ANALYSIS OF VEGF RECEPTORS AND MICROVESSEL DENSITY IN NMU-INDUCED BREAST CANCER UNDER THE INFLUENCE OF PLATELET FACTOR 4 AND RAPAMYCIN

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By

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TABLE OF CONTENTS

LIST OF PUBLICATIONS

1. Effects of rapamycin and platelet factor-4 (PF4) on 1-methyl-1-nitrosourea (MNU)-induced breast cancer.

   Status: Published

   Journal: The Malaysian Journal of Medical Sciences.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

g	Gram
kg	kilogram
mg	miligram
ml	mililiter
mM	milimolar
M	Molar
ng	nanogram
µg	microgram
µl	microliter
µm	micrometer
Ca^{2+}	Calcium ion
cDNA	complementary deoxyribonucleic acid
DAB	diaminobenzidine
DAG	1,2-diacylglycerol
DCIS	ductal carcinoma in situ
DEPC-H_2O	Diethylpyrocarbonate- water
dH_2O	distilled water
DMBA	7,12-dimethylbenz(a)antracene
EDTA	ethylenediamine tetraacetic acid disodium
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor

ERK	extracellular signal-regulated protein kinase
FFPE	formalin fixed paraffin embedded
FGF	fibroblast growth factor
Flk-1	fetal liver kinase-1
H_2O_2	hydrogen peroxide
H&E	hematoxylin and eosin (stain)
HRP	horseradish peroxidase
IDC-NOS	invasive ductal carcinoma-not otherwise specified
L	Liter
LB	lithium boric acid buffer
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NBF	neutral buffered formalin
NMU	N-nitroso-N-methylurea
PEN	polyethylene naphthalate
PF4	platelet factor 4
PI3K	phosphoinositide-3-kinase
PKC	protein kinase C
PLC-	phospholipase C-
PPAR	peroxisome proliferator-activated receptor
PS	phosphatidyl-serine
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
Flt-1	fms like tyrosine-1

Flt-4	fms like tyrosine-4
TBS	tris buffer saline
TDLU	terminal ductal lobular unit
TEB	terminal end bud
UV	ultraviolet

ANALISIS RESEPTOR VEGF DAN KEPADATAN MIKROVASKULAR YANG DIINDUKSI MENGGUNAKAN NMU DI BAWAH PENGARUH PLATELET FACTOR 4 DAN RAPAMYCIN

ABSTRAK

Kanser payu dara merupakan pembunuh utama wanita diseluruh dunia dan merupakan kanser penyebab kematian kedua tertinggi di United States. Angiogenesis merupakan pembentukan salur darah baru daripada salur darah utama yang merupakan proses yang normal dan sangat penting untuk pembesaran dan pertumbuhan serta proses pemulihan dan proses pembentukan tisu bergranul. Angiogenesis memainkan peranan yang penting dalam membekalkan nutrien dan oksigen kepada perkembangan sel dan juga berperanan sebagai laluan bagi metastasis tumor. Pada masa kini, ramai penyelidik sedang menjalankan penyelidikan yang teliti dalam bidang ini yang menjurus ke arah menyekat perkembangan tumor melalui sekatan ke atas proses angiogenesis. Dalam kajian ini, N-nitroso-N-methylurea (NMU) telah diaruh bagi menyebabkan karsinoma kalenjar mamari yang teruk dan dirawat dengan menggunakan dadah anti-angiogenik termasuklah rapamycin iaitu sejenis perencat imun yang berkesan dan Platelet Factor 4 (PF4) iaitu sejenis pengekang angiogenik yang disuntik secara berasingan atau secara kombinasi bagi menentukan tahap keberkesanan sebagai anti-kanser dan anti-angiogenik. Flt-1, Flk-1 and Flt-4 telah dipilih sebagai penanda yang mewakili proses angiogenesis, pembezaan, pembahagian sel dan penelapan vaskular kanser manakala CD34 telah dipilih untuk penakrifan kepadatan mikrovaskular di dalam model NMU. Ekspresi keseluruhan penanda ini samada dalam bentuk gen dan protin

telah dianalisis menggunakan kaedah immunohistokimia dan asai *Real-Time-PCR*. Penemuan daripada kumpulan yang tidak dirawat menunjukkan bahawa darjah penerukan malignan meningkat secara signifikan seiring dengan pertumbuhan kanser payudara tersebut. Penemuan kanser yang terbentuk pada kumpulan tidak terawat menunjukkan keterukan keadaan malignan didorong oleh pembentukan tumor mamari. Pembentukan kanser yang kurang agresif secara pengkelasan histologi sebagai *Invasive Ductal Carcinoma (IDC)-Cribiform* yang mana banyak dijumpai di dalam tisu kanser bersaiz kecil. Sebaliknya, perkembangan lesi yang lebih teruk yang diklasifikasikan secara histologi sebagai IDC-papilari dan "Not Otherwise Specified" (NOS) telah dijumpai pada tumor yang besar. Sementara itu, analisis di dalam kumpulan yang terawat menunjukkan rawatan menggunakan rapamycin berjaya merencat pertumbuhan kanser payudara dan angiogenesis secara signifikan pada peringkat protein dan gen. Semua ekspresi penanda reseptor signal menunjukkan penindasan signifikan yang dikaitan dengan perencatan terhadap tumor. Sebaliknya, rawatan dengan PF4 telah menunjukkan kurang keberkesanan dalam penyekatan angiogenesis tumor yang dicerminkan daripada penindasan ekspresi Flt-1, Flk-1 and Flt-4yang tidak signifikan. Hampir menyerupai rawatan menggunakan rapamycin, kombinasi rawatan menggunakan rapamycin dan PF4 telah menunjukkan penindasan ekspresi Flt-1, Flk-1 dan Flt-4 di peringkat protein dan gen tetapi tidak menunjukkan aktiviti perencatan berganda. Pengiraan kepadatan mikrovaskular telah mendedahkan hubungan diantara kehadiran kapilari dan ekspresi reseptor protein signal VEGF. Kurang kapilari dijumpai pada tumor yang kurang agresif manakala sebaliknya pada tumor yang lebih agresif. Justeru itu, kajian ini mencadangkan bahawa rapamycin bukanlah penggalak atau sinergi dengan PF4. Malahan, PF4 mungkin bertindak sebagai antagonis terhadap aktiviti anti-kanser serta anti-angiogenesis bagi

rapamycin. Kesimpulannya, rapamycin adalah agen anti-angiogenik yang berkesan bagi kanser payudara yang dirangsang oleh NMU di dalam model tikus dan berpotensi digunakan sebagai rawatan untuk kanser payudara peringkat teruk.

ANALYSIS OF VEGF RECEPTORS AND MICROVESSEL DENSITY IN NMU-INDUCED BREAST CANCER UNDER THE INFLUENCE OF PLATELET FACTOR 4 AND RAPAMYCIN

ABSTRACT

Breast cancer is the main killer disease among women worldwide and the second most common cause of cancer death in women in the United States. Angiogenesis is the formation of a new vascular network from the pre-existing vessels. It is a normal and vital process for growth and development as well as in wound healing and in the formation of granulation tissue. Neovasculation or angiogenesis play a pivotal role in the supply of essential nutrients and oxygen for cell growth and also providing passageway for tumour metastasis. Currently, angiogenesis is being intensively studied to inhibit tumour progression by blocking the process. In this study, the rats model were induced N-nitroso-N-methylurea (NMU) to cause invasive mammary tumourigenesis. The rats were divided into 4 groups based on treatment given; rapamycin, PF4 and drug combination. The rapamycin and PF4 were administered as single agent or in combination to determine the anti-tumour and anti-angiogenic effects as well as to evaluate their nature when combined. Flt-1, Flk-1, and Flt-4 were selected as markers for downstream mediators which represent the process of tumour angiogenesis, differentiation, cell proliferation and vascular permeability of NMU-model while CD34 marker had been selected for microvessel density (MVD) counting. The protein expressions and gene expression of these markers were evaluated using immunohistochemistry analysis and Real-time PCR assay. Findings from the control group had demonstrated that the severity of malignancy

significantly increased with the progression of the mammary tumour. Development of less-aggressive lesion that was histologically classified as Invasive Ductal Carcinoma (IDC)-Cribriform subtype was predominant in small sizes of tumour. In contrast, development of more-aggressive lesions that were histologically classified as IDC-Papillary and Not Otherwise Specified (NOS) subtypes were seen in larger sizes of tumour. In the treatment group, rapamycin treatment had been found to show significant inhibition of mammary tumour progression as well as tumour angiogenesis at protein and gene level. All VEGF signaling receptor markers expressions were significantly suppressed which were associated to significant tumour regression. Treatment with PF4 alone is not effective to inhibit the tumour angiogenesis. This was reflected in the insignificant inhibition of Flt-1, Flk-1 and Flt-4 expression. The drug combination had obtained a significant down-regulation of Flt-1, Flk-1 and Flt-4 at protein and gene level but no synergistic effects were seen. Microvessel density counting had revealed that there is correlation ($p<0.01$) between the existing of capillary and the expression of VEGF signaling molecule receptors. Less capillary formation was observed in less aggressive tumours while the converse was seen in aggressive tumours. Thus, the present findings had suggested that rapamycin was not synergistic or additive to PF4. In fact, PF4 might be antagonist towards the action of rapamycin as anti-tumour and anti-angiogenesis. Present findings had concluded that rapamycin is a potent anti-angiogenic agent for the invasive NMU-induced mammary carcinoma in the rat model and has the potential to be applied clinically as an anti-angiogenic therapy for the treatment of advanced stages of breast cancer.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Breast cancer is an uncontrolled growth of epithelial cells lining the ducts and lobules of the breast tissue (Guinebretiere *et al.*, 2005; DeSantis *et al.*, 2009; Bateman, 2010). This uncontrolled growth of cells growth resulted in development of abnormal mass of tissue known as tumour. A tumour of the breast can exist in two types of carcinoma which are carcinoma *in situ* and invasive carcinoma (Russo and Russo, 2000). Tumour cells are confined within ducts or lobules during the carcinoma *in situ* stage and transforms into invasive carcinoma or metastatic when infiltrated into surrounding tissues or into other organs, which is also known as malignant breast cancer (Hanby, 2005; Sontag and Axelrod, 2005; Allred and Medina, 2008). Invasive carcinoma is predominantly diagnosed at an advanced stage of breast cancer, whilst both benign and carcinoma *in situ* are commonly diagnosed at an early stage according to a range of histopathological phenotypes (Hanby, 2005).

Signs of breast cancer may include a lump in the breast, a change in breast shape, dimpling of the skin, fluid coming from the nipple, or a red scaly patch of skin (National Cancer Registry, 2014). In those with distant spread of the disease, there may be swollen lymph nodes, shortness of breath, or yellow skin (Saunders, 2014). Breast cancer has been diagnosed as the most common cause of cancer affecting women and also statistically is the main killer among women worldwide, surpassing

cervical cancer (Parkin and Fernandez, 2006; Porter, 2009; Ferlay *et al.*, 2010; Jemal *et al.*, 2011; Pinder, 2010; Moinfar, 2007). The incidence and mortality rate of breast cancer have been reported to be increasing in most of Asia countries (Sim *et al*., 2006; Takiar *et al.*, 2008; Hirabayashi *et al*., 2009; Medina *et al*., 2010; Park *et al*., 2011). Based on the report by the U.S. National Cancer Institute, it is estimated that 232,670 females and 2360 males will have breast cancer in 2014 and out of this, 40,000 females and 430 males will die from the disease (National Cancer Registry, 2014). Similarly, the incidence of cancer in Malaysia increased from 32,000 new cases in 2008 to about 37,000 in 2012 which comprise of 18.1% of breast cancer incidence (National Cancer Registry, 2013).

Tumour angiogenesis, which refers to the formation of a new vascular network out of primary vasculature (Papetti and Herman, 2002; Harper and Moses, 2006; Otrock *et al.*, 2007) is essential for sustaining tumour growth in breast cancer (Leek and Harris, 2002; Fox *et al.*, 2007; Zelnak and O'Regan, 2007) since breast cancer is known as angiogenic-dependant cancer. Angiogenesis may involves blood capillary or/and lymphatic vessel. Angiogenesis promotes the progression of small localized neoplasm which later proliferates and causes the enlargement of the tumour with the ability to spread by metastasis (Folkman, 1995; Rayson *et al.*, 1999; Harper and Moses, 2006). Pertaining to this, signalling via vascular endothelial growth factor receptor pathway has been implicated as the predominant pathway in signalling tumour angiogenesis (Hicklin and Ellis, 2005; Lohela *et al.*, 2009). This includes promoting cell proliferation via the activation of protein kinase C (PKC) cascade (Cebe-Suarez *et al.*, 2006; Otrock *et al.*, 2007).

Nowadays, there are many treatment and therapies used for cancer which includes amputation, radiotherapy, chemotherapy and also alternative medicine.

Recently, there is an increasing awareness that some cancer preventive agents may act by blocking the process of angiogenesis despite previous investigated mechanisms which directly linked them to cell proliferation and apoptosis (Thompson *et al.*, 2004). Inhibition of tumour angiogenesis suppresses tumour growth in many experimental models (Miller, 2004; Marty and Pivot, 2008; Chen *et al.*, 2011), suggesting that tumour-induced angiogenesis may be a relevant target to inhibit tumour progression. In this regard, the N-nitroso-N-methylurea (NMU)-induced mammary carcinogenesis in rat has been used extensively to evaluate the cancer inhibitory activity of chemopreventive agents and various nutrients (Thompson *et al.*, 1991; Roomi *et al.*, 2005; Goss *et al.*, 2011). This model is also ideal for the investigation of agents that may affect blood vessel formation and/or growth (Thompson *et al.*, 2004).

Rapamycin (sirolimus; Rapamune$^{(R)}$; Wyeth-Ayerst, PA, USA), is a bacterial macrolide with anti-fungal, immunosuppressant that was recently found to have anti-tumour activity by suppressing tumour angiogenesis (Guba *et al.*, 2002; Law, 2005). Rapamycin is produced by the bacterium *Streptomyces hygroscopicus* which was found in Easter Island. Rapamycin is known to target the atypical Ser/Thr kinase mammalian target of rapamycin (mTOR) and inhibits the translation of key mRNA of proteins required for cell cycle progression. The anti-proliferative actions of rapamycin have been demonstrated to be due to its ability to modulate critical signal transduction pathways that link mitogenic stimuli to the synthesis of proteins required for cell cycle traverse from G_1 to S (Wiederrecht *et al.*, 1995). Impressive anti-proliferative activity has been demonstrated following treatment of diverse types of experimental tumors with rapamycin (Eng *et al.*, 1984, Muthukkumar *et al.*, 1995; Seufferlein and Rozengurt, 1996). However, the poor aqueous solubility and

chemical stability of rapamycin precluded its clinical development as an anti-cancer agent. Recently, a series of rapamycin analogs with improved aqueous solubility and stability have been synthesized and evaluated. CCI-779 (Wyeth Ayerst, PA, USA), a soluble ester analog of rapamycin, was selected for development as an anti-cancer agent based on its prominent anti-tumor profile and favourable pharmaceutical and toxicological characteristics in preclinical studies (Gibbons *et al*., 2000).

Platelet Factor 4 (PF4) is a tetrameric, lysine rich member of CXC chemokines family produced almost exclusively by megakaryocytes (Poncz *et al.*, 1987). Under physiological conditions, only a small amount of PF4 is taken up with circulating platelet, therefore a bulk of PF4 protein originates in megakaryocytes (Guzzo *et al.*, 1987). PF4 was originally cloned from a human erythroleukemia cell line and its genetic mapping and polymorphisms were discovered soon there after. PF4 is stored within the granules of platelets and secreted at high concentrations in the vicinity of injured blood vessels following platelet activation. PF4 was discovered to inhibit angiogenesis in 1982. By 1990, it was shown to inhibit tumors in mice. In 1995, PF4 was reported to bind preferentially to vascular endothelium *in vivo* and to bind selectively to regions of active angiogenesis *in vivo*. By 1998, PF4 was revealed to be a marker of new vessel formation in xenografts of human breast cancer (Benny *et al.*, 2008; Kolber *et al.*, 1995).

Since breast cancer is angiogenesis-dependant and the tumour burden has been indicated as a crucial problem in advanced stages of breast cancer treatment, evaluation on the regulation of associated angiogenic markers under tumour-angiogenesis suppressed environment using the invasive breast cancer model is important in order to improve our understanding in this area. Therefore, in the present study, the roles of Flt-1, Flk-1 and Flt-4 as downstream mediators of

angiogenesis were analysed using immunohistochemistry analysis and Real-Time PCR assay to find the correlation between the angiogenic markers and microvessel density of rat's breast tumour induced by NMU with the influenced of rapamycin and PF4 .

1.2 Literature review

1.2.1 Morphogenesis of normal mammary gland

The mammary gland of breasts is a dynamic organ which undergoes continuous changes during pregnancy, lactation and involution (Liu *et al.*, 2005; Hatsell *et al.*, 2005). These changes are controlled under tight hormonal regulation (Rillema, 1994; Lamote *et al.*, 2004). Normal mammary gland is heterogeneously composed of glandular and connective tissues (Guinebretiere *et al.*, 2005). The glandular tissues which are generally built of ducts and lobules are essential in synthesizing milk production which is strictly controlled by hormones prolactin and somatotropin (Lamote *et al.*, 2004; Guinebretiere *et al.*, 2005). These glandular tissues are embedded in connective tissues composed of blood and lymphatic vessels, nerve, adipose and fibrous tissues (Russo *et al.*, 1982; Weigelt and Bissell, 2008). The connective tissues are crucial in supplying nutrition and physical support (Guinebretiere *et al.*, 2005). The entire morphogenesis of mammary gland comprises of several stages including fetal development, growth of the gland during puberty, development of the gland during pregnancy and lactation (Rillema, 1994). In humans, the development of mammary gland begins right after birth during the fourth week of gestation (Polyak, 2001). During this stage, only a few poorly branched mammary ducts are formed (Russo *et al.*, 1982; Rillema, 1994; Polyak,

2001). At the onset of puberty, secretion of hormone estrogen and progesterone stimulate the ductules elongation and branching which takes place at the Terminal End Bud (TEB) (Sternlicht, 2005). The TEB, also referred as Terminal Ductal Lobular Unit (TDLU) is susceptible to carcinogenesis (Russo *et al.*, 1983). During pregnancy, stimulation of estrogen and progesterone as well as prolactin promotes differentiation of existing ductules into lobules and alveoli (Horseman, 1999). Later during parturition, the lobuloalveolar epithelium is converted to a secretory phenotype which begins synthesizing milk production (Binart *et al.*, 2000). At the end of lactation, involution of the lobuloalveolar system occurs in response to milk stasis (Horseman, 1999; Binart *et al.*, 2000).

1.2.2 Breast cancer

1.2.2.1 Breast cancer incidence and prevalence

According to the report compiled by the International Agency on Cancer Research (IACR), breast cancer has been diagnosed as the most common cause of cancer and is the main killer among women worldwide surpassing cervical cancer (Parkin and Fernandez, 2006; Porter, 2009; Ferlay *et al.*, 2010; Jemal *et al.*, 2011). In US alone, the North American Association of Central Cancer Registries (NAACCR) reported 192,370 of new breast cancer cases in 2009 which accounts for 27% of all cancer sites (Jemal *et al.*, 2009). Meanwhile, the National Centre for Health Statistics reported 40,170 death cases which represent 15% of all cancer cases reported in the same year as represented in Figure 1.1 (Jemal *et al.*, 2009). Interestingly, the pattern reported in US concurs with the latest Malaysian Cancer Statistic 2007 which showed 18.1% of breast cancer incidence rate occurring among Malaysian women

compared to other type of cancer (National Cancer Registry, 2006). The data by National Cancer Registry of Malaysia also mentioned that cancer had surpassed heart attack as the most killing disease in Malaysia for the year of 2014 and breast cancer had been highlighted to be the highest incidence in Malaysia for 2007 (Globocan, 2013). In this regard, a rapid increase of breast cancer cases were detected among young women from different parts of Asia which generally in advanced stages compared to their counterpart in developed countries as shown in Figure 1.1 (Parsa *et al.*, 2006; Agarwal *et al.*, 2007; Lin *et al.*, 2009; Leong *et al.*, 2010).

Estimated New Cases*

	Males			Females		
Prostate	192,280	25%		Breast	192,370	27%
Lung & bronchus	116,090	15%		Lung & bronchus	103,350	14%
Colon & rectum	75,590	10%		Colon & rectum	71,380	10%
Urinary bladder	52,810	7%		Uterine corpus	42,160	6%
Melanoma of the skin	39,080	5%		Non-Hodgkin lymphoma	29,990	4%
Non-Hodgkin lymphoma	35,990	5%		Melanoma of the skin	29,640	4%
Kidney & renal pelvis	35,430	5%		Thyroid	27,200	4%
Leukemia	25,630	3%		Kidney & renal pelvis	22,330	3%
Oral cavity & pharynx	25,240	3%		Ovary	21,550	3%
Pancreas	21,050	3%		Pancreas	21,420	3%
All Sites	**766,130**	**100%**		**All Sites**	**713,220**	**100%**

Estimated Deaths

	Males			Females		
Lung & bronchus	88,900	30%		Lung & bronchus	70,490	26%
Prostate	27,360	9%		Breast	40,170	15%
Colon & rectum	25,240	9%		Colon & rectum	24,680	9%
Pancreas	18,030	6%		Pancreas	17,210	6%
Leukemia	12,590	4%		Ovary	14,600	5%
Liver & intrahepatic bile duct	12,090	4%		Non-Hodgkin lymphoma	9,670	4%
Esophagus	11,490	4%		Leukemia	9,280	3%
Urinary bladder	10,180	3%		Uterine Corpus	7,780	3%
Non-Hodgkin lymphoma	9,830	3%		Liver & intrahepatic bile duct	6,070	2%
Kidney & renal pelvis	8,160	3%		Brain & other nervous system	5,590	2%
All Sites	**292,540**	**100%**		**All Sites**	**269,800**	**100%**

Figure 1.1 Occurrence of ten leading cancer types in United States for estimated new cancer cases and death for year 2009 (Jemal *et al.*, 2009)

1.2.2.2 Pathogenesis of breast carcinoma

Breast cancer arises from a series of mutations that accumulates for over many years (Hayat, 2002; Kwei *et al.*, 2010). Generally, cancer cells possessed six essential characteristics to ensure their survival namely (1) self-sufficiency in growth signals, (2) resistance to growth-inhibitory signals, (3) insensitive to apoptosis, (4) unlimited replicative potential, (5) sustained angiogenesis and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000; Liu *et al.*, 2005). One of the key hallmarks of breast carcinoma is the loss of the ability to control growth within organized bilayered ducts as shown in Figure 1.2 (Mallon *et al.*, 2000; Guinebretiere *et al.*, 2005). Normal mammary ducts and alveoli consist of a single layer of epithelial cells lining the lumen and myoepithelial cells lining the basement membrane (Figure 1.3(A)) (Mallon *et al.*, 2000; Polyak, 2001; Guinebretiere *et al.*, 2005). The close cellular contact of luminal and myoepithelial cells enables autocrine and paracrine interaction potentially mediated by chemokines either between luminal and epithelial cells or between luminal epithelial cells and stromal cells including fibroblast, adipocytes, macrophages, eosinophil granulocytes, lymphocytes and endothelial cells (Polyak, 2001). However, aberrant proliferation of epithelial cells and myoepithelial cells lead to the formation of benign lesions such as fibrocystic diseases, sclerosing lesions, epithelial hyperplasia, fibroadenomas, tubular adenomas as well as intraduct papillomas (Courtillot *et al.*, 2005; Bateman, 2010). Generally, these types of lesions are non-detrimental but have the potential to develop into breast carcinoma *in situ*. Carcinoma *in situ* is a pre-malignant proliferation of the breast epithelial cells confined within the basement membrane (Thompson *et al.*, 2000; Russo and Russo, 2000). It can be classified as Ductal Carcinoma *In Situ* (DCIS) (Figure 1.3 (B)) which is originated from the ductal region or Lobular Carcinoma *In Situ* (LCIS) from

the lobular region (Meijnen *et al.*, 2006; Pinder, 2010). DCIS is exhibited in several histological subtypes including cribriform, comedo, solid and micropapillary whilst LCIS does not possess one (Mallon *et al.*, 2000; Guinebretiere *et al.*, 2005). In addition, both carcinomas *in situ* are known to be the precursor of invasive carcinoma (Polyak, 2001; Hanby, 2005). Invasive carcinoma retained similar characteristics as their counterpart in carcinoma *in situ*, but the tumour cells have breached the basement membrane and invade into the surrounding tissue (Russo and Russo, 2000; Bateman, 2010). Invasive ductal carcinoma (IDC) which is the most common breast carcinoma in humans has been histologically classified as special-type and Not-Otherwise Specified (NOS) type (Figure 1.3(C)). Almost 75% of breast carcinoma cases in human were diagnosed as IDC-NOS (Hanby, 2005; Moinfar, 2007) as the tumour exhibits no specific characteristics. Meanwhile, the remaining special-type of invasive carcinoma which have distinctive characteristics such as tubular carcinoma, cribriform carcinoma, solid papillary carcinoma and many more, rarely occur in breast carcinoma cases. In the advanced stage, breast carcinoma commonly metastasize to a distant organ such as lungs, liver, brain, adrenal gland and bones through the nearest lymphatic and vascular invasion (Mallon *et al.*, 2000; Hanby, 2005). Studies have indicated that the development of breast cancer has been attributed to numerous factors including internal such as endocrine or hormonal related factors as well as external such as genetic factors, early menarche, late pregnancy, nulliparity and lifestyle factors for examples obesity, alcohol consumption and reduced physical activity (Polyak, 2001; Parkin *et al.*, 2006).

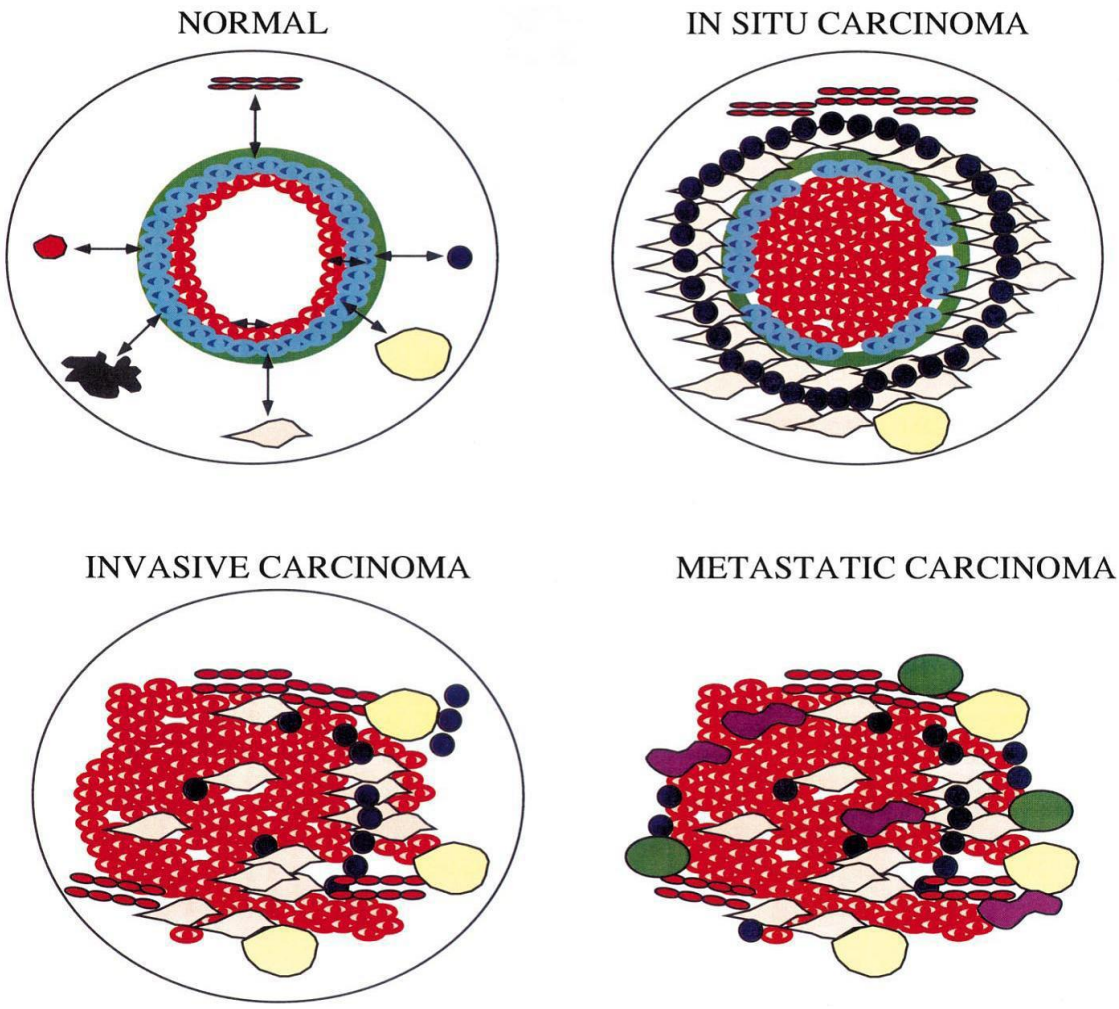


Figure 1.2 Cellular organizations of normal mammary gland and types of mammary carcinoma (Polyak, 2001)

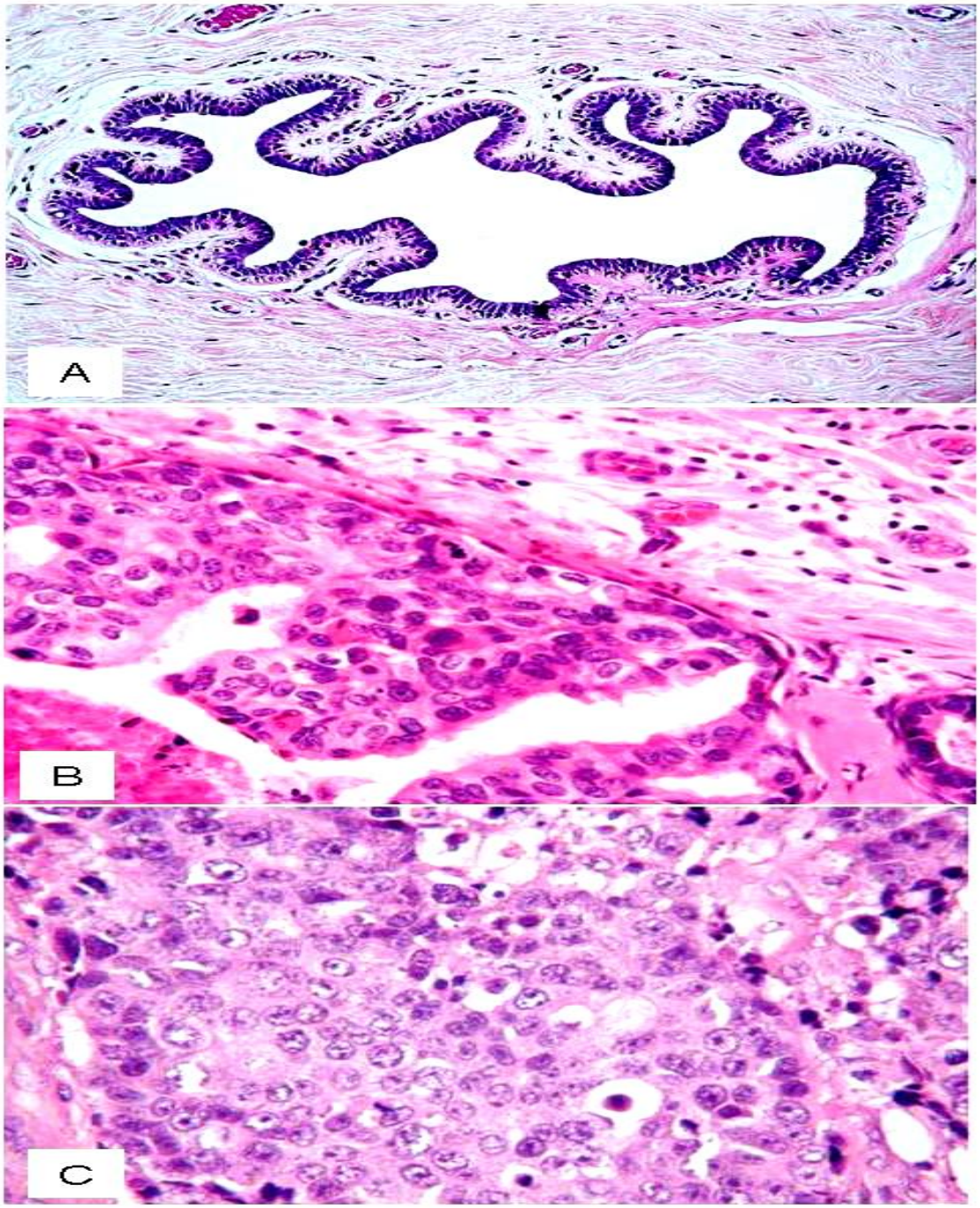
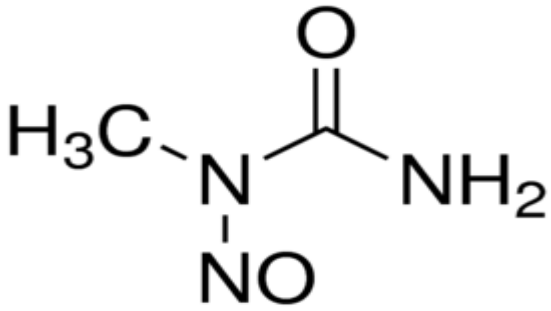


Figure 1.3 Histology of normal and cancerous breast tissue. (A) Normal mammary duct (Guinebretiere *et al.*, 2005), (B) Ductal Carcinoma *In Situ* (DCIS) (Pinder, 2010), (C) Invasive ductal carcinoma-Not-Otherwise Specified (IDC-NOS) (Moinfar, 2007)

1.2.2.3 Breast cancer induced by N-nitroso-N-methylurea (NMU) in rat model

In order to fully understand the mammary carcinogenesis, animal models notably from mammals seem to be the best option. Studies have demonstrated that the histopathological characteristics of mammary carcinoma developed in rat models had close resemblance to the one in human (Thompson and Singh, 2000; Costa *et al.*, 2002; Tsubura *et al.*, 2007; Jaafar *et al.*, 2009). There are variety of animal models for breast cancer study (Cardiff and Wellings, 1999; Tsubura *et al.*, 2007; Medina, 2008), however chemically induced carcinogen rat model has been extensively used in mammary carcinogenesis study for the past several decades (Thompson *et al.*, 1991; Macejova and Brtko, 2001; Roomi *et al.*, 2005; Goss *et al.*, 2011). Out of the various carcinogen models studied so far, NMU and DMBA are the most commonly used and well-characterized (Thompson *et al.*, 2000; Russo and Russo, 2000). NMU is an alkylating agent (Figure 1.4) which exhibits its toxicity by transferring its methyl group to nucleo bases in nucleic acids and leads to mutations. These models offer several advantages including ease of handling, the nature of the carcinogenic response, the histological characteristics of the tumour induced, the simplicity of the tumour induction methodology and the flexibility in the experimental design (Thompson and Singh, 2000). Compared to DMBA, the NMU model has a number of superior advantages. For instance, NMU can be used to demonstrate the development of histologically aggressive mammary carcinoma (Thompson *et al.*, 2000; Liska *et al.*, 2000), has a higher proportion of malignant to benign tumour compared to DMBA (Thompson, 2002) as well as appearance of more estrogen-dependent tumour (Arafah *et al.*, 1980; Thompson *et al.*, 2000; Thompson, 2002; Rajkumar *et al.*, 2004). There are several suggested methods and routes of administration for NMU-induced mammary carcinoma. Intraperitoneal injection at

21 days-old of female Sprague Dawley rats is the most rapid induction method (Thompson, 2002). Induction during this age is ideal because at this time the TEB is in undifferentiated form and susceptible to neoplastic transformation which is attributed to the carcinogen activity (Russo *et al.*, 1983; Thompson and Singh, 2000). This will only occur in young or nulliporous animals, but not in multiporous animals where the TEB is absent due to complete differentiation of the gland (Russo *et al.*, 1983). *In vivo* studies in rats have proven that nulliporous rat is associated with higher risk of developing breast cancer compared to multiporous rat (Thompson *et al.*, 2000; Macejova and Brtko, 2001). Besides the TEB region, studies also indicated another two *in vivo* origin sites of mammary carcinoma which comprised of ducts and ductules (Russo *et al.*, 1982). In humans, the similar pattern was observed with predominant human breast carcinoma originating from ductals (Thompson and Singh, 2000; Thompson *et al.*, 2000; Russo and Russo, 2000; Liska *et al.*, 2000).



(Thompson *et al.*, 2000)

Figure 1.4 The chemical structure of NMU

1.2.3 Angiogenesis

Development and progression of tumour has been associated with a wide range of factors with tumour angiogenesis being identified as one of the factors (Hanahan and Weinberg, 2000; Bluff *et al.*, 2008). In 1960, Folkman and co-workers

initially discovered the role of angiogenesis in supporting the tumour growth (Folkman, 1995; Fan *et al.*, 1995). Angiogenesis, which refers to the formation of new capillaries from the pre-existing vessels is important for both physiological and pathological processes (Papetti and Herman, 2002; Otrock *et al.*, 2007). In normal physiology, angiogenesis is a highly ordered process under tight regulation of angiogenic factors and inhibitors known as pro- and anti-angiogenic molecules, respectively (Papetti and Herman, 2002; Gasparini *et al.*, 2005; Shojaei and Ferrara, 2008). Pro-angiogenic molecules promote vasculature formation; whereas anti-angiogenic molecules do the opposite (Sato, 2003; Hillen and Griffioen, 2007). The amount of pro-angiogenic molecules is exquisitely counterbalanced by those of anti-angiogenic molecules to compel changes in tissue mass and/or metabolic demands in order to maintain adequate oxygen and nutrient delivery (Milkiewicz *et al.*, 2006; Harper and Moses, 2006). This process is important during embryonic development where angiogenesis provides adequate vasculature for growing and developing organs, as well as physiological repair process in wound healing, mammary gland maturation and ovarian cycle during adult phase (Kliche and Waltenberger, 2001; Papetti and Herman, 2002; Sato, 2003). However, deregulated angiogenesis by overexpression or downregulation of angiogenic factors and inhibitors caused aberrant deployment of normal angiogenesis and resulted in various pathological conditions including vascular insufficiency (cerebral ischemia and myocardial infarction), atherosclerosis, diabetic retinopathy, psoriasis, rheumatoid arthritis and tumour growth as well as metastasis (Milkiewicz *et al.*, 2006; Hillen and Griffioen, 2007). In the case of tumour progression, absence of angiogenesis is severely restricting the tumour to grow beyond 1-2 mm^3 (Folkman, 1995; Gasparini *et al.*, 2005). Moreover, angiogenesis also facilitates local invasion and metastasis as the

tissue oxygen diffusion is limited within 100-200 μm, which corresponds to 3–5 cell layers around a blood vessel (Locopo *et al.*, 1998; Gasparini *et al.*, 2005).

1.2.3.1 Regulation of tumour angiogenesis in breast cancer

Breast cancer has been identified as angiogenesis-dependent tumour for past several decades (Atiqur Rahman and Toi, 2003; Fox *et al.*, 2007; Marty and Pivot, 2008). Despite angiogenic factors and inhibitors, there are also other stimuli which have been noted to regulate breast tumour angiogenesis in direct or indirect manners including soluble growth factors, membrane-bound proteins, cell-matrix and cell-cell interactions (Papetti and Herman, 2002; Otrock *et al.*, 2007), hypoxia (Boudreau and Myers, 2003), inflammation, mechanical factors (shear stress and stretch) (Milkiewicz *et al.*, 2006) and oxidative as well as glucose deprivation (Gasparini *et al.*, 2005). Other than that, accumulated clinical and experimental data have also indicated that tumour neovascularisation elicits signalling either via angiogenic mechanisms (i.e. sprouting angiogenesis and lymphangiogenesis) (Otrock *et al.*, 2007b; Lohela *et al.*, 2009) or non-angiogenic mechanisms (i.e. intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option and vasculogenic mimicry) (Cebe-Suarez *et al.*, 2006; Hillen and Griffioen, 2007). In this regard, sprouting angiogenesis or commonly known as angiogenesis has been described as the prominent signalling pathway in breast tumour neovascularisation (Hillen and Griffioen, 2007; Fox *et al.*, 2007). Thus, further details will mainly emphasize more on tumour-induced sprouting angiogenesis instead of the remaining mechanisms.

1.2.3.1.1 Vascular endothelial Growth Factor Receptor (VEGFR)

Basically, the tumour angiogenesis process involves two general phases which begins with prevascular phase (non-angiogenic phenotype), often found in benign tumours, and subsequently switching to vascular phase (angiogenic phenotype), frequently found in malignant tumours (Folkman, 1995; Shinkaruk *et al.*, 2003; Cross *et al.*, 2003). Transformation from avascular to vascular phase will only occur when overwhelming production of pro-angiogenic factors particularly Vascular Endothelial Growth Factor (VEGF) secretes from the tumour cells (Hicklin and Ellis, 2005; Fox *et al.*, 2007). For decades, researchers have implicated that VEGF and its cognate receptors are regularly over expressed in a diverse range of human cancers notably in breast cancer (Atiqur Rahman and Toi, 2003; Salter and Miller, 2007; Schneider and Sledge, 2007). VEGF is the prime pro-angiogenic factor that prominently regulated during tumour angiogenesis development and progression (Miller, 2004; Salter and Miller, 2007; Zelnak and O'Regan, 2007). It exists in multiple isoforms which have distinct physical and biological properties (Tammela *et al.*, 2005; Lohela *et al.*, 2009). VEGFs elicit their angiogenic responses via three specific tyrosine kinase receptors known as: VEGFR-1 (also referred as fms-like tyrosine kinase 1 (Flt-1)), VEGFR-2 (also known as kinase insert domain-containing receptor (KDR) or its murine homolog, fetal liver kinase 1 (Flk-1)) and VEGFR-3 (also known as fms-like tyrosine kinase 4 (Flt-4)) (Takahashi and Shibuya, 2005; Veeravagu *et al.*, 2007). VEGFR-1 and VEGFR-2 are cell surface receptors which were initially discovered on endothelial cells and characterized as specific receptor tyrosine kinases (RTK) (Hicklin and Ellis, 2005). As depicted in Figure 1.5, both receptors share 45% homology sequences and possess similar organizational structure composed of seven immunoglobulin-like domains in the extracellular

domain, a single hydrophobic trans-membrane domain, and an intracellular cytoplasmic domain with tyrosine-kinase activity essential for signal transduction (Shinkaruk *et al.*, 2003; Hicklin and Ellis, 2005; Cebe-Suarez *et al.*, 2006; Lohela *et al.*, 2009). Each receptor was distinguished by a short interrupted sequence with 80% similarity of tyrosine kinase domain (Shinkaruk *et al.*, 2003)

Vascular endothelial growth factors (VEGFs) are crucial regulators of vascular development during embryogenesis (vasculogenesis) as well as blood-vessel formation (angiogenesis) in adults. In mammals, five VEGF ligands, which occur in several different splice variants and processed forms, have been identified so far (Folkman, 1995; Shinkaruk *et al.*, 2003; Cross *et al.*, 2003). These ligands bind in an overlapping pattern to three receptor tyrosine kinases (RTKs), known as VEGF receptor-1, -2 and -3 (VEGFR1–3), as well as to co-receptors (defined as VEGF-binding molecules that lack established VEGF-induced catalytic function), such as heparan sulphate proteoglycans (HSPGs) and neuropilins (Takahashi and Shibuya, 2005; Veeravagu *et al.*, 2007). In certain respects, VEGFs share regulatory mechanisms with other well-characterized RTKs, such as the platelet-derived growth-factor receptors (PDGFRs) and the epidermal growth-factor receptors (EGFRs) (Hicklin and Ellis, 2005). These mechanisms include receptor dimerization and activation of the tyrosine kinase, as well as creation of docking sites for signal transducers. Moreover, the VEGFRs induce cellular processes that are common to many growth-factor receptors, including cell migration, survival and proliferation. However, the VEGFRs also seem to be unique, for example, in their ability to transduce signals that form the three-dimensional vascular tube, and in regulating vascular permeability that leads to oedema and swelling of tissues. VEGFR1 is a positive regulator of monocyte and macrophage migration, and has been described as

a positive and negative regulator of VEGFR2 signalling capacity. Negative regulation is exerted, at least in part, by an alternatively spliced soluble VEGFR1 variant that binds to VEGF and thereby prevents VEGF from binding to VEGFR2 (Shinkaruk *et al.*, 2003; Hicklin and Ellis, 2005; Cebe-Suarez *et al.*, 2006; Lohela *et al.*, 2009). VEGFR2 is implicated in all aspects of normal and pathological vascular-endothelial-cell biology, whereas VEGFR3 is important for lymphatic endothelial cell development and function. Recently, tumour therapies that are based on neutralizing anti-VEGF antibodies and small-molecular-weight tyrosine-kinase inhibitors that target the VEGFRs have been developed. These new strategies for tumour treatment show the clinical relevance of inhibiting VEGF signal-transduction pathways that are exaggerated in pathological angiogenesis.

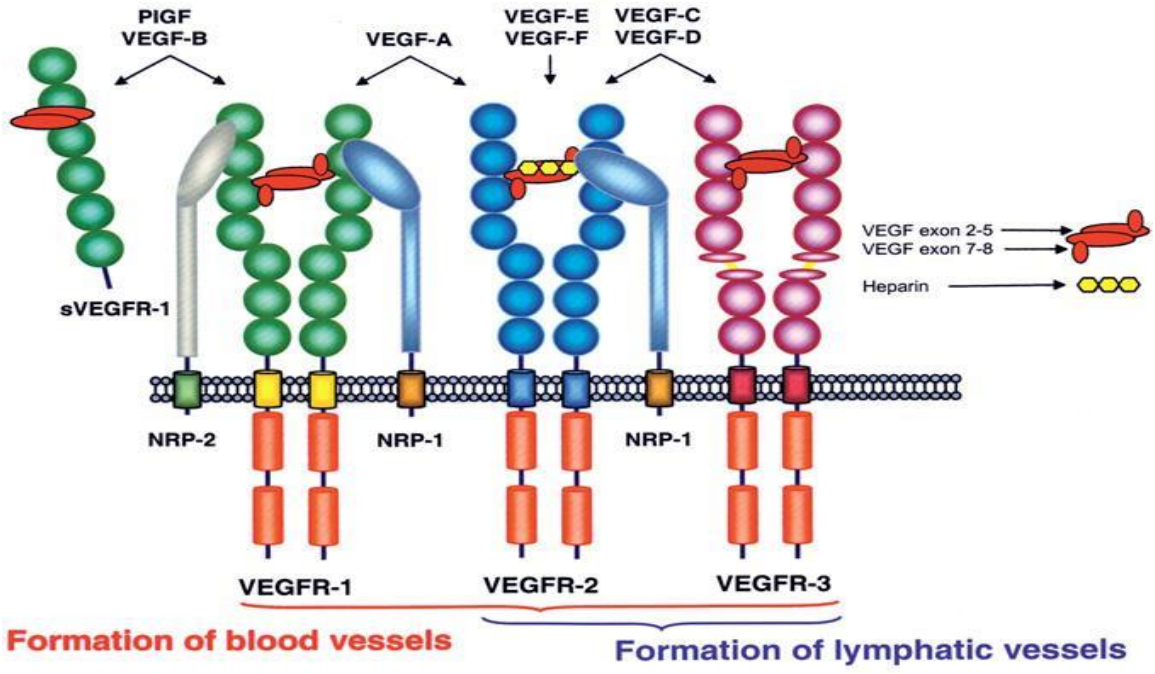


Figure 1.5 Schematic diagrams of VEGF family ligands and their respective receptors (Hicklin and Ellis, 2005)

1.2.3.1.1.1 Flt-1

Vascular endothelial growth factor receptor 1 is a protein that encoded by the Flt-1 gene. In human, the functional relevance of Flt-1 has been subjected of intense debate. Flt-1 binds to VEGF, PlGF, and VEGF-B with at least 10-fold higher affinity than Flk-1 binds to VEGF (Shibuya, 2001). However, Flt-1 has a weaker tyrosine kinase activity (Park *et al.*, 1994), and has been proposed to act as a decoy, regulating the availability of VEGF (Hiratsuka *et al.*, 1998). Other studies indicated, however, that Flt-1 interacts with various signal transducing proteins and generates signals (Luttun *et al.*, 2002; Autiero *et al.*, 2003). The precise role of VEGF-B and Flt-1 in the nervous system is still unclear. Flt-1 is upregulated in astrocytes after administration of VEGF (Mani *et al.*, 2005) or after injury (Choi *et al.*, 2007), and stimulates migration of microglial cells *in vitro* (Forstreuter *et al.*, 2002). Loss of VEGF-B promote stroke, whereas VEGF-B stimulates proliferation of neuronal cultures *in vitro* (Sun *et al.*, 2004) and neurogenesis *in vivo* (Sun *et al.*, 2006), and is protective in injury models, such as axotomy- and NMDA-induced cell death in the retina (Li *et al.*, 2008). VEGF-B has been presumed to exert direct neuroprotective effects, but this evidence was based on anti-apoptotic effects on smooth muscle cells and retinal pericyte cell lines (Li *et al.*, 2008). However, evidence that VEGF-B has direct neuroprotective effects on primary neurons has not yet been provided, nor has long-term delivery been shown to improve the disease course or outcome.

1.2.3.1.1.2 Flk-1

Flk-1 is the most prominent and major mediator in tumour angiogenesis. The mechanism of action of Flk-1 in breast tumour is depicted in Figure 1.6. Flk-1 is a strong ligand-dependent tyrosine phosphorylation (Otrock *et al.*, 2007). Secretion of VEGF from the luminal surface of the vascular endothelium (i.e. circulating cells), or from the abluminal surface (i.e. pericytes, stromal cells and tumour cells) can initiate the signalling once it binds to the extracellular domain of Flk-1 (Weis and Cheresh, 2005). Once the Flk-1 undergoes dimerization and autophosphorylation of the intracellular domain, a cascade of downstream proteins will be activated that will further regulates the endothelial cell proliferation, migration, survival, vascular permeability and dilation (Cross *et al.*, 2003; Hicklin and Ellis, 2005) as well as cell differentiation (Cebe-Suarez *et al.*, 2006). Different cascades will be activated pertaining to different tyrosine-phosphorylation sites (Rahimi, 2006). Currently, there are seven well-described putative tyrosine-phosphorylation sites of Flk-1 from a total of 19 tyrosine residues present in the intracellular domain (Cebe-Suarez *et al.*, 2006). The phosphorylation sites are constructed out of the kinase insert domain (Tyr951 and Tyr996), the kinase domain (Tyr1054 and Tyr1059) (Zachary and Gliki, 2001), the C-terminal tail (Tyr1175 and Tyr1214) (Cross *et al.*, 2003) and Tyr801 (Cebe-Suarez *et al.*, 2006). Phosphorylation of Tyr1175 leads to activation of phospholipase C- (PLC) and protein kinase C (PKC) followed by stimulation of Ras-Raf1-MEK-ERK and results in cell proliferation (Cebe-Suarez *et al.*, 2006; Otrock *et al.*, 2007). Earlier studies demonstrated that phosphorylation of Tyr1175 also promotes cell survival via activation of phosphoinositide 3-kinase (PI3K)-Akt/PKB pathway (Kliche and Waltenberger, 2001; Lohela *et al.*, 2009). The Akt/PKB pathway also induces vascular permeability via activation of endothelial nitric oxide

synthase (eNOS) (Cross *et al.*, 2003; Lohela *et al.*, 2009). In addition, Flk-1 regulates cell migration through several different pathways including Tyr951-TSAd (T-cell-specific adaptor) and p38 MAPK (mitogen-activated protein kinase)-HSP27 (heat-shock protein-27) pathway as well as focal adhesion kinase (FAK) and its substrate, paxilin pathway (Olsson *et al.*, 2006).

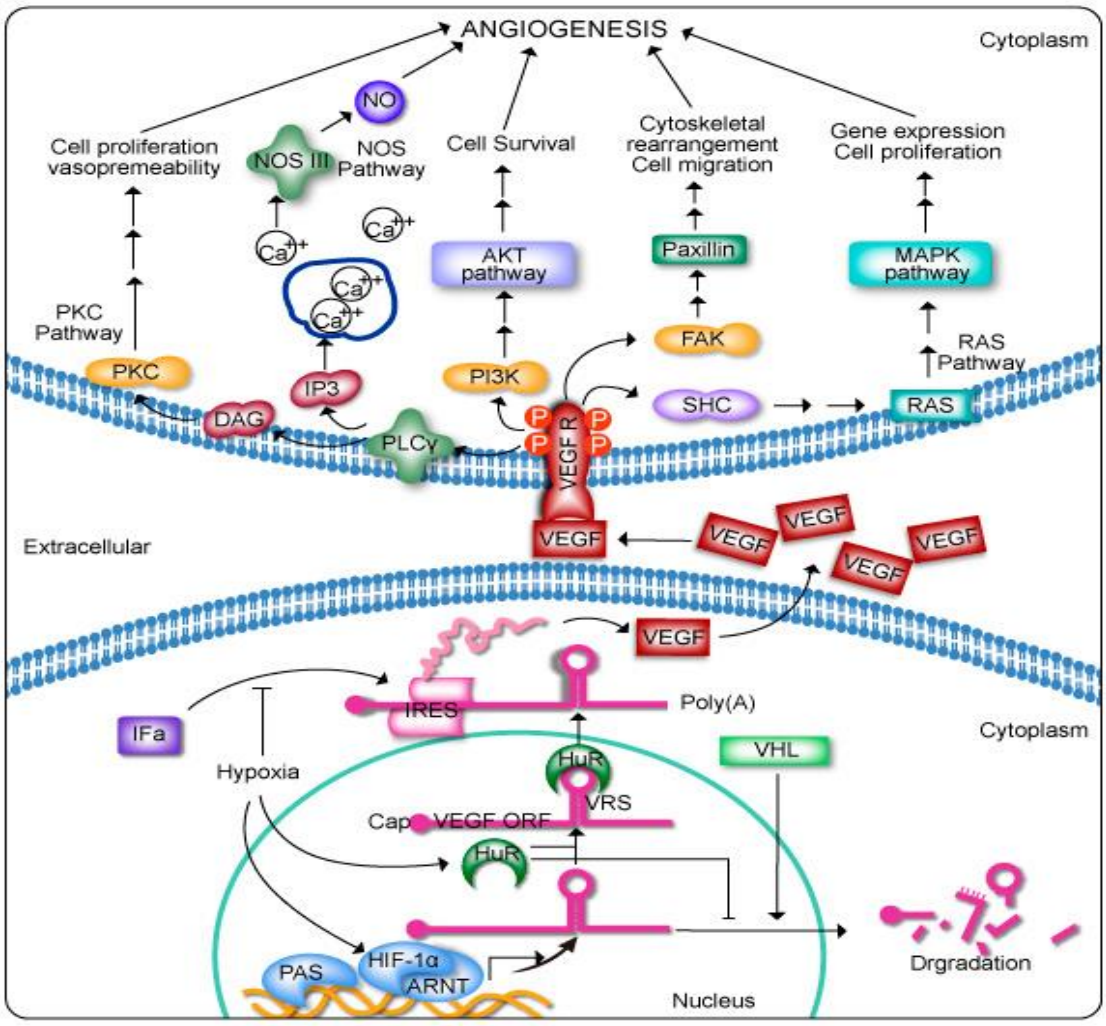


Figure 1.6 Regulation of VEGF signalling pathway (Olsson *et al.*, 2006).

1.2.3.1.1.3 Flt-4

Flt-4 is a third receptor for a ligand of the VEGF family, VEGF-C (Joukov *et al*., 1996). It's expression in adult mice appears predominant in the endothelial cells of lymphatic vessels (Kaipanen *et al*., 1995). The human Flt-4 locus encodes two isoforms. The long form, Flt-4L, differs from the short form, Flt-4S, by the addition of 65 amino acids in the C-terminal region (Pajusola *et al*., 1993, Fournier *et al*., 1995). Another member of the VEGF family, VEGF-C, was isolated as a ligand for the tyrosine kinase VEGFR-3 (Flt4) (Fong *et al*., 1995), a receptor that is expressed in endothelial cell precursors in 8.5 days mouse embryos and later in development is expressed in venous and lymphatic endothelium (De Vries *et al*., 1992). The pattern of VEGF-C gene expression in mouse embryos suggests that VEGF-C may regulate angiogenesis of the lymphatic vasculature (Terman *et al*., 1992). VEGF-C is also a ligand for Flk-1 (Fong *et al*., 1995), but the functional significance of this potential interaction *in vivo* is unknown. The amino acid sequence of VEGF-C has a central region that is related to other members of the VEGF family and exhibits approximately 30% identity to VEGF. In addition, the VEGF-C sequence has N-terminal and C-terminal extensions that are not present in VEGF, PlGF, or VEGF-B (Fong *et al.*, 1995, Quinn *et al.*, 1993). The biosynthesis of VEGF-C involves proteolytic processing that gives rise to a mature secreted protein that essentially consists of the VEGF homology domain (Park *et al*., 1994), i.e., the portion of the molecule that is related in primary structure to all other members of the VEGF family and that contains the cystine knot motif that is found in VEGF family members and in other growth factors (Waltenberger *et al*., 1994).

1.2.3.2 Angiogenic inhibitors

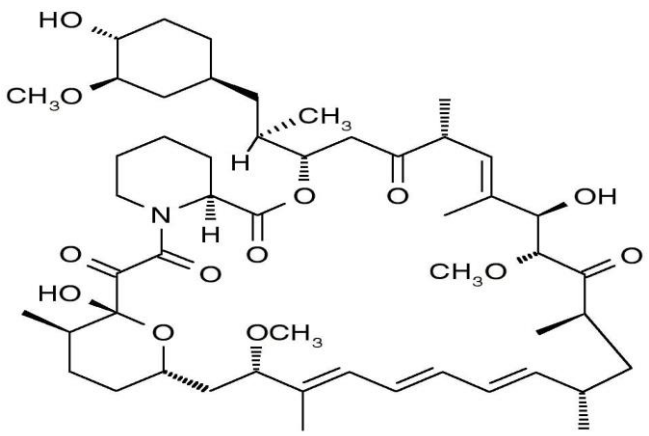
The role of tumour angiogenesis in supporting the tumour development and progression has been well understood. Thus, targeting the tumour angiogenesis seems to be a potential strategy in treating the solid tumour like breast cancer.

1.2.3.2.1 Rapamycin

Rapamycin was first identified as an anti-fungal agent isolated from bacterium *Streptomyces hygroscopicus* at Easter Island (Rapa Nui) in 1975 (Noh *et al.*, 2004; Koehl *et al.*, 2005). Later, it was introduced as a potent immunosuppressant agent and has been applied clinically for treatment in organ transplantation (Sehgal, 1998; Gaumann *et al.*, 2008). However, one of the shortcomings in organ transplant patients is the development of tumour occurrence (Kauffman *et al.*, 2006; Leblanc *et al.*, 2011; Schnitzbauer *et al.*, 2011). Previous studies demonstrated that cyclosporine; the most widely used immunosuppressive drug promotes tumour development, whilst immunosuppressant dosage of rapamycin simultaneously inhibits tumour occurrence (Luan *et al.*, 2002).

Since then, various researches have been conducted to determine the anti-tumour properties of rapamycin and findings have ruled out that rapamycin inhibits tumour growth by halting tumour cell proliferation, inducing tumour cell apoptosis, and suppressing tumour angiogenesis (Guba *et al.*, 2002; Law, 2005; Gaumann *et al.*, 2008). One study suggested that rapamycin inhibits development of highly invasive and metastatic mammary tumour (Met-1) *in vivo* via suppression of p70S6K and 4E-BP1 which are known as downstream effectors of mTOR signalling cascade (Namba *et al.*, 2006). It is well known that activation of PI3K-PDK1-Akt phosporylates

downstream effectors of mTOR, p70S6 kinase and 4E-BP1, and leads to tumour cell proliferation (Law, 2005). Koehl *et al.*, (2005) reported that rapamycin inhibits tumour cell proliferation via disruption of receptors which serves as docking site for p70S6 kinase and 4E-BP1. In addition, rapamycin also act as a pro-survival by inhibiting cyclin dependent kinase 1 (CDK1) which leads to cell cycle arrest at G1 phase in T lymphocytes, suppression of Cyclin D1, D3, A and c-Myc as well as promoting tumour cell apoptosis (Koehl *et al.*, 2005; Law, 2005). Recently, increasing body of evidence implicated that rapamycin exerts anti-angiogenic properties by suppressing VEGF expression in several *in vitro* and *in vivo* tumour models (Guba *et al.*, 2002; Stephan *et al.*, 2004; Molhoek *et al.*, 2008).



(Foster *et al.*, 2010)

Figure 1.7 The chemical structure of rapamycin

1.2.3.2.2 Platelet Factor 4 (PF4)

Platelet Factor 4 (PF4) is one of the angiostatic member of the chemokine family also recognized as CXCL4 (chemokine ligand 4) according to the new nomenclature of chemokines (Strieter *et al.*, 2004). It was first discovered in 1977 as