UNIVERSITI PUTRA MALAYSIA

THE EFFECT OF ETHANOLIC EXTRACT OF CENTELLA ASIATICA ON TRANSDIFFERENTIATION OF RAT FULL-TERM AMNIOTIC FLUID STEM CELLS INTO NEURAL STEM CELLS

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A PROJECT PAPER SUBMITTED AS PARTIAL REQUIREMENT FOR THE DEGREE OF BACHELOR SCIENCES (BIOMEDICAL SCIENCE)

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ABSTRACT

THE EFFECT OF ETHANOLIC EXTRACT OF *CENTELLA ASIATICA* ON TRANSDIFFERENTIATION OF RAT FULL-TERM AMNIOTIC FLUID STEM CELLS INTO NEURAL STEM CELLS

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Background: The incidence of neurodegenerative diseases (NDs), featured with progressive neuronal cell death, is alarming. Unfortunately, there is no definitive treatment for NDs. One potential treatment is neuro-transplantation where neural stem cells (NSCs) have been marked as the prospective source for it. However, obtaining brain-sourced NSCs is challenging. Alternatively, obtaining NSCs from non-brain sources would significantly overcome the challenge. The highly potent stem cells derived from amniotic fluid (AFSCs) can be transdifferentiated into NSCs, but the process needs a specific inducer to generate high-quality NSCs. *Centella asiatica* (CA) consumed traditionally as a memory tonic could be the potential candidate inducer for transdifferentiation of AFSCs into NSCs. **Objective:** This study aims to investigate the effect of ethanolic extract of CA (EECA) treatment in promoting transdifferentiation of rat full-term amniotic fluid stem cell line (R3) into NSCs based on the number of good qualities neurospheres and molecular assessment of specific marker expression on the transdifferentiated NSCs. **Methodology:** R3 was cultured in ESM before being transdifferentiated into NSCs with or without EECA treatment for 48 hours via monolayer adherent culture technique. Four groups were included in this study: 1) untreated R3 (negative control), 2) R3 with 50µM dBcAMP (positive control), 3) 1µg/mL of EECA and 4) 10µg/mL of EECA. The differentiated NSCs from all groups were subjected to form neurospheres for three days and evaluated for their size and number. RNA was extracted from NSCs to assess the expression of the NSC-specific marker *(Sox1*) using RT-qPCR. The housekeeping gene (*GAPDH*) was used to measure the relative expression of the marker. **Results & Discussion:** EECA treatment enhanced the transdifferentiation of R3 into NSCs. EECA treatment groups had greater expression of *Sox1* and a higher number of good-quality neurospheres (of diameter 100 - 150µm) than the untreated and 50µM dBcAMP treatment group. **Conclusion:** Findings of this study suggest the potential of EECA as an inducer for producing quality NSCs from amniotic fluid stem cells, mammalian non-brain cells, as a prospective source for neuro-transplantation.

Keywords: stem cell, amniotic fluid stem cell, rat full-term amniotic fluid, neural stem cell, neurosphere, *Centella asiatica*.

ABSTRAK

KESAN EKSTRAK ETANOLIK *CENTELLA ASIATICA* TERHADAP PENTRANSBEZAAN SEL STEM CECAIR AMNIOTIK JANKA PENUH TIKUS KE SEL STEM NEURAL

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Pengenalan: Insidens penyakit neurodegeneratif (NDs) yang disebabkan oleh kematian sel neuron secara progresif, adalah membimbangkan. Malangnya, tiada rawatan muktamad untuk ND. Satu rawatan yang berpotensi ialah neurotransplantasi dengan menggunakan sel stem neural (NSC) sebagai sumber prospektif sel. Walau bagaimanapun, pemerolehan NSC dari sumber otak adalah mencabar. Sebagai alternatif, NSC boleh diperolehi daripada sumber bukan otak bagi mengatasi cabaran ini. Sel stem yang sangat mujarab yang diperoleh daripada cecair amniotik (AFSC) boleh ditransbezakan kepada NSC, tetapi prosesnya memerlukan pencetus khusus untuk menghasilkan NSC berkualiti tinggi. *Centella asiatica* (CA) yang digunakan secara tradisional sebagai tonik memori boleh menjadi calon penggalak yang berpotensi untuk proses pentransbezaan AFSC ke NSC. **Objektif:** Kajian ini bertujuan untuk menyiasat kesan ekstrak etanolik CA (EECA) dalam menggalakkan pentransbezaan sel stem cecair amniotik jangka penuh tikus (R3) ke NSC berdasarkan bilangan neurosfera berkualiti baik dan penilaian molekul penanda khusus. **Metodologi:** R3 telah dikultur dalam ESM sebelum ditransbezakan kepada NSC dengan atau tanpa rawatan EECA selama 48 jam melalui teknik kultur ekalapisan (*monolayer*) Kajian ini telah dibahagikan kepada empat kumpulan telah dimasukkan dalam kajian ini: 1) R3 yang tidak dirawat (kawalan negatif), 2) R3 dengan 50µM dBcAMP (kawalan positif), 3) 1µg/mL EECA dan 4) 10µg/mL EECA. NSC yang telah melalui process pentransbezaan daripada semua kumpulan telah dikultur untuk pembentukan neurosfera selama tiga hari dan dinilai untuk saiz dan bilangannya. RNA diekstrak daripada NSC untuk menilai ekspresi penanda khusus NSC (*Sox1*) menggunakan RT-qPCR. Gen jaga selia (*housekeeping*) (GAPDH) digunakan untuk mengukur ekspresi relatif penanda. **Keputusan & Perbincangan:** Rawatan EECA meningkatkan pentransbezaan R3 kepada NSC. Kumpulan rawatan EECA mempunyai lebih banyak ekspresi *Sox1* dan bilangan neurosfera berkualiti yang lebih tinggi (diameter 100 - 150µm) daripada kumpulan yang dirawat 50µM dBcAMP dan kumpulan yang tidak dirawat. **Kesimpulan:** Dapatan kajian ini mencadangkan EECA sebagai penggalak berpotensi untuk menghasilkan NSC berkualiti daripada sel stem cecair amniotik, sejenis sel dari sumber bukan otak mamalia, sebagai sumber prospektif untuk neurotransplantasi.

Kata kunci: sel stem, sel stem bendalir amniotik, bendalir amniotik jangka penuh tikus, sel stem neural, neurosfera, *Centella asiatica*.

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ABBREVIATIONS

AD	Alzheimer's disease
AF	Amniotic fluid
AFSC	Amniotic fluid stem cell
ANOVA	Analysis of variance
Aβ	Beta-amyloid
bFGF	Basic fibroblast factor
β-ME	β-mercaptoethanol
CA	*Centella asiatica*
CNS	Central nervous system
Ct	Cycle threshold
dBcAMP	Dibutyryl cAMP
DMSO	Dimethyl sulfoxide
EECA	Ethanolic extract of *Centella asiatica*
EGF	Epidermal growth factor
ESC	Embryonic stem cell
ESM	Embryonic stem cell medium
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GMEM	Glasgow Minimum Essential Medium
HD	Huntington's disease
hMSC	Human mesenchymal stem cell

IL	Interleukin
iNSC	Induced neural stem cell
iPSC	Induced pluripotent stem cell
LIF	Leukemia inhibitory factor
ND	Neurodegenerative disease
NSA	Neurosphere Assay
NSC	Neural stem cell
NTC	Non-template control
PCR	Polymerase chain reaction
PD	Parkinson's disease
p	Significant difference
R3	Rat amniotic fluid stem cell
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
SGZ	Subgranular zone
SHH	Sonic Hedgehog
V-SVZ	Ventricular-subventricular zone

CHAPTER 1
INTRODUCTION

1.1 Background study

Neurodegenerative disease (ND) is a type of disease in which cells of the central nervous system (CNS) stop working or die. Due to progressive neuronal death, NDs usually get worse over time and there is so far no cure (National Cancer Institute, 2018). Statistics show that 1 in 10 people aged over 60 suffers from dementia in Malaysia (National Health and Morbidity Survey, 2018). The number of patients with Parkinson's disease (PD) is expected to increase fivefold from the current estimated 20,000 to 120,000 by 2040 (Hassandarvish, 2019) and the prevalence of dementia that is estimated at 0.126% in 2020 will increase to 0.454% in 2050 (Mat Nuri et al., 2017). Even with today's medical technology, the treatment of these neurodegenerative diseases is mostly symptomatic, which means they help to alleviate symptoms of the disease.

Fortunately, researchers found hope in a potential approach that may be a promising therapy, that is neurotransplantation. Via cell substitution, neurotransplantation could rescue neurons from degeneration, maintain cerebellar reserve, facilitate cerebellar compensation and help reconstruct damaged neural circuits (Cendelin & Mitoma, 2018). In other words, this approach aims to replace the dead cell with a stem cell that is able to self-renew, integrate, differentiate and replenish the nerve cell. To be more specific, these stem cells are neural stem cells (NSCs). NSC is a type of stem cell that possess self-renewal, self-replication and multi-differentiation properties. Under certain conditions,

NSCs may be induced to differentiate into neurons, astrocytes and oligodendrocytes (Hu et al., 2018).

Researchers started to look for the source of NSCs for neurotransplantation. NSCs are found in primary CNS tissues (Pereira et al., 2019). NSCs from the spinal cord were investigated by Abdi and colleagues (2018) but the generation of neurons was not observed in vivo, so it could not be used for neuro-transplantation. Next, NSCs can also be found in the fetal or adult brain. To be specific, it is located in the subgranular of the hippocampus and subventricular zonethe of lateral ventricle in the brain (Obnernier & Alvarez-Buylla, 2019). However, this leads to the greatest challenge of isolating NSCs from the brain, which is inaccessibility. Therefore, it is important to find other sources of NSCs,

Later then, researchers investigated that NSCs can also be obtained through transdifferentiation from somatic cells and multipotent stem cells. Transdifferentiation is defined as the direct reprogramming or conversion of one mature somatic cell type into another cell type without undergoing an intermediate pluripotent state (Pereira et al., 2019). The advantages of converted cells include having a low risk of tumorigenesis, maintaining the capacity of self-renewal and differentiation into particular cell lineages (Barzilay et al., 2009), can be directly converted into other cell types for therapy usage (Nizzardo et al.,2013) and most importantly, these cells are easily accessible (Mollinari et al., 2018).

With the realization of inducing easily accessible cells to inaccessible cells that are lost in degenerative diseases, researchers started to look into the potential in different types of cells that can undergo transdifferentiation. Among the somatic cells or multipotent stem

cells investigated, one interesting type of stem cell caught attention of the reseachers, that is the amniotic fluid stem cells (Baghaban Eslaminejad & Jahangir, 2012a; Bossolasco et al., 2006), which can be collected from the merely discarded amniotic fluid.

Studies showed that AFSCs harbour the potential to differentiate into cell types of the three germ layers and they express pluripotency markers such as Oct-4, SOX2, Nanog and SSEA. They are broadly multipotent and they do not induce tumor formation after transplantation (Rosner et al., 2012; Hamid et al., 2017). These stem cells can be obtained from amniotic fluid, a yellowish fluid that surrounds the fetus contained inside the amnion sac of all mammals throughout the gestation period. Amniotic fluid stem cells can be isolated from the amniotic fluid extracted at mid-term gestation, also known as the second trimester. However, mid-term AF are collected through amniocentesis, which is considered invasive and possesses certain level of risk to harm the fetus. Thus, scientists have later discovered that AFSCs can also be isolated from amniotic fluid obtained from full-term gestation, which is during delivery (Hamid et al., 2017).

According to Mun-Fun and colleagues (2015), amniotic fluid-stem cells can be transdifferentiation into NSCs. However, the efficiency is still low, we believe there is still room for improvement in terms of its efficiency. An enhancer that is neuroprotective, or able to promote nerves and brain regeneration may promote better transdifferentiation procedure. One potential enhancer for transdifferentiation into NSCs would be *Centella asiatica* (CA), also known as "Pegaga" locally. It is a clonal, perennial herbaceous creeper that grows in moist places. It has a wide range of therapeutic value and is actively being used in traditional medicine. In Ayurveda, CA is one of the main herbs for revitalizing the nerves and brain cells (Gohil et al., 2010). Studies have shown that the therapeutic value

of ethanolic extract of CA (EECA) is from the known neuroprotective triterpene that it contains, which are asiatic acid, madecassoside, asiaticoside and madicassic acid (Wong et al., 2019). EECA is able to protect neuron cells against oxidative stress and promote nerve or brain tissue regeneration (Lokanathan et al., 2016). Therefore, EECA is no doubt a potential enhancer in promoting transdifferentiation into neural stem cells.

In conclusion, it is crucial to find an approach in obtaining enough amount of NSCs for neuro-transplantation, a cure for neurodegenerative diseases. Studies revealed that full-term amniotic fluid stem cell is a potential non-brain source of NSCs by undergoing transdifferentiation but an enhancer is needed to improve efficiency of the process. With that, this study is focused on using ethanolic extract of CA (EECA) to enhance the transdifferentiation of rat full-term amniotic fluid stem cell (R3) into NSCs by assessing the number and size of neurospheres formed, as well as the expression level of NSCs specific marker.

1.2 Problem statement

Regenerative medicine can be a solution to the increasing incident of neurodegenerative diseases, especially neuro-transplantation that rescues neurons from degeneration, maintain cerebellar reserve, facilitate cerebellar compensation and help reconstruct damaged neural circuits via cell substitution. However, the isolation of NSCs from brain sources is limited as they are inaccessible in the primary CNS tissue. Thus,

non-brain sources of NSCs in need to meet the demand of NSCs required for neuro-transplantation. In addition, an efficient and effective transdifferentiation procedure requires an appropriate enhancer. *Centella asiatica* that was actively being used in traditional medicine as brain tonic is a possible candidate of the enhancer. Therefore, this study would provide better insights of amniotic fluid stem cells as a potential non-brain source of NSCs and the effect of natural product, specifically ethanolic extract of Centella asiatica as an enhancer of transdifferentiation.

1.3 Hypothesis

EECA treatment enhances the transdifferentiation of R3 into NSCs based on increased number of neurosphere formed and expression of NSC specific markers.

1.4 Objectives

General Objective: To investigate the effect of *Centella asiatica* treatment in promoting transdifferentiation of amniotic fluid stem cells into neural stem cells.

Specific Objectives:

1. To propagate good quality of rat full term amniotic fluid stem cells (R3).
2. To promote transdifferentiation of rat full term amniotic fluid stem cells (R3) into neural stem cells (NSCs) with treatment using ethanolic extract of *Centella asiatica* (EECA).
3. To promote the formation of neurospheres.
4. To molecularly assess specific genetic markers expressed by NSCs using RT-qPCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Neurodegenerative diseases

2.1.1 Definition and characteristic

A neurodegenerative disease is one in which central nervous system cells stop working or die. It usually worsens with time as a result of gradual neuronal loss (National Cancer Institute, 2022). Examples of neurodegenerative diseases include Parkinson's disease (PD), Huntington's disease (HD), Alzheimer's disease (AD) and more. They are distinguished by progressive structural and functional loss of neurons, leading to different clinical symptoms based on their pathological mechanistic pathways (Arbo et al., 2022; K. Poddar et al., 2021).

Neurodegenerative diseases can be categorized based on the anatomical positions in relation to the disease (Kovacs, 2016). For instance, Parkinson's disease is characterized by neuronal degeneration of dopaminergic neurons in the substantia nigra, which results in motor symptoms such as tremor, muscle rigidity, coordination problems, and loss of physical mobility (Kalia & Lang, 2015). In addition, neurodegenerative diseases can be classified according to the proteins that undergo conformational and biochemical modifications (Kovacs, 2016). The discovery of different biochemical stages of Aβ aggregate maturation and the possibility of different variants of Aβ peptides resulting from alternative processing or mutations that cause rare forms of familial AD, has implications for

the understanding of early and late phases of AD, as well as the characterization and interpretation of AD-type pathology (Thal et al., 2015). According to Rijal et al. (2014), there are two biochemical-Aβ stages (1 and 2) in pathologically diagnosed preclinical AD (p pre-AD). Aggregation of $A\beta_{1-40/42}$ alone was detected in biochemical-Aβ stage 1 while additional detection of $A\beta_{N3pE}$ was detected in biochemical- Aβ stage 2. In later phase of AD (symptomatic), aggregation of $A\beta_{1-40/42}$, $A\beta_{N3pE-40/42}$, and phosphorylated $A\beta_{40/42}$ were detected. Their major findings also showed further investigation of AD pathology that phosphorylation of Aβ may promote additional accumulation of Aβ oligomers, protofibrils, and fibrils as pathologically preclinical Alzheimer's disease progress into Alzheimer's disease.

Extending from the previous classification, neurodegenerative diseases can be also grouped according to cellular and subcellular pathology. This refers to whether pathological protein deposits are found in neurons or glial cells (either or both astro- and oligodendroglia), including which compartment of the cells, or are found extracellularly (Kovacs, 2016). For example, the hallmark of HD is the presence of aggregate from huntingtin with an expanded polyQ Track in the cytosolic and nuclear space of neuronal cells (Ruz et al., 2020). As a result, anatomical, cellular and protein vulnerability can be defined in conjunction with a neurodegenerative disorder.

2.1.2 Treatment and possible cure for neurodegenerative diseases

The treatment for neurodegenerative diseases is mostly symptomatic. Some examples among FDA-approved drug regimen include Donepezil and Rivastigmine like acetylcholine that are prescribed to minimize short-term AD progression, as well as a combination of levodopa and carbidopa that showea d therapeutic effect in PD patients by increasinthe g level of dopamine for the first few years after consecutive consumption (K. Poddar et al., 2021).

The future direction for neurodegenerative disease treatment should focus on the complete cure of these diseases, such as the possibility of neurogenesis. In contrast to typical pharmacological therapies, which are designed to treat symptoms and slow the progression of neurodegenerative diseases, neurotransplantation is based on the goal of curing, and restoring nerve tissue function, or regeneration. Neurotransplantation could save neurons from degeneration and retain cerebellar reserve by using cell substitution, as well as help in cerebellar compensation and the repair of damaged neural circuits (Cendelin & Mitoma, 2018).

2.2 Stem Cell

2.2.1 General characteristics and types of stem cells

Stem cells serve as a source of cells in maintaining, replacing and regenerating tissues in humans. In order for a cell to be identified as a stem cell, it must exhibit two essential abilities (Figure 2.1). Firstly, stem cells must be able to self-renew. They divide into daughter cells that are exactly the same as the originating cells, perpetuating the reservoir of stem cells throughout life. Next, stem cells must be able to differentiate into more specialized cells along the developmental stages of an organism (Alison et al., 2002; Biehl & Russell, 2009; Zakrzewski et al., 2019).

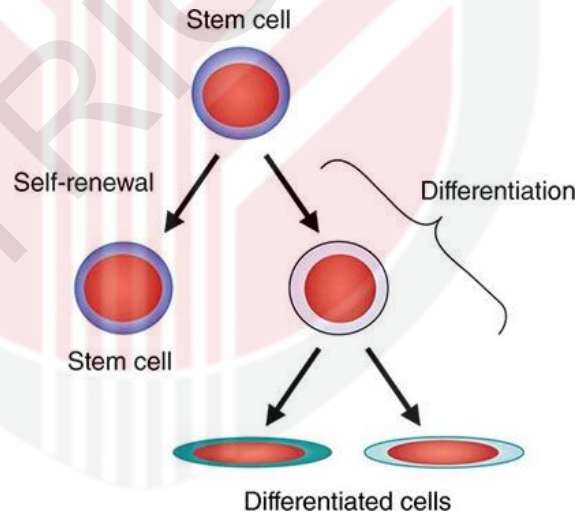


Figure 2.1. Characteristics of stem cells.
Adapted from Themes, U. (2016, November 30). *Applications of Stem Cell Biology in Clinical Medicine*. Basicmedical Key. Retrieved from: https://basicmedicalkey.com/applications-of-stem-cell-biology-in-clinical-medicine/

Based on their differentiation potential, stem cells are classified into 4 main groups: totipotent, pluripotent, multipotent and unipotent (Figure 2.2). The differentiation potency decreases accordingly along embryonic development as the stem cells undergo epithelial-mesenchymal transition, global DNA methylation, shortening of telomere, X-chromosome inactivation and transient developmental genes expression (Takahashi & Yamanaka, 2015). The differentiation potential of stem cells will be further explained in the next section.

2.2.2 Origin and differentiation potential

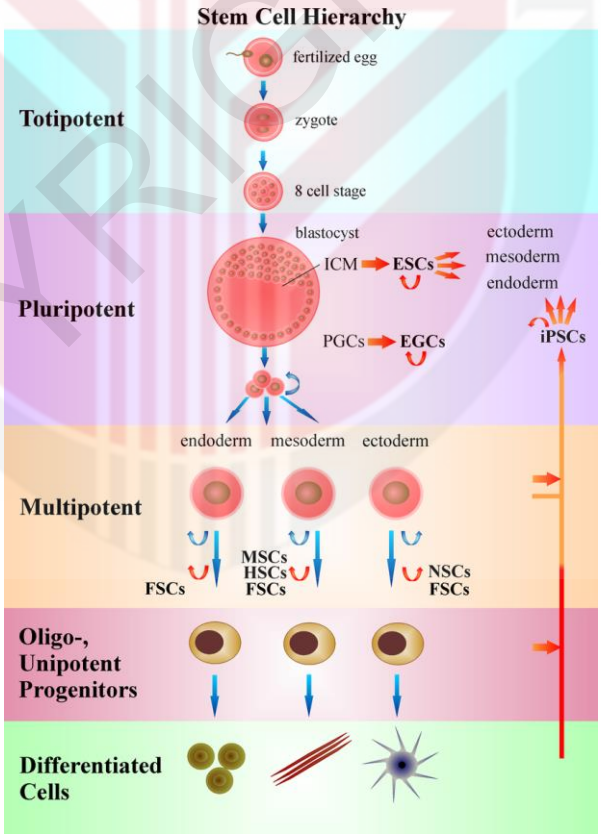


Figure 2.2. Stem cell hierarchy.
Adapted from Forostyak, Oksana & Dayanithi, Govindan & Forostyak, Serhiy. (2016). CNS Regenerative Medicine and Stem Cells. Retrieved from: https://doi.org/10.20388/omp2016.001.0023.

As the importance of regenerative medicine becomes increasingly clear in curing many intractable diseases, the study of stem cells rises in number to learn the therapeutic potential of different stem cells. Researchers obtain their stem cells mainly from these three basic sources: embryonic tissue, fetal and perinatal tissue, as well as adult tissue (Bacakova et al., 2018; Torre & Flores, 2020).

As soon as the oocyte is fertilized, a zygote is formed. Zygote and the descendants of the first two divisions have the potential to differentiate into cells of the whole organism, including embryo and extra-embryonic cells. In other words, zygotes and cells at the morula stage are totipotent embryonic stem cells that can further divide into any of the three germ layers or become cells of the placenta (Alison et al., 2002; Can, 2008). After approximately 3 to 5 days, a blastocyst that is composed of the inner cell mass and the trophectoderm is formed (Zakrzewski et al., 2019). Inner cell mass is a cluster of pluripotent stem cells that will further develop into the embryo through differentiation into cells from the three germ layers (Dulak et al., 2015). In short, totipotent stem cells are the morula while pluripotent embryonic stem cells are derived from the blastocyst.

Although pluripotent stem cells obtained from fetal testis, ovary and lungs were reported (Hua et al., 2009; Kerr et al., 2008a, 2008b), fetal stem cells are usually identified as multipotent stem cells that are able to differentiate into cells of endoderm, mesoderm and ectoderm but not extraembryonic structures (O'Donoghue & Fisk, 2004; Zakrzewski et al., 2019). Under the controversy regarding fetal stem cells, several studies shed light on stem cells obtained from the perinatal

tissues which immediately changed their fate from medical waste to source of precious stem cells (Bailo et al., 2004; Stefańska et al., 2020).

Perinatal stem cells can be obtained from all the extraembryonic tissues such as amniotic membrane and fluid, umbilical cord blood, Wharton's jelly, decidua, chorionic membrane, villi as well as chorionic plate (Torre & Flores, 2020). Studies reported stem cells from amniotic fluid (Roubelakis et al., 2012), amnion, chorion (Chen et al., 2019), and Wharton's jelly (Musiał-Wysocka et al., 2019) with the expression of pluripotency markers such as Oct4, Nestin, Nanog and SSEA4. In comparison, umbilical cord blood contains rather multipotent stem cells (Jaing, 2014), which have lower differentiation potential compared to the first two groups of stem cells (Rajabzadeh et al., 2019) and can only differentiate into cells of specific lineages (Zakrzewski et al., 2019).

Due to minimum ethical issues and the emergence of stem cell reprogramming techniques, the advantages of using adult stem cells are clear for the purpose of cell therapy and tissue engineering. Generally, adult tissues such as skeletal muscle, adipose tissue and skin contain multipotent stem cells for the replacement of dead cells. When these multipotent stem cells commit to differentiation, they become unipotent progenitor cells (Bacakova et al., 2018; Biehl & Russell, 2009). Unipotent stem cells have not gained much attention due to the smallest differentiation capacity, that is only able to differentiate into one cell lineage (Rajabzadeh et al., 2019; Zakrzewski et al., 2019).

2.3 Amniotic Fluid Stem Cells (AFSCs)

2.3.1 Origin and characteristics

When amniotic fluid-derived cells were exposed to supernatant from rhabdomyosarcoma cell lines while in culture, they were found to express the skeletal muscle protein dystrophin. In other words, it was the first indication that stem cells could exist in amniotic fluid (B Streubel et al., 1996). Amniotic fluid (AF) is a clear, yellowish fluid that surrounds the fetus to provide mechanical support and required nutrients during embryogenesis. Other than water as its major component, AF also contains electrolytes, lipids, proteins and morphologically heterogeneous cells that will increase in number with gestational age (Loukogeorgakis & de Coppi, 2017; Roubelakis et al., 2012; Srivastava et al., 2018).

Over ten years, researchers uncovered the characteristics of amniotic fluid stem cells (AFSCs) in terms of molecular characterization, differentiation potential, proliferation ability and genetic stability. Pluripotency-associated markers such Oct-4, Nanog, Sox2, stem cell factor (SCF) and SSEA were observed (Hamid et al., 2017). Study results including high neurogenic capability in human full-term AFSCs (Gao et al., 2016) and differentiation of full-term rat AFSCs into derivatives of the three primary germ layers (Mun-Fun et al., 2015) further supported the potential of AFSCs being broadly multipotent. In addition, AFSCs have been shown to be able to proliferate rapidly without the use of feeders, doubling in 36 hours and not being tumorigenic. Long telomeres and a normal

karyotype were also found in lines that had been cultured for more than 250 population doublings (de Coppi et al., 2007).

2.3.2 Isolation procedure

Most studies have focused on mid-term and full-term AFSCs. Mid-term AFSCs are collected through amniocentesis during the second trimester of gestation. Between weeks 18-24, a fine needle is inserted into the uterus through the mother's abdomen under ultrasound guidance to collect 2ml to 5ml of AF for prenatal test (Hamid et al., 2017; Robinson, 2021). Considering the risks and complications such as cramping, bleeding, infection, injury to the fetus or mother, preterm labor and even miscarriage from this invasive procedure (Johns Hopkins Medicine, 2021; Robinson, 2021), scientists figured out to collect AFSCs from full-term pregnancies. Successful isolation of AFSCs from full-term AF were reported in human, bovine, canine and rat through either one-stage, two-stage culture methods and C-Kit sorting (Hamid et al., 2017).

2.3.4 Application of AFSCs

As scientists studied deeper on different types of stem cells, AFSCs gained more traction for their therapeutic potential in the field of regenerative medicine mainly due to their differentiation capabilities, in vitro culture characteristics and autologous treatment potential, as well as the lack of tumorigenicity and ethical

concerns (Loukogeorgakis & de Coppi, 2017). Studies have shown the promising capability of AFSCs in the regeneration of tissues and organs in the cardiovascular, urinary, gastrointestinal, hematopoietic, musculo-skeletal, respiratory and nervous system (Baghaban Eslaminejad & Jahangir, 2012b; Loukogeorgakis & de Coppi, 2017). With that, AFSCs became a rising star in the field of regenerative medicine as scientists clearly see their capabilities in restoring the function to lost or defective tissue and organs.

From a cancer perspective, Kang et al (2012a) reported decreased cancer cells viability when cocultured with human AFSCs that release cytotoxic factors such as necrosis factor-a, interferon, transforming growth factor-β or ILs. Li et al. (2015) further enhanced the potential of AFSCs as an anti-cancer drug carrier when they found out the high motility of human AFSCs to migrate to ovarian cancer site in nude mice model. Collectively, AFSCs are also believed to have great potential in cell-based therapies for cancer treatment.

2.4 Neural Stem Cells (NSCs)

2.4.1 Source of origin

In the 1960s, a wonderful finding of new neurons generated from hippocampal dentate gyrus of an adult rat brain subverted the assumption that neurons were only generated during embryogenesis (Altman, 1962; Altman & Das, 1965). NSCs can actually be isolated from primary central nervous system (CNS) tissues such as spinal cord tissue, and adult and fetal brain. To be more precise,

brain source NSCs can be found in the periventricular region of the spinal cord, as well as the ventricular-subventricular zone (V-SVZ) on the walls of the lateral ventricles and the subgranular zone (SGZ) of the hippocampus in the brain (Obernier & Alvarez-Buylla, 2019; Pereira et al., 2019).

Considering the difficulties to access NSCs for clinical application, researchers found other ways to generate NSCs through *in vitro* procedures. By understanding the characteristics of pluripotent stem cells being able to differentiate into all cell lineages of the three germ layers (Dulak et al., 2015), scientists are able to differentiate NSCs from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Many techniques were established to generate NSCs from pluripotent stem cells, including cell suspension culturing and monolayer culturing under specific conditions that favors the differentiation of pluripotent stem cells into NSCs (Pereira et al., 2019). Nevertheless, potential risks and ethical issues regarding pluripotent stem cells induced NSCs should never be neglected. With that, transdifferentiation of NSCs from somatic cells or multipotent stem cells brought a glimmer of hope. This will be elaborated in the later section.

2.4.2 Characteristics of NSCs

Neural stem cells (NSCs) are a type of stem cell that has the ability to self-renew, proliferate and differentiate into more specialized neural lineages including

neurons, astrocytes and oligodendrocytes (Hu et al., 2018). They serve as a reservoir to replenish neurons and glial cells throughout a person's life.

During neurogenesis, which is the growth and development of nervous tissue, a series of cell lineages arise according to different stages of the process. They express different intracellular molecules that are responsible for specific purpose. In NSCs, expression of proliferating cell nuclear antigen (PCNA), antigen Ki67, phosphohistone (PH3), paired box 6 (PAX6), neuroectodermal stem cell marker (Nestin), sex determining region Y-box 1 and 2 (SOX1 and SOX2), and Mushashi 1 (MSI1) are closely related to the characteristics of NSCs being actively proliferating, able to self-renew and differentiate into more specialized neural cells (Ludwig et al., 2018; Oikari et al., 2016; Zhang & Jiao, 2015).

The most commonly used markers for NSCs include Nestin and sex determining region Y-box 2 (*SOX2*). Nestin is a class VI intermediate filament protein that is essential for proper survival and self-renewal of NSCs (Park et al., 2010). As NSCs develop into neurons or glial cells, Nestin expression is downregulated (Namiki et al., 2012). *SOX2* is important to preserve NSCs' characteristics in terms of proliferation, self-renewal and differentiation. SOX2 is known as the master regulator of NSCs because it has several responsive genes such as Nestin, PAX6, Brn1, Brn2, *Sonic hedgehog (Shh)* and more to form self-renewal and regulatory mechanisms to maintain the undifferentiated state of NSCs (Kretsovali, 2017; Shimozaki, 2014). With the expression of *SOX2* remains until NSCs commit into neural differentiation, *SOX2* is believed to also play an essential role in neural commitment (Kretsovali, 2017).

In short, the markers that NSCs exhibit alter as they develop into more specialized cells during neurogenesis. Understanding the markers of neural cell lineages is therefore crucial for effective cell characterization.

2.4.3 Isolation of NSCs and generation of neurosphere

In order to isolate NSCs from the brain of mice or rats, sections containing the subventricular zone and hippocampus are collected. Then, NSCs can be expanded in two ways, which are 3D neurosphere assay and 2D monolayer culture (Merck, 2022).

In 2D monolayer culture, coated culture flasks are used for the adhesion and growth of NSCs (Merck, 2022). On the other hand, ultra-low attachment flasks are used to culture NSCs in 3D neurosphere assay. This is to prevent formed neurospheres from adhering to the bottom of culture flasks (Zhou et al., 2020). Neurosphere Assay (NSA) was firstly described by Reynolds and Weiss (1992) to study NSC biology and dynamics in controlled conditions. As respond to epidermal growth factor and basic fibroblast growth factor, NSCs will undergo a period of active proliferation, whereas differentiated cells do not. NSCs form in spherical aggregates termed neurospheres in these settings, which can be passed to extend the pool of these cells (Soares et al., 2020).

2.4.4 Application and limitation of NSCs

Clinical application of NSCs greatly focuses on neurotransplantation. By cell substitution, neurotransplantation could save neurons from degeneration and preserve cerebellar reserve, as well as assist cerebellar compensation and aid in the reconstruction of injured neural circuits (Cendelin & Mitoma, 2018).

However, one great challenge to neurotransplantation is the source of NSCs. There are many active clinical trials of neurotransplantation using bone marrow-derived stem cells (Pendleton et al., 2011). The adult human spinal cord does include cells with *in vitro* stem cell potential. Nonetheless, determining the identity of such cells in humans is difficult due to a lack of research tools, and these cells can scarcely be propagated into more stages *in vitro*. (Liu & Chen, 2019; Sabelström et al., 2014). In short, NSCs source related research should be promoted to enable the acquisition of a sufficient number of NSCs for neurotransplantation.

2.5 Transdifferentiation

2.5.1 Definition and background

The term transdifferentiation was first applied by Selman and Kafatos when they observe the change of the cuticle-producing cells in silkmoth to "transdifferentiate" into salt-secreting cells during metamorphosis to the adult moth (Selman & Kafatos, 1974). It is also known as direct cell reprogramming of

somatic or multipotent cell type directly into another cell type, without undergoing through a pluripotent state (Grath & Dai, 2019; Pereira et al., 2019).

Before discovering the direct conversion of somatic cells into another type of cells, the discovery of reprogramming adult cell types into induced pluripotent stem cells (iPSCs), which are genetically and functionally equivalent to embryonic stem cells (ESCs) was a groundbreaking event in the cell culture world. With iPSCs, any type of cells from the three germ layers can be produced and unlimited source of patient-specific cell types for cell-based therapies can be ensured (Kim et al., 2014; Nizzardo et al., 2013). However, the process of reprogramming somatic cells into iPSCs is time-consuming and epigenetic alterations may occur during the reprogramming process. Furthermore, any residual of iPSC is tumorigenic after transplantation which limits the use of reprogrammed cells through pluripotent state (Kim et al., 2014; Ruggieri et al., 2014).

Therefore, transdifferentiation is clinically significant in cell-based therapies not only for its ability to directly convert somatic or multipotent cell types into another cell type, also because of the low risk of tumorigenesis, the ability to maintain the capacity of self-renewal and differentiation in cells, as well as the easily accessible sources of cells for this procedure (Mollinari et al., 2018; Nizzardo et al., 2013).

2.5.2 Protocol and condition needed for transdifferentiation to NSCs

Reprogramming of somatic cells into homogenous population of iNSCs could be achieved by culturing somatic cells in designated medium with reprogramming factors such as c-*Myc, Klf4, Oct4, Sox2, FoxG1, Brn2* and *E47* in different combinations or through retroviral infection (Ruggieri et al., 2014). Transdifferentiation employing tissue-specific transcription factors and/or chemicals has been shown in recent study to reprogram somatic cells into induced NSCs (iNSCs) or neural progenitor cells. Most TF-induced iNSCs are reprogrammed by targeting the master regulator, *Sox2*, or a single zinc-finger, Zfp521, both of which are expressed in proliferative neural progenitors and are essential regulators of neurogenesis *in vivo*. (Xiao et al., 2018).

Over the years, researchers accomplished improvement in methods of direct reprogramming to iNSCs, but the progress to effective transdifferentiation for cell-based therapy in the future is still a long way to go. Other than somatic cells, researchers are trying to uncover the potential of multipotent or broadly multipotent stem cells and natural product as inducers to improve the efficiency of transdifferentiation to NSCs.

Centella asiatica (CA) is suggested to be used as an alternative nerve stimulant for nerve regeneration as it is shown to be effective to induce the neural differentiation of human mesenchymal stem cell (hMSCs) by stimulating distinct expression of neural protein markers on the differentiated cells (Omar et al., 2019).

2.6 Centella asiatica (CA)

2.6.1 Definition and properties

Centella asiatica (CA) is a clonal, perennial herbaceous creeper that grows to a height of 5 to 15 cm and sprouts between August and September. CA has flowers that are purple-to-pink or white in colour (Figure 2.3), and kidney-shaped or fan-shaped green leaves that have a tobacco aroma. CA belongs to the Umbelliferae/Apiaceae family and grows in damp, swampy areas in most tropical and subtropical countries. CA has various names in different countries. For example, it is commonly known as *pegaga* in Malaysia, Indian Pennywort in the United States of America, *yuhong-yuhong* in the Philippines, *tapak kuda* in Indonesia, *buak bok* in Thailand and other names such as *Gotu kola*, Asiatic pennywort or Spadeleaf (Banerjee et al., 2021; Gohil et al., 2010; Omar et al., 2019; Prakash et al., 2017).

Figure 2.3. Centella asiatica (CA) with white flowers.
Adapted from Sana, M. V. (2017, May 27). *10 Propiedades de la Centella Asiática que te gustarán*. Más Vida Sana. Retrieved from: https://masvidasana.com/propiedades-de-la-centella-asiatica

2.6.2 Phytochemical constituents

The chemical constituents of *Centella asiatica* (CA) was intriguing for its usage as treatment of wide variety of diseases. Using high-performance liquid chromatography, a significant amount of madecassoside, asiaticoside, madecassic acid, and asiatic acid were identified and it is believed that they are major constituents responsible for pharmacological value apart from being rich in flavonoids and terpenoids (Prakash et al., 2017). To be more precise, quantification of CA extract resulted in the high level of pentacyclic triterpenoids, which includes 0.1161mg/g of saponins madecassoside and 0.1411mg/g of asiaticoside. Their aglycones (sapogenins) were also quantified, which includes 0.1437mg/g of madecassic acid and 0.0729mg/g of asiatic acid (Wong et al., 2019).

2.6.3 Therapeutic potential

Since long ago, people around the world had discovered the medicinal use of *Centella asiatica* (CA) in various acute or chronic diseases. The therapeutic potential of CA is clear as researchers successfully found its application in many traditional medicines.

In Indian traditional medicine, CA is known as nerve tonic because it is believed to have the effect of memory enhancement and brain cells stimulation (Alfarra & Omar, 2013). In China, CA was documented as one of the "miracle elixirs of life" for over 2000 years. The oldest records of CA in China may be dated back

to the Song Dynasty's "su wen shi" and "zheng lei ben cao" where CA is described as "bitter, chilly, and nontoxic. Ideal for fevers and skin disorders"(Sun et al., 2020). Besides, CA is also traditionally applied as treatment to skin diseases, rheumatism, syphilis, mental illness, epilepsy in Southeast Asia. However, CA (also known as *Gotu kola*) should not be mixed up with kola nuts that contain caffeine and used as stimulant (Prakash et al., 2017).

Many researches revealed the pharmacological activities of *Centella asiatica* (CA). As reviewed by Prakash et al. (2017), therapeutic benefits of CA include hepatoprotective, anti-cancer, anti-bacterial, anti-fungal, anti-inflammatory, anti-oxidant, wound healing, antidepressant, anti-diabetic, neuroprotective and improve cognitive function. Most investigations focused on the neuropharmacological benefit of CA, which encompasses enzyme inhibition, prevention of amyloid plaque formation in Alzheimer's disease, dopamine neurotoxicity in Parkinson's disease, as well as lowering oxidative stress and inflammatory factors (Orhan, 2012; Sun et al., 2020).

In addition, preclinical and clinical evidence widely supports the cognitive-enhancing and neuroprotective effects of CA (Gohil et al., 2010; Speers et al., 2021). Together, it is clear that *Centella asiatica* (CA) is a natural plant with great potential in treating wide varieties of diseases, including its use in cell-based therapy of regenerative medicine (Lokanathan et al., 2016).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Study Design

This study is divided into four parts according to respective specific objective as shown in Figure 3.1.

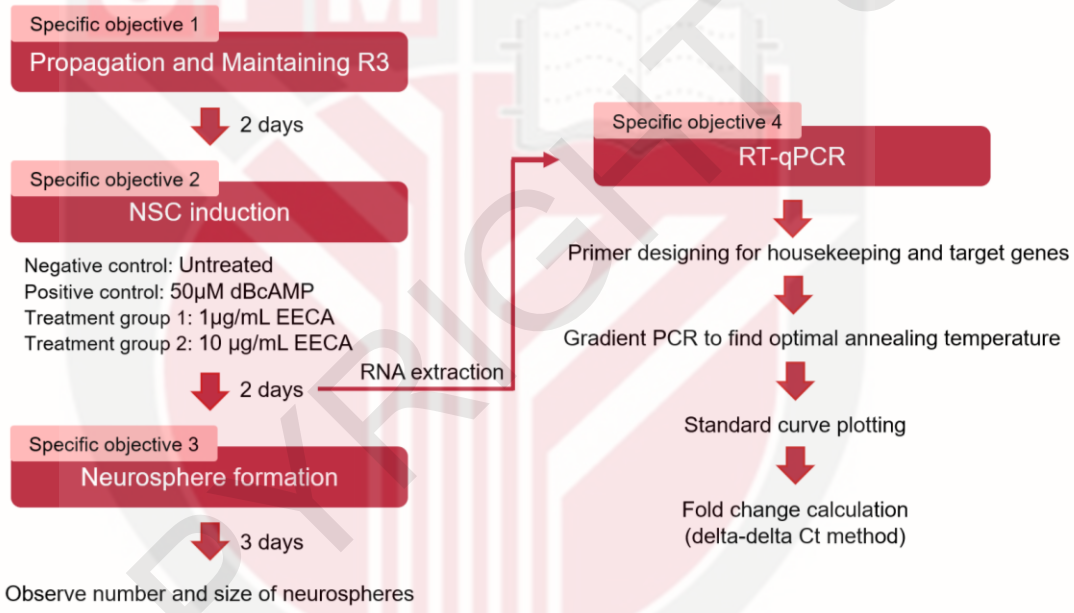


Figure 3.1. Flow diagram showing the general idea of methodology.

3.2 Ethanolic extract of *Centella asiatica* (EECA)

The powdered form of EECA was obtained from Prof Dr. Mohd Ilham Adenan (UiTM). 1mL of sterile water was added to 10mg of the EECA powder for the preparation of stock EECA solution. Then, the concentration of 1.0 µg/mL and 10.0 µg/mL EECA

was prepared by applying dilution formula $M_1V_1 = M_2V_2$, where M_1= initial molarity, V_1= initial volume in liters, M_2= final molarity and V_2= final volume in liters.

3.3 Full-term rat amniotic fluid stem cells (R3)

R3 used in this experiment were established and characterized in-house from amniotic fluid of full-term pregnancy rats by Dr Norshariza Nordin and colleagues from Medical Genetics Unit (Mun-Fun et al., 2015). Passage 36, 38 and 40 of R3 were used throughout the experiment.

3.3.1 Cell culture work

All cell culture work was carried out in class II Bio-safety cabinet (BioAir, EuroClone, Italy). Culture wastes were soaked and washed with 2-4% Decon® 90 after use.

Embryonic stem cell medium (ESM) working solution was prepared by adding 15% fetal bovine serum (FBS) (Gibco, USA) and 20ng/mL leukemia inhibitory factor (LIF) (Gibco, USA) to ESM stock solution, which consist of 1x Glasgow Minimum Essential Medium (GMEM), 7.5% Sodium bicarbonate, 1mM sodium pyruvate, 2mM L-Glutamate, 0.1mM 2-mercaptoethanol (all from Gibco, USA).

3.3.2 Thawing the cells

The cryopreserved R3 from -80°C freezer was quickly thawed in a 37°C water bath by gently swirling the vial for 30 seconds. Thawed cells in the vial were transferred to a 15mL Falcon tube with 9mL pre-warmed ESM working solution. Then, the mixture was centrifuged at 1000 rpm for 5 minutes. The supernatant containing ESM working solution and dimethyl sulfoxide (DMSO) was discarded. The cell pellet was resuspended with appropriate amount of ESM working solution before transferring to 0.1% gelatin (Sigma-Aldrich, USA) pre-coated T25 flask (Thermo Scientific ™ Nunc ™, USA).

3.3.3 Splitting the cells

Once the R3 cells reached 70-90% confluency, the medium was discarded and the cells were washed twice with 1X PBS. The cells were then treated for 2-3 minutes at room temperature in 1-2 mL of 1X trypsin-EDTA (Gibco, USA) until the cells were detached from the flask. After deactivating the trypsin with 10-20mL of 10% Fetal Bovine Serum (Gibco, USA) in 1X PBS, the cells were collected by spinning down the suspension at 1000 rpm for 5 minutes. The supernatant was removed and an appropriate volume of medium was added to resuspend the cell pellet before proceeding to cell counting using haemocytometer under an inverted microscope.

The cells were then seeded into T25 flasks at the seeding density of 3.0×10^4 cells per cm^2 and incubated in 37 °C, 5% CO_2 incubator (AutoFlow IR Water-jacketed CO2 Incubator, USA) until confluent.

3.4 NSC induction via monolayer differentiation

After R3 reached 70-90% confluency, monolayer differentiation method was carried out to promote transdifferentiation into NSCs. According to the experimental groups, undifferentiated R3 was resuspended in respective medium as shown in Table 3.1. Then, R3 was cultured in T25 flask with cell density of density of 3.0×10^4 cells per cm^2 and incubated at 37 °C, 5% CO_2 for two days. All cells were cultured in the NSC medium, which composed of all components of the ESM except LIF. After 3 days, the morphology of the cells was observed.

There are four experimental groups in this study based on the treatment given to the R3. For negative control group, the cells were not given any treatment while for the positive control group; the cells were treated with 50 µM of Dibutyryl cAMP (dBcAMP). As for the treatment groups, the cells were treated with 1.0 µg/mL and 10.0 µg/mL of EECA respectively. The cytotoxicity test of EECA on R3 was carried out previously by the group of Dr. Norshariza Nordin.

Table 3.1 Experimental groups with their respective treatment

Experimental Group	Treatment
Negative control	Resuspended in NSC medium only
Positive control	Resuspended in NSC medium with 50µM of dBcAMP
Treatment 1	Resuspended in NSC medium with 1.0 µg/mL EECA
Treatment 2	Resuspended in NSC medium with 10.0 µg/mL EECA

3.5 Neurosphere Formation

Trypsinization of R3-derived NSCs and R3 in all four experimental groups were carried out. Then, the cells were centrifuged and resuspended with 1mL of neurobasal/B27 medium, supplemented with 20ng/mL of epidermal growth factor (EGF) and basic fibroblast factor (bFGF). Cell counting using haemocytometer was performed before replating the cells at cell density of 3.0×10^4 cells per cm^2 into 100mm uncoated bacteriological grade dish. Neurobasal/B27 medium were topped up to a total of 10mL in the dish and the cells were incubated in 37 °C, 5% CO_2 incubator for 3 days.

The number of neurospheres was observed and recorded using an inverted microscope (Olympus, Japan). Microscopic images of all experimental groups were captured at 4x and 10x magnification in order to proceed with the measurement of the diameter of neurospheres using ImageJ software. Good quality (diameter of 50-100 µm) neurospheres were counted. The data collected was analyzed using SPSS software (One-way ANOVA, Tukey's and Dunnett test).

3.6 RNA extraction

Total RNA from R3-derived NSCs and R3 in all experimental groups was isolated using RNeasy® Mini Kit (Qiagen, Germany) and the manufacturer's protocol under RNAse free conditions. As instructed in the manufacturer's protocol, 10 µL β-mercaptoethanol (β-ME) was added to 1mL Buffer RLT to irreversibly denature RNases by reducing disulfide bonds and destroying the native conformation required for enzyme functionality. Also, 4 volumes of ethanol (96%-100%) was added to Buffer RPE for a working solution.

An approximate of 1.0×10^6 of cells were prepared for RNA extraction. The cells were lysed with 500 µL of Buffer RLT mixed with β-ME, followed by 1 volume of 70% ethanol added to the lysate and mixed well by pipetting. Up to 700 µL of sample, including any precipitate was transferred to a RNeasy Mini spin column placed in a 2mL collection tube. The samples were centrifuged for 15s at ≥8000 x g and the flow-through was discarded. The samples were then added with 700 µL of Buffer RW1, centrifuged for 15s at ≥8000 x g and flow-through was discarded. Next, 500 µL of Buffer RPE was added to the samples. The samples were then centrifuged at 15s at ≥8000 x g and the flow-through was discarded. The same step was repeated with centrifugation condition of 2 minutes at ≥8000 x g. A new 1.5mL collection tube was placed. 30-50 µL of RNase-free water was added directly to the spin column membrane carefully at the center and the samples were centrifuged for 1 minute at ≥8000 x g to elute the RNA. The RNA was collected and stored at -80°C until further analysis.

3.6.1 Assessment of the concentration and integrity of total RNA

The absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) of RNA can be measured using PCRmax Lamda nanodrop (Bibby Scientific, UK). A_{260} quantifies RNA concentration while A_{260} /A_{280} ratio evaluates RNA quality. The RNA's integrity was confirmed using 1% agarose gel electrophoresis. The ratio of 28S and 18S ribosomal (rRNA) bands with 2:1 was regarded to have high integrity (**Appendix A**).

3.7 Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was carried out in two-step method where reverse transcription and quantitative polymerase chain reaction were done separately.

3.7.1 Complimentary DNA (cDNA) synthesis

Total RNA was thawed on ice while components of QuantiTect® Reverse Transcription Kit (Qiagen, Germany) including gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water were thawed at room temperature. A mixture of total RNA (up to 1 µg), 2 µL of gDNA Wipeout Buffer and RNase-free water were prepared, incubated for 2 minutes at 42°C and placed immediately on ice. Reverse-transcription master mix were prepared on ice as instructed in the manufacturer's protocol then added with total RNA mixture as prepared earlier. Samples were

incubated for 15 minutes at 42°C then 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase. All incubation steps were carried out using PeqSTAR 2X Double block thermal cycler (Peqlab, Germany). cDNA was kept at -20°C for long-term storage.

3.7.2 Primer design

Primer pairs of housekeeping (*GAPDH*) and target genes (NSC specific marker, *Sox1*) were designed using NCBI primer designing tool and blast with NCBI primer Blast tool software. All primer sequences were designed to have the length of 18-22 bases, GC content of 40-60%, melting temperature between 52°C to 58°C and at least 4 Guanine or Cytosine nucleotide at the 3' end to promote specific binding. Then, designed primers were produced by the company, Integrated DNA Technologies (IDT). The details of primers for housekeeping and target genes were stated in **Appendix B**.

3.7.3 Optimization of annealing temperature

Once the primers were received, optimization of annealing temperature was carried out by running gradient polymerase chain reaction (PCR) using Polymerase Chain Reaction Kit (Promega, USA). PeqSTAR 2X Double block thermal cycler (Peqlab, Germany) was used to run gradient PCR as it allows the

setting of multitemperaturesture in one run of PCR. The range of annealing temperature for each primer set was stated in **Appendix C (Table C.1)**.

In order to identify the appropriate annealing temperature for each primer pair, gel electrophoresis was performed for 1 hour, at 80V. 1% agarose gel was prepared by heating the mixture of agarose powder (Vivantis) and TAE buffer until it turned transparent. With the lights off, ViSafe Red Gel Stain (Vivantis) was added to the agarose solution right after the heating. After the agarose gel solidified, a mixture of 3 µL of gradient PCR product, 2 µL of 6X loading dye (Vivantis) and 7 µL of TAE buffer was loaded into each well. The optimal annealing temperature was identified with the thickest band in gel electrophoresis images as shown in **Appendix C (Figure C.1)**

3.7.4 Quantitative Polymerase Chain Reaction (qPCR)

After the optimized annealing temperature for both target (*Sox1*) and housekeeping genes (*GAPDH*) was obtained, qPCR standard curve and melting curve for both primer pairs were established (**Appendix D**). qPCR was performed using QuantiTect® SYBR® Green PCR Kit (Qiagen, Germany) in LightCycler® 480 Real-Time PCR system (Roche Life Science). By referring to qPCR standard curve, PCR efficiency that falls between 90 to 110%, with R^2 value ≥ 0.985 were considered optimum condition for the primer. The specificity of the PCR reactions was verified by analysis of melting curves. Reaction mixtures without cDNA

template (NTC) were included as a control to assess contamination or unspecific amplification.

The amplification program started with 15 minutes of PCR initial heat activation at 95°C, followed by 40 cycles of 15 seconds denaturation stage at 95°C, 15 seconds of annealing stage at optimized annealing temperature of primers as shown in **Appendix C (Table C.1)** and 15 seconds of extension stage at 72°C where data acquisition was carried out. Lastly, 30 seconds of cooling stage at 40°C was programmed after 40 cycles of amplification. After Cycle threshold (Ct) values were obtained, fold change calculation using delta-delta Ct method was performed and results from the treatment groups were compared to the negative control (untreated) group.

3.8 Statistical Analysis

The data collected were analyzed with IBM SPSS Statistics (27v.). One-way ANOVA was used to compare the means of two or more independent groups while Tukey's and Dunnett's test were post hoc test used to confirm the experimental group that has significant difference of means. Results with p=value less than 0.05 ($p < 0.05$) was considered as significant.

CHAPTER 4

RESULTS

4.1 Propagation of R3

Morphology of R3 was observed after 2 days of culturing in ESM medium. The propagated R3 had the morphological appearance of a fibroblast-like cell. In the nucleus of each cell, there were two to four nucleoli. Together, it showed that R3 propagated in good condition.

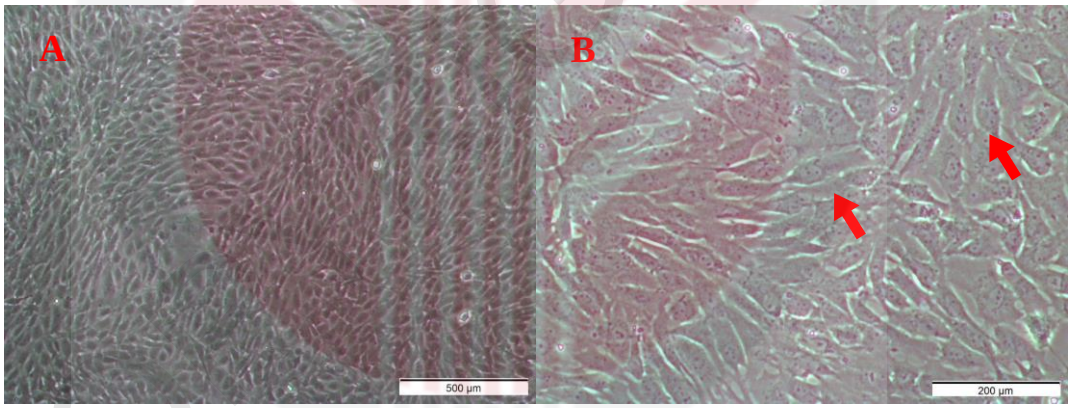


Figure 4.1: Morphology of R3. *The bright field images of R3 under the view of 4x magnification (**A**) and 10 x magnification (**B**).* Arrows indicates the presence of multiple nucleoli in the nucleus of R3.

4.2 Presence of neural rosette after monolayer differentiation

R3 were cultured in NSC medium for 2 days and observed. The morphology of the cells was similar compared to R3 before monolayer differentiation. The presence of neural rosette in all experimental groups was observed and confirmed by comparing to published paper (Ma et al., 2008) as shown in Figure 4.2. Further investigation was carried out to confirm the identity of cells as NSCs.

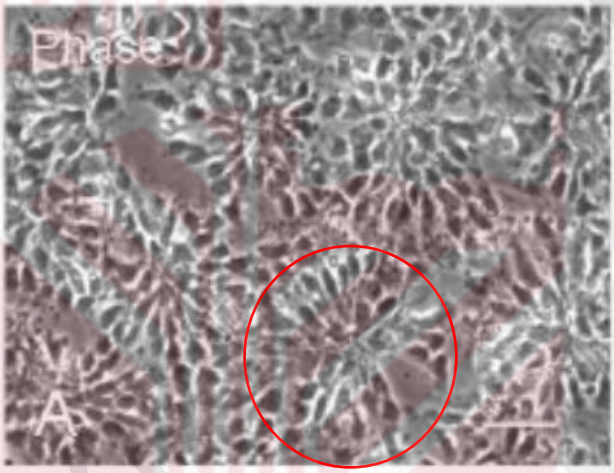


Figure 4.2: Morphology of neural rosette. The bright field images of neural rosette with scale 50µm. Adapted from: Ma, W., Tavakoli, T., Derby, E., Serebryakova, Y., Rao, M. S., & Mattson, M. P. (2008). Cell-Extracellular Matrix Interactions Regulate Neural Differentiation of Human Embryonic Stem Cells. *BMC Developmental Biology*, *8*(1), 90. https://doi.org/10.1186/1471-213x-8-90

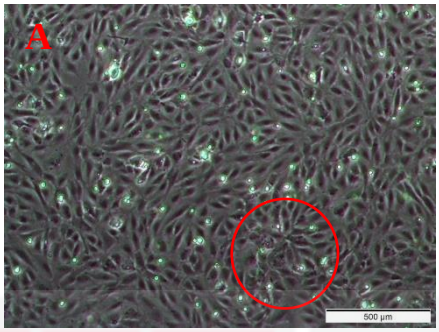
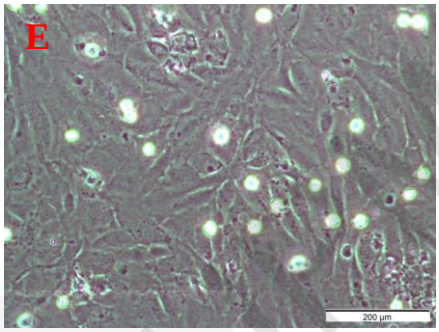
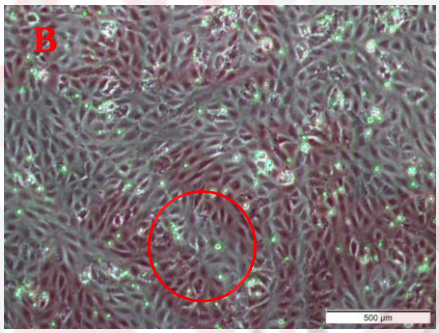
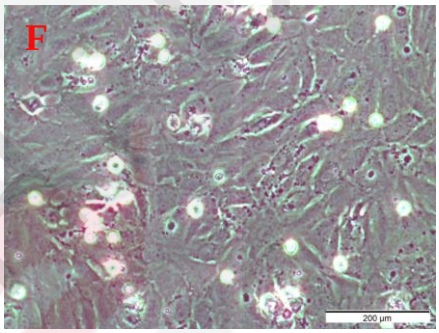
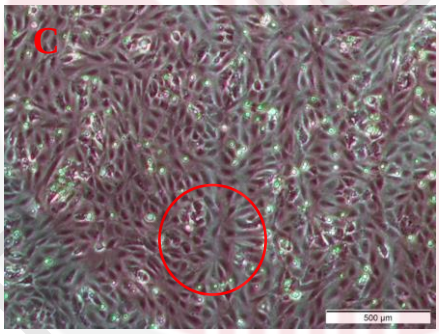
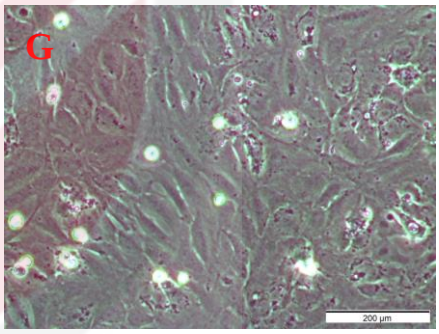
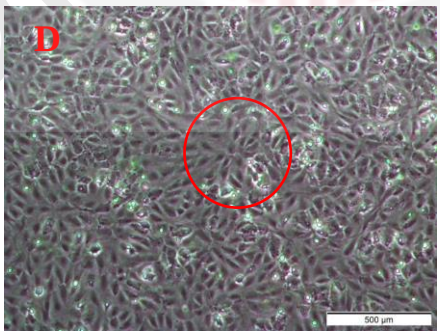
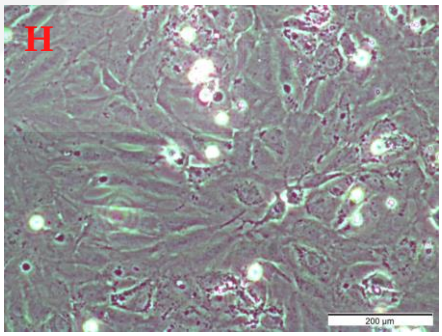
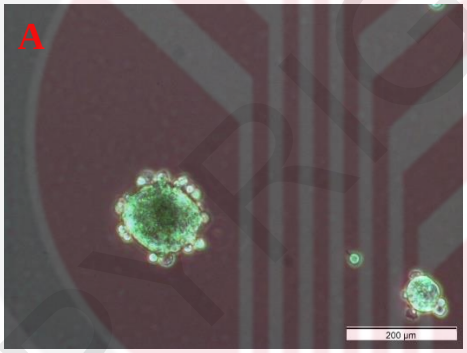
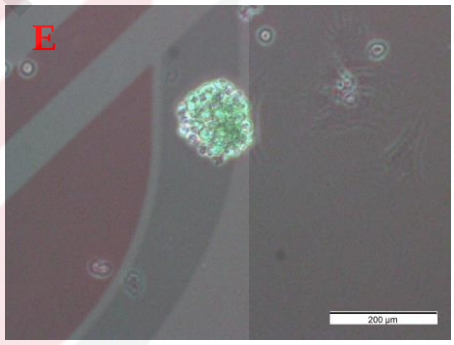
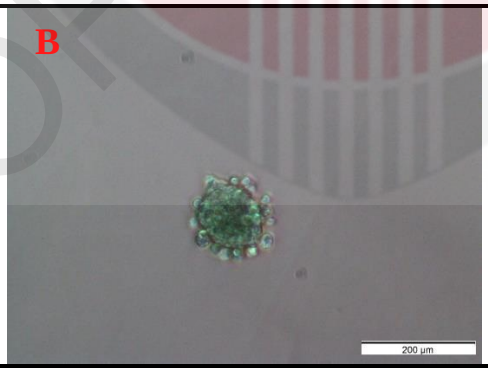
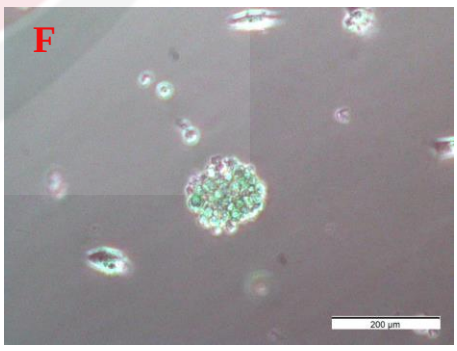
	4x magnification	10x magnification
Untreated (Negative control)		
50 µM dBcAMP (Positive control)		
1 µg/mL EECA		
10 µg/mL EECA		

Figure 4.3: Presence of neural rosette after monolayer differentiation. *The bright field images of the cells under the view of 4 x magnification (left column) and 10 x magnification (right column) in each group: untreated (**A, E**); 50 µM dBcAMP (**B, F**); 1 µg/mL EECA (**C, G**); 10 µg/mL EECA (**D, H**).* Regions indicated in circles shows the presence of neural rosette.

4.3 Effect of EECA on neurosphere formation

Neurospheres formed in first batch (derived from R3 P36) and second batch (derived from R3 P38) are shown in **Figure 4.3**. The morphology of neurospheres were slightly different in two batches. Neurospheres in the first batch had uneven circumferences and sign of cavitation at the center of the structure while smooth circumferences and brighter colour density were observed in neurospheres from the second batch.

	First batch (Derived from R3 P36)	Second batch (Derived from R3 P38)
Untreated (Negative control)		
50 µM dBcAMP (Positive control)		

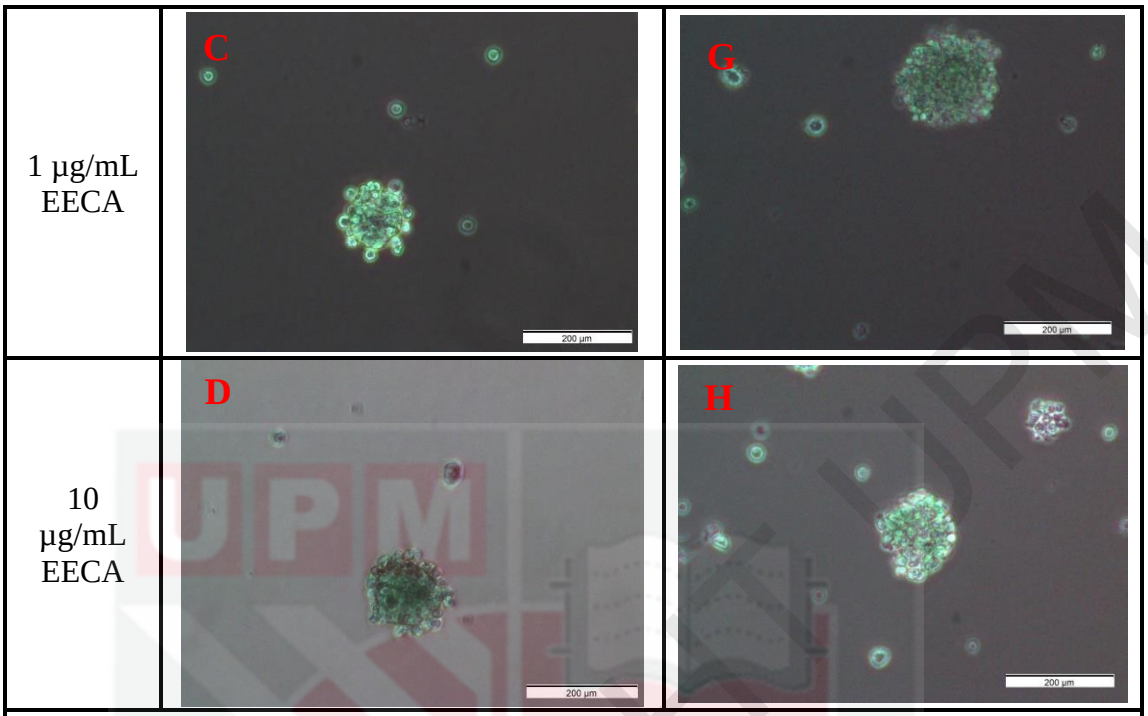


Figure 4.4: The neurospheres. *The bright field images of the cells under the view of 10 x magnification in each group: untreated (**A, E**); 50 µM dBcAMP (**B, F**); 1 µg/mL EECA (**C, G**); 10 µg/mL EECA (**D, H**). Left column shown are neurospheres from the first batch and the right column shown are neurospheres from the second batch.*

The average diameter of neurospheres falls between 70 - 100 µm in both batches. The total number and average diameter of neuropsheres in each group, as well as the number of good quality neurospheres (diameter 50-100µm) are listed in **Table 4.1**. Mean percentage of good quality neurospheres was calculated and the highest percentage is shown in 1 µg/ml EECA treatment group (**Figure 4.4**). Overall, mean percentage of good quality neurospheres is higher in both EECA treatment groups (1 µg/ml EECA and 10 µg/ml EECA). There was no significant result of mean percentage of good quality neurospheres among the groups in one-way ANOVA analysis (n=2, Tukey's and Dunnett's test)

Table 4.1: The total number and average diameter of neuropsheres in two batches (derived from R3 P36 and R3 P38).

Groups	Average diameter (µm)	Total number of neurosphere	Number of neurospheres with diameter 50-100 µm
Untreated	94.10	42	28
50µM dBcAMP	97.78	48	34
1 µg/ml EECA	83.79	103	57
10 µg/ml EECA	74.58	60	34

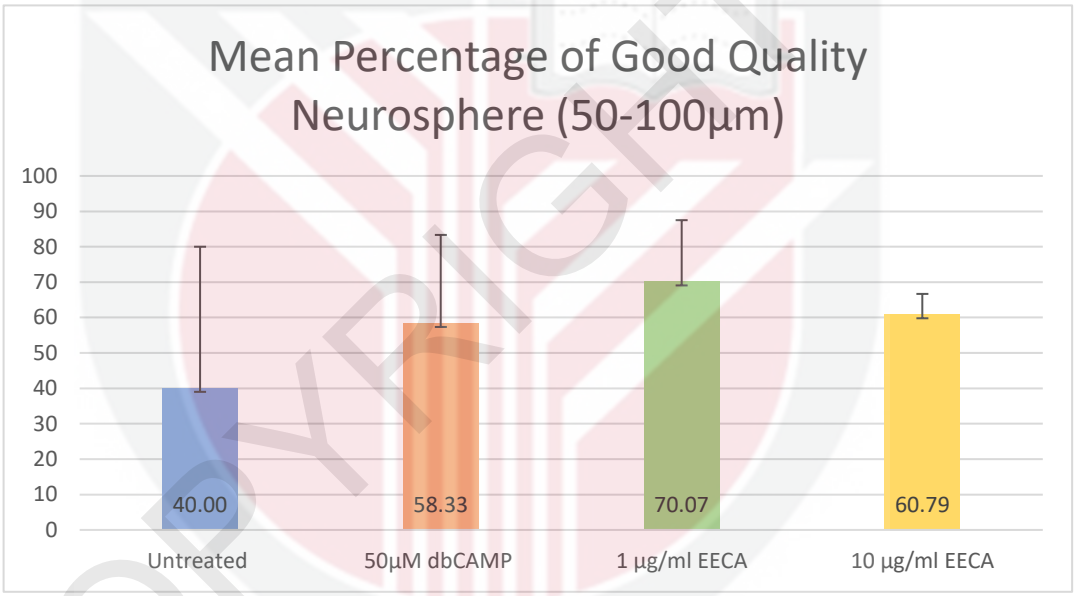


Figure 4.5: Bar graph illustration on mean percentage of good quality neurosphere (diameter 50-100µm). *Error bars show the values of ± SEM (n=2) on 4 group data, one-way ANOVA with Tukey's and Dunnett's post hoc test were performed using SPSS V.27.* Percentage of good quality neurosphere in each batch is shown in **Appendix E.**

4.4 Effect of EECA on NSCs specific marker expression

Based on **Figure 4.5**, highest *Sox1* expression is shown in 1 µg/ml EECA treatment group, which is 13.45 times higher compared to untreated group. In addition, *Sox1* expression in 10 µg/ml EECA treatment group is 2.33 times higher than the untreated group. Generally, both EECA treatment groups showed higher *Sox1* expression compared to negative (untreated) and positive (50µM dBcAMP) control groups.

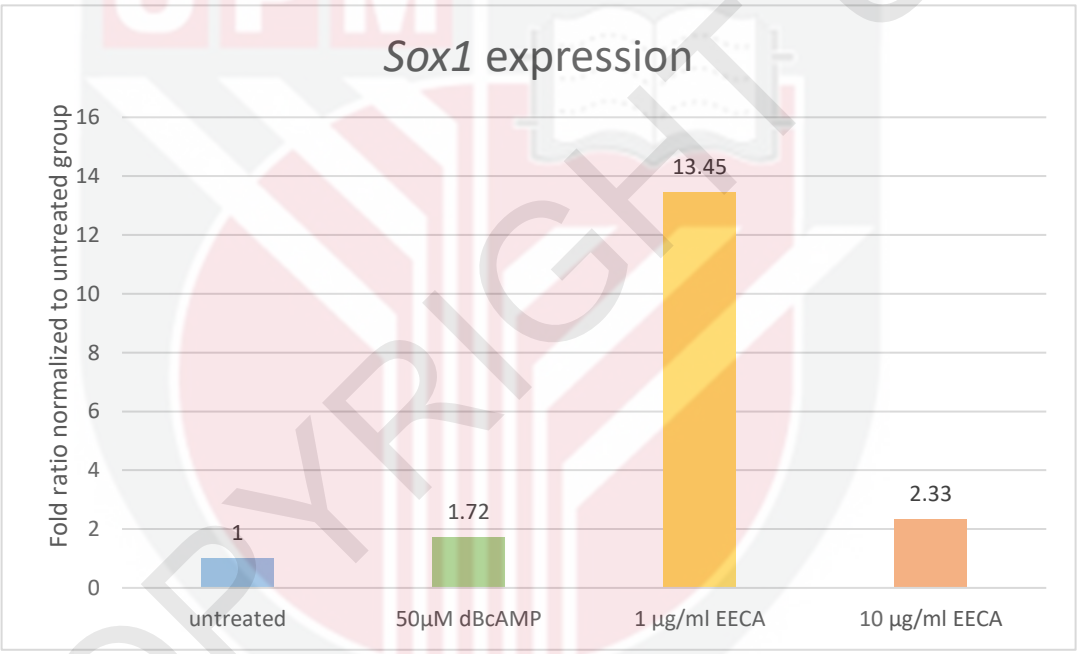


Figure 4.5: Bar graph illustration on RT-qPCR analysis of *Sox1* in R3-derived NSCs after EECA treatment. *Expression levels represented as fold change of treatment groups over the expression of untreated cells. Statistical analysis was not performed due to n=1.*

CHAPTER 5
DISCUSSIONS

5.1 Propagation of good quality R3

At the early phase of the experiment, R3 were cultured in an embryonic stem cell medium (ESM). Culturing method and medium formulation used in this study were referred to in the study done by Mun-Fun et al. (2015). We observed that the majority of the cells had 2 to 4 nucleoli in the nucleus and a fibroblast-like morphology. This observation is in line with the observation by Mun-Fun et al. (2015) during the characterization of R3.

R3 as a broadly multipotent stem cell, multiple nucleoli is one of the characteristics of a good stem cell. Polynucleolarity (the presence of more than one nucleolus within the cellular nucleus) is a well-known phenomenon during the proliferative cell cycle (Gramsbergen et al., 1987). The nucleolus is known to play an important role in cell fate determination, with the hyperactive state of the nucleolus required for pluripotency maintenance (Gupta & Santoro, 2020). Ribosome formation, ribosomal DNA transcription, pre-ribosomal RNA (rRNA) processing, and mature rRNA assembly with ribosomal proteins all take place in nucleoli. (Watanabe-Susaki et al., 2014). Zhang et al (2014) showed that levels of rRNA transcription encourage changes in cell fate, development and proliferation. To be more specific, a high level of rRNA expression promoted the rate of

proliferation. Therefore, the multiple nucleoli in stem cells explain their ability to self-renew and proliferate actively.

5.2 Presence of neural rosette

Based on **Figure 4.2**, the morphology of the cells was similar compared to R3 before monolayer differentiation. However, the presence of neural rosette in all experimental groups could be a clue that monolayer transdifferentiation from R3 to NSCs was successful. During neural development, a crucial morphogenetic process in which neural stem cells are confined in rosette niches to balance proliferation and differentiation (Hříbková et al., 2018). According to Elkabetz et al. (2008), neural rosette cells were shown to represent the first NSC stage identified as being able to respond to patterning cues that lead to differentiation toward region-specific neuronal fates. They express markers associated with NSC fates such as Nestin, Sox2 and 3CB2. Moreover, their self-renewal and proliferative properties are maintained by SHH and Notch signaling pathway. Therefore, the observation of neural rosette after monolayer transdifferentiation showed the presence of NSCs, which further confirmed the ability of R3 to transdifferentiate into NSCs.

5.3 Effect of EECA on the formation of neurospheres

Based on **Figure 4.3**, formation of neurospheres in all groups and in both batches of experiment suggested the presence of NSCs. As respond to epidermal growth factor

and basic fibroblast growth factor, NSCs will undergo a period of active proliferation, whereas differentiated cells do not. NSCs form in spherical aggregates termed neurospheres in these settings, which can be passed to extend the pool of these cells (Soares et al., 2020).

The effect of EECA on the formation of neurospheres was assessed based on the number and diameter of neurospheres formed. Based on **Table 4.1**, the total number of neurospheres in EECA treatment groups (1 µg/ml EECA and 10 µg/ml EECA) were higher than the negative (untreated) and positive (50µM dBcAMP) control group. In this study, neurospheres were observed after 72 hours of incubation and the average diameter of neurospheres in all experimental groups were in the range of 70 - 100 µm. According to Dan Ge et al. (2012), culturing time around 72 hours showed the highest number of cells in the neurospheres where the peak of the growth curve is reached. In terms of neurospheres size, the diameter of neurospheres reached about 100 µm around the same culturing time. In short, the measurement of the neurospheres diameter in this study is in line with the findings by Dan Ge and colleagues (2012) where the highest number of cells (10^5/mL) were expected in neurospheres with a diameter of about 100 µm around 72 hours of culturing time.

The diameter of neurospheres is also useful in determining its quality. According to Dan Ge et al. (2012), good quality neurospheres should be having diameter of 50-100 µm. It is shown that neurospheres with diameter exceeding 100 µm have decreased proliferative ability of cells and negative value of cell-specific growth rate that indicates the reduction of cell number. **Figure 4.4** showed higher mean percentage of good quality neurospheres in both 1 µg/ml EECA and 10 µg/ml EECA treatment groups compared to

the negative (untreated) and positive (50µM dBcAMP) control groups. The highest mean percentage of 70.07% good quality neurospheres is shown in the 1 µg/ml EECA treatment group. In other words, the number of transdifferentiated NSCs is higher in both EECA treatment groups, especially in 1 µg/ml EECA treatment groups.

However, no significant results were found from one-way ANOVA analysis with Tukey's and Dunnett's post hoc test. Thus, all possibilities remain as the sample size was too small to establish a significant difference.

5.4 Effect of EECA on NSC-specific marker expression

In this study, RT-qPCR was done to quantitatively assess the expression of sex determining region Y-box 1 (*Sox1*) after normalizing to the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Housekeeping genes are genes involved in basic cell maintenance and are expected to have a stable expression in varying conditions (Eisenberg & Levanon, 2013). Thereforethe , expression of housekeeping genes is used to measure the relative expression of the markers of interest. As for the NSC-specific genetic marker, *Sox1* is one of the earliest transcription factors expressed in cells committed to the neural fate (Stevanovic et al., 2021). It is also known as the central regulator for neurodevelopment which expresses accordingly to the rate of neurogenesis (Venere et al., 2012). Thus, the expression of *Sox1* confirms the presence of NSCs.

Based on **Figure 4.5**, it was clear that the highest *Sox1* expression was found in 1 µg/ml EECA treatment group, which was 13.45 times higher than the negative control

(untreated) group. 10 µg/ml EECA treatment group also showed slightly higher expression of *Sox1*, which is 2.33 times compared to the negative control (untreated) group. The reason behind the large fold change difference between the 1 µg/ml EECA and 10 µg/ml EECA treatment group remained unclear. It might be due to more improvement needed on the techniques to run RT-qPCR. In addition, statistical analysis was not carried out due to insufficient sample size (n=1). The results showed the potential of EECA in promoting transdifferentiation from R3 to NSCs based on increased *Sox1* expression but other possibilities remain. The experiment should be done three times to ensure validity and reproducibility.

CHAPTER 6

CONCLUSION, LIMITATIONS AND FUTURE RECOMMENDATION

Conclusion

People suffering from neurological illnesses could benefit from neuro-transplantation. Unfortunately, technological limitations in isolating endogenous NSCs from the brain, as well as a lack of evidence on the enhancer in the transdifferentiation process, render neuro-transplantation difficult to achieve. In this study, R3 is shown to be able to transdifferentiatie into NSCs with the presence of neural rosette. EECA is also shown to have great potential to promote transdifferentiation from R3 to NSCs based on the higher percentage of good quality neurospheres (diameter between 50 to 100 µm) in EECA treatment groups compared to negative (untreated) and positive (50 µM dBcAMP) control groups. In addition, increased expression of NSC specific marker, *Sox1*, especially in 1 µg/ml EECA treatment group further suggested the effect of EECA in promoting transdifferentiation of R3 into NSCs. Therefore, the findings of this study suggest the potential of EECA as an enhancer for producing quality NSCs from amniotic fluid stem cells, a mammalian non-brain source, for a prospective application in neuro-transplantation.

Limitations

In this study, there are a few limitations that had been along the experiment. Firstly, the sample size was too small for a valid and reproducible statistical analysis. In this study, only one batch of cells (n=1) was managed to undergo RT-qPCR for the investigation of NSC-specific marker, *Sox1* expression level. Therefore, statistical analysis was not able to carry out, leading to an invalid conclusion of the experiment regarding the expression level of *Sox1*.

Besides, good quality of R3 was only determined by morphology observation. There should be additional characterization methods to further support the statement that the cultured R3 were in good quality. For example, cell population doubling time, immunostaining or RT-qPCR to investigate the expression of R3 specific markers such as *Oct4* and *Nanog*.

Future Recommendations

There are a few recommendations to help in a future experiment. In terms of validity and reproducibility of the experiment, it is encouraged that the experiment should be run three times. To be more specific, there should be three batches of R3 to be cultured and transdifferentiated into NSCs. Also, the investigation of good quality neurospheres and measurement of NSC-specific marker using RT-qPCR should be carried out three times before running statistical analysis.

Other than the characterization of good quality R3 mentioned in the above section, the NSCs should also be characterized with more NSC-specific markers. In this study, only a pair of housekeeping (*GAPDH*) and a target gene (*Sox1*) were used in running RT-qPCR. Due to time constriction, other designed primers were not able to reach in time. The primers include one housekeeping gene (*β-actin*) and two more NSCs specific markers (*Sox2* and *Nestin*) that should be used in RT-qPCR. By using more than one housekeeping genes, to verify if there is any variation in the expression ratios of two housekeeping genes that could reflect the fact that one of them is not constantly expressed. After confirming that the housekeeping genes are working stably, the expression level of more than one NSCs-specific markers such as *Sox2* and *Nestin* should be investigated for a more comprehensive study.

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Appendix A: Concentration and integrity of RNA

Table A.1: Concentration and purity of RNA

RNA Samples	Parameter	Absorbance Reading 1	Absorbance Reading 2	Average Absorbance Reading
Untreated	Concentration (ng/uL)	335.08	329.63	332.36
	$x = \frac{260\lambda - 320\lambda}{280\lambda - 320\lambda}$ ratio	2.007	2.013	2.01
	$x = \frac{260\lambda - 320\lambda}{230\lambda - 320\lambda}$ ratio	2.053	2.047	2.05
dBcAMP	Concentration (ng/uL)	199.85	201.55	200.70
	$x = \frac{260\lambda - 320\lambda}{280\lambda - 320\lambda}$ ratio	1.996	1.983	1.990
	$x = \frac{260\lambda - 320\lambda}{230\lambda - 320\lambda}$ ratio	2.314	2.270	2.292
1ug/mL EECA	Concentration (ng/uL)	103.13	105.93	104.53
	$x = \frac{260\lambda - 320\lambda}{280\lambda - 320\lambda}$ ratio	1.918	1.894	1.906
	$x = \frac{260\lambda - 320\lambda}{230\lambda - 320\lambda}$ ratio	1.961	1.944	1.953
10ug/mL EECA	Concentration (ng/uL)	215.57	217.20	216.39
	$x = \frac{260\lambda - 320\lambda}{280\lambda - 320\lambda}$ ratio	1.984	1.971	1.978
	$x = \frac{260\lambda - 320\lambda}{230\lambda - 320\lambda}$ ratio	1.730	1.721	1.726

Appendix B: List of primer sequences and their properties

Table B.1: Primer sequences.

Gene	NCBI Reference Sequence	Species	Primer sequence (5' – 3')
GAPDH	NM_017008.4	*Rattus Norvegicus*	**F**: GCGAGTTAGTTGGGATCAGT **R**: GCACCTTTTTGTGATGCGTG
Sox1	XR_ 001837382.2		**F**: CCGAGGAACTCAGACCCAAC **R**: AGTGGGATGTGCCCTCTTTG

Table B.2: Properties of each primer sequence.

Gene	Primer sequence (5' – 3')	Product length (bp)	GC content (%)	Annealing temperature (°C)
GAPDH	**F**: GCGAGTTAGTTGGGATCAGT **R**: GCACCTTTTTGTGATGCGTG	144	50 50	57.33 58.87
Sox1	**F**: CCGAGGAACTCAGACCCAAC **R**: AGTGGGATGTGCCCTCTTTG	173	60 55	60.04 59.96

Reaction Conditions:

Nucleic Acid Concentration (nM)	0.25	Monovalent Concentration (mM)	50
Free Mg++ Concentration (mM)	3	Total Na+ Concentration (mM)	269.09

Sense Primer: GCGAGTTAGTTGGGATCAGT

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	54.31	50	1	-1.8	-2.0	0.0

Anti-sense Primer: GCACCTTTTTGTGATGCGTG

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	55.94	50	1	-1.8	-2.0	-2.0

Figure B.1: Quality check for designed *GAPDH* primer pair using online website, Beacon Designer (Premier Biosoft International®).

```
General properties:
-------------------
                    Primer name: foward
                Primer sequence: GCGAGTTAGTTGGGATCAGT
                Sequence length: 20
                    Base counts: G=8; A=4; T=6; C=2; Other=0;
                GC content (%): 50.00
     Molecular weight (Daltons): 6228.12
                      nmol/A260: 5.00
                micrograms/A260: 31.14
            Basic Tm (degrees C): 52
   Salt adjusted Tm (degrees C): 47
Nearest neighbor Tm (degrees C): 62.31

PCR suitability tests (Pass / Warning):
-----------------------------------
                 Single base runs: Pass
          Dinucleotide base runs: Pass
                         Length: Pass
                    Percent GC: Pass
          Tm (Nearest neighbor): Warning: Tm is greater than 58;
                       GC clamp: Pass
                  Self-annealing: Pass
                Hairpin formation: Pass


General properties:
-------------------
                    Primer name: reverse
                Primer sequence: GCACCTTTTTGTGATGCGTG
                Sequence length: 20
                    Base counts: G=6; A=2; T=8; C=4; Other=0;
                GC content (%): 50.00
     Molecular weight (Daltons): 6130.04
                      nmol/A260: 5.54
                micrograms/A260: 33.94
            Basic Tm (degrees C): 52
   Salt adjusted Tm (degrees C): 47
Nearest neighbor Tm (degrees C): 63.40


PCR suitability tests (Pass / Warning):
-----------------------------------
                 Single base runs: Warning: Contains run of T's;
          Dinucleotide base runs: Pass
                         Length: Pass
                    Percent GC: Pass
          Tm (Nearest neighbor): Warning: Tm is greater than 58;
                       GC clamp: Warning: There are more than 3 G's or
C's in the last 5 bases;
                  Self-annealing: Pass
                Hairpin formation: Pass
```

Figure B.2: Quality check for designed *GAPDH* primer pair using online website, Sequence Manipulation Suite, PCR Primer Stats.

Reaction Conditions:

Nucleic Acid Concentration (nM)	0.25	Monovalent Concentration (mM)	50
Free Mg++ Concentration (mM)	3	Total Na+ Concentration (mM)	269.09

Sense Primer: CCGAGGAACTCAGACCCAAC

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	57.01	60	1	-3.4	-1.0	-1.0

Anti-sense Primer: AGTGGGATGTGCCCTCTTTG

Length (bp)	Tm (^{0}C)	GC%	GC Clamp	Cross Dimer (ΔG)	Self Dimer (ΔG)	Hairpin (ΔG)
20	56.87	55	1	-3.4	-2.0	-2.0

Figure B.3: Quality check for designed *Sox1* primer pair using online website, Beacon Designer (Premier Biosoft International®).

```
Global settings:
-The primers do not have a 5'-phosphate group.
-Combined concentration of K+ and Na+ in the reaction = 50 millimolar.
-Mg+2 concentration in the reaction = 1.5 millimolar.
-Primer concentration in the reaction = 200 nanomolar.

------------------------------------------------------------
------------------------------------------------------------
General properties:
-------------------
                    Primer name: forward
                Primer sequence: CCGAGGAACTCAGACCCAAC
                Sequence length: 20
                    Base counts: G=4; A=7; T=1; C=8; Other=0;
                 GC content (%): 60.00
     Molecular weight (Daltons): 6064.99
                      nmol/A260: 5.15
                micrograms/A260: 31.21
          Basic Tm (degrees C): 56
   Salt adjusted Tm (degrees C): 51
Nearest neighbor Tm (degrees C): 65.34

PCR suitability tests (Pass / Warning):
-----------------------------------
                Single base runs: Pass
          Dinucleotide base runs: Pass
                         Length: Pass
                     Percent GC: Pass
           Tm (Nearest neighbor): Warning: Tm is greater than 58;
                        GC clamp: Pass
                  Self-annealing: Pass
               Hairpin formation: Pass
------------------------------------------------------------

------------------------------------------------------------
General properties:
-------------------
                    Primer name: reverse
                Primer sequence: AGTGGGATGTGCCCTCTTTG
                Sequence length: 20
                    Base counts: G=7; A=2; T=7; C=4; Other=0;
                 GC content (%): 55.00
     Molecular weight (Daltons): 6155.05
                      nmol/A260: 5.43
                micrograms/A260: 33.40
          Basic Tm (degrees C): 54
   Salt adjusted Tm (degrees C): 49
Nearest neighbor Tm (degrees C): 65.06

PCR suitability tests (Pass / Warning):
-----------------------------------
                Single base runs: Pass
          Dinucleotide base runs: Pass
                         Length: Pass
                     Percent GC: Pass
           Tm (Nearest neighbor): Warning: Tm is greater than 58;
                        GC clamp: Pass
                  Self-annealing: Pass
               Hairpin formation: Pass
------------------------------------------------------------
```

Figure B.4: Quality check for designed *Sox1* primer pair using online website, Sequence Manipulation Suite, PCR Primer Stats.

Appendix C: Optimization of annealing temperature

Table C.1: Gradient PCR primer details.

Gene	Primer sequence (5' – 3')	Product length (bp)	Annealing temperature (°C)	Range of temperature for gradient PCR (°C)	Optimized annealing temperature (°C)
GAPDH	**F**: GCGAGTTAGTTGGGATCAGT **R**: GCACCTTTTTGTGATGCGTG	144	57.33 58.87	56 - 61	59
Sox1	**F**: CCGAGGAACTCAGACCCAAC **R**: AGTGGGATGTGCCCTCTTTG	173	60.04 59.96	56 - 62	62

Figure C.1: Determining optimal annealing temperature of gradient PCR products using 1% gel electrophoresis. *Gel electrophoresis images for gradient PCR of housekeeping gene, GAPDH (**A**) and NSC specific marker, Sox1 (**B**).*

Appendix D: RT-qPCR Standard curve and melt curve analysis

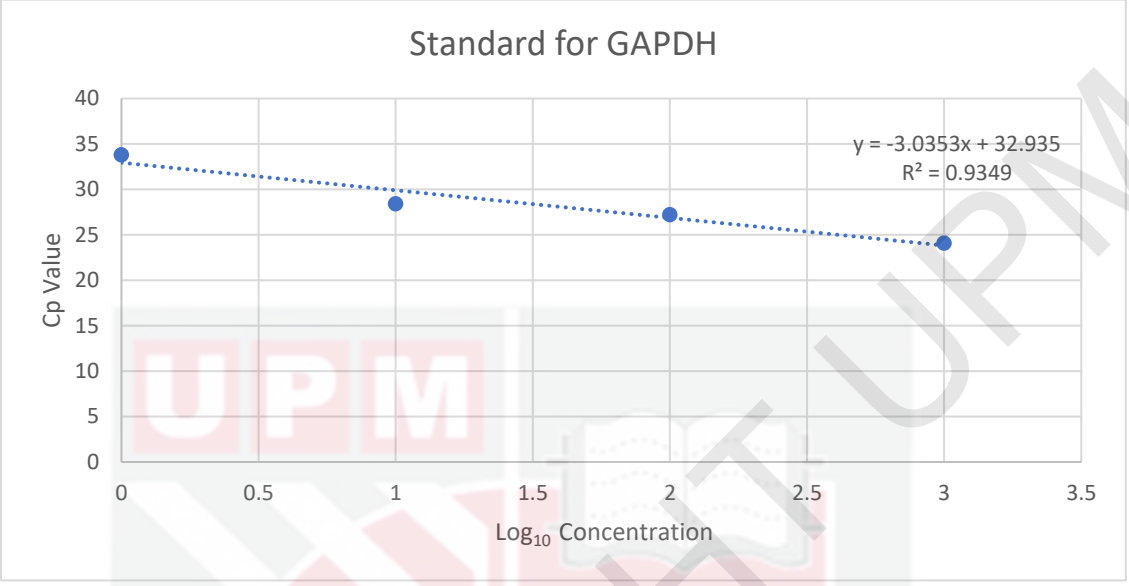

Figure D.1: Standard curve for housekeeping gene, *GAPDH*. *The PCR efficiency is 113.53%, which is calculated using formula, E = -1+10(-1/slope). Equation of standard curve is y = -3.0353x + 32.935 and R^2 value is 0.9349.* PCR efficiency that falls between 90 to 110%, with R^2 value ≥ 0.985 is considered optimum condition for the primer.

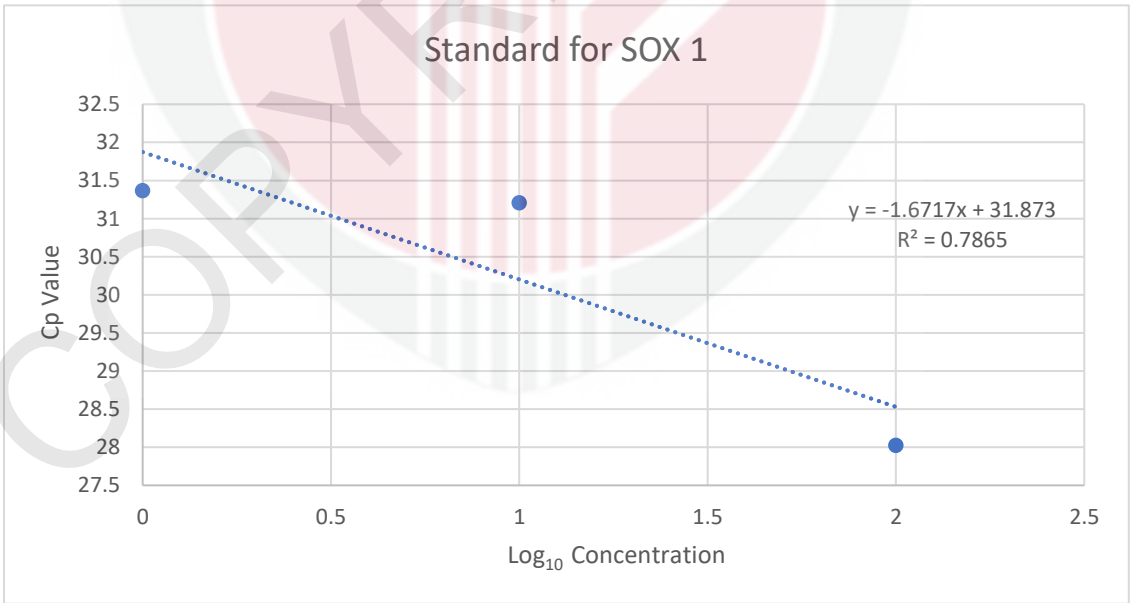

Figure D.2: Standard curve for target gene, *Sox1*. *The PCR efficiency is 296.46%, which is calculated using formula, E = -1+10(-1/slope). Equation of standard curve is y = -1.6717x + 31.873 and R^2 value is 0.7865.* PCR efficiency that falls between 90 to 110%, with R^2 value ≥ 0.985 is considered optimum condition for the primer.

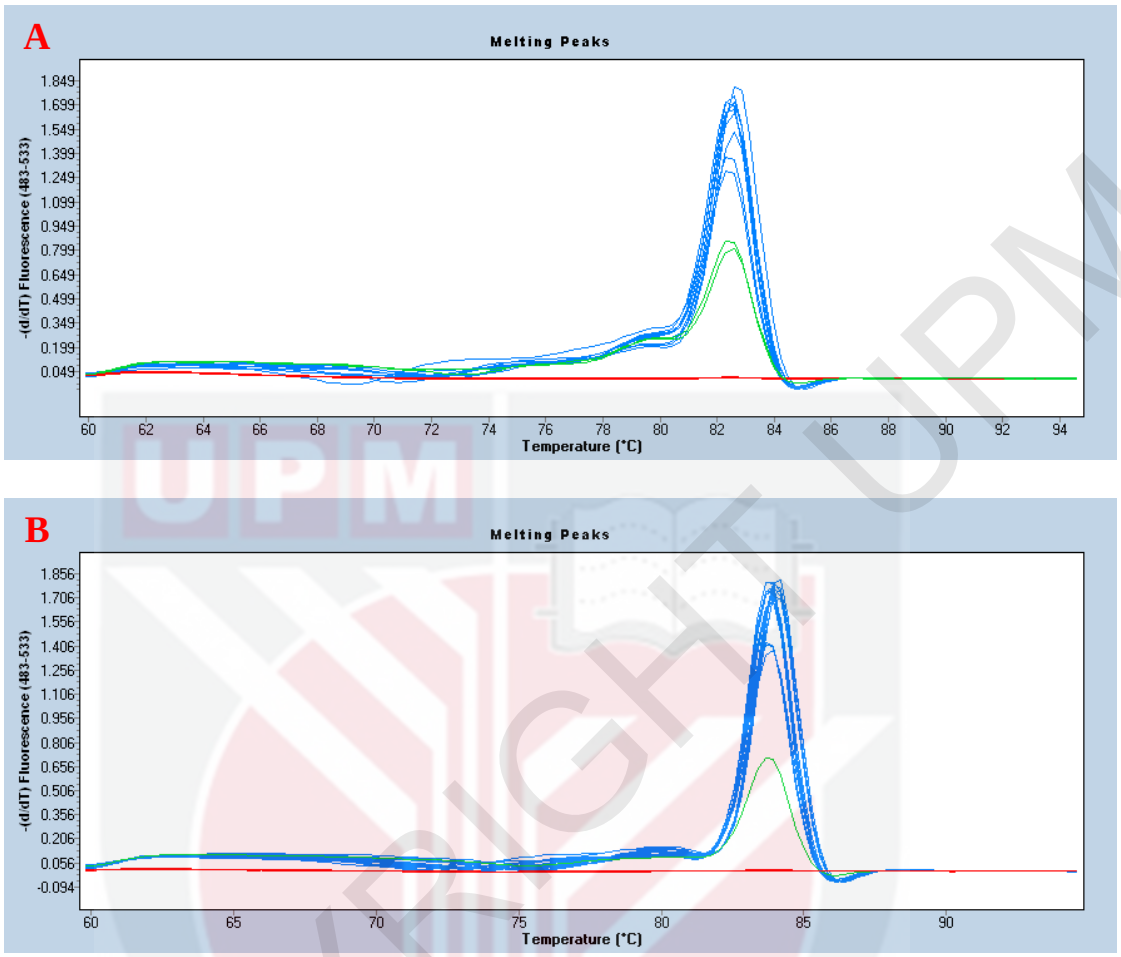

Figure D.1: Melting peak graph. *Melting peaks graph for quantitative PCR of NSC specific marker, Sox1(**A**) and housekeeping gene, GAPDH (**B**).*

Appendix E: Raw data for number and diameter of neurospheres

Table E.1: Raw data in first batch (derived from R3 P36)

Groups	Average diameter (µm)	Total number of neurosphere	Number of neurospheres with diameter 50-100 µm	Percentage of neurospheres with diameter 50-100 µm
Untreated	113.51	7	0	0%
50µM dBcAMP	98.12	12	4	33.33%
1 µg/ml EECA	91.28	8	7	87.50%
10 µg/ml EECA	66.80	9	6	66.67%

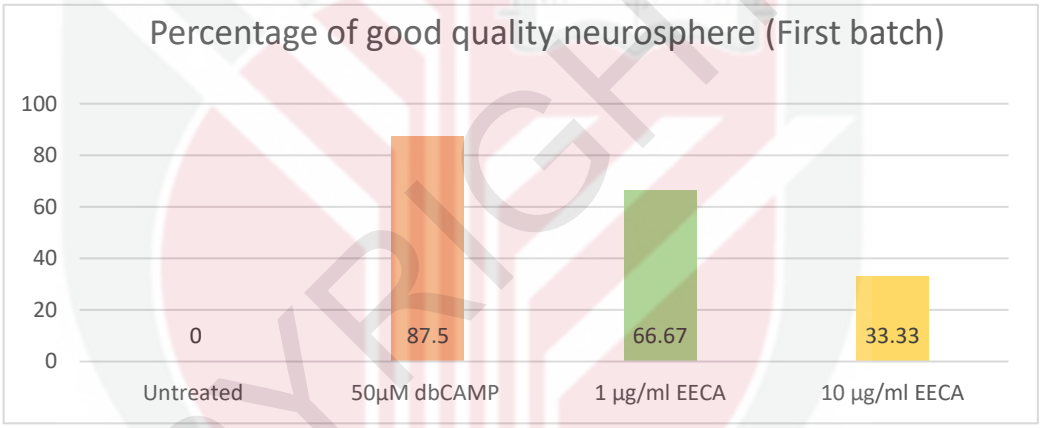

Figure E.1: Bar graph illustration on mean percentage of good quality neurosphere (diameter 50-100µm) in first batch of experiment (derived from R3 P36).

Table E.2: Raw data in second batch (derived from R3 P38)

Groups	Average diameter (µm)	Total number of neurosphere	Number of neurospheres with diameter 50-100 µm	Percentage of neurospheres with diameter 50-100 µm
Untreated	74.68	51	28	54.90%
50µM dBcAMP	76.30	36	30	83.33%
1 µg/ml EECA	82.36	95	50	52.63%
10 µg/ml EECA	97.45	51	28	54.90%

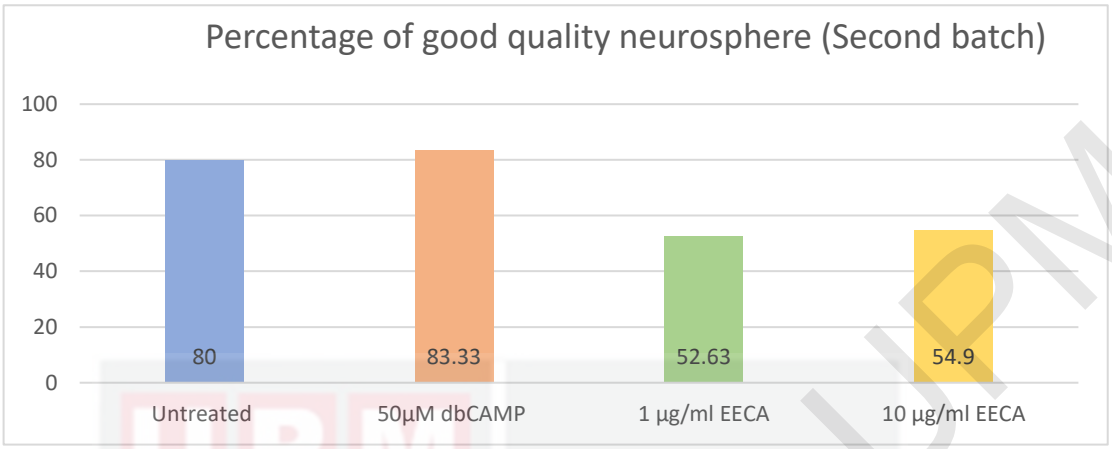

Figure E.1: Bar graph illustration on mean percentage of good quality neurosphere (diameter 50-100µm) in second batch of experiment (derived from R3 P38).

Table E.3: Raw data in third batch (derived from R3 P40)

Groups	Average diameter (µm)	Total number of neurosphere	Number of neurospheres with diameter 50-100 µm	Percentage of neurospheres with diameter 50-100 µm
Untreated	65.433	35	25	71.43%
50µM dBcAMP	59.965	103	74	71.84%
1 µg/ml EECA	52.833	20	9	45.00%
10 µg/ml EECA	46.477	36	13	36.11%

Appendix F: Raw data for RT-qPCR *Sox1* expression analysis

Sample	Average Ct Value		Fold Change Calculation (FC=2^-ΔΔCt)		
	GAPDH	SOX1	ΔCt	ΔΔCt	Fold change
R3 P38	29.67	30.87	1.2	-0.69	1.613284
Untreated NSC	24.70	26.59	1.89	0	1
dBcAMP	27.72	28.83	1.11	-0.78	1.721103
1ug/mL EECA	27.85	25.99	-1.86	-3.75	13.45434
10ug/mL EECA	28.23	28.90	0.67	-1.22	2.329467