Investigation of p75 neurotrophin receptor on human Dental Pulp Stem Cells (hDPSC)

Wenru Pan

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Declaration

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Abbreviations

Alpha- Modified eagles medium (a-MEM)

Analysis of variance (ANOVA)

β-mercaptoethanol (BME)

Brain derived neurotrophic factor (BDNF)

Basic fibroblast growth factor (bFGF)

Bovine serum albumin (BSA)

Butylated hydroxyanisole (BHA)

Calcium (Ca^{2+})

Central nervous system (CNS)

Dental pulp stem cells (DPSC)

Dulbecco's modified eagles medium (DMEM)

Dimethyl sulfoxide (DMSO)

Dorsal root ganglia (DRG)

4', 6-diamidino-2-phenylindole (DAPI)

Epidermal growth factor (EGF)

Embryonic stem cells (ESC)

Enzyme linked immunosorbent assay (ELIZA)

Ethylenediaminetetraacetic acid (EDTA)

Extracellular matrix (ECM)

Fluorescence activated cell sorting (FACS)

Foetal calf serum (FCS)

Glial-derived neurotrophic factor (GDNF)

Glial fibrillary acidic protein (GFAP)

Granulocyte-colony stimulating factor (G-CSF)

Ham's F-12 (F12)

Hanks balanced salt solution (HBSS)

Hematopoietic stem cells (HSC)

Human dental pulp stem cells (hDPSC)

Human immunodeficiency virus (HIV)

3-isobutyl-1- methylxanthine (IBMX)

Insulin, transferring & sodium selenite premix (ITS)

Melanoma cell adhesion molecule (MCAM),

Myenteric plexus (MP)

Mesenchymal stem cells (MSC)

Microelectrode arrays (MEA)

Middle cerebral artery occlusion (MCA-O)

Nerve growth factor (NGF)

Neural crest (NC)

Neural stem cells (NSC)

Neurofilament medium chain (NFM)

Neuronal nuclei (NeuN)

Nitric dioxide (NO)

Neurotrophin 3 (NT3)

Paraformaldehyde (PFA)

Penicillin-streptomycin-glutamine (PSG)

Peripheral nervous system (PNS)

Poly-l-lysine (PLL)

Propidium iodide (PI)

Polysialylated neural cell adhesion molecule (PSA-NCAM)

Recombinant tissue plasminogen activator (r-TPA)

Rostral migratory stream (RMS)

Standard error means (SEM)

Subventricular zone (SVZ)

Subgranular zone (SGZ)

Submucosal plexus (SP)

12-O-tetradecanoylphorbol 13-acetate (TPA)

Transcranial magnetic stimulation (TMS)

Tyrosine kinase receptors (Trk)

Abstract

p75 neurotrophin receptor has recently been suggested as a neural stem cell marker in cells of the brain subventricular zone and brain subgranular zone. Human adult dental pulp stem cells (hDPSC) with the ability to differentiate into neural, chondrocyte, osteocyte and adipocyte lineages contain heterogeneous stem cell populations. p75 is prototypically a neutrophin receptor. hDPSC expressing the neural precursor marker nestin are able to differentiate into functionally active neurons *in vitro* under differentiation protocols, though there is no definitive method established yet. p75 is thus likely to delineate a population of hDPSC that become neural cells. Therefore, this current project aims to characterize p75 expression on hDPSC by immunohistochemistry and flow cytometry (chapter 3); investigate the role of p75 on hDPSC neural potential *in vitro* (chapter 4)*;* and investigate hDPSC neural differentiation *in vitro* through neurosphere formation (chapter 5).

This current study demonstrated that p75 is a neural stem cell marker in hDPSC cultures and defines a cell population with the potential to give rise to neurons and glial cells. Immunohistochemistry showed that $p75^+$ hDPSC had higher expression of SOX1, SOX2, nestin, CD146 and SOX9 (nucleus) when compared to $p75^-$ hDPSC. Neurons generated from $p75^+$ hDPSC exhibited more neuronal-like properties in their morphology, and immunohistochemical expression pattern, in particular with neuronal marker neurofilament medium chain (NFM). This study has also shown that hDPSC are able to differentiate into a neural lineage via neurosphere formation *in vitro*.

The results indicated that $p75^+$ hDPSC are a functional cell population which could mediate the hDPSC neural protection and neural replacement seen in stem cell therapy for stroke brain repair. This study identified a molecular target that could be used to enrich populations through cell

sorting. In addition, the hDPSC neurosphere differentiation provides a superior analysis model to investigate the neural potential of hDPSC *in vitro*.

1. Chapter 1- Introduction

1.1 Stroke

1.1.1 Overview of stroke

Stroke is a sudden vascular disease caused by scarcity of the blood supply into the brain, and leads to brain injury and neuron death (AIHW 2011).There are two categories of stroke events: 1) ischaemic stroke caused by a blood clot (embolus or thrombus) blocking arteries; 2) haemorrhagic stroke caused by the bursting of a blood vessel leading to leakage of blood (Shiber, Fontane & Adewale 2010). In Australia ischaemic stroke is more prevalent, with about 80% of stroke patients having ischaemic stroke and the remaining 20% of patients experiencing a haemorrhagic stroke (National Stroke Foundation 2012). Stroke tends to be a healthy issue in old patients, with70% of stroke patients aged 65 and over (AIHW 2013). Stroke is more prevalent in aged males, with the proportion of males who had had a stroke (72%) being marginally higher than females (68%) (AIHW 2013)

Stroke is the second leading cause of death in Australia. It was estimated that there were 60,000 new and recurrent stroke events in 2010 (McCann et al. 2009), and over 8,300 patients died from stroke in Australia (AIHW 2013). Most of the patients who have a stroke are left with disabilities in motor function, sensory function, cognition and communication (AIHW: Senes 2006). Approximately $2.14 billion is spent annually on stroke in Australia and approximately $50 billion was spent per year on stroke, directly and indirectly, in the USA (Ingall 2004; McCann et al. 2009). Therefore, there is a need for a new therapy for stroke.

1.1.2 Treatment of stroke

Different treatments are given in hospital for stroke patients. Ischaemic stroke patients are usually treated with thrombolysis, in which administration of intravenous recombinant tissue plasminogen activator (r-TPA) is given (Jivan, Ranchod & Modi 2013). To prevent further stroke events, antiplatelet drugs such as aspirin are usually given to patients afterwards (Hankey & Warlow 1999). Due to its sudden and severe properties, treatment for haemorrhagic stroke usually involves medication to control blood pressure and surgery for a bleeding aneurysm (Mak, Lu & Wong 2013).

Though there are various acute stroke treatments, most of the stroke patients are left with some disability (Ingall 2004). For these patients to achieve full recovery from stroke, post-stroke rehabilitation is emphasized. Stroke recovery has been shown to be improved with physiotherapy, speech therapy and electrical stimulation (Hankey 2005). Another promising treatment for stroke that is currently in its early stages of research and patient trial is the use of stem cells to restore the brain neuronal network.

1.2 Stem cells

1.2.1 Overview of stem cells

Stem cells are defined as cells that can maintain self-renewal potential and differentiate into different types of daughter cells (Loeffler 1997). In other words, stem cells can generate daughter cells identical to the mother cells (self-renewal) and daughter cells with more restricted potential (differentiation). Stem cell self-renewal is important for maintaining the stem cell pool population and stem cell differentiation is essential for organ development and cell replenishment.

There are generally two types of stem cells: 1) embryonic stem cells (ESC) which originate from the inner cell mass or the germinal ridge of embryos—these stem cells can differentiate indefinitely

in vitro (self-renewal) and generate all the cell lineages required for an individual development (differentiation); 2) adult stem cells which originate from ESC but keep maintaining the population (self-renewal) in adult tissue throughout the individual's life and can give rise to all the cell lineages required for development of an organ (differentiation) (Loeffler 1997).

1.2.2 Adult neural stem cells

The adult brain neural stem cell (NSC) population resides in two regions of the adult brain: the subventricular zone (SVZ) and the dentate gyrus subgranular zone (SGZ) (Figure 1.1). Endgoenous neurogenesis in adult SVZ has been suggested to be associated with neuronal repair after ischemic brain injury (Romanko et al. 2004). New-born neurons from SGZ regions are integrated into neuronal networks and involved in the formation of memory (Alvarez-Buylla, Herrera & Wichterle 2000; Bruel-Jungerman, Davis & Laroche 2007). NSC are cells which have neurogenic potential. Neurogenesis involves differentiation into neurons and glial cells such as astrocytes and oligodendrocytes (Dulac & Cmeron-Curry 1997). Neurogenesis requires a neurogenic niche/ microenvironment, which is usually restricted to particular regions throughout adulthood.

NSC also remain in a heterogeneous mesenchymal stem cell population (MSC) (Gage 2000). MSC can be stimulated to undergo neurogenesis through a neural differentiation protocol *in vitro*. It has been reported that the neural differentiation pathway can be induced in MSC including bone marrow stem cells (BMSC). Post-differentiated BMSC express mature neuronal nuclear protein (NeuN) and neural glial cell marker glial fibrillary acidic protein (GFAP) through β-mercaptoethanol (BME) promotion or epidermal growth factor (EGF) or brain derived neurotrophic factor (BDNF) activation *in vitro* (Khang et al. 2012; Sanchez-Ramos et al. 2000). Adult skin stem cells also have the ability to differentiate into neurons, glial cells, smooth muscle cells and adipocytes *in vitro*

(Joannides et al. 2004). This indicates that adult-derived stem cells have a promising differentiation potential, rather than being restricted to specific tissue cell type.

Stroke-induced neurogenesis has been suggested to be related to the spontaneous partial or full recovery of stroke patients (Jin et al. 2006). Most neurogenesis is believed to take place in the SGZ of the dentate gyrus and the SVZ. Newly divided beta III tubulin positive neurons are present around the infarct area and preferentially localize in the vicinity of the blood vessels (Jin et al. 2006). This compensatory neurogenesis has provided a target for stem cell therapy in stroke recovery (Jin et al. 2006).

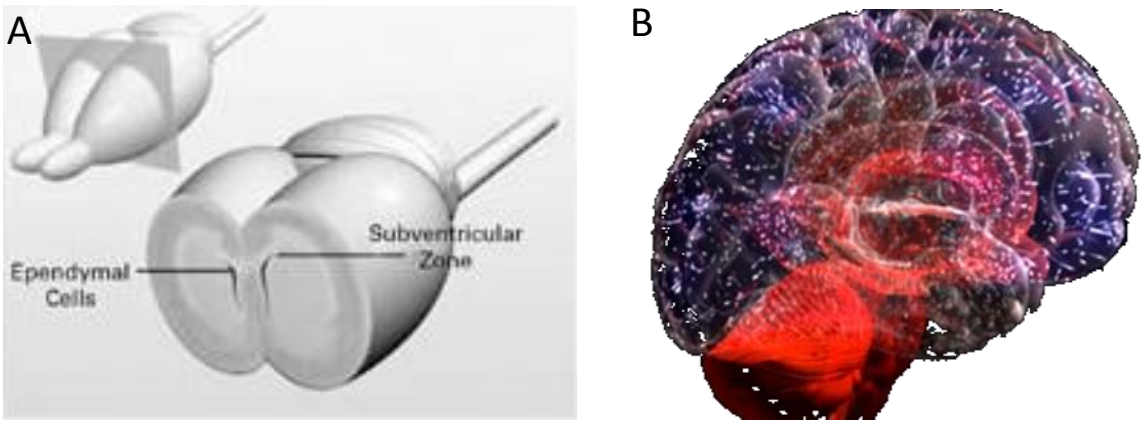


Figure 1.1 Diagram of endogenous neurogenesis in adult brain

A) Brain SVZ neurogenesis is responsible for neuronal repair after brain injury. B) Brain SGZ neurogenesis is responsible for memory formation.

chamber (Figure 1.2). A tooth is structurally described to have two components: the crown and the root (d'Aquino et al. 2009). The crown is exposed into the oral cavity whereas the root is covered under the gum. The layer covering the crown is a hard tissue layer called enamel and the outside of the root is covered with a bone-like tissue cementum. Underlying the enamel and cementum is dentine which is more a bone-matrix like tissue. Odontoblasts and nerve fibers are found in

dentine, these are responsible for maintaining dentine and sensation. Dental pulp stem cells are multipotent stem cells that have the potential to differentiate into a variety of cell types (Fraser & Bronner-Fraser 1991). There are also MSC, neural fibers, blood vessels, and lymphatics present in the dental pulp chamber. The blood vessels and nerve bundles in the dental pulp chamber also provide nutrition and sensation for responding to external stimuli.

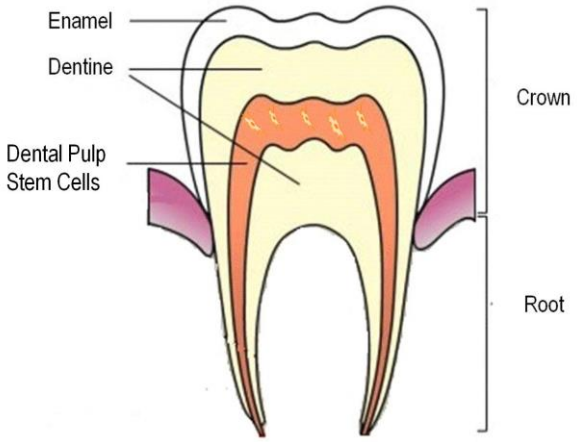


Figure 1.2 Diagram of tooth structure.

Layers of tooth structure including the inner dental pulp chamber which contains dental pulp stem cells. Images adapted from (Cate 1998).

1.2.4.1 Chemically induced neural differentiation

Human dental pulp stem cells (hDPSC), expressing the neural precursor marker nestin, are able to differentiate into functionally active neurons *in vitro* under different protocols, though there is no definitive method yet (Arthur et al. 2008; Kiraly et al. 2009; Vollner et al. 2009). There are two types of neural differentiation methods described in literature for hDPSC. One method is chemically inducing hDPSC to neural differentiation as a cell monolayer by exposing cells to toxic chemicals such as β-mercaptoethanol, 5-azacytidine, dimethyl sulfoxide (DMSO) and putrescine (Kiraly et al. 2009; Osathanon et al. 2013; Takeyasu M 2006; Zhang et al. 2006). The other method is to grow cells as a three-dimensional neurospheres by using the growth factors basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) (Sasaki et al. 2010).

Both chemically induced and growth factor induced neural differentiation methods can generate cells with neuronal morphology that express early neuronal markers such beta III tubulin. One protocol which uses 5-azacytidine (DNA methyltransferase inhibitor) and PKC-cAMP activator (dbcAMP, forskolin, IBMX and TPA) can generate functional neurons with K^+ and Na^+ channel expression, measurable by patch clamping (Kiraly et al. 2009). However, no neuronal network signaling was detected in cells on microelectrode arrays (MEA) differentiated by Aanisma group that use the Kiraly protocol (Aanismaa R 2013). Furthermore, substantial cell death was observed during chemically driven murine DPSC differentiation (Ellis et al. 2014).

1.2.4.2 Growth factor induced neural differentiation

In cell cultures, NSC tend to proliferate as floating neurospheres that maintain their multipotent properties in a serum-free medium, supplemented with EGF and bFGF (Wachs et al. 2003). EGF and bFGF were found to induce cell proliferation and inhibit cell differentiation (Reynolds & Weiss 1992). Other commercial supplements such as N2 or B27 were also used in base medium DMEM/F12.

The neurosphere formation assay was firstly described by Reynolds and Weiss for expansion, purification and isolation of NSC from a heterogeneous stem cell population e.g. embryonic stem cells and adult stem cell population (Reynolds & Weiss 1992). As there are no definitive markers for NSCs, cell pluripotency/ neurogenicity was based on the formation of a neurosphere from a single cell, which is a good indicator of the presence of NSC (Ahmed 2009)

Three types of cell have been identified in SVZ derived neurospheres: 1) astrocytes/type B cells which are GFAP positive; 2) transient amplifying cells/ type C cells which are SOX2, Olig2 positive; 3) Migratory neuroblasts/ type A cells which are beta III tubulin (Tuj) positive (Gil-Perotin et al.

2013). These cells are neural lineage progression cells in different stages that migrate through the rostral migratory stream (RMS) and finally give rise to the olfactory bulb during SVZ neurogenesis (Doetsch, Garcia-Verdugo & Alvarez-Buylla 1997). Neurospheres derived from NSCs provided a physiologically relevant model system for study of neurogenesis and neural development.

The neurosphere differentiation of hDPSC was often associated with neuronal morphology, less cell death (fewer toxic factors applied), expression of mature neuronal markers such as beta III tubulin, neurofilament medium chain (NFM) and glial cell marker GFAP (Osathanon, Nowwarote & Pavasant 2011; Sasaki et al. 2008; Vollner et al. 2009). However, as summarized in Table 1.2, all the differentiation protocols continuously supplemented cells with EGF and bFGF, keeping the cells in a pluripotent state instead of allowing differentiation. An advanced two-step protocol for hDPSC neurogenesis via neurosphere formation and neurotrophin driven differentiation was developed in this study.

Table 1.1 hDPSC neural differentiation methods

Reference	Cell source	Base medium	Growth factors	Protein expression	Gene expression	Electrophysiology
Chemically induced						
Takeyasu et al, 2006	ratDPSC	DMEM/F12	Putrescine	GFAP	βIII-tubulin, Mtap2, GFAP	
Zhang et al, 2006	hDPSC	α-MEM	β-mercaptoethanol, dimethyl sulfoxide (DMSO), butylated hydroxyanisole	NeuN, NSE	-	-
Kiraly, et al 2009	hDPSC	DMEM/F12	5-azacytidine, IBMX, TPA, forskolin dbcAMP	GalC, βIII-tubulin, NeuN, NF-M, GFAP	Musashi, NSE, NF-M, GFAP	Na+ and K+ channel expression by patch clamping
Aanismaa, et al 2012	hDPSC	DMEM/F12	5-azacytidine, IBMX, TPA, forskolin dbcAMP	GalC, βIII-tubulin, Neural specific enolase (NSE), NF-M, GFAP	Musashi, NSE, NF-M, GFAP	No neuronal network detected by MEA, Na+ and K+ channel expression by patch clamping

Reference	Cell source	Base medium	Growth factors	Protein expression	Gene expression	Electrophysiology
Growth factor induced						
Iohara et al, 2006	Porcine DPSC	Neurobasal A	EGF, bFGF,B27, N2, NT3, L-glutamine	neuromodulin	Neuromodulin, neurofilament	-
Vollner et al, 2009	hDFC	Neurobasal A	L-glutamine, G5 (biotin, , EGF, bFGF, insulin, transferring, selenite)	βIII-tubulin, NSE, neurofilament	βIII-tubulin, NSE, neurofilament	-
Govindasamy et al, 2010	hDPSC	Neurobasal A	EGF, bFGF, N2, NT3, L-glutamine	-	βIII-tubulin, NF, GFAP	-
Sasaki et al, 2010	hDPSC	Neurobasal A	EGF, bFGF, B27, L-glutamine, N2, NT3	-	βIII-tubulin	-
Osathaonon et al, 2011 Osathanon et al, 2013	hDPSC	Neurobasal A	EGF, bFGF, B27, L-glutamine, retinoic acid	βIII-tubulin	βIII-tubulin, SOX2, SOX9, GABA receptor α2, β2, β3	-

1.2.5 Stem cell therapy for stroke

There are two clinical approaches to stem cell therapy in stroke: endogenous and exogenous approaches (Banerjee et al. 2011). The endogenous approach aims to stimulate endogenous stem cell migration, for example using granulocyte-colony stimulating factor (G-CSF) to stimulate brain neuron stem cell mobilization and differentiation (Banerjee et al. 2011). The exogenous approach is to transplant stem cells with neurogenic potential intra-cerebrally or systemically into patients.

Many different stem cells can be used as a source for the exogenous stem cell approach. Current stem cells used are embryonic stem cells and fetal stem cells. Fetal stem cells are more restricted in the lineage that they differentiate into, as they are often responsible for particular tissue development in a fetus, and are referred to as pluripotent (d'Aquino et al. 2009). However, acquiring embryonic or fetal stem cells raises serious biological, ethical and legal issues thus limiting widespread use of these stem cells. Therefore, adult stem cells such as those from the dental pulp chamber may be a better source of stem cells for stem cell therapy.

Stem cells derived from dental pulp would be a very attractive alternative source for cell-based stroke therapies for four reasons: 1) DPSC are capable of differentiating into cells of neural lineage (Arthur et al. 2008). Neurons derived from DPSC express the neuronal markers beta III tubulin, NF-M and, upon patch clamping, demonstrate voltage dependent Na+ and K+ channels; 2) DPSC are a safe and easily-accessible resource compared with embryonic and fetal derived stem cells; 3) self-transplantation of SC from the patient's teeth does not raise a transplantation rejection issue; 4) hDPSC proliferate rapidly, therefore large numbers of stem cells are able to be grown up for autologous transplantation.

There is evidence that transplantation of hDPSC improves brain function and cognition in the rat model which has previously undergone middle cerebral artery occlusion surgery (MCA-O) (Leong et al. 2012). It was also found that only 2.3% of the originally transplanted DPSC survived in the rat brain 4 weeks following implantation. Surviving hDPSC differentiated into astrocytes and neurons but rarely differentiated into the endothelial, cardiac or smooth muscle cells(Leong et al. 2012). Most of the hDPSC that survived were NSC and played an important role in post-ischaemic rehabilitation. An effective method is needed to isolate the cell population with neurogenic potential from DPSC. As DPSC are a heterogeneous population, an effective strategy must be developed to isolate, propagate and enrich the cells with neurogenic potential from the whole DPSC population. The study on whether p75 neurotrophin receptor defines a cell population with neurogenic potential may provide an evidence for solving this problem.

1.3 The role of p75 in neurogenesis

1.3.1 The role of p75 on adult SVZ neurogenesis

p75 positive cells were reported as being co-expressed with NSC marker nestin, occasionally co-expressed with polysialylated neural cell adhesion molecule (neuroblasts), but not co-expressed with GFAP (astrocytes), NeuN (neurons) or SOX10 (oligodendrocyte lineage) markers (Giuliani et al. 2004; Young, KM et al. 2007). This suggested a role for p75 in the regulation of neurogenesis in the early stage. Flow cytometry sorted p75 high-expressing SVZ cells have greater neurogenicity than p75 medium-expressing SVZ cells, while p75 low-expressing SVZ cells fail to generate any neural lineage (Young, KM et al. 2007). This is consistent with an *in vivo* study that reported reduced neuroblasts in SVZ of p75 knockout mice compared with p75 wildtype mice (Young, KM et al. 2007).

A study by Young's group also found that p75 neurogenesis is mediated through BDNF binding (Young, KM et al. 2007). Treatment with BDNF induced an 80% increase in the number of neurons generated within p75 high-expressing SVZ cell population, but there was no change in the number of neurons generated from the p75 low-expressing SVZ cell population.

An *in vivo* study by Galvao's group found that infusion of BDNF resulted in no change in neurogenesis in the SVZ (Galvao, Garcia-Verdugo & Alvarez-Buylla 2008). Compared to Young's experiment, a higher concentration of BDNF was applied in Galvao's study. However, the Zigova et al study where Galvao's method was adapted showed that the same high doses of BDNF exerted a positive effect on SVZ neurogenesis *in vivo* (Zigova et al. 1998). A different concentration was not the explanation for the contradiction of BDNF effect on neurogenesis because the two studies also differ in p75 expression. Galvao's group reported no p75 expression in mouse SVZ and low

expression of p75 in rat SVZ, while the Young et al study purified rat p75-high expressing cells, which reconcile to p75 mediated SVZ neurogenesis via BDNF activation. Nonetheless, more research is clearly needed to understand the role of BDNF in neurogenesis through p75 activation.

In Alzheimer's disease, amyloid-beta peptide, which accumulates in amyloid plaques, can also promote neurogenesis of SVZ via p75 both *in vitro* and *in vivo* (Sotthibundhu et al. 2009). During adult neurogenesis, SVZ cells migrate along the rostral migratory stream to become interneurons in the olfactory bulb (Hu, H et al. 1996). The over-stimulated p75 mediated SVZ neurogenesis by secretion of amyloid-beta will lead to depletion of the stem cell reservoir, thus decreasing newly generated neurons in the olfactory bulb. This is correlated with decreased olfaction during the development of Alzheimer's disease (Murphy et al. 2002).

1.3.2 The role of p75 on adult SGZ neurogenesis

Spontaneous neurogenesis takes place in another brain region in the dentate gyrus subgranular zone (SGZ) of the hippocampus during adulthood. The hippocampus neurogenesis, particularly in the SGZ cilia cells, is associated with learning and memory (Acsady & Kali 2007; Aimone, Deng & Gage 2010; Han et al. 2008; Lledo, Alonso & Grubb 2006). p75 and newly-dividing cell marker BrdU are co-expressed in new born neurons and glia cells in brain SGZ (Bernabeu & Longo 2010; Chakravarthy et al. 2010; Colditz et al. 2010). A further study showed that SGZ cilia cells, which account for extensive neurogenesis, have 20-50% higher expression of p75 compared to cells in hippocampal CA1 and CA3 regions (Chakravarthy et al. 2010).

Two studies have clearly described p75 as a NSC marker by comparing the neurogenic potential of SGZ cells from p75 wildtype and p75 knockout mice (Catts et al. 2008; Colditz et al. 2010). p75 knockout mice have reduced hippocampus and dentate gyrus size, particularly the layer of SGZ,

due to a reduction in generation of new born neurons (Catts et al. 2008). A further immunohistochemical analysis demonstrated that these p75 knockout mouse SGZ cells were demonstrated to have a 25% reduction in the number of neuroblast marker polysialylated neural cell adhesion molecule (PSA-NCAM) positive cells, which indicated reduced neurogenic potential in brain SGZ (Catts et al. 2008).

A behavior study revealed that p75 knockout mice have depressive-like behavior in a feed test and a subtle impairment in spatial memory in a Morris water maze test due the reduced neuroblasts and neural regeneration (Catts et al. 2008). p75 knockout mice failed to regain neurogenesis with fluoxetine (an anti-depressive drug) treatment in brain SGZ cells compared to p75 wildtype mice. On the contrary, p75 knockout mice displayed superior spatial memory and learning ability in Barnes maze trials (Barrett et al. 2010). This is because p75 might not only have a regulatory function with respect to SGZ neurogenesis, but may also influence the activity of cholinergic activity, thus affecting synaptic plasticity (Martinowich et al. 2012). Further research is needed to clarify neurogenesis and its related behavior in p75 deficient mice.

1.3.3 The role of p75 on neurogenesis during embryogenesis

p75 is largely expressed by NC stem cells and has been used as a tool to purify and isolate NC cells (Conrad et al. 2011; Rao & Anderson 1997). NC stem cells are responsible for generation of various tissue such as neurons and glia of the peripheral nervous system (PNS), as well as pigment-producing melanocytes of the skin *in vivo* and *in vitro* (Fraser & Bronner-Fraser 1991; Rao & Anderson 1997; Stemple & Anderson 1992).

Several studies have reported p75 is associated with neural generation in NC cells (Lameu et al. 2012; Rao & Anderson 1997; Wilson et al. 2004). During embryo neurogenesis, NC cells migrating

from the dorsal neural tube have been suggested to be heterogeneous, with some cells being multipotent stem cells and some being lineage-specific (Fraser & Bronner-Fraser 1991; Ishii et al. 2012; Sieber-Blum 1989). One *in vivo* study found that $p75^+$ NC cells and Kit^+ NC cells are a separate population in the dorsal region of murine neural tube prior to the migration of NC cells (Wilson et al. 2004). Tracing the Kit^+/ $p75^-$ cells showed that these cells temporarily expressed p75 during migration, but ultimately developed into melanocyte progenitors. However, $p75^+$/Kit^- NC cells migrated into the ventral region of the embryo trunk, indicating their commitment into ventral NC derivatives such as peripheral neurons and glial cells (Becker et al. 2012; Rifkin et al. 2000).

A functional *in vitro* study further revealed that p75 related neurogenesis in NC was through ligand binding of neurotrophin BDNF(Lameu et al. 2012). Treatment with BDNF resulted in up-regulation of p75 gene expression in NC cell culture, which consequently restored normal neural generation level after inhibition of nitric oxide (NO, neurogenesis up-regulator). In fact, up-regulation of p75 gene expression was two to three-fold through co-treatment of BDNF and NO inhibitor, providing a mechanism for BDNF-driven p75 up-regulation in NC stem cell neurogenesis.

1.3.4 The role of p75 neurogenesis with respect to other tissue

Though there has been no conclusive study on p75 as a NSC marker in the peripheral central nervous system (CNS), there is evidence that p75 is associated with neurogenicity in gut and dorsal root ganglia (DRG) (Becker et al. 2012; Joseph et al. 2011; Li, Say & Zhou 2007; Rifkin et al. 2000). p75 is highly expressed in NC cells that give rise to neurons and glial cells in PNS tissue and is down-regulated in mature cells postnatally (Wilson et al. 2004; Yiu & He 2006). Some of the p75 positive NC stem cells may still remain in a particular stem cell niche of these tissues and play a role in replenishment.

Adult stem cells isolated from the myenteric plexus (MP) have higher p75 and SOX10 expression than stem cells from the submucosal plexus (SP), suggesting higher p75 positive NC stem cell content in MP stem cells. These cells have greater self-maintaining, self-renewal abilities and lower apoptosis. Most importantly, MP stem cells exhibited superior neurogenic potential *in vitro* compared with cells isolated from the submucosal plexus (Kruger et al. 2002).

NSC from DRG were p75 positive *in vivo* and *in vitro* (Li, Say & Zhou 2007; Rifkin et al. 2000). p75 and tyrosine kinase (Trk)B receptor (another neurotrophin receptor for BDNF) co-existed *in vivo* on a subset of migrating and mitotic active cells. These $p75^{+}$/$TrkC^{+}$ stem cells were cells involved in the sensory neuron formation in DRG (Rifkin et al. 2000). Results were confirmed by an *in vitro* study that isolated $p75^{+}$/$nestin^{+}$ stem cells from adult rat DRG when cultured in the laboratory, that emigrated out and gave rise to neurons, glial cells and smooth muscle cells (Li, Say & Zhou 2007).

No study has established a clear, systemic mechanism for the p75 signaling pathway in neural differentiation. One possible mechanism might be the p75 regulation of stem cell cycle progression. NGF has been suggested to induce cell cycle arrest by inhibiting the formation of cyclin D1-Cdk4 complexes (Cragnolini et al. 2012). Several studies have already suggested the coupling of cell differentiation with cell cycle withdrawal in myogenic muscle cells (Lassar, Skapek & Novitch 1994; Walsh & Perlman 1997). However, cumulative evidence indicates that mitotic cycle withdrawal and re-entry lead to cell apoptosis (Liu, DX & Greene 2001). The presence of BDNF can prevent cell re-entry and cell death in new born neurons during early stages of development as a consequence of the interaction between NGF and p75 (Lopez-Sanchez & Frade 2002). This was consistent with many studies that have suggested that p75-directed neurogenesis takes place in the presence of BDNF (Galvao, Garcia-Verdugo & Alvarez-Buylla 2008; Gascon et al. 2007; Hosomi et al. 2003; Young, KM et al. 2007). Thus stem cell neural differentiation requires cell cycle withdrawal from its

regular symmetrical proliferation process followed by neural survival under BDNF support. Simultaneous provision of both NGF and BDNF into the stem cell population will result in neural generation through interaction with p75 receptors.

1.3.5 The role of p75 on DPSC neurogenesis

The adult brain SVZ stem cells, SGZ stem cells and stem cells in the PNS actually share a common origin during embryogenesis—neuro-ectodermal stem cells. The SVZ and SGZ cells in CNS were derived from the embryonic neural tube cells. The stem cells in the PNS were differentiated from the NC stem cells. During embryogenesis, the border of the neural plate folds to form neural tube while the roof of the plate cells migrated peripherally as neural crest cells. Dorsal neural tube cells and NC stem cells were all differentiated from neuro-ectodermal stem cells (Cate 1998).

Studies on cranial NC cells and DPSC have put a light on the origin of DPSC (Abe et al. 2012; Chung et al. 2009; Ishii et al. 2012). Post-migratory cranial NC stem cells intensively contributed to formation of craniofacial bone structure including the formation of teeth (Chung et al. 2009). It has been suggested that DPSC exhibit NC cell properties on expression of the cell markers p75, Snail and Slug and its generation of various cell types (Abe et al. 2012; Xiao & Tsutsui 2012).

As DPSC, SVZ cells and NSC from various peripheral nervous tissues share a common embryonic origin and can differentiate into neurons, the question of whether p75 will play the same role in DPSC differentiation into neurons and glial cells arises.

A recent study showed that p75 is expressed by adult human DPSC. By manual counting of immunohistochemistry stained images, approximately 4.3% of adult hDPSC were p75 positive. p75 was co-localized with NSC marker beta III tubulin, S100 and nestin, indicating potential p75 NSC

function in DPSC (Martens et al. 2012; Nam & Lee 2009). There is no current functional study on p75 function on DPSC neurogenesis. One study by a Japanese group has revealed that p75 negative deciduous hDPSC can differentiate into osteocytes and adipocytes while $p75^{+}$ hDPSC cannot do so (Mikami et al. 2011). A functional study aimed at analyzing p75 mechanisms showed that over-expression of p75 in a murine MSC line, C3H10T1/1, inhibited differentiation into the osteocyte and adipocyte lineage. Thus the presence of p75 may suggest an alternative neural differentiation pathway exists for DPSC.

1.4 Marker based stem cell purification

Cell separation technologies are widely used in different disease research areas such as in stem cell therapy, cancer research and immunological research for cell analysis and cell culture. In stem cell therapy, hematopoietic stem cells (HSC) were isolated, which is an essential step offering great potential for the treatment of hematological disorders and immunodeficiency (Peters et al. 2003). In cancer research, tumor cells were depleted from the bone marrow stem cell population, which is an essential step for autologous bone marrow transplantation. In immunological research, T-lymphocytes were positively selected for progression examination on human immunodeficiency virus (HIV) infection and autoimmune disease (Collins, Luebering & Shaut 1998).

Cell separation technologies are grouped into two types. The first group is based on the cell physical criteria such as size, shape and cell density differences, includes technologies such as cell filtration and cell centrifugation. The second group is based on the biochemical characteristics and surface marker expression of cells, which includes the capture of cells on affinity solid matrix(immune panning), fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) (Kamihira & Kumar 2007).

Cell density centrifugation is usually applied for debulking treatment of the samples and usually is used as a pre-enrichment step, for example, separation red blood cells from serum by centrifugation. To achieve separation of $p75^+$ and $p75^-$ hDPSC based on molecular expression difference, affinity based methods such as immune panning, FACS and MACS are chosen by using antibodies against surface p75.

1.4.1 Fluorescence activated cell sorting

FACS is a specialized technology in which cells are analyzed and separated at the same time using a flow cytometer. In short, target cells were firstly labeled with fluorophore conjugated antibody, and then flow through the laser beam in a flow cytometer machine. The fluorescence dyes in the antibody are activated by laser beam and emit fluorescence at a specific wavelength according to the properties of the fluorophore. The cells flow in the center of a liquid stream, which is called sheath flow. The sheath flow enables cells to flow through the laser beam one by one, thus cells are analyzed individually. The emitted light signals from each single cell including fluorescence wavelength, light-scattering and absorbance are measured. At the same time, the liquid flow is cut to form a droplet containing the one cell in one droplet. Since the liquid droplet is electrostatically charged if they form each single cell liquid droplets, these electrostatic droplets/ cell droplets can be collected by sending an electric current to the polarized boards. Therefore, a cell emitting fluorescence at a specific wavelength and at the certain strength is selected and separated instantly.

FACS with its utilization of highly specific antibodies is a technique characterized by high sensitivity and high resolution. However, according to the properties of $p75^+$ hDPSC, FACS is not suitable for hDPSC separation for several reasons. Firstly, cells which flow through the machine are at risks of contamination and undergo a high shear stress, leading to cell death during the procedure.

Secondly, the processing time is estimated to be 3-6 hours including pre-processing steps for immunostaining to ensure the 10^7-10^8 throughput, which takes a long time and cells lose their normal condition for cell recovery after the processing.

1.4.2 Immune panning

Immune panning belongs to the group of cell separation techniques based on the antibody recognition of cell surface marker expression and their affinity to a certain surface (Diogo, da Silva & Cabral 2012). Delta surface treated polystyrene flasks are firstly coated with antibodies against the surface antigen expressed by target cells. Antigen positive target cells and antigen negative cells can be separated by their differences in interaction strength with the coated surface under fluid flow, which terms rolling capacity differences. Antigen positive cells have a lower velocity of rolling/ lower rolling capacity under fluid flow, as they bind to the immobilized antibodies in the surface of polystyrene flasks. Antigen negative cells with higher velocity of rolling/ higher rolling capacity under fluid flow can be washed and collected by gentle washes as they only had adherence to polystyrene.

Immune panning is widely used in the isolation of low numbers of p75 positive embryonic motor neurons from the lumbar spinal cord (Wiese et al. 2010). This technique is characterized by high recovery as it applies lower shear force and no risk of laser beam contamination to cells compared to FACS. This technique is suitable for separation of small and fragile stem cells from the heterogeneous cell population.

1.4.3 Magnetic activated cell sorting

Magnetic activated cell sorting technology belongs to the affinity based stem cell separation group. It is a trademark name of company Miltenyi Biotec, Bergisch Gladbach, Germany. There are three

parts of the equipment: MACS separator, MS column and MACS multistand (Figure 1.3). There are generally two methods for separation of separate target cells from a heterogeneous population—either a positive selection or a negative depletion strategy. In positive selection, the target cells with antigen expression are labeled with antibodies thus labeled with magnetic beads and get retained in the magnetic field. The unbound/ magnetically negative cells are washed and collected. Cells labeled with magnetic beads later are removed from the magnetic field and collected by immediate flushing of buffer solution with a plunger.

In the depletion strategy, instead of the target cells, the unwanted cells are magnetically labeled (pre-analysis is required for the molecular expression of unwanted cells), and trapped in the magnetic field. The target/wanted cells flow through the column and are collected. Unwanted cells can also be flushed out by removal of magnetism and immediate elution of the buffer. The depletion method is usually used in the cases when no specific antibody is available for target cells or labeling of target cells is not desirable. Compared to FACS, MACS is characterized with comparable high sensitivity and potentially lower shear stress to cells. Therefore, MACS separation of $p75^{+}$ and $p75^{-}$ hDPSC was chosen in this project, based on its superiority compared to FACS.

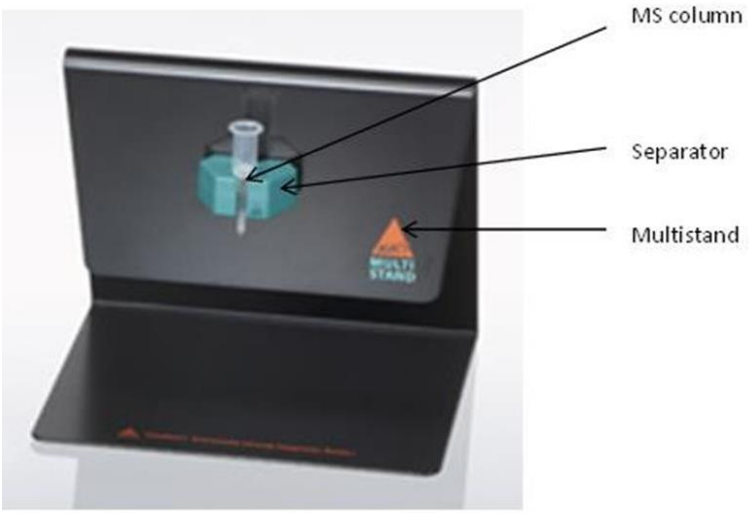


Figure 1.3 Apparatus for the magnetic separation of cells.

Figure adapted from (Miltenyibiotech, 2014)

1.5 Aims and Hypothesis

Based on a review of the literature, it is hypothesized that the p75 neurotrophin receptor defines a cell population with neurogenic potential. The primary objective of this thesis is to address the hypothesis by investigating both the expression and function of p75 in neurogenesis in several hDPSC cell populations. In order to achieve this, several research aims have been developed:

1. To characterize p75 expression on hDPSC *in vitro* by
    a. Flow cytometry
    b. Immunohistochemistry (IHC)
2. To investigate the neurogenic potential of separated $p75^{+}$ hDPSC and $p75^{-}$ hDPSC.
3. To develop a hDPSC neural differentiation protocol via neurosphere formation.

2. Chapter 2- General Materials and Methods

2.1 Materials

This chapter describes the general materials and methods used throughout this thesis.

Methodology and techniques specific to each chapter are described in each chapter.

Table 2.1 Reagents

Reagent	Unit size	Company/ Catalogue number
5-Azacytidine	10mg	Sigma, Australia/ A2385
Alexa Fluor 647 protein labeling kit	-	Invitrogen, Carlsbad/ A-20173
alpha-Modified eagle medium (α-MEM)	500ml	Sigma, Australia/ 12561056
B27	10ml	GibcoBRL, Australia/ 17504-044
BDNF	10 µg	Merk Millipore, Australia/ GF029
bFGF	50µg	Jomar, Australia/ CYT-386
Bovine serum albumin (BSA)	1kg	Sigma, Australia/ A-7906
Collagenase type IV	1gram	Life technology, Australia/ 17104019
dbcAMP	250mg	Sigma, Australia/ D0627
dimethyl sulfoxide (DMSO)	500ml	AnalaR/ 10323.4L

Reagent	Unit size	Company/ Catalogue number
Dispase	1 gram	Sigma, Australia/ D4693
dH_2O	500ml	GibcoBRL, Australia/ 15230-162
Donkey serum	10ml	Sigma, Australia/ D9663
Dulbecco's Modified Eagle Medium (DMEM)	500ml	Sigma, Australia/ 12800017
EGF	100µg	Jomar, Australia/ CYT-217
Ethylenediaminetetraacetic acid (EDTA)	500	AnalaR/ 10424
Forskolin	25mg	Sigma, Australia/ F6886
Ham's F-12 (F12)	500ml	Sigma, Australia/ 11039021
Hanks Balanced Salt Solution (HBSS)	500ml	GibcoBRL, Australia/ 24020117
3-isobutyl-1-methylxanthine (IBMX)	100mg	Sigma, Australia/ I5879
Laminin	1mg/ml	Sigma, Australia/ 23017015
L-ascorbate 2-phosphate		Novachem, Australia
L-glutamine	100ml	GibcoBRL, Australia/ 25030-081
N2 supplement	5ml	GibcoBRL, Australia/ 17502048
Nerve growth factor (NGF)	1mg	Sigma, Australia/ N0513
Neurotrophin 3 (NT3)	10µg	Sigma, Australia/ CYT688
Neurobasal A medium	500ml	Invitrogen, Australia/ 10888022

Reagent	Unit size	Company/ Catalogue number
12-O-tetradecanoylphorbol 13-acetate (TPA)	-	Sigma, Australia/ P8139
Penicillin/ Streptomycin	100ml	GibcoBRL, Australia/ 15070063
Penicillin-streptomycin – glutamine (PSG)	100ml	Invitrogen, Australia/ 10378-016
Poly-l-lysine (PLL)	10µg/ml	Sigma, Australia/ P47097
Prolong Gold antifade reagent	-	Invitrogen, Carlsbad/ P36935
Transferring & sodium selenite premix (ITS)	-	Sigma, Australia/ I1884-IVL
Triton-X	250ml	Sigma, Australia/ T-8787
Trypsin/ EDTA	100ml	GibcoBRL, Australia/ 15400054

Table 2.2 Media

Medium	Supplement
Standard DPSC medium	10% (v/v) foetal calf serum (FCS), 200mM L-glutamine, 100U penicillin 100U streptomycin, 10mM L-ascorbate 2-phosphate in alpha-modified eagle medium (α-MEM)
Kiraly et al Neuronal differentiation	
Neuronal Induction medium	2.5% (v/v) FCS, 1% (v/v) penicillin/streptomycin in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12)
Epigenetic reprogramming medium	10μM 5-azacytidine, 2.5% (v/v) FCS, 10ng/ml bFGF, 100U penicillin/streptomycin in DMEM/F12
Neuronal differentiation medium	250μM 3-isobutyl-1-methylxanthine (IBMX)), 200nM Forskolin, 1mM 12-O-tetradecanoylphorbol 13-acetate (TPA), 10ng/ml dbcAMP 10ng/ml bFG, 30ng/ml NGF, 1% (v/v) insulin, transferrin & sodium selenite premix (ITS), 10ng/ml NT3 in DMEM/F12
Neuronal maturation	1mM dbcAMP, 1% (v/v) N2 supplement 1% (v/v) B27, 30ng/ml NT3 in NeuroBasal A medium.

Medium	Supplement
BSRc and SH-SY5Y medium	10% (v/v) FCS, 1% penicillin-streptomycin – glutamine, 1% L-glutamine in DMEM.
<u>Neurosphere differentiation for hDPSC</u>	
Neurosphere formation medium	20ng/ml bFGF, 20ng/ml EGF, 1% (v/v) Penicillin/ Streptomycin, 1% (v/v) B27 in DMEM/F12.
Neurosphere differentiation medium	10µg/ml BDNF, 1% (v/v) Penicillin/ Streptomycin, 1% (v/v) B27 in DMEM/F12.

Table 2.3 Buffers

Buffer	Components
Blocking buffer A	2% BSA, 0.2% Triton-X in 1XPBS
Blocking buffer B	1% BSA, 0.5% donkey serum in 1XPBS
Depolarization solution	30mM KCl, 0.8% (w.v) NaCl, 2mM $CaCl_2$ in dH_2O
FACS fixation buffer	10% formalin, 20mg/ml glucose, 1M sodium azide
MACS buffer I	0.5% (v/v) BSA, 2mM EDTA in 1XPBS
SAP buffer	0.1% (w/v) saponin, 0.05% (w/v) sodium azide in HBSS

Table 2.4 Antibodies

Antibody	Concentration	Company/ Catalogue number
Rabbit anti-nestin	1: 250	Abcam/ ab22035
Rabbit anti-SOX1	1:300	Merk Millipore/ 071673
Rabbit anti-SOX2	1:1000	Chemicon/ AB5603
Rabbit anti-SOX9	1:250	Sigma/ HPA001758
Mouse anti-p75 mlr2 IgG	4µg/ml	Kindly gifted by Dr Mary-Louise Rogers
Mouse anti-NeuN	1:500	Millipore/MAB377
Mouse anti-NFM chain	1:200	Invitrogen/13-0700
Cy3 anti-mouse IgG	1:400	Merk Millipore/ AP192C
Cy3 anti-rabbit IgG	1:400	Jackson ImmunoResearch/ 711-165-152
Alexa Fluor 647 anti-mouse IgG	1:200	Molecular Probes/ A-21463

Table 2.5 Equipment

Equipment	Company/ Catalogue number
13mm round coverslips	neuVitro, Germany/ 01-115-30
10ml disposal serological pipette	Corning Costar/ 4487
15 ml centrifuge tube polypropylene	Nunc @ Sigma Aldrich/ 339652
24 well plate	Corning Costar/ 3254
25ml disposal serological pipette	Corning Costar/ 4489
50ml centrifuge tube polypropylene	Corning Costar/ 430791
5415C centrifuge	Eppendorf
70µm Cell Strainer	Falcon / Fal 352350
Beckman FC500 Flow Cytometer	Beckman Coulter Inc, Australia
Cryo tubes	Nunc@ Sigma Aldrich/ V7634-500EA
Haemocytometer	Blaubrand
MACS MS columns	Miltenyibiotec/ 130-041-301
MACS MultiStand	Miltenyibiotec / 130-042-303

Equipment	Company/ Catalogue number
MiniMACS Separator	Miltenyibiotec / 130-042-102
Mr. Frosty freezing container	Thermo Scientific/ 5100-0001
Olympus BX51 microscope epifluorescence microscope	Olympus innovation, the USA
Parafilm	Pechiney plastic packaging
TCS SP5 spectral scanning confocal microscope	Leica Microsystems, Wetzlar, Germany
SuperFrost microscope slides	Menzel-Glaser, Thermo Fisher Scientific

Table 2.6 Software

Software	Company/ Catalog number
Adobe Photoshop	Adobe Systems Incorporated
FlowJo	Three Star Inc, Oregon
Prism 5	Graphpad
ImageJ	NIH
NIS elements F package 3.0	Nikon corporation

Table 2.7 Cell lines

Cell lines	Description
H0DPSC, H1A1DPSC, H3DPSC, H4DPSC, AH14DPSC, AH21DPSC, AH23DPSC, AH27DPSC	hDPSC cell populations isolated from different individual patients
H0DPSC-GFP	Cell population transduced with green fluorescence protein (GFP) derived from H0DPSC
BSRc	BSR is a clone of baby hamster kidney fibroblast cells. BSRc stands for BSR control and is p75 negative (Rogers et al. 2006).
SH-SY5Y	A subclone of SK-N-SH cell line which were established from a bone marrow biopsy of a neuroblastoma patient in the early 1970's (Biedler, Helson & Spengler 1973).

2.2 Methods

2.2.1 Ethics

All experiment procedures were carried under approved guidelines set by the University of Adelaide Human Research Ethics Committee (approval number H-2012-164).

2.2.2 Isolation of Human DPSC

hDPSC cell populations used in this study were already established in SRP laboratory from the pulp of adult impacted molars (Figure 2.1). Nine cell populations from different donors were set up and studied as we are looking for hDPSC contains high percentage of $p75^{+}$hDPSC for easy isolation and enrichment. Briefly, discarded teeth were cleaned and cracked to expose the pulp chamber. The pulp tissue was separated from the crown and root and then dissociated in a solution of 3mg/ml collagenase type I and 4mg/ml dispase with 1 hour incubation at 37 degrees. Cells were passed through a 70 μm cell strainer in order to get single cell suspensions. The cells were cultured in standard hDPSC medium under standard conditions (37°C, 100% humidity, 5% CO_2).

2.2.3 Human DPSC maintenance

Nine hDPSC populations were used in the experiments, H0DPSC, H0DPSC-GFP, H1A1DPSC, H3DPSC, H4DPSC, AH14DPSC, AH21DPSC, AH23DPSC, AH27DPSC cultured in standard dental pulp stem cell medium under standard conditions (37°C, 100% humidity, 5% CO_2), in a 75 cm^2 (T75) flask. Sub-confluent colonies were passaged into a new flask to avoid intimate cell contact which may otherwise change their cellular properties.

2.2.4 BSRc and SH-SY5Y cell maintenance

p75 control cells, BSRc p75 negative cell control and SH-SY5Y p75 positive cell control were maintained in high glucose medium under standard conditions (37°C, 100% humidity, 5% CO_2), in a 75 cm^2 (T75) flask. Medium was changed every two days as cells proliferated quickly. Sub-confluent colonies were trypsinized, centrifuged (200g, 2 minutes), resuspended and passaged into a new flask to avoid intimate cell contact which may otherwise change their cellular properties.

2.2.5 Dissociation of cells using trypsin

Trypsin was used to detach cells from culture surfaces when routine cell passaging and harvesting were required. Briefly, cell culture medium was aspirated and cells were incubated with 1-2ml 0.05% Trypsin/ EDTA for 3 minutes at 37°C. An equal volume of medium containing 10% (v/v) FCS was added to neutralize the enzyme and the cell suspension was centrifuged at 200g for 2 minutes in order to remove trypsin containing supernatant.

2.2.6 Cell counts

Cell concentration was calculated using the dye exclusion methods with Trypan blue dye and a haemocytometer (GMBH&Co, Germany). Generally, 10µl of cells were mixed with 90µl trypan blue. 5µl of diluted/mixed cells were put into haemocytometer. Trypan blue is a toxic stain therefore, live cells were colourless and bright under the phase contrast microscope while dead cells stained blue under the phase contract. The number of viable cells in one or more large corner squares were counted and averaged. The cell concentration (cell number/ml) is calculated=

Cell number (in one large square) x10 (dilution factor) x10, 000 (conversion factor)

Desired cell densities/ cell numbers were cultured into coverslips or flasks as required.

2.2.7 Cryopreservation of cell populations

Cryopreservation is used for long term storage of cell populations at sub-zero temperatures. Cells were harvested and resuspended in a cryopreservation mixture of 10% dimethyl sulfoxide (DMSO) in FCS. The 10% DMSO is used in cryopreservations to prevent water crystallization which causes cell membrane rupture during the cryopreservation procedure. 90% serum was added to prevent cells being starved of nutrients. Cells in cryopreservation mixture were aliquoted (<1ml) into CryoTubes and kept into a Cryo 1 degree Mr. Frosty freezing container. The Mr. Frosty achieves a rate of cooling very close to 1 degree/minute, the optimal rate for cell preservation. Cells were kept in -80 °C first and upon long term storage cells were transferred into ultra-low temperatures (-180°C, liquid nitrogen) until required.

2.2.8 Recovery of cells from liquid nitrogen

Frozen cells were recovered by mixing gently with pre-warmed medium. The medium and cell mixture was then centrifuged at 200g for 2 minutes in order to pellet the cells. The supernatant with DMSO was discarded and cells were placed in fresh culture medium for culturing.

2.2.9 hDPSC differentiation (epigenetic reprogramming neural differentiation)

hDPSC were induced to differentiate along a neuronal lineage as described by Kiraly (Kiraly et al. 2009). hDPSC were sub-cultured onto PLL and laminin coated 13mm round coverslips at a density of 50,000 cells per well. hDPSC underwent differentiation by using various differentiation media. hDPSC were firstly cultured in neuronal induction medium overnight for attachment. Cells then underwent epigenetic reprogramming for 48hrs. hDPSC were forced into neuronal lineages by

consecutive stimulation by cAMP and PKC on neuronal differentiation stage for 3 days and matured neurons were protected in maturation medium for 3-7 days (Figure 2.1)

Following the differentiation and maturation, cells were gently rinsed in PBS, fixed in 4% (w/v) PFA for 30 minutes at room temperature and washed in PBS. Bright-field images were captured on a Nikon Eclipse TS100 microscope using NIS Elements.

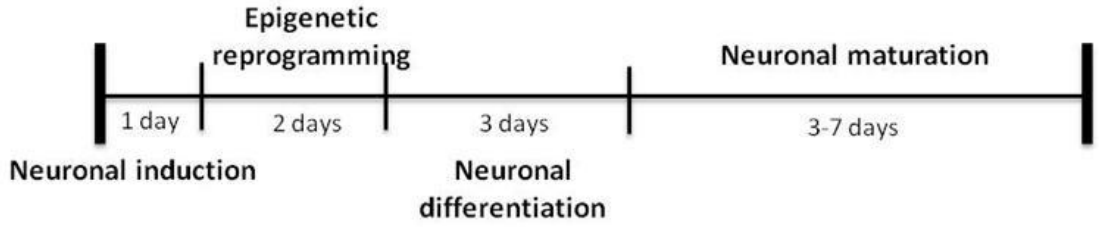


Figure 2.1 Schematic diagram of hDPSC neural differentiation.

Cells underwent a 13-day neuronal differentiation with medium changes through induction, epigenetic reprogramming, neuronal differentiation and neuronal maturation phases.

2.2.10 Immunohistochemistry

Three hDPSC cell populations, H0DPSC-GFP, H3DPSC and H4DPSC were cultured and fixed in 4% paraformaldehyde (PFA) in PBS for 15 minutes. Subsequently, blocking buffer A was used to block the non-specific binding with incubation for 30 minutes at room temperature. Primary antibody mouse anti-p75 mlr2 IgG was diluted in blocking buffer B. Cells were incubated with primary antibody overnight. Secondary antibody cy3 anti-rabbit IgG Alexa Fluor 647 anti-mouse IgG were used to identify bound primary antibody. For double IHC, rabbit anti-nestin, rabbit anti-SOX1, rabbit anti-SOX2 and rabbit anti-SOX9 were diluted in blocking buffer B and applied into cells for overnight

incubation. Secondary antibody cy3 anti-rabbit IgG, was used to identify bound primary antibodies. All coverslips were mounted in Prolong Gold antifade reagent.

2.2.11 Microscopy and image analysis

To estimate the percentage of nestin, SOX1, SOX2 and SOX9 (nucleus) in $p75^+$ and $p75^-$ hDPSC, images were taken in five representative fields using a TCS SP5 spectral scanning confocal microscope (Leica Microsystems, Wetzlar, Germany, http://www.leica-microsystems.com) or Olympus BX51 microscope epifluorescence microscope (Olympus innovation, the USA, http://www.olympusamerica.com/).

Images were processed and analyzed to ensure at least total 500 cells were counted using imageJ (NIH). Images were split into wavelength channels of 'green' (p75), 'red' (nestin/SOX1/SOX2) and 'blue' 4', 6-diamidino-2-phenylindole (DAPI) for analysis. The total number of cells in one image was determined by counting the number of DAPI stained cells. Images in 'green' and 'red' channels were converted to black and white and the threshold of the signal was set until there was no signal/ black in the control image. The number of p75/ nestin/SOX1/SOX2 positive cells was determined by counting black signals in threshold adjusted images (Figure 2.2). The double positive cells (positive for both red and green signals) could be easily determined. The numbers of single positive cells were determined by the total number of green/red positive cells minus the number of double positive cells. For SOX9 nucleus positive cell number counting, the images did not need to convert the black signal as the staining was obvious and easily counted.

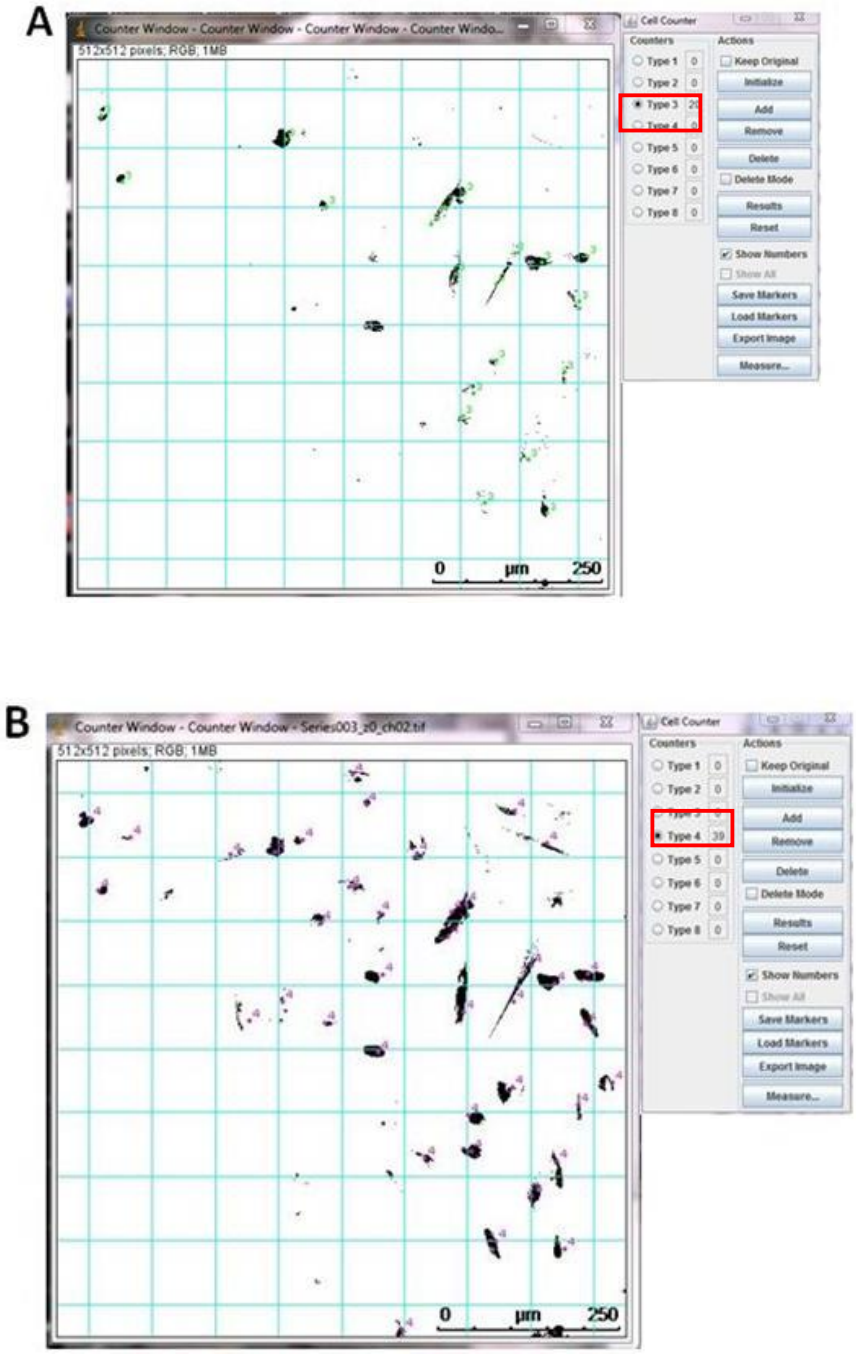


Figure 2.2 Diagram of image processing using imageJ.

(A) Green (p75) signal counting (B) Red (nestin/SOX1/SOX2) counting

2.2.12 Mlr2 anti-p75 IgG labeling with Alexa Fluor 647

2mg/ml mlr2 anti-p75 IgG and reactive dye Alexa Fluor 647 were mixed in 1M sodium bicarbonate solution and incubated for 1 hour at room temperature. The unbound dye and conjugated antibody were separated by gel filtration. Proteins with molecular weight more than 40,000 were eluted first and collected. Unbound dyes with smaller molecular weight were trapped in the gel column and eluted later. Protein absorbance @280nm and @650nm were recorded to calculate the mlr2 anti-p75 Alexa Fluor 647 concentration and degree of labeling. Protein concentration and degree of labeling were determined by the following equations.

Protein concentration (M) =

$$\frac{[\text{A280} - (\text{A650x0.03})] \times \text{dilution factor}}{203{,}000}$$

203,000/ cm.M is the molar extinction coefficient of an IgG

Degree of labeling=

$$\frac{\text{A650} \times \text{dilution factor}}{239{,}000 \times \text{protein concentration}}$$

239,000 is the molar extinction coefficient of Alexa Fluor 647 at A650nm

2.2.13 Flow cytometry

The percentage of hDPSC expressing surface protein p75, nestin, CD146, Major Histocompatibility Complex class I (MHCI) and Major Histocompatibility class II (MHCII) were determined by flow cytometry. As hDPSC is a heterogeneous population, containing MSC and NSC population, CD146, as a MSC marker, was used to assess cells with MSC properties and nestin, as a pan-NSC marker, was used to assess cells with NSC properties. MHCI, as a positive control, was expected to be expressed in all the hDPSC and MHCII, as a negative control, was expected to be negative in all the hDPSC populations tested. For single color staining, cells were incubated with anti-p75 Alexa Fluor 647, anti-MHCI PE, anti-MHCII PE for 30 minutes in the dark at 4°C. For double color p75 and CD146 flow cytometry, cell were washed and incubated with anti-CD146 PE first, washed and then incubated with anti-p75 Alexa Fluor 647 for 30 minutes in the dark at 4°C. For double color p75 and nestin flow cytometry, cells was incubated with anti-p75 Alexa Fluor 647 first, washed and then incubated with anti-nestin PE followed by 4% PFA fixation and SAP buffer permeabilisation. All samples were fixed in 0.5- 1ml flow fixation buffer. The flow fixation buffer ensured labeled cells were preserved for analysis within two weeks. Fluorochrome minus one tubes (FMO/ single color flow cytometry) were used as a negative control for double color flow compensation. Data was analyzed using a Beckman FC500 Flow Cytometer (Beckman Coulter Inc, Australia) and recorded by CXP software. Data was further analyzed using FlowJo software (Three Star inc, Oregon).

2.2.14 Statistical Analysis

All parametric data was assessed using an Analysis of Variance (ANOVA) followed by Bonferroni post-tests to compare replicate means by row and for each row, compare each column to all the

other columns. Column data is presented as mean± standard error of mean (SEM). An alpha value of $p<0.05$ was considered significant.

3. Chapter 3- Characterisation of p75 expression on human Dental Pulp Stem Cells (hDPSC)

3.1 *Introduction*

p75 is prototypically a neurotrophin receptor which binds to all pro-neurotrophin with high affinity and binds to all mature neurotrophin with low affinity. Mature neurotrophin include BDNF, NGF, NT3 and neurotrophin 4/5 (NT4/5) (Hempstead 2002). Besides p75, there is another group of neurotrophin receptors— tropomyosin-related kinase (Trk) family of receptor tyrosine kinase. There are three members in trk receptor family. They are TrkA, TrkB and TrkC. Trk receptors' binding of neurotrophins usually induces cell growth. p75 binding of neurotrophins has various functions which ranges from p75/ p75-sortilin induced neural death (Nykjaer et al. 2004; Nykjaer, Willnow & Petersen 2005) to neural survival and neural differentiation with co-operation of Trk receptors (Culmsee et al. 2002; Verdi et al. 1994).

Besides trophic function, p75 has been proposed to be a NSC marker and defines a cell population with neurogenic potential in the adult brain SVZ and SGZ (Catts et al. 2008; Young, KM et al. 2007). During neuro-embryogenesis, p75 is highly expressed in NC stem cells that give rise to peripheral nervous tissue and is down-regulated in postnatal mature cells (Wilson et al. 2004; Yiu & He 2006). Therefore, it is proposed that some of the p75 positive NC cells may still remain as a particular stem cell population in these tissues. These stem cells are responsible for the tissue regeneration where they are located. For example, hDPSC reside in tooth pulp and are responsible for lifelong maintenance of tooth function and tooth repair (Dimitrova-Nakov et al. 2014).

hDPSC –from the pulp of the tooth, are derived from NC cells (Abe et al. 2012). They have the ability to generate neurons and glial cells *in vitro* by epigenetic reprograming (Kiraly et al. 2009). They also differentiate into cells with neuronal morphology and NFM and beta III tubulin positive when being transplanted into an avian embryo *in vivo*. These NFM and beta III tubulin expressing cells integrated into the host neuronal networks up to 7 days after injection into the avian embryo (Arthur et al. 2008). This provides a mechanism for their function in neural protection and lesion repair when transplanting hPDSC into a post-stroke brain (Leong et al. 2012). It was also found that only a small percentage (~2.3%) of engrafted hDPSC survived in the infarct brain and most of them differentiated into neurons and astrocytes but rarely endothelial cells (Leong et al. 2012). A refined methodology with transplanting only functional hDPSC (hDPSC with neurogenic potential) is in demand to improve stem cell therapy for stroke and other degenerative brain disease. Such methodology requires a NSC marker to enrich and isolate NSC population from the heterogeneous stem cell population. The study outlined in this thesis intends to address the question of whether p75 is a NSC marker in hDPSC.

Before investigating the role of p75 in hDPSC neuronal differentiation, an overview of marker expression in undifferentiated hDPSC is also necessary. Many studies examine the marker expression profile before evaluating marker related functions. p75 has been demonstrated to co-express with NSC marker nestin and PSA-NCAM (neuroblasts), but p75 positive cells did not express GFAP (astrocytes), NeuN (neurons) or SOX10 (oligodendrocyte lineage) (Giuliani et al. 2004; Young, KM et al. 2007). This suggested a role for p75 in regulation of neurogenesis in its early stage. However, little is known about the basal expression levels of p75 in hDPSC. Therefore, this chapter aims to look at p75 co-expression with nestin (NSC marker), SOX1 (neural progenitor

cell marker), SOX2 (pluripotent stem cell marker), SOX9 (NC stem cell marker) and CD164 (MSC marker) by IHC and flow cytometry.

3.2 Results and Analysis

3.2.1 p75 was co-expressed with neural stem cell markers in hDPSC *in vitro*

To characterize the $p75^+$ hDPSC and $p75^-$ hDPSC expression patterns with surface markers *in vitro*, double IHC staining for p75 with nestin (Figure 3.1), SOX1 (Figure 3.2), SOX2 (Figure 3.3) and SOX9 (Figure 3.4) was performed. Using ImageJ analysis, all hDPSC showed a positive immune-reactivity for SOX9 expression, which is consistent with hDPSC NC stem cell origin and properties. Nestin, SOX1 and SOX2 were expressed in a subset of the cell population (Supplementary Figure 7.1). $p75^+$ hDPSC had significantly higher percentage (t-test, $*p<0.001$) of nestin, SOX1 and SOX2 positive cells compared to percentage of nestin, SOX1 and SOX2 positive cells in $p75^-$ hDPSC, consistently across all three hDPSC cell populations (H0DPSC-GFP, H3DPSC and H4DPSC) (Figure 3.5). SOX9 expression remained in the nucleus in hDPSC ranged from 21.60% ±1.34% to 51.61%±20.60% (Supplementary Figure 7.1). SOX9 nucleus expression did not differ between $p75^+$ hDPSC and $p75^-$ hDPSC (t-test, $p>0.05$). The trend was consistent across all three hDPSC populations tested. SOX1 and SOX2 showed a positive immune-reactivity in cytoplasm instead of in the nucleus. This is likely due to the loss of function of these transcription factors when re-culturing of hDPSC reached a certain passage (Liu, L et al. 2011).

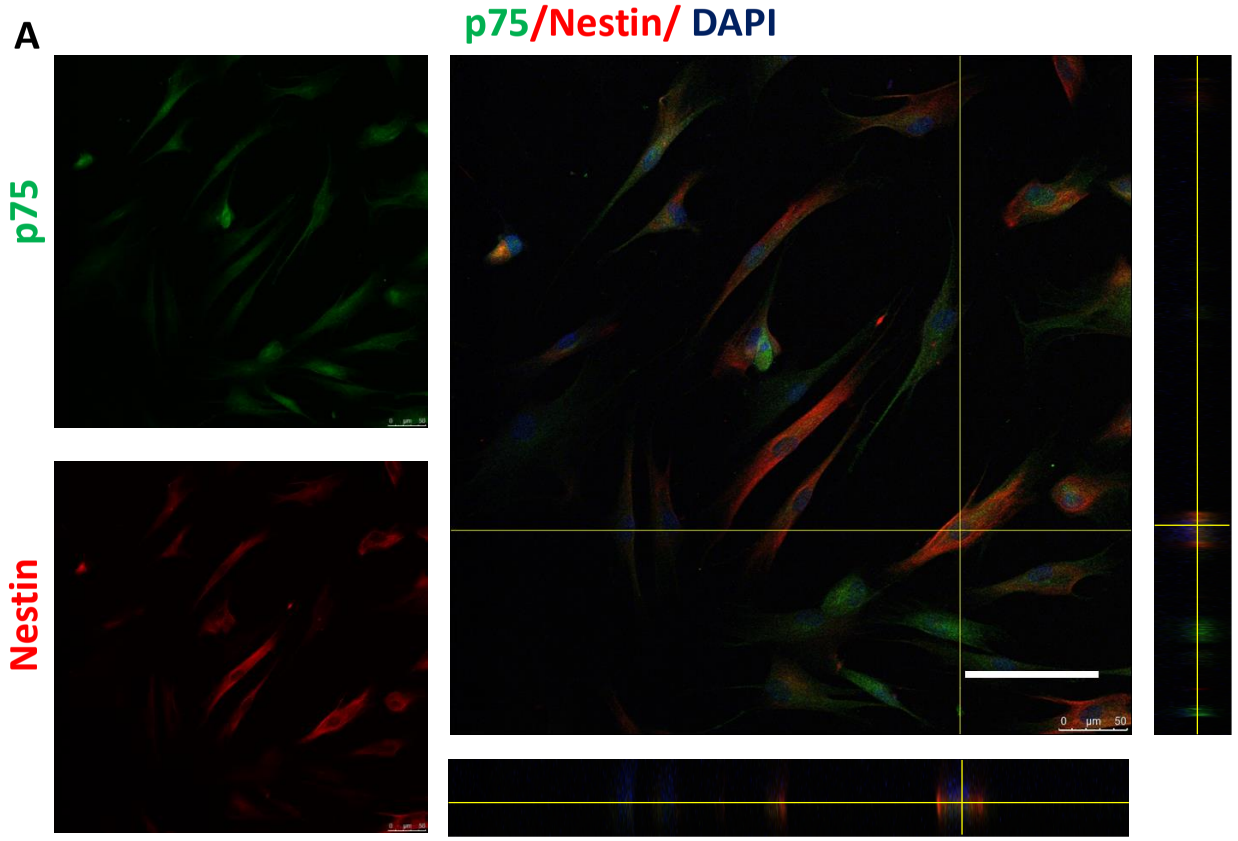


Figure 3.1 IHC staining of p75 and nestin.

(A) The orthogonal view of confocal double immunofluorescence images demonstrated that p75 (green) is co-expressed with potential neural stem cell marker nestin (red).

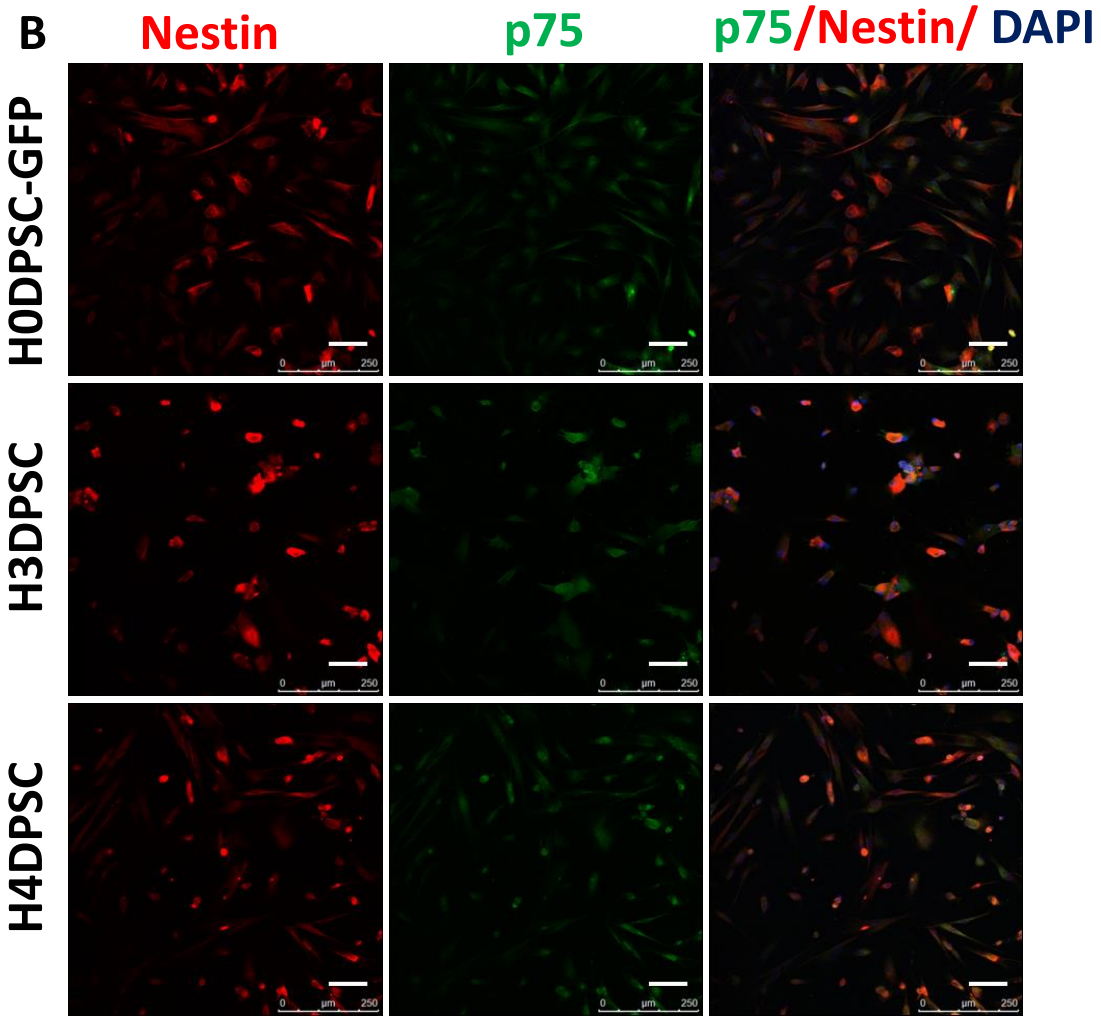


Figure 3.2 IHC staining of p75 and nestin.

(B) Representative images of p75 (green) and nestin (red) staining on three cell populations H0DPSC-GFP, H3DPSC and H4DPSC. Scale bar=100 µm. N=3 experiments.

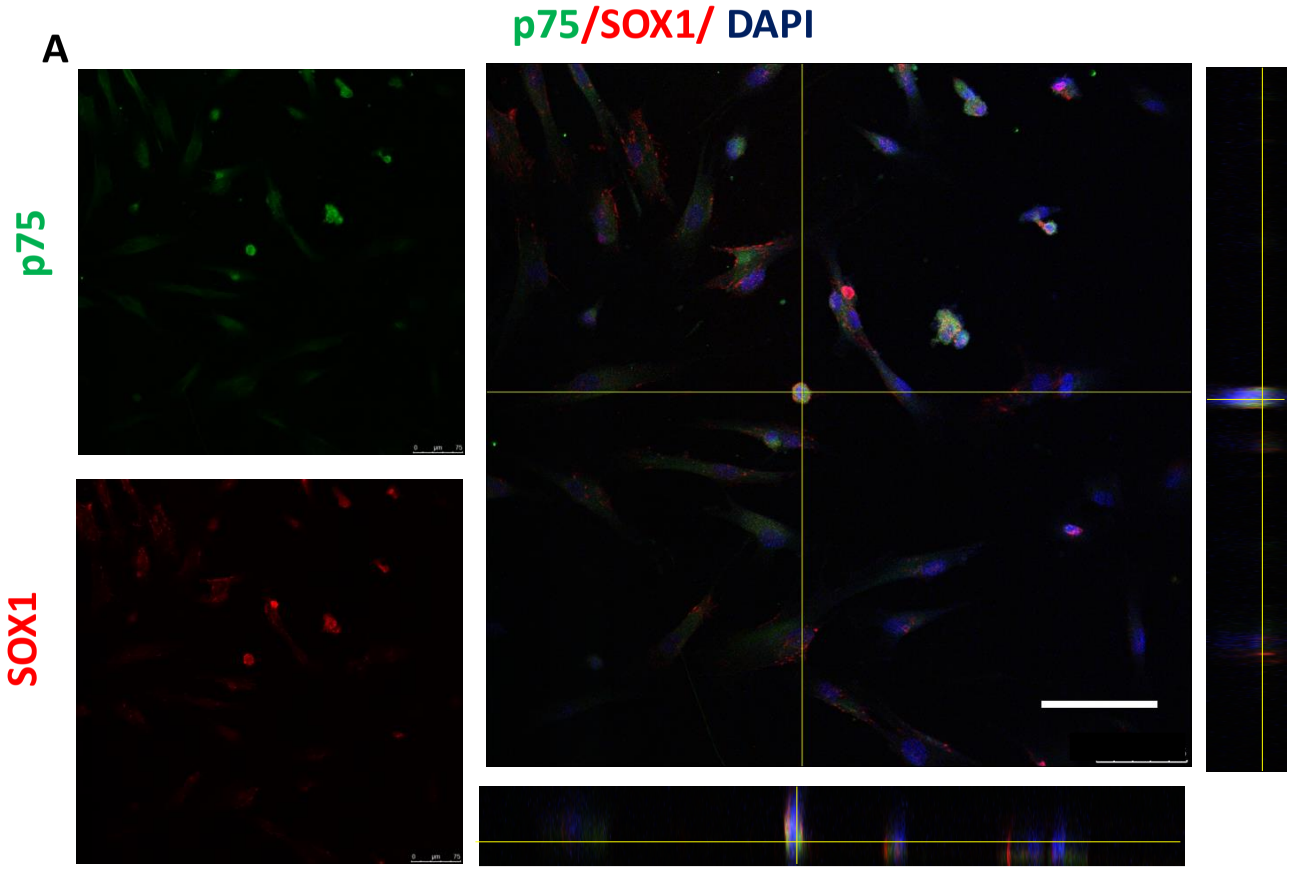


Figure 3.3 IHC staining of p75 and SOX1.

(A) Orthogonal view of confocal double immunofluorescence images demonstrated that p75 (green) is co-expressed with neural precursor marker SOX1 (red).

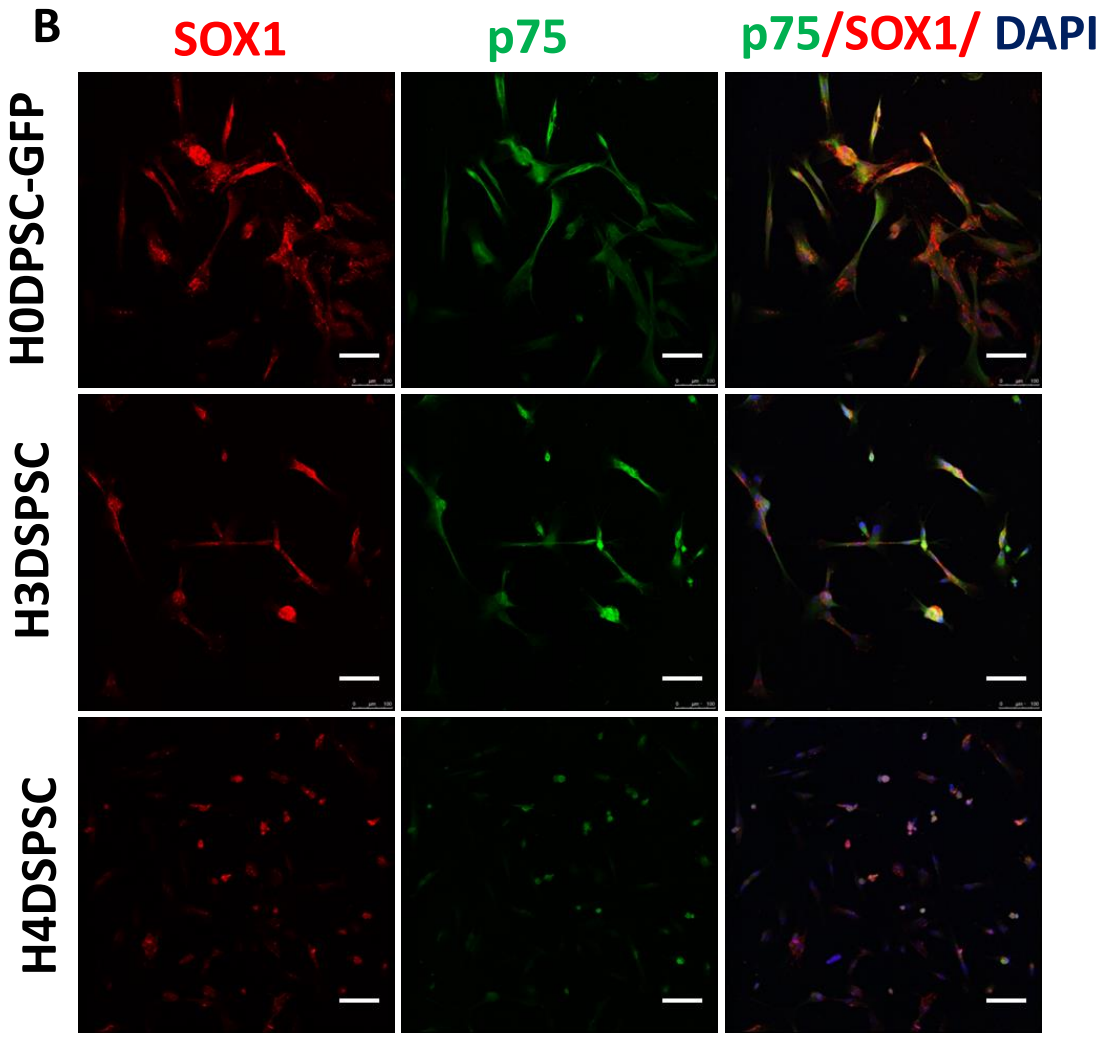


Figure 3.4 IHC staining of p75 and SOX1.

(B) Representative images of p75 (green) and SOX1 (red) staining on three cell populations H0DPSC-GFP, H3DPSC and H4DPSC. Scale bar=100 µm. N=3 experiments.

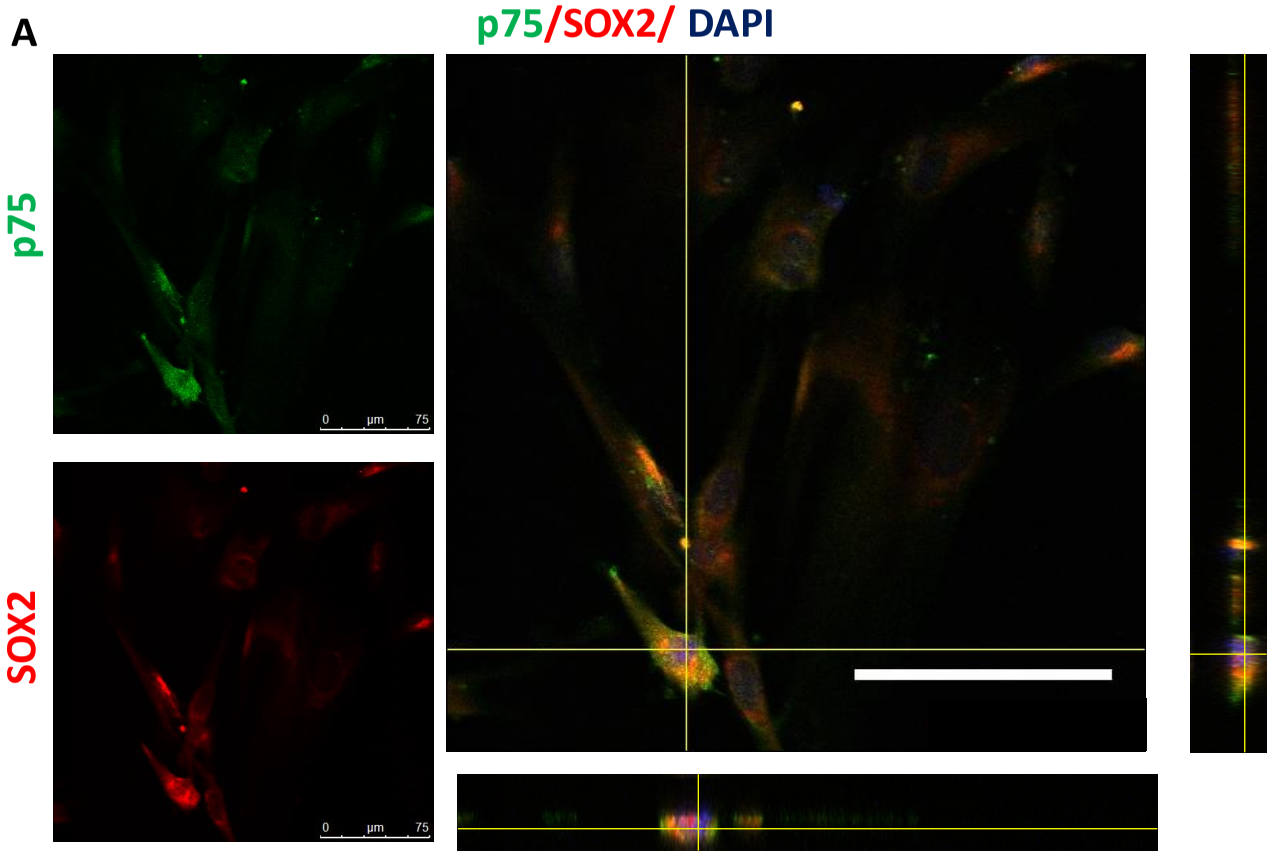


Figure 3.5 IHC staining of p75 and SOX2.

(A) Orthogonal view of confocal double immunofluorescence images demonstrated that p75 (green) is co-expressed with pluripotency marker SOX2 (red).

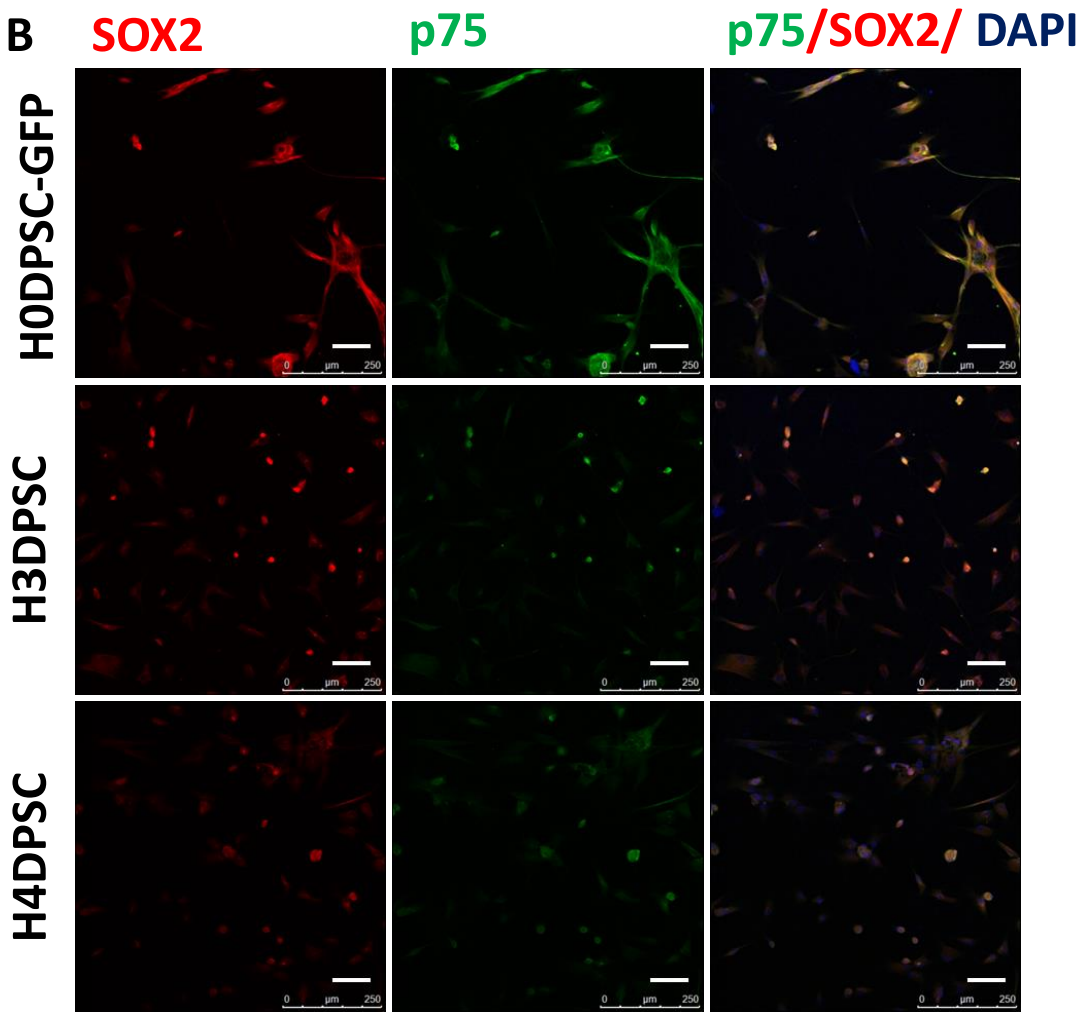


Figure 3.6 IHC staining of p75 and SOX2.

(B) Representative images of p75 (green) and SOX2 (red) staining on three cell populations H0DPSC-GFP, H3DPSC and H4DPSC. Scale bar=100 µm. N=3 experiments.

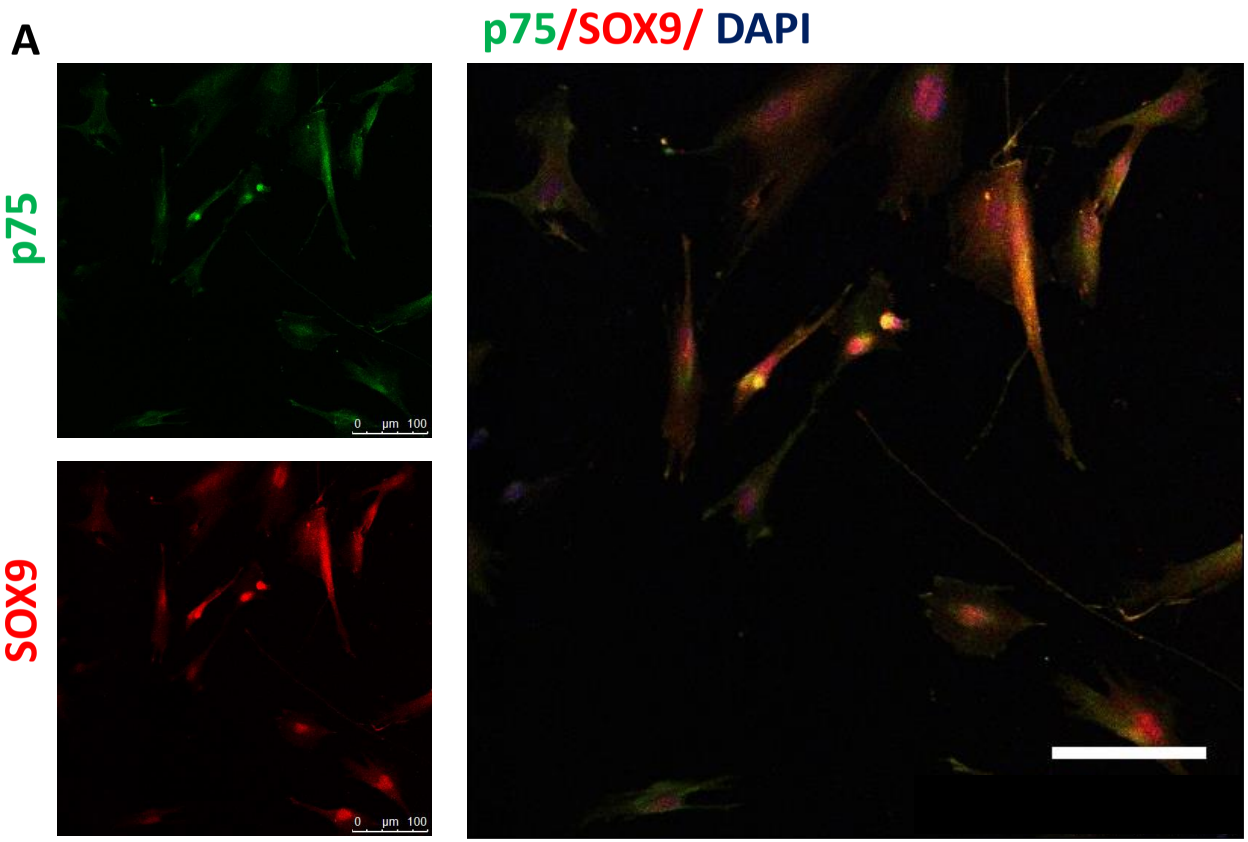


Figure 3.7 IHC staining of p75 and SOX9.

(A) Confocal double immunofluorescence images demonstrated that p75 (green) is expressed in SOX9 (red) nucleus positive cells.

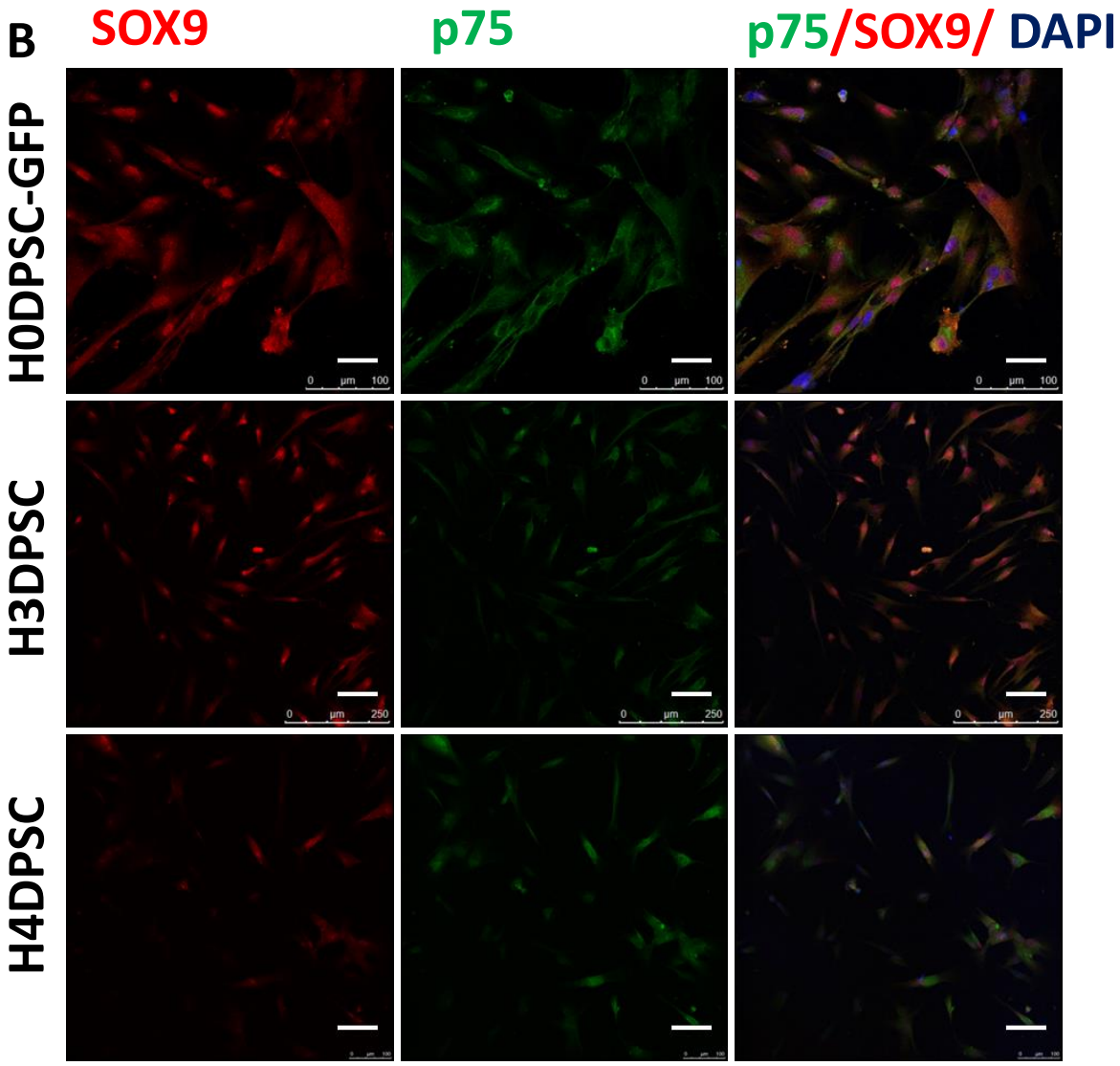


Figure 3.8 IHC staining of p75 and SOX9.

(B) Representative images of p75 (green) and SOX9 (red) staining on three cell populations, all the DPSC were positive for SOX9 either in nucleus or cytoplasm. Scale bar=100 µm. N=3 experiments.

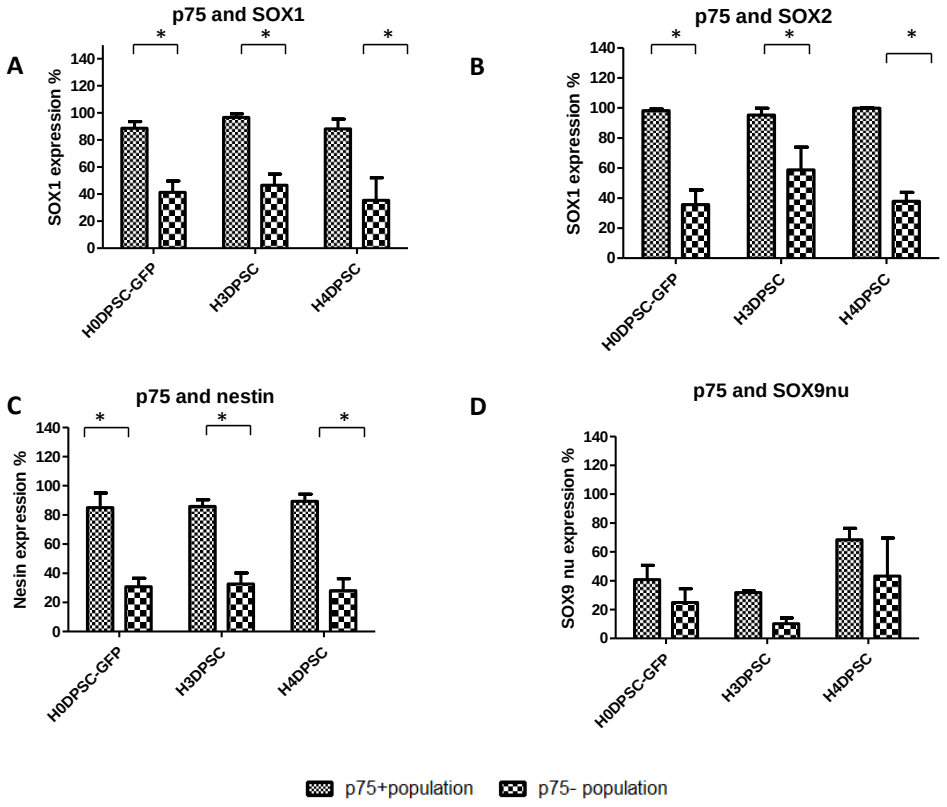


Figure 3.9 Comparison between p75^{+} and p75^{-} populations on expression level of nestin, SOX1, SOX2 and SOX9 (nucleus).

There was a significantly higher expression of (A) nestin, (B) SOX1 and (C) SOX2 on p75^{+} population in comparison to p75^{-} hDPSC. The results were consistently across all the three dental pulp stem cell populations—H0DPSC-GFP, H3DPSC and H4DPSC. *p< 0.05. (D) There were no significant differences of SOX9 expression in the nucleus between the p75^{+} population and the p75^{-} population consistently across all the three dental pulp stem cell populations. p>0.05; mean±SEM; N=3 experiments.

3.2.2 Expression level of p75 in hDPSC

Flow cytometry was carried out to assess the surface expression level of p75, MHCI, MHCII, CD146 (MSC marker) and nestin (NSC marker). MHCI is suggested to be expressed by all nucleated cells and MHCII expression is limited to some antigen presenting cells and B cell lymphocytes. hDPSC were MHCI positive and MHCII negative, consistent with the results reported in other adult stem cells (Figure 3.7 A, B; Figure 3.8 A, B; Figure 3.9 A, B). Down regulation of MHCI was observed in a small percentage of hDPSC. The same MHCI down-regulation has been previously reported in human embryonic stem cell (Schu et al. 2012). p75 was expressed at a low level in hDPSC ranges from 1.43-6.27% (Figure 3.6 , Table 3.1). CD146, a MSC marker, which defines a cell population with the ability to differentiate into chondrocyte, adipocyte and osteocyte lineages, was highly expressed in hDPSC (>80%) (Covas et al. 2008). p75 was found to be co-expressed with CD146, indicating MSC properties of $p75^+$ hDPSC (Figure 3.7 G, Figure 3.8 G and Figure 3.9 G). Nestin as a NSC marker was expressed at high level (>90%) and >80% of $p75^+$ hDPSC expressed nestin (Figure 3.7 F, Figure 3.8 F and Figure 3.9 F). This data indicates both MSC and NSC properties of $p75^+$ hDPSC.

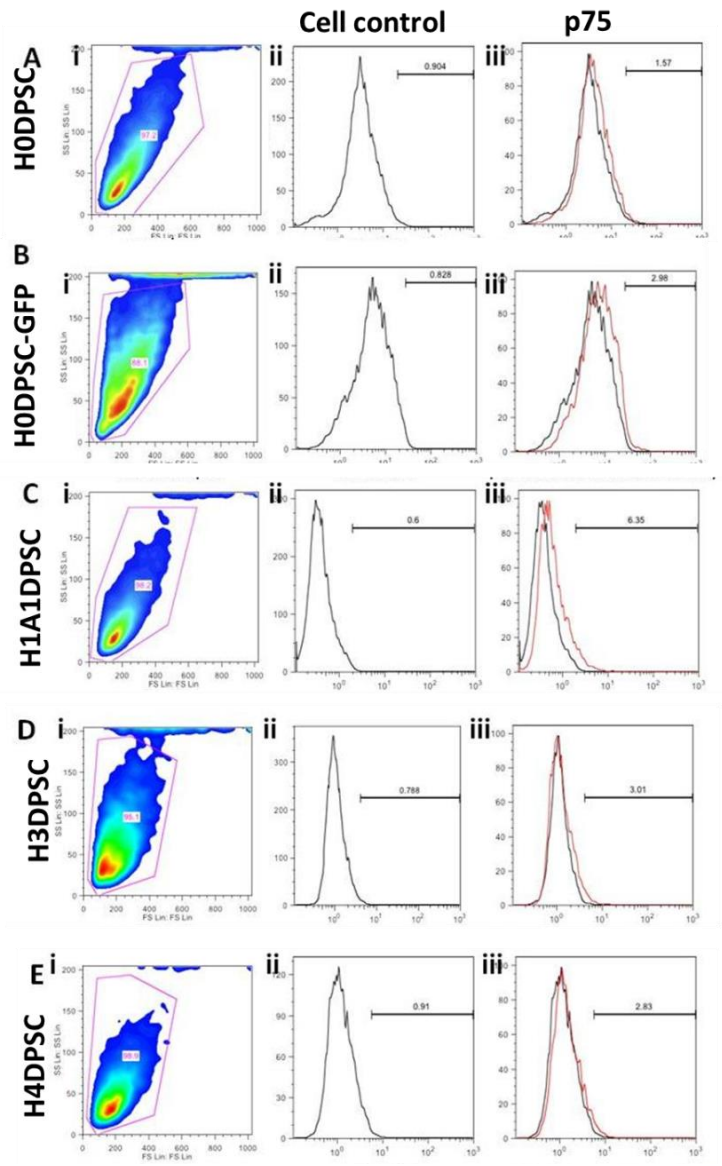


Figure 3.10 hDPSC surface markers expression analysis by flow cytometry.

(A) H0DPSC, (B) H0DPSC-GFP,(C) H1A1DPSC,(D) H3DPSC and (E)H4DPSC. (i) Forward scatter and side scatter graph to present size and cell complexity of dental pulp stem cells. (ii) Cell control histogram to set the positive threshold with >99% of control cells negative(iii) Overlay of cell control and cells with p75 alexa647 labeled histogram to present expression level of p75. N=3 experiments.

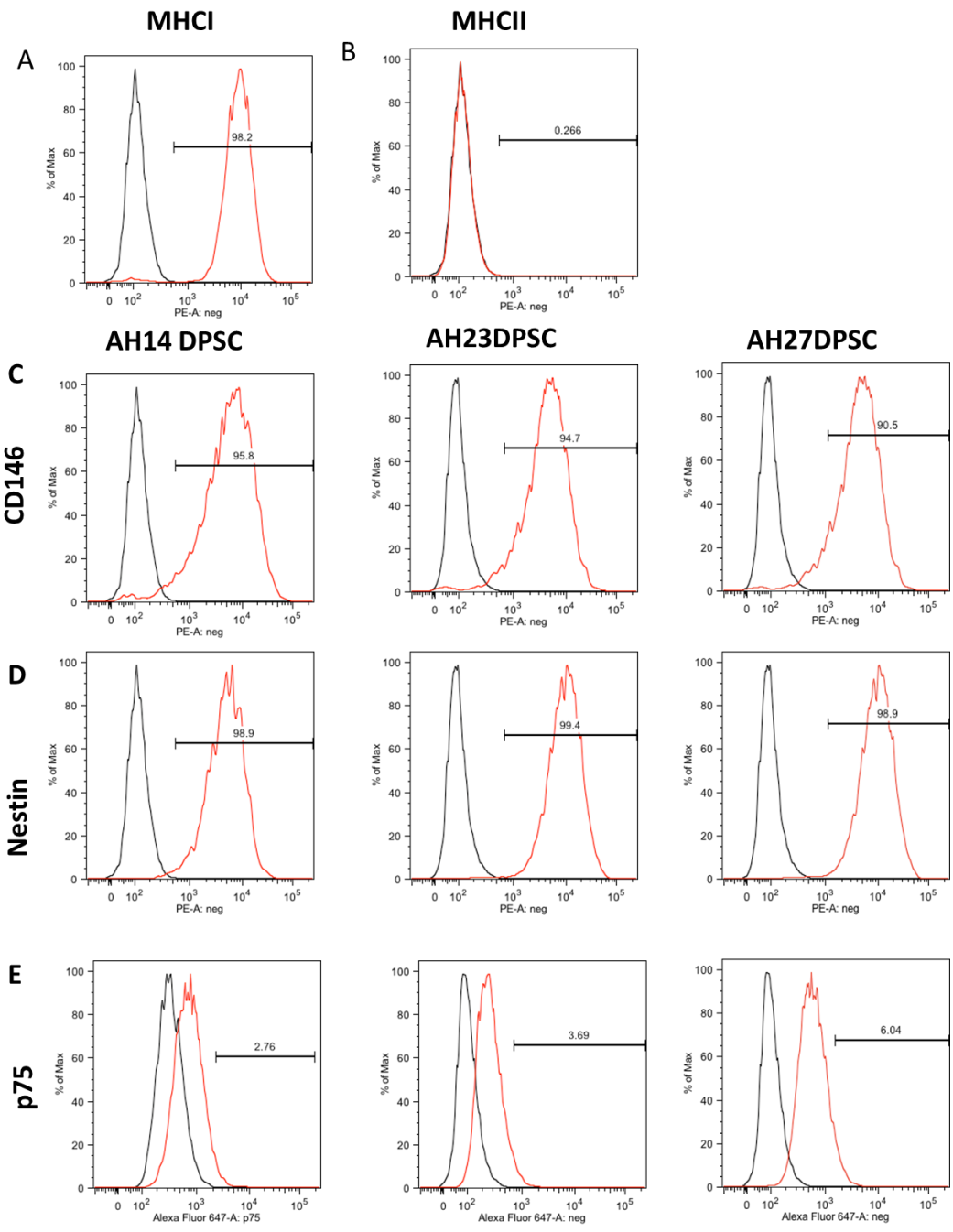


Figure 3.11 Flow cytometry analysis of hDPSC cellular surface marker expression.

hDPSC have (A) high expression of MHCI, >99% , (B) low expression of MHCII, 1%, (C) medium expression of CD146, (D) high expression of nestin, >99%, (E) Low expression of p75, <5%. N= experiments.

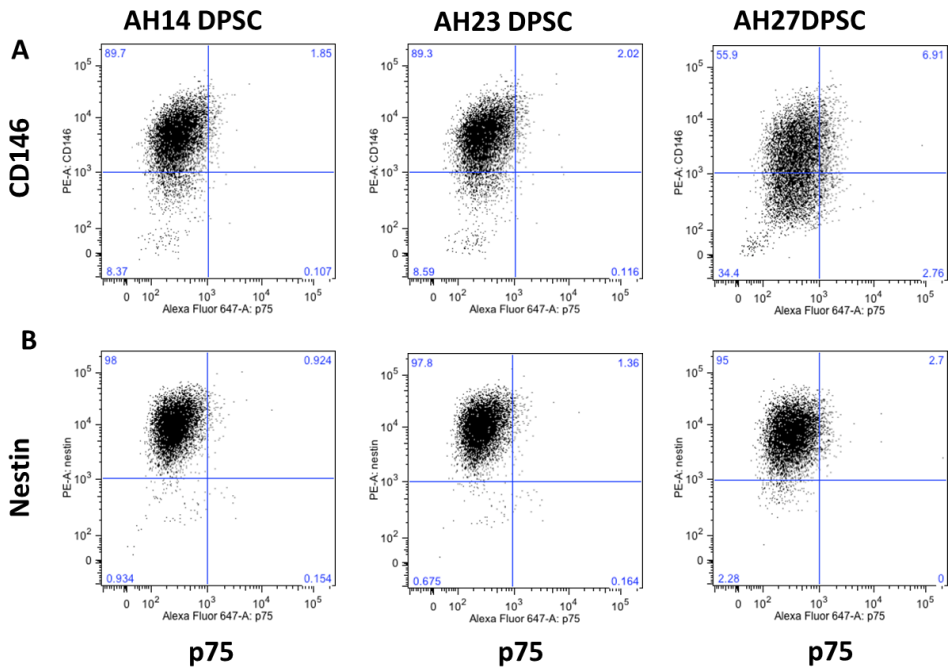
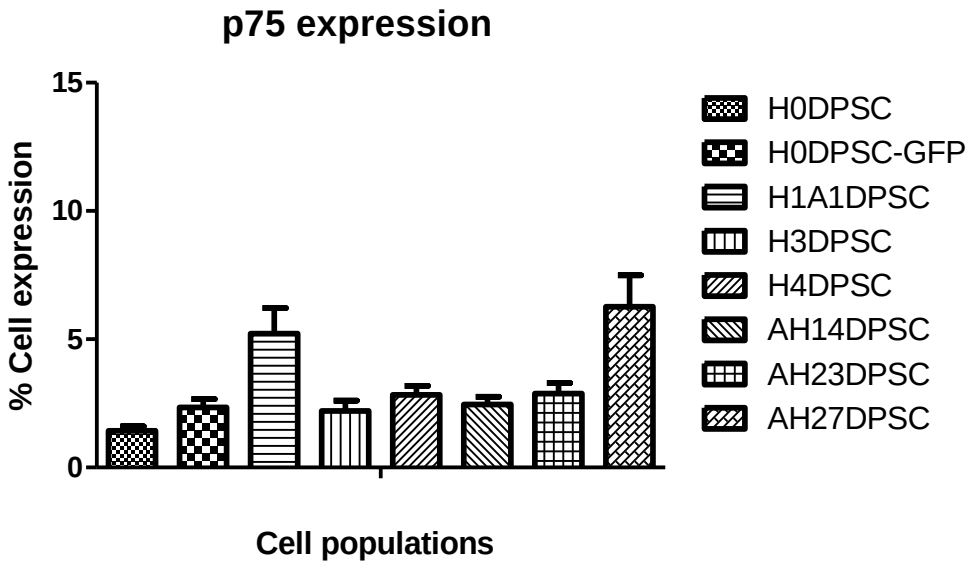


Figure 3.12 p75 co-expression with CD146 and nestin analysis by flow cytometry.

(A) p75 and CD146 double staining. A small subpopulation of hDPSC stained positive CD146^{+}/ p75^{+} (in the right upper square)

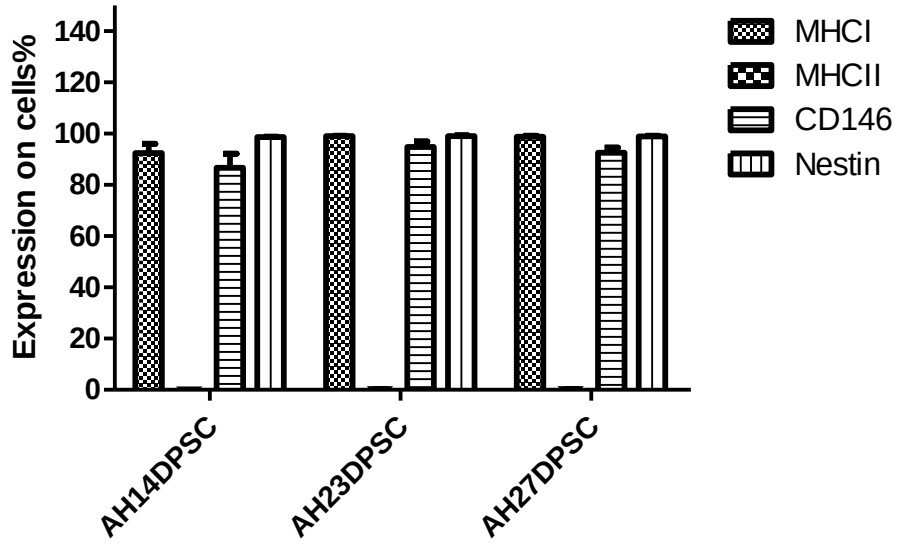
(B) p75 and nestin double staining. A small subpopulation of hDPSC stained positive for nestin^{+}/ p75^{+} (in the right upper square). N=3 experiments.



Cell population	p75 %	SEM	N
H0DPSC	1.43	0.18	3
H0DPSC-GFP	2.34	0.32	3
H1A1DPSC	5.22	1.00	3
H3DPSC	2.21	0.40	3
H4DPSC	2.83	0.35	3
AH14DPSC	2.45	0.30	3
AH23DPSC	2.88	0.41	3
AH27DPSC	6.27	1.23	3

Figure 3.13 Comparison of p75 expression on H0DPSC, H0DPSC-GFP, H1A1DPSC, H3DPSC, H4DPSC, AH14DPSC, AH23DPSC and AH27DPSC.

p75 was expressed at a low level consistently across all the cell populations, $p>0.05$, mean±SEM. p75 expression level ranged from 1.43% to 6.39% on different cell populations.



Mol. exp./Cell	MHCI%			MHCII%		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**
AH14DPSC	92.47	3.65	3	0.20	0.03	3
AH23DPSC	99.07	0.09	3	0.28	0.007	3
AH27DPSC	99.87	0.29	3	0.29	0.07	3

Mol. exp./Cell	CD146%			Nestin%		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**
AH14DPSC	86.73	5.41	3	98.66	0.20	3
AH23DPSC	94.83	2.13	3	96.07	0.29	3
AH27DPSC	92.53	1.98	3	98.90	0.23	3

Figure 3.14 Comparison of MHCI, MHCII, CD146 and Nestin expression analysis on AH14DPSC, AH23DPSC and AH27DPSC.

MHCI and Nestin were expressed at a high level across the three cell populations, $p>0.05$. MHCII was expressed at low level across the three cell populations, $p>0.05$. The expression pattern of molecules is independent of the cell population, $p>0.05$; mean±SEM.

3.3 Discussion

3.3.1 p75^{+} hDPSC are a small subpopulation originating from neural crest stem cells

All the hDPSC populations tested showed positive immune-reactivity for SOX9 either in the nucleus or cytoplasm. 30% of hDPSC expressed SOX9 in the nucleus as a functional transcription factor while remaining 70% had SOX9 expression in the cytoplasm (Data not shown). This might be due to the functional loss of SOX9 in adult hDPSC during *in vitro* culturing. There was no difference in SOX9 expression levels (either in the nucleus or in the cytoplasm) between p75^{+} hDPSC and p75^{-} hDPSC.

The expression of SOX9 in p75^{+} hDPSC and p75^{-} hDPSC is due to hDPSC originating from NC cells in early embryo development. Some properties of NC cells such as pluripotency and neurogenicity in adult dental pulp still remains in adulthood (Abe et al. 2012). The NC stem cells are a stem cell population that have transiently migrated from the dorsal neural folds at the roof of the neural plate into the periphery (Cheung & Briscoe 2003). Following different environmental cues, prospective NC stem cells in the periphery differentiated into multiple cell lineages, including neurons, glia of the peripheral nervous system and skin melanocytes (a type of pigment producing cells) (Bronner-Fraser & Fraser 1988). SOX proteins are a group of transcriptional proteins which share a conserved high-mobility group domain (HGM). The HGM domain serves as a DNA binding domain for these transcription factors. SOX9 belongs to SOX subgroup E and is expressed by NC cells and associated with NC cell pluripotency and migration (Stolt & Wegner 2010). Forced expression of SOX9 in the neural tube cells initiated a programme of neural crest development, suggesting that SOX9 is required for NC development (Spokony et al. 2002). Study has shown that SOX9 transfected NC stem cells migrated from the chicken embryo explant *in vitro* while cells transfected with control vectors did not (Cheung & Briscoe 2003).

p75 is extensively expressed by NC stem cells during neuro-embryogenesis and used as a marker to isolate NSC from NC stem cells. During NC stem cell segregation in the neural tube, $p75^-$ NC cells differentiated into melanocytes while a subpopulation of $p75^+/Kit^-$ cells migrated into the ventral region of embryo trunk, indicating their commitment to ventral NC derived lineages such as neurons in spinal cord (Wilson et al. 2004). p75 was down-regulated at this latter stage. The down regulation was suggested to be after the p75 mediated cell apoptosis involved in CNS size and shape regulation (Haydar et al. 1999). Thereafter, $p75^+$ NC stem cells are reported to be found in low percentages in adult stem cell populations; for example, only 0.3% of cells in the bone marrow stem cell population were $p75^+$ (Poloni et al. 2009).

In this study p75 was also found to be expressed at low percentage in human adult DPSC (<10%, ranging from 1.43% to 6.39%, figure 3.6). This is consistent with the results previously reported that 2.7% of human deciduous DPSC were p75 positive and only 0.3% of $p75^+$ cells are responsible for major neurogenesis in brain SVZ (Mikami et al. 2011; Young, KM et al. 2007).

3.3.2 $p75^+$ hDPSC are predisposed to differentiation into neural lineages

IHC and flow cytometry results demonstrated that p75 was expressed at low level in hDPSC and delineated a small population with higher expression of neural progenitor cell/NSC markers—SOX1, SOX2 and nestin. This indicated their potential for neuronal differentiation compared to $p75^-$ hDPSC.

SOX2 is expressed in embryonic stem cells (ES) and is responsible for maintenance of pluripotency (D'Amour & Gage 2003). SOX2, by formation of a transcription complex with Ocd-3/4, activates transcription of several genes in ES cells. Besides being expressed by ES cells, SOX1 and SOX2 are also expressed in the neuroectoderm during CNS development and responsible for

neural progenitor identity maintenance (Wood & Episkopou 1999). Normal formation of the neuroectoderm was found in either SOX1-deficient or SOX2 deficient mice (Ferri et al. 2004). Mutation in the SOX2 gene caused NSC/neural progenitor cells to lose their pluripotency state and start differentiating into cells with early neuronal differentiation markers (Graham et al. 2003). On the contrary, expression of SOX2 inhibited SVZ neuronal differentiation (Graham et al. 2003).

Nestin is an intermediate neurofilament protein whose expression was observed in neural progenitors and neural stem cells during embryogenesis (Dahlstrand, Lardelli & Lendahl 1995). Many researchers have used it as a marker for neural stem cells or neural progenitor cells. Similar to p75, nestin expression was down-regulated in adult tissue but remained in cells of the brain SVZ (Ernst & Christie 2005) and SGZ (Cameron & McKay 2001), two regions responsible for adult neurogenesis in the brain tissue.

$p75^+$ hDPSC of neural crest origin, have an expression pattern of NSC markers. In other words, $p75^+$ hDPSC with higher SOX1, SOX2 and nestin expression content have a predisposition of differentiation towards the neural lineage.

The loss of SOX1 and SOX2 expression in the nucleus indicates the loss of pluripotency of cells. SOX2 was expressed in hDPSC nucleus in the cells up to 2 passages and was translocated to the cytoplasm afterwards (Liu, L et al. 2011). This might be in relation to the reported altered cell colony-forming capability, cell pluripotency and cell cycle of hDPSC with prolonged passages and cultures *in vitro* (Ma et al. 2009).

3.3.3 $p75^+$ hDPSC had mesenchymal stem cell marker expression

Flow cytometry results revealed that hDPSC were MHC class I positive but MHC class II negative. MHC class I molecules are found in all nucleated cells and MHC class II molecules are normally

restricted in a few specialized cell types such as antigen presenting cells. This was consistent with most of the stem cell populations such as mesenchymal stromal cells from human umbilical cord tissue (Schugar et al. 2009), human osteocyte-derived mesenchymal stem cells and BMSC (Singh et al. 2008) but BMSC were negative for both MHC class I and MHC class II molecules (Jiang et al. 2002). Expressions of MHC class I and MHC class II molecules were related to immunosuppressant properties but no literature have been reported any functional relating to differentiation.

Flow cytometry results demonstrated that p75 was also co-expressed with mesenchymal stem cell marker –CD146 (Figure 3.7 G, Figure 3.8 G and Figure 3.9 G). CD146 is usually expressed in adult stem cells as a MSC marker (Wang & Yan 2013). CD146 is a membrane glycoprotein, which functions as an adhesion molecule in cell to cell interaction (Shih, IM 1999). MSC are a stem cell population showing tri-lineage differentiation giving rise to osteocytes, chondrocytes and adipocytes (Schwarz et al. 1998). Sorted $CD146^-$ HUCPVC could not differentiate into osteocytes, adipocytes and chondrocytes while $CD146^+$ HUCPVC were mesenchymal lineage committed (Baksh, Yao & Tuan 2007). During embryonic development, CD146 is also expressed by vasculature in the developing nervous system and responsible for cell adhesion between neurons and endothelial cells.

On the contrary, one study revealed that p75 expression inhibits mesenchymal stem cell tri-lineage differentiation (Mikami et al. 2011). Therefore, the co-expression of p75 and CD146 in hDPSC made it hard to determine whether hDPSC had a pre-disposition toward neural lineage or mesenchymal lineages. CD146 was also known as melanoma cell adhesion molecule (MCAM), which belongs to a cell-surface IgG superfamily and is expressed in normal tissues expression in normal tissues including in vascular endothelial cells, smooth muscle cells and mesenchymal stem

cells (Ishikawa et al. 2014). The explanation for p75 co-expression with CD146 might be due to the adhesion properties of CD146.

3.4 Summary

hDPSC were SOX9 positive, indicating their NC stem cell origin during embryogenesis. $p75^+$ hDPSC had higher expression of nestin, SOX1 and SOX2. This indicated a neuronal differentiation preposition of $p75^+$ hDPSC compared to $p75^-$ hDPSC. The loss of SOX1 and SOX2 expression in the nucleus indicates the loss of pluripotency of cells. $p75^+$ hDPSC also expressed mesenchymal stem cell marker CD146. The expression of CD146 in $p75^+$ hDPSC was unusual as previous studies have suggested p75 expression inhibits mesenchymal stem cell tri-lineage differentiation. CD146 expression in $p75^+$ hDPSC possible acts as an adhesion molecule for cell interaction.

4. Chapter 4- Separation of p75^{+} cells from hDPSC populations

4.1 Introduction

hDPSC are easily accessible adult stem cells that have been found to improve brain function and cognition in a rat stroke model (Leong et al. 2012). The rat stroke study also found that only a small percentage (2.3%) of transplanted hDPSC survived and that they differentiated into the neurons and astrocytes in the ischemic brain. This is consistent with the suggestion that functional stem cells are usually present in only small quantities in adult tissues and organs (Kamihira & Kumar 2007). Since p75 neurotrophin receptor is expressed at a low level (<5%) (Figure 3.10) on hDPSC and it is co-expressed with NSC marker nestin, neural precursor marker SOX1, pluripotency marker SOX2, NC stem cell marker SOX9 and MSC marker CD146, it is suggested that it has a function associated with pluripotency and neural differentiation potential. Isolation of p75+ stem cells from the heterogeneous hDPSC population is necessary for the assessment of p75 function.

Stem cell separation is different from other cell separation. In order to maintain pluripotency, and achieve high viability and recovery, the separation procedure needs to be handled carefully and rapidly (Kamihira & Kumar 2007). This study aims to select a method for separating the small p75^{+} population from heterogeneous hDPSC populations. FACS with its high sensitivity but long processing time is excluded from this study. Immune panning with low sensitivity but high viability and recovery and MACS with high sensitivity and reasonable viability and recovery have been assessed in this chapter.

4.2 Methods

4.2.1 Flow cytometry: p75 expression on SH-SY5Y and BSRc control cell lines

Flow cytometry analysis was carried out to confirm p75 expression on positive control SH-SY5Y and negative control BSRc cell lines. The protocol as described in section 2.2.13. Cells were harvested when 70% confluent and labeled using an anti-p75 antibody (mlr2 anti-p75 IgG Alexa Fluor 647). Data was analyzed using a Beckman FC500 (Beckman Coulter Inc, Australia) and recorded using CXP software. The data was further analyzed using FlowJo software (Three Star inc, Oregon).

4.2.2 Immune-panning

4.2.2.1 General protocol

p75 high expressing hDPSC and p75 low expressing hDPSC populations were separated by immune panning. hDPSC were trypsinized into a single cell suspension and seeded onto an anti-p75 antibody (mlr2 anti-p75 IgG) coated plate at a cell density 100,000 cells/ well. The cells and antibodies were incubated for 60 minutes at room temperature in the dark. Unbounded cells were then washed with Hanks Balanced Salt Solution (HBSS) three times and the wash collected. It was expected that p75 high-expressing cells would attach onto the plate through an interaction between p75 and mlr2 anti-p75 IgG whereas p75 low-expressing cells would detach during the HBSS wash. The bound p75 high-expressing cells were then depolarized by incubating with depolarization solution (Table 2.3) for 30 seconds and then neutralized with fresh cell culture medium. For hDPSC, cells were neutralized with standard hDPSC medium (Table 2.2). For BSRc and SH-SY5Y cells, cells were neutralized with BSRc and SH-SY5Y cell culture medium (Table 2.2). Cells were pelleted and placed in fresh medium. Cell numbers of both p75 high-expressing and p75-low

expressing hDPSC were determined using a haemocytometer (section 2.2.5). Successful panning was expected to result in approximately 900,000 $p75^-$ hDPSC and approximately 2,000 $p75^+$ hDPSC. Fluorescence emissions were assessed by flow cytometry to confirm the validity of separation (Section 2.2.13).

4.2.2.2 Immune panning of BSRc and SH-SY5Y control cell lines

To assess the validity of the panning procedure, 1x 10^5 BSRc, 1x10^5 SH-SY5Y and 1x10^5 mixture of the two cell populations (in equal cell numbers) were put through the immune panning procedure as above (Section 4.2.2.1). Images were taken of cells remaining on the plate after washing. It was expected that most of the BSRc cells would be washed away and most of the SH-SY5Y would remain in the plate after the HBSS washes.

4.2.2.3 Immune panning of hDPSC

Immune panning was used to separate p75 high-expressing and p75 low-expressing hDPSC (Section 4.2.2.1) and separated cells were sub-cultured in standard hDPSC medium (Table 2.2). Flow cytometry of p75 expression was performed on 70% confluent cells to confirm the efficacy of the separation (Section 4.2.1).

4.2.2.4 Immune panning: optimization of antibody amount

Optimizations of antibody amount was attempted to ensure maximum binding efficiency between cells and the mlr2 anti-p75 IgG antibody. To assess the optimal antibody amount, 24 well plates were treated with 0.6µg or 1.2 µg of the mlr2 anti-p75 IgG antibody and cells were seeded at a density of 100,000 cells/ well on a 24 well plate. The number of cells that remained on the plate after washing was determined.

4.2.2.5 Immune panning: optimization of plate blocking buffer

Optimization of plate blocking was first attempted using 1% BSA. As 1% BSA had an equivalent blocking effect as 10% FCS, further optimization assays were carried with 8% FCS, 4% FCS, 2% FCS or PBS. With each concentration of FCS used, antibody concentrations of 8 µg/mL, 4 µg/mL and 2 µg/mL were tested and compared with PBS only.

4.2.3 Magnetic activated cell sorting (MACS)

4.2.3.1 General protocol

MACS was used to separate p75 high-expressing and p75 low-expressing cells as per the manufacturer's instructions (Miltenyi Biotech, Australia). A total of 1x 10^7 of hDPSC, BSRc and SH-SY5Y cells were labeled with mlr2 anti-p75 IgG Alexa Fluor 647 followed by labeling with anti-Alexa Fluor 647-MACS beads. Cells were then washed and resuspended in 500µL MACS buffer I (Miltenyi Biotech, Australia) and subsequently separated by a strong magnetic field, generated by inserting the MACS MS columns in to the MACS separation magnet (Figure 1.2). The MS column with ferromagnetic spheres, amplified the magnetic field by 10,000-fold, thus inducing a high gradient within the column. The $p75^-$ cells without magnetic labeling migrated through the magnetic field quickly and were eluted first. The $p75^+$ cells labeled with anti-Alexa Fluor 647 MACS beads were trapped by the magnetic field. After collection of $p75^-$ cells, the column was removed from the magnet and trapped $p75^+$ cells were ejected by immediately pushing a plunger firmly into the top of the column.

4.2.3.2 MACS: flow cytometry confirmation of p75 expression after long term culturing

MACS separated $p75^+$ and $p75^-$ hDPSC (H1A1DPSC) (Section 4.2.3.1) were cultured in standard hDPSC culture medium and harvested when cells were 70% confluent. Cells were labeled with

mlr2 anti-p75 IgG Alexa Fluor 647 and assessed by flow cytometry for p75 expression (Section 4.2.1).

4.2.3.3 MACS: flow cytometry confirmation of p75 expression immediately after separation

hDPSC were separated by MACS (Section 4.2.3.1) and expression of p75 was assessed immediately after separation by flow cytometry. In addition, BSRc (negative control) and SH-SY5Y (positive control) cells were separated by MACS at the same time to validate the procedure. Flow cytometry of p75 expression was performed to confirm the efficacy of the separation (Section 4.2.1).

4.2.3.4 MACS: PI assessment of cell death

MACS separated $p75^+$ and $p75^-$ H1A1DPSC (Section 4.2.3.1) were stained with propidium iodide (PI) immediately after separation to assess the viability of the cells, in order to determine whether an additional procedure was required to deplete dead cells. Flow cytometry analysis of PI expression was performed to determine cell survival (Section 4.2.1).

4.2.3.5 MACS: Application of glycophorin A to remove dead cells

H1A1DPSC were first incubated with glycophorin A MACS beads for depletion of dead cells and debris. Cells were then washed and resuspended in 500µL MACS buffer I (Miltenyi Biotech, Australia) and subsequently separated using a strong magnetic field. Cells negative for glycophorin A were eluted first and collected. Cells which were glycophorin A^+ were trapped in the magnetic field and discarded. Glycophorin A^- H1A1DPSC were subsequently labeled with mlr2 anti-p75 Alexa Fluor 647 and anti-Alexa Fluor 647 MACS beads for p75 separation (Section 4.2.3.1).

4.2.3.6 MACS: Confocal microscope confirmation of p75 expression immediately after separation

MACS separated H1A1DPSC (Section 4.2.3.5) with glycophorin A were cultured in poly-L-lysine and laminin coated coverslips overnight for adhesion. The expression of p75 was assessed under a confocal microscope the second day using anti-p75 Alexa Fluor 647.

4.2.4 Neurogenic potential assessment of separated $p75^+$ and $p75^-$ hDPSC

MACS separated $p75^+$ and $p75^-$ H1A1DPSC (Section 4.2.3.5) were seeded on a 24 well plate at a density of 30,000 cells/well. Cells then underwent differentiation by using differentiation media in a progressive neuro-differentiation protocol over 13 days as described in Section 2.2.9. Cells were fixed at the neuronal maturation stage in 4% PFA in PBS for 15 minutes. Subsequently, blocking buffer A (2% BSA, 0.2% Triton-X in 1XPBS) was used to block the non-specific binding with incubation for 30 minutes at room temperature. Primary antibodies mouse anti-NFM (1:200) and mouse anti-NeuN (1:500) were diluted in blocking buffer B (1% BSA, 0.5% donkey serum in 1XPBS) and incubated on cells overnight at 4 °C. After washing in PBS, secondary antibody cy3 anti-mouse IgG (1:400) were used for one hour at 4 °C to identify bound primary antibodies. All coverslips were mounted using Prolong Gold antifade reagent (Invitrogen, Carlsbad, USA). The fluorescence staining was assessed either through a Leica SP5 spectral scanning confocal microscope (Leica Microsystems, Wetzlar, Germany) or Olympus BX51 epifluorescence microscope (Olympus innovation, USA). Images were processed using imageJ (NIH) software.

4.3 Results and analysis

4.3.1 SH-SY5Y and BSRc control cell lines

In order to isolate a $p75^+$ population from hDPSC, it is important that appropriate positive and negative controls are used. SH-SY5Y is a neuroblastoma cell line (Biedler, Helson & Spengler 1973) expressing neurotrophin receptors such as p75 and Trk receptors (Baker et al. 1989). BSRc cells are a clone of baby hamster kidney fibroblast cells (Sato et al. 1977) and are p75 negative (Rogers et al. 2006) (Figure 4.1 B). Flow cytometry confirmed that SH-SY5Y and BSRc cell lines were high and low p75 expressing, respectively (Figure 4.1 A and B). Thus, these cell lines were used to assess the effectiveness of p75 separation techniques in this chapter.

4.3.2 Immune panning

4.3.2.1 Immune panning is an effective separation method for SH-SY5Y and BSRc cells

Both SH-SY5Y cells and BSRc cells were able to settle down into the bottom of the plate effectively by gravity (Figure 4.2 A, B, C). SH-SY5Y cells, with their high expression of p75, attached to the mIr2 anti-p75 IgG coated plate effectively (Figure 4.2 D) while BSRc cells, which are p75 negative, were washed away from the antibody coated plate easily (Figure 4.2 E). Approximately half of the 1:1 mixture of SH-SY5Y cells and BSRc cells remained in the plate and the rest were washed off as expected (Figure 4.2 F). Altogether, this indicates that immune panning based on the expression of p75 can be used to separate $p75^+$ and $p75^-$ cells.

4.3.2.2 Immune panning separation of hDPSC has low sensitivity

As immune panning was able to isolate $p75^+$ cells from a heterogeneous population (Section 4.3.2.1), it was used to separate $p75^+$ hDPSC from $p75^-$ hDPSC (Figure 4.3 C, D). Few hDPSC were still attached into the panning plate after three PBS washes (Figure 4.3 C), which was

consistent with the flow cytometry results that p75 is expressed at a low level (ranging from 1.43% to 6.27%) in hDPSC populations (Figure 3.9). The efficacy of the immune panning procedure was assessed by flow cytometry, and demonstrated that 3.14% of the positively selected cells were p75 high-expressing hDPSC (1.76 fold enrichment) (Figure 4.4 B). The presence of p75 low-expressing^{-} hDPSC in the panned p75^{+} population was likely to be due to non-specific binding of p75^{-} hDPSC onto the plates. Thus, methods to reduce the non-specific binding of p75^{-} hDPSC onto the plastic plates were investigated.

4.3.2.3 1% BSA Blocking buffer blocked the interaction between p75 and mlr2 anti-p75 IgG

DPSC attached onto the plastic surface without blocking buffer without antibody, indicating that there was non-specific binding between hDPSC and the plastic surface (Figure 4.5 A). No hDPSC attached onto the non-coated plastic plate treated with 1% BSA, indicated that the non-specific binding between the hDPSC and the polystyrene surface of the plate was effectively blocked by 1% BSA (Figure 4.5 B). However, 1%BSA also blocked the interaction between p75 and anti-p75 IgG as no cells remained on the mlr2 anti-p75 IgG with 1%BSA group (Figure 4.5 E, F). This suggested that further investigation of blocking solutions was warranted.

4.3.2.4 An antibody amount of 0.6 µg was sufficient for panning for p75 positive hDPSC

hDPSC attached effectively onto plates treated with both 0.6µg (Figure 5.5 C) and 1.2µg (Figure 5.5 D) of mlr2 anti-p75 IgG antibody without blocking. However, altering the antibody amount from 0.6µg to 1.2µg did not improve the interaction between p75 and mlr2 anti-p75 IgG on plates blocked with 1% BSA (Figure 4.5 C, D).

4.3.2.5 An optimal concentration of mlr2 anti-p75IgG was 2µg/ml

hDPSC attached most effectively onto plates treated with 2µg/mL p75 antibody (Figure 5.4 B, F, J and N) compared to other antibody concentrations (4 µg/mL and 8µg/mL) with all FCS blocking

buffer concentrations (2%, 4% and 8%) (Figure 4.6). Interestingly, when plates were treated with 2%FCS or without blocking buffer, cells attached more effective into the plates without antibody coating (Figure 4.6 A and E) than into the antibody coated surfaces (Figure 4.6 B to D and F to H).

4.3.2.6 An optimal concentration of blocking buffer was unable to be determined

hDPSC were demonstrated to attach into the plastic plate non-specifically without antibody present and without blocking (Figure 4.6 M). However, 2%, 4% and 8% FCS failed to stop the cells from attaching into the plate non-specifically (Figure 4.6 A, E, I). Though there was an increased trend of cell binding with a decrease in blocking solution concentration, cells adhered onto the plate blocked with 2% FCS more effectively than PBS only group, suggesting that 2% FCS had an effect on the biological properties of the cells. For example, FCS contains a high amount of fetuin-A. Fetuin-A a glycoprotein that plays a role on embryonic stem cell and mesenchymal stem cell growth and differentiation (Mannello & Tonti 2007).

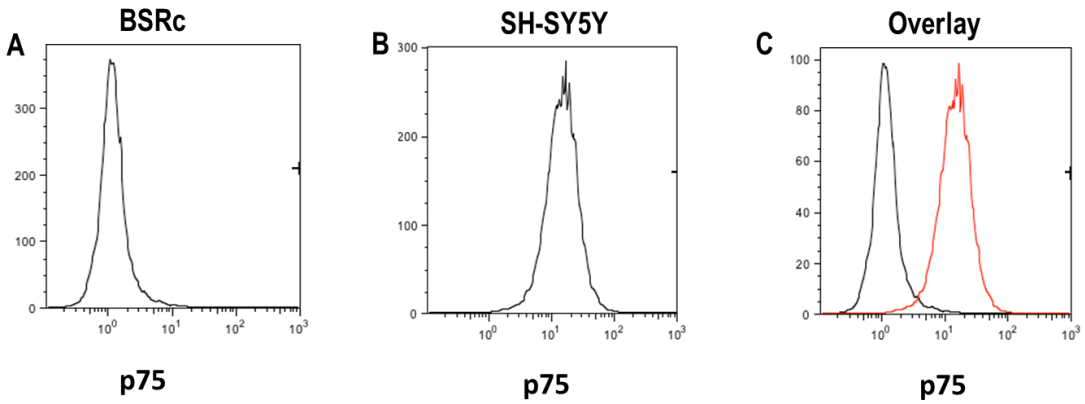


Figure 4.1 Flow cytometry of p75 expression.

Positive and negative control cell lines, SH-SY5Y and BSRc respectively, were labeled using an anti-p75 antibody (mlr2 anti-p75 IgG Alexa Fluor647) and analysed by flow cytometry. (A) no p75 was expressed on BSRc cells (<1%). (B) most of SH-SY5Y cells were p75 positive (>90%). (C) overlay of BSRc (black) and SH-SY5Y (red) (<5%).

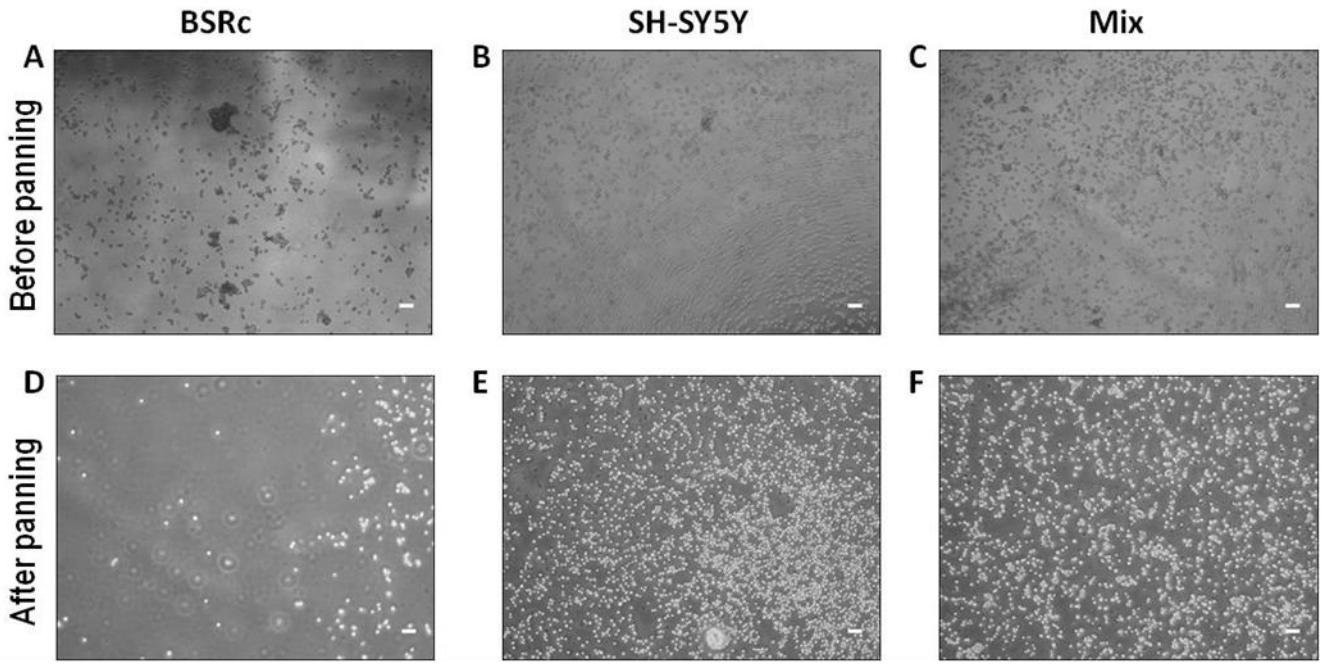


Figure 4.2 Immune panning was performed using a 2µg/ml mIr2 anti-p75 IgG coated plate.

Negative control BSRc cells, positive control SH-SY5Y cells and a mixed population of these two cell types were photographed before the panning procedure (A, B and C, respectively) and after washing (D, E and F, respectively). (D) many of BSRc cells detached from the plate after the panning procedure. (E) most of the SH-SY5Y cells remained on the plate after the panning procedure and (F) the number of mixed cells remaining on the plate was more than the BSRc group but less than the SH-SY5Y group. Scale bar=100µm.

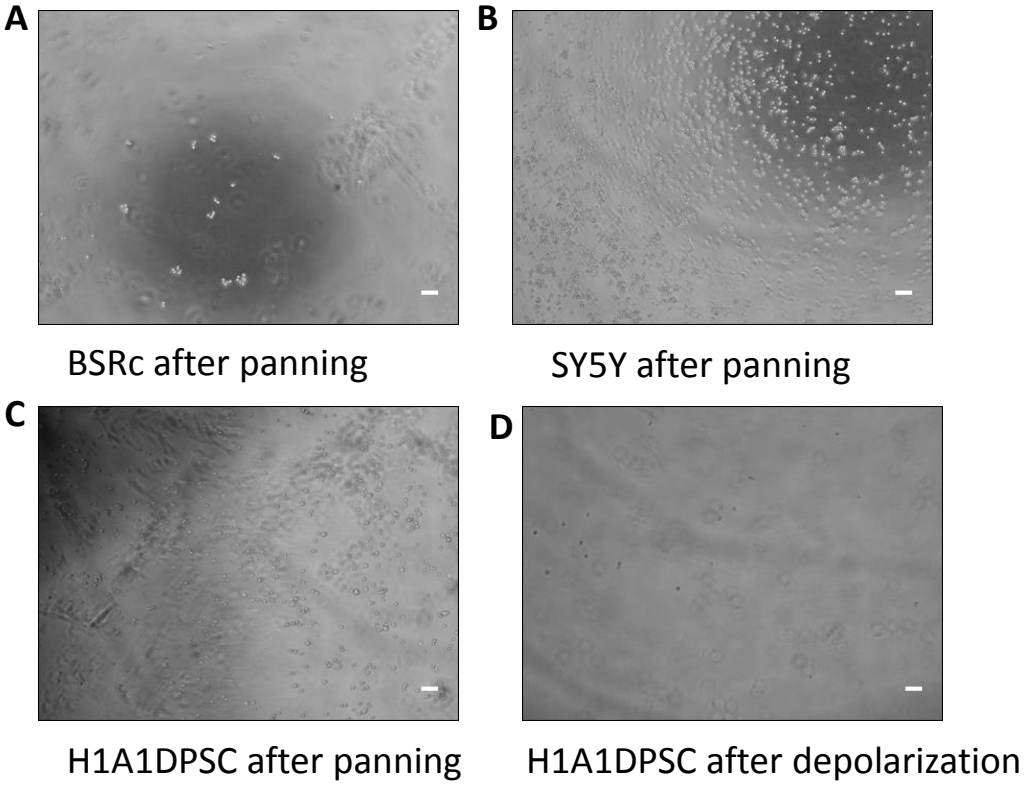


Figure 4.3 Immune panning of H1A1DPSC with p75 negative BSRc and p75 positive SH-SY5Y cell controls.

(A) Most of the BSRc cells were detached from the plate surface after the panning procedure. (B) Most of the SH-SY5Y cells remained on the plate after panning. (C) A few H1A1DPSC cells remained on the plate after the panning procedure and (D) most of the remaining H1A1DPSC cells detached after incubation with the depolarization solution. Scale bar=100µm.

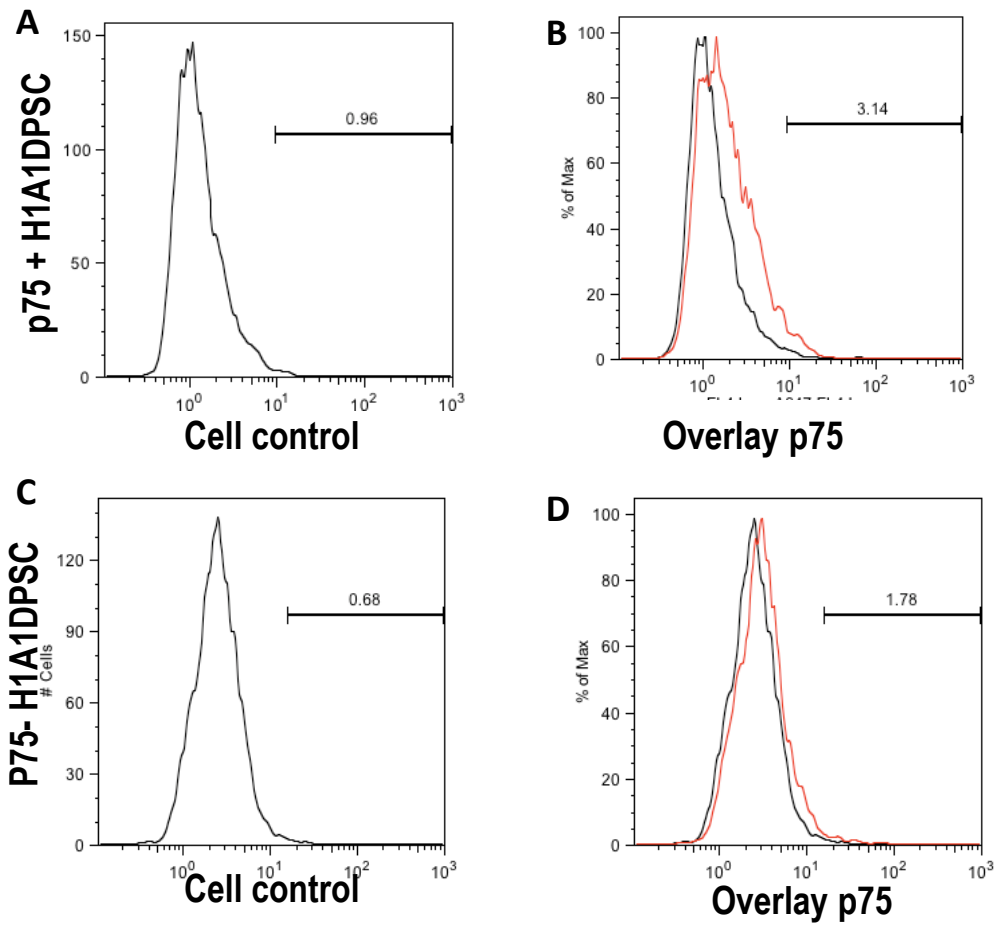


Figure 4.4 Flow cytometry analysis of H1A1DPSC after immune panning.

After immune panning for p75, H1A1DPSC were labeled using an anti-p75 antibody (mlr2 anti-p75 IgG Alexa Fluor647) and analyzed by flow cytometry. (A, C) The cell control without antibody labeling demonstrated a natural fluorescence emitted by cells. A positive threshold was set up as >99% of control cells being negative. (B) 3% of post-panning $p75^{+}$ H1A1DPSC were positive for p75. (D) 1.8% of post-panning $p75^{-}$ H1A1DPSC were positive for p75.

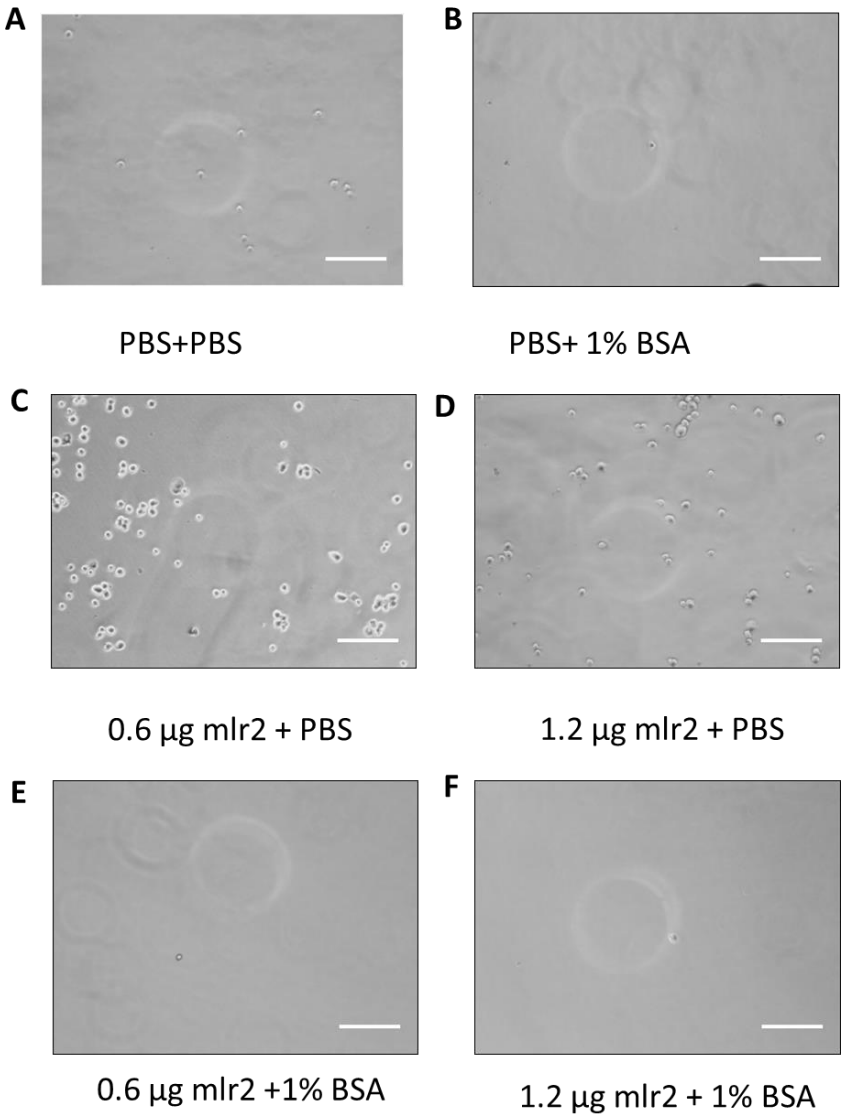


Figure 4.5 Immune panning assays for optimization of the antibody amount with 1% BSA blocking buffer.

Immune panning plates were treated with PBS, 0.6µgof mlr2 p75 antibody or 1.2µg of mlr2 p75 antibody, with (1% BSA) or without (PBS) blocking. A total of 50,000 H1A1DPSC were seeded in each group. (A) H1A1DPSC attached onto the non-coated plastic surface. (B) No H1A1DPSC attached onto the non-coated plastic plate treated with 1% BSA. (C, D) H1A1DPSC attached effectively onto plates treated with both 0.6µg and 1.2µg of mlr2 anti-p75 IgG antibody. (E, F) No H1A1DPSC attached onto mlr2 anti-p75 IgG coated plates blocked with 1% BSA, regardless of the amount of antibody. Scale bar=100µm.

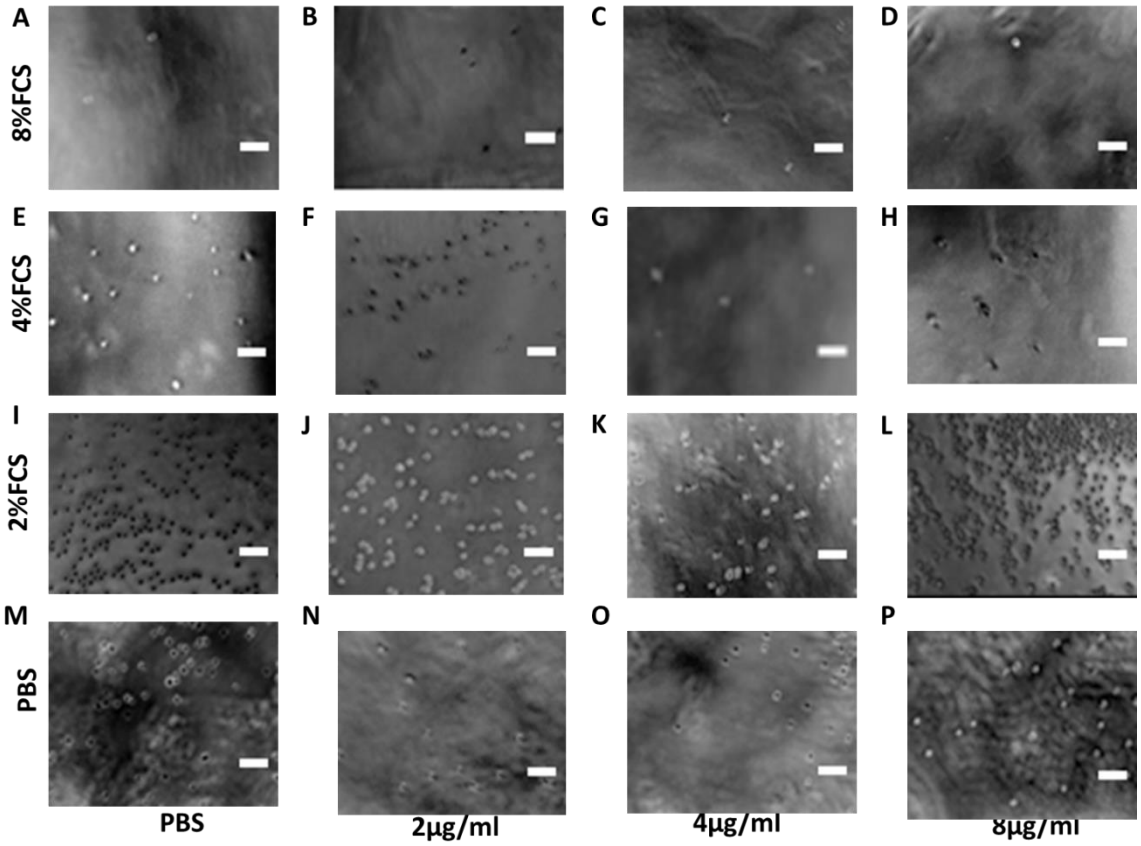


Figure 4.6 Immune-panning for optimization of mlr2 anti-p75 IgG concentration and fetal calf serum blocking buffer concentration.

Immune panning plates were treated with varying concentrations of the mlr2 p75 antibody (0µg/mL, 2 µg/mL, 4 µg/mL or 8 µg/mL) and blocked with varying concentrations of FCS blocking solution (0%, 2%, 4% or 8%), before being seededwith H1A1DPSC (A, E, I, M). H1A1DPSC non-specifically attached onto plastic plates treated with PBS rather than blocking buffer. DPSC attached onto 2µg/mL mlr2 anti-p75 IgG coated plates (B, F, J, N), 4µg/ml mlr2 anti-p75 IgG coated plates (C, G, K, O) and 8µg/ml mlr2 anti-p75 IgG coated plates (D, H, L, P) blocked regardless of FCS concentration. Scale bar=100µm

4.3.3 Magnetic activated cell sorting

4.3.3.1 Long term culturing of MACS separated hDPSC exerted an unexpected effect on cells

MACS separated $p75^+$ hDPSC and $p75^-$ hDPSC were cultured in standard DPSC medium and analyzed via flow cytometry once 70% confluent. Flow cytometry demonstrated a dramatic increase in p75 expression in both $p75^+$ hDPSC (41.8%) (Figure 4.7 B) and $p75^-$ hDPSC (49%) populations (Figure 4.7 D). Due to the presence of high levels of p75 in the negative population these results were suspected to be false positive, a phenomenon that can be caused by the presence of dead cells (Clarke & Davies 2001). After consultation with Miltenyi Biotec it was suggested that cell death may have occurred post-MACS while cells were in culture for an extended period, thus MACS was repeated and flow cytometry performed immediately after the separation.

4.3.3.2 Enrichment of $p75^+$ hDPSC using MACS was low

MACS separated hDPSC were analyzed immediately after separation to avoid cell death with long term culturing. SH-SY5Y positive control cells and BSRc negative control cells were also included to determine the efficacy of the separation method. Flow cytometry of MACS separated SH-SY5Y positive control cells demonstrated that 85.9% of the $p75^+$ SH-SY5Y cell population isolated was p75 positive (Figure 4.8 C), while 10.1% of the $p75^-$ SH-SY5Y cell population was p75 positive (Figure 4.8 D). Flow cytometry of MACS separated BSRc negative control cells demonstrated that there were no $p75^+$ BSRc cells present in either the positively selected or negatively selected cell population (0.64% and 0.95%, respectively; Figure 4.8 A and B), as there was no p75 expressed on BSRc cells. MACS separation of $p75^+$ hDPSC demonstrated that only 2.5% of the $p75^+$ hDPSC

cell population expressed p75 and 0.73% of the $p75^-$ hDPSC population expressed p75. This was only a modest enrichment of 3.4 fold.

4.3.3.3 Glycophorin A was necessary to remove dead cells before separation

It was suggested that magnetic microbeads had non-specific binding to dead cells, which led to the poor separation of p75 positive and negative cell populations. Therefore, PI analysis of cell death in both $p75^+$ hDPSC and $p75^-$ hDPSC was performed. It was found that 50% of $p75^+$ hDPSC were viable (Figure 4.9 A) and 90% of $p75^-$ hDPSC were viable (Figure 4.9 C). The expression of p75 by live cells in the $p75^+$ hDPSC population was 1.51% (Figure 4.9 B) and the expression of p75 by live cells in the $p75^-$ hDPSC population was only 0.12% (Figure 4.9 D). While the expression of p75 was not enriched greatly, the $p75^+$ population was investigated further for its neurogenic potential.

4.3.3.4 MACS separated cells differentiated into cells with neuronal morphology

Separated cell p75 expression was further confirmed by IHC, demonstrating p75 staining in $p75^+$ hDPSC, while no p75 was detected in $p75^-$ hDPSC (Figure 4.10 A, B, D, E). Both $p75^+$ hDPSC and $p75^-$ hDPSC were able to differentiate into cells with neuronal morphology with multiple cellular processes (Figure 4.10 C F). The sizes of differentiated cells derived from $p75^+$ hDPSC were observed to be larger than the ones derived from the $p75^-$ hDPSC.

4.3.3.5 MACS separated cells population differed in their neural marker expression after neural differentiation

IHC demonstrated that MACS separated p75+ hDPSC expressed neural filament medium chain (NFM) (Figure 4.11 B) while MACS separated $p75^-$ hDPSC did not (Figure 4.11 E). This suggested that $p75^+$ hDPSC had a greater propensity towards neural lineage compared with $p75^-$ hPDSC.

Both cells differentiated from p75+ and p75- hDPSC populations were unable to generate NeuN positive cells, indicating that differentiated cells were not mature neurons (Figure 4.11 C, F).

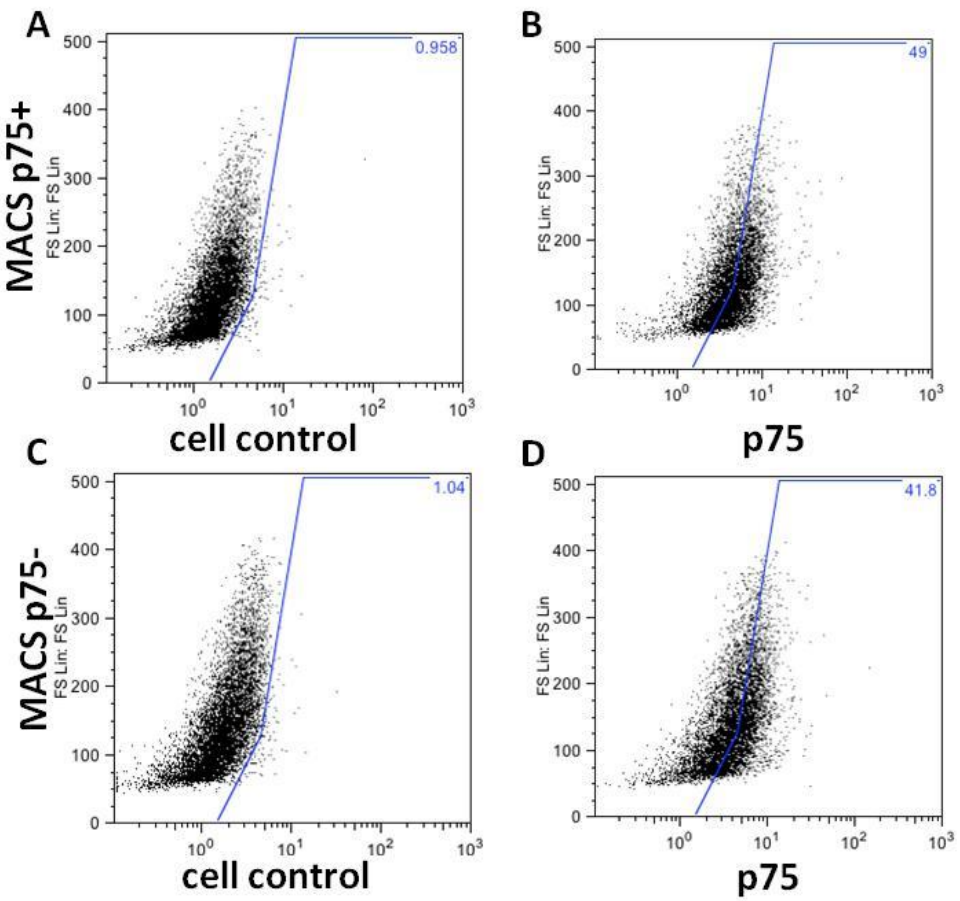


Figure 4.7 Flow cytometry analyses of expression of p75 in p75^{+} and p75^{-} MACS separated cells.

After MACS separation according to p75 expression, cells were cultured in standard DPSC medium and analyzed by flow cytometry once 70% confluent. (A and C) Cell control of MACS isolated p75^{+} cells demonstrated some natural fluorescence emitted by H1A1DPSC. A positive threshold was set up with >99% cells being negative for the analysis of the MACS separated (B) p75^{+} H1A1DPSC population and (D) p75^{-} H1A1DPSC control cell population.

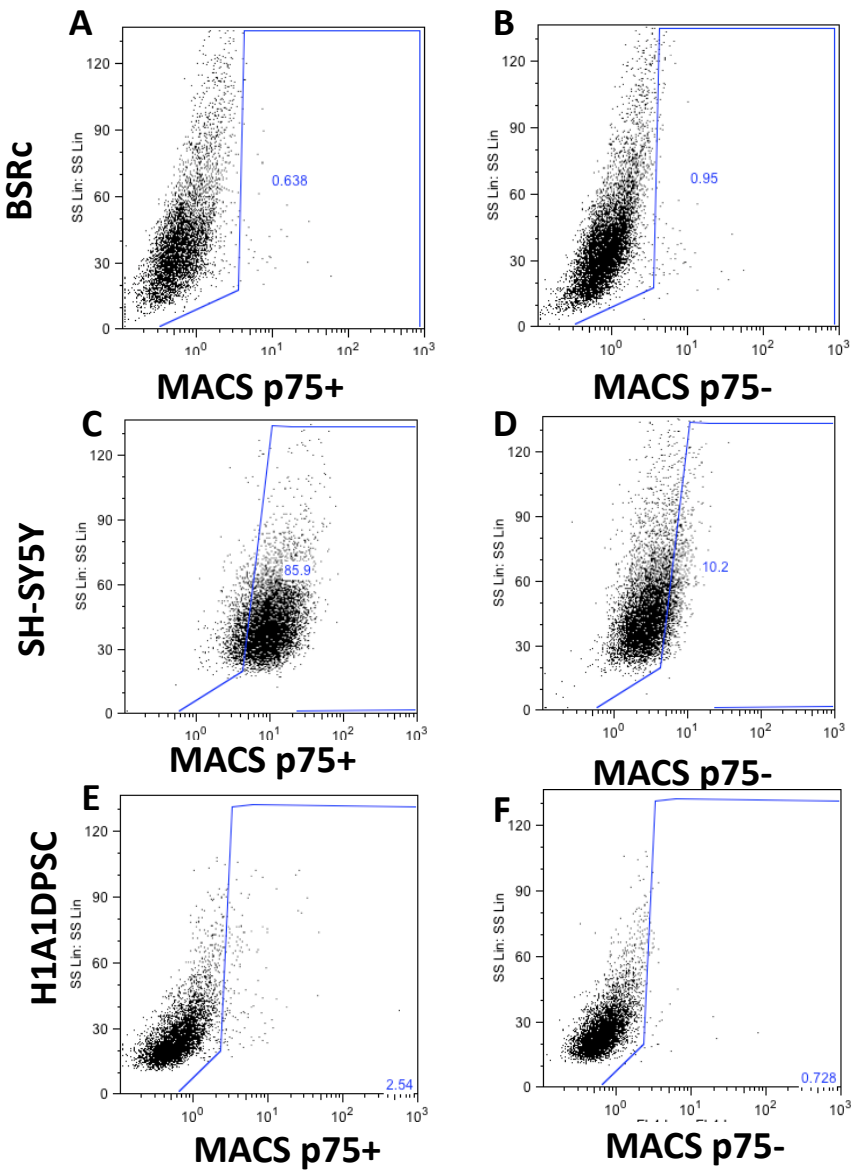


Figure 4.8 MACS separation of p75 negative cell control BSRc, p75 positive cell control SH-SY5Y and H1A1DPSC.

(A, B) p75 negative control BSRc cells, (C, D) p75 positive control SH-SY5Y cells and (E, F) H1A1DPSC were separated into (A, C, E) $p75^{+}$, and (B, D, F) $p75^{-}$ populations by MACS and analyzed by flow cytometry immediately after separation.

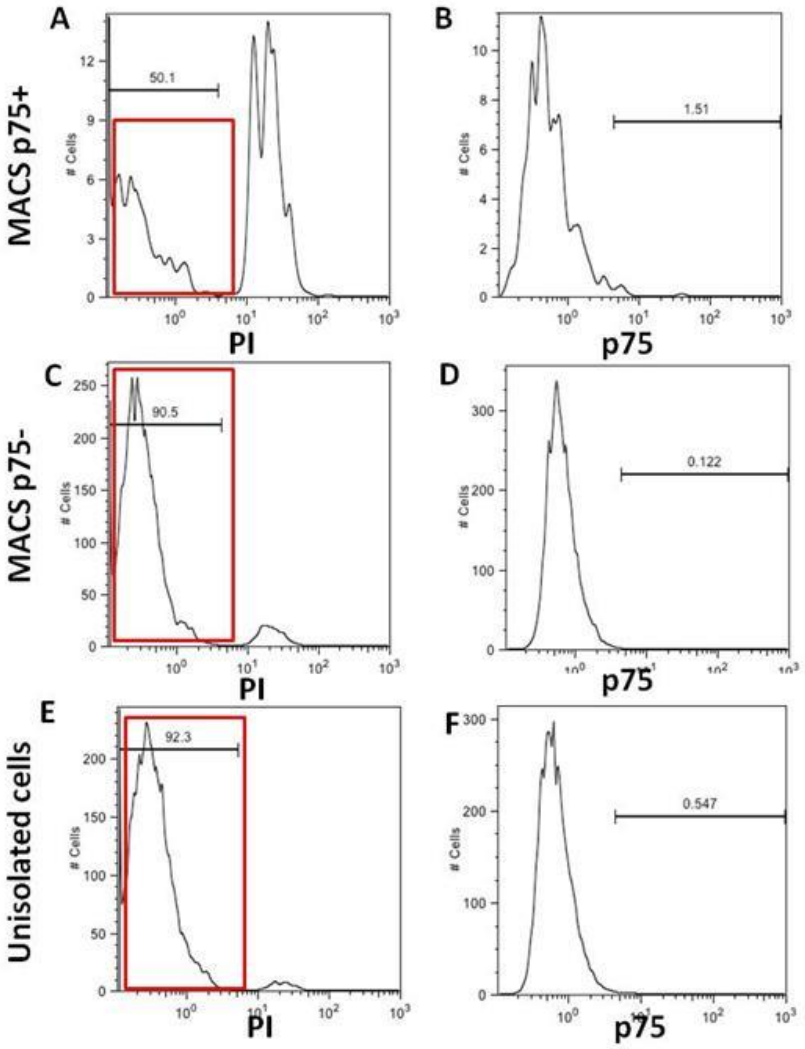


Figure 4.9 Cell viability was determined in MACS separated cells.

Cell viability was determined in MACS separated (A) $p75^+$ H1A1DPSC, (C) $p75^-$ H1A1DPSC and (E) H1A1DPSC that had not been MACS separated using a PI antibody and flow cytometry. (B, D, F) Live cells were further analyzed for p75 expression.

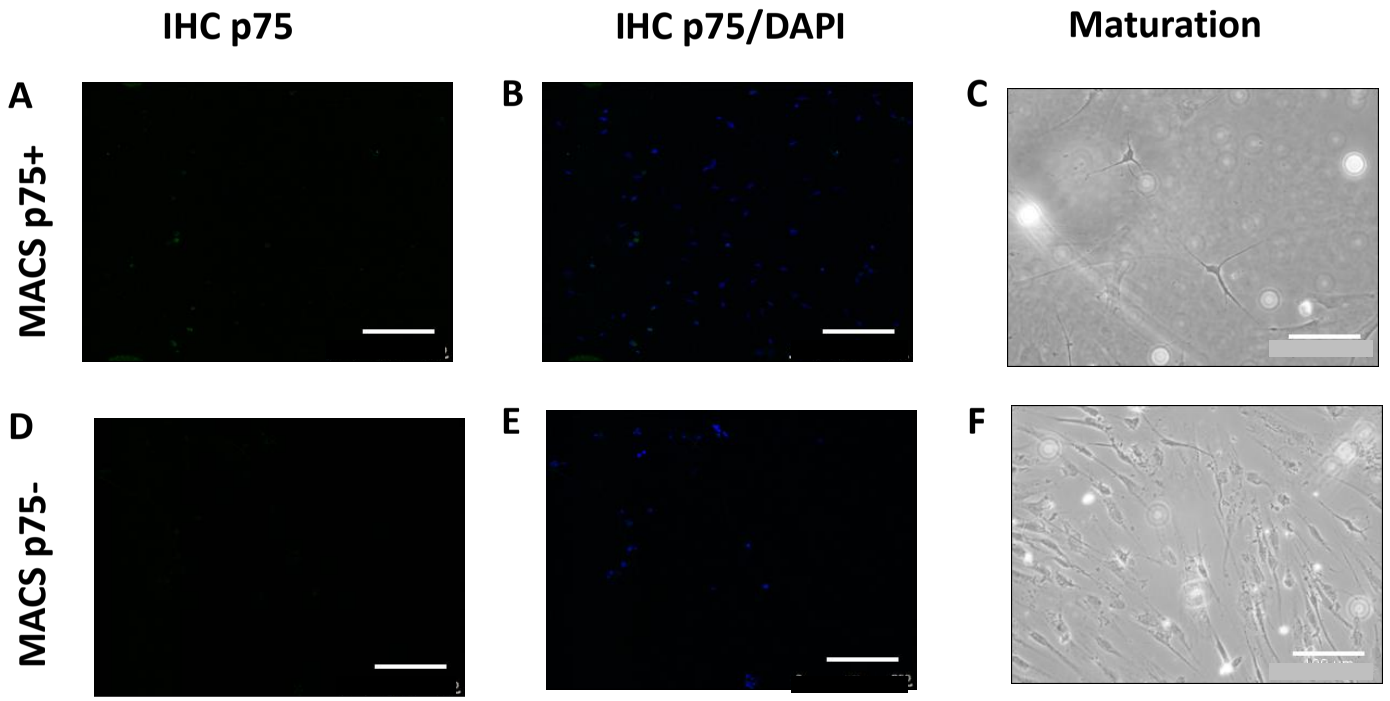


Figure 4.10 Comparison of neurogenicity of MACS separated p75^{+} AH27DPSC.

Assessment of IHC by confocal microscopy demonstrated no expression of p75 (green) in (D, E) MACS p75^{-} AH27DPSC (D, E) and no neuronal morphology after neuronal differentiation (F). Several MACS isolated p75^{+} H1A1DPSC were positive for p75 (green; white arrow) (A, B) and differentiated MACS isolated p75^{+} H1A1DPSC exhibited neuronal like cells of a larger size and with cellular processes(C). Scale bar=100µm.

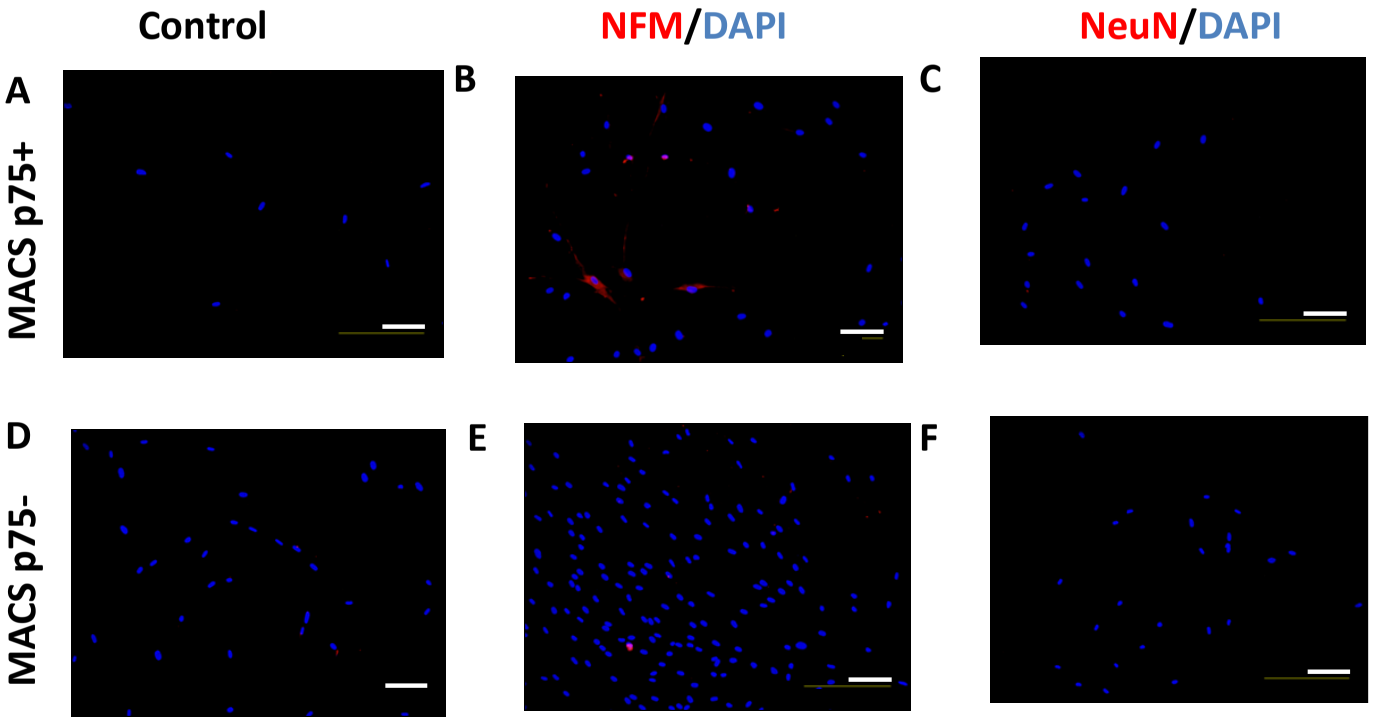


Figure 4.11 Immunohistochemical analysis of differentiated MACS separated $p75^+$ and $p75^-$ hDPSC. Immunohistochemical analysis of differentiated MACS separated (A, B, C) $p75^+$ and (D, E, F) $p75^-$ hDPSC using antibodies specific to (B, E) neurofilament medium chain (NFM) (red) and (C, F) NeuN (red). (A, D) A negative control without primary antibody was also included.

4.4 Discussion

Immune panning and MACS were not effective at separating $p75^+$ hDPSC from $p75^-$ hDPSC. Using the MACS technique, there was some enrichment of $p75^+$ hDPSC by approximately 10 fold. Cells underwent neuronal differentiation and $p75^+$ hDPSC exhibited superior neuronal marker expression compared with $p75^-$ hDPSC.

4.4.1 Immune panning of hDPSC

Compared to FACS, the immune panning technique for stem cell separation avoids potential toxicity caused by exposure to laser light and usually results in a high recovery and viability (Wiese et al. 2010). Immune panning for cell separation is based on the differences in cell rolling velocity/ rolling capacity on a panning surface under fluid flow (Mahara & Yamaoka 2010). Besides non-specific binding between adhesion molecules and the polystyrene surface, the cell interaction strength between p75 and immobilized mlr2 anti-p75 IgG on the panning surface provided additional interaction strength for holding cells onto the plastic surface (Hammer & Apte 1992) (Figure 4.11). Therefore, $p75^+$ cells had lower rolling capacity than $p75^-$ cells and $p75^-$ cells should have dissociated from the plastic plate.

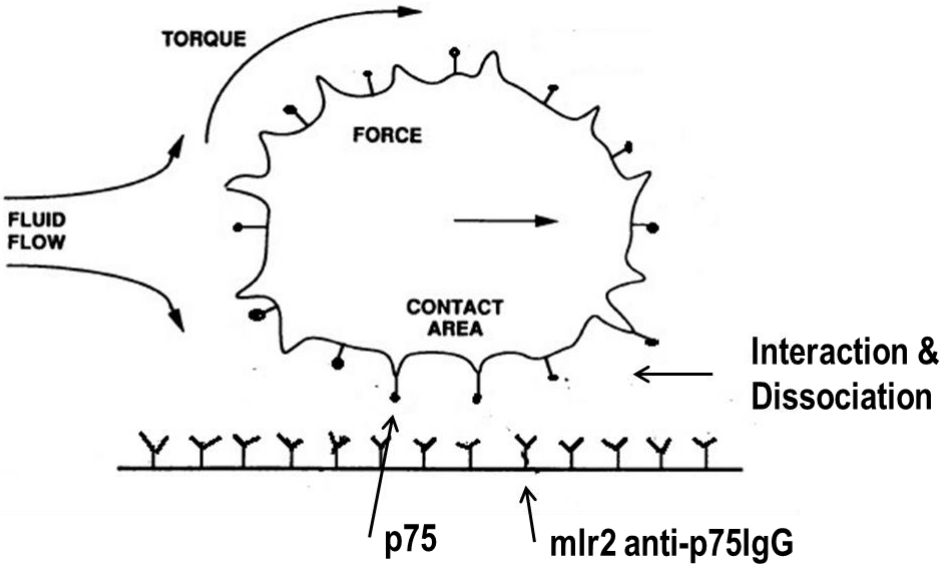


Figure 4.12 A schematic diagram demonstrating the interaction between p75 and immobilized mIr2 anti-p75 IgG and the role of cell rolling capacity under fluid flow.

Figure adapted from (Hammer & Apte 1992).

SH-SY5Y cells with their high expression of p75 on the cell surface had most cells remaining on the panning plate and BSRc cells without expression of p75 were washed away as expected (Figure 4.1 D, E, F). However, flow cytometry analysis (small shift of the histogram of p75 labeled cell from the control cells, Figure 3.6 C) and IHC staining (faint staining, Figure 3.1 A) demonstrated that p75 was expressed in hDPSC at a very low density. The difference in the rolling capacity between $p75^+$ hDPSC and $p75^-$ hDPSC was not great enough to separate them. In other words, the panning rate/fluid flow rate was not able to maintain the $p75^+$hDPSC on the plate and to roll the $p75^-$ hDPSC away. $p75^-$ hDPSC adhered onto the panning surface by non-specific binding between adhesion molecules and the plastic surface. Optimization of immune panning by applying blocking agents BSA or FCS after the mIr2 anti-p75 IgG plate coating was carried out in order to block the non-specific binding sites on the plastic plate.

BSA and FCS have been widely used in many antibody affinity based experiments such as western blot, enzyme linked immunosorbent assay (ELISA) and IHC to reduce non-specific binding (Mahmudi-Azer et al. 1998). FCS/BSA have previously been used in the procedure of immune panning after the coating of the antibody to improve the purity of cell separation (Meyer-Franke et al. 1998; Stallcup & Beasley 1987).

The concentration of 10 % FCS (equivalent to1% BSA) and 8% FCS effectively blocked the non-specific binding between the cells and plastic surface (Figure 4.5 B and Figure 4.6 A), but also blocked the binding between cells and mlr2 anti-p75 Ig (Figure 4.5 E, F and Figure 4.6 B, C, D). FCS concentrations less than 8% (4%FCS and 2%FCS) were unable to block non-specific binding between the cells and the plastic surface (Figure 4.6). Thus, immune panning was unable to be optimized. Besides p75 and mlr2 anti-p75 IgG binding interactions, some other factors may also have played a role in cell rolling capacity such as cell morphology (affects the torque), mechanical properties and rates of reaction between other receptors and ligands.

Immune panning has been widely used in the isolation of motor neurons from the mouse/ rat embryonic spinal cord (Conrad et al. 2011; Graber & Harris 2013). Embryonic motor neurons unlike hDPSC had some characteristics which made them suitable for immune panning. Firstly, p75 is highly expressed on embryonic spinal cord. p75 expression has also been used successfully for embryonic motor neuron enrichment by FACS (Wiese et al. 2010). Motor neurons have morphological differences (being larger in size) from surrounding cells in the panning plate which can be distinguished under a microscope (Graber & Harris 2013). Therefore, the strength of panning can be adjusted during the procedure to achieve a high purity of separation. In addition, embryonic motor neurons are highly vulnerable to damage caused by toxicity such as oxidized metals (during surgery) or toxicity generated by the laser light (FACS) (Graber & Harris 2013).

Thus, immune panning results in better viability and recovery of the fragile embryonic motor neurons compared with FACS.

FCS as a widely used supplement in cell culture medium containing nutrients for cell growth and proliferation. It was interesting that there was an unexpected increase in cell attachment in plates coated with 2% FCS and 4% FCS compared to cells in uncoated plates. However, as the composition is not completely known or constant, FCS has been suggested to have side effects on cultured cells that subsequently affect their properties (Mannello & Tonti 2007). It has previously been found that long term culturing of cells in medium supplemented with 2% or 10% FCS results in chromosome aberration (Suchanek et al. 2013). In this study, it is possible that FCS had an effect on cell adhesion, which resulted in poor cell separation by immune panning.

4.4.2 Magnetic activated cell sorting of hDPSC

MACS separation is a relatively recent technique with some advantages over the traditional FACS and immune panning separation techniques. MACS separation avoids the toxicity of the laser beam used in FACS (Graber & Harris 2013). The MACS separation technique is more sensitive than immune panning as its selection is only based on the expression of the target molecule and is not affected by other cell adhesion molecule expression. The column matrix of the MS columns is composed of ferromagnetic spheres, which amplify the magnetic field by 10,000-fold, when placed in the magnetic field of a MACS® Separato, thus separating trapped cells which are only minimally labeled with MACS® MicroBeads. The space between the spheres is several times larger than most cultured cells allowing the magnetically labeled cells to be held in suspension within the column and cells without label to flow through the column freely.

$p75^+$ SH-SY5Y cells labeled with mlr2 anti-p75 IgG and magnetic microbeads, were trapped by the loosely packed magnetic beads in the column. Some of the $p75^+$ SH-SY5Y cells were found in the p75 negative fraction (Figure 4.8 D). This might have been because the strength generated by the magnetic field was not strong enough to hold all the cell population of SH-SY5Y, given the high expression of p75 in this cell population. The total number of SH-SY5Y cells applied to be separated was around $1x10^7$. Separation results of BSRc cells as a p75 negative control population were as expected. There was no p75 expression on both the trapped BSRc and effluent BSRc fraction (Figure 4. 8 E F). The few trapped BSRc cells in the magnetic column might have been due to the capture on the magnetic surface rather than of the power of the magnetic attraction.

Though MACS separation of SH-SY5Y and BSRc control cells were successful, enrichment of $p75^+$ hDPSC was not so effective. The cells were enriched by only 3.4 fold. There were two possible reasons for this suggested by technicians from Miltenyi Biotec: 1) the dissociation procedure of the hDPSC causes much cell death and debris, which would lead to nonspecific binding and the formation of aggregates; 2) the p75 molecule was negatively affected by the dissociation procedure. This would have resulted in a very dim fluorochrome labeling, leaving many target cells in the negative fraction and positively selected cells that have lost their fluorescent signal.

As PI can bind to intracellular nucleic acids, it is used as a DNA stain for flow cytometry, to evaluate cell viability or DNA content in cell cycle analysis (Lecoeur 2002). PI assessment revealed 50% cell death in the $p75^+$ portion of hDPSC and only 10% cell death in $p75^-$ hDPSC (Figure 4.9 A). As dead cells release nuclear contents and become sticky, cell debris would bind to antibodies and magnetic microbeads nonspecifically. Thus, $p75^-$ hDPSC would be trapped into the column

nonspecifically. Consequently, depletion of cell debris and aggregation was necessary for the improvement of the separation purity.

Glycophorin A is a major sialoglycoprotein that is expressed by human erythrocyte membranes (Zannettino et al. 2007). Cells were magnetically labeled with glycophorin A microbeads specific to erythroid cells and debris and depleted. Though the enrichment was not as high as SH-SY5Y positive control cells separation, IHC results demonstrated that $p75^+$ hDPSC had visible p75 staining while $p75^-$ hDPSC were unstained.

4.4.3 Neurogenic potential of $p75^+$hDPSC and $p75^-$ hDPSC

The current study has demonstrated that p75 prototypically a neurotrophin receptor, delineated a small population with neurogenic potential from heterogeneous hDPSC. Using MACS, $p75^+$ hDPSC were separated from $p75^-$ hDPSC. $p75^+$ hDPSC differentiated into cells which exhibited greater neuronal marker expression than cells derived from $p75^-$ hDPSC. Both cells differentiated from $p75^+$ hDPSC and $p75^-$ hDPSC exhibited neuronal morphology with enlarged cell bodies and multiple cellular processes (Figure 4.10 C, F). Cells differentiated from $p75^+$ hDPSC expressed early neuronal marker NFM while cells differentiated from $p75^-$ hDPSC did not (Figure 4.11 B, E). Neurofilament medium proteins are expressed transiently during embryonic development. Neurofilament light, medium and heavy chain proteins define different stages of neuronal lineage differentiation (Chiu et al. 1995). NFM expression indicated neuronal commitment of cell differentiation (Dahlstrand et al. 1992). Cells differentiated from $p75^+$ hDPSC were able to give rise to cells of the neuronal lineage while cells derived from $p75^-$ hDPSC were not, indicating superior neurogenic potential of $p75^+$ hDPSC compared to $p75^-$ hDPSC.

The mechanism of p75's effect on hDPSC neuronal differentiation was proposed to be through p75 function in cell cycle withdrawal and terminal differentiation promotion in other cell systems (Chittka et al. 2004; Cragnolini et al. 2012). For example, p75 induced arrest of cell proliferation in PC12 cells, which was suggested to be associated with the initiation of PC12 cells differentiation (van Grunsven et al. 1996). p75 regulation of hDPSC neurogenesis was possibly through BDNF as suggested in other stem cell populations by many studies (Gascon et al. 2005; Lameu et al. 2012; Young, KM et al. 2007; Zigova et al. 1998). Further study is needed to elucidate the mechanism of p75 neurogenesis regulation in hDPSC.

Both $p75^+$ hDPSC and $p75^-$ hDPSC failed to generate NeuN positive cells. NeuN is expressed exclusively by nuclei in the nervous system during embryonic development through to the adulthood (Mullen, Buck & Smith 1992). Therefore, NeuN is used as a marker for mature neurons which neurons have exited the differentiation cycle (Bernabeu & Longo 2010). $p75^+$ hDPSC were able to give rise to cells with neuronal morphology and in the early stage of neuronal commitment while $p75^-$ hDPSC did not commit to the neuronal lineages.

4.4.4 Limitations and recommendations

Despite all efforts, the enrichment of $p75^+$ hDPSC was low. There were always 'sticky' cells non-specifically binding onto the panning plate. The limitation of the immune panning technique itself prevented sensitive cell separation. MACS, on the other hand is characterized as a highly sensitive technique.

p75 expression was quite dim on the surface of hDPSC as examined by flow cytometry in chapter 3. Due to the low level of expression, the interaction between the ferromagnetic spheres and magnetic labeled $p75^+$ hDPSC may not have been strong enough to separate them from the

overall cell population, leaving target cells in the negative fraction. This could possibly be improved by using a longer column and stronger magnetic field for separation.

Another potential reason for low p75 enrichment fold could have been $p75^+$ hDPSC death, resulting from the separation procedure. p75 with its death signaling domain, plays a role in cell apoptosis (Reichardt 2006). Manipulation of the p75 receptor on the cell surface might be detrimental to cells, which is supported by the results that 50% of $p75^+$hDPSC were PI positive (Figure 4.9 A). Depletion of the $p75^-$ hDPSC instead of positive labeling of $p75^+$ hDPSC might avoid $p75^+$ hDPSC apoptosis and give more viable cells in the positive fraction. This would be feasible if a marker which labeled $p75^-$ hDPSC could be determined. As it is proposed that hDPSC is a heterogeneous population containing NSC and MSC, depletion of MSC from hDPSC would be helpful to enrich the $p75^+$ NSC population. Several candidate markers for MSC would be recommended for this purpose.

CD73

CD73, also known as ecto-5'-nucleotidase, is an enzyme for conversion of AMP to adenosine (Colgan et al. 2006). CD73 is expressed in lymphoid tissues and interacts with the bone marrow microenvironment to mediate cell to cell interactions (Barry et al. 2001).

CD90

CD90 is a glycosylphosphatidylinositol-linked protein involved in cell to cell adhesion and cell to matrix interaction. It is expressed on various cells such as neurons, hematopoietic stem cells, T-cells and MSC (Lin et al. 2013).

CD105

CD105, also known as endoglin, is a type I membrane protein and is a part of the TGF beta receptor complex. Therefore it interacts with TGF receptor and is involved in cellular localization and migration (Guerrero-Esteo et al. 2002). It also plays a role in the development of the cardiovascular system and vascular remodeling through the cytoskeletal organization (Sanz-Rodriguez et al. 2004).

CD146

CD146 is also known as melanoma cell adhesion molecule (MCAM) and is often used as a marker for the endothelial cell lineage (Kuske & Johnson 1999). It is also expressed on activated T cells, MSC and smooth muscle (Kuske & Johnson 1999). CD146 is strongly expressed on MSC and related to multipotency as MSC with higher pluripotency have higher level of CD146 expression (Covas et al. 2008).

CD166

CD166 is expressed on activated T cells and interacts with CD6 for cell adhesion, T cell proliferation and activation of the immune response (Pinto & Carmo 2013). It is also expressed on MSC, epithelial cells, fibroblasts, neurons and is upregulated in melanoma cells (Mafi et al. 2011).

STRO-1

STRO-1 antigen was first found in bone marrow MSC and defines a multipotent stromal cell lineage. Stro-1$^+$ cells can give rise to multiple mesenchymal lineages such as hematopoietic supportive stromal cells, adipocytes, osteoblasts and chondrocytes (Dennis et al. 2002).

MACS isolation has a common disadvantage: magnetism can exert undesirable biological effects on cells thus interfering with stem cell properties (Diogo, da Silva & Cabral 2012). Long term culturing of MACS processed hDPSC resulted in a dramatic increase in p75 expression from less than 5% to approximately 50% in both $p75^+$ hDPSC and $p75^-$ hDPSC (Figure 4.7). As p75 is associated with neuronal differentiation of hDPSC, magnetism in amplification of p75 expression might be hypothesized to have an effect on promoting hDPSC neuronal differentiation.

Using the neuronal differentiation method by Kiraly et al (Kiraly et al. 2009), hDPSC were unable to generate NeuN positive, mature and functional neurons *in vitro*. A neurosphere differentiation protocol might be used instead of the chemical induction protocol for hDPSC *in vitro* neuronal differentiation (Chapter 5).

4.5 Summary

Immune panning and MACS were trialed for the separation of $p75^+$ hDPSC from $p75^-$ hDPSC. Various optimization methods were investigated to improve immune panning specificity including optimization of antibody concentration and concentration of plate blocking solution. Immune panning was unable to separate $p75^+$hDPSC due to technique limitations. MACS did increase $p75^+$ hDPSC but the enrichment was low. Various trials have been tested to improve the efficacy of MACS separation including analysis immediately after separation and depletion of glycophorin A positive cells and debris. The low enrichment might have been due to the low expression of p75 on hDPSC. Thus, the interaction between the magnetic spheres and magnetic labeled $p75^+$ hDPSC may not have been strong enough to hold the $p75^+$ hDPSC and separated them from the whole population, leaving target cells in the negative fraction. Due to time constraints, further trouble-shooting could not be performed, however, recommendations have been made for protocol optimization in the future with a longer column and stronger magnetic field for separation.

5. Chapter 5- Neurosphere generation

5.1 Introduction

In cell cultures, neural stem cells from brain tissue like to proliferate as floating neurospheres to maintain their multipotencial properties (Wachs et al. 2003). Neurosphere formation is usually achieved by culturing cells as a single-cell suspension on uncoated plastic plates, supplemented with mitogenic factors EGF and bFGF. Therefore, the neurosphere culture system could demonstrate the presence of true neural stem cells in an *in vitro* culture system (Reynolds & Weiss 1992). Neurosphere generation provides a tool for the testing of neural stem cell characteristics such as neurosphere forming capacity after serial passaging followed by examination of differentiation capacity. Despite its function in a neural stem cell assay, the neurosphere also acted as a useful physiological model system for the study of neurogenesis and neural development. The extrinsic cues the cells become exposed to could be easily manipulated by adding the factors of interest into the culture environment.

From chapter 4, it was found that chemically induced/ epigenetic reprogrammed differentiation of hDPSC was unable to generate NeuN positive mature neurons (Figure 4.11 C, F). The neurosphere differentiation of hDPSC which shows neuronal morphology and less cell death, as less toxic factors were applied (Ellis et al. 2014; Sasaki et al. 2008) were compared to the epigenetic reprogramming differentiation of hDPSC using the Kiraly method (Kiraly et al. 2009).

5.2 Materials and Methods

5.2.1 Neuronal differentiation of hDPSC through neurosphere generation

Stage 1: Neurosphere formation

hDPSC were chosen from healthy donors as representative. hDPSC were seeded in 60mm petri dishes at a density of 500,000 per plate. Cells were maintained in sphere formation medium DMEM/F12 supplemented with EGF (20ng/ml), bFGF (20ng/ml), B27 (50x), penicillin (100U/ml) and streptomycin (100µg/ml) for 5-7 days until the formation of neurospheres.

Stage 2: Assay for sphere cells adherence and adaptation

The sphere cells were trypsinized and triturated into single cell suspension. In order to examine the effect of laminin on cell differentiation, these single cells were cultured in either laminin coated or uncoated coverslips at density of 20,000/ well in a 24 well plate. Adherence and adaptation of the cells were allowed for 1-2 days in the sphere formation medium.

Stage 3: Assay for differentiation and maturation of sphere cells

In order to determine the growth supplements of the neuronal maturation medium, triturated sphere cells were differentiated under four different maturation mediums. Each medium was comprised of DMEM/F12 supplemented with BDNF (medium A), BDNF+ N2 (Medium B), BDNF + NT3 (Medium C), BDNF+N2 + NT3 (Medium D). In order to facilitate cell adhesion, a group of coverslips were coated with laminin and PLL.

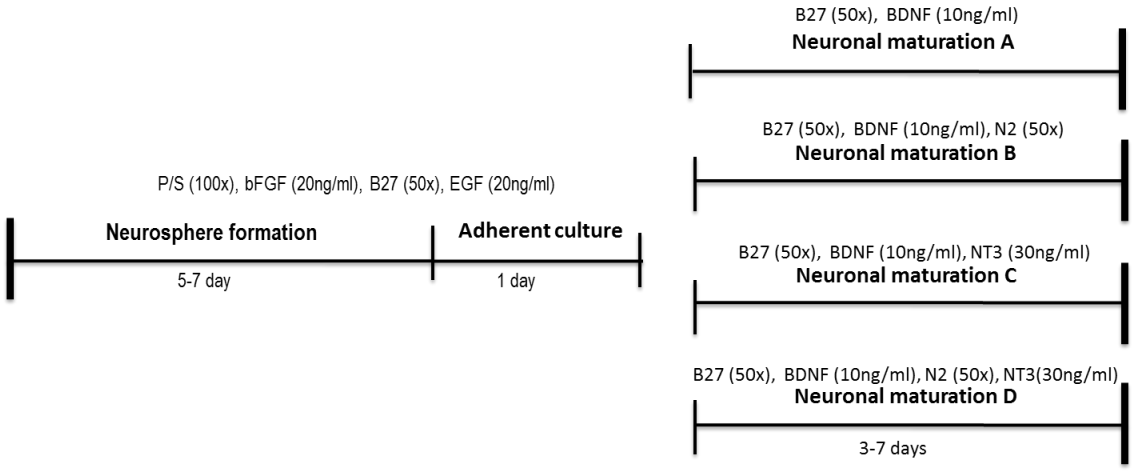


Figure 5.1 DPSC neurosphere differentiation protocols (medium A, B, C, D) and timing of different stages.

5.2.2 Immunohistochemistry

Differentiated cells were fixed in 4% PFA for 15 minutes and permeablised in blocking buffer A (TritonX-100, 0.5% donkey serum, 2% bovine serum albumin in PBS) for 30 minutes. The cells were then incubated with primary antibodies mlr2 mouse anti-p75 mlr2 IgG (1: 750, kindly gifted by Dr Mary-Louise Rogers), mouse anti-nestin (1: 250, Abcam), mouse anti-NFM (1: 200, Invitrogen), mouse anti- beta III tubulin (1:500, Millipore) and rabbit anti-GFAP (1:250, Dako) for overnight incubation. Subsequently, the cells were incubated with secondary antibodies either cy3 anti-rabbit IgG (1:400, Jackson ImmunoResearch) or antibody cy3 anti-mouse IgG for recognition of primary antibodies. Images were taken in five representative fields using an Olympus BX51 epifluorescence microscope (Olympus innovation, the USA, http://www.olympusamerica.com/).

5.3 Results and Analysis

5.3.1 Neurosphere formation

Under mitogenic proliferation, hDPSC formed neurospheres in various sizes. AH14DPSC generated generally small neurospheres sized < 100µm (Figure 5.2 A) while AH21DPSC (Figure 5.2 B) had large neurospheres sized > 100µm. AH27DPSC (Figure 5.2 C) generated both large and small neurospheres. By visualization, AH14 DPSC generated small neurospheres with no clear border, whose cell processes still protruded outside. Neurospheres formed by AH21DPSC had a clear border and floated freely in the culture medium.

5.3.2 Differentiation of sphere-forming cells under different conditions

Neural differentiation potential of sphere forming cells was examined. Cells cultured in laminin coated coverslips adhere better by a dark view of cells (Figure 5.3 A, B, C, D). However, they were unable to differentiate into cell with neural morphology and large amounts of cell death was observed (Figure 5.3 A, B, C, D). Sphere cells derived from hDPSC differentiated into neuronal like cells under differentiation and maturation medium A (BDNF) and medium B (BDNF+ N2) (Figure 5.3 E, F).Neuronal like cells had round cell bodies and multiple cellular processes. Instead of neuronal-like cells, fibroblast-like cells were generated under differentiation and maturation medium C and D supplemented with NT3 (Figure 5.3 G, H).

5.3.3 Neural differentiation potential of sphere-forming cells

Sphere-forming cells derived from hDPSC differentiated into cells with neuronal morphology characterized by rounded and phase-bright cell bodies and multiple cellular projections. Sphere cells also gave rise to a large, non-phase-bright and flattened cell subpopulation (Figure 5.4). Overall, the cells formed into a complex neuronal network. The flattened cells with their cytoplasm

extending underneath the neuronal cells, acted like supporting cells. AH14DPSC mainly gave rise to bipolar neuronal like cells (Figure 5.4 A) while AH21DPSC and AH27DPSC generated neural-like cells with both bipolar and multipolar processes (Figure 5.4 B, C).

Differentiated sphere cells rarely expressed beta III tubulin (Figure 5.5, Figure 5.6, Figure 5.7, A), moderately expressed neural markers NFM (Figure 5.5, Figure 5.6, Figure 5.7, B), and highly expressed GFAP (Figure 5.5, Figure 5.6, Figure 5.7, D) but not NeuN (Figure 5.5, Figure 5.6, Figure 5.7, C). Nestin (Figure 5.5, Figure 5.6, Figure 5.7, E) was down-regulated while p75 was clearly expressed with apparent increase in immunostaining (Figure 5.5, Figure 5.6, Figure 5.7, F).

5.3.4 Kiraly method of differentiation

hDPSC differentiated by epigenetic reprogramming expressed low levels of beta III tubulin (Figure 5.8 A), high levels of NFM and GFAP (Figure 5.8 B and D), but did not express NeuN (Figure 5.8 C). Nestin (Figure 5.8 E) was down-regulated while p75 was clearly expressed with apparent increase (Figure 5.8 F).

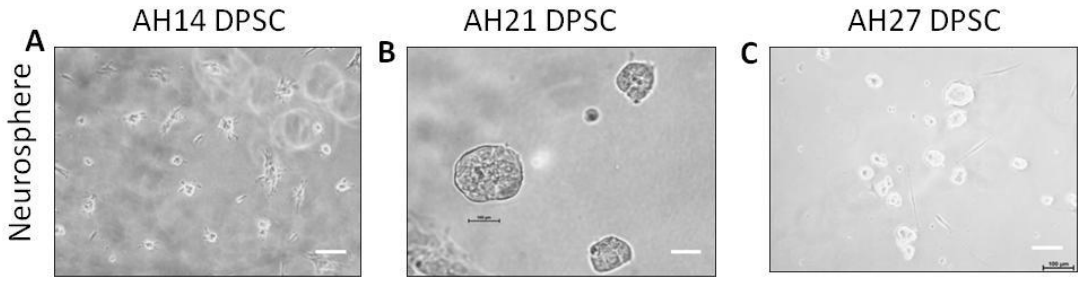


Figure 5.2 Neurosphere formation by culturing in medium containing bFGF and EGF.

(A) Neurosphere formation by AH14 DPSC. (B) Neurosphere formation by AH21 DPSC.

(C) Neurosphere formation by AH27 DPSC. N=3, Scale bar=100 μm.

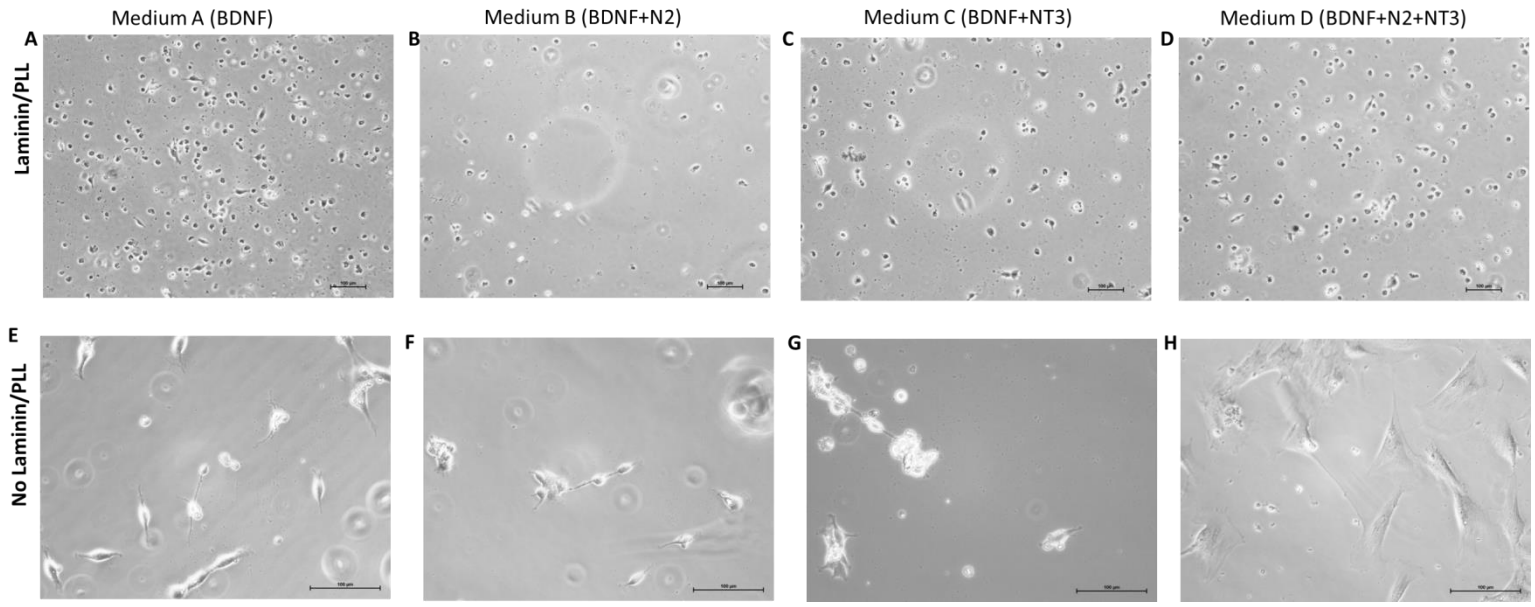


Figure 5.3 Neural differentiation of AH27DPSC under different neurotrophic supplements and on surfaces treated.

A, B, C, D) with laminin. or E,F,G,H) without laminin coating. Scale bar= 100 µm.

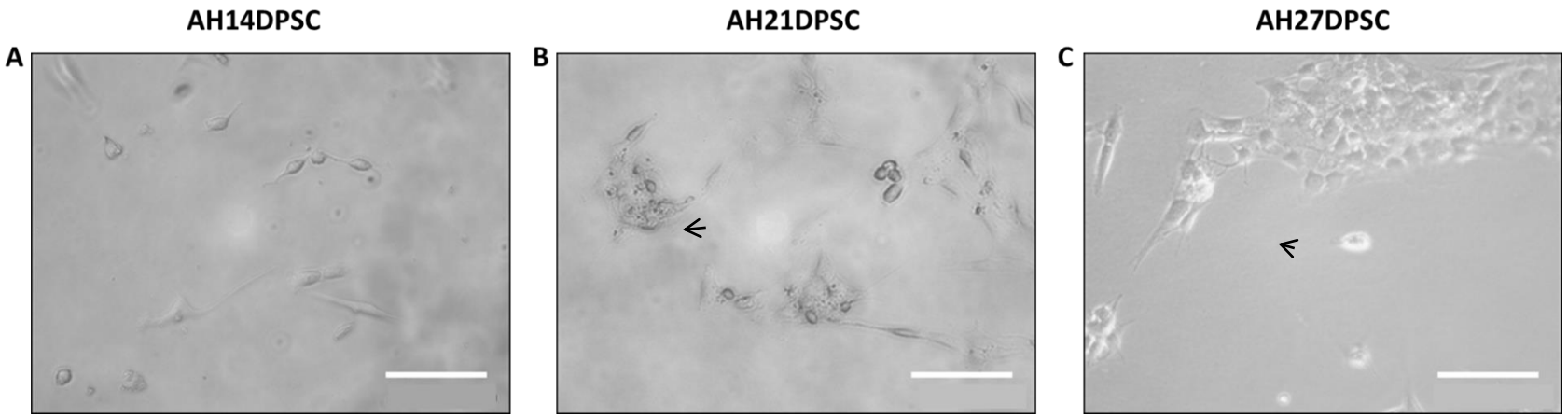


Figure 5.4 Cell morphology of neurosphere differentiated hDPSC.

A) AH14DPSC. B) AH 21DPSC. C) AH27 DPSC differentiated into neuronal- like cells with round cell bodies, thin processes (black arrow). Some AH14DPSC differentiated into cells with flattened morphology (white arrow).N=3 experiments, scale bar= 100 µm.

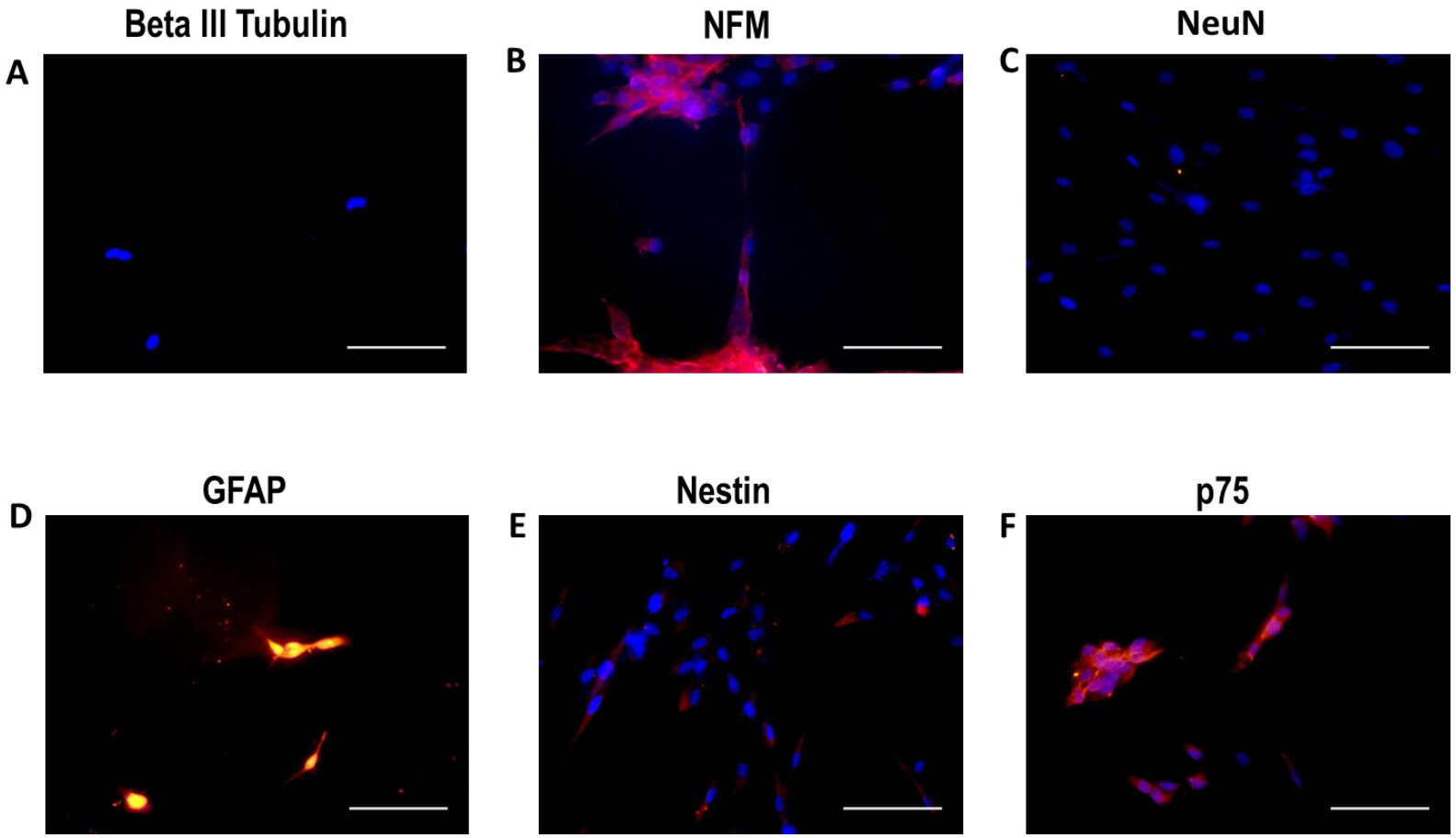


Figure 5.5 Immunohistochemistry staining of neurosphere differentiated AH14DPSC

Differentiated AH14DPSC had A) low expression of β III tubulin (red). B) strong expression of NFM (red). C) no expression of NeuN (red) D) high expression of GFAP (red). E) low expression of Nestin (red). F) High expression of p75 (red). N=3 experiments, scale bar= µm.

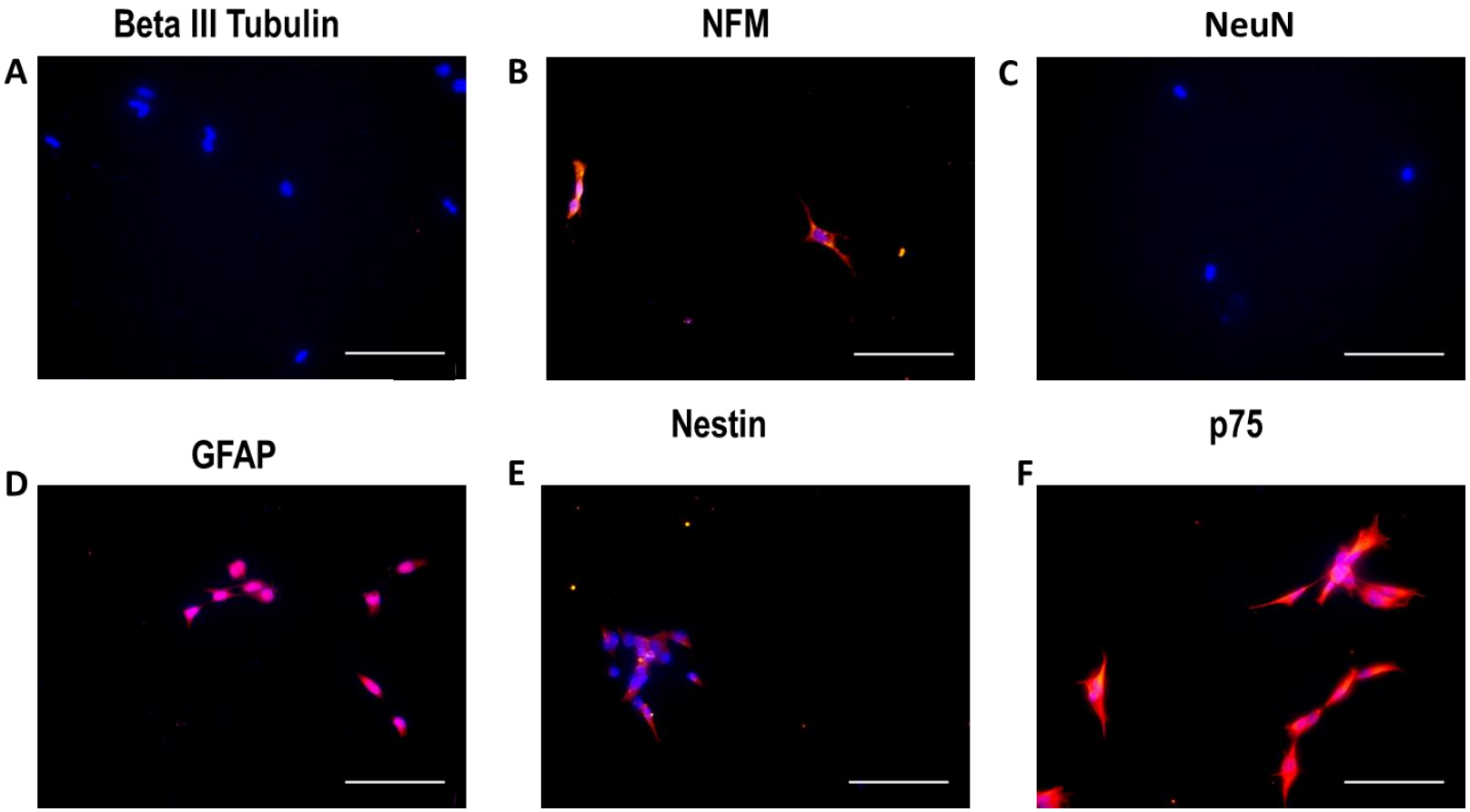


Figure 5.6 Immunohistochemistry staining of neurosphere differentiated AH21DPSC

Differentiated AH21DPSC had had A) low expression of β III tubulin (red). B) strong expression of NFM (red). C) no expression of NeuN (red) D) high expression of GFAP (red). E) low expression of Nestin (red). F) High expression of p75 (red). N=3 experiments, scale bar= µm.

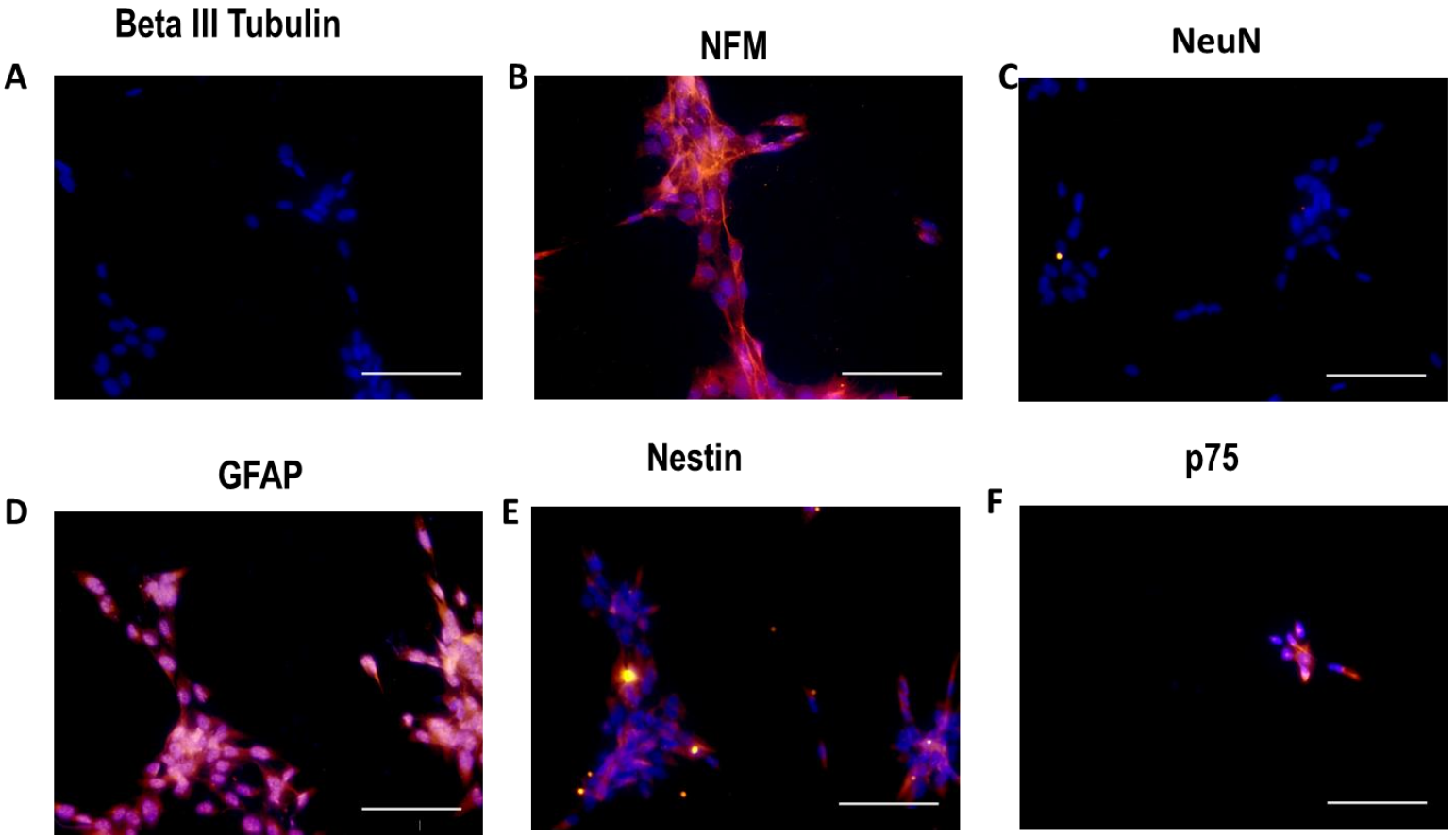


Figure 5.7 Immunohistochemistry staining of neurosphere differentiated AH27DPSC.

Differentiated AH27DPSC had had A) low expression of β III tubulin (red). B) strong expression of NFM (red). C) no expression of NeuN (red) D) high expression of GFAP (red). E) low expression of Nestin (red). F) High expression of p75 (red). N=3 experiments, scale bar= µm.

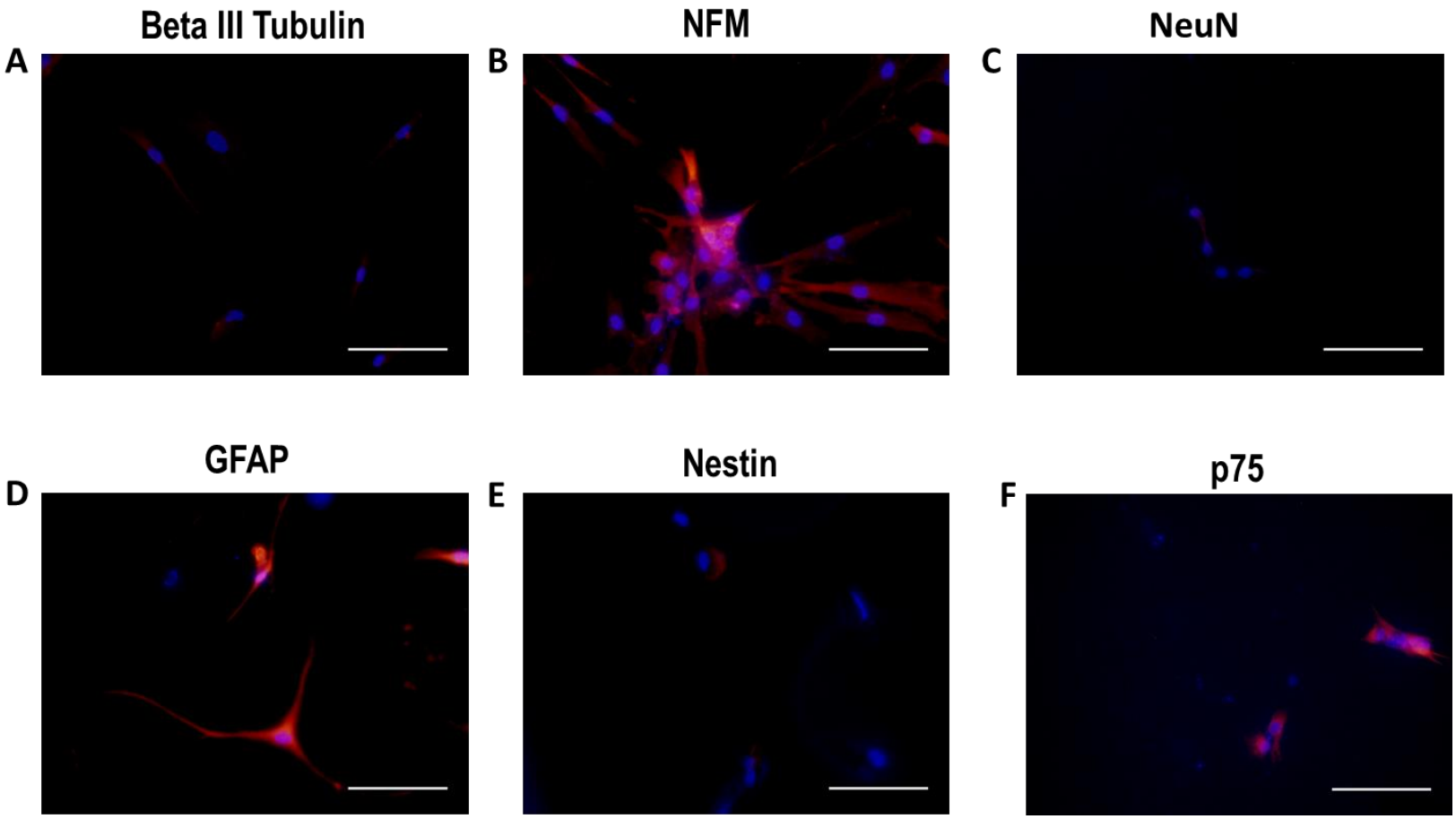


Figure 5.8 Immunohistochemistry staining of Kiraly method differentiated AH21DPSC

AH21DPSC had A) low expression of β III tubulin (red). B) strong expression of NFM (red). C) no expression of NeuN (red) D) high expression of GFAP (red). E) low expression of Nestin (red). F) High expression of p75 (red). N=3 experiments, scale bar= µm.

5.4 Discussion

5.4.1 bFGF and EGF induced sphere formation

In this study, 20ng/ml bFGF and 20ng/ml EGF successfully induced neurosphere formation of hDPSC with B27 supplement in DMEM/F12 (Figure 5.2 A, B, C). bFGF and EGF have been used in numerous studies for neurosphere formation *in vitro,* based on their roles in stem cell pluripotency maintenance and neural differentiation *(Abe et al. 2012; Reynolds & Weiss 1992; Young, KM et al. 2007)*.

bFGF as a cytokine belongs to the family of polypeptide growth factors that play an important role in embryonic parenchymal organ development (Martin 2001). In adult tissue, bFGF is also involved in maintaining tissue homeostasis such as tissue repair and replenishment, metabolism and angiogenesis (Yeoh & de Haan 2007). bFGF in combination with EGF was first described to be used in sphere generation for NSC culturing *in vitro* (Reynolds & Weiss 1992). Many studies have suggested that bFGF and EGF acted as effective cell mitogens in sustaining stem cell pluripotency and stimulating cell proliferation *in vitro* during sphere cell culturing (Hu, F et al. 2013). This was achieved by the role of bFGF and EGF role in regulating and enhancing expression of pluripotency factors Oct4 and Nanong (Xu et al. 2008).

In this study, hDPSC were also differentiated into neuron-like cells using 20ng/ml bFGF and 20ng/ml EGF (Figure 5.4), based on their roles in stem cell neural lineage differentiation (Gu et al. 2014; Jordan et al. 2009). Injection of bFGF promoted SVZ cell proliferation in the ischemic injured rat brain model. bFGF also promoted SVZ cells to undergo neural differentiation into neurons, astrocytes, and oligodendrocytes thus playing a role in neonatal ischemic brain injury repair *in vivo* (Jin-qiao et al. 2009). bFGF and EGF *in vitro* induced differentiation of adult dorsal root ganglia-

derived neural stem cells toward Schwann cells through activation of the ERK1/2 pathway. Their effect in Schwann cell generation was more efficient compared to other factors applied such as NGF and neuregulin 1-beta (Gu et al. 2014). bFGF also facilitated the generation of spinal motor neurons from human neural stem cells derived from fetal brain (Jordan et al. 2009). The effect of bFGF and EGF varied between stemness/pluripotency maintenance and neural differentiation in a dose-dependent manner (Kilpatrick & Bartlett 1993; Tsai & Kim 2005).

The size of the spheres differed between different cell populations in this study (Figure 5.2). It has been suggested that large neurospheres contained more neural stem cells while small neurospheres comprised neural precursor cells (Azari et al. 2011). This can be used to explain the results that AH14DPSC with small neurosphere formation, generated only bipolar suspected sensory neurons (Figure 5.4 A) while AH21DPSC and AH27DPSC which proliferated as large neurospheres, gave rise to both bipolar and multipolar suspected neurons during subsequent differentiation (Figure 5.4 B, C). The spheres derived from AH21DPSC had a clear border, facilitating free floating in the culture medium. The floating sphere structure increased the contact area between sphere cells and culture medium in three dimensions, which provided a superior nutrient absorbing structure. AH14DPSC produced small neurospheres with cellular processes still attached onto the plastic plate, which did not allow the spheres to be free floating.

5.4.2 Laminin and Poly-l-lysine did not facilitate sphere cell differentiation

Laminin and PLL coating facilitated cell adhesion but interrupted sphere cell neural differentiation in this study (Figure 5.2). Laminin, collagen and fibronectin are components of the extracellular matrix (ECM). They are widely used to induce neurite formation, differentiation and neural regeneration (Blong et al. 2010). The results in this study were contradictory to the results of most of the literature that laminin and PLL coated coverslips consistently provided better adherence for cells

and promoted neuron survival and neurite growth during their differentiation. Increased laminin concentration in cell culture led to increased percentage of survival of sympathetic neurons *in vitro* (Edgar, Timpl & Thoenen 1984). Laminin exerted its effect on neurons by both neurite growth stimulation and neuron survival potentiation. PLL as a natural bio-homopolymer of l-lysine is non-toxic to humans and is widely used in food preservation as well as cell culture in order to improve cell adherence (Shih, IL, Shen & Van 2006).

One recent study by Vollner's group demonstrated that a laminin/ poly-l- ornithine substrate coated surface drove human dental follicle cells to form spheres instead of forming monolayers of cells (Vollner et al. 2009). The same sphere effect was observed in cells which were seeded on only PLL coated coverslips.

The interruption of cell neural differentiation and neurite growth in laminin and PLL coated surface might be due to the sphere formation effect of laminin and PLL. The large amount of apoptosis seen might be due to the contradictory signaling activities exerted by BDNF for neurite growth and differentiation against laminin and PLL for sphere maintenance and cell proliferation (Figure 5.3 A, B, C, D). As all the coverslips had undergone acid treatment, which improved cell adhesion by making the surface rough with lumps. It was decided that laminin and PLL were to be removed from the hDPSC neurosphere differentiation protocol.

5.4.3 Neurotrophin induced hDPSC neural differentiation

After generation of neural stem cells from hDPSC via the neurosphere method, application of neurotrophin / neural supplement including BDNF, NT3 and N2 mixture were assayed for sphere cell neural differentiation. From the perspective of cell morphology by visual observation, BDNF promoted sphere cell neuronal differentiation (Figure 5.3E, F) while addition of NT3 impaired the

sphere cell neural lineage commitment (Figure 5.3 G, H). BDNF was first discovered in the brain in mediating fiber outgrowth of embryonic sensory neurons (Barde, Edgar & Thoenen 1982). Subsequently, NT3 was identified in mammalian brain. N2 is a chemically defined supplement for growth and proliferation of neuroblastomas and neurons *in vitro*. In the present study results were consistent with the results of BDNF and NT3 application on cortical and hippocampal neural stem cells *in vitro* (Shetty & Turner 1998). Shetty & Turner found that BDNF supported EGF and bFGF induced neural stem cell survival and differentiation while NT3 and glial-derived neurotrophic factor (GDNF) did not exert such an effect. Based on the current observations, N2 supplement did not improve or impair the neural differentiation established by BDNF (Figure 5.3 E, F).

As neurite formation is an essential and complex process, BDNF was indeed found to be associated with neurite outgrowth in the later stage of sphere cells neural differentiation (Ahmed, Reynolds & Weiss 1995). BDNF deficient mice demonstrated decreased survival of new-born neurons in the brain SVZ and SGZ *in vivo* (Bath et al. 2008; Sairanen et al. 2005). The axon generation effect exerted by BDNF was mediated by an increase of cAMP and protein kinase A in the signaling pathway via its binding to TrkB receptor. This activation of the signaling pathway resulted in the cytoskeletal changes that were associated with neurite formation and outgrowth (Kishi et al. 2005). BDNF regulation of neural differentiation was suggested to be through p75 activation. p75 deficient neural precursor cells had a 25% reduction of PSA-NCAM labeled cells compared to wildtype neural precursor cells. Neurite outgrowth induced by BDNF was interrupted or at least delayed in the p75 knockout neural precursor cells, suggesting that p75 via BDNF activation played a role in the early neural developmental stage (Gascon et al. 2007).

5.4.4 hDPSC gave rise to semi-mature neurons through sphere formation

The current studies examined the expression of markers on sphere differentiated hDPSC by IHC. Neural stem cell marker nestin was down-regulated while neurotrophin receptor p75 was clearly expressed with apparent increase in staining compared to its expression described in chapter 3 (Figure 3.1). This indicated the initiation of hDPSC differentiation towards neural lineages via BDNF signaling. As has been discussed earlier, BDNF regulates neurite growth and neurogenesis of stem cells by binding to p75 and TrkB receptors (Gascon et al. 2005; Hosomi et al. 2003; Young, F, Sloan & Song 2013). By applying BDNF to embryonic NSC, the impaired neural potentiation as a consequence of NO toxicity was restored. Increased p75 expression was suggested to be the mechanism of BDNF neural potentiation reversion (Lameu et al. 2012). This conclusion was also supported by the evidence that up-regulation of p75 was associated with the spontaneous neurogenesis in the rat ischaemic brain (Andsberg, Kokaia & Lindvall 2001). In addition, BDNF was unable to regulate neural commitment of neural precursor cells/ neural stem cells in the p75 knockout forebrain cells *in vivo* (Hosomi et al. 2003). Therefore, association between BDNF and p75 expression is a long stretch but whether these results suggest that there may be an association in these cells that warrant future investigation.

Studies using chemicals for hDPSC differentiation generated nestin positive immature neurons (Aanismaa R 2013; Kiraly et al. 2009) while the current results demonstrated a nestin down-regulation population derived from sphere cells, indicating a successful neural differentiation protocol via growth factor-induced neurosphere formation. Nestin as a pan-neural stem cells marker has been used as an evaluation tool for the presence of neural stem cells/ neural progenitor cells (Dahlstrand, Lardelli & Lendahl 1995). During CNS and PNS development, neural stem cells gradually disappeared, as evidenced by the down-regulation of nestin and up-regulation

of neural marker expression, such as GFAP for glial cells, beta III tubulin, NFM and NeuN for neurons (Gilyarov 2008). These findings may be due to the stem cell differentiation into glial cells and neurons, resulting in up-regulation of GFAP expression and beta III tubulin, respectively.

Neurons derived from hDPSC sphere cells expressed medium stage neural marker NFM but neither expressed beta III tubulin- an early neural differentiation marker, nor NeuN, which is a later mature neuron marker. During embryogenesis, neurofilament chain proteins with different sizes (light, middle and heavy chain) were expressed transitionally, indicating different degrees of neuronal lineage differentiation (Dahlstrand, Lardelli & Lendahl 1995). Cells were suggested to exit from a proliferating cycle into a post-mitotic cell state by a rapid decrease in expression of nestin and increase in intermediate filament chain protein or GFAP. This change in molecular expression was associated with early neural stem cell commitment to neuronal or glial lineages in CNS and PNS development (Ernst & Christie 2005).

Beta III tubulin is a microtubule element of the tubulin family expressed during embryonic neurogenesis or tumor development (Katsetos, Herman & Mork 2003). Beta III tubulin is expressed before or during the terminal mitosis and defined a cell population with neural commitment in CNS development (Menezes & Luskin 1994). Different to beta III tubulin, NeuN was expressed in mature neurons in both the CNS and PNS(Mullen, Buck & Smith 1992). Sphere differentiated cells in the current study did not express either early neuronal marker beta III tubulin or late mature neuronal marker NeuN, but NFM protein was detected. This indicated that cells were presumptive semi-mature neurons in the terminal post-mitotic stage (Dahlstrand, Lardelli & Lendahl 1995). Most of previous studies on hDPSC *in vitro* differentiation also gave rise to immature neurons in which markers such as beta III tubulin, NFM and neuromodulin (expressed in growth cones) were expressed but no NeuN expression was detected (Aanismaa R 2013; Iohara et al. 2006; Kiraly et

al. 2009; Sasaki et al. 2010). Only one study that used application of toxic chemical supplements including BME, DMSO and butylated hydroxyanisole (BHA), hDPSC generated NeuN positive cells. However, it was suggested that chemical induction with its mild toxicity might cause cell shrinkage and cytoskeletal transformation. These changes resulted in cell morphological changes but not a real change in genetic information of cells towards neurogenic lineages. A study by the Lu group demonstrated that, by culturing MSC under different chemical stress, though cells expressed neural markers, mRNA expression could not be detected at genetic level (Lu, Blesch & Tuszynski 2004). Therefore, by comparison to other hDPSC chemical or neurosphere differentiation, our sphere differentiation protocol was an advanced method in marker expression though further experiments need to be carried out to assess protein expression at genetic level and cell function by patch clamping.

hDPSC sphere cells differentiated into cells with GFAP positive. GFAP is well known as an intermediate filament protein in mature astrocytes of the CNS (Eng, Ghirnikar & Lee 2000). Besides being expressed in mature astrocytes, GFAP as intermediate filament protein group is also expressed by early neuroblasts with co-expression of neuronal marker Hu (Liu, Y et al. 2010). The expression of GFAP in sphere differentiated cells most likely to be explained by immature differentiation of sphere cells. As these cells co-expressed GFAP with neuronal marker NFM and neurotrophin receptor p75, which should not be expressed in mature glial cells. Whether the expression indicated a neuronal developmental stage or an *in vitro* artifact is yet to be elucidated.

5.5 Summary

hDPSC formed neurospheres under mitogenic factor supplementation. The neurospheres generated by hDPSC were in varied in size. Sphere forming cells differentiated into neuron-like cells with supplement of BDNF. NT3 and N2 did not have obvious effect on hDPSC sphere cell

differentiation. hDPSC sphere cell differentiated cells were NFM and GFAP positive, beta III tubulin and NeuN negative. Nestin was down-regulated and p75 was clearly expressed with apparent increase in immunostaining. These results indicated that hDPSC sphere cell differentiated into semi-mature neurons. The Kiraly method of chemical differentiation of hDPSC generated cells that were NFM, beta III tubulin and GFAP positive but NeuN negative. Neurosphere differentiated neurons were in a later stage by not expressing early neural marker beta III tubulin compared to the Kiraly chemically differentiated cells.

6. Chapter 6- Future Direction

6.1 Summary of results

p75 is prototypically a neurotrophin receptor, and has been suggested as a neural stem cell marker in adult nervous tissues such as in the SVZ and SGZ cell populations (Catts et al. 2008; Young, KM et al. 2007). It is also highly expressed in embryonic NC stem cells. $p75^+$ NC stem cells migrated to the ventral region of the embryonic trunk, indicating that these cells were responsible for PNS development (Wilson et al. 2004). This was consistent with the results that showed p75 regulation of neurogenesis in adult PNS tissue such as gut and DRG (Kruger et al. 2002; Li, Say & Zhou 2007). Because hDPSC, SVZ, SGZ and neurogenic tissues from PNS share a common embryonic origin— mesoderm stem cells, the research question of whether p75 was expressed by hDPSC and played a role on hDPSC neurogenic potential was raised.

Previous studies have suggested that p75 inhibited hDPSC mesenchymal differentiation, which suggested an alternative neural differentiation pathway, exists for $p75^+$hDPSC. However, no studies have assessed p75 function in hDPSC neurogenesis.

The study outlined in this thesis has demonstrated that p75 was expressed in hDPSC and defines a cell population with neurogenic potential. p75 was expressed on hDPSC at a low level (<10%). $p75^+$ hDPSC had a higher expression level of the neural stem cell marker nestin, the neural progenitor marker SOX1 and the pluripotency marker SOX2 across three hDPSC cell populations. This data suggested that $p75^+$hDPSC had a NC origin and might be a neural stem cell population with inherited embryonic NC properties in adult tissue. This data together indicates $p75^+$hDPSC has a predisposition to neural differentiation.

To confirm p75 function in hDPSC *in vitro*, $p75^{+}$hDPSC and $p75^{-}$hDPSC were separated. Due to the low expression density of p75 on hDPSC, this resulted in a small difference in the rolling capacity between $p75^{+}$hDPSC and $p75^{-}$hDPSC. The immune panning technique was unable to separate the two populations of cells efficiently, and optimization for immune panning in order to achieve high efficiency separation with application of blocking buffer was unsuccessful. The separation efficiency by MACS was low. MACS with glycophorinA pre-separation to remove dead cells generated a 10-fold increase in cell separation based on p75 expression. In addition, p75 expression was visible in a small number of $p75^{+}$ hDPSC and no p75 was detected in $p75^{-}$hDPSC, on examination by confocal microscopy immediately after separation.

MACS separated $p75^{+}$hDPSC were superior to $p75^{-}$hDPSC in neural cell generation. Cells derived from $p75^{+}$hDPSC expressed neuronal marker NFM while cells from $p75^{-}$hDPSC did not. However, $p75^{+}$hDPSC and $p75^{-}$hDPSC failed to generate mature neurons, as indicated by a lack of NeuN expression. All our data suggested $p75^{+}$hDPSC generated NFM positive early semi-mature neurons while cells derived $p75^{-}$hDPSC did not express neuronal marker.

hDPSC neural differentiation through neurosphere formation expressed neuronal marker NFM and astrocyte marker GFAP but did not express early neuronal marker beta III tubulin. While hDPSC, by chemically induction method, differentiated into cells expressing early neuronal marker beta III tubulin. However, both differentiation methods *in vitro* failed to generate NeuN positive cells. These results indicated that hDPSC generated immature neuron via both methods. However, cells derived from hDPSC sphere cells, with no early neuronal marker beta III expression, in a further stage compared with the cells generated via chemically induced neural differentiation. Cells differentiated from spheres expressed neurotrophin receptor p75, indicating association between

BDNF and p75 expression is a long stretch. However, future investigation was needed to warrant that there may be association in hDPSC.

6.2 Limitations and future recommendations

Current studies have demonstrated the importance of p75 on hDPSC neural differentiation *in vitro*. Though experiments in chapter 3 and 5 were done in three times in three cell populations, due to time limitations and some technique limitations, experiments in chapter 4 only were done once in one cell population. Therefore, differentiation of MACS separated $p75^{+}$hDPSC and $p75^{-}$hDPSC was done only once with one cell population and using chemical/ epigenetic factors differentiation methods. Assessments were carried out on three cell populations three times to confirm the validity and that the results were scientifically replicable. Future hDPSC separation and differentiation should be repeated on three cell populations and three times for each cell population.

Due to limitations of the technique, $p75^{+}$hDPSCn and $p75^{-}$hDPSC were unable to be separated by immune panning; they were however able to be separated by MACS but with low efficiency. Several methods for improvement of both techniques have been detailed in section 4.5.4. In summary, the separation efficiency for $p75^{+}$ hDPSC could be improved by applying a longer column and stronger magnetic field for separation. Otherwise, depletion via the MACS methods can be used for the negative separation rather than positive selection. Depletion of mesenchymal stem cells based on the mesenchymal stem cell marker expression could be used to enrich neural stem cells ($p75^{+}$) in the heterogeneous hDPSC. Several mesenchymal stem cell markers were recommended. Flow cytometry analyses were carried out to assess the various mesenchymal stem cell markers outlined in chapter 4 for their co-expression with p75 to be able to use as a selection marker.

Differentiated cells were only assessed for marker expression by IHC in the protein level of the cell surface; experiments with assessment of the genetic aspects- such as RT-PCR likewise are needed to confirm the qualitative differentiation of hDPSC which were not due to cell surface shrinkage or cytoskeletal transformation. The answer to the hypothesis would be more convincing by assessing and comparing MACS separated $p75^+$ hDPSC and $p75^-$ hDPSC neurogenicity by measuring the two cell populations electrophysiological properties, e.g. by patch-clamping.

All the studies of p75 function analyses were *in vitro*. Assessments could be done by comparing the neurogenic potential between p75 wildtype mouse DPSC and p75 knockout mice DPSC (However, mouse DPSC have been shown to be different in their properties compared to hDPSC, as shown in Kylie's paper (Ellis et al. 2014)). As some of the transplanted hDPSC differentiated into NeuN positive neurons or GFAP astrocytes (Leong et al. 2012) after injection into a rodent stroke model, further analysis of implanted hDPSC could include assessing their p75 expression. Therefore, p75 expression and its co-expression with NeuN and GFAP could be examined by IHC on transplanted and survived hDPSC to confirm the potential neurogenic role of $p75^+$hDPSC *in vivo.* As it was suggested that the neural stem cells from the heterogeneous transplanted hDPSC survived and differentiated into neurons and glial cells in the *in vivo* environment, these cells were thus suggested as the main functional cells in brain hDPSC stem cell therapy in a stroke rat model (Leong et al. 2012). A study looking at p75 co-expression with neural markers transplanted hDPSC would provide a mechanism of action for the implanted hDPSC in brain cognition improvement. It also would answer the question that neural stem cell population/ $p75^+$hDPSC population was the main functional population in hDSPC stem cell therapy for stroke.

No mature NeuN positive cells were generated from hDPSC *in vitro* either by neurosphere formation or chemically induced differentiation methods. Neurosphere differentiation with less

toxicity could be considered as an advanced hDPSC neural differentiation *in vitro* and provide a physiological model for hDPSC study. In the current study, uncoated culture plates were used for neurosphere formation. These culture plates have been optimized for cell culturing and adhering to some extent, while free-floating spheres were optimized to obtain nutrients. Neurosphere culturing in a turbulent environment by culturing cells in a cell shaker with proper rate might facilitate sphere movement to obtain the nutrients required. Cells also can be cultured on a super-hydrophobic or super-hydrophilic surface for minimization of cell attachment, which helps to facilitate sphere formation.

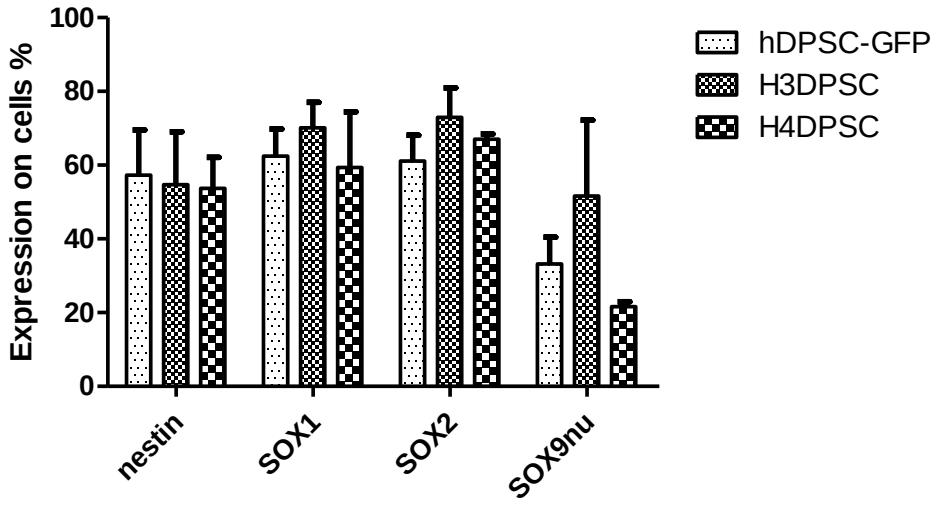
Previous studies have suggested several hDPSC differentiation protocols *in vitro*, with three methods being most recognized: the Kiraly method of epigenetic reprogramming (Kiraly et al. 2009), the Arthur methods using EGF and bFGF mitogenic factors (Arthur et al. 2008) and neurosphere differentiation methods(Sasaki et al. 2008). It would be interesting to assess neurons differentiated under these three major methods, including in surface protein level assessed by IHC, change of molecular expression at a genetic level assessed by RT-PCR and cell electrophysiology assessed by patch clamping.

6.3 Concluding remarks

This current study has revealed that p75 was expressed on hDPSC at a low level and defined a population of cells with neurogenic potential in hDPSC. $p75^+$ hDPSC had a higher expression of nestin, SOX1 and SOX2 than $p75^-$ hDPSC and were SOX9 positive, indicating their neural crest stem cell origin. p75 was co-expressed with nestin and CD146, indicating a role in neural differentiation. MACS separated $p75^+$ hDPSC differentiated into cells with NFM expression while $p75^-$ hDPSC did not. hDPSC generated semi-mature neurons through neurosphere formation.

As NSC was suggested as a functional population in hDPSC stem cell therapy in a rat stroke model (Leong et al. 2012). At the beginning of the thesis, it aims to elucidate p75 role in hDPSC neurogenesis thus providing an evidence of isolation, enriching and separation of NSC population from heterogeneous hDPSC. Due to the time limitation, though the current results did not completely answer the question *in vitro* and *in vivo*, it gives an indication that $p75^+$hDPSC were superior at generating cells with neuronal marker expression than $p75^-$hDPSC. It also gives future directions that experiments could be carried out *in vivo* and *in vitro* study by assessing $p75^+$hDPSC.

7. Appendix



Mol. exp./Cell	Nestin %			SOX1 %		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**
H0DPSC-GFP	57.32	12.24	3	62.46	7.34	3
H3DPSC	54.73	14.32	3	70.14	6.89	3
H4DPSC	53.74	8.40	3	59.40	15.07	3

Mol. exp./Cell	SOX2%			SOX9% (in nucleus)		
	Mean	**SEM**	**N**	**Mean**	**SEM**	**N**
H0DPSC-GFP	61.12	6.99	3	33.22	7.28	3
H3DPSC	72.92	8.00	3	51.61	20.60	3
H4DPSC	67.02	1.43	3	21.60	1.34	3

Figure 7.1 Comparison of nestin, SOX1, SOX2 and SOX9 (in nucleus) expression analysis on H0DPSC-GFP, H3DPSC and H4DPSC.

SOX1 and SOX2 were expressed at a high level across the three cell populations. Nestin was expressed at a moderate level across three cell populations. SOX9 were expressed in nucleus at low level across three cell populations. The expression pattern of molecules is independent of the cell population, $p>0.05$; mean± SEM.

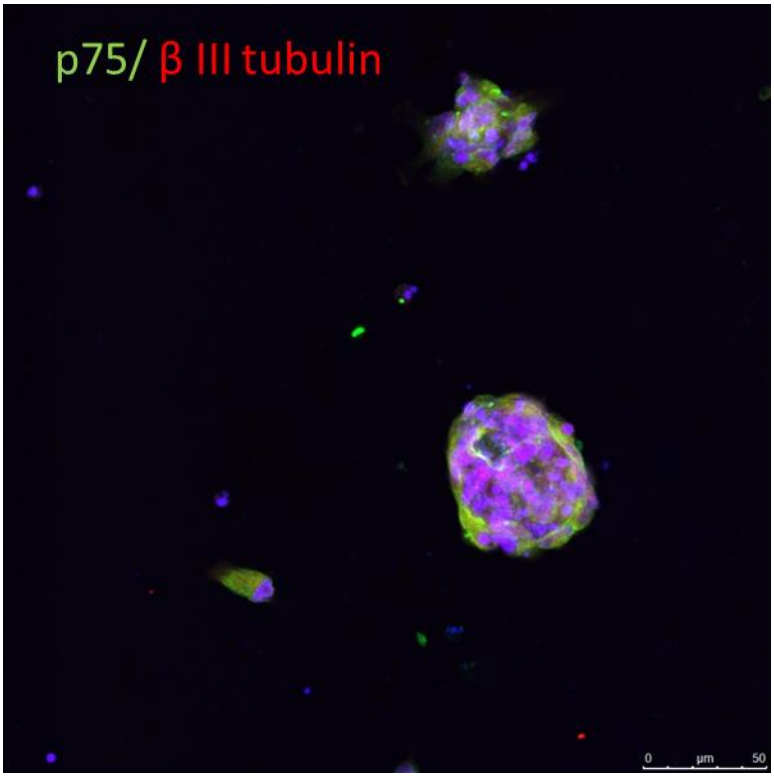


Figure 7.2 IHC staining of p75 and beta III tubulin on neurosphere.

Confocal double immunofluorescence images demonstrated that p75 (green) and beta III tubulin (red) is expressed on neurosphere.

Investigation of p75 neurotrophin receptor on human Dental Pulp Stem Cells (DPSC)

Authors: Wenru Pan[1]; Karlea Kremer[2]; Xenia Kaidonis[2]; Mary-Louise Rogers[3]; Simon A Koblar[2]
[1] *Discipline of Physiology, School of Medical Sciences, University of Adelaide*
[2] *Stroke Research Programme, Robinson Institute, University of Adelaide.*[3] *Department of Human Physiology, Flinders University*

INTRODUCTION

1. p75 neurotrophin receptor has recently been suggested as a neural stem cell marker for cells in the brain subventricular zone (SVZ)[1]
2. p75 is hard to detect as only ~0.3% of cells are p75 positive in SVZ cells[1]; ~0.5% of bone marrow mesenchymal stem cells are p75 positive[2].
3. It is hypothesized that p75 is expressed on hDPSC and defines a neural stem cell population in hDPSC.

AIMS

1. Characterise the expression of p75 in adult hDPSC by Fluorescence-activated cell sorting (FACS) and Immunohistochemistry (IHC).
2. Investigate the function of p75 in hDPSC *in vitro*

METHODS

DPSC CULTURE

Human DPSC were cultured in a standard stem cell medium[3] under standard conditions (37°C, 100% humidity, 5% CO_2).

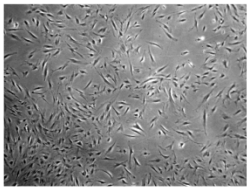


Figure1: Undifferentiated DPSC from one human individual contains heterogeneous precursor cell population

FLUORESCENCE ACTIVATED CELL SORTING (FACS)

The percentage of DPSC co-expressing surface protein p75, CD44 (cell adhesion molecule),CD146 (mesenchymal stem cell marker), PSA-NCAM (polysialylated-neural cell adhesion molecule) MHC-I and MHC-II were determined by FACS, using a Beckman FC500 machine (Beckman Coulter).

IMMUNOHISTOCHEMISTRY (IHC)

The co-localization of p75 with the neuronal stem cell marker nestin (a type VI intermediate filament protein) and neural crest cell marker SOX9 was visualized by IHC using MLR-2 mouse anti-p75 IgG.

SEPARATION OF p75 POSITIVE AND p75 NEGATIVE hDPSC BY IMMUNE PANNING

Cells will be transferred into the prepared panning plate coated with MLR-2 anti-p75 antibody. p75 positive cells will be attached to the panning plate and p75 negative cells will be washed and collected.

ASSESSMENT OF p75 ROLE ON hDPSC NUERAL DIFFERENTIATION

Panning-plate sorted p75 positive cells and p75 negative cells will be cultured in media optimized for neuronal differentiation to assess their neurogenic potential in vitro[3].

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1 Arthur et al (2008). *Stem Cells* 26,1787-95. 2 Poloni et al (2009). *Cytotherapy* 13, 1047-1056. 3.Kiraly et al (2006). *NeuronChem Int* 55, 323-332.

RESULTS

HUMAN DPSC EXPRESS NEUROTROPHIN RECEPTOR p75 AT LOW LEVEL (<5%)

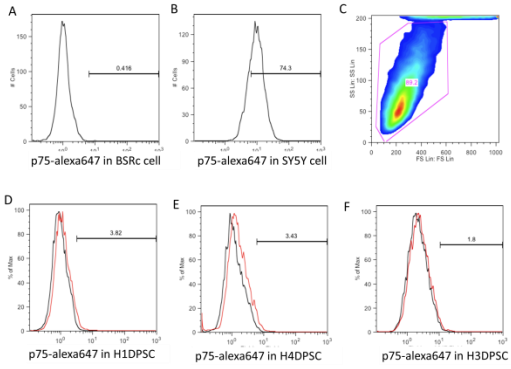

Figure 2: Fluorescence activated cell sorting characterization of p75 expression on hDPSC. A) p75 is not expressed in negative control BSRc cell B) p75 is expressed in positive control SY5Y cell at high level C) Side scatter (SSC) and forward scatter (FSC)of hDPSC without florescence staining D, E, F) p75 is expressed at low levels in three different hDPSC cell population from three different human individuals. (n=3. negative ≦ 1%)

p75 WAS COLOCALIZED WITH NEURAL STEM CELL MARKER NESTIN AND NURAL CREST CELL MARKER SOX9

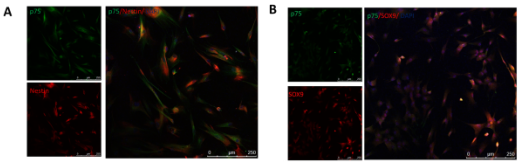

Figure 3: Fluorescent IHC image of A) p75 (green), nestin (red) co-localisation B) p75 (green), SOX9 (red)

CONCLUSION

1. p75 is expressed by hDPSC at low level (<5%).
2. Slightly more p75 is expressed in hDPSC compared to other NSC populations.
3. p75 is colocalized with neural stem cell marker nestin and neural crest cell marker SOX9.

Figure 7.3 Australian Society for Medical Research (ASMR) conference poster.

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