2006

Characterization of neuroblastoma stem cells

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Characterization of Neuroblastoma Stem Cells

Submitted by

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In partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Medical Sciences

Date of Defense:

March 30, 2006

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CHARACTERIZATION OF NEUROBLASTOMA STEM CELLS

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MEDICAL UNIVERSITY OF OHIO

2006
DEDICATION

I would like to dedicate this dissertation to my parents, Qingxiang Ma and Guirong Ma, and my wife, Ying Wang.
ACKNOWLEDGMENTS

I would like to sincerely thank my mentor, Dr. Han-Fei Ding, for his support and guidance throughout my graduate school years. I am also grateful to all my committee members, Drs. Dorothea L. Sawicki, William Anthony Maltese, Kam C. Yeung, and Zijian Xie, for their encouragement and advice. A special thanks goes to Dr. Hongjuan Cui for her great help in my experiments. My appreciation is extended to Dr. Sheila Banerji, Goleeta Alam and Jane Ding for their helpful advice. Last but not the least, I wish to express my gratitude to my wife, Ying Wang, for her love and encouragement.
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INTRODUCTION

A controversial issue in cancer biology today concerns the mechanisms regulating the ability of individual cells in a tumor to form a new tumor (i.e., to have tumor-initiating or self-renewal properties). Rather than being homogeneous and composed of identical progeny of a single tumor cell, it is known that tumors consist of both various differentiated cells and a few immature cells, as determined by their expression of differentiation markers (Auersperg et al., 1989; Adriaansen et al., 1993; Sutherland et al., 1996; Blair et al., 1997; Grabowski, et al., 2004). Further studies show that the differentiated tumor cells are derived from the rare population of immature tumor cells (Blair et al., 1997; Tokar et al., 2005). While the differentiated tumor cells lack the self-renewing ability or tumor-initiating capacity, the immature tumor cell population is able to drive the formation of new tumors consisting of all the cell types in the tumor of origin, including immature cells and heterogeneous populations of differentiated cells.

These findings lead to the cancer stem cell model. This model suggests that tumor cells are heterogeneous, among which only a small cell population has the tumor-initiating capacity (Hamburger and Salmon, 1977; Sutherland et al., 1996; Blair et al., 1997). Within a tumor, only the tumorigenic tumor cells possess self-renewing and differentiating activities, just like normal stem cells that are able to develop new tissues, and thus are called tumor stem cells or cancer stem cells (Reya et al., 2001). Results from several studies suggest that this model readily explains the cell heterogeneity in the tumorigenicity observed in a tumor, and cancer stem cells have been identified from
various human tumors (Lapidot et al., 1994; Bonnet and Dick, 1997; Holyoake et al., 1999; Al-Hajj et al., 2003; Singh et al., 2003, 2004; Matsui et al., 2004; Hotfelder et al., 2005). This cancer stem cell model has broad implications for the biological study of tumor genesis, progression and metastasis as well as the clinical diagnosis and treatment of tumors. Elimination of the cancer stem cells can cure patients with cancers, while their evasion from therapies will allow the reconstitution of tumors even if all non-tumorigenic cancer cells are killed.

Neuroblastoma is a common malignant tumor of infants and children that develops in the sympathetic nervous system. Neuroblastoma represents 8-10% of all childhood malignant tumors and is responsible for 15% of cancer-related deaths in the pediatric population in the United States. An interesting characteristic of human neuroblastoma is that in nearly every tumor, there is a mixture of three different types of cells that are the neuroblastic N-type cells, non-neuroblastic, substrate-adherent S-type cells, and I-type cells that display an intermediate morphology between N- and S-type cells (Biedler et al., 1999; Ross et al., 1995, 2002). All the three types of neuroblastoma cells have been cloned from established cell lines. It was found that I-type neuroblastoma cells could be induced to differentiate into N- or S-type cells (Ross et al., 1995). Furthermore, compared to N- and S-type cells, I-type neuroblastoma cells have much higher colony-forming ability in soft agar assays, which suggests that I-type cells have much higher tumorigenic ability than N- and S-type cells. These findings suggest that a subpopulation of I-type cells may represent cancer stem cells in neuroblastoma. In this study, BE(2)-C I-type neuroblastoma cells have been characterized and a subpopulation
of BE(2)-C I-type cells that possess cancer stem cell-like activities, including
differentiation, self-renewal, and clonogenicity, have been identified.

Having observed cancer stem cell-like activities in a subpopulation of I-type
neuroblastoma cells, I attempted to isolate neuroblastoma stem cells from the BE(2)-C
cell line. A common method to isolate the cancer stem cell population is to employ the
fluorescence-activated cell sorting (FACS) for cells expressing cancer stem cell specific
surface marker(s). The selection of specific surface marker(s) is critical for the success of
this approach. In this study, I have focused on two groups of surface markers. Because of
the similarity between normal stem cells and cancer stem cells, the first group of markers
that were selected are normal crest stem cell specific surface markers, such as p75, HNK-
1 and NCAM. The second group of markers that were selected are those associated with
high tumor malignancy, such as GD2 and CD24 in neuroblastoma. Among all of the
markers, it has been shown that CD24 positive BE(2)-C I-type neuroblastoma cells have
very high clonogenicity. This means that compared to the whole population of I-type
cells and CD24 negative cells, CD24 positive cells may be more tumorigenic and CD24
may be one of the specific markers of neuroblastoma stem cells. However, the difference
between the whole population of I-type cells and CD24 positive cells in clonogenicity
was only three-fold. Thus, in future studies, more specific marker(s) are needed to isolate
a relatively pure population of neuroblastoma stem cells. Nevertheless, my investigation
identified CD24 as a useful marker for the enrichment of neuroblastoma stem cells.

The key role of cancer stem cells in the formation and maintenance of tumors led
me to further study the molecular mechanisms underlying activities of neuroblastoma
stem cells. A major question that I tried to address is what determines the tumorigenic capacity of neuroblastoma stem cells. According to the cancer stem cell model, the self-renewal capacity of cancer stem cells is responsible for their tumorigenicity. A growing number of studies have demonstrated classical oncogenes can play a role in the regulation of self-renewal in both normal stem cells and cancer stem cells. These oncogenes included Wnt, STAT, Notch, Shh and Bmi-1 (Austin et al., 1997; Karanu et al., 2000; Spink et al., 2000; Bhardwaj et al., 2001; Kiger, et al., 2001; Taipale and Beachy, 2001; Tulina and Matunis et al., 2001; Varnum-Finney et al., 2001; Calvi et al., 2003; Lemischka and Moore, 2003; Lessard and Sauvageau, 2003; Molofsky et al., 2003; Reya et al., 2003; Tohdai et al., 2003; Zhang et al., 2003; Dontu et al., 2004; Duncan et al., 2005). Among them, Bmi-1 was of particular interest. As a member of the polycomb family, Bmi-1 has been shown to be involved in the formation and progression of various types of tumors (Hanson et al. 1999). Berns and colleagues (van Lohuizen et al., 1991) found that Bmi-1 could collaborate with c-Myc in tumorigenesis. Importantly, amplification of N-myc, a homolog of c-Myc, is observed in 25% – 30% of patients with neuroblastoma. More interestingly, in a mouse model, Morrison and colleagues (Molofsky et al., 2003) showed that Bmi-1 is required for the self-renewal of neural crest stem cells. As far as we know, neuroblastoma comes from neural crest stem cells or their derived cells in the peripheral nervous systems. Together, these findings suggest that Bmi-1 may be implicated in the regulation of neuroblastoma stem cell activities. In this study, the level of Bmi-1 has been shown to correlate with the stemness of neuroblastoma stem cells. I found that only the intermediate level of Bmi-1 was able to maintain the
stemness of neuroblastoma stem cells, whereas high and low levels of Bmi-1 promote their differentiation into neuron-like and Schwann cell-like tumor cells, respectively. These results shed light on the molecular mechanisms underlying the pathogenesis of neuroblastoma, which may lead to the development of better therapeutic strategies for neuroblastoma.
Cancer Stem Cell Model

A fundamental question in cancer biology is how tumor growth is initiated and maintained at the cellular level. Transplantation assays are used to determine the tumor-initiating ability of various types of cancer cells, during which a certain number of cancer cells are transplanted into experimental animals, usually nude mice or immunodeficient mice. It has been observed that injection of more than $10^6$ cancer cells is usually required for initiating new tumor growth in mice. This finding raised the question of why so many cancer cells are needed to initiate tumor growth. In other words, why does not every cancer cell within the cancer have the tumor-initiating ability? Two models have been proposed to address the question (Reya et al., 2001; Dick, 2003). The stochastic model suggests that every cancer cell is able to initiate new tumor growth, but whether it can do so *in vivo* is determined by some stochastic events with a low probability of occurrence, such as survival from the body defense system (Figure 1). Thus, under a given condition, only a subpopulation of cancer cells can form new tumors. The stochastic model proposes that cancer cells are homogeneous in their ability to form tumors. However, experimental evidence appears to contradict the model. For example, two different neuroblastoma cell lines, SH-SY5Y and SHEP1, were established from the same tumor of a neuroblastoma patient. It was found that injection of $10^6$ SH-SY5Y cells could induce tumor formation in immunodeficient mice while injection of more than $10^7$ SHEP1 cells failed to do so. Thus, different cell types within a tumor vary in their ability to initiate new tumor
All or most cancer cells are homogeneous in tumor-initiating ability, and stochastic events with a low probability of occurrence decide whether they can really form new tumors. A curved block arrow indicates that the cell has self-renewing capacity. Adopted from Reya et al., (2001) Nature, 414, 109.
growth. These and other findings lead to a new model. This model predicts that tumor cells are heterogeneous in tumor-initiating ability and only a few of them have the capacity to unlimitedly divide and form new tumors. This model accounts for the distinct tumor-forming abilities of different cancer cell lines established from the same tumor. The more tumor-initiating cells a cancer cell line contains, the fewer cancer cells are needed to reconstitute tumors in immunodeficiency mice in transplantation assays. If a cancer cell line consists of only non-tumorigenic cells, no new tumors can be produced in mice, no matter how many cancer cells are injected. This model inspired scientists to further explore the features of the limited population of tumorigenic cancer cells. With a clonal origin (Daschner, et al., 1997; Quinn et al., 2001; Adam et al., 2005; Cheng et al., 2005; Jones et al., 2005), a tumor seems to be an abnormal tissue developed from a tumorigenic cancer cell reminiscent of the ability of a stem cell to give rise to a normal tissue. By functional definition, stem cells are primitive cells with the properties of indefinite self-renewal and multipotent differentiation (Weissman, 2000). Briefly, after a division, a stem cell can give rise to an identical stem cell, which is called self-renewal, while in the meantime it also can produce a more specialized cell, which is called differentiation (Figure 2). On one hand, the self-renewing capacity of normal stem cells enables them to generate new tissues. Since tumorigenic cancer cells have the similar ability to initiate abnormal tissues, tumors, they should have the similar self-renewing capacity. On the other hand, normal tissues contain phenotypically diverse cells differentiated from the original stem cells. Similarly, there are heterogeneous populations of cancer cells in
During differentiation, stem cells first give rise to various progenitor cells, which are able to further produce more mature cells. Red cells with a red curved block arrow are stem cells with self-renewing capacity, whereas pink, yellow and brown cells are progenitors and blue and yellow cells are terminally differentiated cells. Black straight arrows indicate differentiation, while red ones mean self-renewal. Adopted from Perez-Losada and Balmain, (2003) Nature Reviews Cancer, 3, 437.
different differentiation states and with different dividing potential, observed in a tumor derived from a tumorigenic cell. Thus, tumorigenic cancer cells have a stem cell-like differentiating ability (Auersperg et al., 1989; Adriaansen et al., 1993; Sutherland et al., 1996; Blair et al., 1997; Grabowski, et al., 2004). That tumorigenic cancer cells possess stem cell-like activities explained the similarities between the mechanisms underlying oncogenesis and those that regulate self-renewal and differentiation of stem cells. It has been shown that many classical tumorigenic pathways also may regulate normal stem cell development and activities. For example, it has been shown that activation of the oncogene Notch (Callahan and Raafat, 2001) can promote self-renewal of hematopoietic stem cells (HSCs) (Karanu et al., 2000; Varnum-Finney et al., 2001). More recently, Reya and colleagues observed that Notch signaling is much more active in HSCs than that in differentiated cells and their downregulation induces the differentiation of HSCs, further suggesting that the oncogene Notch contributes to the maintenance of the activities of HSCs (Duncan et al., 2005). Other signaling pathways implicated in oncogenesis, such as the Sonic hedgehog (Shh), Wnt, receptor tyrosine kinase (RTK), JAK-STAT and Smad signaling pathways, also are involved in the regulation of self-renewal and/or differentiation of stem cells (Austin et al., 1997; van Den Berg et al., 1998; Gat et al., 1998; Korinek et al., 1998; Zhu and Watt, 1999; Kiger et al., 2001; Bhardwaj et al., 2001; Sylvester and Longaker, 2004). Since tumorigenic cells are cancer cells with stem cell like activities including self-renewal and differentiation, they have been named cancer stem cells or tumor stem cells (Figure 3) (Hamburger and Salmon, 1977; Reya et al., 2001). The above new model to explain the different tumor-forming
Similiar to their normal counterparts, tumorigenic cancer stem cells are able to renew themselves and differentiate to give rise to cancer progenitors, which further generate “mature” cancer cells. Red cells with a red curved block arrow are stem cells or cancer stem cells with self-renewing capacity, whereas pink cells are progenitors or cancer progenitors and blue and green cells are terminally differentiated cells or “mature” cancer cells. Red straight arrows indicate self-renewal. Adopted from Reya et al., (2001) Nature, 414, 108.
abilities of cancer cells are called cancer stem cell model or tumor stem cell model (Figure 4). This model proposes that heterogeneous cancer cells vary in their tumor-initiating abilities, and only the rare cancer stem cell population has the capacities to self-renew and drive tumor formation, as shown in clonogenic assays and xenograft transplantation (Reya et al., 2001). In strong support of this model, accumulated evidence indicates that cancer stem cells exist in a number of types of tumors. Dick and his colleagues (Lapidot et al., 1994) provided the first evidence of cancer stem cells in 1994. From human acute myeloid leukemia cells, they isolated a group of leukemia cells expressing the specific surface marker of human hematopoietic stem cells, CD34⁺CD38⁻ (Terstappen et al., 1991). It has been shown that only this group of leukemia cells could initiate the acute myeloid leukemia in the NOD/SCID (non-obese diabetic, severe combined immunodeficiency) mice, while other groups of leukemia cells failed to generate any leukemia (Lapidot et al., 1994). Furthermore, from patients with breast cancer, Clarke and his colleagues identified and isolated a population of tumorigenic cells, CD44⁺CD24⁻/low cells, by flow cytometry (Al-Hajj et al., 2003). They demonstrated with limiting-dilution assays that injection of only 100 CD44⁺CD24⁻/low cancer cells was able to produce new tumors in immunodeficient mice, while injection of tens of thousands of the other populations of cancer cells could not initiate any new tumor, providing the first direct evidence for cancer stem cells in solid tumors. Recently, it was reported that in human brain tumors, only a group of tumor cells with the expression of CD133, a specific surface marker expressed by normal central nervous system stem cells (Uchida et al., 2000), have stem cell-like activities to generate tumor spheres containing cancer stem
Figure 4. Cancer Stem Cell Model

Tumor cells are heterogeneous and only the cancer stem cell population has the ability to self-renew and can initiate new tumors. Red cells with a red curved block arrow are cancer stem cells with self-renewing capacity. Red straight arrows indicate self-renewal or tumorigenesis. Adopted from Reya et al., (2001) Nature, 414, 109.
cells produced by their self-renewal as well as more “mature” cancer cells derived from their differentiation (Singh et al., 2003). It was demonstrated by xenograft assays that as few as 100 CD133⁺ human brain tumor cells caused a tumor in mouse brains, whereas $10^5$ CD133⁻ tumor cells failed to do so (Singh et al., 2004). Up to now, cancer stem cells have been identified in chronic myeloid leukemia, acute lymphoid leukemia, and multiple myeloma by their specific markers (Holyoake et al., 1999; Matsui et al., 2004; Hotfilder et al., 2005). At present, extensive efforts are being made to identify cancer stem cells in skin, lung, pancreatic, ovarian, prostate, and many other cancers (Kim et al., 1990, 2005; Morris, 2004; Bapat et al., 2005; Collins et al., 2005; Fang et al., 2005; Secko, 2005; Tokar et al., 2005). The cancer stem cell model is not only of great significance in basic cancer research, but also has important implications in cancer therapy. Currently, cancer cells are treated as if all of them possess the ability to unlimitedly self-renew and initiate new tumors, no matter how diverse they are in their functions. Among the cancer cells in a tumor, cancer stem cells usually only represent a minor population (Lapidot et al., 1994; Al-Hajj et al., 2003; Singh et al., 2003; Matsui et al., 2004), whereas most cells in a tumor cannot undergo indefinite self-renewal or produce new tumors. If cancer therapies were developed against cancer stem cells, patients with tumors could be cured, even though non-cancer stem cells evaded from the therapies. Unfortunately, almost all the present therapeutics have been selected based on their ability to rapidly reduce tumor volume. Since most cancer cells in a tumor are non-tumorigenic cells without the self-renewing ability, the shrinkage of a tumor usually means the death of cancer cells other than cancer stem cells. Indeed, it has been shown that it is more difficult for
chemotherapeutic drugs to kill stem cells than to eliminate more mature cells of the same tissue origin (Harrison and Lerner, 1991; Berardi, et al., 1995). The mechanisms responsible for the chemotherapeutic resistance of stem cells might include their relatively long cell cycle time, resistance to apoptosis, and strong expression of ABC transporters such as ABCG2/Brcp1 (breast cancer resistance protein 1) to reduce drug absorption (Berardi, et al., 1995; Bouwens and DeBlay, 1996; Peters et al., 1998; Domen et al., 1998; Terskikh et al., 2001; Zhou et al., 2001; Guan et al., 2003). Cancer stem cells may have similar properties (Guan et al., 2003). It has been shown by previous studies that leukemic stem cells with the CD34+CD38− phenotype were obviously more resistant to various chemotherapeutics such as daunorubicin and cytarabine than the other leukemia cells (Costello et al., 2000; Guzman et al., 2002). So, although most cancer cells are killed by normal cancer therapies, surviving cancer stem cells can still drive the formation, progression and metastasis of new tumors through their self-renewal and differentiation, which can explain why most therapies only transiently reduce tumor size but tumors can gradually grow back, and the lives of patients are not significantly extended (Lippman, 2000). The cancer stem cell model suggests that we should look for cancer stem cell-directed therapies for the cure of cancer patients (Figure 5).
Conventional therapies are designed against all the cancer cells, so drug resistance of cancer stem cells usually allow them to escape from therapies. Surviving cancer stem cells can self-renew and differentiate to reconstitute new tumors. On the contrary, cancer stem cell-directed therapies would eliminate cancer stem cells. Even if non-cancer stem cells survive, they do not possess stem cell-like activities to drive tumor formation, thus finally the tumor will disappear. Adopted from Reya et al., (2001) Nature, 414, 110.
Neural Crest Cells and Neuroblastoma

In this study, neuroblastoma was chosen as a model to identify cancer stem cells and clarify the molecular mechanisms regulating their stem cell-like activities. Due to the neural crest origin of neuroblastoma cells, important information about neural crest cells and several neural crest-derived progenitors needs to be introduced. During the evolution of vertebrates, the neural crest is lately developed as a transient population of cells, from which various important derivatives originate (LaBonne and Bronner-Fraser, 1998; Berry, 2002, 2003). Neural crest cells, separating from the closing neural tube, are derived from neural stem cells and have been identified by researchers (Mujtaba et al., 1998). Although neural crest cells are a transient population of cells, they possess both of the two activities of stem cells, and thus also are called neural crest stem cells (Morrison et al., 1997; LaBonne and Bronner-Fraser, 1998). Neural crest cells have self-renewing ability and can differentiate into a wide variety of cells with distinct phenotypes, including melanocytes, craniofacial muscle cells, and the neurons and glial cells in the peripheral nervous system (Bronner-Fraser, 1995; Berry, 2003) (Figure 6). Like other stem cells, neural crest cells give rise to completely mature progeny through more specialized progenitors. So far, some progenitors derived from neural crest cells have been characterized. It is very interesting that Schwann cell progenitors might just be glial progenitors in the peripheral nervous system (Mirsky and Jessen, 1996). Scientists have established a cell line clonally originated from a rat sympathoadrenal neuron progenitor (Vandenbergh et al., 1991), from which adrenal chromaffin cells and sympathetic neurons are derived. It has been suggested that there are multiple lineages of progenitors
Neural crest stem cells are multipotent stem cells. They have self-renewal and multipotent differentiation potentials to give rise to Schwann/glial cells, peripheral neurons, and many other different types of cells. Adopted from LaBonne and Bronner-Fraser, (1998) J. Neurobio. 36, 184.
in the enteric nervous system, which are generated from neural crest cells (Gershon, 1997). Differentiation-inducing assays of neural crest cells also indicate the existence of smooth muscle precursors and myoblasts (Shah et al., 1996).

As a malignant tumor developed in the sympathetic nervous system, neuroblastoma is the most frequently discovered pediatric extracranial solid tumor (Seeger et al., 1982; Eggert et al., 2001; Parham, 2001; Lonergan, et al., 2002; Bartolini et al., 2003; Kushner, 2004). Neuroblastoma usually occurs in the sympathetic nervous system-associated areas, among which the adrenal medulla is the most common site (Pashankar et al., 2005). A feature of neuroblastoma is its diverse clinical behavior and prognosis. On one hand, in some very young children with neuroblastoma, the tumor may spontaneously disappear or differentiate into a benign ganglioneuroma (Brodeur et al., 1992). On the other hand, older children with neuroblastoma usually die from rapidly progressive and metastatic tumors, no matter what intensive therapies have been applied (Matthay et al., 1999). The significant heterogeneity of human neuroblastoma cells accounts for the diversity of clinical behavior of tumors. Although the cellular origin of neuroblastoma cells remains elusive, it is generally believed that neuroblastoma comes from the neural crest (Alexander, 2000; Mora and Gerald, 2004; Tatli et al., 2004).

Within neuroblastomas, there are tumor cells with different neural crest lineage origins, such as neuroblasts, Schwann cells, smooth muscle cells, and melanocytes. In general, all the neuroblastoma cells can be classified into three different cell types. One type of neuroblastoma cells have neuroblast-like morphology with small and rounded cell bodies, characteristic neuritic processes, and pseudoganglion-like cell aggregates, which tightly
adhere to each other but loosely adhere to the substrate. They also exhibit other neuronal
features, such as neurotransmitter biosynthetic enzyme activities and expression of
opioid, muscarinic, and nerve growth factor receptors. Thus, they are called neuroblastic
type (N-type) neuroblastoma cells (De Clerck et al., 1987; Rettig et al., 1987; Sadee et
al., 1987; Tsokos et al., 1987; Ciccarone et al., 1989). On the contrary, another type of
neuroblastoma cell has a fibroblast or epithelial cell-like morphology with large and
flattened cell body, a big oval nucleus, abundant cytoplasm, and no neuritic processes.
They may possess characteristics of various neural crest-derived cells other than
neuroblasts, such as Schwann/glial cells, melanocytes, or smooth muscle cells (De Clerck
and Lee, 1985; Ross and Biedler, 1985; De Clerck et al., 1987; Rettig et al., 1987; Scarpa
et al., 1987; Tsokos et al., 1987; Ciccarone et al., 1989; Slack et al., 1992; Sugimoto et
al., 2000). Due to their highly substrate-adherent property, they are called substrate-
adherent type (S-type) neuroblastoma cells. Nearly all the S-type cells are not able to
form new tumors, so some scientists considered them as normal stromal cells coming
from neighbor tissues rather than real neuroblastoma cells. But results from several
studies have indicated that although N- and S-type neuroblastoma cells isolated from the
same patient exhibit significant differences in their phenotypes, both of them have the
same abnormal gene characteristics and a common progenitor origin (Ciccarone et al.,
1989; Valent et al., 1999; Mora et al., 2001), demonstrating the existence of S-type
neuroblastoma cells. Another type of neuroblastoma cells are intermediate between N-
and S-type cells in morphology and have some proporties of both of them, so called
intermediate type (I-type) neuroblastoma cells (Ciccarone et al., 1989). They have a N-
type cell-like round nucleus and S-type cell-like abundant cytoplasm. They attach intermediately to each other and to the substrate. In addition, they may express both N- and S-type cell-specific markers. Intriguingly, nearly in every single neuroblastoma, there is a mixture of these three types of cells. All the three types of neuroblastoma cells have the differentiating ability to give rise to more mature cells in the neural crest lineage. N-type neuroblastoma cells, resembling sympathetic neural progenitors, can undergo further differentiate into neuronal cells or neuroendocrine cells (Ross et al., 2002). S-type neuroblastoma cells seem to be the equivalents of various progenitors in the neural crest lineage except neuroblast progenitors, such as Schwann/glial progenitors, melanocyte progenitors, or immature smooth muscle cells (Slack et al., 1992; Sugimoto et al., 2000). More importantly, in vitro differentiation-inducing assays have demonstrated that I-type neuroblastoma cells can be induced to differentiate into N- or S-type neuroblastoma cells (Ciccarone et al., 1989; Ross et al., 1995). They appear to be neural crest stem cell-like neuroblastoma stem cells. These findings suggest that the I-type neuroblastoma cell line would be a powerful source for the isolation and study of cancer stem cells. Characterization and isolation of neuroblastoma stem cells will help to elucidate the molecular mechanisms of neuroblastoma tumorigenesis and develop new approaches to treat neuroblastoma.

**Identification of Cancer Stem Cells**

In this study, I attempted to identify cancer stem cells in I-type neuroblastoma cells. In principle, there are two methods to identify cell types. One is to define cell types
by their expression of lineage specific markers. The other is to morphologically or functionally identify cell types, such as by the detection of specific enzyme activities. Since cancer stem cells are undifferentiated cells without specialized physiological functions, the detection of specific markers is mainly used to identify them. However, due to their stem cell-like activities, cancer stem cells have some specific characteristics, which also can be used to identify them. Hogge and colleagues successfully identified cancer stem cells from patients with acute myeloid leukemia through cell cycle analysis since these leukemia stem cells have a slow-growing characteristic (Guan et al., 2003).

An important property of cancer stem cells in the nervous system is their ability to form a sphere-shaped cell cluster in suspension, called the tumor sphere, by which cancer stem cells in pediatric brain tumors and glioblastoma have been identified (Hemmati et al., 2003; Galli et al., 2004). One popular method is to isolate a putative stem cell population, called a side population, by the ability of cancer stem cells to exclude fluorescent dyes such as Hoechst 33342. Cancer stem cells in neuroblastoma and glioma have been enriched using this method (Hemmati et al., 2003; Kondo et al., 2004).

But the most popular and successful approach is to identify cancer stem cells by their specific surface markers. The common strategy is to isolate a population of putative cancer stem cells by their specific markers using fluorescence-activated cell sorter (FACS). The isolated cells are then analyzed for stem cell-like activities, i.e., multipotent differentiation and self-renewal potentials. Finally, these cells are examined for their tumorigenicity in animal models. The success of this approach depends on the selection of surface markers for the specific type of cancer stem cells. Complementary DNA
(cDNA) microarray has been employed to analyze the expression profile of a cell population. By comparing the gene expression profile of cancer stem cells with that of differentiated cancer cells or that of their normal stem cell counterparts, we can identify not only specific surface markers of cancer stem cells, but also differentiation and transformation related genes, helping clarify molecular mechanisms of differentiation and tumorigenesis. Microarray analysis has been implied in the studies of specific gene expression of stem cells (Kelly and Rizzino, 2000; Ramalho-Santos et al., 2002). However, although the application of microarray analysis is widespread, it is costly and highly condition-dependent. Microarray analysis of cells in different culture conditions would show significantly different gene expression patterns. The present identified surface markers of cancer stem cells all come from two sets of known antigens (Lapidot et al., 1994; Bonnet and Dick, 1997; Holyoake et al., 1999; Al-Hajj et al., 2003; Singh et al., 2003, 2004; Matsui et al., 2004; Hotfilder et al., 2005). One set of possible markers for cancer stem cells are the markers of normal stem cells and progenitors. Cancer stem cell model suggests that cancer stem cells may be derived from stem cells with proliferating mutations and/or progenitor cells with additional oncogenic mutations to regain their self-renewing potential (Reya et al., 2001; Al-Hajj and Clarke, 2004). So, cancer stem cells probably maintain the specific surface markers expressed on the same type of normal stem cells and progenitors. It has been demonstrated that cancer stem cells in acute and chronic myeloid leukemia possess the same markers as hematopoietic stem cells, CD34+CD38- (Terstappen et al., 1991; Lapidot et al., 1994; Bonnet and Dick, 1997; Holyoake et al., 1999). In addition, Dirks and colleagues (Singh et al., 2003) found that
cancer stem cells in several types of brain tumors have the same marker, CD133, as neural stem cells (Uchida et al., 2000). Since neuroblastoma comes from neural crest stem cells or their progenitor progeny, neuroblastoma stem cells may express neural crest stem cell-related surface markers, such as low-affinity nerve growth factor receptor (NGFR) p75, HNK-1 and polysialic acid-neural cell adhesion molecule (PSA-NCAM) (Mujtaba et al., 1998; Gage, 2000; Lee et al., 2002). On the other hand, because cancer stem cells may originate from progenitor cells with mutations that activate different self-renewal pathways, they may gain various markers expressed on different types of normal stem cells and progenitors. Thus, specific markers of neuroblastoma stem cells could be selected from markers expressed by other types of stem cells and progenitors. There are many markers commonly expressed by most stem cells since they all possess similar self-renewal and differentiation potentials. For example, c-kit, a marker of hematopoietic stem cells, also is expressed by neural crest stem cells, neural stem cells and melanocyte precursors (Wehrle-Haller, 2003; Walton et al., 2004). Another example is CD133, which is expressed by multiple stem cell populations including hematopoietic stem cells, neural stem cells, skin epidermal stem cells, and prostate cancer stem cells (Uchida et al., 2000; Yu et al., 2002; Collins et al., 2005). The other set of possible markers for cancer stem cells are the markers related to tumor progression and metastasis. According to the cancer stem cell model, only cancer stem cells are self-renewing and tumorigenic. So the malignancy of tumors, characterized by rapid progression and metastasis, is associated with the number of cancer stem cells within tumors. Based on the reasoning that malignancy-related tumor markers could be the markers of cancer stem cells, Clarke and
colleagues (Al-Hajj et al., 2003) have successfully identified cancer stem cells in breast cancer by their expression of malignancy-related tumor markers, CD44⁺CD24⁻. In neuroblastoma, disialoganglioside GD2 and CD24 are markers associated with the malignancy (Poncet et al., 1996; Kristiansen et al., 2004; Reynolds, 2004). Thus, they are candidates for the neuroblastoma stem cell markers.

**Bmi-1 and Molecular Mechanisms for the Stemness of Cancer Stem Cells**

What makes a cancer stem cell so special? More specifically, how do cancer stem cells possess their particular qualities, the stem cell-like activities? Although molecular mechanisms underlying the stem cell-like activities of cancer stem cells needed to be further explored, the cancer stem cell model has suggested several future directions for research in this area. At first, the two essential activities of a stem cell, indefinite self-renewal and multipotent differentiation, are actually inseparable. When a stem cell asymmetrically divides upon stimulation, it generates two different progeny cells. One maintains the stem cell activities and the dividing process is called self-renewal, while the other loses some of the stem cell activities and the process is called differentiation. Although most previous studies of stem cell activities focused on the control of cellular self-renewing capacity, genes and signaling pathways that regulate cellular differentiation must simultaneously regulate their self-renewal, and more attention should be paid to the research in the area. Second, the cancer stem cell model suggests that the self-renewing activity of cancer stem cells contributes to their tumorigenicity, so the genes and signaling pathways revealed to play roles in tumorigenesis, such as oncogenes and tumor
suppressors, should also be involved in the regulation of stem cell-like activities of cancer stem cells. Recent studies have provided the related evidence. For instance, Weissman and colleagues (Jamieson et al., 2004) isolated leukemia stem cells in chronic myeloid leukemia (CML) and observed that their self-renewal is dependent on the activities of an oncogene in Wnt signaling pathway, beta-catenin (Behrens and Lustig, 2004). Third, both normal stem cells and cancer stem cells possess self-renewing and differentiating capacities. The similarities between them suggest that they may share the same molecular mechanisms to regulate their stem cell-like activities. For example, it has been demonstrated that STAT5 regulates stem cell self-renewal in both normal hematopoiesis and leukemia genesis (Kato et al., 2005). Of course, cancer stem cells have some important properties different from normal stem cells. The most important difference is that self-renewal and differentiation of normal stem cells are tightly controlled by extrinsic factors in their environment. A characteristic example is that most adult stem cells are maintained in a silent state and only divide when there is injury. On the other hand, stem cell-like activities of cancer stem cells are deregulated, so that they gain extensive and uncontrolled proliferation potential in addition to the abilities to self-renew and differentiate that are shared with normal stem cells. It means that in cancer stem cells, some genes and signaling pathways implicated in the regulation of the activities of normal stem cells are abnormally activated or inactivated. It has been shown that some genes in the Hedgehog signaling pathway, which is involved in the maintenance of the activities of neural stem cells, are mutated in some brain tumors. The development of medulloblastoma is associated with inactivation of PTCH, a suppressor gene in
Hedgehog signaling pathway (Raffel et al., 1997). Amplification of the gene coding for Gli, a key regulator of Hedgehog signaling pathway, has been observed in glioma (Bigner et al., 1988).

Then, what are molecular mechanisms that maintain the stem cell-like activities of the cancer stem cells in neuroblastoma? To answer this question, the related genes need to be identified. As the cancer stem cell model indicates, they can be selected from a rapidly growing list of genes that have been demonstrated to be associated with oncogenesis and/or play a role in the control of self-renewal and/or differentiation of stem cells (normal stem cells or cancer stem cells). These gene candidates include genes in Notch, Wnt and Hedgehog signaling pathways, some members of the polycomb gene family and many other genes (Austin et al., 1997; Karanu et al., 2000; Spink et al., 2000; Bhardwaj et al., 2001; Kiger, et al., 2001; Taipale and Beachy, 2001; Tulina and Matunis et al., 2001; Varnum-Finney et al., 2001; Ohta et al., 2002; Calvi et al., 2003; Lemischka and Moore, 2003; Lessard and Sauvageau, 2003; Molofsky et al., 2003; Reya et al., 2003; Tohdai et al., 2003; Zhang et al., 2003; Dontu et al., 2004; Kim et al., 2004; Duncan et al., 2005). In this study, we focused on the role of Bmi-1 in the regulation of the stemness of neuroblastoma stem cells. Belonging to the Polycomb group (PcG) of genes (Simon, 2003), Bmi-1 was initially cloned as an oncogene that collaborates with c-myc in the onset of murine B-cell lymphomas (Haupt et al., 1991; van Lohuizen et al., 1991). Bmi-1 encodes a zinc finger protein containing a conserved centrally located helix-turn-helix-turn (H-T-H-T) motif (van Lohuizen et al., 1991), which is responsible for its transcriptional repression but not tumorigenesis (Alkema et al., 1997), and a conserved
ring finger domain at its amino-terminus, which contributes to its growth-promoting ability and transforming capacity (Alkema et al., 1997; Jacobs et al., 1999a; Itahana et al., 2003) (Figure 7). As other PcG proteins, Bmi-1 can form DNA-binding multimeric protein complexes with enzymatic activity and probably function by altering the conformation of chromatin to silence gene expression (Jacobs and van Lohuizen, 1999; Mahmoudi and Verrijzer, 2001; Ringrose and Paro, 2001; Goodrich and Tweedie, 2002; Simon and Tamkun, 2002; Orlando, 2003; Pirrotta et al., 2003; Lund and van Lohuizen, 2004). This epigenetic modification can be inherited during cell divisions, and considered as a cellular memory, supporting a wide variety of physiological and pathological roles of PcG proteins, including the regulation of cellular self-renewal and differentiation as well as oncogenesis (Caldas and Aparicio, 1999; Muller, 1999; Francis and Kingston, 2001; Jacobs and van Lohuizen, 2002; Orlando, 2003; Gil et al., 2005). A critical target of transcription repression by Bmi-1 is the Ink4a/Arf locus (Jacobs et al., 1999a, b), which encodes two proteins with distinct structures, p16Ink4a and p14Arf (Figure 8). These two proteins are translated from a common exon using different reading frames (Robertson and Jones, 1999; Sherr, 2001). Both proteins can inhibit cell progression, but do so through different molecular mechanisms. As a cyclin-dependent kinase (CDK) inhibitor, p16Ink4a inhibits cyclin D-Cdk4/6 complexes, and thus indirectly induces the retinoblastoma (Rb) tumor-suppression pathway (Sharpless and DePinho, 1999; Sherr, 2001). p14Arf acts through inhibition of p53 degradation mediated by MDM2, leading to activation of the p53 pathway (Sharpless and DePinho, 1999; Sherr, 2001). At the cellular level, p16Ink4a and p14Arf play important roles not only in the regulation of senescence,
Figure 8. Molecular Mechanisms of Bmi-1

Bmi-1 downregulates p16Ink4a and p14Arf through transcription repression of Ink4a/Arf locus encoding the two proteins, and finally inhibits RB and p53 signaling pathways. Adopted from Robertson and Jones, (1999) Oncogene, 18, 3812.
which is associated with changes in the self-renewal activities of stem cells (Sharpless, 2004), but also in the control of the genesis of various human tumors as an important tumor suppressor locus (Jacobs et al., 1999a, b; Lowe and Sherr, 2003; Molofsky et al., 2005). Obviously, transcriptional repression of the Ink4a/Arf locus could explain an important part of the functions of Bmi-1 (Jacobs et al., 1999a, b; Molofsky et al., 2005), although other mechanisms also may be involved (Dimri et al., 2002). As a multiple-function protein, Bmi-1 is not only a classical oncogene to induce tumor formation after dysregulation, but also has a large number of physiological functions. On one hand, as an oncogene (Jacobs et al., 1999a), Bmi-1 overexpression in mice promotes cell proliferation by down regulation of the Ink4a/Arf locus (Jacobs et al., 1999a; Itahana et al., 2003). Bmi-1 also collaborates with other oncogenes, such as Ras or Myc, to transform cells and induce tumorigenesis (Jacobs et al., 1999a, b). Bmi-1 itself can immortalize human mammary epithelial cells and enhance their proliferating activities (Dimri et al., 2002). In addition, Bmi-1 amplification and overexpression have been observed in some human tumors, including medulloblastoma, non-small cell lung cancer, mantle cell lymphomas and B-cell non-Hodgkin lymphoma, have been observed (Band and Sager, 1989; Bea et al., 2001; van Kemenade et al., 2001; Vonlanthen et al., 2001). All of these observations indicate an important role for Bmi-1 in human tumorigenesis. Notably, a recent microarray analysis demonstrated that a neural crest stem cell–like expression profile driven by Bmi-1 is strongly associated with high malignancy of various human tumors including prostate cancer and medulloblastoma (Glinsky et al.,
Since tumor malignancy is largely determined by the self-renewing activity and
tumorigenicity of cancer stem cells, this study not only indicates the existence of cancer stem cells in multiple types of tumors but also suggests a key role of Bmi-1 in the control of the stem cell-like activities of various cancer stem cells. On the other hand, Bmi-1 is involved in the control of development decisions and affects cellular fate, including self-renewal, differentiation and senescence, further providing the evidence that Bmi-1 is essential in the regulation of the activities of several types of stem cells. Studies with Bmi-1 knockout mice demonstrate that Bmi-1 functions in the development of a variety of tissues, such as skeleton, blood and neural tissue (van der Lugt et al., 1994; Jacobs et al., 1999a). In addition, Bmi-1 plays a role in the differentiation of lymphocytes and is expressed in immature T and B lymphocytes (Raaphorst et al., 2000, 2001). Sometimes, immature progenitor cells are thought to be a type of stem cells, although they have a limited self-renewing ability and can only give rise to one or few types of cells. Importantly, several studies have identified Bmi-1 as a regulator of self-renewal of hematopoietic stem cells, leukemic stem cells, neural stem cells in the central nervous system, and neural crest stem cells in the peripheral nervous system (Lessard and Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003; Raaphorst, 2003). Bmi-1 may also regulate the activities of neuroblastoma stem cells, because Bmi-1 has been identified as a regulator of self-renewal of neural crest stem cells (Molofsky et al., 2003, 2005), from which neuroblastoma originates. In addition, Bmi-1 can collaborate with c-Myc, a structural and functional homolog of N-myc, in tumorigenesis (Haupt et al., 1991;
van Lohuizen et al., 1991). N-myc amplification occurs in 25%-30% of neuroblastoma patients and is tightly linked to disease progression, metastasis and poor prognosis (Brodeur and Nakagawara, 1992; Schwab, 1993; Goodman et al., 1997). Therefore Bmi-1 may also cooperate with N-myc in the pathogenesis of neuroblastoma. In general, Bmi-1 appears to play a role in the regulation of the activities of neuroblastoma stem cells, and thus was studied in this work.
MATERIALS AND METHODS

Cell Culture

The human I-type neuroblastoma cell line BE(2)-C (ATCC), the human S-type neuroblastoma cell line SHEP1 and the human fibrosarcoma cell line HT1080 (ATCC) were maintained as monolayers in Dulbecco's modified Eagle’s medium (DMEM) (Sigma) supplemented with penicillin/streptomycin (Invitrogen Corp) and 10% fetal calf serum (FBS) (Invitrogen Corp) at 37°C in a humidified 5.0% CO₂ atmosphere.

The 293GPG human kidney retroviral packaging cell line (a gift from Dr. Richard Mulligan) was maintained as monolayers in 293 GPG medium, DMEM supplemented with G418/puromycin/doxycyclin (Invitrogen Corp), penicillin/streptomycin, and 10% heat-inactivated FBS, at 37°C in a humidified 5.0% CO₂ atmosphere.

Cells were passaged every 3 d by treatment with trypsin (Invitrogen Corp).

Differentiation Analysis

10⁻⁵ M 5-bromo-2’-deoxyuridine (BrdUrd) (Sigma) or 10⁻⁵ M all-trans retinoic acid (RA) (Sigma) dissolved in dimethyl sulfoxide (DMSO) (Sigma) was directly added into the medium of BE(2)-C I-type neuroblastoma cells and the cell medium was changed every 3 d. After 7 d of treatment with RA or 14-21 days of treatment with BrdUrd, BE(2)-C I-type cells were photographed under a microscope. After 4 wk of treatment, the altered differentiation status induced in BE(2)-C I-type cells was stable, and then
treatment was ceased. In these studies, 0.1% DMSO-treated BE(2)-C I-type cells were used as negative control.

**Immunocytochemistry Assay**

All the cells cultured on cover glasses (Fisher Scientific) were fixed with a solution of 95% ethanol (Fisher Scientific) and 5% glacial acetic acid (Fisher Scientific) at –20°C for 20 min, permeablized with 0.3% Triton X-100 (Fisher Scientific) in phosphate buffered saline powder (PBS) (Fisher Scientific) at room temperature (RT) for 5 min, and blocked with 5% nonfat milk (Fisher Scientific) for 1 h. Next, cells were incubated with 1:100 mouse HNK-1 monoclonal antibody (BD Pharmingen™), 1:200 rabbit peripherin antiserum (Chemicon) or 1:200 rabbit glial fibrillary acidic protein (GFAP) antiserum (Sigma) at 4°C overnight, washed with PBS, and incubated with Texas red- or fluorescein (FITC)-conjugated secondary antibodies (Molecular Probes) at RT for 30 min. After being washed with PBS again, the cover glasses were put on microscope slides (Fisher Scientific) and then photographed with a microscope (Nikon Eclipse E800) with Image-Pro Plus software for image analysis. In these assays, HT1080, a human fibrosarcoma cell line, was used as negative control.

**Soft Agar Colony Formation Assay and MTT Assay**

About 5X10² or 10³ BE(2)-C cells were suspended as single cells in DMEM supplemented with 10% FBS and 0.3% Noble agar (Sigma) and plated on 6-well plates
containing a solidified bottom layer (0.6% Noble agar in DMEM supplemented with 10% FBS). After 2 wk of incubation at 37°C, 0.5 ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added into each well of the 6-well plate for 30 min at 37°C to stain viable cell colonies. Viable cell colonies that contain more than 40-50 cells were counted manually. The ability of neuroblastoma cells to self-renew and grow in an anchorage-independently manner was assessed as the percentage of cells with colony forming ability. In serial soft agar formation assays, single clones were removed from soft agar with sterile Pastier pipettes. After digested with trypsin, clone cells were mechanically dissociated into single-cell suspensions and replated at 500 cells/well in soft agar on 6-well plates for another 2 wk of incubation. The same operations were repeated five times.

**Plasmid Construction**

To downregulate Bmi-1, two 64-bp oligonucleotides containing the human Bmi-1 siRNA sequences (5’-ATGAAGAGAAGAAGGGATT-3’ and 5’-AATGGACATACCTAATACT-3’, positions 269-287bp and 546-574bp relative to the start codon, respectively) were synthesized by IDT and cloned into the pSuper plasmid (OligoEngine) (Brummelkamp et al., 2002a) digested with BglIII and HindIII (New England Biolabs) to generate psuper/Bmi-1si plamids, according to the previously described procedure (Brummelkamp et al., 2002b).
Retroviral Infection

Ecotropic retroviral supernatants were produced by transfection of the 293GPG packaging cells (Ory et al., 1996) using Lipofectamine Plus reagents (Invitrogen Corp) (Slepak et al., 1995). $5 \times 10^6$ 293GPG cells per 60 mm dish were seeded the day before transfection and maintained in 293 medium, DMEM containing 10% heat-inactivated FBS. During the transfection, 293 medium was changed to DMEM. Next, 2.0 µg of DNA, including psuper/Bmi-1si plamid DNA and pBabepuro/Bmi-1 plasmid (a gift from Dr. Goberdhan P. Dimri) DNA, was mixed with 8 µl of Plus reagent, followed by addition of 12 µl of lipofectamine to each 60 mm dish with retrovirus vectors expressing green fluorescence protein (GFP) as control, and 5 h later, the medium was changed to 293GPG medium. 48 h after transfection, ecotropic retroviral supernatant was harvested, filtered through a 0.45 µm filter, and used to infect BE(2)-C I-type neuroblastoma cells per 12 h for four times. Infected cells were cultured in puromycin selection medium for 3 d and drug-resistant cells were pooled. The percentage of retrovirus-infected cells ranged from 60–80%, as estimated in parallel infections using retroviruses expressing GFP. Expression of relevant genes was confirmed by immunoblotting.

Immunoblot Analysis

Expression levels of Bmi-1 and peripherin were detected by immunoblotting with α-tubulin levels as standard control. Cells were collected after trypsin digestion and low speed centrifugation. After PBS wash, each example was mixed with sample buffer
containing 10 mM Tris-HCl (pH 7.6) (Fisher Scientific), 150 mM sodium chloride (NaCl) (Fisher Scientific), 0.5 mM ethylenediaminetetraacetic acid (EDTA) (Fisher Scientific), 1% sodium dodecyl sulphate (SDS) (National Diagnostics), and a mixture of protease inhibitors (1 mM PMSF, 1 µg/ml pepstatin A, and 1 µg/ml leupeptin) (Boehringer Mannheim) and fractionated on 10% SDS-polyacrylamide gels (National Diagnostics). After electrophoresis, proteins were transferred to nitrocellulose (Millipore), blocked with block solution containing 5% nonfat dry milk in TBST buffer (20 mmol/L Tris-HCl, 500 mmol/L NaCl, and 0.01% Tween 20) (Fisher Scientific), and incubated with 1:600 mouse Bmi-1 monoclonal antibody (Upstate Biotechnology) at 4°C overnight, 1:2000 rabbit peripherin antiserum (Chemicon) at RT for 30 min or 1:10000 mouse α-tubulin monoclonal antibody (Sigma) at RT for 30 min. Immunoreactivity was detected by sequential incubation with horseradish peroxidase conjugated goat anti-mouse or anti-rabbit IgG (Cappel) and enhanced chemiluminescence reagents (Amersham Biosciences) (Kandasamy et al., 2003).

**Flow Cytometry and Fluorescence-Activated Cell Sorting**

Cells were digested with trypsin, dispersed into single cell suspensions, and washed twice with PBS (1ml per 10^6 cells). For labeling of intracellular markers, such as GFAP, cells were fixed in a solution of 95% ethanol and 5% glacial acetic acid at −20°C for 20 min, washed with PBS, and permeablized with 0.3% Triton X-100 in PBS at RT for 5 min. This procedure is not needed to label cell surface markers, such as HNK-1.
Next, cells were resuspended in 1 ml per $10^6$ cells of DMEM with 2% heat-inactivated FBS. Cells then were incubated in a kind of first antibody for 30 min on ice, washed twice with PBS, and then incubated in a respective secondary antibody for 20 min on ice. After PBS wash, cells were resuspended in 1 ml per $10^6$ cells of PBS for flow cytometry analysis or fluorescence-activated cell sorting (FACS) performed on the Epics Elite FACS machine (Beckman-Coulter). For FACS, the whole cell population, marker-positive cells and marker-negative cells are all sorted into separate culture plates. A few of the sorted cells were reanalyzed for purity.
RESULTS

**BE(2)-C cells Possess the Capacity to Differentiate Along Either the Neuroblastic or the Schwann/glial Pathway**

BE(2)-C cell line is one of the common I-type neuroblastoma cell lines, clonally derived from SK-N-BE(2) N-type neuroblastoma cell line (Piacentini et al., 1992). It has been reported that BE(2)-C I-type neuroblastoma cells can be induced to differentiate into N-type- and S-type- like neuroblastoma cells by chemical inducing agents, e.g., all-trans retinoic acid (RA) or 5-bromo-2’-deoxyuridine (BrdUrd) (Ross et al., 1995), suggesting that I-type neuroblastoma cells may have cancer stem cell-like activities. BrdUrd and RA are widely used differentiation-inducing agents, although the underlying mechanism remains unclear. BrdUrd is a DNA intercalating agent and may affect expression of genes. RA can enter into the cell nucleus to associate with its nuclear receptor and regulate expression of many genes. Their ability to regulate gene expression may be responsible for their differentiation-inducing activity (Lee et al., 1992; Kitamura et al., 1997; Minucci et al., 2001; Li et al., 2003). We first wanted to confirm the earlier finding that BE(2)-C I-type cells can differentiate into N- and S-type cells. BrdUrd (10^{-5} M) or RA (10^{-5} M) was directly added into the medium of BE(2)-C I-type neuroblastoma cells and the cell medium was changed every 3d. After 7d of treatment with RA, BE(2)-C cells displayed neuron-like morphologies (Figure 9). The cells had small and rounded cell bodies, relatively little cytoplasm, and long neuritic processes. They loosely attached to
Figure 9. Differentiation of BE(2)-C I-type Neuroblastoma Cells Induced by RA and BrdUrd

After treatment with RA or BrdUrd, differentiated BE(2)-C cells demonstrated neuron-like or Schwann/glial cell-like morphologies, respectively.
the substrate and tended to aggregate together. By contrast, after 2-3 wk of treatment with BrdUrd, BE(2)-C cells displayed Schwann/glial cell-like morphologies (Figure 9). The cells were big and flattened, with abundant cytoplasm and no neuritic processes. They adhered tightly to the substrate, and did not tend to aggregate together. After 4 wk of treatment, the altered differentiation status induced in BE(2)-C I-type cells was stable, and drug treatment was stopped. These findings indicate that similar to neural crest stem cells, a subpopulation of I-type neuroblastoma cells can differentiate into neuron- or Schwann cell-like cells.

After the altered differentiation states induced in BE(2)-C neuroblastoma cells stabilized, we carried out immunocytochemistry assays to determine if there were some characteristic antigenic changes along with differentiation of BE(2)-C I-type cells. HNK-1, peripherin, and glial fibrillary acidic protein (GFAP) were used as characteristic markers for neural crest stem cells, neurons and Schwann cells, respectively (Erickson, 1988; Hafidi et al., 1993; Geuna et al., 2003; Helfand et al., 2003). In these assays, HT1080, a human fibrosarcoma cell line, was used as negative control. After fixation, permeabilization and blocking, cells were incubated with anti-HNK-1, anti-peripherin or anti-GFAP antibodies at 4°C overnight, and with Texas red- or fluorescein-conjugated secondary antibodies at RT for 30 min. As shown in Figure 10, about 21% of BE(2)-C I-type cells expressed HNK-1, about 43% expressed peripherin, and no significant GFAP expression was observed in I-type cells. For BE(2)-C derived neuron-like cells, about 9% expressed HNK-1, about 94% expressed peripherin and about 17% expressed GFAP. For
Figure 10a. Changes of Expression of Markers in BE(2)-C Cells After Differentiation

I: DMSO-treated BE(2)-C I-type cells
N: RA-treated BE(2)-C cells
S: BrdUrd-treated BE(2)-C cells
Figure 10b. Changes of Expression of Markers in BE(2)-C Cells After Differentiation

I: DMSO-treated BE(2)-C I-type cells
N: RA-treated BE(2)-C cells
S: BrdUrd-treated BE(2)-C cells

The figure shows the mean values and standard deviation based on three independent experiments.
BE(2)-C derived Schwann cell-like cells, about 84% expressed GFAP, and no significant expression of HNK-1 or peripherin was observed (Figure 10). These findings further confirm that, similar to neural crest stem cells, a subpopulation of I-type neuroblastoma cells can differentiate into neuron- or Schwann cell-like cells, accompanied by a decrease in the expression level of the stem cell marker HNK-1 and an increase in the expression level of the neuron marker peripherin or of the Schwann cell marker GFAP.

A Subpopulation of BE(2)-C I-type Neuroblastoma Cells Possess Self-renewing Capacity

Self-renewal is an essential property of stem cells. A demonstration of this property in I-type neuroblastoma cells is necessary to verify the hypothesis that they are cancer stem cells. Two in vitro clonogenic assays have been used to determine the self-renewing capacity of a stem cell: The sphere formation assay in culture (Reynolds et al., 1992) and the colony formation assay in soft agar or methylcellulose (Hamburger and Salmon, 1977; Buick et al., 1981; Thomson and Meyskens, 1982). In both assays, a primary sphere or colony is dissociated into single cells and replated. Formation of new spheres or colonies that maintain a multilineage differentiation potential demonstrates self-renewal. As BE(2)-C cells did not form spheres under a variety of culture conditions (data not shown), we examined their self-renewing capacity in soft agar colony formation assays, which is positively correlated to the tumorigenic capacity of cancer cells (Freedman and Shin, 1974).
500 single BE(2)-C cells were plated into individual wells of a 6-well culture plate. Examination of the wells under a light microscope immediately after plating revealed only individual cells and no cell clusters. Colonies, defined as a collection of more than 50 cells, appeared between 10-14d after plating. Based on the values obtained from dividing the number of colonies formed by the number of cells plated, we estimated that a significantly high percentage (about 20%) of BE(2)-C I-type cells were able to give rise to colonies in the soft agar assay (cloning efficiency), in comparison with neuron-like (4.6%) or Schwann cell-like BE(2)-C cells (2.2%) (Figure 11). These results indicate that a large subpopulation of I-type cells have colongenic ability, supporting the notion that they may be cancer stem cells with self-renewing capacity.

Successive soft agar colony formation assays then were performed to determine if a subpopulation of I-type cells have the ability to renew themselves for a long period, just as cancer stem cells do. After the first soft agar colony formation assay (Figure 12a, 1\textsuperscript{0} well), individual primary colonies containing more than 1000 cells were plucked from the agar with sterile Pasteur pipettes, pooled, and dissociated into single-cell suspensions. The cells were replated at 500 cells per well (six-well plate). The single-cell nature of the suspensions was again confirmed by microscopy. After two weeks, secondary (2\textsuperscript{0}) colonies were formed that were similar to the primary colonies in size, morphology, and cloning efficiency (Figure 12a, 2\textsuperscript{0} well). However, cells replated from 2\textsuperscript{0}, 3\textsuperscript{0}, 4\textsuperscript{0} colonies showed a gradual increase in cloning efficiency (from about 20% in 2\textsuperscript{0} colonies to about
Figure 11. Soft Agar Colony Formation Assay for BE(2)-C I-type Neuroblastoma Cells and Their Derived Neuron-like and Schwann Cell-like Neuroblastoma Cells

I: BE(2)-C I-type cells
N: neuron-like BE(2)-C cells
S: Schwann cell-like BE(2)-C cells

The figure shows the mean values and standard deviation based on three independent experiments.
Figure 12a. Self-renewing Assay of BE(2)-C I-type Neuroblastoma Cells

1°, 2°, 3°, 4°, and 5°: BE(2)-C I-type cells
1°': firstly renewing BE(2)-C cells
2°': secondly renewing BE(2)-C cells
3°': thirdly renewing BE(2)-C cells
4°': fourthly renewing BE(2)-C cells
5°': SHEP1 S-type neuroblastoma cells were used as negative control.
Figure 12b. Self-renewing Assay of BE(2)-C I-type Neuroblastoma Cells

1⁰: BE(2)-C I-type cells
2⁰: firstly renewing BE(2)-C cells
3⁰: secondly renewing BE(2)-C cells
4⁰: thirdly renewing BE(2)-C cells
5⁰: fourthly renewing BE(2)-C cells
SHEP1 S-type neuroblastoma cells were used as negative control.
60% in $5^0$ colonies), indicating an expansion of clonogenic BE(2)-C cells during the successive soft agar assays (Figure 12).

In addition to the study with pooled colonies, we have performed the same successive soft agar assays with BE(2)-C cells from individual colonies. In each round of the serial transfer, 10 single colonies of similar size (containing more than 1000 cells) were removed from the soft agar and dissociated into single cells, and the entire progeny of each colony was plated into an individual well of a 6-well plate. In all cases, we observed the formation of colonies within 2 wk (data not shown). Together, these data suggest that clonally derived BE(2)-C colonies contain individual stem-like cells with the ability to self-renew for a long term.

**Clonally Derived BE(2)-C Colonies Maintain the Capacity to Differentiate Along**

**Either the Neuroblastic or the Schwann/glial Pathway**

We next addressed the question of whether the clonally derived BE(2)-C colonies, like their founder cells, could differentiate along specific neural crest lineages. We characterized the clonally derived BE(2)-C colonies by immunofluorescence staining for cell lineage markers HNK-1, peripherin, and glial fibrillary acidic protein (GFAP). Single primary colonies ($n=20$) were transferred onto laminin-coated glass coverslips, allowed to adhere for 1h, and processed for immunofluorescence staining. A lot of cells in the colonies showed strong HNK-1 staining (Figure 13, HNK-1). Some cells expressed significant levels of peripherin (Figure 13, peripherin), and very few cells were immunoreactive for GFAP (Figure 13, GFAP). Notably, most of the cells that were
Figure 13. Spontaneous Differentiation of BE(2)-C Neuroblastoma Cells Derived from a Single Cell
positive for peripherin or GFAP showed negative staining of HNK-1 (Figure 13, HNK-1/peripherin, HNK-1/GFAP), suggesting the presence of more differentiated neuroblasts and Schwann/glial cells in the clonally derived colonies. The same staining patterns were also observed in secondary and later colonies (data not shown). These findings suggest that the single I-type neuroblastoma cells have a spontaneous differentiation potential along either the neuroblastic or the Schwann/glial pathway.

We further determined whether the single I-type neuroblastoma cells could also be induced to differentiate along either the neuroblastic or the Schwann/glial pathway. Individual tertiary colonies were dissociated into single cells and plated into separate wells of a 24-well plate. Cells were either treated with 0.1% DMSO or $10^{-5}$ M of differentiation-inducing agents, RA or BrdUrd, dissolved in DMSO. Most of the DMSO-treated cells showed a typical I-type cell morphology, with a round nucleus and relatively abundant cytoplasm (Figure 14, I-type, phase). Cells treated with RA for 7d exhibited a neuronal morphology, as described above (Figure 14, N-type, phase). By contrast, after treatment with BrdUrd, cells with a Schwann/glial morphology began to appear (Figure 14, S-type, phase).

To confirm the phenotypes of the differentiated N- and S-type cells, we performed immunofluorescence staining of the cells with antibodies against HNK-1, peripherin, and GFAP. Many control DMSO-treated BE(2)-C cells showed strong HNK-1 staining (Figure 15, I-type, HNK-1). A significant number of the DMSO-treated cells were weakly immunoreactive for peripherin, which in general were smaller cells (Figure 15, I-type, peripherin). Very few DMSO-treated cells expressed detectable levels of
Figure 14. Induced Differentiation of BE(2)-C Neuroblastoma Cells Derived from a Single Cell
Figure 15a. Changes of Expression of Markers in Clonally Derived BE(2)-C Cells After Induced Differentiation

I: DMSO-treated BE(2)-C I-type cells
N: RA-treated BE(2)-C cells
S: BrdUrd-treated BE(2)-C cells
Figure 15b. Changes of Expression of Markers in Clonally Derived BE(2)-C Cells After Induced Differentiation

I: DMSO-treated BE(2)-C I-type cells
N: RA-treated BE(2)-C cells
S: BrdUrd-treated BE(2)-C cells

The figure shows the mean values and standard deviation based on three independent experiments.
GFAP (Figure 15, I-type, GFAP). By contrast, in both differentiated N- and S-type cells, the expression of HNK-1 was markedly decreased (Figure 15, N- and S-type, HNK-1). Moreover, all the differentiated N- and S-type cells displayed very strong staining for peripherin and GFAP, respectively (Figure 15, N- and S-type, peripherin and GFAP). These results demonstrate that BE(2)-C colonies contain stem/progenitor cells that are multipotent, giving rise to cells with neuronal or Schwann/glial characteristics.

**Use of HNK-1 Expression did not Allow the Isolation of a Cancer Stem Cell**

**Population from BE(2)-C Cells**

The above results indicate that BE(2)-C I-type cells possess differentiation potential along either the neuroblastic or the Schwann/glial pathway, and about 20% of the I-type cells can sustain long-term growth in soft agar (self-renewal). Importantly, about 20% of BE(2)-C I-type cells express the neural crest stem cell marker, HNK-1. Thus, the HNK-1$^+$ population of BE(2)-C I-type cells may be cancer stem cells. We tried to isolate cancer stem cells form BE(2)-C cells by the expression of HNK-1. BE(2)-C I-type neuroblastoma cells were digested, dispersed into single cell suspension, and stained sequentially with a mouse monoclonal antibody against HNK-1 and a fluorescein-conjugated anti-mouse antibody. The whole cell population and HNK-1$^+$ and HNK-1$^-$ cells were sorted into separate tubes by fluorescence-activated cell sorting (FACS), and then their abilities to self-renew in soft agar were assessed. The results showed that there is no significant difference in the colony forming ability among the whole cell population (20.5%) and HNK-1$^+$ (24%) and HNK-1$^-$ BE(2)-C cells (17.2%) (Figure 16). This means
Figure 16. Colony Formation Assay for BE(2)-C I-type Cells Sorted by Expression of HNK-1

HNK-1⁻: sorted HNK-1 negative BE(2)-C I-type cells
HNK-1⁺: sorted HNK-1 positive BE(2)-C I-type cells
whole population: sorted whole population of BE(2)-C I-type cells
The figure shows the mean values and standard deviation based on three independent experiments.
that the isolation of BE(2)-C cells based on their expression of HNK-1 did not enrich for cancer stem cells. Thus, HNK-1 was not a cancer stem cell marker for BE(2)-C I-type cells.

**Partial Isolation of the Cancer Stem Cell Population from BE(2)-C Cells by the Expression of CD24**

According to the cancer stem cell model, only self-renewing and tumorigenic cancer stem cells are responsible for the malignancy of tumors, which is shown by disease progression, metastasis and prognosis. Thus, the malignancy-related neuroblastoma marker, CD24 may be a specific marker for neuroblastoma stem cells. We attempted to isolate cancer stem cells from BE(2)-C cells by the expression of CD24. As above, we sorted and collected the whole cell population and CD24$^+$ and CD24$^-$ BE(2)-C I-type cells by fluorescence-activated cell sorting (FACS), and then assessed their abilities to self-renew in soft agar. The results showed that compared with the whole cell population (24.1%), CD24$^+$ BE(2)-C cells (49.6%) possess high colony forming ability while CD24$^-$ BE(2)-C cells (13.2%) possess low colony forming ability in soft agar (Figure 17). This means that the isolation of BE(2)-C cells expressing CD24 can enrich for the cancer stem cells and CD24 is a possible cancer stem cell marker for BE(2)-C I-type cells. Of course, the difference in the colony forming ability between the whole cell population and CD24$^+$ BE(2)-C cells was not stringent enough to allow the isolation of a pure population of neuroblastoma stem cells. However, the results showed that CD24 can be used to enrich the neuroblastoma stem cell population.
Figure 17. Colony Formation Assay for BE(2)-C I-type Cells Sorted by Expression of CD24

CD24−: sorted CD24 negative BE(2)-C I-type cells
CD24+: sorted CD24 positive BE(2)-C I-type cells
whole population: sorted whole population of BE(2)-C I-type cells
The figure shows the mean values and standard deviation based on three independent experiments.
Differentiated N- and S-type Cells Show a Disparity in Bmi-1 Expression Levels

Unlike their parental BE(2)-C cells, differentiated N- and S-type cells were unable to form colonies in soft agar (Figure 11), suggesting the loss of clonogenic, self-renewal capacity. Given the recent report showing an essential role of the Bmi-1 in maintaining the self-renewal capacity of neural crest stem cells (Molofsky et al., 2003), we examined the levels of Bmi-1 expression in these cells. In comparison with parental BE(2)-C cells, Bmi-1 expression was significantly downregulated in differentiated S-type cells (Figure 18). However, much to our surprise, the level of Bmi-1 was actually increased in differentiated N-type cells (Figure 18). These results suggest a lineage-specific regulation of Bmi-1 expression in neuroblastoma cells.

Downregulation of Bmi-1 in BE(2)-C Cells Promotes Exit from the Stem Cell State and Entry into Differentiation Along the Schwann/glial Pathway

We next investigated the possibility that the observed differential regulation of Bmi-1 expression in N- and S-type cells might be actively involved in the commitment of neuroblastoma I-type cells to differentiate along a specific lineage pathway. We first examined the effect of downregulation of Bmi-1 on the stem cell and differentiation states of BE(2)-C cells. We generated a Bmi-1 siRNA-expressing retroviral construct to target the Bmi-1-coding sequence. Retroviruses produced from the construct were able to knockdown the levels of Bmi-1 in BE(2)-C cells by more than 50% (Figure 19). Soft agar colony formation assays showed that BE(2)-C cells with reduced levels of Bmi-1 were
Figure 18. Different Expression Levels of Bmi-1 in Different Types of BE(2)-C Cells

I: DMSO-treated BE(2)-C I-type cells
N: RA-treated neuron-like BE(2)-C cells
S: BrdUrd-treated Schwann/glial cell-like BE(2)-C cells
Figure 19. Detection of Expression Levels of Bmi-1 in Infected BE(2)-C Cells

pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
unable to form colonies in soft agar (Figure 20). This finding is consistent with the recent report that Bmi-1\textsuperscript{−/−} neural crest stem cells formed significantly smaller neurospheres that did their wild-type control cells (Molofsky et al., 2003). Thus, Bmi-1 expression levels play a critical role in the control of BE(2)-C cell self-renewal.

BE(2)-C cells with downregulation of Bmi-1 showed no overt morphological changes. However, immunofluorescence staining revealed that in these cells the expression of HNK-1 was markedly decreased, which was accompanied by a significant increase in the expression levels of GFAP and a significant decrease in that of peripherin (Figure 21a and 21b). This expression pattern of molecular markers was identical or very similar to that observed in the S-type cells differentiated from BE(2)-C cells after BrdUrd treatment (Figure 15, S-type). To exclude the possibility that the observed effect was not artificial, we performed flow cytometry analyses and immunoblot analysis and confirmed the changes in the expression levels of HNK-1, GFAP and peripherin in these cells (Figure 21c, 21d, and 21e). Taken together, these results suggest that downregulation of Bmi-1 in BE(2)-C cells is sufficient to initiate differentiation along the Schwann/glial pathway. These also meant that the reduction in Bmi-1 levels in the differentiated S-type cells was not simply a consequence of specific effects of the inducing agent BrdUrd, but rather an integral part of the signaling cascade that drives I-type cells to differentiate into Schwann/glial cells.
Figure 20. Colony Formation Assay for BE(2)-C Cells with Downregulation of Bmi-1

pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
Figure 21a. Immunocytochemistry Analyses of Changes in the Expression of Markers in BE(2)-C Cells after Downregulation of Bmi-1

pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
Figure 21b. Immunocytochemistry Analyses of Changes in the Expression of Markers in BE(2)-C Cells after Downregulation of Bmi-1

pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
Figure 21c. Flow Cytometry Analysis of the Change in the HNK-1 Expression on BE(2)-C Cells After Downregulation of Bmi-1

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pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
Figure 21d. Flow Cytometry Analysis of the Change in the GFAP Expression on BE(2)-C Cells After Downregulation of Bmi-1

FITC control: BE(2)-C cells stained only with FITC conjugated secondary antibody as staining control
pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
Figure 21e. Immunoblot Analysis of the Change of the Peripherin Expression in BE(2)-C Cells after Downregulation of Bmi-1

pSuper: BE(2)-C cells infected with the pSuper empty vector as control
pSuper/Bmi-1si: BE(2)-C cells infected with the pSuper/Bmi-1si construct
Upregulation of Bmi-1 in BE(2)-C Cells Promotes Exit from the Stem Cell State and Differentiation Along the Neuronal Pathway

We next examined the effect of upregulation of Bmi-1 on the stem cell and differentiation states of BE(2)-C cells. The human Bmi-1 gene was introduced into BE(2)-C cells by retroviral-mediated gene transfer, and overexpression of Bmi-1 was confirmed by immunoblot analysis (Figure 22). No overt differences in morphology were observed between the control and Bmi-1-overexpressing BE(2)-C cells. However, Bmi-1-overexpressing BE(2)-C cells displayed an expression pattern of molecular markers that was identical or very similar to that of differentiated N-type cells. Just as differentiated N-type BE(2)-C cells (Figure 15), Bmi-1-overexpressing cells demonstrated a significant reduction in the levels of HNK-1 and a concomitant increase in the levels of peripherin, shown by immunocytochemistry analysis (Figure 23a) and confirmed by immunoblot analysis (Figure 23b). Also like differentiated N-type cells (Figure 11), Bmi-1-overexpressing BE(2)-C cells gave rise to colonies that were much smaller than those generated by the control BE(2)-C cells (Figure 24), suggesting a loss of the clonogenic, self-renewal capacity possessed by undifferentiated BE(2)-C cells. Together, these results suggest that upregulation of Bmi-1 expression can induce BE(2)-C cells to exit from the stem cell state and to differentiate along the neuronal pathway.
Figure 22. Detection of Expression Levels of Bmi-1 in Infected BE(2)-C Cells

pBabe/Bmi-1: BE(2)-C cells infected with the pBabepuro/Bmi-1 construct
pBabe: BE(2)-C cells infected with the pBabepuro empty vector as control
Figure 23a. Immunocytochemistry Analyses of Changes in the Expression of Markers in BE(2)-C Cells After Bmi-1 Overexpression

pBabe: BE(2)-C cells infected with the pBabepuro empty vector as control
pBabe/Bmi-1: BE(2)-C cells infected with the pBabepuro/Bmi-1 construct
Figure 23b. Immunoblot Analysis of the Change of the Peripherin Expression in BE(2)-C Cells After Bmi-1 Overexpression

pBabe: BE(2)-C cells infected with the pBabepuro empty vector as control
pBabe/Bmi-1: BE(2)-C cells infected with the pBabepuro/Bmi-1 construct
Figure 24. Colony Formation Assay for BE(2)-C Cells with Bmi-1 Overexpression

pBabe: BE(2)-C cells infected with the pBabepuro empty vector as control
pBabe/Bmi-1: BE(2)-C cells infected with the pBabepuro/Bmi-1 construct
DISCUSSION

BE(2)-C Cells Possess the Capacity to Differentiate Along Either the Neuroblastic or the Schwann/glial Pathway

According to the cancer stem cell model, cancer stem cells are cancer cells with stem cell-like activities including self-renewal and differentiation (Hamburger and Salmon, 1977). Differentiating ability is one of the essential functions of cancer stem cells. In previous studies, it has been demonstrated that I-type cells derived from several different human neuroblastoma cell lines including BE(2)-C may be induced experimentally to differentiate along different neural crest cell lineages, including neuron and Schwannian/glial/melanoblastic lineages (Walton et al., 2004). I-type cells appear to represent multipotent cancer stem cells in neuroblastoma. This work was not only able to demonstrate that BE(2)-C I-type cells could be induced to differentiate, as found by other groups of researchers, but also showed that there were changes of several specific lineage markers after differentiation of BE(2)-C cells.

HNK-1, originally identified as a carbohydrate epitope recognized by the monoclonal antibody HNK-1, is a marker for developmentally immature avian and human, but not amphibian or mouse, neural crest cells (Bronner-Fraser, 1987; Tucker et al., 1988). Additionally, expression of HNK-1 is observed in undifferentiated neuroblastoma cells, but not in the more mature cells (Hoehner et al., 1998). In this study, the soft agar colony formation assay showed that about 20% of BE(2)-C I-type cells have colony forming ability characteristic of cancer stem cells. Interestingly, nearly the same
proportion of BE(2)-C I-type cells are HNK-1 positive. After differentiating into N-type like cells, HNK-1^+ cells markedly decreased, whereas after differentiating into S-type like cells, HNK-1^+ cells nearly disappeared. Accordingly, a few BE(2)-C N-type cells still had colony forming ability and none of BE(2)-C S-type cells had colony forming ability. This means that HNK-1^+ cells may be the malignant neural crest stem cells or their derived progenitors that drive tumor formation and progression. However, HNK-1^+ neural crest cells are only a subpopulation of neural crest stem cells (Minarcik and Golden, 2003), so neuroblastoma stem cells might include HNK-1^-cells. In the peripheral nervous system, besides neural crest stem cells, there are still some cells known to be HNK-1^+ (Tucker et al., 1984). Thus, some HNK-1^+ neural crest cells may not be cancer stem cells.

Peripherin is an intermediate filament protein marker characteristic of neural crest-derived peripheral neurons (Troy et al., 1990). This study demonstrated that almost all the differentiated BE(2)-C N-type cells are peripherin^+ and nearly all BE(2)-C S-type cells are peripherin^- This means that peripherin is a good specific marker for N-type like neuroblastoma cells. Interestingly, a subpopulation of BE(2)-C I-type cells is peripherin^+, which might represent neuroblast like progenitor cells, derived from original cancer stem cells by spontaneous differentiation. After long-term successive culture, BE(2)-C I-type cells behave more and more like neurons in morphology and they are more and more resistant to BrdUrd-induced differentiation into S-type like cells (data not shown), possibly due to their spontaneous differentiation into N-type like cells. Notably, a few BE(2)-C I-type and N-type cells expressed both HNK-1 and peripherin. They may represent neuroblast like progenitor cells just differentiated from HNK-1^+ / peripherin^-
malignant neural crest stem cells, some of which may still possess cancer stem cell-like activities.

Previously widely used as an astrocyte specific marker in the central nervous system (de Vitry et al., 1981), GFAP is also found to be a marker for immature Schwann/glial cells in peripheral nervous system (Jessen and Mirsky, 2002; Sieber-Blum et al., 2004). Although it has been reported that BE(2)-C I-type cells could be induced to differentiate into Schwannian/glial/melanoblastic like cells, the S-type cells, it still remains elusive as to which kind of S-type cells the differentiated BE(2)-C cells belong. The present study demonstrates that almost all the differentiated BE(2)-C S-type cells were GFAP+ while very few BE(2)-C I-type cells expressed GFAP, which means that BE(2)-C I-type cells can be induced to differentiate into Schwann/glial S-type cells by BrdUrd. Intriguingly, after all-trans retinoic acid induction, there were still a few GFAP+ BE(2)-C cells. The possible reason is that some BE(2)-C I-type cells might spontaneously differentiate along the Schwann/glial pathway irreversibly and would maintain their Schwann/glial phenotype even treated with all-trans retinoic acid.

Based on the previous observation of other laboratories that I-type neuroblastoma cell populations could be induced to differentiate along different pathways, it could not be concluded that I-type neuroblastoma cells are multipotent. It was not excluded that some I-type cells in the population could only differentiate along the neuroblastic pathway and the others only along the Schwannian/glial/melanoblastic pathway, so that after treatment with different inducing agents, different subpopulations of I-type cells would be selectively stimulated and induced to differentiate. It is important to determine
if a single I-type cell can differentiate along at least two different pathways. In our
current study, individual tertiary colonies were plucked from soft agar in successive soft
agar colony formation assays to make sure the colonies came from single BE(2)-C I-type
cells. The findings in this study indicated that colonies derived from single I-type cells
could also be induced to differentiate along different pathways leading to the conclusion
that BE(2)-C I-type cells are multipotent. Furthermore, to reduce possible artifacts that
might arise from the induction, the differentiation status of cells in these colonies was
directly detected without induction. I found that the differentiation status of cells in any
colony was various, even though all of them originated from a single I-type cell. Among
the cells in a single colony, many were HNK-1⁺, some were peripherin⁺, and very few
were immunoreactive for GFAP. This suggests that BE(2)-C I-type cells are not only
multipotent, but also can spontaneously differentiate along different pathways. In
addition, there were still many colony cells that failed to stain for any of the three
markers. These cells might include other subpopulations of cancer stem cells as well as
the cells spontaneously differentiating along pathways other than the neuroblastic and the
Schwannian/glial pathways. It should be noticed that although there were significant
differences in the marker expression among these colony cells, there were not obvious
morphological differences as are observed when differentiation of BE(2)-C cells were
induced by all-trans retinoic acid or BrdUrd. This suggests that during differentiation,
changes in marker expression are not necessarily accompanied by morphological
changes. The latter might occur in the relatively late phase or need additional factors such
as optimum substrate interactions (Tsai et al., 2002). Additionally, at this moment we do
not know whether the I-type neuroblastoma stem cells represent transformed neural crest stem cells or sympathetic progenitor cells, as both of them are expected to be multipotent. Further studies, including identification, isolation, and characterization of the I-type cells with stem cell properties, should shed lights on the origin of neuroblastoma cells.

A Subpopulation of BE(2)-C I-type Neuroblastoma Cells Possess Self-renewing Capacity

Self-renewing capacity is the other essential function of cancer stem cells. Self-renewal of stem cells is the ability to generate progeny cells with the same stem cell activities including self-renewal and differentiation. According to the cancer stem cell model, self-renewal of cancer stem cells is essential for tumor formation. To be cancer stem cells, I-type neuroblastoma cells must possess such property. At present, the commonly used in vitro method to determine the self-renewing capacity of a cell is the sphere formation assay (Reynolds et al., 1992). It has been found that a stem cell from nervous system can still survive, proliferate and form a sphere-like cell colony, called neurosphere, under suspension conditions, while other kinds of cells cannot survive or proliferate in suspension (Molofsky et al., 2003). The exact reason is unclear, although the anchorage-independent growth property of stem cells may be responsible for it (Cifone, 1982). In a neurosphere, there are two kinds of progeny of the original stem cell. Differentiation of the original stem cell produces various differentiated progeny neurosphere cells, which can be identified by detection of respective lineage specific markers, while its self-renewal produces a few stem cell progeny among neurosphere
cells, whose stem cell properties can be determined by secondary neurosphere formation assay. In secondary neurosphere formation assay, a primary sphere is dissociated into single cells and cultured in suspension again to determine if these progeny stem cells are still able to differentiate and self-renew to form similar neurospheres. Self-renewal of neural stem cells and neural crest stem cells has been successfully determined by this assay (Uchida et al., 2000; Molofsky et al., 2003). Recently, similar tumor sphere assays have been applied for cancer stem cells to detect their self-renewing capacity (Singh et al., 2003). Unfortunately, because BE(2)-C I-type cells could not form spheres in a variety of culture conditions, the soft agar colony formation assay was used to examine their self-renewing capacity. Normal cells from solid tissues cannot grow without attachment to a solid surface, whose growth is called anchorage-dependence. Malignant cells from solid tissues are able to grow in semisolid substrate such as soft agar, methylcellulose, or in suspension culture, which means their growth is anchorage-independent (Cifone, 1982). The soft agar or methylcellulose colony formation assay is usually used to detect the anchorage-independent growth and proliferation potential of cancer cells (Hamburger and Salmon, 1977; Buick et al., 1981; Thomson and Meyskens, 1982). It has been found that not all the cancer cells are able to form colonies in soft agar and that the colony-forming ability of cancer cells is positively correlated with their tumorigenic capacity (Freedman and Shin, 1974). According to the cancer stem cell model, only cancer stem cells would have the clonogenicity and tumorigenicity (Reya et al., 2001; Al-Hajj and Clarke, 2004). In the present study, the self-renewal capacity of cells was firstly detected by successive soft agar colony formation assays. So far, five
successive soft agar colony-growth assays were conducted. On average, there were about 5000 cells per harvested colony. To grow into a 5000-cell colony, a cell needs to divide at least 12 times \(2^{12}=4096\). Thus, for each soft agar colony-growth assay, BE(2)-C I-type cells have to proliferate for at least 12 generations, and for five successive soft agar colony-growth assays, BE(2)-C I-type cells have to self-renew for at least 60 generations (12x5). The results, therefore, were the first to demonstrate the long-term self-renewal capacity of human neuroblastoma I-type cells through clonal analyses in vitro. It was found that a subpopulation of BE(2)-C I-type cells were capable of maintaining and expanding themselves over an extended number of clonal passages in culture. Based on both morphology and expression of lineage-specific markers, their clonally derived colonies retained the ability to differentiate into neurons and Schwann/glial cells, two major cell types of the sympathetic nervous system. This provided further evidence of the presence of cancer stem cells in neuroblastoma I-type cell lines and confirmed and extended the original suggestion by Ross et al. (1995) and Walton et al. (2004). Of course, an optimum niche is necessary for stem cells to show their activities (Tsai et al., 2002). There are a lot of differences between in vitro and in vivo environments. In future studies, in vivo transplantation experiments would be performed to determine the tumorigenecity of BE(2)-C I-type cancer stem cells. Of note, in this study, cancer stem cells within I-type neuroblastoma cells were enriched through successive soft agar colony formation assays and their percentage in the I-type neuroblastoma cells were gradually increased from \(~27\%\) to \(~63\%\) (Figure 12). The current study also shows that although all the suggested cancer stem cells have colony forming ability, they formed colonies with
various sizes in soft agar. This indicates that there might be heterogeneous populations of cancer stem cells with different self-renewing ability and tumorigenic capacity.

**Isolation of the Cancer Stem Cell Population from BE(2)-C I-type Cells by the Expression of Surface Markers**

Until now, the expression of specific surface markers has been widely used to isolate various cancer stem cells (Lapidot et al., 1994; Bonnet and Dick, 1997; Holyoake et al., 1999; Al-Hajj et al., 2003; Singh et al., 2003, 2004; Matsui et al., 2004; Hotfilder et al., 2005). The most popular way to isolate cancer stem cells is by the expression of surface markers of normal stem cells and progenitors. The cancer stem cell model indicates that cancer stem cells may come from respective normal stem cells and/or progenitor cells with oncogenic mutations (Reya et al., 2001; Al-Hajj and Clarke, 2004). Thus, cancer stem cells may still express the normal stem cell-related specific markers. As mentioned in the Literature section, several types of cancer stem cells, such as those in acute and chronic myeloid leukemia and brain tumors, have been successfully identified and isolated by their expression of surface markers associated with their respective normal stem cells, CD34^+CD38^- and CD133^+ (Terstappen et al., 1991; Lapidot et al., 1994; Bonnet and Dick, 1997; Holyoake et al., 1999; Singh et al., 2003, 2004).

Since neuroblastoma originates from cells in the neural crest lineage, neural crest stem cell-related surface markers should be on the list of possible neuroblastoma stem cell markers, such as LNGFR p75, HNK-1 and PSA-NCAM (Mujtaba et al., 1998; Gage, 2000). Among them, LNGFR p75 was found to be widely expressed on human and rat...
neural crest stem cells and related progenitor cells, although it also was expressed on Schwann cells (Mujtaba et al., 1998; Vroemen and Weidner, 2003). Furthermore, David J. Anderson and his colleagues (Morrison et al., 1999) have successfully isolated rat neural crest stem cells by the expression of LNGFR p75. Unfortunately, BE(2)-C I-type cells expressed a low level of LNGFR p75 and isolation of cancer stem cells from BE(2)-C cells based upon its expression failed (data not shown). Another marker, PSA-NCAM, has been considered a specific marker for neural crest-derived neuron progenitor cells as opposed to neural crest stem cells (Alonso, G. 1999). Many BE(2)-C I-type cells strongly express PSA-NCAM and neuroblastoma stem cells might originate from sympathetic neuron progenitor cells; however, isolation of cancer stem cells from BE(2)-C cells by the expression of PSA-NCAM also failed (data not shown). Although previous studies about the carbohydrate epitope HNK-1 focused on the avian neural crest, it has been suggested that HNK-1 is also a marker for undifferentiated human and rat neural crest cells (Bronner-Fraser, 1987; Tucker et al., 1988). In this study, a subpopulation of BE(2)-C I-type cells expressed HNK-1 whereas, after induced differentiation along either the neuroblastic or the Schwann/glial pathway, the percentage of HNK-1+ BE(2)-C cells greatly decreased. The percentage of HNK-1+ BE(2)-C I-type cells was very similar to the proportion of cancer stem cells with colony forming ability in soft agar for BE(2)-C cells. Although all these findings suggested that HNK-1 might be a marker for BE(2)-C cancer stem cells, isolation of neuroblastoma stem cells by the expression of HNK-1 was unsuccessful. One of the possible reasons is that environment differences between in vitro and in vivo conditions alter levels of HNK-1 and thus the in vivo neural crest.
marker HNK-1 was not retained by cancer stem cells after in vitro culturing of neuroblastoma cell lines. Indeed, environmental factors play an important, maybe even a decisive role in the cellular fates, which of course includes expression of markers (Tsai and McKay, 2000; Tsai et al., 2002). Another possible reason for this failure is that expression of HNK-1 might not be specific for all the neural crest stem cells. It has been shown that HNK-1+ cells may be only a subpopulation of neural crest stem cells (Minarcik and Golden, 2003). In addition, HNK-1 is also expressed on a few types of neural crest derived cells other than neural crest stem cells (Tucker et al., 1984). These findings argue that all currently known neural crest stem cell-related surface markers may not be suitable for the isolation of neuroblastoma stem cell in BE(2)-C cell line, and isolation of cancer stem cells from in vivo animal neuroblastoma is more significant and will be essential.

Another way to isolate cancer stem cells is to select the malignancy-related surface antigens of tumors as possible markers of cancer stem cells. The cancer stem cell model indicates that only cancer stem cells are tumorigenic, so that the malignancy-related surface markers of tumors may be used to isolate cancer stem cells in tumors (Reya et al., 2001; Al-Hajj and Clarke, 2004). For example, cancer stem cells in breast cancer have been identified by the expression of the malignancy-related markers CD24 and CD44 (Al-Hajj et al., 2003). In this study, two malignancy-related markers, disialoganglioside GD2 and CD24, were selected as candidates for neuroblastoma stem cell markers. GD2 is a tumor-associated carbohydrate antigen expressed on human neuroblastoma, and shown to be responsible for cellular interactions (Schulz et al., 1984;
Schengrund and Shochat, 1988). High-risk neuroblastoma, including metastatic neuroblastoma, has been diagnosed by detecting the expression of GD2, and monoclonal GD2 antibodies have been used in the treatment of high-risk neuroblastoma (Sterba, 2002; Reynolds, 2004). Additionally, GD2 can be thought as a marker for neuron progenitor cells (Klassen et al., 2001). Heat stable antigen CD24, a cell adhesion molecule, binds its ligands such as P-selectin expressed on platelets, endothelial cells or other cells, to mediate cellular interactions, which may support its involvement in the tumor progression and metastasis (Kristiansen et al., 2004). It has been found that CD24 is strongly expressed in undifferentiated neuroblastoma and weakly expressed on immature neuroblasts, but is rarely expressed on mature neurons. This suggests that expression of CD24 is related to cellular differentiation status and neuroblastoma malignancy (Poncet et al., 1996; Kristiansen et al., 2004). In this study, it was found that CD24 and GD2 were strongly expressed on BE(2)-C I-type cells but not on differentiated S-type cells (data not shown), further supporting the interpretation that they are possible neuroblastoma stem cell markers. The present study shows that although the isolation of BE(2)-C cells by the expression of GD2 (data not shown) and CD24 enriched the cancer stem cells in BE(2)-C I-type cells, a nearly pure cancer stem cell population was not obtained. This can be explained by the fact that GD2 and CD24 also may be expressed on the neuroblast-like neuroblastoma cells besides cancer stem cells. However, neuroblastoma stem cells can be enriched using GD2 and CD24.
**Bmi-1 Regulates Self-renewal and Differentiation of Neuroblastoma Stem Cells in a Concentration-dependent Manner**

Our investigation reveals a critical role for Bmi-1 in controlling the stem cell activities of neuroblastoma I-type cells. The novelty of our findings is the demonstration that Bmi-1 acts as a concentration-dependent signal in control of the delicate balance between neuroblastoma I-type cell self-renewal and differentiation (Figure 25). Low concentrations of Bmi-1 biased the cells to choose the Schwann/glial fate, and high concentrations of Bmi-1 instructed the cells to differentiate along the neuronal pathway, whereas intermediate concentrations of Bmi-1 were essential for stem cell maintenance. This mode of Bmi-1 action is reminiscent of morphogen gradients in controlling cell fate decisions during embryonic development (Tabata, 2001). For example, in mammals the morphogen Sonic Hedgehog (Shh) is secreted by two ventral midline signaling centers in the neural tube, the notochord and the floor plate, which leads to the formation of an extracellular, ventral-to-dorsal concentration gradient of Shh. Neural progenitor cells in the ventral half of the neural tube choose between various neural fates that are specified by different thresholds of Shh signaling (Briscoe and Ericson, 1999). However, in this study, although changes of Bmi-1 concentrations induced changes of expression levels of specific cell lineage markers of BE(2)-C I-type cells, there were no obvious morphological changes observed. These results are the same as those observed for the spontaneous differentiation of BE(2)-C I-type cells, but different from those observed when the cells were induced to undergo differentiation. Possible explanations may include the idea that morphological changes might occur later than the marker expression
Figure 25. Bmi-1 Regulates Self-renewal and Differentiation of Neuroblastoma Stem Cells in a Concentration-dependent Manner.
changes during cell differentiation, and/or that additional conditions such as cellular interactions with optimum substrates might be needed for morphological changes (Tsai et al., 2002). To confirm that cellular differentiation is occurring, other studies have monitored the expression of another neuron specific marker, neurofilament 200 (NF200) (Groeneweg et al., 1993), an intermediate filament, after the Bmi-1 level in BE(2)-C I-type cells was upregulated or downregulated. Finding similar expression changes for NF200, as for peripherin, suggested that the changes in the cellular differentiation status with the different Bmi-1 levels may be real (data not shown). In the future, the expression of additional Schwann cell markers and neuroblastoma stem cell marker would need to be monitored under the same experimental conditions to further confirm this conclusion.
CONCLUSIONS

1. A subpopulation of I-type neuroblastoma cells were identified as clonogenic cancer stem cells. They possessed stem cell-like, long-term self-renewing capacity and stem cell-like differentiating ability. They can differentiate along either the neuroblastic or the Schwann/glial pathway.

2. Although the isolation of the cancer stem cell population from BE(2)-C I-type neuroblastoma cells by the expression of the neural crest stem cell marker HNK-1 was unsuccessful, selection of cells based on their expression of malignancy-related CD24 marker successfully enriched (three-fold) the cancer stem cell subpopulation compared to the original I-type cell population, suggesting that CD24 is a neuroblastoma stem cell-related marker.

3. Oncogene Bmi-1 regulates self-renewal and differentiation of neuroblastoma stem cells in a concentration-dependent manner. Low concentrations of Bmi-1 biased the cells to choose the Schwann/glial fate, high concentrations of Bmi-1 instructed the cells to differentiate along the neuronal pathway, and intermediate concentrations of Bmi-1 were essential for stem cell maintenance.
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Identification of the haematopoietic stem cell niche and control of the niche size.


ABSTRACT

Human neuroblastoma I-type cells have been proposed as a population of malignant neural crest stem cells, based on their high tumorigenic activity, expression of stem cell markers, and ability to differentiate to cells of neural crest lineages, including neuroblastic (N-type) and myoblastic/glial/melanocytic (S-type) cells. My findings demonstrated that some of the I-type cells were cancer stem cells with self-renewal capacity and multipotent differentiation potential, and CD24 was a neuroblatoma stem cell-related marker and could be used to enrich cancer stem cells in the BE(2)-C I-type cell line by cell sorting. This study also showed that expression of neuroblastoma stem cell activities required the polycomb family transcriptional repressor Bmi-1. Bmi-1 expression levels were found to exert an “instructive” influence on lineage commitment by the I-type cells. Differentiation of I-type cells to S-type cells was accompanied by a marked decrease in Bmi-1 expression, and experimental downregulation of Bmi-1 in I-type cells was found to promote their differentiation along the Schwann/glial (S-type) pathway. In contrast, N-type cells derived from I-type cells expressed higher levels of Bmi-1, and experimental upregulation of Bmi-1 was found to induce the I-type cells to differentiate along the neuronal (N-type) pathway. Thus, the results of this study identified the existence of a complex mechanism in these cells that was dependent upon the concentrations of a protein, Bmi-1, for both self-renewal property and for the outcomes of their differentiation programs.