UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF CRE/LOXP INDUCIBLE TRANSGENIC WNT5A MOUSE EMBRYONIC STEM CELL LINE

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ABSTRACT

Characterization of Cre/*loxP* Inducible Transgenic Wnt5a Mouse Embryonic Stem Cell Line

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Introduction: Increasing studies have suggested the role of Wnt signalling family members in various aspects of neural development and function. One potential member is Wnt5a, which has been shown to promote neural differentiation of embryonic stem cells (ESCs), including into dopaminergic (DA) neurons. Our group has generated an inducible transgenic ESC line that carries a regulatable Wnt5a construct based on Cre/*loxP* binary system. The transgenic line allows us to study the effect of Wnt5a overexpression at specific time points during the neural differentiation process. However, the stability of the line before and after the induction of the Wnt5a transgene has not been properly evaluated. **Objective:** Our study aims to assess the stability of the Cre/*loxP* inducible transgenic Wnt5a ESC clone at the cellular and molecular levels. Methodology: A Wnt5a transgenic ESC line (NI-L7 line) was revived and propagated in a culture containing GMEM supplemented with 10% foetal bovine serum (FBS), other essential supplements and 20 ng/ml leukaemia inhibitory factor (LIF). The stability of the line was assessed for its pluripotency status and the inducibility of the Wnt5a transgene. The pluripotency status of the NI-L7 line was validated via RT-PCR for the expression of pluripotency-associated markers (*Oct 4, Sox2 and Nanog*) and the ability of the cells to form EBs using the cell suspension method. The cellular morphological assessment was carried out for the non- and tamoxifen-induced transgenic Wnt5a ESC clone (CE-L7). The Wnt5a expression of NI-L7 and CE-L7 lines were semi-quantified using ImageJ, an image analysis program upon RT-PCR. **Results and Discussion:** NI-L7 line successfully expressed all the pluripotency-associated markers and is capable of producing good qualities of EBs indicating that the Cre/*loxP*-Wnt5a construct does not compromise the pluripotency status of the transgenic Wnt5a ESC clone. Cellular morphology was indistinguishable between NI-L7 and CE-L7 lines revealing that the transgenic cells retained the stem cell properties even after tamoxifen induction. CE-L7 line showed higher Wnt5a transgene expression levels compared to NI-L7 line suggesting the functionality of the construct in overexpressing Wnt5a upon induction with tamoxifen. **Conclusion:** Findings of this study signify that genetic manipulation has not compromised the pluripotency status of ESCs and confirmed the functionality of the Cre/*loxP*-Wnt5a construct. Hence, the cells could be fully utilised to unravel the role of Wnt5a during the differentiation process of ESCs into the neural lineage including producing quality DA neurons.

Keywords: stem cell, inducible transgenic embryonic stem cell, embryoid bodies, Wnt5a

ABSTRAK

Pengklasifikasian Cre/*loxP* Induksi Transgenik Wnt5a Sel Stem Embrionik Tetikus

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Pengenalan: Kajian yang semakin meningkat telah mencadangkan peranan ahli keluarga isyarat Wnt dalam pelbagai aspek perkembangan dan fungsi saraf. Satu ahli berpotensi ialah Wnt5a, yang telah ditunjukkan mampu menggalakkan proses pembezaan saraf sel stem embrionik (ESC), termasuk ke sel saraf dopaminergik (DA). Kumpulan kami telah menghasilkan titisan ESC transgenik yang boleh diinduksi untuk mengekspres Wnt5a transgen yang boleh dikawal berdasarkan sistem binari Cre/*loxP*. Titisan sel transgenik Wnt5a membolehkan kajian terhadap kesan mengekspres Wnt5a pada masa spesifik semasa proses pembezaan saraf ESC dapat dijalankan. Walau bagaimanapun, kestabilan titisan sel ini sebelum dan selepas induksi transgen Wnt5a belum dinilai sepenuhnya. **Objektif:** Kajian ini bertujuan untuk menilai kestabilan klon Wnt5a ESC transgenik Cre/*loxP* yang boleh diinduksi pada tahap selular dan molekular. **Metodologi:** Titisan ESC transgenik Wnt5a (NI-L7) telah dihidupkan semula dan dibiakan dalam kultur media yang mengandungi GMEM yang ditambah dengan 10% serum lembu janin (FBS), suplemen penting lain dan 20 ng/ml faktor perencatan leukemia (LIF). Kestabilan titisan sel trangenik ini dinilai untuk kestabilan pluripotensinya dan kebolehdorongan transgen Wnt5a. Status pluripotensi titisan NI-L7 telah disahkan melalui RT-PCR untuk ekspresi penanda berkaitan pluripotensi (*Oct4, Sox2 dan Nanog*) dan keupayaan sel untuk membentuk EB menggunakan kaedah penggantungan sel. Penilaian morfologi selular telah dijalankan untuk klon Wnt5a ESC transgenik yang tidak diinduksi (NI-L7) dan yang diinduksi dengan tamoxifen (CE-L7). Tahap ekspresi transgen Wnt5a dari NI-L7 dan CE-L7 telah dikira secara semi-quantiti berdasarkan analisa imej amplikon RT-PCR menggunakan ImageJ, satu program analisis imej. **Keputusan dan Perbincangan:** Titisan sel NI-L7 berjaya mengekspresi semua penanda yang berkaitan dengan pluripotensi dan mampu menghasilkan kualiti EB yang baik. Ini menunjukkan bahawa konstruk Cre/*loxP*-Wnt5a tidak menjejaskan status pluripotensi klon ESC Wnt5a transgenik. Morfologi selular tidak dapat dibezakan antara titisan NI-L7 dan CE-L7 menunjukkan bahawa sel transgenik mengekalkan sifat sel stem walaupun selepas induksi tamoxifen. Titisan CE-L7 menunjukkan tahap ekspresi transgen Wnt5a yang lebih tinggi berbanding garis NI-L7 membuktikan kefungsian konstruk Wnt5a dalam mengekspresikan Wnt5a secara berlebihan semasa induksi dengan tamoxifen. **Kesimpulan**: Penemuan kajian ini menandakan bahawa manipulasi genetik tidak menjejaskan status pluripotensi ESC dan mengesahkan kefungsian konstruk Cre/*loxP*-Wnt5a. Oleh itu, sel-sel boleh digunakan sepenuhnya untuk membongkar peranan

Wnt5a semasa proses pembezaan ESC ke dalam keturunan saraf termasuk menghasilkan saraf DA yang berkualiti.

Kata kunci: sel stem, induksi transgenik sel stem embrionik, badan embrio, Wnt5a

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LIST OF ABBREVIATIONS

4’-OHT	4’-Hydroxytamoxifen
ATRA	All trans retinoic acid
BSA	Bovine serum albumin
CE	Constitutively expressed
cDNA	Complementary DNA
CNS	Central nervous system
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
ER	Estrogen receptor
ESC	Embryonic stem cell
EBS	Embryoid bodies
FBS	Foetal Bovine Serum
ICM	Inner cell mass
iPSC	Induced pluripotent stem cell
LIF	Leukaemia inhibitory factor
mRNA	Messenger RNA
NPC	Neural precursor cells
NSC	Neural stem cells
Pac	Puromycin resistance gene
PCR	Polymerase chain reaction
Neo	Neomycin resistance gene
RNA	Ribonucleic acid

RT-PCR	Reverse transcription polymerase chain reaction
R26	Rosa 26
TAE	Tris Acetate-EDTA
$MgCl_2$	Magnesium chloride
rpm	Revolutions per minute

CHAPTER 1

INTRODUCTION

1.1 Research background

Neurological disorders are common diseases among the ageing population, especially those in developing and developed countries. According to the description of the World Health Organization (WHO), neurological disorders are diseases that affect the central and the peripheral nervous system. Examples of these disorders are Parkinson's disease (PD), Alzheimer's disease (AD) and motor neuron disease (MND). As reported by the Global Burden of Diseases, Injuries and Risk Factors (GBD) Study, the number of deaths from neurological disorders surged by around 36.7% and the number of disability-adjusted life-years by 7.4% between 1990 and 2015. This finding has ranked neurological disorders as the leading source of disability-adjusted life years in 2015 and the second largest cause of global deaths (GBD 2015 Neurological Disorders Collaborator Group, 2017). PD was also reported to be the fastest growing neurological disorder, in terms of prevalence, disability as well as deaths (GBD 2016 Parkinson's Disease Collaborators, 2018). Notably, about 7 to 10 million people worldwide is having this disease (Parkinson's Disease Statistics, n.d.), and in Malaysia, patients with PD are expected to increase five-fold from the current estimated 20,000 to 120,000 by 2040 (Hassandarvish, 2019).

PD is a progressive and complex neurological disorder, characterized by the gradual degradation and irreversible loss of dopaminergic (DA) neurons within a specific site of the brain known as substantia nigra (Kalia & Lang, 2015). The dopamine deficiency will cause dysregulation of body movement and coordination. Cardinal signs of PD include tremor, rigidity, akinesia, postural instability and

shuffling gait (Frank et al., 2006; Jankovic, 2008). This disease may progress until Stage 5, making one impossible to stand on its own and soon becomes bedridden. When all daily activities are impaired, a round-the-clock caregiver is highly needed. Often, these problematic situations cause a huge economic burden to the patients and their families (Yang et al., 2020).

For decades, the therapeutic strategies for PD are designed to treat the disease's symptoms and complications but are inefficient to stop or reverse the ongoing neurodegeneration, which results in no cure for PD (Li, 2012; Klein & Nikkhah, 2014). Though a commonly prescribed drug namely L-Dopa shows efficacy in improving the symptoms of Parkinsonian patients, unfortunately, it is associated with neuropsychiatric side effects (Morizane et al., 2008). One of the attractive solutions to tackle this disease is through regenerative cell replacement therapy with the utilization of stem cell-derived (DA) neurons. Embryonic stem cells (ESCs) may serve as a potential source of stem cells as ESCs acquire two main characteristics, which are self-renewability upon prolonged culture under appropriate conditions and the capability to differentiate into the three primary germ layers-derived cells including DA neurons.

The potential of ESCs as the source of cells for transplantation was confirmed through previous research (Andersson et al., 2013; Steinbeck et al., 2012), where ESC-derived neurons were shown to have successfully survived and established connections with the host cells. But the current results were too preliminary to be applied in clinical practice. Among the major issues of ESC-based therapies is the time-consuming procedure for the generation of DA neurons. Study shows that it takes about 5 weeks to generate DA neurons with the observable expression of a dopaminergic marker, which is tyrosine hydroxylase (Li et al, 2012). The prolonged culture will downgrade the quality of the neurons in terms of their functionality and

integration, and hence utilization of such neurons is not suitable for neuro-transplantation. Therefore, proper understanding of the underlying mechanisms that govern the neural differentiation process is essential in order to generate quality DA neurons in a shorter period. By manipulating the signalling molecule in the neural differentiation would be a great help in solving the problem.

Increasing studies have suggested the role of Wnt signalling members in various aspects of neural development and function. For instance, their regulatory roles range from the initiation of nervous tissue induction through to the synapses formation (Ciani & Salinas, 2005, as cited in Nordin et al, 2008). Among the 19 family members of Wnt, Wnt5a was selected as the potential member as stimulation of Wnt5a was discovered to enhance the differentiation of neural precursor cells (NPC), instead of maintaining the NPC population, suggesting that Wnt5a promotes cells specification of NPC (Nordin et al., 2008; Yu et al., 2006). Additionally, Wnt5a has been demonstrated to enhance in vivo DA neurons produced from stem cells in terms of differentiation and functional integration (Parish et al., 2008). Wnt5a activity has also been shown to be stage-dependent, with the dynamic expression of Wnt5a transcript observed upon subjecting ESC to neural differentiation via 4-/4+ protocol (Nordin et al., 2008). The high Wnt5a expression at the later stage in this study suggests that Wnt5a is needed for stem cells to differentiate into neurons.

The Wnt5a activity is complex and little is known about its expression during the neural differentiation process. It has been reported that the effect of Wnt5a throughout the differentiation process has been observed using purified Wnt5a protein. However, due to the dilution of its concentration by the medium and other external factors, direct protein may make it more difficult to do a thorough quantitative biochemical analysis [review in (Van Amerongen & Nusse 2009)]. In order to assess

the stage-dependency effect of Wnt5a during the process, a system that enables us to strictly regulate its expression in undifferentiated ESCs as well as upon induction in a closed environment, within the cells, is greatly required.

In this study, our group has established inducible transgenic ESC line that carries a regulatable Wnt5a construct based on the Cre/*loxP* binary system. The transgenic line allows us to study the effect of Wnt5a overexpression at specific time points during the neural differentiation process. The key questions of this study are; 1) Does this genetic manipulation affect the stemness of the transgenic ESC line? 2) Is the Cre/*loxP*-*Wnt5a* construct stable and inducible?

1.2 Hypothesis

The genetic manipulation does not compromise the pluripotency status of the transgenic Wnt5a ESC clone. Hence, the stable and inducible Cre/*loxP*-Wnt5a construct will give rise to high Wnt5a transgene expression in the CE-L7 line.

1.3 Objectives

General Objective: To assess the stability of the Cre/*loxP* inducible transgenic Wnt5a ESC clone at the cellular and molecular levels.

Specific Objectives:

1. To examine the effect of the random integration of the Cre/*loxP*-*Wnt5a* construct on the pluripotency status of the clone.
2. To assess the cell morphological differences of non- and tamoxifen-induced transgenic Wnt5a ESC clone.
3. To examine the inducibility of Wnt5a transgene at RNA level.

CHAPTER 2

LITERATURE REVIEW

2.1 Neurodegenerative diseases

Neurodegenerative disease is defined as progressive degeneration and/or loss of nerve cells, particularly neurons in the central nervous system composed of the brain and spinal cord. The functional regression of these nerve cells will subsequently affect every aspect of a person's life, such as memory and cognitive impairments, mobility and imbalance, and speech difficulty. There are a number of neurodegenerative diseases including Alzheimer's disease (AD), Parkinson's disease (PD), Motor neuron disease (MND), Huntington's disease (HD), and Amyotrophic Sclerosis (ALS).

2.1.1 Parkinson's Disease

PD is the main focus of this study and is the second most common neurodegenerative disease after Alzheimer's disease. Meta-analysis of worldwide data conducted by Pringsheim et al. in 2014 has illustrated the effect of ageing on PD, as rising prevalence with age was discovered. This result agrees with a previously reported study which emphasizes the impact that advancing age has on the chances of having this disease (Wood-Kaczmar et al., 2006). Besides, a consistent male-to-female ratio of age-standardised prevalence of PD (1.4 times higher in males) in 2016 and 1990 also suggests PD is more prevalent in men than in women (GBD 2016 Parkinson's Disease Collaborators, 2018). This result also corresponds to other studies with reported ratios of 1.1: 1 to about 3:1 (Van Den Eeden, 2003; Schrag et al., 2000). This might be attributed to estrogen's protective effects on women (Miller & Cronin-Golomb, 2010).

PD was first medically described as a "shaking palsy" by Dr. James Parkinson in 1817. It is caused by the gradual loss of dopaminergic (DA) neurons within a specific area in the brain known as substantia nigra. Dopamine deficiency as a result of neuronal death is thought to be responsible for the classical parkinsonian motor symptoms. The word parkinsonism refers to the motor symptoms of PD, which include resting tremors, bradykinesia, and muscular rigidity with the appearance of postural instability as the disease progresses. Besides that, PD is also linked to a variety of non-motor symptoms such as sleep disturbance, autonomic dysfunction, and cognitive changes, which may precede the motor symptoms by years or even decades (Kalia & Lang, 2015; Dexter & Jenner, 2013; DeMaagd & Philip, 2015).

2.1.2 Treatments available

An individualized therapeutic approach is required for PD treatment due to its broad spectrum of motor and non-motor features. Till today, therapies available for PD are only to treat the symptom but not preventing the underlying neurodegeneration, which means that there is still no cure for PD. Therefore, medical therapy, particularly drugs that increase concentrations of intracerebral dopamine and activate the dopamine receptor remains the mainstay of treatment for PD-related motor symptoms. These drugs are levodopa, anticholinergics, antiglutamatergics, monoamine oxidase inhibitors (MAOIs), catechol-o-methyl transferase inhibitors (COMTIs), and dopamine agonists. Levodopa therapy is often the first-line treatment for early-stage PD patients and is given in combination with carbidopa to prevent peripheral metabolism and lower the risk of nausea significantly. It has been proven that increasing the carbidopa-to-levodopa ratio from the current standard of 1:4 increases on-time without dyskinesia and decreases off time (Trenkwalder et al., 2019).

However, the increasing concern regarding the occurrence of levodopa-related motor complications has led to hesitation in initiating levodopa therapy, subsequently delaying the needed and effective relief of PD-related motor symptoms. Notably, neither animal nor human studies reported the acceleration of disease progression upon initiation of levodopa therapy, and delayed-onset of dyskinesia followed by delayed initiation of respective therapy (Jankovic & Tan, 2020). Besides that, no evidence of toxicity was reported in a 9-month study, called the earlier vs later L-dopa trial, though about 16.5 percent of patients in the 600mg group developed dyskinesia (Fahn et al., 2004). In order to smooth out fluctuations and prevent the wearing-off symptoms, fractionation of the total daily dose is provided as a strategy for patients with a short duration of response to levodopa. Other effective formulations of levodopa include continuous intrajejunal infusion of levodopa-carbidopa intestinal gel (Lang et al., 2016), subcutaneous delivery of soluble carbidopa/levodopa formula (Rosebraugh et al., 2021), and inhalable levodopa powder (LeWitt et al., 2019).

Treatment for non-motor symptoms should also be considered as an overall treatment for PD patients as these symptoms may have a greater impact on the quality of life than motor symptoms. Cholinesterase inhibitors may minimise hallucinations, increase postural stability, and even reduce the incidence of falls in some individuals, in addition to enhancing cognitive performance (Henderson et al., 2016). Sleep disturbances should be treated using sleep hygiene measures, which may be reinforced with hypno-sedatives, tricyclic antidepressants, mirtazapine, trazodone, quetiapine, or night-time dopaminergic treatment if necessary (Ryan et al., 2019).

Results from a double-blind, randomised clinical study showed the effectiveness of home-based and remotely supervised aerobic exercise in lowering the off-state MDS–UPDRS score (van der Kolk et al., 2018). High or moderate physical

activity was shown to minimise the risk of PD as reported in a meta-analysis of 8 prospective studies up to two thousand PD patients with an average follow-up of 12 years (Fang et al., 2019), thereby suggesting the importance of implementing a regular exercise program.

On the other hand, many patients in the intermediate to late stage of PD have a poor quality of life despite receiving optimal medical treatment, which is due to the fluctuating response, dyskinesia, or levodopa-unresponsive symptoms (Jankovic & Tan, 2020). Therefore, surgical therapies such as stereotactic ablations and deep brain stimulation have been developed to help individuals with advanced Parkinson's disease manage their severe movement problems (Jankovic & Tan, 2020). Customisation of stimulation parameters offered by deep brain stimulation (DBS) has successfully replaced ablative surgery as can optimize the benefits in accordance with patient's needs (Jankovic & Tan, 2020). The most common target for DBS therapy of individuals with PD who have debilitating tremors and/or levodopa-related motor problems is STN or globus pallidus interna (GPi), whereas thalamic DBS is usually used for PD patients with high-amplitude tremor (Jankovic & Tan, 2020). Interestingly, a randomized controlled trial has reported a more robust improvement upon DBS as compared to optimal medical therapy (Weaver et al., 2009). Patients treated with DBS have a mean of 4.6 hours per day of on-time without troubling dyskinesia, compared with 0 hours per day for those who received the optimal medical therapy ($p < 0.001$). This study also reported that 71% of DBS and 32% of medical therapy patients have motor function improved by 5 or more points on the motor UPDRS, in addition to the significant improvement shown in the majority of PD-related health-related quality-of-life measures and minor decrease in the neurocognitive test. But the DBS group also possessed a 3.8 times the greater overall

risk of experiencing a serious adverse event as compared to the medical therapy group. Another randomised trial involving patients with early motor complications has reported a rather positive outcome in patients receiving a combination of STN DBS and medical therapy as compared to medical therapy alone, suggesting the importance to consider optimizing medical treatment prior to early DBS (Schuepbach et al., 2013). Although DBS is a proven successful treatment technique, it is thereby important to note that this greatly relies on several factors such as the selection of the right patient as well as the experience and skill of the stereotactic surgeon to provide the best outcomes and minimise the complications (Kim et al., 2019; Dafsari et al., 2019).

Cell replacement therapy (CRT) is a treatment that aims to enhance long-term motor function by halting or decreasing the rate of disease development (Guo et al., 2021). Cell-based therapy with fetal ventral mesencephalon (fVM) was developed in the early phase of pre-clinical work. The 6-OHDA or MPTP lesion model, which used either human or mouse fetal tissue, provided promising findings in animal studies done between 1977 and 1985. The incredible brain plasticity was suggested followed by observable survival of transplanted cells in the lesion area (Parmar et al., 2019). The first double-blind study conducted in 2001 to compare the efficacy of transplantation of fetal midbrain tissue and sham surgery, has reported no clinical benefit in older patients and has recorded recurring dystonia and dyskinesia in 12% of those who received transplants (Freed et al., 2001). Another double-blind, placebo-controlled trial of fetal nigral transplantation failed to show measurable benefit on the primary and secondary endpoint, and worse, most grafted patients developed graft-induced dyskinesia (Olanow et al., 2003). As a result, CRT was being left dormant for a few years until more related studies suggest that this approach requires a longer time in order to show remarkable clinical improvements (Parmar et al., 2019).

Even so, this approach does not represent a realistic therapeutic option in the future due to the presence of dyskinesia in some patients, lack of standardizations that cause highly variable outcomes, ethical concerns, and the limited availability of the tissues (Parmar et al., 2019). Therefore, the field has been directed to stem cell-based therapies as stem cells are readily available, have the ability to self-renew and differentiate, as well as more likely to be produced on a large scale (Fan et al., 2020). Dopaminergic neurons can be produced by inducing stem cells to differentiate under the right conditions. These neurons can be implanted into patients with Parkinson's disease to replace dopamine levels and provide symptomatic relief. Advances in the creation of dopaminergic neurons from somatic human cells, as well as improvised differentiation methods, have resulted in a comeback of cell transplantation in PD (Barker et al., 2013; Nolbrant et al., 2017; Barker et al., 2017; Jankovic et al., 2020). Indefinite volumes of human embryonic stem cell lines and somatic cells that can be transformed into authentic midbrain dopaminergic cells that meet good manufacturing procedure grade standards can be created for clinical use (Barker et al., 2013; Nolbrant et al., 2017; Barker et al., 2017; Jankovic et al., 2020).

2.1.3 Challenges faced

While stem cell-based therapy offers promising treatment approaches, it is essential to address several challenges before it can be used as the first-line treatment for PD. Since grafting therapy would require a neurosurgical procedure, hence it is foremost important and necessary for the respective procedure to be done safely and with low risk. A period of immunosuppression is required for allogenic grafts in order to suppress the associated risk of infection and malignancy. The use of autologous stem cells for transplantation would be ideal since the risk of tissue rejection would be

remarkably low. This has been proved by Hallett and the team, which performed iPSC-derived dopaminergic neuron transplantation onto the same cynomolgus monkey. They observed that the graft survived for up to two years after transplantation, and that motor function and activity improved without the need for immunosuppressive medicines (Hallett et al., 2015). Even so, this application would require the generation of individually specific neural grafting products, hence raising the potential regulatory issues, along with extensive testing for safety purposes, and these would be expensive (Stoker et al., 2017). Additionally, consistency in iPSC-reprogramming and differentiation protocols between all individuals is necessary before the approval of such a product, suggesting an increase in the expenses.

Another issue with autologous graft transplantation is the genetic risk factors carried which would contribute to the development of PD in the patient. Post-mortem examination of human FVM grafts revealed the development of PD pathology, which may be accelerated in the presence of particular genetic risk factors (Li et al., 2016). This might suggest that the advantages of autologous transplants wear off faster than those of allogenic grafts.

Till today, ethical implications are one of the main challenges of using human fetal tissue, specifically embryonic stem cells (ESCs). This is because the generation of products derived from ESC involves the destruction of the human embryo which means that an invasive procedure would likely be carried out, causing this approach to be ethically unacceptable in some societies. However, using ESCs from human embryos that would otherwise be destroyed for the use of foetal tissue is ethically desirable in most nations, therefore this is unlikely to be a significant barrier to the acceptance of these treatments (Stoker et al., 2017).

Tumorigenesis associated with stem cell-based therapies for PD is a serious safety issue. This can happen owing to graft overgrowth, remaining pluripotent cells in the graft, or the acquisition of tumorigenic mutations during cell culture. Although tumour growth was observed in certain early preclinical experiments (Park et al., 2005; Sonntag et al., 2007), advances in differentiation methods have resulted in more refined, pure cell products that have not generated tumours in animals (Liu et al., 2016; Di Giovannantonio et al., 2014; Samata et al., 2016; Fathi et al., 2018). Since neural grafts are expected to reside in transplanted patients for decades, therefore comprehensive safety data will be required to confirm that tumour risk is minimal even over lengthy periods of time.

2.2 Stem cells

2.2.1 Definition & types of stem cell

Stem cells are the organisational units of biological systems that are responsible for organ and tissue regeneration and development. Stem cells are a population of unspecialized cells that has the potential to proliferate extensively (self-renewal) and differentiate into specialized functional cells that make up the various tissues and organs in the body. Stem cells exist both in embryos as pluripotent stem cells which can differentiate into the three germinal layers and function to generate new organs and tissues, also as multipotent or unipotent stem cells in adult tissues to replace cells during the natural course of cell turnover (Bahmad et al., 2021) (Figure 2.1). The main difference between embryonic stem cells (ESCs) and adult stem cells is the differentiation potential, whereby ESCs can give rise to any cells derived from the primary germ layers and also germ cells, while adult stem cells are capable of

differentiating into only a limited quantity of specialized cell types (Seo, 2011; Bacakova et al., 2018).

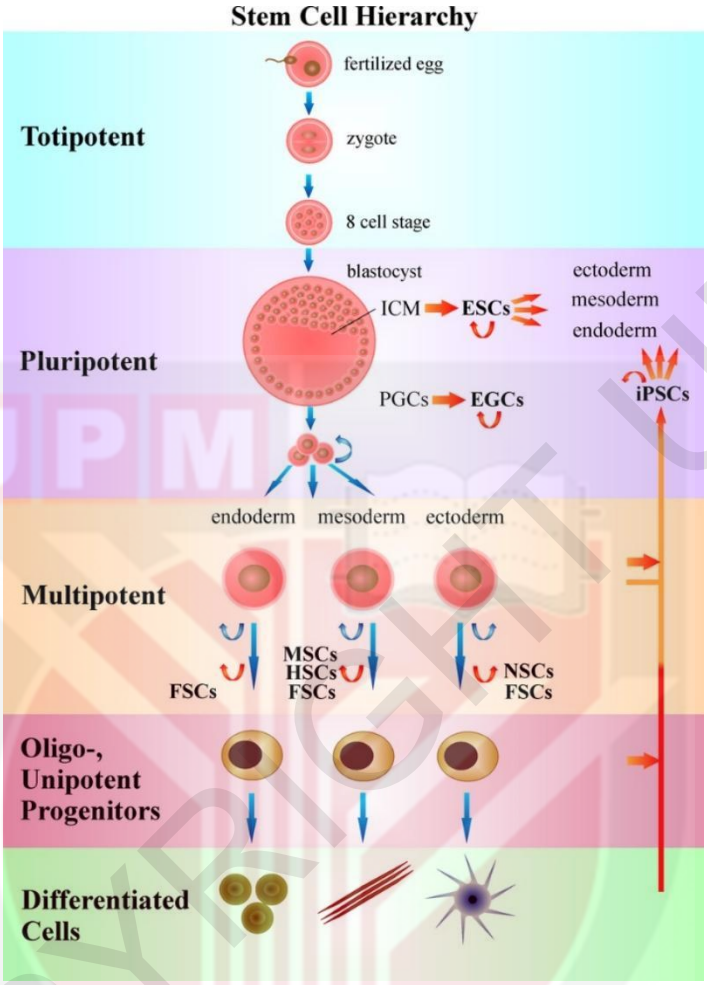

Figure 2.1: Stem cell hierarchy. Classification of stem cell potency.
(Adapted from Forostyak et al., 2016)

2.2.2 Characteristics of stem cell

Three characteristics can be used to classify stem cells, however not all stem cells have these characteristics. Self-renewability is a term used to describe the ability of stem cells to make exact copies of themselves. Notably, a stem cell is not immortal and is endowed with a limited potential to self-renew based on the turnover rate of tissue. This is achieved by a process known as asymmetric cell division, where a stem cell gives rise to 2 daughter cells, one remains as a stem cell whereas the other becomes more committed to forming a particular cell type, also known as committed progenitor

(Chopra et al., 2013). Besides, clonogenicity refers to the capability of a stem cell to divide extensively and forms a colony made up of multiple cell types (Kolios & Moodley, 2013; Chopra et al., 2013). While clonogenicity is an important factor in determining if a cell is a stem cell, note that not all cells that form colonies are stem cells (Chopra et al., 2013). Another characteristic of stem cell is potency. Potency is defined as the ability of the stem cells to differentiate into multiple distinct, specialised cell types (Kolios & Moodley, 2013). Totipotency, pluripotency, and multipotency are the three primary kinds of potency. Stem cells with the highest potency can produce a greater variety of cell lineages than stem cells with lesser potency. A variety of functional assays, as well as the evaluation of numerous molecular markers, may be used to determine the potency of these stem cells. These molecular markers can be used to determine stem cell transcriptional, epigenetic, and metabolic states (Singh et al., 2016). The abovementioned characteristics may differ between various stem cells.

2.2.3 Application and therapeutic potential of stem cell

The value of stem cells in modern medicine is of paramount importance, both for their extensive use in fundamental research and the for the opportunities they provide for the development of new therapeutic strategies in clinical practice. Their unique properties make them useful in numerous biomedical applications such as stem cell-based replacement therapies, 3D bioprinting as well as pharmacology, and toxicology screening (Liras A., 2010; Azkrzewski, W., 2019).

Ever since the first case of successful grafting of genetically modified cells in a damaged brain performed by Rosenberg et al. (1988) to protect the cholinergic cells from dying, it has been a groundbreaking discovery that cleared the door for the use of stem cells as a treatment for neurodegenerative disorders. Three years later, another

study suggested that engrafting neuronal cells into specific brain areas might offer temporary relief from Parkinson's disease by raising neurotransmitter concentrations, although this could not cure the disease (Gage et al., 1991). In 2006, preclinical research was done by Wang et al. to study the effects of neural stem cells (NSCs) transplantation in the cortex of an AD mice model. Behavioral testing was performed on mice after 12 weeks of transplantation, demonstrating that the transplanted neurospheres in the cortex had survived and were able to create more ChAT and serotonin-positive neurons inside and surrounding the grafts. The mice with implanted neurospheres also exhibited significantly decreased working memory errors in the eight-direction maze test (Wang et al., 2006). This study has demonstrated the positive effect produced by NSC in ameliorating the symptoms of AD in mice, and following this, a number of different animal studies were carried out to better understand the impact of transplanted stem cells on AD. Notably, the application of stem cell-based transplantation is not restricted to only neurodegenerative diseases but has also produced promising results in preclinical studies and clinical trials of several other diseases, for example, chronic myeloid leukemia (Gratwohl & Heim, 2009; Hackanson & Waller, 2011), pulmonary fibrosis (Tzouvelekis et al., 2011; Banerjee et al., 2012), diabetes mellitus (Kroon et al., 2008; Trivedi et al., 2008), stroke (Bang et al., 2005; Tang et al., 2007) and spinal cord injuries (Watanabe et al., 2015; Yoon et al., 2007).

The application of stem cells as skin grafts in the case of chronic wounds is quite promising, with the fact that they are capable of producing substances such as chemokines, cytokines, and growth factors, which promote regeneration processes and exhibit paracrine effects (Ho et al., 2018). These factors will trigger the local stem cells to multiply and initiate the healing process when administered to the wound bed (Ho et al., 2018). Despite having a great ability to proliferate, they can enhance

angiogenesis, accelerate the wound closure, and hence, support the natural healing process. Due to their strong adhesion to plastic materials, they are rather easy to be used in therapy as they can be administered directly to the wound or administered systematically through the blood (Yoshikawa et al., 2008).

Three-dimensional (3D) printing technologies have recently been applied to biocompatible materials, cells, and supporting components, resulting in a field called 3D bioprinting that offers enormous potential for artificial organ printing and regenerative medicine, and specifically, playing a central role in addressing the need for tissues and organs suitable for transplantation. In terms of cell sources for bioprinting, it is important for bioprinted cells to expand adequately for bioprinting, besides retaining the ability to recapitulate the biological function of the native tissue (Ong et al., 2018). While the production of artificial organs for transplantation is the ultimate goal, in the meantime, tissue repair has shown to be quite promising. Rather than generating entire functional organs, the small tissue patches can be potentially used to regenerate and treat organs like the heart (Ong et al., 2017) and liver (Ma et al., 2016) as well as bone fractures (Yang et al., 2017). Since products obtained from bioprinting technologies are able to mimic both the biological and functional properties of naturally occurring structures and tissues in the human body, it has led us to various kinds of applications, such as high-throughput 3D-bioprinted tissue models for research, drug discovery, and toxicology (Murphy & Atala, 2014).

For the past 2 decades, several studies have suggested the use of pluripotent stem cells in the field of toxicological screening, specifically as a source of cells for drug discovery, cytotoxicity assessment, and disease modelling (McNeish, 2004; Desbordes & Struder, 2013). Some of them stem cell proliferation and differentiation as parameters to evaluate developmental toxicity (Pal et al., 2011), while others use

stem cell-derived models for drug toxicity assessment (Apáti et al., 2019). Besides reducing the number of test animals and expenses, such in vitro models might be best suited to test a large number of chemicals (Pal et al., 2011). Furthermore, several researchers have been using these cells for the development of high-throughput and high-content screening (Sherman et al., 2011; Desbordes & Struder, 2013). Sherman et al. (2011) also suggested that evaluation of a much broader array of experimental conditions can be achieved at any one time through the combination of high throughput technologies.

2.3 Embryonic stem cells

2.3.1 Origin and properties of ESCs

The embryonic development of a mouse begins with the fertilisation of egg and sperm, which develops into an embryo and all extra-embryonic tissues required for its growth. After implantation, the cell divides quickly and maintains its totipotent status from the zygote to the blastocyst stage, where the latter lasts from embryonic day E3.5 to E4.5 (Pauklin et al.,2010). The cells progressively lose their ability to grow and undergo differentiation. During the early blastocyst stage, the interior cells of the embryo will form the inner cell mass (ICM), residing at one end of the blastocyst and surrounded by the outer cells, which are trophectoderm. The first isolation of ICM from the mouse blastocyst was performed by Evans and Kaufman in 1981. This experiment was done to establish pluripotent cells from the mouse embryos. These cells are termed mouse embryonic stem (ES) cells and are characterized by their indefinite self-renewal ability in vitro while maintaining their pluripotent status under appropriate conditions (Pauklin et al., 2010). With this stemness, ES cells are able to differentiate into germ cells as well as derivatives of the three primary germ layers, which are ectoderm,

endoderm, and mesoderm. These characteristics have made ES cells an extremely attractive cell source for various lineages differentiation for clinical applications and for a better understanding of normal and pathological development in mammals, especially in humans (Nishikawa et al., 2007).

2.3.2 Neurogenic potential of ESCs *in vitro*

With ES cells' pluripotency characteristics, numerous lineage-specific differentiation protocols have been developed. Yet, the generation of a highly homogenous ES cells-derived neural population in a short period remains a challenge, hence restricting its application in pharmaceutical screening and transplantation. As a result, many research groups have put in tremendous efforts to refine and improve the existing protocols which are useful for the regenerative cell replacement therapy intervention of neurodegenerative disorders, such as Parkinson's disease. General approaches that are extensively used to promote ES cell neural differentiation are through the multicellular aggregation of ES cells termed embryoid bodies (EBs) with the addition of neural inducer, co-culture, and monolayer culture.

2.3.2.1 Embryoid bodies (EBs)

Culturing single ES cell suspension in serum-supplemented medium without leukaemia inhibitory factor (LIF) is the most frequently used method of generating embryoid bodies. The ES cells will undergo spontaneous differentiation and forms the three-dimensional multicellular aggregates known as the EBs, which mimic the in-vivo structure of developing embryos. The formation of EBs can be done via the hanging drop method, suspension culture in ultra-low attachment culture vessels, and utilization of hydrogel such as methylcellulose culture.

Cell-cell adhesion of ES cells mediated by E-cadherin, a cell surface protein, is essential for the formation of EBs through cell aggregation (Bedzhov et al., 2013). Notably, N-cadherin is another principal cell-cell adhesion molecule involved in early mouse embryos (Moore et al., 1999). They serve as an essential initiation stage for lineage-specific differentiation into derivatives of the three germinal layers, besides providing a routine means for in-vitro pluripotency test. However, the effect of differentiation greatly depends on the quality of EBs, which is generally affected by the number of cells, size of EBs, and medium conditions generated (Pettinato et al., 2014; Khoo et al., 2005; and Mohr et al., 2010). Early EBs (D2-D4) derived from mouse ES cells recapitulate the morula-like structure *in vivo* (Pettinato et al., 2014) (Figure 4.2). This is the stage where the appearance of the primitive endoderm layer on the early EBs surface is visible. D4-D5 EBs are referred to as cystic EBs, where the primitive endoderm layer has undergone maturation, and become the visceral endoderm. Meanwhile, inner cells will undergo epithelization, with the loss of innermost cells by apoptosis to form the primitive ectoderm, hence resulting in a cavitation process mimicking the *in vivo* blastocoel (Coucouvanis & Martin, 1999; Rodda et al., 2002 as cited in Mohamed Hisham, 2017). D8-D10 EBs are known as the mature EBs, and appeared to be a double-layered structure composed of an inner ectodermal layer and an outer endodermal layer surrounding the cavity (Liyang et al., 2014).

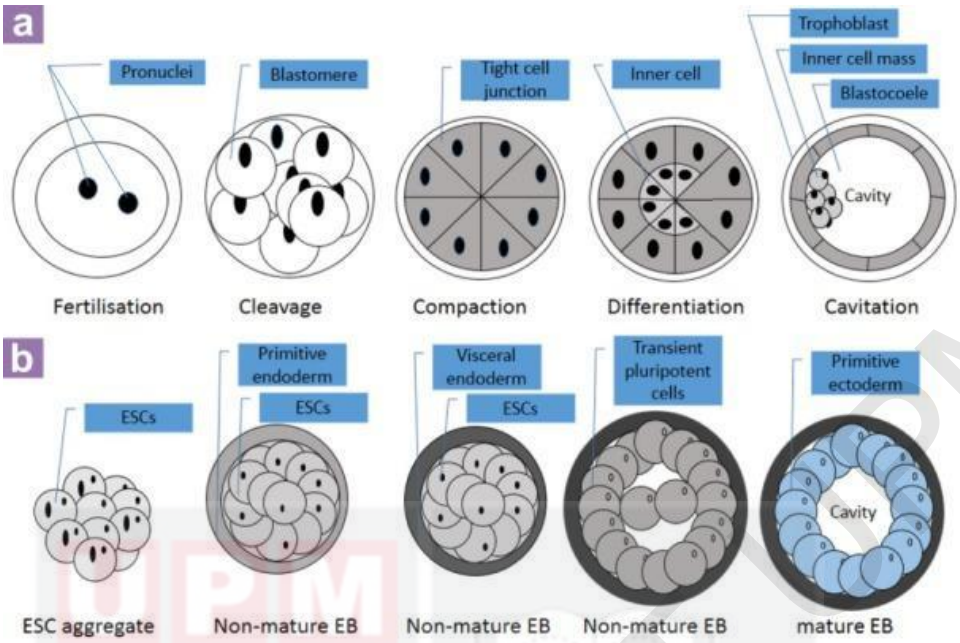

Figure 2.2: The changes of an embryo in early embryogenesis (a) and EBs formation *in-vitro* (b)
(Adapted from Liyang et al., 2014)

2.3.2.2 Monolayer culture

Monolayer culture is a common method for ES cell development into the neural lineage. This approach is advantageous for extensive in vitro characterization, prospective mechanistic and therapeutic screening, in addition to its simplicity and efficiency (Sadegh & Macklis, 2014). Studies have demonstrated that feeder-free adherent monoculture using serum-free media N2B27 has committed ES cells into neural fate with great efficiency (Abranches et al., 2009; Ying et al., 2003). Ying and colleagues also generated the TH neuron subtype by supplementing the culture with Fgf8 and Shh. Monolayer differentiation is not restricted to the generation of NPC only, instead, it has high potential in generating defined neural subtypes such as spinal motor neuron and cortical-motor neuron, as demonstrated by Jha et al. (2015) and Gaspard et al. (2009) respectively. Apart from the advantages, Sadegh & Macklis (2014) also discovered the limitation of monolayer adherent culture upon obtaining a heterogeneous population of ES-derived neocortical with multiple maturation

deficiencies. Their findings show that this approach is extremely reliant on matrix proteins, define inductive media, and the addition of morphogen, all of which have a significant impact on the formation and specialisation of developing cell types. Therefore, further improvement and understanding of the existing protocol is required in order to generate a highly homogenous population of functional neuronal subtype.

2.3.2.3 Co-culture

ES cell will be directed towards specific lineage differentiation upon being in contact with differentiated cells in the co-culture method. The concept of niche as a specialized microenvironment that was postulated by Ray Schofield in 1978, is applied in this method. Schofield and colleagues defined haematopoietic stem cells (HSC) niche as a complex environment in the bone marrow, which is composed of specialized cells with roles in maintaining HSC and its differentiation through cell surface-bound factors and soluble signals. Therefore, it is well understood that the co-culture method mimics the stem cell niche by providing; (i) cell interaction among stem cells, (ii) stem cell interaction with neighbouring differentiated cells, and (iii) stem cell interaction with extracellular matrix adhesion molecule and soluble factors (Yue et al. 2013). The combination of all these cues will determine the differentiation of ES cells into specific lineage cells. A previous study has shown that co-culturing mouse Sertoli cells together with monkey ES cells without induction factor has successfully generated Tyrosine Hydroxylase (TH) positive cells after 3 weeks of induction. This study also explained the crucial role of Sertoli cells in promoting neural differentiation of ES cells, which is through the secretion of two neuron-inducing factors namely cell surface-anchored factor and glial cell-derived neurotrophic factor (GDNF) soluble factor. The co-culture method is technically straightforward, cost-effective, and time-

saving. This, however, frequently results in ambiguous inductive activity, predisposing the system to a heterogeneous differentiated cell population.

2.3.2.4 4-/4+ protocol of neural induction

4-/4+ protocol was developed by Bain and his team in 1996, with the goal to generate mouse ES-derived neural cells. This method begins with the formation of EBs in serum-supplemented media on a non-coated bacteriological-graded culture dish upon the removal of LIF. The 8 days induction procedure was performed by culturing EBs without LIF for 4 days followed by the addition of 1µM retinoic acid (RA) for 4 days, to direct the cells toward neural differentiation and suppressed the mesodermal gene expression (Bain et al., 1996). RA is one of the main neural inducers in the early embryo. Ströbing et al. (1995) further proved the efficiency of RA as neural induction in vitro. According to his study, neuroectoderm differentiation of EBs with RA as a neural inducer has been demonstrated to increase up to 6 times as compared to the ones without RA. A study conducted by Nordin et al. (2008) reported the down-regulation of Oct4 expression on late-stage EBs (D4-D8) upon the RA induction, together with an observable increase of Sox1-GFP expression as early as 6 days in culture and was present until day 14. These findings strongly suggest the differentiation of ES cells and the absence of pluripotent cells. Dissociation of EBs will be performed upon 8 days induction period, followed by plating of the cells on PDL/laminin-coated dish.

2.4 Wnt5a transgenic mouse embryonic stem cell lines

2.4.1 Conditional expression system

A number of site-specific DNA recombination system has been developed and used in genome engineering technologies, which includes bacteriophage-derived Cre/*loxP*

system, yeast-derived Flp/FRT (Ji et al., 2017), Dre/rox system (Li et al., 2020), ParA-MRS (Thomson et al., 2008), Bxb1 recombination system (Thomson et al., 2012), phiC31 (ϕC31) system (Thyagarajan, 2001), and Tet system (T. Das et al., 2016). Cre/loxP system was chosen to be used in this study as it is simple, efficient, and widely used in various gene-editing research (Nagy, 2000; Ray et al., 2000; Reinert et al., 2012).

2.4.1.1 Cre/*loxP* inducible system

Cre/*loxP* inducible system is a powerful genetic editing tool that is generally used to study the specific gene function during development, especially in cases where embryonic lethality may occur as a result of inactivation of the target gene, inhibiting the further investigation of the function of the respective gene in the post-developmental stage. This regulatable system offers the ability to tightly control the expression of the gene of interest through the activation of Cre prior to induction. In this system, Cre recombinase from the P1 bacteriophage is fused to a mutated estrogen receptor (ER) as a transgene (*Cre*-ER), which is only active when exposed to 4'-hydroxytamoxifen (4'-OHT). Once Cre is activated, it will recognise the loxP sites and catalyse the recombination event. Cre has the ability to catalyze three forms of recombination, which are excision, inversion, and translocation (Figure 2.3). Notably, the action of Cre is dictated by the orientation of loxP sites. Cre-mediated excision of floxed region occurs when two loxP sites are similarly oriented on one DNA strand, hence allowing the expression of downstream genes. Meanwhile, loxP sites in reverse orientation on the same DNA strand will result in Cre-mediated inversion whereas recognition of loxP sites in the same direction on both DNA strands results in Cre-mediated translocation.

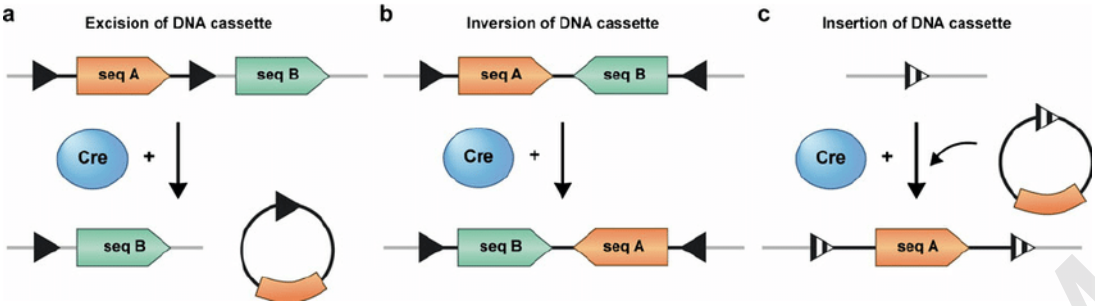

Figure 2.3: The mechanism of Cre/*loxP* system.
(Adapted from Renninger et al., 2011)

2.4.1.2 Establishment and characterization of conditional Wnt5a transgenic ES cell line

To establish a conditional Wnt5a transgenic mouse ES cell line, multiple steps were carried out. Firstly, the validation of Wnt5a plasmid constructs by using Polymerase Chain Reaction (PCR), restriction enzymes, and sequencing analysis. Upon validation, the validated Wnt5a plasmid construct was then transfected into a Cre expressing mouse ES cell line. Dual antibiotics selection by neomycin (G418) and puromycin was carried out to select positive clones carrying the respective construct. Pluripotency characterization of the Wnt5a transgenic ES cell lines was performed followed by the functional characterization of the construct at both RNA and protein levels. The former step is to confirm that the pluripotency status of the Wnt5a transgenic mouse ES cell line is not compromised upon transfection. In this test, the Wnt5a transgenic ES cell line was quantitatively and quantitatively analysed for core ESC pluripotency factors *Oct4*, *Sox2*, and *Nanog* by reverse-transcription polymerase chain reaction (RT-PCR) and immunocytochemistry (ICC). These markers were chosen as the gold standard in determining the pluripotency of ES cells and iPSCs (Yeo & Ng, 2012; Filipczyk et al., 2015; Zhao et al., 2012). Apart from that, studies also proposed the generation of embryoid bodies from ESCs and also iPSCs as a means for in-vitro pluripotency test (Sheridan et al., 2012; Stover et al., 2011).

2.4.2 Role of Wnt5a in neurogenesis

Neurogenesis is defined as the process of generation of functional neurons in the brain, particularly involving the proliferation, migration, and differentiation of neural progenitor cells (NPC) termed radial glia. The interplay of various signaling molecules and transcription factors is crucial for proper brain development. Among all the key signaling pathways, Wnt signaling is one of the primary signaling pathways that is known to have a multitude of significant roles in controlling numerous aspects of neurogenesis. The Wnt family comprises a 19-member group of secreted glycoproteins which is high conserved among various species. Wnt signaling is divided into canonical and non-canonical pathways based on downstream activation of B-catenin pathways. Wnt GSK B-catenin is grouped under the canonical pathway whereas Wnt/PCP and Wnt/CA2+ are grouped under the non-canonical pathway.

Wnt5a, a member which mediates the non-canonical pathway was shown to be involved in the regulation of neurogenesis in a stage-dependent manner. Previous work of Nordin et al. (2008) proved the stage-dependent activity of Wnt5a as a dynamic expression of Wnt5a transcript is observed upon subjecting ES to neural differentiation via the 4-/4+ protocol. Andersson et al. (2008) reported the high expression of Wnt5a spans the whole neuroepithelium of the ventral midbrain at E9.5-E10.5, followed by a gradual restriction to the floor plate and basal plate of the ventricular zone (VZ) at E11.5 -E13.5, suggesting the specific role of Wnt5a on midbrain dopaminergic precursors and its role in the terminal dopaminergic differentiation (Castelo-Branco et al., 2006). This respective study also observed enhanced progenitor proliferation as a result of the deletion of Wnt5a, this was then followed by increased production of Nurr1+ postmitotic cells. This result is consistent with a previously reported study which suggests the main role of the non-canonical Wnt signaling pathway in

differentiation and maturation (Michaelidis & Lie 2008; Bielen & Houart., 2014). Conversely, an *in vitro* study conducted by Lange et al. (2006) reported evidence of Wnt5a supporting the proliferation of neural progenitor cells in the adult subventricular zone.

According to a number of previous studies, Wnt5a is also associated with the generation of dopaminergic neurons in brain development. Findings of Andersson et al. (2013) have shown that Wnt5a promoted the differentiation of DA neural precursor cells to Tyrosine Hydroxylase (TH) positive DA neurons. Furthermore, a significant increase in the number of TH+ cells upon sequential Wnt3a and Wnt5a treatment suggested the interplay of canonical and non-canonical signaling pathways in the regulation of fate specification and terminal differentiation in the brain development during the process of neural differentiation. While removal of Wnt5a had no effect on neurogenesis or the amount of TH+ mDA neurons (Andersson et al., 2008), deletion of both Wnt5a and Wnt1 exacerbated the DA neurogenesis and differentiation impairments were observed in Wnt1-/- animals (Andersson et al., 2013). These results suggested the possible extensive interaction of Wnt1a and Wnt5a in the regulation of mDA neuron development.

The role of Wnt5a in the differentiation of potential stem cells derived from diverse sources has been studied extensively. A study conducted by Li et al. (2020) has shown that both Wnt5a and miRNA200b-3p could promote neural stem cell (NSC) differentiation into neurons and that Wnt5a upregulated miRNA200b-3p expression through MAPK/JNK signaling to promote NSC differentiation into neurons. Besides that, Wnt5a was shown to promote the differentiation as well as the functional integration of stem-cell-derived DA neurons *in vivo*, hence suggesting Wnt5a-treated neural stem cells as a reliable and safe source of DA neurons for cell replacement

treatment in Parkinson's disease (Parish et al., 2008). According to Arredondo et al. (2019), Wnt5a is a neurogenic factor as the findings show that Wnt5a induces neuronal differentiation and dendritic development of newborn neurons through CaMKII, Wnt/JNK, and Wnt/CaMKII pathways respectively.

The neuronal circuit and refinement were constructed once the migrating neurons arrived at their ultimate location by developing long cellular extensions termed neurites, which are made up of axons and dendrites. Wnt5a has been identified as one of the key regulators of axonal development and repulsion events. A finding showed Wnt5a promotes axonal development, DA axon morphogenesis, DA neurogenesis as well as maturation and fasciculation of the medial forebrain bundle (Blakely et al., 2011). Moreover, Wnt5a was identified as an essential key component of nerve growth factor in regulating axonal branching and maturation in the development of sympathetic neurons (Bodmer et al., 2009).

2.5 Application of stem cell-derived dopaminergic neuron

Till today, there were no other applications reported other than the use of stem cell-derived dopaminergic neurons in the cell replacement therapy for the treatment of Parkinson's disease, which has been one of the prospects for curative treatment for Parkinson’s disease. In 2017, a preclinical study conducted by Kikuchi and his team, have reported that human-induced pluripotent stem (iPS) cell-derived dopaminergic progenitor cells able to survive and function as midbrain dopaminergic neurons in a PD primate model. According to the score-based and video recording assessments, a significant increase in spontaneous movement of the monkeys was observed upon transplantation. Furthermore, histological examinations showed the dense neurites extension of mature dopaminergic neurons into the host striatum. He also reported the

absence of tumours in the brains for at least two years (Kikuchi et al., 2017). The above positive results from this preclinical study have suggested that human iPS cell-derived dopaminergic progenitors may be clinically useful in the treatment of PD patients. Meanwhile, in the same year, the first human clinical trial (NYSTEM trial) using midbrain dopamine (mDA) neurons derived from human pluripotent stem cells (hPSCs) was in the mid of preparation. Throughout their preparation, they have successfully demonstrated the generation of in vitro-derived mDA neurons under defined conditions and at a large scale; the capability of the cryopreserved product to reverse PD symptoms in rodent models of PD as well as the successful engraftment of the cells in the brain of PD monkeys (Studer L., 2017). We hope that their research paves the way for the development of an off-the-shelf cell therapy for Parkinson's disease, which might one day become a viable treatment option for PD sufferers.

CHAPTER 3

METHODOLOGY

3.1 Study design

The workflow of this study is summarised in Figure 3.1. The project began with reviving Wnt5a transgenic line (NI-L7) from liquid nitrogen before the cells were propagated for downstream experiments. The downstream experiment started off with the examination of the pluripotency status of the clone in terms of the expression of pluripotency-associated markers (*Oct4, Sox2* and *Nanog*) by RT-PCR, and the ability of the clone to spontaneously differentiate into EBs. Observation on the morphology of EBs formed was recorded every two days until day 8. Next, an assessment of cell morphological differences between non-induced NI-L7 and tamoxifen-induced CE-L7 was performed to ensure the stem cell quality is maintained and are not compromised after tamoxifen induction. Finally, the inducibility of Wnt5a transgene was examined through the expression of Wnt5a using RT-PCR followed by semi-quantification using ImageJ software.

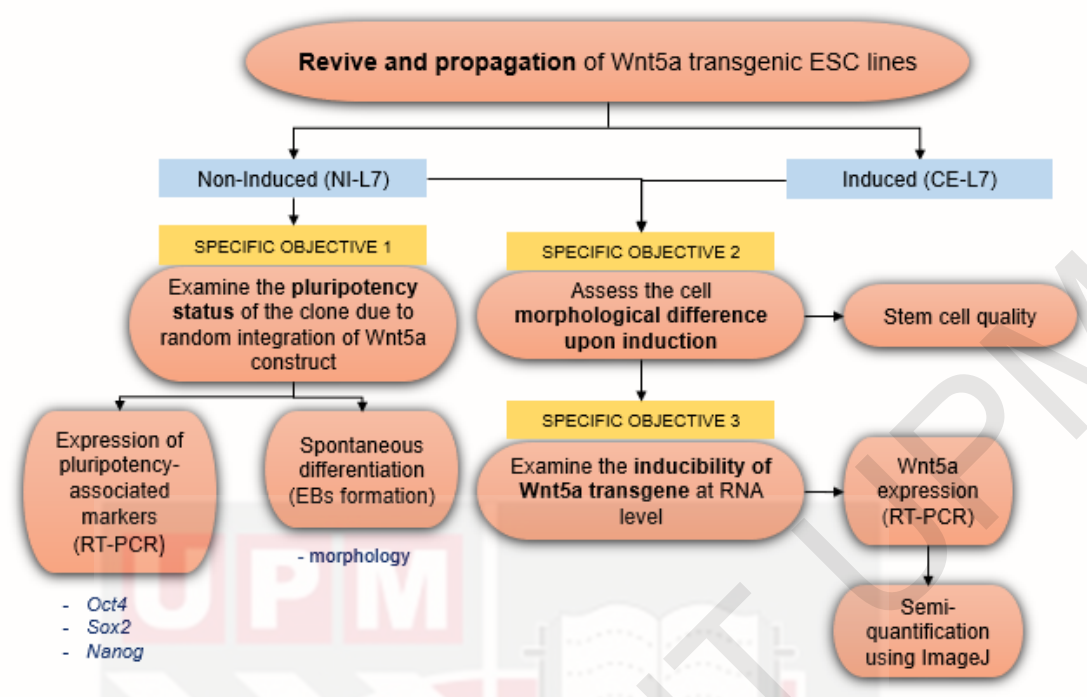

Figure 3.1: Schematic flow diagram of the methodology.

3.2 Routine cell culture of Wnt5a transgenic mouse ESC line

3.2.1 Media and supplement list

The Wnt5a transgenic mouse ESC line was plated on the gelatin-coated flask with 0.1% (w/v) of gelatin (Sigma) diluted in sterile water. Gelatin solution was then autoclaved. The plate was coated for at least 20 minutes before the excess gelatin was pipetted out. The medium was supplemented with 7.5% of sodium bicarbonate (Gibco), 0.1mM of 2-mercaptoethanol (Gibco), 1mM sodium pyruvate (Gibco), 2mM of L-Glutamine (Gibco) and 15% fetal bovine serum (FBS, Gibco). The complete medium was added with 20ng/mL recombinant human leukaemia inhibitory factor (LIF) (Gibco) to promote cell proliferation and inhibit the spontaneous differentiation of ES cells.

3.2.2 Pre-coating plate with gelatin

Plates or flasks for culturing the ESCs were pre-coated with prewarmed 0.1% gelatin for at least 20 minutes at room temperature. The excess gelatin was pipetted out and discarded before plating the cells.

3.2.3 Thawing cells

Frozen cells from -80°C freezer/liquid nitrogen tank was quickly thawed in 37°C water bath for about 45 seconds with shaking. The thawed cells were then gently transferred into 9 ml of pre-warmed complete medium (ESM), resuspended, and centrifuged for 5 minutes at 1000rpm to remove the cryopreserve reagent. The supernatant was removed and the cell pellet was resuspended with an appropriate volume of pre-warmed ESM, depending on the size of the pellet prior to plating the cells into gelatin-coated T25 flasks, or 6-well plate (ThermoFisher). The cells were then grown in 37°C incubator in an atmosphere of 5% CO_2.

3.2.4 Splitting cells

The cells were ready to be split once they have reach 70-80% confluency. The cells were rinsed with 1X PBS once after the old medium had been aspirated off. The cells were then incubated in an appropriate volume of 0.25% trypsin/EDTA (Gibco) in 37°C incubator for 1-3 minutes until the cells were observed to detach from the flask. The trypsin activity was deactivated in an appropriate volume of ESM before the cell suspension was triturated 10 times to dislodge the cells. The dislodged cells were then transferred into a 15mL tube prior to spinning the cell suspension at 1000rpm for 5 minutes at room temperature. The supernatant was pipetted out and discarded, and the pelleted cells were resuspended with an appropriate volume of pre-warmed ESM before being counted using haemocytometer. The cells were then seeded into a gelatin-

coated flask, with a cell seeding density of 4 x 10^4 cells/ cm^2, and incubated in 37°C incubator with an atmosphere of 5% CO_2 until confluent.

3.2.5 Freezing cells

Generally, 70-80% confluent monolayer cultured cells were trypsinised as detailed in section 3.1.4. Upon trypsin deactivation with an appropriate volume of ESM, the cell suspension was subjected to centrifugation at 1000rpm for 5 minutes at room temperature. The cell pellet was then resuspended in an appropriate volume of freezing medium (10% DMSO, 15% FBS in ESM) in 1.0ml aliquot per cryovial, prior to freezing at -80°C. Normally, at least 1×10^6 cells/mL of freezing medium is stored in one cryovial, because a significant number of cells will die while cryopreserved.

3.3 Embryoid bodies formation

The EBs formation was carried out by culturing mouse ES cells at a high cell seeding density (5.0×10^6 cells) in suspension culture (10ml medium) in non-tissue culture-treated 100mm bacteriological grade culture dishes (NEST). The cell density is very crucial in generating high-quality EBs. The cells were cultured in EB medium which contains 1X Glasgow Minimum Essential Medium (GMEM) (Gibco), 7.5% of sodium bicarbonate (Gibco), 0.1mM of 2-mercaptoethanol (Gibco), 1mM sodium pyruvate (Gibco), 2mM of L-Glutamine (Gibco) and 10% foetal bovine serum (FBS, Gibco). The diameter of the EBs formed were manually measured based on the scale bar representing 200µm for micrographs, that were captured during observation under microscope.

3.4 Polymerase Chain Reaction (PCR)

3.4.1 RNA extraction

RNA extraction from frozen cells was carried out using RNAeasy® Plus Mini Kit (Qiagen), a spin-column-based protocol, following the manufacturer's protocol. Generally, cryopreserved cells were thawed and washed trice with 1X PBS. Every washing step is followed by centrifugation at 10,000rpm for 5 minutes in 2.0ml microcentrifuge tube at room temperature to collect cells. The cell pellet could then be stored at -80°C or immediately processed for RNA extraction.

The pelleted cells would then be lysed in 350µL of guanidine-isothiocynate containing lysis buffer [1 ml RLT buffer (Qiagen) + 10 µl 2-mercaptoethanol (Gibco)]. The cell lysate was pipetted and vortex in order to completely lyse to homogenize the lysate. Such thorough cellular disruption is crucial for a good yield and quality RNA. An equal volume of 70% ethanol was added to the lysate to promote selective binding of total RNA to the silica-gel-membrane. The RNA concentrated suspension was added into RNAeasy mini spin column prior to spinning at 10,000 rpm for 15 seconds to allow the binding of RNA onto the silica membrane. Upon discarding the flow-through, the column was first washed with 700µl RW1 buffer (Qiagen), followed by 500ul RPE buffer (Qiagen) twice, prior to eluting the RNA in an appropriate volume (normally 30-40µl) of RNase-free water into spun-dried column membrane. The RNA sample could then be stored in -80°C freezer.

3.4.2 cDNA synthesis

The cDNA was synthesized by using QuantiTect® Reverse Transcription Kit (Qiagen), following the protocol described by the manufacturer with slight modifications, where appropriate. In general, 1µg of extracted RNA was mixed with

2µl of gDNA Wipeout Buffer and an appropriate volume of RNase-free water prior to incubation for 2 minutes at 42°C. The synthesis was then continued in a total volume of 20µl reverse-transcription master mix containing 5x RT Primer Mix, Reverse Transcriptase and RT Buffer. All components were carefully mixed by vortexing prior to quick spin before being loaded into a PCR machine (Bibby Scientific, UK) for 15 minutes of incubation at 42°C. The reverse transcriptase was deactivated by incubating the mixture at 95°C for 3 minutes. Finally, the newly synthesized cDNA was either kept on ice for immediate amplification of cDNA or stored at -20°C until use.

3.4.3 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out in a total volume of 12µl consisting of 1µl of synthesized cDNA, 1.5mM of $MgCl_2$, 0.2mM of dNTPs, 10pmol of both forward and reverse primers, Taq buffer, 1 unit of Taq polymerase enzyme (Promega, USA). -PCR reactions were performed in PeqLab PCR Thermocycler (VWR). Reaction mixtures without cDNA template (NTC) were included as a control to assess contamination and unspecific amplification. The list of primers used to detect the gene expression, the expected size, as well as the RT-PCR parameters for the amplification of each gene, are specifically stated in Appendix A.

3.5 Agarose electrophoresis

DNA and RNA were visualized on the agarose gel. The gel was prepared by heat-dissolving the agarose powder (Vivantis Inc, USA) in 1X TAE (Vivantis Inc, USA) buffer using a microwave oven. The agarose solution was left cooled and red gel stain (Vivantis Inc, USA) was added. The solution was poured onto a gel tray where a flat-tooth comb was used to create wells. The solution took about 20 minutes to solidify before the samples could be loaded into the wells. Samples were run in 1X TAE buffer

at appropriate constant voltage for an appropriate duration of time. The bands were then viewed under ultra-violet (UV) light.

3.6 Semi-quantitative analysis of Image J

ImageJ software was used to compare the band intensity on the agar gel. Semi-quantitative analysis was used in this study to measure the band intensity of conventional PCR amplicons. Based on the digital image of the gel, this software will generate a profile plot that matched the band intensity in the original image. The output will then be generated by picking the peaks in the profile plot. The relative intensity of the band was computed by comparing each peak of the treatment group to the peak of the standard. The ImageJ software was obtained from https://imagej.nih.gov/ij.

CHAPTER 4

RESULTS

4.1 Pluripotency analysis of the inducible transgenic Wnt5a ESC line

4.1.1 RNA expression of pluripotency-associated markers

Total RNA of the non-induced L7 clone (NI-L7) was extracted to analyse the expression of *Oct4*, *Sox2* and *Nanog*. The expected amplicon size for *Oct4, Sox2* and *Nanog* are 312bp, 96bp and 76bp respectively. RT-PCR analysis shows the expression of all the pluripotency-associated genes (Figure 4.1), suggesting that the random integration of the Wnt5a inducible construct into the cell genome does not impair the pluripotency status of the stem cell.

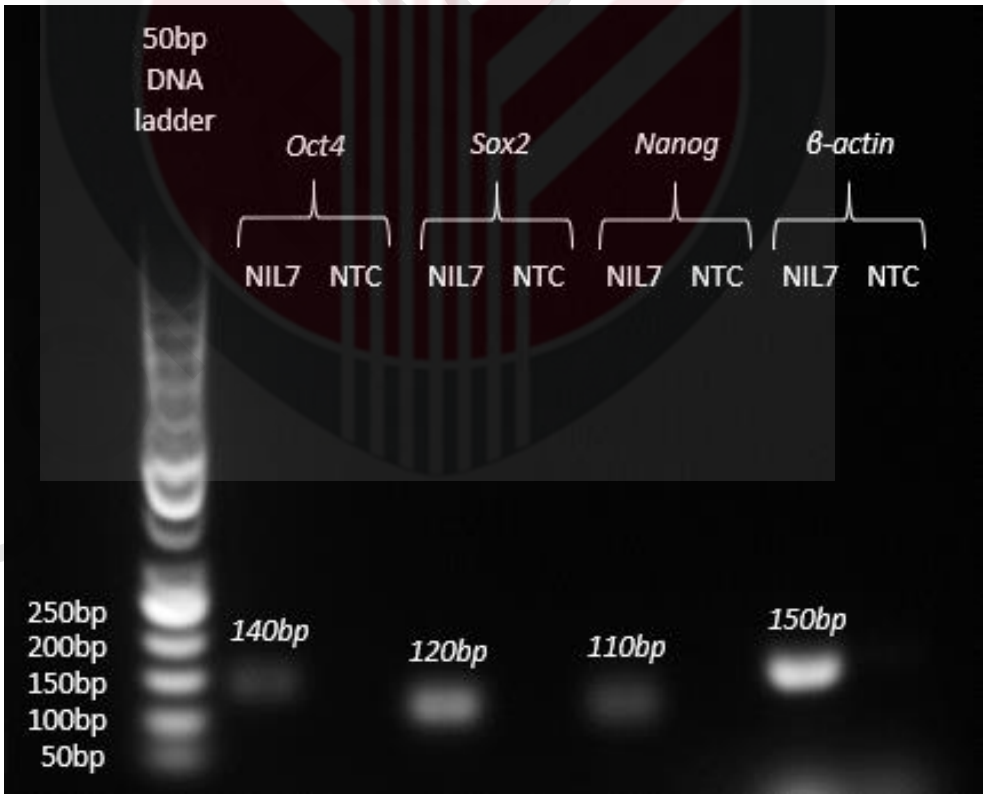

Figure 4.1: Expression of pluripotency-associated markers (*Oct4, Sox2* and *Nanog)* in non-induced NI-L7 cell line by RT-PCR. *β -actin was used as a reference gene control in all RT-PCR experiments.*

4.1.2 *In-vitro* pluripotency test through the formation of EBs

Based on Figure 4.2, the result shows that the non-induced NI-L7 transgenic cell line can undergo spontaneous differentiation and form EBs as soon as 1 day in suspension culture in the absence of LIF. The immature EBs formed on day 2 (Figure 4.2a) through to day 4 (Figure 4.2b) are of rough edges, with no cavitation process. Day 6 EBs showed smoother edges as compared to previous days, having clear boundaries, along with a cavitation process about to form in the middle of the EBs. The EBs formed in this experiment are of good quality because they are in round shape, exhibit smooth boundaries and have a diameter of 250-400µm. Besides that, EBs were observed to have a cavitation process after a few days of culture, showing that the EBs are undergoing a standard developmental process (Figure 4.2d).

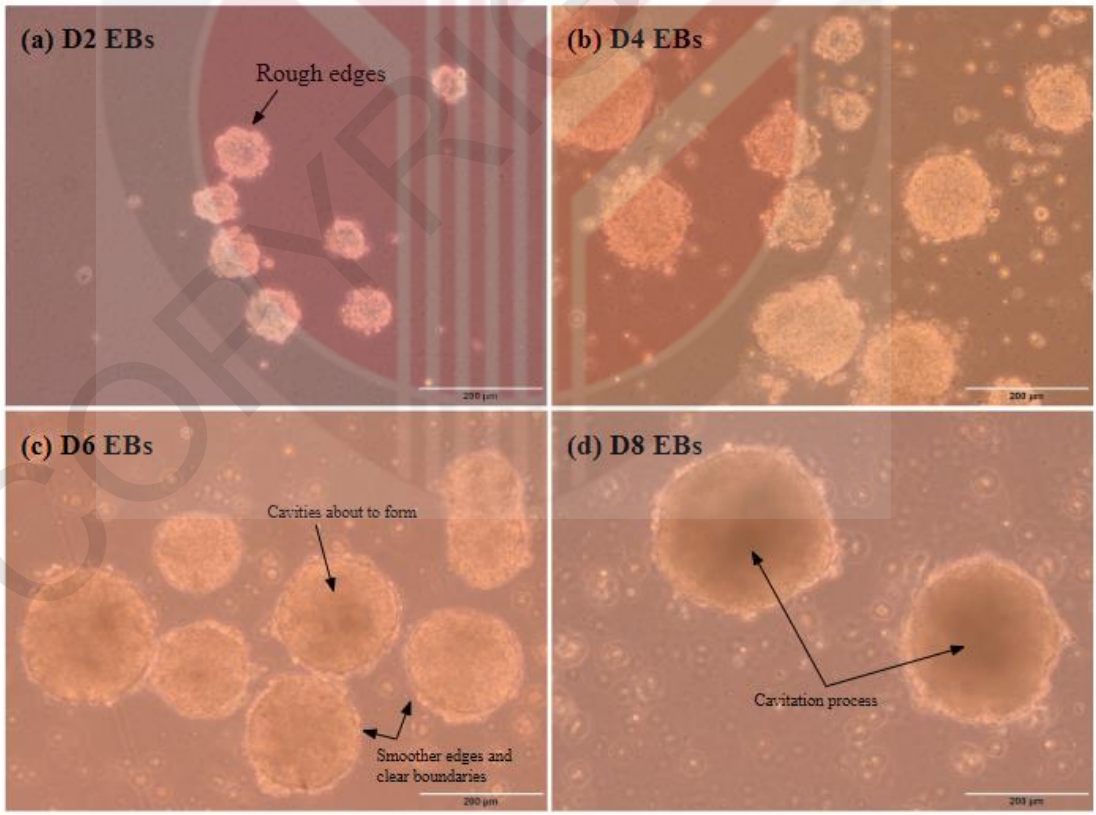

Figure 4.2: *In-vitro* pluripotency test of non-induced NI-L7 cell line through the formation of EBs. *Random micrographs taken on EBs cultured on (a) day 2, (b) day 4, (c) day 6 and (d) day 8. The bright field images of the EBs under the view of 10 x magnification.*

4.2 Assessment of cell morphological differences upon induction

Based on Figure 4.3, the pictures shown are the undifferentiated morphological appearance of the non-induced NI-L7 cell line and tamoxifen-induced CE-L7 cell line observed under 20x magnification upon 2 days of culture in ESM medium. The are no significant morphological differences between these two cell lines. Both cell lines shared similar doomed-like appearance of refractile colonies and have cells with high homogeneity in terms of size and shape. Besides, each individual cell in both cell lines has large nuclei and prominent multinucleoli as well as a high ratio of the nucleus to the cytoplasm.

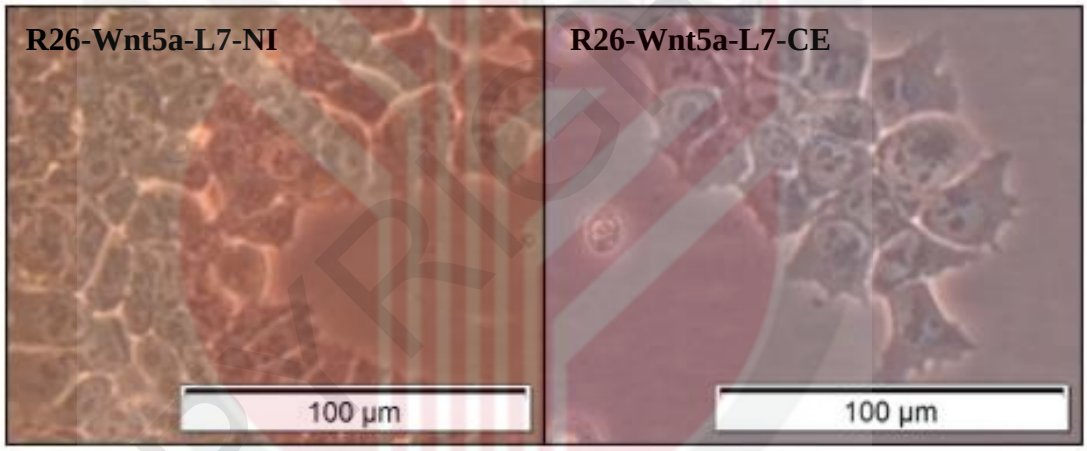

Figure 4.3: Undifferentiated non-induced NI-L7 and tamoxifen-induced CE-L7 transgenic Wnt5a ESC lines exhibit similar morphology cell with high nucleus-cytoplasmic ratio and multiple nucleoli. *The bright field images of the cells under the view of 20x magnification.*

4.3 Inducibility of Wnt5a transgene by RT-PCR

The inducibility of the Wnt5a transgene was examined at the RNA level through the Wnt5a expression using RT-PCR. Specific primer with an amplicon size of 230bp was used to amplify the Wnt5a transgene. Based on Figure 4.4, Wnt5a expression was present in the tamoxifen-induced CE-L7 cell line but was hardly seen in the non-induced NI-L7 cell line. Upon conducting semi-quantification of Wnt5a expression

using ImageJ software, the result in Figure 4.5 showed higher Wnt5a transgene expression in the tamoxifen-induced CE-L7 cell line as compared to the non-induced NI-L7 cell line (more than 7-fold increase).

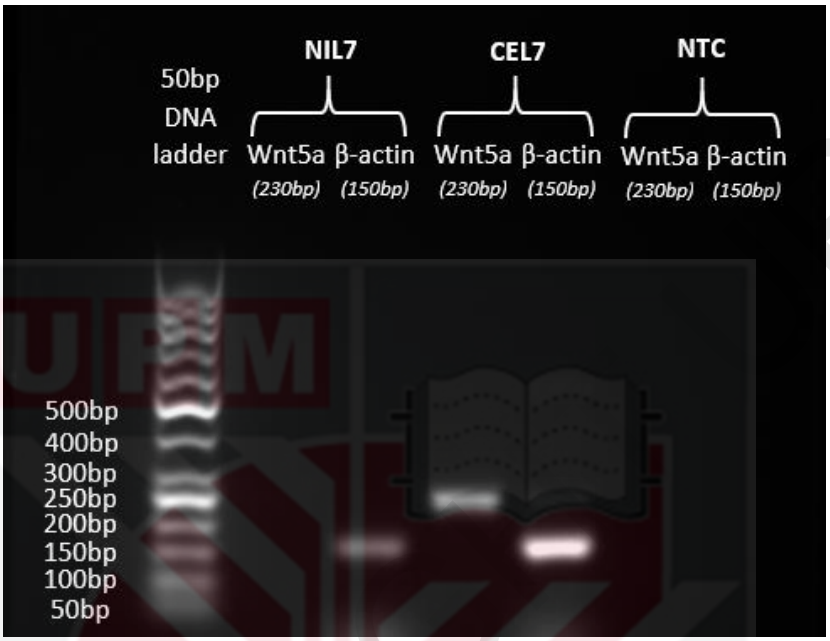

Figure 4.4: Expression of Wnt5a in non-induced NI-L7 and tamoxifen-induced CE-L7 transgenic Wnt5a ESC lines by RT-PCR demonstrating the high expression of Wnt5a in CE-L7. *β -actin was used as a reference gene control in all RT-PCR experiments.*

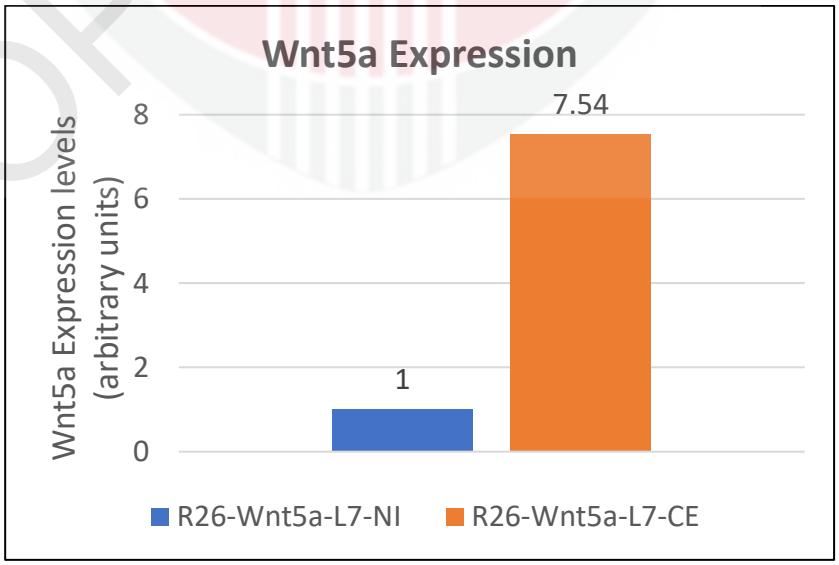

Figure 4.5: Bar graph of semi-quantitative analysis of Wnt5a expression level in non-induced NI-L7 and tamoxifen-induced CE-L7 transgenic Wnt5a ESC lines. *Semi-quantitative analysis was performed using ImageJ software.*

CHAPTER 5

DISCUSSION

5.1 Pluripotency characterization of the inducible transgenic Wnt5a ESC line

In the first phase of the experiment, pluripotency characterization of the inducible transgenic Wnt5a ESC line was carried out to ensure that the random integration of the Wnt5a construct does not compromise the pluripotency status of the established line. The fundamental pluripotency-associated markers; Octamer-binding protein, *Oct4*, SRY-box 2, *Sox2* and homeobox protein, *Nanog* have been discovered to involve in the self-renewability and maintenance of ESCs (Loh et al., 2006; Rodda et al., 2005) besides serving as the standard parameters for the assessment of the pluripotency status of ESCs and iPSCs (Filipczyk et al., 2015). These properties have led us to choose them for the pluripotency assessment of the non-induced NI-L7 cell line in this study. The obvious expression of the three fundamental pluripotency-associated markers, *Oct4*, *Sox2* and *Nanog* was observed in the NI-L7 cell line by RT-PCR suggesting that the pluripotency status of the transgenic Wnt5a ESC line is strongly maintained and is unaffected by the random integration of the Cre/*loxP*-Wnt5a inducible construct (Figure 4.1).

The mouse transcription factor, *Oct4* is encoded by the locus POU5F1 is highly expressed in pluripotent embryonic cells and germline cells during mouse embryonic development, and its expression rapidly declines upon differentiation (Shi & Jin, 2010). The absence of *Oct4* may lead to embryo death upon implantation due to the lack of pluripotent inner cell mass (ICM) cells has signified its importance in early embryonic development (Nichols et al., 1998, as cited in Shi & Jin, 2010). Therefore,

Oct4 is regarded as the master regulator for the generation and maintenance of pluripotent cells during embryogenesis. *Nanog*, a transcription factor from the homeobox DNA binding family and is crucial in germ cells formation. This protein regulates the differentiation of ICM into epiblast as well as maintains its pluripotency (Mitsui et al., 2003; Silva et al., 2009). Sex determining Y-box2 (*Sox2*) is a member of Sox family transcription factor, which is initially expressed at the morula stage and has higher expression at the later stages of ICM of blastocyst and epiblast. This suggests the crucial role of *Sox2* in the development and survival of early pluripotent embryonic cells (Avilion et al., 2003; Zhang & Cui, 2014). Together with *Oct4* and *Nanog*, these three core pluripotency transcription factors regulate and control the pluripotency of the stem cells (Adachi et al, 2010; Kashyap et al., 2009; Zhang and Cui, 2014).

Another important characteristic of the pluripotency potential of cells is their ability to spontaneously form 3D multicellular aggregates, termed the embryoid bodies (EBs) upon the withdrawal of leukaemia inhibitory factor (LIF). EBs mimic the in-vivo structure of developing embryos and serve as an essential initiation stage for lineage-specific differentiation into derivatives of the three germinal layers, besides providing a routine means for in-vitro pluripotency test (Pettinato et al., 2014; Brickman & Serup, 2017). As shown in Figure 4.2, the non-induced NI-L7 cell line began to form cell aggregates as early as day 2 upon the removal of LIF. Good quality of EBs were generated with the smooth boundaries, cavitation process in the middle of the EBs as well as acceptable diameter with a range between 250-400μm. All these findings clearly indicate that the pluripotency status of the transgenic line is retained upon the establishment of the line.

5.2 Effect of tamoxifen induction on the morphology of inducible transgenic Wnt5a ESC line.

The cellular morphological assessment was carried out via microscopic analysis on both non-induced NI-L7 and tamoxifen-induced CE-L7 cell lines to ensure that the tamoxifen induction does not affect the inducible transgenic Wnt5a ESC line in terms of the stem cell quality. As demonstrated in Figure 4.3, there are no significant morphological differences observed between the undifferentiated non-induced NI-L7 and tamoxifen-induced CE-L7 cell lines. Both cell lines are able to retain the morphology of pluripotent ESCs in the presence of LIF and on gelatin-coated dishes shown by the appearance of doomed-like and refractile colonies. High-quality stem cells were demonstrated by NI-L7 and CE-L7 lines, where the cells in both lines exhibit high nucleus-to-cytoplasm ratio, large nucleus with multiple nucleoli as well as homogenous in size and shape. The multinucleated characteristics indicate the good quality of a stem cell should have, in order for them to maintain the progenitor pools in the tissue with a high proliferation rate (Zhang et al., 2014). Moreover, this characteristic also explains the stem cells' ability to self-renew as the nucleoli are highly involved in the synthesis of ribosomal RNA (rRNA) and this component plays a crucial role in the mechanism of protein synthesis (Kalinina et al., 2018). Two crucial factors should be taken into consideration in order to obtain high-quality pluripotent stem cells that is able to be robustly propagated over an extended period: (1) the cell density prior to plating after each split; and (2) the frequency of splitting cells (Mulas et al., 2019). Another factor that may restrict the ESCs from differentiating is the addition of exogenous LIF in our culture, which is important for self-renewability of the cells, signifies the stimulation of Janus kinase-signal transducer and activator of transcription pathway (JAK-STAT) mediated by LIF (Graf et al, 2011; Ohtsuka et al.,

2015; Onishi and Zandstra, 2015; Smith et al., 1988). These observations indicate that tamoxifen induction has no effect on the stem cell quality of the inducible transgenic Wnt5a ESC line.

5.3 Inducibility of Wnt5a transgene upon tamoxifen induction

The last characterization step of the inducible transgenic Wnt5a ESC line is the examination of the inducibility of Wnt5a transgene upon tamoxifen induction. Through RT-PCR analysis, observable Wnt5a gene expression was only observed in tamoxifen induced CE-L7 line, but not the non-induced NI-L7 line (Figure 4.4). This may be due to the very minimal expression that is hardly detectable through naked eye. Semi-quantitative was then carried out the semi-quantify the gene expression based on the band intensity using ImageJ software. Our result demonstrated up to 7-fold higher Wnt5a expression in the tamoxifen-induced CE-L7 cell line compared to the non-induced NI-L7 cell line (Figure 4.5). The result clearly suggests that Wnt5a transgene has been induced upon tamoxifen induction which leads to the complete activation of the regulatable Cre/*loxP-Wnt5a* construct upon tamoxifen induction. Therefore, Cre/*loxP-Wnt5a* construct is confirmed to be stable and inducible. Remarkably, our result obtained is also in agreement with a previous study conducted by Nordin et al. (2008), where they showed very minute Wnt5a expression of the ESCs on Day 0 during the neural differentiation. This hence further confirms that the high expression of Wnt5a in the tamoxifen-induced CE-L7 cell line was solely due to tamoxifen induction and not endogenous factors.

CHAPTER 6

CONCLUSION, LIMITATIONS AND FUTURE RECOMMENDATIONS

Conclusion

Previous research conducted by our group has successfully established an inducible transgenic ESC line that carries a regulatable Wnt5a construct based on Cre/*loxP* binary system. The transgenic line allows us to study the effect of Wnt5a overexpression at specific time points during the neural differentiation process. However, the stability of the line before and after the induction of the Wnt5a transgene has not been properly evaluated. This study has assessed the stability of the Cre/*loxP* inducible transgenic Wnt5a ESC clone. The non-induced NI-L7 cell line was observed to maintain its pluripotency status through the expression of pluripotency-associated genes, which are *Oct4, Sox2* and *Nanog* by RT-PCR. Besides, the cell line has also passed the *in-vitro* pluripotency test with its ability to form good quality EBs. No significant cellular morphological difference between the non- and tamoxifen-induced L7 clone further confirms that the quality of stem cells is maintained. The high level of Wnt5a transgene expression in the tamoxifen-induced CE-L7 cell line strongly confirms the inducibility of the Cre/*loxP*-*Wnt5a* construct.

Taken all these findings together, we conclude that the genetic modification has not compromised the pluripotency status of the Cre/*loxP* inducible transgenic Wnt5a ESC clone at cellular and molecular levels, along with stable inducible transgenic Wnt5a clone that is ready for downstream application. The Wnt5a transgenic ESCs could be fully utilised to unravel the role of Wnt5a during the differentiation process of ESCs

into the neural lineage, particularly as a candidate source for neurotransplantation for PD therapy.

Limitation of the study

This study has demonstrated the characterization of Cre/*loxP* inducible transgenic Wnt5a mouse ESC line. Nonetheless, time constraints have limited the number of independent experiments in assessing the inducibility of the Wnt5a transgene at RNA level. Additional independent replicates would result in more precise, reliable and reproducible results (Blainey et al., 2014). Another limitation of this study is the lack of proper approaches to quantitatively measure and analyse Wnt5a overexpression on both NI-L7 and CE-L7 cell line by RT-qPCR.

Future Recommendations

Research conducted has shown the stability of the Cre/*loxP* inducible transgenic Wnt5a ESC clone, thus the future direction of this study should be studying the effect of inducing Wnt5a at specific time points during the dopaminergic differentiation process. Then, the next study should be focusing on the functional assessment of the DA neurons generated at different stages of Wnt5a induction in terms of the release and reuptake of dopamine *in-vitro* by using the patch-clamp technique. This technique will allow us to determine and analyse the ionic current and membrane potential. The advancement stage of this study can then be focused on the *in-vivo* analysis in terms of the ability of the transplanted mouse ESC-derived DA neurons in innervating and integrating with a complex system, for example parkinsonian mice model.

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APPENDICES

Appendix A: Primers for Reverse-Transcription Polymerase Chain Reaction

Name	Sequence (5'-3')	Size (bp)	Parameters
ß-Actin	*ß-Actin* Forward: CCTGTCAGCAATGCCTGGGT *ß-Actin* Reverse: CCAAGCCCTTCCTTCCTTGGGTA	151	95°C – 3 mins 95°C – 15 secs 60°C – 15 secs 72°C – 20 sec (35 cycles) 4°C – 5 mins
Wnt5a	*Wnt5a* Forward: TCCTATGAGAGCGCACGCAT *Wnt5a* Reverse: CAGCTTGCCCCGGCTGTTGA	230	95°C – 3 mins 95°C – 30 secs 57°C – 30 secs 72°C – 40 sec (35 cycles) 4°C – 5 mins
Oct4	*Oct4* Forward: GGCGTTCTCTTTGGAAAGGTGTTC *Oct4* Reverse: CTCGAACCACATCCTTCTCT	312	95°C – 3 mins 95°C – 30 secs 60°C – 30 secs 72°C – 50 sec (35 cycles) 4°C – 5 mins
Sox2	*Sox2* Forward: CGCCCAGTAGACTGCACA *Sox2* Reverse: CCCTCACATGTGCGACAG	95	95°C – 3 mins 95°C – 30 secs 60°C – 30 secs 72°C – 50 sec (35 cycles) 4°C – 5 mins
Nanog	*Nanog* Forward: GCTTTCATGTCCTGGGACTC *Nanog* Reverse: TGCTTACAAGGGTCTGCTACTG	76	95°C – 3 mins 95°C – 30 secs 60°C – 30 secs 72°C – 50 sec (35 cycles) 4°C – 5 mins