EXPANSION, CHARACTERISATION AND DIFFERENTIATION OF HUMAN NEURAL STEM CELLS

By

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ABSTRACT

Expansion, Characterisation and Differentiation of Human Neural Stem Cells

Stroke irreversibly damages affected brain part, leading to permanent neuronal impairment. Neural stem cell (NSC)-based therapy is a potential stroke treatment due to its ability to self-renew and to differentiate into various viable neuronal cells for damaged brain tissue regeneration. Here, human NSCs were expanded from GIBCO® human NSC line and stroke patients' brain subventricular zone (SVZ) tissue biopsy with ethical approval. NSCs obtained from GIBCO® cell line were used for pilot testing of NSC characterisation and differentiation ability *in vitro*. GIBCO® NSCs were cultured in complete StemPro® NSC SFM while human brain SVZ tissues were cultured in an adherent layer with serum-free medium containing bFGF and EGF. Characterisation of NSC was performed using qRT-PCR and Western blot; while NSC differentiation was performed using Neuron Differentiation Medium and Astrocytes Differentiation Medium. Both assays used human normal brain cell line (SVG-p12) as negative control. qRT-PCR data illustrated that NSCs showed over 4000-fold difference in Nestin and 6000-fold difference in CD133 expression compared to SVG-p12, indicated that Nestin and CD133 were specific to NSC. These results were consistent with the Western blot data in which Nestin protein with approximately 200 kDa was identified in NSCs but was absent in SVG-p12. Upon four days in culture with differentiation media, NSCs did not show morphological changes towards neurons and astrocytes respectively, compared to non-treated cells. This is due to the limited time available to perform this assay in present study. Longer culture duration which is more than one week will be performed in future to obtain more accurate result. Meanwhile, two clonal neurospheres were obtained from SVZ tissue culture, indicated that the culture method used in this study was successful to isolate NSCs. However, neurosphere contains only small amount of true stem cells, thus cell disaggregation and proliferation are suggested in future study to obtain more cells for downstream analysis. Conclusion, NSCs were successfully obtained from both GIBCO® human NSC line and SVZ tissue. However, further expansion, characterisation and differentiation of NSC isolated from SVZ tissue should be performed in future study.

Keywords: Stroke; neural stem cell (NSC); human brain subventricular zone (SVZ) tissue; quantitative real time polymerase chain reaction (qRT-PCR); Western blot; basic fibroblast growth factor (bFGF), epidermal growth factor (EGF); neurospheres.

ABSTRAK

Pengembangan, Pencirian dan Pembezaan Sel Stem Neural Manusia

Strok menyebabkan kemerosotan neuron yang tidak boleh diperbaharui dalam bahagian otak yang terjejas. Terapi yang menggunakan sel stem neural (NSC) merupakan rawatan strok alternatif kerana keupayaan sel berkenaan untuk memperbaharui diri dan membezakan diri kepada pelbagai jenis sel neuron yang matang untuk pertumbuhan semula tisu otak yang terjejas akibat strok. Di sini, NSCs manusia telah dikembang daripada turunan sel manusia GIBCO® NSC dan daripada tisu biopsi yang diperolehi daripada zon subventrikular (SVZ) otak pesakit strok dengan kelulusan etika. NSCs yang diperolehi daripada pengembangan turunan sel manusia GIBCO® NSC telah digunakan untuk ujian pencirian dan ujian kemampuan pembezaan NSC. GIBCO® NSCs telah dikulturkan dalam StemPro® NSC SFM lengkap manakala biopsi tisu SVZ yang diperolehi daripada otak pesakit strok telah dikulturkan dalam media tanpa serum yang mengandungi bFGF dan EGF. Pencirian NSCs telah dilakukan dengan menggunakan cara qRT-PCR dan pemendapan Western, manakala prosedur pembezaan NSCs telah dilakukan dengan menggunakan Medium Pembezaan Neuron dan Medium Pembezaan Astrosit. Kedua-dua ujian ini menggunakan sel otak manusia normal (SVG-p12) sebagai kawalan negatif. Data qRT-PCR menunjukkan bahawa NSCs mempunyai perbezaan lebih 4000 kali ganda dalam ekspresi Nestin dan perbezaan lebih 6000 kali ganda dalam ekspresi CD133 berbanding dengan SVG-p12. Ini menunjukkan bahawa Nestin dan CD133 adalah gen yang khususnya untuk NSCs. Keputusan ini adalah selaras dengan data pemendapan Western di mana protein Nestin dengan saiz lebih kurang 200 kDa telah dikenal pasti hanya dalam NSCs tetapi tidak dalam SVG-p12. Selepas empat hari kultur dalam media pembezaan, morfologi NSCs tidak berubah ke

arah sel neuron dan sel astrosit, berbanding dengan NSCs yang tidak dirawati dengan media pembezaan. Hal ini kerana masa untuk menjalankan ujian ini tidak cukup. Oleh sebab itu, masa untuk kultur NSCs dalam media pembezaan akan dipanjangkan pada masa depan untuk mendapat keputusan yang tepat. Sementara itu, dua neurospheres klon telah didapati daripada kultur tisu SVZ. Keputusan ini menunjukkan bahawa kaedah kultur yang digunakan dalam kajian ini telah berjaya untuk memperolehi NSCs daripada tisu SVZ manusia. Walau bagaimanapun, kuantiti NSCs yang sahih dalam neurosphere adalah rendah, oleh sebab yang demikian, penceraian neurosphere dan proliferasi sel dicadangkan dalam kajian yang akan datang untuk mendapatkan lebih banyak sel untuk analisis lanjutan. Sebagai rumusan, NSCs telah berjaya diperolehi daripada kedua-dua turunan sel GIBCO® NSC dan tisu SVZ. Walau bagaimanapun, perkembangan lanjut, pencirian dan pembezaan NSC yang diasingkan daripada tisu biopsi SVZ harus dilakukan pada masa depan.

Kata Kunci: Strok; sel stem neural (NSC); tisu zone subventrikular otak manusia; kuantitatif tindak balas rantai polimerase masa nyata (qRT-PCR); pemendapan Western; faktor pertumbuhan fibroblast asas (bFGF), faktor pertumbuhan epidermis (EGF); neurosphere

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LIST OF ABBREVATIONS

ADSCs	Adipose-derived stem cells
APS	Ammonium Persulfate
AVM	Arteriovenous malformation
bFGF	Basic fibroblast growth factor
BDNF	Brain-derived neurotrophic factor
BMSCs	Bone marrow stem cells
BSA	Bovine serum albumin
BSC	Biosafety cabinet
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon dioxide
C_t	Cycle threshold
CTS	Cell Therapy System
D-MEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
DPBS	Dulbecco's phosphate buffered saline
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
elF4A	Eukaryotic initiation factor-4A
ESCs	Embryonic stem cells
EtBr	Ethidium bromide
FBS	Fetal bovine serum
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
HRP	Horseradish peroxidase
HUSM	Hospital Universiti Sains Malaysia

iPSCs	Induced pluripotent stem cells
Klf4	Kruppel-like factor 4
Mg^{2+}	Magnesium
MEM	Minimum Essential Medium
NGF	Nerve growth factor
NT3	Neurotrophin
NIH	National Institutes of Health
NSCs	Neural stem cells
Oct-4	Octamer-binding transcription factor 4
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PDL	Poly-D-Lysine
PHDIs	Prolyl-4-hydroxylase inhibitors
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real-time polymerase chain reaction
RNase	Ribonuclease
rRNA	Ribosomal ribonucleic acid
RNA	Ribonuclei acid
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SFM	Serum free medium
SGZ	Subgranular zone
SDF-1	Stromal-derived factor 1
SVZ	Subventricular zone
TAE	Tris-Acetate-Ethylenediaminetetraacetic acid
TBS-T	Tris-buffered saline and Tween 20
TEMED	Tetramethylethylenediamine
tPA	Tissue plasminogen activator
UCBCs	Umbilical cord blood stem cells
USFDA	United States Food and Drug Administration
VEGF	Vascular endothelial growth factor

LIST OF UNITS

µl	Microlitre
ml	Millilitre
l	Litre
ng	Nanogram
µg	Microgram
mg	Milligram
g	Gram
nm	Nanometre
µm	Micrometre
cm	Centimetre
%	Percent
Δ	Delta
°C	Degree celcius
V	Voltage
g	Gravity
A	Absorbance
mM	Millimolar
M	Molar
b	Base
Da	Dalton
w/v	weight/volume
v/v	volume/volume

CHAPTER 1: LITERATURE REVIEW

1.1 Stroke

Stroke is a cerebrovascular disease which attacks the brain. Stroke occurs when blood supply to part of a brain is interrupted, causing the affected brain part deprived of oxygen and nutrients and subsequently leading to irreversible brain cell death. Stroke is a single most common cause of adult severe disability and remains as a serious health problem throughout the world (Hamidon *et al.*, 2012). In Malaysia, stroke is one of the five leading factors of death and one of the major factors of hospitalisation besides ischemic heart disease, pneumonia, septicaemia and chronic lower respiratory disease (Loo and Gan, 2012). In 2010, the stroke mortality rate had increased to 14.36 per 100,000 population (Ministry of Health, 2010) from 12.08 per 100,000 population in 2000 (Ministry of Health, 2000), and the rate is expected to continuously increase for the next 10 years (Ministry of Health, 2010).

Human brain is a complex structure made up of billions of brain cells such as neurons and glial cells. Brain cells are permanent cells, they are incapable to regenerate once matured and they require continuous supply of glucose and oxygen for its heavy workload. These characteristics of brain cells make stroke becomes life-threatening because disruption of blood supply to brain within a few minutes is sufficient to cause the brain cell death and trigger permanent brain damage (Brass, 1992). Brain damage can lead to many devastating effects such as paralyse on one side or whole body, difficulty in speaking, inability to understand speech and many other cognitive and neurological deficits. The adverse effect of stroke depends on which part of the brain being affected whilst the severity of deficits depends on the extent of damage caused by the stroke.

1.2 Types of Stroke

1.2.1 Ischemic Stroke

There are two main types of stroke: ischemic stroke and hemorrhagic stroke. About 87% of stroke cases are ischemic. An ischemic stroke occurs when there is a clot disrupts the blood flow to a particular region of brain (Figure 1.1).

Figure 1.1 Ischemic stroke. (Source: http://www.stroke.org)

There are three major types of ischemic stroke: thrombotic stroke (Figure 1.2A), lacunar stroke (Figure 1.2B) and embolic stroke (Figure 1.2C). Thrombotic stroke occurs when a thrombus (blood clot) develops in the brain larger arteries due to atherosclerosis. The fatty deposits on the artery lining disrupt normal blood flow to brain supplied by the artery (Figure 1.2A). In contrast, lacunar stroke happens when an occlusion which occurs in brain small artery penetrates deep into the brain. Lacunar stroke is often associated with chronic hypertension which causes a small arteriole becomes abnormal and susceptible to occlusion from micro-thrombi (Figure 1.2B) (Shah, 2006). On the other hand, embolic stroke is caused by embolus (clot) formed in elsewhere in the body (heart or carotid arteries) which travels in the bloodstream until it reaches a blood vessel in a brain which blocks its passage. Embolic stroke is usually associated with atrial fibrillation (abnormal heart rhythm in which the atria do not beat effectively) which eventually results in clot formation (Brass, 1992). It is also caused by clot dislodges from the atherosclerotic plaque formed in aorta and carotid artery (Figure 1.2C) (Silva *et al.*, 2011).

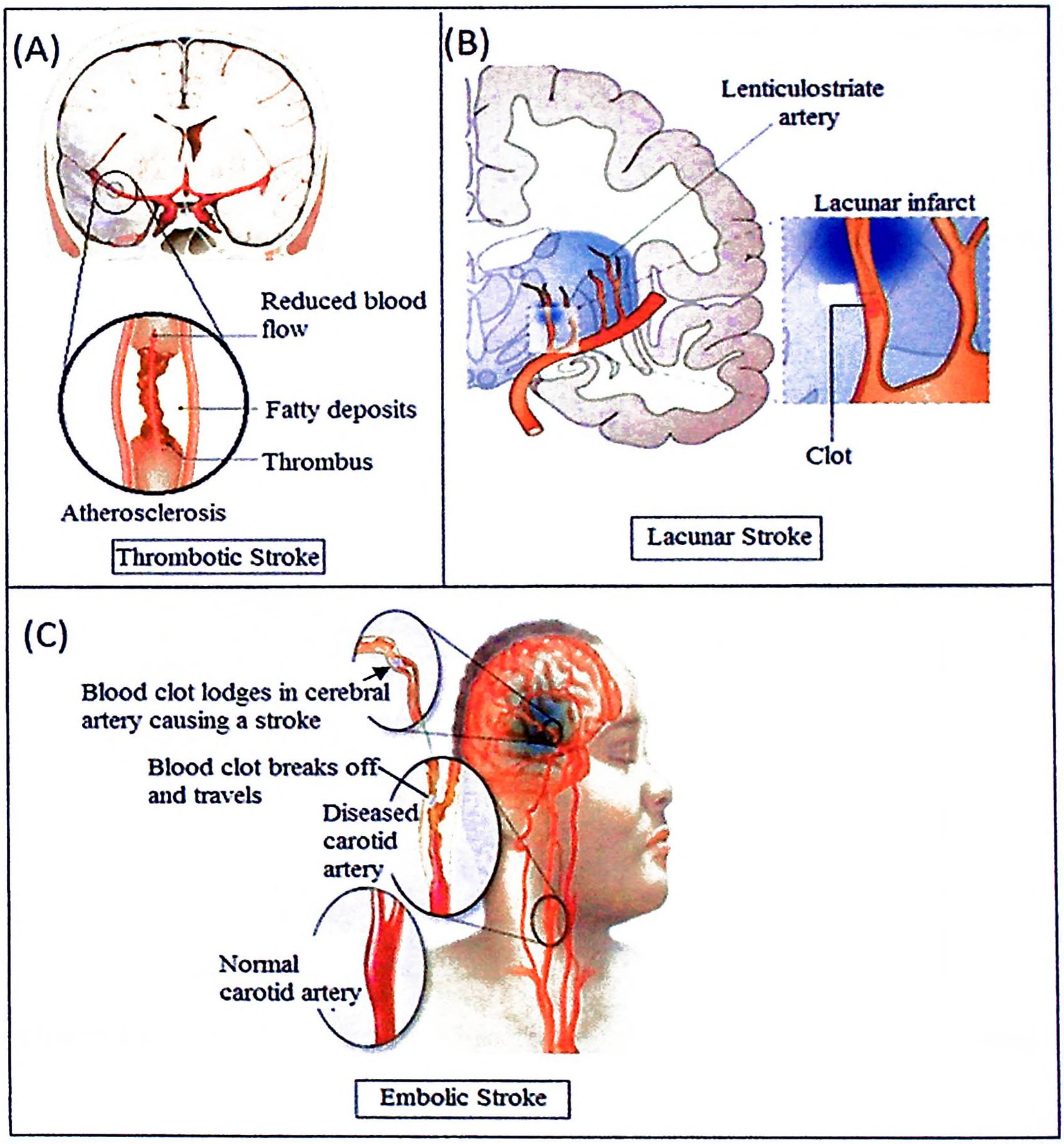

Figure 1.2 Types of ischemic stroke. (A) Thrombotic stroke occurs when thrombus formed from atherosclerosis blocks the blood flow. (B) Lacunar stroke occurs when a clot obstructs normal blood flow in one of the small arteries found deep in the brain. (C) Embolic stroke occurs when clot dislodges from distant site of a brain, travels to and lodges in an artery in the brain. (Source: http://www.mayoclinic.org; Sörös *et al.*, 2013)

1.2.2 Hemorrhagic Stroke

Hemorrhagic stroke is less likely to occur as compared to ischemic stroke. It accounts for only approximately 13% of all strokes incidents. It happens when blood vessel in the brain ruptures and causes bleeding inside or around brain tissue (Figure 1.3). The leaching blood will diffuse into brain tissue and increase the pressure inside the brain. Increased pressure inside the brain will push the adjacent sides of the brain tissue and blood vessels against the skull and results in further damage. Besides this, vasospasm (secondary constriction) of artery adjacent to bleeding site may occur due to irritating by-products from leaking blood. It can interrupt blood flow and cause a secondary ischemic stroke (Brass, 1992).

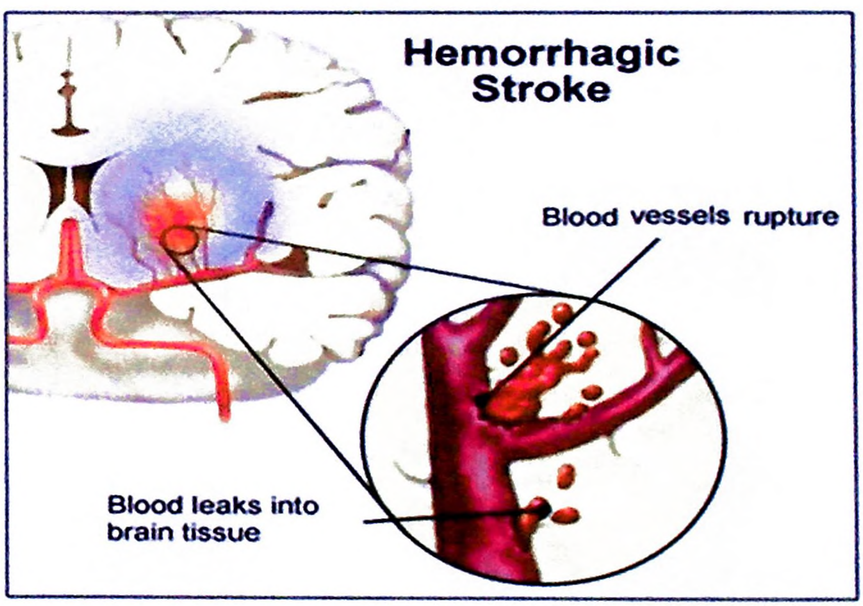

Figure 1.3 Hemorrhagic stroke. (Source: http://www.medicalassessment.com)

Hemorrhagic stroke is subdivided into two main types based on the location of bleeding: intracerebral hemorrhage (Figure 1.4A) and subarachnoid hemorrhage (Figure 1.4B) (Vega, 2014). Intracerebral hemorrhage occurs when blood leaks inside the brain tissue.

It is usually caused by uncontrolled high blood pressure which weakens the wall of small vessel inside the brain and causes the vessel becomes easily rupture (Figure 1.4A). Subarachnoid hemorrhage happens when blood leaks into the subarachnoid space, the area between the arachnoid membrane and pia meter of the brain (Figure 1.4B). It is usually caused by aneurysm and arteriovenous malformation (AVM). Aneurysm is an outpouching of a weak spot in a vessel wall which will rupture and result profuse bleeding in the brain. AVM is an abnormal collection of blood vessels in which arteries directly connected to veins without the capillaries. These vessels are fragile and easily bleed (Brass, 1992).

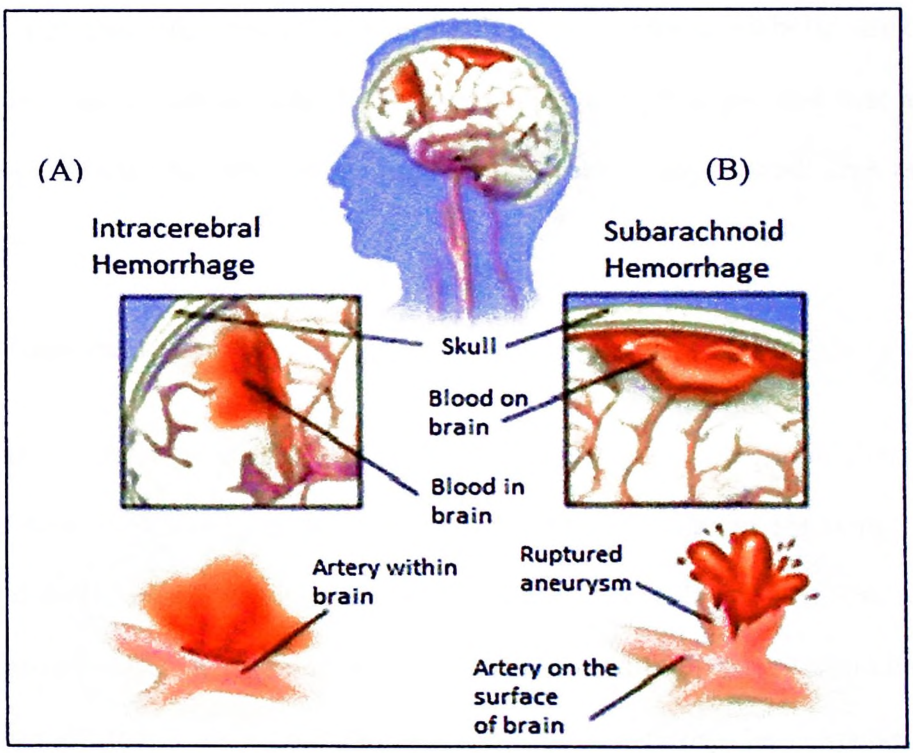

Figure 1.4 Types of hemorrhagic stroke. Panel A: Intracerebral hemorrhage occurs when artery within the brain ruptures and bleeding occurs inside the brain. Panel B: Subarachnoid hemorrhage occurs when a blood vessel ruptures upon the surface (weak point) of the brain, causing blood spills into the subarachnoid space. (Source: http://visihow.com)

1.3 Prognosis of Stroke

Patients' mortality rate after a first stroke is roughly 30% one year after onset and this rate increases sharply to 60% five years after onset. The chance of survival in stroke patients depends on type of stroke suffered. Hemorrhagic stroke patients achieve a higher mortality rate than ischemic stroke because the hemorrhagic stroke does not only cause the death of brain cells but also trigger vasospasm and increase intracranial pressure which may cause additional adverse effects to the patients (Hamidon *et al.*, 2012). As compared among ischemic stroke subtypes, embolic stroke is more serious than the other two ischemic stroke subtypes (thrombotic stroke and lacunar stroke) because recurrence often occurs within the first few weeks in embolic stroke patients. On the other hand, patients with lacunar stroke have highest survival rate and exhibit better recovery than the other types because it involves only a small area of the brain (Brass, 1992).

1.4 Symptoms of Stroke

Stroke can result in neurological and functional deficits. Neurologic deficits include sensation loss, inability to understand speech and difficulty in speaking. Functional disabilities include loss of movement, social disability and depression. The severity and type of deficits suffer depend on the size and location of brain injury. Stroke in the right hemisphere of the brain can result in short-term memory loss, spatial-perceptual problems and left hemiplegia. Injury in the left hemisphere can cause speech and language deficits as well as right hemiplegia. The consequences of stroke in the cerebellum including loss of coordination and slurred speech. Each patient will encounter different problems and require different needs as a result of stroke (Brass, 1992).

1.5 Current Treatment of Stroke

Immediate treatment is required in order to reduce stroke complications. For ischemic stroke, treatment aims at restoring blood flow to the ischemic penumbra in order to minimise mortality and morbidity caused by brain damage and prevent recurrence or secondary complications of stroke. In case of hemorrhagic stroke, emergency treatment aims at avoid further bleeding and reduce increased pressure caused by blood accumulating in brain. Generally, current treatment for stroke can be categorized into three aspects: pharmacological treatment, surgical treatment and rehabilitation treatment.

1.5.1 Pharmacological Treatment

Tissue plasminogen activator (tPA) is the United States Food and Drug Administration (USFDA) approved thrombolytic agent used to treat acute ischemic stroke. tPA will attach and activate plasminogen which aids in thrombus lysis. It is usually injected intravenously into a vein of arm. It had been proved to be effective in reducing complications in patients who suffered ischemic stroke when treatment was given within 3 hours from stroke onset (Hankey, 2007). However, tPA administration is only eligible for patients with stroke occur within 3 hours (Brown *et al.*, 2006) because administration beyond 4.5 hours after stroke onset will cause cerebral hemorrhage transformation (Shinozuka *et al.*, 2013b). This is because blood may burst through damaged blood brain barrier (beyond the occlusion) when blood flow is resumed after 4.5 hours of stroke onset, causing profuse bleeding into the brain which can be fatal to the patients (Hankey, 2007).

Aspirin is the most common antiplatelet agent used for immediate treatment of ischemic stroke or transient ischemic stroke. Blood clot is mainly formed from aggregation of platelets in the blood vessels. Aspirin interferes normal platelet function which is essential for clot formation (Hankey, 2007). Aspirin benefits patients by reducing death and functional dependency among ischemic stroke patients as well as rate of recurrence (Antithrombotic Trialists' Collaboration, 2002). However, aspirin may cause some side effects such as gastrointestinal bleeding and upper gastrointestinal disturbance (Hankey, 2007).

Anticoagulation medications like warfarin are also used in stroke treatment. Anticoagulant acts by disrupting the normal coagulation cascade which can prevent stroke recurrence effectively especially in patients with atrial fibrillation (Brass, 1992; Brown *et al.*, 2006). However, improper use of warfarin could cause excessive bleeding or insufficient protection from stroke. Therefore, frequent monitoring of dosage is required. Also, anticoagulation therapy is not recommended for treating acute ischemic stroke (Hamidon *et al.*, 2012) because evidence showed no overall benefits with anticoagulation therapy as it did not reduce mortality and disability rates but increased the risk of intracranial and extracranial hemorrhage (Gubitz *et al.*, 2004).

Anti-hypertensive drug is given especially to patient with hemorrhagic stroke in order to lower blood pressure which will help to decrease ongoing bleeding from diseased arteries. Anti-hypertensive drugs are usually used to prevent stroke recurrence especially those patients with hypercholesterolemia and hypertension. However, the usage of anti-hypertensive medication during acute stroke treatment is controversial because reduction of blood pressure may compromise cerebral blood supply especially to ischemic penumbra which may lead to further damage (Brown *et al.*, 2006).

1.5.2 Surgical Treatment

Besides pharmacological medications, surgical procedure sometimes is performed when needed. For example, endovascular mechanical thrombectomy is performed especially for stroke patients who are ineligible for thrombolysis or for those patients after a failed thrombolysis treatment (Hamidon *et al.*, 2012). A mechanical device consists of a guide catheter, a microcatheter and a retriever is used to dissociate or remove the clot in blocked artery. In case of hemorrhagic stroke in which increased intracranial pressure is present, craniotomy is performed in order to remove excess fluid or blood in the brain to reduce pressure in the brain. These surgical procedures have successfully reduced the incidence of stroke recurrence (Hankey, 2007) but it does not have extra beneficial effects on ongoing symptoms in patients who have had a stroke (Brown *et al.*, 2006).

1.5.3 Rehabilitation Treatment

Stroke survivors are subjected to rehabilitation therapy which includes physiotherapy, occupational therapy, speech and language therapy as well as psychology and psychiatry treatment. The purpose of rehabilitation is to restore lost function caused by stroke which in turn helps stroke patients to carry out their daily activities as usual as possible and to reduce functional dependence, social impairments as well as stress on the family and carer. Physiotherapy is important to help patients who are partially paralyzed. Exercise will help patients to improve muscle tone and increase the range of motion which in turn enhance the motor recovery. Therapist also helps patients to re-establish the fundamental of language or recall what they learned before the incidence. Stroke survivors often encounter psychological problems such as depression, loss of self-esteem and social interaction with others. Hence, psychotherapy will help stroke survivors who encounter psychological problems (Brown *et al.*, 2006). However, rehabilitation therapy can help them recover from disabilities to certain extent only. The period and extent of recovery is uncertain because they are influenced by a number of factors such as the degree of rehabilitation therapy received, motivation of recovery, severity of stroke and age at the time of stroke onset. The greatest extent of stroke recovery usually occur in the first three to six months from stroke onset (Jørgensen *et al.*, 1999; Hankey *et al.*, 2007). Following six months above after stroke, less functional improvement will be observed (Hamidon *et al.*, 2012).

1.5.4 Limited Recovery Rate after Current Treatment for Stroke

Prognosis after current stroke treatments is very limited. Both pharmacological medications and surgical interventions may help to ameliorate the adverse outcomes caused by stroke, but they are not curative because brain damage is irreversible. Stroke patients still suffer certain extent of disabilities after stroke treatments. According to National Stroke Association, there were only 10% stroke survivors exhibit full recovery, 25% with mild disabilities and 50% with moderate-to-severe disabilities which require special care (National Stroke Association, 2014). This indicates that current stroke treatments are not fully effective and very limited because they are only meant to minimise damage and reduce stroke recurrence. Therefore, there is an urge for alternative treatment for stroke. Currently, stem cell has emerged as a promising therapeutic agent for stroke due to its self-renewal and differentiation potential.

1.6 Stem cell-based Therapy for Stroke

Brain cells are permanent cells and they are incapable to regenerate once they are matured, this can lead to permanent neurological deficits and physical deficits if the brain cells are damaged or dead. Stem cells present in many organs of human body. They are undifferentiated cells which are capable of self-renewal and differentiate into any specific functional cells depend on their lineages in response to suitable stimulation. Stem cells play an important role to regenerate specific cells or tissues in response to body conditions and requirements. For example, neural stem cells (NSCs) can be differentiated into new functional neuron to replace those permanently damaged neuron due to stroke and restore brain function. These cells showed positive signs as functional improvement was observed in stroke models after cells transplantation (Andres *et al.*, 2008). Besides this, NSCs also secrete neural trophic factors such as vascular endothelial growth factor (VEGF) and nerve growth factor (NGF) which have neuroprotective effect that can help to improve functional recovery after ischemic stroke (Hao *et al.*, 2014). Therefore, using stem cells as regenerative treatment for stroke is practicable and it is specially meant for patient in chronic phase of stroke.

Many stem cell types had been investigated to identify feasibility, safety and efficacy of stem cell replacement therapy in stroke model, including: embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adipose-derived stem cells (ADSCs), bone marrow stem cells (BMSCs), umbilical cord blood stem cells (UCBCs), as well as NSCs (Andres *et al.*, 2008).

1.7 Stem Cell Classification

Human body is made up of many types of differentiated cells which are originated from stem cells. Stem cells are usually classified based on their potency to differentiate into different lineage of cell and their origin. Based on the potency, stem cells are classified into five types: totipotent, pluripotent, multipotent, oligopotent and unipotent.

1.7.1 Totipotent

Totipotent stem cell has the greatest potency among all the stem cell types. It is formed from the union of a sperm and an ovum, which is a zygote. Cells which are produced after first few division of an embryo (morula) are totipotent in nature. A totipotent stem cell can proliferate and give rise to any differentiated cells such as brain, heart and blood cells required to form an entire functional human body (Figure 1.5A) (Hui *et al.*, 2011).

1.7.2 Pluripotent

Pluripotent stem cells are derived from totipotent stem cells after four days of fertilization when an early blastocyst is formed. Inner cell mass of blastocyst comprised of pluripotent stem cells will form the three germ layers (ectoderm, mesoderm and endoderm) of embryo and give rise to all cell types found in body except the placenta which will be formed by the outer cell mass of blastocyst. Pluripotent stem cells are incapable to make extraembryonic tissue which will form the placenta and this make it different from totipotent stem cells (Singh *et al.*, 2014). Example of pluripotent stem cell is embryonic stem cell (Figure 1.5B).

1.7.3 Multipotent

Multipotent stem cells are more differentiated cells derived from pluripotent stem cells. This stem cell can give rise to more than one cell types of a few lineages based on their location in the body. For example, hematopoietic stem cells present in the bone marrow can differentiate into both lymphoid stem cells and myeloid stem cells (Figure 1.5C) (Singh *et al.*, 2014).

1.7.4 Oligopotent

Oligopotent stem cells are able to differentiate into only a few cell types of a specific lineage. For example, lymphoid stem cells derived from multipotent hematopoietic stem cell are oligopotent and only able to differentiate into limited cell types such as T cell and B cell (Figure 1.5D) (Singh *et al.*, 2014).

1.7.5. Unipotent

Unipotent stem cells have unlimited reproductive capabilities but can only differentiate into one cell type which perform a specific function. Examples of a unipotent stem cell are muscle stem cells and epithelial stem cells which can only differentiate into muscle cells and epithelial cells, respectively (Figure 1.5E) (Hima and Srilatha, 2011).

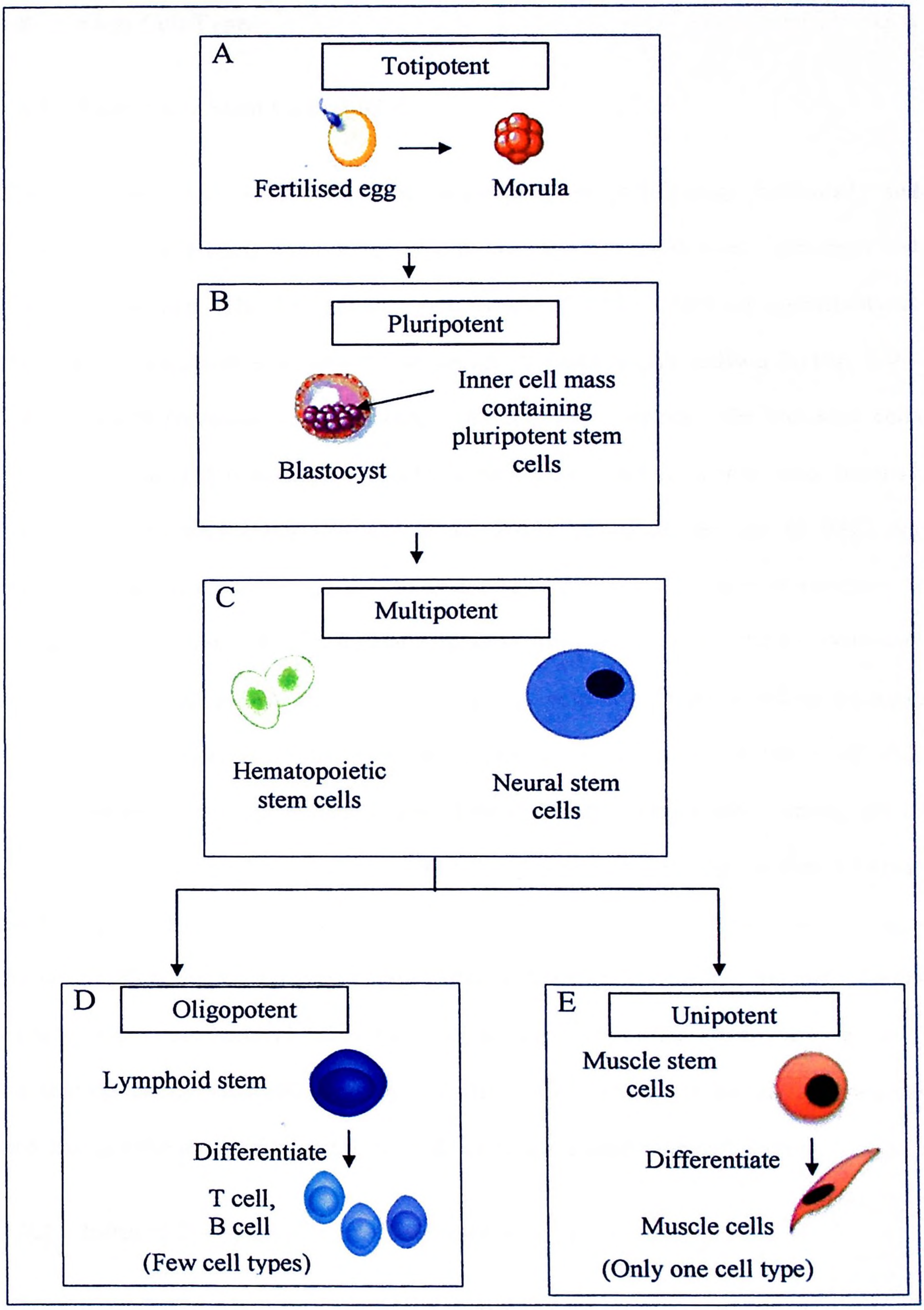

Figure 1.5 Classification of stem cells based on differentiation potential.

1.8 Stem Cell Types

1.8.1 Embryonic Stem Cells (ESCs)

ESCs are pluripotent stem cells which are capable of proliferating indefinitely and differentiate into all cell types of three germ layers. ESCs are derived from inner cell mass of blastocyst. The pluripotent characteristic of ESCs offers an opportunity of establishing regenerative treatment for stroke. A study which utilised human ESC-derived neural precursor was transplanted in stroke mice models. The precursor cells were found to differentiate into electrophysiologically active neuron and improve functionally recovery (Drury-Stewart *et al.*, 2013). However, the use of ESCs for transplantation is controversial due to ethical concerns need to destroy embryos to obtain ESCs and the risk of teratoma formation (Shinozuka *et al.*, 2013a). Although recent literature cited differentiation of ESCs may help to reduce the risk of teratoma formation (Hui *et al.*, 2011), extra efforts are required to direct differentiation of ESCs in order to obtain functional adult cell in desired lineage. Furthermore, feeder cells of animal origin are used in ESC differentiation protocol, increasing the risk of cross contamination in human ESCs culture environment (Singh *et al.*, 2014). In addition, a further purification process was required after differentiation of ESCs on animal feeder cells to obtain pure human NSCs (Hui *et al.*, 2011). This purification process was time-consuming and not cost-effective. The limitations of ESCs had raised ethical concerns and changed the research direction towards using alternative stem cell sources.

1.8.2 Induced Pluripotent Stem Cells (iPSCs)

Induced pluripotent stem cells (iPSCs) are cells derived from somatic cells by meant of reprogramming. iPSCs have features similar to ESCs in which the cells can differentiate into any types of cells of three germ layers that make up of human body. In 2006, Yamakana *et al.* had demonstrated the production of iPSCs from mouse fibroblast by force expression of four pluripotency transcription factors; Oct4, Sox2, c-myc and Klf4 (Yamanaka and Takahashi, 2006). Based on Yamanaka *et al.*, Oct4 and Sox 2 are important factors which can maintain the pluripotency of cells while the function of c-myc and Klf4 is yet to be confirmed (Yamanaka and Takahashi, 2006). These genes have been turned off in somatic cells. The researchers delivered these four transgenes into the host genome by meant of retrovirus transduction, permitting their long term gene expression in somatic cells, reprogramming the somatic cells back to pluripotent state (Yamanaka and Takahashi, 2006). Invention of iPSCs allows researchers to manipulate these cells for various kinds of stem cell therapies because it can be differentiated into any cells types by manipulation. Study showed that iPSCs can direct differentiate into NSCs *in vitro* and these transplanted cells have the potential to improve functional outcome in stroke animal models (Valerio and Sugaya, 2014). Using iPSCs for stem cell therapy does not encounter ethical conflict and the source of cells such as skin cells can be easily obtained from intended patients. This has offered extra benefit in which graft rejection can be avoided due to autologous transplantation. However, using iPSCs may impose a risk of tumorigenesis due to c-myc transgenes (Valerio and Sugaya, 2014) and the reprogramming method and directed differentiation of iPSCs into neuronal lineage are time-consuming and costly.

1.8.3 Adipose-derived Stem Cells (ADSCs)

Adipose tissue contains plenty of adult stem cells which are multipotent in nature. ADSCs can give rise to cells of multiple lineages such as neural, glial, and vascular endothelial cells *in vitro* and secrete trophic factors such as VEGF and hepatocyte growth factor (HGF) (Ikegame *et al.*, 2011). Thus, ADSCs arise as a potential source of stem cells for stroke regenerative treatment. This is further supported by studies which reported attenuation of ischemic damage after ADSCs transplantation in stroke animal model (Ikegame *et al.*, 2011; Li *et al.*, 2012; Gutiérrez-Fernández *et al.*, 2013). Besides that, autologous transplantation of ADSCs also avoids graft rejection and ethical issue as well as no risk of teratoma formation. However, ADSCs are not in neuronal lineage and thus directed differentiation is required to obtain lineage-desired cells.

1.8.4 Bone Marrow-derived Stem Cells (BMSCs)

Bone marrow transplantation is a well-known procedure used in cancer treatment related to blood. Adult bone marrow contains hematopoietic stem cells, mesenchymal stem cells and endothelial progenitor cells (Shinozuka *et al.*, 2013a). Hematopoietic stem cells can differentiate into cells of blood lineage such as erythrocytes, leukocytes, platelets and lymphocytes. Mesenchymal stem cells will differentiate into many cell types such as osteoblasts, chondrocytes, adipocytes and hematopoietic supporting stroma using appropriate inducer (Singh *et al.*, 2014). Endothelial progenitor cells are responsible for the formation of mature endothelial cells that line the blood vessel. Literature cited transplantation of these stem cells helped to improve functional recovery in stroke mice model (Bliss *et al.*, 2007). Using this stem cells source can avoid ethical issue and it enables autologous transplantation which can help to prevent immunogenic reaction. Besides, there is no risk of tumorigenesis associated with BMSCs as compared to ESCs and iPSCs. However, similar to ADSCs, these kinds of stem cells are not in neuronal lineage. Extra efforts are required to direct differentiation of these stem cells into cells of neuronal lineage. Furthermore, the differentiation potential of these stem cells into neuronal tissue is uncertain (Bliss *et al.*, 2007). In addition, painful invasive procedure is required in order to obtain BMSCs from donor.

1.8.5 Umbilical Cord Blood Stem Cells (UCBCs)

Umbilical cord blood contains rich sources of stem cells similar to BMSCs. Moreover, no painful invasive procedure involved because these stem cells can be obtained from placenta and umbilical cord after baby delivery. Therapeutic benefits of UCBCs are associated with trophic factors released by the stem cells. Neurotrophic factor such as VEGF and NGF can induce angiogenesis and stimulate endogenous repair mechanism in injured area. In addition, thrombopoietin present in cord blood promotes proliferation and survival of hematopoietic stem cells (Chen *et al.*, 2001). However, therapeutic potentials of UCBCs are related to trophic factors production rather than neuron replacement. Thus, a cell source with closer lineage to neuron cells is more preferable for stem cell-based treatment for stroke.

1.8.6 Neural Stem Cells (NSCs)

NSCs are undifferentiated cells which are capable of self-renewal and proliferate. NSCs are precursor in neuronal and glial lineage that can differentiate into neuron, oligodendrocytes, astrocytes and microglia. NSCs can be derived from the fetus and adults mammalian brain. Subventricular zone (SVZ) in the lateral ventricles of the brain was found to harbour NSCs which allow continuous neurogenesis in the brain (Andres *et al.*, 2008) (Figure 1.6).

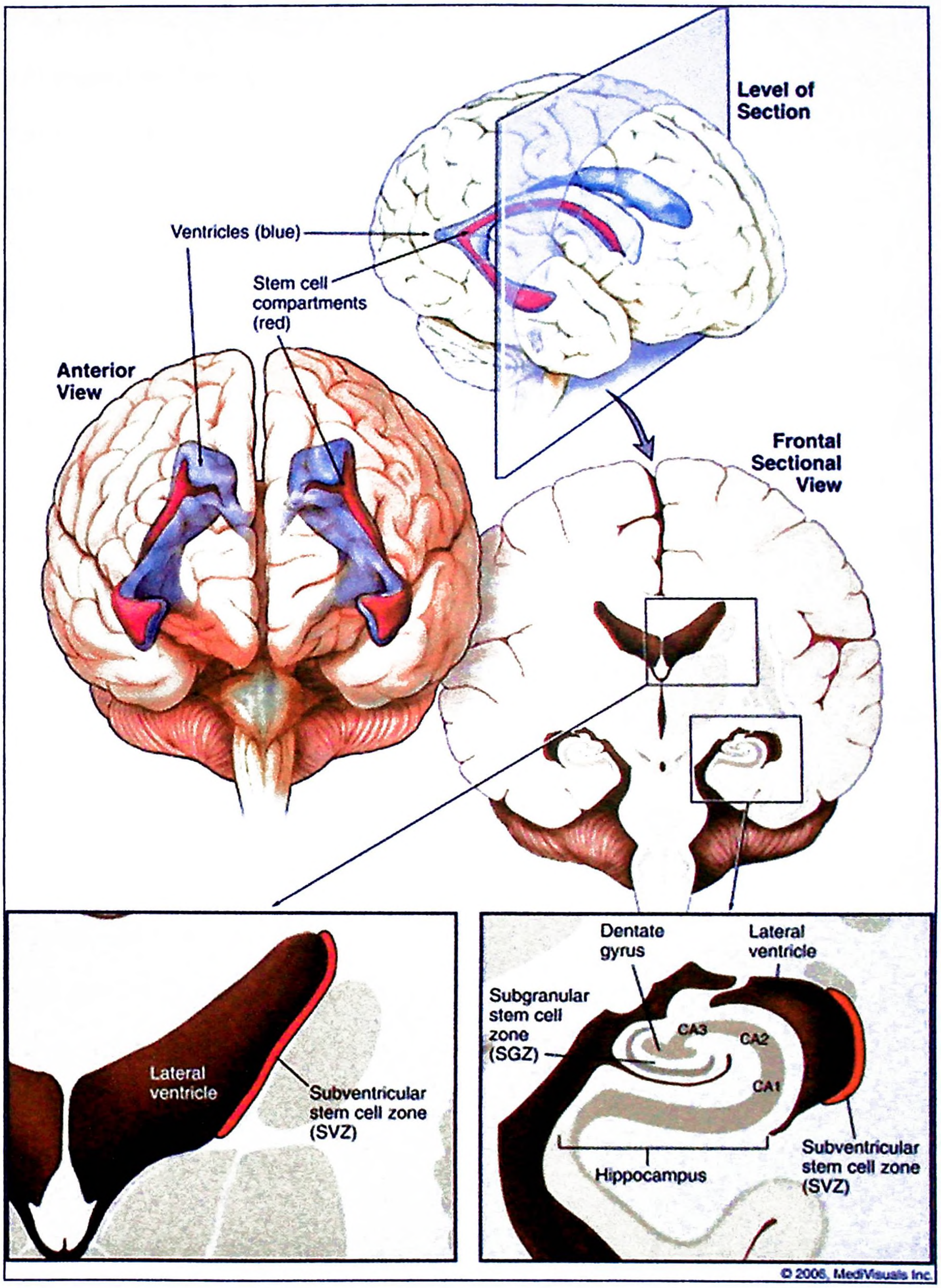

Figure 1.6 The anatomical location of SVZ in human brain which harbour NSCs (red). (Source: beyondthedish.wordpress.com/)

NSCs in SVZ are activated when stroke or hypoxia occurs. These undifferentiated cells will migrate towards the site of injury in response to chemokine signals such as stromal-derived factor 1 (SDF-1), VEGF (Shinozuka *et al.*, 2013a) and neutrophin which promote endogenous repair and regenerative mechanism after stroke (Gincberg *et al.*, 2012). However, endogenous repair may be insufficient or fail in severe cases due to limited number of endogenous NSCs. Therefore, endogenous NSCs were isolated from SVZ and expanded *in vitro* prior to transplantation into stroke animal models to enhance the therapeutic potential of these NSCs in treating stroke disease (Andres *et al.*, 2008). Transplanted NSCs can home to the injured site, secrete neurotrophic factors, promote angiogenesis and differentiate into functional brain cells to replace damaged brain cells and integrate into the existing neuronal circuits (Gincberg *et al.*, 2012). Utilising adult NSCs for stem cell therapy of stroke does not impose risk of teratoma formation in comparison with ESCs. Besides, NSCs can be harvested from brain of intended patient, expanded and differentiated into neuronal specific cells *in vitro* before transplantation. This enables autologous transplantation which eliminates the risk of graft rejection. Selecting NSCs as a source of stem cell therapy is less laborious compared with other stem cells types because NSCs are in neuronal lineage and they will differentiate into brain cells in nature. Less complicated differentiation protocol is required for this stem cell type and this can result in quicker treatment to the patients which can better improve functional outcomes. Thus, in this study, the methods of isolation and expansion of NSC from human SVZ was investigated.

A summary of all stem cell types which had been investigated for its therapeutic potential for stroke disease is listed in Table 1.1.

Table 1.1 Comparison of stem cell types which have been tested for stroke therapy. Cell source, advantages and disadvantages of each cell types are listed.

Cell Type	Source	Differentiation potential	Ethical Conflict	Immunogenic Rejection	Risk of Tumorigenicity	Neuronal Lineage
ESC	Inner cell mass of blastocyst	Yes	Yes	Yes	Yes	No
iPSC	Somatic cells	Yes	No	No	Yes	No
ADSC	Adipose tissue	Yes	No	No	No	No
BMSC	Bone Marrow	Yes	No	No	No	No
UCBC	Umbilical cord blood	Yes	No	No	No	No
NSC	SVZ of human brain	Yes	No	No	No	Yes

1.9 Primary Human Neural Stem Cell (hNSC) Culture from Human Brain Subventricular Zone Tissue

Adult-derived NSCs can be obtained by meant of surgical procedure from SVZ in the brain of intended patient and grow in suitable culture condition. NSCs are naturally present in SVZ during brain development. They prefer to reside in the specialised microenvironment of SVZ because it contains various transcriptional factors which are important to prevent NSC death, to retain their multipotency and to determine their differentiation. Primary cell culture always contains heterogeneous cell population

(Harry *et al.*, 1998). Cell culture under hypoxia or use prolyl-4-hydroxylase inhibitors (PHDIs) to activate hypoxia-inducible factor (HIF) response was shown to improve stem cells survival in hypoxic environment (Tan *et al.*, 2011). However, a relatively pure culture of NSCs is difficult to be obtained due to the presence of cellular impurities and other cell types (Harry *et al.*, 1998). In addition, downstream analysis of NSCs is restricted by limited cell number. Hence, in present study, NSCs were also cultured from established GIBCO® H9 hNSC line (H9-hNSC) in order to obtain sufficient cell number for downstream analysis such as characterisation and differentiation assays prior to primary hNSCs.

1.10 GIBCO® H9 Human Neural Stem Cell (H9-hNSC) Line Culture

H9-hNSCs are multipotent cells produced from National Institutes of Health (NIH) approved human ESC line H9 in which researchers do not need to encounter ethical issue and laborious isolation and derivation process with the use of ESCs. The morphology appearance of H9-hNSCs is shown in Figure 1.7.

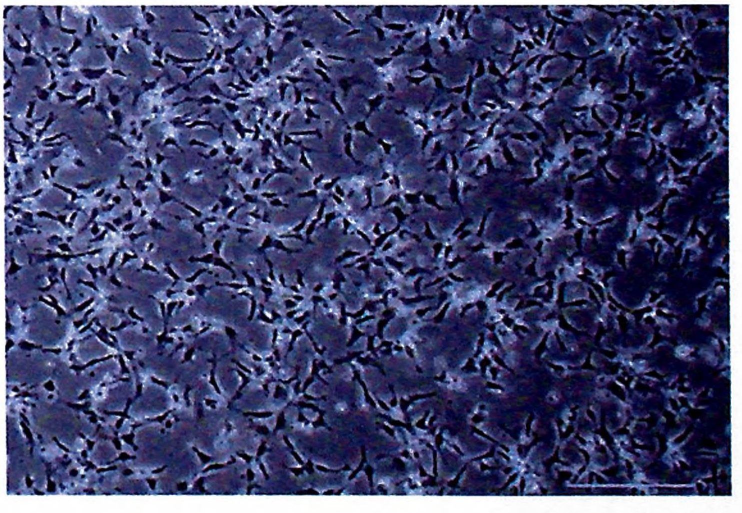

Figure 1.7 Morphology appearance of H9-hNSCs under phase contrast microscope. (Source: Invitrogen, 2009).

This H9-hNSC possesses characteristic of self-renewal. It is able to proliferate rapidly in an undifferentiated state in an adherent culture condition with the presence of complete StemPro® NSC serum free medium (SFM) contains essential growth factors such as epidermal growth factor (EGF) and fibroblast growth factor (FGF). Besides this, H9-hNSC holds a proliferation potential up to 3 passages. Therefore, more H9-hNSCs can be obtained for downstream analysis. These H9-hNSCs can be identified through the detection of undifferentiated NSCs-specific markers such as Nestin and CD133 using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot methods (Invitrogen, 2009).

1.11 Characterisation of NSCs

Characterisation of NSCs by specific markers expression enables the identification of true NSC in primary culture. In this study, Nestin and CD133 were selected to characterise NSCs based on the cell line manufacturer (Invitrogen, 2009). Nestin is an intermediate filament protein (type IV) and usually used as a marker for NSC. Nestin expression plays an important role to the survival and self-renewal characteristics of NSC and its expression was regulated by bFGF and EGF (Park *et al.*, 2010). However, expression of Nestin alone is insufficient to characterise NSCs. Thus, CD133, one of the reliable markers for NSCs which determines the multipotency of stem cells was chosen (Kozubenko *et al.*, 2010). In this study, SVG-p12 (a human glial cell line) was used as a negative control to differentiate the gene expression between H9-hNSCs and SVG-p12 (Figure 1.8). SVG-p12 is a differentiated cell with glial fibrillary astrocytic protein (GFAP) expression. Hence, the absence of GFAP in H9-hNSCs further validates the presence of H9-hNSCs. The analysis of target gene and protein expression were performed by qRT-PCR and Western blot respectively in this study.

Figure 1.8 Morphological appearance of SVG-p12 under inverted light microscope.

1.12 Differentiation of NSCs

NSCs are undifferentiated cells which can generate cells of neuronal lineages. Differentiation of stem cells is regulated by signals present in the microenvironment in adult brain as well as external signalling. Signalling molecules such as neurotransmitters, hormones, and growth factors can control differentiation of these multipotent cells into neurons, oligodendrocytes and astrocytes. Spontaneous differentiation of NSCs *in vitro* can be induced by replacing a medium without growth factor in an adherent culture condition (Bazán *et al.*, 2004; Dottori and Pera, 2008). H9-hNSCs showed potential to differentiate into neuron and glial cells after multiple passages in differentiation medium (Invitrogen, 2009). Different composition (specific factors and/or hormone) in differentiation medium can induce NSC differentiation into different cell types. For instance, glial differentiation from NSCs can be achieved by culturing NSCs in a differentiation medium containing bFGF, EGF as well as platelet-