EFFECTS OF KNOCKING-DOWN HIF-1α (siRNA) ON VGSC EXPRESSION IN THE AGGRESSIVE HUMAN BREAST CANCER CELLS (MDA-MB-231)

by

MOHD ZAKI BIN HUSSIN

Dissertation submitted in partial fulfilment
of the requirement for the degree
of Bachelor of Health Sciences (Biomedicine)
May 2015

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

ACKNOWLEDGEMENT

'In the name of Allah, the Most Gracious and the Most Merciful'

Alhamdulillah, thanks to Allah. This final year project is made possible through the help and support from everyone, including: parents, lectures, friends, and all the personal who directly involve in giving me their support, strength and courage. First of all, please allow me to dedicate my acknowledgment of gratitude toward the following significant advisors and contributors: First and foremost, I would like to thank Dr Noor Fatmawati Mokhtar for her most support, encouragement, supervision, guidance and constant lesson. She kindly offered invaluable detailed advices on every stages of the research progress and make this paper possible. Second, I would like to thank all master and Phd students, especially Miss Hemaniswari Dewi, Miss Nur Sabrina Kamarulzaman and Mr. Mohd Farid Hussin for their valuable advices, training and willingness to share with me their knowledge, experience and skills over the past few month of my pursuit of the bachelor degree. Here also, I want to thanks my research partner, Miss Nur Sabrina Binti Abd Rashid for all her great thought, quick thinking, and excellence idea and for being with me throughout all the time in order to ensure this beautiful project complete. I would also like to thank the institute, INFORMMs for their willingness to prepare the place for me to do my final year project by providing all facilities and equipment. Finally, I sincerely thank to my parents, family, and friends, who provide the advice and financial support. The product of this final year project would not be possible without all of them.

MOHD ZAKI BIN HUSSIN (114077)

ABBREVIATION

ALDA	Aldolase A
ARNT	Aryl hydro-carbon receptor nuclear translocator
µg	Microgram
µl	Microlitre
µm	Micrometer
BSA	Bovine serum albumin
CCND1	Cyclin D1
cDNA	Copy deoxyribose nucleic acid
CTGF	Connective tissue growth factor
CXCR4	C-X-C chemokine receptor type 4
DMEM	Dulbecco's modified medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribose nucleic acid
ENO1	Enolase 1
FBS	Foetal bovine serum
GLUT-1	Glucose transporter-1
GLUT-3	Glucose transporter-3
HK1	Hexokinase 1
HK2	Hexokinase 2
HRE	HIF response element
HIF-1α	Hypoxia inducible factor-1α

IGF-2	Insulin growth factor-2
IGF-BP2	IGF-factor-binding protein 2
LDHA	Lactate dehydrogenase A
LOX	Lysyl oxidase
Ml	Millilitre
mRNA	Messenger ribose nucleic acid
MXI-1	Max interactor 1
Nav	Voltage-gated sodium channels
nNav 1.5	'Neonatal' Nav 1.5
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PDGF-B	Platelet-derived growth factor-B
PDK1,	Pyruvate dehydrogenase kinase 1
PFKL	Phosphofructokinase L
RNAi	RNA interference
RT-PCR	Real-time PCR
SDF-1	Stromal-derived factor 1
SEM	Standard error of mean
siRNA	Small interfering RNA
SCN1A	Sodium channel, voltage gated, type I alpha subunit
SCN1B	Sodium channel, voltage gated, type I beta subunit
TGF-α	transforming growth factor-a
UPAR	Urokinase plasminogen activator receptor

VHL	von Hipple-Lindau
VEGF	Vascular endothelial growth factor
VGSCs	Voltage-gated sodium channels

ABSTRAK

Kebanyakan tisu kanser payudara mengandungi kawasan dengan tahap pengukuran oksigen yang rendah atau lebih dikenali sebagai hipoksia. Kawasan hipoksik diketahui mempunyai kuantiti penanda hipoksia atau lebih dikenali sebagai HIF-1α dalam jumlah yang tinggi. Hipoksia diketahui mampu menghalang keberkesanan rawatan, selain juga berupaya mendorong peningkatan sifat agresif kanser kerana disebabkan oleh peningkatan transkripsi gen-gen yang menyebabkan metastatik. Selain itu, kanser payudara metastatik juga diketahui terlibat dengan aktiviti saluran ion terutamanya VGCSs. Dalam kajian ini, telah dinyatakan secara hipotesis bahawa HIF-1 alpha terlibat dalam mengawal ekspresi VGSCs yang seterusnya menggalakkan proses metastatis dalam kanser payudara. Sehubungan dengan itu, kajian ini dijalankan untuk mengkaji interaksi diantara HIF-1α dan subjenis VGSCs terutamanya Nav1.5, dan bentuk neonatalnya, nNav1.5 menggunakan kaedah siRNA dalam sel-sel kanser payudara, MDA-MB 231. Kaedah siRNA dijalankan untuk 'knock-down' ekspresi HIF-1α dan tahap ekspresi mRNA HIF-1α, Nav 1.5 dan nNav1.5 diukur menggunakan kaedah 'real-time PCR'. Di dapati bahawa, apabila ekspresi HIF-1a di 'knock-down' menggunakan kaedah siRNA, ekspresi Nav1.5 dan nNav 1.5 masing-masing telah berkurang sebanyak ~ 30% dan ~ 11%. Ini dapat disimpulkan bahawa terdapat kemungkinan hubungan di antara HIF-1α dan subjenis VGSCs terutamanya Nav1.5 dan nNav1.5 dalam MDA-MB 231 sel kanser payudara. Dengan memahami hubungan di antara HIF-1α dan VGSC (Nav1.5, nNav1.5) dapat memberikan maklumat berguna dalam memahami metastatis kanser payudara.

ABSTRACT

Most breast cancer tissue contain the region with low level of oxygenic measurement or known as hypoxic region. Hypoxic region known to express large quantities of hypoxic marker, HIF-1α. This hypoxic condition hinders the effectiveness of treatment but also promoting cancer aggressiveness due to transcriptional up-regulation of metastatic related genes. Metastatic in breast cancer was also known to have involved with ion channel mainly the VGCSs. In this study, it was hypothesized that HIF1 alpha regulate VGSC expression which promote in breast cancer metastasis. Therefore, this study was done to investigate the interaction between HIF-1α and VGSCs particularly Nav1.5, and its neonatal splicing form, nNav1.5 in breast cancer cells using siRNA in MDA-MB 231 cell line. siRNA was conducted to knock-down HIF-1α and the mRNA expression level of HIF-1α, Nav 1.5 and nNav1.5 were measured using real-time PCR. When HIF-1a was knock-downed with the siRNA, the expression of Nav1.5 and nNav 1.5 was reduce by ~30% and ~11%. The result conclude the possible relationship between HIF-1α and VGSCs mainly Nav1.5 and nNav1.5 in MDA-MB 231 breast cancer cells. Hence, by understanding the involvement of HIF-1α VGSCs mainly Nav1.5 and nNav1.5 could promise useful information in breast cancer metastasis.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Metastasis in breast cancer is characterized by the ability of the tumor cells to spread and invade into another place in the body and develop into a metastatic lesion at distant site. At early stage, the primary tumor cells growth locally until they start to invade the surrounding tissue, enter the microvasculature of the lymph and blood systems, then translocate through the bloodstream distant tissues via microvessels. This metastatic ability and invasiveness appear when tumor cells having the accumulation of genetic alteration.

Many studies have been conducted to view the ability of these malignant cells to survive at the distant site (Nguyen *et al.*, 2009). In fact, the vascular system in the tumor cell is often poorly formed and irregular with poor nutritional supply. The incomplete and poor supply system is due to the high proliferation of tumor cells that create the distance between cells and the vasculature. These poor vascular systems decrease the availability of oxygen supply and produce the hypoxic environment to the cell. Some studies had recognized hypoxia as one of the factors related to the tumor microenvironment development and tumor progression, which further increase resistance to the treatment and promote metastasis (Brahimi-Horn *et al.*, 2007) It is believed that hypoxic condition has the ability to induce molecular response and activate a key transcription factor; the hypoxia-inducible factor-1α (HIF-1α) . This transcription factor responsible in regulate a number of genes that promote the survivality of tumor cells (Brahimi-Horn *et al.*, 2007).

Besides HIF-1α, ionic channels also known to be involved in the carcinogenic process (Roger *et al.*, 2006). Generally, Voltage-gated sodium channel (VGSC) mediate the cell membrane depolarization and also responsible in conducting the electrical signal in nerves and muscles. Previous study noted that VGSCs may also be expressed in ''non-excitable'' cell types, such as lymphocytes, glia, fibroblasts and also in the metastatic cancer cells of epithelial origin (Diss *et al.*, 2004). Previous study by Fraser *et al.*, 2005 on VGSC expression in the metastatic human breast cancer cells showed the significant up-regulation in the expression. Furthermore, the VGSC activity also responsible in potentiate cell motility, endocytosis and invasion which is associated with tumor cell progression. Therefore, there is possible interaction between HIF and VGSCs due to both scientifically found presences in the metastatic cancer cell.

The further chapter will discuss on the involvement of HIF-1α and how it promote the tumor progression. Several genes which related with the up-regulation of HIF-1α also will be discuss.

In this experiment, in order to investigate the interaction of HIF-1α and VGSC, the level of VGSCs expression after transfect with HIF-1α siRNA was measured. The method real-time PCR was used to measure the gene expression.

1.2 Significance of study

Since the relationship between HIF-1α and VGSCs not yet been discovered in breast cancer, the goal of this study was to determine the effects of VGSC expression on human breast cancer cells after knock down HIF-1α. The expected reduce of VGSC expression after knock down HIF-1α using siRNA, which in turn slower the metastatic of cancer cells provide a better knowledge about the relationship between transcription factor HIF-1α and VGSC.

1.3 Objective of study

1.3.1 General objective

To investigate the interaction of HIF-1α and VGSCs in breast cancer cells using siRNA.

1.3.2 Specific objective

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 study the effect of knocking down HIF-1a on VGSCs expression in breast cancer cells line.

1.4 Conceptual framework

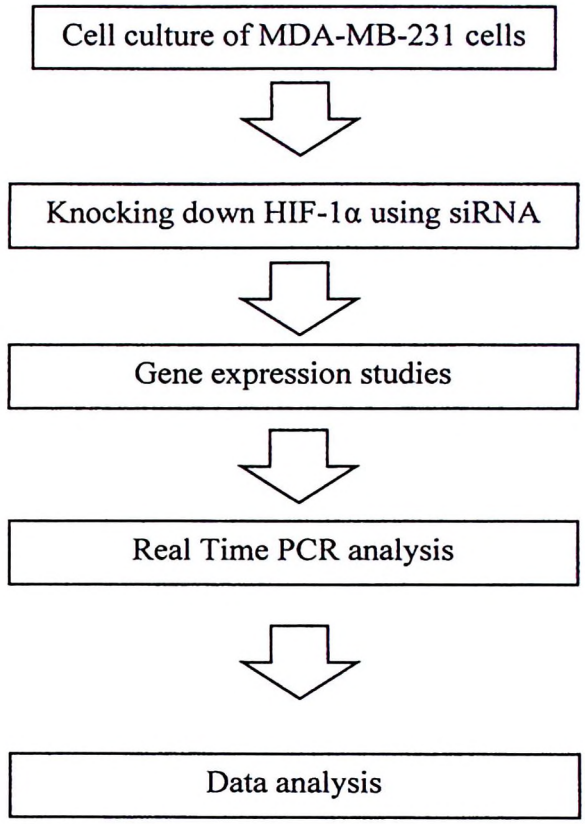

Figure 1: Research framework

CHAPTER 2

LITERATURE REVIEW

2.1 History of breast cancer

Breast cancer has been mentioned in almost every period of recorded history. Breast cancer progressing from a small lump to large tumors and can invade distant organ. The discussion of breast cancer was found since prehistory and the ancient world. William L. Donegan in his article on the history of breast cancer had mention that, during a Greek and Roman period (460 bc–475 ad), the term "karkinoma" was used to describe malignant growths and solid tumors was describe as "scirrhous". Furthermore, the term "Cacoethes" was used for an early malignancy (Donegan, 2006).

Hippocrates (460–375 BC), a Greek physician had described one case of breast cancer diagnosed with a carcinoma of the breast with bloody discharge from her nipple. On his observation, his detailed that hard tumors appear in the breast and become increasingly firm, contain no pus, and spread to other parts of the body. To avoid further causalities, He advised no treatment for hidden breast cancers because treatment was futile and shortened the patient's life (Donegan, 2006).

Until the seventeenth century, physicians keep challenge the theory of the breast cancer by arguing how and what the origin of the cancer. The eighteenth century begins the idea of the breast cancer as a localized disease and the rise of surgery to discover and treat the disease (Donegan, 2006). Physicians became more certain that breast cancer was a

localized disease. Henri Le Dran, a French physician, argued that surgery could actually cure breast cancer as long as the infected axillia lymph nodes were removed. Similarly, Claude-Nicolas Le Cat argued that the scalpel was the only way to cure cancer. Le Cat would amputate the breast, cutting out the lymph nodes as well as the pectoralis major muscle. These physicians were convinced that the presence of a tumor did not necessarily imply a more serious problem, but was a single-site disease that could be surgically removed locally before it spread (Donegan, 2006).

In the nineteenth century, the major advances were made in surgery. The introducing of hand washing by the Hungarian physician, Ignac Semmelweis (1818–1865) and by Oliver Wendell Holmes, the discovery of "putrefying" bacteria by Louis Pasteur's (1822–1895), the surgical mask by the Pole Johannes von Mikuliez-Radecki in 1886, sterile rubber surgical gloves by William S. Halsted in 1890, and successful demonstration of general anesthesia by William T. Morton in Boston in 1846 allowed unprecedented development of surgery (Donegan, 2006). After 1900, blood transfusions became safe when Karl Landsteiner discovered blood groups. All of these technologies make the treatment for breast cancer more promising and the technology upon breast cancer treatment started from this century. The creation of the microscope by Matthias Schleiden (1804–1881) during the early of the nineteenth century has introduced us with the word ''cell''. Later, Johannes Müller (1801–1859) was first to report that cancers were composed of living cells. Müller noted the similarity of cells in a "scirrhus" of the breast and its metastases in the ribs and noted that cancer cells had lost the proportions of normal cells (Donegan, 2006).

The next 100 years, the twentieth century was the creation of mammography and chemotherapy. Mammography, become the most important advance to date in the detection of breast cancer, developed in parallel with surgery. Mammography allowed many breast cancers to be detected when clinically occult, including ductal carcinoma in situ, which was regularly curable. Film-screen mammography involved penetrating the breast with x-rays to activate a rare earth screen that glowed in response. This screen exposed a transparent, photosensitive film in the same cassette which, when developed, provided an image in various shades of gray for interpretation. Mammography was followed by a number of innovative means for imaging the breast (Donegan, 2006).

2.2 Epidemiology

Breast cancer is the most common cancer in women. It is estimated that of the 1.4 million new cases of breast cancer worldwide in 2012, at least 50% will occur in low and middle income countries such as Malaysia (Abdullah *et al.*, 2013). In Malaysia, the incidences of breast cancer deaths may be higher compare to what the statistics report due to the lack of awareness in preventing the disease and because many patients prone to seek traditional therapy and those deaths may be attributed to natural causes. Furthermore, survival outcome in Malaysia is generally poor due to late presentation (Abdullah *et al.*, 2013). Of those who present early, many refuse treatment for complementary therapy. The publish study by Taib and colleagues, 2007 reported that one major factor is the patient's belief in complementary and alternative medicine (CAM) in favor of hospital-based treatments where it was found that 15.5% of women with breast cancer in Malaysia sought

CAM, such as nutritional and herbal medicine, traditional Chinese medicine and spiritual healing, prior to visiting a breast cancer clinic (Abdullah *et al.*, 2013).

The review on the statistic for breast cancer for the Malaysian population was done by The National Cancer Registry (NCR) of Malaysia. Cheng Har Yip and colleagues, 2006 noted that in 2004, data collected from NCR showed an age standardized incidence rate (ASR) of 46.2 per 100,000 women which means that approximately 1 in 20 women in Malaysia develop breast cancer in their lifetime. The data distribution was among three main races, the Malay, Chinese and Indian. The age standardized incidence in Chinese is the highest, with 59.7 per 100,000 (1 in 16), followed by the Indians at 55.8 per 100,000 (1 in 16). The Malays have the lowest incidence of 33.9 per 100,000 (1 in 28). Furthermore, the commonest age at presentation is between 40-49 years, with just over 50% of the cases under the age of 50 years, 16.8% below 40, and 2% under 30. Some 55.7% of all cases were found to be ER positive (Yip *et al.*, 2006).

2.3 Anatomy of the Breast

In a normal female individual, breast development starts between the ages of 9-14 years old when hormonal changes associated with puberty begin to occur. The mammary glands, or milk producing areas, lie between the pectoralis major muscle and the skin and are arranged into rounded areas called lobules. 15-20 of these lobules exists per breast. The breast contains fatty tissue, ducts that carry milk to the nipple, and small amounts of muscle that open and close the nipple. The complete structures of breast consist of skin, fatty tissue and fascia (superficial and deep tissue that is found between muscles and different tissue

layers in the body where it helps hold tissues together), coopers ligament (helps hold the breast up and keeps it from sagging), acini "sacklike" end of the channel that carries milk), areola (nipples), main duct (milk from the 15-20 lobules all collects into this duct), glandular tissue (produces the milk), retromammary space (an area below the breast tissue, but still above the muscle), pectoralis (major and minor, the muscles lying directly beneath the breast) and the axilla (armpit). (Figure 2.1)

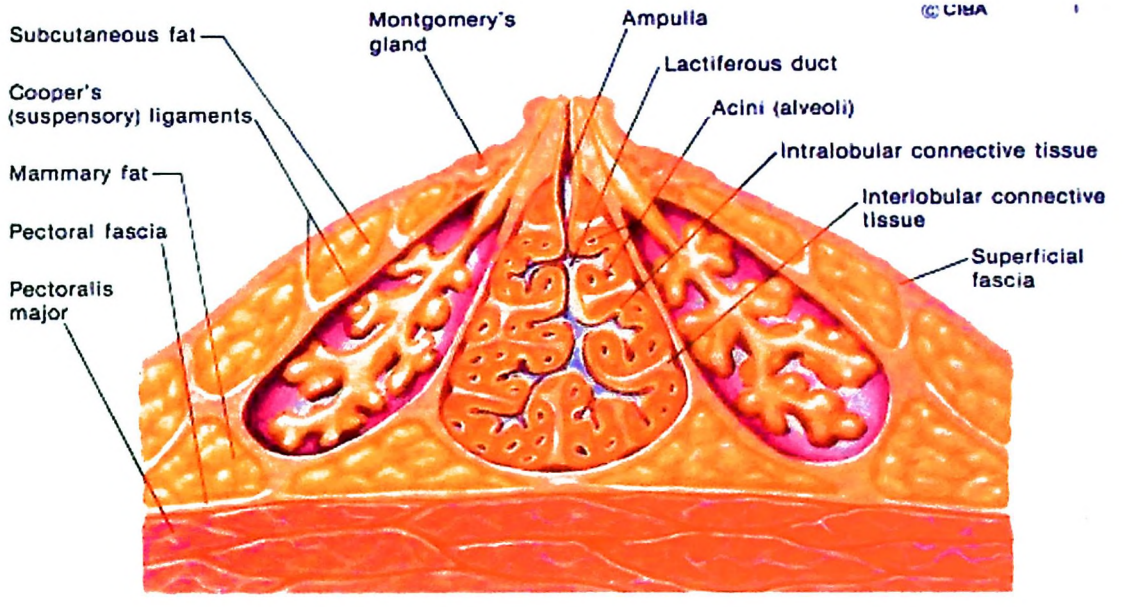

Figure 2: Figure of Breast's Anatomy

Lymphatic system plays a major role in the breast. Lymph vessels in the breast tissue which collect and move lymph fluid away from the breast into lymph nodes are important to fight infections. The breast lymph nodes include: Supraclavicular nodes located above the collarbone, infraclavicular (or subclavicular) nodes which found below

the collarbone, axillary nodes in the armpit and internal mammary nodes which located inside the chest around the breastbone (sternum). The axillary lymph nodes are divided into 3 levels: Level I (low axilla) located in the lower or bottom part of the armpit, along the outside border of the pectoral muscle, level II (mid axilla) located in the middle part of the armpit, beneath the pectoral muscle and the level III (high axilla) are located below and near the centre of the collarbone, above the breast area and along the inside border of the pectoral muscle.

The blood system of the breast includes a branches of the internal mammary artery, lateral branches of the posterior intercostal arteries and several branches from the axillary artery including highest thoracic, lateral thoracic and pectoral branhes of the thorocoacromial artery. Venous drainage of the breast was known to has an important role in breast carcinoma metastasis. The veins basically follow the path of the arteries such as the intercostal veins, axillary veins and also the internal mammary vein perforators.

2.4 Breast Cancer

Body makes new cells to grow, replace worn-out tissue and heal injuries. Sometimes cells don't grow, but divide and die in the usual way. Cells that grow uncontrolably may form a lump called a tumour. A tumour can be benign or malignant. Benign tumour is confined to only one area and are yet not able to spread to other parts of the body and this is not known as cancer yet. Malignant tumour consists of cancerous cells, which have the ability to spread by travelling through the bloodstream or lymphatic system to the distant organs.

Breast cancer occurs when the cells lining the breast lobules or ducts grow abnormally and out of control in the lobules or ducts of the breast. It can affect men and women. Several types of cancer are known: Non-invasive breast cancer, invasive breast cancer and secondary breast cancer.

Non-invasive breast cancer is known as ductal carcinoma in situ (DCIS). It consist of the abnormal cells within the ducts of the breast. Invasive breast cancer means the cancer has invade or spread from the ducts or lobules into surrounding breast tissue e.g to the lymph nodes in the armpit. The most common types of early breast cancer are invasive ductal carcinoma (IDC) and invasive lobular carcinoma (ILC). The other type of the invasive carcinoma is locally advanced breast cancer. The cancer cells has spread to other areas near the breast, such as the chest including the skin, muscles and bones of the chest. For the secondary breast cancer, it is known as metastatic breast cancer where it has spread from the breast to other distant areas of the body, such as the bones, liver or lungs. This is also known as advanced breast cancer (Mothoneos, 2014).

2.5 Metastasize Breast Cancer

Metastasis is the spread of a cancer from one organ or part to another. The metastatic cells spread from the primary tumor to distant organs and it becomes resistant to conventional therapies. Stephen Paget's 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.

In 'seed and soil' hypothesis, there are three principles involve which first is the primary tumor and metastases consist of both tumor cells and host cells include epithelial cells, fibroblast, endothelial cells and infiltrating leukocytes. The second principle is that the process of metastasis is only 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 can have clonal origin and different metastases can originate from the proliferation of different single cells. The third principle is the metastases can only develop in specific organs. It mean that the metastasis process can be influenced by many factors. The different in microenvironments of the different organs, the different cell-surface receptors and different growth factors able to influence the phenotype of metastases that develop there. In other words, the outcome of metastasis depends on multiple interactions of metastasizing cells with homeostatic mechanisms (Fidler, 2003).

2.6 Hypoxia and breast cancer

Oxygen homeostasis is a critical organizing principle of human growth, development, and physiology. Oxygen plays a key role in aerobic cellular metabolism, facilitating the conversion of glucose, protein and lipids into usable energy. The condition

of the inadequate oxygen tension at the cellular level due to the diminished availability of oxygen to the body tissues is known as hypoxia.

Most cancers have a poorly organized blood supply which results in regions of low oxygen levels in the tumor. The condition referring to tissue regions with pO2 values less than 5–10 mmHg, which occurs in the majority of solid human tumors (Chaudary and Hill, 2006). Therefore, hypoxia is actually a stress to tumor cells. However, due to this hypoxic stress, cancer undergoes changes that ultimately increases patient treatment resistance and favors tumors progression (Brahimi-Horn, 2007).

Hypoxia features are commonly found related with the pathogenesis of human disease especially cancer. In the studies by Cormac Taylor and Sean Colgan (2007) on hypoxia and gastrointestinal disease, they summarize the results of analyzing hypoxia-induced gene expression in intestinal epithelial cells and the role of hypoxia in the pathogenesis of inflammatory bowel disease. Other studies conducted Rubin Tuder and colleagues (Tuder *et al.*, 2007) reviewed the role of hypoxia in respiratory distress syndrome, high altitude pulmonary edema, pulmonary arterial hypertension, and chronic obstructive pulmonary disease (Semenza, 2007b).

In breast cancer, research on Hypoxia-inducible factor-1 (HIF-1), a transcription factor that regulates gene expression involved in tumor growth and metastases showed the relationship between level of HIF-1α which is increased during carcinogenesis in breast tissue (Bos *et al.*, 2001). HIF-1 level in cells is depend on the intracellular oxygen concentration. It continuously degrade via the ubiquitin pathway in normal tissue which is in non hypoxic condition. However, under hypoxic conditions, the ubiquitination process of HIF-1α is blocked and stabilized the HIF-1. Previous study by Reinhard Bos and colleagues

,2001 reported that levels of HIF-1α increased as the degree of malignancy increased, suggesting that HIF-1α may be a biomarker of preinvasive human breast cancers (Bos *et al.*, 2001).

2.6.1 Hypoxia-inducible factor 1 (HIF-1)

Hypoxic regions can be found in most solid tumour. It affects a variety of tumor cell properties such as cell growth rate, neovascularization, metastasis and sensitivity to treatment. In the case of breast cancer, nearly half of breast cancer patients treated for localized disease develop metastases and often combinations of local and systemic therapy are not curative and tissue oxygenation measurements in human breast carcinomas have shown large areas of hypoxic tissue and immunolocalized signals of the hypoxic markers, CAIX and HIF-1α, in breast cancer tissue show strong staining around necrotic regions (Chaudary and Hill, 2006).

A wide range of genes associated with breast cancer metastasis have been reported to be upregulated under hypoxic conditions and hypoxic gene signatures are associated with poorer outcome in breast cancer (Bos *et al.*, 2001; Liu *et al.*, 2015; Semenza, 2007a). Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that plays an important role in cellular and systemic responses to reduced oxygen availability. HIF-1 of human cells is a heterodimer composed of HIF-1α and HIF-1β subunits, which dimerize and bind to DNA containing the core sequence 5′-(A/G)CGTG-3′, a hypoxia response element (Semenza *et al.*, 1991). Database searches for proteins that were homologous to HIF-1α led to the

identification of HIF-2α, which can also dimerize with HIF-1β and bind to an overlapping but distinct set of target genes (Semenza, 2007b).

HIF-1α and HIF-2α are each negatively regulated by Oxygen-dependent hydroxylation of key proline and asparagine residues that dramatically reduce protein half-life and transcriptional activity, respectively, under aerobic conditions (Semenza, 2007a) HIFs regulate the expression of genes which then facilitate both oxygen delivery and adaptation to oxygen deprivation. The HIF pathway known as positive regulator of tumor growth as its inhibition often results in tumor suppression. The genes regulated by HIFs involves in many cellular processes, including glucose uptake and metabolism, angiogenesis, erythropoiesis, cell proliferation and apoptosis. They are members of the PAS (PER- ARNT (arylhydrocarbon receptor nuclear translocator)-SIM(single-minded)) family of basic helix-loop-helix (bHLH) transcription factors that bind to DNA as heterodimers composed of an oxygen sensitive α subunit and a constitutively expressed β subunit, also known as ARNT (Rankin and Giaccia, 2008).

When oxygen is available, HIFs are targeted for proteasomal degradation by tumor suppressor gene, which is the von Hippel–Lindau, pVHL. pVHL is the substrate recognition component of an E3 ubiquitin ligase complex that interacts with HIF-a in the present of the oxygen. Under hypoxic condition, pVHL binding is inhibited and HIF-1α become stabilized. The stabilize HIF-1α enter the nucleus, where they heterodimerize with HIF-1β and bind to HRE in target gene, and then transactivate a variety of hypoxia-responsive genes. Table 2.1 show the list of genes that regulated due to the stabilization of HIF-1α in hypoxic condition.

Table 2.1: List of genes regulated by HIF which responsible for tumorigenesis.

Hypoxia-responsive genes			
angiopoietin 1; angiopoietin 2; FLT-1 FLK-1 matrix metalloproteinase-2, matrix metalloproteinase-9 plasminogen activator inhibitor-1 PDGF-B, vascular endothelial growth factor(VEGF,) TIE-2;	ALDA, ENO1 GLUT-1, GLUT-3 HK1 HK2 LDHA, MXI-1, PDK1, PFKL	CCND1 erythropoietin IGF-2, IGF-BP2 TGF-a	CTGF CXCR4, E-cadherin LOX, PAI-1 SDF-1 UPAR
Angiogenesis	metabolism,	proliferation,	invasion, and metastasis

Source:(Rankin and Giaccia, 2008)

List of genes regulated by HIF. HIF regulates the expression of over 100 genes that promote the angiogenesis, metabolism, proliferation, invasion, and metastasis.

2.7 Voltage-Gated Sodium Channels

Ion channels are signaling molecules widely expressed in tissues. Ion channels are very important in determining a variety of cellular process such as cellular proliferation, solute transport, volume control, enzyme activity, secretion, invasion, gene expression, excitation-contraction coupling and intercellular communication (Fraser SP, 2014; Hille B, 1992). Voltage-gated sodium channels, (VGSCs) are macromolecular protein complexes consist of pore-forming α subunit (Nav1.1, Nav1.2, Nav1.3, Nav1.4, Nav1.5, Nav1.6, Nav1.7, Nav1.8, Nav1.9) and smaller non-pore-forming β subunits (β1, β2, β3, β4). The

nine members, Nav1.1–Nav1.9 encoded by genes *SCN1A–SCN11A* and four β subunits, β1-β4, encoded by genes *SCN1B–SCN4B*. VGSCs responsible for action potential initiation and conduction in excitable cells. VGSCs are activated mainly by membrane depolarization and exhibit selectivity for sodium ions and are also expressed in cell types that are considered as "non-excitable," including metastatic cancer cells (Brackenbury, 2012; Catterall, 2000)

VGSCs play an important pathological role during cancer progression and promote metastasis. In breast cancer cells, a dominant type of α subunit has been identified, with the most highly expressed α subunit is Nav1.5. Expression of the Nav1.5 α subunit are associate with poor prognosis in clinical breast cancer specimens, suggesting that VGSCs may have the potential as prognostic markers for cancer progression. VGSCs are expressed during cancer progression by potentiating the cell behaviours linked to metastasis, such as motility, invasion and adhesion. VGSCs become key regulators in cancer progression because their expression is under the control of cancer mechanisms, principally hormones and growth factors (Fraser *et al.*, 2014).

An *in vitro* study has shown that the aggressive human MDA-MB-231 breast cancer cell line consist highly expressed functional VGSCs. Electrophysiology recordings conducted by Fraser SP *et al*, (2014) showed 70% of the strongly metastatic MDA-MB-231 cells tested in laboratory expressed inward currents (representing influx of positive charge) activated by membrane depolarization. The inward currents were abolished in sodium-free medium, consistent with functional VGSC expression. While in less metastatic potential, the normal breast epithelial cell line MCF-10A and the weakly metastatic MCF-7 and MDA-MB-468 breast cancer cells showed no inward currents.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Material

3.1.1 MDA-MB-231 cell line

The cell line used in this study was MDA-MB 231 which was obtained commercially. MDA-MB-231 cell line is an aggressive type of human breast cancer cell line which possess epithelial-like morphology and exhibit invasive properties. MDA-MB-231 often used in the study of cancer cell metastasis, and cell invasion or cell migration.

3.1.2 Chemicals and Reagents

The chemicals and reagents used in this study are listed in Table 3.1.

3.1.3 Laboratory Equipments

Laboratory equipment used in this study are listed in Table 3.2

3.1.4 Commercial Kits and Consumable Items

Commercial kits used in this study are listed in Table 3.3.

3.1.5 Software

List of software used are listed in Table 3.4

Table 3.1: List of chemicals and reagents used in the experiment.

Chemicals/ Reagents
Dulbecco's Modified Eagle Medium (DMEM)
Penicillin-streptomycin (Pen Strep)
L-glutamine
Fetal Bovine Serum
Dimethyl sulfoxide (DMSO)
Trypsin
Phosphate Buffer Saline
Ultrapure DNase/RNase-Free distilled water
Serum free medium
TRIzol LS reagent
Chloroform
100% isopropanol
75% ethanol
Agarose
TAE buffer
5x DNA loading buffer
Ladder hyperladder 4
Dye
Methanol
Crystal violet

Table 3.2: List of laboratory equipment used in this study

Equipment
Autoclave
Biological safety cabinet type II
Centrifuge
Microcentrifuge
NanoDrop spectrophotometer
Incubator
Uv cabinet for master mix and reagent.
Hot plate
Neubouer haemocytometer
Inverted microscope
Freezer (4^0C, -20^0C, -80^0C)
Electrophoresis tank
Microwave

Table 3.3: List of commercial kits used in this study

Commercial kit
siRNA SMARTpool: ON-TARGET plus (Dharmacon)
QuantiTect Reverse Transcription (Qiagen)
Sensi FAST^{TM} SYBR Hi-Rox kit (Qiagen)

Table 3.4: List of software used in this study

Software
ABI prism 7000 SDS software
Microsoft Excel
Microsoft word

3.1.6 Reagent preparation

3.1.6.1 Complete Dulbecco's Modified Eagle Medium (DMEM)

The complete medium for cell cultured consist of 5% fetal bovine serum (FBS), 4 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin added into the DMEM free serum media. Two types of complete media were prepared which is the complete media with the antibiotics and the other was the complete media without the antibiotic for siRNA procedure by excluded the penicillin and streptomycin.

3.1.6.2 75% Ethanol

The 75% ethanol solution was prepared for the RNA wash procedure by adding the 30.15 mL of 99.5% ethanol stock solution into the 9.85 mL of DNase/RNase free distilled water. The solution then stored at 4^0C

3.2 Methodology

3.2.1 Cell culture

The MDA-MB-231 cells were cultured and maintained in Dulbecco's Modified Eagle Medium (DMEM) complete medium supplemented with 5% fetal bovine serum (FBS), 4 mM l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were seeded into tissue culture dishes and were incubated using humidified incubator at 37 °C with 5% CO2. Aseptic technique was applied during the procedure to prevent the contamination.

3.2.1.1 Cell Passaging

The cells passaging were done as the cell maintenance and for the preparation of the siRNA cell treatment. The method conducted by detach the cells using 5 mL of trypsin (trypsinization) after removed the old media and then cells were incubated for 3 minutes. Then, the equivalent amount of DMEM was added for detrypsinized process. From the dish, the detrypsinized cells were transferred into 15 mL Falcon tube prior to the centrifugal process at 1500 rpm for 3 minutes. Cells were resuspended with 2 mL PBS and washed by the centrifugal process for 1500 rpm in 3 minutes time. The supernatant was then removed and the pallet was gently resuspended into 7 mL of antibiotics-free complete medium.

3.2.2 Cell plating

Cell plating was prepared for the siRNA experiment. To plate the cells, cells first were count using the haemocytometer counting chamber. 10 µl of cells was added into the haemocytometer chamber, and were counted under inverted microscope. Below is the image of the chamber field (Figure 3.1)

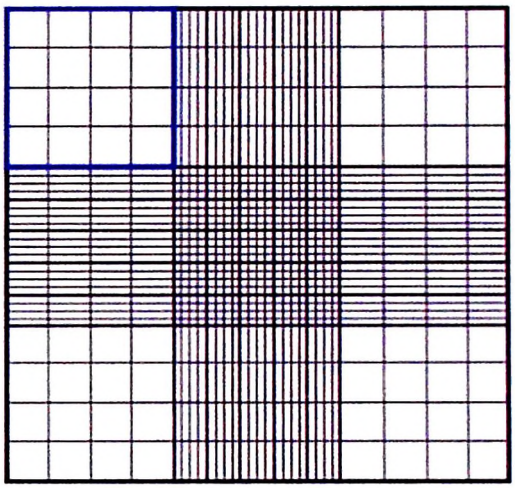

Figure 3: View of haemocytometer counting chamber field

Cells which were resided inside the highlighted (blue) square box were counted. Four similar squares (n1 – n4) at the upper part and lower part of the chamber were used to count the cells.

The cells calculation using haemocytometer counting chamber:

$$\frac{n1+n2+n3+n4}{4} \times 10^4$$