STUDY ON REGULATION OF VGSCS EXPRESSION BY REST USING AZA IN MCF-7 BREAST CANCER CELL LINE.

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Study on regulation of VGSCs expression by REST using AZA in MCF-7 breast cancer cell line.

By

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CERTIFICATE

This is to certify that the dissertation entitle "Study on regulation of VGSCs expression by REST using AZA in MCF-7 breast cancer cell line" is bonafide record of research work done by Nursyuhana Binti Rason during the period from September 2013 to June 2014 under my supervision.

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"In the name of Allah, Most Gracious, Most Merciful"

"Over the knowledgeable, Allah the Most Knowledgeable"

All praises and gratitude is to Allah, the Lord to whom every single creature in the heaven and earth belongs to. Thank Allah for giving me the strength and patient during this challenging time. May peace and blessing be on the leader of all creation, the prophet Muhammad S.A.W., his family and companion.

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LIST OF ABBREVIATIONS

A	Absorbance
AZA	5-Azacytidine
bp	Basepair
CHGA	chromagranin A
DCIS	Ductal Carcinoma In Situ
DMEM	Dulbecco's Modified Eagle Medium
Da	Dalton
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
IDC	Infiltrating Ductal Carcinoma
ILC	Infiltrating Lobular Carcinoma
kDa	Kilo Dalton
LCIS	Lobular Carcinoma In Situ
mM	Milimolar
Na^+	Sodium
nm	Nanometer

nNav1.5	neonatal splice form of Nav1.5
NRSE	neuron-restrictive silencer element
NRSF	neuron-restrictive silencing factor
NTC	no template control
OD	Optical density
PCR	Polymerase chain reaction
psi	Pound per square inch
rcf	Relative centrifugal force
RE1	Repressor element 1
REST	Repressor element 1 silencing transcription factor
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide Gel Electrophoresis
SYP	Synaptophysin
TAE	Tris-Acetic Acid-EDTA
TNF α	Tumor necrosis factor alpha
TTX	tetrodotoxin
VGSC	Voltage-gated sodium channels
V	Volt

μg/mL	Microgram per mililitre
μL	Microlitre
μM	Micromolar
$[Na+]_I$	Concentration of sodium influx

Kajian mengenai pengawalanaturan ekspresi VGSCs oleh REST menggunakan AZA di dalam sel kanser payudara MCF-7.

Abstrak

Faktor transkripsi repressor elemen-1 (REST) menjadi pengantara kepada penekanan beberapa gen neuron di dalam sel bukan neuron. REST menekan gen sasaran melalui penyahasetilan histone, pembentukan semula *chromatin* dan metilasi. Kehilangan metilasi DNA telah dilaporkan sebagai acara awal *tumorigenesis* dan mungkin boleh menjelaskan peningkatan ekspresi gen neuron termasuk *voltage-gated sodium channels* (VGSCs) dalam sel kanser. Persoalannya sekarang, adakah VGSCs dalam sel kanser merupakan gen sasaran REST? Dalam kajian ini, perencat metilasi DNA, 5-azacytidine (AZA) telah digunakan untuk menghalang fungsi REST dan menguji kesannya terhadap proliferasi dan ekspresi gen sasaran REST dan VGSCs di dalam sel kanser payudara metastatik lemah, MCF-7. Ujian MTT telah dijalankan untuk menentukan kesan rawatan AZA pada pertumbuhan sel menggunakan kepekatan yang berbeza, 100 µM – 1 mM selama 24, 48 dan 72 jam. Pengekstrakan RNA total, sintesis cDNA, PCR, gel elektroforesis dan analisis imej telah dijalankan untuk mengkaji kesan AZA pada gen synaptophysin (SYP), chromagranin A (CHGA), kedua-duanya merupakan gen sasaran biasa REST dan VGSCs ($Na_v1.5$ dan $nNa_v1.5$). Keputusan menunjukkan AZA menyebabkan proliferasi MCF-7 menurun dengan meningkatnya dos di mana kesan paling tinggi pada kepekatan tertinggi, 1 mM selepas 72 jam rawatan. Ekspresi gen sasaran REST, SYP dan CHGA mempunyai corak peningkatan selepas rawatan menggunakan AZA. Menariknya, corak ekspresi $Na_v1.5$ dan $nNa_v1.5$ juga didapati meningkat, walaupun kenaikan ekspresi gen ini tidak ketara. Di sini, kemungkinan bahawa $Na_v1.5$ dan $nNa_v1.5$ dalam sel kanser payudara merupakan gen sasaran REST boleh

diandaikan, walaubagaimanpun kerja-kerja selanjutnya diperlukan untuk mengesahkan interaksi ini.

Study on regulation of VGSCs expression by REST using AZA in MCF-7 breast cancer cell line.

Abstract

The repressor element 1-silencing transcription factor (REST) mediates the repression of several neuronal genes in non-neuronal cells. REST represses its target gene through histone deacetylation, chromatin remodelling and methylation. Loss of DNA methylation has been reported as an early event in tumorigenesis and could possibly explained the increased expression of neuronal genes including voltage-gated sodium channels (VGSCs) in cancer cells. The question remain, is REST regulates VGSC expression in breast cancer? In this study, a DNA methylation inhibitor 5-azacytidine (AZA) was used to inhibit REST function and test its effects on proliferation and the expression of REST common target genes and VGSCs in the weakly metastatic breast cancer cells, MCF-7. MTT assay was carried out to determine the effect of AZA treatment on cell growth at different concentration, 100 μM – 1 mM for 24, 48 and 72 h. Total RNA extraction, cDNA synthesis, PCRs, gel electrophoresis and image analysis were conducted to investigate the effects of AZA on the gene expression of synaptophysin (SYP), chromagranin A (CHGA), both REST common target genes and VGSCs ($Na_v1.5$ and $nNa_v1.5$). Results showed that AZA caused a dose-dependent decreased on the viability of MCF-7 cells where the effect was prominent at highest concentration, 1 mM after 72 h of treatment. REST common target genes, SYP and CHGA had an increased pattern in expression after AZA treatment. Interestingly, an increased expression pattern of $Na_v1.5$ and $nNa_v1.5$ was also observed, though the increments of expression of these genes were not significant. Herein, a possibility that $Na_v1.5$ and $nNa_v1.5$ in breast cancer cells are REST target gene could be postulated, though further works are needed to confirm the interaction.

CHAPTER 1: INTRODUCTION

1.1 GENERAL INTRODUCTION

Breast cancer is the most common cancer of women and the second leading cause of female cancer-related deaths worldwide (Jemal *et al.*, 2008). Breast cancer also has become the commonest cancer in Malaysian women with incident rate of 46.2 per 100000 women (Yip et al., 2006). The main cause for the high mortality rate of breast cancer is due to the metastasis.

As shown by previous study, voltage-gated sodium channel (VGSC), one of the hallmarks neuronal genes are widely expressed in metastatic breast cancer. For example, *SCN5A* (encoding Nav1.5), mRNAs have been detected in breast cancer cell lines (Fraser *et al.*, 2005). Of these, a neonatal splice variant of $Na_v1.5$ is most abundant, and its mRNA is >1,800-fold higher in strongly metastatic MDA-MB-231 cells than weakly metastatic MCF-7 cells (Fraser *et al.*, 2005).

The repressor element 1-silencing transcription factor (REST) also known as neuron-restrictive silencer factor (NRSF) mediates the repression of several neuronal genes in non-neuronal cells. REST represses its target gene through histone deacetylation, chromatin remodelling and methylation. Loss of DNA methylation has been reported to be an early event in tumorigenesis and could possibly explained the increased expression of neuronal genes including VGSCs in cancer cells. Thus far, REST, has only been shown to affect VGSCs expression specifically $Na_v1.2$ gene that is in neuron (Kraner *et al.*, 1992; Chong *et al.*, 1995).

Meanwhile, 5-azacytidine (AZA), an analog of cytidine and a useful demethylating agent for epigenetic research (Christman, 2002) work by inhibit DNA methylation. Thus in the present study, we used AZA to examine the transcription of REST target gene and VGSCs ($Na_v1.5$ and $nNa_v1.5$). Treatments of MCF-7 cells with AZA were expected to increase the expression of $Na_v1.5$ and $nNa_v1.5$ considerably via methylation inhibition of promoters. The results presented here will provide a new possibility that $Na_v1.5$ and $nNa_v1.5$ in breast cancer cells could be REST target genes.

1.2 RATIONALE OF THE STUDY

The impact of breast cancer is huge. Breast cancer gives a lot of negative consequences toward patient themselves and their family in term of emotion and financial status. For example, once patient diagnose with breast cancer, they will start become depress as the disease not only can take away her femininity but even her life. Although some patient can be cured, often the healing costs are excessively onerous for the patient and family.

Secondary metastatic disease is the main cause of mortality and therefore targeting metastasis is an ideal therapeutic target (Yang *et al.*, 2012). VGSCs are widely expressed in metastatic cells from a number of cancers, including breast cancer and are highly associated with breast cancer aggressiveness. Hence, VGSCs in breast cancer such as of $nNa_v1.5$ could potentially serves as therapeutic target for breast cancer metastasis in the future.

However, plenty researches are needed to understand the regulation of $nNa_v1.5$ in breast cancer. There are suggestions that increase expression of VGSCs in breast cancer

could possibly due to epigenetic. Therefore, using AZA as a DNA methylation inhibitor, this study was designed to investigate the role and potential interaction of REST on VGSCs in MCF-7 breast cancer cell line.

1.3 RESEARCH OBJECTIVES

Generally this study is aimed to investigate whether an inhibition of DNA methylation using AZA is sufficient to enhance REST target gene transcription in breast cancer MCF-7 cell including VGSCs ($Na_v1.5$ and $nNa_v1.5$).

The specific objectives of this research are:

1. To determine the toxicity effect of the AZA on breast cancer MCF-7 cells.
2. To investigate the effect of AZA on the expression of synaptophysin, chromagranin A (both REST target genes), $Na_v1.5$ and $nNa_v1.5$ in breast cancer MCF-7 cells.

The overview of this study was sketched out as shown in Figure 1.1

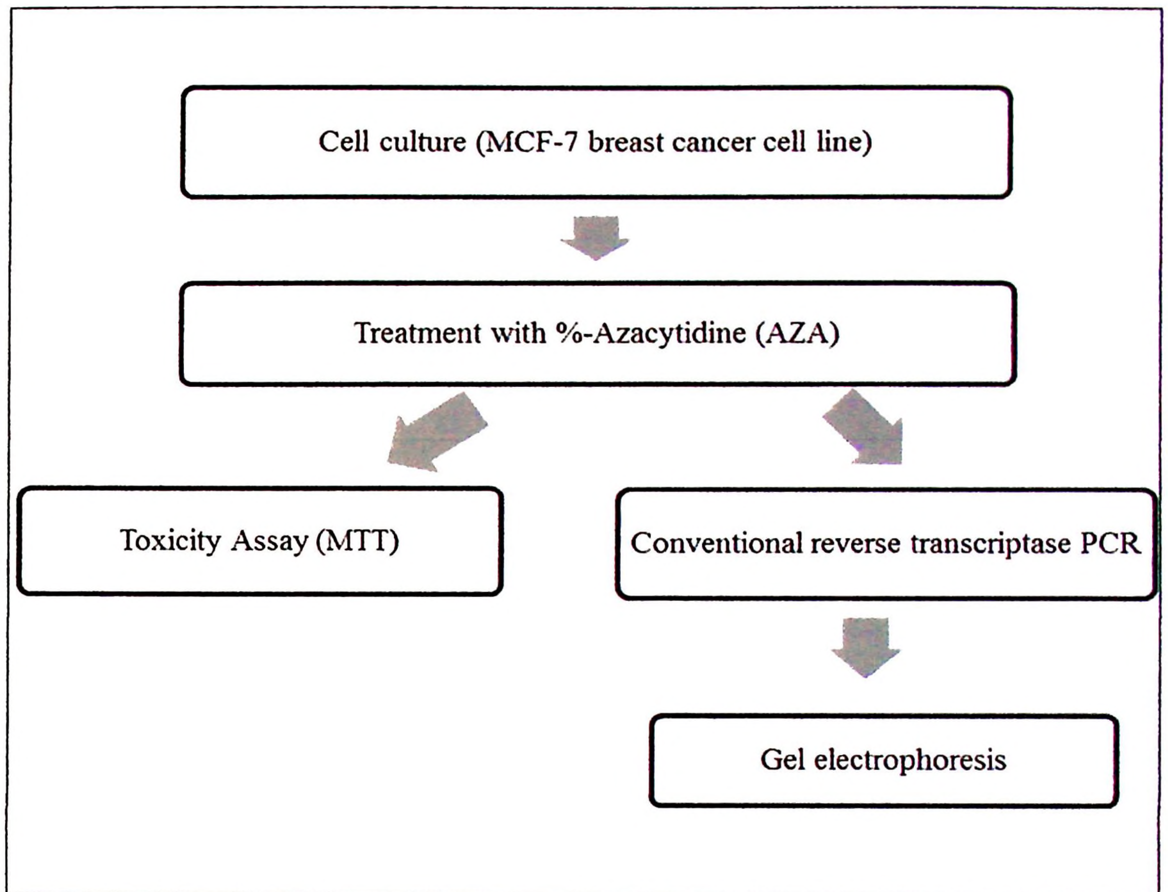

Figure 1.1: Overview of the study carried out.

CHAPTER 2: LITERATURE REVIEW

2.1 BREAST CANCER

2.1.1 Background

The word *cancer* refers to more than 200 related diseases affecting organs or systems of the body. Cancer that starts in the breast is called breast cancer. Cancer is a disease of cells. Normally, in a healthy body, cells replicate themselves by dividing in a regular, orderly manner so that body tissues can grow and repair any damage. If this normal function is disturb, there may be formed a lump called tumor by a group of uncontrolled growth cells. There are two types of tumor: benign and malignant. Benign tumors are not cancer but the malignant ones are called cancer (Peacock, 2001).

Most tumors in the breast are benign. They can be removed and usually do not grow back. They do not disperse and do not threaten a person's life. An example of common benign breast tumor is fibroadenoma. Malignant breast tumors (cancer) can also grow in the breast. These are dissimilar to benign tumor because they can be life threatening as they can detach from the original breast tumor and travel in bloodstream or lymphatic system to vital organs (such as the lung or liver) where they form new tumors known as metastases or secondaries (Pennery *et al.*, 2008).

Even though breast cancer is the second leading cause of female cancer-related deaths worldwide (Jemal *et al.*, 2008), and has become the commonest cancer in Malaysian women with incident rate of 46.2 per 100000 women (Yip *et al.*, 2006). Breast cancer can develop in anyone, including men, but of course the higher risk factors are being female

and aging. On the other hand, the risk factors for men having of breast cancer included growing older, a family history of breast cancer and enlarged breast.

2.1.2 Types of Breast Cancer

There are two main types of breast cancer, fundamental to the internal structure of female breast, which are lobular and ductal carcinomas. Breast cancers are called carcinomas because they derive from the cells lining an organ or system. Female breast have lobules (where women's milk is made and stored) and ducts (tubes which carry milk to the nipple). Breast cancer that is found in the lobules known as lobular carcinoma, and breast cancer found in the lining of milk ducts are called ductal carcinoma. Another form of breast cancer is Pagets's disease of the nipple (Peacock, 2001; Pennery *et al.*, 2008).

According to WHO 1981, breast cancer can be further divided into two main categories, noninvasive and invasive carcinoma. Noninvasive breast cancers are classified as lobular carcinoma in situ or ductal carcinoma in situ. Whereas invasive carcinomas include infiltrating lobular carcinoma, infiltrating ductal carcinoma, inflammatory breast cancer, mucinous carcinoma, tubular carcinoma, etc. (World, 1981)

2.1.2.1 Noninvasive Breast Cancer

Lobular carcinoma *in* situ (LCIS) is an early stage of breast cancer that develop within lobules of the breast and does not penetrate through the wall of the lobules. LCIS is hard to be detected as there are no specific clinical abnormalities, in particular, absence of a palpable lump, and, because most LCIS is not associated with microcalcifications, it is undetectable by mammography (Lakhani *et al.*, 2006).

Ductal carcinoma *in* situ (DCIS) is the most common form of noninvasive breast cancer. It presents in the duct and remain confine (Peacock, 2001). DCIS is mainly treated with mammary resection. Both lymph-node dissection and biopsy techniques are not indicated very often since axillary metastases are rare (Veronesi *et al.*, 2005).

2.1.2.2 Invasive Breast Cancer

Infiltrating lobular carcinoma (ILC) is a cancer that begin from lobules epithelium and accounted for about 10 percent of invasive breast cancer (Peacock, 2001). It is often hard to detect this type of breast cancer by mammogram or even by ultrasonography (Carlson *et al.*, 2011).

Infiltrating ductal carcinoma (IDC) is a cancer that starts in the ducts of the breast and then spreads to the fatty tissue of the breast or the other part of the body. IDC accounted for about 70 percent of breast malignancies (Peacock, 2001). It can be detected by physical examination, mammogram and radiography.

Inflammatory breast cancer is a rare type of infiltrating carcinoma that has spread to lymphatic vessels in the skin covering the breast. Cancer cells blocking lymph vessels cause the skin of the affected breast to look red and feel warm. The skin of breast may also thicken to the consistency of an orange peel. About 1% of invasive breast cancer are inflammatory carcinomas (Lopez and Porter, 1996).

2.1.3 Risk Factor

There is no absolute cause for breast cancer and doctors often cannot explain exactly why a patient develop breast cancer. The reason is breast cancer is a complex

disease that reflects the interaction between environmental and genetic risk factors (Zimmerman, 2004). The major cause of breast cancer is getting older as the breast cancer is more common found in older women. However, breast cancer can attack women at any age. This is significant because the hormonal status of female patients seems to play a role in the behavior of the disease. In younger women the tumors tend to be more aggressive, and the patients may have a less favorable prognosis (Saunders and Jassal, 2009).

Environmental factors that responsible for the increasing incidence of breast cancer are water pollutants, pesticides, radiation, alcohol, stress, oral contraceptives, hormone replacement therapy, and chemicals such as xenoestrogens. Many of these risk factors can be avoided by staying away from known risk factors whenever possible (Zimmerman, 2004).While the link between environment and breast cancer remains unclear, a number of genetic factors that increase the risk of developing breast cancer have been identified. Women who inherit susceptibility to breast cancer (altered BRCA1 and BRCA2 genes) have greater probability of developing this disease at a younger age.

2.2 METASTASIS

2.2.1 Background

Metastasis is the most fearsome aspect of cancer. It can be defined as the spread of cells from the primary tumor to the distant organ and their constant growth (Isaiah, 2003). Cells can disperse via blood vasculature, lymphatics, or within body cavities (Welch *et al.*, 2000). This fear is well founded. Despite significant improvements in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths

from breast cancer are due to metastases that are resistant to conventional therapies (Al-Hajj *et al.*, 2003).

Metastasis is a complex, multi-step process in which cancer cells (i) exit the primary tumor by disassociating from each other, degrade and detach from extracellular matrix, (ii) it is then migrate through local tissue and invade through basement membrane, (iii) intravasate into capillary vasculature or lymphatic vessels (iv) arrest at a distant site and extravasate, and (v) forms secondary tumors after proliferation and induction of angiogenesis (Onkal and Djamgoz, 2009; Alexander and Bendas, 2011). Step by step of the metastatic cascade is shown in Figure 2.1.

2.2.2 Metastasis and Breast Cancer

Based on 'seed and soil' hypothesis of metastasis, it explained that the pattern of metastasis is not random. The primary tumour cells (seed) have a specific affinity for the environment of certain organs (soil). Thus, metastasis formed only when the seed is well matched with the soil (Isaiah, 2003). In other word, there are some genes that mediate the breast cancer metastasis to other organs.

As shown by Bos *et al.*, (2009), the expression of α2,6-sialyltransferase ST6GALNAC5 gene in breast cancer cells enhances their adhesion to brain endothelial cells and their passage through the blood–brain barrier. Apart from that, the finding by Minn *et al.*, (2005) has shown that there are set of genes, denoted as promoting lung metastagenicity. Furthermore, together with bone, the lung is one of the most frequent targets of breast cancer metastasis in humans.

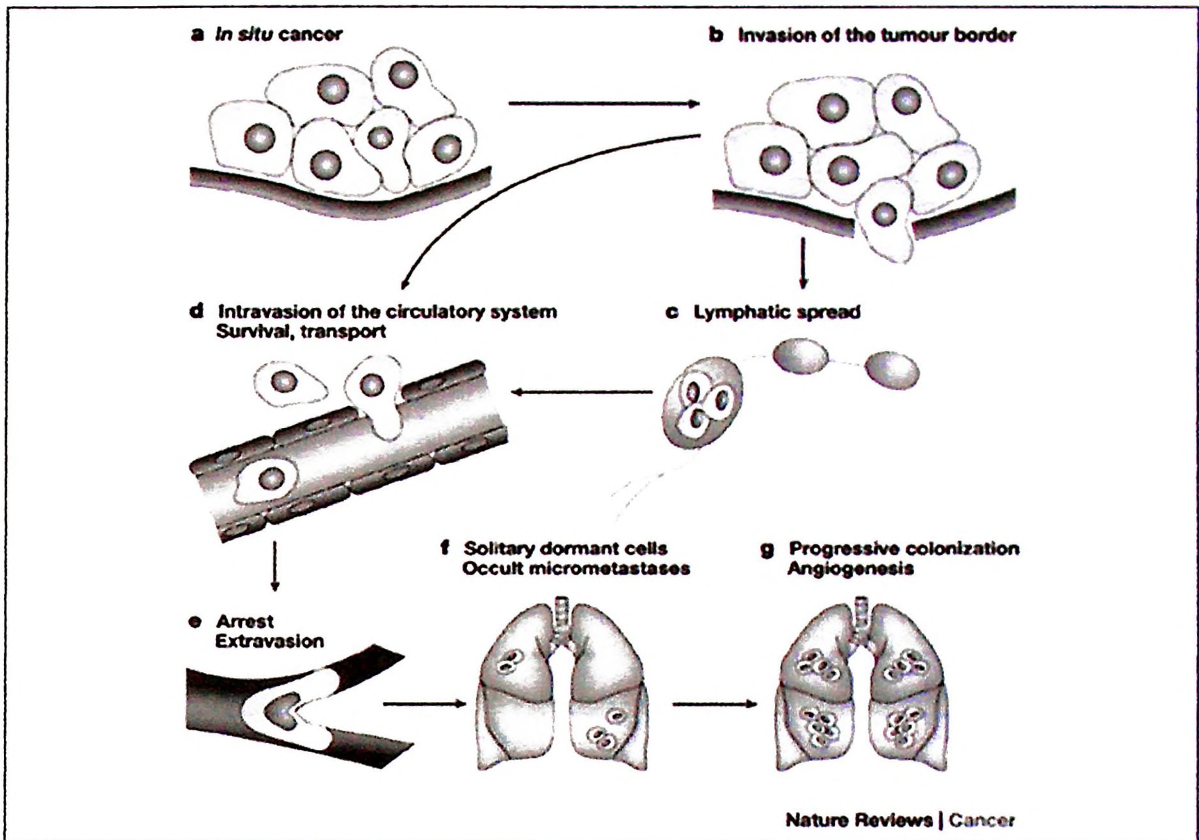

Figure 2.1: Schematic representation of the metastasis cascade.

The illustration gives insights into the step-by-step process of the metastasis: beginning with **a)** transformation of normal epithelial cells results in an in situ cancer surrounded which by an intact basement membrane. Followed by the **b)** detachment of tumor cells from primary tumor; invade the basement membrane. Then the metastasizing cells can intravasate into the **c)** lympatic vessel or **d)** blood system. **e)** Survival and arrest of tumour cells (in blood the cells associate with platelets and leukocytes to prevent shear forces and immune defense) and extravasation of the circulatory system occur. **f)** The next steps are physical arrest or arrest via interaction with the vascular endothel, generation of micrometastases and **g)** angiogenesis to the blood supply. Modified from (Steeg, 2003).

2.3 MCF-7 CELL LINE

The MCF-7 breast cancer cell line was derived from a pleural effusion taken from a patient with metastatic breast cancer and was isolated in 1970 from a 69-year-old Caucasian woman. MCF-7 is the acronym of Michigan Cancer Foundation-7 (Levenson and Jordan, 1997).

The characteristics of MCF-7 cells are the presence of estrogen and progesterone receptors. It also has a proliferative response to estrogens. In mice, MCF-7 is tumorigenic if it is supplemented with estrogen. There is no ERBB2 gene amplification (with Her2/neu protein overexpression) in MCF-7 cells (Ross and Perou, 2001; Charafe-Jauffret *et al.*, 2006). In addition, tumor necrosis factor alpha (TNF alpha) can inhibits the growth of MCF-7 breast cancer cells (Soule *et al.*, 1973; M. Leclercq, 2004).

Study done by Lv *et al.* (2010) has shown that the level of REST was relatively higher in MCF-7 cells and MDA-MB-468 cell lines, and it was relatively low in MDA-MB-231 and MDA-MB-435s cell lines. Apart from that, it was well known that MCF-7 cells were less malignant than MDA-MB-231 and MDA-MB-435s cell.

2.4 VOLTAGE GATED SODIUM CHANNEL (VGSC)

2.4.1 Background

Voltage-gated sodium channels (VGSCs) are responsible for the rising phase of the action potential in electrically excitable cells, including nerve, muscle, and neuroendocrine cell types. They are also expressed at low levels in nonexcitable cells, where their

physiological role is unclear. Sodium channels are the founding members of the ion channel superfamily in terms of their discovery as a protein and determination of their amino acid sequence roles, and pharmacological significance.

2.4.2 Structure of VGSC

VGSCs are large glycoproteins comprise of an α-subunit (250–260 kDa) that constitutes the central pore of the channel and at least two auxiliary β-subunits (30–40 kDa) that adjust the channel function and participate in the targeting to the membrane (Catterall *et al.*, 2005). The basic structure of VGSC is shown in Figure 2.2.

The mammalian VGSCα gene family contains at least 9 functional members (Nav1.1–Nav1.9, coded by genes SCN1A– SCN11A). Each VGSCα (220–260 kDa) that forms a functional ion (Na+-selective) pore with particular tetrodotoxin (TTX) sensitivity, electrophysiological and pharmacological properties, and tissue distribution (Onkal and Djamgoz, 2009).

2.4.3 Role of VGSC in metastasis

In previous study it has been reported that different VGSCs isoforms have been found in different cancers (Roger *et al.*, 2007). Interestingly, in metastatic breast cancer, VGSCs, predominantly the 'neonatal' splice form of Nav1.5 (nNav1.5), are upregulated and potentiate metastatic cell behaviors.

Fraser *et al.* (2005) has stated that in breast metastatic cancer cells, the activity of sodium channels are linked to the invasive properties of these cells as illustrated in Figure 2.3. In addition, Fraser and colleagues, (2005) previously reported that a sodium

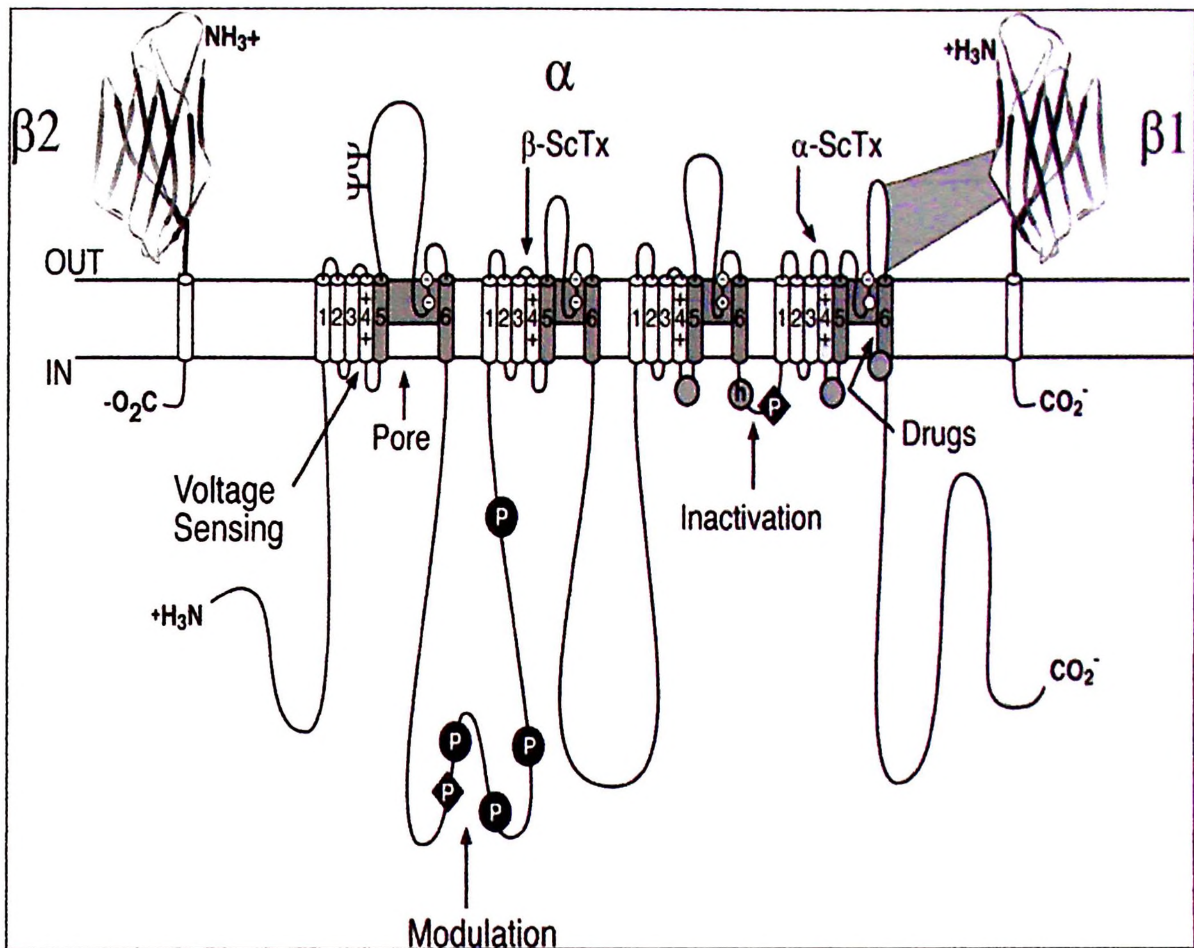

Figure 2.2: Transmembrane organization of sodium channel subunits.

The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane-folding diagrams. Cylinders represent probable α-helical segments. Bold lines represent the polypeptide chains of each subunit, with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the β1 and β2 subunits are shown as immunoglobulin-like folds. ψ, sites of probable N-linked glycosylation; P, sites of demonstrated protein phosphorylation by protein kinase A (circles) and protein kinase C (diamonds); shaded, pore-lining S5-P-S6 segments; white circles, the outer (EEDD) and inner (DEKA) rings of amino residues that form the ion selectivity filter and tetrodotoxin binding site; + +, S4 voltage sensors; h in shaded circle, inactivation particle in the inactivation gate loop; open shaded circles, sites implicated in forming the inactivation gate receptor. Sites of binding ofα- and β-scorpion toxins and a site of interaction between α and β1 subunits are also shown (Catterall *et al.*, 2005).

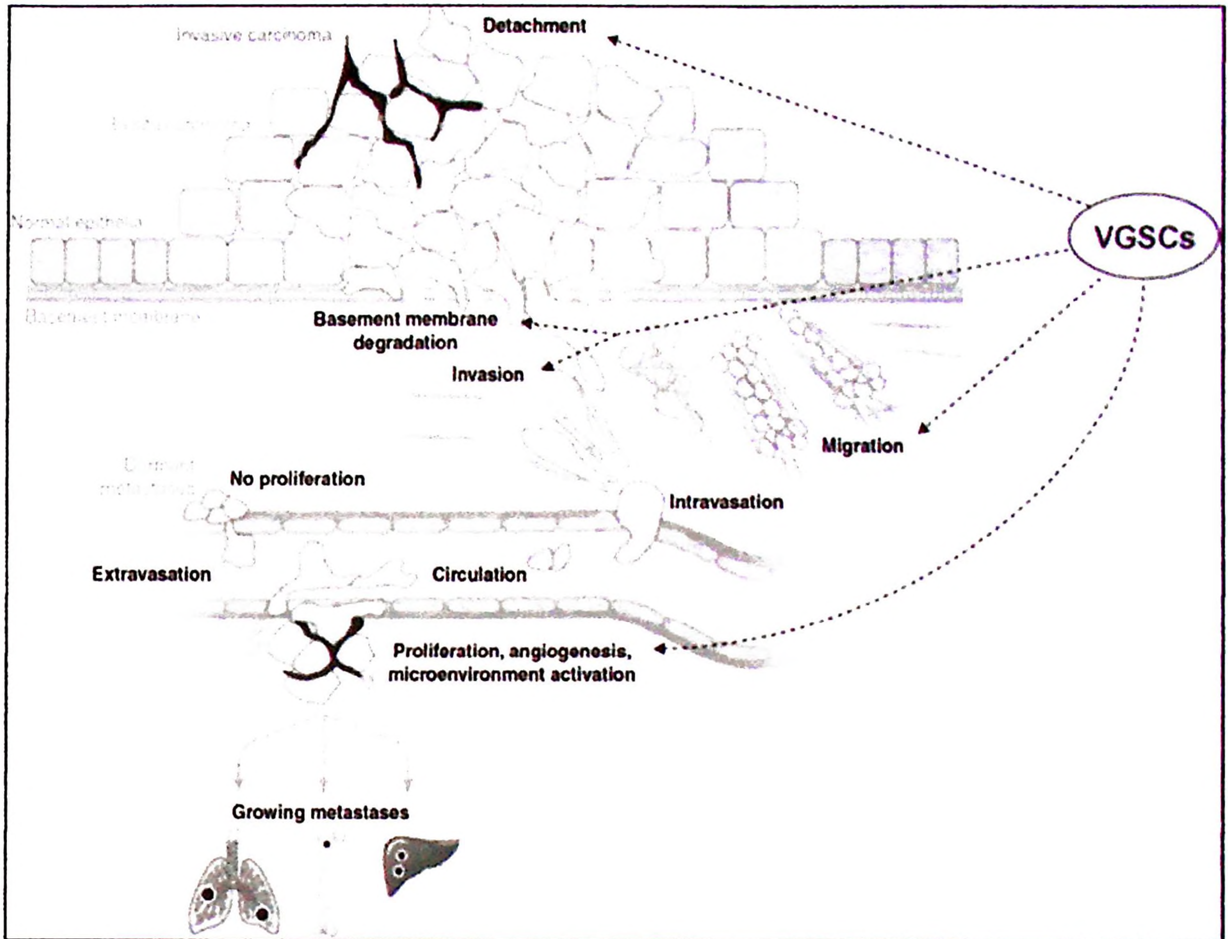

Figure 2.3: The involvement of VGSC in metastasis.

Transformation of normal epithelial cells results in carcinoma in situ. After degradation of the basement membrane, tumour cells invade the surrounding stroma, migrate and intravasate into lymph or blood circulation, and surviving cells arrest in the distant capillaris of an organ. Formation of secondary tumors occurs after proliferation, induction of angiogenesis and microenvironment activation. Voltage-gated sodium channels (VGSCs) have been reported to be involved in controlling various components of the metastatic cascade, as shown by the arrows (Onkal and Djamgoz, 2009).

current was expressed in the highly invasive breast cancer cell line MDA-MB-231 while no such current was observed in MCF-7 cells.

2.4.4 Possible mechanism of VGSC activity in the control of metastatic cell behaviors

The precise mechanism through which VGSC activity enhances metastatic cell behaviors is not fully understood. In this regard, two main possibilities can be considered, as follows.

2.4.4.1 Conductance (Na+ influx) mechanisms

Na+ influx through VGSC (INa) may be responsible for the enhancement of metastatic cell behaviors. Any change in [Na+] influx due to VGSC activity may alter membrane potential, Ca2+ homeostasis, pH balance, and activity of Na+-dependent enzymes (e.g. PKA, cathepsins) in metastatic tumour cells. For example, INa through VGSC (Nav1.5) was shown to acidify the 'perimembrane' pH, thus enhancing the proteolytic activity of secreted cysteine cathepsins towards the surrounding extracellular matrix, ultimately resulting in the invasive phenotype of MDA-MB-231 breast cancer cells (Djamgoz *et al.*, 2001; Roger, 2003; Roger *et al.*, 2007).

2.4.4.2 Non-conductance mechanisms

Alternatively, the role of VGSCs in metastasis could involve a 'nonconducting' function via direct interactions of VGSCαs and/or VGSCβs with other plasma membrane and/or intracellular proteins. For example, VGSC β1 and β2 can associate with tenascin-C and tenascin-R (thereby influencing cell migration) and participate in homophilic cell adhesion, resulting in cellular aggregation and ankyrin recruitment (Shao *et al.*, 2009).

2.5 REST

2.5.1 Background

The repressor element 1-silencing transcription factor (REST, also known as neuron-restrictive silencer factor or NRSF), mediates the repression of several neuronal genes in non-neuronal cells, such as type II sodium channel, SCG10, and synaptophysin (Kim *et al.*, 2006). REST also controls other non-neuronal genes that may impact cell motility, angiogenesis, apoptosis, cell replication, and protein synthesis (Lv *et al.*, 2010).

REST/NRSF work by recognizing the neuron-restrictive silencing element (NRSE) motifs within the control regions of these genes through its zinc finger domain and recruits multiple co-factors via two repression domains, one at the amino terminus and one at the carboxyl terminus (Huang and Bao, 2012). These cofactors will then alter the chromatin structure and regulate transcription through histone deacetylation, chromatin remodelling and methylation (Lunyak *et al.*, 2002; Coulson, 2005) (Figure 2.4).

In addition, REST has a tumour-suppressor function, however in breast cancer cells the function has been loss and resulted in subsequent increase of neuroendocrine genes expression (Gunsalus *et al.*, 2012). In agreement with Gunsalus *et al.*, a study by Huang and Bao (2012) indicated that in non-nervous cancers, loss of REST activity leads to neuroendocrine carcinoma-like tumour, a highly aggressive type of carcinoma, suggesting that REST dysfunction could contribute to tumor development and malignant progression. On the other hand, the level of REST was varied in five different human breast carcinoma cell lines. It was strong in MCF-7 and MDA-MB-468 cell line and relative low expression

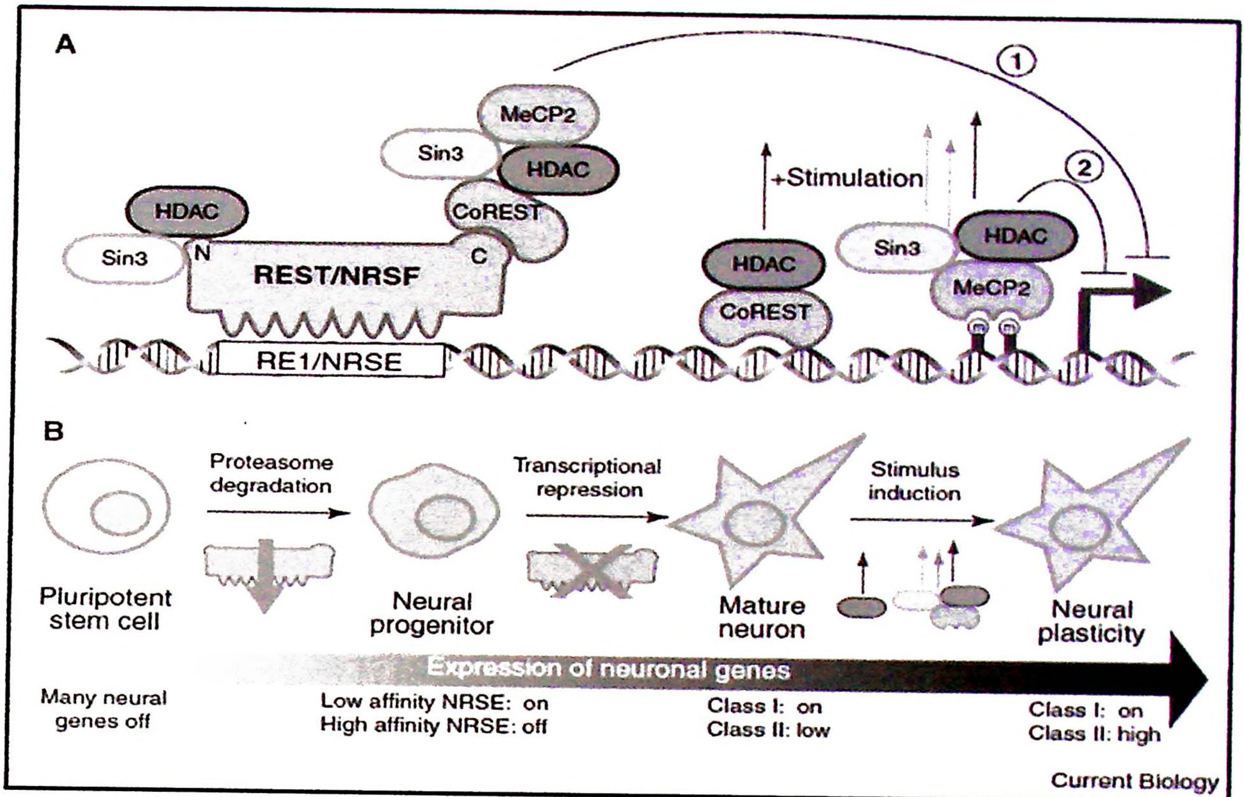

Figure 2.4: The availability of REST/NRSF and dynamic co-factor complexes regulates neuronal gene expression.

(A) REST/NRSF assembles co-factors on target genes. The amino terminus of REST/NRSF recruits Sin3–histone deacetylase (HDAC) to mediate active repression of neuronal genes. The carboxyl terminus recruits a large complex via the specific corepressor CoREST, which can include Sin3–HDAC, SWI/SNF remodelling components (not shown), the methyl-DNA-binding protein MeCP2, histone H3 K9 methyltransferases and HP1 (not shown) to mediate epigenetic silencing in non-neuronal cells. The corepressor complex dependent on bound REST/NRSF represses transcription through mechanism 1, whilst REST/NRSF-directed co-factor association with methylated DNA in mechanism 2 is released in response to stimulation that induces transcription, for example KCl-induced membrane depolarisation of cortical neurons (Ballas *et al.*, 2005). (B) Titration of REST/NRSF levels occurs as neurogenesis proceeds to release repression of individual subsets of neuronal genes, initially those with lower affinity RE1/NRSE motifs, then class I genes that use only mechanism 1 for transcriptional repression. Class II genes that also use repression mechanism 2 may only be maximally expressed by stimulus induction (see above) (Coulson, 2005)

in MDA-MB-231 and MDA-MB-435s cells line (Lv *et al.*, 2010)

.2.5.2 REST and VGSC

Hitherto, only one transcription factor, REST/NRSF has been linked to control VGSC expression. REST/NRSF was first identified as a repressor of the expression of the neuronal Nav1.2 gene (Kraner *et al.*, 1992; Chong *et al.*, 1995).

2.5.3 REST Methylation & Cancer

Abnormal methylation of densely clustered cytosines (so-called CpG islands) occur during aging and during tumor development (Christman, 2002). Since methylation of CpG islands occurs infrequently in normal cells, methylation provides a selective tumor-specific therapeutic target (Baylin, 2005).

Inducing DNA hypomethylation may have short-term anticancer effects, but might also help speed tumor progression from cancer cells surviving the DNA demethylation chemotherapy (Erlich, 2002).

2.6 AZA

5-Azacytidine (AZA) is a potent inhibitor of DNA methyltransferase 1 (DNMT1), known to induce demethylation and reactivation of silenced genes. AZA is soluble in acetic acid:water (1:1 v/v) at 5mg/ml. AZA is also soluble in culture medium at 1.2mg/10ml. AZA has molecular weight of 244.2 with formula $C_8H_{12}N_4O_5$.

Equally important to note is that AZA is a specific inhibitor of DNA methylation. It can be incorporated into both DNA and RNA chains and that, when present in DNA/RNA,

it inhibited DNA/RNA methylation. There is now a revived interest in the use of AZA as a therapeutic agent for cancers in which epigenetic silencing of critical regulatory genes has occurred (Christman, 2002).

CHAPTER 3: MATERIALS AND METHODS

3.1 GENERAL METHODS

3.1.1 Sterilization

Equipment, mediums and solutions such as eppendorf tubes, pipette tips, PCR tubes, etc were autoclaved by using Sun Clave at 15 psi (121°C) for 15 minutes. Whereas, solvents and other solutions were filtered using 0.2 µm or 0.45 µm membrane filter. In addition, UV was used for sterilizing small areas such as biosafety cabinet and PCR cabinet.

3.1.2 Aseptic Techniques

It is crucial to carry out the experiment aseptically in order to avoid contamination of the cell culture. Thus, cell culture work is carried out using a biosafety cabinet (Biological Safety Cabinet, NUAIRE-NU425-400E). Every item that comes into contact with a culture must be sterile. This includes direct contact (e.g., a pipet used to transfer cells) as well as indirect contact (e.g., flasks or containers used to temporarily hold a sterile reagent prior to aliquoting the solution into sterile media). Single-use, sterile disposable item such as pipets were used. Apart from that, 70% ethanol was used for a quick sterilization of surfaces and equipment.

3.1.3 Medium Preparation

3.1.3.1 Complete Dulbecco's Modified Eagle Medium (DMEM) preparation.

MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum, 4 mM L-glutamine and 1%

penicillin/streptomycin. Complete DMEM is prepared under the biosafety cabinet using disposable sterile plastic ware. Both FBS and L-glutamine were thawed at room temperature. 25 ml of 5% FBS and 10ml of 4MM L-glutamine was then added into 500 ml of DMEM. Complete DMEM was pre-warmed before use by placing into a water bath set at 37°C for 15 minutes to 30 minutes. Complete DMEM was stored at 2°C to 8°C when not in use.

3.1.3.2 Preparation of MTT Solution

25 mg of MTT powder was weighed using analytical balance scales and mixed with 5 ml of sterile PBS in 15 ml falcon tube. The MTT solution was kept at 4°C in the dark. Before use, the bottle is pre-warmed for 5 minutes at 37°C and gently mix by inverting the bottle (creating bubbles was avoided).

3.2 CELL CULTURE & MAINTAINANCE

3.2.1 Cell Passaging

All reagents such as complete DMEM and 0.05% Trypsin are pre-warmed at 37°C in water bath. Existing medium were removed from 30 mm culture dish / 96-well plate, the cells then are exposed to Trypsin (3 ml), and the culture dish was rocked gently to ensure that the entire monolayer is covered with the Trypsin solution. The cells were incubated for 5 minutes in the incubator at 37°C with 5% CO_2 until they detached from the plate dish. 3 ml of complete DMEM was added and the cells were pipetted up and down until the cells are dispersed into a single cell suspension.

The cell suspension was transferred into new sterile falcon tube before centrifuged for 3 minutes at 2000 rpm. The supernatant is then removed and the pellet was re-suspended with 4 ml complete DMEM. The cells were counted by hemocytometer for seeding. Appropriate volume of cell suspension was added to a new sterile dish containing complete medium.

3.2.2 Growth Condition of Cell line

All cultured cells were maintained at a constant temperature of 37 °C in 5% CO_2 and 100% humidified atmosphere.

3.2.3 Change Media

Media for all culture cells were changed every day before new treatment was given. To change the media, treatment dissolved in fresh culture media was pre-warmed at 37°C in water bath for at least 30 minutes and vortexed for 2 minutes. Old media from the 30 mm culture dish / 96-well plate was removed into a waste pot containing some disinfectant. Immediately replaced the media with 100 µl of fresh pre-warmed treatment dissolved in culture media and return to CO_2 37°C incubator.

3.3 PHARMACOLOGY

3.3.1 Preparation of AZA

AZA was purchased in a concentration of 150mM whereas the working concentrations needed for this study are 1mM, 100µM, 10µM and 1µM. Thus the drug needed to be diluted. Certain amount of drug and complete media (based on calculation)

was aliquoted into new sterile eppendorf tube. Information about the drug is shown in Table 3.1.

3.3.2 Treatment of Cell line

For cytotoxicity assay MCF-7 cells were cultured in 96-well plates at a concentration of 1 x 10^4 cells/well in complete DMEM. Various concentrations (1 μM, 10 μM, 100 μM and 1 mM) of AZA were added when cells reached 50% confluence. The treatments were done for 24, 48 and 72 hours (Table 3.2). Measurements were performed in triplicate, and percent viability was calculated relative to the untreated samples.

On the other hand, for gene expression assay, MCF-7 cells were cultured in 30mm petri dish at a concentration of 5 x10^5 cells/well in complete DMEM. Various concentrations (1 μM, 10 μM, 100 μM and 1 mM) of AZA were added when cells reached 50% confluence. The treatments were done for 24, 48 and 72 hours (Table 3.2). Measurements were performed in duplicate.

3.4 FUNCTIONAL ASSAY

3.4.1 MTT Assay

To determine cell viability the colorimetric MTT metabolic activity assay was used. MCF-7 cells (1 × 10^4 cells/well) were cultured in a 96-well plate at 37 °C, and exposed to varying concentrations of AZA for 24 h, 48 h and 72 h. Cells treated with medium only served as a negative control group. After removing the old medium, 10 μl of MTT solutions (5 mg/ml in PBS) and 100μl of medium were then introduced. After incubation for another 4 hours

Table 3.1: Information of 5-Azacytidine (AZA)

Drug	Company	Solvent	Storage	Stock Conct.	Working Conct.
AZA	InvivoGen	50% acetic acid	-20 °C	150 mM	1, 10, 100 and 1000 µM

Table 3.2: Treatment of MCF-7 cells.

Time \ Concentrations	Control Media	Acetic Acid	1µM	10µM	100µM	1mM
24 Hours						
48 Hours						
72 Hours						