THE EFFECT OF TRICHOSTATIN A (TSA) ON THE REGULATION OF VOLTAGE-GATED SODIUM CHANNELS (VGSCs) EXPRESSION BY REPRESSOR ELEMENT 1 (RE-1)-SILENCING TRANSCRIPTION FACTOR (REST) USING MCF-7 HUMAN BREAST CANCER CELL LINE

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By

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CERTIFICATE

This is to certify that the dissertation entitled "The effect of Trichostatin A (TSA) on The Regulation of Voltage-gated Sodium Channels (VGSCs) Expression by Repressor Element-1 (RE-1)-Silencing Transcription Factor (REST) using MCF-7 Human Breast Cancer Cell Line" is the bonafide record of research work done by Ms. Nor Izzaty Binti Mas Sudin during the period from September 2013 to June 2014 under my supervision.

ACKNOWLODGEMENT

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 the earth belongs to. Thank Allah for giving me the strength and patience during this challenging time. May peace and blessings be on the leader of all creation, the prophet Muhammad S.A.W, his family and companion.

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LIST OF ABBREAVIATION

~	Approximately
µg/ml	Microgram per milliliter
µl	Microliter
Bp	base pair
BSC	Biological Safety Cabinet
cDNA	complementary Deoxyribonucleic Acid
CHGA	Chromogranin A
CO_2	Carbon dioxide
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
et al.	and others
EtBr	ethidium bromide
EtOH	ethanol
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
gDNA	genomic DNA
GLP	good laboratory practice
HAT	histone acetyltransferases
HDACi	histone deacetylase inhibitor
HP-1	heterochromatin protein 1
HRT	hormone replacement therapy
H	hour(s)
INTDEN	integrated density
LSD1	lysine-specific demethylase-1
MCF-7	Michigan Cancer Foundation-7
Mecp2	methyl-CpG binding protein 2
Mg	Milligram
mg/ml	Milligram per milliliter

$MgCl_2$	Magnesium chloride
min	minute(s)
Ml	Milliliter
mM	Milimolar
Ms	milliseconds
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
ng/ml	Nanogram/microliter
Na+	Sodium ions
N-CoR	nuclear hormone receptor co-repressor
ng/µl	Nanogram per microliter
NRSF	Neuron-restrictive silencer factor
NRSE	Neuron-restrictive silencer element
NSCLC	non-small cell lung cancer
NTC	No Template Control
OD	optical density
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PR	Progesterone receptor
RD	Repressor domain
REST	Repressor Element 1 (RE-1)-Silencing Transcription Factor
RNA	Ribonucleic Acid
S	second(s)
SCLC	small cell lung cancer
SEM	standard error of mean
siRNA	small interfering RNA
SIRs	sirtuins
SYP	Synatophysin
TAE	Tris-Acetate EDTA
TDS	Technical Data Sheet
TM	Trademark
TSA	Trichostatin A
TTX	Tetrodotoxin
UV	Ultraviolet
V	Volt

VEGF	vascular endothelial growth factor
VGSCs	Voltage-gated Sodium Channels

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KESAN TRICHOSTATIN A (TSA) KE ATAS PENGAWAL ATURAN VOLTAGE-GATED SODIUM CHANNELS (VGSCs) OLEH FAKTOR TRANSKRIPSI REPRESSOR ELEMEN 1 (RE-1) (REST) DI DALAM SEL KANSER PAYUDARA MCF-7

ABSTRAK

Faktor transkripsi *repressor elemen 1* (RE-1) (REST) menjadi pengantara kepada penekanan beberapa gen neuron di dalam sel bukan neuron. REST menekan gen sasaran melalui penyahasetilan histone, pembentukan semula kromatin dan metilasi. *Voltage-gated sodium channels* (VGSCs) terutamanya $nNa_v1.5$ diekspreskan dengan tinggi dalam kanser payudara yang agresif. Trichostatin A (TSA) adalah antibiotik antikulat dengan ciri membunuh sel dan pembezaan dalam sel mamalia. TSA adalah perencat yang kuat dan khusus dalam aktiviti penyahasetilan histone. Oleh itu, kajian ini bertujuan untuk menyiasat sama ada perencatan aktiviti penyahasetilan histone oleh TSA cukup untuk meningkatkan pengungkapan transkripsi gen sasaran REST, Synatophysin (SYP) dan Chromogranin A (CHGA), VGSC ($Na_v1.5$ and $nNa_v1.5$) dalam sel MCF-7 yang lemah metastatik. Sel MCF-7 dirawat dengan menggunakan kepekatan TSA yang berbeza (10, 100, 1000 dan 10000 ng/ml) selama 24, 48 dan 72 jam. Kemampuan sel MCF-7 untuk berproliferasi ditentukan dengan pengasaian MTT. Untuk menyiasat kesan TSA ke atas gen pengungkapan (REST, SYP, CHGA, $Na_v1.5$ and $nNa_v1.5$), pengekstrakan RNA, sintesis cDNA, Reaksi Polimerase Berantai (PCR) dan elektroforesis gel telah dijalankan. Pengasaian MTT mendedahkan bahawa TSA merencat pertumbuhan sel-sel MCF-7 mengikut urutan kepekatan TSA dan masa. TSA meningkatkan corak pengungkapan gen SYP dan CHGA mengikut urutan kepekatan TSA dan masa. Terdapat penurunan corak pengungkapan untuk gen $Na_v1.5$ selepas rawatan TSA. Menariknya, terdapat corak peningkatan ekspresi mRNA untuk gen $nNa_v1.5$ selepas rawatan TSA. Penemuan ini menunjukkan bahawa TSA menyebabkan penindasan aktiviti REST melalui perencatan aktiviti penyahasetilan histone dan seterusnya menyebabkan ekspresi gen sasaran meningkat. Di sini satu kemungkinan boleh diandaikan bahawa gen $nNa_v1.5$

dalam sel kanser payudara dikawal oleh REST, walau bagaimanapun kerja-kerja selanjutnya diperlukan untuk mengesahkan interaksi ini.

THE EFFECT OF TRICHOSTATIN A (TSA) ON THE REGULATION OF VOLTAGE-GATED SODIUM CHANNELS (VGSCs) EXPRESSION BY REPRESSOR ELEMENT 1 (RE-1)-SILENCING TRANSCRIPTION FACTOR (REST) USING MCF-7 HUMAN BREAST CANCER CELL LINE

ABSTRACT

The repressor element 1 (RE-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 remodeling and methylation. Voltage-gated sodium channels (VGSCs) especially the neonatal $Na_v1.5$ ($nNa_v1.5$) is predominantly and highly expressed in aggressive breast cancer. Trichostatin A (TSA), an antifungal antibiotic with cytostatic and differentiating properties in mammalian cell culture, is a potent and specific inhibitor of histone deacetylase (HDAC) activity. Hence, this study is aimed to investigate whether an inhibition of histone deacetylation using TSA is sufficient to induce or enhance REST target genes, Syanatophysin (SYP), Chromogranin A (CHGA) and VGSC ($Na_v1.5$ and $nNa_v1.5$) transcription in the weakly metastatic MCF-7 cells. MCF-7 cells were treated with TSA at various concentrations (10, 100, 1000 and 10000 ng/ml) for 24, 48 and 72 h. Then, MCF-7 cells viability were determined by a 3-(4,5-dimethtyl-2-thiazolyl)-2H-tetrazolium bromide (MTT) assay. To investigate the effects of TSA on the expression of genes (REST, SYP, CHGA, $Na_v1.5$ and $nNa_v1.5$), total RNA extraction, cDNA synthesis, Polymerase Chain Reaction (PCR) and gel electrophoresis were conducted. MTT assays revealed that TSA inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. TSA caused an increased pattern of SYP and CHGA genes expression dose- and time-dependently. There was a decreased pattern of $Na_v1.5$ gene expression after treatment with TSA compared to control cells. Interestingly, there was an increased pattern of $nNa_v1.5$ gene expression after TSA treatment. The findings demonstrate that TSA induced loss of REST repression through histone deacetylation inhibition which caused REST target gene CHGA and SYP to increase. A possibility that $nNa_v1.5$ in breast cancer cells are regulated by REST

could be postulated when TSA caused a slight increase in its expression, though further works are needed to confirm the interaction.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

The repressor element 1 (RE-1)-silencing transcription factor (REST) mediates the repression of several neuronal genes in non-neuronal cells (Thiel & Hohl, 2006) in which it blocks the expression of neuronal phenotypic traits in non-neuronal cells. REST represses its target gene through histone deacetylation, chromatin modeling and methylation (Kim et al., 2006). In previous study, a variety of ion channels have been detected in cancer cells. In particular, voltage-gated ion channels (activated by a depolarizing shift in membrane potential), including those permeable to potassium ion (K^+), chloride ion (Cl^-) and sodium ions (Na^+) are widely expressed in a range of cancer cells (Fraser et al., 2002). Among these, up-regulation of voltage-gated sodium channels (VGSCs) has been associated pathophysiologically with several strongly metastatic carcinomas. Expression of VGSC has been reported *in vitro* and *in vivo* in breast cancer, prostate cancer, small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), mesothelioma, neuroblastoma, melanoma and cervical cancer (Djamgoz & Onkal, 2009). Especially, up-regulation of functional VGSCs occurs in a metastatic human breast cancer (Brackenbury et al., 2007). In a study by Fraser et al. (2005), they demonstrated that *SCN5A* (encoding $Na_v1.5$) mRNA have been detected in metastatic breast cancer. The dominant VGSC in breast cancer was found to be embryonic or neonatal splice variant ($nNa_v1.5$). Aberrant function of histone deacetylation in REST regulation could possibly explain the increased expression of VGSCs in cancer cells. Hence, the crucial question is: whether REST regulates VGSC ($Na_v1.5$ and $nNa_v1.5$) expression in cancer cells or not. In this study, TSA which is a well-known histone deacetylase inhibitor (HDACi) was used to inhibit REST function via inhibition of histone deacetylation and test its effect on REST target genes ($Na_v1.5$ and $nNa_v1.5$) in MCF-7 cells. Here, treatment of TSA on MCF-7 cells is expected to increase the expression of REST target genes ($Na_v1.5$ and $nNa_v1.5$).

1.2 Hypothesis

The concentration of 10, 100, 1000 and 10000 ng/ml of TSA will inhibit the function of REST via histone deacetylation inhibition and therefore, increase VGSCs ($Na_v1.5$ and $nNa_v1.5$) expression in MCF-7 cells.

1.3 Objective

1.3.1 General Objective

To investigate the effect of inhibition of histone deacetylation activity by TSA with different concentration of 10, 100, 1000 and 10000 ng/ml at 24, 48 and 72 h is sufficient to enhance VGSC expression by regulation of REST in MCF-7 cells.

1.3.2 Specific Objectives

i) To evaluate the effect of TSA on viability of MCF-7 cells using MTT assay.

ii) To investigate the effect of TSA on the expression of SYP and CHGA genes (REST target genes) and $Na_v1.5$ and $nNa_v1.5$ genes in MCF-7 cells.

1.4 Significance of Study

Breast cancer is the most common malignancy among females affecting approximately one out of ten women. The incidence of cancer has been predicted to increase year by year. The total expected cancer incidence is expected to increase by an additional 45% (from 1.6 million to 2.3 million) (US data) (Smith et al., 2009). The risk of developing breast cancer after 65 years of age is 5.8 times higher than before 65, and 150-fold higher than before 30 years of age (Imyanitov & Hanson 2004).

VGSCs are well described in excitable cells, where they are responsible for the rising phase and the propagation of action potentials (Roger et al., 2007). Study has shown that VGSCs gene is present in most cancerous cell. Interestingly, different VGSCs isoforms (Na_v) have been found in different cancers. In breast and prostate metastatic cancer cells, the activity of VGSCs has been shown to be linked to the invasive properties of these cells (Fraser et al., 2005). VGSCs predominantly the $nNa_v1.5$ is up-regulated in metastatic breast cancer and potentiate metastatic cell behaviors (Chioni et al., 2009). No one dies from cancer that remains in the breast. What kills most people with cancer is metastasis (secondary tumorigenesis). Metastasis is what causes the majority of cancer-related mortality (Nguyen & Massagué, 2007; Hunter et al., 2008).

Reasoning that VGSCs expression were are up-regulated in metastatic breast cancer, we sought to identify the factors that regulate VGSCs expression in breast cancer cell line. Understanding the mechanism by which VGSCs expression is regulated, could provide understanding into how its expression become elevated in cancer tissue, and may also suggest targets for novel pharmacological agents aimed at suppressing its expression.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast Cancer

Breast cancer is a type of cancer originating from breast tissue, most commonly from the inner lining of milk ducts or the lobules that supply the ducts with milk (Sariego, 2010). It occurs when the cells in the lobules or the ducts become abnormal and divide uncontrollably. These abnormal cells begin to invade the surrounding breast tissue and may eventually spread via blood vessels and lymphatic channels (Malaysian Oncological Society, 2012).

Breast cancer is the most common cancer of women and the second leading cause of female cancer mortality, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458, 400) of the total cancer deaths in 2008 (Jemal et al., 2011). During 1998 in Malaysia, breast cancer is the commonest diagnoses among cancer deaths (Lim, 2002). It is the primary cause of death in women worldwide mostly as a consequence of the appearance of metastases (Parkin et al., 2005). The mortality from this disease has decreased through screening programs, education and improved adjuvant treatment. Still, about 20-30% of patients develop metastatic disease that still remains incurable, with median survival between 2-4 years depending on the subtype (Eroles et al., 2011).

The risk factors that are well established for the development of breast cancer includes age, family history, early menarche, late menopause, nulliparity, late age at first full-term pregnancy and use of hormone replacement therapy (HRT) (Ferlay et al., 2008).

Breast cancer is characterized by its molecular and clinical heterogeneity. Studies profiling gene expression have classified breast cancers into six intrinsic subtypes: (i) luminal A, (ii) luminal B, (iii) HER2-enriched, (iv) basal-like, (v) normal breast-like tumors and (vi) claudin-low (Perou et al., 2000). These subtypes express characteristics genes and are associated with varying prognoses (Sotiriou et al., 2003) (Table 2.1). This classification allows us to better understand the gene expressions that may be driving the tumor (Russel, 2014) and allow clinicians to improve treatment of each subtypes.

Table 2.1: Feature of molecular subtypes and associated gene expression in breast cancer (Sotiriou et al., 2003).

Molecular subtype	Frequency	ER,PR, HER2	Proliferative genes	Prognosis
Basal-like	10-20%	ER- PR- HER2-	High	Bad
HER2-enriched	10-15%	ER- PR- HER2+	High	Bad
Normal breast-like	5-10%	ER-/+ HER2-	Low	Intermediate
Luminal A	50-60%	ER+ PR+ HER2-	Low	Excellent
Luminal B	10-20%	ER+/- PR+/ HER2+/-	High	Intermediate/Bad
Claudin-low	12-14%	ER- PR- HER2-	High	Bad

ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth receptor 2

Breast cancer has been recognized as having identifiable targets (biomarkers) for therapy and to identify them, new methods and agents have been created (Russell, 2014). These biomarkers must be specific, measurable, and reliable and linked to specific biological process (Atkinson et al., 2001).

The ER was the first of many targets that has been described in most breast cancers. About 75% of newly diagnosed breast cancers are ER-positive, which strongly predicts for benefit from hormonal manipulation. The second most commonly described target in breast cancer is the HER2 protein (Russell, 2014). HER2 is a member of the human epidermal growth factor family of receptors. This family consists of 4 transmembrane proteins (HER 1 to 4) each of which has different properties. Approximately, 25% of metastatic breast cancers are known to be HER2+. HER2 positivity is strongly associated with large tumor size, higher tumor grade, higher mitotic index, number of lymph node involved, and reduced expression of the PR (Menard et al., 2002).

2.2 Metastasis

Metastasis is defined as spread of cancer cells from the primary neoplasm ultimately to form secondary tumour. Metastasis is what causes the majority with more than 90% of cancer-related mortality (Nguyen & Massagué, 2007; Hunter et al., 2008). Non-malignant cells, under sturdy conditions, proliferate as needed to replace themselves as they age or become injured. However, this process can go awry, resulting in uncontrolled proliferation and tumor formation, which can be benign or malignant. Benign tumors are generally slow growing, enclosed within a fibrous capsule, noninvasive, and more morphologically resemble their cellular precursor. If a benign tumor is not close to critical vascular or neural tissue, diagnosis and treatment frequently results in a cure. In contrast, malignant tumors rarely encapsulate, grow rapidly, invade regional tissues, have morphologic abnormalities and metastasize (Talmadge & Fidler, 2010). The chances of survival from metastatic breast cancer are less than 5%. In many patients, metastasis has occurred by the time of diagnosis, so metastasis prevention may not be relevant (Del Monte, 2009).

2.2.1 The Pathogenesis of Metastasis

The pathogenesis of metastasis comprises a series of steps, reliant on both the intrinsic properties of the tumor cells and the host response (Poste & Fidler, 1979). It consists of a long series of sequential, interrelated steps (Fidler, 2003), in which cancer cells (i) separate from each other and detach from extracellular matrix, (ii) migrate through local tissues and invade through basement membrane, (iii) enter blood or lymph circulation (iv) re-attach at a distant site and extravasate, and (v) form secondary tumors after proliferation and induction of angiogenesis (Fidler 2002a,b, 2003; Hunter, 2004; Bacac & Stamenkovic 2007). Each of these can be rate limiting, as a failure or an insufficiency at any of the steps can stop the entire process (Poste & Fidler, 1979; Fidler, 2002b) (Figure 2.1). Many genetic and epigenetic events have been identified that contribute to metastatic path (Hanahan & Weinberg, 2000).

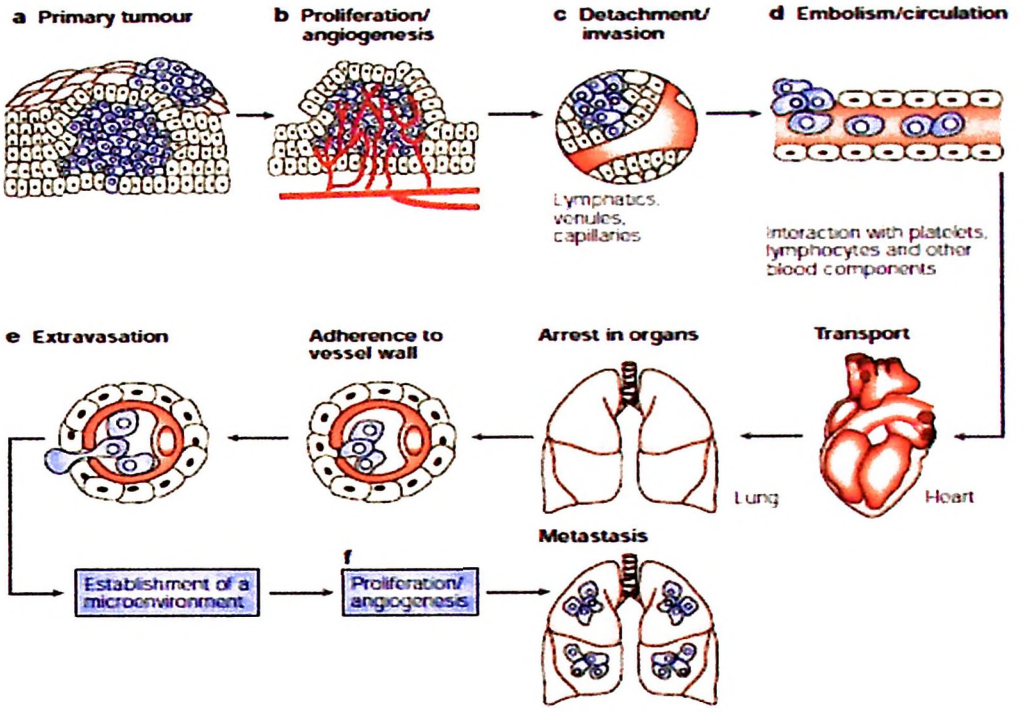

Figure 2.1: The illustration gives insights into step-by-step process of metastasis. These include invasion of the primary tumor border and intravasation of the circulatory system, survival and arrest in the circulation, extravasation to a distant site, formation of a micrometastasis and then progressive colonization to form a life threatening metastasis (Fidler, 2003).

Breast cancer principally metastasizes to the regional lymph nodes, bone, liver, lungs and brain (Fidler et al., 2003) and recurrences are often detected following years or decades of remission (Schmidt-Kittler, 2003).

On the basis of a review of autopsy records from 735 women with fatal breast cancer, Stephen Paget (1889) showed that breast tumor preferentially spread to the liver and bone marrow. These observations identify that the site of secondary tumors is dependent on the tumor cell ("seed") and the target organ ("soil"). Therefore, a metastatic focus is established only if the seed can grow in the soil, that is, if the microenvironment of the target site is compatible with the properties and requirements of the disseminated tumor cell.

2.3 Voltage-gated sodium channels (VGSCs)

2.3.1 Introduction of VGSCs

Ion channels are the main signaling molecules expressed in a wide range of tissues where they have significant involvement in determining a variety of cellular functions: proliferation, solute transport, volume control, enzyme activity, secretion, invasion, gene expression, excitation-contraction coupling and intercellular communication (Hille, 1992). Ion channels, including voltage-gated ion channels (those activated by a change in membrane potential) could similarly have a significant role in cancer (Cuzick et al., 1998). 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 sequences (Hille, 2001).

VGSCs play a crucial role in the initiation and propagation of action potentials in neurons and other electrically excitable cells such as myocytes and endocrine cells (Hodgkin & Huxley, 1952; Hille, 2001).When the cell membrane is depolarized by a few millivolts, VGSCs activate and inactivate within milliseconds (ms). Influx of sodium ions (Na+) through the integral membrane protein comprising the channel depolarizes the membrane further and initiates the rising phase of the action potential (Catterall, 2000). They are also expressed at low levels in non-excitable cells.

2.3.2 VGSCs Subunits and Structure

VGSCs open in response to membrane depolarization, allowing rapid influx of Na^+. These heteromeric membrane proteins are made up of single α-subunit (VGSCα) generally in association with one or more auxiliary β-subunit (VGSCβ) (Catteral, 2000; Diss et al., 2004). The mammalian VGSCα gene family contains at least 9 functional members ($Na_v1.1$- $Na_v1.9$, coded by genes *SCN1A-SCN11A*). In cancer cells, Na^+ current carried by VGSCαs increases migration, invasion and metastasis *in vivo*. In contrast, the VGSCβ mediate cellular adhesion and process extension (Brackenbury & Isom, 2008).

The VGSCα are organized in four homologous domains (I-IV), each of which contains six transmembrane α helices (S1-S6) and an additional pore loop located between the S5 and S6 segments. The pore loops line the outer, narrow entry to the pore, whereas S5 and S6 segments line the inner, wider exit from the pore. The S4 segments in each domain contain positively charged amino acid residues at every third position. The residues serve as gating charges and move across the membrane to initiate channel activation in response to depolarization of the membrane. The short intracellular loop connecting homologous domains III and IV serves as the inactivation gate, folding into the channel structure and blocking the pore from the inside during sustained depolarization of the membrane (Catterall et al., 2005) (Figure 2.2).

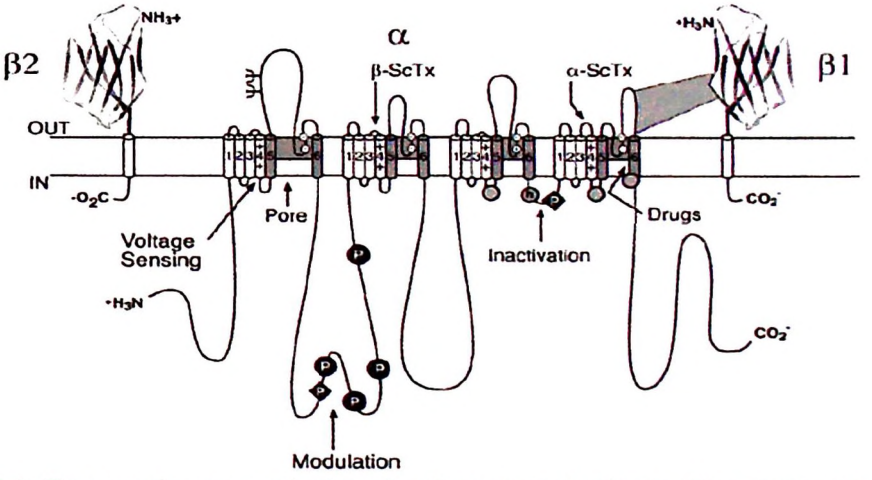

Figure 2.2: Transmembrane organization of sodium channel subunits. The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane-folding diagrams (Catterall et al., 2005).

2.3.3 VGSC Expression in Cancer: VGSC and Its Involvement in Metastasis

VGSC have potentially been associated with mechanisms that are involved in generating and sub-serving the hallmarks of cancer (Fraser & Pardo, 2008; Prevarskaya et al., 2010). They possess a number of remarkable functional properties that enable them to take central role in cellular physiology and consequently, pathophysiology (Djamgoz & Onkal, 2013). The variety of ion channels, including VGSC involved in metastasis as illustrated in figure 2.3.

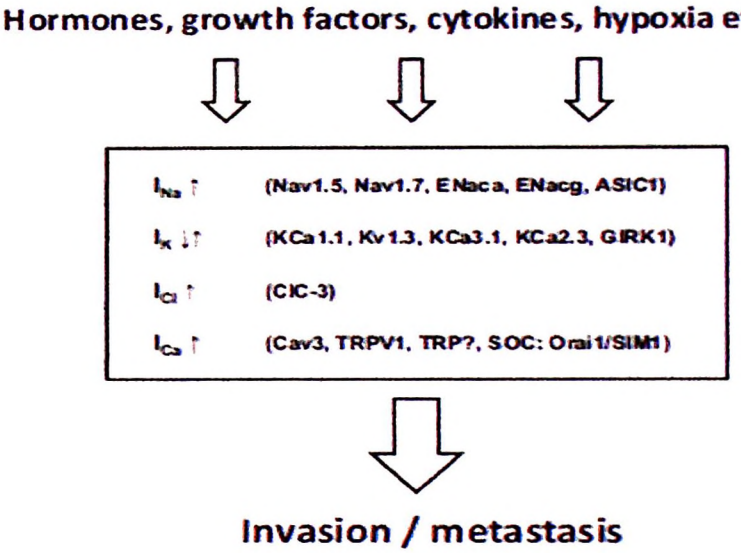

Figure 2.3: Ion channels involved in cancer cell metastasis. A range of ion channels have been shown to control cancer cell invasiveness through mechanisms like motility and secretion of proteolytic enzymes. Upstream of ion channel expression or activity are primary controlling mechanisms including hormones, growth factors and hypoxia. This figure shows how membrane currents (I_{Na} etc.) change in line with invasiveness. The channels in parantheses indicate those that are involved in the generation of those currents (Prevarskaya et al., 2010).

VGSC expression has been predominantly reported to date in carcinomas (cancers of epithelial origin). The VGSCα have been identified in cells from the following carcinomas; breast cancer (Roger et al., 2003; Fraser et al., 2005), cervical cancer (Diaz et al., 2007; Hernandez-Plata et al., 2012) colon cancer (House et al., 2010), melanoma (Carrithers et al, 2009; Allen et al., 1997), mesothelioma (Fulgenzi et al., 2006), neuroblastoma (Ou et al., 2005), NSCLC (Roger et al., 2007), ovarian cancer (Gao

et al., 2010), prostate cancer (Diss et al., 2001; Laniado et al., 1997; Smith et al., 1998; Grimes, 1995) and SCLC (Onganer & Djamgoz, 2005; Blandino et al., 1995).

In several cancers expressing multiple VGSCα , a predominant VGSCα has been identified. For example, in lymphoma and breast cancer cells, the most highly expressed VGSCα is $Na_V1.5$ (gene: *SCN5A*) (Fraser et al., 2005; Fraser et al., 2004) whereas in prostate cancer cells, the predominant VGSCα is $Na_V1.7$ (gene: *SCN9A*) (Diss et al., 2001).

Alternative mRNA splicing enables further functional variation among VGSCα (Diss et al., 2004). An important developmentally regulated splicing event occurs in exon 6, encoding the domain I segment 3 (D1:S3) region, such that the 5' "neonatal" variant is expressed at birth, whereas 3' "adult" variant is expressed later in postnatal development. In lymphoma, neuroblastoma, breast and prostate cancer cells, *SCN5A* and *SCN9A* are mainly expressed in their D1:S3 5' neonatal splice forms (Fraser et al., 2005; Ou et al., 2005; Fraser et al., 2004; Diss et al., 2005). In contrast, the adult *SCN5A* is expressed in colon cancer cells, and the neonatal variant is absent (Brackenbury, 2012).

In has been shown that VGSCα mRNA and protein expression correlates with metastatic potential. For example, in breast cancer, the $nNa_v1.5$ is expressed ~1800-fold higher in metastatic MDA-MB-231 cells than weakly metastatic MCF-7 cells (Roger et al., 2003; Fraser et al., 2005). This $nNa_v1.5$ expression in breast cancer biopsies correlates with occurrence of lymph nodes metastasis. For patients who had recurrence, or died within five years, and associates with increased odds of developing metastasis, it has been demonstrated that $Na_v1.5$ mRNA is prominent (Yang et al., 2012).

The expression of VGSCβ in cancer cells has been less extensively studied. Chioni et al. (2009) has shown expression of VGSCβ in breast cancers with β1 is the most abundant. Interestingly, β1 appears to be inversely correlated with $Na_v1.5$ and metastatic potential: *SCN1B* mRNA (encoding β1) is significantly higher in weakly

metastatic MCF-7 cells than in highly metastatic MDA-MB-231cells. This proposes that VGSCβ may be performing certain functions in cancer cells independent of the pore-forming α subunits.

2.3.4 VGSCs Functional Role

VGSCα potentiate several cellular behaviors associated with metastasis. It has been shown to be involved in controlling various components of metastatic cascade (figure 2.4). . Studies using VGSC pore-blocker tetrodotoxin (TTX) in breast, prostate, and lung cancer cell lines has shown to inhibits behaviors including process out-growth or extension (Fraser et al., 1999), galvanotaxis (Fraser et al., 2005, Mycielska et al., 2001), migration (Fraser et al., 2005; ,Fukgenzi et al., 2006; Gao et al., 2010; Fraser et al., 2003; Brackenbury et al., 2007; Brackenbury & Djamgoz, 2006; Uysal-Onganer & Djamgoz, 2007), endocytosis (Fraser et al., 2005; Onganer & Djamgoz, 2005; Mycielska et al., 2003),vesicular patterning (Krasowska et al., 2004; Krasowska et al., 2009),detachment from substrates (Palmer et al., 2008), gene expression (Brackenbury & Djamgoz, 2006; Mycielska et al., 2005) and invasion (Roger et al., 2003; Fraser et al., 2005; Hernandez-Plata et al., 2012; Carrithers et al., 2009; Roger et al., 2007; Gao et al., 2010; Laniodo et al., 1997; Grimes et al., 1995; Fraser et al., 2004). Specifically, $Na_V1.5$ has been shown to regulate galvanotaxis, lateral motility, transwell migration, endocytic membrane activity, cellular adhesion, gene expression and invasion (Brackenbury, 2012). However, TTX does not inhibit proliferation of cells (Roger et al., 2003; Fraser et al., 2005; Hernandez-Plata et al., 2012; Roger et al., 2007; Gao et al., 2010; Fraser et al., 2003) suggesting that VGSC may be involved mainly in metastatic progression, rather than tumorigenesis. Recent evidence has shown that VGSCs also regulate angiogenic properties of endothelial cells, including vascular endothelial growth factor (VEGF)-induced proliferation, tubular differentiation and adhesion (Andrikopoulos et al., 2011).

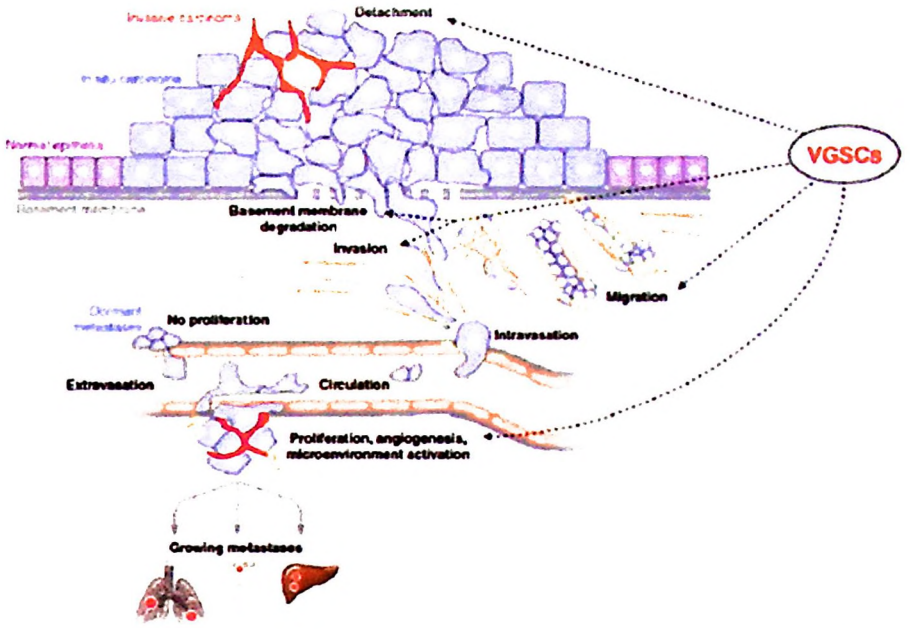

Figure 2.4: VGSC has been shown to be involved in controlling various components of metastatic cascade (Bacac & Stamenkovic, 2007).

Silencing *SCN5A* with small interfering RNA (siRNA) reduces *in vitro* invasion of MDA-MB-231 breast cancer cells (Gillet et al., 2009). Further, specifically targeting the neonatal splice variant, $nNa_V1.5$ decreases the migration and invasion of MDA-MB-231 cells, suggesting that the neonatal form itself may be responsible for VGSC-dependent potentiation of metastatic cell behavior in breast cancer cells (Brackenbury et al., 2007).

2.4 Repressor Element 1 (RE-1)-Silencing Transcription Factor (REST)

2.4.1 Introduction of REST

The RE-1 silencing transcription factor (REST) or neuron-restrictive silencer factor (NRSF) is a transcriptional repressor with a critical role in suppressing expression of neuronal-specific genes in non-neuronal cells. Target genes of REST encode neuronal receptors, ion channels, neuropeptides, synaptic vesicle protein, transcription factors and adhesion molecules (Thiel & Hohl, 2006). The expression of REST was detected in five breast cancer cell lines, MCF-7, MDA-MB-231, MDA-MB-453, and MDA-MB-468. The level of REST was relatively higher in MCF-7 and MDA-MB-468, and it was relatively low in MDA-MB-231 and MDA-MB-435 cell line (Lv et al., 2010).

2.4.2 Modular Structure & Repressor Complex of REST

REST composed of a DNA-binding domain that anchors the protein to DNA, and a repressor domain. The REST protein displays a modular structure (Figure 2.4). The DNA-binding domain has been confined within the cluster of eight zinc fingers at the N-terminal. It was shown that zinc finger 7 of REST is crucial for DNA binding (Shimojo et al., 2001). REST contains two repressor domains (RD) which are RD1 and RD2 located at the N-terminal and C-terminal respectively (Tapia-Ramírez et al., 1997; Thiel et al., 1998; Naruse et al., 1999). The C-terminal RD includes a single zinc finger motif.

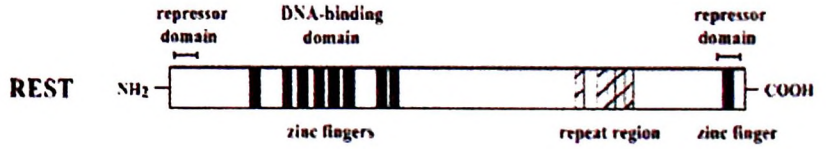

Figure 2.5: The modular structure of REST. A cluster of eight zinc fingers in the N-terminal region functions as DNA-binding domain. Repressor domains have been mapped on the N- and C-termini of the molecule (Tapia-Ramírez et al., 1997; Thiel et al., 1998).

The DNA-binding domain can recognize two types of RE-1 motifs, including the canonical RE-1 motif and non-canonical RE-1 motif. The canonical RE-1 motif consists of two 10 bp conserved sequences separated by a single non-conserved nucleotide, whereas the non-canonical RE-1 motif has variable length of insertion between these two conserved sequences (Otto et al., 2007). The canonical RE-1 motif has higher affinity for REST than non-canonical motif, suggesting that tissue-specific function of REST may depend on binding to different RE-1 motifs. Once REST binds to the RE-1 motifs in target genes, it acts as a molecular scaffold to recruit several cofactors to its N-terminal RD1 and C-terminal RD2 repression domains. This macromolecular complexes in return generated epigenetically modulate target gene expression. The most important transcriptional co-regulators that REST recruits are CoREST and mSin3A (Andres et al., 1999; Ballas & Mandel, 2005; Coulson, 2005; Lunyak & Rosenfeld, 2005; Hsieh & Gage, 2005; Cao et al., 2006; Huang et al., 1999; Majumder, 2006). The N-terminal RD1 domain of REST interacts with mSin3A, subsequently recruits the histone deacetylase complex (HDAC1, HDAC2, HDAC4 and HDAC5) and a nuclear hormone receptor co-repressor (N-CoR) (Huang et al., 1999; Nomura et al., 2005). Meanwhile, the C-terminal RD2 domain of REST associates with CoREST, which also interacts with methyl-CpG binding protein 2 (Mecp2), lysine-specific demethylase-1 (LSD1) and histone H3-lysine 9 methyltransferase G9a (Zheng et al., 2009; Ballas et al., 2005; Mulligan et al., 2008). These studies demonstrated that REST regulates expression of target genes through a dynamic macromelocular complex. The composition of the repressor complex may affect specificity of REST regulation on target genes.

2.4.3 Biological Activity of REST

REST has an intrinsic repression activity that targets chromatin organization of the genome (Thiel et al., 2004). It has been characterized as a dual- specific repressor (Thiel et al., 2004) which induces transcriptional repression via recruitment of HDACs and gene silencing via histone methylation (methyl-CpG binding protein MeCP2) and heterochromatin formation (heterochromatin protein 1 (HP-1)) (Lunyak et al., 2002).

2.4.4 Mechanism of Transcriptional Repression by REST: Modulation of the Chromatin Structure

Nucleosome is the fundamental unit of chromatin with two molecules each of histones H2A, H2B, H3 and H4 building the core histone. The single nucleosomes are linked by short stretches of DNA and the linker H1. The N-terminal regions of the core histone are often modified by acetylation, methylation and phosphorylation. The acetylation of histones by histone acetyltransferases (HAT) involves the transfer of an acetyl group from acetyl coenzyme A to the ε-amino group of a lysine residue (Figure 2.5a). Histone acetylation is of major importance for the regulation of gene transcription because this modification reduces the net positive charges of the core histones, leading to a decrease in their binding affinity for DNA (Thiel & Hohl, 2006). After the nucleosome unfolds and provides access for transcription factors, the termini are subsequently displaced from the nucleosome. Thus, transcriptional activation occurs only after the repressive histone-DNA interaction has been destabilized by HAT (Wade et al., 1997).

In contrast, histone deacetylation by HDACs, removes the acetyl group from the e-amino group of lysine residues of histones, thus permitting ionic interactions between the negatively charged DNA phosphate backbone and the positively charged N-termini of the core histones. This results in a more compact chromatin structure that is not easily accessible for the transcriptional machinery (Figure 2.5b). While histone acetylation and hyperacetylation has been correlated with transcriptionally active chromatin, histone deacetylation is thought to be involved in the repression of transcription. In neurons, REST is expressed at extremely low concentrations (Thiel & Hohl, 2006).

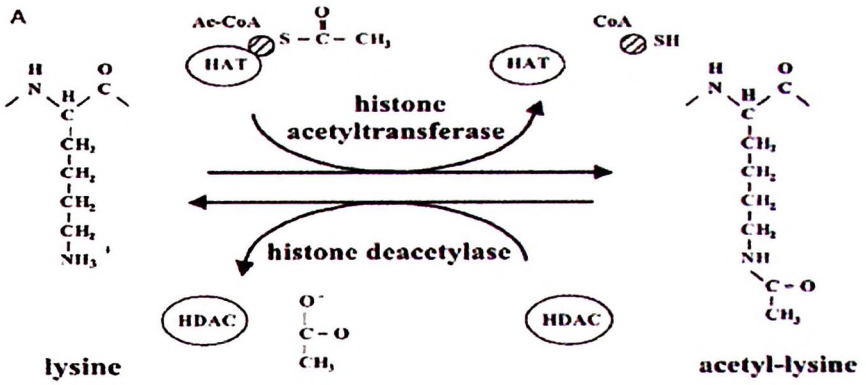

Figure 2.6 (a): Chemical composition of the side chains of lysine and acetyl-lysine. HAT catalyze the transfer of an acetyl group from acetyl coenzyme A to the e-amino group of internal lysine residues of histone N-terminal domains, removing the positive charge of the e-amino group at physiological pH. HDAC catalyze the removal of the acetyl groups.

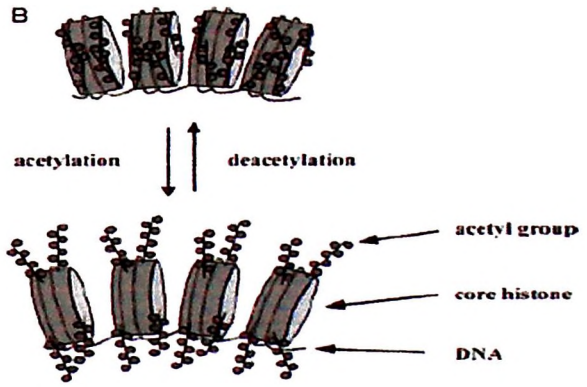

Figure 2.6 (b): Acetylation of histones loosens the contact between DNA and the histone octamer, thus generating an open configuration of the chromatin. Deacetylation, in contrast, stabilizes the DNA/histone binding, leading to chromatin compaction (Thiel et al., 2004).

2.5 HISTONE DEACETYLASES (HDACs)

2.5.1 CLASSIFCATION OF HDACs

There are three major classes of mammalian HDACs based on their structural homologies to the three distinct yeast HDACs: Rpd3 (class 1), Had 1 (class 2), and Sir2/Hst (class III). Class III HDACs consist of the large family of sirtuins (SIRs) that are evolutionary distinct, with a unique enzymatic mechanism dependent on the cofactor NAD^+, and are virtually unaffected by all HDACIs currently under development (Imai et al., 2000; Finnin et al., 2001). In the other hand, class I and II HDACs are evolutionary

similar, contain an active site zinc as a critical component of their enzymatic pocket, have been described in association with cancer, and are thought to be comparably inhibited by most currently available HDACIs. The class I HDACs includes HDAC1, HDAC2, HDAC3, and HDAC8. They are widely expressed in a variety of tissues and are primarily localized in the nucleus. The class II HDACs include HDAC4, HDAC5, HDAC6, HDAC7, HDAC9 (a and b isoforms), and HDAC10, and are structurally much larger in size. Class II HDACs can shuttle between the nucleus and cytoplasm (Kao et al., 2001; Wang & Yang, 2001; Gray & Ekstrom, 2001; Guardiola & Yao, 2002), suggesting different functions and cellular substrates form class I HDACs. Class II HDACs also display a more limited tissue distribution (de Ruijter et al., 2003; Grozinger et al., 1999; Petrie et al., 2003).

2.5.2 Identification of HDAC Inhibitor (HDACi)

There are six structurally distinct classes HDACi which act by binding to various portions of the catalytic domains within class I and II HDACs. These include hydroxamic acid, epoxyketone, carboxylates or short-chain fatty acid, cyclic peptide (either contain epoxyketone group or are devoid from it), and benzamide class (Drummond et al., 2005).

Hydroxamic acid, including TSA, SAHA (Vorinostat), and LAQ824, have three basic components: (a) a hydroxamic acid moiety that chelates the active zinc in a bidentate manner, hydrogen bonds with residues composing the charge relay systems, and displaces the nucleophilic water molecule present in the active site; (b) a hydrophobic spacer that has a length optimal for spanning the length of the hydrophobic pocket and dimensions capable of navigating the narrowest segment of the cavity; and (c) a hydrophobic cap that blocks the entrance to the active site (Drummond et al., 2005).

The study of HDACIs is moving rapidly into a new stage of development producing encouraging results in the field of cancer therapy. SAHA and FK228

(epoxyketone-devoid cyclic peptide class) have already been approved by the US Food and Drug Administration (FDA) for the clinical treatment of cutaneous T-cell lymphoma. SAHA is currently under evaluation in several phase II trials in breast cancer (Pathiraja et al., 2012), including combination therapy with standard cytotoxic agents (for example, paclitaxel), endocrine therapy (tamoxifen) or novel targeted therapy (trastuzumab, bevacizumab). Other HDACIs such as MS-275 (benzamide class) and LBH-589 (hydroxamic acid class) are in phase I/II studies in combination with other agents, such as trastuzumab, in women with metastasic HER2-positive breast cancer (Pathiraja et al., 2012).

2.5.3 Trichostatin A (TSA)

TSA is a well-known HDACI (Vigushin et al., 2001) that come from the hydroxamic acid class. TSA from *Streptomyces hygroscopicus* was initially identified as an antifungal agent (Tsuju et al., 1976) and can cause potent reversible inhibition of mammalian HDAC at nanomolar concentrations both *in vivo* and *in vitro* (Yoshida et al. 1990; Siavoshin et al. 2000). Several investigations have revealed that TSA exerts a dose-dependent anti-tumor activity against breast cancer *in vitro* and *in vivo* (Vigushin et al., 2001; Schmidt et al., 1999). TSA able to reach cytocidal effects at 100 nM (Schmidt et al., 1999).

Nanomolar concentrations of TSA cause a marked accumulation of highly acetylated histones *in vivo* and strongly inhibit the activity of the partially purified HDAC *in vitro*. It have been demonstrated that at extremely low concentrations of TSA caused induction of Friend leukemia cell differentiation as well as inhibition of the cell cycle of normal rat fibroblasts in both the G1 and G2 phases (Yoshida et al., 1990). It has been reported that TSA induces apoptotic death in Jurkat lymphoid cell line and several colorectal carcinoma cell lines (Medina et al., 1997; McBain et al., 1997).

A study by Vigushin et al., (2001) showed that cell lines that express ERα (T-47D, MCF-7, BT474 and ZR-75-1) were more sensitive to TSA than ERα-negative cell lines. This differential sensitivity of the ERα-positive cell lines to TSA suggests that the mechanism of growth inhibition is different from cells that do not express functional ERα.

2.6 MCF-7 Human Breast Cancer Cell Line

Breast cancer cell lines have been used extensively in basic research and most critical components in studying tumor carcinogenesis, signal transduction pathways and new therapeutic for breast carcinoma (Subik et al., 2010).

MCF-7, the acronym of Michigan Cancer Foundation-7 (Levenson & Jordan, 1997), is a breast cancer cell line derived from a pleural effusion taken from a patient with metastatic breast cancer that was isolated in 1970 from a 69-year-old Caucasian woman (Soule et al., 1973). MCF-7 cells retain characteristics of differentiated mammary epithelium. The cells are generally slow-growing although easy to propagate. It is the most widely used breast cancer cell line worldwide (Burdall et al., 2003). MCF-7 cells are ER and PR positive, HER2 negative and fell within Luminal A subtype (Subik et al., 2010).

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. It was well known that MCF-7 cells and MDA-MB-468 cell were less malignant than MDA-MB-231 and MDA-MB-435s cell (Lv et al.,2010). On the other hand, Fraser et al. (2005) stated that the level $Na_v1.5$ in weakly metastatic MCF-7 cells was low compared to strongly metastatic MDA-MB-231 which was up-regulated ~1800-fold at mRNA level. Hence, there is some kind of negative correlation between REST and VGSCs expression. In this present study, we speculated that in aggressive breast cancer cells (MDA-MB-231 cells), the existing VGSCs expression is due to loss of REST expression/activity. In order to test the hypothesis, TSA is treated MCF-7 cells and the treatment is expected to re-express $Na_v1.5$ and $nNa_v1.5$ gene expression in these cells.

CHAPTER 3

METHODOLOGY

The MCF-7 breast cancer cell line was kindly provided by Prof. Dr. Nik Soriani Yaacob (Department of Chemical Pathology, School of Medical Sciences, USM). The TSA (InvivoGen USA) was purchased by our supervisor, Dr. Noor Fatmawati Binti Mokhtar. The mainly practiced procedure in this experiment is the cell culture procedure, which will be done in a strictly sterile condition. Each experimental design requires treatment of MCF-7 cells with each 10, 100, 1000 and 10000 ng/ml TSA. These will be then followed with the procedure of the MTT assay and molecular study. The study design ends with obtaining the expression of VGSCs ($Na_v1.5$ and $nNa_v1.5$) genes through PCR (Figure 3.1).

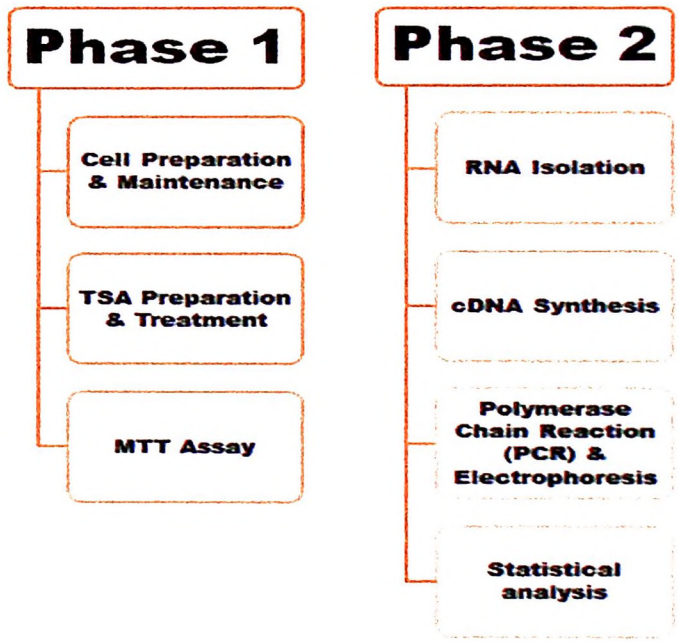

Figure 3.1: The overview of methodology which can be divided into 2 phases. Phase 1 starting from cell preparation and maintenance, TSA preparation and treatment and MTT Assay. Phase 2 starting from RNA isolation, cDNA synthesis, PCR and electrophoresis and finally statistical analysis for gene expression analysis were conducted.

3.1 Cell Culture Preparation & Maintenance

3.1.1 Aseptic Technique

It is crucial to carry out the experiment aseptically in order to avoid contamination of the cell culture. It is practiced in each cell culture procedure using 70% EtOH. The cell culture procedure is carried out in a safety and decontaminated environment inside Biological Safety Cabinet (BSC) (Nuaire, NU425-400E). The surface of the BSC was carefully swapped with 70% EtOH using tissue paper to avoid any possibilities of contamination. Every instruments, items or chemicals that being handled inside the BSC were decontaminated before used inside BSC.

3.1.2 Preparation of Complete Dulbecco's Modified Eagle Medium (DMEM)

MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Fetal Bovine Serum (FBS), 4 mM L-Glutamine and 1% penicillin or streptomycin. This complete DMEM was prepared inside the BSC using disposable sterile plastic ware. Both FBS and L-glutamine were thawed at room temperature. 25 ml of 5% FBS and 10 ml of 4mM glutamine were added into 500 ml of DMEM. This complete DMEM was pre-warmed before used by placing the complete DMEM into a water bath set at 37°C for 15-30 minutes (min). The complete DMEM was stored at 2-8°C when not in used.

3.1.3 Cell Culture and Splitting

All reagents such as complete DMEM and 0.05% Trypsin are pre-warmed at 37°C in water bath before used. The MCF-7 cells maintained at a constant temperature of 37°C in a 5% carbon dioxide (CO_2) and 100% humidified atmosphere were removed from the incubator (Thermo Forma-311). Firstly, the media was sucked from the culture plate using the 100 µl pipette or using the vacuum pump. Then, 3 ml 0.05% of Trypsin was added into the culture plate and rocked gently. The cell was incubated in the incubator (37°C, 5% CO_2) for 5 min. Then, the plate was removed from the incubator and observed under the microscope to ensure all cells have detached from the plate surface. 3 ml of complete DMEM (ratio 1:1 Trypsin: Complete DMEM) was added into the plate to stop the action of trypsin. The whole solution was then transferred into

a 15 ml centrifuge tube and centrifuged at 2000 g for 3 min. The supernatant was discarded carefully without removing the cell pellet. After that, 4 ml of complete DMEM was added into the centrifuge tube and the solution was suspended up and down to allow the cell to homogenize.

The cell was then transferred into two separate culture plates with the addition 1 ml cell mixture into each plate, followed by addition of 5 ml of complete DMEM. The cell was allowed to homogenize. Lastly, the cell was incubated in the incubator (37°C, 5% CO_2) and allowed to grow for 24 h before proceeding with the experimentation.

3.1.4 Cell Counting

The cells were counted by using hematocytometer for each experimental set-up depending on the required amount of cell seeding number for each type of procedure conducted. After obtaining the cell pellet, 4 ml of complete media was added into the centrifuge tube and the mixture was pipetted up and down to make sure the cell homogenized. The hematocytometer kit was prepared and 10 µl of the cells mixture was added onto the hematocytometer glass. The cell was then counted under 10X magnification for each four quadrant.

Calculation:

i) Cell number:

$$X = \frac{n_1 + n_2 + n_3 + n_4}{4} \times 10^4$$

n=number of cells in quadrant

X=cell number

ii) Volume of seeding:

$$\frac{\text{Desired seeding number}}{X} = ? \text{ Ul}$$

3.2 Preparation of TSA

Table 3.1: Information of TSA.

Drug	Company	Solvent	Storage	Stock Concentration	Working Concentration
TSA	InvivoGen	100% EtOH	-20°C	1 mg/ml	10, 100, 1000 and 10000 ng/ml

TSA (Table 3.1) is supplied as a lyophilized powder and it was dissolved in 100% EtOH (2 mg/ml). For this study, 10, 100, 1000 and 10000 ng/ml TSA were needed to be prepared by serial dilution. Certain amount of drug and complete DMEM (based on calculation) was aliquot into a new sterile falcon tube. From the stock solution (1000 µg/ml or 1 mg/ml), 10 µg/ml (10000 ng/ml) of TSA was prepared by adding 12 µl of TSA and 1188 µl of complete DMEM. From 10 µg/ml TSA, 1 µg/ml (1000 ng/ml) TSA was prepared by adding 120 µl of 10 µg/ml TSA and 1080 µl media into a new tube labeled with 1 µg/ml. For the preparation of 100 ng/ml (0.1 µg/ml) TSA, 120 µl of 1 µg/ml TSA and 1080 µl media into a new tube. Finally, for 10 ng/ml (0.01 µg/ml), 120 µl of 100 ng/ml TSA and 1080 media were added into a new tube. This preparation was conducted in the BSC to avoid EtOH evaporation.

3.3 MTT Assay

3.3.1 *Preparation of MTT Solution*

5 ml of MTT solution was prepared aseptically by weighing 25 mg of MTT using analytical balance scales which was then dissolved in 5 ml Phosphate Buffer Saline (PBS). The solution was re-suspended until it dissolved completely. The MTT solution must be kept at 4°C in the dark for no more than 2 weeks. Before use, the MTT solution is pre-warmed at 37°C and gently mixed by inverting the solution without creating bubbles.

3.3.2 Experimental Set-Up

The experiment was set-up by preparing the MCF-7 cells aseptically. The cell pellet was obtained as per cell in the cell splitting procedure. The cell pellet was obtained in a 15 ml centrifuge tube, and then a total of 4 ml of complete media was added into the tube and re-suspend, making sure the cell dissolved completely. The cell was then counted in the