Targeted Differentiation of Embryonic Stem Cells towards the Neural Fate

Yang Tao

A Thesis Submitted in Partial Fulfillment

of the Requirements for the degree of

Doctor of Philosophy

in

Anatomical and Cellular Pathology

November 2008

ACKNOWLEDGEMENT

I am greatly indebted to my supervisor Prof. Ho Keung Ng for providing me with such a precious opportunity to study as a research student in the Department of Anatomical and Cellular Pathology, and his invaluable guidance as well as his trust, understanding and considerate support throughout my study period.

I wish to express my sincere appreciation and gratitude to my co-supervisor Dr. Kent Kam Sze Tsang, for introducing me into stem cell research field, giving detailed guidance on technical methodology, data analysis, thesis writing, presentation skills and in particular his constant encouragement all along. Owing to the constructive guidance and invaluable advice derived from Prof. Ho Keung Ng and Dr. Kent Kam Sze Tsang, I have gained a lot in my research project.

I would like to express my thanks to members in the Brain Disease Laboratory, Mr. Jesse Chung Sean Pang, Dr. Xiaoming Li and Ms. Nellie Yuk Fei Chung, for their technical support and suggestions. Appreciation also goes to Mr. David Lo, Dr. Jin Li, Ms. Yu Mui Lau and Mr. T. T. Lau for the technical assistance in various aspects during the whole research period.

Finally, I would like to express my gratitude to my parents and my wife. I could not finish my research plan without their love and understanding, all of which I will

cherish forever. My appreciation extends to all of my family members and friends who support and encourage me.

LIST OF PRESENTATIONS

Part of the research results in the thesis was submitted for presentation in international and local conferences:

Yang, T., Li, J., Tsang, K.S., Ng, H.K. Attributes of Notch Signaling in the Neural Commitment of Embryonic Stem Cells. In: Abstracts of the 4th Asia Pacific Multidisciplinary Meeting for Cancer Research, Hong Kong. 2008: P48.

Yang, T., Tsang, K.S., Ng, H.K. Targeted differentiation of human embryonic stem cells toward the neural fate by using MS5. In: Abstracts of the 5th Asia Pacific Multidisciplinary Meeting for Nervous System Diseases, Hong Kong. 2008: P22.

Yang, T., Tsang, K.S., Ng, H.K. Therapeutic potential of MS5-induced embryonic stem cell-derived neural lineage cells in a mouse cerebral ischemia model. In: Abstracts of the 5th Annual Meeting of the International Society for Stem Cell Research, Cairns, Australia. 2007: P350.

Yang, T., Tsang, K.S., Ng, H.K. Induction of embryonic stem cells into neural lineage cells through "864" culture method. In: Abstracts of CUHK Research Day Symposium, Hong Kong. 2006: P48.

Manuscripts in preparation for consideration of publication:

Yang, T., Tsang, K.S., Poon, W.S., Ng, H.K. Neurotrophism of bone marrow stromal cells to embryonic stem cells: induction and transplantation to a mouse ischemic stroke model. (Submitted for consideration of publication in Cell Transplantation)

Yang, T., Tsang, K.S., Ng, H.K. Guided neural differentiation of human embryonic stem cells towards the neural fate by an improved culture protocol. (In preparation)

LIST OF ABBREVIATIONS

AFP	α-fetoprotein
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
BrdU	5-bromodeoxyuridine
cDNA	complementary DNA
CM	conditioned medium
CNTF	ciliary neurotrophic factor
CXCL-12	chemokine (C-X-C motif) ligand 12
DAPI	4′, 6′-diamidino-2-phenylindole hydrochloride
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
PBS	phosphate-buffered saline
dpc	days post coitus
EB	embryoid bodies
EDTA	ethylenediamine tetraacetic acid
ES cell	embryonic stem cell
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fn	fibronectin
GABA	gamma-aminobutyric acid
GAPDH	glyceraldehyde phosphate dehydrogenase
GDNF	glial cell-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HS cell	haematopoietic stem cell
IGF	insulin-like growth factor
ITS	insulin-transferrin-sodium selenite medium
ITSFn	ITS supplemented with fibronectin
KO DMEM	Knock-Out Dulbecco's Modified Eagle's medium
LIF	leukemia inhibitory factor
MAP-2	microtubule-associated protein-2

MBP	myelin binding protein
Nestin	neuroepithelial stem cell intermediate filament
NGF	nerve growth factor
NS cell	neural stem cell
NT-3	neurotrophin-3
Oct-4	Octamer binding transcription factor-4
PI	popidium iodide
MEF	mouse embryonic fibroblast
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SDIA	stromal cell-derived inducing activity
SR	serum replacer
SSEA	stage-specific embryonic antigen
Tra-1-81	tumor rejection antigen-1-81
TRITC	tetramethyl rhodamine isothiocyanate
TuJ-III	Class III beta tubulin
VEGF	vascular endothelial growth factor
Wnt3A	wingless-related MMTV integration site 3A

ABSTRACT

Embryonic stem (ES) cells, which possess proliferating and differentiating abilities, are a potential source of cells for regenerative medicine. Nowadays, the challenge in using ES cells for developmental biology and regenerative medicine has been to direct the wide differentiation potential towards the derivation of a specific cell fate. This study is aimed to establish a simple and efficient method to derive ES cells into neural lineage cells and examine the safety and efficacy of derived cells in a mouse ischemic stroke model. To explore the underlying mechanisms responsible for lineage commitment of stem cells, Notch signaling and serotonin responses are also studied.

In a non-contact coculture system, mouse ES cells (D3 and E14TG2a) were cocultured with the stromal cells MS5 for eight days. On the other hand, human ES cells (H9 and H14) were directly cocultured with MS5 in a contact manner for two weeks. Derived cells were further propagated in a serum-free medium and selected subsequently in a differentiating medium. The cell viability, numbers, phenotypes and lineage-specific gene expression profile were evaluated at stages of induction, propagation and selection.

The Notch inhibitor (γ-secretase inhibitor) and serotonin were supplemented into induction cultures to investigate the roles of Notch signaling and the neurotransmitter serotonin in neural differentiation. For in vivo study, mouse ES cell-derived cells were labeled with BrdU and implanted onto the caudate putamens of mice having undergone transient occlusion of bilateral common carotid arteries and reperfusion to induce cerebral ischemia. Spatial learning and memory ability of transplanted mice were

assessed in a water maze system. Histological assessment was also conducted on brain sections of mice three weeks post transplant to examine the migration and homing of implanted cells.

MS5 was noted to express genes encoding neurotrophins and neuroprotective factors. Functional tests showed that MS5 exerted neurotrophism on neuroblastoma cell lines (SK-N-AS, SH-SY5Y, and SK-N-MC) and ES cells. The numbers of viable cells and the proportion of neural subtypes derived from ES cells at three stages of the culture system were significantly higher than those of the control cultures without MS5 induction, respectively. MS5 cocultures generate a relatively higher yield of neural lineage cells but select against the mesodermal and endodermal lineage derivatives. Together with non-contact MS5 coculture, serotonin could further increase the proportion of neural precursors and accelerate maturation of neural progenitor cells in a synergistic manner. During the induction phase with non-contact MS5 coculture, the Notch inhibitor could significantly decrease the number of derived neural precursors and instigate non-neural differentiation. With the supplement of the Notch inhibitor, serotonin could neither promote the expression of neuroectodermal genes nor enhance the proportion of neural precursors in MS5-cocultured ES cells. Notably, in the propagation of undifferentiated human ES cells, Notch signaling was also found to play an active role in maintaining cell survival.

In vivo, behavioral assessments of ischemic mice after transplantation of mouse ES cell derivatives revealed a significant improvement in spatial learning and memory ability as compared to ischemic mice without cell therapy. Histology of brain sections of transplanted mice demonstrated the migration of $BrdU^+$ cells to the CA1 region of the

hippocampus, which was evident of both an increase of pyramidal neuron density and normalized morphology. Teratoma development was found in one out of 17 transplanted mice.

This study describes a simple and efficient differentiation protocol to derive mouse ES cells and human ES cells into neural lineage cells. Derived cells appear to significantly improve cognitive functions in a mouse ischemic stroke model. Data of the study suggest that MS5 cells may exert a neurotrophic effect on ES cells. With MS5 coculture, serotonin synergistically promotes neural commitment and facilitates maturation of derived neural precursors in ES cell cultures. In contact coculture with MS5, Notch signaling is shown to play a role in the directed neural differentiation of human ES cells, whereas in maintenance culture, Notch signaling is also important to cell survival of human ES cells. Thus, Notch signaling through cell-cell interaction may explain, at least partially, the difference between mouse ES cells and human ES cells in cell growth ability when seeded at low cell densities.

摘　要

胚胎幹細胞具有高度的增殖能力及多向分化潛能，有望廣泛應用於再生醫學並成爲重要的細胞供體。目前，在胚胎幹細胞的應用實踐中遇到的主要問題集中於如何有效地定向誘導這類細胞至所需的細胞類型。本研究旨在建立一套簡易而行之有效的技術手段來定向誘導胚胎幹細胞分化爲神經系統細胞，並利用動物模型來檢驗幹細胞誘導產物移植治療缺血性中風的安全性及有效性。此外，爲了深入闡釋幹細胞定向分化的機制，本研究亦將探討 Notch 信號轉導途徑及 5-羥色胺添加物在胚胎幹細胞定向誘導分化過程中的作用，以期更好地操控胚胎幹細胞的分化過程從而爲臨床實踐服務。

基質細胞 MS5 能夠表達多種可編碼不同神經營養因數和神經保護因數的基因。並且，MS5 細胞可促進共培養的神經前體細胞 (神經母細胞瘤細胞系 SK-N-AS，SH-SY5Y，和 SK-N-MC) 分化爲神經元，證實 MS5 細胞對幼稚細胞具有神經營養作用及驅動分化的效應。在序列化的培養過程中，可檢測到胚胎幹細胞的誘導產物向多種神經細胞亞型分化的比率隨著不同的培養階段逐步升高。MS5 細胞的共培養，一方面可顯著增加多種神經細胞亞型的細胞總產量，另一方面抑制了胚胎幹細胞向內胚層及中胚層來源的種系分化。外源性的 5-羥色胺添加物在 MS5 細胞共培養的環境中能夠協同促進神經前體細胞數量的增加，並加速神經前體細胞向神經元分化的進程。但是，若在培養基中加入 Notch 信號抑制劑，則誘導階段所生成的神經前體細胞數量明顯降低，並且來源於內胚層及中胚層的多個種系特異性基因開始顯著表達。在該抑制劑存在的培養環境中，5-羥色胺不再能夠增加神經前體細胞的生成數量或促進神經外胚層相關基因的表達。此外，在人類胚胎幹細胞，Notch 信號抑

制劑可顯著降低未分化狀態下幹細胞的存活率，證實 Notch 信號途徑除了參與定向誘導神經系分化過程，還維持著人類胚胎幹細胞的存活。

將序列化誘導培養後的細胞產物移植於小鼠模型腦組織中的尾殼核。該細胞移植顯著改善了中風小鼠模型的空間學習與記憶能力，後者的恢復程度接近于正常小鼠。組織學的檢測手段顯示，移植的外源性細胞能夠自發地遷移至海馬的 CA1 區，有效地促進了海馬內錐體神經元密度及正常形態構架的恢復。在 17 例接受移植的小鼠中，有 1 例小鼠在移植後發生了畸胎瘤。

本實驗建立了簡易而有效的定向誘導鼠胚胎幹細胞和人胚胎幹細胞向神經系分化的技術手段。經過序列化的誘導培養，胚胎幹細胞來源的誘導產物能夠顯著改善小鼠中風模型的認知功能，預示細胞移植治療中風的良好前景。本實驗顯示，基質細胞 MS5 具有神經營養效應，可驅動胚胎幹細胞向神經系分化。在與 MS5 細胞共培養的環境中，5-羥色胺可協同促進胚胎幹細胞向神經前體細胞分化並加速神經前體細胞的分化成熟。Notch 信號途徑在 MS5 細胞共培養以及外源性 5-羥色胺介導的神經系定向分化過程中發揮著關鍵性的調控作用。此外，Notch 信號途徑通過細胞間接觸，參與維繫未分化狀態下人胚胎幹細胞的存活。這也解釋了低密度接種人胚胎幹細胞時，其存活率及克隆形成率顯著低於鼠胚胎幹細胞的原因。

CONTENT

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1
LITERATURE REVIEW

1.1 Stem cells

The conventional definition of a stem cell is an undifferentiated cell with the capacity for self-renewal, combined with the capacity to produce at least one type of highly differentiated progeny. Research on stem cells during the last decades has provided important information on developmental, morphological and physiological processes that govern tissue and organ formation, maintenance, regeneration and repair after injuries (Bryder et al., 2006; Murry et al., 2005; Bonner-Weir and Weir, 2005).

Recently, significant advancements in understanding of stem cell biology have aroused great interests and held high therapeutic promises based on the *in vitro* and *in vivo* expansion and differentiation of stem cells into functional progenies that could regenerate the injured tissues or organs in the body (Ringdén, 2007; Brunstein et al., 2007; Wu et al., 2007). These studies constitute a significant part of the rapidly developing field of regenerative biology and medicine. In addition to stem cells from embryos, fetal tissues, amniotic fluid and umbilical cord, the multipotent adult stem cells with a self-renewal capacity and multilineage differentiation potential have been identified within specific niches in many human tissues and organs (Bussolati et al., 2005). Among them, there are bone marrow, heart, brain, adipose tissues, muscles, skin, eyes, kidneys, lungs, liver, gastrointestinal tract, pancreas, breast, ovaries, prostate and testis (Herrera et al., 2006; Koblas et al., 2007). The tissue-specific stem cells can generate new further differentiated and specialized cells, and thereby repopulate the tissues in which they reside under homeostatic conditions as well as regenerate damaged tissues after intense injuries.

At present, cells used for tissue engineering are obtained from a small biopsy of tissue which is dissociated in culture. The resulting cell population is expanded, seeded on a matrix, and implanted back into the host. The source of tissues is either allogenic or autologous. In certain circumstances, autologous cells are preferred because they are unlikely to be immuno-rejected by the host's immune system. However, inherent difficulty of in vitro expansion is a major limiting factor for use of some autologous cells. Even though some organs have a very high regeneration rate in vivo (for instance, the liver), cells from these organs can be difficult to expand in vitro, which hinders their clinical potential. By studying the conditions that regulate and guide in vivo regeneration after injury and exploring conditions that promote cell differentiation, one can overcome this limitation and achieve extensive expansion in vitro. While autologous cells are recognized as the ideal cell source for transplantation, some patients with organ disease at an end stage do not produce enough cells for transplantation. In this case, allogenic cells may be advantageous. Furthermore, some primary cells, whether autologous or allogenic, cannot be expanded from particular organs, such as the pancreas. In these situations, pluripotent stem cells are envisioned as an alternative source of cells from which the desired tissue can be derived. Pluripotent stem cells represent an endless source of versatile cells that could lead to novel sources of replacement organs. Under certain circumstances, an organism may require some stem cells to be only self-renewing, without ever differentiating, thus creating other daughter cells that further differentiate and participate as specialized cells within the organism while the originating stem cell keeps proliferating at a slow cycle to protect the telomeres and genetic makeup, and the new daughter cells fulfill the needs of the organism. In this instance, the microenvironment is almost certainly the influencing factor determining the behaviour of the cells (Moore and Lemischka, 2006; Wilson and Trumpp, 2006).

Pluripotent stem cells appear to be the most suitable cell source to consider for treatment through cell therapy. These cells have numerous advantages over terminally differentiated cells, since they are self-renewing, can be readily available, can be produced to indefinite numbers under defined conditions, and can be directed to become cells with distinct phenotypes that yield specific candidates for cell therapies for multiple diseases. However, much research is still needed before their clinical application. A central challenge is to develop methods to direct differentiation of stem cells in a controlled manner to produce individual populations of specific cell types. In addition, elucidation of molecular pathways, which define stem cell pluripotency, self-renewal and differentiation, will be critical to achieving this goal.

1.2 Stem cell types

Basically, there are mainly two categories of stem cells: embryonic stem cells derived from blastocysts, and adult stem cells which reside in differentiated tissues. Stem cells in both categories are distinguished from one another and from their progeny by a combination of location, morphology, cytology, cell surface receptors and transcription factors.

1.2.1 Embryonic stem cells

Embryonic stem (ES) cells are isolated from the inner cell mass of a blastocyst by using an immunosurgical technique (Solter and Knowles, 1975). These cells are characterized by pluripotency, retention of an undifferentiated state and high mitotic capacity (Kim et al., 2007). ES cells may be induced to differentiate into specific progenitor cells or mature and specialized cell lineages of all three embryonic germ layers in vivo and in vitro (Asahara and Kawamoto, 2004). Among them, there are the haematopoietic cell lineages, neuron-like cells, glial progenitors, dentritic cells,

hepatocytes, pancreatic islet-like cells, osteocytes, chondrocytes, adipocytes, cardiomyocytes, and muscular, endothelial, skin, lung, and retinal cells (Leri et al., 2005; Sharma et al., 2006; Ozasa et al., 2007; Shim et al., 2007). The ES cell-derived progeny may constitute an easily available source of transplantable cells for regenerative medicine (Behfar et al., 2007; Bieberich et al., 2004). However, there are many unknowns such as the purity, yields and functions of the cell products. Additional obstacles to the clinical application are the possibility of immune rejection and teratoma development in the recipients in vivo owing to the presence of residual pluripotent and undifferentiated ES cells in transplants (Wu et al., 2007; Andrews et al., 2005). It has been observed that the injection of undifferentiated ES cells into severe combined immunodeficient (SCID) mice might result in the teratoma formation corresponding to the complex structures containing the differentiated cell types from three germ layers (Trounson, 2006; Kim et al., 2007). Thus, elimination of undifferentiated ES cells in transplants is requisite to prevent the teratoma development in the clinical setting.

1.2.2 Adult stem cells

The identification of adult stem cells in most tissues and organs in mammalian organisms, which provide critical functions in hemeostatic maintenance by replenishing the mature cell types within the tissues in which they reside over the lifetime, has caused great interest and enthusiasm for their use in cellular and tissue engineering therapies (Schaffler and Buchler, 2007; Leri et al., 2005; Kim et al., 2005). The establishment of functional properties of adult stem cells and their early progenitors in vitro and in vivo has also indicated that they can actively participate in cell replenishment of mature cell types within the tissue of their origin under pathological conditions (Lim et al., 2007; Herrera et al., 2006; Koblas et al., 2007).

Many adult tissues contain stem cells. The compex interactions between the adult stem cells and host cells and particular specialized microenvironments that are prevalent within niches might influence their behavior (Wilson and Trumpp, 2006; Arai and Suda, 2007). More specifically, the reciprocal interactions of adult stem cells with neighbouring cells via the formation of cell-cell junction, cell-extracellular matrix interaction and the secretion of diverse soluble factors might contribute to their restricted mobility and the adoption of a quiescent or activated state within niches. For regenerative medicine, the challenge is to characterize these adult stem cells, understand their regenerative potential, and to develop useful therapies.

Among various adult stem cells, the neural stem (NS) cells, haematopoietic stem (HS) cells and mesenchymal stem (MS) cells have been demonstrated to be useful for the treatment of certain diseases in humans.

1.2.2.1 Neural stem cells

Over the past decades, convincing evidence emerged that neurogenesis in the adult central nervous system (CNS) is a continuous physiological process. Neurogenesis is present in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus (Kaplan and Bell, 1984; Kuhn et al., 1996). Additionally, recent studies also demonstrated the presence of neural stem (NS) cells in the striatum and neocortex (Palmer et al., 1995; Palmer et al., 1999; Yamamoto et al., 2001), indicating NS cells and more restricted neuronal and glial progenitor cells are dispersed widely throughout the adult vertebrate brain (Gage, 2002; Alvarez-Buylla and Garcia-Verdugo, 2002). Long after fetal development, multipotent NS cells continue to line the forebrain ventricles (Morshead et al., 1994; Weiss et al., 1996), and committed neuronal progenitor cells also remain within the ventricular wall, throughout its extension to the olfactory bulb and the hippocampus (Luskin, 1993; Lois and Alvarez-Buylla, 1993). Initially construed as

precursors of mature neural lineage cells, SVZ and dentate gyrus-derived NS cells are characterized by long-term self-renewal capacity and multipotency, and could become robustly neurogenic in culture (Palmer et al., 1999; Roy et al., 1999). Adult SVZ and dentate gyrus-derived NS cells persist throughout the life span of mammals including humans (Kuhn et al., 1996; Eriksson et al., 1998).

Neurogenesis occurs in a physiological mode or is exogenously modulated by external signals and pathophysiological processes. External global stimulants such as physical activity and stress (Kempermann et al., 2000) or application of defined molecules such as fibroblast growth factors (Kuhn et al., 1997), vascular endothelial growth factor (VEGF; Schänzer et al., 2004), brain-derived neurotrophic factor (BDNF; Chmielnicki et al., 2004) and erythropoietin (EPO; Shingo et al., 2001) differentially modulate adult neurogenesis. Besides, it was noted that seizures and traumatic brain injury in animal models induce neurogenesis (Rice et al., 2003; Zhang et al., 2004).

NS cells, genetically modified NS cells, or their further differentiated progeny could be used to treat and cure a variety of central nervous system disorders and replace neuronal cells lost by new functional cells in brain injuries resulting from ischemic or hemorrhagic stroke or trauma (Chang et al., 2007). Several progressive and neurodegenerative diseases caused by deterioration and loss of neuronal cells, such as multiple sclerosis, Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and Huntington's disease, could also benefit from NS cell-based treatments (Geraerts et al., 2007; Ramaswamy et al., 2007; Lu and Xiao, 2007). The restoration of lost neuronal cells could improve impaired brain functions such as memory loss and abnormal control of movement, sensation, behavior and other autonomic nervous functions (Nakatomi et al., 2002). The delivery of specific growth factors or cytokines such as epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, sonic hedgehog, granulocyte colony-

stimulating factor, and stem cell factor in the damaged brain areas may also stimulate NS cells and neurogenesis in vivo (Kawada et al., 2006).

Multipotent NS cells have been identified by clonal analyses. When cultured in the presence of EGF, these cells form balls of cells called neurospheres that produce both neurons and glia when the EGF is withdrawn. Single cells derived from the spheres proliferate to form new neurons or glial cells, as well as new neurospheres, indicating that neurospheres contain committed neural and glial precursors as well as self-renewing stem cells (Gage, 2000). Immunofluorescence study reveals that the neurosphere is composed of a population of cells which are Nestin$^+$, and do not express differentiated neuronal or glial cell markers, suggesting its developmental potential towards neural lineage (Rietze et al., 2001).

1.2.2.2 Haematopoietic stem cells

The first enriched stem cells to be grafted are those of the haematopoietic system, and haematopoietic stem (HS) cells, which were originally obtained from bone marrow (BM), remain the best characterized of all stem cells. BM-derived HS cells are able to drive haematopoiesis by giving rise to all the haematopoietic cell lineages found in peripheral circulation including leukocytes, erythrocytes and platelets over the lifetime (Bryder et al., 2006; Wilson and Trumpp, 2006). Accordingly, cytokine-mobilized haematopoietic stem cells in peripheral blood have gained support as a less invasive procedure (Carion et al., 2003). The most primitive and undifferentiated human HS cells are characterized by a specific phenotype: CD34$^+$/CD38^{low}/Thy-1$^+$/CD117$^+$/Lin$^-$/CD133$^+$ (Bryder et al., 2006; Wilson and Trumpp, 2006). In addition, committed progenitors in the haematopoietic system may express different levels of lineage-specific markers, such as members of the lymphocyte activation molecule family (Kiel et al., 2005). Notably, in mice, HS cells express CD150$^+$/CD48$^-$/CD244$^-$ cell surface molecules,

whereas non-self-renewing multipotent haematopoietic progenitors are CD150 /CD48 /CD244$^+$ and more restricted lineage progenitor cells are CD150 /CD48$^+$/CD244$^+$ (Kiel et al., 2005).

Immature and quiescent HS cells are localized at the endosteal bone surface, where they interact, via the formation of adherent junctions, with the supporting cells, osteoblasts that regulate their functions (Moore and Lemischka, 2006; Arai and Suda, 2007). Haematopoietic stem cells are also found in BM microvasculature sinusoidal endothelium, where they are colocalized with endothelial cells (Wilson and Trumpp, 2006). The transitions between the quiescent and activated state of BM haematopoietic stem cells as well as their migration are controlled by a complex network of growth factors and cytokines (Arai and Suda, 2007; Moore and Lemischka, 2006). Haematopoietic stem cells can notably migrate from the endosteal surface to the vascular niche under specific stimuli such as tissue injuries (Bhattacharya et al., 2006; Wilson and Trumpp, 2006). Hence, the localization of haematopoietic stem cells within the BM vascular niche may allow their rapid release into BM microvasculature bloodstream and their subsequent migration into the peripheral circulation under physiological and pathological conditions. Besides BM, HS cells can also be found in umbilical cord blood. Umbilical cord blood transplantation has recently been explored in an increasing number of patients (Cohen and Nagler, 2004). The relative ease of procurement and the lower risk of severe acute graft-versus-host disease (GVHD) have made umbilical cord blood an appealing source for cell transplantation therapy (Barker et al., 2002).

A recent investigation on parabiotic mouse models has indicated that the circulating HS cells have a short lifetime in peripheral circulation, and thereby only a small number may be available for the reconstitution of BM niches and the repair of distant tissues and organs (Wright et al., 2001). Mobilized HS cells may establish their homing at distant

extramedullary sites including the sinusoidal endothelium within the spleen in human (Kiel et al., 2005; Mimeault and Batra, 2006). Other investigations have also revealed that BM-derived HS cell-like stem cells and/or their progeny could be localized in the skin, muscle, neural tissues, lung, liver, and gastrointestinal tract (Tavian et al., 2005; Mimeault and Batra, 2006). Hence, these circulating and tissue-resident haematopoietic stem cell-like cells could participate in the regeneration of peripheral tissues by promoting the immune system response and/or transdifferentiating into functional cells involved in the repair of damaged tissues.

1.2.2.3 Mesenchymal stem cells

It is evident that BM contains non-haematopoietic progenitor cells, which harbor the potential to develop into a variety of non-mesenchymal and mesenchymal cells and can constitute the microenvironment for haematopoiesis (Pittenger et al., 1999; Prockop, 1997). Despite the lack of cellular and molecular tools and in vivo assays that permit the rigorous identification of stem cells from mesenchymal tissues, it is known that a cell population with mesenchymal stem (MS) cell-like characteristics can be isolated based on its ability to adhere to plastic and may be partially purified by separation techniques (Smith et al., 2004). Beyond the BM, adherent cells capable of density-independent growth, believed as MS cells, are also found in a number of non-haematopoietic connective tissues, such as periosteum and dental pulp (Gronthos et al., 2002).

Important characteristics of MS cells are their potential to proliferate in culture with the fibroblastic morphology, the presence of specific cell surface marker proteins, and their multilineage differentiation potential. MS cells comprise a population of multipotent progenitors capable of supporting haematopoiesis and differentiating into osteogenic, adipogenic, chondrogenic, myogenic and cardiomyogenic lineages (Deans and Moseley, 2000; Minguell et al., 2000). Thus, MS cells appear to be an attractive tool in the context

of tissue engineering and cell-based therapy. Bone marrow stroma represents the major source for MS cells of different species. In humans, aspirated marrow from the iliac crest is suggested to be most suitable for the isolation of MS cells (Haynesworth et al., 1992, Pittenger et al., 1999).

Recently, several surface antigens for identification of human MS cells were established. The monoclonal antibody of surface binding protein (SB)-10 corresponds to the activated leukocyte cell adhesion molecules (ALCAM) (Bruder et al., 1998) and the monoclonal antibody of Src homology (SH)-2 recognizes an epitope on the transforming gowth factor-β (TGF-β) receptor III (Barry et al., 1999). In addition, the monoclonal antibodies SH-3 and SH-4 recognize two distinct epitopes on the surface of human MS cells (Barry et al., 2001). Most likely, both SH-3 and SH-4 epitopes are present on CD73, which plays a role in the activation of B-lymphocytes and in signal transduction within the haematopoietic compartment of BM. However, no unique antibody profile for mesenchymal progenitor or stem cells exists. MS cells express mesenchymal, muscular, epithelial and endothelial cell surface markers (Minguell et al., 2001). Cultured MS cells display a single phenotypic population of cells (Pittenger et al., 1999). These cells are uniformly positive for SH-2, SH-3, the transferrin receptor CD71, the hyaluronic acid receptor CD44, β-1 integrin CD29 and are negative for haematopoietic antigens such as the lipopolysaccharide receptor CD14, the leukocyte common antigen CD45 and the early haematopoietic stem cell marker CD34. However, single cell-derived colonies of MS cells are not morphologically homogeneous and contain at least three distinct cell types: (1) a large population of large flat barely proliferatively active MS cells, (2) proliferatively active spindle-shaped cells, and (3) very small round cells that rapidly self-renew (Conget and Minguell, 1999; Colter et al., 2001). All of these cell types demonstrate multilineage differentiation potential, even though to a different extent.

Many data showed that various cytokines and their receptors, growth factors and their receptors, extracellular matrix molecules and adhesion molecules could be expressed by MS cells (Minguell et al., 2001; Conget and Minguell, 1999). The vast variety of expressed cytokines and their corresponding receptors suggests that bone marrow mesenchymal stem cells and progenitors take part in the formation and function of the stromal microenvironment, which is responsible for providing signals not only for the development of mesenchymal cells but also for haematopoietic cells. For instance, MS cells supply signals to stimulate platelet and megakaryocyte production from $CD34^+$ HS cells (Cheng et al., 2000).

MS cells exhibit extensive proliferation capacity and differentiation potential. MS cells could differentiate into distinct cell types and tissues, such as bone (Long, 2001), cartilage (Pittenger et al., 1999), fat (Pittenger et al., 1999), muscle (Wakitani et al., 1995), tendon (Awad et al., 1999), neural tissue (Kopen et al., 1999), and haematopoiesis-supporting stroma (Koc and Lazarus, 2001). Hence, BM-derived MS cells, which may be collected from BM aspirate or mobilized in peripheral circulation, could be used in cell transplantation therapies to reconstitute the immune system or to repair damaged tissues in pathological conditions.

1.2.2.4 Stem cells derived from fetal tissues

Stem cells derived from fetuses, which include amniotic mesenchymal stem cells, haematopoietic stem cells, trophoblastic precursors and pluripotent amniotic epithelial cells, are immunologically naive since they do not form teratomas in humans (Marcus and Woodbury, 2008; Miki et al., 2005). These cells were reported to be a stem cell alternative for the cell replacement therapies in certain experimental and clinical settings (Wolbank et al., 2007; Gao et al., 2006). Generally, fetal tissues represent a promising source of stem or progenitor cells to obtain functional haematopoietic cells,

cardiomyocytes, hepatocytes, insulin-secreting β-cells, lung progenitor cells, muscle cells and dopaminergic neurons that could be used for the cell replacement and tissue engineering-based therapies in regenerative medicine (Chan et al., 2007; Gao et al., 2006). It was reported that human amniotic epithelial cells could be induced to differentiate in culture in vitro into diverse mature and specialized cells of three germ layers: pancreatic and hepatic cells (endoderm); cardiomyocytes, myocytes, osteocytes, adipocytes (mesoderm) and keratinocytes and neuronal and glial cells (ectoderm) (Ilancheran et al., 2007; Miki et al., 2005).

The umbilical cord blood (UCB) represent another source of multipotent stem cells that might be readily available for stem cell transplantation or for generating diverse tissue-specific adult stem/progenitor cells and their further differentiated progeny for cellular therapies of diverse disorders in humans (Barrilleaux et al., 2006; Mimeault and Batra, 2006). Several studies have revealed the possibility of differentiating UCB-derived stem cells into diverse functional progenitors, including dendritic cells, cardiomyocytes, MS cell progenitors, neural precursors, keratinocytes, hepatocytes, pancreatic β-cells and endothelial cells in specific culture conditions in vitro and in vivo (Yamada et al., 2007; Oh et al., 2007; Brunstein et al., 2007).

On the other hand, the amniotic membrane from placenta may serve as a scaffold for tissue engineering and stem cell transplantation in vivo (Wu et al., 2006). Limbal biopsy explants cultured on intact amniotic membrane, for instance, were employed in the treatment of total limbal stem cell deficiency or ocular surface reconstruction in chemical and thermal injuries (Tejwani et al., 2007; Maharajan et al., 2007).

1.2.2.5 Organ/tissue-specific stem cells

1.2.2.5.1 Epidermal stem cells

Epidermal stem cells reside in the basal layer of the skin (Stern and Bickenbach, 2007). These cells replace the outer keratinized layers of protective epidermis, which are continually shed, and regenerate new epidermis following an incisional or excisional wound. Epidermal stem cells were noted to express higher levels of the transmembrane Notch ligand, Delta 1 (Lowell et al., 2000) and β-1 integrin than other epidermal cells (Jones et al., 1995). Genetic ablation or activation of the Notch pathway reveals that Notch signalling promotes differentiation of the hair follicle, sebaceous gland and interfollicular epidermal lineages and that Notch acts as an epidermal tumour suppressor (Favier et al., 2000; Nicolas et al., 2003). In addition, integrin-expressing cells are found in epidermis to divide infrequently to give rise to a more profligate transit amplifying cell population that undergoes division within the basal layer (Jensen et al., 1999; Jones and Watt, 1993). Transit amplifying cells spread out from the stem cell patches, migrating over the basement membrane, to fill the rete ridge spaces (Jensen et al., 1999). Recent studies suggest that an epidermal stem cell can divide asymmetrically, giving rise to one basal stem cell and one committed cell (Clayton et al., 2007).

1.2.2.5.2 Muscle stem cells

Muscle stem cells: Skeletal muscle contains stem cells located between the sarcolemma of the myofiber and its overlying basal lamina. These "satellite" cells were first identified by electron microscopy and subsequently shown by DNA-labeling studies in several species to be the source of regenerated muscle (Yablonka-Reuveni et al., 1987). After muscle injury, satellite cells recapitulate the embryonic muscle program by proliferating, fusing, and differentiating into myofibers (Jones, 1982). Quiescent satellite cells do not express the basic helix-loop-helix (bHLH) myogenic factors that regulate embryonic myogenesis, but express them in culture, although in a different sequence than the one observed during muscle embryogenesis (Smith et al., 1994). Specific markers for

quiescent satellite cells are myocyte nuclear factor, which may prevent transcription of the bHLH myogenic factors (Garry et al., 1997), the c-met receptor tyrosine kinase (Cornelison and Wold, 1997), which plays a key role in the activation of proliferation by hepatocyte growth factor (Tatsumi et al., 1998), and p130, a protein that associates with E2F transcription factors to block cell cycle progression and also inhibits the differentiation of cultured myoblasts by blocking the expression of MyoD (Carnac et al., 2000).

1.2.2.5.3 Endothelial stem cells

Blood vessels regenerate primarily by compensatory hyperplasia of endothelial cells in the walls of venules, but endothelial stem cells in the marrow also contribute (Ergün et al., 2007). A small fraction of marrow stromal cells synthesize von Willebrand factor VIII-associated antigen, an endothelial cell marker, facilitating the identification of endothelial stem cells within the BM aspirate (Prockop, 1997). Up to now, endothelial stem cells, identified by their reaction with antibodies to CD34 and Flk1 antigens, have been isolated from human, rabbit, and mouse peripheral blood (Brunt et al., 2007). These precursors differentiate into endothelial cells in vitro (Asahara et al., 1997). In vivo, the endothelial stem cells transplanted were differentiated into mature endothelial cells and incorporated directly in the endothelium of vascular wall locally. On the other hand, the transplanted cells could migrate underneath the endothelium, releasing proangiogenic growth factors and promoting proliferation of nearby resident epithelial cells (Asahara et al., 1997; Roberts et al., 2005).

1.2.2.5.4 Liver stem cells

The liver is believed to have a very slow turnover time, but regenerates rapidly after partial hepatectomy (Michalopoulos and DeFrances, 1997). In the liver, if hepatocytes are

destroyed by toxic chemical injury or prevented by drugs from proliferating after partial hepatectomy, small oval cells appear in the epithelium of the bile ductules and regenerate hepatocytes (Theise et al., 1999; Alison et al., 1996). These cells are thought to arise from liver stem cells in the terminal bile ductules, and thus are equivalent to a transit amplifying population which are bipotential and can differentiate into hepatocytes or bile duct epithelium (Sell, 2001; Sell, 1994). The oval cells express the markers characteristic of hepatoblasts: α-fetoprotein, γ-glutamyl transpeptidase, cytokeratin19, OC-2, OV-6 and Thy-1, only a few of which are expressed by hepatocytes, suggesting that they recapitulate the embryonic differentiation of hepatocytes (Hixson et al., 1997; Petersen et al., 1998). Oval cells also express markers of bile ductile cells, suggesting their probable origin from the terminal ducts (Thorgeirsson, 1996). Further studies demonstrated that the oval cells in the liver could express haematopoietic cell markers, suggesting a potential overlap in the liver stem cell and haematopoietic stem cell phenotypes (Petersen et al., 1998; Thorgeirsson, 1996). This scenario may also suggest the transdifferentiation potential of liver stem cells.

1.3 Potency and plasticity of stem cells

Recent advances in technology have allowed the isolation and culture of multipotent stem cells. Such studies may help elucidate the underlying mechanisms that will further the understanding of cell proliferation and differentiation.

1.3.1 Hierarchical differentiation potency of stem cells

It was noted that the cell line derived from fetal rat calvaria cells can differentiate into myotubes, adipocytes, chondrocytes and osteoblasts, suggesting multipotentiality (Grigoriadis et al., 1990). This cell population contains cells with all four differentiation potencies, as well as tripotent, bipotent, and unipotent cells. If any one of these kinds of

cell differentiation results solely from activation of a specific set of cell-specific genes, one might expect to find only totipotent and unipotent cells in the population. However, the presence of tripotent, bipotent, and committed unipotent cells suggests that the lineage-specific differentiation may be attributed to the restriction of the other differentiation possibilities of a pluripotent cell. The molecular evidence for multipotency of these cells is seen in osteoblastic cell lines isolated from neonatal rat calvarias that show both myogenic and osteogenic cell-specific markers (Timmons et al., 2007; Vertino et al., 2005). The progenies of the rat calvarial stem cell line include adipocytes-chondrocytes, adipocytes-myotubes and chondrocytes-myotubes (Grigoriadis et al., 1990), which suggest random restriction of differentiation potencies. However, analyses on differentiation of multipotent progenitor cells reveal that the potential differentiation that is restricted is evolutionarily older, i.e., arose earlier during evolution, than the type of cell differentiation that occurs, favoring the notion that evolution occurs prior to differentiation (Flickinger, 2005; Yellajoshyula and Brown, 2006).

The timing by which cultured multipotent rat mesenchymal precursor cells differentiate into multinucleated muscle cells, adipocytes, chondroblasts, and osteoblasts is 9–10 days, 12 days, 16 days, 21 days, respectively (Grigoriadis et al., 1988). In developing rats, oligodendrocytes appear at birth, while type 2 astrocytes are found 7–10 days after birth (Williams et al., 1985). In the mouse embryo, erythroid cells appear before macrophages (Keller et al., 1993). The vertebrate retina contains seven major cell types that appear in the same sequence during both evolution and development and rods appear before bipolar cells (Alexiades and Cepko, 1997). Sensory and autonomic neurons appear before glial cells (Anderson, 1995), and smooth muscle cell differentiation occurs before that of enteric or parasympathetic neurons in chick embryos (Ito and Sieber-Blum, 1993). The evolutionarily conservative sympathetic neurons are present by 12.5 dpc in

mouse embryos (Lo et al., 1991), while chromaffin cells appear by birth in rats. Neuronal cells appear before skeletal muscle fibers from multipotent mouse embryonic stem cells (Tajbakhsh and Buckingham, 1995), while neurons form before epidermis in isolated Xenopus gastrula ectoderm (Hemmati-Brivanlou and Melton, 1997). These observations indicate that an evolutionarily more primitive cell type differentiates before an evolutionarily younger one.

1.3.2 Transdifferentiation of lineage-specific stem cells

The normal fate of adult stem cells is to regenerate site-specific tissue and lineage-specific cells. Recent experiments have shown that some of these stem cell types possess a developmental potential much greater than their normal fate, as revealed by transplanting or injecting cells into the embryos of mice and birds, SCID adult mice, or injury environment of adult mice (Kindler, 2005). Muscle stem cells can differentiate into blood cells in irradiated mice (Jackson et al., 1999) and into cardiomyocytes when transplanted into injured myocardium (Horackova et al., 2004; Atkins et al., 1999). NS cells have remarkable developmental plasticity. Fetal human NS cells injected into the ventricles of embryonic or neonatal rats are able to migrate widely into the host brain and differentiate into neurons and glia appropriate to each region of the brain (Brüstle et al., 1998; Flax et al., 1998). Besides, clonally derived NS cells are able to differentiate as blood, hepatocytes, intestine, skeletal muscle and cardiac muscle when injected into irradiated mice or embryos of the mouse and chick (Bjornson et al., 1999; Clarke et al., 2000). Isolated mouse NS cells from the lateral ventricles of the brain differentiate in vitro into skeletal myotubes containing the muscle markers α-actinin-2 and myosin heavy chain, when cocultured with C2C12 cells (Rietze et al., 2001).

Bone marrow cells can differentiate into a wide vatiety of cell types beyond their normal fate. When bone marrow cells were injected into the regenerating tibialis anterior

muscle of SCID, they were noted to give rise to muscle cells (Ferrari et al., 1998). They are also able to differentiate into cardiomyocytes in vitro after treatment with 5-azacytidine (Makino et al., 1999) and in vivo when injected into the ventricular myocardium of rats (Bittira et al., 2002). In rats with injured livers, transplanted bone marrow cells were noted to give rise to hepatocytes (Petersen et al., 1999). Marrow-derived hepatocytes have also been identified in female patients having been transplanted with male bone marrow (Alison et al., 2000; Theise et al., 2000). When injected into the brain ventricles of neonatal mice, marrow cells migrated throughout the forebrain and cerebellum, where they differentiated into astrocytes (Kopen et al., 1999) and neurons (Brazelton et al., 2000; Mezey et al., 2000). Bone marrow cells could also differentiate into neurons in culture in vitro (Sanchez-Ramos et al., 2000; Woodbury et al., 2000).

A single bone marrow cell was shown to home to bone marrow in lethally irradiated mice, repopulate the haematopoietic system, and possess the ability to differentiate into epithelial cells of the liver, esophagus, stomach, intestines, lung, kidney tubules and skin (Krause et al., 2001). The study attested the finding of previous reports in which the bone marrow-derived mesenchymal stem cells could contribute to not only bone and cartilage, but also the epithelial component of the lung (Pereiera et al., 1995). These data suggested the multilineage differentiation potency and transdifferentiation capacity of bone marrow stem cells.

The mechanism by which stem cells of specific tissues change fates under different circumstances is unknown. The cells might dedifferentiate to an earlier, more pluripotent state from which they differentiate along the lineage specified by their new environment. Alternatively, the new environment might instigate a new transcriptional program without reverting to a more primitive state (Weissman, 2000a). The specific sets of signals that attract and guide circulating stem cells into various environments are largely

unknown.

1.3.3 Dedifferentiation

Generally, stem cells or progenitor cells are activated and then participate in the regeneration or repair of injured tissues in mammals. However, in vertebrates with more extensive regenerative abilities, such as the salamanders, new stem cells or progenitor cells are created through a process of cellular dedifferentiation in which differentiated cells can reverse the normal developmental processes and once again become precursor cells (Kumar et al., 2000). Several studies have shown that certain mammalian cell types can be induced to dedifferentiate to progenitor cells when stimulated with the appropriate signals. This discovery opens the possibility that the endogenous regenerative capacity of mammals might be enhanced by inducing cellular dedifferentiation in vivo (McGann et al., 2001; Crescenzi et al., 1995).

At the beginning of dedifferentiation, the initial factor(s) might induce a set of common or cell specific downstream effector genes that, in turn, might repress the expression of differentiation genes, while activating cell cycle genes or their cognate proteins. These combinatorial effects could lead to cellular dedifferentiation and subsequent proliferation of the dedifferentiated cells (McGann et al., 2001). Specific differentiation signals could initiate the redifferentiation process and, in conjunction with the expression of appropriate patterning genes, could lead to the formation of the regenerated structure (Tanaka et al., 1999). From a medical standpoint, identification of genes responsible for dedifferentiation could lead to therapies aimed at enhancing the regenerative processes in mammals, especially in humans.

Ectopic expression of oncogenes can induce mouse myotubes to reenter the cell cycle. Expression of the *E1A* gene can downregulate myogenic genes (Tiainen et al., 1996), while the SV40 large T antigen can induce cleavage of the myotube to form

smaller myotubes or mononucleated cells (Endo and Nadal-Ginard, 1998). Moreover, upregulation of *msx1*, in conjunction with unidentified serum factors, can repress the expression of myogenic differentiation genes, such as *MyoD*, *myogenin*, *MRF4*, and those that encode the contractile proteins. This repression causes the myotube to reverse its differentiation phenotype and eventually cleave to form mononucleated cells (Odelberg et al., 2000). The gene *msx1* also represses the expression of the cyclin dependent kinase 4 (cdk4) inhibitor and indirectly upregulates cyclin D1 expression (Hu et al., 2001). The combination of these two events leads to the activation of cyclin D1-cdk4 complexes, which can then release the transcription factor E2F and induce DNA synthesis within the dedifferentiated cells (Odelberg et al., 2000).

Under certain conditions, mammalian cells can be induced to reprogramme their nuclei to resemble an earlier developmental state (Campbell, 1999). The well-publicized cloning of adult mammals is an example in which a nucleus from an adult cell is reprogrammed to resemble the nucleus of a zygote following the fusion of the adult cell with an enucleated oocyte (Wilmut et al., 1997). It has been reported that oligodendrocyte precursor cells can be induced to dedifferentiate to a cell type that resembles CNS stem cells by sequentially treating the precursor cells with either fetal calf serum or bone morphogenetic proteins and then with fibroblast growth factor-2 (Kondo and Raff, 2000). Based on these researches, cellular dedifferentiation might one day be used as a method for enhancing the regenerative capacity in mammals.

1.3.4 Cell fusion

Under certain conditions, stem cells may fuse with somatic cells and such fusion-derived cells acquire markers of both "parental" cells. Cell fusion of transplanted bone marrow-derived cells with host cells has been shown in some studies that demonstrated "chimerism" of neural, cardiac, or hepatic cells (Alvarez-Dolado et al., 2003).

Furthermore, lineage-committed granulocyte-macrophage or mature macrophages were identified as potential populations of bone marrow-derived fusogeneic cells (Camargo et al., 2004). The phenomenon of cell fusion seems to occur preferentially in cells where polyploidy is commonly seen, such as hepatocytes, Purkinje cells, or cardiomyocytes, and further, it has been suggested that cell fusion may be the mechanism by which these cells become multinucleated or poluploid (Alvarez-Dolado et al., 2003).

When cocultured with pluripotent embryonic stem cells, the mouse brain cells have undergone epigenetic reprogramming. These brain cells carry a transgenic marker and chromosomes derived from the embryonic stem cells (Ying et al., 2002). Therefore, the altered phenotype does not arise by direct conversion of brain cells to embryonic stem cells but rather through spontaneous generation of hybrid cells. The tetraploid hybrids exhibit full pluripotent character, including multilineage contribution to chimeras (Tada et al., 2001). It has been proposed that transdifferentiation consequent to cell fusion could underlie many observations otherwise attributed to an intrinsic plasticity of tissue stem cells (Vieyra et al., 2005).

In addition, neural stem cells were demonstrated to differentiate into virtually every cell type when they were injected into blastocysts in vivo or cultured in vitro with differentiating embryonic stem cells (Clarke et al., 2000). This indicated that the extracellular factor(s) of blastocysts or embryonic stem cells, or cell-cell interaction of neural stem cells with such embryonic cells, might be sufficient for reprogramming adult cells into a more pluripotent status. Furthermore, recent studies have demonstrated that transplanted bone marrow cells can turn into unexpected lineages including myocytes, hepatocytes, neurons and many others (Weissman, 2000b). It was suggested that mouse bone marrow cells can fuse spontaneously with recipient cells in vivo and even embryonic stem cells in culture in vitro. Spontaneously fused bone marrow cells can

subsequently adopt the phenotype of the recipient cells (Terada et al., 2002). Thus, the transdifferentiation of bone marrow stem cells may be partially attributed to spontaneous cell fusion.

1.4 Stem cell therapy and regenerative medicine

The therapeutic application of stem cells is a promising and raipidly emerging branch of regenerative medicine in which stem cell-based treatments could be applied to treat and cure many aggressive and lethal diseases in humans (Lindvall et al., 2004; Barrilleaux et al., 2006). Numerous recent investigations carried out with stem cell-derived functional progenies have provided accumulating evidence supporting their potential use for the treatment of genetic and degenerative disorders (Ringdén, 2007; Brunstein et al., 2007; Behfar et al., 2007; Schaffler and Buchler, 2007). Autologous or allogenic transplantation of stem cells or their further differentiated cells may notably constitute a potential therapeutic strategy, alone or in combination with the conventional treatments, for overcoming the progressive loss of functions, due to aging or degenerative diseases (Deeg et al., 2006; Mimeault and Batra, 2006).

The prevailing view is that stem cells possess the capability of unlimited self-renewal to produce daughter cells, which undergo a terminal differentiation process (Hall and Watt, 1989; Watt and Hogan, 2000). In particular, ES cells are believed to have more diverse plasticity, pluripotency and capacity to differentiate into the whole body-spectrum of cell types, as compared to adult stem cells. In terms of origin, potency, availability, yield and purity of cell products, the difference of ES cells and adult stem cells are categorized in Table 1-1. Thus, ES cells appear to be a promising cell source for regenerative medicine to replace or regenerate the damaged tissues or organs, since highly regulated developmental processes, including cell proliferation, migration, lineage progression, differentiation and maturation, could lead to the formation and remodeling

of most adult tissues (Gage, 2000).

1.4.1 Embryonic stem cells derived from different species

The culturing of mouse or human ES cells in a proliferating and undifferentiated state is a multistep process. First, the inner cell mass of a preimplantation blastocyst is removed from the trophectoderm that surrounds it. Then they are allowed to grow in culture medium supplemented with fetal calf serum (FCS) on small plastic culture dishes pre-coated with a "feeder" layer of non-dividing cells. The feeder cells are often mouse embryonic fibroblasts (MEF) that have been chemically inactivated or irradiated to arrest proliferation. Mouse ES cells can grow *in vitro* without the feeder layer support if the leukemia inhibitory factor (LIF) is added to the culture medium. Human ES cells, however, do not respond to LIF. After several days to a week, cell colonies are removed and passaged into new culture dishes freshly pre-coated with MEF. Under these *in vitro* conditions, some colonies may differentiate. It is difficult to maintain dispersed ES cells of human origin in cultures and prevent the spontaneous differentiation. From mouse ES cells, researchers have learnt much about the culture conditions of human ES cells and their capacity for differentiation (Passier and Mummery, 2003). In 1998, Thomson and co-workers succeeded in isolating human ES cell lines for the first time (Thomson et al., 1998). Starting with 14 inner cell masses, five human ES cell lines were isolated. Like ES cells of other species, human ES cell lines were found to differentiate randomly in vitro into derivatives of all three germ layers, as well as in vivo when injected into tissues of severe combined immunodeficient (SCID) mice (Thomson et al., 1998). Characteristics that are typical for ES cells are shown to be present on the isolated human ES cell lines, which display high levels of telomerase activity and express cell surface markers that characterize non-human primate and human embryonal carcinoma cells, including stage-specific embryonic antigen (SSEA)-3, SSEA-4, tumor rejection antigen (Tra)-1-60, Tra-

1-81 and alkaline phosphatase. The differences between mouse ES cells and human ES cells are shown in Table 1-2.

1.4.2 Potential application of stem cell therapy

Changes in living environments and lifestyle lead to a preponderance of diseases that might not be prevalent previously. The management of these disorders is likely to be beyond the scope of current therapies, in terms of both long-term survival and quality of life. The success of therapies used to treat severe damage in terminally differentiated tissues or organs is limited, due to the donor site morbidity of autologous grafts, the immunogenicity of allogenic grafts and loosening of the alloplastic implants (Mason et al., 2000). Basic and clinical research accomplished during recent years on ES cells has constituted a revolution in regenerative medicine by providing the possibility of generating multiple therapeutically useful cell types, which could be used for treating numerous genetic and degenerative disorders.

1.4.2.1 Neurological diseases

The effects of neurological diseases on quality of life are profound, and they can be very distressing for both patients and their families. Although some drug treatments can relieve symptoms, they do not address the underlying pathology and they are not suitable for all patients, in terms of clinical efficacy or the long-term costs. A key issue in the treatment of neurological diseases is that injurious processes are often irreversible because neurons in the brain are unable to spontaneously regenerate effectively. This is especially frustrating because the adult CNS harbors stem cells with the potential to regenerate, but it fails to recruit them efficiently. Therapeutic approaches that could address this include introducing regenerating cells into the diseased CNS by means of transplantation or, alternatively, enhancing the number and function of endogenous

regenerating cells. Beneficial effects of cell transplantation therapy have been reported in several animal models of different neurologic diseases such as stroke, Parkinson's disease, Alzheimer's disease, multiple sclerosis and amyotrophic lateral sclerosis (Lindvall and Kokaia, 2006). The mechanisms by which transplanted cells are involved in clinical recovery are not fully understood. Previously, the therapeutic potential of cell transplantation therapy had focused primarily on cell replacement for damaged or missing cells. Until recently, studies focusing on reparative mechanisms have shed light on the ways in which transplanted cells in the brain may promote neural recovery.

Stroke, particularly ischemic stroke, strikes millions of people worldwide. Current therapies are mainly for primary or secondary prevention (Krakauer, 2007; Liepert et al., 2000). There is no effective treatment to improve the neurological functions of patients after stroke pertaining to cell death or degeneration. Acute ischemic stroke caused by cerebral artery occlusion or rapid blood loss leading to infarction of brain tissue and death of neurons, astroglia and oligodendroglia, is the most important vascular CNS disorder in the modern world and remains a leading cause of death and disability. Despite significant clinical benefits of systemic thrombolysis, only a minority of patients has timely access to this therapy. A majority of the survivors suffer from long-term to permanent neurological disorders. Under such circumstances, it is crucial to develop new alternative therapeutic strategies. An encouraging approach is to replace infarcted CNS tissue in an organotypic appropriate manner by the cell-based therapy. Lost neurons and glial cells such as astroglia and oligodendroglia need to be replaced to allow the re-establishment of a functional neuronal circuitry. Besides, the implanted cells could provide trophic support to tissue at risk in the penumbra surrounding the infarct area and help promote survival, migration and differentiation of endogenous precursor cells.

It was suggested that neurogenesis is continuous and mediated by multipotent $CD133^+$/$Nestin^+$ NS cells with an astroglia-like cell phenotype found within two specific brain regions: the lateral subventricular zone of lateral ventricle in the forebrain and dentate gyrus in hippocampus (Lim et al., 2007; Mimeault and Batra, 2006). NS cells, which are co-localized in close proximity to blood vessels in the supraventricular zone, can give rise to three principal neural cell lineages: mature neurons, astrocytes and oligodendrocytes (Lindvall et al., 2004; Lim et al., 2007). Neural precursors in the subgranular cell layer of hippocampus may give rise to granule cell projection neurons. Both global and focal ischemia had been shown to induce neurogenesis in the subventricular zone and dentate gyrus in adult mammals (Kee et al., 2001; Zhang et al., 2004; Arvidsson et al., 2001). However, the number of newborn neurons seems to be low. Experimental data suggest that only a very small proportion of destroyed cells are replaced by newly generated neurons and over 80% of newborn neurons die within the first six weeks after stroke (Arvidsson et al., 2002). Thus, neurogenesis appears to depend largely on the presence of exogenous stimuli or direct cell transplantation.

To date, one of the best candidates for mass generation of specialized neural cells is the ES cells (Cedar et al., 2006). By recapitulation of developmental conditions in culture, it is possible to grow ES cells in vitro and to generate cultures enriched with desired neural subtypes, such as motor neurons, dopaminergic neurons, astrocytes and oligodendrocytes among others (Zhang, 2006). Recent data regarding their beneficial effects in animal models of stroke indicate that transplanted neural cells could not only replace damaged neurons, but also attenuate deleterious inflammation, protect the CNS from degeneration, and enhance endogenous recovery process (Savitz et al., 2004; Dobkin, 2007). These early studies provide proof to validate cell therapy for treatment of stroke, although many questions regarding the optimal site of grafting, dose of cell

delivery, graft injection, and adverse effects, were put forward

In Parkinson's disease (PD), in addition to the degeneration of the dopaminergic neurons, there is now clear evidence of alterations in cholinergic, serotonergic, GABAergic, and norepinephrine pathways, in which a direct relationship between degenerative neurons of the locus coeruleus and the degeneration of dopaminergic neurons in the substantia nigra in PD has been demonstrated (Baloyannis et al., 2006; Gesi et al., 2000). The participation of these other pathways in PD changes the underlying premise of how cell transplantation therapies are employed. Although restoring or replacing dopamine-producing cells has improved motor deficits, replenishment of other cell types could be the difference between minimal improvement and reversing the progress of PD. Effectiveness of stem cell therapy, in general, may have more to do with protecting and repairing the degenerating or injured tissue than with the actual replacement of cells (Menendez et al., 2006). This may be accomplished either directly by the stem cells providing the protection or repair through their predetermined properties or genetic alteration of their properties (McLeod et al., 2006; Raymon et al., 1997), or indirectly by the stem cells inducing the endogenous cells to repair or regenerate the damaged cells, or to release the needed neurotrophic or growth factors for repair and protection (Whitton, 2007).

Huntington disease is another candidate for stem cell therapy because it also results from a localized degenerative process involving a specific neuronal subtype, GABAergic neurons. The ease of generation of GABAergic neurons from transplanted stem cells or progenitor cells will contribute to the progress in treatment for Huntington disease (Bosch et al., 2004). Other degenerative diseases of the adult CNS such as Alzheimer's disease and amyotrophic lateral sclerosis may also be suitable for stem cell therapy, since transplanted cells could replace a range of neuronal types, remyelinate axons, and repair

complex neural circuitries (Sugaya, 2005; Appel et al., 2008).

1.4.2.2 Diabetes

Diabetes is the one of the commonest causes of non-accidental death in more developed countries. Health-care costs associated with diabetes have dramatically increased in recent years. Not only diabetes itself, but the complications that arise from it will have an increasing effect on human health. Novel approaches such as islet transplantation offer new opportunities for treatment (Ryan et al., 2001), but the limitations of transplanted tissue are well known and such concerns must be dealt with before this treatment can become generally available for diabetes.

A nomal adult human pancreas contains approximately 10^9 islet cells. The need to develop an unlimited supply of human surrogate β cells has led investigators to attempt islet expansion in tissue culture, as well as differentiation of other cells into insulin-producing cells. So far, islet expansion has proven to be quite chanllenging, because the cells do not grow well and tend to dedifferentiate (Russ et al., 2008). Thus, the focus at present is on differentiation of stem cells, which can be easily expanded in vitro. Manipulation of their culture conditions, as well as the introduction of transcription factor genes capable of reprogramming cell differentiation, hold the promise of inducing stable phenotypic changes in stem cells to convert them into β-like insulin-producing cells (Santata et al., 2006). Furthermore, stem/progenitor cells already committed to other tissues, such as liver and bone marrow, are utilized in an attempt to divert their development into β-like cells, which may allow the use of autologous cells (Jin et al., 2008; Hess et al., 2003). Based on these investigations, cell therapy using stem cell-derived insulin-secreting cells might be a promising approach.

1.4.2.3 Joint replacement and bone regeneration

The use of artificial joint replacements in orthopaedic surgery has been one of the greatest clinical successes in tissue replacement in the past 30 years. Hip replacements have been clinically successful since their introduction, although there were difficulties with knee replacements initially. These implants have also led to remarkable increases in quality of life of patients. However, a plateau in implant survival has been reached at around 15 years (Korda et al., 2008). To cope with future clinical demands, new approaches are needed to attain implant survival in the range of 20~30 years. A particular challenge is to find a biological material or tissue regeneration therapy that behaves equivalently to an autograft, which is the gold standard in bone regeneration for orthopaedic surgeons.

Recent advances in molecular cell biology of morphogenesis will aid in the design principles and architecture for tissue engineering and regeneration of bone. Bone is a prototype model for tissue engineering. Implantation of demineralized bone matrix into subcutaneous sites results in local bone induction. The sequential cascade of bone morphogenesis mimics sequential skeletal morphogenesis in limbs and permits the isolation of bone morphogens. The symbiosis of bone inductive and conductive strategies is critical for regeneration of bone, and is in turn governed by the context and biomechanics. The context is the microenvironment, consisting of extracellular matrix scaffolding, and can be duplicated by biomimetic biomaterials such as collagens, hydroxyapatite, proteoglycans, and cell adhesion proteins. Moreover, when seeded onto the artificial scaffold, stem cells are capable in vitro of forming both bone and cartilage under the appropriate environmental conditions, providing the biomimetic surrogate for the diseased articulus.

1.4.2.4 Cardiovascular diseases

Nowadays, cardiovascular diseases, including coronary heart diseases, cardiac

valvular diseases and hypertension, are severely threating human health throughout the world. Coronary heart diseases are the leading cause of death in many countries. Despite the availability of a diversity of treatment modalities, the tissues die as a result of myocardial infaction. The replacement of dead myocardium by using stem cell-derived cells will be encouraging.

Intense interests have recently focused on regenerative medicine approach: cell transplantation, mobilization of resident stem cells, and tissue engineering as potential strategies for enhancing the repair or regenerative capacity of the injured heart. The progress and achievements in experimental models and initial clinical experience have been decribed in the field of cardiac stem cell therapy and tissue engineering. These therapies illustrate the promise of new technologies that may provide a new tool for reconstructing damaged hearts that previously would have been irreparable.

1.4.3 Clinical potentials of embryonic stem cells

Bioengineering technologies may provide novel tools for regenerative medicine and help overcome the limitations of conventional treatments. Tissue engineering allows the design of functionally active cells within supportive bio-scaffolds combined with the controlled delivery of growth factors to promote the development of new tissues for the restoration of pathologically altered tissues (Sittinger et al., 1996). Basically, common tissue engineering involves functional cells which are compatible with host environments and responsive to distinct environmental cues, suitable carriers for the in vitro cell differentiation and subsequent transplantation, and defined bioactive molecules driving the differentiation and maturation of implanted cells.

The main advantage of ES cells as a source of precursors or differentiated cells is that they can be propagated indefinitely without loss of pluripotency. This means that ES cell lines can potentially supply any type of differentiated cells in the quantities required.

Another advantage of ES cells is the absence of any potential effects of age that might be associated with adult stem cells. The proliferation capacity of many adult organ-specific cells is low and long-term in vitro cultivation, in particular, reduces their functionality. These are the reasons why so much attention has been drawn to pluripotent ES cells. Since adult tissues do not appear to have the full range of signaling systems to direct the differentiation of ES cells to site-specific cell phenotypes, the use of derivatives of ES cells may be suitable for transplantation, in case of abnormal development caused by pluripotent ES cells. The ES cell derivatives have been produced either by random differentiation and subsequent lineage selection in vitro, or in some cases, by directing the differentiation of ES cells in vitro to more restricted precursor cells that can differentiate the rest of the way in response to the remaining signals maintained by intact or injured adult tissues.

Transplantation of ES cell derivatives to correct tissue deficiencies has been reported (Müller et al., 2000). Cardiomyocytes have been derived by random differentiation from mouse embryoid bodies (EB) and grafted into the ventricular muscle of dystrophic mice. The grafts were stably integrated into the host cardiac muscle, as judged by anti-dystrophin staining (Klug et al., 1995; Klug et al., 1996).

Multipotent glial precursor cells, derived by the directed differentiation of mouse ES cells in vitro, were injected into the spinal cord and brain of mutant rats suffering from a myelin-deficiency disease that mimics human Pelizaeus-Merzbacher disease (Brüstle et al., 1999). The injected cells were shown to differentiate into astrocytes and oligodendrocytes, which remyelinated cord and brain axons. A similar result was obtained after grafting ES cell-derived oligodendrocyte precursors into the spinal cord of myelin-deficient mice or the dorsal columns of chemically injured spinal cords of adult rats (Liu et al., 2000). Multipotential neural precursors, derived from the directed

differentiation of mouse ES cells in vitro and injected into spinal cord lesions of paralyzed rats 9 days after injury, migrated throughout the injured areas and differentiated into neurons, astrocytes and oligodendrocytes. The rats were able to achieve partial recovery of motor functions (McDonald et al., 1999). Whether the grafted cells restored some function by forming new neuronal connections, remyelinating demyelinated host axons, secreting factors that neutralized toxic factors inhibitory to host regeneration, or some combination of these, was not clear.

Another example of experimental therapeutics using derivatives of ES cells is insulin-secreting cells (Soria et al., 2000). Insulin secreting cells were selected using a chimeric gene of the human insulin gene regulatory region and drug resistance gene. Insulin-secreting cells emerged after EB formation and subsequent lowering of the glucose concentration. One ES cell-derived insulin-secreting clone showed regulated hormone secretion in vitro in the presence of various stimuli. Clusters obtained from this clone were implanted into the spleen of streptozotocin-induced diabetic animals. After transplantation, hyperglycemia was corrected within one week and body weight was restored in four weeks. This approach opens new possibilities for tissue transplantation for the treatment of type 1 and type 2 diabetes.

A large number of studies have documented the development of various erythroid, myeloid and, to a lesser extent, lymphoid lineages within EBs derived from embryonic stem cells (Keller et al., 1993). Under optimal conditions, haematopoietic development within EBs is efficient and highly reproducible. Commitment to haematopoiesis takes place in the absence of added growth factors with the exception of those present in fetal calf serum (FCS). The fact that bone morphogenetic protein 4 (BMP-4) induces both mesoderm formation and haematopoiesis within the EBs in these defined culture conditions (Johansson and Wiles, 1995) suggests that it could be one of the active

components found in serum. In addition to factors present in FCS, the EBs themselves probably provide an environment that supports haematopoiesis, as genes encoding various growth factors and growth factor receptors are expressed early in EB development (Kennedy et al., 2007; Guo et al., 2006). These findings could be considered a significant step forward in our approach to understanding the molecular events that regulate haematopoietic development and differentiation, simultaneously making ES cells be an alternative tool in treating patients with haematopoietic diseases.

1.4.4 Customized ES cell-based therapies

ES cells, as a cell source for regenerative medicine, have several limitations: (1) Undifferentiated ES cells can give rise to teraromas after direct transplantation (Cao et al., 2007), (2) cultured ES cells exhibit epigenetic instability (Humpherys et al., 2001), and (3) derivatives of ES cells elicit immunorejection unless transplanted to immunoprivileged sites like the brain. To address these issues, "therapeutic cloning" has been designed to customize stem cell-based therapies for the recipients.

1.4.4.1 Somatic cell nuclear transfer

Somatic cell nuclear transfer (SCNT) entails the removal of an oocyte nucleus in culture, followed by its replacement with a nucleus derived from a somatic cell obtained from a patient, resulting in ES cells that are genetically identical to the patient. It has been shown that nucleus-transferred ES cells derived from fibroblasts, lymphocytes and olfactory neurons are pluripotent and generate live pups after tetraploid blastocyst complementation, showing the same developmental potential as fertilized blastocysts (Brambrink et al., 2006; Rideout et al., 2002; Eggan et al., 2004). The resulting ES cells are perfectly matched to the patient's immune system and no immunosuppressants would be required to prevent rejection. It has been shown that blastocysts generated from SCNT

can give rise to a liveborn infant that is a clone of the donor when implanted into a uterus (Wilmut et al., 1997).

The reproductive cloning is banned for human applications. In contrast, therapeutic cloning is used to generate only ES cell lines, the genetic material of which is identical to that of their source. Currently, non-human primate ES cell lines have been generated by SCNT of nuclei from adult skin fibroblasts (Byrne et al., 2007). To test their differentiation potential, the cells were exposed to cardiomyocyte differentiation conditions, and they produced contracting aggregates that expressed markers of cardiac muscle tissue. Neural differentiation resulted in the expression of microtubule-associated protein-2 (MAP-2), β-tubulin and tyrosine hydroxylase. When injected into SCID mice, SCNT-derived ES cells induced teratomas which contained differentiated cell types representing all three embryonic germ layers (Mitalipov et al., 2007). These studies suggested the pluripotency of SCNT-derived ES cells. However, before SCNT-derived ES cells can be used as clinical therapy, careful assessment of quality of the lines must be determined.

1.4.4.2 Parthenogenetic ES cells

Parthenogenesis is the process by which a single egg can develop without the presence of the male counterpart and is a form of reproduction common to many species such as fish, amphibians and lizards, which may routinely reproduce in this manner. Mammals are not spontaneously capable of this form of reproduction. However, mammalian oocytes can successfully undergo artificial parthenogenesis in vitro and can be activated by mimicking the calcium influx induced by sperm at normal fertilization, with the use of a calcium ionophore, and stimulated to divide (Revazova et al., 2007; Allen et al., 1994).

The differentiation capacity of parthenogenetic ES cells is striking. Mouse parthenogenetic ES cells differentiate in vitro and contribute to multiple tissues in chimeric mice. When transplantation compatibility of these cells was tested, it was found that differentiated parthenogenetic cells engrafted only when no mismatch of major histocompability complex (MHC) with the recipient exists (Allen et al., 1994). Similarly, human parthenogenetic ES cells could form embryoid bodies (EB) in culture and teratomas composed of cell types from all the three embryonic germ layers (Revazova et al., 2007). Human parthenogenetic ES cells are histocompatible with the oocyte donor, but they do not necessarily contain two copies of each chromosome. The extent of homozygosity depends on oocyte activation stage. Exactly, activation of metaphase-I arrested oocytes (before first polar body extrusion) gives rise to parthenogenetic ES cell lines that are identical to the donor as they contain the two maternal chromosome homologs. However, these cells are not applicable to clinical use, since experiments in mice showed that most cell lines derived were tetraploid or aneuploid (Henery and Kaufman, 1992). In contrast, metaphase-II arrested mouse and human oocytes (before second polar body extrusion) give rise to parthenogenetic ES cell lines that have the normal karyotype (Allen et al., 1994).

Kim and colleagues found that the most of the genes in parthenogenetic ES cells are heterozygous, but close to the centromere the gene copies show predominant homozygosity (Kim et al., 2007). In contrast, nuclear transfer-derived ES cells contain heterozygosity throughout the genome. Analysis of a human ES cell line that was claimed to be derived from a cloned embryo showed that it contains homozygosity in the MHC loci indicating a hemizygous human leucocyte antigen (HLA) match to the oocyte donor (Revazova et al., 2007).

Parthenogenetic human ES cell lines that do not carry two sets of MHC genes could potentially prove beneficial not only for the oocyte donor but also for genetically related individuals. In this scenario, there is a 50% chance that a cell line which is derived from a patient will be histocompatible to any of her children. Moreover, if a sizable depository of MHC-homozygous parthenogenetic human ES cell lines that carry MHC alleles which are common in the population were to be generated, it may serve as a MHC-matching bank. This would be the case since MHC-homozygous parthenogenetic human ES cell lines would have a much higher matching frequency than heterogeneous ES cell lines (Taylor et al., 2005). Generally, it would be possible to produce parthenogenetic human ES cell lines as efficiently as reported. Such cells may become a major source of therapeutic histocompatible cell lines for fertile women, genetically related individuals and the general public.

1.4.4.3 Reprogramming

Reprogramming is a technique that involves de-differentiation of adult somatic cells to produce patient-specific pluripotent stem cells without the use of embryos. Cells generated by reprogramming would be genetically identical to the somatic cells and would not be rejected by the donor. This method also avoids the technical limitations of nuclear transfer into oocytes. Takahashi and Yamanaka were the first to discover that MEF and adult mouse fibroblasts can be reprogrammed by retroviral transfection of genes *Oct3/4*, *Sox2*, *c-myc*, and *Klf4* (Takahashi and Yamanaka, 2006). Reprogrammed cells possessed the immortal growth characteristics of self-renewing ES cells, expressed genes specific for ES cells, and generated embryoid bodies in vitro and teratomas in vivo. When the induced pluripotent stem (IPS) cells were injected into mouse blastocysts, they contributed to a variety of diverse cell types, demonstrating their developmental potential.

Although IPS cells were pluripotent they were not identical to ES cells. Unlike ES

cells, chimeras of IPS cells did not result in full-term pregnancies. Gene expression profiles of the IPS cells showed that they possessed a distinct gene expression signature compared to ES cells. The epigenetic state of the IPS cells was somewhere between their somatic origins and fully reprogrammed ES cells, suggesting that the reprogramming was incomplete. These results were improved significantly by Wernig and co-workers (Wernig et al., 2007). It has recently been shown that reprogramming by transduction of four defined factors can be done with human cells (Takahashi et al., 2007; Yu et al., 2007).

Yamanaka's group demonstrated that retrovirus-mediated transfection of Oct3/4, Sox2, Klf4 and c-Myc generates human IPS cells that are similar to human ES cells in terms of morphology, proliferation, gene expression, surface markers and teratoma formation (Takahashi et al., 2007). Thomson's group showed that retroviral transduction of Oct4, Sox2, Nanog, and Lin28 could generate pluripotent stem cells without introducing any oncogenes such as c-Myc (Yu et al., 2007). Both studies showed that human IPS cells were similar but not identical to human ES cells. The IPS cells contain three to six retroviral integrations which may increase the risk of tumorigenesis.

It seems that cells that are already multipotent could be reprogrammed with greater efficiency, since the more undifferentiated donor nucleus, the better SCNT performs (Blelloch et al., 2006). Although reprogramming is an exciting phenomenon, current understanding of the mechanisms of reprogramming is limited.

1.5 In vitro differentiation of ES cells towards neural lineage

There are various methods to direct the differentiation of ES cells to neural fates. They include treatment with retinoic acid (RA; Bain et al., 1995), suspension culture of single cells (Tropepe et al., 2001), sequential culture in serum and serum-free media (Okabe et al., 1996), neural lineage selection protocols (Lee et al., 2000), adherent

monoculture protocols (Ying et al., 2003), forced expression of neuroectoderm-related genes through viral transfection (Chung et al., 2002) or coculture with specific stromal cells (Kawasaki et al., 2000). In addition, it is well established that trilineage neural progenitors capable of giving rise to neurons, astrocytes and oligodendrocytes, can be generated from ES cells (Joannides et al., 2007).

1.5.1 Spontaneous neural differentiation by default

Direct neural differentiation protocols are based on the default hypothesis that absence of signals in primitive cells will lead to neural differentiation (Ying et al., 2003). Many studies with mouse ES cells confirmed that under minimal conditions, in the absence of bone morphogenetic proteins but in the presence of endogenous FGF signals, neural induction does occur in cultures.

ES cells cultured in defined, low-density conditions were noted to readily adopt a default mechanism of neural specification. Initially, ES cells were cultured at 20 cells per microliter and seven days later, they formed sphere colonies. Addition of FGF2 and heparin caused a slight but nonsignificant increase in the numbers of primary sphere colonies compared to LIF alone. Under such circumstances, single ES cells differentiate into colony-forming cells in the absence of serum, feeder layers or the formation of EB. In the limiting dilution assays of mouse ES cells, the frequency in which a neurosphere could be derived was around 0.2% (Tropepe et al., 2001). Neurospheres which were generated in the presence of LIF contained cells expressing the neural precursor marker Nestin. When dispersed to fully differentiate, each of the colonies contained MAP-2$^+$ or β-Tubulin III$^+$ neurons, GFAP$^+$ astrocytes and O4$^+$ oligodendrocytes, suggesting that the individual cells giving rise to the neural colonies had neural multilineage potential. Interestingly, ES cells cultured for seven days in the same differentiation conditions at high cell densities do not express the neuronal makers MAP-2 or β-Tubulin III. Thus, at

relatively high cell densities, ES cells must be specified to a neural identity (neural stem cell colonies) in order to differentiate into neurons and glia.

Several studies have demonstrated neural differentiation from EB-derived cells, with tha addition of specific growth factors (Strübing et al., 1995; Okabe et al., 1996; Brüstle et al., 1999). Although these observations clearly demonstrate the potency of such factors to promote neuronal differentiation, each experiment initially utilized EB cultures in the presence of serum. Here, ES cells may be competent to directly differentiate into neural cells at low cell densities and serum-free conditions in the absence of exogenous growth factors, even within the initial 24 hours (Tropepe et al., 2001). This protocol represents an alternative and specific paradigm for neural cell fate specification directly from ES cells in serum-free conditions in the absence of EB formation. Although this paradigm can facilitate the discovery of genes that regulate the transition from an ES cell to a neural cell by utilizing an expression-based gene trap library of ES cell lines (Stanford et al., 1998), the low neural induction efficiency is a big hurdle for application in clinical scale.

1.5.2 Chemical induction

Many chemicals and compounds have been noted to enhance neural differentiation under certain circumstances, such as retinoic acid, ascorbic acid, arabinosylcytosine, hydroxyurea, and dimethyl sulphoxide. These findings suggest a novel role of these chemicals in lineage commitment of ES cells, and present a simple and effective strategy for promoting neural differentiation of ES cells.

1.5.2.1 Retinoic acid

Retinoic acid (RA), a vitamin A derivative, was found to have important regulatory functions during embryonic development (Morriss-Kay and Sokolova, 1996). RA induced malformations in laboratory animals and humans when administered during

pregnancy (Rosa et al., 1986). RA applied to mouse ES or embryonal carcinoma cells induced concentration- and time-dependent differentiation of neuronal, cardiac, myogenic, adipogenic, and vascular smooth muscle cells, respectively (Rohwedel et al., 1999). A significant induction of neuronal differentiation was achieved by the application of high concentrations of RA (10^{-6} to 10^{-7} M) at early stages of development (Bain et al., 1995).

RA has a well-established patterning role during development in posteriorizing CNS tissue (Li et al., 2005) and ES cell derivatives (Zhang, 2006). The yield of neural lineage cells generated by EB can be dramatically increased by the addition of RA (Guan et al., 2001; Gottlieb, 2002). A recent study has reported that RA is required for adult hippocampal neurogenesis (Jacobs et al., 2006). RA promotes the differentiation of pluripotential teratocarcinoma cells into neural progenitors and neurons (Jones-Villeneuve et al., 1983; Bain and Gottlieb, 1994), and this treatment was applied to ES cells to promote neural induction. ES cell-derived EB cultured for 4 days were treated for an additional 4 days with RA and then attached to adhesive substrates for 7 days (Bain et al., 1995). There was a yield of up to 40% neuron-like cells, in comparison to just a few percent observed in the cultures not treated with RA. Related protocols increased the yield of neural derivatives, including glutamatergic neurons and motor neurons, with RA treatment (Wichterle et al., 2002; Bibel et al., 2004; Li et al., 2005). However, subsequent studies have established that the NS cells generated by using the RA protocol may have restricted differentiation potential. When neural progenitors generated by RA induction were transplanted to the embryonic chick neural tube, they showed limited capacity to differentiate, compared with ES cell-derived neural progenitors generated in the absence of RA (Plachta et al., 2004). Other drawbacks of RA are the non-specific effects which attributed to numerous cell types in the final product, and the tumorigenicity of RA when used at high concentration beyond the physiological dose. Despite these, this approach

has been applied to human ES cells (Schuldiner et al., 2001).

1.5.2.2 Ascorbic acid

Ascorbic acid is a derivative of vitamine C. It was reported that in combination with bFGF and EGF, ascorbic acid could induce the generation of neuronal precursors in in-vitro culture of ES cells (Lau et al., 2006). Subsequent culture of the precursor cells in the presence of ascorbic acid together with EGF, FGF8 and Shh, could also promote cell proliferation (Lee et al., 2000; Lau et al., 2006).

The treatment of ES cells with ascorbic acid alone converted them into cells which displayed spontaneous and rhythmic contractile activity and stained positively for sarcomeric myosin and α-actinin, suggesting that ascorbic acid induces cardiac differentiation in ES cells, as confirmed by the induced expression of cardiac genes such as *GATA4*, *Nkx2.5*, *α-MHC*, *β-MHC* and *ANF* (Takahashi et al., 2003). In addition, ascorbic acid promotes osteogenic differentiation in mesenchymal stem cells (Arrigoni and De Tullio, 2002). Further studies are needed to elucidate the mechanisms involved in these distinct effects of ascorbic acid.

1.5.2.3 Arabinosylcytosine

Arabinosylcytosine (Ara-C) is a potent inhibitor of mammalian DNA polymerases α and β that acts through competition with the natural substrate dCTP. On incorporation into nascent DNA chain, Ara-C resulted in accumulation of short strands of DNA, thus inhibiting cell proliferation and causing expression of differentiation markers (Ross et al., 1990). Ara-C promotes erythroid differentiation of human leukemia cells (K562 cells) by irreversible induction of hemoglobin synthesis, loss of cell renewal capacity, and marked decrease of c-myc expression (Bianchi Scarrà et al., 1986). Furthermore, Ara-C promotes monocytic differentiation of human leukemic promyelocytes (HL-60 cells) by inhibiting

cell proliferation (Griffin et al., 1982). However, whether Ara-C, as a differentiating agent, could play a role in the lineage-specific differentiation of embryonic stem cells, requires further investigations.

1.5.2.4 Dimethyl sulphoxide

Dimethyl sulphoxide (DMSO) was used extensively for induction of differentiation of murine erythroleukemic cells (MEL cells) and other cells like HL-60 (Collins et al., 1978). The precise mechanism by which DMSO induces differentiation is not known but may involve perturbation of protein kinase C (PKC) activity, increase in membrane fluidity, elevation of calcium uptake, and alteration in expression of c-myc and p53 genes (Tsiftsoglou and Robinson, 1985). DMSO-mediated differentiation has been associated with G_0/G_1 arrest, activation of cyclin-dependent kinases (Jiang et al., 1994), and generation of DNA strand breaks (McMahon et al., 1984).

Both rodent and human bone marrow stromal cells were found to transdifferentiate into neurons in vitro by means of DMSO induction (Woodbury et al., 2000). Thus, the ability to transdifferentiate an easily accessible cell source into neurons could have substantial potential for promoting neural repair. On the other hand, DMSO could facilitate cardiac differentiation of P19 embryonal carcinoma cells. However, effects of DMSO on lineage commitment of embryonic stem cells remain controversial.

1.5.2.5 Hydroxyurea

As an inhibitor of S-phase DNA replication, hydroxyurea could result in DNA fragmentation and terminal erythroid differentiation in human erythroleukemic K562 cells (Tsiftsoglou et al., 2003). Hydroxyurea was also found to increase fetal hemoglobin in cultured erythroid cells derived from normal individuals (Fibach et al., 1993). However, it remains unclear whether hydroxyurea could affect lineage-specific

differentiation of embryonic stem cells.

1.5.2.6 Azacytidine

The 5-azacytidine, a demethylating agent, has been reported to induce the differentiation of mesenchymal stem cells into cardiomyocytes in vitro (Hakuno et al., 2002; Fukuda, 2003). Xu and co-workers reported that 5-azacytidine induced the differentiation of human ES cells into cardiomyocytes (Xu et al., 2002). This compound can cause extensive demethylation of 5-methylcytosine and reduce DNA methyltransferase activity in the cell (Haaf and Schmid, 2000). Recently, 5-azacytidine was found to reverse the differentiation status of EBs back to ES cells (Tsuji-Takayama et al., 2004). Therefore, 5-Azacytidine is useful for studying the roles of DNA methylation in the mechanisms of gene activation and cell differentiation.

1.5.3 Protocols for neural lineage selection

The serial culture protocols have been widely used and actually combine the use of EB induction and subsequent neural lineage-specific selection steps. Protocols for growth factor-mediated lineage selection of neuronal cells have been established (Okabe et al., 1996), including: (1) formation of cells of all three primary germ layers in EB; (2) selective differentiation of neuroectodermal cells (and inhibition of mesodermal cells) by growth factor removal; (3) proliferation and maintenance of neural precursor cells in the presence of inductive factors such as FGF2, EGF (Okabe et al., 1996), FGF8, and Shh (Lee et al., 2000); and (4) the differentiation induction and maintenance of functional neurons and glial cells by the combined addition of neural differentiation and survival-promoting factors. As a result, a characteristic sequence of expression of neural-specific genes and proteins was described. At an early stage characterized by the proliferation of neural precursor cells, the gene coding for Nestin, which is specifically expressed in

neuroepithelial stem cells, was highly expressed (Lee et al., 2000) in parallel to genes encoding neural transcription factors mash-1 and Engrailed-1 (En-1). Besides, high levels of Otx1, Otx2, Pax2, Pax5 and Wnt1 genes were detected (Okabe et al., 1996; Lee et al., 2000). At the same stage, immunofluorescence analysis revealed that up to 85% of ES cell-derived cells expressed Nestin (Rolletschek et al., 2001). The data suggested that ES cells differentiated according to this protocol showed a progressive restriction to neural precursor cells. After withdrawal of FGF2 and EGF, downregulation of Nestin and mash-1 was followed by upregulated expression of synaptophysin, Nurr1 and TH genes. Decreased formation of Nestin-positive cells was related to an increased number of neuronal cells expressing neuron-specific proteins. Mature neuronal cells are evidenced by the production of neurotransmitters such as dopamine, serotonin, GABA and glutamate. In addition, GFAP-positive cells were identified. These results suggest that, in the presence of neuronal differentiation factors, ES cell-derived, Nestin-positive neural precursor cells differentiated into glial cells and functional dopaminergic, GABAergic, glutamatergic, and serotoninergic neurons (Lee et al., 2000; Rolletschek et al., 2001).

In addition, as indicated by earlier investigations, some survival-promoting factors play an important role in promoting further differentiation towards neuronal fates. By using primary cultures of dopaminergic cells, it has been shown that interleukin-1β (Ling et al., 1998), dibutyryl-Camp (Branton et al., 1998), TGF-β3 (Krieglstein et al., 1998), glial cell line-derived neurotrophic factor (GDNF; Beck et al., 1995; Eggert et al., 1999), and neurturin (NTN; Horger et al., 1998) could promote the differentiation and survival of dopaminergic neurons. The application of these survival-promoting factors, at terminal stages of ES cell differentiation significantly enhanced mRNA levels of Nurr1 and TH, whereas mRNA levels of En-1, mash-1 and synaptophysin were not upregulated. In parallel, TH and dopamine transporter (DAT)-positive neurons were significantly

increased, whereas no specific effects on the amount of GABA- and serotonin-positive neurons were observed (Rolletschek et al., 2001). A significant upregulation of mRNA levels of Nurr1 and TH, but especially of the antiapoptotic gene bcl-2, suggested that the application of survival-promoting factors resulted in long-term increase and maintenance of functional neurons by preventing apoptosis (Rolletschek et al., 2001).

1.5.4 Monolayer differentiation

Undifferentiated ES cells were dissociated and plated onto gelatin-coated tissue culture dishes in N2B27 medium which contains insulin, apotransferrin, progesterone, putrescine, sodium selenite and bovine serum albumin fraction V (Ying et al., 2003). A feature of the adherent monolayer culture is that not all cells behave identically, as indicated by the expression of Sox-1, which is the earliest marker of the neuroectodermal differentiation (Wood and Episkopou, 1999) and expressed discretely throughout the cultures. A majority of cells differentiate into non-neural cell types and about 15% of the cells remain undifferentiated. The undifferentiated ES cells are fully competent for differentiation if trypsinized and replated. The differentiation results suggested that there is a random compotent, a community effect, or both involved in lineage commitment of ES cells.

In adherent monolayer culture, elimination of inductive signals for mesodermal and endodermal fates may be sufficient for ES cells to develop into neural precursors. The monolayer culture process is not a simple default pathway for neural differentiation, however, but requires autocrine FGF (Kunath et al., 2007). Upon induction, the neural precursors can be selected and purified by FACS or drugs.

In adherent monoculture, neither multicellular aggregation (Wiles and Johansson, 1999; Bain et al., 1995) nor coculture (Kawasaki et al., 2000) is necessary for ES cells to commit to a neural fate. The convenience in purification provides a platform for defining

the molecular machinery of neural commitment and optimizing the efficiency of neuronal and glial cell production from pluripotent mammalian stem cells. However, in view of poor survival of discrete human ES cells, this protocol is not applicable to neural commitment of human ES cells.

1.5.5 Gene manipulation for neural commitment

Two approaches have been reported: forced expression of lineage-specific genes and selective ablation of cells not expressing a lineage-restricted gene. Both techniques are effective to yield cells of interest in high purity, relying on expression of the particular promoter construct or transient selection during its expression window (Lakshmipathy et al., 2004).

In the forced differentiation approach, ES cells are transfected to constitutively express lineage-determinated genes. The proneural genes, NeuroD1, NeuroD2 and NeuroD3, act as transcriptional activators of neuronal differentiation genes (Lee, 1997), and gene deletion blocks neuronal differentiation (Ma et al., 1996). These bHLH neural determination genes are among the earliest neuronally restricted genes to be expressed in the neuroepithelium, beginning with NeuroD3, followed by NeuroD1 and NeuroD2. As reported, a number of cell lines can be converted to neurons by misexpressing the bHLH proteins (Kanda et al., 2004). Thus, forced differentiation following overexpression of neural lineage control genes is a complementary approach to obtain neural cell populations.

Other protocols for selective isolation of neuronal cells have been established on the basis of transgenic ES cell lines carrying tissue-specific promoters fused to selectable marker genes. Neural precursors were selectively generated from ES cells transfected with the tissue-specific transcription factor Sox2 (Li et al., 1998). Upon completion of the selection with G418, Sox2-negative nonneural cells were eliminated. Derived cells

expressed Sox2 and Sox1, consistent with the expression of Nestin at early stages. Sox2-selected precursor cells were noted to further differentiate into mature neurons, evidenced by GABA and glutamate neurotransmitters (Li et al., 1998). However, the hurdles for future application are the complicated procedure for filtration after induction, and the possible mutation in transfected cells (Morishita et al., 2007)

1.5.6 Stromal cell-derived inducing activity

Stromal cells, derived from the bone marrow, could be used to induce neural differentiation of ES cells by coculture (Kawasaki et al., 2000). This effect is identified as stromal cell-derived inducing activity (SDIA). In the bone marrow, the concept of the stem cell niche is based largely on studies of HS cells indicating that their survival, proliferation and differentiation rely upon interaction with bone marrow stromal cells (Kaushansky, 2006). Surprisingly, these stromal cells also have the ability to direct the differentiation of NS cells from both mouse and human ES cells (Kawasaki et al., 2000; Barberi et al., 2003).

Stromal cell lines with the highest efficiencies of neural induction are typically at the preadipocytic stage of differentiation and are isolated from the bone marrow (e.g., PA6, MS5, S17) or the aorta-gonad-mesonephros (AGM) region (Vazin et al., 2008; Kawasaki et al., 2002). Throughout this protocol, serum-free conditions are required, and neural differentiation is achieved by reducing endogenous BMP signals via plating cells at low density under minimal medium conditions or in the presence of the BMP antagonist noggin. However, the identity of the stromal cell-derived factors, which are permissive or instructive, has not yet been elucidated, but studies suggest that both soluble factors and interaction between ES cells and stromal cells may be required (Kawasaki et al., 2000). Advantages of this protocol include the relatively pure population of multipotent neural derivatives, and no need of embryoid body formation.

NS cells generated by using this approach have been used to produce many neural derivatives, including dopaminergic and GABAergic neurons (Kawasaki et al., 2000; Barberi et al., 2003).

1.6 Signaling pathways involved in neural differentiation

Embryonic development is characterized by the progressive restriction of differentiation potentials of stem cells in response to a series of intrinsic and extrinsic cues. In vertebrate embryos, the initial commitment of ectodermal cells to a neural fate during gastrulation is followed by their allocation to specific neural cell types in response to signaling factors or developmental cues. Understanding the molecular details of these differentiation processes remains a major focus of developmental biology and has a great impact on ES cell manipulation for therapeutic purposes.

1.6.1 Wnt signaling

The embryonic neural tube of the mouse is patterned along its rostrocaudal and dorsoventral axes (Echevarría et al., 2003). The development of the neuronal population is controlled by two signaling centers: the midbrain/hindbrain boundary (MHB) or isthmic organizer acting along the rostrocaudal axis and the ventral midline of the neural tube or floor plate acting along its dorsoventral axis (Prakash and Wurst, 2006). Wnt1 and the fibroblast growth factor (FGF) 8 are expressed in the caudal midbrain and rostral hindbrain at the MHB, respectively (Martinez, 2001). The floor plate secretes the lipid-modified glycoprotein Sonic hedgehog (Shh; Murdoch et al., 2001). The mammalian dopaminergic neurons arise at around 10.5 dpc of mouse embryonic development from the ventral midline floor plate of the cephalic flexure close to the MHB (Prakash and Wurst, 2006), suggesting that Wnt1 and FGF8 play an important role in the neural tube generation. Wnt1 expression is confined to a ring encircling the neural tube at the rostral

border of the MHB in the caudal midbrain, the roof plate of the mesencephalon and diencephalon, and two stripes adjacent to the FP of the midbrain (Prakash et al., 2006).

Despite Wnt's well-established role as a mitogen, its effect on ES cells appears to be on neural differentiation (Aubert et al., 2002; Otero et al., 2004). Neural differentiation of ES cells can either be inhibited or promoted by Wnt signaling (Nordin et al., 2008) Activation of the Wnt cascade by expression of Wnt1 or a dominant active form of β-catenin, or addition of lithium chloride, which inhibits GSK-3 and thereby β-catenin degradation, inhibits neural differentiation, whereas treatment with the Wnt antagonists SFRP2 and Dkk1, stimulates production of ES cell-derived neural progenitors (Aubert et al., 2002; Haegele et al., 2003; Watanabe et al., 2005). In contrast, the overexpression of Wnt3A or the expression of a dominant-negative form of E-cadherin was noted to increase intracellular levels of β-catenin, and promote neural differentiation under high density conditions that ordinarily block this lineage commitment (Otero et al., 2004). Wnt signals may activate several different downstream cascades with some independent of β-catenin. Some cascades may promote, while others inhibit neural differentiation of ES cells (Montcouquiol et al., 2006). The effect of Wnt signaling perturbation on neural differentiation of ES cells depends on the specific conditions used to induce neural differentiation, and the precise differentiation stage at which the intervention takes place (Nordin et al., 2008).

1.6.2 Sonic hedgehog signaling

During embryogenesis, each cell is specifically situated within a morphogenic field, where it attains a particular developmental fate. This cell fate is determined primarily by the various extracellular signals received by these cells. One example of such an extracellular morphogenic signal is the Sonic hedgehog (Shh), which has been shown to specify numerous cell fates in a concentration-dependent manner (Ingham and McMahon,

2001).

Shh is secreted from the floor plate and basal plate of the ventral midbrain (Ye et al., 1998). It was shown that ectopic expression of Shh and its downstream effector molecule Gli1 in the dorsal neural tube is able to induce ectopic dopaminergic and serotonergic neurons in the dorsal midbrain and hindbrain region, in areas where Wnt1 and FGF8 are expressed normally (Hynes et al., 1997). More recent data have revealed that dopaminergic neurons are not generated in Shh$^{-/-}$ mutants and are considerably reduced by conditionally inactivating the molecule Smoothened, which is the essential positive mediator of Shh, in the mouse embryo at 9.0 dpc (Blaess et al., 2006). It was suggestive that Shh is necessary for induction of the dopaminergic neuron fate in ventral midbrain progenitors but appears to be dispensable for their later differentiation into dopaminergic neurons in vivo (Blaess et al., 2006). In addition to its well-established role in conferring ventral identity to neural tube derivatives, the Shh signal has been implicated in differentiation, survival, and proliferation of many cell types in the embryonic nervous system (Ingham and McMahon, 2001).

Studies also indicate that Shh promotes both the survival and proliferation of NS cells and neuroblasts during adult neurogenesis (Ahn and Joyner, 2005; Palma et al., 2005). A previous study, using ES cells carrying mutations in Shh signaling components, suggested that this signal is critical for the differentiation of primitive ectoderm to neuroectoderm (Maye et al., 2004). Recently, two additional roles for Shh signaling in ES cell neurogenesis have been demonstrated: promoting proliferation of Sox1-negative, FGF5-positive primitive ectoderm precursors, and supporting survival of the Sox1-positive NS cells (Palma et al., 2005). The source of the Shh signal appears to be the differentiating NS cells themselves and this finding is consistent with the observation that NS cells isolated from the neonatal mouse subventricular zone (SVZ) secrete Shh (Rafuse

et al., 2005). These Shh-secreting NS cells were also found to support the survival of dopaminergic neurons when co-cultured with ventral midbrain tissue (Rafuse et al., 2005) and when transplanted into a mouse model of Parkinson's disease (Ostenfeld et al., 1999). Thus, Shh signaling plays an important role in both embryonic development and tissue regeneration within the adult.

1.6.3 FGF signaling

FGF2 is a well-established mitogen for CNS-derived embryonic and adult NS cells. Along with epidermal growth factor (EGF), FGF2 addition promotes the production and passage of CNS-derived neurospheres (Reynolds and Weiss, 1996). FGF2 also plays a role in supporting proliferation of ES cell-derived NS cells (Okabe et al., 1996; Conti et al., 2005) and promotes proliferation of undifferentiated human ES cells (Xu et al., 2005).

A role for FGF4 in the production of ES cell-derived NS cells has also been demonstrated, although the specific mechanism involved is still obscure. Studies suggest that FGF4 produced by undifferentiated mouse ES cells acts in an autocrine manner to promote neural specification (Kunath et al., 2007). It was observed that FGF receptor 1-deficient ES cells produce significantly fewer neural colonies. The readouts were also observed with addition of a function-blocking FGF antibody to wild type cultures (Tropepe et al., 2001). Treatment with an inhibitor of FGF receptor kinases, SU5402, or expression of a dominant-negative FGF receptor inhibits production of nestin-positive NS cells without affecting their growth or survival, whereas addition of FGF4 increases the frequency of NS cells present in the cultures (Ying and Smith, 2003).

In the mouse embryo, FGF5 is transiently expressed by primitive ectoderm before gastrulation, and confers competence to respond to subsequent inductive signals (Hebert et al., 1991). It was shown that FGF5 is also transiently expressed upon primitive ectoderm differentiation of mouse ES cells (Rathjen et al., 1999). The observation that ES

cells gain the competence to respond to a Hedgehog antagonist only after they begin to express FGF5, suggests this growth factor may mediate the transition to primitive ectoderm, which can then respond to additional inductive signals mediating neuroectoderm differentiation (Maye et al., 2004).

1.6.4 Notch signaling

Studies in Drosophila established a role for Notch signaling in neurogenesis, but as an inhibitor of neural specification in favor of epidermal differentiation (Artavanis-Tsakonas et al., 1999). In the vertebrate CNS, Notch maintains NS cells in an undifferentiated and proliferative state through the action of *Hes* genes (Ross et al., 2003). In a neural stem cell, Notch is activated by the ligand Delta. Upon activation, Notch intracellular domain (NICD) is cleaved and transferred into the nucleus. Subsequently, NICD forms a complex with RBP-Jk in the nucleus. This complex induces *Hes1* and *Hes5* expression which could inhibit the activator-type bHLH factors by sequestering E47. In addition, *Hes1* and *Hes5* repress transcription of the activator-type bHLH genes by binding to their promoters (Ishibashi et al., 1994). However, in a differentiating neuron, Notch is not activated and RBP-Jk represses Hes1 and Hes5 expression. Thus, the activator-type bHLH genes are expressed. The activator-type bHLH factors induce expression of *Hes6*, which inhibits *Hes1* functions and reinforces the neurogenic process. The activator-type bHLH factors also induce neuronal specific genes and one of them includes the Notch ligand Delta, which activates Notch signaling in neighboring cells (Kageyama et al., 2008).

Notch- or Hes-deficient mice show premature neural differentiation (Yoon and Gaiano, 2005). It was suggested that Notch may play a role in neural differentiation, since it promotes production of prosensory patches in the chick inner ear (Daudet and Lewis, 2005). Recent work showed that Notch promotes neural lineage specification of

ES cells (Lowell et al., 2006). Expression of the constitutively active Notch intracellular domain (NICD) in ES cells promotes NS cell differentiation, and the transition from primitive ectoderm to neuro-ectoderm. Treatment of ES cells with the γ-secretase inhibitor, which acts by blocking the cleavage of Notch to its active intracellular domain, inhibits NS cell differentiation, regardless of the induction protocols used (Dang and Tropepe, 2006; Lowell et al., 2006). Notch signaling may help homogenize the expression of Sox1 among cells by cell-cell interaction and inhibit differentiation of other cell types (Lowell et al., 2006).

In vivo, Notch signaling plays an important role in the developing vertebrate nervous system. Generally, activation of the Notch response favors the differentiation toward glial cell types, while cells without an activated Notch response differentiate toward neuronal fates (Louvi and Artavanis-Tsakonas, 2006). In addition, in the adult forebrain, neural stem cells are present as a relatively quiescent subpopulation in the subependyma, a remnant of the embryonic germinal zone (Morshead et al., 1994). The generation and the size of the neural stem cell population are tightly regulated during embryogenesis as well as in the adult (Hitoshi et al., 2002). Recent evidence suggests that Notch signaling plays a role in preserving the neural stem cell population. Mice deficient for *Hes1*, one of the downstream effectors of Notch signaling, display a decrease in the number of embryonic neural stem cells (Nakamura et al., 2000). Thus, Notch signaling is essential in neural stem cells both to expand their population size in the developing brain and to maintain the size of the neural stem cell pool in the adult brain.

In the developing embryo, neighbouring cells must continually communicate with each other to coordinate patterning of tissues. Notch signaling elicits lateral induction and lateral inhibition. Lateral induction acts to ensure that cells within a particular region adopt the same fate (Morimoto et al., 2005). On the other hand, lateral inhibition secures

that neighbouring cells commit to different fates, so that one single cell type does not dominate within a particular region (Heitzler and Simpson, 1991). Thus, mediated by cell surface bound ligands *Delta* and *Jagged*, Notch signaling is involved during development to control cellular fate changes organically.

1.6.5 Serotonin signaling

Serotonin (5-hydroxytryptamine, 5-HT) is one of the well known monoamine neurotransmitters, mitogens, and hormones, which mediate a wide variety of physiological effects, including peripheral and central actions, as it is present in diverse body parts such as blood vessels, gastrointestinal tracts, and the central nervous system. The diverse functions of 5-HT are attributable to the complexity of 5-HT receptors which can be classified into different families depending on their signaling pathways. They include the 5-HT_1 and 5-HT_5 receptors interacting negatively with adenylyl cyclase, the 5-HT_2 receptor coupling with the activation of phospholipase C, the 5-HT_4, 5-HT_6 and 5-HT_7 receptors activating adenylyl cyclase, and the 5-HT_3 receptor being a ligand-gated ion channel. 5-HT receptors share similarities in their amino acid sequence, pharmacological properties and signaling mechanisms. These receptors except the 5-HT_3 receptor are G protein-coupled receptors exhibiting typical rhodopsin-like seven transmembrane domains. Some receptor subtypes exhibit multiple potential couplings to different G proteins, depending on cell types and context, and thus have dual signaling properties involving both cyclic adenosine monophosphate and calcium mobilizations, as shown by 5-HT_7 and 5-HT_1 receptors (Raymond et al., 2001).

Data indicate that 5-HT possess developmental functions (Lauder, 1988; Lauder, 1993). The 5-HT receptors are found to be necessary for gamete interactions during fertilization (Falugi, 1993), and 5-HT receptor antagonists can inhibit early embryonal polychaete development (Emanuelsson, 1992). It has been shown that 5-HT is present

early in mammalian embryonic development and is maternally derived (Yavarone et al., 1993a). In mice, the ability to take up 5-HT is detected in the heart myocardium and the rhombencephalic neuroepithelium at early embryonic stages of 8.5 dpc (Shuey et al., 1993). The physiological relevance of 5-HT was attested in studies showing that embryos grown in the presence of high levels of 5-HT uptake blockers develop deficient head mesenchyme, hypoplastic mandibular arches and forebrain, open cranial neural folds and abnormal eyes. Similarly, these malformations also happened in whole embryo cultures following exposure of mouse embryos to 13-*cis*-retinoic acid, which has the ability to enter the nucleus to interact with nuclear retinoid receptors and exerts teratogenic effects (Rühl et al., 2001; Lauder et al., 1988). 5-HT has also been shown to participate in rat craniofacial development (Byrd and Sheskin, 2001), the formation and migration of the cranial neural crest cells (Moiseiwitsch and Lauder, 1995) and the cardiovascular morphogenesis in the chicken and mouse (Kameda, 1990; Yavarone et al., 1993b).

That mammalian oocytes might respond to 5-HT was first suggested by the induced hyperpolarization pulses, associated with calcium surges, when 5-HT was added to isolated metaphase II hamster oocytes (Miyazaki et al., 1990). The fact that 5-HT receptors exist was subsequently confirmed by the expression of 5-HT_{2A} receptor mRNA in hamster oocytes (Amireault and Dubé, 2005). The main subtypes of 5-HT receptors in mouse oocytes are 5-HT_{1D} and 5-HT_7 receptors (Veselá et al., 2003; Amireault and Dubé, 2005). Roughly concomitant with activation of zygotic transcription, the 5-HT_7 mRNA disappears at about the four-cell stage (Amireault and Dubé, 2005), whereas 5-HT_{1D} mRNA is apparently persistent, even up to the blastocyst stage (Veselá et al., 2003). These observations suggest distinct functions and temporal display of these two receptors. The 5-HT_7 receptor acts at earlier stages of oocyte maturation, when local 5-HT may be available from neighbouring granulosa cells, whereas 5-HT_{1D} receptor might come into

play at later stages of embryo preimplantation. Other studies reported that exogenous 5-HT or the agonist sumatriptan may result in unstable changes in cell numbers within the blastocyst (Il'ková et al., 2004; Veselá et al., 2003). Such roles of 5-HT are in agreement with the recent finding that maternal 5-HT is required for normal mouse embryonic development (Côté et al., 2007). These data suggested that preimplantation embryos might remain sensitive to surrounding 5-HT which may have additional functions not yet identified in vivo.

In peripheral tissues, in addition to the effects of 5-HT on vascular tone, cardiac and intestinal functions, 5-HT was found to regulate proliferation of fibroblasts (Van Obberghen-Schilling et al., 1991) and lymphocytes (Abdouh et al., 2001; Abdouh et al., 2004). Recent investigations have reported that 5-HT could also regulate osteoblast differentiation (Gustafsson et al., 2006), liver regeneration (Lesurtel et al., 2006) and mammary gland development (Matsuda et al., 2004), by activation of new signaling pathways, including serotonylation (Walther et al., 2003).

There was increasing evidence that 5-HT acts as a mitogen or neurohormone on a variety of normal and malignant cells (Fanburg and Lee, 1997; Siddiqui et al., 2005; Xu et al., 2006). The mechanisms of 5-HT-induced mitogenesis appear to involve receptor-specific crosstalks between major signaling pathways. In 5-HT_{2B}-transfected mouse fibroblasts, 5-HT induces cell cycle progression through cyclin D1 and Src activation (Nebigil et al., 2000). The signals are mediated by extracellular signal-regulated kinase (ERK) induction, in concert with receptor tyrosine kinase platelet-derived growth factor receptors. It was reported that phosphatidylinositol-3-kinase (PI3K)/Akt pathway is involved in cell mitogenesis induced by 5-HT in smooth muscle (Liu and Fanburg, 2006).

The hurdles in studying small molecule inhibitors and activators in developmental biology are inefficient delivery of the substances to the embryo at relevant concentrations,

and the constraints in obtaining and culturing embryonic tissues in vitro. Currently these issues are alleviated by adopting ES cell cultures. Thus, the roles of 5-HT signaling in proliferation and differentiation of ES cells require further investigations.

Table 1-1 Comparison of cell products derived from embryonic stem cells and adult stem cells

		Somatic/Adult Stem Cells		
	ESC	**NSC**	**HSC**	**MSC**
Origin	Inner Cell Mass (ICM)	Aborted fetus	Bone Marrow & Umbilical cord blood	Bone Marrow
Potency	Pluripotent	Multipotent	Multipotent	Multipotent
Availability	Infinite	Limited	Plentiful	Limited
Feeder support*	±	–	–	–
Product purity	Heterozygous	Homozygous	Heterozygous	Heterozygous
Product yield	Moderate	High	Low	Low
Graft-associated malignancy	Teratoma	Not reported	No teratoma	No teratoma

ESC: embryonic stem cells; NSC: neural stem cells; HSC: haematopoietic stem cells; MSC: mesenchymal stem cells
* "±" represents "Needed" and "–" represents "No need".

Table 1-2 Comparison of characteristics of mouse and human embryonic stem cells

	Embryonic stem cells	
	mouse	**human**
SSEA-1	+	–
SSEA-3	–	+
SSEA-4	–	+
TRA-1-60	–	+
TRA-1-81	–	+
Oct-4	+	+
STAT3	+	+
Nanog	+	+
Alkaline phosphatase	+	+
Telomerase activity	+	+
TGF-β signaling	+	+
Eras and EHox	+	–
Fbx15	+	–
Key factor for self-renewal	LIF	bFGF
Growth status (undifferentiated)	tight, rounded multi-layer clumps; can form EBs	loose, flat aggregates; can form EBs
BMP's effect	undifferentiated	promoting differentiation
Trophectoderm	no spontaneous diff.	spontaneous diff.
Cloning efficiency	good	very low

Cited from "Rao M. 2004"

SSEA: stage-specific embryonic antigen; TRA: tumor rejection antigen; EB: embryoid body
LIF: leukemia inhibitory factor; bFGF: basic fibroblast growth factor; BMP: bone morphogenetic protein
TGF: transforming growth factor; Oct-4: Octamer binding transcription factor-4,
STAT: the signal transducer and activator of transcription

CHAPTER 2
AIMS OF THE STUDY

At current stage, the challenges in studying ES cells for regenerative medicine focus on directing the wide differentiation potential towards the derivation of a specific cell fate. Several in vitro systems allowing derivation of neural progeny from ES cells have been described (Kawasaki et al., 2000; Lee et al., 2000; Tropepe et al., 2001; Ying et al., 2003). However, these differentiation protocols are hampered by the limitations of extended in vitro culture (Lee et al., 2000; Okabe et al., 1996; Brüstle et al., 1999), cell line- and strain-dependent variability of the differentiation results (Lee et al., 2000; Wakayama et al., 2001), low efficiency of neural induction (Tropepe et al., 2001) and the generation of a limited number of neural subtypes (Ying et al., 2003). The establishment of a simple and efficient method to derive embryonic stem cells into neural lineage cells is requisite for both developmental study and clinical need.

After brain ischemia, many types of cells are damaged, including neurons, astrocytes and oligodendrocytes. A majority of surviving patients suffer a significant impairment of neural functions. Besides, the prevalence of stroke-related morbidity is expected to increase as the population ages. However, conventional therapies hardly help reverse these defects. Cell replacement therapy, by using ES cell derivatives (Erdö et al., 2003), neural stem cells (Watson et al., 2003), and bone marrow stromal cells (Chen et al., 2007), may represent a novel treatment for stroke damage (Björklund and Lindvall, 2000; Kondziolka et al., 2002). Directing neural differentiation of human ES cells in vitro has been of particular interest in view of the success of mouse ES cells in neural commitment and preclinical therapy in various diseases. Based on these, aims of this study include:

(1) The establishment of a simple and efficient method to differentiate mouse and

human ES cells into neural lineage cells, and *in-vivo* functional assessment of the derived cells in an mouse stroke model in terms of cognitive recovery, migration and homing of implanted cells and teratoma development.

(2) To elucidate whether the small molecule, serotonin, could enhance neural differentiation of mouse ES cells.

(3) To investigate the role of Notch signaling in the proliferation and differentiation of mouse and human ES cells.

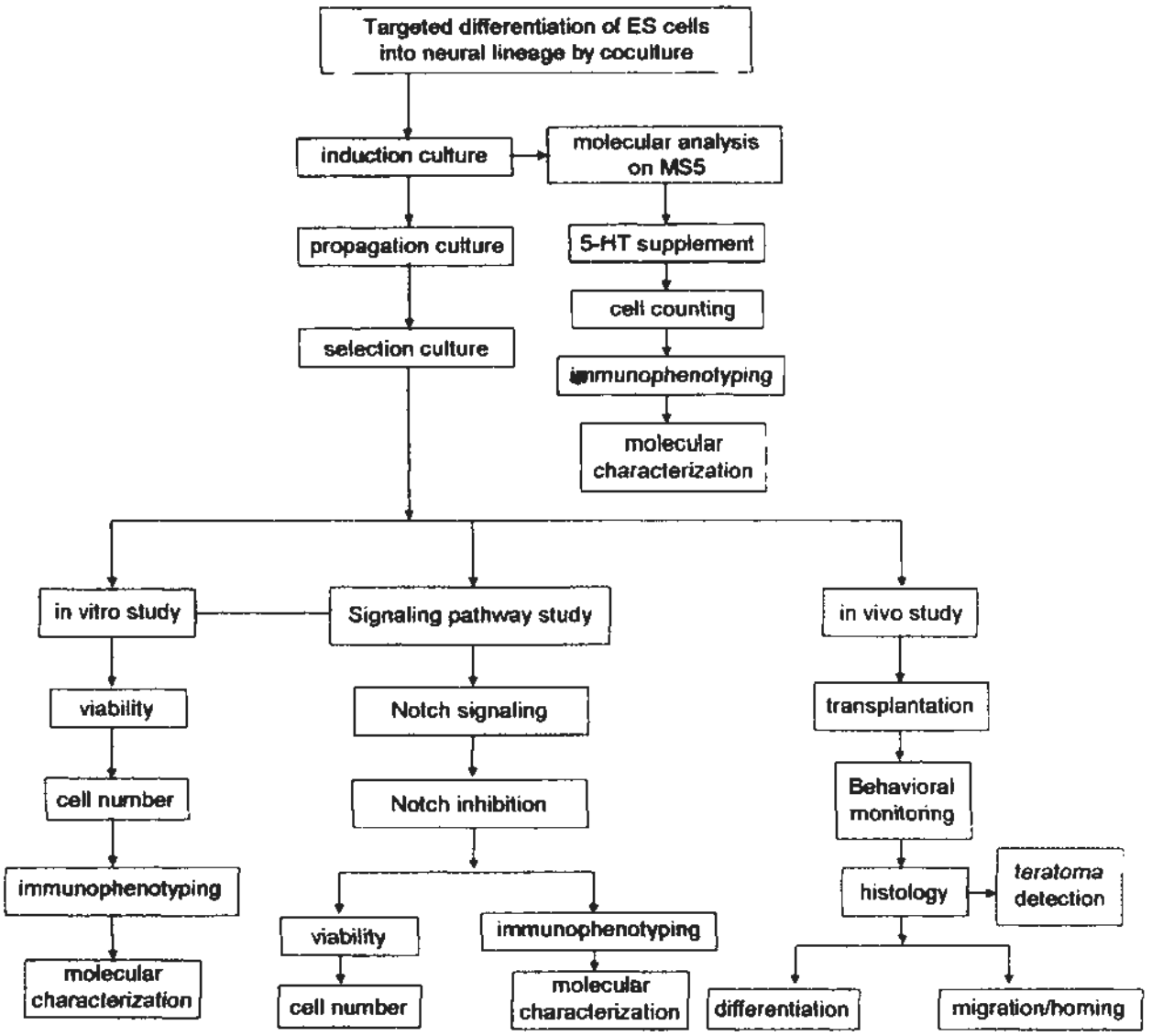

Figure 2-1 The schema of stem cell research in this study

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Cell lines

- Primary mouse embryonic fibroblasts: Cells are isolated from embryos of C57BL/6 pregnant mice 13.5 to 14.5 dpc (The appearance of vaginal plugs was designated as day 0 of gestation).
- Mouse ES cell line D3 (CRL-1934, American Type Culture Collection [ATCC], Manassas, VA, USA): D3 cells are isolated from the blastocyst of the 129/Sv+c/+p strain mouse.
- Mouse ES cell line E14TG2a (CRL-1821, ATCC): E14TG2a cells are isolated from the blastocyst of the 129/Ola strain mouse. E14TG2a cells are deficient in hypoxanthine guanine phosphoribosyl transferase and could be resistant to 0.06 mM 6-thioguanine.
- Mouse neural precursor cell line C17.2: C17.2 is generated by retrovirus-mediated v-myc transfer into progenitors derived from neonatal mouse cerebellum. The cell line C17.2 is a kind gift from the late Dr. David Walsh, Department of Anatomy, University of New South Wales, Australia.
- Murine stromal cell line MS5: MS5, provided by COPE, is established after irradiation of the adherent cells in long-term murine bone marrow culture.
- Human ES cell line H9 (WA9, Wicell Research Institute, Madison, WI, USA): H9 cells, provided by Wicell Institute, are derived from aborted human embryos.
- Human ES cell line H14 (WA14, Wicell Research Institute, Madison, WI, USA): H14 cells, provided by Wicell Institute, are derived from aborted human embryos.
- Human neuroblastoma cell line SK-N-AS (CRL-2137, ATCC, Manassas, VA, USA): derived from human brain neuroblastoma.
- Human neuroblastoma cell line SH-SY5Y (CRL-2266, ATCC, Manassas, VA, USA): derived from human brain neuroblastoma.
- Human neuroblastoma cell line SK-N-MC (HTB-10, ATCC, Manassas, VA, USA): derived from human brain neuroepithelioma.

3.1.2 Experimental animals

Adult male ICR mice of a mean age of 12 weeks, weighing 25 ~ 30 g, were used in this study. Mice were nurtured in micro-isolator cages. They were cared for and handled according to Guidelines of Laboratory Animal Services Centre in The Chinese University of Hong Kong.

3.1.3 Reagents for cell culture

- B27 supplement (50×, Cat. 17504-044, Invitrogen Corporation, Carlsbad, CA, USA)
- Basic Fibroblast Growth Factor (bFGF; Cat. 13256-029, Invitrogen Corporation, Carlsbad, CA, USA)
- Collagenase (Cat. C9722, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Dimethyl sulfoxide (DMSO; Cat. WAK-DMSO-10, Cryosure, Amphi Ltd., Turkey)
- Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12, Cat. 11330-032, Invitrogen Corporation, Carlsbad, CA, USA)
- Dulbecco's phosphate-buffered saline (DPBS; Cat. 14190-136, Invitrogen Corporation, Carlsbad, CA, USA)
- Fibronectin (0.1%, Cat. F1141, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Fetal calf serum (FCS; Cat. 26140-079, Invitrogen Corporation, Carlsbad, CA, USA)
- Fetal calf serum (FCS; ES Cell-Qualified, Cat. 10439-024, Invitrogen, Carlsbad, CA, USA)
- Gamma-Secretase Inhibitor (1Mm, Cat. 565771, Calbiochem, La Jolla, CA, USA)
- Gelatin powder (Cat. 101186M, BDH, Merck & Co., Inc, USA)
- Insulin-Transferrin-sodium selenite medium supplement (Cat. 11884, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- Ketamine (10%, Alfasan, FarmaVet SA, Romania)
- Knockout Dulbecco's Modified Eagle's Medium (KO-DMEM; Cat. 10829-018, Invitrogen Corporation, Carlsbad, CA, USA)
- Knockout serum replacer (SR; Cat. 10828-028, Invitrogen Corporation, Carlsbad, CA, USA)
- Laminin (Cat. L2020, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- Leukemia inhibitory factor (LIF; Cat. ESG1106, Millipore, Billerica, MA, USA)

- L-Glutamine (200Mm, 100×, Cat. 14190-136, Invitrogen Corporation, Carlsbad, CA, USA)
- MEM Non-Essential Amino Acid (10Mm, 100×, Cat. 11140-050, Invitrogen Corporation, Carlsbad, CA, USA)
- Mercaptoethanol beta (Cat. M7522, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- Mitomycin C (Cat. M4287, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- N2 supplement (100×, Cat. 17502-048, Invitrogen Corporation, Carlsbad, CA, USA)
- Neurobasal medium (Cat. 21103-049, Invitrogen Corporation, Carlsbad, CA, USA)
- Penicillin/Streptomycin (Cat. 15140-122, Invitrogen Corporation, Carlsbad, CA, USA)
- Poly-L-ornithine (0.01%, Cat. P4957, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- Serotonin (Cat. H7752, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- Trypan blue dye solution (Cat. 07050, Stem Cell Technologies, USA)
- Trypsin-ethylenediamine tetraacetic acid (EDTA) (0.05%, Cat. 25300-054, Invitrogen Corporation, Carlsbad, CA, USA)
- Xylazine (2%, Alfasan, FarmaVet SA, Romania)

3.1.4 Reagents for immunofluorescence study and histology

- 4',6'-diamidino-2-phenylindole hydrochloride (DAPI, Cat. H1200, Vector Laboratory Inc., CA, USA)
- Aminopropyl triethoxy-silane (APES; Cat. A3648, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- Bovine serum albumin (BSA; Cat. A4919, Sigma-Aldrich Corporation, Saint Louis, MO, USA)
- BrdU (5-bromodeoxyuridine; 1000×, Cat. 1647229, Roche Applied Science, Basel, Switzerland)
- Eosin (Cat. 34197, BDH, Merck & Co. Inc., USA)
- Fluorescein isothiocyanate (FITC)-conjugated anti-BrdU (Cat. 1202693, Roche Applied Science, Basel, Switzerland)
- FITC-conjugated Goat anti Mouse IgG (H+L) (Cat. 816511, Zymed Laboratory Inc., London, UK)

- FITC-conjugated Goat anti Mouse IgM (Cat. 626811, Zymed Laboratory Inc., London, UK)
- FITC-conjugated Goat anti Rabbit IgG (H+L) (Cat. 816111, Zymed Laboratory Inc., London, UK)
- Haematoxylin Hydrate (Cat. 34037, BDH, Merck & Co. Inc., USA)
- Monoclonal mouse anti-beta Tubulin class III (Cat. 11-264-C100, Exbio antibodies, Vestec, Czech Republic)
- Monoclonal mouse anti-glial fibrillary acidic protein (GFAP; Cat. 180021, Zymed Laboratory Inc., London, UK)
- Monoclonal mouse anti-microtubule-associated protein-2 (MAP-2; Cat. 131500, Zymed Laboratory Inc., London, UK)
- Monoclonal mouse anti-neuroepithelial stem cell intermediate filament (Nestin; Cat. 611659, Pharmingen, BD Bioscience, California, USA)
- Monoclonal mouse anti-stage specific embryonic antigen-1 (SSEA-1; Cat. sc-21702, Santa Cruz, California, USA)
- Monoclonal mouse anti-stage specific embryonic antigen-4 (SSEA-4; Cat. sc-21704, Santa Cruz, California, USA)
- Paraformaldehyde (Cat. P6148, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Polyclonal mouse anti-myelin basic protein (MBP; Cat. 559904, Pharmingen, BD Bioscience, California, USA)
- Polyclonal rabbit anti-Musashi1 (Cat. AB5977, Chemicon International Inc., Boronia Victoria, Australia)
- Propidium Iodide (Cat. P3556, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Triton X-100 (Cat. T8787, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)
- Xylene (Cat. 8681, Merck & Co. Inc., USA)

3.1.5 Reagents for molecular biology analyses

- DNA ladder (Cat. SM0243, MBI, Fermentas Life Sciences, Burlington, Canada)
- Deoxynucleoside Triphosphate (Cat. R0192, MBI, Fermentas Life Sciences, Burlington, Canada)
- Eithidium bromide (Cat. 2515, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)

- Magnesium Chloride (50Mm, Cat. Y02016, Invitrogen Corporation, Carlsbad, CA, USA)
- PCR buffer-$MgCl_2$ (10×, Cat. Y02028, Invitrogen Corporation, Carlsbad, CA, USA)
- Platinum Taq DNA polymerase (Cat. 10966-034, Invitrogen Corporation, Carlsbad, CA, USA)
- Reverse Transcriptase (Cat. 600085-51, Stratagene Corporation, California, USA)
- Rnase inhibitor (Cat. N211B, Promega Corporation, Madison, USA)
- Rneasy Extraction Mini Kit (Cat. 74106, Qiagen, Hilden, Germany)
- Stratascript (10×, Cat. 600085-52, Stratagene Corporation, California, USA)
- Superscript II Rnase H (Cat. 18064-071, Invitrogen Corporation, Carlsbad, CA, USA)
- Tris-acetic acid-EDTA buffer solution (TAE, 50×, Cat. 24710-030, Invitrogen Corporation, Carlsbad, CA, USA)
- Tween-20 (Cat. P1379, Sigma-Aldrich Corporation, Saint Louis, Missouri, USA)

3.1.6 Equipments

- Analytic Plus Balance (AP250D, OHAUS, New Jersey, USA)
- Biological safety cabinet (1185, Thermo Forma, Forma Scientific Inc., New York, USA)
- Centrifuge (CR412, Jouan, Thermo Electron's corporation, New York, USA)
- Coulter haematology analyzer (AcT Diff, Coulter Corporation, Miami, USA)
- Cytocentrifuge (Autosmear CF-120, Sakura Seiki Co., Tokyo, Japan)
- Electrophoresis documentation and analysis system (Eastman Kodak company, Rochester, New York, USA)
- Eppendorf centrifuge (5417c, Eppendorf International, Hamburg, Germany)
- Gamma-irradiator (Gammacell 1000 Elite 214, MDS Nordion, Canada)
- Harvard Trip Balance (1450-SD, OHAUS, New Jersey, USA)
- Instant camera (Polaroid DS34, St. Albans, Hertfordshire, England)
- Inverted microscope (IX71, Olympus, Tokyo, Japan)
- Laboratory gasburner (Gasprofi 1SCS 6.103.000, WLD-TEC, Techcomp Ltd., USA)
- Stereotaxic adaptor for mouse brain (Model: 921-E, KOPF, California, USA)
- Mouse Ear Bars (Model: 921-F&G, KOPF, California, USA)
- Improved Neubauer hemocytometer (Cat. 63510-11, EMS corporation, Hatfield, PA, USA)

- Object marker adaptor (Cat. 79031, Nikon, Japan)
- Programmable Thermal Controller (PTC-100, Hoefer Scientific Instruments, San Francisco, USA)
- Rotary Microtome (RM 2035, Leica, Germany)
- Spectrophotometer (GeneQuant II, Pharmacia LKB Biochrom Ltd., Cambridge, England)
- Stereotaxic Frame (KOPF 900, California, USA)
- Vortex Mixer (M37615, Thermolyne, Barnstead International, USA)
- Volvere-GX, handpieces (GX-35EM, Nakanishi Inc., Japan)
- Water-jacketed CO_2 Incubator with HEPA filter (Series II 3111, Thermo Forma, Thermo Electron's Corporation, New York, USA)
- Watermaze System (SH-1, Xi Bo Biotechnology Ltd., Shanghai, China)

3.1.7 Consumables

- Cell culture flask, 25cm^2 with filter, sterile (Cat. 690275, Greiner Bio-one, Frickenhausen, German)
- Cell culture flask, 75cm^2 with filter, sterile (Cat. 658175, Greiner Bio-one, Frickenhausen, German)
- Cell Scraper (Cat. 179707, Nunc, Denmark)
- Cell Strainer (40µm, Cat. 352340, BD Biosciences, California, USA)
- Centrifuge tube (50ml, Cat. 210261, Greiner Bio-one, Frickenhausen, Germany)
- Coverslip 12mm circular (Cat. CM120RA1, Gerhard Menzel, Germany)
- Cryotube polypropylene (Cat. 377267, Nunc, Denmark)
- Culture plate, 12-well with lids, sterile (Cat. 140675, Multidishes Nunclon, Nunc, Denmark)
- Culture plate, 6-well with lids, sterile (Cat. 140674, Multidishes Nunclon, Nunc, Denmark)
- Filter Syringe (0.22µm, Cat. SLGP033RS, Millipore Corporation, Billerica, USA)
- Glass slide (Cat. 7101, Sail Brand, China)
- Parafilm (Cat. PM996, Parafilm, South East Chem. Ltd., HK)
- Pipette 10ml (Cat. PN10E1, Orange, AM Plus Company, HK)
- Pipette 2ml (Cat. PN2E1, Orange, AM Plus Company, HK)
- Pipette 5ml (Cat. PN5E1, Orange, AM Plus Company, HK)

- Serological glass pipets (1ml, Cat. 13-678-27B, Fisher Scientific)
- Serological glass pipets (5ml, Cat. 13-678-27E, Fisher Scientific)
- Serological glass pipets (10ml, Cat. 13-678-27F, Fisher Scientific)
- Syringe 25 gauge (1ml, Cat. BND305125, Becton Dickinson, BD Bioscience, New Jersey, USA)
- Thincert cell culture insert for 12-well plate (pore size 0.4μm, Cat. 16670, Greiner Bio-one, Frickenhausen, Germany)
- Tissue culture dish (diameter 10cm, Cat. 430167, Corning Inc., New York, USA)
- Universal bottle (30ml; Cat. BS128A, Sterilin, Barloworld Scientific Ltd., Staffordshire, UK)

3.1.8 Solution preparation

3.1.8.1 Gelatin solution (0.1%)

Gelatin powder	0.5g
Sterile double-deionized water	500ml

The solution was autoclaved and stored (not more than 1 month) at –80 °C until further use.

3.1.8.2 Enriched KO-DMEM

KO-DMEM	500ml
L-Glutamine (200mM, 100×)	5ml
Penicillin/Streptomycin	5ml

Aliquotes were kept (not more than 1 months) at –80 °C until further use.

3.1.8.3 Working solution of Insulin-Transferrin-Selenite (ITS) supplement

ITS supplement powder	5g
Glacial acetic acid	0.2ml
Sterile double-deionized water	5ml

The solution was filter-sterilized and added into 50ml sterile double-deionized water Aliquotes were kept (not more than 3 months) at –80 °C until further use.

3.1.8.4 β-Mercaptoethanol solution

β-Mercaptoethanol solution (1M)

β-Mercaptoethanol (14.33M)	1ml
Sterile double-deionized water	13.3ml

Aliquotes were kept (not more than 1 months) at –80 °C until further use.

β-Mercaptoethanol solution (0.1M)

1M β-Mercaptoethanol solution	1ml
Sterile double-deionized water	9ml

Aliquotes were kept (not more than 1 months) at –80 °C until further use.

Working solution of β-Mercaptoethanol (0.01M)

0.1M β-Mercaptoethanol solution	1ml
Enriched KO-DMEM	9ml

Aliquotes were kept (not more than 1 months) at –80 °C until further use. Culture medium was freshly supplemented with β-Mercaptoethanol just prior to use.

3.1.8.5 Poly-L-ornithine solution

Poly-L-ornithine solution 0.01%	4ml
Sterile double-deionized water	22.6ml

The solution was freshly prepared prior to use.

3.1.8.6 Laminin solution

Laminin	25µl
DPBS	25ml

The solution was freshly prepared prior to use.

3.1.8.7 Propidium iodide solution

Phenylenediamine	100mg
Glycerol	90ml
DPBS	10ml

Popidium Iodide	10µl

The pH of the solution was adjusted to 8.0 with hydrocarbonate acid (pH 9.0). Aliquotes were kept (not more than 1 year) at –20 °C in the dark environment until further use.

3.1.8.8 Paraformaldehyde solution 4%

Paraformaldehyde	4g
DPBS	100ml

The solution was heated at 60 °C until complete dissolution, and kept (not more than 1 month) at 4 °C in the dark environment.

3.1.8.9 Triton X-100 solutions

Triton X-100 solution 3%

Triton X-100 (100%)	300µl
DPBS	9.7ml

Triton X-100 solution 0.3%

3% Triton X-100	1ml
DPBS	9ml

The solution was kept (not more than 1 month) in dark at room temperature until further use.

3.1.8.10 Maintenance medium for mouse embryonic fibroblasts and MS5 cells

α-MEM	435ml
Fetal Calf Serum (FCS)	50ml
L-Glutamine (200mM, 100×)	5ml
Penicillin/Streptomycin	5ml
Non-essential amino acid (10mM, 100×)	5ml

Aliquots were kept (not more than 1 month) at –80 °C until further use.

3.1.8.11 Maintenance medium for mouse ES cells

KO-DMEM	399.5ml
ES Cell-Qualified FCS	75ml
L-Glutamine (200mM, 100×)	5ml
Penicillin/Streptomycin	5ml
Leukemia inhibitory factor (LIF, 1000U/μl)	0.5ml
Non-essential amino acid (10mM, 100×)	5ml
β-mercaptoethanol (0.1M)	10ml

Aliquots were kept (not more than 1 month) at −80 °C until further use. LIF was supplemented into the medium prior to use.

3.1.8.12 Cryopreservation medium for mouse cells

KO-DMEM	4ml
Fetal calf serum (FCS)	4ml
DMSO	2ml

The freezing medium was freshly prepared on ice prior to use.

3.1.8.13 Medium used for induction culture of mouse ES cells

KO-DMEM	410ml
Knockout Serum Replacer (SR)	75ml
Penicillin/Streptomycin	5ml
L-Glutamine (200mM, 100×)	5ml
Non-essential amino acid (10mM, 100×)	5ml

Aliquots were kept (not more than 1 month) at −80 °C until further use.

3.1.8.14 Medium used for propagation culture of mouse ES cell derivatives

Enriched KO-DMEM	98.9ml
Insulin-Transferrin-Selenite (ITS) supplement	1ml
Fibronectin	100μl

Fibronectin was freshly supplemented into the medium prior to use

3.1.8.15 Medium used for selection culture of mouse ES cell derivatives

Neurobasal medium	39.2ml
Penicillin/Streptomycin	0.4ml
N2 supplement (100×)	320µl
B27 supplement (50×)	80µl

The medium was kept (not more than 1 week) at 4 °C.

3.1.8.16 Maintenance medium for human ES cells

3.1.8.16.1 Preparation of bFGF solution (2µg/ml)

bFGF (0.1%)	10µl
0.1% BSA in PBS	5ml

The solution was stored at –20 °C in 0.5ml aliquots.

3.1.8.16.2 Preparation of L-Glutamine solution supplemented with β-mercaptoethanol (final concentration of β-mercaptoethanol: 20mM)

L-Glutamine (200mM, 100×)	5ml
β-Mercaptoethanol (14.33M)	7µl

3.1.8.16.3 Human ES cell culture medium

DMEM/F12	200ml
Knockout SR	50ml
L-Glutamine solution supplemented with β-mercaptoethanol	1.25ml
Non-essential amino acid (10mM, 100×)	2.5ml
bFGF solution (2µg/ml)	0.5ml

The medium was filter-sterilized and stored at 4 °C within 2 weeks.

3.1.8.17 Collagenase solution

Collagenase Type IV (10%)	0.3ml

DMEM/F12 medium	30ml

The medium was filter-sterilized and stored at 4 °C.

3.1.8.18 Cryopreservation medium for human ES cells

FCS (ES cell-qualified)	6ml
DMSO	2ml
Human ES cell medium	2ml

The medium was freshly prepared on ice prior to use.

3.2 Methods

3.2.1 Cell culture

3.2.1.1 Coating of culture vessels

Coating could facilitate a better adhesion of cells to the culture vessels.

3.2.1.1.1 Gelatin coating

The 0.1% Gelatin in deionized water after autoclaving, was dispensed to completely cover the culture surface of a culture vessel for at least 20 minutes at room temperature The solution was then aspirated off. The flasks or dishes were air-dried prior to use.

3.2.1.1.2 Poly-L-ornithine and laminin coating

Four millilitres of 0.01% poly-L-ornithine were added into a 30ml universal tube containing 22.6ml sterile water to prepare 15 μg/ml working solution. One millilitre (two millilitres) of the working solution was transferred to each well of 12-well plates (6-well plates) with or without circular coverslips (12mm in diameter; Gerhard Menzel, Germany) before incubation for an hour at 37 °C. The supernatant was then removed and culture plates were rinsed three times with PBS for 5 minutes each. Subsequently, one millilitre

(two millilitres) of laminin solution at 1μg/ml was added to each well of 12-well plates (6-well plates) and incubated for an hour at 37°C. Culture plates were washed once with PBS prior to use.

3.2.1.2 Derivation of mouse embryonic fibroblasts from embryos

CD-1 mice were cared for and handled according to Guidelines of Laboratory Animal Services Centre in The Chinese University of Hong Kong. A mouse at 12 13 dpc was anesthetized by intra-peritoneal injection of ketamine (100mg/Kg; FarmaVet, Alfasan, Romania) and xylazine (10mg/Kg; FarmaVet). After sacrificing it by cervical dislocation, the mouse was placed on its back. The skin of its belly was sterilized with 70% ethanol before making an incision.

The uterine horns were dissected off, briefly rinsed with 70% ethanol and placed into a Petri dish containing phosphate-buffered saline (PBS). Embryos were separated from surrounding membranes. After removal of viscus, the remaining embryonic tissue was washed with fresh PBS and finely minced by using a minimal amount of PBS and scissors. Minced tissues were suspended in 0.25% trypsin-EDTA at about 2ml per embryo and incubated at 37°C for 15 minutes with gentle agitation. The resulting cell suspension was essentially free of any larger pieces of tissue. To remove remaining pieces of tissue, the suspension was settled down within a few minutes and the supernatant was carefully pipetted off. In the supernatant containing dissociated cells, effects of trypsin were terminated by adding two volumes of α-MEM supplemented with 10% FCS. The cell pellet was collected after centrifugation, and was resuspended with supplemented α-MEM and plated out at 3 embryos equivalent per T75 flask containing 10ml α-MEM supplemented with 10% FCS and 2mM L-glutamine. Cultures were incubated at 37 °C in a humidified 5% CO_2-incubator until confluence.

3.2.1.3 Cryopreservation of mouse embryonic fibroblasts

Confluent cultures were washed with PBS and trypsinized with 0.05% trypsin-EDTA for 5 minutes at 37 °C. Trituration was performed by pipetting up and down to segregate cells. Digestion was stopped by adding FCS-supplemented medium. Dissociated cells were pelleted by centrifugation. Then, the supernatant was aspirate and cells were resuspended in cold freezing medium containing 10% DMSO. Cell suspension at 1ml of 10^6 cells was aliquoted into each cryo-vial and put in an ultra-low freezer at - 80 °C. Vials were transferred to a liquid nitrogen tank the follwing day for long-term storage.

3.2.1.4 Thawing mouse embryonic fibroblasts

Frozen cells were quickly thawed in a 37 °C waterbath. Ten millimeters of ice-cold α-MEM with 10% FCS were added dropwise. Cells were pelleted by centrifugation to remove the DMSO. Then cells were resuspended in tissue culture medium for further culture.

3.2.1.5 Passage of mouse embryonic fibroblasts

Confluent cultures were washed with magnesium- and calcium-free PBS once before digestion with 0.05% trypsin-EDTA (2 ml per T75 flask). After incubation at 37°C for 5 minutes, dislodged cell aggregates were triturated and trypsinization was stopped by adding FCS-supplemented medium. Cell pieces were pipetted up and down for several times to dissociate aggregates into single cells, and then were pelleted by centrifugation at 200g for 5 minutes. The supernatant was discarded, and cells were resuspended in fresh medium and then plated onto 5 T75 flasks.

3.2.1.6 Preparation of the feeder layer

Cultures of MEF at approximately 90% confluency were washed with PBS and trypsinized with 0.05% trypsin-EDTA, and digestion was stopped by adding 10% FCS-supplemented α-MEM. After centrifugation, cell pellets were resuspended in fresh medium. Cell suspension in a 50ml tube was exposed to 8,000 *rads* (80 *Gy*) γ-irradiation in the γ-irradiator. Irradiated cells of 1.875×10^5 and 3×10^6 were seeded onto each well of 6-well plates and a T75 flask (or 10cm tissue culture dish) pre-coated with gelatin respectively.

3.2.1.7 Establishment of mouse ES cell D3 and E14TG2a cultures

Vials of ES cells were firstly removed from liquid nitrogen and quickly thawed in a 37 °C waterbath. Cell suspension was transferred to a sterile tube. Ten milliliters of mouse ES cell culture medium were added, gently mixed and then pelleted by centrifugation at 200 g for 5 minutes. The supernatant was aspirated off to remove DMSO, and cells were resuspended with fresh ES cell medium and finally plated onto newly prepared MEF feeders. ES cell culture medium was refreshed daily. Upon subconfluence 2 days later, cells were trypsinized for passage or cryopreservation.

3.2.1.8 Passage of mouse ES cells

Mouse ES cells should be routinely passaged every 2 days. Cells were firstly checked under the microscope for differentiation signs before passaging. Reagents were warmed up briefly before use. Basically, the procedure for passaging confluent cultures of adherent cells applied with minor modifications. In view of different ability of mouse ES cells and MEF to attach to tissue culture wares, mouse ES cells in cell suspension could be enriched after incubation at 37°C for 45 minutes, since a majority of adherent cells would be MEF. ES cells in the medium were carefully collected, and distributed onto 2~3 T75 culture flasks pre-coated with irradiated MEF.

3.2.1.9 Cryopreservation of mouse ES cells

Freezing medium containing 10% DMSO was freshly prepared on ice. Spent medium was discarded, and cells were washed with PBS and then trypsinized at 37 °C for 5 minutes. The trypsinized cultures were dissociated into single cells by pipetting, and reactions were stopped by adding FCS-supplemented medium. Cells were pelleted by 200 g centrifugation and the supernatant was removed. Cells were resuspended in a volume of ES cell culture medium which is equivalent to half the desired volume of final cell suspension. Then, an equal volume of pre-cooled freezing medium was added into cell suspension dropwise. The final cell suspension was mixed gently and transferred into cryovials at 1ml per vial. Vials were transferred into a –80 °C ultra-low freezer. One day later, the vials were put into a liquid nitrogen tank for long-term storage.

3.2.1.10 Establishment of human ES cell H9 and H14 cultures

A vial of frozen human ES cells was immersed in a 37 °C waterbath and swirled gently. Cells were gently pipetted into a 15ml conical tube. Four milliliters of human ES cell culture medium were added dropwise to cells, and the tube was agitated gently to mix the content. After pipetting up and down for several times, cells were spun at 200 *g* for 5 minutes. The supernatant was removed, and then the cell pellet was gently resuspended in 2.5ml human ES cell medium. Then, cell suspension was added dropwise into a well of a 6-well tissue culture plate pre-coated with the irradiated MEF feeder layer before incubation at 37 °C. The human ES cell culture medium was refreshed daily.

3.2.1.11 Passage of human ES cells

Prior to passage, cultures were examined under a microscope for differentiation signs. If there are mostly undifferentiated colonies on the plate with only a few differentiated ones, the differentiated colonies are picked to remove by using a glass pipet.

The undifferentiated colonies remain on the plate until they are ready to passage. On the other hand, if there are only a few undifferentiated colonies on the plate with a large number of differentiated colonies, the undifferentiated colonies are picked to propagate onto a new plate until they are ready to passage, whereas the differentiated colonies on the original plate are discarded.

During passage, the spent medium was firstly aspirated off, and one milliliter of 0.1% collagenase solution was added to each well of the 6-well plate. After incubation for 5 minutes, the plate was viewed under a microscope to confirm colony separation from the plate. Second, cells were scraped off the surface of the plate by using a glass pipet, and the collagenase solution was pipetted up and down to wash the cells off the surface while scraping cells off the plate. Dissociated cells were then pooled into a sterile 15ml conical tube. One milliliter of human ES cell medium was used to wash each well of the 6-well plate, and the human ES cell medium wash was transferred to the 15ml conical tube and then pipetted gently to mix. Third, broken cell colonies were pelleted by centrifuging at 200g for 5 minutes. The supernatant was aspirated and the cell pellet was washed with 2-3ml fresh medium in the 15ml conical tube. Cells were again centrifuged and the supernatant was removed from human ES cell pellet. Fourth, the cell pellet was resuspended again with 2-3ml fresh medium, and then a sufficient volume of medium was added to the 15ml conical tube to ensure a total of 2.5ml medium per well. Finally, human ES cells were transferred to another 6-well plate pre-coated with the newly prepared MEF feeder layer before incubation at 37°C.

3.2.1.12 Cryopreservation of human ES cells

Spent medium was aspirated from the 6-well plate. One milliliter of 0.1% collagenase in DMEM-F12 medium was added to each well, and cells were incubated for 5 to 10 minutes. Cultures were examined under a microscope for evidence of peeling

peripheries of colonies. Then cells were scraped off the plate with a 5ml glass pipet and transferred into a 15ml conical tube. The well of the plate was washed with 1ml human ES cell medium, which was pooled with the rest of the cells in the 15ml conical tube. Cells were pelleted by centrifuging at 200 g for 5 minutes. The supernatant was aspirated, and cell aggregates were gently resuspended in 2–3 ml human ES cell medium. Cell washing was repeated by centrifuging again at 200g for 5 minutes and the supernatant was removed. The cell pellet was reconstituted in a volume of human ES cell medium that is equivalent to half the final volume of cell suspension. Then, an equal volume of freezing medium was added slowly and dropwise into the cell suspension. One milliliter of final suspension was transferred to a cryovial and placed in a chamber containing isopropanol for freezing at –80 °C overnight. On the following day, vials were transferred into a liquid nitrogen tank.

3.2.2 Enumeration of cell number and viability

In cell suspension, cells were counted by using the improved Neubauer hemocytometer under a microscope. The hemocytometer was enclosed on the top by a cover slip, and cell suspension after appropriate dilution was charged to the hemocytometer chamber, which has a reflective surface and a grid with nine large squares. Each square of the hemocytometer (with cover slip in place) represents a total volume of 10^{-4} ml. Cells in 4 squares located at corners of the hemocytometer chamber were counted. The average cell count was total cell number divided by number of squares counted. Cells per ml in suspension is the average cell count multiplied by the dilution factor and divided by 10^{-4} ml, and the total cell number in the suspension was the number of cells per ml multiplied by the total suspension volume.

Trypan blue dye exclusion test was used to detect cell viability. One fourth volume of trypan blue solution was added to the cell suspension. At least 300 cells, unstained

viable cells and bluish-stained dead cells, were counted within several microscope fields on the Neubauer hemocytometer. Cell viability was expressed as a percentage of unstained viable cells divided by totally counted cells.

3.2.3 RNA extraction and quantitation

Total RNA was extracted by using the Rneasy extraction kit according to the manufacturer's instruction. Upon washing in PBS, cell pellet was homogenized in 400μl lysis buffer RLT which contains guanidine isothiocyanate to protect RNA from degradation. Cell lysis was applied to a spin column containing silica gel membrane, and centrifuged at 8,000g for 15 seconds. RNA was bound to the resin in the spin column, whereas other components were eluted off. Subsequently, the spin column was cleaned with RW1 buffer by centrifuging at 8,000g for 15 seconds, followed by two additional washes with RPE buffer at 8,000g and 20,000g for 15 seconds and three minutes, respectively. Purified RNA was eluted in 30μl RNase-free water by centrifuging at 8,000g for one minute. RNA samples were stored at −80 °C until further use.

After 100-fold dilution for RNA samples, the absorbance at 260nm and 280nm of each RNA sample was read by using a spectrophotometer. RNA concentration was calculated according to the readout at 260nm and RNA purity was decided by the ratio between the absorbance value at 260nm and that at 280nm.

3.2.4 In vitro differentiation of mouse ES cells

3.2.4.1 Maintenance of mouse ES cells prior to differentiation

Mouse ES cell lines, D3 (CRL-1934, American Type Culture Collection [ATCC]) and E14TG2a (CRL-1821, ATCC), were used. Undifferentiated ES cells were maintained on 80-Gy γ-irradiated primary mouse embryonic fibroblasts pre-established in gelatinized tissue culture dishes (10 cm in diameter) in high-glucose KnockOut Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 15% ES cell-qualified fetal calf serum

(FCS), 2 mM L-Glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, 0.1 mM non-essential amino acid, 0.2 mM β-mercaptoethanol, and 1000 U/ml leukaemia inhibitory factor (LIF). ES cells were passaged on alternate day with 0.05% trypsin containing 0.5 Mm ethylene-diamine-tetra-acetic acid (EDTA).

3.2.4.2 Establishment of irradiated MS5 feeder cells

Cultures of the murine bone marrow stromal cells MS5 at approximately 90% confluency were trypsinized and mitotically inactivated by using 80-Gy γ-irradiation Cells at 1×10^5 in α-MEM supplemented with 2mM L-Glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS were seeded onto tissue culture inserts housed to 12-well ThinCerts tissue culture plates. Culture medium and inserts of newly prepared feeder cells were changed every four days.

3.2.4.3 Cultures of neuroblastoma cells

To confirm neural induction effects of MS5 cells on neural precursors, neuroblastoma cells, as neural precursors, were used to be cocultured with irradiated MS5 cells. Neuroblastoma cell lines, SK-N-AS (CRL-2137, ATCC), SH-SY5Y (CRL-2266, ATCC) and SK-N-MC (HTB-10, ATCC), were plated at 5×10^3 cells/well in 12-well plates with tissue culture inserts of inactivated MS5 and cultured for eight days in supplemented α-MEM. Culture medium and inserts of newly prepared MS5 feeder cells were changed every four days.

3.2.4.4 Preparation of MS5-conditioned medium (CM)

Mitotically inactivated MS5 cells at 1×10^7 in 10 ml serum replacement medium (SRM; KO-DMEM supplemented with 15% serum replacer, 2 mM L-Glutamine, 0.1 mM non-essential amino acid, 100 U/ml penicillin and 100 μg/ml streptomycin) were seeded onto 75-cm^2 tissue culture flasks and maintained at 37°C for four days with daily change of culture medium. The spent medium was collected and centrifuged at 400 *g* for 10

minutes to remove cellular debris. The supernatant was kept at –80°C and six batches of CM were pooled for use thereafter.

3.2.4.5 Induction, Propagation and Selection cultures of ES cells

Mouse ES cells (1×10^5) were induced by non-contact coculture with MS5 cells for eight days in SRM made up of KO-DMEM with 15% serum replacer, 2 mM L-Glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, and 0.1 mM non-essential amino acid. The culture medium was refreshed every two days. Upon completion of the induction, ES cell-derived cells were enzymatically segregated using 0.05% trypsin-EDTA and cells (1×10^5) were further cultured for six days in KO-DMEM supplemented with 2mM L-Glutamine, 100U/ml penicillin and 100µg/ml streptomycin, 5µg/ml insulin, 5µg/ml transferrin, 5ng/ml selenium and 10µg/ml fibronectin, and finally in neurobasal medium supplemented with 100U/ml penicillin, 100µg/ml streptomycin, 0.2× N2 and 0.8× B27 for four days. The medium was changed every other day.

3.2.4.6 RT-PCR

Total RNA was extracted from stromal cells MS5, undifferentiated ES cells, ES cell-derived cells and control tissues/cells (mouse ischemic brain, mouse embryonic heart and liver, mouse embryos, mouse embryonic fibroblasts, neural precursor cell line C17.2 derived from neonatal mouse cerebellum, human brain tissue, human bone marrow and human umbilical cord tissue) by using a Qiagen RNeasy Extraction Kit according to the manufacturer's instructions. The cDNA were transcribed from 2 µg total RNA with 100 ng random hexamers and 200U Superscript reverse transcriptase in reverse-transcription reaction mix. Five microliters of the reverse-transcription product of the first strand cDNA were used for amplification of a specific gene sequence in 25 µl of reaction mix containing 20 mM Tris-HCl buffer (pH 8.4), 50 mM potassium chloride, 1% Tween 20, 100 µM dNTP, 6 pmol of each primer, 0.5 U Platinum Taq DNA polymerase and 1.6 –

2.5 mM magnesium chloride. Table 3-1 and Table 3-2 show details of genes, primer pairs, thermal profiles, cycle numbers and product sizes., RNA integrity was confirmed by RT-PCR of a ubiquitous mRNA *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) for mouse cells or *pyruvate dehydrogenase (PDH)* for human cells. Results were confirmed in at least two separate analyses.

3.2.5 In vivo study of mouse ES cell-derived neural cells

3.2.5.1 Cell labelling

Mouse ES cell-derived cells were labelled with 10 μM bromodeoxyuridine (BrdU) in supplemented neurobasal medium for 24 hours prior to transplantation to ischemic animals. Cells were then trypsinized, washed, and adjusted to 1×10^5/μl in Dulbecco's phosphate-buffered saline (DPBS).

3.2.5.2 Preparation of mice with cerebral ischemia

Animals were cared for and handled according to Guidelines of Laboratory Animal Services Centre in The Chinese University of Hong Kong. Twenty-eight adult male ICR mice of a mean age of 12 weeks, weighing 25 – 30 g, were anesthetized by intra-peritoneal injection of ketamine (100mg/Kg) and xylazine (10mg/Kg). Bilateral common carotid arteries were exposed and then occluded with aneurysm clips for 20 minutes. The incision was sutured after removal of the clips. Eight control mice were sham-operated but bilateral common carotid arteries were not occluded. Mice were nurtured in micro-isolator cages for three days prior to cell transplantation.

3.2.5.3 Cell transplantation

Sedated mouse was fixed onto the Kopf stereotaxic frame. With a Hamilton micro-syringe fitted with a 26-gauge blunt needle, 5×10^5 ES cell-derived cells in 5 μl PBS were slowly injected into each caudate putamen (0.5 mm anterior to the bregma; 2 mm medio-lateral to the mean line and 4 mm ventral to the dura) of 17 mice at a rate of 1 μl

per minute. Equal volume of PBS was injected into seven ischemic control mice. The needle was slowly withdrawn in 10 minutes. The skull hole was cemented and the skin was sutured.

3.2.5.4 Behavioral assessment

The spatial learning and memory ability of the mice were evaluated by using the water T-maze system as reported previously (Fong et al., 2007). The water T-maze was a rectangular labyrinth, with 8 cm-wide pathways and four blind alleys diverging from the through-path. A mouse was placed at the starting point facing the wall and allowed to reach the ladder to exit and escape from the water. Mice which could get out of the maze in 2 – 3 minutes were recruited to *in- vivo* studies. Two weeks post-transplant, mice were trained daily for three days prior to formal assessments on two consecutive days. The time latency in the water maze and episodes of entering blind alleys were recorded.

3.2.5.5 Immunofluorescence study

To prepare cells for immunofluorescence study, cells (1×10^5) were either cyto-spun onto a glass slide or allowed to grow on poly-L-ornithine- and laminin-coated coverslips. They were fixed in 4% paraformaldehyde for 20 minutes. Paraffin-embedded brain sections were dewaxed and antigen retrieval was conducted by microwaving for 20 minutes. Cells/sections were permeabilized for 5 minutes with 0.3% Triton X-100 in PBS and then rinsed thrice with PBS. Non-specific binding was blocked with 10% normal goat serum (NGS) in PBS for 10 minutes. Cells/sections were washed with 1% NGS in PBS and incubated overnight at 4°C with the following primary antibodies diluted in PBS containing 1% NGS: mouse IgM anti-stage specific embryonic antigen (SSEA)-1 (1:100), mouse IgM anti-SSEA-4 (1:100), mouse rabbit polyclonal anti-Musashi-1 (1:100), mouse IgG1 anti-nestin (1:400), mouse IgG1 anti-class III β-tubulin (TuJ-III, 1:1,000), mouse IgG1 anti-glial fibrillary acidic protein (GFAP, 1:50) and mouse IgG2b

anti-myelin basic protein (MBP, 1:100). After thrice washes with PBS, cells/sections were further incubated for 30 minutes with the corresponding secondary antibody: fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (1:100), FITC-conjugated goat anti-mouse IgG (1:100) or FITC-conjugated goat anti-rabbit antibody (1:100). Washed cells were counter-stained with propidium iodide (PI) and visualized by using an inverted fluorescence microscope. The percentage of immunoreactive cells was enumerated by scoring five randomly selected fields in each culture of three to five independent cultures. To visualize BrdU-labelled cells, cells/sections were incubated for 30 minutes with FITC-conjugated anti-BrdU antibody (1:1,000) and then counter-stained with 4', 6'-diamidino-2-phenylindole hydrochloride (DAPI). Cells/sections without primary antibody incubation were processed in the same manner as controls of false-positivity.

3.2.5.6 Tissue processing and histology

Mice were transcardially perfused with 4% paraformaldehyde. Excised brains in either coronal or lateral plane were embedded in paraffin for sectioning at 4 μm thickness. Sections were dewaxed and stained with haematoxylin and eosin or thionin. Images of every fifth serial coronal sections of thionin-stained CA1 region of the hippocampus 1.8 – 2.0 mm posterior to the bregma were captured and overlaid to an image of a counting frame 1 × 0.25 mm derived from the Neubauer hemocytometer. The preponderance of pyramidal neurons with large nuclei, discrete nucleoli and clear cell periphery derived from the hippocampal CA1 region were enumerated (Nakatomi et al., 2002). The morphology of the hippocampal CA1 region was graded (0: no necrosis or < 10% of total cells with pyknotic morphology within the counting frame; I: scattered single cell necrosis or 10 – 40% of total cells with pyknotic morphology; II: 40 – 70% of

total cells with pyknotic morphology; III: almost complete cell necrosis or > 70% of total cells with pyknotic morphology) (Kitagawa et al., 1990).

3.2.6 Neural induction of human ES cells

3.2.6.1 Maintenance of human ES cells prior to neural induction

Human ES cell lines, H14 and H9, were cultured on γ-irradiated mouse embryonic fibroblasts (MEF) in DMEM/F12 medium supplemented with 20% knockout serum replacement, 1mM L-Glutamine, 1% non-essential amino acids, 0.1mM β-mercaptoethanol and 4ng/ml basic fibroblast growth factor (bFGF). Culture medium was refreshed daily. Cultures were passaged every five days by mechanical dissection with glass pipettes, and were passaged onto the freshly prepared MEF feeder (Zhang et al., 2001).

3.2.6.2 Comparison of neural induction efficacies

Neural differentiation of human ES cells was induced by using murine stromal cells MS5 (Auffray et al., 1996). To determine the most efficient MS5-based culture methods for neural induction of human ES cells, the induction efficacies of contact MS5 coculture, non-contact MS5 coculture, and usage of MS5-conditioned medium were compared, in terms of cell viability and quantification of Musashi' neural precursors after induction for eight days.

3.2.6.2.1 Contact coculture with MS5

Irradiated MS5 cells at 2×10^5 in supplemented α-MEM were seeded onto 0.1% gelatin-coated wells of 6-well tissue culture plates one day prior to direct coculture with human ES cells. On the following day, the spent α-MEM was aspirated. Human ES cells at 2×10^5 in DMEM/F12 medium supplemented with 20% KSR, 1mM L-Glutamine, 1% non-essential amino acids and 4ng/ml bFGF were directly seeded onto the MS5 feeder layer. Medium was refreshed every other day. Eight days later, human ES cell-derived

colonies were collected by manual dissection with glass pipettes, and trypsinized into dissociated cells for cell viability detection and quantification of Musashi' cells through immunostaining.

3.2.6.2.2 Non-contact coculture with MS5

Irradiated MS5 cells at 2×10^5 in supplemented α-MEM were seeded onto tissue culture inserts housed to 6-well tissue culture plates one day before non-contact coculture with human ES cells. On the following day, human ES cells at 2×10^5 were seeded onto each well of 6-well tissue culture plates. The spent α-MEM was aspirated from tissue culture inserts, and the whole coculture system was cultured in DMEM/F12 medium supplemented with 20% KSR, 1mM L-Glutamine, 1% non-essential amino acids and 4ng/ml bFGF. Medium was refreshed every other day and the inserts of newly prepared MS5 feeder cells were changed every four days. Eight days after non-contact coculture, cell viability and Musashi' positivity in human ES cell derivatives were examined

3.2.6.2.3 Neural induction by using MS5-conditioned medium

Inactivated MS5 cells at 1×10^7 cells in 10ml supplemented DMEM/F12 were seeded onto 75-cm^2 tissue culture flasks and maintained at 37 °C for four days. The spent media were collected daily for four consecutive days and centrifuged at 400 *g* for 10 minutes to remove cellular debris. The supernatant was pooled and kept at –80 °C. Prior to use, the collected MS5-CM was diluted at the ratios of 1:1 and 2:1 (two-fold volume of MS5-CM plus one-fold volume of freshly supplemented medium) respectively with freshly supplemented DMEM/F12 medium. Human ES cells at 2×10^5 were suspended in diluted MS5-CM and seeded onto each well of 6-well tissue culture plates. Culture medium was changed every other day. Eight days later, cell viability and Musashi' positivity in human ES cell derivatives were examined.

3.2.6.3 Neural lineage selection through serial culture stages

Neural differentiation of human ES cells was induced by coculturing with MS5 feeder cells for two weeks and subsequent incubation in 50% MS5-CM for two weeks, followed by selection culture in supplemented Neurobasal medium for additional two weeks. At the end of cultures in MS5-CM, cells were trypsinized with 0.05% trypsin containing 0.5 mM ethylene-diamine-tetra-acetic acid and 1×10^5 cells were reseeded onto a poly-L-ornithine- and laminin-coated well of 6-well tissue culture plates filled with the Neurobasal medium supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 0.2× N2 and 0.8× B27. The culture medium was changed every other day.

3.2.7 Notch signaling inhibition

The γ-secretase inhibitor was used to block Notch signaling. The γ-secretase inhibitor at 4μM in dimethyl sulfoxide (DMSO) was supplemented into the maintenance medium of human ES cells for eight days in the presence of the MEF feeder.

In the neural induction culture, the same concentration of the inhibitor was applied to the medium for eight days in the presence of MS5 feeder cells. Medium containing same amount of DMSO was used as a solvent control (Noggle et al., 2006)

3.2.8 Serotonin-supplemented cultures

Serotonin at 50, 100, 200, 400 and 800nM in DPBS was applied to mouse ES cell cultures in serum replacement medium for eight days, respectively. Cell viabilities derived from mouse ES cell cultures at various concentrations of serotonin were evaluated.

3.2.9 Statistical analysis

Data derived from at least three independent experiments were presented as mean ± standard deviation (SD), unless stated otherwise. The t-test was used to compare cell viability and relative frequencies of neural cell subtypes derived from cultures with and without MS5 co-cultures. ANOVA in combination with Newman-Keuls post hoc analysis

was used to evaluate cell numbers, viability and relative frequencies of immunoreactive cells derived from different cultures as well as the time latency and error incidence within the water maze, and the neuron density in the hippocampal CA1 regions of normal mice, ischemic mice and transplanted mice. Ranked data of the histological grading of pyramidal neurons in the hippocampi of ischemic mice with and without cell therapy were compared using Kruskal-Wallis test followed by revised t test or Mann-Whitney test. The software SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used and p-values $\leq$ 0.05 were considered statistically significant.

Table 3-1 Primer sequences, thermal profiles and product sizes of the targeted mouse genes

Target genes	Primer sequence (from 5′ to 3′)	Thermal profile and cycles	Size (bp)
m-GAPDH	(F) ACC ACA GTC CAT GCC ATC AC (R) TCC ACC ACC CTG TTG CTG TA	94 °C, 30 sec; 60 °C, 30 sec; 72 °C, 45 sec; Cycles 30	452
m-Oct-4	(F) GGC GTT CTC TTT GGA AAG GTG TTC (R) CTC GAA CCA CAT CCT TCT CT	95 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min; Cycles ·25	312
m-Otx-1	(F) GCT GTT CGC AAA GAC TCG CTA C (R) ATG GCT CTG GCA CTG ATA CGG ATG	94 °C, 30 sec; 61 °C, 1 min; 72 °C, 1 min; Cycles ·35	424
m-Pax-6	(F) TGC CCT TCC ATC TTT GCT TG (R) TCT GCC CGT TCA ACA TCC TTA G	95 °C, 1 min; 54 °C, 1 min; 72 °C, 2 min; Cycles ·33	178
m-Nestin	(F) GGA GTG TCG CTT AGA GGT GC (R) TCC AGA AAG CCA AGA GAA GC	94 °C, 30 sec; 61 °C, 1 min; 72 °C, 1 min; Cycles ·35	327
m-Nurr-1	(F) TGA AGA GAG CGG AGA AGG AGA TC (R) TCT GGA GTT AAG AAA TCG GAG CTG	94 °C, 30 sec; 61 °C, 1 min; 72 °C, 1 min; Cycles ·35	255
m-Brachyury	(F) GAG AGA GAG CGA GCC TCC AAA C (R) GCT GTG ACT GCC TAC CAG AAT G	95 °C, 1 min; 56 °C, 1 min; 72 °C, 1 min; Cycles ·30	230
m-Myf-5	(F) TGC CAT CCG CTA CAT TGA GAG (R) CCG GGG TAG CAG GCT GTG AGT TG	94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 1 min; Cycles ·35	352
m-Nkx-2.5	(F) CAG TGG AGC TGG ACA AAG CC (R) TAG CGA CGG TTC TGG AAC CA	94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 1 min; Cycles ·31	216
m-AFP	(F) ATG TAT GCC CCA GCC ATT CTG TCC (R) GAG ATA AGC CTT CAG GTT TGA CGC	95 °C, 1 min; 54 °C, 1 min; 72 °C, 2 min; Cycles ·30	466
m-GATA-4	(F) AGC CTA CAT GGC CGA CGT GG (R) TCA GCC AGG ACC AGG CTG TT	94 °C, 30 sec; 58 °C, 30 sec, 72 °C, 1 min; Cycles ·30	809
m-CXCR-4	(F) GGT CTG GAG ACT ATG ACT CC (R) CAC AGA TGT ACC TGT CAT CC	96 °C, 30 sec; 56 °C, 1 min; 72 °C, 1 min, Cycles ·35	524
m-BDNF	(F) GTG AGA AGC TTG ATG ACC ATC C (R) AAC AGA ATT CCA CTA TCT TCC C	95 °C, 1 min; 57 °C, 1 min; 72 °C, 2 min, Cycles ·45	768
m-NGF	(F) GGA CTA AGC TTC AGC ATT CCC (R) ACA CTG TTG TTA ATG TTC ACC	95 °C, 1 min; 57 °C, 1 min; 72 °C, 2 min. Cycles ·45	397
m-GDNF	(F) CTG ACC AGT TTG ATG ACG TC (R) TCT AAA AAC GAC AGG TCG TC	95 °C, 1 min; 60 °C, 1 min; 72 °C, 2 min; Cycles ·30	403
m-NT-3	(F) GGT GAA CAA GGT GAT GTG ATG TCC ATC (R) GCT GCC CAC GTA ATC CTC CA	95 °C, 1 min; 57 °C, 1 min; 72 °C, 2 min; Cycles ·45	391
m-CNTF	(F) ATG GCT TTC GCA GAG CAA TCA C (R) CAT AAT GGC TCT CAT GTG CTG	95 °C, 1 min; 60 °C, 1 min; 72 °C, 2 min, Cycles ·30	580
m-GFAP	(F) AAC AAC CTG GCT GCG TAT AG (R) CGC TCT AGG GAC TCG TTC GT	94 °C, 30 sec; 65 °C, 45 sec; 72 °C, 1 min; Cycles ·40	427
m-IGF-I	(F) TCG TCT TCA CAC CTC TTC TAC CTG (R) CTT CTG AGT CTT GGG CAT GTC AGT	94 °C, 1 min; 55 °C, 1 min; 72 °C, 1 min, Cycles ·30	321
m-IGF-II	(F) AGC GGC CTC CTT ACC CAA CT (R) GAA GTC GTC CGG AAG TAC GG	95 °C, 1 min; 60 °C, 1 min; 72 °C, 2 min; Cycles ·33	345
m-EPO	(F) TCC TTG CTA CTG ATT CCT CTG G (R) AAG TAT CCG CTG TGA GTG TTC G	94 °C, 30 sec; 57 °C, 30 sec; 72 °C, 1 min; Cycles ·40	451
m-VEGF	(F) GCG GGC TGC CTC GCA GTC (R) TCA CCG CCT TGG CTT GTC AC	94 °C, 1 min; 65 °C, 1 min; 72 °C, 1.5 min; Cycles ·30	644, 512
m-bFGF	(F) ACA CGT CAA ACT ACA ACT CCA (R) TCA GCT CTT AGC AGA CAT TGG	94 °C, 30 sec; 60 °C, 30 sec; 72 °C, 1 min; Cycles ·35	295
m-CXCL-12	(F) TCT CGG TCC ACC TCG GTG TCC T (R) GCT TTC TCC AGG TAC TCT TGG A	96 °C, 30 sec; 56 °C, 1 min; 72 °C, 1 min; Cycles ·35	325
m-Wnt-3A	(F) GGA ATG GTC TCT CGG GAG TTT G (R) TTC GGG GTT AGG TTC GCA GAA G	94 °C, 30 sec; 60 °C, 30 sec; 72 °C, 45 sec; Cycles ·30	372
m-Delta-1	(F) CCT CGT TCG AGA CCT CAA GGG AG (R) TAG ACG TGT GGG CAG TGC GTG C	94 °C, 45 sec; 62 °C, 45 sec; 72 °C, 45 sec, Cycles ·40	552
m-Delta-3	(F) CAC GCC ATT CCC AGA CGA GTG C (R) GCA GTC GTC CAG GTC GTG CT	94 °C, 45 sec; 58 °C, 45 sec, 72 °C, 45 sec; Cycles ·40	565
m-Jagged-1	(F) CCT GCC AGT GCC TGA ATG GAC G (R) GGC TGT CAC CAA GCA ACA GAC CC	94 °C, 45 sec; 61 °C, 45 sec; 72 °C, 45 sec; Cycles ·40	620
m-Jagged-2	(F) ACC GTG ACC AAG TGC CTC AGG GCA (R) GAG CGG AGC CCA CTG GTT GTT GG	94 °C, 45 sec; 59 °C, 45 sec; 72 °C, 1 min; Cycles ·40	485

Table 3-2 Primer sequences, thermal profiles and product sizes of the targeted human genes

Target genes	Primer sequence (from 5' to 3')	Thermal profile and cycles	Size (bp)
h-PDH	(F) GAA GTT GCC CAG TAT GAT GG (R) TCT CTA GCA CCA CCA CTG G	94 °C, 30 sec; 55 °C, 30 sec; 72 °C, 1 min; Cycles ·30	420
h-Nestin	(F) CTC TGA CCT GTC AGA AGA AT (R) GAC GCT GAC ACT TAC AGA AT	95 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min; Cycles ·35	327
h-Musashi-1	(F) CGA CTC CAA AAC AAT TGA CCC (R) GGT GGA ATG TAA GAA AGC TCA	95 °C, 1 min; 60 °C, 1 min; 72 °C, 1 min; Cycles ·35	300
h-GFAP	(F) TCA TCG CTC AGG AGG TCC TT (R) CTG TTG CCA GAG ATG GAG GTT	95 °C, 15 sec; 65 °C, 30 sec; 72 °C, 15 sec; Cycles ·30	383
h-MAP-2	(F) GCA CTT CAA GGG AAG CTG AT (R) ATC AAA TGG TCC ACT AGG CG	95 °C, 15 sec; 58 °C, 30 sec; 72 °C, 15 sec; Cycles ·30	412
h-Nurr-1	(F) GCA TAC AGG TCC AAC CCA GT (R) GAA TCA ATC CAT TCC CCA AA	95 °C, 15 sec; 65 °C, 30 sec; 72 °C, 15 sec; Cycles ·30	230
h-Zeta-globin	(F) TGA GCG AGC TGC ACG CCT AC (R) GTA CTT CTC GGT CAG GAC AGA	95 °C, 1 min; 62 °C, 1 min; 72 °C, 1 min; Cycles ·35	173
h-Osteocalcin	(F) CAT GAG AAG CCC TCA CA (R) AGA GCG ACA CCC TAG AC	95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 30 sec; Cycles ·35	310
h-Osteopontin	(F) CTA GGC ATC ACC TGT GCC ATA CC (R) CAG TGA CCA GTT CAT CAG ATT CAT C	95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 30 sec; Cycles ·35	347
h-Aggrecan	(F) TCA GGA GGG CTG GAA CAA GTA CC (R) GGA GGT GGT AAT TGC AGG GAA CA	95 °C, 30 sec; 57 °C, 30 sec; 72 °C, 30 sec; Cycles ·35	392
h-α-P2	(F) TAC TGG GCC AGG AAT TTG AC (R) TCA ATG CGA ACT TCA GTC CA	95 °C, 30 sec; 57 °C, 30 sec; 72 °C, 30 sec; Cycles ·35	240
h-Oct-4	(F) CGA CCA TCT GCC GCT TTG AG (R) CCC CCT GTC CCC CAT TCC TA	95 °C, 15 sec; 60 °C, 30 sec; 72 °C, 15 sec; Cycles ·30	577
h-ALP	(F) TGG AGC TTC AGA AGC TCA ACA CCA (R) ATC TCG TTG TCT GAG TAC CAG TCC	95 °C, 30 sec; 55 °C, 30 sec; 72 °C, 30 sec; Cycles ·35	453
h-Notch-1	(F) CAG GTC AGT ACT GTA CCG AG (R) TGG CAC TCT GGA AGC ACT GC	94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min; Cycles ·40	547
h-Notch-2	(F) ACA TCA TCA CAG ACT TGG TC (R) CAT TAT TGA CAG CAG CTG CC	94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min; Cycles ·40	398
h-Notch-3	(F) TGG ATG AGT GTC AGC TGC AG (R) AGG TGC AGC TGA AGC CAT TG	94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min; Cycles ·40	659
h-Notch-4	(F) ACA TAC CCC CAG ATC CAG TG (R) AGA GGC AGG AGA AAG AGC CC	94 °C, 1 min; 61 °C, 1 min; 72 °C, 1 min; Cycles ·40	622

CHAPTER 4

DIFFERENTIATION OF MOUSE ES CELLS TOWARD THE NEURAL LINEAGE

A novel strategy in inducing mouse ES cells into neural lineage cells in a non-contact coculture system with MS5 was developed. Mouse ES cells were induced by using an 8-day coculture with the bone marrow stromal cell line MS5, followed by a 6-day propagation culture and a 4-day selection culture. At each culture stage, Nestin$^+$ neural precursors, class III β-tubulin$^+$ neurons, GFAP$^+$ astrocytes, MBP$^+$ oligodendrocytes and SSEA-1$^+$ undifferentiated ES cells were enumerated by immunofluorescent staining of specific markers. Furthermore, lineage-specific genes of three germ layers were characterized.

4.1 Neurotrophic and neuroprotective effects of MS5 cells

Figure 4-1 shows that genes encoding the brain-derived neurotrophic factor (*BDNF*), ciliary neurotrophic factor (*CNTF*), glia-derived neurotrophic factor (*GDNF*), nerve growth factor (*NGF*), neurotrophin-3 (*NT-3*), insulin-like growth factor-I (*IGF-I*) and insulin-like growth factor-II (*IGF-II*) are present in MS5 cells. Neuroprotective factors, erythropoietin (*EPO*) and vascular endothelial growth factor (*VEGF*), were detected. Besides, it was noted that MS5 cells expressed the growth-promoting basic fibroblast growth factor (*bFGF*) and the chemokine C-X-C motif (*CXCL-12*), but no *Wnt-3A*. These data suggested that MS5 cells may exert neurotrophic and neuroprotective effects.

4.2 Neurotrophism of MS5 on neuroblasts

In 8-day non-contact co-cultures of neuroblastoma cell lines, SK-N-AS, SH-SY5Y and SK-N-MC, with MS5, the mean numbers of viable SK-N-AS, SH-SY5Y and SK-N-

MC in four separate experiments of 16 replicate co-cultures showed 2.0-, 1.6- and 2.2-fold increase, respectively, as compared to control cultures run in parallel (Figure 4-2a). Immunostaining of class III β-tubulin (TuJ-III) demonstrated significantly higher numbers of TuJ-III$^+$ neurons derived from co-culture of neuroblastoma cells with MS5 (Figure 4-2b; SK-N-AS: with vs. without MS5, 34.8 ± 4.2% vs. 25.9 ± 9.2%, p=0.027; SH-SY5Y: 38.2 ± 7.3% vs. 22.4 ± 9.1%, p=0.002; SK-N-MC: 23.7 ± 4.0% vs. 16.8 ± 4.0%, p=0.004), indicating the neurotrophic effect of MS5

4.3 MS5-mediated neural induction of ES cells

4.3.1 Non-contact induction

In four separate experiments of 16 replicate non-contact co-cultures of ES cell D3 with MS5 for eight days, a progressive increase in the number of colonies with protruding processes was noted (Figure 4-3 a-b). There were upsurges of 2.0 and 2.2 folds of nestin$^+$ and Musashi-1$^+$ neurospheres, respectively, as compared to those derived from control cultures (Figure 4-3 c-d). The cell viability was comparable among co-cultures with and without MS5. However, the total numbers of viable cells derived from co-cultures with MS5 were significantly higher than those of the control cultures (D3 with vs. without MS5: 3.23 ± 0.38 × 10^5 vs. 1.19 ± 0.27 × 10^5, p = 0.0003), suggesting the growth-enhancing effects of MS5 co-culture on ES cell-derived cells. Immunocytochemistry on enzymatically segregated D3-derived cells revealed TuJ-III$^+$ neurons, GFAP$^+$ astrocytes and MBP$^+$ oligodendrocytes accounting to 10.6 ± 2.0%, 3.8 ± 1.5% and 3.7 ± 1.4%, respectively, which incidences were significantly higher than those of ES cell D3 control cultures without MS5 induction (Figure 4-4). Similar readouts were noted in non-contact co-cultures of ES cell E14TG2a with MS5 (11.7 ± 2.0% TuJ-III$^+$ neurons, 4.5 ± 1.4% GFAP$^+$ astrocytes and 2.5 ± 1.2% MBP$^+$ oligodendrocytes).

In four separate experiments of induction culture, immunostaining of the undifferentiated ES cell marker SSEA-1 revealed a significant decrease of immuno-reactive cells in ES cell-derived cell products which had been co-cultured with MS5 for eight days (Figure 4-5, SSEA-1$^+$ cells: with vs. without MS5 support; 9.3 ± 1.5% vs. 13.1 ± 2.1%; p=0.001). Readouts suggest the pro-differentiative effect of MS5.

4.3.2 MS5-conditioned medium (CM)

In four individual experiments of 16 replicate studies on MS5 CM, data revealed cell viability and numbers of D3-derived cells in 50% CM for eight days were comparable to those in 25% CM (viability: 50% CM vs. 25% CM, 90.6 ± 5.5% vs. 92.8 ± 3.2%, p=0.173; cell number: 2.85 ± 0.32 × 10^5 vs. 3.08 ± 0.40 × 10^5, p = 0.073), but significantly higher than those in 75% CM (viability: 50% CM vs. 75% CM, 90.6 ± 5.5% vs. 70.9 ± 8.5%, p=0.0001; cell number: 2.85 ± 0.32 × 10^5 vs. 2.01 ± 0.25 × 10^5, p=0.0003) and control cultures without CM supplement (cell number: 50% CM vs. control, 2.85 ± 0.32 × 10^5 vs. 1.19 ± 0.27 × 10^5, p = 0.0004), indicating that dose-dependent growth-inhibiting metabolites accumulated in the CM could be negated by 50% dilution. In terms of the number of Nestin$^+$-colonies derived, 50% CM was demonstrated to be superior to the control culture (Nestin$^+$-colonies: CM culture vs. control, 43.5 ± 5.5% vs. 30.4 ± 5.4%, p = 0.0003). Compared to MS5 co-cultures, cultures in 50% CM showed a significantly less number of Nestin$^+$-colonies (Nestin$^+$-colonies: CM culture vs. MS5 co-cultures, 43.5 ± 5.5% vs. 61.5 ± 4.3%, p = 0.0004). It might be attributable to the addiction of ES cell cultures to labile factors which were not well preserved at low temperature, at which MS5 CM was stored.

4.4 Propagation culture and selection culture of ES cell-derived cells

Upon completion of 8-day induction culture with and without MS5, ES cell-derived cells were allowed to propagate in the serum-free medium supplemented with insulin, transferrin, selenium and fibronectin for six days and further selected in N2B27-supplemented Neurobasal medium for four days. Immunostaining displayed a progressive loss of SSEA-1 expression in MS5-mediated D3-derived cells after 18 days, which was in contrast to the persistent existence of SSEA-1$^+$ cells in control cultures (Figure 4-5b). Semi-quantitative RT-PCR demonstrated no expression of the octamer-binding transcription factor-4 (*Oct-4;* undifferentiated ES cell marker), *Brachyury* (mesodermal) and *α-fetoprotein* (endodermal) genes in the ES cell-derived cells derived from co-cultures after 18 days (Figure 4-6a).

A progressive increase of neural lineage cells was noted in D3-derived cells at three different phases of MS5 co-culture (Fig. 4-7a) and control cultures (Fig. 4-7b). Figure 4-7c shows the preponderance of nestin$^+$ stem cells, TuJ-III$^+$ neurons, GFAP$^+$ astrocytes and MBP$^+$ oligodendrocytes derived from MS5 co-cultures over control cultures. On day 18, neural stem cells, neurons, astrocytes and oligodendrocytes accounted to 73.1 ± 3.0%, 14.2 ± 1.9%, 6.2 ± 1.7% and 5.2 ± 1.5%, respectively, in four separate experiments. Notably, cultures extended for four more days in N2B27-supplemented Neurobasal medium resulted in a significant drop of cell numbers ($1.38 \pm 0.08 \times 10^6$ on day 18 vs. $1.16 \pm 0.16 \times 10^6$ on day 22, $p = 0.003$) and viability (67.6 ± 2.6% on day 18 vs. 61.7 ± 5.3% on day 22, $p=0.015$), suggesting the 18-day optimally defined protocol.

4.5 Lineage-specific gene expression in ES cell-derived cells

MS5-induced ES cell-derived cells were characterized for lineage commitment by RT-PCR. A progressive loss of expressions of *Oct-4* (undifferentiated status-related), *Brachyury* (mesodermal) and *α-fetoprotein* (endodermal) genes was noted in D3-derived

cells upon completion of induction, propagation and selection cultures with MS5, compared to the persistent expressions in control cultures without MS5 support (Figure 4-6a). Data indicate that the co-culture system did not favour non-neural commitment. On day 18, *Pax-6* and *Otx-1* (neuroectodermal), *Nestin* and *GFAP* (neural stem cell-related), *Nurr-1* (neuronal), and *CXCR-4* (chemokine-related) genes were detected in MS5-induced D3- and E14TG2a-derived cells, respectively, whereas *Myf-5* and *Nkx2.5* (mesodermal) and *GATA-4* (endodermal) were not detected (Figure 4-6b). Data suggest that ES cells co-cultured with MS5 cells induced a hierarchical population of neural lineage cells, but selected against mesodermal, endodermal derivatives and undifferentiated ES cells. Besides, gene expressions of neurotrophins, neuroprotective factors and chemokine, *BDNF*, *CNTF*, *GDNF*, *NGF*, *NT-3*, *bFGF*, *IGF-I*, *IGF-II*, *VEGF*, *EPO* and *CXCL-12*, were also evident in 18-day derived cell products (Figure 4-8).

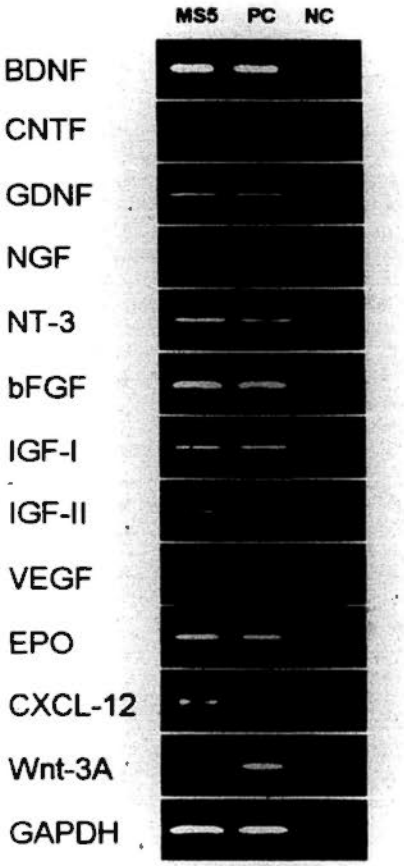

Figure 4-1 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products derived from inactivated MS5 cells. MS5: inactivated MS5 cells; PC: positive control (mouse ischemic brain for BDNF, GDNF, NGF, NT-3, bFGF, IGF-I, IGF-II, VEGF, CXCL-12, Wnt-3A and GAPDH; mouse embryonic fibroblasts for EPO and CNTF); NC: negative control (reagent blanks).

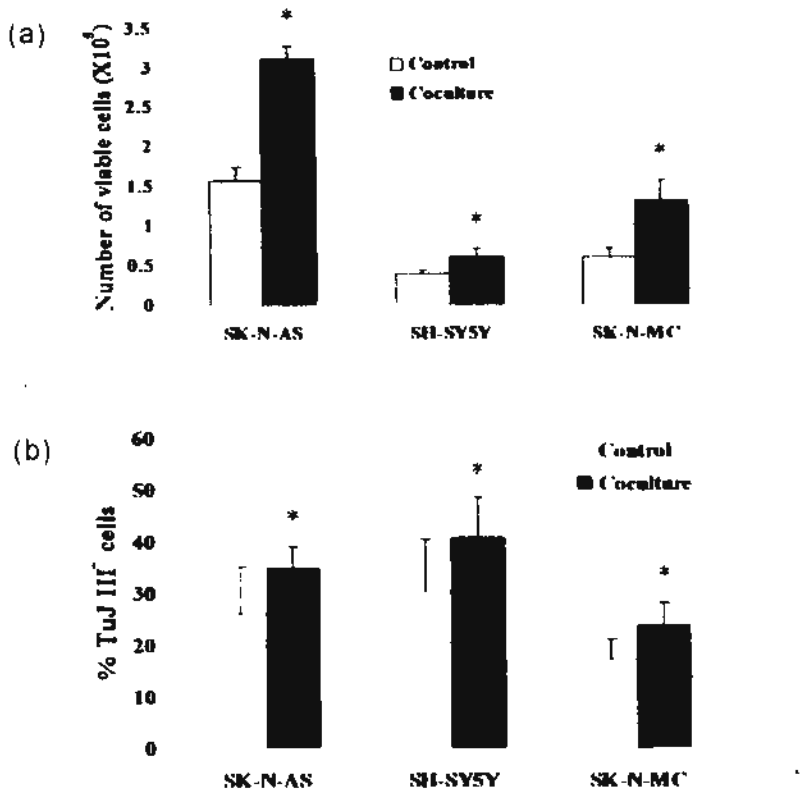

Figure 4-2 Number of viable cells and TuJ-III$^+$ neurons derived from four independent experiments of 16 replicate non-contact co-cultures of neuroblastoma cell lines SK-N-AS, SH-SY5Y and SK-N-MC at 5×10^3 cells originally with MS5 for eight days, respectively. a: number of viable cells (mean ± SD); b: percentages of TuJ-III$^+$ neurons (mean ± SD). * $P < 0.05$ (coculture vs. control without MS5 coculture)

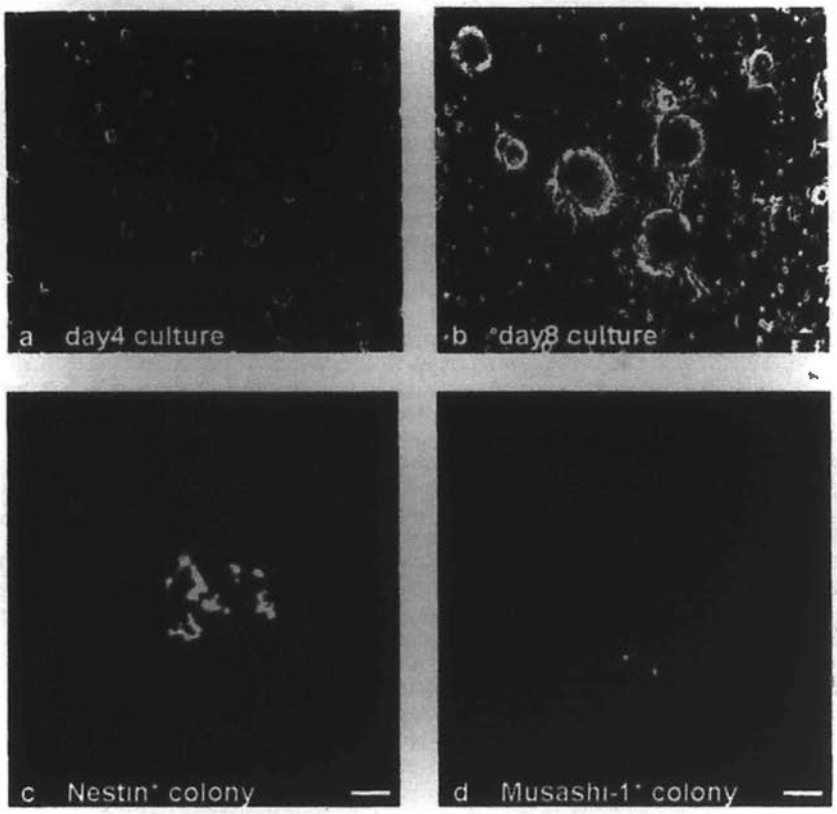

Figure 4-3 Morphology and immunostaining of Nestin and Musashi-1 on ES cell D3-derived cell colonies co-cultured with MS5 for eight days. a: day 4 culture, b: day 8 culture, c: Nestin (green fluorescence) expressed by cells with nuclei counterstained by propidium iodide (red fluorescence) and d: Musashi-1 (green fluorescence) expression. Scale bar, 100 μm for a and b, 200 μm for c and d.

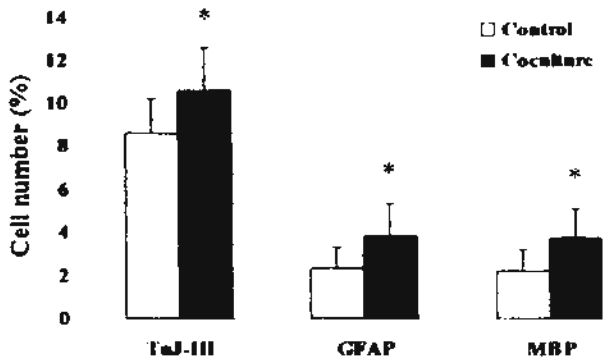

Figure 4-4 The prevalence of TuJ-III$^+$ neurons, GFAP$^+$ astrocytes and MBP$^+$ oligodendrocytes derived from neural induction of ES cells D3 with and without MS5 support. * $P<0.05$ (coculture vs. control)

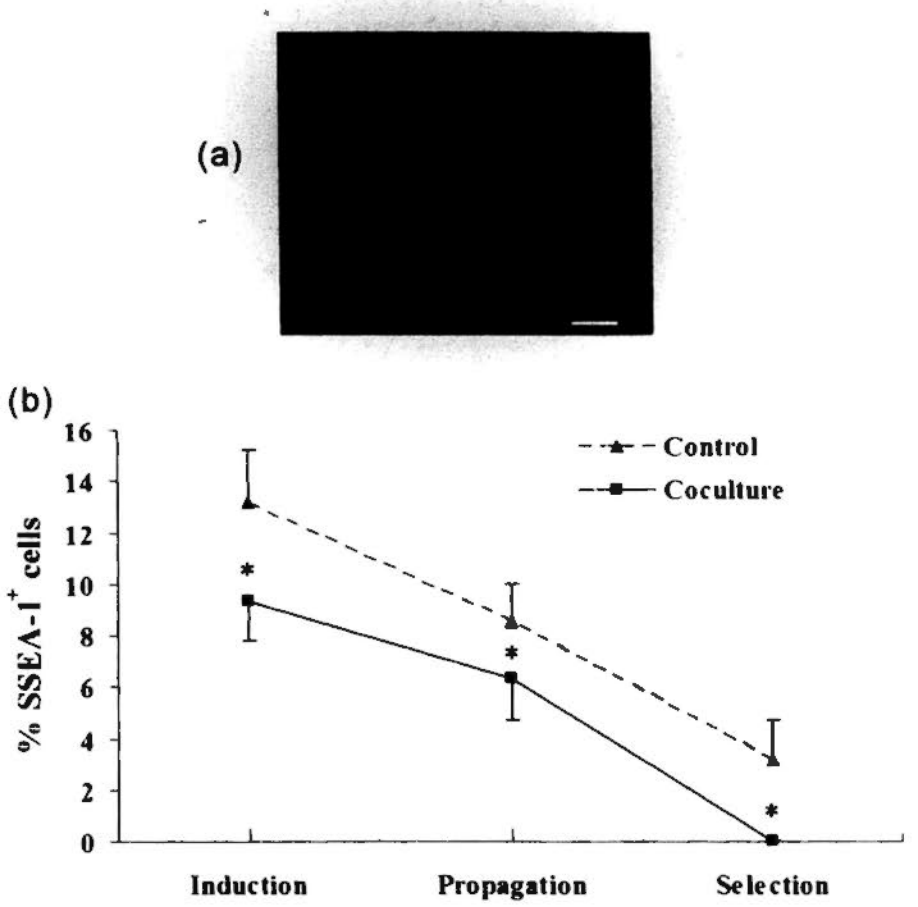

Figure 4-5 Immunostaining of the stage-specific embryonic antigen-1 (SSEA-1) in cell products derived from cultures with and without MS5 supports upon completion of 8-day induction, 6-day propagation and 4-day selection in 4 separate experiments of 16 replicate analyses. a: a representative staining of SSEA-1 (green fluorescence) of ES cell D3-derived cells with nuclei counterstained by propidium iodide (red fluorescence). scale bar, 20μm. b: percentage of SSEA-1^+ cells derived from cultures with and without MS5 supports upon completion of 8-day induction, 6-day propagation and 4-day selection in 4 separate experiments of 16 replicate analyses. * $P<0.05$ (coculture vs. control)

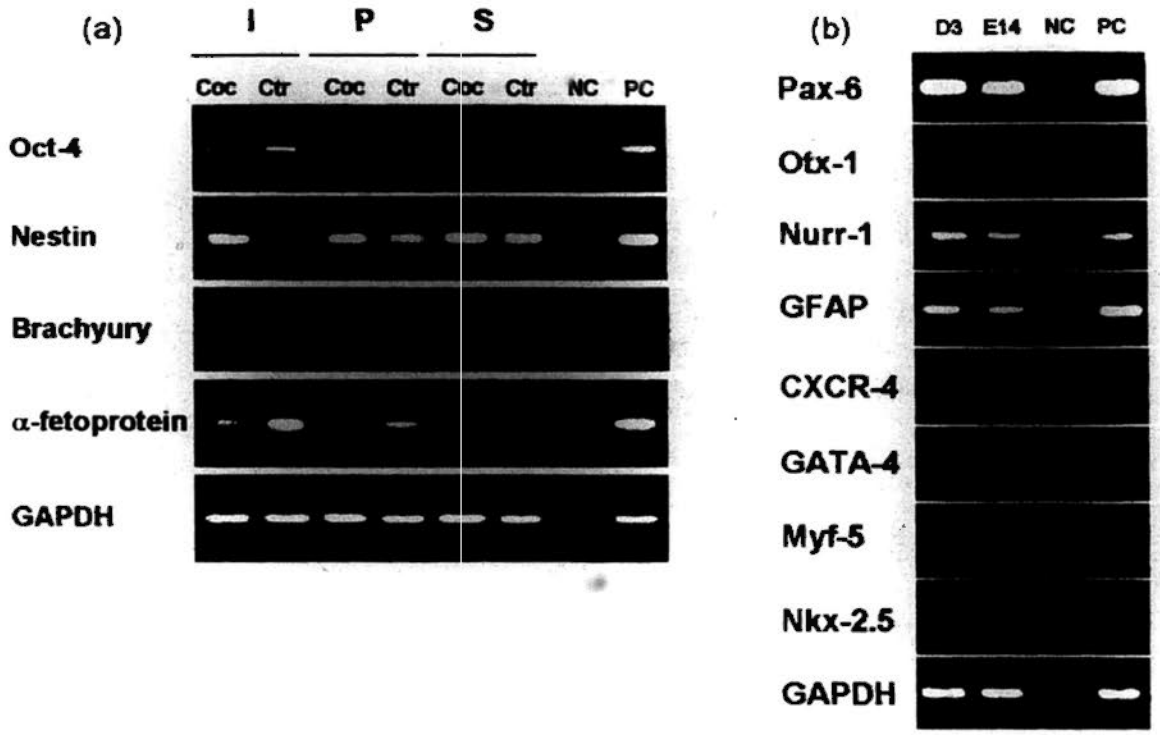

Figure 4-6 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products derived from ES cell-derived cells at different culture stages. a: gene expression of cell derivatives of ES cell D3 upon completion of 8-day induction culture (I), 6-day propagation culture (P) and 4-day selection culture (S). Coc: with MS5 co-culture; Ctr: control culture without MS5 co-culture. b: gene expression of ES cell D3-derived cells (D3) and E14TG2a-derived cells (E14) after 18-day neural induction in co-cultures. PC: positive controls (undifferentiated mouse ES cells for Oct-4; mouse ischemic brain for Nestin, Pax-6, Otx-1, Nurr-1, GFAP, CXCR-4 and GAPDH; mouse embryos for α-fetoprotein; mouse embryonic liver for GATA-4; mouse embryonic fibroblasts for Brachyury; mouse embryonic hearts for Myf-5 and Nkx-2.5); NC: negative controls (reagent blanks).

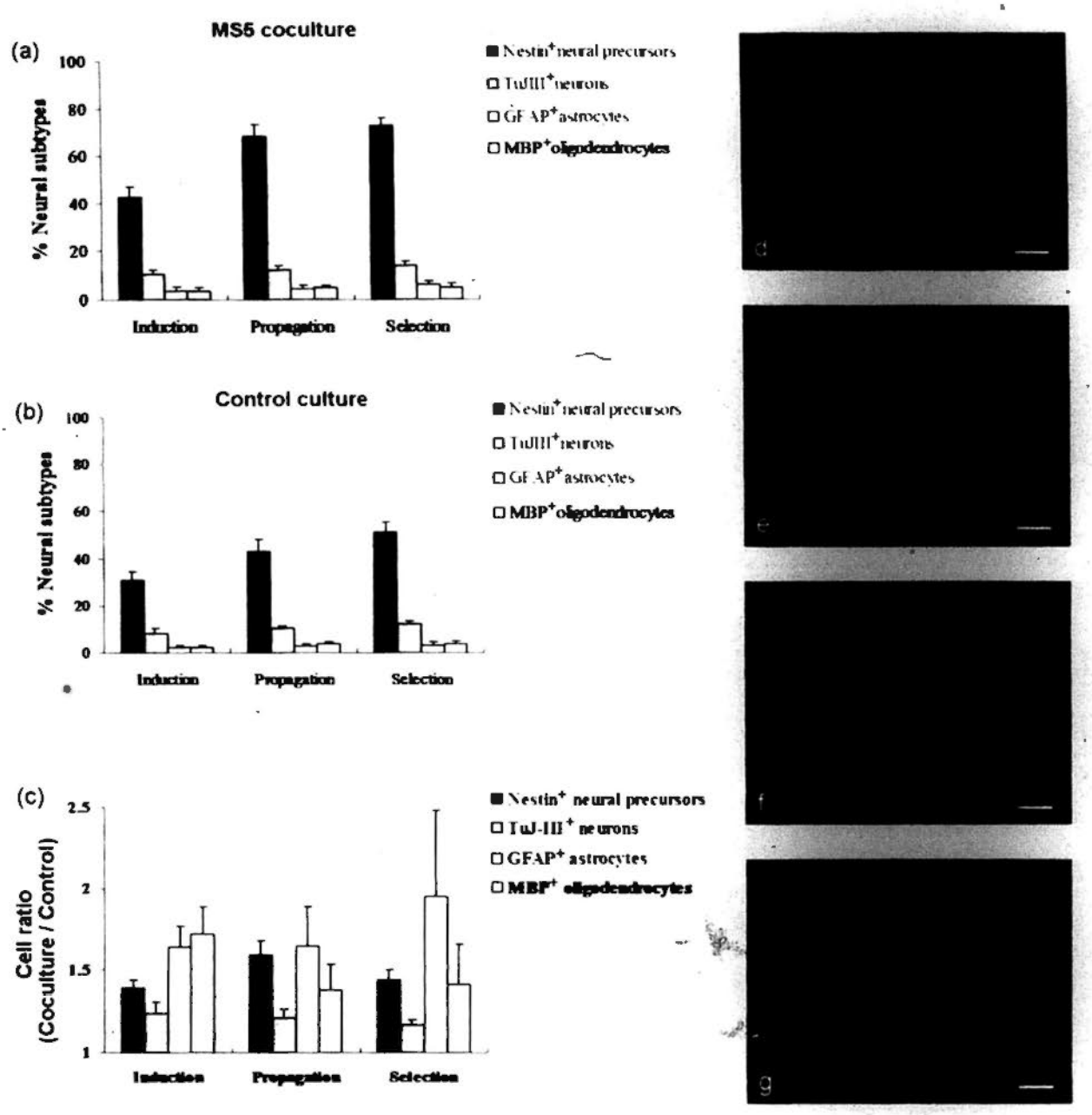

Figure 4-7 A progressive increase of neural lineage cells in MS5-cocultured cell derivatives of ES cell D3. a and b: the relative frequencies of neural cell subtypes derived from MS5 coculture (a) and control culture without MS5 (b); c: ratios of the relative frequencies of neural cell subtypes derived from ES cell D3 upon completion of induction culture, propagation culture and selection culture with and without MS5 co-cultures in four separate experiments; d-g: the representative staining (green fluorescence) of Nestin (d), TuJ-III (e), GFAP (f) and MBP (g) in ES cell D3-derived cells with nuclei counterstained by propidium iodide (red fluorescence). scale bar, 20μm for e and 10μm for d, f, g.

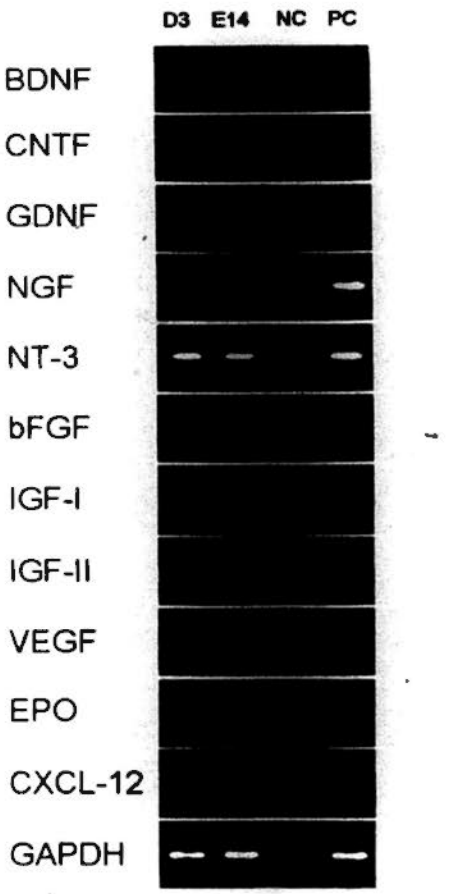

Figure 4-8 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products from 18-day ES cell-derived cell products. D3: ES cell D3-derived cell products; E14: ES cell E14TG2a-derived cell products; PC: positive control (mouse ischemic brain for BDNF, GDNF, NGF, NT-3, bFGF, IGF-I, IGF-II, VEGF, CXCL-12 and GAPDH; mouse embryonic fibroblasts for EPO and CNTF); NC: negative control (reagent blanks).

4.6 Discussion

The generation of functional cells that can be used to replace lost or damagd tissue is a key step in stem cell-based therapies for degenerative diseases. Several different cell sources have been proposed (Mimeault et al., 2007). Based upon their proliferative and pluripotential properties, ES cells are an attractive alternative to somatic stem cells.

The derivation of specific neuronal or glial cell types from ES cells invariably includes the production of NS cells or neural precursors, which are capable of differentiating into neurons, astrocytes and oligodendrocytes (Temple, 2001; Doetsch, 2003). Various protocols have been reported to direct neural commitment of ES cells. Basically, these protocols were deployed to mimick the active neurogenic niche or evoke a specific lineage commitment. The present strategy employed both aspects and elicits neural differentiation of mouse ES cells by stromal cell coculture, followed by neural lineage-specific selection

Bone marrow stromal cells MS5 have been demonstrated to support the proliferation of haematopoietic progenitor cells in long-term cultures (Itoh et al., 1989; Issaad et al., 1993). Recently, stromal cells derived from mouse bone marrow, PA6, M2-10B4 and MS5, were shown to induce neural differentiation of ES cells in co-culture systems (Morizane et al., 2006; Lee et al., 2007; Becerra et al., 2007). In this study it was noted that the neurotrophic effect of MS5 on ES cells was similar to the PA6 stromal cell-derived inducing activity (Kawasaki et al., 2000). MS5-conditioned medium also exerted the neural inducing effect, though to a lesser extent, implying the presence of soluble neurotrophic and neuroprotective factors. These works suggest the potential of employing feeder supports mimicking the micro-environment of neurogenesis for ES cell induction, despite the underlying molecular mechanisms of directed differentiation into neural lineage cells are not fully known.

In normal developmental process, the neural fate in mouse is thought to take place during gastrulation of 6.5 – 8 dpc. *In vitro* neural induction of ES cells was conducted by co-culture with MS5 for eight days to mimic the developmental micro-environment and the duration. Compared to other methods reported in the literature, the non-contact co-culture system established in this study offered a simple and efficient procedure to differentiate ES cells into neural lineage cells and evaded using complex and procedural embryoid body-based differentiation protocols (Barberi et al., 2003, Hayashi et al., 2006, Kawasaki et al., 2000; Morizane et al., 2002). Besides, non-neural feeder-mediated ES cell-derived cells are heterogeneous consisting of neural cells at different stages of differentiation, non-neural lineage cells and undifferentiated ES cells.

By using a non-contact co-culture system, ES cell-derived cells needed not be enzymatically or mechanically segregated from the feeder support in contrast to previously reported contact co-cultures (Hayashi et al., 2006, Barberi et al., 2003, Perrier et al., 2004; Tabar et al., 2005), hence minimizing cell loss and feeder cell contamination prior to transplantation. Subsequent cultures in serum-free medium enriched with insulin, transferrin, selenium and fibronectin, and N2B27-supplemented Neurobasal medium allowed the propagation and selection of neural lineage cells, without impairing their differentiation potential (Lee et al., 2000; Okabe et al., 1996; Rappa et al., 2004; Pachernik et al., 2005). Upon completion of the 8-day induction culture, 6-day propagation culture and 4-day selection culture, stable yields of relatively purified neural lineage cells including neural stem cells, neurons, astrocytes and oligodendrocytes, could be achieved.

Nestin$^+$ neural stem cells accounted to 73% of the derived cells. RT-PCR revealed neuroectodermal and neural related genes, *Otx-1*, *Pax-6*, *Nestin* and *Nurr-1*, but no mesodermal and endodermal genes and ES cell transcriptional factor *Oct-4*. Apparently,

the system did not support non-neural differentiation and maintenance of undifferentiated status of ES cells, which may attribute to abnormal development in vivo post transplant. Thus, the present protocol was able to generate neural subtypes with high efficiency without embryoid body formation The neural lineage cells were derived within a short period without feeder cell contamination

CHAPTER 5

NOTCH SIGNALING IS INDISPENSABLE TO NEURAL COMMITMENT OF ES CELLS

Notch receptors and ligands were identified in ES cells and feeder cells, respectively. The roles of Notch signaling in ES cell proliferation and neural differentiation were studied by using the Notch inhibitor.

5.1 The presence of Notch signaling components in human ES cells and feeder cells

Immunostaining of the neural precursor marker Musashi-1 in human ES cell derivatives cocultured with MS5 in serum replacement medium showed that Musashi-1 cells were interspersed with negatively stained cells in a composite manner, suggesting cell-cell interaction may regulate, or at least play a role in the neural commitment of human ES cells. Thus, Notch signaling which requires cell-cell interaction to function may be involved in neural differentiation of human ES cells.

RT-PCR demonstrated the gene expression of Notch ligands, including *Delta-1*, *Delta-3*, *Jagged-1* and *Jagged-2*, in MS5 (Figure 5-1a), whereas Notch receptors, *Notch-1*, *Notch-2*, *Notch-3*, and *Notch-4* were detected in undifferentiated human ES cells, H14 and H9, and invariably detected in H14 cell-derived cells (Figure 5-1b). Data suggested that Notch signaling may play a role in the neural induction of human ES cells in the MS5 coculture system.

5.2 The role of Notch signaling in maintenance of human ES cells

To ascertain whether Notch signaling was involved in self-renewal and neural lineage commitment of human ES cells, cocultures of human ES cells with or without MS5 cells were supplemented with the γ-secretase inhibitor, which could inhibit the Notch signaling by preventing formation of the Notch intracellular domain (NICD). In the maintenance cultures of H14 and H9 cells in 12 replicate studies from four individual experiments, a significant decrease in cell viability after Notch inhibition was noted after eight days (without vs. with the γ-secretase inhibitor: H14 cells, 86.8 ± 6.0% vs. 52.4 ± 9.3%, $P = 0.0003$; H9 cells, 89.3 ± 6.3% vs. 57.4 ± 10.3%, $P = 0.0003$). Data suggested that Notch signaling may play a role in sustaining growth of human ES cells in the maintenance culture.

After inhibition of Notch signaling of human ES cells H14, the undifferentiated markers, *Oct-4* and *AP* were still strongly expressed, whereas neither neural markers *Nestin* and *MAP-2* nor the non-neural marker *Zeta-globin* was detectable (Figure 5-2). Immunostaining of SSEA-4 in four individual experiments of 12 replicate studies displayed that the mean percentage of SSEA-4$^+$ cells did not change in the maintenance culture of human ES cells after Notch inhibition for eight days (without vs. with Notch inhibition: H14 cultures, 93.2 ± 3.3% vs. 89.7 ± 5.2%, $P = 0.062$; H9 cultures, 92.3 ± 3.6% vs. 90.2 ± 4.5%, $P = 0.228$). These data further suggested that Notch inhibition did not significantly impair the stemness of human ES cells in the maintenance culture.

5.3 The role of Notch signaling in human ES cell differentiation

In the neural induction phase of human ES cells H9 in MS5 coculture, the mean cell viabilities in cultures with and without the γ-secretase inhibitor were comparable (without vs. with the γ-secretase inhibitor for eight days: 84.3 ± 7.2% vs. 80.4 ± 9.1%, $P = 0.254$). Besides, it was also found that Notch inhibition does not impair the cell viability of spontaneously differentiated human ES cells (without vs. with the γ-secretase inhibitor

for eight days: H9-derived cells, 41.4 ± 7.8% vs. 35.4 ± 7.3%, P 0.065, H14-derived cells: 38.1 ± 6.9% vs. 34.7 ± 6.6%, P = 0.237). It was suggested that Notch signaling may not be necessary for cell survival of human ES cell-derived cells, since Notch signaling inhibition did not impede cell viability of human ES cell derivatives under inductive conditions

As indicated by RT-PCR, after Notch inhibition for eight days in both MS5-cocultured human ES cell derivatives and spontaneously differentiated ES cells, a weaker expression of the neural precursor marker *Nestin* was noted, and there was no expression of the neuronal maker *MAP-2* (Figure 5-3). Conversely, the mesoderm-related gene *Zeta-globin* was detected in MS5-cocultured H14 cell derivatives supplemented with the Notch inhibitor for eight days, and a strong expression of the transcription factor *Oct-4* was also noted (Figure 5-3). Immunostaining in four experiments of 12 replicate studies demonstrated that the mean percentage of Musashi-1$^+$ cells dramatically decreased after Notch inhibition in both spontaneously differentiated human ES cells and MS5-cocultured human ES cells (spontaneously differentiated H14 cell derivatives: without vs with Notch inhibition, 24.3 ± 7.2% vs. 10.5 ± 3.1%, P = 0.0002, MS5-cocultured H14 cell derivatives, 46.9 ± 9.2% vs. 22.6 ± 6.5%, P = 0.0002). Besides, after Notch inhibition, the mean number of SSEA-4$^+$ cells significantly increased in cultures of spontaneous differentiation and in MS5-coculture (H14 spontaneous differentiation without vs. with Notch inhibition, 21.7 ± 4.9% vs. 62.3 ± 11.8%, P = 0.0002; MS5-coculture, 13.7 ± 3.6% vs. 41.5 ± 11.0%, P = 0.0001). These data demonstrated that Notch signaling inhibition by the γ-secretase inhibitor resulted in impairment of neural differentiation and initiated non-neural lineage commitment of human ES cells, suggesting Notch signaling may be pivotal to neural differentiation of human ES cells.

5.4 Notch signaling also directs neural lineage entry of mouse

ES cells

The γ-secretase inhibitor was supplemented into the induction culture of mouse ES cells for eight days in serum replacement medium. In three individual experiments of eight replicate studies, the numbers and cell viability of D3-derived cells were comparable (D3 control culture with vs. without γ-secretase inhibitor: cell number, 1.13 ± 0.29 × 10^5 vs. 1.18 ± 0.25 × 10^5, P = 0.739; viability, 91.8 ± 3.4 % vs. 94.8 ± 3.3 %, P = 0.093). Similar readouts were noted in D3 derivatives in cocultures with MS5 (with vs. without γ-secretase inhibitor: cell number, 2.75 ± 0.62 × 10^5 vs. 3.03 ± 0.60 × 10^5, P = 0.379; viability, 90.7 ± 4.0 % vs. 92.6 ± 2.9 %, P = 0.289). These data suggested that the γ-secretase inhibitor did not influence cell survival of mouse ES cell derivatives. However, the γ-secretase inhibitor significantly decreased the Musashi-1 positivity in cell derivatives, regardless of MS5 support (with vs. without γ-secretase inhibitor in D3 control cultures: 13.2 ± 3.7 % vs. 42.4 ± 6.2 %, P = 0.0001; with vs. without γ-secretase inhibitor in D3/MS5 cocultures: 31.2 ± 6.6 % vs. 64.5 ± 8.1 %, P = 0.0003), suggesting Notch signaling may play an important role in neural differentiation of mouse ES cells.

RT-PCR demonstrated the presence of Notch receptors in undifferentiated mouse D3 cells and D3 cell derivatives (Figure 5-4). The addition of the γ-secretase inhibitor to D3/MS5 cocultures enhanced the generation of cells with flat morphology and cell foci without processes (Figure 5-5). With the inhibitor, the expression of neuroectoderm-related genes *Pax-6* and *Nestin*, characteristic of neuroepithelium and neural precursors, was substantially suppressed in both MS5-cocultured cells and control cultures (Figure 5-5e). However, the expression of non-neural genes, such as *Brachyury* and *Nkx2.5* characteristic of mesodermal differentiation, *α-fetoprotein* (*AFP*) and *GATA-4* which specifically derived from endoderm, was upregulated (Figure 5-5f). Besides, the expression of *Oct-4*, a gene specific for undifferentiated ES cells, was also evident after

treatment with the γ-secretase inhibitor (Figure 5-5e). Immunofluorescent staining in three individual experiments of eight replicate studies showed an increase of SSEA1$^+$ cells in D3/MS5 cocultures supplemented with the Notch inhibitor (with vs. without γ-secretase inhibitor: 46.2 ± 8.7 % vs. 9.3 ± 1.5 %, P = 0.0004) and in control cultures (with vs. without γ-secretase inhibitor: 57.7 ± 9.5 % vs. 15.4 ± 4.1 %, P = 0.0007). Data suggested that Notch signaling inhibition by the γ-secretase inhibitor could result in a suppression of neural differentiation but an enhancement of non-neural commitment of mouse ES cells

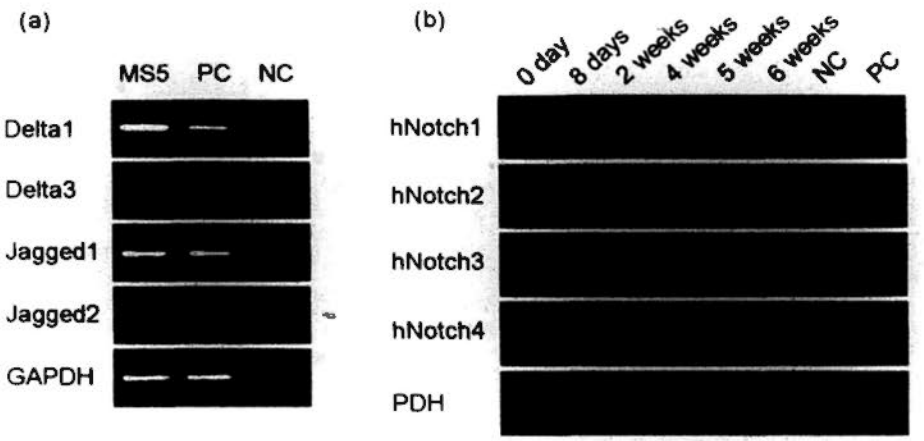

Figure 5-1 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products of Notch ligands and receptors. (a): gene expression of Notch ligands in MS5 cells; (b): gene expression of Notch receptors in undifferentiated H14 cells (0 day) and H14-derived cells at different time points of 8 days – 6 weeks. NC: negative controls (reagent blanks); PC: positive controls (mouse brain tissue for Delta1, Delta3, Jagged1, Jagged2 and GAPDH; human bone marrow for hNotch-1, hNotch-2, hNotch-3, hNotch-4 and PDH).

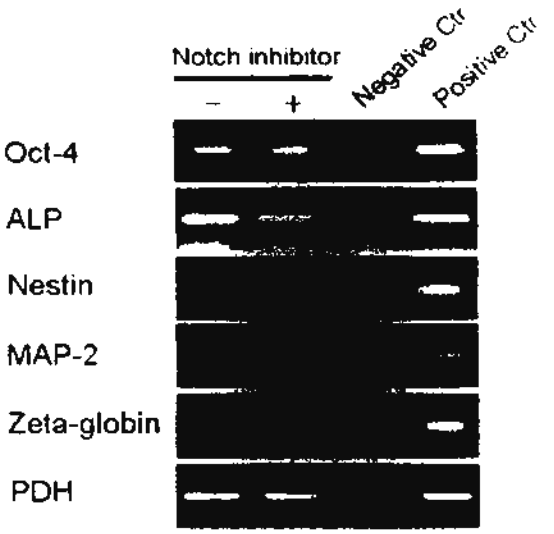

Figure 5-2 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products derived from maintenance cultures of H14 cells without and with Notch inhibition for eight days. Negative Ctr: negative controls (reagent blanks); Positive Ctr: positive controls (undifferentiated H14 cells for Oct-4 and ALP; human brain tissue for Nestin, MAP-2 and PDH; human umbilical cord blood for Zeta-globin).

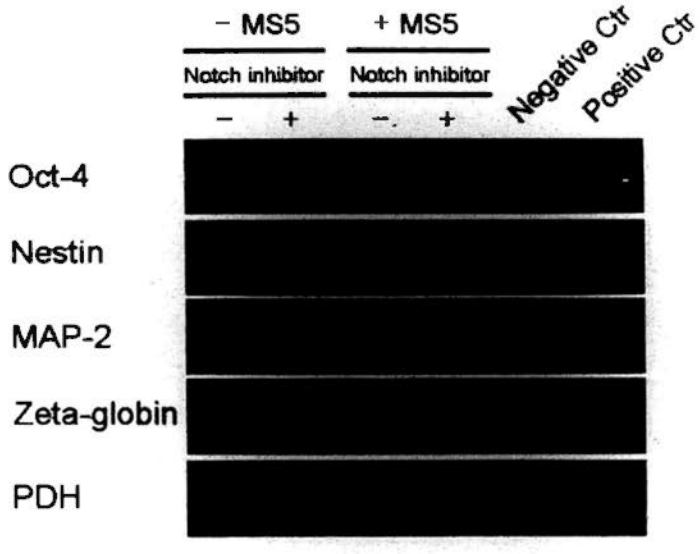

Figure 5-3 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products from MS5-cocultured human ES cell (H14) derivatives and control cultures with and without Notch inhibition for eight days. Negative Ctr: negative controls (reagent blanks); Positive Ctr: positive controls (undifferentiated H14 cells for Oct-4; human brain tissue for Nestin, MAP-2 and PDH; human umbilical cord blood for Zeta-globin).

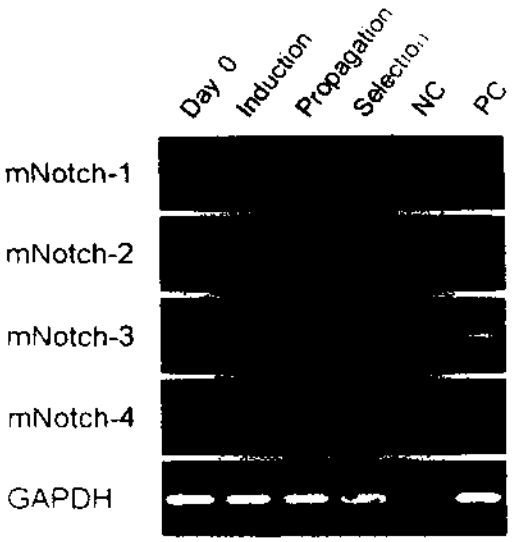

Figure 5-4 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products of Notch receptors derived from undifferentiated mouse ES cells D3 (Day 0) and D3 cell derivatives upon completion of induction culture, propagation culture and selection culture. NC: negative controls (reagent blanks); PC: positive controls (mouse brain tissue).

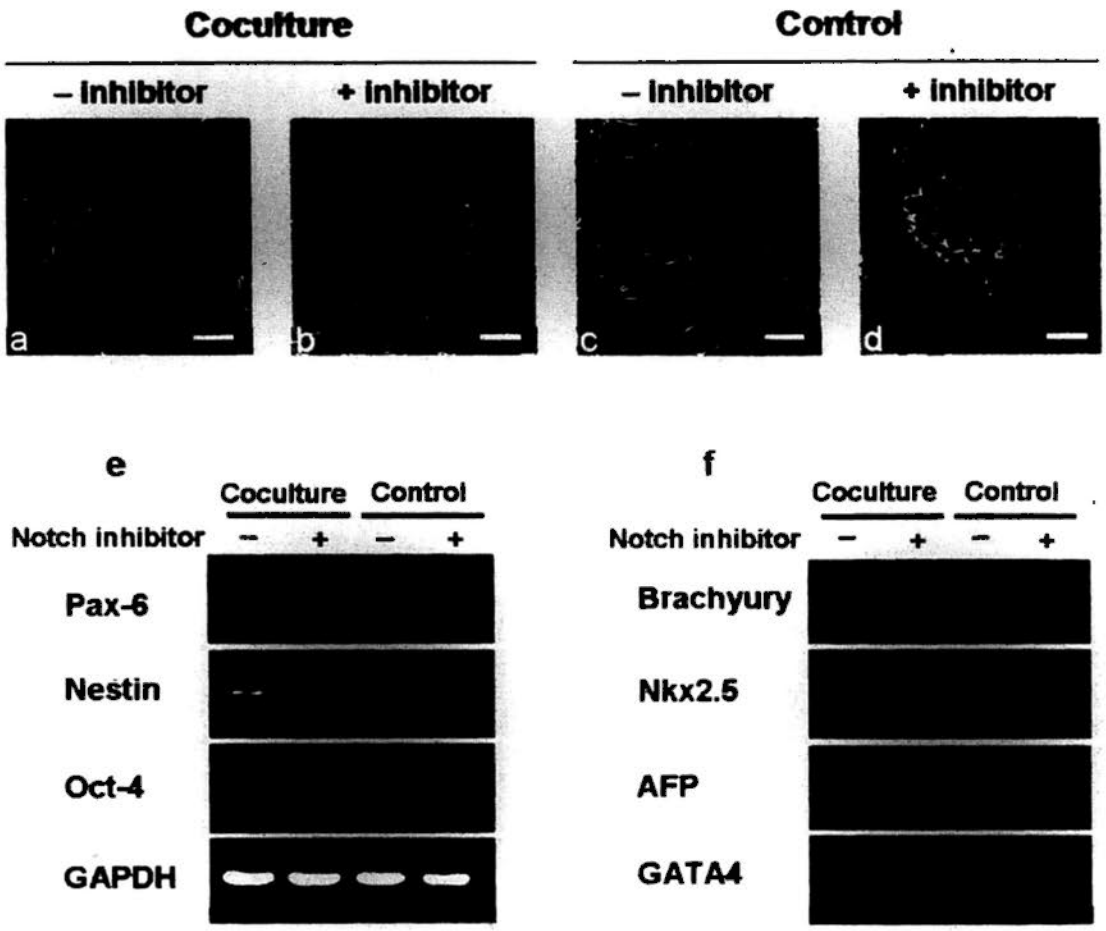

Figure 5-5 Effects of Notch inhibition by the γ-secretase inhibitor on lineage commitment of mouse ES cells D3. a and b: living morphology of MS5-cocultured cell derivatives from D3 cells without (a) and with (b) the supplement of γ-secretase inhibitor; c and d: living morphology of control cultures without (c) and with (d) the supplement of γ-secretase inhibitor; e and f: ethidium bromide-stained agarose gel of electrophoresed RT-PCR products of lineage-specific genes (as indicated) from D3 cell derivatives. Scale bars in a – d: 50μm.

5.5 Discussion

Notch signaling was reported to play an important role in developmental conversion of neural precursors to mature neurons by mediating signaling between adjacent cells (Kageyama et al., 2005) This pathway is hypothesized to restrict the spread of cell differentiation, a process called lateral inhibition (Heitzler and Simpson, 1991) Conversely, Notch is thought to promote neighbouring cells to adopt the same fate, known as lateral induction (Daudet and Lewis, 2005) In the present study, Notch was shown to be employed in neural induction of ES cells The findings indicated that Notch signaling inhibition by the γ-secretase inhibitor significantly impeded the neural induction of ES cells in MS5 cocultures and in control cultures without MS5, suggesting Notch signaling may play an important role in guiding differentiation of ES cells toward the neural fate. On the other hand, in the maintenance culture of human ES cells using MEF feeder support, Notch inhibition was noted to significantly impair cell viability, suggesting Notch signaling may play a role in the survival and growth of human ES cells in the maintenance culture

In the neural induction phase of ES cells using MS5 feeder cells, Notch inhibition arrested neural differentiation, but favoured non-neural lineage commitment of ES cells These data suggested that Notch signaling may be a limiting factor for neural specification in ES cell cultures In vivo, Notch signaling has been shown to act in certain contexts to promote neural competence in tissues. An earlier investigation demonstrated that activated Notch signaling promoted the generation of sensory patches in the developing inner ear of the chick (Daudet and Lewis, 2005). However, the role of Notch signaling might be of greater significance for cultured ES cells in vitro, confronted with conflicting autocrine, paracrine and exogenous differentiation cues, than that in the

gastrulating embryo where differentiation signals are tightly restricted, spatially and temporally in vivo.

A significant difference between mouse ES cells and human ES cells is the growth characteristics at low cell densities. In maintenance cultures, human ES cells tend to propagate in cell clusters and dissociated cells grow poorly, whereas individual mouse ES cells can grow nicely and form clone. Mouse embryos, after knockout of Notch signaling, survive until gastrulation and mouse ES cells derived from these embryos are viable and able to proliferate (Oka et al., 1995), suggesting that the roles of Notch signaling are based on the different circumstances of embryonic development in different species. Previous reports have suggested that poor growth of human ES cells at low density may be attributable to the loss of direct cell-cell contact (Androutsellis-Theotokis, et al., 2006; Rho et al., 2006). The current study demonstrated that the Notch signaling pathway provides a cell-cell proliferation signal for human ES cells. Consistently, it was reported that stimulation of the Notch signaling increases the plating efficiency of human ES cells by activation of the serine/threonine kinase Akt and STAT-3 (Androutsellis-Theotokis, et al., 2006). Phosphatidylinositol-3 kinase/Akt signaling was also noted to prevent apoptosis of human ES cells (Li et al., 2007; Armstrong et al. 2006).

In the context of neural induction from human ES cells, data of the study indicated that Notch signaling augments neural specification. The possible roles of Notch in human ES cell fate choice are to promote neural differentiation, enhance proliferation of derived neural cells, and suppress non-neural commitment. Although neural commitment in vivo is regulated by the interaction of bone morphogenetic proteins, fibroblast growth factors and Wnt signals (Wilson and Edlund, 2001), several yet unidentified signaling events may also be requisite for neural specification (Linker and Stern, 2004). Data of the study suggested that Notch may play a role in neural development and morphogenesis

The challenge in ES cell technology is how to suppress the diversity of embryonic development and precisely impose unitary lineage commitment. The current study suggested that Notch signaling could be deployed to efficiently direct differentiation of ES cells towards the neural fate, and manipulation of the Notch pathway may be a potential strategy to induce neural lineage cells for the treatment of nervous system diseases.

CHAPTER 6

SEROTONIN PROMOTES NEURAL DIFFERENTIATION OF MOUSE ES CELLS

Serotonin (5-HT) was noted to exert a growth-enhancing effect on mouse ES cell derivatives. Besides, it also mediated neural differentiation of mouse ES cells in co-cultures with MS5.

6.1 5-HT promotes proliferation of ES cell-derived cell products

Different concentrations of 5-HT were supplemented into ES cell D3 cultures respectively (Figure 6-1). In serum replacement medium, 5-HT at 200 nM was noted to be well tolerated, as compared to the cell viability derived from 400nM and 800nM 5-HT, after culture for 8 days. In the cultures of D3 with and without non-contact MS5 coculture, 5-HT at 200nM for eight days induced larger cell foci and denser processes (Figure 6-2). There was a significant increase in the total number of viable cells in three individual experiments of eight replicate studies (D3 control cultures: with vs. without 5-HT, $1.33 \pm 0.15 \times 10^5$ vs. $1.18 \pm 0.14 \times 10^5$, $P = 0.049$; D3/MS5 coculture: with vs. without 5-HT, $3.44 \pm 0.23 \times 10^5$ vs. $3.21 \pm 0.13 \times 10^5$, $P = 0.036$; E14TG2a control cultures: with vs. without 5-HT, $1.29 \pm 0.13 \times 10^5$ vs. $1.08 \pm 0.12 \times 10^5$, $P = 0.006$; E14TG2a/MS5 coculture: with vs. without 5-HT, $3.37 \pm 0.13 \times 10^5$ vs. $3.19 \pm 0.11 \times 10^5$, $P = 0.010$), suggesting 5-HT has a growth-enhancing effect on mouse ES cell derivatives.

6.2 5-HT accelerates neural differentiation of ES cells

After eight days with 5-HT supplement in cultures, neural commitment of derived cells was assessed by means of immunofluorescence study for the neural precursor marker Musashi-1. In the control cultures of eight replicate studies in three separate

experiments, there was no significant change in the percentage of Musashi-1$^+$ cells among totally derived cells (D3: with vs. without 5-HT, 44.6 ± 8.0 % vs. 42.4 ± 6.2 %, P = 0.552; E14: with vs. without 5-HT, 45.5 ± 7.3 % vs. 40.5 ± 6.6 %, P = 0.168). Conversely, in MS5-cocultured ES cells, Musashi-1 positivity was significantly enhanced by 5-HT supplement (D3 cocultures: with vs. without 5-HT, 73.6 ± 7.4 % vs. 64.5 ± 8.1 %, P = 0.034; E14TG2a cocultures: with vs. without 5-HT, 76.4 ± 5.6 % vs. 65.5 ± 7.4 %, P = 0.005). RT-PCR attested the gene expression of neuroepithelium and neural progenitor cells, including *Sox-2*, *Pax-6* and *Nestin*, in 5-HT-supplemented D3 cell cultures (Figure 6-3). In the study, it was noted that 5-HT could further promote neural commitment of ES cells, by acting synergistically with MS5 feeder cells.

The 5-HT was also supplemented to the propagation culture and selection culture of ES cell-derived cells for six days and four days, respectively. Upon completion of the serial cultures, immunofluorescent staining of the neuronal marker, β-Tubulin III (TuJ-III), in eight replicate cultures of three separate studies demonstrated a significant increase of TuJ-III$^+$ cells, as compared to control cultures without 5-HT supplement (D3: with vs. without 5-HT, 21.8 ± 3.2 % vs. 14.2 ± 1.9 %, P = 0.0005; E14TG2a: with vs. without 5-HT, 23.4 ± 4.5 % vs. 15.1 ± 2.3 %, P = 0.001). Data of the study suggested that 5-HT further promoted neural commitment of mouse ES cells in cocultures with MS5 cells.

6.3 5-HT-mediated neural differentiation was impeded by the γ-secretase inhibitor

Since Notch signaling was demonstrated to play a role in neural commitment of ES cells, the question was further asked whether there is any crosstalk between 5-HT-mediated neural differentiation and Notch signaling. The γ-secretase inhibitor and 5-HT

were supplemented into D3/MS5 cocultures for eight days. Derived cell foci rarely exhibited processes, in marked contrast to those without the γ-secretase inhibitor (Figure 6-4 a-h). Immunofluorescent staining demonstrated that Musashi-1$^+$ cells were comparable in D3/MS5 cocultures supplemented with the γ-secretase inhibitor (without vs. with 5-HT: 31.2 ± 6.6 % vs. 35.2 ± 6.0 %, P = 0.227, Figure 6-4 i). RT-PCR indicated that the neuroectoderm-related genes *Sox-2* and *Nestin* were weakly expressed in cultures supplemented with 5-HT and γ-secretase inhibitor (Figure 6-4 j). Besides, the expression of *Oct-4*, an undifferentiated status-related gene, was particularly evident in cultures with the γ-secretase inhibitor (Figure 6-4 j). Immunostaining of SSEA-1 in three individual experiments of eight replicate studies indicated that, without Notch inhibition, 5-HT significantly decreased SSEA-1 positivity in D3/MS5 cocultures (without vs. with 5-HT 9.3 ± 1.5 % vs. 6.6 ± 1.3 %, P = 0.002). As more neural precursors were derived under the influence of 5-HT, there was less number of undifferentiated cells. In the presence of γ-secretase inhibitor, however, the effect of 5-HT was negated. SSEA-1 positivities of D3 derivatives in MS5 cocultures were comparable in three individual experiments of eight replicate studies (without vs. with 5-HT: 46.2 ± 8.7 % vs. 42.7 ± 7.0 %, P = 0.38). Data suggested that Notch signaling inhibition by the γ-secretase inhibitor could block the activity of 5-HT in promoting neural commitment of ES cells.

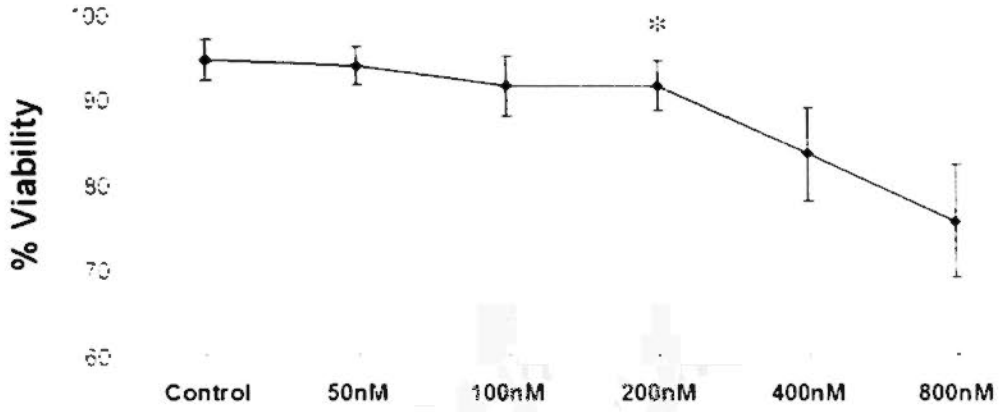

Figure 6-1 Cell viability evaluation of ES cell D3 derivatives in serum replacement medium supplemented with 50 – 800 nM 5-HT. The concentration at 200 nM was shown to elicit no apparent adverse effect on cell viability. * $P < 0.05$ (200 nM vs. 400 nM or 800 nM 5-HT)

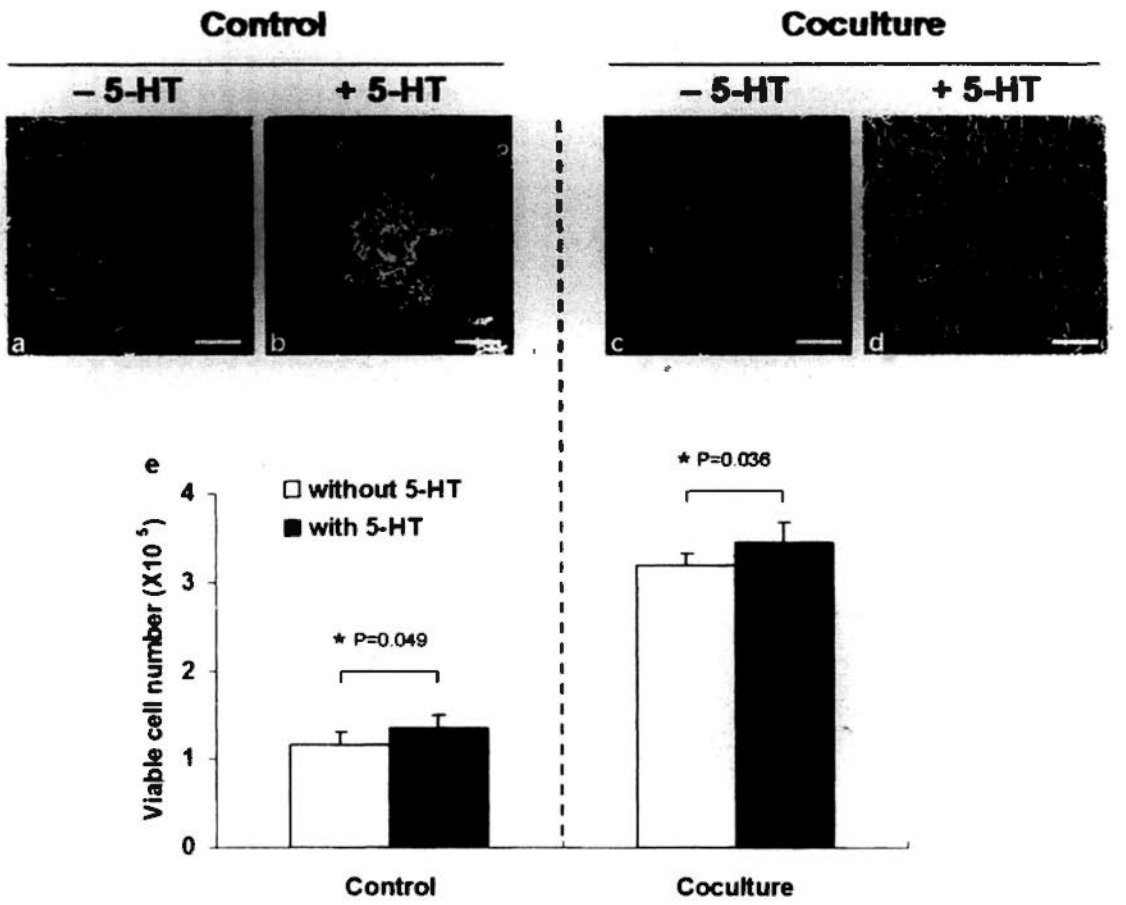

Figure 6-2 5-HT promotes proliferarion of ES cell D3 derivatives regardless of MS5 support. a and b: control cultures without (a) and with (b) 5-HT supplement; c and d: MS5-cocultured D3 cell derivatives without (c) and with (d) 5-HT supplement; e: 5-HT increases the number of ES cell D3-derived cells, regardless of the presence of MS5 coculture. Scale bars, 50μm. * $P < 0.05$

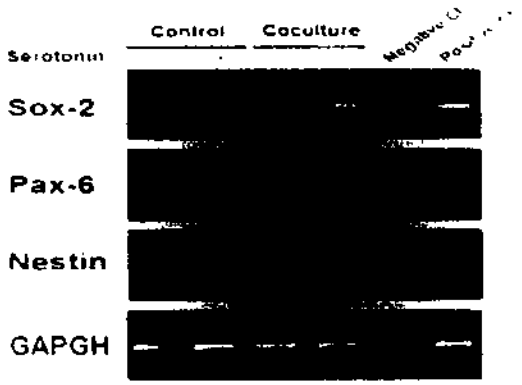

Figure 6-3 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products of neural precursor markers, Sox-2, Pax-6 and Nestin, in cell derivatives from D3 control cultures and cocultures with MS5 in the presence or absence of serotonin (5-HT) supplement. Positive Ctr: positive controls (mouse brain for Sox-2, Pax-6, Nestin and GAPDH); Negative Ctr: negative controls (reagent blanks).

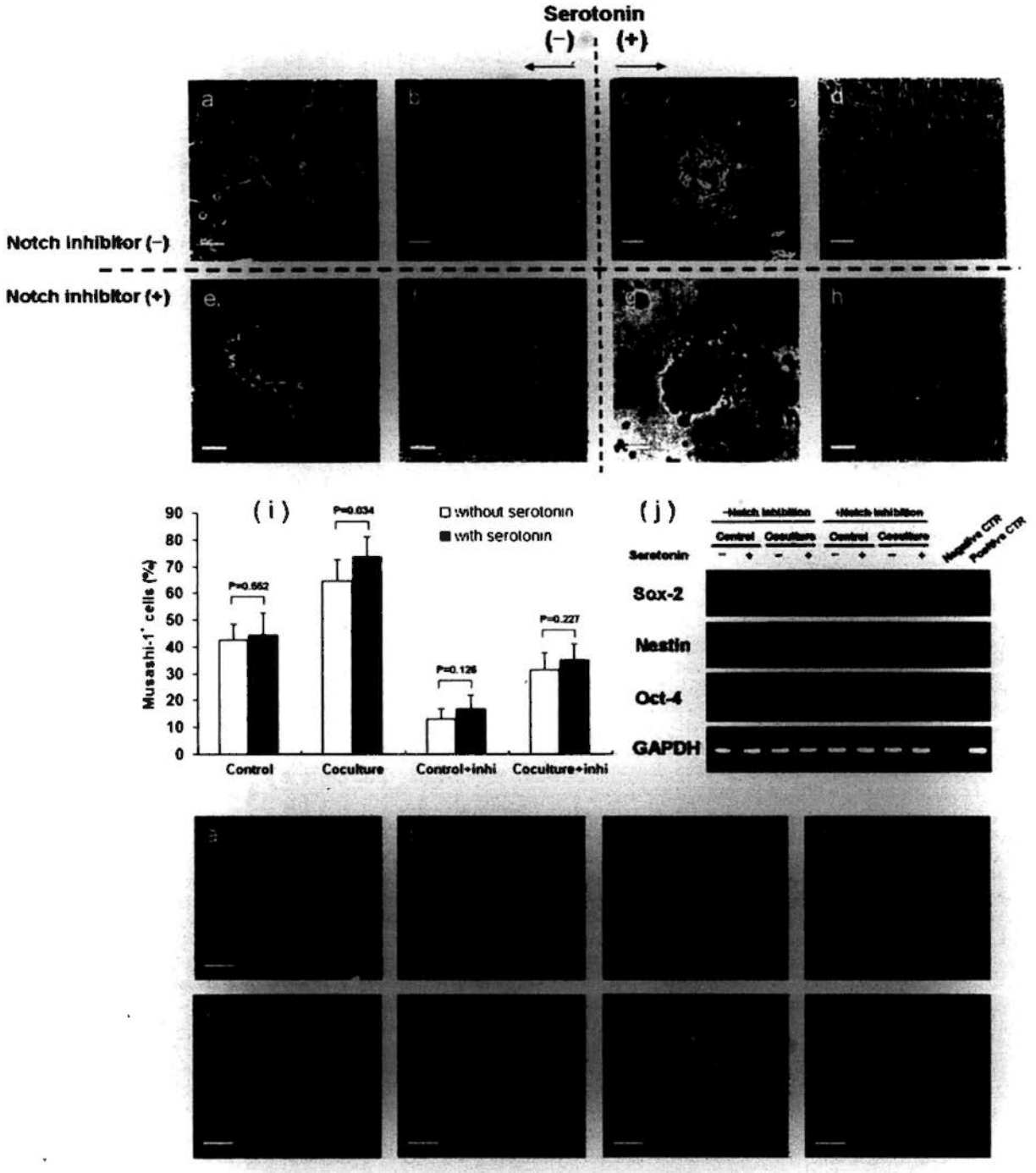

Figure 6-4 The morphology, Musashi-1 immunostaining and RT-PCR analysis of cell foci derived from MS5-cocultured D3 cells with and without supplement of serotonin (5-HT) and γ-secretase inhibitor. a and e: Control cultures without (a) and with (e) the γ-secretase inhibitor; b and f: MS5-cocultured cell derivatives without (b) and with (f) the γ-secretase inhibitor; c and g: 5-HT-supplemented control cultures without (c) and with (g) the γ-secretase inhibitor; d and h: MS5-cocultured cell derivatives in 5-HT-supplemented culture without (d) and with (h) the γ-secretase inhibitor. i: Without γ-secretase inhibitor, 5-HT could significantly enhance the number of Musashi-1$^+$ cells in MS5-cocultured D3 cell derivatives (P = 0.034), whereas 5-HT could not increase Musashi-1$^+$ cells in MS5-cocultured D3 cell derivatives supplemented with the γ-secretase inhibitor (Coculture + inhi, P = 0.227). j: ethidium bromide-stained agarose gel of electrophoresed RT-PCR products of lineage-specific genes from D3 cell derivatives under different conditions as indicated. Negative CTR: negative control (reagent blanks); Positive CTR: positive control (using mouse brain tissue for *Sox-2*, *Nestin* and *GAPDH*, undifferentiated D3 cells for *Oct-4*). a'-h': immunostaining on Musashi-1 for cell derivatives in a-h respectively. Scale bars, 50 μm for a-h and a'-h'.

6.4 Discussion

It was reported that the indoleamine 5-HT is not only a neurotransmitter but also a mitogen with various extraneuronal functions (Veenstra-VanderWeele et al., 2000). The tryptophan hydroxylase (TPH) is the rate-limiting enzyme in its biosynthesis, and the extracellular level of 5-HT is also regulated by a membrane protein transporter (5-HTT). The diversity of 5-HT receptors creates many ways by which 5-HT could affect cell behaviors, such as cell growth, proliferation, differentiation and migration. Fifteen genes that encode 5-HT receptors have been cloned in the mammalian cells (Raymond et al., 2001). Two of these genes encode 5-HT-gated ion channel receptors (5-HT_{3A} and 5-HT_{3B}) and the other thirteen encode G protein-coupled receptors. Generally, activated receptors belonging to the 5-HT_1 and 5-HT_5 classes promote a decrease in cellular cAMP and those belonging to the 5-HT_4, 5-HT_6 and 5-HT_7 classes elicit an increase in cAMP, while 5-HT_2 receptors induce a rise of calcium ions through the phosphatidyl-inositol bisphosphate hydrolysis pathway (van Hooft and Yakel, 2003).

The 5-HT is a potent mitogen and modulates the remodeling of many tissues (Vitalis and Parnavelas, 2003; Matsuda et al., 2004). The platelet-derived 5-HT was shown to mediate liver regeneration through activation of its receptor subtypes 5-HT_{2A} and 5-HT_{2B} (Lesurtel et al., 2006). Recent studies suggested that 5-HT acts as a mitogen on a variety of normal and malignant cells (Siddiqui et al., 2005; Xu et al., 2006). Notably, the mouse ES cell extracts contain easily detectable amounts of 5-HT which are not present in extracts of feeder-cell cultures alone, and TPH transcripts were noted to be more abundant in ES cells than in total mouse brain (Walther and Bader, 1999), demonstrating the presence of 5-HT system in ES cells. Previous investigations suggested that the preimplantation embyo mainly expresses receptor subtypes 5-HT_7 and 5-HT_{1D}. 5-HT_7 mRNA was believed to disappear at about the four-cell stage, roughly concomitant with

activation of zygotic transcription (Amireault and Dubé, 2005), whereas 5-HT_{1D} mRNA is apparently persistent, from unfertilized oocytes up to blastocysts generated in vivo or in vitro (Vesela et al., 2003). The receptor 5-HT_{1D} is coupled to a cAMP-inhibitory G protein, finally resulting in a reduction of cellular cAMP upon activation. An early investigation indicated that significant reduction in cellular cAMP level might be sufficient to give a biochemical signal for cell proliferation (Fanburg and Lee, 1997). It is intuitive that 5-HT_{1D} activation and subsequent reduction in cAMP level are, at least partially, responsible for the effect of 5-HT on cell proliferation in ES cell cultures. In MS5-cocultured ES cell derivatives, the stimulation of cellular proliferation by 5-HT was noted to be intense, which may be attributable to a synergistic effect of 5-HT with growth factors derived from MS5 cells.

Recently, there is increasing evidence that neurotransmitters are used as developmental signals, which modulate the construction and plasticity of brain circuit. 5-HT was the first neurotransmitter for which a developmental role was suspected. Serotonergic neurons are among the earliest neurons to be generated, and 5-HT is released by growing axons before conventional synapses are established (Lauder, 1993; Levitt et al., 1997). Pharmacological studies initially showed that 5-HT can modulate a number of developmental events, including cell division, cell differentiation, neuronal migration and synaptogenesis (Azmitia, 2001; Vitalis and Parnavelas, 2003). In the present study, 5-HT was found to further facilitate neural differentiation of ES cells in MS5 cocultures, whereas 5-HT alone had no direct impact on neural commitment of ES cells in control cultures. The observation suggested that MS5 mediated neural differentiation of ES cells by mimicking the neurotrophic niche (Barberi et al., 2003). As the neural commitment of ES cells was initiated, 5-HT was thought to play a synergistic role with a variety of neurotrophins and growth factors derived from the MS5 cells in

facilitating neural commitment of ES cell derivatives, and promoting further maturation of derived neural progenitors. Previous studies reported that, when the raphe neurons begin to differentiate in vivo, 5-HT is released and could elicit a trophic autocrine effect. In cultures of raphe neurons, 5-HT amplifies its own synthesis, promotes neuronal maturation and increases axon outgrowth (Galter and Unsicker, 2000). In addition, during embryonic differentiation in vivo, the transcriptional network that specifies the 5-HT phenotype in neural crest derivatives (enteric and parafollicular cells) involves *Mash1*, *Nkx2.2* and other factors (Gershon, 1997; Pfaar et al., 2002). These data were in concordance with the observation in the present study that 5-HT promotes neural differentiation in ES cells cocultured with MS5 cells. A recent investigation also reported that 5-HT could facilitate the differentiation of human monocytes into dendritic cells in the presence of stimulatory signals (Katoh et al., 2006), further suggesting the differentiation-promoting effect of 5-HT may be based on the cytokine microenvironment in the local tissue.

In ES cell cultures, identification of the signaling pathways involved in generating and maintaining the differentiated cell types is a key to controlling differentiation to form specific cell types. In the neural induction of ES cells, blockade of Notch signaling by the γ-secretase inhibitor inhibits neural lineage commitment and directs ES cells to a non-neural fate, suggesting the important role of Notch signaling in directing neural differentiation of ES cells. In the study, 5-HT was shown to promote neural differentiation of ES cells cocultured with MS5. However, 5-HT can not revert the γ-secretase inhibitor-mediated inhibition of neural differentiation, suggesting there may be crosstalks between 5-HT signaling and Notch signaling. The 5-HT signaling pathway and Notch signaling pathway may have some signaling molecules in common during signal transduction, and involved molecules owned by both pathways are supposed to act

downstream of the γ-secretase. Recently, accumulating evidence has suggested that 5-HT directs diverse cell behaviors through certain signaling molecules, such as phosphatidylinositol-3-kinase (*PI3K*), *Akt* and mammalian target of rapamycin (*mTOR*) (Katoh et al., 2006; Liu et al., 2004). 5-HT could induce smooth muscle cell (SMC) proliferation by activating *PI3K/Akt* and *mTOR*/p70 ribosomal S6 kinase 1 (*S6K1*) pathways (Liu and Fanburg, 2006). The inhibition of *mTOR* with rapamycin could significantly reduce SMC proliferation caused by 5-HT, and both *mTOR* and *PI3K* are involved in regulation of the downstream effector *S6K1* activation (Aeder et al., 2004; Wang et al., 2001; Liu and Fanburg, 2006). 5-HT, together with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, could promote the differentiation of human monocytes into dendritic cells (Katoh et al., 2006). 5-HT was noted to induce an increase in *Akt* activation in a neuroblastoma cell line expressing 5-HT_{1B} receptors (Hsu et al., 2001). *Akt* was identified as the downstream target of *PI3K* in developmental and survival signaling (Liang and Slingerland, 2003; Lawlor and Alessi, 2001).

The signaling molecules, *Akt* and *mTOR*, were also noted to be involved in Notch signaling and act downstream of the γ-secretase (Androutsellis-Theotokis et al., 2006; Cantley, 2002). Previous investigations showed that Delta family members, as Notch ligands, could induce γ-secretase-dependent phosphorylation of two principal activation sites on *Akt* (Artavanis-Tsakonas et al., 1999; Cantley, 2002), downstream of which *mTOR* is a key regulator of cell behavior (Navé et al., 1999). Likewise, the Notch ligand *Jagged1* could also cause transient phosphorylation of *mTOR* (Androutsellis-Theotokis et al., 2006). In earlier reports, *STAT3* was shown to be a target of *Akt* and *mTOR*, and act as a transcription factor activated at the cell surface by the *gp130 LIF* receptor and the *JAK* tyrosine kinase (Levy and Darnell, 2002). Extracellular ligands that promote

phosphorylation of *STAT3* could stimulate the differentiation of CNS stem cells to a glia fate (Rajan and McKay, 1998), and the γ-secretase inhibitor was reported to reduce the basal amounts of phosphorylated *STAT3*, suggesting that endogenous γ-secretase has a role in *STAT3* phosphorylation which mediates cell behavior upon Notch activation (Kamakura et al., 2004; Artavanis-Tsakonas et al., 1999). Based on these observations, it is intuitive that *Akt/mTOR/STAT3* pathway may be the common pathway involved in both 5-HT signaling and Notch signaling. In view of the fact that 5-HT could not override the inhibitory effect of γ-secretase on neural differentiation of ES cells, the γ-secretase-dependent activation of *Akt* and other signaling molecules was supposed to be a key constituent in the 5-HT signaling and underlie the activity of 5-HT in the induction culture of ES cells.

To our knowledge, this study provided the first evidence that 5-HT could enhance the in vitro expansion of ES cell derivatives, and synergistically promote neural commitment of ES cell derivatives in the presence of MS5.

CHAPTER 7

NEURAL DIFFERENTIATION OF HUMAN ES CELLS

The neural induction effect of MS5 cells observed on mouse ES cells was tested and evaluated by using contact and non-contact cocultures with human ES cells. MS5-conditioned medium (MS5-CM) at different concentrations was also analyzed by comparing the cell viability and immuno-positivity of the neural precursor marker Musashi-1. Human ES cell derivatives from contact cocultures with MS5 were propagated and selected in subsequent cultures. The number of total viable cells was enumerated and derived cells were characterized immunochemically for lineage-specific markers. RT-PCR was also conducted to investigate the expression of lineage-specific genes in the derived cells.

7.1 Contact coculture with MS5 cells

In four independent experiments of 12 replicate studies for H14 cell derivatives after eight days' culture, the cell viability and number of Musashi-1$^+$ cells derived from contact coculture with MS5 were 85.9 ± 3.3% and 46.9 ± 9.2% respectively, significantly higher than those derived from non-contact coculture (cell viability: non-contact coculture vs. contact coculture, 66.5 ± 6.8% vs. 85.9 ± 3.3%, $P = 0.0002$; Musashi-1$^+$ cells: non-contact coculture vs. contact coculture: 35.9 ± 9.1% vs. 46.9 ± 9.2%, $P = 0.002$).

The cell viability and Musashi-1$^+$ cells derived from H14 cell derivatives in contact coculture were also significantly higher than those observed in cultures supplemented with 50% and 67% MS5-CM (viability: 50% MS5-CM vs. contact coculture: 31.9 ± 5.7%

vs. 85.9 ± 3.3%, P = 0.0001; 67% MS5-CM vs. direct MS5 coculture 25.3 ± 6.0% vs 85.9 ± 3.3%; P = 0.0004; Musashi-1$^+$ cells: 50% MS5-CM vs. contact coculture 27.7 ± 8.2% vs. 46.9 ± 9.2%, P = 0.0006, 67% MS5-CM vs. contact coculture 28.6 ± 7.6% vs 46.9 ± 9.2%, P = 0.0001). The inducing effect of MS5 on human ES cells through contact coculture was attested by using another human ES cell line H9 (Figure 7-1), demonstrating the superiority of contact coculture to non-contact coculture and culture in MS5-CM, in terms of neural induction efficiency

7.2 In vitro differentiation of human ES cells directed by the culture system

Undifferentiated human ES cells H14 with a high nucleus-to-cytoplasm ratio and clearly defined colony periphery on the monolayer of MEF were noted (Figure 7-2 a, e). Human ES cell colonies were dissociated and cultured for two weeks on MS5 stromal feeder cells. In human ES cell derivatives, cells located at the center of the colonies displayed a necrotic morphology, whereas living cells gradually developed a columnar morphology and formed neural tube-like rosettes (Figure 7-2 b, f). After two weeks, immunofluorescent staining in four experiments of 12 replicate studies indicated that 54.7 ± 9.6% of the enzymatically segregated cells were immunoreactive to the neural precursor marker Musashi-1, significantly higher than 30.4 ± 7.0% Musashi-1$^+$ cells in the control culture without MS5 (P = 0.0004; Figure 7-2 g). Over the next two weeks of cultures in medium supplemented with 50% MS5-CM, cell foci with rosette morphology grew to small clusters (Figure 7-2 c). Immunostaining showed that 80.5 ± 8.3% of individual cells expressed Musashi-1, which were in marked contrast to 42.6 ± 5.6% Musashi-1$^+$ cells derived from control cultures run in parallel (P = 0.0007; Figure 7-2 g). Subsequently, in the final two-week selection culture in Neurobasal medium

supplemented with N2 and B27, cells with large nuclei, discrete nucleoli and cytoplasmic processes were noted (Figure 7-2 d), which were molecularly positive to the post-mitotic neuronal marker *MAP-2* and midbrain dopaminergic neuronal marker *Nurr-1* as indicated by RT-PCR (Figure 7-3), suggesting the maturation of derived neural cells. Besides, Musashi-1$^+$ cells accounted for 89.3 ± 5.9%, being significantly higher than 65.9 ± 8.4% in the control cultures at the final stage (P = 0.0007, Figure 7-2 g). Compared to the control cultures, the contact coculture with MS5 follwed by subsequent serial cultures exhibited a superior ability in promoting neural differentiation of human ES cells.

In four individual experiments of 12 replicate studies, the numbers of viable cells at the end of the contact coculture stage in the initial two weeks, were significantly higher than those derived from the control culture (H14-derived cells with vs. without MS5 coculture, 4.43 ± 1.01 × 10^5 per well vs. 6.14 ± 1.85 × 10^4 per well, P = 0.0003; H9-derived cells with vs. without MS5 coculture, 3.75 ± 0.73 × 10^5 per well vs. 6.39 ± 1.71 × 10^4 per well, P = 0.0005). Data suggested that MS5 could elicit growth-enhancing effects on human ES cell derivatives. After propagation in cultures supplemented with 50% MS5-CM, MS5-induced H14-derived cells were dissociated enzymatically and 1 × 10^5 cells were seeded onto poly-L-ornithine- and laminin-coated wells. A mean number of 8.95 ± 2.66 × 10^5 cells per well was derived after selection culture in N2B27-supplemented Neurobasal medium, being significantly higher than that derived from the control culture (3.91 ± 1.47 × 10^5 cells per well, P = 0.0009; data derived from four individual experiments of 12 replicate studies). Similarly, after 1 × 10^5 MS5-induced H9-derived cells were seeded onto each well, 8.54 ± 2.25 × 10^5 cells per well were derived after the selection culture stage in Neurobasal medium, being significantly higher than those derived from the control culture (4.13 ± 1.22 × 10^5 cells per well, P = 0.0002; data derived from four individual experiments of 12 replicate studies). These data

demonstrated that, in comparison to the control culture, more neural precursor cells could be derived after MS5 contact coculture and subsequent propagation culture in 50% MS5-CM. Derived precursor cells could further grow and proliferate during the selection culture stage in Neurobasal medium, and form a higher number of viable cells than that derived from the control culture

7.3 Molecular analysis of human ES cell derivatives

RT-PCR for genes in MS5-induced human ES cell-derived cells demonstrated the expression of *Nestin* and *Musashi-1* throughout the cultures since day 8 (Figure 7-3a). The astrocytic gene *GFAP* and the neuronal gene *MAP-2* were noted to express at the end of two-week contact coculture phase (Figure 7-3a). The transcription factor *Nurr-1*, which was required for the development of ventral midbrain and differentiation of neural precursors into dopaminergic neurons, was detected at week four, the end of the propagation culture phase in 50% MS5-CM (Figure 7-3a). Notably, expression of the mesodermal gene *zeta-globin* was also evident on day 8 during coculture with MS5 but disappeared afterwards (Figure 7-3a). The osteogenic differentiation genes, *Osteocalcin* and *Osteopontin*, persisted on day 8 culture until week four, the end of the propagation culture phase in MS5-CM after contact coculture (Figure 7-3a). The expression of the chondrogenic gene *Aggrecan* and the adipogenic gene *α-P2* lost progressively upon completion of induction culture on week two (Figure 7-3a). However, the expression of *Oct4* and alkaline phosphatase (*ALP*) was noted until week two, the end of MS5 contact coculture phase (Figure 7-3a).

Compared to control cultures without MS5 support, MS5 coculture enhanced the expression of the astrocytic gene *GFAP*, the neuronal gene *MAP-2*, and the mature midbrain neuron gene *Nurr-1* (Figure 7-3a). The genes of *Oct-4* and *ALP* were not detected in MS5-induced human ES cell-derived cells, in marked contrast to the evident

expression of *Oct-4* and *ALP* in human ES cell derivatives without MS5 support (Figure 7-3a). Meanwhile, after MS5 coculture phase and subsequent propagation culture in MS5-CM, expression of non-neural genes *zeta-globin*, *Osteopontin* and *α-P2*, was not evident in human ES cell H14-derived cells on week four, as compared to the persistent expression in control cultures without MS5 (Figure 7-3a). Also, the similar observations could be derived by using another human ES cell line H9 (Figure 7-3b). Data indicated that the MS5 coculture strategy could enhance neural differentiation of human ES cells and the non-neural commitment could be suppressed by MS5 contact coculture and subsequent culture in MS5-CM, whereas the selection culture in Neurobasal medium helped promote the maturation of derived neural cell.

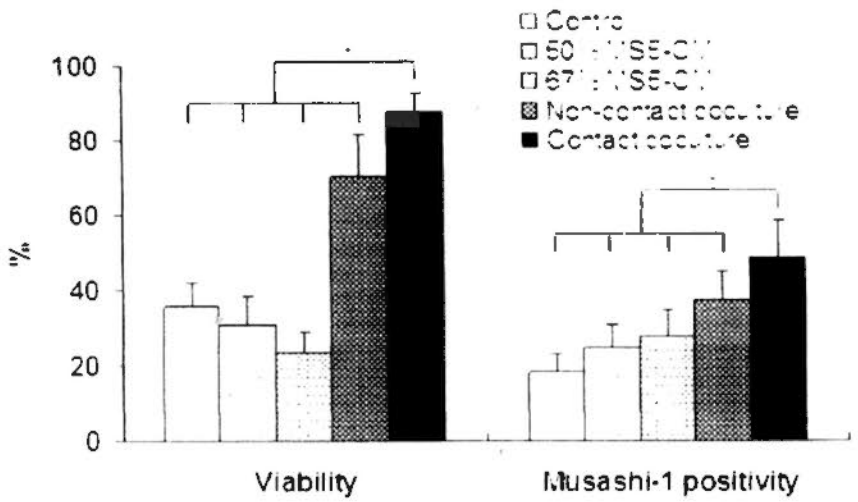

Figure 7-1 Viability and Musashi-1 positivity of human ES cell H9-derived cells after induction for eight days in contact and non-contact cocultures with MS5, and in 67%, 50% MS5-conditioned medium (MS5-CM). * $P < 0.05$ contct coculture vs. any other culture group (data derived from four independent experiments of 12 replicate studies).

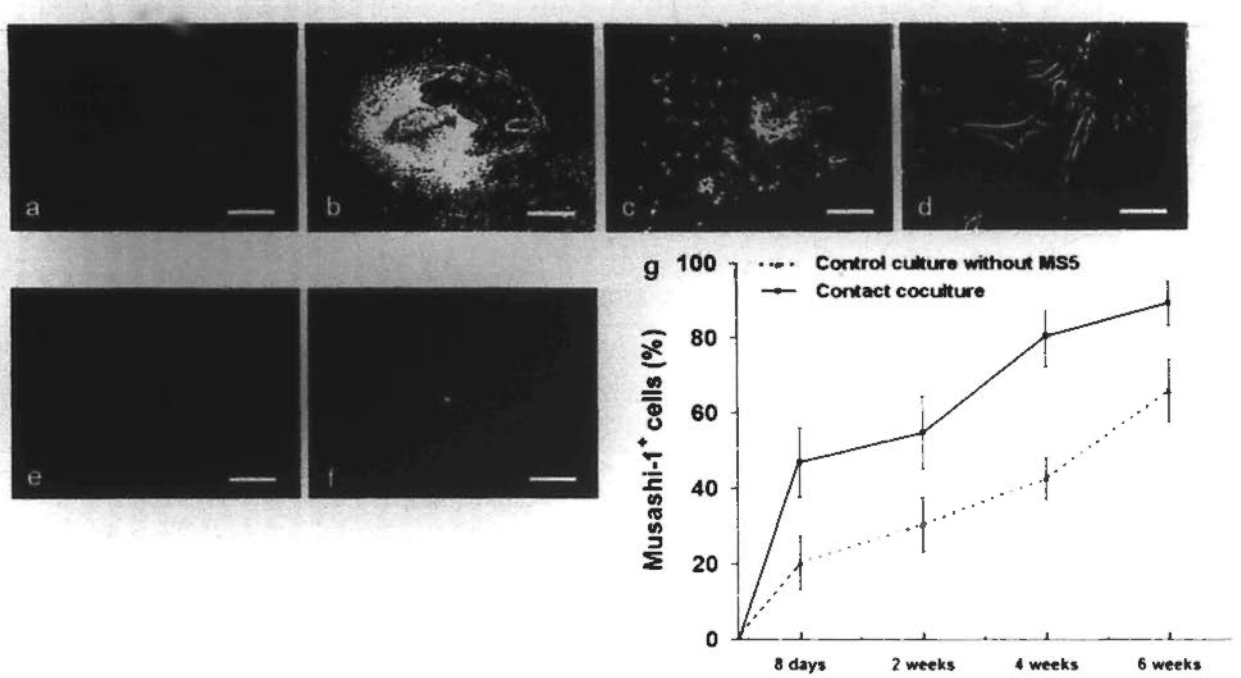

Figure 7-2 Morphology and Musashi-1 immunoreactivity of human ES cell H14-derived cells in cocultures with MS5 for two weeks, followed by two-week propagation culture in 50% MS5-conditioned medium (MS5-CM) and two-week selection culture in Neurobasal medium. Initially, the MEF feeder was used to maintain the undifferentiated human ES cell (H14) colony (a), which exhibited SSEA-4 positivity (e). In the neural induction culture, human ES cells H14 were induced by contact coculture with MS5 for two weeks, followed by two-week propagation culture in 50% MS5-CM and two-week selection culture in Neurobasal medium. Upon completion of coculture, H14 derivatives formed rosette-like structures (b), which were immunoreactive to Musashi-1 (f). During the propagation culture in MS5-CM, H14 derivatives further grew to small clusters (c). Upon completion of selection culture in Neurobasal medium for the final two weeks, cells with the morphology of large nuclei, discrete nucleoli and cytoplasmic processes were noted (d). g: Along the serial cultures, Musashi-1$^+$ cells increased progressively, being significantly higher than those derived from control cultures without MS5 run in parallel. Nuclei in (e, f) were counter-stained with propidium iodide (PI). Scale bars, 50μm for a, b, c, f and 20μm for d, e.

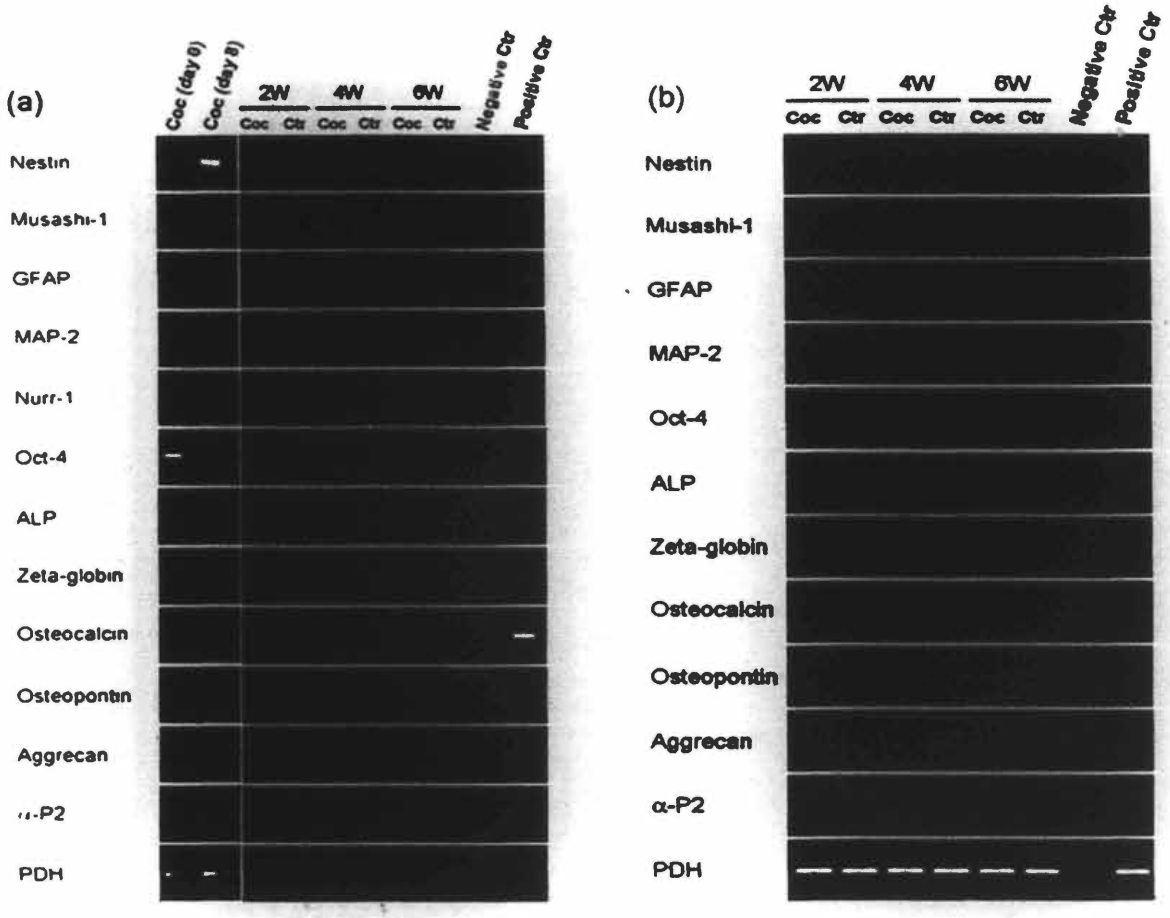

Figure 7-3 Ethidium bromide-stained agarose gel of electrophoresed RT-PCR products derived from human ES cell-derived cells. (a): gene expression of cell derivatives from human ES cell H14 upon completion of maintenance culture (day 0), 8-day induction culture on the MS5 feeder (day 8), 2-week induction culture on the MS5 feeder (2W), 2-week propagation culture in MS5-CM (4W) and 2-week selection culture in supplemented Neurobasal medium (6W); (b): gene expression of cell derivatives from human ES cell H9 upon completion of 2-week induction culture on the MS5 feeder (2W), 2-week propagation culture in MS5-CM (4W) and 2-week selection culture in supplemented Neurobasal medium (6W). Coc: human ES cell derivatives cocultured with MS5; Ctr: control culture of human ES cell derivatives without MS5 coculture. Negative Ctr: negative controls (reagent blanks); Positive Ctr: positive controls (human brain tissue for Nestin, Musashi-1, GFAP, MAP-2 and Nurr-1; undifferentiated Hes cells H14 for Oct-4 and ALP; human umbilical cord tissue for Zeta-globin; human bone marrow for Osteocalcin, Osteopontin, Aggrecan, α-P2 and PDH).

7.4 Discussion

The human ES cells are known for their ability to propagate indefinitely in culture as undifferentiated cells, which can be induced to differentiate in vitro and in vivo into various cell types (Thomson et al., 1998). These features made human ES cells a valuable source of cell types for cell biological investigation, pharmaceutical screening, and cell replacement therapies for degenerative diseases. Currently, there is a great need for novel and efficient therapies which can be used to treat patients with neurological disorders such as multiple sclerosis, Parkinson's disease, Alzheimer's disease, and stroke. The emergence of human ES cells as an experimental and therapeutic resource represents a major opportunity for brain repair. If human ES cells are to be harnessed effectively for these goals, it will be necessary to develop robust methods for directing neural commitment and simultaneously suppressing non-neural differentiation. Insights from induction systems of mouse ES cells provide a platform for studies on human ES cells, notwithstanding inter-species differences (Dahéron et al., 2004; Rajesh et al., 2007).

The protocol presented here offered a simple and efficient method for the targeted differentiation of human ES cells towards the neural lineage. This protocol was distinctive from previous reports. The neural induction mediated by MS5 coculture was further extended by using MS5-conditioned medium (MS5-CM). Usage of MS5-CM for propagation culture replaced the exhausted MS5 feeder cells after two-week coculture. In addition, prior to propagation culture in MS5-CM, there was no mechanical dissection and enzymatic digestion of derived cells from MS5 cocultures. Accordingly, the integrity of human ES cell derivatives was maintained and cell death was minimized. Thus, not only the protocol could provide favorable conditions to induce neural differentiation of human ES cells, but also it would yield cells with lesser *in-vitro* manipulation for study on the molecular mechanism and signal transduction involved in differentiation.

Understanding molecular interactions in mammalian development, particularly those underlying human development, is hindered by the limited accessibility to early embryos and inadequate amount of stage- and cell type-specific materials. These hurders may now be overridden by the use of human ES cells. The establishment of *in-vitro* differentiation models that recapitulate normal development will form the foundation for dissecting molecular interactions. As indicated in previous investigations (Tropepe et al., 2001, Ying et al., 2003), the dissociation of mouse ES cell colonies to individual discrete cells in a chemically defined medium, results in the acquisition of neuroectodermal markers, Sox-1, Nestin and Musashi-1. However, unlike mouse ES cells, discrete human ES cells do not grow well and give a low efficiency for neural induction. Besides, the single cell system does not resemble normal embryonic development in which cell-cell interactions are crucial. The amount of derivatives generated from single cells is often not amenable to the large-scale chemical analysis and clinical application. Thus, the present study employs a modified strategy in which small clusters of human ES cells were differentiated into neural lineage cells in chemically defined media. The present culture system yields a robust neuroectodermal differentiation while preserving complex cellular interactions within the cluster. In view of the fact that human ES cells are derived from a 5 – 6 day-old embryo, the generation of columnar neuroepithelia and the formation of neural rosettes at the end of the initial two-week induction culture reflect the normal *in-vivo* gastrulation of a human embryo at the beginning of the third week. Thus, the present culture system represents a useful *in-vitro* model to study early human brain development, and provides a controlled environment for examining the relationships among extracellular signals and intracellular machineries that lead to neural lineage commitment. Early analysis revealed that human neuroectodermal cells at different developmental stages produce different subclasses of neurons in response to the same set of morphogens,

suggesting that environmental factors affect cell fate (Zhang, 2006). In this study, specific genes of different subtypes of neural lineage cells were demonstrated to express in a time-dependent manner, indicating that the culture system might simulate a neurotrophic niche to induce neural fate of human ES cells.

MS5 have been demonstrated to support the proliferation of haematopoietic progenitor cells in long-term cultures (Issaad et al., 1993). Recently, stromal cells derived from mouse bone marrow, PA6, M2-10B4 and MS5, were shown to induce neural differentiation of ES cells in coculture systems (Lee et al., 2007, Becerra et al., 2007, Morizane et al., 2006). In this study it was noted that the neurotrophic effect of MS5 on ES cells was similar to the PA6 stromal cell-derived inducing activity (Kawasaki et al., 2000; Kawasaki et al., 2002). MS5-CM was also found to exert the neural inducing effect, though to a lesser extent, which may suggest the presence of soluble neurotrophic and neuroprotective factors secreted by MS5. As demonstrated by Figure 5-1, contact coculture with MS5 cells may facilitate activation of Notch signaling cascade in human ES cells and promote their neural differentiation. With respect to neural induction efficiency, contact coculture with MS5 was shown to be superior to non-contact MS5 coculture and culture in MS5-CM. In addition to the neurotrophic and neuroprotective effects of soluble factors mediated by MS5, the cell-cell interaction of MS5 and human ES cells, through the Notch signaling, might also account for the propensity of neural commitment of human ES cells.

The challenge in neural induction of human ES cells is how to precisely suppress the diversity of embryonic development and impose unitary lineage commitment. This study indicated that the culture protocol of MS5 coculture, in combination with downstream propagation and selection culture, could be used to efficiently direct differentiation of human ES cells towards the neural fate.

CHAPTER 8

IN VIVO STUDY OF MOUSE ES CELL-DERIVED NEURAL LINEAGE CELLS

Labelled with BrdU, MS5-induced ES cell-derived neural lineage cells were implanted into the striata of mice having undergone transient occulusion of bilateral common carotid arteries. Cognitive functions in terms of spatial learning and memory ability of normal mice and ischemic mice with and without cell transplant were tested by using a water maze system. Three weeks post transplant, mice were sacrificed. Migration and homing of donor cells were tracked. Histological examination of the hippocampal CA1 region was also conducted to assess the degree of regeneration.

8.1 Ischemic injury to the hippocampus

It has been documented that the hippocampus is the most vulnerable part of the brain to ischemic injury. Accordingly, the number of intact CA1 pyramidal neurons in the hippocampus was used as an index to reflect the severity of ischemic damage. Thionin staining demonstrated a remarkable number and extent of pyknotic cells and necrosis, respectively, in the hippocampal CA1 region at 12 hours shortly after ischemic attack (Figure 8-1). The numbers of morphologically intact pyramidal neurons with large nuclei, discrete nucleoli and clear cell periphery dropped extensively on day three post-induction of ischemia, compared to those derived from intact mice and mice at 12 hours and one day having undergone induction of ischemia (Figure 8-1). There was no significant difference in the numbers of intact pyramidal neurons on day three and day five (Figure 8-1). Thus, cell transplantation was conducted on day three post-induction of ischemia.

8.2 Improvement of cognitive functions in mice with cell

transplant

Immunostaining of BrdU in ES cell-derived neural lineage cells showed that the labelling efficiency was 64.6 ± 4.8%. In four separate experiments, trypan blue dye exclusion tests demonstrated that the mean ± SD cell viability of enzymatically segregated BrdU-labelled D3-derivrd cells was 67.2 ± 4.4% MS5-induced ES cell-derived neural lineage cells (5×10^5) were implanted bilaterally onto the caudate putamens of 17 ischemic mice three days after stroke, at which time an extensive loss of approximately 80% of pyramidal neurons was evident.

Having undergone ischemic induction, 85.7% (24/28) mice survived and exhibited signs of disturbance of consciousness, drowsiness, paucity of movement and coma. Two weeks post-transplant no death was encountered and mice did not display abnormal behaviour or develop dyskinesia, suggesting no obvious adverse reactions of intracranial implantation of ES cell-derived neural cells into the caudate putamens

On day five of behavioural assessment (19 days post-transplant), the episodes of transplanted ischemic mice entering blind alleys of the water maze were significantly less than that committed by ischemic control mice (episodes of error committed by ischemic mice with vs. without cell therapy, 4 ± 3 vs. 11 ± 3, p=0.0001), but comparable to that of sham-operated normal mice (episodes of error: transplanted ischemic mice vs. sham-operated normal mice: 4 ± 3 vs. 2 ± 1, p = 0.074). Figure 8-2 shows the time latency of 17 ischemic mice with cell therapy and eight sham-operated normal mice to escape from water over five days of behavioural assessment. Seventeen transplanted ischemic mice exhibited a progressive reduction of retention time in the water maze, compared to a relatively constant time latency derived from seven ischemic control mice. On day five of behavioural assessment, transplanted mice took significantly less time to get out of the maze (ischemic mice with [n=17] vs. without [n=7] cell therapy, 62.2 ± 17.9 seconds vs.

174.6 ± 33.7 seconds, p = 0.0002). There was no difference in the time latency between transplanted ischemic mice and eight sham-operated normal mice (transplanted ischemic mice [n=17] vs. sham-operated normal mice [n=8]: 62.2 ± 17.9 seconds vs. 54.9 ± 15.4 seconds, p = 0.439). Motor functions of transplanted ischemic mice and sham-operated normal control mice were comparable as evident by a similar swimming speed tracked on an 80-cm straight path of the water maze on day five of testing (transplanted ischemic mice vs. sham-operated normal control mice: 27.2 ± 3.5 cm/second vs. 29.0 ± 4.5 cm/second, p=0.304). Data suggested that cell therapy helped improve spatial learning and memory ability of ischemic mice.

8.3 Donor cell-mediated hippocampal repair

Mice were sacrificed three weeks post-transplant. It was noted that, from the injection site, transplanted cells spread widely into the surrounding parenchyma (Figure 8-3). Immunofluorescence staining of BrdU revealed a substantial number of the donor cells in the caudate putamen. BrdU$^+$ cells were also evident in the cortex, corpus callosum (Figure 8-3) and hippocampus (Figure 8-4). There were significantly greater numbers of intact pyramidal neurons in the hippocampal CA1 regions of ischemic mice with than without cell implants. The densities of pyramidal neurons were comparable between transplanted ischemic mice and sham-operated normal control mice (Figure 8-4). A lesser degree of lesion of the hippocampal pyramidal layer in transplanted ischemic mice than non-transplanted ischemic mice was also seen (Table 8-1). Among 17 transplanted ischemic mouse, one was noted to develop teratoma (p=0.059). Data suggested that transplanted cells helped protect the hippocampal CA1 region from ischemic damage and enhance regeneration.

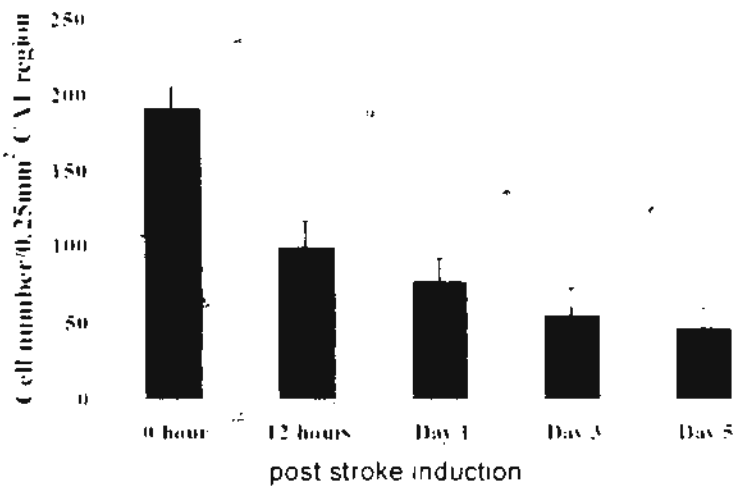

Figure 8-1 Scoring of thionin-stained pyramidal neurons with large nuclei, discrete nucleoli and clear cell periphery in every fifth serial coronal sections of the CA1 region of the hippocampus 1.8 – 2.0 mm posterior to the bregma of 12 mice 12 hours to five days after ischemic stroke and three sham-operated control mice. Data were derived from counting cells in 24 frames of 1 × 0.25 mm dimension from totally 8 – 10 coronal sections per mouse. * $P<0.05$.

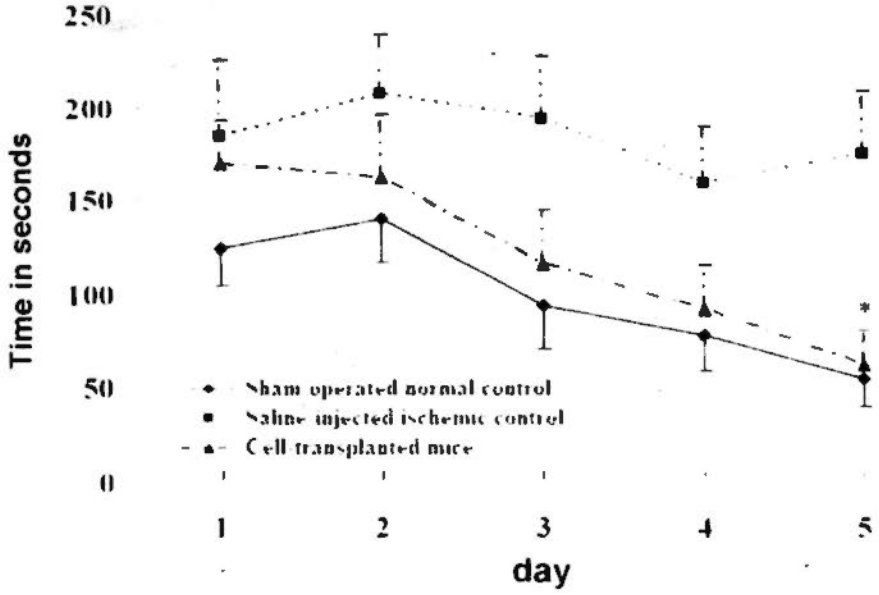

Figure 8-2 Mean ± SD retention time over five consecutive days in the water maze taken by ischemic mice with (n = 17) and without cell therapy (n = 7) and eight sham-operated normal control mice two weeks post transplant. * Ischemic mice with vs. without cell therapy: P = 0.0002.

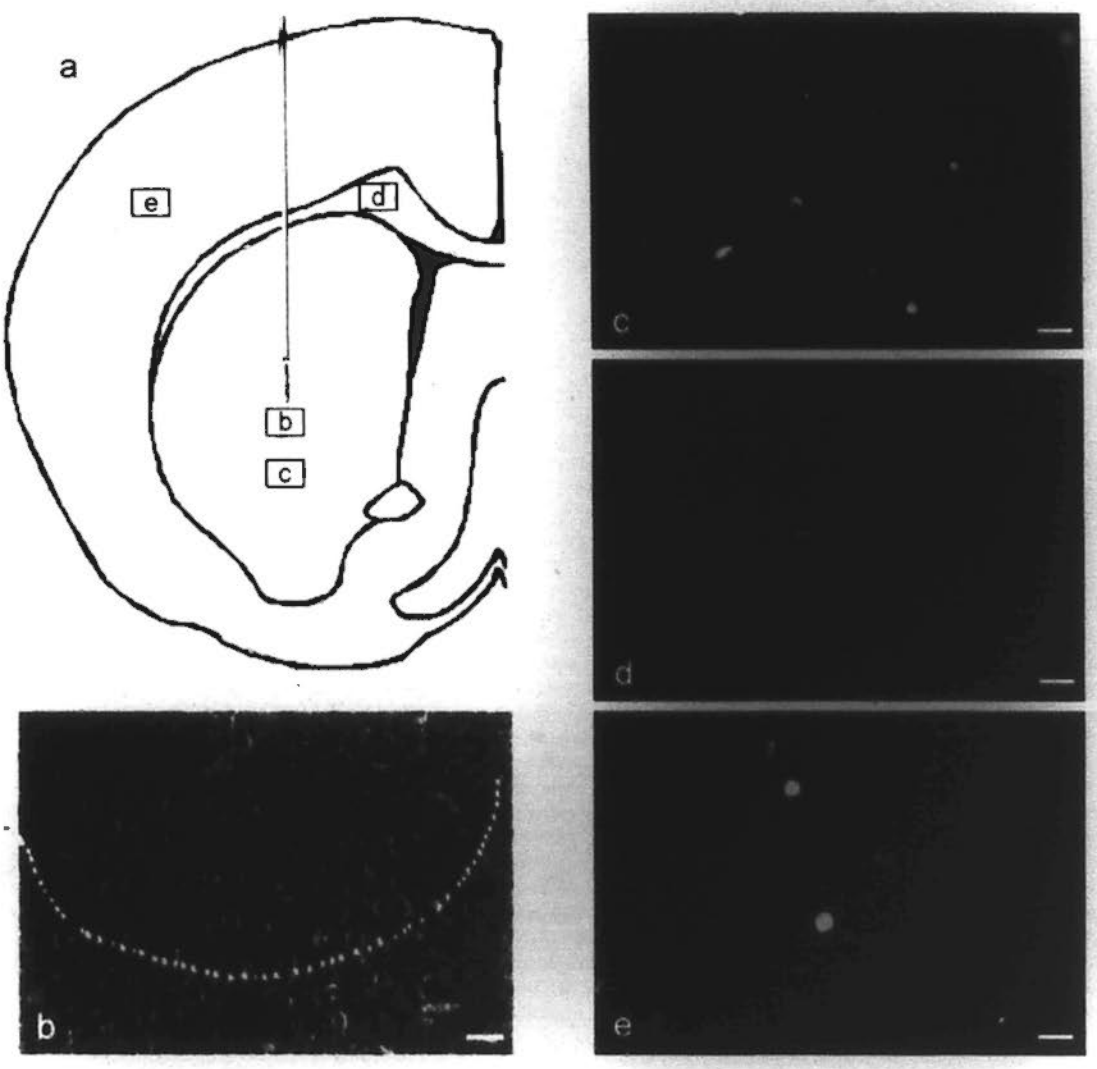

Figure 8-3 Distribution of transplanted cells in the brain parenchyma at three weeks post transplant. a: Illustration of extensive distribution of transplanted cells (green dots) in the coronal section of the mouse brain at 0.5mm anterior to the bregma; b~e in (a) represent the corresponding localities where the images of b~e are taken respectively; b: From the injection site (as indicated by the arrowhead), transplanted cells spread widely into the surrounding parenchyma and the interface between transplanted cells and host cells could be approximately recognized (as shown by the yellow dash line); c, d, e: Immunostaining indicated that BrdU-labeled transplanted cells (with green nuclei) spread widely in the caudate putamen (c), corpus callosum (d) and cortex (e) of the host brain three weeks post transplant, suggesting the survival and migration behavior of transplanted cells. Cell nuclei in c, d, e were counterstained with DAPI; Scale bars, 25μm for b and c, 20μm for d and e.

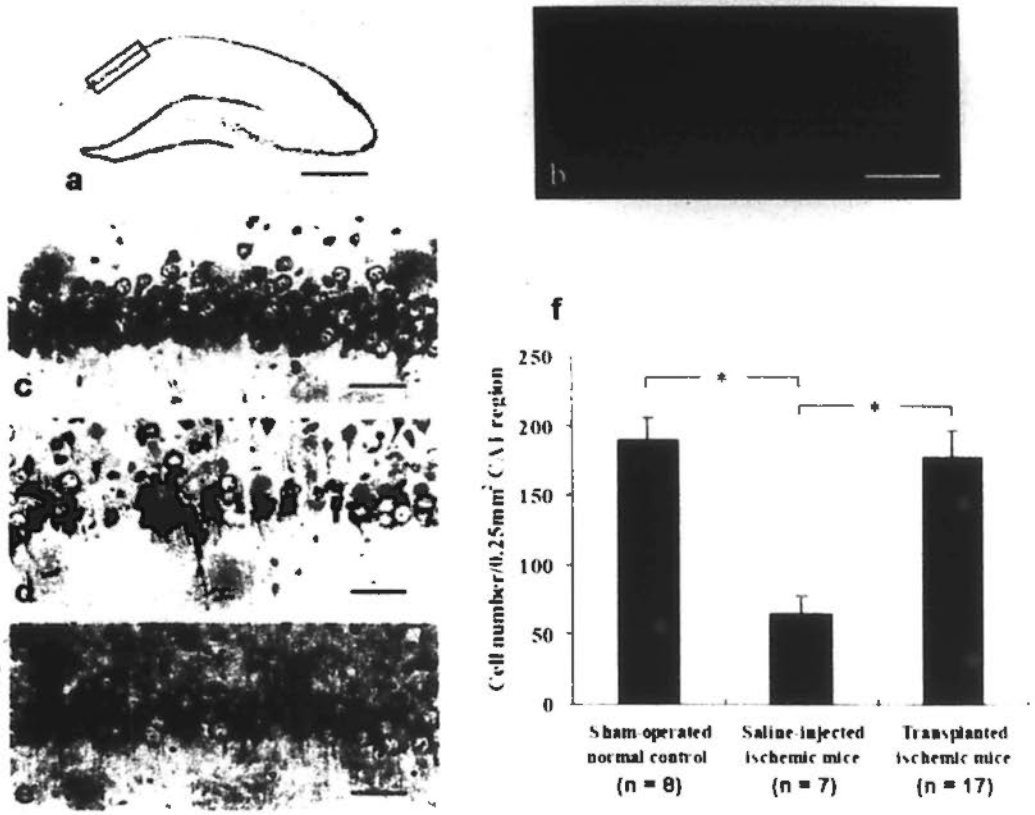

Figure 8-4 Histology of pyramidal neurons in the hippocampal CA1 region at three weeks post transplant. a: a representative coronal section of the entire thionin-stained hippocampus with illustration of a counting frame; b: Immunostaining revealed the homing of few BrdU-labeled ES cell-derived cells (green fluorescent nuclei) to the injured hippocampal CA1 region; c: a representative section derived from sham-operated normal control mice demonstrating intact pyramidal neurons; d: a representative section derived from saline-injected ischemic control mice showing conspicuous necrosis and abundant pyknotic cells; e: a representative section derived from transplanted ischemic mice exhibiting hippocampal CA1 neurons in orderly alignment; f: Quantification of intact pyramidal neurons showed the numbers derived from transplanted ischemic mice were comparable to those of sham-operated normal control mice (CA1 neuron density in transplanted ischemic mice vs. sham-operated normal control: 177.0 ± 19.1 cells/0.25mm^2 vs. 189.9 ± 15.6 cells/0.25mm^2, p = 0.089), but were significantly higher than those of ischemic control mice with saline injection (ischemic mice with vs. without cell therapy: 177.0 ± 19.1 cells/0.25mm^2 vs. 65.6 ± 12.4 cells/0.25mm^2, p = 0.0008). Scale bar, 500 μm for a, 50 μm for b, c, d and e. * P<0.05.

Table 8-1 Histological grading of pyramidal neurons in CA1 region of hippocampus

Group	Score			
	0	I	II	III
Sham-operated control mice (Group A, n=8)	8	0	0	0
Ischemic mice with saline injection l (Group B, n=7)*	0	0	3	4
Ischemic mice with cell therapy (Group C, n=17)#	11	5	1	0

Score	Criteria
0	no necrosis or less than 10% of total cells with pyknotic morphology within the counting frame
I	scattered single cell necrosis or 10 – 40% of total cells with pyknotic morphology
II	40 – 70% of total cells with pyknotic morphology
III	almost complete cell necrosis or more than 70% of total cells with pyknotic morphology

* $P < 0.05$: Group B vs. Group A

# $P < 0.05$: Group C vs. Group B, and $P > 0.05$: Group C vs. Group A

8.4 Discussion

The study demonstrated that intracranial implantation of ES cell-derived neural lineage cells onto the caudate putamens of cerebral ischemic mice could help recover cognitive functions of spatial learning and memory ability. Functional studies of ES cell-derived cells in animal models displayed contradictory results (Kim et al., 2002; Takagi et al., 2005). The interaction of neural progenitors, neurons and glial cells underline the significance of transplantation of neural lineage cells to the ischemic brain, which is characterized by a severe loss of different neural cell types. Previous studies demonstrated that fully maturated neurons survived less efficiently than neural progenitors upon implantation to the brain, which might be related to the susceptibility of mature neurons to mechanical stress and harvesting for transplant (Morizane et al., 2002). Cognitive impairment was found to correlate with the neuronal loss in the hippocampus (Semmler et al., 2007). Cells were transplanted to mice on day three after stroke to explore the therapeutic potential of ES cell-derived neural lineage cells, at which time the cell loss was most severe, accounting to approximately 80% loss in the hippocampal CA1 region. The ES cell-derived cell grafts, which were composed of largely neural stem cells and some post-mitotic neurons and glias (73.1 ± 3.0% nestin$^+$ neural stem cells, 14.2 ± 1.9% neurons, 6.2 ± 1.7% astrocytes and 5.2 ± 1.5% oligodendrocytes), were shown to improve the spatial learning and memory ability of ischemic mice significantly, as demonstrated by the water maze test.

It has been reported that, during normal development of the mouse, approximately 10 to 12 days post coitus are required for the occurrence of neurons in the ventral mesencephalon of the brain (Kawasaki et al., 2002). Transplanted ES cell-derived neural stem cells may exhibit their plasticity and further differentiate *in vivo* in response to the

environmental cues in the host brain in similar time duration. Behavioural assessments of the animals were conducted two weeks post transplant. The reversal of lesion-induced neurological deficits observed in the test animals two weeks post transplant might be attributable to the newly formed functional neurons.

Hippocampus is the major site of memory functions. To a lesser extent the caudate putamen is also critical for the procedural memory (Matthews and Silvers, 2004). Injured caudate putamen was noted to sustain the interaction between transplanted neural cells and striatal neurons (Di Porzio and Zuddas, 1992), making it a target of cell transplantation therapy for cerebral ischemic damage. Tracking of BrdU$^+$ cells in the study demonstrated that a majority of the implanted cells were localized in the caudate putamens, while some were evident in the cortex, corpus callosum and hippocampus. As BrdU intensity of proliferating cells will reduce and lose to an undetectable level, there may be an under-estimation of migration, homing and engraftment of donor cells to different localities of the ischemic brain. It was inconclusive that the small number of ES cell-derived cells engrafted to the lesioned hippocampus responded to the improved cognitive functions.

Data of the present and earlier studies demonstrated genes of neurotrophins, growth factors and chemokine, including *BDNF*, *CNTF*, *GDNF*, *NGF*, *NT-3*, *bFGF*, *IGF-I*, *IGF-II*, *VEGF*, *EPO* and *CXCL-12*, were expressed by neural stem cells, microglias and astrocytes (Fong et al., 2007; Abe, 2000). Intra-cerebral infusion of neurotrophic factors and growth factors was also noted to recruit endogenous progenitors in the peri-ventricular zone near the hippocampus to induce massive regeneration of CA1 pyramidal neurons after ischemia (Nakatomi et al., 2002; Kiprianova et al., 1999). In the study, readouts of the neuronal density and histological grading of pyramidal neurons in the hippocampal CA1 region supported the hypothesis of regeneration of neural stem and

progenitor cells present in the peri-ventricular region near the hippocampus subject to ischemic stress (Bingham et al., 2005). A comparable degree of cell necrosis, incidence of pyknotic cells and neuronal density was noted among sham-operated normal control mice and ischemic mice at three weeks post transplant, whilst a significant extent was evident in ischemic control mice. It is suggestive that the small number of ES cell-derived neural stem and progenitor cells engrafted to the hippocampus might enhance endogenous regeneration through the donor cell-derived neurotrophic and neuroprotective factors.

After *in-vitro* culture for 18 days (see Chapter 4), an increasingly higher percentage of neural lineage cells were derived, in particular Nestin$^+$ neural stem cells which accounted for 73.1 ± 3.0% among totally derived cells. RT-PCR analysis revealed neuroectodermal and neural related genes, *Otx-1*, *Pax-6*, *Nestin* and *Nurr-1*, but no mesodermal and endodermal genes and ES cell transcriptional factor *Oct-4*. Apparently, the culture system did not support non-neural differentiation and maintenance of undifferentiated status of ES cells, which may attribute to a single episode of teratoma development among 17 transplanted ischemic mice examined.

The findings of the study suggested that MS5 cells might exert the neurotrophic and neuroprotective effects to induce the differentiation of ES cells into neural lineage cells, which could effectively improve neurological deficits in the mouse ischemic stroke model. Having implanted onto the caudate putamens of ischemic mice, MS5-induced ES cell-derived neural lineage cells not only enabled to migrate, home and engraft to the injured brain parenchyma but also could enhance endogenous regeneration. Although the significant advancements in mouse stem cell research have provided important information on stem cell biology and offer great promise for developing novel successful stem cell-based medical treatments for neurodegenerative diseases, further investigations

appear to be necessary to translate the basic knowledge into clinical therapeutic applications in humans. Additional studies to optimize the experimental conditions for isolation, expansion, and differentiation of human stem/progenitor cells into specific differentiated cells in vitro and in vivo, are essential before their prospective use in treating human pathological disorders.

CHAPTER 9

CONCLUSION

The project described a novel technique for neural induction of mouse embryonic stem cells by utilizing a non-contact coculture system and the bone marrow stromal cell line MS5. During neural differentiation, the monoamine neurotransmitter serotonin could be used as a supportive agent. Neural induction of human embryonic stem cells could also be achieved by MS5 coculture. It was demonstrated that the promoting effects on neural induction were mediated by the Notch signaling. Derived neural lineage cells were able to engraft in the mouse model of cerebral ischemia and improve the impaired cognitive function.

Neural induction of mouse ES cells was mediated by using an 8-day non-contact coculture with the bone marrow stromal cell line MS5, followed by a 6-day propagation culture and a 4-day selection culture. The culture system yielded a relatively pure population of multipotent neural derivatives that can be further differentiated into neural subtypes with high efficiency. No need to generate embryoid bodies and non-contact coculture allow an efficient generation of neural lineage cells without feeder cell contamination.

Notch signaling was noted to play a role in the proliferation and differentiation of ES cells into neural lineage cells, but suppress non-neural commitment. A significant difference between mouse ES cells and human ES cells is the ability to grow individually in vitro. Discrete human ES cells tend to grow poorly. A dependence on the cell-cell interaction via the Notch signaling accounts for the self-renewal and neural differentiation of human ES cells.

This study provided the first evidence that 5-HT could enhance the proliferation of mouse ES cell-derived neural lineage cells, and synergistically promote neural commitment of mouse ES cells cocultured with MS5

The MS5 coculture system established for the neural induction of mouse ES cells was also applicable to human ES cells with slight modifications

The transplantation of MS5-mediated ES cell-derived neural lineage cells could effectively improve cognitive function in the mouse ischemic stroke model The implanted cells not only enabled to migrate, home and engraft to the injured brain parenchyma but also could enhance endogenous regeneration, suggesting the intracerebral injection of ES cell-derived neural lineage cells might be a potential treatment modality for stroke

CHAPTER 10
FUTURE STUDY

The significant advancements in stem cell research have provided important information on stem cell biology and offer great promise for developing novel ES cell-based regimes. However, a number of unanswered questions need to be addressed before stem cell-based therapies could be applied to the clinics. Further investigstions appear to be necessary to translate the basic knowledge into clinical therapeutic applications in humans.

(1) Animal cell-free culture conditions for human ES cell derivatives

In order to avoid the risk of animal pathogen transmission, human ES cells can be best cultured without animal cells, matrix, proteins or derivates. MEF feeder cells are conventionally used to maintain human ES cells. Several recent reports have demonstrated that human ES cells could be propagated without MEF support on laminin- or Matrigel-coated plastic surfaces (Thomson et al., 1998; Reubinoff et al., 2000, Xu et al., 2001), or by employing human feeder cells (Richards et al., 2002). Works should be done to formulate cell-free and serum-free medium to propagate and differentiate human ES cells.

(2) Decontamination of pluripotent ES cells from the derived cell product before transplantation

Pluripotent ES cells present a safety concern because of the teratoma development after transplantation. Normal or injured adult tissues lack the cues required to fully induce ES cells to form appropriate tissue-specific cell types (Nussbaum et al., 2007). The way to ensure the safety of cell transplantation therapy is to *in-vitro* differentiate ES cells into the desired cell types and purge any undifferentiated ES cells. So far, despite

considerable progress in directing the differentiation of ES cells, the purification of the derived cell product is desirable. Decontamination of undifferentiated ES cells from the derived cell product should be explored to pursue safety in the application of ES cell-derived cell therapy from bench to bedside

(3) Usage of GFP (green fluorescence protein) labeling to facilitate research on cell migration behavior

As BrdU could only label proliferative cells and BrdU intensity of proliferating cells will reduce and lose to an undetectable level, there may be an under-estimation of migration, homing and engraftment of donor cells to different localities of the ischemic brain. Early investigations have demonstrated that endogenous progenitors are abundant in the periventricular region near the hippocampus and these cells are involved in the regeneration of the hippocampus (Nakatomi et al., 2002; Kiprianova et al., 1999) Therefore, it is inconclusive that the small numbers of ES cell-derived cells that engraft to the lesioned hippocampus respond to the improved cognitive functions However, if GFP labeling is adopted subsequently, instead of BrdU labeling, all the derived neural lineage cells are likely to be labeled through transfection On this basis, more accurate number of transplanted donor cells could be detected in different localities of the ischemic brain after transplantation, facilitating the research on cell behaviors of migration and homing

REFERENCES

Abdouh M, Albert PR, Drobetsky E, Filep JG, Kouassi E. 5-HT1A-mediated promotion of mitogen-activated T and B cell survival and proliferation is associated with increased translocation of NF-kappaB to the nucleus. Brain Behav Immun. 2004; 18(1): 24–34.

Abdouh M, Storring JM, Riad M, Paquette Y, Albert PR, Drobetsky E, Kouassi E. Transcriptional mechanisms for induction of 5-HT1A receptor mRNA and protein in activated B and T lymphocytes. J Biol Chem. 2001; 276(6): 4382–8.

Abe K. Therapeutic potential of neurotrophic factors and neural stem cells against ischemic brain injury. J Cereb Blood Flow Metab. 2000; 20(10): 1393–408.

Aeder SE, Martin PM, Soh JW, Hussaini IM. PKC-eta mediates glioblastoma cell proliferation through the Akt and Mtor signaling pathways. Oncogene. 2004; 23(56): 9062-9.

Ahn S, Joyner AL. In vivo analysis of quiescent adult neural stem cells responding to Sonic hedgehog. Nature. 2005; 437(7060): 894–7.

Alexiades MR, Cepko CL. Subsets of retinal progenitors display temporally regulated and distinct biases in the fates of their progeny. Development. 1997; 124(6): 1119–31.

Alison MR, Golding MH, Sarraf CE. Pluripotential liver stem cells: facultative stem cells located in the biliary tree. Cell Prolif. 1996; 29(7): 373–402.

Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. Hepatocytes from non-hepatic adult stem cells. Nature. 2000; 406(6793): 257–61.

Allen ND, Barton SC, Hilton K, Norris ML, Surani MA. A functional analysis of imprinting in parthenogenetic embryonic stem cells. Development. 1994; 120(6): 1473–82.

Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature. 2003; 425(6961): 968–73.

Alvarez-Buylla A, Garcia-Verdugo JM. Neurogenesis in adult subventricular zone. J Neurosci. 2002; 22(3): 629–34.

Amireault P, Dubé F. Intracellular Camp and calcium signaling by serotonin in mouse cumulus-oocyte complexes. Mol Pharmacol. 2005; 68(6): 1678–87.

Anderson DJ. A molecular switch for the neuron-glia developmental decision. Neuron. 1995; 15(6): 1219–22.

Andrews PW, Matin MM, Bahrami AR, Damjanov I, Gokhale P, Draper JS. Embryonic stem (ES) cells and embryonal carcinoma (EC) cells: opposite sides of the same coin. Biochem Soc Trans. 2005; 33(Pt 6): 1526–30.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. Notch ignaling regulates stem cell numbers in vitro and in vivo. Nature. 2006; 442(7104): 823–6.

Appel SH, Engelhardt JI, Henkel JS, Siklos L, Beers DR, Yen AA, Simpson EP, Luo Y, Carrum G, Heslop HE, Brenner MK, Popat U. Hematopoietic stem cell transplantation in patients with sporadic amyotrophic lateral sclerosis. Neurology. 2008; 71(17): 1326–34.

Arai F, Suda T. Maintenance of quiescent haematopoietic stem cells in the osteoblastic niche. Ann N Y Acad Sci. 2007; 1106: 41–53.

Armstrong L, Hughes O, Yung S, Hyslop L, Stewart R, Wappler I, Peters H, Walter T, Stojkovic P, Evans J, Stojkovic M, Lako M. The role of PI3K/AKT, MAPK/ERK and NFkappabeta signalling in the maintenance of human embryonic stem cell pluripotency and viability highlighted by transcriptional profiling and functional analysis. Hum Mol Genet. 2006; 15(11): 1894–913.

Arrigoni O, De Tullio MC. Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta. 2002; 1569(1-3): 1–9.

Artavanis-Tsakonas S, Rand MD, Lake RJ. Notch signaling: cell fate control and signal integration in development. Science. 1999; 284(5415): 770–6.

Arvidsson A, Collin T, Kirik D, Kokaia Z, Lindvall O. Neuronal replacement from endogenous precursors in the adult brain after stroke. Nat Med. 2002; 8(9): 963–70.

Arvidsson A, Kokaia Z, Lindvall O. N-methyl-D-aspartate receptor-mediated increase of neurogenesis in adult rat dentate gyrus following stroke. Eur J Neurosci. 2001; 14(1): 10–8.

Asahara T, Kawamoto A. Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol. 2004; 287(3): C572–9.

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science. 1997; 275(5302): 964–7.

Atkins BZ, Hueman MT, Meuchel JM, Cottman MJ, Hutcheson KA, Taylor DA. Myogenic cell transplantation improves in vivo regional performance in ignaling rabbit myocardium. J Heart Lung Transplant. 1999; 18(12): 1173–80.

Aubert J, Dunstan H, Chambers I, Smith A. Functional gene screening in embryonic stem cells implicates Wnt antagonism in neural differentiation. Nat Biotechnol. 2002; 20(12): 1240–5.

Auffray I, Chevalier S, Froger J, Izac B, Vainchenker W, Gascan H, Coulombel L. Nerve growth factor is involved in the supportive effect by bone marrow-derived stromal cells of the factor-dependent human cell line UT-7. Blood. 1996; 88(5): 1608–18.

Awad HA, Butler DL, Boivin GP, Smith FN, Malaviya P, Huibregtse B, Caplan AI. Autologous mesenchymal stem cell-mediated repair of tendon. Tissue Eng. 1999; 5(3): 267–77.

Azmitia EC. Modern views on an ancient chemical: serotonin effects on cell proliferation, maturation, and apoptosis. Brain Res Bull. 2001; 56(5): 413–24.

Bain G, Gottlieb DI. Expression of retinoid X receptors in P19 embryonal carcinoma cells and embryonic stem cells. Biochem Biophys Res Commun. 1994; 200(3): 1252–6.

Bain G, Kitchens D, Yao M, Huettner JE, Gottlieb DI. Embryonic stem cells express neuronal properties in vitro. Dev Biol. 1995; 168(2): 342–57.

Baloyannis SJ, Costa V, Baloyannis IS. Morphological alterations of the synapses in the locus coeruleus in Parkinson's disease. J Neurol Sci. 2006; 248(1-2): 35–41.

Barberi T, Klivenyi P, Calingasan NY, Lee H, Kawamata H, Loonam K, Perrier AL, Bruses J, Rubio ME, Topf N, Tabar V, Harrison NL, Beal MF, Moore MA, Studer L. Neural subtype specification of fertilization and nuclear transfer embryonic stem cells and application in parkinsonian mice. Nat Biotechnol. 2003; 21(10): 1200–7.

Barker JN, Krepski TP, DeFor TE, Davies SM, Wagner JE, Weisdorf DJ. Searching for unrelated donor hematopoietic stem cells: availability and speed of umbilical cord blood versus bone marrow. Biol Blood Marrow Transplant. 2002; 8(5): 257–60.

Barrilleaux B, Phinney DG, Prockop DJ, O'Connor KC. Review: ex vivo engineering of living tissues with adult stem cells. Tissue Eng. 2006; 12(11): 3007–19.

Barry F, Boynton R, Murphy M, Haynesworth S, Zaia J. The SH-3 and SH-4 antibodies recognize distinct epitopes on CD73 from human mesenchymal stem cells. Biochem Biophys Res Commun. 2001; 289(2): 519–24.

Barry FP, Boynton RE, Haynesworth S, Murphy JM, Zaia J. The monoclonal antibody SH-2, raised against human mesenchymal stem cells, recognizes an epitope on endoglin (CD105). Biochem Biophys Res Commun. 1999; 265(1): 134–9.

Becerra GD, Tatko LM, Pak ES, Murashov AK, Hoane MR. Transplantation of GABAergic neurons but not astrocytes induces recovery of sensorimotor function in the traumatically injured brain. Behav Brain Res. 2007; 179(1): 118–25.

Beck KD, Valverde J, Alexi T, Poulsen K, Moffat B, Vandlen RA, Rosenthal A, Hefti F. Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature. 1995; 373(6512): 339–41.

Behfar A, Perez-Terzic C, Faustino RS, Arrell DK, Hodgson DM, Yamada S, Puceat M, Niederländer N, Alekseev AE, Zingman LV, Terzic A. Cardiopoietic programming of embryonic stem cells for tumor-free heart repair. J Exp Med. 2007; 204(2): 405–20.

Bhattacharya D, Rossi DJ, Bryder D, Weissman IL. Purified haematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. J Exp Med. 2006; 203(1): 73–85.

Bianchi Scarrà GL, Romani M, Coviello DA, Garrè C, Ravazzolo R, Vidali G, Ajmar F. Terminal erythroid differentiation in the K-562 cell line by 1-beta-D-arabinofuranosylcytosine: accompaniment by c-myc messenger RNA decrease. Cancer Res. 1986; 46(12 Pt 1): 6327–32.

Bibel M, Richter J, Schrenk K, Tucker KL, Staiger V, Korte M, Goetz M, Barde YA. Differentiation of mouse embryonic stem cells into a defined neuronal lineage. Nat Neurosci. 2004; 7(9): 1003–9.

Bieberich E, Silva J, Wang G, Krishnamurthy K, Condie BG. Selective apoptosis of pluripotent mouse and human stem cells by novel ceramide analogues prevents teratoma formation and enriches for neural precursors in ES cell-derived neural transplants. J Cell Biol. 2004; 167(4): 723–34.

Bingham B, Liu D, Wood A, Cho S. Ischemia-stimulated neurogenesis is regulated by proliferation, migration, differentiation and caspase activation of hippocampal precursor cells. Brain Res. 2005; 1058(1-2): 167–77.

Bittira B, Kuang JQ, Al-Khaldi A, Shum-Tim D, Chiu RC. In vitro preprogramming of marrow stromal cells for myocardial regeneration. Ann Thorac Surg. 2002; 74(4): 1154–9.

Björklund A, Lindvall O. Cell replacement therapies for central nervous system disorders. Nat Neurosci. 2000; 3(6): 537–44.

Bjornson CR, Rietze RL, Reynolds BA, Magli MC, Vescovi AL. Turning brain into blood: a haematopoietic fate adopted by adult neural stem cells in vivo. Science. 1999; 283(5401): 534–7.

Blaess S, Corrales JD, Joyner AL. Sonic hedgehog regulates Gli activator and repressor functions with spatial and temporal precision in the mid/hindbrain region. Development. 2006; 133(9): 1799–809.

Blelloch R, Wang Z, Meissner A, Pollard S, Smith A, Jaenisch R. Reprogramming efficiency following somatic cell nuclear transfer is influenced by the differentiation and methylation state of the donor nucleus. Stem Cells. 2006; 24(9): 2007–13.

Bonner-Weir S, Weir GC. New sources of pancreatic beta-cells. Nat Biotechnol. 2005; 23(7): 857–61.

Bosch M, Pineda JR, Suñol C, Petriz J, Cattaneo E, Alberch J, Canals JM. Induction of GABAergic phenotype in a neural stem cell line for transplantation in an excitotoxic model of Huntington's disease. Exp Neurol. 2004; 190(1): 42–58.

Brambrink T, Hochedlinger K, Bell G, Jaenisch R. ES cells derived from cloned and fertilized blastocysts are transcriptionally and functionally indistinguishable. Proc Natl Acad Sci U S A. 2006; 103(4): 933–8

Branton RL, Love RM, Clarke DJ. Camp included during cell suspension preparation improves survival of dopaminergic neurons in vitro. Neuroreport. 1998; 9(14): 3223-7

Brazelton TR, Rossi FM, Keshet GI, Blau HM. From marrow to brain: expression of neuronal phenotypes in adult mice. Science. 2000; 290(5497): 1775–9

Bruder SP, Ricalton NS, Boynton RE, Connolly TJ, Jaiswal N, Zaia J, Barry FP. Mesenchymal stem cell surface antigen SB-10 corresponds to activated leukocyte cell adhesion molecule and is involved in osteogenic differentiation. J Bone Miner Res. 1998; 13(4): 655–63.

Brunstein CG, Setubal DC, Wagner JE. Expanding the role of umbilical cord blood transplantation. Br J Haematol. 2007; 137(1): 20–35

Brunt KR, Hall SR, Ward CA, Melo LG. Endothelial progenitor cell and mesenchymal stem cell isolation, characterization, viral transduction. Methods Mol Med. 2007; 139: 197–210

Brüstle O, Choudhary K, Karram K, Hüttner A, Murray K, Dubois-Dalcq M, McKay RD. Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. Nat Biotechnol. 1998; 16(11): 1040–4

Brüstle O, Jones KN, Learish RD, Karram K, Choudhary K, Wiestler OD, Duncan ID, McKay RD. Embryonic stem cell-derived glial precursors: a source of myelinating transplants. Science. 1999; 285(5428): 754–6

Bryder D, Rossi DJ, Weissman IL. Haematopoietic stem cells: the paradigmatic tissue-specific stem cell. Am J Pathol. 2006; 169(2): 338-46

Bussolati B, Bruno S, Grange C, Buttiglieri S, Deregibus MC, Cantino D, Camussi G. Isolation of renal progenitor cells from adult human kidney. Am J Pathol. 2005; 166(2): 545–55

Byrd KE, Sheskin TA. Effects of post-natal serotonin levels on craniofacial complex. J Dent Res. 2001; 80(8): 1730–5

Byrne JA, Pedersen DA, Clepper LL, Nelson M, Sanger WG, Gokhale S, Wolf DP, Mitalipov SM. Producing primate embryonic stem cells by somatic cell nuclear transfer. Nature. 2007; 450(7169): 497–502.

Camargo FD, Chambers SM, Goodell MA. Stem cell plasticity: from transdifferentiation to macrophage fusion. Cell Prolif. 2004; 37(1): 55–65

Campbell KH. Nuclear transfer in farm animal species. Semin Cell Dev Biol. 1999; 10(3): 245–52.

Cantley LC. The phosphoinositide 3-kinase pathway. Science. 2002; 296(5573): 1655–7.

Cao F, van der Bogt KE, Sadrzadeh A, Xie X, Sheikh AY, Wang H, Connolly AJ, Robbins RC, Wu JC. Spatial and temporal kinetics of teratoma formation from murine embryonic stem cell transplantation. Stem Cells Dev. 2007; 16(6): 883–91.

Carion A, Benboubker L, Hérault O, Roingeard F, Degenne M, Senecal D, Desbois I, Colombat P, Charbord P, Binet C, Domenech J. Stromal-derived factor 1 and matrix metalloproteinase 9 levels in bone marrow and peripheral blood of patients mobilized by granulocyte colony-stimulating factor and chemotherapy. Relationship with mobilizing capacity of haematopoietic progenitor cells. Br J Haematol. 2003; 122(6): 918–26.

Carnac G, Fajas L, L'honoré A, Sardet C, Lamb NJ, Fernandez A. The retinoblastoma-like protein p130 is involved in the determination of reserve cells in differentiating myoblasts. Curr Biol. 2000; 10(9): 543–6.

Cedar SH, Cooke JA, Luo Z, Patel MJ, Minger SL. From embryos to embryonic stem cells: biopolitics and therapeutic potential. Reprod Biomed Online. 2006; 13(5): 725–31.

Chan J, Waddington SN, O'Donoghue K, Kurata H, Guillot PV, Gotherstrom C, Themis M, Morgan JE, Fisk NM. Widespread distribution and muscle differentiation of human fetal mesenchymal stem cells after intrauterine transplantation in dystrophic mdx mouse. Stem Cells. 2007; 25(4): 875–84.

Chang YC, Shyu WC, Lin SZ, Li H. Regenerative therapy for stroke. Cell Transplant. 2007; 16: 171–181.

Cheng L, Qasba P, Vanguri P, Thiede MA. Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34(+) haematopoietic progenitor cells. J Cell Physiol. 2000; 184(1): 58–69.

Chmielnicki E, Benraiss A, Economides AN, Goldman SA. Adenovirally expressed noggin and brain-derived neurotrophic factor cooperate to induce new medium spiny neurons from resident progenitor cells in the adult striatal ventricular zone. J Neurosci. 2004; 24(9): 2133–42.

Chung S, Sonntag KC, Andersson T, Bjorklund LM, Park JJ, Kim DW, Kang UJ, Isacson O, Kim KS. Genetic engineering of mouse embryonic stem cells by Nurr1 enhances differentiation and maturation into dopaminergic neurons. Eur J Neurosci. 2002; 16(10): 1829–38.

Clarke DL, Johansson CB, Wilbertz J, Veress B, Nilsson E, Karlström H, Lendahl U, Frisén J. Generalized potential of adult neural stem cells. Science. 2000; 288(5471): 1660–3.

Clayton E, Doupé DP, Klein AM, Winton DJ, Simons BD, Jones PH. A single type of progenitor cell maintains normal epidermis. Nature. 2007; 446(7132): 185–9.

Cohen Y, Nagler A. Umbilical cord blood transplantation how, when and for whom? Blood Rev. 2004; 18(3): 167–79

Collins SJ, Ruscetti FW, Gallagher RE, Gallo RC. Terminal differentiation of human promyelocytic leukemia cells induced by dimethyl sulfoxide and other polar compounds. Proc Natl Acad Sci U S A. 1978; 75(5): 2458–62.

Colter DC, Sekiya I, Prockop DJ. Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells. Proc Natl Acad Sci U S A. 2001; 98(14): 7841–5.

Conget PA, Minguell JJ. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. J Cell Physiol. 1999; 181(1): 67–73.

Conti L, Pollard SM, Gorba T, Reitano E, Toselli M, Biella G, Sun Y, Sanzone S, Ying QL, Cattaneo E, Smith A. Niche-independent symmetrical self-renewal of a mammalian tissue stem cell. PloS Biol. 2005; 3(9): e283.

Cornelison DD, Wold BJ. Single-cell analysis of regulatory gene expression in quiescent and activated mouse skeletal muscle satellite cells. Dev Biol. 1997; 191(2): 270–83.

Côté F, Fligny C, Bayard E, Launay JM, Gershon MD, Mallet J, Vodjdani G. Maternal serotonin is crucial for murine embryonic development. Proc Natl Acad Sci U S A. 2007; 104(1): 329–34.

Crescenzi M, Soddu S, Sacchi A, Tatò F. Adenovirus infection induces reentry into the cell cycle of terminally differentiated skeletal muscle cells. Ann N Y Acad Sci. 1995; 752: 9–18.

Dahéron L, Opitz SL, Zaehres H, Lensch MW, Andrews PW, Itskovitz-Eldor J, Daley GQ. LIF/STAT3 signaling fails to maintain self-renewal of human embryonic stem cells. Stem Cells. 2004; 22(5): 770–8.

Dang L, Tropepe V. Neural induction and neural stem cell development. Regen Med. 2006; 1(5): 635–52.

Daudet N, Lewis J. Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. Development. 2005; 132(3): 541–51.

Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000; 28(8): 875–84.

Deeg HJ, Friedman D, Bohr VA, Constine L, Jones IM, Sigurdson A, Socié G, Van Zant G, Wei Q, Martin P. Transplantation and aging. Biol Blood Marrow Transplant. 2006; 12(9): 893–8.

Di Porzio U, Zuddas A. Embryonic dopaminergic neuron transplants in MPTP lesioned mouse striatum. Neurochem Int. 1992; 20(Suppl): 309S–320S.

Dobkin BH. Behavioral, temporal, and spatial targets for cellular transplants as adjuncts to rehabilitation for stroke. Stroke. 2007; 38(2 Suppl) 832–9

Doetsch F. The glial identity of neural stem cells. Nat Neurosci 2003, 6(11). 1127-34

Echevarria D, Vieira C, Gimeno L, Martinez S Neuroepithelial secondary organizers and cell fate specification in the developing brain. Brain Res Brain Res Rev. 2003; 43(2): 179–91

Eggan K, Baldwin K, Tackett M, Osborne J, Gogos J, Chess A, Axel R, Jaenisch R Mice cloned from olfactory sensory neurons. Nature. 2004; 428(6978) 44-9

Eggert K, Schlegel J, Oertel W, Würz C, Krieg JC, Vedder H. Glial cell line-derived neurotrophic factor protects dopaminergic neurons from 6-hydroxydopamine toxicity in vitro. Neurosci Lett. 1999; 269(3): 178–82

Emanuelsson H. Autoradiographic localization in polychaete embryos of tritiated mesulergine, a selective antagonist of serotonin receptors that inhibits early polychaete development. Int J Dev Biol. 1992; 36(2): 293–302

Endo T, Nadal-Ginard B. Reversal of myogenic terminal differentiation by SV40 large T antigen results in mitosis and apoptosis. J Cell Sci. 1998; 111(Pt 8): 1081 93.

Erdö F, Bührle C, Blunk J, Hoehn M, Xia Y, Fleischmann B, Föcking M, Küstermann E, Kolossov E, Hescheler J, Hossmann KA, Trapp T. Host-dependent tumorigenesis of embryonic stem cell transplantation in experimental stroke. J Cereb Blood Flow Metab 2003; 23(7): 780–5

Ergün S, Tilki D, Hohn HP, Gehling U, Kilic N. Potential implications of vascular wall resident endothelial progenitor cells. Thromb Haemost. 2007; 98(5). 930-9.

Eriksson PS, Perfilieva E, Björk-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH. Neurogenesis in the adult human hippocampus. Nat Med. 1998; 4(11): 1313 7

Falugi C. Localization and possible role of molecules associated with the cholinergic system during "non-nervous" developmental events. Eur J Histochem. 1993; 37(4): 287–94.

Fanburg BL, Lee SL. A new role for an old molecule: serotonin as a mitogen. Am J Physiol. 1997; 272(5 Pt 1): L795–806.

Favier B, Fliniaux I, Thélu J, Viallet JP, Demarchez M, Jahoda CA, Dhouailly D. Localisation of members of the notch system and the differentiation of vibrissa hair follicles: receptors, ligands, and fringe modulators. Dev Dyn. 2000; 218(3): 426–37

Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, Mavilio F. Muscle regeneration by bone marrow-derived myogenic progenitors Science. 1998; 279(5356): 1528–30.

Fibach E, Burke LP, Schechter AN, Noguchi CT, Rodgers GP. Hydroxyurea increases fetal hemoglobin in cultured erythroid cells derived from normal individuals and patients with sickle cell anemia or beta-thalassemia. Blood. 1993; 81(6): 1630–5

Flax JD, Aurora S, Yang C, Simonin C, Wills AM, Billinghurst LL, Jendoubi M, Sidman RL, Wolfe JH, Kim SU, Snyder EY. Engraftable human neural stem cells respond to developmental cues, replace neurons, and express foreign genes. Nat Biotechnol. 1998; 16(11): 1033–9

Flickinger RA. Transcriptional frequency and cell determination. J Theor Biol. 2005, 232(2): 151–6.

Fong SP, Tsang KS, Chan AB, Lu G, Poon WS, Li K, Baum LW, Ng HK. Trophism of neural progenitor cells to embryonic stem cells: neural induction and transplantation in a mouse ischemic stroke model. J Neurosci Res. 2007; 85(9): 1851–62.

Fukuda K. Use of adult marrow mesenchymal stem cells for regeneration of cardiomyocytes. Bone Marrow Transplant. 2003; 32(Suppl 1): S25–7

Gage FH. Mammalian neural stem cells. Science. 2000; 287(5457): 1433–8

Gage FH. Neurogenesis in the adult brain. J Neurosci. 2002; 22(3): 612–3

Galter D, Unsicker K. Sequential activation of the 5-HT1(A) serotonin receptor and TrkB induces the serotonergic neuronal phenotype. Mol Cell Neurosci. 2000, 15(5): 446–55.

Gao J, Prough DS, McAdoo DJ, Grady JJ, Parsley MO, Ma L, Tarensenko YI, Wu P. Transplantation of primed human fetal neural stem cells improves cognitive function in rats after traumatic brain injury. Exp Neurol. 2006; 201(2): 281–92.

Garry DJ, Yang Q, Bassel-Duby R, Williams RS. Persistent expression of MNF identifies myogenic stem cells in postnatal muscles. Dev Biol. 1997; 188(2): 280–94

Geraerts M, Krylyshkina O, Debyser Z, Baekelandt V. Concise review: therapeutic strategies for Parkinson disease based on the modulation of adult neurogenesis. Stem Cells. 2007; 25: 263–70.

Gershon MD. Genes and lineages in the formation of the enteric nervous system. Curr Opin Neurobiol. 1997; 7(1): 101–9.

Gesi M, Soldani P, Giorgi FS, Santinami A, Bonaccorsi I, Fornai F. The role of the locus coeruleus in the development of Parkinson's disease. Neurosci Biobehav Rev. 2000; 24(6): 655–68.

Gottlieb DI. Large-scale sources of neural stem cells. Annu Rev Neurosci. 2002; 25: 381–407.

Griffin J, Munroe D, Major P, Kufe D. Induction of differentiation of human myeloid leukemia cells by inhibitors of DNA synthesis. Exp Hematol. 1982; 10(9): 774–81.

Grigoriadis AE, Heersche JN, Aubin JE. Continuously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal cell line. Dev Biol. 1990; 142(2): 313–8.

Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle, fat, cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. J Cell Biol. 1988; 106(6): 2139–51.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. Stem cell properties of human dental pulp stem cells. J Dent Res. 2002; 81(8): 531–5.

Guan K, Chang H, Rolletschek A, Wobus AM. Embryonic stem cell-derived neurogenesis. Retinoic acid induction and lineage selection of neuronal cells. Cell Tissue Res. 2001; 305(2): 171–6.

Guo Y, Graham-Evans B, Broxmeyer HE. Murine embryonic stem cells secrete cytokines/growth modulators that enhance cell survival/anti-apoptosis and stimulate colony formation of murine hematopoietic progenitor cells. Stem Cells. 2006; 24(4): 850–6.

Gustafsson BI, Thommesen L, Stunes AK, Tommeras K, Westbroek I, Waldum HL, Slørdahl K, Tamburstuen MV, Reseland JE, Syversen U. Serotonin and fluoxetine modulate bone cell function in vitro. J Cell Biochem. 2006; 98(1): 139–51.

Haaf T, Schmid M. Experimental condensation inhibition in constitutive and facultative heterochromatin of mammalian chromosomes. Cytogenet Cell Genet. 2000; 91(1–4): 113–23.

Haegele L, Ingold B, Naumann H, Tabatabai G, Ledermann B, Brandner S. Wnt signalling inhibits neural differentiation of embryonic stem cells by controlling bone morphogenetic protein expression. Mol Cell Neurosci. 2003; 24(3): 696–708.

Hakuno D, Fukuda K, Makino S, Konishi F, Tomita Y, Manabe T, Suzuki Y, Umezawa A, Ogawa S. Bone marrow-derived regenerated cardiomyocytes (CMG Cells) express functional adrenergic and muscarinic receptors. Circulation. 2002; 105(3): 380–6.

Hall PA, Watt FM. Stem cells: the generation and maintenance of cellular diversity. Development. 1989; 106(4): 619–33.

Hayashi J, Takagi Y, Fukuda H, Imazato T, Nishimura M, Fujimoto M, Takahashi J, Hashimoto N, Nozaki K. Primate embryonic stem cell-derived neuronal progenitors transplanted into ischemic brain. J Cereb Blood Flow Metab. 2006; 26(7): 906–14.

Haynesworth SE, Goshima J, Goldberg VM, Caplan AI. Characterization of cells with osteogenic potential from human marrow. Bone. 1992; 13(1): 81–8.

Hebert JM, Boyle M, Martin GR. mRNA localization studies suggest that murine FGF-5 plays a role in gastrulation. Development. 1991; 112(2): 407–15.

Heitzler P, Simpson P. The choice of cell fate in the epidermis of Drosophila. Cell. 1991; 64(6): 1083–92.

Hemmati-Brivanlou A, Melton D. Vertebrate embryonic cells will become nerve cells unless told otherwise. Cell. 1997; 88(1): 13–7.

Henery CC, Kaufman MH. Cleavage rate of haploid and diploid parthenogenetic mouse embryos during the preimplantation period. Mol Reprod Dev. 1992; 31(4): 258–63.

Herrera MB, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus MC, Bussolati B, Camussi G. Isolation and characterization of a stem cell population from adult human liver. Stem Cells. 2006; 24(12): 2840–50.

Hess D, Li L, Martin M, Sakano S, Hill D, Strutt B, Thyssen S, Gray DA, Bhatia M. Bone marrow-derived stem cells initiate pancreatic regeneration. Nat Biotechnol. 2003; 21(7): 763–70.

Hitoshi S, Alexson T, Tropepe V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A, van der Kooy D. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev. 2002; 16(7): 846–58.

Hixson DC, Chapman L, McBride A, Faris R, Yang L. Antigenic phenotypes common to rat oval cells, primary hepatocellular carcinomas and developing bile ducts. Carcinogenesis. 1997; 18(6): 1169–75.

Horackova M, Arora R, Chen R, Armour JA, Cattini PA, Livingston R, Byczko Z. Cell transplantation for treatment of acute myocardial infarction: unique capacity for repair by skeletal muscle satellite cells. Am J Physiol Heart Circ Physiol. 2004; 287(4): H1599–608.

Horger BA, Nishimura MC, Armanini MP, Wang LC, Poulsen KT, Rosenblad C, Kirik D, Moffat B, Simmons L, Johnson E Jr, Milbrandt J, Rosenthal A, Bjorklund A, Vandlen RA, Hynes MA, Phillips HS. Neurturin exerts potent actions on survival and function of midbrain dopaminergic neurons. J Neurosci. 1998; 18(13): 4929–37.

Hsu EH, Lochan AC, Cowen DS. Activation of Akt1 by human 5-hydroxytryptamine (serotonin)1B receptors is sensitive to inhibitors of MEK. J Pharmacol Exp Ther. 2001; 298(2): 825–32.

Hu G, Lee H, Price SM, Shen MM, Abate-Shen C Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. Development 2001, 128(12) 2373 84.

Humpherys D, Eggan K, Akutsu H, Hochedlinger K, Rideout WM 3rd, Biniszkiewicz D, Yanagimachi R, Jaenisch R. Epigenetic instability in ES cells and cloned mice Science. 2001; 293(5527): 95–7.

Hynes M, Stone DM, Dowd M, Pitts-Meek S, Goddard A, Gurney A, Rosenthal A Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene Gli-1. Neuron. 1997; 19(1): 15 -26.

Ilancheran S, Michalska A, Peh G, Wallace EM, Pera M, Manuelpillai U Stem cells derived from human fetal membranes display multilineage differentiation potential Biol Reprod. 2007, 77(3): 577 -88

Il'ková G, Rehák P, Veselá J, Cikos S, Fabian D, Czikková S, Koppel J Serotonin localization and its functional significance during mouse preimplantation embryo development. Zygote. 2004; 12(3): 205–13

Ingham PW, McMahon AP Hedgehog signaling in animal development paradigms and principles. Genes Dev. 2001; 15(23) 3059–87

Ishibashi M, Moriyoshi K, Sasai Y, Shiota K, Nakanishi S, Kageyama R Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural differentiation in the central nervous system. EMBO J. 1994, 13(8): 1799–805

Issaad C, Croisille L, Katz A, Vainchenker W, Coulombel L A murine stromal cell line allows the proliferation of very primitive human CD34++/CD38 progenitor cells in long-term cultures and semisolid assays. Blood. 1993; 81(11): 2916 24.

Ito K, Sieber-Blum M Pluripotent and developmentally restricted neural-crest-derived cells in posterior visceral arches. Dev Biol. 1993; 156(1): 191–200

Itoh K, Tezuka H, Sakoda H, Konno M, Nagata K, Uchiyama T, Uchino H, Mori KJ Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. Exp Hematol. 1989; 17(2): 145–53.

Jackson KA, Mi T, Goodell MA. Haematopoietic potential of stem cells isolated from murine skeletal muscle. Proc Natl Acad Sci U S A. 1999; 96(25). 14482–6

Jacobs S, Lie DC, DeCicco KL, Shi Y, DeLuca LM, Gage FH, Evans RM. Retinoic acid is required early during adult neurogenesis in the dentate gyrus. Proc Natl Acad Sci U S A. 2006; 103(10): 3902–7.

Jensen UB, Lowell S, Watt FM. The spatial relationship between stem cells and their progeny in the basal layer of human epidermis: a new view based on whole-mount labelling and lineage analysis. Development. 1999; 126(11): 2409–18

Jiang H, Lin J, Su ZZ, Collart FR, Huberman E, Fisher PB. Induction of differentiation in human promyelocytic HL-60 leukemia cells activates p21, WAF1/CIP1, expression in the absence of p53. Oncogene. 1994; 9(11): 3397–406

Jin CX, Li WL, Xu F, Geng ZH, He ZY, Su J, Tao XR, Ding XY, Wang X, Hu YP Conversion of immortal liver progenitor cells into pancreatic endocrine progenitor cells by persistent expression of Pdx-1 J Cell Biochem. 2008, 104(1) 224–36.

Joannides AJ, Fiore-Heriché C, Battersby AA, Athauda-Arachchi P, Bouhon IA, Williams L, Westmore K, Kemp PJ, Compston A, Allen ND, Chandran S A scaleable and defined system for generating neural stem cells from human embryonic stem cells Stem Cells. 2007; 25(3): 731- 7

Johansson BM, Wiles MV. Evidence for involvement of activin A and bone morphogenetic protein 4 in mammalian mesoderm and hematopoietic development. Mol Cell Biol. 1995; 15(1): 141 51

Jones PH, Harper S, Watt FM. Stem cell patterning and fate in human epidermis Cell 1995; 80(1): 83–93

Jones PH, Watt FM. Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression Cell 1993, 73(4): 713–24

Jones PH. In vitro comparison of embryonic myoblasts and myogenic cells isolated from regenerating adult rat skeletal muscle. Exp Cell Res. 1982; 139(2): 401–4

Jones-Villeneuve EM, Rudnicki MA, Harris JF, McBurney MW Retinoic acid-induced neural differentiation of embryonal carcinoma cells. Mol Cell Biol 1983; 3(12) 2271-9

Kageyama R, Ohtsuka T, Hatakeyama J, Ohsawa R Roles of bHLH genes in neural stem cell differentiation. Exp Cell Res. 2005; 306(2): 343–8.

Kageyama R, Ohtsuka T, Kobayashi T. Roles of Hes genes in neural development Dev Growth Differ. 2008; 50(Suppl 1): S97–103.

Kamakura S, Oishi K, Yoshimatsu T, Nakafuku M, Masuyama N, Gotoh Y Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling Nat Cell Biol 2004; 6(6): 547–54.

Kameda Y. Ontogeny of the carotid body and glomus cells distributed in the wall of the common carotid artery and its branches in the chicken. Cell Tissue Res 1990; 261(3) 525–37.

Kanda S, Tamada Y, Yoshidome A, Hayashi I, Nishiyama T Over-expression of bHLH genes facilitate neural formation of mouse embryonic stem (ES) cells in vitro Int J Dev Neurosci. 2004; 22(3): 149–56.

Kaplan MS, Bell DH. Mitotic neuroblasts in the 9-day-old and 11-month-old rodent hippocampus. J Neurosci. 1984; 4(6): 1429–41

Katoh N, Soga F, Nara T, Tamagawa-Mineoka R, Nin M, Kotani H, Masuda K, Kishimoto S. Effect of serotonin on the differentiation of human monocytes into dendritic cells. Clin Exp Immunol. 2006; 146(2): 354–61.

Kaushansky K. Lineage-specific haematopoietic growth factors. N Engl J Med 2006, 354(19): 2034–45

Kawada H, Takizawa S, Takanashi T, Morita Y, Fujita J, Fukuda K, Takagi S, Okano H, Ando K, Hotta T. Administration of hematopoietic cytokines in the subacute phase after cerebral infarction is effective for functional recovery facilitating proliferation of intrinsic neural stem/progenitor cells and transition of bone marrow-derived neuronal cells. 2006; Circulation. 113(5): 701-10

Kawasaki H, Mizuseki K, Nishikawa S, Kaneko S, Kuwana Y, Nakanishi S, Nishikawa SI, Sasai Y. Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. Neuron. 2000; 28(1): 31–40

Kawasaki H, Suemori H, Mizuseki K, Watanabe K, Urano F, Ichinose H, Haruta M, Takahashi M, Yoshikawa K, Nishikawa S, Nakatsuji N, Sasai Y Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. Proc Natl Acad Sci U S A. 2002; 99(3): 1580-5

Kee NJ, Preston E, Wojtowicz JM. Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat. Exp Brain Res 2001, 136(3) 313–20

Keller G, Kennedy M, Papayannopoulou T, Wiles MV Haematopoietic commitment during embryonic stem cell differentiation in culture. Mol Cell Biol 1993, 13(1) 473–86.

Kempermann G, van Praag H, Gage FH. Activity-dependent regulation of neuronal plasticity and self repair. Prog Brain Res. 2000; 127 35–48

Kennedy M, D'Souza SL, Lynch-Kattman M, Schwantz S, Keller G Development of the hemangioblast defines the onset of hematopoiesis in human ES cell differentiation cultures. Blood. 2007, 109(7): 2679–87.

Kiel MJ, Yilmaz OH, Iwashita T, Yilmaz OH, Terhorst C, Morrison SJ SLAM family receptors distinguish haematopoietic stem and progenitor cells and reveal endothelial niches for stem cells Cell. 2005; 121(7): 1109–21.

Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. Cell. 2005; 121(6): 823–35.

Kim JH, Auerbach JM, Rodríguez-Gómez JA, Velasco I, Gavin D, Lumelsky N, Lee SH, Nguyen J, Sánchez-Pernaute R, Bankiewicz K, McKay R Dopamine neurons derived from embryonic stem cells function in an animal model of Parkinson's disease Nature. 2002; 418(6893): 50–6.

Kim K, Ng K, Rugg-Gunn PJ, Shieh JH, Kirak O, Jaenisch R, Wakayama T, Moore MA, Pedersen RA, Daley GQ. Recombination signatures distinguish embryonic stem cells derived by parthenogenesis and somatic cell nuclear transfer Cell Stem Cell. 2007. 1(3): 346–52.

Kindler V. Postnatal stem cell survival: does the niche, a rare harbor where to resist the ebb tide of differentiation, also provide lineage-specific instructions? J Leukoc Biol 2005; 78(4): 836–44.

Kiprianova I, Sandkühler J, Schwab S, Hoyer S, Spranger M. Brain-derived neurotrophic factor improves long-term potentiation and cognitive functions after transient forebrain ischemia in the rat. Exp Neurol. 1999; 159(2): 511–9.

Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, Handa N, Fukunaga R, Kimura K, Mikoshiba K, Kamada T 'Ischemic tolerance' phenomenon found in the brain. Brain Res. 1990; 528(1): 21–24.

Klug MG, Soonpaa MH, Field LJ. DNA synthesis and multinucleation in embryonic stem cell-derived cardiomyocytes. Am J Physiol. 1995; 269(6 Pt 2): H1913–21.

Klug MG, Soonpaa MH, Koh GY, Field LJ. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts J Clin Invest 1996, 98(1): 216–24.

Koblas T, Zacharovová K, Berková Z, Mindlová M, Girman P, Dovolilová E, Karasova L, Saudek F. Isolation and characterization of human CXCR4-positive pancreatic cells Folia Biol (Praha). 2007; 53(1): 13–22.

Koc ON, Lazarus HM. Mesenchymal stem cells: heading into the clinic Bone Marrow Transplant. 2001; 27(3): 235–9.

Kondo T, Raff M. Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. Science. 2000; 289(5485). 1754–7.

Kondziolka D, Wechsler L, Achim C. Neural transplantation for stroke J Clin Neurosci. 2002; 9(3): 225–30.

Kopen GC, Prockop DJ, Phinney DG. Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains. Proc Natl Acad Sci U S A. 1999; 96(19): 10711–6.

Korda M, Blunn G, Goodship A, Hua J. Use of mesenchymal stem cells to enhance bone formation around revision hip replacements. J Orthop Res. 2008; 26(6): 880–5.

Krakauer JW. Avoiding performance and task confounds: multimodal investigation of brain reorganization after stroke rehabilitation. Exp Neurol. 2007; 204(2): 491–5.

Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 2001; 105(3): 369–77.

Krieglstein K, Henheik P, Farkas L, Jaszai J, Galter D, Krohn K, Unsicker K. Glial cell line-derived neurotrophic factor requires transforming growth factor-beta for exerting its full neurotrophic potential on peripheral and CNS neurons. J Neurosci. 1998; 18(23): 9822–34.

Kuhn HG, Dickinson-Anson H, Gage FH. Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation. J Neurosci. 1996; 16(6): 2027–33.

Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci. 1997; 17(15): 5820–9.

Kumar A, Velloso CP, Imokawa Y, Brockes JP. Plasticity of retrovirus-labelled myotubes in the newt limb regeneration blastema. Dev Biol. 2000; 218(2): 125–36.

Kunath T, Saba-El-Leil MK, Almousailleakh M, Wray J, Meloche S, Smith A. FGF stimulation of the Erk1/2 signalling cascade triggers transition of pluripotent embryonic stem cells from self-renewal to lineage commitment. Development. 2007; 134(16): 2895–902.

Lakshmipathy U, Pelacho B, Sudo K, Linehan JL, Coucouvanis E, Kaufman DS, Verfaillie CM. Efficient transfection of embryonic and adult stem cells. Stem Cells. 2004; 22(4): 531–43.

Lau T, Adam S, Schloss P. Rapid and efficient differentiation of dopaminergic neurons from mouse embryonic stem cells. Neuroreport. 2006; 17(10): 975–9.

Lauder JM, Tamir H, Sadler TW. Serotonin and morphogenesis. I. Sites of serotonin uptake and -binding protein immunoreactivity in the midgestation mouse embryo. Development. 1988; 102(4): 709–20.

Lauder JM. Neurotransmitters as growth regulatory signals: role of receptors and second messengers. Trends Neurosci. 1993; 16(6): 233–40.

Lauder JM. Neurotransmitters as morphogens. Prog Brain Res. 1988; 73: 365–87.

Lawlor MA, Alessi DR. PKB/Akt: a key mediator of cell proliferation, survival and insulin responses? J Cell Sci. 2001; 114(Pt 16): 2903–10

Lee H, Shamy GA, Elkabetz Y, Schofield CM, Harrsion NL, Panagiotakos G, Socci ND, Tabar V, Studer L. Directed differentiation and transplantation of human embryonic stem cell-derived motoneurons. Stem Cells. 2007; 25(8): 1931–9

Lee JE. Basic helix-loop-helix genes in neural development. Curr Opin Neurobiol. 1997; 7(1): 13–20

Lee SH, Lumelsky N, Studer L, Auerbach JM, McKay RD. Efficient generation of midbrain and hindbrain neurons from mouse embryonic stem cells. Nat Biotechnol. 2000; 18(6): 675–9.

Lepore AC, Fischer I. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. Exp Neurol. 2005; 194(1): 230–42.

Leri A, Kajstura J, Anversa P. Cardiac stem cells and mechanisms of myocardial regeneration. Physiol Rev. 2005; 85(4): 1373–416

Lesurtel M, Graf R, Aleil B, Walther DJ, Tian Y, Jochum W, Gachet C, Bader M, Clavien PA. Platelet-derived serotonin mediates liver regeneration. Science. 2006; 312(5770): 104–7

Levitt P, Harvey JA, Friedman E, Simansky K, Murphy EH. New evidence for neurotransmitter influences on brain development. Trends Neurosci. 1997; 20(6): 269–74.

Levy DE, Darnell JE Jr. Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol. 2002; 3(9): 651–62

Li J, Wang G, Wang C, Zhao Y, Zhang H, Tan Z, Song Z, Ding M, Deng H. MEK/ERK signaling contributes to the maintenance of human embryonic stem cell self-renewal. Differentiation. 2007; 75(4): 299–307.

Li M, Pevny L, Lovell-Badge R, Smith A. Generation of purified neural precursors from embryonic stem cells by lineage selection. Curr Biol. 1998; 8(17): 971–4

Li XJ, Du ZW, Zarnowska ED, Pankratz M, Hansen LO, Pearce RA, Zhang SC. Specification of motoneurons from human embryonic stem cells. Nat Biotechnol. 2005; 23(2): 215–21.

Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003; 2(4): 339–45

Liepert J, Bauder H, Wolfgang HR, Miltner WH, Taub E, Weiller C. Treatment-induced cortical reorganization after stroke in humans. Stroke. 2000; 31(6): 1210–6

Lim DA, Huang YC, Alvarez-Buylla A. The adult neural stem cell niche lessons for future neural cell replacement strategies. Neurosurg Clin N Am. 2007, 18(1): 81-92

Lindvall O, Kokaia Z. Stem cells for the treatment of neurological disorders. Nature 2006; 441(7097): 1094-6

Lindvall O, Kokaia Z, Martinez-Serrano A. Stem cell therapy for human neurodegenerative disorders-how to make it work. Nat Med. 2004; 10(Suppl): S42-50.

Ling ZD, Potter ED, Lipton JW, Carvey PM. Differentiation of mesencephalic progenitor cells into dopaminergic neurons by cytokines. Exp Neurol. 1998; 149(2): 411-23

Linker C, Stern CD. Neural induction requires BMP inhibition only as a late step, and involves signals other than FGF and Wnt antagonists. Development. 2004; 131(22): 5671-81.

Liu S, Qu Y, Stewart TJ, Howard MJ, Chakrabortty S, Holekamp TF, McDonald JW. Embryonic stem cells differentiate into oligodendrocytes and myelinate in culture and after spinal cord transplantation. Proc Natl Acad Sci U S A. 2000; 97(11): 6126-31

Liu Y, Fanburg BL. Serotonin-induced growth of pulmonary artery smooth muscle requires activation of phosphatidylinositol 3-kinase/serine-threonine protein kinase B/mammalian target of rapamycin/p70 ribosomal S6 kinase 1. Am J Respir Cell Mol Biol. 2006; 34(2). 182-91.

Liu Y, Suzuki YJ, Day RM, Fanburg BL. Rho kinase-induced nuclear translocation of ERK1/ERK2 in smooth muscle cell mitogenesis caused by serotonin. Circ Res. 2004, 95(6): 579-86

Lo LC, Johnson JE, Wuenschell CW, Saito T, Anderson DJ. Mammalian achaete-scute homolog 1 is transiently expressed by spatially restricted subsets of early neuroepithelial and neural crest cells. Genes Dev. 1991, 5(9): 1524-37.

Lois C, Alvarez-Buylla A. Proliferating subventricular zone cells in the adult mammalian forebrain can differentiate into neurons and glia. Proc Natl Acad Sci U S A. 1993; 90(5): 2074-7.

Long MW. Osteogenesis and bone-marrow-derived cells. Blood Cells Mol Dis. 2001, 27(3): 677-90

Louvi A, Artavanis-Tsakonas S. Notch signalling in vertebrate neural development. Nat Rev Neurosci. 2006; 7(2): 93-102

Lowell S, Benchoua A, Heavey B, Smith AG. Notch promotes neural lineage entry by pluripotent embryonic stem cells. PLoS Biol. 2006; 4(5): e121.

Lowell S, Jones P, Le Roux I, Dunne J, Watt FM. Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. Curr Biol. 2000; 10(9): 491–500.

Lu CZ, Xiao BG. Neuroprotection of G-CSF in cerebral ischemia. Front Biosci. 2007; 12: 2869–75.

Luskin MB. Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron. 1993; 11(1): 173–89.

Ma Q, Kintner C, Anderson DJ. Identification of neurogenin, a vertebrate neuronal determination gene. Cell. 1996; 87(1): 43–52.

Maharajan VS, Shanmuganathan V, Currie A, Hopkinson A, Powell-Richards A, Dua HS. Amniotic membrane transplantation for ocular surface reconstruction: indications and outcomes. Clin Experiment Ophthalmol. 2007; 35(2): 140–7.

Makino S, Fukuda K, Miyoshi S, Konishi F, Kodama H, Pan J, Sano M, Takahashi T, Hori S, Abe H, Hata J, Umezawa A, Ogawa S. Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest. 1999; 103(5): 697–705.

Marcus AJ, Woodbury D. Fetal stem cells from extra-embryonic tissues: do not discard. J Cell Mol Med. 2008; 12(3): 730–42.

Martinez S. The isthmic organizer and brain regionalization. Int J Dev Biol. 2001; 45(1): 367–71.

Mason JM, Breitbart AS, Barcia M, Porti D, Pergolizzi RG, Grande DA. Cartilage and bone regeneration using gene-enhanced tissue engineering. Clin Orthop Relat Res. 2000; 379(Suppl): S171–8.

Matsuda M, Imaoka T, Vomachka AJ, Gudelsky GA, Hou Z, Mistry M, Bailey JP, Nieport KM, Walther DJ, Bader M, Horseman ND. Serotonin regulates mammary gland development via an autocrine-paracrine loop. Dev Cell. 2004; 6(2): 193–203.

Matthews DB, Silvers JR. The use of acute ethanol administration as a tool to investigate multiple memory systems. Neurobiol Learn Mem. 2004; 82(3): 299–308.

Maye P, Becker S, Siemen H, Thorne J, Byrd N, Carpentino J, Grabel L. Hedgehog signaling is required for the differentiation of ES cells into neurectoderm. Dev Biol. 2004; 265(1): 276–90.

McDonald JW, Liu XZ, Qu Y, Liu S, Mickey SK, Turetsky D, Gottlieb DI, Choi DW. Transplanted embryonic stem cells survive, differentiate and promote recovery in injured rat spinal cord. Nat Med. 1999; 5(12): 1410–2.

McGann CJ, Odelberg SJ, Keating MT. Mammalian myotube dedifferentiation induced by newt regeneration extract. Proc Natl Acad Sci U S A. 2001; 98(24): 13699–704

McLeod M, Hong M, Mukhida K, Sadi D, Ulalia R, Mendez I. Erythropoietin and GDNF enhance ventral mesencephalic fiber outgrowth and capillary proliferation following neural transplantation in a rodent model of Parkinson's disease. Eur J Neurosci. 2006; 24(2): 361–70.

McMahon G, Alsina JL, Levy SB. Induction of a Ca2+, Mg2+-dependent endonuclease activity during the early stages of murine erythroleukemic cell differentiation. Proc Natl Acad Sci U S A. 1984; 81(23): 7461–5.

Menendez P, Bueno C, Wang L. Human embryonic stem cells: A journey beyond cell replacement therapies. Cytotherapy. 2006; 8(6): 530–41

Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Science. 2000; 290(5497): 1779–82.

Michalopoulos GK, DeFrances MC. Liver regeneration. Science. 1997; 276(5309): 60–6

Miki T, Lehmann T, Cai H, Stolz DB, Strom SC. Stem cell characteristics of amniotic epithelial cells. Stem Cells. 2005; 23(10): 1549–59.

Mimeault M, Batra SK. Concise review: recent advances on the significance of stem cells in tissue regeneration and cancer therapies. Stem Cells. 2006; 24(11): 2319–45

Mimeault M, Hauke R, Batra SK. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther. 2007; 82(3): 252–64

Minguell JJ, Conget P, Erices A. Biology and clinical utilization of mesenchymal progenitor cells. Braz J Med Biol Res. 2000; 33(8): 881–7.

Minguell JJ, Erices A, Conget P. Mesenchymal stem cells. Exp Biol Med (Maywood). 2001; 226(6): 507–20.

Mitalipov SM, Zhou Q, Byrne JA, Ji WZ, Norgren RB, Wolf DP. Reprogramming following somatic cell nuclear transfer in primates is dependent upon nuclear remodeling. Hum Reprod. 2007; 22(8): 2232–42.

Miyazaki S, Katayama Y, Swann K. Synergistic activation by serotonin and GTP analogue and inhibition by phorbol ester of cyclic Ca2+ rises in hamster eggs. J Physiol. 1990; 426: 209–27.

Moiseiwitsch JR, Lauder JM. Serotonin regulates mouse cranial neural crest migration Proc Natl Acad Sci U S A. 1995; 92(16): 7182–6

Montcouquiol M, Crenshaw EB 3rd, Kelley MW. Noncanonical Wnt signaling and neural polarity. Annu Rev Neurosci. 2006; 29: 363–86

Moore KA, Lemischka IR. Stem cells and their niches. Science 2006, 311(5769): 1880–5

Morimoto M, Takahashi Y, Endo M, Saga Y. The Mesp2 transcription factor establishes segmental borders by suppressing Notch activity. Nature 2005; 435(7040) 354–9

Morishita R, Nagata K, Ito H, Ueda H, Asano M, Shinohara H, Kato K, Asano T Expression of smooth muscle cell-specific proteins in neural progenitor cells induced by agonists of G protein-coupled receptors and transforming growth factor-beta. J Neurochem 2007; 101(4): 1031–40

Morizane A, Takahashi J, Shinoyama M, Ideguchi M, Takagi Y, Fukuda H, Koyanagi M, Sasai Y, Hashimoto N. Generation of graftable dopaminergic neuron progenitors from mouse ES cells by a combination of coculture and neurosphere methods J Neurosci Res. 2006; 83(6): 1015–27

Morizane A, Takahashi J, Takagi Y, Sasai Y, Hashimoto N. Optimal conditions for in vivo induction of dopaminergic neurons from embryonic stem cells through stromal cell-derived inducing activity. J Neurosci Res 2002; 69(6): 934–9

Morriss-Kay GM, Sokolova N Embryonic development and pattern formation. FASEB J. 1996; 10(9): 961–8

Morshead CM, Reynolds BA, Craig CG, McBurney MW, Staines WA, Morassutti D, Weiss S, van der Kooy D. Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. Neuron. 1994; 13(5). 1071–82.

Müller M, Fleischmann BK, Selbert S, Ji GJ, Endl E, Middeler G, Müller OJ, Schlenke P, Frese S, Wobus AM, Hescheler J, Katus HA, Franz WM. Selection of ventricular-like cardiomyocytes from ES cells in vitro. FASEB J. 2000; 14(15): 2540–8.

Murdoch JN, Doudney K, Paternotte C, Copp AJ, Stanier P. Severe neural tube defects in the loop-tail mouse result from mutation of Lpp1, a novel gene involved in floor plate specification. Hum Mol Genet. 2001; 10(22): 2593–601.

Murry CE, Field LJ, Menasché P. Cell-based cardiac repair: reflections at the 10-year point. Circulation. 2005; 112(20): 3174–83

Nakamura Y, Sakakibara S, Miyata T, Ogawa M, Shimazaki T, Weiss S, Kageyama R, Okano H. The bHLH gene hes1 as a repressor of the neuronal commitment of CNS stem cells. J Neurosci. 2000; 20(1): 283-93.

Nakatomi H, Kuriu T, Okabe S, Yamamoto S, Hatano O, Kawahara N, Tamura A, Kirino T, Nakafuku M. Regeneration of hippocampal pyramidal neurons after ischemic brain injury by recruitment of endogenous neural progenitors. Cell. 2002; 110(4): 429–41

Navé BT, Ouwens M, Withers·DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation. Biochem J. 1999; 344(Pt 2): 427–31.

Nebigil CG, Launay JM, Hickel P, Tournois C, Maroteaux L. 5-hydroxytryptamine 2B receptor regulates cell-cycle progression: cross-talk with tyrosine kinase pathways. Proc Natl Acad Sci U S A. 2000; 97(6): 2591–6

Nicolas M, Wolfer A, Raj K, Kummer JA, Mill P, van Noort M, Hui CC, Clevers H, Dotto GP, Radtke F. Notch1 functions as a tumor suppressor in mouse skin. Nat Genet. 2003; 33(3): 416-21

Noggle SA, Weiler D, Condie BG. Notch signaling is inactive but inducible in human embryonic stem cells. Stem Cells. 2006; 24(7): 1646–53

Nordin N, Li M, Mason JO. Expression profiles of Wnt genes during neural differentiation of mouse embryonic stem cells. Cloning Stem Cells. 2008, 10(1): 37-48

Nussbaum J, Minami E, Laflamme MA, Virag JA, Ware CB, Masino A, Muskheli V, Pabon L, Reinecke H, Murry CE. Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response. FASEB J. 2007. 21(7): 1345-57.

Odelberg SJ, Kollhoff A, Keating MT. Dedifferentiation of mammalian myotubes induced by msx1. Cell. 2000, 103(7): 1099–109

Oh SH, Witek RP, Bae SH, Zheng D, Jung Y, Piscaglia AC, Petersen BE. Bone marrow-derived hepatic oval cells differentiate into hepatocytes in 2-acetylaminofluorene/partial hepatectomy-induced liver regeneration. Gastroenterology. 2007; 132(3): 1077–87.

Oka C, Nakano T, Wakeham A, de la Pompa JL, Mori C, Sakai T, Okazaki S, Kawaichi M, Shiota K, Mak TW, Honjo T. Disruption of the mouse RBP-J kappa gene results in early embryonic death. Development. 1995; 121(10): 3291–301

Okabe S, Forsberg-Nilsson K, Spiro AC, Segal M, McKay RD. Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. Mech Dev. 1996; 59(1): 89–102.

Ostenfeld T, Horn P, Aardal C, Orpen I, Caldwell MA, Svendsen CN. Mouse epidermal growth factor-responsive neural precursor cells increase the survival and functional capacity of embryonic rat dopamine neurons in vitro. Neuroreport. 1999; 10(9): 1985-92

Otero JJ, Fu W, Kan L, Cuadra AE, Kessler JA. Beta-catenin signaling is required for neural differentiation of embryonic stem cells. Development. 2004; 131(15): 3545–57.

Ozasa S, Kimura S, Ito K, Ueno H, Ikezawa M, Matsukura M, Yoshioka K, Araki K, Yamamura KI, Abe K, Niwa H, Miike T. Efficient conversion of ES cells into myogenic lineage using the gene-inducible system. Biochem Biophys Res Commun. 2007; 357(4) 957–63.

Pachernik J, Bryja V, Esner M, Kubala L, Dvorák P, Hampl A. Neural differentiation of pluripotent mouse embryonal carcinoma cells by retinoic acid: inhibitory effect of serum. Physiol Res. 2005; 54(1): 115 -22.

Palma V, Lim DA, Dahmane N, Sánchez P, Brionne TC, Herzberg CD, Gitton Y, Carleton A, Alvarez-Buylla A, Ruiz i Altaba A. Sonic hedgehog controls stem cell behavior in the postnatal and adult brain. Development. 2005; 132(2): 335–44

Palmer TD, Markakis EA, Willhoite AR, Safar F, Gage FH. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. J Neurosci. 1999; 19(19): 8487–97

Palmer TD, Ray J, Gage FH. FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci. 1995; 6(5): 474–86.

Passier R, Mummery C. Origin and use of embryonic and adult stem cells in differentiation and tissue repair. Cardiovasc Res. 2003; 58(2): 324–35

Perrier AL, Tabar V, Barberi T, Rubio ME, Bruses J, Topf N, Harrison NL, Studer L. Derivation of midbrain dopamine neurons from human embryonic stem cells. Proc Natl Acad Sci U S A. 2004; 101(34): 12543–8.

Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. Science. 1999; 284(5417): 1168–70

Petersen BE, Goff JP, Greenberger JS, Michalopoulos GK. Hepatic oval cells express the haematopoietic stem cell marker Thy-1 in the rat. Hepatology. 1998; 27(2): 433–45.

Pfaar H, von Holst A, Vogt Weisenhorn DM, Brodski C, Guimera J, Wurst W. mPet-1, a mouse ETS-domain transcription factor, is expressed in central serotonergic neurons Dev Genes Evol. 2002; 212(1): 43–6.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science. 1999; 284(5411): 143–7.

Plachta N, Bibel M, Tucker KL, Barde YA. Developmental potential of defined neural progenitors derived from mouse embryonic stem cells. Development. 2004, 131(21) 5449–56.

Prakash N, Brodski C, Naserke T, Puelles E, Gogoi R, Hall A, Panhuysen M, Echevarria D, Sussel L, Weisenhorn DM, Martinez S, Arenas E, Simeone A, Wurst W. A Wnt1-regulated genetic network controls the identity and fate of midbrain-dopaminergic progenitors in vivo. Development. 2006; 133(1): 89–98

Prakash N, Wurst W. Development of dopaminergic neurons in the mammalian brain. Cell Mol Life Sci. 2006; 63(2): 187–206

Prockop DJ. Marrow stromal cells as stem cells for nonhaematopoietic tissues. Science 1997; 276(5309): 71–4

Rafuse VF, Soundararajan P, Leopold C, Robertson HA. Neuroprotective properties of cultured neural progenitor cells are associated with the production of sonic hedgehog Neuroscience. 2005; 131(4): 899–916.

Rajan P, McKay RD. Multiple routes to astrocytic differentiation in the CNS. J Neurosci. 1998; 18(10): 3620–9

Rajesh D, Chinnasamy N, Mitalipov SM, Wolf DP, Slukvin I, Thomson JA, Shaaban AF. Differential requirements for haematopoietic commitment between human and rhesus embryonic stem cells. Stem Cells. 2007; 25(2). 490–9

Ramaswamy S, Shannon KM, Kordower JH. Huntington's disease: pathological mechanisms and therapeutic strategies. Cell Transplant. 2007; 16. 301–12

Rao M. Conserved and divergent paths that regulate self-renewal in mouse and human embryonic stem cells. Dev Biol. 2004; 275(2): 269–86.

Rappa G, Kunke D, Holter J, Diep DB, Meyer J, Baum C, Fodstad O, Krauss S, Lorico A. Efficient expansion and gene transduction of mouse neural stem/progenitor cells on recombinant fibronectin. Neuroscience. 2004; 124(4): 823–30

Rathjen J, Lake JA, Bettess MD, Washington JM, Chapman G, Rathjen PD. Formation of a primitive ectoderm like cell population, EPL cells, from ES cells in response to biologically derived factors. J Cell Sci. 1999; 112(Pt 5): 601–12

Raymon HK, Thode S, Gage FH. Application of ex vivo gene therapy in the treatment of Parkinson's disease. Exp Neurol. 1997; 144(1): 82–91

Raymond JR, Mukhin YV, Gelasco A, Turner J, Collinsworth G, Gettys TW, Grewal JS, Garnovskaya MN. Multiplicity of mechanisms of serotonin receptor signal transduction. Pharmacol Ther. 2001; 92(2-3): 179–212.

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A. Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol. 2000; 18(4) 399–404.

Revazova ES, Turovets NA, Kochetkova OD, Kindarova LB, Kuzmichev LN, Janus JD, Pryzhkova MV. Patient-specific stem cell lines derived from human parthenogenetic blastocysts. Cloning Stem Cells. 2007; 9(3): 432 -49.

Reynolds BA, Weiss S. Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell. Dev Biol 1996; 175(1) 1–13

Rho JY, Yu K, Han JS, Chae JI, Koo DB, Yoon HS, Moon SY, Lee KK, Han YM Transcriptional profiling of the developmentally important signalling pathways in human embryonic stem cells. Hum Reprod. 2006; 21(2): 405–12.

Rice AC, Khaldi A, Harvey HB, Salman NJ, White F, Fillmore H, Bullock MR Proliferation and neuronal differentiation of mitotically active cells following traumatic brain injury. Exp Neurol. 2003; 183(2): 406–17.

Richards M, Fong CY, Chan WK, Wong PC, Bongso A. Nat Biotechnol. 2002, 20(9) 933–6.

Rideout WM 3rd, Hochedlinger K, Kyba M, Daley GQ, Jaenisch R. Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. Cell. 2002; 109(1): 17–27.

Rietze RL, Valcanis H, Brooker GF; Thomas T, Voss AK, Bartlett PF. Purification of a pluripotent neural stem cell from the adult mouse brain. Nature. 2001; 412(6848): 736-9.

Ringdén O. Immunotherapy by Allogeneic Stem Cell Transplantation. Adv Cancer Res. 2007; 97C: 25–60.

Roberts N, Jahangiri M; Xu Q. Progenitor cells in vascular disease J Cell Mol Med. 2005; 9(3): 583–91.

Rohwedel J, Guan K, Wobus AM. Induction of cellular differentiation by retinoic acid in vitro. Cells Tissues Organs. 1999; 165(3-4): 190–202

Rolletschek A, Chang H, Guan K, Czyz J, Meyer M, Wobus AM. Differentiation of embryonic stem cell-derived dopaminergic neurons is enhanced by survival-promoting factors. Mech Dev. 2001; 105(1-2): 93–104.

Rosa FW, Wilk AL, Kelsey FO. Teratogen update: vitamin A congeners. Teratology 1986; 33(3): 355–64.

Ross DD, Chen SR, Cuddy DP. Effects of 1-beta-D-arabinofuranosylcytosine on DNA replication intermediates monitored by pH-step alkaline elution. Cancer Res. 1990; 50(9): 2658–66.

Ross SE, Greenberg ME, Stiles CD. Basic helix-loop-helix factors in cortical development. Neuron. 2003; 39(1): 13–25.

Roy NS, Wang S, Harrison-Restelli C, Benraiss A, Fraser RA, Gravel M, Braun PE, Goldman SA. Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. J Neurosci. 1999; 19(22): 9986–95.

Rühl R, Plum C, Elmazar MM, Nau H. Embryonic subcellular distribution of 13-cis- and all-trans-retinoic acid indicates differential cytosolic/nuclear localization. Toxicol Sci. 2001; 63(1): 82–9.

Russ HA, Bar Y, Ravassard P, Efrat S. In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. Diabetes. 2008; 57(6): 1575–83.

Ryan EA, Lakey JR, Rajotte RV, Korbutt GS, Kin T, Imes S, Rabinovitch A, Elliott JF, Bigam D, Kneteman NM, Warnock GL, Larsen I, Shapiro AM. Clinical outcomes and insulin secretion after islet transplantation with the Edmonton protocol. Diabetes. 2001; 50(4): 710–9.

Sanchez-Ramos J, Song S, Cardozo-Pelaez F, Hazzi C, Stedeford T, Willing A, Freeman TB, Saporta S, Janssen W, Patel N, Cooper DR, Sanberg PR. Adult bone marrow stromal cells differentiate into neural cells in vitro. Exp Neurol. 2000; 164(2): 247–56.

Savitz SI, Dinsmore JH, Wechsler LR, Rosenbaum DM, Caplan LR. Cell therapy for stroke. NeuroRx. 2004; 1(4): 406–14.

Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells–basic and clinical implications for novel cell-based therapies. Stem Cells. 2007; 25(4): 818–27.

Schänzer A, Wachs FP, Wilhelm D, Acker T, Cooper-Kuhn C, Beck H, Winkler J, Aigner L, Plate KH, Kuhn HG. Direct stimulation of adult neural stem cells in vitro and neurogenesis in vivo by vascular endothelial growth factor. Brain Pathol. 2004; 14(3): 237–48.

Schuldiner M, Eiges R, Eden A, Yanuka O, Itskovitz-Eldor J, Goldstein RS, Benvenisty N. Induced neuronal differentiation of human embryonic stem cells. Brain Res. 2001; 913(2): 201–5.

Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology. 2001; 33(3): 738–50.

Sell S. Liver stem cells. Mod Pathol. 1994; 7(1): 105–12.

Semmler A, Frisch C, Debeir T, Ramanathan M, Okulla T, Klockgether T, Heneka MT. Long-term cognitive impairment, neuronal loss and reduced cortical cholinergic innervation after recovery from sepsis in a rodent model. Exp Neurol. 2007; 204(2): 733–40.

Sharma AD, Cantz T, Manns MP, Ott M. The role of stem cells in physiology, pathophysiology, and therapy of the liver. Stem Cell Rev. 2006; 2(1): 51–8.

Shim JH, Kim SE, Woo DH, Kim SK, Oh CH, McKay R, Kim JH. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia. 2007; 50(6): 1228–38.

Shingo T, Sorokan ST, Shimazaki T, Weiss S. Erythropoietin regulates the in vitro and in vivo production of neuronal progenitors by mammalian forebrain neural stem cells. J Neurosci. 2001; 21(24): 9733–43.

Shuey DL, Sadler TW, Tamir H, Lauder JM. Serotonin and morphogenesis. Transient expression of serotonin uptake and binding protein during craniofacial morphogenesis in the mouse. Anat Embryol (Berl). 1993; 187(1): 75–85.

Siddiqui EJ, Thompson CS, Mikhailidis DP, Mumtaz FH. The role of serotonin in tumour growth (review). Oncol Rep. 2005; 14(6): 1593–7.

Sittinger M, Bujia J, Rotter N, Reitzel D, Minuth WW, Burmester GR. Tissue engineering and autologous transplant formation: practical approaches with resorbable biomaterials and new cell culture techniques. Biomaterials. 1996; 17(3): 237–42.

Smith CK 2nd, Janney MJ, Allen RE. Temporal expression of myogenic regulatory genes during activation, proliferation, and differentiation of rat skeletal muscle satellite cells. J Cell Physiol. 1994; 159(2): 379–85.

Smith JR, Pochampally R, Perry A, Hsu SC, Prockop DJ. Isolation of a highly clonogenic and multipotential subfraction of adult stem cells from bone marrow stroma. Stem Cells. 2004; 22(5): 823–31.

Solter D, Knowles BB. Immunosurgery of mouse blastocyst. Proc Natl Acad Sci U S A. 1975; 72(12): 5099–102.

Soria B, Roche E, Berná G, León-Quinto T, Reig JA, Martin F. Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice. Diabetes. 2000; 49(2): 157–62.

Stanford WL, Cohn JB, Cordes SP. Gene-trap mutagenesis: past, present and beyond. Nat Rev Genet. 2001; 2(10): 756–68.

Stern MM, Bickenbach JR. Epidermal stem cells are resistant to cellular aging. Aging Cell. 2007; 6(4): 439–52.

Strübing C, Ahnert-Hilger G, Shan J, Wiedenmann B, Hescheler J, Wobus AM. Differentiation of pluripotent embryonic stem cells into the neuronal lineage in vitro gives rise to mature inhibitory and excitatory neurons. Mech Dev. 1995; 53(2): 275–87.

Sugaya K. Possible use of autologous stem cell therapies for Alzheimer's disease. Curr Alzheimer Res. 2005; 2(3): 367–76.

Tabar V, Panagiotakos G, Greenberg ED, Chan BK, Sadelain M, Gutin PH, Studer L. Migration and differentiation of neural precursors derived from human embryonic stem cells in the rat brain. Nat Biotechnol. 2005; 23(5): 601–6.

Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol. 2001; 11(19): 1553–8.

Tajbakhsh S, Buckingham ME. Lineage restriction of the myogenic conversion factor myf-5 in the brain. Development. 1995; 121(12): 4077–83.

Takagi Y, Nishimura M, Morizane A, Takahashi J, Nozaki K, Hayashi J, Hashimoto N. Survival and differentiation of neural progenitor cells derived from embryonic stem cells and transplanted into ischemic brain. J Neurosurg. 2005; 103(2): 304–10.

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007; 131(5): 861–72.

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126(4): 663–76.

Takahashi T, Lord B, Schulze PC, Fryer RM, Sarang SS, Gullans SR, Lee RT. Ascorbic acid enhances differentiation of embryonic stem cells into cardiac myocytes. Circulation. 2003; 107(14): 1912–6.

Tanaka EM, Drechsel DN, Brockes JP. Thrombin regulates S-phase re-entry by cultured newt myotubes. Curr Biol. 1999; 9(15): 792–9.

Tatsumi R, Anderson JE, Nevoret CJ, Halevy O, Allen RE. HGF/SF is present in normal adult skeletal muscle and is capable of activating satellite cells. Dev Biol. 1998; 194(1, 114–28.

Tavian M, Zheng B, Oberlin E, Crisan M, Sun B, Huard J, Peault B. The vascular wall as a source of stem cells. Ann N Y Acad Sci. 2005; 1044: 41–50

Taylor CJ, Bolton EM, Pocock S, Sharples LD, Pedersen RA, Bradley JA. Banking on human embryonic stem cells: estimating the number of donor cell lines needed for HLA matching. Lancet. 2005; 366(9502): 2019–25.

Tejwani S, Kolari RS, Sangwan VS, Rao GN. Role of amniotic membrane graft for ocular chemical and thermal injuries. Cornea. 2007; 26(1): 21–6

Temple S. The development of neural stem cells. Nature. 2001; 414(6859): 112–7

Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW. Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature. 2002; 416(6880): 542–5

Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, Henegariu O, Krause DS. Liver from bone marrow in humans. Hepatology. 2000; 32(1): 11–6.

Theise ND, Saxena R, Portmann BC, Thung SN, Yee H, Chiriboga L, Kumar A, Crawford JM. The canals of Hering and hepatic stem cells in humans. Hepatology. 1999; 30(6): 1425–33

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science. 1998; 282(5391): 1145–7

Thorgeirsson SS. Hepatic stem cells in liver regeneration. FASEB J. 1996; 10(11): 1249–56.

Tiainen M, Spitkovsky D, Jansen-Dürr P, Sacchi A, Crescenzi M. Expression of E1A in terminally differentiated muscle cells reactivates the cell cycle and suppresses tissue-specific genes by separable mechanisms. Mol Cell Biol. 1996; 16(10): 5302–12.

Timmons JA, Wennmalm K, Larsson O, Walden TB, Lassmann T, Petrovic N, Hamilton DL, Gimeno RE, Wahlestedt C, Baar K, Nedergaard J, Cannon B. Myogenic gene expression signature establishes that brown and white adipocytes originate from distinct cell lineages. Proc Natl Acad Sci U S A. 2007; 104(11): 4401–6

Tropepe V, Hitoshi S, Sirard C, Mak TW, Rossant J, van der Kooy D. Direct Neural fate specification from embryonic stem cells: a primitive mammalian neural stem cell stage acquired through a default mechanism. Neuron. 2001; 30(1): 65–78.

Trounson A. The production and directed differentiation of human embryonic stem cells. Endocr Rev. 2006; 27(2): 208–19

Tsiftsoglou AS, Pappas IS, Vizirianakis IS. Mechanisms involved in the induced differentiation of leukemia cells. Pharmacol Ther. 2003; 100(3): 257–90

Tsiftsoglou AS, Robinson SH. Differentiation of leukemic cell lines: a review focusing on murine erythroleukemia and human HL-60 cells. Int J Cell Cloning. 1985; 3(6): 349–66

Tsuji-Takayama K, Inoue T, Ijiri Y, Otani T, Motoda R, Nakamura S, Orita K. Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells. Biochem Biophys Res Commun. 2004; 323(1): 86–90

van Hooft JA, Yakel JL. 5-HT3 receptors in the CNS: 3B or not 3B? Trends Pharmacol Sci. 2003; 24(4): 157–60.

Van Obberghen-Schilling E, Vouret-Craviari V, Haslam RJ, Chambard JC, Pouysségur J. Cloning, functional expression and role in cell growth regulation of a hamster 5-HT2 receptor subtype. Mol Endocrinol. 1991; 5(7): 881–9.

Vazin T, Chen J, Lee CT, Amable R, Freed WJ. Assessment of stromal-derived inducing activity in the generation of dopaminergic neurons from human embryonic stem cells. Stem Cells. 2008; 26(6): 1517–25

Veenstra-VanderWeele J, Anderson GM, Cook EH Jr. Pharmacogenetics and the serotonin system: initial studies and future directions. Eur J Pharmacol. 2000; 410(2-3): 165–181.

Vertino AM, Taylor-Jones JM, Longo KA, Bearden ED, Lane TF, McGehee RE Jr, MacDougald OA, Peterson CA. Wnt10b deficiency promotes coexpression of myogenic and adipogenic programs in myoblasts. Mol Biol Cell. 2005; 16(4): 2039–48

Veselá J, Rehák P, Mihalik J, Czikková S, Pokorný J, Koppel J. Expression of serotonin receptors in mouse oocytes and preimplantation embryos. Physiol Res. 2003; 52(2): 223–8.

Vieyra DS, Jackson KA, Goodell MA. Plasticity and tissue regenerative potential of bone marrow-derived cells. Stem Cell Rev. 2005; 1(1): 65–9.

Vitalis T, Parnavelas JG. The role of serotonin in early cortical development. Dev Neurosci. 2003; 25(2-4): 245–56.

Wakayama T, Tabar V, Rodriguez I, Perry AC, Studer L, Mombaerts P Differentiation of embryonic stem cell lines generated from adult somatic cells by nuclear transfer Science. 2001; 292(5517): 740-3.

Wakitani S, Saito T, Caplan AI Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 1995, 18(12) 1417 26.

Walther DJ, Bader M Serotonin synthesis in murine embryonic stem cells Brain Res Mol Brain Res. 1999; 68(1-2): 55–63

Walther DJ, Peter JU, Winter S, Höltje M, Paulmann N, Grohmann M, Vowinckel J, Alamo-Bethencourt V, Wilhelm CS, Ahnert-Hilger G, Bader M Serotonylation of small GTPases is a signal transduction pathway that triggers platelet alpha-granule release Cell. 2003; 115(7): 851–62.

Wang X, Li W, Williams M, Terada N, Alessi DR, Proud CG Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J 2001, 20(16): 4370-9

Watanabe K, Kamiya D, Nishiyama A, Katayama T, Nozaki S, Kawasaki H, Watanabe Y, Mizuseki K, Sasai Y Directed differentiation of telencephalic precursors from embryonic stem cells. Nat Neurosci. 2005; 8(3): 288–96.

Watson DJ, Longhi L, Lee EB, Fulp CT, Fujimoto S, Royo NC, Passini MA, Trojanowski JQ, Lee VM, McIntosh TK, Wolfe JH. Genetically modified NT2N human neuronal cells mediate long-term gene expression as CNS grafts in vivo and improve functional cognitive outcome following experimental traumatic brain injury J Neuropathol Exp Neurol 2003, 62(4). 368-80

Watt FM, Hogan BL. Out of Eden: stem cells and their niches. Science 2000, 287(5457): 1427-30.

Weiss S, Dunne C, Hewson J, Wohl C, Wheatley M, Peterson AC, Reynolds BA Multipotent CNS stem cells are present in the adult mammalian spinal cord and ventricular neuroaxis. J Neurosci. 1996; 16(23): 7599-609

Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell. 2000a; 100(1): 157–68.

Weissman IL. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. Science. 2000b; 287(5457): 1442–6.

Wernig M, Meissner A, Foreman R, Brambrink T, Ku M, Hochedlinger K, Bernstein BE, Jaenisch R. In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state. Nature. 2007; 448(7151): 318–24.

Whitton PS. Inflammation as a causative factor in the aetiology of Parkinson's disease Br J Pharmacol. 2007; 150(8): 963–76.

Wichterle H, Lieberam I, Porter JA, Jessell TM. Directed differentiation of embryonic stem cells into motor neurons. Cell. 2002; 110(3): 385–97.

Wiles MV, Johansson BM. Embryonic stem cell development in a chemically defined medium. Exp Cell Res. 1999; 247(1): 241–8.

Williams BP, Abney ER, Raff MC. Macroglial cell development in embryonic rat brain: studies using monoclonal antibodies, fluorescence activated cell sorting, and cell culture. Dev Biol. 1985; 112(1): 126–34.

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. Nature. 1997; 385(6619): 810–3.

Wilson A, Trumpp A. Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol. 2006; 6(2): 93–106.

Wilson SI, Edlund T. Neural induction: toward a unifying mechanism. Nat Neurosci. 2001; 4(Suppl): 1161–8.

Wolbank S, Peterbauer A, Fahrner M, Hennerbichler S, van Griensven M, Stadler G, Redl H, Gabriel C. Dose-dependent immunomodulatory effect of human stem cells from amniotic membrane: a comparison with human mesenchymal stem cells from adipose tissue. Tissue Eng. 2007; 13(6): 1173–83.

Wood HB, Episkopou V. Comparative expression of the mouse Sox1, Sox2 and Sox3 genes from pre-gastrulation to early somite stages. Mech Dev. 1999; 86(1-2): 197–201.

Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. J Neurosci Res. 2000; 61(4): 364–70.

Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL. Physiological migration of haematopoietic stem and progenitor cells. Science. 2001; 294(5548): 1933–6.

Wu DC, Boyd AS, Wood KJ. Embryonic stem cell transplantation: potential applicability in cell replacement therapy and regenerative medicine. Front Biosci. 2007; 12: 4525–35.

Wu ZY, Hui GZ, Lu Y, Wu X, Guo LH. Transplantation of human amniotic epithelial cells improves hindlimb function in rats with spinal cord injury. Chin Med J (Engl). 2006; 119(24): 2101–7.

Xu C, Inokuma MS, Denham J, Golds K, Kundu P, Gold JD, Carpenter MK. Feeder-free growth of undifferentiated human embryonic stem cells. Nat Biotechnol. 2001; 19(10): 971–4.

Xu C, Police S, Rao N, Carpenter MK. Characterization and enrichment of cardiomyocytes derived from human embryonic stem cells. Circ Res. 2002; 91(6): 501–8.

Xu RH, Peck RM, Li DS, Feng X, Ludwig T, Thomson JA. Basic FGF and suppression of BMP signaling sustain undifferentiated proliferation of human ES cells. Nat Methods. 2005; 2(3): 185–90.

Xu W, Tamim H, Shapiro S, Stang MR, Collet JP. Use of antidepressants and risk of colorectal cancer: a nested case-control study. Lancet Oncol. 2006; 7(4): 301–8.

Yablonka-Reuveni Z, Quinn LS, Nameroff M. Isolation and clonal analysis of satellite cells from chicken pectoralis muscle. Dev Biol. 1987; 119(1): 252–9.

Yamada Y, Yokoyama S, Wang XD, Fukuda N, Takakura N. Cardiac stem cells in brown adipose tissue express CD133 and induce bone marrow nonhaematopoietic cells to differentiate into cardiomyocytes. Stem Cells. 2007; 25(5): 1326–33.

Yamamoto S, Nagao M, Sugimori M, Kosako H, Nakatomi H, Yamamoto N, Takebayashi H, Nabeshima Y, Kitamura T, Weinmaster G, Nakamura K, Nakafuku M. Transcription factor expression and Notch-dependent regulation of neural progenitors in the adult rat spinal cord. J Neurosci. 2001; 21(24): 9814–23.

Yavarone MS, Shuey DL, Sadler TW, Lauder JM. Serotonin uptake in the ectoplacental cone and placenta of the mouse. Placenta. 1993a; 14(2): 149–61.

Yavarone MS, Shuey DL, Tamir H, Sadler TW, Lauder JM. Serotonin and cardiac morphogenesis in the mouse embryo. Teratology. 1993b; 47(6): 573–84.

Yellajoshyula D, Brown DT. Global modulation of chromatin dynamics mediated by dephosphorylation of linker histone H1 is necessary for erythroid differentiation. Proc Natl Acad Sci U S A. 2006; 103(49): 18568–73.

Ye W, Shimamura K, Rubenstein JL, Hynes MA, Rosenthal A. FGF and Shh signals control dopaminergic and serotonergic cell fate in the anterior neural plate. Cell. 1998; 93(5): 755–66.

Ying QL, Nichols J, Evans EP, Smith AG. Changing potency by spontaneous fusion. Nature. 2002; 416(6880): 545–8.

Ying QL, Smith AG. Defined conditions for neural commitment and differentiation. Methods Enzymol. 2003; 365: 327–41.

Ying QL, Stavridis M, Griffiths D, Li M, Smith A. Conversion of embryonic stem cells into neuroectodermal precursors in adherent monoculture. Nat Biotechnol. 2003; 21(2): 183–6.

Yoon K, Gaiano N. Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat Neurosci. 2005; 8(6): 709–15.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA Induced pluripotent stem cell lines derived from human somatic cells. Science. 2007; 318(5858): 1917-20

Zhang R, Zhang Z, Wang L, Wang Y, Gousev A, Zhang L, Ho KL, Morshead C, Chopp M. Activated neural stem cells contribute to stroke-induced neurogenesis and neuroblast migration toward the infarct boundary in adult rats. J Cereb Blood Flow Metab. 2004; 24(4): 441-8

Zhang SC, Wernig M, Duncan ID, Brüstle O, Thomson JA. In vitro differentiation of transplantable neural precursors from human embryonic stem cells. Nat Biotechnol. 2001; 19(12): 1129–33