Analysis of the ERK5 MAPK Signalling Pathway in Endothelial Cells

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Thesis Summary

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Angiogenesis is the development of new capillary vessels from pre-existing ones. It involves the proliferation, migration and differentiation of endothelial cells. It is activated by different stimuli and requires the coordination of several signalling pathways. The most important growth factor for angiogenesis is VEGF. However, other factors also play an important role. For example, H_2S is a gaseous molecule that exerts its pro-angiogenic effect through activation of the pro-survival Akt signalling pathway. In vitro studies have further shown the ability of H_2S to enhance endothelial cell proliferation, adhesion, migration, and tube formation.

The ERK5 signalling cascade is the most recently discovered mammalian MAPK (mitogen-activated protein kinase) pathway. ERK5 knockout mice die due to impaired heart development and endothelial cell dysfunction. Furthermore, VEGF promotes ERK5 activation in primary endothelial cells and ERK5 is required for VEGF-induced tubular morphogenesis. Importantly, VEGF activates ERK5 in endothelial cells, which in turn activates the AKT pro-survival pathway to suppress apoptosis of endothelial cells. As H_2S also mediates its vasoprotective effect through AKT activation, the hypothesis of this thesis was that H_2S protects endothelial cells by activating the ERK5 pathway. The aim of this thesis was therefore to investigate whether H_2S activates ERK5 and to develop the necessary tools to further investigate the mechanisms involved.

In this thesis, an assay detecting and quantifying an electrophoretic mobility shift by western blotting has been developed to measure ERK5 activation in mammalian cells. Furthermore, ERK5 could be shown to be activated by H_2S in HeLa cells. Importantly, H_2S also activated ERK5 in endothelial cells, demonstrating that ERK5 signalling mediates the pro-survival response of H_2S in endothelial cells. To further analyse ERK5 activation in response to various stimuli, including H_2S, additional tools to determine ERK5 phosphorylation were evaluated, including mass spectrometric determination of phosphorylation sites and the characterisation of a novel phosphor-specific antibody.

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who has directly or even indirectly, contributed to my success, and I incidentally forgot their names.

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List of Abbreviations

a.a.	*Amino acid*
ACE	*Angiotensin-converting enzyme*
Ad-virus	*Adenovirus*
AKT	*Serine/Threonine kinase*
Ang	*Angiopoietins*
ANOVA	*Analysis of variance*
anti-PTM	*anti-immunogen-post-translational modification*
APS	*Ammonium persulfate*
AP-1	*Activator protein-1*
Asp	*Aspartic acid*
AspN	*Aspartic acid N*
ATP	*Adenosine triphosphate*
BCA assay	*Bicinchoninic acid-assay*
BDNF	*Brain-derived neurotrophic factor*
BK_{Ca}	Ca^{2+}*-activated* K^{+} *channel*
BLMECs	*Bovine lung microvascular endothelial cells*
BMK1	*Big mitogen kinase 1*
BSA	*Bovine serum albumin*
°C	*Celsius*
Ca^{2+}	*Calcium*
CAD	*Coronary artery disease*
cAMP	*Cyclic adenosine monophosphate*
CAT	*Cysteine aminotransferase*
Cat.	*Catalogue*
CBS	*Cystathionine β-synthase*
CD	*Common docking*
cdc 37	*Cell division cycle 37*
Cdc42	*Cell division control protein 42 homolog*
CDK1	*Cyclin-dependent kinase 1*
cDNA	*Complementary deoxyribonucleic acid*
CDO	*Cysteine dioxygenase*
CERII	*Cytoplasmic extraction reagent*
cGMP	*cyclic guanosine-3`,5`-monophosphate*
CHD	*Coronary heart disease*

CL	*Cysteine lyase*
CO	*Carbon monoxide*
CO_2	*Carbon dioxide*
CREB	*cAMP response element-binding protein*
CSE	*Cystathionine γ-lyase*
CSE-KO	*Cystathionine γ-lyase knocked out mice*
CVDs	*Cardiovascular Diseases*
CVD	*Cerebrovascular disease*
CVS	*Cardiovascular diseases*
Da	*Dalton*
DAO	*D-amino acid oxidase*
DAPI	*4`,6-Diamidine-2`-phenylindole dihydrochloride*
DMEM	*Dulbecco's Modified Eagle's Medium*
DMSO	*Dimethyl sulfoxide*
DTT	*DL-Dithiothreitol*
DUSP	*Dual specificity phosphatase*
ECL	*Enhanced chemiluminescent substrate*
ECs	*Endothelial cells*
EDTA	*Ethylenediaminetetraacetic acid*
EGF	*Epidermal growth factor*
ELISA	*Enzyme linked immune-sorbent assay*
eNOS	*endothelial nitric oxide synthase*
ERK	*Extracellular-signal regulated kinase*
ET	*Endothelin*
ETB	*Endothelin B receptors*
ET-1	*Endothelin 1*
E9.5	*Embryonic day 9*
FBS	*Fetal bovine serum*
FCS	*Fetal bovine serum*
FGF	*Fibroblast growth factor*
Fe	*Iron*
G-Gly	*Glycine*
GluV8	*Glutamyl endopeptidase*
GSSG	*Oxidized glutathione*
GSH	*Reduced glutathione*
GYY4137	*Dichloromethane complex*
HA-MEK5	*Hemagglutinin-Dual specificity mitogen-activated protein kinase Kinase5*

HASMCs	*Human aortic smooth muscle cells*
hb FGF5	*Basic fibroblast growth factor, human*
HCl	*Hydrochloric acid*
HCT-116	*Colorectal carcinoma cells*
HC-100	*Hydrocortisone 100*
HDMEC	*Human dermal microvascular endothelial cell*
hEGF	*human-Epidermal growth factor*
HEK 293	*Human embryonic kidney 293*
HeLa	*Henrietta Lacks*
Hepes BSS	*Hepes buffered saline solution*
Hep G2	*Hepatocellular carcinoma cells*
hERK5	*Human extracellular-regulated kinase 5*
His	*Histidine*
HL60	*Caucasian promyelocytic leukemia cells*
HO1	*Haeme oxygenase 1*
HPEC	*Human placental endothelial cells*
HS^-	*Sulfanide*
Hsp 90	*Heat shock-protein 90*
H_2S	*Hydrogen sulphide*
HUVECs	*Human umbilical vein endothelial cells*
IL	*Inter-leukine*
IMR-90	*Human foetal lung cells*
K^+	*Potassium*
K_{ATP}	*ATP-sensitive potassium channel*
kb	*Kilo-base*
kDa	*kilodalton*
KLF	*Kruppel-like transcription factor*
LDS	*Lithium dodecyl sulphate*
Leu	*Leucine*
M	*Mole*
mA	*milliampere*
MAPK	*Mitogen-activated protein kinase*
MAPKK	*Mitogen-activated protein kinase kinase*
MAPKKK	*Mitogen-activated protein kinase kinase kinase*
MCF-7	*Human breast (adenocarcinoma) cells*
MEF	*Myocyte enhancer factor*
MEKK3	*Mitogen-activated protein kinase kinase kinase 3*

$MEK5^{DD}$	*Constitutively active human MEK5*
MEK1/2	*Dual specificity mitogen-activated protein kinase kinase 1/2*
MEK5	*Dual specificity mitogen-activated protein kinase kinase 5*
Met	*Methionine*
MI	*Myocardial Infarction*
MKPs	*Mitogen kinase phosphatases*
mm	*Millimetre*
mmol	*millimole*
MS	*Mass-spectrometry*
MV4-11	*Thymus/lymph node cells*
MW	*Molecular weight*
Na_2S	*Sodium sulphide*
NaHS	*Sodium hydrosulphide*
NaCl	*Sodium chloride*
NER	*Nuclear extraction reagent*
NES	*Nuclear export signal*
NFκB	*Nuclear factor kappa-light-chain-enhancer of activated B cells*
NGF	*Nerve growth factor*
NLS	*Nuclear localisation signal*
NO	*Nitric oxide*
No.	*Number*
NOS	*Nitric oxide synthase enzyme*
PAEs	*Porcine aortic endothelial cells*
PAF	*Platelet-activating factor*
PAGE	*Polyacrylamide gel electrophoresis*
PAK	*p-21 activated kinase*
PBS	*Phosphate-buffered saline*
PDGF	*Platelet-derived growth factor*
pERK	*Phospho extracellular-signal regulated kinase*
PGI_2	*Prostacyclin*
Phe	*Phenyl alanine*
PI3-kinase	*Phosphoinositide 3-kinase*
PKA	*Protein kinase A*
PKB	*Protein kinase B*
PKC	*Protein kinase C*
P-Pro	*Proline*
PR	*Proline region*

PR1	*Proline-rich regions*
pS496	*phospho-serine 496*
pS731	*phospho-serine 731*
PTP-SL	*Protein tyrosine phosphatase STEP-like*
pT202	*phospho-threonine 202*
pT218	*phospho-threonine 218*
pT733	*phospho-threonine 733*
pY204	*phospho-tyrosine 204*
pY220	*phospho-tyrosine 220*
RF/6A	*Chorioretinal endothelial cells*
RIPA	*Radioimmunoprecipitation buffer*
r.p.m.	*Revolutions per minute*
R-SH	*Thiol-bearing intermediate*
R3 IGF-1	*Long-arginine 3, insulin-like growth factor*
SAP1a	*a nuclear protein stimulating transcription*
SDS	*Sodium dodecyl sulphate*
sGC	*Soluble guanylyl cyclase enzyme*
SH3	*Src-homology 3*
siRNA	*Small interfering ribonucleic acid*
SO	*Sulfide oxidase*
Src	*SH2*
S-Ser	*Serine*
STAT3	*Signal transducers and activator of transcription 3*
S770	*Serine 770*
TBS	*Tris buffered saline*
TBST	*tris-buffered saline and Tween 20*
TEMED	*Tetramethylethylenediamine*
tERK	*Total extracellular-signal regulated kinase*
TEY	*Threonine tyrosine motif*
Thr	*Threonine*
TIA	*Transient ischemic attack*
TNF-α	*Tumor necrosis factor*
Trp	*Tryptophan*
TSMT	*Thiol-S-methyltransferase*
T-Thr	*Threonine*
TXA2	*Thromboxane*
TXY	*Threonine tyrosine motif*

Tyr	*Tyrosine*
T-75	*Culture flask*
U2OS	*Osteosarcoma cells*
U20S	*Human bone osteosarcoma epithelial cells*
VEGF	*Vascular-epidermal growth factor*
VSMC	*Vascular smooth muscle cell*
(v/v)	*Volume/volume*
vWF	*von Willebrand factor*
WB	*Western blot*
WHO	*World health organisation*
WI 38 cell	*Caucasian fibroblast-like fetal lung cells*
WP-bodies	*Weibel-Palade bodies*
WT	*Wild type*
Y-Tyr	*Tyrosine*
Zn	*Zinc*
µM	*Micromolar*
3-MST	*3-mercaptosulfurtransferase*
3-MP	*3-mercaptopyruvate*

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Chapter 1

Introduction

1. Introduction

1.1. General overview of the cardiovascular system

It is well-known that there is a global epidemic of cardiovascular diseases (CVDs), which have serious implications in public health and comprise an extraordinary amount of healthcare expense. In association to high morbidity and mortality rates, the CVD may represent a significant role in increasing the problems on the global medical and economic levels (Deveza et al. 2012). CVD has been listed as the number one cause of death worldwide, according to the World Health Organization (WHO). It has been documented that, and in the United States of America alone, 32.8% of the approximately 2.5 million deaths are accounted for CVD in 2008 (Roger et al. 2012). In 2016, CVD represented the leading cause of death in the United States, responsible for 840,768 deaths (635,620 cardiac) (Benjamin et al. 2018). CVD has been reported to be one of the 15 leading conditions that induce functional disability for those that have experienced one CVD event, thereby it can be said that the quality of life and the ability to work are both affected (Deveza et al. 2012). Narrowing or even occlusion of blood supply of the vascular beds is a general characteristic of CVD, where it is caused by atherosclerosis (Libby and Theroux 2005, Jawad and Arora 2008). Even though there are a number of modified (controllable) risk factors, such as hypertension, smoking, obesity, lack of physical activity and diabetes, other unmodified risk factors may arise such as age, gender, and family history (Goff et al. 2014).

1.1.1. The cardiovascular system

Three main components can classify the cardiovascular system: heart, blood vessels and blood. Further, three types of distinct blood vessels can be categorized in term of anatomical and functional properties: arteries, veins and capillaries. In order to deliver the oxygenated blood and nutrients from the heart to target organs and tissues, the arteries seem to play a critical role in fulfilling this function. Thicker and more elastic vessel walls are distinctive features of the arteries, where the high blood pressure for blood delivery from the heart

would be complemented. Carrying deoxygenated blood to the heart would be accomplished by the veins, together with waste products and other factors excreted by the tissues. In comparison to the arteries, veins can be distinguished to have larger luminal areas, thinner vessel wall and valves to accommodate the pressure changes. Capillaries play a key role in connecting together the arteries and veins, and allow the direct exchange between the target tissues and the blood of the oxygen and nutrients with carbon dioxide and waste products. In general, three layers are major components of the vessel walls: the tunica intima, tunica media and tunica adventitia (Figure 1.1). The underlying layer is composed of the tunica intima, that contains a single layer of endothelial cells (ECs) and connective tissues, both of which superimpose the internal elastic lamina. It has been found that, based

Figure 1.1: Blood vessels structure.
The figure demonstrates the major anatomical parts of the blood vessels. In general, the blood vessel is composed of three predominant layers: tunica intima, tunica media, and tunica externa. As it appears from the figure that tunica intima is the innermost layer of the blood vessel, which contains endothelium, subendothelial layer, and internal elastic membrane. On the other hand, the outer layer (tunica externa), forms of external elastic membrane. The capillary is only composed of basement membrane and endothelial cells. It can be inferred from the figure that the lumen of artery is narrow and more elastic due to the presence of the internal elastic membrane to meet the pressure changes in delivering the blood from heart to the tissues. The lumen of vein is wider and has valves to deliver the blood back to the heart from the tissues. Image taken from (Kizirian 2020).

on its structure, the tunica intima can perform a distinctive role as a selective permeable barrier between the extravascular space, the vascular wall and the blood. Muscular elements of blood vessels can reside in the tunica media, between the tunica intima and the tunica adventitia, and comprises peripherally organized smooth muscle cells surrounded by a layer of external elastic lamina. Finally, the exterior layer, the tunica adventitia, contains connective tissues and matrix-secreting fibroblasts. It has been found that the tunica adventitia plays a critical role in the protection of vascular structure and anchoring the vessels in the right place in accordance to the surrounding tissues. Capillaries on the other hand compile non-muscular vessels and only consist of an internal elastic lamina preserved by a monolayer of ECs, which provides exchangeable surface area allowing transport of molecules between vessels and tissues.

1.1.2. Endothelial cells and their impairments in CVDs

Vascular endothelial cells (ECs) play an essential role in the integrity of the entire circulatory system as they overlay the innermost layer of the vascular system. Acting as a semi-selective and non-adherent barrier between the lumen of vessels and the underlying tissues is a distinctive property of the vascular endothelial cells, where regulation of tissue perfusion and movement of inflammatory cells can be attained between them (Aird 2007). The vascular endothelial cells (vECs) have been reported to be a major regulator for the vascular permeability, blood flow, vascular tone and blood coagulation as well as their essential role involving vascular remodelling in response to diverse physiological and pathological stimuli (Chiu and Chien 2011). Upon secretion of anti-coagulant factors for instance prostacyclin, nitric oxide (NO) and prostaglandin-E_2, vECs can induce a preliminary role at the physiological level as anti-coagulant and anti-thrombotic. It has been found also that vECs can maintain vascular homeostasis by reducing inflammatory cell adhesion (van Hinsbergh 2012). In order to create vascular remodelling, numerous pathological events have been found to be caused by increasing the vascular permeability, stimulating extravasation of

immune cells, which are activated by vascular injury or pro-inflammatory cytokines. Endothelial nitric oxide (NO) synthase (eNOS) is an enzyme which plays a key role in synthesis and formation of the vasodilator NO (Forstermann and Sessa 2012). Therefore, repressing activity of eNOS results in alleviation of vEC secretion of vasodilator NO, which it has been found to be a major contributor to aggravation of vascular pathogenesis (Alderton et al. 2001). The pathophysiology of many CVDs, for example hypertension and atherosclerosis, is strictly bound to occurrence of endothelial dysfunction, which in turn being provoked by different factors such as environmental and genetic (Favero et al. 2014). Eventually, the vECs dysfunctions display a potential target for drug development in terms of therapeutic approaches (Versari et al. 2009).

1.1.3. Cardiovascular disease

A wide range of problems would arise within the cardiovascular system including endocarditis, rheumatic heart disease, abnormality in the conduction system (Olvera Lopez et al. 2020). CVD or heart disease can be classified into 4 predominant entities. First, coronary artery disease (CAD) which may also be referred to as Coronary Heart Disease (CHD), results from decreased myocardial perfusion that induce angina, myocardial infarction (MI) and/ or heart failure. In general, the previous examples of the CAD may account for the majority of the cases of CVD (one third to one half). Secondly, cerebrovascular disease, that includes stroke and transient ischemic attack (TIA). Third, peripheral artery disease (PAD), which involves particularly arterial disease in the limbs resulting in claudication. Fourth and finally, aortic atherosclerosis that includes thoracic and abdominal aneurysms.

Infectious myocarditis is another problem that may occur within the cardiovascular system. In general, infection of the heart by some microbes, such as cardiotropic viruses and T. cruzi, may procure the manifestation of myocarditis. However, it remains obscure which cell types are particularly infected in many myocarditis-associated viruses. Furthermore, cardiac vasculature represents another target for infection in the cardiovascular system. To be

specific, in order to infect the heart, the endothelium of the cardiac microvasculature would play a key role as a first barrier to encounter infectious agents. Infection of human and / or animal endothelial cells can be acquired by T. cruzi and more than 20 viruses associated with myocarditis. One outstanding observation demonstrates among others that endothelial activation, damage, and permeability can be induced by infection of the cardiac endothelium. For example, it has been shown that in patients with viral myocarditis, induction of endothelial microparticles reflecting endothelial damage would be caused upon the role of cardiac endothelial cells infection. However, it remains unknown how non-viral mechanisms would cause the changes to cardiac (micro) vasculature seen in infectious myocarditis, when encountered in vivo (Woudstra et al. 2018).

1.1.3.1. Aetiology

The aetiology of CVD may directly appear from different causes such as rheumatic fever resulting in valvular heart disease, blood clot in patient with arterial fibrillation causing ischemic stroke (Olvera Lopez et al. 2020). Development of atherosclerosis and other associated factors can be addressed with high importance because it plays an essential role in the pathophysiology of CVD. During the last few decades, a resultant shift to sedentary life style from physically demanding in the industrialized economy, along with consumption demanding and technology-driven culture which require long working hours and less recreational time, may justify the increase in the incidence of CVD. In particular, development of atherosclerosis and other metabolic defects such as metabolic syndrome, diabetes mellitus, and hypertension can arise associating to lack of physical activity, consuming a high calorie-diet, fats, and sugars (Fox et al. 2004, Vasan et al. 2005, Benjamin et al. 2018, Force et al. 2018). Smoking, dyslipidaemia, hypertension, diabetes, abdominal obesity, psychosocial factors, low consumption of fruits and vegetables, regular alcohol consumption, and limited physical activity are all 9 modifiable risks factors concluded from INTERHEART study which recruited subjects from 52 different countries, including high, middle, and low-income countries. The main goals of establishing this study were to detect

the strength of correlation between different risk factors and acute myocardial infarction and to emphasize if there is a significant variations between geographic regions, ethnic origin, sex or age. Another significant objective was to determine the PAR (Population Attributable Risk) for risk factors and their combinations at overall population and in different subgroups (Yusuf et al. 2004). Thus, 90% of the risk of having a first myocardial infarction (MI) is accounted for by those 9 modified risks factors (Yusuf et al. 2004). Affirmative association and predictive value of dyslipidaemia, high blood pressure, smoking, and glucose intolerance have been concluded from other robust studies such as the Farmingham Heart Study and the Third National Health and Nutrition Examination Survey (NHANES III). Therefore, 60% to 90% of CHD occurrence would be attributed in individuals with at least one risk factor.

Family history, age, and gender are non-modified factors that have been addressed in different implications. Suggestive evidence was revealed stating that the presence of CVD risk factors would variably affect gender (Fox et al. 2004, Fox et al. 2008). For example, in women compared to men, increasing CVD risk is typically associated with diabetes and smoking more than 20 cigarettes per day (Njolstad et al. 1996). Significantly, within each decades of life, the risk and prevalence of CVD is increased (Savji et al. 2013). It remains controversial that consumption of specific diet factors like meat, limited fibre, and coffee are associated with CVD, and that is because of the alignment in significance seen in epidemiological studies (Ioannidis 2013, Trepanowski and Ioannidis 2018).

1.1.3.2. Epidemiology

Since 1975, CVD is the second-leading cause of death in the United States with 1 in every 4 deaths. In 2015, heart disease has been categorized as the leading cause of death followed by 595,930 deaths from cancer (Benjamin et al. 2018). According to the WHO, the number 1 cause of death globally is CVD with an estimated 17.7 million deaths in 2015. By

the year 2030, more than 23,3 million people have been projected to die from CVD globally (WHO 2020).

In spite of the incremental declining over time in the age-adjusted rate and acute mortality from MI due to the progress in diagnosis and treatment over the last couple of decades, the calculated percentage of heart disease risk in the general population remains high with 50% risk from the age of 45 (Lloyd-Jones et al. 1999, Fox et al. 2008). The significant increase in incidence is associated with age, with taking into consideration that some variations between genders are likely to occur.

1.1.3.3. Pathophysiology

In term of pathophysiology, atherosclerosis is a prominent example for the CVD. It can be defined as a pathogenic process resulting from decreased or absent blood flow from the blood vessels stenosis in the arteries and the aorta that cause a disease (Libby et al. 2011). Dyslipidaemia, immunological reactions, inflammation, and endothelial dysfunctions are multiple involving factors which are believed to enhance fatty streak formation, leading to development of the atherosclerotic plaque (Figure 1.2) (Davies et al. 1988). This process is complex including thickening of the intima followed by accumulation of lipid-laden macrophage (foam cells) and extracellular matrix, with subsequent aggregation and proliferation of smooth muscle cells comprising the atheroma plaque formation (Sata et al. 2002). It is widely accepted that macrophages play a key role in progression of atherosclerotic lesion. To be specific, the recruitment of monocyte-derived cells into the sub-endothelial space would mediate an immune response, where they eventually differentiate into macrophage that engulf the normal and modified lipoproteins, transforming them into the cholesterol-laden "foam cells". At this stage, macrophages play a decisive role by secreting pro-inflammatory cytokines and chemokines, maintaining the local inflammatory response (Moore et al. 2013). Within the expansion of lesions, occurrence of apoptosis in the deep layers would be promoted, depositing more macrophage that within

time could form atherosclerotic plaque upon their calcification and transition. Intra-plaque haemorrhage and arterial remodelling are other mechanisms that play a decisive role in the acceleration of the progression of atherosclerotic CVD by delay and acceleration.

Figure 1.2: The steps of evolving a fatty streak in atherosclerosis.

Monocytes adhesion to the endothelium may develop upon occurrence of injury, which on turn loses the endothelial cell junctions, and migrating monocytes under the endothelium, to be differentiated into macrophages. Upon permeability of the endothelium, more LDL would be allowed to get in the intima of the artery, thereafter, the LDL would be engulfed by macrophages by phagocytosis. Foam cells are a terminology referred to transformation of macrophage to lipid laden from ingested LDL, where their appearance may create fatty streaks. Also, the figure demonstrates the role of T-lymphocytes in the intima. T-lymphocytes have the ability to secret some cytokines, which eventually may promote migration of smooth muscle cells from the media to the intima. Thereafter, these smooth muscle cells, and under the effect of growth factors, may start to proliferate. In time, there would be a progressive aggregation of lipid and smooth muscle cells in the intima, which eventually may lead to growing of the endothelium, and surpass the lumen of artery. Image from (LaMorte 2016, Kizirian 2020).

1.2. Angiogenesis

The development of functional vessels is complex (Udan et al. 2013). It is important to differentiate between vasculogenesis and angiogenesis. In the early embryo, a new synthesis (de novo) and assembly of endothelial cells into vessels in the absence of a pre-existing vascular system is referred to as vasculogenesis. On the other hand, angiogenesis is defined as the development of capillary vessels from pre-existing ones (Betz et al. 2016). Angiogenesis literally means creation of new blood vessels. The word "angio" means blood vessels while "genesis" means creation. Based on the nature of the vessel, angiogenesis would be defined as either arteriogenesis or lymphangiogenesis. In both cases,

Figure 1.3: The angiogenic process.
Four major steps can illustrate and summarize the angiogenic process. (A) It is important for the endothelial cells localised in the outer surface of a blood vessel to receive initially pro-angiogenic signals from Angiogenin (ANG), Vascular Endothelial Growth Factors (VEGF), Platelet Derived Growth Factor (PDGF), Placental Growth Factor (PGF), Epidermal Growth Factor, Growth Factor Form Fibroblast (FGF), Transforming Growth Factor Beta 1(TGF B), and Tumor Necrosis Factor Alpha (TNF-α). Several receptors have their corresponding ligands (angiogenic growth factors) on the surface of endothelium, for example VEGFR1/2, TGFBR1/2, TNFRS. (B) In order to conduct the degradation of the basement membrane, the MMPs should firstly escape the blood vessels at the same time as increasing the blood vessel pores to form fenestrations. (C) Then the migration and proliferation of endothelial cells would be evolved, through a process named partial endothelial to mesenchymal transition at the place of fenestration, resulting in the budding and formation of a new blood vessel. (D) Once the new tube is obtained, a variety of signals, such as RASIP1 and ARHGAP29, would be received from the environment, which in turn gives the newly formed network, a well-organized and 3-D structure. At the end of this stage, it has been found that the pericytes inhabiting the exterior of the blood vessels, are responsible for contraction of the blood vessel and beginning to establish the newly formed network. Image taken from (Zimta et al. 2019).

angiogenesis is mediated via two distinguished pathways: splitting and sprouting. Increased in micro-vascular shear stress leads to the intraluminal splitting of the micro-vessel linear into two vessel, on the other hand, tissue hypoxia stimulates sprouting angiogenesis and budding of a new capillary sprout laterally from a pre-existing vessel. This process requires a high level of coordination by a series of angiogenic factors and inhibitors (Figure 1.3) (Folkman 2007). Some of the normal physiological situation where angiogenesis is required include those of wound healing and formation of the placenta during pregnancy. (Michiels 2003)

1.2.1. Biological and molecular role of vascular endothelial cells

Blood vessel walls form a unique and selective barrier, while permitting the transport of molecules between the blood and tissues (Rajendran et al. 2013). They form a complex interface structure, with a total area measuring around 350 m^2 in the human body (Augustin et al. 1994). Endothelial cells make up the tunica intima, the interior lining of the vasculature (Mazurek et al. 2017). They exert their function by responding to various neurotransmitters, hormones and vasoactive agents that induce vasomotion, thrombosis, and inflammation. The endothelium is composed of a continuous monolayer structure, with the individual cells linked to each other by various types of cell-to-cell junctions (adhesiveness) (Dorland and Huveneers 2017). Three types of junctions have been identified in endothelial cells: gap junctions, adherens junctions, and tight junctions (Bazzoni and Dejana 2004).

It has long been believed that the endothelium is just a "cellophane wrapper", with no other functions than selective barriers for passaging water and electrolytes between the blood and tissues (Wilson et al. 2001, Rajendran et al. 2013, Kruger-Genge et al. 2019). However, more recent research has allowed a better understanding of endothelial cells, discovering that they play critical roles in controlling many vascular functions. For example, they contribute to the secretion of several mediators that enhance and maintain vascular hemodynamics in the normal physiological state. Endothelial cells also perform a unique

function in fluid filtration processes (e.g., in the glomeruli of the kidneys) (Durand and Gutterman 2013). In spite of their distinctive roles in participating in all aspects of vascular homeostasis, endothelial cells are also a potent contributor to pathological conditions such as thrombosis, inflammation, and vascular wall remodelling (Michiels 2003, Gimbrone and Garcia-Cardena 2016). Furthermore, it has been shown that vECs regulate blood pressure and blood flow upon secreting vasodilator (e.g., nitric oxide and prostacyclin PGI_2) as well as vasoconstrictor molecules (e.g., endothelin and platelet-activating factor) (Sandoo et al. 2010). Endothelial cells have also been shown to regulate the trafficking of immune cells (Barton et al. 2012) as well as contribute to coagulation processes initiated through turbulent blood flow, which is pivotal for the onset of blood vessel repair (Cines et al. 1998, Kruger-Genge et al. 2019). Other areas where endothelial cells play a major role are in inflammatory reactions and in the neoangiogenesis of tumours (Michiels 2003). Overall, a complex interaction between vasoregulatory pathways is required for the vascular response to carry out particular regulatory function, including under pathophysiological circumstances (Rajendran et al. 2013).

1.2.2. Endothelial dysfunction

Endothelial dysfunction is typically associated with inflammatory conditions and reactions (Stenvinkel 2001, Steyers and Miller 2014). Inflammation in general can be defined as an immunological response to injury or destruction at a localized site of body tissues. Inflammation provides the necessary protection by diluting and destroying the injured tissue and the causative agent at the location. The acute form of inflammation can be distinguished from the chronic one by appearance of such common signs as pain (dolor), heat (color), redness (rubor), and swelling (tumor, edema). In the endothelium, inflammatory reactions are mainly initiated upon the migration of leukocytes to the inflammatory location. If the inflammation has not been resolved after some time, it can lead to excessive (chronic) inflammation as a pathological consequence. Interestingly, migration of leukocytes to the site of injury (inflammation) involves a complex series of events and mediators, including

dilation of vessels (venules, arterioles, and capillaries), increased blood flow and permeability, and perfusion of fluids (Nathan 2002, Sumagin and Sarelius 2013). On the other hand, numerous inflammatory mediators such as NFκB, IL-1, and TNF-α play an indispensable role in regulating each individual step of the inflammatory reaction (Lawrence 2009). Atherosclerosis is an example of these inflammatory reactions which leads to the dysfunction of the endothelium (Rajendran et al. 2013, Kany et al. 2019). Vulnerability to atherosclerosis may vary based on several factors, including hypertension, diabetes, and hypercholesterolemia (von der Thusen et al. 2003). Changes in the endothelial cells initiate the proinflammatory and prothrombotic phase of atherosclerosis (Landmesser et al. 2004, Gimbrone and Garcia-Cardena 2016). A reduction of endothelium-derived NO production is noticed in lesions of the aorta and coronary arteries. Increased levels of c-reactive protein promote atherosclerosis by reducing the level of eNOS-mediated NO in the plasma (Rajendran et al. 2013). A number of endothelial mechanisms enhance the atherogenesis process. For example, the inflamed endothelium releases chemoattractant substances that recruit circulating monocytes and activate their endothelin B (ETB) receptors. Upon the activation of macrophages by endothelin 1 (ET-1), inflammatory mediators such as IL-6, IL-8, TNFα and superoxide anion may be secreted (Rajendran et al. 2013). Alongside smooth muscle cell migration effect, ET-1 promotes hypertrophy and the secretion of fibroblast growth factor 2, which in turns provides responsiveness to angiotensin 2. After the stimulation of fibroblast proliferation, chemotaxis, and matrix synthesis by ET-1, P-selectin expression and PKC (protein kinase C) activation would be augmented upon the role of ET-1, as well (Best and Lerman 2000). However, impaired endothelial cells are able to secrete plasminogen activator inhibitor-1, which is highly efficient in suppressing fibrinolysis levels (Behrendt and Ganz 2002).

1.2.3. Signal transduction pathways in angiogenesis

Analyzing the required molecular events that initiate and preserve vascular development, has been investigated in a detailed study of rat embryos in which the genes for polypeptide

growth factors or their trans-membrane receptors (receptor tyrosine kinases), have been switched off. In this study, vascular endothelial growth factor (VEGF) was identified as the most potent and critical factor of vascular development (Ho and Fong 2015). Vascular endothelial growth factor (VEGF), also known as VEGF-A, is a member of the VEGF platelet-derived growth factor (PDGF) family of structurally related mitogens. Other family members including placental growth factor (PIGF), VEGF-B, VEGF-C and VEGF-D, demonstrate some degree of variation of homology with VEGF (Ferrara and Davis-Smyth 1997). VEGF is a homodimeric glycoprotein with a molecular weight of approximately 45 kDa (Carmeliet 2005). Furthermore, it is widely known as a mitogen for vascular endothelial cells derived from arteries, veins and lymphatics, but is deprived of consistent mitogenic activity for other cell types (Ferrara 2000). There are at least four main isoforms of VEGF existing as a result of alternative splicing. These isoforms are 121,165, 189 and 206 amino acids long. It is important to mention that 165-amino acid form exemplifying the major and dominant species of VEGF. Recently, it has been recognized that another family of growth factors known as angiopoietins (Ang) and some members of the ephrin family (e.g., ephrinB2) would also have unique roles in the endothelium by conducting subsequent actions on further remodelling and maturation. Therefore, an incorporated model of known vascular-specific growth factors would include vascular formation and expansion. In order to maintain the quiescence and integrity of the mature vasculature, Ang1 appears additionally to play a continuing role in fulfilling this function (Gale and Yancopoulos 1999, Michiels 2003, Rajendran et al. 2013).

*A number of cellular signalling pathways have been shown to regulate angiogenesis downstream of the VEGF receptor by modulating the proliferation, migration and tube formation of endothelial cells (Patel-Hett and D'Amore 2011) (*Figure 1.4*). For example, VEGF-induced ERK1/2 MAPK activation is essential for endothelial cell proliferation (Takahashi et al. 2001, Kiec-Wilk et al. 2010). Cell proliferation and migration is further*

induced by VEGF-mediated PLC-γ/PKC activation (Wang et al. 2008). VEGF activation also leads to the activation of p38 MAPK, which stimulates endothelial cell migration (Lamalice et al. 2004). In addition, VEGF also activates the PI3K/AKT pathway, which mediates endothelial cell survival (Fujio and Walsh 1999, Dayanir et al. 2001). The ERK5 MAPK pathway has been shown to play a decisive role in angiogenesis through activation of the AKT pathway and the promotion of endothelial cell survival (Roberts et al. 2010).

Figure 1.4: Signal transduction pathways in angiogenesis.
The figure demonstrates the major characteristic pathways activated by VEGF-A/VEGF receptor-2 and how it is distinguished from other pathways induced by the IL-1 and EGF receptor in endothelial cells. In order to elicit the angiogenic/proliferative response, activation of the Ca^{++} /calcineurin and PKC/MAPK pathways has to be induced by VEGF-A coupled with Tyr1175 to PLC-γ, which it turns to procure to the activation of the transcription factor NFAT and EGR-1 that are important for part of the VEGF-induced gene repertoire. On the other hand, promotion of cell survival, migration, and potentially permeability would be attained by activation of other pathways via PI3K/Akt, TsAd, Src and p38. IL-1 receptor, and via NF-κB, would promote genes to a large magnitude. It may also appear that a significant portion of the NF-κB-induced genes is up-regulated by VEGF-A-induced NFAT. It is more relevant to notice that MAPK pathway would be activated by EGF. NF-κB activation is seemingly not activated to a significant extent by VEGF-A or EGF. Image taken from (Hofer and Schweighofer 2007).

1.3. MAPK signalling pathways

1.3.1. MAPK pathways in mammalian cells

MAPKs are thoroughly conserved enzymes in eukaryotic cells that regulate many cellular processes. Examples of those critical processes are cell growth, differentiation, and apoptosis (Cargnello and Roux 2011). MAPK signalling pathways consist of three-component hierarchical cascades and are named according to the downstream protein kinase (Figure 1.5). Classically, 4 pathways are distinguished at the molecular level of eukaryotic cells: ERK1/2 (extracellular signal-regulated kinase 1/2), p38MAPK (with different isoforms α/β/γ/δ), c-Jun NH2-terminal kinases (JNK) 1/2/3 and ERK5 (Krishna and Narang 2008, Morrison 2012).

Each part of this cascade performs a crucial function according to their distinctive structure (Dhillon et al. 2007, Plotnikov et al. 2011). The first protein in the signalling pathway is entitled MAPK kinase kinase (MAPKKK), whose role is to respond to extracellular stimulation, for instance cellular stress or growth factors. Then, at the second level, is MAPKK. These proteins contain particular motifs of serine (Ser; S) and threonine (Thr; T) amino acids that are dual-phosphorylated by the MAPKKK in order to activate the MAPKK. Then, the downstream MAPK would be phosphorylated by the MAPKK, thereby activating it (Cargnello and Roux 2011). MAPK activation then leads to the modification of a set of regulatory molecules such as structural proteins, transcription factors, and phospholipids, which in turn maintain or regulate cellular activity (Krishna and Narang 2008, Cargnello and Roux 2011).

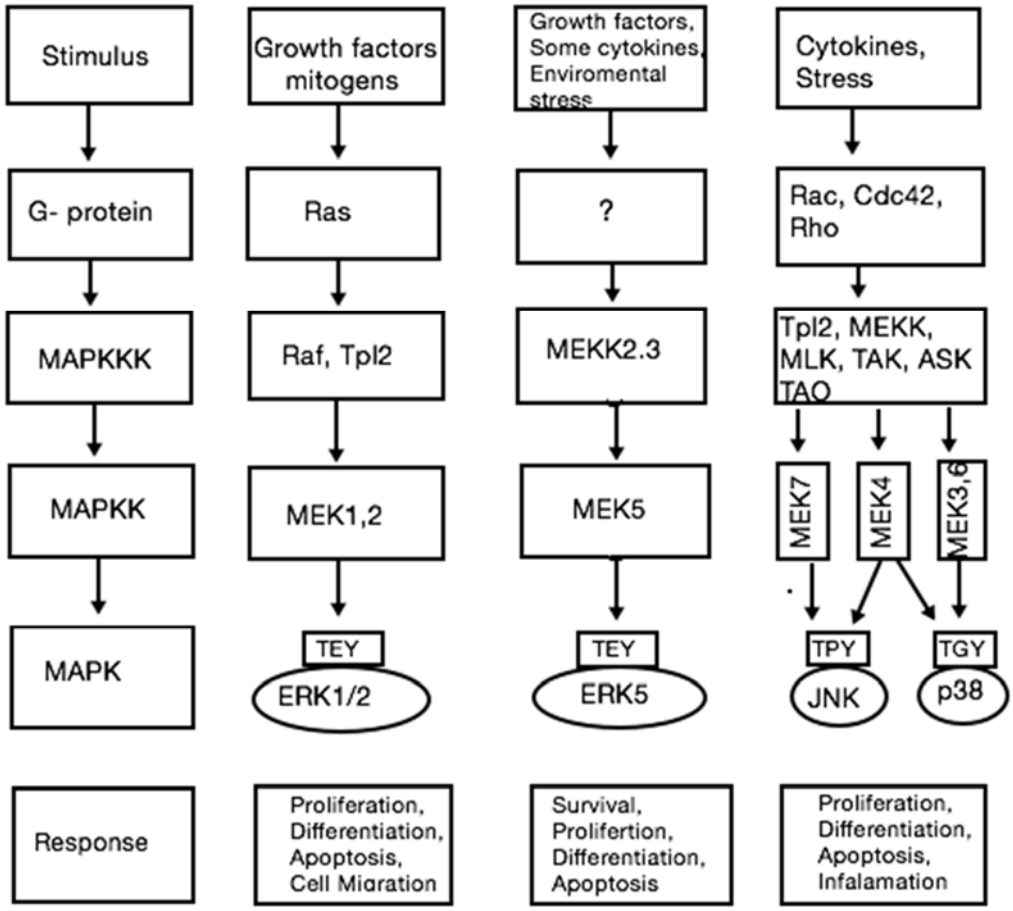


Figure 1.5: Mitogen-activated protein kinase (MAPK) signalling pathways in mammalian cells.

Four distinctive MAPK pathways can be found in mammalian cells, termed ERK1/2, JNK, p38 MAPK, and ERK5 MAPK. Activation and stimulation of MAPK pathways can be attained upon the impact of a variety of extracellular stimuli including: mitogens, cytokines and growth factors, as well as environmental stress factors such as oxidative stress and osmotic stress. Upon activation of the apical MAPKKK, activation of the downstream MAPKK is achieved by dual phosphorylation. The activated MAPKK then phosphorylates the MAPK at specific amino acid motifs (TXY) to activate it. Regulation of a variety of crucial cellular functions (e.g.,cell proliferation, differentiation, apoptosis, cell migration, and inflammation) is a consequence of transcriptional and non-transcriptional regulation by the MAPK pathways.

1.3.2. MAPK activation and inactivation

MAPKs are activated by a large number of environmental stimuli, including osmotic and oxidative stress as well as growth factors. Each MAPK cascade can be stimulated by one or more specific stimuli (Dhillon et al. 2007, Munshi and Ramesh 2013). The ERK1/2 pathway, which was discovered first, is generally activated by mitogenic growth factors such

*as epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF). It has been well-established that the activation of this pathway was governed and well-understood during the 1990s (Meloche and Pouyssegur 2007). In contrast, the p38 and JNK MAPK pathways are characterised as stress-activated MAPK pathways, as they respond preferentially to environmental stress and cytokines (Coulthard et al. 2009). Finally, the ERK5 pathway has been shown to be activated by growth factors (e.g., FGF, VEGF), cytokines (e.g., IL-6, IL-8) and environmental stresses (e.g., osmotic stress, shear stress) (Rose et al. 2010). Upon stimulation of the pathway, activation of the terminal MAPKs is achieved by dual phosphorylation at TXY motifs of the activation loop, which is located in the kinase domain, by the upstream MAPKK (Krishna and Narang 2008). The middle residue of the motif (X) varies between the four MAPKs. The JNK MAPK contains a proline (P) at this position, while p38 contains a glycine (G). Interestingly, both ERK1/2 and ERK5 share the same activation motif (TEY), containing a glutamic acid residue at the equivalent position (*Figure 1.6*).*

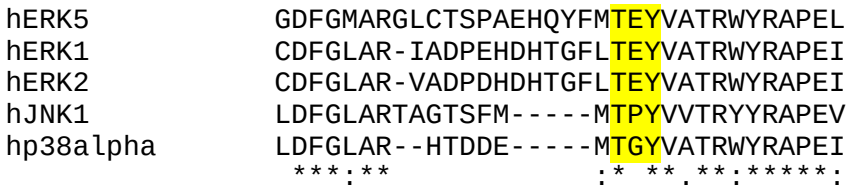


Figure 1.6: Sequence alignment of the activation loop of human MAPKs

The protein sequence of the activation loop of representative members for the 4 mammalian MAPK families is shown and the sequences around the conserved TxY motif (highlighted in yellow) are aligned. While the TxY motif is fully conserved between the different proteins, ERK5 and ERK1/2 share an aspartate residue in the x position.

Inactivation of MAPK signalling can be achieved by various means. The most common mechanism is the dephosphorylation of the TXY motif in the activation loop, as MAPKs require the phosphorylation of both the threonine and tyrosine residues for full kinase activity (Caunt and Keyse 2013). A number of enzymes have evolved to perform this function, including Type 1/2 Ser/Thr phosphatases, protein tyrosine phosphatases and dual-specificity Thr/Tyr phosphatases, which are all able to dephosphorylate and inactivate the

MAPK proteins (Keyse 2000). The largest and best-studied group of MAPK phosphatases (MKPs) that specifically regulate MAPK activity are the dual-specificity MAPK phosphatases (DUSPs) (Caunt and Keyse 2013). In mammalian cells, the nuclear proteins DUSP1/MKP-1, DUSP2, DUSP4/MKP-2 and DUSP5 dephosphorylate ERK1/2, p38 and JNK. Another group includes the cytoplasmically located ERK1/2-specific phosphatases DUSP6/MKP-3, DUSP7/MKP-X and DUSP9/MKP-4 and the final group contains the p38/JNK-specific phosphatases DUSP8 (MKP3/6), DUSP10/MKP-5 and DUSP16/MKP-7. Interestingly, there have not been any MKPs/DUSPs identified to date that can dephosphorylate and inactivate ERK5 (Seternes et al. 2019).

1.4. ERK5

1.4.1. ERK5 discovery

1995 was the discovery of ERK5. Initially, Dixon and co-workers (Zhou et al. 1995) used a yeast two-hybrid assay with MEK5 as bait to identify its binding partners, including ERK5. At the same time, another group discovered BMK1 (big mitogen kinase 1), which eventually was recognised to be identical to ERK5 (Lee et al. 1995). This identification was accomplished by screening a cDNA library from placenta tissue to identify novel MAPKs. The ERK5 gene is located on chromosome 17p11.2, and it extends to 5.79kb. ERK5 shows significant homology with ERK1/2, in particular at the kinase domain level. Expression of ERK5 can be identified in a variety of cell types, including skeletal muscle, heart, brain, placenta, and lung (Lee et al. 1995, Buschbeck and Ullrich 2005). ERK5 and ERK1/2 can both be dual phosphorylated at TEY motifs in the kinase domain, and both proteins contain an oligomerisation domain. On the other hand, ERK5 contains additional sequences, particularly at the C-terminus. These additional sequences make the ERK5 protein the biggest mammalian MAPK and allow it to be regulated in a more complex way (Nithianandarajah-Jones et al. 2012).

1.4.2. ERK5 structure

ERK5 is larger than the other MAPKs and the regulation of ERK5 (also known as MAPK7) is more complex (Nishimoto and Nishida 2006). The ERK5 gene is composed of 5824 bases, with an open reading frame of 2451 base pairs, which in turn encodes a protein with 816 amino acids (Nithianandarajah-Jones et al. 2012). It contains 6 exon and there are 11 transcripts, seven of which are protein coding (Inesta-Vaquera and Cuenda 2010). The unique structure of ERK5 can be divided into several domains, corresponding to their function (Figure 1.7). Firstly, a cytoplasmic targeting domain is located at the N-terminus of ERK5 (a.a.1-77). Then, the predominant part is the kinase domain (a.a. 78-406). The kinase domain contains three other motifs: a MEK5 binding region (a.a. 78-139), an oligomerisation domain (a.a. 140-406), and a common docking (CD) domain (a.a. 350-358). The CD domain is a negatively charged sequence that binds to the substrate and is present in all MAPKs.

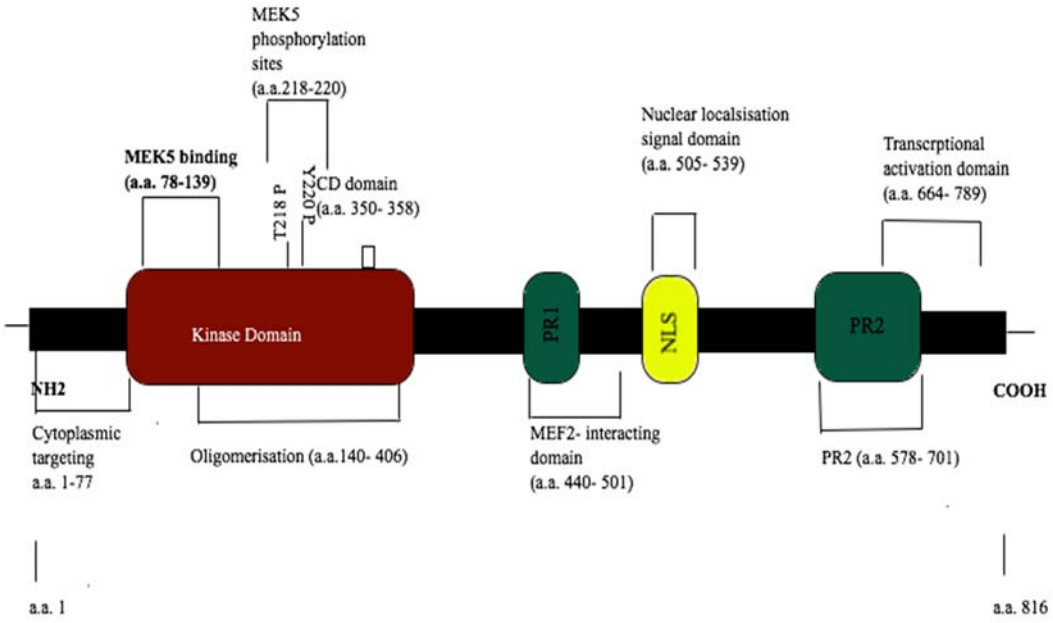


Figure 1.7: ERK5 structure and functional domains.
ERK5 consists of 816 amino acids. It contains an N-terminal kinase domain that includes a region for cytoplasmic targeting (a.a. 1-77). The Kinase domain (a.a. 78-406) contains the activating sites that are phosphorylated by MEK5 (Thr^{218}/Tyr^{220}). In addition, the kinase domain includes a MEK5 binding region (a.a. 78-139), a common docking domain (CD) (a.a. 350-358), and the oligomerisation region. The extended C-terminal tail consists of two proline-rich (PR) domains PR1 (a.a. 434-485) and PR2 (a.a. 578-701), a MEF2 interacting region (a.a.440-501), the nuclear localization signal (NLS) (a.a. 505-539) and the transcriptional activation domain (a.a. 664-789). Figure modified from (Nithianandarajah-Jones et al. 2012).

The kinase domain also contains the activation motif (TEY), which is phosphorylated by the upstream MEK5.

What makes ERK5 twice as big as the conventional MAPKs is the extended C-terminal domain, which performs additional functions. For instance, a nuclear localization signal (NLS; a.a. 505-539) is located in the C-terminal domain, which facilitates nuclear translocation (Morimoto et al. 2007). Interestingly, it has been shown that the C-terminal half of ERK5 also contains a unique transcriptional activation domain. This domain is essential for the activation of gene expression by ERK5 (Morimoto et al. 2007) and is not present in any other mammalian MAPKs. Also, two proline-rich regions, PR1 (a.a. 434-465) and PR2 (a.a.578-701), are found within the C-terminal domain. These regions bind Src-homology 3 (SH3) domain-containing proteins that can further regulate ERK5 activity. Importantly, the PR1 region has been shown to specifically interact with myocyte enhancer factor 2 (MEF2) (a.a. 440-501) (Yan et al. 2001), a major substrate of ERK5 (Roberts et al. 2010, Tubita et al. 2020).

1.4.3. ERK5 activation / inactivation and downstream targets

ERK5 can be activated by a number of stimuli. Laminar-shear-stress and hypoxia are prominent examples for ERK5 being activated by physiological and pathological stress factors (Abe et al. 1996, Pearson et al. 2001, Tubita et al. 2020). In addition, extracellular growth factors, such as VEGF, EGF, FGF, and PDGF, have been shown to activate ERK5 (Kato et al. 1998, Hayashi et al. 2004, Kesavan et al. 2004, Tubita et al. 2020). In neurons, ERK5 could also be activated by brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Cavanaugh et al. 2001, Watson et al. 2001). Furthermore, certain inflammatory cytokines, for instance interleukin 6 (IL-6) and interleukin 8 (IL-8), were identified to activate and stimulate the ERK5 pathway (Carvajal-Vergara et al. 2005).

The activation process of ERK5 is complex (Figure 1.8) (Buschbeck and Ullrich 2005, Kondoh et al. 2006). In the unphosphorylated (inactive) state, ERK5 exists in a folded, closed conformation due to a molecular, non-covalent interaction between the C- and N-terminals. This conformation activates its nuclear export signal (NES) and inhibits its NLS, leading to the cytoplasmic sequestration of ERK5. Once Thr218 and Tyr220 are phosphorylated by MEK5, ERK5 undergoes conformational changes due to a reduction of the interaction between the C- and N-terminals. This conformational change inactivates the NES and exposes the NLS, leading to the translocation of ERK5 to the nucleus. In addition, a number of C-terminal residues are autophosphorylated, leading to the full activation of ERK5 (Pearson et al. 2020).

Figure 1.8: The ERK5 activation process.
In the unphosphorylated (inactive) state, ERK5 exists in a folded, closed conformation due to a molecular interaction between the C- and N-terminals. This conformation activates its nuclear export activity and inhibits its nuclear localisation signal, leading to cytoplasmic sequestration of ERK5. Upon activation by MEK5 phosphorylation, the interaction between the C- and N-terminals is reduced, exposing the nuclear localization signal, leading to the translocation of ERK5 to the nucleus as well as autophosphorylation of a number of C-terminal residues (Figure from Nithianandarajah-Jones, 2012).

Much less is known about the inactivation of ERK5. Typically, MAPKs are inactivated through dephosphorylation of their TXY motif in the activation loop of the kinase domain (Dickinson and Keyse 2006). While a specific DUSP (dual-specificity phosphatase) that dephosphorylates these residues has not yet been discovered, ERK5 has been shown to be dephosphorylated by the phosphotyrosine-specific phosphatase PTP-SL (protein tyrosine phosphatase STEP-like), which leads to its inactivation and inhibition of its nuclear translocation (Morimoto et al. 2007, Tubita et al. 2020).

Significantly, it has been shown that ERK5 regulates the activity of several transcription factors (Kato et al. 1997, Kasler et al. 2000, Barsyte-Lovejoy et al. 2004), for instance MEF2C, a member of the myocyte enhancer factor (MEF) family consisting of MEF2 A, C, and D. MEF2C and the other family members promote the activity of the c-Fos promoter by activating c-Myc, CREB, and Sap1a. KLF2 and KLF4 (krüppel-like transcription factors) are other examples of more recently discovered transcription factors activated by ERK5 (Sunadome et al. 2011). Activation of KLF2 by ERK5 leads to the downregulation of p21-activated kinases (PAKs) (Komaravolu et al. 2015), which play an important role in cardiovascular development and endothelial cell migration, likely by activating Cdc42 and the small Rho GTPase Rac. Interestingly, it has been demonstrated that the transactivation activity of ERK5 is highly dependent on the activated kinase activity of ERK5, implying that autophosphorylation of the C-terminal half of ERK5 is required for its ability to directly activate transcription (Morimoto et al. 2007, Tubita et al. 2020).

1.4.4. Physiological roles of ERK5

At the molecular level, targeting the ERK5 gene by deletion has opened the avenue to identify the physiological significance of the ERK5 signalling pathway. It has been shown that knocking out either the Erk5, Mek5, or Mekk3 gene in mice is embryonic lethal, with death occurring between embryonic day E9.5 and E11.5 as a consequence of irregularity in the development of the cardiovascular system (Yang et al. 2000, Yan et al. 2003, Wang

et al. 2005). Immature vasculogenesis and lack of organization in endothelial cells are lethal consequences, which lead to death due to haemorrhage and leakage in the vascular system. In addition, Mef2c$^{-/-}$ knock-out mice also exhibited embryonic lethality as a result of the loss of vascular integrity (Lin et al. 1997, Lin et al. 1998, Bi et al. 1999). Because mice with gene ablation in all components of the ERK5 signalling axis share the same phenotype, it can be inferred that a crucial role of the ERK5 signalling cascade is to regulate angiogenesis and/or vasculogenesis. Interestingly, knocking out the ERK5 gene globally or conditionally in endothelial cells resulted in the same phenotype: disturbed cardiovascular integrity and vascular haemorrhage disorders (Hayashi et al. 2004). In contrast, knocking out Erk5 specifically in hepatocytes (Hayashi and Lee 2004) and cardiomyocytes (Hayashi et al. 2004, Kimura et al. 2010), displayed no effects on development and maturation. Interestingly, in adult mice, inducible ERK5 ablation generated leakage of blood vessels as a result of endothelial cell apoptosis, resulting in death within 2-3 weeks (Hayashi et al. 2004). Even though ERK5 is expressed in a variety of cell types, the most crucial function therefore appears to be within the context of vascular integrity.

1.4.5. The role of ERK5 in angiogenesis

While the results from the knock-out studies demonstrated a requirement for ERK5 in angiogenesis, these studies did not clarify where in the process ERK5 is needed. VEGF is a well-known inducer of angiogenesis and is important for vascular development in embryogenesis and early life (Carmeliet et al. 1996, Gerber et al. 1999, Ferrara 2000, Haigh 2008). Importantly, a recent study showed that ERK5 plays an essential role as a critical mediator of the VEGF-dependent survival of endothelial cells (Roberts et al. 2010). VEGF induced sustained ERK5 activation in primary endothelial cells (HDMEC; human dermal microvascular endothelial cells). Interestingly, reduction of ERK5 activity by siRNA-mediated gene silencing disrupted tubular morphogenesis of HDMEC cells without reducing proliferation. In contrast, overexpression of constitutively active MEK5 and ERK5 stimulated tubular morphogenesis in the absence of VEGF, clearly demonstrating the ability of ERK5

to induce angiogenesis. An important observation was that ERK5 regulated AKT phosphorylation and activation, leading to the VEGF-mediated phosphorylation (and thus inactivation) of the pro-apoptotic protein BAD and increased expression of the anti-apoptotic gene BCL2. This resulted in decreased caspase-3 activity and the suppression of apoptosis. Together, these results demonstrated that ERK5 is required for the survival of endothelial cells during angiogenic processes (Roberts et al. 2010).

1.5. The role of ERK5 MAPK in the integrity of vascular endothelial cells

Knocking out ERK5 in mice provided outstanding evidence of the role of ERK5 MAPK in the integrity of the endothelium (Hayashi and Lee 2004). Cardiovascular defects and death have been caused in knocked out endothelial ERK5$^{-/-}$ mice around E10.0. This can be explained as enhancing the survival of endothelial cells and inhibition of endothelial apoptosis are major regulatory functions of ERK5 MAPK in endothelial cells (Roberts et al. 2010). MEF2C is a transcription factor attributed to protect endothelial cells from apoptosis (Roberts et al. 2009). It is well-known that ERK5 can phosphorylate and activate MEF2C, thus enhancing the survival of cells. However, under unusual condition such as targeted deletion of the ERK5 or Mef2C gene, cardiac and vascular malformation was acquired, demonstrating the phenotypic similarity between ERK5$^{-/-}$ and Mef2C$^{-/-}$ mice, with embryonic lethality in both mouse lines (Lin et al. 1998, Bi et al. 1999).

It is important to discuss the profound role of ERK5 in connecting the growth factor receptors to cell survival. ERK5 has been reported to regulate PKB/Akt phosphorylation in vitro through different mediators and conditions, such as the phosphorylation of PKB/Akt at Ser^{473} and Thr^{308} in mouse embryonic fibroblast under osmotic stress status (Wang et al. 2006), and activation of PKB/Akt through platelet-derived growth factor (PDGF) in porcine aortic endothelial cells (PAEs) (Lennartsson et al. 2010). Furthermore, in human dermal

microvascular endothelial cells (HDMECs), the mediatory action of VEGF is supported by ERK5, which activates Akt and enhances the expression of pro-survival protein BCL-2 as well as phosphorylation and inactivation of the pro-apoptotic protein BAD (Roberts et al. 2010). In summary, it can be concluded that PKB/Akt activation is regulated by ERK5, although the precise mechanism remains to be resolved. Consequently, cell survival and tubular morphogenesis of the endothelial cells are fully dependent on ERK5 activity, due to inhibition of apoptosis.

Atherosclerosis is an inflammatory condition starting by accumulation of leukocytes in the sub-endothelial area, followed by depositing of fatty streak and other inflammatory agents in the wall of arteries leading to formation of atherosclerotic lesions (Lusis 2000, Rafieian-Kopaei et al. 2014). Inhibition, or even blocking of blood flow is a fatal consequence triggered by atherosclerosis. Therefore, keeping a steady laminar blood flow can be defined as an atheroprotective agent, where harmful factors such as oxidative stress and inflammatory cells would be suppressed (Chiu and Chien 2011). In in vitro studies of both bovine lung microvascular endothelial cells (BLMECs) and human umbilical vein endothelial cells (HUVECs), shear-stress mediated ERK5 activation has been shown to play a remarkable regulatory role by stimulating an anti-apoptotic (BLMECs) and atheroprotective effect (HUVECs) (Figure 1.9). The exact mechanism of this regulatory role can be demonstrated upon mediating flow-dependent inhibition of TNFα signalling (Akaike et al. 2004, Pi et al. 2005). In terms of maintaining vascular quiescence, ERK5 is required for Kruppel-like factor 2 gene expression (KLF2) (Parmar et al. 2006), which induces a prompt decrease in angiogenesis and inflammation processes. Moreover, KLF2 decreases the adhesion of leukocytes and reduces the anti-inflammatory response by inhibiting nuclear factor κB (NF-κB). KLF2 is additionally involved in regulating endothelial cells as it enhances the synthesis of endothelial nitric oxide synthase (eNOS) and thrombomodulin, which possess potent properties as anti-thrombotic and anti-inflammatory agents, influencing vasoprotection (Nithianandarajah-Jones et al. 2014).

Figure 1.9: The role of ERK5 in endothelial cells.
The figure shows that the ERK5 pathway is activated by multiple stimuli in endothelial cells, including VEGF and shear stress. Activation of ERK5 in turn leads to several pro-angiogenic and survival responses through the activation of several effector molecules as shown. Image taken from (Nithianandarajah-Jones et al. 2014).

1.6. Regulation of vascular tone

Vasoactive factors can be classified into two main groups based on their biological effect on the blood vessels. The main source of these vasoactive factors was identified to be the endothelium (Vanhoutte and Mombouli 1996). These vasoactive factors would be grouped as vasodilatory or vasoconstrictory factors. Examples of vasodilatory factors include: nitric oxide (NO) and prostacyclin (PG_2). Thromboxane (TXA_2) and endothelin-1 (ET-1) are examples of vasoconstrictive factors (Sandoo et al. 2010).

Nitric oxide (NO) has long been identified as a vasodilator and regulator of blood vessel tone (Levine et al. 2012). To be precise, the underlying smooth muscle layer of the endothelium is the main target of nitric oxide action (Jin and Loscalzo 2010). Upon

*stimulation of nitric oxide synthase enzyme (NOS), formation of NO is chemically achieved through the conversion of the amino acid L-arginine. Compounds such as acetylcholine, histamine, and angiotensin II were shown to be involved in enhancing NO generation. Three different isoforms of NOS, eNOS (endothelial NOS), nNOS (neuronal NOS) and iNOS (inducible NOS), are distributed through various types of cells (Michiels 2003, Forstermann and Sessa 2012). Various kinds of actions and effects can be concluded of those different inducible isoforms, which would also be allocated in context of neurons, macrophage and endothelium. Cardiac myocytes, platelets, skeletal muscle, and hippocampus are also examples of tissues where these isoforms occur (Prast and Philippu 2001). Production of NO is highly dependent on the level of Ca^{2+} in the cells, whether it is originated from the inner endoplasmic reticulum or from extracellular resource. The importance of Ca^{2+} abundance can be explained in terms of the binding to the eNOS enzyme; where dissociation of eNOS from calcium-calmodulin complex can be observed under the reduction of Ca^{2+} levels. Thereafter, eNOS becomes inactivated via their induced binding to caveolin (Govers and Rabelink 2001, Forstermann and Sessa 2012). Interestingly, shear stress is a consequence of increasing the blood flow in the vessels, which it turns mediates a remarkable effect via protein kinase B (Akt) to phosphorylate eNOS. Upon production, NO diffuses to the endothelium to bind to the soluble guanylyl cyclase enzyme (sGC) that resides in the adjacent smooth muscle. As a result, the level of cGMP in the muscle is dramatically increased (*Figure 1.10*). The well-known actions of increased cGMP in the smooth muscle can be summarized in terms of reducing muscle tension and contraction (Sandoo et al. 2010).*

It is also important to discuss the opposite action of prostacyclin (PGI_2) with thromboxane (TXA_2) in generating vasoconstriction as a regulatory mode for vascular tone. In brief, the level of intracellular Ca^{2+} in the smooth muscle is augmented by cyclooxygenase catalysis (Griendling and Alexander 1997). Homeostasis of healthy vessels can be envisaged in their

regulatory role of balancing the activity of PG_2 and TxA_2. Thus, resting vascular tone is also affected by interaction between endothelial constituted products (e.g., PGI_2 and TXA_2) (Masferrer et al. 1994).

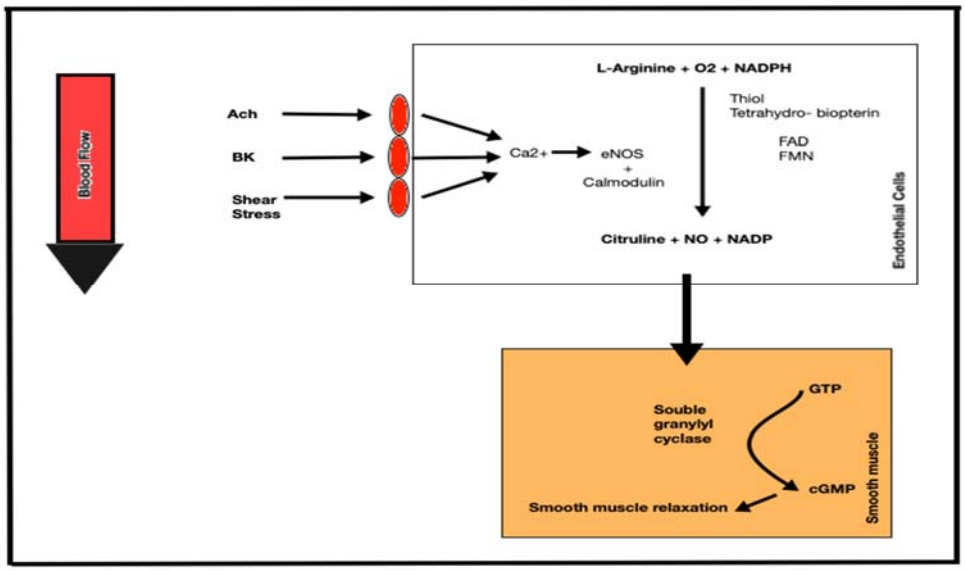


Figure 1.10: Illustrating schematic for nitric oxide production and how it affects the vascular smooth muscle.
ACh= acetylcholine; BK= bradykinin; eNOS= endothelial nitric oxides; sGC= soluble guanylyl cyclase; cGMP= cyclic guanosine-3', 5'-monophosphate.

Endothelin is another agent of regulation of vascular tone, which has been identified as an endothelium-derived 21 amino acid peptide playing a potent role with long lasting effects on vasoconstriction (Yanagisawa et al. 1988). Endothelin synthesis is a de novo phenomenon, dependent on response to various chemicals (e.g., thrombin, angiotensin II, cytokines) and physical agents (e.g., hypoxia, shear stress), as the endothelin is not stored in endothelial cells (Michiels 2003, Kruger-Genge et al. 2019). Three isoforms of endothelin have been identified in the body, ET-1, ET-2, and ET-3 (Alonso and Radomski 2003). $Endothelin_A$ (ET_A) and $endothelin_B$ (ET_B) are both coupled to G proteins with different locations in the blood vessels. ET_A receptor is located on the vascular smooth muscle cells whereas ET_B receptor is situated on the endothelial cells. In terms of affinity, it is well-known that the ET_B receptor has a tendency of binding to the three endothelin isoforms, while the ET_A receptor is constrained with high affinity for binding to both ET-1 and ET-2, excluding ET-3 (Kedzierski

and Yanagisawa 2001). As ET-1 is selectively released from endothelial cells, it is possibly to further explore their exact function. First, endothelin converting enzyme is the main agent responsible for converting big ET-1 to ET-1. The level of secretion and production of ET-1 can be affected by several factors. Inflammatory factors such as interleukins and TNF-α can enhance ET-1 production, while ET-1 release can be diminished by the effects of NO and PGI_2. In addition to the mitogenic activity of ET-1 on smooth muscles cells, vasoconstriction can be exerted by binding to the ET_A receptor, or by activating other growth factors such as platelet-derived growth factor. The phospholipase C pathway appears to be involved in generating smooth muscle cell contraction. However, binding of ET-1 to ET_{B2} in the endothelium causes vasodilation after the release of NO and PGI_2. Overall, it is crucial for vascular response that many vasoactive pathways would interact with each other, creating the proper result of regulation to vascular tone (Sandoo et al. 2010).

The integrity of vascular endothelial cells plays a pivotal role in the stability of circulation. For this reason, vascular endothelial cells were found to be a source of many regulatory substances and chemicals as well as their physical barrier role. Hence, manifestation of cardiovascular diseases such as hypertension and atherosclerosis is a strict consequence of endothelium-dependent regulatory system dysfunction (Rajendran et al. 2013). In addition, upon the efforts of Furchgott and Zawadzki (Furchgott and Zawadzki 1980), it has been shown that the integrity of the endothelium would induce a vasodilation effect on the vascular smooth muscle in response to acetylcholine (Furchgott and Zawadzki 1980). Vascular homeostasis is a remarkable phenomenon requiring the release of specific regulatory substances from the lining of the endothelial cells including endothelium-derived NO and prostacyclin under certain conditions and stimuli, such as physical stimulation, hormones, and platelet-derived substances. Vascular relaxation and platelet function inhibition are the result of those modulations (Denninger and Marletta 1999). However, while inducing vasoconstriction is somehow a response to pathophysiological conditions, it remains an important factor in the regulation of vascular tone. The onset of some

pathophysiological disorders including high blood pressure and hypoxia, the vascular endothelial cells can produce several contracting agents (e.g., endothelin, thromboxane A2, angiotensin II, superoxide). Significantly, the release of such vasoactive substances (e.g., NO, endothelin, and angiotensin II) by the endothelium has also been found to perform a major role in vascular growth processes.

1.7. H_2S: a gaseous signalling molecule

Hydrogen sulphide (H_2S) has been recognized as a toxic agent and environmental hazard for many decades (Szabo 2018). It can be defined as a reactive thiol which has distinctive chemical properties. H_2S is a water-soluble, flammable, colourless gas with offensive odour of rotten eggs (Elsey et al. 2010, Szabo et al. 2013). However, it can be also secreted and synthesized in significant amounts in mammalian cells (including human cells). Synthesis of H_2S can be endogenously attained in different mammalian tissues from metabolizing L-cysteine or L-methionine (Giuffre and Vicente 2018). Cystathionine β-synthase and cystathionine γ-lyase are both pyridoxal-5'-phosphate-dependent enzymes for H_2S synthesis. The relevant substrate could be sourced from endogenous proteins or even from nutrients (Wang 2002, Fiorucci et al. 2006). Trans-sulphuration of L-methionine can be a pathway to produce H_2S with the assistance of homocysteine (i.e. being the mediator). In addition, endogenous H_2S can be synthesized by the 3-MST (3-mercaptosulfurtransferase) enzyme and one of the following substrates: 3-MP (3-mercaptopyruvate) by CAT (cysteine aminotransferase), thioredoxin, dihydrolipoic acid and D-cysteine along with DAO (D-amino acid oxidase) (Pan et al. 2017).

It is crucial to characterize the organ-specific and molecular source of these enzymes (CBS and CSE), where the eventual product of them is going to be H_2S formation. The liver and kidney are the predominant organs for expression of CBS, as well as the nervous system and brain. Meanwhile, the liver is mainly responsible for expression of CSE in additional to

vascular and non-vascular smooth muscle (Elsey et al. 2010). However, thiosulphate and sulphate are major products of sulphide degradation being released by specialized enzymes from intestinal epithelium. Therefore, enterobacterial flora can be a source for H_2S production, perhaps the main function is to hinder excess entry of H_2S to the circulation (Fiorucci et al. 2006, Szabo 2007).It has been brought to the attention with strong evidence that the cardiovascular system (e.g., vasculature and heart) would be the endogenous source of H_2S generation including H_2S-generating enzymes CBS, CSE, and 3-mercaptosulfurtransferase MST (Furne et al. 2001, Pan et al. 2017).

H_2S, together with CO and NO are well known as gas-transmitter molecules implementing various biological effects at key targets (Kolluru et al. 2017). They are typically small molecules with a simple molecular composition and structure. Fast diffusion through the cell membrane due to their lipophilic nature is a distinctive feature of gasotransmitter molecules. In addition, based on the structural similarities with H_2O, it was proposed that aquaporines and some ion channels (e.g., KATP channels, Ca^{2+} channels) may facilitate H_2S transportation across the cellular membrane (Mathai et al. 2009). Interestingly, gasotransmitters share common features (labile gas, rapidly eliminated by mammals) that result in influencing a wide range of cytotoxic and cytoprotective effects (Szabo 2007). It is important to mention the bio-molecular interaction between H_2S and CO at the physiological level, although it is not fully understood how they react with each other as well as with NO synthase (NOSs) (Elsey et al. 2010, Wu et al. 2018). In vitro studies using Na_2S or NaHS have shown that H_2S neutralizes and interacts with a variety of oxyradical species, peroxynitrite, homocysteine and hypochlorous acid and that it can generate cytoprotective actions (anti-necrotic or anti-apoptotic effects) (Whiteman et al. 2004, Whiteman et al. 2005, Rinaldi et al. 2006, Szabo 2007). Interestingly, many researchers have used inorganic sulphide salts (e.g. Na_2S) and sodium hydrogen sulphide (NaHS) as H_2S equivalents. It has been shown that treating cells, tissues, or animals with sulphide salts would induce a protective effect against a number of diseases. It has been confirmed by the Lefer group

that using Na_2S as an exogenous H_2S donor would attenuate ischemia-induced heart failure upon long-term H_2S therapy (Calvert et al. 2010). In addition, sulphide salts have shown a significant role as a protective agent against other diseases such as inflammation and Osteoarthritis (OA). Significantly, treatment of human cells with NaHS is critical to alleviate IL-6 and IL-8 expression levels, which may account for the anti-inflammatory role of H_2S against OA. Also, it is significant to take extra care when NaHS is used as it has been found that pro-inflammatory characteristic would be acquired instead of the anti-inflammatory effects of NaHS upon extended of the incubation from 15 minutes to 1 hour. It is clear that sulphide salts, as H_2S donors, may have a positive effect in boosting H_2S concentration rapidly. However, it would be hard to control H_2S concentration when the compounds release H_2S synergistically at the time the solution is prepared. In addition, under laboratory condition, it has been found that H_2S would be lost from solution because it can be quickly volatilized in aqueous solution. Carefulness has to be taken in purchasing commercial sulphide salts, especially NaHS, where it has been noted that it contains significant amount of impurities (Zhao et al. 2014). The concentration of endogenous H_2S is profoundly significant in modulating the generated actions, for example cytotoxic and cytoprotective effects (Whiteman et al. 2004, Whiteman et al. 2005, Rinaldi et al. 2006). At micromolar concentrations, the cytoprotective effects (antinecrotic or anti-apoptotic) of H_2S have been reported in multiple studies, to be generated in vitro from Na_2S or NaHS (a H_2S donor), that may associate with its capacity to neutralize different reactive species, for example oxyradicals and peroxynitrite, hypochlorous acid and homocysteine. Also, modulation of intracellular caspases and or kinase pathways can be one of these cellular effects of H_2S concentration. On the other hand, in order to upregulate the cytotoxic effect of exogenous H_2S, endogenous H_2S has been shown to be initially inhibited, which shows the cytotoxic effects of low (physiological) levels of endogenous sulphide. Exposure of the cells to high (millimolar) H_2S has a negative impact shown in form of cytotoxicity, and that due to generation of high level of free radical and oxidant, calcium mobilization and glutathione depletion (Szabo 2007).). Moreover, various studies have shown that endogenous

antioxidant systems would be upregulated (activated) upon low levels of H_2S production (low micromolar), examples of those antioxidant agents include N-acetylcysteine, glutathione, and superoxide dismutase (Whiteman et al. 2004, Yang et al. 2004, Ezerina et al. 2018). It has been reported in various studies that H_2S can induce anti-inflammatory action by up-regulation of specific genes such as haeme oxygenase 1 (HO1) (Qingyou et al. 2004, Oh et al. 2006). Thus, anti-inflammatory and cyto-protective effects would be initiated upon triggering the production of CO. Examples of those effects include the inhibition of the Nuclear Factor-κB pathway (NFκB) and downregulation of NO synthase expression (Oh et al. 2006, Ryter et al. 2006). On the other hand, H_2S may also exhibit a pro-inflammatory role (Li et al. 2006). It has been shown that exposure to endogenous H_2S may correlate with toxicity and cell death in the lung and other organs. Currently, supportive evidence of a pro-inflammatory effect of H2S in vivo has been concluded from animal models of endotoxic or septic shock (Li et al. 2006). It has been reported that preliminary role of excessive activation of KATP channels to vasoconstrictor agents may occur in lipopolysaccharide (LPS)-induced hypotension and hyporeactivity, which in turn raise the probabilities that septic or endotoxic shock may be a result of abnormal synthesis or activity of H_2S (a potential natural ligand for KATP channels). Other group has demonstrated that H_2S formation is likely upregulated in various tissues from LPS-injected mice and rats (Hui et al. 2003). Inhibition of CSE-mediated H_2S formation by propargylglycine (PGA), would contribute to reduce both the inflammation and the multiple organ dysfunction in endotoxic shock and employee a protective effect in haemorrhagic shock.

In regard to H_2S metabolism, numbers of different metabolic pathways have been reported to be involved, with oxidation in mitochondria and cytosolic methylation being the most important metabolic pathways (Beauchamp et al. 1984, Cao et al. 2019) . In various studies and reports the concentrations of H_2S in the blood and many tissueswere measured under conditions while around 100 μM in the human blood and 160 μM in the human brain were detected (Elsey et al. 2010). In in vitro studies by Zhao et al (Zhao et al. 2001), the rat liver showed higher production of H_2S than in vascular tissue, which stresses the importance of

the regulation and maintenance of H_2S in the circulation by the liver (Zhao et al. 2003). Under certain intracellular conditions, regulating the H_2S availability can be achieved with sulfane sulfur (Ishigami et al. 2009). In spite of the unreliability of H_2S assay methods, total sulfate and sulfite measurements may demonstrate some efficiency (Elsey et al. 2010). Furthermore, the methylene blue technique has been recently used with high efficiency in the literature to measure hydrogen sulphide in biological samples (Shen et al. 2015). Excretion of H_2S from the body would eventually be regulated by the kidney in both forms (free or conjugated sulfate) (Beauchamp et al. 1984, Cao and Bian 2016).

It is fundamental to discuss the bio-molecular role of H_2S on the cardiovascular system (Figure 1.11). In the cardiovascular domain, it has been found that H_2S may induce its effect by interacting with other signalling pathway components and biological mediators, although a specific thiol receptor has not been detected yet (Elsey et al. 2010). It has been further demonstrated that H_2S properties vary from other gasotransmitters, typically these variations are centred in its dissociation ability. Under normal conditions and in aqueous solutions at pH 7.4, the pKa for H_2S is 6.77, leading to over three quarters of H_2S to dissociate to HS-anion. It has long been believed that the H_2S pool contains H_2S, HS^- and S^{2-}. In order for H_2S to play different biological effects, it has been regarded that protein persulfidation, or protein S-sulfhydration is a significant molecular mechanisms. More precisely, it is important to notice that modification of cysteine residues from the –SH to –SSH group can represent the main differences. The properties of –SH and –SHH groups are profoundly different. In comparison to their corresponding thiols (-SH) and hydrosulphide (-SSH), they have generally distinguished nucleophilic ability, for stronger chemical reactivity (Zhang et al. 2017). It has been found that H_2S gas would be involved as a mediator of physiological changes in many different cell types and tissues. Examples of these key roles including angiogenesis, vascular permeability, inflammatory cell function, vasoconstriction/vasodilation, and smooth muscle growth, as well as apoptosis (Szabo and Papapetropoulos 2011). There is also evidence that H2S is produced in significant amounts

in intrauterine tissues (Patel et al. 2009) and that it can inhibit endometrial contractility in the rat uterus (Sidhu et al. 2001). Evidently, the action of H_2S on the cardiovascular system can be essentially characterized based on its source. In other words, endogenous H_2S can regulate vascular NO production while exogenous H_2S is believed to affect vascular smooth muscle upon activation of K_{ATP} channels (including vasodilator or vasoconstrictor effect), depending on its concentration (Gheibi et al. 2018). However, alteration of H_2S production has been found to be a causative agent in influencing vascular tone associated with disease states (Elsey et al. 2010).

Figure 1.11: Mechanisms of cardioprotection mediated by H_2S.
The figure demonstrates the various exerted effects of endogenous regulation or exogenous administration of H_2S in physiological concentrations, on infarct-sparing, anti-apoptotic, anti-oxidant, anti-inflammatory and pro-angiogenic benefits in the heart secondary to different stressors. These involve myocardial infarction, pressure/volume overload, doxorubicin or alcohol toxicity and cardiac arrest/resuscitation. It can be also recognized from the figure that attenuation of myocardial scar formation and oxidative stress may result from the combined preservative effects of H_2S, promoted angiogenesis and adequate remodelling resulting in protection of function. Image taken from (Salloum 2015).

In terms of angiogenesis, a pro-angiogenic role of H_2S was reported involving activation of the prosurvival PI3-kinase/Akt and ERK1/2 MAPK signalling pathways (Cai et al. 2007). In vitro studies have shown the ability of H_2S to enhance pro-angiogenic factors including proliferation, adhesion, migration, and tube-like formation was recognized using endothelial culture assay. Furthermore, increasing Akt phosphorylation was observed upon exposure to NaHS, whereas the presence of the PI3-kinase inhibitor LY294002 inhibits Akt phosphorylation. Otherwise, it has been demonstrated in vivo using a mouse matrigel plug assay that exogenous H_2S at a concentration of 10-50 μmol kg^{-1} day^{-1} can exert a pro-angiogenic role. Several studies and reports have also shown the inhibitory role of H_2S on smooth muscle proliferation by influencing apoptosis (Elsey et al. 2010). The capacity of H_2S to produce sulphide can attribute to cardio-protective effects by reducing neutrophil adhesion. As reactive nitrogen species exert a pathological effect on myocardial infarction, it has been proven that ischaemia reperfusion is a beneficial outcome of the ability of H_2S to react and neutralize these cytotoxic reactive species (e.g., peroxynitrite). In case of coronary disease, sulphide replacement therapy has been considered, where the level of endogenous sulphide is dropped down in such cardiac disease (Pan et al. 2017). Finally, while it is not clear how does hydrogen sulphide (H2S) work in the body and reacts with other molecules such as NO and CO, there is an explicit evidence that there are some interaction between these gaseous signalling molecules at the molecular level that warrants further studies (Carson et al. 2004).

1.8. Hypothesis and Aims

A number of observations have led to the hypothesis of this thesis. First, ERK5 is well known to play a major role in the vascular system, mainly as a vascular protective factor. Second, H_2S has been shown to protect vascular cells from damage and death. Third, H_2S has been shown to enhance vascular and placental health and to be deficient in vascular disease, including pre-eclampsia. Finally, H_2S and ERK5 signalling have both been shown to activate the AKT pro-survival pathway. The hypothesis of this work is therefore that:

H_2S activates ERK5 to mediate vascular protection.

The specific aims of the thesis are to:

- *Establish a reproducible assay to measure ERK5 activation in endothelial cells and tissues;*
- *determine whether ERK5 can be activated by H_2S in mammalian cell lines*
- *investigate ERK5 activation by H_2S in endothelial cells*
- *develop assays to investigate ERK5 C-terminal phosphorylation*

Chapter 2

Materials and Methods

2. Materials and Methods

2.1. Cell culture

2.1.1. Culture of established cell lines

Established cell lines were purchased from LGC Standards (ATCC lines), where the cells' identity was validated. The cells were stored under liquid nitrogen and used for a maximum of 25 passages. HEK293 and HeLa cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Penicillin/Streptomycin antibiotic (containing 5000 Units /ml of penicillin and 5000 µl/ml of streptomycin) in T-75 cell culture flasks at 37°C in a humidified air incubator containing 5% (v/v) CO_2. The cells were propagated 3-4 times a week by washing with PBS, incubation with trypsin (Lonza, catalogue number BE02-007E; containing 0.5 g/L trypsin and 0.2 g/L Versene EDTA) at 37°C, thereafter, rinsing the surface of culture flask with DMEM media.

2.1.2. Culture of primary endothelial cells (HUVEC)

Primary Human Umbilical Vein Endothelial Cells (HUVEC; purchased from PromoCell, catalogue number C-12203) were maintained in Endothelial Cell Basal Medium 2 (purchased from PromoCell, Cat. Number: C-22211) containing 1x FCS-10, 1x hEGF-2.5, 1x HC-100, 1x VEGF-0.25, 1x hb FGF-5, 1x R3 IGF-1, 1x AA-500, and 1x Hep-11.25 (Supplement Pack Endothelial Cell GM2, purchased from PromoCell, catalogue number: C-37320, C-30224, C-31063, C-30321, C-31700, C-31750, and C-31650, respectively) in T-75 cell culture flasks at 37ºC in a humidified air incubator containing 5% (v/v) CO_2. Cells were propagated by washing with Hepes BSS (PromoCell, catalogue number C-40010), incubation with 0.04% Trypsin/ 0.03% EDTA (PromoCell, catalogue number C-41010), and 0.05% Trypsin Inhibitor, 0.1% BSA (PromoCell, catalogue number C-41110) at 25ºC (room temperature) and dislodging them from the culture flask by rinsing and pipetting the surface several times.

2.1.3. Storage and recovery of cells

The cells were harvested as described above and spun at 200 x g for 5 minutes. The cells were then resuspended in freezing media [10 % DMSO (Sigma, catalogue number D8418); 20 % FCS; 70% DMEM] at a concentration of 5 × 10^6 cells / ml. For HUVECs, the freezing media (Cryo-SFM) were purchased from PromoCell (catalogue number C-29910). 1.5 ml of cell suspension was then transferred into a freezing vial (ThermoFisher, 5000-0020) and placed into a special freezing container (Thermo Scientific, catalogue number 5100-0001), to allow a controlled cooling down period. The closed freezing container was placed at –80°C over night, after which the cells would be transferred to liquid nitrogen storage.

For thawing, the frozen vial was removed from the liquid nitrogen storage and quickly thawed in a 37°C water bath. As soon as the cells are thawed, the cell suspension would be carefully be transferred to a centrifuge tube and 10 ml of warm culture medium addded. The cell suspension would be gently spun at 200 x g for 5 minutes. After removing the medium without disturbing the pellet, the cells were suspended in 10 ml of fresh culture medium, placed in a small T 25 flask and incubated in a cell culture incubator with 5 % CO_2.

2.1.4. Experimental treatments and lysis of cells

The cells were plated on 60 mm dishes after being trypsinised as illustrated above and counted in a haematocytometer. The density of cells was 1.2 x 10^5 cells/well for 6-well dishes and 5.5 x10^5 cells/dish for 60 mm dishes. In order to starve the cells, the media was replaced with Endothelial Cell Basal Medium 2 without the supplemental materials and reagents (HUVEC) or Dulbecco's Modified Eagle's Medium (DMEM) media containing no FBS. After 3 hours, the cells were treated with either epidermal growth factor (EGF; Sigma, catalogue number E9644), Sorbitol (Sigma), GYY4137 (Sigma SML0100-10MG), hydrogen sulfide (NaHS; Sigma 161527-100G) or the appropriate vehicle PBS or DMSO (Sigma, D2650, maximum concentration 0.2%) at the concentration indicated in the results. Upon following the times shown in the experimental section, PBS and 1x RIPA

(Radioimmunoprecipitation) buffer solution (Merck Millipore, catalogue number 20-188; 10x solution containing 0.5 M Tris-HCL, pH 7.4, 1.5 M NaCl, 2.5 % deoxycholic acid, 10% NP-40, 10 mM EDTA), were used to wash and lyse the cells, respectively. Thereafter, the cells were centrifuged at 4°C, 15000 x g for 5 minutes to remove cell debris and stored at -20 °C until needed.

2.2. SDS PAGE and Western Blot analysis

2.2.1. Determination of protein concentration (BCA Assay)

In order to quantify the total protein in a sample, the BCA (Bicinchoninic Acid) protein assay would be used and implemented according to the instructions of the manufacturer. Briefly, samples were mixed with the appropriate amount of the working solution and incubated for 30 minutes at 37°C. The absorbance reading would then be taken at 562 nm. BSA solutions of known concentrations were used to construct a standard curve.

2.2.2. Standard SDS gel electrophoresis

Discontinuous polyacrylamide sodium dodecyl sulphate (SDS) gel was used to separate the proteins of interest based on their molecular weight. Thus, the resolving gels (10 ml per cassette) consists of 2.5 ml 1.5 M Tris/HCL pH 8.8, 2.33 ml (for 7% gels), 3.33 ml (for 10% gels) or 4.21 ml (for 14% gels) acrylamide (30%/0.8%), 100 µl 10% SDS, 90 µl APS (10%), and 10 µl TEMED. The stacking gel (5 ml) contains 0.67 ml acrylamide (30%/ 0.8%), 1.25 ml 0.5 M Tris/HCL pH 6.8, 50 µl 10% SDS, 45 µl APS (10%), and 5 µl TEMED. Then, the gels were loaded with 30 µl of sample containing the lysed protein of interest, 1x NuPAGE LDS Sample Buffer (from 4x concentrated stock; ThermoFisher, #1225644) and 3 µl DTT (0.5 M). 7.5 µl of protein standard (NEB, P7712S) was also used. The gels were run for 1.5 -2.0 hours at 125 volts.

2.2.3. SDS-PAGE gel electrophoresis (large gels)

30 ml of resolving gel 7% were prepared consisting of 7.5 ml 1.5 M Tris/HCL pH 8.8, 6.9 ml of acrylamide (30%/0.8%), 300 µl 10% SDS, 270 µl APS (10%), and 30µl TEMED. The stacking gel (15 ml) contains 2.01 ml acrylamide (30%/0.8%), 3.75 ml 0.5 M Tris/HCL pH 6.8, 150 µl 10% SDS, 135 µl APS (10%), and 15 µl TEMED. 15 µl of a protein standard (NEB, P7712S) was used for the large gel preparations. The gel was run at constant current (10 mA) overnight.

2.2.4. SDS-PAGE gel electrophoresis using PhosTag reagent

Phos-tag reagent is a highly selective phosphate binding molecule in combination with dinuclear metal complexes [e.g., mangane (II) and zinc (II)-Phos-tag]. Based on the number and location of the phosphate group the method allows the differentiation of different isoforms of phosphoproteins. In the presence of divalent metal ions and once the phosphorylated proteins bind to the reagent, their movement on SDS-PAGE gels would slow down. Therefore, 7% resolving gels were prepared by following the standard protocol of SDS gel electrophoresis (see Section 2.2.2). Phos-Tag reagent with a final concentration of 5 mM would be added. The gels were run at 9 mA overnight (at least 17 hours). The gels then were soaked for 10 minutes in transfer buffer containing 5 mmol/L EDTA (with gentle agitation) after electrophoresis in order to remove the metal ions.

2.2.5. Western blotting

Transferring the proteins to nitrocellulose membrane after the gel electrophoresis was performed using the XCell II blot module from Invitrogen. The transfer buffer contained 25 mM Tris, 192 mM Glycine, and 20% methanol. Further, Tris buffered saline TBS (137 mM Sodium Chloride, 20 mM Tris) containing 0.1% Tween-20 (TBST) was used to wash the membrane, which was then incubated for 1 hour at room temperature (or overnight at 4°C) in blocking solution, consisting of 3% BSA in TBST. Three times of washing the membrane

were applied with TBST, and thereafter membranes were incubated in primary antibody as illustrated in Table 2.1. Then, an appropriate secondary antibody conjugated with horseradish peroxidase (Table 2.2), was incubated with the membranes after being washed three times with TBST. ECL reagent, together with a gel documentation system (Genesys) was used to visualize the Western blots.

Table 2.1: Primary antibodies used for western blot.

Antibody	Name of Company	Catalogue Number	Working Concentration	Incubation time	Incubation Temperature
anti-ERK5	*Cell Signalling*	*3372*	*1:1000*	*Overnight*	*4ºC*
anti-ERK5	*Cell Signalling*	*12950*	*1:1000*	*Overnight*	*4ºC*
anti-ERK5 (coupled to agarose beads)	*Santa Cruz*	*sc-1284AC*	*10 µl / ml lysate*	*4 hours*	*4ºC*
anti-phospho ERK5 (T218/Y220)	*Sigma*	*07-507*	*1:1000*	*Overnight*	*4ºC*
anti-ERK1/2 (total)	*Cell Signalling*	*9102*	*1:1000*	*Overnight*	*4ºC*
anti-phospho ERK1/2	*Cell Signalling*	*4377*	*1:1000*	*Overnight*	*4ºC*
anti-(total)MEK5	*Abcam*	*ab171760*	*0.5 µg/ml*	*Overnight*	*4ºC*
anti-phospho MEK5	*Invitrogen*	*RB2154011*	*1:500*	*Overnight*	*4ºC*
anti-phospho S731 ERK5	*Abnova*	*PAB15919*	*1 µg/ml*	*Overnight*	*4ºC*
anti-phospho S496 ERK5	*Abnova*	*PAB15918*	*1µg/ml*	*Overnight*	*4ºC*
anti-Histone H3	*Cell Signalling*	*4499*	*1:1000*	*1 hour*	*25°C*
anti-MEK1/2	*Cell Signalling*	*8727*	*1:1000*	*1 hour*	*25°C*

Table 2.2: Secondary antibodies used for western blot.

Antibody	Name of Company	Catalogue Number	Working Concentration	Incubation time	Incubation Temperature
Goat anti Rabbit	*Cell signalling*	*7074s*	*1:1000*	*1 hour*	*Room Temp.*
Goat anti Rabbit	*Cell signalling*	*A0545*	*1:2000*	*1 hour*	*Room Temp.*
Goat anti Mouse	*Invitrogen*	*31439*	*1:15000*	*1 hour*	*Room Temp.*

2.2.6. Quantification of results

GeneTools software from Syngene was used to analyse the final image obtained from the blotted membrane. Upon selecting the manual quantification track and drawing a rectangle of consistent size around each band, analyzing the quantity of each band of interest was performed by calculating the pixel density of each rectangle, which is relative to the protein quantity. ERK1/2 activation was quantified by dividing the afforded value of activated ERK1/2 (pERK1/2) to the obtained value for the total ERK1/2 protein (tERK1/2). Activation of ERK5 was quantified by dividing the value of activated ERK5 (pERK5, the sum of the 2 upper bands) to the value of the total ERK5 (the sum of all three bands).

2.2.7. Statistical Analyses

The statistical functions of the Excel software were used to analyze statistically the generated values. Unpaired t tests were conducted to analyze the difference between specific time points compared to the control where 2 groups were compared. Thereafter, differences between more than 2 groups would be calculated using a one-way ANOVA test followed by a Tukey's HSD post-hoc test for pairwise comparisons of all means using SPSS Statistic 24.

2.3. Adenovirus

2.3.1. Amplification of Adeno-Virus

After plating the HEK293 cells at 4x10^6 cells per T-75 flask, the required amount of crude adenovirus containing the gene of interest (e.g., 50-150 µl) was added to the cells in 10 ml of DMEM media. Typically, the infected cells in the plate would grow efficiently at 37°C in a humidified air incubator containing 5% (v/v) CO_2 within 2 to 3 days. In order to harvest the cells, both infected cells and cell supernatant containing adenovirus particles were collected to a sterile, capped tube. First, both the harvested cell and cell supernatant containing adenovirus particles were centrifuged for 15 minutes at 3500 x g. Then, the cell supernatant containing the adenovirus particles were pipetted to new, sterile capped tube and store at 4°C (-80°C for longer periods of time). The cell pellet was resuspended in 2 ml of the supernatant and the cells lysed by freezing and thawing (-80°C for 30 minutes followed by 37°C for 15 minutes), three times. The samples were centrifuged for 15 minutes at 2000 x g and the supernatant containing the adenovirus particles were collected and added to the previous supernatant. 1 µl of nuclease enzyme (Benzonase) was added to each 1 ml culture volume. The sample mixture was then incubated at 37°C in a water bath for 30 minutes to digest cellular nucleic acid. The digested cell supernatant was transferred to a Vivaclear Maxi spin column (Yellow capped spin column), and centrifuged for 5 minutes at 500 x g. After adding 10 fold of loading buffer to the collected flow-through, the 10x of washing buffer was prepared in deionized water. In order to equilibrate the AdenoPACK Maxi spin column, 5 ml of diluted washing buffer were added and centrifuged for 5 minutes at 500 x g. Then, the sample volume were loaded into the Adeno PACK Maxi insert and spun for 5 minutes at 500 x g. In order to elute the adenovirus particles containing the sequence of interest, it was necessary before to wash properly the spin column two times with adding 18 ml of washing buffer and spun for 5 minutes at 500 x g. The adenovirus-containing eluent was then collected in a fresh, sterile capped tube after being incubated for 10 minutes, and then centrifuged for 5 minutes at 500 x g in AdenoPACK Maxi membrane. For the titration

purpose, it is significant to concentrate the adenovirus particles containing the sequence of interest using Vivaspin 20 centrifugal concentrator and physiological buffer containing 20 mM Tris/HCL, 25 mM NaCl, 2.5% Glycerol (w/v), pH 8.0 at 22°C.

2.3.2. Adenovirus titration

Adenoviruses were titrated using the Quick Titer ELISA Kit from Cell Biolabs. HEK293 cells were harvested and resuspended in culture medium at 5 x 10^5 cells/ml, 100 µl of the cells were seeded in each well of a 96-well plate and incubated at 37°C, 5% CO_2 for one hour. Then, Ad-β gal positive control and the viral sample were prepared for a serial dilution in culture medium. Dropwise, 50 µl of diluted viral sample were added to each well of 96-well assay plate and performed in duplicate. The infected cells were incubated at 37°C, 5% CO_2 for 2 days. Afterward, the immunoassay was conducted by fixing the infected HEK293 cells in 100 µl of cold methanol, and incubated for 20 minutes at -20°C. After washing the cells three times with 1x PBS, the 293 cells were blocked for one hour with 200 µl of 1% BSA in PBS at room temperature. To probe the fixed cells, 100 µl of diluted 1x anti-Hexon antibody solution were added to each well and incubated for one hour at room temperature. After washing the fixed cells three times with 1x PBS, 100 µl of diluted 1x secondary antibody solution were added to each well and incubated for other one hour at room temperature. Then, the fixed cells were again washed with 1x PBS, five times. In order to add 100 µl of TMB substrate to the fixed cells, TMB substrate was warmed to room temperature and incubated with the fixed cells at room temperature for 5-10 minutes. Before measuring the optical density of the reaction at 450 nm on a 96-well plate reader, the reaction was stopped by adding 100 µl of stop solution to each well. The final calculation of the viral titration was obtained based on the standard curve from Ad-β gal positive control titration.

2.3.3. Transduction with Adenovirus

HeLa or HUVEC cells were plated in p60 (60 mm) dishes after being counted using a haematocytometer at a density of 6x10^5 cells/dish on p-100 (100 mm) dishes or at a density

of 1.5x10^6 cells/dish on p60 (60 mm) dishes. The appropriate full cell culture media was employed in this assay. The cells were left in the incubator at 37°C in a humidified air incubator containing 5% (v/v) CO_2, for two days. Meanwhile, the entire plated cells were transduced with Adeno-Virus ERK5 (wildtype human ERK5) and /or Adeno-Virus MEK5^{DD} (constitutively active human MEK5 α with the following mutations: S313D, T317D) (Raviv et al. 2004). The Adenoviruses were a generous gift from Dr Michael Cross (University of Liverpool). The media were changed after 6 hours with new media containing 5% Penicillin/Streptomycin (containing 5000 Units of penicillin and 5000 µg/ ml of streptomycin), but without fetal bovine serum or growth factors prior to running each experiment within at least 24 hours.

2.4. Immunoprecipitation assay

2.4.1. Cell lysis

The cells were washed with PBS and lysed in 1x RIPA (Radioimmunoprecipitation) buffer solution (Merck Millipore, catalogue number 20-188; 10x solution containing 0.5 M Tris-HCl, pH 7.4, 1.5 M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10 mM EDTA) using 350 µl per 60 mm dish or 700 µl per 100 mm dish. Lysed cells were centrifuged at 4°C, 15000 x g for 5 minutes to remove cell debris.

2.4.2. Preparation of antibody-protein A/G agarose beads

Protein A/G agarose beads (Thermo Scientific, catalogue number: 20423; 20 µl for each sample) were carefully washed and centrifuged three times with 1x RIPA (Radioimmunoprecipitation) buffer solution (1ml and 1 minute at 300 x g). 20µl of protein A/G agarose beads were transferred to each microtube containing the relevant cell lysates. 2µl of Anti-FLAG antibody (Purchased from Sigma, catalogue number F7425-2MG, Concentration ~ 8 mg/ml), were added to the mixture and placed on a rotating shaker at 4°C for 3-4 hours. Afterward, the immunoprecipitates were carefully washed with 1 ml of 1x

RIPA buffer and centrifuged at 500 x g for 30 seconds, five times to remove any non-specific proteins. Finally, 2x NuPAGE LDS Sample Buffer (ThermoFisher, #1225644) was added to the washed immunoprecipitations as well as 50 mM DTT. The mixture was heated for 5 minutes at 95°C.

2.5. Coomassie gel staining

A Coomassie Based Staining Solution (Expedeon, catalogue number: ISB1L) was used to identify the migrated bands of interest. The Coomassie Based Staining Solution was carefully added to the gel and incubated for at least one hour on the orbital shaker, at room temperature. Destaining was performed with several changes of distilled water.

2.6. Immunofluorescent Staining

*After 24 hours of transfection, cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes at room temperature and then permeablized with 0.5% Triton X-100 in PBS for 5 minutes. After being blocked with 3% bovine serum albumin in PBS, the cells were then incubated with indicated primary antibodies (*Table 2.3*) for 1 hour at room temperature and then incubated with a goat anti-rabbit secondary antibody (*Table 2.4*) for 1 hour at room temperature. Cells were finally mounted in Prolong Diamond Antifade Mountant with DAPI (Life technology, reference : p36962, lot: 1796977) and examined using a confocal microscope (Bio-Rad).*

Table 2.3: Primary antibodies used for immunostaining.

Antibody	Name of Company	Catalogue Number	Concentration	Incubation time	Temperature
total ERK5	*Cell Signalling*	*12950*	*1:50*	*1 hour*	*Room Temp. 25ºC*

Table 2.4: Secondary antibody used for immunostaining.

Antibody	Name of Company	Catalogue Number	Concentration	Incubation time	Temperature
Goat anti Rabbit (Alexa488-conjugated)	*Abcam*	*A11034*	*1:500*	*1 hour*	*In the dark Room Temp. 25ºC*

2.7. Antibody affinity purification

2.7.1. Coupling procedure

To prepare the affinity column, the peptide was immobilised onto the resin containing functional aldehyde groups reacting with the primary amines of the peptide. All centrifugation steps were performed at 1,000 x g for one minute unless otherwise indicated. First, the AminoLink Plus Spin Column (Thermo Scientific; catalogue number 20475) and reagents were equilibrated at room temperature. Afterward, the column was placed in a collection tube and centrifuged at 1,000 x g for two minutes. The column was washed twice in 300 µl of Coupling Buffer (PBS: 0.1 M Na phosphate, 0.15 M NaCl; pH 7.2). 140 µl (100 µg) of peptide (unphosphorylated ERK5) dissolved in coupling buffer was added directly into the resin. In the following step and in a fume hood, 2 µl of Sodium Cyanoborohydride Solution were added to the reaction slurry and the reaction was mixed. The column was incubated at room temperature for 4 hours with mixing the reaction every hour. The column was placed in a collection tube and centrifuged to remove the liquid. 300 µl of Coupling Buffer was added and centrifuged to wash the resin. The previous step was repeated two more times.

For blocking the remaining active binding sites, 300 µl of Quenching Buffer (1 M Tris HCl, 0.05% NaN3, pH 7.4) was added onto the resin and mixed. The column was then placed in a collection tube to be centrifuged and flow-through would be discarded. After repeating the previous steps, 200 µl of Quenching Buffer was added onto the resin and mixed. In a fume hood, 4 µl of the Sodium Cyanoborohydride Solution were added to the reaction slurry.

Afterward, the column was mixed thoroughly. It was important to mix the reaction every 15 minutes upon its incubation at room temperature for 30 minutes. The column was then placed in a collection tube to be centrifuged and the flow-through was discarded. For washing and storing purposes, sodium azide would be added to the Coupling Buffer at a final concentration of 0.02 % for long-term storage (i.e. > 2 weeks).

2.7.2. Affinity purification

The resin containing the immobilized antibody was equilibrated to room temperature. 300 µl of Wash Solution (1 M Tris-HCL, 0.05 % NaN_3, pH 7.4) were added and the reaction was mixed. Then the column was placed in a collection tube to be centrifuged, and flow-through were discarded, with taking into consideration to repeat this step two more times. 500 µl of the antibody (phosphor-ERK5; FT2; rabbit 541) to be purified were directly added to the resin. Then, the cap was replaced, and the reaction was mixed. Gentle agitation or end-over-end mixing was applied to the incubated reaction overnight at 4°C). The column was placed in a collection tube to be centrifuged, the flow-through would be saved and represented the phosphor-specific antibody. Reducing the possible nonspecific interaction would be attained by preparing 1 ml of 0.5 M NaCl containing a final concentration of 0.05 % Tween-20. The following few steps would be repeated two times to achieve a highest efficiency. First, adding 300 µl of the solution and 10 times of gentle inversions were applied to the column. Thereafter, the tube was centrifuged and the flow-through was discarded. After repeating the previous steps two more times, 300 µl of Coupling Buffer were added, the cap was replaced, and the tube was inverted 10 times. Centrifugation and discard flow-through would be applied after placing column in a collection tube. Then, 300 µl of Coupling Buffer were added in increments of 100 µl, on the inside surface of the column to wash down the resin. The plug was then removed, and the column was placed in a collection tube to be centrifuged. The flow-through was discarded.

For elution purpose, 100 µl of elution buffer pH2.8 was added along sides of the column onto the resin. The cap and column were replaced and mixed. It is significant to incubate the column at room temperature for 10 minutes. Then, the column was placed in a collection tube to be centrifuged. In order to neutralize the low pH of the eluted fraction, 5 µl of 1 M Tris, pH 9.0 was added. The resulting fraction represents the total anti-ERK5 antibody (not phosphor-specific). Regeneration of the resin has been executed after elution by washing three times with 300 µl of Coupling Buffer containing 0.02 % sodium azide, then storing the column at 4°C.

2.8. DNA sequencing

DNA sequencing was performed by Source Biosciences. For each sequence, 10 µg of plasmid DNA in a total volume of 10 µl was sent to the company in a sealed Eppendorf tube. The specified primers were provided by the company and the results were received by email.

2.9. Mass spectrometry

2.9.1. Protein digestion

Proteins were digested either at the University of Warwick as part of the mass spectrometry service or locally at Bradford University. The gel slice identified by Coomassie staining was diced into small cubes and destained in 50% EtOH / 50 mM ammonium bicarbonate ($(NH_4)HCO_3$) at room temperature. The samples were then dehydrated in 100% EtOH for 5 minutes at room temperature and reduced and alkylated with 50 mM TCEP / 200 mM CAA (Tris-(2-carboxyethyl) phosphine hydrochloride / 2-chloroacetamide) for 5 minutes at 70°C. The gel slices were then washed twice in 50% EtOH / 50 mM $(NH_4)HCO_3$ for 20 minutes at room temperature. Samples were dehydrated in 100% EtOH and the dried gel slices rehydrated with 2.5 ng/µl of the required enzyme (trypsin or pepsin). 50% EtOH / 50 mM $(NH_4)HCO_3$ was added to fully cover the gels and digestion was carried out over night at

37°C. Peptides were extracted twice in 25% acetonitrile / 5% formic acid using a sonication bath for 10 minutes each. Peptides were concentrated in a Speed-Vac dryer and resuspended in 2% acetonitrile / 0.1% tri-fluoroacetic acid (TFA) to a final volume of 50 µl.

2.9.2. Mass spectroscopy

Mass spectroscopy was carried out at the University of Warwick by the WPH Proteomics RTP, Gibbet Hill Road, University of Warwick, UK as a paid service. An aliquot containing 20 µl (from a total of 50 µl) of extracted peptides was analysed by means of nanoLC-ESI-MS/MS using the Ultimate 3000/Orbitrap Fusion instrumentation (Thermo Scientific) using a 60 minute LC separation on a 50 cm column.

2.9.3. Database searching

Tandem mass spectra were analyzed using MaxQuant (Max Planck Institute of Biochemistry, Martinsried, Germany; version 1.6.2.6). MaxQuant was set up to search the Homo_sapiens_uniprot-proteome_AUP000005640_March2017 database assuming the relevant digestion enzyme. MaxQuant was searched with a fragment ion mass tolerance of 0.50 Da. Carbamidomethyl of cysteine was specified in MaxQuant as a fixed modification. Oxidation of methionine, acetyl of the N-terminus and phospho of serine, threonine and tyrosine were specified in MaxQuant as variable modifications.

2.9.4. Criteria for protein identification

Scaffold (version Scaffold_4.10.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95% probability by the Peptide Prophet algorithm (Keller et al. 2002) with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet

algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters.

Chapter 3

Detection of ERK5 activation

3. Detection of ERK5 activation

3.1. Introduction

The elementary goal of this chapter is to establish and characterize an assay to reliably measure ERK5 activation within human cells. ERK5 is activated by dual phosphorylation of T218/Y220 in the kinase activation loop, analogous to other MAPKs. Following this phosphorylation event, ERK5 is further phosphorylated at several sites in the C-terminus, which leads to its translocation to the nucleus (see Figure 1.8). However, there are several challenges associated with detecting ERK5 activation experimentally. While the antibodies recognising the activating phosphorylation sites of ERK1/2 (pT202/pY204) have been very reliable and instrumental in deciphering many functional aspects of ERK1/2 signalling (Lai and Pelech 2016), the corresponding antibodies for ERK5 (pT218/pY220) have given less reproducible results. Several batches of the commercial antibody demonstrate high background in Western Blots and often are not sensitive enough to reproducibly detect phosphorylated (activated) ERK5, particularly the endogenous protein. In addition, the antibodies cannot be used in assays like immunofluorescence as they cross-react with activated ERK1/2 (Honda et al. 2015). While it is principally possible to measure ERK5 activity directly using in vitro kinase assays (Müller and Morrison 2002), this technique is complex and not easily adaptable to larger sample sizes. It was therefore necessary to establish a technique to measure ERK5 activity in a reproducible fashion that is suitable for larger sample sizes. This included testing commercially available antibodies specifically recognising residues in the phosphorylated C-terminus of ERK5, antibodies recognising activated MEK5, as well as indirect measurements such as gel mobility shifts.

3.2. Results

3.2.1. Establishment of required controls

3.2.1.1. ERK5 is well detected in the HEK293 cell line

In order to test different methods for detecting ERK5 activation, it was necessary to assure that ERK5 was detectable in the cell line used. For these initial experiments HEK293 cells were used. This cell line is well described in the literature and is well known to be easy to maintain and to express a large variety of proteins. ERK5 expression was therefore tested in HEK293 cells by western blotting. HEK293 cells were grown on 60 mm dishes (p60) and serum-starved overnight. On the following day, the cells were treated with 50 ng/ml or 100 ng/ml EGF (or PBS as a negative control) for 15 minutes to stimulate ERK5 activity. Cells were lysed in RIPA buffer and the proteins were separated on a 10% polyacrylamide gel and analysed by Western blotting using an antibody directed against ERK5 that recognises ERK5 regardless of its activation status (Figure 3.1). The results demonstrate that ERK5 can be efficiently detected by the antibody and that the levels of ERK5 does not change when the cells are stimulated with EGF. It should be noted that ERK5 is known to be detected as a band of approximately 105 kDa on SDS-PAGE, despite its theoretical MW of approximately 88 kDa.

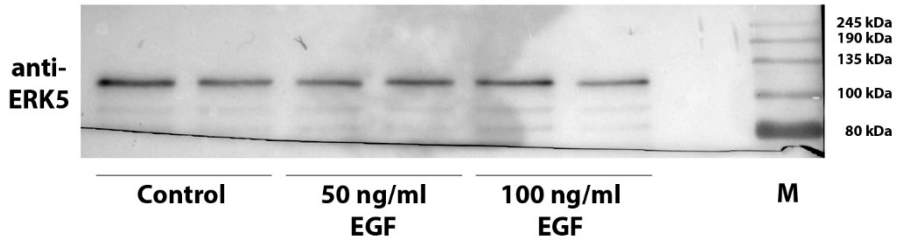


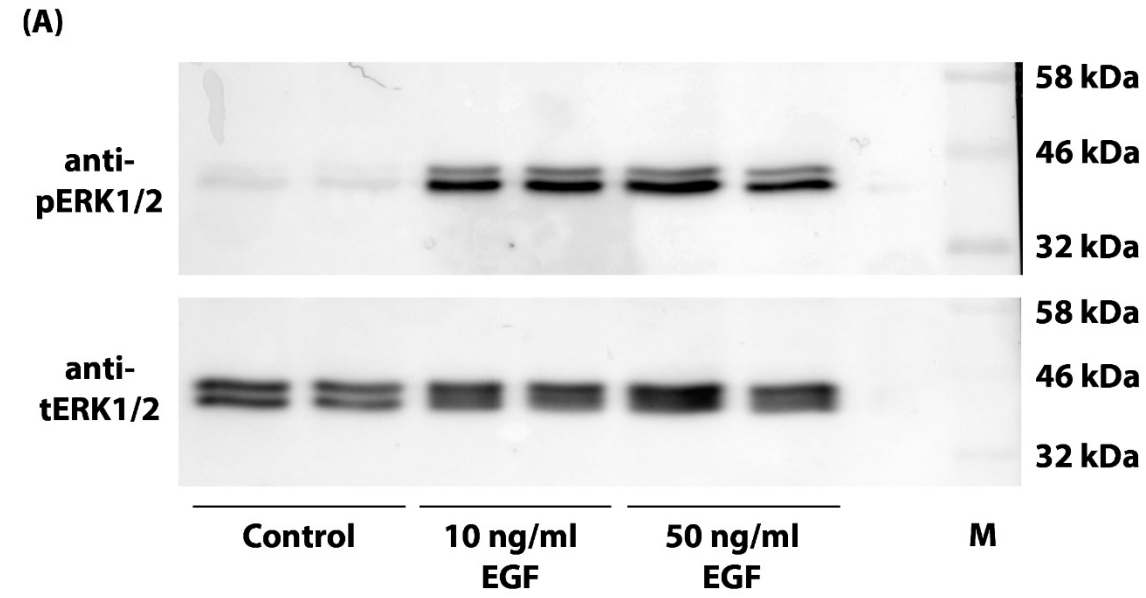
Figure 3.1: ERK5 expression in HEK293 cells.

HEK293 cells were grown in 60mm dishes, serum-starved and stimulated with EGF at the indicated concentrations (PBS was used as negative control). After 15 minutes, the cells were washed with PBS and lysed by adding 250 µl of RIPA buffer. Cells were separated on a 10% gel before Western blotting with antibodies directed against ERK5 (Cell Signalling Technology). n=1

3.2.1.2. ERK1/2 MAPK is efficiently activated by EGF in HEK293 cells

EGF has been shown to activate both MAPK pathways: ERK1/2 (Gao et al. 2005) and ERK5 (Drew et al. 2012). Before further investigating methods to detect ERK5 activation, EGF stimulation of MAPK signalling in HEK293 cells was validated under the conditions used. The cells were therefore grown in p60 dishes as above and serum-starved overnight. The cells were then treated with 10 ng/ml or 50 ng/ml EGF (or PBS as a negative control) for 15 minutes to stimulate ERK1/2 (and ERK5) activity and lysed in RIPA buffer, as above. Cell lysates were separated on a 10% polyacrylamide gel and analysed by western blotting using antibodies directed against the activating sites of ERK1/2 (pERK1/2). This experiment demonstrates strong and equal activation of ERK1/2 by EGF at both concentrations of EGF (Figure 3.2).

It is well described in the literature that ERK1/2 activation by EGF is normally only temporary, demonstrating fast activation with full activation in less than 5 minutes and returning to baseline levels after 30-60 minutes (Shu et al. 2019). To verify that this is also the case in the planned experiments, HEK293 cells were grown in p60 dishes as described, serum-starved overnight and then stimulated with 50 ng/ml EGF (or PBS as a negative control) for 5, 15, 30 and 60 minutes before lysis in RIPA buffer. Proteins were separated on a 10% polyacrylamide gel (SDS-PAGE) and Western blot analysis was performed with antibodies directed against activated ERK1/2 (pERK1/2) and total ERK1/2. The result clearly demonstrates that ERK1/2 MAPK is strongly activated by EGF at 5 and 15 minutes, with its activity returning to baseline levels at around 30 minutes (Figure 3.3). These results demonstrate that EGF effectively activates MAPK signalling and that the dynamics of the cellular response are intact. Similarly treated cells were therefore used to analyse ERK5 activation in the HEK293 cell line.



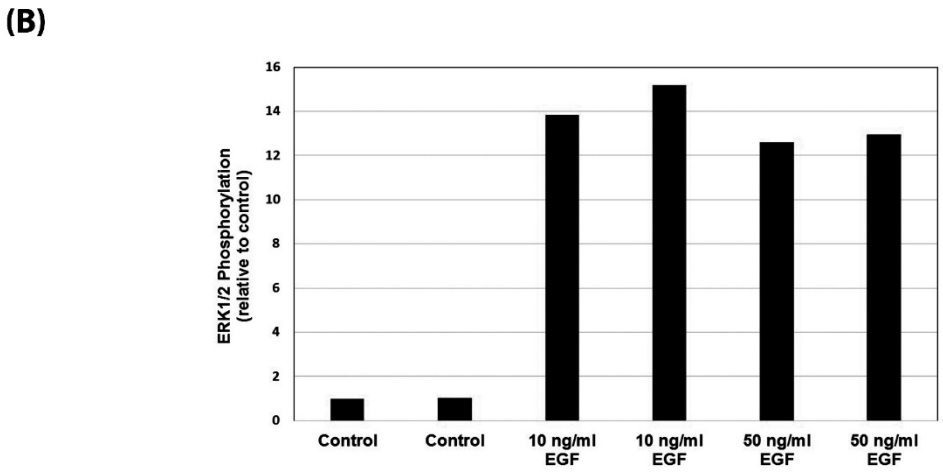


Figure 3.2: ERK1/2 MAPK is efficiently activated by EGF at 15 minutes.
HEK293 cells were grown in 6-well plates. The ERK1/2 MAPK pathway was stimulated by EGF for 15 minutes at the concentrations shown. PBS was used as negative control. The cells were washed with PBS and lysed by adding 250 µl of RIPA buffer. A. Proteins were separated on a 10% gel before western blotting with antibodies directed against activated ERK1/2 (pERK1/2; top panel) and total ERK1/2 (tERK1/2; bottom panel). B. ERK1/2 MAPK activation was quantified by using GeneTools software from Syngene. The quantification of ERK1/2 activation was accomplished by dividing the value obtained for the activated ERK1/2 (pERK1/2) by the value for the total ERK1/2 protein (tERK1/2). Expected molecular weights: ERK1=44kDa; ERK2=42kDa. Representative image of n=2

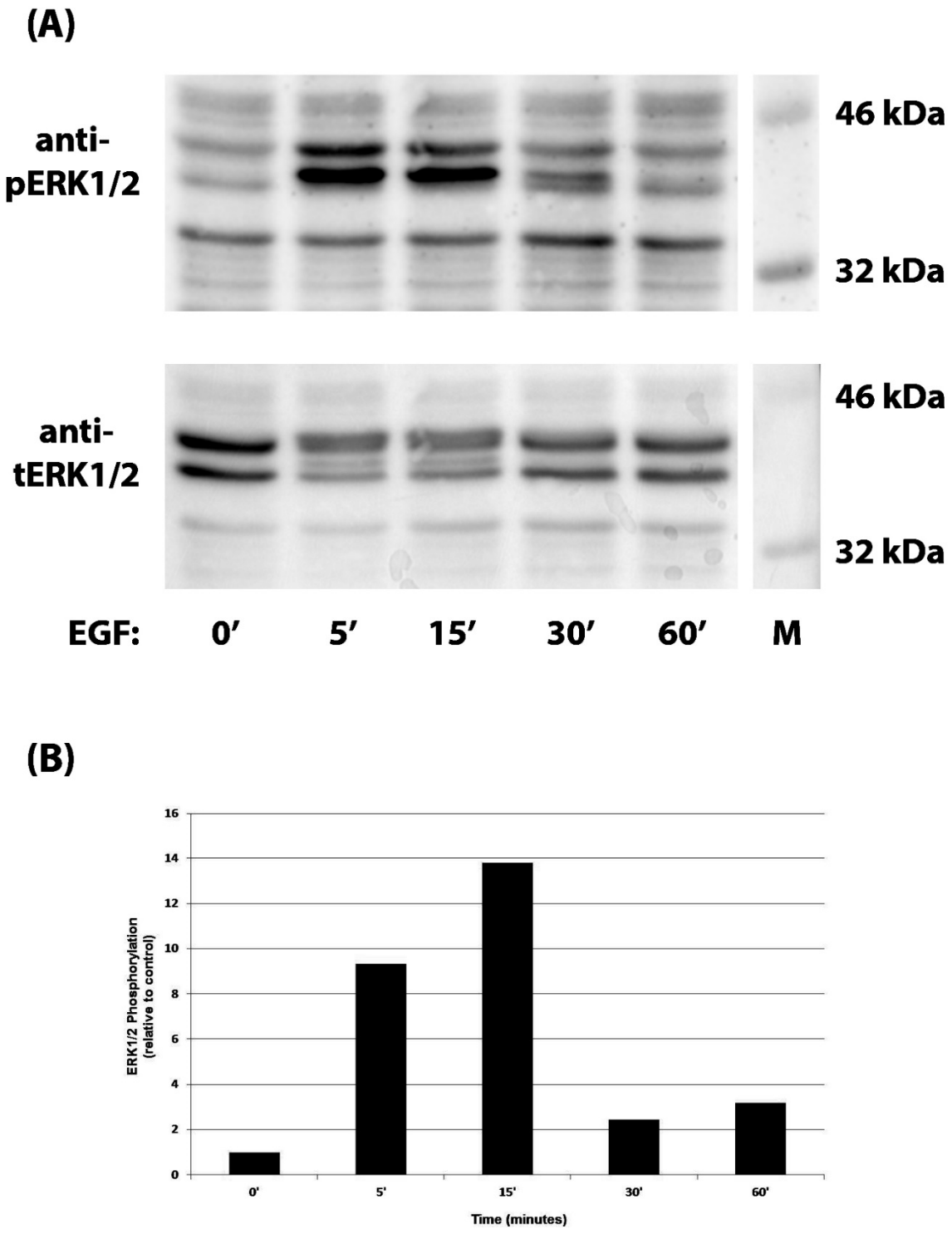


Figure 3.3: ERK1/2 MAPK is efficiently activated by EGF in a temporal fashion.

HEK293 cells were grown in 6-well plates. The ERK1/2 MAPK pathway was stimulated with 50 ng/ml EGF for the indicated times. PBS was used as negative control. The cells were washed with PBS and lysed by adding 250 µl of RIPA buffer. A. Proteins were separated by 10% SDS-PAGE before western blotting with antibodies directed against activated ERK1/2 (pERK1/2; top panel) and total ERK1/2 (tERK1/2; bottom panel) as indicated. B. ERK1/2 MAPK activation was quantified by using GeneTools software from Syngene. The quantification of ERK1/2 activation was accomplished by dividing the value obtained for the activated ERK1/2 (pERK1/2) by the value for the total ERK1/2 protein (tERK1/2). Representative image of n=2

3.2.2. Phosphor-specific antibodies to detect ERK5 activation

3.2.2.1. Antibodies directed against the phosphorylated activation sites of ERK5 (T218/Y220)

*For ERK1/2, antibodies recognising the phosphorylation sites located in the activation loop of the kinase (pT202/pY204) have been extremely valuable in characterising the pathway in numerous conditions (see also Figure 3.2 and Figure 3.3). Similar to ERK1/2, ERK5 is activated by MEK5 through phosphorylation at threonine 218 and tyrosine 220 in the activation loop of the ERK5 kinase domain. An equivalent antibody exists for ERK5 (Sigma 07-507), recognising the phosphorylated residues T218 and Y220. This antibody was therefore tested to determine whether it could be used to monitor endogenous ERK5 activation in human cells. HEK293 cells were grown in 60 mm dishes as described above. To activate ERK5, the cells were treated with EGF (50 or 100 ng/ml) as indicated in Figure 3.2. Cell lysates were prepared and separated by SDS-PAGE and Western blots performed with the anti-pERK5$^{T218/Y220}$ antibody. The experiments demonstrate that the antibody does not efficiently recognise the endogenous activated ERK5 (*Figure 3.4*), even though ERK5 is present in the cells (see Figure 3.1). Repeated experiments confirmed that this antibody (or at least the current batch purchased) does not reliably recognise endogenous activated ERK5 and could thus not be used in further studies.*

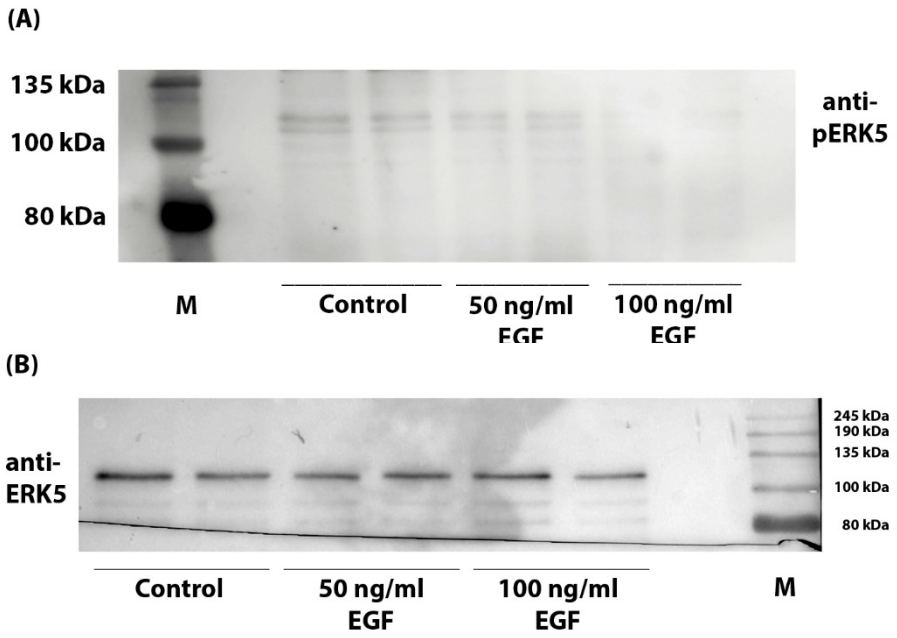


Figure 3.4: Using phosphoERK5 (pT218/pY220) antibodies to detect ERK5 activation.

(A) HEK293 cells were grown in 6-well plates. The ERK5 MAPK pathway was stimulated by EGF for 15 minutes at the concentrations shown. PBS was used as negative control. The cells were washed with PBS and lysed by adding 250 µl of RIPA buffer. Proteins were separated on a 7% gel before western blotting with antibodies directed against activated phosphoERK5 (pT218/pY220). Representative image of n=2

(B) The same proteins as in (A) were separated on a 10% gel before Western blotting with antibodies directed against ERK5 (Cell Signalling Technology) to demonstrate the presence of ERK5 in the samples (reproduced from Figure 3.1). n=1

3.2.2.2. Antibodies directed against the C-terminal phosphorylation sites

It has been shown that multiple residues in the C-terminal domain of ERK5 undergo autophosphorylation upon ERK5 activation by MEK5, leading to the transactivation of transcription (Morimoto et al. 2007, Honda et al. 2015). Commercial antibodies directed against the phosphoS496 (pS496) and phosphoS731/phosphoT733 (pS731/T733) autophosphorylation sites were employed to test whether these phosphorylation events could be used to measure ERK5 activation. HeLa cells were grown on 60 mm dishes, serum-starved overnight, and stimulated with EGF or Sorbitol as indicated. The cells were lysed with RIPA buffer and subjected to SDS-PAGE and western blotting. Unfortunately, these antibodies did not recognise ERK5 and cannot be used to monitor endogenous ERK5 activity in lysates of human cells (Figure 3.5).

3.2.2.3. Antibodies detecting MEK5 phosphorylation

MEK5 directly activates ERK5 by phosphorylation of the activation loop of the kinase domain. Furthermore, MEK5 is the only known activator of ERK5. It would therefore be feasible to use MEK5 activity as an indirect marker for ERK5 pathway activation. Cell lysates prepared from HeLa cells serum-starved and stimulated with EGF or Sorbitol as above were therefore tested by western blotting using phosphoMEK5 (pMEK5) antibodies. However, Figure 3.6 shows that these antibodies also do not reliably detect endogenous, activated MEK5 (and thus ERK5) activation under these conditions.

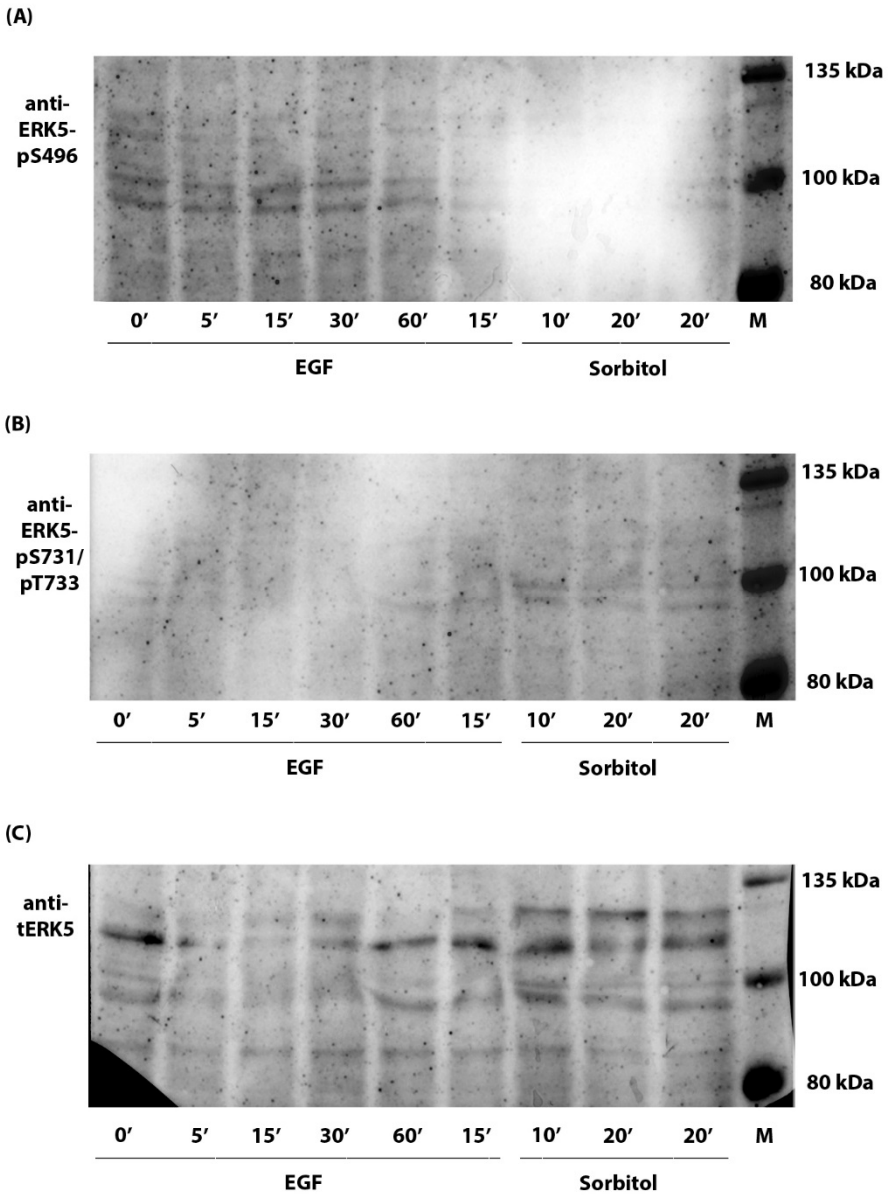


Figure 3.5: Using antibodies directed against the C-terminal phosphorylation sites to detect ERK5 activation.

HeLa cells were grown in 60 mm cell culture dishes. The ERK5 MAPK pathway was stimulated with 50 ng/ml EGF for the times shown. Cells were also stimulated with 400 mM (lanes 8-9) or 300 mM (lane 10) Sorbitol. PBS was used as negative control. The cells were washed with PBS and lysed by adding 350 µl of RIPA buffer. Proteins were separated on three 7% gels before western blotting with antibodies directed against ERK5 phosphorylated at pS496 (A) or pS731/pT733 (B) or total ERK5 (C). Representative images of n=2

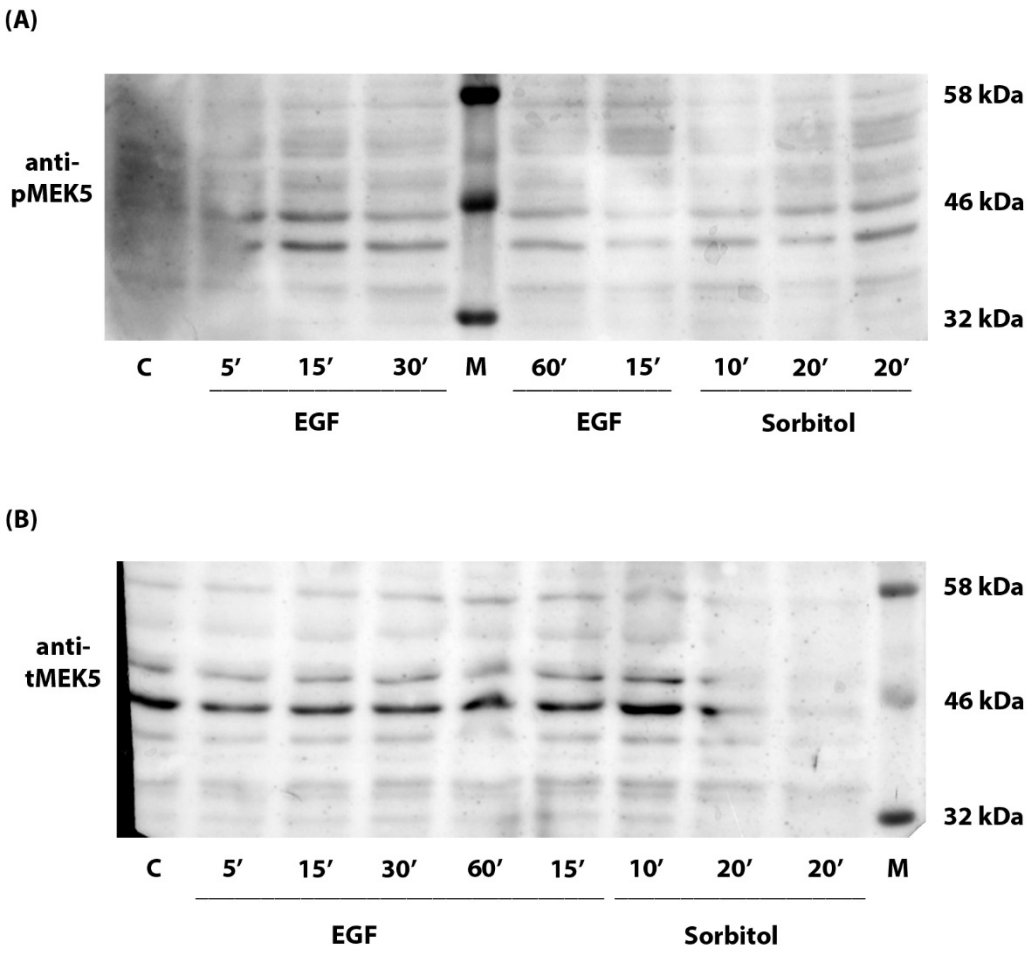


Figure 3.6: Using antibodies directed against the MEK5 activation sites (pMEK5) to detect ERK5 activation.

HeLa cells were grown in 60 mm cell culture dishes. The ERK5 MAPK pathway was stimulated with 50 ng/ml EGF for the times shown. Cells were also stimulated with 400 mM (lanes 8-9) or 300 mM (lane 10) Sorbitol. PBS was used as negative control. The cells were washed with PBS and lysed by adding 350 µl of RIPA buffer. Proteins were separated on a two 10% gels before western blotting with antibodies directed against (A) phosphorylated (activated) MEK5 (pMEK5) or (B) total MEK5 (tMEK5). Representative image of n=2

3.2.3. Detecting ERK5 activation using electrophoretic mobility shifts

3.2.3.1. ERK5 activity measurement using a gel retardation assay

Unfortunately, the commercial antibodies directed against a variety of activation sites in ERK5 and MEK5 were not able to reliably detect any activation of endogenous ERK5 in the performed assays. ERK5 has a unique feature in the elongated C-terminal domain, which becomes hyperphosphorylated upon ERK5 activation (Drew et al. 2012), in parallel with the activation loop of the kinase domain. It has therefore been shown that under certain conditions, ERK5 may undergo a visible electrophoretic mobility shift upon activation (due to the presence of a large number of phosphorylation sites) (Duff et al. 1995, Abe et al. 1996, Abe et al. 1997). Therefore, it was tested whether this assay could be used to measure endogenous ERK5 activation.

HeLa cells were grown in 60 mm dishes as above and stimulated with EGF (50 ng/ml) for 5, 15, 30 and 60 minutes and Sorbitol (10, 20 minutes). A 7% polyacrylamide gel was used to separate the proteins after lysing the cells with RIPA buffer. To achieve a better separation of the potential ERK5 bands, the gel was run until the 80 kDa protein marker reached the bottom of the gel. The gel was blotted and the membrane probed with an ERK5 antibody facilitating detection of the activation status of the ERK5 protein. From Figure 3.7, it can be seen that separation of ERK5 protein into species with diverse electrophoretic mobility was clearly visible. The lower band represents the non-phosphorylated (inactive) form of ERK5, while the upper bands represent the phosphorylated one (activated). This assay system was clearly capable of detecting the activation of endogenous ERK5 and was utilized in the successive experiments to monitor ERK5 activation in cell lines and primary cells.

It is worthwhile to emphasize that ERK5 MAPK is activated by EGF between 15 and 30 minutes of exposure (Figure 3.7); in sequence activity of ERK5 back down to baseline.

Unlike ERK1/2, EGF activates ERK5 slightly later (compare to Figure 3.3), in which it shows the role and importance of diversified dynamics on activation of protein kinase in spite of using the same growth factor. In addition, osmotic stress plays a key role in activation of ERK5 as indicated by the retardation of the ERK5 band upon treating the cells with 400 mM Sorbitol for 20 minutes.

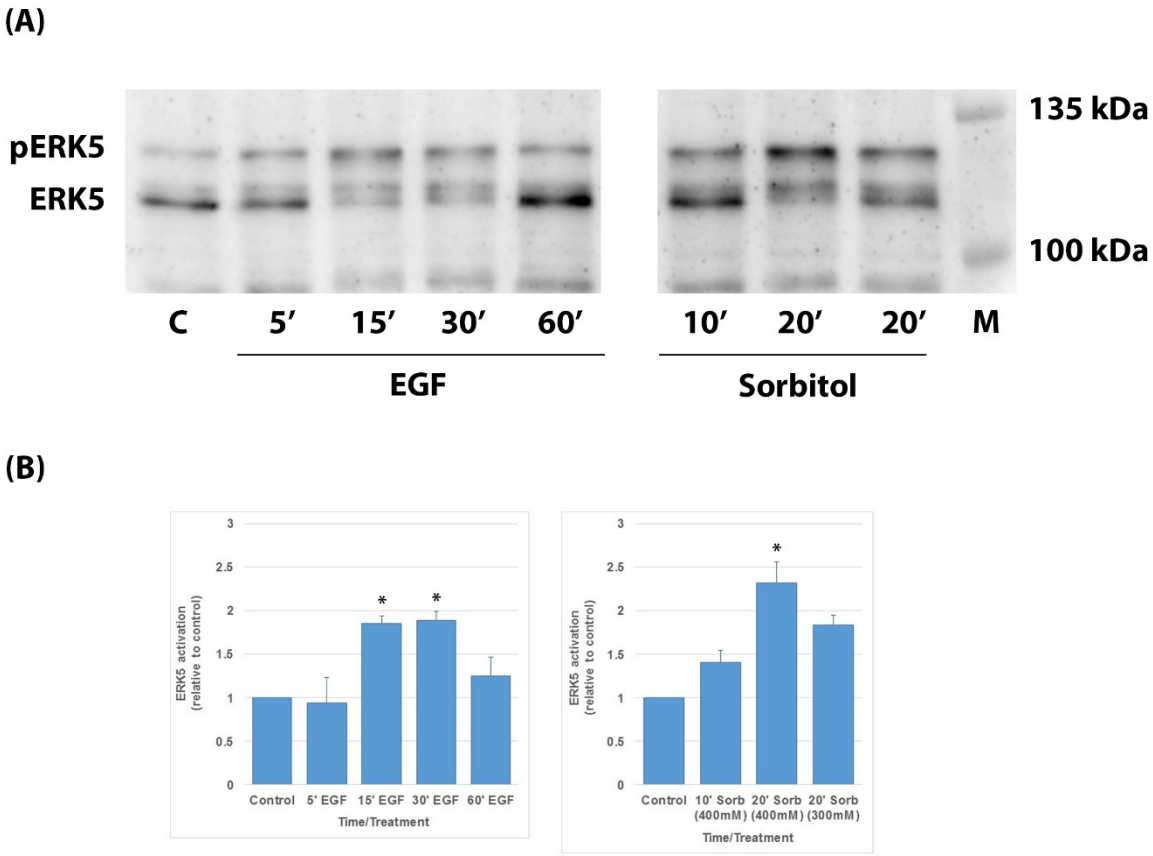


Figure 3.7: Activation of ERK5 by growth factors and stress can be identified as a shift in electrophoretic mobility.

HeLa cells were grown in 60 mm dishes. The ERK5 MAPK pathway was stimulated with 50 ng/ml EGF for the times shown. Cells were also stimulated with 400 mM (lanes 8-9) or 300 mM (lane 10) Sorbitol. PBS was used as negative control. The cells were washed with PBS and lysed by adding 250 µl of RIPA buffer. Proteins were separated on a 7% gel before western blotting with antibodies against ERK5 (Cell Signalling Technologies) (A). ERK5 MAPK activation was quantified using GeneTools software from Syngene (B). Determination of ERK5 activity was accomplished by dividing the value for the upper band (phosphoERK5) by the sum of all bands (total ERK5). The resulting value for the control band was arbitrarily set to 1 and the other results normalised accordingly. Statistical analysis (n=3) was performed using a one-way ANOVA test, demonstrating significant differences between the samples (P<0.001). Subsequently, the Tukey HSD posthoc test was performed, confirming significant differences for treatment with EGF for 15 and 30 minutes (P=0.048, P=0.046) as well as for Sorbitol treatment (20 minutes at 400 mM; P=0.002).

3.2.3.2. A gel shift assay to analyse ERK5 activation using the phosTag gel system

The PhosTag reagent has been shown to further decrease the mobility of phosphorylated proteins, thus improving the resolution between phosphorylated and non-phosphorylated isoforms (Kinoshita et al. 2006). In order to test whether the PhosTag reagent can improve the detection of ERK5 activation, HeLa cells were serum-starved overnight and stimulated with EGF to activate MAPK signalling. Cells were lysed in RIPA buffer and subjected to SDS-PAGE. ERK5 activation was investigated by western blotting using an antibody detecting the ERK5 protein. The results show two bands with different electrophoretic mobility (Figure 3.8). However, it is not clear what isoforms these bands represent. In addition, the PhosTag reagent did not improve on the good detection of ERK5 activation achieved by a simple gel retardation assay (Figure 3.7). Therefore, the simple gel retardation assay described above was used in future experiments.

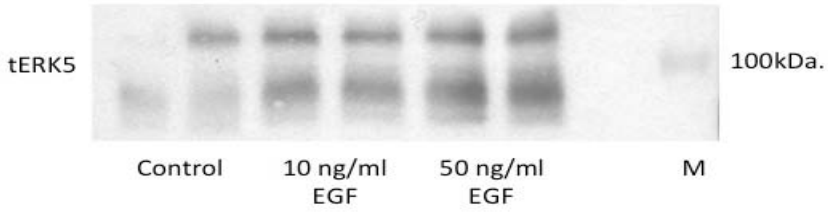


Figure 3.8: Establishing a gel shift assay to analyze ERK5 activation (phosTag gel).
HeLa cells were grown in 6-well plates. The ERK5 MAPK pathway was stimulated with 10 and 50 ng/ml EGF for 15 minutes; PBS was used as negative control. The cells were washed with PBS and lysed by adding 250 µl of RIPA buffer. Proteins were separated on a 7% gel containing 5 mM PhosTag reagent before western blotting with antibodies directed against ERK5. Representative image of n=2

3.3. Discussion

A reliable model to detect ERK5 activation and dynamics is central to research into the role of H_2S in the vascular system. As it has been articulated above, ERK5 is part of the MAPK family, which can be activated by certain growth factors (e.g., EGF, FGF), cytokines (e.g., IL-6 and IL-8), and cellular and mechanical stress (Nithianandarajah-Jones et al. 2012). Detecting the phosphorylation of the TEY kinase activation motif is theoretically sufficient to gauge the kinase activity of ERK5. However, based on the findings shown here, the current methods and available antibodies are all unable to reliably detect phosphorylation of those residues for the endogenous protein (not overexpressed).

In contrast to ERK5, the dynamics of ERK1/2 activation by EGF (50 ng/ml) in HEK293 cells stimulated for different amount of time ranging from 0, 5, 15, 30, 60 minutes were clearly detected. The highest peak of activation was at 15 minutes, which ERK1/2 activity steadily declining after that time point. It is obvious and clear from the results (Figure 3.2 and Figure 3.3) that endogenous ERK1/2 activity can be measured in HEK293 cells using antibodies directed against the TEY activation motif of ERK1/2. On the other hand, while endogenous ERK5 levels were sufficiently high to be detected in HEK293 cells (Figure 3.1), it was not possible to determine the kinase activity using the equivalent phosphor-specific antibodies for ERK5. In fact, commercial antibodies directed against phosphorylation sites located in either the activation loop of the kinase (pERK5$^{T218/Y220}$) or the C-terminal domain (phosphoS496 and phosphoS731/phosphoT733) were not effective in the experiments to measure ERK5 activation, even though ERK5 was present and EGF efficiently stimulated the cells (shown by ERK1/2 activation). An important aspect that also needs to be taken into account is the dynamics of ERK5 signalling. While the phosphorylation of the activation loop precedes C-terminal (auto-) phosphorylation, the exact timing may vary between experiments. However, the results in Figure 3.7 *demonstrate significant C-terminal phosphorylation after 15 minutes of EGF treatment, demonstrating that ERK5 has been*

significantly activated at that time. As all experiments in this Chapter have been performed using either 15 minutes stimulation or a time course covering this time window, it is unlikely that the failure of the antibodies to detect ERK5 activation is due to missing the correct time at which ERK5 has been activated.

MEK5 is a major activator of ERK5 by phosphorylation of the activation loop of the kinase domain. As MEK5 catalyses dual phosphorylation at the TEY domain within the activation loop of ERK5 (Lee et al. 1995, Zhou et al. 1995, Kato et al. 1997), an antibody against activated MEK5 to detect ERK5 pathway activation was also used. However, this antibody was not suitable for reliable detection of ERK5 pathway activity.

Ultimately, the establishment of a gel shift assay to detect and measure ERK5 activity was efficient and reproducible. While phosphorylation of ERK5 at both the activation loop of the kinase domain (by MEK5) and the C-terminus (by autophosphorylation) can lead to some conformational changes in the electrophoretic mobility of the ERK5 protein, it was therefore feasible to monitor ERK5 activity on the basis of a clearly visible electrophoretic mobility shift. (Figure 3.7). ERK5 activation has been previously linked to a gel mobility shift and this method has been determined to provide a good estimation of ERK5 activation (Nithianandarajah-Jones and Cross 2015). This assay will therefore be used in the remainder of this thesis to monitor ERK5 pathway activation.

Overall, it has increasingly become clear that monitoring ERK5 activation is a challenging process. While phosphor-specific antibodies have been used to detect the phosphorylation of the TEY motif in a number of publications, most of these have been performed using overexpressed ERK5 (Obara et al. 2009). In addition, we and others have also observed a lack of consistency with the antibody, resulting in some batches to be better than others. While it is sometimes possible to use the phosphor-TEY motif antibody for endogeneous protein (Roberts et al. 2010), many batches of this antibody only detect overexpressed, but

not endogenous ERK5 (see figure 3.4). The finding that ERK5 MAPK activity could be potentially measured by electrophoretic mobility shift is therefore highly valuable, not only for the present research but also for other studies, where ERK5 MAPK pathway is involved in the development of disease. Examples of such diseases include: aggressiveness of cancer (e.g., breast cancer, prostate cancer), inflammation, endothelial dysfunction in vascular system (Stecca and Rovida 2019).

Chapter 4

ERK5 is activated by H_2S, a gaseous signalling molecule

4. ERK5 is activated by H_2S, a gaseous signalling molecule

4.1. Introduction

Altogether, the gaseous signalling molecules hydrogen sulfide (H_2S), nitric oxide (NO) and carbon monoxide (CO) – also called gasotransmitters or gasomediators - are composed of a group of biologically active gases. They can permeate the cellular membrane freely, and their movements in the intracellular or intercellular domains do not depend on associated membrane receptors or other active transportation mechanisms. The generation of these gaseous signalling transmitters in mammalian cells can be attained endogenously, in parallel with particular substrates and enzymes, and their production is controlled to accomplish signalling messenger functions. It is worth emphasizing that they all have particular functions at the physiological levels. In addition to their involvement in signalling transduction, they can fulfil their functions by targeting specific cellular and molecular processes, such as the regulation of BK_{ca} channels or enhancing cGMP production (Wang 2014).

Gasotransmitters can be defined in many different ways, but in general they are best known as endogenously generated gaseous signalling molecule (Wang 2002). The first recognized gaseous signalling molecule was NO. Stimulation of NO production in a variety of mammalian cell types can be attained, in conjunction with using L-arginine as a substrate, by the role of different isoforms of NO synthase. Recently a better understanding of mammalian NO metabolism has occurred. It has illustrated that a molecule of gas can exert extraordinary signalling to maintain cellular functions. In addition to its plentiful biological implications, NO is well defined as an endothelium-derived relaxing factor (EDRF) (Erdmann et al. 2013).

Carbon monoxide (CO) is another gaseous signalling molecule produced from heme metabolism (Ferreira et al. 2011). Production of CO can be acquired by the role of two major enzymes: heme oxygenase-1 (HO-1) and HO-2. Intriguingly, HO-1 is identified as an inducible enzyme whereas HO-2 is present in a constitutive form. In terms of functions, there are several similarities between CO and NO (Wu and Wang 2005); for example, CO enhances relaxation of vascular tissue, decreases blood pressure, and preserves the heart from ischemia/re-perfusion damage (Wu and Wang 2005).

After NO and CO, hydrogen sulfide (H_2S) has been discovered to be the third gasotransmitter. It has been shown that hydrogen sulfide (known as rotten-egg gas) can be generated in mammalian cells in specific amounts by the enzymatic roles of cystathionine γ-lyase, cystathionine β-synthase, and 3-mercaptopyruvate sulfurtransferase (Kimura 2014). L-cysteine and homocysteine or their derivatives play a crucial role as substrates of these H_2S-generating enzymes (Wang et al. 2013, Wang 2014). The indispensable role of H_2S would be envisaged in regulating many physiological functions at the levels of body organs and systems (Yang et al. 2008). Further, H_2S plays an essential role as a signalling molecule in neurotransmission and neuromodulation, and is included in cognition processes such as learning and memory (Wang 2014). Similar to the EDRF role for NO, H_2S has been shown to be an endothelium-derived hyper-polarizing factor (EDHF) (Mustafa et al. 2011, Tang et al. 2013).

From around 1984 research on NO biology and physiology attained a vigorous interest from the scientists around the globe, followed by research toward CO and H_2S, with the majority of publications being related to the toxicological and environmental concepts. (Wang 2014). Interestingly, the term 'gasotransmitter' for gaseous signalling molecules was conceived in 2002 (Wang 2002), however, this term was not generally accepted. Nevertheless, a field of research has been started investigating novel cellular signalling mechanisms. Following the acknowledgement of an important role of gaseous signalling molecules in cellular

regulation, the physiological significance of the gaseous signalling molecules to the human body has started to be revealed in more detail (Wang 2014).

*Synthesis and generation of H_2S in mammalian tissues can be attained via endogenous biological enzymes and non-enzymatic pathways, such as the reduction of thiols (*Figure 4.1*). For example, hydrogen sulpfide can be generated in vascular endothelial cells and vascular smooth muscle through a chemical reaction catalyzed mainly by cystathionine γ-lyase (CSE) (Li et al. 2011). Although much is known about H_2S production and synthesis in the body, less is understood about its metabolism. To date, much effort has been centred*

Figure 4.1: Synthesis and degradation of hydrogen sulfide.
Synthesis of H_2S inside the cells can be attained by the action of cystathionine γ-lyase (CSE), cystathionine β-synthetase (CBS), or 3-mercaptopyruvate sulfurtransferase (3-MST) from cysteine. Degradation and metabolism of hydrogen sulfide could be acquired by the action of cysteine aminotransferase (CAT), cysteine dioxygenase (CDO), and cysteine lyase (CL) from sulfur-containing amino acids. Altogether, GSSG, GSH, and R-SH represent oxidized glutathione, reduced glutathione, and a thiol-bearing intermediate, respectively. In addition, TSMT is thiol S-methyltransferase and SO represents sulfide oxidase. Figure taken from (Li et al. 2011).

toward H_2S and its role on individual body systems (Li et al. 2011). The cardiovascular system and inflammation have enticed the majority of the attention in this field (Elsey et al. 2010). Surprisingly, it has been reported in entire animals that administration of NaHS or other H_2S donors such as ADT-OH, S-diclofenac and GYY4137 can reduce the chronic and acute symptoms of raised blood pressure, and high concentration of administrated NaHS is able to dilate blood vessels in vitro (Li et al. 2011). NaHS has also been shown to reduce heart rate and left ventricular developed pressure, while increasing coronary flow in the isolated perfused rat heart (Hussain et al. 2011).

Discussing the main roles of hydrogen sulfide on cell signalling is important, as it is being reclassified from being an environmental poison/toxin to a mammalian intracellular signalling molecule (Li et al. 2011). Exposing cells to H_2S displays some attenuation in ATP biosynthesis and oxidative phosphorylation. The K_{ATP} channel was the first reported target molecule of H_2S in spite of the cytochrome c oxidase exception (Cooper and Brown 2008). Sulfhydration of the K_{ATP} channel is the only known mechanism by which the K_{ATP} channel is activated (Kiss et al. 2008). Recently, it has been reported that relaxation of the smooth muscle by NaHS administration occurs not only via opening of K_{ATP} channel, but also upon the effect of activation of myosin-light-chain phosphatase (Gastreich-Seelig et al. 2020).

It has been reported that hydrogen sulfide can have a biological role on the regulation of kinases (Li et al. 2011). However, the effect of H_2S on mitogen-activated protein kinase (MAPK) signalling varies in different cell types and H_2S concentrations (Du et al. 2004). For example, administration of NaHS to cultured rat aortic vascular smooth muscle cells inhibits endothelin-provoked rises in p38 MAPK activity. However, it enhances p38 MAPK activity in cultured human umbilical vein endothelial cells (Li et al. 2011). Phosphorylation of ERK1/2 can be attained in pigs exposed to coronary artery occlusion and reperfusion due to the action of H_2S (Osipov et al. 2010). It has been reported that H_2S induces a sustained activation/ phosphorylation of ERK1/2 in HEK-293 cells as a consequence of overexpression of CSE (Yang et al. 2004). H_2S can also activate Akt in endothelial cells

(Elsey et al. 2010). In addition, it has been found that a brief administration of NaHS in isolated hearts predisposed to ischemic pre-conditioning would result in activation of both cardiac Akt and protein kinase C (PKC); which improves the mechanical performance of the heart and alleviates injury (Yong et al. 2008). Finally, the H_2S-induced attenuation of cell apoptosis can inhibit caspase-3 activity, a downstream target of the H_2S-ERK interaction (Yang et al. 2004). Interestingly, it has been shown in a recent study using human aortic smooth muscle cells (HASMCs) that ERK1/2 activation is also critical for H_2S-induced apoptosis (Yang et al. 2004). Significantly, upon involvement of the ERK1/2 inhibitor U0126 during H_2S-induced apoptosis, it has been shown that ERK1/2 plays a key role in the process of apoptosis in many cell types.

The importance of these gases underlines their critical role in cell signalling pathways; including the ERK5 MAPK pathway (Papapetropoulos et al. 2009). H_2S exposure and ERK5 activation share several downstream effects, such as AKT activation and cell and tissue protection from death. However, activation of the ERK5 MAPK pathway by H_2S has not been shown. Thus, finding the link between ERK5 MAPK activation and H_2S is an important question. Identification of the missing links in the ERK5 MAPK activation by H_2S will lead to a refined understanding of the cellular and molecular mechanisms underlying H_2S–induced cellular proliferation and angiogenesis. Novel mechanisms for many diseases linked to H_2S–related abnormal cellular proliferation and angiogenesis may be revealed.

4.2. Results

4.2.1. Activation of ERK5 by NaHS

The main goal of the work conducted in this chapter is to test whether the ERK5 MAPK signalling pathway could be activated by the gaseous molecule H_2S. To test this hypothesis, HeLa cells were treated with hydrogen sulfide. First, the cells were treated with 400 mM NaHS for the indicated times (Figure 4.2). NaHS is known to release H_2S relatively quickly when exposed to an aqueous environment. Both EGF (50 ng/ml) and Sorbitol (400 mM) were employed in the experiment as a positive control for ERK5 activation. Figure 4.2 demonstrates that EGF and Sorbitol treatment effectively activated ERK5. NaHS treatment resulted in some ERK5 stimulation, albeit to a lower degree. The differences were not statistically significant and ERK5 activation also did not change during the time course, up to 2 hours of treatment.

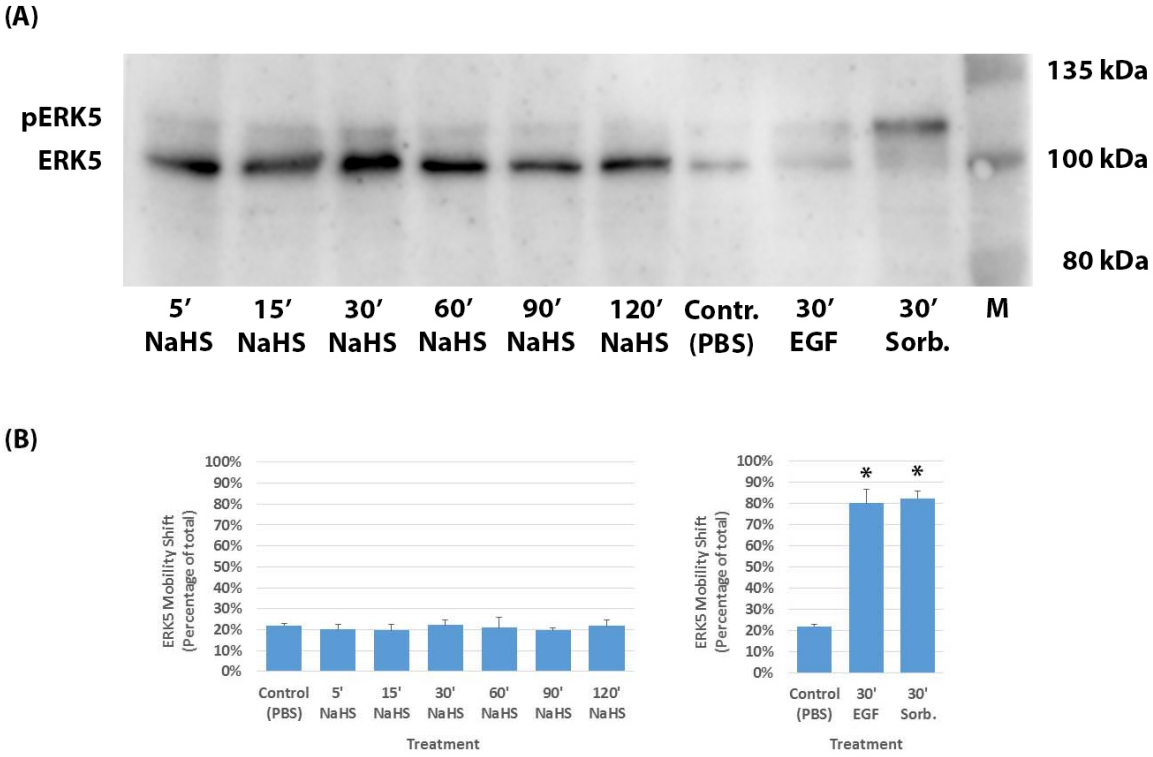


Figure 4.2: Activation of ERK5 bAy NaHS, a H_2S donor molecule.

HeLa cells were grown in 60 mm dishes. The ERK5 MAPK pathway was stimulated with 50 ng/ml EGF, 400 mM Sorbitol, 400 mM NaHS for the indicated times. PBS was used as a negative control. The cells were washed with PBS and lysed by adding 350 µl of RIPA buffer. Proteins were separated on a 7% gel before western blotting with antibodies directed against ERK5. Quantification of ERK5 activity was accomplished by dividing the value for the upper band (phosphoERK5) by the sum of all bands (total ERK5) to yield a value for relative (percentage) activation. Statistical analysis (n=3) was performed using a one-way ANOVA test, which showed significant differences between samples. A subsequent Tukey HSD test demonstrated that both positive controls were significantly different from the control (P<0.001). However, NaHS treatment did not result in any statistically relevant differences.

4.2.2. Activation of ERK5 by GYY4137

In addition to NaHS, GYY4137 was also used to stimulate the HeLa cells. GYY4137 is a water-soluble chemical that is well known to slowly release H_2S over a longer time, up to several hours. It can be observed from Figure 4.3 that the ERK5 MAPK signalling pathway shows a trend of activation by GYY4137, as demonstrated by the ERK5 bands showing reduced electrophoretic mobility, although the difference was not statistically significant. The potential activation of ERK5 is most clearly seen at the 30 minutes time point (p=0.056). Thereafter, the degree of activation is sequentially dropped down starting from 60 minutes of treatment.

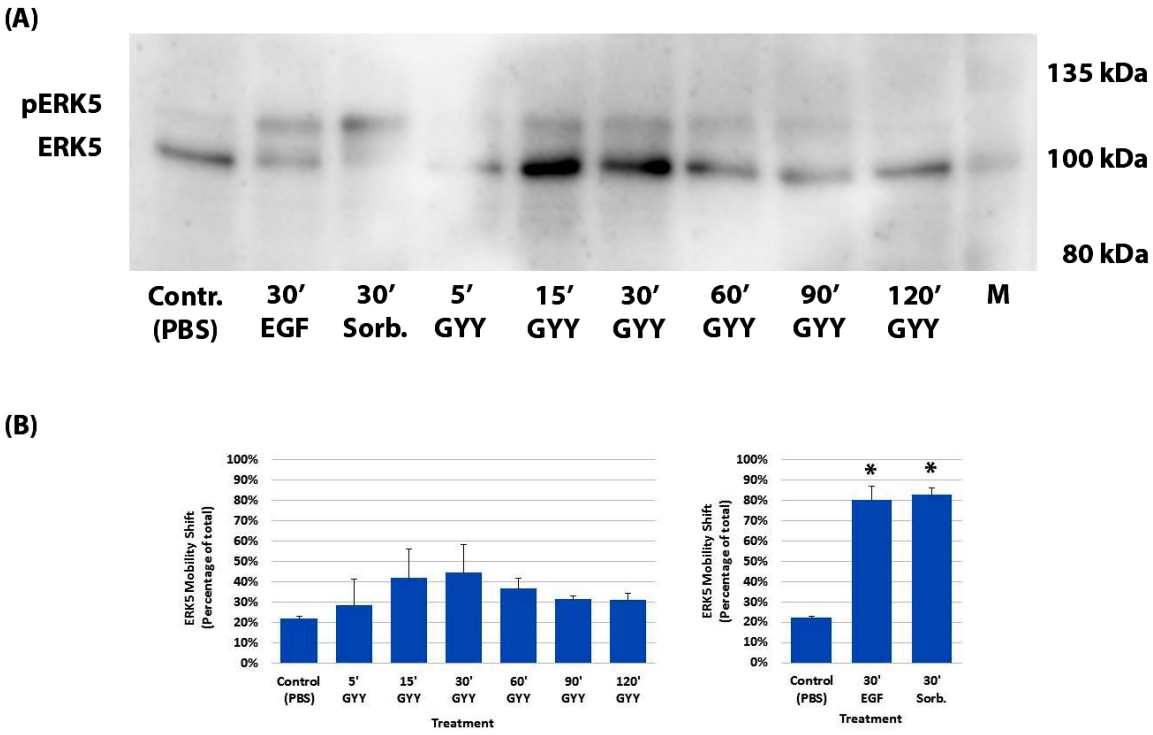


Figure 4.3: Activation of ERK5 by GYY4137, a H_2S donor molecule.

HeLa cells were grown in 60 mm dishes. The ERK5 MAPK pathway was stimulated with 50 ng/ml EGF, 400 mM Sorbitol, 400 µM GYY4137 for the indicated times. PBS was used as a negative control. The cells were washed with PBS and lysed by adding 350 µl of RIPA buffer. Proteins were separated on a 7% gel before western blotting with antibodies directed against ERK5. Quantification of ERK5 activity was accomplished by dividing the value for the upper band (phosphoERK5) by the sum of all bands (total ERK5) to yield a value for relative (percentage) activation. Statistical analysis (n=3) was performed using a one-way ANOVA test, which showed significant differences between samples. A subsequent Tukey HSD test demonstrated that both positive controls were significantly different from the control (P<0.001). However, GYY4137 treatment did not result in any statistically relevant differences. Representative image of n=3

4.2.3. Subcellular localization of ERK5 after treatment with H_2S

Activated ERK5 has been shown to be translocated to the nucleus upon activation (Kondoh et al. 2006). It was therefore also analysed whether ERK5 is present in the cytoplasm or the nucleus upon stimulation of HeLa cells with H_2S. The cells were plated on cover slips and transduced with an Adeno-Virus expressing wildtype ERK5. Cells were then serum-starved and treated with 400 µM GYY4137 for 60 minutes. Cells were stained with antibodies for ERK5 and with DAPI to stain the nucleus. Figure 4.4 *clearly shows that while ERK5 MAPK is primarily located in the cytoplasm under resting conditions, it is efficiently translocated into the nucleus upon GYY4137 stimulation for 60 minutes (Figure 4.4). This further demonstrates that H_2S activated the ERK5 MAPK, leading to nuclear translocation.*

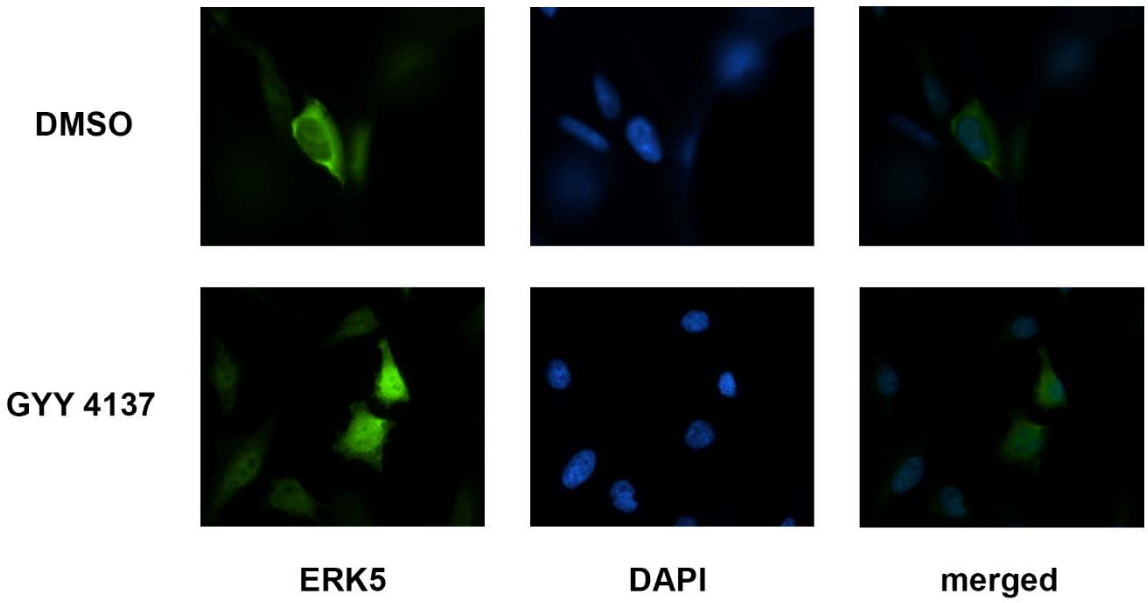


Figure 4.4: Activation of ERK5 by H_2S leads to its translocation to the nucleus.
HeLa cells were plated on cover slips and transduced with an Adeno-Virus expressing ERK5. The cells were serum-starved for 16 hours and stimulated with GYY4137, a slow-releasing H_2S donor for 60 minutes. Immunocytochemistry was performed using ERK5 primary and Alexa488-conjugated secondary antibodies and DAPI was added with the mounting medium. Dimethyl sulfoxide (DMSO, 2µl) was used as the vehicle control. The results show that ERK5 MAPK is translocated into the nucleus upon GYY4137 stimulation, but not after DMSO treatment. Representative image of n=7

4.3. Discussion

The findings of this study suggest that ERK5 MAPK may be activated by hydrogen sulphide (H_2S) in HeLa cells. While the differences are not significant and require additional investigations, there is an apparent reduction in the electrophoretic mobility of ERK5 in a gel-shift assay (Figure 4.3) in response to the H_2S-releasing chemical GYY4137. In addition, in initial experiments, GYY4137 also led to the translocation of ERK5 to the nucleus of the cell (Figure 4.4), anindication that ERK5 is being activated. ERK5 MAPK pathway activation plays a critical role in various cellular functions, such as the prevention of cellular apoptosis, cell survival, proliferation and differentiation. Activation of ERK5 MAPK can be attained by osmotic and oxidative stress, a range of growth factors (e.g., EGF, VEGF), and certain cellular cytokines (e.g., IL-6, LIF) (Drew et al. 2012). Both EGF and sorbitol were employed here to standardize the results, as they surely activate the ERK5 MAPK pathway. GYY4137, a chemical that slowly releases hydrogen sulphide, was mainly the source of hydrogen sulphide due to its reproducible effects on the treated cells. It has been shown in a previous study using two strata of endothelial cells (primary endothelial cells and transformed endothelial cells), that H_2S has the capability to stimulate cell proliferation and migration once the targeted signalling pathway was activated. Furthermore, pro-angiogenic effects were demonstrated in an in vitro study of endothelial cells in response to H_2S where there was some augmentation in tube-like formation (Papapetropoulos et al. 2009).

In contrast to GYY4137, activation of ERK5 MAPK by NaHS was weak and would require some optimization in terms of the concentration and incubation time. GYY4137 activated ERK5 in a more sustained way and remained higher than baseline for a longer period of time than NaHS. The most likely reason for this difference are the temporal differences in H_2S release. While NaHS releases H_2S very quickly once in an aqueous environment, GYY4137 acts much more slowly, releasing H_2S over several hours. This is similar to an in vitro study of three forms of cancerous cells (Lee et al. 2011). In this study, a panel of human

cancer cell lines were generated (HeLa; HCT-116; Hep G2; U2OS) and compared with two normal human diploid fibroblast cell lines (WI-38 and IMR90). GYY4137 at a concentration of 400 μM efficiently reduced cell proliferation and survival, whereas NaHS was completely inactive. (Lee et al. 2011)

There are plenty of known molecular targets for hydrogen sulphide, which can be classified broadly into effects on (a) protein (cysteine thiol), (B) intracellular cell signalling, (c) ion channels, and (d) metabolism (effects on mitochondria and ATP production) (Li et al. 2011). Interestingly, the effect of hydrogen sulphide on cell signalling is more likely related to the subject of this thesis, where the related molecular pathway associated with them needs to be explained. For example, the targeted molecular pathway of hydrogen sulphide activation could include phosphorylation and activation of phosphatidylinositol 3 kinase (PI3K) and signal transducer and activator of transcription 3 (STAT3). Upon activation of these pathways, numerous cellular primary functions such as prosurvival and preventing apoptosis would be evoked. Protein kinase C signalling pathway has been reported to be activated upon the action of hydrogen sulphide stimulation (Li et al. 2011). However, it is impracticable to predict the final outcomes of the intracellular events (e.g., final biological response and nuclear transcription) with receptor activation due to emerging of cross-talk (interaction) between cellular signalling pathway inside the cells (Robinson-White and Stratakis 2002).

Also, inhibition of many cellular receptors and channels mediating transmission of the signalling pathway may be associated with ERK5 activation (e.g., K_{ATP} channels) (Modis et al. 2013). Thus, it would be of great advantages for the future research to test and determine if hydrogen sulphide can activate other signalling pathway rather than ERK5, and recognize the role of cross-talk between signalling pathway in activation of ERK5 MAPK. On the other hand, the unique C-terminal domain of ERK5 would also have a critical role in activation of ERK5 by hydrogen sulphide as they harbour threonine and serine residues that can be

activated by other signalling pathways and upstream effectors, CDK1 and ERK1/2 are examples. It has been reported that H_2S can promote and phosphorylate ERK1/2 in pigs exposed to coronary artery occlusion and reperfusion (Li et al. 2011). Therefore, the mechanism by which H_2S activates the ERK5 pathway needs to be investigated in more detail.

ERK5 has been reported to translocate to the nucleus under a number of conditions, although its localisation within the cellular domain is variable (Buschbeck and Ullrich 2005). For example, the predominant nuclear patterns can be identified in COS-7, HeLa BT474 and SKBR3 cells (Kondoh et al. 2006), on the other hand, ERK5 can be allocated in an overall diffuse patterns in MCF7 cells (Esparis-Ogando et al. 2002). Several mechanisms have been described in its nuclear translocation, including MEK5-mediated phosphorylation, phosphorylation by CDK1 (cyclic-dependent kinase 1), MEK1/2-ERK1/2 –dependent mechanism and regulation of ERK5 nuclear shuttling by SUMOylation at the N-terminus (Tubita et al. 2020). Under basal conditions, i.e. in unstimulated cells, cytosolic ERK5 presents in unphosphorylated inactive folded form, where the C-terminal half is bound to the N-terminal, resulting in concealing the NLS (nuclear localisation signal) and preventing nuclear translocation (Yan et al. 2001). Following ERK5 activation by MEK5-dependent phosphorylation at the TEY region, activated ERK5 is able to phosphorylate itself in the C-terminus, thereby, leading to an open conformation and exposing the NLS sequence that facilitates ERK5 nuclear translocation (Erazo et al. 2013). Thus, it seems necessary for ERK5 to be phosphorylated at the MEK5 consensus for transporting to the nucleus. Beside MEK5-dependent activation, there are other mechanisms driving ERK5 nuclear translocation including overexpression of CDC37 in cancer cell lines, phosphorylation at the C-terminal half by other kinase and phosphorylation of ERK5 by ERK1/2 (Tubita et al.

2020). The exact mechanism by which H2S may facilitate the nuclear translocation of ERK5 remains to be determined.

Altogether, activation of ERK5 MAPK by hydrogen sulphide was noticeable, although not statistically significant. The highest level of activation was at 30 to 60 minutes time basis, then the degree of activation went down gradually till the baseline at 120 minutes. The subcellular localization of the MAPK components to the nucleus is well-known by its role in regulating the transmitted signals by MAPK cascade (Pouyssegur et al. 2002). Thus, it was suggested from the presented studies that ERK5 MAPK translocated to the nucleus upon its activation by hydrogen sulphide. The exact mechanism is yet to be revealed.

Chapter 5

ERK5 is activated by H_2S in primary endothelial cells

5. ERK5 is activated by H_2S in primary endothelial cells

5.1. Introduction

After the observation that H_2S may activate ERK5 in an established cell line (see previous chapter), it was now investigated if H_2S also has an effect on ERK5 in primary endothelial cells. The integrity of endothelial cells is very important in terms of maintenance of blood vessel function. In general, all blood vessels, ranging from the largest arteries and veins to the smallest capillary venules, are lined by endothelial cells (Ruoslahti and Rajotte 2000). The function of the endothelial cells is dependent on the localization of their corresponding blood vessels (Bouis et al. 2001). However, angiogenesis is a complicated biochemical process characterized by continuous changes in endothelial cell behaviour associated with remodelling of extracellular matrix, which enhances cellular growth and migration to be assembled and thus promotes capillary formations.

Overall, there are two types of experimentally utilizable endothelial cell (EC) lines available: (a) prolonged life span of endothelial cell and (b) immortalized cell lines. In general, the overall requirements for human EC cell line should meet the following standards: (1) the life span of the ECs should cover the duration of the experiment (2) the consistent presentation of at least two specific endothelial markers, for instance WP-bodies (Weibel-Palade), vWF (von Willebrand Factor), ACE activity (Angiotensin-converting enzyme) (3) experimental expression of relevant markers (4) minimal indication of tumorigenic conversion of the cells (Bouis et al. 2001).

There are a number of different endothelial cell types available. The overall behaviour of endothelial cells varies based on their vascular origin. For example, the morphology of human placental endothelial cells (HPEC) is different from HUVEC(human umbilical vein

endothelial cells); the cells are more elongated, form networks in sparse cultures and grow in expanded spiral structures of elongated cells at confluence (Schutz and Friedl 1996). HUVEC cells are commonly used for biochemical experiments. This is due to the abundance of the available information about in vitro experiments with human endothelial cells derived from umbilical veins (Jaffe et al. 1973). On average, HUVECs can be maintained up to 10 serial passages and kept in culture for up to 5 months, after which the cells enter senescence (Bouis et al. 2001).

It has been reported that endothelial cells are a target and source of H_2S (Papapetropoulos et al. 2009). The biological importance of each pathway modified by H_2S is varied depending on several aspects including: cell types, organs, species. Examples of these pathways include: effects on metal ions (Fe, Zn), S-sulfhydration (NF-κB), MAPK family, and cAMP/PKA (Szabo 2007). As MAPK has an experimental relevance to the work in this thesis, it would be significant to reveal the outstanding literature that articulates the biological role of H_2S on the MAPK family (Li et al. 2011). Four main members can describe the big MAPK superfamily: ERK1/2, p38, JNK and ERK5 (Cowan and Storey 2003). Otherwise, it has been shown that the mitogen activated protein kinase (MAPK ERK1/2 and p38) can be stimulated and phosphorylated upon exposure to Na_2S, which in turn enhances the cell migration in HUVEC (Papapetropoulos et al. 2009).

In terms of angiogenic activation, it has been described that K_{ATP} (upstream of MAPK pathway) channels can play a major role in the angiogenic pathway upon their activation. Profoundly, two independent researching groups (Cai et al. 2007, Papapetropoulos et al. 2009) have reported the factual phenomenon that H_2S plays a crucial role in proliferation and migration of endothelial cell in vitro (whether transformed or primary endothelial cells). Overall, angiogenesis response to H_2S would undoubtedly follow a bell-shaped dose-response pattern: the impact of H_2S can be ranged from physiological, cytoprotective effects (which happen at low concentrations) to cytotoxic effects (which may occur apparently at

higher concentrations) (Li et al. 2011). Exposing endothelial cells to exogenous H_2S has been shown to induce activation of multiple signalling pathways including: PI-3K/AKT axis and phosphorylation of members of the MAPK pathway (e.g., ERK1/2 and p38), with different time course. Once PI-3K/Akt is activated, tube-like structure formation in RF/6A cells would be regulated; meanwhile, activation of MAPK has been reported to play a crucial role in cell migration in HUVECs (Papapetropoulos et al. 2009).

The present study aims to reveal the precise role of exogenous H_2S on angiogenesis via ERK5 MAPK activation. In vascular tissues, the pro-angiogenic effect involves production of endogenous H_2S by VEGF. The exact mechanism of interactions is yet to be elucidated. In analogy to endogenous H_2S, an applied exogenous H_2S would induce a pro-angiogenic effect via interaction of VEGF/H_2S, leading to the activation of K_{ATP} channels. Suppression of those channels may inhibit VEGF-induced angiogenesis. In overall, it can be said that the major effect of H_2S on cellular signalling pathway would be the activation, with little exception (Szabo and Papapetropoulos 2011). Incubation of endothelial cells with H_2S can stimulate network like formation of ECs in vitro via capillary morphogenesis. Moreover, appearance of angiogenic phenotype can be a consequence of exerted positive effects of H_2S on endothelium migration (Papapetropoulos et al. 2009).

5.2. Results

5.2.1. ERK5 is activated by H_2S in primary endothelial cells

To study whether H_2S administration exerts activation effects on ERK5 MAPK in endothelial cells, HUVECs were treated with 400 µM GYY4137 - a water-soluble chemical that slowly releases H_2S - for 60 minutes, after which the cells were serum-starved for at least 3 hours. Transient transfection of HUVEC cells was performed using Adenoviral Expression System containing the cDNA of wild-type (WT)-hERK5. A constitutively active MEK5 ($MEK5^{DD}$) was used as a positive control for ERK5 activation.

To determine whether ERK5 MAPK was phosphorylated and activated by H_2S in HUVECs, lysates obtained from HUVECs were subjected to western blot analysis using an anti-ERK5 antibody. Treatment of HUVECs with GYY4137 resulted in an activation of ERK5 MAPK, as indicated by enhanced gel retardation (Figure 5.1). In consequence, this experiment suggests that ERK5 might be activated by H_2S in HUVECs. It is interesting to note that activation by H_2S leads to a smaller mobility shift than co-transduction with activated MEK5. This observation suggests that not all C-terminal phosphorylation sites are phosphorylated in response to H_2S, in contrast to full activation by $MEK5^{DD}$. This potential difference will be analysed in the future, when reliable methods (e.g., antibodies) exist to fully map C-terminal phosphorylation.

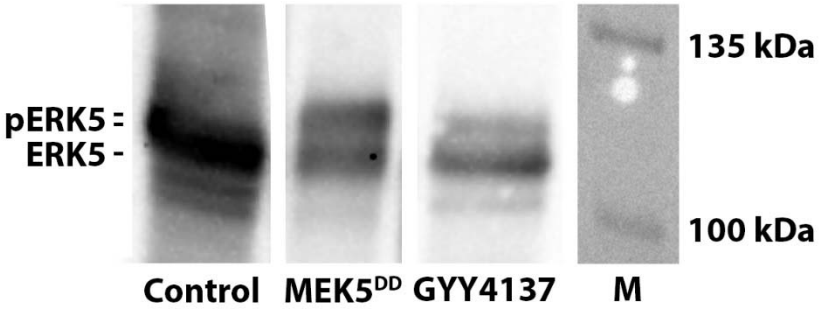


Figure 5.1: Activation of ERK5 by GYY4137.

HUVEC cells were plated on 60mm dishes and transduced with Adenoviruses expressing wildtype ERK5. Positive control cells also received MEK5^{DD}-expressing Adenovirus where indicated as a positive control. Cells were starved and one set was treated with GYY4137 for 60 minutes. Cells were lysed with RIPA buffer, lysates separated by SDS-PAGE and proteins visualized by western blotting using an antibody recognizing total ERK5 protein. Non-relevant lanes from the same gel have been removed for clarity. The shift in electrophoretic mobility signifies phosphorylation. Representative image of n=6

5.3. Discussion

Gaseous signalling molecules s (e.g., H_2S) play a pivotal role in angiogenesis (Altaany et al. 2013) and blood vessel formation requires a high degree of coordination associated with endothelial cell proliferation, migration, and differentiation (Patel-Hett and D'Amore 2011). Lately, the research on the roles of H_2S in the homeostasis and pathogenesis of endothelial cells has been emphasized dramatically. Via different signalling pathways, H_2S can protect the vascular system from onset of inflammation by inhibition or activation; including inhibition of p38 and nuclear factor kappa-light chain enhancer of activated B cells (NF-κB), or activation of HO-1 expression as well as activation of K_{ATP} and BK_{Ca} channels (Fiorucci et al. 2005, Zuidema et al. 2010). The endothelium also regulates angiogenesis and the processing of vascular remodelling. H_2S orchestrates angiogenesis in the vascular system by inhibiting of vascular smooth muscle cell (VSMC) proliferation and phenotypic changes at one side, and on the other hand, it promotes endothelium replication and migration (Papapetropoulos et al. 2009). In vitro studies from aortic rings from CSE-KO mice have shown that VEGF-induced angiogenesis can be markedly repressed (Papapetropoulos et al. 2009).

In the present study, it was found that H_2S can activate ERK5 MAPK in the HUVEC cell line. The mechanism for H_2S action is mainly attributed to the stimulation and phosphorylation of the ERK5 MAPK. It was also shown in chapter 4 (Figure 4.3) that H_2S activates and phosphorylates ERK5 MAPK in HeLa cells in a dynamic way, which means the highest degree of activation was observed at 60 minutes, then the degree of activation started to reduce gradually from 60-90 minutes. Whether this is also the case in primary endothelial cells remains to be investigated.

The finding of Moore and colleagues (Lee et al. 2011) has shown that H_2S could induce a major effect on angiogenesis using Matrigel modulation (Papapetropoulos et al. 2009). The

finding that H_2S promotes angiogenesis is also in agreement with the finding where ERK5 MAPK is activated by H_2S in primary endothelial cells. A new pathway downstream of effectors of H_2S in ECs has therefore been recognized. It is well-documented that H_2S can change the activation status of both ERK1/2 and p38 in a cell-type and stimulus-dependent way. In this study, it was initially described that ERK5 MAPK appears to be activated by H_2S and the degree of activation can time-dependent in HeLa cells. While H_2S did not affect ERK1/2 phosphorylation in transformed ECs (Papapetropoulos et al. 2009), the preliminary data from this study may suggest this it does activate ERK5. While more detailed experimentation will be necessary to address this hypothesis, this is an interesting finding. It has been shown that H_2S activates the PI3K/AKT pathway to promote cell survival. ERK5 activation also leads to AKT activation and the promotion of endothelial cell survival (Roberts et al. 2010). It is therefore possible that ERK5 activation represent the link between H_2S signalling and the AKT-dependent anti-apoptotic signal of H_2S. Future experiments will further investigate this potential link using a range of pharmaceutical treatments, including drugs modifying KATP channels and the AKT response.

Chapter 6

Analysis of ERK5 phosphorylation

6. Analysis of ERK5 phosphorylation

6.1. Introduction

Activation of ERK5 requires a dual phosphorylation of the TEY motif by upstream, activated MEK5 (MAPK kinase) in response to EGF stimulation or different stresses. Thereafter, active ERK5 is phosphorylated at its C-terminal tail, which results in generating a series of events including: (1) segregation of the ERK5-Cdc37 complex from the cytosolic anchor Hsp90; (2) conformational changes in the ERK5 molecule, where the NLS motif is exposed; and (3) translocation to the nucleus (Gomez et al. 2016).

ERK5 is a member of the MAPK family whose N-terminal domain is similar to the prototypical ERK1/2 MAPKs, and varies in having a unique C-terminal domain that is not present in other MAPK family (Nishimoto and Nishida 2006). This C-terminal domain has two proline rich regions that control the nucleo-cytoplasmic trafficking of ERK5, and shares no known predominant features with other proteins (Buschbeck and Ullrich 2005, Borges et al. 2007). Furthermore, this region has the ability to activate the transcription of a number of genes (e.g., c-Myc, Sap1a, c-Fos, Fra-1 and MEF2 family members) due to the presence of transcriptional activation domain (Kasler et al. 2000). It has been reported recently that the C-terminal region may undergo autophosphorylation and this phenomenon is important to regulate gene expression and promote the AP-1 activity (Morimoto et al. 2007). Deletion of the C-terminal coding region of the ERK5 gene, beyond the codon for amino acid 699, enhance the retention of ERK5 in the nucleus, and this grants resistance to death receptor-induced apoptosis (Diaz-Rodriguez and Pandiella 2010). However, the exact molecular pathways regulating activity of the transcriptional activation of the ERK5 C-terminal region remains unclear (Morimoto et al. 2007).

Using purified recombinant active ERK5 it has been shown that several Thr/Ser residues within the C-terminal region can be autophosphorylated by ERK5, including Ser421,

Ser433, Ser731, Thr733 (Mody et al. 2003), Ser760, Ser764 and Ser766 (Mody et al. 2003, Morimoto et al. 2007). However, it has been reported that the C-terminal tail of ERK5 can also be phosphorylated and activated by other kinases in a MEK5-independent pathway. For example, ERK5 is phosphorylated during mitosis at residues Ser567, Ser720, Ser731, Thre733, Ser753, and Ser830 and this is thought to be ERK5 kinase-independent (Diaz-Rodriguez and Pandiella 2010). Significantly, such phosphorylation would enhance migration of a kinase-inactive form of ERK5, from cytoplasm toward the nucleus resulting in a second pathway of ERK5 phosphorylation (Gomez et al. 2016). Phosphorylation of these sites has been shown to be fulfilled by cyclin-dependent kinase-1 (Diaz-Rodriguez and Pandiella 2010, Inesta-Vaquera et al. 2010).

Overall, different signals work together to phosphorylate the C-terminal domain of ERK5. shows the known C-terminal phosphorylation sites of ERK5, whose phosphorylation can result in nuclear translocation and activation of transcription by ERK5. Therefore, it can be inferred that ERK5 C-terminal phosphorylation can be classified into two major mechanisms. A MEK5-dependent mechanism would exemplify one pathway that combines signals such as growth factors (EGF) and oxidative and osmotic stresses to activate the MEK5-ERK5 pathway. However, phosphorylation of the C-terminus of ERK5 can also proceed by other kinase (e.g., CDK1), which induces ERK5-mediated activation of transcription as well as nuclear translocation, without implementing ERK5 kinase activity (Gomez et al. 2016).

Table 6.1: Known C-terminal phosphorylation sites of ERK5.

List of published known C-terminal phosphorylation sites of ERK5. For better comparison, the position of phosphorylation is given relative to the human protein [the position for the homologous residues vary slightly for the mouse protein (Morimoto et al. 2007)]. The published article and a brief description of the involved methodology is also given.

Phosphorylation sites (residues in human ERK5)	*Experiment*	*Reference*
• **Ser 567** • **Ser 720** • **Ser 731/Thr 733** • **Ser 803**	*Mitotic phosphorylation*	*(Inesta-Vaquera et al. 2010)*
• **Thr 28** • **Ser 421** • **Ser 433** • **Ser 496** • **Ser 731** • **Thr 733**	*Autophosphorylation in vitro*	*(Mody et al. 2003).*
• **Thr 733** • **Ser 770** • **Ser 774** • **Ser 776**	*Mutagenesis of potential phosphorylation sites (COS7 and NIH3T3 cells)*	*(Morimoto et al. 2007)*
• **Ser 567** • **Ser700-Ser800** *(at least one site)* • **Ser 706** • **Ser 720** • **Ser 731** • **Thr 732** • **Thr 733** • **Ser 753** • **Ser 773** • **Ser 830**	*Mitotic phosphorylation*	*(Diaz-Rodriguez and Pandiella 2010)*
• **Ser 706** • **Ser 719** • **Ser 730** • **Thr 732** • **Ser 753** • **Ser 769** • **Ser 773** • **Ser 775**	*Mutational analysis (isogenic HeLa cell lines)*	*(Pearson et al. 2020)*

6.2. Results

6.2.1. Analysis of ERK5 phosphorylation by mass spectrometry

The changes in ERK5 phosphorylation are still incompletely understood. While it is clear that the TEY motif is phosphorylated by MEK5 upon pathway activation, the phosphorylation of the C-terminus is not well understood. Which specific residues become phosphorylated under which environmental conditions needs further investigation, particularly in endothelial cells. To enable such studies the aim was to identify the residues that are phosphorylated upon ERK5 stimulation and to establish a method to monitor the phosphorylation of those sites. The best method to unambiguously identify specific phosphorylation events is using mass spectrometry. To test and establish this method, ERK5 protein was produced in HeLa cells to yield a sufficient quantity of ERK5 to perform the analyses.

6.2.1.1. Overexpression and purification of ERK5

To obtain sufficient material for the mass spectrometric analyses of ERK5, 20 p100 dishes of HeLa cells were transduced with an adenovirus expressing Flag-tagged wildtype ERK5. After two days, the cells were lysed with RIPA buffer. ERK5 was immunoprecipitated with an anti-FLAG antibody coupled to agarose beads, . After separation on a 7% SDS polyacrylamide gel, ERK5 was visualised by staining with colloidal Coomassie. This protocol resulted in the purification of a substantial quantity of ERK5 (Figure 6.1). In addition, three bands of ERK5 were visible, corresponding to the unstimulated (inactive) ERK5 (lower band) as well as to the two hyperphosphorylated forms (middle and upper band) (Komaravolu et al. 2015). The bands were excised with a clean scalpel and sent to the University of Warwick for analysis by mass spectrometry.

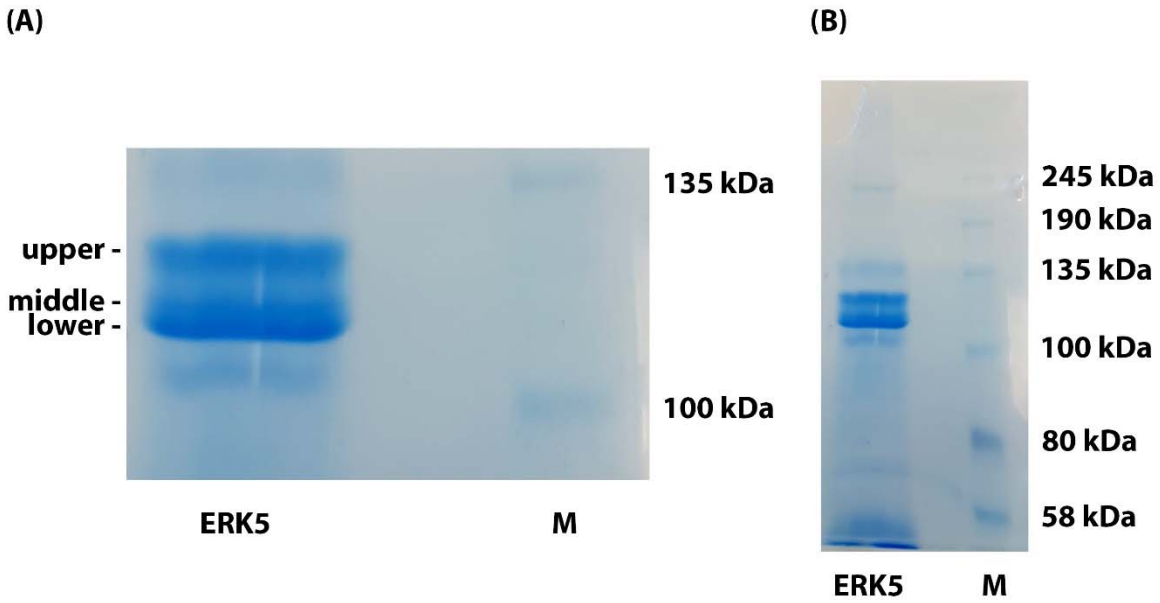


Figure 6.1: Immunoprecipitation of ERK5.

FLAG-tagged ERK5 was expressed in HeLa cells and immunoprecipitated with an agarose-coupled anti-FLAG antibody. Purified ERK5 was separated by SDS-PAGE and stained with colloidal Coomassie. In (A), the image has been enlarged to illustrate the three bands of ERK5 that can be identified. (B) shows the whole gel to allow an estimate of protein purity. M = molecular weight standard. Representative image of n=4

6.2.1.2. Mass spectrometric analysis of ERK5

The mass spectrometric analyses of the resulting tryptic peptides from the three individually investigated bands identified 29.5% of the total ERK5 protein between the samples (Figure 6.2). Unfortunately, no phosphorylation sites could be identified. Close inspection of the ERK5 sequence reveals that most known ERK5 phosphorylation sites are in tryptic peptides that have not been identified by the MS analysis of any of the three bands (in either phosphorylated or non-phosphorylated form), including the most abundant lower band, indicating that this result is not due to limited sample quantities (see underlined residues in Figure 6.2). A potential reason for this lies in the distribution of the cleavage sites for trypsin. Table 2.1 *shows all theoretical peptides arising from the digestion of ERK5 with trypsin. 11 of these peptides are larger than 2 kDa and are likely to be missed by mass spectrometry. Indeed, the largest peptide comprises 145 amino acids and has a theoretical MW of almost 14 kDa. The majority of the known C-terminal phosphorylation sites are located within this peptide, which is the likely reason that no phosphorylations could not be identified.*

To overcome the problem with limited tryptic digestion sites, the ERK5 sequence was inspected for other options regarding proteolytic cleavage. Unfortunately, cleavage sites for common enzymes such as GluV8 or AspN were also mostly absent within the larger tryptic peptides. The ERK5 protein was therefore digested with chymotrypsin, which cleaves proteins at the following amino acids: Trp, Tyr and Phe (high specificity) and also at Leu, Met and His (low specificity). ERK5 digested with chymotrypsin was then subjected to mass spectrometry as before. While cleavage with chymotrypsin is relatively unspecific and often results in excessive cleavage, several peptides could be identified with an overall protein coverage of 17.6% (Figure 6.3). This included 10.4% of ERK5 that was not detected in the tryptic digest, resulting in a total ERK5 coverage of 39.9%. Nevertheless, while Y220 (of the TEY motif) was shown to be phosphorylated in all samples (red residue in Figure 6.3*), none of the C-terminal phosphorylation sites were identified. As above, this is unlikely due to*

limited sample quantity, as most of the corresponding peptides (even in the unphosphorylated form) were still not detected in the most abundant lower band (Figure 6.4).

Overall, these experiments demonstrate that a comprehensive mapping of ERK5 phosphorylation by mass spectrometry is very challenging and may not be possible with conventional analysis methods.

MAEPLKEEDGEDGSAEPPGPVKAEPAHTAASVAAKNLALLKARSFDVTFDVGDEYEIIET

IGNGAYGVVSSARRRLTGQQVAIKKIPNAFDVVTNAKRTLRELKILKHFKHDNIIAIKDI

LRPTVPYGEFKSVYVVLDLMESDLHQIIHSSQPLTLEHVRYFLYQLLRGLKYMHSAQVIH

RDLKPSNLLVNENCELKIGDFGMARGLCTSPAEHQYFMTEYVATRWYRAPELMLSLHEYT

QAIDLWSVGCIFGEMLARRQLFPGKNYVHQLQLIMMVLGTPSPAVIQAVGAERVRAYIQS

LPPRQPVPWETVYPGADRQALSLLGRMLRFEPSARISAAAALRHPFLAKYHDPDDEPDCA

PPFDFAFDREALTRERIKEAIVAEIEDFHARREGIRQQIRFQPSLQPVASEPGCPDVEMP

SPWAPSGDCAMESPPPAPPPCPGPAPDTIDLTLQPPPPVSEPAPPKKDGAISDNTKAALK

AALLKSLRSRLRDGPSAPLEAPEPRKPVTAQERQREREEKRRRRQERAKEREKRRQERER

KERGAGASGGPSTDPLAGLVLSDNDRSLLERWTRMARPAAPALTSVPAPAPAPTPTPTPV

QPTSPPPGPLAQPTGPQPQSAGSTSGPVPQPACPPPGPAPHPTGPPGPIPVPAPPQIATS

TSLLAAQSLVPPPGLPGSSTPGVLPYFPPGLPPPDAGGAPQSSMSESPDVNLVTQQLSKS

QVEDPLPPVFSGTPKGSGAGYGVGFDLEEFLNQSFDMGVADGPQDGQADSASLSASLLAD

WLEGHGMNPADIESLQREIQMDSPMLLADLPDLQDP

Legend:
Lower only: grey
Middle only: dark yellow
Lower + Middle: yellow
Lower + Upper: pink
Lower + Middle + Upper: blue

Figure 6.2: Tryptic ERK5 peptides identified by mass spectroscopy

The entire amino acids comprising the ERK5 protein is shown (full peptide sequence of ERK5). The highlighted yellow boxes represent the resulting peptides that were recognized by mass-spectrometry analysis, which it turns to comprise about 29.5% of the total ERK5 protein. The possible proteolytic cleavage sites for trypsin enzyme have been highlighted in the bolded letters of the peptide sequencing. Known phosphorylation sites are underlined. Raw data are available in the appendix.

Table 6.2: Theoretical peptides arising from the digestion of ERK5 with trypsin sorted by size.

There are 76 cleavage sites that can be identified by trypsin enzymes. 11 peptides of these cleavage sites are bigger than 2 kDa, which it turns to complicate their identification by mass spectrometry. The largest peptide comprises 145 amino acids, having theoretically heavy molecular weights of almost 14 kDa, which it turns to be inferred that it is almost impossible to recognize this sequence by mass spectrometry. It is important to demonstrate that many known phosphorylation sites (highlighted in yellow) exist along these large peptides sequencing, whereas their identification by mass-spectrometry was almost unattainable.

Position of cleavage site	Resulting peptide sequence	Peptide length [aa]	Peptide mass [Da]
719	MARPAAPALTSVPAPAPAPTPTPTPVQPTSPPPGPLAQPTGPQPQSAGSTSGPVPQPACPPPGPAPHPTGPPGPIPVPAPPQIATSTSLLAAQSLVPPPGLPGSSTPGVLPYFPPGLPPPDAGGAPQSSMSESPDVNLVTQQLSK	145	13957.89
466	FQPSLQPVASEPGCPDVEMPSPWAPSGDCAMESPPPAPPPCPGPAPDTIDLTLQPPPPVSEPAPPK	66	6718.617
797	GSGAGYGVGFDLEEFLNQSFDMGVADGPQDGQADSASLSASLLADWLEGHGMNPADIESLQR	62	6402.852
258	APELMLSLHEYTQAIDLWSVGCIFGEMLAR	30	3394.966
160	SVYVVLDLMESDLHQIIHSSQPLTLEHVR	29	3359.845
73	SFDVTFDVGDEYEIIETIGNGAYGVVSSAR	30	3211.443
293	NYVHQLQLIMMVLGTPSPAVIQAVGAER	28	3036.601
369	YHDPDDEPDCAPPFDFAFDR	20	2369.46
225	GLCTSPAEHQYFMTEYVATR	20	2304.577
816	EIQMDSPMLLADLPDLQDP	19	2141.436
566	GAGASGGPSTDPLAGLVLSDNDR	23	2127.252
197	DLKPSNLLVNENCELK	16	1829.098
735	SQVEDPLPPVFSGTPK	16	1697.906
318	QPVPWETVYPGADR	14	1614.778
22	EEDGEDGSAEPPGPVK	16	1612.624
131	DILRPTVPYGEFK	13	1534.775
391	EAIVAEIEDFHAR	13	1499.643
505	DGPSAPLEAPEPR	13	1335.436
97	IPNAFDVVTNAK	12	1288.466
181	YMHSAQVIHR	10	1241.433
35	AEPAHTAASVAAK	13	1223.351
168	YFLYQLLR	8	1115.34

304	AYIQSLPPR	9	1044.219
84	LTGQQVAIK	9	957.138
513	KPVTAQER	8	928.056
118	HDNIIAIK	8	923.08
476	DGAISDNTK	9	919.944
205	IGDFGMAR	8	866.003
326	QALSLLGR	8	857.021
343	ISAAAALR	8	771.915
349	HPFLAK	6	711.862
335	FEPSAR	6	705.769
265	QLFPGK	6	688.825
6	MAEPLK	6	687.852
41	NLALLK	6	670.85
571	SLLER	5	616.715
374	EALTR	5	588.662
400	QQIR	4	543.624
228	WYR	3	523.592
485	AALLK	5	514.666
524	RRR	3	486.578
396	EGIR	4	473.53
574	WTR	3	461.521
527	QER	3	431.449
538	QER	3	431.449
110	HFK	3	430.507
329	MLR	3	418.555
520	EEK	3	404.42
480	AALK	4	401.506
101	TLR	3	388.467
104	ELK	3	388.464
488	SLR	3	374.44
107	ILK	3	372.508
75	RR	2	330.39
171	GLK	3	316.401
376	ER	2	303.318
517	ER	2	303.318
531	ER	2	303.318
540	ER	2	303.318
543	ER	2	303.318
515	QR	2	302.333
492	LR	2	287.362
533	EK	2	275.305
295	VR	2	273.335
490	SR	2	261.281
378	IK	2	259.349
43	AR	2	245.282
529	AK	2	217.268

98	*R*	*1*	*174.203*
259	*R*	*1*	*174.203*
392	*R*	*1*	*174.203*
521	*R*	*1*	*174.203*
534	*R*	*1*	*174.203*
535	*R*	*1*	*174.203*
85	*K*	*1*	*146.189*
467	*K*	*1*	*146.189*
541	*K*	*1*	*146.189*

MAEPLKEEDGEDGSAEPPGPVKAEPAHTAASVAAKNLALLKARSFDVTFDVGDEYEIIET

IGNGAYGVVSSARRRLTGQQVAIKKIPNAFDVVTNAKRTLRELKILKHFKHDNIIAIKDI

LRPTVPYGEFKSVYVVLDLMESDLHQIIHSSQPLTLEHVRYFLYQLLRGLKYMHSAQVIH

RDLKPSNLLVNENCELKIGDFGMARGLCTSPAEHQYFMTEYVATRWYRAPELMLSLHEYT

QAIDLWSVGCIFGEMLARRQLFPGKNYVHQLQLIMMVLGTPSPAVIQAVGAERVRAYIQS

LPPRQPVPWETVYPGADRQALSLLGRMLRFEPSARISAAAALRHPFLAKYHDPDDEPDCA

PPFDFAFDREALTRERIKEAIVAEIEDFHARREGIRQQIRFQPSLQPVASEPGCPDVEMP

SPWAPSGDCAMESPPPAPPPCPGPAPDTIDLTLQPPPPVSEPAPPKKDGAISDNTKAALK

AALLKSLRSRLRDGPSAPLEAPEPRKPVTAQERQRERREEKRRRRQERAKEREKRRQERER

KERGAGASGGPSTDPLAGLVLSDNDRSLLERWTRMARPAAPALTSVPAPAPAPTPTPTPV

QPTSPPPGPLAQPTGPQPQSAGSTSGPVPQPACPPPGPAPHPTGPPGPIPVPAPPQIATS

TSLLAAQSLVPPPGLPGSSTPGVLPYFPPGLPPPDAGGAPQSSMSESPDVNLVTQQLSKS

QVEDPLPPVFSGTPKGSGAGYGVGFDLEEFLNQSFDMGVADGPQDGQADSASLSASLLAD

WLEGHGMNPADIESLQREIQMDSPMLLADLPDLQDP

Legend:
Lower only: grey
Middle only: dark yellow
Upper only: green
Lower + Middle: yellow
Lower + Middle + Upper: blue

Figure 6.3: Chymotryptic ERK5 peptides identified by mass spectroscopy

The entire amino acids comprising the ERK5 protein can be identified easily through the outlined letters (full peptide sequence of ERK5). The highlighted regions represent the resulting peptides that were cleaved by chymotrypsin enzyme, and then identified by mass spectrometry analysis, comprising about 17.6% of the total protein. It is worthy to emphasize that 10.4 % of ERK5 that was not identified in the tryptic digestion, have been tested using the chymotrypsin digestion. In addition, Y220 was identified as a phosphorylated residue (red). Raw data are available in the appendix.

MAEPLKEEDGEDGSAEPPGPVKAEPAHTAASVAAKNLALLKARSFDVTFDVGDEYEIIET

IGNGAYGVVSSARRRLTGQQVAIKKIPNAFDVVTNAKRTLRELKILKHFKHDNIIAIKDI

LRPTVPYGEFKSVYVVLDLMESDLHQIIHSSQPLTLEHVRYFLYQLLRGLKYMHSAQVIH

RDLKPSNLLVNENCELKIGDFGMARGLCTSPAEHQYFMTEYVATRWYRAPELMLSLHEYT

QAIDLWSVGCIFGEMLARRQLFPGKNYVHQLQLIMMVLGTPSPAVIQAVGAERVRAYIQS

LPPRQPVPWETVYPGADRQALSLLGRMLRFEPSARISAAAALRHPFLAKYHDPDDEPDCA

PPFDFAFDREALTRERIKEAIVAEIEDFHARREGIRQQIRFQPSLQPVASEPGCPDVEMP

SPWAPSGDCAMESPPPAPPPCPGPAPDTIDLTLQPPPPVSEPAPPKKDGAISDNTKAALK

AALLKSLRSRLRDGPSAPLEAPEPRKPVTAQERQREREEKRRRRQERAKEREKRRQERER

KERGAGASGGPSTDPLAGLVLSDNDRSLLERWTRMARPAAPALTSVPAPAPAPTPTPTPV

QPTSPPPGPLAQPTGPQPQSAGSTSGPVPQPACPPPGPAPHPTGPPGPIPVPAPPQIATS

TSLLAAQSLVPPPGLPGSSTPGVLPYFPPGLPPPDAGGAPQSSMSESPDVNLVTQQLSKS

QVEDPLPPVFSGTPKGSGAGYGVGFDLEEFLNQSFDMGVADGPQDGQADSASLSASLLAD

WLEGHGMNPADIESLQREIQMDSPMLLADLPDLQDP

Figure 6.4: Total peptide coverage of ERK5.

Full peptide sequence of ERK5. The highlighted gray boxes represent the resulting peptides that were identified after either trypsin or chymotrypsin cleavage. Total protein coverage is 39.9%.

6.2.2. Characterisation of a phosphor-specific antibody recognizing the ERK5 C-terminus

Several residues in the C-terminus of ERK5 have been recognized to be phosphorylated, supposedly by autophosphorylation by activated ERK5 (Morimoto et al. 2007) (Figure 1.8). The aim of this section was to develop an antibody that recognises the phosphorylated C-terminus of ERK5 to develop a new tool to detect ERK5 activation and C-terminal phosphorylation. As demonstrated in Chapter 3, the phosphor-specific antibodies currently available are of limited use for this purpose. It was therefore decided to develop a novel antibody recognising the phosphorylated C-terminus. The S770 site was chosen because it is known to be specifically phosphorylated (Gilley et al. 2012).

6.2.2.1. Peptide design and antibody production

The peptide was designed in collaboration with Eurogentec around the sequence of S770. A cysteine was added to the C-terminus to facilitate coupling to the affinity column, resulting in the final peptide: ac-GPQDGQAD-pS-ASL+C-nh2. The polyclonal antibodies were produced by Eurogentec using the Speedy anti-PTM protocol. Briefly, two rabbits were immunized with the peptide, intermediate control samples were taken, and the animals were sacrificed after 28 days. The antibodies were purified by Eurogentec using two consecutive affinity columns. The first column consisted of the phosphorylated peptide, with the eluate containing antibodies against both phosphorylated and unphosphorylated ERK5. The second purification step used the unmodified peptide to remove those antibodies that recognise the ERK5 protein but not ERK5 phosphorylated at S770, as those would be bound to the column. The flow-through of this step thus contained only the antibodies recognising phosphorylated, but not unphosphorylated, ERK5.

6.2.2.2. Testing of the pS770 antibody by western blotting

Of the two antibodies obtained, the antibody with the higher affinity, based on the ELISA data provided from by Eurogentech, was further evaluated. First, the antibody was titrated to establish the dilution required for further experiments. In HeLa cells, transient transfection with Adenoviral Expression System containing the cDNA of wildtype ERK5 as well as a constitutively active MEK5, $MEK5^{DD}$. Cell lysates were then separated by SDS-PAGE and western blots and probed with the anti-Ser770 antibody. The results shown in Figure 6.5 demonstrate that the antibody recognised ERK5 and that the strength of the bands was directly proportional to the dilution of the antibody. Based on these results, the antibody was used at a dilution of 1:100 for subsequent experiments.

The next step was to characterise the antibody regarding its specificity for the phosphorylated ERK5. For this purpose, HeLa cells were transduced with an adenovirus expressing wildtype ERK5. The cells then were serum starved for at least 18 hours prior to running the experiment. To standardize the experiment and to activate ERK5, cells were also transduced with a gene encoding constitutively active MEK5: $MEK5^{DD}$. The cells were lysed and ERK5 analysed by SDS PAGE and western blotting with the pS770 antibody (specific for ERK5 phosphorylated at S770). The results confirm that the antibody recognises ERK5 (Figure 6.6). However, it became clear that this was not specific for the phosphorylated ERK5 (upper bands), but that the antibody also recognised ERK5 from the serum-starved cells, which likely represents the unphosphorylated form (lower band).

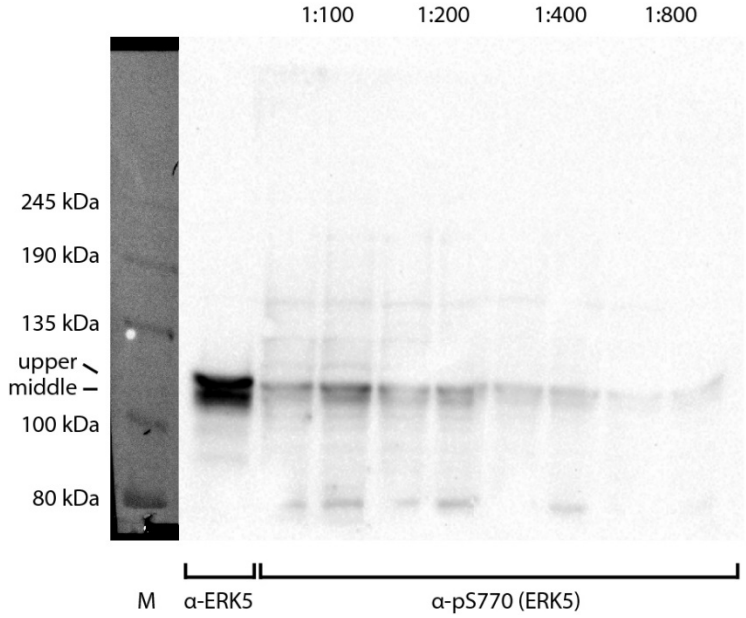


Figure 6.5: Detection of activated ERK5 and C-terminal phosphorylation using ERK5 pS770 antibody.

HeLa cells were grown and maintained in p60 dishes. The cells were transient transduced with Adenoviral Expression System containing the cDNA of wildtype ERK5 and a constitutively active MEK5 (MEK5^{DD}) to activate ERK5. The cells then were serum-starved for 16 hours, washed with PBS and lysed by adding 350 µl of RIPA buffer. Proteins were separated on a 7% gel before western blotting with the specified antibodies. Note that the marker lane has been obtained through illumination with reflective light in a separate exposure. The pS770 antibody was specifically able to identify activated ERK5. Also, there were some proportionality between the strength of bands and the dilution of antibody. Representative image of n=4

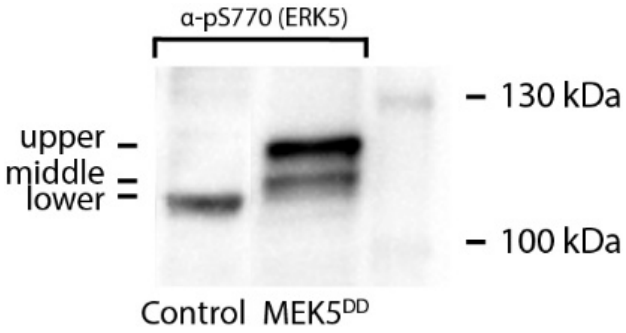


Figure 6.6: Specificity of the pS770 ERK5 antibody (before purification).
HeLa cells grown on 60mm plates were transduced with an Adenovirus expressing ERK5 (left and right lanes) as well as MEK5^{DD} (right lane only). After serum-starvation for 16 hours, the cells were lysed and subjected to SDS-PAGE and western blotting with the pS770 antibody. All forms of ERK5 (non-phosphorylated and phosphorylated) are detected by the antibody. Representative image of n=4

6.2.2.3. Antibody purification and characterisation

Based on the above results indicating that the antibody was likely not specific for the phosphorylated ERK5, it was decided to further purify it using an affinity column. For this purpose, the non-phosphorylated ERK5 peptide was coupled to agarose beads according to the instructions of the manufacturer (MicroLink Protein Coupling Kit, Pierce). The antibody was then incubated with the beads to bind the unspecific antibodies, i.e. those that recognize unphosphorylated ERK5. The flow-through containing the phosphor-specific antibodies was collected. The purified antibody was then tested again using ERK5 protein from HeLa cells expressing ERK5 (with or without MEK5^{DD}) as above. The results demonstrate that this purified antibody does recognise ERK5 as before, but that it is now specific for the phosphorylated isoforms (middle and upper bands) and only weakly recognises the non-phosphorylated form (lower band) (Figure 6.7).

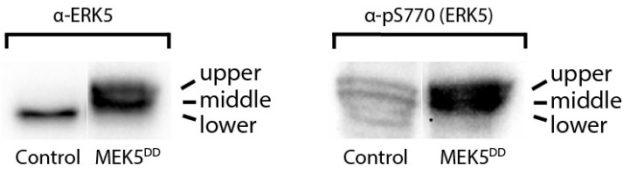


Figure 6.7: Characterization of the purified pS770 ERK5 antibody.

HeLa cells were grown and maintained in p60 dishes. The cells were transient transduced with Adenoviral Expression System containing the cDNA of wildtype ERK5 and a constitutively active MEK5 (MEK5^{DD}). The cells then were serum-starved for 16 hours. The cells were washed with PBS and lysed by adding 350 µl of RIPA buffer. Proteins were separated on a 7% gel before western blotting with ERK5 antibodies (total α-ERK5 at 1:1000 and purified α-pS770 at 1:100). Note that the purified antibody (right panel) recognises the middle and upper band, but only weakly the lower one (control lane). Representative image of n=3

6.3. Discussion

*This chapter aimed at developing a method to monitor the C-terminal phosphorylation of ERK5. The first approach was to use mass spectrometry to monitor C-terminal phosphorylation. ERK5 was successfully purified and the three differentially phosphorylated bands were isolated (Figure 6.1). This method did specifically identify the ERK5 protein within all three bands (*Figure 6.4*). It has also detected phosphorylation of Y220, one of the activating phosphorylation sites within the activation loop of ERK5 (Figure 6.3). However, no C-terminal phosphorylation sites were identified. This is most likely due to the absence of suitable trypsin recognition sites within the C-terminus of ERK5. In addition, recognition sites for other common proteases were also absent. While it was possible to identify additional ERK5 peptides by using chymotrypsin, this enzyme has variable specificity depending on the enzymatic conditions and it did not reveal any additional C-terminal peptides harbouring known phosphorylation sites. While it may be possible to develop more sophisticated ways to fragment ERK5 that may yield usable peptides, it is clear that mass spectrometry will not be suitable for routine comprehensive monitoring of ERK5 phosphorylation on a larger scale.*

It was therefore decided to develop a phosphor-specific antibody for one of the C-terminal phosphorylation sites. In this study, it was demonstrated that the activated ERK5 MAPK phosphorylates ERK5 at Ser770 upon transfection of the cells with a constitutively active form of MEK5^{DD}. However, the role of phosphorylation at Ser770 by ERK5 remains unclear. There are some probabilities, where such site may encourage the nuclear translocation of ERK5, or promote efficient phosphorylation of ERK5 upon its binding to transcription factors. The results showed ERK5 phosphorylation at Ser770 using phosphor-specific antibodies. Interestingly, in association with phosphorylation status of the ERK5 TEY motif or ERK5 kinase activity, ERK5 phosphorylation at Ser770 is induced. In addition to the canonical MEK5-dependent pathway, there is other pathway of ERK5 activation involved

phosphorylation of multiple serine and threonine residues in the ERK5 C-terminal by a cyclin-dependent kinase 1 including Ser706, Thr732, Ser753 and Ser773. These series of phosphorylation are significant for detention of ERK5 in the nucleus (Honda et al. 2015).

ERK5 is the only MAPK containing a C-terminal domain that follows the kinase domain (Buschbeck and Ullrich 2005). In this study, the question of whether the presence of such phosphorylation sites along the C-terminal tail could be used to monitor ERK5 activity was addressed. For this reason, a specific antibody (anti-Ser770) was designed that can detect and bind to its correspondence amino acid in the C-terminal domain. Based on this method, it was clear that phosphorylation of ERK5 would be dependent on the activity of MEK5, as the cells were transfected with the active constitutive form of MEK5^{DD}. This finding, besides demonstrating that ERK5 phosphorylation was MEK5 dependent, opened up the avenue to detect a specific residue involved in ERK5 C-terminal phosphorylation. In addition to what has been reported by two independent researching groups (Mody et al. 2003, Morimoto et al. 2007) that several Thr/Ser residues in the C-terminal tail would be autophosphorylated by ERK5, it was found here that Ser770 in the C-terminal domain could be autophosphorylated by active ERK5, and availed in monitoring ERK5 activity. This antibody represents therefore a new opportunity to evaluate ERK5 activation and has the potential to open up new avenues of research.

Chapter 7

Discussion

7. Discussion

This thesis is based on the hypothesis that H_2S activates ERK5 to mediate vascular protection. This hypothesis is grounded on a number of observations. First, ERK5 has been shown to play a major role in the vascular system, for example, through the analysis of knock-out mice (Hayashi and Lee 2004). Secondly, ERK5 acts as a vascular protective factor by protecting endothelial cells from apoptosis (Roberts et al. 2010). Thirdly, H_2S has been shown to protect vascular cells from damage and death and to enhance vascular and placental health (Elsey et al. 2010, Szabo and Papapetropoulos 2011). Studies using CSE knockout mice have also highlighted the critical role of hydrogen sulfide in angiogenesis and cardio-protection, with the defect discovered to occur within the endothelium. Finally, H_2S and ERK5 signalling have both been shown to activate the AKT pro-survival pathway (Cai et al. 2007, Roberts et al. 2010). The aim of this thesis was therefore to investigate the activation of the ERK5 pathway by H_2S and to initiate investigations into methods to monitor ERK5 MAPK activation in both human cell lines and endothelial cells. As a consequence, several preliminary discoveries have been revealed, which are discussed promptly.

7.1. Development of assays to detect ERK5 activation

The shortage in the current tools and methods to measure ERK5 activity required an investigation into the most reliable technique for detecting ERK5 activation within the context of cells. Currently, all the present methods to detect ERK5 activation in cells are dependent on in vitro kinase assays and phosphor-specific antibodies (Esparis-Ogando et al. 2002). However, there are some inconsistent data that could be produced upon using antibodies against phosphorylated proteins, as shown in chapter 3.2.2. Theoretically, ERK5 activation requires dual-phosphorylation of Thr^{218}/Tyr^{220} residues in the activation loop of the kinase domain of the ERK5 by sole upstream molecule MEK5 (Pearson et al. 2020). Then, numerous residues in the C-terminal domain of the ERK5 would undergo an autophosphorylation process resulting in enhancement of the transcriptional activity of

ERK5. Thus, the presence of these numerous phosphorylation sites in both kinase domain and the C-terminal domain of ERK5 is a unique feature (Honda et al. 2015).

To test different methods to detect ERK5 MAPK activation, it was initially tested whether ERK5 protein could be produced in an established cell line (HEK 293) at a level that can be easily detected using an affordable technique (e.g., western blotting technique). Sufficient ERK5 levels were observed in the HEK 293 cells by western blotting using antibody directed against total ERK5. In addition, it was showed that EGF stimulated the intracellular signalling pathway by demonstrating the successful activation of ERK1/2 MAPK. Lysates from cells similarly treated were then used to investigate ERK5 activation.

*The most direct method to monitor the activation of a protein kinase is an in vitro kinase assay. It is also suitable for monitoring ERK5 kinase activity, but it is only valuable for a small sample sizing (Müller and Morrison 2002). To facilitate the identification and quantification of protein kinase activation, the basis of generating and exploiting phosphor-specific antibodies against the phosphorylation sites of many pathways in different conditions has been proposed. For example, the activation of the ERK1/2 MAPK pathway can be efficiently monitored using a phosphor-specific antibody directed against the TEY activation motif. Unfortunately, the analogue pERK5$^{T218/Y220}$ antibody directed against the TEY motif of ERK5 did not detect endogenous ERK5 activation (*Figure 3.4*). This antibody has been mainly used in the past to detect the activation of overexpressed ERK5 and has shown variable results with the detection of endogenous ERK5 (Sanchez-Fernandez et al. 2016), which is consistent with the results presented here. In addition, pS496 and pS731/pT733, antibodies directed against the C-terminal phosphorylation sites were not efficiently able to recognize endogenous ERK5 activation too (Figure 3.5). Another phosphor-specific antibody tested was directed against activated MEK5, the upstream activator of ERK5. Unfortunately, this antibody also did not efficiently recognize endogenous*

MEK5 (Figure 3.6). Thus, inconsistent and unreliable results are a major drawback of using phosphor-specific antibodies to detect endogenous ERK5 pathway activation (Kondoh et al. 2006). Unfortunately, it was demonstrated in this thesis that these commercial antibodies directed against a variety of activation sites on ERK5 and MEK5 have no efficiency in characterizing and analyzing the endogenous ERK5.

ERK5 is unique among the mammalian MAPKs in that it contains a large C-terminal domain (Nishimoto and Nishida 2006). Upon activation of ERK5 kinase activity by MEK5, the C-terminus of ERK5 becomes significantly hyperphosphorylated. In consequence, it has been shown previously that this can result in a visible electrophoretic shift under some conditions. It was clearly shown here that this assay succeeded in measuring and monitoring ERK5 phosphorylation, which relates to its activity (Esparis-Ogando et al. 2002, Buschbeck and Ullrich 2005). The assay was optimised for better resolution for the potential ERK5 bands by allowing the gel to be run until the 80 kDa protein marker reached the bottom of the gel (note that ERK5 runs at an apparent MW of approximately 105 kDa on SDS-PAGE). In addition, it was determined that a 7% polyacrylamide gel gave the best resolution for the different ERK5 isoforms. Using this optimised assay, the lower band could be clearly distinguished from 2 upper bands representing the activated ERK5. It was even possible to detect ERK5 proteins with different degrees of phosphorylation in endothelial cells (Figure 5.1). The efficiency of gel shift assay is tightly connected to reducing both time and cost of the conducted experiment. Therefore, it was decided to use this assay in the successive experiments to monitor ERK5 activation in both cell lines and primary cells.

Interestingly, ERK5 activation followed a different pattern compared to ERK1/2 activation in the experiments presented. In HeLa cells, ERK1/2 activation was rapid and quickly returned to baseline activity (Figure 3.3), while ERK5 activation was delayed but lasted longer (Figure 3.7). This demonstrates significant differences in regard to the dynamicity of the activation of the two different MAPK pathways. Similar results have been obtained previously in

endothelial cells, where ERK1/2 activation by VEGF preceded the activation of ERK5 (Roberts et al. 2010).

Overall, an efficient assay has been established to measure ERK5 activation that was able to truthfully reproduce ERK5 activation kinetics and that was able to efficiently detect the activation of endogenous ERK5. This assay was therefore used in the remainder of the thesis to monitor the dynamics of ERK5.

7.2. Activation of the ERK5 MAPK pathway by H_2S

*The present study suggests a potential activation of ERK5 MAPK by hydrogen sulfide (H_2S). First, addition of GYY4137 (a donor of H_2S**)** promoted a qualitative shift in the electrophoretic mobility of ERK5 (Figure 4.3). Secondly, translocation of the ERK5 MAPK from the cytoplasm to the nucleus is attained by GYY4137 stimulation (Figure 4.4). In addition, this study showed a similar gel shift in HUVECs, i.e. endothelial cells (Figure 5.1).*

The increased electrophoretic mobility of ERK5 induced by H_2S followed a similar time course as activation by VEGF (Roberts et al. 2010). The optimum dose of GYY4137 (400 μM) promoted ERK5 MAPK phosphorylation maximally at 60 minutes, then the degree of activation dropped down till the baseline at 120 minutes. This indicates that ERK5 activation by H_2S is transient. Secondly, treatment of the cells with 400 μM of GYY4137 led to the translocation of the ERK5 MAPK into the nucleus. These results suggest that H_2S induces ERK5 MAPK activation as well as its transcriptional activation function.

Another interesting observation of this study was that, in contrast to stimulation by GYY4137, no gel shift could be observed following NaHS treatment. While this could be potentially related to differences in the dose required to activate ERK5 signalling, this is unlikely as a range of concentrations was tested for both chemicals (data not shown). A

more likely explanation lies in the different kinetics of H_2S release by the two different chemicals. NaHS releases H_2S almost instantly when in contact with an aqueous solution and H_2S release stops when the chemical is exhausted, usually within 15-30 minutes. In contrast, it is well known that GYY4137 releases H_2S over a much longer time in aqueous solutions. The results therefore indicate that ERK5 activation requires a sustained exposure to H_2S rather than a temporary one. The ERK5 pathway may therefore only respond to H_2S when the exposure is due to chronical changes in the body.

The ERK5 MAPK pathway and its inhibitors are regulated by wide range of mitogenic stimuli (e.g., growth factors, G protein coupled receptor agonists, cytokines) and cellular stresses (e.g., hypoxia, shear stress) (Lin et al. 2016). To date, it has been found that, in cultured rat aortic vascular smooth muscle cells, NaHS (sodium hydrogen sulphide) can inhibit an endothelin-provoked rise in p38 MAPK activity, yet upregulation in p38 MAPK activity can be attained in cultured human umbilical vein cells, where stimulation of angiogenesis was associated with these effects (Li et al. 2011). In summary, H_2S-induced cell proliferation and angiogenesis may be (partly) mediated by the activation of ERK5 MAPK pathway, leading to upregulation of phosphorylated ERK5 MAPK.

While this study indicates that H_2S may activate the ERK5 pathway in endothelial cells, the exact mechanism of activation ERK5 MAPK in human umbilical vein endothelial cells (HUVECs) by H_2S is unclear. However, it has been reported in other studies that H_2S actions may be due to K_{ATP} channel opening. Expression of K_{ATP} channels in endothelial cells occurs in the plasma membrane and in intracellular organelles. Inhibition of K_{ATP} channels by glibenclamide or 5-HD may attribute to block the migratory response to H_2S. In addition, a concentration-dependent migratory response in endothelial cells can be induced by incubation of cells with the K_{ATP} channel opener SG209, where K^+ efflux per se can control EC motility (Umaru et al. 2015).

It is established that angiogenic responses to VEGF are essential under both normal and pathophysiological conditions (Felmeden et al. 2003). Since inhibition of VEGF-stimulated angiogenic properties of endothelial cells can be acquired by the CSE inhibitor PAG or by CSE silencing, this observation suggests that exposure of endothelial cells to VEGF enhances H_2S release (Asimakopoulou et al. 2013). Indeed, it has been shown in a previous study that the output of H_2S would be increased upon incubation of human ECs with VEGF (Katsouda et al. 2018). Combined with the observation that ERK5 stimulates angiogenesis (Roberts et al. 2010), the ERK5 MAPK pathway may be mediating at least some of the effects of VEGF on endothelial cell survival as well as angiogenesis through H_2S.

If further experiments support the hypothesis that H_2S activates ERK5 MAPK, two novel hypothesis could be inferred (a) ERK5 MAPK activation by H_2S in primary endothelial cells could be a useful model in diseases states associated with poor diagnosis of reducing angiogenesis (e.g., pre-eclampsia) and (b) stimulation of endogenous ERK5 activation may be a useful target in conditions of pathological angiogenesis, particularly under conditions of deficiencies of H_2S release.

7.3. C-Terminal phosphorylation of ERK5

It is well established that the residues Threonine 218 and Tyrosine 220 in the activation loop of the ERK5 kinase domain may undergo dual phosphorylation by the upstream, activated MEK5 in response to some growth factors, cytokines and stressing events (Drew et al. 2012). However, in order to understand the mechanisms involved in ERK5 activation, particularly in endothelial cells, it was necessary to be able to evaluate the C-terminal phosphorylation of ERK5. This is important as this mechanism leads to the translocation of ERK5 to the nucleus and the activation of transcription of genes downstream of ERK5. The aim was therefore to monitor some residues in the C-terminal domain of the ERK5 that may undergo phosphorylation upon ERK5 stimulation.

Arguably, the best way to identify those phosphorylation sites is to use the mass-spectrometry (Mody et al. 2003). For this reason, mass-spectrometry was applied to recognize those phosphorylation sites in the ERK5 (especially those that reside in the C-terminal domain). In order to do this the ERK5 protein was over-produced in HeLa cells, resulting in an optimum quantity to be analyzed. In order to precipitate the ERK5 in the cell lysate, the immunoprecipitation technique was performed using an antibody against ERK5 coupled to agarose beads. Three bands were obtained after Coomassie staining, where the lower band corresponding to the inactive ERK5, and the upper two bands corresponding to the activated one. The two upper bands likely have different degrees of C-terminal phoshporylation. In general, the mass spectrometry analysis of the resulting peptides tested using two different enzymes, trypsin and chymotrypsin, cleaving the ERK5 sequence between the sample into many short peptides. The overall ERK5 sequence identified by mass spectrometry was about 39.9% of the total ERK5 protein. Unfortunately, no phosphorylation sites apart from Y220 could be identified. Indeed, the distribution of the cleavage sites for trypsin was the major problem. The majority of the predicted phosphorylation sites are located within very large tryptic (and chymotryptic) fragments that were not suitable for analysis by mass spectrometry. In addition, other fragmentation techniques such as chemical treatment (e.g., CnBr) are less reliable and in silico digestions using PeptideCutter (Expasy) indicate that the resulting fragments would still be problematic for mass spectrometric analyses. Overall, it can be inferred from the previous examples that the process of comprehensive mapping of ERK5 by mass spectrometry is challenging.

This thesis demonstrated that there are some limitation in using commercial antibodies (phosphor-specific antibodies) to measure and monitor ERK5 activation, but it was the only way for the experimental purpose to develop a novel phosphor-specific antibody that may recognize and bind to some phosphorylation sites on the C-terminal, and test its efficiency. The S770 site was used as it has been shown in the literature that it is one of the known

phosphorylation sites on the C-terminal that has not been investigated much yet (Gilley et al. 2012). To design and develop this antibody (anti-serine p770), a well-documented protocol was followed in collaboration with Eurogentec to fulfil this mission. The results in this thesis demonstrate that the antibody recognizes ERK5 effectively. However, to specifically recognize only the phosphorylated form, it was necessary to further purify the antibody using an affinity column. After purification, the anti-phospho Ser770 selectively recognized the upper (phosphorylated) bands with only weak detection of the lower band (Figure 6.7). However, it would be useful for further research to implement in vitro kinase assays using E.coli purified recombinant ERK5 (where it should not be phosphorylated) to unambiguously show whether this antibody does or does not recognize the non-phosphorylated form. Indeed, it might be interesting for further studies to rule out the fact that the lower band might be phosphorylated. The final conclusion obtained from the result using the purified antibody has showed evidently that the phosphorylated site (S770) located on the C-terminal could be clearly identified and used to monitor ERK5 phosphorylation. This antibody will be valuable to analyse the C-terminal phosphorylation of ERK5 in response to H_2S in endothelial cells in the future.

7.4. Conclusion

Overall, this thesis has shown that an optimised gel shift assay is well suited to monitor ERK5 dynamics in different cell types, including endothelial cells. In addition, it was shown that H_2S may activate ERK5 in HeLa and endothelial cells, which will be the subject of further studies. Finally, a new antibody was developed that recognizes ERK5 C-terminal phosphorylation that will be used in the future to monitor ERK5 dynamics.

Using these tools will enable more detailed studies to define the role of ERK5 in human disease as well as investigating signalling crosstalk between different MAPK pathways. Ultimately we expect that this will unravel a key role for ERK5 as a viable target for

therapeutic intervention particularly in diseases associated with deficient angiogenesis, for example preeclampsia, and in targeting cancer.

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9. Appendix: Mass spectrometry raw data

Trypsin lower (Experiment 1)

Sequence	Prob	P-score	Modifications	Observed	Stop	Actual Mass	Charge	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start
(K)AEPAHTAASVAAK(N)	100%	102.95		612.3226	35	1,222.63	2	0.000038	0.03105	19.206	1.22E+07	14,085.60	23
(K)AEPAHTAASVAAK(N)	100%	86.189		612.3226	35	1,222.63	2	0.000038	0.03105	19.635	1.22E+07	13,872.90	23
(R)RRLTGQQVAIK(K)	100%	85.958		423.9298	84	1,268.77	3	-0.000178	-0.1402	20.493	2888200	18,103.30	74
(R)RLTGQQVAIK(K)	100%	96.665		557.3406	84	1,112.67	2	-0.000182	-0.1634	22.435	2.06E+07	7,134.87	75
(R)RLTGQQVAIKK(I)	100%	139.38		621.3881	85	1,240.76	2	-0.000222	-0.1788	19.908	5412400	12,579.90	75
(R)RLTGQQVAIKK(I)	100%	97.452		414.5945	85	1,240.76	3	-0.000218	-0.1756	19.924	4594500	22,094.20	75
(R)LTGQQVAIKK(I)	100%	155		543.3375	85	1,084.66	2	-0.000222	-0.2045	21.082	1.72E+07	86,667.90	76
(K)KIPNAFDVVTNAK(R)	100%	127.83		708.8959	97	1,415.78	2	-0.000102	-0.07199	36.084	3721900	6,408.30	85
(K)KIPNAFDVVTNAK(R)	100%	116.84		708.8959	97	1,415.78	2	-0.000102	-0.07199	36.507	3721900	3,434.66	85
(K)KIPNAFDVVTNAKR(T)	100%	122.66		524.9667	98	1,571.88	3	-0.000108	-0.06866	32.478	1768200	6,223.29	85
(K)KIPNAFDVVTNAKR(T)	100%	87.184		786.9465	98	1,571.88	2	-0.000102	-0.06485	32.874	341990	1,324.75	85
(K)KIPNAFDVVTNAKR(T)	99%	75.02		524.9667	98	1,571.88	3	-0.000108	-0.06866	32.901	1768200	4,761.85	85

(K)IPNAFDVVTNAK(R)	100%	152.04		644.8484	97	1,287.68	2	-0.000062	-0.04811	41.676	1.14E+07	8,819.45	86
(K)IPNAFDVVTNAK(R)	100%	136.52		644.8484	97	1,287.68	2	-0.000062	-0.04811	41.255	1.14E+07	6,776.62	86
(K)IPNAFDVVTNAKR(T)	100%	158.47		722.899	98	1,443.78	2	-0.000062	-0.04291	36.35	2114400	6,577.04	86
(K)IPNAFDVVTNAKR(T)	100%	103.97		482.2684	98	1,443.78	3	-0.000068	-0.04707	36.301	4945400	11,060.20	86
(K)IPNAFDVVTNAKR(T)	100%	102.87		482.2684	98	1,443.78	3	-0.000068	-0.04707	36.72	4945400	4,811.46	86
(K)IPNAFDVVTNAKR(T)	97%	59.265		722.899	98	1,443.78	2	-0.000062	-0.04291	36.77	2114400	1,862.08	86
(K)DILRPTVPYGEFK(S)	99%	71.268		512.2803	131	1,533.82	3	-0.000238	-0.1551	40.761	537180	3,556.46	119
(R)YFLYQLLR(G)	99%	84.359		558.316	168	1,114.62	2	-0.000062	-0.05557	50.192	274760	2,832.09	161
(R)GLKYmHSAQVIHR(D)	99%	72.652	Oxidation (+16)	778.4117	181	1,554.81	2	-0.00007701	-0.0495	20.831	351260	1,514.93	169
(K)YMHSAQVIHR(D)	100%	131.62		621.314	181	1,240.61	2	0.000038	0.03061	20.729	3499400	7,027.24	172
(K)YmHSAQVIHR(D)	100%	120.45	Oxidation (+16)	419.8768	181	1,256.61	3	0.00003699	0.02941	18.983	3654500	25,181.70	172
(K)YmHSAQVIHR(D)	100%	93.143	Oxidation (+16)	629.3115	181	1,256.61	2	0.00004299	0.03418	18.948		8,100.79	172
(K)IGDFGMAR(G)	99%	134.66		433.7131	205	865.4116	2	-0.000142	-0.1639	32.553	1.01E+07	9,074.43	198
(R)ISAAAALRHPFLAK(Y)	100%	89.189		489.2928	349	1,464.86	3	-0.000058	-0.03957	31.663	1788500	5,107.48	336
(R)ISAAAALRHPFLAK(Y)	100%	83.137		489.2928	349	1,464.86	3	-0.000058	-0.03957	32.09	1788500	4,103.06	336
(R)ISAAAALRHPFLAK(Y)	100%	80.014		733.4355	349	1,464.86	2	-0.000042	-0.02865	31.673	418190	1,822.00	336

(K)EAIVAEIEDFHAR(R)	97%	59.426		750.3781	391	1,498.74	2	-0.000062	-0.04134	41.301	99,111.00	1,084.43	379
(R)REGIRQQIR(F)	99%	75.043		385.895	400	1,154.66	3	-0.000138	-0.1194	18.825	455170	5,481.29	392
(R)EGIRQQIR(F)	99%	138.37		500.2883	400	998.5621	2	-0.000142	-0.1421	20.351	3.64E+07	9,469.55	393
(K)AALKAALLK(S)	100%	113.89		449.8078	485	897.6011	2	-0.000122	-0.1358	26.87	1208000	7,480.71	477
(R)SRLRDGPSAPLEAPEPR(K)	98%	62.528		924.4898	505	1,846.97	2	-0.000202	-0.1093	26.034	363560	2,030.42	489
(R)SRLRDGPSAPLEAPEPR(K)	97%	71.286		616.6623	505	1,846.97	3	-0.000218	-0.118	25.977	1627300	3,822.94	489
(R)LRDGPSAPLEAPEPR(K)	98%	70.332		802.9232	505	1,603.83	2	-0.000242	-0.1508	29.311	1295400	3,318.44	491
(R)DGPSAPLEAPEPR(K)	100%	129.88		668.3306	505	1,334.65	2	-0.000222	-0.1662	32.636	4.99E+07	18,706.30	493
(R)DGPSAPLEAPEPR(K)	100%	108.31		668.3306	505	1,334.65	2	-0.000222	-0.1662	34.937	1181100	8,716.26	493

Trypsin lower (Experiment 2)

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start
(M)sRQFSSRSG(Y)	100%	122.33	Acetyl (+42)	527.2572	1,052.50	2	10	0.00009129	0.08665	20.238		12,675.40	2
(R)SGGGFSSGSAGIINYQR(R)	100%	223.08		829.4001	1,656.79	2	29	-0.000062	-0.0374	34.43	7366300	14,715.70	13
(R)FSScGGGGGSFGAGGGFGSR(S)	100%	182.72	Carbamidomethyl (+57)	883.371	1,764.73	2	65	-0.0002073	-0.1174	32.348	1.87E+07	22,593.40	46

(C)GGGGGSFGAGGGFGSR(S)	100%	123.26		642.7894	1,283.56	2	65	-0.000262	-0.204	28.307	2017500	19,676.30	50
(G)GGGGSFGAGGGFGSR(S)	100%	92.943		614.2787	1,226.54	2	65	-0.000222	-0.1808	28.342	590910	10,614.20	51
(R)SLVNLGGSKSISISVAR(G)	100%	112.91		563.3282	1,686.96	3	82	-0.000038	-0.02251	36.629	329460	8,852.13	66
(R)SLVNLGGSKSISISVAR(G)	99%	82.908		844.4887	1,686.96	2	82	-0.000042	-0.02488	36.609	317260	3,285.96	66
(L)GGSKSISISVAR(G)	100%	112.36		581.3329	1,160.65	2	82	-0.000042	-0.03616	24.353	446050	7,177.60	71
(K)SLNNQFASFIDKVR(F)	100%	102.33		546.9581	1,637.85	3	199	-0.000008	-0.004881	42.471	248130	7,524.24	186
(R)FLEQQNQVLQTK(W)	100%	182.46		738.3963	1,474.78	2	211	-0.000182	-0.1233	31.692	8705600	10,318.50	200
(R)NKYEDEINKR(T)	100%	183.72		654.8308	1,307.65	2	277	-0.000082	-0.06266	19.98	2963300	38,111.80	268
(R)NKYEDEINKR(T)	100%	140.78		436.8896	1,307.65	3	277	-0.000098	-0.07489	19.987	2213800	34,496.60	268
(N)KYEDEINKR(T)	100%	131.62		398.8753	1,193.60	3	277	-0.000128	-0.1071	18.236	4349900	50,244.40	269
(K)YEDEINKR(T)	99%	124.59		533.7618	1,065.51	2	277	-0.000082	-0.07689	18.954	2400000	65,138.80	270
(R)TNAENEFVTIK(K)	100%	139.43		633.3222	1,264.63	2	288	-0.000042	-0.03318	32.812	3950100	12,396.70	278
(R)TNAENEFVTIKK(D)	100%	208.15		697.3697	1,392.72	2	289	-0.000082	-0.05883	26.694	9986200	32,100.50	278
(R)TNAENEFVTIKK(D)	100%	166.11		465.2489	1,392.72	3	289	-0.000068	-0.04879	26.723	3372300	44,039.90	278
(K)KDVDGAYMTK(V)	100%	132.25		564.2737	1,126.53	2	298	-0.000202	-0.1792	20.712		6,697.09	289

(K)DVDGAYMTKVDLQAK(L)	100%	80.318		827.4113	1,652.81	2	304	-0.000302	-0.1826	34.201	213380	2,672.46	290
(K)VDLQAKLDNLQQE(I)	100%	99.5		757.3965	1,512.78	2	311	-0.000282	-0.1863	37.47	589280	5,434.65	299
(E)LSQmQTQISE(T)	100%	134.25	Oxidation (+16)	590.7794	1,179.54	2	332	-0.000137	-0.1161	26.376	3675500	17,823.90	323
(E)TNVILSmDNNR(S)	100%	180.09	Oxidation (+16)	646.8168	1,291.62	2	343	-0.00003701	-0.02863	26.603	1.25E+07	64,898.30	333
(K)AQYEDIAQKSK(A)	100%	166.86		640.8277	1,279.64	2	366	-0.000142	-0.1109	20.972	462140	6,841.85	356
(K)AQYEDIAQKSKAEAE(S)	100%	127.52		840.9074	1,679.80	2	370	-0.000122	-0.07258	24.225	1144300	6,981.99	356
(K)AQYEDIAQKSKAEAE(S)	100%	98.253		560.9407	1,679.80	3	370	-0.000128	-0.07615	24.195	1360100	10,553.70	356
(K)SKAEAESLYQSK(Y)	100%	187.58		670.8383	1,339.66	2	376	-0.000002	-0.001492	21.323	1898600	20,203.20	365
(K)SKAEAESLYQSKYEE(L)	100%	171.99		881.4125	1,760.81	2	379	-0.000002	-0.001135	25.788	2178800	7,615.18	365
(K)AEAESLYQSK(Y)	100%	195.05		563.2748	1,124.54	2	376	-0.000002	-0.001777	24.161	1452300	19,755.50	367
(K)AEAESLYQSKYEE(L)	100%	160.91		773.849	1,545.68	2	379	0.000018	0.01164	29.562	1226900	10,208.90	367
(E)AESLYQSKYEE(L)	100%	136.99		673.8092	1,345.60	2	379	-0.000002	-0.001485	27.625	1330200	12,664.70	369
(K)YEELQITAGR(H)	100%	200.54		590.3039	1,178.59	2	386	-0.000122	-0.1034	32.311	8426500	30,279.50	377

(R)LRSEIDNVKK(Q)	100%	125.71		601.3486	1,200.68	2	417	-0.000142	-0.1182	19.159	4251400	22,082.70	408
(R)LRSEIDNVKK(Q)	100%	128.51		401.2348	1,200.68	3	417	-0.000138	-0.1148	19.155		20,651.10	408
(K)QISNLQQSISDAEQR(G)	99%	96.64		858.9292	1,715.84	2	432	-0.000122	-0.07106	34.287	504280	4,157.30	418
(R)GSGGGSSGGSIGGRGSSSGGVK(S)	100%	151.11		876.417	1,750.82	2	624	-0.000262	-0.1496	16.954	7701500	43,121.40	603

Trypsin lower (Experiment 3)

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start
(K)AEPAHTAASVAAK(N)	99%	106.16		612.3226	1,222.63	2	35	0.000038	0.03105	1,130.46	1973100	12,170.10	23
(K)AEPAHTAASVAAK(N)	99%	76.24		408.5508	1,222.63	3	35	0.000032	0.02615	3,248.22	2.52E+07	120003	23
(R)RLTGQQVAIK(K)	100%	104.01		557.3406	1,112.67	2	84	-0.000182	-0.1634	3,252.48	2.66E+07	133076	75
(R)LTGQQVAIK(K)	100%	145.04		479.29	956.5655	2	84	-0.000182	-0.1901	3,245.88	6.14E+07	94,829.20	76
(R)LTGQQVAIK(K)	100%	110.12		479.29	956.5655	2	84	-0.000182	-0.1901	1,454.58	1480500	3,910.66	76
(K)IGDFGMAR(G)	99%	143.02		433.7131	865.4116	2	205	-0.000142	-0.1639	3,288.60	3.15E+07	159314	198
(K)IGDFGMAR(G)	99%	138.37		433.7131	865.4116	2	205	-0.000142	-0.1639	3,263.10	3.15E+07	177934	198

(R)QALSLLGR(M)	98%	134.06		429.2638	856.513	2	326	-0.000122	-0.1423	3,282.36	5.77E+07	488397	319
(R)ISAAAALR(H)	99%	163.84		386.7374	771.4603	2	343	0.000018	0.0233	3,274.20	1.38E+08	929069	336
(R)ISAAAALR(H)	99%	173.85		386.7374	771.4603	2	343	0.000018	0.0233	3,248.76	1.38E+08	140639	336
(R)LRDGPSAPLEAPEPR(K)	100%	120.87		802.9232	1,603.83	2	505	-0.000242	-0.1508	3,317.10	1.82E+07	51,597.20	491
(R)LRDGPSAPLEAPEPR(K)	100%	106.58		802.9232	1,603.83	2	505	-0.000242	-0.1508	3,342.24		32,689.90	491
(R)LRDGPSAPLEAPEPR(K)	97%	102.9		802.9232	1,603.83	2	505	-0.000242	-0.1508	3,493.50	7107100	58,721.80	491
(R)LRDGPSAPLEAPEPR(K)	95%	89.136		535.6179	1,603.83	3	505	-0.000258	-0.1608	3,493.44	1.91E+07	203389	491
(R)DGPSAPLEAPEPR(K)	100%	114.86		668.3306	1,334.65	2	505	-0.000222	-0.1662	3,142.98	9431200	37,248.10	493
(R)DGPSAPLEAPEPR(K)	100%	90.05		668.3306	1,334.65	2	505	-0.000222	-0.1662	3,168.42	9431200	51,098.80	493
(R)DGPSAPLEAPEPR(K)	100%	93.561		668.3306	1,334.65	2	505	-0.000222	-0.1662	3,246.00	3.27E+08	112869	493
(R)DGPSAPLEAPEPR(K)	100%	103.08		668.3306	1,334.65	2	505	-0.000222	-0.1662	3,296.82	3.27E+08	131637	493
(R)DGPSAPLEAPEPR(K)	100%	106.4		668.3306	1,334.65	2	505	-0.000222	-0.1662	3,193.68	9431200	52,563.90	493
(R)DGPSAPLEAPEPR(K)	100%	106.62		668.3306	1,334.65	2	505	-0.000222	-0.1662	3,271.32	3.27E+08	798500	493
(R)KPVTAQER(Q)	99%	135.77		464.7642	927.5138	2	513	-0.000082	-0.08831	3,269.82	2.86E+08	1279030	506
(R)KPVTAQER(Q)	99%	148.99		464.7642	927.5138	2	513	-0.000082	-0.08831	3,244.62	2.86E+08	125396	506

(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	116.58		1,064.02	2,126.02	2	566	-0.000322	-0.1514	3,330.36		18,268.40	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	140.03		1,064.02	2,126.02	2	566	-0.000322	-0.1514	3,356.10		10,730.80	544
(K)SQVEDPLPPVFSGTPK(G)	100%	122.46		849.4409	1,696.87	2	735	-0.000262	-0.1543	3,350.58		18,081.90	720
(K)SQVEDPLPPVFSGTPK(G)	100%	98.368		849.4409	1,696.87	2	735	-0.000262	-0.1543	3,325.08	1.15E+07	31,253.90	720

Trypsin middle

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start
(K)AEPAHTAASVAAK(N)	100%	144.55		612.3226	1,222.63	2	35	0.000038	0.03105	1,124.70	6.09E+07	27,754.10	23
(K)AEPAHTAASVAAK(N)	98%	115.57		612.3226	1,222.63	2	35	0.000038	0.03105	1,150.62	6.09E+07	26,166.60	23
(R)RLTGQQVAIK(K)	98%	128.6		557.3406	1,112.67	2	84	-0.000182	-0.1634	1,324.38		97,102.60	75
(R)LTGQQVAIK(K)	100%	172.03		479.29	956.5655	2	84	-0.000182	-0.1901	1,452.48	6.41E+07	30,016.90	76
(R)LTGQQVAIK(K)	96%	107.59		479.29	956.5655	2	84	-0.000182	-0.1901	1,479.00	6.41E+07	14,456.60	76
(R)LTGQQVAIKK(I)	100%	136.27		543.3375	1,084.66	2	85	-0.000222	-0.2045	1,227.84	1.72E+07	66,700.60	76
(K)KIPNAFDVVTNAK(R)	100%	119.03		708.8959	1,415.78	2	97	-0.000102	-0.07199	2,177.04		16,467.40	85

(K)KIPNAFDVVTNAK(R)	99%	113.69		708.8959	1,415.78	2	97	-0.000102	-0.07199	2,259.96	2008200	13,547.80	85
(K)KIPNAFDVVTNAKR(T)	100%	117.48		524.9667	1,571.88	3	98	-0.000108	-0.06866	2,266.56		16,952.00	85
(K)KIPNAFDVVTNAKR(T)	99%	109.07		524.9667	1,571.88	3	98	-0.000108	-0.06866	2,204.10		16,686.10	85
(K)KIPNAFDVVTNAKR(T)	99%	108.58		524.9667	1,571.88	3	98	-0.000108	-0.06866	2,177.52		15,302.20	85
(K)IPNAFDVVTNAK(R)	100%	144.55		644.8484	1,287.68	2	97	-0.000062	-0.04811	2,415.48	6203200	30,868.20	86
(K)IPNAFDVVTNAK(R)	100%	125.68		644.8484	1,287.68	2	97	-0.000062	-0.04811	2,455.44	6203200	16,919.20	86
(K)IPNAFDVVTNAK(R)	100%	115.7		644.8484	1,287.68	2	97	-0.000062	-0.04811	2,442.24	6203200	25,694.50	86
(K)IPNAFDVVTNAK(R)	99%	107.45		644.8484	1,287.68	2	97	-0.000062	-0.04811	2,389.74	6203200	11,820.70	86
(K)IPNAFDVVTNAKR(T)	99%	109.15		482.2684	1,443.78	3	98	-0.000068	-0.04707	2,218.26		20,946.00	86
(K)IPNAFDVVTNAKR(T)	96%	102.33		482.2684	1,443.78	3	98	-0.000068	-0.04707	2,105.58		25,123.20	86
(K)YmHSAQVIHR(D)	100%	115.49	Oxidation (+16)	629.3115	1,256.61	2	181	0.00004299	0.03418	1,125.12	1097100	12,029.90	172
(K)IGDFGMAR(G)	99%	179.16		433.7131	865.4116	2	205	-0.000142	-0.1639	1,920.42	2.11E+07	24,947.90	198
(K)IGDFGMAR(G)	99%	185.22		433.7131	865.4116	2	205	-0.000142	-0.1639	1,894.92	2.11E+07	62,174.20	198
(K)IGDFGMAR(G)	99%	98.418		433.7131	865.4116	2	205	-0.000142	-0.1639	1,869.60	2.11E+07	16,146.60	198
(R)AYIQSLPPR(Q)	99%	111.01		522.7955	1,043.58	2	304	-0.000082	-0.0785	1,875.30		15,953.30	296

(R)AYIQSLPPR(Q)	99%	111.82		522.7955	1,043.58	2	304	-0.000082	-0.0785	1,936.98		15,979.50	296
(R)AYIQSLPPR(Q)	96%	91.626		522.7955	1,043.58	2	304	-0.000082	-0.0785	1,835.10	1747000	17,482.40	296
(R)AYIQSLPPR(Q)	96%	96.492		522.7955	1,043.58	2	304	-0.000082	-0.0785	1,900.74		15,296.90	296
(R)QPVPWETVYPGADR(Q)	100%	120.77		807.8992	1,613.78	2	318	-0.000162	-0.1003	2,563.56	2184500	9,208.08	305
(R)QPVPWETVYPGADR(Q)	99%	113.69		807.8992	1,613.78	2	318	-0.000162	-0.1003	2,538.24	2184500	7,452.46	305
(R)QALSLLGR(M)	98%	117.93		429.2638	856.513	2	326	-0.000122	-0.1423	2,116.68	2.66E+07	157101	319
(R)QALSLLGR(M)	97%	107.32		429.2638	856.513	2	326	-0.000122	-0.1423	2,091.24	2.66E+07	129823	319
(R)MLRFEPSAR(I)	100%	128.61		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,971.12	6990500	40,792.90	327
(R)MLRFEPSAR(I)	100%	114.89		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,919.88	6990500	46,406.00	327
(R)MLRFEPSAR(I)	100%	116.37		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,758.42	2.06E+07	54,154.60	327
(R)MLRFEPSAR(I)	99%	126.52		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,869.12		50,659.40	327
(R)MLRFEPSAR(I)	99%	99.136		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,945.74	6990500	44,723.10	327
(R)MLRFEPSAR(I)	98%	101.25		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,996.32	6990500	34,713.30	327
(R)MLRFEPSAR(I)	98%	101.25		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,894.38	6990500	47,134.20	327
(R)MLRFEPSAR(I)	98%	113.71		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,843.74		65,133.70	327

(R)MLRFEPSAR(I)	98%	98.033		553.7924	1,105.57	2	335	-0.000022	-0.01988	2,046.84	6990500	25,813.70	327
(R)MLRFEPSAR(I)	97%	78.113		553.7924	1,105.57	2	335	-0.000022	-0.01988	2,072.34	6990500	23,697.40	327
(R)MLRFEPSAR(I)	97%	101.64		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,707.06	2.06E+07	60,523.50	327
(R)MLRFEPSAR(I)	97%	99.802		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,672.92	2.06E+07	96,325.50	327
(R)MLRFEPSAR(I)	97%	107.66		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,732.32	2.06E+07	66,010.40	327
(R)MLRFEPSAR(I)	95%	104.44		553.7924	1,105.57	2	335	-0.000022	-0.01988	1,811.22		72,111.60	327
(R)ISAAAALR(H)	99%	148.21		386.7374	771.4603	2	343	0.000018	0.0233	1,546.56	8.48E+07	24,120.90	336
(R)ISAAAALR(H)	99%	133.47		386.7374	771.4603	2	343	0.000018	0.0233	1,572.36	8.48E+07	51,590.00	336
(R)EGIRQQIR(F)	97%	147.69		500.2883	998.5621	2	400	-0.000142	-0.1421	1,195.98	2.97E+07	53,350.90	393
(K)KDGAISDNTKAALK(A)	100%	178.67		477.9316	1,430.77	3	480	-0.000258	-0.1802	1,192.08	9688300	21,824.90	467
(K)KDGAISDNTKAALK(A)	100%	139.78		716.3937	1,430.77	2	480	-0.000262	-0.183	1,223.28	606290	6,257.61	467
(K)KDGAISDNTKAALK(A)	99%	258.78		716.3937	1,430.77	2	480	-0.000262	-0.183	1,191.60	9456700	10,200.10	467
(K)DGAISDNTKAALK(A)	100%	139.32		652.3463	1,302.68	2	480	-0.000222	-0.1703	1,389.48	2.21E+07	13,668.80	468
(K)DGAISDNTKAALK(A)	99%	113.61		435.2333	1,302.68	3	480	-0.000218	-0.1672	1,391.28	5300900	16,986.60	468
(K)AALKAALLK(S)	95%	100.19		449.8078	897.6011	2	485	-0.000122	-0.1358	1,575.24	7841400	14,308.70	477

(K)AALKAALLK(S)	95%	116.9		449.8078	897.6011	2	485	-0.000122	-0.1358	1,601.10	7841400	45,752.10	477
(R)SRLRDGPSAPLEAPEPR(K)	100%	151.11		616.6623	1,846.97	3	505	-0.000218	-0.118	1,680.42	7372300	52,526.70	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	132.15		616.6623	1,846.97	3	505	-0.000218	-0.118	1,757.28	1.31E+07	45,947.20	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	137.27		616.6623	1,846.97	3	505	-0.000218	-0.118	1,626.36	1.84E+07	56,658.60	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	147.83		616.6623	1,846.97	3	505	-0.000218	-0.118	1,782.48	1.31E+07	63,677.70	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	127.05		616.6623	1,846.97	3	505	-0.000218	-0.118	1,731.84	7503900	55,977.60	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	145.94		616.6623	1,846.97	3	505	-0.000218	-0.118	1,807.80	1.31E+07	72,104.00	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	138.81		616.6623	1,846.97	3	505	-0.000218	-0.118	1,858.32	1.71E+07	59,012.40	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	138.05		616.6623	1,846.97	3	505	-0.000218	-0.118	1,599.84	1.84E+07	87,440.60	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	133.23		616.6623	1,846.97	3	505	-0.000218	-0.118	1,883.46	1.40E+07	95,425.90	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	119.92		616.6623	1,846.97	3	505	-0.000218	-0.118	1,574.52	1.84E+07	150065	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	115.58		616.6623	1,846.97	3	505	-0.000218	-0.118	1,833.18	1.31E+07	69,076.80	489
(R)SRLRDGPSAPLEAPEPR(K)	99%	110.84		616.6623	1,846.97	3	505	-0.000218	-0.118	1,651.74	1.84E+07	46,986.00	489
(R)SRLRDGPSAPLEAPEPR(K)	99%	120.6		924.4898	1,846.97	2	505	-0.000202	-0.1093	1,856.16	6913200	16,671.40	489
(R)SRLRDGPSAPLEAPEPR(K)	99%	109.44		924.4898	1,846.97	2	505	-0.000202	-0.1093	1,574.64	6482800	33,787.60	489

(R)SRLRDGPSAPLEAPEPR(K)	99%	102.72		616.6623	1,846.97	3	505	-0.000218	-0.118	1,706.58	7372300	46,311.40	489
(R)SRLRDGPSAPLEAPEPR(K)	98%	104.24		924.4898	1,846.97	2	505	-0.000202	-0.1093	1,624.92	6482800	10,256.70	489
(R)SRLRDGPSAPLEAPEPR(K)	98%	95.273		924.4898	1,846.97	2	505	-0.000202	-0.1093	1,830.84	3738000	16,237.70	489
(R)SRLRDGPSAPLEAPEPR(K)	97%	108.14		616.6623	1,846.97	3	505	-0.000218	-0.118	1,908.90		71,116.10	489
(R)SRLRDGPSAPLEAPEPR(K)	97%	92.295		924.4898	1,846.97	2	505	-0.000202	-0.1093	1,650.78	6482800	9,791.89	489
(R)SRLRDGPSAPLEAPEPR(K)	95%	77.894		924.4898	1,846.97	2	505	-0.000202	-0.1093	1,805.70	3738000	13,858.20	489
(R)LRDGPSAPLEAPEPR(K)	96%	117.93		802.9232	1,603.83	2	505	-0.000242	-0.1508	2,103.78		11,591.10	491
(K)ERGAGASGGPSTDPLAGLVLSDNDR(S)	99%	114.34		804.7298	2,411.17	3	566	-0.000378	-0.1567	2,498.76	3839200	13,114.00	542
(K)ERGAGASGGPSTDPLAGLVLSDNDR(S)	99%	111.3		804.7298	2,411.17	3	566	-0.000378	-0.1567	2,575.20	2382500	9,435.18	542
(K)ERGAGASGGPSTDPLAGLVLSDNDR(S)	96%	104.77		804.7298	2,411.17	3	566	-0.000378	-0.1567	2,550.24		12,551.70	542
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	134.58		709.6819	2,126.02	3	566	-0.000378	-0.1777	2,811.30	8.43E+07	365971	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	129.77		709.6819	2,126.02	3	566	-0.000378	-0.1777	2,862.30	8.43E+07	30,444.30	544

(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	130.47		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,920.44	3.08E+08	12,737.80	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	121.47		709.6819	2,126.02	3	566	-0.000378	-0.1777	2,785.98	8.43E+07	77,351.90	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	139.64		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,807.10	3.08E+08	659468	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	123.63		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,858.94	3.08E+08	77,175.60	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	122.33		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,863.68	3.08E+08	82,558.20	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	119.56		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,832.96	3.08E+08	142114	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	184		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,895.30	3.08E+08	28,444.00	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	117.37		1,064.02	2,126.02	2	566	-0.000322	-0.1514	2,780.22	3.08E+08	23,147.90	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	99%	108.12		709.6819	2,126.02	3	566	-0.000378	-0.1777	2,837.10	8.43E+07	66,679.70	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	98%	108.25		709.6819	2,126.02	3	566	-0.000378	-0.1777	2,886.54	8.43E+07	18,855.50	544

(K)SQVEDPLPPVFSGTPK(G)	100%	168.82		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,629.32	1.31E+08	101123	720
(K)SQVEDPLPPVFSGTPK(G)	100%	177.42		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,552.64	1.31E+08	11,567.30	720
(K)SQVEDPLPPVFSGTPK(G)	100%	172.76		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,577.78	1.31E+08	448601	720
(K)SQVEDPLPPVFSGTPK(G)	100%	120.6		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,761.50	986320	7,719.61	720
(K)SQVEDPLPPVFSGTPK(G)	100%	198.97		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,603.58	1.31E+08	193656	720
(K)SQVEDPLPPVFSGTPK(G)	100%	113.95		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,668.98	1.31E+08	55,368.60	720
(K)SQVEDPLPPVFSGTPK(G)	99%	122.18		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,643.84	1.31E+08	120640	720
(K)SQVEDPLPPVFSGTPK(G)	99%	120.55		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,684.28	1.31E+08	34,146.10	720

Trypsin upper

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start
(K)AEPAHTAASVAAK(N)	100%	118.03		612.3226	1,222.63	2	35	0.000038	0.03105	2,662.74	3318800	21,453.00	23
(R)LTGQQVAIK(K)	100%	138.24		479.29	956.5655	2	84	-0.000182	-0.1901	2,373.72	1122700	14,113.20	76
(R)LTGQQVAIK(K)	100%	129.68		479.29	956.5655	2	84	-0.000182	-0.1901	2,348.34	1122700	11,750.90	76
(R)LTGQQVAIK(K)	100%	115.46		479.29	956.5655	2	84	-0.000182	-0.1901	2,477.04	923050	17,972.90	76
(R)LTGQQVAIK(K)	100%	117.16		479.782	957.5495	2	84	0.9838	-19.73 (+1	2,040.18		8,850.75	76

									neutron corrected from 1026)				
(R)LTGQQVAIK(K)	100%	115.46		479.29	56.5655	2	84	-0.000182	-0.1901	2,151.06		10,019.70	76
(R)LTGQQVAIK(K)	100%	111.65		479.29	956.5655	2	84	-0.000182	-0.1901	2,247.30		12,572.90	76
(R)LTGQQVAIK(K)	100%	108.09		479.29	956.5655	2	84	-0.000182	-0.1901	2,297.76		8,327.39	76
(R)LTGQQVAIK(K)	100%	107.59		479.29	956.5655	2	84	-0.000182	-0.1901	2,323.02	612460	9,227.60	76
(R)LTGQQVAIK(K)	97%	96.19		479.29	956.5655	2	84	-0.000182	-0.1901	2,527.86		13,038.70	76
(R)LTGQQVAIK(K)	96%	93.143		479.29	956.5655	2	84	-0.000182	-0.1901	2,502.54		15,632.00	76
(R)LTGQQVAIK(K)	96%	101.28		479.29	956.5655	2	84	-0.000182	-0.1901	2,553.42		12,856.00	76
(R)LTGQQVAIKK(I)	100%	141.52		543.3375	1,084.66	2	85	-0.000222	-0.2045	1,223.28	3662100	20,145.20	76
(K)IGDFGMAR(G)	99%	128.86		433.7131	865.4116	2	205	-0.000142	-0.1639	2,755.38		26,754.10	198
(K)IGDFGMAR(G)	99%	157.99		433.7131	865.4116	2	205	-0.000142	-0.1639	2,806.32		31,068.10	198
(K)IGDFGMAR(G)	99%	124.59		433.7131	865.4116	2	205	-0.000142	-0.1639	1,861.14	1329500	10,456.20	198
(K)IGDFGMAR(G)	99%	111.04		433.7131	865.4116	2	205	-0.000142	-0.1639	2,725.68		21,808.00	198
(K)IGDFGMAR(G)	99%	115.46		433.7131	865.4116	2	205	-0.000142	-0.1639	2,781.24		27,476.60	198

(K)IGDFGMAR(G)	99%	115.46		433.7131	865.4116	2	205	-0.000142	-0.1639	2,701.14		19,037.60	198
(K)IGDFGMAR(G)	99%	89.369		433.7131	865.4116	2	205	-0.000142	-0.1639	2,646.00	715630	18,319.70	198
(K)IGDFGMAR(G)	98%	94.662		433.7131	865.4116	2	205	-0.000142	-0.1639	2,771.88	1376700	26,328.10	198
(K)IGDFGMAR(G)	97%	90.777		433.7131	865.4116	2	205	-0.000142	-0.1639	2,729.52		23,013.30	198
(K)IGDFGMAR(G)	95%	89.369		433.7131	865.4116	2	205	-0.000142	-0.1639	2,669.76		17,861.50	198
(R)QALSLLGR(M)	99%	120.49		429.2638	856.513	2	326	-0.000122	-0.1423	2,921.94		65,351.10	319
(R)MLRFEPSAR(I)	100%	134.48		553.7924	1,105.57	2	335	-0.000022	-0.01988	3,081.96	9162100	74,011.70	327
(R)MLRFEPSAR(I)	100%	131.22		553.7924	1,105.57	2	335	-0.000022	-0.01988	3,107.16	9162100	53,155.60	327
(R)MLRFEPSAR(I)	100%	139.88		553.7924	1,105.57	2	335	-0.000022	-0.01988	3,056.46	9162100	68,000.60	327
(R)MLRFEPSAR(I)	100%	113.66		553.7924	1,105.57	2	335	-0.000022	-0.01988	3,031.32	9162100	53,240.20	327
(R)ISAAAALR(H)	99%	128.86		386.7374	771.4603	2	343	0.000018	0.0233	2,571.06		38,953.90	336
(R)ISAAAALR(H)	99%	128.38		386.7374	771.4603	2	343	0.000018	0.0233	1,537.50	2.62E+07	12,649.70	336
(R)ISAAAALR(H)	99%	119.56		386.7374	771.4603	2	343	0.000018	0.0233	2,736.48		58,537.80	336
(R)ISAAAALR(H)	98%	108.98		386.7374	771.4603	2	343	0.000018	0.0233	2,647.02		55,185.40	336
(R)ISAAAALR(H)	98%	105.2		386.7374	771.4603	2	343	0.000018	0.0233	2,613.72		51,122.60	336
(R)ISAAAALR(H)	97%	108.98		386.7374	771.4603	2	343	0.000018	0.0233	2,584.14		39,581.90	336

(R)ISAAAALR(H)	96%	97.473		386.7374	771.4603	2	343	0.000018	0.0233	2,264.52	948270	21,590.50	336
(R)ISAAAALR(H)	96%	97.473		386.7374	771.4603	2	343	0.000018	0.0233	2,621.58		54,154.70	336
(R)ISAAAALR(H)	96%	101.21		386.7374	771.4603	2	343	0.000018	0.0233	2,490.18	2302000	35,570.00	336
(R)ISAAAALR(H)	95%	97.473		386.7374	771.4603	2	343	0.000018	0.0233	2,673.84		49,616.10	336
(R)ISAAAALR(H)	95%	97.473		386.7374	771.4603	2	343	0.000018	0.0233	2,520.24		45,634.80	336
(R)ISAAAALR(H)	95%	97.473		386.7374	771.4603	2	343	0.000018	0.0233	2,486.22	2302000	36,594.60	336
(R)EGIRQQIR(F)	99%	173.59		500.2883	998.5621	2	400	-0.000142	-0.1421	1,193.64	1.22E+07	68,365.30	393
(K)KDGAISDNTKAALK(A)	100%	159.58		477.9316	1,430.77	3	480	-0.000258	-0.1802	2,973.90	2673000	37,124.30	467
(K)KDGAISDNTKAALK(A)	100%	183.28		477.9316	1,430.77	3	480	-0.000258	-0.1802	1,192.92		14,546.90	467
(K)KDGAISDNTKAALK(A)	98%	106.55		477.9316	1,430.77	3	480	-0.000258	-0.1802	2,999.40	3030300	35,221.40	467
(R)SRLRDGPSAPLEAPEPR(K)	100%	127.05		616.6623	1,846.97	3	505	-0.000218	-0.118	3,082.02	1.04E+07	99,480.30	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	102.29		616.6623	1,846.97	3	505	-0.000218	-0.118	3,028.98	1.04E+07	33,765.90	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	99.711		616.6623	1,846.97	3	505	-0.000218	-0.118	3,056.52	1.04E+07	51,568.00	489
(R)SRLRDGPSAPLEAPEPR(K)	100%	104.78		616.6623	1,846.97	3	505	-0.000218	-0.118	3,107.10	1.04E+07	64,321.60	489
(R)LRDGPSAPLEAPEPR(K)	100%	157.52		802.9232	1,603.83	2	505	-0.000242	-0.1508	2,643.90	2176000	10,088.90	491

(R)LRDGPSAPLEAPEPR(K)	97%	109.15		802.9232	1,603.83	2	505	-0.000242	-0.1508	3,075.54	2156200	16,466.70	491
(R)DGPSAPLEAPEPR(K)	98%	100.55		668.3306	1,334.65	2	505	-0.000222	-0.1662	1,939.74	1788000	15,443.90	493
(R)KPVTAQER(Q)	99%	117.4		464.7642	927.5138	2	513	-0.000082	-0.08831	2,566.20		32,537.80	506
(R)KPVTAQER(Q)	98%	109.01		464.7642	927.5138	2	513	-0.000082	-0.08831	2,692.20		36,902.90	506
(R)KPVTAQER(Q)	96%	105.14		464.7642	927.5138	2	513	-0.000082	-0.08831	2,667.00		36,450.70	506
(R)KPVTAQER(Q)	95%	103.74		464.7642	927.5138	2	513	-0.000082	-0.08831	2,743.26		30,025.90	506
(R)KPVTAQER(Q)	95%	98.629		464.7642	927.5138	2	513	-0.000082	-0.08831	2,540.88		33,718.40	506
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	151.9		1,064.02	2,126.02	2	566	-0.000322	-0.1514	3,001.74	2397000	5,964.60	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	100%	127.71		1,064.02	2,126.02	2	566	-0.000322	-0.1514	3,004.74	2397000	5,616.92	544
(R)GAGASGGPSTDPLAGLVLSDNDR(S)	98%	103.2		709.6819	2,126.02	3	566	-0.000378	-0.1777	3,037.44	2290400	14,733.30	544
(K)SQVEDPLPPVFSGTPK(G)	95%	102.73		849.4409	1,696.87	2	735	-0.000262	-0.1543	2,957.58	1689300	8,695.23	720

Chymotrypsin lower

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start

(F)DVGDEYEIIETIGNGAY(G)	99%	154.84		929.4231	1,856.83	2	66	-0.000262	-0.141	3,289.50	1.59E+08	177872	50
(Y)EIIETIGNGAY(G)	98%	97.798		590.2982	1,178.58	2	66	-0.000142	-0.1204	2,585.58	1.14E+08	62,448.20	56
(Y)EIIETIGNGAY(G)	95%	84.743		590.2982	1,178.58	2	66	-0.000142	-0.1204	2,611.32	1.14E+08	36,809.70	56
(F)KHDNIIAIKDILRPTVPY(G)	100%	215.96		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,262.12	4.45E+09	340306	110
(F)KHDNIIAIKDILRPTVPY(G)	100%	184		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,312.76	4.45E+09	228153	110
(F)KHDNIIAIKDILRPTVPY(G)	100%	176.43		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,287.62	4.45E+09	1581060	110
(F)KHDNIIAIKDILRPTVPY(G)	100%	155.56		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,338.08	6.70E+07	97,723.20	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	137.21		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,363.58	2.66E+07	95,432.20	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	121.18		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,422.74	5357800	29,202.70	110
(F)KHDNIIAIKDILRPTVPYGEF(K)	98%	97.203		1,220.17	2,438.33	2	130	-0.000322	-0.132	2,531.58	8.82E+07	79,093.90	110
(Y)FLYQLLRGLKY(M)	100%	178.99		707.4163	1,412.82	2	172	-0.000162	-0.1146	2,705.70	3.55E+07	63,181.20	162
(F)LYQLLRGLKY(M)	99%	134.81		633.8821	1,265.75	2	172	-0.000182	-0.1437	2,249.88	1.29E+08	94,910.30	163
(Y)FMTEyVATRW(Y)	100%	150.36	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,808.42	8.02E+07	63,205.30	217
(Y)FMTEyVATRW(Y)	100%	141.52	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,833.92	8.02E+07	101209	217
(Y)FMTEYVATRW(Y)	100%	137.14		652.3106	1,302.61	2	226	0.000018	0.01381	2,480.76	6.00E+07	117482	217

(Y)FMTEyVATRW(Y)	99%	128.36	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,867.88		22,763.90	217
(Y)FmTEyVATRW(Y)	99%	118.26	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,506.80	1.87E+07	58,587.50	217
(F)MTEyVATRW(Y)	100%	144.09	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,306.52	9.68E+07	95,146.20	218
(F)mTEyVATRW(Y)	99%	122.19	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,124.54	3.57E+07	49,854.90	218
(F)MTEYVATRW(Y)	99%	118.74		578.7764	1,155.54	2	226	0.000018	0.01556	2,065.74	1.02E+08	136560	218
(F)mTEyVATRW(Y)	99%	111.95	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,176.92		46,407.00	218
(F)mTEYVATRW(Y)	99%	99.139	Oxidation (+16)	586.7739	1,171.53	2	226	0.000002991	0.002551	1,916.94	2.66E+07	101160	218
(F)mTEyVATRW(Y)	98%	89.355	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,151.18	3.57E+07	38,142.90	218
(F)mTEyVATRW(Y)	98%	87.639	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,150.28	3.57E+07	37,234.20	218
(F)MTEyVATRWY(R)	100%	140.11	Phospho (+80)	700.2912	1,398.57	2	227	0.00004652	0.03324	2,603.52	1.09E+07	69,982.60	218

(W)YRAPELMLSLHEY(T)	99%	140.78		811.4058	1,620.80	2	239	-0.000062	-0.03823	2,368.14	6.40E+08	309889	227
(Y)RAPELMLSLHEY(T)	95%	82.029		729.8741	1,457.73	2	239	-0.000082	-0.05621	2,206.26		252829	228
(Y)IQSLPPRQPVPW(E)	99%	150.27		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,315.04	4.15E+09	1.59E+07	298
(Y)IQSLPPRQPVPW(E)	99%	142		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,289.60	4.15E+09	1.06E+07	298
(Y)IQSLPPRQPVPW(E)	99%	133.99		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,365.74	4.15E+09	7006050	298
(Y)IQSLPPRQPVPW(E)	99%	121.6		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,340.48	4.15E+09	1.07E+07	298
(Y)IQSLPPRQPVPW(E)	99%	117.31		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,205.56	3314900	21,864.20	298
(Y)IQSLPPRQPVPW(E)	98%	106.88		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,033.00		24,023.00	298
(Y)IQSLPPRQPVPW(E)	98%	106.2		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,005.88		28,031.80	298
(Y)IQSLPPRQPVPW(E)	98%	105.98		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,976.60		26,514.90	298
(Y)IQSLPPRQPVPW(E)	98%	105.52		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,198.00		25,495.00	298
(Y)IQSLPPRQPVPW(E)	98%	103.55		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,566.64	237020	4,126.58	298
(Y)IQSLPPRQPVPW(E)	98%	103.26		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,264.16	4.15E+09	1679970	298
(Y)IQSLPPRQPVPW(E)	98%	100.02		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,760.00	6959200	43,271.00	298
(Y)IQSLPPRQPVPW(E)	98%	98.048		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,058.68		24,948.10	298

(Y)IQSLPPRQPVPW(E)	98%	98.048		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,270.48		20,967.80	298
(Y)IQSLPPRQPVPW(E)	97%	96.143		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,437.28		14,256.60	298
(Y)IQSLPPRQPVPW(E)	97%	94.85		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,084.12		25,285.30	298
(Y)IQSLPPRQPVPW(E)	97%	90.15		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,162.06		20,787.90	298
(Y)IQSLPPRQPVPW(E)	96%	88.948		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,816.64	7229400	42,350.00	298
(Y)IQSLPPRQPVPW(E)	96%	85.676		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,911.44		38,384.90	298
(Y)IQSLPPRQPVPW(E)	95%	83.783		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,244.68		19,557.00	298
(Y)IQSLPPRQPVPW(E)	95%	82.831		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,468.24	560010	9,520.78	298
(F)LAKYHDPDDEPDcAPPFDF(A)	98%	89.663	Carbamidomethyl (+57)	1,124.98	2,247.94	2	365	-0.0004873	-0.2167	2,370.24	2.24E+08	196344	347
(Y)HDPDDEPDcAPPFDF(A)	99%	110.53	Carbamidomethyl (+57)	887.3385	1,772.66	2	365	-0.0004073	-0.2296	2,781.24	9.24E+07	137162	351
(Y)HDPDDEPDcAPPFDF(A)	98%	96.665	Carbamidomethyl (+57)	887.3385	1,772.66	2	365	-0.0004073	-0.2296	2,755.68	9.24E+07	50,559.90	351
(F)QPSLQPVASEPGcPDVEMPSPW(A)	97%	90.759	Carbamidomethyl (+57)	1,204.55	2,407.08	2	423	-0.0002873	-0.1193	3,070.86	4.23E+07	108169	402
(F)SGTPKGSGAGY(G)	99%	111.06		491.2354	980.4563	2	741	-0.000142	-0.1447	1,115.82	6.09E+07	418293	731

(F)SGTPKGSGAGYGVGF(D)	99%	111.86		671.3253	1,340.64	2	745	-0.000182	-0.1357	1,842.90	1.79E+08	67,801.10	731

Chymotrypsin middle

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start
(F)DVGDEYEIIETIGNGAY(G)	100%	183.03		929.4231	1,856.83	2	66	-0.000262	-0.141	3,289.98	1.32E+08	131556	50
(Y)EIIETIGNGAY(G)	98%	98.458		590.2982	1,178.58	2	66	-0.000142	-0.1204	2,583.84	2.00E+08	57,247.50	56
(F)KHDNIIAIKDILRPTVPY(G)	100%	167.61		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,339.46	3.02E+07	56,817.40	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	162.46		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,263.50	8.24E+09	134894	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	130.66		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,364.66	3.87E+07	106209	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	126.71		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,288.76	8.24E+09	1426020	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	118.52		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,314.14	8.24E+09	174313	110
(F)KHDNIIAIKDILRPTVPY(G)	97%	96.707		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,426.22	6506800	26,673.30	110
(F)KHDNIIAIKDILRPTVPYGEF(K)	99%	130.06		1,220.17	2,438.33	2	130	-0.000322	-0.132	2,531.70	5.39E+07	55,144.80	110
(F)LYQLLRGLKY(M)	99%	148.04		633.8821	1,265.75	2	172	-0.000182	-0.1437	2,253.96	1.34E+07	79,449.90	163
(Y)FMTEyVATRW(Y)	100%	180.44	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,832.84	3.98E+08	924369	217

(Y)FmTEyVATRW(Y)	100%	138.06	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,502.12	9.06E+07	110367	217
(Y)FMTEyVATRW(Y)	100%	118.33	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,807.34	3.98E+08	55,787.00	217
(Y)FmTEyVATRW(Y)	100%	117.53	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,646.66	4.67E+07	54,807.60	217
(Y)FmTEyVATRW(Y)	100%	113.22	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,568.24	4.67E+07	37,754.90	217
(Y)FMTEyVATRW(Y)	100%	112.11	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,858.04	3.98E+08	78,446.50	217
(Y)FmTEyVATRW(Y)	100%	104.01	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,594.46	4.67E+07	130910	217
(Y)FmTEyVATRW(Y)	100%	92.051	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,620.56	4.67E+07	69,875.80	217
(Y)FMTEYVATRW(Y)	100%	152.47		652.3106	1,302.61	2	226	0.000018	0.01381	2,479.20	9788600	63,418.80	217
(Y)FmTEyVATRW(Y)	99%	70.552	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,701.38	6969200	32,095.30	217

(Y)FmTEyVATRW(Y)	99%	68.846	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,674.86		41,896.60	217
(Y)FmTEyVATRW(Y)	98%	57.598	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,540.40	2866600	32,599.50	217
(F)MTEYVATRW(Y)	100%	130.56		578.7764	1,155.54	2	226	0.000018	0.01556	2,070.00	2.74E+07	147281	218
(F)MTEyVATRW(Y)	100%	164.96	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,305.02	4.97E+08	145808	218
(F)MTEyVATRW(Y)	100%	128.36	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,331.00	4.97E+08	167102	218
(F)mTEyVATRW(Y)	100%	124.98	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,158.08	1.47E+07	81,984.40	218
(F)mTEyVATRW(Y)	100%	121.96	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,132.58	1.58E+08	658446	218
(F)mTEyVATRW(Y)	99%	114.5	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,184.06	1.47E+07	80,127.40	218
(F)MTEyVATRW(Y)	99%	93.143	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,376.72		36,360.10	218
(F)mTEyVATRW(Y)	98%	89.355	Oxidation (+16),	626.757	1,251.50	2	226	0.00001151	0.009189	2,210.22	1.47E+07	51,338.90	218

			Phospho (+80)										
(W)YRAPELMLSLHEY(T)	99%	104.82		811.4058	1,620.80	2	239	-0.000062	-0.03823	2,366.16	8.78E+08	91,196.80	227
(W)YRAPELmLSLHEY(T)	99%	95.483	Oxidation (+16)	819.4032	1,636.79	2	239	-0.00005701	-0.03481	2,206.20	3.77E+08	218129	227
(W)YRAPELMLSLHEY(T)	98%	88.134		811.4058	1,620.80	2	239	-0.000062	-0.03823	2,395.62	8.78E+08	56,004.20	227
(Y)RAPELmLSLHEY(T)	99%	115.23	Oxidation (+16)	737.8716	1,473.73	2	239	-0.00007701	-0.05222	2,056.44	2.04E+08	324882	228
(Y)RAPELMLSLHEY(T)	96%	75.652		729.8741	1,457.73	2	239	-0.000082	-0.05621	2,209.92	2.84E+08	211733	228
(Y)IQSLPPRQPVPW(E)	100%	170.47		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,362.26	6.31E+09	9824990	298
(Y)IQSLPPRQPVPW(E)	99%	134.05		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,286.30	6.31E+09	1.37E+07	298
(Y)IQSLPPRQPVPW(E)	99%	128.86		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,311.50	6.31E+09	1.83E+07	298
(Y)IQSLPPRQPVPW(E)	99%	125.97		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,235.38		18,711.60	298
(Y)IQSLPPRQPVPW(E)	98%	117.31		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,005.58	6641900	27,893.80	298
(Y)IQSLPPRQPVPW(E)	98%	111.46		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,336.88	6.31E+09	1.50E+07	298
(Y)IQSLPPRQPVPW(E)	98%	109.66		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,086.46		28,446.90	298
(Y)IQSLPPRQPVPW(E)	98%	106.88		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,057.96		27,598.80	298
(Y)IQSLPPRQPVPW(E)	98%	105.1		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,262.74		24,485.70	298

(Y)IQSLPPRQPVPW(E)	98%	104.2		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,779.80	9243600	60,167.60	298
(Y)IQSLPPRQPVPW(E)	97%	101.65		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,975.16	7861700	33,151.00	298
(Y)IQSLPPRQPVPW(E)	97%	101.65		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,031.44	6641900	33,042.70	298
(Y)IQSLPPRQPVPW(E)	97%	101.65		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,181.80	4497400	25,196.60	298
(Y)IQSLPPRQPVPW(E)	97%	96.665		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,887.20		38,793.90	298
(Y)IQSLPPRQPVPW(E)	97%	96.143		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,860.98		40,938.80	298
(Y)IQSLPPRQPVPW(E)	96%	94.309		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,751.42	9243600	58,016.10	298
(Y)IQSLPPRQPVPW(E)	96%	93.598		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,207.84	4497400	27,309.80	298
(Y)IQSLPPRQPVPW(E)	96%	93.111		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,806.68	7458000	57,227.60	298
(Y)IQSLPPRQPVPW(E)	96%	93.111		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,833.32	9652100	47,763.70	298
(Y)IQSLPPRQPVPW(E)	96%	91.265		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,524.64		10,383.50	298
(Y)IQSLPPRQPVPW(E)	96%	91.087		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,898.48		55,476.00	298
(Y)IQSLPPRQPVPW(E)	96%	90.906		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,441.72		11,536.20	298
(Y)IQSLPPRQPVPW(E)	96%	89.886		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,387.40	6.31E+09	8580340	298
(Y)IQSLPPRQPVPW(E)	96%	89.886		709.4012	1,416.79	2	309	-0.000202	-0.1425	3,495.00		8,127.80	298

(F)LAKYHDPDDEPDcAPPFDF(A)	99%	93.766	Carbamidomethyl (+57)	1,124.98	2,247.94	2	365	-0.0004873	-0.2167	2,368.86	2.61E+08	135160	347
(Y)HDPDDEPDcAPPFDF(A)	99%	103.88	Carbamidomethyl (+57)	887.3385	1,772.66	2	365	-0.0004073	-0.2296	2,784.78	1.10E+08	74,260.20	351
(Y)HDPDDEPDcAPPFDF(A)	97%	77.379	Carbamidomethyl (+57)	887.3385	1,772.66	2	365	-0.0004073	-0.2296	2,758.32	1.10E+08	70,321.40	351
(F)QPSLQPVASEPGcPDVEMPSPW(A)	99%	117.03	Carbamidomethyl (+57)	1,204.55	2,407.08	2	423	-0.0002873	-0.1193	3,071.34	8.31E+07	49,823.50	402
(F)QPSLQPVASEPGcPDVEmPSPW(A)	95%	88.552	Carbamidomethyl (+57), Oxidation (+16)	1,212.55	2,423.08	2	423	-0.0004023	-0.166	2,865.60	3.31E+07	56,764.20	402
(F)SGTPKGSGAGY(G)	99%	159.28		491.2354	980.4563	2	741	-0.000142	-0.1447	1,123.80	3.49E+07	172296	731
(F)SGTPKGSGAGYGVGF(D)	99%	126.66		671.3253	1,340.64	2	745	-0.000182	-0.1357	1,831.50	1.18E+08	78,660.70	731
(F)SGTPKGSGAGYGVGFDLEEF(L)	97%	99.481		987.9576	1,973.90	2	750	-0.000282	-0.1428	2,760.48	1.79E+07	40,065.90	731

Chymotrypsin upper

Sequence	Prob	P-score	Modifications	Observed	Actual Mass	Charge	Stop	Delta Da	Delta PPM	Retention Time	Intensity	TIC	Start

(F)DVGDEYEIIETIGNGAY(G)	100%	113.93		929.4231	1,856.83	2	66	-0.000262	-0.141	3,295.92	6.00E+07	52,445.20	50
(Y)EIIETIGNGAY(G)	100%	126.28		590.2982	1,178.58	2	66	-0.000142	-0.1204	2,582.04	1.75E+07	76,961.00	56
(F)KHDNIIAIKDILRPTVPY(G)	100%	172.09		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,258.88	2.10E+09	881711	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	130.47		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,361.84	1.77E+07	50,341.10	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	126.71		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,336.40	2.10E+09	55,910.00	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	116.58		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,285.04	2.10E+09	794492	110
(F)KHDNIIAIKDILRPTVPY(G)	99%	114.7		1,053.61	2,105.20	2	127	-0.000422	-0.2004	2,310.54	2.10E+09	223703	110
(F)KHDNIIAIKDILRPTVPYGEF(K)	100%	115.8		1,220.17	2,438.33	2	130	-0.000322	-0.132	2,544.96	2.13E+07	106670	110
(F)LYQLLRGLKY(M)	100%	161.9		633.8821	1,265.75	2	172	-0.000182	-0.1437	2,242.14	7.64E+07	108831	163
(F)LYQLLRGLKY(M)	99%	90.37		633.8821	1,265.75	2	172	-0.000182	-0.1437	2,274.72		18,806.80	163
(Y)QLLRGLKY(M)	99%	144.29		495.8084	989.6022	2	172	-0.000162	-0.1635	1,744.02	8.67E+08	881887	165
(Y)FMTEyVATRW(Y)	100%	129.42	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,804.22	1.27E+08	129570	217
(Y)FMTEyVATRW(Y)	100%	125.16	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,829.72	1.27E+08	259594	217
(Y)FMTEyVATRW(Y)	100%	120.12	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,855.40		60,895.60	217
(Y)FMTEyVATRW(Y)	100%	116.52	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,882.64	3983800	50,345.00	217

(Y)FMTEYVATRW(Y)	100%	109.71		652.3106	1,302.61	2	226	0.000018	0.01381	2,472.72	8695200	86,260.20	217
(Y)FMTEyVATRW(Y)	100%	107.69	Phospho (+80)	692.2938	1,382.57	2	226	0.00002652	0.01917	2,803.62	1.27E+08	108923	217
(Y)FmTEyVATRW(Y)	99%	106.42	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,520.54	4.34E+07	105741	217
(Y)FmTEyVATRW(Y)	99%	89.247	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,493.78	4.34E+07	106202	217
(Y)FmTEyVATRW(Y)	97%	78.113	Oxidation (+16), Phospho (+80)	700.2912	1,398.57	2	226	0.00003151	0.02251	2,593.32	8534500	199592	217
(F)MTEyVATRW(Y)	100%	157.91	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,316.96	1.72E+08	109433	218
(F)MTEyVATRW(Y)	100%	146.81	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,291.46	1.72E+08	67,002.80	218
(F)mTEyVATRW(Y)	100%	146.81	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,130.24	7.54E+07	62,872.90	218
(F)mTEyVATRW(Y)	100%	138.54	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,156.16	1.23E+07	67,320.30	218
(F)MTEyVATRW(Y)	100%	136.8	Phospho (+80)	618.7596	1,235.50	2	226	0.000006518	0.005271	2,342.52		25,423.40	218

(F)mTEyVATRW(Y)	100%	120.07	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,182.86	1.23E+07	41,134.10	218
(F)MTEYVATRW(Y)	99%	104.44		578.7764	1,155.54	2	226	0.000018	0.01556	2,054.28	1.18E+07	64,395.90	218
(F)mTEyVATRW(Y)	99%	101.54	Oxidation (+16), Phospho (+80)	626.757	1,251.50	2	226	0.00001151	0.009189	2,209.14	1.23E+07	27,406.00	218
(W)YRAPELMLSLHEY(T)	100%	129.89		811.4058	1,620.80	2	239	-0.000062	-0.03823	2,364.42	2.56E+08	138356	227
(Y)RAPELMLSLHEY(T)	99%	109.83		729.8741	1,457.73	2	239	-0.000082	-0.05621	2,197.14	7.81E+07	108402	228
(Y)IQSLPPRQPVPW(E)	100%	134.25		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,354.34	2.43E+09	4573710	298
(Y)IQSLPPRQPVPW(E)	100%	128.86		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,328.72	2.43E+09	6502530	298
(Y)IQSLPPRQPVPW(E)	99%	109.66		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,252.46	2.43E+09	664244	298
(Y)IQSLPPRQPVPW(E)	99%	109.66		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,302.98	2.43E+09	8782000	298
(Y)IQSLPPRQPVPW(E)	99%	107.21		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,379.72	2.43E+09	2698400	298
(Y)IQSLPPRQPVPW(E)	99%	106.88		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,277.72	2.43E+09	8356940	298
(Y)IQSLPPRQPVPW(E)	99%	91.265		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,410.74	2.43E+09	1999810	298
(Y)IQSLPPRQPVPW(E)	98%	87.258		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,951.52		27,838.40	298

(Y)IQSLPPRQPVPW(E)	98%	84.615		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,384.88	2.43E+09	3343420	298
(Y)IQSLPPRQPVPW(E)	97%	79.148		709.4012	1,416.79	2	309	-0.000202	-0.1425	2,882.04		42,883.70	298
(F)EPSARISAAAALRHPF(L)	96%	172.58		565.3093	1,692.91	3	346	0.000012	0.007084	1,835.94	7046300	58,078.00	331
(F)LAKYHDPDDEPDcAPPFDF(A)	99%	103.14	Carbamidomethyl (+57)	1,124.98	2,247.94	2	365	-0.0004873	-0.2167	2,363.40	7.05E+07	41,651.90	347
(F)SGTPKGSGAGYGVGF(D)	100%	132.17		671.3253	1,340.64	2	745	-0.000182	-0.1357	1,830.72	6912700	35,164.80	731