EFFECTS OF KNOCKING-DOWN HIF-1α ON MIGRATION IN HUMAN BREAST CANCER CELL MDA-MB-231

by

NUR SABRINA BINTI ABD RASHID

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NUR SABRINA BINTI ABD RASHID (112035)

ABBREVIATION

µg	Microgram
µl	Microlitre
µm	Micrometer
mL	Millilitre
%	Percentage
<	Less than
ARNT	Aryl hydro-carbon receptor nuclear translocator
ASR	Age standardised incidence rate
Bca	Breast cancer
cDNA	Copy deoxyribose nucleic acid
CTCs	Circulating tumor cells
DCIS	Ductal carcinoma in situ
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
EMT	Epithelial-mesenchymal transition
FBS	Foetal bovine serum
HRE	HIF response element
HIF-1α	Hypoxia inducible factor-1α
mRNA	Messenger ribose nucleic acid
Nav	Voltage-gated sodium channels
nNav 1.5	'Neonatal' Nav 1.5
NCR	National Cancer Registry

PBS	Phosphate buffered saline
PCa	Prostate cancer
PHD	Prolyl hydroxylase
PTEN	Phosphate and tensin homolog
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-PCR	Real-time PCR
SEM	Standard error of mean
siRNA	Small interfering RNA
VHL	von Hipple-Lindau
VEGF	Vascular endothelial growth factor
VGSCs	Voltage-gated sodium channels

ABSTRAK

Kanser payudara merupakan satu kebimbangan kesihatan global di seluruh dunia. Telah dianggarkan bahawa lebih daripada satu juta wanita telah disahkan menghidapi kanser payudara setiap tahun dan lebih daripada 410,000 wanita mati disebabkan oleh kanser ini. Keupayaan sel kanser payudara untuk metastasis menjadi barah kedua (secondary tumor) adalah aspek yang paling berbahaya yang boleh membawa kepada kematian. Ciri-ciri ini amat berkait rapat dengan keadaan yang dipanggil sebagai hipoksia yang memainkan peranan penting dalam perkembangan penyakit malignan. Tumor pepejal seperti kanser payudara telah dikaitkan dengan hipoksia Di bawah hipoksia, akan terjadinya penstabilan faktor transkripsi 'hypoxia-inducible factor-1α (HIF-1α)' yang boleh mengawal ekspresi banyak gen termasuk yang terlibat di dalam proses metastasis tumor. Peningkatan ekspresi "voltage-gated sodium channels (VGSCs)" terutamanya isoform kardium Nav 1.5 dan nNav 1.5 telah terlibat dalam membuatkan kanser payudara cenderung menjadi lebih agresif. Dalam kanser payudara agresif, terdapat kolerasi positif antara HIF-1α dan Nav 1.5 serta nNav 1.5. Dalam usaha untuk mengkaji interaksi antara kedua-dua molekul ini, satu kajian telah dijalankan untuk mengkaji kesan 'knocking down' HIF-1α pada penghijrahan kanser payudara meggunakan sel MDA-MB-231. Aktiviti HIF-1α telah disenyapkan dengan mensasarkan HIF-1α menggunakan 'small interfering RNA (siRNA)'. Kemudian, tahap mRNA HIF-1α, Nav 1.5 dan nNav 1.5 diukur meggunakan kaedah 'real-time PCR' dan kesan ke atas penghijrahan sel ditentukan menggunakan kaedah penghijrahan melintang. siRNA HIF-1α telah berjaya menggurangkan tahap Nav 1.5 dan nNav 1.5 sebanyak ~31 % dan ~12 % masing-masing. Manakala, siRNA juga telah meggurangkan penghijrahan sel MDA-MB-231 sebanyak ~37 %. Dari hasil siRNA di atas, dapat disimpulkan bahawa HIF-1α

dan VGSCs mempunyai perkaitan dan apabila perkaitan ini diganggu, ia turut menyebabkan penghijrahan tumor juga tertindas.

ABSTRACT

Breast cancer is a global health concern worldwide. It is estimated that more than one million women are diagnosed with breast cancer every year and more than 410,000 will die from the diseases. The ability of breast cancer cell to metastases to a secondary tumor is the most dangerous aspect that leads to death. These characteristics of breast cancer cells are closely related to the condition called hypoxia which plays an important role in the progression of malignant disease. Solid tumor such as breast cancer has been associated with hypoxia. Under hypoxia, the stabilization of the key transcription factor hypoxia-inducible factor-1α (HIF-1α) can regulate the expression of many genes including those that involve in tumor metastasis process. Increase expression of voltage-gated sodium channels (VGSCs) particularly the cardiac isoform Nav 1.5 and its neonatal splice variant, nNav 1.5 have been implicated in the promotion of breast cancer aggressiveness. In aggressive breast cancer, there is positive correlation between HIF-1α and Nav 1.5 as well as nNav 1.5. In order to study the interaction between the two molecules, this study is aimed at investigating the effects of knocking down HIF-1α on the migration of breast cancer, MDA-MB-231 cells. The activity of HIF-1α was silenced by targeting HIF-1α using small interfering RNA (siRNA). The HIF-1α, Nav 1.5 and nNav 1.5 mRNA level were measured using real-time PCRs and effects on cell migration were determined using transverse migration. The HIF-1α siRNA reduced the level of Nav 1.5 and nNav 1.5 mRNA by ~31 % and ~12% respectively. Importantly, the siRNA supressed *in vitro* migration of MDA-MB-231 cells by ~37 %. From the siRNA result, it was concluded that HIF-1α and VGSCs are associated and that when this association was interrupted, tumor migration was also suppressed.

CHAPTER 1

1.0 INTRODUCTION

1.1 Background of study

Breast cancer (BCa) is the most common cancer diagnosed in women and the first cause of death among women worldwide. The most dangerous aspect of the BCa that could lead to death is their ability to metastases. Therefore, metastasis is proven to be a key step in the progression of malignant disease. Since 1994, the role of ionic channels in breast cancer has been studied which mainly focus on potassium channels. However, recent studies found that voltage-gated sodium channels (VGSCs) were upregulated in metastatic human BCa that have significant role in BCa progression.

Recent *in vitro* study shows that strongly metastatic human MDA-MB-231 breast cancer cell line expressed functional VGSCs (Roger *et al.*, 2004; Fraser *et al.*, 2005). Importantly, this VGSC activity contributes to many cellular behaviour related to metastasis including cellular process extension, lateral motility, galvanotasis, transverse invasion and secretory membrane activity (Fraser *et al.*, 2005). These proteins are comprised of a core α-subunit together with one or more auxiliary, modulatory β-subunit. In MDA-MB-231 cells, the Nav1.5 α-subunit was predominantly expressed about 80 % at mRNA level. Interestingly, the Nav1.5 was expressed primarily in its 'neonatal' DI:S3 3'-splice form (nNav1.5) and it was proven that nNav1.5 was significantly upregulated during BCa progression and can potentiate a series of cell

behaviours integral to the metastatic cascade (Fraser *et al.*, 2005; Brackenbury *et al.*, 2007).

One factor that may play an important role in the progression of malignant disease including BCa is the presence of hypoxia. It is an important indicator of cancer prognosis associated with aggressive growth, metastasis and poor response to treatment. Studies on gene expression have demonstrated that hypoxia-inducible factor-1α (HIF-1α) is a key transcription factor that is upregulated in hypoxic condition (Wykoff *et al.*, 2000; Harris, 2002). It activation resides in the inhibition of post-translational hydroxylation of the α-subunit that permits stabilization, heterodimerization and binding to hypoxia response element (HRE) in target genes (Brahimi-Horn *et al.*, 2007). Expression profiling studies have highlighted several classes of genes that are up-regulated through the activation of HIF-1α which include angiogenic factors, proliferation, cell-adhesion, cell survival/death, metabolism, pH regulation, migration and metastasis (Harris, 2002; Brahimi-Horn *et al.*, 2007). Furthermore, there is evidence that VGSC ion channels also respond to hypoxia by abnormal Na^+ influx (Hammarström and Gage, 2002).

1.2 Significance of study

The study on interaction of HIF-1α and VGSCs in controlling the metastasis is to provide knowledge about the correlation between transcription factor HIF-1α and VGSCs. In addition, this study also provides better understanding on BCa metastasis pertaining to the effects of the cells migration. The ability of tumor cells to survive under hypoxic conditions is the main problem to combating with BCa progression. Currently, the correlation between HIF-1α and VGSCs in BCa has not yet been

investigated and since HIF-1α is of prognostic significance, there is a possibility for HIF-1α to be target for therapies downregulating the proliferation rate of BCa.

1.3 Objective of study

1.3.1 General objective

The general objective of this study is to investigate the interaction of HIF-1α and VGSCs in breast cancer migration.

1.3.2 Specific objectives

The specific objective of this study as below:

1. To knock-down HIF-1α in MDA-MB-231 breast cancer cells using siRNA.
2. To investigate the effect of HIF-1α knock-down on the migration of MDA-MB-231 cells using transverse migration assay.
3. To study the relationship between HIF-1α and VGSCs on cancer cell migration.

1.4 Conceptual framework

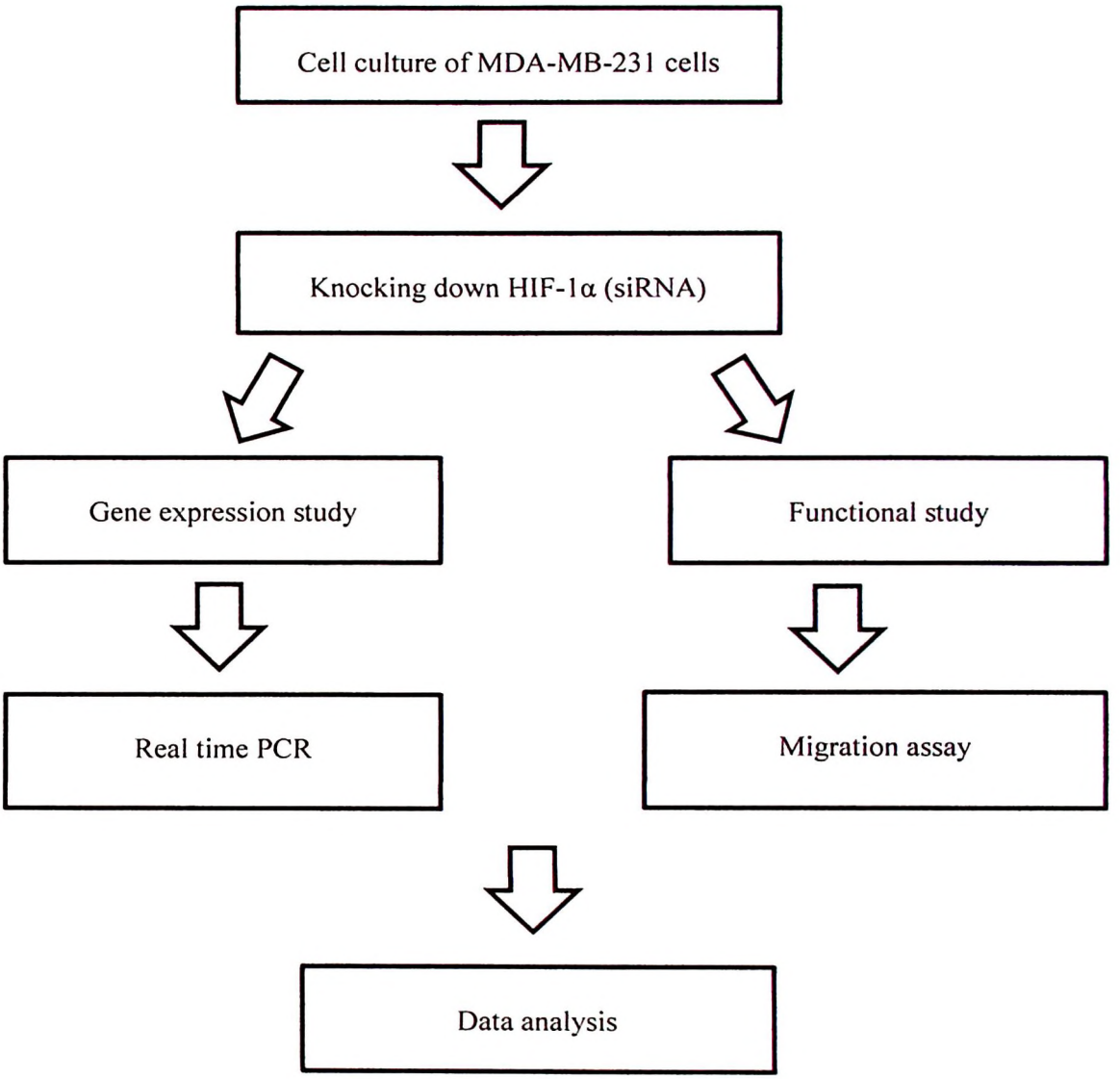

Figure 1: Research framework for this study

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Breast Cancer

2.1.1 History of breast cancer

BCa is an ancient disease which has been described by the Egyptians 3,000 years ago by Edwin Smith and George Ebers papyri. One of the descriptions refers to bulging tumors of the breast that has no cure. In 460 B.C., Hippocrates, the father of Western Medicine, described breast cancer as a humoral disease by hard tumors appearing in the breast, become increasingly firm, contain no pus, and spread to other parts of the body (Donegan, 2006). While Galen (129-200 BC) viewed BCa as a systemic disease caused by an excess of black bile in the blood and postulated that some tumors were more dangerous than others. Each anatomic discovery generated new theories about BCa. In seventeenth centuries, the father of investigative surgery, John Hunter conceived that coagulation of lymph rather than black bile was responsible for carcinoma of the breast and the associated cancerous nodes.

While in nineteenth century, the microscope became a key toward progress in pathology which made Johannes Müller first report that cancers were composed of living cells that had lost the proportions of the normal cells. Müller was the first to suspect that spread of malignant cells constituted the mechanism of metastasis which later was confirmed by the microscopic work of Carl Thiersch (1822-1895) and Wilhelm von Waldeyer (1836-1921) that supported the concept that breast cancer

spread from a local origin (Donegan, 2006). Until the twentieth century, breast cancer was recognized as a major health problem worldwide, thus leading to the development of more effective treatments.

2.1.2 Epidemiology

Cancer is the leading cause of death in many developed countries and the second leading cause of death in developing countries. Based on the data released by GLOBOCAN 2008, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred which contribute about 56 % of the cases and 64 % of the deaths occurred in the economically developing world. Among that, BCa is the most frequently diagnosed cancer and one of the leading causes of cancer death in females worldwide. Public health data indicate that the global burden of breast cancer in women measured by incidence, mortality, and economic costs is substantial and increasing, accounting for 23% (1.38 million) of the total new cancer cases and 14% (458,400) of the total cancer deaths in 2008. BCa incidence rates have been reported to be increasing by up to 5% per year in many populations of developing countries. There is marked geographical variation, with the highest incidence in Western and Northern Europe; intermediate in South America and Northern Africa; and low in sub-Saharan Africa and Asia (Figure 2.1). These examples clearly indicate that a disease once called a disease of the western world' has gone global.

In Malaysia, 3,738 new cases of BCa were reported to the National Cancer Registry (NCR) of Malaysia, giving an age standardised incidence rate (ASR) of 46.2 per 100,000 women in 2003 (Yip *et al.*, 2006). This means that 1 in 20 women in Malaysia will develop breast cancer in their lifetime. However the rate differs between the three main races Malays, Chinese and Indians. The age standardized incidence in

Chinese is the highest, with 59.7 per 100,000 followed by the Indians at 55.8 per 100,000. The Malays have the lowest incidence of 33.9 per 100,000. This translates into both 1 in 16 Chinese women and Indian women and 1 in 28 Malay women will develop BCa at some stage in their lives (Lim GCC; Halimah Y, 2004). While according to the age, the mean age was 48.1 years in Malays, 51.4 years in Chinese and 52.3 years in Indians (Yip *et.al*, 2006). When compared to data in the developed countries such as USA and Europe, the incidence of breast cancer in Malaysia is less than half that of USA.

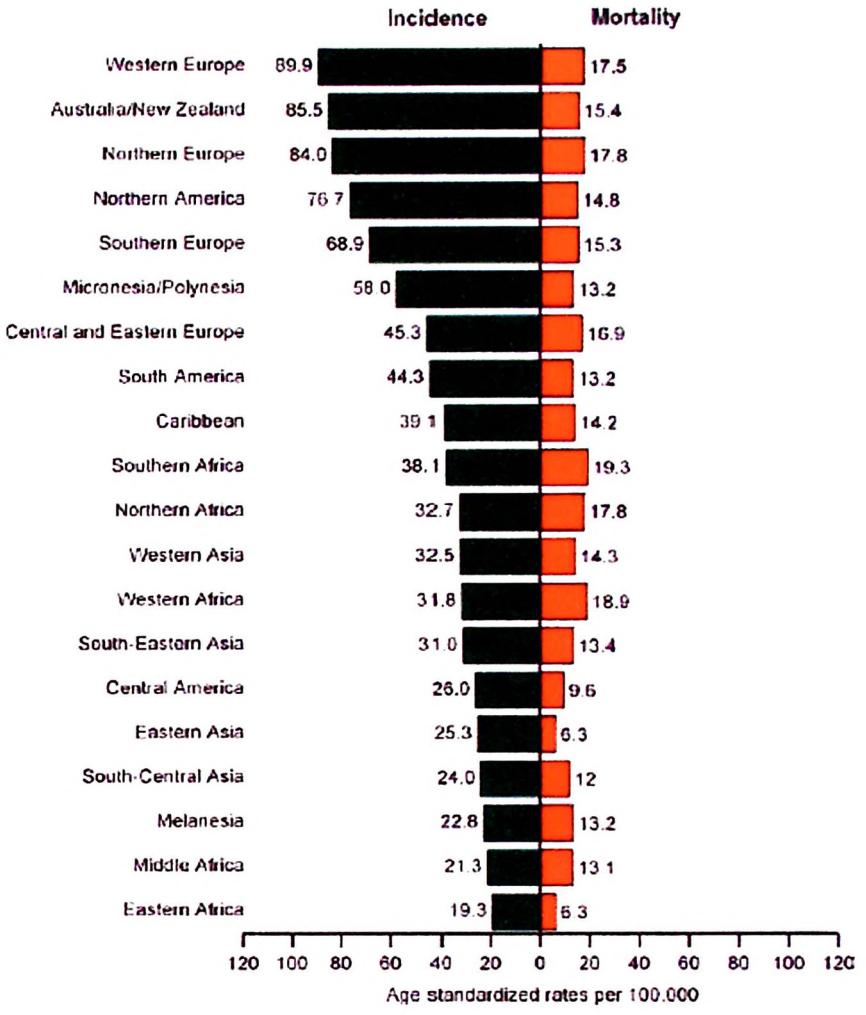

Figure 2.1: Breast Cancer Incidence and Mortality Rates by World Area.

Source: GLOBOCAN (2008)

2.1.3 Risk factor

BCa can arise due to several risk factors. Experimental data had strongly suggested that estrogens have a role in the development and growth of breast cancer. The risk of getting BCa also depends on increasing age, geographical variation, age at menopause, age at first pregnancy, radiation, previous benign breast disease, lifestyle, etc. (McPherson *et al.*, 2000; Clemons and Goss, 2001). Up to 10 % of BCa incidence is due to genetic predisposition. Two BCa genes, *BRCA1* and *BRCA2* have been identified for a substantial proportion of high-risk individuals (McPherson *et al.*, 2000). Among all the risk factors, environmental factors are of greater importance than genetic factors in developing BCa. Yip *et al*, (2006) suggested that the lower incidence in Malaysia and other countries in Asia and Africa could be due to the differing risk factors associated with lifestyle (*e.g* smoking, alcohol intake, weight, etc.), reproductive factors and diet. The predictive value of these factors in assessing the risk of BCa is increased by their combination as it provides more accurate assessment of risk than each factor assessed individually.

2.2 Breast Cancer Metastasis

Metastasis is the spread of cells from the primary tumor to distant organs and their relentless growth. It is responsible for about 90 % of cancer-associated mortality as it becomes resistant to conventional therapies. Stephen Paget proposed that metastasis depends on cross-talk between selected cancer cells and specific organ microenvironments in his 'seed and soil' hypothesis (Fidler, 2003). The potential of tumor cell to metastasize depends on its interactions with the homeostatic factors that promote tumor cell growth, survival, angiogenesis, invasion and metastasis.

There are three principles involve in 'seed and soil' hypothesis. Firstly, the primary tumor and metastases consist of both tumor cells and host cells including epithelial cells, fibroblast, endothelial cells and infiltrating leukocytes. The second principle is that the process of metastasis is selective for cells that succeed in invasion, embolization, survival in the circulation, arrest in a distant capillary bed and extravasation into and multiplication within the organ parenchyma. This causes the survival and growth of a few subpopulations of cells that pre-exist within the parent tumor. Therefore, metastases could have clonal origin and different metastases can originate from the proliferation of different single cells. The most important principle is that metastases could only develop on specific organs such as lungs, pleura, brain, liver and bones (e.g vertebrae, legs and ribs) (Plunkett *et al.*, 2000; Fidler, 2003). This microenvironment of organs is biologically unique that influences the phenotype of metastases develops.

2.2.1 Mechanism of metastasis

The process of metastasis is a sequential multistep process that can be simplified into two major phases (Figure 2.2). The first phase involves the physical translocation of cancer cells from the primary tumor to the microenvironment of a distant tissue (Eccles and Welch, 2007; Chaffer and Weinberg, 2011). In order for the individual cancer cell to break away from the primary tumor, these cells must acquire the ability to migrate and invade. These cells move through the extracellular matrix of the surrounding tissue toward blood and lymphatic vessels for their passage to secondary sites. Normal epithelial cells are tightly bound to neighboring cells and to underlying basement membranes by adherent junctions, tight junctions, desmosomes and hemi-desmosomes which effectively immobilizing them in a sheets. However, as tumor progresses, cells

disseminate themselves from these associations and begin to dissolve underlying basement membrane and invade adjacent stromal compartments. Therefore causes cells to both intravasate and subsequently extravasate (Chaffer and Weinberg, 2011).

The second phase is the adaptation of the disseminated cell to the microenvironment at the metastatic site or term as colonization. The circulating tumor cells transiting from the primary tumor to metastatic site can arrive at their destination via variety of mechanisms such as become lodged in the capillary beds of specific organs by displaying specific adhesion molecules that enable them to adhere to microvessels in specific organs or by respond to a chemoattractive gradient arise from a particular tissue. The tumor cells also may preferentially home to organs where premetastatic niche has prepared a microenvironment conductive for their survival. When the cells have extravasated they first experience a period of quiescence or dormancy while they adapt to their new microenvironment (Pantel and Brakenhoff, 2004; Eccles and Welch, 2007; Chaffer and Weinberg, 2011). These dormant cells may progress to micrometastatic deposits in response to the recruitment of appropriate stroma and proliferative signals present in the host microenvironment. At this stage, the size of the cells are kept balance by proliferation, apoptosis and phagocytosis by host-tissue immune system. Later, when cancer cells have recruited an adequate blood supply, they develops into a macrometastasis (growth beyond 1 to 2 mm).

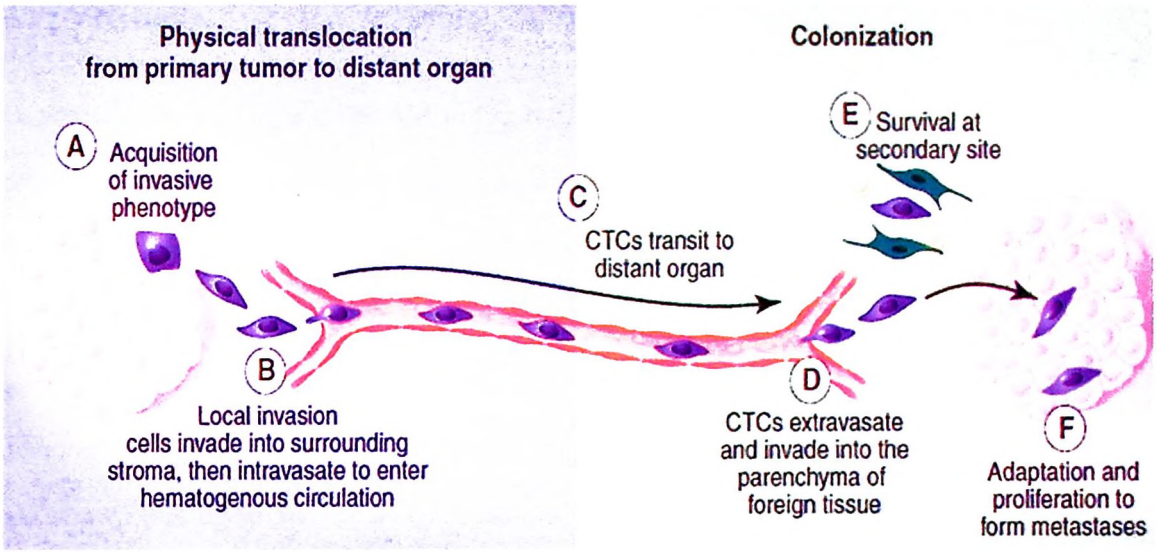

Figure 2.2: The diagram of metastatic cascade

Source: "A Perspective on Cancer Cell Metastasis" by Chaffer and Weinberg (2011)

Metastasis occurs in two major phases; (i) physical translocation of cancer cell from primary tumor to a distant organ and (ii) colonization of the translocated cells. (**A**) The cascade begin when primary tumor exhibit an invasive phenotype. (**B**) Cancer cells invade into surrounding matrix toward blood vessels to enter the circulation. (**C**) Cancer cells travelling through the circulation as CTCs. (**D**) At the distant organ, CTCs exit the circulation and invade the microenvironment of foreign tissue. (**E**) At the secondary site, cancer cells must able to survive as a single cell. (**F**) Adaptation to the new microenvironment results in the cell proliferation for macrometastatic deposit.

2.3 Hypoxia

Hypoxia is defined as a reduction in the normal level of tissue oxygen tension which could occur during acute or chronic vascular disease, pulmonary disease and cancer (Harris, 2002). It is the important factor that plays a role in the progression of malignant disease. Hypoxic areas arise as a result of an imbalance between the supply and consumption of oxygen. Tumor cells become hypoxic because new blood vessels develop are aberrant and have poor blood flow (Harris, 2002; Vaupel, 2004). Evidence has shown that up to 50 - 60 % of locally advanced solid tumors may exhibit hypoxic tissue areas that are heterogeneously distributed within the tumor mass. This is due to the oxygen consumption rate of tumor cells that may outweigh a restricted oxygen supply (Vaupel, 2004).

There are several pathogenic mechanism involve in the development of hypoxia in solid tumor which are: (i) severe structural and functional abnormalities of tumor microvessels such as leakiness, irregular architecture, lack of endothelial linings and others (Chaudary and Hill, 2007) that term as *perfusion-limited O_2 delivery* or 'acute hypoxia'. Hypoxia also may develop through; (ii) deterioration of diffusion geometry where it causes increase in diffusion distances, so that the cells far away from nutritive blood vessels receive less oxygen than needed (*diffusion-limited hypoxia*) or term as 'chronic hypoxia'; (iii) tumor-associated or therapy-induced anemia can also contribute to the development of hypoxia by reducing oxygen transport capacity of the blood (Vaupel, 2004).

There was evidence showing the association between hypoxia and metastasis *in vitro* and *in vivo* (Dachs and Tozer, 2000; Goonewardene *et al.*, 2002; Vaupel, 2004) through regulating the expression of molecules required at various steps of the

metastatic process. For example, hypoxia disrupts the epithelial-mesenchyal transition (EMT) through the loss of the transmembrane molecule Epithelial (E)-cadherin, a cellular adhesion molecule that regulates cell-cell adhesion. Besides that, hypoxia also increased the expression of extracellular matrix protein, LOX which promote the invasive and metastatic potential of BCa. Studies also have shown that hypoxia through hypoxia inducible factor (HIF) is a potent inducer for CXCR4 gene that involved in directional migration of metastatic tumor cells (Rankin and Giaccia, 2008).

2.3.1 Hypoxia-inducible factor (HIF)

One way that cells respond to reduced oxygen levels is through the hypoxia –inducible factor (HIF) pathway. There are three HIF that are already identified which is HIF-1, HIF-2 and HIF-3. Among the three, HIF-1 is a key transcription factor that is induced by hypoxia (Harris, 2002; Rankin and Giaccia, 2008) since it is ubiquitously expressed. HIF-1 is a heterodimeric protein that consists of a constitutively expressed HIF-1α subunit and HIF-1β subunit (Harris, 2002; Semenza, 2003; Vaupel, 2004). The level of HIF-1α expression is determined by the rates of the protein synthesis and protein degradation where their synthesis is regulated via O_2-independent mechanism whereas degradation is regulated primarily via O_2-dependent mechanism (Semenza, 2003).

Under normal condition (normoxia), HIFs are targeted for proteosomal degradation by the von Hippel-Lindau (VHL) tumor suppressor. HIF-1α will bound to the VHL protein and become ubiquitylated and then targeted to the proteoseome where it is degraded (Goonewardene *et al.*, 2002; Harris, 2002; Rankin and Giaccia, 2008). Whereas, under low oxygen level (hypoxia), HIF-1α subunits are stabilized by heterodimerize with the β-subunits and translocated to the nucleus. In the nucleus, the

complex will bind to hypoxia-response elements (HREs) where it activates transcription by recruiting the transcriptional activators p300 and CBP and start to activate expression of hypoxia-response genes (Rankin and Giaccia, 2008). There are numbers of HIF-regulated genes that promote key aspects in tumorigenesis including angiogenesis (PDGF-B, VEGF-A, etc.), metabolism (GLUT-1, GLUT-2, ALDA, etc.), proliferation (EPO, TGF-α, etc.) and in invasion and metastasis (CXCR4, LOX, E-CADHERIN, etc.) (Vaupel, 2004; Rankin and Giaccia, 2008).

This transcription factor has emerged as an important transcription factor in Bca and prostate cancer (PCa) biology that are expressed in the early stages of mammary and prostate carcinogenesis. In BCa, the upregulation of HIF-1α is observed in both Her2/erb2 overexpressing and Her2/erb2 negative tumors (Bos *et al.*, 2004). High level of HIF-1α expression in human BCa occurs in the pathogenesis of BCa and change the molecular pathogenesis of BCa. In normal breast epithelium, there is an absence of high levels of HIF-1α protein expression, while in early stages of mammary carcinogenesis their expression is correlates with areas of nearby necrosis in ductal carcinoma in situ (DCIS) and also associated with VFGF-C expression in invasive ductal carcinoma (Kimbro and Simons, 2006). The association of HIF-1α with most of the proliferation markers (bFGF, PDGF-BB and EGFR) shows that it enhanced expression of growth receptors and increase glycolic activity in BCa (Bos *et al.*, 2004; Bos *et al.*, 2005).

The upregulation of HIF-1α is not only determined by the hypoxic condition of the microenvironment of tumor cells but also through oxygen-independent regulation, RAS-ERK pathway (Semenza, 2003) where it effects VEGF expression through HIF-1α via mutations and allelic loss of the Phosphatase and tensin homolog (PTEN) tumor suppressor gene (Zhong *et al.*, 2000; Gomez-Manzano *et al.*, 2003; Majumder *et al.*, 2004). The losses of tumor suppressor gene function can also up-regulate HIF-1α as

well (Zagzag *et al.*, 2005). In addition, oxygen-free radicals have also been stabilize HIF-1α protein by reducing prolyl-4-hydoxylase domain (PHD) enzymatic actions on HIF-1α (Kaelin Jr, 2005) (Figure 2.3). Since, there is evidence indicating that HIF-1 mediates resistance to chemotherapy and radiation, targeting its activity could therefore represent an important component of cancer therapies.

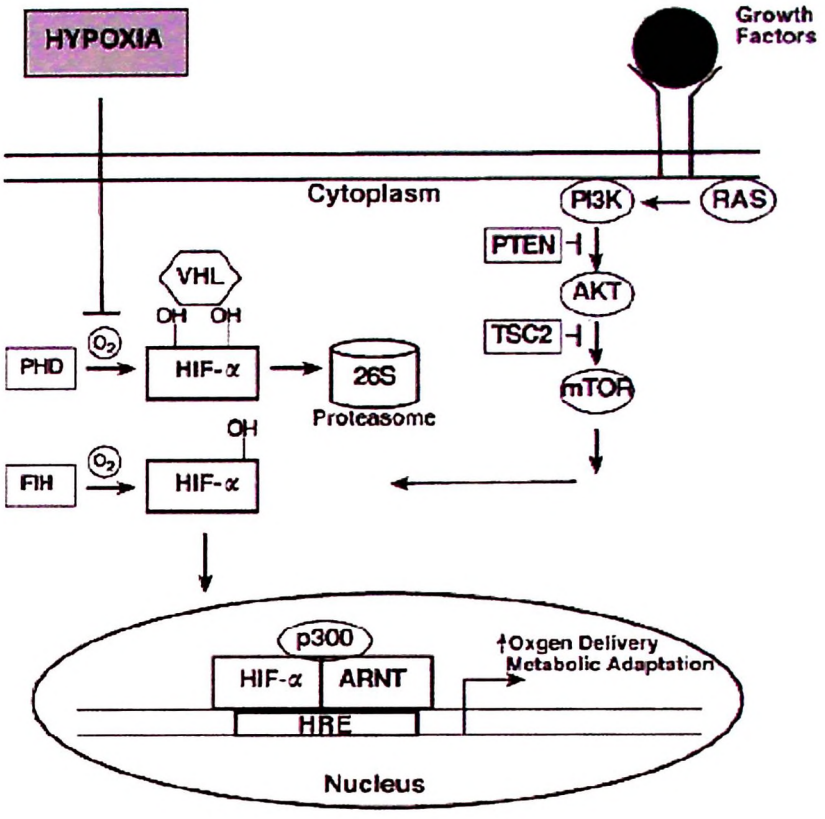

Figure 2.3: Mechanism of HIF activation in cancer cells under hypoxic condition

Source: "The Role of Hypoxia-inducible factors in Tumorigenesis" by Rankin and Giaccia (2008)

Low oxygen inhibits both PHD and factor inhibiting HIF-1 (FIH-1) activity which negatively regulates HIF stability and cofactor (p300/CBP) recruitment. Under normal condition, hydroxylation of proline residue within HIF-1α mediates pVHL binding and subsequent ubiquitination and degradation by 26S proteasome. However, in hypoxic condition, loss of pVHL stabilized HIF-1α and translocated to the nucleus where it heterodimerizes with ARNT and bind to hypoxia response elements (HREs) in target genes upon cofactor recruitment. HIF activity can also be induced through growth factor signalling, oncogenic Ras activation or inhibition of negative regulators (e.g PTEN).

2.4 Voltage-gated Sodium Channels (VGSCs)

Voltage-gated sodium channels (VGSCs) are macromolecular protein complexes containing a highly processed α-subunit which is approximately 260 kDa associated with at least two auxiliary β-subunits which are approximately 33-38 kDa. The VGSC-α protein contains four repeat domains (DI to DIV), each are composed of six transmembrane segments (S1 to S6) (Diss *et al.*, 2004) (Fig 2.4). The pore-forming α-subunit is sufficient for functional expression, but the channel and current density, kinetics and voltage-dependence of gating are modified by the β-subunit. There are ten genes that encoding for the VGSC-α subunits have been identified and nine of these genes constitute a single family named Na_v1 according to their phylogeny and are designated into $Na_v1.1$ to $Na_v1.9$. While, they also can be further classified based on their tetrodotoxin (TTX) sensitivity, where they are classified as TTX-sensitive ($Na_v1.1$ to $Na_v1.4$, $Na_v1.6$ and $Na_v1.7$) and TTX-resistant ($Na_v1.5$, $Na_v1.8$ and $Na_v1.9$) (Frank and Catterall, 2003; Roger *et al.*, 2006).

The expression of VGSCs is common in classically 'excitable' cells within the central nervous system (CNS), peripheral nervous system (PNS) and muscle where they involve in generation and conduction of action potentials (Catterall, 2000). However, recently it has been shown that VGSCs also expressed in 'non-excitable' cells including glia, lymphocytes, osteoblasts, fibroblasts and endothelial cells (Diss *et al.*, 2004). In addition, recent studied has shown the correlation of these VGSCs with metastatic cancer cells of epithelial origin (carcinomas), particularly in those of breast, cervix, colon, lung (small-cell, non-small-cell and mesothelioma), skin, ovary and prostate (Catterall *et al.*, 2005; Fraser *et al.*, 2014) where they involve in disease progression that lead to metastasis. In the beginning of the 2000's, two group of researches (Roger S *et al* and Djamgoz M) showed that VGSCs (Nav 1.5, Nav 1.7 and Nav 1.6) were

expressed in a highly metastatic breast cancer cell line, MDA-MB-231 cells which ~80 % were contributed by Nav 1.5. In one study, Nav 1.5 was identified to present predominantly in its neonatal form, nNav 1.5 (Fraser *et al.*, 2005). These finding was supported by the hypothesis that embryonic genes may be re-expressed in cancer cells (Monk and Holding, 2001).

2.4.1 Regulation of VGSC expression in cancer cells

The expression of VGSC can be regulated from the transcription level to the post-translational level by specific mechanism (e.g miRNAs, intracellular trafficking, etc.) (Fraser *et al.*, 2014). It was found that primary regulators such as hormone, growth factor and intracellular Na^+ concentration are all associated with VGSC regulation (Brackenbury and Djamgoz, 2006; Fraser *et al.*, 2014). In BCa, the nNav 1.5 allows greater entry of Na^+ into the cell which in turn will regulate the intracellular and extracellular pH, enzyme activity and Ca^{2+} homeostasis. In addition, hypoxia that has been discussed earlier is also associated with VGSCs activity. Recent finding showed that hypoxia could causes abnormal Na^+ influx (Hammarström and Gage, 2002). The VGSC activity has shown to contributed to many cell behaviour including in cells migration, invasion, colony formation in three-dimensional Matrigel, process extension, galvanotaxis, adhesion, gene expression, endocytic membrane activity, vesicular patterning, nitric oxide production and invadopodia formation (Fraser *et al.*, 2014). Since, VGSC plays a significant role in cancer progression, thus it can be a potential novel therapeutic target against metastatic diseases.

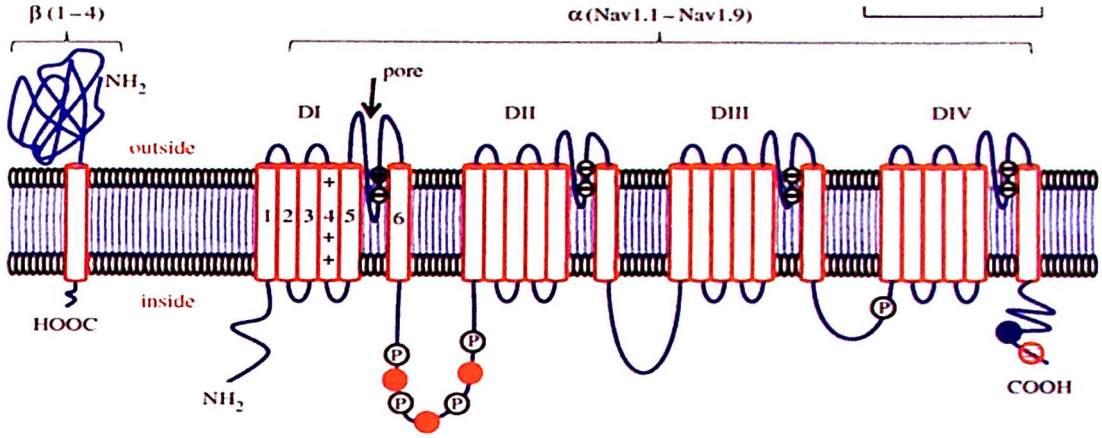

Figure 2.4: A schematic diagram of VGSC structure

Source: "Regulation of voltage-gated sodium channel expression in cancer: hormones, growth factors and autoregulation" by Fraser *et al.* (2014)

The α-subunits have four domains (DI-DIV) which each composed of six transmembrane segments. While, β-subunits are composed of N-terminal extracellular immunoglobulin loop with one transmembrane segment.

CHAPTER 3

3.0 MATERIALS AND METHODOLOGY

3.1 Material

3.1.1 MDA-MB-231 Cell

The MDA-MB-231 cell line used in this study was isolated from human mammary gland. It is an epithelial cell type that adheres to the culture plate when cultured. These cell lines were used as it is an aggressive type of breast cancer cells. HIF-1α was two to three times higher in MDA-MB-231 cell lines (Hiroko Bando, 2003), therefore this cell lines was used in this study.

3.1.2 Chemicals and Reagents

The following chemicals have been used in this experiment: are Dulbecco's Modified Eagle Medium (DMEM), Penicillin-streptomyocin, L-glutamine, Fetal bovine serum (FBS), Dimethyl sulfoxide (DMSO), Trypsin, Phosphate buffer saline (PBS), Ultrapure DNase/RNase-free distilled water, Serum-free medum, Sepasol-RNA I (Nacalai), Chloroform, 100 % isopropanol, 75 % ethanol, methanol and crystal violet.

3.1.3 Primer

The sequences of primer are listed in Table 3.1.

3.1.4 Commercial Kits

i. siRNA SMARTpool: ON-TARGET plus (Dharmacon)

ii. QuantiTect Reverse Transcription (Qiagen)

iii. Sensi FAST™ SYBR Hi-Rox kit (Qiagen)

3.1.5 Laboratory Instrument and Equipment

The instrument used in this experiment including BD falcon tube (15 mL and 50 mL), 1.5 mL microcentrifuge tube, culture dish, 24 well plate, 8 µm migration insert, MicroAmp Optical strip, MicroAmp Optical cap, pipette, filtered pipette tips and serological pipette .

The list of equipment that have been used are biological safety cabinet type II, centrifuge, refrigerated microcentrifuge, Nanodrop spectrophotometer, incubator, DNA/RNA UV-clearner, hot plate, Neubouer haemocytometer, inverted microscope and freezer (4^0C, -20^0C, -80^0C).

3.1.6 Software

i. ABI prism 7000 SDS software

ii. ImajeJ

iii. Microsoft Excel

Table 3.1: Sequences of the primer pairs used for real-time PCR

Target gene	Primer sequence	Amplicon size (bp)	Accsession number.
HIF-1α	5'-CAACCCAGACATATCCACCTC-3' (F) 5'-CTCTGATCATCTGACCAAAACTCA-3' (R)	104 bp	NM_001530
CA9	5'-CCTTTGCCAGAGTTGACGAG-3' (F) 5'- TGGAAGAAATCG CTGAGGAAG -3' (R)	135 bp	NM_001216.2
NaV1.5	5'-TTGCTTGTTATGGTCATTGGC-3' (F) 5'-GTTGTTCATCTCTC TGTCCTCAT-3' (R)	117 bp	NM_001160160
nNAv1.5	5'-CTGCACGCGTTCACTTTCCT-3' (F) 5'-GACAAATTGCCTAGTTTTATATTT-3' (R)	101 bp	NM_001160160.1
β-actin	5'-ATTGCCGACAGGATGCAGAAG-3' (F) 5'-TAGAAGCATTTGCGGTGGACG-3' (R)	203 bp	NM_001101
Cyclophilin B	5'-CTCTCCGAACGCAACATGAAG -3' (F) 5'-ACCTTGACGGTGACTTTGGG -3' (R)	128 bp	NM_000942.4

3.1.7 Reagent preparation

3.1.7.1 Complete Dulbecco's Modified Eagle Medium (DMEM)

The complete medium was prepared by adding 25 mL of 5 % FBS together with 10 mL of 4 mM L-glutamine and also 5 mL of 1 % penicillin-streptomycin into 500 mL of DMEM. The media were stored at 4^0C.

3.1.7.2 75 % Ethanol

Ethanol solution (75 %) was prepared by mixing 30.15 mL of 99.5 % ethanol (stock) with 9.85 mL of DNase/RNase free distilled water and stored at 4^0C.

3.1.7.3 Crystal Violet

Crystal violet solution was prepared by dissolving 0.05 g of crystal violet powder into 100 mL 0.25 % methanol. The solution were then stored at room temperature.

3.1.7.4 1 % FBS medium

To prepare 40 mL of 1 % FBS medium, 400 µL of FBS were added into 38.8 mL of serum free medium. Then followed by addition of 800 µL 200 mM L-glutamine to give final concentration of 4 mM L-glutamine. The medium was mixed well and stored at 4 ^{0}C.