial-Mesenchymal Transition Pathway	101

p-value = probability that the association between the genes in the observed values and the canonical pathway is explained by chance alone using right-tailed Fisher's exact test

4.3.4 Validation of selected miRNAs using qPCR analysis

Of the top ten commonly up-regulated and down-regulated miRNAs that identified, three of each category (up-regulated: let-7a, miR-22, and miR-485-5p; down-regulated: miR-302, miR-373, and miR-520e) were selected for quantitative validation by using qPCR. As shown in Figure 4.6, let-7a, miR-22 and miR-485-3p showed up-regulated in SHED and WJSCs at P6 in compared to P3, there were significantly difference was observed between samples. On the other hand, miR-302a and miR-373 showed down-regulated in SHED and WJSCs at P6 in compared to P3, no significant difference was observed between samples. Results of qPCR validation here were alike to what was observed in the array data, however miR-520e had no significant differential expression.



	miRNA					
hMSCs	let-7a	miR-22	miR-485-5p	miR-302a	miR-373	miR-520e
SHED	8.96±0.29	4.28±0.25	3.42±0.26	-9.34±0.10	-6.57±0.12	0.11±0.09
WJSCs	7.11±0.10	3.37±0.04	1.40±0.50	-9.44±0.20	-6.70±0.24	-0.27±0.33

Figure 4.6. Validation of the miRNAs array data using qPCR analyis
Six selected commonly expressed miRNAs in between SHED (red) and WJSCs (blue) were validated using qPCR. The data were normalized to RNU48 and were expressed in Log_2(fold change) comparing P6 to P3, bars represent average from three biological repeats for each cell types.

4.4 Discussion

The key element of maintaining stem cell characteristics is the balanced combination of growth factors and signaling molecules between self-renewal and differentiation (Viatour, 2012; Aponte and Caicedo, 2017). It is important to note that, inactivation of any signaling molecules contribute to the physiological changes of stem cells. miRNA plays a major role in the regulation of translational machinery of mRNA. Several works have been tried to identify the possible targets of miRNA that may explain the broad signaling network in orchestrating the stem cells biological processes (Maroney *et al*., 2006; Thomson *et al*., 2006; Filipowicz *et al*., 2008; Heinrich and Dimmeler, 2012; Zhao *et al*., 2014; Ba *et al*., 2016). It has been reported that deregulation of miRNAs would have a profound impact on the mRNA levels which directly affecting the signaling activities and resulting in cellular phenotype alterations (Thomson *et al*., 2006, Filipowicz *et al*., 2008). Although substantial progress has been made in microRNomics profiling aiming to uncover the role of miRNAs in developmental and stem cells biology (Tang *et al*., 2006; Ren *et al*., 2009; Guo *et al*., 2011; Sharma and Wu, 2013; Meng *et al*., 2016), the exact and further insights into miRNA regulation in stem cell ageing remains to be explored.

As miRNAs regulation is one of the key regulatory networks governing many cellular processes including cellular ageing, here in this study differential miRNA expression and their possible targets correlating with *in vitro* passaging induced ageing like characteristic in SHED and WJSCs were explored. It is speculated that deregulation of miRNAs during *in vitro* passaging induced ageing like characteristics in the later passages. Indeed, this study demonstrated that numerous of miRNAs have showed differentially expressed in P6 compared to P3 of hMSCs from two different tissue sources. Interestingly, various overlapping or commonly differentially expressed miRNAs in between SHED and WJSCs

were also identified. This analysis suggested that both SHED and WJSCs might share the common miRNAs regulatory mechanisms on cellular ageing during *in vitro* passaging.

Of the highly differentially expressed miRNAs identified, miR-22 showed a remarkable increase in its expression from P3 to P6. Indeed, miR-22 is known to regulate multiple cellular processes including cell proliferation, senescence, apoptosis, and epigenetic through their target genes. For example, previous studies have reported that miR-22 is found to be up-regulated in aged cells and exhibit suppressive effect on CDK6 and SIRT1 which are related to cell senescence and proliferation (Xu *et al*., 2011; Jazbutyte *et al*., 2013). Furthermore, over-expression of miR-22 resulting in down-regulation of tumor suppressor protein PTEN in cancer cell line has been noted previously (Bar and Dikstein, 2010).

Furthermore, the miR-22 was been reported to inhibit the expression of epigenetic regulator histone deacetylase 4 (HDAC4), which plays an important role in regulate transcriptional genes expression (Wang *et al*., 2014). Of note, HDAC4 induce deacetylation to chromatin structures whereby promotes chromatin condensation that leads to transcriptional gene repression of many biological processes including senescence and apoptosis. In particular, works by Mottet *et al*., (2009) and Kang *et al*., (2014) clearly showed that inhibition of HDAC4 activities induces expression of p21. These observations are in line with the earlier findings in Chapter 3, which demonstrating the increased expression of *CDK4* and *p21* in WJSCs at P6 in compared to P3.

Additionally, some other miRNAs that confer the inhibition of their target mRNAs which are involved in proliferation related cellular processes were also found to be differentially expressed namely, let-7a and miR-485-5p were shown up-regulated in both cell types.

Evidence from previous studies revealed that growth related genes such as high mobility group AT-hook 2 (HMGA2) (Yu *et al*., 2013; Wu *et al*., 2015) and *c-Myc* (Sampson *et al*., 2007) were direct target of let-7a. Also, study from Lou *et al*., (2016) demonstrated that over-expression of miR-485-5p suppressed tumor growth and proliferation. Overall, data in this study was consistent with the notion that aberration of cell cycle or growth related signaling pathway will affect cell proliferation (Schäuble *et al*., 2012).

The molecular significance of stemness related transcriptional factors in stem cell biology have been well reported (Takahashi and Yamanaka, 2016; Filipczyk *et al*., 2015), for instance, the expression level of stemness factors; organic cation/carnitine transporter 3/4 (Oct3/4), SRY (sex determining region Y)-box 2 (Sox2), Kruppel like factor 4 (Klf4) and c-Myc in embryonic stem cell and induced pluripotent stem cells (iPSCs) play a crucial role in regulating self-renewal and differentiation capability (Takahashi and Yamanaka, 2006; Tiemann *et al*., 2014). Of particular importance from previously reported study on expression and role of miRNA in stem cell biology was showing a comparable observation, in which miR-302 clusters were employed to mediate iPSCs reprogramming (Anokye-Danso *et al*., 2011). This implicated that deregulation of miR-302 has great impacts on genes that associated with stem cell regenerative potential. For instance, evidence from previous report demonstrated that methyl-CpG binding domain protein 2 (MBD2), DNA methylation proteins that repress transcription of Nanog in somatic and partially reprogrammed cells via binding to methylated Nanog promoter region is direct target of mir-302 cluster (Lee *et al*., 2013).

Interestingly, a similar role as miR-302 in related to cell growth arrest has been proposed for miR-520e as they had shared a similar consensus seed sequence: AAGUGC at the 5' end seed region (Ren *et al*., 2009). Similar to this observation, miRNAs including miR-

302a, miR-373 (member of miR-302 family) and miR-520e in this study were showed down-regulated at P6 compared to P3 in both cell types, in which support the notion that reduced expression of miR-302 and miR-520 cluster may lead to reduction in stemness (Ren *et al*., 2009; Anokye-Danso *et al*., 2011; Subramanyam *et al*., 2011), therefore confers lower regenerative potential as evident and discussed in the previous chapter (Chapter 3). Collectively, this study revealed that the importance of miRNAs as regulator in counterbalancing the transcriptional circuitry to support the maintenance of stem cell characteristics.

Furthermore, the performed functional clustering analysis using the putative predicted miRNA-targets generate by IPA system based on bio-informatics prediction algorithms in order to determine their prominently corresponding biological functions and pathways. Most significant biological functions that associated include cell growth, cell proliferation, cell death, cell survival and others. These findings are in agreement with previous studies as described in Chapter 3 showing decline in growth rate as well as deregulation of genes that associated with pluripotency, cell cycle and stress markers in both SHED and WJSCs during *in vitro* passaging. Additionally, this observation was further supported by canonical pathway analysis showed the predicted miRNA-targets were involved in inflammatory (NF-κB), proliferation (PTEN, mTOR), stemness (STAT3, role of Nanog in mammalian embryonic stem cell pluripotency), cell survival (integrin), cellular growth (AMPK), and others, which have been found to play critical role in regulating stem cell homeostasis (Dalton, 2013; Bieberich and Wang, 2013; Takase *et al*., 2013; Ito and Suda, 2014; Qi *et al*., 2015). Results of the current study suggesting that cellular ageing are relatively a cellular progressive deterioration process that involves many biological function pathways. It is worth of note that some molecules that involved in above mention signaling pathways

might converge on the activation of stress related biological processes such as DNA damage, telomeres shortening or oxidation stress which are the hallmarks of cellular senescence. For instance, previous reports indicated that PTEN is vital for the activation of DNA repair mechanism in respond to DNA damage; impairment of PTEN expression compromised the chromosomal integrity (Shen *et al*., 2007; Ming and He, 2012), which may explain the inefficient repair activation and ageing-like phenomena.

Interestingly, inflammatory related regulation NF-κb signaling pathway in the predicted miRNA-targets analysis which has been reported to mediates immune-surveillance and promotes cell senescence or apoptosis in preventing the damaged cell from progression into malignancies (Chien *et al*., 2011). Also, the predicted miRNA-targets were categories associated to 14-3-3 mediated signaling pathway which play a critical role in coordinating progression of cell cycle event to balance between cell survival and apoptotic processes (Hermeking and Benzinger, 2006; Morrison, 2009). Additionally, the predicted miRNA-targets were showed to be associated with IGF-1 and mTOR signaling pathway, which in line with previous finding that cellular ageing have also been demonstrated to be associated with deregulation of regulators of nutrient sensing pathways (Harries *et al*., 2012). Study by Jung *et al*., (2010) reported that the autophagy process is executed when cell under glucose starvation condition through mTOR in order to degrade and recycling its unnecessary or dysfunctional cellular component. The deprivation of ATP and AMP in cell activate AMPK, which in turn inhibits mTORC1 via phosphorylation of TSC2, and then trigger the autophagy process (Inoki *et al*., 2003).

The overall results here explained the different signaling pathway activation at different stage in the lifespan of hMSCs orchestrates the cellular processes controlling their self-renewal features. On top of these, the predicted miRNA-targets of commonly expressed

miRNAs between SHED and WJSCs were also subjected for IPA analysis, whilst cross cell types comparison would allow us to identify specific conserved miRNAs and related biological processes in support cellular ageing. Results here revealed that the biological functions of the commonly expressed miRNAs were more specificity and refined to cell death and survival. However, the same canonical pathways trend was observed in the predicted miRNA-targets of commonly expressed miRNAs in compared to the total differentially expressed miRNAs. Finally, six miRNAs including let-7a, miR-22, miR-485-5p, miR-302, miR-373 and miR-520e were subjected for quantitative validation by qPCR. It was confirmed that expression of miR-22, let-7a and miR-485-5p was up-regulated, whereas miR-302a and miR-373 were reduced at P6 in compared to P3. These results were consistent with the expression patterns of array data in the previous experiments, except miR-520e which shown not to be differentially expressed.

Altogether, these comparative miRNA analyses imply that differentially expressed of miRNAs have great implication in cell proliferation, survival, death, senescence and apoptosis which ultimately lead to cellular ageing. Further investigation is necessary to delineate the role of some key miRNAs identified in this study for their regulatory role and functions. By comprehending the miRNA regulatory network implicated in stem cells ageing paves a path for the development of strategies to overcome deregulation of miRNAs that induced cellular ageing. Thus, this could be used as a promising tool to delay or reverse the ageing of MSCs during their expansion, thus producing higher quality cellular resources for therapeutic applications.

4.5 Conclusion

1. The differential expression of miRNA was evident between SHED and WJSCs culture at P6 and P3 and numerous of miRNAs including miR-22, miR-485-5p, let-7a that associated with cellular ageing induced during *in vitro* passaging were identified.
2. Functional clustering (e.g., cell proliferation, cell survival, cell death, and apoptosis) and canonical pathway analysis (e.g., NF-κB, PTEN, mTOR, and AMPK) via IPA support the relevance of miRNAs in cellular ageing induced during *in vitro* passaging.
3. The array data of selected differentially expressed miRNAs was validated by the qPCR for SHED and WJSCs.

CHAPTER 5: COMPARATIVE mRNA EXPRESSION OF HUMAN MESENCHYMAL STROMAL CELLS ISOLATED FROM THE PULP OF EXTRACTED DECIDUOUS TEETH AND WHARTON'S JELLY DURING *IN VITRO* PASSAGING

5.1 Introduction

Despite having many breakthrough in stem cell research and therapeutic development in the past decades, clinical applications of mesenchymal stem cell (MSCs) have been hampered by their tendency towards senescent and ageing-like characteristics upon long-term *in vitro* expansion (Hayflick, 1965; von Zglinicki and Martin-Ruiz, 2005; Wagner *et al*., 2010b). In the previous chapter, it was evident that human mesenchymal stromal cells (hMSCs) isolated extracted deciduous tooth (SHED) and Wharton's jelly (WJSCs) underwent cellular ageing-like characteristic changes during *in vitro* passaging. These including changes in their cell morphology from spindle-shaped to a flattened and enlarged morphology, reduction in their proliferation capability as well as deregulation of genes expression associated with cell cycle, stemness and stress. It is of note that, these changes were found associated with deregulation of expression of many micro ribonucleic acids (miRNAs). We hypothesized that those differentially expressed miRNAs during passaging as described in Chapter 4 could lead to the deregulation of expression of their targets and signaling pathways that are involve in the coordinated control of cellular ageing.

Indeed, recent evidence indicates that stem cells undergo highly regulated signaling and intrinsic mechanism orchestrate the cellular programs including cell differentiation, self-renewal, senescence, apoptosis, cell fate and other key processes (Bieberich and Wang, 2013; Dalton, 2013). All of these biological programs have been reported cross talk to each other and are intricately regulated by miRNAs. For instance, expression of miR-15 has been showed to inhibit proliferation by targeting *Cyclin D* (Cai *et al*., 2012; Sun *et al*.,

2014), *Cyclin E* genes (Luo *et al*., 2013) as well as the anti-apoptotic gene *Bcl-2* (Cimmimo *et al*., 2005). It is known that cell signaling is a communication system that directs basic cellular activities and biological processes. It plays a key role in governing almost all biological events in response to either the extrinsic signaling from the microenvironment around the cell or its own intrinsic signaling from signaling transduction, protein interaction, and cell-cell communication.

Therefore, alteration in gene expression of any signaling pathway frequently impinges on cell physiology as whole and indeed this notion apply to stem cell biology as well (Dalton, 2013). For instance, the phosphatidylinositol 3-kinase / Protein Kinase B (PI3K/Akt) signaling pathway that has been reported as a mechanism that controlling the balance between self-renewal and differentiation of stem cell (Huang *et al*., 2015). Study from Singh *et al*., (2012) illustrated that an active PI3K/Akt signaling promotes self-renewal via activation of Nanog, one of the downstream targets of Activin A/Smad 2,3. Conversely, low PI3K/Akt signaling promotes conjunction of Wnt effector and Smad 2,3 in which promote differentiation (Singh *et al*., 2012; Shoni *et al*., 2014).

Despite the efforts in uncovering the possible gene targets that interact and regulate cellular ageing in MSCs using molecular approaches (Wagner *et al*., 2009; Laschober *et al*., 2010; Brandl *et al*., 2011), there are still gaps in need of filling to explain the board underlying mechanism involved in cellular ageing. Given the importance of gene expression in coordinating signaling network of any biological processes in stem cell fate, here in this study, we aimed to assess the changes in messenger ribonucleic acids (mRNAs) expression during *in vitro* passaging in SHED and WJSCs. In this study, gene expression analysis using microarray approach was employed to catalog the transcriptomic profiles of the samples in a high-throughput manner. Uncovering the gene expression in this study would

allow us to gain better insights on the cellular regulatory networks which are implicated in cellular ageing of hMSCs induced by passaging. Moreover, it would also provide an insightful information needed in the attempt to correlate miRNAs expression with cellular ageing in the following chapter. Thus, this could serve as framework for future experimental investigation to determine how miRNAs contribute to the regulation of mRNA that associates with the progression cellular ageing.

The research objectives of this study are as follows;

1. To determine differentially expressed mRNAs of SHED and WJSCs at selected late passage (P6) compared to early passage (P3).
2. To assess the characteristics of differentially expressed mRNA profiles through their functional clustering.
3. To conduct quantitative validation study of selected differentially expressed mRNAs involved in cellular ageing by qPCR analysis.

5.2 Materials and methods

5.2.1 RNA extraction

Figure 5.1 illustrates the work flowchart for the analysis of mRNA profiling. First, total RNA isolation was performed for the SHED and WJSCs (one biological samples for each) at passage 3 and 6 by using the QiagenRNeasy® Plus Mini Kit as per the manufacturer's instructions. In brief, 350 µL of RLT buffer was directly added to the cell culture before lysing the cells by pipetting the solution up and down few times. The lysate was then collected into the gDNA Eliminator spin column placed in a 2 mL collection tube, and proceed to centrifugation at 8000 ×*g* for 30 s. Then, 1 volume (350 µL) of 70% molecular grade ethanol was added to the flow-through and mixed by pipetting, followed by the transfer of mixture into RNAeasy spin column and centrifugation at 8000 ×*g* for 15 s. The collected RNA in the RNAeasy spin column was then washed by flow-through with 700 µL RW1 once and 500 µL RPE buffer twice. To eliminate the residual, the RNAeasy spin column was then centrifuged at full speed for 1 min. Finally, 30 µL of RNase-free water was added into the column to elude the RNA by centrifugation at 8000 ×*g* for 1 min. RNA integrity was analyzed with the Agilent 2100 bioanalyzer to ensure the average RNA integrity value (RIN) was above 8.5. The RNA concentration and quality was measured by using the Nanodrop 2000 spectrophotometer, with acceptable A_{260}/A_{280} ratio range of 1.7 to 2.1.

5.2.2 Gene expression microarrays

5.2.2.1 First-strand complementary DNA synthesis

The total RNA samples were processed for hybridization to Human Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) using the GeneChip WT PLUS Reagent Kit (Affymetrix) following the manufacturer's instruction. First, 100 ng of total RNA were reverse transcribed to single-stranded complementary (cDNA) using primers with a T7 promoter sequence at the 5' end. A diluted premixed poly-A RNA control was added to the total RNA prior to the first-strand cDNA synthesis to monitor the labeling process. The first-strand cDNA reaction mix was prepared as shown in Table 5.1 and incubated with the following thermal cycle condition: 1 h at 25ºC, then1 h at 42ºC, followed by 2 min at 4ºC.

Table 5.1. First-strand cDNA reaction mix

Component	Volume for one reaction
Total RNA (100 ng)	variable
Diluted poly-A RNA control	2 µL
Nuclease-free water	variable
First-strand buffer	4 µL
First-strand enzyme	1 µL
Total volume	10 µL

5.2.2.2 Double-strand cDNA synthesis

Next, the single-stranded cDNA was converted to double-stranded cDNA by adding second-strand master mix (consist of DNA polymerase and RNase H to simultaneously degenerate the RNA) as showed in Table 5.2 and incubated with the following thermal cycle condition: 1 h at 16ºC, then 10 min at 65ºC, followed by 2 min at 4ºC.

Table 5.2. Second-strand cDNA reaction mix

Component	Volume for one reaction
First-stranded cDNA	10 µL
Second-strand buffer	18 µL
Second-strand enzyme	2 µL
Total volume	30 µL

5.2.2.3 Complementary RNA synthesis and purification

Following that, the complementary RNA (cRNA) was synthesized and amplified from the cDNA template generated earlier using IVT master mix (Table 5.3) which consist of T7 RNA polymerase (T7 *in vitro* technology known as the Eberwine or RT-IVT technique) by incubated for 16 h at 40ºC. The enzyme, inorganic phosphates, and unincorporated nucleotides were removed by using 100 µL of Purification Beads. The captured cRNA on the Purification Beads against to the magnetic stand were washed three times with 200 µL of 80% ethanol. The, purified cRNA was eluted using 27 µL of preheated (65ºC) nuclease-free water.

Table 5.3. IVT reaction mix

Component	Volume for one reaction
Second-stranded cDNA	30 µL
IVT buffer	24 µL
IVT enzyme	6 µL
Total volume	60 µL

5.2.2.4 2nd-cycle single-stranded cDNA synthesis

Subsequently, 625 ng/µL of purified cRNA (in final volume of 24 µL) was primed with 4 µL of 2nd-cycle primers and incubated with the following thermal cycle condition: 5 min at 70ºC, then 5 min at 25ºC, followed by 2 min at 4ºC. The 2nd-cycle of single-stranded cDNA (2nd-cycle s-s cDNA) (incorporated with deoxyuridine triphosphate (dUTP)) was then synthesized by reverse transcription of cRNA using the reaction mix as shown in Table 5.4 and incubated with the following thermal cycle condition: 10 min at 25ºC, 90 min at 42ºC, 10 min at 70ºC, followed by 2 min at 4ºC.

Table 5.4. 2nd-cycle s-s cDNA reaction mix

Component	Volume for one reaction
cRNA/2nd-cycle primers	28 µL
2nd-cycle s-s cDNA buffer	8 µL
2nd-cycle s-s cDNA enzyme	4 µL
Total volume	40 µL

5.2.2.5 cRNA hydrolysis and 2nd-cycle s-s cDNA purification

Next, the remaining cRNA in the reaction mix was hydrolyzed using 4 µL of RNase H and incubated with following thermal cycle condition: 45 min at 37ºC, then 5 min at 95ºC, followed by 2 min at 4ºC. Immediately after incubation, 11 µL of nuclease-free water was added to bring the final volume up to 55 µL. Then, the 2nd-cycle s-s cDNA was purified in order to remove the enzyme, inorganic phosphates, and unincorporated nucleotides residues by 20 min incubation aster addition of 100 µL of Purification Beads and 150 µL of 100% ethanol. The capture 2nd-cycle single-stranded cDNA on the Purification Beads against to the magnetic stand were washed three times with 200 µL of 80% ethanol. Then, the purified cRNA was eluted using 30 µL of preheated (65ºC) nuclease-free water.

5.2.2.6 Fragment and label 2nd-cycle s-s cDNA

The purified 2nd-cycle s-s cDNA was fragmented by uracil-DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1) at the unnatural dUTP residues. Approximately, 176 ng/µL of 2nd-cycle s-s cDNA (in volume of 31.2 µL) was mixed with the fragmentation reaction mix as shown in Table 5.5 and incubated with the following thermal cycle condition: 1 h at 37 ºC, then 2 min at 93ºC, followed by 2 min at 4ºC.

Table 5.5. Fragmentation reaction mix

Component	Volume for one reaction
176 ng/µL of 2nd-cycle s-s cDNA	31.2 µL
Nuclease-free water	10.0 µL
10× cDNA fragmentation buffer	4.8 µL
UDG, 10 U/µL	1.0 µL
APE1, 1000 U/µL	1.0 µL
Total volume	48.0 µL

In the labeling procedure, 45 µL of fragmented 2nd-cycle s-s cDNA was mixed the labeling reaction mix as shown in Table 5.6 and incubated with the following thermal cycle condition: 1 h at 37ºC, then 10 min at 70ºC, followed by 2 min at 4ºC.

Table 5.6. Labeling reaction mix

Component	Volume for one reaction
Fragmented 2nd-cycle s-s cDNA	45 µL
5× TdT buffer	12 µL
DNA labeling reagent, 5mM	1 µL
TdT, 30 U/µL	2 µL
Total volume	60 µL

5.2.2.7 Hybridization

Subsequent hybridization, wash, and staining were carried out using the AffymetrixGeneChip Hybridization, Wash, and Stain Kit following the manufacturer's protocols. Briefly, approximately 3.5 µg of fragmented and labeled 2nd-cycle s-s cDNA mixed with the hybridization reaction mix (Table 5.7) was hybridized to a GeneChip Human Gene 1.0 ST Array at 45°C for 16 h in AffymetrixGeneChip Hybridization Oven 645.

Table 5.7. Hybridization reaction mix

Component	100 format	Final concentration
Fragmented 2nd-cycle s-s cDNA	41 µL	23 ng/µL
Control Oligo B2 (3 nM)	2.5 µL	50 pM
20× Hybridization Control (*bioB, bioC, bioD, cre*)	7.5 µL	1.5, 5, 25, and 100 pM respectively
20× Hybridization Mix	75.0 µL	1×
DMSO	10.5 µL	7%
Nuclease-free water	13.5 µL	
Total volume	109.0 µL	

After hybridization, the array chips were stained and washed using an Affymetrix Fluidics Station 450 followed by scanned on AffymetrixGeneChip Scanner 3000 7G (Affymetrix, USA). The microarray experimental procedure is illustrated in Figure 5.1.

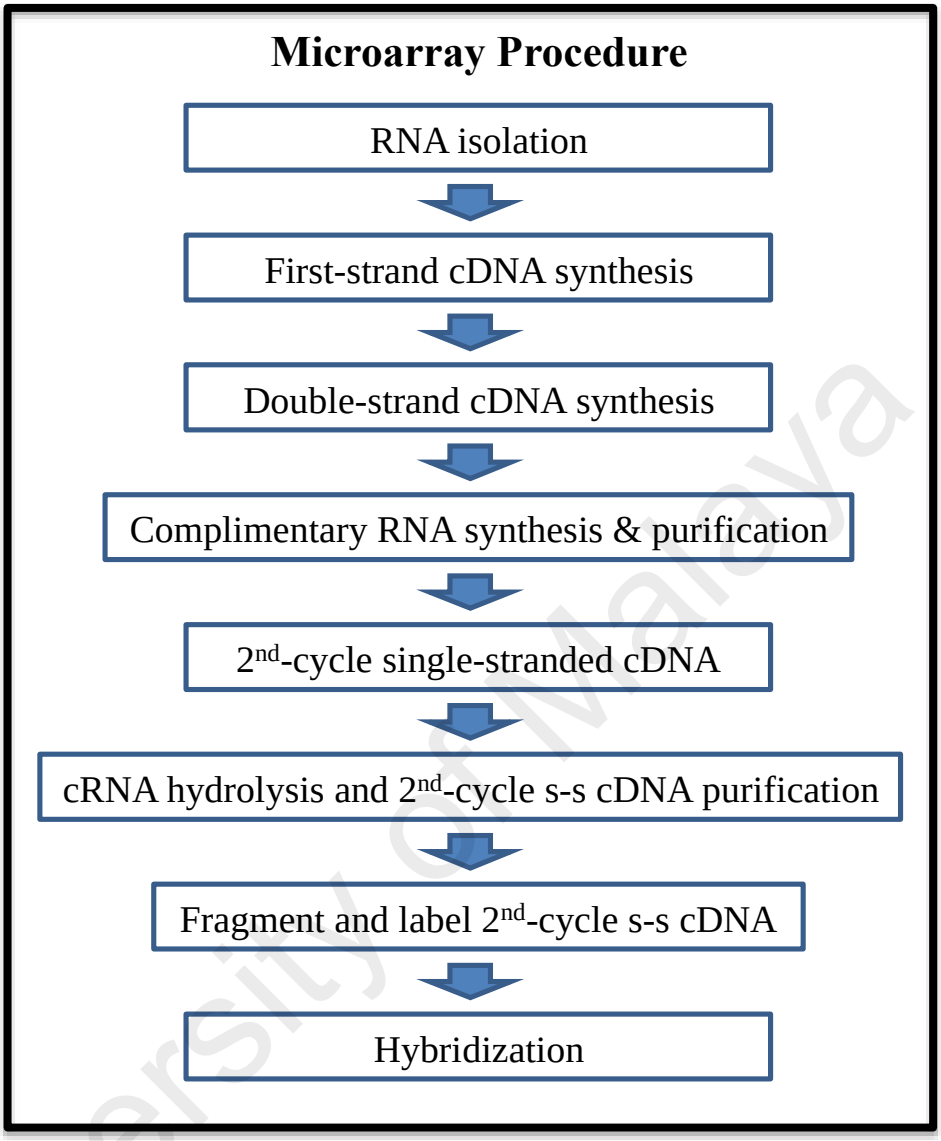


Figure 5.1. Flowchart for the microarray procedure
(RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; cRNA, complementary ribonucleic acid; s-s, single-stranded)

5.2.3 Data Analysis

Figure 5.2 illustrates the work flowchart for the analysis of mRNA profiling data. In brief, the image (.DAT) files that generated in this study were preprocessed using the AffymetrixGeneChip Command Console (AGCC) software v.4.0 to produce cell intensity (.CEL) files. The raw data intensity was assessed using the Robust Multichip Average (RMA) and Tukey's bi-weight average algorithms at threshold of ≥ 1.5 prior to data analysis by Affymetrix Expression Console software v.1.3. The quality assessment metrics (including spike-in controls during target preparation and hybridization) were found within boundaries.

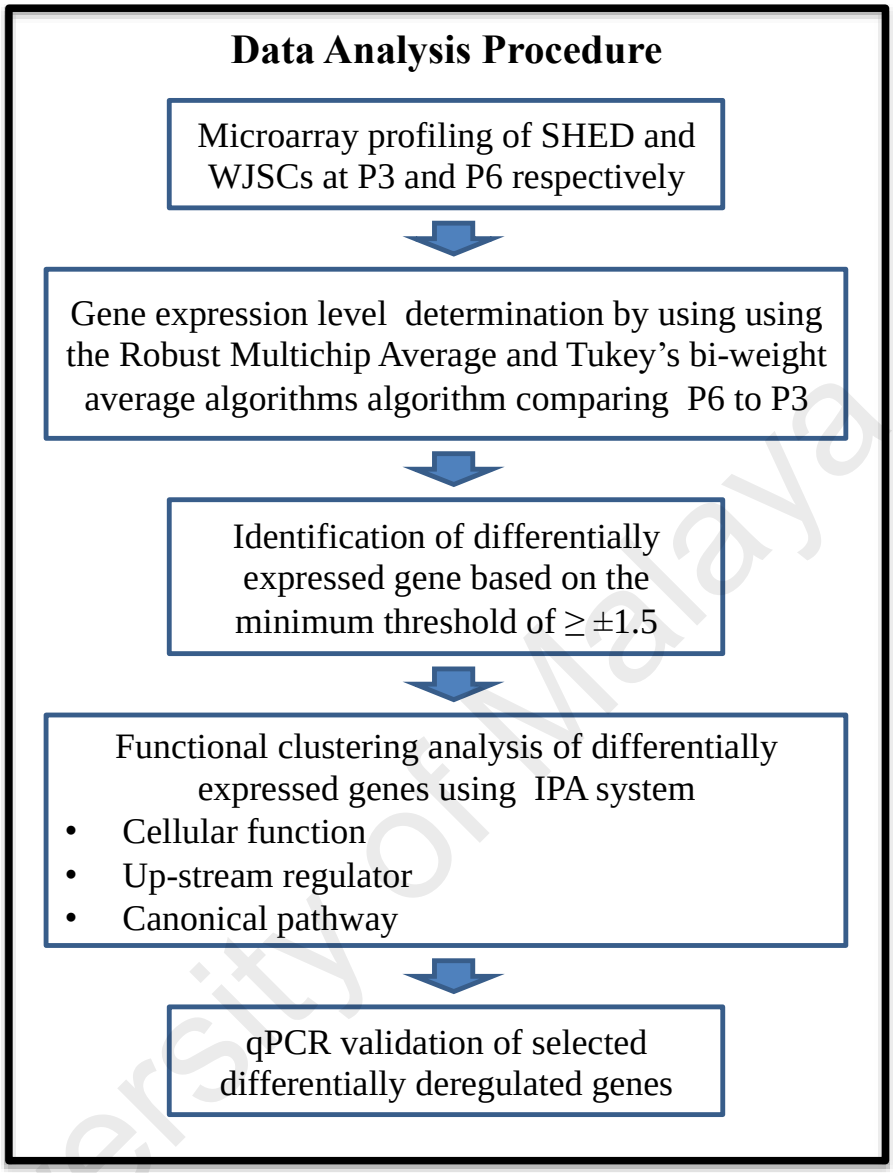


Figure 5.2. Flowchart for the analysis of mRNA profiling
(SHED, hMSCs from the pulp of extracted deciduous teeth; WJSCs, hMSCs from Wharton's jelly; IPA, Ingenuity Pathway Analysis; qPCR, quantitative polymerase chain reaction)

5.2.3.1 Ingenuity pathway analysis

Ingenuity pathway analysis (IPA) software was used to examine the cellular function clustering, up-stream regulator, canonical pathways of differentially expressed genes in SHED and WJSCs comparing P6 to P3. Expression levels of well-annotated mRNAs with 1.5 fold up-regulation and 1.5 fold down-regulation were used as inputs to perform all IPA analyses. Fisher's Exact Test was used to calculate p-value by IPA system to determine the significant of the association between the genes in the dataset and the biological function or pathway in the Ingenuity Knowledge Base in random chance. A regulation *z*-score (statistical measure of the match between expected relationship direction and observed gene expression) is computed to infer the activation state of a predicted canonical pathway.

5.2.4 mRNA validation by qPCR analysis

After initial microarray screening, differential expression for four selected genes was quantitative validated using qPCR technique. These genes were selected based on features observed previously in experiment; 1) genes fall into the canonical pathways that identified by IPA; 2) genes associated with the cell cycle, proliferation or stem cell stemness in referring to published data as mentioned in the earlier discussion (Chapter 4). The extracted RNA from SHED and WJSCs at P3 and P6 (three biological samples for each) were reverse transcribed into cDNA using MultiScribe^{TM} RT (Applied Biosystems, USA) with TaqMan® Reverse Transcription reagentsaccording to manufacturer's instruction. The cDNA conversion was performed in 20 µL reaction mixtures which consist of reagents as listed in Table 5.8. Reaction mixtures were incubated on a thermal cycler (Applied

Biosystems Veriti Thermal Cycler, USA) and the following thermal-cycling conditions were performed: 25ºC for 10 min, 37ºC for 30 min followed by 95ºC for 5 min.

Table 5.8. Components of reverse transcription reaction mixture

Components	Volume for one reaction (µL)
Random hexamers (50 µM)	1.00
$MgCl_2$ (25 mM)	1.40
dNTPs (10 mM)	4.00
MultiScribe Reverse Transcriptase (50 U/µL)	1.00
RT Buffer (10×)	2.00
RNase Inhibitor (20 U/µL)	1.00
Nuclease-free water + total mRNA (1000 ng)	9.60
Total	20.00

Next, qPCR were performed in a 20 µL reaction mixture which consist of 2 µL of reverse-transcribed product (cDNA template), 10 µL of 2× TaqMan® Fast Advanced Master Mix, 7 µL of nuclease-free water and 1 µL of 20× TaqMan® Gene Expression Assay (CKS1B: Hs02518862, CCNB1: Hs01030099, CDK1: Hs00938778, MAP3K1: Hs00394890, GAPDH: Hs02786624). The existence of targets in the sample were amplified using 7500 Fast Real-Time PCR system (Applied Biosystems, USA) which was performed with 7500 Fast system default thermal cycling conditions as followed: 50ºC for 2 min and 40 cycles at 95ºC for 3 s followed by 60ºC for 30 s. The C_T values that generated from qPCR were employed to determine relative expression levels of target mRNA by using $2^{-\Delta\Delta CT}$ algorithm according to the protocol as described earlier in sections 3.2.9, where GAPDH was the designated endogenous control. All chemicals were purchased from Applied Biosystems (USA) unless stated otherwise.

5.3 Results

5.3.1 Analysis of mRNAs expression profiles of SHED and WJSCs at P6 compared to P3

In order to investigate the differential gene expression profiles during *in vitro* passaging, microarray analysis of SHED and WJSCs at P3 and P6 were performed using Affymetrix designed Human Gene 1.0 ST array (coverage a total of 36079 RefSeq transcripts). Differentially expressed genes at P6 compared to P3 were identified using the threshold/cutoff values of ≥ 1.5. The analysis lead to the identification a total of 2972 differentially expressed genes in SHED and of which 1760 genes are well-annotated; in which the up-regulation and down-regulation annotated genes were found in nearly equal proportions (Table 5.9). On the other hand, 1851 differentially expressed genes were identified in WJSCs and of 892 genes are well-annotated; in which 56% of total differentially expressed annotated genes showed an increased, whereas 44% of them showed a decreased in their expression level. The list of differentially expressed genes can be found in Appendix E and F.

Table 5.9. Differentially expressed of annotated genes in P6 compared to P3 of SHED and WJSCs

Level of gene expression in P6 compared to P3	Gene over the total of 36079 transcripts	
	SHED	WJSCs
Up-regulation	873	497
Down-regulation	887	395

5.3.2 Functional clustering analysis of differentially expressed mRNA in SHED and WJSCs during *in vitro* passaging

In order to determine the biological roles associated with the differentially expressed annotated genes, cellular function analysis was carried out using IPA software. The top five significant cellular functions of differentially expressed annotated genes in SHED and WJSCs at P6 compared to P3 are shown in Table 5.10. The clustered cellular function with highest ranking generated by IPA revealed that both SHED and WJSCs underwent deregulation of genes were related to cellular movement function with p-value of 1.54×10^{-10} and 1.01×10^{-6} respectively. Also, numerous differentially expressed of annotated genes in SHED were found to be involved in the cell assembly, DNA replication, cellular development governing processes as well as gene growth and proliferation. On the other hand, the expression profiles of annotated genes in WJSCs were found related to cell movement, cellular compromise as well as cellular function and maintenance. The list of genes that associated to the specific cellular functions in SHED and WJSCs have been tabulated in Appendix G and H respectively.

In additional, up-stream regulator analysis (generated by IPA system) was conducted to predict the cascade of upstream transcription regulators among the observed known genes (Table 5.11). This analysis identified the transcription regulators of the genes from the dataset and also compared their expression changes to what was expected from the literature of Ingenuity® Knowledge Base in order to predict possible significant transcriptional regulators.

Table 5.10. Top five cellular functions of differentially expressed of known genes of SHED and WJSCs

SHED		Rank	WJSCs	
Cellular functions	p-Value, #Molecules		p-Value, #Molecules	Cellular functions
Cellular Movement	1.54×10^{-10}, 181	1	1.01×10^{-6}, 21	Cellular Movement
Cell Assembly and Organization	1.70×10^{-9}, 37	2	6.07×10^{-6}, 13	Cell Morphology
DNA Replication, Recombination, and Repair	1.70×10^{-9}, 45	3	1.16×10^{-5}, 30	Cell-To-Cell Signaling and Interaction
Cellular Development	5.56×10^{-7}, 191	4	1.16×10^{-5}, 13	Cellular Compromise
Gene Growth and Proliferation	5.56×10^{-7}, 241	5	1.09×10^{-4}, 13	Cellular Function and Maintenance

Table 5.11. Top 10 up-stream regulator of differentially expressed of known genes of SHED and WJSCs

SHED		Rank	WJSCs	
p-Value	Up-stream regulator		Up-stream regulator	p-Value
1.21×10^{-11}	ERBB2	1	IL17A	2.13×10^{-7}
7.82×10^{-11}	FOXM1	2	IL25	7.34×10^{-7}
9.75×10^{-11}	NUPR1	3	IL1A	1.21×10^{-6}
1.6×10^{-11}	TGFB1	4	TRAF6	1.24×10^{-6}
1.37×10^{-9}	SMARCA4	5	CDK	1.44×10^{-6}
1.82×10^{-9}	CDK4	6	TNFRSF12A	1.44×10^{-6}
9.18×10^{-9}	CCND1	7	TNFSF12	1.16×10^{-5}
4.08×10^{-8}	RABL6	8	TNIP3	2.21×10^{-5}
1.23×10^{-7}	ESR1	9	IL1B	4.68×10^{-5}
3.44×10^{-7}	CNOT7	10	IL10	5.04×10^{-5}

The identified differentially expressed annotated genes were further subjected to canonical pathway analysis in order to facilitate the understanding of its related intricate signaling pathways. IPA analysis showed that the differentially expressed annotated genes of SHED were predicted to be associated with canonical pathways as shown in Figure 5.3. Canonical pathways such as interferon signaling and cell cycle G2/M DNA damage checkpoint regulation were predicted to be deregulated in SHED at later passages with an overall increase in the activity (orange bar: positive *z*-score). It is also noted that there is decreased activity (blue bar: negative *z*-score) of few canonical pathways including Eicosanoid, tumor necrosis factor receptor (TNFR) 2 and TNFR1 signaling pathways. Subsequently, the candidate genes of selected canonical pathway including interferon signaling, cell cycle G2/M DNA damage checkpoint regulation, Eicosanoid and, TNFR 1/2 as well as their predicted activities that are generated through IPA were summarized in Table 5.12 to 5.17 respectively.

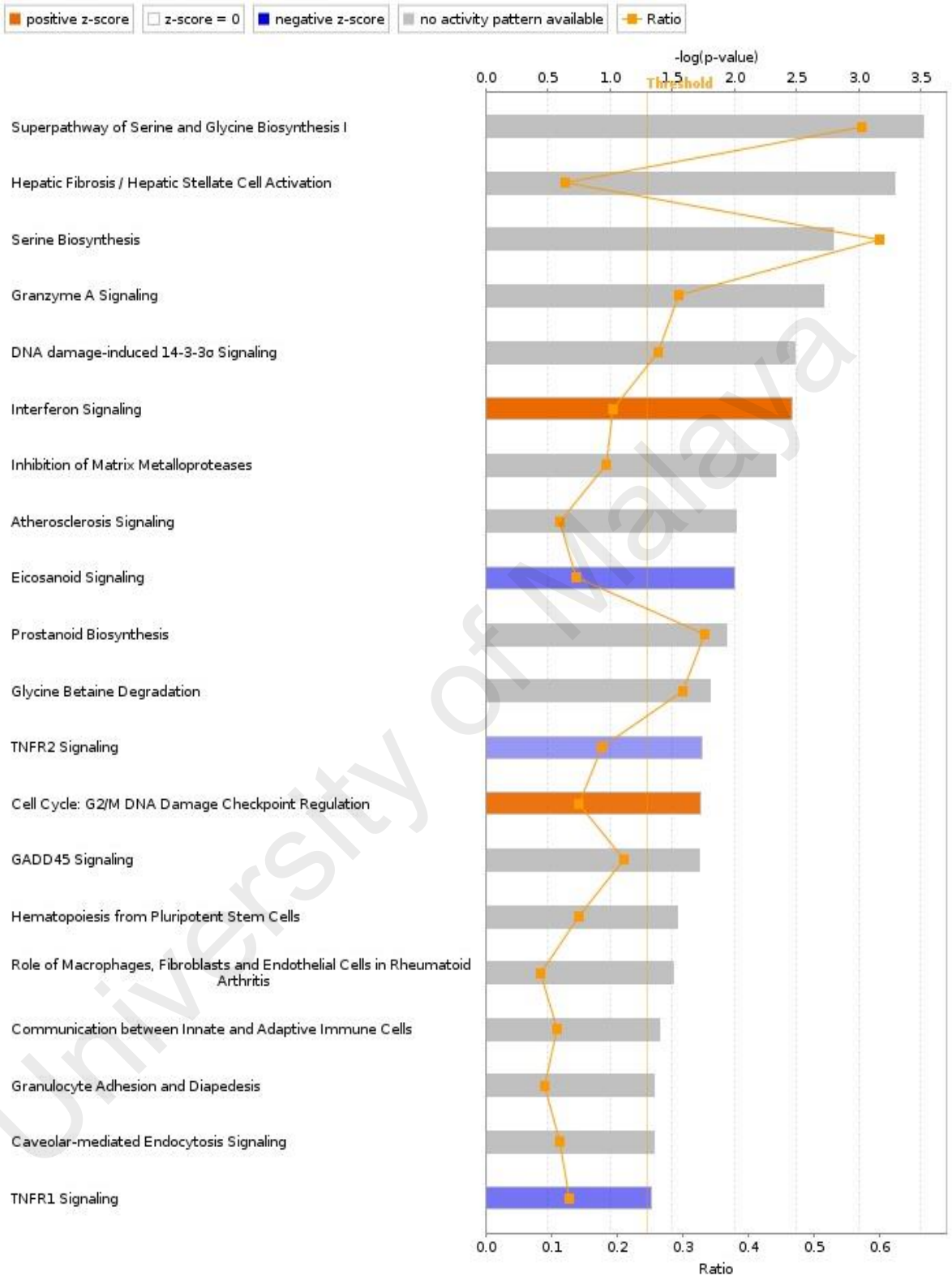


Figure 5.3. Major canonical pathways deregulated in SHED
The calculated *z*-score indicates prediction of a pathway with genes exhibiting overall increased mRNA levels (orange bars), decreased mRNA levels (blue bars) or no mRNA activity (grey bars).

Table 5.12. Genes associated with interferon signaling pathway with positive *z*-score (n=1)

Gene symbol	Entrez gene name	Fold change	Expected regulation by IPA	Location	Types/ category
IFI6	interferon alpha inducible protein 6	1.85	Up	Cytoplasm	other
IFIT1	interferon induced protein with tetratricopeptide repeats 1	2.29	Up	Cytoplasm	other
IFIT3	interferon induced protein with tetratricopeptide repeats 3	2.22	Up	Cytoplasm	other
IFNA1/ IFNA13	interferon alpha 1	1.79	Up	Extracellular Space	cytokine
OAS1	2'-5'-oligoadenylate synthetase 1	2.01	Up	Cytoplasm	enzyme
SOCS1	suppressor of cytokine signaling 1	-1.57	Down	Cytoplasm	other
STAT1	signal transducer and activator of transcription 1	2.39	Up	Nucleus	transcription regulator

Table 5.13. Genes associated with G2/M DNA damage checkpoint regulation with positive *z*-score (n=1)

Gene symbol	Entrez gene name	Fold change	Expected regulation by IPA	Location	Types/ category
CCNB1	cyclin B1	-1.52	Down	Cytoplasm	kinase
CCNB2	cyclin B2	-1.7	Down	Cytoplasm	other
CDC25C	cell division cycle 25C	-1.53	Down	Nucleus	phosphatase
CDK1	cyclin dependent kinase 1	-1.57	Down	Nucleus	kinase
CKS2	CDC28 protein kinase regulatory subunit 2	-1.77		Other	kinase
CKS1B	CDC28 protein kinase regulatory subunit 1B	-1.7	Down	Other	kinase
PLK1	polo like kinase 1	-1.86	Down	Nucleus	kinase

Table 5.14. Genes associated with Eicosanoid signaling pathway with negative *z*-score (n=1)

Gene symbol	Entrez gene name	Fold change	Expected regulation by IPA	Location	Types/ category
AKR1C3	aldo-ketoreductase family 1, member C3	1.56		Cytoplasm	enzyme
LTB4R	leukotriene B4 receptor	-1.6	Up	Plasma Membrane	G-protein receptor
PAFAH1B3	platelet activating factor acetylhydrolase 1b catalytic subunit 3	-1.74	Up	Cytoplasm	enzyme
PLA2G4C	phospholipase A2 group IVC	-2.49	Up	Plasma Membrane	enzyme
PLBD1	phospholipase B domain containing 1	-2.08	Up	Extracellular Space	enzyme
PNPLA3	patatin like phospholipase domain containing 3	2.29	Up	Cytoplasm	enzyme
PTGDS	prostaglandin D2 synthase	1.57		Cytoplasm	enzyme
PTGIS	prostaglandin I2 (prostacyclin) synthase	-2.61		Cytoplasm	enzyme
PTGS1	prostaglandin-endoperoxide synthase 1	1.73		Cytoplasm	enzyme

Table 5.15. Genes associated with TNFR1 and TNFR2 signaling pathways with negative *z*-score (n=1)

Symbol	Entrez gene name	Fold change	Expected regulation by IPA	Location	Types/ category
BIRC3	baculoviral IAP repeat containing 3	-1.78	Down	Cytoplasm	enzyme
IKBKE	inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase epsilon	-1.67	Up	Cytoplasm	kinase
MAP3K1	mitogen-activated protein kinase kinasekinase 1	-1.56	Up	Cytoplasm	kinase
NAIP	NLR family apoptosis inhibitory protein	1.56	Down	Cytoplasm	other
PAK6	p21 (RAC1) activated kinase 6	-1.56	Up	Cytoplasm	kinase
TNFAIP3	TNF alpha induced protein 3	-1.92		Nucleus	enzyme

On the other hand, the determined differentially expressed annotated genes in WJSCs sample were found related to inflammation signaling, given that triggering receptor expressed on myeloid cells 1 (TREM 1) signaling and role of interleukin 17F (IL-17F) in allergic inflammatory airway diseases showing a positive *z*-score, whereas liver X receptor/retinoid X receptor (LXR/RXR) activation signaling showing a negative *z*-score (Figure 5.4). Then, the candidate genes of selected canonical pathway TREM1 and LXR/RXR and their predicted activities that are generated through IPA were summarized in Table 5.16 and Table 5.17 respectively.

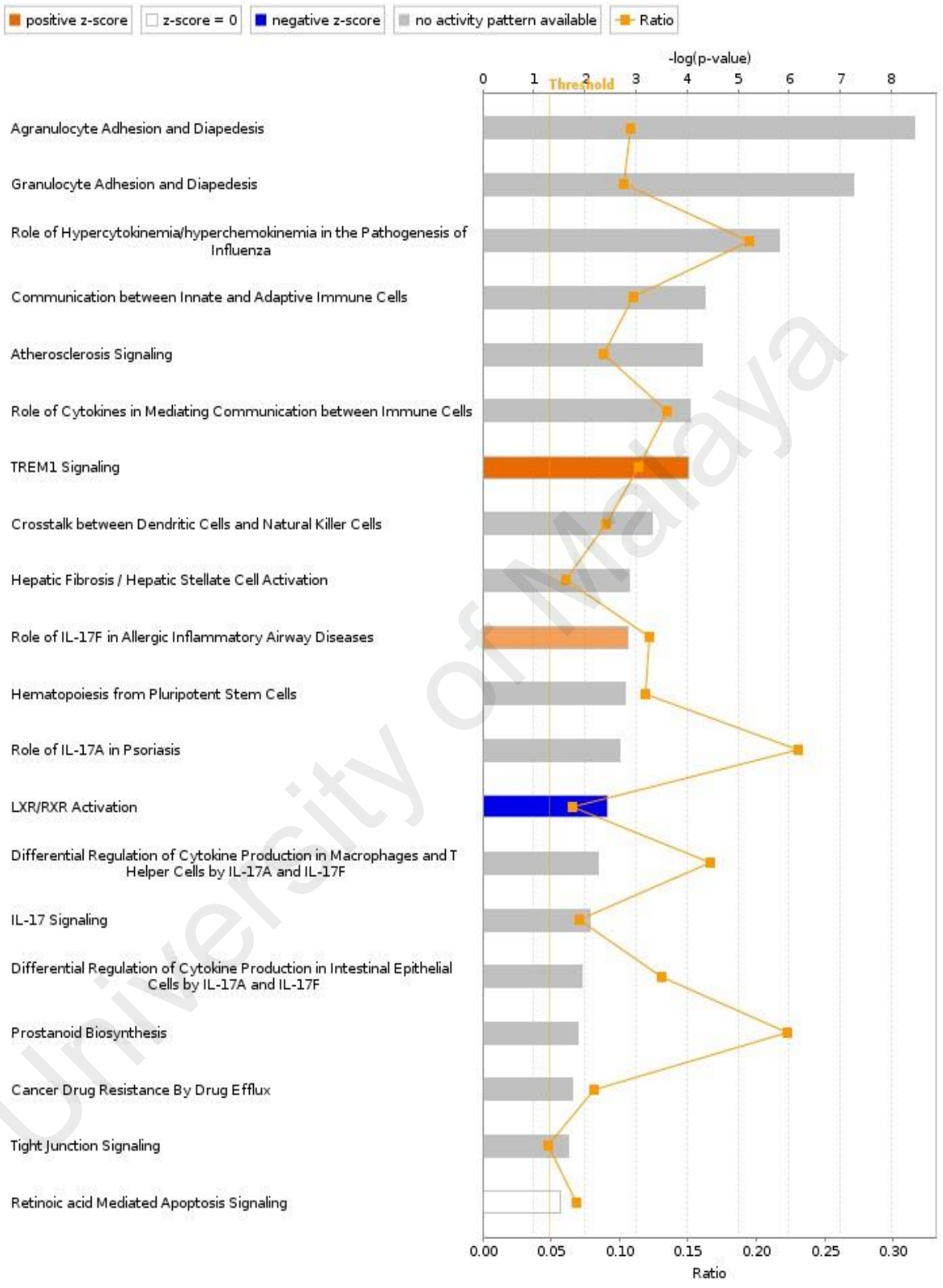


Figure 5.4. Major canonical pathways deregulated in WJSCs

The calculated *z*-score indicates prediction of a pathway with genes exhibiting overall increased mRNA levels (orange bars), decreased mRNA levels (blue bars) or no mRNA activity (grey bars).

Table 5.16. Genes associated with TREM1 signaling pathway with positive *z*-score (n=1)

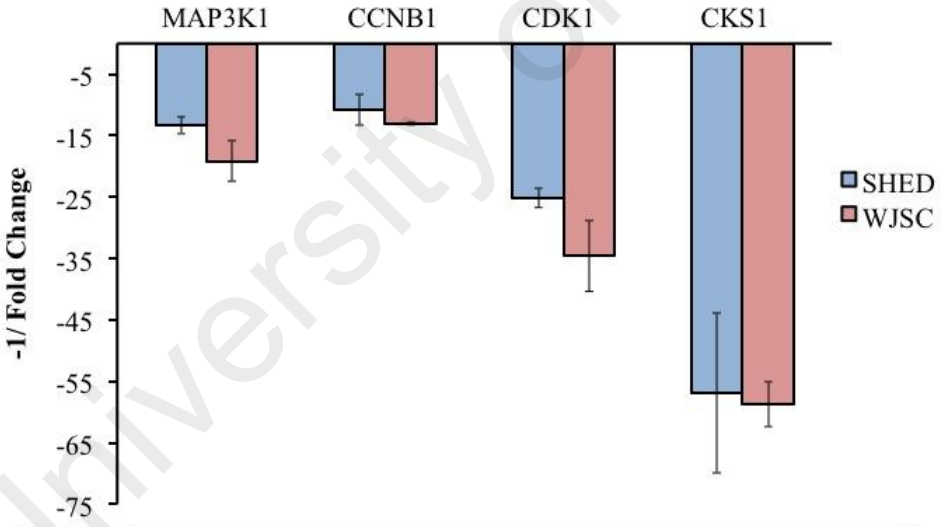
Symbol	Entrez fene name	Fold change	Expected regulation by IPA	Location	Types/ category
CCL2	C-C motif chemokine ligand 2	1.72	Up	Extracellular Space	cytokine
CCL7	C-C motif chemokine ligand 7	1.69	Up	Extracellular Space	cytokine
CXCL8	C-X-C motif chemokine ligand 8	1.83	Up	Extracellular Space	cytokine
DEFB4A /DEFB4B	defensin beta 4A	-1.64	Up	Extracellular Space	other
FCGR2B	Fc fragment of IgG receptor IIb	1.56	Up	Plasma Membrane	transmembrane receptor
IL6	interleukin 6	2.72	Up	Extracellular Space	cytokine
NLRP10	NLR family pyrin domain containing 10	1.82	Up	Other	other
TLR4	toll like receptor 4	1.55	Up	Plasma Membrane	transmembrane receptor

Table 5.17. Genes associated with LXR/RXR signaling pathway with negative *z*-score (n=1)

Symbol	Entrez gene name	Fold change	Expected regulation by IPA	Location	Types/ category
APOA2	apolipoprotein A2	1.68	Up	Extracellular Space	transporter
CCL2	C-C motif chemokine ligand 2	1.72	Down	Extracellular Space	cytokine
CCL7	C-C motif chemokine ligand 7	1.69	Down	Extracellular Space	cytokine
IL6	interleukin 6	2.72	Down	Extracellular Space	cytokine
IL33	interleukin 33	3.48	Down	Extracellular Space	cytokine
IL1F10	interleukin 1 family member 10 (theta)	1.69	Down	Extracellular Space	cytokine
SERPINA1	serpin family A member 1	1.52	Up	Extracellular Space	other
TLR4	toll like receptor 4	1.55	Down	Plasma Membrane	transmembrane receptor

5.3.3 qPCR validation of selected miRNAs

Based on the canonical pathway analysis performed using IPA system, *CDK1*, *CKS1B*, *CCNB1*, and *MAP3K1* were selected for quantitative validation by using qPCR. As shown in Figure 5.5, these selected genes for qPCR validation showed down-regulated in SHED and WJSCs at P6 in compared to P3. The relative gene expression pattern of those selected genes in SHED at later passage compare to earlier passage was comparable to the microarray analysis. However, most genes were decreased in expression more than 5-fold. On the other hand, result of qPCR validated genes of WJSCs were in disagreement with the microarray data, in which the genes of interest were found no differently expression using microarray approach, but were found differentially expressed in qPCR validation.



hMSCs	Gene			
	MAP3K1	CCNB1	CDK1	CKS1
SHED	-13.39 ± 1.37	-10.81 ± 2.53	-25.08 ± 1.62	-56.92 ± 12.99
WJSC	-19.123 ± 3.35	-13.01 ± 0.24	-34.54 ± 5.81	-58.66 ± 3.71

Figure 5.5. Validation of the microarray data using qPCR analysis
Four selected genes were validated using qPCR, data were normalized to *GAPDH* and were expressed in -1/ Fold Change comparing P6 to P3, bars represent average from three biological repeats for SHED and WJSCs.

5.4 Discussions

The mechanisms of gene signaling networks that mediated cellular senescence has remained poorly understood, especially with regards to its importance in the interference of MSCs phenotypes during *in vitro* passaging. The purpose of this study was to determine the gene expression profiles of SHED and WJSCs at P6 compared to P3 using microarray analysis. This study demonstrated that both cell types at P6 exhibit substantial difference in gene expression profiles when compared with those in P3. Of the highly differentially expressed genes identified, CXCL14 and PARM-1 (Appendix E and F) showed a remarkable decrease in its expression from P3 to P6 in SHED and WJSCs respectively. Both genes are known to regulate cell proliferation (Fladeby *et al*., 2008; Charfi *et al*., 2013; Park et al., 2013; Lin et al., 2014). On the other hand, senescence-associated secretory phenotype (SASP) associated genes *ERAP2* and *IL-33* were showed up-regulation in SHED and WJSCs respectively (Accornero *et al*., 2017; Daniels *et al*., 2017). The specific functional roles of these differentially expressed genes were further analyzed using IPA software and found to be associated with multiple cellular functions, among which the cellular movement, cell assembly and organization, DNA replication, cell morphology, cell to cell signaling and interaction being top-ranked for both SHED and WJSCs.

These findings are in agreement with other studies that proposed that cellular senescence process not only involved in cell growth arrest, it also participate in the biological process such as cellular movement or migration (Campisi and d'Adda di Fagagna, 2007; Rodier and Campisi, 2011). It has also been previously reported that cellular movement is a fundamental cellular process that underlies the development and functioning of stem cells in many biological and physiological events including immune response, embryogenesis, wound healing and cellular repair (Weidt *et al*., 2007; Smart and Riley, 2008). Often,

MSCs that resided in the stem cell niches migrate to the injury site for the regeneration or repair purposes (Rennert *et al*., 2012), which require the support of chemokines such as stromal cell-derived factor-1α (SDF-1α) and C-X-C chemokine receptor type 4 (CXCR4) (Marquez-Curtis and Janowska-Wieczorek, 2013). A part from that, many of the differentially expressed genes in SHED and WJSCs during *in vitro* passaging were found to be clustered to the cellular function associated with gene growth and proliferation as well as cellular function and maintenance respectively. These findings provide further support for our hypothesis that these detected differentially expressed genes in SHED and WJSCs during *in vitro* passaging may have a direct influence on the hMSCs phenotypes as shown in previous study in Chapter 3. This is due to the activation or interference with these cellular functions can dramatically alter their stemness (Weissbein *et al*., 2014).

Additionally, upstream regulators analysis was performed in order to identify the possible transcriptional factors governing biological events in SHED and WJSCs during *in vitro* passaging. IPA showed that the predicted up-stream regulators of annotated genes detected in SHED was associated with ERBB2, CNOT7, CDK4, CCND1 and others. Interestingly, some genes have been found to be involved in mediating proliferation and cell cycle progression activities. For instance, CNOT7 has been reported to facilitate anti-proliferation activities via interactions with BTG/Tob proteins (Doidge *et al*., 2012). Also, previous reports illustrated that CCND1 form a complex with CDK4 to phosphorylate repressor retinoblastoma protein to allow de-repressing of E2F, through which promoting the transcription of E2F-target genes which are essential for DNA synthesis (Aguilar and Fajas, 2010; Duronio and Xiong, 2013). Inhibition of cyclin D expression can impacts CDK 4/6 activities, which lead to cell cycle arrest and reduces proliferation (Yang *et al*., 2006; Duronio and Xiong, 2013). This finding was consistent with the previous study in Chapter

3, which demonstrated that the proliferative characteristics of SHED were declining as indicated by the decreased expression of *CDK 4* and *Cyclin D*.

On the contrary, it is found that a set of inflammatory genes which predicted to serve as the up-stream regulators of differentially expressed annotated genes in WJSCs. Particularly, IL-17A and IL-1B that has been reported to be involved in the activation of NF-κB signaling in cellular responses to stress stimuli (Sønder *et al*., 2011; Liu *et al*., 2017). Indeed, there is evidence documenting the major role of NF-κB signaling favouring cellular senescence (Rovillain *et al*., 2011). Overall, this finding suggests that it is likely that the stress signal activated the inflammatory chemokine, which may contribute to the ageing-like phenotypes alteration in WJSCs during *in vitro* passaging.

Next, the key regulatory and canonical pathways that mediate cellular ageing through differentially expressed genes in SHED and WJSCs cultured *in vitro* were explored. It has been extensively studied that cells undergoing progressive growth arrest in response to external stress and inflammatory stimuli induces DNA damage, oxidation stress, oncogenic stimulation, telomeres shortening and presence of inflammatory factors (Coppé *et al*., 2010; Kidane *et al*., 2014; Zhang *et al*., 2016). In accordance with this notion, the differentially expressed genes in both SHED and WJSCs along the passaging were found to be related to inflammatory event. IPA analysis in this study showed that the interferon signaling related genes such as signal transducers and activators of transcription 1 (*STAT1*) and the IFN-induced protein with tetratricopeptide repeat 3 (*IFIT3*) were shown to be up-regulated in SHED. This shows the importance of *STAT1* and *IFIT3*, given that both genes belong to the down-stream proteins of interferon receptor 1 (IFNAR1) (Niess *et al*., 2015). It has been reported that, stress stimuli such as DNA damage can activate cell-autonomous induction of interferon signaling which play a key role in promoting cell senescence (Yu *et al*., 2015).

Beside, the crosstalk of interferon signaling with cell cycle arrest was studied in cancer cells (Xiao *et al*., 2006). For instance, cellular senescence event which is caused by cell cycle arrest is accompanied by the expression of cyclin dependent kinase inhibitors CDKN1B (p27) (Satoh & Kaida, 2016). Reports from Xiao *et al*., (2006) demonstrated that the expression of IFIT3 could indirectly cause an increase level of CDKN1B protein in nucleus, whereby IFITS inhibits the binding of COPS5 to CDKN1B in nucleus and exports it to cytoplasm for degradation. Overall, these observations provided further evidence on the role of inflammatory cytokines in promoting cellular senescence corresponding to stress stimuli during *in vitro* culture.

For the pass decades, many efforts are devoted to study the complex process of cellular ageing in order to understand the self-renewal characteristic of stem cells. Studies have shown a consensus regarding the molecular characteristics of cellular ageing in MSCs is accompanied by a diminished capacity of DNA damage repair (Wagner *et al*., 2008; Wagner *et al*., 2009; Yu and Kang, 2013). The maintenance of stem cell characteristics depends on the regulation of DNA repair mechanisms (Jung and Brack, 2014; Pan *et al*., 2016). Accumulation of DNA damage activates checkpoint response such as up-regulation of cell cycle checkpoints/regulators and inhibitors including p53 and p16 that promote cellular senescence which contribute to impairment of the stem cell functionality (Shibata *et al*., 2007; Solozobova and Blattner, 2011). Considering the importance of DNA damage repair in the maintenance of stem cell characteristics, it is found in this study that genes involved in G2/M DNA damage checkpoint regulation (including *CCNB1*, *CCNB2*, CDC25C, CDK1, *CKS1B*, *CKS2* and *PLK1*) were down-regulated in SHED during passaging. This finding may in fact connect the inactivation or aberration of DNA damage repair related signaling having an impact on cell cycle that directs cellular proliferation.

Furthermore, some of identified differentially expressed genes being clustered under Eicosanoid signaling pathway in SHED. *PTGDS* gene encodes enzyme prostaglandin-H2 D isomerase that serves as an important enzyme that catalyzes the conversion of prostaglandin H2 (PGH2) to prostaglandin D2 (PGD2) (Adams and McLaren, 2002; Moniot *et al*., 2009). PGD2 has been reported as a mediator with opposing effects in immune system as pro-inflammatory and anti-inflammatory (Lone and Taskén, 2013) suggesting its involvement in stress and inflammatory pathways. For instance, 15-d-PGJ_2, a PGD_2 metabolite that has been demonstrated to both stimulate and inhibit the expression of inflammatory mediators including TNF-α and IL-1β (Harris *et al*., 2002). In this study, it is found that cellular ageing-like characteristics caused by passaging lead to interruption of TNFR1 and TNFR2 signaling pathways in SHED with concomitant lower level expression of *MAP3K1*. Both these pathways play important roles in various physiological and pathological processes, including cell proliferation, survival, and apoptosis (Parameswaran and Patial, 2010; Fischer *et al*., 2011; Rauert *et al*., 2011). Inhibition of MAP3K in turn will block the attenuation of C-Jun (JNK) cooperates with NF-κB in the TNFR1 as well as TNFR2 signaling pathways and thus increases their susceptibility to apoptosis (Nakagawa and Maeda, 2012; Sharma *et al*., 2016), which may explain the lower proliferative capacity of SHED in the later passages.

On the contrary, it was observed that the differentially expressed genes in WJSCs were associated with TREM1 and LXR/RXR signaling pathway. The SASP such as chemokine (C-C mofit) ligand 2 (CCL2), chemokine (C-C mofit) ligand 7 (CLL7), chemokine (C-X-C mofit) ligand 8 [CXCL8; also known as interleukin 8 (IL-8)] and IL-6 were showed up-regulated during P6. Apart from the cell-autonomous mechanism such as DNA damage repair, which prevent of cell damage in response to stress, senescent cell can secrete

cytokines to induce immune-mediator thus triggering senescence in the neighboring cells (van Deursen, 2014; Pérez-Mancera *et al*., 2014; Eggert *et al*., 2016). It is worth noting that the highlighted signaling pathways in SHED were not identical to WJSCs, yet they might be equally important in the induction of cellular senescence. SHED and WJSCs could have dissimilar differentiation potentials which could lead to different phase of cell ageing, thus resulting in different pathways to induce cellular senescence. This observation suggests that cell ageing of SHED and WJSCs during *in vitro* passaging may influenced by their physiological function and tissue origin, event through the phenotypic changes are very much the same.

Finally, four genes including *CNNB1*, *CDK1*, *CKS1* and *MAP3K* were validated using qPCR analysis. It was confirmed that relative gene expression pattern of those selected genes in SHED at P6 compared to P3 were comparable to the microarray analysis and most genes decreased in expression more than 5-fold. In contrast, no correlation was observed between qPCR validation and microarray data for WJSCs. It can be reasoned that the analytical platform, procedures and data normalization approach are fundamentally different between qPCR and microarray analysis. Nevertheless, experimental replication is one of a key contribution factors that determines the reliability and accuracy of experimental data. This is because an adequate replication is essential to produce a high statistical power that enables us to correctly determine gene expression level. Therefore, it is ought to include more biological samples of observation in future research study.

In summary, this study revealed the putative signaling pathways that are deregulated in two different stem cell populations; SHED and WJSCs in associated with cellular ageing caused by *in vitro* passaging. Through the IPA, it has been shown that the potential of inflammatory factors and SASP that involved in interferon, eicosanoid, TREM1 and

LXR/RXR signaling pathways play an integral as stress inducer that lead to cellular ageing. This study also highlight that the importance of cell cycle regulation in cellular ageing, where deregulation of G2/M DNA damage checkpoint regulation, TNFR1 and TNFR2 signaling pathways were observed in SHED, thereby allowing an interrupt in cell cycle progression, which in turn inhibits their proliferation. Altogether, this study constitutes a resource that may guide others to investigate the function of genes in cellular ageing and how these factors might affect the quality of hMSCs culture as a step forward toward clinical application. Further investigation of these genes-specific deviations might be informative for elaborating the miRNA-mRNA integration that associated to cellular ageing.

5.5 Conclusion

1. Differentially expressed genes were found in SHED when cultured at P6 and P3. Numerous of genes including *CCNB1*, *CKS1B* which found deregulated in SHED are associated with cellular ageing induced during *in vitro* passaging were identified.
2. IPA analysis predicted a set of proliferation and cell cycle related genes (*ERBB2*, *CNOT7*, *CDK4*, *CCND1*) as up-stream regulator in SHED, whereas inflammatory related gene (*IL-17A* and *IL-1B*) as up-stream regulator in WJSCs. These explain the differences in stress inducers that potentially mediate cellular ageing during *in vitro* passaging between SHED and WJSCs.
3. Functional clustering analysis results of differentially expressed genes in SHED (cell movement, cell assembly, DNA replication, cellular development governing processes, gene growth and proliferation) and WJSCs (cell movement, cellular compromise as well as cellular function and maintenance) support the relevance of genes in cellular ageing induced during *in vitro* passaging.
4. Canonical pathway analysis results of differentially expressed genes showed differ between SHED (G2/M DNA damage checkpoint regulation, TNFR1 and TNFR2 signaling pathway) and WJSCs (interferon, eicosanoid, TREM1 and LXR/RXR signaling pathway). Results suggested that both SHED and WJSCs could have dissimilar potentials in which would lead to different phase/ kinetics of cell ageing, thus resulting in different pathways to induce cellular senescence.
5. Quantitative validation results on selected differentially expressed mRNAs were in line with the array data in SHED, but no correlations of qPCR validation and microarray data in WJSCs.

CHAPTER 6: MIRNA-mRNA INTERACTION ON CELLULAR AGEING DUE TO *IN VITRO* PASSAGING OF HUMAN MESENCHYMAL STROMAL CELLS ISOLATED FROM THE PULP OF EXTRACTED DECIDUOUS TEETH AND WHARTON'S JELLY

6.1 Introduction

The maintenance of stem cell phenotype is tightly regulated by various internetworking molecular signaling pathways and cellular events which are entailed by the synergistic and antagonistic interplay of many molecular entities. One of such key interplay is between miRNAs and mRNAs in regulating gene expression. As an important mechanism of gene expression regulation, miRNA binds to mRNA targets which lead to degradation of messenger transcripts and inhibition of translation. For instance, miR-200 direct targets zinc finger E-box-binding homeobox 1 (*ZEB1*) (Park *et al*., 2008), which can repress expression of E-cadherin; a trans-membrane protein that play a critical role in governing stem cell fates and activities (Redmer *et al*., 2011; Soncin and Ward, 2011).

In the two previous chapters, findings on differentially expressed of miRNA and mRNA in SHED and WJSCs during *in vitro* passaging were discussed. Hence, we hypothesized that these molecules might have significant interactions which may be the underlying mechanism of *in vitro* ageing of stem cells cultured in expansion media. Although recent studies have greatly advanced, our understanding of biological influences of miRNAs, the mechanisms involved in maintenance, regulation and modulation of their signal integration are not fully documented. The complexity of miRNA-mRNA interaction remained to be a challenge in stem cell studies as single miRNA can target hundred different mRNAs that it is of significance, hence this study aimed to unravel the miRNA regulation *in vitro* stem cell ageing. Therefore, deeper understanding of the relationships between miRNA-mRNA,

could allow us to identify the potential miRNA and its translation repression targets that is involved in the biological processes that concerned particularly *in vitro* cellular ageing.

In continuation of this research, we aim to analyse miRNA-mRNA interactions of differently expressed miRNAs and mRNAs in association with *in vitro* ageing of SHED and WJSCs during passaging via the integration analysis. For this purpose, several analysis works were conducted including the sequence-based prediction algorithms (web-based miRanda algorithms software) to search for putative miRNAs that are complementary with genes of selected canonical pathways that are previously identified. Subsequently, the negative correlation of selected miRNA-mRNA pairs were evaluated by Pearson correlations coefficient based on their expression levels.

Taken together, it was intended that this study would contribute to the understanding of the molecular process of miRNA-mRNA interaction in driving the cellular ageing in SHED and WJSCs. This may pave a path for the development of strategies aimed to impede stem cells from undergoing senescence and ageing in culture, providing valuable insight for future research and development of effective stem cell based therapy. The research objectives of this study are as follows;

1. To correlate cellular ageing related miRNAs and mRNAs
2. To validate the selected miRNA-mRNA interactions by negative linear regression analysis.
3. To model the highly differentially expressed miRNAs which are involved in the regulation of cellular ageing.

6.2 Materials and methods

6.2.1 miRNA-mRNA interaction analysis and functional mapping

Putative miRNAs that are complementary with genes of selected canonical pathways as previously identified via IPA system were predicted using genomic coordinates retrieved from online database (www.microrna.org). The miRNA-mRNA interaction was assessed based on the evolutionary conservation of the target mRNA sequence and the miRNA-mRNA sequence alignment. The miRNA-mRNA interaction prediction is presented using miRNA support vector regression (mirSVR) scoring algorithm, where the miRNA target sites efficiency was predicted and identified in referring to experimentally determined reports on the mRNA expression changes following miRNA transfections. Cutoff mirSVR score of $\leq$ -1 were used in this study as it corresponds to the top 7% significant prediction of miRNA and mRNA correlations. Additionally, parameters including conservation, alignment and energy score were obtained from this system to predict the potential miRNA targets. Subsequently, schematic diagram of the miRNA and mRNA interactions of G2/M DNA damage checkpoint regulation and TNFR2 signaling pathway were derived using IPA system.

6.2.2 Pearson correlation coefficient analysis

In order to measure the strength of a negative correlation interaction between the identified miRNA-mRNA pairs, the expression levels of qPCR validated miRNA and of its mRNA targets were subjected for Pearson correlation coefficients evaluation. Pearson correlation coefficient is designated by r and is by design constrained as $-1 \leq r_s \leq 1$, where r value is

closer to ±1 the stronger the correlation interaction. The equation of Pearson correlation coefficient as follow:

$$r = N\sum xy-(\sum x)(\sum y))/\sqrt{[N\sum x^2-(\sum x)^2][N\sum y^2-(\sum y)^2]}$$

where x is the fold change of miRNA, y is the fold change of mRNA, N is the number of pairs of data.

6.3 Results

6.3.1 miRNA-mRNA interaction prediction

In order to understand the miRNA-mRNA regulatory relationship of those differentially expressed miRNA and mRNA in SHED and WJSCs during *in vitro* passaging, genomic coordinates retrieved from online database (www.microrna.org) was employed to predict the potential miRNA-mRNA interactions. The genes from selected canonical pathway analysis which are associated with cellular ageing including G2/M DNA damage checkpoint regulation and TNFR2 signaling pathway (as previously discussed in Chapter 5) were subjected to miRNA-mRNA interactions prediction using web-based miRanda algorithms software. The miRNAs that showed mirSVR score of ≤ -1 in the interactions with their corresponding target mRNAs associated with G2/M DNA damage checkpoint regulation and TNFR2 signaling pathway were tabulated in Table 6.1 and 6.2 respectively. The miRanda software also estimates other metrics (conservation, align and energy score) to predict the correlation between putative miRNA-mRNA interaction. These results indicated that genes including *CDK1*, *CKS2*, *CKS1B*, *PKL1*, *MAP3K1*, *NAIP* and *TNFAIP3* have negative correlations with selected miRNAs in both SHED and WJSCs.

Table 6.1. List of correlated miRNA-mRNA pairs in G2/M DNA damage regulation canonical pathway. The table includes gene symbol and miRNA name with their expression level from array dataset (pink box), the conservation, align score, energy as well as mirSVR score retrieved from miRanda online database. The highlighted boxes in red represent miRNAs detected in both SHED and WJSCs.

Gene symbol (fold change)	miRNA name	Log_2(fold change)		Conservation	Align score	Energy	mirSVR score
		SHED	WJSCs				
CCNB1 (-1.52)	hsa-miR-379	-	-	0.4966	147	-15.09	-1.1179
CDC25C (-1.53)	hsa-miR-142-3p	-	-	0.5663	160	-14.13	-1.3013
	hsa-miR-136	-	-1.2543	0.6969	155	-14.46	-1.2518
CDK1 (-1.57)	hsa-miR-31	1.1025	1.6578	0.6067	154	-17.44	-1.2528
	hsa-miR-217	-	-3.8378	0.5475	132	-19.34	-1.0169
	hsa-miR-219-5p	-	-	0.6801	160	-19.16	-1.0599
	hsa-miR-140-5p	-	-	0.7867	146	-14.74	-1.0223
	hsa-miR-143	-	2.7062	0.5475	140	-17	-1.1476
	hsa-miR-191	-	-	0.8292	122	-16.23	-1.231
	hsa-miR-371-5p	-	-	0.6022	123	-14.47	-1.1476
	hsa-miR-490-3p	-	-	0.8292	163	-20.43	-1.2519
	hsa-miR-455-5p	-	5.4722	0.794	124	-13.6	-1.0033
	hsa-miR-539	-	1.9562	0.8367	156	-15.92	-1.1276
	hsa-miR-590-3p	-2.4220	-	0.5933	122	-2.25	-1.2804
	hsa-miR-599	-	-	0.779	147	-11.43	-1.1522
CKS2 (-1.77)	hsa-miR-26a	-	1.0697	0.6592	161	-14.2	-1.1067
	hsa-miR-26b	-	1.4557	0.6592	159	-14.76	-1.0884
	hsa-miR-148a	-	1.7842	0.6151	123	-14.66	-1.074
	hsa-miR-152	1.1990	3.3108	0.6151	122	-12.98	-1.074
	hsa-miR-371-5p	-	-	0.5839	142	-19.55	-1.0092
	hsa-miR-148b	-	3.1462	0.6151	123	-15.69	-1.074
CKS1B (-1.77)	hsa-miR-22	7.2615	8.4297	0.6225	120	-14.51	-1.0772
	hsa-miR-9	-	-	0.7382	156	-15.38	-1.2346
	hsa-miR-361-5p	2.1140	2.4163	0.5899	145	-14.32	-1.1639
	hsa-miR-485-5p	4.0650	3.9162	0.6225	141	-15.09	-1.2285
	hsa-miR-494	-1.0895	-	0.6458	152	-12.89	-1.1605
PLK1 (-1.86)	hsa-miR-181a	1.7470	2.7133	0.6165	120	-9.27	-1.1627
	hsa-miR-181b	-	-	0.6165	124	-15.3	-1.1627
	hsa-miR-181d	-	-	0.6165	124	-15.3	-1.1608

Table 6.2. List of differentially expressed miRNA-target relationship in TNFR2 signaling. The table includes gene symbol and miRNA name with their expression level from dataset (pink box), the conservation, align score, energy as well as mirSVR score retrieved from miRanda online database. The highlighted boxes in red represent miRNAs detected in both SHED and WJSCs.

Gene symbol (fold change)	miRNA name	Log_2(fold change)		Conservation	Align score	Energy	mirSVR score
		SHED	WJSCs				
MAP3K1 (-1.56)	hsa-let-7a	2.2065	6.6852	0.8364	159	-23.11	-1.2313
	hsa-let-7b	-	-1.0822	0.8364	157	-24.28	-1.2326
	hsa-let-7c	-4.4500	1.3812	0.8364	156	-25.35	-1.2313
	hsa-let-7d	-	2.3418	0.8364	159	-19.58	-1.2313
	hsa-let-7e	-	-	0.8364	154	-20.91	-1.2313
	hsa-let-7f	-	1.8073	0.8364	163	-23.07	-1.2299
	hsa-miR-18a	-	4.7937	0.7794	146	-17.17	-1.1455
	hsa-miR-21	2.2225	6.1102	0.7253	144	-12.69	-1.2518
	hsa-miR-23a	2.1370	6.2627	0.7525	148	-11.89	-1.0063
	hsa-miR-26a	-	1.0697	0.5999	124	-7.84	-1.1335
	hsa-miR-33a	-	-	0.7525	144	-11.94	-1.2669
	hsa-miR-98	-	-	0.8364	159	-18.57	-1.2313
	hsa-miR-203	-	-	0.7658	148	-10.59	-1.0582
	hsa-miR-200b	-1.6540	1.6663	0.7658	148	-13.24	-1.0922
	hsa-let-7g	-1.1480	-	0.8364	155	-21.45	-1.2313
	hsa-let-7i	-	-	0.8364	155	-23.03	-1.2326
	hsa-miR-1	-	-	0.7132	156	-14.51	-1.0465
	hsa-miR-23b	2.3550	3.4242	0.7525	148	-14.55	-1.0063
	hsa-miR-144	-5.2870	-3.9853	0.8364	133	-15.82	-1.1327
	hsa-miR-9	-	-	0.8364	140	-11.77	-1.1075
	hsa-miR-206	-	-	0.7132	152	-13.54	-1.0465
	hsa-miR-200c	-	-	0.7658	142	-15.15	-1.0922
	hsa-miR-340	-1.6070		0.8036	163	-11.06	-1.2214
	hsa-miR-18b	1.3705	5.9782	0.7794	152	-19.7	-1.1294
	hsa-miR-429	-	-	0.7658	146	-12.47	-1.0922
	hsa-miR-202	-2.5410	-2.0778	0.8364	126	-18.78	-1.0864
	hsa-miR-494	-1.0895	-	0.7132	161	-14.57	-1.1615
	hsa-miR-505	-4.1735	-	0.717	124	-13.72	-1.0615
	hsa-miR-590-5p	-2.3315	-	0.7253	147	-7.89	-1.2541
	hsa-miR-590-3p	-2.4220	-	0.8308	151	-2.99	-1.2849
	hsa-miR-613	-4.2035	-	0.7132	140	-11.54	-1.0492
	hsa-miR-33b	-	-	0.7525	140	-9.85	-1.2669
	hsa-miR-411	-	-	0.7945	149	-15.39	-1.0958

Table 6.2 (continue)

Gene symbol (fold change)	miRNA name	Log_2(fold change)		Conservation	Align score	Energy	mirSVR score
		SHED	WJSCs				
NAIP (-1.56)	hsa-miR-15a	1.6000	1.7077	0.5279	133	-17.4	-1.1449
	hsa-miR-15a	-	-	0.5189	133	-17.4	-1.1343
	hsa-miR-30a	-	-	0.5747	146	-10.81	-1.2206
	hsa-miR-30c	-	3.8727	0.5747	145	-9.26	-1.2177
	hsa-miR-30d	1.0175	2.4717	0.5747	145	-10.81	-1.2206
	hsa-miR-34a	1.0695	5.6563	0.5619	148	-19.7	-1.0565
	hsa-miR-15b	1.0210	7.9647	0.5279	125	-14.06	-1.1469
	hsa-miR-30b	-	3.6572	0.5747	145	-9.26	-1.2177
	hsa-miR-140-5p	-	-	0.5279	152	-12.26	-1.0505
	hsa-miR-145	-	4.4612	0.542	154	-15.77	-1.2775
	hsa-miR-34c-5p	-	2.3737	0.5619	147	-12.68	-1.0591
	hsa-miR-30e	-	-	0.5747	146	-11.5	-1.2177
	hsa-miR-424	-	5.5922	0.5279	129	-13.92	-1.1429
	hsa-miR-449a	-	-	0.5619	149	-17.77	-1.0643
	hsa-miR-433	-	-	0.5747	142	-12.56	-1.0161
	hsa-miR-497	-	-	0.5279	122	-15.28	-1.1489
	hsa-miR-505	-4.1735	-	0.6586	148	-14.71	-1.211
	hsa-miR-449b	-2.3385	-	0.5619	149	-15.46	-1.0643
TNFAIP3 (-1.92)	hsa-miR-18a	-	4.7937	0.598	168	-15.69	-1.164
	hsa-miR-19a	-	-	0.7007	156	-15.1	-1.3099
	hsa-miR-19b	-	-	0.7007	156	-15.1	-1.3099
	hsa-miR-23a	2.1370	6.2627	0.7145	147	-11.1	-1.2941
	hsa-miR-23b	2.3550	3.4242	0.7145	150	-11.1	-1.2941
	hsa-miR-374a	-	-	0.7007	144	-7.04	-1.2804
	hsa-miR-340	-1.6070	-	0.598	140	-8.11	-1.0451
	hsa-miR-18b	1.3705	5.9782	0.598	163	-15.65	-1.164
PAK6 (-1.56)	hsa-miR-185	-	4.4417	0.6145	145	-16.87	-1.1108
	hsa-miR-302a	-11.8225	-4.2588	0.6264	129	-13.52	-1.0321
	hsa-miR-302b	-	-4.2588	0.6264	123	-9.28	-1.0321
	hsa-miR-302d	-1.4810	-	0.6264	122	-10.29	-1.0321
	hsa-miR-373	-7.9405	-5.2968	0.6264	128	-15.05	-1.0321
	hsa-miR-520e	-10.6935	-5.1203	0.6264	131	-12.48	-1.0321
	hsa-miR-520a-3p	-	-	0.6264	122	-8.78	-1.0349
	hsa-miR-520b	-	-5.4933	0.6264	123	-8.58	-1.0376
	hsa-miR-520c-3p	-14.4515	-	0.6264	124	-9.54	-1.0376
	hsa-miR-520d-3p	-1.6970	-1.6728	0.6264	125	-8.62	-1.0349
BIRC3 (-1.78)	hsa-miR-155	-1.0460	-	0.543	160	-12.3	-1.0062
	hsa-miR-374a	-	-	0.4901	162	-14.41	-1.1549
	hsa-miR-590-3p	-2.4220	-	0.4987	149	-6.61	-1.1004

6.3.2 miRNA-regulated network integration

To further visualize the miRNA-mRNA interactions, the miRNA-regulated network integrations were built based-on results obtained from canonical pathways analysis (Chapter 5) and the correlated miRNA-mRNA pair prediction analysis (Section 6.3.1). The selected canonical pathways that associated with cellular ageing and their predicted corresponding miRNA-miRNA pairs were mapped using IPA software. Figure 6.1 illustrated that the miRNA-mRNA interaction in G2/M DNA damage regulation canonical pathway. One of the miRNA-targeted hub genes that highlighted in this study is the cyclin-dependent kinases regulatory subunit 1 (*CKS1*), cyclin-dependent kinase 1 (*CDK1*) and *Cyclin B*, whereby down-regulation of *CKS1* direct affect *CDK1* and *Cyclin B* expression which lead to the interruption of G2/M DNA damage progression in cell cycle. *CKS1* was predicted to be direct target of miR-22, miR-361-5p, miR-485-5p in both SHED and WJSCs. Furthermore, IPA predicted that interruption of TNFR2 signaling is concomitant with the lower level expression of mitogen-activated protein 3 kinases 1 (MAP3K1) (Figure 6.2). MAP3K1 was predicted to be direct target of let-7a, miR-21, miR-23a, miR-23b, miR-18b, miR-15a in both SHED and WJSCs.

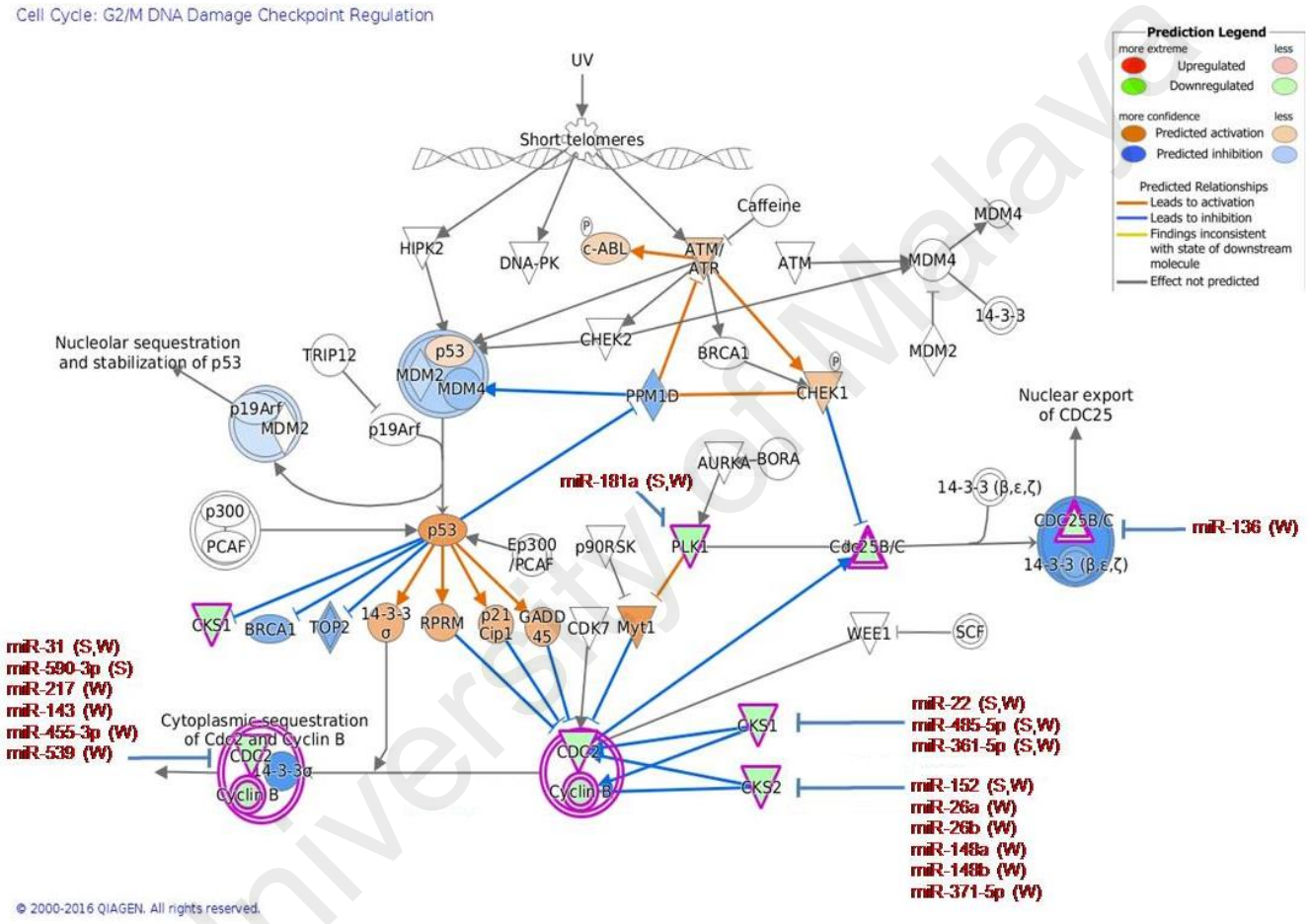


Figure 6.1. Visualization of miRNA-mRNA interaction in G2/M DNA damage regulation canonical pathway
miRNA-mRNA interaction were prediction using web-based miRanda algorithms software. Red shading indicates increased expression in P6 relative to P3 (control), green shading indicates decreased expression in P6 relative to P3 (control), orange shading and arrow indicates predicted activation, blue shading and arrow indicates predicted inhibition, white and gray shading indicates non-expression and non-differential expression respectively, S=SHED and W=WJSCs.

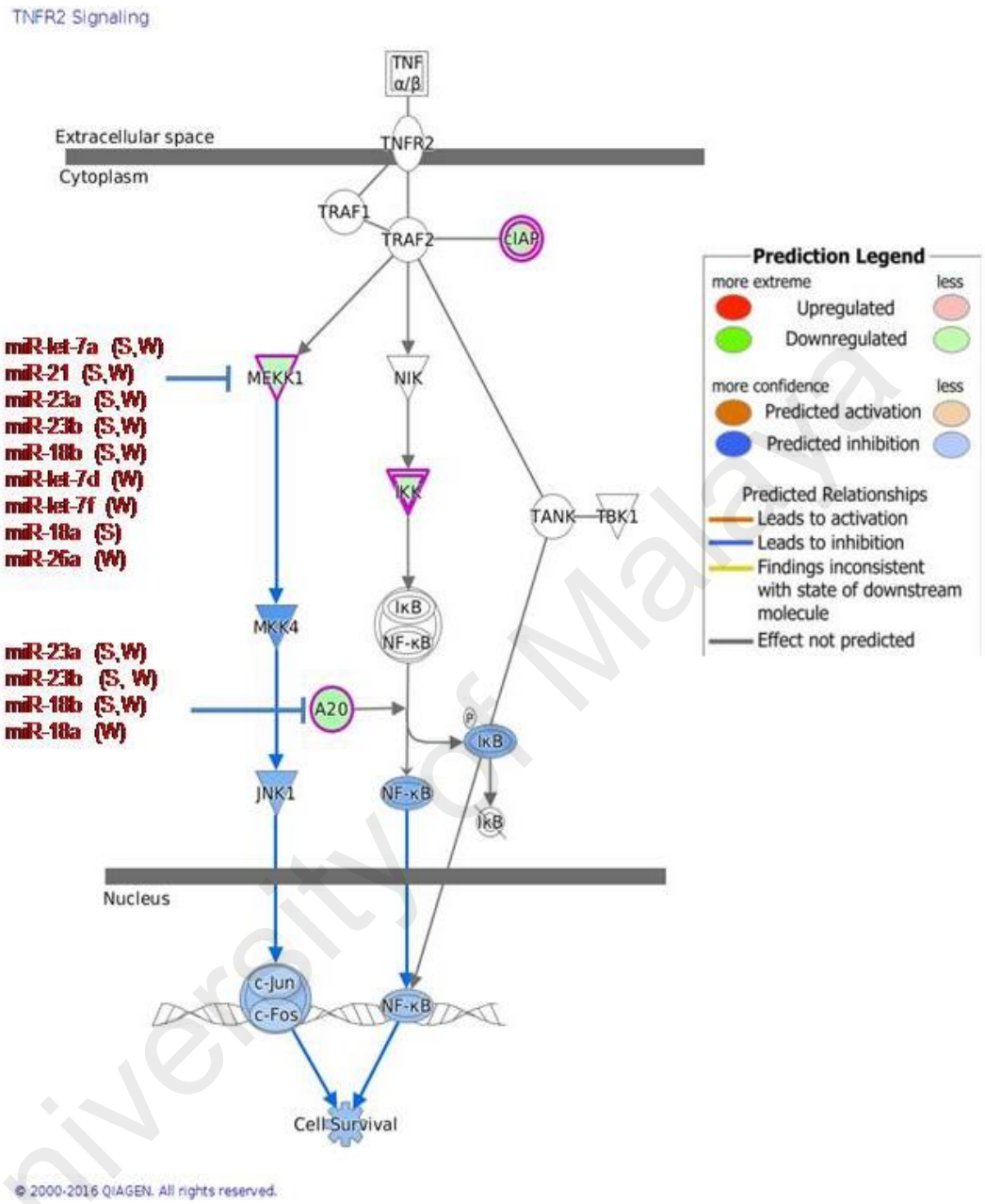


Figure 6.2. Visualization of miRNA-mRNA interaction in TNFR2 canonical pathway

miRNA-mRNA interaction was prediction using web-based miRanda algorithms software. Red shading indicates increased expression in P6 relative to P3 (control), green shading indicates decreased expression in P6 relative to P3 (control), orange shading and arrow indicates predicted activation, blue shading and arrow indicates predicted inhibition, white and gray shading indicates non-expression and non-differential expression respectively, S=SHED and W=WJSCs.

6.3.3 Negative correlation coefficient examination of miRNA-mRNA pairs

Based on the miRanda miRNA-mRNA interaction prediction analysis, it was found that many differentially expressed miRNAs shown to interact with the genes that associated with cellular ageing signaling pathway. In order to measure the strength of correlation interaction between predicted miRNA-mRNA pairs, Pearman correlation coefficient analysis was applied to the qPCR validated data of selected miRNAs (results generated from Chapter 4) and mRNAs (results generated form Chapter 5) of SHED and WJSCs respectively. As shown in Table 6.3, there were four out of six miRNA-mRNA pairs demonstrated a negative correlation (< -0.5), which indicating that they are more likely to be promising interactions.

Table 6.3. Pearman correlation coefficient analysis of selected miRNAs and mRNAs of SHED and WJSCs respectively

Sample	Differentially expressed mRNA	Fold change (n=3)	Differentially expressed miRNA	Fold change (n=3)	Pearman correlation
SHED	*CKS1*	-53.6881 -45.8531 -71.2116	miR-22	20.0709 22.5384 16.0655	-0.99
			miR-485-5p	11.7999 11.9080 8.6973	-0.96
	MAP3K	-14.8157 -13.2701 -12.0781	let-7a	8.6228 9.1571 9.0986	-0.85
WJSCs	*CKS1*	-54.6222 -61.8998 -59.4717	miR-22	10.6281 10.3286 10.0895	-0.70
			miR-485-5p	2.0565 2.2761 3.9199	0.29
	MAP3K	-17.8218 -16.6321 -22.9318	let-7a	139.0534 128.1997 146.3462	0.89

6.4 Discussion

The aim of this study is to improve our understanding of the molecular mechanism and signaling events that negatively interfere with maintenance of stem cell characteristics which resulting in stem cell ageing. Studies from previous chapters shown that the phenotypic changes in SHED and WJSCs culture during *in vitro* passaging were controlled by complex and integrated molecular regulatory events, which was reflected in the alterations in their proliferation capacity as well as the stemness, cell cycle progression and arrest related gene expression. Previous studies have underscored the differentially expressed miRNAs and mRNAs were associated with signaling pathways related to cellular apoptosis, cell cycle, growth and proliferation, stress and injury, growth factors as well as transcriptional factor.

In continuation to this finding, the interaction of previously identified differentially expressed miRNAs and mRNAs were delineated using miRNA-mRNA integration approach. As shown previously in Chapter 5, the IPA revealed that differentially expressed mRNAs in both SHED and WJSCs were likely linked to the induction of the inflammatory signaling pathways, which is evidently implicated in the regulation of cellular senescence. Additionally, the differentially expressed genes in SHED culture during *in vitro* passaging were also found associated with G2/M DNA damage checkpoint and TNFR2 signaling, however this pathway was not noticed in the WJSCs sample and this may due to the differences of lineage origin.

G2/M DNA damage checkpoint signaling was arose to be an important signaling pathway through analysis that this pathway is implicated as one of the checkpoints in the cell cycle, whereby a gap between DNA replication phase (S phase) and cell division phase (M phase) (Branzei and Foiani, 2008). It is a crucial stage in cell cycle where cell can undergo DNA damaged repair and preparation for the next phase of cell

cycle which is the mitosis event (Li and Zou, 2005; Hyka-Nouspikel *et al*., 2012). During the cell cycle progression, CDK1 and Cyclin B complex act as a key regulators that responsible for the transition of G2 phase to M phase followed by triggering the down-stream signaling to promote mitotic mechanism (Rhind and Russell, 2012; Shaltiel *et al*., 2015). Studies from Westbrook *et al*., 2007 reported the depletion of CDK1 interrupt on the entry into M phase.

In this study, the microarray profiling analysis demonstrated that the key genes that acting in this pathway such as *CDK1*, *Cyclin B* (*CCNB1*), *CKS1* and others also showed down-regulation. This results were in agreement with the study by Androic *et al*., (2008) which demonstrated that knockdown of *Cyclin B* has led to inactivation of *CDK1* and consequently inhibited cell proliferation in various cancer cell lines. Indeed, CDK1 characterized as an indispensable key component in G2/M checkpoint as disruption of CDK1 expression cannot be restored by its close family protein CDK2 (Satynarayana *et al*., 2008). On the other hand, CKS1 was proposed as an essential cofactor to stabilize CDK1/Cyclin B complex (Tsai *et al*., 2005, Westbrook *et al*., 2007). A decrease of CDK1/Cyclin B activity resulting in G2/M phase arrest can be observed in the degradation of CKS1 protein.

Given the importance of G2/M checkpoints in regulating cell cycle progression, next, we pursued our analysis to explore the potential miRNA regulators of the corresponding genes in this pathway through the miRNA-mRNA interaction prediction using genomic coordinates retrieved from online database. The miRNA-mRNA correlation analysis predicted that the down-regulation of *CKS1* in SHED is likely due to the hindrance by the differentially expressed of miRNAs miR-22, miR-9, miR-361-5p, miR485-5p, and miR-494. It was also found that *CKS1* were the direct target of these miRNAs which have mirSVR score less than -1. Of the miRNA candidates identified, miR-22, miR-

361-5p and miR-485-5p were found up-regulated in both SHED and WJSCs from P3 to P6 based on miRNA profiling analysis, whereas miR-494 was found down-regulated only in SHED and no differentially expression of miR-9 in either sample. In accordance with this finding, it is reasonable to speculate that the depletion of *CKS1* upon up-regulation of miR-22, miR-485-5p and other miRNAs in SHED in which affecting the G2/M checkpoint might be one of key factors that induce cell cycle arrest, which explains the lowering in proliferation capacity of stem cells cultured in expansion media (Chapter 3).

Our finding is consistent with observation whereby over expression of miR-22 and miR-485-5p inhibited proliferation via direct targeting *SIRT1* and *PGC-1α* expression respectively in human cancer cells (Chen *et al*., 2016; Lou *et al*., 2016), suggesting the possible involvement of epigenetic and metabolic pathways in this ageing models. It has been reported that SIRT1, a histone deacetylase, that play a key role in regulating cellular ageing via direct modification of numerous key transcriptional factors (Wakeling *et al*., 2009). Previous studies demonstrated that activation of SIRT1 leads to inhibition of p53 (Luo *et al*., 2001) and NF-κB (Yeung *et al*., 2004), which is then decrease the pro-apoptotic and pro-inflammatory event respectively. Also, SIRT1 activates PGC-1α expression in responses to metabolic stress then promotes increase of glucose levels (Nemoto *et al*., 2005).

Furthermore, IPA also demonstrated that the differentially expressed genes of SHED culture during *in vitro* passaging were associated with both tumor necrosis factor receptor (TNFR)1 and TNFR2 signaling pathways with decreased activity z-score. Although both TNFR1 and TNFR2 signaling pathway have some degree of common and crosstalk intermediate molecules, they have distinct functions. It is noteworthy that, when tumor necrosis factor (TNF) bind to TNFR 1 will induces apoptosis via TNFR1-

associated death domain (TRADD) and Fas-associated death domain (FADD), whereas activated TNFR2 by TNF will trigger cell survival related functions through NF-κB. (Naudé *et al*., 2011; Rauert *et al*., 2011; Faustman and Davis, 2013). Based on the miRNA-mRNA correlated pair analysis, *MAP3K* appear to be direct target of 33 different miRNAs with mirSVR score less than -1, indicating high confidence binding site for *MAP3K* to various miRNAs. Of the miRNA candidates identified, let-7c, let-7d, let-7f and miR-18a found to be up-regulated only in WJSCs in P6 compared to P3 via miRNA profiling analysis, whereas let-7a, miR-21, miR-23a, miR-23b and miR-18b were found to be up-regulated in both SHED and WJSCs. The results imply that inhibition of *MAP3K* by these above mentioned miRNAs in turn will block the attenuation of C-Jun (*JNK*) cooperates with *NF-κB* in the TNFR2 signaling pathway and resulting in increasing their susceptibility to apoptosis. Taken these together, it is suggested that these are the possible miRNA candidates which negatively regulate *MAP3K* expression in SHED.

A similar observation also reported by other studies in which the expression level of miRNAs let-7 family increases during cell development and differentiation (Zhao *et al*., 2010; Kuppusamy *et al*., 2015). let-7 family is a cluster miRNA that evolutionarily conserved across various animal species including nematode and mammals. In human, let-7 family comprises of more than 8 mature let-7 miRNAs including let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, let-7h and let-7i, which encoded by 12 different genomic loci (Roush and Slack, 2008; Wang *et al*., 2012). It has been reported that the let-7 family targeting multiple genes that involves in the regulation of various biological processes including cell proliferation, for instance E2F2 a transcriptional factor that plays a critical role in cell cycle progression (Iwasaki *et al*., 2015). Indeed, report from Kim *et al*., 2014 has showed that the maintenance of self-renewal and pluripotency characteristics of stem cells can be accomplished by blocking the let-7 expression. Of

note, these observation further support our assumption that differentially expressed of let-7a is a critical contribution factor for stem cell ageing.

Furthermore, miR-21 was reported as oncogene miRNAs that promote anti-apoptosis function, which was found highly expressed in cancer cells including orpharyngeal cancer (Zhang *et al*., 2016), prostate cancer (Dong *et al*., 2015), breast cancer (Yan *et al*., 2011), gliblastoma (Gaur *et al*., 2011). This finding is in contrary to other studies revealed that over expression of miR-21 lead to increase in proliferation, promote tumor formation and growth via direct target of programme cell death 4 (*PDCD 4*) gene (Lu *et al*., 2008; Krichevsky and Gabriely, 2009).

In order to measure the strength of the negative correlation interaction, Pearson correlation coefficient analysis was done between the expression levels of selected miRNA and mRNA that were determined by qPCR validation. The analysis revealed a strong negative correlation between *CDK1* with miR-22 in both SHED and WJSCs. On the other hand, *CDK1* and *MAP3K* were found to be negatively correlated with miR-485-5p and let-7a in SHED, on the contrary, the two were found to have positive correlation in WJSCs. However, evidence of absolute association of above mentioning miRNA- mRNA pair remains to be further investigated. These findings suggest that manipulating the function or expression of desire miRNAs and as such it can directly interfere with the expression of the miRNA targets.

Notwithstanding the above-mentioned miRNA-mRNA interaction promoting cellular ageing, additional reports have been recapitulated the influence of other identified highly differentially expressed miRNAs (Chapter 4) as gene expression regulators in modulating cellular phenotypes alteration (Table 6.4). Particularly, miR-22, miR-485-4p, let-7a, miR-302a, and miR-373 has been elucidated to participate in the regulation of various biological functions including cell proliferation, cell cycle stress, epithelial-

mesenchymal transition (EMT), cell survival, epigenetic regulation and pluripotency through their target genes (Figure 6.3). These findings may in fact connect the deregulation of miRNAs and mRNAs to the phenotypic alteration of SHED and WJSCs during *in vitro* passaging observed in Chapter 3. These observations are inline with previous studies reported that p53 and p21 play an important role to direct a damaged cell either undergo cell survival or apoptosis by regulating the expression of CDKs and Cyclins (Gulappa *et al*., 2013; Schwermer *et al*., 2015).

Table 6.4. Summary of target genes of miR-22, miR-485-4p, let-7a, miR-302a, miR-373, miR-520e and its associated functions in cellular ageing

Differentially expressed miRNA	Target gene	Associated function/pathway	Reference
miR-22	Mediator of DNA damage checkpoint protein 1 (*MDC1*)	DNA damage response pathway	Lee *et al*., 2015
	Histone deacetylase 4 (*HDAC4*)	Epigenetic regulator	Huang *et al*., 2013; Wang *et al*., 2014
	Cyclin dependent kinase 6 (*CDK6*)	Cell cycle	Xu *et al*., 2011
	Sirtuin 1 (*SIRT1*)	Regulate stress and apoptosis related function p53	Huang *et al*., 2013; Jazbutyte *et al*., 2013
	Phosphatase and tensin homolog (*PTEN*)	AKT signaling pathway	Bar and Dikstein, 2010
	Specificity protein 1 (*Sp1*)	Cell growth, cell cycle progression, cell apoptosis	Xu *et al*., 2011; Kong *et al*., 2014; Guo *et al*., 2013;
	Tet methylcytosine dioxygenase 2 (*TET2*)	Epigenetic regulator, DNA demethylation	Song *et al*., 2013
miR-485-5p	High mobility group AT-hook 2 (*HMGA2*)	Cell proliferation, epithelial-mesenchymal transition	Chen *et al*., 2015
	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (*PGC-1α*)	Promotes cell survival upon metablic stress and oxidation stress	Lou *et al*., 2016
let-7a	High mobility group AT-hook 2 (*HMGA2*)	epithelial-mesenchymal transition	Wu *et al*., 2015
	c-Myc	Stem cell self-renewal	Sampson *et al*., 2007
	Tripartite motif containing 71 (*Trim71*)	Cell proliferation	Chang *et al*., 2012
	E2F2	Cell cycle	Iwasaki *et al*., 2015
miR-302a / miR-373	Transforming growth factor, beta receptor II (*TGFβR2*)	Tgfβ signaling	Subramanyam *et al*., 2011; Faherty *et al*., 2012; Kekkikoglou *et al*., 2012
	Lefty	Wnt signaling	Barroso-delJesus *et al*., 2011; Zhou *et al*., 2012; Rosa *et al*., 2014
	Methyl-CpG binding domain protein 2 (*MBD2*)	DNA methylation that repress Nanog	Lee *et al*., 2013; Chen *et al*., 2012
	Large Tumor Suppressor Kinase 2 (*LATS2*)	Hippo signaling pathway	Tian *et al*., 2015; Lee *et al*., 2009
miR-520e	No identify published information		

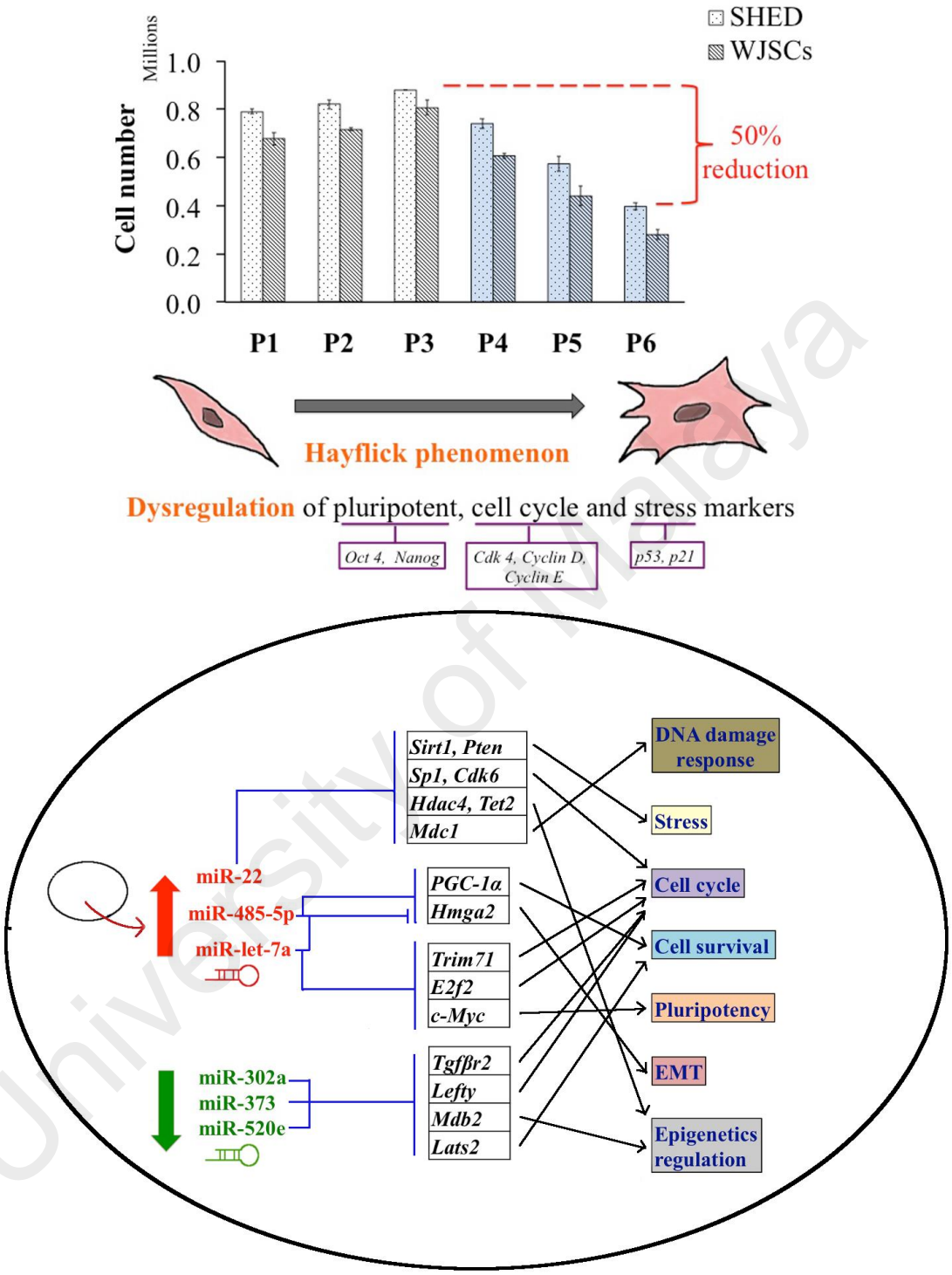


Figure 6.3. The antagonistic actions of miRNAs in regulating the expression of gene associated with cellular ageing

The hMSCs phenotype can be dramatically altered (including reduce in proliferation capacity, Hayflick limited phenomenon as well as deregulation of pluripotent, cell cycle and stress markers) by differentially expressed of miRNAs (up-regulation, red; down-regulated, green) by regulating biological processes (box in colours) through their targeted genes.

Furthermore, these observations implicate the combinations of miRNAs act synergistically to control cellular ageing. Here, a proposed molecular model of "miRNAs switch" in regulate stem cell fate into ageing illustrating how miRNAs exert counterbalancing actions on cellular ageing phenotype. As shown in Figure 7, miRNAs miR-302a/373/520e cluster, miR-22, miR-485-5p and let-7 cluster regulate multiple cellular processes including self-renewal, pluripotency, epigenetic regulation, stress, apoptosis through their target genes. The antagonistic actions of these miRNAs on its target genes dynamically alter cellular phenotypes. Down-regulation of miR-302a/373/520e cluster confers the lost of stem cell regenerative potential via affecting DNA methylation that repress Nanog, as well as growth and proliferation biological processes via WNT and and TGFRβII signaling pathways. However, up-regulation of miR-22 and miR-485-5p activates negative cellular processes associated with ageing events in stem cells by affecting cell cycle via G2/M DNA damage regulation signaling pathway, whereas apoptosis via PTEN signaling. On the other hand, let-7a could impact stem cell pluripotency via c-Myc pathway; EMT signaling via HMGA2; cell apoptosis via TNFRII signaling as well as cell growth and proliferation via AMPK signaling.

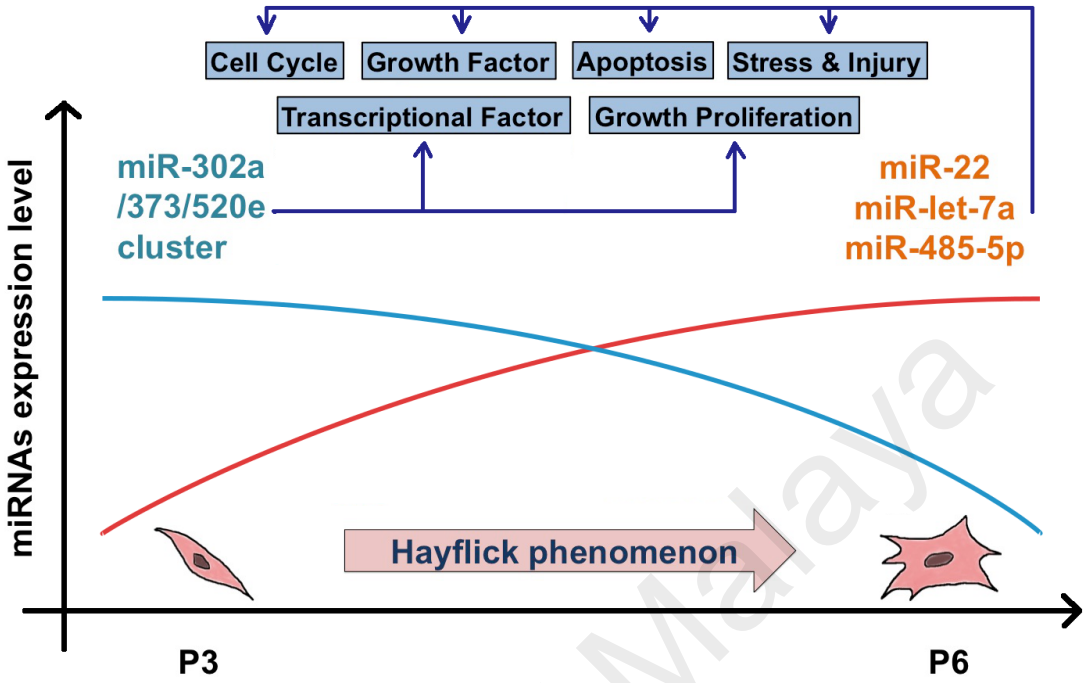


Figure 6.4. Proposed model of "miRNAs switch" which is likely to regulate stem cell fate into ageing

Commonly differentially expressed top miRNAs, miR-302a/373/520e cluster, miR-485-4p, let-7a and miR-22 in SHED and WJSCs are known to regulate multiple cellular processes including self-renewal, pluripotency, epigenetic regulation, stress, apoptosis through their target genes (listed in the blue boxes). The down-regulation of miR-302a/373/520e cluster confers the lost of their stem cell regenerative potential, meanwhile up-regulation of miR-485-5p, let-7a and miR-22 activates negative cellular processes associated with ageing events in stem cell.

Altogether, SHED and WJSCs in culture undergo rapid cell division for limited passages before entering a state of growth arrest. Cells lost its capability to proliferate shown flattened and enlarged morphology. This phenotype is signified as replication senescence, in which has been shown triggered by dysfunction of telomerase that lead to telomere shortening and DNA damage (Serakinci *et la*., 2008). As hMSCs continue to hold great promise for the success of cell-based therapy, it is crucial for us to comprehend the molecular mechanisms underlying replication senescence. Through a detailed comparative analysis of the miRNA and mRNA expression profiles as well as integration analysis have shed further light on the specification mechanism underlying the differentially expressed miRNA and mRNA in SHED and WJSCs during *in vitro* passaging. This study has shown that phenotype changes in both cultures during *in vitro* passaging was characterized by the up-regulation of a subset of miRNAs and create a negative correlation to inhibit the expression of mRNA that related to cellular senescence and ageing.

The identified miRNA-mRNA pairs in this study might be informative for elaborating the cellular ageing in others hMSCs cultures that faces the similar challenge which may limit its application in regenerative medicine research and therapy. We suggested that the identified miRNA-mRNA pairs could be valuable information and may lay the foundation for the development strategies to enhance the quality of various hMSCs cultures for the purpose of clinical application. Our work highlights the potential reason for SHED underwent proliferation arrest may due to the direct impinge on the expression of CKS1 by miRNAs particularly miR-22 and miR-485-5p which lead to down regulation of CDK1 and Cyclin B. This finding may point to an important role of miRNAs as alternative mechanism to interfere DNA damage repair. In this regard, miRNA knockdown approach such as antagomir, chemically engineered oligonucleotides can be used to block the translation of miR-22 and miR-485-5p would

be of interest. Here, we postulated that the expression of CKS1 might be restored by miR-22 and miR-485-5p suppression in which can prevent G2M accumulation. Westbrook *et al*., (2007) showed that CKS1 regulates CDK1 expression at mRNA level, depletion of CDK1 interrupt on the entry into M phase. This approach may be relevant when considering strategies to enhance the quality of hMSCs in cultures for the purpose of clinical application.

The biological influences of replication senescence in cultured cells have been acquired over the past few decades and become the model system for investigating the molecular causes or effectors of senescence-related disease. The findings of our study provide implications as follows. First, understanding senescence and ageing of MSCs in culture set a precaution for the quality of the cells to be transplanted. Furthermore, *in vitro* ageing study provides a control experimental setting in understanding the *in vivo* scenario and precise cellular and molecular processes underlying ageing associate disorder. It is an attractive subject that worth futher exploration particularly the potential therapeutic interventions to counteract the detrimental effects in senescence-related disease such as cancer, heart diseases, diabetes and others. Cells are frequently exposed to exogenous and endogenous stressors which result in inducing phenotype changes. The phenotype alteration can be the result of a few factors, such as telomere shortening, DNA damage, faulty of DNA damage response and others. One example of senescence-related disease is cancers, which are associated with faulty of DNA damage responses that lead to accumulate of DNA damage and genetic instability. Indeed, dynamic efforts have been made to validate DNA damage response as well as to identify potential DNA repair targeted agents for cancer treatment (Kelley *et al*., 2014; Gavande *et al*., 2016).

6.5 Conclusion

1. Integration analysis revealed that the possible interaction of miR-22 and miR-485-5p with *CKS1* which contribute to disruption of G2/M checkpoint in cell cycle progression, indicating the differentially expressed of these miRNA may have a great impact on the hMSCs proliferation capacity.
2. Correlation coefficient analysis revealed a strong negative correlation between miR-22 with *CKS1* in both SHED and WJSCs as well as miR-485-5p with *CKS1* in SHED respectively, indicative of high confidence miRNA target prediction of an activating effect on G2/M checkpoint signaling pathway.
3. Integration analysis also revealed that the possible interaction of let-7a with *MAP3K* which contribute to disruption of TNFR2 signaling pathway and may have impaired the cell survival.
4. Correlation coefficient analysis also revealed that *MAP3K* expression was negatively correlated with let-7a in SHED, indicative of high confidence miRNA target prediction of an activating effect on TNFR2 signaling pathway.
5. A proposed model of "miR-302a and miR-22 switch" derived from the data of this study in the regulation of cellular ageing implicated the miRNAs exert counterbalancing actions in the maintenance of stem cell characteristics.

CHAPTER 7: CONCLUSION

7.1 Summary of research findings

A substantial progress has been made over the past decades to expand our understanding of stem cell with the hope of maximizing its potentials for therapeutic benefits. However, the application of human mesenchymal stem cells (hMSCs) in research and clinical setting is greatly hampered by the scarcity of suitable hMSCs resources as their tissues of origin may be difficult to obtain and the yield of isolated hMSCs is usually inadequate. Hence, it is essential that hMSCs to be expanded *in vitro* to meet the research and therapeutic needs. As hMSCs propagate in expansion culture media to obtain adequate number of cells, distinct phenotypic changes resembling senescence and cellular ageing were often observed along passaging (Wagner *et al*., 2008; Wagner *et al*., 2010b; Estrada *et al*., 2013; Carlos Sepúlveda *et al*., 2014). Previous studies have reported that MSCs culture lost it proliferation capacity during *in vitro* long-term cultivation (Bentivegna *et al*., 2016; Legzdina *et al*., 2016). Hence, these changes limit the maintenance of regenerative potential in long-term culture and thus impair their therapeutic values. However, the knowledge of molecular mechanisms underlying cellular ageing included during *in vitro* passaging in hMSCs remains to be explored. Therefore, this necessitates the comprehensive investigations of cellular and molecular characteristics of MSCs during *in vitro* passaging to uncover other critical and yet unidentified mechanisms that are responsible for maintenance of stem cell characteristics.

In this research, hMSCs from extracted deciduous pulp (SHED) and Wharton's jelly (WJSCs) were isolated, cultured, expanded and characterized for cellular and molecular changes. With its stemness characteristic, deciduous pulp and Wharton's jelly tissues

could be taken into consideration as promising hMSCs sources for the purpose of cell-based therapy can be easily obtained by non-invasive methods. The data presented in this study have also led to a number of interesting findings in regard to phenotypic and molecular changes of SHED and WJSCs upon *in vitro* passaging. Cells from both sources which were passaged in expansion media showed flattened and enlarged morphology alteration remarkably at P3 onwards indicating the emergence of senescence-like cells as passage number increases. Meanwhile, growth kinetics of SHED and WJSCs showed approximately 50% reduction in cell number as well as an increase in cumulative population doubling level (cPDL) from approximately 10 to 20 cPDL from passage (P) 3 to 6, reflecting the decline in their proliferative capacity during passaging. In addition to that, significant reduction was detected in the expression of senescence and stemness associated genes in SHED and WJSCs at passage 6 compared to the earlier passage. Taken these together, reduction in proliferative capacity and stemness markers, in association with increased expression of senescence and cell cycle arrest markers in both SHED and WJSCs during their *in vitro* passaging was evident, which is also a common observation in hMSCs from other sources as reported by many other groups (Wagner *et al*., 2008; Wagner *et al*., 2010a; Estrada *et al*., 2013; Carlos Sepúlveda *et al*., 2014).

Subsequently, this led to the key research question of study on the molecular mechanisms underlying cellular ageing during *in vitro* passaging in these cell types. Given the importance of miRNAs as post-transcriptional regulators in gene expression that coordinate various and most cellular processes, miRNAs expression profiling study using SHED and WJSCs at both passage 6 and passage 3 revealed those differentially expressed miRNAs. Profoundly, the predicted targets of identified differentially expressed miRNAs were clustered to be in the following functions; regulation of proliferation, growth, cell fate, survival and death. Among those differentially expressed

common 108 miRNAs identified, miR-22, let-7a and miR-485-5p, miR-302a, miR-373 were found to be highly differentially expressed in SHED and WJSCs at P6 compared to P3. These miRNAs are known to regulate cellular processes essential and involved in regulation of stem cell characteristics; self-renewal, pluripotency, senescence, epigenetic regulation, oxidative stress which were previously reported by other research groups (Ren *et al*., 2009; Anokye-Danso *et al*., 2011; Subramanyam *et al*., 2011; Lee *et al*., 2013; Song *et al*., 2013; Bu *et al*., 2017).

Furthermore, this work also demonstrated that the mRNA expression profiling of WJSCs at P6 compared to P3 were primarily associated with inflammatory signaling pathways such as interferon, eicosanoid, TREM1 and LXR/RXR signaling pathways which play an integral role as stress inducer that lead to cellular ageing. On the other hand, mRNA related to G2/M DNA damage checkpoint regulation, TNFR1 and TNFR2 signaling pathways were shown to be highly differentially expressed in SHED are known to play critical role in cell cycle progression, in turn govern the proliferation and self-renewal of the stem cells. It is worth noting that the highlighted signaling pathways in SHED were not identical to WJSCs, suggesting existence of different pathways and mechanisms which may be cell type dependent, yet they might be equally important in the induction of cellular senescence and ageing. SHED and WJSCs could have dissimilar differentiation potentials which could lead to different phase of cell ageing, thus resulting in different pathways to induce cellular senescence. We postulated that senescence triggers changes in gene expression might be differs in both SHED and WJSCs, despite both cell types have mutually exclusive expression of miRNAs. One of the possible reasons to explain this discrepancy is that a single miRNA can regulate several mRNA targets and a single transcript can be regulated by many miRNAs. Besides, the replication senescence can differ depending upon the stimulus that induces senescence. For instance, cellular senescence induced by dysfunctional mitichodria is

documented in post-mitotic cells (damaged tissue which cannot be replaced such as neurons and muscle cells) (Lee et al., 2010; Wallace, 2010), whereas human fibroblast is rendered senescent by oncogene activation (Nelson *et al*., 2014). These findings may in fact analogous to what was observed in previous studies that showed that hMSCs undergoing phenotyping alteration due to *in vitro* passaging. Despite the two data set of miRNA and microarray shows different cluster of functions which are differentially expressed in P6 in compare to P3, a comprehensive view of this data indicate interconnected nature of this cellular process leading to cell cycle arrest, DNA damage, senescence and ageing.

Lastly, this study also highlighted the potential miRNA and mRNA pairs that might be involved in regulating *in vitro* cellular ageing. The findings from this study suggested that the possible interaction of miR-22 and miR-485-5p with *CKS1*, in which up-regulate miR-22 and miR-485-5p and inversely correlation with *CKS1* might contribute to the disruption of G2/M checkpoint in cell cycle progression. Besides, it was predicted that the possible interaction of let-7a with *MAP3K* transcript, in which up-regulate the let-7a and favor the interference of TNFR2 signaling pathway by direct targeting of *MAP3K*. This molecular clues warrants future research in understanding the role of those differentially expressed miRNAs which was implicated in the possible miRNA-mRNA pairs to further elucidate the complexity of cellular ageing especially *in vitro* stem cell ageing.

Collectively, all findings in this study led to a key conclusion that cellular ageing is inevitable for both SHED and WJSCs during *in vitro* passaging. The miRNAs and mRNA profiling analysis identified several potential miRNA-mRNA pairs that associated with cellular senescence, stress and ageing associated signaling pathways. Results presented here support the notion of miRNAs as regulators in the

counterbalancing of transcriptional circuitry to support the maintenance of stem cell characteristics. Infer the key miRNA would greatly impact the cellular homeostatic.

7.2 Limitations of study

The findings of this study provide insight into the miRNAs and mRNAs mediating the regulation of cellular ageing in hMSCs during *in vitro* passaging. However, there were limitations and weaknesses that could be avoided in order to produce a more robust experimental data. One of limitation in this study is that the molecular expression profiles may not provide accurate descriptions of the cell behavior in our samples due to the total RNAs samples originated from heterogeneous cell populations. Therefore, analysis at single cell level or in-situ hybridization approach could elucidate the complexcity of molecular expression in regulation of multitude biological functions that represent the whole cell population. Another limitation is that the differentiation potential of hMSCs into adipogenic, chrondorgenic and osteogenic lineages were conducted via trilineage differentiation assay. However, this assay is a subjective assessment as the degree of differentiation potential was not determined. Therefore, it may require further analysis in order to verify these findings such as qPCR and staining intensity evaluation using software such as by ImageJ (Wayne Rasband, retired from National Institutes of Health).

Methods to indicate cell senescent in our study are limited to growth kinetic study and cell cycle genes. This approch lack specificity, therefore additional analytical approaches are necessary to enhance the ability to determine cell senescence *in vitro*, for example the senescence-associated beta-galactosidase (SA-β-gal) assay (Chen *et al*., 2013).

As previously discussed, RNA microarrays analysis was employed to determine gene expression, nonetheless the value of gene expression data that acquired are influence by the number of biological replications. Despite promising findings generated in this study, there were limitations and weaknesses that could be avoided in order to produce a more robust experimental data. For instance, adequate replications are recommended in order to produce a high statistical power that enables us to correctly produce experimental data with higher reliability and accuracy. However, the experimental cost often dictates the replication number that can be accomplished. Therefore, suggest that we ought to include more biological samples of our observation in future research study. Furthermore, bioinformatics tool was employed to translate the differentially expressed miRNAs and genes data obtained from array analysis into functional categories. However, functional interpretation result may differ across different existing analytical platform. Therefore, it is suggested to compare and to validate the generated data using multiple analytical tools in order to obtain accurate functional interpretation result. For instance, validation of protein expression by western blotting and immunocytochemistry techniques

7.3 Future perspective

Overall, our study revealed the importance of miRNAs as regulator in counterbalancing the transcriptional circuitry to support the maintenance of stem cell characteristics, alterations in miRNAs expression can greatly impact cellular homeostatic. Therefore, the potential roles of miRNAs in regulation stemness in stem cells merit further exploration. A detailed understanding of the miRNA regulatory network implicated in stem cells ageing pave a path for the development of strategies to delay or reverse the ageing of hMSCs obtained from primary sources during their expansion aiming to

produces higher quality and quantity of cellular resources for clinical applications. With this study, we hope to provide a framework to identify the key miRNAs that are potential to antagonize the senescence related pathways in stem cells by repress the expression of targeted miRNAs by knockdown or knockout approach.

Lastly, there are several potential directions that could be extended in this study. One of the important and promising avenues for future work is the use of these identified miRNAs as biomarkers to detect premature cell senescence. Besides, further exploration and understanding of the deregulated miRNAs in human stem cells is necessary to translate such valuable information into practical applications for the benefits in regenerative medicine. Furthermore, this application could be extended in other cell types, particularly those which are related to ageing-associated diseases. The proposed model incorporates in this study provides a better insights into the key miRNAs regulation mechanism in cellular ageing and we recommends that this findings to be validated and further explored in future research study using many other cellular models.

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