The role of ERK5 in tumour angiogenesis and drug resistance

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

By

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Declaration

I declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research

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This research was carried out in the Department of Molecular and Clinical Pharmacology, Institute of Translational Medicine, University of Liverpool.

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Extracellular regulated signal protein kinase 5 (ERK5) is the most recently identified member of the MAPK family which are involved in cellular proliferation, differentiation, migration. ERK5 differs from other MAPKs, in that contains an unusually long carboxyl-terminal tail. ERK5 is activated by growth factors and cellular stresses. Targeted deletion of *erk5* in mice results in death around E9.5-11.5 due to severe defects in vasculature revealed the importance of the ERK5 signalling pathway. Aberrant ERK5 signalling has already been reported in several different cancers such as breast, prostate, hepatocellular and melanoma.

This project investigated the differences in ERK5 activation between VEGF in endothelial cells and EGF in HeLa cells. It was revealed that VEGF has only the ability to induce the dual phosphorylation of the kinase domain of ERK5, without any effect on the phosphorylation of the C-terminal residues suggesting that phosphorylation of Thr^{218}/Tyr^{220} in the kinase domain and C-terminal phosphorylation are not mutually inclusive events and that ERK5 can be activated in the absence of C-terminal phosphorylation. Furthermore, this study also investigated the role of ERK5 in drug resistance and tumour angiogenesis. It was showed that inhibition of MEK5/ERK5 signalling pathway by BIX02189 or XMD8-92 in combination with anti-cancer drug increased the sensitivity of melanoma and ovarian cancer cells to vemurafenib and doxorubicin respectively. VEGF plays a key role in angiogenesis and it has been reported that ERK5 is required for VEGF-mediated survival and tubular morphogenesis. Targeting MEK5/ERK5 signalling cascade resulted in inhibition of VEGF-induced angiogenesis in HDMEC/NHDF/cancer cells co-culture angiogenesis *in vitro* assay.

Taken together, this data has suggested that the ERK5 signalling axis is a viable target to restore sensitivity to chemotherapy in drug-resistant cells and inhibit aberrant angiogenesis which could present a possibility of ERK5 serving as a therapeutic target for drug resistance in cancer.

Dedicated to my father Abdullah Aljasir and my mother Badriah Alodheib and my wife Lolwah Almarzooq and my little girls Danah & Ghadah

{الْحَمْدُ لِلَّهِ رَبِّ الْعَـٰلَمِينَ}

All thanks are due purely to God, Lord of the universe

(QS. Al Fatihah 2) Quran

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Table of Contents

Abbreviations

AKT	v-akt murine thymoma viral oncogene homolog (PKB)
Ala	Alanine (A)
ABCG2	ATP-binding cassette sub-family G member 2
ALK	Anaplastic lymphoma kinase
AP-1	Activator protein-1
AR	Amphiregulin
Arg	Arginine (R)
Asp	Aspartic acid (D)
ATP	Adenosine triphosphate
B16F10	Murine melanoma tumour cell line
BAD	BCL2-antagonist of cell death
BAEC	Bovine aortic endothelial cell
BCL2	B-cell lymphoma-2
BDNF	Brain-derived neurotrophic factor
Bim	Bcl-2 interacting mediator
BLMEC	Bovine lung microvascular endothelial cell
BMK1	Big MAPK-1
BRCA	Breast cancer gene
BSA	Bovine serum albumin
BT474	Breast cancer cell line
BTC	Betacellulin
C-	Carboxy-
CA	Constitutively-active

CD	Common docking
CDK	Cyclin-dependent kinase
cDNA	Complementary DNA
CD31	Cluster of differentiation 31
COS7	Monkey kidney tissue-derived fibroblast-like, CV-1 cell line
CR1	Cysteine rich domain
CREB	cAMP-responsive element binding-protein
CT	Carboxyl terminal domain
Cys	Cysteine (C)
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DRG	Dorsal root ganglia
dsRNA	Double-stranded RNA
DUSP	Dual-specificity protein phosphatase
E	Embryonic day
EC	Endothelial cell
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	EGF receptor
EPR	Epiregulin
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGM	Full growth medium

FasL	Fas ligand
FCS	Foetal calf serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FGFR	FGF receptor
FIGO	International Federation of Gynaecology and Obstetrics
Flk-1	Foetal liver kinase-1
Flt	Fms-related tyrosine kinase
FoxO3a	Forkhead box O3a
Gab1	Grb2-associated binder-1
Glu	Glutamic acid (E)
Grb2	Growth-factor-receptor-bound 2
GST	Gastrointestinal stromal tumour
GSTP1	Glutathione S-transferase P1
HB-EGF	Heparin-binding EGF-like growth factor
HDMEC	Human dermal microvascular endothelial cell
HEK293	Human embryonic kidney 293 cells
HeLa	Henrietta Lacks-derived immortalised epithelial cervical cancer cell line
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia-inducible factor-1 alpha
HIF-1β	Hypoxia-inducible factor-1 beta
HP-EGF	Heparin-binding EGF
HUVEC	Human umbilical vein endothelial cell
IC50	Half maximal inhibitory concentration

IF	Immunofluorescence
IFN-α	Interferon-alpha
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin-6
IL-8	Interleukin-8
IP	Immunoprecipitation
JM	Juxta membrane domain
JNK	c-Jun NH_2-terminal kinase
KDR	Kinase-insert domain-containing receptor
KLF2	Krüppel-like factor 2
KLF4	Krüppel-like factor 4
L1	Ligand binding domain
Lad	Lck-associated adaptor
Lck	Lymphocyte-specific protein tyrosine kinase
LL/2 Lewis	Lewis lung carcinoma cells
Lys	Lysine (K)
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MCF-7	Michigan cancer foundation-7 breast cancer cell line
MDR-1	Multidrug resistance protein 1
MEF	Myocyte enhancer factor
MEK	MAPK/ERK kinase
MEKK	MEK kinase

MKP	MAP kinase phosphatase
MM	Malignant mesothelioma cells
MMP	Matrix metalloproteinases
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
mTORC	mTOR complex
N-	Amino-
NES	Nuclear export signal
NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor-kappaB
NGF	Nerve growth factor
NHDF	Normal human dermal fibroblast
NLS	Nuclear localisation signal
Nrf2	NF-E2 related factor 2
NRG	Neuregulin
NRP	Neuropilin
$p90^{RSK}$	90 kDa ribosomal S6 kinase
PAGE	Polyacrylamide gel electrophoresis
PB1	Phox and Bem1p
PBS	Phosphate-buffered saline
PC12	Pheochromocytoma 12
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGFR	PDGF receptor

PDK	Phosphoinositide-dependent kinase
PI3K	Phosphoinositide 3’-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLCγ	Phospholipase C-gamma
PlGF	Placental growth factor
PMA	Phorbol 12-myristate 13-acetate
PML	Promyelocytic leukaemia protein
PP2A	Protein phosphatase 2A
PPAR	Peroxisome proliferation activator receptor
PR	Proline-rich
Pro	Proline (P)
PTB	Phosphotyrosine-binding
PTEN	Phosphatase and tensin homologue
PTP	Phosphor-tyrosine specific phosphatases
qRT-PCR	Quantitative real-time PCR
RAF	Rapidly accelerated fibrosarcoma kinase
RAS	Rat sarcoma protein
RCC	Renal cell carcinoma
RIPA	Radio-immunoprecipitation assay
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature

RTK	Receptor tyrosine kinase
SAPK	Stress-activated protein kinase
Sap1a	Serum response factor accessory protein 1a
Sck	Shc-like protein
SDS	Sodium dodecyl sulphate
Ser	Serine (S)
SGK	Serum- and glucocorticoid-inducible kinase
SH2	Src homology 2
SH3	Src homology 3
Shb	Src homology 2 protein B
siRNA	Small-interfering RNA
SKOV-3	Human ovarian carcinoma cell line
STAT	Signal transducer and activator of transcription
SUMO	Small ubiquitin-related modifier
TBS	Tris-buffered saline
TBS-T	TBS Tween
TGF-α	Transforming growth factor-alpha
Thr	Threonine (T)
TK	Tyrosine kinase domain
TM	Transmembrane domain
TNF-α	Tumour necrosis factor-alpha
TKD	Tyrosine kinase domain
TSAd	T-cell specific adapter
TSP-1	Thrombospondin-1

Tyr	Tyrosine (Y)
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VEGFR	VEGF receptor
VPF	Vascular-permeability factor
VRAP	VEGF receptor-associated protein
WB	Western blot
Wt.	Wild-type

Chapter One: Introduction

1.1 Vascular system

The human body requires a mechanism to deliver oxygen and nutrients to the tissues and organs adequately and remove toxic metabolites. The vascular system accomplishes these functions and also carries immune cells to sites of infection and inflammation, and transports endocrine signals to responder cells (Rossant and Howard, 2002)

The vascular system functions via two main parts, blood vessels and lymphatic vessels. The lumens of these vessels are lined by the endothelium, a monolayer of endothelial cells. The endothelial cells of capillaries and small vessels are surrounded by a basement membrane and covered by smooth muscle cells, pericytes, giving the vessels stabilization (Figure 1.1 B). The large vessels such as arteries and veins consisted of three layers. The inner layer is composed of the endothelium, as *tunica intima*, surrounded by extra cellular matrix proteins. This layer is surrounded by two layers; a thick layer of smooth muscle cells that promote the stabilization called *tunica media* and the tight outermost layer, the *tunica adventia*, which comprises connective tissue, collagen and elastic fibres (Figure 1.1 A). The vascular system is formed by the process of vasculogenesis and modified and remodelled by the process of angiogenesis (Gerhardt and Betsholtz, 2003).

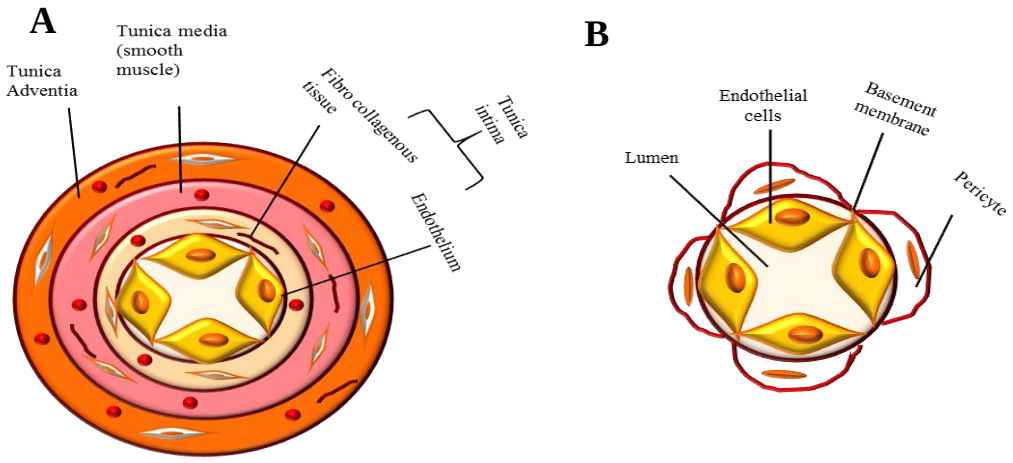


Figure 1.1 Blood Vessel structure. (A) Illustration of a cross section of large blood vessel such as artery or vein. **(B)** Illustration of a cross section of a small blood vessel such as a capillary or arteriole.

1.1.1 Vasculogenesis

Vasculogenesis is defined as the process by which new blood vessels form from endothelial precursor cells (Risau and Flamme, 1995). In the yolk sac, the mesoderm cells differentiate into haemangioblasts, precursor cells, which in turn differentiate into angioblasts and aggregate to form blood islands. The blood islands consist of an outer layer of endothelial precursor cells that surround the inner mass of hematopoietic precursor cells. The angioblasts differentiate into endothelial cells and form a primitive vascular plexus of blood vessels. The haematopoietic precursor cells differentiate to form mature hematopoietic precursor cells. The fusion of blood islands and the differentiated angioblasts and haematopoietic precursor cells to form primary vascular plexus of blood vessels in yolk sac that surrounds the embryo (Choi et al., 1998). New capillaries appear by either sprouting or intussusception from the first vessels and the plexus will then expand remodel through angiogenesis.

1.1.2 Angiogenesis

Angiogenesis is the formation of blood vessels from pre-existing tissues (Risau, 1997). Angiogenesis is important in embryonic development. It important in the female reproductive cycle, during pregnancy and in wound healing (Groothuis, 2005). The body tightly regulates angiogenesis by producing a precise balance of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and anti-angiogenic factors such as angiostatin, endostatin and thrombospondin. Disturbance of this balance results in abnormal growth of blood vessels by either excessive or insufficient angiogenesis (Figure 1.2). Abnormal angiogenesis is implicated in many diseases and deadly conditions such as cancer, diabetic retinopathy, cardiovascular disease and stroke result of insufficient angiogenesis (Carmeliet, 2005) (Figure 1.2).

Angiogenesis occurs through two distinct mechanisms; sprouting angiogenesis and intussusceptive or splitting angiogenesis. In sprouting angiogenesis, new blood vessels are formed from pre-existing vessels (Risau, 1997). The balance of growth factors regulates endothelial cell quiescence. An increase in pro-angiogenic factors such as VEGF or decrease in anti-angiogenic factors, such as thrombospondin, induces changes in the phenotype of endothelial cells which switch on the angiogenic switch and endothelial cells become activated (Carmeliet and Jain, 2000). The blood vessels have oxygen and hypoxia-induced receptors to supply a sufficient amount of oxygen to tissues. The endogenous signals or hypoxia activate cells to release signalling factors, for example VEGF, bFGF, to develop new blood vessels from pre-existing vessels. VEGF, a pro-angiogenic factor, binds to its cognate receptor on the endothelial cell membrane called VEGFR (Carmeliet and Jain, 2011). An endothelial tip cell releases proteases such as matrix metalloproteinases, MMP, to degrade the basement membrane surrounding the endothelial cell and remodel the extracellular matrix. The tip cell migrates toward stimuli that release VEGF by extending various filopodi. Stalk endothelial cells extend the sprouting and proliferate to establish junctions with neighbouring endothelial cells and regulate formation of vascular lumen. Two tip endothelial cells encounter each other and constitute endothelial cell-endothelial cell junctions and form the lumen (Lamalice et al., 2007). The extracellular matrix forms a new basement membrane and the pericytes are recruited to stabilize the newly-formed tubes. When the blood flow is established, the resting state of blood vessels is re-established (Carmeliet and Jain, 2000). Intussusceptive angiogenesis is the grow of new blood vessels from the pre-existing vasculature via insertion of pillars into the lumen of capillaries, small arteries and veins which subsequently fuse to form new capillary network (Djonov and Makanya, 2005). The formation of pillar, which is the hallmark of intussusceptive angiogenesis, progresses through a multistep process; the endothelial walls of the opposite side of a vessel protrude until they contact each other to form intraluminal pillar. The

interendothelial junctions are reorganized by which the endothelial bilayer is perforated centrally in the core of the pillar. The pericytes and myofibroblasts invade the pillar and deposit extracellular matrix into it. The endothelial cells retract and the new pillars increase in size and fuse with each other and finally splitting up the original capillary into two new capillaries (De Spiegelaere et al., 2012, Makanya et al., 2009).

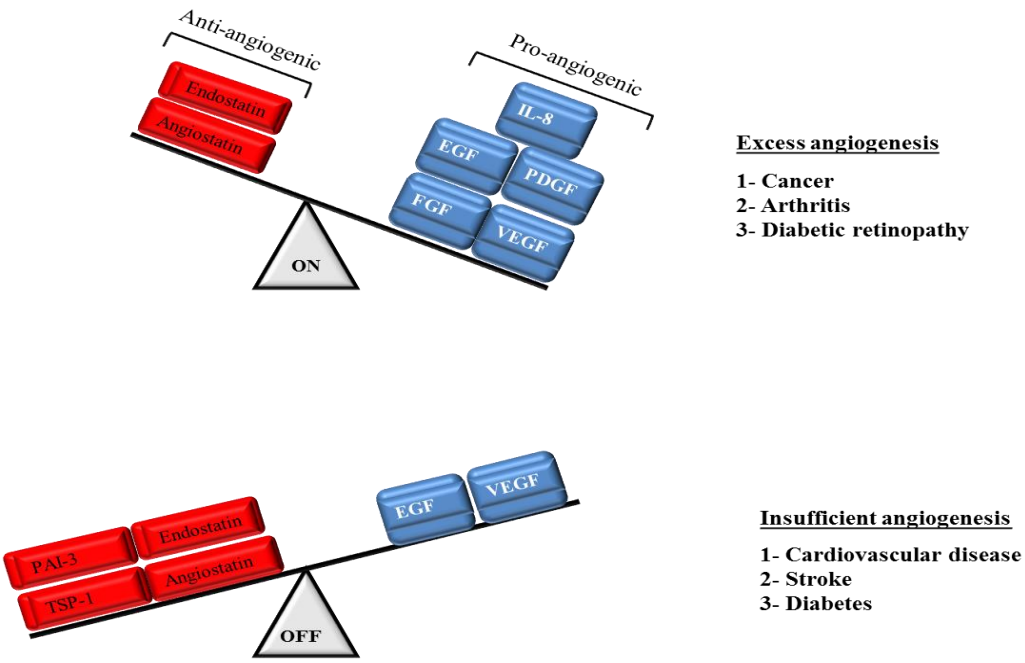


Figure 1.2 Angiogenic balance in physiological angiogenesis. The pathologies distinguished by excessive and insufficient angiogenesis which are associated with up-regulation of pro-angiogenic factors and/or down regulation of anti-angiogenic factors.

1.1.3 Tumour angiogenesis

Cancer represents a progression from normal cellular homeostasis to a neoplastic condition with the cellular acquisition of a number of defined hallmarks: sustained proliferative signalling, evading suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, reprogramming energy metabolism and evading

immune destruction (Hanahan and Weinberg, 2011). Cancer cells require an adequate blood supply that contains nutrients and oxygen. Tumour cells are able to penetrate the cardiovascular system and proliferate to any organ of the body forming metastasis (Folkman, 1971). Metastasis is angiogenesis-dependent as the formation of vascular networks is a vital step in its process and in supplying oxygen and nutrients to the tumour (Folkman, 1971). Angiogenesis is essential for tumour growth progression as without an adequate blood supply tumours will stop growing and become apoptotic (Parangi et al., 1996). Thus, a tumour without blood supply grows to 1-2 mm^3 and then stops while with a blood supply it grows beyond 2 mm^3 (Muthukkaruppan et al., 1982). Hypoxic conditions that result from insufficient new blood vessels induce the expression of different pro-angiogenic growth factors such as VEGF and bFGF in tumour cells which bind to their cognate growth factor receptors on the surface of the endothelial cells of neighbouring vessels (Bottaro and Liotta, 2003). Pro-angiogenic growth factors activate endothelial cells that launch the angiogenic responses which result in tumour angiogenesis (Figure 1.3). The newly-formed vessels are disorganised, leaky and lack the quiescent state compared with normal vessels (Bergers and Benjamin, 2003). Therefore, tumour vessels lose their proper functions and induce more angiogenesis to supply the tumour. Angiogenesis depends on the type of tumour and the cancerous organ as each organ has different stromal cells that release various pro- and anti-angiogenic factors (Carmeliet and Jain, 2000).

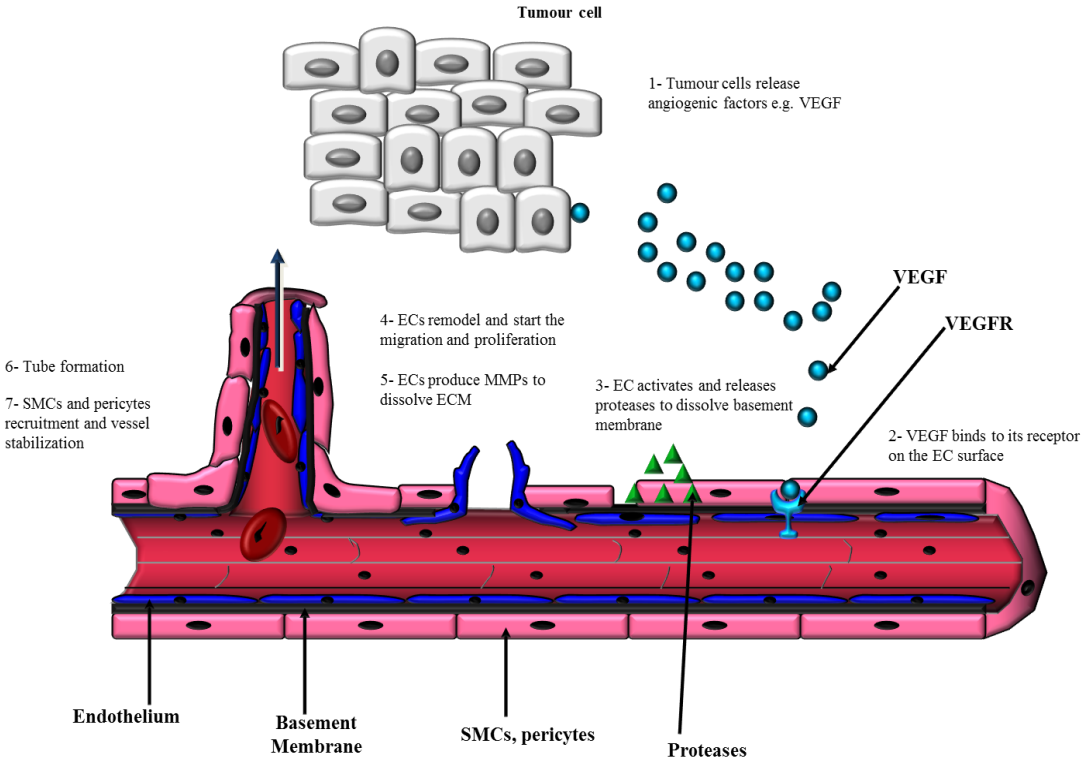


Figure 1.3 Steps of tumour angiogenesis. Tumour cells release angiogenic factors such as VEGF and FGF (1) which in turn bind to their receptors (e.g. VEGFR-2 or FGFR-1) on the surface of the endothelial cells neighbouring capillary blood vessel (2). Binding of these growth factors to their receptors on ECs surface, activates ECs and direct angiogenic response by activating many intracellular signalling pathways (3). Activated endothelial cells secrete proteases to dissolve the basement membrane (3). Degradation of basement membrane and tip cell formation, EC migrate, followed by stalk cell proliferation to form unstable lumen (4). Endothelial cells secrete extracellular matrix protein such as matrix metalloproteinases (MMPs) and growth factors for degradation of most component of extra cellular matrix (ECM) and reconstruct the basement membrane (5). ECs differentiate to form a lumen containing vessel (6). The new vessel is stabilised by recruitment of pericytes (7).

1.1.4 Therapeutic Angiogenesis

In ischaemic diseases, angiogenesis inducers stimulate therapeutic angiogenesis while in cancer, angiogenesis inhibitors can be used to debilitate therapeutic angiogenesis. Therapeutic angiogenesis that targets treating ischaemic disorders by inducing growth of new blood vessels has had success

in animal models. However, it is yet to be proven as relevant as a human therapy (Chu and Wang, 2012). In 1977 the Food and Drug Administration (FDA) approved becaplermin as the first angiogenesis stimulator as therapeutic angiogenesis (Regranex, recombinant human PDGF-BB) for treating diabetic foot ulcer disease (Yoo and Kwon, 2013).

1.1.5 Anti-angiogenic therapy

The last 20 years has seen an intensive research interest in developing anti-angiogenic agents for the treatment of diseases with aberrant angiogenesis (Ferrara and Kerbel, 2005). Targeting VEGF and its cognate VEGFR is the most common strategy to inhibit angiogenic signal transduction. In 2004, bevacizumab (Avastin®), a recombinant humanized monoclonal antibody that specifically targets the VEGF-A isoform of VEGF, became the first angiogenesis inhibitor to be approved by the U.S. FDA for cancer treatment. It prevents human VEGF from binding to its receptors by binding to VEGF (Jain, 2005). Combination of anti-angiogenic treatment, bevacizumab, with chemotherapeutic agents has been shown to increase the efficacy of treatment (Jain, 2005). It is believed that blocking VEGF signalling normalizes the tumour vasculature and improves therapeutic agents to the tumour and increases the beneficial aspects for chemotherapy treatment (Bergers and Hanahan, 2008). In 2006, the FDA approved sunitinib (Sutent®) which is a small molecule receptor tyrosine kinase (RTK) inhibitor that specifically inhibits VEGFR-2 tyrosine kinase activity and other RTKs such as platelet derived growth factor (PDGF) to treat renal cell carcinoma (Motzer et al., 2006). Nonetheless, although the anti angiogenic agents, bevacizumab and sunitinib, have shown relative success for treating cancers, there is a limitation due to the development of drug resistance (Bergers and Hanahan, 2008).

Two mechanisms can cause drug resistance, by classical multidrug resistance, drug efflux as a resistance mechanism, and mutations of the growth factor

signalling receptor (Broxterman et al., 2009). These types of mutations target the tumour endothelium instead of the tumour itself. Therefore, the development of drug resistance to anti-angiogenic treatment by these mutations can be escapable as the tumour recruits endothelial cells for vascularization to survive and these endothelial cells are more stable genetically compared with tumour cells, making them less sensitive to development of drug resistance (Abdollahi and Folkman, 2010). Despite the development of drug resistance associated with anti-angiogenic treatment such as bevacizumab and sunitinib, promising results have been seen and also cooperative effects when combined with chemotherapy and radiation therapy (Abdollahi and Folkman, 2010). Therefore, these complications need to be investigated to identify new promising targets of anti-angiogenic treatment.

1.2 The role of growth factors in angiogenesis

Growth factors are polypeptides that induce cell proliferation and affect cell growth and differentiation either positively and/or negatively (Steed, 1997). These soluble growth factors bind to specific cell surface receptors to control the cellular processes (Steed, 1997). Growth factors usually act in a paracrine and autocrine manners but not in an endocrine manner to maintain biochemical processes required for cell proliferation (Goustin et al., 1986, Favoni and de Cupis, 2000). Growth factors play an important role in wound healing *in vivo* and are released by multiple cells types and from platelets in clotting (Goustin et al., 1986). Recently, researchers identified many cancer molecular targets that are protein kinases. Protein kinases control protein activity and are found on the cell surface as transmembrane receptors or inside the cell as intracellular stimulators or even inside the nucleus to regulate transcription (Yasui and Imai, 2008). Thus, protein kinases are considered as an important target for cancer therapy. As a result of this, a number of growth factor receptors such as epidermal growth factor receptor

(EGFR) and VEGFRs have been studied as potential drug targets (Bennasroune et al., 2004).

1.2.1 Epidermal Growth Factors (EGFs)

Epidermal growth factors were discovered when researchers tried to identify the active agent in a tumour extract (Cohen, 1962). Subsequently, Cohen investigated EGF in the mouse salivary gland and named it EGF due to its mitogenic effect on chick embryo epidermal cells (Cohen, 1965). The EGF family consists of more than 10 related peptide growth factors that activate epidermal growth factor receptors. EGF family ligands contain one or more repeats of six conserved cysteine residues that have the ability to form three intramolecular disulphide bond loops that are required for high affinity binding to the receptors (Groenen et al., 1994, Harris et al., 2003). These ligands are derived from type1 transmembrane precursor which signal via the juxtacrine pathway resulting in proteolytic cleavage within the cell surface and release of a soluble structure of growth factors (Harris et al., 2003). Among the EGF ligand family are the following: EGF, transforming growth factor (TGF-α), amphiregulin (AR), epiregulin (ER), betacellulin (BTC), heparin-binding EGF (HP-EGF) and neuregulin 1,2,3,4 (NRG-1, 2, 3, 4) (Harris et al., 2003). These ligands bind to a special set of receptors as the binding affinity of EGFRs is changeable. Therefore, EGF, TGF-α and AR bind exclusively to EGFR1 while BTC, ER and AR use both EGFR1 and EGFR4 and factors like NRGs bind specifically to EGFR3 and EGFR4 while EGFR2 lacks a known ligand, making it the only orphan receptor in the ErbB family (Holbro and Hynes, 2004) (Figure 1.4).

1.2.2 Epidermal growth factor receptors (EGFRs)

EGF receptor family or ErbB, derived from the erythroblast leukaemia viral oncogene to which this receptor is homologous, is a transmembrane receptor tyrosine kinase (RTK). Four structurally related EGF receptors are found in mammals: ErbB1 (also called Her1 or EGFR1), ErbB2 (Her2 or c-Neu),

ErbB3 (Her3) and ErbB4 (Her4) (Yarden and Sliwkowski, 2001) (Figure 1.4). EGF receptors form homo- or hetero-dimers in response to ligand-dependent activation (Jorissen et al., 2003). They are involved in different biological processes such as metabolism, proliferation and differentiation. The prototypic member of EGF receptor family, EGFR1, is a transmembrane glycoprotein receptor of 170 KDa and expressed by most cells such as epithelial cells. It consists of three domains; an amino-terminal 622 amino acid extracellular domain, transmembrane domain and carboxyl-terminal 542 amino acid intracellular domains (Figure 1.5).

1.2.2.1 Extracellular domain

The extracellular domain consists of four subdomains referred to as L1 and L2, ligand binding domains and CR1 and CR2, cysteine rich domains. At a low affinity state, ligand binds L1 resulting in conformational change of the receptor extracellular domain. Consequently, ligand binds L2 resulting in high affinity binding (Pralhad et al., 2003). In a closed conformation, low affinity, the CR1 is in contact with CR2 while in opened conformation, high affinity, due to ligand binding CR1 exposes a loop from the back interacting with a second ligand bound EGF receptors (Mottet et al., 2007) (Figure 1.5). Furthermore, CR2 presumed to play a role in dimerization and stabilisation (Pralhad et al., 2003). Also CR2 is considered to involve in targeting of the EGFR to the caveolae/raft component of the plasma membrane (Yamabhai and Anderson, 2002).

1.2.2.2 Transmembrane domain (TM)

EGFR uses a single 23 amino acid alpha helical domain to pass the cell membrane. The TM domain has a role in the regulation of receptor dimerization and spanning membrane (Shpitz et al., 2003). Bargmann and his colleagues found that mutation in the transmembrane of EGFR2 could increase the receptor dimerization (Cheng et al., 2014). The TM domain reportedly plays a role in regulating EGFR activity via blocking the receptor

auto phosphorylation and downstream signalling after exposing cells to TM domain peptides (Arjaans et al., 2013).

1.2.2.3 Intracellular domain

The intracellular domain consists of three subdomains; a juxtamembrane domain (JM), a tyrosine kinase domain (TK) and a carboxyl terminal domain (CT) (Figure 1.5). This CT domain has a number of tyrosine residues that regulate EGFR mediated signal transduction after phosphorylation where there are binding sites to proteins containing src homology 2 (SH2) (Chen and Bonaldo, 2013). Beside the existence of tyrosine residues, there are serine/threonine residues that are phosphorylated by downstream kinases (Schlessinger, Plotnikov et al. 2000). The JM domain has regulatory functions as it regulates downstream proteins and ligand internalisation (Ohta et al., 2009).

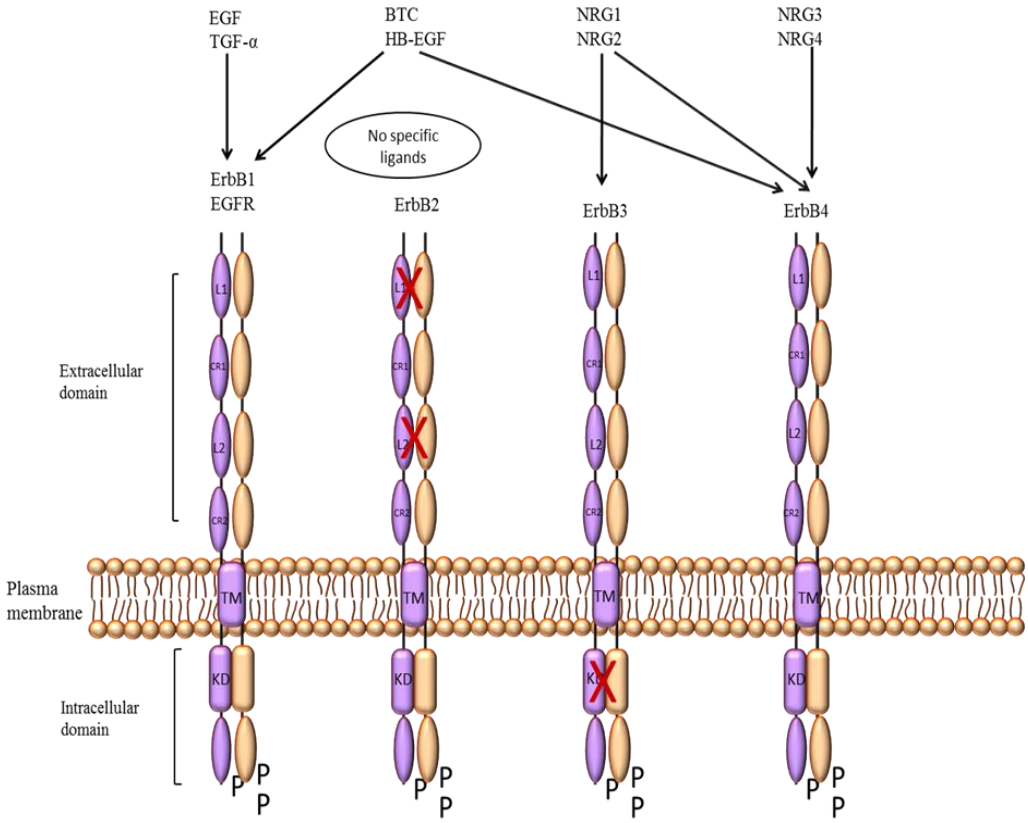


Figure 1.4 ErbB receptors. The ErbB receptor tyrosine kinases consist of four related members; ErbB1, ErbB2, ErbB3 and ErbB4. The ErbB receptor tyrosine kinases are activated by the EGF family of ligands. The EGF family of ligands share most of the identity in their EGF-like domain, however the binding affinity of ErbB receptors is highly variable (Hedlund et al., 2009). EGF and TGF-α bind specifically to ErbB1 (EGFR) while BTC and HB-EGF bind to ErbB1 and ErbB4. The neuregulin (NRG) consist of two subclasses; NRG1 and NRG2 which bind to ErbB3 and ErbB4 and the second subclass is NRG3 and NRG4 which bind to ErbB4. The inactive ligand binding domains of ErbB2 and inactive kinase domain of ErbB3 are marked with an X. L1 and L2, ligand binding domains; CR1 and CR2, cysteine rich domains; TM , transmembrane domain; KD, kinase domain. Diagram adapted from (Shepard et al., 2008).

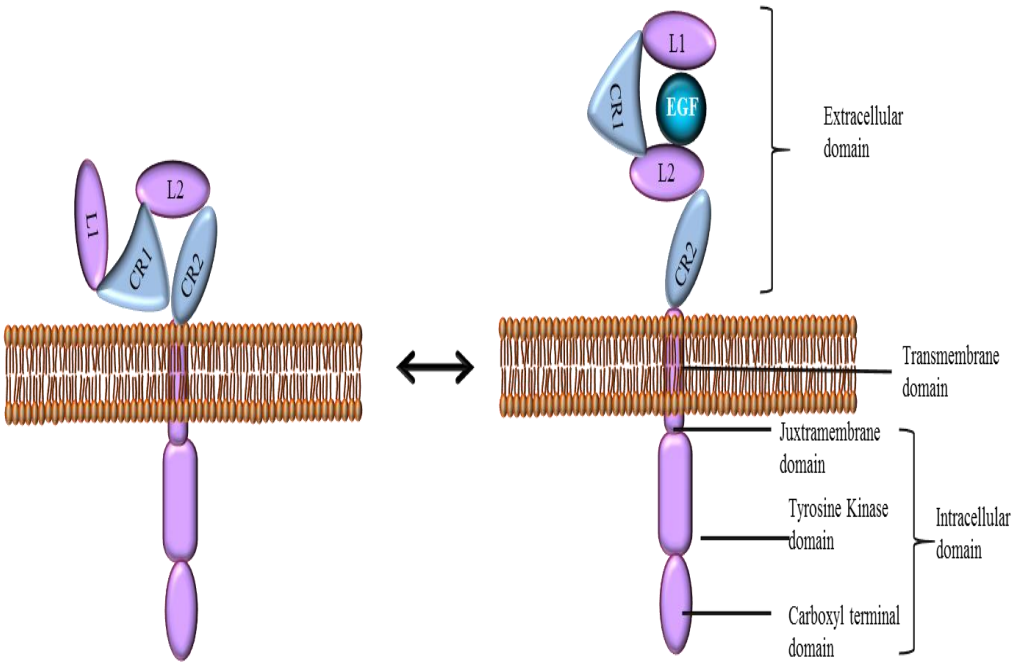


Figure 1.5 Structure of ErbB receptors and activation. Each of the EGF receptors consists of three subdomains; Extracellular domain (EC), Transmembrane domain (TM) and Intracellular domain (IC). The extracellular domain is divided into four sections; ligand binding domains L1 and L2 and cysteine rich domains CR1 and CR2. The transmembrane domain is followed by the intracellular domain which consists of the juxtramembrane (JM) domain, tyrosine kinase (TK) domain and carboxyl terminal domain. The EC present in two conformations; low-affinity or closed conformation, (left) and high-affinity or opened conformation (right). In closed conformation, the CR1 contacts CR2 while in opened conformation CR1 locates to expose a dimerization arm. In the absence of ligands such as EGF, TGF-α, ErbB receptors present in an inactive state, closed conformation. EGFR is activated by binding of ligands (e.g. EGF) in an opened conformation between L1 and L2 which exposes the dimerization arm leading to formation of homo- or hetero-dimer via interacting with another ErbB receptor. The TM domain is followed by the IC domain which has JM, TK and CT domains responsible for regulation and phosphorylation of proteins in signalling pathways that are stimulated by ErbB receptors. Diagram adapted from (Burgess et al., 2003).

1.2.3 Epidermal growth factor receptor and cancer

EGFR activation has been reported to trigger numerous cellular events including cellular differentiation, proliferation, migration, blocking apoptosis and angiogenesis. These processes have a role in uncontrolled cell division in

conditions such as cancer beside their vital role in healthy cells (Pralhad et al., 2003). In the last 20 years, EGFR has been a target for intensive research as a cancer therapy as it is overexpressed in many types of human cancer such as breast, lung, prostate and ovarian cancer (Baselga, 2002, Mendelsohn, 2002).The progressions of some types of cancers and lack of treatment response shows a correlation with expression of EGFR (Baselga, 2002). Furthermore, the overexpression of EGFR in tumour cells has been correlated with increased release of ligands such as EGF and TGF (Mendelsohn, 2002). There are claims that EGFR also plays a role in malignant melanoma as Zhu and his colleagues showed upregulation of EGFR in cells that were taken from metastatic melanoma patient biopsies (Cohen et al., 2000). In response to hypoxia, colon cancer release TGFα which binds to its receptor (EGFR) and activates the cell survival programmes (Rajput et al., 2007). Furthermore, increased TGFα expression in ovarian cancer may implicated in ovarian resistance to cisplatin, and also the increased EGF-related proteins is associated with advanced stage of ovarian cancer (Niikura et al., 1997b). The activation of EGFR signalling pathway stimulates the intracellular pathways MAPK and PI3K which play a role in cell proliferation and differentiation (Mendelsohn, 2001). Overexpression of EGFR in breast cancer has been reported to associate with poor differentiation and poor prognosis with large tumour size (Sainsbury et al., 1987).

1.2.4 Vascular Endothelial Growth Factors (VEGFs)

VEGFs are a family of dimeric glycoproteins of about 40 KDa that are secreted from many cell types such as endothelial, fibroblast and tumour cells (Olsson et al., 2006). In mammals, the VEGF family is comprised of five members: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factor (PLGF). Structurally related VEGFs are parapoxvirus (VEGF-E) and snake venom (VEGF-F) (Hug et al., 1984, Ohuchi, 2005, Nemeth et al., 1988). VEGF-A which is also named as vascular permeability factor (VPF), was the

first described VEGF, and has the ability to trigger vascular leakage (Kiritsy et al., 1993). Alternative splicing of a human VEGF-A gene leads to generate six different variants of 121, 145, 165, 183, 189 and 206 amino acids in length assigned $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$ and $VEGF_{206}$ respectively (Lynch et al., 1987, Lippman et al., 1986, Jingjing et al., 1999, Robinson and Stringer, 2001). Furthermore, these isoforms have overlapping functions because they have different abilities to bind to heparin sulphate and neuropilins. The heparin binding domain is the area encoded by exon 6 and exon 7. VEGF-A_{121} does not contain exon 6 and exon 7 so that it does not bind heparin sulphate and is freely diffusible (Zhang et al., 2004). VEGF-A_{145} has the heparin sulphate domain and the ability to bind to extracellular matrix (Lei et al., 1998). VEGF-A_{165} is the predominant isoform and is the most extensively and biologically active VEGF isoform in humans and is released by different types of cells such as tumour cells, vascular smooth muscle and macrophages (Berse et al., 1992). In addition, VEGF-A_{165} contains the heparin sulphate domain (Zhang et al., 2004). VEGF-A_{189} and VEGF-A_{206} bind to heparin sulphate and extracellular matrix at the cell surface more strongly than the other isoforms (Zhang et al., 2004).

1.2.5 Vascular Endothelial Growth Factor Receptors

VEGFs make their biological effects by binding to three highly homologous receptors denoted VEGF receptor-1 (Flt-1), VEGF receptor-2 (KDR/Flk-1) and VEGF receptor-3 (Flt-4), as well as two transmembrane glycoproteins that act as co-receptors for VEGFs termed neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2). VEGFRs are members of the RTK family and are closely related to the PDGF receptor family (PDGFR). Unlike PDGFRs that contain 5 immunoglobulin-like domains at the extracellular domain, VEGFRs have 7 Ig-like domains on their extracellular domain. VEGFRs are expressed in different cell types; VEGFR-1 is expressed on ECs, pericytes, macrophages, renal mesangial cells and osteoblasts (Zachary and Gliki, 2001). Beside the vascular ECs, VEGFR-2 is expressed on pancreatic duct

cells and retinal progenitor cells (Ferrara et al., 2003). VEGFR-3 is specifically expressed on lymphatic endothelial cells which are specialised ECs which line the lymphatic system (Partanen et al., 2000). Different types of VEGF bind to the different VEGFR; VEGF-A binds to both VEGFR-1 and -2 and it also binds to NRP-1 and NRP-2 while VEGF-B and PLGF are the ligands for VEGFR-1 and NRP-1 (Klagsbrun et al., 2002, Olofsson et al., 1999). Both VEGF-C and VEGF-D have a high affinity for VEGFR-2 and VEGFR-3 whereas VEGF-E binds to VEGFR-2 (McColl et al., 2004, Suto et al., 2005). VEGFRs structurally consist of three domains; the extracellular ligand-binding domain, transmembrane domain and intracellular domains containing two kinase domains (Figure 1.6).

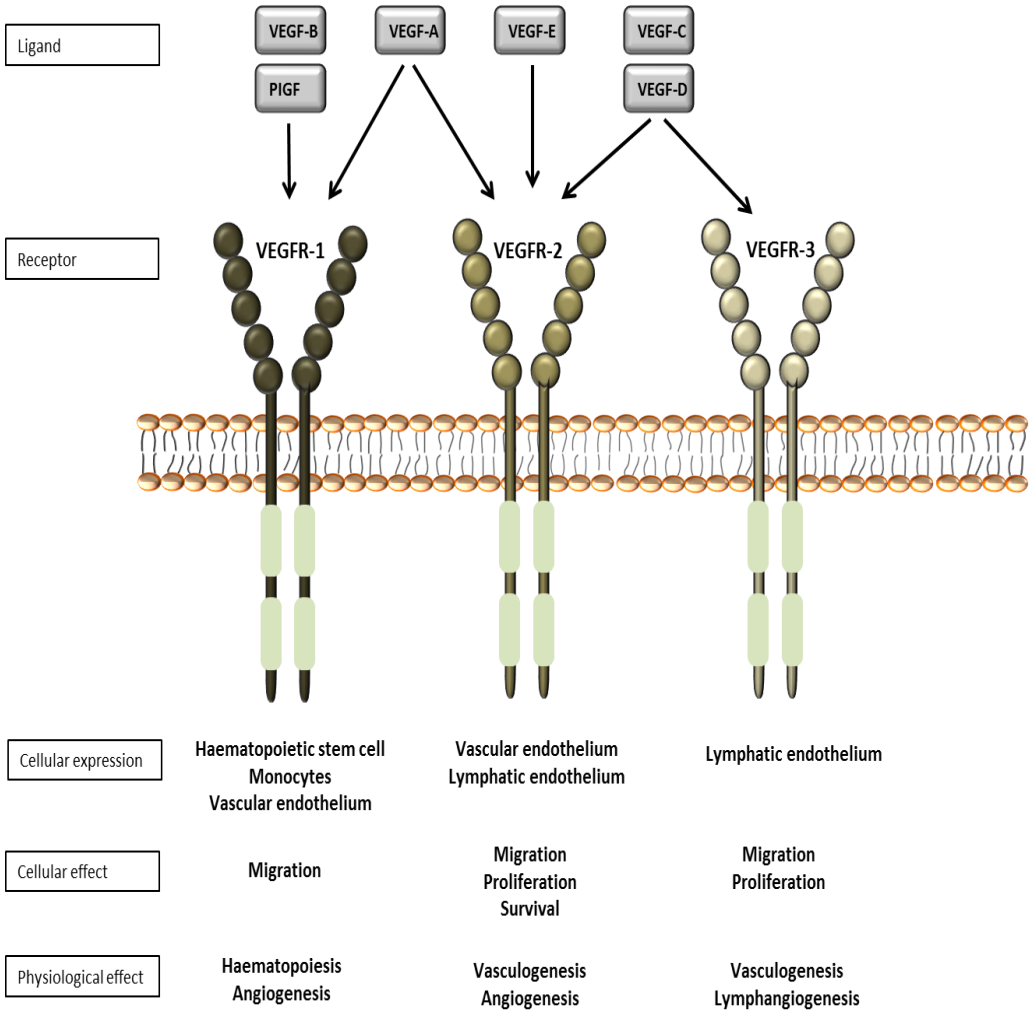


Figure 1.6 Diagram of VEGF family and VEGFRs. Each VEGF ligand binds to specific VEGFRs. PLGF and VEGF-B bind to VEGFR-1 while VEGF-A binds to both VEGFR-1 and VEGFR-2. VEGF-C and –D bind to VEGFR-3 and VEGFR-2 whereas VEGF-E only binds to VEGFR-2. VEGFR-1 is expressed on haematopoietic stem cells and vascular endothelium. VEGFR-2 is expressed on both vascular and lymphatic endothelium whereas VEGFR-3 is only expressed on lymphatic endothelium. The downstream effects of VEGFR-2 activation involve cell proliferation, migration and survival resulting in vasculogenesis and angiogenesis. Diagram adapted from (Holmes et al., 2007).

1.2.5.1 VEGFR-1

VEGFR-1, also known Fms-like tyrosine kinase 1 (Flt1), is expressed on haematopoietic stem cells, monocytes and vascular endothelial cells and can bind to VEGF-A, VEGF-B and PLGF. A splice variant of VEGFR-1 which

lacks the transmembrane and internal signalling domains of VEGFR-1 is called soluble VEGFR-1 (sVEGFR-1). sVEGFR-1 acts as trap receptor to bind VEGF and negatively regulate VEGFR-2 signalling (Kendall and Thomas, 1993, Roberts et al., 2004). Knockout of the *vegfr-1* gene in mice resulted in death at E8.5- E9.0 due to overproduction of haemangioblasts leading to disorganised vasculature (Fong et al., 1995). It has been demonstrated in endothelial cells that the extracellular domain of VEGFR-1 and transmembrane domain were essential for normal development while downstream signalling of the VEGFR-1 c-terminal domain was disposable for normal vascular development (Hiratsuka et al., 2005).

1.2.5.2 VEGFR-2

VEGFR-2 is considered to be the main VEGF receptor expressed on vascular endothelial cells and plays a major role in transducing the intracellular signalling cascade leading to physiological and pathological angiogenesis (Waltenberger et al., 1994). VEGFR-2 is involved in vascular development as knockout of *vegfr-2* gene in mice results in embryonic lethality between E8.5-9.5 due to defects of development of endothelial cells and lack of blood vessels (Shalaby et al., 1995). VEGFR-2 is also named in humans as kinase-insert domain receptor (KDR) and foetal liver kinase (Flk)-1 in mice. Although Flk-1 is 2 amino acids shorter than KDR, it shares about 85% sequence homology with KDR (Holmes et al., 2007). In early embryogenesis, VEGFR-2 is widely expressed in vascular endothelial progenitors; however the expression is low in quiescent ECs in adults.

1.2.5.3 VEGFR-3

VEGFR-3 is expressed in all endothelial cells during development, and this expression is restricted in lymphatic endothelial cells in adults (Olsson et al., 2006, Lohela et al., 2009). Ablation of *Flt-4* in mice led to fluid accumulation in pericardial cavity and cardiovascular failure which led to embryonic lethality at E9.0 prior the beginning of lymphangiogenesis (Dumont et al.,

1998). VEGFR-3 (Flt-4) only binds to VEGF-C and VEGF-D ligands and the ablation of *vegfc* gene leads to complete absence of lymph vessels in mouse embryos demonstrating a pivotal role of *vegfc* in lymphangiogenesis (Karkkainen et al., 2002, Karkkainen et al., 2004).

1.2.6 VEGFs/VEGFRs and tumour angiogenesis

As previously mentioned, tumour growth and metastasis events depend on angiogenesis. VEGF plays an important role in tumour angiogenesis such as breast cancer, ovarian cancer, prostate cancer and lung cancer and as a result, tumour angiogenesis has become a novel therapeutic target for cancer (Yoshiji et al., 1996, Santin et al., 1999, Grivas et al., 2016, Herbst et al., 2005). The VEGF neutralizing monoclonal antibody, bevacizumab, was the first anti-angiogenic drug approved for treatment of colorectal cancer (Willett et al., 2004).

The expression of VEGF-B and PLGF has a role in tumour angiogenesis via VEGFR-1. Highly activation of PIGF has been shown in non-small cell lung cancer cells while increased VEGF-B expression is associated with oral squamous cancer cells (Zhang et al., 2005, Hanahan et al., 2003). As the lymphatic vasculature provides another path to tumour metastasis, VEGF-C, VEGF-D and their receptor VEGFR-3 are associated with tumour lymphangiogenesis and metastatic cancer (Stacker et al., 2002). Furthermore, VEGFR-2 can drive tumour angiogenesis in response to VEGF-C and VEGF-D binding (Zachary and Gliki, 2001). VEGF-A is activated in both normal and malignant cells in response to hypoxia which is a key mediator for hypoxic responses (Semenza, 2003). As a result of triggering VEGF-A gene expression via hypoxia, VEGF-A gene transcription is increased through mediation of hypoxia inducible factor-1 (HIF-1) (Forsythe et al., 1996).

1.3 Mitogen-activated protein kinases signalling pathway

Mitogen activated protein kinases (MAPKs) are a highly conserved family of signalling proteins that mediate a multitude of intracellular processes such as gene transcription, differentiation, migration, proliferation and apoptosis. MAPKs are combined with a broad array of stimuli including growth factors, environmental stress, hormones and cytokines (Kyriakis and Avruch, 2012). Furthermore, MAPKs are the last terminal member of a three tiered hierarchical kinase cascade and they are activated by this canonical kinase cascade. Extracellular stimuli activate MAPK kinase kinase (MAPKKK) (also recognized as MAP3K, ERK kinase kinase/MEKK) which in turn phosphorylates its downstream object, MAPK kinase (MAPKK) (also known MAP2K, ERK kinase/MEK) on specific serine and threonine residues. Consequently, MAPKK induces the activation of MAPK by phosphorylating the threonine and tyrosine residues in a conserved T-X-Y motif which is located on the activation loop of MAPK (Zhang and Dong, 2007). Downstream effector proteins of MAPK generate a specific intracellular response in response to activation and phosphorylation of MAPK by a sequential cascade. These specific intracellular proteins involve other protein kinases, transcription factors, cytoplasmic enzymes and phospholipases (Yoon and Seger, 2006). MAPKK can be activated by more than one MAPKKK and that explains the complexity and diversity of MAPK (Chang and Karin, 2001). The scaffold proteins and specific docking interaction between individual components of each MAPK cascade maintain the specificity of signal transfer to MAPK which results in required cellular response via efficient signal transfer (Tanoue and Nishida, 2003, Raman et al., 2007). Dual specificity protein phosphatases (DUSPs) also known as MAP kinase phosphatases (MKPs) maintain the phosphorylation of MAPK via inducing de-phosphorylation of threonine and tyrosine in the activation loop of MAPKs to inactivate them (Keyse, 2008).

1.3.1 Classification of MAPKs

The MAPK pathway consists of two subdivisions; conventional and atypical pathways. The conventional mammalian MAPK pathway divides into four distinct pathways; extracellular signal-regulated kinases (ERK) 1/2, c-Jun NH 2 -terminal kinases (JNK) 1/2/3, p38 proteins α/β/γ/δ and ERK5 (Payne et al., 1991, Cuenda and Rousseau, 2007, Nakamura and Johnson, 2007, Zhou et al., 1995). The kinase domain of MAPKs is activated by dual phosphorylation of threonine and tyrosine residues within a T-X-Y motif in the activation loop. ERK1/2 contains the activation motif threonine-glutamic acid-tyrosine (T-E-Y) whereas JNK contains a dual phosphorylation motif threonine-proline-tyrosine (T-P-Y) and p38 MAPK contains a threonine-glycine-tyrosine (T-G-Y) activation motif (Widmann et al., 1999). Finally, ERK5 which is the most recently identified MAPK cascade and is similar to ERK1/2 as it contains a T-E-Y dual phosphorylation motif but is larger than ERK1/2 and has a unique structure as it has a carboxyl-terminal (Nishimoto and Nishida, 2006) (Figure 1.7).

The atypical MAPK pathways are not organised in the classical three tiered kinase cascades, divided into four subdivisions; ERK3, ERK4, ERK7/8 and Nemo like kinase (NLK) (Cargnello and Roux, 2011b). The atypical MAPKs are also distinct from the conventional MAPKs as they do not contain the T-X-Y dual phosphorylation motif in ERK3, ERK4 and NLK, while ERK7/8 has a T-G-Y activation motif but these residues are not phosphorylated by an upstream MAPKK as it is in the conventional (Cargnello and Roux, 2011b). The conventional and atypical MAPK pathways both phosphorylate other protein kinases called mitogen activated protein kinase-activated protein kinase (MAPKAPKs) which consist of 11 serine and threonine kinases and contain five groups; ribosomal S6 kinases (RSKs), mitogen and stress activated kinases (MSKs), MAPK-interacting kinases (MNKs), MAPK

activated protein kinases 2/3 (MK2/3) and MAPK activated protein kinase 5 (MK5) (Cargnello and Roux, 2011b). Although the MAPKs are widely expressed in all mammalian cells, due to the differential expression in transcription factors in distinct cell types the function for each MAPK can be different between organisms and also between cell types in the same organism (Donovan et al., 2001).

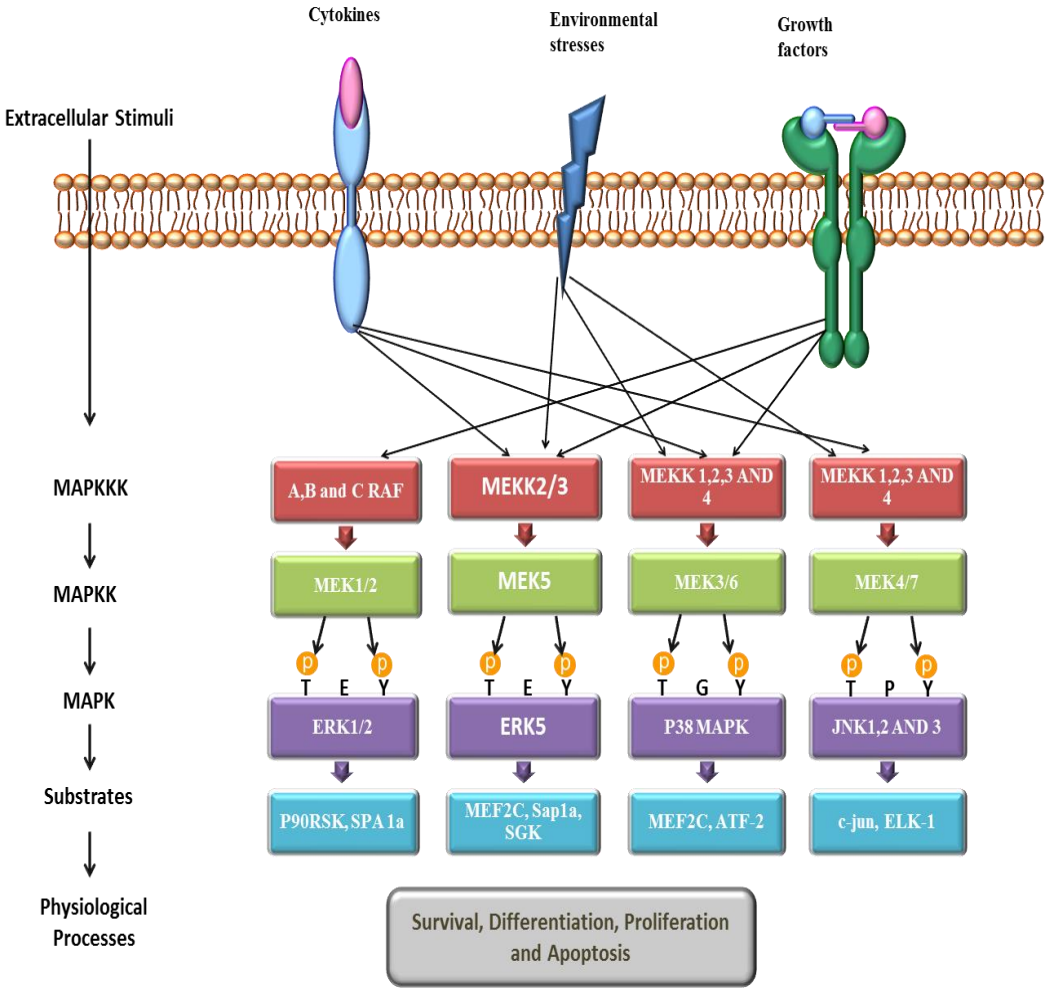


Figure 1.7 Mitogen activated protein kinase (MAPK) signalling pathways in mammalian cells. There are four distinct MAPK cascades for mammalian cells termed; ERK1/2, p38 MAPK (p38α, β, γ and δ), JNK 1/2/3 and ERK5. These cascades are arranged into a hierarchal three tier system. Mitogens such as growth factors, cytokines and environmental stress and extracellular stimuli, stimulate the activation of MAPK cascade by acting on specific receptors. Distinct extracellular stimuli phosphorylate one or more MAPKKK which sequentially activates MAPKK. The activated MAPKK phosphorylates MAPKs on T-X-Y motif in the activation loop of MAPKs. X corresponds to a glutamic acid in ERK1/2, a glycine in p38 MAPKs, a proline in JNKs and a glutamic acid as well in ERK5. Phosphorylated MAPKs activate multiple downstream cytosolic or nuclear substrates including P90RSK and SGK which are protein kinases and MEF2 and Sap1a which are transcription factors. Activated substrates lead to specific cellular responses such as cell survival, differentiation, proliferation and apoptosis. Diagram adapted from (Nithianandarajah-Jones et al., 2012b).

1.3.2 Extracellular-signal-regulated kinase 1/2 (ERK1/2)

ERK1/2 was the first mammalian protein kinase of MAPKs to be discovered and since then it has been extensively studied as the best characterised MAPK (Sturgill and Ray, 1986, Rubinfeld and Seger, 2005). ERK1 and ERK2 are individual kinases so ERK1 in humans has 379 amino acids whereas ERK2 consists of 360 amino acids, however they are usually referred to as ERK1/2 because they share about 84% sequence identity and most of the same signalling activity (Boulton et al., 1991). ERK1/2 is activated by numerous stimuli including growth factors such as PDGF, EGF and NRG in response to insulin and also they are activated by ligands for heterodimeric G protein coupled receptors, osmotic stress and cytokines (Raman et al., 2007). Receptor tyrosine kinases (RTKs) initiate the activation of ERK1/2 where the RAF kinases which are the primary MAPKKKs in the ERK1/2 module phosphorylate the dual specificity kinases MEK1/2 (MAPKK). In turn, phosphorylation of ERK1/2 occurs within T-E-Y in the activation loop which allows ERK1/2 to activate its downstream substrates (Cargnello and Roux, 2011b). ERK1/2 has an important role in various pathologies such as cardiovascular diseases, cancers and diabetes as it is widely expressed and regulates various cellular and physiological events (Rose et al., 2010).

Although ERK1 and ERK2 are approximately similar in amino acid sequence, they are distinctly different in expression pattern and biological functions during development when phenotypic studies in mice knock out the respective components of MEK/ERK cascade. When ERK2 is deleted, mice die early in development between E10.5 and E11.5 while in case of deleting ERK1, mice are viable and develop normally, suggesting that ERK1 cannot compensate for lack of ERK2 activity (Pages et al., 1999, Yao et al., 2003). Furthermore, deletion of both ERK1 and ERK2 in developing mice in endothelial cells prompted embryonic lethality at E10.5 as the angiogenesis became defective (Srinivasan et al., 2009).

1.3.3 p38 mitogen activated protein kinase

The p38 MAPK family consists of four isoforms in mammalian cells; p38α (SAPK2a), p38β (stress activated protein kinase 2b, SAPK2b), p38γ (SAPK3) and p38δ (SAPK4). p38α is ubiquitously expressed in most cell types at a significant level while other isoforms are expressed in specific tissues. p38β is expressed in the brain, p38γ is expressed in skeletal and cardiac muscle and p38δ is expressed in endocrine glands (Cuadrado and Nebreda, 2010). Furthermore, p38β shares about 73% identities with p38α whereas p38γ and p38δ are 70% identical with p38α (Li et al., 1996, Denise Martin et al., 2012). The p38 MAPK cascade is activated by different environmental and cellular stress factors and inflammatory cytokines such as UV radiation, oxidative stress, hypoxia, chemical shock, interleukin 1 (IL-1) and tumour necrosis factor alpha (TNF-α) (Cuadrado and Nebreda, 2010).The p38 MAPK is important in the regulation of many biological functions including differentiation, cell migration, apoptosis and cell survival (Cargnello and Roux, 2011a).

MKK3 and MKK6 activate and phosphorylate p38 MAPKs in the dual phosphorylation Thr-Gly-Tyr (T-G-Y) motif of the activation loop. MKK4 can also phosphorylate p38 (Cuenda et al., 1995). The p38 family is present in the cytosol and nucleus. Once the p38s are activated, they phosphorylate their various substrates. The substrates in the cytoplasm as targets for p38s include cytosolic phospholipase A2 (c-PLA2), MAPK interacting serine/threonine kinase 1 (MNK 1/2), MK2, MK3, MK5, Bcl-2 and Bax. The nuclear targets of p38s comprise activation transcription factor-1 -2 -3 (ATF-1/2/3), ETS domain containing protein like transcription factor (Elk-1), MEF-2, GADD153, p53 and MSK1/2 (Cargnello and Roux, 2011b, Denise Martin et al., 2012).

The functional redundancy of the p38 MAPK family has been demonstrated (Sabio et al., 2005). However, genetic ablation of p38α in mice leads to

embryonic lethality at E11.5 due to angiogenesis defects in the placenta while deletion of p38β, p38γ and p38δ MAPK isoforms did not result in any phenotypic abnormalities in mice which suggest that p38α is important for normal embryonic development and has a role in vascular development (Mudgett et al., 2000).

1.3.4 C-Jun-N-terminal kinases (JNKs)

In the early 1990s, the first c-Jun-N-terminal kinase was identified as a cycloheximide activated microtubule associated protein-2 (MAP-2) kinase (Kyriakis and Avruch, 1990). Three isoforms of JNK have been discovered, termed; JNK1 (SAPKβ), JNK2 (SAPKα) and JNK3 (SAPKγ), also named MAPK8, MAPK9 and MAPK10 respectively (Cargnello and Roux, 2011b, Kyriakis and Avruch, 2012). The JNKs family are over 85% identical in sequence and are encoded by 3 different genes which lead to more than 10 spliced variants (Gupta et al., 1996). JNK3 is expressed only in neuronal, testis and cardiomyocytes while JNK1 and JNK2 are ubiquitously expressed. JNK1 is the major c-Jun kinase after stimulation in response to environmental stresses, inflammatory cytokines and growth factors while JNK2 plays a role in c-Jun degradation after binding to c-Jun in unstimulated cells, suggesting that they have functional differences (Raman et al., 2007). The JNK family has been implicated in various biological processes including cell survival, cell proliferation, apoptosis and differentiation and has a physiological role in immune responses and inflammation (Nakamura and Johnson, 2007). Besides the physiological role, JNKs have pathological roles in arthritis, neurological disorders, liver diseases, cardiac diseases and cancer (Rose et al., 2010).

The JNKs are activated by various stress stimuli such as heat shock, ionizing radiation, oxidants, UV radiation and to a lesser extent are activated in response to growth factors (Kyriakis and Avruch, 2012, Bode and Dong, 2007). MKK4 and MKK7 phosphorylate JNKs by dual phosphorylation of Thr and Tyr residues within Thr-Pro-Tyr motif in the activation loop.

Furthermore, JNKs are localized from the cytoplasm to the nucleus where they interact with different transcriptional factors such as c-Jun, ATF-2, p53, Elk-1 and activator protein-1 (AP-1) (Rose et al., 2010). The JNKs have a critical role in an intrinsic apoptosis pathway; deletion of both *JNK1* and *JNK2* from mice resulted in a defect in the mitochondrial death signal pathway and failure to release cytochrome c (Tournier et al., 2000). However, genetic ablation of *JNK1*, *JNK2* or *JNK3* individually in mice has been shown that mice are still viable and develop normally (Dong et al., 1998).

1.4 Extracellular signal regulated kinase 5 (ERK5)

Extracellular regulated signal protein kinase 5 (ERK5) is the most recently identified member of the MAPK family. ERK5 is the largest member of the MAPK family and is twice as large as other members. The specific function of ERK5 is not clear, despite studies showing that ERK5-deficient mice die at around E10.5 because of cardiovascular defects and angiogenesis failure in embryonic and extra-embryonic tissue (Hayashi et al., 2004a).

1.4.1 Identification of ERK5

Two research groups originally cloned ERK5 (Zhou et al., 1995, Lee et al., 1995). Dixon and co-workers first identified MEK5, the catalyst of ERK5; they isolated MEK5 cDNA and found around 40% identity to known MEKs. Subsequently, they identified ERK5 as a binding partner by specific interactions with MEK5 in a yeast two-hybrid assay (Zhou et al., 1995). In a separate study, Lee et al. used a PCR assay of placental cDNA and identified the complete cDNA of a new human MAPK termed Big MAP kinase (BMK1). This was found to be different to ERK1 and ERK2 due to its unique C-terminal structure. Both groups stated that BMK1 and ERK5 were derived from the same gene, which ultimately meant that both were the same protein (Lee et al., 1995). In mammals, ERK5 is expressed in various tissues and is

abundant in heart, skeletal muscle, placenta, lung and kidney (Zhou et al., 1995, Lee et al., 1995).

1.4.2 Structure of ERK5

The human ERK5 gene (MAPK7) is located on chromosome 17p11.2, spanning 5.6 kb, with an open reading frame of 2445 base pairs; the gene encodes 816 amino acids with a molecular mass of approximately 98 KDa (Figure 1.8) (Yan et al., 2001). ERK5 has an N-terminal domain that consists of two regions: cytoplasmic targeting (amino acids 1-77) and a kinase domain (amino acids 78-406). The N-terminal kinase domain shows the highest homology to ERK1 and ERK2, with a sequence identity of about 60%. It is important for upstream interactions with the kinase MEK5 (amino acids 78-139) and for oligomerisation (amino acids 140-406) and also includes the MEK5 dual phosphorylation sites (T^{218} and Y^{220}) (Yan et al., 2001). Adjacent to the kinase domain, ERK5 has a common docking (CD) domain which involved in the interaction of ERK5 with its substrates (Tanoue and Nishida, 2003). In addition, ERK5 has a unique C-terminal domain in comparison with other MAPKs (400 amino acids) which gives it a relatively large size, more than twice that of conventional MAPKs (Buschbeck and Ullrich, 2005). The C-terminal tail contains nuclear localisation signals (NLS) which are key for nuclear import; it also has a nuclear export signal (NES) which plays a significant role in the cytoplasmic retention of ERK5 (Kondoh et al., 2006). The N-terminal tail of ERK5 binds to the C-terminal half to retain ERK5 in the cytoplasm and generate NES (close conformation). Dual phosphorylation of Thr^{218} and Tyr^{220} by MEK5 in the N-terminal domain causes the interruption of this binding and exposes NLS in the C terminal domain of ERK5 to import ERK5 into the nucleus (open conformation) (Kondoh et al., 2006) (Figure 1.9). Furthermore, the C-terminal portion of ERK5 contains a Myocyte enhancer factor 2 (MEF2) interacting region (amino acids 440-501), which is regulated by an effective transcriptional activation domain (amino acids 664-789) via auto-phosphorylation (Kasler et al., 2000). Moreover, the

C-terminal truncation of ERK5 results in an increase in the kinase activity of ERK5, suggesting that the C-terminal half of ERK5 has an auto-inhibitory capacity (Buschbeck and Ullrich, 2005). The C-terminal portion of ERK5 also has two proline-rich domains, called PR1 (amino acids 434-465) and PR2 (amino acids 578-701), which play an important role as binding sites for Src3 homology 3 (SH3) domain containing proteins (Borges et al., 2007)(Figure 1.8).

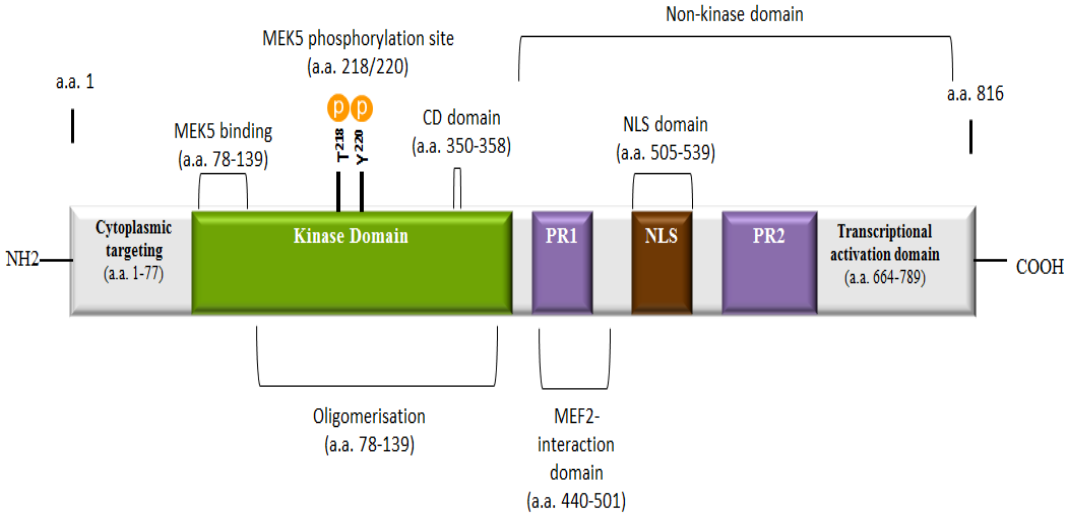


Figure 1.8 Structure of ERK5. ERK5 protein divided in to N-terminal domain and large C-terminal domain. The N-terminal kinase domain contains cytoplasmic region and MEK5 binding region in the kinase domain and oligomerisation site. ERK5 is phosphorylated via MEK5 on residues Thr218 and Tyr220 which are located in the kinase domain. C-terminal contains two prolin-rich domains (PR1 and PR2), nuclear localization signal and transcriptional activation domain. Diagram adapted from (Nithianandarajah-Jones et al., 2012b).

1.4.3 Activation of ERK5

ERK5 is activated by serum and a range of growth factors such as VEGF, EGF, FGF-1 and PDGF and also by certain inflammatory cytokines like IL-6 beside the osmotic and oxidative stresses that were originally identified as stimulators of ERK5 (Hayashi and Lee, 2004, Kato et al., 1998b, Abe et al., 1996). Furthermore, trophic factors in neurons such as nerve growth factor

(NRG) and brain derived neurotrophic factor (BDNF) are also known to activate ERK5 (Finegan et al., 2009, Cavanaugh et al., 2001). Laminar shear stress, hypoxia and ischaemia as physiological and pathological stresses respectively, have the ability to phosphorylate ERK5 (Yan et al., 1999, Sohn et al., 2002).

MEKK2 and MEKK3 are the two best characterised MAPKKKs that are activated by extracellular stimuli which in turn phosphorylate the MEK5/ERK5 signalling cascade by phosphorylating Ser^{313} and The^{317} of MEK5 (Chao et al., 1999, Sun et al., 2001). Hetero-dimerization adjusts the specificity of signal transduction between MEKK2/MEKK3 and MEK5 by phox and Bem1p (PB1) domains within the N-terminal of MEK5 and MEKK2/MEKK3 (Nakamura and Johnson, 2003). MEKK2 and MEKK3 share 94% sequence identity and also contain PB1 domains in their N-terminal tail (Blank et al., 1996). Furthermore, they are abundantly divergent in their N-terminal region to support the differential regulation of ERK5 signalling pathway. Based on this, MEKK2 binds to the adaptor Src homology 2 (SH2) domain containing scaffold protein Lck associated adaptor (Lad) which establishes a mechanism by which EGF activates ERK5 by Src and also enhances ERK5 activation via complexes with MEK5 (Sun et al., 2003). MEKK2 has a great affinity to MEK5 compared with MEKK3 and triggers the phosphorylation of ERK5 to a greater extent than MEKK3, demonstrating that MEKK2 is a more powerful activator of ERK5 than MEKK3 (Sun et al., 2001). Once MEK5 is activated by MEKK2 and MEKK3, it phosphorylates the Thr^{218} and Tyr^{220} residues within the T-E-Y dual phosphorylation motif in the activation loop of the ERK5 kinase domain (Mody et al., 2003). MEK5 is the only upstream MAPKK that activates ERK5 directly. The phosphorylation of ERK5 on Thr^{218} via MEK5 is not enough for full catalytic activity, thus phosphorylation of both Thr^{218} and Tyr^{220} is required for full activation of ERK5 (Mody et al., 2003). The activation of ERK5 is followed by phosphorylation of MEK5 and the auto-

phosphorylation of various residues in the C-terminal of ERK5 resulting in increased transcriptional activity of ERK5 (Morimoto et al., 2007a).

MEK5 shares 48% identity in sequence with MEK1 and contains a Raf-1 phosphorylation motif $S^{311}XXXT^{315}$ analogous to the activation motif of MEK1 and the cascade Ras > Raf-1> MEK1/2> ERK1/2 suggesting that Ras/Raf-1 may be able to activate ERK5; there are no interactions or activation of protein in the MEK1/ERK1 cascade and MEK5/ERK5 cascades (Zhou et al., 1995, English et al., 1998). The small molecular weight GTPase protein Ras has been implicated in the regulation of ERK5 by EGF manifested by the ability of a dominant negative (DN) mode of Ras to inhibit ERK5 activation in response to EGF (Kamakura et al., 1999). However, the dominant active (DA) form of Ras induces the phosphorylation of ERK5 in human embryonic kidney 293 (HEK293) cells (English et al., 1998). The phosphorylation of ERK5 in HeLa cells in response to EGF is independent of Ras, suggesting that, Ras mediated activation of ERK5 is likely dependent on the cell type (Kato et al., 1998b, Kamakura et al., 1999) (Figure 1.9).

1.4.4 Inactivation of ERK5 cascade

A MAP Kinase phosphatase (MKP) subfamily of dual specificity phosphatases (DUSPs) dephosphorylates the T-X-Y motif of MAPKs to inactivate them (Dickinson and Keyse, 2006). However, the DUSP that dephosphorylates Thr^{218} and Tyr^{220} residues within the T-E-Y activation motif of ERK5 has not been identified. On the other hand, it has been stated that phosphor-tyrosine specific phosphatases PTP-SL (protein tyrosine phosphatase STEP-like) may able to dephosphorylate ERK5 at Tyr^{220} residue which interrupts its translocation to the nucleus (Buschbeck and Ullrich, 2005). ERK5 can phosphorylate PTP-SL but this action compared to the increased phosphatase activity of PTP-SL binding to ERK5 is considered to be a minor interaction (Buschbeck and Ullrich, 2005). Moreover, post-translational modifications have the ability to regulate the activation of

ERK5. A small ubiquitin-like modifier 3 (SUMO3) protein covalently binds to ERK5 on Lys_6 and Ly_{22} following exposure to advanced glycation end-stage products (AGE) or H2O2 in HUVEC (Woo et al., 2008). Consequently, SUMOylation of ERK5 inhibits the transcriptional activity induced via shear stress without affecting ERK5 phosphorylation in HUVEC (Woo et al., 2008) (Figure 1.9).

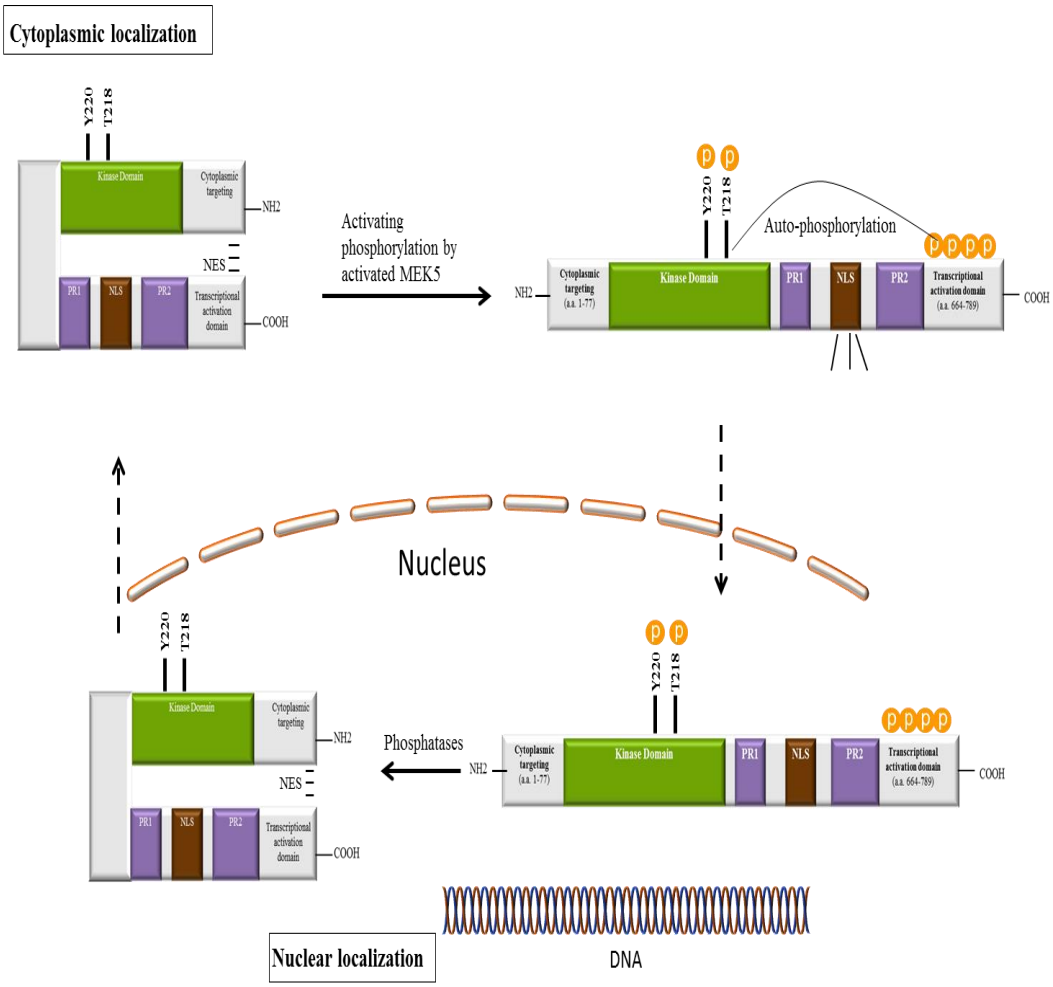


Figure 1.9 ERK5 intracellular localization. Under the basal condition (un-phosphorylated state) the N and C-terminal of ERK5 interact with each other leading to masked NLS and release of NES to export ERK5 from the nucleus. Upon phosphorylation of ERK5 by MEK5 in T-E-Y motif in the activation loop, the conformation is changed which results in the disruption of NES and unmasking NLS to trigger the translocation of ERK5 into the nucleus. Diagram adapted from (Nishimoto and Nishida, 2006).

1.4.5 Substrates of ERK5

Regulation of downstream transcription factors is considered the most important role of ERK5. Relatively few physiological substrates of ERK5 have been identified; myocyte enhancer factor 2A, C, D (MEF2A, MEF2C

and MEF2D) are the best characterised ERK5 substrates (Kato et al., 1997). Once activated, ERK5 phosphorylates MEF2C on serine 387 which enhances the transcriptional activity of MEF2C and then increases c-Jun gene expression (Kato et al., 1997). MEF2A and MEF2C are regulated by both ERK5 and p38 MAPK, while MEF2D is a specific substrate for ERK5 (Ornatsky et al., 1999, Kato et al., 2000). The MEF2 interacting region and a transcriptional activation domain in ERK5's C-terminal region play an important role in the regulation of MEF2 activity (Kasler et al., 2000). The stimulation of MEF2 activity by ERK5 is affected by truncation of the C-terminal tail of ERK5 mutant (Yan et al., 2001).

Other substrate targets of ERK5 that are well-characterised include the MEF2 family; serum response factor accessory protein 1a (Sap1a), c-Myc and pro-apoptotic protein Bcl2 antagonist of cell death (Bad) (English et al., 1998, Kamakura et al., 1999, Pi et al., 2004). Like MEF2 substrates, ERK5 activates Sap1a and subsequently improves transcriptional activity (Kamakura et al., 1999). However, unlike MEF2 and Sap1a, ERK5 phosphorylates c-Myc at serine 62 and increases the stability of c-Myc protein (Sears et al., 2000). Furthermore, ERK5 activation results in the phosphorylation of c-Fos promotor on serine 387 and alternative sites in its C-terminal tail (Terasawa et al., 2003). ERK5 and all other MAPK members have the ability to phosphorylate substrates on Ser/Thr residues that precede a proline residue (Mody et al., 2003). However, residues Thr^{28} in the N-terminal tail of ERK5 and residues Ser^{421}, Ser^{433}, Ser^{496}, Ser^{731} and Thr^{733} in the C-terminal region of ERK5 undergo auto-phosphorylation of ERK5 but are not followed by proline residues, suggesting that the specificity of ERK5 may differ from other MAPK family members (Mody et al., 2003)

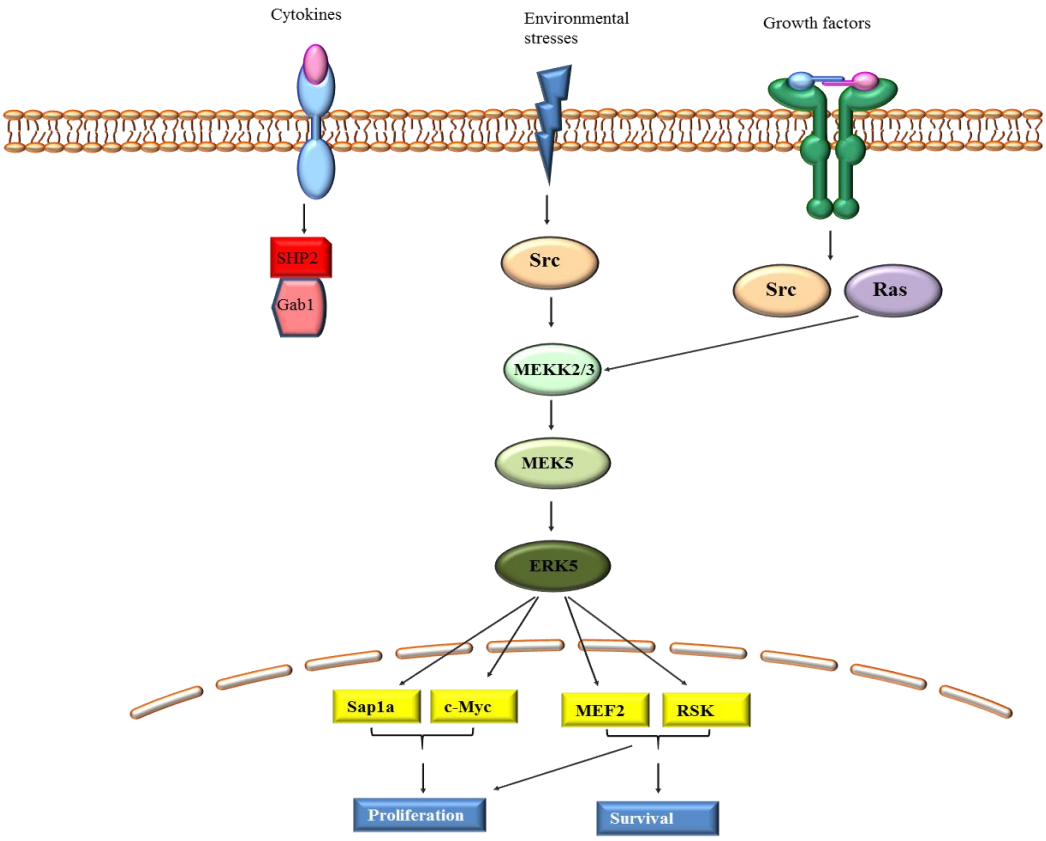


Figure 1.10 Schematic representation of ERK5 signalling pathway. Cytokines, growth factors and environmental stresses are the main stimuli that activate the signalling cascade; MEKK2/MEKK3 phosphorylates MEK5 which in turn activates ERK5. The activated ERK5 phosphorylates several downstream targets to regulate cell survival and proliferation. Diagram adapted from (Wang and Tournier, 2006).

1.4.6 Cellular physiology of ERK5

ERK5 is ubiquitously expressed in many cell types; however, some studies propose that ERK5 has particular roles in specific cell types (Regan et al., 2002b, Hayashi et al., 2004a). ERK5 has been involved in the regulation of various cellular functions such as cell survival, cell adhesion and cell proliferation (Kato et al., 1998a). In addition, ERK5 has been implicated in progression of some diseases for example cancer, cardiac hypertrophy and ischaemia (Montero et al., 2009a).

1.4.6.1 ERK5 and cell survival

ERK5 plays a role in the activation of the pro-apoptotic Bcl-2 interacting mediator of cell death (Bim), Bcl-2 homology (BH3)-only member of the Bcl-2 family (Girio et al., 2007). When ERK5 is inactivated, Bim becomes de-phosphorylated and segregated in the mitochondria which subsequently interacts with Bax (Bcl-2 associated X protein) to induce apoptosis pathway through mitochondria, resulting in caspase activation and cell death (Girio et al., 2007). During mitosis in HeLa cells, the phosphorylation and de-phosphorylation of Bim is mediated by EGF stimulation of ERK5 activation (Girio et al., 2007) These findings suggest that ERK5 may able to regulate other Bcl-2 family proteins to promote cell survival.

Many studies have shown that ERK5 plays a pivotal role in neuronal cell survival in response to various neurotrophic pro-survival stimuli (Wang and Tournier, 2006).Watson and colleagues revealed that ERK5 in rat dorsal root ganglia (DRG) mediates the activation of the transcriptional factor cAMP responsive element binding protein (CREB) in response to neurotrophins BDNF and NGF to help neuronal cell survival (Watson et al., 2001). ERK5 mediates the protective effect of BDNF in newly-generated cerebellar granule neurones giving rise to MEF2 dependent transcription of the neurotrophin 3 (NT3) to facilitate neuronal cell survival, suggesting that MEF2 is implicated in ERK5-mediated survival in neuronal cells (Shalizi et al., 2003).

1.4.6.2 ERK5 and cell proliferation

Various studies have shown that ERK5 is implicated in the regulation of cell proliferation in certain cell types. The activation of ERK5 mediates the proliferation of MCF10A cells in response to EGF stimulation (Kato et al., 1998a). Hayashi and co-workers demonstrated that the activation of serine/threonine kinase SGK to S phase entry required ERK5 which subsequently facilitate cell proliferation following EGF stimulation in MCF10A cells (Hayashi et al., 2001).

On the other hand, the cell cycle progression and cell proliferation of CCI39 fibroblasts in response to EGF stimulation were blocked when cells were treated with MEK1/2 inhibitor PD184252 to inhibit the activation of ERK1/2 but not ERK5 activation (Squires et al., 2002). Furthermore, the cell proliferation of vascular smooth muscle cells in response to aldosterone is independent of the ERK5 pathway (Ishizawa et al., 2005). In addition, knockdown of ERK5 with shRNA in leukaemia T-cells had no effect on cell cycle progression and cell proliferation suggesting that ERK5 is not necessary for cell proliferation in response to growth factor induction in certain cell types (Garaude et al., 2006).

1.4.6.3 ERK5 and cell migration and adhesion

ERK5 mediates cell migration of renal epithelial cells in response to growth factor stimulation (Izawa et al., 2007). However, ERK5 mediates the phosphorylation of FAK to facilitate focal adhesion in hepatic stellate cells leading to shrink migration in these cells (Rovida et al., 2008). Several studies have been demonstrated that the relationship between ERK5 and FAK depends on the cell types; ERK5 seems to be an upstream activator for FAK in some cell types and downstream of FAK in other cell types (Villa-Moruzzi, 2007, Rovida et al., 2008, Sawhney et al., 2009). In breast and prostate cancer, ERK5 forms a complex with FAK to regulate cell migration and adhesion in these cancerous cells (Sawhney et al., 2009). Furthermore, overexpression of MEK5 accelerates the phosphorylation of FAK (Sawhney et al., 2009). These data suggest that ERK5 is important in the regulation of cell migration and adhesion.

1.4.7 ERK5 and endothelial cell function

1.4.7.1 Inhibition of ERK5 signalling axis

The physiological role of the ERK5 signalling pathway *in vivo* was established by using gene ablation of specific components of the pathway in mice (Hayashi et al., 2004a)(Table 1.1). ERK5 deficient-mice showed defects

in the development of the heart and maturation of vasculature and angiogenesis in embryonic tissues which lead to lethality around E10.5 (Regan et al., 2002b, Sohn et al., 2002, Hayashi et al., 2004b, Kesavan et al., 2004a). Furthermore, overexpression of VEGF in these mice led to normal development up to E12.5 with abnormalities in cardiovascular which subsequently resulted in observed lethality at E12.5-E14.5 (Miquerol et al., 2000). Studies have shown that deregulation of hypoxia inducible factor-1 α (HIF-1α) may play a role in increased VEGF expression and preventing its ubiquitination and proteolysis which consequently causes aberrant angiogenesis (Pi et al., 2005). The phenotypic defects of *erk5* gene deletion in mice include immature vasculature, disorganised and rounded endothelial cells resulting in increased vessel leakiness and loss of vascular integrity leading to embryonic death by haemorrhage (Regan et al., 2002b, Sohn et al., 2002, Yan et al., 2003). Similar phenotypic abnormalities have been shown in MEKK3, MEK5 and MEF2 knockout mice, suggesting that the ERK5 signalling pathway is significant in angiogenesis (Table 1.1) (Wang et al., 2005).

1.4.7.2 Inhibition of endothelial apoptosis

To address the initial defects caused by ERK5 gene ablation, generation of endothelial-specific ERK5 knockout mice confirmed the primary defects occurred in the endothelium which subsequently caused cardiovascular abnormalities and death around E10, similar to the global ERK5 knockout mice (Hayashi et al., 2004a). Nevertheless, specific knockout of ERK5 in hepatocytes, neuronal and cardiomyocytes cells has no effect on the development (Hayashi and Lee, 2004, Hayashi et al., 2004a) (Table 1.1). Vascular integrity in adult mice is affected by knockout of ERK5 followed by death within 2-3 weeks due to leaky blood vessels resulting from endothelial cell apoptosis (Hayashi et al., 2004a). Together, these data suggest that ERK5 is critical for endothelial cell function.

Table 1.1 Phenotypic results in mice after global ablation of specific components of ERK5 signalling pathway.

Genotype	Phenotype	Reference
Mekk2$^{-/-}$	Mice develop normally and are fertile and viable. Mice exhibit altered cytokine expression in thymocytes but develop normally and are viable. Mice are viable and develop normally, but exhibit reduced cytokine expression in embryonic stem cell-derived mast cells.	(Kesavan et al., 2004b) (Guo et al., 2002) (Garrington et al., 2000)
Mekk3$^{-/-}$	Embryonic death at E11.0 due to severe abnormalities in early angiogenesis. Vasculogenesis was not affected.	(Yang et al., 2000)
Mek5$^{-/-}$	Defects in cardiac development, decreased proliferation and increased apoptosis in heart, dorsal and head lead to embryonic lethality at E10.5	(Wang et al., 2005)
Erk5$^{-/-}$	Abnormalities in vascular maturation, angiogenesis, heart development and looping resulted in embryonic death at E9.5-E10.5 Underdevelopment of vasculature within the yolk sac, embryonic death at E9.5-E10.5 Defects angiogenesis in the embryo and placenta, embryonic death at E10.5-E11 Embryonic death at E10.5-11.5 with growth retardation in the head and impaired angiogenesis in the embryo and placenta.	(Regan et al., 2002b) (Hayashi et al., 2004a) (Yan et al., 2003) (Sohn et al., 2002)
Erk5$^{-/-}$ *Hepatocyte*	Mice develop normally and are viable	(Hayashi and Lee, 2004)
Erk5$^{-/-}$ *Cardiomyocytes*	Mice are viable and develop normally.	(Hayashi et al., 2004b)
Erk5$^{-/-}$ *Endothelial*	Cardiovascular defects lead to embryonic death at E9.5-10.5	(Hayashi and Lee, 2004)

The phenotype of *Mef2c* $^{-/-}$ is similar to that of *Erk5*$^{-/-}$ in mice leading to cardiac and vascular defects which eventually results in embryonic lethality (Hayashi and Lee, 2004, Roberts et al., 2009). Furthermore, *Erk5*$^{-/-}$ murine endothelial cells exposed to overexpression of a constitutively active form of MEF2C are partially protected from apoptosis (Hayashi et al., 2004b, Olson, 2004). Recent *in vitro* studies revealed that ERK5 is required for VEGF induced phosphorylation of AKT in HDMECs to suppress apoptosis and support cell survival in tubular morphogenesis via VEGFR-2 (Roberts et al., 2010c).

1.4.7.3 Shear-stress and atheroprotection

Fluid shear stress is important in modulating the structure and function of blood vessels and also plays a pivotal role in hemodynamic forces recognised and transduced via endothelial cells (Yan et al., 1999). The integrity of ECs is critical for maintenance of blood vessels function. Various factors can contribute to impairment of endothelial cells such as high cholesterol, smoking, hypertension and diabetes which subsequently lead to dysfunction of endothelial cells, low lamina shear stress, endothelial apoptosis and finally atherosclerosis (Traub and Berk, 1998). Laminar shear stress induces ERK5 activation in endothelial cells (Yan et al., 1999). Fluid shear stress and peroxisome proliferator activated receptor (PPARγ) have atheroprotective effects, since ERK5 is activated by shear stress; phosphorylation of PPARγ is induced via activated ERK5 in endothelial cells (Akaike et al., 2004). Furthermore, ERK5 activation via fluid shear stress has the ability to confer an atheroprotective effects by negatively regulating the inflammatory cytokines such as tumour necrosis factor-α (TNF-α) mediated activation of nuclear factor κB (NF-κB) and adhesion molecule expression including vascular cellular adhesion molecule-1 (VCAM-1) and E-selectin in endothelial cells (Akaike et al., 2004).

The atheroprotective role of activated ERK5 by steady laminar shear stress is demonstrated by inhibiting bovine lung microvascular endothelial cells (BLMECs) apoptosis by activation of Bad (Pi et al, 2005). In addition, activation of ERK5 by constitutively active-MEK5 (CA-MEK5) in these cells increased the cell viability and reduced the apoptotic cells, while inhibiting the activation of ERK5 via overexpression of dominant negative ERK5 (DN-ERK5) increased cell apoptosis and reduced the protective effect (Pi et al., 2005). Using a novel MEK5 inhibitor, BIX02188, showed that in BLMECs, the MEK5/ERK5 pathway mediates flow dependent blocking of TNF-α signalling (Li et al., 2008). Studies of transcriptional profiles in human umbilical vein endothelial cells (HUVECs) identified Kruppel like factor 2 (KLF2) which is induced by laminar shear stress (SenBanerjee et al., 2004). KLF2 has been involved in negatively regulating inflammation and angiogenesis which improved vascular stability (Dekker et al., 2002, SenBanerjee et al., 2004). Moreover, activation of ERK5 is required for flow induced expression of KLF2 in HUVECs (Parmar et al., 2006). Beside the activation of KLF2, phosphorylation of KLF4-dependent gene expression has been shown to be provoked by ERK5 and this complex MEK5/ERK5/KLF4 plays an important role in flow-dependent protective functions in endothelial cells (Ohnesorge et al., 2010). ERK5 activation increased transcriptional activity of Nrf2 (Kim et al., 2012). In contrast, depletion of ERK5 by siRNA or chemical compounds such as the MEK5 inhibitor, BIX 01289, inhibit laminar flow-induced the activation of Nrf2 dependent gene expression (Kim et al., 2012). More recently, it has been shown that the transcriptional activity of ERK5 in HUVECs is inhibited via MAP-activated protein kinase-1 (MAPAPK-1 also known p90RSK) which in turn induces the inflammatory response in the endothelial cells and promotes vascular dysfunction (Le et al., 2013).

1.4.7.4 Hypoxic responses

The levels of hypoxia inducible transcription factor HIF-1α in BLMECs is regulated via ERK5 by promoting HIF-1α ubiquitination and proteolysis resulting in a reduction in hypoxia induced gene expression of VEGF (Pi et al., 2005). Hypoxic conditions induce ERK5 activation, which in turn negatively regulate VEGF expression in murine fibroblasts (Sohn et al., 2002). In addition, it has been shown that increased VEGF production leads to recognised angiogenic defects in ERK5 $^{-/-}$ embryo (Sohn et al., 2002). Analysis of gene expression profiles found that several hypoxic responsive genes were activated in the absence of ERK5, Sohn and co-workers suggest that the absence of ERK5 plays a repressor role increasing the hypoxic inducible responsiveness of some genes and also increases expression of genes that are normally unresponsive to hypoxia (Sohn et al., 2005).

1.5 ERK5 and cancer

Cancer is the cellular progression from normal homeostasis to neoplastic status with acquisition of a number of hallmark traits; evading suppressors, enabling replicative immortality, resisting cell death, sustained proliferative signalling, activating invasion and metastasis, inducing angiogenesis, reprogramming energy metabolism and evading immune destruction (Hanahan and Weinberg, 2011). Activation of the MEK5/ERK5 pathway is implicated in cancer development and disease progression through its role in angiogenesis and blood vessel homeostasis (Lochhead et al., 2012). Increased attention to the potential role of ERK5 in cancer has resulted from the discovery that the inhibitors used to demonstrate a potential role for ERK1/2 in cancer also targeted ERK5 (Kamakura et al., 1999, Mody et al., 2001a). The activation of ERK5 is implicated in proliferation of the cervical cancer cell line HeLa and non-tumourigenic breast epithelial cell line MCF10A in response to EGF sttimulation (Kato et al., 1998b). In neuroblastoma, ERK5 is

phosphorylated by anaplastic lymphoma kinase (ALK), a molecular target in neuroblastoma, which induced the transcription factor MYCN and induced cell proliferation, suggesting that ERK5 with ALK are important targets in neuroblastoma (Umapathy et al., 2014).

In addition to EGF, various oncogenes have the ability to phosphorylate ERK5, suggesting that ERK5 is a key downstream effector of these cascades (Lochhead et al., 2012). Anlaysis of cancer tissues or cancer cell lines has revealed that the MEK5/ERK5 pathway is overexpreed in many human cancers including breast cancer, prostate cancer, hepatocellular carcinoma, melanoma and ovarian cancer correlates with poor prognosis (Mehta et al., 2003a, Song et al., 2004, Ostrander et al., 2007, Sticht et al., 2008, Montero et al., 2009b).

The ability of ERK5 to promote cell proliferation and survival contributes to cancer progression, thereby facilitating metastasis via promoting cell migration and invasion (Figure 1.11). The ERK5 pathway promotes metastasis by transcriptional upregulation of the matrix metalloproteinase-9 (MMP-9) via AP-1 transcription factor (Mehta et al., 2003a). As MMPs degrade extracellular matrix proteins (ECM), they play a sigificant role in metastasis (Foda and Zucker, 2001).

The Ras oncogene is mutated in a number of cancer types, such as thyroid (50%), colon (50%), lung (30%), ovarian (15%), breast, skin and liver. The ERK5 cascade is suspected to be implicated in mediating the oncogenic effect of RAS. In PC12 cells and C2C12 cells, the activation of ERK5 requires RAS activation (Kamakura et al., 1999). Ras exists in dependent and independent pathways for the activation of ERK5; for example, the inhibition of EGF-induced ERK5 in PC12 cells by RAS was complete, while in COS17 cells it was about 50%, indicating that the results depend on the cell type (Kamakura et al., 1999). In addition to RAS, there are many other oncogenes

that can activate ERK5, such as Cancer Osaka Thyroid (Cot), which requires JNK, p38 and ERK5 to induce the c-Jun promoter to subsequently stimulate neoplastic transformation (Chiariello et al., 2000). Src is another oncogene that mediates ERK5 activation in response to H_2O_2 (Abe et al., 1997). Similar to RAS, activated Src tyrosine kinase activates the ERK5 pathway, which in turn contributes to transformation via Src; this suggests that ERK5 plays an important role in neoplastic transformation (Barros and Marshall, 2005).

1.5.1 Tumour angiogenesis

ERK5 is implicated in tumour-associated angiogenesis; this was suggested by analysis of the deletion of the *erk5* gene in mice, resulting in embryonic lethality at E9.5-10.5, and the histological and immune histological analysis of mutant embryos, which revealed serious defects in cardiac development and angiogenesis (Regan et al., 2002a). Furthermore, ablation of ERK5 in a human tumour xenograft model resulted in regression of tumour vasculature and reduction in tumour growth in mice (Hayashi et al., 2005b). In addition, it is known that pro-angiogenic factors such as VEGF, FGF-2 and EGF have the ability to stimulate ERK5 activation in HUVECs (Hayashi et al., 2004a). Roberts and co-workers have shown that ERK5 is required to mediate VEGF stimulated tubular angiogenesis in HDMECs (Roberts et al., 2010b). Together, these data indicate that ERK5 could be a potential anti-angiogenic drug target.

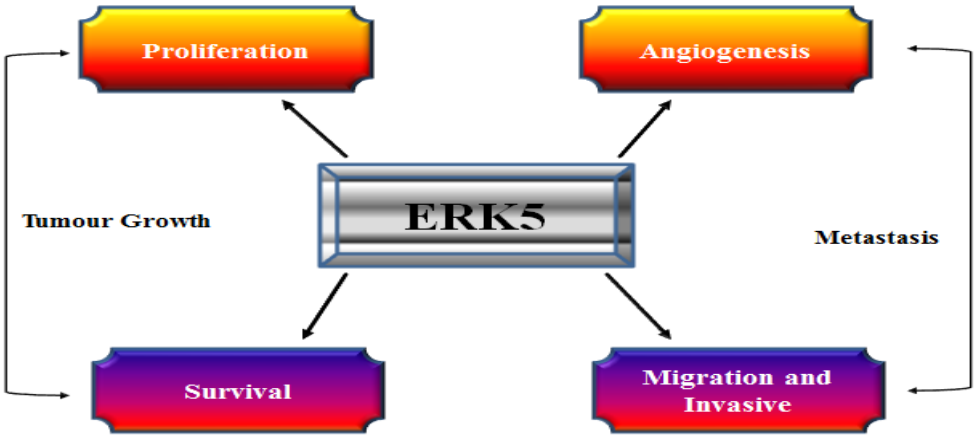


Figure 1.11 ERK5 promotes cancer progression. ERK5 regulates tumour growth by triggering un-controlled cell proliferation and survival. In addition, ERK5 induces angiogenesis to control tumour development and also it increases the mobility and the invasiveness of cancer cells to facilitate the metastasis. The abundance of ERK5 functions makes this cascade attractive as a therapeutic target for cancer.

1.5.2 Breast and Prostate cancers

Studies have examined MEK5 in breast cancer and found that the constitutive activation of signal transducer and activator of transcription (STAT3) induces and up-regulates MEK5. STAT3 is commonly detected in breast cancer cell lines but not in breast epithelial cells (Song et al., 2004). STAT3 is regulated by ErbB/HER, which suggests that, as MEK5 is up-regulated by STAT3, the phosphorylation of downstream ERK5 is, induced as well (Song et al., 2004). Furthermore, ERK5 is a cellular mediator of EGF-induced proliferation in an immortalised breast epithelial cell line showing the growth of breast cancer cells (Esparis-Ogando et al., 2002a). The second member of the epidermal growth factor (EGF) family, Neuregulin (NRG), binds to ErbB3/HER3 and ErbB4/HER4 and induces the ERK5 pathway, which in turn participates in responses of proliferation that are induced by NRG receptors (Esparis-Ogando et al., 2002a). Mutations or increased activation of these receptors (ErbBs) is associated with poor prognosis, a high risk of metastases and potentially chemotherapy-resistant cancers (Esparis-Ogando et al., 2002a). In addition, the expression of a dominant-negative form of ERK5 represses the

proliferation of breast cancer cells through a noticeable decrease in NRG-induced proliferation. Overexpression of HER2/ErbB2 constitutively activates the dual phosphorylation site of ERK5 in breast cancer cell lines such as SKBR3 and BT474 (Esparis-Ogando et al., 2002a). ERK5 has been assessed in 84 samples of primary breast cancer tumours; the results indicate that 17 samples (20%) had an overexpression of ERK5 (Montero et al., 2009b). MEK5 is implicated in survival signalling and apoptotic regulation in breast cancer and inhibition of MEK5 signalling sensitised breast cancer cells to chemotherapy (Weldon et al., 2002).

Based on the observation of phosphorylation of ERK5 induced by the EGFR family in breast cancer cells and the constitutively activated form of MEK5, a study has assessed the role of the abnormal activation of the MEK5/ERK5 pathway in prostate cancer. The results indicate that ERK5 protein expression is up-regulated in high grade prostate cancer (Mehta et al., 2003a). Overexpression of MEK5 in prostate cancer is correlated with an unfavourable prognosis and tumour metastases (Mehta et al., 2003a). ERK5 activation induces the transcriptional activation of c-Jun via MEF2, which in turn increases the activator protein AP-1; this up-regulates the matrix metallopeptidase-9 (MMP-9) resulting in degradation of the extracellular matrix (ECM) surrounding the cancer cell and allows them to invade and metastasise as mentioned (section 5.1) (Mehta et al., 2003a). The expression of MMP-9 plays a role in metastasis in various tumours including breast cancer, prostate cancer and ovarian cancer (Scorilas et al., 2001, Sehgal et al., 1998). In addition, overexpression of ERK5 in PC3 cells increases the levels of proliferation, invasion and motility which in turn accelerate tumour formation, confirming the role of ERK5 in the aggression of prostate cancer (McCracken et al., 2008a).

1.5.3 Ovarian carcinoma

Folic adhesion kinase (FAK) plays a functional role in cell motility and cell survival, as well as recruiting signalling molecules to focal adhesions (Hanks et al., 2003). The overexpression of FAK has been found in many different types of tumours and its over-activity is associated with invasion and metastasis (Parsons et al., 2008). Protein tyrosine phosphatase (PTP) and dual specificity phosphatase (DUSP) are known to have a role in cellular motility that is maintained by HER2; also, PTPN12 is a regulator of adhesion, migration and FAK (Villa-Moruzzi, 2011). ERK5 is a downstream target for FAK and the receptor tyrosine kinase HER2 (Esparis-Ogando et al., 2002a, Sawhney et al., 2009). Based on these facts, Villa-Moruzzi assessed the role of the ERK5 target FAK in the HER2-dependent pathway; the results indicated that HER2-driven motility is mediated by ERK5 and PTPN12 in an ovarian cancer cell line (SKOV-3) (Villa-Moruzzi, 2011).

1.5.4 Skin cancer

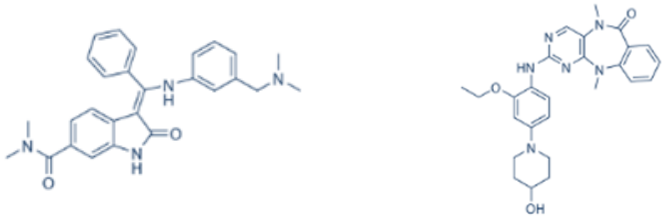
The knockdown of ERK5 in human malignant melanoma cells A375 abolished their ability to form invadopodia (Ramsay et al., 2011). Furthermore, the deletion of ERK5 in B16F10 melanoma and LL/2 Lewis xenograft models affected tumour angiogenesis and reduced tumour volumes in these xenograft models (Hayashi et al., 2005b). Inactivation of ERK5 in epidermal keratinocytes blocked inflammation driven tumourigenesis (Finegan et al., 2015). Furthermore, the ablation of epidermal ERK5 weakened the migration of neutrophils and mast cells to the skin in response to tetradecanoylphorbol acetate (TPA) (Finegan et al., 2015). Recent data has shown that in contrast to ERK1/2, ERK5 does not drive proliferation in melanoma cells with KRAS or BRAF mutations (Lochhead et al., 2016).

1.5.5 Pharmacological inhibitors of ERK5

Two pharmacological inhibitors of the MEK5/ERK5 pathway have been discovered, BIX02188 and BIX02189 (Figure 1.12) (developed by the

Boehringer Ingelheim), which belong to indoline kinase inhibitors series and selectively inhibit MEK5 without affecting MEK1/2 (Tatake et al., 2008). BIX02188 and BIX02189 have been shown to prevent sorbitol induced ERK5 activation and increase the transcriptional activity of MEF2C in co-transfected cells with MEK5 DA and ERK5 (Tatake et al., 2008). Osmotic stress-induced ERK5 and MEF2C activation in HeLa and HEK293 cells were inhibited by these inhibitors (Tatake et al., 2008). Utilising these inhibitors to block ERK5 activation in response to NRG stimulation in PC12 cells, inhibits the differentiation of these cells (Obara et al., 2009). Based on reports indicating that CDK1 is proposed to be the kinase responsible for ERK5 activation during mitosis, using BIX02188 and BIX02189 is pivotal to distinguish whether the phosphorylation of ERK5 is MEK5-dependent or-independent (Inesta-Vaquera et al., 2010). These findings suggest that the inhibition of MEK5 via BIX inhibitors is not sufficient to block ERK5 activation in mitotic tumour cells (Inesta-Vaquera et al., 2010).

The modification of adenosine triphosphate (ATP)-competitive polo kinase inhibitor (BI-2536) that led to loss of polo kinase inhibitor activity, resulted in a XMD8-92, a highly selective inhibitor for ERK5 phosphorylation (Figure 1.12) (Yang et al., 2010b). XMD8-92 inhibits ERK5 activation in response to EGF stimulation in HeLa cells without an inhibitory effect on ERK1/2 (Yang et al., 2010b). The treatment of mouse lung and cervical xenograft tumours with XMD8-92 reduced the tumour growth by 95% by a reduction of tumour cell proliferation by suppression of the promyelocytic leukaemia protein (PML) and by inhibition of the contribution of ERK5 in tumour associated angiogenesis (Yang et al., 2010b). In contrast with the loss of vascular integrity after deletion of *erk5* in endothelial cells of adult mice, it has been noted that the stability of blood vessels of mice did not appear to be detrimentally affected by XMD8-92 treatment (Yang et al., 2010b).



BIX02189 XMD8-92

Figure 1.12 Chemical structure of BIX02189 and XMD8-92

1.5.6 Anti-cancer drugs

The anti-cancer treatments that used in this project are doxorubicin and cisplatin in ovarian cancer cell lines and vemurafenib in malignant melanoma cell lines.

1.5.6.1 Doxorubicin

In 1957, the first isolated anthracycline antibiotics was daunorubicin from the actinobacterium Streptomyces peucetius (Dimarco et al., 1981). In 1969, doxorubicin, analogue daunorubicin, was discovered from Streptomyces peucetius var. caesius, a mutagenic Streptomyces peucetius, and since then many research reports relating to pharmacodynamics and pharmacokinetics of doxorubicin have been published (Arcamone et al., 1969).

Doxorubicin consists of an amino sugar called daunosamine which is linked to the tetracyclic aglycone structure and this amino sugar produces a hydrophilic centre for doxorubicin (Figure 1.1). A short chain with a carbonyl group at C-13 and a hydroxyl group at C-14 attaches to ring A at C-9 and a quinine group presents in ring C and a hydroquinone group presents in ring B (Arcamone et al., 1969, Minotti et al., 2004). The structural difference between daunorubicin and doxorubicin is that it is a methyl group at C-14 in daunorubicin, while in doxorubicin it is a hydroxyl group at C-14 (Figure 1.1). The complex structure of doxorubicin is closely related to its main mechanisms of action and its metabolism. Since doxorubicin was introduced into clinical practice in the

1970s, it has become one of the most effective chemotherapy treatments for several tumours, such as breast cancer, ovarian cancer and mesothelioma (Ogura, 2001). The various mechanisms of action of doxorubicin-induced cytotoxicity have been discovered include inhibition of topoisomerase II triggered via DNA intercalation, apoptosis induced by p53 dependent/independent pathway, oxidative damage mediated by free radicles and transport doxorubicin into the nucleus via interactions with proteasome (Minotti et al., 2004).

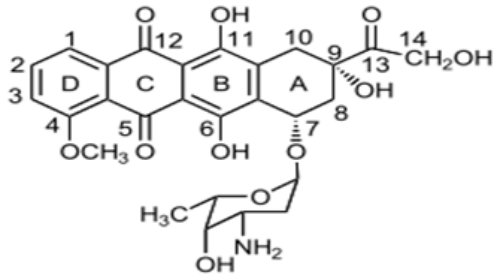


Figure 1.1 The molecular structure of doxorubicin

1.5.6.2 Cisplatin

The inorganic platinum complex, cis-diamminedichloroplatinum (II), commonly known as cisplatin, was the first platinum compound to be discovered. It was first described by Peyrone in 1845, but its antitumor activity was only noticed after 121 years (Peyrone, 1844). The physicist Barnett Rosenberg and colleagues accidentally observed that Escherichia coli when grown in an electrical chamber with platinum electrodes stopped dividing and started to elongate. This inhibition of cell division without an apparent reduction in growth was attributed to the elution of platinum compounds from the platinum electrodes used in the chamber (Rosenberg et al., 1965). Further experiments with platinum compounds led to the determination of cisplatin as the compound that exerted the anti-proliferative actions. It is composed of a doubly charged platinum ion surrounded by four ligands, two amine ligands and two chloride ligands (Figure 1.12). Cisplatin was approved

by the US Food and Drug Administration (FDA) for treatment of testicular and bladder cancer in 1978 (Kelland, 2007). Cisplatin is now a widely used chemotherapeutic drug in the treatment of a variety of cancers, including lung, bladder, testicular, ovarian and gastric cancers (Schiller et al., 2002, von der Maase et al., 2000, Einhorn, 2002, Hennessy et al., 2009, Cunningham et al., 2006). In spite of the extensive clinical experience with cisplatin, the exact molecular mechanism of the cytotoxicity induced by cisplatin is not clearly understood, but it is generally accepted that the main target for the drug is DNA (Kelland, 2007). Once inside cytoplasm, cisplatin undergoes hydrolysis, the chloride atoms are replaced by water molecules and cisplatin becomes a potent electrophile which allows the platinum atom to bind to DNA and form cisplatin-DNA adducts inducing intra-strand and inter-strand cross-linking. The interaction of cisplatin with DNA to form DNA adducts mediates its cytotoxic effects by inhibiting DNA replication and transcription by inducing programmed cell death (Trimmer and Essigmann, 1999).

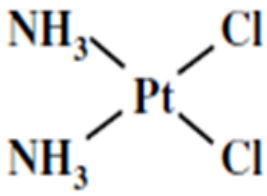


Figure 1.12 Structure of cisplatin Cis-diamminedichloroplatinum (II)

1.5.6.3 Vemurafenib

The vemurafenib was approved by the US Food and Drug Administration (FDA) in 2011 and by Health Canada and the European Commission in 2012 for the treatment of adult patients with metastatic melanoma harbouring $BRAF^{V600E}$ mutations, gets its name from **V**600**E** **mu**tated **BRAF** inhibition and is also known under the names PLX4032 and Zelboraf®. It is developed by Plexxikon Inc. and commercialised by Hoffmann-La Roche (Sharma et al.,

2012). Vemurafenib is a novel and highly selective serine/threonine kinase inhibitor with the ability to block proliferation in tumours carrying the mutant BRAF gene (Figure 1.13) (Nijenhuis et al., 2014).

Vemurafenib inhibits the protein synthesised by the mutant $BRAF^{V600E}$ and prevents the constitutively activation of the MAPK signalling pathway (Sharma et al., 2012). Vemurafenib binds reversibly to $BRAF^{V600E}$ and reduces the activity of the kinase by blocking the activating domain, which results in decreased activation of MEK and ERK, which in turn slows down the cell growth and proliferation (Flaherty et al., 2011). Despite the strong initial response, patients almost always experience tumour relapse within one year of treatment and become resistant to vemurafenib and this suggests the existence of intrinsic BRAF inhibitor resistance. Also, an acquired resistance after initial response to the drug was observed (Kudchadkar et al., 2012).

PLX4720 is a structurally related progenitor of vemurafenib/PLX4032 that displays similar inhibition of mutant BRAF protein in biochemical assays and it differs only in one phenyl ring from PLX4032 (Tsai et al., 2008, Michaelis et al., 2014). Vemurafenib was selected for further clinical development over PLX4720, due to a more favourable pharmacokinetic profile, with greater bioavailability in beagle dogs and monkeys (Bollag et al., 2010). However, PLX4720 is the preferred compound for use in pre-clinical mouse models, due to better pharmacokinetics in rodents (Bollag et al., 2012). PLX4720 was found to inhibit $BRAF^{V600E}$ at low nanomolar concentrations, and was 10-fold more selective for B-Raf (V600E) than for the wild-type B-Raf. It was even more selective for $BRAF^{V600E}$ than for other kinases (Bollag et al., 2010).

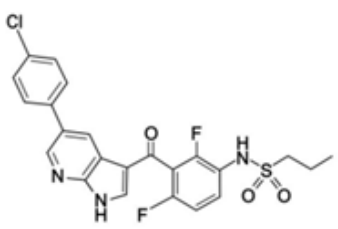


Figure 1.13 The molecular structure of vemurafenib.

1.5.6.4 Oncogene addiction

Cancer cells are known to contain multiple genetic and epigenetics abnormalities and are notorious for harbouring several mutations. Despite this, they often display dependence on a single oncogene or pathway for their growth and survival, a phenomenon called oncogene addiction (Weinstein, 2002; Weinstein and Joe, 2006). As a consequence of this addiction, inactivation of the oncogene which tumour cells are dependent on results in regression of the tumour.

The oncogene addiction phenomenon has been verified in many mouse models. Switching on the *c-myc* oncogene in hematopoietic cells resulted in the development of myeloid leukemia and T-cell; however, switching off this gene the leukemia cells stopped dividing and showed differentiation and apoptosis (Felsher and Bishop, 1999). Furthermore, oncogene addiction has also been shown to occur in conditional mouse models of *h-ras* in melanoma (Chin et al., 1999), *bcr-abl* induced leukemia (Huettner et al., 2000) and *k-ras* induced lung adenocarcinoma (Fisher et al., 2001).

Oncogene addiction is also relevant to tumour-stromal interaction, tumour metastasis and tumour invasion. Therefore, activation of *EGFR* or *RAS* leads to stimulation of signalling pathways in cancer cells which promote the expression of MMPs and VEGF (Wittekind and Neid, 2005). Inactivation of these genes in cancer cells inhibits tumour invasion, angiogenesis (Weinstein and Joe, 2006). RAS mutations can drive ERK1/2 pathway addiction; in tumour cells with KRAS mutations, inhibition of MEK1/2 is still a viable strategy (Little et al., 2013). The ERK1/2 signalling pathway is often de-regulated in human cancer attributable to mutations in *BRAF* or *KRAS* and also *NRAS* and *HRAS* and activating mutations in RTKs such as EGFR and FGFR1. As a consequence of the addiction of tumour cells to these oncogenic drivers to maintain their cancer specific traits, mutant oncoprotiens and their signalling pathway are important in drug targets. The ERK1/2 cascade has attracted more

interest in new cancer therapeutics research and some inhibitors are currently undergoing clinical evaluation, such as the RAF inhibitor, vemurafenib, which selectively inhibits $BRAF^{V600E}$ and the MEK1/2 inhibitor, selumetinib (Ji et al., 2012; Joseph et al., 2010). Cell lines derived from melanoma, thyroid and colorectal cancer that have BRAF mutations exhibit high activation of ERK1/2 and these cell lines are frequently addicted to the ERK1/2 signalling pathway. A s a consequence of this addiction, inhibition of ERK1/2 signalling pathway sensitises tumour cells to insults they are relatively resistant to (Balmanno and Cook, 2009).

1.6 Projects aims

The objective of this project was to elucidate the role of ERK5 in drug-induced resistance in cancer cells and tumour angiogenesis.

In order to achieve this objective, this study aimed to:

1- Characterise the activation and phosphorylation of ERK5 in endothelial cells and tumour cells (HeLa) in response to pro-angiogenic factors and small molecule inhibitors of the ERK5 signalling axis XMD8-92 and BIX02189. Assess the role of these inhibitors on VEGF induced angiogenesis using a 3-D collagen gel model and co-culture *in vitro* angiogenesis assay.
2- Assessment of the activation of ERK5 in vemurafenib-resistant melanoma cells and the effect of inhibitors of the MEK5/ERK5 signalling pathway. Determine the role of ERK5 in aberrant angiogenesis via inhibition of melanoma cell-mediated angiogenesis by targeting the MEK5/ERK5 cascade in HDMEC/NHDF/melanoma cell co-culture *in vitro* angiogenesis assay.
3- Investigate the ERK5 activation status in doxorubicin and cisplatin-resistant ovarian cancer cells and the effect of inhibitors of the MEK5/ERK5 signalling pathway in potentially restoring chemo sensitivity in resistant cells. Assessment the effect of targeting the ERK5 signalling axis to prevent tumour cell-induced angiogenesis by using HDMEC/NHDF/ovarian cancer cell co-culture *in vitro* angiogenesis assay.

Chapter Two: Materials and Methods

2.1 Materials

2.1.1 Reagents

Recombinant human vascular endothelial growth factor VEGF-A_{165} and basic fibroblast growth factor (FGF-2) were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Recombinant human hepatocyte growth factor (HGF), epidermal growth factor (EGF), dimeric platelet-derived growth factor (PDGF)-BB and insulin like growth factor (IGF) were bought from Peprotech EC (Rocky Hill, NJ, USA).

Aprotinin, leupeptin, pepstatin, phenylmethylsulphonyl fluoride (PMSF), agar, agarose (electrophoresis grade), glycerol, 3-(N-morpholino)-propanesulphonic acid (MOPS), polyoxyethylenesorbitan monolaurate (Tween-20) and sodium dodecyl sulphate (SDS) 20% (w/v) solution, were purchased from Melford (Ipswich, UK). Prestained protein marker (broad range 10-230 kDa) was purchased from New England Biolabs (UK) Ltd, (Hitchin, UK).

Ammonium chloride, ammonium peroxodisulphate (APS), ampicillin solution, bicinchoninic acid solution, copper (II) sulphate solution, dimethyl sulphoxide (DMSO), sterile DNase- and RNase-free dH_2O, ethidium bromide (EtBr), ethylenediaminetetraacetic acid (EDTA), gelatin from porcine skin, kanamycin solution, Luria Bertani (LB) broth powder, manganese (II) chloride tetrahydrate, sodium orthovanadate (Na3VO4), N,N,N',N'-tetramethylethylenediamine (TEMED), Tris-EDTA (TE) buffer solution (pH 8.0) and Triton X-100 were purchased from Sigma-Aldrich (Poole, UK).

Enhanced chemiluminescence (ECL from Pierce) western blotting detection reagents, glycine, sodium chloride (NaCl), Tris-Base were bought from Thermo Fisher Scientific™ (Loughborough, UK).) Bovine serum albumin (BSA) was purchased from *Boehringer*. Full-range (12-225 kDa) rainbow molecular weight markers and Hybond ECL nitrocellulose membrane were purchased from GE Healthcare (Amersham, UK). Ultrapure ProtoGel®

solution was bought from Geneflow Ltd. (National Diagnostics, Staffordshire, UK). NuPAGE® 4-12% Bis-Tris gels, LDS sample buffer [4X] were purchased from Novex®, Life Technologies™ (Paisley, UK). BIX 02189 was purchased from Selleck Chemicals (Stratech Scientific Ltd., Suffolk, UK). XMD 8-92 was bought from Tocris Bioscience (Bristol, UK).

RNeasy mini RNA extraction kit, QIAshredder kit, RNase-Free DNase set and QIAprep Spin Miniprep/Maxiprep kits were purchased from Qiagen (Crawley, UK). Oligonucleotide primers and 2x Power SYBR ® Green mastermix were purchased from Life Technologies™ (Paisley, UK). Moloney murine leukemia virus (M-MLV) reverse transcriptase, 0.1 M dithiothreitol (DTT), 5 x first strand buffer, 10 mM PCR grade dNTP mix, RNaseOUT™ recombinant ribonuclease inhibitor. GoTaq® Flexi DNA polymerase, MgCl 2 (25 mM), 5X colourless GoTaq® Flexi Buffer; ultra-pure deoxynucleotide triphosphates (dNTPs) (100 mM of each dNTP in ddH_2O) and CellTiter-Glo™ luminescent cell viability assay were from Promega. Optical-grade sealing film for 96-well qRT-PCR plates was purchased from Bioline (London, U.K.).

Paraformaldehyde, Triton X-100, 2-mercaptoethanol for electrophoresis, and SIGMA FAST TM BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) tablets were purchased from Sigma-Aldrich (Poole, U.K.).

2.1.2 Antibodies

Table 2.1 Primary antibodies used in this study

Antibody	Source	Cat. No.	Dilution	Host Species	Appl.
Actin (I-19)	Santa Cruz Biotechnology (CA, USA)	sc-1616-R	1:2000	Rabbit	WB
AKT	New England Biolabs (Hitchin, UK)	#9271	1:1000	Rabbit	WB
EGF-R1	New England BioLabs (Hitchin, UK)	#2232	1:1000	Rabbit	WB
ERK5	New England Biolabs (Hitchin, UK)	#3372	1:1000 1:100 1:50	Rabbit	WB IF IP
GAPDH XP	New England Biolabs (Hitchin, UK)	#5174	1:2000	Rabbit	WB
p44/42 MAPK (ERK1/2)	New England Biolabs (Hitchin, UK)	#9102	1:1000 1:100	Rabbit	WB IF
VEGF Receptor 2 (55B11)	New England Biolabs (Hitchin, UK)	#2479	1:1000 1:200	Rabbit	WB IF
Phospho- AKT (S473) XP	New England Biolabs (Hitchin, UK)	#4060	1:2000	Rabbit	WB
Phospho- EGFR1	New England Biolabs (Hitchin, UK)	#2234	1:1000	Rabbit	WB
Phospho- ERK5 (T218/Y220)	New England Biolabs (Hitchin, UK)	#3371	1:1000	Mouse	WB
Phospho- p44/42 MAPK (T202/Y204) XP	New England Biolabs (Hitchin, UK)	#4370	1:2000	Rabbit	WB
Phospho-SAPK/JNK (Thr^{183}/Tyr^{185}) Antibody	New England Biolabs (Hitchin, U.K.)	#9251	1:1000	Rabbit	WB
Cleaved Caspase-3 (Asp^{175}) Antibody	New England Biolabs (Hitchin, U.K.)	#9661	1:1000	Rabbit	WB
Monoclonal Mouse Anti-Human CD31, Endothelial Cell Clone JC70A	Dako Cytomation (Glostrup, Denmark)	M 0823	1:750	Mouse	IHC

WB, Western blotting; IF, immunofluorescence; Immunohistochemistry (IHC)

Table 2.2 Secondary antibodies used in this study

Antibody	Source	Cat. No.	Dilution	Appl.
Alexa Fluor® 488 Donkey Anti-Rabbit IgG (H+L)	Molecular Probes® (Life Technologies™; Paisley, UK)	#A21206	1:1000	IF
Alexa Fluor® 568 Donkey Anti-Rabbit IgG (H+L)	Molecular Probes® (Life Technologies™; Paisley, UK)	#A10042	1:1000	IF
Hoechst 33342	Molecular Probes® (Life Technologies™; Paisley, UK)	#H1399	1:5000	IF
HRP-conjugated AffiniPure Donkey Anti-Goat IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc. (PA, USA)	#705-035-147	1:10000	WB
HRP-conjugated AffiniPure Goat Anti-Mouse IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc. (PA, USA)	#115-035-166	1:5000	WB
HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L)	Jackson ImmunoResearch Laboratories, Inc. (PA, USA)	#111-035-144	1:5000	WB
Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate antibody	Sigma-Aldrich (Poole, U.K.)	#250	A3688	IHC

WB, Western blotting; IF, immunofluorescence; Immunohistochemistry (IHC)

2.1.3 Cell lines

Table 2.3 Cell lines used in this study

Abbr. Name	Cell type and details	Source
A375	Malignant melanoma cancer cell line	From Prof. Richard Marais (Manchester Cancer Institute)
A375-R	Malignant melanoma cancer cell line resistant to PLX4027	From Prof. Richard Marais (Manchester Cancer Institute)
SKMel 5	Malignant melanoma cancer cell line	From Prof. Richard Marais (Manchester Cancer Institute)
SKMel 5-R	Malignant melanoma cancer cell line resistant to PLX4027	From Prof. Richard Marais (Manchester Cancer Institute)
HeLa	Human epithelial cervical cancer cell line Isolated from adult, African-American female	European Collection of Cell Cultures supplied by Sigma- Aldrich (Poole, UK)
A2780	Malignant ovarian cancer cell line	From Prof. Robert Brown and Carol McCormik (Institute of Cancer Science, Glasgow University)
A2780-Cis	Malignant ovarian cancer cell line resistant to cisplatin	From Prof. Robert Brown and Carol McCormik (Institute of Cancer Science, Glasgow University)
A2780-ADR	Malignant ovarian cancer cell line resistant to doxorubicin	From Prof. Robert Brown and Carol McCormik (Institute of Cancer Science, Glasgow University)
NHDF	Normal Human Dermal Fibroblasts	PromoCell (Heidelberg, Germany).
HDMEC	Human Dermal Microvascular Endothelial cells (Lot No. 6060707.1) Isolated from juvenile, Caucasian male	PromoCell (Heidelberg, Germany)

2.1.4 Cell culture media and solutions

Table 2.4 Cell culture media and solutions used in this study

Name	Composition	Source
EBM MV2 basal medium containing 1% (v/v) FCS	EBM MV2 basal medium supplemented with 1% (v/v) fetal calf serum (FCS), (Cat. No. C-22221).	PromoCell (Heidelberg, Germany).
Fibroblast growth medium	Fibroblast growth medium supplemented with 1.0 ng/ml bFGF and 5.0 µg/ml insulin, (Cat. No.: C-23110).	PromoCell (Heidelberg, Germany).
EBM MV2 growth medium	EBM MV2 basal medium supplemented with 5% (v/v) fetal calf serum (FCS), EGF (5.0 ng/ml), hydrocortisone (0.2 µg/ml), VEGF (0.5 ng/ml), FGF-2 (10.0 ng/ml), insulin-like growth factor-1 (20.0 ng/ml) and ascorbic acid (1.0 µg/ml), (Cat. No. C-22121).	PromoCell (Heidelberg, Germany).
DMEM	Dulbecco's Modified Eagle Medium (DMEM) (Cat. No. D6429) containing 4500 mg/L glucose, L-glutamine, sodium pyruvate and sodium bicarbonate Supplemented with 10% (v/v)	Gibco® (Life Technologies™; Paisley, UK)
RPMI 1640	RPMI medium 1640 (1X) + GlutaMAX™ -I	Gibco® (Life Technologies™; Paisley, UK)
Serum-free medium	EBM MV2 basal medium with no added growth factors or serum, (Cat. No. C-22221).	PromoCell (Heidelberg, Germany)
Trypsin	0.05% Trypsin-EDTA (1X), Phenol Red (Cat. No. 25300-054)	Gibco® (Life Technologies™; Paisley, UK)
Gelatin	0.5% (w/v) gelatin from porcine skin in ddH_2O, autoclaved	Sigma-Aldrich® (Poole, UK)
Versene	Dulbecco's phosphate buffered saline without Ca^{2+}/Mg^{2+} supplemented with 0.5 mM EDTA	Lonza (Basel, Switzerland)
PBS Cell Wash	Dulbecco's phosphate buffered saline with Ca^{2+}/Mg^{2+}	Lonza (Basel, Switzerland)
Ice-cold PBS Lysis Wash	Dulbecco's phosphate buffered saline without Ca^{2+}/Mg^{2+} stored at 4ºC	Lonza (Basel, Switzerland)
HEPES buffer solution (1M)	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid in distilled water (pH 7.2 to 7.5).	Gibco® (Life Technologies™; Paisley, UK)
PureCol collagen	97% type I collagen (3 mg/ml), 3% type III collagen from bovine hide in 0.01 M HCl, pH 2.0.	Inamed Biomaterials (Leimuiden, The Netherlands)
Sodium bicarbonate solution 7.5% (w/v)	Sodium bicarbonate in ddH_2O, 7.5% (w/v).	Gibco® (Life Technologies™; Paisley, UK)

2.1.5 Cell culture materials

The 10 centimetre diameter polystyrene tissue-culture dishes, 6-, 12-, 24- and 96-well cell-culture plates, white-walled 96-well cell culture plates, 96-well polypropylene PCR microplates, centrifuge tubes, cell scrapers, cryovials, microfuge tubes, filter pipette tips and serological pipettes were bought from Greiner Bio-One (Stonehouse, U.K.). Glass coverslips (16 mm diameter) were bought from Agar Scientific (Stansted, U.K.).

2.1.6 Oligonucleotide primers

The oligonucleotide primer pairs used in this study are provided in Appendix I. Primers were purchased from Invitrogen (Paisley, U.K.) and designed via Invitrogen OligoPerfect™ designer facility using the following parameters: Primer size (bases): min 18, opt 20, max 27. Primer melting temperature (T m (°C)): min 57, opt 60, max 63. Primer GC content (%GC): min 40, opt 50, max 60. Product size (bp): min 100, max 150. Salt conc.: 50 mM Primer conc.: 50 nM.

Oligonucleotide primers were reconstituted to 100 µM using Tris-EDTA (TE) buffer solution (pH 8.0). Qiagen primers were reconstituted to 100 µM by adding 55 µl of TE buffer solution. Primers were incubated at room temperature (r.t.) for 2 min to allow re-hydration to occur, vortexed for 15s and stored at -80°C. Aliquots of the reconstituted primers were diluted 40-fold in sterile, RNase-free ddH_2O (Sigma-Aldrich), to a final concentration of 2.5 µM prior to use in qRT-PCR reactions.

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Cell Culture technique

Sterile conditions were maintained by wearing a Howie coat and nitrile gloves, using sterile cell culture equipment and working a TriMAT^2 Class II microbiological safety cabinet. Culture media were warmed in a water bath to 37°C prior to use. All work surfaces were disinfected using 70% (v/v) ethanol before and after cell culture work.

2.2.1.2 Gelatine coating of cell culture dishes and plates

NHDF and HDMEC were seeded and cultured on sterile 10 cm diameter polystyrene cell culture dishes, plates and cover slips coated with 0.5% (w/v) gelatine and incubated at 37 °C in a humidified, 5% (v/v) CO_2, atmosphere in a Sanyo MCO-17AC incubator (SANYO E&E Europe BV, Loughborough, U.K.) for at least 10 min before use. Prior to plating out NHDF and HDMEC, gelatine was aspirated off from the dishes or/and plates.

2.2.1.3 Thawing of cryopreserved cell stocks

Cryopreserved cells stored in liquid nitrogen at -196 °C were thawed in a 37 °C water bath for 2-3 min and the cryovials were disinfected with 70% (v/v) ethanol before transferring the cells to a 15 ml capped tube containing 9 ml culture medium. The cells were centrifuged at 1500 rpm for 5 min and the supernatant was aspirated off and the pelleted cells resuspended in 10 ml culture medium. After that, NHDF and/or HDMEC were transferred into 10 cm gelatin coated dish whereas the cancer cell types (A375, SKMel5 and A2780) were transferred to a non-gelatinized T75 cm^2 flask.

2.2.1.4 Routine cell culture

Cells were grown at 37 °C for 2-3 days until the confluence reached 80-90%, the cells were then split to the next passage. Medium was aspirated and cells were washed with 10 ml Versene (DPBS without Ca^{2+}/Mg^{2+}) before adding

1 ml of 0.05% Trypsin-EDTA. Cells were incubated at 37 °C for 3-5 minutes, after which, dishes/flasks were tapped gently to aid cell detachment. 90-100% of cells will have detached, which can be viewed under an inverted light microscope. Cells were re-suspended with appropriate cell culture medium and re-seeded in the splitting ratio as described in **Table 2.5.** Finally, the resistant cancer cell lines (A2780-Cis, A2780-ADR, A375 R and SKMel5 R) were treated cisplatin, doxorubicin and vemurafenib (PLX4720) respectively at an appropriate concentration to maintain their phenotypic resistance.

Table 2.5 Cell splitting ratio

Cell type	Culture medium	Splitti
HDMEC	**EBM MV2 growth medium.**	**1:5**
NHDF	**Fibroblast growth medium**	**1:5**
HeLa	**D-MEM containing 10% (v/v)**	**1:5**
A2780	**RPMI containing 10% (v/v) FBS**	**1:18**
A2780-	**RPMI containing 10% (v/v) FBS**	**1:10**
A2780-	**RPMI containing 10% (v/v) FBS**	**1:8**
A375	**RPMI containing 10% (v/v) FBS**	**1:8**
A375-R	**RPMI containing 10% (v/v) FBS**	**1:10**
SKMel 5	**RPMI containing 10% (v/v) FBS**	**1:5**
SKMel 5-	**RPMI containing 10% (v/v) FBS**	**1:6**

2.2.1.5 Cell Counting

Cells were counted to ensure an accurate number of cells for each experiment. As described in section 2.2.1.4, cells were trypsinised in 1 ml trypsin to detach from the dish/flask and re-suspended in 7 ml of appropriate cell culture medium after incubated the trypsinised cells for 3 min. Approximately 100 µL of cells were pipetted onto a Neubauer Improved haemocytometer (Hecht-Assistant, Sondheim/Rhon, Germany) for counting under a light microscope.

2.2.2 Cell treatment

2.2.2.1 Growth factor stimulation of cells

Prior to stimulation with growth factors, all the cells (HDMEC, NHDF, A2780s, A375s and SKMel5) were serum-starved in appropriate low serum medium overnight at 37 °C. The growth factors used can be found in section 2.1.1. Vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) were the main growth factors utilised in this study to stimulate HDMEC and cancer cell lines respectively. Sterile filtered PBS containing 0.1% (w/v) BSA was used to dilute the growth factors making 100 mg/ml stock concentrations which were stored in aliquots at -80 °C. For experimental use, growth factors were diluted with low serum medium to 50 ng/ml final concentrations and incubated for different periods of time.

2.2.2.2 Intracellular kinase inhibition using small-molecule inhibitors

The small molecule kinase inhibitors used in the experiments, BIX 02189, XMD8-92, TRAMETINIB and lapatinib, were diluted in sterile dimethyl sulphoxide (DMSO) to stock solutions of 50 mM, 30 mM, 10 mM and 30 mM respectively and stored in aliquots at -80 °C. The inhibitors were diluted in appropriate low-serum media to give 0.1% (v/v) final concentration of DMSO in each condition. In control experiments, 0.1% (v/v) DMSO in appropriate low serum was added as a vehicle control. Prior to stimulation of cells with growth factors (section 2.2.2.1) and cell lysis (section 2.2.2.4), cells were incubated with inhibitors for one hour at 37 °C.

2.2.2.3 Anti-cancer drugs

The anti-cancer drugs used in this study, cisplatin, adriamycin (doxorubicin) and PLX4720, were diluted in DMSO into stock solutions of 1 mM, 30 mM and 50 mM respectively and stored in aliquots at -80 °C. For experimental use, the drugs were diluted in appropriate cell culture medium containing 10% FCS to result in 0.1% (v/v) final concentration of DMSO in each case.

Cells were incubated with different concentrations of anti-cancer drugs for 24h or 48h prior to cell lysis (section 2.2.2.4).

2.2.2.4 Cell lysis

Unless otherwise stated in the text, cell lysates were prepared by placing cell culture plates on ice and then washing cells with ice-cold PBS (Table 2.4). After that, modified radio immunoprecipitation assay (RIPA) buffer, 20 mM Tris-HCl; pH 7.5, 150 mM NaCl, 2.5 mM EDTA, 10% (v/v) glycerol, 1% Triton-X-100, 1 mM Na_3VO_4, 10 µg/mL Aprotinin, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 1 mM PMSF, 0.1% (w/v) SDS and 0.5% (w/v) sodium deoxycholate, was added to generate the lysates for Western immunoblotting and then the lysed cells were scraped and transferred into centrifuge tubes and the cell debris cleared by centrifugation at 14000 rpm for 20 min. The supernatant was transferred to fresh centrifuge tubes and mixed with 1/3 volume of 4x LDS. Protein samples were then boiled at 90 °C for 5 min and vortexed and finally frozen at -80 °C prior to use in Western immunoblotting.

2.2.3 RNA extraction

The RNA extraction process was carried out on a clean bench using RNase free filter tips and RNase free water. To minimize RNA degradation, RNA samples were kept on ice all the time. The RNA was extracted from the cells using an RNeasy mini kit (Qiagen. UK).

The cells were washed with PBS (without Ca^{2+}/Mg^{2+}) and 600 µl of RLT buffer containing 1% (v/v) 2-mercaptoethanol was added to each 10 cm dish. Cells were collected by scraping in RLT buffer and the lysates were transferred to QIAshredder spin column and centrifuged for 2 min at 15000 rpm. Samples were transferred into RNeasy mini column and spun at 15000 rpm for 30 seconds and then the flow through was discarded. RNeasy mini columns were washed with 350 µl of RW1 and centrifuged at 15000 rpm for 30 sec, the flow-through was discarded. To eliminate genomic DNA

contamination, on-column DNase digestion was applied by adding 80 µl of DNase 1 solution to the column membrane of each RNeasy mini column and incubated for 15 min at room temperature to ensure complete digestion of any contaminating genomic DNA. Each membrane was washed by adding 350 µl of RW1, spun at 15000 rpm for 30 sec and flow through discarded. Each RNeasy mini column was washed with 500 µl of RPE and centrifuged for 2 min at 15000 rpm. The RNeasy columns were transferred to new centrifuge tubes and 30 µl of RNase free water was added to each column membrane to elute total RNA. The RNA quality was assessed using a Nano drop ND-1000 spectrophotometer (Laptech international, Lewes, UK). A 260/280 ratio > 2.0 was required to confirm RNA purity.

2.2.4 Reverse transcription of mRNA (cDNA synthesis)

The reverse transcription reaction procedure was carried out in sterile, RNase and DNase free PCR tubes. In each tube, 1 µl of total RNA was mixed with 1 µl oligo d (T)18 (Invitrogen Custom primer, 500 µg/ml), 1 µl dNTP mix (10mM, Invitrogen) and nuclease free water to give a final reaction volume of 12 µl. The reaction was mixed by pipetting gently, tubes were heated to 65 ºC for five minutes using a PCH-1 heating unit and placed on ice to cool. A master mix containing 4 µl of 5x first strand buffer, 2 µl 0.1 M DTT, 1 µl RNaseOUT and 1 µl M-MLV Reverse Transcriptase (Invitrogen) was prepared and 8 µl of this master mix was added to each tube and incubated at 25 ºC for 5 min followed by a further 37 ºC for 60 min and finally by 15 min at 70 ºC to inactive the reaction. At the end, samples were diluted with 130 µl of ddH_2O to give a cDNA concentration of 6.7ng/µl.

2.2.5 Real Time PCR

RT-PCR reaction mixtures contained 1.5 µl (10 ng) cDNA template, 6.7ng/µl sterile nuclease free ddH2O, 10 µl 2x Power SYBR Green Mastermix (Applied Biosystem), a highly specific dye SYBR Green that fluoresces when bound to dsDNA, and 2 µl of each of the forward and reverse primers at 250

nM in a final volume of 20 µl for each reaction. All the RT-PCR reactions were mixed by vortexing and then loaded into a 96-well PCR plate, which was then sealed with optically clear sealing film. Thereafter, the plate was run on an ABI 7000 PCR system (Applied Biosystem, CA, USA) with the following parameters; 50˚C for two minutes and 95˚C for 10 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for one minute.

2.2.5.1 Interpreting RT-PCR results

The relative expression of the gene of interest was determined by using the comparative cycle threshold (Ct) method. The mean and the standard deviation (SD) of three replicate samples were calculated for the first stage.

Secondly, the Ct value of the target gene was subtracted from the Ct value of the reference gene, a housekeeping gene such as GAPDH or β-Actin, to calculate the ΔCt for each sample as shown in the equation below:

$$\Delta Ct = Ct \text{ target gene} - Ct \text{ reference gene}$$

Thirdly, the standard deviation of ΔCt value was calculated from the standard deviation for the target and reference gene Ct values, as shown below:

(s=standard deviation)

s_1= standard deviation of reference gene Ct values s_2= standard deviation of target gene Ct values $s(\Delta Ct)=(s_1^2+s_2^2)^{1/2}$

In the fourth stage, the ΔΔCt value was calculated by subtracting ΔCt of the stimulated samples from the ΔCt of the basal samples as shown below:

$$\Delta\Delta Ct = \Delta Ct \text{ stimulated samples} - \Delta Ct \text{ basal samples.}$$

Finally the range in fold increase was calculated as follows using this equation:

$$2^{-(\Delta\Delta Ct+2)} \text{ to } 2^{-(\Delta\Delta Ct-s)}$$

2.2.6 Cell viability assay

As mentioned in 2.2.6, a Cell-Titre Glo® luminescent cell viability kit (Promega, UK) was used to determine the viable cells in culture based on the quantification of ATP. Cells were seeded at 5000 per well in a 96-well plate in appropriate cell culture medium and incubated at 37 ºC for 24 hours. After incubation, the cells were treated with inhibitors, growth factors and/or anti-cancer drugs in an appropriate cell culture medium and incubated for 72 hours at 37 ºC. Cells were washed twice with PBS and 100 µl of PBS was added to each well followed by 25 µl Cell-Titre Glo mix and incubated for 10 minutes at room temperature on the shaker. After that, 100 µl of lysate was transferred to a white 96-well plate and luminescence was measured using a Varioskan plate reader. Finally Graph Pad Prism 5 software (Graph Pad Prism software, Inc. San Diego, CA) was used to determine and analyze an IC50 for each cell type and condition.

2.2.7 Western Blotting

2.2.7.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was accomplished using two methods, the first was pre-cast 15-well NuPAGE® Novex® 4-12% Bis-Tris polyacrylamide gels in the XCell SureLock™ Mini-cell electrophoresis system, with 800 ml 1x MOPS running buffer (0.05 M MOPS, 0.05 M Tris, 1 mM EDTA, 0.1% (v/v) SDS). The second type was self-cast, made in XCell SureLock™ Mini-Cell, XCell II™ Blot Module, 1.5 mm (Invitrogen, UK). The desired acrylamide gel percentage was prepared as shown in Table 2.6 with 800 ml 1x SDS running buffer (25 mM Tris base, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3 was prepared as a 10X stock solution and diluted 1:10 in ddH_2O prior to use. To ensure an even edge to the top of the gel and remove air bubbles, 1 ml of isopropanol was overlaid on top of the gel and allowed to polymerise for 45 min at room temperature. After 45 min, the isopropanol was poured off and

the stacking gel (Table 2.6) was added to a 15-well plate. A 1.5 mm comb was inserted into the top cassette and the stacking gel allowed polymerising for 45 min.

One or two gels (pre-cast or self-cast) were loaded in the Mini-Cell running unit and placed into the tank and secured by locking the tension wedge. The combs were removed and rinsed with running buffer to eliminate any un-polymerised acrylamide. Up to 25µl of protein lysates in LDS was loaded into each well with 2 µl of full range rainbow marker that added to 23 µl 1X LDS sample buffer and loaded into the first well of each SDS-PAGE gel. Then, the running buffer was poured into the tank and the lid was placed on to the running unit. Finally, the apparatus was connected to power supply to run the gels at constant current of 50 mA, 250 V, 15 W for MOPS and 35 mA, 125 V, 15 W for Tris-Glycine for up to 2 hours.

Thereafter, the plastic cassettes containing the electrophoresed gels were opened after disassembling the Mini-Cell. The stacking gel was removed and the resolving gel soaked in 25 ml 1X Tris-Glycine transfer buffer (12 mM Tris, 96 mM Glycine, 20% (v/v) Methanol) before transfer.

Table 2.6 Composition of SDS-PAGE gels

Mini Gel	**Units**	**Stacker**	**Running Gel**						
			5%	6%	7%	8%	10%	12%	15%
30% acrylamide/0.8%Bis	ml	0.7	1.7	2.0	2.3	2.7	3.3	4.0	5.0
2.0M Tris/HCl pH8.8	ml	-	2.0	2.0	2.0	2.0	2.0	2.0	2.0
0.5M Tris/HCl pH6.8	ml	0.7							
87% Glycerol	ml	-	0.7	0.7	0.7	0.7	0.7	0.7	0.7
dH_2O	ml	3.6	5.6	5.3	5.0	4.6	4.0	3.3	2.3
Total Volume	ml	5	10	10	10	10	10	10	10
10%APS	µl	2.5	22.9	22.9	22.9	22.9	22.9	22.9	22.9
TEMED	µl	5	5	5	5	5	5	5	5

2.2.7.2 Western Blot analysis

Proteins resolved by SDS-PAGE or Phos-tag™ SDS-PAGE were transferred onto Hybond-ECL nitrocellulose membrane in 1X transfer buffer. In the transfer unit, the electrophoresed gel along with nitrocellulose membrane, measuring 7.5 cm x 8.5 cm, was sandwiched between two pre-soaked sponges and filter papers, measuring 7.5 cm x 8.5 cm. The blotting chamber was filled with 1X transfer buffer and the rest of tank with cold dH_2O and then the lid was placed on top. Finally, the apparatus was connected to power supply to run at constant current of 140 mA, 250 V, 15 W for MOPS and 125 mA, 300 V, 15 W for Tris-Glycine for up to 2 hours.

After protein transfer, the nitrocellulose membrane was washed with 1X TBS-T to remove methanol. The membrane was blocked in 5% (w/v) Bovine Serum Albumin (BSA) in TBS-T and incubated at room temperature for 1 hour to block non-specific binding sites. After that, the membrane was incubated with appropriate primary antibody diluted in 2% (w/v) BSA in TBS-T at 4 ºC overnight. The membrane was then washed six times with 1X TBS-T prior to incubation with appropriate horseradish peroxidase (HRP) - linked secondary antibody diluted in 2% (w/v) BSA in TBS-T at 4 ºC for 90 minutes. Following incubation with secondary antibody, the membrane was washed 5 times with 1X TBS-T followed by a wash with TBS before incubating the membrane with a 1:1 solution of enhanced chemiluminescence (ECL) reagent 1 and reagent 2 at room temperature for 2-4 minutes. The membrane was then sealed in a plastic wallet within an X-ray film cassette before transfer to a dark room to expose the membrane to X-rays for different times. Films were soaked in developing and fixing solution for 1 minute before drying.

2.2.7.3 Quantification of protein expression by densitometry

The relative amount of specific proteins was determined by quantifying the immune reactive bands detected on X-ray film. The X-ray films were scanned

using an Epson® 4490 photo scanner and the Densitometric analysis was accomplished using ImageJ (National Institute of Health (NIH), Version 1.47n). Actin or GAPDH were used as a loading control.

2.2.8 3-D collagen matrix tube formation assay

This assay was utilised to induce the tubular morphogenesis process by placing endothelial cells between two layers of collagen gels and exposing these cells to appropriate growth factors.

Under sterile conditions and on ice, the lower layer of collagen mixture, 8 parts PureCol™ type I collagen (final concentration 2.4 mg/ml), 1 part sterile-filtered 0.1 M NaOH (final concentration 10 mM), 1 part X10 conc. Ham's F-12 medium without L-glutamine or sodium bicarbonate, 1:50 dilution of HEPES buffer solution (1 M) (final concentration 20 mM), 1:64 dilution of sodium bicarbonate (7.5%) liquid (final concentration 0.117%), 1:100 dilution of GlutaMAX™-I supplement (200 mM) (final concentration 2 mM) was prepared and mixed gently prior adding 300 µl of collagen mix to each well of 24-well plate and incubating at 37 ºC for overnight allowing collagen to set. After incubation, 0.5 ml of EBM MV2 basal medium was added to each well to facilitate HDMEC adhesion to the collagen layer.

HDMECs which had been placed in EBM MV2 basal medium containing 1% FCS overnight prior to seeding, were added to each well of 24-well plate at 90,000 cells/well in EBM MV2 basal medium containing 1% FCS and incubated at 37 ºC for 2 hours to allow cellular attachment. The cells were investigated under an inverted light microscope to confirm cell adhesion to the lower layer. Once the cells had adhered, media was aspirated off and 2nd collagen layer added (200 µl) and the plate incubated at 37 ºC for 1.5-2 hours allowing the upper collagen layer to set. After incubation, 0.5 ml of EBM MV2 basal medium with 1% FCS containing 2X appropriate growth factors and/or inhibitors was added gently without disturbing the upper collagen layer to each well and incubated at 37 ºC for 16-24 hours. Following the

tubular morphogenesis assay, media was aspirated and the cells were fixed by adding 0.5 ml of 4% (w/v) paraformaldehyde in PBS to each well.

2.2.8.1 Visualisation and quantification of tubes within 3D-collagen gels

The tubular morphogenesis assay was imaged using an inverted light microscope attached to a Nikon DS-Fil-L2 digital camera (Nikon, UK). Tubes were defined as structures containing a number of fused cells counted in three randomly chosen fields to each well from triplicate condition. For image quantification, AngioQuant image analysis software was used.

2.2.9 NHDF/HDMEC co-culture *in vitro* angiogenesis assay

2.2.9.1 Normal NHDF/HDMEC -/+ cancer cells co-culture

On day 1, NHDFs were seeded at 20,000 cells per well in a gelatine-coated 24-well plate in 1 ml fibroblast growth medium and incubated at 37 ºC for 3 days. On day 4 after ensuring that the NHDF had grown to confluence, the NHDF layer was washed once with PBS and then HDMECs were seeded at 45,000 cells per well in 0.5 ml of EBM MV2 growth medium and incubated for 24 hours. After incubation for 24 hours, cells were washed with PBS and 0.5 ml of EBM MV2 basal medium with 1% FCS containing growth factors and/or inhibitors were added to each well and the plate was returned to the incubator for 72 hours. On day 8, media was aspirated off and replaced with fresh EBM MV2 basal media containing 1% FCS and growth factors -/+ inhibitors and returned to the incubator for a further two days. On day 10, media was removed from the wells and the co-culture was washed twice with PBS and then fixed in 1 ml of ice-cold 70% (v/v) ethanol per well and incubated at room temperature for 30 minutes.

In the case of cancer cell co-culture, appropriate densities of cancer cells (Table 2.7) were seeded in 0.5 ml of appropriate cell culture medium on top of the NHDF layer and mixed gently on day three and incubated for 24 hours.

Table 2.7 Cell density for co-culture of cancer cells

Cell Type	24-well plate (cells/well) in 0.5 ml of media
HeLa	10000
A375 WT	10000
A375 R	10000
SKMel5 WT	10000
SKMel5 R	10000
A2780 WT	5000
A2780-ADR	5000

2.2.9.2 Inhibition of co-culture assay with MEK5 and ERK5 inhibitors at different times

To assess the functional effects of BIX02189 and XMD8-92 in angiogenesis, the NHDF/HDMEC -/+ cancer cells co-culture assay was treated with these inhibitors and fixed at different times. As described above (2.2.9.1), NHDFs were plated on the first day and incubated for 3 days. On day 3, cancer cells were added to the NHDF monolayer and incubated for 24 hours. One day prior to seeding HDMECs on the co-culture, HDMECs growing on 10 cm gelatine-coated dishes were treated with BIX02189 and XMD8-92 and incubated for 24 hours at 37 ºC. On day 4 of the assay, HDMECs treated with inhibitors were seeded at 45,000 cells per well in 0.5 ml EBM MV2 growth medium and incubated for 24 hours. On the fifth day, the NDHF/HDMEC -/+ cancer cells co-culture was treated with inhibitors -/+ VEGF (50 ng/ml) in 0.5 ml EBM MV2 growth medium containing 1% FCS after the cells were washed with PBS and incubated for two days. On day 7, cells were fixed as described above (2.2.9.1). In certain experiments, the treated HDMECs with

BIX02189 and XMD8-92 were seeded on the co-culture on day 7 and incubated for 24 hours in EBM MV2 growth medium. After that, the co-culture was treated with BIX02189 and XMD8-92 -/+ VEGF in EBM MV2 containing 1% FCS and incubated for 24 hours on day 8. Cells were fixed in 1 ml of ice-cold 70% (v/v) ethanol per well and incubated at room temperature for 30 minutes on day 10.

2.2.9.3 Staining of HDMECs in NHDF/HDMEC -/+ cancer cells co-culture

After fixing the co-culture for 30 min in ice-cold ethanol 70%, the ethanol was tipped off and the plate was blotted on blue roll to remove residual ethanol. One millilitre of blocking buffer (1% BSA in PBS w/v) was added to each well and incubated at 37 ºC for 30 min. After incubation, the blocking buffer was poured off and the plate was blotted on the blue roll and then 200 µl of Monoclonal Mouse Anti-Human CD31, Endothelial Cell Clone JC70A Antibody (Dako Cytomation, Glostrup, Denmark) antibody diluted in blocking buffer (1:750) was added to each well and incubated at 37 ºC for 60 minutes. The primary antibody solutions were tipped off and the plate was washed with 1 ml blocking buffer three times. For colorimetric assay, 250 µl of Anti-mouse IgG (whole molecule) alkaline phosphatase conjugate antibody was added in blocking buffer (diluted 1:200) to each well and incubated for 1 hour. After secondary antibody incubation, the plate was washed 3 times with distilled water after aspirating the secondary antibody from the plate. 250 µl of substrate (1 SIGMA Fast BCIP/NBT tablet per 10 ml dH2O) was added to each well and incubated for 10 min at 37 ºC to form a purple colour precipitate in tubule-containing wells. The substrate solution was removed and dH_2O added to each well for 5 minutes. The dH_2O was aspirated and this procedure repeated 3 times.

2.2.9.4 Immunofluorescence staining of HDMEC/NHDF/HeLa co-culture assay

Cell culture was fixed in 2% paraformaldehyde (PFA)/PBS for minutes at RT on day 10. 500 microliters of DPBS containing 0.2% (v/v) Triton-X-100 was added per well and the plates were incubated at room temperature for 10 min. After that, the plates were blocked in TBS containing 0.1% Tween-20 and 1% BSA and 5% serum (from animal source of 2° antibody) for 1 hour at room temperature. A 1:750 dilution of monoclonal mouse anti-human CD31, endothelial cell clone JC70A antibody, 1:100 Rabbit anti-human collagen-1 antibody and 1:50 mouse anti-human muc16 antibody in 1% BSA in TBST for 1 hour at RT. Alexa Fluor® 488 conjugate, 568 and 680 were prepared in antibody dilution buffer 1% BSA in TBST. 50 microliters of the antibody dilution was added to each well, and plates were incubated at RT for 1 hour in the dark. 20µl of Hoechst was added to each well in dilution 1:5000 in 1% BSA in TBST and incubated for 10 min at RT. After that, each well was washed twice in TBST. Then plates were analysed using an inverted Zeiss Axio Observer microscope and the associated Zen software.

2.2.9.5 Image analysis and quantification of tube formation in NHDF/HDMEC -/+ co-culture

Tube formation in co-culture was analysed by taking three random images in different fields from triplicate wells per condition. The co-culture assay was examined by using an inverted light microscope attached to a Nikon DS-Fil-L2 digital camera and the low magnification (X4 objective). For image quantification, AngioQuant image analysis software was used.

2.2.10 Statistical analysis

Data are presented as means ± SD. IC50s and densitometric values from cell viability assays and western blot experiments respectively that had been repeated 3 times or more were subjected to statistical analysis. Data analysis for IC50s was performed using GraphPad Prism (GraphPad software Inc. San Diego, CA) while ImageJ software was utilised to analyse for densitometry to quantify the level of signal from western blotting images. Two-tailed unpaired Student's t-test was used to compare the means of two independent groups. In all cases, differences with p values less than 0.05 were considered as significantly different.

CHAPTER THREE: Characterisation of the role of ERK5 in tumour angiogenesis *in vitro*

3.1 Introduction

MEK5 and ERK5 proteins are expressed in different tissues and cell lines (Zhou et al., 1995, Buschbeck and Ullrich, 2005). ERK5 is involved in various cellular functions including cell proliferation, differentiation, adhesion, migration and survival (Kato et al., 1998a, Wang and Tournier, 2006, Sawhney et al., 2009). Furthermore, ERK5 has been implicated in progression of some diseases such as cancer, ischemia and cardiac hypertrophy (Wang and Tournier, 2006, Takeishi et al., 2002, Montero et al., 2009b). Targeted deletion of *erk5* (Regan et al., 2002b, Yan et al., 2003), *mek5* (Wang et al., 2005) or *mekk3* (Yang et al., 2000) in mice results in death around E9.5-11.5 due to severe defects in vasculature revealed the importance of the ERK5 signalling pathway. Notably, developmental defects due to global *erk5* knockout in mice was also revealed in endothelial-specific deleted *erk5* in mice, but not in other cell types such as cardiomyocytes and hepatocytes revealing the significant role of ERK5 in endothelial cell function (Hayashi et al., 2004b, Hayashi and Lee, 2004, Roberts et al., 2009). However, the heterogeneity of endothelial cells rising from different vascular beds resulted in a little useful information regarding study the role of interesting protein in angiogenesis by using large-vessel endothelial cells such as HUVEC (Conway and Carmeliet, 2004). Angiogenesis is a process confined to microvasculature. Thus, *in vitro* study of microvascular endothelial cells gives appropriate representation of physiological events during angiogenesis *in vivo* (Cines et al., 1998, Hewett, 2009).

ERK5 is activated in response to pro-angiogenic growth factors VEGF, EGF and FGF-2 in HUVEC and has a potential role in mediating growth factor-stimulated angiogenesis (Hayashi et al., 2004a). Previous work in the group has shown that ERK5 plays an important role in HDMEC via regulating VEGF-mediated AKT phosphorylation and inhibiting apoptosis to facilitate tubular morphogenesis in cells (Roberts et al., 2010c). In the context of

tumour development, ERK5 has been implicated in tumour angiogenesis and integrity of vascularisation during development (Hayashi et al., 2005a). Over expression of ERK5 has implicated in tumourigenesis in many cancers, increased activation of ERK5 in oral squamous cell carcinoma is associated with advanced tumour stage (Sticht et al., 2008). ERK5 over-expression correlated with decreased disease-free survival in breast cancer and furthermore, MEK5 over-expression in prostate cancer is associated with bone metastases, poor survival and invasion (Montero et al., 2009a, Mehta et al., 2003b). In addition, high levels of ERK5 are involved in the development and progression of hepatocellular carcinoma (HCC) (Zen et al., 2009).

This chapter describes experiments performed in order to characterise ERK5 activation in both HDMEC and HeLa cells to analyse the role of ERK5 in tumour angiogenesis by utilising an *in vitro* model that is more representative of *in vivo* angiogenesis. Small molecule inhibitors of the MEK5/ERK5 signalling pathway (BIX02189 and XMD8-92) and other intracellular signalling molecules were used to define potential regulators and effectors of ERK5. Specifically, the effect of these inhibitors on angiogenesis in NHDF/HDMEC/HeLa co-culture was assessed to determine whether ERK5 represents a potential therapeutic target for tumour angiogenesis.

3.2 Characterisation of ERK5 activation

3.2.1 ERK5 protein expression in HDMEC and HeLa cells

Since the discovery of ERK5 in 1995, detection of ERK5 activation mostly involved protein separation by SDS-PAGE on an 8% acrylamide gel followed by western blotting using a polyclonal anti-BMK1/ERK5 antibody. During SDS-PAGE, phosphorylated ERK5 displays reduced electrophoretic mobility, thus migrating slower than the non-phosphorylated form of ERK5 resulting in a bandshift with the appearance of a separated band above the

main ERK5 protein band (Abe et al., 1996, Abe et al., 1997, Duff et al., 1995). This bandshift assay was used to confirm ERK5 activation. Since VEGF had been reported to induce ERK5 activation in HUVECs and MLCECs and EGF triggered ERK5 activation in HeLa cells, these agonists were used to determine the activation of ERK5 in HDMEC and HeLa cells (Hayashi et al., 2004a, Kato et al., 1998a, Esparis-Ogando et al., 2002b). Furthermore, the activation of ERK1/2 by VEGF in various endothelial cells and EGF in HeLa cells were known, thus ERK1/2 phosphorylation was assessed to determine whether these growth factors were biologically active (Dangelo et al., 1995, Mody et al., 2001b). HDMECs were stimulated or not with 50 ng/ml of pro-angiogenic growth factor, vascular endothelial growth factor (VEGF) and HeLa cells were also stimulated or not with 50 ng/ml of epidermal growth factor (EGF) for 10 min and then cells were lysed with RIPA lysis buffer. The total cell lysates were separated on 8% acrylamide SDS-PAGE gel. Western blot analysis using anti-ERK5 in EGF-stimulated HeLa cells revealed a slower migrating band above the main band at approximately 130 kDa suggesting that ERK5 is activated in HeLa cells by EGF (Figure 3.1). In contrast, VEGF stimulation of HDMECs did not show a mobility bandshift of ERK5 phosphorylation. To analyse this further, phosphorylation of Thr^{218}/Tyr^{220} residues of the T-E-Y motif located on the activation loop of ERK5 was probed with phospho-ERK5 antibody in order to detect ERK5 activation. The phosphorylation of ERK5 in HDMECs was detected by p-ERK5 antibody and clearly induced upon stimulation via VEGF (Figure 3.1). Furthermore, the use of p-ERK5 antibody on HeLa lysates confirmed the ERK5 activation results obtained from the mobility bandshift assay. VEGF and EGF stimulation resulted in ERK1/2 activation in HDMECs and HeLa cells suggesting that these growth factors are biologically active in these cells (Figure 3.1). Individual quantification of the stimulated upper p-ERK5 mobility bands were compared to an arbitrary value of 1.0 set for p-ERK5 bands in the basal condition.

Western blotting showed that ERK5 is activated in HDMECs and HeLa cells in response to VEGF and EGF respectively. In order to analyse this further the mRNA expression of VEGFR-2, EGFR-1, MEK5 and ERK5 in HDMECs and HeLa was also analysed by qRT-PCR. The *VEGFR2* mRNA was highly expressed in HDMECs by 8000-fold increase compared to HeLa cells while in contrast; EGFR-1 expression was highly increased in cancer cells (HeLa) by a 45-fold increase. The expression of *MEK5* and *ERK5* genes in cancer cells, represented here by HeLa cells was higher in comparison with HDMECs (Figure 3.2).

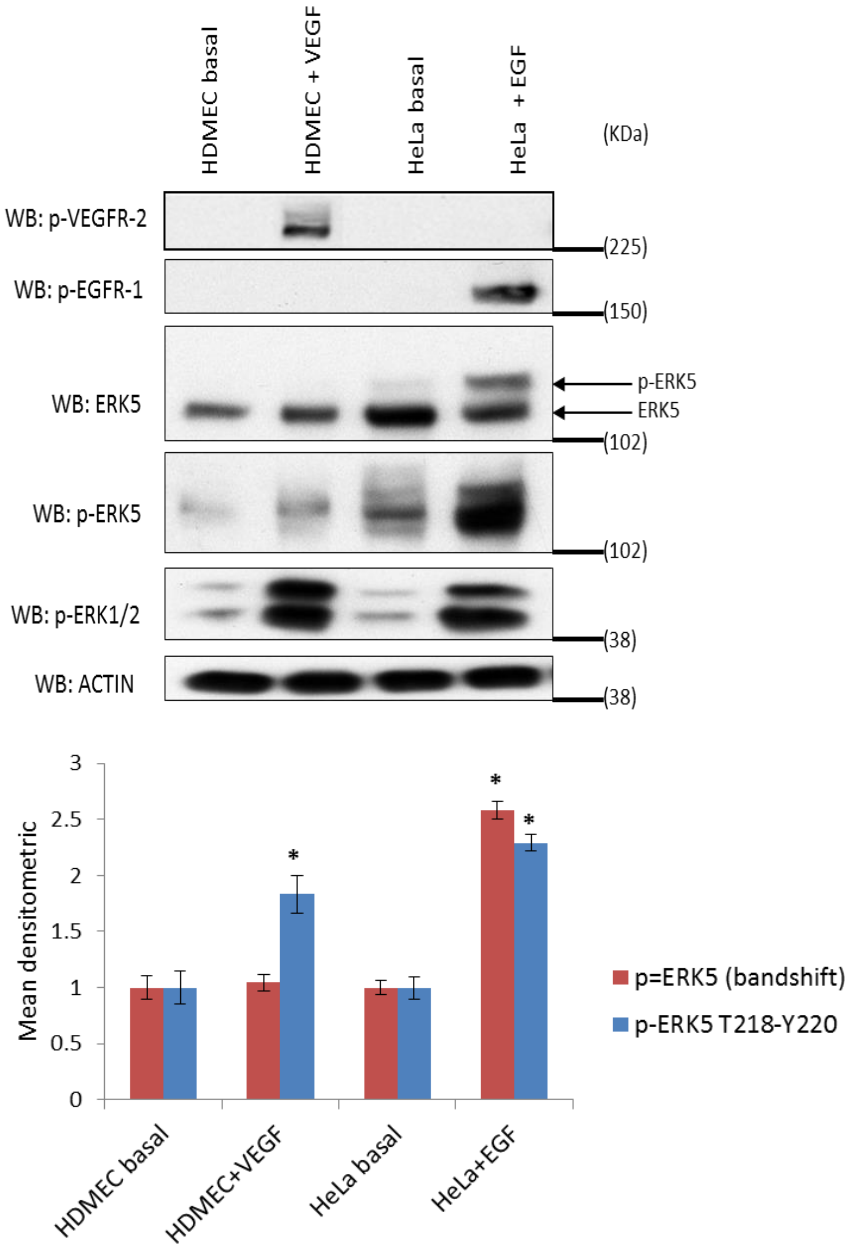


Figure 3.1 Characterisation of ERK5 activation in HDMECs and HeLa cells. HDMECs and HeLa cells were seeded on 12-well plates for 24 h, prior to overnight serum starvation. Cells were stimulated with 50ng/mL of VEGF and EGF for 10 min, followed by RIPA lysis. Protein lysates from HDMECs and HeLa cells were resolved on an 8% acrylamide gel, followed by Western blotting (WB) with antibodies against ERK5, p-ERK1/2, p-VEGFR, p-EGFR and actin as a loading control. Densitometric analysis of protein phosphorylation is relative to the basal control condition. The basal control condition was set arbitrarily as 1.0. This result is representative of three independent experiments (*$p<0.05$).

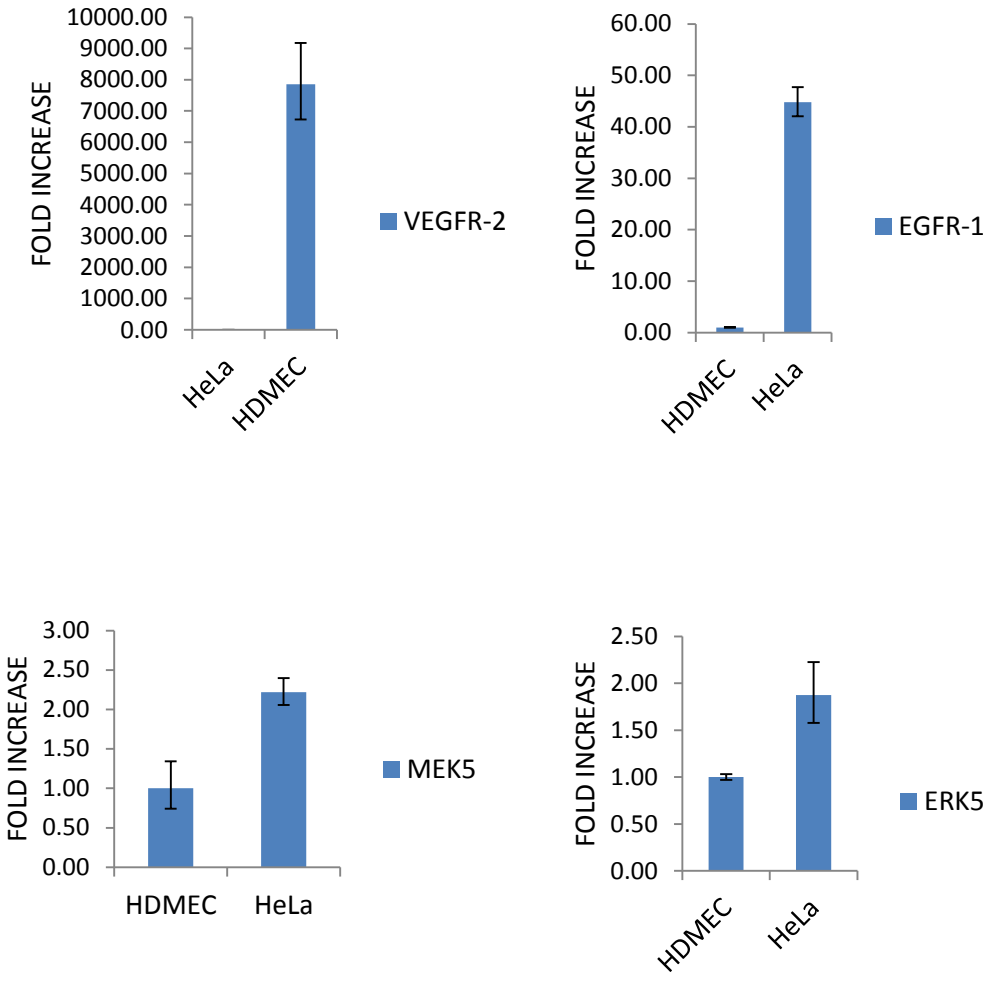


Figure 3.2 Analysis of gene expressions by qRT-PCR. The bar charts show RT-PCR analysis for VEGFR-2, EGFR-1, MEK5 and ERK5 mRNA level in HDMECs and HeLa cells. RNA of these cells was extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to β-actin expression and illustrated as fold change.

3.3 Small-molecule kinase inhibitors

BIX02189 (Tatake et al., 2008) and XMD8-92 (Yang et al., 2010c) are two small-molecule kinase inhibitors used for blocking the activation of MEK5 and ERK5 respectively. BIX02189 selectively inhibits MEK5 over MEK1/2 whereas XMD8-92 inhibits EGF stimulated ERK5 activation (Tatake et al., 2008, Yang et al., 2010b). These two inhibitors were validated by dose

response experiments conducted in HDMECs and HeLa cells to assess the effect on intracellular signalling pathways.

3.3.1 ERK5 activation was inhibited in response to XMD8-92

In HeLa cells, EGF-stimulation resulted in increased ERK5 activation compared to non-stimulated cells (Figure 3.3). Adding 0.1 μM XMD8-92 and subsequent doses 0.3 μM, 1 μM and 3 μM appeared to gradually decrease the phosphorylation of ERK5 in comparison with basal or 0.1% DMSO control. In addition 10 μM and 30 μM concentrations were able to reduce the activation of ERK5 to the level of the unstimulated control condition (Figure 3.3). The phosphorylation of AKT and ERK1/2 appeared unaffected by any concentration of XMD8-92. Also, EGF-mediated ERK5phosphorylation was not inhibited by XMD8-92 even at higher concentration 30 μM.

In HDMECs, stimulation with VEGF increased ERK5 activation in comparison with unstimulated cells (Figure 3.4). The addition of XMD8-92 displayed marginal reduction at 0.3 μM and 1 μM increasing from 3 μM up to 30 μM which nearly reached the level of ERK5 phosphorylation in basal or 0.1% DMSO untreated controls (Figure 3.4). The activation of AKT was slightly increased from the untreated condition to XMD8-92-treated condition at a concentration of 3 μM and then decreased with high concentrations back to control levels. The addition of XMD8-92 to HDMECs did not affect the phosphorylation of ERK1/2 whereas VEGF-mediated ERK5 phosphorylation was slightly inhibited at 30 μM compared to basal control (Figure 3.4).

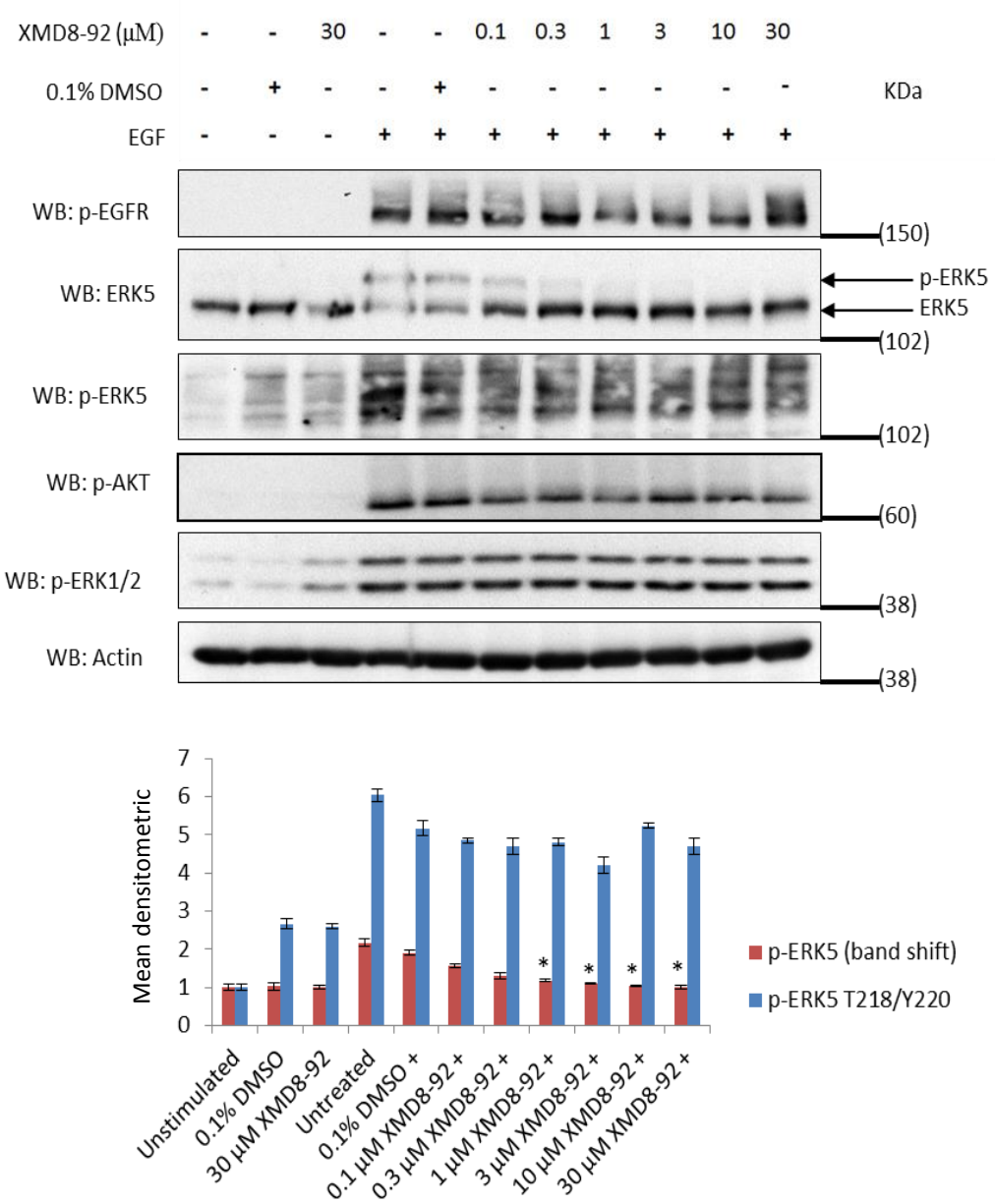


Figure 3.3 XMD8-92 dose responses in HeLa cells. HeLa cells were plated on 12-well plates for 48 h prior to overnight serum starvation. Cells were then pre-incubated with 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM and 30 µM XMD8-92 or 0.1% DMSO for 1 h, prior to EGF stimulation (50ng/mL) for 10 min and RIPA lysis. Proteins were separated on an 8% acrylamide gel followed by incubating with antibodies against p-EGFR, ERK5, p-AKT, p-ERK1/2 and Actin for western blotting (WB). Densitometric analysis of protein phosphorylation or protein expression relative to the basal control condition (set arbitrarily as 1.0).This result is representative of three independent experiments (*$p<0.05$) related to untreated cells.

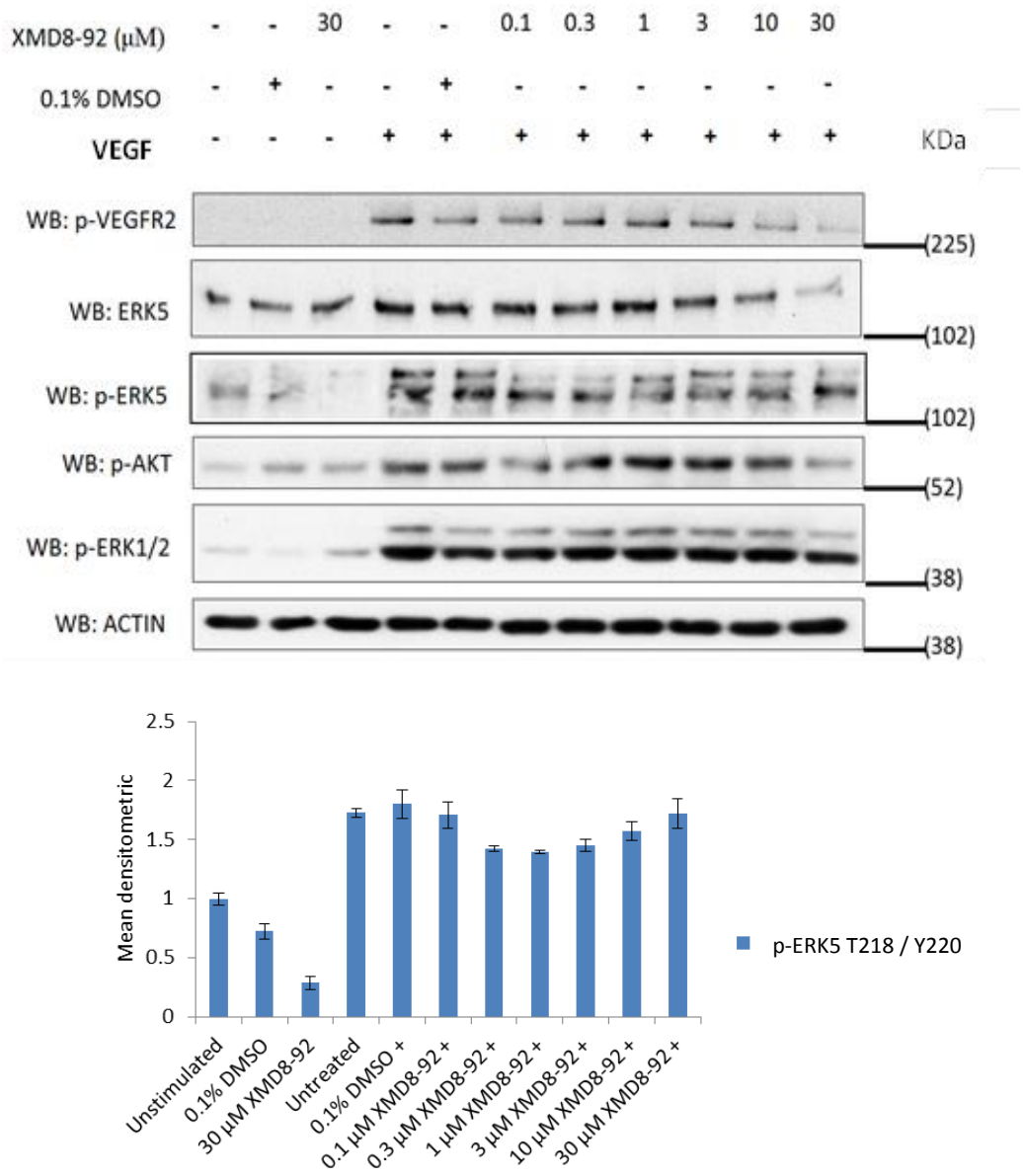


Figure 3.4 XMD8-92 dose responses in HDMECs. HDMECs were plated on 12-well plates for 48 h prior to overnight serum starvation. Cells were then pre-incubated with 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM and 30 µM XMD8-92 or 0.1% DMSO for 1 h, prior to VEGF stimulation (50ng/mL) for 10 min and RIPA lysis. Proteins were separated on an 8% acrylamide gel followed by incubating with antibodies against p-VEGFR2, ERK5, p-AKT, p-ERK1/2 and Actin for western blotting (WB). Densitometric analysis of protein phosphorylation or protein expression relative to the basal control condition (set arbitrarily as 1.0).This result is representative of three independent experiments.

3.3.2 BIX02189 inhibits ERK5 activation

In HeLa cells, stimulation with 50ng/ml EGF resulted in more than a 3-fold increase in ERK5 phosphorylation compared to unstimulated basal and 0.1% DMSO controls (Figure 3.5). The addition of 0.1 μM, 0.3 μM and 1 μM BIX02189 appeared to decrease the activation of ERK5 in comparison with EGF-stimulated basal or 0.1% DMSO controls while 3 μM, 10 μM and 30 μM concentrations of BIX02189 reduced the phosphorylation of ERK5 to the unstimulated basal level (Figure 3.5). Phosphorylation of EGFR and ERK1/2 did not appear to be effected at any concentration of BIX02189, while activation of AKT appeared to be slightly increased at 3 μM and reached 7-fold increase at 30 μM compared to EGF-stimulated basal (Figure 3.5).

In HDMECs, VEGF-stimulation produced an increase in ERK5 activation compared to unstimulated cells (Figure 3.6). Treatment with BIX02189 decreased the phosphorylation of ERK5 in comparison with stimulated basal or 0.1% DMSO controls detected by Thr^{218}/Tyr^{220} specific p-ERK5 antibody (Figure 3.6). Phosphorylation of VEGFR2 was reduced at 0.1 μM BIX02189 and started to gradually increase at 1 μM until it reached 3-fold increase at 30 μM compared to the stimulated control suggesting that MEK5 may play a negative regulation role in VEGFR2 signalling. ERK1/2 activation was not affected by BIX02189 concentrations up to 10 μM; however activation of ERK1/2 was inhibited at 30 μM reducing the level to that in the unstimulated BIX02189-treated control and that could relate to the potential non-specific and toxic effect of this inhibitor at this concentration. BIX02189 did not appear to have an effect on the activation of AKT up to 3 μM. However the higher concentrations 10 μM and 30 μM decreased the phosphorylation of AKT to 1.5 and 2-fold respectively in comparison with stimulated control and that could suggest that VEGF-mediated ERK5 activation is required for activation of AKT and ERK1/2 (Figure 3.6).

These data suggested that 3 µM BIX0218 is sufficient to inhibit the MEK5/ERK5 signalling pathway without affecting the parallel ERK1/2 pathway in HDMEC and HeLa cells.

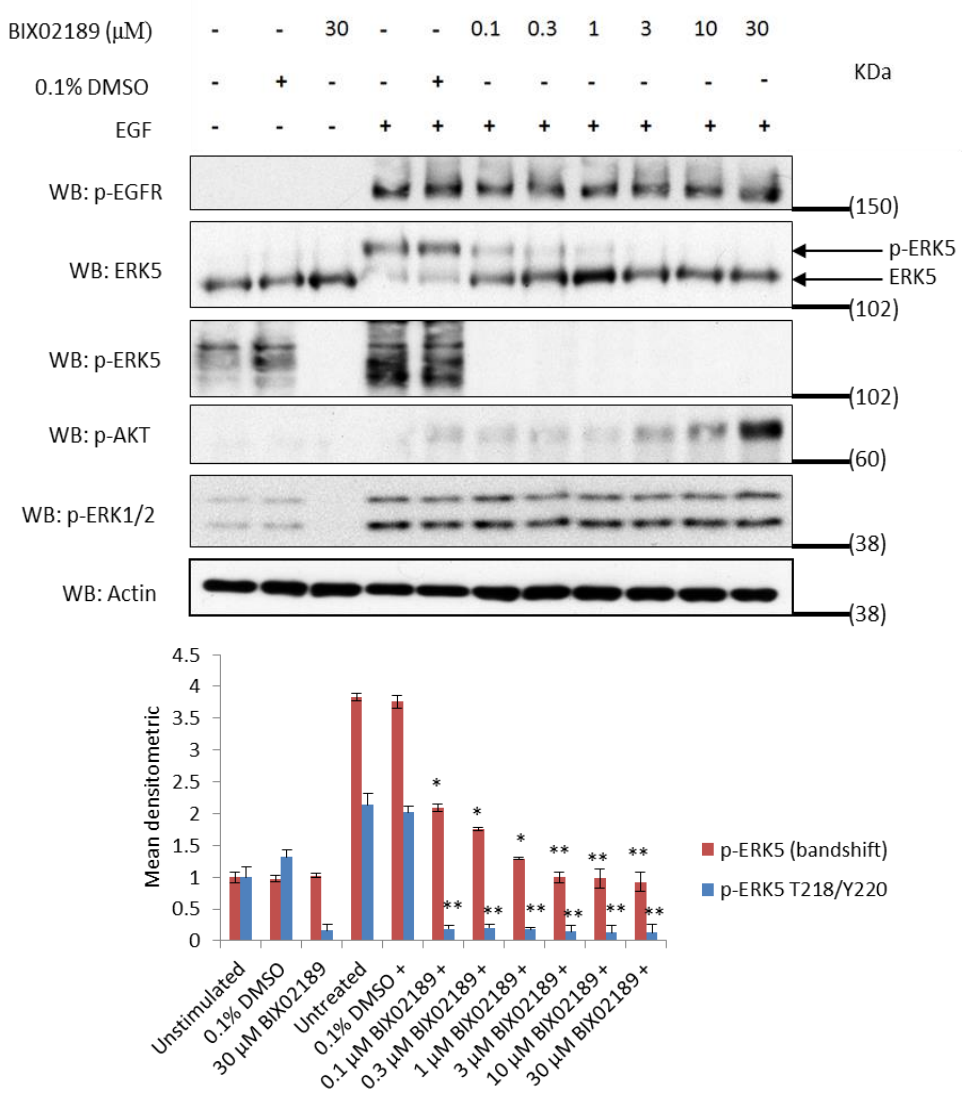


Figure 3.5 BIX02189 dose responses in HeLa cells. HeLa cells were plated on 12-well plates for 48 h prior to overnight serum starvation. Cells were then pre-incubated with 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM and 30 µM BIX02189 or 0.1% DMSO for 1 h, prior to EGF stimulation (50ng/mL) for 10 min and RIPA lysis. Proteins were separated on an 8% acrylamide gel followed by incubating with antibodies against p-EGFR, ERK5, p-AKT, p-ERK1/2 and Actin for western blotting (WB). Densitometric analysis of protein phosphorylation or protein expression relative to the basal control condition (set arbitrarily as 1.0).This result is representative of three independent experiments (*p<0.05, **p<0.01) related to untreated cells.

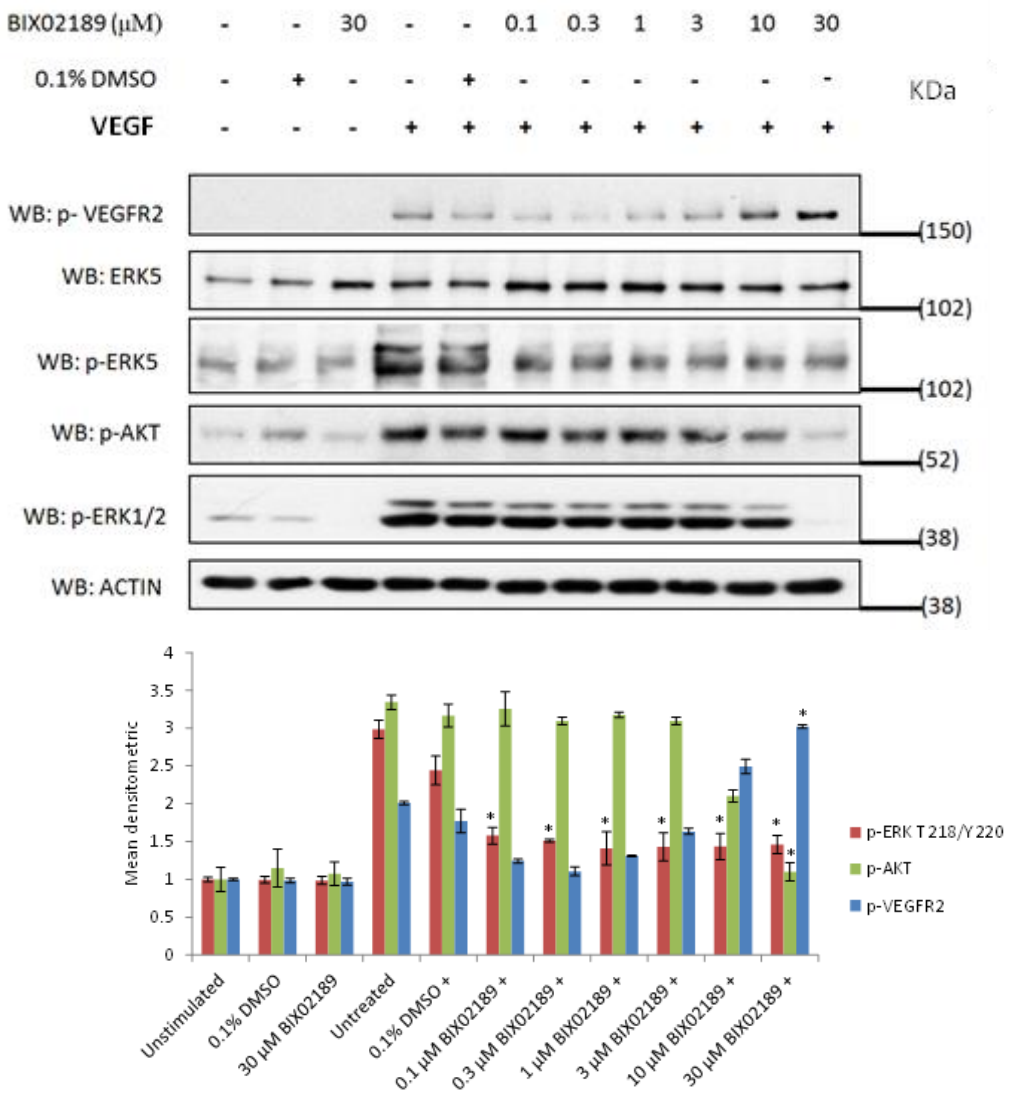


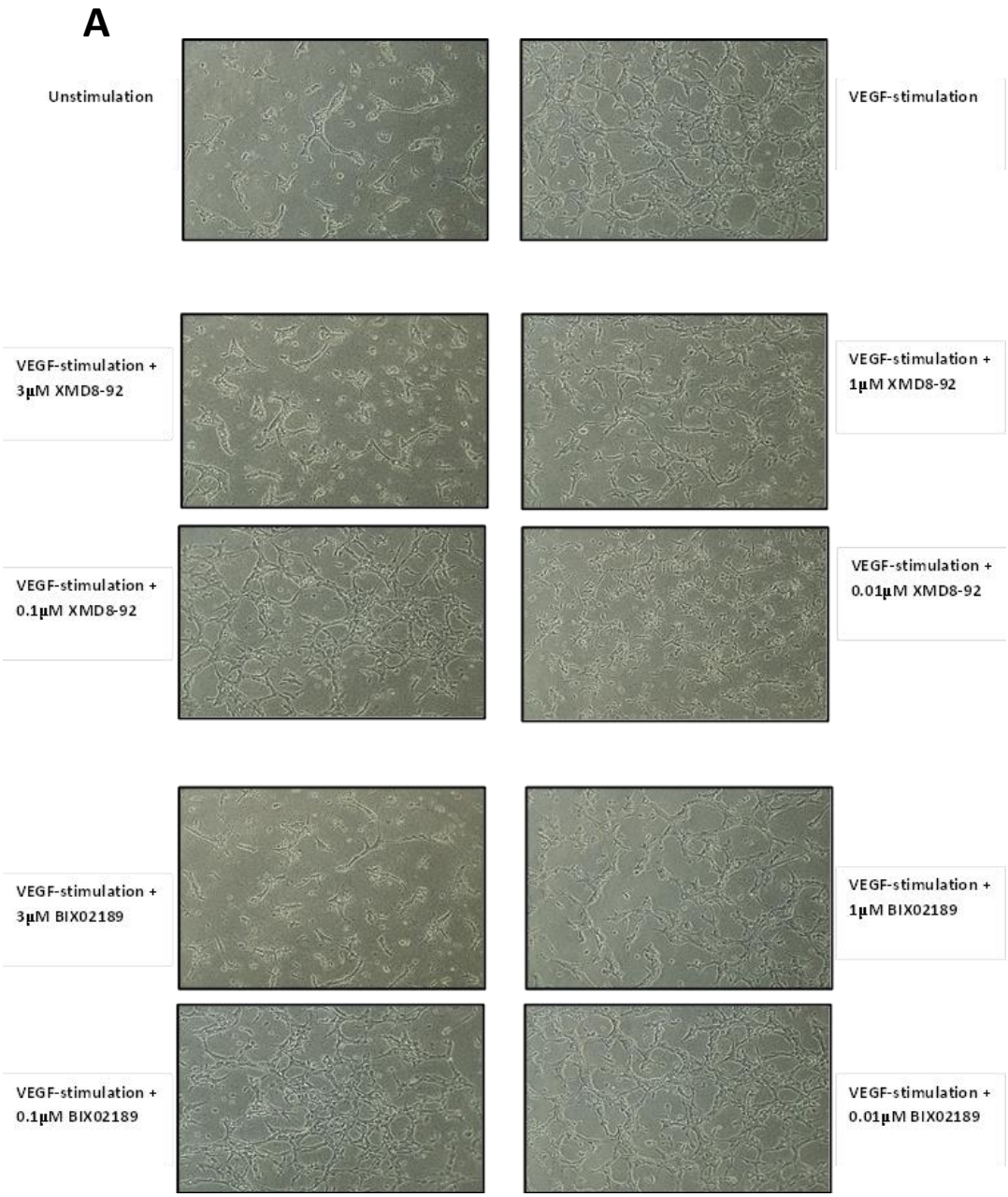
Figure 3.6 BIX02189 dose responses in HDMECs. HDMECs were plated on 12-well plates for 48 h prior to overnight serum starvation. Cells were then pre-incubated with 0.1 μM, 0.3 μM, 1 μM, 3 μM, 10 μM and 30 μM BIX02189 or 0.1% DMSO for 1 h, prior to VEGF stimulation (50ng/mL) for 10 min and RIPA lysis. Proteins were separated on an 8% acrylamide gel followed by incubating with antibodies against p-VEGFR2, ERK5, p-AKT, p-ERK1/2 and Actin for western blotting (WB). Densitometric analysis of protein phosphorylation or protein expression relative to the basal control condition (set arbitrarily as 1.0).This result is representative of three independent experiments (*$p<0.05$) related to untreated cells

3.4 Assessment of the effect of MEK5 inhibitor BIX02189 and ERK5 inhibitor XMD8-92 on VEGF-stimulated tubular morphogenesis in HDMECs

Stimulation of endothelial cells with pro-angiogenic growth factors such as VEGF and FGF-2, results in tubular morphogenesis to make capillary-like structures in a 3-D collagen gel (Montesano et al., 1983, Bohman et al., 2005). To determine wither growth factor stimulation could trigger tubular morphogenesis in HDMECs; endothelial cells were seeded between two layers of collagen (section 2.2.9.1) and stimulated with vascular endothelial growth factor (VEGF) for 24 hours. HDMECs without stimulation with VEGF, failed to form tube-like structure. However, stimulation with VEGF induced HDMECs to undergo tubular morphogenesis (Figure 3.7).

The importance of the MEK5/ERK5 signalling pathway in VEGF-mediated tubular morphogenesis in endothelial cells was assessed by using different concentrations of the kinase inhibitors BIX02189 and XMD8-92 to inhibit MEK5 and ERK5 expression respectively. Treatment of HDMECs with 3μM XMD8-92 had a profound effect upon VEGF-stimulated tubular morphogenesis by decreasing the tubes to the level of unstimulated control. Furthermore, even the 1μM, 0.1μM and 0.01μM concentrations produced a clear effect on VEGF-induced tubular morphogenesis in HDMECs in comparison with the stimulated control (Figure 3.7). Similarly, HDMEC treatment with 3μM MEK5 inhibitor (BIX02189) also abolished VEGF-induced HDMEC tube-like structures over 24 h. Moreover, adding 1μM BIX02189 to HDMECs suppressed the formation of tubular morphogenesis while 0.1μM and 0.01μM had a marginal effect in comparison with stimulated control (Figure 3.7).

This data suggested that 3μM of MEK5 and ERK5 inhibitors (BIX02189 and XMD8-92 respectively) is able to abolish VEGF-induced tubular morphogenesis in HDMECs in 3D collagen gel. Furthermore, these results show that ERK5 is required for VEGF-induced HDMEC tubular morphogenesis in agreement with previous data by using MEK5 and ERK5 siRNA (Roberts et al., 2010c).



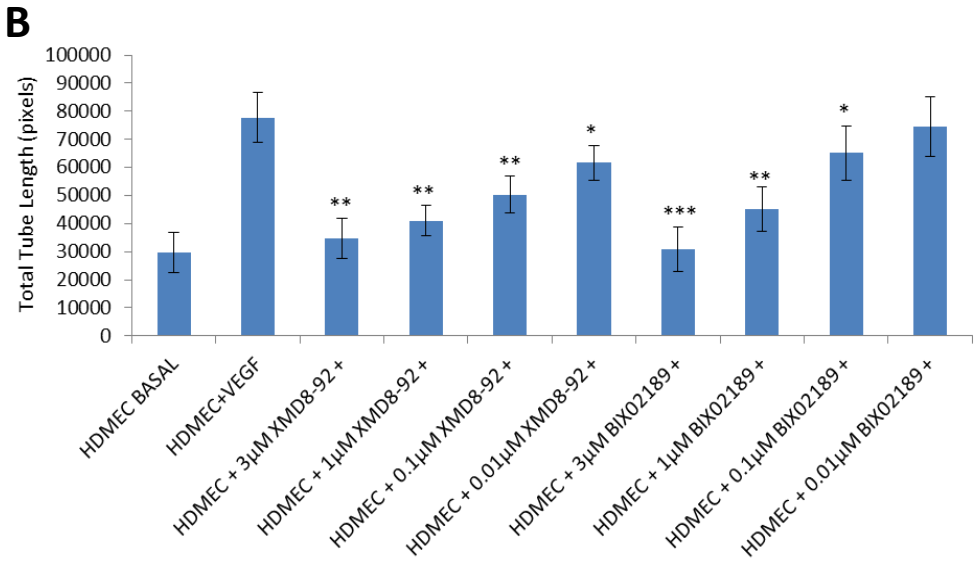


Figure 3.7 MEK5 and ERK5 inhibitors prevent VEGF-stimulated HDMEC tubular morphogenesis within 3-D collagen matrix. (A) HDMECs were seeded at 8.25 x 10^5 cells per dish in 10 cm dishes in 10 ml of EBM MV2 growth medium, and grown for 24 h. Cells were serum-starved in EBM MV2 basal medium containing 1% (v/v) FCS for 20 h prior to seeding between two layers of collagen I gel at 90,000 cells per well in 24-well cell-culture dishes (section 2.2.9.1). Cells seeded within the collagen I gels were treated with the MEK5 inhibitor BIX02189 and the ERK5 inhibitor XMD8-92 at different concentrations and incubated for 30 minutes. Cells were then stimulated with EBM MV2 basal medium containing 1% (v/v) FCS supplemented with 50ng/ml VEGF and incubated for 16-24 h at 37 °C. After that time, cells were fixed in 4% paraformaldehyde and visualised by inverted light microscope (section 2.2.9.2). (B)The total length of tubular structures was quantified from three fields (X 10 objective) per well using AngioQuant image analysis software. *p<0.05; **p<0.01; ***p>0.005

3.5 Assessment of the effects of MEK5/ERK5 signalling pathway inhibitors (BIX02189, XMD8-92) on tumour angiogenesis by using an *in vitro* endothelial / fibroblast/ tumour cell co-culture assay.

Angiogenesis involves the coordinated proliferation, migration and differentiation of endothelial cells to form a lumen containing vessel. This process is regulated by a balance between pro- and anti-angiogenic factors and when the balance is disrupted, pathological conditions can appear such as cancer (Folkman, 1995). Pro-angiogenic factors such as VEGF and bFGF

induce the formation of tumour blood vessels (Ferrara et al., 1992, Galzie et al., 1997). These growth factors directly bind to the receptor tyrosine kinases on the endothelial cell surface and stimulate endothelial cells to release proteolytic enzymes that degrade the extracellular proteins, allowing cell invasion and metastasis. Thus, inhibiting angiogenesis provided a promising strategy for the treatment of solid tumours (Folkman et al., 1971).

Although the 3-D collagen gel *in vitro* angiogenesis assay represents the early stage of *in vivo* angiogenesis, a disadvantage of this model is that tubular structures usually regress within 48 hours of formation (Montesano et al., 1983, Bohman et al., 2005, Matsumoto et al., 2002). However, endothelial cells in co-culture with fibroblasts undergo differentiation over several days to form well-established tube-like structures that remain stable for many days after formation and represent the capillary structure *in vivo* (Montesano et al., 1993, Bishop et al., 1999, Donovan et al., 2001, Roberts et al., 2010b, Richards and Mellor, 2016). Furthermore, endothelial/fibroblast co-culture assay is more easily quantified by automated computer analysis than a 3-D collagen assay.

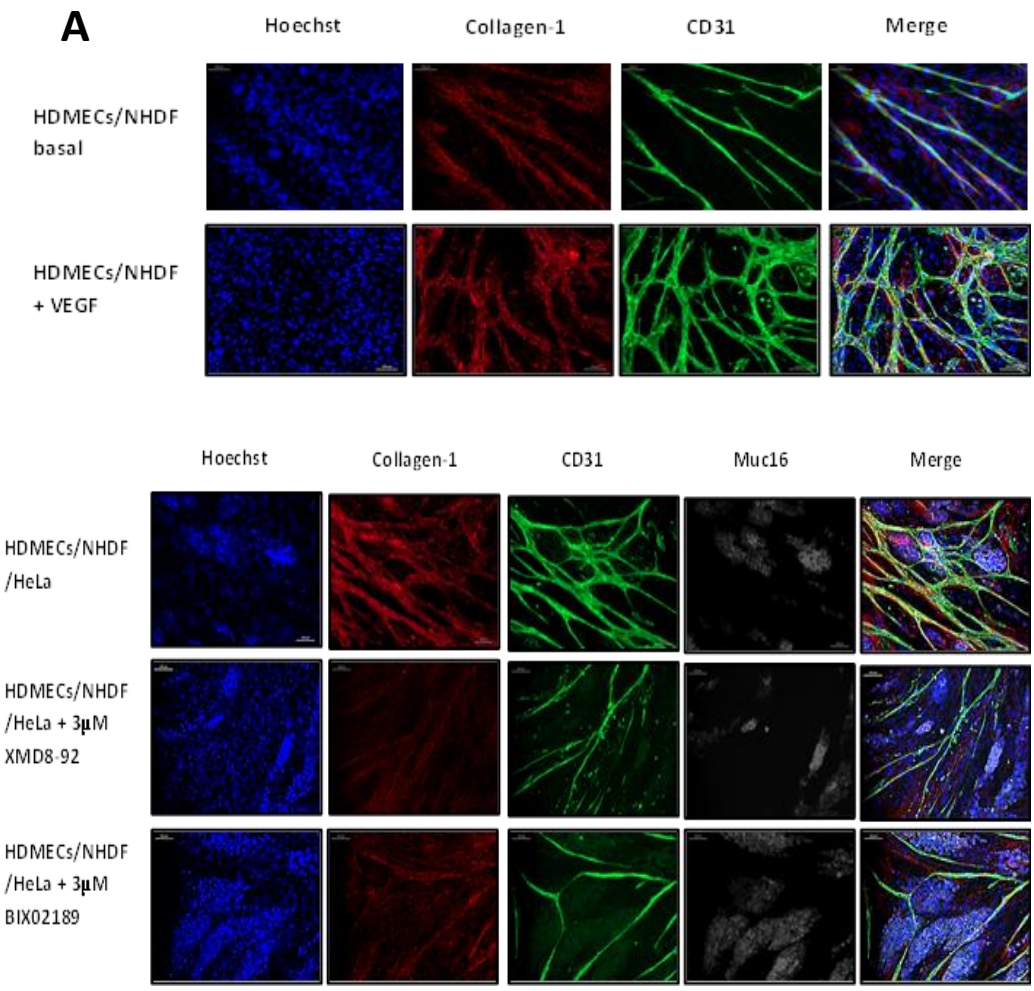
In this experiment, endothelial cells represent key cells involved in angiogenesis which are dependent on the interaction with mural cells such as fibroblasts. Fibroblasts produce extracellular matrix proteins which provide a base to promote endothelial cell migration and facilitate vessel remodelling (Staton et al., 2009). The pro-angiogenic growth factors that are released from tumour cells, bind to fibroblast and endothelial cells to assist the progress of 3-D organisation and tubulogenesis of endothelial cells (Sorrell et al., 2007). Therefore, endothelial cells co-cultured with fibroblasts provide a more realistic concept to the collagen tubular morphogenesis assay *in vitro* representation of *in vivo* angiogenesis (Donovan et al., 2001). Optimisation of the number of endothelial cells, HDMEC, seeded on the confluent fibroblast, NHDF, layers and the length of HDMEC/NHDF assay was conducted

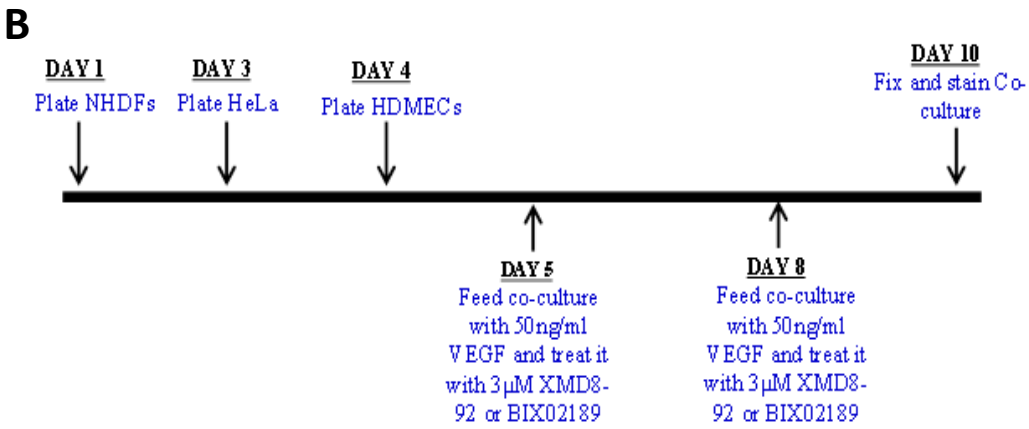
previously in our lab. In this study, the image analysis software termed 'AngioQuant' was used for quantification of tubule formation in HDMEC/NHDF/HeLa cells.

3.5.1 BIX02189 and XMD8-92 inhibit tumour cell-induced tube formation in a HDMEC/NHDF/HeLa co-culture assay

To determine the effect of MEK5 and ERK5 inhibitor treatment upon angiogenesis of HeLa cells in a HDMECs/NHDF/HeLa co-culture assay, HeLa cells were plated on a confluent monolayer of fibroblast cells prior to plating endothelial cells (HDMECs) on these layers on day 4 and the co-culture then treated with 3μM BIX02189 and/or XMD8-92 on day 5 and 8 and finally fixed on day 10 (as illustrated in Figure 3.8 b). Analysis of HDMECs co-cultured with NHDFs revealed that l VEGF stimulated tube-like networks compared to unstimulated basal control (Figure 3.8). The addition of tumour cells (represented by HeLa cells) to this co-culture induced tube formation by increasing the total tube length in comparison with unstimulated basal control which supports the ability of tumour cells to trigger networked vessels (Figure 3.8). Treatment of the HDMECs/NHDF/HeLa co-culture assay with 3μM XMD8-92 on day 5 and 8 of this assay had a notable effect upon tube formation by decreasing tube length compared to the untreated basal control. Similarly, treatment of this co-culture with a MEK5 inhibitor (BIX02189) impaired angiogenesis in this assay by shortening the tube length about 2-fold compared to the untreated basal control (Figure 3.8).

This data revealed that the HeLa cervical carcinoma cells are able to induce the tube formation. However, treatment with ERK5 inhibitor (XMD8-92) or its upstream MEK5 inhibitor (BIX02189) is able to inhibit the tumour angiogenesis.


A
Hoechst
Collagen-1
CD31
Merge
HDMECs/NHDF basal
HDMECs/NHDF + VEGF
Hoechst
Collagen-1
CD31
Muc16
Merge
HDMECs/NHDF /HeLa
HDMECs/NHDF /HeLa + 3µM XMD8-92
HDMECs/NHDF /HeLa + 3µM BIX02189


B
DAY 1
Plate NHDFs
DAY 3
Plate HeLa
DAY 4
Plate HDMECs
DAY 10
Fix and stain Co-culture
DAY 5
Feed co-culture with 50ng/ml VEGF and treat it with 3µM XMD8-92 or BIX02189
DAY 8
Feed co-culture with 50ng/ml VEGF and treat it with 3µM XMD8-92 or BIX02189

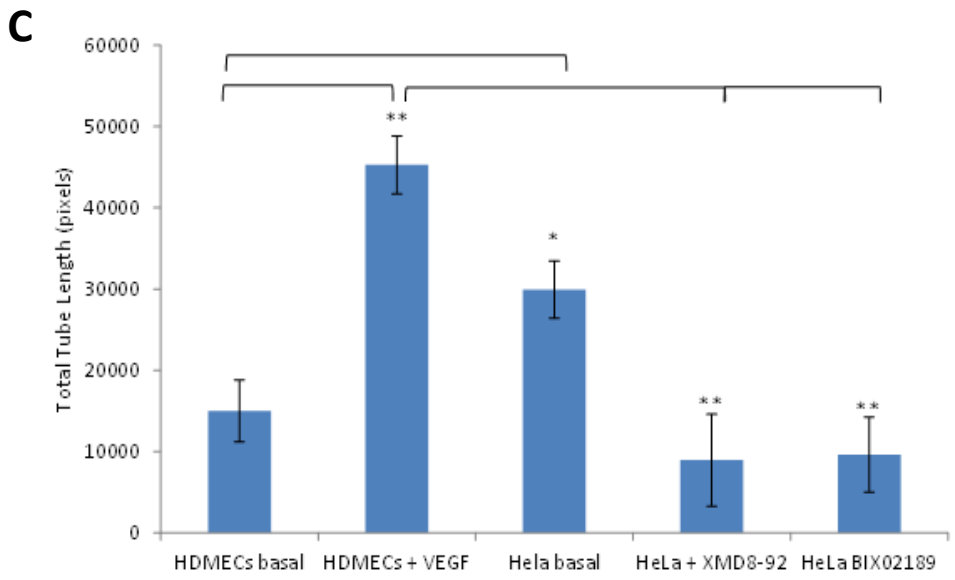


Figure 3.8 Effect of ERK5 and MEK5 inhibitors on tumour angiogenesis in HDMECs/NHDF/HeLa co-culture assay. (A) NHDF were seeded in in fibroblast growth medium at 20,000 cells per well on gelatine-coated 24-well plates and incubated for 48 hours. On day 3 HeLa cells were plated at 10,000 cells per well on NHDF layer and incubated for 24 hours. On day 4 of the assay, HDMECs were plated at 45,000 cells per well onto the confluent NHDF and HeLa layers. On day 5 and 8, co-cultures were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone or with indicated amount of BIX02189 and XMD8-92, as shown in the treatment schedule (B). On day 10, cells were fixed and stained with anti-CD31 to detect endothelial cells (green) or anti-collagen-1 to detect fibroblast (red) or anti-muc16 to detect HeLa cells (grey) (as described (section 2.2.9.4) , and total tube length was quantified using AngioQuant image analysis software. (C) Data is presented as total tube length from triplicate (n=3, mean ±SD) compared to basal. The result shown is representative of three independent experiments (*p< 0.05, **p<0.01).

3.5.2 The effect of MEK5/ERK5 signalling cascade inhibition on early vessels development of normal and tumour angiogenesis in HDMECs/NHDF -/+ HeLa co-culture assay

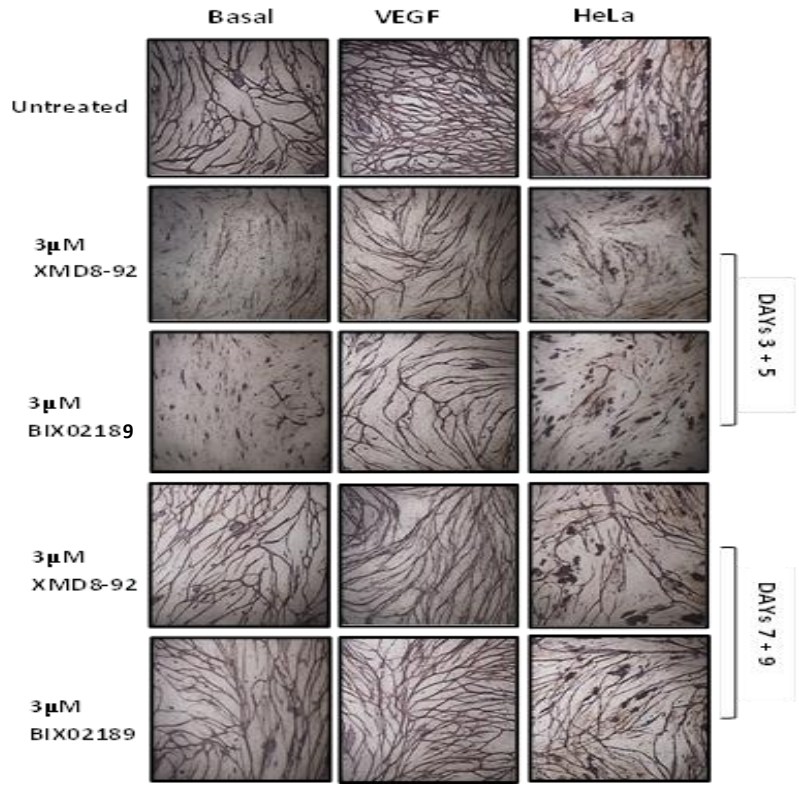
3-D collagen gel assay results revealed that ERK5 is critical for tubular morphogenesis below 24 hours which suggests that ERK5 may be required for early angiogenesis in response to pro-angiogenic factors such as VEGF. However, deletion of *erk5* in adult mice induced lethality due to endothelial apoptosis leading to cardiovascular defects (Hayashi et al., 2004b) suggesting

that ERK5 is also important for endothelial cell physiology in established vasculature. To compare the effect of XMD8-92 and BIX02189 at early neovascularisation and on established vessels, HDMECs were treated with an ERK5 inhibitor (XMD8-92) or MEK5 inhibitor (BIX02189) prior to plating onto a confluent NHDF or NHDF/HeLa layer on day 4 and the co-culture treated with these inhibitors prior to stimulation of the HDMEC/NHDF co-culture with VEGF on day 6 (Figure 3.9b). To analyse effects on established vessels, the co-culture was stimulated with VEGF on day 6 and treated with XMD8-92 and BIX02189 on day 7 and 9 before fixing on day ten. Treatment of HDMECs with 3μM XMD8-92 prior to plating onto NHDF notably decreased VEGF stimulated tube formation by 3-4 times compared to stimulated cells (Figure 3.9a). Similar to XMD8-92, treatment of HDMECs and co-culture with BIX02189 prior to stimulation with VEGF reduced tube formation by 3-4 fold in comparison with stimulated basal control (Figure 3.9a). By contrast, treatment of the co-culture with XMD8-92 or BIX02189 on day 7 and 9 of the assay only reduced VEGF stimulated tube formation 2 and 1 fold respectively in comparison to stimulated basal control. In the HDMEC/NHDF/HeLa co-culture there was a clear induction of tube formation from tumour cells by increasing the tubes two folds compared to unstimulated cells (Figure 3.9a and c). Treatment of HDMEC prior to plating onto NHDF/HeLa on day 3 and HDMEC/NHDF/HeLa co-culture on day 5 of the assay with 3μM of ERK5 inhibitor had a noticeable effect upon tumour-stimulated tubule formation compared to untreated basal control. However, treatment of HDMEC/NHDF/HeLa co-culture with XMD8-92 on day 7 and 9 of the assay had less effect upon network formation compared to untreated cells (Figure 3.9a). Quantification of total tube length showed that treatment of ERK5 inhibitor at early time points reduced tumour-induced tube formation by 4-fold while treatment the established vessels decreased tube-like network by 3-folds in comparison with untreated basal control (Figure 3.9c). Similar to XMD8-92, treatment of the co-culture with BIX02189 on

day 3 and 5 of the assay diminished tumour-stimulated tubule formation 4-times compared to the untreated basal control (Figure 3.9a and c). By contrast, treatment HDMEC/NHDF/HeLa co-culture with MEK5 inhibitor on day 7 and 9 on the assay reduced only about a single fold in comparison with the untreated basal control. Also this experiment was repeated and confirmed by using immunofluorescence staining of HDMEC/NHDF/HeLa co-culture which gave the same findings (Figure 3.10).

This data suggest that tubule formation in a HDMEC/NHDF or HDMEC/NHDF/HeLa co-culture assay is most sensitive to ERK5 and/or MEK5 inhibitor (XMD8-92 and BIX02189 respectively) treatment during early vessel development.

A



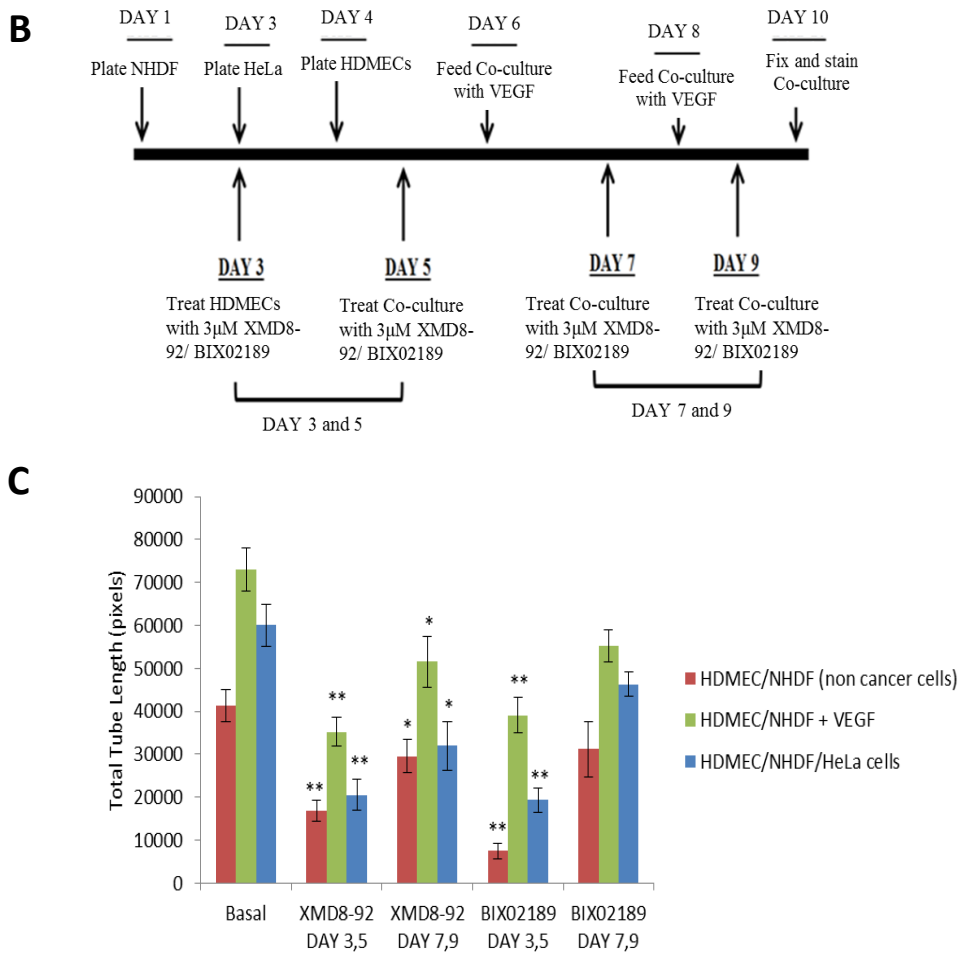
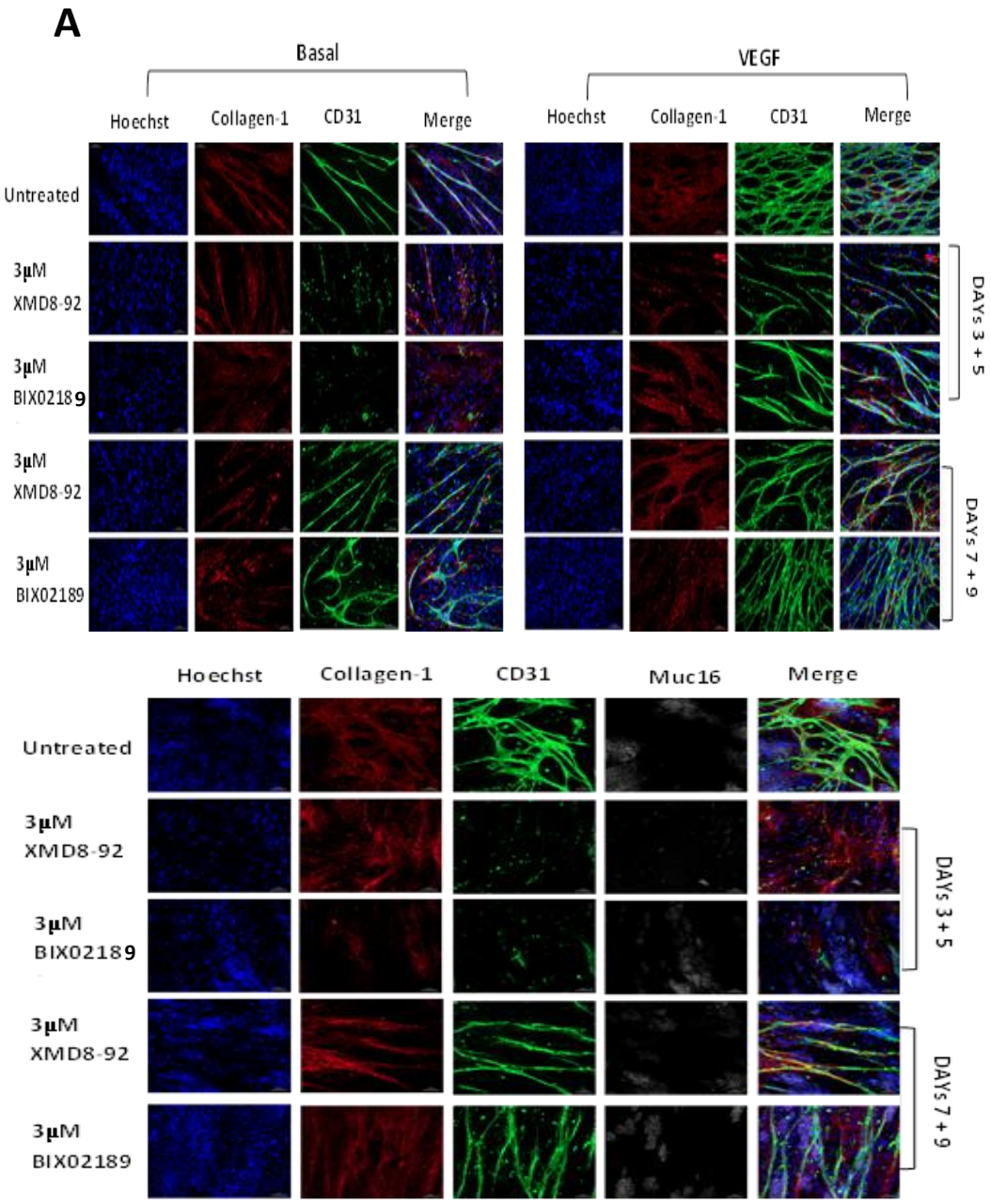


Figure 3.9 The effect of MEK5/ERK5 inhibitors, BIX02189 and XMD8-92, on tumour angiogenesis in a HDMEC/NHDF/HeLa co-culture assay (A) NHDF were seeded in fibroblast growth medium at 20,000 cells per well on gelatine-coated 24-well plates and incubated for 48 hours. On day 3, HDMECs were treated with 3µM MXD8-92 and BIX02189; HeLa cells were plated at 5,000 cells per well on NHDF layer and incubated for 24 hours. On day 4, HDMECs were plated at 45,000 cells per well onto the confluent NHDF and HeLa layers with EBM MV2 basal medium FGM. On day 5, co-culture media were changed to EBM MV2 basal medium containing 1% (v/v) FCS and the co-culture was treated with inhibitors. Day 7 and 9, co-cultures were treated with the indicated amount of BIX02189 and XMD8-92, as shown in the treatment schedule (B). On day 10, cells were fixed and stained as described (section 2.2.9.3.3), and total tube length was quantified using AngioQuant image analysis software. (C) Data is presented as total tube length from compared to basal. The result shown is representative of three independent experiments (*p< 0.05, **p<0.01).


A
Basal
VEGF
Hoechst
Collagen-1
CD31
Merge
Hoechst
Collagen-1
CD31
Merge
Untreated
3µM XMD8-92
3µM BIX02189
3µM XMD8-92
3µM BIX02189
DAYs 3 + 5
DAYs 7 + 9
Hoechst
Collagen-1
CD31
Muc16
Merge
Untreated
3µM XMD8-92
3µM BIX02189
3µM XMD8-92
3µM BIX02189
DAYs 3 + 5
DAYs 7 + 9

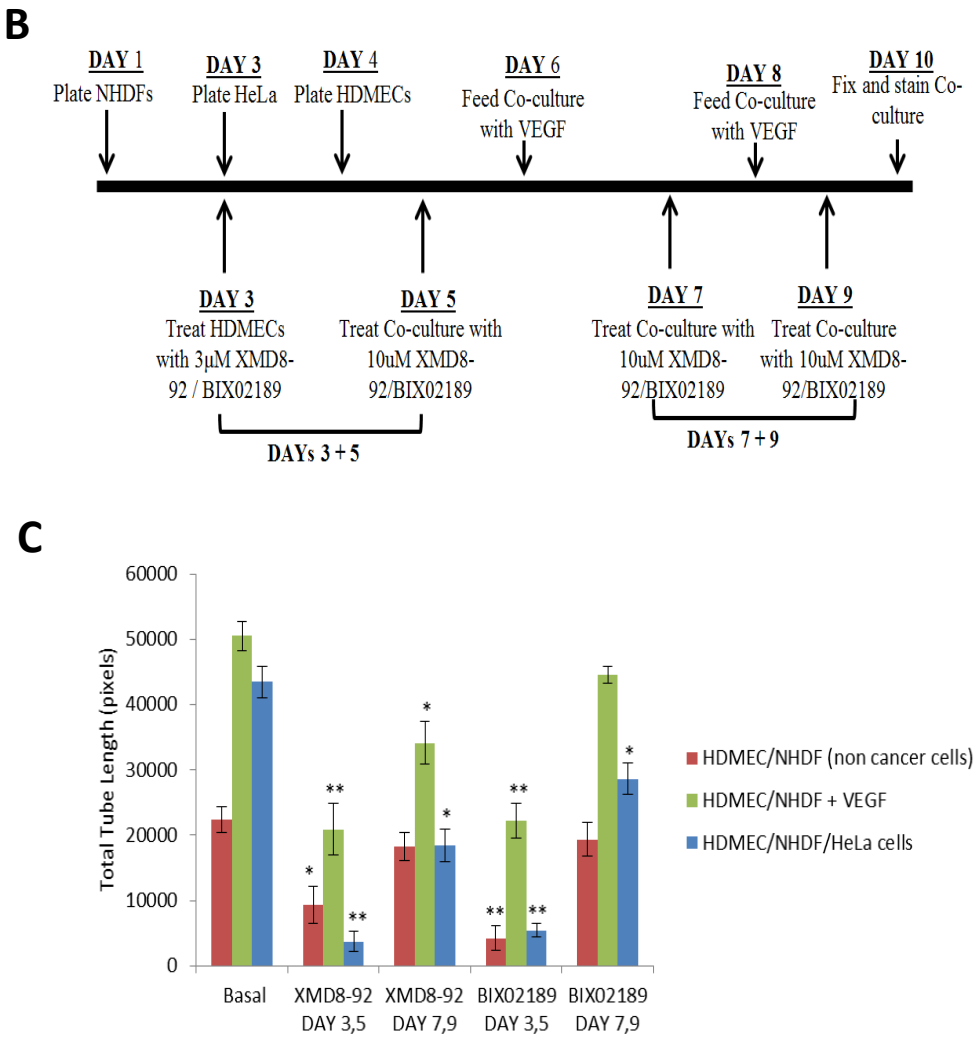


Figure 3.10 The effect of MEK5/ERK5 inhibitors, BIX02189 and XMD8-92, on tumour angiogenesis in a HDMEC/NHDF/HeLa co-culture assay. (A) NHDF were seeded in fibroblast growth medium at 20,000 cells per well on gelatine-coated 24-well plates and incubated for 48 hours. On day 3, HDMECs were treated with 3µM MXD8-92 and BIX02189; HeLa cells were plated at 5,000 cells per well on NHDF layer and incubated for 24 hours. On day 4, HDMECs were plated at 45,000 cells per well onto the confluent NHDF and HeLa layers with EBM MV2 basal medium FGM. On day 5, co-culture media were changed to EBM MV2 basal medium containing 1% (v/v) FCS and the co-culture was treated with inhibitors. Day 7 and 9, co-cultures were treated with the indicated amount of BIX02189 and XMD8-92, as shown in the treatment schedule (B). On day 10, cells were fixed and stained with anti-CD31 to detect endothelial cells (green) or anti-collagen-1 to detect fibroblast (red) or anti-muc16 to detect HeLa cells (grey) (as described section 2.2.9.4) and total tube length was quantified using AngioQuant image analysis software. (C) Data is presented as total tube length from compared to basal. The result shown is representative of three independent experiments (*p< 0.05, **p<0.01).

3.6 Discussion

The clear activation of ERK5 upon EGF stimulation in HeLa cells (Figure 3.1) confirms data from previous studies on ERK5 in these cells (Kato et al., 1998a, Kondoh et al., 2006, Yang et al., 2010c). In HeLa cells, the ERK5 protein underwent a mobility bandshift with conventional SDS-PAGE as a consequence of phosphorylation. ERK5 is known to undergo phosphorylation on the Thr^{218} / Tyr^{220} residues in the activation loop of the kinase domain which then facilitates C-terminal phosphorylation (Figure 3.1). In contrast, it was difficult to detect VEGF-mediated ERK5 activation in HDMECs via mobility bandshift with conventional SDS-PAGE (Figure 3.1). However, using p-Thr^{218} / Tyr^{220} antibody facilitated the detection of ERK5 phosphorylation in HDMECs upon VEGF stimulation (Figure 3.1).

The use of small molecule kinase inhibitors targeting MEK5 and ERK5 upon VEGF and EGF stimulation of ERK5 phosphorylation revealed that XMD8-92, which is an ATP-competitive inhibitor, prevented the phosphorylation of ERK5 in C-terminal residues in HeLa cells (Figure 3.3) while the dual phosphorylation of ERK5 in HDMECs was not affected (Figure 3.4) which means that XMD8-92 could affect the kinase activity of ERK5 which activate the transcriptional activation of C-terminal tail by auto-phosphorylation (Morimoto et al., 2007a). Furthermore, the inhibition of MEK5 with BIX02189 decreased the phosphorylation of ERK5 on Thr^{218} / Tyr^{220} in VEGF-stimulated HDMECs (Figure 3.6). In addition, the phosphorylation of C-terminal residues of ERK5 in HeLa cells was inhibited by BIX02189 (Figure 3.5). Thus, pharmaceutical inhibition of ERK5 only targets kinase activity in comparison with ERK5 knockout which leads to unfavourable effects on vascular integrity (Yang et al., 2010c). The siRNA mediated silencing of ERK5 expression has been previously shown to inhibit VEGF-stimulation tube formation in HDMECs (Roberts et al. 2010). Similar results were obtained using a small molecule ERK5 inhibitor.

To analyse the effect of small molecule kinase inhibitors XMD8-92 and BIX02189 upon VEGF stimulated tube formation in human dermal microvascular endothelial cells, a 3-D collagen angiogenesis assay which is highly representative of angiogenesis *in vivo* was applied. As is well established, VEGF is able to induce tubular morphogenesis, migration and survival of HDMECs in various angiogenic assays. In this chapter, VEGF induced HDMEC tube formation in a 3-D collagen gel (Figure 3.7). Treated HDMEC with different concentrations of XMD8-92 or BIX02189 decreased VEGF-induced tube formation in HDMEC in especially with 3μM and 1μM in comparison with untreated HDMEC with inhibitors (Figure 3.7) and thus could support the observation that the knockout of ERK5 in mice declined the blood vessels significantly in a Matrigel implant (Hayashi et al., 2005a). Also these findings confirmed that MEK5/ERK5 is required for VEGF-induced tubular morphogenesis in endothelial cells (Roberts et al., 2010c).

The ability of the MEK5/ERK5 inhibitors to prevent VEGF-stimulated angiogenesis may have therapeutic value in the treatment of aberrant angiogenesis. The 3-D collagen *in vitro* angiogenesis assay showed that XMD8-92 and BIX02189 MEK5/ERK5 inhibition prevented VEGF-stimulated HDMEC tube formation. To investigate the role of ERK5 inhibitor in VEGF-induced capillary like structure in NHDF/HDMEC -/+ tumour cell co-culture *in vitro* angiogenesis assay was applied, which more highly represented *in vivo* angiogenesis. Treatment of the HDMEC/NHDF/HeLa cells co-culture with dose response of MEK5/ERK5 pharmaceutical inhibitors (BIX02189 and XMD8-92) prevented tube-like structure formation at a concentration of 3 μM and demonstrating the potent anti-tumour effect of XMD8-92 and BIX02189 in tumour angiogenesis which support data that revealed that XMD8-92 blocks angiogenesis *in vivo* (Figure 3.8) (Yang et al., 2010c). Treatment of HDMECs with XMD8-92 or BIX02189 at an early stage of tubule formation (day 3 and 5) and at established capillary like-structures (day 7 and 9) provided an opportunity to study the role of ERK5 in

both early and established tubule formation. Treatment of HDMEC with XMD8-92 and BIX02189 on day 3 before plating on NHDF/HeLa and then treatment of NHDF/HDMEC/HeLa co-culture with XMD8-92 and BIX02189 on day 5 led to more potent inhibition of endothelial capillary formation which suggests that tube formation in HDMEC/NHDF/HeLa co-culture is most sensitive to MEK5/ERK5 signalling pathway inhibitor treatment during early angiogenesis (Figure 3.9).

Whilst there has been extensive study of the role of MEK5/ERK5 signalling pathway in tumour in the last decade, the current study is the first to investigate the inhibitory effect of the pharmaceutical inhibitors (BIX02189 and XMD8-92) on inhibition of the MEK5/ERK5 signalling cascade in NHDF/HDMEC/tumour cell co-culture.

CHAPTER FOUR: The role of ERK5 in tumour angiogenesis and drug-resistance in metastatic melanoma cell

4.1 Introduction

Melanoma is the most deadly skin cancer which originates from the melanocytes. Melanoma is known for its high metastatic potential, enhanced heterogeneity and also the ability to resist chemotherapy. It is the least common form of skin cancer (less than 5% of cases) but is considered the most deadly. Skin cancer is ranked the fifth most common cancer among men and sixth among women (National Cancer Institute, 2015). In 2015, an estimated 73,870 people will be diagnosed with melanoma in the USA and an estimated 9,940 of those diagnosed will die as a result of this malignancy (National Cancer Institute, 2015). In the UK, 100,000 new cases are diagnosed each year and this disease kills over 2500 patients each year (British Skin Foundation, 2015). Furthermore, Caucasian white populations are at high risk and are more likely to develop melanoma than black populations (Boyle, 2011).

The risk factors correlated with increased development of melanoma include environmental factors such as UV light and increased exposure to the sun. Family history as a genetic factor also plays an important role in the development of melanoma (Gray-Schopfer et al., 2005). Genetic mutations in melanocytes such as the p53 tumour suppressor gene and BRAF, NRAS and oncogenes, increase the risk of melanoma (Tran et al., 2008, Zaidi et al., 2012). BRAF plays a central role in the development of melanoma. Approximately 60% of melanoma cases have the BRAF mutation in the gene encoding the serine–threonine protein kinase B-RAF and about 20% have undergone an activating mutation in one of the *RAS* genes while the remaining 20% of melanomas are unknown (Flaherty et al., 2010, Davies et al., 2002). BRAF mutations are also found in about 55% of papillary thyroid and 18% of colorectal cancers as well as about 14% of ovarian cancers (Flaherty et al., 2010, Davies et al., 2002, Downward, 2003) . More than 75 mutations have been described in BRAF but the BRAF gene with valine to

glutamic acid substitution (V600E) is considered the most common mutation in BRAF (90%) which leads to constitutive activation of BRAF and the downstream MAPK signalling cascade (Davies et al., 2002). The RAS-RAF-MEK-ERK MAPK pathway plays a pivotal role in the progression of melanoma and is considered a therapeutic target for melanoma treatment (McCubrey et al., 2006). In August 2011, the FDA approved vemurafenib/PLX4032 (Zelboraf®) and PLX4720 (preclinical analog of PLX4032) which are selective BRAF V600E inhibitors for treatment of metastatic melanoma. This agent benefits as many as 80% of patients and improves overall survival compared to other chemotherapy agents such as dacarbazine (Chapman et al., 2011). Although these patients' responses to vemurafenib were encouraging, unfortunately virtually all patients acquired resistance to the agent within about 7 months and the disease relapsed (Chapman et al., 2011). Therefore, acquired resistance to BRAF inhibition is considered a challenge to long term survival for melanoma patients.

The melanoma cells acquire resistance to vemurafenib due to reactivation of the MAPK pathway. Alternative spliced forms of BRAF V600E and also activation of RTK (PDGFRβ) have been shown to play a role in acquired resistance to vemurafenib (Su et al., 2012).

This chapter describes the experiments performed in an effort to characterise the activation of ERK5 in BRAF V600E melanoma cancer cell lines and vemurafenib resistant counterparts. In addition, by utilising the pharmacologic inhibitors of the MEK5/ERK5 signalling pathway in combination with PLX4720 in a cell viability assay, the ability to reverse resistance in melanoma cells for these inhibitors has been defined. Furthermore, the effect of XMD8-92 and BIX02189 in combination with vemurafenib in tumour angiogenesis was analysed by using a tumour cell/endothelial/fibroblast co-culture assay.

4.2 Characterisation of ERK5 activation in A375 and A375R melanoma cancer cell lines

4.2.1 MAPKs signalling pathways in A375 and A375R

In mammals, mitogen-activated protein kinases (MAKPs) play a role in cell responses to growth factors and stress leading to proliferation, survival, motility and differentiation of cells (Cargnello and Roux, 2011b). MAPKs include ERK1/2, p38 (α, β, γ, and δ), JNK1 to -3 and ERK5.

In order to assess the profile of MAPK activity in A375 and A375R in response to 1μM PLX4720 and test whether these kinases in addition to AKT interfered with ERK5 activation in the melanoma resistant cell line, protein expression using western blot was conducted. A375 and A375R were incubated with 1μM PLX4720 for 24 hours and then lysed with RIPA lysis buffer prior to resolution on 8% SDS-PAGE acrylamide gel and western blot analysis with phospho antibodies directed against p-AKT, p-SAPK/JNK, p-p38 and p-ERK1/2.

Treatment with vemurafenib totally abrogated the phosphorylation of ERK1/2 in A375 cells whereas the same concentration, 1μM, of the drug in vemurafenib-resistant A375R cells incompletely decreased the phosphorylation of ERK1/2, in agreement with a previous report (Su et al., 2012). The p-JNK level was increased in the vemurafenib-resistant cells compared with vemurafenib-sensitive cells whereas activation of p38 MAPK and p-AKT were observed in both cell types (Figure 4.1). The receptor tyrosine kinase EGFR was activated in the vemurafenib-resistant cells but not in vemurafenib-sensitive cells (Figure 4.1). These results revealed that ERK5, SAPK/JNK and EGFR showed increased phosphorylation in A375R compared to A375.

4.2.2 ERK5 activation is increased in the PLX4720 resistant melanoma cell line (A375 R)

ERK5 is involved in many cellular functions including cell survival, cell proliferation and differentiation and has been implicated in the pathogenesis of many types of cancer (Lochhead et al., 2012). ERK5 is activated by a variety of growth factors and it has been reported that EGF and NGF activate ERK5 in many cell types (Kato et al., 1998b). All growth factors in Figure 4.3 especially EGF stimulated ERK5 phosphorylation in the A375 cell lines. ERK5 up-regulation was investigated in A375 and A375R cells in presence or not of PLX4720 to study the role of ERK5 in the resistant A375R cells.

The A375 and A375R cells were incubated with 1µM of PLX4720 or with 0.1% (v/v) DMSO vehicle control for 24 hours and then lysed with RIPA prior to SDS-PAGE using an 8% gel, followed by immunoblotting with anti-ERK5 (New England BioLab, Hitchin, UK). It was shown that ERK5 was activated in A375R in the absence of PLX4720 compared to A375 at the same state (Figure 4.1). In the present of 1µM of PLX4720, ERK5 was phosphorylated in the A375R resistant cell line two fold higher compared with A375 (Figure 4.1). Furthermore, EGFR was observed to be activated in A375R without any stimulation with EGF suggesting that EGFR could be implicated in the resistance of A375 cells to vemurafenib.

RT-PCR was also used to analyse the expression of *MEK5* and *ERK5* in A375 and A375R. The expression of MEK5 mRNA in A375R was increased slightly above A375 (Figure 4.2). In case of the expression of ERK5 mRNA, the A375R cells showed that ERK5 was more expressed in these cells compared to the basal (Figure 4.2). These data suggest that activation of ERK5 may play a role in vemurafenib resistant in melanoma cells.

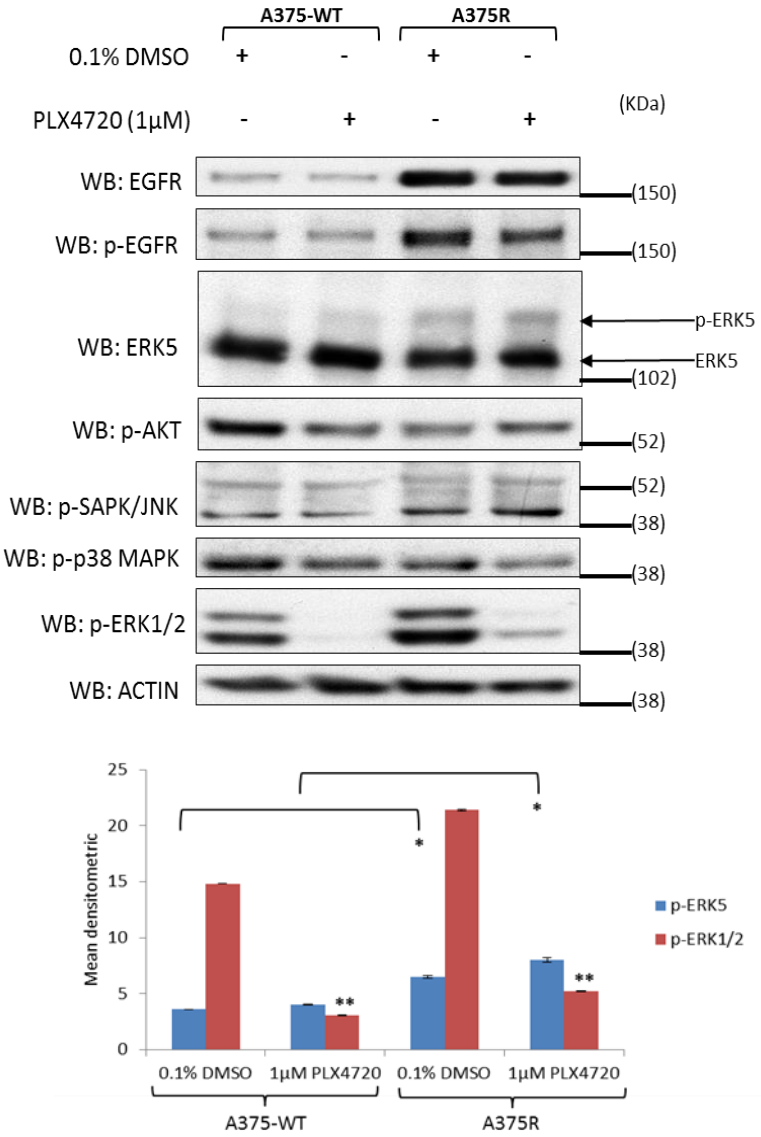


Figure 4.1 Intracellular signalling in vemurafenib-sensitive and resistant A375 cells. A375 and A375R were seeded in 12-well plate for 24 hours and then incubated with 1μM PLX4720 or 0.1% DMSO vehicle control prior to lysis of the cells with RIPA lysis buffer. Protein lysates from A375 and A375R were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-EGFR, p-AKT, p-SAPK/JNK, p-p38 MAPK, p-ERK1/2 and Actin. Analysis of densitometry of protein phosphorylation or protein expression is relative to basal control condition of each cell type. This experiment is representative of three independent experiments (*p< 0.05, **p<0.001).

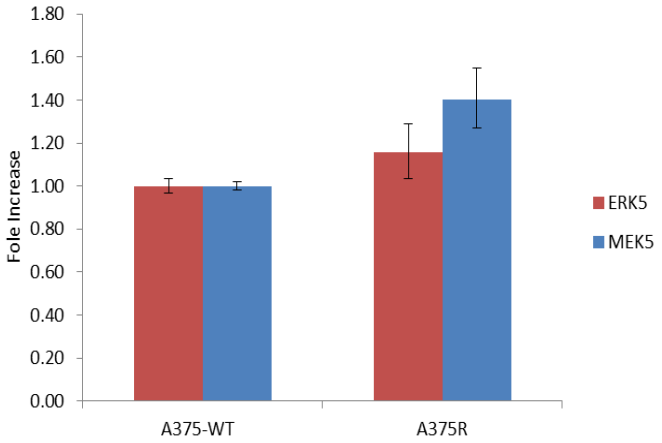


Figure 4.2 MEK5 and ERK5 mRNA expression in A375 and A375R. The bar chart shows RT-PCR analysis for MEK5 and ERK5 mRNA level in A375 and A375R. The melanoma cells RNA were extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to β-actin expression and illustrated as fold change. Both experiments is representative of four individual experiments (*p<0.05).

4.2.3 ERK5 is activated in A375 and A375R in response to growth factors

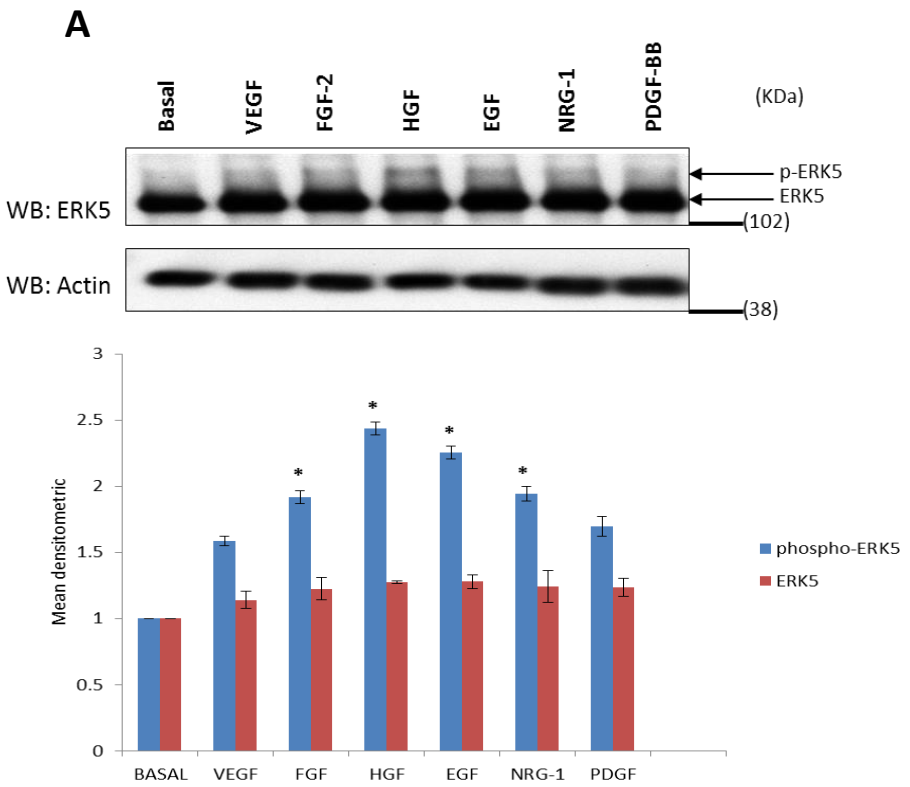
A375 are human malignant melanoma cells derived from the skin of a 54 year old female patient with malignant melanoma (Yang et al., 2010a). These cells were kindly provided by Prof Richard Marais, CRUK Manchester Cancer Institute. A375 cells express the BRAF (V600E) mutation. The A375R cells are vemurafenib resistance and were generated by chronic exposure of A375 cells to increasing levels of PLX-4720 (0.01μM-0.1μM over 8 weeks).

As mentioned in Chapter Three, phosphorylated ERK5 on an 8% SDS-PAGE gel exhibited reduced electrophoretic mobility, migrating slower than the non-phosphorylated form of ERK5, a phenomenon called bandshift.

Various growth factors, VEGF, FGF-2, HGF, EGF, NRG-1 and PDGF-BB were applied to evaluate the profile of ERK5 activation in A375 and A375R, by means of a mobility bandshift. The cells were stimulated or not (basal)

with growth factors for 10 minutes and then lysed with RIPA lysis buffer. The total cell lysates were separated on 8% acrylamide SDS-PAGE gel and analysed by western blot using a commercially available ERK5 antibody (New England BioLab, Hitchin, UK) (Figure 4.3).

It was found that in A375 and A375R cells, all the utilised growth factors stimulated ERK5 activation compared to the control basal condition as their stimulation resulted in an ERK5 mobility bandshift. In A375 cells, both HGF and EGF were the agonists that most increased the activation of ERK5 compared to the other agonists (Figure 4.3a). EGF stimulation of ERK5 activation in A375R was greater than the other agonists (Figure 4.3b).
Quantification of the stimulated lower ERK5 and upper ERK5 mobility bands were compared to an arbitrary value of 1.0 set for both ERK5 and p-ERK5 bands in the basal condition.



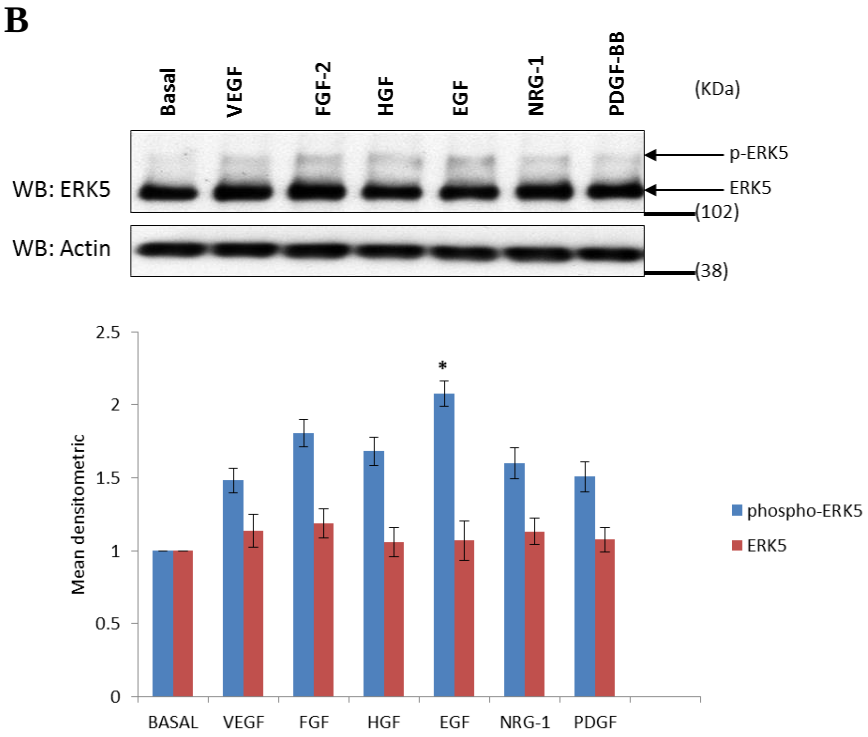


Figure 4.3 Characterisation of ERK5 activation in A375 and A375R in response to growth factors using electrophoretic mobility band shift. (A) A375 cells were seeded in 12-well plate for 24 hours and then serum starved overnight. Cells were stimulated with 50ng/ml of VEGF, FGF-2, HGF, EGF, NRG-1 and PDGF-BB followed by RIPA lysis. (B) A375R cells were seeded in 12-well plate for 24 hours and then serum starved overnight. Cells were stimulated with 50ng/ml of VEGF, FGF-2, HGF, EGF, NRG-1 and PDGF-BB followed by RIPA lysis. Protein lysates from A375 and A375R were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5 and Actin. Analysis of densitometry of protein phosphorylation or protein expression was relative to basal control condition of each cell type which was set arbitrarily as 1.0. This experiment is representative of three independent experiments (*p< 0.05) compared to basal control.

4.3 Small molecule kinase inhibitors

BIX02189 and XMD8-92 are two small-molecule kinase inhibitors used for blocking the activation of MEK5 and ERK5 respectively. BIX02189 selectively inhibits MEK5 over MEK1/2 whereas XMD8-92 inhibits EGF stimulated ERK5 activation (Tatake et al., 2008, Yang et al., 2010b). TRAMETINIB (trametinib) inhibits MEK1/2 kinase activity and blocks RAF-dependent MEK1/2 phosphorylation (Gilmartin et al., 2011). Lapatinib

is a dual EGFR-1/HER2 TK inhibitor which has the ability to act as an ATP competitor and targets the kinase domain of HER2 and EGFR-1 (D'Amato et al., 2015).

4.3.1 PLX4720 combined with XMD8-92 or BIX02189 but not trametinib or lapatinib inhibits ERK5 activation in A375 R

In the vemurafenib-sensitive A375 cells, the addition of 1μM PLX4720 combined with small molecule kinase inhibitors (BIX02189, XMD8-92 or lapatinib) appeared to inhibit ERK5 activation (Figure 4.4). In contrast, the combined treatment of PLX4720 with trametinib increased the phosphorylation of ERK5 in the parental cell line.

As A375 cells are a vemurafenib-sensitive cell line, the treatment of these cells with PLX4720 abolished the phosphorylation level of ERK1/2 due to the inhibition of its upstream BRAF activity (Figure 4.4). The combination of a B-RAF inhibitor (PLX4720) with XMD8-92, BIX02189 or lapatinib but not trametinib induced the activation of ERK1/2 in the A375 cells (Figure 4.4). In addition, PLX4720 combined with these kinase inhibitors did not appear to affect the phosphorylation of AKT which is important in cell survival and apoptosis (Figure 4.4). BIX02189 and XMD8-92 combined with PLX4720 blocked the phosphorylation of EGFR-1 compared to the 0.1% DMSO vehicle control (Figure 4.4).

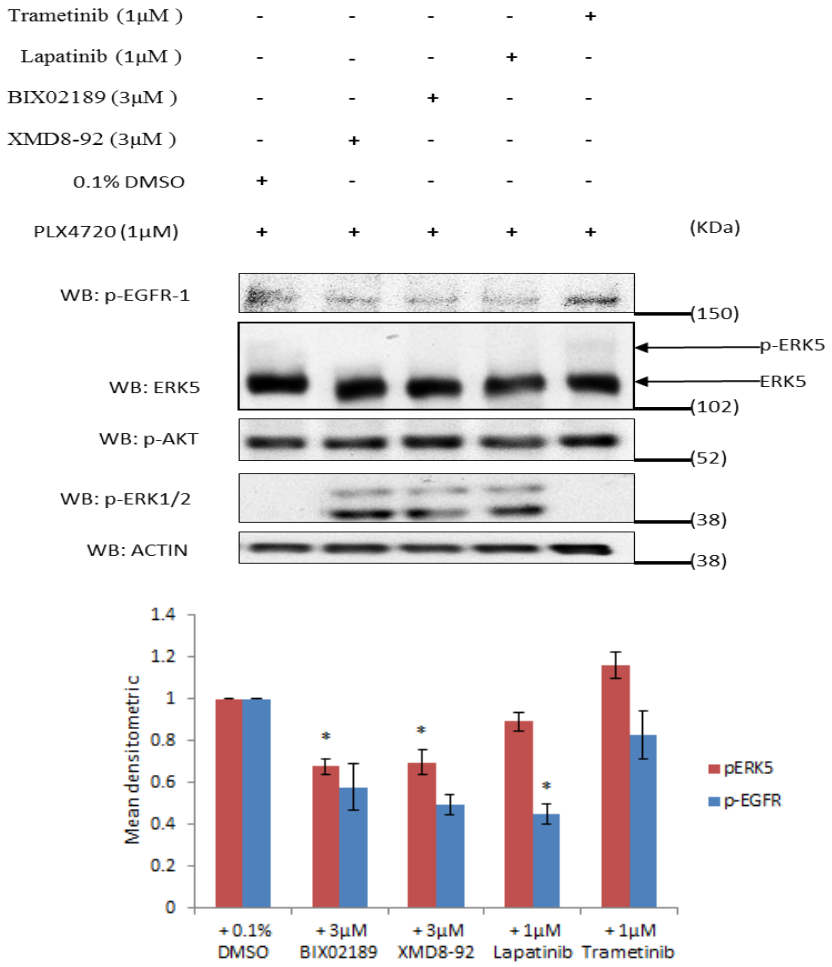


Figure 4.4 Small-molecule kinase inhibitors in A375. A375 cells were seeded in a 12-well plate for 24 hours and then incubated with 1μM PLX4720 for 24 hours and 0.1% DMSO vehicle control, 3μM XMD8-92, 3μM BIX02189, 1μM lapatinib or 1μM trametinib were added to the drugged cells and incubated for 1 hour prior to lyse of the cells with RIPA lysis buffer. Protein lysates from A375 cells were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-EGFR, p-AKT, p-ERK1/2 and Actin which was used as a loading control. Analysis of densitometry of protein phosphorylation or protein expression is relative to basal control condition of each cell type which was set arbitrarily as 1.0. This experiment is representative of three independent experiments (*p< 0.05).

In the vemurafenib-resistant A375R cells, the combination of 1μM PLX4720 with 3μM XMD8-92 and BIX02189 inhibited p-ERK compared with the 0.1% DMSO vehicle control (Figure 4.5). The high sequence identity and significant similarity between MEK1/2 and MEK5 suggested that the first generation of MEK1/2 inhibitors such as PD98059 could inhibit MEK5 and

ERK5 MAPK activity (Fremin and Meloche, 2010), however 1µM trametinib combined with PLX4720 did not appear to suppress ERK5 activation in A375R as the slower electrophoretic band (p-ERK5) on the 8% SDS-PAGE acrylamide gel was detected (Figure 4.5). Furthermore, ERK5 phosphorylation in vemurafenib-resistant A375R cells increased even in the presence of lapatinib and PLX4720, suggesting that ERK5 is not dependent upon EGF for activation in PLX4720-resistant melanoma cells.

The phosphorylation of ERK1/2 and AKT did not have an effect by the combination of PLX4720 and kinase inhibitors except trametinib which blocked p-ERK1/2 as an inhibitor for this kinase (Figure 4.5).

XMD8-92 and BIX02189 with PLX4720 appeared to induce the activation of EGFR with an increase in XMD8-92/PLX4720 compared to the 0.1% DMSO vehicle control (Figure 4.5). Trametinib/PLX4720 inhibited p-EGFR compared to 0.1% DMSO/PLX4720. These data together suggest that the ERK1/2 signalling pathway and EGFR do not play a role in MEK5/ERK5 signalling activation in the PLX4720-resistant melanoma cells.

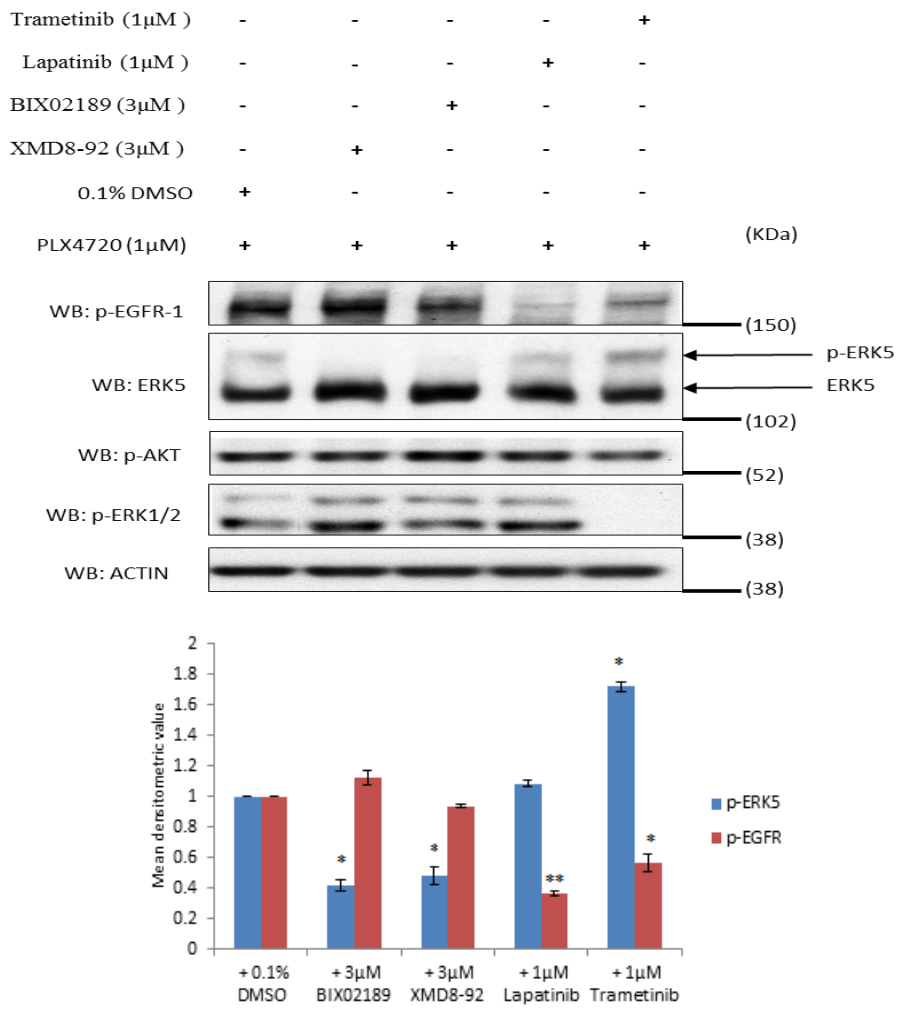


Figure 4.5 Small-molecule kinase inhibitors in A375R. A375R cells were seeded in a 12-well plate for 24 hours and then incubated with 1μM PLX4720 for 24 hours and 0.1% DMSO vehicle control, 3μM XMD8-92, 3μM BIX02189, 1μM lapatinib or 1μM trametinib were added to the drugged cells and incubated for 1 hour prior to lyse of the cells with RIPA lysis buffer. Protein lysates from A375R cells were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-EGFR, p-AKT, p-ERK1/2 and Actin which was used as a loading control. Analysis of densitometry of protein phosphorylation or protein expression is relative to basal control condition of each cell type which was set arbitrarily as 1.0. This experiment is representative of three independent experiments (*p< 0.05, **p<0.01).

4.4 Increased ERK5 activation in vemurafenib-resistant melanoma cells.

In order to confirm the role of the MEK5/ERK5 signalling axis in vemurafenib-resistant BRAF V600E mutated melanoma cancer cells,

investigations of the activation of ERK5 in different cell lines were conducted. Pairs of vemurafenib-sensitive and resistant cells from SKMel5 and SKMel5R cell were used for this study. All these cells express mutant BRAF V600E and they are melanoma cell lines established from patient-derived tumour samples.

4.4.1 ERK5 is activated in SKMel5-WT and SKMel5R cells

SKMel5 is a melanoma cell line harbouring the BRAF V600E mutation, and was established in 1974 from a 24-old Caucasian female with malignant melanoma (Memorial Sloan Kettering Cancer Center, 2015). SKMel5 and SKMel5R were treated with 1μM PLX4720 or 0.1% DMSO as vehicle control for 24 hours and lysed with RIPA prior to resolution on 8% SDS-PAGE acrylamide gel followed by immunoblotting with anti ERK5. Western blot showed that a mobility bandshift of ERK5 appeared in vemurafenib-resistant melanoma cells SKMel5R (Figure 4.6). The quantification of stimulated upper p-ERK5 mobility bands in SKMel5R compared to SKMel5 showed that ERK5 is more phosphorylated in PLX4720-resistant cells than PLX4720-sensistive cells (Figure 4.6).

The analysis of RTK and MAPK activity in SKMel5-WT and SKMel5R in response to PLX4720 revealed that EGFR-1 was up-regulated in vemurafenib-resistant cells compared with vemurafenib-sensitive cells (Figure 4.6). Treatment of SKMel5 and SKMel5R with 1μM PLX4720 showed that the phosphorylation of ERK1/2 was decreased in both cell lines compared to the DMSO vehicle control. The activation of AKT in the resistant cell line appeared to be lower compared with sensitive cells. The incubation of SKMel5 and SKMel5R with 0.1% DMSO vehicle control or 1μM PLX4720 had a negligible effect on the phosphorylation of JNK and p38 MAPK (Figure 4.6).

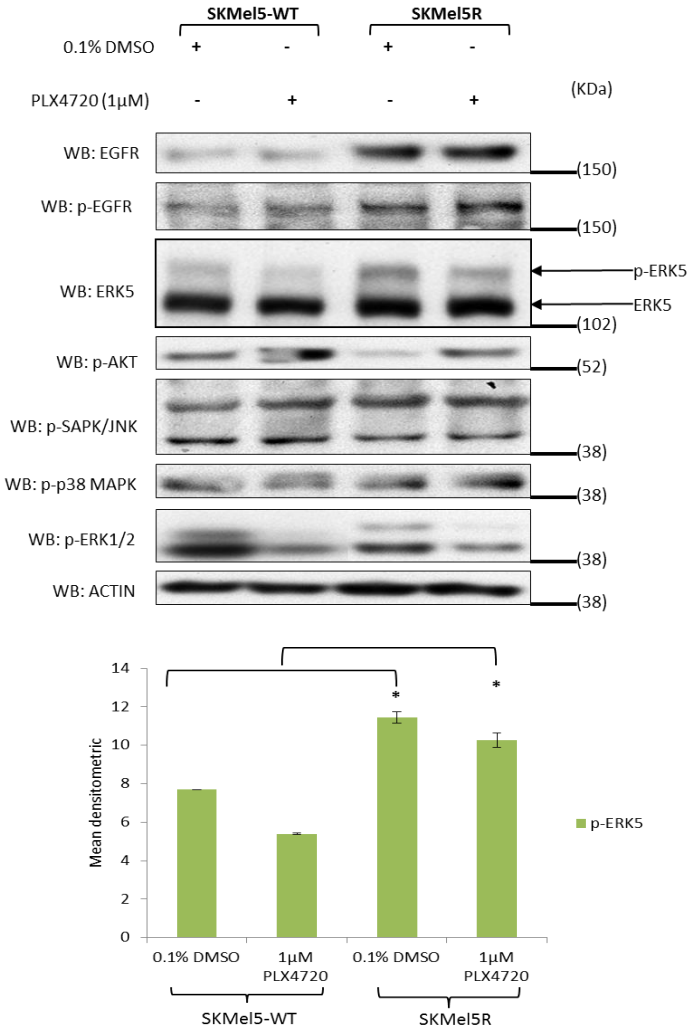


Figure 4.6 Intracellular signalling in vemurafenib-sensitive and resistant SKMel5 cells. SKMel5-WT and SKMel5R were seeded in a 12 well plates for 24 hours and then incubated with 1μM PLX4720 or 0.1% DMSO vehicle control prior to lysis of the cells with RIPA lysis buffer. Protein lysates from SKMEL5 and SKMel5R were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-EGFR, p-AKT, p-SAPK/JNK, p-p38 MAPK, p-ERK1/2 and Actin. Analysis of densitometry of protein phosphorylation or protein expression is relative to basal control condition of each cell type. This experiment is representative of three independent experiments (*p< 0.05).

The analysis of *MEK5* and *ERK5* mRNA in vemurafenib-sensitive and resistant cells in A375 and SKMel5 in response to 0.1% DMSO vehicle control or 1μM PLX4720 revealed that, the *erk5* gene expression data in resistant cell lines was approximately compatible with the ERK5 protein

expression data which indicated that ERK5 was activated in resistant cell lines compared with parental cells (Figure 4.7). Treatment with PLX4720 induced gene expression of MEK5 in both sensitive and resistant cell conditions in A375 and SKMel5 whereas cells incubated with the 0.1% DMSO vehicle control expressed MEK5 about one-fold in SKMel5R and slightly less in A375R compared to the PLX4720-sensitive cells A375 and SKMel5 (Figure 4.7).

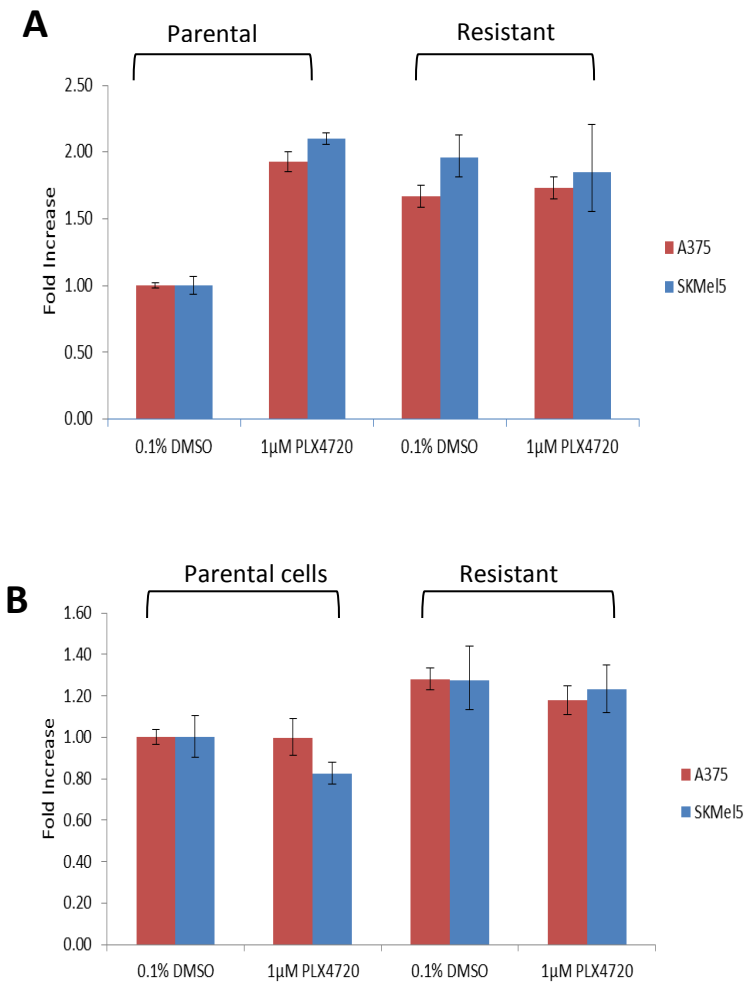


Figure 4.7 *MEK5* and *ERK5* mRNA expression in A375 and SKMel5 cells. The bar chart shows RT-PCR analysis for (A) MEK5 and (B) ERK5 mRNA level in A375 & A375R and SKMel5 & SKMel5R. The melanoma cells RNA were extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to β-actin expression and illustrated as fold change. The result shown is representative of three independent experiments.

4.5 Inhibition of ERK5 signalling cascade prevents vemurafenib resistance

The increased activation of ERK1/2 in vemurafenib melanoma has led to rational use of MEK1/2 inhibitor treatments in combination with vemurafenib to treat melanoma patients. Data showing increased ERK5 activation in vemurafenib resistant cells suggests that inhibition of the ERK5 signalling cascade may also potentially inhibit drug resistance in these cells. The effect of BIX02189 and XMD8-92 on cell viability in A375 and A375R cells was analysed.

4.5.1 Vemurafenib-sensitive and resistant melanoma cells do not show difference in response to the small-molecule kinase inhibitors

In order to analyse the effect of the small-molecule kinase inhibitors of the MEK5/ERK5 axis in combination with vemurafenib on the resistant melanoma cells, validation and determination of the concentration of these inhibitors and PLX4720 in A375 and A375R is required to chose a concentration of drug that is not toxic to cells. The examination of the effect of the single-agent PLX4720 and the small-molecule kinase inhibitors, BIX02189, XMD8-92, lapatinib and trametinib against A375 and A375R was conducted by using an *in vitro* CellTiter-Glo® Luminescent Cell Viability Assay, which indirectly determines the number of viable cells by quantifying the amount of ATP present in a metabolically active cell, after 72 hours of treatment. The PLX4720, BIX02189, XMD8-92, lapatinib and trametinib dose response curves in vemurafenib-sensitive A375 cells and vemurafenib-resistance A375R cells are shown in figure 4.8. The parental cells showed increased sensitivity to PLX4720 with an IC50 at 1.084 μM compared to the resistant cells A375R with an IC50 at 10.17 μM which gave 9.38-fold more resistance than parental cell line A375 (Table 4.1). Treating the vemurafenib-sensitive and resistant melanoma cell lines with dose-response of four small-

molecule kinase inhibitors alone for 72 hours showed no changes between the two cell lines (Figure 4.8).

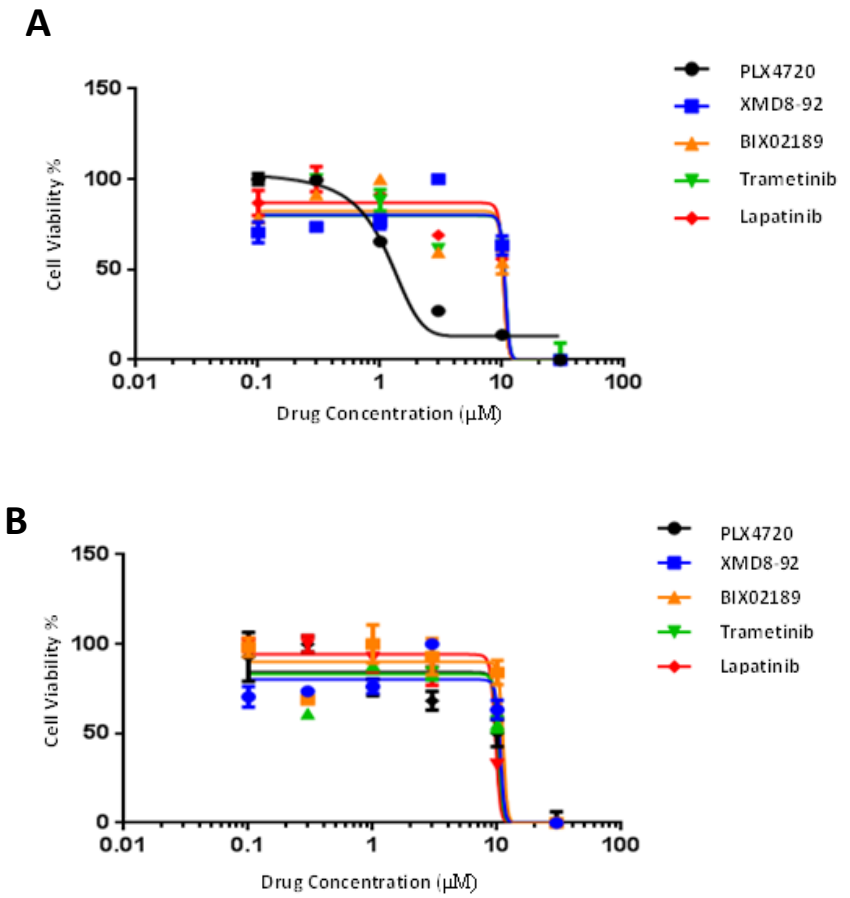


Figure 4.8 Dose response curves of PLX4720 and four small-molecule kinase inhibitors in A375 and A375R. (A) A375 and (B) A375R cells seeded in a 96-well plate for 24 hours and then treated with different concentrations of PLX4720, BIX02189, XMD8-92, lapatinib and trametinib and incubated for 72 hours. Cells were then analysed by using a *CellTiter-Glo® Luminescent Cell Viability Assay. This result is representative of three independent experiments.*

Table 4.1 The sensitivity of A375 and A375R to PLX4720, BIX02189, XMD8-92, lapatinib and trametinib

Compound	IC50 (µM)		
	A375	A375R	Fold resistance
PLX4720	1.084	10.17	9.38
BIX02189	10.28	11.14	1.03
XMD8-92	10.57	10.59	1.00
Lapatinib	10.29	9.7	0.95
Trametinib (GSK1120212)	10.59	10.29	0.97

Fold resistance is defined as the IC50 of the resistant cell line / IC50 of the parental cell line

4.5.2 Combination treatment of PLX4720 with BIX02189 reverses the PLX4720 resistance

The parental cell lines, A375 and SKMel5-WT, and their acquired vemurafenib-resistant sublines, A375R and SKMel5R, were examined for their sensitivity to PLX4720 alone and the combination of PLX4720 with BIX02189 or XMD8-92.

The results shown in Figure 4.9 show that, as expected, in contrast to the parental cell lines, the PLX4720-resistant cell lines are significantly resistant to PLX4720. The parental cell lines A375 and SKMel-WT were sensitive to PLX4720 with an IC_{50} of 0.784 and 1.075 μM respectively, while the resistant cells A375R and SKMel5R were more resistant to PLX4720 with an IC_{50} of 10.02 and 9.960 μM respectively which gave a 12.87-fold increase in A375 and 9.26-fold increase in SKMel5 in IC_{50} (Figure 4.9 and table 4.2). The combination of PLX4720 with 3μM BIX02189 or XMD8-92 in the A375 cells did not show a change in IC_{50} values compared with PLX4720 alone, whereas the SKMel5-WT cells were sensitive to BIX02189 or XMD8-92 with PLX4720 compared to PLX4720 alone (Figure 4.9 and Table 4.2). In the resistant cells A375R, incubation of 3μM BIX02189 or XMD8-92 with in the presence of PLX4720, increased sensitivity to PLX4720 and shifted the dose response curves to the left compared to PLX4720 alone. In addition, SKMel5R cells were more sensitive to PLX4720 when treated with 3μM BIX02189 or XMD8-92 in combination with PLX4720 and the resistance to PLX4720 were reversed (Figure 4.9).

This result suggests that the combination of PLX4720 with BIX02189 or XMD8-92 could potentially reverse the resistance of A375 or SKMel5 to vemurafenib.

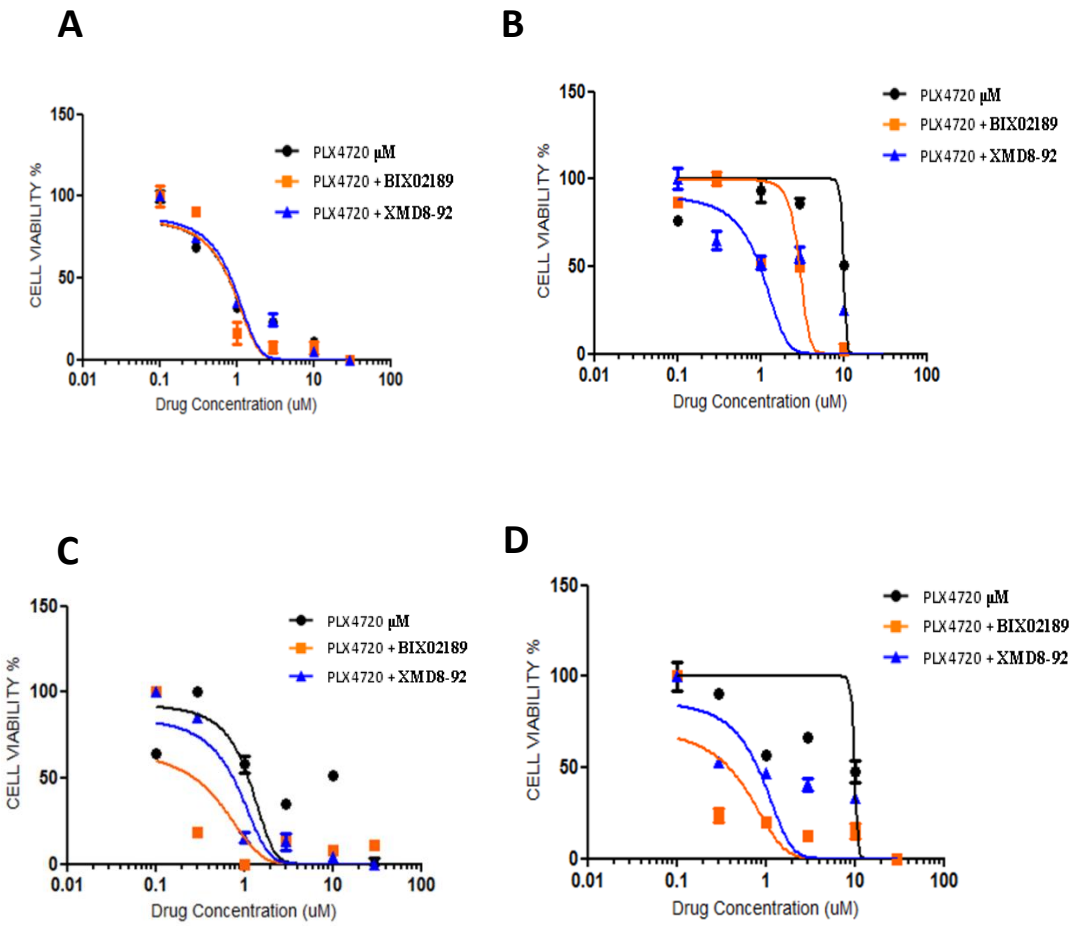


Figure 4.9 Dose response curves of PLX4720 combined with/without MEK5 inhibitor BIX02189 or ERK5 inhibitor XMD8-92 in A375, A375R, SKMel5-WT and SKMel5R. (A) A375, (B) A375R (C) SKMel5-WT and (D) SKMel5R cells seeded in a 96-well plate for 24 hours and then treated with different concentrations of PLX4720 on its own and with 3µM BIX02189 or XMD8-92 and incubated for 72 hours. Cells were then analysed by using a CellTiter-Glo® Luminescent Cell Viability Assay. This result is representative of three independent experiments.

Table 4.2 The sensitivity of A375 and A375R to PLX4720, combined with BIX02189, or XMD8-92

Compound	IC50 (µM)		
	A375	A375R	Fold resistance
PLX4720	0.784	10.02	12.87
PLX4720 + 3µM BIX02189	0.796	2.97	3.73
PLX4720 + 3µM XMD8-92	0.85	0.987	1.16
	SKMel5-WT	SKMel5R	
PLX4720	1.075	9.960	9.26
PLX4720 + 3µM BIX02189	0.274	0.388	1.41
PLX4720 + 3µM XMD8-92	0.685	0.817	1.19

Fold resistance is defined as the IC50 of the resistant cell line / IC50 of the parental cell line (Lidsky et al., 2014)

4.6 Development of an *in vitro* endothelial cell/fibroblast/melanoma cancer cell co-culture assay to assess the effect of BIX02189 and XMD8-92 inhibition of ERK5 on tumour angiogenesis

Tumour angiogenesis is characterized by an imbalance of pro-angiogenic and anti-angiogenic factors, endothelial cell proliferation, migration and differentiation and also the remodelling of extracellular matrix which eventually results in sprouting of new blood vessels. This process is triggered by the tumour cells which release pro-angiogenic factors such VEGF and bFGF which directly bind to the receptor tyrosine kinases on the surface of endothelial cells to support invasive sprouting to the tumour. Thus, inhibiting tumour angiogenesis provides a promising strategy for treatment of solid tumours (Folkman et al., 1971).

In this experiment, endothelial cells represent key cells involved in angiogenesis which depend on the interaction with mural cells such as fibroblasts. Fibroblasts produce extracellular matrix (ECM) proteins which provide a base to promote endothelial cell migration and facilitate vessel remodelling (Staton et al., 2009). The pro-angiogenic growth factors that are released from tumour cells, bind to fibroblast derived ECM to assist the progress of 3D organisation and tubulogenesis of endothelial cells (Sorrell et al., 2007). Therefore, endothelial cells co-cultured with fibroblasts provide a precise *in vitro* representation of *in vivo* angiogenesis (Donovan et al., 2001).

Optimisation of the number of endothelial cells, HDMEC, seeded on the confluent fibroblast, NHDF, layers and the length of HDMEC/NHDF assay has been conducted previously in our lab. In this study, the image analysis software termed ‘AngioQuant’ was used for quantification of tubule formation in HDMEC/NHDF/A375s.

4.6.1 Combination of PLX4720 with BIX02189 or XMD8-92 inhibits tube- formation in a HDMEC/fibroblast/A375s co-culture assay

It was reported that ERK5 is required for VEGF stimulated tubular morphogenesis in a HDMEC/NHDF assay (Roberts et al., 2010a). Furthermore, deletion of *erk5* in adult mice induces lethality due to endothelial apoptosis resulting in cardiovascular degeneration (Hayashi et al., 2004a), which means that ERK5 is critical to endothelial cell survival in established vessels. To determine the effect of MEK5/ERK5 inhibitors upon tumour cell stimulated angiogenesis in human microvascular endothelial cells in co-culture assay, normal human dermal fibroblasts (NHDFs) were plated on day one to obtain a confluent monolayer followed by plating of A375 and A375R cells on day 3. On day 4, HDMECs were plated on the NHDF/A375s layers and incubated overnight. Co-cultures were treated with the selective BRAF inhibitor, PLX4720, MEK5 inhibitor, BIX02189, and ERK5 inhibitor, XMD8-92. Single-dose treatment with 1μM PLX4720, 3μM BIX02189 and 3μM XMD8-92 and combined PLX4720 with BIX02189 or XMD8-92 was performed on day 5 and day 8. Co-cultures were later fixed and stained for expression of endothelial cell specific marker CD31 and visualised by immunohistochemistry (section 2.2.9.2 and 2.2.9.3).

Quantification of total tube length revealed that tumour cells in HDMEC/NHDF/A375s induced more tube-like structures than HDMEC/NHDF. The ability of resistant cells A375R to increase tube formation compared to sensitive cells A375 relates to their secretion of more VEGF-A and VEGF-C to induce endothelial cells to increase tubule formation (Figure 4.11). In addition, VEGFR-2 expressed in HDMEC but not A375 or A375R cells.

Single-dose treatment with 1μM PLX4720 or 3μM BIX02189, XMD8-93 alone marginally lowered melanoma cell-stimulated endothelial capillary morphogenesis in PLX4720-sensitive and -resistant cells, compared to basal

control (Figure 4.10). Treatment of a HDMEC/NHDF/A375R co-culture with 1μM PLX4720 in combination with 3μM BIX02189 resulted in a significant decrease in A375R-induced tube formation compared to basal control and treatment with PLX4720 alone (Figure 4.10). Similarly, combination treatment with low dose PLX4720 and BIX02189 in vemurafenib-sensitive cells co-cultured with HDMEC and NHDF has given approximately the same effect. 3μM XMD8-92 in combination with a low single dose of PLX4720 on day 5 and day 8 profoundly inhibited A375R-driven angiogenesis compared to basal control and PLX4720 or XMD8-92 alone (Figure 4.10). Vemurafenib-sensitive cells co-cultured with HDMEC/NHDF displayed a great reduction in tumour angiogenesis when treated with PLX4720 combined with XMD8-92. This data suggested that continuous inhibition of the MEK5/ERK5 signalling axis reduced tumour cell-stimulated endothelial capillary network formation in HDMEC/NHDF/A375s co-cultures. Also, these data imply that, melanoma cancer cell-induced tube formation is more susceptible to PLX4720 combined with BIX02189 or XMD8-92 treatment than PLX4720 or BIX02189, XMD8-92 alone.

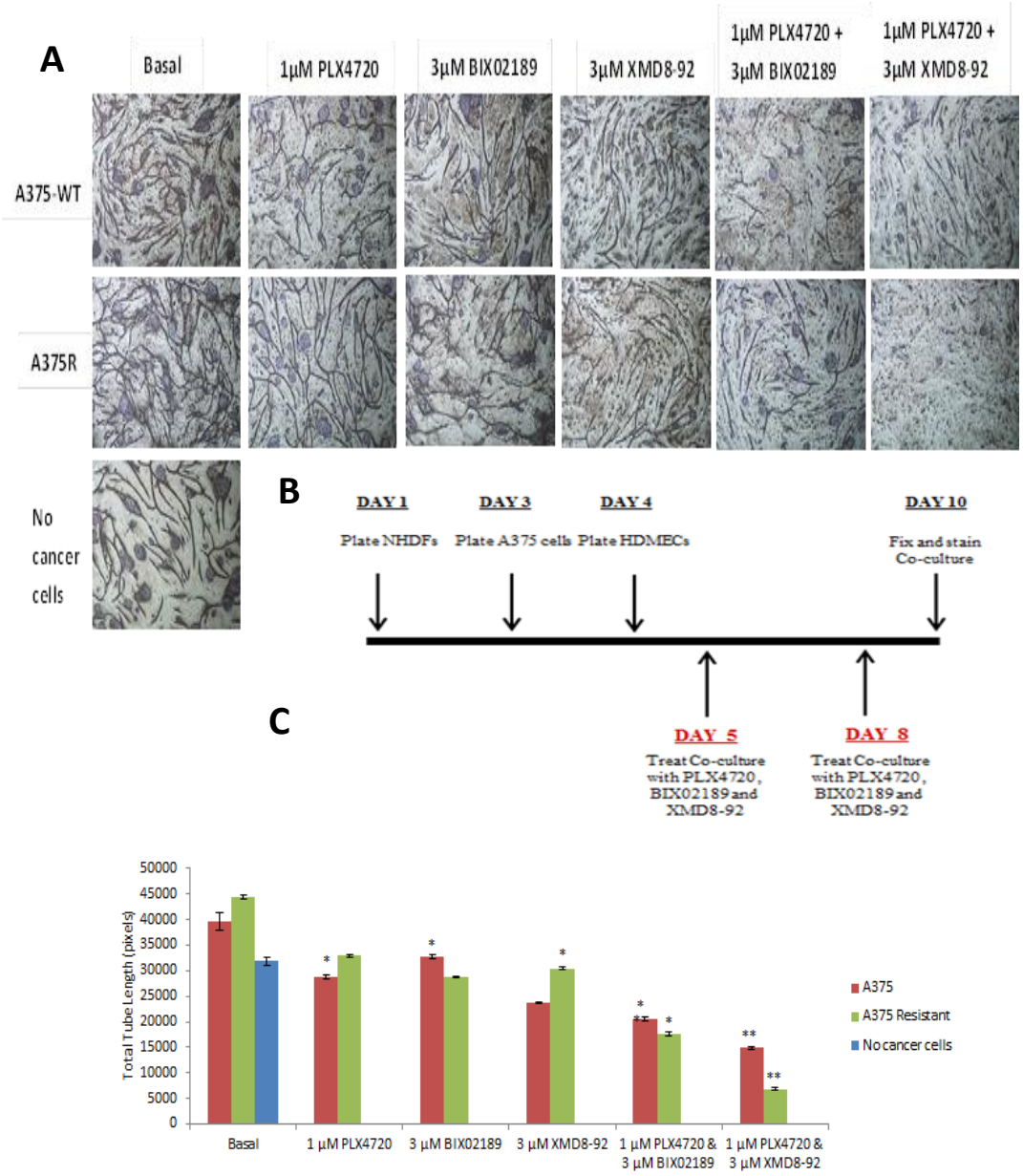


Figure 4.10 The effect of MEK5/ERK5 inhibitors, BIX02189 and XMD8-92, on tumour angiogenesis in a HDMEC/NHDF/A375s co-culture assay. (A) NHDF were seeded in fibroblast growth medium at 20,000 cells per well on gelatine-coated 24-well plates and incubated for 48 hours. On day 3, A375 and A375R cells were plated at 10,000 cells per well on NHDF layer and incubated for 24 hours. On day 4 of the assay, HDMECs were plated at 45,000 cells per well onto the confluent NHDF and A375 layers. On day 5 and 8, co-cultures were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone or with indicated amount of PLX4720, BIX02189 and XMD8-92, as shown in the treatment schedule (B). On day 10, cells were fixed and stained as described (section 2.2.9.3.3), and total tube length was quantified using AngioQuant image analysis software. (C) Data is presented as total tube length from triplicate (n=3, mean ±SD) compared to basal. The result shown is representative of three independent experiments (*p< 0.05, **p<0.01).

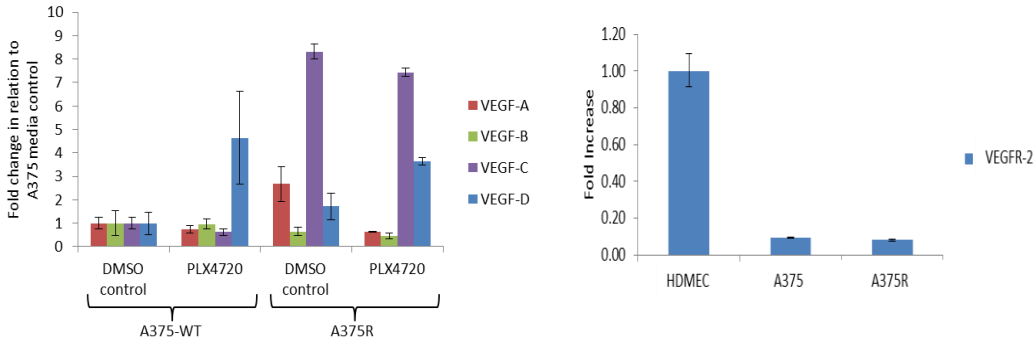


Figure 4.11 mRNA expression of VEGFs and VEGFR-2 in malignant melanoma A375 and A375R cells. The bar chart shows RT-PCR analysis of *vegf-a*, *vegf-b*, *vegf-c*, *vegf-d* and *vegfr-2* mRNA expression in A375 and A375R cells. The malignant melanoma cancer cells RNA were extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to GAPDH expression and illustrated as fold change.

4.6.2 Tumour angiogenesis in a HDMEC/fibroblast/SKMel5 co-culture assay is abolished by treatment of the combination of PLX4720 with BIX02189 or XMD8-92

To further investigate the role of the MEK5/ERK5 signalling axis in tumour angiogenesis by using MEK5 and ERK5 inhibitors combined with BRAF inhibitor, PLX4720, in a HDMEC/NHDF/SKMel5 co-culture assay, NHDFs were plated on 24-well plates on day 1 to produce a confluent monolayer. On day three, SKMel5 and SKMel5R were plated on the NHDF monolayer and incubated for 24 hours followed by plating human dermal microvascular endothelial cells on the NHDF/tumour cell layer for 24 hours. On day 5 and 8, the cells were treated with low doses of PLX4720, BIX02189 and XMD8-92 alone or in combination therapy followed by fixing and staining on day ten.

Similar to the HDMEC/NHDF/A375 co-culture, SKMel5 cells induced tube-formation when co-cultured with fibroblasts and endothelial cells compared with HDMEC/NHDF co-culture without cancer cells, which confirms that tumour cells produce massive amounts of growth factors which bind to

endothelial cells to trigger invasive sprouting to the tumour (Figure 4.12). Treating the co-culture with a single agent (PLX4720, BIX02189 and XMD8-92) decreased total tube length in PLX4720-sensitive cells SKMel5-WT compared with PLX4720-resistant cells SKMel5R, and tube-formation in both cell lines were decreased compared with basal control (Figure 4.12). Combination therapy using PLX4720 with MEK5 inhibitor BIX02189, or ERK5 inhibitor XMD8-92, revealed that malignant melanoma cell-induced tube-formation in the HDMEC/NHDF co-culture was decreased by 9-10 fold compared with the basal control and 7-8 fold compared with PLX4720 alone (Figure 4.12).

This data combined with data in section 4.6.2 suggests that the dual combination of a BRAF inhibitor (PLX4720) and the MEK5/ERK5 signalling pathway inhibitor may give hope to inhibit tumour angiogenesis resulting in starvation and oxygen and finally shrinkage of the malignant cells.

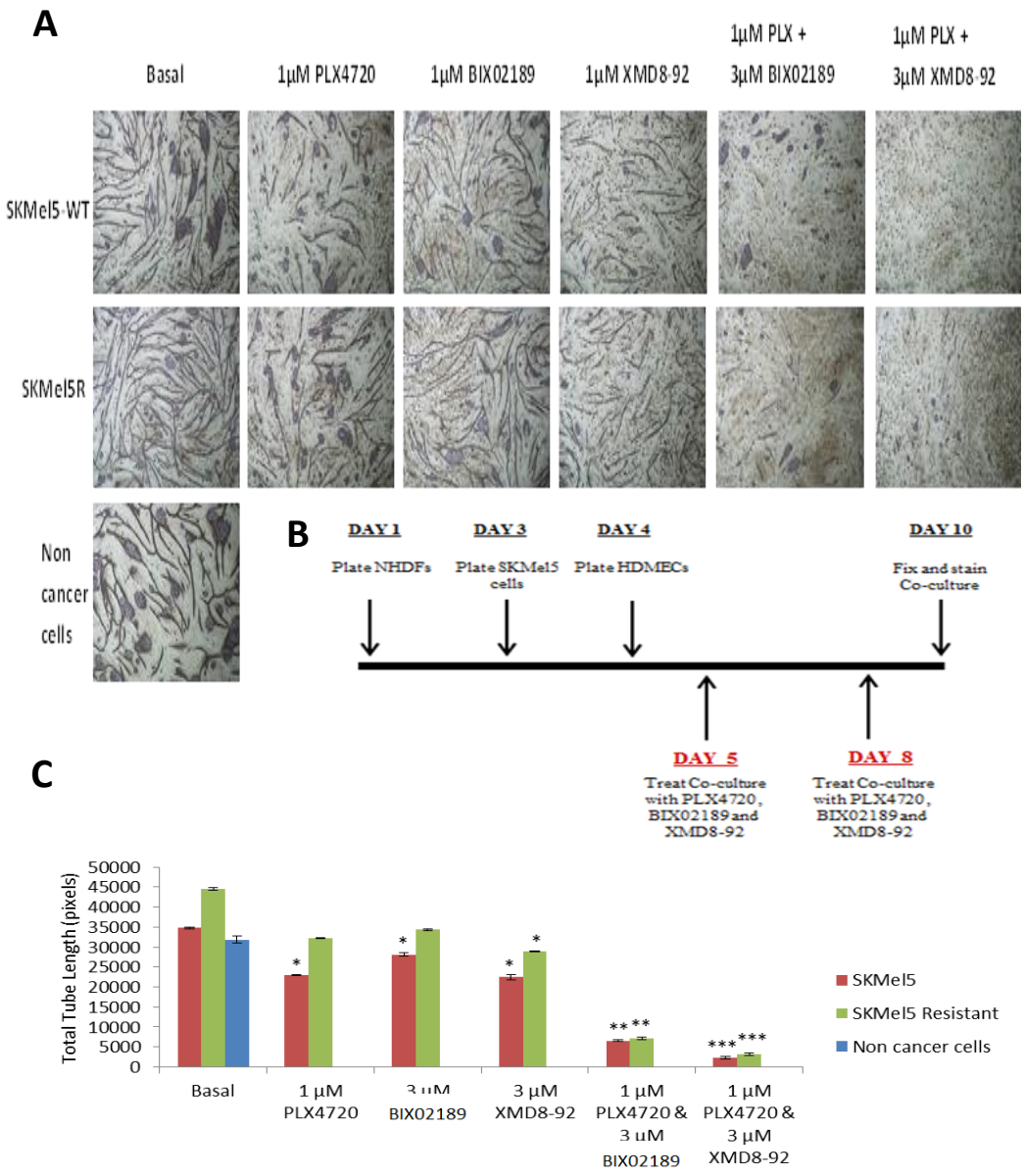


Figure 4.12 The effect of MEK5/ERK5 inhibitors, BIX02189 and XMD8-92, on tumour angiogenesis in a HDMEC/NHDF/SKMel5s co-culture assay. (A) NHDF were seeded in fibroblast growth medium at 20,000 cells per well on gelatine-coated 24-well plates and incubated for 48 hours. On day 3, SKMel5 and SKMel5R cells were plated at 10,000 cells per well to the NHDF layer and incubated for 24 hours. On day 4 of the assay, HDMECs were plated at 45,000 cells per well onto the confluent NHDF and SKMel5 layers. On day 5 and 8, co-cultures were treated with EBM MV2 basal medium containing 1% (v/v) FCS alone or with indicated amount of PLX4720, BIX02189 and XMD8-92, as shown in the treatment schedule (B). On day 10, cells were fixed and stained as described (section 2.2.9.3.3), and total tube length was quantified using AngioQuant image analysis software. (C) Data is presented as total tube length from triplicate (n=3, mean ±SD) compared to basal. The result shown is representative of three independent experiments (*p< 0.05, **p<0.01, ***p<0.001).

4.7 Discussion

The study of the MEK5/ERK5 signalling pathway in cancer is important because of the role of this pathway in cell proliferation, cell survival and angiogenesis (Wang and Tournier, 2006). The abnormal expression of MEK5/ERK5 has been demonstrated in many human cancers such as breast cancer, prostate cancer and oral squamous cell carcinoma (Montero et al., 2009b, McCracken et al., 2008a, Sticht et al., 2008).

The aim of this study was to analyse and investigate the role of ERK5 in malignant melanoma by exogenous activators such as growth factors and anti-cancer agent PLX4720. The activation of ERK5 in A375 vemurafenib-sensitive or resistant cells by a variety of growth factors, VEGF, FGF-2, HGF, EGF, NRG-1 and PDGF-BB, with potent stimulation by EGF (Figure 4.1), corroborates data from previous studies in different cell types (Kato et al., 1998b, Hayashi et al., 2004a). The extensive phosphorylation of ERK5 in these cells resulted in a mobility bandshift from ERK5 with conventional SDS-PAGE.

The expression and activation of ERK5 in tumour cells correlates with relapsed patients which is associated with aggressive conditions and poor prognosis (Mehta et al., 2003a, Jin et al., 2011). MEK5 expression via STAT3 in breast cancer cells was reported to provide critical survival signals to tumour cells and lead to chemo-resistance (Song et al., 2004, Weldon et al., 2002). The development of resistance to therapeutic agents is considered a major barrier to effective cancer treatment. At the molecular level, drug resistance is distinguished by changes in signalling pathways and gene expression which support cell survival and proliferation and a more malignant phenotype. After preliminary data revealed that ERK5 was activated in malignant melanoma cells in response to growth factors, analysis of the role of ERK5 in resistant cells in response to PLX4720 was conducted. ERK5 phosphorylation was increased in PLX4720-resistant cells compared to

the parental cell line (Figure 4.2 and 4.6). Based on EGF-induced ERK5 activation in melanoma cells (Figure 4.1), inhibition of EGFR, MEK5, ERK5 and ERK1/2 was used to investigate the correlation between ERK5 activation and these kinases after incubation of the cells with PLX4720. This study showed that selective inhibition of EGFR activity using lapatinib inhibited EGFR activation as expected but did not prevent ERK5 activation in PLX4720-resistant cells, revealing that ERK5 activation in a melanoma resistant cell line is not mediated by EGFR (Figure 4.5). The first generation of ERK1/2 inhibitors (U0126, PD98059 and PD184352) have been reported to inhibit endogenous ERK5 in HeLa cells (Mody et al., 2001a). Therefore it was possible that an ERK1/2 inhibitor (trametinib) may block ERK5 activation in A375R melanoma cells. As expected, trametinib was found to be an effective inhibitor of ERK1/2 activation in PLX4720-sensitive and resistant cells. By contrast, the phosphorylation of ERK5 was increased by trametinib treatment in both cell lines (Figure 4.4 and 4.5). These data suggest that trametinib-mediated inhibition of ERK1/2 activation leads to ERK5 activation in malignant melanoma cells (A375 and A375R). This effect is similar to the reported effect of PD184352 upon EGF-induced ERK5 activation in HeLa cells (Mody et al., 2001a) Therefore, ERK1/2 may have a negative regulatory effect upon the MEK5/ERK5 signalling pathway and provides further evidence of cross-talk between the ERK5 and ERK1/2 signalling pathway as first suggested by Mody et al. (2001). Furthermore, a recent study revealed that inhibition of the ERK1/2 signalling pathway results in phosphorylation of ERK5 as an alternative pathway that rescues cell proliferation in colorectal cancer (de Jong et al., 2016).

After determining the role of ERK5 in vemurafenib-resistant A375 cells, the second aim was to attempt to reverse drug resistance in the A375R cell line by inhibition of the MEK5/ERK5 signalling pathway. Previous studies have focused on dual inhibition to overcome resistance in melanoma (Heppt et al., 2015). As reactivation of the MAPK pathway is involved in acquired

resistance in more than half of cases of BRAF V600E melanoma, and ERK1/2 is involved in the RAS-RAF pathway, the combination of BRAF and MEK inhibitors such as dabrafenib and trametinib has improved the progression free-survival and response rates compared with the use of individual inhibitors (Shi et al., 2014, Wagle et al., 2014). Inhibition of the MEK5/ERK5 signalling pathway by BIX02189 and XMD8-92 synergistically with the BRAF inhibitor, PLX4720, exhibited a vital role in the reversal of PLX4720-resistance in melanoma A375 and SKMel5 cells (Figure 4.9). Concurrent targeting of BRAF and MEK5/ERK5 in cells with BRAF V600-mutated malignant melanoma reversed resistance to PLX4720 in both melanoma cell lines A375R and SKMel5R (figure 4.9). The reversed resistance levels were approximately similar to treated PLX4720-sensitive A375 and SKMel5 cells with PLX4720 alone, which mean the combination of these agents in resistant cells, reversed the cells back to the vemurafenib sensitive state (Table 4.2).

These results demonstrate that ERK5 expression was up-regulated in vemurafenib-resistant melanoma cancer cells. In addition, use of the MEK5/ERK5 signalling pathway inhibitors, BIX02189 and XMD8-92, in combination with a BRAF inhibitor, PLX4720, could reverse vemurafenib-resistance.

The third aim was to assess the role of the MEK5/ERK5 signalling pathway inhibitors in combination with a BRAF inhibitor in tumour angiogenesis by using the melanoma tumour cells in a HDMEC/NHDF co-culture *in vitro* angiogenesis assay.

In tumour progression, pro-angiogenic factors induce quiescent vasculature to sprout new blood vessels and maintain tumour growth by supplying nutrients and oxygen (Lochhead et al., 2012). The role of MEK5 and ERK5 in blood vessel formation has been demonstrated by deletion of *mek5* and *erk5* in mice (Wang et al., 2005). In B16F10 murine melanoma cells, the target deletion of

ERK5 in endothelial cells decreased the tumour mass and vascular density (Hayashi et al., 2005b). ERK5 is required for VEGF induced tubular morphogenesis in an *in vitro* angiogenesis system (Roberts et al., 2010a). These findings demonstrate the central role of ERK5 in tumour angiogenesis.

Comparison of the inhibitory effects of the BRAF inhibitor PLX4720 or the MEK5/ERK5 signalling pathway inhibitors BIX02189 and XMD8-92 respectively alone or in combination with PLX4720 on malignant melanoma cell induced tumour angiogenesis, suggests that the effect of these agents are both additive and synergistic (Figure 4.10 and 4.11). Combination of BRAF inhibitor and MEK5/ERK5 inhibitor treatment was sufficient to reverse the resistance of melanoma cells to the BRAF inhibitor (Figure 4.9). Treatment with the same agents was able to reduce tumour cell-stimulated tube development in a HDMEC/NHDF/A375 or SKMel5 co-culture *in vitro* angiogenesis assay (Figure 4.10 and 4.11). It would be interesting to investigate the effect of siRNA mediated silencing of *MEK5* and *ERK5* expression in HDMEC/NHDF/tumour cells to further validate this model.

These findings together demonstrate that ERK5 expression is up-regulated in vemurafenib-resistant A375R and SKMel5R melanoma cells, and inhibition of the MEK5/ERK5 and BRAF could reverse PLX4720 resistance, and prevent tumour angiogenesis, presenting a possibility of ERK5 serving as a therapeutic target to overcome vemurafenib resistance in melanoma patients.

CHAPTER FIVE: The role of ERK5 in drug resistance and angiogenesis in ovarian cancer cells

5.1 Introduction

Ovarian cancer is the fourth common cancer in women after breast, bowl and lung cancer (Cancer Research UK, 2016). In the UK, 7000 cases of ovarian cancer are diagnosed yearly with approximately 4300 deaths from this cancer (Cancer Research UK, 2016). The reason for poor prognosis in females with ovarian cancer is that more than 50% of women are diagnosed at an advanced stage making treatment difficult. The diagnosis of ovarian cancer at the late stage is due to the lack of a regular screening test compared to breast and cervical cancer. The current five-year survival rate for patients diagnosed with advanced stage ovarian cancer is 46% compared to 90% of those diagnosed at early stage of this disease (Ovarian Cancer Awareness Month, 2016). According to the International Federation of Gynaecology and Obstetrics (FIGO), ovarian cancer is classified into four stages based on the level of progression.

- Stage I is confined to the ovary or fallopian tubes
- Stage II has spread into pelvic tissue
- Stage III has extended from the ovary to the peritoneal lining
- Stage IV has penetrated the peritoneum and metastasised outside of the abdomen (Prat and Oncology, 2015)

There are many contributing risk factors for ovarian cancer. A family history of ovarian cancer increases the risk of developing this disease. The appearance of ovarian cancer in a first degree relative such as a mother or sister increases the possibility of ovarian cancer by more than 5 times (Soegaard et al., 2009) and is a potent risk factor for earlier onset ($\leq$ 50 years) ovarian cancer (Soegaard et al., 2009). Analysis of gene mutations in relation to ovarian cancer risk has shown that mutations in the BRCA1/2 genes play a major role in ovarian cancer susceptibility in addition to their role in breast cancer (Wooster et al., 1995, Modan et al., 2001). In addition, the tumour

suppression gene p53 is mutated in 50% of ovarian cancer cases especially in the late stage (Berchuck et al., 1994). Certainly, the risk of developing ovarian cancer increases with age (Chan et al., 2008).

Treatment of ovarian cancer by using chemotherapy in the last few decades has had minimal effect on patient survival. Thus, new treatment approaches are needed to improve the outcomes in ovarian cancer. Patients with ovarian cancer are treated with chemotherapy; e.g. taxane or cisplatin. Most patients respond initially, however many of them relapse with drug-resistant disease within the first 5 years after initial diagnosis (Greimel et al., 2006).

Angiogenesis, which is the formation of new blood vessels from a pre-existing vasculature, is a crucial step in tumour proliferation and metastasis and is a promising target for cancer treatment. For tumours to grow larger than a few hundred microns in diameter, there must be growth of new blood vessels (Carmeliet and Jain, 2000). Angiogenesis is a complicated process which is tightly regulated by a balance between pro-angiogenic and anti-angiogenic factors, which are synthesised by a range of cell types. Growth factors such as VEGF, PDGF and EGF beside IL8 represent the pro-angiogenic factors while Ang1 and 2 and thromobospondin represent the anti-angiogenic factors. Furthermore, angiogenesis plays a role in normal ovary physiology as new blood vessel formation and regression in the female reproductive system occurs in each cycle via complex interactions between steroid hormones and angiogenic factors (Ramakrishnan et al., 2005). Therefore, pre-clinical and clinical research studies have suggested that angiogenesis and its effectors have a pivotal role in ovarian cancer (Abu-Jawdeh et al., 1996).

It has recently been shown that ERK5 is critical in angiogenesis. Previous results from our group have revealed that the knockdown of ERK5 in HDMECs prevents tubular morphogenesis in vitro (Roberts et al., 2010a,

Nithianandarajah-Jones et al., 2014). In addition, the ablation of erk5 in mice results in embryonic lethality in vivo (Hayashi et al., 2004a).

This chapter analyses the role of the MEK5/ERK5 signalling pathway in doxorubicin and cisplatin resistant ovarian cancer cells.

5.2 Characterisation of ERK5 phosphorylation in drug resistant ovarian cancer cells

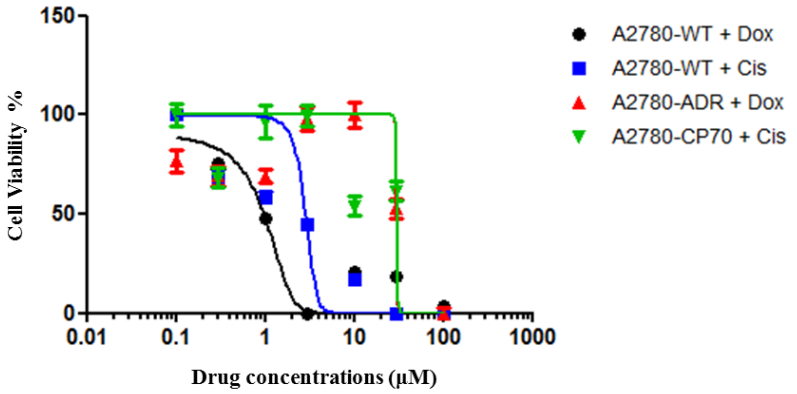
5.2.1 ERK5 is activated in A2780-ADR and A2780-CP70 resistant ovarian cancer cell lines

Human ovarian carcinoma A2780 cells and their doxorubicin-resistant substrain A2780-ADR and cisplatin-resistant substrain A2780-CP70 were obtained from Prof. Robert Brown, University of Glasgow, UK. The A2780 human ovarian cancer cell line was established from tissue obtained from an untreated patient. The doxorubicin and cisplatin resistant cells were generated by exposure of the parental cell line to multiple and increasing concentrations of doxorubicin and cisplatin (Behrens et al., 1987, Brown et al., 1993, Rogan et al., 1984).

Cisplatin is a platinum analogue that intercalates with DNA strands causing cross-linking; interfering with the DNA repair mechanism which subsequently induce apoptosis in cancer cells (Dasari and Tchounwou, 2014). It has been used in treatment of many types of cancer including head, neck, ovarian and bladder cancer. Doxorubicin is an anthracycline which interferes with DNA and prevents its replication and transcription (Jurisicova et al., 2006). It is used to treat a variety of cancers such as breast, lymphoma, ovarian and leukaemia. Chemotherapeutic agents can trigger the signalling pathways that are implicated in drug resistance, for example the constitutive activation of ERK1/2 appears in many types of cancers such as mesothelioma (de Melo et al., 2006). ERK5 has been involved in the regulation of cellular

proliferation, cell death and tumourigenesis and with its upstream activator MEK5; is essential in vasculogenesis (Wang and Tournier, 2006).

To validate the resistance of doxorubicin and cisplatin in A2780 ovarian cancer cell lines, an ATP viability assay was applied. The parental cell line and doxorubicin resistant cells were treated with dose response of doxorubicin and incubated for 72 hours. Also, A2780 and cisplatin resistant cells were treated with dose response of cisplatin and incubated for 72 hours. In the A2780 cells, the IC50 for doxorubicin is 0.976 µM while for cisplatin is 2.76 µM while in the A2780-ADR and A2780-CP70 cell lines, the IC50 of doxorubicin and cisplatin are 30.05 µM and 30.20 µM respectively (Figure 5.1). The IC50 drug doses for the two cell lines A2780 and A2780-ADR differ by 31-fold for doxorubicin whereas the IC50 drug doses for A2780 and A2780-CP70 differ by 11-fold for cisplatin (Figure 5.1).



Compound	IC50 (µM)		
	A2780-WT	A2780-ADR	Fold resistance
Doxorubicin	0.976	30.05	30.8
	A2780-WT	A2780-CP70	
Cisplatin	2.76	30.20	10.9

Figure 5.1 Dose response curves of doxorubicin and cisplatin in A2780-WT, A2780-ADR and A2780-CP70. A2780-WT, A2780-ADR and A2780-CP70 cells seeded in a 96-well plate for 24 hours and then treated with different concentrations of doxorubicin (A2780-WT and A2780-ADR) or cisplatin (A2780-WT and A2780-CP70) and then incubated for 72 hours. After that, cells were analysed by using CellTiter-Glo® Luminescent Cell Viability Assay. This result is representative of three independent experiments.

The ovarian cancer cell lines, A2780 and A2780-ADR (ovarian cancer cells resistant to doxorubicin), were incubated with/without doxorubicin, whereas A2780 and A2780-CP70 (ovarian cancer cells resistant to cisplatin) were incubated with/without cisplatin for 1 hour.

The cells were lysed and protein resolved on an 8% SDS-PAGE acrylamide gel. The band shift shown in Figure 5.2 revealed that ERK5 phosphorylation was increased in both resistant cell lines compared with the parental cell line (Figure 5.2a). Moreover, the densitometry of phosphorylated ERK5 bands confirmed that the cells incubated with 1μM of anti-cancer agents showed an increase in ERK5 activation in both resistant cell lines (Figure 5.2b).

The levels of mRNA expression of *mek5* and *erk5* in A2780-WT, A2780-ADR and A2780-CP70 were also assessed and showed that *mek5* and *erk5* expression was increased in doxorubicin resistant cells compared to parental cells (Figure 5.3). These data together revealed that ERK5 could play a role in the resistance of ovarian cancer cell lines to doxorubicin and cisplatin.

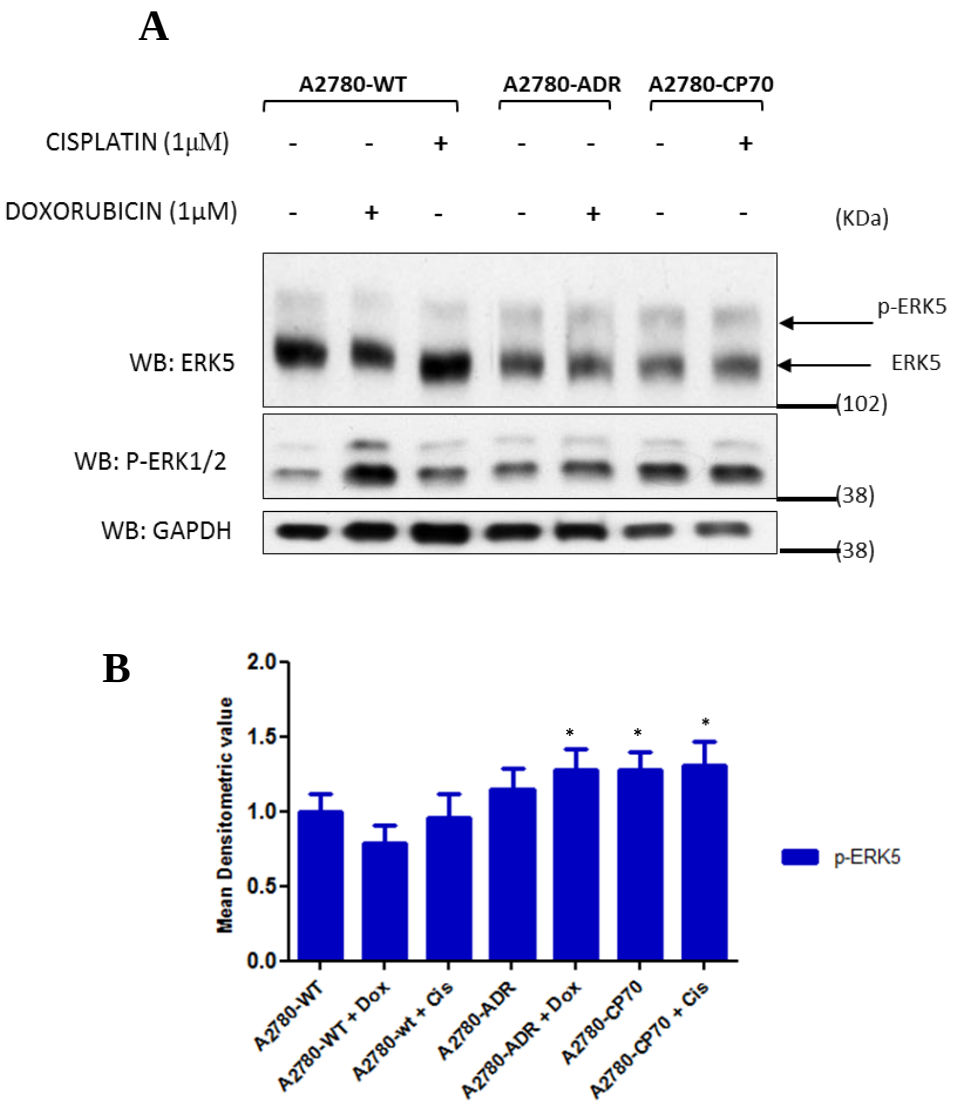


Figure 5.2 ERK5 activation in A2780 ovarian cancer resistant cell lines. (A) A2780-WT, A2780-ADR and A2780-CP70 were seeded in a 12-well plate for 24 hours and then incubated with 1μM doxorubicin or cisplatin prior to lyse the cells with RIPA lysis buffer. Protein lysates from A2780-WT, A2780-ADR and A2780-CP70 were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-ERK1/2 and GAPDH. (B) Analysis of a densitometry of protein phosphorylation or protein expression relative to basal control condition of each cell type was (set arbitrarily as 1.0). This result is typical of four independent experiments (*$p<0.05$).

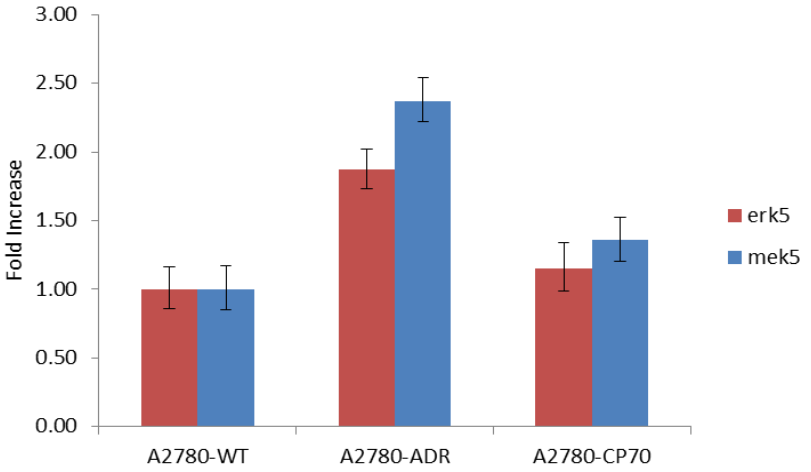


Figure 5.3 mRNA expression of *mek5* and *erk5* in A2780 ovarian cancer resistant cell lines. The bar chart shows RT-PCR analysis for MEK5 and ERK5 mRNA level in A2780-WT, A2780-ADR and A2780-CP70. The ovarian cancer cells RNA were extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to GAPDH expression and illustrated as fold change. Both experiments are representative of four individual experiments.

5.2.2 ERK5 is activated in response to a variety of other growth factors in all the A2780 ovarian cancer cell lines

ERK5 is activated by a range of growth factors and cellular stresses including VEGF, FGF-2, EGF, NRG-1 PDGF-BB and HGF. Kato and colleagues revealed that EGF mediates cell proliferation in HeLa cells through ERK5 signalling (Kato et al., 1998b). Furthermore, studies have shown that MEKK2 regulates the activation of ERK5 in response to FGF-2 in embryonic stem cells (Kesavan et al., 2004a). The overexpression of NRG receptors are involved in cell proliferation in breast cancer cells through the ERK5 signalling pathway (Esparis-Ogando et al., 2002a). In addition, the role of HGF in cell proliferation in malignant mesothelioma cells is dependent on ERK5 signalling (Shukla et al., 2013b).

It was previously shown that drug resistance in ovarian cancer cells leads to upregulation of RTKs in these cells and increased responsiveness to growth factors. The response to a range of growth factors was studied in the ovarian

cancer cells. The cells of ovarian cancer (A2780-WT, A2780-ADR and A2780-CP70) were stimulated with 50ng of VEGF, FGF-2, HGF, EGF, NRG-1 and PDGF-BB for 10 minutes and analysed by western blot. In the parental A2780, EGF and NRG-1 were the potent stimulators of ERK5 activation with about 2-fold more compared to the basal control (Figure 5.4a).

In doxorubicin and cisplatin resistant cells the NRG-1 and EGF also have a strong effect on ERK5 phosphorylation compared to the basal control (Figure 5.4b, c).

In contrast, PDGF-BB was the weakest ERK5 activator in both A2780 WT and A2780-CP70 compared with other growth factors. However, PDGF-BB in A2780-ADR was the third most potent inducer of ERK5 activation after NRG-1 and EGF while the HGF was the weakest stimulator of ERK5 activity in these cell line (Figure 5.4b). On the other hand, EGFR was phosphorylated in all three cell lines in response to epidermal growth factor at different levels.

Altogether, these findings demonstrated that EGF and NRG-1 may play an important role in ERK5 activation in A2780 cell lines, especially in ADR and CP70 resistant cell lines.

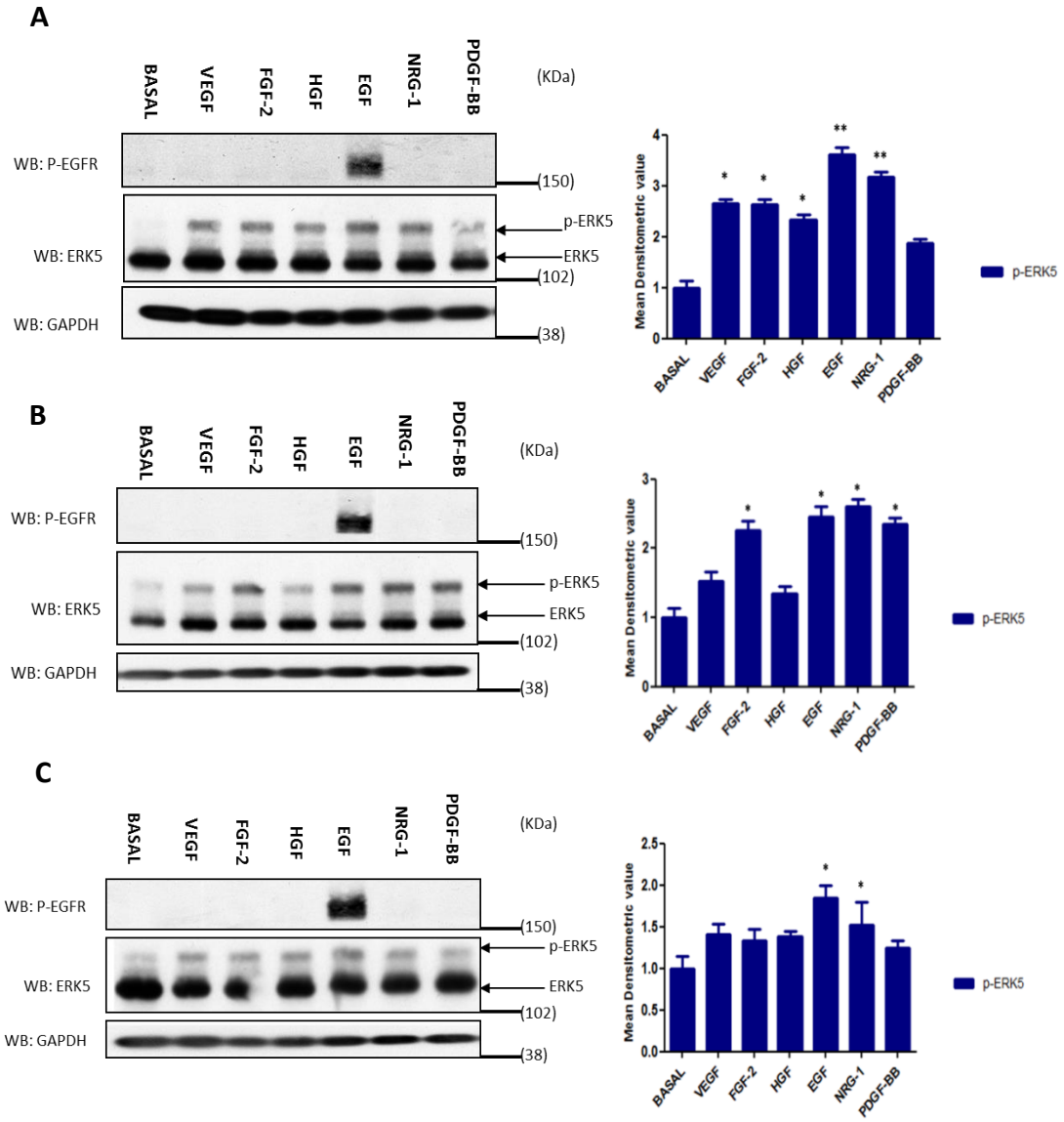


Figure 5.4 Characterisation of ERK5 activation in A2780-WT, A2780-ADR and A2780-CP70 in response to growth factors using electrophoretic mobility band shift. (A) A2780-WT, (B) A2780-ADR and (C) A2780-CP70 cells were seeded in 12-well plate for 24 hours and then serum starved overnight. Cells were stimulated with 50ng/ml of VEGF, FGF-2, HGF, EGF, NRG-1 and PDGF-BB followed by RIPA lysis. Protein lysates from A2780-WT, A2780-ADR and A2780-CP70 were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against p-EGFR, ERK5 and GAPDH. Analysis of densitometry of protein phosphorylation or protein expression was relative to basal control condition of each cell type which was (set arbitrarily as 1.0). This result is typical of three independent experiments (*p<0.05, **p<0.01)

5.2.3 EGF and NRG-1 mediate ERK5 activation in A2780 ovarian cancer cell lines

On average, 48% of ovarian cancer cases are reported to express aberrant EGFR receptors and ligands (Lafky et al., 2008). The overexpression of EGFR in ovarian cancer has been associated with poor prognosis (Bull Phelps et al., 2008). NRG-1 is an important member of the neuregulin family which consists of a broad family of EGF-like signalling molecules (Britsch, 2007). NRG-1 interacts with transmembrane tyrosine kinase receptors of ErbB family (Britsch, 2007). NRG-1 induced ErbB3 activation and promotes cell proliferation in human ovarian cancer cells (Sheng et al., 2010).

Figure 5.5 showed that stimulation of ovarian cancer cells with EGF and NRG-1 for 10 minutes induced the activation of ERK5 in all three cell lines. In contrast, the stimulation of these cells with both ligands, EGF and NRG-1, for 1 hour induced ERK5 phosphorylation but with less effect compared with 10 min stimulation. ERK5 activation in the parental cell line displayed more phosphorylation in response to EGF and NRG-1 compared with resistant cell lines ADR and CP70 (Figure 5.5).

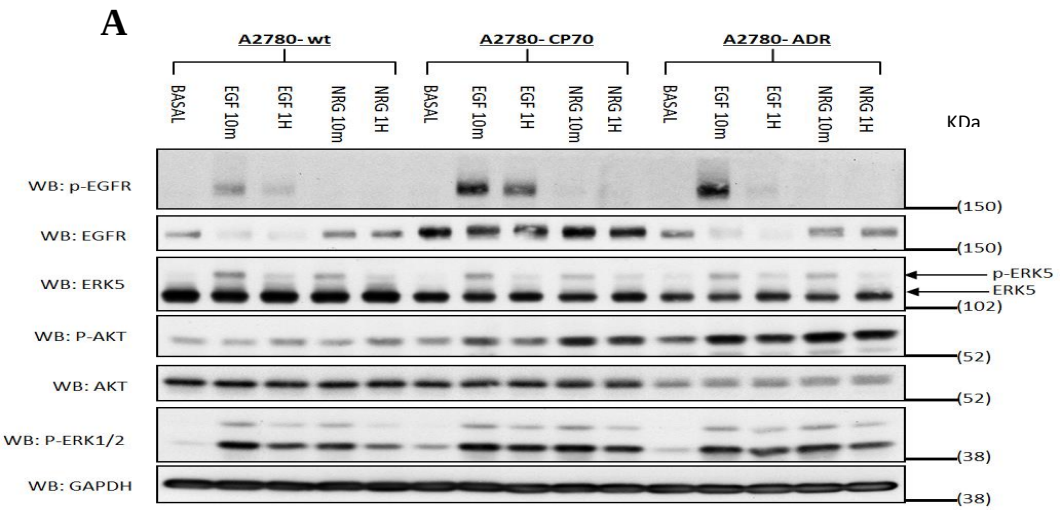
Four sites (Ser124, Thr450, Thr308 and Ser473) on AKT have been identified that are phosphorylated *in vivo* (Alessi et al., 1996); Ser124 and Thr450 are basally phosphorylated whereas Thr308 and Ser473 are required for AKT activation and phosphorylated in response to a range of extracellular stimuli (Datta et al., 1999). AKT was phosphorylated on Ser473 in A2780-ADR and A2780-CP70 in response to growth factors compared to the basal control (Figure 5.5).

BRAF and KRAS are associated with ovarian carcinogenesis and mutations in these genes lead to phosphorylation of the downstream target, MEK1/2, which in turn activates ERK1/2 resulting in phosphorylation of downstream cellular targets in ovarian cancer (Wan et al., 2004, Hsu et al., 2004).

Activation of the ERK1/2 pathway in response to EGF and NRG-1 was observed in all three ovarian cancer cell lines compared with the basal control (Figure 5.5). Furthermore, the ovarian cancer cell lines show a variable expression of EGFR activation in response to EGF (Figure 5.5).

Overexpression of EGFR has been linked with poor prognosis in advanced stage ovarian tumour (Figure 5.5) (Psyrri et al., 2005, Niikura et al., 1997a). EGFR was activated in response to EGF in resistant ovarian cancer cells (A2780-ADR and A2780-CP70) (Figure 5.5 a). To assess the expression of the EGFR family in ovarian cancer cells, quantitative RT-PCR was used. The mRNA expression assay displayed a high expression of *egfr-1* in CP70 and ADR in comparison to A2780-WT cells (Figure 5.5 c).

In addition, gene expression of *egfr-3* was expressed in both resistant cell lines and that may be associated with the finding that ErbB3 is present in a significant proportion of human ovarian cancers driven by NRG-1 which suggest that ErbB3 and NRG-1 are a possible treatment target in ovarian cancer (Figure 5.5 c) (Sheng et al., 2010).



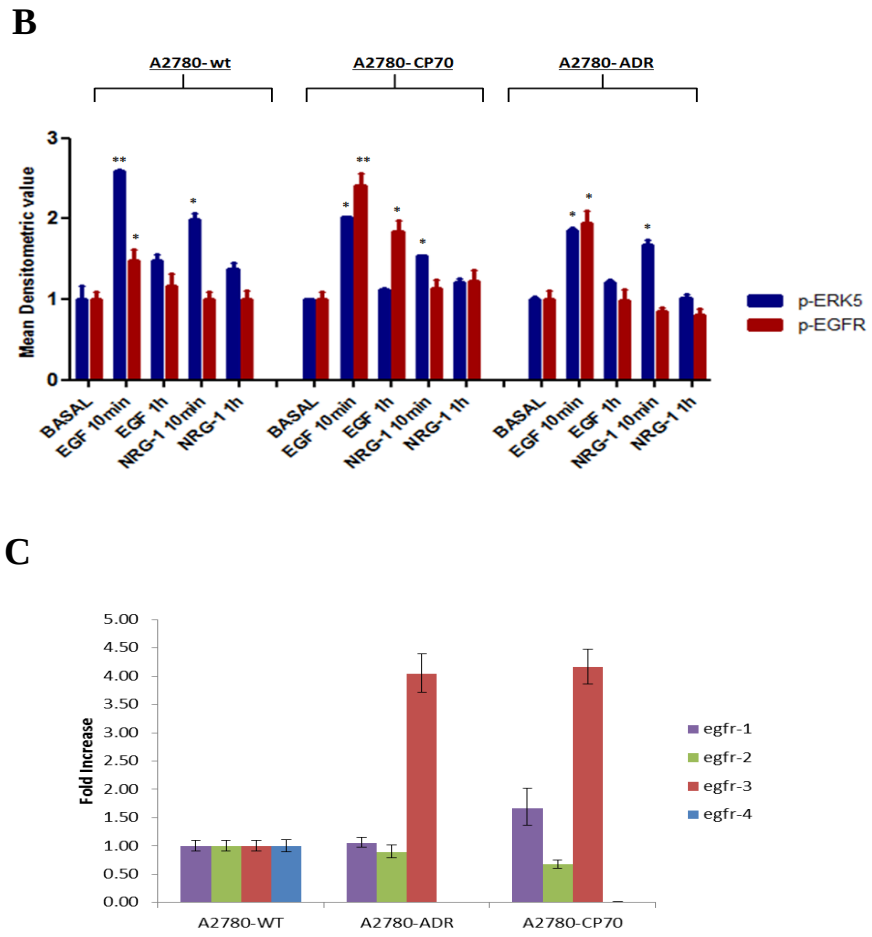


Figure 5.5 ERK5 activation in response to EGF and NRG-1 for 1 hour and 10 minutes. (A) A2780-WT, A2780-ADR and A2780-CP70 cells were seeded in a 12-well plate for 24 hours and then overnight serum starvation. Cells were stimulated with 50ng/ml of EGF and NRG-1 for 1 hour and 10 minutes followed by RIPA lysis. Protein lysates from A2780-WT, A2780-ADR and A2780-CP70 were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against p-EGFR, EGFR, ERK5, p-AKT, AKT, p-ERK1/2 and GAPDH. (B) Analysis of densitometry of protein phosphorylation or protein expression was relative to basal control condition of each cell type which was (set arbitrarily as 1.0). (C) The bar chart shows RT-PCR analysis for EGFR-1, 2, 3, 4 mRNA level in A2780-WT, A2780-CP70 and A2780-ADR. The ovarian cancer cells RNA were extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to GAPDH expression and illustrated as fold change. This result is typical of three independent experiments (*p<0.05, **p<0.01).

5.3 Small molecule kinase inhibitors

5.3.1 Treatment combination of doxorubicin with MEK5/ERK5 inhibitors decreased ERK5 activation in A2780-ADR

In order to investigate the role of combination treatment on activation of MEK5/ERK5 pathway in ovarian cancer cells, western blotting was applied to examine the phosphorylation of ERK5, ERK1/2 and EGFR proteins from extracts of cells treated with doxorubicin alone and in combination with small molecule kinase inhibitors of EGFR, ERK1/2, MEK5 and ERK5.

Treatment of A2780-WT and A2780-ADR cells with doxorubicin confirmed the result in Figure 5.1 which revealed that ERK5 was more activated in resistant cells than in the parental cells (Figure 5.6a).

Phosphorylation of ERK1/2 was increased in A2780-WT via doxorubicin compared to untreated cells, while in doxorubicin resistant cells there was no effect on ERK1/2 phosphorylation. In the parental cells, A2780-WT, the combination of doxorubicin with MEK5 inhibitor BIX02189, ERK5 inhibitor XMD8-92, EGFR inhibitor lapatinib and ERK1/2 inhibitor, trametinib, did not show a clear effect on the phosphorylation of ERK5 in comparison with the basal control (Figure 5.6a). The phosphorylation of ERK1/2 in A2780-WT appeared to be decreased by treatment of doxorubicin with lapatinib and as expected was inhibited by doxorubicin with trametinib. The densitometric data showed that the level of EGFR-1 phosphorylation was not clearly affected by doxorubicin with BIX02189, XMD8-92 or trametinib (Figure 5.6b).

Applying dual treatment in A2780-ADR showed that the MEK5 and ERK5 inhibitors, BIX02189 and XMD8-92 respectively, combined with doxorubicin, decreased the phosphorylation of ERK5 in comparison with the basal control. Furthermore, there were no obvious changes in ERK5 activation in A2780-ADR cells in response to the combination of doxorubicin

with lapatinib compared to the basal control, while the combination of doxorubicin with trametinib appeared to increase the activation of ERK5 (Figure 5.6).

Interestingly, phosphorylation of ERK1/2 was increased when the cells was treated with doxorubicin and BIX02189 or XND8-92 in comparison with the basal control (Figure 5.6). The combination of doxorubicin with lapatinib decreased the activation of ERK1/2 as seen in the parental cells as well. The densitometry of EGFR-1 phosphorylation revealed that doxorubicin increased the phosphorylation of EGFR-1 in doxorubicin resistant cells (Figure 5.6b). The combination of doxorubicin with BIX02189, XMD8-92 or trametinib increased the activation of EGFR-1 in A2780-ADR.

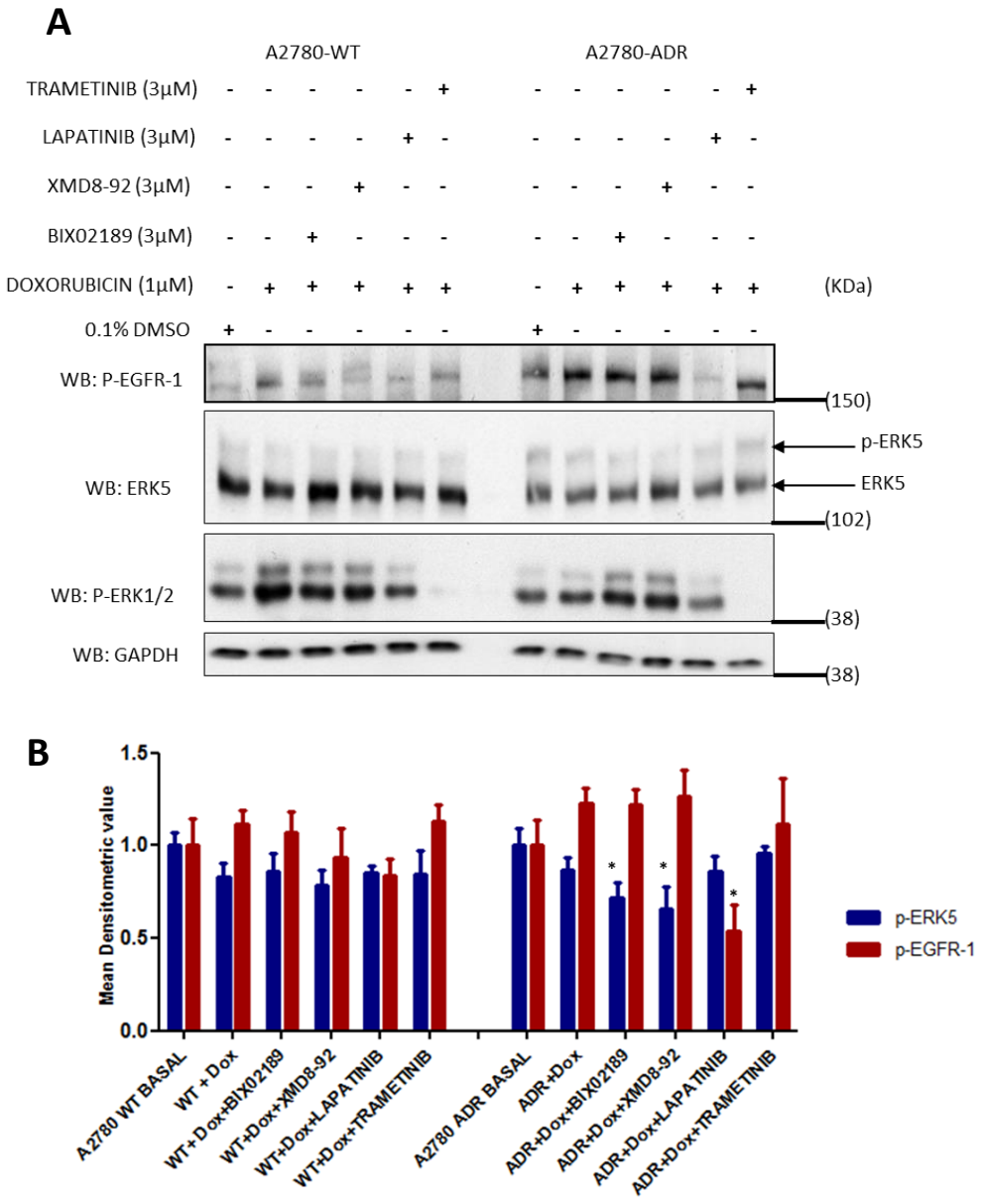


Figure 5.6. Small-molecule kinase inhibitors in A2780-WT and A2780-ADR. (A) A2780-WT and A2780-ADR cells were seeded in 12-well plate for 24 hours and then incubated with 1μM doxorubicin for 24 hours and 0.1% DMSO vehicle control, 3μM XMD8-92, 3μM BIX02189, 3μM lapatinib and 3μM trametinib were added to the drugged cells and incubated for 1 hour prior to lysis of the cells with RIPA lysis buffer. Protein lysates from A2780-WT and A2780-ADR cells were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-EGFR, p-ERK1/2 and GAPADH which was used as a loading control. (B) Analysis of densitometry of protein phosphorylation or protein expression is relative to basal control condition of each cell type which was (set arbitrarily as 1.0). This result is typical of three independent experiments (*p<0.05)

5.3.2 Small molecule kinase inhibitors reduced ERK5 phosphorylation in A2780-CP70

Treatment of A2780-WT and A2780-CP70 cells with cisplatin confirmed that the phosphorylation of ERK5 was increased in cisplatin resistant ovarian cancer cells compared with cisplatin-sensitive ovarian cancer cells (Figure 5.7a).

The combination of cisplatin with BIX02189 or XMD8-92 in A2780-WT did not show a clear effect on the activation of ERK5 compared with the basal control, while the effect of these treatment combinations clearly increased the phosphorylation of ERK1/2 (Figure 5.7a).

The phosphorylation of ERK5 in A2780-WT was slightly increased in response to cisplatin with lapatinib or trametinib (Figure 5.7a). EGFR-1 phosphorylation was increased in response to cisplatin in A2780-WT, whereas it decreased in response to the combined treatment of cisplatin with BIX02189 or XMD8-92.

In cisplatin resistant cells (A2780-CP70), the phosphorylation of ERK5 was decreased in response to treatment combinations of cisplatin with BIX02189, XMD8-92, lapatinib or trametinib compared to cells treated only with cisplatin (Figure 5.7a).

The combination of cisplatin with BIX02189, XMD8-92 or lapatinib in A2780-CP70 increased the activation of ERK1/2 compared with the basal control. As expected, the combination of cisplatin with trametinib abolished the activation of ERK1/2 in both cell lines (A2780-WT and A2780-CP70) to confirm its act (Figure 5.7a). In contrast to the parental cells, the cisplatin resistant ovarian cancer cells showed an increase in EGFR-1 phosphorylation level in response to the treatment combinations of cisplatin with MEK5 inhibitor BIX02189, ERK5 inhibitor XMD8-92 or ERK1/2 inhibitor trametinib (Figure 5.7a and b).

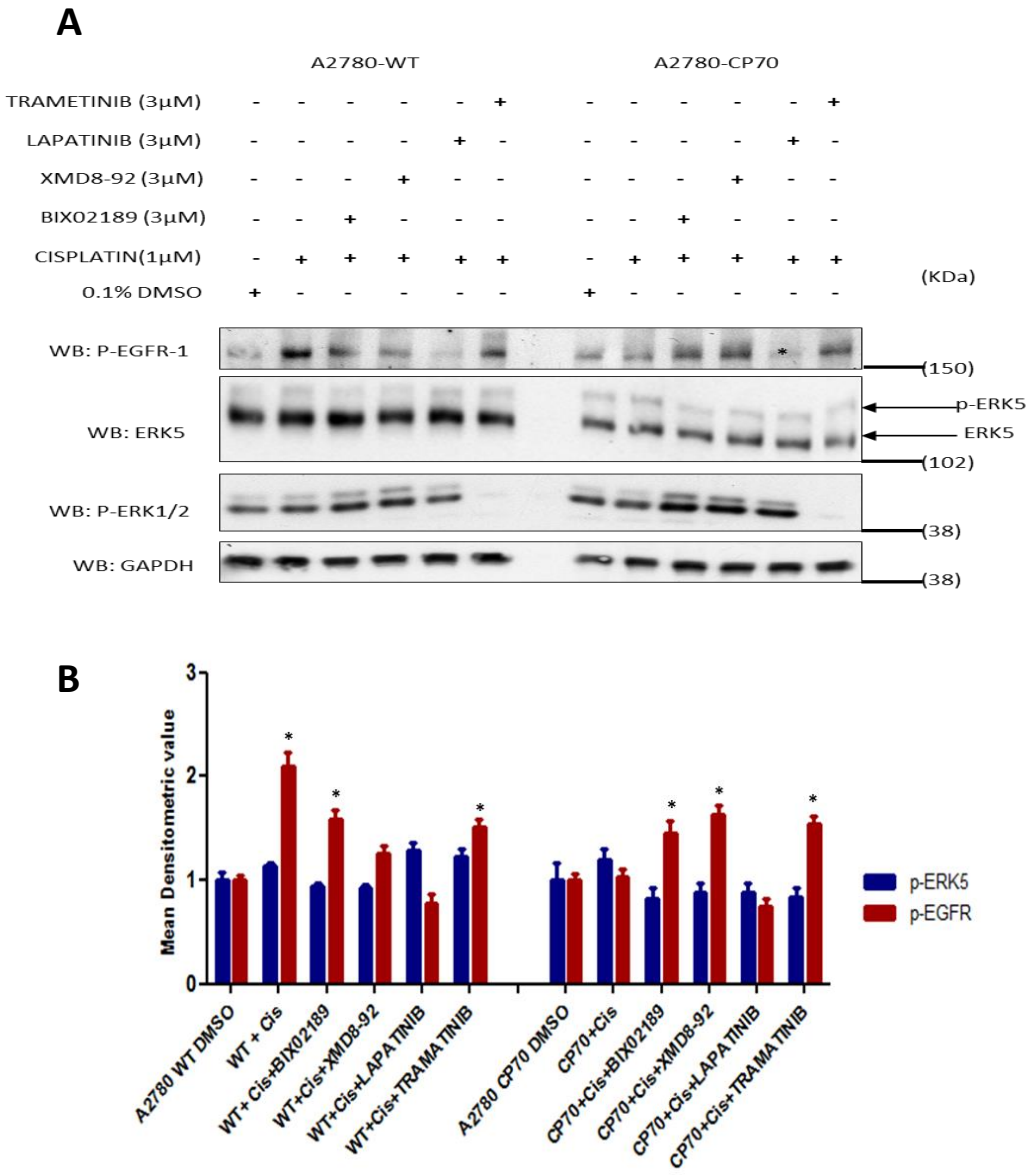


Figure 5.7 Small-molecule kinase inhibitors in A2780-WT and A2780-CP70. (A) A2780-WT and A2780-CP70 cells were seeded in 12-well plate for 24 hours and then incubated with 1μM cisplatin for 24 hours and 0.1% DMSO vehicle control, 3μM XMD8-92, 3μM BIX02189, 3μM lapatinib and 3μM trametinib were added to the drugged cells and incubated for 1 hour prior to lysis of the cells with RIPA lysis buffer. Protein lysates from A2780-WT and A2780-CP70 cells were resolved on 8% acrylamide gel and analysed with western blot by using antibodies against ERK5, p-EGFR, p-ERK1/2 and GAPADH which was used as a loading control. (B) Analysis of densitometry of protein phosphorylation or protein expression is relative to basal control condition of each cell type which was (set arbitrarily as 1.0). This result is typical of three independent experiments (*p<0.05).

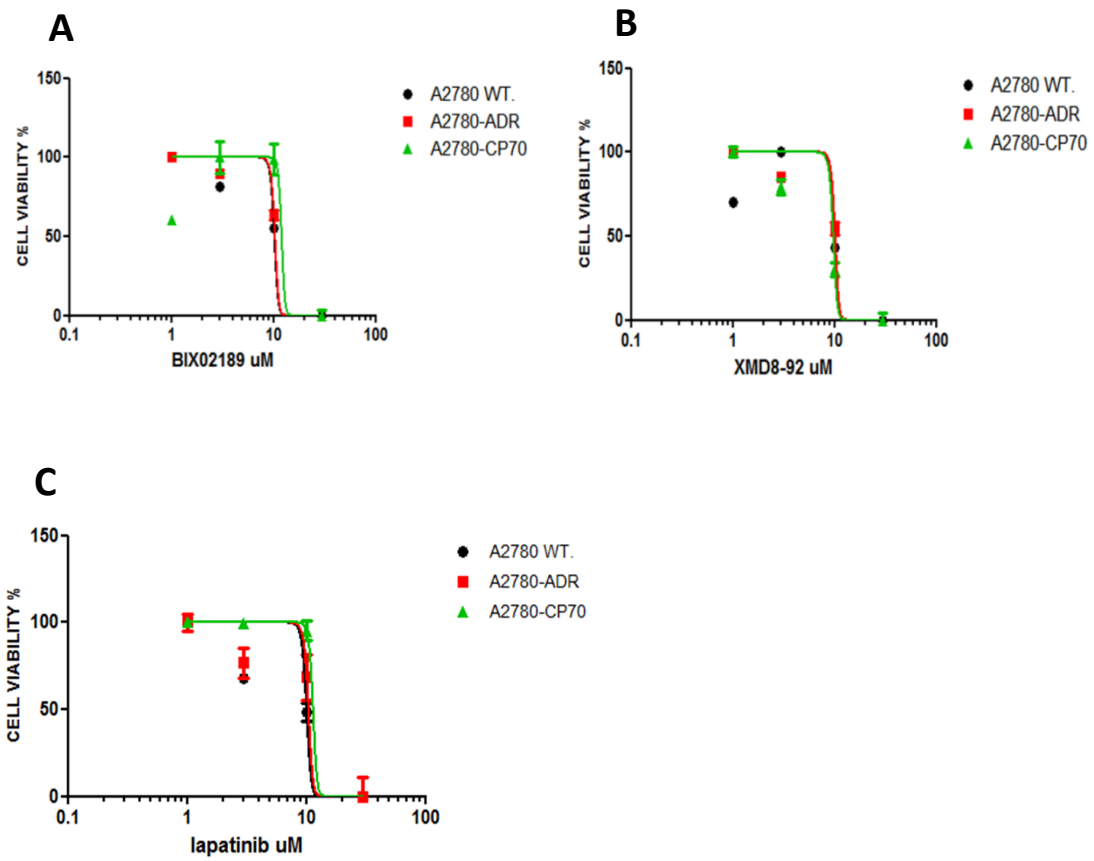
5.4 Doxorubicin resistance is prevented by inhibition of the MEK5/ERK5 signalling pathway

Ovarian cancer treatment consists of a combination of surgery and chemotherapy, but patients usually experience disease relapse within two years of initial treatment due to the development of drug resistance (Mody et al., 2003). Thus, a better understanding of the mechanisms underlying drug resistance could improve treatment and extend survival time.

The A2780-WT and doxorubicin resistant cell line A2780-ADR were investigated for their sensitivity to doxorubicin on its own and in combination with MEK5/ERK5 inhibitors, BIX02189 and XMD8-92, or the EGFR-1 inhibitor, lapatinib. To assess the appropriate dose of inhibitors for combination with doxorubicin and avoid toxicity, dose response of BIX02189, XMD8-92 and lapatinib was applied in the A2780-WT, A2780-ADR and A2780-CP70 cells. The IC_{50} of all of these inhibitors was around 10 μM (Figure 5.8). Therefore 3 μM was selected as a minimal cell toxicity level. The parental cell line A2780-WT was sensitive to doxorubicin with an IC_{50} of 0.412 μM compared with the A2780-ADR cells which were more resistant to doxorubicin with an IC_{50} of 9.75 μM, which is a 23.7-fold increase in IC_{50} (Figure 5.9).

There was no change in the sensitivity to doxorubicin with A2780-WT cells in combination with BIX02189, XMD8-92 or lapatinib (Figure 5.9a). In contrast, incubation of A2780-ADR cells with BIX02189, or XMD8-92, or lapatinib appeared to reverse sensitivity to doxorubicin by potentially reversing resistance (Figure 5.9b).

These findings suggested that a combination therapy of doxorubicin with MEK5/ERK5 cascade inhibitors (BIX02189 and XMD8-92) or EGFR-1 inhibitor (lapatinib) reduced the resistance of A2780-ADR cells to doxorubicin.



Compound	IC50 (μM)		
	A2780-WT	A2780-ADR	A2780-CP70
BIX02189	10.09	10.24	11.89
XMD8-92	10.11	10.14	9.66
lapatinib	9.79	10.34	11.28

Figure 5.8 Dose response curves of MEK5 inhibitor BIX02189, ERK5 inhibitor XMD8-92 and EGFR-1 inhibitor lapatinib in A2780-WT, A2780-ADR and A2780-CP70 cells. A2780-WT, A2780-ADR and A2780-CP70 cells seeded in 96-well plate for 24 hours and then treated with different concentration of inhibitors (A) BIX02189, (B) XMD8-92 and (C) lapatinib and then incubated for 72 hours. After that, cells were analysed by using *CellTiter-Glo® Luminescent Cell Viability Assay. This result is representative of three independent experiments.*

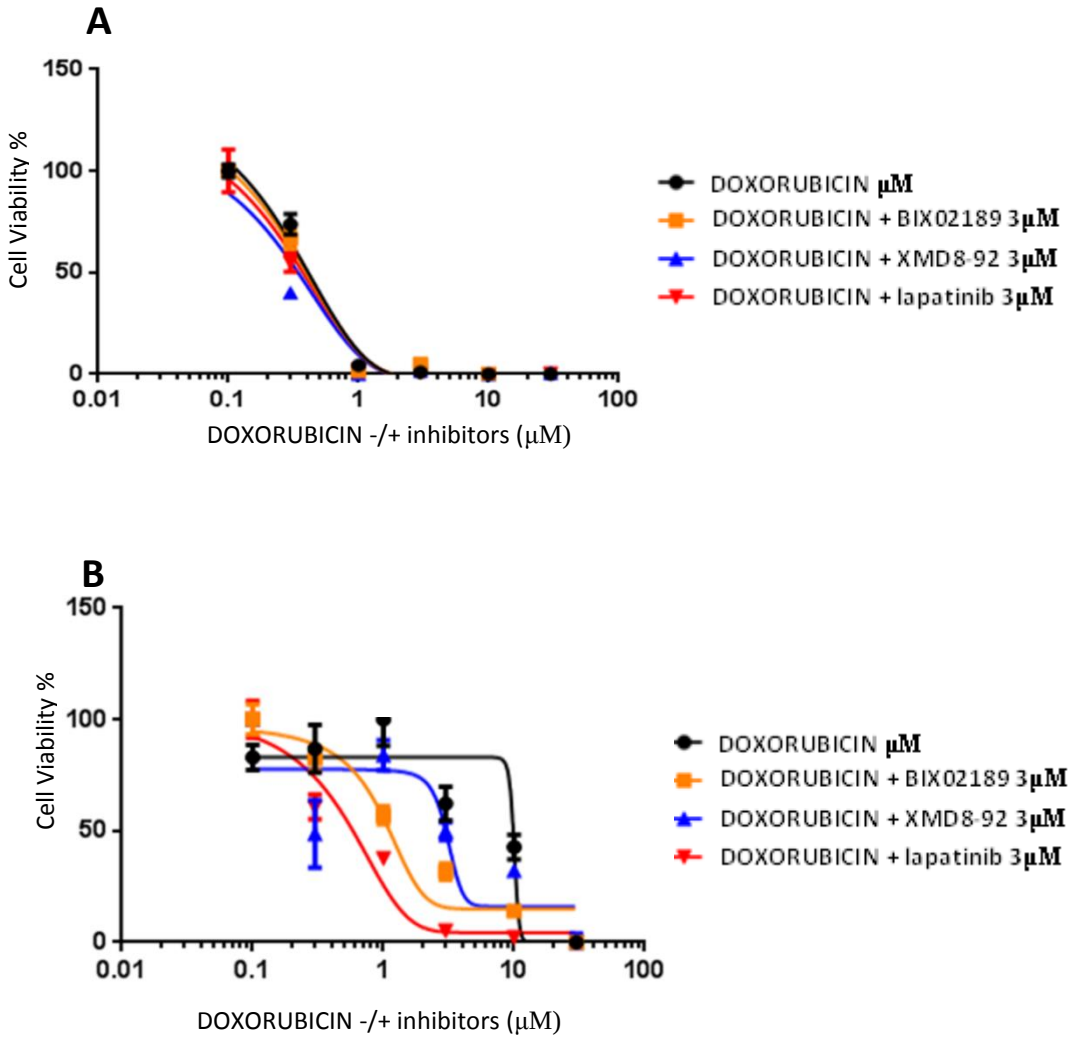


Figure 5.9. Dose response curves of DOXORUBICIN combined with/without MEK5/ERK5 signalling pathway inhibitors and EGFR inhibitor in A2780-WT and A2780-ADR. (A) A2780-WT and (B) A2780-ADR cells seeded in 96-well plate for 24 hours and then treated with different concentration of doxorubicin on its own and with 3µM BIX02189, or XMD8-92 or lapatinib and then incubated for 72 hours. After that, cells were analysed by using CellTiter-Glo® Luminescent Cell Viability Assay. This result is representative of three independent experiments.

Table 5.1 The sensitivity of A2780-WT and A2780-ADR to doxorubicin -/+ BIX02189 or XMD8-92 or lapatinib

Compound	IC50 (µM)		
	A2780-WT	A2780-ADR	Fold resistance
DOXORUBICIN	0.412	9.75	23.7
DOXORUBICIN + 3µM BIX02189	0.390	1.12	2.9
DOXORUBICIN + 3µM XMD8-92	0.340	3.80	11.2
DOXORUBICIN + 3µM lapatinib	0.370	0.574	1.6

Fold resistance is defined as the IC50 of the resistant cell line / IC50 of the parental cell line (Lidsky et al., 2014).

5.4.1 Inhibition of EGFR reduced cisplatin resistance in A2780-CP70 cells

The resistant cell line A2780-CP70 and parental cell line A2780-WT were treated with dose response of cisplatin alone and in combination with 3μM of BIX02189, XMD8-92 or lapatinib to analyse the effect of these kinase inhibitors on cisplatin resistance using a cell viability assay. The concentration was determined by using dose response of these inhibitors alone in all three ovarian cancer cell lines via a cell viability assay (Figure 5.8).

To assess the effect of the treatment combination of cisplatin with BIX02189, or XMD8-92 or lapatinib on cisplatin resistant ovarian cancer cells, cell viability of A2780-WT and A2780-CP70 cells in response to cisplatin alone and in combination with these inhibitors was applied.

The parental cell line A2780-WT was sensitive to cisplatin with an IC_{50} for 0.615 μM, while the cisplatin resistant cells A2780-CP70 were more resistant to cisplatin with an IC_{50} of 9.917 μM which gave 16.12-fold increase in IC_{50} (Figure 5.10).

In the parental cell lines A2780-WT, combination treatment of cisplatin with BIX02189, or XMD8-92 or lapatinib did not change the sensitivity of these cells to cisplatin (Figure 5.10a). However, incubation of A2780-CP70 cells with lapatinib reversed the resistance of these cells to cisplatin with an IC50 of 0.830 μM compared to an IC50 of 9.917 μM for treatment with cisplatin alone (Figure 5.10b).

This finding demonstrated that, the EGFR-1 inhibitor (lapatinib) in combination with cisplatin could potentially reverse the resistance of A2780-CP70 to cisplatin.

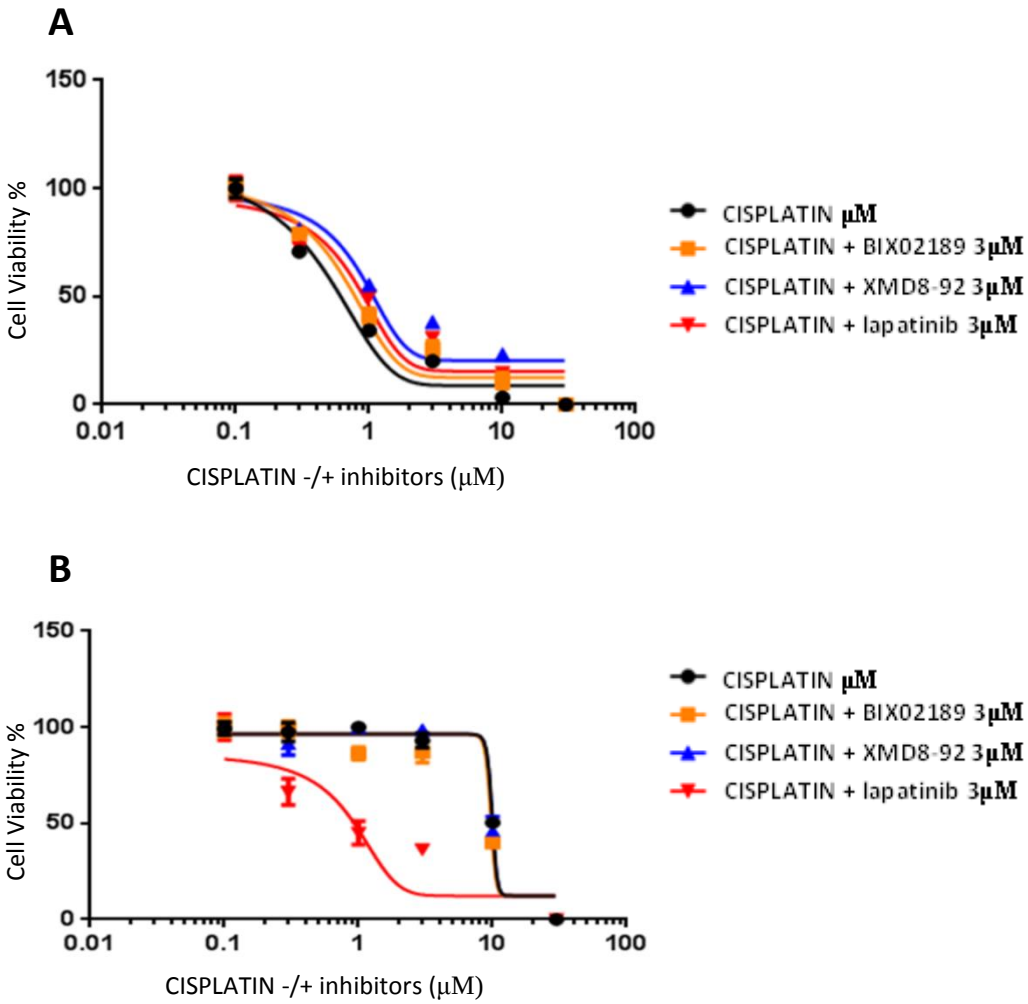


Figure 5.10. Dose response curves of cisplatin combined with/without MEK5/ERK5 signalling pathway inhibitors and EGFR inhibitor in A2780-WT and A2780-CP70. (A) A2780-WT and (B) A2780-CP70 cells seeded in 96-well plate for 24 hours and then treated with different concentration of cisplatin on its own and with 3µM BIX02189, XMD8-92 and lapatinib and then incubated for 72 hours. After that, cells were analysed by using CellTiter-Glo® Luminescent Cell Viability Assay. This result is representative of three independent experiments.

Table 5.2 Sensitivity of A2780-WT and A2780-CP70 to cisplatin -/+ BIX02189, or XMD8-92 or lapatinib

Compound	IC50 (µM)		
	A2780-WT	A2780-CP70	Fold resistance
CISPLATIN	0.615	9.917	16.1
CISPLATIN + 3µM BIX02189	0.760	9.685	12.7
CISPLATIN + 3µM XMD8-92	1.03	9.825	9.5
CISPLATIN + 3µM lapatinib	0.902	0.830	0.9

Fold resistance is defined as the IC50 of the resistant cell line / IC50 of the parental cell line (Lidsky et al., 2014).

5.5 MEK5/ERK5 signalling pathway inhibitors decrease angiogenesis stimulated by A2780 ovarian cancer cells

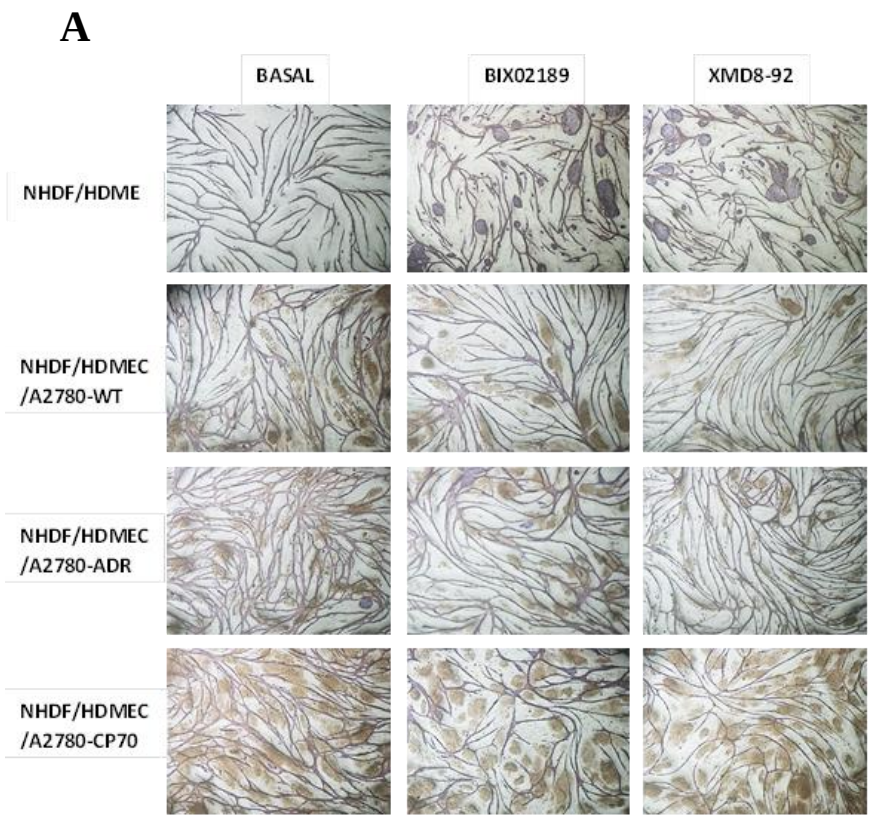
Angiogenesis is a crucial factor in tumour growth and invasion, and the formation of metastases. Clinical studies have suggested a specific role of angiogenesis and its effectors in ovarian cancer development (Abu-Jawdeh et al., 1996).

In order to assess the effect of MEK5 and ERK5 inhibitor (BIX02189 and XMD8-92 respectively) treatment upon the angiogenic potential of A2780-WT, A2780-ADR and A2780-CP70 ovarian cancer cell lines, a co-culture assay of NHDF/HDMEC/A2780s was performed. After plating the fibroblast cells on day 1, the ovarian cancer cells were added onto the fibroblast monolayer on day 3. HDMECs were added to the NHDF/ovarian cancer cells after 24 hours. On day 5, the co-culture of NHDF/HDMECs/A2780s was treated with BIX02189 or XMD8-92 and incubated at 37 ºC until day 8. Treatment of the co-culture with inhibitors was repeated and incubated in 37 ºC for 48 hours. On day 10, the co-culture was fixed. Furthermore, a NHDF/HDMEC co-culture assay without tumour cells was run as a control.

It was found that tumour cells induced tube-formation in the NDHF/HDMEC/A2780 co-culture assay compared to the co-culture without cancer cells (Figure 5.11). Moreover, the inclusion of doxorubicin and cisplatin resistant cells in the co-culture assay prompted the development of more networked capillary vessels compared to the basal control assay and the A2780-WT sensitive tumour cell assay, which could mean that the expression levels of angiogenic factors (VEGF-A, B, C and D) from tumour cells are reflective of the degree of aggressiveness of the tumour cells (Figure 12d).

Quantification of total tube length revealed that the MEK5 inhibitor BIX02189 reduced tubule formation in the doxorubicin and cisplatin resistant cell lines by 3-fold respectively compared to non-treated cells (Figure 5.11b).

In addition, treatment with the ERK5 inhibitor XMD8-92, decreased tube-formation more than 2-fold in A2780-ADR cells and A2780-CP70 cells compared to untreated cells. The decrease in the level of tube formation in resistant ovarian cancer cells after treatment with MEK5/ERK5 signalling pathway inhibitors, BIX02189 and XMD80-92, when compared to the sensitive ovarian cancer cells or basal control assay without tumour cells could indicate that ERK5 plays a role in resistant cancer cells and suggests that ERK5 is responsible in resistant cancer cell lines for inducing more capillary like-structures.



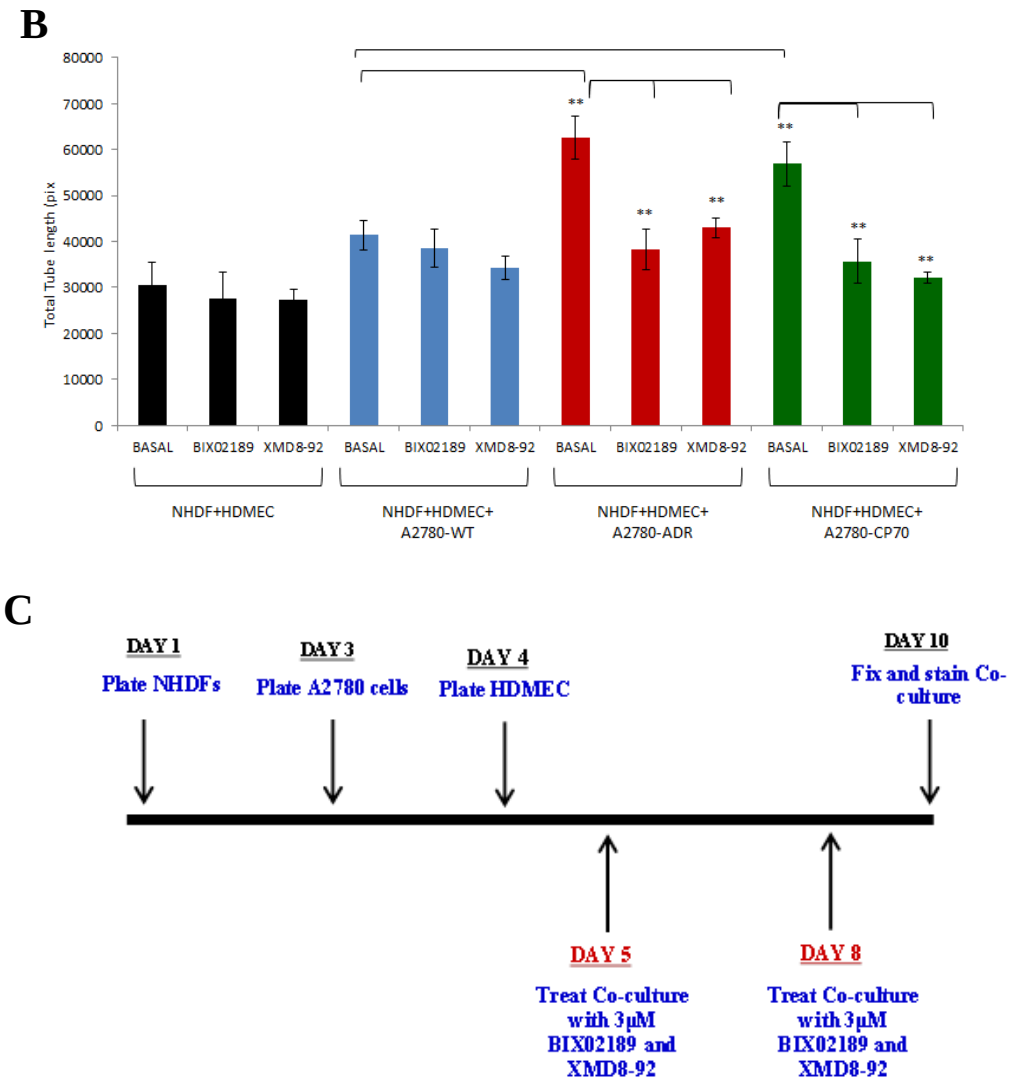


Figure 5.11. The effect of MEK5/ERK5 inhibitors, BIX02189 and XMD8-92, on tumour angiogenesis in a HDMEC/NHDF/A2780s co-culture assay. (A) NHDF were seeded in fibroblast growth medium at 20,000 cells per well on gelatine-coated 24-well plates and incubated for 48 hours. On day 3, A2780-WT, A2780-ADR and A2780-CP70 cells were plated at 5,000 cells per well on the NHDF layer and incubated for 24 hours. On day 4 of the assay, HDMECs were plated at 45,000 cells per well onto the confluent NHDF and A2780s layers with EBM MV2 basal medium FGM. On day 5, co-culture media were changed to EBM MV2 basal medium containing 1% (v/v) FCS and treated with indicated amount of BIX02189 and XMD8-92, as shown in the treatment schedule (C).The treatment was repeated on day 8. On day 10, cells were fixed and stained as described (section 2.2.9.3.3), and total tube length was quantified using AngioQuant image analysis software. (B) Data is presented as total tube length compared to basal. This result is typical of two independent experiments (**$p<0.01$).

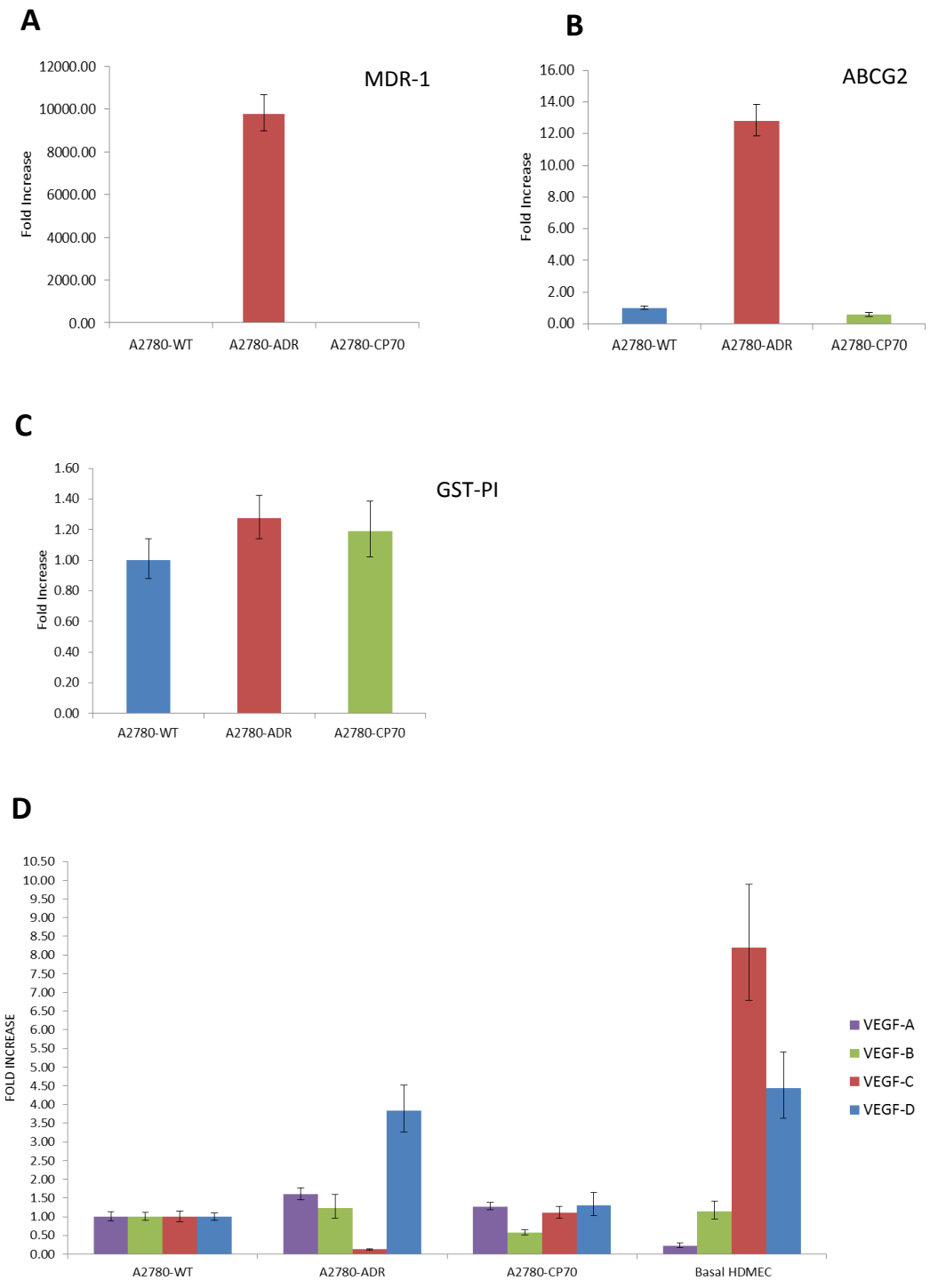


Figure 5.12 mRNA expression of in ovarian cancer cells. The bar chart shows RT-PCR analysis of (A) *mdr-1*, (B) *abcg2*, (C) *gst-pi* and (D) *vegf-a*, *vegf-b*, *vegf-c* and *vegf-d* mRNA expression in A2780-WT, A2780-CP70 and A2780-ADR. The ovarian cancer cells RNA were extracted and cDNA prepared. Data were analysed by the ΔΔCt value method and the expression was normalized to GAPDH expression and illustrated as fold change.

5.6 Discussion

The purpose of conducting the experiments detailed in this chapter was to assess the role of the MEK5/ERK5 signalling pathway in doxorubicin and cisplatin resistant ovarian carcinoma cells by using the small molecule kinase inhibitors BIX02189 and XMD8-92.

In this study, the ovarian carcinoma cell lines (A2780-WT, A2780-ADR and A2780-CP70) were exposed to doxorubicin and cisplatin to assess the effect of these chemotherapeutic drugs on ERK5 activation.

Increased ERK5 activation was observed in the resistant cell lines in comparison with the sensitive cells which mean that the MEK5/ERK5 signalling pathway could play a role in drug resistance in ovarian carcinoma (Figure 5.2).

Furthermore, the resistant cells exposed to chemotherapeutic agents displayed increased ERK5 activation, which is considered an important contributor to the cell survival mechanism, suggesting that doxorubicin and cisplatin activate survival pathways in ovarian carcinoma cells (Wang and Tournier, 2006).

ERK5 activation is increased in response to growth factors (Mody et al., 2003). Thus, Figure 5.4a, b and c showed that phosphorylation of ERK5 increased in response to a variety of growth factors in all three ovarian cancer cell lines. Receptor tyrosine kinases of the ErbB family are activated when it interacts with ligands of the EGF family (Wang and Tournier, 2006).

Resistance to chemotherapy, metastasis and poor prognosis in cancer are associated with over expression of EGFR receptors (Wang and Tournier, 2006). EGFR is induced via epidermal growth factor (EGF) and other related ligands, whereas EGFR-2, 3 and 4 are activated by EGF-like ligands called neuregulins (NRGs) (Esparis-Ogando et al., 2002a). Analysis of ERK5

activation in human derived cancer cell lines such as breast cancer has illustrated that constitutive activation of ERK5 is associated with activated forms of ErbB2, 3 and 4 (Esparis-Ogando et al., 2002a). Knockdown of ERK5 via siRNA induced apoptosis and decreased chemo resistance in HL-60 acute myeloid leukaemia cells (Hayashi et al., 2005b).

In this chapter, EGF and NRG-1 were the most potent ligands that increased ERK5 activation in ovarian cancer cell lines (Figure 5.4). EGFR was activated in ovarian cancer cells in response to EGF. The gene expression of *erbb1* and *erbb3* were increased in ovarian cancer resistant cell lines which demonstrated their role in poor prognosis and resistance to chemotherapy agents in cancer patients (Figure 5.5c).

Inhibition of the MEK5/ERK5 signalling pathway by the small kinase inhibitors BIX02189 and XMD8-92 in combination with doxorubicin or cisplatin was investigated in doxorubicin and cisplatin resistant ovarian cancer cell lines (Figure 5.6 and 5.7). Combination therapies using chemotherapeutic drugs (doxorubicin and cisplatin) with MEK5/ERK5 signalling pathway inhibitors (BIX02189 and XMD8-92) or EGFR inhibitor (lapatinib) were applied to evaluate the effect of these combinations on the resistance of A2780 ovarian cancer cell lines.

BIX02189, XMD8-92 or lapatinib in combination with doxorubicin shifted the resistance in doxorubicin-resistant cells compared to cells treated with doxorubicin alone (Figure 5.9). The inhibition of EGFR combined with doxorubicin in A278-ADR cells restored sensitivity to doxorubicin to approximately the same level as the parental cell line (Figure 5.9). Moreover, treatment of cisplatin-resistant cells with a combination of lapatinib and cisplatin restored sensitivity to cisplatin to approximately the level of the parental cell line (Figure 5.10).

Different mechanisms have been considered for doxorubicin resistance, including the expression of many membrane efflux pumps; the gene expression of MDR-1 and ABCG-2 in A2780-ADR cells was increased more than 3500-fold and 20-fold respectively in comparison with A2780-WT (Gottesman et al., 2002) (Figure 5.12a and b). Furthermore, increased drug metabolism enzymes such as glutathione S-transferase P1 (GSTP1) affect doxorubicin cytotoxicity in cancer and the gene expression of GSTP1 in A2780-ADR cells was increased compared to the parental cell line (Harbottle et al., 2001) (Figure 5.12c).

There are several mechanisms implicated in cisplatin resistance including increased DNA repair, decreased drug accumulation and increased detoxification via GSTP1 which is considered as a key limiting factor in cisplatin cytotoxicity (Pasello et al., 2008). Gene expression of GSTP1 in A2780-CP70 cells has been investigated and showed an increase compared to parental cell line (Figure 5.12c).

Thus in future works, inhibition of certain ABC transporters such as MDR-1 and ABCG-2 by MEK5/ERK5 could be a mechanism to assess the role of the MEK5/ERK5 signalling pathway in the regulation of chemoresistance in tumour cells that overexpress many ABC transporters. These findings suggested that the MEK5/ERK5 signalling pathway, along with EGFR, could be a possible therapeutic target to reduce drug resistance in ovarian cancer.

Deletion of the *erk5* gene revealed its involvement in tumour associated angiogenesis and its effect in the vasculature development of melanoma and lung carcinoma xenograft (Hayashi et al., 2005b). In addition, deletion of *erk5* in flank region of mice reduced the number of large blood vessels in a growing tumour (Hayashi et al., 2005b).

In this chapter, the role of the MEK5/ERK5 pathway in tumour angiogenesis was assessed by inhibition of this cascade via BIX02189 and XMD8-92

inhibitors in a NHDF/HDMEC/A2780s co-culture *in vitro* angiogenesis assay.

Initially, embedding ovarian cancer cells in either sensitive or resistant state in NHDF/HDMEC co-culture revealed that tumour cells induced more capillary network vessels compared to co-cultures without tumour cells (Figure 5.11). Previous data from our group revealed that ERK5 is required for AKT phosphorylation, and also involved in VEGF-mediated survival and tubular morphogenesis in HDMECs (Roberts et al., 2010c). The doxorubicin or cisplatin resistant cells triggered more tubular formation compared with A2780-WT cells which demonstrates the aggressiveness of resistant cells.

The ability of tumour cells to increase tube-formation relates to increased secretion of VEGF-A to induce endothelial cells and increase tubule formation (Figure 5.12d). Treatment of the co-culture with MEK5 and ERK5 inhibitors (BIX02189 and XMD8-92) revealed that tube formation was reduced, and this effect was more evident in resistant cancer cells.

These data suggest that ERK5 plays a role in tumour angiogenesis and is required in resistant cancer cells to induce networked blood vessels; this may provide a possible means for therapeutic inhibition of angiogenesis *in vivo*. Future study should aim to investigate the effect of siRNA-mediated silencing of MEK5 and ERK5 expression in a NEHDF/HDMEC/cancer cells co-culture to further validate this model.

Chapter Six: General Discussion

6.1 The role of ERK5 signalling axis in tumour angiogenesis and drug resistance

The ERK5 protein is ubiquitously expressed and plays different roles in many cell types (Hayashi et al., 2004a, X and Tournier, 2006, Spiering et al., 2009). In addition to its role in regulating cellular processes such as survival, proliferation and differentiation, ERK5 has been involved in the progression of a number of pathologies such as cancer and ischaemia (Kato et al., 1998a, X and Tournier, 2006, Takeishi et al., 1999, Montero et al., 2009a).

Gene targeting studies in mice highlighted the critical role of ERK5 in cardiovascular development and maintenance of vascular integrity (Hayashi and Lee, 2004, Hayashi et al., 2004b). Moreover, some studies have characterised the role of ERK5 activation in regulating intracellular signalling and gene expression in human endothelial cells (Abe et al., 1996, Yan et al., 1999, Roberts et al., 2010b). In addition, several studies focused on the role of ERK5 activation in response to EGF in case of tumour cell proliferation and intracellular localisation (Kato et al., 1998a, Kamakura et al., 1999, Raviv et al., 2004, Kondoh et al., 2006, Yang et al., 2010d). Aberrant ERK5 signalling has been already reported in various cancers such as breast cancer, hepatocellular, prostate cancer and malignant mesothelioma and associated with poor prognosis (Montero et al., 2009a, Zen et al., 2009, McCracken et al., 2008b, Shukla et al., 2013a). ERK5 appears to be critical factor in tumour-associated angiogenesis and has been presented to be vital for angiogenesis in melanoma and lung carcinoma, as well as prostate cancer metastasis (Hayashi et al., 2005a, Mehta et al., 2003b).

This thesis has addressed the effect of the therapeutic inhibition of ERK5 signalling in tumour angiogenesis by using melanoma and ovarian carcinoma as tumour cell models, as well as assessing the combination therapy of MEK5/ERK5 signalling inhibitors with chemotherapy agents in drug-resistant cancer cells.

6.1.1 pro-angiogenic factors induce phosphorylation of ERK5 in HDMECs and HeLa cells

The characterisation of ERK5 activation was investigated in this project by using VEGF stimulation of HDMECs and EGF stimulation of HeLa cells based on previous findings (Roberts et al., 2010c, Mody et al., 2001b). It was confirmed that VEGF and EGF stimulated ERK5 phosphorylation in HDMECs and HeLa cells respectively (Figure 3.1). In this project, the detection of ERK5 activation by phospho-ERK5 (Thr^{218}/Tyr^{220}) antibody for western blotting and through electrophoretic mobility bandshift on conventional SDS-PAGE was used. The activation of ERK5 by phosphorylation by its upstream MEK5 preferentially phosphorylates the threonine residue of the T-X-Y motif which is similar that of JNK activation by its upstream MEK4/7 (Mody et al., 2003), opposite to p38 MAPK and ERK1/2 which are phosphorylated on the tyrosine residue of the T-X-Y (Haystead et al., 1992, Fleming et al., 2000). However, ERK5 is distinct in comparison with JNK, p38 MAPK and ERK1/2, in addition to dual phosphorylation of threonine or tyrosine residues of T-X-Y motif, ERK5 is able to undergo autophosphorylation of its C-terminal tail residues (Mody et al., 2003, Buschbeck and Ullrich, 2005).

This project in line with current studies, confirmed that EGF stimulated HeLa cells undergo phosphorylation of ERK5 on Thr^{218}/Tyr^{220} residues in the activation loop of the kinase domain and residues in the C-terminal tail which resulted in a mobility bandshift with conventional SDS-PAGE. In endothelial cells (HDMECs), VEGF stimulated HDMECs analysed by conventional SDS-PAGE did not induce an ERK5 mobility bandshift, with ERK5 activation only detected by using phospho-ERK5 (Thr^{218}/Tyr^{220}) antibody for Western blotting. This data suggested that, VEGF has only the ability to induce the dual phosphorylation of the kinase domain of ERK5, without any effect on the phosphorylation of the C-terminal residues. Taken together this data suggests that phosphorylation of Thr^{218}/Tyr^{220} in the kinase domain and

C-terminal phosphorylation are not mutually inclusive events and that ERK5 can be activated in the absence of C-terminal phosphorylation. This suggests that in endothelial cells, ERK5 may play a different intracellular signalling role than in epithelial cells. Interestingly, the phenotype of *erk5 -/-* mice shows cardiovascular defects with loss of cardiac vascular integrity suggesting that endothelial cells have a unique requirement for ERK5 activation (Nithianandarajah-Jones et al., 2014)

The dose response data with the ERK5 inhibitor XMD8-92 on HeLa cells suggested that phosphorylation of ERK5 on Thr^{218}/Tyr^{220} residues was not affected by the inhibitor; however a decrease in bandshift was observed suggesting that C-terminal phosphorylation was inhibited. This is consistent with XMD8-92 affecting ERK5 kinase activity rather than phosphorylation of the T-E-Y motif which is accomplished by MEK5. The use of XMD8-92 in HDMECs showed that VEGF-stimulated AKT activity was not affected by use of this inhibitor (Figure.3.4).

The dose response data of MEK5 inhibitor BIX02189 on HDMECs revealed that the phosphorylation of AKT requires VEGF-mediated MEK5 kinase activity and subsequently ERK5 activation to some degree (Figure 3.6). Previous data from the group has shown that in HDMECs, siRNA mediated silencing of ERK5 reduces VEGF-mediated AKT phosphorylation by approximately 50% when cells are on a gelatin matrix (Roberts et al., 2010b); this would appear to agree with the data generated with the BIX02189. In contrast, EGF-mediated ERK5 phosphorylation in HeLa with BIX02189 or even XMD8-92 did not affect AKT activity (Figure 3.3 and 3.5). XMD8-92 inhibits ERK5 activity and supresses tumour growth in lung and cervical tumour model without inducing vasculature abnormalities (Yang et al., 2010c, Yang and Lee, 2011). One potential concern with the development of ERK5 inhibitors is the profound cardiovascular abnormalities seen in the erk5 $^{-/-}$ gene KO studies (Nithianandarajah-Jones et al., 2012a). Considering that XMD8-92 appears to preserve VEGF-mediated AKT activation in contrast to

siRNA mediated gene silencing (Roberts et al., 2010b) it is interesting to speculate that inhibition of ERK5 kinase activity in endothelial cells may not have the detrimental effects seen with the erk5 $^{-/-}$ gene knockout and that XMD8-92 may be a viable drug for use in patients.

6.1.2 Activation of ERK5 increases tumour resistance

All types of cancer virtually characterised by their ability to progress for increase malignancy. There are number of different methods for cancer treatment. Chemotherapy is one of the most important for treatment of cancer, but the major problem that limits the effectiveness of chemotherapy to treat cancer is drug resistance. Tumour cells can be intrinsically resistant to chemotherapy before receiving the treatment. However, drug resistance can be acquired and develop during treatment that were initially sensitive to chemotherapy and that can be caused by mutations during treatment or increased expression of therapeutic target and activation of alternative signalling pathway (Holohan et al., 2013). Mitogen activated protein kinases (MAPKs) are serine threonine kinases mediating intracellular signalling associated with different cellular activities including cell differentiation, proliferation and migration. The mammalian MAPK family is composed of ERK, p38 MAPK, JNK and ERK5 (Dhillon et al., 2007). MAPK plays a key role in development and progression of cancer (Kohno and Pouyssegur, 2006, Galabova-Kovacs et al., 2006, Bradham and McClay, 2006, Bubici and Papa, 2014, Ortiz-Ruiz et al., 2014). ERK5 has been shown to mediate resistance to cytotoxic chemotherapy-induced apoptosis (Weldon et al., 2002). In this project, the role of ERK5 in drug resistance was investigated by using malignant melanoma A375 and SKMel5 cell lines harbouring mutation of BRAF V600E, as well as ovarian carcinoma A2780 cell lines that are resistant to doxorubicin and cisplatin.

ERK5 is activated in malignant melanoma cells in response to various growth factors such as EGF (Figure 4.1). Increased phosphorylation of ERK5 is

significant (*p< 0.05) in resistant cells in comparison with sensitive cells (Figure 4.2 and 4.6). As vemurafenib can inhibit BRAF (V600E) →MEK→ERK signalling sufficiently, treatment of A375 or SKMel5 cells with PLX4720 inhibited ERK1/2 activation, while increase ERK5 activation in resistant cells (A375R and SKMel5R). Investigation of interplay between ERK1/2 and MEK5/ERK5 signalling pathway was applied by inhibiting ERK1/2 via trametinib aMEK1/2 inhibitor, in combination with PLX4720. Interestingly, inhibition of ERK1/2 increased the phosphorylation of ERK5 in A375R cells which could consider as alternative pathway that allows cancer growth to continue (Figure 4.5). This finding correlates with recent research which revealed that inhibition of ERK1/2 increased ERK5 activation providing a bypass route to rescue cell proliferation in colorectal cancer (de Jong et al., 2016). In addition, incubating A375 cells with XMD8-92 or BIX02189 in presence of PLX4720 increased ERK1/2 activation (Figure 4.4 and 4.5). Furthermore, dose response curves of BIX02189, XMD8-92, lapatinib and trametinib via cell viability assay shows there is no effect of these inhibitors on A375R cells (Figure 4.8). Taken together, these data could confirm the recent study showing that ERK5 signalling is unlikely to play a role in cell proliferation in tumour cells with *KRAS* or *BRAF* mutations (Lochhead et al., 2016). Moreover, Lochhead and colleagues revealed that cancer cells that overexpress ERK5 are not addicted to MEK5/ERK5 signalling pathway for proliferation and suggests that anti-tumour effects seen with ERK5 pathway targeted therapies are likely through other mechanisms (Lochhead et al., 2016).

The development of tumour resistance to single- targeted treatment, vemurafenib, seems to be unavoidable and it is important to identify an alternative way to overcome this resistance. Different mechanisms are being investigated that may have a role in the resistance to BRAF inhibitors which frequently reactivates the MAPK pathay through MEK (Van Allen et al., 2014). Thus, appropriate drug combination strategies that target oncogenic

pathways along with BRAF are needed. There are preclinical and clinical studies show promising results to treat this problem such as a combination of BRAF inhibitor, dabrafinib and MEK inhibitor, trametinib which was approved by the FDA in patients with BRAF V600E mutated metastatic melanoma in 2014 which prolong progression-free and overall survival compared to dabrafinib alone. Here we investigated the up-regulation of ERK5 in vemurafenib-resistant melanoma cells and using MEK5/ERK5 inhibitors, BIX02189 and XMD8-92 in combination with vemurafenib to overcome resistance. Based on the role of ERK5 as a compensatory pathway that rescue tumour proliferation upon inhibition of ERK1/2 pathway (de Jong et al., 2016), the combination of BRAF inhibitor (PLX4720) with MEK5 inhibitor (BIX02189) or ERK5 inhibitor (XMD8-92) was analysed. Treatment combination of PLX4720 and BIX02189 or XMD8-92 increased the sensitivity of melanoma resistant cells to PLX4720 in both cell lines A375R and SKMel5R and shifting the dose response curve to the lift compared to PLX4720 alone (Figure 4.9). Therefore, inhibition of MEK5/ERK5 signalling in combination with vemurafenib can significantly reverse the resistance and increase the sensitivity to this chemotherapeutic agent in malignant melanoma cells and could provide a potential therapeutic stratgey for vemureafenib resisatance in malignant melanoma. The activation of ERK1/2 induces the activation of negative feedback mechanisms that supresses the upstream kinases of ERK pathway such as RTK and Raf (de Jong et al., 2016). At cellular levels, vemurafenib efficiently inhibits ERK1/2 activation and cell proliferation in most BRAF V600E melanoma cell line (Joseph et al., 2010). ERK5 is activated in response to EGF via a separate RAS-dependent pathway which in trun activates MEK5-ERK5 signalling cascade (Kamakura et al., 1999). Therefore, activation of ERK5 upon inhibition of ERK1/2 pathway could be a consequence of rewiring of signalling pathways downstream of RAS which suggests that ERK5 plays a role as a bypass route downstream of EGFR-RAS and activated upon inhibition of ERK1/2 and continue the nuclear transduction in melanoma

resistant cells (A375R and SKMel5R), suggesting that MEK5/ERK5 signalling pathway could play a pivotal role in vemurafenib resistance and inhibition of this pathway in combination with PLX4720, which targets BRAF V600E mutation and MEK-ERK signalling, could provide a potential therapeutic strategy to overcome drug resistance (Figure 6.1).

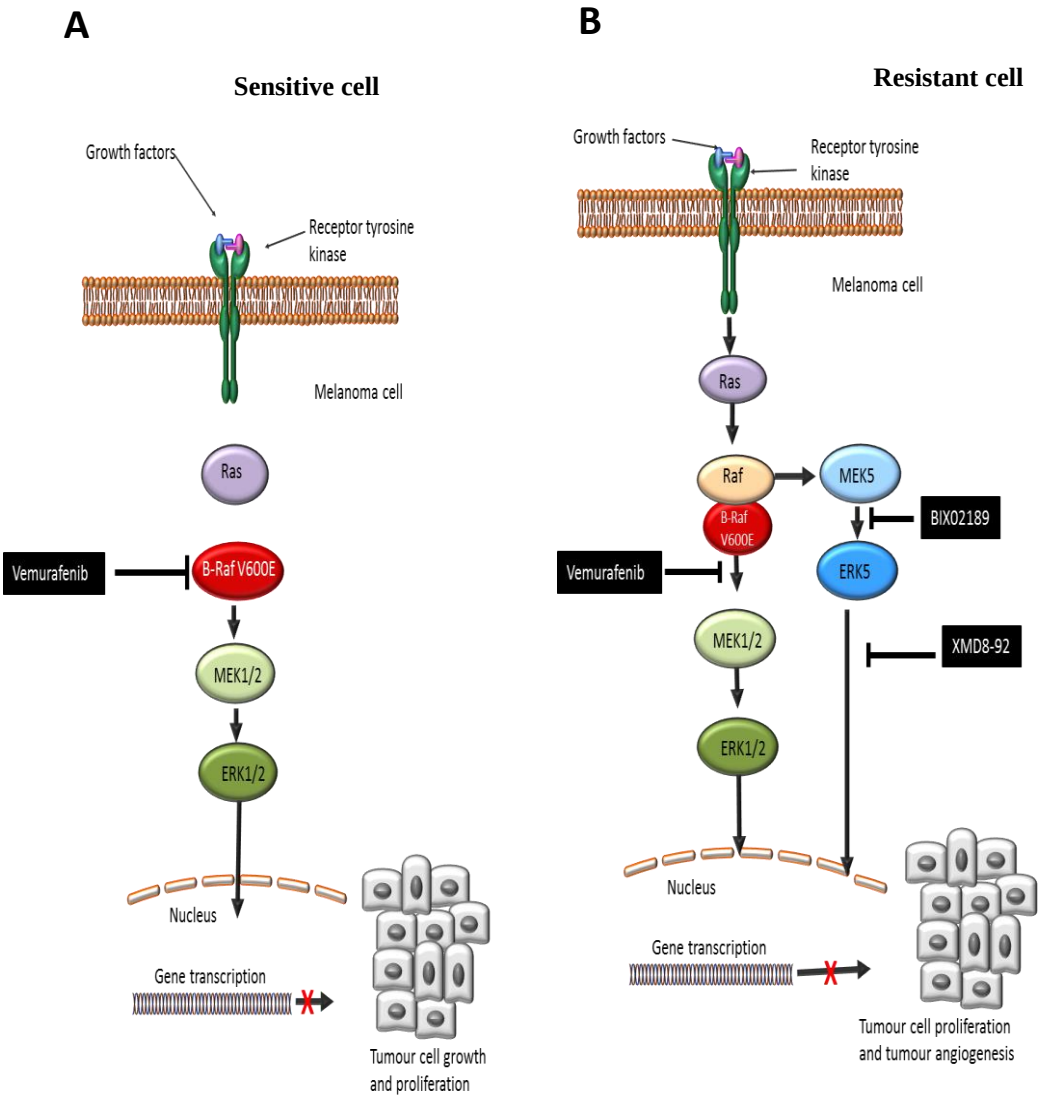


Figure 6.1 The role of MEK5/ERK5 signalling pathway in vemurafenib drug resistance in malignant melanoma cells. (A) In vemurafenib sensitive cells, BRAF V600E mutant melanoma is constitutively active and no need for signalling between RAS and RAF which is disconnected because the constitutive active of BRAF mutant. Inhibition of BRAF V600E mutant by addition of vemurafenib inhibits downstream activation of ERK pathway, and thereby decreasing cell proliferation and inducing apoptosis. (B) In resistant cells, inhibition of MEK1/2-ERK1/2 pathway by vemurafenib leads to lack of negative feedback which in turn activates RTK-RAS-RAF-MEK5-ERK5 pathway which results in continues tumour cell proliferation in malignant melanoma. Treatment combination of vemurafenib with BIX02189 or XMD8-92 can reverse the compensatory upregulation of MEK5/ERK5 pathway and also prevent the angiogenic and lympangiogenic effect of increased VEGF-A and VEGF-C secretion in the drug-resistant melanoma cells.

In ovarian carcinoma cells, the phosphorylation of ERK5 was increased in response to growth factors specially EGF and NRG-1 as well as activation of ERK1/2 increased in response to these growth factors (Figure 5.4 and 5.5a). ERK5 was activated in resistant cell lines A2780-ADR and A2780-CP70 compared to parental cells A2780-WT and also the expression of *MEK5 mRNA* and *erk5 mRNA* in resistant cell lines was higher in comparison with sensitive cell (Figure 5.2 and 5.3). In addition, adding doxorubicin or cisplatin to tumour cells increased activation of ERK1/2 which correlates with studies showning that ERK1/2 is up-regulated in several types of cancer in response to DNA-dmaging chemotherapeutic agents such as doxorubicin and cisplatin (Taherian and Mazoochi, 2012, Tekedereli et al., 2012, Abrams et al., 2010, Tentner et al., 2012). This suggests that chemotherapeutic agents (doxorubicin and cisplatin) activate survival pathways in ovarian cancer cells.

The activation of ERK5 in resistant ovarian cancer cells may be a consequence of activation of another pathway such as ERK1/2 or RTKs such as EGFR. The kinase domain in N-terminal tail of ERK5 shares approximately 50% homolgy with ERK1/2 (Nishimoto and Nishida, 2006). Compared to ERK1/2, ERK5 has a unique C-terminal tail which has two prolin-rich regions, nuclear localisation signal (NLS) domain and a transcriptional activation domain (Nithianandarajah-Jones et al., 2012b). The molecular mechanism regulating transcriptional activation activity of ERK5 C-terminal lies in that activated N-terminal (ERK5N) phosphorylates C-terminal (ERK5C) at multiple autophosphorylation sites on ERK5C and the phosphorylation of C-terminal tail of ERK5 is important for improvement the transactivation activity of AP-1 (Morimoto et al., 2007b). Basid on this, Honda and colleagues investigated the interaction between ERK5 and ERK1/2 and they suggested that ERK1/2 may phosphorylates autophosphorylation of ERK5 on its C-terminal, ultimately they revealed that ERK1/2 phosphorylates ERK5 at Thr_{732} on transcriptional activation domain on C-terminal half of ERK5 (Honda et al., 2015). In doxorubicin resistant

cells A2780-ADR, treating cells with BIX02189 or XMD8-92 in combination with doxorubicin decreased ERK5 activation and increased ERK1/2 phosphorylation. However, adding trametinib with doxorubicin to these cells, completely inhibited ERK1/2 activation and increased the phosphorylation of ERK5 compared to treated cells with doxorubicin alone (Figure 5.5a). This finding is similar to that in malignant melanoma resistant cells which means that ERK5 could potentially play a role in doxorubicin resistant ovarian cancer cells when ERK1/2 was abrogated. Moreover, inhibition of MEK5/ERK5 cascade via its inhibitors (BIX02189 and XMD8-92) or ERK1/2 by trametinib increased the activation of EGFR in A2780-ADR cells compared to treated cells with vehicle control (DMSO). Cell viability assay was applied to assess the role of MEK5, ERK5, EGFR and ERK1/2 inhibitors in A2780-WT and A2780-ADR alone as a single agent or in combined therapy with doxorubicin, there was no change or effect for inhibitors BIX02189, XMD8-92, lapatinib and trametinib alone on cancer cells compared to doxorubicin (Figure 5.8). However, the combination of doxorubicin with BIX02189 or XMD8-92 increased the sensitivity of A2780-ADR to doxorubicin and could potentially reversed the resistance. Thus, ERK5 could play a role in doxorubicin ovarian cancer resistant cells but not cell proliferation which ERK1/2 could play this role in A2780-ADR and increase of ERK5 activation which in turn increase the resistance of A2780 cells to doxorubicin.

The most important and common mechanism of drug resistance is the ability of cancer cells to eject the chemotherapeutic agents from the cells through transport proteins such as MDR-1 and ABCG2. Increased expression of MDR-1 (ABCB1) and ABCG2 is observed in A2780-ADR cells and this is likely responsible for drug resistance (Figure 5.12). Shukla revealed that inhibition of ERK5 decreased ATP-binding cassette (ABC) transporters and increased doxorubicin retention in malignant mesothelioma (Shukla et al., 2013a). In cisplatin resistant cells (A2780-CP70), the combination of

BIX02189 or XMD8-92 did not affect the sensitivity of A2780 cells to cisplatin compared to cisplatin alone (Figure 5.10). This finding suggests that MEK5/ERK5 may not play a central role in cisplatin resistant cells and there is another activated pathway that increases the resistance to cisplatin. Treatment of CP70 cells with cisplatin in combination with trametinib decreased the activation of ERK5 to the same level of the effect of BIX02189 or XMD8-92 (Figure 5.7). Therefore, triple combination BIX02189 or XMD8-92 with trametinib and cisplatin could be a potential target to overcome cisplatin drug resistance in ovarian cancer cells.

Epidermal growth factor receptor-1 (EGFR-1) is expressed in ovarian cancer cells (Niikura et al., 1997a). Stimulation of EGFR-1 on the cell membrane leads to activate two important pathways; MAPK pathway and PI3K/AKT pathway which drive cell proliferation and survival (Grant et al., 2002, Normanno et al., 2006), and owing to this role in tumour (Corkery et al., 2009), lapatinib (EGFR1-2 inhibitor) was approved for treatment of Her2 in breast cancer in combination with chemotherapeutic agents (Mukherjee et al., 2007). The important interaction of lapatinib when combined with anticancer agents appears to be associated with its ability to inhibit ABCG2 and MDR-1 activity (Dai et al., 2008, Chun et al., 2015). Consistent with this studies, our findings indicate that lapatinib in combination with doxorubicin or cisplatin increased the cytotoxic effect of these chemotherapeutic agents in ovarian cancer cells and reversed the resistance of A2780 cells to doxorubicin or cisplatin in comparison to lapatinib alone (Figure 5.9 and 5.10). This finding suggests that combination of lapatinib with doxorubicin or cisplatin may offer more effective target for treatment of drug resistance in cancers. Doxorubicin inhibits tumour growth via intercalation with DNA and resulting to DNA damage and blocking cell replication. However, ovarian cancer cells up-regulate pro-survival signals ERK1/2, ERK5 and EGFR-1 as a consequence of doxorubicin toxicity and this suggests that these signalling pathways could be implicated in doxorubicin resistance in ovarian cancer.

6.1.3 Targeting the MEK5/ERK5 signalling axis results in inhibition of VEGF-mediated angiogenesis

Tumours require oxygen and nutrients to maintain cell growth and survival. In the absence of angiogenesis, tumour will be quiescent and limited at the early satges (Naumov et al., 2006). Tumour angiogenesis is charactarised by an imbalance between pro-angiogenic and anti-angiogenic, extracellular matrix remodeling, endithelial cell migartion, proliferation and differentiation which eventually results in sprouting new blood vessel. Tumour and stromal cells initiate this proccess by suppling an extensive pro-angiogenic growth factors and most notable is vascular endothelial growth factor (VEGF) which interact directly with receptor tyrosin kinase on the endothelial cell surface to promot invasive sprouting into the tmour and forming new blood vessel that proliferates tumour (Kerbel, 2008, Hanahan and Folkman, 1996a). Therefore, inhibition of tumour angiogenesis could be a promising strategy for treating solid tumours (Folkman, 1971). Gene ablation of *erk5* in mice resulted in death at embryonic day E9.5-11.5 as a result of disruption of cardiovascular development and vascular integrity (Hayashi et al., 2004a). ERK5 is required for VEGF-mediated tubular morphogenesis in human microvascular endothelial cells (Roberts et al., 2010c). In this project, the role of ERK5 in tumour angiogenesis was characterised to assess the possibility of targeting MEK5/ERK5 signalling pathway for therapeutic inhibiting of tumour angiogenesis by using in vitro angiogenesis co-culture assay. Judah Folkman was the first to demostrate *in vitro* angiogenesis that capillary endotheial cells were cultured on gelatin and stimulated to form tubule network when added tumour-conditioned media (Folkman and Haudenschild, 1980). Here, different cell types (endothelial, fibroblast and tumour) were successfully incorporated into controlled *in vitro* environment, demostrating reproducible angiogenic sprouting and inhibition in response to anti-angiogenic. Data presented in this study shows that inhibition of MEK5/ERK5 signalling pathway by the pharmacological inhibitors (BIX02189 and XMD8-92)

decreased VEGF-induced neovascularisation in a HMDEC/NHDF/tumour co-culture *in vitro* angiogenesis model.

Inhibition of MEK5/ERK5 cascade via BIX02189 or XMD8-92 treatment in a co-culture assay preferentially inhibited the growth of vessels undergoing angiogenesis compared to established tubes in HDMEC/NHDF/HeLa co-culture (Figure 3.9 and 3.10). This finiding sugests that early blood vessels are more sensitive to inhibition of ERK5 than the established capillary network. Based on the critical role of ERK5 for VEGF-induced endothelial cell survival by mediating activation of AKT (Roberts et al., 2010c), inhibition of MEK5/ERK5 signalling pathway *in vivo* by BIX02189 or XMD8-92 could prevent angiogenesis by preventing endothelial cell survival in response to stimulation with growth factors via inhibiting AKT activation. Furthermore, co-culture of A375 melanoma cancer cells with HDMEC/NHDF induced more tubule formation than HDMEC/NHDF co-culture which demonstrates the ability of tumour cells to secrete more VEGF which is a potent inducer of angiogenesis (Hanahan and Folkman, 1996b) (Figure 4.11). Analysis of the MRNA expression of different VEGF family members revealed that in vemurafenib-resistant melanoma cells increased levels of *VEGF-A mRNA* and *VEGF-C mRNA* were observed (Figure 4.11). This data has been confirmed at the protein level with VEGF family members measured by ELISA (R.Marais, personal communication). This suggests that the drug-resistant cells have potential to regulate angiogenesis via VEGF-A secretion and lymphangiogenesis via VEGF-C secretion. It is interesting to speculate that secretion of these VEGFs may be a potential biomarker of drug resistance in patients receiving vemurafenib and warrants further investigation by analysing patients plasma for VEGFs before and during vemurafenib treatment. The VEGFR-2 data shows that VEGFR-2 is only expressed on HDMEC and therefore strengthens the argument that VEGF-A secreted by drug resistant cells is not acting back on the melanoma cells

but on other cells such as endothelial cells in a paracrine manner (Figure 4.11).

Based on the role of ERK5 in VEGF-mediated tubular morphogenesis in HDMEC (Roberts et al., 2010b) and the role of combined therapy of MEK5/ERK5 inhibitors (BIX02189 or XMD8-92) with PLX4720 in reversing drug resistance in malignant melanoma cells one could speculate that ERK5 inhibitors directly target both the tumour cells and the angiogenic endothelial cells. This consistent with study revealed that combination of ERK5 inhibition with anticancer drug attenuated malignant mesothelioma (MM) tumour and decrease the level of many central cytokines and growth factors such as IL-8 and VEGF (Shukla et al., 2013a). In addition, the same finding was observed in ovarian cancer cells when they co-cultured with HDMEC/NHDF and increased the tube formation compared to HDMEC/NHDF co-culture without tumour cells and the resistant cells were more inducers of tubular network compared to sensitive cells and that could also prove the secretory VEGF from resistant cells to induce angiogenesis (Figure 5.11 and 5.12d). Treatment of HDMEC/NHDF/A2780 co-culture with BIX02189 or XMD8-92 decreased the tube formation compared to basal control (Figure 5.11). Shukla study the effect of ERK5 inhibition in MM tumour growth and found that inhibition of ERK5 significantly decreased tumour and this effect is far better when combined with doxorubicin (Shukla et al., 2013a). It would be interesting to analyse the effect of combiantion therapy of BIX02189 or XMD8-92 with doxorubicin or cisplatin on angiogenesis with ovarian cancer cells.

Overall, this data has suggested that the ERK5 signalling axis is a viable target to restore sensitivity to chemotherapy in drug-resistant cells. This effect was observed in vemurafenib-resistant melanoma cells and doxorubicin-resistant ovarian cancer cells. It will be interesting to see what

other drug-resistant cancers show increased activation of the ERK5 signalling axis as this could offer a potential mechanism to reverse drug resistance in many different cancers.

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Appendices

Appendix I

Primers sequence used in this thesis

Primer name	Forward sequence	Reverse sequence
VEGF-A	CCTTGCTGCTCTACCTCCACC	TCCTCCTTCTGCCATGGG
VEGF-B	GGG CAC ACA CTC CAG GCC ATC	CCT TGA CTG TGG AGC TCA TGG GCC
VEGF-C	TCAGGCAGCGAACAAGACC	TTCCTGAGCCAGGCATCTG
VEGF-D	TCCAGGAACCAGCTCTCTGT	TTTTTGGGGTGCTGGATTAG
VEGFR-2	CAAACGCTGACATGTACGGTCT	CCAACTGCCAATACCAGTGGA
EGFR-1	TATGTTCCCTCCAGGTCAGC	GCACCTGTAAAATGCCCTGT
EGFR-2	CTACGGCAGAGAACCCAGAG	CTTGATGCCAGCAGAAGTCA
EGFR-3	CTTATCCGAGGGCAAATTCA	TTTCCCTTAGTTCCCCATCC
EGFR-4	TGTGTTCCAGTGATGGCTGT	CCATTCTCAAACTCCCGAAA
MEK5	ACGTGAAGCCCTCCAATATG	GGCGCCATATAAGCATTTGT
ERK5	TGTTCTCAGGCACACCAAAG	GAGGCTGAGAGAGAGGCTGA
MDR-1	GTGGGGCAAGTCAGTTCATT	TTCCAATGTGTTCGGCATTA
ABCG2	CCTGAGATCCTGAGCCTTTG	AAGCCATTGGTGTTTCCTTG
GST-PI	ACCTCCGCTGCAAATACATC	GACAGCAGGGTCTCAAAAGG