NEUROGENESIS OF ADULT STEM CELLS FROM THE LIVER AND BONE MARROW

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2005

This work is dedicated to my daughter, Catherine L. Deng,
from whom my life is being regenerated.

ACKNOWLEDGMENTS

I would like to express my gratitude to my mentor, Bryon Petersen, for almost four years of unfaltering guidance — he has, finally, beat the word “control” into my brain. I feel very lucky to have spent my time in his laboratory to study stem cells, and have the opportunity to work with a group of intelligent and fun fellow lab-mates. Additionally, I need to thank all the wonderful people with whom I was lucky to interact in Steindler’s lab, especially Eric Laywell who was my mentor by the bench.

I would also like to thank the invaluable members of my dissertation committee, Edwin Meyer, Edward Scott, Naohiro Terada and especially Dennis Steindler who has provided not only the outstanding mentorship to my graduate education, but also funding for all the projects included in this dissertation. My thanks also go to Gerry Shaw and Ronald Mandel, who have opened me the doors to the neuroscience and neurodegenerative disorder research.

Next I would like to thank my parents, Jialie Deng and Yeufang Yuan, who gave me not only a healthy body, but also a firm heart to sustain my unceasing curiosity. Also, I need to recognize my wife of ten years, Zhengqing Luo, for all the support and inspiration she provided to me during these years together.

Last, but certainly not least, I need to thank my fellow students and friends during the course of my graduate study at the University of Florida. They were, and will continue to be my true treasure for life.

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May 2005

Chairman: Bryon E. Petersen
Major Department: Pathology, Immunology and Laboratory Medicine

Recent reports of the adult stem cell multipotencies have generated tremendous interest regarding their potential therapeutic value, while bypassing ethical concerns surrounding the use of human embryonic stem cells. Among the different adult stem cells, the bone marrow-derived mesenchymal stem cells (MSCs) may represent the best hope for cell replacement therapy since, in addition to their multipotency and accessibility, MSCs may also be used in autologous transplantations to minimize immune rejection. The isolation of a large number of hepatic oval cells (HOCs) holds tremendous promise as a source for liver transplantation in treating both acute and chronic liver failure. With the recent reports of the trans-differentiation of oval cells into the insulin-producing pancreatic cells, as well as neural-like cells *in vivo*, using HOCs in treating diseased tissues other than liver may also be possible.

The use of stem cell therapy in treating neurodegenerative disorders has attracted considerable attention lately. In Parkinson's disease (PD), the engraftment of fetal

mesencephalic neurons, which are rich in postmitotic dopaminergic neurons, has significantly improved the patient symptoms. But limited by ethical issues as well as short of supplies in utilizing embryonic tissue, the alternative to use adult stem cells has moved to the forefront of the research. The trans-differentiation of MSCs into neural cell types has been explored extensively, with several groups reporting that these stem cells can trans-differentiate into neurons, astrocytes and microglias. However, controversies of the adult stem cell multipotency arose after reports of failures to repeat several significant previous experiments, as well as confounding factors of possible cell contamination or fusion.

In an attempt to clarify these issues, I studied two types of adult stem cells, the mesenchymal bone marrow derived cells (BMDCs) and the hepatic oval cells. There is a significant difference between these two types of adult stem cell in their capability to differentiate into neural phenotypes. While the BMDCs spontaneously generate neurogenic and astrogenic progenitors, HOCs showed little sign of neural trans-differentiation capability *in vitro*. However, both BMDCs and HOCs differentiated and expressed neural specific proteins after they were grafted into the neurogenic subependymal zone of the neonatal mouse brains.

CHAPTER 1
INTRODUCTION

Highlighted by several historical breakthroughs, stem cell biology saw its rebirth at the end of the last century. In 1997, the world was surprised by Wilmat *et al.* (1997), who demonstrated that the nucleus of a somatic cell showed full genetic potential by giving birth to Dolly sheep after injecting it into a denucleated oocyte. A year later, Thomson *et al.* (1998) developed an isolation and culture method to maintain human embryonic stem cells *in vitro*. In the field of adult stem cell research, Ferrari *et al.* (1998) first reported the trans-differentiation of bone marrow stem cells into muscle tissue in1998. The same year Shi *et al.* (1998) followed by reporting the endothelial tissue from bone marrow. A year later, Petersen *et al.* (1999) reported the bone marrow derived hepatocytes following hepatic injury, and in 2000, Brazelton *et al.* (2000) reported neural regeneration from bone marrow source. These reports of the adult stem cell multipotency changed the view of the old paradigm in cell biology and opened new possibilities for treating human diseases. With the findings of adult stem cell plasticity, it becomes possible to replace the injured, or senile tissues by either stimulating the proliferation of endogenous adult stem cells, or grafting allogenic progenitors derived from an exogenous source. However, questions about the genuineness and potential application of the adult stem cell trans-differentiation phenomenon quickly arose, and calls for re-evaluation began to appear (Holden & Vogel, 2002). The low number of trans-differentiation seen *in vivo*, as well as cell fusion and failure to repeat some experiments, puzzles scientists.

From the initial wide-range of reported plasticity to the later reexamination of those findings with more stringent criteria, in the span of only five years, the study of stem cell biology experienced the usual ups-and-downs of a new scientific field. In spite of the unclear future of the adult stem cell biology, the closer and broader re-evaluations of various issues are generally agreed on and pursued by scientists.

1.1 The Definition of Stem Cell

Defining stem cell is one of the most difficult tasks in the field of cell biology, because it is a cell type defined by its functional attributes - to generate different cell types in making of organisms - rather than the physical property. It immediately implies an inevitable paradox of maintaining cell stability in the process of functionality evaluation. Although still under debate, a working definition of stem cell is a *clonal, self-renewing* entity that is functionally *multipotent* and thus can generate multiple differentiated cell types (Melton & Cowan, 2004). Based on the development stages, there are embryonic stem (ES) cells and adult stem (AS) cells. ES cells exist in the embryogenesis stage that can eventually give rise to a whole animal. The term “adult stem cell” is used to refer to stem cells found in the tissue of an adult animal. Different names are normally given to AS cells following the tissue types in which they reside, such as hematopoietic stem cell (HSC), liver 'stem' cell, or neural stem cell (NSC), etc.

1.1.1 Three Criteria of Stem Cell Definition

The *self-renewal* of a stem cell is the ability to maintain its own numbers without input from another cell stage. It is rather hard to evaluate this property under *in vitro* conditions since tissue culture itself may alter the maintenance of the cell being cultured. A vague criterion to use, in this case, is the extensive proliferation capability in base

culture medium without apparent morphological change (Melton & Cowan, 2004). However, some somatic cells would appear to fit into this standard even *in vivo*, but the number of the passages (*in vitro*), or divisions (*in vivo*) is normally limited in somatic cells. Young cells that may be derived from stem cells have to replenish the senile cells to maintain a stable population in the tissue *in vivo*. For the stem cells, the *in vitro* passage number should be higher than eighty, an upper limit of most somatic cells (Melton & Cowan, 2004), to "unlimited." *In vivo*, stem cells should be able to last throughout the lifetime of the tissue in which they reside.

Although *clonality* is regarded as the "gold standard" (Melton & Cowan, 2004) to evaluate a stem cell, it is practically difficult to use in defining AS cells. ES cells are clonogenic: the ability to create all the cell types of an animal that is demonstrated repeatedly by chimeric animals. With a careful experimental design, a single hematopoietic stem cell has also showed its clonogenicity by generating all the progenies of the hematopoietic lineage as well as many other cell types (Krause *et al.*, 2001). Other AS cell types that reside in solid organs are much more difficult to be tested for their clonality, simply because it is impossible to repeat the developmental process of these solid organs in the adult animals. Some researchers argue that, although defined as stem cells by functional studies, mesenchymal stem cells from bone marrow will always be a heterogeneous population, because a portion of the cells differentiate spontaneously during normal culture (Quesenberry *et al.*, 2004). Under these circumstances, they argue that the clonality is an idol standard and is logically impossible to achieve for these types of stem cells.

Stem cell *multipotency* describes the functional aspect of this unique cell type that is distinct from that of a somatic cell. It refers to the multi-lineage differentiation capability of stem cells. It should be noticed that, in addition to the morphologic and immuno-phenotypic characteristics, the *functional evaluation* of a cell should be included to claim a successful differentiation. Different terms are normally used in corresponding to the potentiality hierarchies that exist in different types of stem cells. A fertilized egg is call 'totipotent' because it can generate full functional organism, as well as the placenta and other supporting tissues. Embryonic stem cells are called "pluripotent," illustrated by their capability to generate every tissue of an animal after being grafted into the ovary of a viable female foster animal. The term "multipotent" is used to describe the progenitors of different germ layers that have lost the ability to generate a whole embryo after ovary implantation. Finally, a group of terms such as "bipotent, unipotent, or monopotent" is used to describe stem cells with more restricted potencies. Included in this group are most of the AS cells that are generally tissue specific, and only give rise to one or two mature cell types of the tissue they reside. For example, hepatic oval cells are usually called 'bipotential' for the reasons that they only differentiate into hepatocytes and cholangiocytes in the adult liver. However, under the recent development of adult stem cell biology, the term *trans-differentiation* is adopted to describe the multipotency of AS cells demonstrated *in vivo* and *in vitro*. Trans-differentiation refers to the phenomenon that the progenitors of one cell lineage can differentiate into cell types of other lineages either being treated with specific, sometimes non-physiological level of induction reagents *in vitro*, or being grafted into tissue that is different from their origin *in vivo*. In this context, the hepatic oval cells may also be called multipotent, because they have been

shown to differentiate into pancreatic and neural lineages (Yang *et al.*, 2002; Deng *et al.*, 2003).

1.1.2 Embryonic Stem Cell

Embryonic stem cells are derived directly from the inner cell mass of preimplantation embryos after the formation of a cystic blastocyst. The inner cell mass would normally produce the epiblast and eventually all adult tissues, which may help to explain the developmental plasticity exhibited by ES cells. In fact, ES cells appear to be the *in vitro* equivalent of the epiblast, as they have the capacity to contribute to all somatic lineages, and in mice, to produce germ line chimeras (Papaioannou, 2001). In animal species, *in vivo* differentiation can be assessed rigorously by the ability of ES cells to contribute to all somatic lineages and produce germ line chimerism. In the purpose of obtaining suitable cell lines for the regenerative medicine, extensive efforts have been dedicated to generate different cell types *in vitro* from ES cells. However, the major obstacles are to isolate and purify the differentiated cells, and to eliminate the uncommitted ES cells after differentiation. As the result of their pluripotency, the uncommitted ES cells tend to give rise to teratomas when grafted *in vivo*.

1.1.3 Adult Stem Cell

The term adult stem cell refers to the cells found in adult animal that constantly replenish the somatic cells in the tissue of their origin. Although the existence of these AS cells is beyond doubt in most cases, the isolation and identification of these cells proved to be difficult. A rigorous assessment of the adult stem cells is to prospectively purify a population of cells using cell surface markers, transplant a single cell from the purified population into a syngeneic host without any intervening *in vitro* culture, and

observe self-renewal and tissue, or organ regeneration for multipotency. However, as discussed above, this type of *in vivo* reconstitution assay is not well defined, and impossible to do for the cells in solid organs. Therefore, it is important to assess the *in vitro* differentiation capability of the AS cells, which may reflect their developmental potential. In recent studies, the concept of multipotency of AS cells has moved to the forefront of stem cell research. It is suggested that the restrictions in cell fate are not permanent, but flexible (trans-differentiation) or reversible (de-differentiation) (Ferrari *et al.*, 1998; Shi *et al.*, 1998; Petersen *et al.*, 1999; Brazelton *et al.*, 2000). This concept has generated new ideas in the adult stem cell research, and infused new avenues into the promising stem cell therapy.

1.1.4 A Changing View on Stem Cell

Along with the findings of stem cells in various tissues in the adult animal, two models start to emerge that may be used to explain the origin and nature of the AS cells. The traditional model believes that there is a stem cell pool residing in the tissue of each organ of the adult animal that may have been preserved from the tissue specific progenitor cells during the development. The hematopoietic stem cell is an example that may be best explained by this model. Hepatologists also believe that the hepatic oval cells, the 'stem' cells of the liver that have been known for over fifty years, originate locally within the canal of herring in the liver (Alison *et al.*, 1996). However, recent findings of bone marrow derived hepatocytes suggest that oval cells may have derived from a bone marrow derived precursors (Petersen *et al*., 1999; Lagasse *et al*., 2000). These reports, along with many others that have demonstrated the bone marrow derived allogeneic tissues in solid organs, suggest a second model of the AS cell origin. This

model proposed that there might be a master adult stem cell source in the bone marrow of the adult animal. These master cells can circulate and differentiate into lineage-specific progenitors, and eventually reconstitute the damaged tissue of the solid organ (Hennessy *et al.*, 2004).

1.2 Brief History of Stem Cell Research

1.2.1 Early Research of Embryonic Stem Cell

The systematic analysis of ES cells began in the1960s, when Finch and Ephrussii (1967) established the first pluripotent embryonic carcinoma (EC) cell line from the undifferentiated compartment of murine and human germ cell tumors (Andrews, 2002). Based on experience with the culture of EC cells, the first murine ES cells were isolated from the inner cell mass of the blastocyst in 1981 (Evans & Kaufman, 1981; Martin, 1981). Bradley *et al.* (1984) later developed a technique to reconstitute early mouse embryos by injecting ES cells into blastocyst, which has formed the basis for the hundreds of “knock out” and “knock in” transgenic animals (Thomas and Capecchi, 1987). Embryonic stem cells have also allowed *in vitro* studies of the initial stages of the mammalian development, without the need to harvest peri-implantation embryos, and dissection of the basic mechanisms underlying plauripotency and cell lineage specification. In the following years, significant efforts have been made to isolate ES cells from other species including rabbits (Graves and Moreadith, 1993), pigs (Li *et al.*, 2003) and primates (Thomson *et al.*, 1996), and highlighted by the isolation of human ES cell in 1998 (Thomson *et al.*, 1998; Amit *et al.*, 2000; Reubinoff *et al.*, 2001; Richards *et al.*, 2002; Hovatta *et al.*, 2003). The establishment of various ES cell lines from different species has largely expanded our means to understand the mechanistical aspects of the

stem cell self-renewal and differentiation in the culture dish. But the generation of human ES cell lines has sparked a great deal of controversy particularly in certain religious communities (Orive *et al.*, 2003).

1.2.2 Early Development of Adult Stem Cell

The history of AS cell research goes back to early studies of each individual stem cell type resides in different organs of the adult animal. These cells possess strong regenerative capability to replenish the senile or sick cells of the tissue in which they reside under physiological condition or injury, and the study of these stem cells appeared to be unrelated from each other. Hematopoietic stem cell (HSC) in bone marrow is one of the first-known and most-studied adult stem cells. It is also the most successful example of "stem cell therapy," a term that has been give new meaning and hope in the past couple of years. The ground breaking work by Till and McCulloch (1961) in the early 1960s provided the first clear evidence that mouse bone marrow contained stem cells capable of repopulating hematopoietic tissues following cellular depletion by exposure to a cytotoxic agent, e.g., radiation. They demonstrated that grafted exogenous tissue can invade the hematopoietic organ spleen, and form colony-forming units (CFU). This experiment provided the scientific basis for subsequent human bone marrow transplant studies and dramatically expanded our knowledge of HSCs. Liver stem cell is another type of adult stem cell that has been well studied, but yet poorly understood. Grisham and Hartroft (1961) first described oval cells in the recovering liver in 1961. The rat oval cell model developed by Evarts *et al.* in 1987 (Evarts *et al.*, 1987a) and the murine oval cell model by Preisegger *et al.* in 1999 (Preisegger *et al.*, 1999) have dramatically enhanced our knowledge of HOCs. However, since oval cells cannot be found in large quantity

under normal physiological condition, or most forms of liver injuries including partial hepatectomy, the precursors of oval cell become the focal point of the controversy surrounding the liver stem cells. Neural stem cell (NSC) in the adult brain is one of the latest stem cells to be identified. For years, the central nervous system in adult animals was regarded as mitotically dormant. In the early 1990s, Reynolds and Weiss (1992) first reported the neurogenesis in the subventricular zone of adult mouse brain. Subsequently, several reports showed that NSCs exist in the dentate gyrus of the hippocampus (Gage *et al.*, 1995; Palmer *et al.*, 1997), as well as in the spinal cord (Shihabuddin *et al.*, 2000). Reynolds *et al.* (1992) also developed the widely used neurosphere culture system, which allows clonal NSCs to grow into sphere-like colonies. The use of neurospheres has given scientists a quantitative tool to study the function aspect of NSCs, and has, in some way, placed the research of NSCs ahead of many other AS cells. Utilizing neurosphere culture, Kondo and Raff (2000) and Laywell *et al.* (2000) reported that astrocytic stem cells might be the NSCs in the adult brain, and further revealed the identity of neural stem cells.

1.2.3 Recent Development of Adult Stem Cell Biology

The recent development of multipotency exhibited by a variety of AS cells, especially the multipotency of bone marrow derived stem cells, has dramatically changed the course of stem cell research in the past five years. Several studies have made a significant contribution during this period. Utilizing the well established bone marrow reconstitution of irradiated recipient in combination of genetic tracing markers, Ferrari *et al.* (1998) first reported the transdifferentiation of bone marrow stem cells into muscle in1998. Later the same year, Shi *et al.* (1998) reported the endothelial generation from

bone marrow following the similar design. Petersen *et al.* (1999) reported the bone marrow derived liver regeneration after injury in 1999, and Brazelton *et al.* (2000) reported neural regeneration from bone marrow source in 2000. Plasticity has also been found in cells isolated from other tissues including skeletal muscle (Jackson *et al.*, 1999) and brain (Bjornson *et al.*, 1999). The underlined impact of these paradigm-shifting work has changed our view on the long-believed forward development biology, the way the animal body function, as well as how we may be able to treat diseases in the future.

1.2.4 A Time for Reappraisal

Despite the wide range of reports of AS cell plasticity from different tissue and species, the initial enthusiasm of the possible clinical application quickly gave way to rigorous critical evaluation of the trans-differentiation phenomenon. The genuineness of the newly found neurogenesis in the human neocortex (Shankle *et al.*, 1998) was the first to be challenged by Korr and Schmitz in 1999, and followed by Rakic *et al.* in 2002. Several groups then showed that the hematopoietic cells that were proposed to have been derived from trans-differentiation of muscle cells were in fact *bona fide* hematopoietic cells resident within muscle tissue (McKinney-Freeman *et al.*, 2002; Issarachai *et al.*, 2002). Terada et al. (2002) and Ying et al. (2002) independently demonstrated that when bone marrow cells are cultured with ES cells, they fuse with each other, and the hybrid cells take on an ES cell phenotype. These studies may suggest that the adult stem cells thought to be trans-differentiating might have fused with host cells within various local tissue microenvironments. This was further confirmed when it was demonstrated that it might also be an event *in vivo* in the liver (Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003), brain (Weimann *et al.*, 2003a; Weimann *et al.*, 2003b), and heart (Alvarez-Dolado *et al.*,

2003). Recently, Zhang *et al.* (2004) demonstrated that trans-differentiation and cell fusion might co-exist in the process of cardiomyocyte regeneration from $CD34^+$ bone marrow derived stem cells. Under the current circumstances, it is obvious that more serious evaluation of the trans-differentiation phenomenon with more stringent criteria is needed for the future of stem cell-based regenerative medicine.

1.3 Bone Marrow Derived Mesenchymal Stem Cells

Bone marrow derived mesenchymal stem cell (MSC) was first described by Petrakova *et al.* (1963) some 40 years ago. It was demonstrated that pieces of bone marrow transplanted under the renal capsule of mice formed an osseous tissue over a period of several weeks that was invaded by hematopoietic cells (Petrakova *et al.*, 1963). Mesenchymal stem cells can be extensively expanded *in vitro* and readily differentiate into mesenchymal lineage including osteocytes, chondrocytes and stromal cells with little to no specific inductions. In the recent development of regenerative medicine, MSCs have been the favorite cell source for transplantation because of their potent differentiation capability, and also because of the accessibility and possible autologous transplantation to eliminate immuno-rejection (Awad *et al.*, 1999; Dezawa *et al.*, 2004). Despite the great potential to differentiate into many useful cell types, the identity of MSCs, or even whether MSC are true stem cells or not, remains questionable (Javazon *et al.*, 2004). Limited by our current knowledge of the MSC surface marker, there has not been a globally agreed context for characterizing MSCs.

1.3.1 The Bone Marrow Niche

Bone marrow stroma is a complex tissue with the function of supporting hematopoiesis. It hosts a number of cell types and maintains the undifferentiated HSC

and supports differentiation of erythroid, myeloid, and lymphoid lineages. There are adherent macrophages and other mononuclear cells of hematopoietic lineage, including some phagocytic cells and other antigen-presenting (dendritic) cells. There are mesenchymal cells, such as osteoblasts and adipoblasts. There are endothelial cells, which may arise from a hemangioblast or other endothelial cell precursor. Bone marrow stroma promotes cellular differentiation to these specific lineages while also maintaining stem and progenitor cells. Bone marrow also actively maintains the undifferentiated state of HSCs and MSCs.

1.3.2 Isolation and Characteristics of Mesencymal Stem Cells

Mesenchymal stem cells can be isolated from a bone marrow aspirate, and readily cultured via methodology similar to that originally used by Friedenstein, and optimized by Caplan *et al.* in 1991, utilizing their adhesive properties (Goshima *et al.*, 1991b; Friedenstein, 1995). Mesenchymal stem cells are spindle-shaped and fibroblast-like in their undifferentiated state of *in vitro* culture. In the rodent experimental animals, bone marrow aspirates are normally taken from the tibias and femurs. In human marrow donors, they are often harvested from the superior iliac crest of the pelvis. Frequently, the marrow sample is subjected to fractionation via density gradient centrifugation and cultured in a medium such as Dulbecco's modified Eagle's medium (DMEM), containing 10-20% fetal bovine serum. Primary cultures are usually maintained for 12-16 days, and are then detached by trypsinization followed by sub-culturing.

An important property, but not a defining feature, of the MSC population *in vitro* is their ability to form colonies after low-density plating or single-cell sorting (Brockbank *et al.*, 1985; Kuznetsov *et al.*, 1997; Colter *et al.*, 2000; Javazon *et al.*, 2001). As

demonstrated by Owen and Friedenstein (1988) and DiGirolamo *et al.* (1999), colonies derived from CFU-F assays are extremely heterogeneous in both appearance (morphology and size) and differentiation potential. One of the difficulties in defining MSCs is that there are no immunophenotypic markers that are uniquely expressed by MSCs (Haynesworth *et al.*, 1992; DiGirolamo *et al.*, 1999). In order to identify a culture derived from whole bone marrow cell suspension as MSCs, an array of immunophenotypic profile has to be used. MSCs express neither a hematopoietic marker such as CD45, CD34, CD14, nor a endothelial marker such as CD31. They do express a large number of adhesion molecules such as CD44, SH-4, and some stromal cell markers such as SH-2, SH-3 and SH-4, with significant variations reported by different laboratories (Haynesworth *et al.*, 1992; Majumdar *et al.*, 1998; Deans & Moseley, 2000; Peister *et al.*, 2004). As discussed previously, perhaps the most useful approach for presumptive identification of the MSC remains functional. The capacity for induced *in vitro* differentiation of MSCs to bone, fat, and cartilage is perhaps the single critical requirement to identify putative MSC populations (Pereira *et al.*, 1994; Pittenger *et al.*, 1999). It is important to emphasize that currently all MSC populations analyzed by clonal assays are heterogeneous, with individual cells capable of varying differentiation potential and expansion capacity (Owen & Friedenstein, 1988; DiGirolamo *et al.*, 1999).

1.3.3 Mesengenesis of Mesenchymal Stem Cells

The differentiation of MSCs into bone, cartilage, and fat *in vitro* has been well-described (Barry, 2003). Osteogenic activation requires the presence of β-glycerol-phosphate, ascorbic acid-2-phosphate, dexamethasone, and fetal bovine serum (Barry, 2003). When cultured in monolayer in the presence of these supplements, the cells

acquire an osteoblastic morphology with up-regulation of alkaline phosphatase activity and deposition of a hydroxyapatite mineralized extracellular matrix (Barry, 2003). Chondrogenic differentiation occurs when MSCs are cultured under certain conditions, including 1) a three-dimensional culture format, 2) a serum-free nutrient medium, and 3) the addition of a member of the transforming growth factor-β superfamily. MSCs cultured in monolayer in the presence of isobutylmethylxanthine become adipocytes with the production of large lipid-filled vacuoles (Suzawa *et al.*, 2003).

The *in vivo* differentiation capability of MSCs is demonstrated by their contribution to the repairing process of the injured tissue after transplantation. Mesenchymal stem cells implanted in an osseous defect, such as a large segmental gap in the femur, stimulate formation of new bone (Bruder *et al.*, 1998). Similarly, Ponticiello *et al.* (2000) showed that scaffolds loaded with MSCs and implanted in an osteochondral lesion on the medial femoral condyle give rise to both cartilage and bone cells. In addition, Toma *et al.* (2002) reported that human MSCs, when delivered by infusion to an immunocompromised mouse, could engraft to the normal myocardium and differentiate into a cardiomyocyte phenotype. Significantly, greater injury-specific cardiac homing of infused MSCs occurs when the cells are delivered within 10 mins of infarction, compared to 2 weeks post-infarction (Toma *et al.*, 2002).

1.3.4 Trans-differentiation of Mesenchymal Stem Cells

In the past several years, various groups reported that bone marrow derived stem cells differentiate into hepatic, muscle, kidney, lung, as well as neural lineages *in vivo* (Factor *et al.*, 1990; Ferrari *et al.*, 1998; Brazelton *et al.*, 2000; Orlic *et al.*, 2003). However, since bone marrow contains both HSCs and MSCs, the use of whole bone

marrow cells in most of these experiments cannot distinguish which of the two populations contributed to the newly generated tissue. *In vitro* experiment using cultured MSCs to induce differentiation may provide better demonstration of MSC plasticity, since HSC has not been described to endure long-term culture without differentiating toward a mature phenotype. However, it may be argued that multipotency demonstrated by *in vitro* experiments may not reflect the nature of MSCs *in vivo*, since the culturing process normally involves a non-physiological level of growth factors or chemicals. Nevertheless, several groups have demonstrated that long-term cultured MSCs can be induced to differentiate into hepatic, pancreatic and neural lineages (Woodbury *et al.*, 2000; Sanchez-Ramos *et al.*, 2000; Deng *et al.*, 2001; Lee *et al.*, 2004; Shu *et al.*, 2004; Tang *et al.*, 2004). Furthermore, engraftment of cultured MSCs into neonatal or fetal mouse brain have demonstrated a migration and trans-differentiation of MSCs into neural lineage (Azizi *et al.*, 1998; Kopen *et al.*, 1999).

1.3.5 Multipotent Adult Progenitor Cells and Marrow-isolated Adult Multilineage Inducible Cells

Jiang *et al.* (2002) reported bone marrow derived stem cells, namely multipotent adult progenitor cells (MAPCs) from the postnatal marrow of mice and rats, following the typical MSC isolation protocol. The MAPCs can be cultured indefinitely in a relatively nutrient-poor medium. They are highly plastic and differentiate into cells bearing endodermal, mesodermal, or ectodermal markers under induction *in vitro*. The MAPCs also display their broad differentiation potential *in vivo*. For these assays, ROSA-26-derived MAPCs injected into murine blastocysts resulted in chimeric mice with ROSA-26 cells contributing to nearly all somatic tissues, including brain, lung, myocardium, liver, intestine, and kidney. After intravenous administration into a

sublethally irradiated immunodeficient mouse, MAPCs differentiate, in varying degrees, into hematopoietic cells in the marrow, blood and spleen, and into epithelial cells in liver, lung, and intestine.

Similar to MAPC, D'Ippolito *et al.* (2002) isolated a bone marrow derived population of postnatal young and old human cells with extensive expansion and differentiation potential to generate chondrocyte, adipoctye, neuron, and insulin producing cells *in vitro*. They named their cells marrow-isolated adult multlineage inducible (MIAMI) cells. Like the MAPCs, the cell surface antigen profile of MIAMI cells demonstrate MSC characteristics, indicating both types belong to mesenchymal cells. Because of the concerns about the manipulation in cell culture process, critics may still question whether these highly potent stem cells are the *real* MSCs exist in the bone marrow of an animal or not. Nevertheless, the existence of MAPCs and MIAMIs may have proved the therapeutic value of bone marrow derived stem cells as the potential cell source in the stem cell therapy.

1.4 Hepatic Oval Cells

Hepatic oval cell is a transit cell type during the liver regeneration when hepatocyte proliferation is impeded (Pack *et al.*, 1993). Hepatic oval cells differentiate into hepatocytes and bile duct cells, and can be isolated from the animal models in large quantity. Cultured oval cells are self-renewable and have been shown to become pancreatic cells when challenged with high glucose in an *in vitro* system (Yang *et al.*, 2002), and neural cells after transplantation into the mouse brain (Deng *et al.*, 2003).

1.4.1 Hepatic Oval Cell As The Adult Stem Cell In The Liver

The potent regeneration ability of the liver after injuries has been known for centuries. The ancient legend of Prometheus should be mentioned to illustrate this historically well-known phenomenon. Prometheus was punished severely for stealing the secret of fire and giving it to man. Zeus banished him to Mt. Caucasus, where he endured the torture of a bird of prey pecking out his liver on a daily basis. Every night the liver would repair itself only to be pecked out the next day. Despite the mythic quality of this story, the liver does indeed have the remarkable ability to regenerate. In general, hepatocytes maintain their potential to divide and will respond to elevated growth factors such as hepatocyte growth factor (HGF), acidic fibroblast growth factor (aFGF) after liver injury (Kan *et al.*, 1989; Lindroos *et al.*, 1991). However, when hepatocyte proliferation is blocked by chemicals such as allyl alcohlol (AA) and carbon tetrachloride (CCl_4), stem cell-involved liver regeneration is initiated to recover the lost liver function (Rechnagel & Glende, Jr., 1973; Badr *et al.*, 1986; Belinsky *et al.*, 1986). Although no "hepatic stem cell" has been convincingly isolated from health animal liver, a "transit" type, the so-called hepatic oval cell has been successfully isolated in large quantity under protocols causing liver injuries while inhibiting the hepatocyte proliferation (Shinozuka *et al.*, 1978; Evarts *et al.*, 1987b; Evarts *et al.*, 1989). Oval cells were first described by Grisham and Hartroft (1961) et al in the recovering liver. They are bi-potential in that they can differentiate into mature hepatocytes and biliary epithelial cells *in vitro* and *in vivo* (Sirica *et al.*, 1990; Sirica, 1995). However, the progenitors of HOCs, the presumable "hepatic stem cell", remains enigmatic and under debate currently. There are two major ideologies: 1) Alison *et al.* (1996) detected so-called "facultative liver stem

cells" at the canal of herring that could have given rise to the oval cells and thus they are native to the liver; and 2) Petersen *et al.* (1999) demonstrated that, after certain liver injuries, rats that received bone marrow transplantation hosted mature hepatocytes of donor cell origin, suggesting that oval cells may have derived from an extra hepatic source. Theise *et al.* (2000) also provided evidence that human hepatocyte could also derived from bone marrow. Crosbie *et al.* (1998) provided *in vitro* evidence to show that there are hematopoietic stem cells exist in the human liver. Besides the known observation of their bipotential differentiation ability, the oval cells express some proteins that mark its 'stem' cell identity. Oval cells express many hematopoietic stem cell markers, such as Thy1, c-kit, and CD34 in the rat, and flt-3 in the mouse (Omori *et al.*, 1997a; Omori *et al.*, 1997b; Petersen *et al.*, 1998a). Recently, it is also reported that mouse oval cells also express Sca-1 as well as CD34 (Petersen *et al.*, 2003).

1.4.2 Induction and Isolation of Hepatic Oval Cells

In the rat model, several protocols have been described to induce the oval cell proliferation (Evarts *et al.*, 1989). The most common protocols are so-called two-step induction, in which rats are given a chemical such as 2-acetylaminofluorene (2AAF), which hinders hepatocyte proliferation, and physical damage such as PHx or CCl_4 (Petersen, 2001). 2-AAF is a chemical that, when metabolized by hepatocytes, blocks the cyclin D1 pathway in the cell cycle. The oval cells then arise in the periportal region of the liver. In the mouse, the HOC proliferation does not respond to 2-AAF/PHx protocol the same way as in the rat. Preisegger *et al.* (1999) developed a model using the chemical 3,5-diethoxycarbonyl-1,4-dihydrpcollodine (DDC) in a standard diet at a concentration of 0.1%. The mouse oval cells are also different from the rat oval cells in their marker

expression profiles. Instead of OV6 antibody as that of rat, the mouse oval cells are positive to A6 antibody (Factor *et al.*, 1990).

The early methods to isolate the oval cells from the rat and mouse livers are based on gradient centrifugation of the non-parenchymal cell (NPC) fraction after collagnease perfusion of the liver. Several other methods such as Metrizamide gradient (Sells *et al.*, 1981), Percoll gradient (Sirica & Cihla, 1984) and centrifugal elutriation (Yaswen *et al.*, 1984; Pack *et al.*, 1993) have also been used in the past two decades. However, the purity of the oval cell population from these isolation techniques cannot exceed 90% based on marker testing. Recently, the oval cells have been found to highly express the hematopoietic stem cell marker Thy-1 in rat (Petersen *et al.*, 1998a). Based on this finding, Petersen *et al.* (1998a) described an isolation method to utilize this feature of the oval cells, in combination of the flow cytometry technique. This method yields a 95-97% enriched population of Thy-1.1^{+} cells, which were also showed to express the traditional oval cell markers of α-fetal protein (AFP), cytokine 19 (CK-19), gamma glutamyl-transferase (GGT) and OV6. Using centrifugal elutriation, Pack *et al.* (1993) has been able to establish three cell lines from DL-ethionine-fed rats. They've demonstrated that the oval cells can be maintained in culture for at least two years. In their culture, rat HOCs have a size of about 10-15 μm in diameter, positive for CK-19, GGT immunocytochemistry staining. However, CK-19 became negative after 10 passages, demonstrating a transformation of the oval cells at *in vitro* culture condition. In our culture medium that contains high level of stem cell growth factors, such as stem cell factor (SCF), leukemia inhibitory factor (LIF), IL-3 and IL-6, HOCs started to proliferate in about a week, and appear typical oval cell morphology. If lower the growth factor

concentration by ten times, the oval cells transformed into a morphology resembling marrow stromal cells. Mouse oval cells can also be enriched by using their cell surface antigen such as Sca-1 (Petersen *et al.*, 2003). The cells isolated with this method express the mouse oval cell specific proteins such as AFP and A6, and can be culture for about two months.

1.4.3 The Multipotency of Hepatic Oval Cells

The multipotency of HOCs to differentiate into cell lineage other than hepatocyte and bile duct cells has not been explored adequately. A few studies showed that HOCs might adopt different cell types when cultured under various conditions (Pack *et al.*, 1993). When co-cultured with porcine microvascular endothelial cells (PMEC), HOCs give a strong epithelial morphology. If the HOCs in culture (3-day colonies) are overlaid with Matrigel, they appear to become stellate cells 7 days later (Petersen, 2001). Recently, Yang et al (2002) trans-differentiated a purified rat oval cell line into endocrine pancreas capable of insulin-secretion *in vitro*, when challenged with high concentration of glucose and nicotinomide. It has also been demonstrated that the mouse HOCs express multiple neural specific proteins, and exhibit phgocytosis activity of functional microglias after transplanted into the mouse brain (Deng *et al.*, 2003).

1.5 Developmental Neurogenesis

The nervous system is the most complex of all the organ systems in the animal embryo. In mammals, for example, billions of neurons develop a highly organized pattern of connections, creating the neuronal network that makes up the functioning brain and the rest of the nervous system. During embryogenesis, an orchestration of delicately balanced signaling molecules are involved to develop this complex network. And yet, recent

evidences show that neurogenesis also exists in the adult brain, a concept against the long-held belief of developmental biologists and neurologists.

As in other developing systems, nerve cell specification is governed both by external signals and by intrinsic differences generated through asymmetric cell division. During neurogenesis, multiple biological processes function in concert to ensure that the diverse neurons and glias proliferate, differentiate, migrate and form synapses at the appropriate time and place. These processes rely on the precise control of temporal and spatial expression of genes that encode secreted and membrane associated proteins. Proteins destined for secretion or for transport to locations within the membrane (e.g. neurotransmitters, growth factors, guidance cues, ion channels, etc.) convey fundamental information necessary for the cells to respond to the evolving intra- and extra-cellular environments during development.

1.5.1 Singling Pathways during the Developmental Neurogenesis

Vertebrate neurogenesis involves several progressive steps mediated by multiple signaling pathways that eventually sculpt the gene expression profile of specific subtypes of neuron. As the first step, the neural induction defines the neural plate, which consists of neural precursors that express pan-neural genes. In general, three signaling pathways have been implicated in the neural induction: repression of bone morphogenetic protein (BMP) signaling, and activation of the fibroblast growth factor (FGF) as well as Wnt pathways (Knecht & Harland, 1997; Baker *et al.*, 1999). The apparently autonomous acquisition of neural character on removal of inhibitory BMP signaling in the frog has led to the proposal that the neural cell state occurs by default (Hemmati-Brivanlou & Melton, 1997; Tropepe *et al.*, 2001). The well-known BMP signaling antagonists are secreting

proteins encoded by *noggin*, *chordin*, *follistatin* and *Xnr3* (Diez & Storey, 2001). Wnt pathway has also been shown to reduce BMP signaling, and promote neural cell fate (Baker *et al.*, 1999). More recently, FGF signaling has been shown to initiate (Alvarez *et al.*, 1998; Storey *et al.*, 1998) and to be required (Wilson *et al.*, 2000) for neural induction in chick, acting in part by suppressing *BMP4* transcription (Streit *et al.*, 1998). The second major event during the vertebrate developmental neurogenesis is the dorsoventral, anteroposterior, and segmentation patterning of the central nervous system. Signaling molecules at this stage include retinoic acid, Krox20, eFGF for anteroposterior determination, and sonic hedgehog for dorsoventral determination (Franco *et al.*, 1999). The last step is the neuron subtype specification, which involves a combination of homeodomain transcription factors such as Dbx1, Dbx2, Nkx2.2, Pax6, and Pax7 (Briscoe *et al.*, 2000). As the neuron maturation reaches to the final stage, these transcription factors are down-regulated (Walther & Gruss, 1991; Scardigli *et al.*, 2001), replaced by commonly known neuronal markers such as Tuj1, NF-L, and NeuN (Diez & Storey, 2001). Overlaps of developmental stages may exist, and one signaling molecule may be involved in multiple pathways.

1.5.1.1 Bone Morphgenetic Protein and Noggin/Chordin system

The bone morphgenetic protein 4 (BMP-4), and its inhibitors noggin and chordin forms one of the most important external signaling system throughout the embryonic neurogenesis (Hemmati-Brivanlou & Melton, 1997). An enormous amount of efforts in early twentieth century was devoted to identify the signals involved in neural induction in amphibians and birds (Waddington, 1950; Spemann & Mangold, 2001). The results indicated that the inducing molecules do not act directly on the cells that will form neural

tissue, but act instead on molecules that inhibit the cells from forming neural tissue (Hemmati-Brivanlou & Melton, 1997). BMP-4 was later found to play a pivotal role in the neural induction, since it inhibits cells from forming neural tissue. The inhibitors of BMP signaling are proteins encoded by the genes *noggin* and *chordin*. Noggin and chordin are secreted proteins unrelated to any of the known growth factor families. When added into isolated blastula animal caps, the noggin and chordin proteins induced neural markers expression in the culture (Hemmati-Brivanlou & Melton, 1997).

1.5.1.2 Retinoic Acid Signaling

Retinoic acid (RA) is a small hydrophobic molecule- a derivative of vitamin A- which has an important role in local signaling in vertebrate development. The precursor of RA, retinol, has been described as a hormone, released from its storage sites in the liver and kidney. However, unlike other hormones, there are no reported regulatory factors that control retinol's release into the circulation. Homeostatic controls exist solely to maintain steady levels of plasma retinol. During embryogenesis, RA play a critical role in patterning, segmentation, and neurogenesis of the posterior hindbrain and it has been proposed that they act as a posteriorizing signal during hindbrain development (Durston *et al.*, 1997). The endogenous RA is a precisely regulated factor that controls many aspects of embryonic development. RA binds to and activates transcriptional regulators of the nuclear receptor family that also includes the receptors for thyroid and steroid hormones. Two types of binding proteins are thought to be involved in the intracellular regulation of retinoids; cellular retinol binding protein (CRBP) types I and II, and cellular retinoic acid binding protein (CRABP) types I and II (Ong *et al.*, 2000). CRBP I binds retinol and is involved in the storage as well as in the oxidation of retinol via retinol to

RA (Carson *et al.*, 1984; Eriksson *et al.*, 1987; Posch *et al.*, 1992). The role of CRBP II, which is mainly found in the enterocytes of the gut, may be to handle retinoids after dietary uptake for further metabolism and transport to the liver (Porter *et al.*, 1985).

1.5.1.3 Fibroblast Growth Factor in neurogenesis

There are at least 23 different members of the fibroblast growth factor (FGF) family. These FGFs are classified as a family on the basis of a conserved 120 amino acid core region and share a 30-60% amino acid identity across the family. Fibroblast growth factor family members have diverse functions, being potent modulators of cell proliferation, migration, differentiation and survival (Goldfarb, 1996; Ornitz, 2000). There are four FGF receptor genes, *FGFR-1-4*, and within these, alternative splicing creates receptor isoforms with distinct specificities for different FGFs. Expression studies demonstrate that members of the FGF family are highly expressed early in the developing central nervous system (CNS) (Ford-Perriss *et al.*, 2001). Among them, FGF-1, FGF-2 and FGF-15 are more generally expressed throughout the developing neural tube in both the embryonic and adult CNS. Noticeably, FGF-8 and FGF-17 are tightly localized to specific regions of the developing brain and are only expressed in the embryo during the early phases of proliferation and neurogenesis (Ford-Perriss *et al.*, 2001). There is accumulating evidence that FGFs have a critical role in the initial generation of neural tissue at the stage of neural induction. Fibroblast growth factor-1 has been reported to stimulate neuronal process regrowth in retinal ganglion cell cultures (Lipton *et al.*, 1988), spiral ganglion explants (Dazert *et al.*, 1998) and adult dorsal root ganglion cells (Mohiuddin *et al.*, 1996). Fibroblast growth factor-3 is down-regulated in the hindbrain

by E11, but expression continues in the vestibular sensory epithelia and organ of Corti at later ages (Mansour *et al.*, 1993; McKay *et al.*, 1996).

1.5.2 Neurogenesis in the Adult Brain

Increasing evidence has demonstrated that generation of new neurons is not entirely restricted to prenatal development, but continues throughout adult life in certain regions of the mammalian brain (Steindler *et al.*, 1996; Gage, 2002). The demonstration of neurogenesis in the human brain makes this phenomenon of particular relevance to treating neurological injury and disease, with the hope that the ability to generate new neurons may be utilized for structural brain repair (Eriksson *et al.*, 1998). Neurogenesis is not widespread within the adult mammalian brain, but restricted to the two germinal centers, the hippocampal dentate gyrus and the anterior subependymal zone (SEZ) of the lateral ventricles (Thomas *et al.*, 1996; Peretto *et al.*, 1999). Transient neurogenesis may also occur in the cerebral cortex (Gould *et al.*, 2001). Similarly, limited neurogenesis may occur in the substantia nigra, although this is disputed (Lie *et al.*, 2002; Frielingsdorf *et al.*, 2004). As a preserved niche for neural stem cells, SEZ and hippocampus also provide an ideal environment for testing the neurogenecity of other adult stem cell recently in the postnatal and adult brain (Zheng *et al.*, 2002; Deng *et al.*, 2003; Hudson *et al.*, 2004).

1.5.2.1 Hippocampal neurogenesis

In the hippocampus of the adult brain, a certain rate of cell proliferation has been described in the dentate gyrus granular layer, giving rise to granule neurons (Altman & Das, 1965; Kaplan & Bell, 1984). In the rat, such neurogenesis has been observed up to 11 months of age. Newly generated hippocampal granule cells extend dendrites and axons; the latter grow through the hilus and CA3 region of the Ammon's horn (Stanfield

and Trice, 1988), thus representing an example of long-distance axonal pathfinding through a mature brain neuropil. Unlike the olfactory receptor neurons, newly generated cells of the dentate gyrus substantially increase with age (Bayer *et al.*, 1982). It has been proposed that hippocampal granule cells can originate during adulthood both from local proliferation in the granule cell layer and after short migration from the hilus (Cameron *et al.*, 1993), in a manner similar to that described during postnatal development (Schlessinger *et al.*, 1975). Despite accumulating evidence for the existence and modulation of adult neurogenesis, there is still limited data elucidating the functional contribution of these newly generated neurons (Kempermann *et al.*, 2004). However, there is evidence that individual neurons can become functionally integrated (van Praag *et al.*, 2002). In addition to forming appropriate anatomical connections (Markakis & Gage, 1999), newly generated hippocampal neurons have been shown to exhibit appropriate electrical activity (van Praag *et al.*, 2002).

1.5.2.2 Subependymal zone/olfactory bulb neurogenesis

The olfactory bulb is another area of the mammalian CNS where neurogenesis has been described during adulthood (Altman & Das, 1965; Hinds, 1968; Steindler *et al.*, 1996). In early studies this neurogenesis was correlated with an adjacent region of the forebrain known as the subependymal zone (SEZ), a remnant of the primitive forebrain. Proliferating cells within the SEZ migrate along a defined pathway, the rostral migratory stream (RMS), where cell proliferation continues until reaching the olfactory bulb where they integrate into the granule and glomerular cell layers (Luskin, 1993; Lois & Alvarez-Buylla, 1994). The SEZ, which undoubtedly constitutes the major site of cell proliferation in the adult mammalian brain (Tzeng *et al.*, 2004), has been indicated as the

source of cell precursors, known as ‘brain marrow’ (Steindler *et al.*, 1996). Thus, cell proliferation in the SEZ and neurogenesis in the olfactory bulb form a complex system spanning the length of the forebrain (about 5–6 mm in rodents (Altman & Das, 1965; Lois & Alvarez-Buylla, 1994)). The newly generated neurons in the olfactory bulb also show evidence of functional integration into neural circuitry involved in processing sensory input (Carleton *et al.*, 2003). While these newly generated neurons appear capable of functioning and participating in established circuitry, recent studies carried out on this system provided evidence for several morphological and functional peculiarities. For example, the persistence of long-distance migration and multipotent stem cell compartment appear qualitatively and quantitatively different from those described in other neurogenetic areas of the adult mammalian nervous system (Peretto *et al.*, 1999).

1.6 Neural Induction *In vitro*

In vitro neural induction offers an ideal system for testing theories of neurogenesis during development. The recent progress within stem cell biology has infused a high interest in developing effective protocols to drive stem cells into a neural phenotype, in the hope that they may be used to replace the lost neural tissues in the neurodegenerative diseases.

1.6.1 Neural Induction from Embryonic Carcinoma and Embryonic Stem Cell Lines

The early efforts of neural differentiation *in vitro* are mostly contained within ES and embryonic carcinoma (EC) cell types, in the purpose of understanding the basic mechanism of cell differentiation during neurogenesis. As discussed above, RA is an important growth factor during the embryogenesis, it is also commonly used to induce EC or ES cells into neuron phenotype in culture. Pleasure *et al.* (1992) used RA to treat NT2-N cells, a human teratocarcinoma cell line, and achieved a 95% pure neuron

population. Bain *et al.* (1995) used a so-called '4+/4-' protocol, in which ES cell aggregates were treated with RA for four days and cultured without RA for four days in non-adhesive culture condition. The cell aggregates were then plated on an adhesive substrate and were differentiated into neurons, while aggregates not treated with RA differentiate into various lineages (Bain *et al.*, 1996). Gottlieb and Huettner (1999) found a significant upregulation of RA receptor- (RAR) and RAR mRNA, but a rapid down-regulation of RAR and retinoid X receptor- (RXR) mRNA during RA-induced neuronal differentiation of mouse EC cells (Gottlieb & Huettner, 1999). These support the hypothesis that *in vitro* and *in vivo* pathways may be comparable.

Besides RA, other pro-neuronal growth factors have also been used, either by direct addition to the medium, or through gene transfer into the cells. Pevny *et al.* (1998) demonstrated that P19 cells started to express neuronal markers in 4-5 days after transfected with the *sox1* gene. They also showed that Sox1 and neurofilament proteins are mutually exclusive in the mature neurons, a phenomenon also exists in the developing brain. Sox1 is expressed throughout the neural plate and early neural tube, but is down regulated in a stereotyped manner in cells along the dorsoventral axis of the neural tube later in the development stage (Pevny *et al.*, 1998). As a potent antagonizing factor of BMP signaling, noggin has also been shown to convert embryonic stem cells into primitive neural stem cells by inhibiting BMP signaling (Gratsch & O'Shea, 2002).

1.6.2 Neural Induction from Mesenchymal Stem Cell Line

Mesenchymal stem cells are the most-studied adult stem cells in terms of differentiation induction *in vitro*, because they are easy to obtain and culture (Azizi *et al.*, 1998; Kopen *et al.*, 1999; Brazelton *et al.*, 2000). The neural induction methods of MSCs

range widely, from the use of neurotrophic factors, co-culturing with neural tissue, to the so-called ‘chemical inductions’.

1.6.2.1 Neurotrophic Growth Factor induction

Neurotrophic growth factors are polypeptide hormones that are essential for the development and maintenance of the central nervous system. During the period of target innervation, limiting amounts of neurotrophic factors regulate neuronal numbers by allowing survival of only some of the innervating neurons, the remaining being eliminated by apoptosis (Kirkland & Franklin, 2003; Yeo & Gautier, 2004; Wiese *et al.*, 2004). Several lines of evidence indicate that various neurotrophic factors also influence the proliferation, survival and differentiation of precursors of a number of neuronal lineages (Kirkland & Franklin, 2003; Wiese *et al.*, 2004). In the adult brain, neurons continue to be dependent on trophic factor support, which may be provided by the target or by the neurons themselves. Their ability to promote survival of peripheral and central neurons during development and after neuronal damage has stimulated the interest in these molecules as potential therapeutic agents for the treatment of nerve injuries and neurodegenerative diseases. They are also widely used in the neural induction from adult stem cells *in vitro*. Sanchez-Ramos *et al.* (2000) treated mouse marrow stromal cells with epithelial growth factor (EGF) and brain derived neurotrophic factor (BDNF), as well as co-culture with fetal midbrain tissue, and successfully detected neuronal markers such as NeuN and MAP2 expression. In combination with 5-azacytidine, a demethylating agent capable of altering the gene expression pattern, Kohyama *et al.* (2001) treated the marrow stroma-derived mature osteoblasts with noggin, and induced neural

differentiation. Several commonly utilized members of the neurotrophic factors in neural differentiation protocols are listed below.

Nerve growth factor (NGF) is the prototype for the neurotrophin family of polypeptides which are essential in the development and survival of certain sympathetic and sensory neurons in both the central and peripheral nervous systems. Nerve growth factor was discovered when mouse sarcoma tissue transplants into chicken embryos caused an increase in the size of spinal ganglia. In the course of attempting to characterize the agent responsible for this action, snake venom, used as a phosphodiesterase, was found to be a rich source of NGF (Angeletti *et al*., 1968).

Brain-Derived Neurotrophic Factor (BDNF) is important in development and maintenance of neuronal populations within the central nervous system or cells directly associated with it. BDNF has been shown to enhance the survival and differentiation of several classes of neurons *in vitro,* including neural crest and placode-derived sensory neurons, dopaminergic neurons in the substantia nigra, basal forebrain cholinergic neurons, hippocampal neurons, and retinal ganglial cells (Larsson *et al.*, 2002; Gustafsson *et al.*, 2003).

Neurothophin3 (NT-3) is a member of the neurotrophin family. Neurothophin3 is important in development and maintenance of neuronal populations and promotes differentiation of neural crest derived sensory and sympathetic neurons. Neurothophin3 is critical for proprioceptive 1a afferent neurons, which relay information from peripheral muscle spindles to motorneurons, sending projections to spinocerebellar neurons. It is also critical in the superior cervical and nodose ganglia.

1.6.2.2 Chemical induction

The term “chemical induction” was first adopted by Lu *et al.* (2004) to describe a group of neural induction methods that use potent chemical reagents to achieve rapid and dramatic neuron-like morphology acquisition in adult stem cells. Woodbury *et al.* (2000) was the first to report that dimethylsulfoxide (DMSO) and butylated hydroxyanisole (BHA) could induce rat and human marrow stromal cells to differentiate into neurons. Deng *et al.* (2001) treated human marrow stromal cells with isobutyl mehtylxanthine (IBMX) and dibutyral cyclic AMP (dbcAMP) to elevate cytoplasm cAMP and observed morphological change from stromal cell-like to neuron-like (Deng *et al.*, 2001). Since then, there have been many groups using similar methods to induce mesenchymal stem cells from a variety of sources with essentially the same observation (Lambeng *et al.*, 1999; Black & Woodbury, 2001; Safford *et al.*, 2002; Jori *et al.*, 2004; Lopez-Toledano *et al.*, 2004; Lu *et al.*, 2004). However, the next section will discuss in depth the effect of chemical induction is currently very controversial. Several commonly used reagents listed below.

Cell-permeable *Dibutyryl Cyclic Adenosine Monophosphate* (dbcAMP) analog activates cAMP dependent protein kinase A (PKA). It affects cell growth and differentiation by altering gene expression. It can inhibit cell proliferation and induce apoptosis, yet reported to improve the survival of dopaminergic neurons in culture (Mourlevat *et al*., 2003). It has been shown to block free radical production in response to parathyroid hormone, pertussis toxin or ionomycin (Graves and Moreadith, 1993).

3-Isobutyl-1-Methylxanthine is a non-specific inhibitor of cAMP and cGMP phosphodiesterase. The increase in cAMP level as a result of phosphodiesterase

inhibition by IBMX activates PKA, leading to decreased proliferation, increased differentiation, and induction of apoptosis. 3-Isobutyl-1-Methylxanthine inhibits phenylephrine-induced release of 5-hydroxytryptamine from neuroendocrine epithelial cells of the airway mucosa (Li *et al.*, 2003).

Dimethyl Sulfoxid is a common cryoprotective agent to keep most mammalian cells from mechanical injury caused by ice crystals from freezing, concentration of electrolytes, dehydration, pH changes and denaturation of proteins. It has been shown that DMSO induces differentiation and function of leukemia cells of mouse (Thomson *et al.*, 1996), rat (Amit *et al.*, 2000), and human (Reubinoff *et al.*, 2001). Dimethyl Sulfoxid is also found to stimulate albumin production in malignantly transformed hepatocytes of mouse and rat and to affect the membrane-associated antigen, enzymes, and glycoproteins in human rectal adenocarcinoma cells (Richards *et al.*, 2002).

1.6.2.3 Controversies in neural trans-differentiation from mesenchymal stem cells

Because of the inadequate characterization of the MSCs currently, there are significant inconsistency, even controversies among the reports of neural trans-differentiation from MSCs in different laboratories. In spite of wide range reports of trans-differentiation of MSCs to generate neurons and astrocytes, Wehner *et al.* (2003) argued that bone marrow-derived cells do not generate astroctyes. They used a transgenic mouse strain that contains GFP gene expression cassette under GFAP promoter control, and failed to observe GFP expression in all three experiments both in cell culture and in vivo engraftment. Recently, two independent groups, Lu, *et al.* (2004) and Neuhuber, *et al.* (2004), re-evaluated the rapid and robust neural-like neurofilament formation in differentiated MSCs under the DMSO/BHA induction protocol reported earlier. They

applied time lapse imaging analysis, and compared cells of different types including rat epidermal fibroblasts, PC12 in response to chemical stressers such as triton or sodium hydroxide, and observed identical changes in both MSCs and fibroblasts. They concluded that the neuron-like morphological and immunocytochemical changes of MSCs, following the so-called 'chemical induction', are not the result of genuine neurofilament extension but represent actin cytoskeleton retraction in response to chemical stresses (Lu *et al.*, 2004).

Summarizing recent work on neural trans-differentiation of MSCs, there are four uncertainties among the reported results: 1) the early reports rely the neuronalization conclusion mostly, if not only, on the immunophenotypic characteristics of the differentiated MSCs. As evidence start to show that stem cells express many markers spontaneously, to rely on this criterion solely may not reflect the real induction processes (Tondreau *et al.*, 2004); 2) the process-bearing morphological characteristics has been overvalued to indicate the neuronal differentiation. Detailed inspection of the differentiated cells revealed little resemblance to typical neuron morphology in most of the reports (Lu *et al.*, 2004; Neuhuber *et al.*, 2004); and 3) many of the trans-differentiation clams rely only on *in vitro* data, few has reported the fate of cells upon transplanted into the CNS *in vivo*, which makes it hard to evaluate the functionality, and therapeutic value of differentiated cells.

1.7 Potential Application of Stem Cell Therapy in Parkinson's Disease

Neurodegenerative diseases belong to a group of neurological disorders that are caused by the loss of neurons in a defined fashion regionally, or globally in the central nervous system. Such diseases include Parkinson's Disease (PD), Alzheimer's Disease

(AD), stroke, amyotrophic lateral sclerosis (ALS) and Huntington's Disease (HD) etc. Transplantation of stem cells or their derivatives and mobilization of endogenous stem cells within the adult brain, has been proposed as future therapies for neurodegenerative diseases. It may seem unrealistic to induce functional recovery by replacing cells lost through disease, considering the complexity of human brain structure and function. Studies in animal models have nevertheless demonstrated that neuronal replacement and partial reconstruction of damaged neuronal circuitry is possible (Piccini *et al*., 2000). There is also evidence from clinical trials that cell replacement in the diseased human brain can lead to symptomatic relief (Lindvall *et al.*, 2004).

1.7.1 A New Hope for Parkinson's Disease Patients

Among the common neurodegenerative diseases, the pathology of PD is relatively better understood. The loss of dopaminergic (DA) neurons confined mostly to the relatively defined nigrostriatal pathway in the basal ganglia region of the brain. Parkinson's Disease is the second most-common neurodegenerative diseases affecting around 2% of the population over 65 years of age in the world. There are 500,000 new cases each year in the United States alone. As the PD onset has close connection with aging, the increase of number of incidences is expected due to continuous improvement of living standard in the future. Cell replacement therapy has come into sight along with the rapid development of the stem cell biology. Among the common aging related neuro-degenerative disorders, PD patients hold the highest expectation to be benefited in the upcoming area of regenerative medicine. It has been shown that the symptoms of patient with severe PD can be significantly improved by using fetal mesencephalic neurons, which are rich in postmitotic dopaminergic neurons (Freed *et al.*, 1992; Freed *et al.*,

1993; Bjorklund *et al.*, 2003). The use of fetal tissue has been one of the clinical options for the PD patient, but is unlikely to become a routine procedure due to the ethical concerns in using human embryos. More profoundly, the short of tissue supply largely limits the application of this approach at the moment. Under this situation, stem cells, perhaps adult stem cells, appear to be the best solution. Indeed, the easily accessible bone marrow derived stem cells have been extensive studied to differentiate into DA neuron phenotype, since the realization of the therapeutic potential of adult stem cells (Pavletic *et al.*, 1996; Schwarz *et al.*, 1999; Jiang *et al.*, 2003; Yoshizaki *et al.*, 2004; Hermann *et al.*, 2004; Dezawa *et al.*, 2004). The key issue is to identify a proper cell type that is capable to transform into DA neurons, and lacks the propensity to cause tumor. It has been shown that the risk of forming teratoma is reduced if the ES cells are differentiated *in vitro* before transplantation, which implies that somewhat committed adult stem cells may be safer in terms of tumorigenesis when used clinically (Erdo *et al.*, 2003).

1.7.2 Current Challenges of Embryonic Tissue and Cell Therapy in Parkinson's Disease Treatment

Although the use of fetal mesencephalic tissue has produced promising improvement for the PD patients, the present cell replacement procedures are still far from optimal (Freed *et al.*, 1993; Bjorklund *et al.*, 2003). Some recent sham surgery-controlled trials showed only modest improvement in relief the patients' symptoms, compare to some early reports (Freed *et al.*, 2001; Olanow *et al.*, 2003). After transplantation, 7–15% of grafted patients also developed dyskinesia (Hagell *et al.*, 2002; Olanow *et al.*, 2003), similar to the L-dopa therapy. However, this adverse effect is not due to dopaminergic overgrowth (Hagell *et al.*, 2002), but may have been caused by uneven and patchy reinnervation (Ma *et al.*, 2002), giving rise to low or intermediate

amounts of striatal dopamine. Another major problem in using ES cells is the risk for teratoma after grafted into the brain. In one particular study, it was reported that implantation of mouse ES cells into rat striatum caused teratomas in 20% of the animals (Bjorklund *et al.*, 2003), and ES cells seem more prone to generate tumors when allografted into the same species (Erdo *et al.*, 2003).

1.7.3 Building an Adult Stem Cell Therapy For Parkinson's Disease

While the use of ES cells encounters problems and needs to be improved dramatically, working towards applying adult stem cells in treating PD is certainly an appealing strategy. There have been reports to induce MSCs into tyrosine hydroxylase (TH) positive neurons in culture dish (Jiang *et al.*, 2003; Tondreau *et al.*, 2004), but the current work is too preliminary. To develop a clinically competitive adult stem cell therapy, it must provide advantages over current L-dopa treatments for PD. Cell-based approaches should induce long-term improvements of mobility and suppression of dyskinesia. On the basis of results obtained from fetal transplants in both animal and human studies, several aspects need to be considered for clinically suitable adult stem cell-derived DA neurons: i) the cells should release dopamine in a regulated manner and should show the molecular, morphological and electrophysiological properties of substantia nigra neurons; (ii) the cells must be able to reverse the motor deficits in animals that resemble the symptoms in persons with PD; (iii) the yield of cells should allow for at least 100,000 grafted dopaminergic neurons to survive over the long term in each human putamen; (iv) the grafted dopaminergic neurons should re-establish a dense terminal network throughout the striatum; and (v) the grafts must become functionally integrated into host neural circuitries (Hagell *et al.*, 2002).

Although controversies still exist in multipotency of adult stem cells, there is growing body of evidence points to the genuineness of these discoveries reported in the past few years. The issues of cell fusion or technical error that brought cautions to this field should be used wisely to move our detection and evaluation methods forwards, not to deter further discoveries. After all, we cannot afford to ignore the potential of adult stem cells' possible role in the future medical practice; the revolutionary idea of regenerative medicine may in large part depend on how much we know about adult stem cells either in their native niche, or in a grafted host environment. With the increasing number of experiments designed to test the therapeutic value of MSCs in the animal model of neurodegenerative diseases, our knowledge about their potential in both neural trans-differentiation and clinical application will certainly grow. For a field that is young and active as adult stem cell biology, we should let future to decide its fate while investing our best efforts.

CHAPTER 2
THE NEURAL PROPERTY OF BONE MARROW DERIVED CELLS FROM ADULT MOUSE

2.1 Background and Introduction

Previous studies have shown that adult stem cells exist in various tissues of adult animals, and that these tissue-specific stem cells may have the capacity for trans-differentiating into cell types of different lineages (Wetts & Fraser, 1988; Jones *et al.*, 1995; Ferrari *et al.*, 1998; Brazelton *et al.*, 2000; Petersen, 2001; Hughes, 2002). The apparent multipotency of adult stem cells has generated tremendous interest regarding their potential therapeutic value, while bypassing ethical concerns surrounding the use of human embryonic stem cells. Furthermore, adult stem cells are more restricted in their differentiation potential, and thus are thought to be less tumorigenic than embryonic stem cells. However, because some laboratories failed to repeat several significant experiments (Wagers *et al.*, 2002; Wehner *et al.*, 2003), the early exuberance surrounding the first reports of adult stem cell plasticity has given way to serious concerns about whether what was being described was true trans-differentiation, or an epiphenomenona mediated, perhaps, by cell contamination or fusion (McKinney-Freeman *et al.*, 2002; Terada *et al.*, 2002; Ying *et al.*, 2002; Issarachai *et al.*, 2002).

The bone marrow-derived mesenchymal stem cell (MSC) has been known since 1963, when Petrakova and colleagues (Petrakova *et al.*, 1963) demonstrated that pieces of bone marrow transplanted under the renal capsule of mice formed an osseous tissue over a period of several weeks that was invaded by hematopoietic cells. The MSC may

represent the best hope for stem cell-base replacement therapy since, in addition to their potency and accessibility, it may be possible to use MSCs in autologous transplantations to minimize immune rejection (Awad *et al.*, 1999; Dezawa *et al.*, 2004). For this reason, the MSC is one of the most extensively-studied adult stem cells with respect to trans-differentiation potential (Kadiyala *et al.*, 1997; Ferrari *et al.*, 1998; Bruder *et al.*, 1998; Pittenger *et al.*, 1999; Awad *et al.*, 1999). However, despite this great interest the MSC remains enigmatic as both its identity and qualification as a true stem cell remains uncertain (Javazon *et al.*, 2004; Baksh *et al.*, 2004). This uncertainty results primarily from the lack of universally defined cell surface markers to characterize the MSC in the manner of the hematopoietic stem cell (Devine, 2002; Javazon *et al.*, 2004; Baksh *et al.*, 2004). Additionally, the relatively unrefined MSC isolation methodology, that has remained essentially unchanged for 40 years, has no doubt also contributed to the weak characterization of the MSC.

The high incidence of age-related neurological disorders has spurred interest in the ability of the MSC to trans-differentiate into neural lineage (Torrente *et al.*, 2002; Chopp & Li, 2002; Sugaya, 2003). Among various protocols to induce neural transdifferentiation of MSCs, the use of chemicals including dimethylsulfoxiede (DMSO), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), as well as dibutyral cyclic AMP and isobutylmethylazanthine (IBMX) has become popular, as they induce a rapid and robust neuron-like morphological transformation from the normally flat, fibroblast-like appearance of MSCs (Woodbury *et al.*, 2000; Sanchez-Ramos *et al.*, 2000; Deng *et al.*, 2003). Since then, there has been a series of studies using similar methods to induce neural differentiation of stem cells from a variety of other sources

(Lodin *et al.*, 1979; Lambeng *et al.*, 1999; Safford *et al.*, 2002; Lopez-Toledano *et al.*, 2004). However, recent studies cast doubt on the 'neuralization' of bone marrow-derived stem cells. Wehner, *et al.* (2003) utilized a transgenic mouse line carrying a green fluorescence protein (GFP) expression vector under the control of the glial fibrilary acidic protein (GFAP) promoter to examine the capacity of MSCs to undergo neuralization. After three *in vivo* and *in vitro* experiments, they concluded that bone marrow-derived cells could not differentiate along the astrocytic lineage. Recently two independent groups, Lu, *et al.* (2004) and Neuhuber, *et al.* (2004) , reevaluated the rapid and robust neural-like neurofilament formation by MSCs under the DMSO/BHA induction protocol reported earlier. They applied time lapse imaging analysis, and compared cells of different types including rat epidermal fibroblasts, PC12 in response to chemical stressers such as triton or sodium hydroxide, and observed identical changes in both MSCs and fibroblast. They concluded that the neuron-like morphological and immunocytochemical changes of MSCs following the treatments are not the result of genuine neurofilament extension but represent actin cytoskeleton retraction in response to chemical stress. Although these results can not account for all the neural trans-differentiation of MSCs reported so far, and they can not explain the *in vivo* neuralization of the MSCs, they do raise serious questions about the generality of the neural differentiation potential of the MSCs, and temper the hope of potentially applying MSCs in the treatment of brain disorders.

In an attempt to further clarify these issues, we established long-term cultures of bone marrow-derived cells (BMDCs) from whole bone marrow, using a common protocol for mesenchymal stem cell culture (Goshima *et al.*, 1991a; Friedenstein, 1995).

We assessed BMDC "stemness" by examining the clonality, and cell division patterns (symmetric vs. asymmetric). To assess the previous protocol of using dbcAMP/IBMX (Deng *et al.*, 2001) for rapid neuronal induction, we examined the expression of neural specific proteins in BMDCs and NIH3T3 pre- and post-treatment. To further test the multipotency, and their potential for cell-replacement therapy for neurological disorders, we transplanted BMDC into the neonatal mouse brain and exam their *in vivo* performance as a neuro-progenitor cell. We also applied confocal scanning imaging system, and Y-chromomsome painting technique to confirm the authenticity of immuno-labeling of the neural specific protein expressions by BMDCs, and to assess the cell fusion events *in vivo*.

2.2 Materials and Methods:

2.2.1 BMDC Culture

Eight weeks old C57/B6 and C57/B6GFP mice were used to establish BMDC cultures, utilizing the physical property of plastic adherence (Goshima *et al.*, 1991a; Friedenstein, 1995). In brief, mice were given a lethal dose of phenobarbital, and the tibias and femurs were removed. A 22-gauge needle filled with Dulbecco's Modified Eagle's Medium (DMEM) was used to flush out whole bone marrow. The recovered cells were then mechanically dissociated, filtered through a 70μm mesh, and plated in 35mm tissue culture dishes containing DMEM supplemented with 20% fetal bovine serum (FBS), 0.5% gentamycin, and 1000units/ml of Leukemia Inhibitory Factor (LIF), as per Jiang, *et al.* (2002). After 24hrs, the non-adherent cells were removed, and the culture medium was completely replaced. After reaching confluency, BMDCs were passaged (1:3 dilution) twice a week with fresh medium.

In order to generate clonal cultures, we grew single BMDCs, in conditioned medium collected from confluent BMDC cultures derived from whole bone marrow. Conditioned medium was centrifuged at 2,600*g* for 10min., and then filtered through a 0.22μm mesh. We created a dilution series with BMDCs to reach a cell density of one to two cells per 5μL, and plated 5μL of the cell suspension in each well. Immediately after plating, we examined each well with phase microscopy, and excluded those wells containing more than one cell. We then added 100μL of mixed medium (50% conditioned medium + 50% fresh medium). In order to ensure single-cell clonality, we again examined each well after an additional 24 hours, and discarded those containing more than one cell. Clonal BMDC cultures were maintained in the mixed medium until confluent, at which point the cells were maintained in fresh, unconditioned medium.

2.2.2 FACs Analysis of BMDCs

Immunofluorescence with a variety of antibodies against surface antigens was used to characterize BMDCs. These antibodies included directly-conjugated anti-Sca1, anti-CD34, anti-CD45, and directly-conjugated anti-mouse IgG_{2a} (PharMingen; 1:500), as a control. In addition, the following unconjugated antibodies were used: anti- c-Kit, anti-CD9, anti-CD31, anti-CD105 (PharMingen; 1:500), anti-CD11b (Serotec; 1:300), and anti-rat IgG_{2a} (PharMingen; 1:500), as a control. Primary antibodies were applied for 30min. at room temperature, followed by washing and application of fluorescently-conjugated secondary antibodies for an additional 30min for the un-conjugated antibodies. Cells were then centrifuged at 200*g*, and washed twice in PBS to eliminate unbound antibodies. Approximately 10^6cells/mL cell suspension was run through a flow cytometer (CELLQuest, Becton Dickinson FACScan).

2.2.3 Immunolabeling and Cell Counting

Immunolabeling was performed on glass coverslips plated with BMDCs. Cells were fixed in ETOH:acetic acid (95:5) for 15mins., washed with PBS containing 0.1% Triton (PBST), and blocked for 30min in PBST supplemented with 10%FBS. Cells were then incubated with primary antibodies overnight at 4°C, washed, and incubated in secondary antibodies for 1hr at RT.

Free-floating, 40μm brain sections were immunolabeled, as previously described (Deng *et al.*, 2003), with the following antibodies: nestin (Developmental Studies Hybridoma Bank, University of Iowa; 1:250), glial fibrillary acidic protein (GFAP; from Immunon (monoclonal and polyclonal; 1drop/0.5ml), neurofilament medium subunit (NFM; EnCor Biotech. Inc.; 1:500), βIII tubulin (Promega; 1:1500), S100 and MAP2ab (Sigma; 1:500), Polysailic Acid-NCAM (PSA-NCAM; Chemicon; 1:100). Confocal laser scanning microscopic analysis of the immunolabeling was done on the University of Florida Cancer Center's Leica TCS SP2 confocal laser imaging system (Leica Microsystems, Wetzlar, Germany).

Cell counting was performed under a fluorescence microscope (Olympus BX51). The ratios of positive cells were obtained by averaging three different experiments for both control and treatment groups. In each experiment, five randomly chosen views were counted and averaged.

2.2.4 Neural Induction by Elevating Cytoplasmic cAMP

Our protocol for neural induction by elevating intracellular cAMP was modified from Deng, *et al.* (2001). In addition to primary induction medium (0.5mM isobutylmethylxanthine (IBMX)/1mM dibutyryl cyclic AMP (dbcAM) (Sigma) in

DMEM/F12) used for the first 24hrs of treament, a cocktail of growth factors (10ng/mL of Brain-Derived Neurotrophic Factor (BDNF; Pepro Tech.), Nerve Growth Factor (NGF; Invigtren), Epidermal Growth Factor (EGF; Pepro Tech) and basic Fibroblast Growth Factor (bFGF; Pepro Tech), and N2 Supplements (Gibco)) has been added to the primary induction medium for treatments longer than 24hrs.

2.2.5 *In situ* Hybridization for GFAP mRNA

To generate the GFAP riboprobes, we used RT-PCR to amplify a 401bp DNA fragment of the GFAP gene (gi: 26080421) from mouse brain tissue with a pair of primers designed using the Primer 3 program (forward: GCCACCAGTAACATGCA AGA; reverse: ATGGTGATGCGGTTTTCTTC). The PCR product was then cloned into the PCR4 TOPO vector (Invitrogen). After linearization, plasmids extracted from clones of both directions were used as templetes to synthesize digoxigenin (DIG)-labeled GFAP sense and antisense probes using T7 RNA polymerase. *In situ* hybridization followed the protocol of Braissant and Wahli (1998) (Braissant & Wahli.W, 1998) with small modifications. The probe concentration was 400ng/ml and the hybridization temperature was set at 45ºC.

2.2.6 Western Blotting

For western blotting, approximately 20μg of protein from cell lysates was electrophoretically separated by 8% SDS-PAGE. After transfer to a nitrocellulose membrane, we applied anti-GFAP (Immunon; 1:30) antibody, and a chemiluminecence method for detection (ECL, Amersham). We then incubated the membrane in striping solution at 56°C for 30mins, and incubated it again using anti-actin (Abcam; 1:2,000) antibody.

2.2.7 Transplantation of BMDCs into Neonatal Mouse Brain

BMDCs were trypsinized and labeled with the fluorescent carbocyanine dye, DiI (Molecular Probes), according to a protocol adapted from Paramore *et al.* (1992). Briefly, cells were centrifuged for 5min. at 1000 rpm, and resuspended in fresh medium. DiI was dissolved in absolute ethanol (2.5mg/ml), and added to the cell suspension such that the final concentration of DiI was 40μg/mL. The cells were incubated in the DiI-containing medium for 30min. at 37°C before being washed three times in PBS.

DiI-labeled BMDCs were transplanted into the lateral ventricle of postnatal day 1-4 wild-type C57BL6 mice as described previously (Deng *et al.*, 2003). Approximately 10^5 BMDCs in 1μL of PBS were injected into the left lateral ventricle. After 10 days survival, mice were euthanized with an overdose of Avertin and perfused transcardially with 4% paraformaldehyde in PBS. The brain tissue was excised, post-fixed overnight in perfusate, and sectioned through the coronal plane into 40μm slices with a vibratome.

2.2.8 Y-chromosome Painting for Cell Fusion Detection

Twenty micron vibratome sections were used for assaying possible fusion events associated with DiI-labeled donor BMDCs in the neonatal mouse brain. Brain sections were first treated with 0.2N HCl for 30mins., and retrieved in 1M Sodium Thiocyanate (NaSCN) for 30mins. at 85°C. The sections were then digested with 4mg/mL pepsin (Sigma; diluted in 0.9% NaCl pH2.0) for 60mins. at 37°C. After equilibrating in 2X SSC for 1min., the sections where dehydrated through graded alcohols. The tissue was then incubated with FITC-conjugated Y-chromosome probes (Cambio, UK; denatured for 43mins at 37°C) using Hybrite (Vysis, IL) for 20.5hrs. following a denaturing step of 6mins. at 75°C. After hybridization, cells were washed first in 1:1 formamide:2xSSC,

then in 2xSSC before being re-coverslipped in mountant containing DAPI (Vector, Burlingame, CA).

2.3 Results

2.3.1 BMDC Cultures Can Be Derived from the Bone Marrow of Adult Mice

We established viable cultures of BMDCs, from the tibia and femur of adult mice according to the adhesive property of mesenchymal stem cells (MSC) described before (Goshima *et al.*, 1991a; Friedenstein, 1995). About 30 days after plating, the appearance of fast growing BMDC with fibroblast-like morphology can be observed in amid of slow growing, round or polygonal cell types that appeared firstly in the initial bone marrow dissociates culture. At around day 45, stable fibroblast-like BMDC lines can be achieved (Fig. 2-1A). Besides the morphological change, we also observed GFP silencing concomitantly in all of the three GFP transgenic mice we used to establish BMDCs, indicating there was also a change of gene expression profile in the process of establishing BMDC from its original cell types in the bone marrow (Fig. 2-1A).

To characterize the BMDCs, we performed flow cytometry analysis using a battery of markers for characterizing mesenchymal stem cells (Fig. 2-1B). We found that BMDCs are negative for the hematopoietic markers CD34, CD45, and Mac1; negative for the stem cell marker c-kit, but partially positive (18.6%) for the stem cell marker Sca1; negative for the endothelial marker CD31, partially positive for CD105 (19.1%), and 97% positive for CD9. These results, along with morphological characteristics, indicate that BMDCs are mesenchymal stem cells, and are similar to the MAPCs isolated by Jiang *et al.* (2002).

2.3.2 BMDC Cultures Normally Express Neural Markers

To evaluate the neural property, we tested the BMDCs on the expression of several neural specific proteins, including the neural progenitor marker nestin. We found that BMDCs are highly positive for nestin (close to 100%); partially positive for several neuron specific proteins, including βIII tubulin (12%), neurofilament-M (NFM; 13.2%) and Map2ab (9.6%); negative for PSA-NCAM, a surface protein expressed on migratory neuroblasts; partially positively for the astrocyte specific protein, S100β (15%), but negative for the astrocyte intermediate filament proteins, GFAP and Vimentin (Fig. 2-2B).

2.3.3 Astrocyte, but not Neuronal Proteins, Are Upregulated by cAMP Elevation

Several studies have used cytoplasmic elevation of cAMP to induce neural differentiation from mesenchymal stem cells (Deng *et al.*, 2001; Jori *et al.*, 2004; Lambeng *et al.*, 1999; Lopez-Toledano *et al.*, 2004). To test the same protocol on our BMDCs, we treated the cells with 0.5mM IBMX/1mM dbcAMP. We found that, as reported (Deng *et al.*, 2001), cytoplasmic cAMP elevation does induce a significant morphological change of BMDCs, where the cells become neural-like with rounded somas, and long processes. However, we saw no evidence for a change in the expression of most neural markers before and after the treatment (Fig. 2-2B). Furthermore, when we treated NIH 3T3 cells with the same protocol, we observed similar morphological change without detecting neural marker expression (Fig. 2-2B,C). A significant upregulation of GFAP was, however, observed after treatment with dbcAMP/IBMX (Fig. 2-3A). The enhanced expression of GFAP was confirmed using both *in situ* hybridization with

digoxinin-labeled GFAP riboprobes (Fig. 2-3B), and western immuno-blotting (Fig. 2-3C).

2.3.4 Single-Cell BMDC Clones Show Plasticity by Generating both Neuronal and Astrocytic Lineages

We cloned BMDCs from single cells by limiting dilution in conditioned medium. Single cell-derived BMDCs recapitulated the cell surface marker expression profile of their ancestor population by flow ctyometry analysis. When we tested the cloned BMDCs with NFM immunostaining, we did not observe any positivity in clones of smaller than five cells (n=10), but did see labeling with this marker in clones of ten or more cells (n=13) (Fig. 2-4A). This may imply that there is a symmetric and asymmetric division in BMDCs that is cell density or division number dependent (See working model in Figure 4Ab, and Discussion)**.** Furthermore, when we performed double immunostaining, we found that both neuronal and astrocyte cells existed in the cloned population (Fig. 2-4B).

2.3.5 BMDCs Exhibit Neural Differentiation upon Grafting into the Neonatal Mouse Brain

To test the *in vivo* trans-differentiation capacity of BMDCs, we grafted the cells into the neonatal mouse brain. We observed a migration of BMDCs along the rostral migratory stream (RMS) from the lateral ventricle to the olfactory bulb. While most of the grafted cells maintained a spindle-like appearance similar to their *in vitro* morphology, some cells exhibited morphological characteristics of astroctyes around the ventricle, and penetrated into the overlying parenchyma (Fig. 2-5A). Immunostaining shows that these cells are positive for GFAP antibody (Fig. 2-5B). A significant number of cells within the RMS were immunopositive for the neuronal marker βIII tubulin (Fig. 2-5B), and more significantly, we consistently observed a small number of BMDCs

possessing typical characteristics of granule cells within the granule cell layer (GCL) of the olfactory bulb (Fig. 2-5A). Immunolabeling reveals that these cells are positive for PSA-NCAM (Fig. 2-5B). To confirm the expression of neuronal proteins by these donor BMDCs, we used confocal laser scanning microscopy to verify that the expression of the proteins are indeed in the same focal layers of the DiI used to label the cells (Fig. 2-6). To control for the possible leakage of the DiI, we grafted identically-labeled NIH3T3 cells into the ventricles of a different set of animals. In these cases, we only observed labeled cells within the subependymal zone of the lateral ventricle, near the site of injection where the cells where grafted (n=4).

2.3.6 Chromosome Analysis Reveals no Evidence of BMDC Fusion

To evaluate the possibility that cell fusion between donor BMDC and differentiated host cells is responsible for the co-expression of neuronal proteins and DiI, we grafted DiI-labeled, male BMDCs into neonatal male mouse brain, and analyzed tissue sections for the presence of cells with more than one Y-chromosome. We optimized the Y-chromosome painting such that a high efficiency of detection (>99%) was achieved in cells with an intact nucleus, using Dapi counterstaining and confocal microscopy**.** From analysis of three different animals, we observed that all DiI labeled cells contain only one Y-chromosome, as shown in Figure 2-8. We therefore conclude that there is no sign of fusion of grafted cells.

2.4 Conclusion and Discussion

We have demonstrated that BMDCs from adult mice constitutively express several neural markers *in vitro* under standard culture conditions. The neural induction protocol of applying dbcAMP/IBMX to elevate the cytoplasmic cAMP does not

significantly result in the upregulation of neural specific proteins from their uninduced state in BMDCs**.** Single-cell BMDC clones undergo symmetric and asymmetric division with or without induction, generating neuronal marker expressing cell, and inducible astrocytic marker-expressing cells *in vitro*. Non-fused BMDCs also have the capacity to generate neurons and astrocytes upon grafting into the neonatal mouse brain. These cells seemingly behave normally, as donor cells are seen to migrate along the RMS to the olfactory bulb, where they differentiate into granule cells.

We believe that the BMDC we described in the present study is equivalent to the MSC; however, we are reluctant to give it the name because of the current incomplete characterization of MSCs. The surface marker expression profile accords well with previous studies (Colter *et al.*, 2000; Javazon *et al.*, 2001), and the absence of CD34, CD45, and CD11b has been widely accepted as the major difference between MSCs' and hematopoietic stem cells (HSC) (Colter *et al.*, 2000). The expression of some endothelial cell markers, including CD105 and CD9, has also been reported in MSCs (Tanio *et al.*, 1999; Hayashi *et al.*, 2000; Jones *et al.*, 2002; Sun *et al.*, 2003). While the expression of the stem cell marker Sca1 mirrors other reports (Jiang *et al.*, 2002), the lack of c-kit expression by our BMDCs is anomalous (Sun *et al.*, 2003). This discrepancy may reflect the loose definition of MSCs currently in vogue. While there is a wide range of surface markers that have been tested to characterize MSCs, there is currently no single set of phenotypic markers used to unequivocally identify a MSC. As a result, there may be subtypes of MSCs that differ slightly from each other, and this may account for the variation of marker expression, as well as the inconsistent results regarding the trans-differentiation of MSCs from different laboratories. The use of FBS as the main, and

only, source for growth factors to establish the cell population has been adapted since the pioneering work of Petrakova (1963). It is simple and effective, but the lack of positive selection markers -as used for hematopoietic stem cells- may result in the inclusion of undefined cell types, which may underlie the interlaboratory variability seen with these types of protocols. We noticed that BMDCs in culture have an irregular growth rate at different periods of the culture (data not shown). We also noticed that BMDCs derived from GFP transgenic mouse lose the GFP expression during the course of culture.

The constitutive expression of neural specific proteins demonstrated by our BMDCs casts doubt on some previously reported protocols that claim neural induction, but fail to show the pre-induction level of neural specific proteins. However, this clarification did not weaken the recognition of MSC as a genuine stem cell type, but rather strengthen it by showing its vigorous, spontaneous neural differentiation property. The neural property exhibited by BMDCs may be explained by the neural propensity of stem cells reflected in the development of nervous system during embryogenesis. It is generally believed that unspecified ectoderm cells differentiate into neural lineage by default unless inhibited by ventralizing factors, such as bone morphogenetic protein-4 (BMP4) (Wilson & Hemmati-Brivanlou, 1995). So-called neuralizing factors such as noggin, chordin, and follistatin promote neuro-ectoderm specification by inhibiting BMP4 (Streit & Stern, 1999). The embryonic stem cell also shows active neural differentiation unless inhibited by BMP *in vitro* (Hemmati-Brivanlou & Melton, 1997; Finley *et al.*, 1999). Therefore, it is not surprising that BMDCs, as multipotent stem cells, may partially exhibit a neural property in their default state of differentiation *in vitro*, where there are no pro-mesoderm inhibitors such as BMP4. The expression of some

neural markers by uninduced MSCs is a matter of some controversy. Woodbury, *et al.* (Woodbury *et al.*, 2000) did not observe any neural specific protein expression except neuron-specific enolase (NSE). Sanchez-Ramos, *et al.* (Sanchez-Ramos *et al.*, 2000) reported low levels of NeuN, nestin and GFAP expression detectable with immunocytochemistry. Deng *et al.* (2001) have previously reported expression of vimentin, Map1b and β-III tubulin, but no NFM, GFAP and S-100. A more recent paper by Tondreau, *et al.* (2004) corroborates our finding by reporting significant expression of several neuronal markers, including nestin, β-III tubulin, Map2, and tyrosine hydroxylase (TH) flow cytometry analysis using non-induced MSCs. As pointed out above, the variation in MSC subtypes may be due to differing isolation and culturing protocols from different laboratories (Javazon *et al.*, 2004).

Despite causing a vigorous neuron-like morphological change, we have demonstrated that the use of the dbcAMP/IBMX induction protocol does not change the neuron-specific protein expression profile in BMDCs. Furthermore, we have also observed a rapid and dramatic morphological change in NIH3T3, similar to that of BMDCs upon dbcAMP/IBMX treatment, but without expression of neuronal marker. In the initial paper describing the protocol (Deng *et al.*, 2001), Deng *et al.* found no expression of NFM, which differs from our results, but they did report equal levels of MAP1b and βIII tubulin with and without induction, as we have found in our BMDCs. Tondreau, *et al.* (Tondreau *et al.*, 2004) recently reported an over 90% unchanged nestin expression pre- and post-induction, as we have demonstrated, but reported a decreased βIII tubulin expression level from over 90% to about 30% after ten days of treatment with lower concentration of dbcAMP (5μM). Our results, together with previous reports,

suggest that the dramatic neuron-like morphological transformation of MSCs under dbcAMP/IBMX treatment is an unreliable indicator of neuronalization, supporting a previous analysis of DMSO/BHA induction protocol reported by Neuhuber, *et al.* (2004) and Lu, *et al.* (2004).

Along with many other inconsistent reports of neural specific protein expression with or without induction in MSCs, the expression of GFAP has also been controversial. Despite the early findings of MSC trans-differentiation into GFAP expressing astrocyte *in vitro* and *in vivo* (Sanchez-Ramos *et al.*, 2000; Jiang *et al.*, 2002; Zhao *et al.*, 2002), Wehner, *et al.* (2003) reported that there was no GFAP expression from MSCs derived from a mouse strain carrying a GFP expression vector driven by the GFAP promoter cassette. The original paper that described the cytoplasmic cAMP elevation to induce neural differentiation from MSCs did not detect GFAP expression before or after the treatment (Deng *et al.*, 2001), and this partially supported Wehner, *et al.* However, our data from immunolabeling, *in situ* hybridization as well as western blotting unequivocally demonstrates that GFAP expression is up-regulated by cytoplasmic cAMP elevation. In agreement with this, Tondreau, *et al.* (2004) also report the up-regulation by MSCs of GFAP following prolonged exposure to a low concentration of dbcAMP/IBMX**.** Besides the demonstration of GFAP expression *in vitro*, we also observed BMDC differentiation into GFAP-expressing cells following transplantation into the neonatal mouse brain. As we showed in Fig. 1A, BMDC undergo GFP gene silencing during the establishment of the long-term culturing population. It, therefore, may be speculated that the gene-silencing event could have interfered with the GFP expression cassette in the previous

study of Wehner, *et al.* (2003), and thus resulted in a failure to detect GFAP expression in MSCs.

We have shown that clonal BMDC cultures give rise to populations that are identical to the parent population. These clones exhibit multipotency by differentiating into cells of neuronal and astrocytic lineages. Pittenger, *et al.* (1999) reported a similar clonal property of bone marrow-derived multipotent human MSCs in differentiating into adipogenic, chondrogenic and osteogenic lineage. Based on the immunophenotyping of BMDC clones of different sizes, we propose a working model that may reflect the symmetric and asymmetric cell division pattern in the BMDCs (Fig 4Ab). We suggest that at least three cell types exist in the BMDC population, each with different potency: multipotent, neuron restricted, and astrocyte restricted. The fact that we did not observe neural marker expression in the small clones (<5 cells) may mean that only multipotent cells, not expressing neural markers, can renew themselves by symmetric division, while neuron restricted and astrocyte restricted cells do not survive or proliferate under clonal culture conditions. The fact that we start to observe neural-specific protein expression in larger clones (>10 cells) may mean that there is a cell division number, or cell density that triggers asymmetric division that generates cells with restricted potentials.

Although the neural differentiation capability of MSCs *in vitro* has been widely explored, the *in vivo* response of this cell type upon direct engraftment into the brain has not been adequately assessed. Our finding that BMDCs integrate into the postnatal neurogenic pathway of the RMS/olfactory bulb system by migrating appropriately and differentiating into olfactory granule cells supports the conclusion that the bone marrow derived adult stem cell indeed possesses neural trans-differentiation capability under the

influence of environment cues from the brain. The fact that BMDCs can migrate along RMS, and differentiate into mature neurons at a distant site may also imply their therapeutic potential in acting as neural progenitor cells and replacing lost neural tissue after injuries. Munoz-Elias *et al.* (2004) reported a wide scope of migration of MSCs after transplanted into the embryonic rat brain, and transplanted cells appeared to express the neuron marker calbindin at the olfactory bulb. Zhao, *et al.* (2002) demonstrated that human MSCs expressed astrocytic markers and some neuronal markers after grafting to the site of ischemic injury in rat brains. Further work in various injury models, designed to fully assess the ability of BMDCs to functionally integrate into neural circuitry, will determine the potential therapeutic value of these cells in the treatment of neurological injury and disease.

In summary, we have demonstrated that BMDC, a MSC cell type, possesses neural progenitor-like property by expressing neuron- and astrocyte- specific proteins spontaneously or inducibly. Although the previously reported neural induction protocol using dbcAMP/IBMX does not seem to promote neuronal differentiation in BMDCs, it may be able to drive an astrocyte differentiation by showing a GFAP up-regulation. However, the neuron-like morphological transformation under the protocol may not be a reliable criterion for evaluating neuronalzation, due to the fact the NIH3T3 also acquired identical change without neuron-specific protein expression. We have also used confocal scanning imagine system, and Y-chromosome painting techniques to demonstrate, unequivocally, that neural trans-differentiation from stem cell of mesenchymal origin does exist, and may be able to develop into the ideal cell type for cell-replacement therapy in the future.

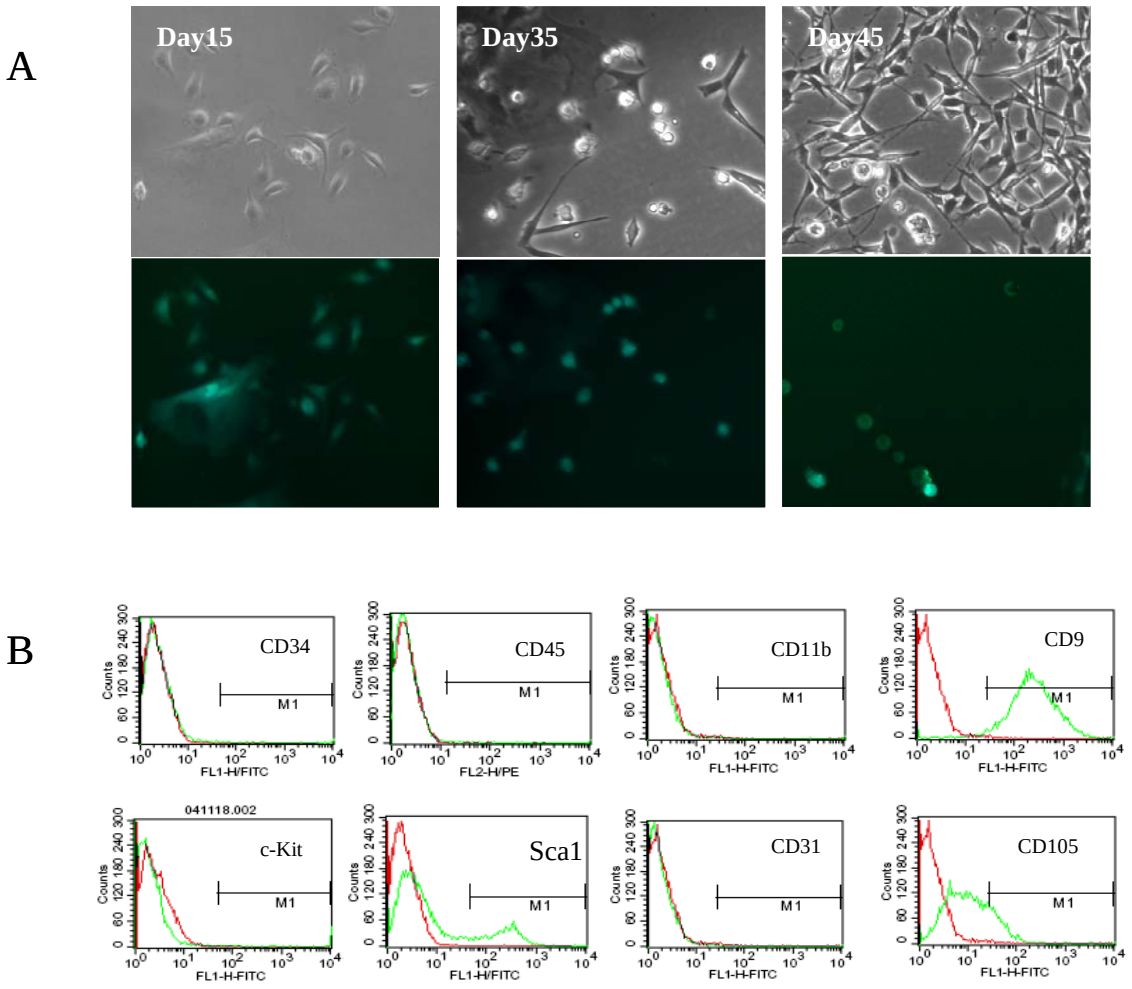
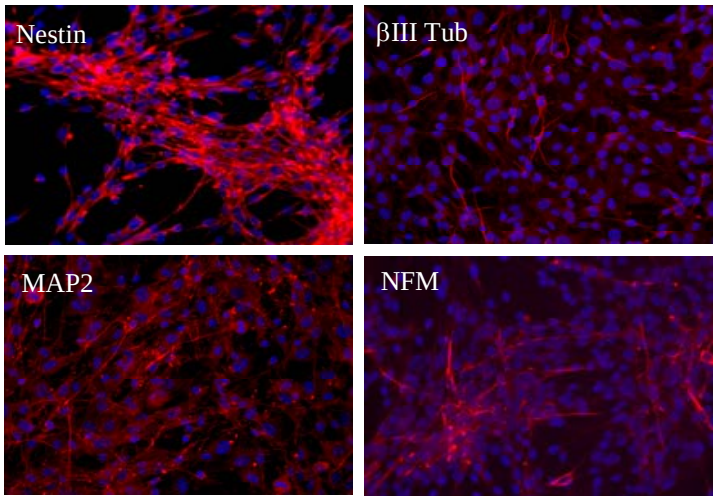


Figure 2-1. BMDC culture and characterization. Wild type and GFP transgenic C57/B6 mice of 8 weeks old have been used to isolate BMDCs. A) The establishment of long-term culturable BMDC. There is a clear transition from short polygonal to long fibroblast-like morphology in BMDCs during the establishment stage. The bottom pictures are the GFP fluorescent imagines of the same picture above, showing the loss of GFP expression when fibroblast-like BMDCs appear in the culture while the unchanged cells retain the GFP expression. B) The flow cytometry analysis of BMDCs on the cell surface antigen characteristics. BMDCs of over 50 passages isolated from wt C57/B6 mice were incubated with different antibodies. The BMDCs are completely negative for CD34, CD45, CD 11b, CD31 and c-kit; partially positive for Sca1 (18.7%) and CD105 (19.1%); and strongly positive for CD9 (97.5%). The red line indicate IgG isotype control corresponding to the antibodies in which they are generated. The green lines are counts of cell population that is positive for the antibody indicated in the each individual figure. M1 is the gating.

A



B

Neural Specific Protein	BMDC		NIH3T3	
	CM	DbcAMP/IBMX	CM	DbcAMP/IBMX
nestin	>99%	>99%	0	0
betaIII Tubulin	12.0%	11.4%	0	0
NFM	13.2%	13.7%	0	0
Map2	9.6%	8.9%	14.5%	16.5%
PSA-NCAM	0	0	0	0
GFAP	0	10.3%	0	0
S100	15.0%	15.5%	0	0
Vimentin	0	0	0	0

C

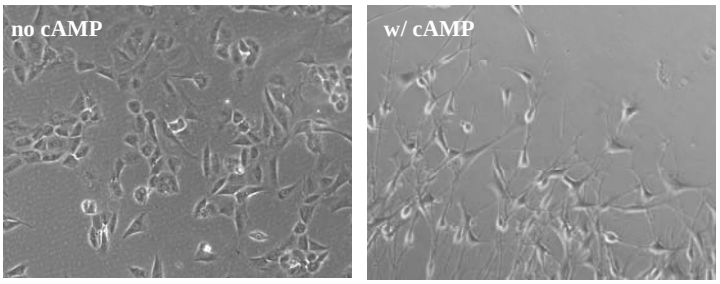


Figure 2-2. BMDCs express neuron specific proteins spontaneously under normal culture condition. A) Immunocyto-labeling of BMDCs using anti- nestin, βIII tubulin, Map2ab, and NFM antibodies. B) The quantification of neural marker expression on BMDCs and NIH3T3 pre- and post dbcAMP/IBMX treatment for two days. Numbers in the table is the portion (in percentages) of cells positive for each antibody labeling. C) NIH3T3 acquisition of neuronal morphology after treated with dbcAMP/IBMX for two days.

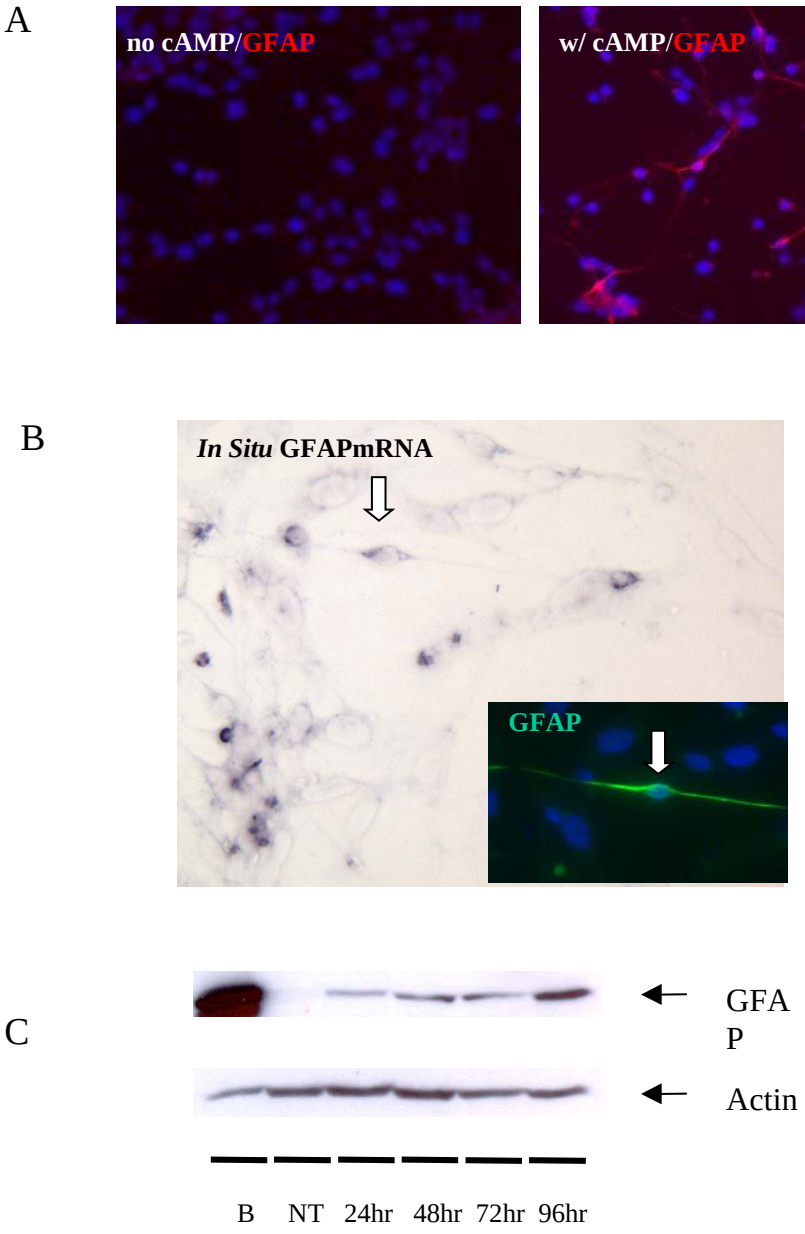


Figure 2-3. Cytoplasmic cAMP elevation promote GFAP expression in BMDCs. A) GFAP immunolabeling of BMDCs pre- and post- dbcAMP/ IBMX treatment. B) *In situ* hybridization in BMDCs treated with dbcAMP/IBMX for two days. The inset indicates the same cell (arrow) is also labeled with GFAP immuno-fluorescence (green). Noticed that not every *in situ* labeled cell is positive for GFAP immunolabeling; this is likely caused by the harsh treatment of *in situ* hybridization procedure. C) Western immuno-blotting using GFAP monoclonal antibody in BMDC treated by dbcAMP/IBMX. Actin antibody has been used as internal control. B- Brain tissue as positive control; NT- no treatment; hr- hours of treatment.

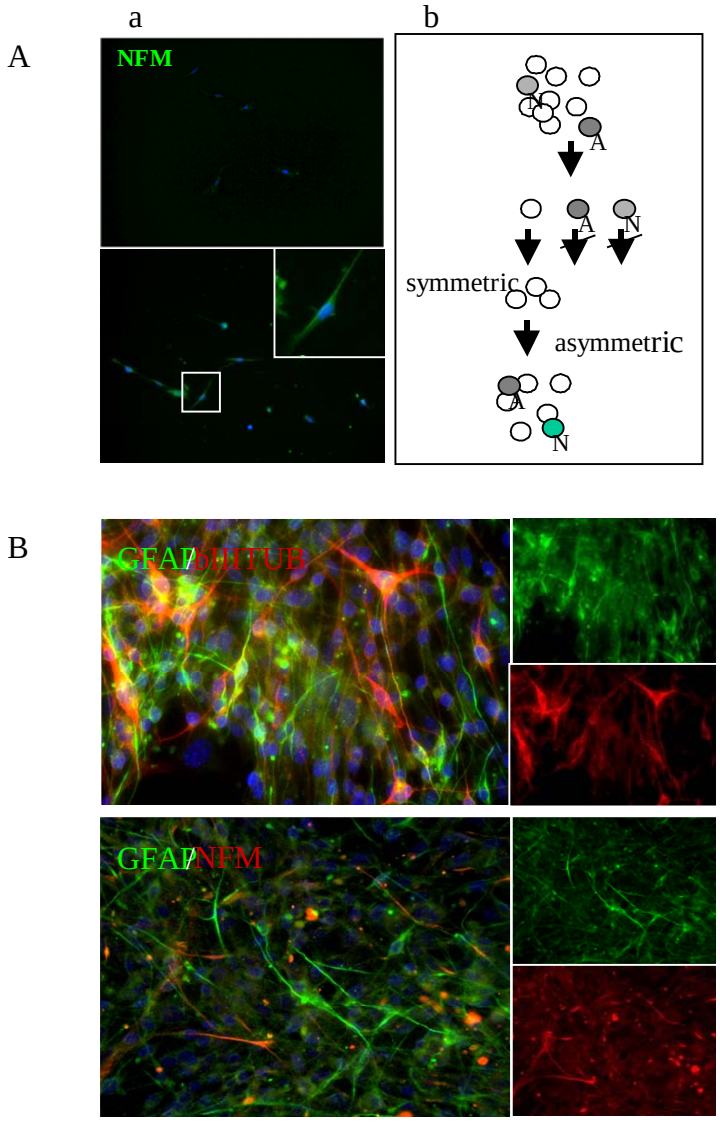


Figure 2-4. Single cell cloned BMDCs exhibit mutlipotency by generating progenies of different property through symmetric and asymmetric division. A) Cloned BMDCs exhibit symmetric and asymmetric division. a, immunolabelling of cloned BMDCs with anti-NFM antibody. Top: representative imagine of clones around five cells; no NFM positive cells are observed in these clones (n=10). Bottom: representative imagine of clones with more than ten cells; small portion of the cells start to express NFM as showing in the inset in these clones (n=13). b, a working model of the symmetric and asymmetric division of BMDCs: at least three different cell types existed in the original population, primitive cell with full potential (clear circle), neuron potential (filled circle denoted with N), and astrocyte inducible (filled circle denoted with A). B) Double immuno-labeling of BMDCs treated with dbcAMP/IBMX using antibodies against neuronal and astrocyte specific proteins. The expression of GFAP is labeled with green fluorescence; βIII tubulin and NFM are labeled with red fluorescence; blue fluorescence is Dapi stain for nucleus.

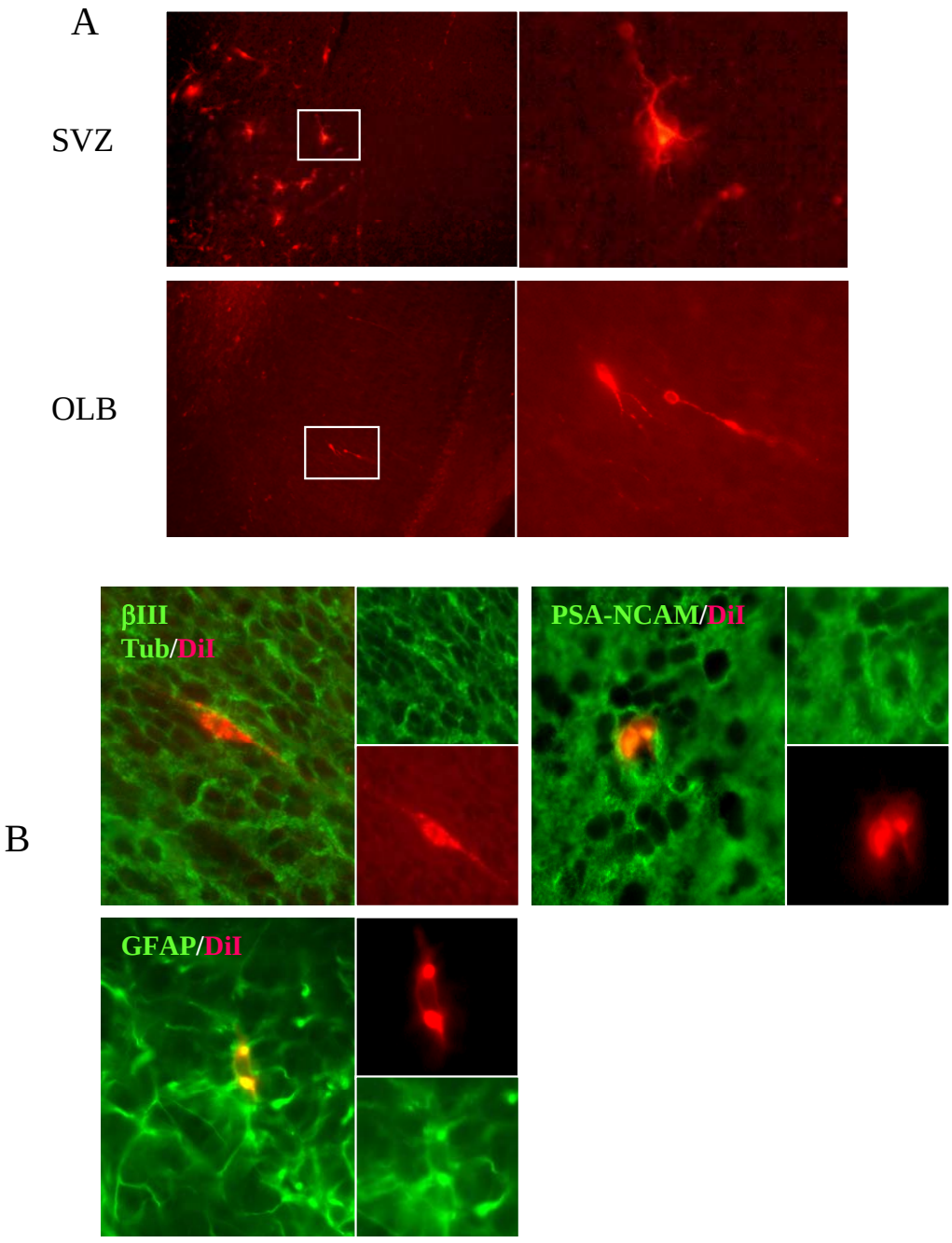


Figure 2-5. BMDCs differentiate into neurons and astrocytes upon transplantation into the lateral ventricle of the neonatal mouse brain. A) Transplanted DiI-labeled BMDCs (red) exhibit morphological characteristics of astrocyte at the sub-ventricular zone (SVZ; top), and typical granule cell at the granule cell layer (GCL) of olfactory bulb (bottom). Picture at right shows the enlarged area of the framed inset at the. B) BMDCs (red) show immnuo-phenotypes of neurons and astrocyte in the brain. The neural specific protein βIII tubulin, PSA-NCAM, and GFAP are immuno-labeled with green fluorescence. Insets in each picture show individual channel.

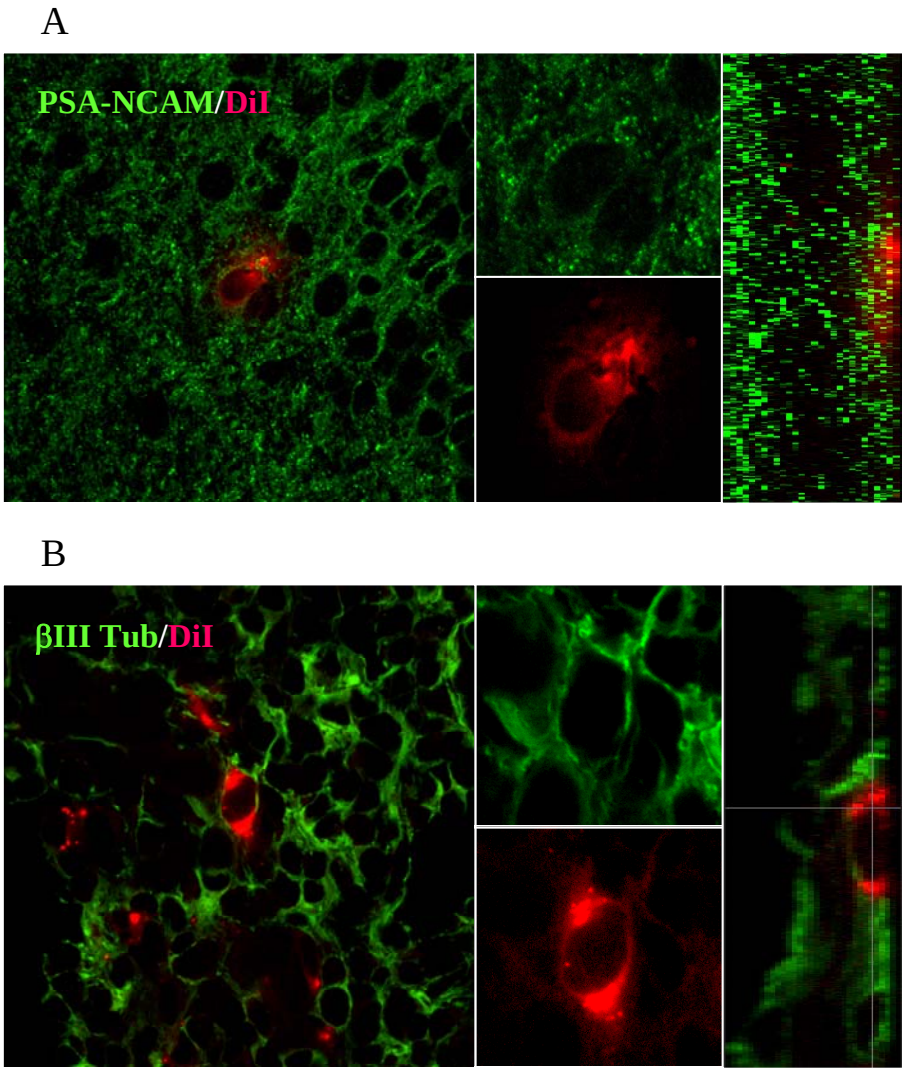


Figure 2-6. Confocal scanning microscopic imagines demonstrate the immuno-labeling of BMDCs with neuronal specific proteins. Confocal imaging analysis of BMDCs (DiI; red) immuno-labeled with PSA-NCAM (A) and βIII tubulin (B) (FITC; green). Left: merged confocal imagine in the GCL (A) and RMS (B) of olfactory bulb. Middle: separate channel of the cell indicated on the left picture, showing the green immuno-fluorescence labeling fall into the same plate of the BMDC (red); right: the side-view of the confocal imagine of the same cell on the left, showing the BMDC (red) site on the top of the tissue section, and it has been truncated at the mid-plane of the cell.

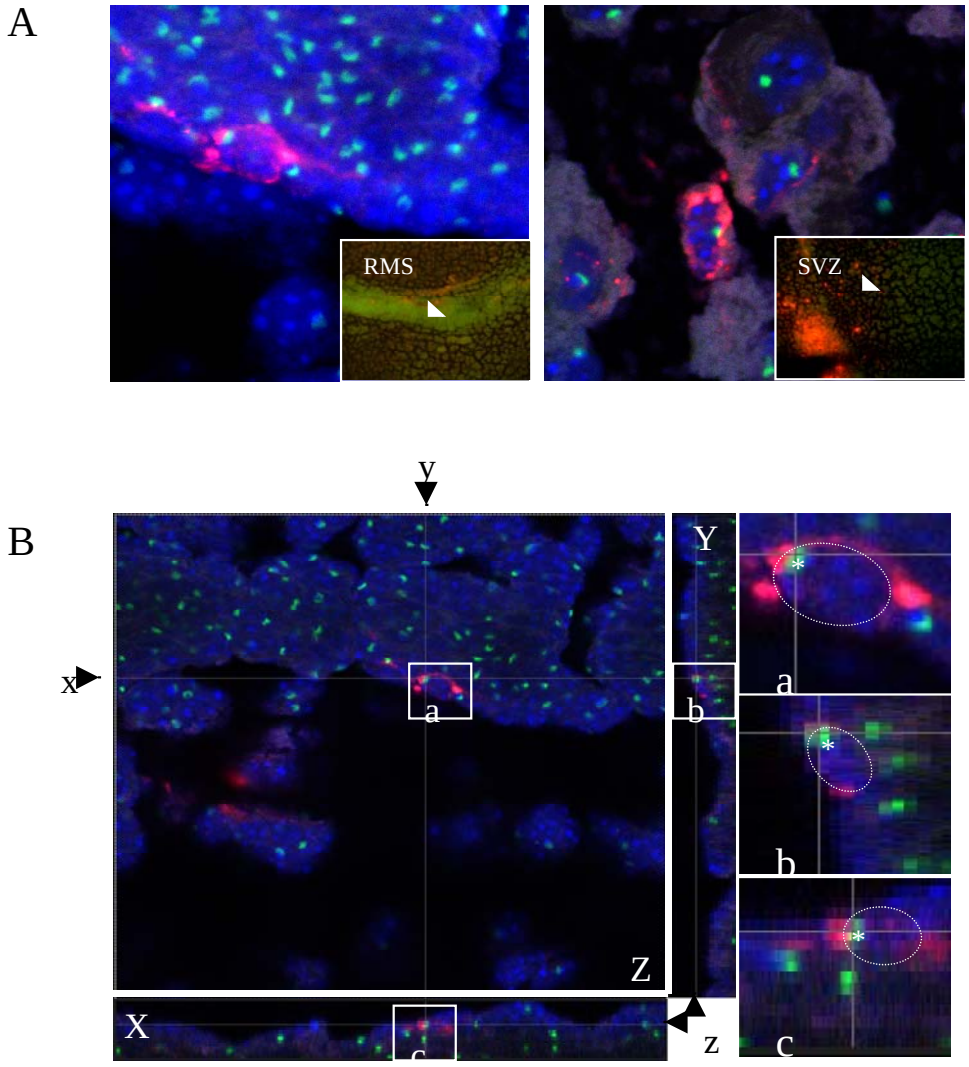


Figure 2-7. Confocal scanning microscopic imagine evaluation of cell fusion between male animal derived BMDCs and endogenous cells of male recipient mouse in the brain. A) Montage picture of confocal scanning imagines from two cells located at RMS and SVZ separately. There is only one Y-chromosome (FITC; green) within the cell boundary (DiI; red). Inset shows the overview of the cell location (arrowhead). B) Three-dimensional confocal analysis of Y-chromomsome locality in the nucleus of a BMDC shown in the left picture of A. X, Y and Z are the cross-section planes taken from three different angles indicated by x, y and z (arrowhead and gray lines); a, b and c are high magnification imagines of insets in X, Y and Z planes. The white dotted lines in a, b and c delineate the nucleus boundaries from different angle.

CHAPTER 3
NEURAL TRANSDIFFERENTIATION OF MOUSE HEPATIC OVAL CELL *IN VIVO*

3.1 Background and Introduction:

Stem cells have recently been characterized in a variety of tissues of adult animals, including liver, blood, skin, brain, and heart (Wetts & Fraser, 1988; Spangrude *et al.*, 1988; Jones *et al.*, 1995; Petersen, 2001; Hughes, 2002). Their plasticity, as demonstrated by the multipotency to differentiate into mature, tissue-specific cell types, may offer new therapeutic tools for a variety of diseases. Hepatic oval cells (HOCs) are considered the stem cells of the liver, having been shown to be capable of giving rise both to hepatocytes and bile duct cells (Petersen *et al.*, 1998a). The majority of HOC studies have been conducted in various rat models; however, a mouse model was recently developed which allows for the isolation of large quantities of HOCs. This model incorporates the chemical 3,5-diethoxycarbonyl-1,4-dihydrocollidin (DDC) at a 0.1% concentration in the normal chow (Preisegger *et al.*, 1999). Development of this mouse model also led to the characterization of an antibody -termed A6- that recognizes a specific epitope on mouse HOCs (Factor *et al.*, 1990). In conjunction with this new mouse oval cell model and the two step liver perfusion technique (Seglen, 1979), Petersen *et al.* have developed a enrichment protocol which allow us to isolate a greater than 90% pure Sca-1+ oval cell population from the DDC treated mouse liver (Petersen *et al.*, 2003).

Trans-differentiation -the ability of stem cells from one tissue to generate cells characteristic of an entirely different tissue- is of interest not only because in it lies the true answer to the multipotent capabilities of the adult "stem" cells, but also because it may provide an easily accessible, non-controversial source of cells for future autologous transplantation therapies. The use of stem cell therapy in treating neurodegenerative disorders has attracted considerable attention lately. The trans-differentiation of bone marrow-derived stem cells (BMSC) into neural cell types has been explored extensively, with several groups reporting that these stem cells can trans-differentiate into neurons, astrocytes and microglia (Azizi *et al.*, 1998; Kopen *et al.*, 1999; Brazelton *et al.*, 2000). The trans-differentiation of BMSC into microglia was thought to recapitulate microglia ontogeny (Rio-Hortega del, 1932; Eglitis & Mezey, 1997); however, the functionality of these trans-differentiated microglia cells has not been reported thus far.

Yang *et al.* have recently reported that oval cells can trans-differentiate into insulin-producing pancreatic cells in culture when challenged with high glucose (Yang *et al.*, 2002). In order to further characterize the HOC and its potential plasticity, we transplanted isolated HOCs - derived from GFP transgenic mice- into the lateral ventricles of neonatal wild-type mouse brain, according to a model of intracerebral transplantation recently described for assaying stem cell behaviors of neural cells (Zheng *et al.*, 2002). We asked whether the oval cells could trans-differentiate into cells of a neural phenotype.

3.2 Materials and Methods

3.2.1 Hepatic Oval Cell Induction and Enrichment from Mouse Liver

According to the protocol established by Preisegger *et al.* (1999), we fed adult C57BL6 /GFP$^{+/+}$ transgenic mice a normal diet supplemented with 0.1% DDC (BioServe, Frenchtown NJ) for six weeks. To isolate HOCs, we performed a two step liver perfusion according to Seglen *et al.* (1979), collecting the non-parenchyma fraction (NPC) using gradient centrifugation. We incubated the NPC fraction with the Sca-1 antibody conjugated to micro-magnetic beads, processing the cell suspension through magnetic columns to enrich the oval cell population positive for Sca-1, the stem cell antigen-1 (MACs, Miltenyi Biotec).

3.2.2 FACs Analysis for Purity on MACs Sorted Sca-1$^{\pm}$ Oval Cells

Wild-type Sca-1^{+} and Sca-1^{-} oval cells, obtained from MACs magnetic sorting, were incubated with Fluorescein Isothiocyanate (FITC)-Sca-1 and FITC-rat IgG_{2a} antibodies (PharMingen) (1:500) for 30mins at room temperature. Cells were then pelleted by centrifugation at 200g and washed twice in PBS to eliminate unbound antibodies. Approximately 10^6cells/ml-cell suspension was run through a flow cytometer (CELLQuest, Becton Dickinson FACScan).

3.2.3 Immunocytochemistry of MACs Sorted Oval Cells

Wild-type Sca-1^{+} oval cells, obtained from MACs magnetic cell sorter, were cytocentrifuged to slides, fixed with 4% paraformaldehyde in PBS, and examined for mouse oval cell markers as described (Petersen *et al.*, 1998a). A6 antibody (a gift from Dr. Valentina Factor of the NIH) (1:20) and anti-α-fetal protein (AFP) (Santa Cruz Biotechnology) (1:200) were used for the immuno-characterization of oval cells.

3.2.4 Culture of Mouse Oval Cells

Approximately 10^6 Sca-1+ mouse oval cells, obtained from MACs cell sorting were cultured in a 35mm culture dish (Costar, Corning In.) in HOC culture medium (89% Iscove's Modified Dulbecco's Medium, 10% FBS, 1% Insulin, 1000 U/ml of leukemia inhibitory factor, 20ng/ml granulocyte macrophage colony stimulating factor, 100ng/ml each of stem cell factor, interleukin-3 and interleukin-6).

3.2.5 Cell Transplantation into Neonatal Mouse Brain

Sca-1^+ MACs sorted primary dissociates of GFP^+ oval cells were transplanted into the lateral ventricle of postnatal day 1 wild-type C57BL6 mice within the first 24 hours after birth. Briefly, newborn pups were anesthetized by hypothermia and placed in a clay mold. The head was trans-illuminated under a dissection microscope, and a Hamilton syringe with a beveled tip was lowered through the scalp and skull immediately anterior to bregma. Approximately $2.5x10^5$ GFP^+ HOCs in 1μl volume of Dulbecco's Modified Eagle Medium/F12 (DMEM/F12, Gibco) were then slowly pressure-injected into the left lateral ventricle. Immediately after injection, pups were warmed in a 37°C incubator, and returned to the mother after approximately 30 min. At ten days post-transplantation, mice were sacrificed with an overdose of Avertin, and perfused transcardially with 4% paraformaldehyde in PBS. The brain tissue was excised, post-fixed overnight in perfusate, and sectioned through the coronal plane into 40μm slices with a vibratome.

3.2.6 *In vivo* Phagocytosis Assay

An *in vivo* phagocytosis assay of microglia was performed by adding fluorescent latex microbeads to the graft bolus immediately prior to transplantation. Latex microbeads (Sigma L-0530, 0.5μm in diameter, fluorescent blue conjugated) were added

into the cell suspension (~2.5 x 10^5 cells/μl in DMEM/F12) at a concentration of 15% (0.15μl bead solution/0.85ul cell suspension). One microliter of cell/bead mixture was injected into the lateral ventricle of newborn pup brains as described above. Hosts were then allowed to survive for ten days before the brains were fixed and processed for immuno-characterization.

3.2.7 Immunolabeling of Brain Sections

Forebrains were cut with a vibratome into 40μm coronal sections exhaustively, and processed free-floating for immuno-fluorescence. After blocking in PBS with 10% goat serum, sections were incubated overnight at 4°C in primary antibodies directed against the following proteins: nestin, a marker of neuronal stem and progenitor cells (Developmental Studies Hybridoma Bank, University of Iowa; 1:250); the astrocyte-specific markers glial fibrillary acidic protein (GFAP; from Gerry Shaw University of Florida,1:200), and S100β (Sigma;1:250); the microglia marker CD11b (Serotec;1:200); and the neuronal markers neurofilament medium subunit (NFM; from Gerry Shaw University of Florida,1:500), α-internexin (α-IN; from Gerry Shaw University of Florida,1:200), and MAP2ab (Sigma;1:500). The tissues were then washed in PBS, followed by incubation in appropriate secondary antibodies conjugated to R-phycoerythrin (R-PE) (Molecular Probes) at room temperature for 1hr. After a final wash in PBS, brain slices were mounted onto glass slides, viewed, and counted with a fluorescence microscope.

3.2.8 Quantification of Grafted Cells

Cell counting was performed under a fluorescence microscope (Olympus BX51). Every sixth section through the forebrain was selected for counting of grafted cells. A

cell was counted if the cell body could be identified. Total number of cells was then obtained by multiplying the counted result by a factor of six. The standard deviations were obtained using Microsoft Office Excel statistics software.

3.3 Results

3.3.1 Hepatic Oval Cell Enrichment with Sca-1 Antibody

In order to verify the purity obtained with our sorting method, we performed FACs analysis on MACs sorted Sca-1^+ cells. After MACs sorting, only 20% of the Sca-1 epitopes were occupied by the Sca-1 conjugated magnetic beads, which allowed us to use the remaining epitopes to perform the FACs analysis for purity. Figure 1A and B represent histograms of FACs analysis showing a distinct population of cells. MACs sorted cells are over 90% positive for Sca-1 antibody (Fig. 3-1A), while the flow-through cells were Sca-1 negative (Fig. 3-1B). Immunocytochemistry revealed that the Sca-1^+, MACs sorted cell were also positive for A6 and AFP- known markers for mouse oval cells (Fig. 3-1C). When cultured *in vitro*, HOCs started to proliferate in about 5 days, and formed colonies after about two weeks (Fig. 3-1D). The HOCs in culture appeared to be a homogeneous and undifferentiated cell population.

3.3.2 Hepatic Oval Cells Survive and Differentiate in the Neonatal Mouse Brain

Ten days after transplantation of HOCs, intensely fluorescent GFP^+ cells were seen within the host brain. The majority of surviving donor cells was located in periventricular areas in all of the mice with successful cell delivery (Fig. 3-2A, B and C). GFP^+ cells were most frequently observed superficially along the walls of the lateral ventricle, but numerous grafted cells were also found to migrate laterally within the white matter of the corpus callosum (data not shown). At points along the ventricular wall,

grafted cells penetrated into the parenchyma of the brain, a phenomenon previously described following intraventricular transplantation of multipotent astrocytes (Zheng *et al.*, 2002). The survival rate of the transplanted HOCs averaged 0.56% (SD=0.36%, n=9) of the total injected cells (Table 1). Approximately 11.5% (SD=2.5%, n=3) of grafted cells remained undifferentiated, and were characterized by a small, rounded, non-process bearing morphology. The remainder displayed varying degrees of differentiation and process extension. Seven of 36 animals receiving transplants did not contain any detectable donor cells.

3.3.3 Grafted Hepatic Oval Cells Express Neural Antigens

The filament protein nestin has frequently been considered indicative of neural progenitor cells (Lendahl *et al.*, 1990). We found that 22.1% (SD=11.6%, n=4) of surviving donor cells were immuno-positive for nestin (Fig. 3-3A, Table2), suggesting that HOCs may be able to assume the phenotype of early neural lineage. Of the donor cells that differentiated, the majority exhibited a typical amoeboid or ramified microglia morphology (Fig. 2D-G). A smaller fraction displayed the stellate, process-rich characteristics of astrocyte morphology (Fig. 3-2H-K). Immuno-labeling with the Mac-1 antibody, directed against the CD11b epitope characteristic of macrophages, showed that 60.6% (SD=10.5%, n=3) of the GFP^+ donor cells express this microglial marker (Fig. 4A and B, Table 2). Additionally, 34.7% (SD=9.0%, n=4) and 27.2% (SD=5.7%, n=3) of donor cells express the astrocyte specific proteins GFAP and S100, respectively (Fig. 3-3B-D, Table 2). Many of the cells expressing astrocyte proteins were located within the corpus callosum, and their processes could be seen intertwining with the processes of native astrocytes.

A small number of donor cells were also seen to be immunopositive for neuron specific markers. 6.5% (SD=1.3%, n=3) of the grafted cells expressed the neuronal marker NF-M (Fig. 3-3E, Table 3-2), and a comparable number expressed α-IN (Fig. 3-3F). A considerably larger percentage, 19.9% (SD=2.5%, n=3), of donor cells were immuno-positive for MAP2 (Fig. 3-3G, Table 3-2). Although these grafted cells do have an antigenic profile consistent with neurons, suggesting that HOCs can generate cells belonging to the neuronal lineage, their morphologies are ambiguous and do not resemble typical *in vitro* or *in vivo* neurons.

3.3.4 Donor-Derived Cells Have Functional Properties of Microglia

Grafted cells with the antigenic profile of microglia also display appropriate phagocytic activity, since co-transplanted fluorescent microbeads were incorporated into their cytoplasm at high efficiency (Fig. 3-4C, Table 3-3). 58.7% of grafted GFP^+ cells, as well as numerous indigenous microglia were seen to incorporate microbeads, and these cells were subsequently shown to express the CD11b antigen, characteristic of macrophages, including brain microglia.

3.4 Conclusion and Discussion

Our results indicate that a portion of the HOCs from adult mouse liver can survive transplantation to the neonatal mouse brain, and can differentiate into cells that share certain phenotypic characteristics with neurons, astrocytes, and microglia. Furthermore, donor cells that express microglial antigens can also display functional properties characteristic of microglia, i.e. active phagocytosis.

We believe that our quantification of donor cell survival and differentiation characteristics is extremely conservative, in light of the fact that we have observed that

HOCs acutely isolated from the liver of the GFP transgenic C57BL6 have largely variable levels of GFP expression, with approximately 50% of them not expressing detectable GFP. We have also observed that long-term cultured GFP+ HOCs gradually lose GFP expression over a three-month period (data not shown). Therefore, the actual survival rate of grafted HOCs in our study may likely to be higher than that we have quantified. We also observed a large variation of HOC survival between different animals. Survival ranged from 0% (7 of 36 animals had no detectable GFP^+ cells) to 1.0%, with a standard deviation of 0.36%. This inconsistency is likely the result of technical failure during the transplant procedure. For instance, the lumen of the transplant needle can become fully or partially blocked during the penetration through the scalp and skull, resulting in reduced numbers of cells being delivered. Additionally, in some animals we observed extrusion of the graft bolus up the needle tract, which again would greatly decrease the delivery of cells to the ventricle.

The fact that a percentage of donor cells was seen to be immunopositive for nestin is provocative since this protein has been considered to be a marker of primitive neural stem/progenitor cells (Lendahl *et al.*, 1990), and it is reasonable to suggest that trans-differentiation from hepatic to brain lineage would involve a transition through an early neural stage.

The adoption of microglial phenotype by grafted HOCs is clearly the most frequent occurrence in our transplantation paradigm, and seems intuitively consistent given the close relationship among hematopoietic cells, liver cells, and microglia. It has been shown that hematopoietic stem cells and HOCs share considerable antigenicity, and donor hematopoietic stem cells can contribute to liver regeneration (Omori *et al.*, 1997a;

Omori *et al.*, 1997b; Petersen *et al.*, 1998a; Petersen *et al.*, 1999). Furthermore, it is generally accepted that most brain microglia originate from the hematopoietic system (Rio-Hortega del, 1932; Eglitis & Mezey, 1997).

Recently, studies describing the trans-differentiation of bone marrow and other types of stem cell into neurons have come under criticism by Holden *et al.* (Holden & Vogel, 2002), with one of the concerns being the lack of complete neuron morphology. Previously reported trans-differentiated cells did not display long process-bearing morphology, with axons and dendrites. Morphological characteristics are still the primary criteria for assessing the neuron generating potential of a differentiating progenitor cell, since it is an integral part of neuronal function to efficiently and remotely transmit electric signals. Another weakness of these reports is the lack of functionality of the trans-differentiated cells. Unlike the use of stem cells to reconstitute the full function of an immune-deprived blood system in rodents, the functional assay for a single neuron in the brain is much more difficult. We have shown that a small portion of oval cells, which have been transplanted into the neonatal mouse brain express some neuronal markers and start to show limited neuron morphology. While additional morphological and electrophysiological data are required to definitively prove trans-differentiation, our results strongly indicate that liver-derived HOCs can, under certain environmental influences, adopt some characteristics of neuronal cells. The vastly greater number of MAP2 labeled donor cells could indicate that this marker is associated with neurons in a different stage of differentiation, or more likely reflects the fact that the Map-2 antigen can be expressed by non-neuronal cells under certain circumstances, such as brain injury

which certainly occurs in our transplants due to the penetration of the needle (Lin & Matesic, 1994).

The origin of the hepatic oval cells is still a controversial issue. A precursor cell type is believed to exist that generates oval cells when certain liver injury occurs. The traditional view holds that there is an endoderm-derived liver stem cell, possibly in the Canal of Hering (Theise *et al.*, 1999). While another view is supported by recent reports showing that circulating stem cells originating from the bone marrow can contribute to the precursors of oval cells in the liver (Petersen *et al.*, 1999). The second view is supported by the immunochemical characteristics that oval cells and hematopoietic stem cells have in common. Oval cells express many hematopoietic stem cell markers, such as Thy1, c-kit, and CD34 in the rat, and flt-3 in the mouse (Omori *et al.*, 1997a; Omori *et al.*, 1997b; Petersen *et al.*, 1998a). Recent work from our laboratory has reported that mouse oval cells also express Sca-1 and CD34 (Petersen *et al.*, 2003). Our present results show the trans-differentiation potential of oval cells in becoming cell types of the brain. We presented data that microglia differentiation from oval cells is possible, showing a complete morphology and the phagocytosis activity of the trans-differentiated cells.

It is possible that nuclear fusion of donor cells with host astrocytes and neurons might be a factor in the putative trans-differentiation we observed, as we have reported previously in an *in vitro* model (Terada *et al.*, 2002). However, since cell fusion involving stem or progenitor cells has not yet been confirmed *in vivo*, and based upon the large number of GFP^+ cells we observed, it seems unlikely. Nevertheless, future studies will need to be conducted to rule out this phenomenon as a confounding factor. Sex mismatching of donors and hosts would allow us to detect fusion events via *in situ*

hybridization for X and Y chromosomes, and we have used this technique successfully in looking at potential trans-differentiation events in archived human brain sections (unpublished observation).

The isolation of large number of oval cells holds tremendous promise as a source for liver transplantation in treating both acute and chronic liver failure. With the recent report of the trans-differentiation of oval cells into the insulin-producing pancreatic cells, using this cell type in treating diseased tissues other than liver may be possible (Yang *et al.*, 2002). Our studies show that these HOCs may also hold potential plasticity for becoming neural cells, which may help our understanding of stem cell differentiation. Since a large number of oval cells appear to differentiate into GFAP positive astrocyte-like cells, and since astrocytes have been previously reported by us and others to exhibit multipotency under certain conditions (Laywell *et al.*, 2000; Seri *et al.*, 2001), it will be important to determine if oval cells might give rise to glial cells with neuronal differentiation potential. Future studies will be needed to further characterize this intriguing cell type, but the data presented here on oval cell behavior following intraventricular transplantation, in line with that described by others looking at bone marrow hematopoietic and stromal cells, suggest a potential common origin for these plastic cells.

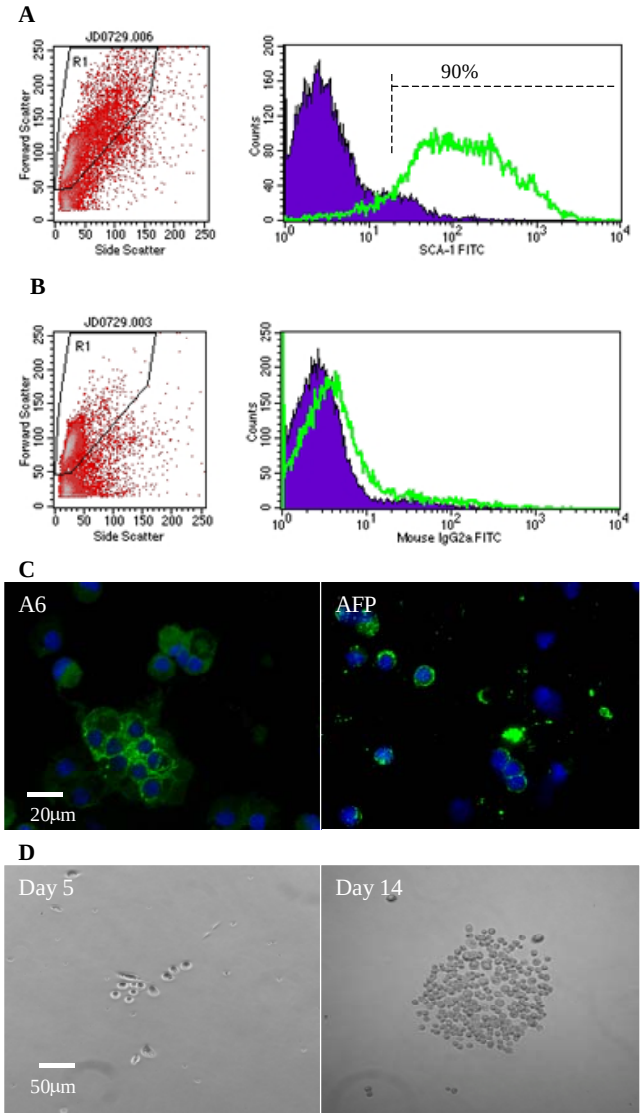


Figure 3-1. Characteristics of mouse hepatic oval cells enriched using MACs magnetic beads. MACs sorted cells were subjected to FACs analysis to obtain purity levels. A) Histogram showing positive cells (green line) above the 90% level at the gate of maximum overlap with the control (blue fill). R1 in scatter plots demarcates the analyzed cell population. B) Negative flow through cells (green line) from the magnetic column are overlapped with the control (blue fill). Immunocytochemistry was performed to verify that the Sca-1^{+} cells isolated by MACs are indeed oval cells. C) MACs enriched cells are positive for the A6 epitope and AFP, known markers for murine oval cells. FITC conjugated (green) secondary antibody was used to visualize the positive cells with DAPI (blue) to show the nuclei of the cells. D) *In vitro* culture of HOCs. HOCs started to proliferate in about 5 days, and formed colonies in about two weeks. The HOCs in culture appear to be a homogeneous, undifferentiated cell population.

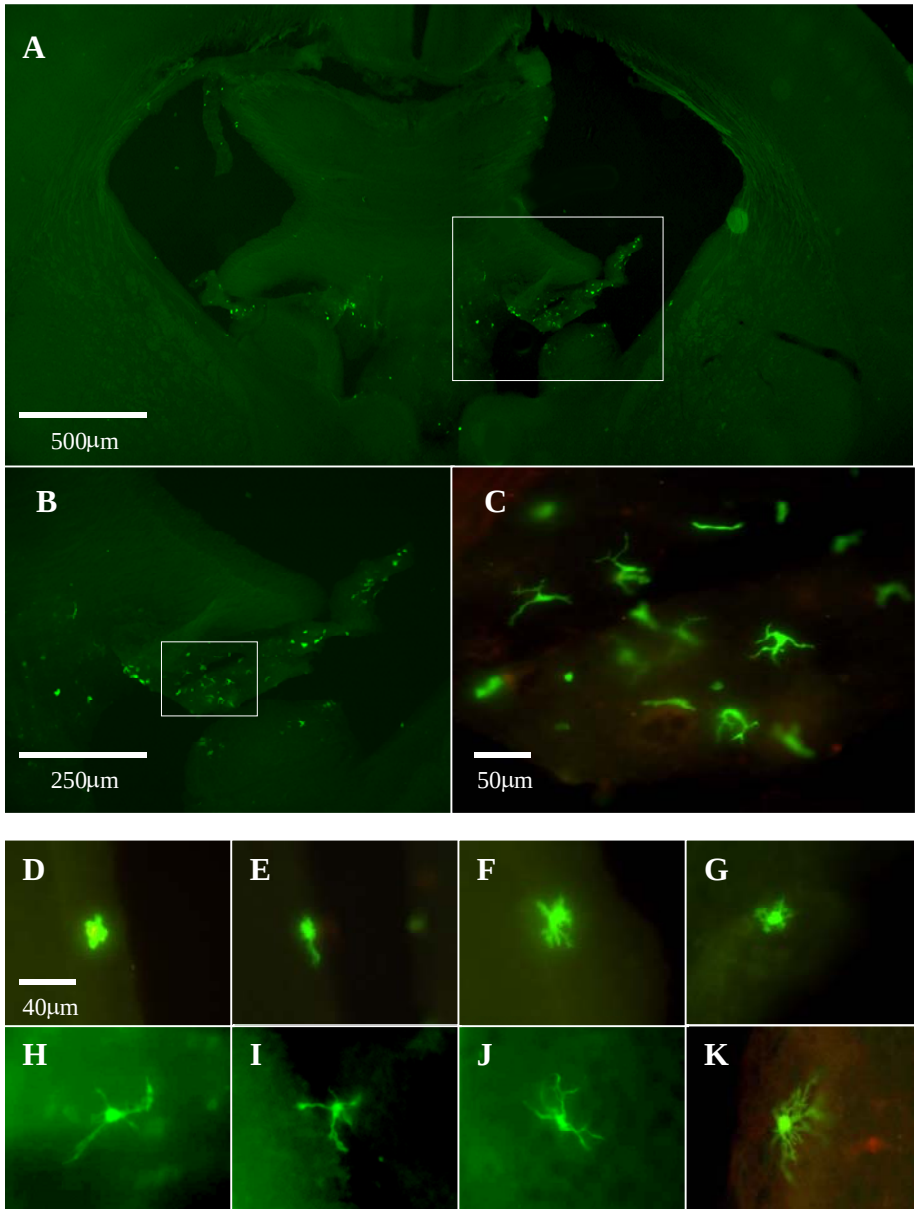


Figure 3-2. Hepatic oval cells survive and differentiate in the neonatal mouse brain. A) Oval cells reside along the lateral ventricle ten days after transplantation. Most of the cells are clustered around the fimbria (Fi). Some are seen dispersed within forebrain parenchymal sites along the ventricular walls. B) Higher magnification of the box in A, showing the local distribution of the transplanted oval cells at the fimbria region. C) An inset in B, showing differentiation of oval cells. D-K) Variety of differentiated oval cell morphologies: (D)-(E), amoeboid microglia-; (F)-(G), ramified microglia-; (H)-(K), astrocyte-like morphology. The scale bar in (D) applies to (E)-(K).

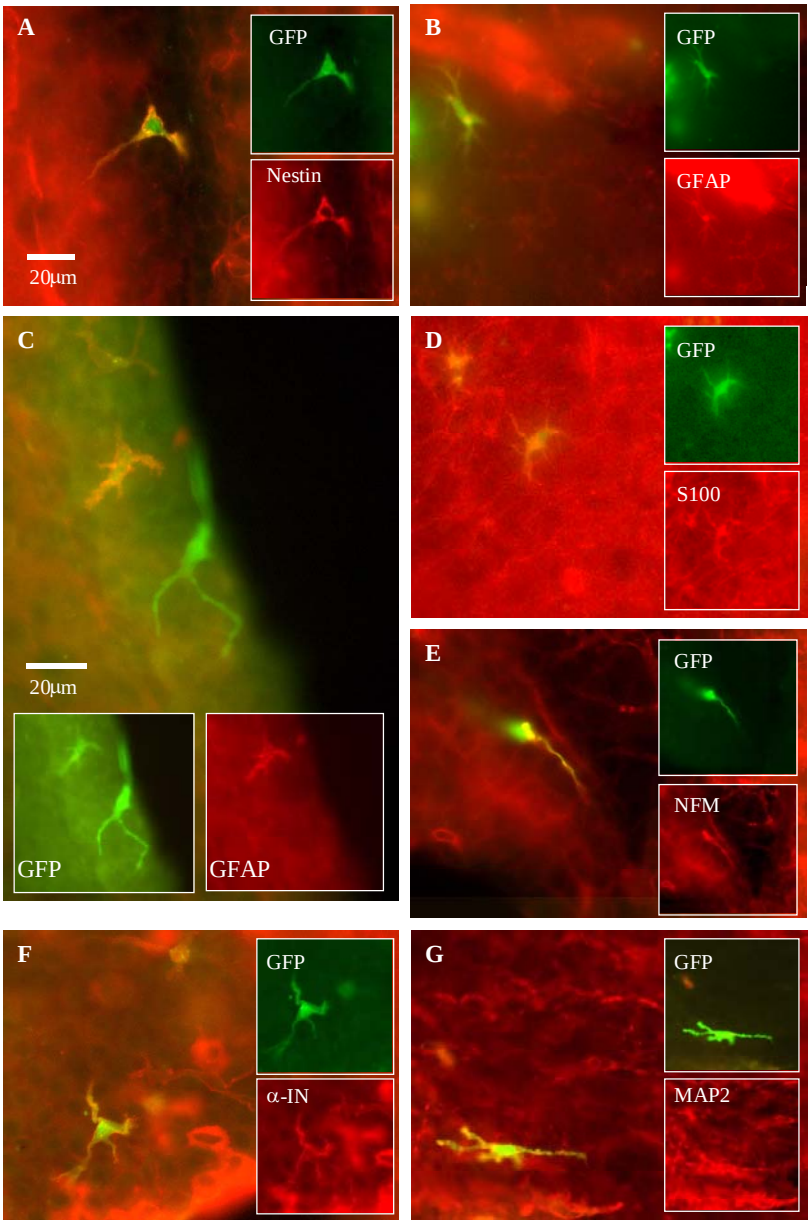


Figure 3-3. Differentiated GFP$^+$ hepatic oval cells express neural-specific proteins in the neonatal mouse brain. GFP expression of oval cells (green) co-localize with immunostaining with antibodies to neural-specific proteins, visualized with secondary antibody conjugated to PE (red). A) Oval cells are positive for nestin, a marker of primitive neural stem/progenitor cells. B)-D) Oval cells are positive for the astrocytic markers GFAP and S100. Oval cells are seen to intertwine with native astrocytes in the surrounding tissue. Note in (C), only one of the two GFP$^+$ cells expresses GFAP. E)-G) Oval cells are positive for antibodies against several neuron specific proteins NFM, α-internexin (α-IN), and MAP2. The scale bar in (A) applies to (B) and (D)-(G).

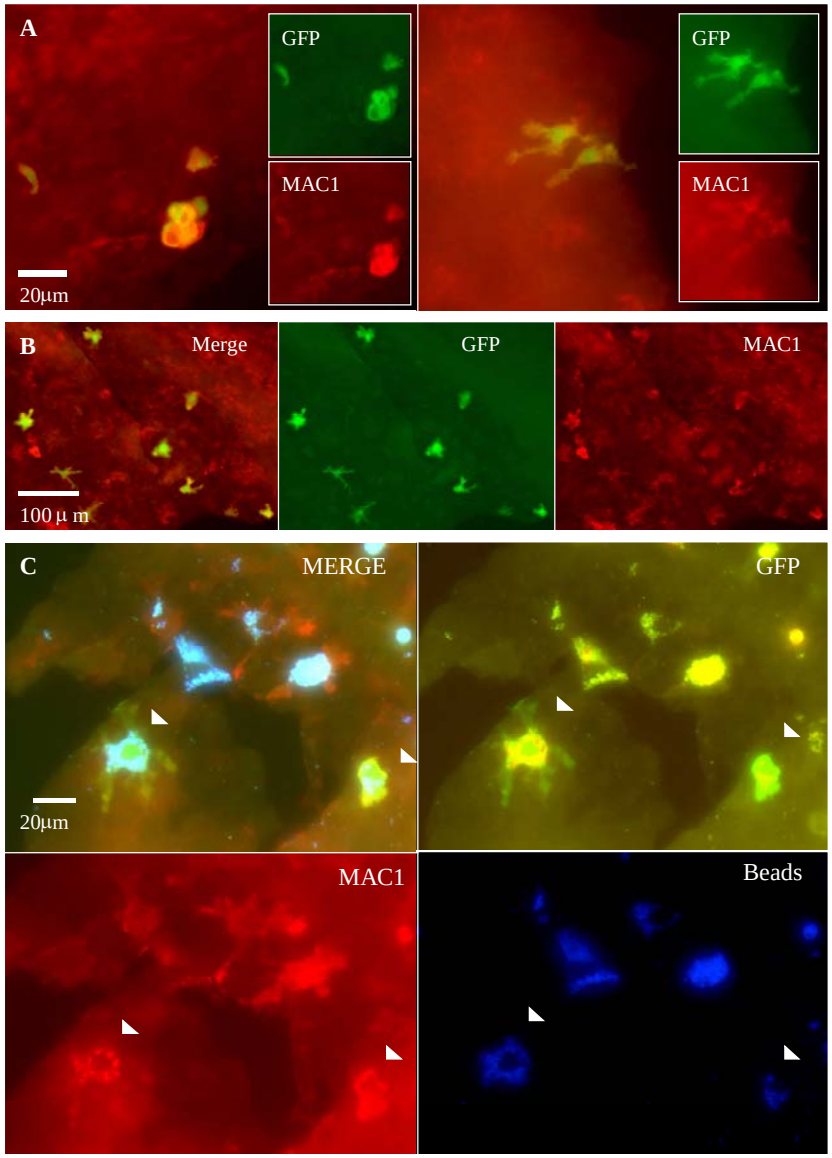


Figure 3-4. Oval cells acquire microglia phenotype and phagocytosis activity in the mouse brain. GFP expression of oval cells (green) co-localize with immunostaining with Mac1 antibody against mouse microglia specific protein CD11b, visualized with secondary antibody conjugated to PE (red). A) $Mac1^{+}$ oval cells have amoeboid- and ramified-microglia morphology. B) Many $Mac1^{+}$ oval cells coexist with native microglias. C) Differentiated hepatic oval cells show phagocytosis activity. Latex microbeads, conjugated with florescent blue were co-injected into the mouse brain with the isolated oval cells. Brain sections were immuno-stained with Mac1 antibody. Two differentiated GFP^{+} oval cells (arrowheads) with ramified and amoeboid microglia morphology are co-localized with Mac1 staining (red, R-PE) and microbeads (blue). The native microglia are also seen to take up microbeads.

Table 3-1. Survival Rate of Transplanted HOCs in the Neonatal Mouse Brain.

Animal No.	Number of Injected Cells ($x10^5$)	Number of GFP^+ Cells	Percentage of Survival
5.3	2.5	680	0.27%
13.1	2.5	390	0.16%
14.6	2.5	2,250	0.90%
14.7	2.5	468	0.19%
15.4	2.0	2,022	1.01%
15.5	2.0	1,962	0.98%
15.6	2.0	1,302	0.65%
15.7	2.0	1,584	0.79%
15.9	2.0	546	0.27%
Average	**2.2**	**1,245**	**0.56%**

Nine mouse brains were counted. The GFP^+ cells of every sixth section of the forebrain were counted for each brain. The total numbers of survived HOCs were obtained by multiplying the counted results by a factor of six. The standard deviation is 0.36%.

Table 3-2. Composition of the Neural Markers in the Transplanted HOCs in the Neonatal Mouse Brain

Markers	Number of Animals	Number of Positive Cells	Number of GFP^+ Cells	Percentage of Positive Cells	StdV
Nestin	4	25.5	115.3	22.1%	11.6%
Mac1	3	102.7	169.3	60.6%	10.5%
GFAP	4	78.8	227.0	34.7%	9.0%
S100	3	68.0	250.0	27.2%	5.7%
NFM	3	11.0	168.0	6.5%	1.3%
Map2	3	55.0	276.0	19.9%	2.5%

Every sixth section of the forebrain was counted for each animal. The average numbers of cells positive for each marker, and the GFP^+ cells, as well as the percentages of the number of positive cells among the total GFP^+ cells, and their standard deviations (StdV) among all the mice inspected are shown.

Table 3-3. The Percentage of Cells Taking up Microbeads among the Total GFP+ Cell.

Animal No.	**GFP^+ w/ Beads**	**Total GFP^+**	**% of GFP^+ w/ beads**
21.5	52	78	66.7%
21.6	23	37	62.2%
21.7	14	25	56.0%
21.8	14	28	50.0%
Average	**26**	**42**	**58.7%**

Every sixth section of the forebrain was inspected for each animal. The standard deviation is 7.3% among all four mice

CHAPTER 4
NEURAL INDUCTION OF HEPATIC OVAL CELLS *IN VITRO*

4.1 Introduction

The neural differentiation of hepatic oval cells *in vitro* is of interest, because it may provide information on the mechanisms of neural trans-differentiation observed *in vivo* previously reported by Deng et al (2003), and it may also reveal the correlations of HOC with MSC by comparing their responses to the previously used neural induction protocols in MSCs.

The hypothesis of a bone marrow origin of HOCs has been suggested previously. Crosbie *et al.* (1998) provided *in vitro* evidence to show that there are hematopoietic stem cells exist in the human liver. Oval cells express many hematopoietic stem cell markers, such as Thy1, c-kit, and CD34 in the rat, and flt-3 in the mouse (Omori *et al.*, 1997a; Omori *et al.*, 1997b; Petersen *et al.*, 1998a). Recent work from our laboratory has reported that mouse oval cells also express Sca-1 and CD34 (Petersen *et al.*, 2003). Petersen *et al.* (1999) demonstrated that, after 2AAF/PHx liver injury, rats that received bone marrow transplantation had mature hepatocytes of donor cell origin within the regenerating liver, suggesting that oval cells may have also been derived from the bone marrow source. Theise *et al.* (2000) also provided evidence that human bone marrow can be transplant into liver directly and reconstitute its function. Under this context, it would be very interesting to compare the difference of HOC and MSC, in the purpose of understanding the genesis of these two types of stem cells.

In vitro neural differentiation is a critical steps towards the future stem cell application in neurological disorders. Hepatic oval cell may not be the best choice as a candidate for the use in cell transplantation, because they do not exist in large quantity under normal condition, and to obtain HOCs involves invasive procedures. However because of the sheer numbers that can be obtained in experimental animal, it may provide some insights to the mechanisms of neural differentiation of adult stem cells in general. Yang *et al.* (2002) reported that rat HOCs can be induced to produce insulin in the culture condition, demonstrating the multipotency of HOCs *in vitro*. Deng *et al*. (2003) demonstrated that mouse HOCs trans-differentiated into neural lineage when grafted in to the neonatal mouse brain. Trans-differentiation from HOCs to pancreatic lineage may not come out as a surprise, because both cell types are endoderm tissues. The effort of inducing HOCs into a neural phenotype *in vitro*, the ectoderm lineage phenotype would further prove HOC multipotency, which could qualify it as a "true stem" cell.

Utilizing this rational, we applied a variety of methods which have been shown effective to induce a neural phenotype from MSCs, in the purpose of demonstrating the neural differentiation of HOCs *in vitro*.

4.2 Materials and Methods

4.2.1 Isolation and Culture of Oval Cell

Rat HOCs (rHOCs) were induced by utilizing procedures as previously described by Petersen *et al.* (1998b) using the 2-acetylamino-fluorene/partial hepatectomy injury model. Oval cells were then isolated from the rat livers by using the two-step collagenase perfusion protocol of Seglen (1979), and purified by fluorescence activated cell (FAC) sorting for the Thy-1.1-positive cell population (Petersen *et al.*, 1998a). The FITC-

conjugated anti-rat Thy1.1 antibody was purchased from PharMingen. This technique resulted in hepatic oval cell populations with a purity of higher than 95%, and were tested to express the hepatic stem cell markers α-fetoprotein, albumin, γ-glutamyl transpeptidase, cytokeratin-19, and OV6 (Petersen *et al.*, 1998a). The purified Thy-1.1-positive hepatic oval cells were cultured in serum-free Iscove's modified Delbecco's medium (IMEM; GIBCO/BRL) supplemented with leukemia inhibitory factor (10 ng/ml), IL-3 (10 ng/ml), stem cell factor (10 ng/ml), and Flt-3 ligand (10 ng/ml), and they have been culture over 50 passages (Yang *et al.*, 2002).

The mouse HOCs (mHOCs) induction and isolation have been described in the previous chapter (see 3.2.1)

4.2.2 Neurospheres Generation and Culture

Neurospheres (NS) were generated from postnatal day 5-7 mouse or rat brains. In brief, the pups were decapitated under deep anesthesia (IP injection of sodium phenobarbital), and the brains were removed from the skull. After removed the olfactory bulb and the cerebellum, tissue was minced to small pieces, washed in PBS, and trysinized at 37°C for 10mins to dissociate the cells completely. After further washed, cells were re-suspended in 2% Methyl Cellulose which is dissolved in DMEM/F12 and supplemented with N2 and growth factor cocktail (10ng/ml basic FGF and 20ng/ml EGF). The cultures were maintained up to a month, during which time NSs would become visible and grow up to about 200μm in diameter.

4.2.3 Organotypic Brain Slice Culture

Organotypic brain slice cultures were generated from postnatal E15.5 embryos, postnatal day 4, and 8 weeks adult mice. Briefly, animals were euthanized and quickly

decapitated. The brains were cut into two saggital halves and immersed in a preparation medium (DMEM, L-ascorbic acid, L-glutamate, and pen/strep). The halves were then super-glued to the vibratome stage, medial surface down, and covered with cool molten 2% agar. The stage was then placed in the vibratome chamber and filled with preparation medium. Slices were cut between 300-400 μM, placed in cold preparation medium. Slices from the appropriate levels were then immediately transferred to a transwell (Falcon), placed in a 6 well plate, and incubated at 35 degree °C and 5% CO_2. Each transwell was suspended in 1.8 mL of "A" medium, DMEM F12 with B27 and N2 supplements containing 25 % serum. The medium was changed the next day and feeding was done every other day. For long-term cultures "A" medium was phased out replaced by a serum free "B" medium, DMEM F12, B27, and N2. To avoid serum deprivation effects, a mixture of 2/3's "A" and 1/3 "B" was used on day 3 and on day 5 a mixture of 2/3's "B" and 1/3 "A" was used. On day 7 medium was completely replaced with "B" medium and replaced every other day (Benninger *et al.*, 2003; Scheffler *et al.*, 2003).

4.2.4 Immunocytochemistry

The cells, grown on coverslips or culture dishes, were fixed in 4% paraformaldehyde, or ETOH:acetic acid (95:5) for 15mins. After washed 3x5 mins with PBS, the cells were blocked in 10% goat serum in PBS for 30 mins. The cells were then incubated with primary antibodies for one hour at room temperature. Several antibodies have been used in the study, which included βIII tublin (Promega), NeuN (Phamingen), neurofilament associated protein medium (NFM; Encor), Nestin, α-internexin (α-IN) and MAP2ab (Sigma) antibodies against neuron specific proteins, and glia fibrilary acidic protein (GFAP; Immunon), and S100 (Sigma) antibodies against astrocytes, and Mac1

(Serotec) antibody against mouse microglia specific protein CD11b. After washed in PBS three times, the cells were then incubated with a florescent conjugated secondary antibody for one hour at room temperature. Finally, cells were washed in PBS three times before mounted with florescent mounting medium (Vectashild).

4.2.5 Neuronal Induction Using IBMX and dbcAMP

One day before the experiment, rHOCs were replated into 35mm well plates at 60% confluence and grew overnight in the oval cell culture medium. For induction, the culture medium was replaced with induction medium which contained 0.5mM IBMX and 1mM dbcAMP. Cells were then cultured for 7, 14, 21days, during which time the medium was changed once a week. After terminating the inductions at the designated time points by fix the cells with 4% paraformaldehyde, immunocytolabeling were performed to detect neuronal specific proteins.

4.2.6 Neural Induction Using BME, DMSO and BHA

One day before the induction, rHOCs were plated in 35mm dishes with a confluence of 60% in HOC culture medium. For the induction, the culture medium was first replaced with induction medium A, which contains IMDM (80%), FBS (20%) and BME (1mM), and culture for 24 hrs. The medium A was then replaced with medium B that contained only IMDM and BME (5mM) and culture for 24 hrs. Finally, the medium B was replaced with medium C that contains DMSO (2%) and BHA (0.2mM) in IMEM, and cells were treated for prolonged one to two weeks depending on the morphological changes.

4.2.7 Neural Induction Using Retinoic Acid under 4+/4- Protocol

Rat HOC aggregates were induced by introducing scratches on an over-confluent rHOC monolayer culture. We then cultured the aggregates with 0.5 μM RA to normal culture medium without LIF in an anti-adhesive petridish for four days. The rHOC aggregates were then cultured in normal medium without RA for an additional four days. Finally, the aggregates were then moved onto cover slips coated with laminin to induce neural differentiation for 7-14 days. The neural differentiation was then evaluated by immunocytolabeling.

4.2.8 Neural induction of Hepatic Oval Cell by Over-Expressing Chordin and Noggin

Chordin and noggin plasmid constructs were gifts from Dr. O'Shea of University of Michigan (Gratsch & O'Shea, 2002). FuGENE6 (Roche) transfection kit was used for the gene delivery. The cells were passed into a 35mm 6-well plate with a confluence of 50-80% the day before transfection. For transfeciton, 100μL of serum-free IMEM and 6μl FuGENE6 reagent were mixed in a sterile tube and incubated for 5 mins at room temperature. And then 2μg DNA were added into the reaction mixture, and incubated for 30 mins at room temperature. The reaction mixtures were then laid onto the cells with 2mL culture medium, and incubated for 48hrs.

4.2.9 Neural Induction of HOC by Co-Culturing with Differentiating Neurospheres

Rat HOCs were induced to express green fluorescent protein (GFP) using lentiviral vectors developed in Dr. Razaida's laboratory at the Physiology Department of University of Florida. For the co-culture, the neurospheres derived from neonatal mouse brain were first induced to differentiate by plating on laminin coated cover slips. The

GFP expressing rHOCs were then plated on the differentiating neurospheres for up to four weeks.

4.2.10 Neural Induction of HOCs by Micro-injecting into Neurospheres

For this experiment, 10-20 GFP^+ rHOCs were injected into a neurosphere of 50-100μm in diameter. The rHOCs were microinjected into the center of the neurospheres in the University of Florida Cancer Center. Following microinjection, the neurospheres were placed into the neurosphere culture medium, and cultured for two weeks. The neurospheres incorporated with GFP^+ rHOCs were then placed on laminin coated cover slips to induce neural differentiation.

4.2.11 Neural Induction of HOC by Incorporating into the Embryoid Body

R1 ES cells:GFP^+ rHOC cells (3:1) were mixed in ES differentiation medium (20% FBS, 15μL Monothioglycerol (Sigma) in IMEM) with a density of 100cells/μL. Thirty micro liters of the cell mixture were gently laid on the cover of a petridish, and cultured in a hanging-drop to induce embryoid body (EB) formation. After two days culture, the EBs were removed from the hanging-drops, and maintained in ES culture medium for additional two days. The EBs incorporated with GFP^+ rHOCs were then cultured in the ES differentiation medium on an adhesive culture dish to induce differentiation.

4.2.12 Neural Induction of Hepatic Oval Cells by Implanting into and Explanting out of Neonatal Mouse Brain

Freshly isolated mGFP^+HOCs were grafted into the lateral ventricles of neonatal mouse brains as described in 3.2.5. Ten days post-transplantation, the mouse brains were processed to generate neurospheres. Using inverse fluorescence scope, GFP^+

neurospheres were selected for further culture. After growing into about 100μm in diameter, the GFP^+ neurospheres were induced to differentiate by growing on laminin coated cover slips.

4.2.13 Neural Induction Using 5-Azacytidine

Rat HOCs were replated in a 35mm 6-well plate with a 60% confluence one day before the induction. Cells were then treated with 10μM 5-Azacytidine mixed in IMEM supplemented with 10% FBS containing 50ng/ml of NGF, BDNF and NT-3 for 96 hrs. After induction, the medium was replaced with N2-supplemented DMEM/F12 containing 50 ng/ml of NGF, BDNF and NT-3.

4.3 Results

4.3.1 IBMX /dbcAMP Treatment to the HOC Causes Neural-like Morphological Change

After two days treatment in IBMX /dcAMP, rHOCs had dramatic morphological change from fibroblast-like to neural-like (Fig 4-1A). This phenomenon has been observed in stromal cells derived from bone marrow (Deng *et al.*, 2001). However, immuno-labeled with neural specific proteins, rHOCs were negative for most of the markers except βIII tubulin and S100 (Fig 4-1B). The Expression of βIII tubulin was also limited to very small portion of the cells, which included rHOCs with neural-like as well as non-neural-like cells.

4.3.2 BME/BHA Does not Induce Neural-like Change in HOC

BME/BHA have been used to induce the bone marrow derived stem cells into neural differentiation (Woodbury *et al.*, 2000; Black & Woodbury, 2001). After about two weeks treatment, significant morphological change was observed in the rHOCs (Fig

4-2A). When tested with neural markers, rHOCs were not stained positive for any of the markers including βIII tubulin (Fig 4-2B).

4.3.3 4+/4- Retinoic Acid Protocol Does not Induce Neural Differentiation in rHOC

To test the neuronal trans-differentiation potential of HOCs, a previously reported 4+/4- RA treatment protocol was used. This protocol has been shown to be affective to induce neuronal differentiation in various cell lines including embryonic cells (Bain *et al.*, 1996). For induction, rHOC aggregations were induced to form by scratching the surface of a confluent HOC monolayer (Fig 4-3A). However, neither the morphological change nor the neural marker expression was observed in the rHOCs that have been treated with the protocol (Fig 4-3B).

4.3.4 Over-Expressing Chordin and Noggin Does not Induce Neural Differentiation in rHOC

Rat HOCs were transfected with two plasmid constructs containing transcription factors chordin and noggin that are responsible for the embryonic nervous system development, following Kohyama *et al.* (Gratsch & O'Shea, 2002). Despite that the cells transfected with noggin and chordin had different morphology from cells with plain vector control (Fig 4-4A, B, C), no neuronal marker expression was observed in the cells in the subsequent immunocytochemistry (Fig 4-4D).

4.3.5 Co-Culturing with Differentiating Neurospheres Does not Induce Neural Differentiation in HOC

Rat HOCs were co-cultured with the differentiating NSs, under the rational that the secreted growth factors or chemockines by the NSs would recourse the differentiation of rHOCs. After five days of co-culture, we observed limited morphological change of rHOCs labeled with green fluorescent protein (GFP) (Fig 4-5A). The cells were also

positive for α-internexin, a protein reported to be highly up-regulated in regenerating neurons (Fig 4-5B) (Evans *et al.*, 2002). The cells were not positive, however, for the immuno-staining of other neural specific proteins including NFM, βIII tubulin, Map2, GFAP.

4.3.6 Internalization of HOC with Neuropheres Contribute Little to HOC Neural Differentiation

Neurospheres have been used to test various theories of neural stem cell (NSC) differentiation, and have been intensively studied in the past decade (Laywell *et al.*, 1999; Kukekov *et al.*, 1999). The outer layer of the NS contains partially committed progenitors and differentiated neural cells, while the center of the NS is believed to accommodate truly multipotent NSCs. There are rich growth factors to keep the NSC pool in the core area in the NSs. We hypothesized that the core of the NS is able to de-differentiate HOCs, and the neural differentiation condition of the NS after plated on a laminin coated substrate would drive HOCs into neural differentiation. To test this hypothesis, approximately ten GFP^+ rHOCs were injected into a NS with 50-100μm in diameter (Fig 4-6A). However, the experimental system proved to be a failure at the early stage, because of the poor survival of GFP^+ rHOCs inside of the packed NS core. Alternatively, the GFP^+ rHOCs were incorporated into the NS by co-culturing them with NSs in non-adhesive culture dish (Fig 4-6B). During five days incorporation co-culture period, the number of HOCs were seen to expand dramatically, but the subsequent differentiation induction yield no GFP+ neural cells judged by morphological criteria (data not shown).

4.3.7 Internalization of HOCs into the Embryoid Body Does not lead to Neural Differentiation of HOCs

Hybrid embryoid bodies (EBs) of ES cells and GFP^+HOCs were generated by hanging-drop culture system (Fig 4-7A). However, despite the vigorous differentiation of ES cells within the hybrids to become various of tissues, including cardiomyocytes that exhibit synchronized pulsing, GFP^+HOCs remained unchanged morphologically from normal culture state (Fig 4-7B). The immuno-phenotyping of the differentiated hybrid EBs reveals no GFP^+ neural cells, while non-GFP neural lineage derived from ES cells has been observed.

4.3.8 Brain Tissue Transplantated with $GFP^\pm$ HOC Generate $GFP^\pm$ Neurospheres

Under the same rational of the previous experiments (see 4.3.6 and 4.3.7), we further test the de-differentiation and re-differentiation theory by grafting the GFP^+ mHOCs into the neonatal mouse brain, and generating NSs from the subventricle zone of the recipient brain (Fig 4-8A). In this system, HOCs were given the chance to live in the neural stem cell niche at the SEZ of the neonatal mouse brain, a region called ‘brain marrow’ (Steindler *et al.*, 1996) and believed to contain the NSC pools even in the adult animal. Ten days after transplantation, GFP^+ NSs were generated following typical neurosphere generating protocol (Fig 4-8B, C). However, after induced for differentiation by plating on laminin coated substrate, the differentiated cells at the outer rim of the NSs lost the GFP expression, while the core, where undifferentiated facultative NSCs reside, maintain the GFP expression (Fig 4-8D). Immunophenotyping showed that these GFP^+ NSs generated both neurons and astrocytes (data not showing).

4.3.9 Hepatic Oval Cells Survive Poorly in the Organotypic Brian Slice Culture

A key prerequisite for transplant-based neural repair strategies is the functional interaction of the grafted cells with host neurons and glias. Conventional transplant strategies provide only limited experimental access to donor cell migration, integration, and function. The organotypic brain slice culture system provides a direct visualization and functional analysis of transplanted GFP^+ HOCs. However, it was found that mHOCs survived poorly on the 350μm brain slices (Fig 4-9A, B) in our study. By day10 post-graftment, no GFP^+ mHOCs were observed in the slices processed from E15.5 mouse embryo, as well as adult animals (Fig4-9C).

4.4 Conclusion and Discussion

Although engraftment of HOCs into the neonatal mouse brains has shown neural trans-differentiation of HOCs, the *in vitro* inductions tested in this study showed little sign of trans-differentiation (Deng *et al.*, 2003). This difference may be explained by the complexity of signaling molecules involved in the cell lineage determination *in vivo*, which is difficult to be recapitulated *in vitro*.

The use of chemical reagents such as IBMX, dbcAMP, BME and BHA has indeed induced neural morphological acquisition in HOCs, as has been reported in the MSCs after treated with similar induction protocols (Woodbury *et al.*, 2000; Deng *et al.*, 2001; Black & Woodbury, 2001). But as has been shown in the previous chapter, same effect was also observed when using same protocol in NIH3T3 fibroblast cells. Similar to what has been shown in NIH3T3, there is little to no sign of neural specific protein upregulation from HOCs under the induction of IBMX/dbcAMP and BME/BHA protocols, including the astrocyte specific GFAP that has been upregulated in response to

the IBMX/dbcAMP treatment in BMDCs (see Chapter 2). The similar response to the so-called ‘chemical induction’ exhibited in the BMDCs and HOCs, along with the observation from NIH3T3, may further support previous conclusion that the neuron-like morphological change under such treatment is the result of cell stress, rather than *true* neuronalization (Lu *et al.*, 2004; Neuhuber *et al.*, 2004).

The use of RA to induce neuronal induction is effective in the ES cells (Bain *et al.*, 1995; Shum *et al.*, 1999), but has been ineffective in driving HOCs toward neural phenotype lineage *in vitro*. RA is an important growth factor for neural determination during the embryogenesis. The HOCs are progenitors of hepatocytes and cholangiocytes in the liver of adult animals. It is, therefore, not surprising that they are not responsive to the RA. Further work to exam the existence of RA receptors on the HOCs cell surface would be helpful to interpret the outcome. *Chordin* and *noggin* are proneural genes that can drive neuroplate formation from ectoderm by inhibiting BMP-4 signaling (Hemmati-Brivanlou & Melton, 1997). Proteins encoded by these genes have been shown to been effective to drive ES cells to become neurons *in vitro* (Gratsch & O'Shea, 2002). It has also been reported that they could promote neural differentiation in MSCs (Kohyama *et al.*, 2001). Retinoic acid binds to and activates transcriptional regulators of the nuclear receptor family, and noggin and chordin act as transcription factors themselves in the nucleus that regulate a set gene expressions at the early stage of the development to control the neurogenesis. The non-responsiveness of HOCs to RA, chordin and noggin may collectively reflect the different multipotency between HOCs and MSCs, and suggesting that HOCs are more determined than the ES cells and MSCs.

The limited effect of neurosphere co-culturing in guiding HOCs toward neural-like morphology through may suggest a preliminary neural induction effect of neural stem cells (Fig 4-5). Co-culture with neural stem cells has been widely used to provide an *in vitro* neural induction environment that is impossible to completely recapitulated by synthetic culture medium (Llado *et al.*, 2004). Alpha-internexin has been reported to associate with neuro-regeneration (McGraw *et al.*, 2002; Evans *et al.*, 2002). The expression of α-internexin in HOCs seems suggest that there is indeed a neuro-differentiation of HOCs through co-culturing with the neural stem cells. However, the fact that only α-internexin, but not other neural specific proteins has been detected in the GFP^+ rHOCs weakens this possibility.

That GFP^+rHOCs lived poorly, and eventually died inside the neurospheres through microinjection may be caused by the oxygen and nutrient deprivation at the center of the densely packed cells (Fig 4-6A). However, the healthy growth of HOCs inside of the neurospheres through spontaneous incorporation argues against this suggestion (Fig 4-6B). Rat HOCs also exhibit normal viability of inside embryoid bodies, which further indicates that the HOCs died of other causes after micro-injected into the neurospheres(Fig 4-7). The colony formation of stem cells may be one of the unique properties that have been observed in different types of stem cells. The NSCs develop into neurospheres, while colony-forming units (CFU) have been used to isolate mesenchymal stem cells regularly (Short *et al.*, 2003). Hepatic oval cells will also aggregate when a confluent monolayer culture is maintain unpassaged over long period of time (unpublished observation). It may be speculated that the tight cell-cell contact creates a unique condition for stem cell maintenance. However, the failure of HOCs to

respond to the internal environment of EB and NS may suggest that HOCs are not 'plastic' enough, but it may also imply that stem cell aggregates, generated either by clonal growth (e.g. NS) or cell aggregation (e.g. EB), do not create an *in vivo*-like condition through cell - cell contact. Further study to exam these cell - cell contact signaling in those aggregates is of interesting to understand the nature of the colony forming property of stem cells.

The generation of GFP^+NS from young mouse brains after grafted with GFP^+HOCs may suggests that hepatic oval cell experienced a de-differentiation to re-differentiation process to become a neural stem cells. It is an important and significant demonstration of the plasticity of HOCs, as well as the *in vivo* induction condition. However, the alternative explanation of this result may be cell fusion. It is possible that the GFP^+ mHOCs fused with endogenous NSCs after grafted into the neurogenic lateral ventricles, and enable the NSCs to express GFP. The loss of GFP expression in the HOC derived neurospheres after being induced for neural differentiation may have resulted from the reprogramming of genetic materials during the differentiation. Future studies to use X, Y-chromosome painting or other evaluation methods to further resolve the issue would be necessary for understanding the plasticity modulation of HOCs in the lateral ventricles of the neonatal mouse brains.

The organotypic brain slice culture has been previously used to partially recapitulate the *in vivo* condition while allow *in vitro* manipulation (Benninger *et al.*, 2003; Scheffler *et al.*, 2003). However, the mHOCs survived poorly comparing to direct transplantation into the brain, and showed little sign of differentiation. The special medium that is required for maintaining the brain slices may have contributed to the poor

survival of HOCs, since HOC requires rich growth factors for normal maintenance. More tests may be needed to justify this technique in culturing HOCs successfully.

In summary, the *in vitro* neural trans-differentiate capability of HOCs is very restricted. Despite the variety of induction methods tested that have been shown to be effective to induce ES or MSCs to differentiate into neural lineage, we only observed limited effect in driving HOCs to differentiate into neural phenotype. As has been suggested previously that HOCs may have derived from bone marrow stem cells (Petersen *et al.*, 1999; Theise *et al.*, 2000), this sheer difference in terms of *in vitro* neural trans-differentiation potency between them clearly put HOCs behind MSCs in the differentiation potential hierarchy. Our results may not negate the *in vitro* neural trans-differentiation capability of HOCs, but they certainly demonstrate that HOCs are restricted hepatic progenitors.

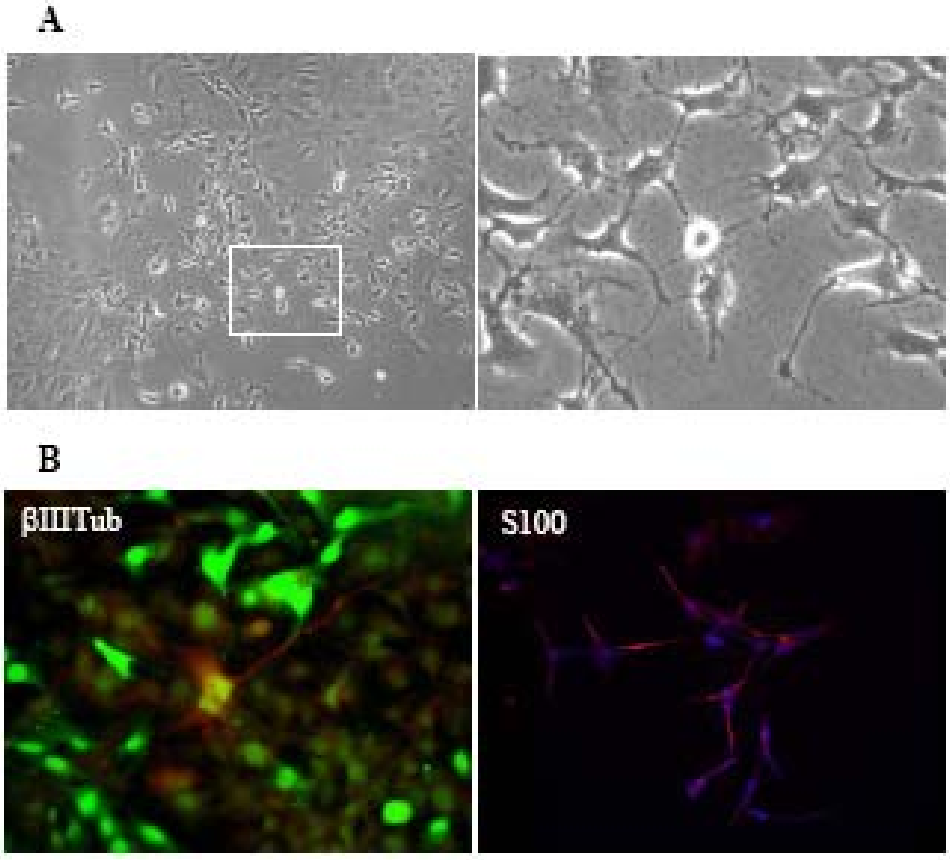


Figure 4-1. Neural induction of HOCs using Isobutyl-methylxanthine and dibutyryl cyclic AMP (IBMX /dcAMP). A) Oval cells undergo neuron-like morphological change after one day of treatment. Picture at the right is the enlarged images of the framed area in the left imagine. B) Immunostaining of neuronal marker βIII tubulin. Small number of cells shows the positivity (red); Cells has been induced with GFP expression vector (green). C) Immunostaining of astrocyte marker S100. Blue shows the nucleus counterstaining.

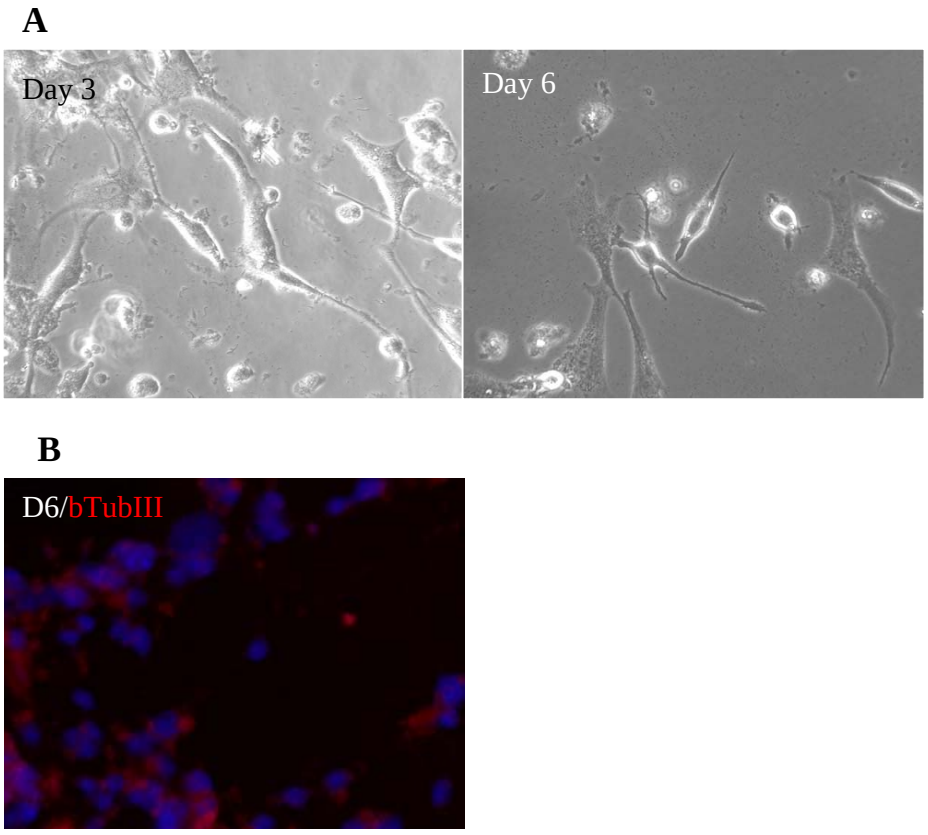


Figure 4-2. Neural induction of HOCs using Beta-Mercaptoethanol and Butylated Hydroxyanisole (BME/BHA). A) After 3 days treatment, elongated neuron-like cells can be observed. By day 6, more cells show neuron-like small cell body with long processes. B) Immunostaining of neuron specific protein βIII tubulin shows negativity (red). Nucleus has been counterstained with Dapi (blue).

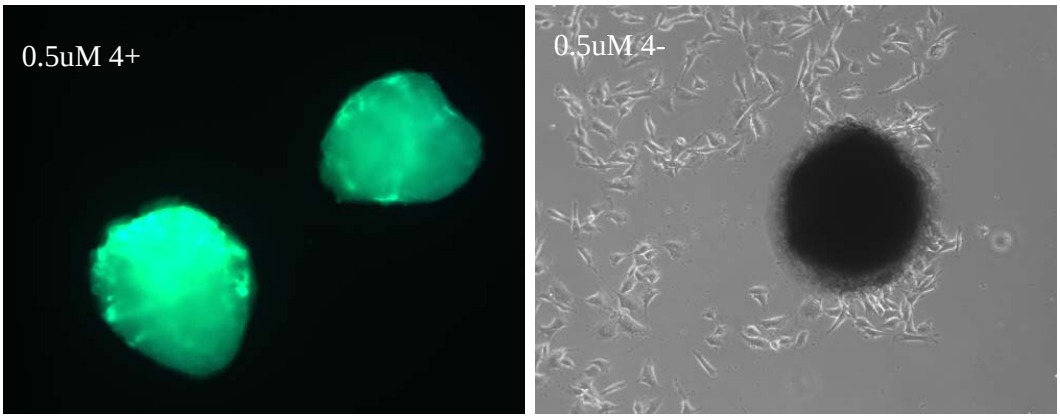


Figure 4-3. Neural induction of HOC using Retinoic Acid (RA) under 4+/4- Protocol. A) GFP^{+} HOC aggregates were treated with 0.5μM RA for four days. B) After culture the HOC aggregates in anti-adhesive petridish for four days, cells were plated on adhesive culture dish to induce differentiation.

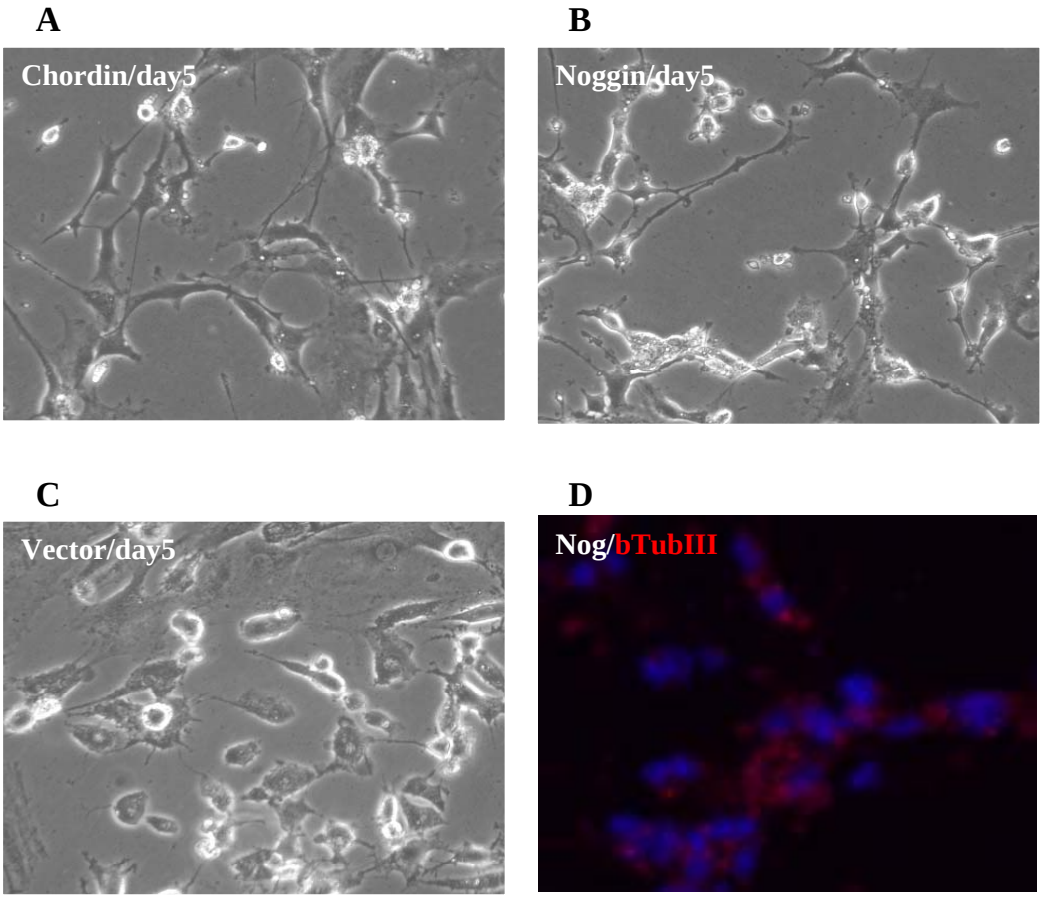


Figure 4-4. Neural induction of HOC by over-expressing chordin and noggin (CHD/NOG). Five days after rat HOCs were transfected with CHD (A) and NOG (B), small number of cells showed neuron-like morphology. There was no similar change in the null vector transfected cells (C). D) NOG transfected HOCs did not express neuron specific protein βIII tubulin.

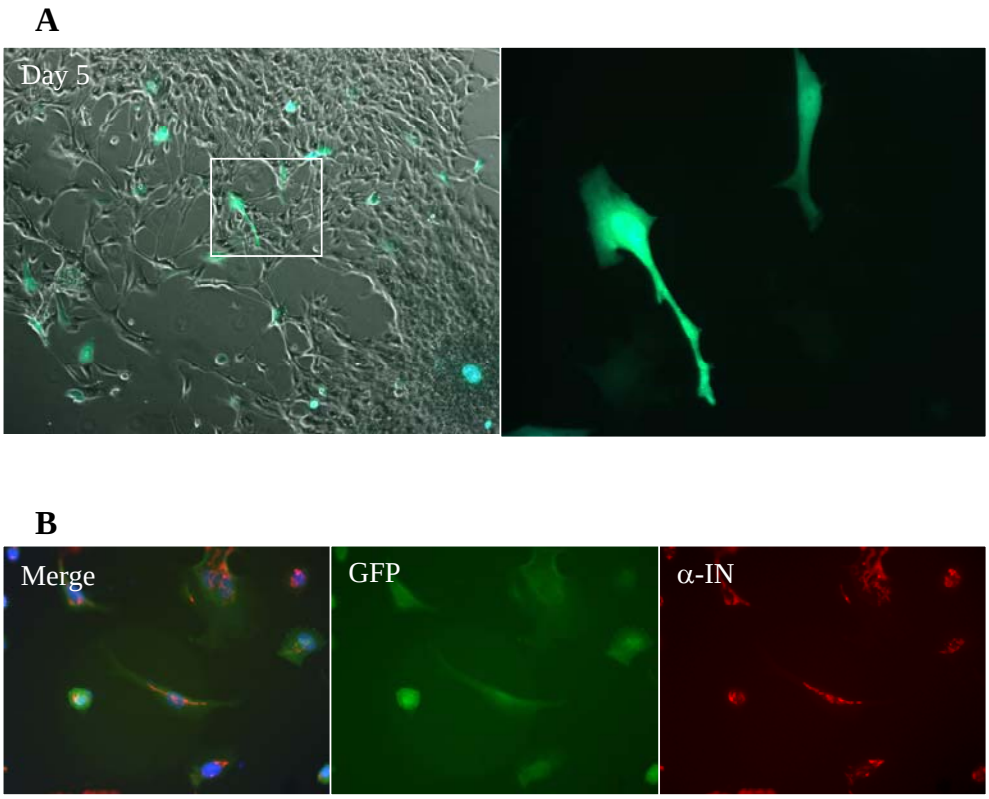


Figure 4-5. Neural induction of HOC by co-culturing with differentiating neurospheres. A) Small number of GFP^{+}HOCs form processes after five days co-culture with neural cells differentiated from NSs. Picture on the right shows the enlarged area in the inset on the left. B) Many cells express α-internexin (red) after co-culturing.

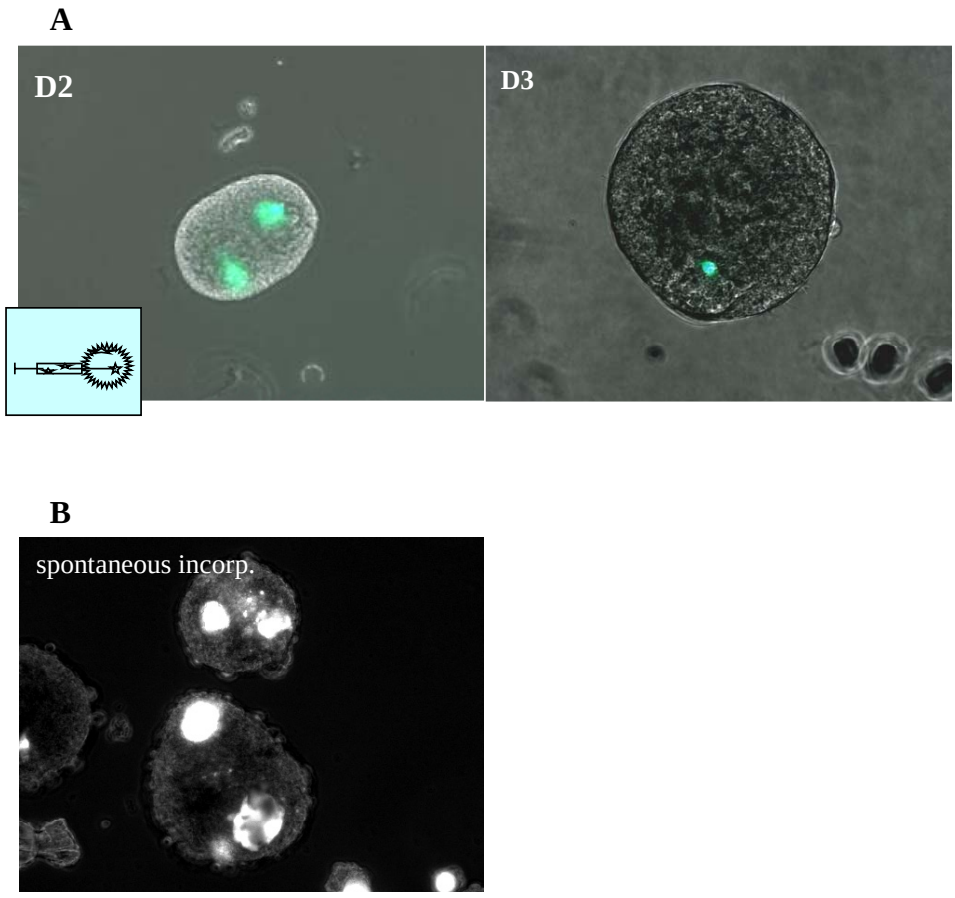


Figure 4-6. Neural induction of HOC by incorporating into the core of neurospheres. A) Micro-injection of GFP^{+}HOCs into the core of the NSs. HOCs survived poorly inside of NSs, disappeared from the NSs in about a week. B) Spontaneous incorporation of GFP^{+}HOCs into NS's resulted in fast growing HOC aggregates inside of NSs.

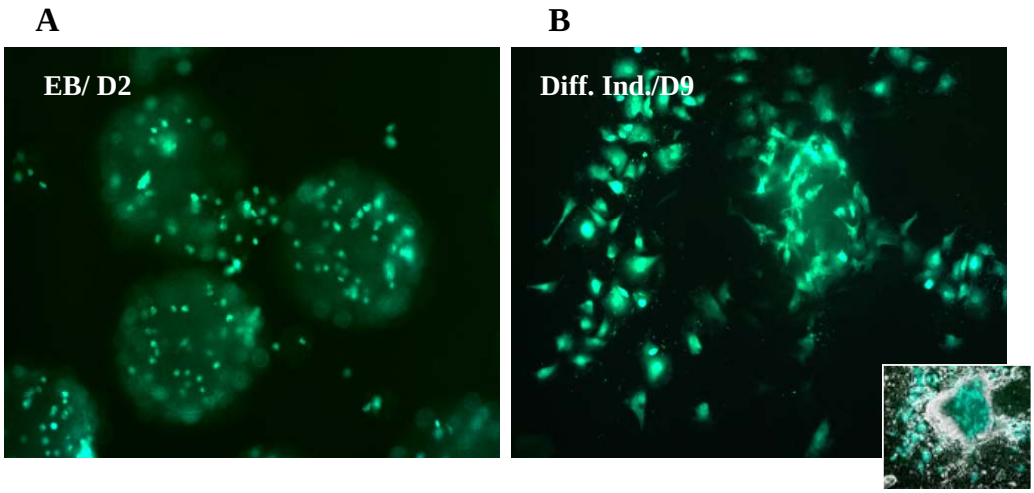


Figure 4-7. Neural induction of HOC by incorporating into embyoid bodies (EBs). A) Two days after plating the GFP^{+} HOCs incorporated EBs on to non-adhesive petridish. B) Nine days after inducing the EBs for differentiation. Morphologically, HOCs were not different from the normal culture condition.

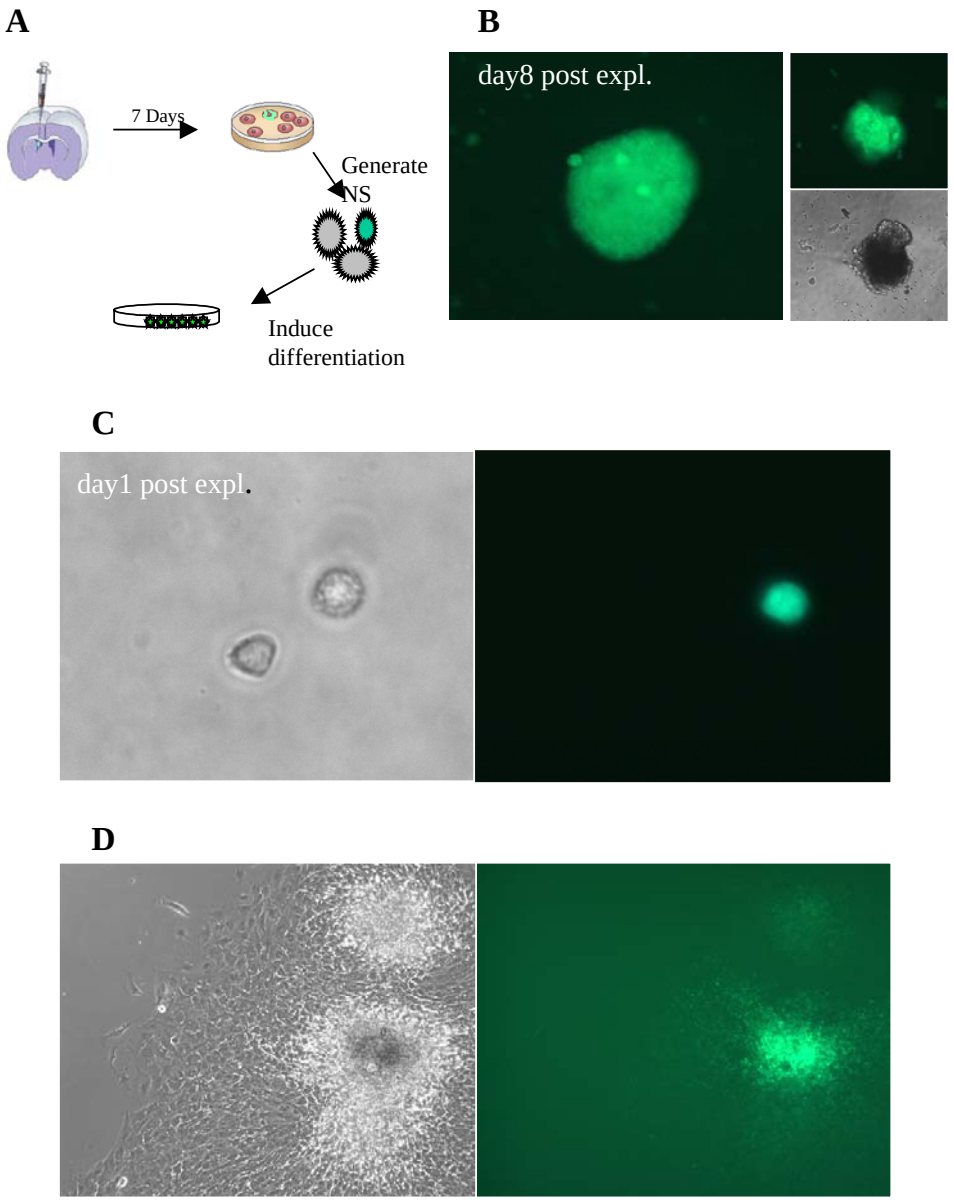
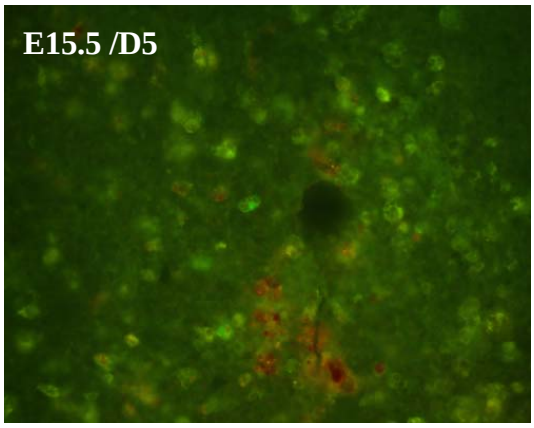
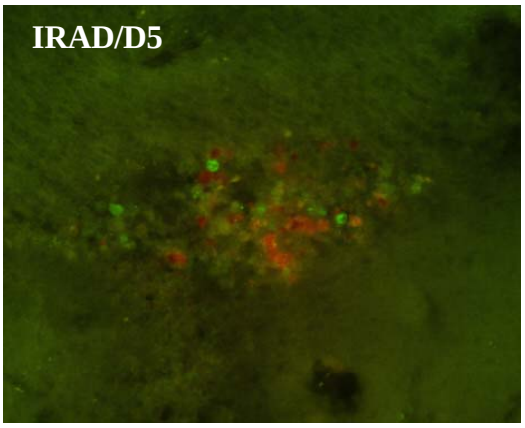


Figure 4-8. Neural induction of HOCs transplanting into and explanting out of the neonatal mouse brain. A) A schematic diagram of experimental procedure. B) Eight days post-explantation, GFP^+ NS's were observed in the culture. Two pictures on the right showing a GFP^+ NS fused with a GFP^- NS by florescent and phase-contrast imaging. C) A GFP^+ cell and a GFP^- at day 1 post-explantation. D) Differentiation of a GFP^+ NS and a GFP^- NS showing by phase-contrast (left) and florescent (right) imaging. The loss of GFP express is observed after cell differentiated and left the center of the GFP^+ core.

A



B



C

	Day5	Day10	Day15
E15.5 (n=3)	+	-	-
PU1-/- E15.5(n=3)	+	-	-
PN (n=4)	+	-	-
IRAD (n=3)	-/+	-	-
NormAD(n=4)	-/+	-	-

Figure 4-9. HOCs culture on organotypic brain slices. A) Five days after culturing HOCs on a brain slice processed from embryonic day 15.5 tissue. B) Five days after culturing HOCs on a brain slice from a sub-lethally irradiated, 8 weeks old mouse brain. C) A table to show the survival of HOCs on brain slices processed from different group of animals. '+' –fair survival; '-/+' –poor survival; '-' –no survival; E15.5 –embryonic day 15.5; PU1-/- E15.5 –embryonic day 15.5 tissue from PU1 knockout mouse; PN – postnatal; IRAD –sublethally irradiated 8 weeks mouse; NormAD –normal 8 weeks mouse.

CHAPTER 5
SUMMARY AND CONCLUSIONS

We have studied two types of adult stem cells, the bone marrow derived cells and the hepatic oval cells. There is a significant difference between these two types of AS cell in their capability to differentiate into neural cell type. While the BMDCs spontaneously generate neurogenic and astrogenic progenitors under normal culture, HOCs showed little sign of neural trans-differentiation capability *in vitro*. However, neurogenic subependymal zone (SEZ) provides strong induction incentives to stem cells, guiding both BMDCs and HOCs to express neural specific proteins after engraftment. Whether HOC or BMDC relate to each other or not is still a matter of debating, but what have been demonstrated in the current study show that BMDC is more potent, and probably more primitive than HOC.

Despite the doubts and disbelieves surrounding the trans-differentiation phenonmenon of adult stem cells, We have demonstrated that the two types of adult stem cell, BMDC and HOC, indeed showed neural trans-differentiation capability *in vivo* or *in vitro*. In particular, the bone marrow derived BMDCs show neural property spontaneously, exhibiting an asymmetric division to become neurogenic, and astrogenic progenitors, in addition to symmetric division to maintain the multipotent cell population. *In vivo*, both BMDC and HOC differentiated into neural lineage by expressing the neural specific proteins. Besides showing strong phenotypic and morphological characteristics of microglia, HOCs also exhibited vigorous phagocytosis capability, as did the functional

endogenous microglias in the brain. The BMDCs have stronger neural trans-differentiation potential than HOCs, showing neural progenitor-like migration along rastrol migratory pathway (RMS), and differentiate into morphologically and phenotypically mature granule cells in the olfactory bulb. Evaluated by Y-chromosome painting technique, it can be concluded that cell fusion event did not contribute to the neural trans-differentiation of BMDCs *in vivo*.

Although they both are adult stem cells, BMDCs and HOCs have contrast difference in the differentiation plasticity. MSCs have also been reported to differentiate into most of the lineages including brain, liver, heart, skeletal muscle kidney, pancreas, lung, skin, gastrointestinal tract (Herzog *et al.*, 2003). HOCs have only been reported to differentiate into pancreatic insulin-producing cells *in vitro* (Yang *et al.*, 2002), and neural phenotypes *in vivo* (Deng *et al.*, 2003), besides hepatic lineages. In the current study, we have not been able to show the neural differentiation capability of HOCs *in vitro*, while the BMDCs demonstrated neural property spontaneously under normal culturing by expressing variety of neural specific proteins. Besides the different potentialities showing in the *in vitro* induction, BMDCs also had stronger performance after grafted into the neonatal mouse brain than HOCs. BMDCs were shown to migrate along RMS and differentiating into granule cells at the olfactory bulb, but HOCs mostly penetrated and retained at SEZ. As suggested previously that hepatic oval cells may have generated from progenitors reside in the bone marrow (Petersen *et al.*, 1999; Theise *et al.*, 2000), the exact nature of this facultative progenitor remains unclear. Krause, *et al.* (2001) reported that hematopoietic stem cells (HSC) could be the source for bile duct epithelial cells in the liver, which may suggest a HSC origin of hepatic oval cells, since

the anatomical origin of oval cells was reported at the cannel of herring where bile duct terminated in the liver (Theise *et al.*, 1999). Besides the differentiation potential between them, BMDCs is more accessible than HOCs, which makes them a better source for the purpose of clinical application. HOCs may be observed in large quantity only under certain injuries, and have to be isolated through invasive hepatectomy. It's unlikely to treat the brain disorder of a patient in the expanse of his liver. The BMDCs, on the other hand, are more obtainable from multiple sources of a patient without risking his life, and can be extensively expanded in culture.

Although we have shown the perspectives of BMDCs as a possible source for neurological disorders treatment, many issues of the BMDCs have not yet understood for the application of this type of adult stem cells in clinical phase. The next step is to apply BMDCs, both induced or uninduced *in vitro*, into the animal models of different disease, such as the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD). In this step, MSCs could be either directly injected into the lesion sites alone, or engineered *ex vivo* to obtain functional units of the tissue before engraftment. The combination of stem cell therapy with gene therapy is also one of the alternatives currently under investigations in many laboratories, and has shown promising results (Kurozumi *et al.*, 2004). Li *et al.* (2001) delivered MSCs directly into the striatum of the MPTP mouse brain, and observed moderated reduction of PD symptom of the injured mice. But they observed very few exogenous dopaminergic neurons at the grafted site. Zhao *et al.* (2002) used a ischemic rat brain model to test their MSCs in repairing the damaged cortex, and also observed behavior improvements in limb placement test. Kurozumi *et al.* (2004) injected brain derived neurotrophic factor (BDNF) expressing

MSCs into a rat stroke model and observed functional recovery and reduced infarct size at the site of lesion. Lu *et al.* (2005) also used MSCs that have been transduced to express BDNF in a spinal cord injury model, and observed a significant increase in the extent and diversity of host axonal growth. All these work contribute significantly to the next phase of MSC research (Kurozumi *et al.*, 2004).

In summary, BMDCs are shown to be a promising candidate for the potential use in treating neurological disorders. With additional effort in testing the effect of this cell type in the animal model of various neurodegenerative diseases, their potential therapeutic value will come clear. In spite of the neural trans-differentiation in the neonatal mouse, HOCs are not likely to be developed into a therapeutic agent in treating brain disorders. However, the HOC trans-differentiation demonstration has largely increased our understanding of the neurogenicity potential of SEZ in guiding an endodermal progenitor cells into neural differentiation. Utilizing the large quantity of the uncultured HOCs that can be obtained form animal, additional experiments may be used to test the mechanistical aspect of the neurogenicity property of SEZ.

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BIOGRAPHICAL SKETCH

Jie Deng was born in Sichuan Province, the People's Republic of China, on March 13, 1967 (lunar calendar). From 1979 to 1984, he attended high school at the Fushuan No. 2 Middle School in Sichuan. In 1985 Jie began his college education at the SiChuan University in Chengdu, SiChuan. He was awarded a Bachelor of Science degree in zoology in 1989. From 1990 to 1993, Jie worked at National Bird Banding Center of China, serving as biologist. From 1994 to 1995, Jie worked at CITES Management Authority in China, serving as wildlife officer. In 1996, Jie was accepted by the graduate program of Wildlife Ecology and Conservation Department at the University of Florida to work on the master's degree, and graduated in May 1998. He then joined the Interdisciplinary Program (IDP) of the University of Florida, College of Medicine, to work on his Ph.D. degree in 1999.