The common basis of sympathetic nervous system and neuroblastoma development

Huilin Shi

Medical University of Ohio

Follow this and additional works at: http://utdr.utoledo.edu/theses-dissertations

Recommended Citation
http://utdr.utoledo.edu/theses-dissertations/1134

This Dissertation is brought to you for free and open access by The University of Toledo Digital Repository. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of The University of Toledo Digital Repository. For more information, please see the repository's About page.
Investigation of common bases of sympathetic nervous system and neuroblastoma development

Submitted by:
Huilin Shi

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

<table>
<thead>
<tr>
<th>Examination Committee</th>
<th>Signature/Date</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Major Advisor:</strong></td>
<td></td>
</tr>
<tr>
<td>Han-Fei Ding, Ph.D.</td>
<td></td>
</tr>
<tr>
<td><strong>Academic Advisory Committee:</strong></td>
<td></td>
</tr>
<tr>
<td>William Maltese, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Manohar Ratnam, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Ivana de la Serna, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Zi-jian Xie, Ph.D.</td>
<td></td>
</tr>
<tr>
<td>Hongjuan Cui, Ph.D.</td>
<td></td>
</tr>
</tbody>
</table>

Senior Associate Dean
College of Graduate Studies
Michael S. Bisesi, Ph.D.

Date of Defense: April 30, 2009
The common basis of sympathetic nervous system
and neuroblastoma development

Huilin Shi
Cancer Biology Track
College of Medicine
University of Toledo
2009
ACKNOWLEDGMENTS

I would like to sincerely thank my major advisor, Dr. Han-Fei Ding, for his patience, encouragement, support and incredible guidance in my five years Ph.D. study. His perpetual enthusiasm, abundant knowledge, outstanding scientific thinking and hardworking attitude have always motivated me to make progress in the research and in the future career.

I would like to especially thank my co-advisor Dr. William Maltese for his supervision, guidance and invaluable support in the fifth year of my study and helping me complete the work in manuscript two.

I would like to express my gratitude to my committee members: Dr. Manohar Ratnam, Dr. Zi-Jian Xie, Dr. Ivana de la Serna and Dr. Hongjuan Cui, for their precious and extensive personal and professional guidance in my study. They teach me diverse thinking styles in science and in real life that make me more open-minded.

I would like to acknowledge Dr. William Gunning for teaching me solid knowledge of microscopy and making me adequate for doing the research in my first manuscript while providing pathological supplies in my research. I would also like to thank Dr. David Giovannucci and Dr. Andrea Nestor for their help in confocal microscopy. I am grateful to Dr. Ming Zhang for his advice and help in the histological analysis of my first paper.
I would also like to give my appreciation to all the members in Dr. Maltese’s lab: Dr. Jean Overmeyer, Ashley Young, Dr. Aparna Kaul, Kristen Koterba and Haymanti Bhanot, for their kindness, patience and generous technical assistance.

I would like to thank Jane Ding for her hard work and providing us an excellent research support. I’m very grateful to other lab colleagues, Goleeta Alam, Dr. Baochun Zhang, Dr. Zhe Wang, Dr. Jun Ma, Liqun Yang, who have supported me with technical assistance, encouragement and friendship.

I would like to give my special thankfulness to my English teacher and friend, Dr. Andy Chermak and his wife Dr. Shelley Green for their warm support in my personal life and helping me dissolving a lot of language problems.

At last, I would like to give my gratefulness to my parents for their complete care and understanding.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iv</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature</td>
<td>8</td>
</tr>
<tr>
<td>Manuscript One</td>
<td>52</td>
</tr>
<tr>
<td>Nestin expression defines both glial and neuronal progenitors in postnatal sympathetic ganglia</td>
<td></td>
</tr>
<tr>
<td>Manuscript Two</td>
<td>90</td>
</tr>
<tr>
<td>GATA3 regulation of human neuroblastoma stem cell activities</td>
<td></td>
</tr>
<tr>
<td>Summary/Discussion</td>
<td>148</td>
</tr>
<tr>
<td>Conclusions</td>
<td>152</td>
</tr>
<tr>
<td>Bibliography</td>
<td>154</td>
</tr>
<tr>
<td>Abstract</td>
<td>195</td>
</tr>
</tbody>
</table>
INTRODUCTION

The sympathetic nervous system originates from ventrally migrating neural crest cells that are derived from the dorsal neural tube, including sympathetic ganglia and adrenal medulla. Two chains of sympathetic ganglion primordia are formed along the both sides of the dorsal aorta in the early embryonic development. Through a series of differentiation processes, noradrenergic neuroblasts and glial progenitors are developed from neural crest cells and coalesce to form definitive sympathetic ganglia. Sympathetic ganglia have been widely used as a model in studying the neurogenesis (Schafer et al., 1997; Stanke et al., 1999; Tsarovina et al., 2004), such as mechanisms under the autonomic neuronal differentiation (Goridis and Rohrer, 2002; Howard, 2005), signals controlling the sympathetic ganglion development (Glebova and Ginty, 2005) and functions of ganglion cells (Hirst and McLachlan, 1984). In 1991, Hall and Landis found that in the embryonic rat superior cervical ganglia (SCG), the development of neuronal and glial cell precursors occurs with temporal differences. Neurogenesis peaks around embryonic day 14.5 (E14.5) and is completed around E17.5 (Hall and Landis, 1991). However, glial progenitor cells proliferate intensely during E16.5 to E18.5 and thereafter continue proliferating but at a slower rate (Hall and Landis, 1992). These previous studies propose the following questions: Are there any neural progenitor cells in the postnatal sympathetic ganglia? What is the pattern of gliogenesis and neurogenesis in the postnatal sympathetic development? Are there any specific molecular markers to identify progenitor cells in the sympathetic ganglia?
To answer these questions, we investigated the proliferation and differentiation status in the postnatal mouse SCG at different time points from newborn to two months. Nuclear antigen Ki67 was used as a proliferation marker to detect proliferating cells in SCG, which is expressed in the G1, S, G2 and M phases of the cell cycle, but not in G0 (Sawhney and Hall, 1992). The differentiation process was determined by bromodeoxyuridine (BrdU) tracing. The major action of BrdU is to incorporate into DNA at S phase through acting as a thymine analogue and can transfer to progeny cells during cell division (Matsuoka et al., 1990). Colocalization of BrdU with different cell type markers in a sequential time course identifies cell differentiation.

We found that Ki67-positive proliferating cells show a gradually decreasing process during postnatal three weeks. The majority of Ki67-positive cells are coexpressed with brain lipid-binding protein (BLBP), a glial cell marker labeling both glial progenitors and fully differentiated mature glial cells. The expression level of BLBP remains in a stable status during the postnatal period. Therefore, postnatal development in the mouse SCG is characterized by gliogenesis. We also use another glial cell marker, S100, to trace postnatal gliogenesis. S100 expression exhibits a gradually increasing pattern postnatally. Its expression starts to be detected around postnatal day 4 (P4), peaks around P18 and persists thereafter. During P7 to P18, a small number of Ki67 and S100 double-positive cells are observed which confirms that S100 specifically labels the mature glial cell group. We used tyrosine hydroxylase (TH) as a neuronal marker that is a specific marker for sympathetic neurons because it is the first and rate-limiting enzyme in synthesizing the catecholamine neurotransmitters. To our surprise, there are still a small percentage of
Ki67 and TH double-positive cells during postnatal week one. These observations delineate different proliferation patterns of gliogenesis and neurogenesis in the postnatal sympathetic ganglia. During the differentiation study by BrdU tracing, we observed that a population of BrdU and BLBP double-positive cells can differentiate into a group of BrdU and S100 double-positive mature glial cells, confirming the dominant role of gliogenesis in postnatal sympathetic development.

We also identified a new glial progenitor cell marker, nestin, previously a neural progenitor cell marker in the central nervous system (CNS) and in neural crest cells (Lendahl et al., 1990; Lothian and Lendahl, 1997). The expression of nestin is highest at birth and gradually decreases during postnatal three weeks similar to the pattern of Ki67 expression. The majority of nestin positive cells are also Ki67 positive, which indicates that nestin-positive cells represent a progenitor proliferating cell population in postnatal sympathetic development. In the differentiation study, similar to Ki67, approximately 95% of the BrdU positive cells are co-stained with nestin. BrdU and nestin double-positive cells are extensively coexpressed with BLBP and sparsely colocalized with TH which demonstrates that nestin is a marker for both glial and neuronal progenitors.

Our findings provide nestin as a molecular marker that defines the distinct sympathetic progenitor cell population. The detailed characterization of the cellular basis for the postnatal sympathetic development using different population markers provides a foundation for the further investigation of genes and signaling pathways that regulate the developmental process.

Neuroblastoma is the most common solid malignant tumor in infancy and childhood which originates from the sympathetic nervous system, occurring in
sympathetic ganglia and adrenal medulla (Brodeur, 2003). 90% of children with this disease are diagnosed before 6 years of age (Schwab et al., 2003). Therefore the tumorigenesis of neuroblastoma may represent an abnormal sympathetic development in the embryonic or postnatal phase.

The cancer stem cell model reflects a new viewpoint on tumorigenesis. Cancer stem cells are cancer cells that generate tumors through their stem cell abilities – self renewal and differentiation – and give rise to all the cell types found in a particular tumor. These cells are proposed to persist in tumors as a distinct population to give rise to new tumors and cause relapse and metastases. Since neuroblastoma is one of the few cancers occurring in infancy and childhood, it is possible that neuroblastoma originates from transformed neural crest stem cells or malignant sympathetic progenitor cells that obtain the stem cell properties through mutations.

Neuroblastoma stem cells have been demonstrated to exist in neuroblastoma tumors (Hirschmann-Jax et al., 2004; Walton et al., 2004). I-type (intermediate) neuroblastoma cell lines have been proven to be one kind of neuroblastoma stem cells based on their self-renewal and differentiation ability. I-type neuroblastoma cells exhibit dramatically higher tumorigenicity in both soft agar assays and immunodeficient mice compared with N-type (neuroblastic) and S-type (non-neuronal, substrate-adherent) neuroblastoma cell lines (Cui et al., 2006; Spengler et al., 1997).

Therefore, it is important to investigate the mechanisms under the regulation of neuroblastoma stem cells in order to better understand the origin and development of this disease and establish effective clinical therapies targeting the root of neuroblastomas. Of the thousands of regulators, we chose GATA3 as the one to
investigate its regulation in neuroblastoma stem cell activities. I chose GATA3 based on following reasons:

First, GATA3 belongs to the GATA family that contains 6 members of zinc finger transcription factors (GATA1 to 6) in vertebrates (Patient and McGhee, 2002). GATA family members play important roles in vertebrate development. The mutation and expression modulation of GATA family members can induce human diseases. A GATA1 mutation results in dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000). The HDR syndrome – hypoparathyroidism, deafness, and renal dysplasia – is caused by a GATA3 mutation (Van Esch et al., 2000). A GATA4 mutation induces congenital heart defects (Garg et al., 2003). GATA3 plays an essential role in the development of many tissues in vertebrates, including the Th2 cell differentiation, the development of the kidney, the inner ear, embryonic and pubertal mammary glands, adipocytes and neurons, et al. (Asselin-Labat et al., 2007; Grote et al., 2008; Kouros-Mehr et al., 2008; Lillevall et al., 2006; Smith et al., 2002; Tong et al., 2005; van Doorninck et al., 1999; Yamashita et al., 2005).

Second, GATA3 is essential for normal sympathetic development. The bone morphogenetic proteins (BMPs) secreted from cells of the dorsal aorta triggers the expression of downstream transcription factors – Phox2b, Mash1, Phox2a, dHand and GATA3 in the neuronal progenitor cells and leads the cells to gain the characteristics of differentiated sympathetic neurons (Howard, 2005). GATA3 controls sympathoadrenal differentiation by mutual modulations with other sympathetic regulators (Moriguchi et al., 2006). In the mouse sympathetic system, GATA3 knockout embryos die by E11 due to noradrenaline deficiency and loss of tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH) (Lim et al., 2000). Loss of
GATA3 results in the shrinkage of the ganglion size and induces apoptosis in the sympathetic ganglia and adrenal glands (Moriguchi et al., 2006; Tsarovina et al., 2004).

Third, GATA3 also plays different roles in tumorigenesis in multiple tumors, such as T cell acute lymphoblastic leukemias (T-ALL), breast and pancreatic cancers, cervical and esophageal carcinomas (Gulbinas et al., 2006; Minegishi et al., 1997; Shiga et al., 1993; Steenbergen et al., 2002; Usary et al., 2004).

Since GATA3 is an essential regulator in the vertebrate development and plays an important role in the sympathetic development and tumorigenesis, we proposed the hypothesis that GATA3 has important functions in regulating the neuroblastoma stem cell activities. We use BE(2)-C cells as an I-type neuroblastoma stem cell model to investigate the role of GATA3 in this system.

We found that loss of GATA3 induces the increased tumorigenicity of BE(2)-C cells, whereas overexpression of GATA3 decreases the tumorigenic ability of BE(2)-C cells as evidenced by soft agar assays. The clonogenic assay, MTT assay and cell growth assay were performed to confirm the effects of the increased proliferative potential due to GATA3 deficiency in BE(2)-C cells. Through western blot analyses between GATA3 knockdown cells and the control cells, we observed that Cyclin D1 expression is highly upregulated and the E2F1 expression is dramatically downregulated in GATA3 knockdown cells. Therefore, Cyclin D1 and E2F1 may be the downstream targets of GATA3 in the increased proliferative status. In the differentiation study, we demonstrated that loss of GATA3 induces glial differentiation in BE(2)-C cells, as evidenced by the upregulation of the glial cell marker glial fibrillary acidic protein (GFAP) and the downregulation of the neuronal
marker SNAP25. Additionally, GATA3 overexpression promotes neuronal
differentiation in BE(2)-C cells as indicated by the downregulation of GFAP and the
upregulation of SNAP25. The regulation of the differentiation might be controlled by
the modulations between GATA3 and other sympathetic regulators, such as Phox2b
and Mash1.

In summary, GATA3 deficiency induces glial differentiation in BE(2)-C cells
with increased tumorigenicity and GATA3 overexpression promotes neuronal
differentiation accompanied by a decreased proliferative potential in BE(2)-C cells.
These findings delineate the role of GATA3 in the regulation of the BE(2)-C cell
tumorigenic and differentiative status. The role of GATA3 in the I-type
neuroblastoma stem cell activities indicates that loss of GATA3 may contribute to the
tumorigenesis, dissemination and metastasis of neuroblastoma.
Tumorigenesis

Tumorigenesis is a multiple gene mutation process caused by interactions between genetics and environment which creates progressively unrestrained proliferation of transformed malignant cells (Kinzler and Vogelstein, 1996). Though derived from normal cells, cancer cells acquire 6 distinguished capabilities “self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential (immortalization), evading programmed cell death (apoptosis), sustained angiogenesis, and tissue invasion and metastasis” (Hanahan and Weinberg, 2000). Through these acquired abilities, cancer cells undermine the balanced regulation between cell birth and death and induce neoplastic transformation.

The basis of loss of growth regulation that gives rise to cancer is genetic mutation, which involves two broad classes of genes: proto-oncogenes and tumor suppressor genes. Functionally, the proteins encoded by both classes of genes mostly regulate the cell cycle, apoptosis and damaged DNA repair. Regulation of the eukaryotic cell cycle is maintained through complex and delicate mechanisms. Both positive- and negative-acting regulators tightly control the progression through each phase of the cell cycle - G1, S, G2 and M phase - which are prime targets for cancer-causing mutations. The restriction point is a late G1 phase checkpoint in the cell cycle. Cells that progress through this point are irreversibly committed to enter S phase, where DNA synthesis and replication will occur. D-type cyclins, cyclin-dependent kinases (CDKs), and the retinoblastoma protein (pRb) are the regulators that control
the passage through the restriction point. The Cyclin D expression is induced upon stimulation by many growth factors or mitogens. The CDK4 and CDK6 can partner with Cyclin D to form catalytically active cyclin-CDK complexes, which promotes the passage through the restriction point. Active Cyclin D/CDK4, Cyclin D/CDK6 and other cyclin/CDK complexes catalyze the complete phosphorylation of pRb (Diehl, 2002). The completely phosphorylated pRb releases the active E2F transcription factors, which are important in the regulation of gene transcription required for DNA synthesis, therefore cells irreversibly enter into S phase (Weinberg, 1995). The p16 functions as a CDK inhibitor and specifically binds to CDK4 and CDK6 thus leading to G1 arrest by inhibiting their kinase activities (Aprilikova et al., 1995) (Figure 1). Genetic mutations occurring in this pathway or the mutations in upstream regulators of this pathway which lead to unregulated passage from G1 to S phase are oncogenic.

Amplification or overexpression of Cyclin D1 is important in the development of many cancers including parathyroid adenoma, lymphoma, breast and prostate cancers (de Boer et al., 1997; Drobnjak et al., 2000; Hsi et al., 1996; Musgrove et al., 1994). The p16 belongs to the family of tumor suppressor genes. The loss-of-function of p16 occurs in several human cancers, such as melanoma, breast and lung cancers (Belinsky et al., 1998; Freedberg et al., 2008; Milde-Langosch et al., 2001). Mutations of both Rb alleles lead to retinoblastoma in childhood. Studies stated that only one working allele of the Rb gene is necessary for its function, so both need to be mutated before the cancer phenotype appears. Loss of the Rb gene function in later life commonly combines with other gene mutations in cancers and plays a less important role (Chau and Wang, 2003). The E2F genes are transcriptionally regulated by several
repressors, such as pRb, Swi/Snf complexes (Gunawardena et al., 2007). Increased proliferative effects of E2F proteins due to loss-of-function of repressors induce passage through the cell cycle and lead to oncogenesis. The G1 checkpoint is important for inhibiting cells with damaged DNA from entering the S phase. As “the guardian of the genome”, p53 plays an important role in this checkpoint by activating the expression of genes encoding proteins that sustain arrest in G1 and G2, promote apoptosis or exert DNA repairs. The loss-of-function mutation of p53 occurs in multiple cancers due to uncontrolled regulation of the G1 checkpoint and apoptosis (Davidoff et al., 1991; Nozaki et al., 1999). Therefore, tumorigenesis is controlled by a complicated regulatory network inducing unbalanced control of cell birth and death which provides the foundation for anti-cancer therapies.
Unphosphorylated Rb protein binds transcriptional factors collectively called E2F and thereby prevents E2F-mediated transcriptional activation of many genes whose products are required for DNA synthesis (e.g., DNA polymerase). The kinase activity of cyclin D-CDK4 phosphorylates Rb, thereby activating E2F; this kinase activity is inhibited by p16. Overproduction of cyclin D, a positive regulator, or loss of the negative regulators p16 and Rb, commonly occurs in human cancers. Adopted from Lodish, H. F. (2003). Molecular cell biology, 5th edn (New York, W.H. Freeman and Company), 958.
**Stem Cells**

The classic definition of a stem cell is that a cell has both capacities of self-renewal and differentiation into progeny cells. According to the differentiation potency, stem cells can be classified into four groups. Totipotent stem cells are fertilized oocytes (the zygotes) that have the capability to form all embryonic and extraembryonic cell types. Pluripotent stem cells are the cells arising from any of the three germ layers and are the descendants of totipotent stem cells, which have the ability to differentiate into almost all types of cells. Multipotent stem cells can differentiate into a limited range of cell lineages, based on which, most tissues are formed. Unipotent stem cells are able to produce only one cell type, such as muscle stem cells and epidermal stem cells, whereas they still keep the ability of self-renewal which distinguishes them from non-stem cells (Preston et al., 2003). There are usually several intermediate cells of increasing differentiated status, which are progenitor cells, between stem cells and their terminal progeny cells. Therefore, stem cells reflect a relatively undifferentiated cell status and are the source of committed cells, whereas they are not able to exert the special functions of progeny cells.

In addition to self-renewal and differentiation, stem cells also have other properties which may only apply to certain tissues, including the capability to go through asymmetric cell divisions; display extensive self-renewal ability; exist in a mitotically quiescent status; and regenerate all types of cells of the tissue in which they exist (Hall and Watt, 1989; Potten and Loeffler, 1990). These aspects exhibit the variety of stem cell properties besides most fundamental questions. The variety of stem cell properties reflects the difficulties in reaching a comprehensive definition of a stem cell.
Symmetric Versus Asymmetric Divisions

The self-renewal ability of a stem cell is often considered through the asymmetric division which produces one stem cell (itself) and one committed progenitor cell (Figure 2b). But this kind of division is not exclusive in the stem cell self-renewal. Some stem cells can undergo symmetric divisions (Figures 2a and 2c), allowing the pool expansion of stem cells or the production of two differentiated daughter cells at expense of the stem cell depletion (Potten and Loeffler, 1990). The clear examples are including the divisions in Hirudo medicinalis, Drosophila melanogaster, Caenorhabditis elegans and the mammalian system. These two kinds of symmetric divisions exist in one population and are regulated by the factors that control the probability of self-renewal versus differentiative divisions. If the probability is 0.5, the proportion of stem cells and differentiated cells remains in a steady state in this population which can’t be distinguished from the population behavior in Figure 2b. However, there is plenty of evidence that the size of stem cell population changes in natural environments and the probability of self-renewal versus differentiative divisions could be different from 0.5. For example, the fetal liver hematopoietic stem cells (HSCs) double their absolute number daily during mid-gestation phase (Morrison et al., 1995a); the long-term self-renewing HSCs increase their absolute number more than five-fold during adult life in mice (Morrison et al., 1996). Therefore, the symmetric divisions are fundamental for the stem cell number regulation which is impossible in strictly asymmetric divisions. Lastly, stem cells can exist in a quiescent status under the control of niche factors, exhibiting no self-renewal and differentiation (Figure 2d).
A stem cell can self-renew by dividing symmetrically to generate two stem cells (a), or asymmetrically to generate a stem cell and a restricted progenitor (b). A loss of either developmental potential (c) or the ability to proliferate (d) results in a failure of stem cells to self-renew. Maintenance of developmental potential means maintenance of pluripotentiality in all kinds of stem cells. Adopted from Molofsky, A. V., Pardal, R., and Morrison, S. J. (2004). Diverse mechanisms regulate stem cell self-renewal. Curr Opin Cell Biol 16, 700-707.
**Self-Renewal Capacity**

There is an assumed concept that the self-renewal ability of stem cells lasts for the whole life of the organism. However, there is ample evidence to support that not all stem cells possess unlimited potential of self-renewal (Abkowitz et al., 1990; Margolis and Spradling, 1995; Morrison et al., 1995b). First, though there are adult stem cells in the organisms, some of them simply persist in a quiescent state under most conditions, as shown by the astrocyte-like stem cells in the mouse hippocampus (Ermini et al., 2008). Second, the active stem cells during the development exist transiently. For example, oocytes and sperms which are both derived from primordial germ cells start to behave differently from birth, though their stem cell properties in early gestation can’t be distinguished from females and males. Oocyte number is stable since birth, while sperm number keeps increasing into adulthood (Donovan, 1994). Thus, no matter whether they are embryonic or adult stem cells, their self-renewal states are different during the life, but do not last for life.

**Mitotic Quiescence**

Dividing slowly and rarely seems a characteristic of stem cells, but it is not completely true, as evidenced by the mammalian intestinal crypt stem cells and the Drosophila ovary somatic stem cells. Their dividing rate is demonstrated to be once every 12 hours (Margolis and Spradling, 1995; Potten and Loeffler, 1990). It is generally true that adult stem cells divide slowly, such as the stem cells in the skin (Lavker et al., 1993) and bone marrow (Morrison and Weissman, 1994), but which can’t include all the stem cell division statuses in adults.

**Regenerative Capacity**
It is well known that some stem cells only display regenerative capacity conditionally, such as in the case of tissue damage. However, it doesn’t mean that there are no stem cells in non-regenerative tissues, like the brain (Alvarez-Buylla and Lois, 1995; Gage et al., 1995). Reasons for the regeneration failure may derive from lack of space, connective tissues or factors inducing the rebuilding of the cell arrangement or promoting differentiation in the injured tissues, but not because of the absence of pluripotent stem cells.

The regulation of stem cell self-renewal is controlled by both extrinsic and intrinsic factors and involves multiple pathways at the genetic level. Some factors are also related to differentiation pathways, whereas others are not.

The interplay network among Nanog, Oct4, leukemia inhibitory factor (LIF) signaling and bone morphogenetic protein (BMP) signaling is essential for the regulation of embryonic stem (ES) cell self-renewal and the decision whether to self-renew or differentiate (Chambers et al., 2003; Mitsui et al., 2003; Ying et al., 2003). Oct4 and Nanog are intrinsic transcription factors that are only expressed in the inner cell mass of blastocysts and in the germline in vivo (Palmieri et al., 1994) and are necessary for the maintenance of pluripotency in the inner cell mass (Chambers et al., 2003; Mitsui et al., 2003). Other transcription factors, like Foxd3, can regulate self-renewal in both ES cells and somatic stem cells at a later developmental stage, such as neural crest cells (Hanna et al., 2002). The underlying mechanisms for Foxd3 to promote self-renewal are to inhibit differentiation in both pluripotent and multipotent stem cell contexts (Dottori et al., 2001; Kos et al., 2001). In vitro studies proven that LIF activity is dependent on BMP level in the serum. LIF promotes the self-renewal of mouse ES cells in cultures through Stat3 activation (Niwa et al., 1998), whereas
BMP exerts its function through inhibiting the neurogenic transcription factors which promote neuronal differentiation (Ying et al., 2003).

The Notch, Wnt and Sonic hedgehog (Shh) signaling pathways can regulate self-renewal in many kinds of stem cells and their functions are usually context-dependent. Notch is an important factor in diverse stem cell niches in regulating self-renewal abilities. Notch activation promotes the neural stem cell self-renewal under some conditions (Hitoshi et al., 2002), whereas it promotes glial differentiation under other conditions (Morrison et al., 2000; Scheer et al., 2001). The Wnt signaling pathway regulates the self-renewal of the intestinal epithelial and skin stem cells by controlling their proliferation and migration (Batlle et al., 2002; Huelsken et al., 2001). The Shh signaling is essential for maintaining neural stem cells in multiple regions of the mammalian central nervous system (CNS) (Lai et al., 2003; Machold et al., 2003), which is independent of its patterning effect on the nervous system.

The Mel-18, Rae-28 and Bmi-1 belong to polycomb family members, which assemble into large protein complexes to repress transcriptions by modulating chromatin structures (Jacobs and van Lohuizen, 2002). Although Mel-18, Rae-28 and Bmi-1 are components of the polycomb complex, they play different roles in self-renewal regulation. The self-renewal ability of fetal HSCs increases in Mel-18 deficient mice (Antonchuk et al., 2002) whereas decreases in Rae-28 deficient mice (Ohta et al., 2002). Bmi-1 has little effect on the embryonic development but plays an essential role in the postnatal maintenance of hematopoietic and neural stem cells through cell cycle regulators p16\(^{ink4a}\) and p19\(^{arf}\) (Jacobs et al., 1999a; Molofsky et al., 2003; Park et al., 2003).
The CDK inhibitors which regulate the cell cycle progression into G1 phase are also important in regulating self-renewal in multiple kinds of stem cells. The early G1-phase regulator \(p18^{\text{Ink4C}}\) and the late G1 phase regulator \(p21^{\text{cip1}}\) both decrease the self-renewal in HSCs (Cheng et al., 2000b; Yuan et al., 2004). The difference is that \(p18^{\text{Ink4C}}\) deficiency induces unlimited expansion of stem cells, whereas loss of \(p21^{\text{cip1}}\) leads to transient stem cell expansion (Cheng et al., 2000b). The \(p21^{\text{cip1}}\) and \(p27^{\text{kip1}}\) regulate different hematopoietic cell populations. The \(p21^{\text{cip1}}\) regulates the self-renewal of HSCs, but not the proliferation of restricted hematopoietic progenitor cells, whereas the \(p27^{\text{kip1}}\) doesn’t control the self-renewal of HSCs but does regulate the proliferation of restricted hematopoietic progenitor cells (Cheng et al., 2000a; Cheng et al., 2000b).
Cancer Stem Cell and Tumorigenesis

A cancer stem cell can be defined as “a cancer cell that has the ability to self-renew, dividing into another malignant stem cell and a cell that gives rise to the phenotypically diverse tumor cell population” (Bjerkvig et al., 2005). As we know, stem cells in different tissues can self-renew and differentiate into multiple tissue specific cell types based on their intrinsic properties (Al-Hajj and Clarke, 2004). It is also well known that most tumors are heterogeneous, comprised of diverse cell populations which are different in the morphology, proliferative potentials, metastatic capacities as well as tumor reconstitution abilities on transplantation.

Cancer stem cells were first identified in leukemias in 1997 by John Dick and colleagues at the University of Toronto. They harvested leukemic cells from patients and found that only a small subset of cancer cells was capable of forming the same cancer in immunodeficient mice (Bonnet and Dick, 1997). Cancer stem cells have been isolated and amplified by surface makers on their membranes (Blair et al., 1997; Jordan et al., 2000). A subpopulation of cells from human acute myeloid leukemia (AML) expressing a CD^{34+}/CD^{38-} phenotype were identified as leukemia-initiating cells by transplantation assay. Another subpopulation of cells - CD^{34+}/CD^{38+} leukemic cells couldn’t initiate leukemia in immunodeficient mice in most cases, though they exhibit a leukemic blast phenotype (Bonnet and Dick, 1997; Sutherland et al., 1996). On transplantation, cancer-initiating cells produce tumors composed of both new cancer stem cells and diverse heterogeneous differentiated non-tumorigenic cells with limited proliferative potentials. The constitution of tumor is similar to the developmental hierarchy in the tissues from which the cancers arose. Since then, cancer stem cells have been identified in multiple cancers, such as brain, breast, colon,
ovary, pancreas and prostate cancers (Al-Hajj et al., 2003; Li et al., 2007; Maitland and Collins, 2008; O'Brien et al., 2007; Singh et al., 2003; Zhang et al., 2008). Cancer stem cells are widely recognized as the tumor-initiating source as opposed to the conventional cancer model.

As shown in Figure 3, in the conventional model of cancer, tumors are composed of different cell types with diverse genetic backgrounds and each type of cells can divide and initiate tumors. However, this can’t explain why tumors which had been shrunk to nothing after the first round treatment would soon recur. The mysterious cell population which researchers and therapists overlooked was possibly cancer stem cells. In the cancer stem cell model, tumors comprise diverse cell types including cancer stem cells and only cancer stem cells can initiate tumors, but not other cell types (Abbott, 2006). According to this model, cancer stem cells have particular properties which distinguish them from other cell types in tumors and these stem cells are resistant to typical therapies. They lurk for months or years, re-seed cancers and cause the recurrence usually in a more aggressive way. This theory sheds light on the “the root of the problem (tumorigenesis)” and provides a new way for tumor therapies - treatments to target the cancer stem cell subpopulation according to their specific properties, without damaging normal stem cells, and thus prevents tumor recurrence.
Conventional model of cancer is based on the ability of every cancer cell to form new tumors and current therapy targets the main body of tumor cells (a). But new cancer stem cell model suggests that only a subset of tumor cells which possess the stem cell properties can form new tumor. If target this specific cell group, the tumor recurrence will be prevented. Adopted from Abbott, A. (2006). Cancer: the root of the problem. Nature 442, 742-743.
The exact origin of cancer stem cells in tumors is not quite clear yet. They could derive from normal stem cells that have accumulated oncogenic mutations over time and are malignantly transformed. This idea is proven by the evidence that many kinds of cancer stem cells express the cell surface markers which are also found in normal stem cells (Fillmore and Kuperwasser, 2007; Reiter et al., 1998). But normal stem cells might not be the only origin of cancer stem cells. The origin of tumor cells could be a more differentiated cell which acquires the continuous self-renewal ability and gains stem cell properties (Cozzio et al., 2003). Krivtsov and colleagues found a leukemia-associated fusion gene that functions to turn non-stem cells into cells acquiring stem cell behaviors and thus induce tumor formation (Krivtsov et al., 2006). This gene is produced by the ‘mixed lineage leukemia’ gene (MLL) fusing with AF9 gene and is closely related to the development of AML (Daser and Rabbitts, 2004).

Myeloid progenitor cells are derived from HSCs and differentiate into further specialized progenies, so they don’t possess self-renewal ability and are normally destined to produce mature white blood cells or myeloid cells (Akashi et al., 2000). The MLL-AF9 fusion gene induces myeloid progenitor cells to aberrantly express a specific set of ‘stem cell genes’ and turns them into cancer stem cells capable of initiating, maintaining and propagating the leukemia (Krivtsov et al., 2006).

It has been proven that several pathways that regulate normal stem cell self-renewal are shared by neoplastic proliferation, but in a deregulated mutated style, such as Notch, Wnt, Shh, Pten, Bmi-1 signaling pathways. These signaling pathways have been demonstrated to regulate self-renewal in somatic stem cells and also contribute to the carcinogenesis in the same tissue when these pathways are
deregulated (Table 1) (Di Cristofano and Pandolfi, 2000; Lessard and Sauvageau, 2003; Pear et al., 1996; Polakis, 1999; Wetmore, 2003).

Notch activation has been shown to consistently increase the amount of primitive progenitor cells in the HSC culture, which suggests that Notch signaling upregulates the HSC self-renewal or maintains the multipotentiality of hematopoietic progenitor cells (Karanu et al., 2000; Varnum-Finney et al., 2000). Moreover, the constitutively activated Notch pathway is linked to a subset of acute T-cell lymphoblastic leukemias (Pear et al., 1996). β-catenin is the downstream molecule of Wnt signaling and located in the cytoplasm. Constitutive activation of β-catenin promotes the self-renewal of CNS and keratinocyte stem cells and induces CNS and skin tumors (Gat et al., 1998; Zurawel et al., 1998). In the intestinal epithelial stem cell niche, Wnt signaling regulates stem cell self-renewal primarily through two downstream pathways. One is EPH-family adhesion molecules (Batlle et al., 2002) which control the migration out of niche (crypt) and induce differentiation. The other is c-Myc (van de Wetering et al., 2002) which promotes proliferation. Wnt signaling activates the same downstream molecules in colorectal cancers (van de Wetering et al., 2002) and causes the hyperproliferation of crypt progenitor cells, producing polyps comprising multiple lineage progenies (Moser et al., 1992; Powell et al., 1992). Wnt signaling is also implicated in hematopoietic malignancies based on its property to promote self-renewal in HSCs (Chung et al., 2002; Qiang et al., 2003). Shh signaling is essential for the cerebellum development. Shh proteins are secreted by Purkinje cells and support the proliferation of granule-cell precursors at a high rate while inhibit their terminal differentiation (Kenney and Rowitch, 2000; Wechsler-Reya and Scott, 2001). In the normal development, however, most granule-cell precursors cease
to proliferate around postnatal 14 days even in the continuous presence of Shh, which is possibly due to the accumulation of \( p27 \) (Miyazawa et al., 2000). Medulloblastoma originates from granule-cell precursors and is characterized by neural progenitor expansion, (Pietsch et al., 1997; Wechsler-Reya and Scott, 2001) which might be caused by Shh signaling to maintain the unlimited proliferative capacity in progenitor cells through suppression of pRb (Marino et al., 2000).

The phosphatase and tensin homologue deleted on chromosome 10 (PTEN) tumor suppressor gene is one of the most mutated genes in glioblastoma (Li et al., 1998). PTEN deficiency induces PIP\(_3\) accumulation and activates signaling pathways that regulate cell size, cell migration, cell death, cell proliferation and differentiation which are closely related to tumorigenesis (Stiles et al., 2004). PTEN deletion leads to increased capacity to the neural stem/progenitor cell self-renewal through promoting exit from the G0/G1 phase into S phase. So PTEN might play an essential role in brain tumorigenesis (Groszer et al., 2006). Recently, Yilmaz et al. found that in the hematopoietic system, PTEN deletion leads to generation of leukemic stem cells but depletion of normal HSCs, which implies that the underlying mechanisms maintaining pools of leukemic stem cells and normal HSCs are distinct (Yilmaz et al., 2006). This distinction may realize the goal that therapies are designed to combat leukemia targeting the PTEN pathway without affecting normal stem cell pools. Bmi-1 is one member of the polycomb family which works as a transcriptional repressor through chromatin remodeling. Bmi-1 is essential for self-renewal of HSCs as well as for that of leukemic stem cells partly through two CDK inhibitors, \( p16^{ink4a} \) and \( p19^{arf} \) (Jacobs et al., 1999a; Jacobs et al., 1999b). Bmi-1 is also required for self-renewal of stem cells in the peripherin nervous system (PNS) and CNS but not for their survival
or differentiation (Molofsky et al., 2003). This relates Bmi-1 to the generation of multiple brain tumors, such as medulloblastoma (Leung et al., 2004), glioblastoma (Godlewski et al., 2008) and PNS tumors, such as neuroblastoma (Cui et al., 2007). Bmi-1 overexpression was found in the majority of human medulloblastomas analyzed, which is correlated to the overexpression of PTCH1, a reliable indicator of Shh pathway activation (Leung et al., 2004). Bmi-1 is also demonstrated as an important regulator in balancing differentiation and clonogenic self-renewal in I-type neuroblastoma cells, which are considered as a population of malignant neural crest stem cells (Cui et al., 2006).
<table>
<thead>
<tr>
<th>Pathway</th>
<th>Stem cell</th>
<th>Reference</th>
<th>Cancer</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Keratinocyte stem cells</td>
<td>Hueiskan et al. (2001)</td>
<td>Pilomatrixoma</td>
<td>Chan et al. (1999)</td>
</tr>
<tr>
<td></td>
<td>Cerebellar granule-cell progenitors</td>
<td>Cui et al. (1998)</td>
<td>Medulloblastoma</td>
<td>Zurawel et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Cerebellar granule-cell progenitors</td>
<td>Wechsler-Reya et al. (1999)</td>
<td>Medulloblastoma</td>
<td>Raffel et al. (1997)</td>
</tr>
<tr>
<td>Bmi-1</td>
<td>Hematopoietic stem cells</td>
<td>Park et al. (2003)</td>
<td>B-cell lymphomas</td>
<td>Jacobs et al. (1999)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Medulloblastoma</td>
<td>Leung et al. (2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glioblastoma</td>
<td>Godlewski et al. (2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Neuroblastoma</td>
<td>Cui et al. (2007)</td>
</tr>
<tr>
<td></td>
<td>Mammary epithelial stem cells</td>
<td>Chepko et al. (2003)</td>
<td>Breast cancer</td>
<td>Jhapan et al. (1992)</td>
</tr>
<tr>
<td>PTEN</td>
<td>Neural stem cells</td>
<td>Groszer et al. (2001)</td>
<td>Gliomas</td>
<td>Deutt et al. (1998)</td>
</tr>
<tr>
<td></td>
<td>Hematopoietic stem cells</td>
<td>Zhang et al. (2006)</td>
<td>AML</td>
<td>Yilmaz et al. (2006)</td>
</tr>
</tbody>
</table>

AML, acute myeloid leukaemia. CNS, central nervous system. PTEN, phosphatase and tensin homologue deleted from chromosome 10. Shh, Sonic hedgehog.
Various stem cells are characterized by expressing drug-resistance proteins, such as MDR1 and ABC transporters (Chaudhary and Roninson, 1991; Zhou et al., 2001), which might be the reasons for less sensitivity to chemotherapy and apoptosis induction (Johnstone et al., 1999; Pallis and Russell, 2000). If cancer stem cells also express these proteins at a higher level than do differentiated cancer cells, chemotherapy may not be able to kill these resistant cells. Chemotherapy that kills more differentiated cancer cells with limited proliferative potential would lead to the shrinkage of tumor. But the surviving cancer stem cells still will induce tumorigenesis. Therefore, identification of agents that particularly target cancer stem cells is an essential way of preventing tumor recurrence. The gene expression profile of cancer stem cells, differentiated cancer cells with limited proliferative potential and stem cells is indicated to be different in a given patient (Al-Hajj et al., 2003; Bonnet and Dick, 1997; Lapidot et al., 1994). So comparison of subpopulation RNA profiles in the same tumor, rather than using the whole tumor’s RNA will help to identify therapeutic targets that are preferentially expressed in cancer stem cells. The stem cells and cancer stem cells tend to depend on signal pathways at different levels to maintain self-renewal or neoplastic proliferation because the extent of activation of a certain pathway is dependent on the mitotic activity of cells, the amount of regenerative activity in the tissue and the stage of development. The status of stem cells and cancer stem cells is usually different in these aspects, which provides the possibility of targeting cancer stem cells without harming normal stem cells. If cancer stem cells can be specifically eliminated, the possibility of cancer recurrence will be dramatically decreased.
Neural Crest Cells and Sympathetic Development

Neural crest stem cells (NCSCs) are a transit population of cells from which diverse derivatives originate (LaBonne and Bronner-Fraser, 1998). Trunk NCSCs that migrate dorsally develop into pigment-synthesizing melanocytes and dorsal root ganglia containing sensory neurons. Ventrally migrating NCSCs differentiate into sympathetic ganglia, adrenal medulla, vertebral cartilage and nerve clusters around aorta. In rodents, migrating and post-migratory NCSCs can be identified by their expression of the markers Sox10, FoxD3, nestin and p75, the low affinity nerve growth factor receptor (NGFR). Sox10, a member of the SRY related HMG box gene family of transcription factors, is essential for maintaining the stem cell state of NCSCs (Kim et al., 2003). More recent studies revealed that Bmi-1, a member of the Polycomb Group family of transcription repressors, is required for the self-renewing proliferation of NCSCs. Bmi-1-deficient mice display progressive loss of NCSCs, and Bmi-1−/− NCSCs show reduced ability to self-renew in cultures (Molofsky et al., 2005; Molofsky et al., 2003).

Sympathetic ganglia are derived from ventrally migrating NCSCs arising from the trunk region of the neural tube. Two columns of sympathetic ganglion primordia are formed along the dorsa aorta during the early embryonic development. Through a series of differentiation processes, NCSCs give rise to noradrenergic neuroblasts and glial progenitors that coalesce to form definitive sympathetic ganglia. In rodent superior cervical ganglia (SCG), neurogenesis peaks on embryonic day 14.5 (E14.5) and is completed by E17.5 (Hall and Landis, 1991), and gliogenesis peaks around E16.5-E18.5 and continues at a lower level during the first 3 weeks after birth (Hall and Landis, 1992; Shi et al., 2008).
Sympathetic neurogenesis has been widely used as a model for the study of the neuronal development. A commonly used marker for monitoring sympathetic neurogenesis is tyrosine hydroxylase (TH), a rate-limiting enzyme in the biosynthesis of noradrenaline that characterizes the noradrenergic trait of sympathetic neurons. BMPs, produced by and secreted from the dorsal aorta, serve as an extracellular signal essential for sympathetic neuronal differentiation. Addition of BMP2, 4, or 7 or overexpression of a constitutively active BMP receptor dramatically increases the number of TH-expressing cells in NCSC cultures (Reissmann et al., 1996; Varley et al., 1998). More importantly, implantation of beads soaked in the BMP antagonist Noggin in the vicinity of the dorsal aorta prevents the expression of noradrenergic and neuronal markers in NCSCs, demonstrating an essential role of BMPs in the generation of sympathetic neurons in vivo (Schneider et al., 1999). At molecular levels, the binding of BMPs to their receptors on NCSCs initiates a cascade of events that eventually leads to the expression of a group of transcription factors that controls the differentiation of sympathetic neurons (Figure 4). This group of transcription factors includes Mash1, Phox2a, Phox2b, dHand and GATA3.
The specification is initiated by BMPs and transcription factors Mash1, Phox2b, dHand and GATA3, which in turn control the expression of noradrenergic (TH and DBH) and generic neuronal (NF160 and SCG10) properties. Adopted from Goridis, C., and Rohrer, H. (2002). Specification of catecholaminergic and serotonergic neurons. Nat Rev Neurosci 3, 531-541.
In NCSC cultures, addition of BMP2 can induce the expression of Mash1 and Phox2a (Lo et al., 1998). Mash1 is a helix-loop-helix transcription factor and is transiently expressed around E10.5 during mouse sympathetic neurogenesis (Guillemot and Joyner, 1993). In Mash1−/− mouse embryos, NCSCs assemble normally at the dorsal aorta, but most of them fail to express pan-neuronal markers or noradrenaline biosynthetic enzymes TH and dopamine β-hydroxylase (DBH) (Guillemot et al., 1993). Mash1 appears to function upstream of Phox2a because the constitutive expression of Mash1 in NCSCs induces expression of Phox2a and neuronal differentiation (Lo et al., 1998). Also in Mash1−/− embryos, expression of Phox2a in sympathetic ganglia is strongly reduced, but the expression of Phox2b, dHand, neurofilaments and neuron-specific tubulin is not affected (Guillemot et al., 1993; Lo et al., 1998). BMPs can also induce the expression of the homeodomain protein Phox2b, independently of Mash1. Phox2b is expressed earlier than Phox2a and also plays a role in regulation of Phox2a expression (Pattyn et al., 1997; Pattyn et al., 1999; Schneider et al., 1999). Mice deficient in Phox2b display more severe defects in sympathetic neurogenesis. Although in Phox2b-null embryos, NCSCs can assemble at the dorsal aorta on E10.5, these cells fail to express dHand, neurofilaments and neuron-specific tubulin, indicating a block in generic neurogenesis which is not affected by Mash1 mutation (Pattyn et al., 1999). In addition, Mash1 expression is downregulated in Phox2b−/− mouse embryos (Pattyn et al., 1999). The dHand basic helix-loop-helix transcription factor plays an essential role late in the sympathetic neurogenesis. The expression of dHand depends on Phox2b but not on Mash1 (Howard et al., 2000; Morikawa et al., 2005). Forced expression of dHand induces the expression of noradrenergic biosynthetic enzymes and pan-neuronal markers in NCSCs (Howard et al., 2000; Morikawa et al., 2005). GATA3 is a zinc
finger transcription factor. In GATA3 mutant embryos, sympathetic ganglia express Phox2a and Phox2b, as well as pan-neuronal markers. However, the neurons fail to express TH and DBH, indicating a block in the maturation of sympathetic neurons (Lim et al., 2000).

It is generally thought that gliogenesis represents a default pathway of NCSCs differentiation. Sox10 is the only gene known to be essential for the generation of glial cells from truck NCSCs. Sox10 expression persists in sympathetic glial progenitors and fully differentiated glial cells (Jessen and Mirsky, 2005). One function of Sox10 in gliogenesis might be to preserve the ability of NCSCs and/or glial progenitors to respond to neuregulin 1 (NRG1) by maintaining the expression of ErbB3, the NRG1 receptor (Britsch et al., 2001). NRG1 and Notch ligands are two extracellular factors with the gliogenic promoting function. Both appear to function indirectly inhibiting neurogenesis (Jessen and Mirsky, 2005). Several markers are available for the identification of sympathetic glial cells and their progenitors. The mostly commonly used markers are glial fibrillary acidic protein (GFAP) and S100 for differentiated glial cells, and brain lipid binding protein (BLBP) for both progenitor and differentiated glial cells.

**Neuroblastoma**

Neuroblastoma is a common childhood solid cancer and originated from NCSCs or sympathetic precursor cells (Brodeur, 2003). 90% of children with this disease are diagnosed before the age of 6 years (Schwab et al., 2003). Neuroblastoma can either regress spontaneously, usually in infants, or mature into a benign
ganglioneuroma by undergoing apoptosis and/or differentiation, whereas in children over 1 year old, the tumors are mostly aggressive and fatal, and their overall prognosis has been poor (Brodeur, 2003; Nakagawara, 1998). Neuroblastoma has diverse clinical features because of its variable origin sites, inclination to metastasis, hormone secretion and manifestation as a paraneoplastic syndrome (Schwab et al., 2003). The enigmatic clinical behavior of neuroblastoma is also based on its heterogeneous cellular composition, genetic mutations and biological pathways.

Three distinct types of cell lines were established from human neuroblastomas based on their morphologies, biochemical properties and growth patterns. They are N for neuroblastic, S for non-neuronal, substrate-adherent and I for intermediate cell types (Ross et al., 2003). N-type cells have immature neuroblastic features with small and rounded bodies, a high nuclear to cytoplasmic ratio and short neurites. They attach better to other cells than to substrate and form cell aggregates in culture. They express neurofilaments, specific noradrenergic neuronal enzymes, such as TH and DBH, and cell surface receptors in developing neuroblasts, like the norepinephrine uptake transporter and the low affinity nerve growth factor receptor (Ross et al., 2002). S-type cells show characteristics opposite to those of N-type cells. They are large and flattened cells with abundant cytoplasm. They may have long filopodia but no neurites. These cells adhere tightly to substrate, grow as a monolayer in culture and present contact inhibition of growth. S-type cells express no neuronal markers and have phenotypes of Schwann/glial cells, melanocytes and smooth muscle cells (Ambros and Ambros, 1995; Sugimoto et al., 1991). They express glial cell markers like vimentin and GFAP and other proteins such as the epidermal growth factor receptor, melanocyte enzyme tyrosinase and alpha-smooth muscle actin. I-type cells
exhibit an intermediate status between N-type and S-type cells. They attach equally well to both the substrate and other cells. They may or may not have neurites and form multilayers with focal aggregates in cultures. They have nuclei similar to N-type cells and more cytoplasm like S-type cells. I-type cells express both N- and S- type cell markers and stem cell markers, such as CD133 and c-kit (Walton et al., 2004). It has been proposed that I-type cells may represent a population of neuroblastoma stem cells based on its differentiation and clonogenic ability. I-type cells can differentiate into both N-type and S-type cells under different conditions (Acosta et al., 2009). N-type cells can be induced to neuronal or neuroendocrine cells (Ross et al., 2002) whereas S-type cells can further differentiate into Schwann/glial cells, melanocytes and smooth muscle cells (Slack et al., 1992; Sugimoto et al., 2000). I-type cells have the greatest clonogenic activity in soft agar and tumorigenic potential in immunodeficient mice. S-type cells are non-tumorigenic and N-type cells exhibit tumorigenic ability depending on particular cell lines (Spengler et al., 1997). Pathological analyses of primary neuroblastoma samples exhibit that tumors are comprised of neuroblast-like cells and stroma cells (Mora et al., 2001). I-type cells are hidden in these two kinds of cells morphologically and can be identified by stem cell markers or combination with tumor markers (Kristiansen et al., 2004; Poncet et al., 1996; Reynolds, 2004). It has been demonstrated that I-type like cells are detectable in tumors of all stages and related a high possibility in tumor progression (Ross et al., 2003). Therefore, I-type cells in primary tumors may present a subpopulation of cancer stem cells that are the origin of tumorigenesis.

Neuroblastoma cells, like cells of many other tumor types, suffer from extensive, non-random genetic mutations at multiple genetic loci that provide genetic
basis for their diverse clinical behaviors. The most common genetic alterations in
neuroblastoma are MYCN amplification, gain of chromosome 17q distal region, and
loss of heterozygosity (LOH) for chromosomes 1p36 and 11q23 (van Noesel and
Versteeg, 2004). The relevant genes that are affected by 17q gain, 1p36 or 11q23
LOH in neuroblasoma remain to be identified. MYCN amplification is found in 20-
25% of all neuroblastomas and is related to a poor prognosis (Canete et al., 2009).
The amplification values are generally around 50 to 100 fold in tumors, but may also
range from 10 to 500 fold (Schwab et al., 2003). MYCN has tumorigenic potential as
evidenced by co-operating to transform primary cells, converting established cell lines
to possess tumorigenic capacities and initiating tumorigenicity in genetically
engineered mice (Schwab et al., 1985; Small et al., 1987; Weiss et al., 1997). The
mechanisms underlying MYCN malignant properties are its ability to promote
proliferation (Lutz et al., 1996) and prime cells for apoptosis by sensitizing them to an
array of damages. The evasion of MYCN primed apoptosis is required for tumor
aggression in neuroblasts with MYCN amplification (Hogarty, 2003). The evasion is
likely to result from co-operating mutations that activate survival pathways or inhibit
apoptosis pathways. In survival pathways, constitutive neurotrophin signaling may
suppress apoptosis in neuroblasts and the signaling pathway may involve
neurotrophins and their receptors- TrkA, TrkB and TrkC (Evangelopoulos et al.,
2004). The mutations from both mitochondrial and death receptor apoptosis pathways
contribute to evasion from MYCN primed apoptosis, such as Bcl2 overexpression and
Caspase 8 inactivation (Schmitt and Lowe, 2001; Teitz et al., 2000).

Neuroblastoma is one of few cancers originating from the embryonic
development, which implies that this tumor may be initiated by transformed
developing NCSCs or malignant sympathetic precursor cells that obtain the stem cell properties through mutations. This neuroblastoma stem cell model can adequately interpret the heterogeneous characteristic of human neuroblastoma tumors, based on the self-renewal and differentiation ability of malignant NCSCs or sympathetic precursor cells. I-type neuroblastoma cell lines have been identified as cancer stem cells based on their special phenotypic characterization, differentiation potential and malignant potential (Ross and Spengler, 2007). The existence of stem-like cells in neuroblastoma tumors was demonstrated using dye exclusion with fluorescence activated cell sorting (FACS) analysis (Hirschmann-Jax et al., 2004) and co-expression of proteins from different neural crest cell lineages (Walton et al., 2004). It was observed that in high mortality rate neuroblastomas, usually in children over 1 year old, many tumor stem cells exist and differentiated tumor cells are less than those of favorable prognosis tumors (Cassady, 1984). Since neuroblastoma cancer stem cells play an essential role in the tumor initiation, it is necessary to investigate the regulatory mechanisms underlying self-renewal and differentiation capacities of neuroblastoma stem cells, which provide the foundation for appropriate future therapies in neuroblastomas. The research in our lab indicated that Bmi-1, a NCSC self-renewal regulator, controls the delicate balance of clonogenic self-renewal and differentiation of I-type neuroblastoma stem cells in a concentration-dependent manner (Cui et al., 2006). My research demonstrated that GATA3, a transcription factor in normal sympathetic neurogenesis, also plays an important role in the regulation of I-type neuroblastoma cell activities.
GATA3, a Member of GATA Family Transcription Factors

The GATA family is a group of evolutionary conserved transcriptional factors that have been shown to play crucial roles in the development, including regulation of differentiation, proliferation, cell-fate specification and cell movement, et al. These factors have been found in all eukaryotic organisms from fungi to plants and from invertebrates to vertebrates (Lowry and Atchley, 2000). In vertebrates, the GATA family contains 6 six members (named GATA1 to 6) which belong to zinc finger transcription factors. They bind to the common consensus DNA sequence (A/T)GATA(A/G) from which the family name originated (Patient and McGhee, 2002).

The vertebrate GATA family proteins contain a conserved DNA-binding domain comprised of two multifunctional zinc fingers. The C-terminal zinc finger is responsible for site-specific recognition and DNA-binding to the core GATA motif, whereas the N-terminal zinc finger contributes to the specificity and stability of the DNA-binding (Figure 5) (Martin and Orkin, 1990; Omichinski et al., 1993; Yang and Evans, 1992). Since the GATA family members share a highly conserved DNA-binding domain, they all show similar DNA-binding properties (Ko and Engel, 1993; Merika and Orkin, 1993). The specificity of GATA is partly governed through protein-protein interaction with other transcription factors (Charron and Nemer, 1999; Molkentin, 2000). There is an extensive list of ubiquitously expressed or cell-restricted factors that are identified as cooperating with GATA factors to control tissue specific transcription in the hematopoietic system, the heart, the pituitary, the adrenal glands, the gonads and many other tissues (Durocher et al., 1997; Gordon et al., 1997; Jimenez et al., 2003; Merika and Orkin, 1995; Morin et al., 2000). The
multitype zinc finger proteins named Friend of GATA1 (FOG1) and Friend of GATA2 (FOG2) are the most notable GATA-interacting factors, which were originally identified as GATA-specific cofactors interacting with N-terminal zinc fingers of the GATA family members (Holmes et al., 1999; Tsang et al., 1997). FOG1 is highly coexpressed with GATA1 in hematopoietic cell lineages (Tsang et al., 1997). FOG2, with GATA4, is highly expressed in the heart, brain, and gonads (Laitinen et al., 2000; Lu et al., 1999). FOG proteins act as either enhancers or repressors of GATA transcription activity in different cell contexts. Although FOG proteins don’t bind to DNA directly, they still play crucial roles in the vertebrate development just as GATA factors (Fox et al., 1999; Robert et al., 2002; Svensson et al., 2000a). FOG1 knockdown results in the failure in erythroid and megakaryocytic differentiation (Tsang et al., 1998), while loss of FOG2 leads to defects in the heart morphogenesis and the coronary vascular development (Crispino et al., 2001; Svensson et al., 2000b; Tevosian et al., 2000), as well as the impaired gonad development (Tevosian et al., 2002). The activity of GATA factors is also regulated by posttranslational modifications such as sumoylation, acetylation and phosphorylation. The effect of these modifications includes enhanced transcriptional activities due to changes in the nuclear localization, DNA-binding, protein stability, and cofactor recruitment of GATA family factors (Viger et al., 2008).
Figure 5. Structures and homology of the vertebrate GATA proteins

All GATA factors share a similar zinc finger DNA-binding domain, a feature that defines this family of transcription factors. The zinc finger region is also involved in protein interactions with cofactors and/or other transcriptional partners. Transactivation domains are located in the N-terminal and C-terminal regions. The percent homology among the different GATA proteins (as deduced from the mouse sequences) in the N-terminal, C-terminal and zinc finger domains is indicated. Adopted from Viger, R. S., Guittot, S. M., Anttonen, M., Wilson, D. B., and Heikinheimo, M. (2008). Role of the GATA family of transcription factors in endocrine development, function, and disease. Mol Endocrinol 22, 781-798.
The six GATA family members can be divided into two subgroups based on their spatial and temporal patterns (Figure 5). GATA1/2/3 are expressed in hematopoietic cell lineages and play an essential role in the proliferation of HSCs, the development of T lymphocytes and the differentiation of erythrocytes and megakaryocytes (Weiss and Orkin, 1995). However, their expression is not limited in the hematopoietic system, but is also shown in the nervous system, kidney, inner ear, et al., and is important in the development of these organs (George et al., 1994; Labastie et al., 1995; Lillevall et al., 2004; Nardelli et al., 1999). GATA4/5/6 are mainly expressed in the tissues originated from the mesoderm and endoderm, such as the heart, gut and gonads (Molkentin, 2000). The mutation and expression modulation of GATA family factors can cause human diseases. GATA1 mutation results in dyserythropoietic anemia and thrombocytopenia (Nichols et al., 2000). Hypoparathyroidism, deafness, and renal dysplasia -HDR syndrome- is caused by a GATA3 mutation (Van Esch et al., 2000). A GATA4 mutation induces congenital heart defects (Garg et al., 2003).

GATA3 plays an essential role in the development of many tissues in vertebrates, including Th2 cell differentiation, the development of the kidney, the inner ear, embryonic and pubertal mammary glands, adipocytes and neurons, et al.

GATA3 is essential for differentiation of T helper type 2 (Th2) cells in the immune system. T helper type 2 (Th2) cells secrete IL-4, IL-5, IL-10, and IL-13 and mediate allergic and asthmatic diseases. GATA3 is required for optimal Th2 cytokine production in vitro and in vivo and must be sustained to maintain the Th2 phenotype (Pai et al., 2004). T cell receptor-mediated activation of the Ras-ERK-MAPK cascade stabilizes GATA3 protein in developing Th2 cells through inhibiting the ubiquitin-
proteasome pathway and facilitates GATA3-mediated chromatin remodeling at Th2 cytokine gene loci leading to successful Th2 cell differentiation (Yamashita et al., 2005). The differentiation of T helper progenitors to Th1 or Th2 effector cells requires the action of two opposing transcription factors, T-bet and GATA3. T-bet is essential for the development of Th1 cells, comparable to the similar role of GATA3 in the Th2 development. T-bet represses Th2 lineage commitment through tyrosine kinase-mediated interaction between the two transcription factors that interferes with the binding of GATA3 to its target DNA (Hwang et al., 2005), while GATA3 expression in developing Th1 cells suppresses the Th1 development through downregulation of Stat4 (Usui et al., 2003).

Human GATA3 haploinsufficiency leads to HDR (hypoparathyroidism, deafness and renal dysplasia) syndrome, demonstrating the essential role of GATA3 in the kidney and inner ear development. Nephric duct-specific inactivation of GATA3 leads to massive ectopic ureter budding which results in a spectrum of urogenital malformations including kidney adysplasia, duplex systems, and hydrourerter, as well as vas deferens hyperplasia and uterine agenesis. GATA3 inactivation also causes premature nephric duct cell differentiation (Grote et al., 2008). In the inner ear development, GATA3 deficiency leads to severe and unique abnormalities during otic placode invagination. Loss of GATA3 also alters the expression of several cell adhesion mediating genes, which suggests that GATA3 controls adhesion and morphogenetic movements in the early otic epithelium (Lillevali et al., 2006). Hearing loss following GATA3 haploinsufficiency is peripheral in origin and is caused by a cochlear disorder (van der Wees et al., 2004).
GATA3 plays essential roles in the development of mammary glands in both embryos and adults. In embryos, loss of GATA3 leads to an expansion of luminal progenitors and a concomitant block in differentiation. Introduction of GATA3 into a stem cell-enriched population induces maturation along the alveolar luminal lineage (Asselin-Labat et al., 2007). In pubertal mice, GATA3 is found in the luminal cells of mammary ducts and the body cells of terminal end buds (TEBs). Upon conditional deletion of GATA3, mice exhibit severe defects in the mammary development due to failure in TEB formation. After acute GATA3 loss, adult mice display undifferentiated luminal cell expansion with basement-membrane detachment, which leads to caspase-mediated cell death in the long term (Kouros-Mehr et al., 2006). These studies provide evidence for GATA3 as a critical regulator of luminal differentiation.

GATA3 is crucially involved in epidermal and hair follicle differentiation (Kaufman et al., 2003). A conditional GATA3^{−/−} mouse in which GATA3 is specifically deleted in the epidermis and hair follicles shows aberrant postnatal growth and development, delayed hair growth and maintenance, abnormal hair follicle organization and irregular pigmentation. After the first hair cycle, the germinative layer surrounding the dermal papilla is not restored, whereas proliferation is pronounced in basal epidermal cells (Kurek et al., 2007).

GATA2 and GATA3 are expressed in adipocyte precursors and control the preadipocyte-to-adipocyte transition. Constitutive expression of both GATA2 and GATA3 suppresses the adipocyte differentiation primarily with two mechanisms. One is through direct binding to the peroxisome proliferator-activated receptor gamma (PPARgamma) promoter and suppression of its basal activity (Tontonoz et al., 1994).
The other is through forming protein complexes with CCAAT/enhancer binding proteins alpha (C/EBPalpha) and C/EBPbeta, members of a family of transcription factors that are integral to adipogenesis (Tong et al., 2005).

GATA3 is an important transcription factor in the development of both CNS and PNS. In the CNS, for example, GATA3 is expressed in the developing serotonergic neurons of the caudal raphe nuclei. Absence of GATA3 affects the cytoarchitecture of serotonergic neurons and knockout mice show a serious defect in their locomotor performance on a rotating rod (van Doorninck et al., 1999). GATA3 and Stem Cell Leukemia (SCL) gene, a basic helix-loop-helix (bHLH) transcription factor gene, are coexpressed in V2 interneurons of the spinal cord and regulate their differentiation (Smith et al., 2002). In the PNS, GATA3 is essential for the development of sympathetic nervous system. GATA3 knockout mouse embryos show reduced synthesis of tyrosine hydroxylase (TH) (Lim et al., 2000).

In addition to the above functions, GATA3 is also involved in the early stage of the embryonic development, such as the regulation of trophoblast-specific gene expression and placental function (Ma et al., 1997).

GATA3 and Sympathetic Neuronal Development

In 1995, Pandolfi et al. found that GATA3−/− embryos die by E11 and display massive internal bleeding, marked growth retardation, severe deformities of the brain and spinal cord, and gross fetal liver hematopoietic aberrations (Pandolfi et al., 1995). In 2000, Lim et al. reported that this GATA3-induced embryonic lethality can be partially rescued by a catecholamine supplement, which suggests noradrenaline
deficiency in the sympathetic nervous system might cause embryonic lethality in these mutants (Lim et al., 2000). They discovered that null mutation of GATA3 leads to dramatic downregulation of TH and DBH mRNA, whereas the expression of Phox2a, Phox2b, neurofilaments, p75 NGFR and β-tubulin III in the sympathetic nervous system is not affected. Due to the important roles of GATA3 in the development of the cephalic neural crest cells, metanephric kidney, thymus and heart, pharmacologically rescued GATA3−/− embryos exhibit severely hypoplastic mandibles, serious metanephric and thymic hypoplasia, loosely dispersed myocardium and severely hypoplastic ventricular walls as well as blood congestion. These defects may constitute other reasons for embryonic lethality.

The generation of sympathetic neurons from NCSCs is induced by the extrinsic signal BMPs, which initiates the expression of a network of transcription factors that, in turn, control sympathetic neuronal differentiation (Goridis and Rohrer, 2002). Mash1 is the first transcription factor identified to be essential for sympathetic neurogenesis (Guillemot et al., 1993). Phox2b is another essential transcriptional factor which is required for Mash1 expression and independent of Mash1 (Pattyn et al., 1999). Mash1 and Phox2b are genetically upstream of Phox2a and dHand (Flora et al., 2001; Stanke et al., 2004). Forced expression of Phox2a and dHand induces the expression of the upstream genes Mash1 and Phox2b, so the interactions are reciprocal (Goridis and Rohrer, 2002). Phox2a, Phox2b and dHand bind directly to the promoters of the TH or DBH gene and activate their transcription (Rychlik et al., 2003; Xu et al., 2003). These previous researches propose the questions: Is GATA3 one of the transcription factors in this network? What is the relationship between
GATA3 and other factors? Does GATA3 bind directly to the TH or DBH promoter to induce their expression?

In 2004, Tsarovina et al. were trying to answer these questions in their research. They used chicks as the model to investigate the essential role of GATA factors in the sympathetic development (Tsarovina et al., 2004). They found that GATA2 rather than GATA3 is involved in chick noradrenergic differentiation, whereas GATA2 and GATA3 are expressed at the equivalent levels in mouse sympathetic ganglia. It was found that temporally, GATA2 is expressed after the onset of Cash1, Phox2a, Phox2b and dHand expression, but before the noradrenergic marker genes TH and DBH. GATA2 expression is also dependent on BMP signaling as evidenced by BMP loss-of-function experiments. These facts suggest that GATA2/3 might also be involved in the network of BMP induced transcription factors. Further experiments proved that the expression level of GATA2 and TH is upregulated in gain-of-function studies of Phox2b, Mash1 and Phox2a. In Phox2b knockout mice, the expression of GATA2 and GATA3 in E10.5 sympathetic ganglion primordia can’t be detected. Therefore, GATA2/3 may act downstream of Phox2b in the BMP-induced network. In GATA2 knockout chick embryos, TH expression is strongly reduced by 50%, which is consistent with the effects of GATA3 knockout on the noradrenergic gene expression in mice shown in Lim et al.’s research in 2000. They further found a reduction in sympathetic ganglion size in GATA2 knockout chick embryos, reflected by a smaller area of Phox2b- and SCG10-expressing cells, which suggests a more general role in the sympathetic development than reported for mouse GATA3. In order to find more facts, Tsarovina et al. re-investigated the effects in the GATA3 knockout mouse model. On E10.5, the sympathetic ganglion primordia shows normal size in GATA3’
mice, as demonstrated by the expression of transcriptional control genes (Mash1, Phox2b, Phox2a, dHand), noradrenergic markers (DBH, TH), the GFL receptor signaling subunit (Ret) and generic neuronal markers (TUJ1). The only differences compared with the control ganglia are lack of GATA2 expression and substantially lower TH expression levels, whereas DBH expression is intact. On E11.5, the size of sympathetic ganglia is dramatically reduced in GATA3−/− mice due to a strong increase in the number of apoptotic cells. On E13.5, only rudiments of ganglia are left.

In 2006, Moriguchi et al. confirmed that there are increased apoptosis and significant impairment of sympathoadrenal differentiation in adrenal chromaffin cells and sympathetic neurons in GATA3 knockout mouse embryos. Through mRNA analyses of purified chromaffin cells from GATA3 mutants, the relative expression levels of Mash1, dHand and Phox2b (postulated upstream regulators of GATA3) as well as downstream products TH and DBH, are markedly downregulated. The researchers made another transgenic mouse model that specifically expresses GATA3 in sympathoadrenal lineages under the human DBH promoter. This lineage specific restoration of GATA3 recovers sympathoadrenal function almost to the normal level and phenotypically restores downstream, as well as the putative upstream genes, which provides solid evidence that sympathoadrenal differentiation is controlled by mutually reinforcing feedback transcriptional interactions between GATA3, Mash1, dHand and Phox2b. Although full function restoration in the sympathoadrenal system, transgenic-rescued mutant embryos still exhibit hypoplastic mandibles and glandular aplasia (the parathyroid glands and thymuses) as well as inner ear and kidney deficiencies, which suggests the significance of GATA3 in the development of these
organs. However, the hearts of transgenic-rescued mutant embryos have normal cardiac thickness that is indistinguishable from wild-type littermates, which implies that the heart development is dependent more on noradrenalin than GATA3 itself (Moriguchi et al., 2006).

In 2006, Hong et al. found that the mechanism underlying GATA3 regulation of TH expression is not through directly binding to the TH promoter as other transcription factors like Phox2a, Phox2b and dHand. GATA3 interplays with transcription factor CREB by protein-protein interaction. CREB binds to a cAMP response element (CRE) in the TH promoter area and promotes the TH expression (Hong et al., 2006). In 2008, Hong et al. further discovered that GATA3 also activates through protein-protein interaction with DBH gene expression in primary NCSC cultures. GATA3 binds to the transcription factors, Sp1 and AP4, respectively and the protein complexes bind to the DBH gene promoter to activate transcription (Hong et al., 2008). Therefore, GATA3 via a novel and distinct protein-protein interaction directly contributes to noradrenalin phenotype specification.

**GATA3 and Tumorigenesis**

GATA3 is an important regulator of cellular proliferation and differentiation in the development. So whether the GATA3 expression in tumor cells has any effect on tumorigenesis is a question of interest. The effects of GATA3 expression have been widely investigated in T cell-related tumors and breast cancers.

GATA3 is expressed in human T cell acute lymphoblastic leukemias (T-ALL) (Minegishi et al., 1997). GATA3 is capable of forming a complex with the
transcription factors LIM-only domain protein Lmo2 and the basic-helix-loop-helix (bHLH) protein Tal1, which are often shown to be aberrant expression pattern in human T-ALL. Lmo2 and Tal1 act as cofactors for GATA3 to activate the transcription of retinaldehyde dehydrogenase 2 (RALDH2) gene in T-cell tumorigenesis (Ono et al., 1998). Antisense GATA3 treatment can inhibit the expression of TNF-α and Th2 cytokines in tumor cells and also depress tumor growth in tumor-bearing mice (Yao et al., 2005). Direct evidence for a role of GATA3 in T-ALL is that enforced expression of GATA3 during the T cell development in CD2-GATA3 transgenic mice induces CD4+CD8+ double-positive (DP) T cell lymphoma (Nawijn et al., 2001). Enforced GATA3 expression induces high c-Myc expression and converts DP thymocytes into a pre-malignant state, whereby subsequent induction of Notch1 signaling cooperates to establish malignant transformation (van Hamburg et al., 2008).

The expression of GATA3 is associated with estrogen α receptor in breast cancers (Hoch et al., 1999). GATA3 is involved in growth control and the maintenance of the differentiated state in mammary epithelial cells, and loss of GATA3 function may contribute to breast cancer tumorigenesis (Usary et al., 2004). GATA3 also contributes to the metastasis of breast cancers through its capability to regulate differentiation. Breast tumors with high GATA3 expression tend to be well differentiated with low metastatic potential, whereas tumors with low GATA3 expression tend to be poorly differentiated with high metastatic potential (Hoch et al., 1999; Jacquemier et al., 2005; Jenssen et al., 2002; Mehra et al., 2005). Loss of GATA3 marks progression from adenoma to early carcinoma and onset of tumor dissemination in breast cancers. Restoration of GATA3 in late carcinomas induces
tumor differentiation and suppresses tumor dissemination. Targeted deletion of GATA3 in early tumors leads to apoptosis of differentiated cells, which suggests that the loss of GATA3 is not sufficient for malignant conversion, while an expanding GATA3-negative tumor cell population accompanies malignant progression. These facts demonstrate that GATA3 regulates tumor differentiation and suppresses tumor dissemination in breast cancers (Kouros-Mehr et al., 2008).

GATA3 has been shown alter its expression patterns in other types of cancers, such as pancreatic cancers, cervical and esophageal carcinomas. In pancreatic cancers, the mRNA and protein levels of GATA3 are markedly upregulated. The strong specific upregulation of GATA3 impairs nuclear translocation and its cooperative action with the TGF-β pathway, suggesting that GATA3 plays an important role in human pancreatic cancers (Gulbinas et al., 2006). In cervical cancers, a progressive downregulation of GATA3 expression is observed during carcinogenesis. Human papillomavirus-mediated immortalization of cervical cell lines leads to a loss of GATA3 expression (Steenbergen et al., 2002). In contrast, a clear upregulation of GATA3 expression is observed in human esophageal cancer cell lines, whereas no expression of GATA3 is observed in normal esophageal mucosal cells and primary carcinoma cells (Shiga et al., 1993).

GATA3 has been shown to express in multiple neuroblastoma cell lines (George et al., 1994). But no further researches have been investigated.
Nestin expression defines both glial and neuronal progenitors in postnatal sympathetic ganglia

Huilin Shi,1 Hongjuan Cui,1 Goleeta Alam,1 William T. Gunning,1,2 Andrea Nestor,2,3 David Giovannucci,2,4 Ming Zhang,5 and Han-Fei Ding1*

1Department of Biochemistry and Cancer Biology, 2Advanced Microscopy and Imaging Center, 3Department of Surgery, and 4Department of Neurosciences, University of Toledo Health Science Campus, Toledo, Ohio 43614

5Department of Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand

Running title: Postnatal sympathetic progenitors

Associate Editor: John L. R. Rubenstein

Key words: noradrenergic neurons; satellite cells; superior cervical ganglia; postnatal sympathetic development
*Correspondence to: Han-Fei Ding, Department of Biochemistry and Cancer Biology, BHSB 461, University of Toledo Health Science Campus, 3035 Arlington Avenue, Toledo, Ohio 43614. E-mail: han-fei.ding@utoledo.edu

Grant sponsor: the National Cancer Institute; grant number: R01 CA124982.
ABSTRACT

Sympathetic ganglia are primarily composed of noradrenergic neurons and satellite glial cells. Although both cell types originate from neural crest cells, the identities of the progenitor populations at intermediate stages of the differentiation process remain to be established. Here we report the identification in vivo of glial and neuronal progenitor cells in postnatal sympathetic ganglia, using mouse superior cervical ganglia as a model system. There are significant levels of cellular proliferation in mouse superior cervical ganglia during the first 18 days after birth. A majority of the proliferating cells express both nestin and brain lipid-binding protein (BLBP). BrdU fate-tracing experiments demonstrate that these nestin and BLBP double positive cells represent a population of glial progenitors for sympathetic satellite cells. The glial differentiation process is characterized by a marked downregulation of nestin and upregulation of S100, with no significant changes in the levels of BLBP expression. We also identify a small number of proliferating cells that express nestin and tyrosine hydroxylase, a key enzyme of catecholamine biosynthesis that defines sympathetic noradrenergic neurons. Together, these results establish nestin as a common marker for sympathetic neuronal and glial progenitor cells and delineate the cellular basis for the generation and maturation of sympathetic satellite cells.
INSTRUCTION

The sympathetic nervous system is composed of sympathetic ganglia and the adrenal medulla, a specialized sympathetic ganglion containing secretory chromaffin cells. Sympathetic ganglia of mammals are organized into two paravertebral chains that span from cervical to sacral regions, with the ganglia being interconnected with pre- and postganglionic sympathetic nerve fibers. Sympathetic ganglia contain two major cell types, neurons and glial cells. Most mammalian sympathetic neurons use noradrenaline as a neurotransmitter and, thus, are called noradrenergic neurons. These neurons are commonly marked by their expression of tyrosine hydroxylase (TH) that catalyzes the rate-limiting step in the biosynthesis of catecholamines including dopamine, noradrenaline, and adrenaline. Sympathetic glial cells include Schwann cells and satellite cells. Schwann cells provide myelin to insulate axons of the peripheral nerves whereas satellite cells line the exterior surface of sympathetic neurons. Within a sympathetic ganglion, the majority of glial cells are satellite cells and Schwann cells are generally associated with intra-ganglionic nerve fibers. A common marker for the sympathetic glial cells is S100, an acidic calcium-binding protein (Cocchia and Michetti, 1981).

It is well established that sympathetic neurons and glia are derived from neural crest cells (Anderson, 1989; LaBonne and Bronner-Fraser, 1998; Le Douarin and Dupin, 1993), a transient, highly migratory population of multipotent stem/progenitor cells. The neural crest can be divided into four regions along the anterior–posterior axis: cranial, vagal, trunk, and lumbosacral neural crest. During sympathetic development, neural crest cells, mainly from the trunk region of the neural crest, migrate ventrally...
and aggregate adjacent to the dorsal aorta to form the primary sympathetic chain. A subpopulation of the cells then undergo dorsal migration to form the paravertebral sympathetic ganglia where they differentiate into sympathetic neurons and glial cells (Francis and Landis, 1999; Kirby and Gilmore, 1976).

The generation of sympathetic neurons (neurogenesis) and glia (gliogenesis), which is best studied in rat superior cervical ganglia (SCG), occur during different periods of sympathetic development. While neurogenesis peaks around embryonic day 14.5 (E14.5) and is essentially completed at the time of birth, gliogenesis begins around E16.5 and continues postnatally (Hall and Landis, 1991; Hall and Landis, 1992; Hendry, 1977). Consistent with the temporal pattern of in vivo sympathetic neurogenesis and gliogenesis, in vitro fate tracing experiments revealed that proliferating cells isolated from the E14.5 rat SCG gave rise predominantly to clones containing only neurons, whereas those from the E17.5 rat SCG generated mostly clones that contained only glial cells (Hall and Landis, 1991). These findings have led to the suggestion that post-migratory neural crest cells commit to a neuronal or glial fate at a very early stage of the sympathetic development (Hall and Landis, 1991). However, the identities of sympathetic neuronal and glial progenitors have not been clearly defined.

In this study, we examined the development of mouse sympathetic ganglia during the first eight weeks after birth, with the goal of identifying molecular markers that define distinct sympathetic progenitor populations. A detailed characterization of the cellular
basis for postnatal sympathetic development should facilitate the investigation of
genes and signaling pathways that control the developmental process.
MATERIALS AND METHODS

Mice

Male and female C57BL/6J mice 1-day to 8-week old were used in this study. Mice were obtained from the Jackson Laboratory (Bar Harbor, ME) and maintained under Specific Pathogen Free conditions in the animal facility of University of Toledo Health Science Campus. All animal experiments were pre-approved by the Institutional Animal Care and Use Committee.

BrdU injection and SCG collection

The thymidine analog 5-bromo-2’-deoxyuridine (BrdU, Sigma, St. Louis, MO) was dissolved in saline at 10 mg/ml, and mice were injected intraperitoneally with BrdU at 50 mg/kg (body weight) 2 hours prior to being euthanized by CO₂ inhalation on postnatal day 4, or they were injected once daily for 3 days on postnatal day 4, 5 and 6, and sacrificed on day 1, 7, 14, 21, 28, and 56 after the final BrdU injection. C57BL/6J mice without BrdU injection were also euthanized by CO₂ inhalation on postnatal day 1, 4, 7, 10, 14, 18, 22, 26, 30, 42 and 56. For each time point, at least 3 mice were used. Mouse SCG were collected, fixed in 10% neutral buffered formalin and embedded in paraffin. The paraffin tissue blocks were stored at 4°C until sectioned.

Immunofluorescence
Sections of 4-µm were cut from the SCG paraffin blocks and mounted on 3-aminopropyltriethoxysilane-treated slides (Sigma). After de-paraffinization and rehydration, the sections were treated in a microwave oven at 95 degree for 20 minutes in 10 mM citrate buffer (pH= 6) for antigen retrieval and washed in PBS. Sections were blocked with 10% horse serum for 1 hour and incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: mouse anti-Ki67 (1:100, BD Pharmingen, San Jose, CA), rat anti-BrdU (1:200, Abcam, Cambridge, MA), mouse anti-TH (1:5000, Sigma), rabbit anti-TH (1:1000, Chemicon, Temecula, CA), rabbit anti-S100 (1:200, Dako, Carpinteria, CA), rabbit anti-BLBP (1:2000, Chemicon), and chicken anti-nestin (1:1000, Novus Biologicals, Littleton, CO). For double or triple immunostaining, various combinations of primary antibodies were used. After washing in PBS, the sections were again blocked with 10% goat serum for 1 hour, followed by incubation with secondary antibodies for 2 hours. All secondary antibodies were purchased from Invitrogen (Carlsbad, CA) and used at 1:400 dilutions. The secondary antibodies used were: goat anti-mouse (FITC or Texas-Red), goat anti-rabbit (Alexa fluor 594 or Alexa Fluor 647), goat anti-chicken (Alexa Fluor 488), and goat anti-rat (Alexa Fluor 488). After secondary antibody incubation, the sections were washed in PBS and counterstained with DAPI to stain nuclei. Sections were mounted in Fluorescence Mounting Medium (Dako) and ProLong Gold antifade reagent (Invitrogen), coverslipped, and examined using a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems, Bannockburn, IL) or a Nikon ECLIPSE E800 fluorescence microscope (Nikon Instruments Inc., Melville, NY). Images were processed using Image J (NIH shareware) and Adobe Photoshop CS (Adobe Systems, Mountain View, CA). Figures were assembled and labeled in Canvas 9 (ACD Systems, Miami, FL).
**Antibody characterization**

The clone B56 anti-Ki67 mouse monoclonal antibody (BD Pharmingen, #550609, Lot #32659) was raised against human Ki67 protein. It has the same antigen specificity as the well-characterized clone MIB 1 anti-Ki67 monoclonal antibody (Key et al., 1993). Both MIB 1 and B56 antibodies recognize a double band of 345 and 395 kD on Western blot. Flow cytometry analysis reveals that the binding of Phycoerytherin-conjugated B56 can be blocked by purified MIB 1 antibody. Immunohistochemistry analysis demonstrates that B56 gives the same staining pattern as MIB 1 on both frozen and paraffin-embedded tissue sections (manufacturer’s Technical Data Sheet).

The specificity of the monoclonal rat anti-BrdU antibody (Abcam, ab6326, Lot #163833) has been confirmed by several assays. It reacts with free BrdU, BrdU in single-stranded DNA, or BrdU attached to a carrier protein, but not with thymidine (manufacturer’s technical information). In our study, no staining was seen when the antibody was used to stain tissue sections from mice that had not been injected with BrdU.

The mouse monoclonal anti-TH antibody (clone TH-2, Sigma, T1299) was raised against rat TH. It recognizes an epitope present in the N-terminal region (amino acids 9-16) of both rodent and human TH. The rabbit polyclonal anti-TH antibody (Chemicon, AB152, Lot #0603025098) was prepared against denatured TH from rat pheochromocytoma. Both antibodies detect a single band of ~60 kD on Western blot.
(manufacturer’s technical information), and only stained sympathetic cells with the morphology of neurons.

The rabbit polyclonal anti-S100 antibody (Dako, Z0311, Lot #00015317) was raised against S100 isolated from cow brain. In western blotting of purified recombinant human S100 proteins, the antibody stains S100B strongly, S100A1 weakly, and S100A6 very weakly. No reaction was observed with S100A2, S100A3 and S100A4 (manufacturer’s technical information). Also according to the information provided by Dako, Z0311 only labels glial cells in the brain and gives the same staining pattern as a monoclonal anti-S100 antibody previously reported (Hagen et al., 1986). In our study, we found that Z0311 only stained cells with the morphology and distribution of glial cells in adult mouse SCG (Fig. 3).

The rabbit polyclonal anti-BLBP antibody (Chemicon, AB9558, Lot #0603024945) was raised against recombinant BLBP. It stains a single band of ~15 kD on Western blot and only glial cells in immunohistochemistry (manufacturer’s technical information). We also found that the antibody only stained cells with the morphology and distribution of glial cells in mouse SCG (Fig. 2).

The chicken anti-nestin antibody (Novus Biologicals, NB100-1604, Lot #0305) was raised against mouse nestin. According to the information provided by the company, the antibody stains neural stem cells of the ventricular zone of the mouse brain by immunofluorescence. In Western blotting, this antibody reacts with recombinant
mouse nestin and detects a single band of approximately 250 kD in mouse brain homogenate. No staining was seen in Western blotting of mouse liver homogenate. We also found that the antibody only stained neural crest cells, but not their neuronal progeny cells (unpublished data), which was identical with previous descriptions (Stemple and Anderson, 1992).

**Quantification**

For sections from each mouse at each time point, a total of 1000 cells (DAPI-positive) were counted from at least 4 randomly selected 400x fields, and the percentages of proliferating cells (Ki67- or BrdU-positive) and progenitor- and lineage-marker expressing cells were determined. All data were presented as mean ± standard deviation (SD).
ACKNOWLEDGMENTS

We thank Jane Ding for the assistance in maintaining the mouse colony.
RESULTS

Cell proliferation in postnatal mouse SCG

Progenitor cells are generally defined as populations of dividing cells with the capacity to differentiate (Smith, 2006). To identify the progenitor cell populations in postnatal mouse SCG, we first performed Ki67 immunofluorescence staining of SCG sections (Fig. 1A). Ki67 is a nuclear protein specifically expressed in cells undergoing active proliferation (Sawhney and Hall, 1992). From postnatal day 1 (P1) to P4, approximately 20% of ganglionic cells in mouse SCG expressed Ki67. The number of Ki67-expressing cells decreased gradually with time, and by the end of the third postnatal week, only 0.4% of ganglionic cells were stained positively for Ki67 (Fig. 1). Thus, during normal development, sympathetic cell genesis in mice continues at high levels for approximately 2 to 3 weeks after birth. Importantly, the proliferating cells in early postnatal mouse SCG were evenly distributed and no specialized proliferation zones were observed (Fig. 1A). These observations indicate that postnatal development of the sympathetic nervous system is markedly different from that of the central nervous system where continuous neurogenesis persists throughout adulthood in specialized proliferation zones, such as the subventricular zone, the olfactory bulb and the dentate gyrus of the hippocampus (Gross, 2000; Ming and Song, 2005).

Identification of proliferating cells in postnatal mouse SCG

To establish the identities of the Ki67-expressing cells in postnatal mouse SCG, we examined these cells for their expression of lineage markers. For glial markers, we
chose S100 and brain lipid-binding protein (BLBP, also called brain fatty acid-binding protein, BFABP). It has been shown previously that sympathetic satellite cells express S100 (Cocchia and Michetti, 1981); BLBP is a marker for Schwann cell precursors and immature Schwann cells of the peripheral nervous system (Jessen and Mirsky, 2005). We found that all satellite cells in mouse SCG expressed high levels of BLBP, and the intensity of its expression did not change significantly from P1 to P30 (Fig. 2). This finding indicates that BLBP is an early marker for sympathetic satellite cells. By contrast, P1 SCG contained no detectable S100-expressing cells, which were first seen in P4 SCG (Fig. 3, A,B). The number of S100-expressing satellite cells increased gradually thereafter, and by P22 all satellite cells were stained positively for S100 (Fig. 3). The expression of S100 was maintained in sympathetic satellite cells throughout adulthood (data not shown). Thus, S100 appears to be a late marker for sympathetic satellite cells.

Double immunofluorescence staining and quantification revealed that between P1 and P18, a period of high levels of postnatal sympathetic cell synthesis, approximately 80% of Ki67-positive cells expressed BLBP (Fig. 2 and 4A), demonstrating that gliogenesis is a predominant event during postnatal sympathetic development. The observation also suggests that early BLBP-expressing satellite cells possess substantial proliferation potential. During the same period, there was a gradual increase in the percentage of Ki67-positive cells that expressed S100, which peaked at the level of ~27% between P14 to P18 (Fig. 3 and 4B). The timing of their appearance suggest that these Ki67 and S100 double positive cells probably represent a subpopulation of immature satellite cells undergoing final rounds of proliferation...
before differentiation into mature satellite cells. There were a few Ki67-positive cells in P22 and P30 SCG that stained negatively for either BLBP or S100 (Fig. 2-4), and their identity is unknown at the moment.

We also examined Ki67-positive cells for expression of TH, a marker for sympathetic noradrenergic neurons. We found that approximately 5% of Ki67-positive cells expressed TH in mouse SCG of P1, P4, and P7 (Fig. 5), indicating that sympathetic neurogenesis continues at low levels for at least one week after birth. We were not able to detect consistently Ki67 and TH double positive cells in SCG from older mice (data not shown). Together, the data from our immunostaining studies suggest that most proliferating cells in early postnatal mouse SCG are of glial or neuronal lineage.

**Differentiation potential of proliferating cells in postnatal mouse SCG**

To confirm that the proliferating cells are sympathetic progenitors with the capacity to differentiate into mature glial cells or neurons, we traced the fates of the cells that were proliferating during the first postnatal week. We injected 4-day-old neonatal mice with BrdU intraperitoneally once daily for 3 days to label all cycling cells; the mice were euthanized at various time points after the final BrdU injection. We then performed triple immunofluorescence staining of SCG sections for BrdU, TH, and BLBP or S100 to follow the fates of BrdU-labeled cells. Quantification of BrdU-positive cells at different time points showed a gradual decrease in their numbers, with approximately 50% of them retaining the BrdU label after 8 weeks (Fig. 6A). This result suggests that about half of the progenitors continued to proliferate
extensively after BrdU labeling, while the remaining half underwent limited cell divisions and thus retained the BrdU label.

Consistent with our Ki67 studies, we observed that a small number (~5%) of BrdU-positive cells expressed high levels of TH with the morphology of mature neurons in mouse SCG collected within a week after BrdU labeling (Fig. 6B,C), indicating the presence of neuronal progenitors and continuation of neurogenesis in early postnatal mouse sympathetic ganglia.

Staining for BLBP revealed that approximately 80-85% of BrdU-labeled cells expressed BLBP throughout the time points examined (Fig. 6D-J). Using S100 as a marker for glial differentiation, we found that an average of 15% of the BrdU-labeled cells stained positively for S100 one day after the final round of BrdU injection (Fig. 6D,K). Thereafter, there was a gradual increase in the number of BrdU and S100 double positive cells. The number peaked by the end of 3 weeks after BrdU labeling, with ~80% of BrdU-labeled cells expressing S100 (Fig. 6D,K-P). These findings indicate that most of the ganglionic cells that were proliferating during the first postnatal week are BLBP-expressing satellite cell progenitors, which eventually differentiate into S100-expressing satellite cells. The data from our fate-tracing studies also provide further evidence that the generation of satellite cells is a predominant process of postnatal sympathetic development.
Nestin expression marks both glial and neuronal progenitor cells in postnatal mouse SCG

The postnatal sympathetic glial and neuronal progenitors express the lineage markers BLBP and TH, respectively. However, neither marker is specifically associated with sympathetic progenitor cells, as BLBP expression is maintained in differentiated satellite cells and TH in mature neurons. To understand the molecular mechanisms that control the progenitor cell differentiation during postnatal sympathetic development, it is essential to identify the molecular markers whose expression marks distinct stages of the developmental process. We focused our studies on nestin, a filament protein initially identified as a maker for neural stem cells in the central nervous system (Lendahl et al., 1990). Importantly, it has been shown previously that nestin is expressed in neural crest cells from which all sympathetic neurons and glia originate (Stemple and Anderson, 1992).

Immunofluorescence staining revealed that a large number of cells in P1 and P4 SCG expressed significant levels of nestin, which often formed a thin layer of filamentous structure around nuclei (Fig. 7A,B). Nestin expression was gradually downregulated thereafter and was largely undetectable around P18 (Fig. 7C-F). This temporal pattern of nestin expression closely matches that of Ki67 (Fig. 1). Indeed, double immunofluorescence staining for Ki67 and nestin revealed that approximately 92-93% of Ki67-positive cells in P1 and P4 SCG also expressed nestin (Fig. 7G,H,I). The percentage of Ki67 and nestin double positive cells decreased with time, and by P18 only about 13% of Ki67-positive cells showed detectable levels of nestin expression (Fig. 7I). Given that most of Ki67-positive cells in P1 SCG differentiated into glia or
neurons by P18 (Fig. 2-5), the Ki67+nestin− cells in P7-P18 SCG probably represent a population of proliferating cells undergoing differentiation or immature glial cells and neurons. Taken together, our data suggest that nestin is a specific marker for sympathetic progenitor cells.

We next determined whether nestin expression is specific for glial or neuronal progenitor cells. We injected mice at P4 with BrdU and euthanized them 2 hours later to minimize the chance of BrdU-labeled cells to undergo extensive differentiation. Triple immunofluorescence staining of SCG sections was performed with antibodies against BrdU, nestin, and BLBP or TH, and the sections were examined with a confocal microscope. Consistent with the results of our Ki67 and nestin double staining experiments, approximately 95% of the BrdU-labeled cells expressed nestin (data not shown). Importantly, almost all of BrdU- and nestin-positive cells that were examined expressed either BLBP (Fig. 7J,K) or TH (Fig. 7L,M). These findings demonstrate that nestin is a marker for both glial and neuronal progenitor cells.
DISCUSSION

In this paper, we report three major findings from our in vivo examination of the postnatal development of mouse sympathetic ganglia, using mouse SCG as a model system. First, we show that nestin is a molecular marker for both sympathetic glial and neuronal progenitors. Second, we identify a population of nestin and BLBP double positive cells as the direct precursors of sympathetic satellite cells. Third, our study reveals a temporal expression pattern of glial lineage markers that defines the cellular intermediates in the process of satellite cell differentiation (Fig. 8). These findings define the cellular basis of postnatal sympathetic development, which should facilitate the investigation of the genes and signaling pathways that regulate the developmental process.

Nestin as a marker for both glial and neuronal progenitors in postnatal sympathetic ganglia

Nestin is an intermediate filament originally identified in neural stem cells (Lendahl et al., 1990). In the central nervous system, nestin expression marks the proliferating state of neurogenesis and is rapidly downregulated during differentiation (Dahlstrand et al., 1995). Neural crest cells isolated from rat embryonic trunk neural tubes have also been shown to express nestin (Stemple and Anderson, 1992), which give rise to both neurons and glia of the sympathetic nervous system (Le Douarin and Kalcheim, 1999). Our study reveals that during postnatal sympathetic development, nestin expression levels are at the highest at P1 and gradually decline thereafter, becoming largely undetectable by P18. This temporal expression of nestin correlates closely
with the proliferating phase of postnatal sympathetic development. In addition, triple immunofluorescence staining of multiple lineage markers and BrdU-based fate-tracing experiments show that nestin is expressed in both glial-restricted and neuron-restricted progenitor cells. These observations, coupled to the previously reported nestin expression in neural crest cells (Stemple and Anderson, 1992), identify nestin as a molecular link between neural crest cells and sympathetic progenitors. Also importantly, our findings provide nestin as a molecular marker for identifying genes and signaling pathways that promote the differentiation and maturation of sympathetic neurons and satellite cells.

The identities of neuronal and glial progenitors in postnatal sympathetic ganglia

Noradrenergic neurons and satellite glial cells are the two main cell types that constitute sympathetic ganglia. The progenitors for sympathetic neurons have been extensively studied. It is generally accepted that both sympathoadrenal progenitors from the trunk neural crest and sympathoenteric progenitors from the anterior vagal neural crest contribute to the generation of sympathetic neurons (Anderson, 1993; Birren and Anderson, 1990; Carnahan and Patterson, 1991; Durbec et al., 1996; Francis and Landis, 1999). These progenitors are characterized by their nonneuronal morphology and expression of neurofilaments (NF68 and NF160) and c-Ret (Guillemot et al., 1993; Sommer et al., 1995), a protein tyrosine kinase co-receptor for glial cell-derived neurotrophic factor (Plaza-Menacho et al., 2006). It is not known whether these early neuronal progenitors are present in postnatal sympathetic ganglia. Our study reveals that in postnatal mouse SCG, approximately 5% of proliferating cells express nestin and TH. These cells are morphologically indistinguishable from
the rest of post-mitotic sympathetic neurons, suggesting that they may represent a population of progenitor cells late in the neurogenic process.

In contrast, very little is known about the identity of the glial progenitors that give rise to sympathetic satellite cells. Using Ki67 to mark cells in an active proliferation state, a property of progenitor cells, we found that the vast majority of Ki67-positive cells in postnatal mouse SCG express BLBP, a nervous system-specific member of the lipid-binding protein family that was first identified in radial glial cells and immature astrocytes of the central nervous system (Feng et al., 1994; Kurtz et al., 1994). Our BrdU fate-tracing experiments further show that the BLBP-positive proliferating cells eventually differentiate into S100-expressing satellite cells, demonstrating that they function as progenitors of sympathetic satellite cells. However, BLBP is not an exclusive marker of the glial progenitors, as mature satellite cells maintain high levels of BLBP expression. In contrast to BLBP, nestin is expressed at high levels in proliferating glial progenitors but is markedly downregulated in differentiated satellite cells. Also, very few S100-positive satellite cells express detectable levels of nestin (data not shown). Thus, nestin and BLBP expression defines a population of satellite progenitors in mouse postnatal sympathetic ganglia.

**Postnatal sympathetic development**

The embryonic development of mammalian sympathetic ganglia has been extensively studied, which in rat SCG is characterized by the generation of neurons which peaks around E14.5, followed by a wave of gliogenesis that begins around E16.5 (Hall and
Landis, 1991; Hall and Landis, 1992; Hendry, 1977). For postnatal sympathetic development, the predominant event is the proliferation of nonneuronal cells, which, in rat SCG, lasts for at least two weeks after birth (Hendry, 1977). We confirm this observation in postnatal mouse SCG, which shows significant levels of proliferation up to 18 days after birth. We further demonstrate that, based on both morphology and expression of lineage markers, the vast majority of the proliferating cells are glial progenitors that give rise to satellite cells. Thus, the continuing generation and further differentiation of satellite cells characterize the postnatal development of mouse sympathetic ganglia.

During the postnatal gliogenic process in mouse SCG, both glial progenitors and mature satellite cells maintain high levels of BLBP expression, whereas nestin expression is downregulated and S100 expression is upregulated (Fig. 8A). Based upon lineage marker expression profiles and proliferation states, we suggest a model for the glial cell populations that may represent distinct stages of sympathetic gliogenesis (Fig. 8B). The glial progenitors are defined by the expression of both nestin and BLBP, and they possess the capacity to undergo multiple rounds of proliferation. The mature satellite cells are defined by the expression of both BLBP and S100, and they are post-mitotic cells. There is also a population of BLBP-positive glial cells with no or low levels of nestin and S100 expression, which may represent immature satellite cells with limited proliferation potential. The identification of the distinct glial populations and the developmental stages that they represent should help uncover the genes and signaling pathways that control the generation of satellite cells in the sympathetic nervous system.
LITERATURE CITED


Birren, S. J., and Anderson, D. J. (1990). A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. Neuron 4, 189-201.


FIGURE LEGENDS

**Fig. 1.** Identification of proliferating cells in postnatal mouse SCG. A-J: Immunofluorescence staining for Ki67-positive cells (red) in SCG from P1 to P56. Sections were counterstained with DAPI (blue) to visualize nuclei. There were significant levels of proliferation in SCG during the first 18 days after birth. Scale bar = 100 µm. K: Quantification of Ki67-positive cells in SCG from P1 to P30. At least 1000 cells were counted from each ganglion and 3-4 ganglia were examined for each time point. Data are presented as mean ± SD.

**Fig. 2.** BLBP immunofluorescence staining for lineage identification of proliferating cells in postnatal mouse SCG. Sections of SCG from P1 to P30 were stained with antibodies against Ki67 (green) and BLBP (red). All satellite cells stained positively for BLBP. The majority of Ki67-positive cells expressed BLBP (yellow), indicating that the postnatal development of sympathetic ganglia is predominantly a gliogenic process that was essentially completed by P22. Scale bar = 50 µM.

**Fig. 3.** S100 immunofluorescence staining for lineage identification of proliferating cells in postnatal mouse SCG. Sections of SCG from P1 to P30 were stained with antibodies against Ki67 (green) and S100 (red). Postnatal sympathetic development in SCG is characterized by a gradual increase in the number of S100-expressing satellite cells, which peaked by P22. In SCG from P7 to P18, a subpopulation of Ki67-positive cells expressed S100 (yellow). Scale bar = 50 µM.
**Fig. 4.** Quantification of Ki67-positive cells that express the glial lineage marker BLBP or S100. For each time point, 3-4 ganglia were examined, and Ki67 single positive and Ki67-BLBP (A) or Ki67-S100 (B) double positive cells were counted from at least 4 randomly selected fields (400X). Data are presented as mean ± SD.

**Fig. 5.** TH immunofluorescence staining for lineage identification of proliferating cells in postnatal mouse SCG. Sections of SCG from P1 to P7 were stained with antibodies against Ki67 (green) and TH (red), and counterstained with DAPI (blue) to visualize nuclei. Arrows indicate Ki67 and TH double positive cells, which represented approximately 5% of Ki67-positive cells. The Ki67-positive neurons are morphologically indistinguishable from the rest of post-mitotic neurons. Scale bar = 25 µM.

**Fig. 6.** BrdU fate-tracing study of proliferating cells in postnatal mouse SCG. Mice at P4 were injected with BrdU once daily for 3 days and sacrificed on day (D) 1, 7, 14, 21, 28, 56 after the final round of BrdU injection. Mouse SCG sections were stained with antibodies against BrdU (green), TH (blue), and BLBP or S100 (red). **A:** Quantification of BrdU label-retaining cells in SCG from D1 to D56. For each time point, 3-4 ganglia were examined. BrdU-positive cells were counted from at least 4 randomly selected fields (400X) and the average number of BrdU-positive cells per field from D1 SCG was defined as 100%. Data are presented as mean ± SD. **B,C:** Confocal immunofluorescence images showing postnatal mouse SCG at D1 (B) and
D7 (C) contained BrdU and TH double positive neurons (arrows), which account for approximately 5% of total BrdU-positive cells. Scale bar = 25 µm. D: Quantification of BrdU-positive cells that express the glial lineage (Lin+) marker BLBP or S100 at the indicated time points. For each time point, 3-4 ganglia were examined. BrdU single positive and BrdU-BLBP or BrdU-S100 double positive cells were counted from at least 4 randomly selected fields (400X). Data are presented as mean ± SD. E-P: Confocal immunofluorescence images of SCG sections from mice at different time points (D1-D56) following the final round of BrdU injection. The sections were stained with antibodies to BrdU, TH, and BLBP (E-J) or S100 (K-P), showing that BrdU-labeled, BLBP single-positive cells in D1 SCG differentiated into satellite cells expressing both BLBP and S100 in a time-dependent manner. Scale bars = 50 µm.

Fig. 7. Nestin is a common marker for glial and neuronal progenitors in postnatal mouse SCG. A-F: Time-course study of nestin expression in mouse SCG from P1 to P18. Sections were stained with an antibody against nestin (green), counterstained with DAPI (blue), and examined with a confocal microscope. Nestin expression was essentially undetectable by P18. Scale bar = 50 µm. G,H: Confocal immunofluorescence images of SCG sections from mice at P1 (G) and P4 (H). The sections were stained with antibodies against nestin (green) and Ki67 (red), showing nestin expression in Ki67-positive proliferating cells. Scale bar = 25 µm. I: Quantification of Ki67 and nestin double positive cells in mouse SCG at the indicated time points (P1-P18). For each time point, 3 ganglia were examined. Ki67 single-positive and Ki67-nestin double positive cells were counted from 3 randomly selected fields (400X). Data are presented as mean ± SD. J-M: Confocal immunofluorescence
images of SCG sections from mice at P4. The mice were injected with BrdU and sacrificed after 2 hours. **J,K:** Sections were stained with antibodies against BrdU (blue), nestin (green) and BLBP (red), showing nestin expression in BrdU and BLBP double positive glial progenitors (arrows). Scale bar = 25 µm. **L,M:** Sections were stained with antibodies against BrdU (blue), nestin (green), and TH (red), showing nestin expression in BrdU and TH double positive neuronal progenitors (arrows). Scale bar = 25 µm.

**Fig. 8.** A model for the generation and differentiation of satellite cells in postnatal sympathetic ganglia. **A:** A diagram for the temporal expression pattern of molecular markers (nestin, BLBP and S100) during postnatal sympathetic gliogenesis. **B:** A model for the putative glial cell populations that represent distinct stages of satellite cell differentiation (see text for detail).
Figure 4

A

B

Ki67+BLBP+Ki67+ (%) vs Postnatal days

K167+SO100+Ki67+ (%) vs Postnatal days
Figure 5
Figure 6

A

BrdU-positive cells (%)

B

TH-BrdU

C

D1

D7

D21

D28

D56

Post BrdU injection (day)

D

BrdU*BLBP*+  
BrdU*S100*

BrdU*TH-BrdU (%)

Post BrdU injection (day)

E, F, G, H, I, J

BLBP-TH-BrdU

K, L, M, N, O, P

S100-TH-BrdU
GATA3 regulation of human neuroblastoma stem cell activities

Huilin Shi\textsuperscript{1}, Hongjuan Cui\textsuperscript{1}, Goleeta Alam\textsuperscript{1}, Jane Ding\textsuperscript{2}, Jean H. Overmeyer\textsuperscript{1}, William A. Maltese\textsuperscript{1}, Han-Fei Ding\textsuperscript{2*}

\textsuperscript{1}Department of Biochemistry and Cancer Biology, University of Toledo, Health Science Campus, Toledo, Ohio 43614, USA.

\textsuperscript{2}Department of Pathology and Cancer Center, Medical College of Georgia, Augusta, GA 30912, USA.

*Corresponding author: Han-Fei Ding hding@mcg.edu

Subject categories: Cancer initiation
Abstract

Neuroblastoma is a common childhood malignant tumor of the sympathetic nervous system. BE(2)-C cells, a human neuroblastoma cell line enriched with tumorigenic stem cells, can be induced to undergo either neuronal or glial differentiation. We use BE(2)-C cells as a system to identify the genes that regulate neuroblastoma stem cell activities, such as self-renewal and differentiation. One of these genes is GATA3, a zinc-finger transcription factor with an essential role in the sympathetic development. Downregulation of GATA3 by siRNA promotes BE(2)-C cell proliferation and overexpression of GATA3 decreases the proliferation of BE(2)-C cells. GATA3 regulates cell proliferation through distinct pathways involving Cyclin D1 and E2F1, as evidenced by an increased expression of Cyclin D1 and a decreased expression of E2F1 in GATA3 knockdown cells. In addition, GATA3 knockdown induces BE(2)-C cells to undergo glial differentiation, as indicated by an increase in the expression of GFAP, a glial cell marker, and a decrease in the expression of neuronal marker SNAP25. GATA3 overexpression promotes neuronal differentiation of BE(2)-C cells, as indicated by a reduction of GFAP expression and an upregulation of SNAP25 expression. GATA3 knockdown also downregulates Phox2b and GATA3 overexpression upregulates Mash1, two transcription factors that are expressed in neuronal progenitor cells and are essential for the sympathetic development. Together, these findings suggest that GATA3, Phox2b and Mash1 may function in a regulatory network in the control of neuroblastoma cells in a stem/progenitor cell state.

Keywords: Neuroblastoma/Cancer stem cell/GATA3/BE(2)-C/Cyclin D1/E2F1
Neuroblastoma is a common childhood solid cancer and originated from neural crest stem cells (NCSCs) or sympathetic precursor cells (Brodeur, 2003), occurring in sympathetic ganglia and adrenal medulla. 90% of children with this disease are diagnosed before the age of 6 years (Schwab et al., 2003). Neuroblastoma can either regress spontaneously, usually in infants, or mature into a benign ganglioneuroma by undergoing apoptosis and/or differentiation. However in children over 1 year old, the tumors are mostly aggressive and fatal and their overall prognosis has been poor (Brodeur, 2003; Nakagawara, 1998). The enigmatic clinical behavior of neuroblastoma is based on its heterogeneous property. Histopathologically, neuroblastoma is characterized by heterogeneous composition, from tumors consisting of dominant undifferentiated neuroblasts to those comprised of largely fully differentiated neurons with a dense stroma of Schwann cells (Castleberry, 1997).

The cell lines established from human neuroblastomas also exhibit heterogeneity.

Three distinct types of cell lines have been identified based on their morphologies, growth patterns and biochemical properties. They are N for neuroblastic, S for non-neuronal, substrate-adherent and I for intermediate cell types (Ross et al., 2003). N-type cells have immature neuroblastic features with small and rounded bodies, a high nuclear to cytoplasmic ratio and short neurites. They attach better to other cells than to substrate and form cell aggregates in culture. They express neurofilaments, specific noradrenergic enzymes and receptors (Ross et al., 2002). S-type cells show
characteristics opposite to those of N-type cells. They are large and flattened cells with abundant cytoplasm. They may have long filopodia but no neurites. These cells adhere tightly to substrate, grow as a monolayer in culture and present contact inhibition of growth. They express glial cell markers like vimentin or glial fibrillary acidic protein (GFAP) (Acosta et al., 2009). I-type cells exhibit an intermediate status between N-type and S-type cells. They attach equally well to both the substrate and to other cells. They may or may not have neurites and form multilayers with focal aggregates in cultures. They have nuclei similar to N-type cells and more cytoplasm like S-type cells. I-type cells express both N- and S-type cell markers and stem cell markers, such as CD133 and c-kit (Walton et al., 2004).

It has been proposed that I-type cells may represent a population of neuroblastoma stem cells based on their differentiation and clonogenic ability. I-type cells can differentiate into both N-type and S-type cells under different conditions (Acosta et al., 2009). N-type cells can be induced to neuronal or neuroendocrine cells (Ross et al., 2002) whereas S-type cells can further differentiate into Schwann/glial cells, melanocytes and smooth muscle cells (Slack et al., 1992; Sugimoto et al., 2000). I-type cells have the greatest clonogenic activity in soft agar and tumorigenic potential in immunodeficient mice. S-type cells are non-tumorigenic and N-type cells exhibit tumorigenic ability depending on particular cell lines (Spengler et al., 1997). Neuroblastoma is one of few cancers originating during the embryonic development, which implies that this tumor may be initiated from transformed developing NCSCs or malignant sympathetic precursor cells that obtain stem cell properties through mutations. This neuroblastoma stem cell model can adequately interpret the
heterogeneous characteristic of human neuroblastoma tumors, based on the self-renewal and differentiation ability of malignant NCSCs or sympathetic precursor cells. I-type neuroblastoma cell lines have been identified as cancer stem cells based on their special phenotypic characterization, differentiation potential and malignant potential (Ross and Spengler, 2007).

Since neuroblastoma cancer stem cells play an essential role in the tumor initiation, it is necessary to investigate the regulatory mechanisms underlying self-renewal and differentiation capacities of neuroblastoma stem cells, which provide the foundation for appropriate future therapies in neuroblastomas. In this study, we investigated the GATA3 regulation of BE(2)-C cell – an I-type neuroblastoma cell line – activities, including tumorigenicity and differentiation. In vertebrates, the GATA family contains 6 six members (named GATA1 to 6) which belong to zinc finger transcription factors and bind to the common consensus DNA sequence (A/T)GATA(A/G) (Patient and McGhee, 2002). GATA3 plays an essential role in the development of many tissues in vertebrates, including Th2 cell differentiation, the development of the kidney, the inner ear, embryonic and pubertal mammary glands, adipocytes and neurons, et al. (Asselin-Labat et al., 2007; Grote et al., 2008; Kouros-Mehr et al., 2008; Lillevall et al., 2006; Smith et al., 2002; Tong et al., 2005; van Doorninck et al., 1999; Yamashita et al., 2005). The haploinsufficiency of the GATA3 gene in chromosome 10p causes HDR (hypoparathyroidism, deafness and renal dysplasia) syndrome (Van Esch et al., 2000). In the sympathetic nervous system, GATA3−/− mouse embryos die by embryonic day 11 (E11) due to noradrenaline deficiency. Loss of GATA3 leads to dramatic downregulation of the TH and DBH
expression (Lim et al., 2000). GATA3 controls sympathoadrenal differentiation by mutual modulation with the other sympathetic regulators Mash1, Phox2b and dHand (Moriguchi et al., 2006). GATA3 also contributes to tumorigenesis in multiple tumors, such as T cell acute lymphoblastic leukemias (T-ALL) and breast cancers (Minegishi et al., 1997; Usary et al., 2004). Enforced expression of GATA3 during the T cell development in CD2-GATA3 transgenic mice induces CD4⁺CD8⁺ double-positive (DP) T cell lymphoma (Nawijn et al., 2001). Loss of GATA3 in breast cancers induces poorly differentiated tumors cells and promotes dissemination (Kouros-Mehr et al., 2008). Therefore, it is a question of interest to investigate the role of GATA3 in the regulation of neuroblastoma stem cell activities to uncover the mechanisms underlying tumorigenesis of neuroblastoma.
Results

*Loss of GATA3 increases the clonogenic capacity of BE(2)-C cells*

Cancer cells can grow in a semi-solid medium, such as soft agar or methylcellulose whereas non-cancer cells grow only when attached to a surface. The semi-solid medium can prevent the migration of cells and lead to the formation of spatially distinct colonies (Thomson and Meyskens, 1982). The anchorage-independent growth is an important characteristic of cancer cells and is correlated closely with the tumorigenic capacity of cancer cells. BE(2)-C-pSIH1-H1-puro-GFPsi (abbr BE(2)-C-GFPsi) and BE(2)-C-pSIH1-H1-puro-GATA3si (abbr BE(2)-C-GATA3si) cells were plated at 1000 cells per well (6-well culture plates). Viable colonies were stained with MTT 11 days after plating. By dividing the number of colonies by the number of cells plated, it is estimated that the colonies formed by BE(2)-C-GATA3si cells are higher than that of BE(2)-C-GFPsi by 44.9% (Figure 1AB). Therefore, loss of GATA3 expression significantly increases the cloning efficiency in soft agar and increases the clonogenic capacity of BE(2)-C cells. The soft agar colony formation assay performed in GATA3 overexpression cells further confirms this conclusion. The colonies formed by BE(2)-C-pBabe-puro-GATA3 cells are 31.85% fewer than those formed by BE(2)-C-pBabe-puro cells (Figure 1CD), which suggests that the function of GATA3 is to suppress the clonogenic capacity of BE(2)-C cells.

Clonogenic assays were performed by plating 1000 cells in a 100mm plate and staining with 0.4% crystal violet after 14 days. Based on the percentage of the number
of colonies formed in that of plated cells, BE(2)-C-GATA3si cells give rise to more colonies than BE(2)-C-GFPsi cells by 57.55%, whereas BE(2)-C-pBabe-puro-GATA3 cells produce fewer colonies than those of BE(2)-C-pBabe-puro cells by 26.65% (Figure 2). Clonogenic assay reflects long term cell survival of the specific cell line, but can’t exhibit the clonogenic capacity of the cell line because this technique is feasible to any attached cells including non-cancer cells and cancer cells. The crystal violet staining is chemical staining and is not specific for viable cells. Even though, these results still indicate that loss of GATA3 increases the proliferative potential of BE(2)-C cells.

In the MTT assay, the number of viable cells is reflected by the OD value. The viable cell numbers of the GATA3 knockdown or overexpression cell line were measured in continuous 7 days in cultures at initially 500 or 750 cells. There is an increase in the number of viable cells in the cultures of BE(2)-C-GATA3si cells and a decrease in the BE(2)-C-pBabe-puro-GATA3 cell cultures compared with their respective controls (Figure 3). Though the MTT assay can reflect the dynamic process for proliferative potentials of cells, it still has technical limitations which are caused by the ratio of MTT amount to cell numbers, culture time, the frequency for medium change, et al. Therefore, the cell growth assay was performed to confirm the MTT results. In this assay, the exact cell numbers in cultures at indicated time points were examined, which compensates the limitations of the MTT assay and reflects the dynamic process of cell proliferation. As shown in Figure 4, the growth rate of GATA3 knockdown cells is markedly increased compared with control cells, whereas a decreased rate of cell proliferation is detected in GATA3 overexpression cells. To summarize, these
four assays are highly consistent and demonstrate that loss of GATA3 increases the clonogenic capacity and proliferative potential of BE(2)-C cells.

**Cyclin D1 and E2F1 are the downstream genes of GATA3 responsible for the increased clonogenicity of BE(2)-C cells**

Since the loss of GATA3 increases the proliferative potential of BE(2)-C cells, it is interesting to investigate the mechanisms underlying the increased clonogenic capacity. The expression of candidate downstream genes was investigated in the GATA3 knockdown cell line and its control.

The first group of genes - p16, E2F1 and the retinoblastoma protein (pRb) - is the regulators closely involved in controlling the late G1 phase checkpoint in the cell cycle. Cyclin D/CDK4 or Cyclin D/CDK6 complexes can phosphorylate pRb and the phosphorylated pRb releases E2F transcription factors which regulate gene transcription required for DNA synthesis and promote cells to enter into S phase irreversibly (Diehl, 2002; Weinberg, 1995). The p16 functions as a cyclin-dependent kinase (CDK) inhibitor and specifically binds to CDK4 and CDK6 thus leading to G1 arrest by inhibiting their kinase activity (Aprelikova et al., 1995). In our research, p16 is expressed equally in the GATA3 knockdown and control cells (Figure 5B), which suggests that GATA3 is not acting through p16 to regulate cell proliferation and p16 is not responsible for the expression alteration of pRb and E2F1. The pRb expression is dramatically upregulated in GATA3 knockdown cells compared with the control (Figure 5B). Because pRb has two forms, phosphorylated and unphosphorylated, it’s
difficult to distinguish the difference in the expression alteration of these two forms using general pRb antibodies. Therefore, the upregulation of pRb reflects the change of total pRb expression and implies that it may be a downstream gene of GATA3 to promote cell cycle progression. The expression pattern of E2F1 in our research is not consistent with the reported function of this gene in promoting cell proliferation through the Cyclin D-pRb-E2F pathway. The E2F1 expression is significantly downregulated in GATA3 knockdown cells compared with the control (Figure 5B). However, the E2F family also includes other proteins, such as E2F2 to E2F8; E2F3a is also a downstream target of pRb promoting the cell cycle (Chong et al., 2009). The overexpression of E2F1 has been proven to increase apoptosis (Wu and Levine, 1994). Therefore, our results are consistent with the decreased E2F1 expression causing an increased clonogenicity of BE(2)-C cells through apoptosis suppression.

The second group of genes - p27, p21 and p53 - belongs to tumor suppressor genes while the p21 and p27 are CDK inhibitors, which play important roles as negative regulators during progression of the cell cycle. The p27 can bind to the Cyclin D/CDK4 complex and prevent CDK4 from phosphorylating its substrate - pRb. The increased level of p27 typically causes cells to arrest in the G1 phase of the cell cycle. The p27 can also bind to the Cyclin A/CDK2 and Cyclin E/CDK2 complexes (Toyoshima and Hunter, 1994). The p21 expression is induced by p53 during a DNA damage-induced G1-phase checkpoint response and inhibits both CDK4 and CDK2 activities (He et al., 2005). The loss of the CDK inhibitors occurs in multiple tumors. The p27 mutation has been proven to exist in the breast, pituitary and colon cancers (Galizia et al., 2004; Spirin et al., 1996; Takeuchi et al., 1998), et al. The decreased
expression of p21, p27 and p53 is expected to occur in poor prognosis cancers (Klopfleisch and Gruber, 2009; Koljonen et al., 2006). In our research, the expression levels of p21, p27 and p53 in GATA3 knockdown cells are not significantly different from control cells (Figure 5C). This implies that these CDK inhibitors are not downstream targets of GATA3 responsible for the increased proliferation while they are not involved in the regulation of the pRb or E2F1 level.

Cyclins are a family of proteins which control the progression of cells through the cell cycle by regulation of CDKs (Galderisi et al., 2003). Cyclins themselves have no enzymatic activity. There are two main groups of cyclins: G1/S cyclins are essential for the control of G1/S transition in the cell cycle; G2/M cyclins are required for the G2/M transition. G1/S cyclins include Cyclin A/CDK2, Cyclin D/CDK4, Cyclin D/CDK6 and Cyclin E/CDK2 while G2/M cyclins contain Cyclin B/CDK1 (Galderisi et al., 2003). The overexpression of cyclins promotes cell cycle progression and occurs in diverse cancers (Collecchi et al., 2000; Knudsen et al., 2006; Lotayef et al., 2000; Schildkraut et al., 2008; Wang et al., 1997). In our research, GATA3 deficiency induces the upregulation of Cyclin D1 (Figure 5D), which suggests that Cyclin D1 is responsible for the increased proliferative potential and is the downstream target of GATA3. GATA3 suppresses the cell proliferation through inhibiting the Cyclin D1 expression. In other three cyclins, Cyclin B1 and Cyclin E are equally expressed between the GATA3 knockdown cell line and its control whereas the Cyclin A expression is significantly decreased under GATA3 deficiency, which is inconsistent with the context (Figure 5D).
**GATA3 expression is closely related to the differentiation status of BE(2)-C cells**

The essential role of GATA3 in the sympathetic nervous system is to induce the differentiation of sympathetic neurons by promoting TH synthesis. Loss of GATA3 will result in mouse lethality around E11 due to noradrenergic deficiency (Moriguchi et al., 2006). Since BE(2)-C cells are potential neuroblastoma stem cells and have the capacity to differentiate into both neurons and glial cells (Acosta et al., 2009), GATA3 may closely regulate the differentiation of BE(2)-C cells and determine the differentiation status of neuroblastoma stem cells.

To investigate the effects of GATA3 on neuronal differentiation in BE(2)-C cells, the expression of a group of neuronal markers is investigated, including TH, peripherin, SNAP25, Rab5A and Rab1B.

Tyrosine hydroxylase (TH) is a specific marker for sympathetic neurons because it is the first and rate-limiting enzyme in synthesizing the catecholamine neurotransmitters. In the rat embryonic study, TH is started to express in the cells of thoracic sympathetic ganglia on E11, and occurs in abdominal and lumbar ganglia on E12~13 (Teitelman et al., 1979). Embryonic mouse superior cervical ganglia show a similar developmental time course in the neuronal differentiation by TH detection (Coughlin et al., 1977). In BE(2)-C cells, GATA3 overexpression induces the upregulation of TH expression (Figure 6B), which is consistent with the role of GATA3 in the sympathetic development whereas the alteration of TH expression is not significant in the GATA3 deficiency context.
SNAP25 is a palmitoylated protein which assembles with syntaxin as a key component of the “t-SNARE” complex and locates at the synaptic plasma membrane (Chapman et al., 1994; McMahon and Sudhof, 1995). Proteins of the synaptic vesicle membrane (such as synaptobrevin, synaptophysin, synapsin and synaptotagmin) interact with their plasma membrane-located counterparts (such as SNAP25 and syntaxin) to facilitate synaptic vesicle docking and calcium dependent exocytosis (Kretzschmar et al., 1996). The expression of these vesicle exocytosis regulators is an important characteristic of neuronal cells. For example, postmitotic NT2N cells, which are considered to be a good in vitro model for mature neurons in the human central nervous system (CNS), are derived from human NT2 teratocarcinoma cells by a retinoic acid (RA) treatment. The vesicle exocytosis proteins are dramatically expressed in NT2N cells and can’t be detected in untreated NT2 cells or cells exposed to RA for only 6 days (Sheridan and Maltese, 1998). Based on these facts, the phenomena that the SNAP25 expression is decreased under GATA3 deficiency and is increased during GATA3 overexpression in our experiments (Figure 6B) provide solid support for the role of GATA3 in the induction of neuronal differentiation in BE(2)-C cells.

Peripherin is a peripheral neuronal marker which is a type of III intermediate filament (IF) protein mainly found in the neurons of the peripheral nervous system (PNS) such as motor, sensory and sympathetic neurons as well as neuroendocrine carcinomas of the skin and certain melanomas (Baudoin et al., 1993; Huttenbach et al., 2002; Portier et al., 1983). The fact that peripherin expression remains constant in both the GATA3
deficiency and overexpression context (Figure 6B) is consistent with the previous study that loss of GATA3 doesn’t influence generic neuronal properties (Moriguchi et al., 2006).

Rab proteins are a family of Ras-related GTPases that regulate vesicular traffic in mammalian cells (Novick and Zerial, 1997). Around 70 different Rab proteins have been identified in humans so far. The different Rab GTPases are localized to the cytosolic face of specific intracellular membranes, where they regulate multiple steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks and membrane fusion. These processes constitute the route through which cell surface proteins are trafficked from the Golgi to the plasma membrane and are recycled (Stenmark and Olkkonen, 2001). Rab5A is related to a active status of the endocytic pathway in recycling of synaptic vesicles in neurons (Sudhof, 1995). The expression of Rab5A in differentiated NT2N cells is 4 to 5 fold higher than in undifferentiated NT2 cells (Sheridan and Maltese, 1998). However, in BE(2)-C cells, the expression level of Rab5A is not influenced by GATA3 expression (Figure 6B), which indicates that Rab5A is not a suitable neuronal marker in this system. Rab1B functions in constitutive transport of proteins between the endoplasmic reticulum and Golgi apparatus (Plutner et al., 1991) and may relate to the cell proliferative status. In our results, upregulation of Rab1B under loss of GATA3 is related to a higher proliferative status, whereas downregulation of Rab1B during GATA3 overexpression is committed to a decreased proliferative status (Figure 6B), which further confirms the role of GATA3 in the regulation of the BE(2)-C cell proliferation (Figure 1 to 4).
Two glial cell markers, GFAP and vimentin, are investigated to confirm the role of GATA3 in differentiation. GFAP and vimentin both belong to Class III family of IF proteins and distribute in diverse glial cells. The GFAP is specifically expressed in astrocytes in the CNS; in the PNS, it has been detected in the satellite cells and non-myelinating Schwann cells (Sancho-Tello et al., 1995; Stephenson and Byers, 1995; Triolo et al., 2006). Neural stem cells also strongly express GFAP (Imura et al., 2003). Vimentin is also expressed in both early and mature glial cells in the nervous system (Schnitzer et al., 1981). In BE(2)-C cells, increased GFAP expression is shown under loss of GATA3, while decreased GFAP expression is induced by GATA3 overexpression (Figure 6C), which is consistent with the role of GATA3 in the neuronal differentiation in BE(2)-C cells.

In summary, GATA3 deficiency induces the glial differentiation while GATA3 itself promotes the neuronal differentiation in BE(2)-C cells. Interestingly, we found that GATA3 expression is not shown in the typical S-type neuroblastoma cell line - SHEP1, but is highly exhibited in I-type and most N-type cell lines (Figure 7A). This result further confirms the role of GATA3 in the neuroblastoma differentiation. SHEP1 cells can represent an extreme model of GATA3 deficiency. However, we also observed that GATA3 is downregulated during the RA-treated neuronal differentiation (Figure 7B). In the RA treatment time course study, GATA3 expression starts to dramatically decrease after 3 days, thereafter, the expression can’t be detected. The RA-induced strong neuronal differentiation in BE(2)-C cells as is shown in Figure 7C. The neuronal differentiation also begins after 3-day treatment
and progresses in the differentiation status thereafter until fully differentiated. So the pattern of GATA3 downregulation is consistent with the neuronal differentiation process in the time course. These observations suggest that GATA3 plays an important role in neuronal progenitors. If cells exist in a terminal differentiation status, GATA3 expression is not expected.

Finally, a progenitor cell marker nestin was investigated in BE(2)-C cells. Nestin is one kind of IF protein (Hockfield and McKay, 1985). Nestin was first identified as a specifically expressed gene in neuroepithelial stem cells and the nestin expression distinguishes stem cells from more differentiated cells in the neural tube (Lendahl et al., 1990). Later, it was found that nestin is widely distributed in most neuronal progenitor cells in the CNS (Dahlstrand et al., 1995), in migrating neural crest cells (Lothian and Lendahl, 1997; Stemple and Anderson, 1992) and in adult radial glial cells (Chanas-Sacre et al., 2000). Following transition to differentiated cells, nestin expression decreases and is substituted by other tissue-specific IFs (Dahlstrand et al., 1995). In the PNS, nestin is expressed in both neuronal and glial progenitor cells (Shi et al., 2008). In BE(2)-C cells, nestin is highly expressed but its expression is not affected by the alteration of GATA3 expression (Figure 6D). This suggests that BE(2)-C cells, as a kind of neuroblastom stem cells, are still in a progenitor status.

**The modulation between GATA3 and other sympathetic development regulators**

GATA3 was first identified as a gene specifically required for the expression of adrenergic traits because in GATA3 mutant mice, sympathetic ganglia form and
express pan-neuronal markers and Phox2 proteins, but fail to express TH and DBH
(Lim et al., 2000). GATA3 is genetically downstream of Phox2b because its
expression in sympathetic precursor cells is abolished in Phox2b mutants (Tsarovina
et al., 2004).

In 2006, Moriguchi et al. found that the expression levels of Mash1, dHand and
Phox2b (postulated upstream regulators of GATA3) as well as downstream products,
TH and DBH, are markedly downregulated in the developing sympathoadrenal
system of GATA3 mutant mice. In the transgenic mouse model that specifically
expresses GATA3 in the sympathoadrenal lineages under the human DBH promoter,
the restoration of GATA3 recovers the sympathoadrenal function almost to the
normal level and phenotypically restores the downstream as well as the putative
upstream genes, which suggests that the sympathoadrenal differentiation is controlled
by mutually reinforcing feedback transcriptional modulations between GATA3,
Mash1, dHand and Phox2b (Moriguchi et al., 2006).

However, there were no in vitro studies performed before to confirm the modulation
between GATA3 and other sympathetic regulators. In our study, the direct
modulations between GATA3 and Mash1, GATA3 and Phox2b were investigated in
BE(2)-C cells under the GATA3 knockdown or overexpression context. It is
demonstrated that Mash1 is significantly upregulated in GATA3 overexpression cells,
while Phox2b is significantly downregulated in GATA3 knockdown cells (Figure 8).
These results provide the direct evidence that GATA3 directly regulates the
expression of Mash1 and Phox2b at the cellular level. Therefore, these postulated upstream genes actually have a bi-directional modulation with GATA3.
Discussion

To investigate the role of GATA3 in neuroblastoma stem cells, it is important to learn the GATA3 function in the normal development of the sympathetic nervous system as well as other tissues. In the normal sympathetic development, loss of GATA3 induces embryonic lethality around E11 due to noradrenaline deficiency and leads to increased apoptosis and significant impairment of sympathetic differentiation. The size of sympathetic ganglia is dramatically reduced in GATA3\(^{-/-}\) mice due to a strong increase in the number of apoptotic cells (Tsarovina et al., 2004). However, in the development of the kidney, nephric duct-specific inactivation of GATA3 leads to massive ectopic ureter budding and premature nephric duct cell differentiation which suggests that loss of GATA3 promotes both proliferation and differentiation of nephric progenitor cells (Grote et al., 2008). In the development of breast epithelial cells, GATA3 deficiency leads to an expansion of luminal progenitors and a concomitant block in differentiation, while introduction of GATA3 into a stem cell-enriched population induces maturation along the alveolar luminal lineage (Asselin-Labat et al., 2007). Based on the above phenomena, the effects of GATA3 loss in the nephric development are completely opposite to those in the sympathetic development, whereas in the mammary development GATA3 deficiency induces proliferation as in nephric ducts and blocks differentiation as in the sympathetic system. Therefore, though GATA3 plays essential roles in the mammalian development in multiple organs, the underlying mechanisms are diverse. But the differences are presented in the cellular level; in the organic level, loss of GATA3 consistently results in developmental defects, including shrinkage of sympathetic ganglia, a spectrum of
urogenital malformations and the mammary undifferentiated luminal cell expansion with basement-membrane detachment that leads to the caspase-mediated cell death in the long term. In summary, GATA3 is active in stem cells or progenitor cells in different organs and contributes to the normal mammalian development.

In our I-type neuroblastoma cell system, loss of GATA3 results in increased proliferation potential and blocking commitment to neuronal differentiation. Compared with the normal sympathetic development, GATA3 shows a similar function in differentiation but has opposite effects on proliferation. The conflict in the effects on proliferation may reflect the difference between in vivo and in vitro studies. The sympathetic proliferation in vivo is not only influenced by the local loss of GATA3 but also controlled by more integral factors such as defects from other organs due to GATA3 deficiency. The defects in the kidney, heart, thymus and hematopoietic system et al. would result in an abnormal status in the whole body and further have effects on local organs. The apoptosis in the sympathetic ganglia and adrenal medulla might be induced by some specific factors produced in the abnormal development, but not by the direct loss of GATA3. Another possibility is that GATA3 is indispensable in maintaining the neuronal progenitor cell survival. If GATA3 expression is deleted, these neuronal progenitor cells can’t survive and apoptosis is induced. This possibility can be proven by deleting the GATA3 expression in N-type neuroblastoma cell lines, such as SK-N-DZ or SK-N-AS et al. If apoptosis is induced in these N-type neuroblastoma cell lines, this possibility might be true. In our BE(2)-C cell system, the external factors are eliminated, so the increased proliferation induced by GATA3 deficiency might reflect the direct effect on the sympathetic development. However,
the BE(2)-C cell line belongs to the intermediate I-type neuroblastoma stem cells, so its situation might be different from N-type neuroblastoma cell lines. Based on observations, GATA3 deficiency induces increased proliferation in SK-N-AS cells, but leads to apoptosis in SK-N-DZ cells. Therefore, even in N-type neuroblastoma cell lines, the effects of GATA3 loss are diverse; this might be derived from the delicate differentiated status of N-type cells.

Previous studies demonstrated that breast cancers might originate from breast cancer stem cells (Dontu, 2008). Similarly, loss of GATA3 function contributes to the tumorigenesis of breast cancers (Usary et al., 2004). Loss of GATA3 marks a progression from adenoma to early carcinoma and the onset of tumor dissemination in breast cancers. Restoration of GATA3 in late carcinomas induces tumor differentiation and suppresses tumor dissemination. Targeted deletion of GATA3 in early tumors leads to apoptosis of differentiated cells, which suggests that the loss of GATA3 is not sufficient for malignant conversion, while an expanding GATA3-negative tumor cell population accompanies malignant progression (Kouros-Mehr et al., 2008).

In summary, loss of GATA3 in cancer stem cells promotes tumor progression. The expression level of GATA3 in cancer stem cells can be a good prognosis marker of tumors. Low expression of GATA3 in tumors might predict a more progressive and malignant tendency in the tumor development. The prediction would help to start suitable treatments in the early stage of tumor growth.
Amplification or overexpression of Cyclin D1 is important in tumorigenesis in multiple organs including the parathyroid adenoma, lymphoma, breast and prostate cancers (de Boer et al., 1997; Drobnjak et al., 2000; Hsi et al., 1996; Musgrove et al., 1994). Recently, Dr. Richard Pestell and colleagues at Thomas Jefferson University found that Cyclin D1 is required for the breast cancer growth in mice. The therapy targeting Cyclin D1 may block the expansion of breast cancer stem cells and effectively control tumor growth. Amplification of the Cyclin D1 gene has been also found in some neuroblastoma tumors (Molenaar et al., 2003). In our study, the role of GATA3 in the increasing clonogenic capacity of neuroblastoma stem cells might through the downstream Cyclin D1 gene, which suggests that targeting Cyclin D1 in the therapy might be an effective approach to decreasing the proliferative potential of neuroblastoma stem cells and effectively cure this cancer.

Cyclin D1 promotes cell cycle progression via the typical Cyclin D1-pRb-E2F pathway. The active E2F factors become detached from pRb and induce the synthesis of transcription factors required for entering the S phase. However, in our study the significant downregulation of E2F1 expression accompanied by the increased proliferation suggests that there must be another pathway to promote proliferation by reduced E2F1 in addition to the typical Cyclin D1-pRb-E2F pathway. Actually, the role of E2F1 in tumorigenesis is complex and not completely clear. In addition to promoting proliferation, E2F1 can also potently induce apoptosis when it is overexpressed or deregulated through pRb inactivation (Tsai et al., 1998; Wu and Levine, 1994). Various mouse models have been reported to show an oncogenic or
tumor suppressive property for E2F1. Transgenic mice overexpressing E2F1 in squamous epithelial tissues or in the liver are predisposed to developing tumors (Conner et al., 2000; Pierce et al., 1998; Pierce et al., 1999). On the other hand, mice lacking a functional E2F1 allele are also predisposed to the tumor development and in this case E2F1 may inhibit tumorigenesis by promoting apoptosis (Yamasaki et al., 1996). Our research implies that the primary function of E2F1 in this I-type neuroblastoma stem cell system is to induce apoptosis, but not promote proliferation. E2F1 downregulation induced by loss of GATA3 results in blocking apoptosis and contributes to increased clonogenicity. This second pathway might expand the effects of GATA3 deficiency on proliferation (Figure 9).

In differentiation studies, GATA3 promotes the neuronal differentiation in BE(2)-C cells, which is consistent with the GATA3 function in the normal sympathetic development. Interestingly, loss of GATA3 not only blocks the commitment to neuronal differentiation, but also promotes glial differentiation in BE(2)-C cells as evidenced by the GFAP upregulation. It is well known that the transcription factors Mash1, Phox2b, Phox2a, dHand and GATA3 are necessary for sympathetic neuronal differentiation, but there are no reports that they also have effects on glial differentiation. One possibility is that loss of GATA3 induces apoptosis and masks further effects in normal sympathetic ganglia or adrenal medulla. Since sympathetic neurons and glial cells are both originated from NCSCs, it is possible that suppression of GATA3 and other sympathetic neuronal regulators in glial progenitors is essential for glial differentiation in the sympathetic nervous system. Lack of GATA3 expression in the typical S-type neuroblastoma cell line SHEP1 supports this point.
Although in 2006, Moriguchi et al. already demonstrated that GATA3 is not only a downstream gene of Mash1, Phox2b and dHand but also possesses mutual modulations with these sympathetic regulators (Moriguchi et al., 2006), no in vitro study has been investigated to prove this since then. In the mouse study, the reduced expression of Mash1, Phox2b and dHand induced by GATA3 deficiency is accompanied by the shrinkage of sympathetic ganglia or adrenal medulla due to apoptosis, so it’s difficult to judge if the decreased expression is directly derived from loss of GATA3 or other integral factors. From the data derived from BE(2)-C cells, we can confirm that the modulations between GATA3 and Mash1, GATA3 and Phox2b are directly at the cellular level. In addition, the Phox2b and Mash1 promoters have multiple (A/T)GATA(A/G) sequences, which provides the possibility that GATA3 might bind to their promoters to promote transcription.

The cancer stem cell model demonstrates the origin of tumorigenesis. Only if therapies targeting killing cancer stem cells are employed, tumors can be eliminated, or else new tumors will recur repeatedly. Therefore, it’s important to investigate the physiology of cancer stem cells. In this study, we used BE(2)-C cells as a neuroblastoma stem cell model and investigated the GATA3 regulation in this system. The mechanisms underlying enhanced clonogenicity induced by GATA3 deficiency provide more information for new neuroblastoma therapies.
Materials and methods

Cell culture

The human neuroblastoma I-type BE(2)-C cells (ATCC CRL-2268) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) / F12 (1:1) with L-Glutamine (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Atlantic Biologicals) and 1% penicillin/streptomycin (Fisher) at 37°C in a humidified 5% CO₂ incubator. BE(2)-C cells were subcultured at the split ratio of 1:3 every 3 days.

The 293TN cells (a gift from Dr. William Maltese), a pseudoviral particle packaging cell line, were cultured in DMEM with 2% heat-inactivated FBS. The 293GPG human kidney retroviral packaging cells (a gift from Dr. Richard Mulligan) were grown in 293 GPG medium which contains DMEM supplemented with G418/puromycin/doxycyclin (Invitrogen), penicillin/streptomycin, and 10% heat-inactivated FBS.

GATA3 knockdown in the BE(2)-C cell line

Construct

The lentiviral expression vector – pSIH1-H1-puro shRNA Expression Lentivector (SBI) – was provided by Dr. William Maltese. A short hairpin RNA was designed to target a 19 bp sequence specific to human GATA3 mRNA (Genebank NM
The oligonucleotide sequence, 5’-CCAAGACGTCCATCCACCA-3’, was designed by Oligoengine and synthesized by IDT. The sequence is followed by a 12-nucleotide non-complementary spacer (CTTCCTGTCAGA) and the reverse complement of the initial 19-nucleotide sequence. The sticky ends of the hairpin RNA were specifically cloned into the BmHI and EcoRI sites in the pSIH1-H1-puro vector to generate pSIH1-H1-puro-GATA3si. The pSIH1-H1-puro-GFPsi vector was used as the control.

**Infection**

Lentivirus was produced in 293TN packaging cells. For transfection, the 293TN cells were seeded at 3.75×10⁶ per 100mm dish in DMEM containing 10% heat-inactivated FBS. 24 hours later, the cells were transfected with the pSIH1-H1-puro-GATA3si vector and three lentiviral packaging plasmids (pPack-Rev, pPack-Gag and pPack-VSVG, provided by Dr. William Malese) by Lipofectamine (Invitrogen). After 24 hours, cells were started growing in DMEM containing 2% heat-inactivated FBS. 48 hours after transfection, the virus-enriched medium was collected and passed through a 0.45µm filter to infect target cells (BE(2)-C cells) in the presence of 4µg/ml polybrene (Sigma).

**Selection**

72 hours after infection, the medium was replaced on the infected BE(2)-C cells by DMEM / F12 (1:1) with L-Glutamine. After being cultured for three days, 2µg/ml puromycin (MP Biomedicals) was added to the medium and kept for 6 days. After 6-
day selection, the surviving cells were pooled and used for studies in the following sections.

**GATA3 overexpression in the BE(2)-C cell line**

The pBabe-puro-GATA3 construct was obtained from Dr. Charles M. Perou. A pBabe-puro vector was used as the control. 293GPG packaging cells were used to produce retrovirus. For transfection, the 293GPG cells were seeded at $5 \times 10^6$ per 60mm dish in DMEM containing 10% heat-inactivated FBS. After 24 hours, the cells were transfected with the pBabe-puro-GATA3 or pBabe-puro vectors by Liferfectamine. 5 hours later, cells were started growing in the 293GPG medium. 48 hours after transfection, the retroviral supernatant was harvested and passed through a 0.45 μm filter to infect BE(2)-C cells every 8 hours for 4 times in the presence of 4μg/ml polybrene. One day after the final round of infection, cells were cultured in the presence of 2.5μg/ml puromycin for 10 days, and drug-resistant cells were pooled and used for studies in the following sections.

**Soft agar colony formation assay**

1,000 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 10-14 days of incubation at 37°C, viable clones were stained with 5 mg/ml 3-
[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma). The colonies were photographed and counted.

**Clonogenic assay**

For the clonogenic assay, cells were seeded at 1,000 per dish in 100-mm dishes. The medium was replaced in the cultures every 3 days. Colony formation was assessed after 14 days by washing the cultures with PBS, fixing the cells for 15 min in a mix with 10% acetic acid and 10% methanol, and staining for 15 min with 0.4% crystal violet in 20% methanol. The colonies were photographed and counted.

**MTT assay**

The viability of cell populations in culture was quantified by metabolic activity assay, measuring the conversion of MTT to the formazan derivative. The formazan derivative was quantified in a continuous 7-day period by measuring its absorbance at 570 nm and 690 nm with a Spectra Max 384 Plus plate reader. The net absorbance (OD$_{570}$ minus OD$_{690}$) is the MTT reading.

**Cell growth**

To assess cell growth, GATA3 knockdown or overexpression cells were seeded in 35 mm dishes at 1000 or 1500 cells per dish on the initial day respectively. On the day 3,
6, 9, 12, 15 and 18, the cells were harvested from three parallel dishes and counted with a Coulter Z-series particle counter (Beckman-Coulter Corporation).

**Immunoblot**

Cells were suspended in standard sodium dodecyl sulfate (SDS) sample buffer. Protein concentrations were determined with a Bio-Rad protein assay kit, using bovine serum albumin as a reference. 50µg of protein was separated on 8%, 10% or 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes, which were then probed with antibodies, visualized by enhanced chemiluminescence (ECL) and quantified with an Alpha Innotech FluorChem HD2 imaging system. The following primary antibodies and dilutions were used: mouse anti-GATA3, 1:100 (Santa Cruz); rabbit anti-p16, 1:2000 (Santa Cruz); mouse anti-E2F1, 1:200 (Santa Cruz); rabbit anti-pRb, 1:1000 (Santa Cruz); rabbit anti-p21, 1:200 (Santa Cruz); rabbit anti-p27, 1:200 (Santa Cruz); mouse anti-p53, 1:50 (Calbiochem); mouse anti-Cyclin A, 1:2000 (Santa Cruz); mouse anti-Cyclin B1, 1:200 (Santa Cruz); mouse anti-Cyclin D1, 1:200 (Santa Cruz); rabbit anti-Cyclin E, 1:200 (Santa Cruz); mouse anti-TH, 1:10,000 (Sigma); rabbit anti-peripherin, 1:10,000 (Chemicon); mouse anti-SNAP25, 1:2500 (Serotec); rabbit anti-Rab5A, 1:1000 (Santa Cruz); rabbit anti-Rab1B, 1:2000 (Zymed); rabbit anti-GFAP, 1:2500 (DAKO); mouse anti-vimentin, 1:2000 (Santa Cruz); mouse anti-nestin, 1:1000 (BD); mouse anti-Mash1, 1:250 (BD); rabbit anti-Phox2b, 1:2000 (Jabs lab); mouse anti-α-tubulin, 1:8000 (Sigma). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit was used as secondary antibodies (ICN) at dilution 1:4000.
**Differentiation analysis**

For differentiation assays, retinoic acid (RA) was dissolved in Me$_2$SO and 10 mM stock solutions were prepared. BE(2)-C cells were treated with 10 µM RA for 1, 3, 7, 10 and 14 days to induce neuronal differentiation. Phase contrast pictures were taken at each time point. BE(2)-C cells treated with Me$_2$SO were used as a negative control.

**Statistical Analysis**

Values are represented as Mean ± SD. A Two-tailed Student’s $t$-test was performed for two groups (the control group versus the GATA3 overexpression or downregulation group). A $P$ value $0.05$ is considered statistically significant.
Acknowledgements

We thank Dr. William Maltese for providing techniques to establish the GATA3 knockdown cell line. We thank Dr. Jean Overmeyer and Ashley Young for the troubleshooting in the process of GATA3 knockdown cell line establishment. We thank Dr. William Maltese for providing the SNAP25, Rab5A and Rab1B primary antibodies and Dr. Roy Collaco for providing pRb and p27 primary antibodies. This work is supported by the National Cancer Institute grant CA124982 (H.-F.D.).
References


Figure Legends

Figure 1  Soft agar colony formation assay for BE(2)-C cells with knockdown and overexpression of GATA3.  (A) The pictures of the soft agar colony formation assay of BE(2)-C-pSIH1-H1-puro-GFPsi (abbr BE(2)-C-GFPsi) and BE(2)-C-pSIH1-H1-puro-GATAsi (abbr BE(2)-C-GATA3si) cell lines. 1000 individual BE(2)-C-GFPsi or BE(2)-C-GATA3si cells were grown in soft agar for 11 days respectively. The clone number of BE(2)-C-GATA3si cells in soft agar is higher than that of BE(2)-C-GFPsi cells.  (B) The percentage of clones growing from 1000 cells in the BE(2)-C-GFPsi and BE(2)-C-GATA3si cell lines, represented as a mean ± SD (4 dishes). The percentage of colony formation in BE(2)-C-GATA3si cells is significantly higher than that of BE(2)-C-GFPsi cells (P<0.0001).  (C) The pictures of the soft agar colony formation assay of BE(2)-C-pBabe-puro and BE(2)-C-pBabe-puro-GATA3 cell lines. 1000 individual BE(2)-C-pBabe-puro or BE(2)-C-pBabe-puro-GATA3 cells were grown in soft agar for 14 days respectively. The clone number of BE(2)-C-pBabe-puro-GATA3 cells in soft agar is less than that of BE(2)-C-pBabe-puro cells.  (D) The percentage of clones growing from 1000 cells in BE(2)-C-pBabe-puro and BE(2)-C-pBabe-puro-GATA3 cell lines, represented as a mean ± SD (4 dishes). The percentage of colony formation in BE(2)-C-pBabe-puro-GATA3 cells is significantly lower than that of BE(2)-C-pBabe-puro cells (P<0.0001).

Figure 2  Clonogenic assay for BE(2)-C cells with knockdown and overexpression of GATA3.  (A) The pictures of the clonogenic assay of BE(2)-C-GFPsi and BE(2)-C-
GATA3si cell lines. 1000 individual BE(2)-C-GFPsi or BE(2)-C-GATA3si cells were grown in 100 mm cell culture plates for 14 days respectively. The clone number of BE(2)-C-GATA3si cells is higher than that of BE(2)-C-GFPsi cells. (B) The percentage of clones growing from 1000 cells in BE(2)-C-GFPsi and BE(2)-C-GATA3si cell lines, represented as a mean ± SD (6 dishes). The percentage of colony formation in BE(2)-C-GATA3si cells is significantly higher than that of BE(2)-C-GFPsi cells \((P<0.0001)\). (C) The pictures of the clonogenic assay of BE(2)-C-pBabe-puro and BE(2)-C-pBabe-puro-GATA3 cell lines. 1000 individual BE(2)-C-pBabe-puro or BE(2)-C-pBabe-puro-GATA3 cells were grown in 100 mm cell culture plates for 14 days respectively. The clone number of BE(2)-C-pBabe-puro-GATA3 cells is less than that of BE(2)-C-pBabe-puro cells. (D) The percentage of clones growing from 1000 cells in BE(2)-C-pBabe-puro and BE(2)-C-pBabe-puro-GATA3 cell lines, represented as a mean ± SD (6 dishes). The percentage of colony formation in BE(2)-C-pBabe-puro-GATA3 cells is significantly lower than that of BE(2)-C-pBabe-puro cells \((P<0.0001)\).

**Figure 3** MTT assays for BE(2)-C cells with knockdown and overexpression of GATA3 in a continuous 7-day period. (A) MTT assays exhibit an increase in the number of viable cells in cultures of the BE(2)-C-GATA3si cell line in comparison with the BE(2)-C-GFPsi cell line. OD value is represented as a mean ± SD. Points, mean determined from six separate cultures in a 96-well plate initially seeded at 500 cells per well; bars, SD. (B) MTT assays exhibit a decrease in the number of viable cells in cultures of the BE(2)-C-pBabe-puro-GATA3 cell line compared with the BE(2)-C-pBabe-puro cell line. OD value is represented as a mean ± SD. Points, mean
determined from four separate cultures in a 96-well plate initially seeded at 750 cells per well; bars, SD. MTT was added directly to the medium to avoid losing detached cells.

**Figure 4** The growth rate of BE(2)-C cells with knockdown and overexpression of GATA3. (A) BE(2)-C-GATA3si cells exhibit a marked increase in the growth rate compared with the control group of BE(2)-C-GFPsi cells. Cells were seeded in 35 mm dishes on day 0 at an initial density of 1000 cells per dish. On each of the indicated days, cells were harvested from three dishes in each group and counted with a Coulter Z1 particle counter (mean ± SD). (B) BE(2)-C- pBabe-puro-GATA3 cells show a dramatic decrease in the growth rate in comparison with the control group of BE(2)-C-pBabe-puro cells. Cells were seeded in 35 mm dishes on day 0 at an initial density of 1500 cells per dish. On each of the indicated days, cells were harvested from three dishes in each group and counted with a Coulter Z1 particle counter (mean ± SD).

**Figure 5** Immunoblot analyses of the expression of indicated cell cycle regulation genes in BE(2)-C-GFPsi and BE(2)-C-GATA3si cell lines by representative samples. P value is indicated on the left of each panel. P value is calculated from the normalized units (gene expression level/loading control level) between the two groups. Each group contains 3 to 4 samples and represented as a mean ± SD. (A) The GATA3 expression is significantly decreased in GATA3 knockdown cells (P<0.05). The level of α-tubulin is shown as the loading control. (B) The expression levels of p21, p27 and p53 in BE(2)-C-GATA3si cells show no significant difference from that
of BE(2)-C-GFPsi cells. (C) The expression levels of p16, E2F1 and pRb in BE(2)-C-GFPsi and BE(2)-C-GATA3si cells. The E2F1 expression is significantly decreased ($P<0.05$) compared with that of the control, whereas the pRb expression is significantly increased ($P<0.05$). (D) The expression levels of Cyclin A, B1, D1 and E in BE(2)-C-GFPsi and BE(2)-C-GATA3si cells. Cyclin B1 and Cyclin E are expressed equally in the control and the GATA3 knockdown cell line. The Cyclin D1 expression is significantly upregulated ($P<0.05$) compared with that of the control, whereas the Cyclin A expression is significantly downregulated ($P<0.05$).

Figure 6   Immunoblot analyses of the expression of indicated differentiation marker genes in GATA3 knockdown and overexpression cell lines by representative samples. $P$ value is indicated on the left of each panel. $P$ value is calculated from the normalized units (gene expression level/loading control level) between the two groups. Each group contains 3 to 4 samples and represented as a mean ± SD. (A) The GATA3 expression is significantly decreased in GATA3 knockdown cells and increased in GATA3 overexpression cells ($P<0.05$). The level of $\alpha$-tubulin is shown as the loading control. (B) The expression levels of neuronal marker genes and Rab GTPase genes are presented in GATA3 knockdown and overexpression cell lines. The TH expression is significantly upregulated in the GATA3 overexpression cell line in comparison with its control ($P<0.05$), whereas there is no significant difference of the TH expression between the GATA3 knockdown cell line and its control. The SNAP25 expression is significantly decreased in GATA3 knockdown cells ($P<0.05$) while it is significantly increased in GATA3 overexpression cells ($P<0.05$). One of the Rab GTPase genes, Rab 1B, shows a significant increase in GATA3 knockdown
cells ($P<0.05$) while it exhibits a significant decrease in GATA3 overexpression cells ($P<0.05$). Peripherin and Rab5A are expressed equally in four lanes. (C) The expression of glial marker genes in GATA3 knockdown and overexpression cell lines. The GFAP expression is significantly upregulated in GATA3 knockdown cells ($P<0.05$) and significantly downregulated in GATA3 overexpression cells ($P<0.05$). Vimentin shows no difference of expression levels in four lanes. (D) The expression of the progenitor cell marker gene, nestin, shows equal expression in GATA3 knockdown and overexpression cell lines.

**Figure 7** Immunoblot analyses of GATA3 expression levels in different neuroblastoma cell lines and the retinoic acid (RA) – treated BE(2)-C cell line. (A) The expression of GATA3 in 11 neuroblastoma cell lines, including the I-type cell line, BE(2)-C; the S-type cell line SHEP1; others are N-type cell lines. The $\alpha$-tubulin levels are shown as the loading control. (B) The time course study of GATA3 expression in RA-treated BE(2)-C cells. Cells were collected on day 1, 3, 7, 10, 14 after RA treatment parallel with untreated controls at same time points. (C) The morphology of BE(2)-C cells on day 1, 3 and 14 after RA treatment.

**Figure 8** Immunoblot analyses of the expression of indicated sympathetic development regulator genes in GATA3 knockdown and overexpression cell lines by representative samples. $P$ value is indicated on the left of each panel. $P$ value is calculated from the normalized units (gene expression level/loading control level) between the two groups. Each group contains 3 to 4 samples and represented as a
mean ± SD. The Mash1 expression shows a significant increase in the GATA3 overexpression cell line ($P<0.05$). In the GATA3 knockdown cell line, even though the Mash1 expression exhibits an inclination to decrease, the difference is insignificant compared with the control. In contrast, the Phox2b expression is significantly downregulated in the GATA3 knockdown cell line ($P<0.05$), whereas the increase of its expression in the GATA3 overexpression cell line is insignificant in comparison with the control.

**Figure 9** A simplified schematic diagram for the mechanisms underlying GATA deficiency induced enhanced clonogenicity.
Figure 1

A

GFPsi  GATA3si

B

Clone number in soft agar (%)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>GFPsi</th>
<th>GATA3si</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40</td>
<td>90</td>
</tr>
</tbody>
</table>
Figure 3

A

Viable cells (MTT OD\textsubscript{570-OD\textsubscript{690}})

- GFPsi
- GATA3si

Days

B

Viable cells (MTT OD\textsubscript{570-OD\textsubscript{690}})

- pBabe-puro
- pBabe-puro-GATA3

Days
Figure 4

A

Cell Number (x 10^5)

Days

0 3 6 9 12 15 18

GFPsi

GATA3si

B

Cell Number (x 10^5)

Days

0 3 6 9 12 15 18

pBabe-puro

pBabe-puro-GATA3
Figure 5

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA3</td>
<td></td>
<td></td>
<td>*P=0.009</td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Protein</th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p16</td>
<td></td>
<td></td>
<td>P=0.2899</td>
</tr>
<tr>
<td>E2F1</td>
<td></td>
<td></td>
<td>*P=0.0052</td>
</tr>
<tr>
<td>pRb</td>
<td></td>
<td></td>
<td>*P=0.0371</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Protein</th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td></td>
<td></td>
<td>P=0.3296</td>
</tr>
<tr>
<td>p27</td>
<td></td>
<td></td>
<td>P=0.0627</td>
</tr>
<tr>
<td>p53</td>
<td></td>
<td></td>
<td>P=0.0902</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th>Protein</th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclin A</td>
<td></td>
<td></td>
<td>*P=0.0025</td>
</tr>
<tr>
<td>Cyclin B1</td>
<td></td>
<td></td>
<td>P=0.4777</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td></td>
<td></td>
<td>*P=0.0016</td>
</tr>
<tr>
<td>Cyclin E</td>
<td></td>
<td></td>
<td>P=0.1875</td>
</tr>
</tbody>
</table>
Figure 6

A

<table>
<thead>
<tr>
<th></th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>pBabe-puro</th>
<th>pBabe-puro-GATA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA3</td>
<td></td>
<td>*P=0.0053</td>
<td></td>
<td>*P=0.028</td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>pBabe-puro</th>
<th>pBabe-puro-GATA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>TH</td>
<td></td>
<td>P=0.1387</td>
<td></td>
<td>*P=0.01</td>
</tr>
<tr>
<td>Peripherin</td>
<td></td>
<td>P=0.3401</td>
<td></td>
<td>P=0.288</td>
</tr>
<tr>
<td>SNAP25</td>
<td></td>
<td>*P=0.0307</td>
<td></td>
<td>*P=0.0185</td>
</tr>
<tr>
<td>Rab5A</td>
<td></td>
<td>P=0.3623</td>
<td></td>
<td>P=0.1198</td>
</tr>
<tr>
<td>Rab1B</td>
<td></td>
<td>*P=0.0401</td>
<td></td>
<td>*P=0.0001</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th></th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>pBabe-puro</th>
<th>pBabe-puro-GATA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFAP</td>
<td></td>
<td>*P=0.0473</td>
<td></td>
<td>*P=0.0065</td>
</tr>
<tr>
<td>Vimentin</td>
<td></td>
<td>P=0.9718</td>
<td></td>
<td>P=0.2927</td>
</tr>
</tbody>
</table>

D

<table>
<thead>
<tr>
<th></th>
<th>GFPsi</th>
<th>GATA3si</th>
<th>pBabe-puro</th>
<th>pBabe-puro-GATA3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nestin</td>
<td></td>
<td>P=0.3793</td>
<td></td>
<td>P=0.6855</td>
</tr>
</tbody>
</table>
Figure 7

A

<table>
<thead>
<tr>
<th>SK-N-F1</th>
<th>LAN6</th>
<th>SK-N-SH</th>
<th>SH-SY5Y</th>
<th>SK-N-AS</th>
<th>IMR-32</th>
<th>SK-N-DZ</th>
<th>BE(2)C</th>
<th>SHEP1</th>
<th>SK-N-BE(2)-1</th>
<th>SK-N-BE(2)-2</th>
<th>SMS-KCNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubulin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Control</th>
<th>RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day1</td>
<td></td>
</tr>
<tr>
<td>Day3</td>
<td></td>
</tr>
<tr>
<td>Day7</td>
<td></td>
</tr>
<tr>
<td>Day10</td>
<td></td>
</tr>
<tr>
<td>Day14</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GATA3</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulin</td>
<td></td>
</tr>
</tbody>
</table>

C

RA 1 day  RA 3 day  RA 14 day

144
Figure 8

Mash1

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GFPsi</td>
<td>GATA3si</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>P=0.0598</td>
</tr>
<tr>
<td>Phox2b</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>*P=0.0085</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P=0.0028</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P=0.1228</td>
</tr>
</tbody>
</table>

Figure 9

GATA3

BE(2)-C

Cyclin D1

E2F1

Suppression of apoptosis + Entering S phase

Increased tumorigenicity

pRb

E2F

pRb

E2F

pRb
SUMMARY/DISCUSSION

This dissertation includes two parts of work. In the first part, we investigated the postnatal development of mouse sympathetic ganglia and found that it is characterized predominantly by gliogenesis and a small amount of neurogenesis. Nestin is identified as a molecular marker for both sympathetic glial and neuronal progenitors. In the second part, we demonstrated the role of a GATA family zinc finger transcription factor, GATA3, in the regulation of human neuroblastoma stem cell activities. We use BE(2)-C cells, a I-type neuroblastoma cell line, as the neuroblastoma stem cell model. We found that GATA3 deficiency induces increased clonogenicity in BE(2)-C cells through the upregulation of Cyclin D1 and the downregulation of E2F1 whereas overexpression of GATA3 results in decreased clonogenicity of BE(2)-C cells. In the differentiation study, we demonstrated that loss of GATA3 induces gliogenesis and overexpression of GATA3 promotes neurogenesis in BE(2)-C cells. GATA3 regulates differentiation in BE(2)-C cells through the modulations with the other sympathetic regulators, Mash1 and Phox2b, at the cellular level.

In the relationship, the first part of work is the basic research for the second part. A detailed characterization of the cellular basis for the postnatal sympathetic development facilitates the investigation of tumorigenesis in neuroblastoma. It is well established that neuroblastoma originates from the developing sympathetic nervous system. Based on the cancer stem cell model, neuroblastoma stem cells are the origin of neuroblastoma formation. Neuroblastoma stem cells are most likely derived from
transformed neural crest stem cells or sympathetic progenitor cells that obtain stem cell properties through mutations.

Since 90% of children with this disease are diagnosed before 6 years old, there are two possible phases in which neuroblastoma stem cells can be generated – the embryonic and postnatal developmental phase. In the embryonic development, sympathetic ganglia are composed of intensely proliferating neural crest stem cells, neurons and glial cells, though their temporal developmental differences are dramatic. Therefore, neuroblastoma stem cells can be derived from any type of cells in any time during the embryonic sympathetic development due to genetic mutations. However, in the postnatal sympathetic ganglia, the situation is different. During this phase, the neural crest stem cells are either consumed or hibernating in the niche; the majority of neurogenesis has completed and only a very small amount of neurons are proliferating; gliogenesis continues after the embryonic development and dominates the postnatal development, whereas it shows a gradually decreasing proliferating tendency and the proliferation is completed by the adult phase. Therefore, the origin of neuroblastoma stem cells in this phase is difficult to judge because the three types of cells are either resting or going down in the proliferative ability. The cellular mutations or external signals that can induce increased proliferation or gain of stem cell properties in the three cell types need to be investigated to explore the formation of neuroblastoma stem cells in the postnatal sympathetic development.

GATA3 deficiency has been demonstrated to increase clonogenicity in the I-type neuroblastoma cell line, BE(2)-C. Loss of GATA3 might be a promoting factor in the transformation of neural crest stem cells or sympathetic progenitor cells to form neuroblastoma stem cells. Previous studies indicated that GATA3 deficiency induces
a progenitor cell expansion in the kidney and mammary development (Asselin-Labat et al., 2007; Grote et al., 2008). Loss of GATA3 promotes the dissemination and metastasis in breast cancers (Kouros-Mehr et al., 2008). These facts suggest that GATA3 deficiency might play a similar role in the neuroblastoma formation.

To prove this point, a series of experiments can be designed to investigate the role of GATA3 in the tumorigenesis of neuroblastoma. For example, we can specifically knockout the GATA3 expression in the migrating neural crest stem cells, neuronal or glial progenitors during embryonic development, then observe if there is upregulated proliferation or transformed characteristics in these cells. This would determine if loss of GATA3 is sufficient to induce tumorigenesis in the developing sympathetic nervous system.

In this study, we also found Cyclin D1 and E2F1 are the downstream targets of GATA3 in the proliferation regulation. Upregulation of Cyclin D1 and downregulation of E2F1 are responsible for the increased proliferation induced by GATA3 deficiency. We can further investigate if GATA3 can directly bind the promoters of Cyclin D1 and E2F1 to regulate their expression. Are there any other underlying mechanisms responsible for the increased clonogenicity induced by GATA3 deficiency in BE(2)-C cells? Do the therapies targeting Cyclin D1 block the expansion of neuroblastoma stem cells and effectively control the tumor growth?

It has been demonstrated in this research that GATA3 deficiency not only suppresses neuronal differentiation, but also promotes glial differentiation. There are no previous studies reporting that GATA3 plays a role in gliogenesis. GATA3 has only been well established as a sympathetic neuronal regulator modulating with other sympathetic neuronal regulators like Phox2b, Mash1, Phox2a, dHand. Our study
suggests that loss of GATA3 or other sympathetic neuronal regulators might also be necessary for gliogenesis in the normal sympathetic development. The loss of GATA3 expression in the typical S-type neuroblastoma cell line (similar to glial/Schwann cells) – SHEP1 – has confirmed this point and suggests that SHEP1 cells might represent an extreme status of GATA3 deficiency. It would be interesting to explore what are the mechanisms of the suppressed GATA3 expression in the gliogenesis which expands the GATA3 functions in the sympathetic development. GATA3 has been proven in our study to modulate Phox2b and Mash1 at the cellular level in regulating the BE(2)-C cell differentiation, it is of interest to investigate if GATA3 binds to their promoters to regulate the expression at the genetic level?

Together with previous studies of the GATA3 function in the normal sympathetic system and our data in I-type neuroblastoma stem cells, we can determine the role of GATA3 in the regulation of activities in the normal sympathetic development and in the tumorigenesis of neuroblastoma, providing a good foundation for future studies and clinical applications.
CONCLUSIONS

1. Nestin is a molecular marker for both sympathetic glial and neuronal progenitors in the mouse postnatal sympathetic development.

2. A population of nestin and BLBP double-positive cells in the mouse postnatal sympathetic ganglia is the direct precursors of sympathetic satellite cells. The mouse postnatal sympathetic development is characterized predominantly by gliogenesis and a very small amount of neurogenesis.

3. Our study reveals a temporal expression pattern of glial lineage markers that define the cellular intermediates in the process of satellite cell differentiation in mouse postnatal sympathetic development.

4. GATA3 deficiency induces increased clonogenicity of BE(2)-C cells, a type of I-type neuroblastoma stem cell line, whereas GATA3 overexpression results in decreased clonogenicity of BE(2)-C cells.

5. The upregulation of Cyclin D1 and downregulation of E2F1 are possibly the downstream mechanisms that are responsible for the increased clonogenicity induced by GATA3 deficiency.
6. GATA3 deficiency induces glial differentiation in BE(2)-C cells whereas GATA3 overexpression promotes neuronal differentiation.

7. GATA3 modulates with other sympathetic regulators Mash1 and Phox2b at the cellular level to regulate the BE(2)-C cell differentiation.


expressed by neuroendocrine carcinomas of the skin, their xenograft on nude mice, and the corresponding primary cultures. Cancer Res 53, 1175-1181.


Birren, S. J., and Anderson, D. J. (1990). A v-myc-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. Neuron 4, 189-201.


Garg, V., Kathiriya, I. S., Barnes, R., Schluterman, M. K., King, I. N., Butler, C. A.,
mutations cause human congenital heart defects and reveal an interaction with TBX5.
Nature 424, 443-447.

morphogenesis and hair tumors in mice expressing a truncated beta-catenin in skin.
Cell 95, 605-614.

George, K. M., Leonard, M. W., Roth, M. E., Lieuw, K. H., Kioussis, D., Grosveld, F.,
and Engel, J. D. (1994). Embryonic expression and cloning of the murine GATA-3
gene. Development 120, 2673-2686.


Godlewski, J., Nowicki, M. O., Bronisz, A., Williams, S., Otsuki, A., Nuovo, G.,
of the Bmi-1 oncogene/stem cell renewal factor by microRNA-128 inhibits glioma

cooperate to activate the thyrotropin beta-subunit promoter. J Biol Chem 272, 24339-
24347.


human melanoma assessed by (monoclonal) antibodies reactive on paraffin sections. Histopathology 10, 689-700.


factor GATA-1 binds specifically to DNA as a zinc or iron complex. Proc Natl Acad Sci U S A 90, 1676-1680.


Rychlik, J. L., Gerbasi, V., and Lewis, E. J. (2003). The interaction between dHAND and Arix at the dopamine beta-hydroxylase promoter region is independent of direct dHAND binding to DNA. J Biol Chem 278, 49652-49660.


Hearing loss following Gata3 haploinsufficiency is caused by cochlear disorder. Neurobiol Dis 16, 169-178.


ABSTRACT

Sympathetic ganglia are primarily composed of noradrenergic neurons and satellite glial cells. Although both cell types originate from neural crest cells, the identities of the progenitor populations at intermediate stages of the differentiation process remain to be established. Here we report the identification in vivo of glial and neuronal progenitor cells in postnatal sympathetic ganglia, using mouse superior cervical ganglia as a model system. There are significant levels of cellular proliferation in mouse superior cervical ganglia during the first 18 days after birth. A majority of the proliferating cells express both nestin and brain lipid-binding protein (BLBP). BrdU fate-tracing experiments demonstrate that these nestin and BLBP double positive cells represent a population of glial progenitors for sympathetic satellite cells. The glial differentiation process is characterized by a marked downregulation of nestin and upregulation of S100, with no significant changes in the levels of BLBP expression. We also identify a small number of proliferating cells that express nestin and tyrosine hydroxylase, a key enzyme of catecholamine biosynthesis that defines sympathetic noradrenergic neurons. Together, these results establish nestin as a common marker for sympathetic neuronal and glial progenitor cells and delineate the cellular basis for the generation and maturation of sympathetic satellite cells.

This research in the normal postnatal sympathetic development provides the basis for the neuroblastoma study. Neuroblastoma is the most common childhood malignant tumor which originates from sympathetic nervous system. 90% of children
with this disease are diagnosed before 6 years old. Therefore, the tumorigenesis of neuroblastoma may represent an abnormal embryonic or postnatal sympathetic development. Transformed neural crest stem cells or malignant sympathetic precursor cells that obtain stem cell abilities are recognized as neuroblastoma stem cells that might be the origin of neuroblastoma. BE(2)-C cells, a human neuroblastoma cell line enriched with tumorigenic stem cells, can be induced to undergo either neuronal or glial differentiation. We use BE(2)-C cells as a system to identify the genes that regulate neuroblastoma stem cell activities, such as self-renewal and differentiation. One of these genes is GATA3, a zinc-finger transcription factor with an essential role in sympathetic development. Downregulation of GATA3 by siRNA promotes BE(2)-C cell proliferation and overexpression of GATA3 decreases the proliferation of BE(2)-C cells. GATA3 regulates cell proliferation through distinct pathways involving Cyclin D1 and E2F1, as evidenced by an increased expression of Cyclin D1 and a decreased expression of E2F1 in GATA3 knockdown cells. In addition, GATA3 knockdown induces BE(2)-C cells to undergo glial differentiation, as indicated by an increase in the expression of GFAP, a glial cell marker, and a decrease in the expression of neuronal marker SNAP25. GATA3 overexpression promotes neuronal differentiation of BE(2)-C cells, as indicated by a reduction of GFAP expression and an upregulation of SNAP25 expression. GATA3 knockdown also downregulates Phox2b and GATA3 overexpression upregulates Mash1, two transcription factors that are expressed in neuronal progenitor cells and are essential for sympathetic development. Together, these findings suggest that GATA3, Phox2b and Mash1 may function in a regulatory network in the control of neuroblastoma cells in a stem/progenitor cell state.