EVALUATION OF DENTAL PULP STEM CELLS' GROWTH PHASES ON THEIR DIFFERENTIATION TOWARDS DOPAMINERGIC-LIKE CELLS

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Evaluation of dental pulp stem cells' growth phases on their differentiation towards dopaminergic-like cells

Field of Study: Regenerative Dentistry

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ABSTRACT

Parkinson's disease (PD) is a debilitating, incurable neurodegenerative movement disorder that is defined by the gradual emergence of motor as well as non-motor symptoms and affects approximately 3% of people over sixty-five years old. The two pathological hallmarks of PD include progressive deterioration of dopaminergic (DA-ergic) neurons within the substantia nigra (SN) and the widespread distribution of Lewy bodies. As a result, a large numbers of DA-ergic neurons are needed to replace the depleted neurons to revert the symptoms presented by PD patients. Cell replacement therapy (CRT) has been suggested to be the future possible treatment modality since current treatments were deemed ineffective in managing symptoms of PD. Among the widely described cell source, dental pulp stem cells from extracted deciduous tooth (SHEDs) has been regarded as the optimal cell source owing to the fact that they are originate from neural crest and display high neuronal differentiation capacity, migratory activity rate and regenerative potential. Furthermore, the inherent characteristics of cells such as growth phase are hypothesized to play significant roles in determining their regenerative potential.

In this study, both *in vitro* and *in vivo* capabilities of SHEDs at selected growth phases were investigated, firstly differentiation towards DA-ergic-like cells, followed by determination of respective gene as well as protein expression. It was demonstrated that neuronal markers such as nestin and β-tubulin-III as well as matured DA-ergic markers like tyrosine hydroxylase (TH) were highly up-regulated in SHEDs-Day 7 which was also reflected in their functional behaviour ($p<0.05$). The differential output (i.e differentiation capacity and functionality) obtained from various growth phases were believed to be directly involved with their cell cycle profile.

Further, the immunomodulatory behaviour of SHEDs was also evaluated in a microenvironment depicting neuro-inflammation of typical PD scenarios. The cells were shown to significantly alter the profile of inflammatory cytokines secreted such as interleukins-1α, 6, 8, interferon-gamma, as well as tumor necrosis factor-α, but were unable to regulate the levels of nitric oxide and reactive oxygen species ($p<0.05$). This findings indicate that they exhibit some degree of neuro-protective ability over damaged neurons.

Finally, SHEDs were evaluated for their homing capacity as well as their regenerative behaviour when they were transplanted via intrathecal in a MPTP-induced mice model. Behavioural assessments were measured from the perspective of sensorimotor as well as olfactory functions. Our findings revealed that PD-induced mice, transplanted with SHEDs displayed improved behavioural changes (approximately 60%) as early as week 8 post-transplantation ($p<0.05$). The same pattern was also observed in the immunostaining of TH, DA transporter and DA decarboxylase in SN as well as striatum of their brain. In addition, immunohistochemistry analysis revealed homing capacity of SHEDs at areas related to SN which was identified from the expression of matured DA marker, TH.

In conclusion, this study has highlighted the regenerative capacity of SHEDs from the perspective of differential growth phases. It is suggested that the growth phase of cells to be considered as one of many important parameter when designing personalised cell replacement therapies in the future.

ABSTRAK

Penyakit Parkinson (PD) merupakan penyakit yang didiagnos dengan kemunculan simptom berasaskan keupayaan motor serta bukan motor yang menjejaskan hampir 3% dari pesakit yang berumur lebih dari enam puluh lima tahun. Dua petanda yang jelas dalam PD ialah kemerosotan neuron dopaminegik (DA-ergic) secara progresif dan kemunculan 'Lewy bodies' secara menyeluruh. Sehubungan dengan itu, penambahan neuron DA-ergic perlu dibuat untuk menggantikan neuron yang telah berkurangan. Terapi penggantian sel (CRT) telah dikenalpasti sebagai satu kaedah terkini yang mungkin boleh mengubati gejala yang ditunjukkan oleh pesakit PD memandangkan kaedah rawatan sekarang kurang berkesan. Antara pelbagai jenis sel-sel stem yang terdapat di tubuh manusia, sel stem dari pulpa dari gigi desidus (SHED) yang telah dicabut boleh merupakan suatu sumber yang perlu dipertimbangkan memandangkan keupayaan proliferasi and migrasi yang tinggi serta kebolehan menjana semula neuron. Tambahan pula, keadaan dalaman sel-sel seperti fasa pertumbuhan dianggap sebagai faktor yang memainkan peranan penting dalam menentukan potensi penjanaan semula sel stem. Dalam peyelidikan ini, kedua-dua kajian secara *in vitro* dan *in vivo* telah dijalankan, pertamanya pengolahan keadaan fizikal dan fungsi ke arah sel seumpana DA-ergic, diikuti dengan pengesanan ekspresi gen dan protein. Tanda-tanda perubahan dari segi gen serta protein seperti Nestin, beta-tubulin-III, dan petanda matang bagi DA-ergic iaitu 'tyrosine hydroxylase' (TH) jelas menunjukkan keupayaan mereka secara signifikan berbanding dengan kumpulan fasa pertumbuhan yang lain ($p<0.05$).

Seterusnya, keupayaan mereka dari segi mengubah rembesan faktor-faktor immunologi juga telah dikaji secara teliti. SHED didapati menujukkan potensi untuk memodulasikan rembesan sitokin inflamasi seperti interleukin-1α, 6, 8, interferon

gamma, dan juga tumor nekrosis faktor-alpha, selain mengawal tahap oksida nitrik dan spesies oksigen reaktif ($p<0.05$). Penemuan ini menunjukkan bahawa SHED berpotensi untuk melidungi neuron dari dicedrakan dalam situasi inflamasi.

Akhir sekali, keupayaan SHED untuk menjana semula dan mengawal tingkah laku tikus yang telah diberi penyakit PD menggunakan MPTP secara 'intrathecal'. Penilaian tingkah laku tikus dibuat dari segi sensorimotor serta olfaktori. Penemuan menunjukkan bahawa tingkah laku tikus-tikus yang menerima SHED berubah secara signifikan seawal minggu ke-lapan (hampir 60%) berbanding dengan kumpulan-kumpulan yang lain ($p<0.05$). Corak yang sama juga diperhatikan dalam ujian pengesanan sel melalui antibodi TH, DA 'transporter' dan DA 'decarboxylase' di substantia nigra (SN) serta striatum. Tambahan pula, analisis secara terperinci telah mendedahkan keupayaan SHED untuk diintegrasikan di kawasan SN yang berkaitan.

Kesimpulannya, kajian ini telah menggambarkan keupayaan penjanaan semula SHED dari perspektif fasa pertumbuhan yang berbeza dan faktor ini perlu diambilkira apabila CRT dijalankan.

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A2A	:	Adenosine A_{2A} receptor
AADC	:	Aromatic l-amino acid decarboxylase
AD	:	Alzheimer's Disease
ANOVA	:	Analysis of Variance
ATP	:	Adenosine Triphosphate
β-TUB	:	Beta-Tubulin
BBB	:	Blood Brain Barrier
BDNF	:	Brain-Derived Neurotrophic Factor
bFGF	:	Basic Fibroblast Growth Factor
BMP	:	Bone Morphogenetic Proteins
BMSC	:	Bone Marrow Mesenchymal Stem Cells
Brn4	:	Brain-4
BSA	:	Bovine Serum Albumin
c-Myc	:	Cellular Myelocytomatosis
c-kit	:	Cellular Based Tyrosine-Protein Kinase Kit
CD	:	Cluster of Differentiation
cDNA	:	Complementary DNA
CDNF	:	Cerebral Dopamine Neurotrophic Factor
ChAT	:	Choline Acetyltransferase
CK	:	Cytokeratin
CM	:	Conditioned Media
CNC	:	Cranial Neural Crest
CNTF	:	Ciliary Neurotrophic Factor
COMT	:	Catechol-O-methyl Transferase
CRT	:	Cell Replacement Therapy
CSF	:	Cerebrospinal Fluid

CT	:	Computed Tomography
CXCL	:	Chemokine (C-X-C motif) ligand 1
CXCR	:	CXC chemokine receptors
DA-ergic	:	Dopaminergic
DAB	:	3,3'-diaminobenzidine tetrahydrochloride
DAPI	:	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DAT	:	Dopamine Transporter
DBS	:	Deep Brain Stimulation
DDCIs	:	Dopamine Decarboxylase Inhibitors
DEPC	:	Diethylpyrocarbonate
DFPCs	:	Dental Follicle Progenitor Stem Cells
DG	:	Dentate Gyrus
DMEM	:	Dulbecco's Modified Eagle Medium
DNA	:	Deoxyribonucleic acid
DPBS	:	Dulbecco's phosphate-buffered saline
DPC	:	Dental Pulp Cells
DPSCs	:	Dental Pulp Stem Cells
DPX	:	Distyrene tricresyl phosphate xylene
DSP	:	Dentin Sialoprotein
DTT	:	Dithiothreitol
ECM	:	Extracellular matrix
EDTA	:	Ethylene-diamine-tetra acetic acid
EGF	:	Epidermal Growth Factor
ESCs	:	Embryonic Stem Cells
FBS	:	Fetal Bovine Serum
FFPE	:	Formalin Fixed Paraffin Embedded
FGF	:	Fibroblast Growth Factor

FITC	:	Fluorescein isothiocyanate
GABA	:	Gamma-Aminobutyric acid
GAD	:	Glutamic Acid Decarboxylase
GDNF	:	Glial Cell Line-derived Neurotrophic Factor
GID	:	Graft Induced Dyskinesia
GMP	:	Good Manufacturing Practice
GPe	:	External Globus Pallidus
GPi	:	Internal Globus Pallidus
hDPCs	:	Human Dental Pulp Cells
HLA-DR	:	Human Leukocyte Antigen - D Related
IA	:	Intra-arterial
ICV	:	Intracerebroventricular
IFN	:	Interferon
IgG	:	Immunoglobulin G
IL	:	Interleukin
IP	:	Intraperitoneal
IPSCs	:	Induced Pluripotent Stem Cells
IT	:	Intrathecal
IV	:	Intravenous
KCl	:	Potassium Chloride
kd	:	Kinase domain
Klf4	:	Kruppel-like factor 4
KO	:	Knock Out
L-DOPA	:	L-3,4-dihydroxyphenylalanine
LANGFR	:	Low-Affinity Nerve Growth Factor Receptor
LB	:	Lewy Bodies
LGP	:	Lateral Globus Pallidus
MAP-2	:	Microtubule-associated Protein-2

MGP	:	Medial Globus Pallidus
MAO	:	Monoamine oxidase
MOG	:	Myelin Oligodendrocyte Glycoprotein
MPP^+	:	1-methyl-4-phenylpyridinium
MPTP	:	1-Methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine
MSCs	:	Mesenchymal Stem Cells
NCAM	:	Neural Cell Adhesion Molecule
NADPH	:	Nicotinamide adenine dinucleotide phosphate
Nanog	:	Homeobox transcription factor
NESTIN	:	Neuroectodermal stem cell marker
NeuN	:	Neuronal specific nuclear protein
NGF	:	Nerve Growth Factor
NICD	:	Notch1 Intracellular Domain
NMDA	:	N-methyl-D-aspartate
NR4A2	:	Nuclear receptor related 1 protein
NSCs	:	Neural Stem Cells
NURR1	:	Nuclear receptor related 1 protein
ODHA	:	6-hydroxydopamine
OCT-4	:	Octamer-binding transcription factor 4
PBS	:	Phosphate-Buffered Saline
PBST	:	Phosphate Buffered Saline With 0.05% Tween-20
PCR	:	Polymerase Chain Reaction
PD	:	Parkinson’s Disease
PDGF	:	Platelet-Derived Growth Factor
PDLSCs	:	Periodontal Ligament Stem Cells
PE	:	Pulmonary embolism
PET	:	Positron Emission Tomography

PFA	:	Paraformaldehyde
PKH26	:	Paul Karl Horan 26
PLGA	:	Poly (lactic-co-glycolic acid)
p75	:	Nerve Growth Factor Receptor
RNA	:	Ribonucleic acid
rRNA	:	Ribosomal ribonucleic acid
ROS	:	Reactive Oxygen Species
rpm	:	Revolutions per minute
RT	:	Room Temperature
SCAPs	:	Stem Cells From Apical Papilla
SD	:	Standard Deviation
SDF	:	Stromal Cell-Derived Factor
SDS	:	sodium dodecyl sulfate
sec	:	Seconds
SHEDs	:	Stem Cells from Human Exfoliated Deciduous Teeth
SN	:	Substantia Nigra
SNpc	:	Substantia Nigra Pars Compacta
SNpr	:	Substantia Nigra Pars Reticularis
SOX	:	SRY-related HMG-box
SPSS	:	Statistical Package for the Social Sciences
SSEA	:	Stage-Specific Embryonic Antigen
STN	:	Subthalamic Nucleus
STRO-1	:	stromal precursor antigen-1
SVZ	:	Subventricular Zone
TH	:	Tyrosine Hydroxylase
TNFα	:	Transforming Growth Factor-Alpha
TRA	:	T Cell Receptor Alpha Locus

TUBB	:	Tubulin, Beta Class
UCB	:	Umbilical Cord Blood
UiTM	:	University Teknologi Mara
VGCC	:	Voltage Gated Calcium Channels
VM	:	Ventral Mesencephalon
VMAT	:	Vesicular Monoamine Transporter
WNT5A	:	Wingless-Type MMTV Integration Site Family, Member 5A
3-0-MD	:	3-0-methyldopa

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CHAPTER 1: INTRODUCTION

1.1 Study background

In our body, stem cells (SCs) serve as cellular source for repair due to their capabilities for extensive proliferation (self-renewal) and differentiation. These regenerative capacities have made them to be useful especially for the treatment of degenerative diseases and disorders in form of cell replacement therapy (CRT). The benefit of such therapy include enhanced functional recovery and less rejection or allergic issues as compared to treatment modality using synthetic drugs (Mount, Ward, Kefalas, & Hyllner, 2015). Despite numerous studies demonstrating the efficacy of stem cells both in *in vitro* and *in vivo* models, their effectiveness in clinical trials however do not truly reflecting the aforementioned advantages.

There are several factors that contribute to successful clinical application of stem cells. These include; a suitable cell source, optimal cell count for transplantation, optimized culturing method, presence of genetic modification (on case-to-case basis), route of delivery and also post-transplantation procedures (L. Liu et al., 2015). Various factors have made their manipulation difficult to be controlled leading to variability in their therapeutic/clinical outputs. In the neurological disorder such as Parkinson disease (PD) for instance, the variability of a number of parameters are acknowledged and thoroughly investigated with utmost importance given to degree of disease, cell preparation as well as transplantation procedures (Lindvall, 2015). This shows that changes in any of these parameters can lead to significant shifts in the outcome of clinical trials.

In this study, a parameter that is equally important but has not been given its due significance namely the growth phase of stem cells from exfoliated human deciduous tooth (SHED) was proposed to be investigated. In any transplantation studies, cell count and viability are two major factors being considered and their quantification/association

with clinical outcome is not routinely measured but especially, their intrinsic condition is left unnoticed most of the time (Lee et al., 2015). In general, the cell culture confluency rate (known as population doubling time) is briefly highlighted but again the rate varies from one laboratory to another simply due to lack of standardization in culture technique. Having said that, we believe that by controlling the growth phase of cells, the general outcome of any transplantation work can be unified and positively be improved. Owing to their proliferative behaviour, cells are considered to be present in their cell cycle phases such as in G0/G1, S and G2/M phases (Pauklin & Vallier, 2013). By understanding the cell cycle phases at a particular time point, their directed differentiation capability could possibly be enhanced which in turn could serve as optimal cell condition for transplantation.

On another note, among the widely available cell sources, dental pulp stem cells from human extracted deciduous teeth (SHEDs) have been regarded as the next promising candidate for CRT due to their ease of access and having less ethical hurdles. They also display excellent proliferative behaviour and multi-potentiality towards various cell lineages. In particular, they can also be deployed for CRT with regards to neurological disorders because of their neural crest origin. We have previously reported the ability of SHEDs to differentiate into dopaminergic (DA-ergic)-like cells with distinct functionality profiles (N. Gnanasegaran, V. Govindasamy, & N. H. Abu Kasim, 2015). By utilizing SHEDs as cell source, a thorough analysis to demonstrate the influence played by their intrinsic condition with regards to application for CRT in PD will be conducted.

The following research questions are formulated for further investigation:

1) How does growth phase influence the differentiation efficiency of SHEDs into DA-ergic-like cells?
2) Can the pre-selected SHEDs cells (based on cell cycle phases) modulate immuno-modulatory behaviour when subjected to conditions mimicking PD?
3) Are the pre-determined cells (based on cell cycle phases) able to home and exhibit their regenerative potential upon transplantation in an animal model?

1.2 Aim and objectives

The aim of this study was to study the role of cells' kinetics (growth phase, differentiation efficiency and regenerative potential) of SHEDs as a potential stem cell source for the treatment (repair/regeneration) of PD. Therefore, the objectives of this study are to:

1) investigate the distinct influence of growth phases of SHEDs in their differentiation potential towards DA-ergic like cells
2) elucidate the immuno-modulatory behaviour of SHEDs from selected growth phase in an *in vitro* model of PD
3) study the neuro-restoration and neuro-protection ability conferred by SHEDs from selected growth phase in PD mice model.

The integration provided from both 'on the bench' and 'in living body' models in this study will help us to understand the necessity of considering cells' growth phase for CRT purposes. The workflow as shown below provides an overview of this study.

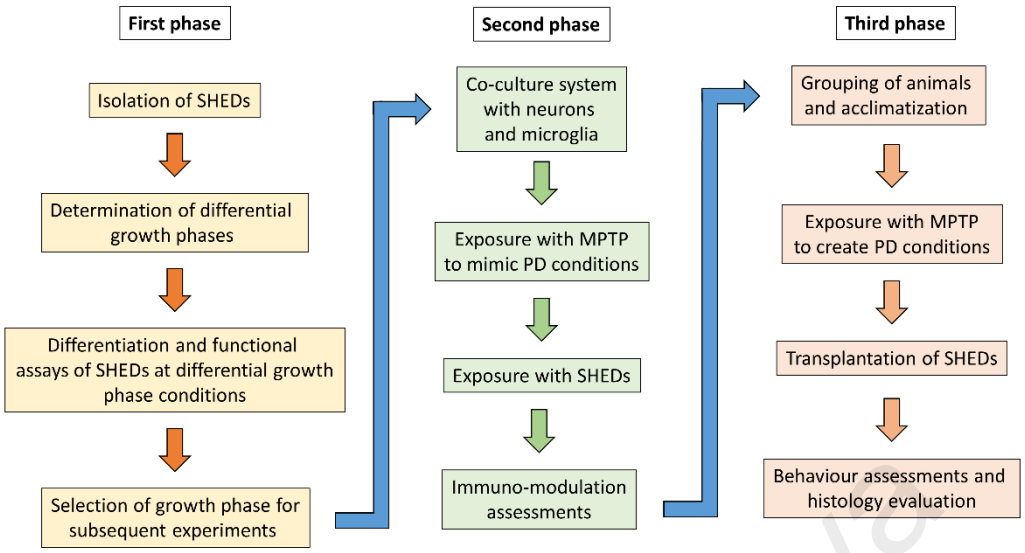

Figure 1-1: Workflow of the study. This study comprises of three inter-linked phases that would possibly answer the applicability of SHEDs as a treatment modality for PD

CHAPTER 2: LITERATURE REVIEW

2.1 Stem cell and its niche

It is needless to say that SCs are prevalent throughout our body. They play pivotal role in organogenesis as early as during embryonic development and persists throughout adulthood and aid tissue repair and regeneration. Essentially, they have exclusive ability to renew themselves and also to differentiate into variety cell types. There are two naturally occurring SC categories, namely embryonic stem cells (ESC) and adult stem cells (ASC) (Aron Badin et al., 2016). The former arises from inner cell mass of blastocysts with the potential to generate all three embryonic germ layers namely ectoderm, mesoderm and endoderm. Following to this, the embryo would then additionally form either somatic stem cells (SSCs) for organogenesis or germ line stem cells (GSCs) for reproduction. Despite displaying distinct differences, their main feature is self-renewal. They would give rise to multiple cell lineages (an ability known as multipotent), committed to their respective lineage or even generating single lineage cells (known as unipotent) which is specific for certain tissues (Lee, Zhang, & Le, 2014).

During development, these cells (GSCs and SSCs) tend to harbour themselves in a specific location or microenvironment known as ‘niche’ that varies among tissues. Here they serve as an integral component for maintaining the internal control mechanisms (homeostasis) such as to sustain on-going tissue regeneration via replacement of injured or dead cells. This function requires a distinct balance between self-renewal and differentiation. This fine line presents itself as the critical factor determining the fundamentals of tumour formation, SC regulation as well as their therapeutic usages in human disorders (Tatullo, Marrelli, Shakesheff, & White, 2014).

2.1.1 The stem cell biology in tooth organogenesis

During embryogenesis especially around six weeks post-gestation, the embryo has yet to form its typical recognizable figure with length of less than an inch. Despite this, cross talks that facilitate initiation and guidance of tooth formation have already begun. The complexity involved in signalling pathways is one main reason for inability to grow teeth and other organs in the laboratory settings (Mayo, Sawatari, Huang, & Garcia-Godoy, 2014).

Classically, the generation of organs would arise with the interaction of at least two different cell types such as mesenchymal and epithelial, and this pattern holds true in teeth formation as well. The first inductive cues instructing mesenchymal stem cells (MSCs) to trigger tooth formation and related tissues such as jawbone are secreted mainly by oral epithelial cells which are intended to form lining of oral cavities. In return, they would respond to epithelial cells by sending similar molecular signals and this would persists throughout development of embryonic tooth. In the beginning, the embryonic oral epithelium would get thickened and they begin to infiltrate the underlying mesenchymal tissues. By the time the embryo reaches seventh week, the epithelial tissues would condense and develop the tooth bud (J. Liu et al., 2015).

At about 14 weeks, the epithelial tissues of tooth bud penetrate further and condense around the mesenchyme to form bell-shaped structure. Eventually, around six to twelve months after birth the epithelial tissues of tooth bud form the outer layer of enamel which erupts from baby's gum line. Subsequently the nonvisible portions of the tooth, like dental pulp, dentin, cementum and also periodontal ligament which bridges the tooth to the jawbone emerge from the mesenchymal portion of developing tooth (Varga & Gerber, 2014).

It should be noted that the epithelial signals that trigger odontogenesis are also partly responsible for activating vital genes that facilitate formation of jaw mesenchyme. During embryogenesis, homeobox genes take part in establishing the formation and setting of organs as well as their supporting tissues. Activation of homeobox genes are location-dependant, as this would give rise to activation of distinct pathways and form either premolar, molar, incisor or canine. For instance, when Barx1 gene is switched on, mesenchymal cells differentiate into incisors teeth. *In vivo* studies have shown that the knockdown of Barx1 gene leads to formation of incisors instead of molar teeth (Lymperi, Ligoudistianou, Taraslia, Kontakiotis, & Anastasiadou, 2013).

From the early stages of evolution, i.e the time when pharyngeal teeth were developed in jawless fish, the preservation of general gene network is prevalent (Fraser et al., 2009). As such, the regulatory network is well conserved throughout different species (Richman & Handrigan, 2011; Tummers & Thesleff, 2009). As well as gene and regulatory network conservation, odontogenesis is also similar to other ectodermally derived developing appendages such as hair, nails and exocrine glands (Mikkola, 2009). The progress of tooth development occurs in several morphological stages, beginning from dental epithelium deriving from ectoderm interacting with the underlying cranial neural crest-derived mesenchyme (Chai et al., 2000) (Fig. 2.1).

Emerging from the thickening of ectoderm, the dental epithelium or dental lamina would first form a dental placode. Proliferation of cells within placode would take place to form a bud which will be termed as bud stage subsequently. The cells in the bud continue proliferating and the developing tooth transforms into the cap stage. At this stage, the enamel knot would be formed specifically on the border between the epithelium and mesenchyme. This knot comprises of cells that do not proliferate and governs growth of epithelial and shaping of tooth. It should be noted that during cap stage, the epithelium

partially encapsulate the dental mesenchyme. With the formation of cervical loops at the apical part of the epithelium, a dental papilla is then formed. As the mesenchyme is condensed between the outer parts of the cervical loops, the dental follicle eventually generate osteoblasts, cementoblasts, and periodontal ligament. In brief, osteoblast will deposit alveolar bone matrix while cementoblasts will cover the root surfaces with cement. On the other hand, periodontal ligaments will attach the root of the tooth to the alveolar bone (Egusa, Sonoyama, Nishimura, Atsuta, & Akiyama, 2012).

The epithelial part of the developing tooth during cap stage is termed as enamel organ. This part consists of three different of cell layers. The core is the stellate reticulum, with star-shaped cells, surrounded by the inner enamel epithelium and the outer enamel epithelium (IEE and OEE). Further on, the tooth organ acquires the form of a bell and consequently, this stage is called as the bell stage. New cell types appear and hard matrix starts to form. The cells in the dental papilla that oppose the IEE differentiate into odontoblasts, which eventually deposit dentin matrix. The epithelial cells adjacent to the odontoblasts differentiate into ameloblasts and produce enamel matrix. Mineralization of the enamel and dentin matrix starts at the cusp tips and moves towards the base. Nerve fibers, although surrounding the developing tooth anlage in basket like-formations at much earlier stages, begin to enter the dental papilla at the late bell/early mineralizing phase (Fried, Lillesaar, Sime, Kaukua, & Patarroyo, 2007).

At the base of the bell-shaped developing tooth, the IEE and OEE form a bilayer called the Hertwig's epithelial root sheet (HERS). This bilayer grows apically, and directs the growth of the root. It is also responsible to stimulate the adjacent dental mesenchyme to form odontoblasts, as part of root dentin. However, the HERS does not promote ameloblast differentiation, and thus no enamel would be formed at the root (Jernvall & Thesleff, 2000).

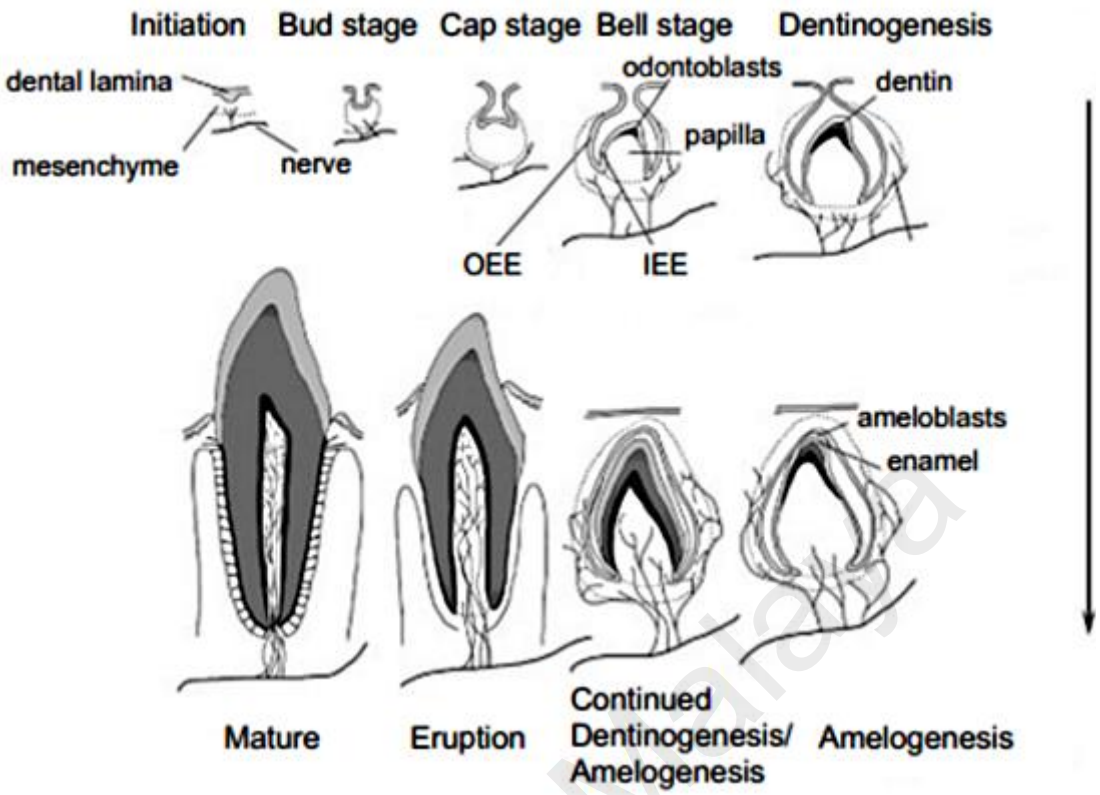

Figure 2-1: Schematic illustration of human tooth development. Modified from Li et al (2015)

It should be noted that the entire process of tooth formation takes a long time in the human dentition. The initiation of tooth morphogenesis normally starts on the fifth week of gestation, and the first deciduous tooth starts erupting at around 6 months post-partum. In the mouse dentition however, teeth are initiated at embryonic day (E) 12 and the incisor erupts at postnatal day 10-12, i.e. a rather fast process (Duailibi et al., 2011; J. Li et al., 2015; Zegarelli, 1944). Pioneering studies have demonstrated that the initial and inducing competence resides in the epithelium. In mouse, this is before E12, the stage when a placode is visible. After E12, the competence has switched to the underlying mesenchyme. Thus, at this stage this mesenchyme can be combined with epithelium from other sites, e.g. the second branchial arch, and teeth would still be formed (Mina & Kollar, 1987). Hence, there are two pre-requisites, an epithelium and a neural crest-derived mesenchyme (Lumsden, 1988). A major signalling pathway for tooth initiation is the

Wnt/beta catenin system. When this pathway is suppressed by Dkk1, no teeth are formed (Wang et al., 2009). Induction of the Wnt pathway in mutant embryos gives rise to supernumerary teeth, but only in the oral region because the competence to form teeth resides only there (Liu et al., 2008; Wang et al., 2009).

Surprisingly, it is not known how the dental lamina is established, and even more striking is the fact that there are no mutant embryos that are unable to form one. Thus, the initiating factor(s) remain to be identified. The WNT/beta catenin pathway signalling induces Bmp4 expression in the mesenchyme, which in turn induces sonic hedgehog (SHH) expression in the epithelium. The condensation of the underlying mesenchyme during the placode stage, is controlled by signalling with FGF8 and Semaphorin 3 from the dental lamina. When important mesenchymal transcription factors are depleted, tooth development is arrested at placode or bud stage (Bei, 2009). Also, when mutant mice are stimulated with different signalling pathways, supernumerary teeth are formed in the diastema region. Such manipulations of signalling pathways and their downstream genes involve overexpression of the gene Ectodysplasin in K14-Eda mouse line, enhanced FGF signalling in Sprouty gene mutants, enhanced SHH signalling in Polaris mutants and mutation of Sostdc1, which is a gene that modulates both BMP and WNT signalling pathways (Ahn, Sanderson, Klein, & Krumlauf, 2010; Ohazama et al., 2009).

The gene p63 has been shown to be important for the initiation of ectodermal placode formation. When p63 is deleted, the mutant mice do not develop any type of ectodermal placodes. Nevertheless, there is a dental lamina formed, but the tooth development stops at this stage. The signalling pathways that are impaired are BMP, Eda, FGF and Notch (Laurikkala et al., 2006). Another important signalling pathway in tooth formation is Eda (ectodysplasin). Impairment of genes in Eda pathways often contributes to defects in

ectodermal appendages. It manifests itself as either missing or imperfect teeth (humans and mice), or defects in hair and sweat glands (humans) (Mikkola, 2009).

2.2 Types of dental derived stem cells

Until recently, interest in the potential application of MSCs of dental origin for tissue repair and regeneration have emerged. In line with this, a number of stem cell populations were discovered. These include stem cells from periodontal ligament (PDLSC) (Seo et al., 2004), dental pulp stem cells (DPSCs) (Gronthos et al., 2002; Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Huang, Pelaez, Dominguez-Bendala, Garcia-Godoy, & Cheung, 2009), apical papilla derived stem cells (SCAP) (Morsczeck et al., 2005), stem cells from pulp of human exfoliated deciduous teeth (SHED) (Miura et al., 2003), as well as dental follicle precursor cells (DFPC; Fig. 2.2). These groups of cells are well known for their potency in adhering to plastic surfaces, manifestation of mesenchymal stem cell markers, multipotent capacity to differentiate, tendency to form colonies in culture as well as their capability to regenerate dentin in *in vivo* settings (Bluteau, Luder, De Bari, & Mitsiadis, 2008; Yen & Sharpe, 2008).

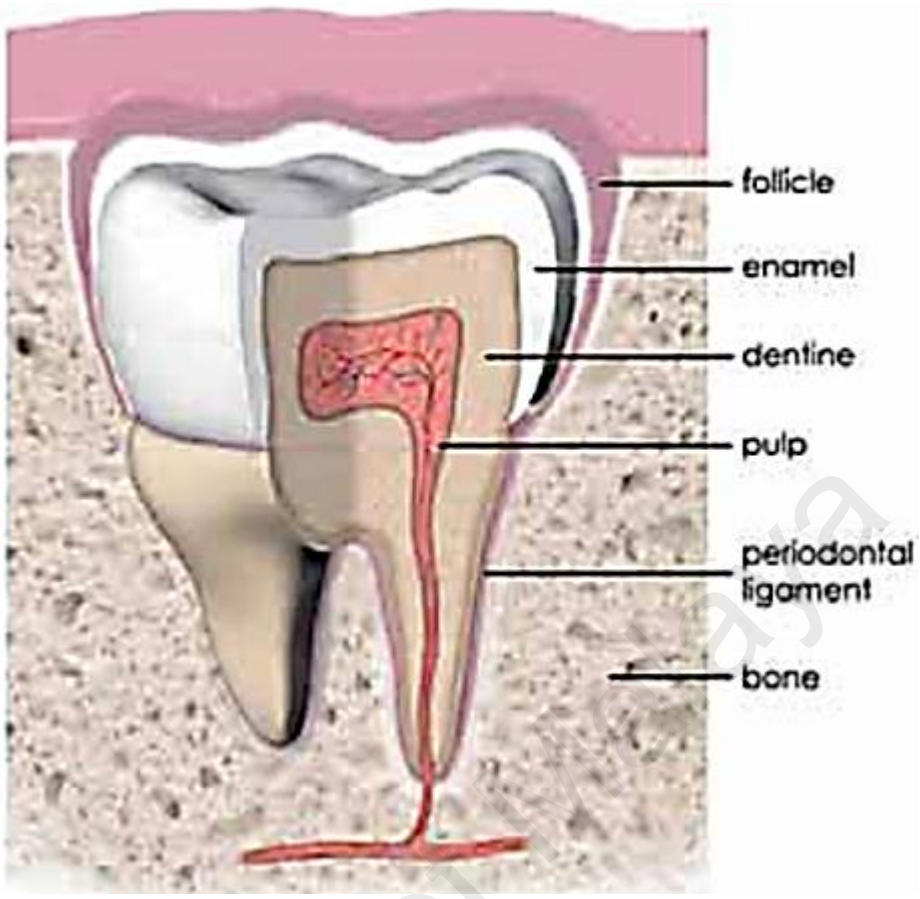

Figure 2-2: Schematic representations of developing tooth structures. Modified from an image produced by Alqahtani (2010).

DPSCs reside in the central cavity of the pulp tissues (Figure 2.2). They inherently undergo phenotypic transformation towards osteoblastic, odontoblastic, adipocytic as well as neuronic cell types *in vitro* (Alqahtani, Hector, & Liversidge, 2010; Nuti, Corallo, Chan, Ferrari, & Gerami-Naini, 2016; Yu et al., 2015).

Periodontal ligaments form connective tissue layers around the dental root that hold it in place (Seo et al., 2004) (Figure 2.2). From this tissue, a heterogeneous cell population with ability to differentiate into cementoblastic, osteoblastic and adipogenic lineages known as PDLSCs can be obtained (Morsczeck et al., 2005). In addition, dental follicle is another vital structure that surrounds the developing tooth germ as loose connective tissues (DFPC). The eruption of tooth and placement of progenitor cells for periodontium development are coordinated by this follicle (Lee, Chambers, Tomishima, & Studer,

2010; Wise, Frazier-Bowers, & D'souza, 2002) (Figure 2.2). Nevertheless, the differentiation aptitude of DFCs have not been comprehensively investigated.

The aforementioned cell sources especially those deriving from third molars have great implication for utilization in cell therapy simply because they represent as ever-propagating tissue that can be isolated with minimal surgical interventions under local anesthesia (Ikeda et al., 2008). Furthermore, they could be exploited by means of tissue engineering to generate more cellular materials for tissue repair as compared to those which can be generated *in situ* during their lifetime (Gronthos et al., 2000). Despite this, it should be noted that further studies are imperative to elucidate their properties as well as their regenerative capacity.

Stem/progenitor cells are present in both deciduous and permanent tooth pulps. Gronthos and collaborators were the first to isolate stem cells in the pulp of permanent third molars. These cells were transplanted into immune-compromised teeth, where they differentiated and produced dentin (Gronthos et al., 2000). They termed these cells (that came from permanent teeth) Dental Pulp Stem Cells (DPSCs). Subsequently, pulpal SCs from human exfoliated deciduous teeth (SHED), were isolated (Miura et al., 2003).

2.2.1 Stem cells from human exfoliated deciduous teeth (SHEDs)

SHEDs is known to be different from DPSCs though they share similar features and differentiation capabilities. First and foremost, they are unable to form a complete pulp-dentin complex when transplanted in vivo and instead recruit osteoblasts into the site with new bone formation as a result. Another feature which differs, is that they can be cultivated not only on plastic with adherence, but also as neurospheres, much like neural SCs (Miura et al., 2003).

2.2.2 Dental Pulp Stem Cells (DPSCs)

Previous studies have indicated that DPSCs have characteristics similar to bone marrow mesenchymal SCs (BMMSCs). They share the ability to adhere to plastic culture surfaces and to form clonogenic cultures (which indicates a potential to self-renew). When using the clonogenic assay on plastic culture dishes, only twenty percent of the seeded cells are clonogenic. Flow cytometry sorting for STRO-1, 3G5 and CD146 in dissociated pulp cells and culture of these cells, has resulted in clonogenic colonies (Gronthos et al., 2002; Shi & Gronthos, 2003). These markers are typical for smooth muscle cells and pericytes, and these DPSCs have been highly associated with blood vessels. They also share some bone marrow stem cell-associated markers such as CD44, STRO-1, CD146 and CD105 (Huang et al., 2009). The difference between these two sources is that the latter can differentiate into odontoblast-like cells and contribute in formation of pulp-dentin complex when transplanted *in vivo* (Gronthos et al., 2000; Huang, Shagramanova, & Chan, 2006). One study on dogs has shown that DPSCs can be used to regenerate the pulp after pulpectomy (Iohara et al., 2011). DPSCs are multipotent and could differentiate into myocytes, chondroblasts/chondrocytes as well as osteoblasts/osteocytes. Another vital and prospective potential of DPSCs is their ability to differentiate into neuronal-like cells, which could be useful for axonal guidance (Arthur et al., 2009). In addition to this, SCs which are isolated from inflamed pulp are called as DPSCs-IP. These cells share many characteristics similar to those from normal pulps and express markers such as CD73 and CD146. Moreover, they can also differentiate and contribute to deposition of dentin, but their potential for this use is often reduced, probably due to the inflammatory processes (Alongi et al., 2010).

One of the pioneer researcher indicating the presence of BrdU which is a marker of dividing cells within cells of dental pulp is Harada et al. (1999). Complementarily, DPSCs per se were first isolated by Gronthos and colleagues (Gronthos et al., 2000) via plastic

adherent assay and were demonstrated to possess two defining features of SCs namely self-renewal and multi-lineage differentiation capabilities. Three years later, another report was published by the same group reporting successful isolation of a SC population from human exfoliated deciduous teeth, referred to as SHED (Miura et al., 2003). Apart from having similar differentiation capacity like DPSCs, their higher proliferation suggested their immature state. DPSCs have been widely reported to display mesenchymal marker STRO-1 but not the hematopoietic indicator CD45 (Sakai et al., 2012) and similar discovery was also noted in DPSCs from mice (Guimarães et al., 2011). Furthermore, genes in which both SHED and DPSCs express include Nestin, GFAP, CD90 and βIII-tubulin (Sakai et al., 2012). There is a dispute whereby Gronthos group has reported that DPSCs are positive for the expression of βIII-tubulin only post-differentiation; not prior to differentiation as claimed by Arthur et al. (2009). Owing to its irregularities particularly when studied in various labs, it can be considered that these cells have not been fully characterized as of yet.

2.3 Potential uses in regenerative medicine

2.3.1 Justification of using SHEDs in neurodegenerative diseases

A number of studies have indicated the usefulness of oral and dental SCs in various treatments such as in osseous integration of titanium implants (Nakamura, Saruwatari, Aita, Takeuchi, & Ogawa, 2005), periodontal and maxillofacial regeneration (Aimetti, Ferrarotti, Cricenti, Mariani, & Romano, 2014; Alkaisi et al., 2013), and dental pulp regeneration after endodontic treatment (Zhu, Wang, Liu, Huang, & Zhang, 2014), another growing body of evidence suggest that adult SCs have vast capacity for varied non-dental biomedical applications (Ding, Niu, & Wei, 2015; Xiao & Nasu, 2014; Zhao & Chai, 2015). The focus of research has shifted to neural lineages derived from oral and dental SCs, simply due to the fact that they possess an innate neurogenic prospect and

differentiation capacity as compared to other ASCs due to their derivation from the embryonic neural crest (Achilleos & Trainor, 2012; La Noce et al., 2014).

2.3.2 SHEDs as neural crest derived SCs

During vertebrate embryogenesis, the neural crest which is a transient distinct structure that gives rise to the CNS (Mayanil, 2013), besides assorted multitude of other lineages which include dental mesenchyme (Miletich & Sharpe, 2004; Mitsiadis, Feki, Papaccio, & Catón, 2011). The vital role demonstrated by this structure has often left it regarded as the 'fourth germ layer' in vertebrate embryogenesis (Hall, 2008) apart from endoderm, mesoderm and ectoderm. During the process of neurulation, the closing of neural tube at the junction of epidermal ectoderm had triggered the formation of the neural crest. These mixed population of SCs within the neural crest had initiated them to migrate along distinct pathways to specified locations within the developing vertebrate embryo, transiting from epithelial-to-mesenchymal lineage along the process (Hall, 2008; Rinon et al., 2011) and differentiating into multitude well-designed lineages such as those of the CNS and the dental mesenchyme (Mayanil, 2013; Mitsiadis et al., 2011). The interaction between stomodeal exoderm lining the interior of emerging oral cavity and dental mesenchyme would generate tooth and its corresponding tissues like the periodontium and its associated oral and dental-derived ASCs (Harada & Ohshima, 2004; Huang et al., 2009). The pioneering study which utilizes transgenic reporter genes such as R26R and Wnt1 to identify the progeny of cranial neural crest during tooth and mandible development. Additionally, it was demonstrated that during mammalian embryogenesis, the migrating neural crest cells give rise to the condensed dental mesenchyme (Chai et al., 2000). This was further verified by another study which had utilized a transgenic reporter gene (LacZ) to trace the cells of neural crest origin in the developing tooth (Yamazaki, Tsuneto, Yoshino, Yamamura, & Hayashi, 2007).

2.4 Mechanisms contributing to regenerative efficacy

2.4.1 Cell procurement and up-scaling

2.4.1.1 Good Manufacturing Practice (GMP) requirement

As gene and cellular therapies are more apparent with the advancement of technology, clinical laboratories which are able to conduct cellular engineering with adherence to good manufacturing practices (GMPs) policies are extremely vital. The need for monitoring laboratory processes have become more extensive, in which possible adverse events to the recipient would occur if conducted otherwise (Burger, 2000). Particularly in Malaysia, the National Pharmaceutical Regulatory Agency (NPRA) is taking pro-active measures in monitoring the compliance of cGMP regulations in clinical laboratories with strict adherence to requirements set by major regulatory bodies such as United States of America Food and Drug Administration (USA-FDA) and European Medicine Agency (EMA).

Regulatory bodies such as the FDA and EMA have immense attention in regards to such techniques conferring to the extent of manipulation involved as well as the adverse effects of post-processing-related events (Harvath, 2000; Rehmann & Morgan, 2009). In instances of very slight handling like cryopreservation of peripheral blood progenitor cells (PBPC) from autologous donors may be conducted via good tissue practices (GTPs), which already has similar control level as those being practiced in clinical laboratories. However, when more-than-minimal manipulation is concerned, an elevated degree of laboratory complexity on top of process is deemed necessary, which in turn requires current good manufacturing practices (GMPs). Examples of more-than-minimal manipulations include *ex vivo* expansion, transduction, activation, combination with non-tissue components, for the usage of other than the tissue's typical role, as well as transplantation of discrete allogeneic tissues and cells (Harvath, 2000).

The application of advanced clinical cell engineering from cGMP point of view require diverse demands especially on laboratory design and operation. More often than not, applications of cGMP are custom-made, with more focus given on processed cell product for individual patients. For instance in Europe, MSCs such as BMMSCs and ASCs are regarded as advanced therapeutic products, as clearly described by the European Regulation. Besides depending on the source, manufacturing processes and proposed indications, MSCs are also considered as somatic-cell therapy or tissue-engineered products on occasions (Rehmann & Morgan, 2009).

Looking at the importance of adhering to cGMP compliance, the modification that are necessary to convert the current protocols which help produce clinical grade ASCs require careful assessments (Sensebe, Krampera, Schrezenmeier, Bourin, & Giordano, 2010). One important aspect which warrants strict control is quality control. This include process controls which qualify the methods involved in production of cells as well as their corresponding functional tests. The integration of various analyses such as karyotype, quantitative expression of telomerase, fluorescent in situ hybridization (FISH), and c-myc would ensure that cells did not undergo any sort of transformation behaviours. In due course, the quality controls must also take viability and phenotype tests such as differentiation capacity of the cells and proliferation rate into account, so that they are compatible with a rapid release of the graft (Sensebe et al., 2010).

Thus, *in vitro* expansion is the technique used to obtain sufficient cell number for large-scale production of MSCs. To achieve this, optimization of factors like the culture conditions and maintenance of phenotypic as well as genotypic stability of MSCs during multiple passages are indeed highly concentrated. Even though the optimal condition of culturing MSCs is yet to be defined, α-MEM or DMEM are basically/commonly used, on top of supplementation with serums such as FBS, human serum or plasma as well as

growth factors. However in GMP production, the implementation of FBS necessitates separate certification to control the risk associated to transmission of infectious disease. In the following sub-heading, a number of factors pertaining to culture media and supplements for the utilization in GMP production will be thoroughly discussed (Inamdar & Inamdar, 2013).

2.4.1.2 Stem cell culture conditions

The utilization of culture media for *in vitro* culture have vital impacts on proliferation and differentiation of SCs. Usually, ASCs are cultivated in traditional culture media like the minimal essential media (MEM), Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640 and Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F-12 (DMEM:F-12) containing balanced salt solutions with the supplementation of serum (V. Govindasamy et al., 2010; Lohmann et al., 2012).

From a viewpoint of cell culturing, the addition of serum supplements have great benefits as they provide vital nutrients, growth factors and attachment factors to the cells. Nevertheless, it should be noted that different species origin and serum concentrations have variable influence the proliferation of ASCs (Sundberg & Isacson, 2014). As an example, FBS is known to be rich in growth factors and stimulates protein accretion in cell cultures. However, the introduction of human serum derivatives like human platelet lysate, allogeneic AB serum and thrombin-activated platelet-rich plasma have been reported to provide equal or probably even higher proliferation rates and multi-lineage differentiation capacity (Lohmann et al., 2012; Vasanthan et al., 2014).

However, from the perspective of culturing cells aimed for clinical therapy, FBS has been indicated as an unsuitable option. This is based on the possibility that xenogeneic antibodies, such as Neu5GC which can be introduced when culturing, could be accidentally transmitted during transplantation thereby increasing the risk of initiating

severe immune response (Govindasamy et al., 2011). Furthermore, there are instances though being rare where severe anaphylaxis and immune reactions have been reported in patients transplanted with human cells exposed to xenogeneic reagents (Colombo, Moore, Hartgerink, & D'Souza, 2014; Shi et al., 2014). The possibilities of transmitting bacterial or viral infections, prions, and as well as unidentified zoonoses remain highly elevated too. Interestingly, Spees and co-workers have reported that the xenogeneic antigens can be possibly removed by up to 99.99 percent. They have demonstrated the removal of xenogeneic internalized antigens from BMSCs initially cultivated in 20% fetal calf serum (FCS) by incubating cells in AHS^+ (autologous human serum) supplemented with10 ng/mL basic fibroblast growth factor (bFGF) and 10 ng/mL EGF. It has also been demonstrated that extended cultivation of hMSCs in AHS^+ for 5–10 days could reduce contamination rate of FCS (Spees et al., 2004).

Looking at the importance of eliminating the problem of introducing xenogeneic or allogeneic antibodies into the patient, it is suggested that AHS^+ can be used as the optimal selection for clinical applications. Despite of this, there are presence of conflicting results in terms of proliferation rate and differentiation potential with AHS^+ as compared to FBS (Stute et al., 2004). Some authors have reported higher proliferation rates using AHS^+ with BMSCs (Dahl et al., 2002; Nimura et al., 2008), while others have exhibited yields of AHS^+ similar to that of FBS (Spees et al., 2004; Yamamoto et al., 2003). From the perspective of differentiation, a study has described an improved differentiation ability towards osteogenic and adipogenic using AHS^+ as compared to FBS (Oreffo & Triffitt, 1999), while another study has revealed similar outcome for osteogenic differentiation using AHS^+ in comparison to FBS (Yamamoto et al., 2003). Moreover, due to limited access and availability as well as presence of high variability have significantly impeded to usage of autoHS for large-scale stem cell production (Bieback et al., 2009; Brinchmann, 2008). The composition of serum include variable amounts of growth

factors and cytokines, like epidermal growth factor (EGF), bone morphogenetic proteins (BMPs), platelet derived growth factor (PDGF) just to name a few and they are mostly uncharacterized with distinct lot-to-lot variation which may influence their reproducibility (Guimarães et al., 2013; Herrera & Inman, 2009; Salvade et al., 2009).

2.4.1.3 Ideal cell conditions or preparation for usage of regenerative medicine

In simple terms, regenerative medicine is a multidisciplinary research field which has evolved hand-in-hand with the biotechnology. It comprises the incorporation of stem cells, growth factors and biomaterials to replace, repair, or regenerate tissues/organs which are impaired by injuries or diseases (Das, Sundell, & Koka, 2012; Sundelacruz & Kaplan, 2009).

The underlying factors to a successful tissue engineered constructs would differ among various applications. As an example, tissues that are designed to improve the quality of life may allow for a higher margin of errors in comparison to those that are designed to prolong life. For instance, regeneration or replacement of blood vessels or even bone would expect to last for an extended period of time whereas cartilage replacement would be considered sufficient if it delays the total joint replacement procedure for another five to ten years (Wichmann, DeLong, Guridi, & Obeso, 2011).

SCs are indeed an ideal source for regenerative medicine due to their capability to self-renew and their differentiation commitments towards multiple lineages (Gimble, Katz, & Bunnell, 2007; Yu et al., 2011). Therefore, the optimal criteria of SCs especially for regenerative medicine applications are as follow:

1. To be obtained in abundant quantity (from millions to billions of cells)

2. Can be isolated by a marginally invasive technique with minimal morbidity rate

3. Have the capability to differentiate into multiple cell lineages in a controlled and reproducible manner

4. Can be transplanted safely and effectively to either an allogeneic or autologous host

5. Can be mass-propagated in accordance with cGMP requirements

2.5 Degenerative disease: Parkinson's disease (PD)

Parkinson's disease (PD) is a chronic, neurodegenerative disease which affects at least one million people in the United States (U.S.). World-wide estimates suggest there are seven to 10 million people living with PD. A slow progressive decline in functioning of people with PD requires ongoing care often exceeding a decade. In the advanced stages of PD, people with PD may require more supportive care due to increased discomfort from functional limitations and cognitive decline (Bunting-Perry, 2006). As the majority of care for people with PD in the U.S. is provided at home by family members, family caregivers play vital roles in the care of advanced PD (Aarsland, Larsen, Tandberg & Laake, 2000; Goetz & Stebbins, 1993). Caregivers of people with PD have reported their unmet need for detailed information about the prognosis of the disease to make necessary decisions for future care and assistance with physical tasks and emotional stress (Goy et al, 2008).

2.5.1 Aetiology of PD

As briefly described earlier, one of the main pathophysiological features of PD is the progressive degeneration of the nigrostriatal pathway in the basal ganglia, namely the DA-ergic neurons and terminals of the SN and the striatum respectively. Additionally, nigrostriatal pathway which locates the non-DA-ergic systems are also thought to be affected in the pathology of PD. Lewy bodies' formation is also another

pathophysiological feature. Recently, the involvement of neuro-inflammation has been indicated as a causative factor of PD (Barker et al., 2016).

2.5.1.1 Nigrostriatal Degeneration

Although PD encompasses a spectrum of both motor and non-motor manifestations, our understanding of the pathophysiologic origin of these non-motor alterations remains very limited. DA-ergic neuron loss has been identified as a pathological hallmark of PD and is thought to underpin the motor aspects of the disease, in particular the cardinal features of bradykinesia and akinesia (Wichmann et al., 2011). It is therefore necessary to define the role of the basal ganglia in modulating cortical motor function in order to better understand how loss of DA may affect motor control (Kopin, 1993; Lindvall, 2016).

2.5.1.2 The basal ganglia

The basal ganglia refers to a collection of subcortical nuclei that are important in the control of movements including: the caudate nucleus and caudate putamen (together deemed the neostriatum); the globus pallidus pars externa (GPe) and the globus pallidus pars interna (GPi); the SN pars compacta (SNpc) and the SN pars reticulata (SNpr) and the subthalamic nucleus (STN) (Fox & Brotchie, 2010). The inhibitory neurotransmitter gamma-aminobutyric acid (GABA) plays a major role in the functioning basal ganglia where the striatum is composed of approximately 95% GABAergic medium spinal neurons (MSNs) (Alavian et al., 2014). There are two major basal ganglia pathways in the motor circuitry: a ‘direct’ pathway connecting the striatum to the GPi and SNr, whereas an ‘indirect’ pathway which links the striatum to the output nuclei of the basal ganglia; however in this instance the fibers first pass through synaptic connections in the external segment of the GPe followed by the STN (Walker et al., 2015).

The direct (with high expression of excitatory DA D1 receptors) and indirect (with high expression of inhibitory DA D2 receptors) pathways work in synergy to facilitate normal motor function (Fig. 2.3). Activation of D receptors, by release of DA in the striatum, activates the direct pathway resulting in the inhibition of GPi and SNr neurons that reduces inhibition of thalamocortical projection neurons and thus facilitates of movement. Activation of D2 receptors of the indirect pathway leads to augmented inhibition of the GPe, subsequently inhibition of the thalamus (via excitation of the STN), which ultimately reduces the glutamatergic excitation of motor cortical areas that would compete with voluntary movement, a role which is equally essential for normal motor function (Barker et al., 2016).

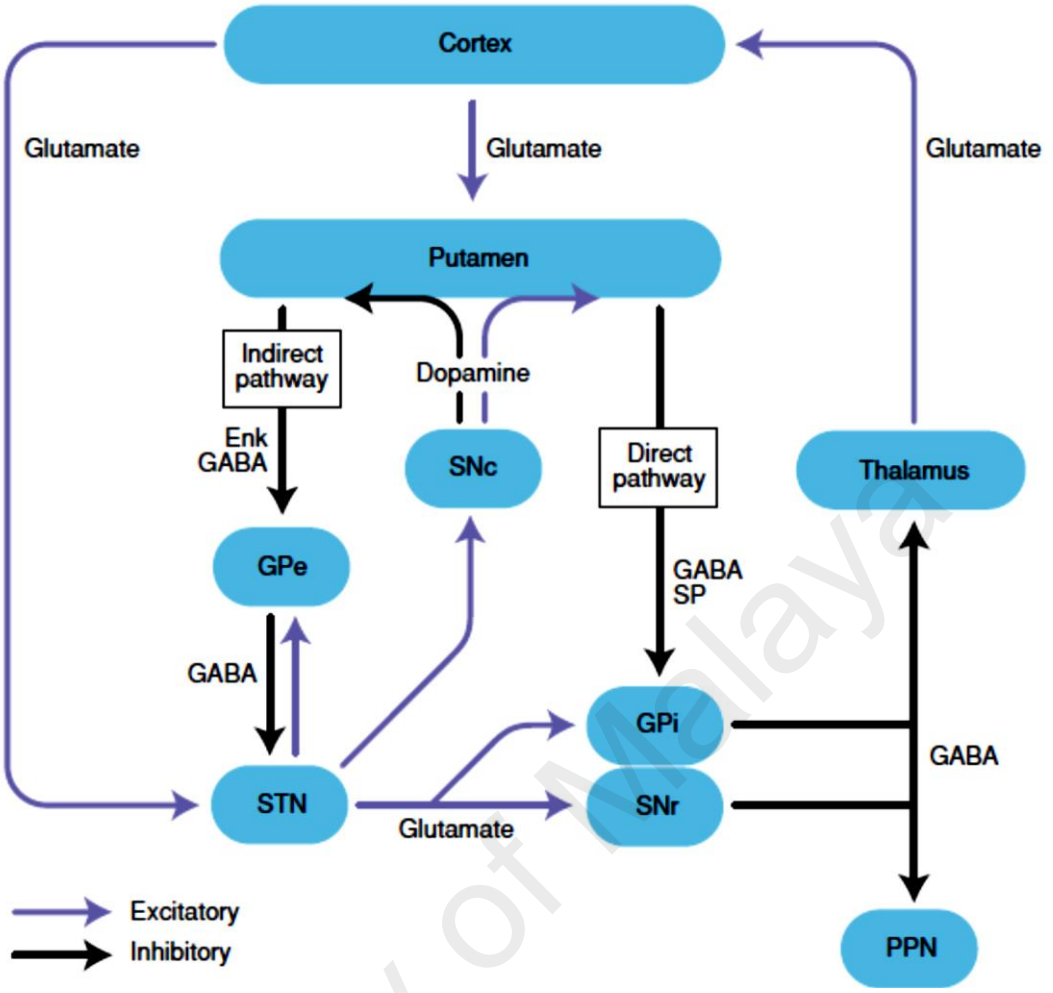

Figure 2-3: The typical conditions of basal ganglia circuitry. In the brain, parallel neuronal networks of striatum link and assimilate functions between basal ganglia nuclei, various regions of the cerebral cortex and the thalamus. The SN is the source of the striatal input of the neurotransmitter DA, which plays an important role in basal ganglia function. Areas of the motor cortex project to the posterolateral putamen, where they synapse through excitatory glutamatergic neurons onto the medium spiny striatal neurons. With GABA as their primary neurotransmitter and substance P (SP) or enkephalin (Enk) as co-transmitters, they are organized into two pathways namely the 'direct' and the 'indirect' pathway. The former connects the striatum to the internal segment of the GPi as well as SNr. The GPi and SNr are the output nuclei of the basal ganglia (GPi/SNr) and project to the brainstem as well as the thalamus to the cortex. The influence of the GPi and SNr on the thalamus is inhibitory, whereas the thalamic projection to the cortex is excitatory. The indirect pathway also connects the striatum to the output nuclei of the basal ganglia but these fibres first pass through synaptic connections in the external segment of the GPe and then the STN. Adapted from Blesa, Trigo-Damas, Quiroga-Varela, and Jackson-Lewis (2015).

In the Parkinsonian brain (Fig. 2.4), decreased levels of DA result in reduced activation of the direct pathway and augmented activation of the indirect pathway. Excessive stimulation of the D2 receptors of the indirect pathway increases the inhibition of the GPe, reduces the inhibition of the STN and therefore, increases excitation of the GPi and SNr. Overall this reduces thalamo-cortical glutamatergic excitation of the motor cortex and thereby reduces movement (Blesa et al., 2015). The decreased levels of DA which induce this dysregulation in the basal ganglia is due to a severe loss of nigrostriatal DA-ergic neurons.

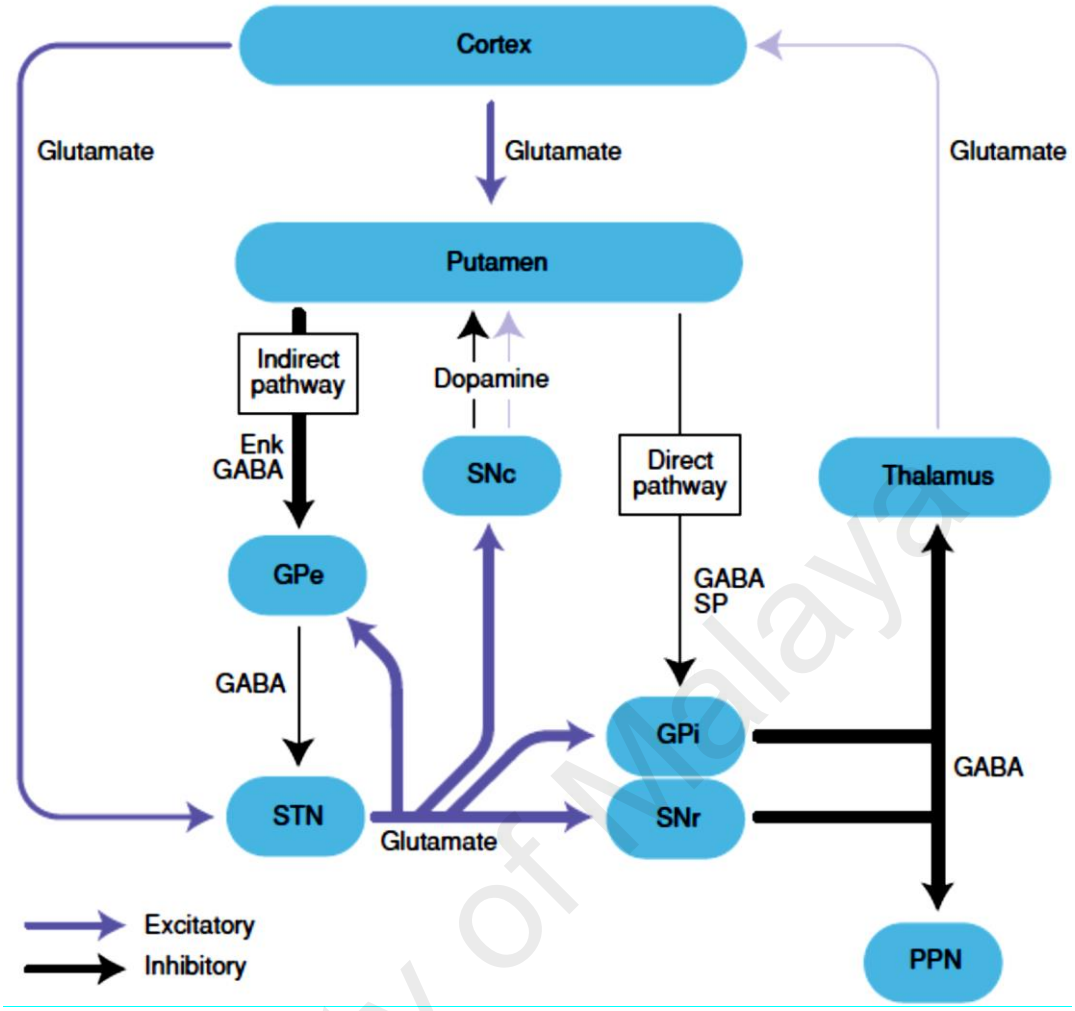

Figure 2-4: Basal ganglia circuitry in PD. In PD, the natural balance of the circuit shown earlier is lost owing to the depletion of DA in the striatum. Both pathways (direct and indirect) function via the GPi/SNr output nuclei and their influence is inhibitory on the thalamus. Thus, increased activity in the output nuclei leads to increased inhibition on the glutamatergic excitation of the motor cortex and a subsequent reduction in movement, observed in patients as bradykinesia. These changes in the PD brain are shown here by the differing thickness of arrows, which represents the relative degree of activation in each projection. Adapted from (Blesa et al., 2015).

2.5.1.3 Loss of DA-ergic nigrostriatal neurons

The main pathological hallmark of PD is progressive death of the nigrostriatal pathway in the basal ganglia, as described above, namely the DA-ergic neurons and terminals of the SN and the striatum, respectively (Fig. 2.5). The loss of neurons in the disease appears to be heterogeneous and whilst it includes the DA-ergic neurons of the SNpc, other neuronal populations such as selected catecholaminergic and serotoninergic nuclei in the

brain-stem; neurons in the olfactory bulb; sympathetic ganglia and parasympathetic neurons in the gut are also affected (Verstraeten, Theuns, & Van Broeckhoven, 2015).

The loss of neurons within the SNpc also appears to be diverse, with greatest tendency being at ventrolateral tier (estimated loss of 60-70% at the onset of symptoms), followed by partial damage in the medial ventral tier and dorsal tier (Michel, Hirsch, & Hunot, 2016) representing a pattern of cell loss relatively specific to PD. This neuronal loss subsequently results in are duction of striatal DA, most prominently in the dorsal and intermediate subdivisions of the putamen, believed to be responsible for akinesia and rigidity generally observed clinically in PD (Choi et al., 2015).

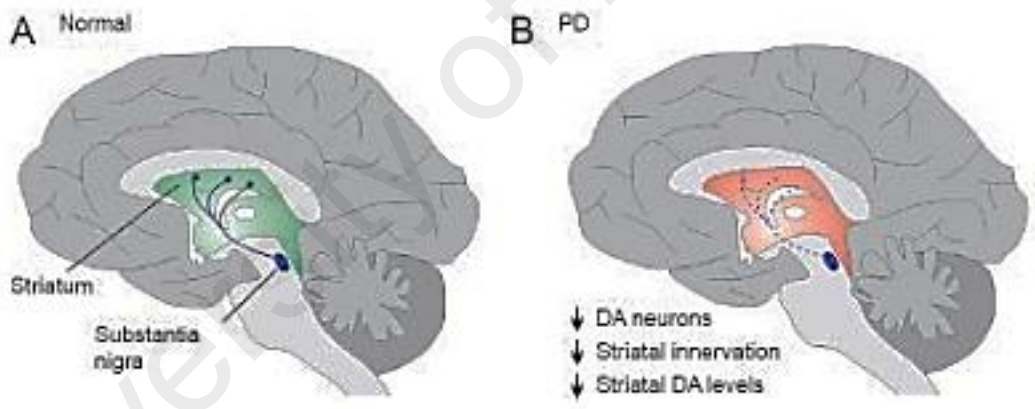

Figure 2-5: Graphical representation of the intact nigrostriatal pathway. The ascending axons (blue) of DA-ergic neurons originating in the SN innervating the striatum. (B) In PD, the progressive deterioration of these neurons contributes to a loss of DA-ergic innervation of the striatum and as a result, a reduction in striatal DA levels, which is one of the pathological hallmarks of PD. Adapted from Hegarty et al. (2014).

The deterioration of the DA-ergic neurons in the basal ganglia can often progress for years prior to the manifestation of motor symptoms. Typically, when patients first present with motor impairment, approximately 40% of DA-ergic neurons in the SN have already been lost and DA levels in the striatum have already been reduced by up to 80% (Daadi,

Grueter, Malenka, Redmond Jr, & Steinberg, 2012). The reason for the delay between the initiation of the disease process and the appearance of clinical symptoms is believed to be due to compensatory mechanisms. These compensatory mechanisms in the pre-symptomatic period of the disease effectively mask the underlying degenerative process via hyperactivity of the remaining DA-ergic neurons to maintain DA homeostasis (Lindvall, 2015), as well as increased DA receptor number and sensitivity in the striatum (Ko et al., 2015). Another pathological process that may be ongoing in the pre-symptomatic phase of PD is the development of Lewy pathology.

2.5.1.4 Lewy bodies

In addition to the progressive degeneration of the nigrostriatal pathway of PD, the other primary pathological hallmark of the disease is the presence of Lewy bodies and/or Lewy neurites. First identified in 1913 by Frederic Lewy, these eosinophilic proteinaceous inclusion bodies accumulate in the degenerating brain of PD patients (Lewy, 1913) as well as in other neurodegenerative disorders (Kopin, 1993). The accumulation of these proteinaceous deposits, while a common feature of many neurodegenerative diseases, is evident in both genetic and sporadic PD patients with very little exception (Poulopoulos, Levy, & Alcalay, 2012), and thus proposes a role for the involvement of Lewy pathology in the progression and development of the disease (Luk & Lee, 2014).

A typical Lewy body is approximately 8-30 μm in diameter and exhibits a densely stained granular core surrounded by a lightly stained halo of radiating filaments when stained with eosin. The primary components of Lewy bodies are the protein α-synuclein, ubiquitin and ubiquinated proteins, and neurofilaments (McNaught, 2004; Sugama, Conti, & Kakinuma, 2015).

Seminal research performed by Braak and colleagues has led to the increased appreciation and knowledge of the staging and spread of Lewy body pathology in PD.

Braak and colleagues have suggested a theory for the progression of the disease from the stage where patients exhibit Lewy pathology but are asymptomatic, to those with late stage clinical symptoms of the disease using a total of 110 α-synuclein positive patients (69 incidental and 41 clinically diagnosed PD patients) (Lecture, 2005; Luk & Lee, 2014). The theory proposed that the Lewy pathology advances via cell-to-cell contact, beginning in the periphery and moving into the medulla oblongata and olfactory system. This initiates the onset of autonomic and olfactory defects, followed by the progression of the pathology to the brainstem, causing motor disturbances. Advancement to the limbic system succeeds until finally the neocortical regions are affected, causing neurobehavioral and cognitive impairment (Fig. 2.6) (Halliday, Lees, & Stern, 2011).

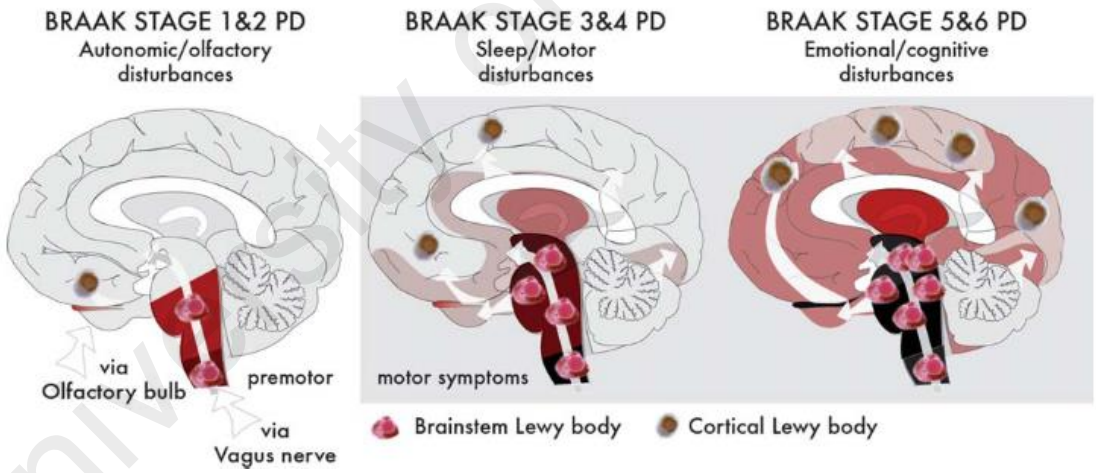

Figure 2-6: Schematic representation of the Braak staging of Parkinson's disease. Schematic representation of the Braak staging of Parkinson's disease showing the initiation sites in the medulla oblongata and olfactory bulb through to the later infiltration of Lewy pathology into the cortical regions. Associated symptoms at each stage are also shown. Adapted from Halliday et al. (2011).

Further reiterating the theory of cell-to-cell transfer of Lewy pathology, is recent evidence showing the ability of α-synuclein to spread from cell-to-cell, thus introducing the prion hypothesis of Lewy body pathology in PD. Although the function of α-synuclein is not well understood, studies suggest that it plays a role in maintaining a supply of synaptic vesicles in presynaptic terminals by clustering synaptic vesicles This prion hypothesis is supported by the observation of Lewy body pathology in embryonic DA neurons transplanted into the putamen of PD patients (Kordower, Chu, Hauser, Freeman, & Olanow, 2008; Li et al., 2008; Li & Gregory, 2008) and by compelling evidence that α-synuclein is capable of pathologically spreading from one cell to another in both *in vitro* and *in vivo* studies (Hansen et al., 2011). This highlights the role of α-synuclein in the pathogenesis of PD and has drawn attention to the possibility of targeting this transfer of α-synuclein as a neuroprotective strategy for the disease (Visanji, Brooks, Hazrati, & Lang, 2013).

2.5.1.5 Neuro-inflammation

Until recently, the role of inflammation as a factor in the pathogenesis of PD has been extensively researched. The involvement of inflammation to the pathogenesis of PD was first suggested by McGeer and colleagues in 1988 after the detection of activated microglial cells and increased major histocompatibility complex (MHC) molecules in the Parkinsonian brain (McGeer, Itagaki, Boyes, & McGeer, 1988). Neuro-inflammation is not only specific to PD and is believed to contribute to other neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis (Ma et al., 2013; McGeer & McGeer, 2004).

It is believed extensively that development of PD is due to exposure of infectious agents with pro-inflammatory characteristics leading to neuro-inflammation (Lima et al., 2006). Nevertheless the degeneration of DA as a consequence of neuro-inflammation

remains highly debatable. In theory, it is believed that chronic neuro-inflammation which was established by neurodegeneration may contribute to continuous deterioration of the DA-ergic neurons. As such, a continuous cycle (Fig 2.7) of neuro-inflammation and neurodegeneration occurs (Tansey & Goldberg, 2010; Tzeng, Hsiao, & Mak, 2005).

There is a huge number of evidences displaying the involvement of neuro-inflammatory processes in the pathogenesis of PD. For instance, microglial activation, NF-κB activation, and astrocytic upregulation have been identified in both patients and animal models of PD. In addition, elevation of IL-6, IL-2, IL-1β, and TNF-α were documented in many areas including the brain, serum and cerebrospinal fluid (CSF) (Ghosh et al., 2015; Ueda et al., 2014). In post-mortem and *in vivo* analysis of PD patients, both cellular and molecular evidence have been identified signalling the presence of neuro-inflammatory activities in the pathogenesis of the disease.

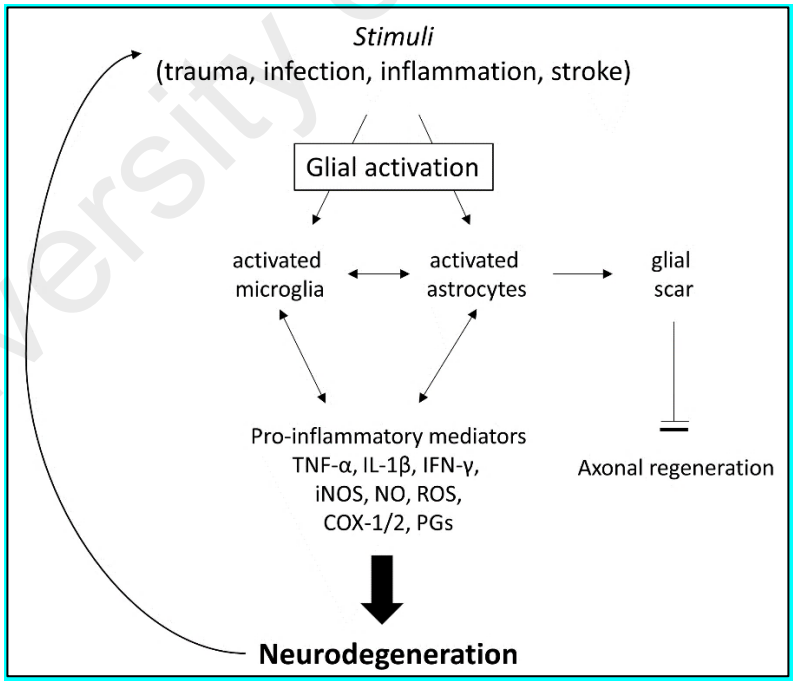

Figure 2-7: A schematic diagram showing the role of glial activation and glial scar in neurodegeneration and the eventual self-sustaining cycle of neuro-inflammation and neurodegeneration (Tzeng et al., 2005).

Microglial activation is a pathological hallmark for neuro-inflammation and has been associated with the pathogenesis of PD as well as other progressive neurodegenerative disorders (Kim et al., 2000). Microglia are a population of innate immune cells resident in the brain and constitute approximately 5-20% of all glial cells. They are thought to function by monitoring the surrounding microenvironment and thus become activated via Toll-like receptor signalling when such a threat is detected (Qin et al., 2007). It has been shown that microglial activation encompasses a morphological change from a quiescent resting or ramified state to an activated or amoeboid state (Streit et al., 1988). This step is essential for CNS integrity but its' over-activation can lead to detrimental and neurotoxic effects to the CNS (Qin et al., 2007). The over-activation of microglia contributes to over-production of pro-inflammatory mediators which in turn stimulate neuronal damage and death. It was further suggested that excessive activation result in DA neuron loss in the progression of PD (Chung et al., 2010). Apart from inducing pro-inflammatory cytokines to protect the CNS against infection, this process also triggers inflammation, and the cycle of inflammation and degeneration can persist even when the instigating stimulus has been eliminated (Qin et al., 2007).

As compared to other brain areas, the SN contains approximately four to five times the amount of microglia (Shi et al., 2014). Furthermore, DA-ergic neurons tend to be more sensitive to microglial activation in comparison to other neurons because of their low intracellular glutathione (Sidiq et al., 2011). In addition, it has been reported that microglial activation may also be triggered by protein aggregation due the interference of the ubiquitin-proteasome system (Jansen, Reits, & Hol, 2014) and mutations in α-synuclein, or by viral or bacterial infections of which are thought to be involved in the etiology and pathogenesis of PD (Deleidi, Hallett, Koprich, Chung, & Isacson, 2010).

2.5.2 Incidence, Prevalence and Healthcare Cost of PD

PD is a progressive neurodegenerative disorder with neuropathological features including the chronic degeneration of the DA-ergic nigrostriatal neurons in the brain accompanied by the abundance of Lewy bodies in the surviving DA-ergic neurons (Barker, Drouin-Ouellet, & Parmar, 2015). It is a known fact that PD is one of the most common neurological diseases encountered in the clinic and is the second most common neurodegenerative disease in the world with an annual occurrence of 0.1 - 0.5%, after Alzheimer's disease (Lindvall, 2016). PD affects about 3% of people over the age of 65 and approximately 4-5% of people over the age of 85. Sporadic idiopathic PD accounts for approximately 90% of diagnosed patients with just 10% of cases resulting from hereditary factors or genetic links (Tarazi, Sahli, Wolny, & Mousa, 2014).

Absolute certainty of the diagnosis of PD cannot be confirmed during life as there is no conclusive test to distinguish the diagnosis of PD from a diagnosis of closely related diseases such as other forms of Parkinsonism (Verstraeten et al., 2015). The pathological examination of post-mortem brain tissue from PD patients remains the superior choice to confirm PD diagnosis and the identification of Lewy bodies in the midbrain is a definitive feature of the disease (Michel et al., 2016). However, it is primarily the appearance of clinical symptoms that is used for diagnosis, despite the fact that onset of clinical symptoms only occurs once 80% of DA levels have depleted and when 40-50% of DA-ergic neurons have deteriorated (Wenker, Leal, Farías, Zeng, & Pitossi, 2016).

The diagnosis of the disease is primarily based on the clinical presentation of numerous motor symptoms along with the recording of a detailed patient history. The cardinal symptoms of PD are bradykinesia, rigidity, tremor at rest, and postural instability along with a myriad of secondary motor symptoms such as loss of spontaneous movements (akinesia), shuffling or festinating gait, changes in speech fluency (dysarthria), freezing,

reduced facial expressions (hypomimia) and dystonia (Michel et al., 2016). Non-motor symptoms are also a consequence of PD however they are often under-recognized and under-reported which leads to delays in treatment and increased treatments costs and hospitalizations for PD patients (Pagano, Ferrara, Brooks, & Pavese, 2016). The non-motor symptoms of PD include neuropsychiatric symptoms such as depression, anxiety and dementia; sleep disorders such as restless legs and insomnia; autonomic symptoms such as dry eyes and bladder disturbances along with gastrointestinal and sensory disturbances. Some of these non-motor symptoms precede the onset of the clinical symptoms of the disease and therefore are said to occur in the sub-clinical phase (Arenas, Saltó, & Villaescusa, 2015).

2.5.3 Current status and treatment approaches for PD

While PD remains as an incurable disease, various agents or treatments to improve the physical and psychological symptoms as well as the quality of life of PD patients have been introduced. Early diagnosis is paramount since the treatment modules in early PD are less challenging as compared to advanced PD. Most treatments modalities are purely to treat the symptoms of PD since no protective drugs are available to stop or even slow disease progression (Salawu, Olokoba, & Danburam, 2010). An ideal drug for PD would include those that could be delivered at early stages of disease and exerts neuroprotective effects. This in turn could prevent or at least slow the disease progression, improve the prognosis of PD patients, and also decrease the cost of treatment. Treatment regimens, while thus far being purely symptomatic, are ultimately patient-specific and highly dependent on the individual's predicted risk for side effects and necessity for symptoms' improvement (Hauser, 2010).

2.5.3.1 Levodopa, the golden treatment of PD

In 1967, oral levodopa therapy was developed, accounting for one of the most significant developments in pharmacotherapy for PD (Cotzias, Van Woert, & Schiffer, 1967). Levodopa, also known as L-dopa, is today regarded as the gold standard of PD treatment and its efficacy has not been surpassed since its discovery over 50 years ago. When L-dopa was first tested in Parkinsonian patients (intravenous administration), it was found to have few short term side effects and essentially abolished the rigidity and bradykinesia symptoms. The mechanism behind this effect has been explained by the conversion of L-dopa to DA via decarboxylation by DOPA-decarboxylase (Fahn, 2008). It should be noted that as a therapeutic intervention, peripheral conversion of L-dopa to DA was found to induce various cardiovascular and gastrointestinal side effects. Subsequently L-dopa was administered with a peripherally-restricted DOPA-decarboxylase inhibitor to ameliorate the side effects of peripheral DA. The most common DOPA-decarboxylase inhibitor in clinical use is carbidopa and numerous studies have demonstrated its ability to double the plasma half-life and increase the bioavailability of L-dopa, therefore increasing the central therapeutic effect of the drug (Arenas, Denham, & Villaescusa, 2015).

2.5.3.2 DA Agonists

DA agonists act by directly stimulating post-synaptic DA receptors and effectively mimicking the effect of the neurotransmitter and have revealed a moderately positive effect on symptoms of PD (Alonso et al., 2014; Pahwa & Lyons, 2010). DA agonists are typically most useful as a monotherapy for treatment of early PD but are also used as an adjunctive therapy with levodopa to delay the onset of dyskinesia, ultimately improving the patient's quality of life (Salawu et al., 2010). It was found that with early treatments, motor fluctuations and dyskinesia were less likely to develop. Moreover DA agonists that have been developed are such as ergot derivatives bromocriptine, lisuride,

pergolide, and cabergoline, as well as other non-ergot agents like apomorphine, pramipexole and ropinirole (Albright, Stojkovska, Rahman, Brown, & Morrison, 2016).

2.5.3.3 MAO/COMT Inhibitors

Inactivation of DA occurs via the action of three enzymes: aldehyde dehydrogenase (ALDH), monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT), producing inactive metabolites like 3, 4-dihydroxyphenyl-acetaldehyde (DOPAC), homovanillic acid (HVA) and 3-methoxytyramine (3-MT). Manipulation of DA metabolism via the use of enzyme inhibitors in order to prolong the availability of DA has been implemented in PD treatment (Michel et al., 2016).

Selegiline and rasagline are two clinically approved MAO-B inhibitors that exert their action by preventing the degradation of DA. These inhibitors provide mild symptomatic relief in early PD and are of therapeutic use as an adjunctive therapy to reduce motor fluctuations resulting from L-dopa treatment (Schapira, 2011). Entacapone is an example of an approved COMT inhibitor for the treatment of PD. While not effective as a monotherapy, there is evidence to show that L-dopa/carbidopa/entacapone combinations are effective in reducing 'end of dose' motor fluctuations of L-dopa (Quittet et al., 2015).

2.5.3.4 Non-DA-ergic Treatments

The anti-Parkinsonian effect of anti-cholinergic drugs was first discovered in 1867. These remained the only drugs used to address the symptoms of the disease for almost a century, and are still used today despite the discovery of other treatments with less risk of adverse events. The most commonly used anticholinergics in the US are the muscarinic antagonist trihexyphenidyl, benztropine, and procyclidine (Rezak, 2007). Adverse events or side effects associated with cholinergic antagonists include confusion, agitation, hallucinations and dementia (Tarazi et al., 2014). Despite this, many clinicians still use anti-cholinergic drugs (either as a monotherapy or in combination therapy) as a first-line

therapeutic option for treatment of the disease. However, because this class of medication is burdened with side effects, it is usually reserved for tremor that is resistant to DA-ergic treatment (Pagano et al., 2016).

Amantadine, originally used as an anti-viral agent, has been shown to offer minor symptomatic control of motor features in PD patients via its action as a non-competitive NMDA receptor antagonist, although it is considerably less effective than DA-ergic therapy (Olson & Gendelman, 2016). Treatment with amantadine is most beneficial in the earlier stages of PD and appears to be of particular use in treating tremor (Rezak, 2007). Emerging data also suggests that chronic amantadine treatment improves L-dopa-induced dyskinesia, and that withdrawal of amantadine treatment can exacerbate L-dopa-induced dyskinesia (Ory-Magne et al., 2014). However, there are various side effects associated with amantadine treatment including confusion, worsening of hallucinations, nausea and oedema (Lindvall, 2016).

2.5.3.5 Deep Brain Stimulation (DBS)

DBS involves the implantation of a device that sends electrical pulses to specific regions of the brain (usually the STN or the GPi) via implanted electrodes (Aron Badin et al., 2016). DBS candidacy is considered when motor problems produced in Parkinson's disease (slowness, stiffness, shaking, walking problems, wearing off of medication, fluctuations of motor symptoms, dyskinesia) are no longer sufficiently treated by an optimized medication regimen (Lee et al., 2016). Similar to available pharmacological treatments, DBS does not slow or halt disease progression, but rather offers symptomatic benefits (Macchi et al., 2015). In addition, DBS is an invasive therapy and in contraindicated in some patients, such as those with a high surgical risk.

2.5.3.6 Limitations of Current Treatments

Despite the obvious symptomatic relief experienced by patients on L-dopa/carbidopa treatment, it is important to note that there are various limitations to the treatment. It has been reported that for some, even high dose carbidopa administered with L-dopa may not be sufficient to prevent the peripheral side effects of nausea, vomiting and hypotension (Barker et al., 2015; Fahn, 2008). Long term treatment with L-dopa has a high tendency to contribute to side effects such as motor fluctuations (on-off periods) and dyskinesias. Effectiveness of the drug on motor symptoms is also curtailed with prolonged treatment and progression of the disease. L-dopa therapy is therefore usually reserved for treatment for advanced PD despite being the most beneficial treatment by far (Hauser, 2010).

In spite of the benefits of their use, DA agonists have been shown to result in non-motor adverse effects such as oedema, sudden onset of sleep, constipation, nausea, dizziness, hallucinations and impulse control disorders (Siri et al., 2010). MAO-B inhibitors are therapeutically limited since they are less efficacious than both L-dopa and DA agonists but conversely they produce less side effects which makes their application desirable in early treatment of the disease (Pahwa & Lyons, 2010). Use of COMT inhibitors, namely entacapone, is hampered by its propensity to increase L-dopa-induced dyskinesia, thereby limiting the permissible daily dose of L-dopa (Rascol, Lozano, Stern, & Poewe, 2011).

Despite the many advances that have occurred in PD therapy, it is important to note that there are critical unmet clinical needs. All the pharmacological treatments used in routine clinical practice have failed to provide disease modification and only contribute to symptomatic relief. The ability of a specific therapeutic agent, or indeed a combination of treatments, to adequately address the symptoms of the disease, reduce motor complications of treatment, halt disease progression and provide functional

neuroprotection and/or restoration of the DA-ergic neurons in the degenerating brain remains elusive to this day (Barker et al., 2015).

The stark insufficiencies in current therapeutic options have prompted a determined effort to identify novel therapeutic agents or strategies to address the degeneration of the nigrostriatal pathway and improve the current inevitably poor prognosis for patients diagnosed with PD.

2.5.4 SHEDs as a prospective therapeutic solution

2.5.4.1 *In vitro* studies: Markers in identifying DA-ergic-like cells

It should be noted that undifferentiated SCs were applied in transplantation studies involving in vivo models that have confirmed the neuro-regenerative capacity of dental and oral derived SCs. A major limitation to this method however is their tendency to undergo spontaneous differentiation upon *in vivo* transplantation. Therefore, the transplantation efficacy could be distinctly reduced since only a small sub-population of transplanted SCs were directly contributing by differentiating into neural lineages. Besides, the risk imposed by undifferentiated SCs in giving rise to undesired lineages at the site of transplantation may probably hamper regeneration capacities. On another note, pre-differentiating SCs towards neuronal lineages *in vitro* could possibly allow neuronal-related adhesion molecules and surface receptors to be expressed, and foster integration with the host, thus improving the therapeutic outcome. Differentiated neuronal lineages from SCs *in vitro* have indeed exhibited great prospects for the treatment of various neurodegenerative diseases (Siniscalco, Bradstreet, Sych, & Antonucci, 2013), stroke (Abeysinghe et al., 2015), traumatic spinal cord and brain injuries (Pavlova et al., 2012; Wilems, Pardieck, Iyer, & Sakiyama-Elbert, 2015), as well as peripheral nerve regeneration (Luo, Zhu, Zhang, & Jin, 2014). Apart from clinical therapy, competent *in vitro* protocols for neuronal differentiation can essentially contribute to diverse non-

therapeutic applications such as *in vitro* modelling of neurodegenerative diseases and *in vitro* neurotoxicity as well as neuro-pharmacological drug screening (Srikanth & Young-Pearse, 2014; Yap et al., 2015).

Since SHEDs are of neural crest-derived origin, it is expected that undifferentiated dental and oral-derived SCs would express baseline levels of neuronal markers, even in the absence of neuronal induction. It has been reported that spontaneous expression of mature genes neuronal nuclei (NeuN) and β-III-tubulin, as well as the neuronal SC gene nestin were observed in undifferentiated human DPSCs and PDLSCs (Foudah et al., 2014). Another study has reported that both DPSCs and SHEDs to be expressing neuronal markers, such as Nestin, microtubule-associated protein 2 (MAP2), β-III-tubulin, and tyrosine hydroxylase (TH), with expression levels being lower in former in comparison to latter (Feng et al., 2013). Furthermore since both DPSCs and SHEDs originated from similar histological source i.e. dental pulp, the only differential factor between them would be the donor's age (SHEDs are derived from younger children whereas DPSCs are derived from mature adults). On top of that, studies have indicated the presence of markers such as PAX6, GBX2, and Nestin which are of neuroectodermal classification in DPSCs as compared to SHEDs both in gene as well as protein level (Vijayendran Govindasamy et al., 2010; Karaoz et al., 2011; Tamaki, Nakahara, Ishikawa, & Sato, 2013).

2.5.4.2 Methods to identify DA-ergic-like cells

It should be noted that due to the heterozygosity in SHEDs population and the unequal inclination to differentiate into the neuronal lineage, it is indeed advantageous to isolate the cellular sub-population via magnetic-affinity or fluorescence-activated cell sorting. It has been proposed the that sub-population of isolated SHEDs in particular those with p75

neurotrophin receptor could give rise to superior therapeutic potential as compared to those unsorted ones (Jie-wen, Hao, Shun-yao, & Jing-ting, 2013).

There are numerous studies depicting successful differentiation of SHEDs into neuronal lineages *in vitro* via variety set of protocols. Different protocols distinguish themselves by accommodating differential components ranging from growth factors, small molecules as well as other culture supplements. In addition, careful considerations were also given pertaining to their surface coatings, total culture duration, presence of multiple culture stages, seeding density, as well as whether neurosphere formation is required (Gervois et al., 2014; Osathanon, Sawangmake, Nowwarote, & Pavasant, 2014).

Moreover, there are reports indicating the capability of SHEDs as spheroids in suspension culture to spontaneously differentiate into neural lineages even without neural induction, as established by the presence of numerous neuronal markers (Karbanová et al., 2010; Xiao & Nasu, 2014).

Additionally, the presence of multi-stage neural induction protocols have been thoroughly reported as well (Chang, Chang, Tsai, Chang, & Lin, 2014; Kanafi, Majumdar, Bhonde, Gupta, & Datta, 2014). For instance, two separate 4-stage protocols have been developed for guiding differentiation of SHEDs into the DA-ergic and motor neuron sub-lineages. The notable difference between these two correspond primarily to the application of all-trans retinoic acid for inducing SHEDs into motor neurons, which is absent in DA-ergic differentiation. The present *in vitro* differentiation studies of ASCs however tend to focus on more complex multi-stage culture protocols, as these would closely recapitulate the dynamic and specific micro-environment of SCs especially during neuronal differentiation (Chang et al., 2014; Feng et al., 2014).

Another major variable that plays crucial role in neurogenesis differentiation in SHEDs is the presence of surface coating while conducting cell culture works. Non-adherent culture dishes are usually utilized for neurosphere suspension culture whereas typical monolayer culture can be cultivated on plates with plasma treated polystyrene (TCPS) (Gervois et al., 2014; Karbanová et al., 2010). Additionally, other substrates such as poly-L-ornithine, laminin, poly-D-lysine, collagen type I, poly-L-lysine, hydroxyapatite, and chitosan can also be employed depending on their endpoint application (Feng et al., 2014; Ge et al., 2015).

A comparative study to shed light on the applicability of various substrata for neural induction have revealed that poly-L-ornithine has distinctly augmented the neuronal SCs propagation and differentiation, in comparison to fibronectin and poly-L-lysine. The underlying mechanism of this phenomena primarily involves the ERK (extracellular signal-regulated kinase) signalling pathway (Ge et al., 2015).

From the perspective of basal media, the commonly utilized ones are include Neurobasal A, Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), DMEM-Knockout (DMEM-KO), and α-MEM (Osathanon et al., 2014; Pisciotta et al., 2015). Neurobasal was initially developed for optimized survival of neural cells *in vitro* with the synergistic effects of B27 supplements (Kiraly et al., 2011). Apart from B27, other supplements that enhances SHEDs differentiation are include N2, non-essential amino acids (NEAA) and ITS (Insulin-Transferrin-Selenium) (Chang et al., 2014; Mainzer et al., 2014).

In addition, growth factors are other vital components which deserve much credit for the enhanced neurogenic potential in SHEDs. The most commonly used ones are basic fibroblast growth factor (bFGF) and epidermal growth factors (EGF). These are important for proliferation of neural precursors. Supplementary factors that co-enhance

differentiation include Transforming Growth Factor β3 (TGF-β3), Bone Morphogenetic Protein 2 (BMP-2), Neurotrophin 3 (NT-3), Glial cell line-Derived Neurotrophic Factor (GDNF), Nerve Growth Factor (NGF), Sonic Hedgehog (SHH), Brain Derived Neurotrophic Factor (BDNF), Fibroblast Growth Factor 8 (FGF-8), Wnt-1, noggin and Insulin-like Growth Factor 1 (IGF1). Apart from playing key roles in neurogenesis, these factors are known for their contribution in regulating propagation, differentiation and other functions in various cell types (J. Li et al., 2015; Yuan, Huang, Xiao, Lin, & Han, 2013; Zhao & Chai, 2015). The role of SHH has been demonstrated in the differentiation of motor and DA-ergic neurons within the embryonic neural tube (Kanafi et al., 2014). The roles played by FGF-8 and Wnt-1 have been elaborated previously for their contribution in neuronal induction in BMMSCs (X. Zhang et al., 2015) and the same pattern was also noted in differentiation of SHEDs (Feng et al., 2014).

The functionality of differentiated SHEDs are often checked via few methods namely electrophysiology analysis of potassium (K^+) and sodium ion (Na^+) channels (N. Gnanasegaran, V. Govindasamy, & N. Abu Kasim, 2015; Kiraly et al., 2011) intracellular calcium (Ca^{2+}) release upon stimulus by neurotransmitters, (Kanafi et al., 2014; Osathanon et al., 2014) and transplantation into *in vivo* models followed by immunohistochemical detection of neuronal markers expressed by the engrafted cells (Kiraly et al., 2011).

2.5.4.3 Pre-clinical approaches

After describing the efficacy of SHEDs as elaborated in previous sub-chapters, their ability to differentiate in animal model has high importance as they would serve as proof of concept regarding their neuro-regenerative capacity. Transplantation of nuclear receptor related-1 (Nurr-1) positive cells have demonstrated to be effective to repair neuronal damage in a stroke model (Yang, Chen, Liao, Pang, & Lin, 2009). Another study

has described the homing capacity of SHEDs in PD rat model whereby significant recovery with improved behavioural scores was noted (Wang et al., 2010). The enhancement with scaffolds have further augmented their potential as shown with biodegradable poly-DL-lactide-co-glycolide tubes, which were used for repairing gaps between nerves upon grafting (Sasaki et al., 2011). SHEDs were also transplanted in transected intercostal nerves and it was found that distinct morphological and functional recovery were observed (Spyridopoulos et al., 2015). In addition, studies have reported that transplanted SHEDs were also demonstrated to stimulate axon regeneration and exert neuroprotective effect in retinal ganglion cells (Beigi et al., 2014; Li, Li, & Jiang, 2015; Mead, Logan, Berry, Leadbeater, & Scheven, 2013).

In addition, presence of numerous evidence indicating success of transplantation could be linked with the paracrine effects of the growth factors secreted by SHEDs such as SDF1-CXCR4, apart from having the cells themselves to differentiate into neurons (Arthur et al., 2009). A study which utilized SHEDs for transplantation into the brain of healthy mice (hippocampus region) was shown to induce migration and homing of neuronal progenitors as well as mature neurons to the site of transplantation, apart from stimulating proliferation of endogenous neural cells (Huang, Snyder, Cheng, & Chan, 2008). Another study using stroke model have demonstrated enhanced post-stroke recovery in rats, even though only 2.3% of the transplanted SHEDs had engrafted and survived in the host (Leong et al., 2012).

2.5.4.4 Clinical studies

Though extensive studies were carried out using SHEDs for cell transplantation studies for PD, the translation from bench to bedside has yet been clearly justified. To date, there are only two studies had been carried out which applied MSCs in PD patients (Venkataramana et al., 2012; Venkataramana et al., 2010). These studies had employed

BM-MSCs and they have reported that PD patients after therapy had testified a mean improvement of 31.21% during 'off' period and 17.92% during 'on' period on the UPDRS scoring system. Interestingly, none of the patients was observed to have increased their medication during the follow-up period. Additionally the patients reported clarity in speech, reduction in rigidity, tremors, and freezing attacks on individual basis. Moreover, correlation with the duration of the disease was also noticed whereby patients transplanted in the early stages of the disease (less than 5 years) presented distinct enhancement with no disease progression in comparison with those from later stages (11–15 years) (Venkataramana et al., 2012).

Nevertheless, the usage of SHEDs for such cases is not highlighted as extensive as Venkataramana. This probably resides in the fact that there are still issues which require further clarification and thorough investigation. It is hoped that in near future, all remaining concerns can be taken care of and the era of cell transplantation using SHEDs would then emerge.

2.5.4.5 Challenges: Optimal doses and condition of cultured cells

As the name implies, the amount of SCs to be transplanted is indeed vital for efficient outcome. Some studies utilized MSCs in mere thousands (Chelluboina & Veeravalli, 2015; J. Li et al., 2015) and can go up to millions (Cerri et al., 2015; Li et al., 2013). For human subjects, perhaps different amount of SCs would be required. In both Venkataramana's studies, cells were administered using the patients' body weight as the parameter.

Another parameter which is worth exploring is the intrinsic properties of SCs. This means that apart from making sure the amount of cells being transplanted, it is also useful to understand their intrinsic behaviour such as the growth phases of those cells (Pauklin, Madrigal, Bertero, & Vallier, 2016). Having cells of pre-mature stage or at senescent

stage would render cell transplantation inefficient. As such, a thorough study on this subject is paramount before SHEDs can be employed to treat PD.

2.5.4.6 Duration of transplantation

Looking at the advancement of technology, it is indeed possible to treat SCs as drugs. As such, frequency of treatment can also affect the outcome of any procedure. Similarly for cell transplantation works, frequency of cell transplantation can play significant especially to repair damages in PD patients. Some studies have delivered the SCs in a single dose format (Vega et al., 2015) and some even employed multiple delivery frequency in an effort to augment the therapeutic effects (Mancardi et al., 2015).

2.5.4.7 Route of delivery

Apart from determining the doses and duration of SC delivery, another vital factor to ensure increased efficiency is the route of delivery. Again, this factor seems another puzzle to solve as there are various ways on how cell administration can take place. These include intracranial (Cerri et al., 2015; Venkataramana et al., 2012), intranasal (Danielyan et al., 2011), intrathecal (Kabu, Gao, Kwon, & Labhasetwar, 2015) or even intravenous (Suzuki et al., 2015). Despite the variations, there is no clear cut answer as to which delivery route is the best for cell administration. As such thorough investigations are paramount to identify the best delivery route.

2.5.4.8 Ways to improve – genetic modification

The advent of iPSC has opened a new dimension in stem cells research as this method provides an avenue to induce the pluripotency of the cells of study. Although NSCs displayed some promise, they however require often invasive biopsies and only helping in neuronal survival. In Japan a research group led by Shinya Yamanaka (Takahashi & Yamanaka, 2006) had invented a technique of developing pluripotent cells from adult mouse fibroblasts. The technique involves expression of transcription factor (TF) genes

such as Sox2, Oct4, c-Myc and Klf4, and the cells would acquire pluripotent features of ESCs, and were subsequently known as induced pluripotent stem cells (iPSCs). Few years later, similar methods were utilized to generate human cells-derived-iPSCs. This method has indeed been a blessing due to the fact that ethical dilemmas of using embryonic stem cells derived from fertilised ova can be circumvented apart from retaining their multi-lineage differentiation ability. DA-ergic-like cells generated from iPSCs have been demonstrated to improve PD-related motor symptoms in *in vivo* model (Wernig et al., 2008). Another advantage of iPSCs is the need for immunosuppression can be eliminated simply because patient-specific cells can be generated and subsequently transplanted. Despite showing promising hopes, iPSCs still pose some setbacks which include random integration of TF genes into cell genome. Due to the oncogenic potential of these TFs, tumor formation can be rather damaging if they are to be transplanted in patients. Latest technologies have offered improvisations to overcome these challenges such as with the utilization of mRNA instead of DNA to induce pluripotency (Brändl et al., 2015; Yee, 2010). However there is still huge caveat which require detailed experimentation to fully understand these cells prior to any transplantation works.

2.5.4.9 The role of intrinsic condition of cells – cellular growth phases

The role of growth phase, especially those pertaining to cell cycle has been thoroughly explored in human embryonic stem cells (hESCs) works as it represent an advantageous model system for investigating the cross-talk between cell fate choice and cell cycle. These cells can indefinitely propagate while maintaining their *in vitro* differentiation capability towards three primary germ layers namely endoderm mesoderm, and neuroectoderm. Additionally hESCs are characterized by a definite cell cycle profile with a short G1 phase, while their differentiation is associated with an increase in G1 phase length (Coronado et al., 2013) which made them to be a good model to study. A recent study has shown that complexes of Cyclin D1–3/CDK4/6 which control the length and

progression of G1 phase, also limit the transcriptional activity associated with Activin/Nodal signalling during the late G1 phase. This mechanism results in a cell cycle-dependent capacity of differentiation in which hESCs can only differentiate into neuroectoderm in their late G1 and into endoderm in their early G1 phase (Pauklin & Vallier, 2013). Finally, S- and G2/M-related mechanisms appear to control the inhibition of pluripotency upon differentiation, suggesting the existence of differentiation checkpoints during cell cycle progression in stem cells (Gonzales et al., 2015). These reports collectively demonstrate that cell cycle modulators have direct roles in early events pertaining to differentiation by controlling the activity of extracellular signals. Interestingly, cell cycle regulators are also known to function at the chromatin level and control gene transcription (Roccio et al., 2013; Sicinski, Zacharek, & Kim, 2007). For instance, Cyclin D proteins have been found on DNA in mouse cancer cell lines but also in mouse retinal tissue (Bienvenu et al., 2010; Casimiro & Pestell, 2012). This clearly indicates the interference made by different growth phases in the downstream application of SC therefore requires further elucidation.

2.5.4.10 Co-culturing/Pre-conditioning

The cells which are to be transplanted have to be in a position where they can respond positively to the microenvironment. Promising potentials have been demonstrated via methods such as cellular reprogramming and preconditioning by physical, chemical, genetic, or even pharmacological manipulation of the cells. This technique essentially 'prime' the cells to the 'state of readiness' to withstand the rigors of lethal ischemia during *in vitro* as well as during post-transplantation (Haider & Ashraf, 2010). To increase the efficacy of transplantation outcome, conditioning the cells by exposing them in an environment with selected factors or parameters that would make them to behave as intended is extremely vital. In fact, this method has been widely tested in cardiac stem

cells, mesenchymal stem cells, neural stem cells and many other types (George, Bliss, Mehta, Sun, & Steinberg, 2015; Sun et al., 2015; Teng, Bennett, & Cai, 2016).

2.5.4.11 Combination of factors

Apart from aforementioned ways to improve our current cell transplantation, it is worthy to note that a combination of these can also be employed for achieving the greatest possibility demonstrated by CRT.

CHAPTER 3: EFFECTS OF G0/G1 PHASE AND NEUROPOEITIC FACTORS IN THE DIFFERENTIATION OF DENTAL PULP STEM CELLS TOWARD DA-ERGIC LIKE CELLS

3.1 Introduction

The applicability of adult stem cells (ASCs) for CRT has always been a challenge. From the perspective of PD for instance, CRT has been suggested to be the future alternative treatment in which stem cells (SCs) or progenitor cells are transplanted at selected brain areas where loss of DA-ergic neurons are prevalent. These cells are then expected to respond in the microenvironment either by differentiating into a particular cell lineage or via secondary effects such as secreting relevant cytokines/chemokines that would then allow innate progenitor cells to flourish and subsequently improve the condition (Connolly & Lang, 2014; Lindvall, 2015).

Despite numerous studies (both *in vitro* and *in vivo*) indicating the effectiveness of SCs from various sources to be advantageous from the perspective of PD, the outcomes are still not readily translatable in human trials (Arenas, Saltó, et al., 2015; Lindvall, 2016). This is echoed by a search on Pubmed that revealed at least 300 studies signifying the potentiality of SCs for CRT purposes (search terms: 'cell replacement therapy for Parkinson's disease') but search on clinical trials however revealed only four studies (search terms: cell replacement therapy for Parkinson's disease). The huge gap between basic research work and clinical translation has prompted us to revisit some of the key aspects at basic research and pre-clinical level.

Putting aside challenges such as amount of SCs to be transplanted, duration of SCs treatment, dosage required and method of SCs delivery, we believe that the actual challenge is within the SCs themselves. Cells can exhibit variation in their respective growth phase, to differentiation stimuli, would consequently contribute to their inability

to respond effectively despite how conducive the micro-environment is (Machado, Fernandes, & Gomes Pde, 2012; Mao & Prockop, 2012; Shilpa, Kaul, Sultana, & Bhat, 2013). Hence, we postulate that the cell cycle phases of SCs have distinct influence when they are exposed to microenvironments due to their differential cell cycle profiles (G0/G1, S, as well as G2/M phases) (Chang et al., 2015; Fong et al., 2012; Gonzales et al., 2015). It is widely known that these phases are vital for mitosis to take place and correlates highly with the genetic content of cell population under study (Kong et al., 2015; Ramos-Gomez & Martinez-Serrano, 2016).

Though little is known about the influence of cell cycle profile in the differentiation potential of SCs, a study by Beerman, Seita, Inlay, Weissman, and Rossi (2014) has however demonstrated the link between cell aging and their respective cell cycle profile. They have experimented on hematopoietic stem cells (HSC) and have observed that aging cells tend to accumulate DNA damage and subsequently upregulate DNA damage response (DDR) and repair the damage upon exit from G_0 phase. This fits the concept whereby cell aging reflects their cell cycle profile, which further contribute towards their differentiation potential.

With that notion, this study was thus undertaken to investigate the role of cell cycle phases in influencing the differentiation potential towards DA-ergic like cells using SHEDs as our study model. It is believed that this study would enlighten us on the major influences carried by these cell cycle phases in the event of SCs being transplanted for CRT applications. Therefore, the objectives of this study are:

1. To determine the optimal sub-culture of SHEDs for downstream experiments
2. To evaluate the differentiation potential of SHEDs toward DA-ergic like cells at different growth phases

3.2 Materials and methods

3.2.1 Dental pulp collection and isolation of stem cells

Upon getting informed consent from donors' parents/guardians prior to the sample collection, primary cultures deriving from healthy deciduous tooth (n=5) were established as described previously (N. Gnanasegaran et al., 2015) (Medical Ethics Clearance Number DF CO1107/0066[L]). In brief, surfaces of root were cleaned with Povidone-iodine (Sigma Aldrich, St. Louis, MO, USA), the pulp were extirpated within two hours post-extraction, and processed subsequently. The extracted pulp tissue were cut into small fragments (physical digestion) prior to digestion in a solution of three mg/mL collagenase type I (Gibco, Grand Island, NY) for 40 minutes at 37°C (chemical digestion). Upon neutralization with 10 % Fetal Bovine Serum (FBS, Hyclone, Utah, USA), the cells were then centrifuged and seeded in culture flasks.

SHEDs were primarily cultured in identical culture condition, namely in T75 cm^2 culture flasks (BD Pharmingen, San Diego CA, USA) with cell seeding of 1000 cell/cm^2 as well as culture medium containing 1X KO-DMEM, 200 units/mL and 200 µg/mL of penicillin/streptomycin (Invitrogen); 0.01X Glutamax (Invitrogen) and 10 % FBS with humidified atmosphere of 95 % of air and 5 % of CO_2 at 37 °C. The medium was replaced every three days until the cells reached 80-90 % confluency. The following downstream experiments were carried out using five different samples and were conducted in a cGMP certified facility with clean environment conforming to guidelines by Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S).

3.2.2 Growth kinetics

The proliferation rate of SHEDs were determined by plating 1000 cells per cm^2 into separate 35mm culture dish (BD Pharmingen) as previously described (Gnanasegaran, Govindasamy, Musa, & Kasim, 2014). Cells were harvested by trypsinization after

reaching 90% confluency prior to assessment for viability by means of trypan blue dye exclusion before the next sub-culture. Cells were re-plated for subsequent sub-culture (SC), and a total of five SCs were studied in this experiment. Growth kinetics was analysed by calculating population doubling (PD) time. The PD time was obtained using:

$$PDT = tplg_2 / (lgNH - lgNI)$$

NI: the inoculums cell number; NH is the cell harvest number and t is the time of the culture (in hours)

3.2.3 Analysis on the β-galactosidase activity in SHEDs

SHEDs were tested for senescence-associated β-galactosidase (SA-βgal) activity according to the manufacture's instruction (Cell Signalling Technology, Beverly, MA). The development of the blue colour was observed under a phase-contrast microscope and the quantitative analyses of the SA-β-gal staining was done by counting the percentage of blue-stained cells that represent senescence cells in the selected field of each sample. The calculation of senescent cells was performed using ImageJ software (US National Institutes of Health).

3.2.4 Cell cycle analysis

Cell cycle profiling of SHEDs was performed with Fx Cycle PI/RNAse (Invitrogen) as described in manufacturers' protocol. A total of 5 x 10^5 cells was used for every acquisition. Analysis was performed on BD FACS Calibur flow cytometer (BD Biosciences, San Jose, California, USA) with excitation wavelength of 488nm and emission wavelength of 585nm based on 10,000 events using BD Cell Quest analysis software (Becton Dickinson).

3.2.5 Differentiation of SHEDs into DA-ergic-like cells

A total of 5 x 10^5 SHEDs at three distinct phases were subjected to DA-ergic-like cell induction at SC3 using chemically-defined media as previously described (N. Gnanasegaran et al., 2015). In the first phase, cells were exposed to induction media (Dopa Media A) consisting of Neurobasal A, 1X B27 supplement, 20ng/mL basic Fibroblast Growth Factor (bFGF), 20ng/mL Epidermal Growth Factor (EGF) for a period of nine days. Following this, cells were then treated with second phase of differentiation (Dopa Media B) in Dopa Media A with the addition of 200ng/mL sonic hedgehog (SHH), 100ng/mL Fibroblast Growth Factor 8 (FGF8), 10ng/mL brain-derived neurotrophic factor (BDNF) and 10µM forskolin (Sigma-Aldrich) for seven days. All chemicals were purchased from Invitrogen unless stated otherwise.

3.2.6 Quantitative gene expression via Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions and was subjected to DNase I treatment (Ambion) to eliminate any traces of genomic DNA. Amplification of cDNA was performed using Superscript II reverse transcriptase (Invitrogen) after treatment with RNase-OUT ribonuclease inhibitor according to the manufacturer's instructions. The expression levels of the genes were quantified in duplicates, using SYBR Green Master Mix (Applied Biosystems). PCR reactions were carried out on ABI ViiA7 qPCR system (Applied Biosystems). For estimation of the fold change when the initial transcript levels were undetectable, the initial cycle threshold (CT) value was assigned to be 35. Gene expressions were analyzed via comparative CT method (ΔΔCT) and were normalized to 18s rRNA against their corresponding control samples. The primer sequences are listed in Table 3.1.

Table 3-1: List of genes with primer sequence and their product size

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Base pair size
MCM4	ACAGGAATGAGTGCCACTTCTCGT	GCCCCTCGGACTGACCCAACGCTGCCCGG	145
MCM3	AGGAAGACTCATGCCAAGGAT	TGGGCTCACTGAGTTCCACTTTCT	172
Cyclin D1	AGGGGAGTTTTGTTGAAGTTGC	GCACTTCTGTTCCTCGCAGA	416
Cyclin T1	GGCGTGGACCCAGATAAAG	CTGTGTGAAGGACTGAATCAT	152
Cyclin F	ATATGCACACGCTTTATTAGC	ATGGACGTGCTTGTCTGTGC	133
CDK 4	GATCACGGGCCTTGTACACT	TTTGTTGCTGCAGGCTCATAC	165
CDK7	CAGTTTGCGACGGTCTATAAGG	GCTTTATCTCCCTTAAGGCTGTTC	124
CDC16	CGCCATGAACCTAGAGCGGC	TCACGTGCTGTGGTCTGACA	152
Pitx3	GACACTGGCCGCCCAAGG	AGGCCCCACGTTGACCGA	171
Lmx1a	ATGGAGGAGAACTTCCAAAGC	CCCGCTCCTTCTCATAGTC	182
Musashi	CTTTGATTGCCACAG CCTTC	CTCCAGCTATGCACAAATCC	148
Pax 6	ATGAACAGTCAGCCAATGGG	CACACCAGGGGAAATGAGTC	163
Nestin	GTAGCTCCCAGAGAGGGGAA	CTCTAGAGGGCCAGGGACTT	206
β-tubulin III	GCGAGATGTACGAAGACGAC	TTTAGACACTGCTGGCTTCG	115
Nurr 1	CGCCTGTAACTCGGCTGAA	AGTGTTGGTGAGGTCCATGC	169
TH	TCATCACCTGGTCACCAAGTT	GGTCGCCGTGCCTGTACT	125
DAT	CTGGTGTCTGGAAGATCTGC	AGCTGTCTCCACTGGAGTCA	219
18s rRNA	CGGCTACCATCCAAGGAA	GCTGGAATTACCGCGGCT	186

3.2.7 Real time Polymerase Chain Reaction using RT^2 Profiler^{TM} PCR Array

Similarly, gene expression of SHEDs at three distinct phases were matched using RT^2 Profiler^{TM} PCR Array Human Cell Cycle as previously described (N Gnanasegaran et al., 2015). Briefly, a total of 1 µg of RNA was reverse-transcribed into cDNA using RT^2 First Strand Kit (Qiagen, Venlo, Limburg, Netherlands) and RT^2 SYBR Green qPCR Mastermix (Qiagen, Venlo, Limburg, Netherlands) was added prior to real-time PCR as per manufacturer's protocol.

3.2.8 Gene expression analysis using computational tool

The "Core Analysis" function included in IPA (Ingenuity® Systems Inc., California, USA) was utilized to interpret the data in the context of biological processes, pathways and networks. The genes that were selected for analysis were of RQ value > 2.0 as compared to SHEDs cultured at Day 0. The selected genes were overlaid on functions or pathways which are relevant to development/differentiation of neurons to assess the cross talk behaviour. After the analysis, generated networks are ordered by a significance score. On the other side, significance of the biological functions and the canonical pathways were tested by the right-tailed p-value from the Fisher Exact test. Selected networks were then converted to form pathways via Path Designer to show relationships between transcription factors, genes or proteins.

3.2.9 Protein expression assay via immunocytochemistry (ICC) and Western blot

Both undifferentiated and differentiated cells were fixed for 20 min in ice cold 4% paraformaldehyde, treated with 0.1% Triton-X for optimal penetration of cell membranes, and incubated at RT in a blocking solution (0.5% BSA; Sigma Aldrich) for 30 min. The details on the primary antibodies, secondary antibodies and their corresponding dilution factors are listed in Table 2. Slides were counterstained with 4',6'-diamidino- 2-phenylindole dihydrochloride (DAPI, Chemicon, Temecula, CA, USA) for

5 min. Fluorescent images were captured using Olympus BX63 microscope (Olympus, Tokyo, Japan).

Table 3-2: List of antibodies with corresponding details

Name	Brand	Dilution factor
Primary antibodies		
Mouse monoclonal anti-nestin	Abcam - ab22035	1:200
Mouse monoclonal anti-beta-tubulin III	Merck Milipore - MAB1637	1:400
Rabbit polyclonal anti tyrosine Hydroxylase	Abcam - ab112	1:1000
Rabbit polyclonal to DOPA Decarboxylase	Abcam - ab3905	1:500
Secondary antibodies		
Anti-rabbit IgG conjugated to Alexa Fluor	Abcam - ab197505	1:200
Goat Anti-Mouse IgG H&L (FITC)	Abcam - ab6785	1:1000

Western blot analysis was also performed after extracting the whole cell lysate using Cytobuster (Novagen) lysis buffer. The proteins were loaded on 10% sodium dodecyl sulfate-polyacrylamide gels, and then transferred to polyvinylidene fluoride membranes. Blocking was done using PBS with Tween 0.1%. The membranes were left overnight with rabbit anti-human tyrosine hydroxylase and alpha tubulin as internal control (Abcam, Cambridge). After that, the membranes were incubated with peroxidase-conjugated secondary antibody (Abcam, Cambridge). The blots were visualized using a chemiluminescence detection system (Li-Cor® Odyssey, Li-Cor Biosciences, Lincoln, NE, USA).

3.2.10 DA secretion assay via enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants from both control and induced SHEDs were collected at respective days to estimate the DA release in these conditions. The DA secretion was measured by a DA ELISA kit (LDN, Germany) according to the manufacturer's protocol. Briefly, the cells were first stimulated with 56mM KCl (Sigma, USA) and 100μM adenosine tri-phosphate (ATP; Sigma, USA) for 5 minutes and 1 minute respectively in 4-(2-hydroxyethy1)-1-piperazineethanesulfonic acid (HEPES)-buffered salt solution (HBSS) at pH 7.4 at 37°C according to manufacturer's protocol. The cell culture supernatants were collected immediately after the stimulation and stored at -80°C for further quantification using ELISA. Absorbance was subsequently measured at 450 nm in a multilabel counter (MultiSkan GO, Thermo Scientific, USA). Culture supernatants from differentiated ReNCell VM cells for both constitutive and inducible release of DA, were used as positive control.

3.2.11 Detection of TH^+ differentiated DPSCs via flow cytometry

The percentage of TH^+ cells in both undifferentiated and differentiated SHEDs were detected via fluorescence-activated cell sorting (FACS) as previously described by our

group (Yazid, Gnanasegaran, Kunasekaran, Govindasamy, & Musa, 2014) using rabbit polyclonal anti tyrosine hydroxylase (Abcam) as primary antibody and anti-rabbit IgG conjugated to Alexa Fluor 488 (Abcam) as its' corresponding secondary antibody. All analyses were standardized against negative control cells incubated with isotype-specific IgG1-PE and IgG1-FITC (BD Pharmingen). Cells were acquired on Guava® easyCyte flow cytometer (Guava Technologies, Hayward, CA, US) and were identified by light scatter for 10,000 gated events and analysed using Cytosoft v 5.2 (Guava Technologies).

3.2.12 Detection of action potential via Multi electrode array (MEA)

Multi electrode arrays (MEAs) and recording stage were supplied by Multi Channel Systems (MCS, Reutlingen, Germany). Signal acquisitions were managed under MC_DataTool Software control and sampled at a frequency of 50 kHz. DPSCs were first seeded onto the centre of microelectrode arrays in a 40 µL droplet containing 20,000 cells and kept in a humidified incubator at 37°C with 5% CO_2. MEAs were then sealed with a Teflon membrane lid (MCS) to minimize evaporation. The cells were allowed to settle for one hour before flooded with 1 mL culture medium. Cultures were then exposed to DA-ergic-like cells induction according to the protocol described above. The external electrophysiology of the cultures was assessed at day 20 to determine spontaneous array-wide activity. To record from cultures, MEAs were placed in an electrically grounded recording stage and allowed to settle for 30 minutes. Three 100 second recordings were taken from all 59 electrodes for each MEA.

3.2.13 Statistical analysis

Results were presented as comparison of mean ± standard deviation (SD) using five independent samples (n=5). The data were analysed using SPSS, version 19.0 (SPSS Inc, Chicago, IL, USA). Statistical test using 2-way ANOVA were carried out and a p-value of < 0.05 was considered to be significant.

3.3 Results

3.3.1 Identification of optimal sub-culture

The optimal sub-culture that will give an overall better yield for SHEDs in terms of cell count, proliferation behaviour and ageing properties was first investigated. In order to achieve this, the cells were cultured for a total of five sub-cultures. Overall, the cells demonstrated rapid growth with fibroblast-like features. Supplementary experiments to describe their growth kinetics presented highly proliferative behaviour whereby an average of $2 \times 10^5 \pm 1.1 \times 10^4$ cells at SC1 was observed and remained elevated at SC5 with an average of $2.7 \times 10^5 \pm 1.4 \times 10^4$ cells (Fig. 3.1A). Similarly their population doubling time (PDT) also revealed rapid turnover time in which the time taken to double their initial cell population at SC1 and SC5 was 49.94 ± 1.03 hours and 48.74 ± 0.2 hours, respectively. Likewise, the population doubling (PD) at SC1 and SC5 was also observed as 2.39 ± 0.2 and 2.86 ± 0.07, respectively indicating their highly proliferative behaviour (Fig. 3.1B). Next, cell ageing properties among these five SCs further revealed that the percentage of senescent cell was increased by almost five-fold from 2.52 ± 0.18 % at SC1 to 12.56 ± 0.3 % at SC5 (Fig. 3.1C). Despite this, it was interesting to note that these characteristics deviated across SCs with distinct proliferation and cell ageing profile even though originating from same culture batch. Investigating these parameters are important in order to identify the optimal cell condition for downstream applications. With these notions, SC3 was carefully selected as the working sub-culture owing to its relatively early condition and highly proliferative performance (cell count of $2.84 \times 10^5 \pm 9.8 \times 10^3$, PDT of 44.5 ± 0.3 hours, and PD of 2.95 ± 0.1) in addition to having relatively low percentage of senescent cells (5.32% ± 0.14).

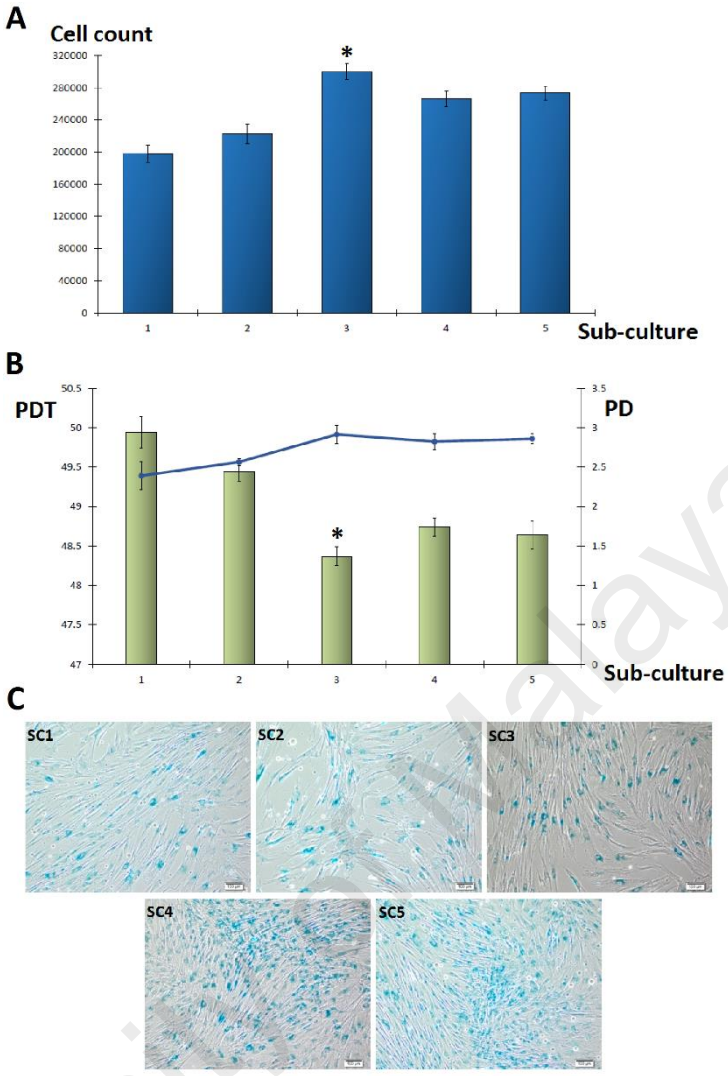

Figure 3-1: Characterizations of SHEDs. (A-B) Cell count and Population Doubling Time (PDT) of SHEDs at five consecutive sub-cultures. Cell counts were represented in bar graphs while the line graphs represented PDT. Data were presented as mean ± SD based on five independent samples. (C) Senescence assay as depicted by β-galactosidase staining of SHEDs culture from SC1 till SC5. The presence of blue-coloured regions indicate senescent cells. Micrographs were taken at 20x magnification and scale bar = 100 µm.

3.3.2 Cell cycle analysis revealed distinct phases based on cell cycle state

Cell cycle analysis was performed on daily basis for a period of ten days to assess the fluctuations of G0/G1, S and G2/M cell cycle phases of cells from SC3 (Fig. 3.2A). It is very well known that G0/G1 phase is the key modulator in cell proliferation. As such, the proportion of G0/G1 phase showed progressive increment from 42.36% ± 3.15 at Day 1 to 85.26% ± 3.21 at Day 7 indicating cell growth and proliferation. It should be noted that

although an increment pattern was noted, the surge of G0/G1 phase from Day 1 to Day 3 was the highest (almost two-fold) possibly indicating distinct mechanism being activated. However, the proportion of cells in this phase began to drop from Day 8 onwards and remained at 75.75% ± 3.42 at Day 10. Correspondingly, S phase also displayed an incremental pattern from Day 1 to Day 3 (from 13.28% ± 2.95 to 17.23% ± 1.21) reflecting DNA synthesis process being carried out and began to vary from 13.07% ± 1.85 at Day 4 onwards to 16.42% ± 4.74 at Day 10. On the contrary, G2/M phase revealed a decrement pattern from 44.36% ± 5.65 at Day 1 to 10.26% ± 3.2 at Day 3 complementing the increment that took place in earlier two phases. Again, series of fluctuations in G2/M phase was noted from Day 4 onwards till Day 10. Though the changes with respect to cell cycle phases indicating their proliferative feature, their morphology nevertheless did not reveal any significant changes and maintained as fibroblast-like cells throughout the culture period (Fig. 3.2B).

Next, after observing the variations in regards to G0/G1 phase within the same batch culture, three time points namely Day 3, Day 7 and Day 10 were tentatively selected based on their corresponding cell cycle profile. These phases are believed to have distinct responses when they are subjected to subsequent downstream experiments.

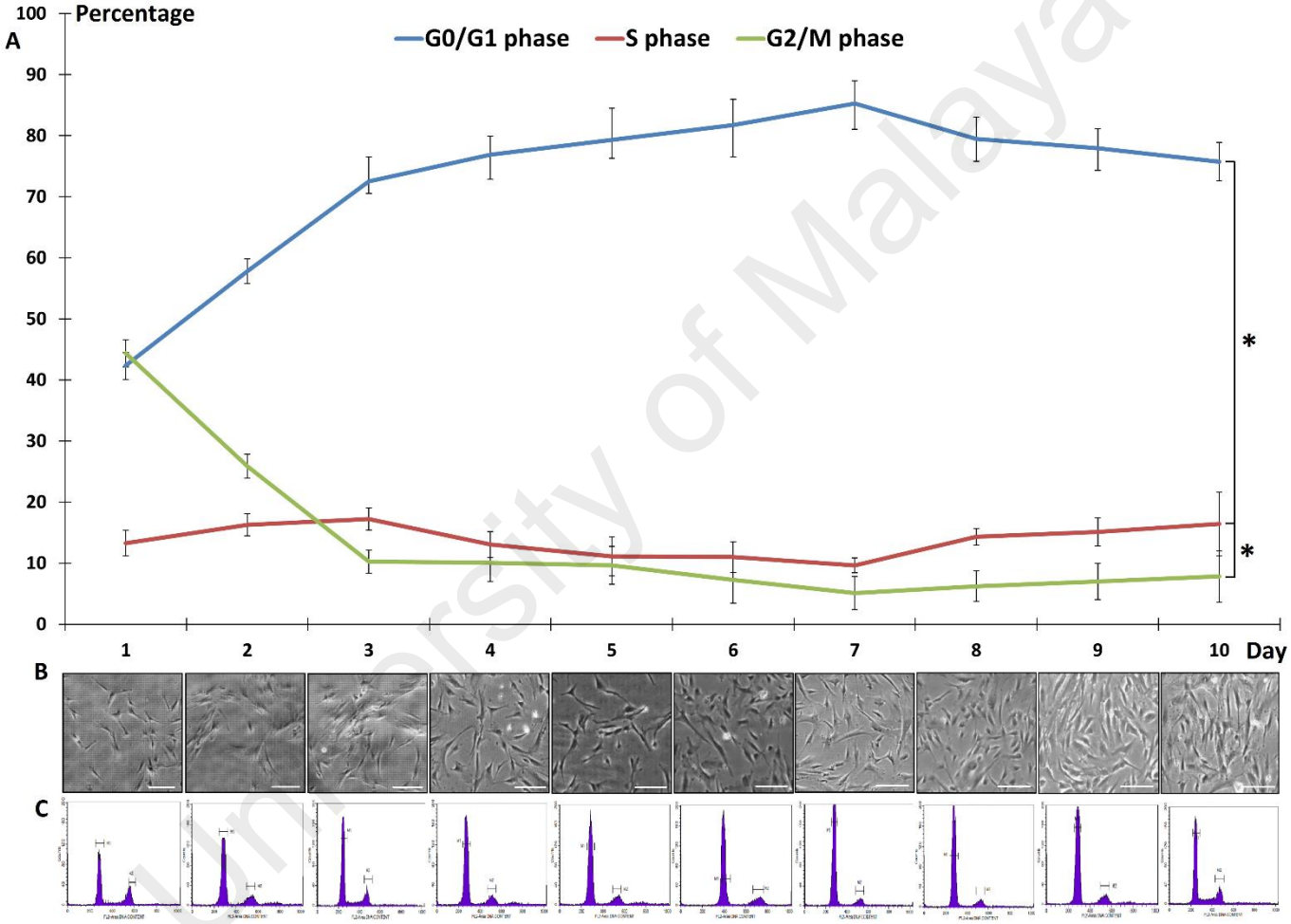

Figure 3-2: Differential proportions of cells' growth phases. (A) Levels of growth phases of SHEDs over ten-day period. (B) Representative phase contrast micrographs of SHEDs depicting their morphology taken at 10x magnification. Scale bar = 200µm. (C) Representative cell cycle analysis data via PI/RNAse staining. A total of 5 x 10^5 cells were used for this acquisition. * indicates $p<0.05$.

3.3.3 Gene expression profile of cell cycle related molecular markers

With the newly selected time points depicting their internal profile, a comparative gene expression assay was then performed using a commercially available PCR array (Human Cell Cycle) before and after directed differentiation procedures. The outcome of the expression profiles were then converted into a heat map for better comparison and viewing (Fig. 3.3). As expected, each growth phase was observed to have significant gene expression profile when compared to one another. For instance on overall basis, the number of up-regulated genes across the time points (from Day 3 to Day 10) was 14, 20, and 25 respectively. In addition, those with unchanged expression across the time points respectively revealed 34, 20, and 25 while the number of down-regulated genes were noted as 36, 44, and 34 respectively. It was noted that the significantly up-regulated genes in SHEDs-Day 7 were those pertaining to cyclin along with cyclin dependant proteins such as Cyclin D1, Cyclin E1, Cyclin T1, CDK1, CDKN1B, as well as CDKN2A ($p<0.05$) which are known to play significant roles in differentiation potential of stem cells. However the expression pattern of these genes post-differentiation were mostly down-regulated if not remained were unchanged, possibly suggesting pathway activation has taken place. To validate these gene expression profile obtained from PCR array, additional qRT-PCR on selected genes was performed. It was observed that the expressions were in accordance to the pattern observed in PCR array (Fig. 3.4). After inspection, it was revealed that cell cycle regulation and checkpoint related genes were mostly expressed by Day 7 and Day 10 SHEDs cultures (> 60% on average) whereas Day 3 cultures expressed those of more related to G2/M phase, conforming further to the pattern observed in cell cycle analysis performed later (Fig 3.5).

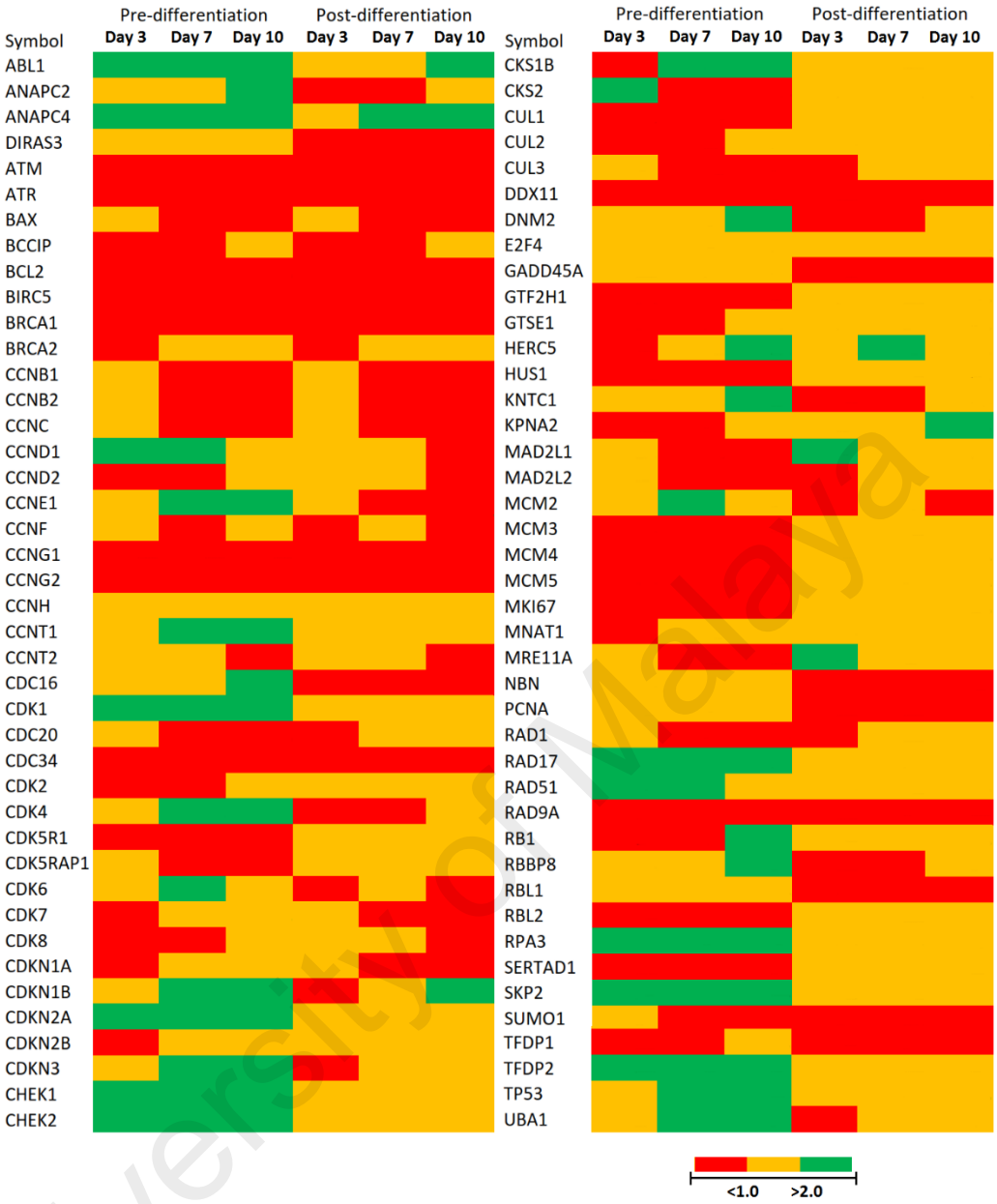

Figure 3-3: Heat map analysis. Heat map analysis revealing gene expression profile of SHEDs in regards to cell cycle at three selected time points. Analysis was performed on SHEDs prior to directed differentiation as well after the differentiation**.**

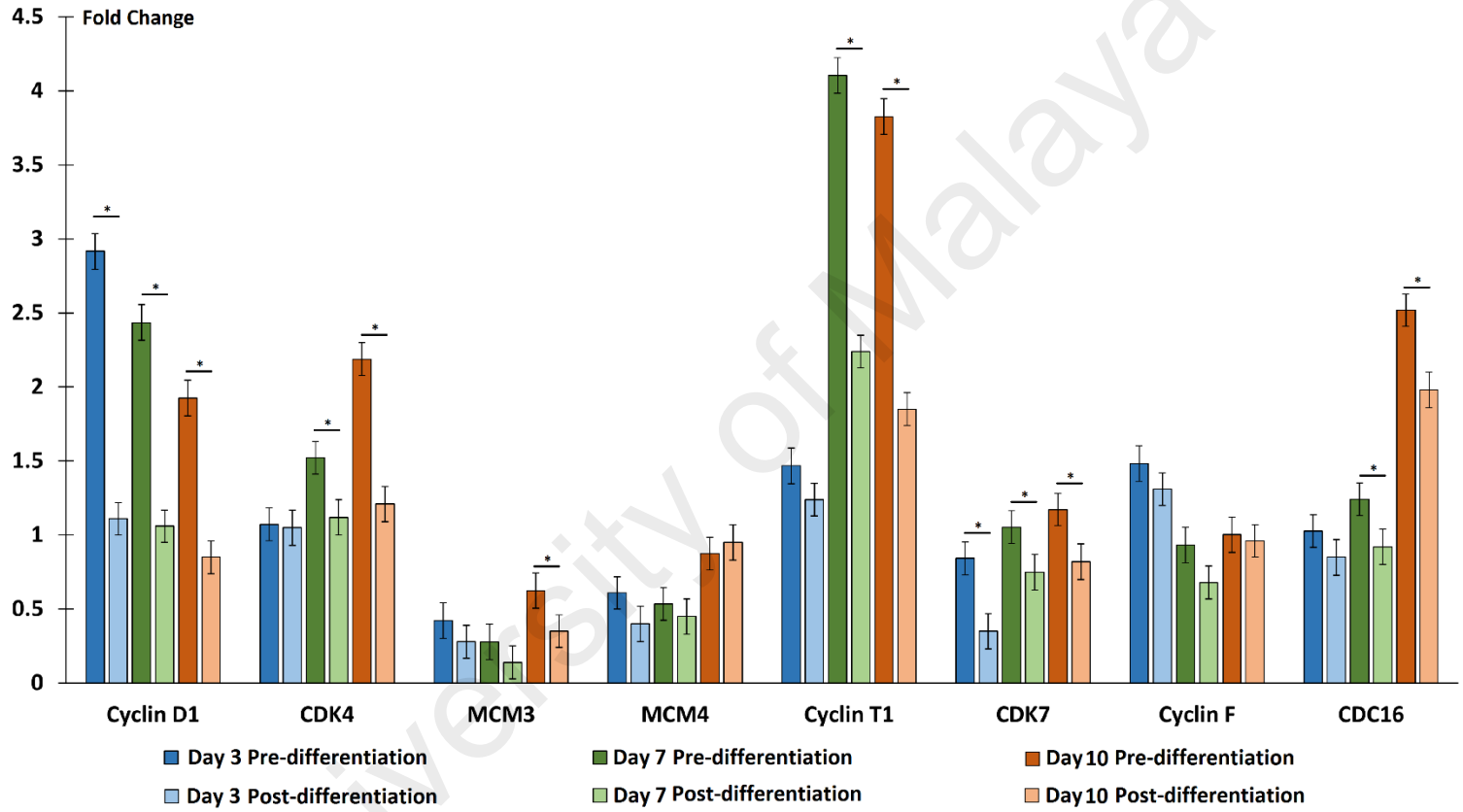

Figure 3-4: Validation of PCR array outcomes via qPCR gene expression assay. Presence of selected markers was performed before and after differentiation with 18s rRNA as housekeeping gene. * indicates $p<0.05$.

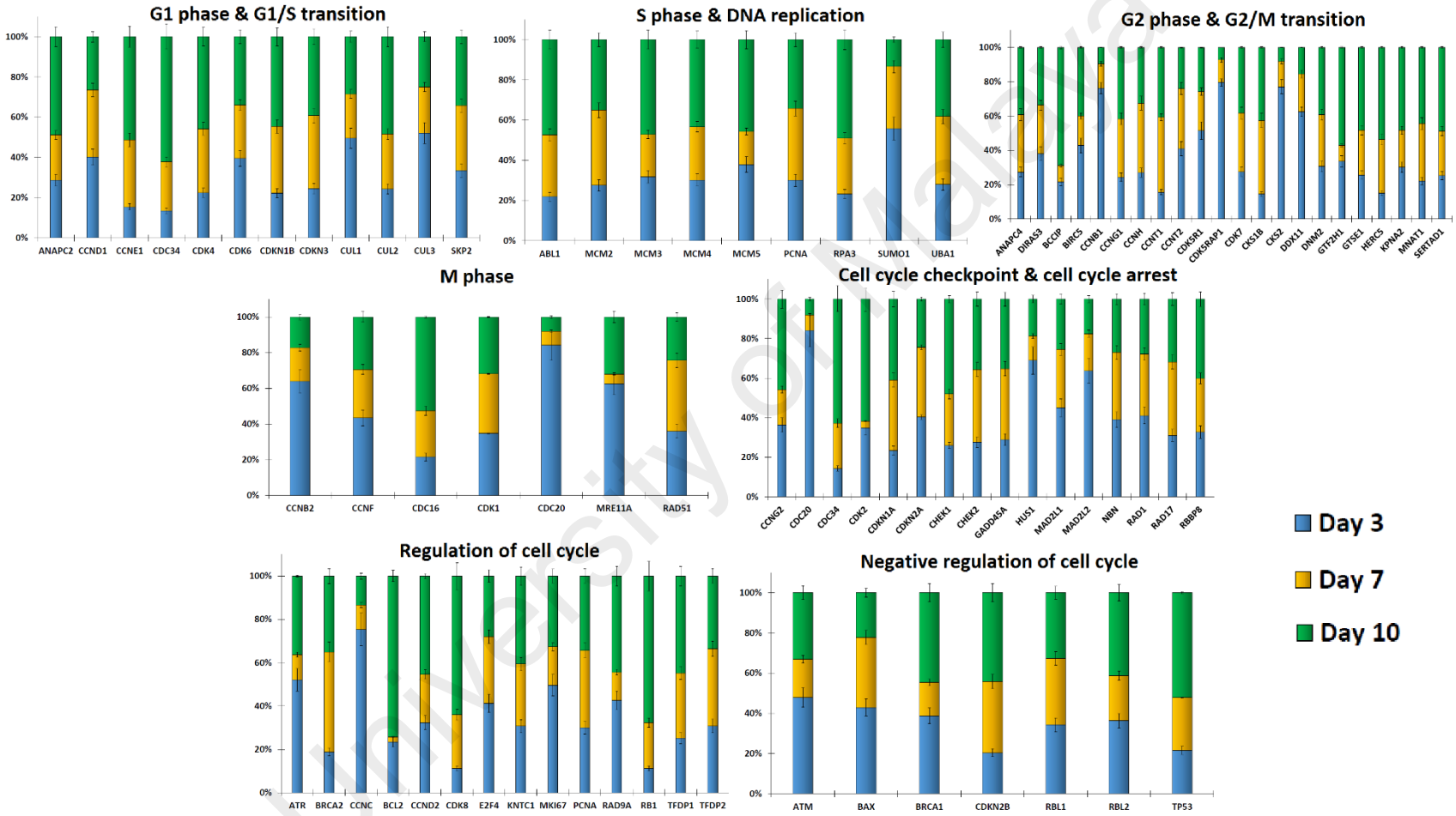

Figure 3-5: Gene expression profiles via PCR array depicting their relative amount of mRNA pertaining to cell cycle related biological functions.

3.3.4 Activated canonical pathway and predicted functional activities by Ingenuity Pathway Analysis (IPA)

With the expression profile obtained from qPCR array, computational analysis was subsequently performed using Ingenuity Pathway Analysis software to understand further the possible interactions that might have triggered during these time points. IPA has identified significant canonical pathways and top functions associated with the differentially expressed genes for each comparison analysed. It was revealed that across all time points, 'Cyclin and Cell Cycle Regulation' (respective z-score: 1.732, 0.577, and 1.213) 'Cell Cycle: G2/M DNA Damage Checkpoint Regulation' (respective z-score: 0.632, 0.905, and 0.905) as well as 'Cell Cycle: G1/S Checkpoint Regulation' (respective z-score: 1.265, 0.905, and 0.258) were all activated though in different sequence indicating that these pathways were differentially activated at different time points (Fig. 3.6). Additionally, the predicted functional activities by IPA revealed that despite common function such as 'Cell Cycle', 'Cancer', 'Hereditary Disorder', 'Organismal Injury and Abnormalities' were present, their respective significance were distinct to one another (Fig. 3.7). This again reflected their differential behaviour although originating from same culture batch. Next, the genes which are involved in selected functions from the IPA's database were checked. Functions such as 'Nervous System Development' and 'Tissue Development' were selected as our parameters and it was shown that all three time points share same group of genes such as Cyclin D1, CDK1, CDK 4, CDK6, and RAD51 (Table 3.3). Again, via retrospective analysis, it was then revealed that all these gene correspond to cell cycle regulation and checkpoint which further conforms the notion that cell cycle phases do play vital roles in determining their cell fate. Additional analysis looking specifically at possible cross talks between cell cycle and neurogenesis was performed using gene expression data from all three time points based on their distinct functional categories (Table 3.4). It was observed that despite the cells originating

from batch of cells, they possess quite different profile in regard to pathway activation (Fig. 3.8). For instance, SHEDs-Day 7 revealed activation of p38 MAPK and SHH pathway which is the key signalling pathways leading to neuronal differentiation. Presence of similar pathways among the three time points such as PI3K/AKT and p53 could probably indicate housekeeping-related activities (Table 3.4). Apart from signalling pathways, it was also shown that SHEDs at different time points took part in specific functions which could possibly indicate their innate behaviour (Fig. 3.9). For example, SHEDs-Day 7 were shown to take part in activities such as cell transformation and cell viability. Interestingly, SHEDs-Day 3 were more inclined towards cell survival while SHEDs-Day 10 were more inclined towards senescence of cells.

With such interesting observations, this led to a question on how their respective response would be when all these phases were subjected to directed differentiation. Owing to the fact that SHEDs are of neural crest origin, directed differentiation towards neurons, especially DA-ergic-like cells was performed and checked for their response accordingly.

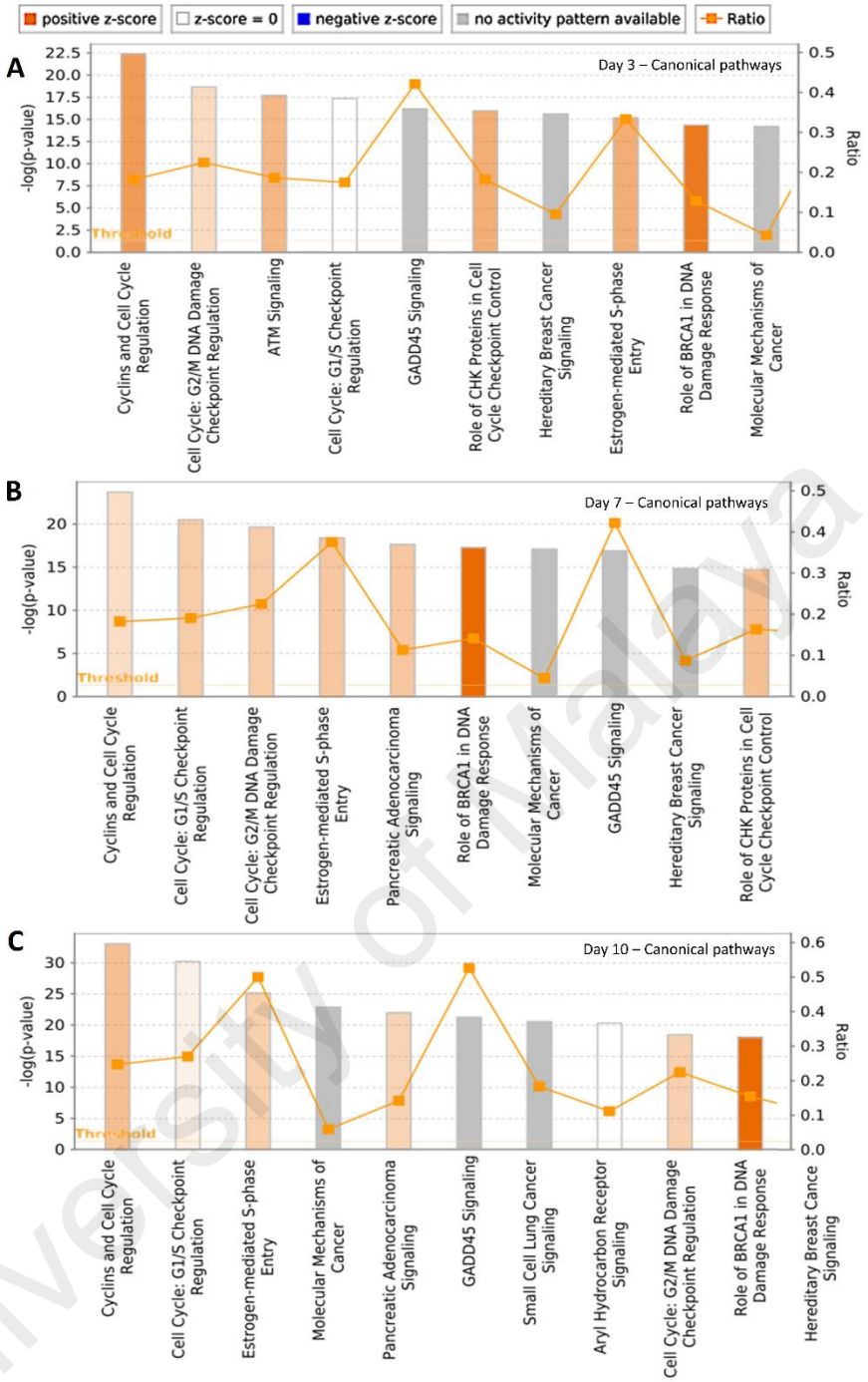

Figure 3-6: Canonical pathways generated from significantly regulated genes using Ingenuity Pathway Analysis (IPA). (A) Significant canonical pathways at SHEDs cultures for three days. (B) Significant canonical pathways at SHEDs cultures for seven days. (C) Significant canonical pathways at SHEDs cultures for ten days.

Table 3-3: List of significant genes involved in Nervous Tissue Development and Tissue Development based on Ingenuity Pathway Analysis

Culture period	List of genes
Day 3	ABL1, BAX, CCNB1, CCNB1, CCND1, CCNE1, CDK1, CDK4,CDK6, CDKN1, CDKN1B, CDKN2A, CDKN3, CHEK2, CKS2, MAD2L1, MRE11A, NBN, PCNA, RAD1, RAD17, RAD51, SKP2, TP53
Day 7	ABL1, BRCA2, CCND1, CCNE1, CDK1, CDK4, CDKN1A, CDKN1B, CDKN2A, CDKN2B, CDKN3, CHEK2, NBN, RAD17, RAD51, SKP2, TP53
Day 10	ABL1, BRCA2, CCND1, CCND2, CCNE1, CDK1, CDK2, CDK4, CDK6, CDKN1A, CDKN1B, CDKN2A, CDKN2B, CDKN3, CHEK2, NBN, RAD17, RAD51, RB1, SKP2, TP53

Table 3-4: List of possible signalling pathways and specific functions due to crosstalk between genes of cell cycle and relevant functional categories as depicted by IPA software

Expression of genes from SHEDs-Day 3		
Functional categories	**Signalling pathways**	**Specific functions**
Interphase, cell cycle progression, arrest in interphase, G2 phase, S phase, mitosis, G1 phase, arrest in G2 phase, G2/M phase, checkpoint control, arrest in G1 phase, G1/S phase transition, arrest in S phase, G2/M phase transition	Wnt/β-catenin signalling, PI3K/AKT signalling, p53 signalling, HGF signalling, GADD45 signalling	cell survival
Expression of genes from SHEDs-Day 7		
Functional categories	**Signalling pathways**	**Specific functions**
Interphase, arrest in interphase, G2 phase, S phase, G1 phase, arrest in G1 phase, arrest in G2 phase, cell cycle progression, arrest in proliferation of cells, G2/M phase, proliferation of cells, arrest in S phase, G1/S phase transition	p53 signalling, GADD45 signalling, PI3K/AKT signalling, SHH signalling, HGF signalling, AMPK signalling, ILK signalling, SAPK/JNK signalling, JAK/Stat signalling, p38 MAPK signalling, CXCR4 signalling, CDK5 signalling	cell transformation, repair of cells, proliferation of stem cells, lifespan of cells, cell viability,

Table 3-4, continued

Expression of genes from SHEDs-Day 10		
Functional categories	**Signalling pathways**	**Specific functions**
Interphase, arrest in interphase, G2 phase, S phase, G1 phase, arrest in G1 phase, arrest in G1 phase, cell cycle progression, arrest in proliferation of cells, arrest in G2 phase, repair of DNA, G1/S phase transition, G2/M phase	P53 signalling, GADD45 signalling, PI3K/AKT signalling, HGF signalling	cell transformation, senescence of cells, repair of cells

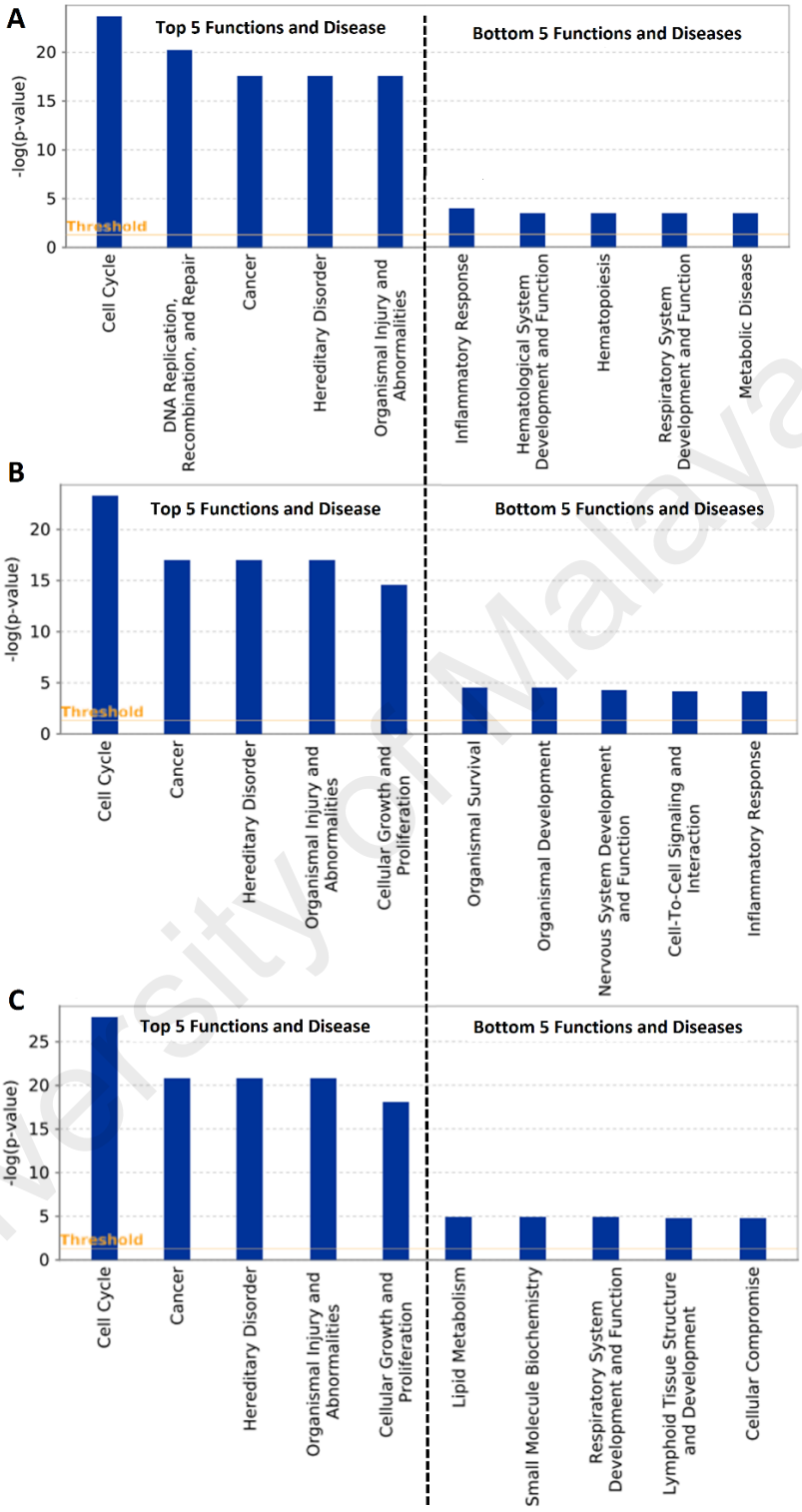

Figure 3-7: Top as well as bottom five biological functions and diseases as generated by Ingenuity Pathway Analysis (IPA) based on significantly regulated genes. (A) Significant biological functions and diseases at SHEDs cultures for three days. (B) Significant biological functions and diseases at SHEDs cultures for seven days. (C) Significant biological functions and diseases at SHEDs cultures for ten days.

3.3.5 Differentiation analysis of SHEDs into DA-ergic like cells revealed discrete pattern from perspectives of morphology and gene expression

The first observation with regards to directed differentiation was in terms of morphology. Interestingly in SHEDs-Day 7's trans-differentiation development, higher proportion of cells with neuronal morphology with extensive neurite outgrowths was observed, followed by those from Day 10. However in SHEDs-Day 3's trans-differentiation development, the neurite outgrowths were lesser and most of them remained fibroblastic by the end of induction period (Fig. 3.10A). Moreover to ascertain that the cells have transformed into DA-ergic like cells, their gene level were also checked. As expected, presence of transcription factors and neural progenitor cues such as Pitx3, Lmx1a, Nestin, Musashi and Pax6 was observed which are collectively responsible to trigger development of neuronal progenitor cells before directing them towards DA-ergic like cells ($p< 0.05$; Fig. 3.11 i-ii). Apart from neural progenitor cues, NURR1 gene which is well known to induce the formation of DA-ergic cells as well as tyrosine hydroxylase (TH) and DA acetyl transferase (DAT) which are specific markers for DA-ergic cells were also significantly expressed ($p < 0.05$; fold change > 2 fold) in SHEDs-Day 7 as compared to other groups (Fig. 3.11 iii). Collectively these data demonstrated the ability of SHEDs to efficiently transform into DA-ergic like cells

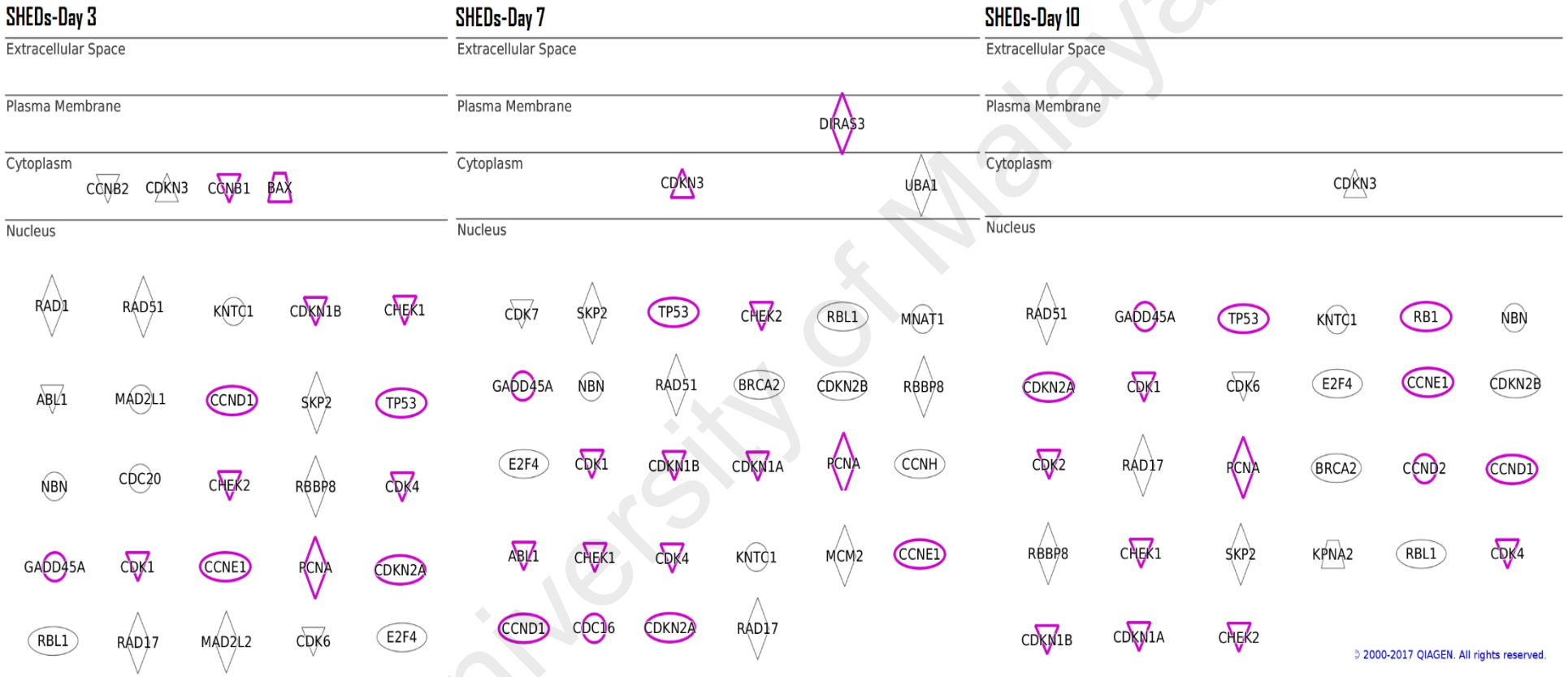

Figure 3-8: Presence of activated genes possibly indicating crosstalk between cell cycle-related functions and signalling pathways. Bolded patterns revealed the significantly up-regulated genes, both common and unique ones pertaining to signalling pathways selected from IPA's database as summarized in Table 3.4.

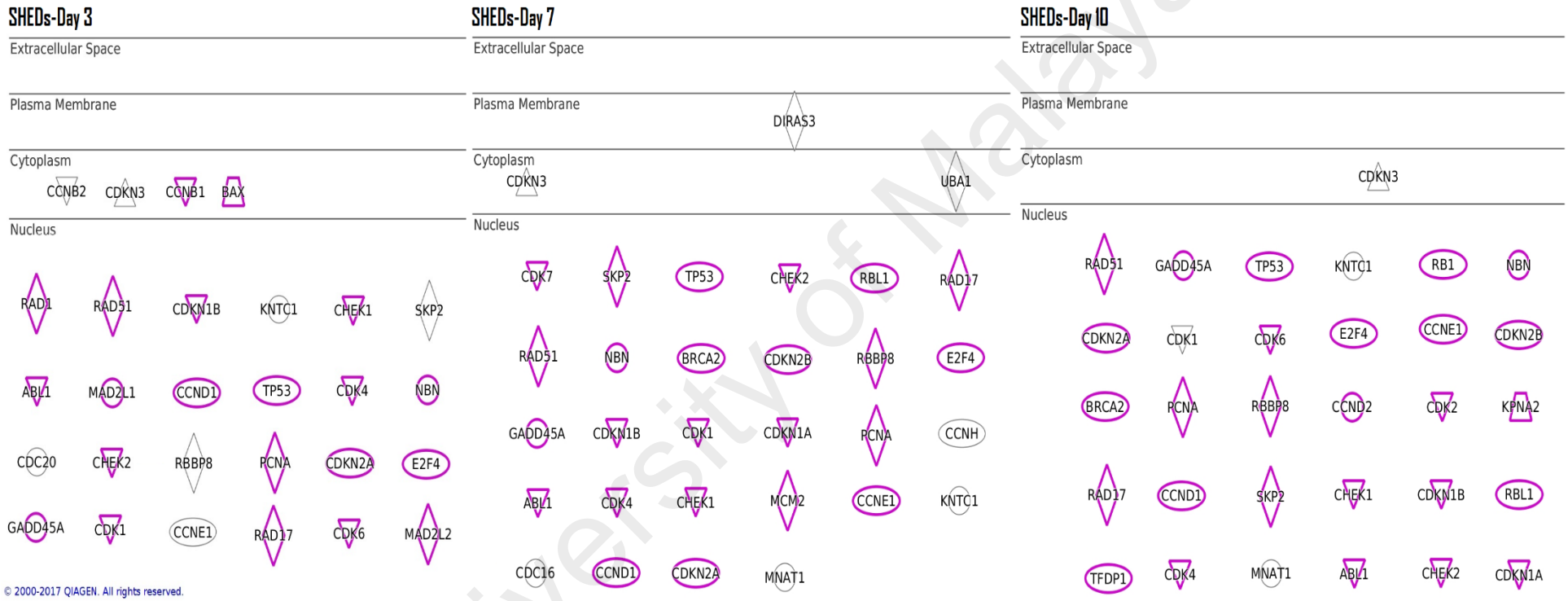

Figure 3-9: Presence of activated genes possibly indicating crosstalk between cell cycle-related functions and specific function. Bolded patterns revealed the significantly up-regulated genes, both common and unique ones pertaining to specific functions selected from IPA's database as summarized in Table 3.4.

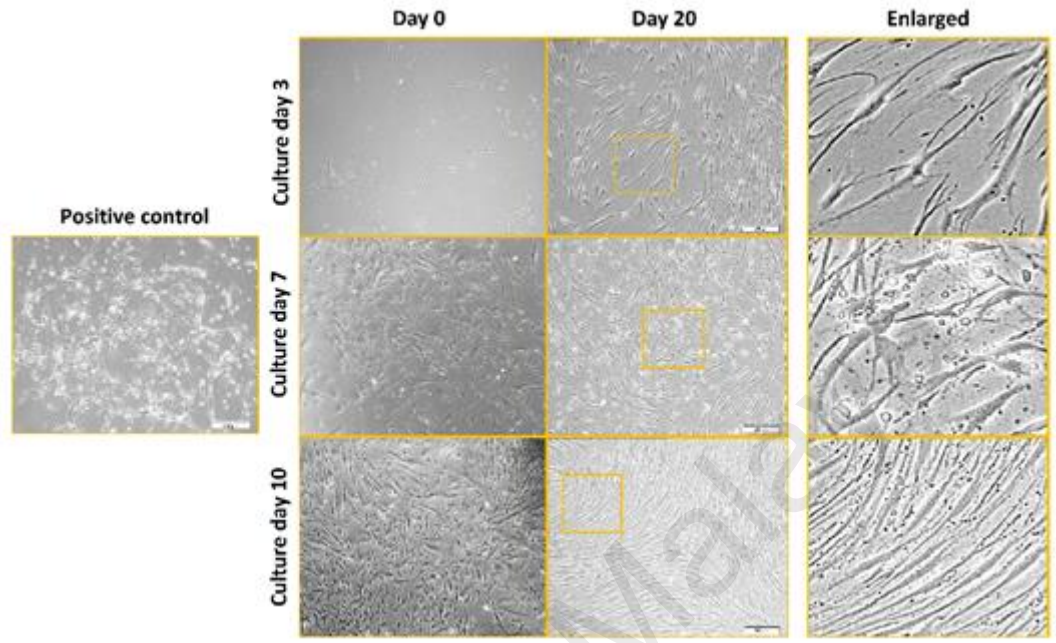

Figure 3-10: Morphology changes of SHEDs at respective time points upon directed differentiation. Micrographs were taken at 10x magnification.

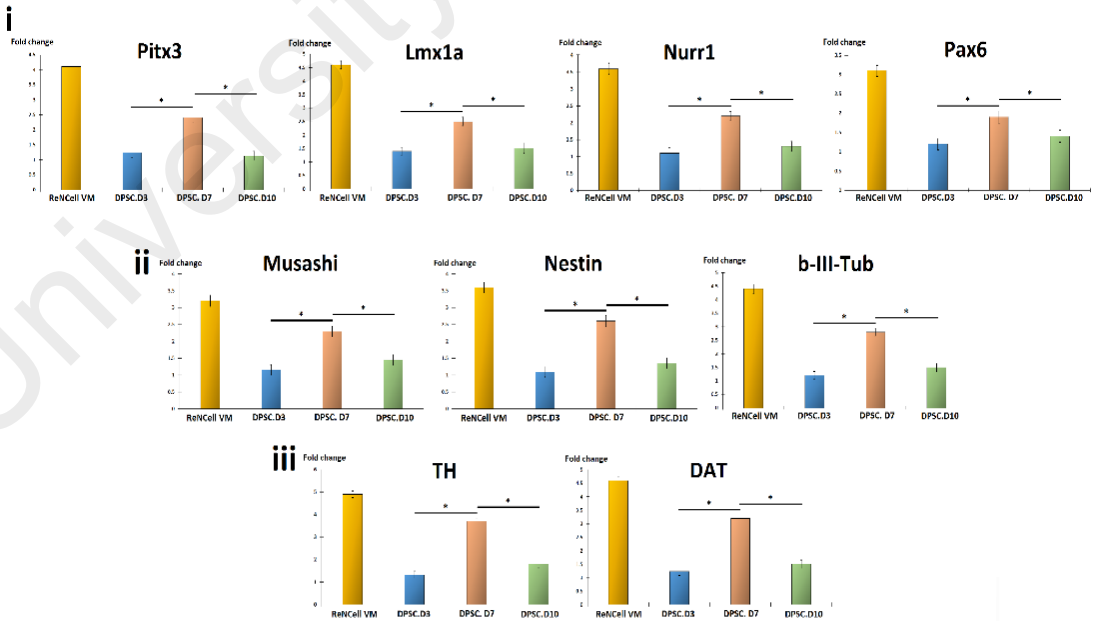

Figure 3-11: Gene expression profiles. Assessment of presence of (i) Transcription factors with respect to neuronal differentiation; (ii) Early neuronal markers, and (iii) Matured DA-ergic like cells markers. 18s gene was utilized as housekeeping gene from five independent samples. * indicates $p<0.05$.

3.3.6 Protein expression profile and functional analysis of SHEDs transformed into DA-ergic like cells

Similar observations were also noted in the cells' protein expression via immunocytochemistry which revealed that differentiated SHEDs-Day 7 were expressing significantly more markers especially Nestin, β-III-Tub, TH as well as DAT as compared to other culture period ($p < 0.05$; Fig. 3.12A i-ii). DA secretion assay too displayed similar phenomenon whereby differentiated SHEDs-Day 7 expressed highest amount of DA, followed by SHEDs-Day 10 and SHEDs-Day 3. As such, the DA release at SHEDs-Day 3 culture prior to differentiation, KCl and ATP stimulation were 99.82 ± 24.5 pg/mL, 108.72 ± 22.4 pg/mL, and 115 ± 26.2 pg/mL, respectively. Upon differentiation and chemical stimulation, the DA release were reported as 349.75 ± 34.35 pg/mL, 461.13 ± 24.3 pg/mL, and 473.83 ± 23.18 pg/mL respectively ($p < 0.05$). In a similar fashion, the DA release in SHEDs-Day 7 culture prior to differentiation, KCl and ATP stimulation were 152.16 ± 44.35 pg/mL, 179.58 ± 23.54 pg/mL, 188.4 ± 27.6 pg/mL, respectively. Again, upon differentiation and chemical stimulation, the DA release were reported as 704.52 ± 55.86 pg/mL, 1214.26 ± 22.96 pg/mL, and 1412 ± 23.15 pg/mL respectively ($p < 0.05$). Finally, the DA release for SHEDs-Day 10 cultures prior to differentiation and stimulation were 103.7 ± 10.2 pg/mL, 151.7 ± 22.3 pg/mL, and 115.9 ± 25.21 pg/mL, respectively. Upon differentiation and stimulation, the DA release were reported as 462.58 ± 35.28 pg/mL, 952.51 ± 25.3 pg/mL, and 1013.95 ± 35.29 pg/mL, respectively ($p < 0.05$). (Fig. 3.12B). Additionally, their action potential profile via multi electrode assay revealed similar trend in which SHEDs-Day 7 fired more action potentials, followed by SHEDs-Day 10 and SHEDs-Day 3 with spike amplitude readings of -42.82 ± 15.12 μV, -34.74 ± 19.73 μV, and -32.25 ± 21.65 μV, respectively ($p<0.05$; Fig. 3.12C-D). In addition, Western blot analysis also presented similar expression in which differentiated SHEDs-Day 7 expressed TH enzyme significantly as compared to other

culture period in line with their respective gene expression (Fig. 3.12E). Similar findings were also observed in the detection of differentiated TH^+ cells via flow cytometry where differentiated SHEDs-Day 7 presented the highest TH^+ cells of 65.7% ± 0.82, followed by SHEDs-Day 10 (48.7% ± 0.92) and SHEDs-Day 3 (34.2% ± 0.21) as compared to pre-differentiated DPSCs (Fig 3.12F). In a nutshell, the information presented by IPA complemented the differentiation analysis indicating that despite these phases originate from same batch of cells, yet they have respective differentiation profiles.

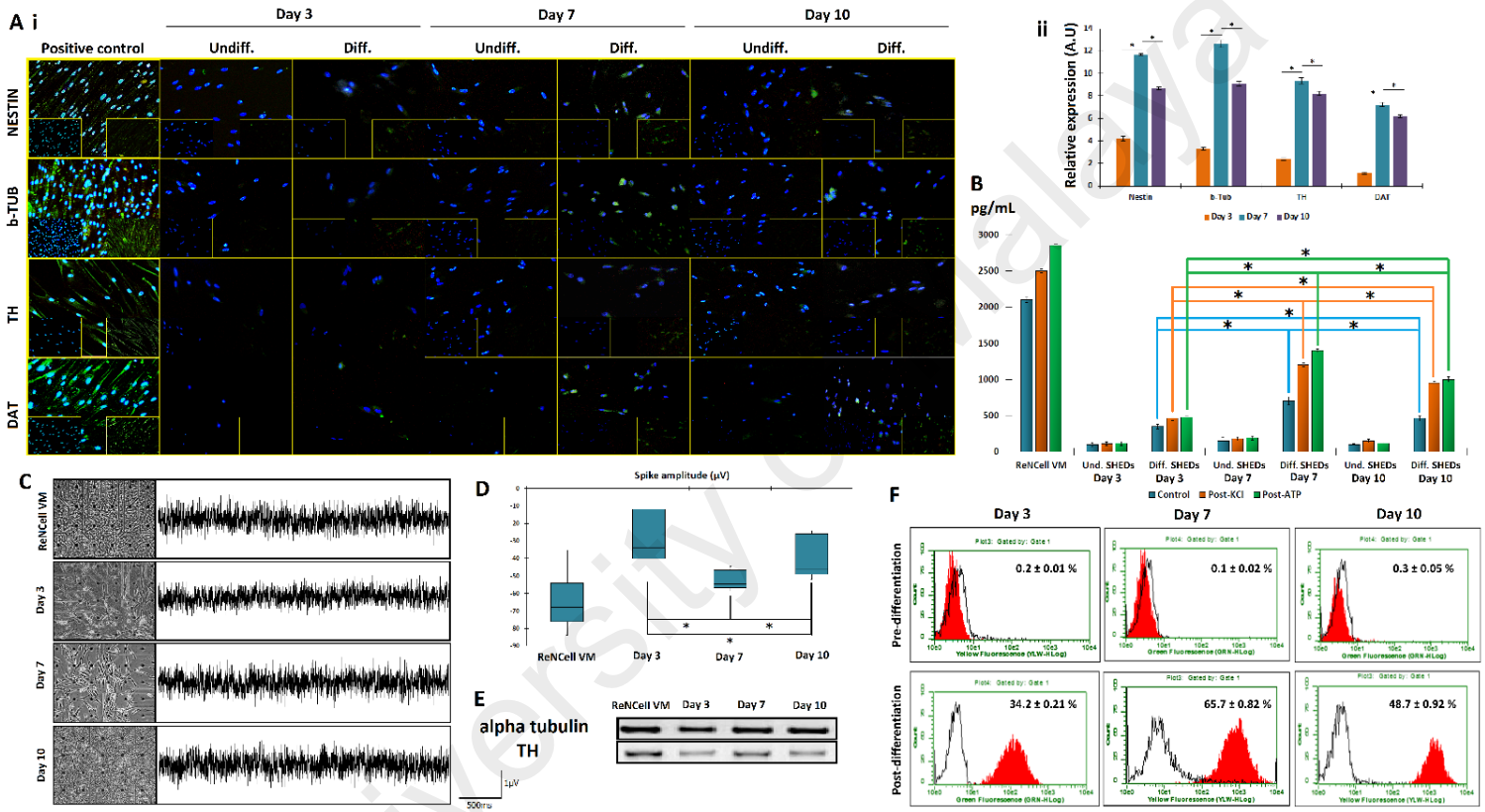

Figure 3-12: Functional behaviour of differentiated SHEDs. (Ai-ii) Protein expression via immunocytochemistry with differentiated ReNCell VM was used as positive control and their corresponding relative expression. (B) DA secretion assay at three time points with KCl and ATP as stimulating factors (C) Cell morphology of differentiated SHEDs from three time points upon seeding on chamber slides as well as impulse generated after activation using ATP (D) Spike amplitude from the generated impulse showing significant functionality of differentiated SHEDs at three time points. Differentiated ReNCell VM was used as control in this experiment. (E) Western blot analysis highlighting the expression of TH in differentiated SHEDs at three time points. It should be noted that the expression of alpha tubulin were almost constant in SHEDs from all time points. (F) Flow cytometry analysis depicting percentage of TH^+ cells upon directed differentiation of SHEDs at three time points.

3.4 Discussion

This study was initiated in an effort to describe the differential cell cycle state of SHEDs and their resultant influence in differentiation potential into DA-ergic like cells. Investigation on the suitable working passage to perform the subsequent downstream experiments was first initiated. Some of the criteria involved in selecting this include cell count, viability, PDT and their corresponding cellular ageing properties, i.e. senescence. These are paramount to ensure that cells are abundant and importantly, are in optimum condition, which would facilitate the SHEDs to respond effectively (Eslaminejad, Nazarian, Shariati, Vahabi, & Falahi, 2015; Roselli et al., 2013; Tavakolinejad et al., 2014). In this study, SC3 was chosen after taking into account of their relatively high cell count, low PDT and percentage of senescent cells as supported by previous studies (Black, Woodbury, Prockop, & Schwarz, 2013; Tatullo et al., 2014).

After selecting SC3 as our working sub-culture, the distinct phases present in SHEDs by cell cycle analysis was established. In general, SHEDs took an average of ten days to become fully confluent in an *in vitro* setting (Eslaminejad, Vahabi, Shariati, & Nazarian, 2010; Tatullo et al., 2014). Generally, as cells begin to proliferate, the adaptation towards culture environment took place and the activation of internal mechanism (either direct or indirect pathways) would led subsequently to Log phase (Maldonado-Soto et al., 2014). This pattern was reflected in their cell cycle profiles as noted in this study which was in tandem with previous studies (Jitschin et al., 2013; Pisciotta et al., 2015; Robey, Kuznetsov, Riminucci, & Bianco, 2014). Notably, cell cycle analysis is a simple yet vital parameter as it mirrors the DNA content of cell population of interest. The differential percentage of G0/G1 and G2/M phases found in this study could be attributed to the intrinsic mechanisms (either deterministic or stochastic factors) within these stages that might have led to diverse outcomes especially in differentiation assay (Gonzales et al., 2015; Sandler et al., 2015).

Strikingly, data from our current study also suggest similar phenomenon as described by Sandler et al. (2015) and Gonzales et al. (2015) whereby distinct cell cycle related gene expression gave rise to significant outcome in cell fate determination. Lengthened G0/G1 region which was the accumulative effect as noted in cell cycle analysis coupled with elevated expression of G1-phase genes such as Cyclin D1, Cyclin E1, CDK4, CDKN1B, CDKN3 and SKP3 in SHEDs-Day 7 might have facilitated the differentiation pathways when the neuronal growth factors were introduced in the culture system (Bertoli, Skotheim, & de Bruin, 2013; Das-Bradoo & Bielinsky, 2010). Moreover, a study by Pauklin et al. (2016) revealed that cell cycle regulators, particularly Cyclin D1–D3 controlled cell fate decisions in human embryonic stem cells (hESCs) by recruiting transcriptional co-repressors and co-activator complexes onto neuro-ectoderm, mesoderm, and endoderm genes. These activities resulted in the blocking of core transcriptional network necessary for endoderm specification while promoting neuro-ectoderm factors. The genomic location of Cyclin Ds was determined by their interactions with the transcription factors SP1 and E2Fs, which resulted in the assembly of cell cycle-controlled transcriptional complexes (Hindley & Philpott, 2013). These results revealed how cell cycle orchestrates transcriptional networks and epigenetic modifiers to instruct cell fate decisions. It is noted that however these studies had employed hESCs as their study model and the current study to date, is the first to have described the role of growth phase in this regard especially on multipotent stem cells such as SHEDs.

As observed in the Results, cells from Day 3 with its distinct growth phase profile would invariably took part in activation of genes pertaining to S and G2 phases such as ABL1, MCM3, MCM5, RPA1, BCCIP, and Cyclin G1 and together, they would retain the pluripotency behaviour and trigger extensive proliferation (Dupin & Coelho-Aguiar, 2013; Gonzales et al., 2015). This could be the underlying reason for the increment pattern of S phase during the first three days possibly indicating DNA synthesis taking

place. Furthermore, since this phase is regarded as 'adaptation phase' which provided the cells a buffer period for them to get used to the culture niche, it makes sense for them to sustain by proliferating and at one point overcome the adaptation 'barrier' and enter the next phase. Moreover, the fact that perhaps there are intrinsic controllers such as chromatin remodelling regulators or chromatin modifiers that govern these mechanisms (via either direct or indirect pathways) and facilitate their respective transitions cannot also be ruled out (Chen & Dent, 2014; Pauklin & Vallier, 2013). These regulators might hold a clue to controlling the cell proliferation/differentiation as well as cell fate determination, which is worth to be extensively studied in future.

Apart from acknowledging the roles played by cell cycle phases in the differentiation capacity of SHEDs, it is also worthy to pay attention on the vice versa implications of the neuropoeitic factors which were introduced during culture that might have manipulated their cell cycle state and possibly enhance the differentiation process. It has been previously stated that growth factors such as FGF2 and EGF are essential for stem cells differentiation into neuronal progenitors and their proliferation by inducing S-phase re-entry (Baguma-Nibasheka, MacFarlane, & Murphy, 2012; Bressan et al., 2014). DA-ergic maturation factors such as SHH, FGF8, BDNF as well as forskolin were reported to regulate levels of cyclin D and cyclin E. For instance, both SHH and FGF8 are responsible for maintenance of G1 phase via G1 cyclin-retinoblastoma axis (Jarrin, Pandit, & Gunhaga, 2012; Li et al., 2010). On the other hand BDNF and forskolin were reported to prevent cell cycle re-entry by delaying G2/M progression (Boutahar, Reynaud, Lassabliere, & Borg, 2010; He et al., 2011). Collectively, these neuropoeitic factors have a degree of influence during the differentiation and from this, it is evident that the introduction of such factors to a cell population with varying cell cycle phases have resulted in distinct outcomes.

Moreover, despite the distinct profile of the phases present in SHEDs, it should be noted that cells from all three phases were able to differentiate into DA-ergic-like cells and even functionally sound. All general assessments including morphology changes, gene as well as cellular protein expression were reflecting the aforementioned configuration thus conforming to the linkage between cell cycle phase and cell lineage commitment. For example, typical DA-ergic like cells are usually characterized with extensive branching and these features were most prominent in SHEDs-Day 7 culture followed by SHEDs-Day 10 and SHEDs-Day 3. Similarly all neuronal related markers such as NANOG, NESTIN, β-III-TUB, NCAM, TH, DAT were markedly expressed in SHEDs-Day 7 culture indicating their progressive development which took place during the course of differentiation (N. Gnanasegaran et al., 2015).

Likewise, their functional behaviour also obeys the same pattern whereby DA release were significantly enhanced ($p<0.05$) in the sequential pattern as described previously (SHEDs-Day 7 > SHEDs-Day 10 > SHEDs-Day 3), signifying the presence of purinergic receptors and K^+ ion channels in fully differentiated cells. In addition, the generation of action potential further showed that these cells were fully functional with their varied spike amplitudes and in tandem with aforementioned pattern.

Though it would be interesting to pin-point the exact mechanism leading to differential outcomes in terms of differentiation and functional assay, such study would indeed involve series of knock-down and over-expression investigations. This is due to the fact that complex mechanisms such as differentiation into neuronal like cells are not as a result of a single gene manipulation but rather a multi-genes interaction studies. Numerous optimization will have to be performed especially in the gene selection and upon conducting an in-depth study from this aspect, probably a better understanding on the roles played by the genes in the culture niche would be appreciated.

At present, one of many limitations faced in relation to the application of SHEDs for regenerative medicine is the small starting cell source material, which impedes the acquisition of large quantity of cells for transplantation. Another likely drawback is to maintain and store the cells of a particular phase and subsequently being able to retrieve them whenever needed for transplantation application. These hurdles can probably be rectified with hypoxia culturing (Peng, Shu, Lang, & Yu, 2015), application of bioreactors (Duan et al., 2014), usage of mitogenic factors such as human platelet lysate (HPL) and basic fibroblast growth factors (bFGF) (Vasanthan et al., 2014) or combination of scaffolds (Woloszyk et al., 2014), and perhaps by having proper storage/maintenance system adhering to cGMP (current Good Manufacture Practices) to ensure the delivery of a 'cell drug' that is not only safe but also reproducible as well as efficient (Potdar & Jethmalani, 2015). With the advancement of technology, the culturing can possibly be performed with automated devices, which will reduce batch-to-batch variation when cells are scheduled to undergo up-scaling for clinical applications.

To the best of our knowledge, this is the first report of its kind which involves the functionality of DA-ergic like cells differentiated from SHEDs from the perspective of cell cycle phases and the results further conformed to our hypothesis that cells from different cell cycle phase have significant impact on their end point differentiation. It is suggested that cells to be checked for their cell cycle phases as part of their routine check and those with lengthened G0/G1 phase (due to accumulative effect during culture period) are the most suitable batches for transplantation works. This information is very valuable particularly in studies involving cell replacement therapies as they can employ the cells according to every arising situation.

3.5 Conclusions

Within the scope of this study, the following conclusions can be made:

1. SHEDs at SC3 was found to be optimum for downstream applications based on their high cell count and relatively low PDT, PD and percentage of cells with senescent characteristics.
2. SHEDs with high percentage of G0/G1 phases (i.e. SHEDs-Day 7) as illustrated by cell cycle analysis revealed significant higher differentiation capability towards DA-ergic-like cells.

CHAPTER 4: NEURO-IMMUNOMODULATORY PROPERTIES OF SHEDS IN AN *IN VITRO* MODEL OF PARKINSON'S DISEASE

4.1 Introduction

Inflammatory responses are typical circumstances that would occur when any traumatic injury incurs a respective environment. In neurodegenerative diseases such as in Parkinson disease (PD) for instance, there are many theories that would explain progressive degeneration of neurons. One of them including neuro-inflammation leading to cell death (Gordon et al., 2016; Sugama et al., 2015). Post mortem studies have confirmed the involvement of innate as well as adaptive immunity in the affected brain regions in PD patients (Aron Badin et al., 2016; Olson & Gendelman, 2016). Furthermore, activated microglial cells and T lymphocytes have also been detected in the SN of patients concomitantly with an increased expression of pro-inflammatory mediators (Macchi et al., 2015; Walker et al., 2015). On another note, CRT has been proposed to be the next solution in clinical settings when the current treatment modalities such as L-Dopa medications and procedures like deep brain stimulation are viewed as clinically ineffective in eradicating the symptoms associated with PD (Barker et al., 2015). Among the widely available cell sources, SHEDs have been in the limelight owing to their ease of accessibility and less ethical hurdles (Abu Kasim et al., 2012).

This group of cells which are derived from neural crest are a population of multipotent stem cells with the capacity to differentiate into several different cells lineages *in vitro* and *in vivo*, including glial and nerve cells (Cho et al., 2016; Kanafi et al., 2014). We have previously shown that these cells were able to differentiate into DA-ergic like cells (N Gnanasegaran et al., 2015; Gnanasegaran et al., 2014) and a number of studies have also utilized them as a treatment modality for PD (Chun, Soker, Jang, Kwon, & Yoo, 2016; Mead, Logan, Berry, Leadbeater, & Scheven, 2016). Even though studies have

indicated that SHEDs were able to accommodate in the brain upon transplantation, their roles in manipulating the inflammatory environment in PD remain to be elucidated.

Apart from neurons, microglia is another prevalent cell type in the brain which is among the most versatile cells in the body (Nayak, Roth, & McGavern, 2014). They possess the capacity to morphologically and functionally adapt to their ever-changing surroundings. Even in a resting state, the processes of microglia are highly dynamic and perpetually scan the central nervous system (CNS). They are in fact vital participants in CNS homeostasis, and dysregulation of these sentinels can give rise to neurological disease, as often noted in PD (Bilimoria & Stevens, 2015). Studies have also reported augmentation of pro-inflammatory mediators when microglia are activated (Herrera Carmona et al., 2015; Peterson & Flood, 2012). Such scenario would often result in disruption of homeostasis and further damage during course of the disease (Perry, 2012). The ideal CRT regime should consists of suppression of inflammation apart from maintaining neuron population and it is believed that SHEDs would be able to carry out these tasks.

With that notion, the present study was undertaken to investigate if SHEDs could influence the inflammatory microenvironment when neurons and microglia were exposed to neurotoxin, 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), which mimics the inflammatory conditions and contribute to degeneration of DA-ergic neurons. It is hoped that this study would enlighten on the possible activities of SHEDs when they are to be transplanted in actual PD cases. Specifically, the objectives are:

1. To determine the suitability of MPTP to trigger inflammatory processes.
2. To investigate the effect of SHEDs on differentiated neurons in an inflammatory *in vitro* microenvironment.

4.2 Materials and methods

4.2.1 Pulp collection and isolation of cells

The propagation and cultivation of SHEDs were carried out as described in sub section 3.2 1 and cells at SC3 with high G0/G1 phase were acquired as described in 3.2.4. All subsequent analyses were performed in cGMP certified facility with clean environment conforming to guidelines by Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S).

4.2.2 Cultivation of IMR-32 and EOC2 cell lines

IMR-32 (neuroblastoma) and EOC2 (microglia) cells were purchased from American Type Culture Collection (ATCC, Virginia, USA). IMR-32 cells were routinely grown in DMEM supplemented with 10% FBS (Hyclone, USA) whereas EOC2 requires an addition of 20% of conditioned media from another cell line (LADMAC: bone marrow culture; ATCC, USA). All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

4.2.3 Differentiation of IMR-32 with retinoic acid

Undifferentiated cultures were harvested by means of trypsinization (0.05% Trypsin-EDTA, Invitrogen) and cultured subsequently in 96 and 24 well plates at 1000 cells/cm^2. After 24 hrs of seeding, IMR-32 cells were allowed to differentiate by adding retinoic acid (RA; Sigma-Aldrich, St. Louis, MO, USA) to the culture medium to a final concentration of 10 µM for a period of 6 days. The medium was replaced every two days. The differentiated IMR-32 were utilized for all subsequent experiments.

4.2.4 Treatment of cell lines with MPTP and LPS

Both RA differentiated IMR-32 and EOC2 cultures were pre-treated with MPTP at 200 µM to mimic inflammation microenvironment as described previously (Aymerich et al., 2016) and their cells were respectively pelleted for DNA damage analysis while their corresponding supernatant was tested for the presence of inflammatory cytokines, reactive oxygen species as well as nitric oxide. Similar treatment using lipopolysaccharide from LPS (Sigma-Aldrich, St. Louis, MO, USA) was performed at concentration of 1µg/mL to serve as control as it is the widely employed study model depicting inflammation. The treatment was conducted in single culture system and also in 1:1 ratio of neurons:microglia co-culture system, as illustrated in Fig. 4.1.

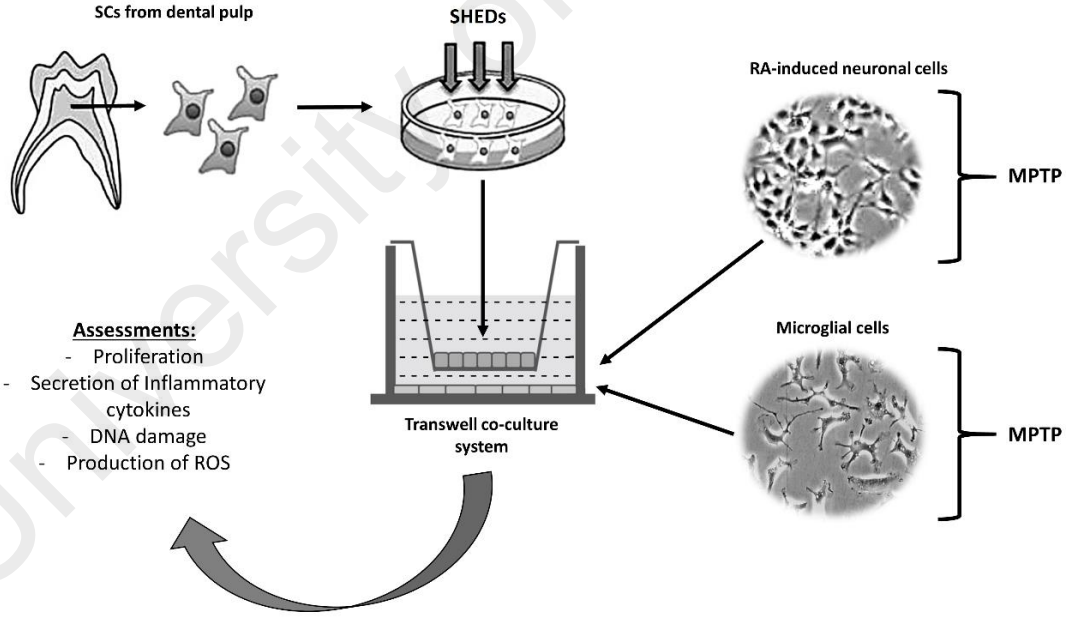

Figure 4-1: Schematic representation of co-culture system. Schematic representation of co-culture system using Transwell. Neurons and microglia were exposed to MPTP before introducing SHEDs and their immuno-modulatory behaviour was evaluated.

4.2.5 Determination of cell viability

The viability of MPTP exposed cells for 24, 48 and 72 hrs were determined via MTT assay as described by the manufacturer (Cell Signalling Technologies, USA). In brief, the culture medium was removed and replaced with fresh culture medium containing MTT (0.5 mg/mL). After 4 h incubation at 37°C, the solution was removed and the resulting blue formazan was solubilized in 100 µL of DMSO. The corresponding optical density was read at 595 nm using microplate reader (Multiskan PLUS, Thermo Scientific, VA, USA).

4.2.6 Determination of reactive oxygen species (ROS) via ELISA

The intracellular ROS of cells were detected using DCHF-DA as described by the manufacturer. Briefly after treatment, the cultured cells were incubated for 30 min in 5 mM DCHF-DA diluted in DMEM-KO basal medium. The ROS levels were detected by using a microplate reader with an excitation of 488 nm and emission of 525 nm (Multiskan PLUS, Thermo Scientific, VA, USA).

4.2.7 Determination of nitrite species

Nitric oxide (NO) was detected in the supernatant of cultures as described by the manufacturer. In brief, 50 µL culture supernatant from each sample was transferred to a 96-well plate in triplicate and added with an equal volume of Griess reagent (Promega, Madison, Wisconsin, USA). Absorbance was read at 530 nm (Multiskan PLUS, Thermo Scientific, VA, USA) after 10 minutes incubation. Nitrite concentration was calculated with reference to a standard curve of freshly prepared sodium nitrite (0 to 100 µM). The results are displayed as concentration of NO^{-}_{2} in µM.

4.2.8 Detection of inflammatory cytokines via Q-Plex technology (multiplex ELISA)

The multiplex ELISA was performed based on placement of immobilized capture antibody in 350–500 μm spots at the bottom of polypropylene 96-well plates to capture target proteins (Rosser, Dai, Miyake, Zhang, & Goodison, 2014).

Samples and standards (50 μL) were loaded onto the Q-Plex™ plate and allowed to incubate for two hours. Subsequently, the sandwich immunoassay complex that forms was incubated with biotin-streptavidin/Horse Radish Peroxidase reagents for an additional two hours. Antigen standard curves were performed in duplicate diluting the antigen standard 1:3 for 11 points with a single negative point. Samples were diluted at ratios of 1 to 2 (sample to buffer) (50%), 1 to 20 (5%) and 1 to 200 (0.5%). Each dilution was loaded into three wells and measured in triplicate, a total of 9 wells per sample. Standard curves were constructed using Q-View Software™, which allows for the selection of multiple non-linear and linear equations to fit the standard curve.

4.2.9 Alkaline Comet assay to identify DNA damage

DNA strand breaks in MPTP treated cells were evaluated using Trevigen Comet Assay™ kit (Trevigen Inc., Gaithersburg, MD). Cells were resuspended in ice-cold PBS (Ca^{2+} and Mg^{2+} free) to a concentration of $1x10^5$ cells/mL. Briefly, an aliquot of 50 μL of cells ($1x10^5$ cells/mL) was added to 500 μL of molten LM Agarose (1% low-melting agarose) kept at 42°C. Fifty microliters were immediately pipetted and evenly spread onto an area of the comet slides. The slide was incubated at 4°C in the dark for 10 min to accelerate gelling of the agarose disc and then transferred to pre-chilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 1% Triton X-100, pH 10) for 60 min at 48°C. A denaturation step was performed in alkali solution (300 mM NaOH, 1 mM EDTA, pH > 13) at room temperature for 20 min, in the dark. The slide was then transferred to pre-chilled alkaline electrophoresis solution pH > 13

(300 mM NaOH, 1 mM EDTA) and subjected to electrophoresis at 1 V/cm, 300 mA for 40 min in the dark at 4°C. At the end of the electrophoresis, the slides were washed with neutralization buffer (0.4 M Tris–HCl, pH 7.4) and immersed in ice-cold 100% ethanol at room temperature for 5 min and air dried. DNA was stained with EtBr (Sigma-Aldrich, St. Louis, MO, USA) for 20 min in the refrigerator and immediately analysed using an Olympus digital camera attached to an Olympus BX51 epifluorescence microscope.

4.2.10 Co-culture of in vitro model with SHEDs

Both cell lines which were treated with 200uM MPTP were then co-cultured with Log phase derived SHEDs for 48 hours using Transwell system as described by the manufacturer (Corning). After 48 hours, the cells and their corresponding supernatant were collected and tested for viability, presence of cytokines and ROS as well as DNA damage, as described earlier.

4.2.11 Gene expression via qRT-PCR

SHEDs which were exposed in the co-culture system were harvested via trypsinization. Their corresponding RNA was extracted using Trizol (Invitrogen) and subsequent gene expression analysis was performed as described previously (N Gnanasegaran et al., 2015; Yazid et al., 2014). Gene expressions were analysed via comparative C_T method ($\Delta\Delta CT$) and were normalized to 18s rRNA against their corresponding control samples. The primer sequences are listed in Table 4.1.

Table 4-1: List of genes with primer sequence and their product size

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Base pair size
Musashi	CTTTGATTGCCACAG CCTTC	CTCCAGCTATGCACAAATCC	148
Pax 6	ATGAACAGTCAGCCAATGGG	CACACCAGGGGAAATGAGTC	163
Nestin	GTAGCTCCCAGAGAGGGGAA	CTCTAGAGGGCCAGGGACTT	206
β-tubulin III	GCGAGATGTACGAAGACGAC	TTTAGACACTGCTGGCTTCG	115
Vimentin	GAGAACTTTGCCGTTGAAGCA CTACCAAGA	TCCAGCAGCTTCCTGTAGGTT CTCAATGTC	170
Nurr 1	CGCCTGTAACTCGGCTGAA	AGTGTTGGTGAGGTCCATGC	169
18s rRNA	CGGCTACCATCCAAGGAA	GCTGGAATTACCGCGGCT	186

4.2.12 Statistical analysis

Results were presented as comparison of mean ± standard deviation (SD) from five independent samples (n=5). The data were analysed using SPSS, version 19.0 (SPSS Inc, Chicago, IL, USA). Statistical test using 2-way ANOVA were carried out and a p-value of < 0.05 was considered to be significant.

4.3 Results

4.3.1 Proliferative capacity of single- and co-culture system decreases with time

We first set out to understand the impact of both toxins (LPS and MPTP) in the proliferation of neurons as well as microglia at 24, 48 and 72 hour post exposure. This is vital as it facilitates the determination of the best time point for conducting subsequent analysis. The morphology features of differentiated IMR-32 and EOC2 microglia has been widely explained in which RA-induced IMR-32 cells would generally be observed as elongated cells with extensive neurite formations (Fig. 4.2A). On another note, EOC2 culture is highly characterized with presence of cell body rounding and their original spindle or stellate appearances. In this study, the microbial toxin LPS was incorporated primarily due to its widely accepted model to simulate inflammation and associating MPTP side-by-side would provide comparative analysis between these two. As expected, the proliferation of differentiated IMR-32 presented a detrimental effect over time (Fig. 4.2B). In terms of sensitivity, differentiated IMR-32 cells were more responsive to LPS as compared to MPTP in which their proliferation rate was decreased to 71% ± 1.39 as compared to 79% ± 1.79 at 24 hour, respectively ($p<0.05$). Proliferation at 48th hour displayed 10% decrement to 61% ± 1.31 in LPS exposed culture whereas MPTP exposed culture displayed 71% ± 1.71 ($p<0.05$). At 72nd hour, proliferation of both culture was the lowest approximately 50%, thereby rendering it to be incompetent to be selected as the appropriate time point for this study. Surprisingly, when microglia culture was exposed to either LPS or MPTP, their proliferation rate was augmented whereby an increment of

at least 20% to 124% ± 0.82 and 122% ± 0.79%, respectively was noted in both cultures at 24th hour ($p<0.05$). However, beginning from 48th hour, their proliferation especially in LPS induced culture started to decrease to 116% ± 0.73 and subsequently reduced to 108% ± 0.58 at the 72nd hour as compared to control culture ($p<0.05$). On the contrary, MPTP significantly stimulated their proliferation from 122% ± 0.82 to 133% ± 0.73 at 48th hour, however their proliferation dropped to 113.4% ± 0.58 ($p<0.05$) at 72nd hour. Subsequent exposure of each toxin to 1:1 ratio of neuron:microglia demonstrated an increment in their proliferation by at least 10%. In LPS introduced culture system, their proliferation rate at 24th hour, 48th hour and 72nd hour was 116.45% ± 1.26, 106% ± 1.35, and 98% ± 1.45, respectively. In MPTP induced culture however, their corresponding proliferation rate at 24th hour, 48th hour, and 72nd hour was noted as 110.6% ± 1.58, 106.5% ± 1.48, and 99.45% ± 1.27, respectively. From these proliferation patterns, it was apparent that MPTP presented slow progressive changes while LPS presented sudden variations. Next, we extended this study to look at their proliferation behaviour when SHEDs were introduced in the co-culture system after the damage by MPTP has commenced. It was demonstrated that SHEDs were able to suppress the proliferation of co-culture system significantly almost to their basal level and this pattern was noted at all time points. The proliferation rate at 24th hour, 48th hour and 72nd hour were noted as 88.9%, 88.1% and 83.5%, respectively ($p<0.05$). Looking at the variations in their proliferation at various time points, 48th hour have been carefully selected as time point of study for subsequent experiments as it provides sufficient amount of time with distinct propagation behaviour.

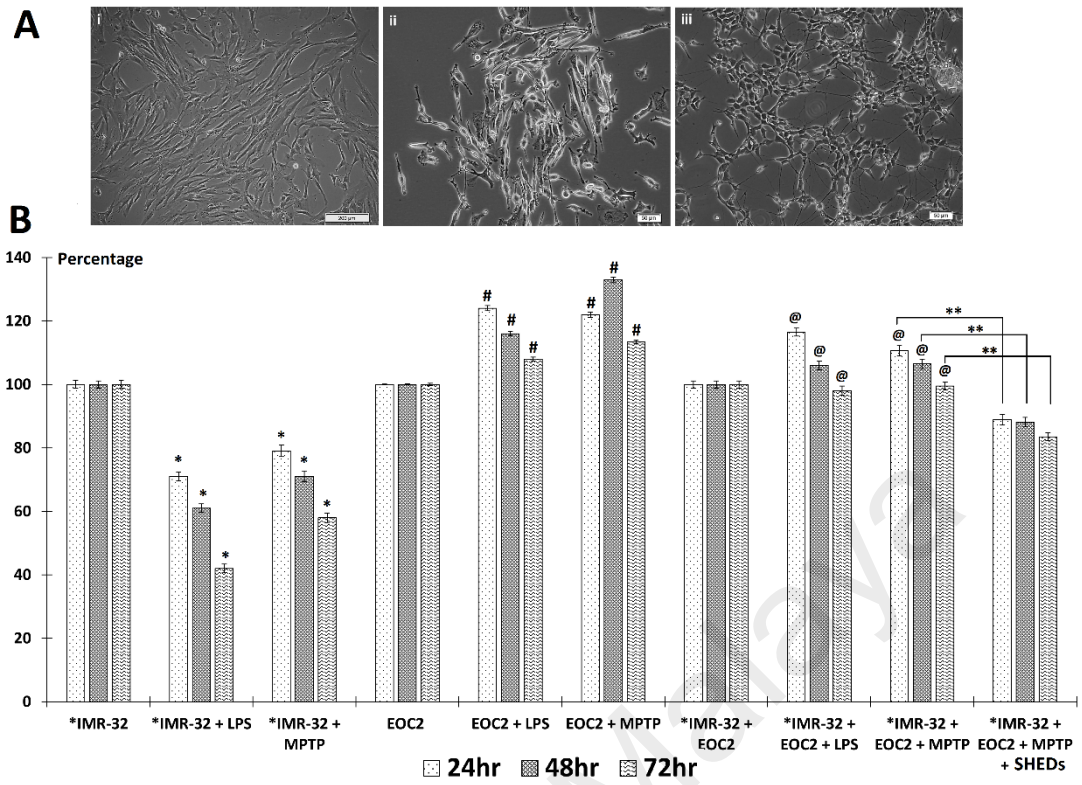

Figure 4-2: Morphology and proliferation profiles of neuronal and microglia cell lines. (A) Morphology features of SHEDs, differentiated IMR-32 and microglia EOC2 (i, ii, iii, respectively). Micrographs were taken at 10x magnification. (B) The proliferative behavior of differentiated IMR-32, EOC2 and SHEDs in single as well as co-culture systems. * indicate $p<0.01$ as compared to differentiated IMR-32, # indicate $p<0.05$ as compared to EOC2, @ indicate $p<0.05$ as compared to co-culture of IMR-32 and EOC2, and ** indicate significance ($p<0.05$) when SHEDs were introduced in the co-culture system.

4.3.2 Attenuation of DNA damage by SHEDs was insignificant

In order to understand the reparative effect of SHEDs from the perspective of DNA damage, they were exposed in MPTP induced inflammation model. Via Comet assay, a clear round shape of DNA would suggest that DNA is still intact while presence of comet-like-smear would indicate DNA damages (Fig. 4.3A). Similar to proliferation behaviour, DNA damage analysis revealed a significant increase in tail moment when either toxins were introduced to neuronal-microglia culture ($p<0.05$; Fig. 4.3B). It should be noted that the damage caused by LPS was more apparent as compared to MPTP which is also reflected in the percentage of DNA in the comet tail (Fig. 4.3C). The introduction of

SHEDs however revealed a 14.8% decrement pattern in tail moment (from 31.67 ± 3.1 to 26.97 ± 2.2) and 13.5% decrement in DNA percentage in tail (from 42.5 ± 6.5 to 36 ± 6.9). Despite portraying insignificance, this decrement pattern might perhaps suggest that the cells were preventing further damages elicited by the toxins in a slow manner and can be attributed to involvement of complex mechanism.

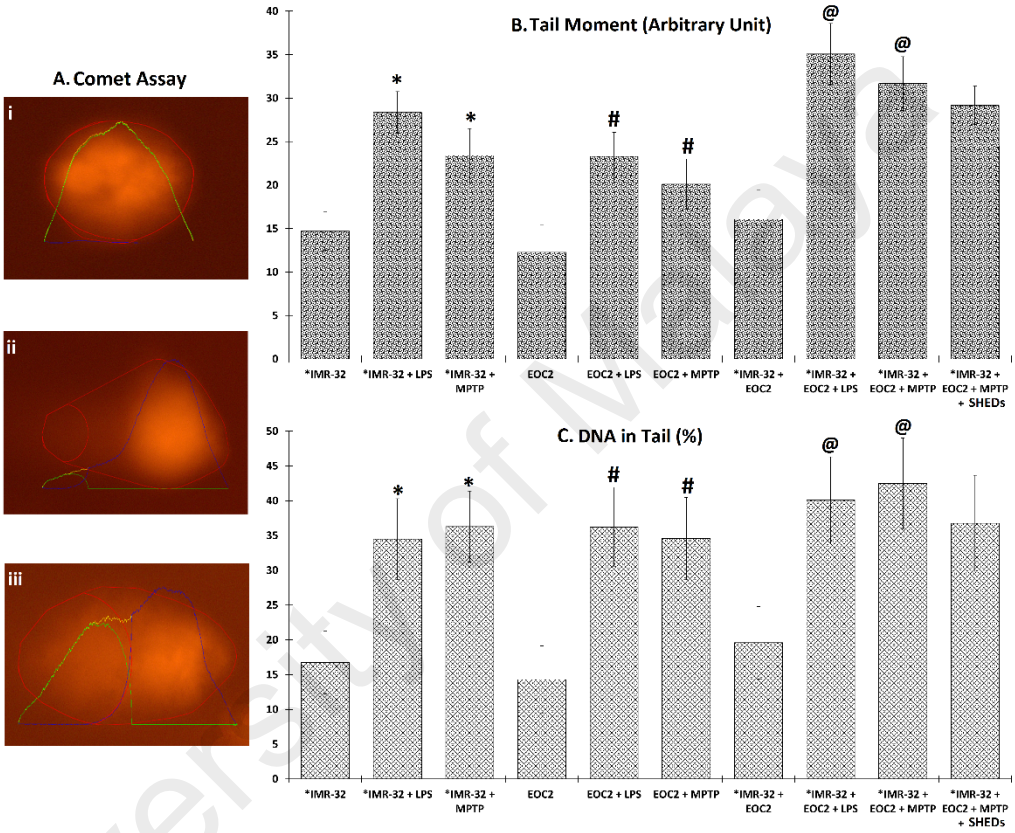

Figure 4-3: DNA damage analysis. (A) Output obtained from Comet assay ranging from intact DNA (i) to severely damaged DNA (iii). Micrographs were taken at 40x magnification. (B) Tail moment when toxins were added and when SHEDs were introduced. (C) Percentage of DNA in tail when toxins were added and when SHEDs were introduced. * indicate $p<0.05$ against differentiated IMR-32, # indicate $p<0.05$ against EOC2, and @ indicate $p<0.05$ against co-culture of IMR-32 and EOC2.

4.3.3 SHEDs attenuates reactive oxygen species and reactive nitrogen species significantly

Additionally, the presence of reactive oxygen species and its corresponding nitrogen species upon exposure with MPTP were also tested (Fig. 4.4). As noted previously, the

presence of either toxins (LPS or MPTP) augmented the levels of ROS as well as NO significantly in both single culture (neurons or microglia) as well as co-culture system ($p<0.05$). With the introduction with SHEDs for 48 hours in the system, the levels of ROS and NO were noted to have distinctly decreased though not to their respective basal level which was 1.98 ± 0.13 for ROS and 37.8 ± 1.79 for NO ($p<0.05$).

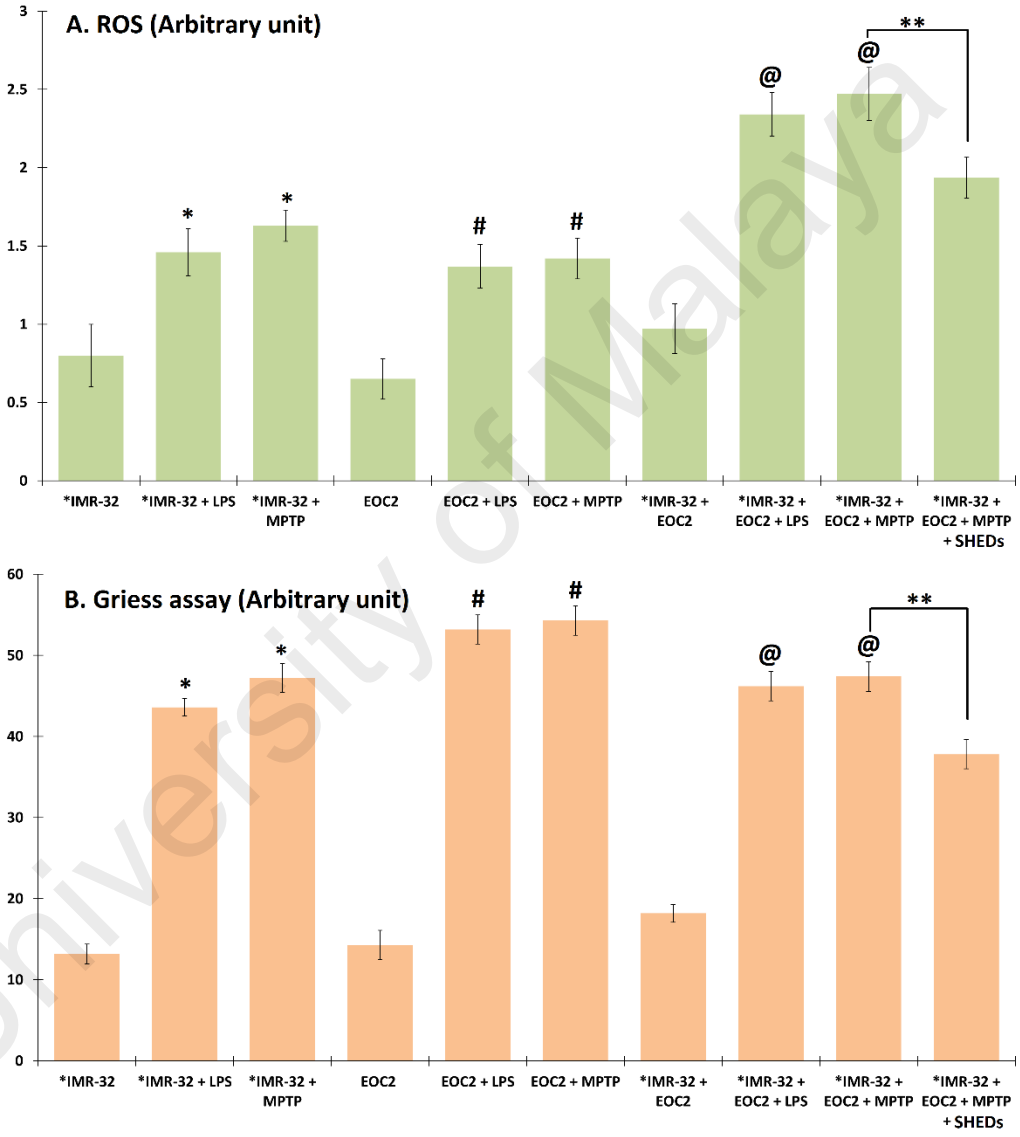

Figure 4-4: Presence of ROS and NO in culture system. (A) Levels of ROS increased when toxins were added and reduced significantly when DPSCs were introduced for 48 hours. (B) Levels of ROS increased when toxins were added and reduced significantly when SHEDs were introduced for 48 hours. * indicate $p<0.05$ against differentiated IMR-32, # indicate $p<0.05$ against EOC2, and @ indicate $p<0.05$ against co-culture of IMR-32 and EOC2.

4.3.4 SHEDs significantly regulates secretion of inflammatory cytokines

The fluctuations of cytokines especially those pertaining to inflammatory were also investigated. As such, the conditioned media from all conditions were thoroughly collected and checked for presence of inflammatory cytokines using multiplex ELISA platform. Again, it was shown that pro-inflammatory cytokines such as IL-1α, IL-1β, IL-5, IL-6, IL-8, IL-12p70, IL-15, IL-17, IL-23, IFNγ, and TNFα were markedly increased with exposure of MPTP as compared to its corresponding basal level ($p<0.05$; Fig 4.5A) in single culture system (neurons or microglia). It should be noted that the expression of these proteins upon exposure to MPTP were lower as compared to those of LPS. As expected, the release of pro-inflammatory cytokines in co-culture system were also in accordance to the pattern observed earlier whereby the introduction of toxins augmented their secretory profiles. When SHEDs were introduced in the co-culture system, a decrement pattern was noted in all cytokine secretion except in IL5, IL6, and IL17. This could possibly suggest that these cytokines were not immediately affected as how the other cytokines have responded.

On the other hand, the expression profile of anti-inflammatory cytokines namely IL-2, IL-4, IL-10, IL-13, and TNFβ had displayed mixed responses (Fig. 4.5B). For instance in the presence of MPTP, a general increment of secretion profile was noted in both single culture system (neurons or microglia), except for TNFβ. Contrastingly in co-culture system, exposure to MPTP displayed general decrement of expression profiles of all cytokines mentioned earlier except for IL10. When the same co-culture system were exposed to LPS, a general increment was noted in all expression profile except for IL2 and TNFβ. In the presence of SHEDs, the expression of anti-inflammatory cytokines were significantly augmented at least by 40% ($p<0.05$). This phenomena is indicative of immunomodulation behaviour of SHEDs in an inflammatory mediated microenvironment.

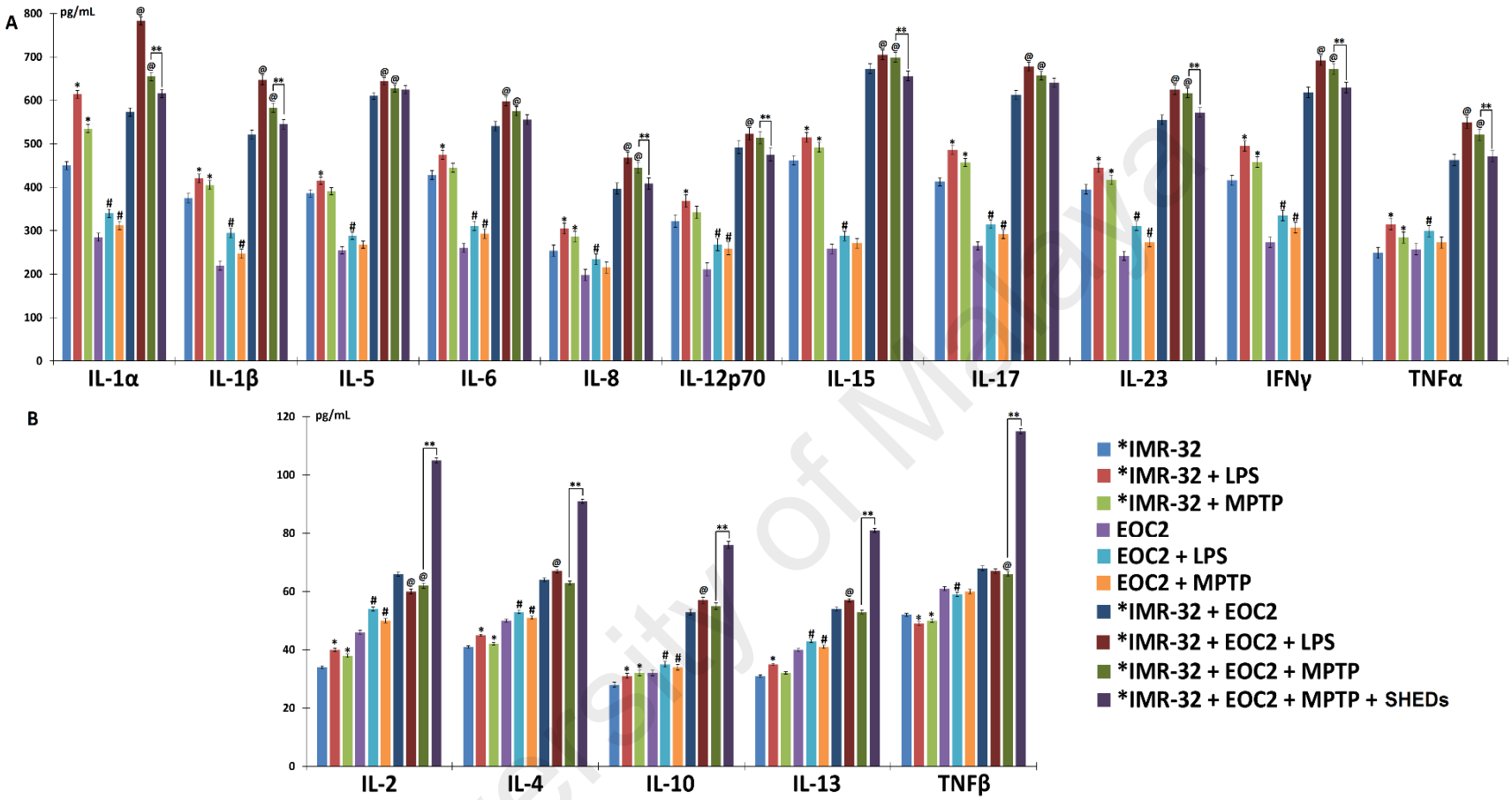

Figure 4-5: Secretion of inflammatory mediators. (A) Secretion of pro-inflammatory factors upon exposure with toxins and their expression regulation with introduction of SHEDs. (B) Secretion of anti-inflammatory factors upon exposure with toxins and their regulation with introduction of SHEDs. * indicate $p<0.05$ against differentiated IMR-32, # indicate $p<0.05$ against EOC2, and @ indicate $p<0.05$ against co-culture of IMR-32 and EOC2.

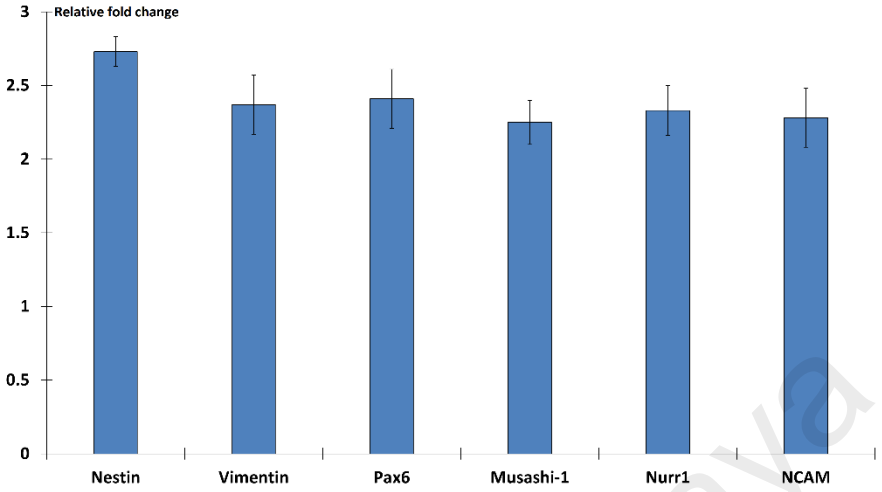

Figure 4-6: Presence of early neuronal markers in SHEDs despite being exposed to harsh microenvironment. Expression of 18s gene was utilized as housekeeping gene

4.4 Discussions

In a typical CRT scenarios, the transplanted cells are expected to perform certain therapeutic activities. These include undergoing self-renewal, proliferation and differentiation sufficient enough to elicit significant changes that would lead to healing/restoration (Barker et al., 2016). In addition, they are also expected to differentiate into a particular cell lineage while controlling the inflammatory conditions present in the traumatic region (J. Liu et al., 2015). In PD for instance, inflammatory processes have been regarded as one of underlying reasons triggering death of DA-ergic neurons (Barker et al., 2015). It has been hypothesized that controlling these processes would facilitate neuro-restoration by the stem cells (Gonzalez, Woynarowski, & Geffner, 2015). In an *in vitro* setting, inflammatory processes can be mimicked via various methods with LPS being the most commonly employed mechanism (Said, Bock, Müller, & Weindl, 2015; X. Zhang et al., 2015). This bacterial toxin has been implicated with the activation of NF-κB via TLR4 receptors which result in elevated expression of pro-

inflammatory mediators such as IL1B, leading to mitochondrial dysfunction and activation of caspase-related apoptotic pathways (Guo, Callaway, & Ting, 2015; Shi et al., 2014). On a separate note, MPTP is widely applied neurotoxin to simulate PD and its action mechanism in an *in vitro* setting is quite similar to that LPS. This neurotoxin has been reported to have activated nucleotide-binding domain, leucine-rich repeat containing proteins (also known as NOD-like receptors, NLRs), especially NLRP3 to augment the expression of pro-inflammatory mediators and induce mitochondrial dysfunction as well as pyroptosis, an inflammatory form of cell death (Lamkanfi & Dixit, 2014; Vanaja, Rathinam, & Fitzgerald, 2015). Moreover, studies have indicated presence of nuclear-factor-E2-related factor 2 (Nrf2) activation in response to MPTP, leading to depletion of glutathione, decreased cell viability, and inhibition of mitochondrial oxygen consumption as well as glycolysis rates in a dose-dependent manner (Ahuja et al., 2016; Lee et al., 2016).

In this study, the presence of both toxins had inhibited the proliferation of neurons and this phenomena could be highly attributed to the activation of apoptosis and pyroptosis, as suggested by previous studies (Adamczak et al., 2014; Fann et al., 2013). On the contrary, proliferation of microglia was distinctly observed upon exposure with these toxins and this could be due to release of pro-inflammatory cytokines by the damaged neurons, thereby leading to co-activation of TLR4 and IFN-γ receptors, which are responsible for their proliferation. Next, they would probably get activated and initiate mechanisms leading to neuronal cell death (Kim, Na, Myint, & Leonard, 2016; Russo & McGavern, 2015).

Apart from negatively regulating proliferation of neurons, activated microglia has also been associated with augmentation of DNA damage, increment of reactive oxidative stress and nitric oxide as well as regulation of inflammatory mediators' secretion (Blesa

et al., 2015; Von Bernhardi, Eugenín-von Bernhardi, & Eugenín, 2015). These would probably lead to the re-activation of NF-κB (positive feedback mechanism) together with MAPK pathway, resulting pro-inflammatory mediators to be further released and instigate related mechanisms (Park et al., 2015).

The introduction of SHEDs in co-culture system has revealed positive co-relation especially in reducing the proliferation rate of microglia, attenuation of ROS, NO as well as regulating secretion of inflammatory cytokines. The reduction in proliferative behaviour as noted in this study could be reflecting the inhibitory effect elicited on microglia as SHEDs has immuno-modulatory properties as we have previously reported (Yazid et al., 2014). The secretory factors by SHEDs could probably contributed to the reversal of inflammatory processes that both LPS and MPTP had initiated (Ahuja et al., 2016; Lee et al., 2016). We believe that the anti-inflammatory factors which were observed in this study could be originated from SHEDs since their release was much elevated as compared to their basal level. With the elevation of anti-inflammatory cytokines, it is speculated that these factors neutralize the inflammatory processes and perhaps with other growth factors secreted by SHEDs, the neuron population would be allowed to sustain themselves (Mead et al., 2016; Strojny, Boyle, Bartholomew, Sundivakkam, & Alapati, 2015). When these features are to be extrapolated in actual PD scenario, we can possibly expect SHEDs to confer protection over DA-ergic neurons by limiting the proliferative behaviour of microglia as illustrated in Fig. 4.6. In addition, their secretory profiles which mainly include anti-inflammatory mediators can be one of many driving forces that would increase the efficiency of CRT (Kong et al., 2016). The SHEDs were also assessed after exposing them in 'neuro-inflammation' microenvironment and surprisingly, they still retain their ability to express neuronal related genes such as Nestin, Pax6, and Nurr1 (Fig. 4.6). This further conforms the applicability of SHEDs as a suitable cell source for CRT especially in PD cases.

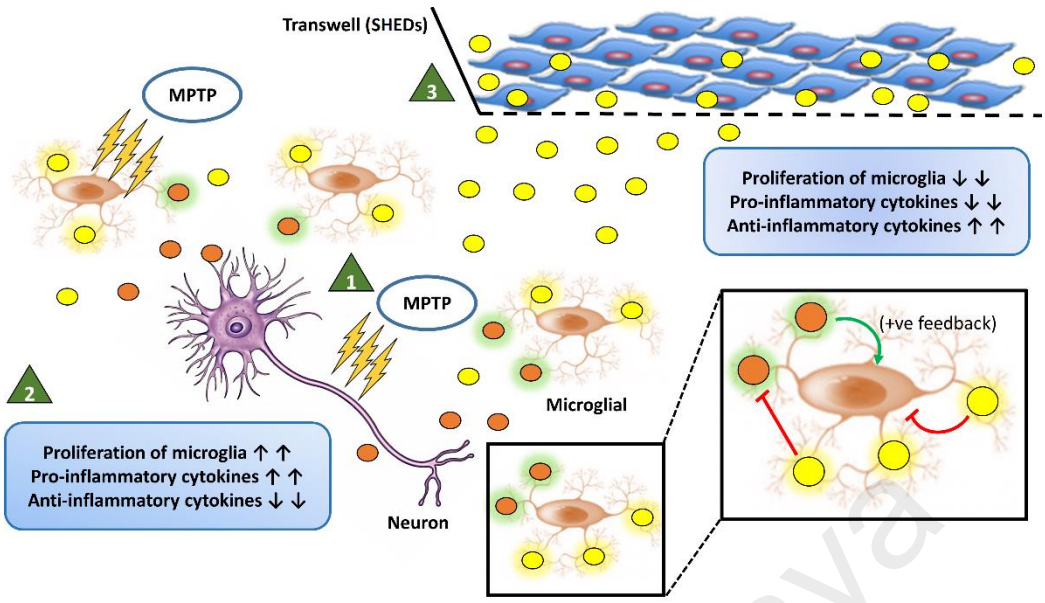

Figure 4-7: Possible mechanism on the immuno-modulatory behavior of SHEDs upon introduction in MPTP-induced neuro-inflammation. Brown circles represent factors secreted by damaged neurons whereas yellow spheres indicate factors released by SHEDs to manipulate the microenvironment. Insert: possibility of positive feedback mechanism by the factors secreted damaged neurons to stimulate microglia proliferation.

As a summary, this study has briefly demonstrated the immuno-modulatory as well as neuro-protective properties, possibly carried out by SHEDs in an *in vitro* setting of PD. This property of SHEDs would be favourable for the application of CRT.

4.5 Conclusions

Within the scope of this study, the following conclusions can be made:

1. The changes in the levels of inflammatory markers indicated that the inflammatory process triggered by treatment using MPTP was comparable to that of LPS.
2. SHEDs at pre-selected phase were shown to possibly regulate the activities and might have indirectly rescued the neurons from further damage and perhaps modulate the expression of inflammatory markers ($p<0.05$).

CHAPTER 5: EFFECTS OF DENTAL PULP STEM CELLS IN MPTP INDUCED DAMAGES IN PARKINSON DISEASE MICE MODEL

5.1 Introduction

Geriatric population remains as a risk group in acquiring diseases primarily due to multifaceted problems or conditions such as degradation of immune system, lower regenerative capacity, hormonal imbalance, and genetic mutations. These conditions collectively impact their quality of life in a drastic manner. Amongst all medical conditions, degenerative conditions/disorders related especially to neurological such as PD and Alzheimer's disease present as the top causal factors in determining their life quality especially to those above the age of 60 years old (Berendzen, Durieux, & Dillin, 2016). In PD which is characterized by the progressive death of dopaminergic (DA-ergic) neurons in the substantia nigra (SN) that project to the striatum, it has been reported that the onset rate of developing PD is 1% in individuals above 60 years old and the rate increases to 3% when their age extend beyond 75 years old as compared to younger individuals (Pagano et al., 2016). Studies have also indicated the interplay of other possible contributors such as genetic changes as well as environmental to be co-influencer in development of PD. Therefore this multifactorial condition presents as a complex threat which calls for a viable solution (Michel et al., 2016).

Currently for geriatric population, PD symptoms are being managed with either surgical intervention (deep brain stimulation) or medications (L-DOPA or dopamine D2 receptor agonists) and despite these methods displayed positive outcome in controlling the symptoms, their effects were rather short-lived thus requiring higher dosage of same treatment to mimic similar recovery latency (Piccoli et al., 2015; Verstraeten et al., 2015). Lately, cell replacement therapy (CRT) has been regarded as the next possible solution to such medical conditions. Although transplantation with fetal mesencephalic tissue has been proposed for decades, the inconsistencies of their outcome have created alarming

thoughts apart from considering the ethical hurdles (Rumpel et al., 2015). Transplantation via SC therapy on the other hand has been widely accepted due to their highly proliferative and excellent multi-potentiality capacity. These are ideal characteristics, which would contribute to improved transplantation consequence. In addition, transplanted SCs are also expected to modulate the inflammatory processes that take place in the targeted area to facilitate regeneration and cell renewal. Though studies have reported the homing capacity of SCs in transplanted area, their secondary effects however are not thoroughly investigated (Lindvall, 2016; Wenker et al., 2016).

Among all available cell sources, one promising candidate for CRT is SHEDs. Apart from carrying aforementioned features, these cells are inherently non-immunogenic as well as non-tumorigenic and therefore can escape from immune surveillance *in vivo* (Mita et al., 2015). Furthermore, numerous studies have invariably depicted the neuro-ectoderm origin of DPSCs and their excellent features in differentiating as well as functioning neurons like DA-ergic like cells (N. Gnanasegaran et al., 2015; Kanafi et al., 2014) . This opens up new promising avenues for the use of SHEDs not only for autologous, but also for allogeneic-cell therapies. Furthermore, delivery via intrathecal has been widely applied especially in cases pertaining to oncology as drug delivery route and similar approach can also be applied with stem cells (Routray, Ravi, & Mishra, 2015). This less invasive technique not only offers direct/immediate entry to the central nervous system, but it also avoids contact with blood brain barrier that would possibly trigger adverse immune response. Otherwise, delivery via intracerebral which is considered to be risky and invasive might be inappropriate to perform especially on geriatric population. Additionally, studies have also reported the applicability of this method as delivery route of SCs and thus could serve as a suitable delivery route for treating PD (B. K. Chen et al., 2015; Nizzardo et al., 2016).

As such, this study was initiated to investigate the therapeutic efficacy of SHEDs in 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced old aged mice. From this study, it is expected to obtain some understanding regarding the neuro-restoration and neuro-immuno-modulation executed by SHEDs in such harsh microenvironment. These information would be particularly useful while designing optimal SHEDs-based CRT modules. Specifically, the objectives are:

1. To establish intrathecal as an alternative route for SHED administration.
2. To evaluate the efficacy of SHEDs in controlling MPTP-induced PD symptoms upon transplantation via intrathecal.

5.2 Materials and Methods

5.2.1 Pulp collection, isolation and cultivation of cells

The pulp collection, isolation and cultivation of SHEDs were carried out as described in sub section 3.2 1 and cells at SC3 with high G0/G1 phase were acquired as described in 3.2.4. All subsequent analyses were performed in cGMP certified facility with clean environment conforming to guidelines by Pharmaceutical Inspection Convention and Pharmaceutical Inspection Co-operation Scheme (PIC/S).

5.2.2 Animal grouping and acclimatization

The ethical approval to conduct this study was provided by the Institutional Animal Care and Use Committee (IACUC) of University of Malaya (PR/01/04/2015/NG (R)). A total of 55 old Swiss albino mice (male; age: 18 months) were purchased from Synertec Enterprise (Kuala Lumpur, Malaysia) and acclimatized for 14 days under controlled environment and were fed with food pellets and water was supplied on *ad libitum* basis. All the animals were kept in animal quarters that have automatic temperature (22 ± 1°C) and light (12 h light/dark cycle) as well as humidity ranging from 65-70%. After acclimatization, they were randomized into groups as follows: (i) G1: untreated group,

untreated; G2: MPTP-induced and treated with saline; and (iii) G3: MPTP-induced and treated with SHEDs via intrathecal. Mice were injected with MPTP at a total dosage of 80mg/kg via i.p at four time intervals by 2 hour each following to the methods described previously (Zhou et al., 2015). Administration of SHEDs via intrathecal was performed as described earlier (Chen, Park, Xie, & Ji, 2015). Briefly, a spinal cord puncture was made with a 30-gauge needle between L5 and L6 levels to deliver either PBS (10 μL) or cells (1×10^6 cells in 10 μL PBS) to the cerebrospinal fluid (CSF). Prior to injection, SHEDs were tagged with PKH26 (a fluorescent dye that stably incorporates into the lipid regions of the cell membrane; .Sigma-Aldrich) as described previously (Lee et al., 2015), washed 3 times with PBS, centrifuged for 5 minutes at 1,000 g, and then re-suspended in PBS. Administration of cyclosporine A (CyA; Wako Pure Chemicals; Osaka, Japan) via subcutaneous was carried out at a dose of 10 mg/kg/day. Treatment with CyA began 2 days before transplantation and continued throughout the experimental period for the first 2 weeks. At subsequent weeks, CyA was administered every other day until end of experimental procedure.

5.2.3 Assessment on homing capabilities of SHEDs in brain

Mice were euthanized with overdose of ketamine/xylazine and perfused with chilled PBS-heparin, and their corresponding CSF was collected from the cisterna magna as previously described (DeMattos et al., 2002; Yamada et al., 2011). The brain sections were collected for hematoxylin and eosin (H&E) staining as well as for immunostaining and fixed overnight in 4% paraformaldehyde at 4°C and then transferred to 30% sucrose. Next, the brain samples were fixed in 10% formalin and further processed for paraffin embedment as FFPE samples prior to sectioning using microtome (Leica) at 4μm thickness on poly-L-lysine slides. Processed slides were then stained using H&E stain and visualized under bright field microscope (Olympus IX63, Japan). Similarly, another set of slides were prepared and were counter-stained with 4',6-diamidino-2-phenylindole

(DAPI) and were visualized under fluorescent microscope (Olympus BX63, Japan) for detection of tagged SHEDs.

5.2.4 Detection of pro-inflammatory factors via multiplex ELISA

The collected CSF was then evaluated for the presence for inflammatory cytokines as described previously (Rosser et al., 2014). In brief, samples and standards (50 μL) were loaded onto the Q-Plex™ plate and allowed to incubate for two hours. Subsequently, the sandwich immunoassay complex that forms was incubated with biotin-streptavidin/Horse Radish Peroxidase reagents for an additional two hours. Antigen standard curves were performed in duplicate diluting the antigen standard 1:3 for 11 points with a single negative point. Samples were diluted at ratios of 1:2 (sample to buffer) (50%), 1 to 20 (5%) and 1 to 200 (0.5%). Each dilution was loaded into three wells and measured in triplicate, a total of 9 wells per sample. Standard curves were constructed using Q-View Software™, which allows for the selection of multiple non-linear and linear equations to fit the standard curve.

5.2.5 Behavioural assessments on MPTP-induced PD models

Behaviour assessments such as 'Challenging beam traversal test' (Khairnar et al., 2015), 'Spontaneous Activity in the Cylinder Procedure' (Maurice et al., 2015), 'Adhesive Removal test' (Quittet et al., 2015) , 'Buried Pellet Test' (Fleming, Ekhator, & Ghisays, 2013), and 'Block Test' (Zhang, Xiao, & Le, 2015) were performed as thoroughly described in previous studies to evaluate their sensorimotor as well as olfactory performances upon introduction of MPTP and treatment with SHEDs. Behaviour assessments were performed every fortnight and their corresponding progress were recorded and analysed.

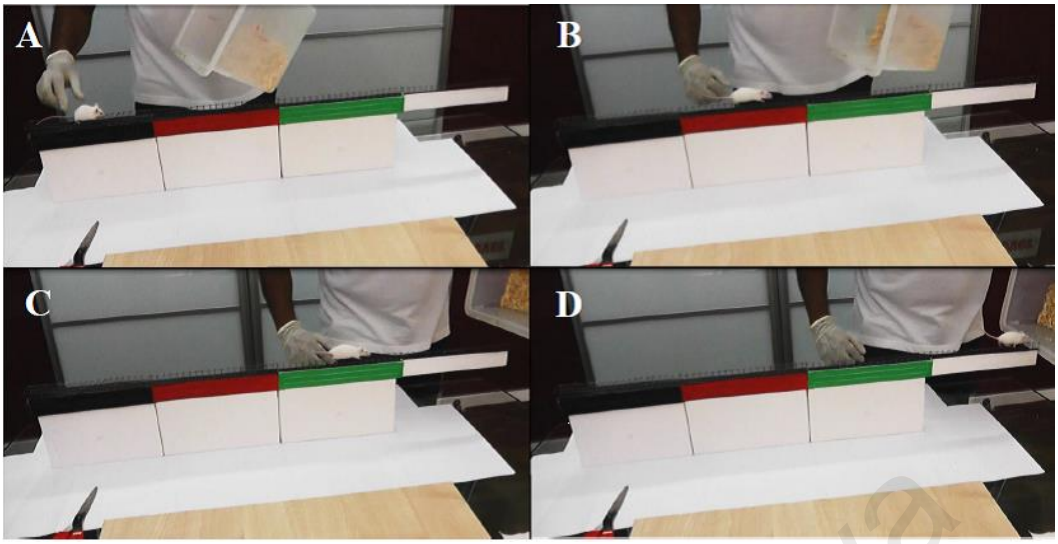

Figure 5-1: Challenging beam traversal test. Motor performance was measured with a novel beam test adapted from traditional beam-walking tests. The beam was constructed and consisted of four sections (25 cm each, 1 m total length), each section having a different width. The beam started at a width of 3.5 cm and gradually narrowed to 0.5 cm increments. Underhanging ledges (1 cm width) were placed 1.0 cm below the upper surface of the beam. Animals were trained to traverse the length of the beam starting at the widest section (A) and ending at the narrowest (D), most difficult section. The narrow end of the beam led directly into the animal's home cage.

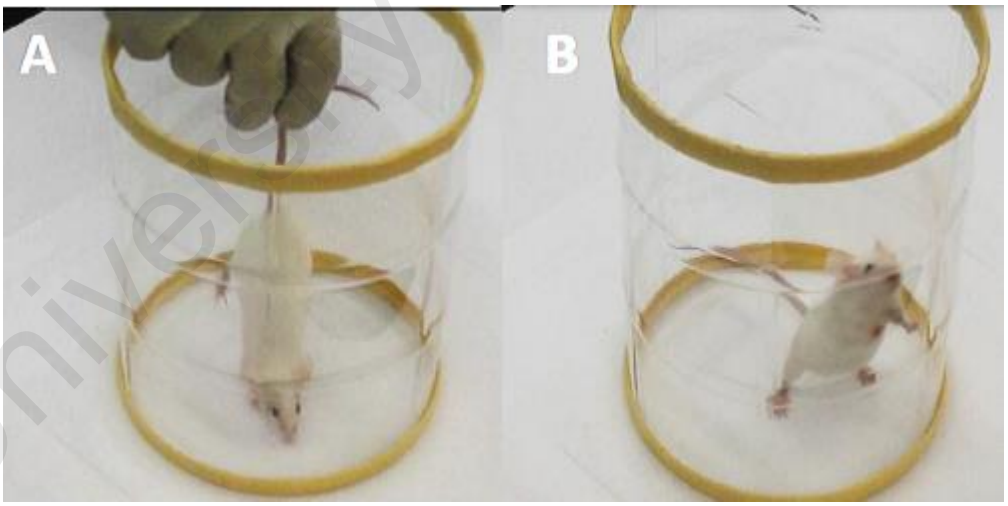

Figure 5-2: Spontaneous Activity in the Cylinder Procedure. Spontaneous movement was measured by placing animals (A) in a small transparent cylinder (height, 15.5 cm; diameter, 12.7 cm). Spontaneous activity was videotaped for 3 minutes. In this experiment, the cylinder was placed and the number of rears (B); forelimb and hind limb steps were measured.

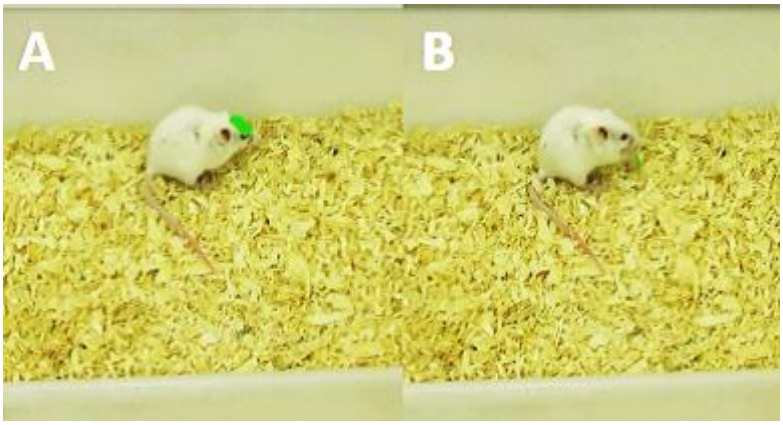

Figure 5-3: Adhesive Removal test. An adhesive stimuli was placed on the snout of the mouse (A), and the time to make contact and remove the stimuli (B) was recorded.

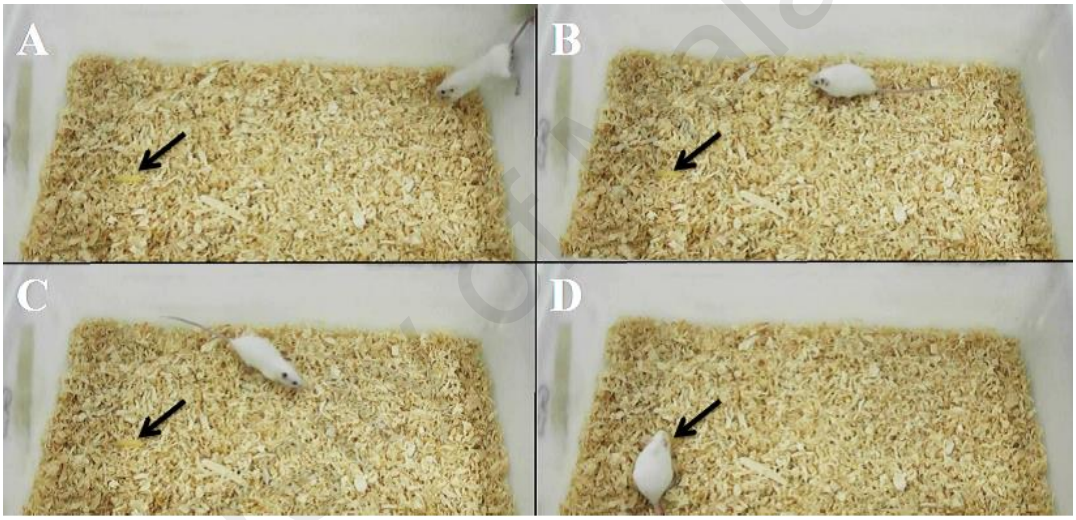

Figure 5-4: Buried Pellet Test. The buried pellet test measures how quickly an overnight-fasted animal can find a small piece of familiar palatable food (marked by black arrow) that is hidden underneath a layer of bedding. The assumption is that food-restricted mice which fail to use odor cues to locate the food within a 5 minute period are likely to have deficits in olfactory abilities. The majority of mice with normal olfaction can find the hidden food piece within a few minutes (A - D).

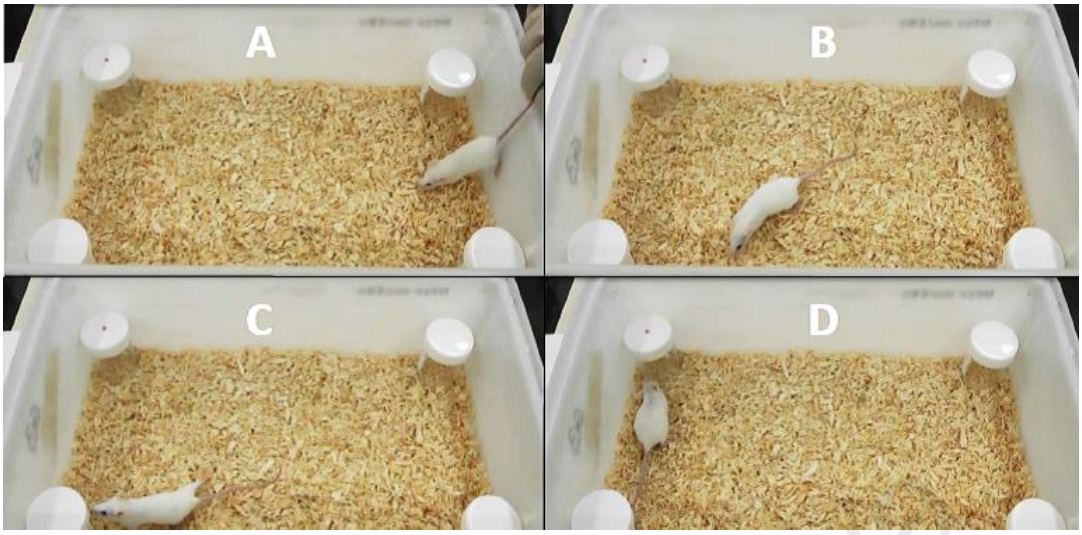

Figure 5-5: Block Test. The block test was conducted by first arranging four plastic containers along the edges of the test cage. The container with a red marking on the top was obtained from another cage. The amount of time taken to identify this foreign container (marked with a red dot) was videotaped using a stopwatch.

5.2.6 Immunohistochemistry analysis on brain sections

Briefly, mouse brains were fixed via intracardiac perfusion with 4% paraformaldehyde in phosphate-buffered saline (PBS) and post-fixation in the same buffer. Brain samples were then embedded in paraffin, and sections were processed for immunohistochemistry staining with antibody against human specific tyrosine hydroxylase (TH; 1:1000), human specific DA transporter (DAT; 1:500) and human specific DA decarboxylase (AADC; 1:1000, all from Merck Millipore, USA). Slides were then scanned at ×20 using a Leica SCN400 slide scanner (Leica Microsystems Inc., Buffalo Grove, IL) and the images were digitalized with Pannoramic Viewer software (3D Histech, v 1.17).

5.2.7 Statistical analysis

Results were presented as comparison of mean ± standard deviation (SD) from five independent samples (n=5). The data were analysed using SPSS, version 19.0 (SPSS Inc, Chicago, IL, USA). Statistical test using 2-way ANOVA were carried out and a p-value of < 0.05 was considered to be significant.

5.3 Results

5.3.1 Significant restoration of DA-ergic markers were observed with administration of SHEDs

Administration of MPTP in old-aged mice severely decreased the brain regions associated with DA-ergic neurons especially in striatum and SN (Fig. 5.6). In TH for instance, their depletion was noted as high as 80% as compared to Sham group (Fig. 5.6Ai-ii; Fig. 5.6Bi). With the administration of SHEDs, progressive developments were noticed and by 12^{th} week post-transplantation, about 60% increment with regards to TH immunostaining was discovered. Similar recovery capacity was also noted for DA Transporter and DA Decarboxylase. These altogether presented the ability of SHEDs to efficiently provide recovery effect on the damaged neurons in old mice upon exposure with neurotoxic MPTP.

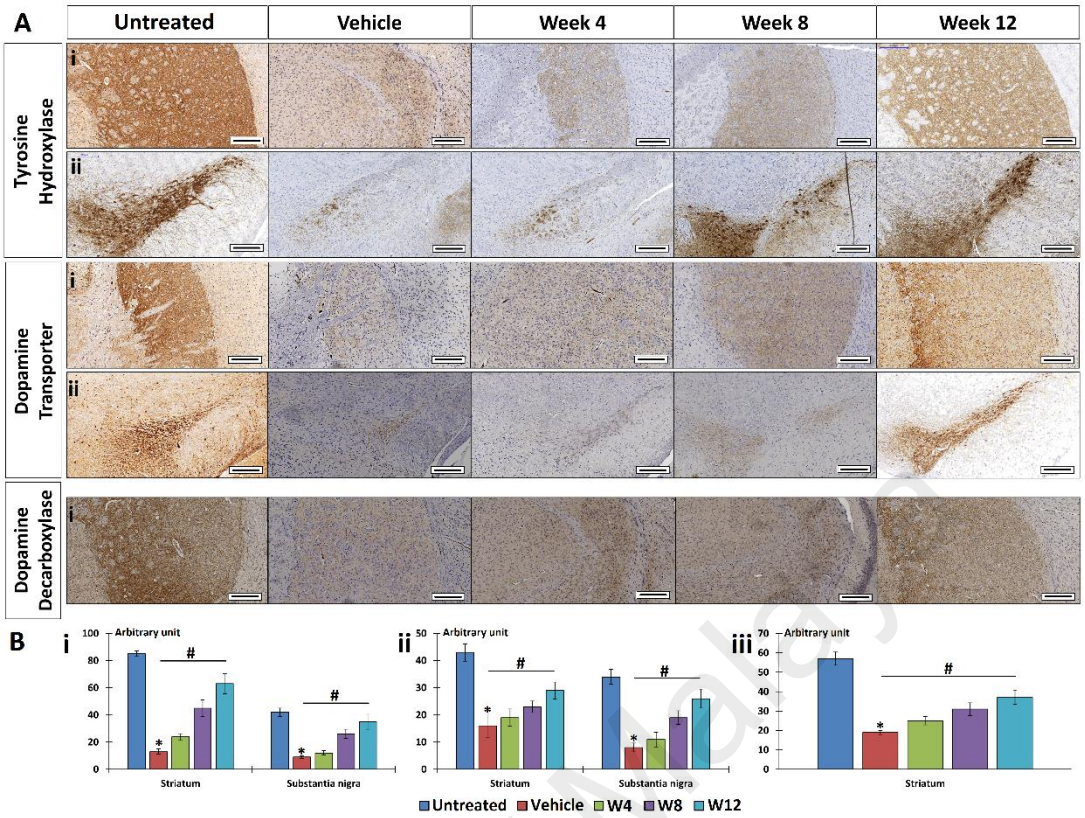

Figure 5-6: Restoration of DA-ergic neurons in striatum and SN and their coverage percentage upon transplantation. (A) Immunohistochemistry revealing presence of TH, DAT, and AADC at striatum and SN (i and ii, respectively) upon intrathecal transplantation of SHEDs for 12 weeks. (B) Percentage depicting coverage of TH, DAT, and AADC (i, ii and iii, respectively) upon intrathecal transplantation of SHEDs on aforementioned locations. # indicate $p<0.05$ between Vehicle group and the 12 weeks SHED-transplanted group.

5.3.2 SHEDs augmented MPTP-induced damages in brain regions and modulates expression of inflammatory related factors

Further analysis was also performed whereby brain sections of three most commonly investigated brain regions namely cortex, thalamus and midbrain were assessed for any improvements due to SHEDs administration. As noted previously, MPTP had decreased the neuronal population, which was exhibited via lack of nucleus-stained areas in all three regions (Fig. 5.7). With the introduction of SHEDs for 12 weeks, a decent recovery with distinct neuronal population was observed. In addition, the transplanted cells were also shown to have regulated the levels of inflammatory mediators in the CSF (Fig. 5.8). It

was distinctly observed that pro-inflammatory factors such as IL1α, IL1β, IL6, IL8, and TNFα were significantly attenuated with the presence of SHEDs ($p<0.05$). Complementarily, the expression levels of anti-inflammatory factors such as IL2, IL4, and TNFβ were also noted to be augmented distinctly when SHEDs were transplanted and retained for a period of 12 weeks ($p<0.05$; Fig. 4B). This phenomena had re-called the secondary effects that SCs transplantation were thought to have as thoroughly investigated in Chapter 4.

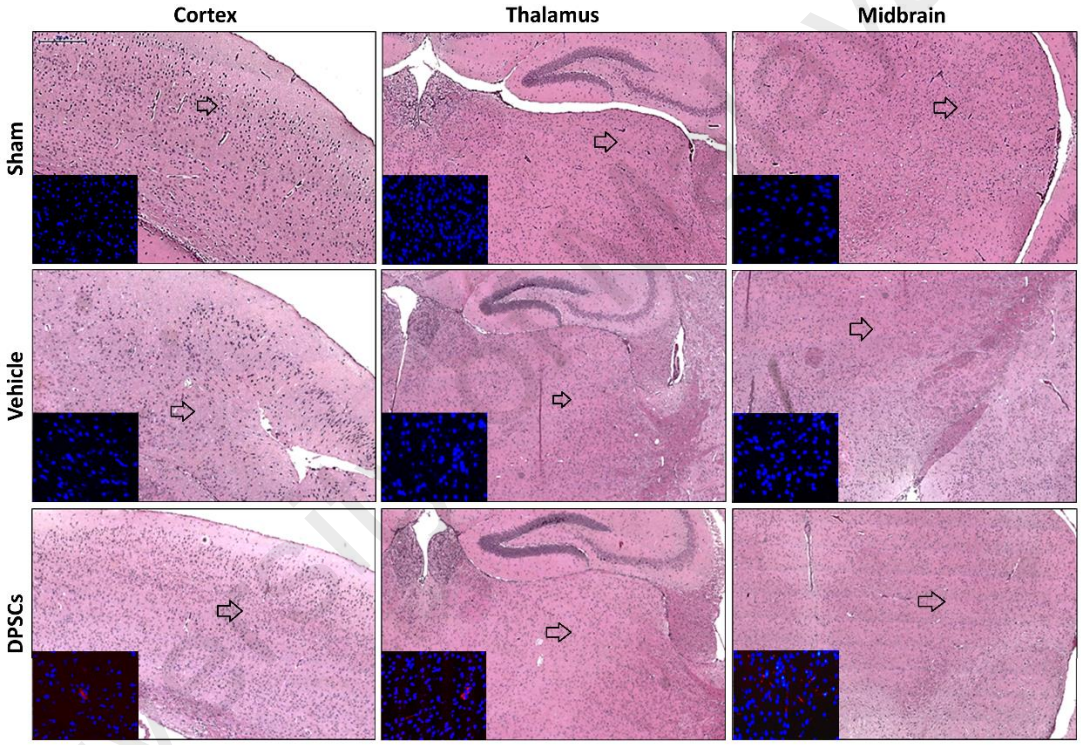

Figure 5-7: Recovery of main brain structures with the administration of SHEDs for 12 weeks. H&E stain revealed distortion of brain structures, demonstrated by lack of nucleus as indicated by arrow due to toxic effect of MPTP, followed by their recovery with transplantation of SHEDs. Insert diagrams depicted the homing capacity of PKH26-tagged SHEDs at respective brain locations.

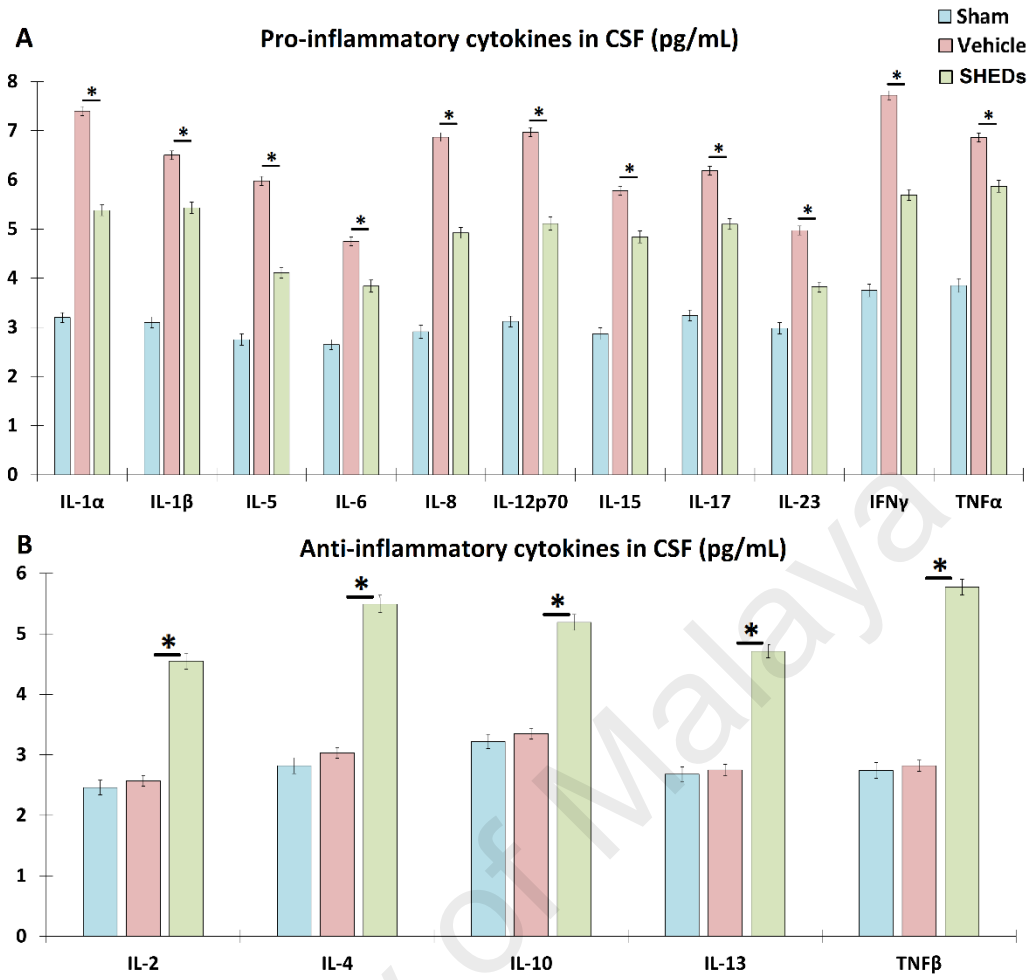

Figure 5-8: Expression of inflammatory mediators in their CSF. (A) Levels of pro-inflammatory mediators in SHEDs-transplanted groups displayed significance as compared to Sham and Vehicle groups. * indicate $p<0.05$ between SHEDs-transplanted group and Vehicle group. (B) Levels of anti-inflammatory mediators in SHEDs-transplanted groups displayed significance as compared to Sham and Vehicle groups. * indicate $p<0.05$ between SHEDs-transplanted group and Vehicle group.

5.3.3 Transplantation of SHEDs ameliorates recovery of MPTP-related behavioural deficits

Behavioural analysis was performed to investigate the therapeutic improvement conferred by SHEDs from the perspective of sensorimotor and olfactory related activities. In 'Challenging beam traversal test', it was observed that SHEDs have significantly improved their performance by reducing the number of errors made while crossing the beam. With similar effect, the time taken to cross the beam also presented distinct improvement ($p<0.05$; Fig. 5.9A). Again the administration of SHEDs have shown to

improve the performance of old mice in 'Spontaneous Activity in the Cylinder Procedure' and 'Adhesive removal test'. As previously observed, SHEDs administration have demonstrated significant improvement in frequency of standing with hind limbs whereas the time taken to remove adhesive also presented similar pattern ($p<0.05$; Fig. 5.9B).

Likewise in olfactory related challenges, transplantation of SHEDs have demonstrated distinct improvement in time taken to identify buried pellet as well as to identify new, foreign scent which were placed in their cage. Corresponding to their sensorimotor profile, their olfactory behaviour have revealed significant improvement as early as Week 8 ($p<0.05$; Fig. 5.9C-F). Regardless of whichever arrangement of blocks (three variations in total) were tested on them, their ability to identify new scent which were lost during MPTP administration, have revealed significant improvement and were able to recognise at a shorter period of time. In addition, the integration of transplanted SHEDs was investigated via antibody staining specific to TH (Fig. 5.9G-H). Although TH was present in both Sham and Vehicle group, their presence was distinct to each other whereby a reduction by 60 % was observed in Vehicle group. However with the administration of SHEDs, double staining of PKH26 and TH gave rise to yellowish population indicating their SHEDs origin. This further strengthens the notion that SHEDs could indeed be potentially suitable candidate for CRT especially in regards to PD. Representative videos depicting the behavioural assessments are appended in form of compact disc in Appendix C.

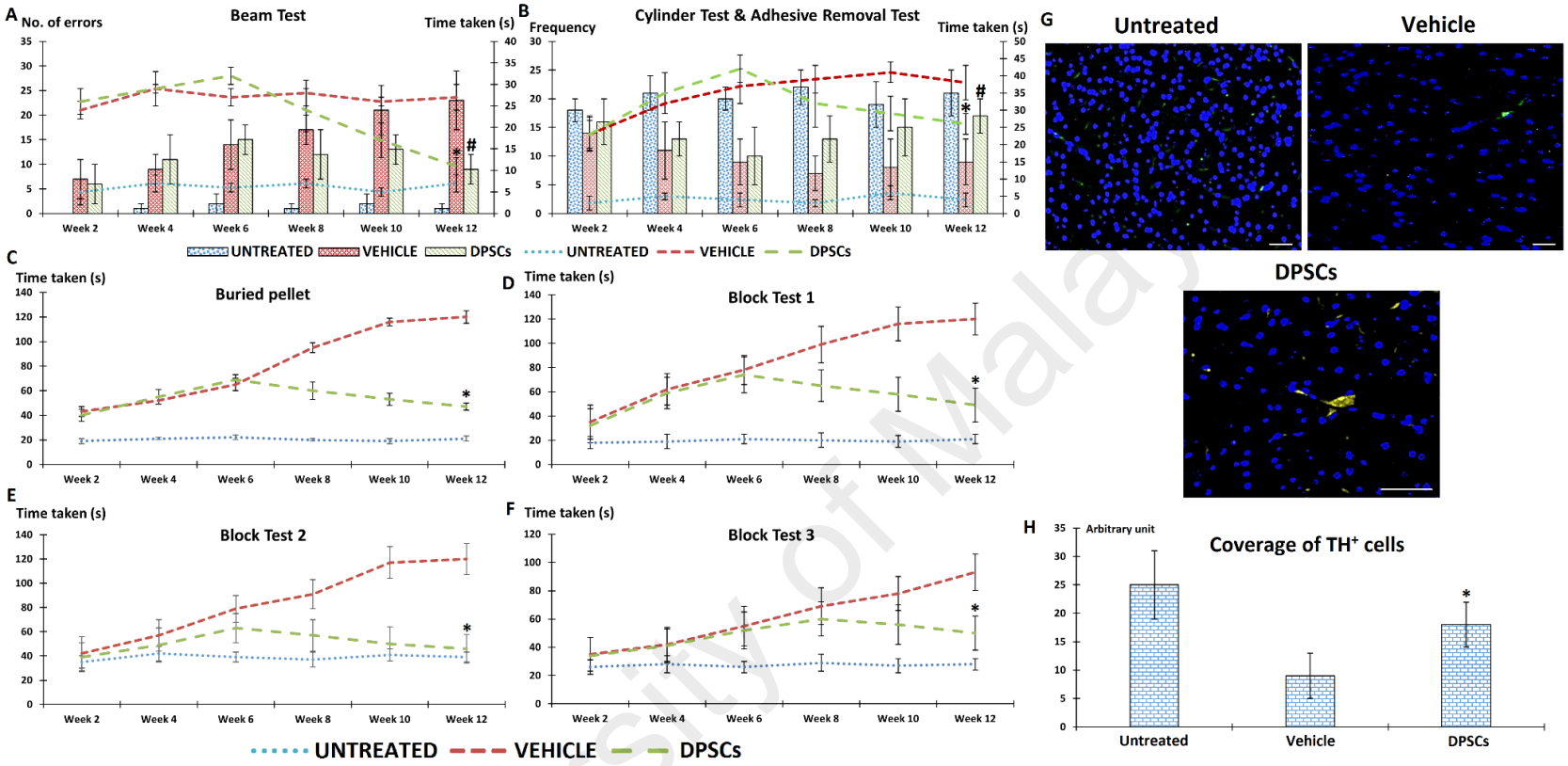

Figure 5-9: Behavioural improvements upon transplantation of SHEDs. (A-B): Sensorimotor behaviour improvements with the transplantation of SHEDs as early as Week 8. * indicate $p<0.05$ of time taken against Vehicle group, and # indicate $p< 0.05$ in terms of frequency against Vehicle group. (C-F): Olfactory related behaviour depicted distinct improvement with SHEDs beginning Week 8. * indicate $p<0.05$ of time taken against Vehicle group. (G): Double staining depicting presence of PKH26-tagged SHEDs (yellow) in SN which expresses TH (green). (H): Fluorescent intensity coverage by transplanted SHEDs. * indicate $p<0.05$ against Vehicle group.

5.4 Discussions

In geriatric population, the risk of developing chronic neurodegenerative diseases with distinct neuronal loss such as Alzheimer's, PD, Huntington disease increases with age. These progressive insults would contribute to marked neural system dysfunction associated with absence of neurotransmitters. In PD particularly, loss of DA-ergic neuronal loss corresponds with depletion of DA. As such, CRT for PD must be targeting at replacing DA-ergic neurons or at least to offer neuro-protection to prevent them from succumbing to additional damages (Gonzales et al., 2015). The applicability of MSCs for such therapy has been widely described. Sources such as from bone marrow (Oron & Oron, 2016), adipose tissue (Choi et al., 2015), endometrium tissues (Wolff et al., 2015), and umbilical MSCs (Ko et al., 2015) have revealed marked improvement both in neuro-restoration and behaviour analyses. Until recently, SHEDs has been regarded as the next suitable cell source for neurodegenerative disease owing to their neural crest origin and with less ethical hurdles (Mead et al., 2016). It has also been reported that the mode of action by which SHEDs confer therapeutic benefits may comprise of paracrine-mediated processes which involve a wide array of secreted trophic factors and is increasingly considered as the principal predominant mechanism.

Previous study has reported the application of SHEDs as a treatment modality in PD in which the transplantation of SHEDs promoted the proliferation of native neural cells and resulted in the recruitment of pre-existing $Nestin^+$ neural progenitor cells (NPCs) and β-tubulin-III^+ mature neurons to the site of the graft (Huang et al., 2008). Additionally SHEDs also were shown to protect neurons from damage induced by MPP^+ (Nesti et al., 2011). Moreover, transplantation of SHED into the striatum of Parkinsonian rats were shown to partially improve the apomorphine-evoked rotation of behavioural disorders with 70.67 ± 14.25 TH^+ cells/section (Wang et al., 2010). It should be noted that they have performed transplantation via intracerebral which is very risky to carry out on

human subjects. In this study, although the percentage of TH cells was approximately 60%, but the intrathecal delivery of SCs have highlighted to be a much practical route of transplantation as compared to intracerebral which can be very challenging and risky especially for geriatric population (Nösslinger, 2016).

In addition, the efficacy of SHEDs were distinctly noticed both in the animal as well as in neuronal cell line models as shown in Chapter 4. Another aspect worth discussing is the fact that the transplanted SHEDs were of high G0/G1 phase as elaborated in Chapter 3. The therapeutic benefit seen as early as Week 8 post-transplantation in old age mice models further strengthen the notion that SHEDs with high G0/G1 phases can confer neuro-protection and restore the functionality of brain to approximately 60% as elucidated by TH immunostaining. The possible immuno-modulatory properties as seen in the *in vitro* model in previous chapter seconded the paracrine effects that probably mediated by SHEDs. It has been reported that outcomes of transplantation can be increased with suppression of pro-inflammatory and augmentation of anti-inflammatory markers (Ratanavaraporn, Furuya, & Tabata, 2012). Furthermore it should be noted that the functional recovery by 8 weeks post-transplantation in mice model can be equivalent to several years in human subjects. However with the advent of technology, this requires further investigation and it is hoped that this duration can be brought sooner.

At the moment, one of many limitations faced with the application of SHEDs for regenerative medicine is the small starting material which impedes the acquisition of large quantity of cells for transplantation. These hurdles can probably be rectified with hypoxia culturing (Peng et al., 2015), application of bioreactors (Duan et al., 2014), usage of mitogenic factors such as human platelet lysate (HPL) and basic fibroblast growth factors (bFGF) (Vasanthan et al., 2014) or combination of scaffolds (Woloszyk et al., 2014), and perhaps by having proper storage/maintenance system adhering to cGMP

(current Good Manufacture Practices) to ensure the delivery of a 'cell drug' that is not only safe but also reproducible as well as efficient (Potdar & Jethmalani, 2015). With the advancement of technology, the culturing can possibly be performed with automated devices, which will reduce batch-to-batch variation when cells are scheduled to undergo up-scaling for clinical applications.

As a summary, this study is the first to have described the appropriateness of utilizing SHEDs as a treatment modality against PD using MPTP-induced mice model via intrathecal route of transplantation. With this information, it is believed that proper CRT strategies can be planned to accommodate the well-being of geriatric population.

5.5 Conclusions

Within the scope of this study, the following conclusions can be drawn:

1. SHEDs of high G0/G1 phase were able to home to affected brain region upon administration via intrathecal.
2. Administration of SHEDs were able to significantly alter the expression of inflammatory cytokines and recover the affected regions in SN and striatum as early as week 8 post-transplantation ($p<0.05$).
3. Transplanted cells were able to significantly restore the behaviour of induced Parkinsonian mice compared to vehicle group ($p<0.05$).

CHAPTER 6: CONCLUSIONS

6.1 Summary of findings

In the present study, the influential role played by the growth phase of SHEDs as a vital parameter that govern the therapeutic level to treat PD was investigated. It was shown that SHEDs cultured at SC3, for 7 days which is characterized by having elevated G0/G1 phases at day 7 was the optimal condition for enhanced differentiation towards DA-ergic like cells (Chapter 3). Similar cell condition was also noted to possibly have some degree of immuno-modulatory behaviours by suppressing pro-inflammatory markers but at the same time augmenting anti-inflammatory mediators that could lead to attenuation of microglia activation in *in vitro* model (Chapter 4). Last but not least, transplantation of SHEDs of selected growth phase in PD mice model have not only demonstrated integration into the brain region but significantly improved their behavioural performances as early as Week 8 post-transplantation (Chapter 5). In a nutshell, SHEDs of high G0/G1 phases could potentially be optimal for CRT application especially for neurodegenerative diseases such as PD.

6.2 Conclusions

Within the limitation and scope of this study, the following conclusions can be drawn:

1. SHEDs of high G0/G1 phases as depicted by cell cycle analysis was seen as the suitable cell condition for CRT application in PD.
2. Apart from differentiating towards DA-ergic-like cells, SHEDs could potentially regulate the inflammatory processes by modulating expression of pro-inflammatory and anti-inflammatory markers.
3. It was also evident that SHEDs were able to integrate with the brain region and subsequently improve the behavioural parameters in induced Parkinsonism mice.

6.3 Clinical significance

In this study, the applicability of intrinsic cell condition by means of cell cycle state has revealed to be a valid parameter which could contribute significantly to efficacy of SCs as a treatment modality for CRT applications. Although studies have shown the relevance of cell intrinsic conditions during embryonic organogenesis, this study has invariably highlighted that this factor indeed plays vital role in CRT as well. However, it should be noted that there are many factors which contribute to successful CRT such as dosage, frequency of transplantation, route of transplantation, time interval between transplantation and so on, but this study just focussed on one of them. Therefore it is highly recommended to incorporate this parameter in CRT to test its efficacy with real patients.

With these findings, dental pulp has been shown to be a potentially good cell source for neurodegenerative diseases. Hence, tooth banking could be of great significance in which SCs from tooth samples can be utilized either for autologous or allogeneic purposes in the future. As such, institutions which provide banking facility can promote aggressively on the possibility of banking deciduous teeth.

6.4 Limitations of current study and future recommendations

The findings of this study have demonstrated that the SHEDs of aforementioned growth phase were able to ameliorate MPTP-induced PD conditions. However, understanding the methods on how to acquire the desired cell conditions (high G0/G1 phases) would be imperative as this can be applied on other cells in order to simulate a similar behaviour. This might include detailed study on the pathway activation and their corresponding genes which facilitated to achieve such conditions. This would then can help us increase the success rate of clinical trials in regards to PD.

In addition, the application of such cell condition other than PD warrants further research. Likewise, the inherent cell conditions as noted in SHEDs may not be the same in cell source apart from SHEDs. As such, a similar study but looking at different aspects of growth phase of different cell lines (Lag, Log, and Stationary) would probably hold the key to enhance differentiation towards specific cell lineage.

Next, other important aspects in CRT such as the dosage, frequency of dosage, time interval between dosages were not investigated in this study. However, within the concepts of CRT, the implications of other relevant factors requires further investigation and perhaps might hold the key to performing successful CRT.

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Zhu, X, Wang, Y, Liu, Y, Huang, GT-J, & Zhang, C. 2014. Immunohistochemical and histochemical analysis of newly formed tissues in root canal space transplanted with dental pulp stem cells plus platelet-rich plasma. *J Endod, 40:*1573-1578.

LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of relevant publications

1. **Gnanasegaran N,** Govindasamy V, Kasim NHA (2016). Differentiation of stem cells derived from carious teeth into dopaminergic-like cells. International Endodontic Journal, 49(10):937-49.

2. **Gnanasegaran, N**., Govindasamy, V., Musa, S., & Kasim, N.H.A. (2016). ReNCell VM conditioned medium enhances the induction of dental pulp stem cells into dopaminergic like cells. Cytotechnology, 68(2), 343-353.

3. **Gnanasegaran, N.,** Govindasamy, V., Kathirvaloo, P., Musa, S., and Abu Kasim, N. H. (2017) Effects of cell cycle phases on the induction of dental pulp stem cells toward dopaminergic-like cells. Journal of Tissue Engineering and Regenerative Medicine, doi: 10.1002/term.2401.

4. **Gnanasegaran, N**., Govindasamy, V., Musa, S., & Kasim, N.H.A. (2016). Neuro-immunomodulatory properties of DPSCs in an *in vitro* model of Parkinson's disease. Journal of Neuroscience Research. (Under Review).

5. **Gnanasegaran, N.,** Govindasamy, V., Simon, C., Gan, Q.F., Vincent-Chong, V.K., Mani, V., Krishnan Selvarajan, K., Subramaniam, V., Musa, S., Kasim, A. and Hayaty, N., 2017. Effect of dental pulp stem cells in MPTP-induced old aged mice model. European Journal of Clinical Investigation, doi: 10.1111/eci.12753.

List of other publications

1. Vasanthan, P., Jayaraman, P., Kunasekaran, W., Lawrence, A., **Gnanasegaran, N**., Govindasamy, V., ... & Kasim, N. H. A. (2016). Generation of functional hepatocyte-like cells from human deciduous periodontal ligament stem cells. The Science of Nature, 103(7-8), 1-12.
2. **Gnanasegaran, N.,** Govindasamy, V., Nathan, P., Musa, S., & Kasim, N. H. A. (2016). Dental Stem Cell Differentiation toward Endodermal Cell Lineages: Approaches to Control Hepatocytes and Beta Cell Transformation. In Dental Stem Cells (pp. 243-268). Springer International Publishing.
3. Jayaraman, P., Govindasamy, V., **Gnanasegaran, N**., Kunasekaran, W., Vasanthan, P., Musa, S., & Kasim, N. H. A. (2016). Expression patterns of immune genes in long-term cultured dental stem cells. Clinical Oral Investigations, 20(1), 109-116.
4. Kasim, A., Hayaty, N., Govindasamy, V., **Gnanasegaran, N**., Musa, S., Pradeep, P. J., ... & Aziz, Z. A. C. A. (2015). Unique molecular signatures influencing the biological function and fate of post-natal stem cells isolated from different sources. Journal of Tissue Engineering and Regenerative Medicine, 9(12), E252-E266.
5. Vasanthan, P., Govindasamy, V., **Gnanasegaran, N**., Kunasekaran, W., Musa, S., Kasim, A., & Hayaty, N. (2015). Differential expression of basal microRNAs' patterns in human dental pulp stem cells. Journal of Cellular and Molecular Medicine, 19(3), 566-580.
6. Vasanthan, P., **Gnanasegaran, N**., Govindasamy, V., Abdullah, A. N., Jayaraman, P., Ronald, V. S., ... & Kasim, N. H. A. (2014). Comparison of fetal bovine serum and human platelet lysate in cultivation and differentiation of dental pulp stem cells into hepatic lineage cells. Biochemical Engineering Journal, 88, 142-153.

7. Osman, A., **Gnanasegaran, N**., Govindasamy, V., Kathivaloo, P., Wen, A. S., Musa, S., & Abu Kasim, N. H. (2014). Basal expression of growth-factor-associated genes in periodontal ligament stem cells reveals multiple distinctive pathways. International Endodontic Journal, 47(7), 639-651.
8. **Gnanasegaran, N**., Govindasamy, V., Musa, S., & Kasim, N. H. (2014). Different isolation methods alter the gene expression profiling of adipose derived stem cells. International Journal of Medical Sciences, 11(4), 391-403.
9. Yazid, F. B., **Gnanasegaran, N**., Kunasekaran, W., Govindasamy, V., & Musa, S. (2014). Comparison of immunodulatory properties of dental pulp stem cells derived from healthy and inflamed teeth. Clinical Oral Investigations, 18(9), 2103-2112.
10. Abdullah, M., Rahman, F. A., **Gnanasegaran, N**., Govindasamy, V., Abu Kasim, N. H., & Musa, S. (2014). Diverse effects of lead nitrate on the proliferation, differentiation, and gene expression of stem cells isolated from a dental origin. The Scientific World Journal, 2014.
11. Ahmad, H., Thambiratnam, K., Zulkifli, A. Z., Lawrence, A., Jasim, A. A., Kunasekaran, W., ... & Kasim, N. H. (2013). Quantification of Mesenchymal Stem Cell Growth Rates through Secretory and Excretory Biomolecules in Conditioned Media via Fresnel Reflection. Sensors, 13(10), 13276-13288.

List of Papers Presented

1. **Gnanasegaran N**, Govindasamy V, Kasim NHA. Evaluation of dental pulp stem cells' growth phases on their differentiation towards dopaminergic-like cells. Stem Cell Singapore Society (SCSS) Symposium 2016, Matrix building, Biopolis, Singapore.
2. **Gnanasegaran N**, Govindasamy V, Kasim NHA. Neuro-protective capacity of dental pulp stem cells in an *in vitro* model of Parkinson's disease. 16th International Union of Biochemistry and Molecular Biology (IUBMB), Vancouver, British Columbia, Canada.
3. **Gnanasegaran N**, Govindasamy V, Kasim NHA. Differential expression patterns of genes upon *ex vivo* cultivation of dental pulp stem cells. HOPE Meeting 2016, Tsukuba, Japan.
4. **Gnanasegaran N**, Govindasamy V, Kasim NHA. Temporal expression of cell cycle related genes upon *ex vivo* cultivation of dental pulp stem cells. Stem Cell Singapore Society (SCSS) Symposium 2015, Matrix building, Biopolis, Singapore.
5. **Gnanasegaran N**, Govindasamy V, Kasim NHA Neurogenesis potential of dental pulp stem cells from carious tooth. International Conference of Innovative Dentistry (ICID) 2015, Petaling Jaya, Malaysia.
6. **Gnanasegaran N**, Govindasamy V, Musa S, Kasim NHA. Trans-differentiation capacity of dental pulp stem cells into dopaminergic-like cells at different growth phases. International Postgraduate Research Awards Seminar 2014 (InPRAS), Kuala Lumpur, Malaysia.

List of awards and recognitions

1. Won Best Oral Presentation for Colgate Award 2017 in 16th International Association of Dental Research-MalSec, Kuala Lumpur, Malaysia.
2. Awarded with Travel Fellowship to participate in Stem Cell Society Singapore (SCSS) 2016 Symposium from 7-8th November 2016.
3. Awarded with Postgraduate Travel Award to participate in 14th Meeting of the Asian-Pacific Society for Neurochemistry (APSN), Kuala Lumpur, Malaysia from 27th-30th August 2016.
4. Selected to represent Malaysia and participate in Young Scientist Program as well as 16th IUBMB Conference in Vancouver, Canada from 14-21st July 2016.
5. Selected to represent Malaysia and participate in 8th Hope Meeting in Tsukuba, Japan from 7-11th March 2016.
6. Awarded with Travel Fellowship to participate in Miami Winter Symposium 2016, organized by International Union of Biochemistry and Molecular Biology (IUBMB) from 24-27th January 2016, Miami, Florida, United States of America.
7. Awarded with Travel Fellowship to participate in Stem Cell Society Singapore (SCSS) 2015 Symposium from 17-18th November 2015.
8. Participated and awarded with Consolation Prize in Oral Presentation in International Conference of Innovative Dentistry (ICID) 2015, Petaling Jaya, Selangor.
9. Awarded as a Bright Spark Scholar under High Impact Research Bright Sparks Programme (HIR-BSP), University of Malaya.
10. Selected to be featured in Marquis Who's Who in the World® 2016 (33rd Edition) by Thomson Reuters.
11. Was featured in 'Thaimozhi' newspaper as the 'Successful Person of the Week' on 15th January 2015 edition.

12. Won First prize in International Postgraduate Research Awards Seminar 2014 (InPRAS) under Biotechnology category, Kuala Lumpur, Malaysia.
13. Awarded Best Paper in International Association of Dental Research – South East Asia Association for Dental Education (IADR-SEAADE) 2014 Conference, Kuching, Sarawak.
14. Awarded Best Paper in International Conference on Emerging Trends in Scientific Research 2014, Kuala Lumpur, Malaysia.

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

UNIVERSITI MALAYA

UM.D/PD211/11
11 August 2011

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

Dear Madam,

ETHICS APPROVAL

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

Thank you.

Yours Sincerely,

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

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

NHAK/ish/etik11

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

APPENDIX B : ETHICAL APPROVAL FROM INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE, UNIVERSITY OF MALAYA

UNIVERSITI MALAYA

UM.C/TNC2/625/16/9/1

1 April 2015

Nareshwaran Gnanasegaran
Department of Restorative Dentistry
Faculty of Dentistry
University of Malaya

Mr/Madam,

Title Project: **THE EFFICACY OF DENTAL PULP STEM CELLS (DPSC) TO TREAT PARKINSON'S DISEASE (PD) IN MICE MODEL**

This is to kindly inform you that the Institutional Animal Care and Use Committee, University of Malaya (UM IACUC) has approved your Animal Research Protocol Application (ARPA) for duration of two (2) years effective from **1 April 2015** until **1 April 2017**.

Your Ethics Reference no. : **PR/01/04/2015/NG (R)**

Thank you.

Yours sincerely,

Durriyyah SHA

Assoc. Prof. Dr. Durriyyah Sharifah Hasan Adli
Chairperson
Institutional Animal Care and Use Committee (IACUC)
University of Malaya

ASSOC. PROF. DR. DURRIYYAH SHARIFAH HASAN ADLI
Chairperson
Institutional Animal Care and Use Committee, University of Malaya (UM IACUC)

JAWATANKUASA INSTITUSI PENJAGAAN DAN PENGGUNAAN HAIWAN UNIVERSITI MALAYA (UM IACUC)
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APPENDIX C : REPRESENTATIVE VIDEOS DEPICTING THE BEHAVIOUR ASSESSMENTS CARRIED OUT IN CHAPTER FIVE

APPENDIX D : PUBLISHED JOURNAL ARTICLES PERTAINING TO RESEARCH STUDY

Cytotechnology
DOI 10.1007/s10616-014-9787-z

ORIGINAL RESEARCH

ReNCell VM conditioned medium enhances the induction of dental pulp stem cells into dopaminergic like cells

Nareshwaran Gnanasegaran · Vijayendran Govindasamy · Sabri Musa · Noor Hayaty Abu Kasim

Received: 18 March 2014 / Accepted: 13 September 2014

Abstract Among the debilitating diseases, neurological related diseases are the most challenging ones to be treated using cell replacement therapies. Recently, dental pulp stem cells (SHED) were found to be most suitable cell choice for neurological related diseases as evidenced with many preclinical studies. To enhance the neurological potential of SHED, we recapitulated one of the pharmacological therapeutic tools in cell replacement treatment, we pre-conditioned dental pulp stem cells (SHED) with culture medium of ReNCell VM, an immortalized neuron progenitor cell, prior to neurogenesis induction and investigated whether this practice enhances their neurogenesis potential especially towards dopaminergic neurons. We hypothesed that the integration of pharmacological practices such as co-administration of various drugs, a wide range of doses and duration as well as pre-conditioning into cell replacement may enhance the efficacy of stem cell therapy. In particular, pre-conditioning is shown to be involved in the protective effect from some membrano-tropic drugs, thereby improving the resistance of cell structures and homing capabilities. We found that cells pre-treated with ReNCell VM conditioned medium displayed bipolar structures with extensive branches resembling putative dopaminergic neurons as compared to non-treated cells. Furthermore, many neuronal related markers such as NES, NR4A2, MSI1, and TH were highly expressed (fold changes >2; $p < 0.05$) in pre-treated cells. Similar observations were detected at the protein level. The results demonstrate for the first time that SHED pre-conditioning enhances neurological potential and we suggest that cells should be primed to their respective environment prior to transplantation.

Keywords Neural progenitor cells · Mesenchymal stem cells · Neurogenesis · Tyrosine hydroxylase · Dopamine acetyl-transferase

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Published online: 17 October 2014

Introduction

Stem cells (SCs) refer to a group of cells which are capable of self-renewal and able to differentiate into many different types of tissue. Traditionally, these cells are found in bone marrow-mesenchymal stem

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Differentiation of stem cells derived from carious teeth into dopaminergic-like cells

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Abstract

Gnanasegaran N, Govindasamy V, Abu Kasim NH. Differentiation of stem cells derived from carious teeth into dopaminergic-like cells. *International Endodontic Journal*.

Aim To investigate whether dental pulp stem cells from carious teeth (DPSCs-CT) can differentiate into functional dopaminergic-like (DAergic) cells and provide an alternative cell source in regenerative medicine.

Methodology Dental pulp stem cells from healthy (DPSCs) and carious teeth (DPSCs-CT) were isolated from young donors. Both cell lines were expanded in identical culture conditions and subsequently differentiated towards DAergic-like cells using pre-defined dopaminergic cocktails. The dopaminergic efficiencies were evaluated both at gene and protein as well as at secretome levels.

Results The efficiency of DPSCs-CT to differentiate into DAergic-like cells was not equivalent to that of DPSCs. This was further reflected in both gene and protein generation whereby key neuronal markers such as nestin, NURR1 and beta-III-tubulin were expressed significantly lower as compared to differentiated DPSCs ($P < 0.05$). In addition, expressions of transcriptomes related to neurogenesis revealed down-regulation of more than 50% of the genes as compared to differentiated DPSC ($P < 0.05$). Amongst the notable genes were those from the transcription factors family (FLNA, MEF2C, NEUROG2), signalling pathway family (DLL1, Notch1, TGF-β1), neuro-inducer family (BDNF) and cell communication family (APBB1).

Conclusions DPSCs-CT were able to differentiate into DAergic-like cells but not as efficiently as DPSCs. As such, prior to use in regenerative medicine, stem cells from any source should be thoroughly investigated beyond conventional benchmarks such as that proposed by the International Society for Cellular Therapy (ISCT).

Keywords: gene expression, mesenchymal stem cells, neuro-ectoderm, neuronal lineage cells.

Received 13 November 2014; accepted 5 September 2015

Introduction

Dental pulp stem cells (DPSCs) are amongst the cell sources that have emerged for use in regenerative medicine due to their high proliferation rate and multilineage differentiation as well as the reduced number of ethical issues involved in the tissue procurement process (Govindasamy *et al.* 2010, Lee *et al.* 2014a,b, Mayo *et al.* 2014).

Apart from healthy pulp tissue, dental pulp stem cells from carious teeth (DPSCs-CT) have also been identified and actively explored as a potential source for regenerative medicine (Alongi *et al.* 2010, Lee *et al.* 2014a,b). The isolation of these cells is mainly based on the severity of inflammation namely in regard to reversible and irreversible pulpitis. Viable cells could still be harvested under the former condition, whereas cells obtained from the latter are often contaminated (Ueda *et al.* 2014). Despite this limitation, such cells still offer huge prospects as potential candidates in regenerative medicine. Current studies

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Effects of cell cycle phases on the induction of dental pulp stem cells toward dopaminergic- like cells

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Article type : Original Paper

Effect of dental pulp stem cells in MPTP-induced old aged mice model

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