Preoptic Regulatory Factor 2 Inhibits Proliferation and Enhances Drug Induced Apoptosis in Neural Stem Cells

A dissertation presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Doctor of Philosophy

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March 2009

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This dissertation titled
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Apoptosis in Neural Stem Cells

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ABSTRACT

MA, SHUANG, Ph.D., March 2009, Biological Sciences

Preoptic Regulatory Factor 2 Inhibits Proliferation and Enhances Drug Induced Apoptosis in Neural Stem Cells (111 pp.)

Director of Dissertation: Felicia V. Nowak

Neural stem cells (NSCs) exist in both the developing and adult brain. In the developing central nervous system (CNS), NSCs shape the structural and functional layout of the brain. After development, NSCs still contribute to low level neurogenesis in several brain areas including the subventricular zone (SVZ) and hippocampal dentate gyrus (DG). NSCs are important in cancer research and treatment. NSCs are a possible origin of brain-cancer stem cells when the strict control of cell growth is disturbed. They are also important resources for therapeutic transplantation in diverse diseases, including neurodegenerative disorders, brain and spinal cord injuries, stroke and epilepsy. Thus elucidating the growth-regulatory mechanism of NSCs will be helpful for understanding brain development, tumorigenesis and providing a platform for NSCs’ clinical application. Preoptic regulatory factor-2 (Porf-2) is a Rho GTPase activator protein (GAP) domain-containing protein found in the CNS. It has been proposed to have a role in gender-related brain development and function. However, the direct effects of Porf-2 on NSCs are not known. The current studies were designed to knock down the expression of Porf-2 in C17.2 cells, a mouse cerebellar NSC line, and investigate the effects of Porf-2 on cell proliferation, apoptosis and differentiation. The mechanisms responsible for the effects of Porf-2 on cell proliferation and apoptosis were also studied. Knockdown of
Porf-2 was performed by transducing short hairpin RNA (shRNA) lentivirus into C17.2 cells and confirmed by quantitative RT-PCR and Western blot analysis. Porf-2 knockdown cells exhibited increased proliferative activities and decreased drug induced apoptosis compared to control cells (p<0.05). There was no difference in differentiation directions between Porf-2 knockdown cells and control cells. Mechanistic studies yielded three findings. First, knockdown of Porf-2 lowered the expression level of cyclin kinase inhibitor p21 and expedited G1 to S cell cycle transition. Second, bleomycin, a genotoxic reagent, caused an elevation in p53 transcriptional activity, p21 expression and Bax expression in C17.2 cells. Knockdown of Porf-2 partially blocked the changes caused by bleomycin. Third, staurosporine (STS), a broad-spectrum kinase inhibitor, enhanced the expression of Bax but did not change the transcriptional activity of p53 or expression of p21 in C17.2 cells. Knockdown of Porf-2 had no influence on the enhancement of Bax expression in response to STS treatment. Three conclusions were drawn from these data. First, Porf-2 inhibits NSC proliferation by enhancing p21 expression followed by G1 cell cycle arrest. Second, Porf-2 plays pro-apoptotic roles in response to drug treatment in NSCs through both p53 transcription dependent and independent pathways. Third, Porf-2 shows no influence on NSC differentiation directions.

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ACKNOWLEDGEMENTS

First of all, I would like to express my deepest sense of gratitude to my advisor, Dr. Felicia V. Nowak, for her invaluable guidance, support and encouragement throughout my research. She helped me learn not only the advanced techniques but also the basics in neuroscience. In addition, she helped me a lot in improving my academic writing. She is always a source of great inspiration of my research.

My gratitude is also due to Drs. Robert Colvin, Xioazhuo Chen and Allan M. Showalter for serving on my doctoral advisory committee and their abundant help and advice on my work. Without their knowledge and assistance, my study would not have been successful.

I am thankful to the Department of Biological Sciences and the Department of Biomedical Sciences for providing me with financial assistantship and a good research environment so that I can finish my dissertation. I am thankful to Dr. Kelly McCall for Luminometer LB 9507 orientation, Dr. Yan Qin and Wei Lin for helping me use the fluorescence microscope and FluoroMax, Dr. Yuriy Slyvka for helping with Western Blot, Wei Liu for helping me with flow cytometry. I also would like to thank our former lab technician Paul Wiehl, my labmates Ning Yuan and Zhenchao Wang for their generous help in the lab. Working with them is a pleasant and unforgettable experience.

Finally, I am indebted to my parents, my husband and my brother for their understanding, help and support.
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LIST OF ABBREVIATIONS

AB: AlamarBlue™

APAF1: adaptor apoptotic protease activating factor-1

BCA: bicinchoninic acid

bp: base pair

BSA: bovine serum albumin

Cdc 42: Cell division cycle 42

CDK: cyclin-dependent kinase

CIP/KIP: CDK interacting protein/kinase inhibitory protein

CNS: central nervous system

d: day

DG: dentate gyrus

DISC: death-inducing-signaling-complex

DMEM: Dulbecco’s modified Eagle’s medium

DMPC: dimethyl pyrocarbonate

DLC: frequently deleted in liver cancer

dsDNA: double strand DNA

ECL: enhanced chemiluminescence

FBS: fetal bovine serum

FI: fluorescence intensity

GAP: GTPase activator protein

GnRH: gonadotropin releasing hormone
GRAF: GTPase regulator associated with the focal adhesion kinase pp125(FAK)

Grit: GTPase regulator interacting with TrkA

h: hour

HRP: horseradish peroxidase

INK: inhibitors of CDK

kD: kilodalton

LB agar plate: Luria broth agar plate

min: minute

MyTH4: Myosin Tail Homology 4

NOMA-GAP: neurite outgrowth multiadaptor RhoGAP

NSC: neural stem cell

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PCNA: proliferation-cell nuclear antigen

PI: propidium iodide

pLKO.1- C17.2: control C17.2 (transduced with pLKO.1 lentivirus)

184-C17.2: Porf-2 knockdown C17.2 (transduced with 184-pLKO.1)

Porf-1: preoptic regulatory factor 1

Porf-2: preoptic regulatory factor 2

PRP 48: pre-mRNA processing protein 48

PS: phosphatidylserine

PVDF: Polyvinylidene difluoride
Rac: Ras-related C3 botulinum toxin substrate
Rb: retinoblastoma susceptibility protein
REDOX: oxidation-reduction
Rho: Ras homolog gene
RT-PCR: reverse transcriptase-polymerase chain reaction
SDS: sodium dodecyl sulphate
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec: second
shRNA: short hairpin RNA
STS: staurosporine
SVZ: subventricular zone
TB: terrific broth
TBS: Tris buffered saline
TDT: terminal deoxynucleotidyl transferase
TUNEL: Terminal deoxynucleotidyl transferase -mediated dUTP Nick-End Labeling assay
×g: times gravity (relative centrifuge force)
CHAPTER 1 INTRODUCTION

Neural stem cell

Neural stem cells (NSCs) are undifferentiated cells having the ability to divide symmetrically to generate high numbers of identical cells, and to divide asymmetrically to produce progenitor cells that give rise to different cell types such as neurons and glia (Mckay 1997; Gage 2000). NSCs are present in both the developing and adult brain. In the developing central nervous system (CNS), NSCs shape the structural and functional layout of the brain. After development, NSCs are still found proliferating and giving rise to new neurons throughout life in several brain areas. Subventricular zone (SVZ) and hippocampal dentate gyrus (DG) are two main neurogenic zones. In addition to these two areas, NSCs were isolated and cultured from other regions including cortical parenchyma and spinal cord. It is possible that NSCs exist dormant throughout the mature CNS and contribute to low level neurogenesis (Kokovay et al. 2008).

NSCs have great significance in both biological research and clinical application. The long-lived and self-renewal properties make NSCs prone to undergo cancer-inducing mutation and become cancer stem cells (Clarke 2004). In cancer stem cell theory, not all the cells in the tumor contribute to tumor growth by proliferation; instead only a small fraction of cells expressing markers of stem/progenitor cells, termed cancer stem cells, are capable of extensive proliferation and self-renewal (Wicha et al. 2006). Cancer stem cells have been identified in a variety of human cancers, such as myeloid leukemia (Hope et al. 2004) and breast cancer (Al-Hajj et al. 2003). In the nervous system, brain-cancer stem cells have also been isolated from diverse brain tumors, including glioma and
pilocytic astrocytoma (Hemmati et al. 2003; Singh et al. 2003; Galli et al. 2004). The excessive expansion of the NSC pool caused by dysregulation of NSC proliferation and apoptosis is involved in brain cancer development (Beachy et al. 2004; Dirks 2005; Dirks 2006; Galderisi et al. 2006). Therefore, brain-cancer stem cells are possibly derived from NSCs when the normal constraint of NSC self-renewal and proliferation is lost. (Singh and Dirks 2007; Dirks 2008a; Dirks 2008b) Thus, clarifying the factors regulating NSC proliferation and apoptosis will give insight into the mechanism of normal brain development. In the future this may have an impact on prevention and treatment of nervous system developmental and behavioral abnormalities. It will also give insight into the mechanisms of brain tumorigenesis which may influence the way brain cancer is treated.

In addition to cancer research, NSCs are important in therapeutics of central nervous system diseases. Transplantation of NSCs or their derivatives has been considered a promising therapeutic strategy for neurodegenerative disorders, such as Parkinson, Alzheimer’s disease and Huntington (Shimazaki 2003; Oliveira and Hodges 2005; Sanberg 2007). Moreover, stem cell therapy also supplies hope for treating diverse other CNS diseases, including brain and spinal injury, stroke and epilepsy (Selden 2008). However, our understanding of NSCs is still limited. Many basic issues must be resolved before clinical utility can be realized. Exploring growth regulation mechanisms in NSCs will help build a platform for clinical application. Therefore, in this research NSCs were chosen to study the functions of a putative growth regulator Porf-2.
Porf-2 gene

Discovery

Preoptic regulatory factor-2 (Porf-2) was first discovered by Dr. Nowak when she was looking for the gonadotropin releasing hormone (GnRH) related genes. (Nowak 1990) Two unique cDNAs, which contain regions homologous to the active GnRH decapeptide, were identified by screening a cDNA library derived from mRNA isolated from the preoptic area of the hypothalamus of the castrated male rat. They were named preoptic regulatory factor 1 (Porf-1) and Porf-2 respectively. Later, it was found that the synthetic Porf-1 and Porf-2 decapeptides do not exhibit GnRH agonist or antagonist activity in a pituitary cell culture gonadotropin assay system. In addition, they seem to have very different structures and potential functions. Thus, they are studied separately.

My research focuses on studying Porf-2.

Expression pattern

Porf-2 is widely expressed in diverse tissues including the CNS and peripheral tissues. In the CNS of the rat, it is highly expressed in the hippocampus and hypothalamus. It is also expressed in the cerebral cortex. In addition to intrinsic regional differences, the expression of Porf-2 in the CNS is influenced by several factors including developmental stage, aging, gender, sex steroids, and pituitary and testicular factors (Hu and Nowak 1994; Hu and Nowak 1995; Nowak 1997; Nowak et al. 1997; Nowak and Gore 1999; Nowak et al. 1999). Its responses to these factors are region specific and may be specified by individual cell phenotypes. The neurons in different brain regions may respond differently to Porf-2 and different neurons in the same brain region may respond
differently to Porf-2. *In situ* hybridization analysis reveals that Porf-2 mRNA is only expressed in a subpopulation of cells at a given point in time (Nowak et al. 1999). It is not known yet whether this is a stable subpopulation and if there are any other distinguishing features of these cells. In addition to CNS, Porf-2 is also expressed in peripheral tissues (Nowak 1997) including several which are characterized by rapid cell division, such as skin and the placental growth cone of mouse, and dividing germ cells in the rat testes (Nowak 1997, Ko 1998, Konno 1999).

**Evolutionary conservation**

Porf-2 has been conserved across a broad evolutionary span. By using antibodies raised against in vitro synthesized rat Porf-2 protein, the native Porf-2 proteins were detected in rat brain extracts (Nowak, unpublished data), fetal mouse brain sections (S. Tobet, personal communication) and the human neuronal hNT cell line (I. Guillemain, personal communication). In addition, genomic Southern Blotting experiments revealed that Porf-2 gene loci also exist in pig, sheep, cow, chicken, and zebrafish (Schmerr et al. 2002). The high-level conservation of this gene implies its useful functions.

**Structure**

Rat Porf-2 gene is located on chromosome 7 (Nowak 1990). Analysis of the structure of rat Porf-2 mRNA based on experimental data demonstrates that it has two exons, which are 111 base pair (bp) and 1245 bp respectively, and an intron, which is 147 bp. It contains an open reading frame, which encodes a 75 amino acid peptide (Fig.1.1). When an in vitro translation was done using rabbit reticulocyte lysate and an in vitro transcribed Porf-2 mRNA, an 8.3 kd peptide was synthesized, consistent with the size of
the predicted open reading frame (Nowak 2003). However, the genome sequence analysis without experimental support suggests that there are 10 more exons upstream of these two experimentally verified exons. The length of a predicted rat Porf-2 mRNA containing all 12 exons would be 4612 bp. The NCBI Reference Sequence of this hypothetical rat mRNA is NM_173122.3.

**Figure 1.1** The 75 amino acid open reading frame of Porf-2 in rat.

Homologues to rat Porf-2 are found both in mouse and human using the BLAST program in gene bank. In mouse, the Porf-2 homologue with NCBI reference sequence NM_198420 is located on chromosome 15. It is also known as D15Wsu169e. It contains 11 exons and is 4495 bp long. In human, the Porf-2 homologue with NCBI reference sequence NM_025251 is located on chromosome 8. It is also called KIAA1688. It contains 12 exons and is 5147 bp long. The similarity between rat, mouse and human Porf-2 homologues is more than 80% (Fig. 1.2).
Functional conserved domains

The conserved domain analysis was done for Porf-2 proteins predicted from the known rat, mouse and human cDNAs. Mouse and human Porf-2 homologues contain a Myosin Tail Homology 4 (MyTH4) domain, which is a conserved domain in the tail of several different unconventional myosins and a plant kinesin-like protein. This domain recently has also been found in several non-motor proteins. Although the function of MyTH4 is not yet fully understood, evidence shows it could bind to microtubules and provide a link between an actin-based motor protein and the microtubules cytoskeleton. Human Porf-2 has an additional Pre-mRNA processing protein 48 (PRP48) domain, which encodes a splicing factor for RNA processing and modification. The common
conserved domain for all three Porf-2 homologues is RhoGAP_KIAA1688 (Fig. 1.3), which is a branch of RhoGTPase activator protein (GAP) domain family.

Figure 1.3 Conserved domains for Porf-2 proteins predicted from the known cDNAs of rat, mouse and human. The cDNA used to predict rat Porf-2 protein is the experimentally verified one. It is either a partial cDNA or one of several alternatively spliced transcripts. RhoGAP_KIAA1688 is a GTPase-activator protein (GAP) domain for Rho-like GTPases. MyTH4 is the domain in Myosin and Kinesin Tails. PRP48 is a splicing factor for RNA processing and modification.

The RhoGAP domain is about 150 amino acids conserved in Rho GTPase-activator proteins (GAP). The presence of the RhoGAP domain is a criterion for defining RhoGAP family members (Moon and Zheng 2003). GTPases work as molecular switches. They are active in the GTP-bound form and inactive in the GDP-bound form. The cycle between GTP-bound state and GDP-bound state is controlled by several regulators. GAP is one of them. GTPases are able to hydrolyze GTP to GDP by themselves. But the intrinsic GTP hydrolytic activity of Rho-like GTPases generally is low. Rho GAPs can enhance the rate of GTP hydrolysis by several orders of magnitude (Zhang and Zheng 1998). Thus, Rho GAPs are inactivators of Rho GTPases (Fig. 1.4). The highly conserved residues Arg^{85}, Lys^{122}, and Asn^{194} in the RhoGAP domain appear
involved in the activity of Rho GAP by directly interacting with GTPases and stabilizing the transitional state of the GTPase reaction (Musacchio et al. 1996; Barrett et al. 1997; Rittinger et al. 1997) Thus, RhoGAP domain is a functional domain responsible for turning off GTPases.

![Figure 1.4](image_url)

**Figure 1.4** Inactivation of Rho GTPase by Rho GAP. (Modified from Tcherkezian and Lamarche-Vane 2007.) GAP catalyzes the hydrolysis of GTP to GAP. Rho GTPases are inactive in GDP bound form and active in GTP bound form. Thus GAPs are inactivators of RTPases.

**Functions of Rho GAPs**

Rho GTPases are part of the Ras related small G protein family including Ras homolog gene (Rho), Ras-related C3 botulinum toxin substrate (Rac) and Cell division cycle 42 (Cdc42) (Symons 1996; Etienne-Manneville and Hall 2002; Pruitt and Der 2001). They are molecular switches of several cellular processes including regulating actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathway and gene transcription (Etienne-Manneville and Hall 2002). They have also been implicated in tumor cell growth and invasion (Evers et al. 2000; Schmitz et al. 2002; Frame and Brunton 2002; Sahai and Marshall 2002). Rho GAP can inactivate Rho GTPases by
accelerating GTP hydrolysis. From this point of view, the biological activity of Rho GAP could be similar to tumor suppressor. Some Rho GAPs, such as neurofibromin, Cdc42GAP, chromosome 13q12 encoded Rho GAP and tGAP1, have already been proved to regulate cell proliferation and apoptosis (Dasgupta et al. 2005; Wang et al. 2005; Nagaraja and Kandpal 2004; Modarressi et al. 2004; Ching et al. 2003). In addition, some Rho GAPs are implicated in actin assembly related cell processes, such as neuronal migration, neuronal morphogenesis including axonal growth and guidance, formation of synapses, and differentiation (Luo 2000; Moon and Zheng 2003). Given the presence of two functional domains, RhoGAP and MyTH4, within the same protein, it is interesting to speculate that MyTH4 further enhances the microtubule associated function of KIAA 1688.

It has been demonstrated that the mutations of some Rho GAPs are related to human diseases. An X-linked Rho GAP gene, ARHGAP6, is implicated in microphthalmia with linear skin defects (Schaefer et al. 1997). The mutation of Rho GAP oligophrenin-1 has been associated with non-specific X-linked mental retardation (Billuart et al. 1998). The biallelic mutation of GRAF encoded Rho GAP was found in three cases of 5q-minus myeloid leukemia (Borkhardt et al. 2000). The DLC1 gene, which encodes a Rho GAP, is always deleted or epigenetically silenced in hepatocellular carcinoma (Wong et al. 2003). With the discovery of association between Rho GAP and human diseases, more and more studies have been done to explore Rho GAPs. Now about 54 RhoGAP domain-containing proteins have been found in human. However, our understanding of their functions is still rudimentary (Peck et al. 2002). The functional
study of Porf-2, a novel RhoGAP domain-containing protein, will help to build our knowledge in this field.

**Cell cycle and p21**

The cell cycle (Fig. 1.5) is the succession of events including cell growth, replication, and division into identical daughter cells. The time required for completion of a cell cycle determines the rate of cell proliferation. Functionally cell cycle is divided into four phases: gap phase 1 (G1), DNA synthesis phase (S), gap phase 2 (G2) and mitosis phase (M).

*Figure 1.5 Schematic diagram of cell cycle regulation. (Adapted from image distributed by Sapphire Bioscience Pty. Ltd. www.sapphirebioscience.com/images.) CDK 4/6 associated with cyclin D and CDK2 associated with cyclin E are in control of G1 to S phase transition. CDK2 associated with cyclin A is involved in S phase progression. CDK1 associated with cyclin A/B is in control of G2 to M phase transition. INK family members inhibit G1 to S transition by binding to CDK4/6. CIP/KIP family members inhibit G1 to S transition by binding to cyclin D, cyclin E and CDK 2/4/6.*
When cells sense a growth signal or mitogen, they exit quiescent phase (G0), where they are out of the cell cycle and stop dividing, and enter G1 phase, in which various enzymes required in S phase are synthesized. When the environment, including the availability of nutrients, growth factors and hormones, is appropriate for DNA replication, cells pass the G1/S checkpoint and enter S phase. After DNA is synthesized and chromosomes are replicated in S phase, cells enter G2 phase, in which proteins required during the process of mitosis are synthesized. At the G2/M checkpoint, cells monitor the DNA replication and if DNA replication is correct, enter M phase. In M phase, when the chromosomes are correctly attached to spindles, the spindle checkpoint is passed and nuclear and cytoplasmic division occurs.

Progression of cells through cell cycle checkpoints is mediated by assembly and activation of cyclin-dependent kinases (CDKs) (Nigg 1995). When CDKs are bound by the regulatory subunit cyclins and phosphorylated, they are activated. There are two main classes of cyclin. The G1 cyclins, including cyclins C, D, and E, control the rate of cell cycle progression from the G1 to S phase. The mitotic or G2 cyclins, including cyclin A and B, are involved in the control of G2 to M phase transition and mitosis. Cyclin D, the first cyclin produced in cell cycle, activates CDK 4/6. Activated CDK 4/6 phosphorylates and thus deactivates retinoblastoma susceptibility protein (Rb). The hyperphosphorylated Rb dissociates from the E2F/DP1/Rb complex and activates transcription factor E2F, which further results in transcription of several genes such as cyclin E, cyclin A, DNA polymerase, and thymidine kinase. Subsequently, cyclin E forms a complex with CDK2 to phosphorylate and activate the downstream proteins required for cell cycle transition.
from G1 to S phase. Cyclin A forms the cyclin A-CDK1 complex and initiates the G2 to M phase transition (Fig 1.5).

The activities of CDKs are carefully regulated at multiple levels including negative control by two families of CDK inhibitors, inhibitors of CDK4 (INK4) family and CDK interacting protein/kinase inhibitory protein (CIP/KIP) family (Fig. 1.5). The members of INK4 family, including p16\textsuperscript{INK4A}, p15\textsuperscript{INK4B}, p18\textsuperscript{INK4C}, p19\textsuperscript{INK4D}, specifically bind to and inhibit CDK 4/6. The CIP/KIP family members, including p21\textsuperscript{CIP1/WAF1}, p27\textsuperscript{KIP1} and p57\textsuperscript{KIP2}, inhibit a broader spectrum of CDKs by binding to both cyclins and CDKs (Mainprize et al. 2001). p21\textsuperscript{CIP1/WAF1} (p21) is a G1 cyclin-dependent kinase inhibitor (Harper et al, 1993). It inhibits phosphorylation and activation of CDK4/6 associated with cyclin D and therefore allows RB to sequester E2F, which is a transcription factor required for entry of the cell into S phase. It also directly inhibits CDK2 associated with cyclin E, which is necessary for G1 to S transition. It is a major mediator of G1 arrest.

Functional studies have demonstrated that Rho GTPases stimulate G1 to S phase cell cycle transition by inhibiting p21 expression and inducing cyclin D1 expression in mid-G1 (Olson et al. 1995). Correspondingly, human chromosome 13q12 encoded Rho GAP exhibits inhibitory function in cancer cell proliferation by affecting p21. As a RhoGAP domain-containing protein, Porf-2 may also impact cell proliferation by affecting p21.
Cell apoptosis and p53

Intrinsic and extrinsic apoptotic pathways operate in almost all cases of programmed cell death. The extrinsic pathway is mediated by death receptors including Fas, DR5 and PERP. When pro-apoptotic ligands bind to the death receptors, the death-inducing-signaling-complex (DISC) is formed, which leads to activation of caspase 8 and 10. The active caspases 8 and 10 lead to a cascade of activation of other caspases including caspase 3, 6 and 7, which in turn induce apoptosis. The intrinsic pathway, also called mitochondrial apoptosis pathway is dominated by the Bcl-2 family proteins. There are two different kinds of Bcl-2 family members, pro-apoptotic proteins such as Bax and Bak; and pro-survival proteins such as Bcl-2 and Bcl-XL. The balance between these proteins governs the release of cytochrome c from the mitochondria. Released cytochrome c binds the adaptor apoptotic protease activating factor-1 (APAF1), forming a large multi-protein structure known as the apoptosome. The apoptosome recruits and activates caspase 9, which further leads to a cascade of activation of downstream effector caspases 3, 5 and 7 and in turn induces apoptosis (Fig. 1.6).
Figure 1.6 Regulation of apoptotic pathways by p53 transcriptional activity (Modified from Ashkenazi 2002). P53 stimulates extrinsic apoptosis and intrinsic apoptosis by inducing the expression of death receptors and Bcl-2 pro-apoptotic proteins respectively.
P53 is a famous transcription factor and tumor suppressor. When p53 is activated by external and internal stress signals, such as DNA damage, expression of oncogenes, hypoxia and nucleotide depletion, it can promote both intrinsic and extrinsic apoptotic pathways by transcriptional regulation of components of these pathways (Haupt et al. 2003) (Fig. 1.6). P53 stimulates the extrinsic apoptotic pathway by inducing the expression of death receptors Fas, DR5 and PERP and promoting trafficking of Fas to the cell membrane. P53 impacts the intrinsic apoptotic pathway by inducing the expression of Bcl-2 pro-apoptotic family members and shifting the balance towards pro-apoptotic effects. Bax is a p53-responsive pro-apoptotic Bcl-2 family protein. Bax can form homodimers and insert into the mitochondrial membrane to release cytochrome c to induce apoptosis. It also can form heterodimers with anti-apoptotic protein Bcl-2. By forming heterodimers with Bcl-2, Bax’s pro-apoptotic function is prevented. In response to stresses, p53 strongly induces the expression of Bax, increases the ratio of Bax to Bcl-2 and therefore promotes the formation of Bax homodimers to induce apoptosis. In addition to its transcription factor activity, p53 is able to promote apoptosis through transcription-independent mechanisms. In response to apoptotic signals, p53 translocates to mitochondria and enhances the permeabilization of the outer mitochondrial membrane by forming complexes with Bcl-2 and Bcl-XL (Schuler and Green 2005).

Due to the extensive effects of p53 on apoptosis, it is widely studied in apoptotic mechanisms. Both p53 transcription-dependent and independent roles have been observed in NSCs (Geng et al. 2007). Thus, if Porf-2 shows influence on NSC apoptosis, it is reasonable to speculate the involvement of p53 in this process. As a primary target
gene of p53 and a main pro-apoptotic protein in Bcl-2 family, Bax should also be
detected to address the contribution of p53 transcription factor activity to apoptotic
changes.

Hypotheses for this dissertation research

Since Porf-2 is a RhoGAP domain containing protein, it may have similar
functions to those of known Rho GAPs. Preliminary data suggested Porf-2 was a growth
regulator (Nowak and Gilham 2004). When NIH 3T3 cells were transfected with a Porf-2
expression plasmid, P2ME6, thymidine incorporation was increased under challenging
conditions [low fetal bovine serum (FBS), low density] while it was decreased under
favorable conditions (high FBS, high density) compared to controls transfected with
plasmid alone. Based on the above observations, three hypotheses were made. First, Porf-
2 is a growth regulator. It inhibits cell proliferation and promotes apoptosis in neural stem
cells. Second, Porf-2 impacts cell proliferation and apoptosis by affecting p53, p21 and
Bax signaling pathway. Third, Porf-2 may
promote differentiation of neural stem cells to
neuronal cells.

Specific aims of this dissertation research

To test these hypotheses, the first specific aim was to knock down expression of
Porf-2 in vitro by RNA interference to establish a stable cell clone with low expression
levels of Porf-2. The second specific aim was to measure cell proliferation in the Porf-2
knockdown cell clone and compare it with control cells to identify the role of Porf-2 in
cell proliferation. The third specific aim was to detect cell apoptosis in the Porf-2
knockdown cell clone and compare it with control cells to identify the role of Porf-2 in
cell apoptosis. Since Porf-2 influenced both cell proliferation and apoptosis, the fourth specific aim was to study the changes of expression of p53, p21 and Bax to clarify the mechanisms by which Porf-2 impacts cell proliferation and apoptosis. The fifth aim of this study was to induce differentiation, observe the direction of differentiation in the Porf-2 knockdown cell clone and compare it with control cells to identify the function of Porf-2 in NSC differentiation.

Because of the availability of the mouse NSCs and short hairpin RNAs (shRNAs) and because a similar gene is found in mouse, rat and human, a mouse system was chosen to accomplish these aims.
CHAPTER 2 MATERIALS AND METHODS

Creating a Porf-2 knockdown neural stem cell clone

Cell culture

C17.2 cells are stable, multipotent NSCs originally derived from neonatal mouse cerebellar external granular layer and immortalized by introduction of v-myc (Snyder et al. 1992). They can differentiate into neurons, astrocytes, and glial precursors in vitro (Yan et al. 2004). The C17.2 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma, St. Louis, MO) containing 4mM L-glutamine (Sigma), 10% fetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA), 5% horse serum (Gibco, Invitrogen) and 1x penicillin/streptomycin (Cambrex Bio Science, East Rutherford, NJ). The cultures were maintained in a standard humidified incubator in 5% CO₂ at 37°C, with fresh medium replaced every 2 d, and split 1:4 when the cells reached 90% confluence.

Confirming the expression of Porf-2 in C17.2 cells by PCR

Isolation of total RNA

Total RNA was isolated from C17.2 cells using RNeasy Mini Kit (QIAGEN, Valencia, CA). Briefly, cells were washed with phosphate buffered saline (PBS) and then detached by 0.05% trypsin-EDTA (Sigma). The collected cell suspension was centrifuged at 300 ×g for 5 min. The cell pellet was washed and suspended in RNeasy Mini Kit Buffer RLT to disrupt cell membranes. Homogenization was done by adding cell lysate into a QIAshredder column and spinning for 2 min at 16,000 ×g. One volume of 70% ethanol was added to the flow through to create conditions that promote selective binding
of RNA to the RNeasy membrane. Then the sample was applied to the RNeasy Mini spin column. After centrifuging for 15 sec at 8000 ×g, total RNA bound to the RNeasy silica-gel membrane. Then contaminants were washed away by RNeasy Mini Kit Buffer RW1. DNase I was pipetted directly onto the membrane and incubated at room temperature (RT) for 15 min to digest DNA. Then RNA was eluted in RNase-free water.

**RNA quantitation**

RNA quantitation was performed by Quant-iT Ribogreen RNA reagent and Kit (Molecular Probes, Invitrogen). This assay is based on the fact that when ribogreen, which is weakly fluorescent in unbound form, binds to nucleic acids, it gains 1000-fold fluorescence enhancement. Briefly, the ribosomal RNA standards provided with the kit and the sample were separately mixed with Ribogreen reagent and incubated at RT in the dark for 2-5 min. Then they were transferred to the VersaFluor™ cuvette fluorometer (Bio-Rad, Hercules, CA) to measure fluorescence. The excitation wavelength used was 480 nm and the emission wavelength was 520 nm. A standard curve of fluorescence versus RNA concentration was generated from the ribosomal RNA standards. The RNA concentration of the sample was determined from the curve.

**Synthesis of cDNA**

cDNA was synthesized by reverse transcription by advantage RT-for-PCR kit (BD Biosciences, San Jose, CA) according to manufacturer’s instruction. Briefly, 1 µl oligo (dT) primer was added into 1 µg total RNA sample and the mixture was heated to 70°C for 2 min followed by quenching rapidly on ice. Then the reagent mixture of 4 µl of 5X reaction buffer, 1.0 µl of dNTP mix (10 mM), 0.5 µl of Recombinant RNase inhibitor
and 1.0 µl of MMLV reverse transcriptase was added to the sample and incubated at 42°C for 1 h. The cDNA synthesis reaction was stopped by heating at 94°C for 5 min. The reaction was diluted to 100 µl by adding DMPC-treated H2O. The cDNA in the reaction was ready for immediate use or storage at -80°C.

**Polymerase Chain Reaction (PCR)**

Two sets of mouse Porf-2 specific primers were designed by Beacon Designer software (PREMIER Biosoft International). The sequences of the primers were: 5’-TTC CTC CAG GTG TTC GTG-3’ and 5’-TGG TAT CTA AAT GCT GAA TGA G-3’ (for zw4); 5’-CCA GCC AAT GTA GCC ATC ACC AAA-3’ and 5’-TGT CAC CCT AGG AGT CCA TAG CC-3’ (for p2). The reaction mixture consisted of 5 µl 10X buffer for Accu Taq LA DNA Polymerase, 2.5 µl 10mM dNTP mix, 2.5 µl Jumpstart REDAccu Taq DNA polymerase, 1 µl 20 µM forward primer, 1 µl 20 µM reverse primer, 10 µl cDNA template and 28 µl sterile H2O. Then the PCR microtube was placed in an iCycler thermal cycler (Bio-Rad) with the following settings: 96°C for 30 sec; 30 cycles of 94°C for 15 sec, 57°C for 30 sec and 68°C for 1 min; 68°C for 7 min. To evaluate the amplified DNA, 10 µl PCR products were mixed with 4 µl 5X loading dye, 2 µl 0.89 M Tris, 0.89 M boric acid, 0.02 M EDTA (10X TBE) and 4 µl H2O. DNA Ladder I (GeneChoice Inc. Frederick, MD) was used as molecular weight marker. The mixture was loaded on a 1% TBE agarose gel and run at 85 volts for 1 h. Then the gel was stained by 0.5 µg/ml ethidium bromide for 5 min and destained in 1 mM MgSO4 for 10 min. Image analysis was done using a BioRad GelDoc2000 imaging system.
**Constructs of Porf-2 shRNA lentivirus**

The expression of Porf-2 in C17.2 was knocked down by short hairpin RNA (shRNA) lentiviral particles. Five frozen bacterial glycerol stocks harboring predesigned sequence-verified shRNAs were purchased from Open Biosystems (Huntsville, AL). These five shRNAs target different exons of the mouse Porf-2 gene. They are named as 184, 185, 186, 187, and 188 shRNA. Each of them includes a hairpin of 21 base-pair sense and antisense stem and a 6 base-pair loop. Their sequences and targeting regions are shown in Table 2.1.

**Table 2.1** The sequences and targeting regions of five Porf-2 shRNAs

<table>
<thead>
<tr>
<th>shRNA clone ID</th>
<th>sequence</th>
<th>Targeting region</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRCN0000097184</td>
<td>CGCCGACGTCTAAAGATTGCTGCTAATCCGGTCATGAAAAGTTGTTA</td>
<td>Exon 11</td>
</tr>
<tr>
<td>TRCN0000097185</td>
<td>CGGGCGATGGAATCTCTGATGTTA</td>
<td>Exon 5</td>
</tr>
<tr>
<td>TRCN0000097186</td>
<td>CGCCCTCATGAAATTACTGCAATCCGGTCTGTTAATGAGTGTGACGTTCTGTTGTTGTTG</td>
<td>Exon 10</td>
</tr>
<tr>
<td>TRCN0000097187</td>
<td>CGGGCCAAGAACTGGAAATCATCATTTGTCGAAATGATGTCGCAATGTTCTGTTGTTGTTG</td>
<td>Exon 3</td>
</tr>
<tr>
<td>TRCN0000097188</td>
<td>CGGGCTCTCTGCTATTTTCCGAAGATCTCGGGATCAGTATCGGAGGCAGCGAGGTTTTG</td>
<td>Exon 11</td>
</tr>
</tbody>
</table>

Note: The two segments of black letters represent sequences for sense and antisense strands. The three segments of yellow letters represent sequences for ligation regions, loop and ligation regions respectively.

Each shRNA is cloned into a self-inactivating (SIN) lentiviral vector, pLKO.1-puro expression vector, which contains a deletion in the U3 region of the 3’ long terminal repeat (Fig. 2.1). When these vectors are co-transfected with compatible packaging plasmids into packaging cells, self-inactivating replication incompetent viral particles can be produced. The resultant lentiviral particles permit efficient infection and integration of the specific shRNA construct into both differentiated and non-dividing cells. In addition,
using the puromycin selectable marker permits stable gene silencing selection, which makes long-term in vitro knockdown and phenotypic observation possible.

**Figure 2.1** pLKO.1-puro vector map and explanation of vector elements. (Adapted from vector map provided by Sigma. http://www.sigmaaldrich.com/life-science/functional-genomics-and-rnai/shrna/library-information/vector-map.html)
**Preparation of Porf-2 shRNA plamid**

Two packaging plasmids, pCMVDR8.9 and pMD.G, and packaging cell HEK293T were generously provided by Dr. Wu (Johns Hopkins Medical Institute). ShRNA-pLKO.1 plasmid and blank-pLKO.1 plasmid without shRNA (for control cells) was extracted from a bacterial glycerol stock.

**Extraction of plasmid DNA**

Perfectprep® Plasmid Midi kit (Eppendorf North America Inc, Westbury, NY) was used to extract shRNA-pLKO.1 plasmid DNA. The frozen glycerol stock of shRNA was streaked onto the LB agar plate containing 100 µg/ml carbenicillin. After overnight incubation at 37°C, a well-separated colony was picked and transferred into 1 ml TB broth containing 100 µg/ml carbenicillin and incubated for 6 h at 37°C with rapid agitation. Then the starter culture was used to inoculate 40 ml pre-warmed TB containing the 100 µg/ml carbenicillin. After incubation at 37°C for 14 to 16 h with rapid agitation, OD$_{600}$ of the culture was examined with a BioMate™ 3 spectrophotometer (ThermoSpectronic, Madison, WI). When OD$_{600}$ reached 1.5 to 2.5, the bacterial cells were pelleted by centrifugation at 10,000 ×g for 5 min, resuspended in Perfectprep® Plasmid Midi kit Solution 1, lysed by Solution 2 and neutralized by Solution 3. The neutralized lysate was centrifuged at 15,500 ×g for 30 min at 4°C to pellet cell debris. The supernatant was collected and mixed with 10 ml DNA Binding Matrix. The matrix-bound DNA mixture was poured into a spin column and centrifuged at 2,000 ×g for 5 min. DNA bound to the spin column was washed twice by Diluted Purification Solution
and then eluted with 6 ml of Elution Buffer. The DNA eluate was mixed with 140 μl of 5 M NaCl and 120 μl mussel glycogen, then precipitated by adding 12 ml of 95-100% ethanol. Plasmid DNA was collected by centrifuging at 15,500 × g for 25 min at 4°C. The DNA pellet was washed twice with 70% ethanol, air dried and resuspended in 200 μl Elution Buffer.

**DNA quantitation**

The DNA concentration was determined with a Fluorescent DNA quantitation kit (Bio-Rad). This assay is based on the fact that when bis-benzimide fluorescent dye Hoechst 33258, which is weakly fluorescent in solution, specifically binds to the A-T base pairs in dsDNA, there is an increase in fluorescence and a shift in the emission maximum from 500 to 460 nm. Briefly, the dsDNA standards provided with the kit and the sample were separately mixed with Hoechst 33258 and placed in the VersaFluor cuvette fluorometer (Bio-Rad) to measure fluorescence. The excitation wavelength used was 360 nm and the emission wavelength was 460 nm. A standard curve of fluorescence versus DNA concentration was generated from the dsDNA standards. The DNA concentration of the sample was determined from the curve.

*Production of Porf-2 shRNA lentivirus*

HEK293T cells were seeded on a 6-well plate with 6.25 x 10⁵ cells in 2 ml DMEM containing 10% FBS and 5% horse serum per well 24 h before transfection. When the cells reached 70% confluence, shRNA-pLKO.1 or blank-pLKO.1 plasmid was mixed with two packaging plasmids, pCMVD8.9 and pMD.G, at a ratio of 3:2:1. Then 1 μg of this plasmid mixture and 6 μl of Lipofectamine LTX™ Reagent (Invitrogen) were
added into each well for co-transfection. Cells were incubated at 37°C in a 5% CO₂ incubator for 16-18 h, then the media was replaced with DMEM containing 30% FBS 1.5ml per well. Viral particles were harvested 42 h post-transfection by collecting media and filtering through a 0.45 μm filter. The particles were immediately used for transduction.

**Tranduction of the shRNA lentivirus into C17.2 cells**

C17.2 cells were seeded into a 24-well plate with 2 x 10⁴ cells in 500 μl culture media, DMEM containing 10% FBS and 5% horse serum per well, 24 h before transduction. When the cells reached 20% confluence, 1 ml viral particles and 1.5 μl polybrene were added to each well, the plate rocked gently to mix and the cell-viral particle mixture incubated at 37°C, 5% CO₂ for 6 h. Media was removed and replaced with 500 μl fresh growth media without puromycin, then incubated for another 42 h. Then media was replaced with media containing 1 μg/ml puromycin 48 h post-infection and cells incubated for at least 48 h to perform puromycin selection. The surviving cells were successfully transduced cells. They were ready for detecting Porf-2 expression and subsequent phenotype observation. For each shRNA-pLKO.1 lentiviral particle and blank-pLKO.1 lentiviral particle, viral transduction was made in triple wells and one well was treated only with transduction reagent without lentiviral particles as a control.

**Confirmation of Porf-2 knockdown at mRNA level**

Quantitative PCR (Real time PCR) was performed to detect Porf-2 mRNA levels in transduced C17.2 cells. Total RNA was isolated, quantified and reverse transcribed to
cDNA as described in section “confirming the expression of Porf-2 in C17.2 cells by PCR” (Page 34).

Primers for mRNA expression level of the Porf-2 gene were designed to flank the intron region so the PCR product from RNA and contaminating DNA were of different sizes (Fig. 2.2). Contamination by DNA can be recognized by observing the melting curve. The primer set for Porf-2 was 5’-TTC CTC CAG GTG TTC GTG-3’ and 5’-TGG TAT CTA AAT GCT GAA TGA G-3’. The primers for β-actin as the reference gene (internal control) were 5’-GGG AAA TCG TGC GTG ACA-3’ and 5’-GCG GCA GTG GCC ATC TC-3’. The primer efficiency was determined by MyiQ Software using a series of diluted primers including 1:1, 1:3, 1:9, 1:27, and 1:81 dilutions.

![Figure 2.2 A schematic model showing primer design in RT-PCR of Porf-2. Primers were designed to flank an intron of the gene. The product of RT-PCR using RNA as template has a smaller size than the one using DNA as template.](image)

SYBR Green was used in real time PCR. SYBR Green binds to only double strand DNA (dsDNA) not single strand DNA. When SYBR Green binds to dsDNA, it gains fluorescence. Thus the amount of dsDNA can be determined by measuring the
number of fluorescence units. The threshold is the point at which the amplification of cDNA is logarithmic and has not yet reached a plateau, and a real difference between RNA levels can be recognized. This threshold is set by the MyiQ Software. The cycle number at which the fluorescence crosses the threshold is referred as Ct.

IQ SYBR Green Supermix (Bio-Rad) was mixed with cDNA template and nuclease free H2O to make a master mix. Primer set (Final concentration = 300 nM) was pipetted into individual wells of a 96 well plate for real time PCR (VWR) followed by adding one aliquot of master mix. Then the plate was subjected to MyiQ™ Single-Color Real-Time PCR Detection System (Bio-Rad) with the following settings: 95°C for 2 min; 40 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 1 min. Ct value was acquired by MyiQ Software for the sample in each individual well. The relative quantitation of targeted gene, Porf-2, was calculated based on the reference gene, β-actin, by Pfaffl method (Pfaffl 2001). Briefly, each sample was run in triplicate and the Ct value was averaged. The relative expression ratio of target gene in experimental cells versus control cells was calculated in the following equation:

\[
\text{ratio} = \frac{(E_{\text{target}})^{\Delta C_{t \text{target}} \text{ (control-experimental)}}}{(E_{\text{ref}})^{\Delta C_{t \text{ref}} \text{ (control-experimental)}}}
\]

In this equation, \(E_{\text{target}}\) is the primer efficiency of the target gene transcript; \(E_{\text{ref}}\) is primer efficiency of a reference gene transcript; \(\Delta C_{t \text{target}}\) is the Ct deviation between control cells and experimental cells.
Confirmation of Porf-2 knockdown at protein level

Extraction of total protein

Total protein was prepared following the method of Rudolph et al. (1999). Briefly, cells were harvested, washed with PBS, and lysed in FT lysis buffer (600 mM KCl, 20 mM pH 7.8 Tris-Cl, 20% glycerol, 1mM Pefabloc, 10 μg/ml Leupeptin, 10 μg/ml Pepstatin, 5 μg/ml Aprotinin). The suspension was frozen briefly in liquid N₂ and was allowed to thaw slowly on ice (about 10 min). The freeze/thaw cycle was repeated once and then benzonase (Novagen, Madison, WI) was added to digest DNA.

Protein quantitation

Protein concentrations were determined by the BCA assay (Pierce, Rockford, IL) according to manufacturer’s instructions. This assay is based on the ability of bicinchoninic acid (BCA) to chelate Cu⁺, which is reduced from Cu⁺² by protein in an alkaline medium, forming a purple product that exhibits a strong absorbance at 562 nm. Briefly, a series of dilutions of bovine serum albumin (BSA) were used as standards. Each protein standard and protein sample was separately mixed with BCA working reagent and incubated at 37°C for 30 min. Then the mixture was examined in a BioMate™ 3 spectrophotometer (ThermoSpectronic) to measure the absorbance at 562 nm. For each sample and standard, triplicates were made and average values were used. A standard curve of absorbance versus protein concentration was plotted from BSA standards. The concentration of protein in each sample was determined using this standard curve.
**Western blot**

Eighty micrograms of protein extract were mixed with an equal volume of 2x Laemmli Buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue, 0.125 M Tris HCl) and heated to 95°C for 10 min. Then the mixture was loaded on 4-12% gradient SDS-PAGE gel (Bio-Rad). Precision Plus Protein™ Western C™ standards (Bio-Rad) were also loaded on the gel as molecular weight markers. Electrophoresis was performed in the running buffer [100 ml of 10x Tris/Glycine/SDS buffer (Bio-Rad) and 900 ml H₂O] at 40 V until the protein samples migrated into the resolving gel, then the voltage was increased to 120 V until the front band of molecular weight marker reached the bottom of the gel.

Polyvinylidene difluoride (PVDF) 0.45µm membrane (Pierce) was soaked in methanol for 10 sec to be activated and then rinsed in water. A transfer package was assembled in the order of sponge slab, whatman paper, gel, PVDF membrane, Whatman paper and sponge slab in the transfer buffer (100 ml of Bio-Rad’s 10x Tris/Glycine/SDS buffer, 200 ml ethanol and 700 ml H₂O). Transfer apparatus (Bio-Rad) was assembled. The transfer package was clamped in a black-white plastic plate with the gel-side facing black plate and inserted into the tank with black plate facing the black electrode. The electrotransfer was performed in transfer buffer at 90 mV overnight at 4°C.

After transfer, the PVDF membrane was blocked in blocking buffer (1x TBS, 5% w/v dry milk) at RT for 1 h and subsequently probed overnight with rabbit anti-Porf-2 (1:1500, F. V. Nowak’s lab) in 1x TBS and 0.5% dry milk (Bio-Rad). After rinsing, the membrane was incubated with horseradish peroxidase (HRP) conjugated goat anti-rabbit
IgG antibody (1:2000, Alpha Diagnostics International Inc, San Antonio, TX) and Precision Plus StrepTactin-HRP conjugate (1:15,000, Bio-Rad). Following the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected on ChemiDoc (Bio-Rad) using an enhanced chemiluminescence kit (Amersham ECL Plus Western Blotting Detection reagents, GE Healthcare, Pittsburgh, PA) according to the manufacturer’s instruction. The images were captured and analyzed by Quantity One software (Bio-Rad).

After detection, the membrane was stripped by incubating in 55°C stripping buffer (100 mM β-mecaptoethanol, 2% SDS and 62.5 mM Tris HCl, pH 6.8) for 30 min. Then the membrane was rinsed three times with TBS/Tween, detected by enhanced chemiluminescence (ECL) to confirm complete stripping, washed twice with TBST, air dried, and activated again by soaking in ethanol followed by rinsing. Then the membrane was blocked, probed again with mouse anti-β-actin IgG antibody (1:2000, Sigma) as primary antibody and HRP conjugated rabbit anti-mouse IgG antibody (1:40,000, Sigma) as secondary antibody, and detected by ECL on ChemiDoc as mentioned in the first detection.

**Measuring proliferation in Porf-2 knockdown cells and control cells**

In order to quantitatively measure cell proliferation, the metabolic activity of viable cells was detected by the AlamarBlue™ assay as described by the manufacturer (AbD Serotec, Kidlington, U.K.) with modifications. This assay incorporates an oxidation-reduction (REDOX) indicator which exhibits both a fluorescent and colorimetric change in response to chemical reduction of the growth medium resulting
from cell growth. As cells being tested grow, innate metabolic activity results in a chemical reduction of AlamarBlue™ (AB). Continued growth maintains a reduced environment, while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator to change from an oxidized (non-fluorescent, blue) form to a reduced (fluorescent, red) form. Then data can be collected using fluorescence-based or absorbance-based instrumentation.

In brief, Porf-2 knockdown C17.2 cells and control cells were seeded into 96-well plates at 5,000 cells per well in 110 µl growth medium containing 10% AB. For each cell clone, 32 wells were prepared. Growth medium, 110 µl containing 10% AB, without cells was added into 4 wells and used as control. The plates were incubated at 37°C, 5% CO₂ for 6 h. Growth medium containing 10% AB without cells was also autoclaved for 15 min to produce 100% reduced AB solution and 110 µl was added into 4 wells as another control. Then the plates were examined in a FluoroMax plate reader (HORIBA Jobin Yvon, Edison, NJ) to determine fluorescence intensity (FI) at a 560 nm excitation wavelength and a 580 nm emission wavelength. Percentage reduction of AB was calculated from FI by the following equation:

\[
\text{Percentage reduction of AB} = \frac{\text{FI} 580 \text{ of cells} - \text{FI} 580 \text{ of control}}{\text{FI} 580 \text{ of 100% reduced AB} - \text{FI} 580 \text{ of control}}
\]

Three different growth media were used respectively: DMEM containing 1% FBS; DMEM containing 5% FBS; DMEM containing 10% FBS plus 5% horse serum. Every second day, measurement of AB reduction was repeated by removal of the old growth medium, substitution with 10% AB-containing new growth medium and detection
of FI 6 h after addition of the AB substrate. The proliferative activity is presented as percent reduction of AB.

**Analyzing cell cycle progression in Porf-2 knockdown cells and control cells**

Cell cycle populations were identified by DNA concentration which reflects the degree of strand replication. Cells in different cell cycle phases (G1, S or G2/M phase) are characterized by different DNA content. Diploid G1 cells have 1X fluorescence intensity (FI). Tetraploid G2/M phase cells have 2X FI. Cells in S phase have FI values between the 1X and 2X populations (Fig. 2.3). The fluorescent dye propidium iodide (PI), which can bind to DNA strongly at a ratio of 1:1, was used to determine the DNA level distribution.

![Cell Cycle Populations](Image)

**Figure 2.3** DNA content in different stages of cell cycle. (Modified from [www.ucl.ac.uk/wibr/services/docs/cellcyc.pdf](http://www.ucl.ac.uk/wibr/services/docs/cellcyc.pdf)) Cells in G0/1, S and G2/M phase have 1, 1-2, and 2 copies of DNA respectively. Thus, the FI value for cells in G0/1, S and G2/M phase is 1X, 1-2X and 2X respectively.

Briefly, cells kept in liquid nitrogen were resuscitated and cultured in 10 cm culture dishes for 24 h. The cells were observed with an inverted microscope (Nikon, Eclipse TS100). Then they were incubated for another 6 to 24 h. When they reached 70% confluence, cells were harvested by trypsinization, washed twice in cold PBS, and fixed
in 70% ethanol at -20°C overnight. Cell pellets were resuspended in staining buffer containing 100 µg/ml RNase (Sigma) and 40 µg/ml PI (Sigma), incubated at 37°C in the dark for 30 min, and subjected to flow cytometry (FACSort, Becton Dickinson, Franklin Lakes, NJ). For each individual sample, data were acquired from 20,000 cells using CellQuest™ software (BD Biosciences). ModFit LT software version 3.2 (Verity Software House Inc., Topsham, ME) was used to evaluate cell cycle.

**Measuring apoptosis in Porf-2 knockdown cells and control cells**

*Treatment with bleomycin and staurosporine (STS)*

Staurosporine (STS), a broad-spectrum kinase inhibitor and bleomycin, a genotoxic reagent, were used to induce apoptosis. For each reagent, three different concentrations and three durations of treatment were tried. STS at concentrations of 30 nM, 100 nM and 300 nM was used to treat C17.2 cells for 2 h, 4 h and 6 h respectively. Bleomycin at concentrations of 0.003 U/ml, 0.01 U/ml and 0.03 U/ml was used to treat C17.2 cells for 12 h, 24h and 48h. The minimum concentration with the shortest duration, which led to obvious apoptosis of C17.2 cells, was chosen for subsequent experiments. Apoptosis was induced in Porf-2 knockdown C17.2 and control cells by incubating them in 100 nM STS for 4 h or 0.01 U/ml bleomycin for 24 h.

*TUNEL assay*

Terminal deoxynucleotidyl transferase (TdT) -mediated dUTP Nick-End Labeling assay (TUNEL) was performed using an In Situ Cell Death Detection Kit, fluorescein (Roche, Indianapolis, IN) according to manufacturer’s directions. TdT catalyzes polymerization of FITC-labeled nucleotides to free 3’-OH ends of the oligonucleosomal
nicks/strand breaks of DNA in apoptotic cells. Thus the apoptotic cells can be recognized by fluorescein labeling.

Briefly, treated cells were collected, washed twice in ice-cold PBS and resuspended at a concentration of $2 \times 10^7$ cells/ml. Then $100 \, \mu$l cells were added to a microcentrifuge tube for each sample and fixed by mixing with $100 \, \mu$l 4% paraformaldehyde. The mixture was incubated on a shaker at RT for 30 min and then centrifuged at $300 \times g$ for 10 min to remove fixative. Cell pellets were washed once with $200 \, \mu$l PBS and resuspended in $100 \, \mu$l permeabilization buffer (0.1% Triton X100, 0.1% sodium citrate, freshly prepared) for 2 min on ice. After washing twice with PBS, cells were mixed with $50 \, \mu$l TUNEL reaction mixture including TdT and fluorescein-dUTP and incubated for 60 min at $37^\circ$C in humidified air in the dark followed by washing twice as above. The percentage of FITC labeled cells was counted by flow cytometry (FACSort, Becton Dickinson). For each individual sample, data were acquired from 10,000 cells and analyzed by CellQuest™ software.

**Annexin V assay**

An Annexin V-FITC apoptosis detection kit I (BD Biosciences) was used to detect apoptotic cells according to manufacturer’s instructions. In the early stage of apoptosis, phosphatidylinerine (PS) translocates from inner leaflet to outer leaflet of the plasma membrane. Annexin V-FITC can bind to exposed PS in cells of early stage apoptosis. Propidium iodide (PI) is membrane impermeable and generally excluded from cells with an intact membrane. It binds to DNA in the cells losing integrity of the cell membrane, including cells in late stages of apoptosis and dead cells.
In brief, treated cells were washed twice with ice-cold PBS and resuspended in 1X Binding Buffer at a concentration of $1 \times 10^6$ cells/ml. Then 100µl of the solution was transferred to a 5 ml culture tube and 5 µl of Annexin V-FITC and 5 µl PI were added. The solution was vortexed gently, incubated for 15 min at RT in the dark followed by the addition of 400 µl 1X Binding Buffer. Samples were subjected to flow cytometry (FACSort, Becton Dickinson) within one h. For each individual sample, data were acquired from 10,000 cells and analyzed using CellQuest™ Software. The cells are distributed in four quadrants (Fig. 2.4). The following controls were used to set up compensation and quadrants: (1) unstained cells, (2) cells stained with Annexin V alone, (3) cells stained with PI alone.

**Figure 2.4** Schematic explanation of Annexin V assay results. Cells in lower left quadrant (LL) are nonapoptotic. Cells in lower right quadrant (LR) are undergoing early stage of apoptosis. Cells in upper right quadrant (UR) are undergoing late stage of apoptosis. Cells in upper left quadrant (UL) are end-stage apoptotic, dead or necrotic cells.
Measuring the expression levels of p21, p53 and Bax in Porf-2 knockdown cells and control cells

Preparation of 12% SDS-PAGE gel

A mixture of 4 ml 30% acrylamide/Bis (29.2% acrylamide, 0.8% N’N’-bis-methylene-acrylamide), 2.5 ml 1.5 M Tris-HCl (pH 8.8), 0.1 ml 10% SDS and 3.4 ml deionized H₂O was degased for 15 min to make a 10 ml monomer solution. The gel mold (Bio-Rad) was set up and 50 µl 10% ammonium persulfate (APS) and 5 µl TEMED were added to the monomer solution and swirled gently to initiate polymerization. The gel mixture was poured into gel mold until 1 cm below the comb position followed by overlay with 500 µl 0.1% SDS. After 30 min, the 12% resolving gel was formed. Then the overlay solution was removed and the unpolymerized residue was washed away with H₂O. Ten ml 5% stacking gel monomer solution was made using 1.7 ml 30% acrylamide/Bis, 2.5 ml 0.5 M Tris-Hcl (pH 6.8), 0.1 ml 10% SDS and 5.7 ml deionized H₂O. After degassing for 15 min, the monomer solution was mixed with 50 µl 10% APS and 10 µl TEMED. The gel mixture was poured on top of the resolving gel, the comb inserted and the gel allowed to stand for 30 min to polymerize.

Western blot

Total proteins were extracted, quantified, separated on a 12% SDS-PAGE gel, probed and detected as described in the section “Confirmation of Porf-2 knockdown at protein level” (page 44 ) with the following minor changes. The transfer of the proteins to PVDF membrane was performed at 350 mA for 1 h and 15 min at 4°C. The primary antibodies used were rabbit anti-p53 IgG (1:500, Santa Cruz Biotechnology, CA, USA),
rabbit anti-p21 IgG (1:500, Santa Cruz Biotechnology) and rabbit anti-Bax IgG (1:500, Santa Cruz Biotechnology) The secondary antibody used was HRP conjugated goat anti-rabbit IgG antibody (1:2000, Alpha Diagnostics International Inc). For p53 detection, a protein extract was prepared from MCF7, a human breast cancer cell line, and used as positive control. The internal control was β-actin.

**Detecting p53 transcriptional activity in Porf-2 knockdown cells and control cells**

In the p53 luciferase assay, the p53 luciferase reporter vector is used to report the binding activity of transcription factor p53. The p53 reporter vector contains a cis-acting DNA binding element which is recognized by p53. Binding of p53 at this site results in the expression of firefly luciferase, an enzyme capable of catalyzing the oxidative carboxylation of beetle-luciferin. The luminescence from this chemical reaction can be quantified by a luminometer and correlates directly with the binding activity of p53. In a dual luciferase reporter assay, pRL-CMV renilla luciferase vector is co-transfected with p53 firefly luciferase vector as an internal control serving as the baseline response. The constitutively expressed renilla luciferase catalyzes the luminescent reaction of coelenterate-luciferin. The luminescence detected is used to eliminate variabilities of pipetting volumes, transfection efficiency, cell lysis efficiency and assay efficiency.

Porf-2 knockdown C17.2 cells and control cells were seeded in a 24-well plate at 1 x 10⁵ cells per well and allowed to grow to 70% confluent. Then cells in each well were transfected with 200 ng of p53 firefly luciferase reporter plasmid (p53-luc, Panomics, Redwood City, CA) and 20 ng of Renilla luciferase control plasmid (pRL-CMV, Promega, Madison, WI) in Fugene6 transfection reagent 1.2 µl (Roche Molecular
Biochemicals). After 24 h, STS or bleomycin treatments were performed in different wells. After treatment, the cells were washed by PBS and lysed by Passive Lysis Buffer (Promega), and dual luciferase assays were performed as described by the manufacturer (Promega) on a luminometer, Lumat LB 9507 (Berthold Technologies, Oak Ridge, TN). Luciferase activity was normalized to Renilla luciferase control according to protocols derived from the manufacturer (Promega). The ratio between light units of firefly luciferase and renilla luciferase control was used to represent p53 transcription factor activity.

**Observing the differentiation direction of Porf-2 knockdown cells and control cells**

*Preparation of glass coverslips*

Glass coverslips (VWR International Inc., San Diego, CA) were sterilized by dipping in 70% ethanol for 1 h, air dried in the hood and inserted into 6-well culture plates. The plates were then coated by adding 2 ml 0.1% polyethyleneimine (Sigma) in borate buffer into each well and letting them stand overnight at RT. Then the coating solution was removed and the wells washed with PBS. The plate was sterilized by exposure to UV light for 30 min.

*Induction of differentiation*

PLKO.1-C17.2 and 184-C17.2 cells were seeded onto the coated coverslips in the 6-well plates at $1 \times 10^5$ cells per well in 2 ml DMEM containing 10% FBS and 5% horse serum. When the cells reached 70% confluence, the culture medium was replaced by serum-free medium, DMEM/F12 1:1 plus N2 supplement (Gibco, Invitrogen). The serum-free medium was replaced every two days. The cell differentiation was induced
gradually by serum starvation for six days (Yan et al. 2004). Six days after changing into serum-free medium, the cells were ready for morphological observation and immunocytochemistry.

**Morphological observation**

For cells before and after induction of differentiation, the cellular morphologies were visualized with an inverted microscope (Nikon, Eclipse TS100) equipped with a NCB-11 filter and 40× LWD phase Achromat objective. The images were captured by a Coolsnap CF2 camera (Nikon) and processed by NIS-Elements F2.20 software. Then the cells were subjected to immunocytochemistry (ICC).

**Immunocytochemistry (ICC)**

Primary antibodies were chosen to address cell type-specific markers including nestin, class III β-tubulin-specific epitope TU20, glial fibrillary acidic protein (GFAP) and type II astrocytes and ganglioside antigen A2B5.

Nestin is an intermediated filament protein expressed predominantly in stem cells of the central nervous system. It plays a role in structural organization of cells (Michalczyk and Ziman 2005). Upon differentiation, nestin is downregulated and replaced by neurofilaments. It is used as a marker for multipotent neural stem cells.

β-tubulin is a globular protein, which can be assembled together with α-tubulin to form microtubules. There are seven β-tubulin isotype classes identified on the basis of highly conserved variable domains. They have characteristic cell type distribution (Sullivan 1988). Class III β-tubulin isotype is most abundant in cells of neuronal origin (Burgoyne et al. 1988). It is one of the earliest appearing markers of neuronal
differentiation (Easter et al. 1993). Thus class III β-tubulin is used as a marker for neuronal lineage.

Glial fibrillary acidic protein (GFAP) is one of the major intermediate filament proteins of mature astrocytes. It plays a role in mitosis by adjusting the filament network in the cell (Tardy et al 1990). It is also involved in long term upkeep of CNS myelination (Goss et al. 1991). It is used as a marker to distinguish mature astrocytes from other glial cells.

A2B5 is a cell surface ganglioside epitope expressed in O2A cells, which are oligodendrocyte progenitors and type II astrocytes. It is absent in type I astrocytes. So it allows one to distinguish between these different types of cells. A2B5 is used as a marker for common glial precursors.

The primary and secondary antibodies used in ICC to characterize cell types are listed in Table 2.2.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSC</td>
<td>Mouse anti-nestin monoclonal IgG antibody, MAB353 (1:200, Chemicon, Temecula, CA)</td>
<td>Alexa Fluor 488 labeled donkey anti-mouse IgG antibody (1:1000, Invitrogen)</td>
</tr>
<tr>
<td>neuron</td>
<td>Mouse anti β-tubulin III monoclonal IgG antibody, CBL412 (1:200, Chemicon)</td>
<td>Alexa Fluor 488 labeled donkey anti-mouse IgG antibody (1:1000, Invitrogen)</td>
</tr>
<tr>
<td>Astrocyte (Type II)</td>
<td>Rabbit anti-GFAP polyclonal IgG antibody (1:400, Dako, Carpinteria, CA)</td>
<td>Alexa Fluor 594 labeled donkey anti-rabbit IgG antibody (1:1000, Invitrogen)</td>
</tr>
<tr>
<td>Glia precursor</td>
<td>Mouse anti A2B5 monoclonal IgM antibody, MAB312 (1:400, Chemicon)</td>
<td>Cy3 conjugated donkey anti mouse IgM antibody (1:500, Jackson Immunoresearch, West Grove, PA)</td>
</tr>
</tbody>
</table>
The cells with or without induction of differentiation were rinsed in the 6 well-plate with PBS twice and then fixed by incubation in 4% paraformaldehyde for 20 min at RT. After three rinses with PBS, permeabilization was performed by incubating cells in 0.1% Triton X-100 in PBS for 15 min. Following three additional rinses with PBS, the cells were incubated with 5% donkey serum for 1 h at RT to block non-specific binding. Then they were incubated in primary antibodies diluted in 5% donkey serum for 2 h at RT. After rinsing three times with PBS, the cells were incubated in secondary antibody diluted in 5% donkey serum containing 300 ng/ml of the nuclear dye DAPI for 2 h at RT in the dark. The coverslips were mounted and sealed on labeled glass slides. The slides were visualized with an epifluorescence microscope (Nikon, Diaphot 300) equipped with a Nikon 20× objective, FITC-HYQ filter and TRITC filter (Chroma Technology Corp, Rockingham, VT) and the images were acquired by a CCD camera (Spot, RT ES, model 9.1 Monochrome w/IR-6). For each sample, five random fields were counted to calculate the percentage of cells expressing nestin, β-tubulin III, GFAP or A2B5. The negative controls were made by carrying out the procedure without adding primary antibodies. There was no fluorescence signal detected in any of the negative controls. Therefore, all cells with a fluorescence signal are considered positive for the respective antigens. For each randomly chosen field, the number of DAPI stained nuclei was counted and used as total cell number. The number of TRITC or FITC positive cells was counted and used as the number of antigen positive cells. The total cell numbers and antigen positive cell numbers in all five fields were added and the ratio between the antigen positive cell number and total cell number was calculated as the percentage of antigen positive cells.
Each experiment was replicated five times using a different preparation of cells. The percentage of antigen positive cell obtained in each of the five replicated experiments was used for statistic analysis. In data analysis, \( n \) refers to the number times the experiment was replicated.

**Statistical analysis**

An independent t test was performed on data obtained from quantitative RT-PCR of Porf-2 mRNA levels, the AlamarBlue™ assay for cell proliferation, cell cycle analysis, TUNEL and Annexin V assay for apoptosis, Western blot for Bax and p21 expression, p53 luciferase assay for p53 transcriptional activity and ICC for cell differentiation. The Statistical Package for the Social Sciences (SPSS) was used to determine statistical significance. P values less than 0.05 were considered statistically significant.
CHAPTER 3 RESULTS

Creating the Porf-2 knockdown neural stem cell line

*Confirmation of the expression of Porf-2 in C17.2*

Before mouse C17.2 cells were chosen to study the function of Porf-2 in NSCs, the expression of Porf-2 gene was examined using two sets of mouse Porf-2 primers, zw4 and p2, which generated two different products. The PCR product generated using zw4 was 195 bp while the one generated using p2 was 214 bp (Fig. 3.1). Both PCR products were consistent with the predicted sizes. Thus it was verified that Porf-2 mRNA was expressed in C17.2 cells.
Confirmation of Porf-2 expression in C17.2 cells by PCR. **A.** schematic diagram for location of primers on mouse Porf-2 and predicted sizes of PCR products. **B.** Gel images for PCR products.

M: molecular weight marker, DNA Ladder I.
Lane 1: PCR product using Porf-2 specific primer set zw4.
Lane 2: PCR product using Porf-2 specific primer set p2.

**Confirmation of Porf-2 knockdown at both mRNA and protein levels**

Knockdown of Porf-2 was performed by transduction of C17.2 cells with five different Porf-2 shRNA lentiviruses. The expression of Porf-2 in five transduced clones was first detected at the mRNA level by real time PCR. In each individual experiment, the melting curve showed a single peak, which indicated no DNA contamination in the PCR reaction. The efficiency of zw4 (Porf-2 specific primer) and β-actin (reference gene primer) was 91.6% and 104.2% respectively (Fig. 3.2), which are within the acceptable
range of 90-110% for primer efficiency. The $E_{zw4}$ and $E_{β-actin}$ used in the Pfaffl calculation was 1.9 and 2.0 respectively.

![Figure 3.2](image)

**Figure 3.2** Results of primer efficiency tests. **A.** The primer efficiency of zw4 is 91.6%. **B.** The primer efficiency of p2 is 104.2%.

The relative expression of Porf-2 was presented as the ratio of Porf-2 in experimental cells versus control cells. In C17.2 cells transduced by 184, 185, 186, 187 and 188 shRNA, the relative expression level of Porf-2 was 28.5%, 81.7%, 52.9%, 61.7%, and 56.4% respectively. The reduction of Porf-2 mRNA level was statistically significant in four transduced clones, including 184-C17.2, 186-C17.2, 187-C17.2 and 188-C17.2 (Fig. 3.3 A). The 184-C17.2 clone, which exhibited the highest reduction of Porf-2 mRNA, was chosen to confirm reduction of Porf-2 at the protein level. A Western blot showed the level of Porf-2 protein in 184-C17.2 was only 29.6 % of that in control
C17.2, which further proved the efficient knockdown of Porf-2 (Fig. 3.3 B). Thus, the 184-C17.2 cell clone was used to generate Porf-2 knockdown cells for all later experiments. The C17.2 transduced with blank-pLKO.1 (pLKO.1-C17.2) was used as control.

Figure 3.3 Confirmation of Porf-2 knockdown. A. Quantitative PCR analysis of Porf-2 mRNA expression in Porf-2 shRNA transduced C17.2 cells. The relative expression levels of Porf-2 were represented by the ratio of Porf-2 in experimental cells versus control cells (pLKO.1-C17.2). There was significant reduction of Porf-2 mRNA in 184-C17.2, 186-C17.2, 187-C17.2 and 188-C17.2. β-actin was used as internal control. The results were compared by the independent t test. * p < 0.05, n=9. Error bars indicate SD of the mean. B. Western blot analysis of Porf-2 protein expression level in 184-C17.2 cells showed obvious reduction compared to control. β-actin was used as loading control.
Knockdown of Porf-2 potentiates cell proliferation in C17.2 cells.

To evaluate the effects of Porf-2 on cell proliferation, Porf-2 knockdown cells and control cells were analyzed by the Alamar Blue assay every two days. In three different conditions, Porf-2 knockdown cells showed consistently higher proliferative activities than control cells (Fig. 3.4). When cultured in DMEM with 10% FBS plus 5% horse serum, Porf-2 knockdown C17.2 increased cell proliferation which was 17.2% more in two days and 28.8% more in four days than control cells. When cultured in DMEM with 10% FBS, Porf-2 knockdown C17.2 increased cell proliferation which was 2.64% more in two days and 6.01% more in four days than control cells. When cultured in DMEM with 5% FBS, Porf-2 knockdown C17.2 increased cell proliferation which was 1.01% more in two days and 3.96% more in four days than control cells. It seems that the richer the culture media, the bigger difference in proliferating activities existed between the Porf-2 knockdown cells and control cells. These data indicate that Porf-2 inhibits cell proliferation in C17.2 cells. The more favorable condition the cells are in, the stronger inhibitory role Porf-2 plays.
Figure 3.4 Cell proliferation detected in Porf-2 knockdown cells and control cells in different culture media. Figures in left panel show the observed degree of cell proliferation on 0, 2 and 4th days. Figures in right panel show the increase of cell proliferation in two days and four days. The results were compared by the independent t test. * $p < 0.05$, n=32. All values are mean ± SEM.
Knockdown of Porf-2 facilitates cell cycle transition from G1 to S phase.

To further investigate how the knockdown of the Porf-2 gene potentiates cell proliferation in C17.2 cells, the cell cycle stages in which Porf-2 exerts its growth inhibitory function were examined. To measure this, cell cycle distribution in Porf-2 knockdown cells and control cells was analyzed with flow cytometry after staining of DNA content by propidium iodide (PI). The distribution of cells in different stages of the cell cycle was changed by knockdown of Porf-2 (Fig 3.5). In the Porf-2 knockdown group, 41.8% of cells were in the G1 phase, 45.3% of cells were in the S phase, and 12.9% of cells were in the G2/M phase. In the control group, 51.1% of cells were in the G1 phase, 37.0% of cells were in the S phase, and 11.9% of cells were in the G2/M phase. Porf-2 knockdown cells showed a smaller percentage of cells in G1 phase and a larger percentage of cells in S phase compared to control cells. There was no significant difference in cells distributed in G2/M phase between Porf-2 knockdown cell and control cells. Thus, knockdown of Porf-2 resulted in a significant decrease of G1 population and a significant increase of S population. This result suggests that knockdown of Porf-2 expedites a rapid transition of G1 to S of the cell cycle and that Porf-2 plays a role in causing G1 cycle arrest.
Figure 3.5 Effect of Porf-2 knockdown on cell cycle. **A.** There was a smaller population in the G1 phase and a larger population in the S phase for Porf-2 knockdown cells compared to control cells. The results were compared by the independent t test. * p < 0.05, n=5 for all groups. Error bars indicate SD of mean. **B.** shows a representative data plot for control cells from one of five experiments. **C.** shows a representative data plot for Porf-2 knockdown cells from one of five experiments.

Knockdown of Porf-2 inhibits drug induced apoptosis in C17.2 cells.

To clarify the influence of Porf-2 on cell apoptosis, the Annexin V and TUNEL assays were performed to detect apoptosis in Porf-2 knockdown cells and control cells. Two different kinds of reagents, bleomycin and STS, were used respectively to induce apoptosis. Bleomycin and STS initiate apoptosis in C17.2 by different mechanisms (Geng et al. 2007). Bleomycin is a genotoxin, which initiates cell apoptosis by damaging DNA in cells. STS is a broad spectrum protein kinase inhibitor, which prevents ATP from binding to the kinase catalytic domain.

Apoptosis induced by bleomycin

According to the results obtained from Annexin V assay, the percentage of early stage apoptosis, late stage apoptosis and total apoptosis in Porf-2 knockdown cells was 20.4%, 7.80% and 28.2% respectively. The percentage of early stage apoptosis, late stage apoptosis and total apoptosis in control cells was 30.5%, 11.1% and 41.6% respectively.
Thus, knockdown of Porf-2 resulted in a significant reduction of apoptosis (19.4%) in response to 0.01 U/ml bleomycin treatment (Fig. 3.6). The TUNEL assay showed that the percentage of apoptosis in Porf-2 knockdown cells and control cells was 51.2% and 66.5% respectively. There was a significant decrease of apoptosis (15.2%) in response to 0.01 U/ml bleomycin treatment by knockdown of the Porf-2 gene (Fig. 3.7). The data obtained from Annexin V assay and from TUNEL assay were in agreement with each other. They indicate that knockdown of Porf-2 inhibits bleomycin induced apoptosis in C17.2 cells. Thus, Porf-2 enhances bleomycin induced apoptosis in NSCs.
Figure 3.6 Porf-2 increases bleomycin induced cell apoptosis detected by the Annexin V assay. A. The percentage of apoptosis in Porf-2 knockdown cells (184-C17.2) was significantly lower than in control cells (pLKO.1-C17.2) in response to 0.01 U/ml bleomycin treatment. The results were compared by the independent t test. * $p < 0.05$, n=3 for all groups. Error bars indicate SD of mean. B. shows a representative data plot for control cells from one of three experiments. C. shows a representative data plot for Porf-2 knockdown cells from one of three experiments.

Quad= quadrant. UL= upper left. UR= upper right. LL= lower left. LR= lower right.
LL, LR, UR and UL represent nonapoptotic cells, early stage apoptotic cells, later stage apoptotic cells and dead or necrotic cells respectively.
Figure 3.7 Porf-2 increases bleomycin induced cell apoptosis measured by the TUNEL assay. A. The percentage of apoptosis in Porf-2 knockdown cells (184-C17.2) was significantly lower (15.2%) than control cells (pLKO.1-C17.2) in response to 0.01 U/ml bleomycin. The results were compared by the independent t test. * \( p < 0.05 \), n=5. Error bars indicate SD of mean. B. shows a representative data plot for control cells from one of five experiments. C. shows a representative data plot for Porf-2 knockdown cells from one of five experiments. The M1 region represents nonapoptotic cells. The M2 region represents apoptotic cells. The shaded regions under the curve in B and C represent bleomycin treated cells, while the unshaded curve represents cell distribution before induction of apoptosis.
Apoptosis induced by STS

According to the results obtained from the Annexin V assay, the percentage of early stage apoptosis, late stage apoptosis and total apoptosis in Porf-2 knockdown cells treated with STS was 13.09%, 6.64% and 19.74% respectively. The percentage of early stage apoptosis, late stage apoptosis and total apoptosis in control cells was 21.46%, 9.68% and 31.14% respectively. Thus, knockdown of Porf-2 resulted in a significant reduction of apoptosis (11.4%) in response to 100 nM STS treatment (Fig. 3.8). The TUNEL assay showed that the percentage of apoptosis in Porf-2 knockdown cells and control cells was 37.6% and 43.7% respectively. There was a significant decrease of apoptosis (6.1%) in response to 100 nM STS treatment by knockdown of the Porf-2 gene (Fig. 3.9). The data obtained from the Annexin V and TUNEL assays are consistent with each other. They suggest that knockdown of Porf-2 inhibits STS induced apoptosis in C17.2 cells. Thus, Porf-2 promotes apoptosis induced by STS in NSCs.
Figure 3.8 Porf-2 increases STS induced cell apoptosis detected by the Annexin V assay. 
A. The percentage of apoptosis in Porf-2 knockdown cells (184-C17.2) was significantly lower than in control cells (pLKO.1-C17.2) in response to 100 nM STS treatment. The results were compared by the independent t test. * $p < 0.05$, n=5 for all groups. Error bars indicate SD of mean. B. shows a representative data plot for control cells from one of five experiments. C. shows a representative data plot for Porf-2 knockdown cells from one of five experiments.
Quad= quadrant. UL= upper left. UR= upper right. LL= lower left. LR= lower right. LL, LR, UR and UL represent nonapoptotic cells, early stage apoptotic cells, later stage apoptotic cells and dead or necrotic cells respectively.
Figure 3.9 Porf-2 increases STS induced cell apoptosis measured by the TUNEL assay. A. the percentage of apoptosis in Porf-2 knockdown cells (184-C17.2) was significantly lower (6.1%) than control cells (pLKO.1-C17.2) in response to 100 nM STS treatment. The results were compared by the independent t test. * \( p < 0.05 \), n=10. Error bars indicate SD of mean. B. shows a representative data plot for control cells from one of ten experiments. C. shows a representative data plot for Porf-2 knockdown cells from one of ten experiments. The M1 region represents nonapoptotic cells. The M2 region represents apoptotic cells. The shaded regions under the curve in B and C represent STS treated cells, while the unshaded curve represents cell distribution before induction of apoptosis.
Knockdown of Porf-2 results in differential expression of p21 and Bax

To determine how Porf-2 expression affects cell apoptosis, several important factors involved in apoptotic signaling pathways of C17.2, including p53, p21 and Bax (Geng et al. 2007) were quantified.

Expression of p53

Western blot analysis did not reveal the expression of p53 in C17.2 cells. While there was an intense band of p53 shown for the positive control, MCF7 cells, there was nothing detected by p53 specific antibody for C17.2 cells indicating that the expression level of p53 was very low in C17.2 cells. A more sensitive method, the p53 luciferase assay, was therefore applied later to detect p53 transcription factor activity.

Figure 3.10 Western blot analysis of p53 in untreated C17.2 cells and C17.2 treated with either STS or bleomysin. p53 protein was not detected in C17.2 cells. The positive control MCF7, a breast cancer cell line, showed a 53 KDa band with strong intensity.
Expression of Bax

When the C17.2 NSCs were cultured under normal conditions without any treatment, Bax was rarely expressed in either Porf-2 knockdown cells or control cells. The exposure of cells to STS for 4 h enhanced the expression of Bax but there was no significant difference in the enhancement of Bax expression between Porf-2 knockdown cells and control cells in response to STS treatment (Fig 3.11). After exposure to 0.01 U/ml bleomycin for 24 h, Bax was dramatically increased in both Porf-2 knockdown cells and control cells. In this case, however, the increment of Bax expression in Porf-2 knockdown cells was significantly less than that observed in control cells. These data indicate that expression of Bax was stimulated by both bleomycin treatment and STS treatment, but knockdown of Porf-2 only inhibited the enhancement of Bax expression in response to bleomycin.

Figure 3.11 Western blot analysis of Bax in untreated C17.2 cells and C17.2 treated with either STS or bleomycin. Bax expression was enhanced by both STS treatment and bleomycin treatment. The enhancement of Bax expression was lower in Porf-2 knockdown cells than in control cells in response to bleomycin. * $p < 0.05$, n=3 for all groups. Error bars indicate SD of mean.
Expression of p21

Under normal untreated culture conditions, the expression of p21 in Porf-2 knockdown cells was lower than that in control cells. There was no change of p21 expression after STS treatment for 4 h; it was again higher in control than in Porf-2 knockdown cells. Exposure of cells to 0.01 U/ml bleomycin for 24 h resulted in elevation of p21 expression in both Porf-2 knockdown cells and control cells. The p21 expression in Porf-2 knockdown cells was still lower than that observed in control cells after bleomycin treatment (Fig. 3.12). These data indicate that bleomycin but not STS stimulates the expression of p21 in C17.2 cells and Porf-2 enhances the expression of p21 in C17.2 cells under normal untreated culture conditions, in STS induced apoptosis and in bleomycin induced apoptosis.

**Figure 3.12** Western blot analysis of p21 in untreated C17.2 cells and C17.2 treated with either STS or bleomycin. P21 expression was lower in Porf-2 knockdown cells than control cells under normal untreated conditions, STS induced apoptosis and bleomycin induced apoptosis. Bleomycin treatment stimulated the expression of p21. * p < 0.05, n=3 for all groups. Error bars indicate SD of mean.
Knockdown of Porf-2 inhibits the enhancement of p53 transcriptional activity in response to bleomycin treatment

Since the expression p53 in C17.2 cells was too low to be determined by Western Blot, a p53 luciferase assay, a more sensitive method, was used to detect the transcriptional activity of p53. When C17.2 cells were cultured under normal conditions without any treatment, there was no difference in transcriptional activity of p53 between Porf-2 knockdown cells and control cells. Exposure of cells to STS for 4 h did not change the transcriptional activity of p53 in either Porf-2 knockdown cells or control cells (Fig. 3.13). However, exposure of cells to 0.01 U/ml bleomycin for 24 h led to the enhancement of p53 transcriptional activity in both Porf-2 knockdown cells and control cells. The enhancement was 34.9% in Porf-2 knockdown cells and 99.2% in control cells. The elevation of p53 transcriptional activity in Porf-2 knockdown cells was significantly less than observed in control cells. These data indicate that bleomycin treatment but not STS stimulates the transcriptional activity of p53 in C17.2 and knockdown of Porf-2 inhibits the elevation of p53 transcriptional activity in response to bleomycin treatment. Thus, Porf-2 could potentiate the enhancement of p53 transcriptional activity due to DNA damage.
Figure 3.13 p53 luciferase assay in untreated C17.2 cells and C17.2 treated with STS or bleomycin. Bleomycin treatment resulted in significant increase of p53 transcription activity in both Porf-2 knockdown cells and control cells. Knockdown of the Porf-2 gene inhibited elevation of p53 transcription activity in response to bleomycin treatment. The results were compared by the independent t test. * p < 0.05, n=3 for all groups. Error bars indicate SD of mean.

Knockdown of Porf-2 shows no influence on C17.2 differentiation

Another potential function of growth regulatory factors in NSC is potentiating differentiation into specific cell types in the CNS. Thus cellular differentiation was monitored in Porf-2 knockdown cells and control cells by morphological observation and immunocytochemistry (ICC).

Morphological observation

Cell morphology is an important indicator of cell differentiation. Before induction of differentiation by serum starvation, Porf-2 knockdown C17.2 and control C17.2 showed similar morphology. They were polygonal flat cells containing prominent nuclei and several cytoplasmic extensions. After induction of differentiation, both Porf-2 knockdown C17.2 and control C17.2 cells lost their uniform shape and even distribution.
They formed clusters of cells with condensed soma and nuclei. Some cell bodies were elongated and became spindle shape. There was no obvious difference in morphology before and after induction of differentiation between Porf-2 knockdown cells and control cells (Fig. 3.14).

**Figure 3.14** Morphological observations of Porf-2 knockdown C17.2 and control C17.2 cells before and after induction of differentiation by serum starvation. **A.** Control C17.2 cells before induction of differentiation. **B.** Control C17.2 cells 6 days after induction of differentiation. **C.** Porf-2 knockdown C17.2 cells before induction of differentiation. **D.** Porf-2 knockdown C17.2 cells 6 days after induction of differentiation. Scale bar = 500 µm.
Immunocytochemistry (ICC)

Expression of cell-type specific markers before induction of differentiation

Prior to induction of differentiation, almost all cells expressed the NSC marker, nestin, in both Porf-2 knockdown C17.2 and control C17.2 groups (Fig. 3.15). No cells expressed differentiated markers, including β-tubulin III, GFAP and A2B5 (Fig. 3.16, Fig. 3.17, Fig. 3.18).
Figure 3.15 Expression of nestin in control and Porf-2 knockdown C17.2 cells before induction of differentiation. All control C17.2 and Porf-2 knockdown C17.2 cells expressed nestin before induction of differentiation. **A.** DAPI staining for nuclei in control C17.2 cells. **B.** Anti-nestin staining in control C17.2 cells. **C.** Merge image of A and B images. **D.** DAPI staining in Porf-2 knockdown C17.2 cells. **E.** Anti-nestin staining in Porf-2 knockdown C17.2 cells. **F.** Merge image of D and E images. Scale bar = 100 µm.
Figure 3.16 Expression of β-tubulin III before induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. A. DAPI staining for nuclei in control C17.2 cells. B. Anti-nestin staining in control C17.2 cells. C. Merge image of A and B images. D. DAPI staining in Porf-2 knockdown C17.2 cells. E. Anti-nestin staining in Porf-2 knockdown C17.2 cells. F. Merge image of D and E images. Scale bar = 100 µm.
Figure 3.17 Expression of GFAP before induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. A. DAPI staining for nuclei in control C17.2 cells. B. Anti-GFAP staining in control C17.2 cells. C. Merge image of A and B images. D. DAPI staining in Porf-2 knockdown C17.2 cells. E. Anti-GFAP staining in Porf-2 knockdown C17.2 cells. F. Merge image of D and E images. Scale bar = 100 µm.
Figure 3.18 Expression of A2B5 before induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. A. DAPI staining for nuclei in control C17.2 cells. B. Anti-A2B5 staining in control C17.2 cells. C. Merge image of A and B images. D. DAPI staining in Porf-2 knockdown C17.2 cells. E. Anti-A2B5 staining in Porf-2 knockdown C17.2 cells. F. Merge image of D and E images. Scale bar = 100 µm.
Expression of cell type specific markers after induction of differentiation for 6 days

Differentiation was induced by serum starvation for 6 days. Then the cell types were characterized by ICC. There were very few cells expressing nestin in both Porf-2 knockdown C17.2 and control C17.2 (Fig. 3.19). A few cells expressed the neuronal marker β-tubulin III (Fig. 3.20). A greater number of cells expressed GFAP, a marker for type II astrocytes (Fig. 3.21). The majority of cells expressed A2B5, a marker for general glial precursors (Fig. 3.22). The proportions of neuron, glial precursor and type II astrocyte are listed in Table 3.1. There was no significant difference between Porf-2 knockdown C17.2 and control C17.2. These data indicate that Porf-2 does not play a role in the direction of C17.2 differentiation when induced by serum starvation.

### Table 3.1 Proportion of different cell types detected after induction of differentiation

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Control C17.2</th>
<th>Porf-2 knockdown C17.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>neurons</td>
<td>8% ± 3%</td>
<td>8% ± 2%</td>
</tr>
<tr>
<td>Type II astrocyte</td>
<td>21% ± 9%</td>
<td>22% ± 6%</td>
</tr>
<tr>
<td>Glial precursor</td>
<td>75% ± 12%</td>
<td>78% ± 17%</td>
</tr>
</tbody>
</table>

Note: There was no significant difference between Porf-2 knockdown C17.2 and control C17.2 for each type of cell. $P < 0.05$. n = 5
Figure 3.19 Expression of nestin after induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. **A.** DAPI staining for nuclei in control C17.2 cells. **B.** Anti-nestin staining in control C17.2 cells. **C.** Merge image of A and B images. **D.** DAPI staining in Porf-2 knockdown C17.2 cells. **E.** Anti-nestin staining in Porf-2 knockdown C17.2 cells. **F.** Merge image of D and E images. Scale bar = 100 µm.
Figure 3.20 Expression of β-tubulin III after induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. After induction of differentiation, 8% of control C17.2 and Porf-2 knockdown C17.2 cells expressed β-tubulin III. A. DAPI staining for nuclei in control C17.2 cells. B. Anti β-tubulin III staining in control C17.2 cells. C. Merge image of A and B images. D. DAPI staining in Porf-2 knockdown C17.2 cells. E. Anti β-tubulin III staining in Porf-2 knockdown C17.2 cells. F. Merge image of D and E images. Scale bar = 100 µm.
Figure 3.21 Expression of GFAP after induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. After induction of differentiation, 21% of control C17.2 and 22% of Porf-2 knockdown C17.2 cells expressed GFAP. A. DAPI staining for nuclei in control C17.2 cells. B. Anti-GFAP staining in control C17.2 cells. C. Merge image of A and B images. D. DAPI staining in Porf-2 knockdown C17.2 cells. E. Anti-GFAP staining in Porf-2 knockdown C17.2 cells. F. Merge image of D and E images. Scale bar = 100 µm.
Figure 3.22 Expression of A2B5 after induction of differentiation in control C17.2 and Porf-2 knockdown C17.2 cells. After induction of differentiation, 75% of control C17.2 and 78% of Porf-2 knockdown C17.2 cells expressed A2B5. 

A. DAPI staining for nuclei in control C17.2 cells.

B. Anti-A2B5 staining in control C17.2 cells.

C. Merge image of A and B images.

D. DAPI staining in Porf-2 knockdown C17.2 cells.

E. Anti-A2B5 staining in Porf-2 knockdown C17.2 cells.

F. Merge image of D and E images. Scale bar = 100 μm.
CHAPTER 4 DISCUSSION

Understanding the regulation of neural stem cell proliferation, apoptosis and differentiation will contribute to our understanding of normal brain development, as well as pathophysiology and response to diseases. It will also help to engineer stem cell based therapies to optimize risk/benefit ratios. Mouse C17.2 cells are NSCs widely used in therapeutic transplantation in CNS diseases. They can integrate in compromised CNS mouse models and differentiate into neurons and glial cells (Snyder et al. 2004). When they were transplanted into injured spinal cords of rats, the behavioral outcome was improved (Teng et al. 2002). When they were engrafted into murine cortical and cerebellar defects, they replaced compromised neurons (Richards et al. 1992; Rosario et al. 1997). In a Parkinson’s disease model of aged mice, C17.2 cell not only resulted in nigral reconstitution but also rescued the dysfunctional host neurons (Ourednik et al. 2002). However, when C17.2 cells were transplanted to repair peripheral nerves in rats, there was a high rate of tumor formation (Johnson et al. 2008). It is likely that the tumorigenesis that occurred in transplantation can be prevented by strictly controlling cell growth. Therefore, it is important to clarify the regulatory mechanisms of C17.2 cell growth.

In the present study, the growth regulatory function of Porf-2 was examined in C17.2 cells. Porf-2 was found to inhibit the cell proliferation of C17.2. This result is consistent with previous data that Porf-2 suppressed cell growth of NIH3T3 cells under favorable conditions (Nowak and Gilham 2004). The present study also showed that, under baseline culture conditions, Porf-2 enhanced the expression of p21 independent of
p53 transcriptional activity and slowed the G1 to S cell cycle progression. These observations suggest the mechanism by which Porf-2 inhibits proliferation of C17.2 cells. The rate of cell proliferation is determined by cell cycle progression, which is modulated by several regulators. P21 is one of them. P21 is a cyclin-dependent kinase (CDK) inhibitor which causes G1 cell cycle arrest by inhibiting CDKs required for transition from the G1 to the S phase (Harper et al. 1993; El-Deiry et al. 1993). P21 also inhibits DNA replication by interacting with proliferation-cell nuclear antigen (PCNA), an accessory factor for DNA polymerase δ and ε (Waga et al. 1994). In the event of DNA damage, the expression of p21 is up-regulated by the transcriptional activity of p53 (El-Deify et al. 1994; Di Leonardo et al. 1994). Therefore, p21 is always considered a part of the p53 mediated cell cycle arrest pathway. However, the level of p21 expression does not completely depend on p53. In some situations, such as normal tissue development, the regulation of p21 expression is independent of p53 (Macleod et al. 1995). Thus, it is not surprising to find that in normal culture media, Porf-2 up-regulated the expression of p21 in untreated C17.2 cells without changing p53 transcriptional activity. The elevated p21 expression caused by Porf-2 could account for the G1 cell cycle arrest and the consequent growth inhibitory function of Porf-2.

When the effects of Porf-2 on C17.2 cell apoptosis were investigated, two more intriguing findings were obtained. First, there are two different apoptotic pathways that can be stimulated in C17.2 cells, a p53 transcription dependent pathway and a p53 transcription independent pathway. This result is in agreement with previous publications, which showed that genotoxin increased p53 nuclear immunoreactivity and
upregulated the expression of a p53 transcriptionally regulated gene PUMA to induce apoptosis of C17.2 cells, while STS triggered p53 cytoplasmic accumulation and induced C17.2 apoptosis without requiring PUMA expression (Akhtar et al. 2006; Geng et al. 2007). Second, Porf-2 has pro-apoptotic effects through both p53 transcription dependent and independent pathways.

Since previous studies had shown that the extrinsic apoptotic pathway, which involves death receptors, did not operate in NSCs (Ceccatelli et al. 2004), the effect of Porf-2 was only investigated in mitochondrial apoptosis in C17.2. When bleomycin was used to treat C17.2 cells, the transcriptional activity of p53 increased and the expression levels of p21 and Bax were up-regulated.

P53 is a transcription factor that responds to aberrant cell growth by inducing cell cycle arrest or apoptosis (Gottlieb and Oren 1996; Ko and Prives 1996; Levine 1997; Jin and Levine 2001). In unstressed cells, p53 is expressed at low levels, degraded shortly after expression and kept in a latent form which needs modification to be activated. When the cells are exposed to certain stresses, the level and/or functional activity of p53 can dramatically increase. The active p53 performs sequence specific transactivation of several components in apoptotic pathways. P21 and Bax are two primary p53-responsive genes. While the upregulation of p21 tends to cause cell cycle arrest followed by repair of damaged DNA, the upregulation of Bax tends to induce apoptosis by forming homodimers to release cytochrome c from the mitochondria and activate caspase-9 (Skulachev 1998; Adam and Cory 1998). P21 and Bax coordinate with other p53
effectors to determine the cellular fate, survival or death (Amundson et al. 1998) (Fig 4.1).

Figure 4.1 Flow diagram of p53 triggered apoptosis pathways. (Adapted from Gillham et al. 2007) The balance between two primary p53-responsive genes, p21 and Bax determine the cellular fate, survival or death.

Bleomycin is a genotoxin causing DNA damage in cells. This type of damage has been shown to be a p53 responsive stress (Kastan et al. 1991; Nelson and Kastan 1994). Thus, it is reasonable to see that bleomycin induced C17.2 apoptosis is dependent on the enhancement of p53 transcriptional activity followed by the elevated expression of p21 and Bax.
When STS was used to treat C17.2 cells, there was no change in either the transcriptional activity of p53 or the expression of p21 but only elevation of Bax expression. A similar phenomenon was observed in NG108-15, a hybrid cell of mouse neuroblastoma and rat glioma (Zhang et al. 2003). STS is a potent, broad-spectrum kinase inhibitor (Rüegg et al. 1989). Although it is extensively used to induce apoptosis in different cell types, the mechanism involved is poorly understood. Results of this research imply that STS induced-C17.2 apoptosis is independent of p53 transcriptional activity but dependent on elevated Bax protein. P53 is the main, but not exclusive, modulator of Bax protein levels. Several other proteins, such as c-myc, insulin-like growth factor-binding protein-3 and Bcl-2, are regulators of Bax protein levels (Miyashita et al. 1995; Mitchell et al. 2000; Butt et al. 2000). It is possible that STS stimulates the expression of Bax by affecting one or more of these other regulators or some other unknown regulators. The mechanism by which STS induces elevation of Bax protein still needs to be clarified.

The pro-apoptotic role of Porf-2 presented in the two different apoptotic pathways occurs through different mechanisms. In the p53 transcription dependent pathway induced by bleomycin, Porf-2 enhances apoptosis by elevating p53 transcriptional activity and Bax expression level in response to DNA damage. With STS treatment, although the expression level of Bax was enhanced, there was no difference in the elevated Bax expression level between Porf-2 knockdown C17.2 cells and control C17.2 cells. Therefore, in p53 transcription independent pathway induced by STS, Porf-2 enhances apoptosis without influencing Bax expression. Several mechanistic possibilities
might account for this phenomenon. Without changing the Bax level, Porf-2 could facilitate homodimerization and translocation of Bax into the mitochondrial membrane to promote apoptosis. Alternatively, Porf-2 could bypass Bax and directly act on the downstream effectors, such as caspases, to increase apoptosis. Since a caspase-independent pathway was also demonstrated to be involved in STS-induced apoptosis in addition to a mitochondrial pathway (Belmokhtar et al. 2001; Zhang et al. 2004), it is also possible that Porf-2 executes pro-apoptotic function through a caspase-independent pathway rather than the mitochondrial pathway in response to STS treatment. To determine the exact mechanism of the pro-apoptotic role of Porf-2 in p53 transcription independent apoptosis, further studies are required.

Sequence analysis shows that Porf-2 contains a conserved RhoGAP domain, indicating Porf-2 is a putative Rho GAP. Rho GAPs are inactivators of Rho GTPases, which regulate diverse cellular events, including cell cycle progression and apoptosis (Etienne-Manneville and Hall 2002). Rho GTPases, including Rho, Rac and Cdc42, exhibit their essential role in stimulating cell cycle progression through G1 in fibroblasts and epithelial cells (Olson et al. 1995). Although the signaling pathways involved are not clear, two roles of Rho, including inhibition of p21 expression and induction of cyclin D1 expression in mid-G1, have been demonstrated (Olson et al. 1998; Welsh et al. 2001). In addition, Rac is also implicated in regulating cyclin D1 expression to control G1 cell cycle progression (Mettouchi et al. 2001). Presumably, as the inactivator of Rho GTPases, Rho GAP could slow down the cell cycle by upregulating p21 expression or downregulating cyclin D1 expression. This hypothesis is already supported by some
evidence. Some Rho GAPs show the role of suppressing cell proliferation (Ching et al. 2003; Modarressi et al. 2004). Human chromosome 13q12 encoded RhoGAP demonstrates a p21 involved cell proliferation inhibitory role in cancer cells (Nagaraja and Kandpal 2004). The cell growth inhibitory role of Porf-2 and its related mechanism discovered in the present study are similar to these known Rho GAPs. This strengthens the idea that Porf-2 is a novel Rho GAP and also supplies the first evidence for the function of a RhoGAP domain-containing protein in NSCs.

In addition to their role in cell cycle progression, Rho GTPases also interfere with the apoptotic process. Rac1 protects tumor cell lines or transformed fibroblasts from apoptosis (Joneson and Bar-Sagi 1999; Pervaiz et al. 2001; Coniglio et al. 2001). Rac2 activates the pro-survival kinase Akt in mast cells (Yang et al. 2000). Rho prevents p53-dependent apoptosis (Costello et al. 2000). As the inactivator of Rho GTPases, it is consistent to observe that Rho GAP induces apoptosis (Modarressi 2004). In parallel with the above observations, the pro-apoptotic role of Porf-2 was established in our study, which further indicates Porf-2 functions as a Rho GAP in NSCs.

One of the main and earliest known functions of RhoGTPases and their regulators is controlling organization of the actin cytoskeleton (Mackay and Hall 1998; Kaibuchi et al. 1999). Presumably, any cellular processes driven by filamentous actin dynamics could be regulated by RhoGAPs. P190RhoGAP is implicated in regulating oligodendrocyte differentiation and neuronal differentiation, both of which are processes that include actin fiber assembly (Wolf et al. 2001; Brouns et al. 2001). Other RhoGAPs, such as neurite outgrowth multiadaptor RhoGAP (NOMA-GAP) and GTPase regulator interacting with
TrkA (Grit), also function in the regulation of neuronal differentiation by promoting neurite outgrowth and extension (Nakamura et al. 2002; Rosario et al. 2007). Based on these facts, it was hypothesized that Porf-2 might impact C17.2 differentiation. However, no influence of Porf-2 on C17.2 differentiation was demonstrated. After induction of differentiation by serum starvation, the proportion of different cell types including neurons, common glial precursors and type II astrocytes in C17.2 was not changed by knockdown of Porf-2. Thus, Porf-2 does not play a role in determining directions of in vitro differentiation of C17.2 cells when this differentiation is induced by serum starvation.

Taken together, the results show that the RhoGAP domain-containing protein, Porf-2, exhibits no effect on the in vitro differentiation of C17.2 cells. It functions as a growth inhibitor by modulating p21 expression independent of p53 transcriptional activity in NSCs. Porf-2 also plays a pro-apoptotic role through both p53 transcription dependent and independent pathways in NSCs. Although the specific GTPase substrates for Porf-2 are not known yet, it is reasonable to speculate that the multiple functions of Porf-2 are realized by Porf-2 inactivating certain GTPases. To clarify the GTPases regulated by Porf-2 and the mechanism by which Porf-2 promotes apoptosis independent of P53, further studies are required. This research provides the first evidence for the function of a RhoGAP-containing protein and its mechanism of action in C17.2 NSCs.
CHAPTER 5 CONCLUSIONS AND FUTURE STUDIES

Three conclusions were drawn from the present study. First, Porf-2 plays a growth-inhibitory role in C17.2 neural stem cells. This function is independent of p53 transcriptional activity as this is the same in untreated control and Porf-2 knockdown cells. This growth-inhibitory role is obtained through enhancement of p21 expression and subsequent G1 cell cycle arrest caused by Porf-2. Second, Porf-2 enhances both bleomycin-induced and STS-induced C17.2 cell apoptosis. In bleomycin-induced apoptosis, Porf-2 potentiates the elevation of p53 transcriptional activity and consequent elevated expression of Bax and p21, which are the results of bleomycin treatment. Thus the pro-apoptotic role of Porf-2 in bleomycin-induced apoptosis is dependent on p53 transcriptional activity. In STS-induced apoptosis, p53 transcriptional activity and expression of p21 do not change, but Bax expression is elevated. There is no difference in the elevated Bax expression level between Porf-2 knockdown C17.2 cells and control C17.2 cells. Thus, the pro-apoptotic role of Porf-2 in STS-induced apoptosis is neither dependent on p53 transcriptional activity nor on the Bax expression level (Fig. 5.1). Third, Porf-2 does not influence the relative distribution of cell types after C17.2 differentiation by serum starvation.

In the future, more studies need to be done to clarify the p53 transcription-independent pathway by which Porf-2 enhances apoptosis in response to STS and to identify the GTPases regulated by Porf-2. In addition, in vitro functional studies should be extended to cortical and hippocampal primary neurons to determine the function of Porf-2 in different cell types. Finally, functional studies can be performed in vivo by
establishing a Porf-2 knockout mouse model and observing the changes in gross anatomy and histology of the CNS and in animal development and behavior.

**Figure 5.1** Schematic diagram of mechanisms by which Porf-2 inhibits cell proliferation and enhances drug induced apoptosis in NSCs. Porf-2 inhibits cell proliferation by enhancing p21 expression and causing cell cycle arrest. Porf-2 enhances drug induced apoptosis through both p53 transcription dependent and independent pathways.
REFERENCES


APPENDIX A: SOLUTIONS

Note: All pH adjustments were made with NaOH or HCl.

30% Acrylamide/Bis-Acrylamide (29.2: 0.8) 

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
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<td>Acrylamide</td>
<td>29.2 g</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>0.8 g</td>
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</table>

Filter and store at -4ºC

10% Ammonium Persulfate (APS)

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<td>Ammonium persulfate</td>
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Blocking buffer (for Western blot)

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Borate buffer (for coating plates)

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<td>Boric acid</td>
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<tr>
<td>Borax</td>
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pH to 8.2 and store at -4ºC

DMEM (for C17.2 cells)

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<td>DMEM (sigma D5030)</td>
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<td>D-Glucose</td>
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<tr>
<td>L-glutamine</td>
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<tr>
<td>NaHCO₃</td>
<td>3.7 g</td>
</tr>
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pH to 7.0-7.4, pass through 0.22 µm filter
### FT LYSIS BUFFER

**Base solution:**
- KCl: 20 ml 3 M KCl
- Tris-HCl (pH 7.8): 2 ml 1 M Tris-HCl (pH 7.8)
- Glycerol: 20 ml glycerol

To 160 µl of base solution, add fresh each time:
- Pefabloc: 1.6 µl of 25 mg/ml (0.1M) stock
- Leupeptin: 1.6 µl of 1 mg/ml stock
- Pepstatin: 3 µl of 0.52 mg/ml stock
- Aprotinin: 0.5 µl of 1.666mg/ml stock

### 6X Loading Dye

- Glycerol: 30 mL
- Bromophenol Blue: 0.5 g
- Xylene Cyanol: 0.5 g

### 1X PBS

- NaCl: 4 g
- KCl: 0.1 g
- Na$_2$PO$_4$: 0.72 g
- KH$_2$PO$_4$: 0.12 g
- pH to 7.5

### Resolving gel buffer (1.5M Tris-HCl, pH 8.8)

- Tris base: 18.15 g
- pH to 8.8

### Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

- Tris base: 6 g
- pH to 6.8

### 10% SDS

- SDS: 10 g
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<td>Tris-HCl</td>
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<td>yeast extract</td>
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