The effects of JNK isoform knockdown on cell growth and death in HUVECs and MCF-7 cells.

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This thesis is the result of the author's original research. It has been composed by the author and had not been previously submitted for examination which has led to the award of a degree.

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Abstract

Cardiovascular disease (CVD) and cancer are two of the leading causes of mortality worldwide. The JNK pathway has been shown to play key roles at various stages of both of these diseases and therefore is an important protein to try and understand. In animal models of atherosclerosis and breast cancer, inhibition of JNK has been demonstrated to reduce pathogenesis and therefore targeting this protein may be key for developing treatments. JNK exists as three individual proteins, JNK1, JNK2 and JNK3 and studies are now showing that not only can these proteins work independently but also opposingly in some instances and therefore understanding the function of the individual isoforms is becoming critical to fully understanding this pathway. Although more research is focusing on JNK isoform function, characterisation of each JNK protein has not yet been carried out in human primary vascular cells or a human breast cancer cell line, an investigation which must be carried out to understand the role of this pathway in the pathogenesis of these diseases. In the current study lentiviral shRNA was used to target and knockdown JNK1 and JNK2 in both human umbilical vein endothelial cells (HUVECs) and MCF-7 breast cancer cells and the effects of knockdown on cell growth and cell death processes were analysed.

In HUVECs knockdown of JNK2 caused an increase in pc-Jun levels and an increase in the percentage of multinucleated cells was observed, suggesting JNK2 may play a role in HUVEC cell growth. Unfortunately, the lentiviral infection itself caused detrimental effects which made it difficult to continue experiments and explore these findings further. In MCF-7 cells JNK knockdown did not produce any changes in cell growth or induced cell death when compared to non-target controls, suggesting that JNK does not play a key role in this breast cancer cell line.

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A PhD is definitely a journey and having the best people around you really helps make it a good one.

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Abbreviations

AAA Abdominal aortic aneurysm

AD Alzheimer disease

AJ Adheren junction

AMPK Adenosine monophosphate-activated protein kinase

AP-1 Activator protein - 1

APO Apolipoprotein

APP Amyloid precursor protein

APS Ammonium persulfate

ASK Apoptosis-signal regulating kinase

ATF Activating transcription factor

ATP Adenosine triphosphate

ATRAP Angiotensin II type 1 receptor associated protein

BAD Bcl-2-associated death

BAEC Bovine aortic endothelial cell

Bax Bcl2 associated X

Bcl-Xl B-cell lymphoma-extra-large

Bid BH3 interacting-domain

BSA Bovine Serum Albumin

cAMP cyclic mononucleotide adenosine-3', 5'-phosphoric acid

CDK Cyclin dependent kinase

CDK2 Cyclin-dependent kinase 2

cIAP1 cellular inhibitor of apoptosis 2

CIN Chromosomal instability

CSC cancer stem cell

CVD Cardiovascular disease

DAPI 4',6-diamidino-2-phenylindole

DEN Diethylnitrosamine (DEN).

DMEM Dulbecco's Modified Eagle Medium

DUSP dual specificity phosphatase

E.coli *Escherichia coli*

EC Endothelial cells

ECL Enhanced chemiluminescence

ECM Extracellular matrix

EGM^{TM}-2 Endothelial Growth Media -2

EMB-2 Endothelial Cell Basal Medium -2

EMT Epithelial-mesenchymal transition

eNOS endothelial nitric-oxide synthase

ERK Extracellular Signal -Related Kinase

FACS Fluorescence Activating Cell Sorting

GFP Green fluorescent protein

Grb2 Growth factor receptor-bound protein 2

H_2O_2 Hydrogen peroxide

HAEC Human aortic endothelial cell

HASMC Human aortic smooth muscle cell

HCC Hepatocellular carcinoma

HEK Human Embryonic Kidney

HLMVEC Human lung microvascular endothelial cell

HUVEC Human umbilical vein endothelial cells

ICAM-1 Intercellular adhesion molecule – 1

IGF-1 Insulin-like growth factor - 1

IL-2 Interleukin – 2

iNOS inducible NOS

JIP JNK interacting protein

JNK c-Jun-N-terminal kinase

KO Knockout

LB Luria Broth

LDLs Low density lipoproteins

LMP-1 Ep- stein–Barr virus latent membrane protein-1

LSB Laemmli's sample buffer

MAP2 Microtubule-associated protein 2

MAPK Mitogen activated protein kinase

MAPKK MAP Kinase Kinase

MAPKKK MAP Kinase Kinase Kinase

MIF Migration inhibitory factor

MKP Map kinase phosphatase

MM Multiple myeloma

MMP Metalloproteinase

mTOR mammalian target of rapamycin

MTT 3-(4,5-dimethylthiaxolyl-2)-2,5-diphenyltetrazolium bromide

NAP Novel proangiogenic glycoprotein

NFAT Nuclear factor of activated T-cell

NF-κB Nuclear factor – κB

NO Nitric oxide

NSCLC Non-small cell lung cancer

NT Non-target

oxLDLs Oxidised LDLs

PARP Poly (ADP-ribose) polymerase

PASMC pulmonary artery smooth muscle cell

pATF2 Phosphorylated ATF2

PBS Phosphate Buffered Saline

PD Parkinson's Disease

PDGF Platelet derived growth factor

PH Pulmonary hypertension

PI Propidium Iodide

PPARγ Proliferator-activated receptor γ

RNAse A Ribonuclease A

ROS Reactive oxygen intermediates

RT Room temperature

SEK-1 SAPK/ERK kinase -1

SEM Standard errors of the mean

SGK serum and glucocorticoid-regulated kinase 1

shRNA Short hairpin RNA

siRNA Small interfering RNA

Smac Second mitochondria-derived activator of caspase

TF Transcription factors

Thr-X-Tyr Threonine-X-Tyrosine

TNF Tumour necrosis factor

TRAF2 TNF receptor associated factor 2

TRAIL TNF related apoptosis inducing ligand

TrxR Thioredoxin reductase

UV Ultraviolet

VCAM-1 vascular cell adhesion protein -1

VEGF Vascular endothelial growth factor

VMSC Vascular smooth muscle cell

WDR62 WD40-repeat protein 62

WT Wild type

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Chapter One

Introduction

1.1 Cellular signalling

For multicellular organisms to function correctly they rely on complex networks of intracellular signalling pathways within cells to assemble and execute coordinated and specific physiological responses to various stimuli. When defects occur in these pathways, signalling can stop functioning properly which may result in changes in the response and outcome. These changes can underpin the development of disease and therefore over the years a huge amount of research has gone into trying to understand these pathways so that these changes can be prevented or symptoms can be treated.

Signalling can be activated by a number of different stimuli including, but not limited to: ligands such as epidermal growth factor (Barrandon and Green, 1987), soluble factors such as; cyclic mononucleotide adenosine-3', 5'-phosphoric acid (cAMP) (Rall and Sutherland, 1957) and direct contact with intracellular structures such as the cytoskeleton via integrins (Jones and Walker, 1999), which can all activate cell receptors. The type of stimuli and receptor activated determines the downstream pathway in which the signal is transduced. Different types of receptors include: cytokine receptors (Bagley *et al.*, 1997), G protein coupled receptors (Lagerström and Schiöth, 2008), ligand gated ion channels (Ortells and Lunt, 1995), integrins (Calderwood, Shattil and Ginsberg, 2000) and receptor tyrosine kinases (Ullrich and Schlessinger, 1990). Many pathways are linked to specific cell processes, for example p53 signalling is linked to cell survival (Lukin *et al.*, 2015) and mammalian target of rapamycin (mTOR) signalling has been linked to cell metabolism (Hagiwara *et al.*, 2012). On the other hand, signalling via c-Jun-N-terminal kinase (JNK) is involved in a variety of different cellular processes from cell growth (Zhang *et al.*, 2005), to immune responses (Conze *et al.*, 2002) to cell death (Deng *et al.*, 2003).

Receptors can recruit adaptors proteins to amplify a signal or link to downstream targets such as mitogen activated protein kinase (MAPK) signalling cascades. These cascades are key in the development, survival and regulation of cells and provide a network for cell to cell communication. Therefore, defects in these signalling pathways have been shown to contribute greatly to a number of different diseases. This chapter focuses on MAPK signalling and more specifically on the stress activated protein kinase pathway, also known as the JNK pathway.

1.2 The MAP kinase pathway

The first MAPK was identified in the 1980s by Ray and Sturgill who used extracts from adipocytes to demonstrate that a kinase transduced a signal from the insulin receptor to microtubule-associated protein 2 (MAP2) through phosphorylation and therefore named this enzyme MAP2 kinase (Ray and Sturgill, 1987). Confirming this initial observation they used hydrophobic interaction chromatography from extracts of the same cells to show that the MAP2 kinase was activated through phosphorylation itself on tyrosine and threonine residues (Ray and Sturgill, 1988). The group produced the same results using fibroblasts and revealed that MAP2 kinase comigrated with pp42, a kinase involved in cell cycle regulation. Both kinases responded to the same mitogenic stimulants and it was thus concluded that these kinases were the same protein and the name subsequently changed from microtubule associated protein 2 kinase to MAPK (Rossomando *et al.*, 1989). Soon after this, researchers began investigating these proteins at the genetic level and the assembly of the first MAPK pathway commenced (Boulton *et al.*, 1990; Ahns *et al.*, 1991; Crews, Alessandrini and Erikson, 1992). For a more detailed discussion of the discovery of MAPK please see review by Joseph Avruch (Avruch, 2007).

The MAPK signalling pathways are present in all cells throughout the human body. These pathways are conserved throughout eukaryotic cells and the signalling cascades are activated by stimuli such as stress, inflammation, infection, growth factors and hormones (Kyriakis and Avruch, 2011). The four major MAPK pathways are; The Extracellular Signal -Related Kinase (ERK) 1/2, ERK5, p38 MAPK and JNK pathways. All of these pathways follow the same core structure of signalling which begins with an external signal initiating activation of a MAP Kinase Kinase Kinase (MAPKKK), which in turn activates a MAP Kinase Kinase (MAPKK) through phosphorylation followed by activation of a MAPK by phosphorylation. Finally, the MAPKs can phosphorylate specific transcription factors (TFs) or other cellular proteins which leads to activation of cellular processes.

The activation of the MAPKs is largely consistent through all the pathways, where the MAPKK performs dual phosphorylation on Threonine and Tyrosine residues within a conserved Threonine-X-Tyrosine (Thr-X-Tyr) motif located in the kinase domain

(Ray and Sturgill, 1988)(Cargnello and Roux, 2011). In ERK for example, the amino acid represented by X is glutamic acid, whereas in p38 MAPK it is Glycine (Wilson *et al.*, 1996). However one of the few exceptions concerning the conserved activation motif is the atypical MAPK ERK3 which contains Serine – Glutamic acid – Glycine in place of Thr-X-Tyr (Gonzalez *et al.*, 1992). The differences in sequence and topology of the different MAPKs allow the targeting of specific downstream substrates by each pathway (reviewed in (Cargnello and Roux, 2011), a critical component in specificity.

The individual MAPK pathways are involved in a variety of different physiological responses and processes which are important for cell survival and regulation. For example, the ERK MAPK pathway plays a major role in cell proliferation through regulation of the cell cycle (Chambard *et al.*, 2007) and also in cell metastasis (Lee *et al.*, 2013). The p38 MAPK pathway has been shown to be involved in cell apoptosis (Potapova *et al*.,2013) and to play a role in regulation of the immune response (Conze *et al.*, 2000), whereas the ERK5 MAPK pathway is involved in anti-apoptotic signalling and cell survival (Drew *et al*., 2011) . In contrast the JNK pathway has an overlapping but distinct functional profile dependent on cell type and extracellular stimuli, for a general overview see (Dhanasekaren and Reddy, 2008; Ma *et al.*, 2012). This will be integrated more later in this chapter.

A key aspect of MAPK regulation not studied here is the dephosphorylation by a number of different proteins including the dual specificity phosphatases (DUSPs) also known as map kinase phosphatases (MKPs) (Peti and Page, 2013). Studies have demonstrated that regulation of MAPKs by MKPs can be beneficial. For example MKP-1 has been linked to neuroprotection in a model of Huntington's disease (Taylor *et al.*, 2013) and overexpression of MKP-2 in breast cancer cells was shown to increase sensitivity to tamoxifen, a drug used to prevent recurrence of breast cancer (Haagenson *et al.*, 2014). MKP-3 has also been linked to the survival of patients with non-small cell lung cancer (NSCLC) and is a possible marker for relapse free patients (Chen *et al.*, 2007). In contrast to these positive roles of MKPs, studies have demonstrated a role for MKP-1 in promoting tumorigenicity in pancreatic cancer cells, where downregulation of MKP-1 resulted in decreased cell proliferation and tumorigenicity (Liao *et al.*, 2003). Along with regulatory proteins, the duration of activation

(Chalmers *et al.*, 2007), cellular location and presence of different interacting proteins can also determine the outcome of MAPK signalling (McKay and Morrison, 2007). This is particularly true for ERK where adapter protein GRB2 is required for transmission of signals from the membrane to activate the downstream pathway (Tanaka *et al.*, 1995). The reader here is directed to reviews by Patterson and Imajo for more information on the regulation of MAPK signalling by phosphatases (Patterson *et al.*, 2009) (Imajo, Tsuchiya and Nishida, 2006).

1.2.2 MAPK in disease

As mentioned previously, the MAPK pathways are involved in many important physiological signalling processes within cells, this means that they have the potential to play significant roles in a large number of different diseases including; diabetes and obesity (Zhao and Stephens, 2013), neurodegenerative disease (Dapper *et al.*, 2013), kidney disease (Yamaguchi *et al.*, 2004) and cancer (Dhillon *et al.*, 2007). MAPK pathways also play a key role in inflammatory response induction through activation of inflammatory gene expression and a considerable amount of work has been accumulated in this area (Kaminska, 2005). This has linked MAPK signalling to the development of inflammatory diseases including; rheumatoid arthritis (Paunovic and Harnett, 2013), septic shock (Lugrin *et al.*, 2013), lung inflammation (Pan *et al.*, 2014) intestinal inflammation (Bobo *et al.*, 2013) and Alzheimer's disease (Ghasemi *et al.*, 2014).

For the diseases mentioned above, each kinase pathway has been shown to play specific roles. For example, the JNK and p38 signalling pathways are most commonly linked to diseases where induction of inflammation or cell death leads to pathogenesis whereas ERK signalling tends to play a role in diseases where an increase in cell survival signalling is detrimental. The balance between these pathways can be observed by examining a number of conditions such as Parkinson's Disease (PD) and Alzheimer's disease. JNK signalling contributes to the neuropathology of Parkinson's Disease through induction of neuronal cell death (Mathiasen *et al.*, 2004). This was demonstrated by using neuroblastoma cells with a dominant negative form of MLK3, a regulator upstream of JNK. The inhibition of JNK signalling prevented cell death mediated by the neurotoxic metabolite $MPP^{+,}$ suggesting JNK would be a promising target for treatment. JNK signalling also mediates apoptosis in transplanted dopamine

neurons which are used for the treatment of Parkinson's Disease, this contributes to the low survival rate of transplanted cells and limits the use of this procedure as a treatment (Rawal *et al.*, 2007).

In contrast to the dominant role of JNK in Parkinson's disease, all three MAPK are activated in Alzheimer disease (AD) at different stages of disease progression (Zhu *et al.*, 2001). This would suggest that both mitogenic and stress signalling is required for Alzheimer pathogenesis. More specifically JNK signalling has been shown to regulate the cleavage and degradation of amyloid precursor protein (APP) which produces beta-amyloid peptides, one of the main pathological features of Alzheimer's disease (Colombo *et al.*, 2009). In addition, p38 signalling has been linked to tau tangle formation, another pathological feature of AD (Kelleher *et al.*, 2007). In this study levels of p38 were increased in a mouse model of AD which correlated with the amounts of tau present in abnormal conformation, linking p38 with the development of taupathy. In contrast to the visible pathogenesis caused by p38 and JNK, dysregulated ERK signalling has been linked to cognitive impairment in humans and animal models of AD (Jahrling *et al.*, 2014). This group demonstrated a novel interaction between pERK and the nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) which correlated with a higher mental state score in patients. These publications demonstrate the different roles each MAPK pathway can play in the pathogenesis of one disease and highlight the importance of investigating each pathway in different disease settings.

A large amount of research has investigated the role of ERK in cancer. This is because in approximately one third of cancers there is a mutation in intermediates of the ERK signalling pathway, which leads to cell growth and promotion of survival and tumorigenesis, detailed review in (Samatar and Poulikakos, 2014). A clear example of this is mutations in the Ras-Raf-MEK-ERK signalling pathway. Mutations in Ras activation occurs in many different cancers, with a prevalence of approximately 90% in pancreatic cancer and 50% in colon and thyroid cancer (Roberts and Der, 2007). Similarly, mutations in BRAF (Raf gene) also occur in many cancers. One study, which screened different cancer cell lines for BRAF mutations, found that approximately 59% of melanoma, 18% of colorectal and 3% of lung cancer cell lines

contained mutations in BRAF (Davies *et al.*, 2002). Mutations in Ras and Raf can lead to dysregulation in the signalling cascade which can promote tumorigenesis and growth.

In contrast to cancer, where ERK signalling is targeted and inhibited for treatment, for example the drug Vemurafenib is used to treat melanoma patients (Chapman *et al.*, 2011), it has been demonstrated that activating ERK signalling in a mouse model of arthritis can suppress inflammation (Arce *et al.*, 2011). In this study a mouse model of arthritis was produced with a constitutively active MEK-1, an upstream activator of ERK. When ERK was selectively activated there was an increase in the anti-inflammatory Treg immune cells which caused inhibition of the arthritis developed in the animal models. The disease dependent roles of ERK signalling further highlights the complexity of MAPK signalling in disease.

Another example of dysregulated MAPK signalling in disease, is the role of p38 in inflammatory function. This kinase has been shown to regulate genes involved in the inflammatory response which contributes to the chronic intestinal inflammation of Crohn's disease. In this condition, inhibition of p38 by the inhibitor SB203580 resulted in suppression of the transcription of pro-inflammatory cytokines, IL-2, TNF-α and IFN-γ, in a mouse model of Crohn's disease (Hollenbach *et al.*, 2005). The role of p38 in inflammatory signalling has also been investigated in inflammatory bowel disease (Feng and Li, 2011), rheumatoid arthritis (Page *et al.*, 2010) and multiple sclerosis (Huang *et al.*, 2012). All of which demonstrate the possible detrimental effects of p38 signalling in disease.

Although there is evidence for all three of the MAPK pathways to play important roles in both survival and disease processes, the JNK pathway has many overlapping roles and more evidence is accumulating for this stress activated protein kinase to play a more mitogenic role. The remainder of this thesis will focus on the JNK signalling pathway and more specifically the role of JNK in disease.

1.3 The JNK pathway

Initially, the JNK protein was known as pp54, a hepatic protein kinase which had Ser/Thr activity and was purified from cycloheximide-treated rat liver (Kyriakis and Avruch, 1990). Since then, the JNK pathway has been identified as a stressed activated cascade which has been shown to be activated by different environmental stresses and involved in the control of apoptosis and cell cycle arrest in many cell types. For a relevant review see (Kyriakis and Avruch, 2011).

The general characteristics of the JNK pathway follows a three tiered kinase cascade (summarised in figure 1.1) consisting of an external or internal stimuli including apoptotic stimuli, growth factors, cellular stress or cytokines activating a membrane receptor. The receptor activates the signalling cascade starting with a MAP3K such as apoptosis-signal regulating kinase (ASK) -1, followed by the MAP2Ks, MKK4 or 7 and then the MAPK JNK (Cui *et al.*, 2007). Whilst the activation of the pathway follows a three tiered cascade, there are several different kinases at each tier level that amplify signals through the cascade to promote different outcomes (Davis, 2000). For example, MKK4 and MKK7 prefer to activate JNK through phosphorylation of different residues, with MKK4 showing preference for the tyrosine residue and MKK7 the threonine residue (Lawler *et al.*, 1998). These MAP2K have also been shown to regulate JNK differently, for example, disruption of gene MKK7 but not MKK4 in mice prevented JNK activation by pro-inflammatory cytokines (Tournier *et al.*, 2001).

As mentioned previously, JNK signalling can be downregulated through dephosphorylation by MKPs. It was recently discovered, through the use of crystallography, that this is via an FXF-docking motif interaction between MKP and JNK, where one phenylalanine is responsible for substrate binding and the other for active site alignment (Liu *et al.*, 2016). The pathway also has a number of different adaptor and scaffolding proteins involved in ensuring the correct signal is transmitted, for example the JNK interacting protein (JIP) helps facilitate signal transmission through linking JNK with its upstream activators and forming complexes to assist activation (Yasuda *et al.*, 1999) This was demonstrated through coimmunoprecipitation assays where JIP was found to selectively bind to MKK7 and JNK. Other known scaffold proteins include Axin, Dvl and Epstein–Barr virus latent

membrane protein-1 (LMP-1), all of which help facilitate JNK activation by MKK4 and 7 (Zou *et al.*, 2007).

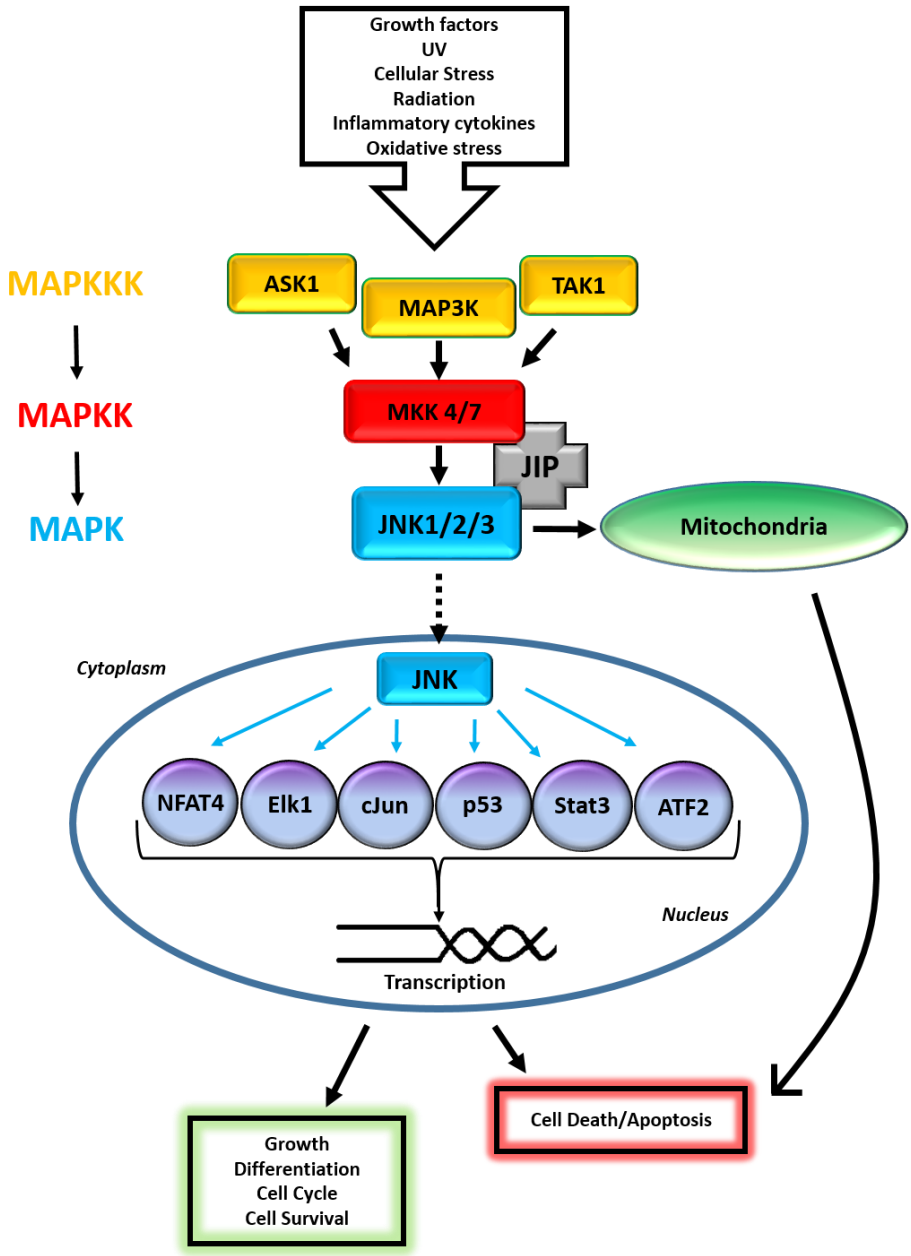


Figure 1.1 The JNK pathway
A simplified model diagram of the JNK pathway which can be activated by many different internal and external stimuli including: growth factors, UV radiation, cellular stress, inflammatory cytokines and oxidative stress. These stimuli can activate MAPKKKs such as ASK1, TAK1 and other MAP3K and initiate a cascade of phosphorylation. MAPKKKs phosphorylate MAPKKs (MKK4/7) which then phosphorylate the MAPKs (JNK1/2/3). This signalling cascade can be amplified and assisted through adapter proteins such as JIP1/2/3. JNK then goes on to directly interact with cytosolic proteins such as mitochondrial proteins (see figure 1.4), or translocates to the nucleus to activate transcription factors. This can then lead to either cell survival or cell death signalling depending on the stimuli, time and location of pathway activation. Adapted from (Cui et al. 2007 and Davis 2000).

Within mammals there are three genes which encode the three JNK proteins JNK1, JNK2 and JNK3. These three genes can undergo alternative splicing at the mRNA level which results in the production of ten different variants, four for JNK1, four for JNK2 and two for JNK3, which have a molecular weight located between 46kDa to 55kDa. The JNK1 and JNK2 proteins are located throughout all tissues within the body, whereas JNK3 is localised to specific tissues such as the testes, heart and brain (Bogoyevitch and Kobe, 2006). All three proteins contain eleven protein kinase subdomains (Figure 1.2) and the conserved threonine and tyrosine activation loop is located between domains VII and VIII. Located between domains IX and X is an alternative splicing site, the products of which demonstrate differences in substrate specificity (Manning and Davis, 2003).

The crystal structure of JNK1 (Kragelj *et al.*, 2015), JNK2 (Shaw *et al.*, 2008) and JNK3 (Xie *et al.*, 1998) have all been determined, allowing similarities and differences in structure to be analysed. All three proteins have similar homology (figure 1.3) with an N-terminal lobe consisting mainly of β-strands and a C-terminal lobe consisting mainly of α-helices. The lobes are connected by a "hinge" region where the binding site for substrates is located, this is also close to the ATP binding site (Bogoyevitch and Kobe, 2006). The reader here is directed to the above reviews by Manning and Bogoyevitch for a more detailed discussion about the structure of JNK proteins and their spliced variants (Bogoyevitch and Kobe, 2006).

Even prior to crystal structure studies, it was hypothesised that the different JNK isoforms have individual selectivity for the different transcription factors they activate. Gupta *et al* proved that substrate binding and phosphorylation are two individual characteristics of JNK which differ between the different isoforms. They demonstrated that the transcription factor JunB binds to JNK but is not a substrate whereas the transcription factor JunD binds weakly to JNK but is a substrate (Gupta *et al.*, 1996). Another example of binding selectivity was demonstrated by Ito *et al* where they found that JNK3 showed a higher binding affinity to the scaffold protein JSAP1 when compared to JNK1 and JNK2 in cos-7 cells using co-transfection experiments (Ito *et al.*, 1999).

Another specific attribute of the JNK pathway that has been discovered is that translocation to the nucleus upon activation is agonist specific. For example, hydrogen peroxide (H_2O_2) but not tumour necrosis factor alpha (TNF-α) has been shown to induce JNK1 translocation to the nucleus in endothelial cells (EC) (Robinson, Sloss and Plevin, 2001). The induction of JNK signalling is also stimulant source specific, with extracellular stimulation such as H_2O_2 unable to activate a specific pathway involving Pyk2 and JNK in cardiac monocytes (Dougherty *et al.*, 2004). Dougherty et al proposed that the source of the stimulant may be important, where activation of the specific pathway requires an internal stress such as reoxygenation, as opposed to an external stimulus. Another example of differential JNK activation was demonstrated by Song and Lee, where ultraviolet (UV) light and glucose deprivation was used to activate JNK. They found that each stimulant activated a different signalling cascade which led to JNK activation. The stimuli of UV light activated a cascade starting with epidermal growth receptor, followed by Grb2-MEKK1-SEK1 and then JNK, whereas glucose deprivation activated ASK1 (apoptosis signal-regulating kinase 1) which directly activated SEK-1 (SAPK/ERK kinase -1) and then JNK. This again demonstrates the different outcomes produced by stimulant location on JNK signalling. Finally, it has also been shown that the levels of expression and/or activity of the JNK isoforms can vary between cell type and subcellular location (Xu *et al.*, 1997). This evidence would suggest that JNK protein function is not only dependent on location and level of expression but also may vary between cell type and stimuli type.

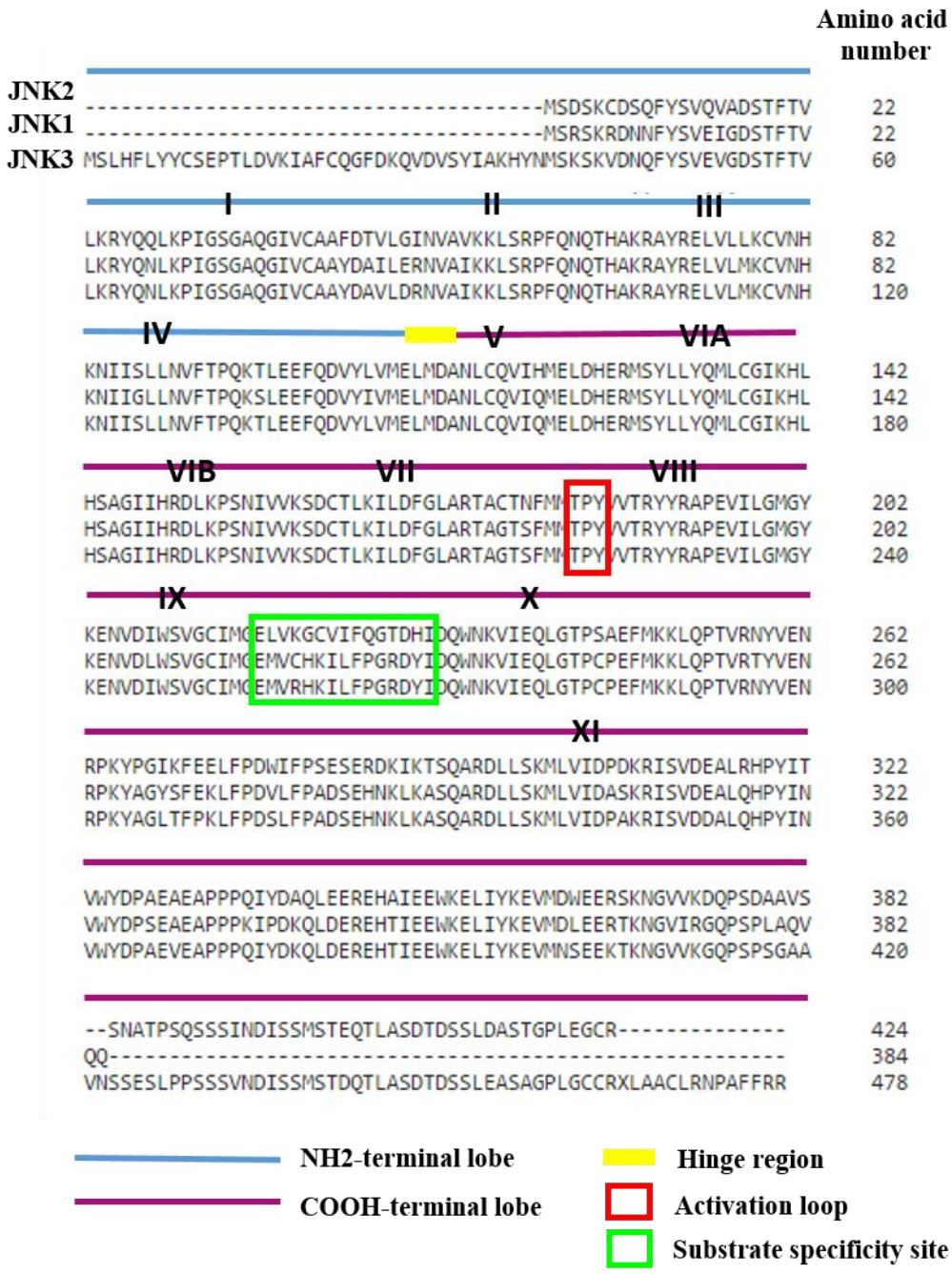


Figure 1.2 Alignment of linear sequence of JNK1, JNK2 and JNK3
Multiple sequence alignment by Clustal Omega software of JNK1, JNK2 and JNK3. The C-terminal lobe and N-terminal lobes are represented by a purple and blue line respectively. The 11 kinase domains are labelled I-XI with the hinge region displayed as yellow. The conserved TYP activation loop is highlighted in red and the alternative splicing sites in green.

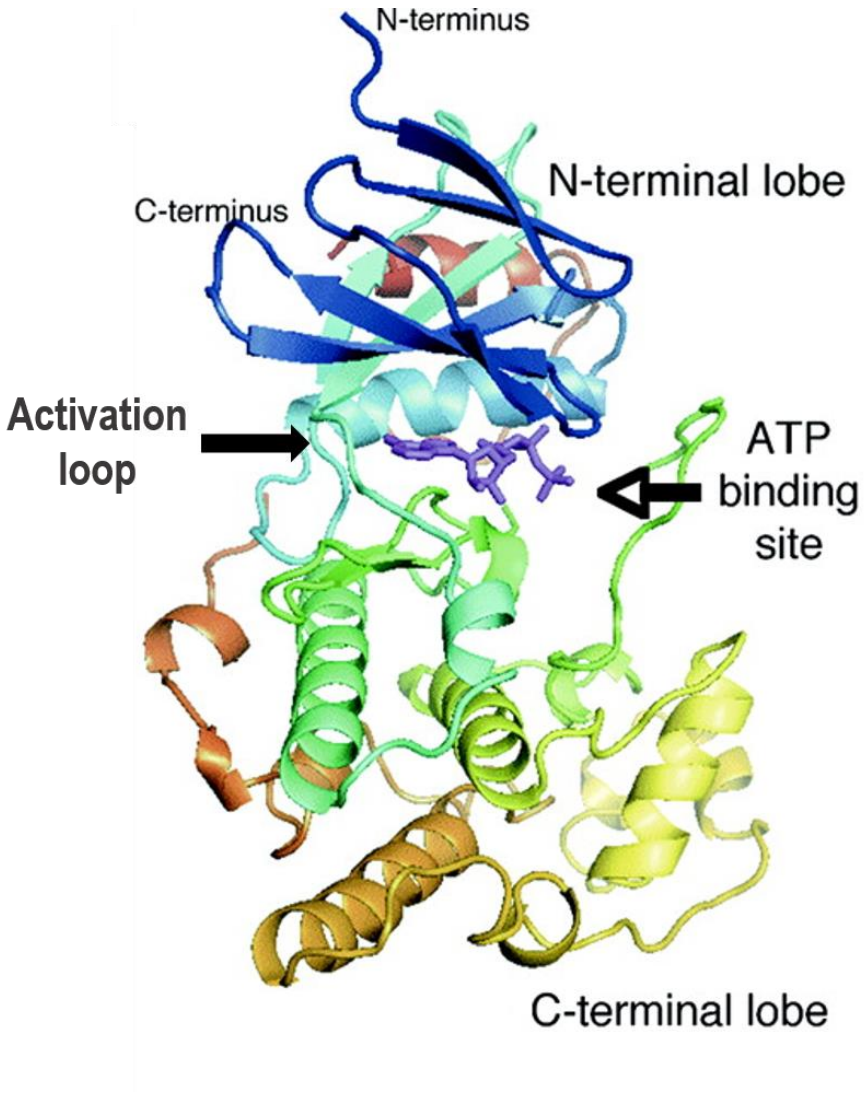


Figure 1.3 Crystal structure of JNK3
Ribbon representation of JNK3 with adenylyl imidodiphosphate bound shown in purple. The N-terminal lobe contains blue and green beta sheets which link onto the green, yellow and red alpha helices of the C-terminal lobe. Activation loop contains dual phosphorylation motif TXY (Bogoyevitch and Kobe, 2006).

1.3.1 JNK in cell death signalling

Since its discovery there has been a great volume of research examining the involvement of the JNK pathway in apoptosis. As mentioned in the previous section different external and internal stimuli can result in the activation of specific and different signalling cascades that lead to JNK activation. After activation of JNK, initiation of apoptosis can occur through either JNK translocation to the nucleus and activation of the transcription of apoptotic proteins or through direct interaction with apoptotic proteins (Björkblom *et al.*, 2008). Known external apoptotic stimuli of JNK include UV light (Tournier *et al.*, 2000), TNF-α (Tang *et al.*, 2002) and serum starvation (Caricchio, D'Adamio and Cohen, 2002), while examples of intracellular stimuli include reactive oxygen species (ROS) (Shi *et al.*, 2014), DNA damage (Yoshida *et al.*, 2005) and ER stress (Nishitoh *et al.*, 2002). Once activated and translocated to the nucleus JNK can activate transcription factors (Figure1.1), for example, UV radiation can cause JNK activation of Elk-1 transcriptional activity (Cavigelli *et al.*, 1995) which has been shown to promote activation of proteins involved in cell cycle arrest and apoptosis (Shin *et al.*, 2011). Whereas in contrast treatment with the chemotherapeutic drug vinblastine causes apoptosis via JNK-AP1 (activator protein–1) signalling (Kolomeichuk *et al.*, 2008). JNK has also been shown to play a role in regulating apoptotic signalling mediated by the tumour suppressor p53 (Kim *et al.*, 2010). In this context, p53 can induce the expression of Bax, a pro-apoptotic protein involved in mitochondrial membrane permeabilisation.

As well as activating the transcription of apoptotic proteins, JNK can also interact directly with cytosolic proteins to regulate cell death (Aoki *et al.*, 2002) (summarised in figure 1.4). For example, JNK can directly interact with the anti-apoptotic proteins Bcl2 and Bcl-Xl (B-cell lymphoma-extra-large) and antagonize their anti-apoptotic activity. Another member of the Bcl2 family is Bax (Bcl2 associated X). JNK can promote translocation of Bax to the mitochondria through phosphorylation of 14-3-3 proteins which retain Bax to the cytoplasm (Tsuruta *et al.*, 2004). Bax can then dissociate from the 14-3-3 protein and translocate to the mitochondria where it can induce cytochrome C release and apoptosis. In agreement with this finding Tournier *et al* demonstrated in fibroblasts that JNK is required for the activation of cell death mediated by UV radiation, as JNK knockout cells had disrupted mitochondrial

depolarisation and cytochrome-c release, this showed that JNK signalling is also an important factor in mitochondrial cell death signalling (Tournier et al. 2000 and Jiang & Wang 2004). Finally, Deng *et al* demonstrated that JNK can cleave the Bcl2 family member Bid (BH3 interacting-domain) to form jBid which translocates to the mitochondria to induce the release of Smac (second mitochondria-derived activator of caspase). This leads to the disruption of the TRAF2-cIAP1 (TNF receptor associated factor 2 – cellular inhibitor of apoptosis 2) complex, which inhibits apoptosis, allowing apoptosis to occur via the caspase pathway (Deng *et al.*, 2003).

The involvement of the JNK pathway in activating apoptosis has been shown to play a very important role in physiological processes, including brain development. Through the use of knockout (KO) mice it has been demonstrated that unlike other areas of the body, the JNK pathway within the brain is constitutively active and is required for neuronal cell death and migration, axon elongation, axon maintenance and dendrite formation (Yamasaki, Kawasaki and Nishina, 2012). JNK1 KO mice are viable, however they have abnormalities in cortical development, axon maintenance and microtubule stability, which effects the overall structure of the developing embryo brain. JNK1 and JNK2 double KO mice are not viable due to a reduction in apoptosis and consequential defects in neural tube closure. Interestingly, JNK2 and JNK3 single KO mice do not share the same deficiencies as JNK1 KO mice and instead suffer from alterations in apoptosis induced by neuronal stress, suggesting they may have a central role in neurodegenerative diseases (for full review see (Yamasaki, Kawasaki and Nishina, 2012)). In addition to showing the importance of the JNK pathway in brain development, the Yamasaki review also proposed that the different JNK isoforms are unique in function, an important feature discussed later in this chapter.

Another important process in which JNK apoptotic signalling has been shown to play a key role is thymocyte selection, a process which helps to prevent an autoimmune response (Rincón *et al.*, 1998). Here, inhibition of JNK signalling, produced by a dominant negative JNK mouse model, was shown to increase the resistance of thymocytes to T-cell receptor signalling. This resulted in reduced deletion of double positive cells and therefore an increase in the population of these cells. In agreement with these findings, Jin *et al* recently showed that suppression of the redox regulator thioredoxin reductase (TrxR)1, a protein upregulated during selection, caused an

increase in JNK apoptotic signalling and TCR-mediated apoptosis in double positive thymocytes, *in vivo* and *in vitro* (Jin *et al.*, 2015). These two studies demonstrate the importance of apoptosis in development and that JNK is a key mediator of this signalling.

Activation of apoptosis via JNK can differ between cell types and also be determined by what stimuli is present (Xu *et al.*, 1997). JNK1, 2 and 3 have all been shown to play a role in apoptosis, with the involvement of JNK 3 restricted to the specific cell types in which it is expressed. Both nuclear and cytosolic JNK apoptotic signalling is important in the regulation of a number of physiological events, this is also the case in a number of disease conditions. Earlier in this chapter, the role for JNK in PD and Alzheimer's disease pathogenesis was discussed. Another example of a disease where JNK plays a key role in development is diabetes (Brozzi *et al.*, 2016). Brozzi *et al* group linked inflammatory signalling in pancreatic beta cells to the activation of JNK apoptotic signalling via Ubiquitin D/IRE1-alpha, a process which is thought to be key in the pathogenesis of type 1 diabetes. Through the use of JNK inhibitors, JNK apoptotic signalling has also been shown to be a key mediator in hepatic ischemia reperfusion injury (Uehara *et al.*, 2005) and myocardial ischemia reperfusion injury (Ferrandi *et al.*, 2004). In both of these studies, inhibition of JNK resulted in a significant decrease in the amount of cell death, a key feature of both disease states.

Finally, another factor of JNK apoptotic signalling which has been highlighted is that activation of apoptosis by JNK is time dependent. Initial activation of JNK can lead to cell survival whilst sustained activation leads to apoptosis (Ventura *et al.*, 2006). Yun *et al* demonstrated this in human prostate carcinomas during glucose deprivation where the activation of adenosine monophosphate-activated protein kinase (AMPK) inhibited ROS generation and late stage pro-apoptotic JNK activation, keeping the pathway activated in its early stage form which is pro-survival (Yun *et al.*, 2009). JNK's role in survival signalling will be discussed more in the next section.

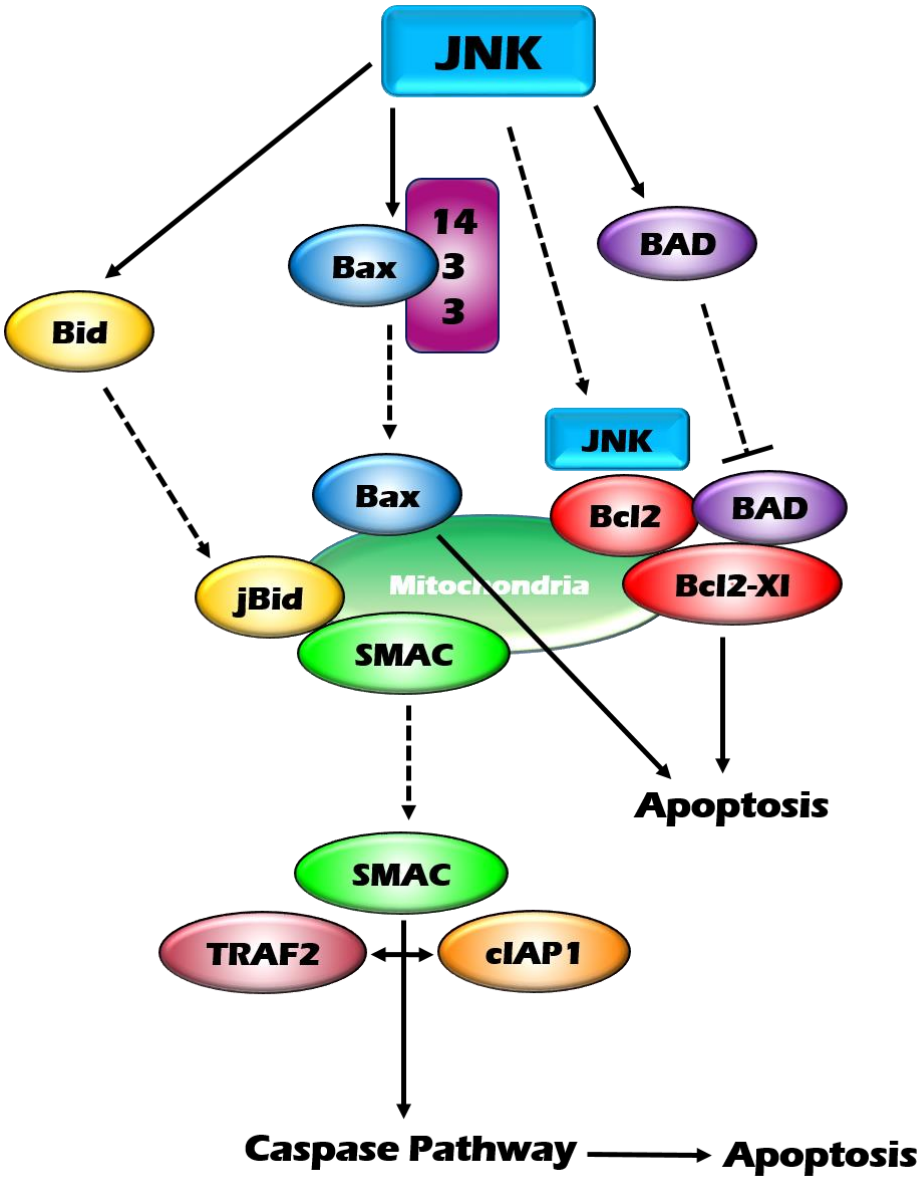


Figure 1.4 JNK signalling in apoptosis
Example of the role of JNK signalling in apoptosis. JNK can negatively regulate apoptosis by phosphorylating BAD and preventing the interaction between BAD and Bcl2-Xl, which would result in activation of apoptosis. JNK can also directly interact with Bcl2 to prevent its anti-apoptotic activity. The cleavage of Bid by JNK to form jBid allows it to translocate to the mitochondria and activate apoptosis via SMAC-TRAF2/cIAP1-caspase signalling. Finally, JNK can also induce the release of Bax from the cytoplasmic anchor 14-3-3 protein, allowing translocation to the mitochondria and activation of apoptosis. Adapted from (Dhanasekaren and Reddy, 2008).

1.3.2 JNK cell survival signalling

While a considerable amount of research has focused on JNK in cell death, the pathway is also involved in cell survival processes such as proliferation (Pathria *et al.*, 2016), anti-apoptotic signalling (Yu *et al.*, 2004) and extracellular matrix (ECM) signalling during serum starvation (Almeida *et al.*, 2000). This also includes regulation of defined phases of the cell cycle.

The Gutierrez group have demonstrated in HeLA cells a role for JNK in regulating cell cycle progression to the G2/M phase through phosphorylation of the phosphatase Cdc2 (Gutierrez *et al*., 2010a). This in turn prevents dephosphorylation and consequential activation of the cell cycle progression regulating protein cyclin dependent kinase (CDK)1. The group then went on to show that JNK also phosphorylates the adaptor protein Cdh1 preventing the early activation of the cyclosome during G2 phase of the cell cycle. In addition the cyclosome was found to regulate JNK through ubiquitination and subsequent proteasomal degradation (Gutierrez *et al*., 2010b). JNK has also been shown to negatively regulate cdc2/cyclin B kinases, which plays an important role in the G2/M phase of the cell cycle (Goss *et al.*, 2003).

A recently published paper showed that JNK signalling is a regulator for melanoma cell proliferation through maintaining c-Jun activity and expression (Pathria *et al.*, 2016). In this study JNK inhibitors were used to demonstrate that JNK was required for c-Jun but not cyclin D1 activity in cell proliferation. On the other hand the ERK pathway was required for regulation of cyclin D1 where inhibition of ERK signalling downregulated Cyclin D1 but did not block c-Jun activity. The group conclude that in the case of melanoma cell proliferation, inhibition of both JNK and ERK signalling pathways is required to reduce proliferation. In contrast to this finding, Schwabe *et al* demonstrated in hepatocytes and also in an animal model of partial hepatectomy that inhibition of JNK caused reduced proliferation of cells and a decrease in cyclin D1 expression (Schwabe *et al.*, 2003). Collectively this work highlights an important role for the JNK pathway in the regulation of the cell cycle which may be important for therapeutic targeting in preventing cell growth.

As well as regulating cell cycle check points JNK also plays a role in regulating microtubule dynamics, an important event in the cell cycle, where interaction between

kinesin-1, a molecular motor, and JNK results in activation of JNK and sequential microtubule elongation (Daire *et al.*, 2009). JNK has also been shown to be activated by the Wnt signalling pathway which, combined with the downregulation of GSK3-beta, is important in microtubule stabilisation (Ciani and Salinas, 2007). In neural progenitor cells deletion of JNK can cause defects in spindle formation and mitotic division (Xu *et al.*, 2014). It has also been demonstrated that JNK is required for chromosomal instability (CIN), a characteristic of many different types of tumour (Wong *et al.*, 2014). This study demonstrated a role for JNK in cells with CIN but not normal cells, where JNK activation was thought to promote DNA repair in G2 phase of the cell cycle. These studies highlight the pro-survival functions of JNK.

The ability of JNK to interact with different substrates at different levels of specificity has led to the discovery of conflicting evidence of the role the pathway plays in many conditions. In cancer JNK has been shown to promote both carcinogenesis through activation of proliferation via c-Jun and also positively regulating autophagy via Bcl-2. However depending on the complex Bcl2 forms, for example with Beclin-1 or the apoptosis regulator protein Bax, JNK can alternatively cause a switch to blocking autophagy and promoting cell death (Tournier, 2013). JNK can also directly interact with the Bcl2 family of proteins to suppress apoptosis (Yu *et al.*, 2004). In this study JNK phosphorylated the protein BAD (Bcl-2-associated death) which prevented BAD interacting with Bcl-Xl and suppressing its anti-apoptotic activity (Figure 1.4). The JNK pathway has also been shown to play an important role in mice lung carcinogenesis by causing an increase in tumour cell proliferation, induced by signalling through the GTPase protein Ras (Cellurale et al. 2011). Along with tumour growth, the process of tumour angiogenesis is also influenced by JNK and therefore the pathway is seen as a possible target for cancer therapy (Ennis *et al.*, 2005). Many cancers occur through mutations in genes encoding proteins that are involved in important cell signalling pathways. Kan *et al* found that a mutation of MKK4, an activator of JNK, is found in many cancers and plays a role in oncogenesis (Kan *et al.*, 2010).

Drawn together most publications share a common finding that the outcome of JNK signalling is dependent on several elements including cell type, location, stimulant, stimulant source, cell cycle stage and kinetics. It is also true that JNK is an ambiguous

kinase which can promote either cell survival or cell death, making the signalling an intricate system to try and interpret.

1.4 JNK in disease

As mentioned above the JNK pathway plays important roles in the pathogenesis of many different types of disease. This includes, but is not limited to, neurodegenerative disease (Borsello and Forloni, 2007), autoimmunity (Kitabatake *et al.*, 2015), cardiovascular conditions (Sumara, Belwal and Ricci, 2005) and cancer (Bubici and Papa, 2014). Since cardiovascular disease is a leading cause of mortality (Townsend *et al.*, 2015) and it has been predicted that one in every two people will be diagnosed with cancer in their lifetime (Ahmad, Ormiston-Smith and Sasieni, 2015), the remainder of this chapter will specifically focus on JNK signalling in cardiovascular disease and cancer. The first sections will discuss research focusing on JNK as a single protein, which is often the way evidence is presented, particularly due to pharmacological inhibitors that are available. The final section will explore the research which has demonstrated that JNK isoforms can function specifically and independently.

1.4.1 Cardiovascular disease

Research in cardiovascular disease (CVD) has become necessary over recent years due to the fact that it is one of the leading causes of death worldwide (Jiang *et al.*, 2013) and childhood obesity is now becoming an increasing concern (Sypniewska, 2015). The term cardiovascular disease covers a broad range of diseases, including but not limited to; coronary heart disease, pulmonary hypertension (PH), cardiac hypertrophy, aortic aneurysm and thrombosis; for detailed reviews on the pathophysiology of these diseases the reader is directed to the following reviews (Libby & Theroux 2005, Tuder et al. 2013, Tham et al. 2015, Guo et al. 2006 and Esmon 2009). Many CVDs have common pathophysiological features which ultimately result in a malfunction in the vasculature and/or a reduction in oxygen received by the heart. In many cases the development of one CVD can lead to the development of another. For example, patients who have hypertension are at high risk of developing coronary artery disease (Rosendorff *et al.*, 2007) and patients with coronary artery disease are at risk of developing thrombosis (Davies, 2000).

The mechanisms responsible for the pathophysiology differs between diseases however a shared feature involves abnormalities in endothelial cell (EC) and vascular

smooth muscle cell (VSMC) function. The key roles of VSMCs under normal circumstances are to alter the luminal diameter of the blood vessel to maintain blood pressure levels and to remodel the vessels when they undergo damage (Rensen, Doevendans and van Eys, 2007). During CVD the function of VSMC can be disrupted and this can cause VSMCs to play a role in the pathogenesis of the disease. For example, in abdominal aortic aneurysms (AAAs), VSMCs have been shown to lose control of MMP signalling resulting in an increase in the production of MMPs which contributes to the degradation of the aortic matrix (Airhart *et al.*, 2014). Proliferation and migration of VSMC into the intima in response to an inflammatory environment results in the formation of a neointima, a key feature of atherosclerosis and restenosis (Duran-Prado *et al.*, 2013). Apoptosis of VSMCs during vessel injury can also activate VSMC migration and proliferation which contributes to vessel remodelling (Yu *et al.*, 2011). Finally, VSMC apoptosis has been shown to drive atherosclerosis in an inducible apoptotic VSMC (Apo)$E^{-/-}$ mouse model, where induction of apoptosis produced features of atherosclerosis in younger mice and prevented remodelling of the vessel (Clarke *et al.*, 2008).

Like VSMC, ECs are crucial in maintenance of vascular function. Under normal circumstances ECs form the inner layer of the blood vessel (intima) and are crucial in regulating vascular structure and tone. They also play a protective role by secreting cardio protective substances such as nitric oxide (NO) which helps to prevent events such as leukocyte adhesion and smooth muscle cell proliferation which can lead to the development of atherosclerosis (Versari *et al.*, 2009). Under disease conditions ECs can become dysfunctional which results in them becoming key players in the development and progression of some CVDs. For example, when EC dysfunction occurs due to oxidative stress, the levels of NO decrease because the oxides scavenge the NO and produce harmful radicals. This in turn can cause more damage to the ECs preventing regulation of VSMCs and leading to the development of hypertension (Bhatt, Lokhandwala and Banday, 2011). Under conditions of shear stress, caused by changes in blood flow, ECs express leukocyte adhesion molecules such as intercellular adhesion molecule – 1 (ICAM – 1), which can facilitate the infiltration of macrophages into the intima, a feature of early stage atherogenesis (Nagel *et al.*, 1994). Finally, apoptosis of ECs plays a key role in late stages of atherogenesis where the

atherosclerotic plaque can rupture and ultimately cause thrombosis (Xu *et al.*, 2009). In this study apoptosis was induced in the abdominal aorta of a rabbit model of atherosclerosis. It was also demonstrated that as the apoptosis score increased the thrombotic score also increased, providing evidence that EC apoptosis plays a key role in this disease. For detailed reviews on VSMC and EC dysfunction see (Michel et al. 2012 & Deanfield et al. 2007).

The role of JNK in different vascular cells has been investigated and results have shown that the JNK pathway can be activated by different stimulants such as; cytokines during infection (Verma and Ihler, 2002), ROS (Tian *et al.*, 2012) and oxygen deprivation during ischemia (Wallace, Jelks and O'Donnell, 2012). Studies have also shown that JNK signalling is involved in the cell migration and proliferation of a number of different cell types (Huang, Jacobson and Schaller, 2004, Mingo-Sion *et al.*, 2004 & Kimura *et al.*, 2013) with more recent studies demonstrating this in VSMCs (Zhang *et al.*, 2012) and ECs (Jun *et al.*, 2012). Understanding the different roles the JNK pathway plays in cellular processes and more specifically within vascular cells is key in recognizing important links between JNK signalling and the pathophysiology of CVD. For the remainder of this section studies demonstrating JNK signalling in cardiovascular cells will be discussed, particularly the role of JNK in VSMCs and ECs.

1.4.1.1 Inflammation induced JNK signalling in CVD

Inflammation greatly contributes to the pathogenesis of CVD with many of the key processes of atherogenesis resulting from an increase in pro-inflammatory signalling. During stress, infection or high fat levels in the vasculature, an inflammatory response can be initiated which results in the recruitment of immune cells from the blood stream to the site of induction. These immune cells include monocytes and T cells that produce cytokines such as TNF-α, IL-6 and angiotensin II which in turn can damage ECs and cause endothelial dysfunction. As mentioned previously, EC dysfunction can lead to monocyte infiltration, altered signalling to VSMC and the promotion of atherogenesis.

The JNK pathways may have the potential to link the immune response to vascular remodelling either directly or indirectly. Macrophage migration inhibitory factor

(MIF) is a pro-inflammatory cytokine that is expressed in increased amounts in tissues from rats with hypoxic pulmonary hypertension (PH) and is involved in pulmonary vascular remodelling. It has been suggested that MIF may stimulate pulmonary artery SMC (PASMC) proliferation via the JNK pathway, contributing to the pathophysiology of PH (Zhang et al., 2012). This was demonstrated using the JNK inhibitor SP600125, which reduced the increase in the total number of cells produced through stimulation with MIF alone. It has been understood for a long time now that TNF-α plays an important role in cardiovascular disease, through promoting inflammation and contributing to EC dysfunction (Ferrari, 1999). JNK can be activated by TNF to induce apoptosis and the nuclear factor - κB (NF-κB) pathway has been demonstrated to negatively regulate this activation and thus promote cell survival (Tang *et al.*, 2001). Recently the reverse of this has been demonstrated in ECs, where endocytosis of TNF-α receptors was shown to modulate the balance of NF-κB and JNK signalling (Choi, Nguyen and Lamb, 2014). In this study inhibition of JNK increased NF-κB activity in response to TNF-α for up to 48 hours and also increased expression of the downstream target ICAM-1, a key mediator of leukocyte binding in atherosclerosis. This data shows that JNK can supress NF-κB signalling in endothelial cells which may play a role in endothelial dysfunction to promote a switch from cell survival to cell death.

Another common feature of many CVDs is damage to the ECM occurring due to an imbalance in proteins that regulate ECM degradation (Raffetto and Khalil, 2008). Inflammatory signalling via JNK has been linked to ECM destruction in an abdominal aortic aneurysm (AAA) mouse model where drug inhibition of JNK not only prevented damage to the ECM, shown by a reduction in aortic diameter and MMP-9 expression, but augmented tissue repair and reduced inflammatory signalling shown by a reduction in macrophage infiltration (Yoshimura *et al.*, 2006). In agreement with these findings it was demonstrated in human aortic SMCs (HASMCs) that JNK is activated by adenosine triphosphate (ATP) which stimulates the release of matrix metalloproteinase (MMP) -2, a protein responsible for causing degradation of ECM components (Iii *et al.*, 2006).

In response to alterations in blood flow, shear stress in the vessel can occur in various CVDs and has been shown to activate KLF2 transcription, an initiator of cytoskeleton

rearrangement and cell alignment. This rearrangement has been shown to prevent inflammation through inhibition of JNK phosphorylation by the protein FAK and consequential activation of the transcription factors c-Jun and ATF2 (Boon *et al.*, 2010). In contrast to these pathogenic examples, JNK has been shown to play a protective role in the pathogenesis of cardiac hypertrophy. Through the use of dominant negative JNK1/2 mice and JNK1/2 gene targeted mice Liang *et al* have shown the ability of JNK to regulate cardiac growth where inhibition of JNK enhanced hypertrophy. JNK is thought to regulate nuclear factor of activated T-cell (NFAT) localisation to the cytoplasm preventing nuclear translocation and subsequent immune gene transcription and growth (Liang *et al.*, 2003). These findings again show the complexity of JNK signalling where the pathway is linked to promoting and preventing pathogenesis of different subsets of CVD.

1.4.1.2 JNK Activation by ROS and angiotensin II

There is now a substantial body of evidence which demonstrates that ROS can contribute to the development and progression of CVDs. ROS has also been shown to activate JNK signalling via the MAP3K, ASK1 (Tobiume *et al.*, 2001) and inhibit the negative regulators of JNK, the MKPs, which promoted sustained JNK activation (Kamata *et al.*, 2005) In the vasculature, a study by Fei *et al* demonstrated that endothelian-1, a vasoconstrictor produced by ECs, can induce the generation of ROS in SMC which in turn can activate the JNK pathway. This leads to the activation of AP-1 which can regulate the transcription of inflammatory cytokines and promote atherogenesis (Fei *et al.*, 2000). Within the vasculature the endothelial nitric-oxide synthase (eNOS) protein produces NO at low levels to maintain vascular homeostasis. As mentioned previously, changes in the levels of NO can contribute to various forms of CVD. Inflammation also initiates production of NO by inducible NOS (iNOS) creating an abundance of NO which can result in the production of ROS that contributes to vascular damage. Recently eNOS has been shown to produce a high amount of NO in human lung microvascular endothelial cells (HLMVECs) under inflammatory conditions. The peptide bradykinin is thought to activate Gα, a G-protein coupled receptor, and initiate JNK signalling which stimulates the generation of NO by eNOS (Lowry *et al.*, 2013). This is a novel signalling pathway for NO production that may further link JNK and ROS to endothelial and SMC damage during CVD,

however since this is the only publication to date, more evidence is needed to confirm this theory.

Different products produced during CVD pathogenesis have been shown to activate JNK signalling. Reoxygenation of cardiac monocytes after exposure to hypoxia causes the production of reactive oxygen intermediates (ROI) and an increase in intracellular calcium produced by the mitochondria. This was found to induce the phosphorylation and consequent activation of the proline rich protein kinase Pyk2, the recruitment of GTPase protein Rac1 and the resulting activation of the JNK signalling cascade (Dougherty *et al.*, 2004). Another stimulant of the JNK pathway is angiotensin II which has been shown to activate the JNK pathway in human mesangial cells, which share similar features with VSMCs, and induce proliferation (Zhang *et al.*, 2005). Since angiotensin II plays an important role in controlling blood pressure and vasoconstriction it has been shown to be involved in various types of CVD where these processes are affected. The angiotensin II/JNK pathway has been demonstrated to be partially inhibited by angiotensin II type 1 receptor associated protein (ATRAP) which results in a decrease in ROS production and inhibition of pathological vascular hypertrophy in mice (Wakui *et al.*, 2013). These studies provide further insight into how JNK, inflammation and ROS together contribute to CVD.

1.4.1.3 VSMC proliferation and migration

As mentioned previously, cellular processes such as migration and proliferation of VSMC contribute to the pathogenesis of CVDs. JNK has been shown to be involved in cell migration and to have a minor role in proliferation in smooth muscle cells when stimulated by platelet derived growth factor (PDGF) (Zhan *et al.*, 2003). In agreement with this finding, inhibition of JNK by the specific inhibitor SP600125 in rat aortic SMCs has been shown to reduce cell migration and proliferation (Kavurma and Khachigian, 2003). In the vasculature there are two different phenotypes of SMCs, the elongated, spindle-like contractile phenotype which is associated with healthy cells and the rounded synthetic phenotype which is quite often found in CVD. The study by Kavurma and Khachigian demonstrated that there are differences in migration between the two phenotypes, however both require JNK for the process (Kavurma and Khachigian, 2003). In this study SMCs from young and old rats were used to correlate with contractile and synthetic SMCs. Using the JNK inhibitor, it was demonstrated

that both cell types migrated less when treated with the inhibitor when compared to control cells. The reader is directed to the following paper for a more information on SMC phenotypes (Qiu *et al.*, 2013). Taken together these studies along with others (Izumi et al. 2001 and Zhang et al. 2012), provide a clear link between JNK and the migration and proliferation of SMC.

1.4.1.4 Atherosclerosis

Where inflammation, VSMC and EC dysfunction are features of different CVDs, they all occur in atherosclerosis. Atherosclerosis is a process that involves the thickening of blood vessels, which can ultimately lead to the depletion of the oxygen supply to the heart or brain. Initiation of atherosclerosis can occur through many different sources including but not limited to infection (Rosenfeld and Campbell, 2011) and hyperlipidaemia (Kerenyi *et al.*, 2006). The use of *in vitro* studies has allowed for the signalling involved within the different vascular cells to be analysed and a role for JNK signalling has been demonstrated in both VSMC and ECs. *In vivo* mouse models of atherosclerosis containing JNK knockout have also been used to investigate JNK function in the progression and development of the disease and at almost every stage of the atherogenic process, JNK has been shown to play a role (summarised in Figure 1.5). For the remainder of this section, *in vitro* and *in vivo* studies demonstrating the role of JNK at the different stages of atherogenesis will be discussed in more detail.

Adhesion of leukocytes

As mentioned previously, once an inflammatory response occurs at a site of injury in the vessel, dysfunctional ECs can express chemoattractants such as MCP-1 or adhesion molecules such as ICAM-1 and VCAM-1 (vascular cell adhesion protein -1) which attract and then bind to circulating leukocytes. The leukocytes attach onto the endothelial surface and roll along the inside of the vessel until they can migrate between a gap into the intima. For a more in-depth discussion on leukocyte-endothelial cell interactions the reader is directed to the following review (Langer and Chavakis, 2009).

Activating transcription factor (ATF)2 is a downstream target of JNK and has been shown to be required for endothelial VCAM-1 gene expression when stimulated with vascular endothelial growth factor (VEGF)-A (Fearnley *et al.*, 2014). However, in this

study only p38 and ERK signalling pathways were investigated, leaving JNK function unresolved. Nevertheless another group investigated the role of JNK signalling in HUVECs in response to VEGF-A and found that VEGF-A stimulated JNK, p38 and ERK in HUVECs (Shen *et al.*, 2012). When JNK was knocked down using siRNA *in vitro* or inhibited with SP600125 *in vitro* and *in vivo* there was a reduction in cell migration and angiogenesis, providing evidence that JNK was key in the induction of these events. VEGF-A has been shown to be pro-atherogenic using gene-transfer experiments as transfer of VEGF-A to $ApoE^{-/-}$ mice, a model of atherosclerosis, increased lesion areas in aortas when compared to control mice. Finally, inhibition of JNK by SP600125 in TNF-α stimulated human aortic ECs (HAECs) was shown to reduce VCAM-1 and ICAM-1 expression (Lin *et al.*, 2005). Since JNK has been shown to regulate expression of VCAM-1 and ICAM-1 in HAECs and VEGF-A is pro-atherogenic and has been shown to activate JNK, JNK may also regulate adhesion molecule expression in ECs in response to VEGF-A.

Infiltration of leukocytes

Once the leukocytes find a gap in the endothelium, they can migrate into the intima of the vessel. This can result in more endothelial dysfunction, more inflammation and the recruitment of more leukocytes. During hyperlipidaemia, a high concentration of low density lipoproteins (LDLs) are found in the bloodstream and have been shown to cause activation of c-Jun (Zhu *et al.*, 1998). During hyperlipidaemia proteasome activity is reduced preventing activated c-Jun degradation and allowing it to enhance MMP-9 expression, a protein which can increase leukocyte infiltration into atherosclerotic plaques (Sozen *et al.*, 2014).

Plaque formation

Once leukocytes have infiltrated the vessel, they can keep activating the inflammatory response through production of cytokines and also contribute to atherosclerotic plaque formation. The atherosclerotic plaque is made up of macrophages which have taken up LDLs (foam cells), lipids and leukocytes including T cells. T cells can secrete more inflammatory cytokines causing further inflammation. As mentioned previously EC dysfunction can cause alterations in NO signalling and induce proliferation and migration of VSMC. These cells migrate to form a fibrous cap on top the plaque which

becomes calcified overtime. Oxidised LDLs (oxLDLs) can activate the scavenger receptor CD36 on macrophages, which in turn can activate JNK signalling and promote the uptake of LDLs (Rahaman *et al.*, 2006). A study using ApoE$^{-/-}$ mice with either JNK1 or JNK2 simultaneously knocked out demonstrated that foam cell formation was dependent of JNK2. Mice with JNK2 but not JNK1 knockout displayed defective uptake of LDL by macrophages, providing evidence that JNK signalling is key to foam cell formation in this model (Ricci *et al.*, 2004). The differences in JNK isoform function demonstrated in this study will be discussed later in this chapter.

Along with foam cell formation, JNK also plays a role in VSMC migration to the intima of the vessel. After induction of balloon injury in the rat carotid artery, a model used to emulate blood vessel injury, there is an induction of SMC migration and proliferation resulting in the formation of the neointima. This process has been shown to involve the JNK pathway where introduction of a double negative mutant form of JNK into the model artery inhibited SMC proliferation and prevented neointima formation (Izumi *et al.*, 2001). In contrast to this the study by Ricci *et al* showed that VSMC from JNK knockout mice showed similar rates of proliferation and migration when compared to WT mice (Ricci *et al.*, 2004). This contradicting evidence may be the result of the different disease models used in each study. Finally, a study which looked at atherosclerotic prone sites in mice found that JNK promoted EC proliferation at susceptible sites, which was reduced in mice lacking JNK1(Chaudhury, Zakkar, Boyle, Cuhlmann, van der Heiden, *et al.*, 2010). Together these findings further highlight that JNK not only plays a role in the initial stages of atherogenesis but also may be key in plaque formation.

Apoptosis, necrosis and plaque rupture

Within the atherosclerotic plaque, foam cells, VSMC, ECs and immune cells can become apoptotic in the inflammatory environment, which can create a necrotic core. This necrotic core can lead to calcification of the fibrous cap and also rupture of the plaque itself (Badimon and Vilahur, 2014). Chaudhury et al demonstrated that JNK1 is important for pro-apoptotic signalling and expression of proteins such as RIP1 and procaspase-3 in atherosclerotic prone sites. In addition they showed that the JNK regulatory protein MKP1 was located in sites protected against atherosclerosis but not

in susceptible sites and protection was lost in the MKP1$^{-/-}$ model (Chaudhury, Zakkar, Boyle, Cuhlmann, van der Heiden, *et al.*, 2010). This study provides evidence that not only is JNK involved in apoptosis in atherosclerosis but is downregulated in protected areas of the vessel. Supporting this work, a different study using HUVECs infected with adenovirus to cause overexpression of MKP-2 showed that MKP-2 overexpression resulted in depletion of JNK signalling and apoptosis in endothelial cells, shown by a reduction in caspase signalling (Al-Mutairi *et al.*, 2010)

As mentioned earlier, during atherosclerosis macrophages infiltrate the site of atherogenesis and produce the inflammatory cytokine TNF-α. Inflammatory cytokines can act on different regulatory proteins of the extracellular matrix, resulting in a weakened and unstable structure. The JNK pathway has been linked to the inflammatory signalling associated with extracellular matrix dysregulation via downregulation of an enzyme which synthesises collagen, prolyl-4-hydroxylase, which may contribute to atherosclerotic plaque rupture (Zhang *et al*., 2007). There have not been any in vivo studies investigating the role of JNK in plaque rupture due to the lack of established mouse models for this process. However, as mentioned previously, JNK plays an important role in apoptosis and has been linked to ECM degradation. This could suggest that JNK may play an important role in the late stages of atherogenesis too. These findings bring together a bigger picture of how JNK signalling is involved in the different progressive stages of atherosclerosis, suggesting JNK could be an attractive target for possible treatments.

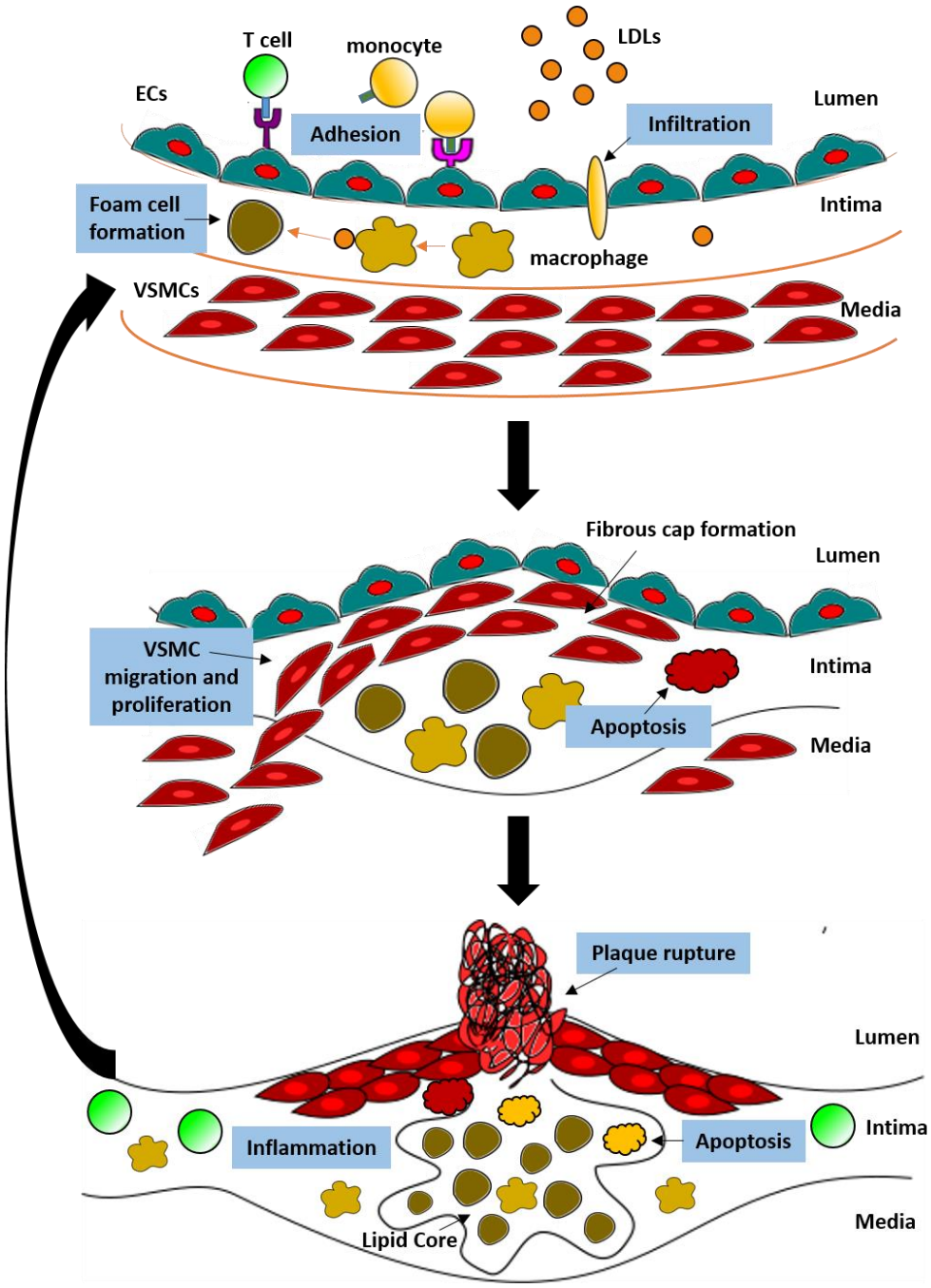


Figure 1.5 JNK in atherosclerosis
Image represents a summary of JNK signalling in atherosclerosis. The different processes which JNK signalling has been linked to are highlighted in blue throughout the different stages of atherogenesis. Starting with leukocyte adhesion and infiltration, inflammatory response, VSMC migration and proliferation, foam cell formation and apoptosis. These processes repeat until finally plaque rupture occurs. Adapted from (Libby, Ridker and Hansson, 2011).

1.4.2 JNK in cancer

The definition of cancer has changed over the years from a tumour described as a hard lump with extending vasculature in the early 1800s (Bell, 1822) to a defect in normal cell growth regulation which results in the production of an abnormal collection of heterogeneous cells in a microenvironment with complex individual properties in 2016. A noted review by Hanahan and Weinberg explored a number of key hallmarks of cancer, drawing together six key strategies cancer uses to thrive. These are; limitless replicative potential, sustained angiogenesis, evasion of apoptosis, self-sufficiency in growth signalling, insensitivity to anti-growth signals and tissue invasion and metastasis (Hanahan and Weinberg, 2000). It is now also known that genetics can play a huge part in the development of cancer, for example, the discovery of the pathogenic variants of the BRCA1/2 genes which have been linked to the increased risk of breast and ovarian cancer (Antoniou *et al.*, 2003). Although a huge amount of progress has been made over the years, there are still many key events in cancer development, progression and treatment which are still not fully understood.

Not surprisingly, the JNK pathway has been studied in many different cancer cell lines and animal models, where the influential role of JNK on cancer development, progression and resistance or susceptibility to cancer treatments is becoming more and more evident (Bubici and Papa, 2014). JNK could be an attractive target for drug development for the treatment of cancer, however because the pathway is involved in many essential cellular processes throughout the body, it is a difficult protein to target without detrimental effects. The evidence demonstrating that JNK has the ability to promote both tumour development and tumour suppression, (see review Kennedy & Davis 2003) makes JNK signalling in cancer even more complicated and difficult to pin point. In the next section of this chapter the role of JNK signalling in cancer will be discussed in more detail.

1.4.2.1 JNK in cancer development and progression

Early studies linked the oncogene ras to JNK activation (Binetruy, Smeal and Karin, 1991) (Adler *et al.*, 1996), however the JNK pathway can be activated by a number of different oncogenes including but not limited to; Met (Rodrigues, Park and Schlessinger, 1997), Bcr-Abl (Raitano *et al.*, 1995), gep (Kashef *et al.*, 2011) and NPM-ALK (Leventaki *et al.*, 2007). JNK itself has also been demonstrated to be highly

expressed in or implicated in the development of a number of different cancers such as breast cancer (Wang *et al.*, 2010), pancreatic cancer (Okada *et al.*, 2014), liver cancer (Chang, Zhang, *et al.*, 2009) and brain cancer (Antonyak *et al.*, 2002). JNK has also been shown to be required for lung tumour formation initiated by Ras signalling in mice (Cellurale et al. 2011).

The role of Ras-JNK signalling in cancer has been investigated for many years now. In 1991 a dominant-negative c-Jun was used to demonstrate the role of c-Jun in ras-transformed cells, where the mutant form of c-Jun reduced tumorigenicity (Lloyd, Yancheva and Wasylyk, 1991). With all of the evidence drawn together JNK could be seen as a promising target for treatment of cancer. This was recently confirmed in a study where the new ATP-competitive JNK inhibitor AS602801 was shown to cause cytotoxicity in cancer stem cells derived from four difference types of cancer (pancreatic, lung, ovarian and brain) and also inhibit self-renewal and tumour-initiating capacity of these cells (Okada *et al.*, 2016). This data clearly demonstrates that JNK could be a promising target for treatment of cancer and highlights the need for JNK signalling in cancer to be fully understood for further advances to be made.

Many different types of approaches have been used to investigate cancer and the processes involved in its development. These include *in vitro* studies (Wang *et al.*, 2010), tissue staining (Khatlani *et al.*, 2007), xenograft models (Okada *et al.*, 2013) and animals models (Eferl *et al.*, 2003), all of which have their limitations. In this next section, studies carried out to investigate the role of JNK in cancer development, progression and suppression, and the different types of JNK function induced by anti-cancer drugs will be discussed in more detail.

Liver Cancer

Over recent years a substantial amount of research has been carried out investigating the role of JNK in hepatocellular carcinoma (HCC), where JNK has been linked to the pathogenesis and poor prognosis of HCC (Das *et al.*, 2011). Much of this work has been demonstrated using animal models of HCC, where HCC is induced though intraperitoneal injection of Diethylnitrosamine (DEN). For example, using the DEN induced HCC rat model it was demonstrated that c-Jun was required for tumour development where inactivation of c-Jun resulted in protection from tumour

development (Eferl *et al.,* 2003). In the same model another group showed that inhibition of JNK signalling using SP600125 caused a switch from oncogenic to tumour-suppressive signalling (Nagata *et al.,* 2009). These studies link well together suggesting the oncogenic signalling by JNK may be through c-Jun. Eferl and co - workers suggested that c-Jun may inhibit the tumour suppressive signalling by p53, where susceptibility to induced apoptosis caused by KO of c-Jun was reversed when p53 was also knocked out (Eferl *et al.,* 2003) . Other studies have also linked the regulation of p53 with JNK signalling (Tafolla *et al.,* 2005) and c-Jun with hepatocyte proliferation and survival (Behrens *et al.,* 2002), connecting together a possible mechanism of HCC development and progression.

Along with inhibition of apoptosis, the JNK pathway has been implicated to pro-inflammatory signalling in myeloid cells which is thought to promote liver pathology and HCC development (Han *et al.,* 2016). In this study a DEN induced HCC mouse model with JNK deficiency specific for myeloid cells only was established. Mice with the JNK deficiency had decreased tumour size when compared to their WT counterpart, however there was not a significant change in the number of tumours. The group suggested that JNK was not required for the formation of tumours but may act to promote tumour development in the liver (Han *et al.,* 2016). Finally, JNK has also been demonstrated to play a role in HCC cell migration, where inhibition of JNK by SP600125 in human HCC derived cells inhibited cell migration (Matsushima-Nishiwaki *et al.,* 2016). Taken together these studies suggest that JNK not only plays a role in HCC tumour formation and survival but also in tumour invasion, suggesting JNK is a key component of HCC. Studies have been carried out on individual JNK isoform function in HCC, this will be discussed in detail later in this chapter.

Breast Cancer

Another cancer which JNK has been linked to is breast cancer. However, unlike HCC where a lot of work has been carried out in animal models, studies tend to be mostly performed in breast cancer cell lines. For example, Wang *et al* investigated the effects of hyperactive JNK on breast cancer cell function. To do this they expressed a constitutively active JNK in MDA-MB-468 breast cancer cells and looked at the effect this had on different cellular processes and signalling pathways. Hyperactive JNK

activity caused an increase in breast cancer cell invasion and migration without causing activation of apoptosis. The JNK signalling involved in the increase in cell invasion was thought to be dependent on AP-1 as siRNA knockdown of AP-1 inhibited cell invasion. Interestingly inhibition of ERK caused a decrease in AP-1 activity in cells with hyperactive JNK suggesting ERK activation was playing a role downstream of JNK and upstream of AP-1. JNK markers of Epithelial-mesenchymal transition (EMT), a process which causes alterations in cell structure to increase cell motility and invasiveness, were greatly upregulated in cells with constitutively active JNK, suggesting that JNK may play a role in the EMT process. They also found that levels of JNK were sustained in the mouse breast cancer cell line, 4T1, which is used to model stage IV breast cancer due to their metastasizing ability. This confirmed a role for JNK in cell invasion since JNK levels were not sustained in the non-metastasising cells analysed. Finally hyperactive JNK decreased apoptosis induced by taxol, this was impaired by addition of an ERK inhibitor, again suggesting a link for JNK-ERK/AP-1 signalling in breast cancer (Wang *et al.*, 2010).

Similar approaches in MDA-MB-231 breast cancer cells showed that activation of JNK via the G protein G12 promoted breast cancer cell invasion (Juneja, Cushman and Casey, 2011). Cells treated with siRNA targeting JNK showed a significant reduction in cell invasion when compared to cells treated with control siRNA. This provides evidence that activation of breast cancer cell invasion through G12 requires JNK. As well as invasion of breast cancer cells, JNK has been shown to play a role in tumour angiogenesis which involves the formation of new blood vessels to provide oxygen and nutrients to the tumour (Belugali Nataraj and Salimath, 2012). In this study a protein called novel proangiogenic glycoprotein (NAP) was shown to induce migration of breast cancer cells *in vitro* and angiogenesis *in vivo*. NAP induced activation of JNK signalling, but not ERK, in MDA-MB-231 breast cancer cells suggesting JNK may play a part in angiogenesis induced by NAP. Finally, in a xenograft JIMT-1 (HER2 inhibitor resistant cell model) mammary tumour model system, inhibition of JNK itself impaired tumour growth in comparison to control conditions (Phelps-Polirer *et al.*, 2016). Taken together this evidence demonstrates that JNK plays a role in various stages of breast cancer.

1.4.2.2. JNK in cancer suppression

In contrast to promoting cancer, evidence has shown that JNK can also be involved in the suppression of cancer through activation of key regulatory mechanisms. This normally means that before a cell has a chance to become mutated and develop into a tumour, cellular processes are in place to promote cell cycle arrest and/or cell death. Processes are also in place to prevent pro-oncogenic signalling, for example, JNK has been demonstrated to activate the tumour suppressor protein p53 in breast cancer and colon cancer cells, which in turn inhibits Mcl1, elF4E, PI3 kinase, three oncogenes which promote cancer survival. The kinase also inhibits Wip1 and MdmX, inhibitors of p53, while activating pro-oxidative genes PUMA and PIGs, which creates a positive feedback loop of enhancing JNK activation and therefore activation of p53 (Shi *et al.*, 2014). In line with this work, JNK has also been shown to play a suppressive role in a breast cancer animal model, where JNK deficiency increased the branching of mammary ducts *in vivo* and promoted basal like breast cancer tumours (Cellurale *et al.*, 2012).

Along with the tumour suppressor protein p53, JNK has also been linked to suppressing cancer via the transcription factor ATF2. ATF2 is a substrate of JNK which has been demonstrated to be highly expressed in different cancers and has been linked in its active form as a possible marker for melanoma progression (Berger *et al.*, 2003). Similarly in mammary carcinomas expression of ATF2 was shown to be linked to poor prognosis, however the phosphorylated ATF2 protein was not linked to promoting cancer progression (Knippen *et al.*, 2009). More recently a link has been made between JNK-ATF2 signalling playing a role in the suppression of liver cancer (Gozdecka *et al.*, 2014). Gozdecka *et al* used MEFs and hepatoblasts from ATF2 KO mice that were susceptible to transformation and compared colony formation to their WT counterparts. KO cells formed a greater number of colonies which was impaired through re-expression of ATF2, suggesting ATF2 may be a suppressor of cell growth *in vitro*. This was also confirmed *in vivo* where recipient mice received hepatoblasts from either ATF2 WT or KO mice injected into the spleen, with livers then assessed for tumour growth and size. Again mice injected with ATF2 KO cells produced more tumours which were also of a greater size, suggesting ATF2 is a suppressor of liver cancer development. Finally, the group used transcriptomic data and microarray to

examine transcriptional targets of ATF2 in human tumours. They found that not only was there low expression of ATF2 target genes in cancers including breast, brain, and lung cancer, but these genes were also shown to be induced by JNK-ATF2 signalling.

Taken together, this evidence along with the studies in the previous section would suggest that JNK may play important roles in both pro-cancer and anti-cancer signalling in breast and liver cancer. Interestingly, studies promoting cancer seem to suggest a more prominent role for the transcription factor c-Jun, whereas signalling in cancer suppression was shown to involve activation of ATF2, suggesting that possibly the progressive or suppressive role of JNK could be determined to some extent by what transcription factors are activated. Noting these differences in JNK function between cancers promotes the need for great care to be taken when targeting this pathway in these diseases.

1.4.2.3. Chemotherapy damage

Following on from the role of JNK in cancer suppression, JNK has also been shown to be involved in the treatment of cancer by certain chemotherapy treatments. Chemotherapeutic drugs or anti-cancer drugs are used to target cancer cells and modulate important processes required for tumour growth and ultimately lead to cell death and tumour reduction. Many different chemotherapeutic drugs have been shown to activate the JNK pathway, some of which are summarised in the review by Fan and colleagues, along with the different cancer cell lines that were used. These include but are not limited to: Vinblastine, Vincristine and Paclitaxel (Taxol) in ovarian, breast and leukaemia cells. The review also summarises a list of studies that inhibit the JNK pathway and demonstrate that micro-tubule inhibiting drugs activate apoptosis via this pathway, however it is also mentioned that these drugs do not always activate JNK, and activation may be dependent on cell type (Fan and Chambers, 2001).

The drugs mentioned above, known as microtubule interfering or inhibiting agents, are used to suppress microtubule dynamics which play important roles in many different cellular functions including cell cycle, which can lead to cell cycle arrest and apoptosis. A study was carried out using five different microtubule-interfering agents (paclitaxel, docetaxel, vinblastine, nocodazole and colchicine) in two different cancer cell lines (MCF-7 breast cancer cells and BR ovarian cancer cells). This was the first

study to demonstrate that these agents activate the JNK pathway via Ras and ASK1, a MAP3K, and suggest that internal activation of the pathway was initiated by disruption of the microtubules (Wang *et al.*, 1998). Another study that was carried out in the MCF-7 breast cancer cell line demonstrated that activation of the JNK pathway by microtubule-interfering agents inhibited Insulin-like growth factor - 1 (IGF-1) signalling, which in turn reduced survival and growth of the breast cancer cells (Mamay *et al.*, 2003).

Finally, combination treatment of different anti-cancer agents is used to try and enhance their effect on cancer cells. A combination of the drugs doxorubicin and gamitrinib was shown to activate JNK and reduce growth of tumours in breast xenograft models (Park *et al.*, 2014). Breast cancer cells also exposed to the dual treatment *in vitro* accumulated apoptotic proteins Bim and Bax. This was reduced in cells treated with the JNK inhibitor SP600125, suggesting JNK plays an important role in apoptotic signalling induced by the combination treatment.

1.4.2.4 Chemotherapy resistance

Although there are many studies which report that anti-tumour therapies activate pro-apoptotic signalling via the JNK pathway, evidence has also accumulated which suggests that in the case of treatment with cisplatin, JNK activation may result in a protective role through the activation of downstream target c-Jun/AP-1 (Vasilevskaya and O'Dwyer, 2003). The authors do conclude that it is c-Jun and not JNK itself which should be considered to enhance cancer treatment as JNK itself may also promote pro apoptotic signalling via a different downstream target. For a recent review on cisplatin - JNK signalling please see (Yan, An and Kuo, 2016).

Along with activation of transcription factors, two other cellular processes which have been linked to chemotherapy resistance are hypoxia (Shannon *et al.*, 2003) and autophagy (Yoon *et al.*, 2012). JNK signalling has been linked to both of these processes where hypoxia has been demonstrated to activate JNK (Laderoute *et al.*, 1999) and JNK has been thought to play a role in autophagy activation (Zhou *et al.*, 2015). A study was carried out in MDA-MB-231 breast cancer cells to investigate the signalling involved in the resistance to taxol during hypoxia (Notte *et al.*, 2013). Cells that were treated with taxol alone showed apoptotic signalling after 16h, however the

cells which were incubated in hypoxic conditions had reduced apoptosis and cell death. Hypoxic conditions were shown to enhance autophagy in cells which in turn promoted cell survival. The JNK pathway was also activated which caused phosphorylation of BCl2 and Bcl-XL proteins. This can lead to either activation of autophagy or activation of apoptosis depending on location within the cell. Notte *et al* also showed autophagy was activated independently of JNK signalling and possibly through the classical mTOR pathway. This would suggest that taxol was activating apoptosis via the JNK pathway, however in contrast they also showed using siRNA that JNK silencing caused an increase in apoptotic signalling and cell death under normoxic and hypoxic conditions, suggesting that JNK signalling had a protective effect in breast cancer cells treated with taxol. However, this is the only study to demonstrate this, where most studies show a role for JNK in Taxol - induced apoptosis.

Hypoxia has also been shown to activate Nrf2 signalling which is responsible for maintaining balanced antioxidant levels (Syu, Chi and Kung, 2016). In this study chemoresistance against cisplatin treatment occurred during hypoxia, due to activation of Nrf2 and consequential detoxification which lead to cell survival. As mentioned above evidence has demonstrated that hypoxia can activate JNK signalling (Jin *et al.*, 2000). JNK signalling has also been linked to the activation of Nrf2 (Keum *et al.*, 2003 & Varì *et al.*, 2015), this could be a possible alternative route to the chemoresistance mentioned earlier by Notte *et al*. In agreement with this theory, JNK signalling has been reported to play a role in chemoresistance through suppression of the generation of ROS in pancreatic cancer (Suzuki *et al.*, 2014). Pancreatic cancer stem cells (CSCs) treated with anti-cancer drugs 5-fluorouracil and gemcitabine showed chemoresistance. Once treated with the JNK inhibitor SP600125 cells showed a significant increase in cell death and production of ROS.

Finally, Lui *et al* demonstrated that inhibition of JNK in the HCC cell line HEPG2 can reduce resistance to treatment with cisplatin, causing an increase in apoptosis and decrease in cell viability. The study suggests that JNK can be activated by cisplatin and may play a role in promoting expression of the gene MDR1. MDR1 then promotes transcription of P-glycoprotein, a drug efflux protein, which would promote the cells resistance to cancer (Liu *et al.*, 2016). As mentioned previously JNK signalling has been demonstrated to be upregulated in HCC, this has also been linked to

chemoresistance in HCC when cancer cells were treated with tumour necrosis factor – related apoptosis inducing ligand (TRAIL) (Mucha *et al.*, 2009). JNK signalling was demonstrated to be enhanced in cancer cell lines when compared to their primary counterpart. JNK/c-Jun signalling was activated by TRAIL in a dose dependent manner, which in turn caused inhibition of apoptosis. Once JNK was inhibited, cell cycle arrest was observed alongside a significant increase in apoptosis. This evidence would suggest JNK inhibition could be a possible target in combination with this kind of therapy as currently, in some cases, the therapy is activating JNK pro-oncogenic signalling.

In summary JNK signalling has been demonstrated to be key in many different stages of cardiovascular disease and cancer. The integral complexity of JNK signalling has proven to be a challenge for researchers to be able to target the pathway for treatment of disease. Furthermore the differences in JNK signalling between cell types and environments means that JNK signalling must be investigated separately in each new scenario, if a clear mapping and understanding of this pathway is desired. More recently, investigations into the specific functions of the individual isoforms has been carried out and it is now suggested that JNK should not be looked at as a single protein as there are not only differences between isoform function but also opposite functions have been demonstrated. For the remainder of this chapter, research on JNK isoform function will be discussed, highlighting the importance for studying each isoform individually in the future.

1.5 Differences in the function of JNK isoforms

Research on the JNK pathway over the years has had a tendency to group the individual JNK isoforms (JNK1, 2 and 3) together and to treat them as one. This may partly be due to the convenience of using the JNK drug inhibitors, for example SP600125, which unfortunately targets all three JNK isoforms together. However there have been a few groups which have used different approaches to determine the function of each isoform individually and have shown why it is important to look at each isoform separately. In 2004 the Sabapathy group demonstrated the different roles JNK1 and JNK2 play in the activation and regulation of the transcription factor c-Jun through the use of genetically modified cells lacking either isoform (Sabapathy *et al.*, 2004). The group highlighted the role of JNK1 in the phosphorylation of c-Jun and showed that upon cell stimulation the amount of JNK1 interacting with c-Jun increased. In contrast JNK2 was mostly bound to c-Jun in unstimulated cells and regulated its activity through involvement in ubiquitination and targeting c-Jun for degradation (Sabapathy *et al.*, 2004).

Assessing the roles of individual JNK isoforms has become a lot more accessible over the years, due to the availability of isoform specific knock out (KO) cell lines, mice and tissues, as well as knockdown techniques, studies have been carried out to identify the specific and redundant functions of the JNK isoforms. For example, using plasmids encoding GFP tagged JNK and immunofluorescence imaging, a protein which is required for normal cell division during brain development, WD40-repeat protein 62 (WDR62), has been shown to recruit JNK1 but not JNK2 to the spindle pole to negatively regulate WDR62 in neural development (Lim *et al.*, 2015). In monocytes siRNA was used to target JNK1 and JNK2 to demonstrate that these isoforms have opposing roles in the regulation of monocyte differentiation, with JNK2 being a negative regulator and JNK1 promoting differentiation (Chen-Deutsch *et al.*, 2009). Finally, in a mouse model of steatohepatitis (fatty liver disease) JNK1 and JNK2 have been shown to have distinct roles where JNK1 promoted the development and progression of steatohepatitis, whilst in contrast, JNK2 played no role in disease development (Singh *et al.*, 2009).

Although JNK isoform function is beginning to be investigated in many different settings like the ones mentioned above, with respect to individual isoform function in cancer and CVD, a large amount of evidence has accumulated demonstrating clear independent function. The remainder of this section will focus more specifically on the studies that have demonstrated differences in JNK isoform function in CVD and cancer.

1.5.1 Cardiovascular disease

Due to the important roles JNK plays in CVD, as demonstrated by the studies discussed previously, investigations into JNK isoform function in cardiovascular cells and disease models are becoming more common. The assumption was that JNK proteins functioned together and therefore targeting them together would have the desired effect. However, because JNK plays key roles in so many essential cellular processes, targeting the proteins is not always easy. Through the generation of knockdown systems and knockout animal models, evidence is clearly demonstrating that the JNK isoforms can work independently of each other and this has opened up new strategies for targeting JNK signalling in CVD. This evidence will be discussed in more detail during this section.

As mentioned earlier inflammation contributes to various CVDs and can induce alterations to the ECM. It has been shown that in fibroblasts alterations to the ECM component fibronectin can cause increased phosphorylation of JNK1 but not JNK2 or JNK3 (Tafolla *et al*., 2005). In the same model it was demonstrated that p53 expression was regulated positively by JNK2 and negatively by JNK1 (Tafolla *et al*., 2005), highlighting important differences between isoform function in this cell type. In contrast to these findings, JNK2 has been shown to be activated in bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) that were exposed to laminar shear stress. JNK2 was also thought to be involved in the EC alignment, which occurs due to disturbed blood flow to prevent atherogenesis (Hahn *et al*., 2011). This group also demonstrated that JNK2 activation was integrin dependent and stimulated by fibronectin, however the difference in results between both groups surrounding the ECM and JNK activation could be due to differences in cell type or model.

A common feature of vascular disease is vascular wall thickening and stiffening which is regulated through ECs (Krishnan *et al.*, 2010). During atherosclerosis disruption to the endothelial barrier occurs due to changes in cell to cell junctions, allowing infiltration of immune cells and progression of pathogenesis (Sun *et al.*, 2012). Using various epithelial cells JNK has been proven to play a role in the negative regulation of adheren junction (AJ) formation, a process which is important for cell organisation (Lee *et al.*, 2011). Through the use of JNK KO mice or lentiviral knockdown it was demonstrated that JNK prevents AJ formation in keratinocytes (you *et al* 2013). In addition, JNK activity has been proven to correlate inversely with substrate rigidity. Where in 2D and 3D culture models, along with *in vivo* models, increasing the firmness of the substrate decreased the level of JNK phosphorylation and increased the number of adheren junctions formed. Interestingly keratinocytes from JNK1 KO mice epidermis contained fewer cell layers compared to JNK2 KO mice which contained a similar number of layers as wild type mice (you *et al* 2013). This could be explained by the opposite roles JNK1 and JNK2 have been shown to play in the regulation of c-Jun (Sabapathy *et al.*, 2004), where knocking down JNK1, a positive regulator of c-Jun phosphorylation, would result in a reduced number of cells.

Individual JNK isoform function has been implied in the regulation of eNOS. In particular JNK2 has been shown to directly phosphorylate eNOS in BAECs, playing a role in negatively regulating NO production and possibly creating a link between JNK2 and prevention of endothelial dysfunction (Park *et al.*, 2012). However in contrast to the proposed protective role, through the use of JNK2 specific KO mice, JNK2 has been found to contribute to endothelial dysfunction through regulating the production of ROS and having an involvement in the antioxidant defence systems during hypercholesterolemia (Osto *et al.*, 2008). These findings demonstrate the importance of characterising the individual JNK isoforms in different models when investigating the pathogenesis of CVD.

In 2004 Ricci *et al* demonstrated using double KO mice that JNK2 was required for atherogenesis in ApoE KO mice (Ricci *et al.*, 2004). Mice with the JNK2 knockout developed smaller atherosclerotic plaques in the aorta compared to control ApoE KO mice. Interestingly JNK1 KO mice had little change in plaque development, with similar findings produced when looking at foam cell formation. A separate study

investigated the role of JNK1 in endothelial cell injury using a hypercholesterolemic $LDLR^{-/-}$ model with JNK1 deletion (Amini *et al.*, 2014). Deletion of JNK1 promoted survival of endothelial cells shown by a reduction in caspase-3 activation and DNA fragmentation. Also lesion area in the aorta was reduced in the JNK1 knockout mice when compared to their $LDLR^{-/-}$ single knockout counterpart. These studies demonstrate major differences in the roles played by JNK1 and JNK2 in atherogenesis and are another example as to why JNK 1, 2 and 3 should be investigated individually.

Finally, Hirosumi *et al* used JNK1 and JNK2 knockout mice to investigate the role played by JNK signalling in obesity and insulin resistance. Total body adiposity blood insulin levels were reduced in JNK1 but not JNK2 KO mice. JNK1 but not JNK2 mice were protected from the development of obesity induced insulin resistance, showing an important role for JNK1 in type 2 diabetes and again a clear difference in isoform function (Hirosumi *et al.*, 2002).

1.5.2 Cancer

Similar to CVD where JNK has been linked to key stages of pathogenesis in different disease, JNK has also been demonstrated to play important roles at various stages and in many different types of cancer. Therefore, investigation into isoform function may be key in obtaining specific targets for the development of treatment.

HCC

As mentioned earlier in this chapter, one of the most studied cancers demonstrating a key role for JNK is HCC, where JNK activity has been shown to be increased in HCC and this has led to many studies looking into individual isoform function in this cancer. For example, Hui *et al* analysed tissue samples from 53 HCC patients and showed that there was an increase in activity of JNK1 but not JNK2 (Hui *et al.*, 2008). The group also used a HCC cell line where they used lentivirus to knockdown JNK1, JNK2 and JNK1/2 together to look at the effects of JNK knockdown on cell proliferation. JNK1 and JNK1/2 knockdown produced a decrease in proliferation where as JNK2 knockdown had no significant effect on proliferation. When these cells were implanted into nude mice, again JNK1 and JNK1/2 knockdown cells developed smaller tumours than control and JNK2 knockdown mice, linking JNK1 but not JNK2 to the tumorigenesis of HCC cells, possibly through the induction of proliferation. Finally,

this study also showed that JNK1 but not JNK2 promoted cancer development, independently of c-Jun, by down regulating p21expression and upregulating c-Myc expression, two proteins involved in cell cycle and growth.

Complimenting this work, Chang *et al* analysed human hepatocellular carcinoma samples paired with non-cancerous tissue and found that JNK1 activation was enhanced in over half of the cancer samples analysed where as JNK2 activation remained relatively equal between control and cancer samples. They also demonstrated a role for JNK1 in the development of HCC where JNK1 was found to enhance proteins which play a critical role in cell growth (Chang et al. 2009a). Furthermore the same group used gene expression profiling to link high JNK1 levels with over-expression of many genes associated with cancer progression and down regulation of genes required for normal liver function (Chang et al. 2009b). The group also used patient survival data which revealed that patients with high JNK1 levels had a lower average survival rate than patients with low JNK1 levels and this correlated with the Kaplen-Meier estimation of overall survival which indicated that survival probability at one year was 91% for patients with low JNK1 and only 18% for patients with high JNK1 levels. This data is a clear example of differences in JNK isoform function in HCC. The importance of JNK1 in HCC development has also been shown through inhibition of the NF-kappaB pathway (Sakurai *et al.*, 2006). Where lack of an NF-kB enhanced mice susceptibility to hepatocarcinogenesis through promotion of sustained JNK1 activation. Taken together JNK1 but not JNK2 can be seen to play a prominent role in HCC and may represent a potential target for treatment in the future.

Skin cancer

While JNK1 has been linked to promoting HCC, JNK2 has been linked to skin cancer development where skin tumorigenesis has been shown to be suppressed in JNK2-defiencent mice in comparison to wild type mice (Chen *et al.*, 2001) and JNK2 but not JNK1 was shown to work with Ras to promote malignancy of epidermal cells (Ke *et al.*, 2010). In contrast JNK1 has been connected to the suppression of skin cancer where JNK1 knockout mice develop a greater number of carcinomas than wild-type or JNK2 knockout mice (She *et al.*, 2002). JNK1 also plays a role in apoptosis induced by the treatment of skin cancer by UVA-radiation (Choi *et al.*, 2009). The transcription

factor and downstream target of JNK signalling, ATF2, has also been shown to play a key role in suppressing skin cancer (Bhoumik *et al.*, 2008) which could possibly be linked to the suppression caused by JNK1 signalling. JNK2 has also been linked to multiple myeloma (MM) where JNK2 was demonstrated to be constitutively activated in MM cell lines and primary tumours (Barbarulo *et al.*, 2013). This group also suggest that JNK2 inhibits JNK1 pro-apoptotic activity in MM through activation of poly (ADP-ribose) polymerase (PARP) 14 which binds to JNK1 and prevents further action. Not only do these studies represent individual JNK isoform function but also opposing functions between isoforms in skin cancer.

Lung, brain and breast cancer

In comparison to HCC and skin cancer little research has been carried out looking into JNK isoform function in other cancers. Nitta *et al* found that expression of JNK2, specifically the alpha variant, was enhanced in non-small cell lung carcinoma tumours and that shRNA knockdown of JNK2 reduced tumour growth in mice. The group also conclude that the phosphorylation of STAT3 by JNK2 plays an important role in NSCLC tumorigenicity (Nitta *et al.*, 2011). JNK2 has also been shown to promote development of glioblastoma tumours through activation of AKT signalling (Cui *et al.*, 2006). A study looking at JNK3 expression in brain tumour cell lines reported that 10 out of 19 cell lines showed a loss of expression of the JNK3 gene, suggesting an important role for JNK3 in brain cancer (Yoshida *et al.*, 2001). There is conflicting evidence demonstrating the role of JNK2 in breast cancer. One study has demonstrated using a JNK2 knockout mouse model of mammary cancer that JNK2 is important in tumour suppression through regulation of the cell cycle (Chen *et al.*, 2010). Whereas Kaoud *et al* used a JNK2 signalling inhibitor to demonstrate the role of JNK2 in promoting breast cancer cell migration (Kaoud *et al.*, 2011). These results highlight the requirement for further studies into JNK isoform function in breast cancer, to allow a clear understanding of the signalling involved.

As well as disease progression itself, individual isoform function has also been demonstrated during the treatment of cancer. In an investigation of cell death signalling induced by the microtubule inhibitors vinblastine and taxol, both drugs induced cell death in MEFs via the JNK pathway (Kolomeichuk *et al.*, 2008).

Interestingly vinblastine treated cells required either JNK1 or JNK2 for cell death to be induced, however in taxol treated cells JNK1 showed a more prominent role in the induction of cell death. This data represents differences in JNK signalling produced by different stimuli within the same cell type and also differences in isoform function. In line with JNK signalling in hypoxic cancer mentioned previously but in contrast to the pro apoptotic signalling of JNK1 induced by taxol, JNK1 but not JNK2 was found to relinquish colon cancer cells more sensitive to treatment with the chemotherapeutic drug oxaliplatin under normoxic and hypoxic conditions (Vasilevskaya *et al.*, 2015).

Taken together, these studies display clear evidence for promoting the investigation into individual JNK isoform function in cancer. The fact isoform signalling can not only differ between cancer types but can also be opposing indicates more care must be taken when targeting this pathway. It also highlights that chemotherapeutic drugs need to be specific to different cancer types as the signalling induced can vary between locations.

Taken together the research discussed in this review provides evidence demonstrating the importance of understanding the functions of the JNK pathway in cardiovascular disease and cancer. The JNK pathway has been linked to the pathophysiology of various CVDs and cancers, and has been shown to be cell, cell location, stimulant, stimulant source, cell cycle stage and time dependent. The pathway has also been shown to promote both cell survival and cell death which adds to the complexity of the signalling when trying to interpret the role of JNK in different models. Finally, this review highlights the great importance of studying the different JNK isoforms individually, with research demonstrating different and opposing roles played by each isoform within different models. The requirement to find better therapeutic targets for the treatment of CVD and cancer highlights even more the necessity of JNK isoform characterisation within these diseases.

1.6 Aims

Although some studies have demonstrated individual characteristics of the JNK isoforms where they can act independently or even opposingly in different scenarios, characterisation of the JNK proteins has not yet been carried out in a human vascular cell line or a human breast cancer cell line. Since JNK function can differ between cell lines, this characterisation is critical if the JNK pathway is to be fully understood and targeted appropriately in the future.

The objective of this current study was to develop approaches to target and knockdown the individual JNK isoforms in a human vascular and human breast cancer cell line and to investigate the effect of isoform knockdown on a range of cellular processes to allow their role to be determined.

The key aims are outlines below:

- Generate lentivirus to knockdown JNK1 and JNK2 in human umbilical vein endothelial cells (HUVECs).
- Assess the effects of JNK isoform knockdown in HUVECs on JNK signalling and cell growth.
- Generate stable breast cancer cell lines containing JNK1, JNK2 and JNK1/2 knockdown using lentivirus in MCF-7 breast cancer cells.
- Investigate the effects of JNK isoform knockdown on both cell death and cell growth in MCF-7 cells to determine the roles of each isoform.

Chapter Two
Materials and Methods

2.1 Materials

2.1.1 General Reagents

All materials and reagents used were obtained from Sigma Aldrich Chemical Company Ltd. (Pool, Dorset, UK) or at the highest grades possible, unless otherwise stated.

Thermo Fisher Scientific UK Ltd (Leicestershire, UK)

Bovine Serum Albumin (BSA)

L – Glutamine

Gibco™ Penicillin-Streptomycin

Gibco™ Fetal Bovine Serum

Dulbecco's Modified Eagle Medium (DMEM)

Cell culture plates and dishes

GE Healthcare Ltd (Buckinghamshire, UK)

Amersham™ Protram™ – ECL nictocellulose membrane

Lonza (Slough, UK)

Endothelial Cell Basal Medium -2 (EMB-2)

Endothelial Growth Media (EGM™-2) SingleQuots

Bio-Rad Laboratories (Hertfordshire, UK)

Bio-Rad AG® 1-X8 Resin, pre-stained SDS-page molecular weight markers

Santa Cruz Biotechnology

Recombinant human TNF-α

Corning B.V (Buckinghamshire, UK)

All tissue culture flasks, 10cm dishes, graduated pipettes and falcon tubes

Whatmann (Kent, UK)

Nitrocellulose membrane, 3MM blotting paper

2.1.2 FACS Analysis

BD Biosciences

BD Pharmingen™ APC Annexin V

BD Pharmingen™ PE Annexin V Apoptosis Detection Kit I

BD Calibrite™ 3 Beads

Annexin V Binding Buffer, 10X concentrate

Thermo Fisher Scientific UK Ltd (Leicestershire, UK)

Propidium Iodide - 1.0 mg/mL Solution in Water

Sigma – Aldrich

Ribonuclease A from bovine pancreas (RNAse A)

2.1.3 Imaging

Thermo Fisher Scientific UK Ltd (Leicestershire, UK)

4',6-diamidino-2-phenylindole **(**DAPI)

VWR

VECTASHIELD® Mounting Medium

Ibidi

µ-Dish $^{35\ mm,\ high}$ Glass Bottom

2.1.4 Clonogenics

Thermo Fisher Scientific UK Ltd (Leicestershire, UK)

60mm Nunclon™ Delta Surface plates

2.1.5 UVC

UVP

CL-1000 Ultraviolet Crosslinker

2.1.6 Spheroids

Corning®

96 Well Clear Round Bottom Ultra Low Attachment microplates

2.1.7 Lentivirus

Thermo Fisher Scientific UK Ltd（Leicestershire, UK)

GIPZ lentiviral transfer vector glycerol stocks

Thermo Scientific™ TurboFect transfection reagent

One Shot Mach1- T1 Chemically Competent *E.coli* kit

Puromycin Dihydrochloride

Addgene

Packaging plasmid pCMV – dR8.2 dvpr

Envelope plasmid pCMV-VSV-G

Scrambled shRNA plasmid pLKO.1

pLVTHM plasmid

Takara Clontech

Lenti-X™ Concentrator

Parafilm M

Parafilm

QIAGEN

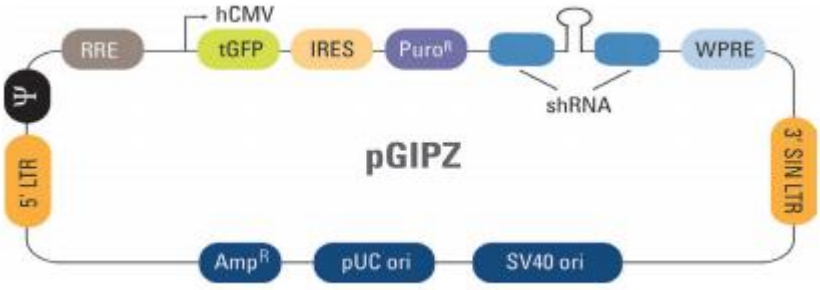
Qiagen Endofree Plasmid maxi kit

Merck Millipore

Millex-HP Syringe Filter Unit, 0.45 µm

2.1.7.1 Lentiviral constructs

For shRNA knockdown of MAPK8 (JNK1) and MAPK9 (JNK2) glycerol stocks of pGIPZ lentiviral vectors (Figure 2.1) containing shRNA targeting JNK1 or JNK2 were purchased from Thermoscientific. For each isoform six vectors targeting a different sequence were purchased.



(Thermoscientific)

Figure 2.1 pGIPZ Lentiviral Vector

pGIPZ Lentiviral Vector (Thermoscientific) containing puromycin resistance gene and green fluorescent protein (GFP) allowing selection of virus containing cells and visual marking.

Target Sequences of shRNA:

MAPK8 (JNK1)	
Lentivirus – 499	TATTACTGGGCTTTAAGTC
Lentivirus – 501	TTCTCAAAGCTATATCCAG
Lentivirus – 503	TAGATGCATCTATTACCAG
Lentivirus – 928	TCTTCTAGGGATTTCTGTG
Lentivirus – 930	TTAGGTCTGTTTTCAACGT
Lentivirus – 931	TCATGATCTAGCTCCATCT
MAPK9 (JNK2)	
Lentivirus – 130	TATTACTGGGCTTTAAGTC
Lentivirus – 169	TTCTCAAAGCTATATCCAG
Lentivirus – 170	TAGATGCATCTATTACCAG
Lentivirus – 511	TCTTCTAGGGATTTCTGTG
Lentivirus – 513	TTAGGTCTGTTTTCAACGT
Lentivirus – 515	TCATGATCTAGCTCCATCT
Lentivirus - NT	ATCTCGCTTGGGCGAGAGTAAG

Table 2.1 JNK1 and JNK2 shRNA targeting sequences
Sequences targeting JNK1 and JNK2 as stated on Thermoscientific website.

Packaging, Envelope and non-target (NT) plasmids were purchased as glycerol stocks from Addgene.

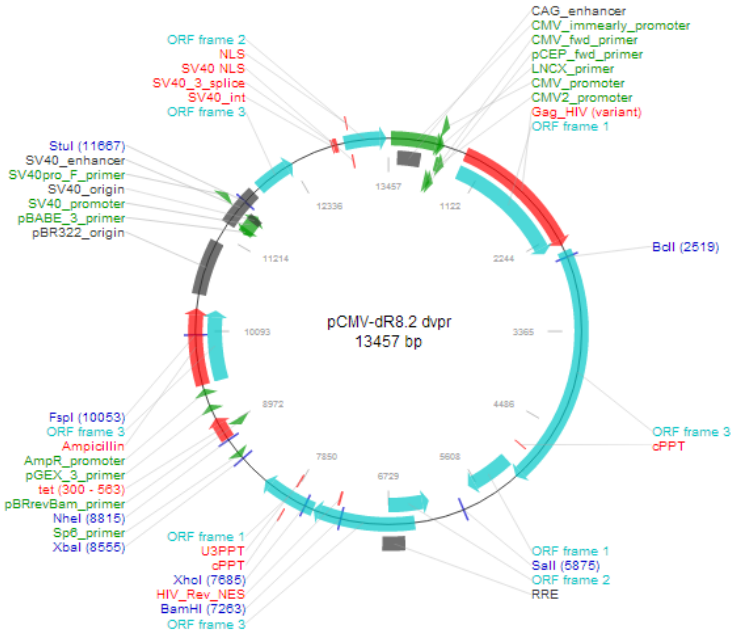


Figure 2.2 Lentivirus Packaging Plasmid

Packaging plasmid pCMV – dR8.2 dvpr (Addgene).

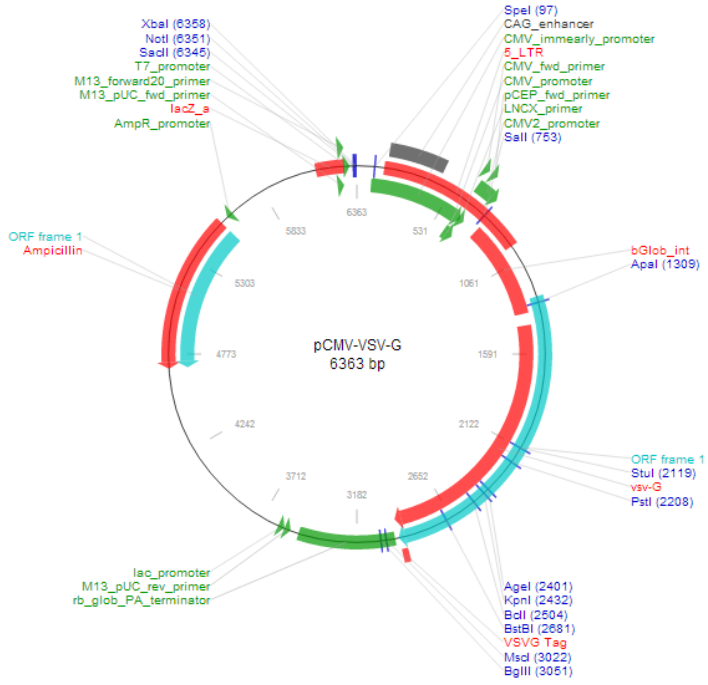


Figure 2.3 Lentivirus Envelop Plasmid

Envelope plasmid pCMV-VSV-G (Addgene).

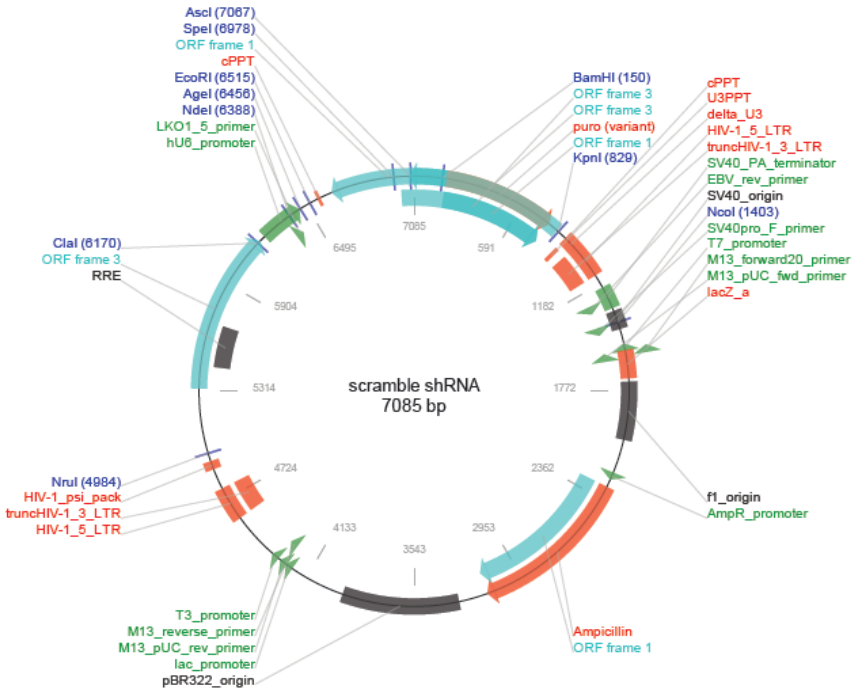


Figure 2.4 Non-target Lentiviral transfer vector (Addgene)

Scramble shRNA plasmid with sequence – CCTAAGGTTAAGTCGCCCTCGCTCG AGCGAGGGCGACTTAACCTTAGG.

Lentivirus transfer vectors were also gifted by Dr Andreadis Stelios from the University of Buffalo which targeted JNK1 and JNK2 individually and also JNK1 and JNK2 (JNK1/2) together (You. H., *et al*. (2013) JNK regulates compliance-induced adherens junctions formation in epithelial cells and tissues. *J Cell Sci*. 126, 18-29) (Figure 3C). The plasmid backbone (Figure 2.5) and shRNA sequences (Table 2.2) can be found below.

Gifted lentivirus is referred to as USA lentivirus throughout this thesis.

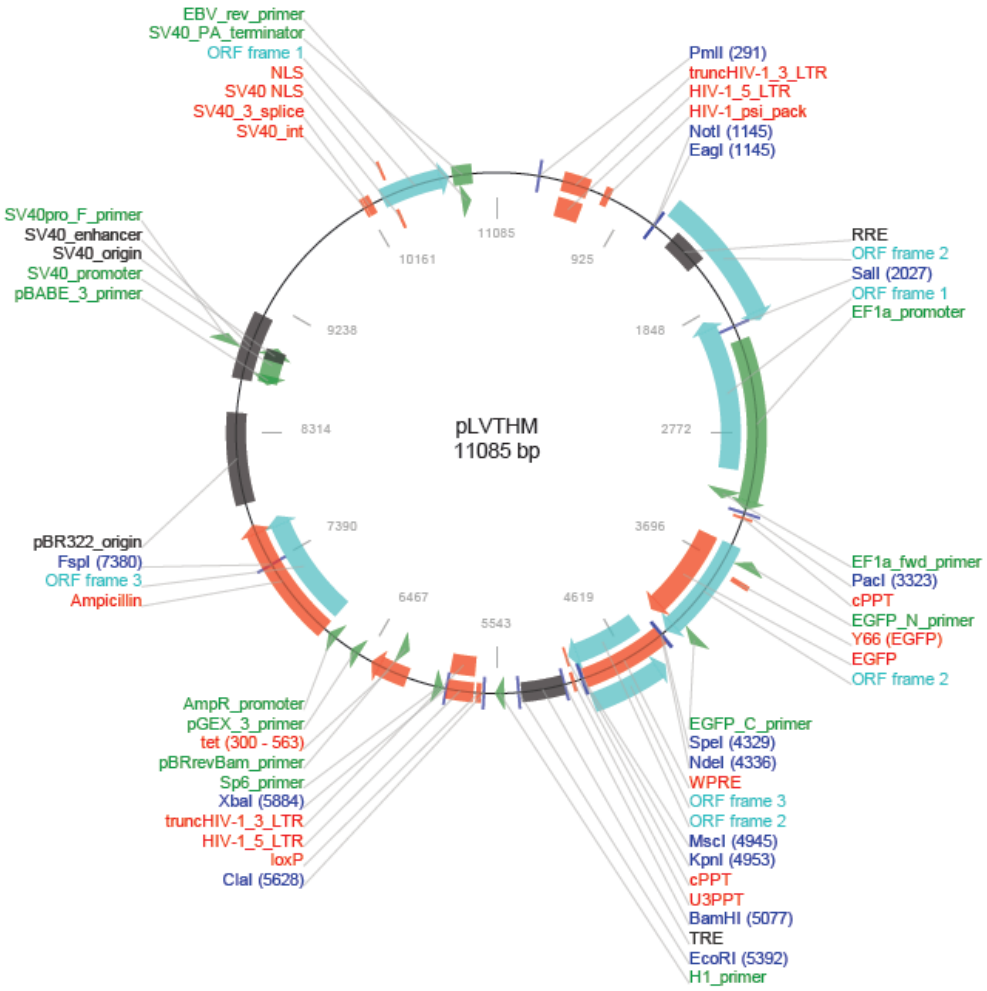


Figure 2.5 Backbone of USA transfer vector

Back bone of USA transfer vector plasmid – pLVTHM (Addgene).

USA shRNA sequences	
JNK1	GGGCCTACAGAGAGCTAGTTCTTAT
JNK2	GCCAACTGTGAGGAATTATGTCGAA
JNK1/2	AAAGAAUGUCCUACCUUCU

Table 2.2 USA shRNA sequences

Sequences for targeting JNK1, JNK2 and JNK1/2 as stated in (You *et al.*, 2013).

2.1.8 Antibodies

Name	Company	Dilution-Incubation
Peroxidase-conjugated Affinipure Goat Anti-Rabbit IgG	Jackson Immuno Research Laboratories Inc.	1/7500 (RT)
Peroxidase-conjugated Affinipure Donkey Anti-Mouse IgG	Jackson Immuno Research Laboratories Inc.	1/7500 (RT)
JNK1 (2C6) Mouse MAb	Cell Signalling Technology	1/15000 (RT)
JNK2 Rabbit Ab	Cell Signalling Technology	1/15000 (RT)
JNK3 (55A8) Rabbit mAb	Cell Signalling Technology	1/3000 (4ºC)
GAPDH (14C10) Rabbit	Cell Signalling Technology	1/60000 (RT)
pc-Jun (KM-1) SC-822 mouse monoclonal IgG	Santa Cruz	1/1000 (4ºC)
P-SAPK/JNK (T183/Y185) Rabbit Ab	Cell Signalling Technology	1/1000 (4ºC)
Phospho-ATF2 (Thr71) Antibody	Cell Signalling Technology	1/1000 (4ºC)
Cleaved PARP (Asp214) (D64E10) XP® Rabbit mAb	Cell Signalling Technology	1/1000 (4ºC)
JNK	Santa Cruz	1/7500 (RT)

Table 2.2 Antibodies.
Information on antibodies used including name, company name and the best concentration and incubation temperature used.

2.2 Methods

2.2.1 Cell Culture

All cell culture work was carried out in a class II cell culture hood (Haraeus Instruments) under aseptic conditions.

2.2.1.1 Human Umbilical Vein Endothelial Cells

Cryopreserved primary Human Umbilical Vein Endothelial Cells (HUVECs) were purchased from Cascade Biologics. Cells were cultured routinely in 75cm^2 cell culture flasks in Endothelial Basal Media (EBM-2) supplemented with EGM-2 single quots (2% Foetal calf serum, 0.2ml Hydrocortisone, 2ml nFGF-B, 0.5ml VEGF, 0.5ml R^3-Insulin like Growth factor-1, 0.5ml hEGF, 0.5ml GA 1000, 0.5ml Ascorbic Acid and 0.5ml Herapin). Cells were cultured at 37^{o}C in a humidified atmosphere of 5% CO_2. Cells were used up to passage six and plated into six or twelve well plates for experimentation. Medium was replaced every 2 days to maintain healthy cells.

2.2.1.2 293 HEK T cells

Human Embryonic Kidney (HEK) 293 T Cells were cultured routinely in 75cm^2 cell culture flasks with medium consisting of Dulbecco's Modified Eagle Medium (DMEM), 10% HI-FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells were plated in 10cm^2 sterile dishes for production of Lentivirus (see section 2.2.3). Cells were cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 and medium was replaced every two days to maintain healthy cells.

2.2.1.3 MCF- 7 cells

MCF-7 (human breast adenocarcinoma cell line) cells were routinely cultures in 75cm^2 cell culture flasks with medium consisting of Dulbecco's Modified Eagle Medium (DMEM) containing sodium pyruvate, pyridoxine hydrochloride and high glucose (Invitrogen, Paisley, UK), 10% HI-FCS, 2mM L-glutamine, 100U/ml penicillin and 100µg/ml streptomycin. Cells were plated in various sizes of sterile dishes and plates for experimentation. Cells were cultured at 37^{o}C in a humidified atmosphere of 5% CO_2 and medium was replaced every two days or three days to maintain healthy cells.

2.2.2 Lentivirus

2.2.2.1 Transformation of *E.coli* competent cells

1 50µl vial of *Escherichia coli (E.coli)* from the One Shot Mach1- T1 chemically competent *E.coli* kit was thawed on ice and 5µg of DNA construct added directly to the vial containing competent cells, mixed gently and incubated on ice for 30 minutes. The vial was then heat-shocked in the water-bath for 45 seconds at exactly 42^{o}C then placed on ice for 2 minutes. 1ml of S.O.C media (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM Glucose) was added directly to the vial and the top was sealed with parafilm (Parafilm M). The vial was then incubated at 37^{o}C on a shaker for 1 hour.
After incubation 100µl of the vial contents was spread onto 10cm plates containing sterile Luria Broth (LB) Agar mix (1% (w/v) Tryptone, 0.5% (w/v) yeast Extract, 1% (w/v) Sodium Chloride, 1.5% (w/v) Agar and 100µg/ml Ampicillin) until dry. Plates were inverted and incubated overnight at 37^{o}C after which colonies could be visualised.

2.2.2.2 Maxi Prep

Competent *E.coli* containing the specific plasmid of interest was grown in media, spread on LB agar plates containing ampicillin and grown overnight to produce colonies. Individual colonies were picked and placed into 5ml of sterile LB broth with ampicillin (1% (w/v) Tryptone, 0.5% (w/v) Yeast Extract, 1% (w/v) Sodium Chloride and 100µg/ml ampicillin) within a 50ml universal tube. The tube was then placed into the incubator at 37^{o}C shaking at 200rpm until the broth became turbid. An aliquot (50µl) of turbid broth was then added to a fresh flask of 150ml sterile LB broth with ampicillin and placed in the incubator overnight at 37^{o}C 200rpm. A Maxi Prep procedure was carried out the next day using the Qiagen Endofree Plasmid maxi kit. The overnight bacterial culture was transferred to a 500ml bottle and centrifuged at 4^{o}C for 15 minutes at 6000rpm. The supernatant was discarded and the pellet formed in the base of the bottle left to dry. Once dry the pellet was re-suspended in 10ml of P1 buffer containing RNase. 10ml of P2 buffer was then added to lyse the cells, the bottle was inverted to mix and then incubated at room temperature for 5 minutes. 10mls of chilled P3 buffer was then added to separate cell fractions, the bottle was inverted to mix and then incubated at room temperature for 10 minutes. After

incubation the mixture was passed through a filter tube into a 50ml falcon tube. 2.5ml of Endofree buffer which removes endotoxins was added to the filtered mixture and incubated on ice for a further 30minutes. During this time an equilibrium column was set up and 10ml of equilibrium buffer was ran through the column. The bacterial solution was then passed through the column followed by two 30ml washes using the wash buffer from the kit. The DNA was then eluted using 15ml of elution buffer and collected in a sterile 50ml bacterial tube. 10.5ml of isopropanol was added to the elutant, mixed thoroughly and then centrifuged for 30minutes at 4^{o}C 12000rpm. Supernatant was carefully discarded and the formed pellet was left to dry and then re-suspended in 5ml of 70% ethanol. The suspension was centrifuged for 8 minutes at 4^{o}C 12000rpm, the supernatant then discarded and pellet left to dry for 15-20 minutes. The pellet containing plasmid DNA was then re - suspended in 100-400µl of RNase free water depending on pellet size. The plasmid DNA was then transferred to a 1.5ml tube and stored in -20^{o}C.

2.2.2.3 Measuring DNA and RNA concentration

Concentrations were measured using NanoDrop 2000c spectrophotometer (R&D Systems, Minneapolis, MN, USA) and the compatible computer software called Nanodrop.

2.2.2.4 Transfection of HEK T cells for Lentivirus Production

A number of 10cm plates were coated in Poly-L-Lysine for 20-30 minutes, washed twice with sterile Phosphate Buffered Saline (PBS) (0.9% (w/v) sodium chloride, 0.04% (w/v) potassium chloride, 0.115% (w/v) sodium phosphate dibasic, 0.02% (w/v) potassium phosphate monobasic anhydrous, adjusted to pH 7.4) and left open to dry in the cell culture hood. HEK T cells were then seeded onto the plates and incubated at 37^{o}C 5% CO_2 until 60-80% confluence was achieved. The media in each 10cm dish was then replaced with 10ml of fresh media (DMEM with 10% FCS, 1% L/G and antibiotics removed). One 1.5ml tube was prepared per 10cm^2 dish containing 600µl of media with serum, L-glutamine, penicillin and streptomycin removed, 2µg p CMV-dR8. 2 dvpr plasmid (Figure 2.2), 2µg pCMV-VSV-G envelope plasmid (Figure 2.3), 2µg pGIPZ shRNA plasmid (Figure 2.2) and 12µl Turbofect (Thermoscientific). 1.5ml tubes were then incubated at room temperature for 20 minutes before the contents of each tube were added drop-wise to the specific 10cm^2 plate. Plates were

incubated for 12-16 hours at 37ºC 5% CO_2. After incubation media containing transfection mixture was removed and replaced with 5ml of complete media and cells were incubated overnight at 37ºC 5% CO_2.

2.2.2.5 Collection and Filtration of Lentivirus particles

Following transfection (2.2.4) and incubation overnight, media containing lentivirus particles was collected from the 10cm plates and stored at 4ºC. 5ml of fresh complete media was added to the plates and they were incubated for a further 24 hours at 37ºC 5% CO_2. After incubation media was collected and pooled with the media collected the previous day. The pooled media was then passed through a 0.45µm filter and either aliquoted into one use volumes and stored in -80ºC or concentrated (see 2.2.6).

2.2.2.6 Lentivirus Concentration

The media containing lentivirus particles that was collected and filtered was then concentrated using the Lenti-X Concentrator kit. 1 volume of Lenti-X-Concentrator was added to 3 volumes of filtered media in a 50ml falcon tube and incubated for 2 hours at 4ºC. The tubes were then centrifuged at 1500 x g for 45 minutes at 4ºC. Supernatant was discarded and the formed pellet was re-suspended in sterile PBS to result in approximately a 100x concentration of lentivirus. The concentrated virus was then aliquoted into single use volumes and stored in -80ºC.

2.2.2.7 Lentivirus Titre

HEK T cells were seeded at 4 x 10^5 cells per well in a 6 well plate and incubated overnight at 37ºC, 5% CO_2. Lentivirus was prepared as 10-fold serial dilutions from 10^{-1} to 10^{-4} in media containing 8µg/ml of polybrene. 1ml of media containing polybrene was added to one well as a control. 1ml of each dilution of lentivirus was then added to the remaining wells. Media was changed after 18-20 hours with complete media only and replaced every 2-3 days for up to a week. Cells were trypsinized and resuspended in PBS for FACS analysis. The percentage of GFP cells were measured using the FITC channel and normalised to the control well. The titre of lentivirus was calculated using the following equation, using values from wells containing between 0-20% GFP positive cells and taking the average titre.

$$Titre = \left\{\frac{F\ x\ Cn}{V}\right\} x\ DF$$

F: The frequency of GFP-positive cells determined by flow cytometry

Cn: Total number of cells infected

V: The volume of the inoculum

DF: The virus dilution factor

Example of titre: $4.8x10^5$ units/ml

2.2.2.8 Infection of HUVECs

HUVEC cells were seeded onto 6 or 12 well plates in complete media and incubated at 37ºC 5% CO_2 until 50-70% confluence was obtained. Media was aspirated off and lentivirus was then added at a specified volume along with media to make up a final total volume of 1ml. Polybrene was also added to each infected well to aid infection at a final concentration of 8µg/ml. Plates were then incubated for 4-6 hours at 37ºC 5% CO_2. After incubation, 1ml of complete media was added to each well and the cells were incubated for a further 24 hours at 37ºC 5% CO_2 and media was changed to complete media. Following 48 hours the media was changed again to complete media. Cells were ready to stimulate or harvest the next day. For Cells that were selected during infection see 2.2.8 or harvested for analysis see 2.5.1.

2.2.2.9 Selection of Cells

Following 48 hours after initial infection with Lentivirus the media was changed in each well to complete media containing puromycin at a final concentration of 1µg/ml. Cells were incubated for 48 hours and only cells containing Lentivirus survived the selection process.

2.2.2.10 MCF-7 cell line production

MCF-7 cells were grown in $75cm^2$ flasks until approximately 50% confluency was reached. An infection mix (5ml complete media, 8µg/ml polybrene and 200µl lentivirus) was added and cells were incubated for 4-6 hours. 5ml of complete media was added and cells were incubated for 48 hours. Media was then replaced with 10ml

of complete media containing 1µg/ml of puromycin for a further 48 hours. Media was replaced once more with media containing puromycin for 24 hours and then cells were either split into new 75cm^2 flasks or frozen down and stored in liquid nitrogen. For cultured cells, media was replaced with complete media every two-three days and selection with puromycin was carried out at every passage to maintain a targeted stock of cells.

2.2.3 Western Blotting

2.2.3.1 Preparation of whole cell extracts

Cells were grown in 6 or 12 well plates and subjected to stimulation by appropriate agonists and/ lentiviral knockdown. Plates were placed on ice and monolayers were washed once with ice cold PBS before adding between 100-200µl of Laemmli's sample buffer (LSB) (63mM Tris-HCL, (pH6.8), 2mM $Na_4P_2O_7$, 5mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50mM DTT, 0.007% (w/v) bromophenol blue). The cells were then scraped from the wells using the rubber end of a plunger and DNA was sheared by the repeated passing of the sample through a 21 gauge syringe. The sample was transferred to 1.5ml tubes, boiled for 5 minutes to denature sample proteins and then stored at -20^{o}C until required for use.

2.2.3.2 BCA protein assay

Cells were washed with PBS and then collected in lysis buffer (50mM Tris-HCL, 0.5% triton x100, pH 7.4) containing 0.1% pepstatin, 0.1% leupeptin and 0.1% aprotinin. Cells were scraped into 1.5ml tubes and rotated for 1 hour at 4^{o}C. Samples were centrifuged at 13000rpm for 5 minutes and supernatants were collected. 5µl of each sample was added in duplicate to a 96 well plate alongside BSA protein standards (0-2000µg/ml). 100µl of reagent B from the BCA protein assay kit (Pierce) was added to 5ml of reagent A and 100µl of the mixed reagent was added to each well. Cells were incubated at 37^{o}C for 30 minutes and then plates were read by a plate reader at 562nm wavelength. Sample protein concentrations were then calculated and samples were prepared with equal protein concentrations using LSB for western blotting.

2.2.3.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Resolving gels were prepared containing 10% (w/v) acrylamide: [N, N' – methilenebis-acrylamide (30:0.8), 375mM Tris (pH 8.8), 0.4% (w/v) SDS and 10%

(w/v) ammonium persulpafe (APS)]. Polymerisation was initiated by the addition of 0.05% (v/v) N, N, N', N' – tetramethylethylenediamine (TEMED). The mixture was added between two glass plates assembled vertically in a frame according to the manufacturers instructions (Bio-Rad) and topped with 300µl of 0.1% (w/v) SDS to level the surface. The SDS was removed after gel polymerisation and a stalking gel (10% (v/v) acrylamide: N-methylenebis-acrylamide (30:0.8) in 125mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED) was added directly on top of the resolving gel with a Teflon comb immediately inserted into the mixture between the glass plates. After stacking gel polymerisation the comb was removed and the glass plates containing the gels were assembled in a Bio-Rad Mini-PROTEAN II^{TM} electrophoresis tank, with reservoirs filled with electrophoresis buffer (25mM Tris, 129mM glycine, 0.1% (w/v) SDS).

2.2.3.4 Electrophoretic Transfer of Proteins to Nitrocellulose Membrane

Proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electrophoretic blotting following a standard protocol (Towbin *et al*., 1979). The gel was pressed firmly against a nitrocellulose sheet and assembled in a transfer cassette sandwiched between two pieces of Whatman 3MM paper and two sponge pads. The cassette was immersed in blotting buffer (12M Tris, 19mM glycine, 20% (v/v) methanol) in a BIO-Rad Mini Trans-Blot ^{TM} tank and the tank cooled by inclusion of an ice reservoir. A constant current of 280mA was applied for 110 minutes.

2.2.3.5 Immunological Detection of Protein

Following transfer of the proteins to the nitrocellulose membrane, the membrane was removed and any remaining protein blocked by incubation in a solution of 2-5% (w/v) bovine serum albumin (BSA) in TBS-T (150mM NaCl, 20mM Tris (pH 7.5), 0.1% (v/v) Tween-20) for 2h with gentle agitation on a platform shaker. The blocking buffer was removed and membranes incubated overnight with antiserum specific to the target protein appropriately diluted with TBST-T respectively containing 0.2-0.5% (w/v) BSA. On the following day membranes were washed with TBST-T three times for 5 minutes each wash. Immunoreactive protein bands were detected by incubation with enhanced chemiluminescence (ECL) (reagents from Amersham International Plc) for 3 minutes. The membranes were blotted on a paper towel, mounted onto an exposure cassette and covered with cling film. The membranes were then exposed to Kodak X-

OMAT LS film for the appropriate time under darkroom conditions and developed by KODAK M35-M X-OMAT processor.

2.2.3.6 Reprobing of Nitrocellulose Membrane

The used nitrocellulose membranes were stored at 4^{o}C in a sealed container containing TBS-T buffer, until reprobing was desired. Antibodies were then stripped from the nitrocellulose membrane by incubating for 1 hour in 15ml of stripping buffer (100 mM β-mercaptoethanol, 2% sodium dodecyl sulphate, 62.5 mM Tris-HCl, pH 6.7) at 70^{o}C on a shaker (Stuart Science Equipment). After the incubation period the stripping buffer was discarded and membranes were rinsed in TBS-T buffer (pH = 7.5) three times at 5 minute intervals to remove residual stripping buffer. Finally immunological detection of protein was carried out as described in section 2.3.4 however the blocking step was not required after stripping.

2.2.3.7 Scanning Densitometry

Western blots were scanned on an Epson perfection 1640SU scanner using Adobe photoshop 5.0.2 software. The captured images were then normalised to a control and quantified using Scion Image (Scion Corp., Maryland, USA).

2.2.4 Flow Cytometry Assay of Apoptosis using Fluorescence Activating Cell Sorting (FACS)

2.2.4.1 Apoptosis

HUVECs were plated in 6 well plates, grown until 70-80% confluent and then stimulated for 24 hours prior to analysis. Non-adherent cells were collected and placed into one 15ml tube per well and the remaining cells were trypsinised and added to the tube. Cells were centrifuged at room temperature for 5 minutes at 1000rpm, washed twice in cold PBS and then the pellet was re-suspended in 100µl of 1x annexin binding buffer (10mM HEPES/NaOH (pH7.4), 140nM NaCl and 2.5mM $CaCl_2$) and placed in FACS tubes. 5µl of Phycoerythyrin-Annexin V and 5µl of 7-AAD were added to the cells and tubes were covered in foil and incubated at room temperature for 15 minutes. For sampled containing lentivirus 5µl of APC-Annexin V was added alone to avoid specteral overlap. Samples were read in FACSCanto flow cytometer and data was analysed using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). A total of 10000 events were measured per sample and gating was determined using PE-

Annexin V FL-2 and 7-AAD FL-3 standards attached to beads or cells individually stained.

2.2.4.2 Cell Cycle

HUVECs were plated in 6 well plates, grown until 70-80% confluent and then stimulated for 24 or 48 hours prior to analyses. Cells were collected in 15ml tubes and centrifuged for 5 minutes at 1000rpm room temperature, washed in cold PBS and then the pellet was re-suspended in 150µl of cold PBS. 100% ethanol was added to fix cells and cells were kept in 4^{o}C until day of analysis. Cells were washed in 1ml of cold PBS, centrifuged and resuspended in 250µl of PBS. 5µl of RNAse A (final concentration 50µg/ml) was added to each sample and incubated in foil at 37^{o}C for 30minutes-1hour. 13.5µl of Propidium Iodide (PI) (final concentration 50µg/ml) was added to each sample and tubes were vortexed before analysis. Samples were read in FACSCanto flow cytometer and data was analysed using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). A total of 10000 events were measured per sample and gating was determined using PI stained populations. Cell cycle events were gated on G1, S, G2/M and sub G1and the % of total events in each phase was measured.

2.2.5 Imaging

Images were either taken on an Epifluorescent Inverted Microscope with a Nikon Eclipse TE300 camera attached using Metamorph software, or the Confocal microscope with a Leica, SP5 camera attached using Velocity software.

2.2.5.1 Multinucleate staining

Cells were seeded onto 10mm round coverslips in a 6 well plate and lentiviral infection was carried out as detailed in section 2.1.2.8. Cells were washed 2 times with PBS and then fixed in ice cold 100% methanol for 5 minutes at room temperature. Cells were then washed three times with PBS. 4',6-diamidino-2-phenylindole (DAPI) (final concentration 10µM) was then added to the coverslips, the plate was wrapped in foil for 5 minutes at room temperature. Cells were washed a final time in PBS. The coverslips were then mounted onto a glass slide using vectashield mounting medium (VWR).

2.2.5.2 Spheroid imaging

Spheroids were imaged on a Leica inverted LED microscope using Lasez software.

2.2.6 MTT assay

Cells were seeded to specific density into a 96 well plate and incubated overnight at 37ºC, 5% CO_2. Cells were then stimulated for specific time. After stimulation media was replaced with 100µl of fresh complete media and 10µl of 3-(4,5-dimethylthiaxolyl-2)-2,5-diphenyltetrazolium bromide (MTT) (10mg/ml) (Sigma-Aldrich). Cells were incubated for 2 hours at 37ºC, 5% CO_2. Media was removed and 100µl of Dimethylsulfoxide (DMSO) (Sigma-Aldrich) was added and cells were incubated for a further 5minutes at 37ºC, 5% CO_2. Plates were quantified using the POLARstar Omega plate reader at 570nm.

2.2.7 UVC exposure

Cells were seeded into either 6 well plates or 35mm^2 dishes and incubated overnight at 37ºC, 5% CO_2. Media was then changed to serum free media in each dish and cells were starved for 24h. Cells were exposed to doses of Ultraviolet C light energy by placing the plate of cells with the lid off inside the UV crosslinker box and typing in the energy value in J/m^2. The doses ranged from 1 – 60 J/m^2. Cells could then be incubated again for specific times after exposure and collected for analysis by either FACS (see 2.2.4) or western blotting (see 2.2.3).

2.2.8 Clonogenics

Cells were plated to a specific density into 60 mm^2 dishes in triplicate for each dose of tested Ultraviolet (UV) radiation and incubated in a 37ºC and 5% CO_2. 24h prior to exposure media was replaced with serum free media. Cells were then exposed, with the lid removed, to a dose 10 J/m^2 of UV. Media was immediately replaced by 5ml of complete media and cells were incubated for 10 – 15 days with media replaced every 3 days. Following 13-15 days of incubation, colony formation was assessed to make sure at least 50 colonies developed in control dishes. Then medium was removed and cells were washed with PBS, fixed with methanol for 10 minutes and stained with giemsa for 20 minutes. The stain was carefully removed and dishes were rinsed with tap water. Formed colonies were counted manually. Plating efficiency (PE) and survival fraction (SF) were calculated from the following equations:

$$\mathrm{PE} = \frac{\text{average no. of control colonies formed}}{\text{no. of seeded cells}}$$

$$\mathrm{SF} = \frac{\text{average no. of colonies formed after treatment}}{\text{no. of seeded cells X PE}}$$

2.2.9 Spheroids

In 96 well round bottom ultra-low attachment plate, cells were added at specific densities, e.g. 4000 cells/well, and incubated at 37ºC, 5% CO_2 for up to 15 days with media replenished on days 5 and 10. Images (see section 2.2.5.2) of spheroid growth were taken throughout the 15 days and analysed on ImageJ. The maximum and minimum diameter of each spheroid was measured and from the average of these two measurement the spheroid volume was calculated using the following equation.

$$V = \frac{4}{3}\pi r^3$$

For knockdown experiments, the average volume of spheroid from each condition was then calculated as a fold of the initial volume measure for each sphere and plotted against time in days.

2.2.10 Statistical Analysis

All statistics were calculated using GraphPad Prism version 5.01. Datasets were analysed for statistical significance by using either a one-or-two way analysis of variance (ANOVA) or an unpaired t test. P values <0.05 were considered significant and means +/− standard errors of the mean (SEM) are depicted in all figures.

Chapter Three

Lentivirus Optimisation and the Effect of JNK Isoform Knockdown on Downstream Signalling and HUVEC cell growth

3.1 Introduction

Cardiovascular disease has become an increasing cause of death, with ischemic heart disease (atherosclerosis) now the leading cause of mortality worldwide. The JNK pathway is involved at multiple/various stages of atherogenesis including vascular cell proliferation and migration (Kavurma and Khachigian, 2003), foam cell formation (Ricci *et al.*, 2004) and plaque formation and rupture (Zhang *et al.*, 2007). The involvement of the JNK pathway in these key stages of atherogenesis highlights JNK as a possible target for drug development and treatment. However, JNK is expressed throughout the body and is an integral component of many important signalling events including cell cycle regulation (Gutierrez, Tsuji, Cross, *et al.*, 2010) and regulation of cell death (Dhanasekaren and Reddy, 2008). Therefore, targeting JNK may result in various off target effects which could be detrimental to the host.

As mentioned in chapter one, JNK exists as three individual isoforms: JNK1, JNK2 and JNK3, which have a tendency to be characterised together as one protein due to the ease of using drug inhibitors. However, research targeting the individual isoforms has demonstrated some differences in protein function. For example, JNK1 and JNK2 have been shown to differentially regulate the transcription factor c-Jun, resulting in either increased or decreased cell proliferation (Sabapathy *et al.*, 2004). More specific to atherosclerosis, foam cell formation was reduced in APO-E/JNK2 double knockout (KO) mice when compared to APO-E KO mice alone. In contrast APO-E/JNK1 double KO mice showed no significant change in this stage of atherogenesis (Ricci *et al.*, 2004).

Distinguishing variations in JNK isoform function in human vascular cells could lead to the discovery of new targets for atherogenesis prevention or treatment. To investigate individual JNK isoform function in human vascular cells, each of the JNKs must be targeted independently of each other. Studies which have looked at JNK isoform function in the vasculature tend to use knock out animal models and not human cells as primary cells are more difficult to manipulate successfully. Lentiviral technology is an efficient gene delivery technique which can be used for many different applications, including gene silencing (Sakuma, Barry and Ikeda, 2012). Lentivirus is a modified version of the *human immunodeficiency virus* which has had

genes removed to prevent replication without affecting the virus's ability to infect cells. Lentivirus can infect both dividing and non-dividing cells and can integrate into the host genome, producing a sustained gene delivery.

The individual function of each JNK isoform has not been fully investigated in vascular cells. Therefore, in this chapter lentiviral technology was optimised to target and knockdown the individual JNK isoforms in Human Umbilical Vein Endothelial Cells (HUVECs) and the effects of knockdown on JNK signalling were subsequently investigated.

3.2 Results

3.2.1 JNK isoform knockdown in HUVECs

Before optimising JNK knockdown, an initial experiment was carried to determine JNK expression in HUVECs. Both JNK1 and JNK2 were clearly detected in samples (data not shown). For JNK3, samples of HUVEC cells alone were prepared and a Western blot was carried out alongside a positive control sample of homogenised mouse brain (gifted by the Jiang group) (Figure 3.1). JNK3 was not detected in the HUVEC samples when compared to the positive control. The nitrocellulose membrane was re-probed for JNK1 which was detected in the lanes containing HUVEC samples, demonstrating that there was a sufficient amount of protein loaded to be detected at Western blot level (Figure 3.1). Therefore, for the remainder of this thesis JNK3 was not analysed and the work focuses on JNK1 and JNK2 in this cell type.

To optimise the knockdown of JNK, six different transfer vectors were compared for each JNK isoform to allow the best lentivirus to be selected for future work. Lentivirus was produced as detailed in chapter two for each of the different transfer vectors. HUVECs were infected with non-concentrated (see chapter 2) lentivirus targeting either JNK1 or JNK2 for 48 hours. Cells were then selected using puromycin for a further 48 hours before being collected for Western blot analysis.

JNK1 knockdown was successfully achieved with all six lentivirus targeting JNK1 (results not shown). The four most efficient lentivirus; 501 (1), 503 (2), 928 (3), and 931(4) were then taken forward and compared further along with combinations of the lentivirus 1-2, 3-4 and 1-4 added during infection of the cells. Knockdown of JNK1 was achieved by all four individual lentiviruses as well as each of the three combinations (Figure 3.2A) The specificity of the knockdown was confirmed by Western blotting using an antibody against JNK2 which showed minimal change in protein expression. In addition, total GAPDH was used as a means of normalising any differences between protein loading (Figure 3.2A). All four lentivirus produced significant knockdown (501 (74.87% ± 7.2), 928 (74.4% ± 5.9), 931 (63.3 ± 8.2), however lentivirus 503, target sequence ‘TAGATGCATCTATTACCAG’, consistently produced the most efficient knockdown with an average of 83.6% (± 7.7) when compared to the non-target (NT) control (Figure 3.2B).

A similar result was produced by the lentivirus targeting JNK2, with all six different lentiviruses 130 (1), 169 (2), 170 (3), 511 (4), 513 (5) and 515 (6) producing knockdown of JNK2 (Figure 3.3A). Combinations of lentivirus 1-3, 4-6 and 1-6 targeting JNK2 were also compared and again all three achieved knockdown when compared to the NT control (Figure 3.3A). Stable JNK1 expression was observed, confirming specificity of the lentivirus for targeting only JNK2. Total GAPDH was again used to determine differences in protein loading and to normalise data (Figure 3.3A). When compared to the NT control all six lentivirus produced a significant level of knockdown, (130 (43.98% ± 2.3), 169 (43.48% ± 1.5), 170 (47.71% ± 9.2), 511 (45.83% ± 1.2) and 513 61.02% ± 3.7) with lentivirus 515, target sequence 'ATGATAACATCATGATGGC', being consistently the most effective at knocking down JNK2 producing an average of 84.4% (± 1.5) knockdown (Figure 3.3B).

Although combinations of the different lentivirus produced good knockdown, lentivirus 503 and 515 alone produced better and more consistent knockdown and therefore were taken forward for future experiments.

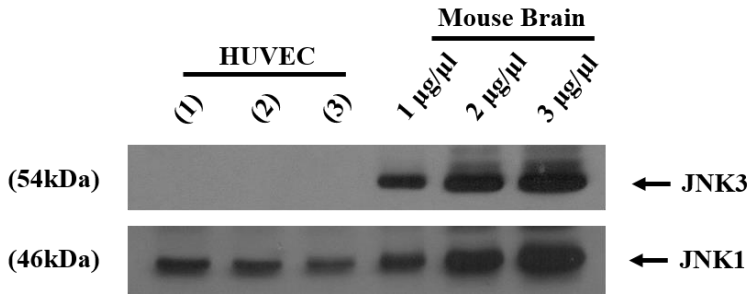


Figure 3.1 JNK3 expression in HUVECs

Confluent HUVECs in six well plates were collected in LSB and homogenized mouse brain was diluted in LSB to a concentration of (1µg/µl). Samples of HUVECs from three individual experiments (1), (2) and (3) were analysed by Western blot along with increasing concentrations of mouse brain as a positive control. Results show, Western blot analyses of JNK3 expression and JNK1 was used to confirm protein loading.

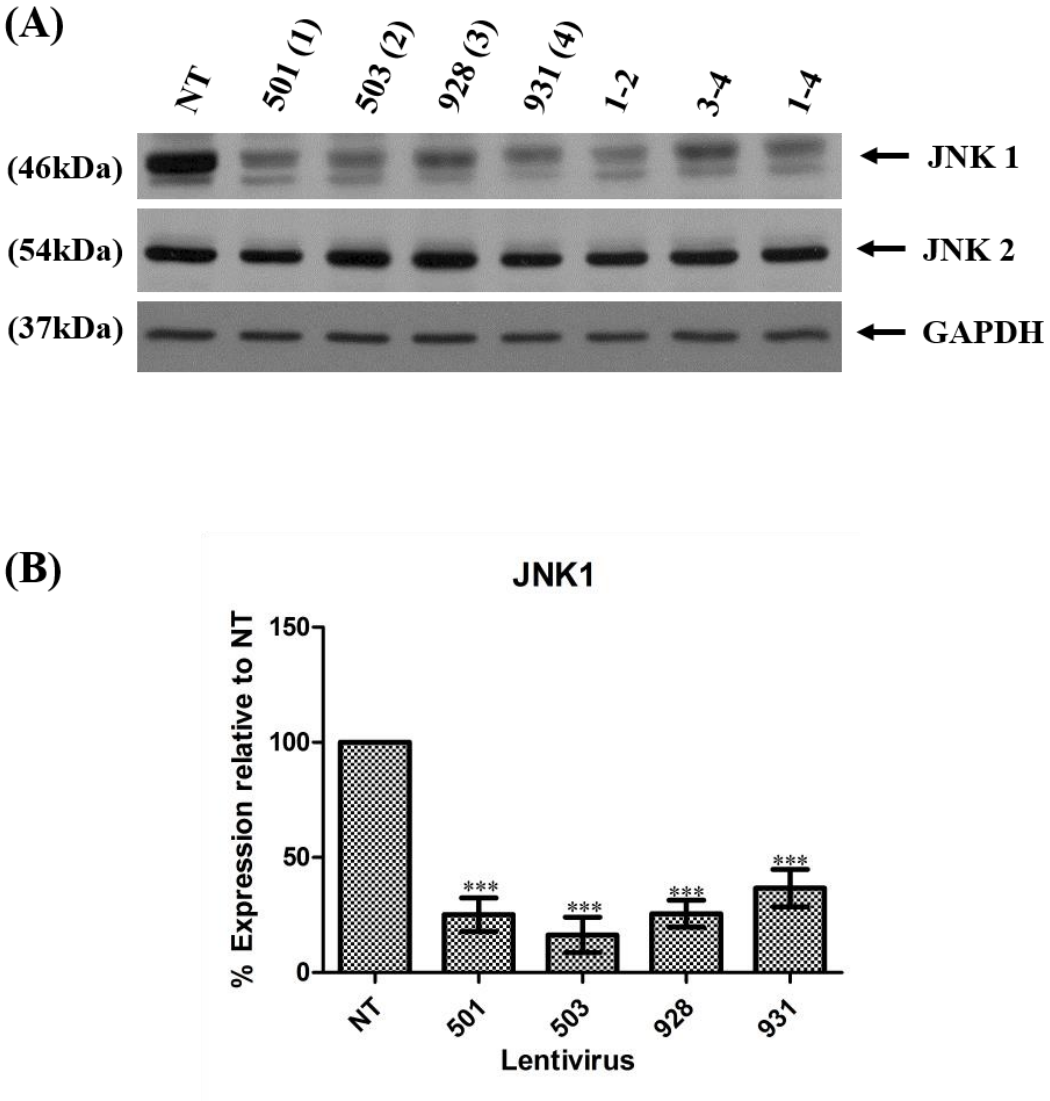


Figure 3.2 JNK1 Knockdown in HUVECs

HUVECs in six well plates grown to 60-70% confluence were infected with a total of 500µl of lentivirus with 4µg/µl of polybrene and 500µl of FBS free media for 4-6 hours. Following infection, 1ml of complete media was added and cells were incubated for 48 hours. Cells were selected with media containing puromycin (1µg/µl) for a further 48 hours and whole cell extracts were analysed using Western blotting and antibodies specific for each isoform. Results show (A) Western blot of JNK1 and JNK2 protein expression. GAPDH was used to determine differences in protein loading. (B) Percentage knockdown compared to NT control. Values represent mean % knockdown ± SEM, n=3, ***P<0.001 compared to NT.

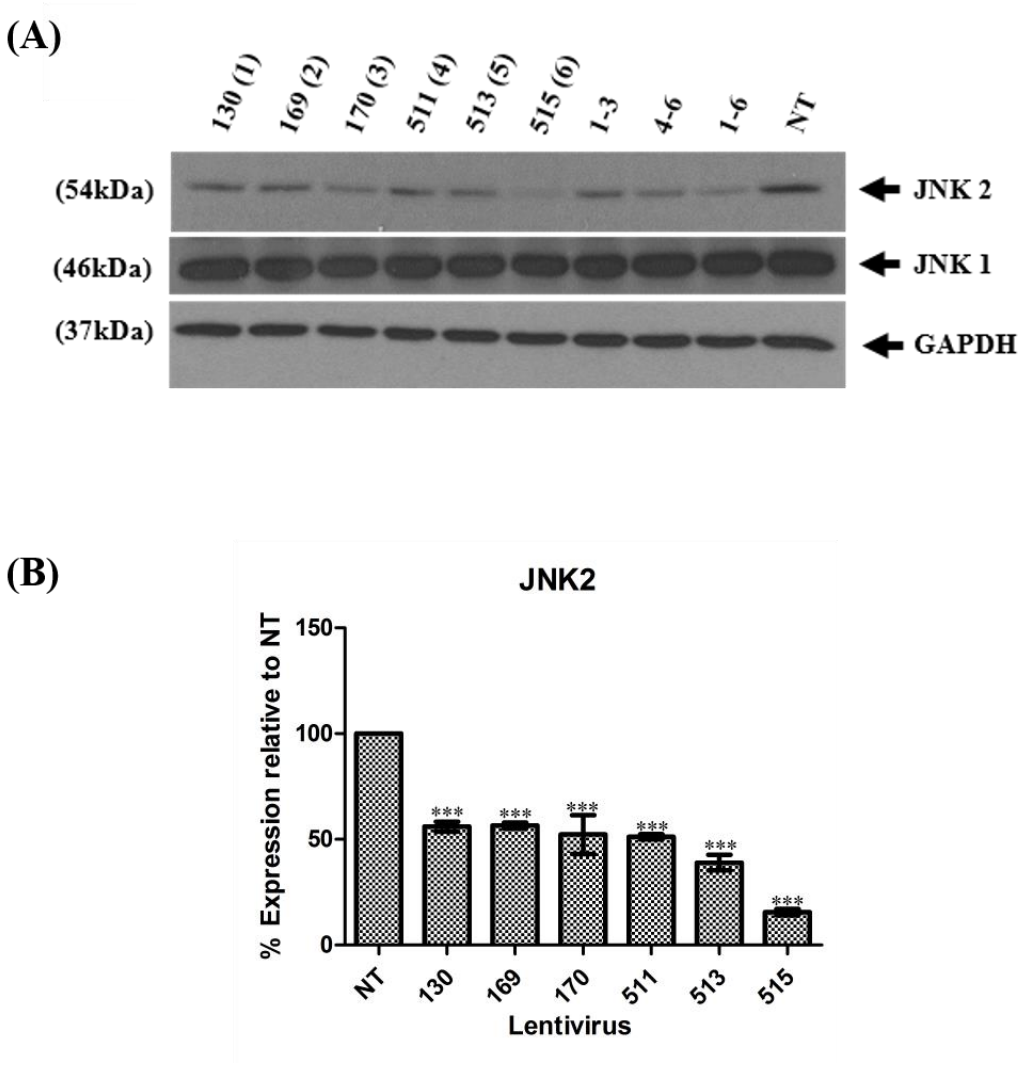


Figure 3.3 JNK2 knockdown in HUVECs

HUVECs were grown in six well plates until 60-70% confluence was attained followed by infection with a total of 500µl of lentivirus with 4µg/µl of polybrene and 500µl of FBS free media for 4-6 hours. Following infection 1ml of complete media was added and cells were incubated for 48 hours. Cells were selected for a further 48 hours and whole cell extracts were collected and analysed by Western blotting and antibodies specific for each isoform. Results show (A) Western blot of JNK1 and JNK2 protein expression. GAPDH was used to determine differences in protein loading. (B) Percentage knockdown compared to NT control. Values represent mean % knockdown ± SEM, n=3, ***P<0.001 compared to NT.

3.2.2 Lentivirus Optimisation

Although knockdown was achieved by each lentivirus, selection by puromycin resulted in the infected cells looking stressed in appearance. This was perhaps due to HUVECs being a primary cell line which tend to be more fragile to work with. To carry out infection without selection, the lentivirus was concentrated approximately 100x using the Lenti-X-Concentrator kit (detailed in section 2.2.2.6). This allowed more virus to be used without altering the total volume of infection mix added to the cells.

Three lentivirus transfer plasmids were also gifted by Dr Andreadis Stelios from the University of Buffalo which targeted JNK1 and JNK2 individually and also JNK1 and JNK2 (JNK1/2) together. These plasmids were used to make up three new lentiviruses to allow a comparison of the JNK isoform knockdown achieved previously by another group (You *et al.*, 2013).

3.2.2.1 Optimisation of Lentivirus Infection

HUVECS were infected with either non-concentrated or concentrated lentivirus containing shRNA targeting JNK1 (503, USA JNK1), JNK2 (515, USA JNK2) or JNK1 and JNK2 (USA JNK1/2). After 48 hours' cells infected with non-concentrated lentivirus were selected with complete media containing 1µg/µl of puromycin while all other cells were replenished with complete media alone. Following a further 48 hours' whole cell extracts were collected and analysed by Western blot. Both lentivirus 503 and 515 showed reduced expression of JNK1 and JNK2 respectively with or without puromycin selection (Figure 3.4A). Selected cells infected with lentivirus 503 and 515 produced a more successful average knockdown by approximately 21% and 5% respectively when compared to cells infected with concentrated lentivirus alone (503 selected 67.45% ± 12.2, 515 selected 88.82% ± 4.12, 503 alone 46.88% ± 6.01 and 515 alone 83.99% ± 11.84).

In contrast, the USA lentivirus without selection produced relatively similar knockdown of both JNK1 (74.4% ± 2.94) and JNK2 (83.99 ± 10.04) isoforms as the selected 503 and 515 samples. The USA JNK1/2 double knockdown also successfully reduced expression of both isoforms together (47.98% ± 13.10 and 82.41 ± 13.75) without selection, therefore these lentiviruses were taken forward and used for all

future experiments. Throughout the rest of this chapter lentivirus USA JNK1, USA JNK2 and USA JNK1/2 will be referred to as JNK1, JNK2 and JNK1/2 respectively.

To visualise infection, images were taken of HUVECs infected with 100µl of lentivirus (Figure 3.5). JNK1, JNK2 and JNK1/2 lentivirus contain a GFP construct and therefore infected cells were visualised to display GFP internally. The observed ratio of GFP to nucleus stained by DAPI indicated that almost 100% of cells were infected in each condition. A limitation to this study was that the NT lentivirus did not contain GFP and therefore could not be visualised alongside the other infections.

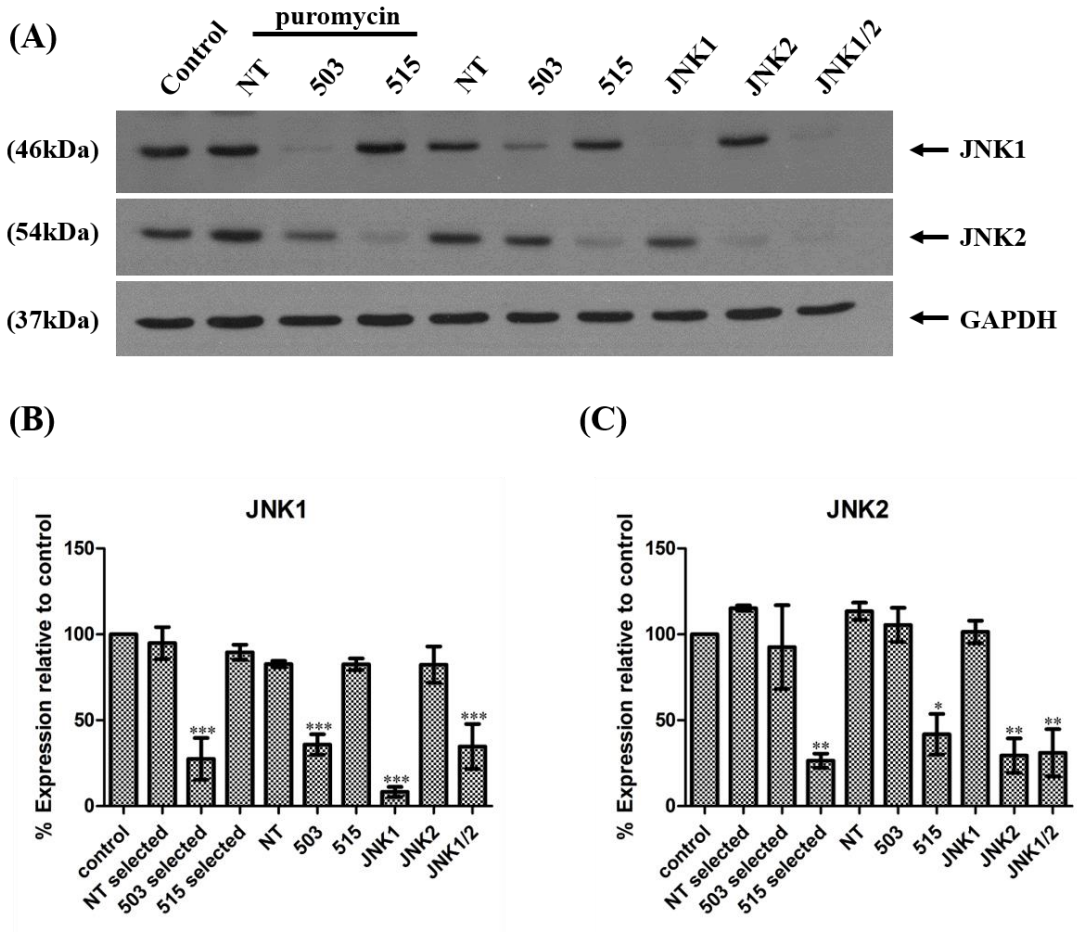


Figure 3.4 Knockdown of JNK in HUVECs using concentrated lentivirus

HUVECs were infected as stated in methods (section 2.2.2.8) with 500µl of non-concentrated lentivirus for selected wells and 100µl of concentrated virus for non-selected wells. Control of serum free media and polybrene. Cells were selected as stated in section 2.2.2.9 of methods chapter. Results show (A) Representative Western blot of JNK1 and JNK2 protein expression. GAPDH was used to normalise data due to differences in sample loading. (B) % knockdown of JNK1 relative to the control sample and (C) % knockdown of JNK2 relative to the control sample. Values represent mean % knockdown ± SEM, n=3, *P<0.05, **P<0.01, ***P<0.001 compared to NT.

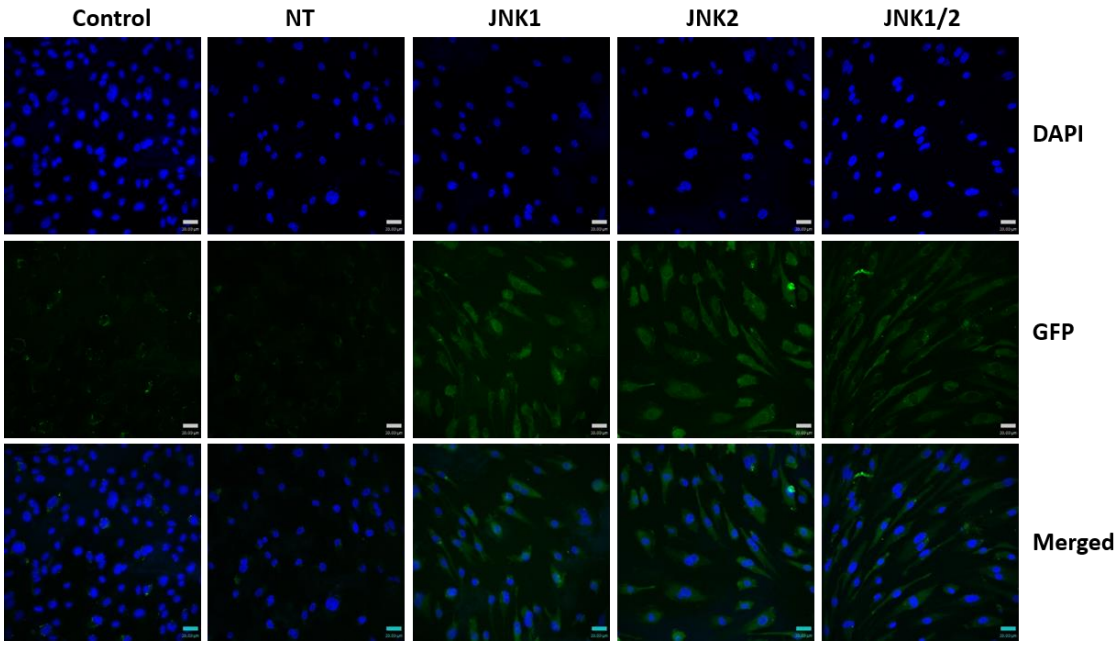


Figure 3.5 Lentiviral infection in HUVECs

HUVECS plated on coverslips were infected with 100µl of lentivirus in 900µl of serum free media containing 8µg/µl of polybrene. Serum free media containing polybrene was used as a control. 1ml of complete media was added after 4h of incubation. Media was then changed at 24 and 72 hours after infection. Cells were fixed with 100% ice cold methanol and stained with DAPI 1/1000 in PBS. Cells were then visualised on the confocal microscope. Results represent Immunofluorescence analysis of HUVECs with or without lentiviral infection. DAPI was used for nuclear staining (blue) and GFP represents infected cells (green). Images are representative of 3 separate experiments with multiple images taken for each condition. Scale bar = 30µm.

3.2.2.2 Optimising Lentivirus Concentration

Although infecting cells with 100µl of lentivirus without selection was producing efficient knockdown of the JNK isoforms with less stress than with selection, the cells continued to display signs of stress compared to non-infected cells. To determine whether the volume of lentivirus used for infection could be reduced, a concentration curve was carried out.

HUVECs were infected with either 25µl, 50µl, 75µl or 100µl of lentivirus targeting JNK1, JNK2 or JNK1/2 together. The level of knockdown produced by each volume of lentivirus was measured by densitometry and compared to the NT control. For JNK1 an average knockdown of 69.07% ± 12.43 was achieved using 50µl, which produced a similar average knockdown as 75µl (63.97% ± 13.60) and 100µl (66.54% ± 13.01) of JNK1 lentivirus (Figure 3.6 A, B). JNK2 lentivirus produced an average knockdown of 75.55% ± 1.94 when infected with 75µl of lentivirus, which was similar to the level of knockdown produced by 100µl (73.33% ± 4.63) (Figure 3.6 A, C). Infection with 100µl of JNK1/2 produced the most efficient knockdown for both JNK1 and JNK2, resulting in a knockdown of 57.51 ± 8.43 and 81.51 ± 3.96 respectively (Figure 3.6 A-C).

From this experiment it was concluded that the volumes of lentivirus used to infect HUVECs could be changed to a minimum of 50µl of JNK1, 75µl of JNK2 and 100µl of JNK1/2 to produce efficient knockdown of the different isoforms.

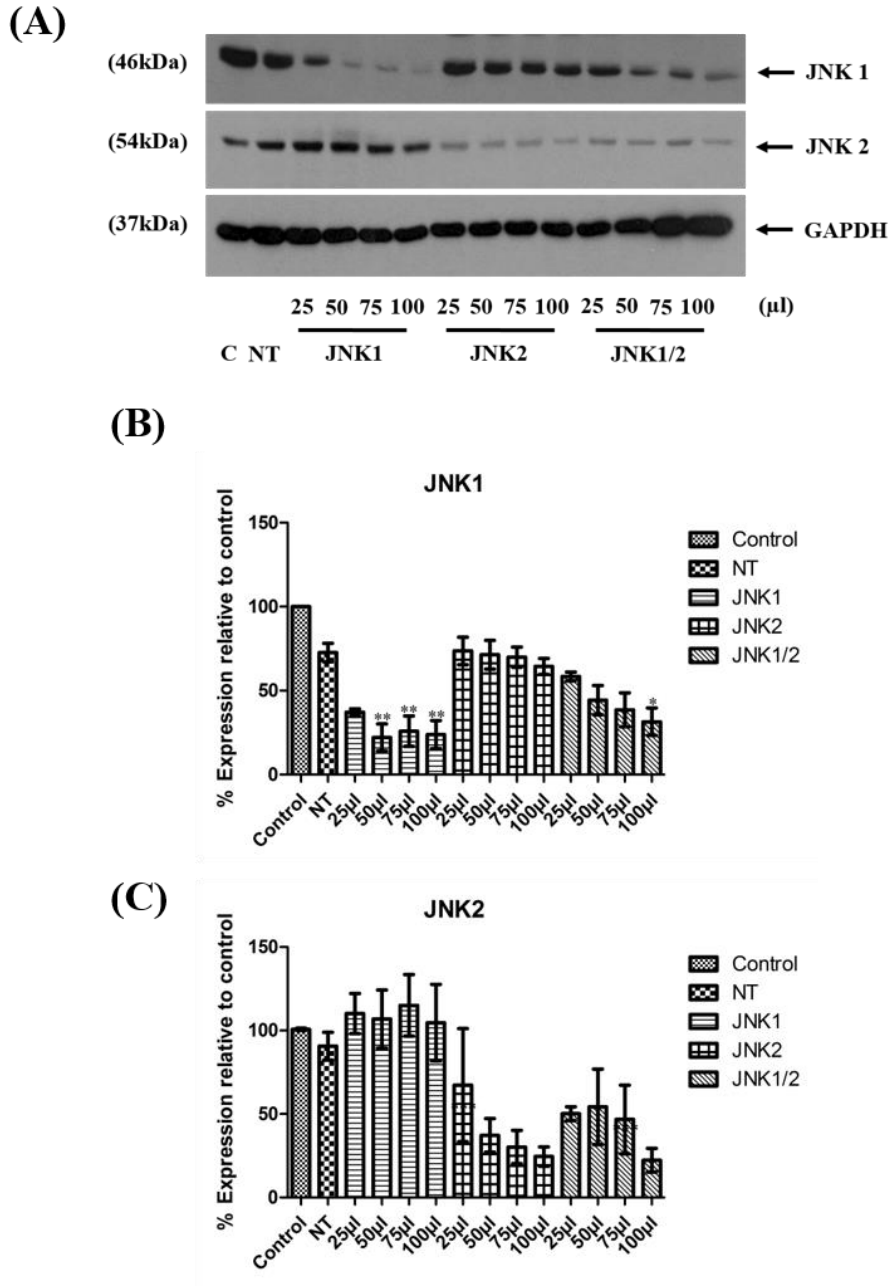


Figure 3.6 Optimum volume of Lentivirus

HUVECs were infected with increasing concentrations of lentivirus as stated in methods section 2.2.2.8, targeting either JNK1, JNK2 or JNK1/2. Controls were either serum free media only or 100µl of NT lentivirus. After infection whole cell extracts were analysed by Western blotting and semi quantified using densitometry. Results show (A) Western blot of JNK1 and JNK2 expression. GAPDH was used to determine differences in sample loading between lanes. Fold change in protein expression compared to NT for (B) JNK1 and (C) JNK2. Data represents the mean percentage knockdown ± SEM of three different experiments *P<0.05 **P<0.01 ***P=0.001 compared to NT.

3.2.3 The effect of JNK knockdown on JNK mediated signalling

Downstream targets of JNK signalling have been shown to play different roles in various stages of atherogenesis. It has been suggested that the transcription factor c-Jun is activated more during hypercholesterolemia and contributes to matrix metalloproteinases expression, a protein that plays a key role during atherogenesis (Sozen *et al.*, 2014). C-Jun has also been linked to cell proliferation and inhibition of apoptosis in endothelial cells when stimulated by epoxyeicosatrienoic acids, an important regulator within the vasculature (Ma *et al.*, 2012). In contrast, evidence has shown c-Jun to play a role in vascular smooth muscle cell (VSMC) apoptosis which contributes to plaque weakness and rupture (Zhang *et al.*, 2014). Another downstream target of JNK is activating transcription factor - 2 (ATF2), which has been demonstrated to be involved in leukocyte binding to endothelial cells, a key process of atherogenesis (Fearnley *et al.*, 2014).

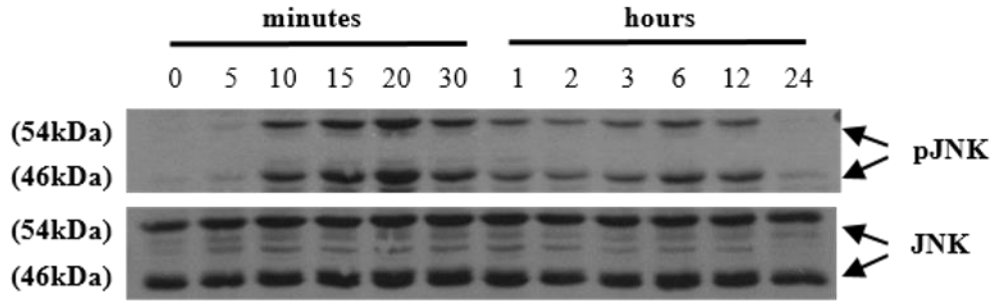
It has previously been demonstrated that JNK1 and JNK2 differentially regulate the downstream targeted transcription factor c-Jun and that JNK1 but not JNK2 binds to ATF2 in fibroblasts (Sabapathy *et al.*, 2004). The group used mutant fibroblasts isolated from JNK1, JNK2 or double isoform knockout embryos and clearly demonstrated differences in cell proliferation between the different knockout cells.

In this section, using the conditions established, the effect of lentiviral knockdown of individual JNK isoforms on the phosphorylation of JNK and transcription factors ATF2 and c-Jun was investigated.

3.2.3.1 TNF-α stimulation in HUVECs

To confirm TNF-α activated the JNK pathway in HUVECs, HUVECs were seeded onto 12 well sterile plates and stimulated with TNF-α at a final concentration of 10ng/µl for a time course of 0 – 24 hours. Both pJNK 54kDa and 46kDa showed early activation which peaked at 15 and 20 minutes. The response then decreased slightly between 30 minutes and 2 hours and then increased again between 3 and 12 hours. The peak stimulation was at the earlier time points however, the pJNK signal remained increased over all times points when compared to expression at 0 minutes and therefore JNK activation induced by TNFα was sustained over the 24 hours. It was also observed that pJNK 54kDa produced a slightly higher increase in fold stimulation when compared to pJNK 46kDa over the 24 hours (Figure 3.7 A-B).

(A)



(B)

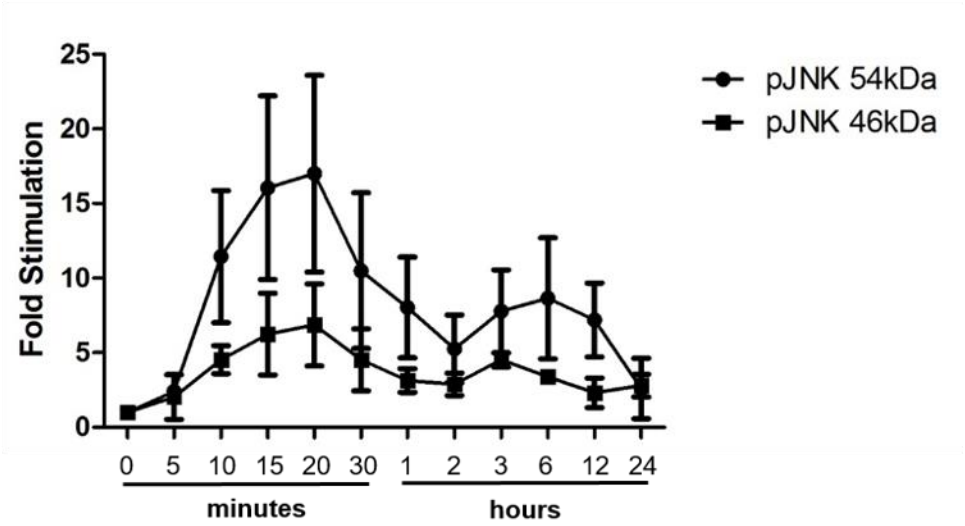


Figure 3.7 TNF-α stimulation in HUVECs.

HUVECs were seeded into 12 well plates and stimulated with TNF-α (10ng/µl) for 5-24 hours. Protein levels were analysed by Western blotting and semi quantified using densitometry. Results show (A) Western blot of pJNK 54kDa and 46kDa phosphorylation. Total JNK was used to confirm equal sample loading between lanes. (B) Graph of pJNK 54kDa and 46kDa phosphorylation between 0-24 hours, n=3.

3.2.3.2 The effect of JNK isoform knockdown on JNK signalling.

Once repeatable knockdown was accomplished, the effect of isoform knockdown on the activation of downstream targets of JNK was investigated. As mentioned previously, the transcription factors ATF2 and c-Jun have both been linked to stages of atherogenesis and therefore these substrates were chosen to be studied.

HUVECs were stimulated with 10ng/µl of TNF-α for 15minutes and expression of phosphorylated c-Jun (pc-Jun), phosphorylated ATF2 (pATF2), phosphorylated JNK (pJNK), total JNK1 and total JNK2 were analysed by Western blot (Figure 3.8 A). Phosphorylation was measured by semi quantitative densitometry and normalised using GAPDH to ensure equal sample loading. Data was then normalised to the control for each data set, where each figure represents the mean of data from three separate experiments (Figure 3.8 B-G).

After 15 minutes' stimulation with TNF-α there was, on average, a 12-fold increase in pc-Jun phosphorylation when compared to unstimulated cells (Figure 3.8 B). HUVECs infected with JNK1 lentivirus showed a slight increase in pc-Jun relative to cells infected with NT lentivirus, however this was not significant. In contrast knockdown of JNK2 in combination with TNFα stimulation, increased levels of pc-Jun to 17.77 fold ± 1.43 when compared to unstimulated cells. This was also significantly higher than cells infected with NT control (10.57 fold ± 0.71). Cells with JNK1/2 knockdown showed an average decrease of approximately 33% in levels of pc-Jun when compared to NT, however due to inconsistences, this was not significant (7.7 fold ± 1.5).

In contrast to c-Jun, TNF-α stimulated the phosphorylation of ATF2 where protein levels increased by just over 2 fold (2.19 ± 0.48) when compared to unstimulated cells (Figure 3.8 C). Infection with JNK1 (1.31 fold ± 0.23), JNK2 (1.56 fold ± 0.11) or JNK1/2 (1 fold ± 0.03) lentivirus had no significant effect on pATF2 phosphorylation when compared to the NT control (1.48 fold ± 0.09). However, JNK1/2 knockdown did show a trend of lower levels of pATF2.

When analysing the effects of JNK1 and JNK2 knockdown on pJNK, JNK1 reduced expression of the 46kDa band and JNK2 reduced the expression of the 56kDa band, therefore, each band was measured separately. TNFα caused an average increase of 25.47 fold ± 11.70 fold of pJNK 46kDa in stimulated HUVECs when compared to

unstimulated cells (Figure 3.8 D). However, cells infected with NT lentivirus produced approximately 56% lower average level of protein expression (11.09 fold ± 2.92). JNK1 and JNK1/2 knockdown also produced a lower phosphorylation of pJNK 46kDa of 78.6% and 50.4% respectively when compared to NT, however due to inconsistences neither were significant (JNK1 2.64 fold ± 1.34 and JNK1/2 5.46 fold ± 2.73). In contrast JNK2 knockdown produced an average fold increase of 23.84 fold ± 8.97 similar to stimulated control cells, though this was again not significant.

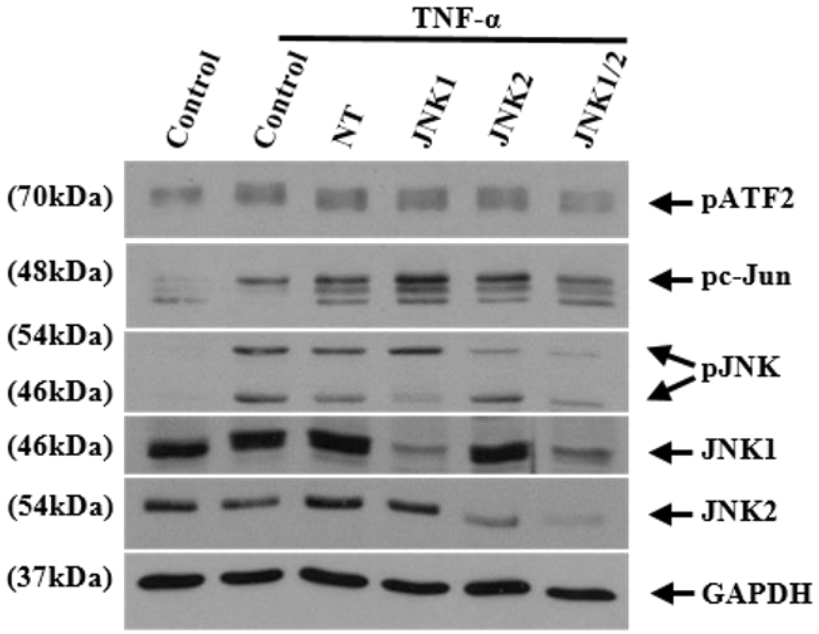
A largely similar pattern was observed for the 54kDa variant of pJNK when stimulated with TNF-α (Figure 3.8 E). HUVECs stimulated with TNF-α produced a 46.34 fold ± 14.56 fold increase in pJNK in comparison to unstimulated cells. Similar to the 46kDa variant, cells infected with the NT lentivirus had a lower phosphorylation of pJNK 54kDa by approximately 38.5% when compared to the stimulated control (28.52 fold ± 2.95). Knockdown of JNK2 and JNK1/2 produced a trend of reduced levels of pJNK 54kDa of 65.8% and 73.4% respectively when compared to NT control, however these values were not significant (JNK2 9.75 fold ± 1.27 and JNK1/2 7.60 fold ± 2.24). In contrast knockdown of JNK1 in HUVECs increased pJNK 54kDa expression to 73.66 fold ± 22.6. This was expected after observing a decrease of pJNK 46kDa expression during JNK1 knockdown and an increase during JNK2 knockdown.

To confirm that there was reproducible knockdown, levels of JNK1 (Figure 3.8 F) and JNK2 (Figure 3.8 G) were normalised to expression levels of JNK1 or JNK2 in control cells and the percentage knockdown of each isoform was calculated. Stimulated HUVECs and HUVECs infected with NT or JNK2 lentivirus maintained a similar level of JNK1 expression averaging 107.6% ± 3.42, 90.97% ± 6.36 and 94.10% ± 12.66 respectively. Cells infected with JNK1 or JNK1/2 lentivirus expressed lower levels of JNK1 at 19.68% ± 7.47 and 38.41% ± 3.72, producing significant knockdowns of 71.29% and 52.56% respectively when compared to the NT control (Figure 3.8 F).

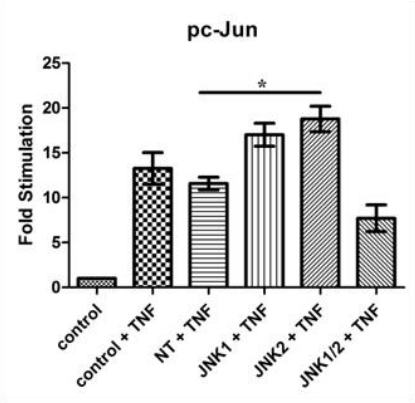
JNK2 expression was maintained at a level similar to the control in stimulated cells (84.98% ± 2.91) and cells infected with NT (99.75% 1.79) or JNK1 (89.44% ± 3.52) lentivirus (Figure 3.8 G). Significant knockdown of JNK2 was achieved in cells infected with either JNK2 or JNK1/2 lentivirus, achieving a knockdown of 78.89%

(20.86% ± 5.54) and 78.69% (21.06% ± 0.70) respectively in comparison to the NT control (Figure 3.8 G).

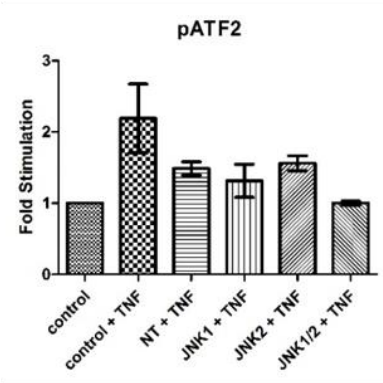
(A)


TNF-α
Control
Control
NT
JNK1
JNK2
JNK1/2
(70kDa)
pATF2
(48kDa)
pc-Jun
(54kDa)
(46kDa)
pJNK
(46kDa)
JNK1
(54kDa)
JNK2
(37kDa)
GAPDH

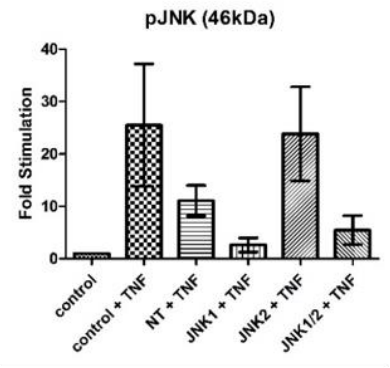
(B)


pc-Jun
Fold Stimulation
25
20
15
10
5
0
*
control
control + TNF
NT + TNF
JNK1 + TNF
JNK2 + TNF
JNK1/2 + TNF

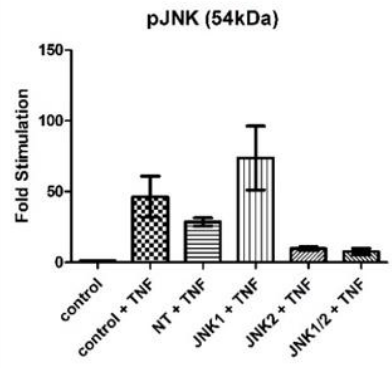
(C)


pATF2
Fold Stimulation
3
2
1
0
control
control + TNF
NT + TNF
JNK1 + TNF
JNK2 + TNF
JNK1/2 + TNF

(D)


pJNK (46kDa)
Fold Stimulation
40
30
20
10
0
control
control + TNF
NT + TNF
JNK1 + TNF
JNK2 + TNF
JNK1/2 + TNF

(E)


pJNK (54kDa)
Fold Stimulation
150
100
50
0
control
control + TNF
NT + TNF
JNK1 + TNF
JNK2 + TNF
JNK1/2 + TNF

(F) **(G)**

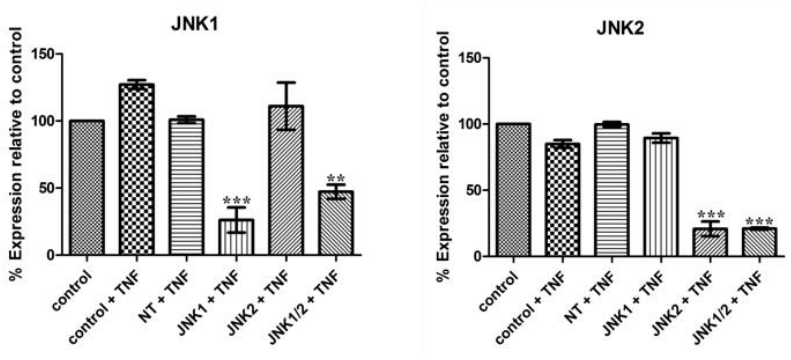


Figure 3.8 The effects of JNK isoform knockdown on the phosphorylation of JNK, c-Jun and ATF2 in HUVECs.

HUVECs were seeded onto 6 well plates and infected with 100µl of lentivirus as stated in methods section 2.2.2.8. Cells were then stimulated with 10ng/µl of TNF-α for 15minutes. Whole cell extracts were analysed by Western blotting and semi quantified using densitometry. Results show (A) Western blot of pc-Jun, pJNK, pATF2, JNK1 and JNK2. GAPDH was used to confirm equal sample loading. Graphical representation of fold change in protein expression for (B) pc-Jun, (C) pATF2, (D) pJNK 46kDa and (E) pJNK 56kDa were normalised to control. Knockdown of (F) JNK1 and (G) JNK2 are represented as a percentage of the control. Data represents the mean ± SEM of three different experiments. *P<0.05 **P<0.01 ***P<0.001.

3.2.4 JNK function in cell proliferation and cell death

As mentioned in chapter 1, JNK signalling has been shown to play a role in cell proliferation. This has been clearly demonstrated in endothelial cells where JNK knockout caused a reduction in Ki-67 expression, a known marker of cell proliferation (Chaudhury, Zakkar, Boyle, Cuhlmann, Van Der Heiden, *et al.*, 2010). Uchida et al also demonstrated a role for JNK in endothelial cell proliferation where inhibition of JNK by SP600125 significantly reduced the fold increase in endothelial cell number (Uchida *et al.*, 2008). In contrast to this, JNK plays a key role in apoptotic signalling in endothelial cells where knockdown of JNK has been shown to reduce caspase-3 signalling induced by treatment with high glucose (Ho *et al.*, 2000). This has also been demonstrated in a JNK knockout mouse model of ischemic reperfusion where knockout animals displayed less caspase-3 cleavage and a significant decrease in cell death (Kaiser *et al.*, 2005).

It is now understood that JNK can either lead to pro survival or pro death signalling, with increasing evidence that JNK1 and JNK2 can work independently of each other. The aim of the remainder of this chapter was to investigate the effects of JNK isoform knockdown on cell survival and cell death processes, and to determine any individual function of each isoform. However, knocking down JNK in HUVECs proved to be challenging and therefore only a small number of experiments were completed in this study.

3.2.4.1 The effect of H_2O_2 on HUVEC viability

To investigated the effects of JNK knockdown on cell viability after treatment with hydrogen peroxide (H_2O_2), a preliminary experiment was carried out to determine which concentration would be most appropriate. HUVECs were seeded into 96 wells plates and treated for 24 hours with either 100 – 800 µM of H_2O_2 or media or water for controls. An MTT assay was then carried out as described in methods section 2.2.6. Data was represented as a percentage of viability relative to a control of cells treated with media alone (Figure 3.9A). At 100µM percentage cell viability dropped significantly to 78.33% ± 12.91. The percentage of viable cells continued to decrease (53.76 ± 7.55, 45.45 ± 7.31, 31.57 ± 12.42, 18.09 ± 13.41, 12.69 ± 8.07) as the concentration of H_2O_2 increased (200µM, 300µM, 400µM, 500µM and 600µM respectively) until the concentration of 700µM was used. Cells treated with 700µM and 800µM of H_2O_2 showed an average of 5.07 ± 0.32% and 4.80 ± 0.27% viability which was comparable to the negative control of water only (5.05 ± 0.36). This demonstrates that HUVEC cell death caused by H_2O_2 is concentration dependent.

After managing to produce a significant decrease in cell viability by treating cells with 200µM of H_2O_2, this concentration was used for the following experiments. To determine the effect of JNK knockdown on cell viability after treatment with H_2O_2, HUVECs were seeded into 6 well plates and infected with lentivirus as stated in methods section 2.2.2.8. After infection cells were trypsinized, reseeded at equal densities into a 96 well plate in replicates of 4 and incubated overnight at 37^{o}C, 5%, CO_2. Cells were treated with either media alone or 200µM of H_2O_2 for 24 hours, followed by an MTT assay as stated in methods section 2.2.6. Data was represented as the percentage of viable cells treated with H_2O_2 to the number of viable cells treated with media alone control for each condition (Figure 3.9B). Results show that infection with lentivirus caused an increase in the number of viable cells when treated with H_2O_2. Unfortunately, this was also true for the NT infected cells and therefore it cannot be assumed that this effect was due to JNK knockdown, but may possibly be due to the infection alone.

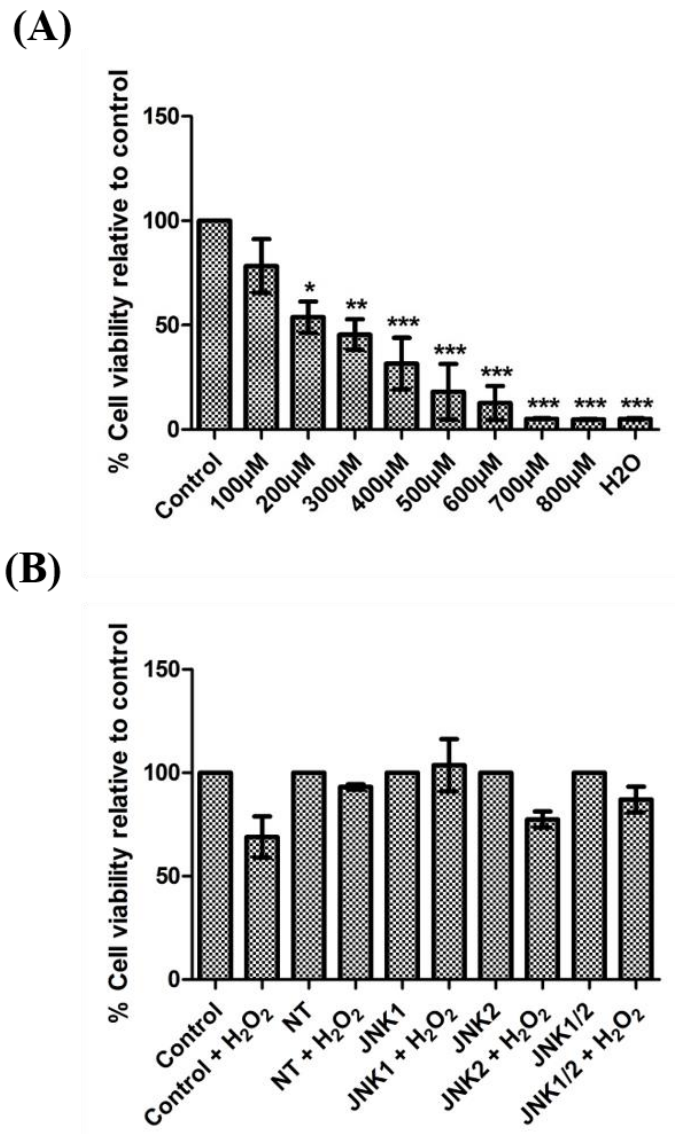


Figure 3.9 Decrease in HUVEC cell viability by H_2O_2 is concentration dependent

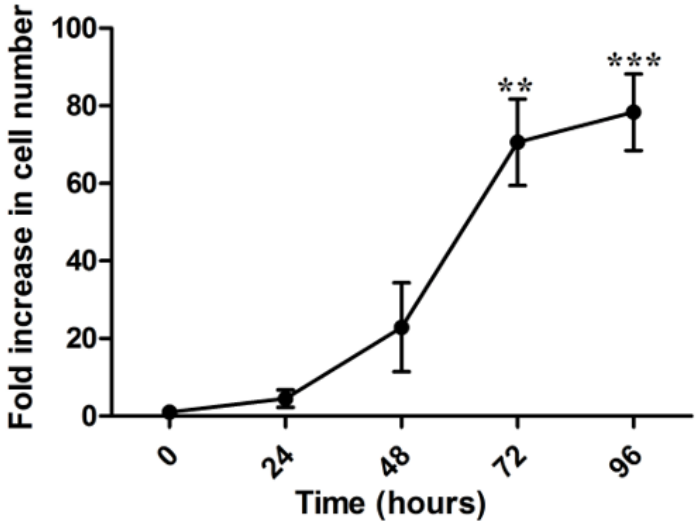
(A) HUVECs were seeded at $5x10^4$ cells/ml into a 96 well plate. Cells were treated with 100-800µM H_2O_2, media alone for control or H_2O alone for positive control for 24 hours. Cell viability was measured using a MTT assay. Data represents the mean ± SEM of three different experiments where *P<0.05 **P<0.01 ***P<0.001. (B) HUVECs were infected as stated in methods section 2.2.2.8 and then seeded into a 96 well plate. Cells were treated with 200µM H_2O_2 or media alone for 24 hours and then viability was measured using a MTT assay. Data represents the number of viable cells treated with H_2O_2 as a percentage of viable cells treated with media alone for each condition, n=2.

3.2.4.2 The effect of JNK knockdown in HUVECs on proliferation

To understand the effects of knocking down JNK in HUVECs on proliferation, a preliminary experiment was carried out to confirm that growth in cell number could be measured over time. For proliferation experiments HUVECs were seeded at a specific concentration into a six well plate, labelled as 0 hours. Every 24 hours a well of cells was trypsinized and cells were counted using a haemocytometer. Media was replenished in the remaining wells at 48 hours. After 96 hours the total cell number for each day was calculated and represented as a fold increase of the starting cell number plated at 0 hours (Figure 3.10A). At 24 and 48 hours there was a trend of increasing cell number, however at 72 and 96 hours this became significant with a fold increase of 70 ± 11.12 and 78 ± 9.84 respectively, demonstrating that HUVEC cell number increases over time and this can be measured.

To look at JNK knockdown, HUVECs were seeded and infected in six well plates with lentivirus targeting JNK1, JNK2, JNK1/2 and NT as stated in methods section 2.2.2.8. Cells from each of these wells, along with control cells were seeded at specific densities into new 6 well plates. A well for each condition was counted every 24 hours and media was replaced in the remaining wells at 48 hours. After 96 hours the fold increase in cell number was calculated for each time point from the starting cell number (Figure 3.10B). Cells infected with lentivirus targeting JNK1, JNK2 and JNK1/2 showed a great reduction in cell number when compared to control cells with an approximate decrease of 83.8%, 73.4% and 71.6% respectively. However, similar to the cell viability assay, the cells infected with NT also showed an approximate decrease of 84.4% when compared to control cells. This would suggest again that the differences observed may be due to the lentiviral infection itself and not the knockdown of JNK.

(A)



(B)

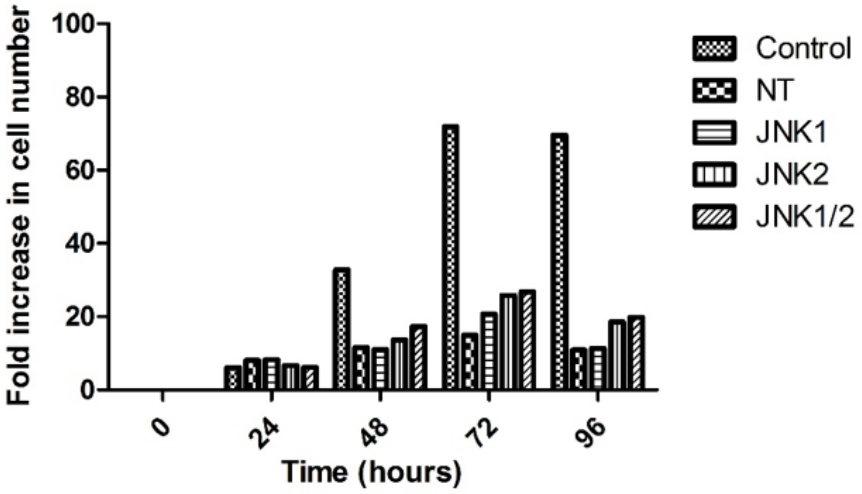


Figure 3.10 The effect of JNK knockdown in HUVECs on proliferation

(A) HUVECs were seeded at specific densities onto six well plates and were counted at 24, 48, 72 and 96 hours. Data represents the fold increase in cell number over 96 hours using the mean ± SEM of three independent experiments, **P<0.01 ***P<0.001. (B) HUVECs were infected with lentivirus at stated in methods 2.2.2.8 and seeded at specific densities onto six well plates and counted at 24, 48, 72 and 96 hours. Data represents the fold increase in cell number over 96 hours, n=2.

3.2.4.3 Lentiviral infection affects cell numbers and induces multinucleation

The cell proliferation and viability assays were carried out on HUVECs with lentiviral JNK knockdown, however results produced were inconsistent or experiments failed. This may be due to the fragility of the primary cells and possibly the infection with the lentivirus was having too much of a detrimental effect. Imaging of infected cells was carried out to try and understand the effects of infection on HUVECs.

HUVECs were plated onto coverslips and infected with lentivirus as mentioned in methods. Cells were stained with DAPI and then visualised using a confocal microscope (Figure 3.11A). The total number of HUVECs per field of view were counted (Figure 3.11B) and also the total number of cells containing multiple nuclei were counted and represented as a percentage of total cell number (Figure 3.12A).

Coverslips containing infected cells (Figure 3.11A) contained almost half the number of cells (NT 25.35 ± 3.67, JNK1 25.24 ± 4.3, JNK2 28.24 ± 5.28, 23.19 ± 3.74) when compared to the control (64.06 ± 6.63). This included the NT infected cells which suggests that the reduction in cell number is due to infection and not JNK knockdown. Interestingly infected cells also contained a higher percentage of multinucleated cells (example shown on Figure 3.12B) with a trend showing JNK2 (17.04% ± 7.76) and JNK1/2 (20.57% ± 3.76) knockdown cells having a slightly higher percentage than the other conditions (Control 4.36% ± 2.04, NT 8.6% ± 4.49 and JNK1 9.06% ± 2.66). This would suggest that lentiviral infection is possibly causing a malfunction in cell division, resulting in cells retaining multiple nuclei.

Due to the generated HUVEC model producing unreliable data, no further work was carried out in this model for the remainder of this thesis.

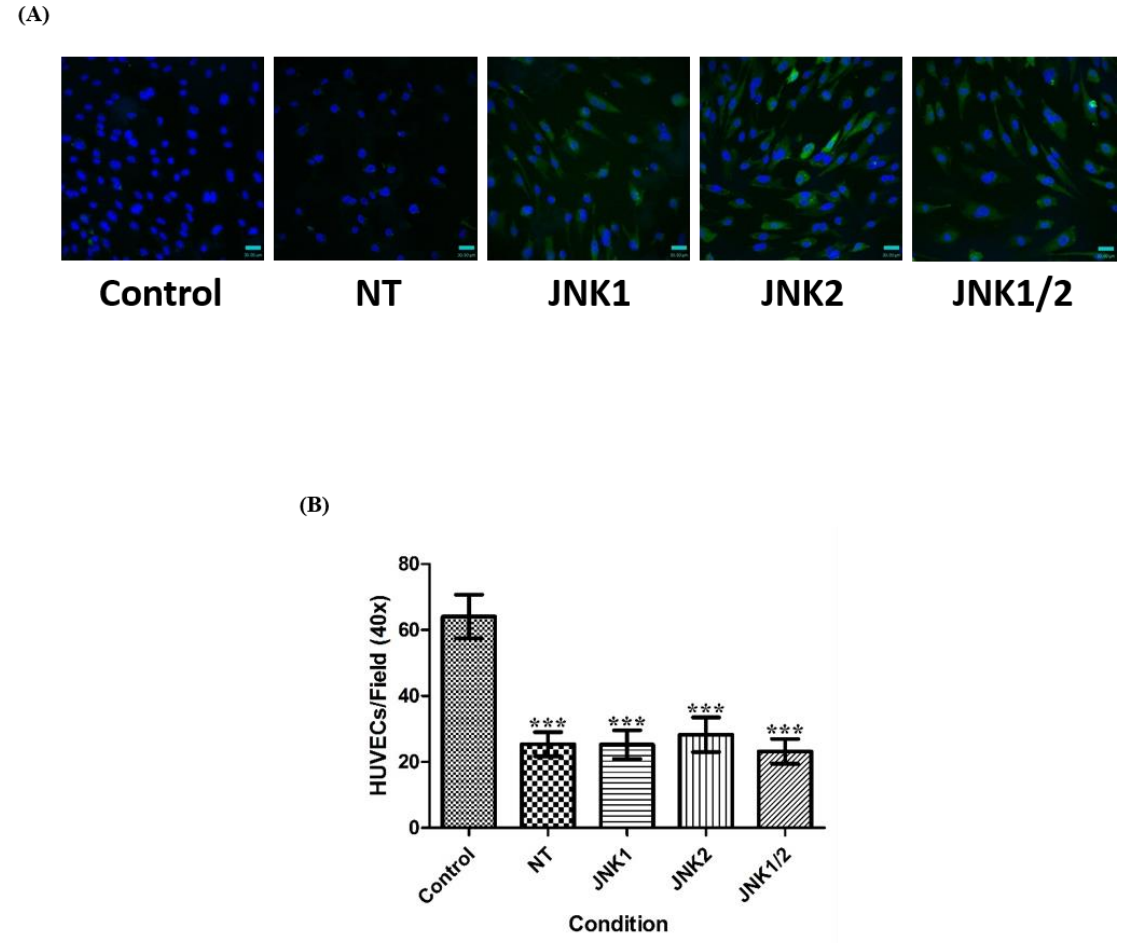
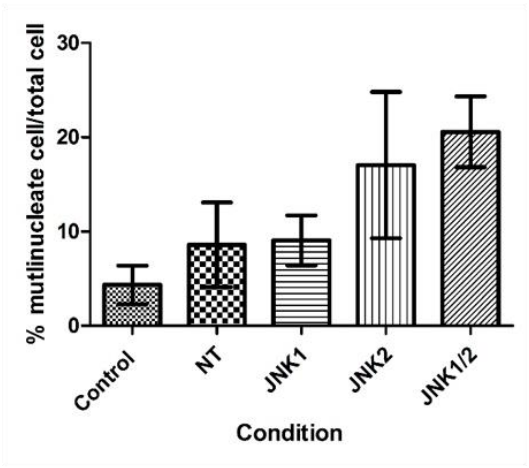


Figure 3.11 Lentiviral infection affects HUVEC cell number

HUVECS plated on coverslips were infected with 100µl of lentivirus in 900µl of serum free media containing 8µg/µl of polybrene. Serum free media containing polybrene was used as a control. 1ml of complete media was added after 4h of incubation. Media was then changed at 24 and 72 hours after infection. Cells were fixed with 100% ice cold methanol and stained with DAPI 1/1000 in PBS. Cells were then visualised on the confocal microscope. Results represent (A) representation of images taken for each condition (B) total number of HUVECs per field of view at 40x magnification. Data represents the mean ± SEM of three different experiments analysing 3-7 coverslips per condition, where ***P<0.001.

(A)



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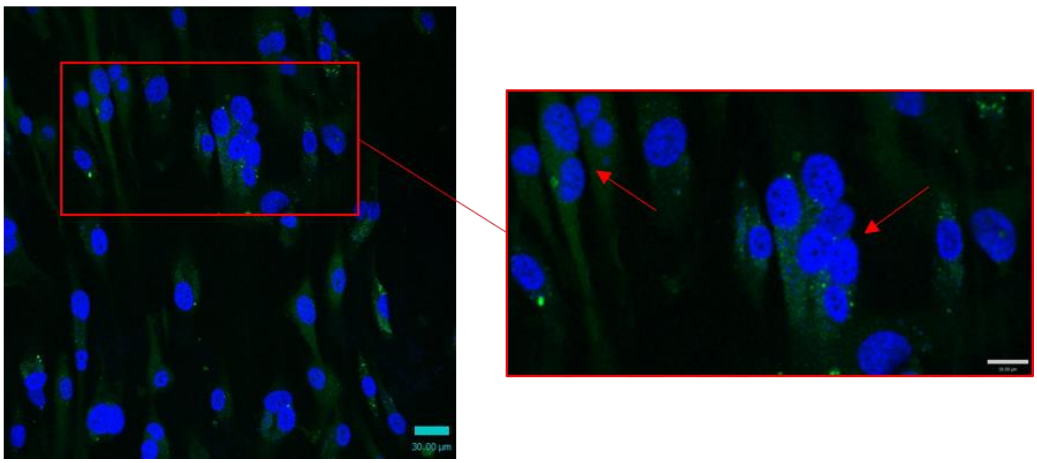


Figure 3.12 Infection with lentivirus induces multinucleation in HUVECs

HUVECS plated on coverslips were infected with 100µl of lentivirus in 900µl of serum free media containing 8µg/µl of polybrene. Serum free media containing polybrene was used as a control. 1ml of complete media was added after 4h of incubation. Media was then changed at 24 and 72 hours after infection. Cells were fixed with 100% ice cold methanol and stained with DAPI 1/1000 in PBS. Cells were then visualised on the confocal microscope. Results represent (A) percentage of multinucleate cells relative to total cell number per field of view (B) Image of JNK1/2 infected cell demonstrating clear multinucleation. Data represents the mean ± SEM of three different experiments analysing 3-7 coverslips per condition.

3.3 Discussion

JNK has been demonstrated to play a role at various stages of atherogenesis, including endothelial dysfunction. Differences in JNK isoform function have been demonstrated in animal models of atherosclerosis, however investigation into JNK isoform function in human vascular cells has not been carried out. In this study HUVECs were used to investigate endothelial cell function and lentivirus was used to target and knockdown JNK to analyse the effects of JNK knockdown on JNK signalling and cell proliferation.

Lentiviral technology is now a widely used tool in different areas of research and is utilised for a variety of techniques such as gene overexpression (Cao *et al.*, 2011), *in vivo* gene transfer (Carbonaro *et al.*, 2006), stem cell production (Sommer *et al.*, 2009) and gene silencing (Chen *et al.*, 2014), which was also used in the current study. Most commonly gene silencing in mammalian cells is carried out using small interfering RNA (siRNA) (Andersen *et al.*, 2007) or short hairpin RNA (shRNA) (Yang *et al.*, 2014). It has been demonstrated that shRNA is more efficient when compared to siRNA (McAnuff, Rettig and Rive, 2007) and it can be synthesized by the target cell allowing stable and long-term silencing (Manjunath *et al.*, 2009), whereas siRNA has a transient silencing effect (Brummelkamp, Bernards and Agami, 2002). In the present study shRNA targeting JNK1, JNK2 and JNK1/2 was used for the high knockdown efficiency and also to allow passaging of treated cells.

There are different viral delivery methods for introducing shRNA into cells including retrovirus, adenovirus and lentivirus. However, only lentivirus can infect both dividing and non-dividing cells for long term gene silencing and also produce less of an immunogenic response within cells when compared to other viral delivery systems (Sliva and Schnierle, 2010). A lot of research into optimising lentiviral technology has been carried out including investigating toxicities caused by infection (Manjunath *et al.*, 2009) and optimising protocols for lentiviral production and delivery (Moore *et al.*, 2010). It has also been demonstrated that concentrating lentivirus removes impurities and improves transduction into cells (Cribbs *et al.*, 2013). In the current study lentivirus was concentrated, which improved transduction and also permitted the removal of antibiotic selection of transduced cells, allowing the cells to remain healthy and less stressed throughout experiments. Very few studies have investigated JNK

isoform function in primary human vascular cells as many methods can be compromising to the cell. In this study lentivirus was used to successfully knockdown JNK1 and JNK2 in HUVECs and the effects of knockdown on JNK signalling and proliferation were investigated.

To investigate the effects of knockdown on JNK signalling, TNFα a well-known activator of the JNK pathway (Yang *et al.*, 2013), (Bas *et al.*, 2015), (Ventura *et al.*, 2006) was used to stimulate HUVECs and activate JNK. Previously it has been demonstrated that JNK activation by TNFα can be either transient or sustained, where each activation resulted in contrasting cell survival or cell death signalling (Ventura *et al.*, 2006). In this study both transient and sustained activation of JNK was produced by stimulation with TNFα. However, transient JNK activation was used to investigate the effects of JNK isoform knockdown on the phosphorylation of downstream targets c-Jun and ATF2.

The transcription factor c-Jun has been demonstrated to play key roles in different stages of atherogenesis, including proliferation and apoptosis of vascular cells. It has previously been demonstrated that activation of c-Jun plays an important role in vascular smooth muscle cell (VSMC) proliferation in vitro (Zhan *et al.*, 2002) and in vivo (Yasumoto *et al.*, 2001). In contrast c-Jun has also been shown to play a role in cell death (Reddy *et al.*, 2013) which has also been demonstrated in vascular endothelial cells (Wang *et al.*, 1999). c-Jun has been shown to be activated by low density lipoproteins, an element of atherogenesis, (Zhu *et al.*, 1998) and has also been investigated in hypercholesterolemic rabbits where it was shown to play a role in atherogenesis through matrix metalloproteinase activation (Sozen *et al.*, 2014). Due to the accumulating evidence of JNK/c-Jun signalling in CVD the effects of JNK knockdown on the phosphorylation of c-Jun was investigated.

Interestingly, there was no significant changes in phosphorylated c-Jun levels in HUVECs with JNK1 knocked down when compared to the NT infected cells. However, in cells containing JNK2 knockdown there was a significant increase in phosphorylated c-Jun levels which would correlate more with the findings produced by Sabapathy and colleagues. Sabapathy *et al* used wild type (WT) and mutant mouse embryonic fibroblasts (MEFs) from $JNK1^{-/-}$ or $JNK2^{-/-}$ mice and compared cell

proliferation between cell types (Sabapathy *et al.,* 2004). They demonstrated that fibroblasts lacking JNK1 produced a lower level of c-Jun phosphorylation and less cell proliferation, whereas JNK2 KO cells had higher levels of phosphorylated c-Jun when compared to WT cells and an increase in cell proliferation. In support of this, another study used $CD8^+$ T cells lacking either JNK1 or JNK2 and showed that JNK1 KO cells proliferated less compared to WT cells whereas proliferation increased in JNK2 KO cells (Conze *et al.,* 2002). The study demonstrates that this was due to JNK1 KO cells having a lower expression of the alpha chain of interleukin – 2 (IL-2α) receptor, which is suggested to be linked to reduced levels of c-Jun in the JNK1 KO cells. IL-2 production was increased in JNK2 KO cells, however this could be due to c-Jun levels as c-Jun has been associated with IL-2 transcription (Walters *et al.,* 2013).

The differences observed between the current study and previous studies when looking at JNK1 function specifically could be due to the fact that lentiviral knockdown does not achieve 100% knockdown whereas the other studies mentioned used mutant cells lacking JNK1. This may suggest that only a small amount of JNK1 expression is required to activate downstream targets. Another possible explanation could be that JNK activity may vary between cell types and therefore JNK1 signalling in HUVECs and fibroblasts may play different roles. Finally c-Jun is a known substrate of other MAPK pathways such as ERK (Deng *et al.,* 2012) and therefore compensatory signalling may have played a role in the increase of pc-Jun expression produced.

ATF2 is another substrate of JNK which plays an important role in inflammatory signalling (Yu *et al.,* 2014) and has been linked to signalling in atherosclerosis (Boon *et al.,* 2010). During early atherogenesis, immune cells in the blood stream adhere to the endothelial cell monolayer of the vessel via adhesion molecules expressed on both the cell surface and vascular endothelium (Collins *et al.,* 2000). Fearnley *et al* demonstrated a role for ATF2 in endothelial-leukocyte interactions through regulation of vascular cell adhesion molecule 1 (VCAM-1) expression (Fearnley *et al.,* 2014). The study mentioned previously carried out by Sabapathy *et al* also investigated the effects of JNK1 and JNK2 knockout on ATF2 expression in fibroblasts (Sabapathy *et al.,* 2004).

The findings in that study were comparable to the current study where no significant differences were found in ATF2 phosphorylation when comparing cells with either JNK1 or JNK2 knocked down. However, Sabapathy *et al* did show that mutant cells lacking both JNK1 and JNK2 simultaneously produced a significantly lower level of pATF2. Contrasting results were observed in the current study, where no significant difference in pATF2 expression was produced in cells containing JNK1 and JNK2 knockdown. Again this may possibly be due to the low levels of JNK1 and JNK2 that remain within the population of stimulated cells. Another observation made was that basal levels of pATF2 were quite high to begin with resulting in a stimulation of approximately only 2 fold. This could be another reason as to why no differences were observed between cells containing NT and JNK knockdown as HUVECs may contain higher levels of pATF2 when compared to the different cells used in other studies and therefore the stimulation and differences produced may be more difficult to observe.

Throughout all experiments, levels of pJNK expression showed great fluctuation for both the 46 and 54 kDa variants. This could perhaps be due to the differences in stress placed on the HUVECs during infection and stimulation which may lead to variances in signalling. Another possible cause could be variation in knockdown between experiments, however this was not represented in the figures of percentage knockdown so seems to be an unlikely explanation for such a large variation. An interesting observation made during this experiment was that knockdown of JNK1 reduced the level of the 46kDa variant of pJNK and knockdown of JNK2 reduced the expression of the 54kDa variant of pJNK. This would suggest that these bands represent JNK1 and JNK2 specifically and therefore can be used to investigate the phosphorylation of each isoform. In the current study phosphorylation of JNK2 seemed to be greater than JNK1, suggesting TNFα may stimulate each isoform to a different level.

Unfortunately, when the effect of JNK knockdown on HUVEC proliferation was investigated it was clear that the lentiviral infection may have been having a detrimental effect on HUVEC cell growth. This was not observed during the signalling experiments, most likely because once infected, cells were immediately stimulated and collected for Western blot analyses. In the proliferation experiment, cells were infected and then trypsinized and plated as specific densities, before being left to grow. This is where the defect in HUVEC cell growth was observed and because the NT infected

cells produced a similar reduction in cell growth as the cells with JNK knockdown, it was concluded that this was due to the lentiviral infection and not the knockdown of protein.

An interesting observation made during the imagining experiments was that lentiviral infection caused an increase in the percentage of multinucleate cells relative to the total number of cells per field of view, in comparison to uninfected cells. This seemed to occur even more in cells containing JNK2 and JNK1/2 knockdown, suggesting that the multinucleation occurred more in cells lacking JNK2. The results were not statistically significant, however there was a clear trend throughout all experiments. JNK has been linked to the regulation of the cell cycle (Gutierrez, Tsuji, Cross, *et al.*, 2010), therefore it is possible that JNK knockdown would cause defects in cell growth. The fact abnormalities in growth were found in all imaged cells which were infected with lentivirus, confirmed that the infection of lentivirus may have been having detrimental effects on HUVEC growth and therefore the technique may not be optimised for use in primary cells. This meant that further studies to investigate differences in HUVEC cell growth caused by JNK2 knockdown were not able to be carried out.

Successful lentiviral knockdown studies have been carried out in HUVECs targeting different proteins (Guangqi *et al.*, 2012) (Bajaj *et al.*, 2012). It is quite common for studies to show results only against the non-target lentivirus infected cells and not include uninfected cells alone, therefore it is difficult to conclude whether the same effects of infection may have occurred in other studies. For primary cells the gentlest procedure of knockdown is required to ensure the least amount of side effects caused by the infection process. Unfortunately, in this study cells were too affected by infection to proceed with further experiments. Cells infected with the non-target lentivirus also developed abnormalities in growth and therefore it can be concluded that it was the infection with lentivirus and not the knockdown of protein which produced these results. This is possibly due to too the amount of virus used for infection or the protocol used may need to be fully optimised for this cell type.

Future studies may benefit from using smaller volumes of virus to infect cells, coupled with multiple selections with a low concentration of antibiotic. This may allow the cells to maintain a more natural phenotype and provide more reliable results.

Due to the challenges encountered with these experiments as described throughout the chapter, the data shown here unfortunately remains somewhat inconclusive. However, other groups have previously demonstrated differences in isoform function in the vasculature, where prolonged activation of JNK1, but not JNK2, by TNF-α and ultraviolet (UV) light resulted in apoptosis in fibroblasts (Liu, Minemoto and Lin, 2004). JNK1 but not JNK2 has been linked to obesity induced insulin resistance whereas JNK2 but not JNK1 has been linked to foam cell formation in atherosclerotic prone mice (Hirosumi *et al.*, 2002) (Ricci *et al.*, 2004). Although work has been carried out to determine differences in JNK isoform function, detailed characterisation of JNK1 and JNK2 isoforms has not been carried out in a human vascular cell line. JNK signalling plays important roles in the development of CVD and therefore further investigation into the roles played by each individual isoform could lead to the development of new therapeutic targets. To do this however, a reliable and successful way to target the individual isoforms without having too many secondary effects in primary cells must be developed.

Chapter Four

The Effects of JNK isoform knockdown on cell growth and cell death in MCF-7 breast cancer cells

4.1 Introduction

After finding that lentiviral knockdown caused detrimental side effects in HUVECs, a primary cell line, the technique was then tried in a human breast cancer cell line to determine whether it could be used in a more durable cell type. The role of JNK in breast cancer has been investigated however contrasting findings on JNK function in breast cancer have been published. In 2010, two studies were carried out by different research groups, investigating the role of JNK proteins in mouse models of breast cancer. In the study by Cellurale *et al* JNK1 and JNK2 were knocked out in a Trp53 dependent mouse model of breast cancer. This model was used to represent breast cancer in humans promoted by the mutation of the p53 gene. Interestingly, knockout of either JNK1 or JNK2 increased the percentage of mice which developed mammary carcinomas when compared to control mice. Mammary carcinoma free survival was also calculated for each of the models and again JNK1 and JNK2 knockout mice had a significant reduction in mammary carcinoma free survival when compared to control mice. These findings suggest that JNK1 and JNK2 may play a suppressive role in mammary tumour development (Cellurale *et al.*, 2010).

In contrast to that study, Chen *et al* used the Polyoma Middle T Antigen (PyV MT) mouse mammary tumour model which represents early and late stages of human breast cancer. They then knocked out JNK2 in this model and investigated the effects of JNK2 knockout on mammary tumorigenesis. Mice with JNK2 knockout had a shortened latency period and also higher tumour multiplicity, this would suggest JNK2 may play a role in suppressing tumorigenesis which would agree with the previous study. However, after tumours were formed, cell proliferation was significantly decreased in the JNK2 knockout mice and on further investigation it was demonstrated that $JNK^{-/-}$ tumour cells underwent more DNA damage and replicative stress when compared to control cells, suggesting JNK2 may play a role in preventing replicative stress and promoting cell cycle progression in mammary tumours (Chen *et al.*, 2010). Taken together these studies demonstrate different roles for JNK proteins in mouse models of breast cancer. Whether this is down to the use of different models or that different processes were investigated, it still remains unclear whether JNK1 and JNK2 are promoters of breast cancer or suppressors.

As mentioned in chapter one, much of the work to determine JNK isoform function and signalling pathways has been carried out in mouse embryonic fibroblasts (MEFs) from JNK knockout models. Although this has allowed a better understanding of the diverse functions of JNK isoforms, studies in human cells are required for this work to progress. In this chapter stable MCF-7 breast cancer cell lines containing JNK1, JNK2 and JNK1/2 knockdown were generated and the effect of JNK isoform knockdown on cell death and cell growth processes were investigated.

JNK has been demonstrated to play an important role in apoptotic signalling and cell death. In cancer cells many studies have shown that JNK plays a key role in cell death induced by anti-cancer drugs. However, similar drugs have been demonstrated to activate cell death via different JNK activated pathways. For example, in the study by Kolomeichuk *et al* a comparison was made between two microtubule inhibitors, vinblastine and Taxol. In cervical cancer cells, treatment with either drug in combination with the inhibition of JNK by SP600125 caused a significant reduction in cell death, demonstrating that both drugs required JNK to induce cell death. Interestingly, vinblastine induced cell death was also shown to be dependent on c-Jun/Ap-1 activity, whereas treatment with Taxol had no effect on AP-1 activity and inhibiting c-Jun had no effect on Taxol - induced cell death (Kolomeichuk *et al.*, 2008).

Differences in JNK induced cell death have also been demonstrated between breast cancer cell lines. Mingo-Sion *et al* used the JNK inhibitor SP600125 in MCF-7 and MDA-MB-231 cells to investigate the effects of JNK inhibition on Taxol and UV - induced cell death. Inhibition of JNK in MCF-7 cells reduced cleaved PARP expression in both Taxol and UV stimulated cells. However, in MDA-MB-231 cells there was less of a reduction in cleaved PARP expression observed, suggesting that JNK may not play as key a role in apoptosis in MDA-MB-231 cells when compared to MCF-7 cells (Mingo-Sion *et al.*, 2004). These studies clearly demonstrate that the outcome of JNK signalling is dependent on stimulant and cell type. In the current study the effect of JNK isoform knockdown on MCF-7 cell death was investigated in response to treatment with UV radiation and Taxol.

UV radiation exposure can cause damage to DNA, cell cycle arrest and ultimately induction of apoptosis, for a detailed review on the mechanisms involved in UV -

induced DNA damaged please refer to the review by Rastogi *et al* (Rastogi *et al.*, 2010). UV mediated cellular stress has been shown to activate cell death in MCF-7 cells (Ferguson, Marietta and Van Den Berg, 2003). Although JNK has been demonstrated to play a key role in UV - induced cell death in MCF-7 cells, the function of JNK1 and JNK2 individually has never been investigated in this cell line.

The same can be said for Taxol - induced cell death, Paclitaxel, also known as Taxol, is a microtubule interfering agent, which binds to microtubules and causes stabilization resulting in cell cycle arrest in the G2/M phase and consequential activation of cell death (Wang, Wang and Soong, 2000). Taxol has been demonstrated to activate JNK signalling in a variety of different human cell lines, including MCF-7 breast cancer cells (Wang *et al.*, 1998). In this study, an interaction between Taxol and the microtubules was required to activate JNK and this was thought to be through both Ras and ASK-1 signalling pathways. Overexpression of JNK has been shown to enhance PARP cleavage induced by treatment with Taxol in MCF-7 cells (Mamay *et al.*, 2003) and inhibition of JNK by SP600125, had been shown to reduce Taxol - induced MCF-7 cell death (Sunters *et al.*, 2006). These studies provide clear evidence that JNK plays a key role in MCF-7 cell death in response to UV and Taxol, however the individual function of JNK1 and JNK2 in MCF-7 cells is still unknown.

In addition to suppressing breast cancer JNK has been shown to promote breast cancer, this has been demonstrated through cell cycle progression, proliferation and clonogenic assays. For example, when both MCF-7 and MB-231 breast cancer cells were treated with the JNK inhibitor SP600125 a large population of cells became arrested in the G2/M phase of the cell cycle (Mingo-Sion *et al.*, 2004). In the same study, MCF-7 cells treated with anti-sense (AS) oligonucleotides to JNK1 or JNK2 or MB-231 cells treated with SP600125 showed a decrease in cell proliferation over 4 days when compared to control cells. Both JNK isoforms produced a similar amount of cell cycle arrest and reduction in proliferation in each experiment. These findings suggest that JNK is key for the successful progression of the cell cycle and proliferation in MCF-7 cells.

Another study used siRNA to target and knockdown JNK1 and JNK2 in MCF-7 breast cancer cells. Similar to the findings by Mingo-Sion *et al*, cells were monitored and

counted over 8 days and knockdown of JNK1 or JNK2 caused a reduction in total cell number when compared to control cells (Parra and Ferreira, 2010). A clonogenics assay was also carried out in this study and again JNK1 and JNK2 knockdown both produced a reduction of approximately 50% in the percentage of colonies formed relative to control cells. Taken together both of these studies highlight a role for JNK in breast cancer cell growth.

Many studies carried out in human cell lines use transient methods of inhibition or knockdown to investigate protein function. The effect of stable JNK isoform knockdown on MCF-7 cell death or growth has not yet been investigated. In this chapter MCF-7 cells containing stable knockdown of JNK1, JNK2 and JNK1/2 were generated and the effects of knockdown on cell death and cell growth was analysed.

4.2 Results

4.2.1 JNK isoform function in MCF-7 cell death

4.2.1.1 Generation of MCF-7 cell lines containing JNK1, JNK2 and JNK1/2 knockdown

Before investigating the role of JNK knockdown on cellular processes, the expression of all three JNK proteins was measured in MCF-7 cells. JNK1 and JNK2 expression was easily detected (data not shown) however, JNK3 expression was not detected in samples of MCF-7 cells (Figure 4.1) when compared to the positive control of homogenised mouse brain. This finding was consistent with studies which show a restricted expression of JNK3. Since the lack of JNK3 expression was confirmed, the remainder of this chapter focused on the function of JNK1 and JNK2 in MCF-7 cells.

To begin investigations of the function of JNK isoforms in MCF-7 cells, four cell lines were generated using lentivirus as stated in section 2.2.2.10. Cells were selected with puromycin every passage and Western blots were carried out to confirm knockdown was consistent throughout all experiments. Knockdown of JNK1 was achieved in the JNK1 and JNK1/2 cells lines at an average of 93.31% ± 2.0 and 70.54% ± 6.4 knockdown respectively (Figure 4.2A). No significant difference in JNK1 expression was observed between control, NT and JNK2 cells lines confirming the lentivirus had high specificity for JNK1. Similarly, JNK2 knockdown was achieved in the JNK2 and JNK1/2 cell lines at an average of 88.76% ± 6.5 and 92.46% ± 3.7 respectively (Figure 4.2B). Again there was no significant difference in JNK2 expression between the control, NT and JNK1 cell lines, confirming that the lentivirus was specific for targeting JNK2 only.

These results confirmed JNK1 and JNK2 knockdown in the MCF-7 generated cell lines which allowed experiments to be carried out confidently and direct comparisons to be made. The remainder of this chapter will focus on the effects of JNK1 and JNK2 knockdown on cell death and cell growth in MCF-7 cells.

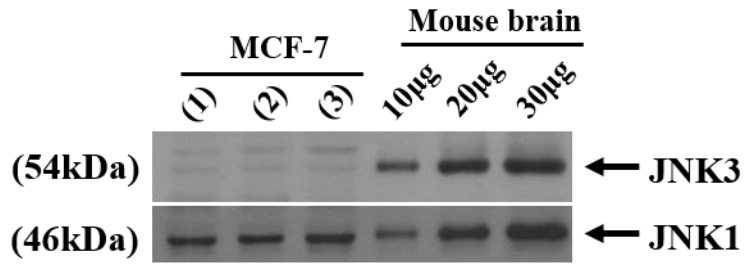


Figure 4.1 JNK3 is not expressed in MCF7 cells

MCF-7 cells were grown in 6 well plates until confluent and collected in LSB. Homogenised mouse brain was diluted with LSB to a concentration of (1µg/µl). Samples of MCF-7 cells from three individual experiments were ran alongside increasing amounts of homogenised mouse brain (10-30 µg) and expression of JNK3 was analysed. JNK1 expression was used to confirm protein loading.

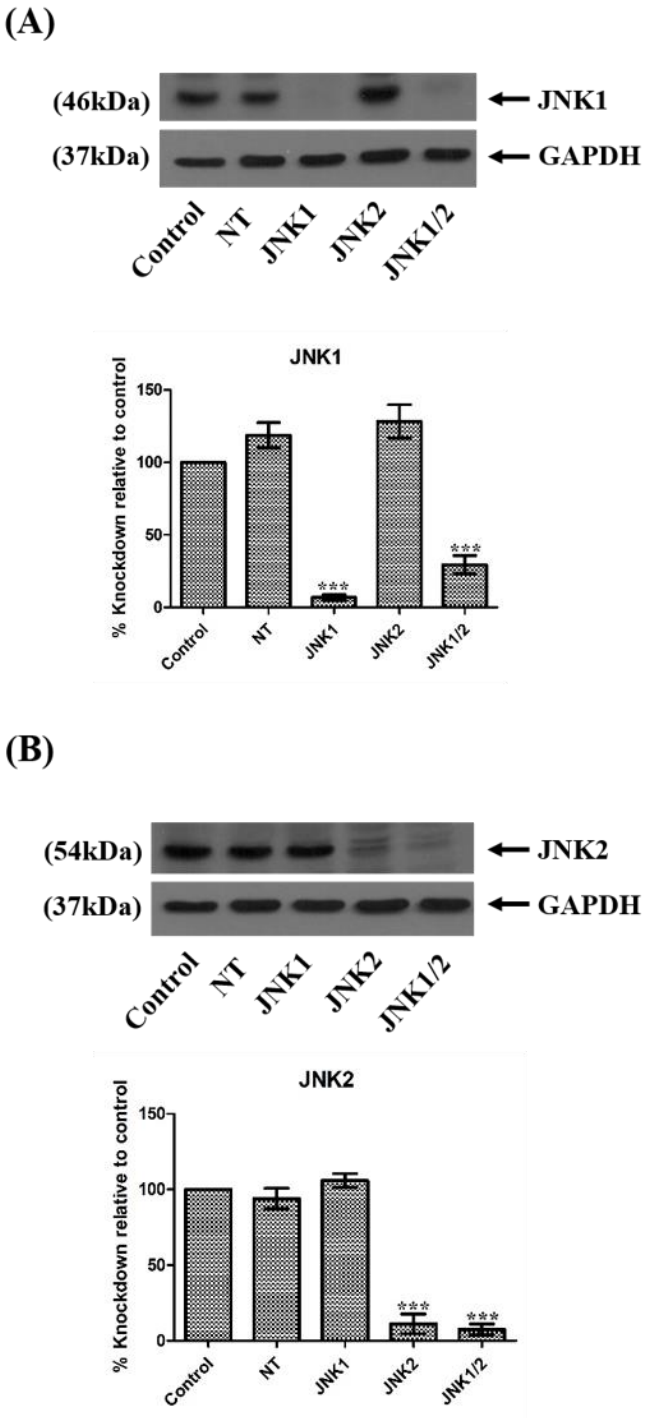


Figure 4.2 Generation of MCF7 cell line with JNK1, 2 and 1/2 knockdown

MCF-7 cell lines were generated as stated in methods section 2.2.2.10 using non-targeting and JNK1, JNK2 and JNK1/2 targeting lentiviruses. Results show Western blot of (A) JNK1 (B) JNK2 expression in all 4 cell lines plus a control line with GAPDH as a loading control. Quantitative analyses of the % of (A) JNK1 or (B) JNK2 expression relative to control cells is represented in graph format. Data represents the mean ± SEM of n=3, ***P<0.001.

4.2.1.2 The effects of JNK isoform knockdown on UV - induced cell death

To establish JNK signalling in MCF-7 cells, UV, a known activator of JNK, was used to investigate the effects of JNK isoform knockdown on cell death signalling. To determine the optimum dose to activate JNK and UV - induced cell death, a dose response curve was carried out. Cells were starved for 24 hours and then exposed to doses of 0 - 60 J/m^2 of UV. After exposure cells were incubated for a further 24 hours and then collected as samples to run in a Western blot. Although JNK was activated at earlier time points (data not shown), 24 hours exposure was used to clearly see the effects of knockdown on the expression of markers of cell death. Levels of pJNK and cleaved PARP were analysed to determine activation of JNK and also activation of cell death respectively. GAPDH was also used to confirm equal loading (Figure 4.3A).

Levels of the 54kDa variant of pJNK began to increase by 2.10 fold ± 0.9 after exposure to 10J/m^2 of UV and continued to increase by approximately 4 fold at 30 and 60J/m^2 (3.87 fold ± 1.9 and 4.15 fold ± 1.9 respectively) (Figure 4.3B). However, due to variation between experiments this increase was not statistically significant when compared to the dose of 0 J/m^2. Similarly, levels of the 46kDa variant of pJNK also showed an increase in expression of 1.23 fold ± 0.3 after exposure to 10J/m^2, this continued to increase to 2.34 fold ± 0.5 and 2.62 fold ± 0.5 after exposure to 30J/m^2 and 60J/m^2 respectively (Figure 4.3C). Again this increase was not significant due to variation between experiments, however a clear trend was observed where increasing the dose of UV caused an increase in pJNK expression. Interestingly the levels of the 54kDa variant of pJNK was almost double that of the 46kDa variant after exposure to 10, 30 and 60J/m^2 of UV suggesting that the 54kDa variant is more sensitive to this stimulant.

To determine the most efficient dose of UV to activate cell death, expression of cleaved PARP was analysed as it is a clear marker of cell death. Low levels of cleaved PARP were observed at 5 J/m^2 of UV however this increased after exposure to 10, 30 and 60 J/m^2 with average fold stimulations of 68.66 ± 57.37, 356.4 ± 260.1 and 390.1 ± 237.1 (Figure 4.3D). Due to the huge variation observed between experiments, these values were not significant when compared to the control samples. However, the fold stimulation observed for cells exposed to 30 and 60J/m^2 of UV were 42.33 fold and

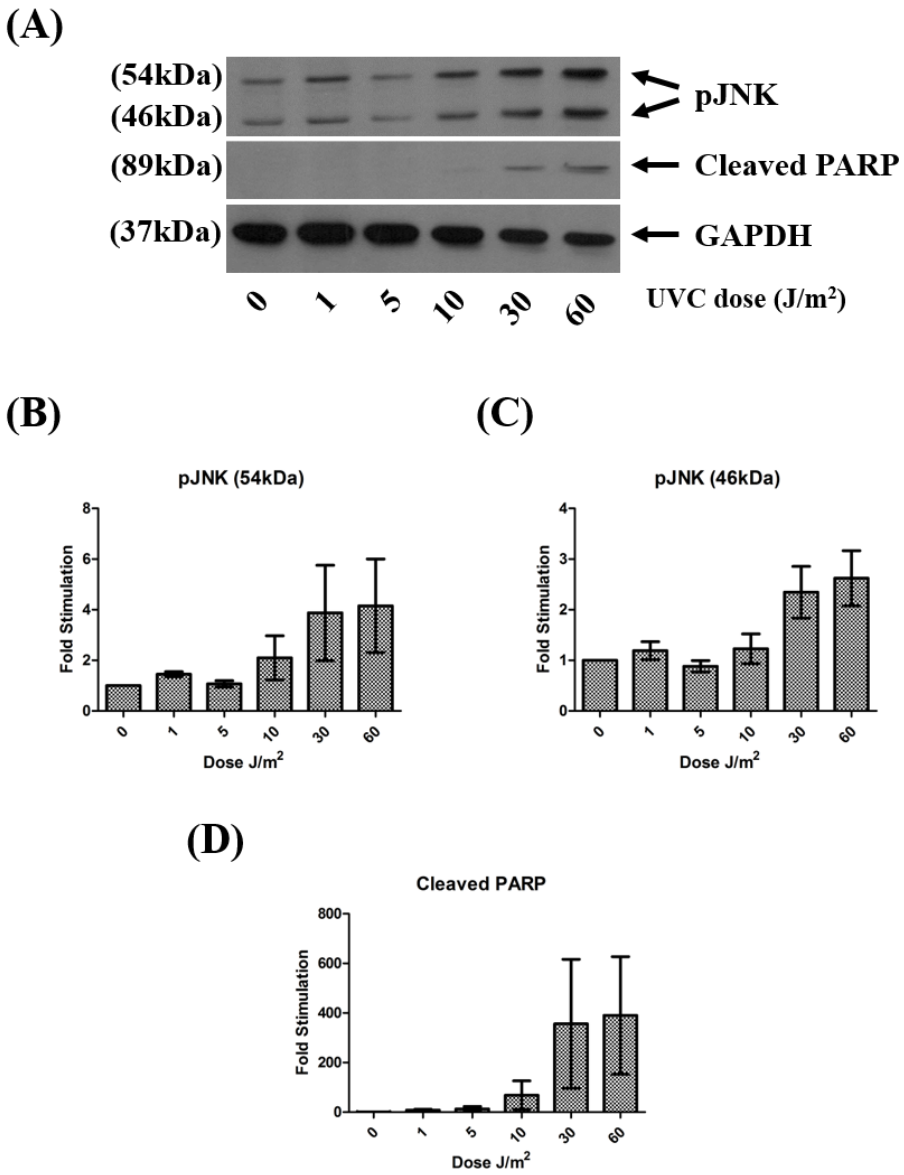


Figure 4.3 UV activation of JNK and cell death is concentration dependent

MCF-7 cells were treated with a dose of UV radiation ranging from 0 – 60 J/m^2 for 24 hours. Results show (A) Western blot of pJNK and cleaved PARP expression, GAPDH was used to confirm equal loading. Protein expression levels for (B) pJNK (54kDa), (C) pJNK (46kDa) and (D) cleaved PARP were semi-quantified using densitometry and presented as a fold stimulation relative to the control. Data represents the means ± SEM of n=3. Data was analysed using a one-way ANOVA test.

33.73 fold respectively and therefore it was concluded that UV was a good activator of cell death in MCF-7 cells at these doses.

The variation observed in these experiments may be due to differences in film exposure which can lead to major differences in quantifying the densitometry values. However, clear activation of JNK was observed along with activation of cell death at doses 30 and 60 J/m^2 of UV. To prevent the cell from undergoing too much stress, the lower dose of 30J/m^2 was used to investigated the effects of JNK knockdown on UV - induced cell death.

Cells from all five cell lines were starved for 24 hours, exposed to UV for 24 hours and then collected. After cells were collected, samples were analysed using Western blotting and antibodies against pJNK and cleaved PARP, JNK1, JNK2 and GAPDH (Figure 4.4 A). Levels of JNK1 and JNK2 were analysed to confirm knockdown and GAPDH was used to confirm equal loading. Protein expression levels were semi quantified by densitometry and presented as a fold stimulation of the control from each cell line (Figure 4.4B-D).

After treatment with UV, levels of both the 46kDa and 54kDa pJNK variants increased to approximately 12 and 5 fold respectively in NT treated cells (12.12 ± 5.6 and 5.78 ± 2.0) (Figure 4.4B-C). The basal level of pJNK 54kDa seemed to be slightly higher than pJNK 46kDa (Figure 4.4A) this might explain the differences in stimulation, however pJNK levels of both variants were similar in control cells suggesting that variation in experiments or analysis may have more likely contributed to this difference. Similar to the experiments carried out in the previous chapter, the levels of pJNK 46kDa were decreased in JNK1 and JNK1/2 knockdown cells and pJNK 54kDa were reduced in JNK2 and JNK1/2 knockdown cells, suggesting these bands represent the phosphorylation of each isoform (Figure 4.4B-C)

To determine the effects of JNK knockdown on UV - induced cell death, the expression levels of cleaved PARP were analysed. UV exposure induced an increase in cleaved PARP expression in all five cell lines. Control, NT, JNK1, JNK2 and JNK1/2 expressed a fold increase of approximately 7, 6, 5, 5 and 7 fold respectively however none of these values were statistically different over the three experiments carried out (7.55 ± 0.8, 5.96 ± 1.4, 5.23 ± 1.3, 4.93 ± 0.7 and 7.10 ± 1.5).

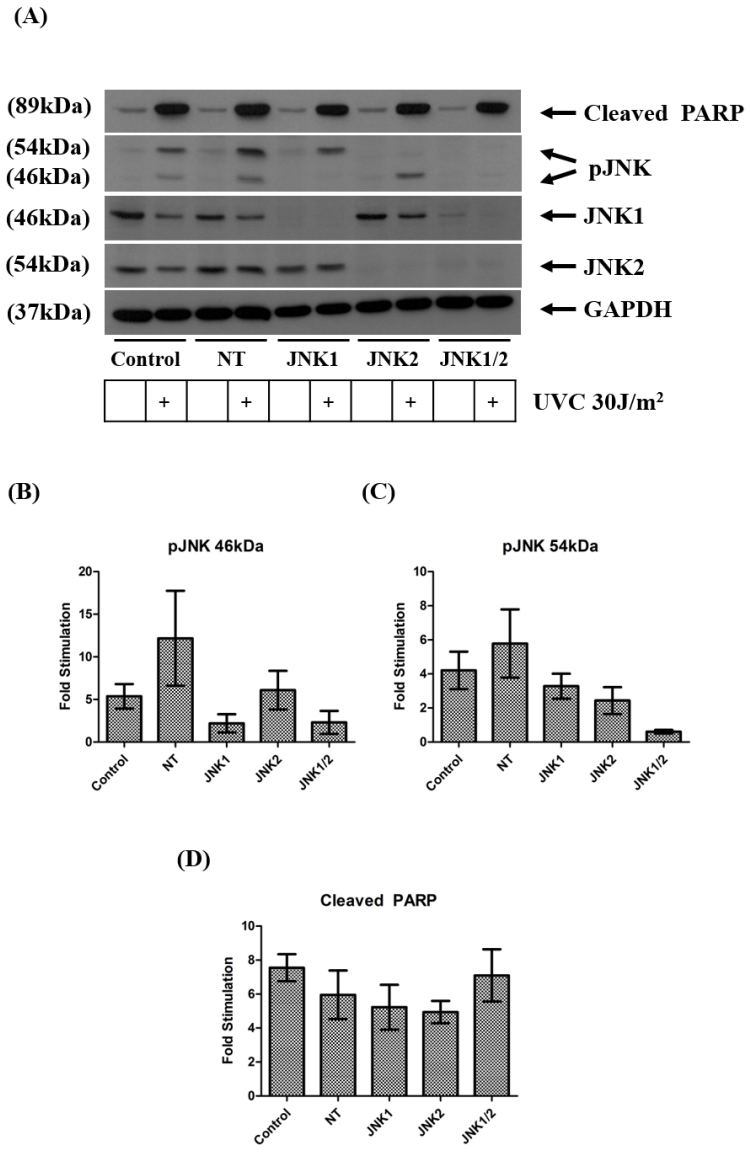


Figure 4.4 UV - induced PARP cleavage is independent of JNK signalling

Control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded at equal densities and starved for 24 hours. Cells were then exposed to 30 J/m^2 of UV for 24 hours. Results show (A) Western blot of pJNK, cleaved PARP, JNK1, JNK2 and GAPDH was used to confirm equal loading. Protein expression levels were semi quantified for (B) pJNK (46kDa), (C) pJNK (54kDa) and (D) cleaved PARP using densitometry and presented as a fold stimulation relative to the control of each cell line. Data represents the mean ± SEM of three independent experiments. Data was analysed using a one-way ANOVA test.

This would suggest that in MCF-7 cells UV - induced cell death was independent of JNK. To analyse cell survival, a clonogenic assay was carried out to determine the effects of JNK knockdown on colony formation after treatment with UV radiation. Firstly, a preliminary experiment was carried out to confirm that MCF-7 cells could form colonies and that UV radiation would cause a reduction in the number of colonies formed (Figure 4.5A-B).

Cells were seeded at a density of 120 cells/ml and left to adhere overnight. Cells were then starved for 24 hours, exposed to $10J/m^2$ of UV radiation, immediately replenished with complete media and then incubated for 12-15 days. After 12-15 days cells were washed in PBS, fixed in methanol, stained with Giemsa and then the number of colonies were counted for each dish. Each condition was carried out in triplicate and this was repeated 3 times. Control cells were placed into the UV crosslinker box with lids removed for 15 seconds with no exposure. The lower dose of $10J/m^2$ was used to prevent cell systems from being exerted too much since cells were grown for approximately 2 weeks after exposure in comparison to the 24 hours used in previous experiments.

After staining successful colonies were formed in both control and treated dishes (Figure 4.5A). However, cells exposed to UV produced approximately 60% less colonies than control cells (Figure 4.5B), confirming that UV radiation does cause a reduction in colony formation (average number of colonies, control 66 ± 2.5 and UV 26 ± 1.5).

To investigate the effects of JNK1 and JNK2 knockdown on colony formation at exposure to UV, all 5 cell lines were seeded at a density of 120 cells/ml and a clonogenics assay was carried out as in the preliminary experiment. Again, successful colony formation was achieved by all 5 cell lines (Figure 4.6A). The survival fraction was calculated as mentioned in methods section 2.2.8 for each cell line. UV exposure successfully decreased the survival fraction in all cell lines with no significant difference in survival fraction observed between control and NT cell lines, confirming the lentiviral infection did not have any detrimental effects itself. Interestingly, the other 3 cell lines produced no differences in the survival fraction with Control, NT,

JNK1, JNK2 and JNK1/2 producing a similar survival fraction of 0.53 ± 0.02, 0.64 ± 0.05, 0.51 ± 0.08, 0.68 ± 0.06 and 0.49 ± 0.03 respectively (Figure 4.6B).

Together the results from both the Western blot and clonogenics assay suggest that UV - induced cell death is independent of JNK signalling in MCF-7 cells.

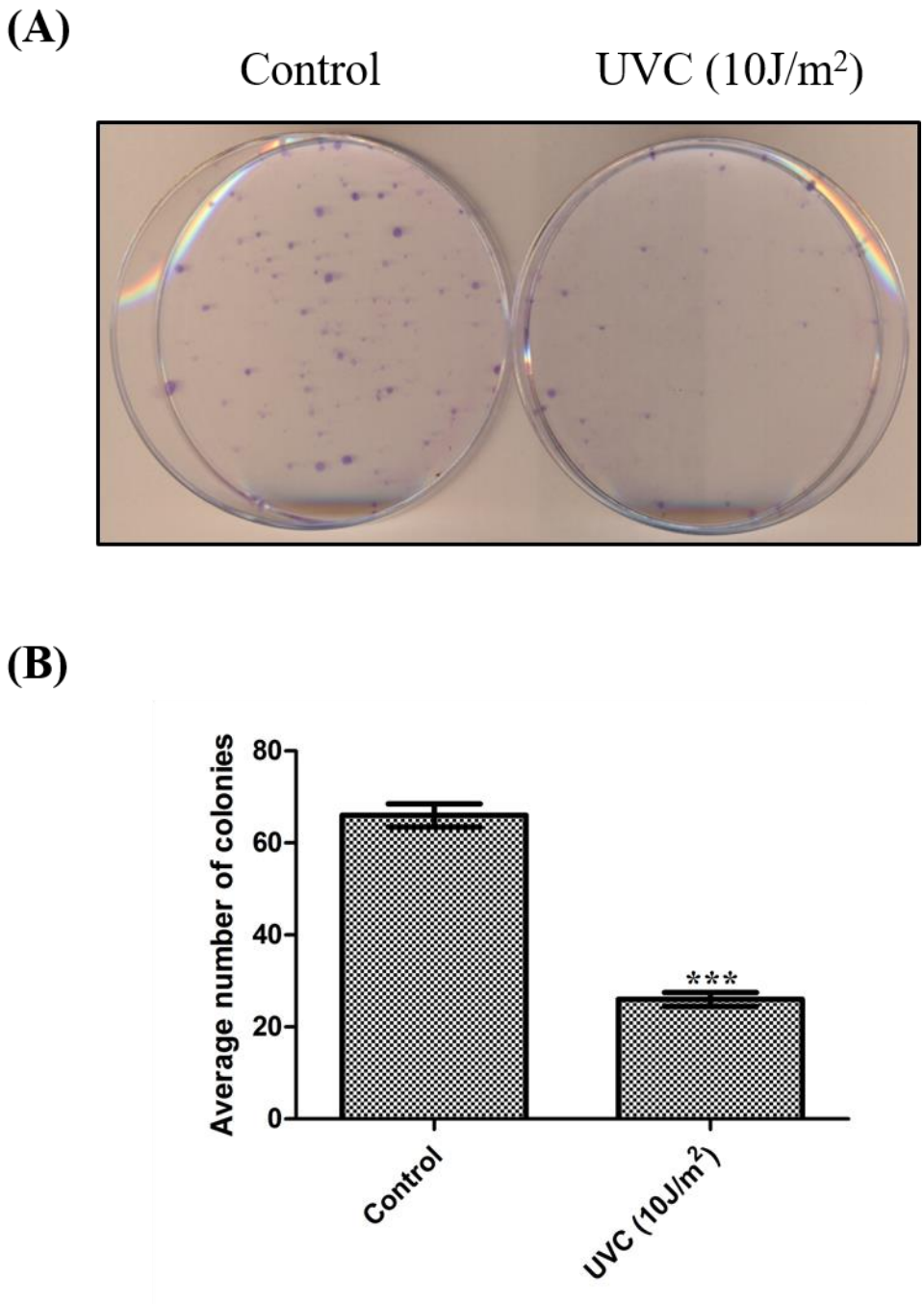


Figure 4.5 UV decreases MCF7 colony growth

MCF-7 cells were seeded at a density of 120 cells/ml, left to adhere for 24 hours, starved for 24 hours and then exposed to 10J/m^2 of UV. Media was immediately replenished and cells were incubated for 12-15 days at 37ºC, 5% CO_2, with media replenished every 4 days. Results show (A) representative image of clonogenic assay and (B) Average number of colonies formed. Data represents the means ± SEM of n=3. Data was analysed using a students t-test, ***P<0.001.

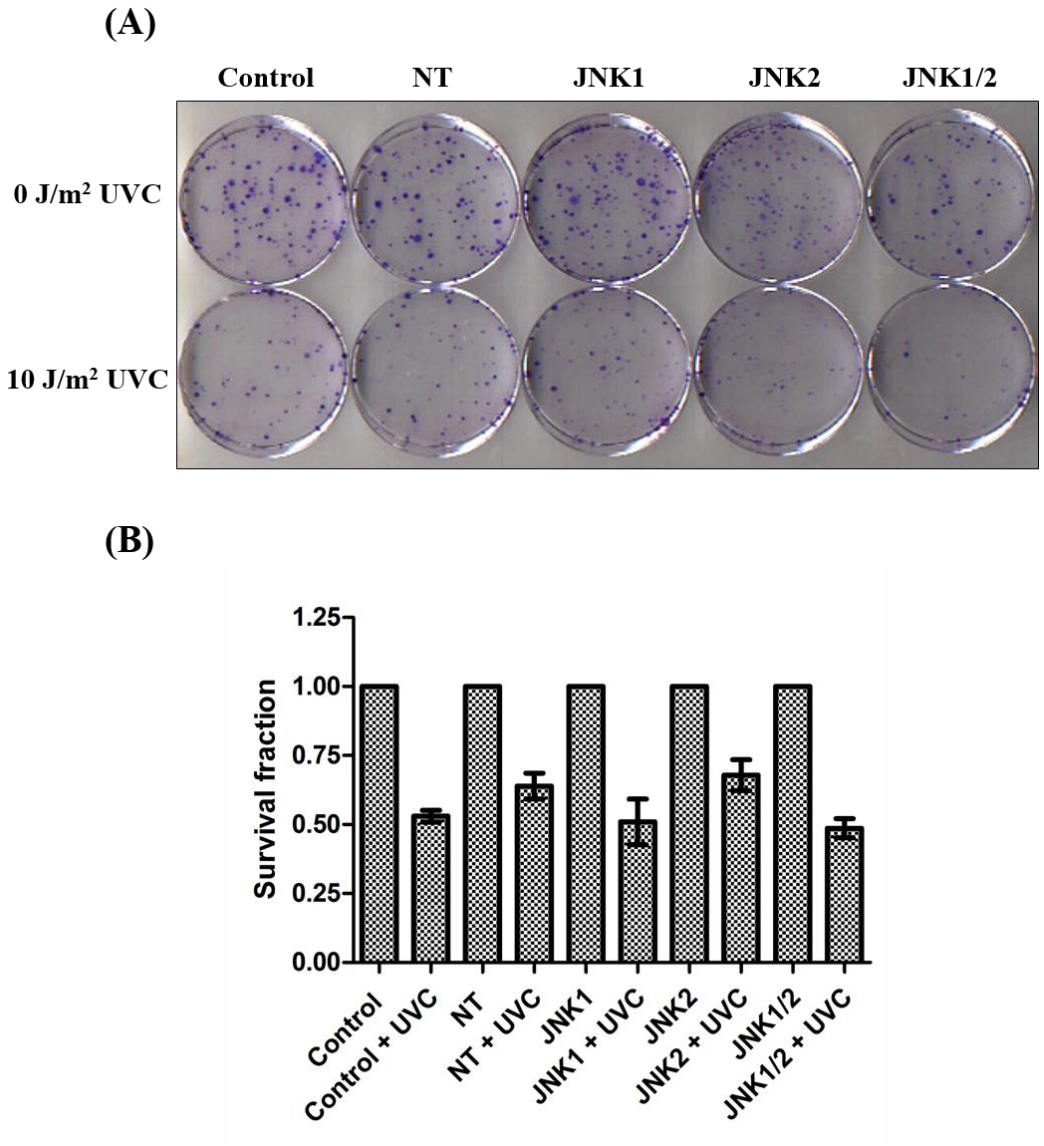


Figure 4.6 UV- induced inhibition of MCF-7 colony survival is independent of JNK

Cells from control, NT, JNK1, JNK2 and JNK1/2 cell lines were treated for a clonogenic assay as stated in methods section 2.2.8. Results show (A) Representative image of colony formation in control and treated cells for each cell line. (B) Survival fraction of each cell line after treatment with UV. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

4.2.1.3 The effect of JNK isoform knockdown on Taxol - induced cell death

After confirming that JNK could be activated in MCF-7 cells by UV radiation, the effect of JNK knockdown on MCF-7 cell death was investigated further by using a different simulant. As mentioned previously, Taxol is an anti-cancer drug used for the treatment of breast cancer and JNK has been demonstrated to play a role in Taxol - induced cell death (Kolomeichuk *et al.*, 2008).

To begin, a preliminary experiment was carried out to determine the optimum concentration of Taxol required to activate the JNK pathway and cell death. MCF-7 cells were treated with either DMSO or Taxol for 24 hours and samples were analysed by Western blot (Figure 4.7A). Levels of pJNK were analysed to confirm JNK activation and cleaved PARP was used to confirm activation of cell death. Levels of pJNK (54kDa) were increased after treatment with 10, 20 and 30nM of Taxol by approximately 2 fold and after treatment with 50nM this increased to 4.6 fold (10nM (2.19 ± 0.4), 20nM (2.68 ±0.7), 30nM (2.06 ± 0.7) and 50nM (4.61 ± 2.6). A similar trend was observed for the 46kDa variant of pJNK with a fold stimulation of approximately 2 for all concentrations of Taxol (10nM (1.95 ± 0.4), 20nM (2.24±0.5), 30nM (2.27±0.4) and 50nM (2.73±0.9)). This confirms that both JNK1 and JNK2 are activated by treatment with Taxol.

All four concentrations of Taxol induced cell death in MCF-7 cells, represented by a large increase in cleaved PARP expression. Cleaved PARP levels increased by 46.34 fold ± 12.0 in cells treated with 10nM of Taxol, this increased further and became significant in cells treated with 20nM, 30nM and 50nM to 71.46 fold ± 21.9, 98.94 ± 23.6 and 100.2 ± 18.5 respectively. Combined these results confirm that Taxol activates JNK signalling in MCF-7 cells and also induces cell death.

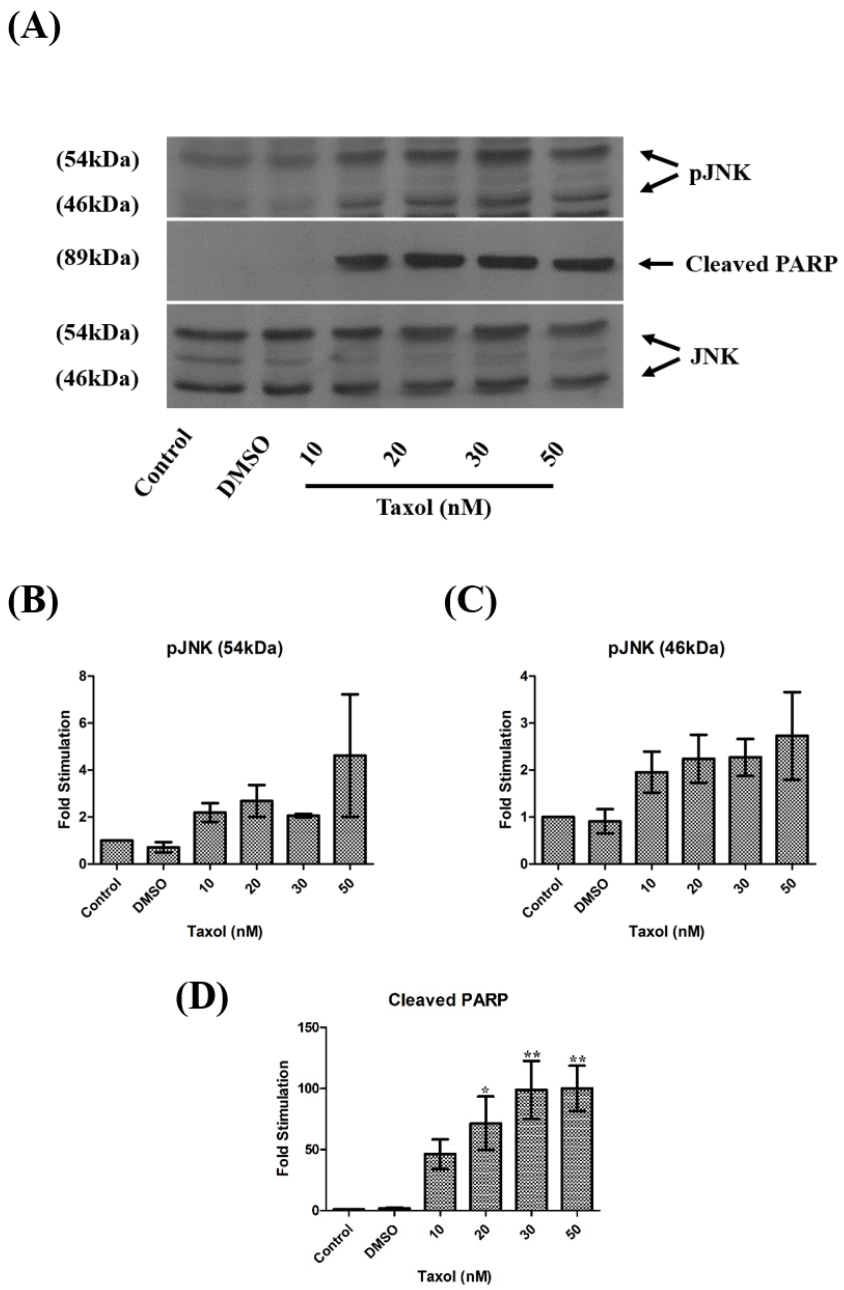


Figure 4.7 Activation of JNK and apoptosis is concentration dependent

MCF-7 cells were seeded into 6 well plates and treated with either media alone, DMSO, 10, 20, 30 or 50nM of Taxol for 24 hours. Results show (A) Western blot of pJNK, cleaved PARP and total JNK was used to confirm equal loading. Expression levels were semi quantified using densitometry and presented as a fold stimulation relative to the control for (B) pJNK (54kDa), (C) pJNK 46 (kDa) and (D) cleaved PARP. Data represents the mean ± SEM of n=3. Data was analysed using a one-way ANOVA test, *P<0.05, **P<0.01.

To investigate the effects of JNK knockdown on Taxol - induced cell death, control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded at equal densities and treated with either DMSO as a control or 20nM of Taxol for 24 hours. Samples were collected and analysed by Western blot (Figure 4.8A). Levels of JNK1 and JNK2 were analysed to confirm knockdown and GAPDH was used to confirm equal loading. Levels of pJNK increased by approximately 5-fold in all cells (Figure 4.8 B-C). Again, cells with JNK1 and JNK1/2 knockdown had lower levels of the 46kDa variant of pJNK (Figure 4.8C) whilst the JNK2 and JNK1/2 knockdown lines had lower levels of the 54kDa variant (Figure 4.8B). This confirms that Taxol activates both JNK1 and JNK2 to a similar level. No significant difference was observed between control and NT cell lines, confirming that lentiviral infection did not cause any major off target effects.

Interestingly, cell death occurred in all five cell lines with levels of cleaved PARP increasing to 26.20 fold ± 10.6, 32.78 fold ± 12.1, 25.15 fold ± 1.4, 19.98 fold ± 9.4 and 28.62 fold ± 6.6 for control, NT, JNK1, JNK2 and JNK1/2 cells respectively. There was no significant difference in levels of cleaved PARP between control and NT cell lines confirming that Taxol can successfully induce cell death in both control and infected cells. Similar to the levels of cleaved PARP produced by UV exposure, no significant difference was observed between cells containing knockdown, again suggesting that Taxol - induced cell death may be independent of JNK in MCF-7 cells.

To analyse MCF-7 cell death another way, an MTT assay was carried out to investigate the effect of JNK knockdown on cell viability following treatment with Taxol. To determine whether Taxol would cause a decrease in cell viability, a preliminary experiment was carried out using a range of concentrations from 5nM – 300nM of Taxol to treat cells for 24 hours (Figure 4.9). After treatment, an MTT assay was carried out as mentioned in methods section 2.2.6. Treatment with all concentrations of Taxol caused a significant decrease in the percentage of cell viability when compared to control cells, while treatment with DMSO showed no significant decrease. The % viability showed a concentration dependent decrease which ranged from 72.52% ± 5.7 at 5nM to 53.18% ± 7.3 at 300nM treatment. Since all concentrations produced a significant decrease in % cell viability, three central

concentrations 10, 20 and 50nM which caused a decrease of 65.68% ± 4.9, 62.71% ± 6.5 and 59.32% ± 6.1 respectively, were selected for the knockdown experiments.

To investigate the effects of JNK isoform knockdown on the decrease in cell viability caused by Taxol. Cells from control, NT, JNK1, JNK2 and JNK1/2 cells lines were seeded at equal densities into 96 well plates and treated with either media alone, DMSO, Taxol or H_2O_2 alone for 24 and 48 hours and then a MTT assay was carried out. As expected all three concentrations successfully produced a decrease in % cell viability at 24 hours in control and NT cell lines (Figure 4.10A) which decreased further after 48 hours (Figure 4.10B). When comparing this between the cell lines containing knockdown, there was no significant difference in % cell viability between the different cell lines after 24 or 48 hours' treatment, suggesting that the reduction in cell viability induced by Taxol was independent of JNK. This result links with the Western blot results which also suggested that Taxol - induced cell death was independent of JNK signalling in MCF-7 cells.

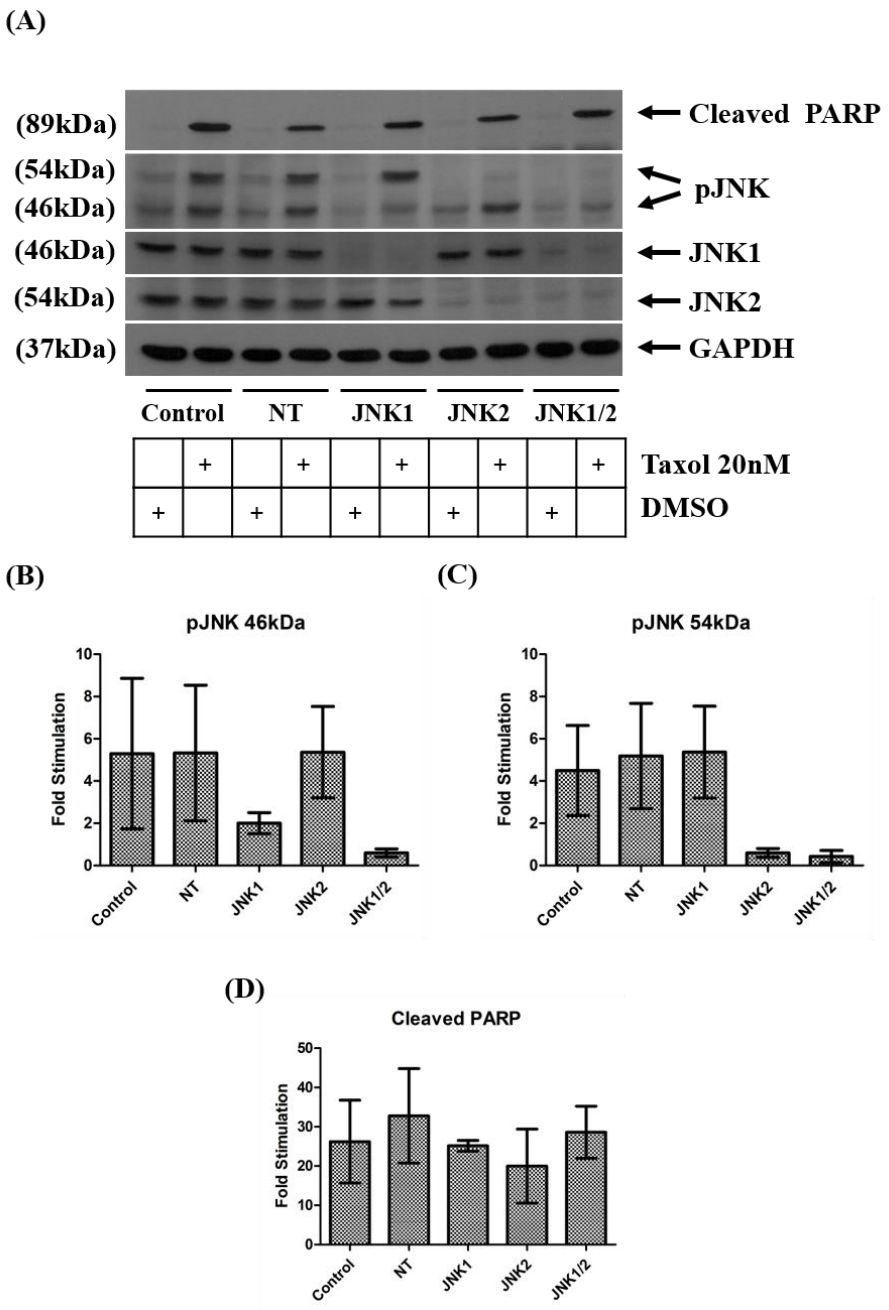


Figure 4.8 Taxol - induced PARP cleavage is independent of JNK

Control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded into 6 well plates and treated with either DMSO or Taxol for 24 hours. Samples were collected and analysed by Western blot. Results show (A) Western blot of pJNK, cleaved PARP, JNK1, JNK2 and GAPDH. Blots were semi quantified by densitometry and expressed as a fold stimulation relative to control for (B) pJNK (46kDa), (C) pJNK (54kDa) and (D) cleaved PARP. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

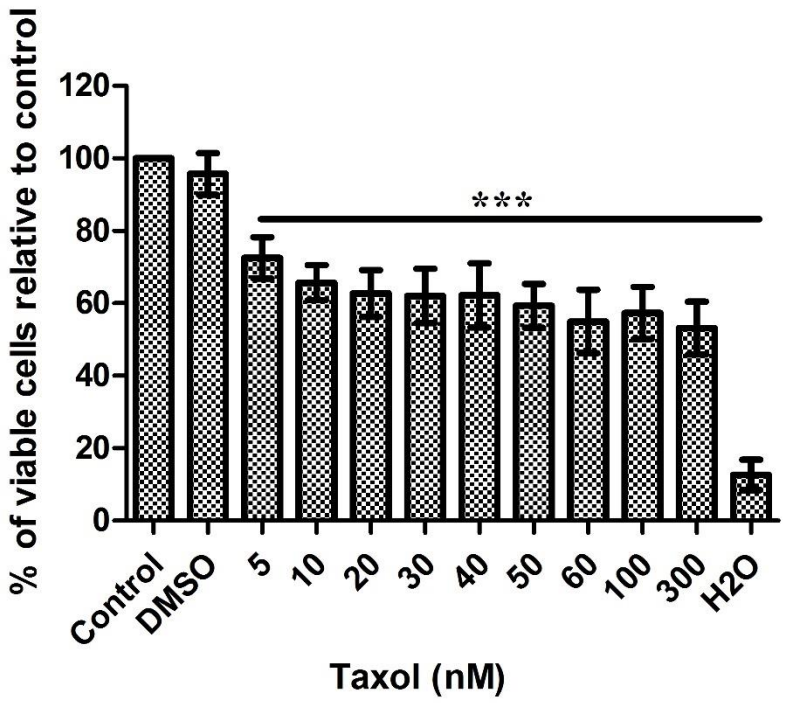
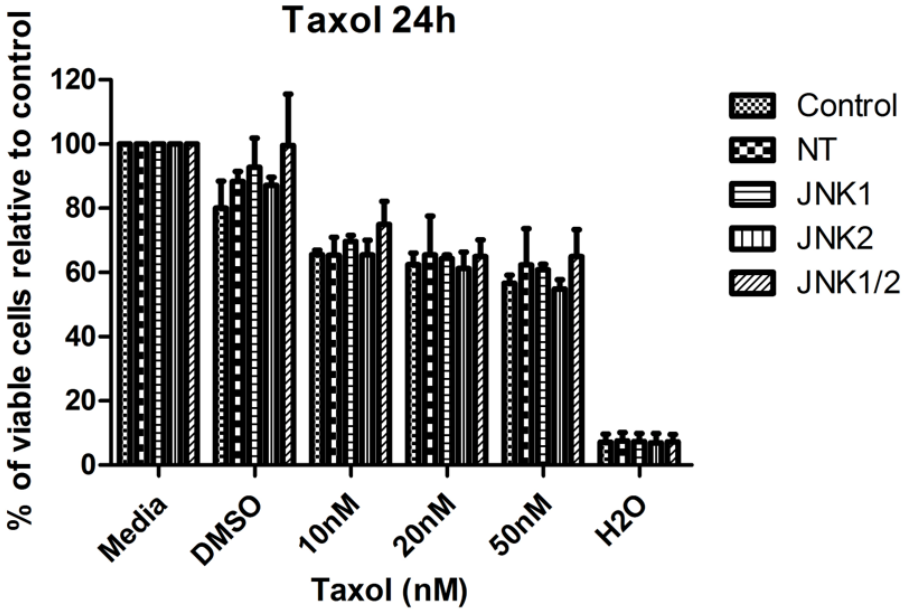


Figure 4.9 Treatment with Taxol decreases cell viability

MCF-7 cells were seeded into a 96 well plates and treated with either media alone, DMSO, 5-300nM of Taxol or H_2O alone for 24 hours. An MTT assay was carried out as mentioned in Methods Section 2.2.6. Results show % of viable cells relative to control. Data represents the mean ± SEM n=3. Data was analysed using a one-way ANOVA test, ***P<0.001.

(A)



(B)

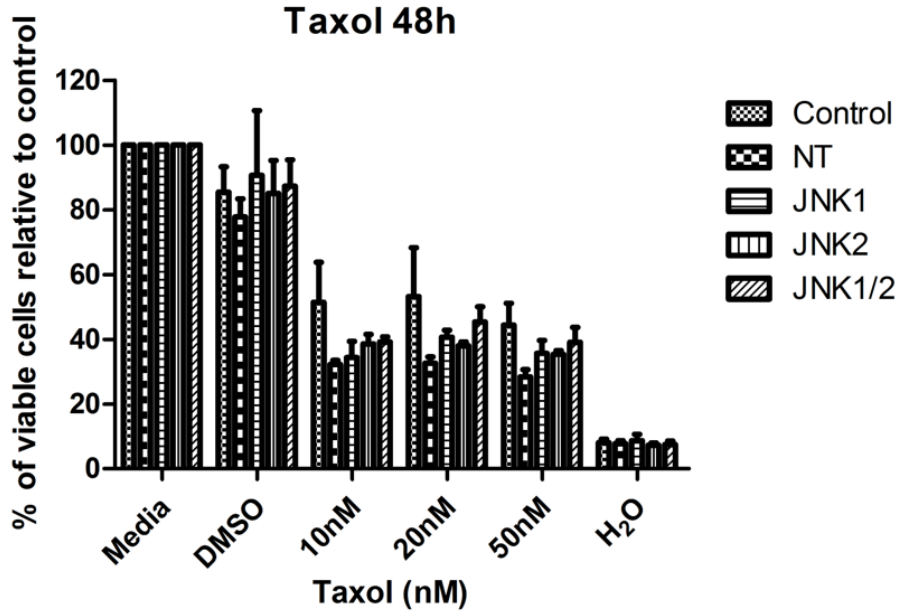


Figure 4.10 Decrease in cell viability produced by Taxol is JNK independent

Control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded at an equal density into 96 well plates and treated with media alone, DMSO, 10, 20 or 50nM of Taxol or H_2O alone for 24 or 48 hours. Plates were then analysed by MTT. Results show the % viability relative to control (media) for each cell line after (A) 24 hours and (B) 48 hours of treatment with Taxol. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a two-way ANOVA test.

Finally, to examine Taxol - induced apoptosis in MCF-7 cells, FACS was used (methods section 2.2.4.1) to analyse the percentage of apoptotic cells after treatment with Taxol. Control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded treated with either DMSO, Taxol (20nM) or H_2O_2 (500µM) for 24 or 48 hours. Cells were analysed by FACS and results were presented as the number of annexin V positive cells as a % of total events for each condition (Figure 4.11 and 4.12).

H_2O_2 was used as a positive control where an increase of approximately 25-30 % of annexin V positive cells was observed across all cell lines, confirming apoptotic cells were clearly detected (Figure 4.11C). After 24 hours of Taxol, there was a small increase of approximately 2-3 % of annexin V positive cells across all 5 cell lines, when compared to the DMSO control for each cell line (Figure 4.11B). Again, no significant difference was observed between control and NT cell lines, demonstrating that apoptosis can be induced in both infected and non-infected cells. This % of annexin V positive cells increased further after 48h of treatment to around 10-15 % of total cells, however similar to the other experiments, there was no significant difference in the % of apoptotic events between cell lines (control (29.03 ± 5.5), NT (30.47 ± 4.8), JNK1 (30.5 ± 7.3), JNK2 (26.7 ± 3.6) and JNK1/2 (25.4 ± 5.6) (Figure 4.12). Combined with the Western blot and MTT results, these findings suggest that JNK signalling is not required for Taxol - induced cell death in MCF-7 cells.

Although knockdown of JNK had no effect on Taxol - induced cell death, whether JNK played a role in the cell cycle arrest induced by Taxol was still unknown. FACS analysis (methods section 2.2.4.2) was used to investigate the effects of JNK isoform knockdown on cell cycle arrest. To confirm Taxol caused cell cycle arrest in MCF-7 cells, a preliminary experiment was carried out which demonstrated that 20nM of Taxol produced a clear decrease in the % of cells in the G1 phase of the cell cycle (64.67 ± 2.1 to 38.67 ± 5.8) and an increase in the G2/M phase (13.43 ± 1.8 to 30.07 ± 6.3) when compared to the DMSO control (Figure 4.13). This data confirmed that Taxol was causing cell cycle arrest in MCF-7 cells.

To investigate the effects of JNK knockdown, control. NT, JNK1, JNK2 and JNK1/2 cell lines were treated with either DMSO or 20nM of Taxol for 24h. FACS analysis was carried out as stated in methods section 2.2.4.2 and results were presented as a %

of total events for each phase of the cell cycle (Figure 4.14). Similar to the preliminary experiment, after treatment with Taxol, the % of cells in the G1 and S phases of the cell cycle decreased by approximately 40% and 8% respectively (Figure 4.14A-B). While the % of cells in the G2/M and Sub G1 phases increased by around 33% and 12% (Figure 4.14C-D). These values demonstrate that Taxol effectively induces cell cycle arrest in both infected and non-infected MCF-7 cells. Interestingly JNK knockdown did not cause any changes in the cell cycle arrest produced by Taxol, suggesting that JNK does not play a role in the cell cycle arrest induced by Taxol.

Taken together these findings demonstrate that UV - induced cell death and the cell cycle arrest and cell death induced by Taxol are independent of JNK signalling in MCF-7 cells.

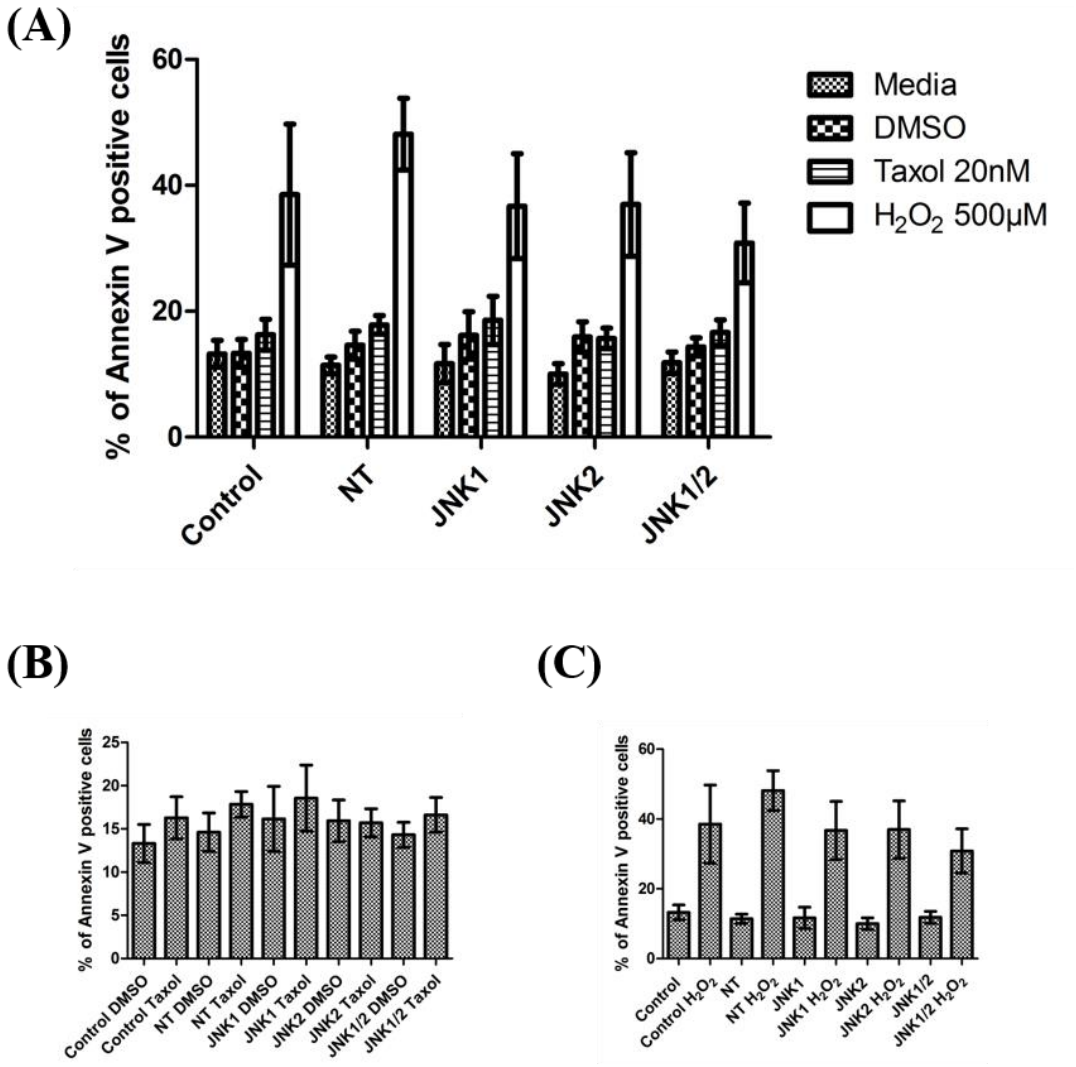


Figure 4.11 Taxol - induced apoptosis at 24 hours is independent of JNK

Cells from control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded into 6 well plates and treated with either DMSO, 20nM of Taxol or 500µM of H_2O_2 for 24 hours. Cells were analysed by FACS analysis (methods 2.2.4.1). Results show % of annexin V positive cells after 24 hours of treatment with (A-B) Taxol (A-C) H_2O_2. Data represents mean ± SEM of 5 individual experiments. Data was analysed using a one-way ANOVA test.

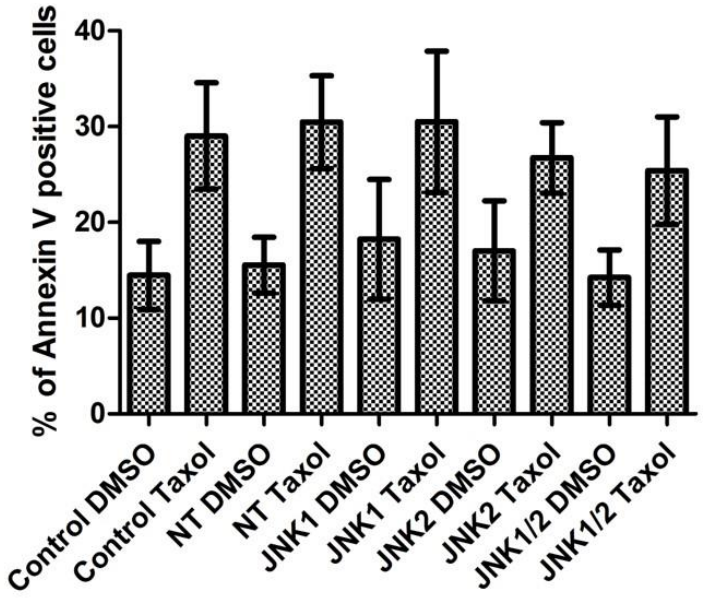


Figure 4.12 Taxol - induced apoptosis at 48 hours is independent of JNK

Cells from control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded into 6 well plates and treated with either DMSO or 20nM of Taxol for 48 hours. Cells were analysed by FACS analysis (methods 2.2.4.1). Results show % of annexin V positive cells after 48 hours of treatment with Taxol. Data represents mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

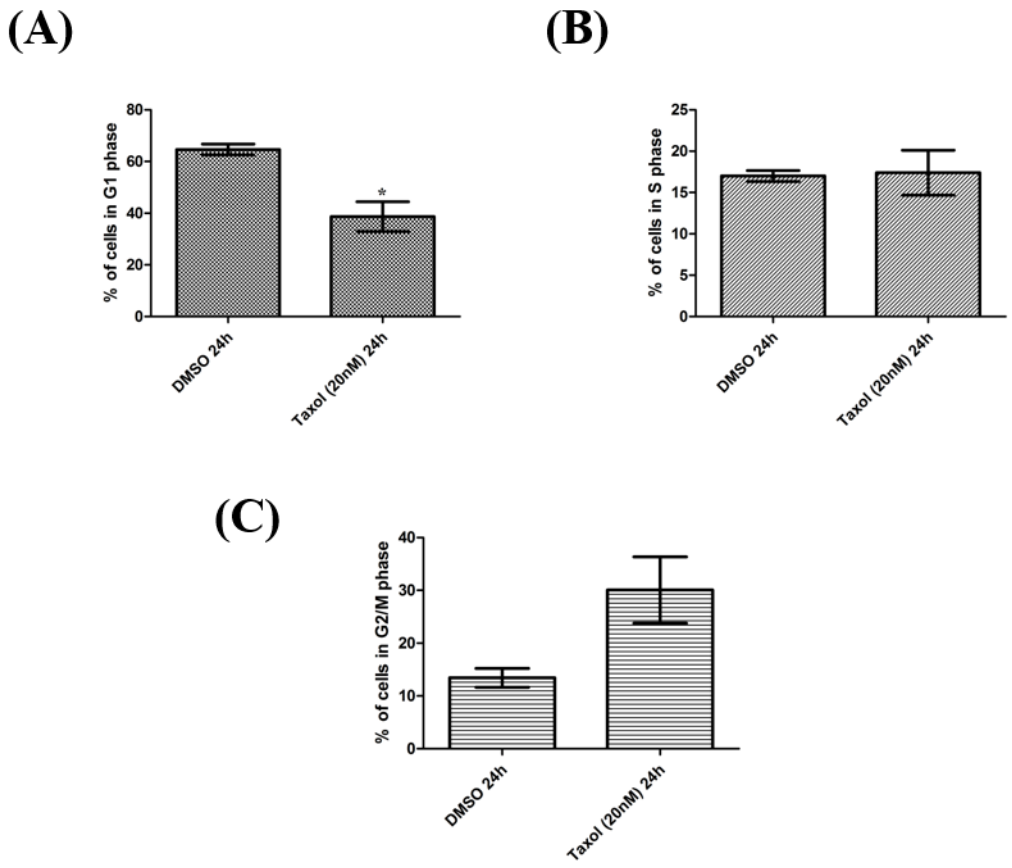


Figure 4.13 Taxol - induced G2/M cell cycle arrest in MCF-7 cells

MCF-7 cells were treated with either DMSO or Taxol (20nM) for 24 hours and analysed by FACS analysis (methods 2.2.4.2). Results show the percentage of total events in (A) G1 phase, (B) S phase and (C) G2/M phase of the cell cycle for each condition. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test, *P<0.05.

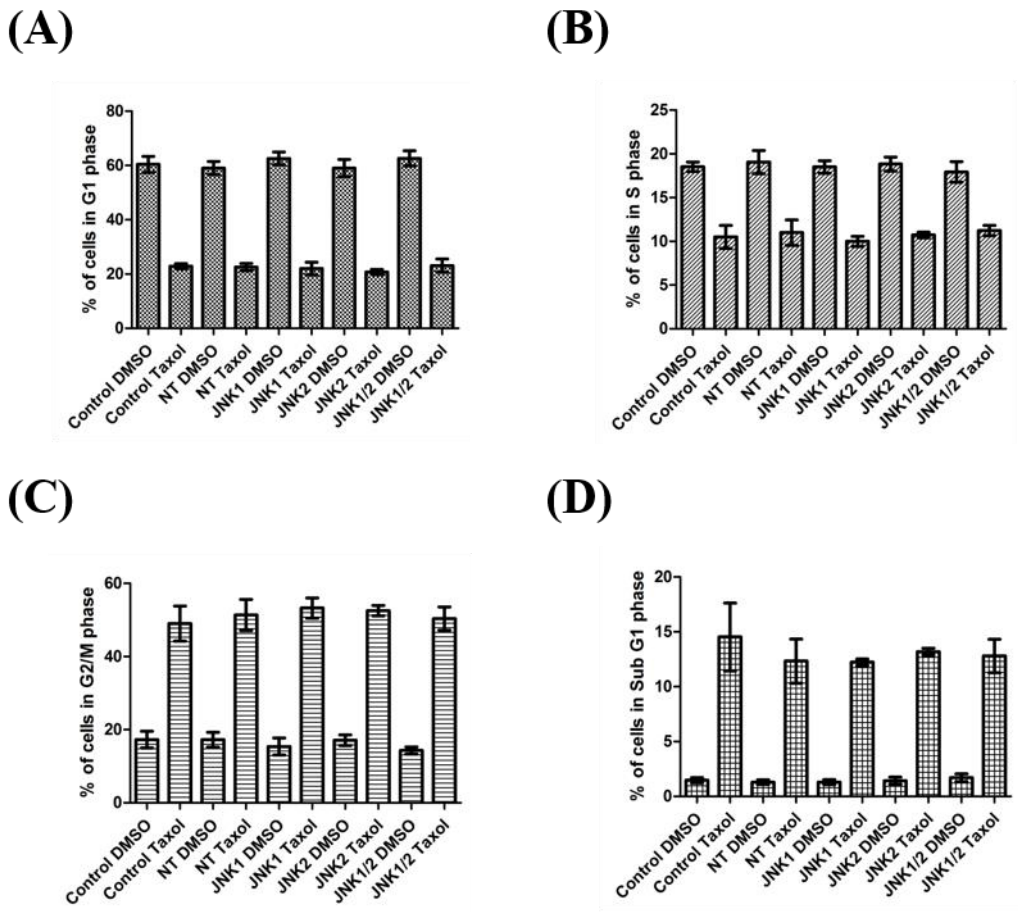


Figure 4.14 Taxol - induced cell cycle arrest at 24h is independent of JNK

Control, NT, JNK1, JNK2 and JNK1/2 cells were treated with either DMSO or Taxol (20nM) for 24 hours and analysed by FACS analysis (methods 2.2.4.2). Results show the percentage of total events in (A) G1 phase, (B) S phase (C) G2/M phase and (D) Sub G1 phase of the cell cycle for each condition. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

4.2.2 JNK isoform function in MCF-7 cell growth

4.2.2.1 The effects of JNK knockdown on the activation of JNK substrates

To begin the investigation into the role of JNK isoforms in MCF-7 cell growth, the effects of JNK knockdown on JNK signalling itself was first examined. As mentioned in chapter one, the transcription factors and downstream targets of JNK ATF2 and c-Jun have both been shown to play a role in the growth and progression of cancer cells. To investigate the role of JNK in the activation of these proteins in MCF-7 cells, FCS was used to activate JNK signalling and Western blotting was used to analyse the levels of pATF2 and pc-Jun.

To determine the best concentration of FCS to use, a concentration curve was carried out where MCF-7 cells were seeded into 6 well plates and starved for 24 hours. Cells were then treated with media containing 0 - 50% FCS for 1 hour and samples were collected and analysed by Western blot for pJNK, pc-Jun and total JNK (Figure 4.15A). Control cells were maintained in complete media to determine any effects caused by the starvation of cells.

Levels of protein were semi-quantified using densitometry and presented as a fold stimulation relative to control cells. Starvation caused no significant difference in protein levels when compared to control cells (Figure 4.15B-D). Interestingly pJNK levels increased to a similar level after treatment with all concentrations of FCS, with the 54kDa variant producing an increase just over 10 fold (Figure 4.15B) and the 46kDa variant around 5 fold (Figure 4.15C). Although there was a clear increase in pJNK 54kDa, this was not statistically significant due to variations in expression between experiments. However, the increase in pJNK 46kDa was significant for all concentrations of FCS with increases of 4.97 ± 0.5, 5.59 ± 0.5, 5.05 ± 0.5, 4.72 ± 0.2, 5.50 ± 0.2 and 5,47 ± 0.4 for treatment with 5, 10, 20, 30, 40 and 50% FCS respectively.

Expression of pc-Jun also increased after treatment with all concentrations of FCS, however, this increase was more concentration dependent. Expression levels increased by approximately 21 fold after treatment with 5% FCS to 53 fold with 50% FCS. This data confirms that JNK signalling is activated by FCS in MCF-7 cells, the concentration of 10% FCS was selected to be used for future experiments as it achieved

good activation of the pathway (28.70 fold ± 9.8) and is the same concentration of FCS used in the complete media used to maintain the cells.

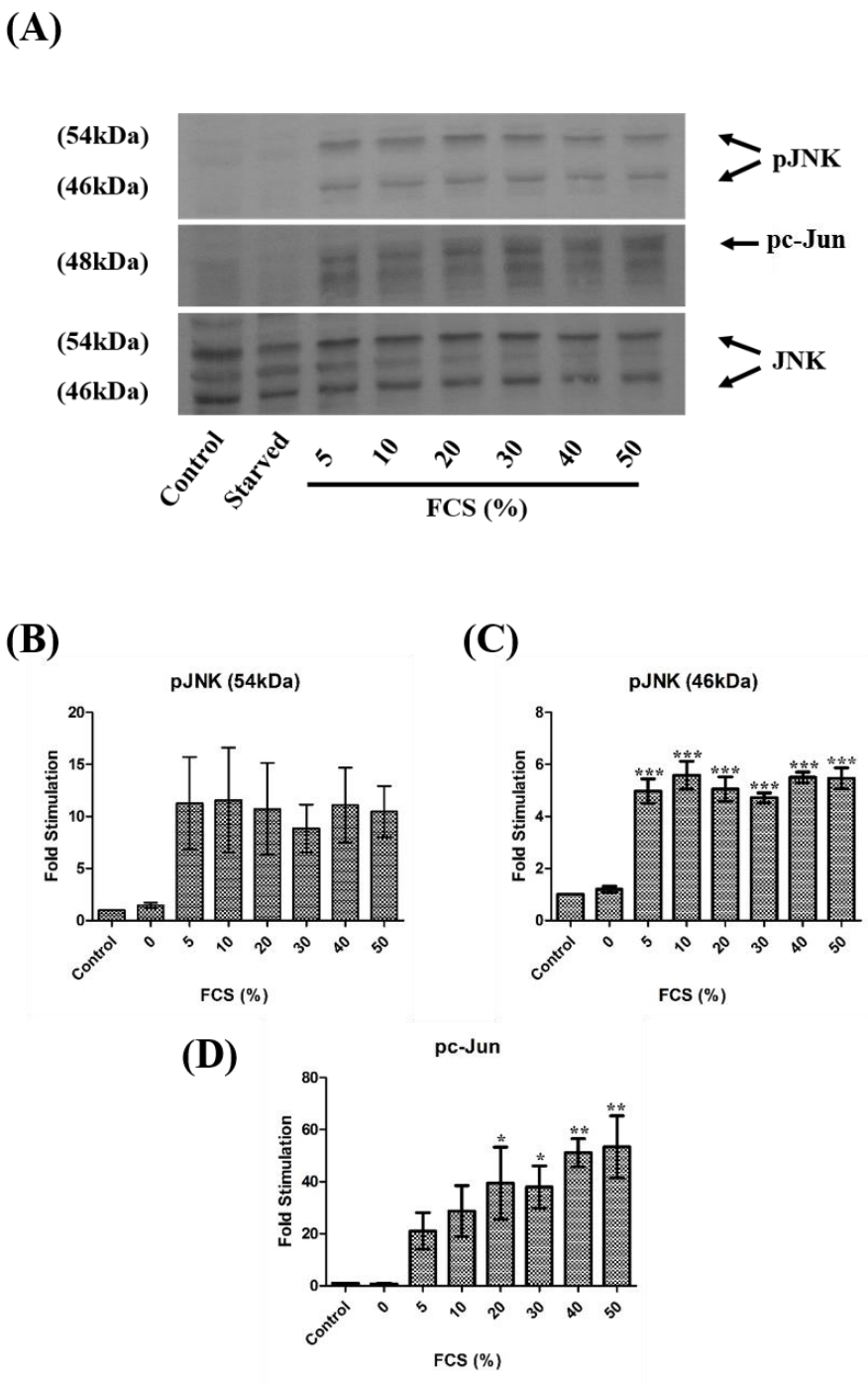


Figure 4.15 Stimulation with FCS activates JNK signalling

MCF-7 cells were starved for 24 hours and then stimulated with 0, 5, 10, 20, 30, 40 or 50% FCS for 1 hour. Control cells were in complete media for the full experiment. Results show (A) Western blot of pJNK, pc-Jun and total JNK. Expression levels were semi quantified by densitometry and presents as a fold stimulation of the control for (B) pJNK 54kDa, (C) pJNK (46kDa) and (D) pc-Jun. Data represents the mean ± SEM for 3 individual experiments. Data was analysed using a one-way ANOVA test, *P<0.05, **P<0.01, ***P<0.001.

To determine whether 1 hour of treatment with FCS was the optimum stimulation time to activate JNK signalling, a time course was carried out using 10% FCS. MCF-7 cells were starved for 24 hours and then stimulated with 10% FCS for time points ranging between 0 - 6 hours and then samples were collected and analysed for pJNK, pc-Jun and total JNK using Western blotting (Figure 4.16A). Control cells were maintained in complete media to observe any effects caused by starvation. As expected, no statistical difference was observed between control and starved cells (Figure 4.16B-D). Levels of pJNK and pc-Jun were semi quantified using densitometry and presented as a fold stimulation relative to control cells.

Phosphorylation of JNK and c-Jun occurred after 15 minutes and sustained activation was observed over the 6 hours (Figure 4.16B-D). Expression levels of pJNK 54kDa increased by around 5-10 fold after stimulation with FCS, however again due to variation between experiments this was not deemed significant (Figure 4.16B). Levels of pJNK 46kDa produced a slightly lower increase of approximately 3-4 fold which was significant at 0.5, 1 and 3 hours (0.5 (4.05 ± 0.5), 1 (3.5 ± 0.6), 3 (4.0 ± 0.8)) (Figure 4.16C). Finally, FCS produced an increase in pc-Jun expression levels between 13 fold and 53 fold over the 6 hours of treatment (Figure 4.16D). Although pc-Jun levels peaked at 3 hours of stimulation (53.66 fold ± 28), 1 hour (39.95 fold ± 17.0) of stimulation was selected for knockdown experiments to allow good activation of both pJNK and pc-Jun.

To investigate the effects of JNK1 and JNK2 knockdown on the activation of JNK substrates, control, NT, JNK1, JNK2 and JNK1/2 cells were starved for 24 hours, treated with 10% FCS for one hour and then samples were collected and analysed for pc-Jun, pATF2, JNK1, JNK2 and GAPDH (Figure 4.17A). Levels of JNK1 and JNK2 were analysed to confirm knockdown and GAPDH was used to confirm equal loading. Levels of protein were semi quantified using densitometry and presented as a fold stimulation relative to the control for each cell line.

Phosphorylation of c-Jun occurred in all 5 cell lines with a similar increase of around just over 1 fold (Figure 4.17B). Oddly this was consistently a lot lower when compared to the preliminary experiments, possibly due to higher background levels. Both c-Jun and ATF2 were phosphorylated in control and NT cell lines confirming this

stimulation worked in lentiviral infected cells. When comparing the activation of c-Jun between all of the cell lines there was no significant difference between NT and knockdown lines, suggesting that the activation of c-Jun may not be dependent on JNK. Similar findings were observed in the pATF2 levels, where although levels of ATF2 did fluctuate between the cell lines, these differences were not significant when compared to NT (control (0.74 fold ± 0.2), NT (1.90 fold ± 0.1), JNK1 (2.53 fold ± 0.4), JNK2 (1.61 fold ± 0.4) and JNK1/2 (3.31 fold ± 0.8) (Figure 4.17C).

Taken together these results suggest that phosphorylation of ATF2 and c-Jun in MCF-7 cells does not depend on activation by JNK.

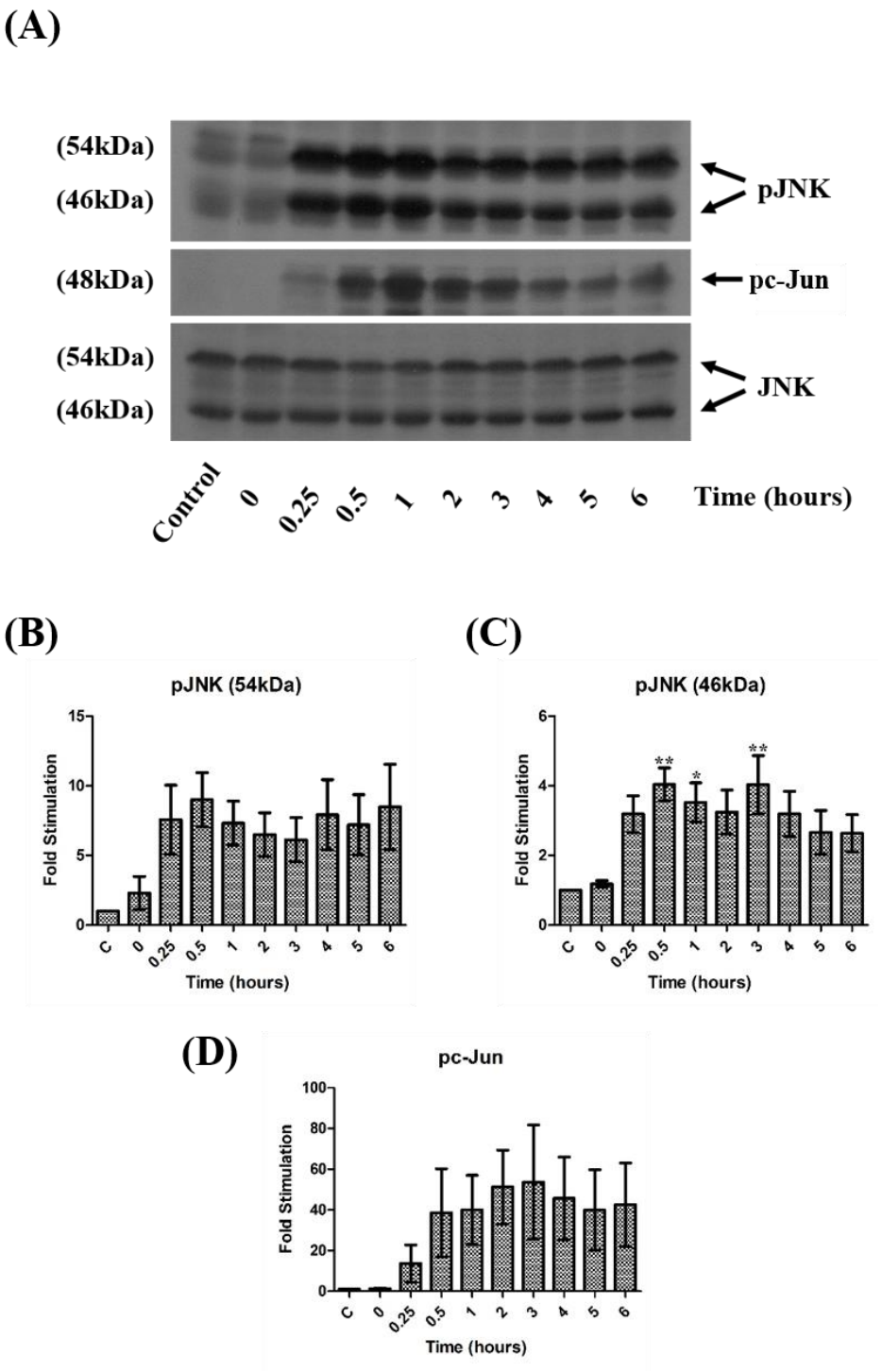


Figure 4.16 FCS induces early JNK activation

MCF-7 cells were starved for 24 hours and then stimulated with 10% FCS for 0-6 hours. Control cells were in complete media for the full experiment. Results show (A) Western blot of pJNK, pc-Jun and total JNK. Expression levels were semi quantified by densitometry and presents as a fold stimulation of the control for (B) pJNK 54kDa, (C) pJNK (46kDa) and (D) pc-Jun. Data represents the mean ± SEM for 3 individual experiments. Data was analysed using a one-way ANOVA test, *P<0.05, **P<0.0.

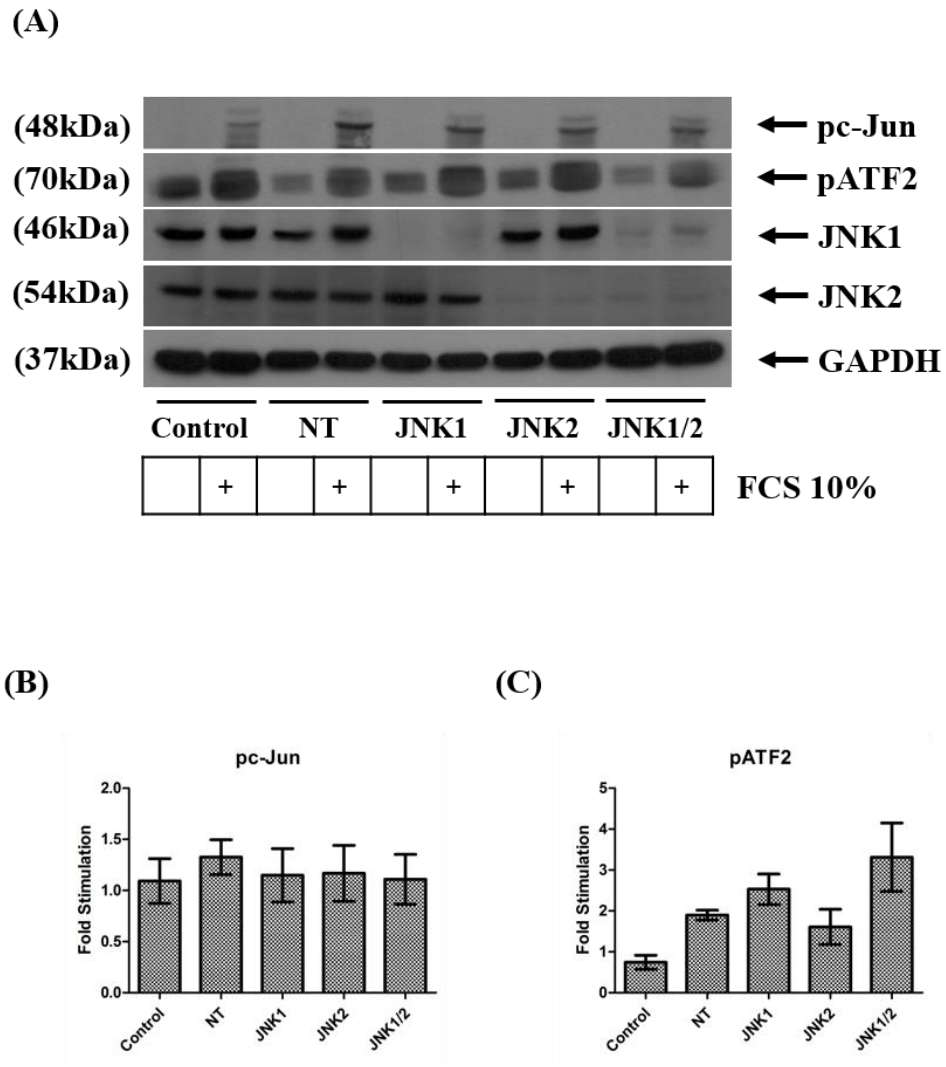


Figure 4.17 Activation of c-Jun and ATF2 was not affected by JNK knockdown

Cells from control, NT, JNK1, JNK2 and JNK1/2 cell lines were starved for 24 hours and stimulated with 10% FCS for 1 hour. Results show (A) Western blot of pc-Jun, pATF2, JNK1, JNK2 and GAPDH was used to confirm equal loading. Expression levels were semi quantified using densitometry and presented as a fold stimulation of the control for each cell line for (B) pc-Jun and (C) pATF2. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

4.2.2.2 The effects of JNK knockdown on cell cycle progression and cell growth

As mentioned previously, JNK has been shown to be important for cell cycle progression, however whether JNK plays a role in cell cycle progression in MCF-7 cells is still unknown. To determine whether cell cycle progression could be induced and analysed in MCF-7 cells a preliminary experiment was carried out. MCF-7 cells were seeded starved for 24 hours, replenished with complete media for either 24 or 48 hours and then analysed by FACS (methods section 2.2.4.2). Cell were gated and presented as a percentage of total events in the G1, S and G2/M phase of the cell cycle (Figure 4.18).

After cells were replenished with media, the % of events in the G1 phase of the cell cycle decreased by approximately 20% after both 24 and 48 hours (Figure 4.18A). The % of events in the S phase of the cycle increased from 6% to 20% at 24 hours and 15% at 48 hours (Figure 4.18B). Similarly, G2/M events also increased after media replenishment by around 9% at 24 hours and 12% at 48 hours (Figure 4.18C). These results confirm that cell cycle progression can be analysed in MCF-7 cells through starvation and serum replenishment.

To investigate the effects of JNK knockdown on cell cycle progression, the same experiment was carried out using control, NT, JNK1, JNK2 and JNK/2 cell lines for 24 (Figure 4.19) or 48 hours (Figure 4.20). As expected, cell cycle progression was clearly observed in the control and NT cell lines, demonstrating that lentiviral infection did not affect the FACS analysis. At 24 hours the replenishment of media caused a decrease in the % of events in the G1 phase of the cell cycle of around 20% (Figure 4.19A), and an increase in the S and G2/M phases of approximately 20% and 4% respectively (Figure 4.19B-C). After 48 hours, events in the G2/M phase increased by a further 5% (Figure 4.20C), demonstrating clear progression through the cell cycle phases over the 48 hours. When comparing the different cell lines, there was no significant difference caused by JNK knockdown at any stage of cell cycle at 24 or 48 hours, suggesting that JNK does not play a key role in cell cycle progression in MCF-7 cells.

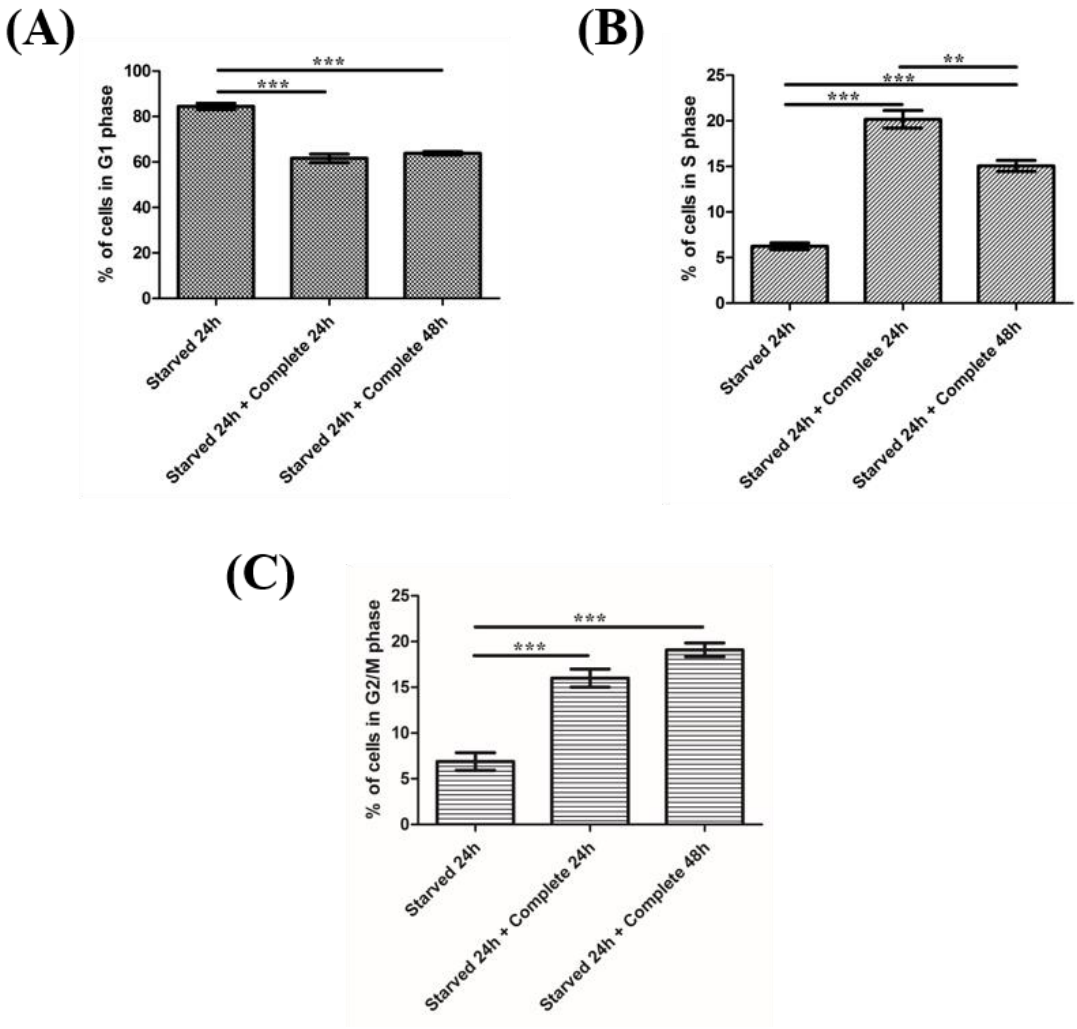


Figure 4.18 Cell cycle progression in MCF-7 cells

MCF-7 cells were seeded into 6 wells plates, starved for 24 hours and then replenished with complete media for 24 or 48 hours. Cells were collected and analysed by FACS analyses (methods 2.2.4.2). Results show the number of cells as a % of total events in the (A) G1 phase, (B) S phase and (C) G2/M phase of the cell cycle. Data represents the mean ± SEM of n=3. Data was analysed using a one-way ANOVA where **P<0.01, ***P<0.001.

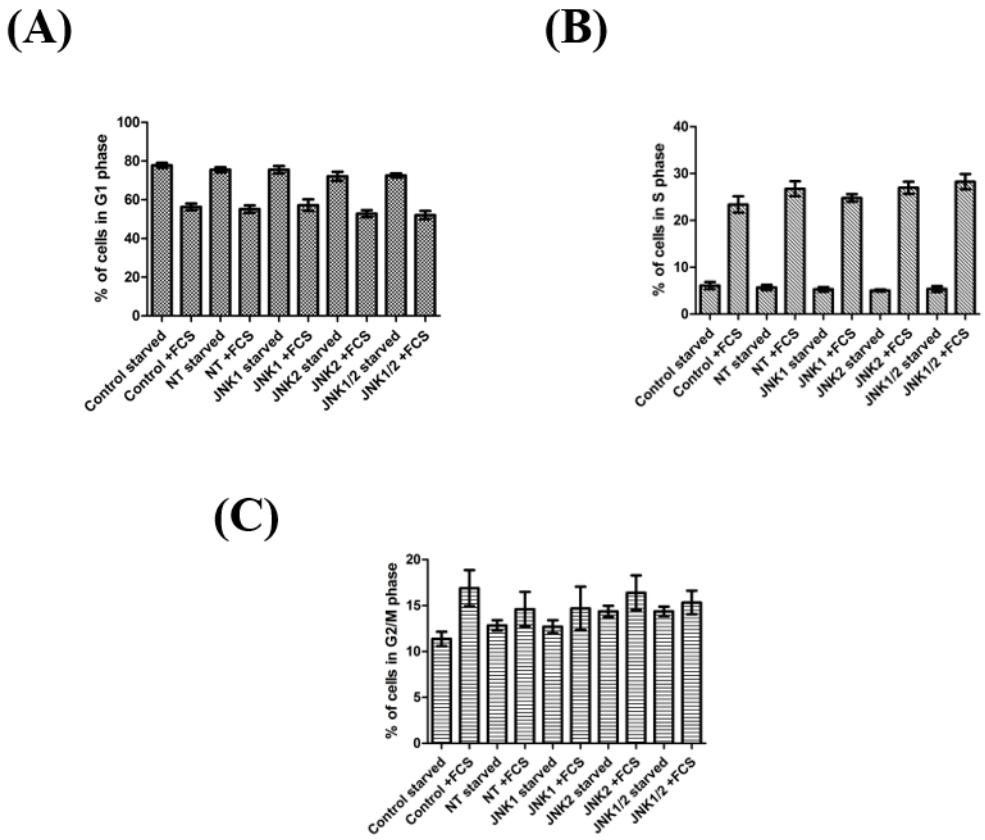


Figure 4.19 FCS induce cell cycle progression at 24 hours is independent of JNK

Control, NT, JNK1, JNK2 and JNK1/2 cells were starved for 24 hours, replenished with complete media for 24 hours and then analysed by FACS analysis (methods 2.2.4.2). Results show the percentage of total events in (A) G1 phase, (B) S phase and (C) G2/M phase of the cell cycle for each condition. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

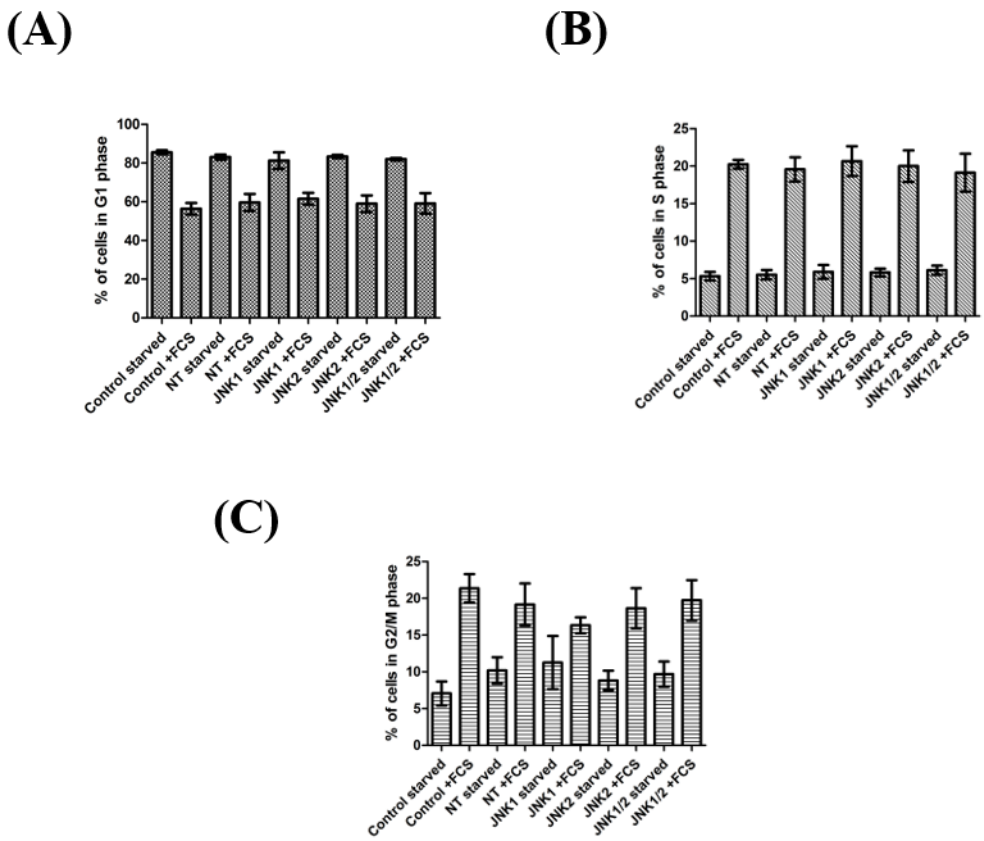


Figure 4.20 FCS induced cell cycle progression at 48 hours is independent of JNK

Control, NT, JNK1, JNK2 and JNK1/2 cells were starved for 24 hours, replenished with complete media for 48 hours and then analysed by FACS analysis (methods 2.2.4.2). Results show the percentage of total events in (A) G1 phase, (B) S phase and (C) G2/M phase of the cell cycle for each condition. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a one-way ANOVA test.

Although JNK did not play a role in cell cycle progression in MCF-7 cells, the effect of JNK knockdown on MCF-7 proliferation was still to be investigated. To examine whether JNK was important for MCF-7 cell proliferation, control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded at equal densities into 96 well plates and grown for 8 days with media replenished on day 5. An MTT assay was carried out on days 2, 4, 6 and 8 to monitor cell proliferation over the 8 days. For each plate, 10 replicates of each cell line was measured, any outliers were excluded and an average value was calculated for each cell line. Over the 8 days the number of cells measured increased steadily, producing an increase of approximately 1 fold from day 2 to day 4, this roughly increased by another 1 fold by day 6 and then a further 3 folds by day 8 (Figure 4.21).

Both control and NT cell lines produced a steady increase in viable cells, with no significant difference between values. Unlike the primary cells used in the previous chapter, MCF-7 cell proliferation was successfully measured in both non-infected and infected cells. Although there were slight variations by day 8 in the number of cells measured for each cell line, none of these differences were significant when compared to NT cells. This confirmed that knockdown of JNK has no significant effect on MCF-7 cell proliferation.

Another way to measure cancer cell growth is through monitoring the growth of multicellular tumour spheroids (Mikhail, Eetezadi and Allen, 2013). To determine whether MCF-7 cells could form spheroids, a preliminary experiment was carried out where MCF-7 cells were seeded at specific densities into non-adherent, round bottomed 96 well plates and grown for 12 days (Figure 4.22). Images were taken every 2 days (Figure 4.22A) and measurements were taken of the sphere diameter to calculate the volume of each sphere each day. The average increase in volume was plotted each day for each density of cells seeded. Densities of 50 – 4000 cells/well-formed successful spheroids, whereas 8000 cells/well was too high a number to produce a round spheroid in the well (Figure 4.22A). Out of all the densities, 4000 cells/well produced the most consistant increase in spheroid growth over the 12 days, which increased steadily from $1.5x10^7$ μm^3 on day 4 to $7.3x10^7$ μm^3 on day 12 (Figure 4.22B), so this density was selected for future experiments.

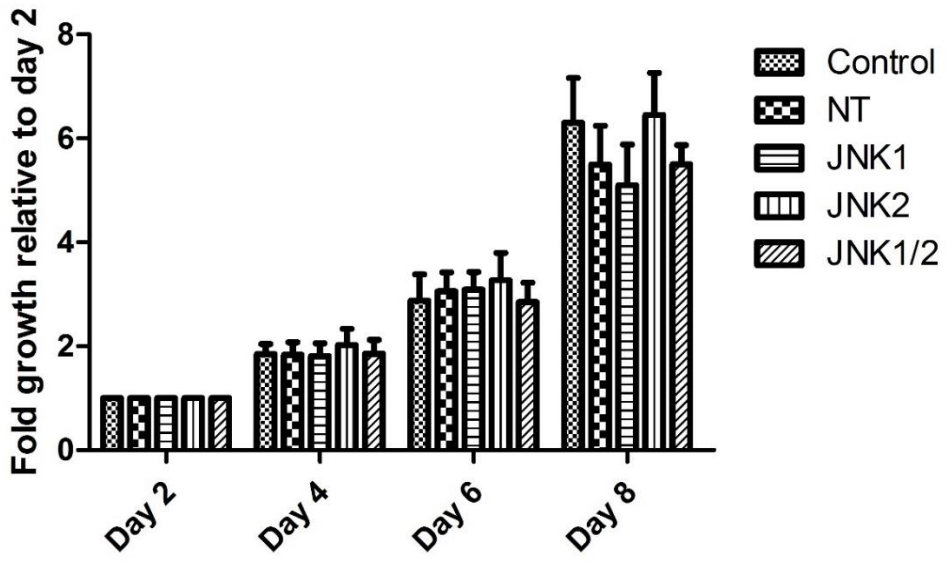


Figure 4.21 MCF-7 cell growth is independent of JNK

Cells from control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded at a density of 10,000 cells/ml in 96 well plates and incubated for 8 days at 37ºC, 5% CO_2, with media replaced on day 5. Cell viability was measured by MTT assay (methods 2.2.6) on days 2, 4, 6 and 8. Data represents the mean ± SEM of 3 individual experiments, where each value was calculated as an average of 10 replicates per plate with outliers excluded. Data was analysed using a two-way ANOVA.

Another way to measure cancer cell growth is through monitoring the growth of multicellular tumour spheroids (Mikhail, Eetezadi and Allen, 2013). To determine whether MCF-7 cells could form spheroids, a preliminary experiment was carried out where MCF-7 cells were seeded at specific densities into non-adherent, round bottomed 96 well plates and grown for 12 days (Figure 4.22). Images were taken every 2 days (Figure 4.22A) and measurements were taken of the sphere diameter to calculate the volume of each sphere each day. The average increase in volume was plotted each day for each density of cells seeded. Densities of 50 – 4000 cells/well-formed successful spheroids, whereas 8000 cells/well was too high a number to produce a round spheroid in the well (Figure 4.22A). Out of all the densities, 4000 cells/well produced the most consistant increase in spheroid growth over the 12 days, which increased steadily from $1.5x10^7$ μm^3 on day 4 to $7.3x10^7$ μm^3 on day 12 (Figure 4.22B), so this density was selected for future experiments.

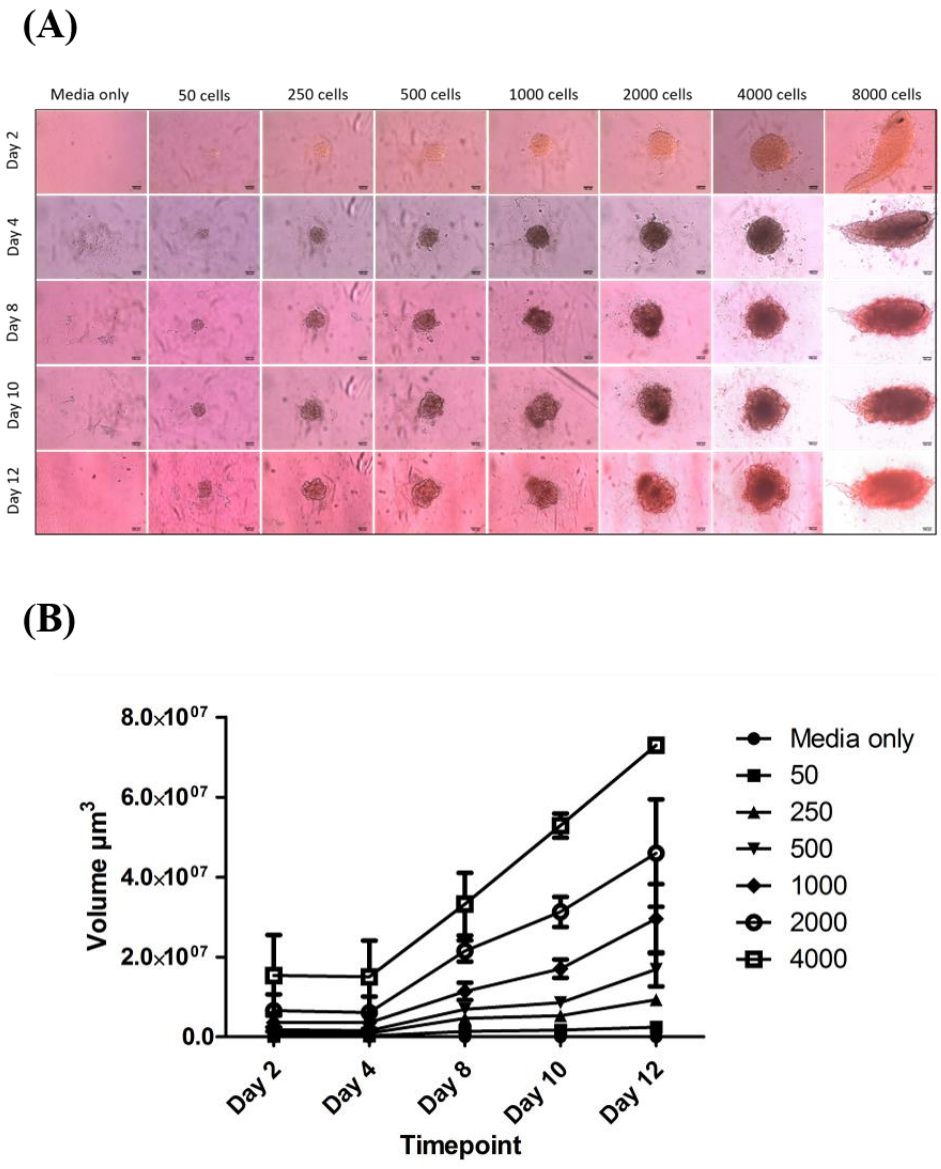


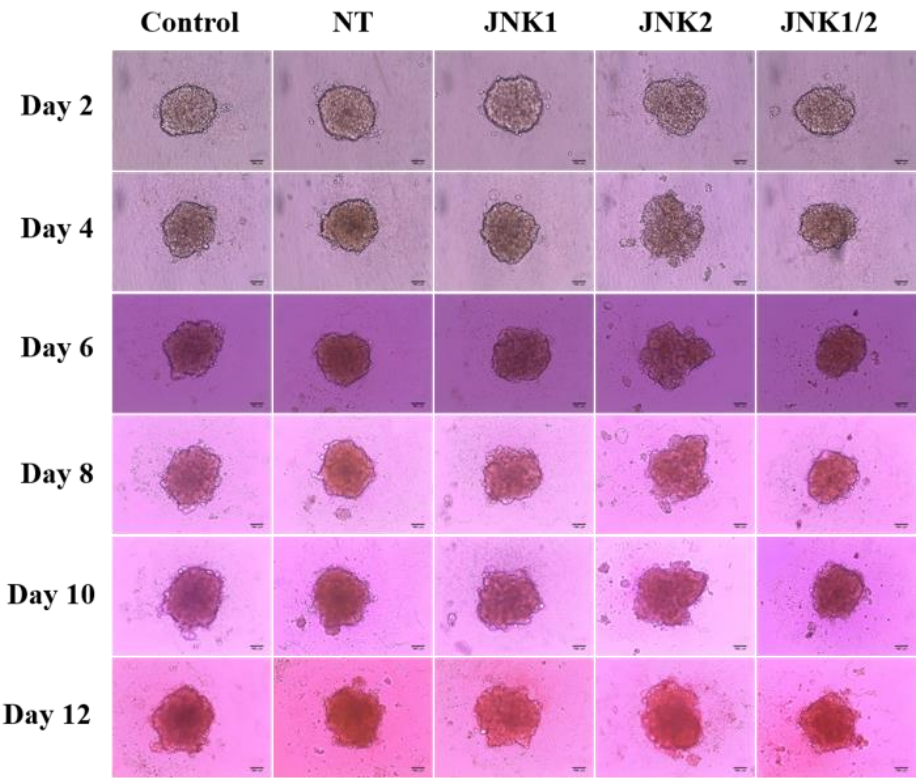
Figure 4.22 MCF-7 spheroid growth correlates with time

MCF-7 cells were plated at densities of 50 – 8000 cells/well in a 96 well non adhesive round bottomed plate. Images of spheroids were taken over the course of 12 days and the volume was calculated. Figure (A) Images of one spheroid at each density followed over 12 days. (B) shows the measured volume of each spheroid monitored over 12 days. Each point represents the average of three spheroids per condition on each day, n=1.

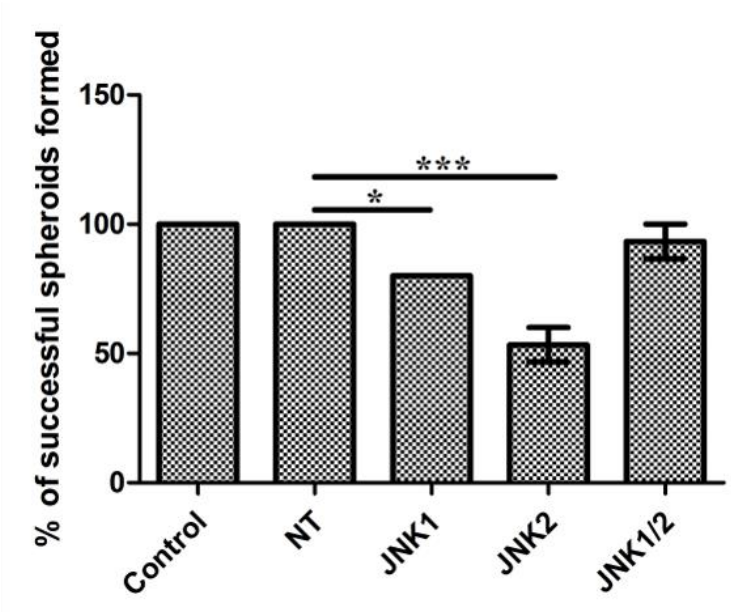
To investigate how JNK knockdown affected spheroid growth, Control, NT, JNK1, JNK2 and JNK1/2 cells were seeded at a density of 4000 cells per well into non-adherent round bottomed plates and incubated for 12 days. Images were taken every 2 days (Figure 4.23A) and a single blind study was carried out by a colleague who took measurements of the minimum and maximum diameter of each sphere and calculated the volume. 5 spheres were plated per cell line for each experiment and the average fold increase measured per sphere from day 6 was calculated (Figure 4.23C).

As expected control and NT cells produced round spheres, similar to the preliminary experiment, which steadily increased in size over the 12 days with no significant differences in sphere formation or fold increase in size (Figure 4.23A-C). Interestingly, when comparing spheres produced from the JNK knockdown cell lines, JNK1 and JNK2 knockdown cells did not seem to form spheres as successfully as the other cell lines (Figure 4.23B). This was more apparent in the JNK2 knockdown cells where only 50% of spheroids successfully formed compact spheres. This made it more difficult to measure the formed spheroids and calculate their volumes. The volume from the 3 roundest spheres from each cell line were used to calculated the fold increase in sphere volume for each cell line. Similar to the proliferation experiments no significant difference in spheroid growth was observed between the different cell lines (Figure 4.22C) over the 12 days. Again like in the proliferation experiment, slight variations were observed between cell lines by the later time points of measurement (days 10 and 12) however these was not significant when compared to NT. The fold increase produced by all cell lines was relatively small over the 6 days with an approximate average of between 0.3 – 0.8 fold increase for each cell line. These results, along with the findings from the cell cycle progression and proliferation experiments, suggest that JNK does not play a key role in MCF-7 cell growth.

(A)


Control
NT
JNK1
JNK2
JNK1/2
Day 2
Day 4
Day 6
Day 8
Day 10
Day 12

(B)


% of successful spheroids formed
150
100
50
0
***
*
Control
NT
JNK1
JNK2
JNK1/2

(C)

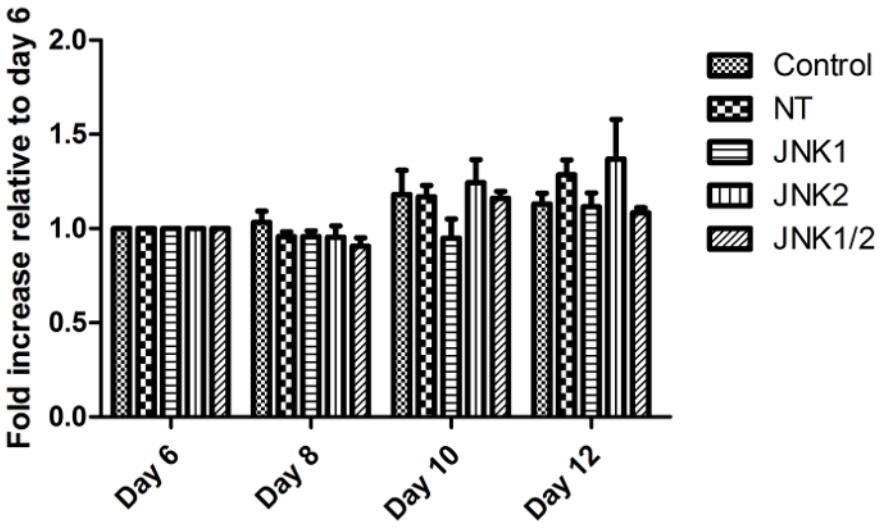


Figure 4.23 MCF7 spheroid growth is independent of JNK

Cells from control, NT, JNK1, JNK2 and JNK1/2 cell lines were seeded at 4000 cells/well into non-adherent round bottom 96 wells plates. Images of spheres were taken every 2 days and the volume was calculated. Results show (A) Representative images of sphere growth over 12 days. (B) the % of successful spheres formed for each experiment and (C) the fold increase in sphere volume relative to Day 6 measurement. Data represents the mean ± SEM of 3 individual experiments. Data was analysed using a (B) one-way ANOVA or (C) two-way ANOVA where *P<0.05, ***P<0.001.

4.3 Discussion

The JNK pathway has been demonstrated to play important roles in both the promotion and the suppression of cancer. It is now known that in some cancers JNK1 and JNK2 have different and even opposing roles in the regulation of cancer progression, however these studies have mainly been carried out in animal models or in cell lines using transient knockdown methods or inhibitors. In this study MCF-7 breast cancer cell lines containing stable knockdown of either JNK1, JNK2 or JNK1/2 were generated and used to investigate the effect of JNK isoform knockdown on both cell death and cell growth processes. Unlike the transient models used in other studies, stable knockdown of JNK did not produce any significant effects on any of the MCF-7 cell growth or death processes investigated in this study, suggesting JNK does not play a key role in this cell line.

Previous studies in MCF-7 cells have demonstrated that inhibition of JNK by SP600125 produces a significant reduction in the amount of cell death induced by UV radiation and Taxol (Mingo-Sion *et al.*, 2004). In the current study cells were treated with UV radiation or Taxol for 24 hours and the expression of cleaved PARP was analysed to investigate the effects of JNK isoform knockdown on cell death. Both stimulants successfully activated JNK signalling and induced cell death, represented by an increase in cleaved PARP expression. Interestingly in the cell lines containing JNK isoform knockdown there was no significant differences in the levels of cleaved PARP expression, suggesting that all cell lines produced a similar level of cell death.

For UV stimulated cells, this was confirmed by a clonogenics assay, where exposure to $10J/m^2$ of UV radiation produced a significant reduction in the survival fraction of cells after 2 weeks of incubation. Again JNK knockdown had no effect, shown by the similar survival fractions produced by all five cell lines (control, NT, JNK1, JNK2, JNK1/2) when compared to the controls. For Taxol stimulated cells an MTT viability assay and FACS analysis were used to confirm that Taxol - induced cell death was also independent of JNK signalling. Although there was slight variation in the reduction of viable cells in the MTT assay and increase in apoptotic events in the FACS experiment when cells were treated with Taxol, no significant difference was observed after JNK knockdown. These results were consistent with the results from the Western

blot suggesting that JNK did not play a role in UV or Taxol - induced cell death in MCF-7 cells. These results were unexpected, since other studies have demonstrated that JNK plays a key role in both of these processes.

A lot of research investigating JNK isoform function has been carried out in mouse embryonic fibroblasts (MEFS) from JNK1 or JNK2 knockout mice (Liu, Minemoto and Lin, 2004). Tournier *et al* focused their study on the role of JNK in UV - induced cell death in MEFS. They found that JNK2 knockout MEFS experienced death similar to wild type (WT) cells, whereas JNK1 knockout cells had a slightly higher percentage of viable cells after treatment with UV radiation and also less DNA fragmentation. JNK1/2 knockout MEFS were almost completely protected from UV - induced cell death and DNA fragmentation, demonstrating clearly that at least one JNK isoform is required for cell death to occur in MEFS. In the current study lentiviral knockdown of JNK did not cause a reduction in the cell death induced by UV radiation, a possible reason for this was that maybe the dose used or the time of exposure was not optimised to observe any differences between cell lines. However, in the Tournier study, JNK1/2 knockout protected MEFS from cell death during exposure to 0 - 240 J/m^2 of UV radiation and for 12 to 28 hours of exposure to $60J/m^2$ of UV radiation (Tournier *et al.*, 2000). This would suggest that conditions used in the current experiment were sufficient to investigate the effects of JNK knockdown on UV - induced cell death.

Another reason as to why contrasting results were observed could be that lentiviral knockdown was not enough to produce the protective effects observed by knockout cells or inhibition of JNK by SP600125. Knockdown of JNK1, JNK2 and JNK1/2 was successfully achieved in the MCF-7 cell lines generated during this study, however the expression of JNK isoforms were only knocked down to approximately 90% in JNK1 and JNK2 cell lines and different levels of knockdown was achieved in the double knockdown cells of 90% for JNK2 and 70% for JNK1. The small amount of JNK expressed in these cells may be enough to allow induction of UV - induced cell death. However, if JNK did play a key role as shown in MEFS in the Tournier study, it would be expected to see at least a small change in cell death induced by UV in the MCF-7 cells. Also other studies have demonstrated that knockdown of JNK expression by 80% can have an effect on JNK apoptotic signalling (Parra and Ferreira, 2010) and therefore 90% would be expected to also have an effect. As it stands, no difference

was observed in any of the cell death experiments carried out in this study, which strongly suggests that JNK does not play an important role in UV - induced cell death in this cell line.

Similar findings were observed when examining Taxol - induced cell death in MCF-7 cells. Taxol successfully induced cell death in MCF-7 cells and this was consistent throughout all cells lines, including cells containing JNK1/2 knockdown. The finding that Taxol - induced cell death was independent of JNK signalling was confirmed by Western blot, MTT assay and FACS analysis. All 5 cell lines (control, NT, JNK1, JNK2 and JNK1/2) produced a similar increase in cleaved PARP expression, decrease in cell viability and increase in the percentage of apoptotic events in cells treated with Taxol. Interestingly, these findings again differed from the outcome of studies found in the literature. As mentioned previously, MCF-7 cells treated with SP600125 show a decrease in the percentage of apoptosing cells, compared to wildtype cells after treatment with Taxol (Sunters *et al.*, 2006). JNK overexpression has also been demonstrated to increase Taxol - induced apoptosis in MCF-7 cells (Mamay *et al.*, 2003). These studies indicate a strong link between JNK and Taxol - induced cell death in MCF-7 cells. Similar conditions were used in the current study as were used in the study by Sunters *et al* for investigating Taxol - induced cell death, the difference in the current experiment was that stable knockdown of JNK was used instead of transient inhibition of JNK. This would suggest that different targeting techniques may possibly produce different results when investigating protein function.

Interestingly, a study published in 2012 investigating the role of the gene ZEB1 in malignant pleural mesothelioma (MPM) produced similar findings to this study where transient but not stable knockdown of ZEB1 caused a reduction in MPM cell growth (Horio *et al.*, 2012). This group discussed the possibility of compensatory mechanisms, where stable knockdown allowed more time for other processes within the cell to step in to reduce changes caused by the knockdown of ZEB1. Whereas transient knockdown was quick and did not give time for the compensation mechanisms to intervene and therefore any effects caused by the protein knockdown were more clearly observed. In the current study no transient inhibition or knockdown was tested and therefore it is difficult to conclude whether compensatory mechanisms

may have caused the lack of effect caused by JNK knockdown in cells treated with UV or Taxol.

After it was concluded that JNK did not play a role in the cell death experiments, the effects of JNK isoform knockdown on cell growth was then investigated. Other studies have shown that inhibition (Mingo-Sion *et al.*, 2004), knockdown (Parra and Ferreira, 2010) and knockout (Sabapathy *et al.*, 2004) of JNK can cause a reduction in activity levels of downstream substrates such as c-Jun and ATF2. Interestingly, lentiviral knockdown of JNK in MCF-7 cells did not cause a significant effect on levels of pc-Jun or pATF2 after activation with FCS. This may be caused by compensatory signalling by other MAPK pathways since p38 has been demonstrated to activate ATF2 (Breitwieser *et al.*, 2007) and ERK has been shown to activate c-Jun (Sanchez *et al.*, 1994). However, the fact that studies using JNK knockout cells can demonstrate differences in c-Jun and ATF2 phosphorylation, makes compensatory signalling a less likely explanation.

As well as JNK signalling the effects of JNK knockdown on MCF-7 cell cycle progression, proliferation and cell growth was also assessed in the current study. Similar to the cell death experiments JNK knockdown did not seem to have any significant effects on MCF-7 cell growth. Cell cycle progression was successfully analysed using FACS analysis, where starvation of cells induced the majority of cells to remain in the G1 phase of the cell cycle and replenishment of media produced a shift into the S and G2/M phase of the cell cycle. However, knockdown of JNK1, JNK2 and both JNK1/2 together produced the same results as control and NT cells. This was surprising as inhibition of JNK by SP600125 has been demonstrated to cause arrest of the cell cycle in the G2/M phase (Mingo-Sion *et al.*, 2004). Again this was transient inhibition and therefore it is possible that stable knockdown of JNK produces a different outcome in the regulation of cell cycle progression.

In the current study proliferation of MCF-7 cells was analysed by MTT viability and spheroid growth assays. Both experiments showed a gradual increase in either viable cells or spheroid volume over the 8 and 12 days of incubation respectively. Once more this was demonstrated for all cells lines (control, NT, JNK1, JNK2, JNK1/2) and therefore it was concluded that JNK did not play a key role in MCF-7 proliferation.

One interesting observation made in the spheroid growth assay was that differences in spheroid formation occurred between the different cell lines. Control and NT cell lines successfully formed a sphere which was roundish and the cells were tight in formation. This was also observed in JNK1/2 knockdown cell line, however in the JNK1 and JNK2 single knockdown lines it was common to observe the cells not quite arranged into a sphere formation and instead look more scattered. This was particularly noticeable in the JNK2 cell line where almost half of the spheres did not form correctly.

The role of JNK in spheroid formation has not previously been investigated, however in the current study JNK2 knockdown decreased the ability of MCF-7 cells to form a round organised structure. A study published by Kenny *et al* investigated the different morphologies which cancer cells can grow in 3-dimensional cultures. MCF-7 cells were shown to grow in a mass like structure where cells grow together in a group with a round outline but have a disorganised nuclei (Kenny *et al.*, 2007). This mass like structure was observed for the control, NT and most of the JNK1 and JNK1/2 spheroids analysed in the current study. Cells containing JNK2 knockdown tended to form a more grape like structure which is described in the study by Kenny *et* al as cells which group together but have poor cell to cell adhesion and therefore do not form a round shape. Taken together this may suggest that JNK2 plays a role in the formation of MCF-7 spheroids where knockdown of JNK2 produces a lack of organisation of cells. However, only bright field images of spheroids were taken in the current study so it is difficult to conclude whether the difference in JNK2 knockdown cells is due to changes in cell adherence or not. Also JNK1/2 double knockdown did not have any effect on spheroid formation, which was surprising considering both single knockdown cell lines demonstrated variation in spheroids formed.

In summary, stable knockdown of JNK1 and JNK2 in MCF-7 cells did not have a significant effect on cell growth or cell death induced by Taxol or UV radiation. This study demonstrates the importance of investigating the effects of stable knockdown in comparison to transient as contrasting outcomes have been shown in this study when compared to other studies using transient methods. In this study MCF-7 cell growth and MCF-7 cell death induced by Taxol and UV radiation occurred independently of JNK signalling. Finally, JNK2 may play a role in MCF-7 spheroid formation, where knockdown of JNK2 caused slight disorganisation of cells. Further work must be done

to investigate the effects of JNK2 knockdown on cell to cell interactions to help to determine how exactly JNK2 is involved in the organisation of cells within the spheroid. JNK has been linked to the regulation of α-catenin binding to adheren junctions (Lee *et al.*, 2011) and also to the formation of adheren junctions between cells (You *et al.*, 2013) therefore the role that JNK isoforms play in cell to cell adhesion may be an area to focus on in the future.

Chapter Five

Discussion

5.1 Discussion and Future Work

JNK signalling has been demonstrated to play a role in both cell survival and cell death processes. The outcome of this signalling is determined by a number of variables including cell type (Fan and Chambers, 2001), cell location (Xu *et al.*, 1997), stimulant type (Dougherty *et al.*, 2004), length of stimulation (Ventura *et al.*, 2006) and protein location (Notte *et al.*, 2013). Due to the plethora of variables involved, characterisation of the JNK proteins can be difficult as every model, cell type or technique can produce a different result. The ultimate goal for many cell signallers is to discover a protein which can be successfully targeted to help prevent or treat a disease. An abundance of work has been carried out to demonstrate that the JNK pathway, and now more specifically the individual JNK isoforms themselves, play key roles in both CVD (Sumara, Belwal and Ricci, 2005) and cancer (Tournier, 2013). Although this makes JNK an attractive target, there is yet to be a successful drug developed which can inhibit this protein without off target effects (Cicenas, 2015). That being said, due to the increasing evidence that JNK isoforms can work independently of each other, it is now disputed whether or not grouping the JNK proteins together would be considered a good target for drug development.

In this current study, lentiviral knockdown of JNK1 and JNK2 was achieved in both HUVECs and MCF-7 cells. Transient knockdown was used in the primary endothelial cells where knockdown of JNK2 produced an increase in levels of phosphorylated c-Jun when compared to the non-target control. Multinucleation was also observed in all cells infected with lentivirus, however this was increased further in cells containing JNK2 or JNK1/2 knockdown, suggesting that JNK2 may play a role in HUVEC cell growth. Unfortunately, lentiviral infection prevented HUVECs from growing at a normal rate. This was also the case with cells infected with the non-target control and therefore it impacted plans for further proliferation and cell death experiments being carried out. It was decided that the infection with lentivirus may not have been fully optimised for primary cells and therefore characterisation of JNK1 and JNK2 in HUVECs still remains inconclusive.

It is well known that primary vascular cells are difficult to infect, however the Baker group have demonstrated successful lentiviral infection in both ECs and VSMCs

(Dishart *et al.*, 2003), proving that these techniques can be used in primary vascular cells. In 2012, lentiviral vectors made up only 2.9% of the vectors used in current clinical trials worldwide (Konishi *et al.*, 2008), this has since increased to 5.8% in 2016 (www.abedia.com/wiley, 2016). It is now clear that lentiviral technology can be used for gene therapy and therefore it is even more important to fully understand the proteins we are choosing to target.

Studies have demonstrated that knockout of JNK2 decreases foam cell formation in atherosclerosis prone mice (Ricci *et al.*, 2004) and JNK1 knockout reduces EC apoptosis (Amini *et al.*, 2014), however in humans it is not possible to knockout a protein throughout the whole body without causing major side effects. This is where cell specific targeted work would be beneficial, for example the Baker group have also managed to successfully generate adenovirus which is selective for VSMCs (Work *et al.*, 2004) reducing the off target effects on other cell types. Lentiviral gene transfer has been shown to be less inflammatory than adenoviral gene transfer and is also thought to be better for long term treatment or study (Bursill *et al.*, 2009) suggesting that this method would be preferential for the study of long term disease states such as atherosclerosis. Using specific promotors, lentivirus selective for VSMC has been generated to inhibit VSMC proliferation whilst allowing EC growth in the same vessel (Jing *et al.*, 2015) demonstrating that lentivirus could possibly be used in the future to develop treatments for CVDs.

Before treatments can be developed the protein targets themselves must be fully understood. For JNK to be characterised in human vascular cells, a robust technique that minimises cellular growth defects is required. In the current study, too high a concentration of lentivirus may have been used, causing detrimental effects to the endothelial cells. For primary cells, lentiviral technology is still one of the best techniques to use for protein knockdown. Primary cells only remain phenotypically stable at low passage numbers in comparison to immortal cell lines which makes the development of knockout human primary cells very difficult. To develop a knockout cell line *in vitro* a system such as CRISPR (clustered regularly interspaced short palindromic repeats) is used to target and remove a specific gene from the cell. The stages required for this process include transfection, cell sorting and then the production of a line from single-cell derived clones (Bauer, Canver and Orkin, 2014)

which can take many passages to complete. By the time a knockout cell line was developed in primary cells, the phenotypically stable part of their life will have ended and therefore the cells could not be used for further experiments.

With shRNA clone isolation is not required and therefore the cells can immediately be used after knockdown is achieved. For the primary cells in the current study, lentiviral delivery of shRNA targeting JNK was the best technique to use due to its high knockdown efficiency and ability to be used in short lived cells (Unniyampurath, Pilankatta and Krishnan, 2016). Unfortunately, although successful knockdown of JNK1 and JNK2 was achieved in HUVECs, the detrimental effects on cell growth caused by the infection suggests that the lentiviral infection itself requires more optimisation to allow the cells to be used for further experiments. The cells were not able to be passaged after infection which created problems when trying to investigate proliferation, cell cycle progression or cell death in more detail. For future studies, the infection of HUVECs by lentiviral shRNA must be optimised further to ensure full knockdown of JNK is achieved with the least amount of stress and effect on normal cell growth caused by the infection itself.

After concluding that the protocol used for lentiviral knockdown in HUVECs was not optimised for primary cells we decided to try and use the lentivirus to knockdown JNK1 and JNK2 in an immortalised breast cancer cell line, MCF-7. These cells were a lot less fragile and were able to be infected with lentivirus and selected with antibiotic with minimal stress observed. Also with the ability to passage the cells infinitely, we were able to generate cell lines containing individual JNK knockdown which improved overall experiment output and produced consistent knockdown of the proteins throughout the study.

In MCF-7 cells JNK knockdown had no effect on cell death induced by UV radiation or Taxol, which was confirmed by cell viability assay, Western blotting and FACS analysis. This result was unexpected as JNK has been demonstrated to play a role in both UV (Ferguson, Marietta and Van Den Berg, 2003) and Taxol (Mamay *et al.*, 2003) induced cell death in MCF-7 cells. Similarly, knockdown of JNK had no effect on cell growth when compared to control cells. This was observed for both proliferation and cell cycle progression experiments and also in the phosphorylation

of JNK substrates c-Jun and ATF2 analysed by Western blotting. Again these results were surprising as JNK signalling has been linked to both proliferation (Parra and Ferreira, 2010) and cell cycle progression (Mingo-Sion *et al.*, 2004) in MCF-7 cells and is a known regulator of both c-Jun and ATF2 in a number of different cell lines (Gozdecka et al. 2014, Sabapathy et al. 2004).

As mentioned throughout this thesis, many studies have demonstrated differences in JNK isoform function, with a lot of the characterisation being carried out in MEFs from JNK knockout animals (Sabapathy et al. 2004). For example, JNK1 but not JNK2 was shown to be required for UV mediated death in MEFs (Liu, Minemoto and Lin, 2004). However, in the current study JNK knockdown had no effect on MCF-7 cell death induced by UV exposure. Whilst western blotting confirmed a knockdown of approximately 90% for JNK1 and JNK2 in the MCF-7 cells used in the current study, the 10% difference between protein knockdown and protein knockout may explain the difference in findings between the current experiments and ones found in the literature using cells from knockout animals. To compare knockdown vs knockout CRISPR could be used to knockout the JNK isoforms in MCF-7 cells. CRISPR has been demonstrated to be more consistent and have less off-target effects when compared to shRNA (Evers *et al.*, 2016), further highlighting why this may be the best technique for producing stable knockout cell lines in the future. The other way to make this comparison would be to use lentivirus to knockdown the JNK isoforms in MEFs which would allow a comparison to be made to the studies using cells from JNK knockout animals. This would confirm whether the knockdown produced by lentiviral shRNA was enough to produce the same changes or whether full knockout is required.

If JNK isoform knockdown in MEFs can produce changes in cell growth and cell death processes, then it could be possible that the different results produced in MCF-7 cells is down to differences in JNK function between cells types. For example is has been demonstrated before that JNK1 promotes HCC cell growth (Hui *et al.*, 2008) but inhibits skin cancer cell growth (She *et al.*, 2002). However in a study using siRNA knockdown of JNK1 or JNK2, knockdown itself increased apoptotic signalling demonstrated by a reduction in Bcl-2 expression and an increase in Bax protein expression in MCF-7 cells (Parra and Ferreira, 2010), suggesting that complete knockout of JNK isn't required in this cell line to produce a change in response.

As mentioned in chapter 4, there is a possibility that the results produced by stable knockdown may differ from results produced by transient knockdown. Since no other study has carried out stable knockdown of JNK isoforms in MCF-7 cells it is difficult to compare these findings to other studies. Therefore, transient knockdown methods must be used alongside stable knockdown in future experiments to determine any differences between the results produced by these techniques. Also a gene array could be carried out to investigate if any other signalling pathways are upregulated as a result of the knockdown of JNK (Yang *et al.*, 2007). This would help to erase or highlight the possibility of compensation which has been demonstrated for other MAPK pathways (Mendoza, ER and Blenis, 2012).

One transient method that is commonly used to inhibit JNK signalling is the use of drug inhibitors which either block JNK from being phosphorylated (Chen *et al.*, 2009) or compete with ATP to prevent JNK from phosphorylating a substrate (Bennett *et al.*, 2001). However it is difficult to develop an inhibitor with high specificity, for example, a lot of studies investigating JNK function have used the JNK inhibitor SP600125 and although this inhibitor does block JNK signalling it has also been demonstrated to inhibit 13 other protein kinases, including AMPK, CDK2 and SGK (Bain *et al.*, 2003). This makes it difficult not to question studies using only this inhibitor and highlights a benefit of gene silencing. Trying to determine JNK isoform function in cancer is going to be challenging due to the differences in JNK function between cancer types. This means that every cancer will have to be looked at individually to determine whether inhibiting JNK would reduce cancer growth and progression or enhance it. Once understood, JNK could be a good target for cancer treatment itself or in combination to enhance other treatments.

Radiotherapy is a cancer therapy which uses radiation to kill cancer cells and is commonly used to treat different types of cancer including breast cancer (Njeh, Saunders and Langton, 2010) and prostate cancer (Teh and Ishiyama, 2012). Over the years there has been an accumulation of evidence which shows that this type of therapy can cause injury to blood vessels which can promote the onset of CVD (Weintraub, Jones and Manka, 2010). Other work in our lab has demonstrated that JNK is activated after exposure to radiation in ECs (Hargrave, unpublished observations). This finding along with the evidence which demonstrates a role for JNK in EC dysfunction (Osto

et al., 2008) may link JNK signalling to radiation induced vessel damage and therefore JNK may be a good target for this off target effect. A combination of lentiviral knockdown of JNK in ECs during radiation treatment could be a possible solution to preventing this side effect. Lentivirus has successfully been used *in vivo* to over express extracellular superoxide dismutase in arteries to reduce vessel damage in rabbits with balloon-injured carotid arteries (Qian *et al.*, 2006) and therefore it may be possible to use the same method to knockdown JNK and see if it can reduce the off target effects of radiotherapy on ECs.

Although most experiments carried out in the current study showed that JNK isoform knockdown had no effect on the processes investigated, during the spheroid assays, one interesting observation was made. JNK2 knockdown cells did not produce spheres as uniformed as spheres produced by the control, NT, JNK1 or JNK1/2 cell lines. The disordered structure could be due to changes in adherence between cells, which JNK has been shown to play a role in (Lee *et al.*, 2011). Another theory could be that instead of forming a tight sphere shape, the cells are instead metastasising outward since JNK2 has been linked to breast cancer metastasis (Nasrazadani and Van Den Berg, 2011). Cdc25C, a phosphatase which activates CDK1 and therefore mitosis progression, has also been demonstrated to play a role in breast cancer metastasis (Feng *et al.*, 2011). JNK has been shown to phosphorylate Cdc25C, regulating its ability to dephosphorylate CDK1 which causes a DNA damage checkpoint, a stage required for successful cell cycle progression (Gutierrez, *et al.*, 2010). The interaction between JNK and Cdc25C could further link JNK with breast cancer metastasis in more detail. For future work Western blotting should be used to investigate the effects of JNK2 knockdown on the levels of phosphorylated Cdc25C to determine whether they are increased. If they are this may explain the increased metastasis.

To examine the extent of cell metastasis a lower magnification should be used to visualise a larger area surrounding the spheroid and also migration assays could be used to examine differences in cell migration between the generated cell lines (Hulkower and Herber, 2011). A higher magnification of imaging could help to identify differences in cell to cell contact between NT and JNK2 knockdown cells by staining for adheren junction proteins cadherin and catenin (Millán *et al.*, 2010). These proteins are also markers for EMT, a carcinogenic process which JNK has been linked

to that promotes invasion of cancer cells (Wang *et al.*, 2010). In the HUVEC experiments JNK2 and JNK1/2 knockdown caused an increase in the number of multinucleated cells, this would link JNK2 and not JNK1 to regulating cell growth. As mentioned earlier, it was concluded that the lentiviral infection may not have been optimised for the infection of primary cells. To determine whether JNK2 knockdown was the cause of the multinucleation observed in this study, passaging of cells containing knockdown would be required to perform time lapse experiments which would allow any differences in cell cycle and division to be observed more clearly (Uetake and Sluder, 2004). As shown with the MCF-7 cells in the current study, the experiments to investigate both cell growth and cell death can be carried out in cells infected with lentivirus, therefore once knockdown is achieved in the endothelial cells without detrimental effects from infection, characterisation of the role of JNK in ECs and other vascular cells could be easily carried out.

All of the preliminary experiments investigating JNK isoform function in MCF-7 cells worked successfully, demonstrating that both cell growth and cell death processes can be analysed in this cell line. Although JNK isoforms were efficiently knocked down in each cell line, all of the experiments produced consistent results which demonstrated that JNK did not play a role in MCF-7 cell death or growth. Why the results obtained differ from similar studies in the literature is unknown and therefore thorough comparisons using MEFs and transient knockdown methods are required to determine this answer. Finally, CRISPR could be used to generate MCF-7 knockout cells to further compare the lentiviral shRNA technique and confirm whether JNK isoforms play a key role in MCF-7 cell growth or death.

5.2 Conclusion

JNK isoforms play crucial roles in cell proliferation, cell cycle progression and apoptosis, however our understanding of individual JNK protein function is limited. Studies are now demonstrating that targeting JNK isoforms in diseases such as HCC and atherosclerosis may be important for the development of treatments in the future. For this to happen a clear understanding of JNK signalling must be gained in each different setting and characterisation of each isoform will help to do this. In the current study JNK2 knockdown in HUVECs caused an increase in the phosphorylation of the transcription factor c-Jun and also produced an increase in the percentage of multinucleated cells similar to JNK1/2 double knockdown. This would suggest that JNK2 may play an important role in HUVEC cell growth however due to technical difficulties this work remains inconclusive and further investigation must be carried out for characterisation of JNK1 and JNK2 in HUVECs. In MCF-7 cells JNK did not seem to play an important role as demonstrated by no change in the cell growth or cell death experiments performed after JNK isoforms were knocked down. Further work must be carried out to confirm this finding and to determine if different methods of protein targeting may produce different results.

Chapter Six

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