A Study On The Transcription Factor Brn-3b, The Cell Cycle Regulation and The Cause Of Elevated Brn-3b Expression in Cancers

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Contents

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List of Abbreviations

5-FU	5-fluorouracil
APS	Ammonium persulphate
Bap	Benzo(a)pyrene
BRCA-1	Breast cancer-1
Brn-3b(l)	Brn-3b long form
Brn-3b(s)	Brn-3b short form
cDNA	Complementary deoxyribonucleic acid
CDK	Cyclin-dependent kinase
COL	Colchicine
DEPC	Diethylpyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Doxyribonucleic acid
DNase I	Deoxyribonuclease I
dNTP	A mixture containing 2'-deoxyadenosine 5'-triphosphate, 2'-deoxycytidine 5'-triphosphate, 2'-deoxyguanosine 5'-triphosphate and 2'-deoxythymidine 5'-triphosphate
DOX	Doxorubicin
ECL	Enhanced chemiluminescence system
ER	Estrogen receptor
ETOP	Etoposide
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum

FGM	Full growth medium
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HBBS	Hank's Balanced Salt Solution
HRP	Horseradish peroxide
HU	Hydroxyurea
MPT	1% non-fat milk and %5 tween-20 in PBS
NS	No serum/Serum free
PBS	Phosphate buffer saline
POU	**P**it-**O**ct-**U**nc
POU_S	POU specific domain
POU_{HD}	POU homeodomain
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
SFM	Serum free medium
TEMED	N,N,N',N'-Tetramethylethylenediamine

Abstract

Cyclin D1, a cell cycle regulator, plays an important role in cell cycle progression. As overexpression of cyclin D1 has been observed in various cancers, it has been considered that high level of cyclin D1 may cause uncontrolled cell proliferation and thus give rise to cancer. Interestingly, we showed that a POU transcription factor Brn-3b, which has been shown to contribute to uncontrolled cell growth and cause tumourigenesis when expressed at a high level, correlates to the level of cyclin D1 mRNA--when the level of Brn-3b mRNA increased, the levels of cyclin D1 mRNA increased; when the level of Brn-3b mRNA decreased, the level of cyclin D1 mRNA also decreased. Thus, the strong correlation between Brn-3b and cyclin D1 indicates that Brn-3b, at least in part, contributes to uncontrolled proliferation by regulating the gene expression of cyclin D1.

Chapter one

Introduction

1.1 Gene expression and transcription

Gene expression is of paramount importance as it controls growth, development and the normal function of a cell. Changes in gene expression may affect those events and may contribute to diseases (Alberts et al. 1994; Lewin 2000). Thus, understanding gene expression is the key to understand how a cell is regulated during various processes such as growth and differentiation.

Generally, gene expression involves converting genetic information (genotype) into corresponding characteristics (phenotype). In this process, which is also known as the fundamental dogma, DNA (genotype) is transcribed into RNA and RNA is translated into proteins which produce the corresponding characteristics (phenotype) of an individual. Therefore, the process of transcription, whereby RNA products are produced from DNA, is an essential and crucial element in controlling gene expression (Latchman 1998).

The process of transcription is complex. In eukaryotic cells, initiation of transcription involves the sequential binding of general transcription factors including TF2B, D, E, F, H and RNA polymerase II to a specific DNA sequence called promoter (Alberts et al. 1994;

Lewin 2000). Through this process, the general transcription factors regulate the rate of transcription initiation by either speeding up or slowing down the assembly in response to regulatory signals. Apart from the general transcription factors, there are other transcription factors regulating transcription by activating or repressing the process of transcription (Alberts et al. 1994). The POU transcription factor is such factor acting which regulates gene expression.

1.2 The POU transcription factors

The initial identification of three mammalian transcription factors, the pituitary-specific Pit-1, the octamer-binding proteins Oct-1 and Oct-2, and a nematode factor Unc-86 led to the identification of the POU (**P**it-**O**ct-**U**nc) transcription factor family (Latchman 126-33;Andersen and Rosenfeld 2-35;Samady et al. 317-23). These four transcription factors, as well as other transcription factors discovered subsequently, are characterized by a unique DNA-binding domain which is referred as the POU domain (Andersen and Rosenfeld 2-35;Wegner, Drolet, and Rosenfeld 488-98).

1.2.1 POU domain

The POU domain is a unique bipartite DNA-binding domain (Andersen and Rosenfeld 2-35;Wegner, Drolet, and Rosenfeld 488-98) which consists of approximately 150-160 amino acids (Latchman 126-33). Based on the sequence homology within the domain, it can be further subdivided into two subdomains--the POU-specific domain (POU_S) and the POU homeodomain (POU_{HD}) (Figure 1.1) (Klemm et al. 1994; Wegner et al. 1993).

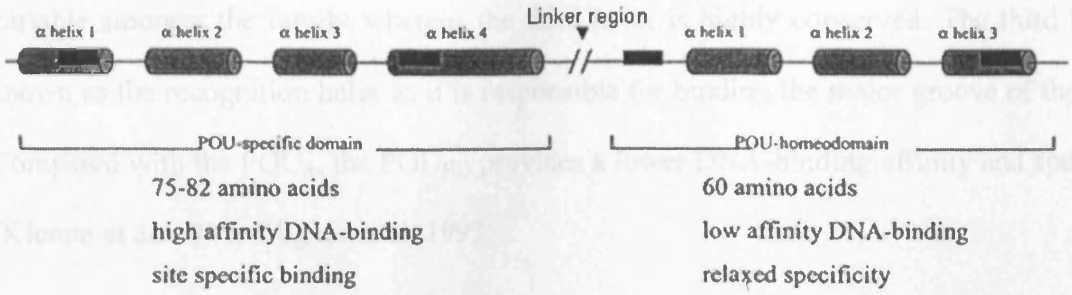

Figure 1.1 schematic representation of the secondary structure elements of the POU domain

The POU domain can be subdivided into the highly conserved POU_S domain and the POU_{HD} separated by a linker region that varies in length (14-25 amino acids). The POU_S domain consists of 4 α-helices (cylinders) while the POU_{HD} consists of 3 α-helices. Conserved regions of basic amino acids are found in both the POU_S and POU_{HD} (indicated by black boxes). (Adapted from Wegner et al. 1993)

1.2.1.1 The POU-specific domain (POU_S)

The POU_S is unique to POU transcription factors. It is a highly conserved amino-terminal sequence of 75-82 amino acids (Figure 1.1). This domain contains four α-helices which are

arranged around a hydrophobic core. This subdomain provides a high affinity site specific DNA-binding property to the POU domain. Helix 3 is responsible for DNA recognition (Klemm et al. 1994; Wegner et al. 1993).

1.2.1.2 The POU homeodomain (POU_{HD})

The POU_{HD} shows an unambiguous similarity to the DNA-binding homeodomain of various developmental regulators in *Drosophila* (Andersen & Rosenfeld 2001). It is a highly conserved carboxyl-terminal sequence of 60 amino acids (Figure 1.1). Similar to the classic homeodomain, the POU_{HD} consists of three α-helices. The first two helices are relatively variable amongst the family whereas the third helix is highly conserved. The third helix is known as the recognition helix as it is responsible for binding the major groove of the DNA. Compared with the POU_S, the POU_{HD} provides a lower DNA-binding affinity and specificity (Klemm et al. 1994; Wegner et al. 1993).

1.2.1.3 Linker region

Apart from the POU_S and POU_{HD}, there is another protein sequence called the linker region within the POU domain (Figure 1.1). The linker region is less conserved amongst the family. This region is responsible to link the POU_S and POU_{HD} and acts as a flexible tether which allows the two sub-domains to bind cooperatively to the DNA (Klemm et al. 1994; Wegner et al. 1993).

1.2.2 Family members of POU transcription factors

The POU domains are classified into six different groups based on the sequence homology of the linker region and the main cluster at the amino-terminus of the POU_{HD} (Andersen and Rosenfeld 2001). Table1.1 provides brief information about all the identified POU transcription factors, however, for the purpose of this thesis, only the class IV mammalian POU domain transcription factor, which is also known as Brn-3, will be discussed.

Class	Factor	Chromosome location	expression	Knock out phenotype
I	Pit-1	Mouse 16	Pituitary	Dwarfism
II	Oct-1	Mouse 1 Human 1	Ubiquitous	Not reported
	Oct-2	Mouse7 Human 19	Nervous system, lymphoid cell, testis and kidney	Aberrant B-cell maturation
III	Brn-1	Mouse 1	CNS, Kidney	Not reported
	Brn-2	Mouse 4 Human 6	CNS	Defects in differentiation/migration of hypothalamic neurons
	Brn-4	Mouse X	CNS	Defects in inner ear development
	Tst-1	Mouse4	Nervous system, testis	Defective myelination
IV	Brn-3a	Mouse 14 Human 13	Nervous system, reproductive system, heart and lungs	Lack of specific sensory and autonomic neurons, non-viable
	Brn-3b	Mouse 8 Human 4	Nervous system, reproductive system and heart	Loss of retinal ganglian cells, blind
	Brn-3c	Mouse 18 Human 5	Nervous system, reproductive system	Loss of inner ear cells, Deaf
V	Oct3/4	Mouse 17 Human 6	Testis, ovaries, germ cells, ES cells	All cells in the early embryo become trophoblasts
VI	Brn-5	Mouse 15 Human 12	Nervous system, skeletal muscle, heart, lung	Not reported

Table 1.1 The six classes of POU transcription factors. The chromosome location in both mouse and humans and expression pattern is indicated. The phenotype of the knock out mouse models, where known, is also briefly discussed. (CNS-central nervous system, ES-embryonic stem cell). (Adapted from Andersen and Rosenfeld 2001).

1.2.2.1 Class IV /Brn-3

Three different proteins have been identified in this group; they are Brn-3a (Brn-3.0), Brn-3b (Brn-3.2) and Brn-3c (Brn-3.1). These members are expressed in both the central and peripheral nervous systems where they regulate neuronal growth and differentiation (Budhram Mahadeo et al. 1996;Milton et al. 1995).

The transcription factors belonging to this group have a short homologous region at the N-terminal and a highly conserved POU domain at the C-terminal of the protein. This short sequence within the N-terminal, which contains about 40 amino acids, is referred as the POU IV box (Figure 1.2)(Samady et al. 317-23;Gerrero et al. 10841-45). The POU IV box and the POU domain are the activation domains of the transcription factor and are able to act on different promoters to regulate transcription (Smith et al. 460-70;Smith et al. 4100-07). A detailed discussion of all three members would be out of the scope of this thesis. For the purpose of the thesis, only Brn-3b is discussed.

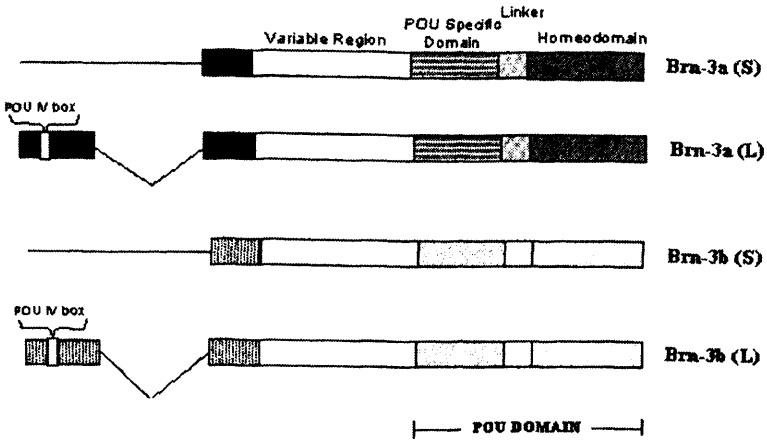

Figure 1.2 Schematic representation of the Brn-3a and Brn-3b transcription factors and their two isoforms. The long isoform [Brn-3a(l) and Brn-3b(l)] of both transcription factors contain an extra N-terminal domain that contains a highly conserved region, the POU IV box. The highly conserved POU IV box is only found in the long isoforms of both proteins while the POU domain is found in both long and short isoforms of each protein.

1.3 The Brn-3b transcription factor

Brn-3b was first detected in ND7 cells using degenerative oligonucleotides to amplify by novel primers (Lillycrop et al. 1992). It has been found to play a role in neuronal growth and differentiation. The expression of Brn-3b induces cell proliferation and represses cell differentiation (Latchman 1999).

Structurally, there are two forms of Brn-3b, the shorter 30kDa form referred as Brn-3b short form (Brn-3b(s)) whereas the longer form is 43-46kDa and is called Brn-3b long form (Brn-3b(l)) (Latchman 1999;Xiang et al. 1993). The expression of the Brn-3b(s) and Brn-3b(l) are the results of the alternate splicing of the Brn-3b RNA.

1.3.1 Alternative splicing of Brn-3b

Brn-3b is encoded by a gene located at chromosome 4 (4q31.2) in human cells (Xiang et al. 689-701). The gene contains 2 exons spanning approximately 4 kb of genomic DNA, and is subject to alternative splicing. When the Brn-3b gene is transcribed from a downstream promoter, mRNA is produced which in turn is translated to Brn-3b(s).

The Brn-3b(s) does not have the POU 4 box. However, when the gene is transcribed from an upstream promoter, a long mRNA with an additional exon is produced. Following translation, Brn-3b(l), which is a 410-amino acid polypeptide consisting of an additional N-terminal sequence, is produced (Dennis et al. 2001;Theil et al. 1993;Xiang et al. 1993).

1.3.2 Function of Brn-3b

Brn-3b has been showed to play a critical role in cell proliferation and differentiation (Budhram-Mahadeo et al. 1994). High levels of Brn-3b promotes proliferation and represses differentiation. As a result, overexpression of it promotes the maintenance of the non-differentiated proliferative phenotype of reduced process outgrowth, which is a normal response of differentiation. However, this inhibitory effect ceases by decreasing the expression level of Brn-3b (Smith et al. 1997).

As a transcription factor, Brn-3b regulates cell proliferation and differentiation by transcriptional regulation. It promotes proliferation by upregulating expression of specific target genes involved in the cell cycle including CDK4 and cyclin D1

(Samady et al. 2004) and by downregulating expression of a tumour suppressor gene, BRCA-1 (Dennis et al. 2001). On the contray, it represses differentiation by downregulating the expression of certain genes which are responsible for differentiation such as SNAP-25 (Lakin et al. 1995), neurofilament (Smith et al. 1997) and α-internexin (Budhram-Mahadeo et al. 1995). Brn-3b appears to regulate cell proliferation, therefore high level of Brn-3b expression may cause uncontrolled cell proliferation, which is one of the characteristics of cancer cells.

1.4 Brn-3b and cancer

Brn-3b is highly expressed in various cancer cell lines, including neuroblastoma and breast cancer cell lines (Dennis et al. 2001; Irshad et al. 2004;Sonia et al. 2005). As well as increasing cell proliferation, Brn-3b also promotes the invasiveness and drug resistance of cancer cells (Irshad et al. 2004;Sonia et al. 2005). In this section, the basic concepts of cancer and the possible roles for Brn-3b in cancer cells are discussed.

1.4.1 Definition of cancer

Cancer refers to diseases that exhibit two main characteristics that enable them to develop and spread: (1) uncontrolled cell growth in the absence of mitogenic stimulus and (2) the ability to metastasize to normal tissues either locally or at distant sites in the body. Indeed, it is the combination of these features which makes cancer dangerous. When the proliferation of cells is out of control, it will give rise to a tumour. However, if the cells in the tumour cluster together to form a single mass, the tumour is said to be benign as a complete cure can be achieved by surgically removal of the mass. A tumour is classified as a cancer when the uncontrolled proliferating cells have the ability to spread and invade other tissues to form secondary tumours. This type of tumour is called a malignant tumour (Alberts et al. 1994).

1.4.2 Types of cancer

The human cancers arising from the epithelium (the layers of cells covering the body's surface and lining internal organs and various glands) are called carcinomas. Cancers

occur in the supporting tissues of the body, such as bone, muscle and blood vessels are called sarcomas(Alberts et al. 1994). Cancers derived from immature progenitor blast cells are called neuroblastoma (Noesel and Versteeg 2004). Cancers of the blood and the lymph glands are called leukemias and lymphomas, respectively (Alberts et al. 1994).

1.4.3 The causes of cancer

The formation of cancer is complex; it can be caused by various factors such as exposure to chemical carcinogens, ionizing radiation, virus and alteration of cellular internal signals. However, in principle, it is caused by the alteration of genetic material (mutation) due to the factors mentioned. As a result of the mutation, the gene expression pattern changes and this causes cells to alter their size, shape, surface characteristics and behavior. The cells are transformed so that they grow indefinitely and become independent of certain growth factors which are required for normal cell growth. Finally, the cells spread and invade normal tissue to establish a new colony (Alberts et al. 1994;Lewin 2000).

1.4.4 Metastasis of cancer cells

The process of the cancer cells migration is called metastasis. In this process, the cells of a solid tumour loosen their adhesion at original site, escape from the tissue origin and burrow through other tissues so that they can reach the circulatory system. In the circulatory system, they can migrate to distant sites. After traveling to a new organ, the cancer cells burrow out of the blood or lymph vessels and invade the surrounding tissues. There they continue to multiply, forming secondary tumours. Eventually, these tumours disrupt the body's normal functions and often lead to death (Alberts et al. 1994).

1.4.5 Brn-3b and various cancer cell lines

The whole process of transformation and metastasis is a result of change of normal gene expression (Lewin 2000). Therefore, transcription, which is an essential element of gene expression, plays an important role in cancer formation. Indeed, overexpression of Brn-3b, which has been observed in various cancer cells including

neuroblastoma and breast cancer cell lines, has been shown to be related to tumourigenesis (Dennis et al. 2001;Irshad et al. 2004).

1.4.6 Brn-3b and neuroblastoma

Brn-3b is expressed at high levels in various neuroblastoma cell lines and human neuroblastoma tumours. It was shown that an increase of the level of Brn-3b enhances cell growth and proliferation. Moreover, overexpression of Brn-3b increases the invasiveness and tumor formation ability of neuroblastoma cells whereas reduction of the Brn-3b expression decreases the invasiveness and tumor formation ability of neuroblastoma cells. These findings indicate that Brn-3b is a potent enhancer of tumourigenesis (Irshad et al. 2004).

Brn-3b exerts the effect of tumourigenesis by regulating its target genes. It has been observed that Brn-3b stimulates the expression of neuronal nicotinic acetyl choline receptor alpha II gene (Milton et al. 1995) and the synapsin I (Smith et al. 1997) which are responsible for cell proliferation whereas it represses the expression of SNAP-25 (Lakin et al. 1995), neurofilament (Smith et al. 1997) and α-internexin

(Budhram-Mahadeo et al. 1995) which are responsible for differentiation. Therefore, increased expression of Brn-3b enhances the target genes that are responsible for proliferation and inhibits the target genes that are responsible for differentiation.

1.4.7 Brn-3b and breast cancer

Apart from neuroblastoma cells, Brn-3b is expressed at a high level in MCF7 human breast adenocarcinoma cells and a number of human breast tumor samples compared to the level in normal mammary epithelium (Samady et al. 317-23;Budhram-Mahadeo et al. 6684-91). It has been shown that Brn-3b promotes cell growth and proliferation through its ability to inhibit the expression of the tumour suppressor gene BRCA-1 and through its interaction with the estrogen receptors ((Samady et al. 317-23;Budhram-Mahadeo et al. 6684-91).

BRCA-1 gene is associated with numerous cell functions, including cell cycle control, controlling protein degradation, DNA damage repair, and transcriptional (gene activity) regulation (Kenemans et al. 2004). Mutation of BRCA-1 has been found in 45% of hereditary breast cancer patients (Dennis et al. 2001). Though there is no evidence of mutation of the BRCA-1 gene in sporadic breast cancer, it has been

observed that in invasive breast cancer BRCA-1 expression is decreased compared to normal mammary epithelial tissue. Therefore, repression of the BRCA-1 expression leads to enhanced risk of tumor formation (Dennis et al. 2001;Kenemans et al. 2004).

The estrogen receptor (ER) plays a role in breast cancer progression. It activates proliferation by binding to its responsive element such as the vitillogenenin promotor. To bind to a promoter, indeed ER needs to interact with an accessory protein. It has been shown that Brn-3b has the ability to interact the ER via a protein-protein interaction (Budhram-Mahadeo, Parker, and Latchman 1029-41;Samady et al. 317-23)).

1.5 Brn-3b and Cell cycle

One of the important aspects of Brn-3b in cancer is that it promotes proliferation (Irshad et al. 2004;Sonia et al. 2005). Previous studies have shown that Brn-3b plays an important role in regulating proliferation by upregulating expression of factors related to cell cycle progression (Samady et al. 2004). In this section, the basic concept of the cell cycle is described. Also, the role of brn-3b in the cell cycle is discussed.

1.5.1 Overview of the Cell cycle

The cell cycle consists of several clearly defined phases: G1, S, G2 and M. G1 phase is a gap phase during which the cell prepares for DNA replication. In this phase, the cell makes the decision to proceed, pause, or exit the cell cycle based on the mitogenic and growth inhibitory signals. S phase is a phase when DNA synthesis and chromosome duplication occurs. G2 phase is a phase that cell prepare for cell division. M phase is the phase that chromosomes a re segregated into separate nuclei and cytokinesis occurs to form two daughter cells. If the cells do not continuously divide, there is an additional resting stage termed G0 phase between phases M and G1 (Figure 1.3) (Johnson and Walker 1999; Stacey 2003).

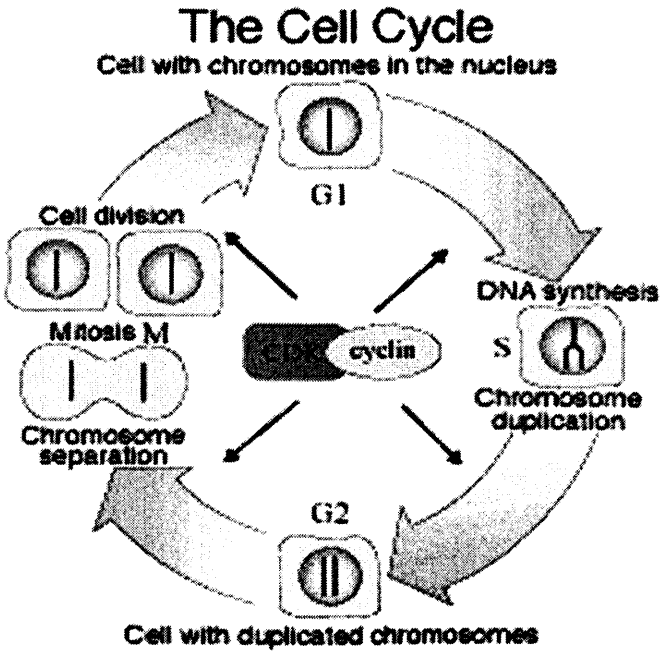

Fig.1.3 The basic morphology of cell cycle.

1.5.1.1 Cyclin-dependent kinases

The transition from one cell cycle phase to another occurs in an orderly fashion and is regulated by different cellular proteins. Key regulatory proteins are the cyclin-dependent kinases (CDKs), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle. There are four CDKs being active during the cell cycle, they are CDK4, CDK6 (active during G1 phase), CDK2 (active during G1 and S phase) and CDK1 (cdc2) (active during the G2 and M phase). When activated, CDK induce downstream processes by phosphorylating selected proteins. CDK protein levels remain stable during the cell cycle, in contrast to their activating

proteins, the cyclins (Arellano and Moreno 1997;Swanton 2004;Vermeulen et al. 2003).

1.5.1.2 Cyclins

Cyclins are the target substrates of the CDKs. They bind to CDKs and control the ability of the CDKs to phosphorylate their target proteins (Vermeulen et al. 2003). During the cell cycle progression, the levels of cyclins oscillate due to periodic synthesis and degradation (Fig. 1.4). In this way, cyclins can control the activation of the CDKs in different phases (Arellano and Moreno 1997;Swanton 2004;Vermeulen et al. 2003).

In mammalian cells, the cell cycle starts progressing through G1 phase when CDK4 or CDK6 associats with the D-type cyclins (cyclins D1, D2 and D3). Cyclin E is expressed in late G1, the formation of CDK2/cyclin E complexes is required for G1 to S transition (Ohtsubo et al. 1995;Resnitzky et al. 1994) and initiation of DNA synthesis (Jackson et al. 1995;Krude et al. 1997;Ohtsubo et al. 1995). After activation

by cyclin E, CDK2 is subsequently activated by the A-type cyclins (cyclin A1 and A2) during S phase and this complex is required for the progression through S phase. The activation of CDK1 or ddc 2 by cyclin B triggers transition from G2 to M (Jeong et al. 2003;Elledge 1996).

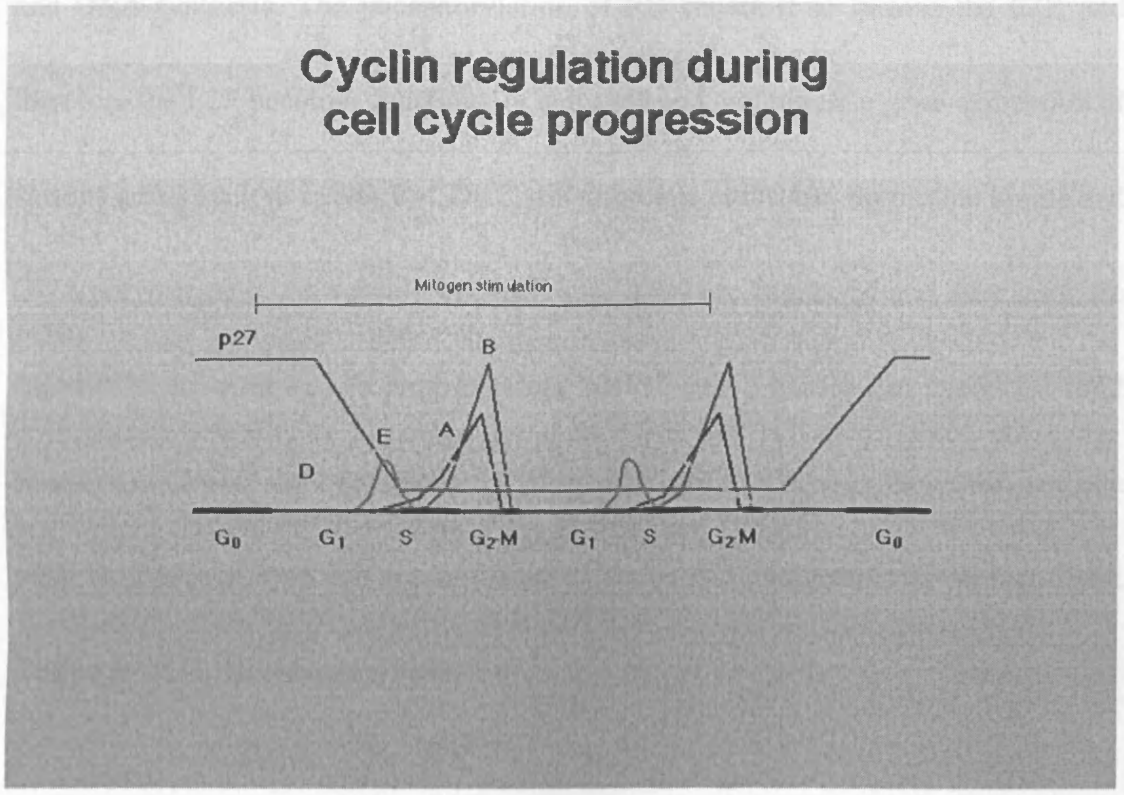

Figure 1.4 The relative amount of cyclins in different phase. A refers to cyclin A, B refers to cyclin B, D refers to cyclin D and E refers to cyclin E.

1.5.2 The G1/S transition

G1/S transition is important in regulating cell growth and proliferation. As mentioned, cyclin D1, CDK4, cyclin E and CDK2 are responsible for the transition. In mid G1

phase, CDK4 which associates with Cyclin D1 phosphorylates a protein called retinoblastoma protein (RB). Before the phosphorylation, the hypophosporylated RB inhibits cell cycle progression by binding to a transcription factor E2F which regulates the expression of many genes that encodes proteins involved in cell cycle progression and DNA synthesis. The phosphorylation of RB causes it to release the E2F, and therefore the E2F becomes functionally activated and can regulate gene expression of various genes such as cyclin E, CDK2, dihydrofolate reductase, thymidine kinase and DNA polymerase α. As a result, cyclin E and CDK2 are expressed and they associate together to drive the cell to progress from late G1 into S phase. The cyclin E/CDK2 complex maintains the hyperphosphorylated state of the RB, thus they take part in a positive feedback loop for accumulation of active E2F (Johnson and Walker 1999; Resnitzky et al. 1994;Stacey 2003).

The G1/S transition is an important cell cycle checkpoint. In the G1 phase, the cell is preparing itself for DNA synthesis. G1 arrest has been observed when the cell is subjected to DNA damage. The arrest prevents the cells from uncontrolled growth. However, overexpression of cyclin D1 and E, which have been observed in various cancer cells, prevents the cell cycle arrest (Resnitzky et al. 1994;Noesel and Versteeg 2003).

1.5.3 Relationship between brn-3b, cyclin D1 and CDK4

It has been observed that CDK4 and cyclin D1 are target genes of Brn-3b. An increase in Brn-3b expression correlated to an increase of the CDK4 and cyclin D1 expression level. Interestingly, both the Brn-3b(s) and Brn-3b(l) can activate CDK4 expression, this indicates that it is the POU domain of Brn-3b that acts as a transcriptional activation domain (Samady et al. 317-23).

Cyclin D1 and CDK4 play a critical role in tumourigenesis. Indeed, overexpression of both cyclin D1 and CDK4 has been shown to relate to some human tumors (Samady et al. 317-23;Zhang et al. 169-75). Moreover, their overexpression of them prevents growth arrest and results in the immortalization of primary cells (Samady et al. 317-23;Holland et al. 3644-49). As Brn-3b is an upstream regulator of both the cyclin D1 and CDK4, high level of Brn-3b expression, which has been observed in various cancer cells, increases the expression of these two genes and thus induces tumourigenesis.

1.6 Objectives of the study

Alteration of normal cell cycle progression has been shown to be implicated in cancer formation. It has been shown that deregulated high expression of cyclin D1 directly contributes to tumourigensis. As Brn-3b plays a role in regulating the expression of cyclin D1, thus Brn-3b is responsible, as least in part, at initiating tumourigenesis through this pathway. However, the expression pattern of Brn-3b in different phases of the cell cycle and when it activates its target gene cyclin D1 remain to be elucidated. Therefore, this study aims to find out the expression pattern of Brn-3b at different phases of the cell cycle and when it activates its target gene cyclin D1 (refer to section 2.2 for experimental set up).

Another aim of this study is to discover the factor which causes the level of Brn-3b to increase in cancer cells. Exposure to carcinogens is a know cause of cancer. It has been shown that exposure to benzo(a)pyrene increases the risk to have cancer (Davis et al. 2002). As Brn-3b is also highly expressed in cancer cells lines and regulates various genes which cause tumourigenesis, it may be possible that exposure to benzo(a)pyrene may increase the expression of Brn-3b which leads to tumourigenesis. Therefore, an experiment was carried out to investigate whether the expression of

Brn-3b changed when the cells were exposed to benzo(a)pyrene (refer to section 2.2 for experimental set up).

Chapter Two

Materials and Methods

Various methods were carried in this study. They are methods related to cell culture (section 2.1), setting up the experiments in this study (section2.2), fluorescence activated cell sorting (FACS) analysis (section 2.3), quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (section 2.4) and western blotting (section 2.5).The details of those methods are described in this chapter.

2.1 Cell culture

Cell culture is a very important and invaluable tool for biological investigation. It allows analysis of biological properties and processes that are not readily assessable at the level of the intact organism (Helgason & Miller 2005). Therefore, it is important to master the cell culture techniques before starting any experiment.

In this section, the cell lines used in the experiments, ND7 and MCF7, are discussed. Moreover, the procedures for culturing, passaging, thawing, cryopreservation, determination of cell numbers and viability are described. It should be stressed that all materials used were sterilized and all the procedures were performed in a laminar

flow hood, which provides a sterile environment. All the reagents were warmed up to 37 °C before use. The surface of all the flasks and bottles were decontaminated with 70% alcohol before taking into a laminar flow hood. Gloves and laboratory coat were always worn. 70% alcohol was sprayed on the gloves to decontaminate every time before working in the hood.

2.1.1 Cell lines

ND7 cells were hybrid cells made by fusing the N18 TG2 azaguanine-resistant neuroblastoma and the primary sensory neurons of the rat dorsal root ganglion (Wood et al. 1990). This allows the cells to bear the characteristics of immortalization and sensory neurons(Lillycrop et al. 1992;Suburo et al. 1992;Wood et al. 1990). Moreover, the cells can be induced to differentiate to non-dividing and process-bearing cells with the characteristics of mature sensory neurons under various treatments(Lillycrop et al. 1992;Suburo et al. 1992).

MCF7 cells were breast cancer line obtained from American Type Culture Collection (Manassas, VA). MCF7 cells are epithelial-like human breast adenocarcinoma derived from pleural effusion. The cells are highly rearranged, where translocations occur in

all chromosomes except chromsome 4(Promochem 2005).

2.2 Basic cell culture techniques

ND7 and MCF7 cells were cultured in complete Liebovitz medium (L15) (refer as full growth medium for ND7 cells)(Gibco) (Table 2.1) (see appendix 1 for the preparation) and complete Dulbeco's Modified Eagles medium (D-MEM) (refer as full growth medium for MCF7 cells)(Gibco) (Table 2.2) (see appendix 2 for the preparation) respectively at 37°C in humidified atmosphere in 5% carbon dioxide. The cells were periodically monitored and passaged when they reached 80%-90% confluency.

Ingredients of the completed L15:	Proportion:
L15	1X
Fetal calf serum (gold)	10%
D-Glucose (Sigma)	1%
Sodium bicarbonate (Gibco)	4.8%
L-Glutamine (Gibco)	0.2mM
100 U/ml penicillin and 100 µg/ml streptomycin (P/S) (Gibco)	1%

Table 2.1 The components and their concentrations required for making complete L15 culture medium for ND7.

Ingredients of the completed L15:	Proportion:
D-MEM	1X
Fetal calf serum	10%
100 U/ml penicillin and 100 μg/ml streptomycin (P/S) (Gibco)	1%

Table 2.2 The components and their concentrations required for making a complete D-MEM culture medium for MCF7.

2.2.1 Cell passaging

Regular cell passage is necessary to maintain viable cells. This ensures that there are sufficient nutrients in the medium for cell growth and that the pH of the medium is not too acidic as a result of large amounts of cellular waste (Helgason & Miller 2005). The cell passage procedures for ND7 and MCF7 are slightly different, and are described below.

2.1.1.1 Cell passaging for ND7

ND7 cells were split when they reached 80%-90% confluency. Firstly, the flask was hit by hand on the side where the cells attached so that the cells would detach from the flask. The flask was then put under a light microscope to observe whether all the

cells were detached. If not the flask would be hit again until all the cells came off. One tenth of the cells were seeded to a new flask. This means, for example, 2ml of the cells were taken out from 20ml of the cells and were seeded into a new flask. Full growth medium (Complete L15) were added so as to fill the flask up to its suggested optimum volume.

2.1.1.2 Cell passaging for MCF7

MCF7 cells were split when they reached 80%-90% confluency. To start with, the culture medium was removed from the cells. The cells were washed twice with Hank's Balanced Slat Solution (HBBS) (Gibco). Trypsin/EDTA (Gibco) was added to the flask after HBBS was removed. The volume of trypsin added depends on the size of the flask. Usually, 3ml of trypsin were added to a 175cm^2 culture flask while 2ml of trypsin were added to a 125cm^2 culture flask. The aim is to allow the trypsin to cover the whole surface of the flask where the cells are attached. The flask was then hit by hand on the side where the cells are attached so that the cells would detach from the flask. Whether all the cells were detached was checked under a light microscope. If not the flask would be hit again until all the cells came off. Afterwards, the cells were transferred to a centrifugation tube and were pelleted at 1000 r.p.m. at

room temperature for 5 minutes. The supernatant was removed and the pellet was resuspended in full growth medium (completed D-MEM). One fourth of the cells were split to a culture flask. Completed D-MEM was added to fill up the flask to its suggested optimum volume.

2.1.2 Cryopreservation

Continuous cell culture increases the chances of cell contamination by bacteria and other unwanted organisms. Moreover, continuous cell culture can lead to the accumulation of karyotype alternations(Helgason & Miller 2005). Therefore, in order to prevent loss of the cell line, it is advisable to cryopreserve some of the cells for future use. The cryopreservation processes of ND7 and MCF7 cells are described in this section.

2.1.2.1 Cryopreservation of ND7 cells

To start with, the cells were detached from the flask. The procedures of it are same as for passaging cells (please refer to section 2.1.1.1) except all the cells were transferred to a centrifugation tube and were pelleted at 1000 r.p.m. at room temperature for 5

minutes. The supernatant was removed and the pellet was resuspended in the 1 ml of 5% Dimethyl sulfoxide (DMSO) (Sigma) in FBS. The cells were transferred to a vial and were stored in -80 °C freezer inside an isopropanol rack overnight. The cells were then transferred to liquid nitrogen for long term storage.

2.1.2.2 Cryopreservation of MCF7 cells

To start with, the cells were detached from the flask. The procedures of it are same as for passaging cells (please refer to section 2.1.1.2) except that the pellet was resuspended in 1ml of 5% DMSO in FBS. The cells were transferred to a vial and were stored in -80 °C freezer inside an isopropanol rack overnight. The cells were then stored in liquid nitrogen the day after.

2.1.4 Thawing cells

Cell thawing is an important procedure to recover cells for experimental use. Improper thawing leads to unwanted cell damage or even death (Helgason & Miller 2005). The procedure of cell thawing of ND7 and MCF7 is the same, and is described in this section.

To thaw cells, firstly, the vial containing the cells was taken out from the liquid nitrogen and was put into a water bath set at 37°C for 5 minutes. The cells were then transferred to a centrifugation tube and the 9 ml of required medium (complete L15 for ND7 and completed D-MEM for MCF7) was added slowly to prevent sudden dilution of DMSO which can cause severe osmotic damage to the cells (Helgason & Miller 2005). The cells were pelleted at 1000 r.p.m. at room temperature for 5 minutes. The supernatant was removed and the pellet was resuspended in the required medium so as to remove the DMSO which is toxic to the cells. The cells were then transferred to a culture flask and the required medium was added to fill up the flask to its suggested optimum volume. The cells were cultured at 37°C in humidified atmosphere in 5% carbon dioxide.

2.1.5 Cell number determination

Every cell line has its own optimal density for maintaining growth and viability (Helgason & Miller 2005). As these factors affect the experimental settings, it is therefore important to determine the cell number and cell viability before carrying out experiments. The processes of cell number determination for both ND7 and MCF7

cells are the same, and are described in this section.

This first step is to detach the cells from the culture flask. This step is same as that in cell passaging (please refer to section 2.1.1.1 for ND7 and section 2.1.1.2 for MCF7). Once the cells were detached, the flask was swirled so that the cells in the flask were evenly distributed. 100µl of the cell suspension was taken out and transferred to the two chambers of a hematocytometer covered with a cover slip. The cell number was counted under a light microscope. In one chamber, there are nine 1 mm^2 squares. The cell numbers were counted from ten of the 1 mm^2 squares from both chambers and average was taken. The cell number in every ml equals to the average value of 10^4.

2.2 Setting up experiments

Generally, there were three different sets of experiments in this study. The first set was the experiment on cell cycle arrest of ND7 and MCF7 cells following various treatments to analysis mRNA levels of Brn-3b and cyclin D1. The second set was the experiment of synchronization of ND7 cells to analyze for Brn-3b and cyclin D1 mRNA levels. The third set was the experiment on Benzo(a)pyrene exposure to ND7 cells to analyze for Brn-3b and mRNA levels. The details of setting up the experiments are described in this section.

2.2.1 The experiment on cell cycle arrest of ND7 and MCF7 cells following various treatments

In this experiment, $1X10^6$ ND7 and $2X10^6$ MCF7 cells were firstly seeded on $64cm^2$ petri dishes. Drugs were added after 24 hours of seeding. Five cell cycle arresting drugs were used. They were 5-flurouracil, hydroxyurea, colchicine, doxorubicin and etoposide and are described below. All the cells were harvested 12 hours after addition of drugs for FACS analysis, quantitative RT-PCR and western blotting. The details of the whole experiment are described in this section.

2.2.1.1 Treatments

5-fluorouracil

5-fluorouracil (5-FU) is an antimetabolites which induces G1 or G1/S cell cycle arrest in cancer cells (Johnson and Walker 1999). However, the exact molecular actions of these anticancer drugs on cell cycle regulation have not been fully explained yet.

Hydroxyurea

Hydroxyurea (HU) induces cell cycle arrest at the G1 and S phases. This is achieved by its specific action on ribonucleotide reductase. Ribonucleotides are reduced by ribonucleotide reductase to deoxyribonucleotides; this reduction reaction is impeded by HU, which limits DNA biosynthesis (Yarbro 1992).

Colchicine

Colchicine (Col) is an antimitotic agent. It induces cell cycle arrest in M phase by disrupting microtubules through binding to tubulin. This prevents tubulin from undergoing polymerization during M phase and hence causes M phase arrest (Wilson et al. 1999).

Doxorubicin

Doxorubicin (Dox) induces cell arrest in G2/M phase (Ling et al. 1996). It intercalates into DNA, blocking both the replication and transcription of the DNA (Katzung 1989). Moreover, it inhibits topoisomerase II activity by occupying space which is normally inhabited by base pairs in DNA. This process could adversely affect the formation of the DNA-topoisomerase II complex and the function of this enzyme leading to alternation of the DNA topology and consequently interference with the global structure in chromation (Foye 1995).

Etoposide

Etoposide (Etop) induces cell cycle arrest in late S and G2 phase. Etoposide causes DNA damage through inhibition of topoisomerase II and activates oxidation-reduction reactions to produce derivatives that bind directly to DNA. Topoisomerase II carries out breakage and reunion reactions of DNA which are necessary for normal cellular function. (Kaufmann 1998).

2.2.1.2 Cell seeding

Cells were seeded on a 64cm^2 petri dish (Falcon). The amount of cells seeded on the dish depends on the cell type and how long the experiment would last. For this experiment, 1X10^6 ND7 cells were seeded in a dish whereas 2X10^6 MCF7 were seeded in one dish. More MCF7 cells have to be seeded because MCF7 cells have a longer doubling time. In order to seed the correct number of cells, the number of the cells was first counted (please refer to section 2.1.5). The correct number of cells were then transferred to the dish and full growth medium (complete L15 for ND7 and complete D-MEM for MCF7) was then added respectively to make the total volume up to 10 ml. The cells were incubated at 37°C in humidified atmosphere in 5% carbon

dioxide. For each treatment, 3 petri dishes of cells were seeded for FACS analysis, RT-PCR and western blotting. This experiment was carried out in triplicate.

2.2.1.3 Addition of drugs

The concentration of each drug is shown in Table 2.3. The drugs were prepared in a specific stock concentration so that a specific amount of drugs were added to each Petri dish which contained 10 ml of medium.

	Molar mass	**Target concentration/Final concentration**	**Stock concentration**	**Solvent**	**Volume added to each dish**
5-Fluorouracil	**130.1**	**1&2.5μg/ml**	**1mg/ml**	**PBS**	**10&25μl**
Hydroxyurea	**76.05**	**1&10mM**	**1M**	**H_2O**	**10&100μl**
Colchicine	**399.4**	**1μM**	**1mM**	**DMSO**	**10μl**
Doxorubicin	**580**	**1μM**	**1mM**	**PBS**	**10μl**
Etoposide	**588.56**	**10μM**	**10mM**	**DMSO**	**10μl**

Table 2.3 The five drugs used in this study. The drugs were prepared in a stock concentration and were added to Petri dishes containing 10ml of full growth medium so as to achieve the target concentration. According to manufacturer's instructions, different solvents were used to dissolve different drugs.

2.2.1.3.1 Preparation of drugs

Generally, the drugs, which were in powder form, were weight with an electrical balance. Their respective solvents were added so as to make them into their respective stock concentration. The amount of the solvent should be added was calculated by the following formula:

The amount of solvent added = (Mass/Molar mass)/Stock concentration

2.2.1.4 Controls of the experiment

For the experiment, two controls were used. One of the controls was the untreated cells whereas the other one was cells treated with 10µl of DMSO as DMSO, which may be toxic to the cell, was used as a solvent for some of the drugs.

2.2.1.5 Harvesting cells

All the cells were harvested after 12 hours of drugs addition/24 hours after full growth medium for FACS analysis, qRT-PCR and western blotting.

2.2.2 Study on synchronization of ND7 cells

This experiment aimed to synchronize ND7 cells in G1 phase of the cell cycle by transferring the cells in serum free medium (see appendix 4 for preparation) for an optimum period of time (see section 2.2.2.1). After the synchronization, the cells were transferred back to full growth medium (complete L-15) containing 20% FCS so that the cells resume cycling in the cell cycle (Budhram-Mahadeo et al. 1994). The cells were harvested at 3, 6, 12, 24, 36 and 48 hours of being transferred back to the full growth medium containing 20% FCS. Similar to the set one experiment, 3 Petri dishes were prepared for one treatment so that samples could be prepared for FACS analysis, qRT-PCR and western blotting. This experiment was carried out in triplicate.

2.2.2.1 Optimization of the time period for cell growth in serum free medium

An initial experiment was carried out on ND7 to determine the optimum period of time for serum free exposure as not all the cells will undergo cell cycle arrest if the exposure time in serum free medium is too short whereas if the exposure time of cells in serum free medium is too long, increased cell death would occur so that fewer cells will be available for analysis.

Six sets of cells were grown; they were cells grown in full growth medium and serum free medium for 24, 48 and 72 hours. The first step of the experiment was to seed cells in 64cm^2 Petri dishes (please see also section 2.2.1.2). After 12 hours of seeding, the full growth medium was removed and serum free medium was added to cells. Cells were harvested at different time points; the untreated cells were harvested at 24, 48, 72 hours after seeding whereas the cells grown in serum free medium were harvested after 24, 48 and 72 hours after addition of serum free medium. Samples were prepared for FACS analysis and qRT-PCR. The results of the optimization showed that 48 hours of serum free exposure was the best period of time for subsequent experiments.

2.2.2.2 Addition of full growth medium containing 20% FCS to ND7 cells after 48 hours of serum starvation of the ND7 cells

In this experiment, ND7 cells were cultured in serum free medium for 48 hours before full growth medium containing 20% FCS was added back to the cells. The cells were harvested at 3, 6, 12, 24, 36 and 48 hours after the addition of 20% serum medium. This experiment was done in triplicate and samples were prepared for FACS analysis and qRT-PCR.

The first step of the experiment was to seed cells in Petri dishes (please see also section 2.2.1.2). After 12 hours of seeding, all the cells were transferred to serum free medium except the untreated control. The full growth medium containing 20% FCS was added to all the cells except the 48 hours serum free control. This was done by removing the serum free medium and addition of 10ml of full growth medium containing 20% FCS to each dish. The cells were harvested at 3, 6, 12, 24, 36 and 48 hours after addition of the full growth medium containing 20% FCS.

2.2.2.3 The controls

Two controls were used in the experiment; they were untreated cells for 48 hours and cells grown in serum free medium for 48 hours. The untreated cells were harvested after 48 hours of seeding whereas the cells growth in serum free medium were harvested 48 hours after addition of the full growth medium containing 20% FCS.

2.2.3 Benzo(a)pyrene exposure to ND7 cells

In this experiment, different concentrations of benzo(a)pyrene were added to ND7 cells to investigate the effect of this carcinogen on Brn-3b mRNA level and cell cycle

progression.

Benzo(a)pyrene is carcinogenic and is a polycyclic aromatic hydrocarbons which forms by incomplete combustion. Therefore, it is abundant in the environment. When it is metabolically activated to form benzo(a)pyrene 7,8-diol 9,10 epoxide, it reacts mainly with the N2 position of guanine and to a lesser extent with N6 position of adenine to form covalent adduct (Davis et al. 2002).

The first step of the experiment was to set up the experiment was to seed cells on Petri dishes (please see also section 2.2.1.2). The dishes included two controls and three different concentrations of benzo(a)pyrene treatments. The two controls were untreated cells and cells treated with 10µl acetone, which was used as the solvent for benzo(a)pyrene. The three concentrations of benzo(a)pyrene used were 2.5µg/ml, 5µg/ml and 10µg/ml. The cells were harvested after 12 hours of addition of benzo(a)pyrene.

2.3 Methods related to FACS analysis

2.3.1 Introduction to FACS analysis

Fluorescence activated cell sorting (FACS) machine can separate, classify and quantify living cells in a suspension on the basis of size and the color of their fluorescence (Kimball 2003). In principle, cells suspension was passed through a narrow dropping nozzle so that each cell is in a small droplet. One or more laser beams are passed through the cell, causing light to scatter and fluorescent dyes to emit light at various frequencies. Photomultiplier tubes (PMT) receive the scattered light signals and convert them to electrical signals. As a result of this, cell data are collected (Parks et al. 1998).

Generally, there are three types of data; they are data of forward scatter, side scatter and fluorescence. The forward scatter data estimate the approximate cellular size while the side scatter data identify cell complexity and granularity. With these data, the dead cells and debris are excluded (Parks et al. 1998).

The data from fluorescent labeling allows investigation of cell structure and function.

Fluorescent dyes are used to label cell structure to generate cell autofluorescence. FACS machine collects fluorescent signals from one to several channels corresponding to different laser excitation and fluorescent emission wavelength (Parks et al. 1998).

In this study, Propidium iodide (PI) was used as a dye for cell cycle analysis. PI dye works by binding to DNA in living cells so as to emit fluorescent signals. Following this, the relative DNA content of the cells was recorded. As cells in different phase have different DNA content, the data shows the proportion of cells in G1, G2 and S phases (Parks et al. 1998).

2.3.2 PI staining if the cells

The cells were scraped off from the plates and were transferred to a 15ml centrifugation tubes and were pelleted at 1000 r.p.m. at 4°C for 5 minutes. The cells were washed with cold phosphate buffer saline (PBS) (see appendix 4 for the preparation) and were pelleted again at 1000 r.p.m. at 4°C for 5 minutes. Afterwards, the pellets were resuspended in 300µl of cold PBS. 1ml of ice-cold 100% ethanol were added dropwise to the cells whist vortexing so as to prevent an osmotic shock

which can damage or even cause unwanted cell death. The cells were stored in -20 °C until the day of analysis.

2.3.3 FACS analysis

On the day of analysis, the cells were pelleted at 1000 r.p.m. at 4°C for 5 minutes. The pellets were resuspensed in 300μl of PI buffer (see appendix 5 for the preparation) containing 100μg/ml of PI and 100μg/ml of ribonuclease A. The cells were then transferred to FACS tubes and were then incubated in the darkness at 37 °C for 30 minutes. Afterward, the cells were analyzed by Epics XL flow cytometer (Beckman Coulter). Fluorescence was excited by laser beam at 488nm and the signals generated were collected at 575nm by a band pass filter. Around 30000 cells were collected for analysis. Double discrimination was used to exclude cell clumps. Cell cycle distribution was estimated by generating a mathematical model using software Multicycle (Phoenix Flow Systems, San Diego, USA).

2.4 Methods related to qRT-PCR

2.4.1 TRIZOL method of RNA isolation

TRIZOL method is a convenient method for the isolation of total RNA from cells and tissues. It is improved from the classical method developed by Chomczynski and Sacchi using a mono-phasic solution of phenol and guanidine isothiocyanate. TRIZOL Reagent can maintain the integrity of the total RNA while disrupting cells and dissolving cell components during sample homogenization or lysis.

The medium in a plate was removed and the cells were washed twice with 5 ml of HBBS. 1 ml of TRIzol solution (invitrogen) was added to the cells after the HBBS was removed. The cells were scraped off and were transferred to an autoclaved eppendorf. The homogenate was incubated for 5 minutes at room temperature. Afterward, 200µl of chloroform was added and the eppendorf was inverted several times to mix. Vortexing in this step is not recommended as this may shear genomic DNA which can cause excessive DNA contamination of the RNA preparation (Dieffenbach & Dveksler 2003). The mixture was pelleted at 13000 r.p.m and 4°C for 15 minutes. The upper aqueous phase, in which RNA remains exclusively, was

transferred to an autoclaved eppendoff to hich 500µl of isopropanol was added. The eppendorf was vortexed to ensure thorough mixing. The mixture was then incubated at room temperature for 10 minutes to allow the RNA to precipitate. The mixture was pelleted again at 13000 r.p.m and 4°C for 30 minutes. The supernatant was carefully removed and the pellet was washed with 1ml of 75% ethanol. The mixture was pelleted at 13000 r.p.m and 4°C for 15 minutes. The supernatant was removed and the pellet was allowed to air dry for 10 minutes at room temperature. The pellet was then resuspended in 30µl DEPC water (see appendix 6 for the preparation). 2µl of the sample was taken for estimation of RNA concentration by spectrometry (see section 2.4.2.1) and 2µl of the RNA was run on a 2% agarose gel to check the quality of the RNA (see section 2.4.2).

2.4.2 RNA yield and quality determination

The main goal of RNA isolation is to obtain good yield and quality RNA for successful downstream experiments. Generally, there are two ways of doing this; one way is by spectrometry and the other way is by agarose gel electrophoresis. Both the methods were used in the study and are described in this section.

2.4.2.1 Spectrometry

Spectrometer is a powerful tool to detect the concentration and the relative quality of RNA and DNA. Generally, it works by recording the absorbance of 260nm (A_{260}) and 280nm (A_{280}) to determine the concentration of the nucleic acid and the protein content respectively. The RNA content can be calculated by the following formula:

RNA concentration (μg/μl) = A_{260} X 0.04μg/μl X dilution factor

The RNA quality can be estimated by the A_{260}/A_{280} ratio. Good quality RNA should give the ratio between 1.5 and 2 (Dieffenbach & Dveksler 2003).

The program of the muti-wavelength was chosen in the spectrometer. 600μl of the MilliQ water was added to a cuvette and put into the spectrometer as a blank. The calibration button on the screen was selected so that the machine was calibrated in accordance with the blank. The water from the cuvette was removed. 2μl of RNA solution was mixed with 598μl of the same MilliQ water which used as a blank. The mixture was added to the cuvette. The cuvette was put into spectrometer again. The button of sample running was selected and reading was recorded. The mixture was

removed and the cuvette was cleaned by the same MilliQ water. If more than one sample was used, the procedures were repeated starting from setting up a blank to clean the cuvette.

2.4.2.2 Agarose gel electrophoresis

Gel analysis is a good method to estimate the yield and quality of RNA. It can be used to estimate the average size distribution of RNA and the RNA degradation in a sample. It resolves RNA by the rate of migration which depends on the size of RNA. Ethidium bromide can be used to detect RNA in a gel. A RNA ladder (invitrogen) was run in gel so that the yield of the RNA can be compared during visualization. The yield and quality can be visualized by using a computer imaging software or directly under UV light. Generally, good quality of the RNA should be resolved in two sharp bands which correspond to the 18S and 28S of ribosomal RNA (Dieffenbach & Dveksler 2003).

Generally, the percentage of the agarose gel was determined by the kilo base (kb) of the nucleic acid to be resolved. 1 % agarose gel can resolve fragment greater than 1kb while 2.5% can resolve fragment shorter than 1 kb (Dieffenbach & Dveksler 2003). In

this study, 2.5% agarose gel was used to resolve the nucleic acid sample. To start with, agarose power (invitrogen) was dissolved in 1X TAE buffer. 2.5% agarose gel means 2.5g of the power should be dissolved in every 100ml of the 1X TAE. The solution was then heated in a microwave oven for 3 minutes to facilitate the power to dissolve and remove any dissolved gas. The solution was cooled down for 5 minutes and 2μl of ethidium bromide was added to every 100ml of the solution. The solution was poured into a gel casting apparatus. Gel combs were inserted to the gel casting apparatus. The solution was then left at room temperature for 40 minutes to allow the gel to solidify.

Once the gel had formed, it was put into a gel tank. 1X TAE buffer was poured into the gel tank so that the gel was covered by the buffer. The combs were removed. 1μg RNA marker was added to one well of the gel. RNA sample, which mixed with 1X loading dye (invitrogen) by 1 to 1 ratio, were added to other wells. The gel tank was connected to a power supply and the gel was run under 100V. The gel should be placed in a right orientation so that the negative RNA could run to the positive pole through the gel. The gel was checked periodically by looking at the "solvent font" to prevent the RNA from running off the bottom of the gel. Generally, it takes around 90 minutes to run the RNA. The gel was taken to a computer imaging system (Syngene,

Synoptics) for image capture. After putting the gel in the system, the Syngene program in the computer was selected. The button of the auto-freeze was selected and the image of the gel was displayed on the screen. The exposure time could be varied so as to change to intensity of the signal detected. Once when the signal detected was optimized, the button of the manual freeze was selected and the image could be printed out.

2.4.3 DNase 1 treatment of RNA

Isolation of total RNA invariably contain trace amount of genomic DNA which may affect downstream application such as real time PCR. Therefore, it is important to remove the genomic DNA. This can be achieved by treating the total isolated RNA with RNase-free DNase 1 so as to remove any genomic DNA contamination. The procedures are decribed in this section.

Firstly, 20μg of total RNA, 10μl of the DNase buffer and 1.5μl of the RNase-free DNase 1 (Amersham) were added to an autoclaved eppendorf. DEPC-treated water was added to make the final volume to 100μl. The mixture was incubated at 37°C for 30 minutes, then at 70 °C for 10 minutes to stop the reaction. DEPC-treated water was

added to the mixture increase the final volume to 200µl. 100µl of phenol and 100µl of chloroform were added to the mixture. The mixture was mixed and pelleted at 13000r.p.m for 15 minutes. The aqueous upper phase was transferred to an autoclaved eppendorf and 200µl chloroform was added. The mixture was mixed and pelleted again at 13000r.p.m for 15 minutes. The aqueous upper phase was transferred to an autoclaved eppendorf. RNA was precipitated with 0.1 volume of 5M NaCl (20µl) and 2.5 volumes of 100% ethanol (500µl) for at least 2.5 hours at -20°C. The sample was pelleted at 13000 r.p.m and 4°C for 30 minutes. The supernatant was removed and the pellet was washed with 180µl of 100% ethanol to remove extra salts. The sample was pelted again at 13000 r.p.m and 4°C for 10 minutes. The supernatant was removed and the pellet was allowed to air dry for 10 minutes. 20µl of DEPC-treated water was added to resuspend the pellet. 2µl of the sample was taken out for the spectrometer described in section 2.4.2.1 and 0.5µg of RNA was run on a 2% agarose gel to check for the quality (see section 2.4.2.2).

2.4.4 cDNA synthesis

cDNA was prepared from RNA for subsequent PCR reactions. To do this, 1µg of RNA was transferred to an autoclaved eppendorf for cDNA synthesis. 2.5µl of

random hexamer primers (100μg/ml) was added. DEPC-treated water was added to bring the final volume to 11μl. The sample was heated at 70°C for 10 minutes and then cooled on ice immediately. After brief spinning, 8μl of master mix (Table 2.4) was added. The mixture was then mixed and briefly spun. The mixture was then incubated at room temperature for 2 minutes. 1μl of superscript 2 (invitrogen) was added to the mixture. The mixture was mixed by pipetting up and down several times and was incubated at room temperature for another 10 minutes. Afterwards, the mixture was incubated at 42°C for 1 hour. Finally, the mixture was incubated at 70 °C to stop the enzyme reaction. The mixture was then stored at -20 °C.

Reagents:	Volume for one reaction
5 X first strand buffer	4μl
0.1 DDT	2μl
10mM dNTP	1μl
RNasin	1μl

Table 2.4 The volume of the reagents used to formulate a master mix for a 20μl reaction of the cDNA synthesis.

It is important to set up a negative control for each sample to see whether there is still some genomic contamination. A negative control can be set up by following the whole processes of cDNA synthesis except 1μl DECP water is added rather 1μl of

superscript 2. If DNA amplification is recorded in the negative control in the subsequent quantitative PCR experiment, this means genomic DNA is present and the sample should be reprocessed from the DNA digestion.

2.4.5 Quantitative RT-PCR

Quantitative RT-PCR (qRT-PCR) was carried out to quantify the amount of cDNA synthezed form the mRNA of Brn-3b, cyclin D1, cyclin E and GAPDH in ND7 and MCF7 from various sets of experiments. Generally, the qRT-PCR technology allows the absolute quantity of mRNA found in a sample to be ascertained by the incorporation of light emitting dyes into the PCR product.

2.4.5.1 Sequence-specific Taqman probes

In the experiment, Taqman probe assay system (quantiTect assay system-Qiagen) was used. This assay system utilizes a sequence specific dual labeled probe, which has a fluorophore dye incorporated to the 5' end of the sequence and a quencher dye at the 3' end. During the extension phase if the PCR the probe is cleaved and the fluorescent and quenching dyes are separated. This results in the emission of a detectable

fluorescent signal that is produced in a proportional manner to the accumulating PCR product.

As the probe system cannot distinguish the product resulting from a cDNA template or a genomic DNA template, it was necessary to ensure that the PCR products were not a result of contamination. Therefore as with all the RT-PCR reactions carried out, cDNA samples prepared without inclusion of the reverse transcriptase were included in each run to determine if there was any genomic DNA contamination present.

2.4.5.2 Standard curve

Standard curve was generated as a reference to estimate the amount of the cDNA present in each sample. Four different standard curves were generated for the four different gene of interest in the study—Brn-3b, cyclin D1, cyclin E and GAPDH. DNA of the genes of interest was collected and amplified by PCR as described in section 2.4.5.4. The products for the standard curve were resolved by a 2.5% agarose gel (please see section 2.4.2.2). The products were directly visualized under UV light and were cut out from the gel to recover the DNA for generation of standard curve (please see section 2.4.5.3).

2.4.5.3 Gel extraction

The gel slice was put in an autoclaved eppendorf and the weight of the gel was estimated by using an electronic balance. 3 volumes of buffer QG was added to 1 volume of gel. For example, 300µl of buffer QG should be added to 100mg. The mixture was incubated at 50°C for 10 minutes. In order to help dissolve the gel, the mixture was vortexed every 2 minute during the incubation. 1 gel volume of isopropanol was added and mixed after the gel was dissolved. This means 100µl was added to 100mg of gel. The mixture was transferred to a QIAquick spin column which was placed in a 2ml collection tube. The mixture was then centrifuged for 1 minute at 13 r.p.m. The flow-through was discarded and 0.5ml of buffer QG was added to the column to remove all traces of agarose. The column was centrifuged for 1 minute at 13 r.p.m. again. The flow-through was discarded and 0.75 ml of buffer PE was added to the column. The column was centrifuged for 1 minute at 13 r.p.m. again and the flow-through was discarded. The column was centrifuged for 1 minute at 13 r.p.m again to remove residual ethanol from buffer PE. The column was placed into an autoclaved eppendorf. 50µl DEPC water was added to the center of the member in the column and was centrifuged for 1 minute at 13 r.p.m. This step is to elute DNA into the eppendorf. The DNA was stored in -20°C.

The concentration of the DNA isolated was quantified by spectrometry (please see section 2.4.2.1) and gel eletrophoresis (please see section 2.4.2.2). Serial dilution was performed subsequently to prepare DNA with different concentrations. Four types of standard curve samples were prepared; they are cyclin D1, cyclin E, GAPDH and Brn-3b(total). The range of concentration prepared for the standard curve samples was based on the level of the gene expression in cells (Table 2.4). These standard samples were used in every experiment so that variations between experiments would be minimized.

50000fg	Cyclin D1; Cyclin E; GAPDH
10000fg	Cyclin D1; Cyclin E; GAPDH
1000fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)
500fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)
100fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)
1fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)
0.1fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)
0.001fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)
0.00001fg	Cyclin D1; Cyclin E; GAPDH; Brn-3b(total)

Table 2.4 The concentrations of DNA prepared for standard curve for real time PCR experiment. Four types of standard curve samples were generated; they were cyclin D1, cyclin E, GAPDH and Brn-3b(total).

2.4.5.4 qRT-PCR reaction

The reactions were performed in a final volume of 20µl in thin walled microcentrifuge tubes. 10µl master mix (Qiagen) was added to a well of the tubes. Following 1µl of the Taqman probe was added to the well, 8µl of DEPC water and 1µl of cDNA were added.

The tubes were then placed in a controlled heat block in the qRT-PCR machine (MJ research) and subjected to a thermal cycle as shown in Table 2.5. The results obtained from the reactions were analyzed using Opticon (MJ research).

Step	Temperature	Duration	Process
1	95°C	15 min	Activate of enzyme
2	95°C	30 sec	Denature of cDNA
3	60°C	30 sec	Annealing of cDNA
4	72°C	30 sec	Extension of cDNA
5	Plate read		

Table 2.5 The thermal cycling profile required for the Taqman probe assay system.

In order to compare the mRNA levels of Brn-3b, cyclin D1 and cyclin E measured, the mRNA levels of GAPDH from each samples were quantified and the values were used to normalize the values obtained from mRNA levels of Brn-3b, cyclin D1 and cyclin E measured from each sample. GAPDH is used as a reference for comparison because it is a house-keeping gene and is assumed that the mRNA level of GAPDH in the same type of cells is more or less the same.

2.5 Methods related Western Blotting

2.5.1 Preparation of samples for protein assay

Cells seeded on the Petri dish was washed twice with 5ml of HBBS. 150μl of 2X sample buffer (see 7 appendix for the preparation) was added to the dish and then the cells were scraped off into the buffer by using a scraper. The mixture was transferred to an autoclaved eppendorf and 16μl of β-mercaptoethanol was added before being boiled at 100°C for 5 minutes. 10μl was taken for protein assay.

2.5.2 Protein assay

Protein concentration in the suspension was determined by Bradford method. 40μl of Bradford Reagent were added into the wells containing standard or samples. Samples were subjected to absorbance measurement by spectrophotometer at 595nm.

2.5.3 SDS-PAGE

The percentage of acrylamide used depends on the size of the protein to be separate. Lower percentage of gel was used to resolve larger size of protein.

12.5% SDS resolving gel solution was prepared by mixing 15 ml of 30% acrylamide stock solution, 9 ml of gel running buffer (see appendix 8 for preparation) and 12 ml of MilliQ water. 147 µl of 10 % fleshly prepared ammonium persulphate (APS) (see appendix 9 for preparation) and 15µl TEMED were then added to the mixture. A gel casting apparatus was set up according to manufacturer's instructions, and the mixture was then loaded into the gel casting apparatus. Water-saturated n-butanol was added on the top of the gel to eliminate formation of meniscus gel and to exclude oxygen, which would otherwise inhibit polymerization on the gel surface. The gel was allowed to stay at room temperature for 1 hour for polymerization. Stacking gel solution was prepared by mixing 2ml of 30% acrylamide stock solution, 3ml of gel stacking buffer (see appendix 10 for preparation) and 7ml of MilliQ water. 100 µl of 10 % fleshly prepared APS and 10µl TEMED were then added to the mixture.

The water-saturated n-butanol on the resolving gel was removed and a comb was inserted between the glasses. Stacking gel mixture was loaded on the top of the resolving gel. The gel was incubated at room temperature for 1 hour for polymerization of the stacking gel. Afterwards, the comb was removed from the gel and wells were rinsed with tank buffer. 60 μg of protein extract (10 μl of extract and sample buffer mixture) and protein marker (10μl) (invitrogen) were loaded into the wells and the gel eletrophoresis chamber was filled with running buffer (see appendix 11 for preparation). Proteins with different size were resolved at a constant current (40-50mA/gel) at room temperature for 2-3 hours.

2.5.4 Western Blotting

After the gel running process had completed, the gel was removed from the gel apparatus and the stacking gel was removed. The separating gel, which resolved the proteins, was used for western blotting.

Proteins resolved by the gel were transferred to Hybond-C membranes using a wet-transfer method. Generally, the membrane and two pieces of 3mm Whatman paper were cut to the size of the gel. The membrane, the 3mm paper, two sponges

were soaked in 1X transfer buffer (see appendix 12 for preparation), and were placed into the transfer gel in the following sequence:

Sponge → 3mm paper → gel → membrane → 3mm paper → Sponge

Special care was taken to ensure that all air bubbles were removed so that the transfer process was not affected. The transfer apparatus was assembled and was putted in a transfer chamber filled up with transfer buffer. The transfer apparatus must be placed in an orientation that the gel was faced to the negative pole while the membrane was faced to the positive pole so that the proteins were able to migrate to the membrane through the electric field. The transfer process was carried out overnight under 30mA at 4°C.

2.5.5 Probing with antibodies

After the transfer process, the membrane was taken out from the apparatus and was blocked by incubation with 1% non-fat milk in PBS and 0.1% of tween 20 (MPT) (see appendix 13 for preparation) for 1 hour at room temperature. The blot was subsequently incubated with primary antibody (see appendix 14 for preparation) for 1

hour at room temperature. The choice of primary antibody depends on the protein of interest. The blots were then washed five times with MPT in order to remove unbounded antibody. Each wash was for 5 minutes. The blot was then incubated with a Horseradish peroxide (HRP) conjugated secondary antibody (see appendix 15 for preparation) for 1 hour at room temperature. Afterwards, the blot was washed four times with MPT to remove the unbounded secondary antibody and was washed one time with 0.1% tween 20 in PBS to remove the non fat milk. Again, each wash was for 5 minutes.

2.5.6 Visualization of proteins

The membrane was placed on a sheet of cling film with protein side up, after the excess washed buffer was drained. Two reagents of the enhanced chemiluminescence system (ECL) were mixed in a ratio of 1:1 and were added to the membrane. Usually, 3 ml of the ECL mixture was added to a membrane, but the actual volume was dependent on the size of the membrane. After 1 minute incubation with the solution at room temperature, the excess reagent was drained off and the blot was wrapped and placed protein side up in an X-ray film cassette (Hypercasstte). The cassette was then taken over to a dark room for autoradiography film development.

In a dark room, a sheet of autoradiography film was placed on top of the membrane for detection of the resulting light emission. The exposure times were dependent on the signal strength and therefore were highly variable. It could vary from 30 seconds to 1 hour. Afterwards, the film was put in a developer and protein bands were visualized.

2.5.7 Striping the antibody

Stripping of the membrane allows the membrane to be re-probed with another antibody. It was simply done by washing the membrane in 0.2M sodium hydroxide 3 times. The wash was 5 minutes for each time. Afterwards, the membrane was washed with PBS for 3 times. Again, the wash was 5 minutes for each time. The membrane was blocked and reprobed as described (please refer to section 2.5.6).

Chapter Three

Results

Previous studies have showed that high level of Brn-3b promotes cell proliferation in cancer cells (Irshad et al. 2004;Sonia et al. 2005). Moreover, Brn-3b has been found to upregulate the expression of CDK4 and cyclin D1 (Samady et al. 2004). Therefore, Brn-3b may enhance cell proliferation by regulating the expression of various cell cycle regulators. However, the expression pattern of Brn-3b during the cell cycle and when it activates its target genes such as cyclin D1 are not known. Therefore, in this study, the expression pattern of Brn-3b and the relationship between Brn-3b and cyclin D1 in ND7 and MCF7 cells were studied. The relationship between Brn-3b and cyclin E was also investigated.

A high level of Brn-3b expression has been observed in various cancer cells (Irshad et al. 2004;Sonia et al. 2005). However, the cause of the increase expression of Brn-3b in the cancer cells it is still to be elucidated. In this study, a carcinogen benzo(a)pyrene was used to treat the cells to investigate whether this carcinogen could increase the level of Brn-3b in the cells or not.

Thus, in this study, three different sets of experiments were carried out on ND7 cells; they were the cell cycle arrest of the cells following various treatments (refer to section 3.1), the addition of full serum medium after cell growth in serum free medium (refer to section 3.2) and cell exposure to a carcinogen benzo(a)pyrene (refer to section 3.3). FACS analysis, quantitative RT-PCR and western blotting were conducted in parallel for each experiment.

The same sets of experiments were to be carried out in parallel on MCF7 cells. However, due to the limitation of time, only the experiment regarding to the cell cycle arrest of the cells under various treatments was performed (refer to section 3.4).

3.1 The experiment on cell cycle arrest of ND7 cells following various treatments

In this study, the relationship of Brn-3b, cyclin D1 and cyclin E in a specific phase of the cell cycle were studied. To do this, $1X10^6$ ND7 cells were seeded on Petri dishes and were subjected to various treatments including 5-fluorouracil, hydroxyurea, colchicines, doxorubicin and etoposide. The concentrations of the various drugs were chosen based on experiment currently undertaken in our laboratory that were shown to effectively arrest cells in a specific phase without causing massive cell death. The

cells were harvested after 12 hours of treatments and were processed for FACS analysis, quantitative RT-PCR and western blotting (please refer to chapter 2 for details).

3.1.1 The changes in percentage of ND7 cells in different stages of the cell cycle following various treatments

To confirm the ND7 cells were arrested in a particular phase in the cell cycle following specified treatments, FACS analysis was carried out. In addition, cells growing in DMSO were also used as a control for cells treated with colchicine, doxorubicin and etoposide. The reason for this was because DMSO was the solvent of those drugs. Thus, this control was used to ensure that the effects observed in cells treated with colchicine, doxorubicin and etoposide were not caused by the solvent.

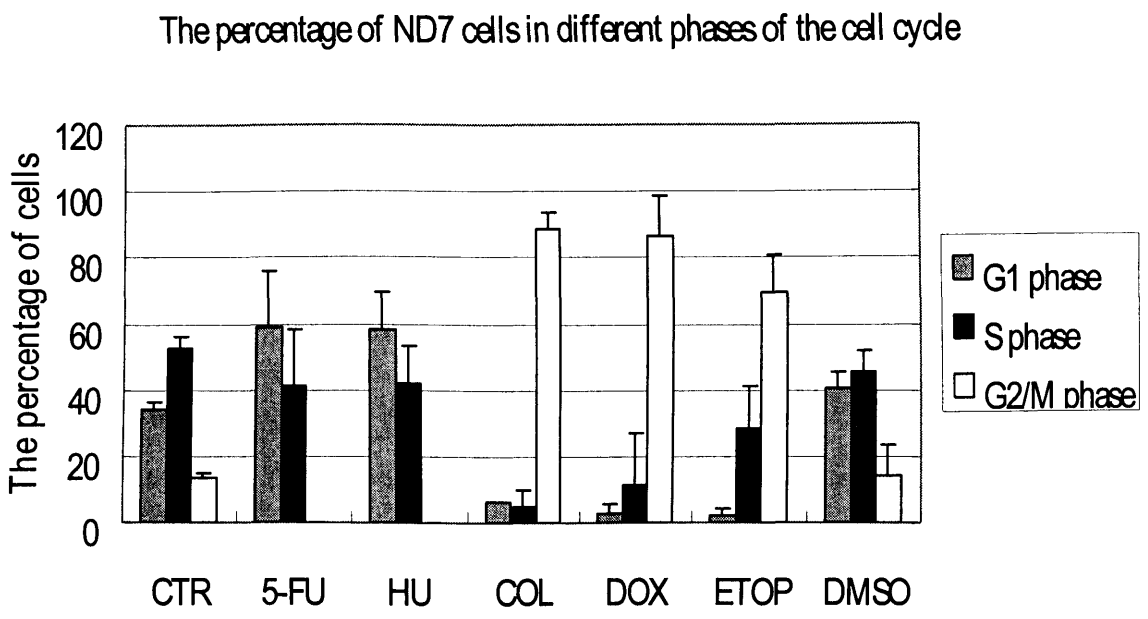

Figure 3.1 The percentage of ND7 cells in different phases of the cell cycle following various treatments. $1X10^6$ of cells were plated out in full serum medium and were left on for 12 hours. Treatments including 5-fluorouracil (5-FU), hydroxyurea (HU), colchicine (COL), doxorubicin (DOX) and etoposide (ETOP) were added for 12 hours. DMSO, which was used as a control, was added to the cells for 12 hours. All the cells were harvested at the same time for FACS analysis.

As shown in Figure 3.1, ND7 cells treated with 5-fluorouracil and hydroxyurea were arrested in the G1 phase when compared with the untreated control, where 58.7% and 58.4% of cells were arrested in G1 phase respectively. These drugs were the most effective at arresting cells in the G1 phase of the cell cycle.

From the results of the FACS analysis (Figure 3.1), cells treated with colchicine, doxorubicin and etoposide were arrested in G2/M phase when compared with the untreated and DMSO controls. Colchicine, doxorubicin and etoposide were able to

arrest 88.5%, 86.5% and 70% of cells respectively in the G2/M phase.

3.1.1.1 Optimization of the concentrations of 5-fluorouracil and hydroxyurea to arrest more ND7 cells in G1 phase of the cell cycle

From the results of FACS analysis, 60% of cells were arrested in G1 phase following the treatments with 5-fluorouracil and hydroxyurea. As we wished to arrest up to 80% of ND7 cells in the G1 phase of the cell cycle, further experiments were carried out to test whether higher concentrations of treatments with the 5-fluorouracil and hydroxyurea could arrest more cells in G1 phase of the cell cycle. In this experiment, the concentration of 5-fluorouracil used to treat cells was increased from 1μg/ml to 2.5μg/ml whereas the concentration of hydroxyurea used to treat cells was increased from 1mM to 10mM.

From the results (Figure 3.2), an increase in the concentrations of 5-fluorouracil and hydroxyurea did not induce more cell arrest in G1 phase of the cell cycle. Different concentrations of 5-fluorouracil gave a very similar cell cycle distribution results. Interestingly, an increase in the hydroxyurea concentration even reduced the percentage of cells distributed in the G1 and S phase. Therefore, for the subsequent

study, 1μg/ml of 5-fluorouracil and 1mM of hydroxyurea were used to treat cells.

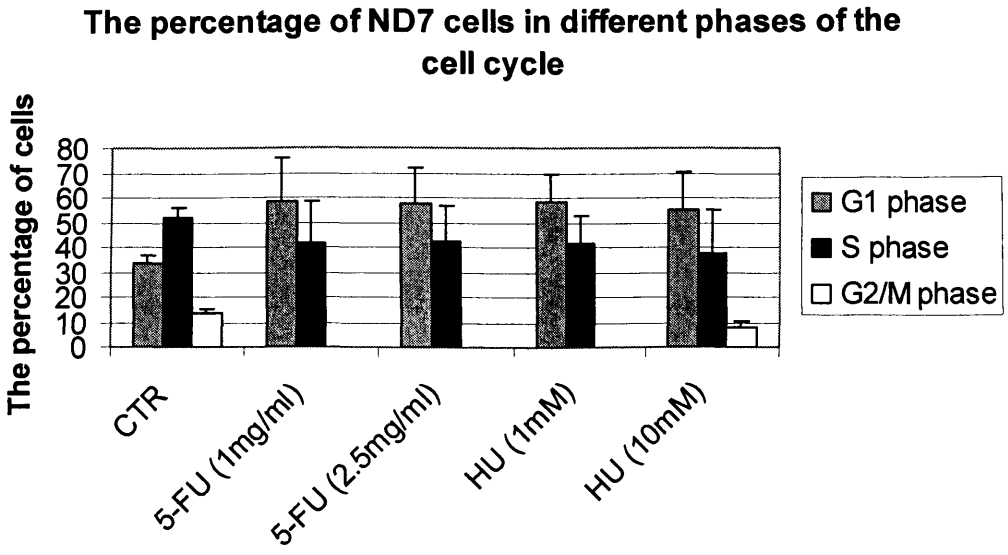

Figure 3.2 The percentage of ND7 cells in different phases of the cell cycle following treatments with 5-fluorouracil and hydroxyurea at different concentrations. $1X10^6$ of cells were plated out in full serum medium and were left for 12 hours. Treatments including 1mg/ml of 5-fluorouracil, 2.5mg/ml of 5-FU, 1mM of hydroxyurea (HU), and 10mM of HU, were added for 12 hours. All the cells were harvested at the same time for the FACS analysis.

In summary (refer to Table 3.1), a higher percentage of ND7 cells treated with 5-fluorouracil and hydroxyurea were arrested in G1 phase whereas a higher percentage of ND7 cells treated with colchicine, doxorubicin and etoposide resulted in cell arrest in the G2/M phase. These results confirmed that the drugs were able to arrest cells in a specific phase of the cell cycle, providing a model for further study of the mRNA levels of Brn-3b, cyclin D1 and cyclin E in cells arrested at different stages of the cell cycle.

	Cell cycle arrest (%)		
	G1	S	G2/M
Full growth medium for 24 hours	34	52.3	13.7
5-fluorouracil (1μg/ml)	**58.7**	41.3	0
Hydroxyurea (1mM)	**58.4**	41.6	0
Colchicine (1μM)	6.1	5.4	**88.5**
Doxorubicin (1μM)	2.5	11	**86.5**
Etoposide (10μM)	2	28	**70**
DMSO (0.1%)	40.7	45.3	14

Table 3.1 The summary of the cell arrest of ND7 cells following various treatments.

3.1.2 Changes in the mRNA levels of Brn-3b, cyclin D1 and cyclin E in ND7 cells following various treatments

In parallel with the FACS analysis, mRNA levels of Brn-3b, cyclin D1 and cyclin E in ND7 cells following various treatments were investigated by quantitative RT-PCR. The cells were seeded and treated in the same conditions as that for FACS analysis. RNA were extracted from the cells and were used for cDNA synthesis. By quantitative RT-PCR, the mRNA levels of Brn-3b, cyclin D1 and cyclin E were normalized against the GAPDH control.

As shown in Figure 3.3, the mRNA levels of Brn-3b in ND7 cells following treatments with 5-fluorouracil and hydroxyurea increased when compared to the untreated control. In particular, the cells treated with 5-fluorouracil showed a higher level of Brn-3b level than the cells treated with hydroxyurea. However, upon treatment with colchicine, doxorubicin and etoposide, the mRNA levels of Brn-3b were low compared with the untreated and DMSO control.

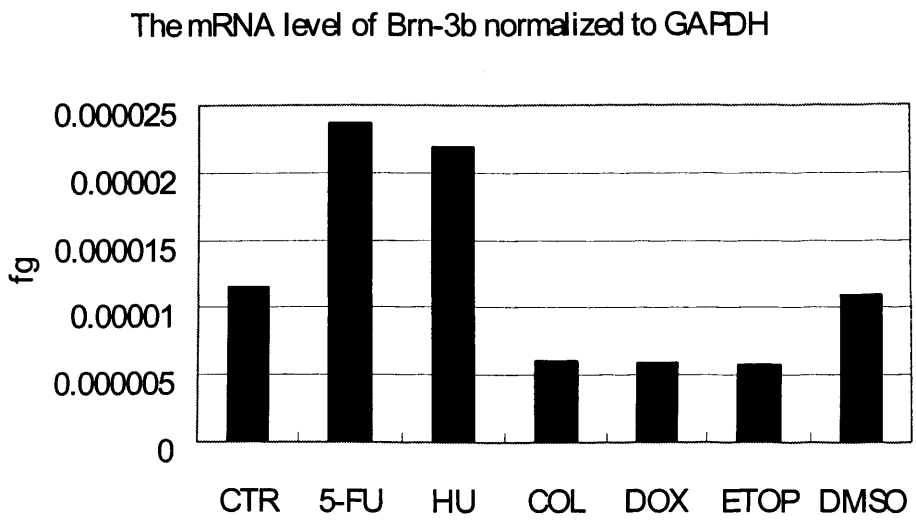

Figure 3.3 The mRNA level of Brn-3b in the ND7 cells following various treatments. The mRNA level of the Brn-3b was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.1. The mRNA levels of Brn-3b and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

The mRNA levels of cyclin D1 in these treated cells are shown in Figure 3.4. Following the treatment with 5-fluorouracil and hydroxyurea, the mRNA levels of cyclin D1 in the ND7 cells increased when compared to the untreated control. Interestingly, the increase in cyclin D1 mRNA level with these treatments was similar to the increase of Brn-3b in these cells. The mRNA level of cyclin D1 in the ND7 cells treated with colchicine, doxorubicin and etoposide was low when compared with the untreated and DMSO controls.

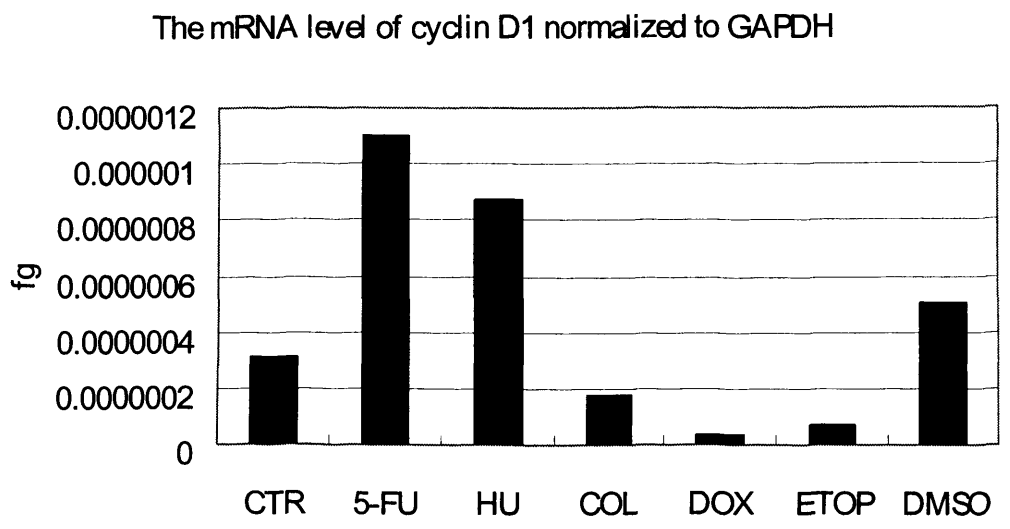

Figure 3.4 The mRNA level of cyclin D1 in the ND7 cells following various treatments. The mRNA level of the cyclin D1 was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.1. The mRNA levels of cyclin D1 and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

The mRNA levels of cyclin E in the ND7 cells were also measured. From Figure 3.5, it shows that the mRNA level of cyclin E were higher in the cells treated with 5-fluorouracil and hydroxyurea when compared to the untreated control. Treatment with hydroxyurea induced a higher increase in cyclin E mRNA level in the ND7 cells than that of the treatment with 5-fluorouracil. Again, the mRNA levels of the cyclin E in the cells treated with colchicine, doxorubicin and etoposide was low when compared with the untreated and DMSO controls.

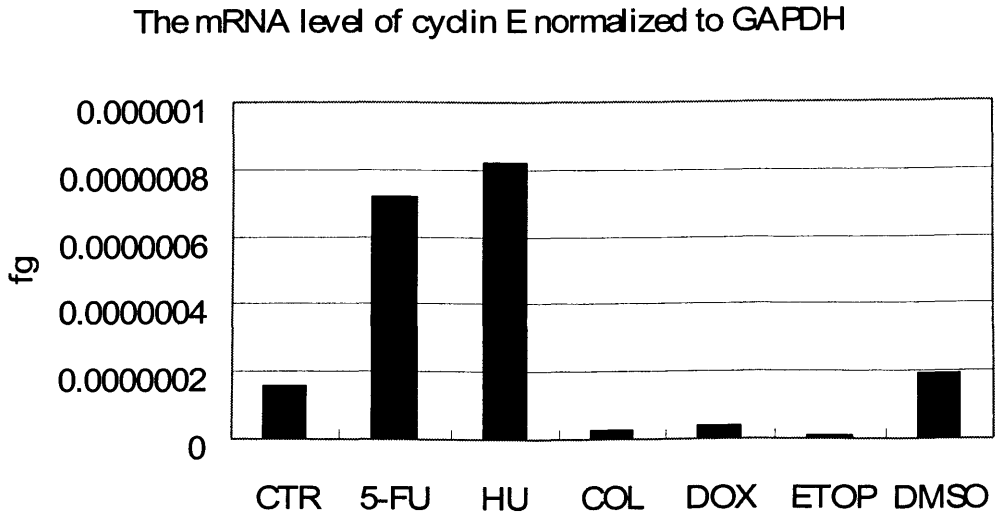

Figure 3.5 The mRNA level of cyclin E in the ND7 cells following various treatments. The mRNA level of the cyclin E was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.1. The mRNA levels of cyclin E and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

Taken together, high mRNA level of Brn-3b was observed when a high percentage of cells were arrested in the G1 phase of the cell cycle whereas the mRNA level of Brn-3b was low in the G2/M phase. This result suggests that Brn-3b levels changes as the cells progress in the cell cycle. Importantly, from the results of the quantitative RT-PCR, it was observed that the changes in the mRNA levels of cyclin D1 and cyclin E also correlated with the mRNA level of Brn-3b. When Brn-3b mRNA level increased, the cyclin D1 and cyclin E mRNA levels also increased whereas when the Brn-3b mRNA level decreased, the cyclin D1 and cyclin E mRNA levels decreased. The similarity in pattern of the mRNA levels of Brn-3b, cyclin D1 and cyclin E at different stages of the cell cycle suggests that Brn-3b may play a role in regulating the

expression of cyclin D1 and cyclin E.

3.1.3 The expression of Brn-3b in ND7 cells following various treatments

Western blotting was carried out to investigate the protein levels of Brn-3b in the ND7 cells following the specified treatments. As before the cells were seeded and treated under the same conditions as that for FACS analysis and quantitative RT-PCR. Proteins were extracted from the cells as described in section 2.3. Brn-3b was probed for and visualized using ECL. β-Actin was also probed to show that same amount of proteins were loaded.

From the results, no clear signals were observed (data not shown). The reason for this might be due to the fact that not enough proteins were loaded. However, due to the limitation of time, the western blotting analysis was not repeated.

3.2 Synchronization of cells to analyze for Brn-3b levels

The previous experiment to analyze changes in the mRNA levels of Brn-3b, cyclin D1 and cyclin E at different phases of the cell cycle following specified treatments

showed that the mRNA level of Brn-3b correlated to that of cyclin D1 and cyclin E. To further investigate whether the mRNA levels of Brn-3b and cyclin D1 change in synchronized cells to allow cells to progress in a uniform manner through the cell cycle, this study was untaken. Cells were synchronized at G1 phase by transferring serum free medium for an optimum time period to induce cell cycle arrest (refer to section 3.3.1), and then transferred back to full serum medium afterwards so that the cells resumed their cell cycle progression in a synchronized manner. Serum free medium was used to synchronize the cells because previous studies demonstrated that after transferred of ND7 cells into serum free medium, they are able to undergo cell cycle arrest at G1 phase of the cell cycle. The cells are then able to resume cycling in the cell cycle once full growth medium was added (Budhram-Mahadeo et al. 1994; Budhram-Mahadeo et al. 1995). In this experiment, the cells were harvested at 3, 6, 12, 24, 36 and 48 hours after being transferred back to full serum medium for FACS analysis, quantitative RT-PCR and western blotting.

3.3.1 Optimization of the time period for cell growth in serum free medium in order to induce cell cycle arrest without excessive cell death

The period of growth of ND7 cells in serum free medium is critical as not all the cells will undergo cell cycle arrest if the exposure time in serum free medium is too short whereas if the exposure time of cells in serum free medium is too long, increased cell death will occur so that fewer cells will be available for analysis. The optimum exposure time of cells in serum free medium depends on two factors—the percentage of cells that survive but arrest in the cell cycle and the percentage of cell death. To address this, an initial experiment was conducted to find out the optimum exposure time of cells in serum free medium.

3.3.1.1 The percentage of ND7 cells arrested in G1 phase and the percentage of cell death after transferring to serum free medium for 24, 48 and 72 hours

In this experiment, ND7 cells were cultured in full growth medium and serum free medium for 24, 48 and 72 hours respectively. The cells grown in full growth medium were used as controls to compare changes seen in the cells grown in the serum free medium at the three time points. Cells were harvested at appropriate time points for

FACS analysis and quantitative RT-PCR for testing the Brn-3b mRNA level.

As shown in Figure 3.6, the cells cultured in serum free medium for 48 hours showed the highest percentage of cells arrested in G1 phase whereas cells cultured in serum free medium for 72 hours showed the lowest percentage of cells arrested in G1 phase but the highest percentage of cells arrested in the S phase. For the ND7 cells cultured in the full growth medium for 24, 48 and 72 hours, they showed a similar cell cycle distribution pattern with more cells distributed in the S phase.

The percentage of cell death of ND7 cells cultured in full growth medium and serum free medium for 24, 48 and 72 hours were also determined by FACS analysis. As shown in Table 3.2, the highest cell death was recorded in cells cultured in serum free medium for 72 hours, and the lowest cell death was recorded in cells cultured in serum free medium for 24 hours. The percentage of cell death of ND7 cells cultured in full growth medium for 24, 48 and 72 hours remained low, approximately 2%.

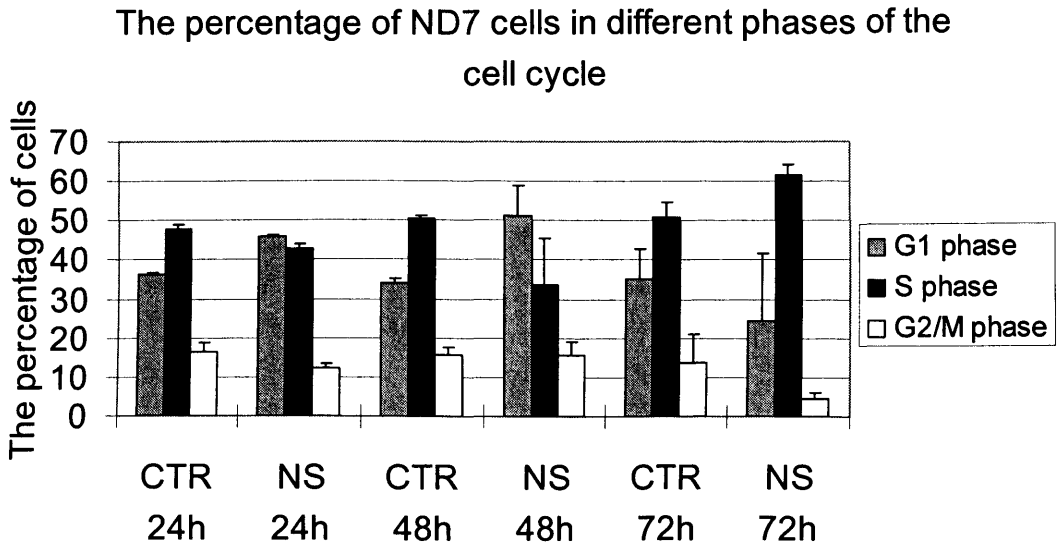

Figure 3.6 The percentage of ND7 cells in different phases of the cell cycle after culturing for 24, 48 and 72 hours in full growth medium and serum free medium respectively. $1X10^6$ of cells were plated in full growth medium for 24 hours and in serum free medium for 24, 48 and 72 hours respectively. $2X10^5$ cells were plated in full growth medium for 48 and 72 hours. The cells were harvested at appropriate time points for FACS analysis.

	24 hours	48 hours	72 hours
% of cell death in FGM	2%	3%	2.5%
% of cell death in SFM	2.5%	23%	47%
% of cells arrested in G1 in FGM	36.3	33.9	35.5
% of cells arrested in G1 in SFM	45.8	51.35	24.35

Table 3.2 The percentage of cell death and the G1 arrest in the cell cycle induced by growing in full growth medium (FGM) and serum free medium (SFM) for 24, 48 and 72 hours respectively.

Since the main aim of the experiment was to synchronize cells at a particular phase of the cell cycle and then allow the cells to go back into the cell cycle, serum free exposure for 48 hours was the optimum time period as it resulted in high percentage of cells arrest in G1 phase and the cell death rate was acceptable.

3.2.1.3 The mRNA levels of Brn-3b and cyclin D1 in ND7 cells cultured in full growth and serum free media for 24, 48 and 72 hours

The levels of Brn-3b mRNA and cyclin D1 mRNA in the ND7 cells cultured in both full growth medium and serum free medium for 24,48 and 72 hours respectively were investigated by quantitative RT-PCR. The levels of Brn-3b mRNA and cyclin D1 mRNA were normalized to GAPDH.

The results (Figure 3.7) clearly showed that the level of Brn-3b mRNA decreased when cells were cultured in serum free medium compared with the level of Brn-3b mRNA of cells cultured in full growth medium for the same period of time. Moreover, the level of cyclin D1 mRNA followed the same trend as the level of Brn-3b mRNA (Figure 3.8)—the level of cyclin D1 mRNA increased when the level of Brn-3b mRNA increased; the level of cyclin D1 mRNA decreased when the level of Brn-3b mRNA decreased. This observation was consistent with the observation in the previous experiment to analyze changes in the mRNA levels of Brn-3b, cyclin D1 and cyclin E at different phases of the cell cycle following specified treatments.

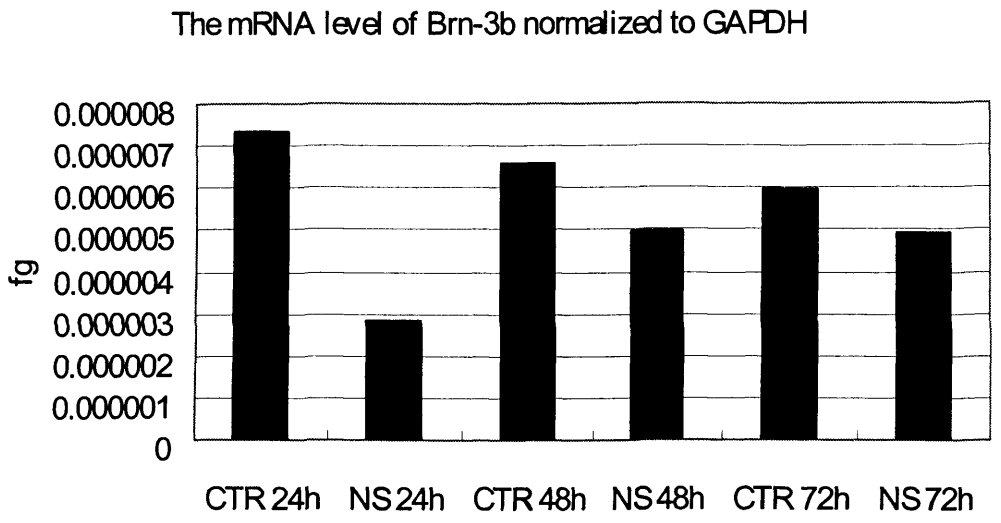

Figure 3.7 The mRNA level of Brn-3b in the ND7 cells after culturing for 24, 48 and 72 hours in full growth medium and serum free medium respectively. The mRNA level of the Brn-3b was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.6. The mRNA levels of Brn-3b and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

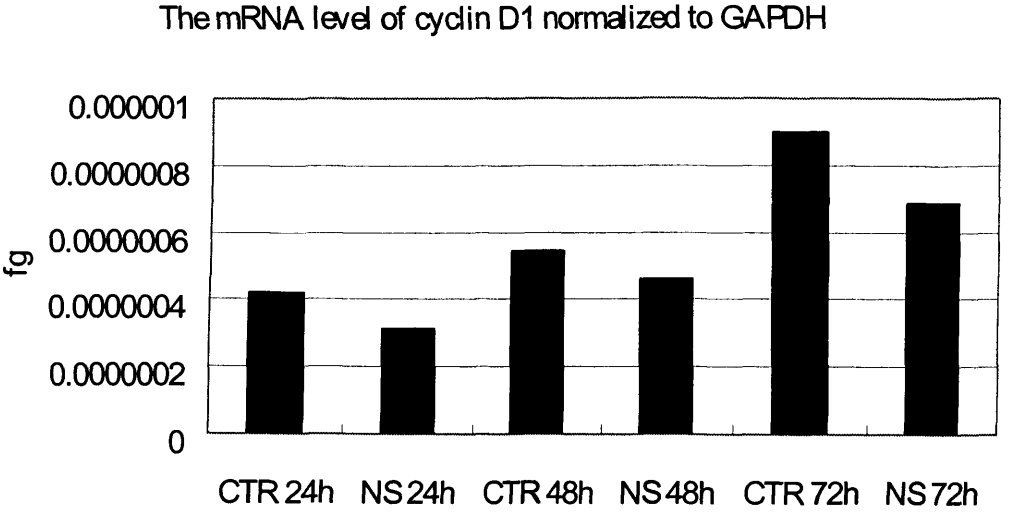

Figure 3.8 The mRNA level of cyclin D1 in the ND7 cells after culturing for 24, 48 and 72 hours in full growth medium and serum free medium respectively. The mRNA level of the Brn-3b was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.6. The mRNA levels of cyclin D1 and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

3.2.2 The percentage of ND7 cells distributed in different phases of the cell cycle after being transferred back to full growth medium

Following the optimization for treating cells in serum free medium, the experiment of transferring ND7 cells back to full growth medium was performed. After 48 hours of growth in serum free medium, the ND7 cells were transferred back to full growth medium containing 20% FCS for 3 (+FGM 3h), 6 (+FGM 6h), 12 (+FGM 12h), 24 (+FGM 24h), 36 (+FGM 36h) and 48 (+FGM 48h)hours respectively. The cells were then harvested at appropriate time points for FACS analysis, quantitative RT-PCR and western blotting.

The distribution of the cells at different phases of the cell cycle after they were transferred back to full growth medium at different intervals was estimated by FACS analysis. As shown in Figure 3.9, after 48 hours in serum free medium (NS 48h), 43% of cells were arrested in the G1 phase. After being transferred back to full growth medium, the cells started progressing through the cell cycle again. From the results, the cells being transferred to full growth medium containing 20% FCS for 3 and 6 hours showed that the percentage of cells in G1 phase decreased while a similar percentage of cells in G2/M phase increased. This suggested that the cells

synchronized in the G1 phase of the cell cycle induced by serum starvation were progressing from G1 phase to S phase while similar amount of cells were progressing from the S phase to the G2/M phase. For +FGM 12h, as the percentages of cells in G1 phase of the cell cycle were higher than that of the +FGM 6h while the percentages of cells in S phase of the cell cycle were lower than that of the +FGM 6h, this suggested that some cells were progressing from the S phase to the G2/M phase while some of the cells were progressing from the G2/M back to the G1 phase of the cell cycle. From the results of +FGM 24h, higher percentages of cells in the S phase and the G2/M phase were observed compared with the +FGM 12h, this suggested that the some of the cells of +FGM 24h were progressing from the G1 phase to the S phase of the cell cycle while some of the cells of +FGM 24h were progressing from the S phase to the G2/M phase of the cell cycle. Moreover, as the percentage of cells of +FGM 24h distributed in G1 phase was lower than that of +FGM 12h, this suggested that most of the cells were not progressing back to G1 phase of the cell cycle. The cells of +FGM 36h showed a similar cells distribution pattern to the CTR. However, as the percentage of cells of +FGM 36h in the S phase of the cell cycle was higher than that of the CTR, this suggested that some of the cells were progressing through the S phase of the cell cycle. For +FGM 24h, it showed a high percentage of cells distributed in the S phase, this indicated that most of the cells were progressing

through the S phase of the cell cycle.

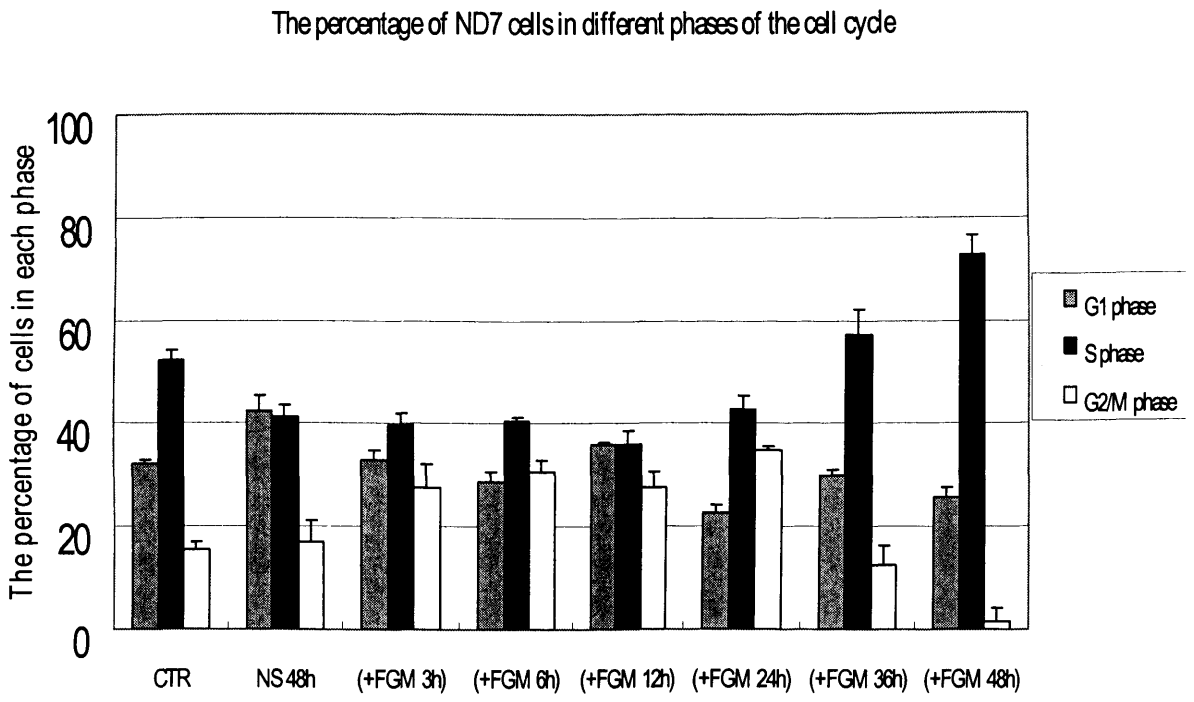

Figure 3.9 The percentage of ND7 cells in different phases of the cell cycle after the cells were cultured in full growth medium for 48 hours (CTR), serum free medium for 48 hours (NS 48h) and full growth medium containing 20% FCS for 3 (+FGM 3h), 6 (+FGM 6h), 12 (+FGM 12h), 24 (+FGM 24h), 36 (+FGM 36h) and 48 hours (+FGM 48h) respectively after being transferred back from serum free medium. $2X10^5$ of cells were plated out in full growth medium for 48 hours whereas $1X10^6$ of cells were plated out in serum free medium for 48 hours. The cells were transferring back to full growth medium containing 20% FCS for 3, 6, 12, 24, 36 and 48 hours respectively after growing in serum free medium for 48 hours. The cells were harvested at appropriate time points for FACS analysis.

3.2.3 The changes of mRNA levels of Brn-3b, cyclin D1 and cyclin E in ND7 cells transferred back to full growth medium after 48 hours of growth in serum free medium

The mRNA levels of Brn-3b and cyclin D1 in the ND7 cells after being transferred back to the full growth medium were estimated by quantitative RT-PCR in parallel to the FACS analysis. The cells were seeded and treated in the same conditions as that for FACS analysis. RNA were extracted from the cells and were used for cDNA synthesis. By quantitative RT-PCR, the mRNA levels of Brn-3b and cyclin D1 were analyzed and normalized against GAPDH.

From the results (Figure 3.10), the mRNA level of Brn3b decreased when the cells were cultured in serum free medium when compared with the untreated control. The mRNA level of Brn3b increased after 3 and 6 hours of cells being transferred back to full growth medium. However, the mRNA levels of Brn3b remained low after 12, 24, 36 and 48 hours after transferred back to full growth medium.

As shown in Figure 3.11, the mRNA level of cyclin D1 decreased when the cells were cultured in serum free medium when compared with the untreated control. After 3 and

6 hours of cells being transferred back to full growth medium, the mRNA level of cyclin D1 increased in both cases. However, the mRNA levels of cyclin D1 were low after 12, 24, 36 and 48 hours of cells being transferred back to full growth medium. Again, the mRNA level of cyclin D1 followed the trend of the mRNA level of Brn-3b.

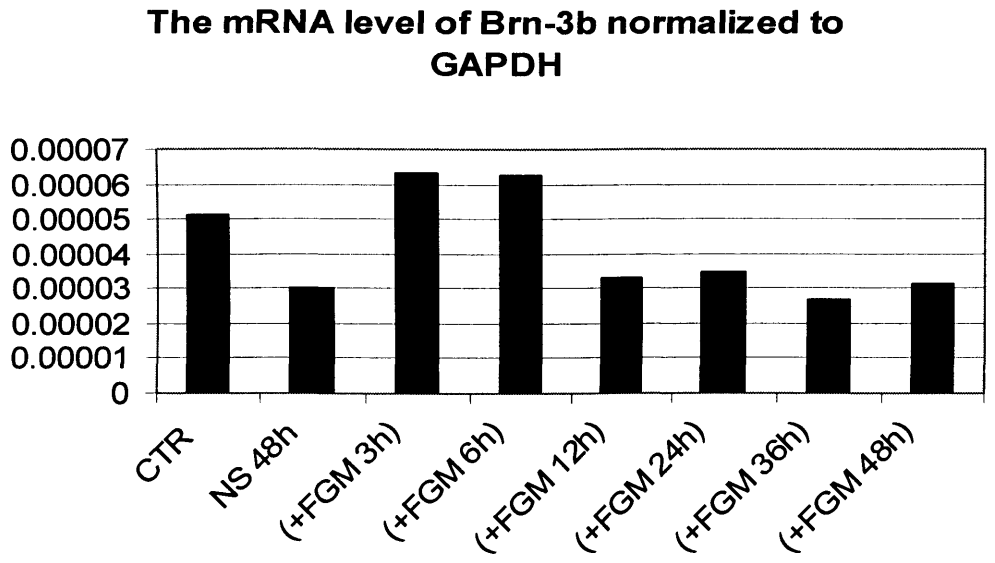

Figure 3.10 The mRNA level of Brn-3b in ND7 cells after the cells were cultured in full growth medium for 48 hours (CTR), serum free medium for 48 hours (NS 48h) and full growth medium containing 20% FCS for 3 (+FGM 3h), 6 (+FGM 6h), 12 (+FGM 12h), 24 (+FGM 24h), 36 (+FGM 36h) and 48 hours (+FGM 48h) respectively after being transferred back from serum free medium. The mRNA level of the Brn-3b was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.9. The mRNA levels of Brn-3b and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

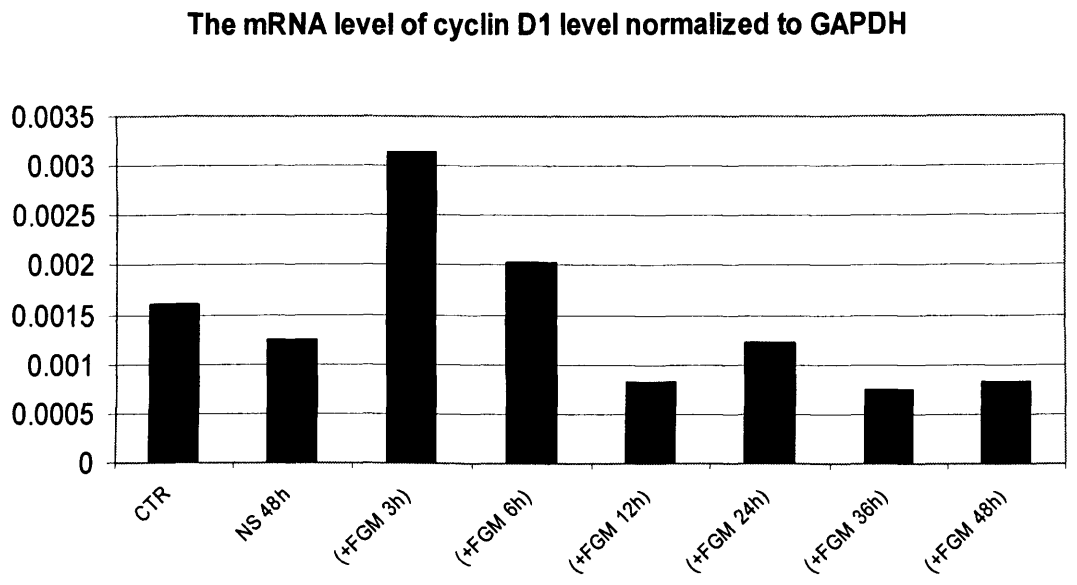

Figure 3.11 The mRNA level of cyclin D1 in the ND7 cells after the cells were cultured in full growth medium for 48 hours (CTR), serum free medium for 48 hours (NS 48h) and full growth medium containing 20% FCS for 3 (+FGM 3h), 6 (+FGM 6h), 12 (+FGM 12h), 24 (+FGM 24h), 36 (+FGM 36h) and 48 hours (+FGM 48h) respectively after being transferred back from serum free medium. The mRNA level of the cyclin D1 was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Figure 3.9. The mRNA levels of cyclin D1 and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

3.3 Benzo(a)pyrene exposure to ND7 cells

Benzo(a)pyrene is an carcinogen found as the environmental pollutant. Exposure to benzo(a)pyrene would increase the risk of tumorgenesis (Davis et al. 2002). Previous findings have shown that elevation of Brn-3b level correlates with changes that would contribute to tumorgenesis. Therefore, Benzo(a)pyrene may induce tumorgenesis by elevating the Brn-3b in cells. In this experiment, we test the idea of whether benzo(a)pyrene could increase Brn-3b in ND7 cells. Therefore, $1X10^6$ of ND7 cells were plated and incubated for 12 hours in full growth medium. After 12 hours, three different concentrations of benzo(a)pyrene were added to the medium—2.5, 5 and 10μg/ml. Acetone, which was used as a control as it was used to dissolve benzo(a)pyrene, were also added. After the incubation of different concentrations of benzo(a)pyrene and acetone for 12 hours, the cells were harvested for FACS analysis, quantitative RT-PCR and western blotting.

3.3.1 The changes in percentage of ND7 cells in different stages of the cell cycle following benzo(a)pyrene exposure

Due to the discrepancy between the data from set one and two, the data were analyzed separately. Nevertheless, the results (Table 3.3) showed that both cells subjected to addition of acetone and benzo(a)pyrene of different concentrations were arrested in the S phase of the cell cycle. This indicated that the cells arrested at the S phase of the cell cycle when cultured with benzo(a)pyrene were caused by acetone, which dissolved benzo(a)pyrene, rather than benzo(a)pyrene. If benzo(a)pyrene could arrest cells in S phase, more cells should have accumulated in the S phase of the cell cycle when the concentrations of benzo(a)pyrene were increased. Hence, the FACS result showed that benzo(a)pyrene did not cause cell cycle arrest.

	G1 Phase (%)		S phase (%)		G2/M phase (%)	
	Set 1	Set 2	Set 1	Set 2	Set 1	Set 2
Acetone	36.7	12	44.1	77.6	19.2	10.4
Benzo(a)Pyrene(2.5μg/ml)	38.3	22.9	44.8	77.1	17.1	0
Benzo(a)Pyrene(5μg/ml)	38.6	7.9	44.8	54.5	18.7	37.6
Benzo(a)Pyrene(10μg/ml)	37.9	29.2	44	70.8	18	0

Table 3.3 The percentage of ND7 cells in different phases of the cell cycle following exposure to benzo(a)pyrene at various concentrations. $1X10^6$ of cells were plated in full serum medium and were left for 12 hours. Subsequently, benzo(a)pyrene was added for 12 hours. Acetone, which was used as a control, was added to the cells for 12 hours. All the cells were harvested at the same time for the FACS analysis.

3.3.2 The mRNA level of Brn-3b in ND7 cells under Benzo(a)pyrene exposure

In this experiment, whether different concentrations of benzo(a)pyrene changes the mRNA level of Brn-3b was investigated using quantitative RT-PCR. RNA were extracted from the cells cultured in the same condition as the cells for FACS analysis, and the RNA were used for cDNA synthesis. By quantitative RT-PCR, the mRNA level of Brn-3b was estimated and normalized against GAPDH.

From the results (Figure 3.12), the mRNA level of Brn-3b failed to correlate with different concentrations of benzo(a)pyrene exposure. Among the different

concentrations, the concentration of 5μg/ml of benzo(a)pyrene seemed to induce an increase of mRNA level of Brn-3b, however, the mRNA level of Brn-3b in the cells following this exposure showed a lower mRNA level of Brn-3b than the untreated control. Therefore, no conclusive relationship between benzo(a)pyrene and Brn-3b could be drawn.

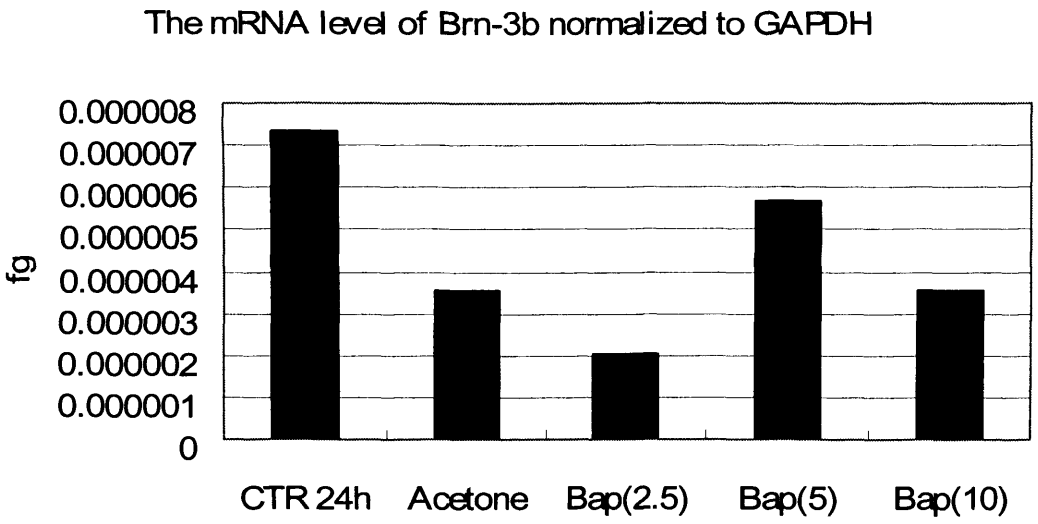

Figure 3.12 The mRNA level of Brn-3b in ND7 cells following exposure to benzo(a)pyrene at various concentrations. The mRNA level of the Brn-3b was normalized to the mRNA level of GAPDH in the same sample used. The experiment was carried out in the same conditions as the FACS analysis as stated in Table 3.4. The mRNA levels of Brn-3b and GAPDH in the ND7 cells were tested by quantitative RT-PCR.

3.4 The experiment on cell cycle arrest of MCF7 cells following various treatments

The experiments of cell cycle arrest were carried in parallel with MCF7 cells using the same set of drugs with the same condition. However, from the result of the FACS analysis (data not shown), an abnormal high percentage of cells in G1 phase was observed in the untreated control, indicating that the cells were not actively proliferating. Also, the results of the FACS analysis of the various treatments showed a high discrepancy, and no mRNA levels of Brn-3b, cyclin D1 and cyclin E were tested due to the limitation of time, therefore, the results of this experiment are not presented as no clear conclusion can be drawn.

Chapter Four

Discussion

Brn-3b was firstly identified from ND7 using novel degenerative oligonucleotides primers (Lillycrop et al. 1992). Subsequently, it has been found that Brn-3b is expressed in regions of the central and peripheral nervous system (Turner et al. 1994), and it plays an important role in regulating survival and development of specific subset of neurons in the retinal ganglia. Studies using Brn-3b knockout mice showed that lack of Brn-3b caused blindness in the mice because of a significant loss of retinal ganglion cells (Gan et al. 1996). Brn-3b has also been found to be expressed in other tissues such as the testis and breast (Budhram-Mahadeo et al. 2001) and has been showed to play a role in regulating growth and proliferation of these cells. Therefore, expression of Brn-3b is important for regulating normal cellular functions such as growth and proliferation. However, it has been shown that high levels of Brn-3b may contribute to uncontrolled proliferation in breast cancer cells and neuroblastoma cell lines, and therefore may cause tumorigenesis (Budhram-Mahadeo et al. 1999;Dennis et al. 2001;Irshad et al. 2004).

Previous studies have shown that increased expression of Brn-3b enhances

proliferation in neuroblastoma and breast cancer cells (Irshad et al. 2004;Sonia et al. 2005). Moreover, it has been found that Brn-3b upregulates CDK4 and cyclin D1 in MCF7 breast cancer cell lines. These suggest that Brn-3b controls cell proliferation, at least in part, by activating expression of cell cycle regulators such as cyclin D1 and CDK4, which cooperate to drive cell progression from the G1 phase to the S phase of the cell cycle. However, whether expression of Brn-3b changed at different phases of the cell cycle and when it activates its target gene cyclin D1 remain unclear. Thus, in this study, we aim to analyze the changes in mRNA levels of Brn-3b, cyclin D1 and cyclin E under different conditions.

Neuroblastoma ND7 and human breast cancer cell MCF7 were chosen for this study. Brn-3b is highly expressed in neuroblastoma cells if cells are proliferating but decreased if cells stop dividing and start differentiating (Latchman 2004). It has been observed that neuroblastoma cells that expresses high levels of Brn-3b proliferate more rapidly but also failed to respond to growth inhibitory effect signals such as retinoic acid and continued to proliferate in the presence of retinoic acid. Elevation of Brn-3b not only increases cell proliferation rate, but also enhances tumour *in vivo*. Increased Brn-3b in neuroblastoma cells have been observed to enhance growth in monolayer and under anchorage-independent conditions which is a critical

characteristic that is required for tumour cells to progress *in vitro*. A study using *in vivo* xenograft models showed that athymic nude mice with high expression levels of Brn-3b resulted in rapid growth of tumours. Moreover, a significant increase in the number of invading cells arising from clones had high Brn-3b expression, indicating that elevated Brn-3b expression enhances the invasiveness of the neuroblastoma cells (Irshad et al. 2004). As a transcription factor, Brn-3b changes the growth and behaviours of cells by regulating gene expression at the transcriptional level. At the molecular level, Brn-3b can repress the promoters of neuronal genes such as α-internexin (Budhram-Mahadeo et al. 1995) and neurofilament (Smith et al. 1997) which are important for neuronal differentiation so that the cells proliferate and fail to differentiate under appropriate conditions (Budhram-Mahadeo et al. 1995; Smith et al. 1997).

The significance of changing Brn-3b protein level on cellular growth and behaviour is further supported by the results of studies in the breast cancer cell line MCF7. Breast cancer cells with increased Brn-3b demonstrated increased anchorage-independent growth, invasiveness and resistance to chemotherapeutic drugs (Sonia et al. 2005). It appears that these effects are regulated by Brn-3b through its ability to alter expression of target genes. Increased Brn-3b upregulates the gene expression of

various target genes related to cell proliferation such as c-myc (Sonia et al. 2005), cyclin D1 (Samady et al. 2004) and estrogen receptor (Dennis et al. 2001). Increasing expression of these target genes are associated with increased proliferation in many breast cancers. Also, high level of Brn-3b elevates the gene expression of HSP27 and cathepsin D which have been shown to significantly enhance invasiveness and drug resistance of breast cancers (Sonia et al. 2005). Plakoglobin, which is associated with cell adhesion, is downregulated with increased Brn-3b, further supporting the role of elevated Brn-3b at enhaning tumourigenesis (Sonia et al. 2005).

In this study, as we aim to understand other mechanism that may cause increased proliferation or tumourigenesis triggered by Brn-3b, we set up a model to test whether the mRNA level of Brn-3b in ND7 cells changes in different phases of the cell cycle. To do this, the cells were arrested at different phases of the cell cycle following the specified treatments. It was observed that the mRNA level of Brn-3b in the cells increased compared with the control when the cells were arrested in G1 phase of the cell cycle, whereas the mRNA level of Brn-3b in the cells decreased compared with the control when the cells were arrested in G2/M phase of the cell cycle. This result suggested that Brn-3b protein may express in a cell cycle dependent manner.

The mRNA level of cyclin D1 and cyclin E were also measured in parallel to the level of Brn-3b mRNA in the cells following the specified treatments. Interestingly, the mRNA levels of cyclin D1 and cyclin E followed a similar trend to the mRNA level of Brn-3b in the cells; when the mRNA level of Brn-3b increased, the mRNA levels of cyclin D1 and cyclin E increased, and vice versa. These results indicated that Brn-3b, as a transcription factor, may regulate the expression of cyclin D1 and cyclin E through a direct or indirect pathway.

Having shown that the mRNA level of Brn-3b correlated to that of cyclin D1 and cyclin E when cells were arrested at different phases of the cell cycle following specified treatments, a further study to investigate whether the mRNA levels of Brn-3b and cyclin D1 change in synchronized cells to allow cells to progress in a uniform manner through the cell cycle was undertaken. Cells were synchronized by using serum free medium because previous studies have shown that deprivation of serum growth factor induces cell cycle arrest while addition of serum back to these synchronized cells resume progression through the cell cycle normally (Budhram-Mahadeo et al. 1994). The result clearly showed that the mRNA level of Brn-3b in the cells correlates to the mRNA level of cyclin D1 in these cells; when mRNA level of Brn-3b increased, the mRNA of cyclin D1 increased accordingly, and

vice versa. This observation, in agreement with the observation made with the cells arrested at a specific phase of the cell cycle following specified treatments, further confirmed the correlation between mRNA levels of Brn-3b and cyclin D1. This positive correlation of changes in the mRNA levels of Brn-3b and cyclin D1 suggested that Brn-3b, as a transcription factor, may upregulate the mRNA level of cyclin D1. Indeed, Brn-3b has been observed to activate the cyclin D1 expression by binding to the cyclin D1 promoter (Personal communication with Dr. Budhram-Mahadeo).

Cyclin D1 is crucial in cell cycle progression. It cooperates with CDK4 to allow the cell to progress through G1 phase by phosphorylating the retinoblastoma protein (RB). This in turn activates the E2F transcription factor and allows expression of genes such as cyclin E to drive cell cycle progression (Stacey 2003). Interestingly, overexpression of cyclin D1 accelerates the G1/S transitions (Resnitzky et al. 1994). As a result, this shortens the length of G1 phase and has implication to cancer (Eastman 2004).

The G1 phase is important for controlling cell fate. In this phase, cells decide whether to prevent cycling when, for example, there is significant DNA damage or lack of appropriate signals. This is crucial to prevent damaged DNA from passaging to the

daughter cells (Eastman 2004;Lewin 2000). The cell will undergo apoptosis if the damage cannot be rescued. Therefore, shortening the G1 phase shortens the time for cells to the make decision of either arresting or undergoing apoptosis when necessary. Thus, high levels of cyclin D1 is directly related to tumorgenesis (Johnson and Walker 1999). Indeed, RNA and protein levels of cyclin D1 have recently been found to be highly expressed in neuroblastomas, indicating its role in tumours (Noesel and Versteeg 2004).

Cyclin D1 protein overexpression is found in up to 50% of human breast cancers. Interestingly, overexpressed cyclin D1 has been shown to bind directly and activate the estrogen receptor in CDK and pRB-indpendent fashion. A large majority of cyclin D1-overexpressing breast cancers are ER positive. Cyclin D1 directly interacted with ER to trigger binding of ER to an estrogen responsive element and increased target gene transcription. Moreover, this effect is independent to CDK4 and pRB as cyclin D1 mutants that cannot bind to CDK4 or pRB can still exert this effect (Arnold and Papanikolaou 2005).

In this study, Brn-3b also appeared to regulate expression of cyclin E. Indeed, it has been suggested that high level of cyclin E play an important role in tumorigenesis by

causing a neuploidy (gains and losses of one or more chromosomes of a diploid genome), which is a form of genetic instability frequently observed in neuroblastoma (Noesel and Versteeg 2004). Generally, incorrect segregation of chromatids and aneuploidy are considered to result in amplification of centrosomes (Noesel and Versteeg 2004). Elevated cyclin E expression can enhance such aberrations of the centrosomes, and this leads to chromosomal instability in tumours (Fukasawa et al. 1996;Mussman et al. 2000).

Previous studies have shown that high level of Brn-3b in cancer cells inhibits expression of tumour suppressor genes such as BRCA-1 (Dennis et al. 2001). In this study, along with the unpublished results from communication with Dr. Budhram-Mahadeo, it is found that Brn-3b regulates gene expression of cyclin D1. Taken together, high level of Brn-3b increases the risk of tumourigenesis by regulating various gene expressions. However, the reason of the high expression level of Brn-3b remains unclear. Previous studies showed that exposure to carcinogens such as benzo(a)pyrene increases the risk for tumourigenesis (Davis et al. 2002). Taking this in mind, therefore it is possible that exposure of carcinogen is a factor to increase the expression of Brn-3b which in turn cause tumourigenesis.

In this study, we tested whether different concentrations of benzo(a)pyrene treatment would induce an increase of Brn-3b mRNA level in ND7 cells. However, it was found that the benzo(a)pyrene did not increase the Brn-3b mRNA level as there was no clear relationship between different concentrations of benzo(a)pyrene and changes in the level of Brn-3b mRNA .

Further experiments

As overexpression of cyclin D1 has been observed in various cancer cell lines, apart from the neuroblastoma and breast cancers, such as esophageal, bladder, lung, squamous carcinomas (Johnson and Walker 1999), and Brn-3b has also been detected to express in various cell types, thus it would be worthy to investigate whether Brn-3b upregulates cyclin D1 in other cancers.

Also, based on the finding of this study, further experiment could be carried out to investigate how Brn-3b regulate cyclin D1 at various stages of the cell cycle, for example early G1, mid G1 or late G1 phase of the cell cycle. Moreover, as shown in this study, mRNA level of Brn-3b also correlates to the mRNA level of cyclin E, therefore, further experiment could be carried out to confirm whether Brn-3b could

also regulate cyclin E or not.

Though it is clear that high level of Brn-3b protein causes tumourigenesis, however, the cause it is still unclear. Thus, further experiment can be carried out to investigate the cause of such elevation observed in tumours.

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Appendices

Appendix 1: Preparation of 10% FCS in L-15 with P/S

The 50ml of FCS(gold) was filtered by a filter with 45μm pore size and was added to 500ml of L-15. 4.3ml of 35% D-glucose solution was also filtered by a filter with 45μm pore size and was added to 500ml of L-15. 26.4ml of sodium bicarbonate and 585μl of L-glutamine were added to the medium. Finally, 5ml of P/S (100 U/ml penicillin and 100 μg/ml streptomycin) were added to medium.

Appendix 2: Preparation of 10% FCS in D-MEM with P/S

FCS was filtered by a filter with 45μm pore size and 50ml of it was added to 500ml of D-MEM to make up 10% FCS in D-MEM. 5ml of P/S (100 U/ml penicillin and 100 μg/ml streptomycin) were added to medium

Appendix 3: Preparation of serum free medium in L-15 with P/S

The procedures of preparing the serum free medium are same as that of preparing the L-15 full growth medium (please refer the appendix 1) except 10ml of filtered 10% bovine serum albumin was added instead of the FCS.

Appendix 4: Preparation of PBS

The PBS (Gibco) was prepared by dissolving the PBS tablet 500ml of MilliQ water. It should be highly mentioned that the PBS used in the tissue culture was not prepared in that way as they needed to be sterile.

Appendix 5: Preparation of PI buffer

250mg of sodium citrate was added to 250ml of MilliQ water. 250µl of TritonX100 was then added to the mixture.

Appendix 6: Preparation of DEPC

0.5ml of DEPC was added to 500ml of MilliQ water. The mixture was left on at room temperature for 2 hours, and was taken to autoclave.

Appendix 7: Preparation of 2X sample buffer

2X sample buffer was prepared by mixing 6g of sodium dodecyl sulphate (SDS), 1.4g of tris(hydroxymethyl)methylamine (Tris) and 20ml of glycerol to 100ml of MilliQ water.

Appendix 8: Preparation of gel running buffer

In order to prepare the gel running buffer, 2g of SDS, 90.9g of Tris were added to 300ml of MilliQ water. Finally, MilliQ water was then added to bring the final volume to 500ml.

Appendix 9: Preparation of 10% of APS

10% of APS was prepared by dissolving 20mg of APS powder (Sigma) into 200µl of MilliQ water.

Appendix 10: Preparation of gel stacking buffer

In order to prepare the gel running buffer, 2g of sodium dodecyl sulphate (SDS) and 30.25g of tris(hydroxymethyl)methylamine (Tris) were added to 300ml of MilliQ water. Finally, MilliQ water was then added to bring the final volume to 500ml.

Appendix 11: Preparation of running buffer

In order to prepare the running buffer, 30.3g of Tris, 144.2g of glycine and 10g of SDS were added to 600ml of MilliQ water. Finally, MilliQ water was then added to bring the final volume to 1000ml.

Appendix 12: Preparation of transfer buffer

In order to prepare the transfer buffer, 30.3g of Tris and 144.2g of glycine were added to 600ml of MilliQ water. Finally, MilliQ water was then added to bring the final volume to 1000ml.

Appendix 13: Preparation of MPT

The MPT was prepared by mixing 10g of non-fat milk powder (Saintsbury) with 1000ml of PBS and 1ml of Tween-20 (Sigma).

Appendix 14: Preparation of primary antibody

Primary antibody for western blotting was preparing by mixing the primary antibody chosen for the experiment with MPT. The volumes of the antibody and the MPT used depend on the ratio required to probe for a specific protein. Normally, the ratio of 1:1000 was used. To prepare this, 50µl of the primary antibody was added to 50ml of the MPT.

Appendix 15: Preparation of secondary antibody

Secondary antibody for western blotting was preparing by mixing the secondary antibody chosen for the experiment with MPT. The volumes of the antibody and the MPT used depend on the ratio required to probe for the primary antibody. Normally, the ratio of 1:3000 was used. To prepare this, 20µl of the secondary antibody was added to 60ml of the MPT.