MOLECULAR REGULATION OF JNK IN TNF-α-STIMULATED HUMAN HEPATOCARCINOMA (HEPG2) CELLS VIA THE mTOR/AKT PATHWAY

By

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ABSTRACT

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The c-Jun N-terminal kinases (JNKs) are serine/threonine protein kinases which can respond to a wide range of external stimuli and proinflammatory cytokines including Tumour Necrosis Factor–α (TNF-α). They have been implicated in cell death, cell survival and cell growth. The working hypothesis in this research is that TNF-α may signal to JNK via the mTOR/Akt pathway. No direct connections between the JNK and mTOR/Akt pathway have thus far been reported. In this study, it is shown that 20 ng/mL of TNF-α treatment was sufficient to increase significantly both mRNA and proteins of JNK in human hepatocarcinoma cells (HepG2). The mammalian target of rapamycin (mTOR) was also identified in this study as a potential upstream regulator of JNK. Specifically, 100 ng/mL of rapamycin, an mTOR inhibitor, significantly inhibited activation of JNK1 when stimulated with TNF-α. Correspondingly, the activation of its cognate transcription factor, c-Jun was also inhibited. Without rapamycin inhibition, mTOR was phosphorylated at the serine 2448 residue, suggesting that TNF-α signals to JNK1 via the mTORC1. With rapamycin inhibition, phosphorylation of mTOR at its serine 2448 residue was abrogated. In contrast, Akt was phosphorylated at

its serine 473 residue, suggesting that mTORC2 signals downstream when mTORC1 is inhibited. This activated Akt molecule may interfere with JNK1 scaffolding, preventing JNK1 from being activated during rapamycin treatment. Notably, phosphorylated MKK7 could only be detected during rapamycin treatment, suggesting that MKK7 can be scaffolded with JNK1 only in its unphosphorylated form. This study also showed that TNF-α treatment reduces both the nuclear hormone receptors, *LXR-α* and *PXR* gene expression. However, pre-treatment with rapamycin prior to TNF-α stimulation significantly induced *LXR-α* and *PXR*, up to ~17- and ~2-fold, respectively. This suggests that mTOR may act as a negative regulator that inhibits the induction of LXR-α and PXR as anti-inflammatory genes. It is also shown here that inhibition of JNK1 via the mTOR/Akt pathway corresponded with the up-regulation of *LXR-α* and *PXR*. Together, these observations suggest that JNK1 may act downstream of mTOR as an LXR-α and PXR inhibitor. From the results gleaned in this study, rapamycin (and its analogues) may be used to reduce acute inflammation by promoting the induction of LXR-α and PXR as anti-inflammatory genes via the inhibition of JNK1 activation.

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APPROVAL SHEET

This thesis entitled "**MOLECULAR REGULATION OF JNK IN TNF-α-STIMULATED HUMAN HEPATOCARCINOMA (HEPG2) CELLS VIA THE mTOR/AKT PATHWAY**" was prepared by NG DI LIN and submitted as partial fulfillment of the requirement for the degree of Master of Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

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I hereby declare that the project is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVIATIONS

4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
β-actin	Beta-actin
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	base pair
cDNA	Complementary deoxyribonucleic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclase
dNTPs	Deoxyribonucleoside triphosphates
EDTA	Ethyl diaminetetraacetic acid
EtBr	Ethidium bromide
FBS	Fetal bovine serum
g	Acceleration of gravity (~9.8 m/s^2)
GTP	Guanidine triphosphate
HepG2	Hepatocellular carcinoma
IL-1	Interleukin-1
IL-1β	Interleukin-1-beta
IL-6	Interleukin-6
I*κ*B	Inhibitory protein of NF-*κ*B
I*κ*Bα	Inhibitory protein of NF-*κ*B-alpha

K63	Lysine 63
kb	Kilobase
kPa	Kilo pascal
L	Litre
LPS	Lipopolysaccharide
M	Molar
MAPKKK	Mitogen-activated protein kinase kinase kinase
MEF	Mouse embryonic fibroblasts
MEM	Eagle's Minimum Essential Medium
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MOPS	3-[N-Mopholino] propanesulphonic acid
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NFκB	Nuclear factor-kappa B
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRAS40	Proline-rich Akt substrate 40kDa
RIP	Receptor-interacting protein
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
RNase	Ribonuclease
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Svedberg units
SDS	Sodium dodecyl sulfate

Taq	*Thermus aquatics*
TBE	Tris-borate-EDTA
TLR	Toll-like receptor
T_m	Melting temperature
TNFR1	Tumour necrosis factor-receptor 1
TNFR2	Tumour necrosis factor-receptor 2
TNF-α	Tumour necrosis factor-alpha
TRADD	TNF-receptor-associated death domain
TRAF2	TNF-receptor-associated factor 2
U	Unit
UV	Ultraviolet
V	Volt
v/v	Volume/volume
w/v	Weight/volume

LIST OF PUBLICATIONS

1. Ng, D.-L., Tie, S.-W., Ong, P.-C., Lim, W.-S., Tengku-Muhammad, T.-S., Choo, Q.-C., Chew, C.H. (2011). Rapamycin pre-treatment abrogates Tumour Necrosis Factor-a (TNF-a) down-regulatory effects on LXR-a and PXR mRNA expression via inhibition of c-Jun N-terminal kinase 1 (JNK1) activation in HepG2 cells. *Elec. J. Biotech.,* **14(3)**.

2. Ng, D.-L., Tie, S.-W., Ong, P.-C., Lim, W.-S., Tengku-Muhammad, T.-S., Choo, Q.-C., Chew, C.H. (2011). Rapamycin pre-treatment followed by Tumour Necrosis Factor-α (TNF-α) stimulation up-regulate *LXR-α* and *PXR* expression in HepG2 cells. *Paper presented at the First National Postgraduate Conference on Molecular Medicine, Kota Bharu, Malaysia.*

CHAPTER 1

INTRODUCTION

The c-Jun N-terminal kinases (JNKs) are members of a larger group of serine/threonine (Ser/Thr) protein kinases known as the mitogen-activated protein kinase (MAPK) family. The diversity of mediators upstream of JNK genes allows activation by a range of external stimuli (Davis, 2000) and proinflammatory cytokines, such as tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) (Dong *et al*., 2002).

Of particular interest is the JNK response to proinflammatory cytokines such as the TNF-α. In addition to genetic alterations, inflammatory cells and circuits characterise the tumour microenvironment and represent crucial players in the tumour development and progression (Balkwill *et al*., 2005; Balkwill & Mantovani, 2001). Chronic inflammation represents a major pathological basis for tumour development. Although inflammation acts as a host defence mechanism against infection or injury and is primarily a self-limiting process, inadequate resolution of inflammatory responses lead to various chronic disorders associated with cancers. Therefore, it is imperative to discover alternative treatments which may avert chronic inflammation by JNK activation.

JNKs have shown some complexity in their role in cell death, cell survival and cell growth. JNKs have been proven to be critical for the initiation and/or execution of cell death following cellular stress. Observations of persistent activation of the JNKs (either achieved through the overexpression of upstream activators or following exposure to appropriate external stimuli) are frequently associated with apoptotic cell death in a variety of cells (Chen *et al*., 1996; Hu *et al*., 1999; Wang *et al*., 1998; Widmann *et al*., 1997).

Conversely, it is also proposed that JNK may act to enhance cell survival following exposure to the stress such as TNF-α, ultraviolet exposure and nitric oxide (Andreka *et al*., 2001; Assefa *et al*., 1999; Roulston *et al*., 1998). Although paradoxical, there must be many cell survival pathways activated if a cell is to survive environmental stress. Therefore, a balance between these signalling pathways was proposed to dictate the cellular decision of death or survival. To rationalise these observations, it is likely that there are other events accompanying JNK activation that will contribute to the cellular outcome. One potential target for inhibition that will affect JNK activation in this study is mTOR (Kim *et al*., 2001).

The mammalian target of rapamycin, mTOR, is a potential target for anti-cancer therapy as it is a key molecule in the Akt/mTOR signalling pathway and plays a critical role in controlling cell proliferation and survival. Inhibition of mTOR can enhance the efficiency of a broad range of

chemocytotoxic agents, including cisplatin and doxorubicin, in various types of human cancers (Mondesire *et al*., 2004; Wu *et al*., 2005). Indeed, it would be interesting if mTOR is found to be implicated in another pathway such as the MAPK cascade, in which JNK1 and JNK2 are vital components; or if it affects the expression of other potential anti-inflammatory nuclear hormone receptors, such as Liver X Receptor-α (LXR-α) and Pregnane X Receptor (PXR), which may be downregulated by the pro-inflammatory action of TNF-α.

Therefore, the objectives of this study were:

i) to investigate the dose and time-course response expression of JNK1 and JNK2 mRNA and protein in human HepG2 cells under TNF-α stimulation,

ii) to investigate the effects of mTOR inhibition on TNF-α-stimulated JNK activation,

iii) to study the effects of mTOR inhibition on the regulation of *LXR-α* and *PXR* nuclear hormone receptors expression after stimulation with TNF-α.

CHAPTER 2

LITERATURE REVIEW

2.1 c-Jun N-terminal Kinase

2.1.1 Brief history

The c-Jun N-terminal kinases (JNK) are a sub-family of mitogen-activated protein kinases (MAPK). Other MAPKs sub-families include the Big MAPK (BMK/ERK5) and the 38 kDa protein kinases (p38s) (Lewis *et al*., 1998; Widmann *et al*., 1999). These different MAPKs can be activated in response to specific stimuli (specifically stress) and can subsequently regulate specific substrates and phosphorylate transcription factors. Other MAPKs, such as ERK, respond to growth factors or mitogenic stimuli and can directly phosphorylate transcription factors such as Elk-1 (Chen *et al*., 2001).

JNK was identified and cloned when a mammalian stress-activated protein kinase (SAPK) was discovered following intraperitoneal injection of the protein synthesis inhibitor cycloheximide (Kyriakis & Avruch, 1990). Activation required the phosphorylation of threonine and tyrosine residues (Kyriakis *et al*., 1991). Affinity purification using the N-terminal transactivation domain of c-Jun identified c-Jun protein kinases that were activated following cell exposure to ultraviolet (UV)-radiation. These protein kinases were 46 and 55 kDa and termed the JNKs (Hibi *et al*., 1993). The

regulation of these activities paralleled the regulation of c-Jun phosphorylation in intact cells, suggesting an important role for these protein kinases in the regulation of c-Jun transcriptional activity (Minden *et al*., 1994).

2.1.2 The general structure of JNK

JNKs are serine/threonine protein kinases containing all 11 protein kinase subdomains (Bogoyevitch & Kobe, 2006). These motifs consist of conserved features of protein kinases, namely binding of ATP and peptide substrates, and maintain the conserved protein kinase three-dimensional fold. Similar for all protein kinases, the small N-terminal lobe (predominantly anti-parallel β-sheets; subdomains I–IV) aids in the orientation and binding of ATP. The larger C-terminal lobe (predominantly α-helices; subdomains VIA–XI) acts in peptide substrate recognition as well as anchoring the phosphate of Mg^{2+} ATP (Figure 2.1).

The defining motif of JNK is the amino acid sequence Thr–Pro–Tyr (T–P–Y) within the activation loop in kinase subdomain VIII. Post-translational modification by phosphorylation of residues within this activation loop activates all MAPKs currently known. As shown for ERKs, it would be expected that phosphorylation within the T–P–Y motif permits C-terminal lobe rotation, therefore aligning the active site residues for efficient transfer of the γ-phosphate of ATP to the hydroxyl residue (serine or threonine) of its protein substrate (Canagarajah *et al*., 1997).

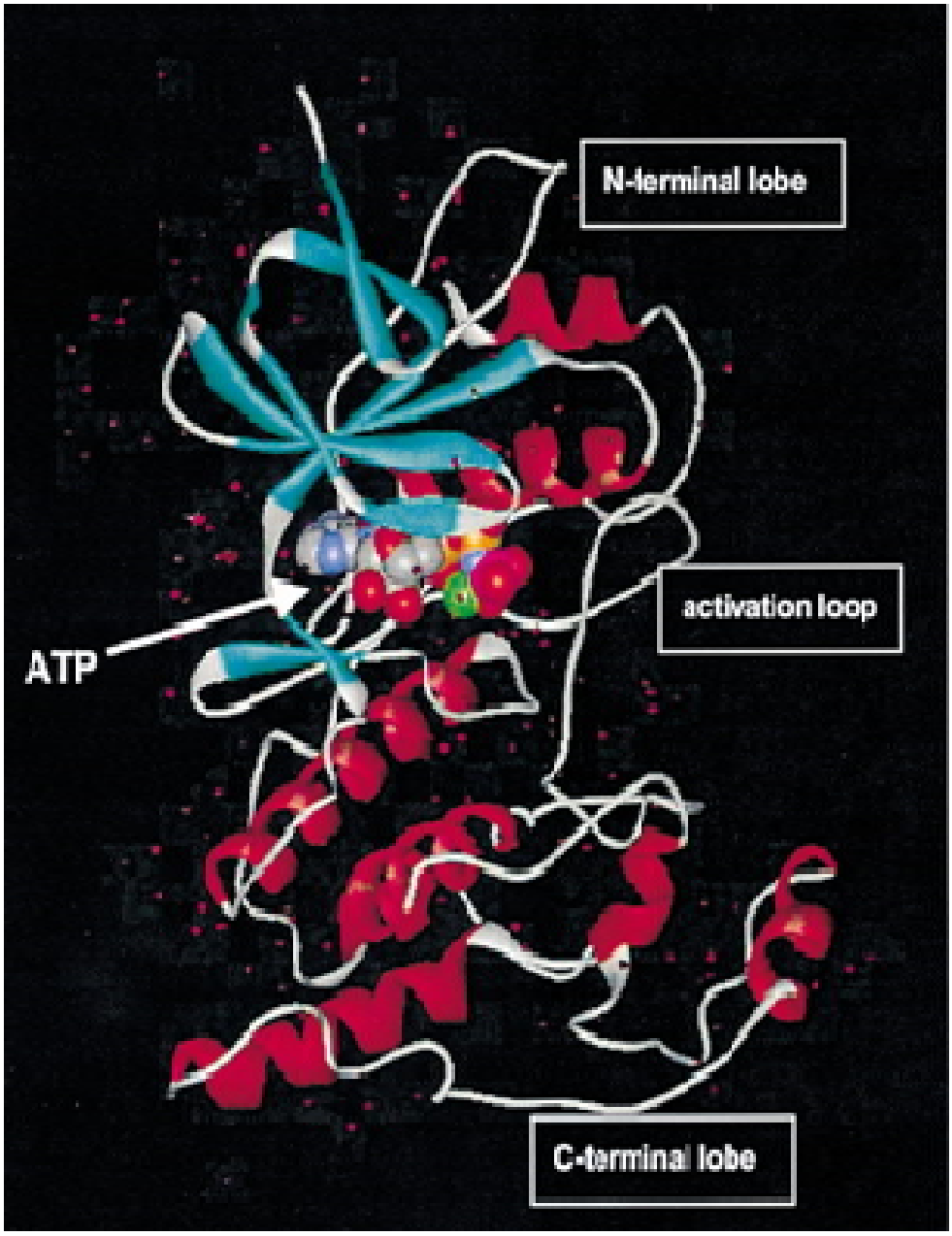

Figure 2.1: The crystal structure of JNK3α1.
The N- and C-terminal lobes as well as the activation loop are indicated. The arrow shows the position of the bound Mg^{2+}. AMP–PNP, indicating the ATP-binding site (Bogoyevitch & Kobe, 2006).

Apart from amino acid sequences and transcript modifications, the crystal structures of JNK1 (Heo *et al*., 2004) and JNK3 as shown in Figure 2.1 (Xie *et al*., 1998) have been published. The crystal structure of JNK2 was determined with a peculiar conformation of the activation loop; when JNK2 adopts this conformation it cannot be phosphorylated by upstream kinases (Shaw *et al*., 2008). Indeed, studies on epithelial morphogenesis revealed that differences in the variable region (amino acids 173-190) of JNK1 and JNK2 determine their structural conformation and in consequence the status of phosphorylation (Takatori *et al*., 2008). Thus, it is not the mere presence of activated upstream kinases, but also the protein structure and conformation which determine the activity of JNKs.

2.1.3 Isoform structure

There are three JNK-encoding genes, JNK1 (Derijard *et al*., 1994), JNK2 (Kallunki *et al*., 1994) and JNK3 (Gupta *et al*., 1996). JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 expression is restricted predominantly to the brain and testes (Bode & Dong, 2007; Cuevas *et al*., 2007). Based on Figure 2.2, there are ten JNK isoforms which result from the alternative splicing of three JNK genes (Gupta *et al*., 1996). This splicing yields proteins of 46 and 55 kDa which are essentially the products with and without a C-terminal extension (Figure 2.2). The other form of alternative splicing (α or β) only occurs for JNK1 and JNK2 and is the result of selection for one of two alternative exons that encode the region between subdomain IX and X of the protein kinase domain (Figure 2.2).

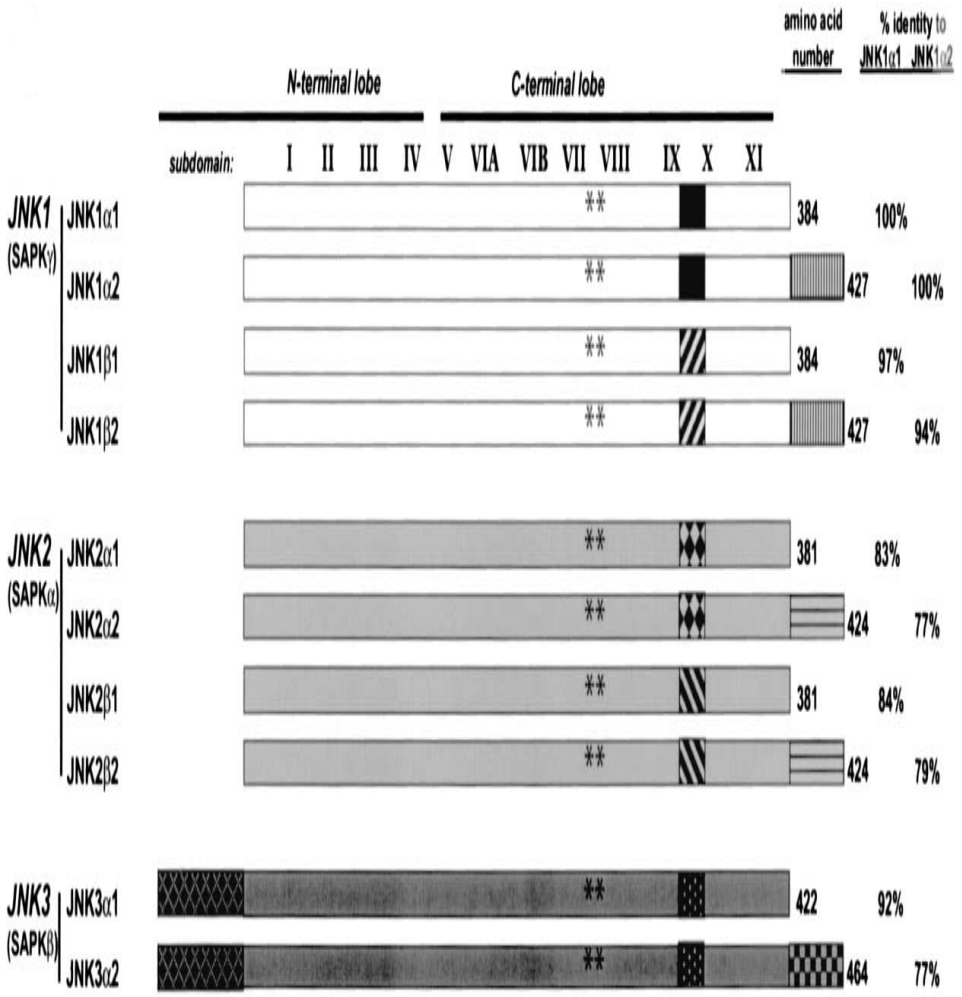

Figure 2.2: The JNKs are alternatively spliced JNK1 (white), JNK2 or (light grey), JNK3 (dark grey) to produce ten different isoforms.

The differences are indicated by the patterned rectangles. There is one splicing site, between subdomain IX and X of the JNK1 and JNK2 gene and the resulting splice forms show altered substrate specificity. The second alternative splicing occurs at the C-terminus of the protein, producing proteins which differ in length by 42 or 43 amino acids (i.e. the typical 46 and 55 kDa proteins originally noted, and the total number of amino acid in each isoform is indicated). Thus JNK1α2 is the longer form of JNK1α1. The similarity of the shorter forms has been estimated and is summarised by percent identity of JNK1β1, JNK2α1, JNK2β1 and JNK3α1 with JNK1α1 as indicated. Similarly, the longer forms can be compared and percent identity of JNK1α2 with JNK1β2, JNK2α2, JNK2β2, and JNK3α2 is shown (Gupta *et al*., 1996).

2.1.4 Substrate specificity

The JNKs were first identified by their ability to phosphorylate the transcription factor c-Jun (Hibi *et al*., 1993). c-Jun, acting within the activator-protein-1 (AP-1) complex, has been implicated in various cellular processes, ranging from cell proliferation and differentiation to neoplastic transformation. c-Jun activity can be post-translationally upregulated by JNK-mediated phosphorylation of serine 63 and serine 73 within its N-terminal activation domain (Pulverer *et al*., 1992). The phosphorylation of these same sites was also suggested to increase the stability of c-Jun, resulting in a modest increase in its steady state level (Musti *et al.,* 1997). Although c-Jun is an exclusive JNK substrate, JNKs can also phosphorylate and activate other transcription factors.

ATF2 is a JNK substrate that heterodimerises with c-Jun and stimulates expression of the c-Jun (van Dam *et al*., 1995). Therefore, through activation of both c-Jun and ATF2, JNKs can regulate the amount and activity of c-Jun. Elk-1 is another JNK target (van Dam *et al*., 1995) and it is involved in the induction of the c-fos, which forms the AP-1 heterodimer with c-Jun (Treisman, 1995). This shows that JNK has the potential to regulate transcriptional activity at several levels.

The structural differences within isoforms have been demonstrated to alter the interaction of JNK with its substrates (Gupta *et al.*, 1996). Although possessing a high level of homology (80%), the differences in their domain assembly such as C-terminal extension or different exon usage between sub-domains can be associated with functional specificity (Gupta *et al.*, 1996). JNK3 isoforms, for example, are characterised by an N-terminal extension as shown in Figure 2.2, which contains the binding domain for the scaffold protein, arrestin-2, a kinase responsible for regulating G protein-coupled receptors (Guo & Whitmarsh, 2008).

There are patterns emerging showing that different JNK isoforms, despite their high levels of sequence similarity, may show substrate preferences. JNK2, rather than JNK1, preferentially interact with the substrate protein doublecortin (Gdalyahu *et al.*, 2004). JNK2 also phosphorylates a larger number of sites in *tau* proteins, and this is correlated with its ability to inhibit microtubule assembly *in vitro* (Yoshida *et al.*, 2004). In *in vitro* knockout studies, the use of JNK2-deficient cells has suggested a role for JNK2, rather than JNK1, in the inactivation of TIF-IA and subsequent downregulation of rRNA synthesis (Mayer *et al.*, 2005). There may also be some degree of isoform selectivity in JNK localisation, with the report of JNK2 translocation to the mitochondria in a PC12 model of dopaminergic cell death (Eminel *et al.*, 2004).

In contrast to JNK2's role to regulate c-Jun stability under non-stressful conditions, JNK1's primary function is to elicit the activation of c-Jun after stress (Sabapathy *et al*., 2004). JNK1 is a more efficient Itch kinase than JNK2 (Gao *et al*., 2004) and has been shown to be critical in the death following cardiac ischaemia-reperfusion (Hreniuk *et al*., 2001). T-cell function was found to be defective in mice lacking JNK1 (Dong *et al*., 2000).

In summary, JNKs phosphorylate various targets to alter their activity, and elicit biological effects. Due to the high sequence identities of the JNK isoforms and the broad peptide specificity of JNKs, it is clear that a number of subtle mechanisms (including transcriptional regulation, cellular localisation, and specific JNK-substrate interactions) must cooperate in the cell to preferentially associate a JNK isoform with a particular substrate. This partially accounts for the controversy surrounding the biological effects of the JNK cascade. Currently, it is still considered a major challenge to identify the molecular networks regulated by the individual JNK family members (Rincon & Davis, 2009; Weston & Davis, 2007).

2.2 Tumour Necrosis Factor-α Signalling

Upon initiation, TNF-α binds with similar affinity to two receptor types known as the TNF-R1 (also called p55 or p60) and the TNF-R2 (also called p80 or p75) (Figure 2.3). These receptors differ substantially in their intracellular signalling domains, notably the 'Death Domain' in the type I

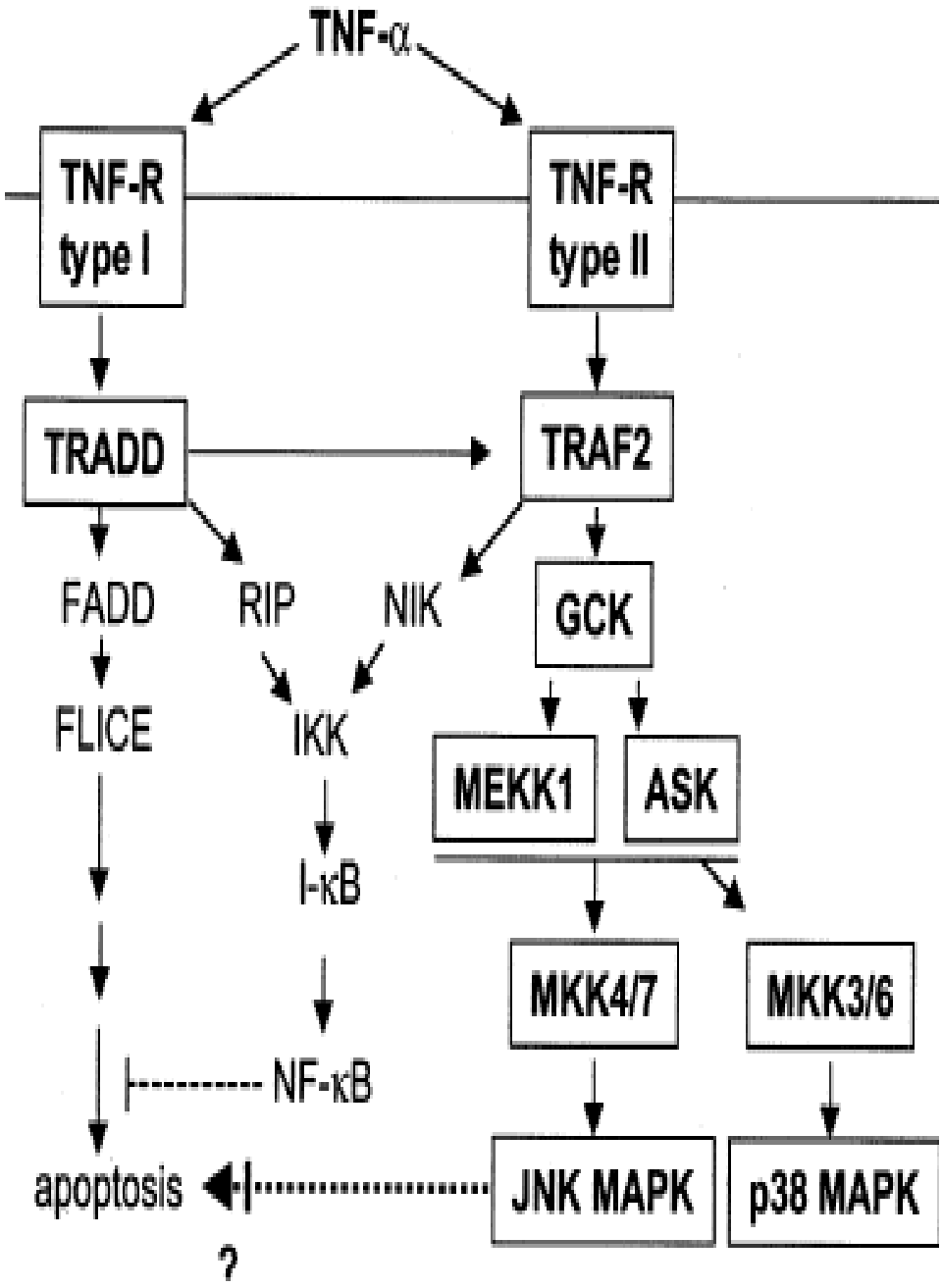

Figure 2.3: **TNF-α pathway leading to JNK activation.**
JNK is activated via MKK4/7 in the canonical TNF-α pathway. However, there are still doubts as to whether JNK actually cause or prevent apoptosis, as denoted by the question mark in the above figure (Bogoyevitch & Kobe, 2006).

receptor is absent in the type II receptor. The binding of TNF to TNF-R1 type I causes trimersation of the receptor and recruitment of the adapter protein TRADD to the cytoplasmic domain of the receptor (Chen & Goeddel, 2002). TRADD acts as a scaffold protein that recruits FADD, RIP1, and TRAF2. In contrast, TNF-R2 directly binds TRAF1, which subsequently recruits TRAF2.

These adapter proteins act to initiate signal transduction pathways within a complex that is bound to the receptor. Thus, apoptosis and anti-inflammatory responses are mediated by the activation of caspase 8 by TRADD (Yeh *et al*., 1998). In contrast, anti-apoptosis and inflammatory responses are mediated by activation of MAPK pathways by TRAF2 (Yeh *et al.,* 1997) and also the NF-*κ*B pathway by TRAF2 plus RIP1 (Devin *et al*., 2000; Kelliher *et al*., 1998). Despite these differences, both type I and II receptors can activate JNKs via the indirect and direct recruitment of the TRAF2 adaptor protein, respectively.

The inflammatory response of cells to TNF-α is also mediated, in part, by the regulation of gene expression by the AP-1 and NF-*κ*B groups of transcription factors (Baud & Karin, 2001). The NF-*κ*B signal transduction pathway is directly engaged by the TNF receptors. In contrast, the AP-1 group of transcription factors is activated by an indirect mechanism that involves MAPK. For example, the transcriptional induction of c-Jun expression may be mediated by several MAPK-responsive elements in the promoter, including

MEF2, a consensus AP-1 site that binds c-Jun, and a non-consensus AP-1 site (Jun2TRE) that binds c-Jun/ATF2 heterodimers (Shaulian & Karin, 2002). The p38 can phosphorylate and activate MEF2 (Han *et al.*, 1997), JNK can phosphorylate and activate c-Jun (Derijard *et al.*, 1994, Kyriakis *et al.*, 1994), and both p38 MAPK and JNK can phosphorylate and activate ATF2 (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; Raingeaud *et al.,* 1996; van Dam *et al.*, 1995). Together, these data suggest that the regulation of AP-1 activity by TNF may involve the concerted actions of MAPK.

However, the link between TRAF2 and further events in the JNK pathway remains incompletely understood. Overexpression of TRAF2 activates JNK (as well as p38 and IκBα kinase) without requirement for TNF-R engagement (Baud *et al.*, 1999). Furthermore, TRAF2 interacts with GCK, a Ste20-like MAPKKK (Yuasa *et al.*, 1998). It should be noted that this complexity, including redundancies or contradictions between various reports, could reflect the different cell types used. For example, GCK is relatively restricted in its expression patterns. It is found mainly in germinal center B cells, brain, lung, and placenta (Kyriakis, 1999). Cells without GCK must, therefore, use other Ste20-like kinases to elicit JNK activation following TNF exposure.

In human physiology, TNF-α has a critical role in chronic inflammatory diseases such as rheumatoid arthritis (Feldmann & Maini, 2008). It has also been implicated as promoting tumours as demonstrated in mouse cancer models (Balkwill, 2009; Egberts *et al.,* 2008; Popivanova *et al.,* 2008). In these models and in human cancers, TNF-α is produced by malignant and/or host cells within the tumour microenvironment (Kulbe *et al*., 2007, Petersen *et al*., 2007). The mechanisms of action of TNF-α in the tumour microenvironment are not fully elucidated, but may include a central role in a cancer-cell autonomous tumour-promoting network of other cytokines and chemokines (Kulbe *et al*., 2007), stimulation of epithelial to mesenchymal transition in malignant cells (Bates & Mercurio, 2003), or further DNA damage to malignant cells (Yang *et al*., 2006). The actions of TNF-α on other cells in the tumour microenvironment include promotion of angiogenesis (Kulbe *et al*., 2007) that could be via induction of a proangiogenic phenotype in recruited monocytes (Yang *et al*., 2006) and inhibiting the cytotoxicity of activated macrophages (Hagemann *et al*., 2008).

2.3 Signalling Pathway of JNK

Activation of JNK has been routinely measured by protein kinase activity towards the transcription factor substrate, c-Jun. Mammalian JNKs have been shown to be activated in response to various extracellular stimuli including growth factors, cytokines and cellular stresses such as heat shock, hyperosmolarity, UV-radiation and ischemia/reperfusion (Bogoyevitch *et al*., 1995; Bogoyevitch *et al*., 1996; Kallunki *et al*., 1994; Minden *et al*., 1994;

Shirakabe *et al*., 1997). Cytokine receptors have been suggested to act as an important initiating event in stress response.

The sequence of signalling events begins with receptor activation, leading to recruitment of adaptor molecules and activation of small GTP-binding-proteins (G-proteins). This is usually followed by three to four tiers of protein kinases. Specifically, the MAPKKKKs (typically of the Ste20-family) phosphorylate and activate the MAPKKKs which, in turn, phosphorylate and activate the MAPKKs (or MEKs) (Figure 2.4). These dual-specificity kinases then phosphorylate and activate the MAPKs which in turn, phosphorylate and activate their specific target proteins such as transcription factors. Figure 2.4 shows the JNK signalling pathway in the wider context of MAPK signalling.

Many different MAPK pathways follow this typical signalling arrangement, and each individual MAPK pathway possesses highly-specific signalling intermediates that maintain accurate signalling. In the specific example of the JNK pathway, these MAPKs are activated by two MAPKKs, MKK4 and MKK7 (Fleming *et al*., 2000). At levels upstream of MKKs, the MAPKKKs leading to JNK activation include MEKKs, mixed lineage kinases (MLKs; including MLK1-3 and DLK), Tpl2, ASK and TAK (Dorow *et al*., 1993; Ichijo *et al*., 1997; Salmerojan *et al*., 1996; Shibuya *et al*., 1996; Shirakabe *et al*., 1997; Tanaka & Nanafusa, 1998; Tibbles *et al*., 1996).

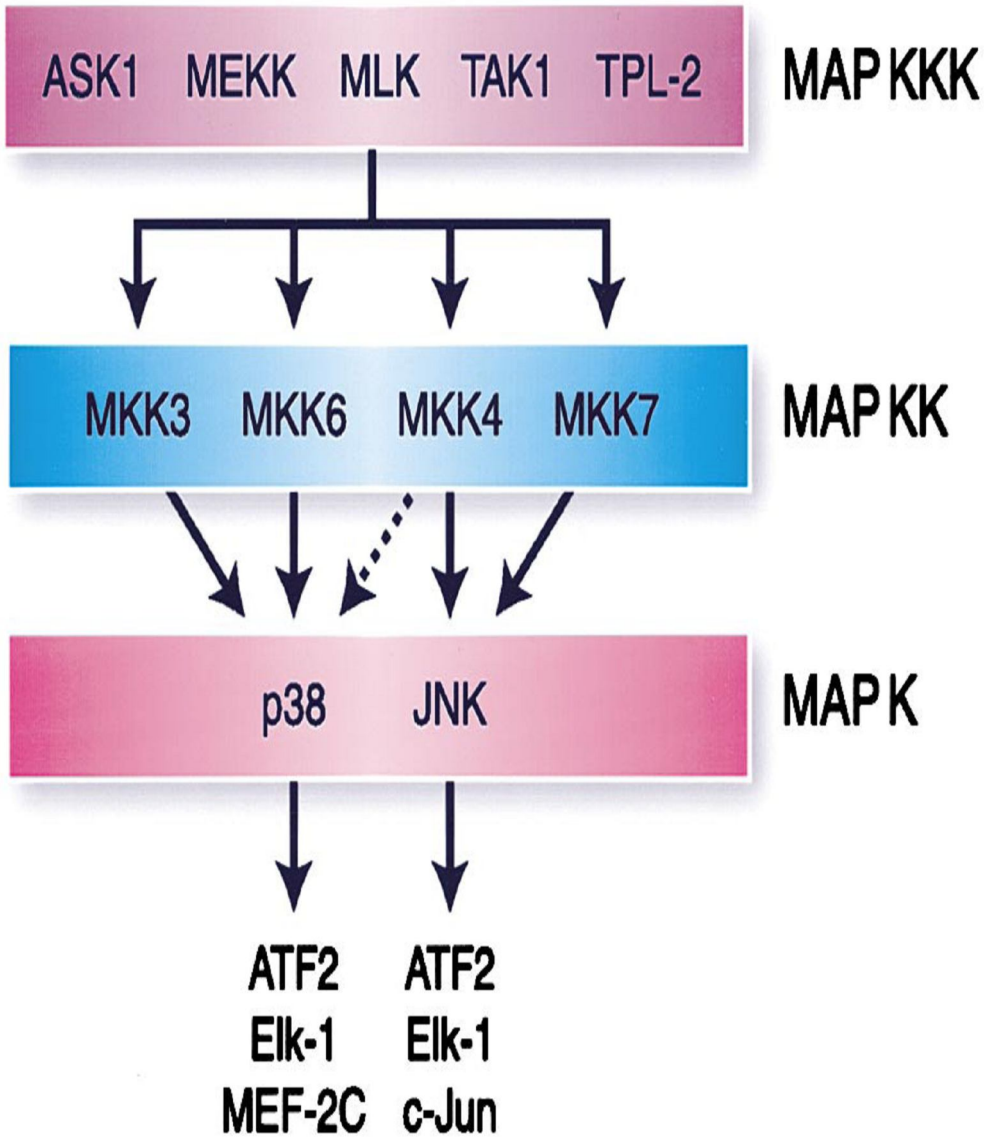

Figure 2.4: **Stress-activated MAPK signalling modules.**
JNK is activated by both MKK4 and MKK7. However, there are many other kinases which can activate MKK4/7. The canonical substrate for JNK is c-Jun, however there are other transcription factors which are JNK substrates as well (Bogoyevitch & Kobe, 2006).

There are other well-established activators of the JNK that contribute to the complexity of upstream events. For example, UV radiation was previously shown to activate JNK (Hibi *et al*., 1999). Since then, UV has been shown to use, in part, the pathway components involved in TNF signalling. Specifically, GCK-like MAPKKKKs can be activated by either TNF or UV (Diener *et al*., 1997; Shi & Kehrl, 1997) and simultaneous clustering of receptors for TNF, EGF and IL1 mimics the UV-induced activation of JNKs (Rosette & Karin, 1996). Thus, it is not surprising that a diversity of upstream regulators such as the small G-protein Ras (Adler *et al*., 1996) or phosphatidylinositol 3'-kinase (PI3K) (Kawakami *et al*., 1998) have been implicated in UV-activation of JNKs.

2.3.1 Effects of JNK activation

Persistent activation of the JNKs (either achieved through the over-expression of upstream activators or following exposure to appropriate external stimuli) has now been frequently associated with apoptotic cell death in a variety of cells (Widmann *et al*., 1997, Zhu *et al*., 2001, Zanke *et al*., 1996). JNK-mediated apoptosis following TNF-α signalling involves caspase 8-independent cleavage of BH3-interacting domain death agonist (BID), which relieves inhibition of caspase 8 activation by TNF receptor-associated factor 2 (TRAF2)– IAP1 (Deng *et al*., 2003).

Other evidence that suggests JNK could induce apoptosis came from the observation that *jnk*1$^{-/-}$*jnk*2$^{-/-}$mice were resistant to apoptosis induced by UV irradiation and anisomycin (Tournier *et al*., 2000). Since UV was unable to induce cytochrome C release or depolarisation of mitochondrial membrane potential in *jnk1*$^{-/-}$*jnk2*$^{-/-}$mice, it was proposed that JNK is an intrinsic component of the mitochondrial-dependent death pathway during stress-induced apoptosis (Davis, 2000). In another report, it was also observed that in Chinese Hamster Ovary (CHO) cells, constitutively active JNKK2-JNK1 fusion protein was sufficient to induce apoptosis by activating the intrinsic death pathway (Lei *et al*., 2002). Likewise, inhibition of JNK prevents apoptosis of multiple myeloma cells (Xu *et al*., 1998), the death of sympathetic neurones upon NGF-withdrawal (Eilers *et al*., 2001), or the IL-1-induced apoptosis of pancreatic-cells (Bonny *et al*., 2001). Overexpression of a dominant-negative JNK1 mutant has been shown to develop resistance to UV-induced apoptosis of a small cell lung carcinoma cell line (Butterfield *et al*., 1997).

However, there have been suggestions that JNK may function as a modulator of apoptosis, rather than as a discrete genetic component of the apoptotic machinery to induce apoptosis. The best example is the regulation of TNF-α-induced apoptosis by JNK activation. NF-κB suppresses TNF-α induced apoptosis by inhibiting caspases and preventing prolonged JNK activation (Lin, 2003). In the absence of NF-κB activation, TNF-α induces apoptosis via activation of caspases and prolonged JNK activation (Tang *et al*.,

2002). However, prolonged JNK activation alone did not induce apoptosis, but was able to promote TNF-α- induced apoptosis in the absence of NF-κB activation (Karin & Lin, 2002; Lin, 2003). It also remains to be determined whether the model of "breaking the brake" on apoptosis can explain the proapoptotic role of JNK in apoptosis induced by other death insults.

On the other hand, JNK activation following exposure to environmental stresses has not always been associated with enhanced apoptotic death. For example, JNK activation is not involved in the apoptotic death following detachment of Madin-Darby Canine Kidney (MDCK) epithelial cells (Khwaja & Downward, 1997) and is not required for Fas-induced apoptosis of a proB cell line (Low *et al*., 1999). Furthermore, JNK activation does not always accompany apoptotic cell death, as has been observed in the case of growth factor withdrawal-induced death of cerebellar granule cells (Gunn-Moore & Tavare, 1998) or surface IgM-mediated apoptosis of WEHI 231, a murine B cell lymphoma line (Lee & Koretzky, 1998). In mice deficient in both *jnk*1 and *jnk*2, the hindbrain and forebrain regions (Kuan *et al.,* 1999; Sabapathy *et al*., 1999) had enhanced apoptosis. These observations suggest that JNK may be involved in cell survival in certain brain regions during development, although the underlying molecular mechanism remains to be elucidated.

To further complicate matters, JNK activation might actually enhance cell survival under some circumstances. Thus, as examples, JNK activation enhances cell survival in response to TNF-α (Roulston *et al.*, 1998), protects HeLa cells from apoptosis following photodynamic therapy (Assefa *et al.*, 1998) and protects myocytes from nitric oxide-induced apoptosis (Andreka *et al.*, 2001). JNKs have also been implicated in proliferation of the IL3-dependent BAF3 cells (Smith *et al.*, 1997), prolactin-activation of proliferation of PC12 cells (Cheng *et al.*, 2000) and EGF-induced transformation of human lung carcinoma cells (Bost *et al.*, 1999).

JNK may also function as an anti-apoptotic protein kinase in certain tumours. Specific anti-sense oligonucleotides of JNK (JNKAS), which inhibits JNK activation, inhibited growth of certain types of tumour cells, probably by promoting apoptosis (Bost *et al.* 1999; Potapova *et al.,* 2002). The anti-apoptotic function of JNK may be related to the status of p53 since JNKAS inhibited growth of certain p53-deficient, but not p53 positive, tumour cells (Potapova *et al.*, 2002). It is likely that activation of the JNK pathway inhibits p53-induced cell cycle arrest and therefore promotes p53-induced apoptosis (Shaulian & Karin, 2002). Thus, JNK may only exert its anti-apoptotic function in p53-deficient tumour cells. The anti-apoptotic function of JNK in tumor cells suggests that JNK may be involved in tumorigenesis. Indeed, it has been reported that JNK functions as an anti-apoptotic molecule in an isoform-specific manner in certain tumors. Tumour promoter 12-O-tetradecanolyphorbol-13-acetate (TPA)-induced skin papillomas were

significantly suppressed in *jnk2* null mice due to enhanced apoptosis when compared to wild-type mice (Chen *et al.,* 2001). In contrast, TPA-induced skin tumours were augmented in *jnk1* null mice (She *et al.,* 2002).

2.3.2 JNK scaffolding

Specificity in JNK activation from cytokine receptors or even stresses can also be achieved through use of scaffolding proteins. The scaffolding proteins will coordinate MAPKKK binding to specific proteins for upstream inputs as well as specific downstream MAPKK-MAPK complexes. The specificity is due to the role of specific scaffolding proteins, which lack enzymatic activity but act as important organisers of each specific pathway. This requirement for such an "organiser" was originally exemplified by the Ste5 scaffold in the yeast pheromone-responsive pathway (Figure 2.5). By binding to multiple members of the cascade into a multimolecular complex, Ste5 organises a functional MAPK pathway module with concomitant increases in signalling specificity, efficiency and amplitude. Hence, Ste5 facilitates proper functioning of the pheromone responsive MAPK pathway by promoting efficient activation of its specific components as well as by minimising interference from components of other MAPK modules (Choi *et al*., 1994).

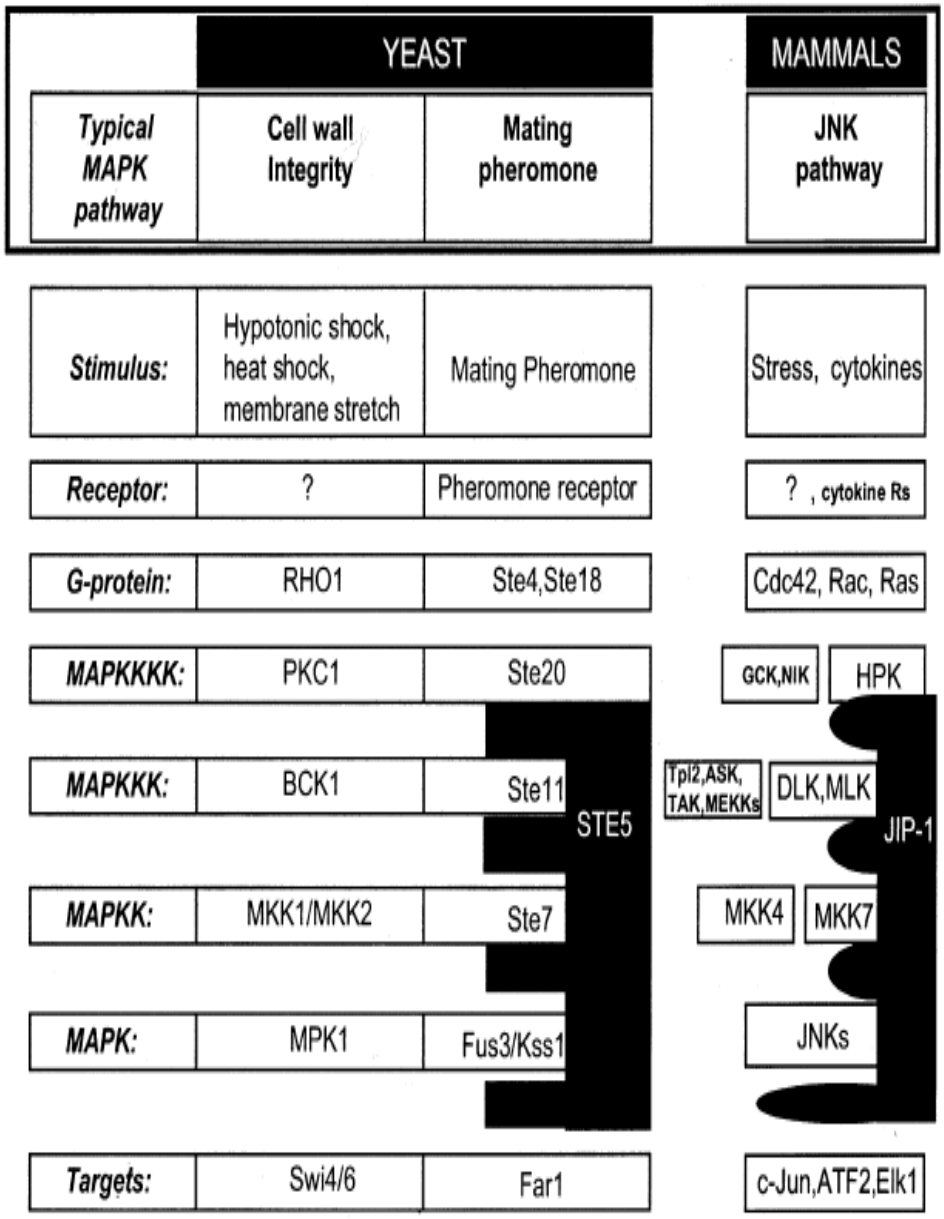

Figure 2.5: The JNK scaffold, JIP-1 and its yeast counterpart, STE5. Similar to its yeast counterpart, the JIP-1 assemble JNK and its upstream kinases MKK4/7 and other MAPKKKs in scaffold to facilliate cellular signaling (Bogoyevitch & Kobe, 2006).

Scaffolding molecules for two mammalian MAPK signalling cascades have been identified. These include, of particular interest, the JNK-interacting protein 1 (JIP-1) that acts in the JNK pathway (Dickens *et al*., 1997). JIP-1 was first identified as a binding partner of JNK1 in yeast two-hybrid analysis. In fact, JIP-1 was originally shown to interact directly with JNK1 and JNK2 (but not ERK or p38) and could neither bind nor enhance the activity of other MAPKs (Dickens *et al*., 1997; Bonny *et al*., 1998). Furthermore, JNK bound JIP-1 with greater affinity than its transcription factor substrates, c-Jun and activating transcription factor-2 (ATF2) (Dickens *et al*., 1997). This high affinity interaction was most likely mediated by a region of JIP-1 showing similarity to the JNK-binding domains (JBDs) of c-Jun and ATF2.

The scaffold protein JIP-1 has been shown to facilitate signal transmission through a specific set of protein kinases that activate JNKs. Apparently JIP-1 scaffolding was specific for the signalling pathway formed by the JNKs and their upstream kinase MKK7 (Whitmarsh *et al*., 1998; Whitmarsh *et al*., 2006). Thus, JIP-1 is likely to allow efficient signal transduction by concentrating JNK pathway components in a restricted area of the cell. Although JNKs are located in both the cytoplasm and nucleus of quiescent cells, activated JNKs can translocate to the nucleus (Cavigelli *et al*., 1995). However, JIP-1 overexpression retains JNKs in the cytoplasm despite the presence of activating stimuli. This prevents the phosphorylation of transcription factors such as c-Jun and ATF2 (Dickens *et al*., 1997). It has also been demonstrated that Akt1 and JIP1 associate with each other directly in

primary neurons, connecting Akt, a pro-survival kinase into the MAPK signalling cascade as well (Kim *et al.,* 2002). Thus, a balance of protein kinases and scaffolding molecules is critical for mediating kinase activation or inhibition.

2.4 Upstream Kinases of JNK

2.4.1 MKK4/7

The catalytic domains of MKK4 and MKK7 exhibit around 40% sequence similarity with the other members of the MKK family. It is composed of 11 sub-domains, which fold into a small, mainly β-stranded, N-terminal lobe and a larger helical C-terminal lobe (Sanchez *et al.,* 1994; Yao *et al*., 1997). The ATP binding site is located in the cleft formed between the two lobes and is surrounded by conserved residues. The main difference between the MKK4 and MKK7 catalytic activities resides in the ability of MKK4 to activate both JNKs and p38 MAPKs, while MKK7 is a specific activator of JNKs (Sanchez *et al.,* 1994; Yao *et al*., 1997).

The specificity of MKK4 and MKK7 for JNK has been observed in knockout mice experiments. Specifically, the disruption of MKK4 was embryonic lethal in mice following embryonic day 12.5 and was associated with abnormal liver development (Ganiatsas *et al*., 1998). This phenotype was similar to embryos lacking c-Jun, thus suggesting that MKK4 was critical for the phosphorylation and activation of c-Jun during liver development. The

deletion of MKK7 was also embryonically lethal and was caused by JNK (Dong *et al*., 2000).

As for their catalytic abilities, the activities of MKK4 and MKK7 are increased following phosphorylation at serine and threonine residues within a Ser-X-Ala-Lys-Thr motif in their activation loops. This phosphorylation is mediated by various MAPKKKs, including mixed lineage protein kinases (MLKs) and MAPK/ERK kinase (MEK) kinase (MEKK1) (Davis, 2000). Activated MKK4 and MKK7 in turn activate JNK by dual phosphorylation of the Thr-Pro-Tyr motif located in JNK's activation loop. Although MKK4 and MKK7 are dual specificity kinases (threonine and tyrosine kinases), previous studies of JNK activation have shown that MKK4 preferentially phosphorylates the tyrosine residue whereas MKK7 phosphorylates the threonine residue. This activation mechanism was supported by *in vivo* studies of mouse embryonic stem (ES) cells bearing targeted disruptions of the *MKK4* and/or *MKK7* genes (Kishimoto *et al*., 2003; Wada *et al*., 2001). Biochemical analyses of JNK signalling in living ES cells from these animals have demonstrated that tyrosine phosphorylation by MKK4, followed by threonine-phosphorylation by MKK7, leads to synergistic JNK activation in response to stress (Nishina *et al*., 2004).

With regard to TNF-α signalling, TNF-α has been shown to activate MKK4 in mouse bone marrow-derived macrophages (Winston *et al*., 1997). Mutant MKK4 has been shown to increase the rate of TNF-induced apoptosis

(Roulston *et al.*, 1998). Several gene products that mediate suppression of apoptosis and are induced by TNF-α are down-modulated in MKK4 knocked-out cells (Sethi *et al.*, 2007). The expression of TNF-α-induced anti-apoptotic proteins such as survivin, IAP1, XIAP, Bcl-2, Bcl-xL, and cFLIP was inhibited in kinase-deleted cells. All these results indicate that MKK4 has a pro-survival role. These results are consistent with another report that MKK4 promotes cell survival by activating PI3K through an NF-κB/PTEN-dependent pathway (Xia *et al.*, 2007).

2.4.2 Cross-talk with Akt

Akt is a serine/threonine kinase with diverse roles related to the regulation of cell growth, proliferation, migration, glucose metabolism, transcription, protein synthesis, angiogenesis and cell survival (Brazil & Hemmings, 2001). There are three isoforms (Akt1, Akt2 and Akt3) which are widely expressed, but Akt1 is predominantly expressed in most tissues (Kandel & Hay, 1999). The Akts are in the family of the AGC kinases (PKA, PKG, and PKC related kinases), which are characterised by a central kinase domain and a C-terminal hydrophobic motif.

Activation of Akt occurs following the binding of a protein growth factor to its receptor on the surface of the cell (Figure 2.6). Ligand binding induces autophosphorylation of tyrosine residues in the cytoplasmic portion of the receptor, resulting in the recruitment and activation of phosphatidylinositol

3-kinase (PI3K). More specifically, PI3K phosphorylates phosphatidylinositol 4,5 biphosphate (PIP2) to phosphatidylonositol 3,4,5-triphosphate (PIP3), which mediates the localisation of Akt to the inner surface of the cell membrane by interaction with its pleckstrin homology domain (PH). PIP3 can be dephosphorylated by phosphatase and tensin homologue deleted on chromosome ten (PTEN), which then serves as a negative regulator of Akt activation (Fayard *et al*., 2005).

Once localised to the inner surface of the cell membrane, Akt is activated by phosphorylation at 2 critical residues: threonine 308 in the kinase domain and serine 473 in the hydrophobic motif. The threonine 308 kinase is 3-phosphoinsotide-dependent kinase 1 (PDK1). Like Akt, it is localised to the inner surface of the cell membrane by an interaction between PIP3 and its PH (Hanada *et al*., 2004). The kinase for the serine 473 residue in the hydrophobic motif had been elusive and the subject of debate for many years, but Sarbassov and colleagues provided compelling evidence that it is a complex consisting of mammalian target of rapamycin (mTOR), G-protein S-subunit-like protein (mLST8/GβL) and rictor (Sarbassov *et al*., 2005a; Bayascas & Alessi, 2005).

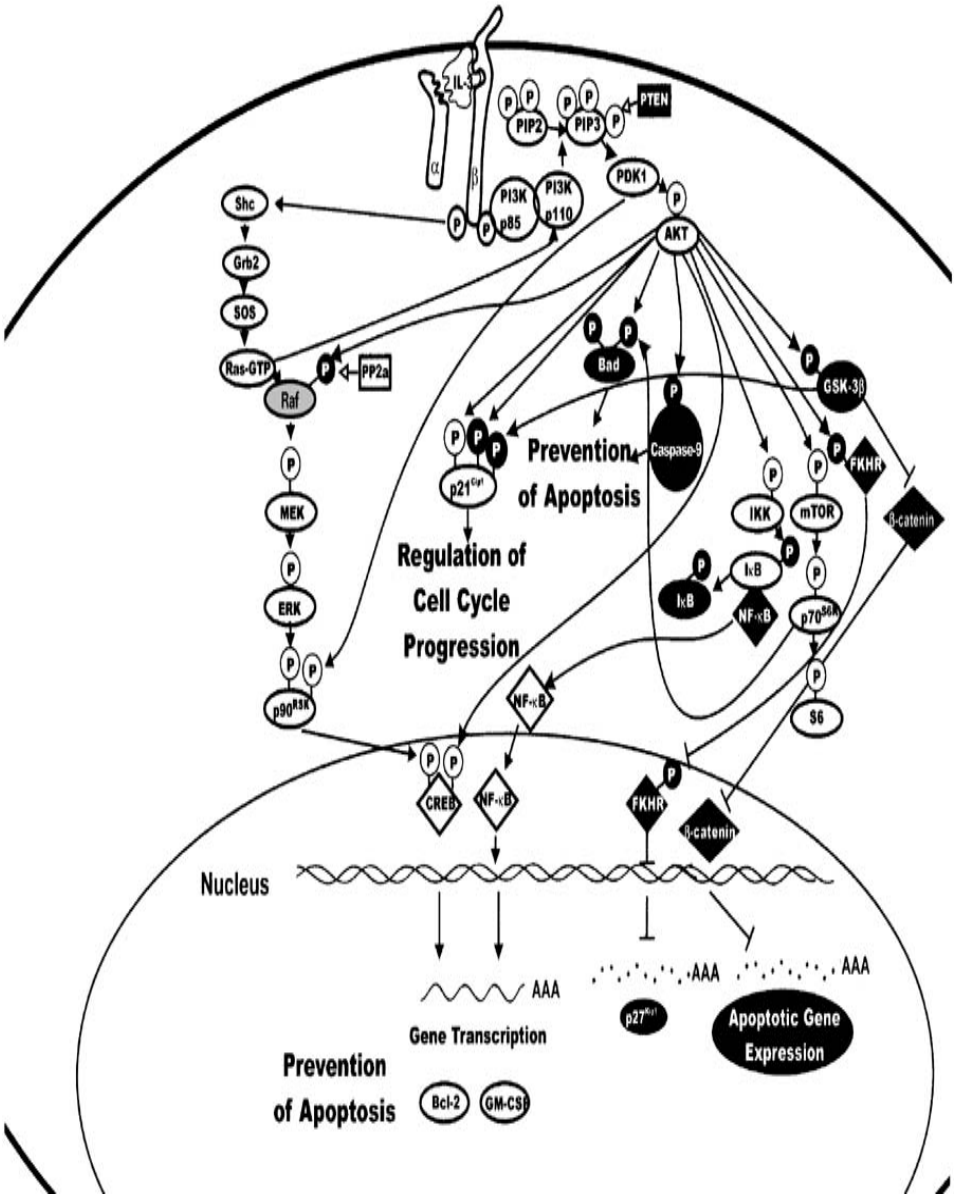

Figure 2.6: Schematic representation of Akt pathway.
The Akt pathway is activated by the membrane localization of PI3K. PIP2 is phosphorylated by PI3K to yield PIP3, which promotes membrane localization of PDK1 through its PH domain. PDK1 then phosphorylates and activates Akt. Proteins activated by S/T or Y phosphorylation are indicated with a black P and a clear circle. Proteins inactivated by S/T or Y phosphorylation are shown with a white P in a black circle. Transcription factors are indicated by diamonds. Phosphatases are indicated by rectangles. Phosphatases that inhibit activity, such as PTEN and SHIP, are indicated in black rectangles. Open arrows indicate phosphate groups hydrolyzed by phosphatases (Chiang & Abraham, 2005).

The first evidence that PI3K/Akt signalling plays a role in supporting the survival of neurons was obtained in studies of NGF-treated PC12 cells (Yao & Cooper, 1995). Subsequently, other investigators confirmed that PI3K/Akt signalling could prevent cell death in a variety of other tissue culture models utilising cerebellar, sympathetic (Crowder & Freeman, 1998), sensory, cortical and motor neurons (Kaplan & Miller, 2000).

Akt was also implicated in mediating neuronal survival as demonstrated by Dudek *et al*. (1997). They further demonstrated that survival was enhanced by transfection with wild-type Akt. Conversely, Luo *et al*. (2003) also showed that in a variety of tissue culture models, deactivation of Akt accompanies cell death. Since these initial observations, a large number of studies has demonstrated that Akt protects from apoptosis due to a wide variety of death-inducing stimuli, including the withdrawal of growth factors, UV irradiation, matrix detachment, cell cycle disturbance, DNA damage, and treatment of cells with anti-Fas antibody (Datta *et al*., 1999).

In the context of JNK signalling, Akt negatively regulates the phosphorylation and activation of c-Jun by a number of mechanisms. In some cellular contexts, MAPKKK are activated by the small GTP binding proteins Rac1 or Cdc42. Both of these signalling proteins have been shown to participate in NGF withdrawal-induced apoptosis in sympathetic neurons (Bazenet *et al*., 1998) and in PC12 cells (Xu *et al*., 2001). Akt has been shown

to deactivate Rac1 by phosphorylating it at serine 71, and thereby reducing its ability to bind GTP (Kwon *et al*., 2000).

Downstream of Rac1 and Cdc42, Akt also negatively regulates MLK3. Barthwal *et al*. (2003) have demonstrated that Akt deactivates MLK3 directly, via a C-terminal domain. Within this domain, Akt phosphorylates serine 674, resulting in diminished JNK activation by MLK3, and decreased cell death. The ability of insulin to attenuate MLK3, MKK7 and JNK activation was dependent on PI3K (Barthwal *et al.,* 2003). Thus, in this system, the prosurvival effects of insulin appear to be mediated by PIK3/Akt signalling, and are dependent on Akt phosphorylation of MLK3. Further downstream of the MLK, Akt also interacts with and phosphorylates MKK4 at serine 78 (Park *et al*., 2002). Phosphorylation at this residue inhibits MKK4-mediated apoptosis. MLK3 is not the only MAPKKK target of Akt. ASK1 is a MAPKKK which activates both JNK and p38 (Ichijo *et al*., 1997). Kim *et al*. (2001) have demonstrated that Akt binds to ASK1, phosphorylates it at serine 83, and reduces its kinase activity. This modification of ASK1 results in a reduced activation of JNK, and a reduction of apoptosis in cell lines.

Moreover, Akt activation has been linked in cancer cells with increased resistance to apoptosis induced by TRAIL/APO-2L (TNF-Related Apoptosis-Inducing Ligand), a member of the TNF superfamily that has been shown to have selective anti-tumour activity. Similarly, Akt is activated by the

phosphorylation of the transcription factor cyclic AMP response element-binding protein (CREB), and the IκB kinase (IKK), a positive regulator of NF-κB. Both of them regulates the expression of genes with anti-apoptotic activity (Chen *et al*., 2001; Kandasamy & Srivastava, 2002; Wang *et al*., 2002; Yuan & Whang, 2002). An anti-apoptotic effect of Akt has been demonstrated to be mediated through activation of mTOR (McCormick, 2004; Wendel *et al*., 2004). Wendel *et al*. (2004) have demonstrated in a murine lymphoma model that the anti-apoptotic effects of Akt can be blocked by rapamycin, an inhibitor of mTOR. However, it has been shown that rapamycin inhibition of mTOR actually occurs at mTORC1, a complex which consists of mTOR, Raptor, mLST8 and Rheb (Sarbassov *et al*., 2005a). To complicate matters, mTOR is also a component of mTORC2, which consists of mTOR, Rictor, mLST8 and Sin1 which is insensitive to rapamycin (Sarbassov *et al*., 2005b). The anti-apoptotic effect of Akt could be restored by eIF-4E, and this effect was not blocked by rapamycin, indicating that eIF-4E is downstream of Akt. Akt is also activated in a positive feedback loop by mTORC2, which suggests another level of regulation, since mTOR was previously merely recognised as an Akt substrate (Sarbassov *et al*., 2005b).

2.4.3 Cross-talk with mTOR

mTOR is a member of the phosphoinositide-3-kinase-related kinase (PIKK) family, which plays central roles in cell growth- and stress-related signalling pathways (Abraham, 2004). mTOR forms two complexes, namely the mTORC1 and mTORC2. A pivotal upstream signal for mTORC activation

involves the stimulation of Class I PI3Ks by members of the receptortyrosine kinase family (Figure 2.7). Membrane-localised Akt is subject to two activating phosphorylation events, one (at threonine 308) executed by PDK1, and the second (at serine 473) mediated at least in part by mTORC2. The complexity of the mTOR signalling network is underscored by the observation that mTOR in one complex (mTORC2) is an upstream activator of Akt. However, in another form (mTORC1), mTOR is a downstream recipient of Akt dependent stimulatory signals (Abraham, 2004).

Activated Akt stimulates mTORC1 through the heterodimeric tuberous sclerosis complex (TSC), which is a heterodimer that comprises TSC1 and TSC2 subunits (Dann *et al*., 2007; Wullschleger *et al*., 2006). TSC functions as a GTPase activating protein (GAP) for the small, Ras-related GTPase, Rheb (Ras homolog enriched in brain). Recent evidence suggests that the active, GTP-bound form of Rheb binds directly to and stimulates mTORC1 (Long *et al*., 2005; Sancak *et al*., 2007). Due to the fact that Rheb-GTP selectively targets mTORC1, it seems likely that raptor is involved in connecting Rheb to mTOR. As a Rheb-specific GAP, TSC acts as a signal-modulated suppressor of mTORC1.

According to one model, Akt stimulates the accumulation of GTP-bound Rheb, which in turn promotes the activation of mTORC1 by disrupting the TSC complex through phosphorylation of the TSC2 subunit. However, this

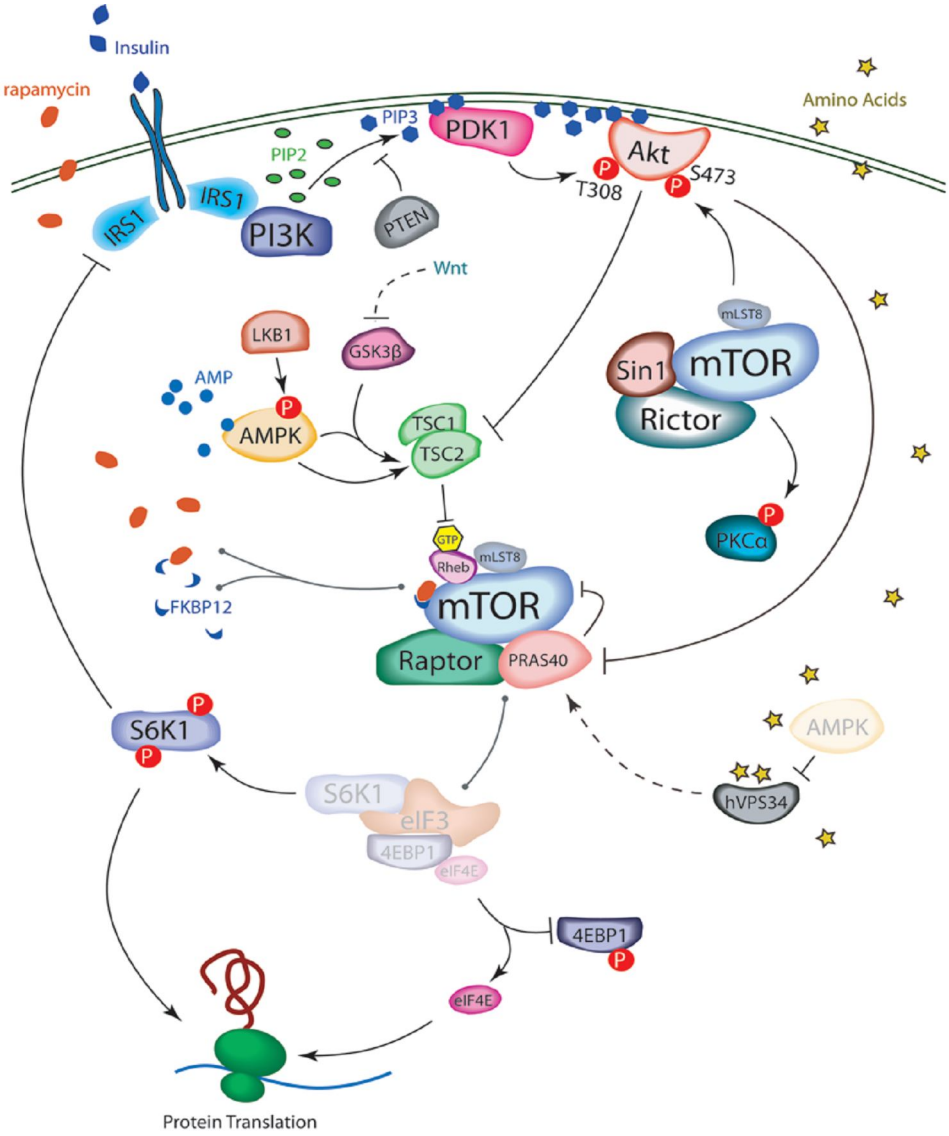

Figure 2.7: **mTOR signalling network in mammalian cells.** mTORC1, the rapamycin-sensitive complex, consists of mTOR, Raptor, mLST8, and PRAS40. TSC1/2-Rheb is the major upstream regulator of mTORC1. Through the TSC1/2-Rheb axis, mTORC1 integrates cellular energy levels, growth factors, and Wnt signals to regulate protein translation by phosphorylating S6 Kinase 1 (S6K1) and 4EB-P1. Phosphorylated S6K1 (active) inhibits IRS1 function and thus attenuates insulin/PI3K signalling. The mTORC2 subunits include mTOR, Rictor, Sin1, and mLST8. mTORC2 controls cell structure and survival by regulating PKCα and Akt. The upstream regulation of mTORC2 remains unclear (Chiang & Abraham, 2005).

mechanism of mTORC1 activation by Akt might not be universally applicable as evidences from both *Drosophila* and mammalian model systems suggest that Akt regulates Rheb-mTORC1 through pathways that do not require TSC2 phosphorylation (Dong & Pan, 2004; Hahn-Windgassen *et al*., 2005).

Activation of mTOR by Akt is achieved indirectly by Akt phosphorylation and inhibition of tuberin (tuberous sclerosis complex [TSC2]). Tuberin/TSC2 functions in a complex with hamartin/TSC1 as a GTPase-activating protein (GAP) to inhibit a Ras-related small GTPase Rheb (Manning & Cantley, 2003). Rheb is positive regulator of mTor signaling. Thus, Akt ultimately activates mTOR by blocking negative regulation of Rheb (Manning & Cantley, 2003).

In this study, mTOR was selected as a potential JNK regulator in HepG2 cells as it has been implicated as both a substrate and an activator of JNK in other cell models (Fujishita *et al.,* 2011; Hiratani *et al.,* 2005; March & Winton *et al.,* 2011). In a study by Huang *et al.* (2003), mTOR was also implicated as a JNK suppressor in human rhabdomyosarcoma cells. Observations from this study may enable other researchers to further unravel the complexity of cell signalling and interaction, specifically on JNK and mTOR cross-talk.

2.5 Transcription factors

2.5.1 c-Jun

c-Jun is a major component of the AP-1 transcription factor complex and together with JunB and JunD forms the family of mammalian Jun proteins (Mechta-Grigoriou *et al*., 2001). While the Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) do not form homodimers but can heterodimerise with members of the Jun family, the Jun proteins (c-Jun, JunB and JunD) can both homodimerise and heterodimerize with other Jun or Fos members to form transcriptionally active complexes (Hai & Hartman, 2001). In addition to Fos proteins, Jun proteins can also heterodimerise efficiently with other transcription factors, such as members of the activator transcription factor (ATF) family (Hai & Hartman, 2001) and other basic zipper containing transcription factors such as CBP, MyoD, NFAT or c-Rel (Herdegen & Leah, 1998).

A key regulatory component in the post-translational function of the c-Jun is the serine (and possibly the threonine) phosphorylation of the N-terminal transactivation domain of this transcription factor. Phosphorylation increases the transcriptional activity of c-Jun as well as that of the related protein JunD (Yazgan & Pfarr, 2002). Studies on the phosphorylation of c-Jun and related transcription factors have provided many useful insights into the mechanisms of JNK-mediated phosphorylation (Gupta *et al*., 1996; Katz *et al*., 2001). The phosphorylation sequences in c-Jun conform to the general consensus motif (Pro)-X-Ser/Thr-Pro [(P)-X-S/T-P], as originally defined by substrate

phosphorylation studies using the archetypical MAPKs, the ERKs (Bogoyevitch & Kobe, 2006). This consensus sequence indicates the ability of MAPKs to phosphorylate either serine or threonice residues (S/T) within a proline (P)-containing sequence.

The initial studies on JNK-mediated phosphorylation of c-Jun also revealed a requirement for amino acid sequences, known as the δ domain or the JNK-binding domain (JBD), distant from the amino acids to be phosphorylated (Kallunki *et al*., 1996; May *et al*., 1998). These distant targeting domains mediate interactions of other MAPKs with their substrates, upstream activators, phosphatases, and scaffold proteins (Tanoue *et al*., 2000) and thus are more generally termed common docking (CD) domains. The use of docking domains by MAPKs can enhance the efficiency and specificity of substrate phosphorylation (Galanis *et al*., 2001).

2.5.2 Liver X Receptor (LXR)

The Liver X Receptors (LXRs) are cholesterol sensors that respond to elevated sterol concentrations, and transactivate a set of genes that govern transport, catabolism, and elimination of cholesterol (Lu *et al*., 2001). LXRs also regulate a number of genes involved in fatty acid metabolism (Repa *et al*., 2000; Schulz *et al*., 2000). LXR has two isoforms, namely LXR-α and LXR-β. LXR-α is abundantly expressed mostly in liver (hence its selection in this study), and in other tissues associated with lipid metabolism, including adipose,

kidney, intestine, lung, adrenals, and macrophages, whereas LXR-β is ubiquitously expressed (Lu *et al*., 2001).

LXRs act as sterol sensors, protecting the cells from cholesterol overload by stimulating reverse cholesterol transport and activating its conversion to bile acids in the liver. In rodents, LXRs increase hepatic cholesterol catabolism (Peet *et al*., 1998) and to promote hepatic lipogenesis by activating sterol regulatory element-binding protein 1c (SREBP-1c) and its target genes (Chu *et al*., 2006; Joseph *et al*., 2003; Talukdar & Hillgartner, 2006). Other LXR target genes that have been identified include the bile acid-detoxifying sulfotransferase (Sult2a9/2a1) (Uppal *et al*., 2006), estrogen sulfotransferase (Est/Sult1e1) (Song, 2001) and fatty acid transporter (Cd36). Cd36 has been shown to be an LXR target gene and an intact expression of Cd36 plays an important role in the steatotic effect of LXR agonists. It was recently reported that the loss of both LXR isoforms in mice resulted in increased basal expression of Cyp3a11 and 2b10, two most abundantly expressed drug-metabolizing enzymes and primary target genes of pregnane X receptor (PXR) (Gnerre *et al*., 2005). However, the mechanism by which LXRs affect the expression of CYP enzymes remains largely unknown.

In addition, LXR also has an anti-inflammatory role, which is relevant to this study. Other than their canonical role in cholesterol homeostasis, LXRs have emerged as important regulators of inflammatory gene expression and

innate immunity. Ligand-activated LXR blunts the induction of classical inflammatory genes such as iNOS, COX-2, MMP-9, and various chemokines in response to LPS, TNF-α, and IL-1β stimuli (Castrillo *et al.,* 2003a; Castrillo *et al.,* 2003b; Ogawa *et al.,* 2005). LXRs also have been shown to positively regulate expression of arginase II, a gene that may have anti-inflammatory effects through antagonism of NO signaling (Marathe *et al.,* 2006). LXR ligands have also been proven effective in the amelioration of inflammation in a number of *in vivo* assays, including models of contact dermatitis and atherosclerosis (Fowler *et al.,* 2003; Joseph *et al.,* 2002). It is therefore interesting to see if these anti-inflammatory reactions of LXR coincides with, or inhibits, JNK, which is a key regulator in inflammatory reactions in HepG2 cells.

2.5.3 Pregnane X Receptor (PXR)

The pregnane X receptor (PXR) is a member of the nuclear receptor gene superfamily of ligand-activated transcription factors expressed most prominently in hepatocytes and gut epithelium (Kliewer *et al*., 1998). The ligand binding site of the PXR is activated by a range of structurally diverse xenobiotics such as antibiotic rifampicin (RIF) and endobiotics (e.g. bile acids) (Kliewer, 2003). On activation, the PXR regulates the expression of a subset of genes encoding drug metabolising (e.g. CYP3A) and drug transporter proteins (e.g. MRP2), that contain response elements within their promoters (Kast *et al*., 2002). The canonical function of the PXR is therefore to sense elevations in

xenobiotics and endobiotics and to orchestrate a response that promotes xenobiotic/endobiotic metabolism and excretion (Kliewer, 2003).

A major regulator of inflammation is the transcription factor NF-κB which regulates the expression of a diverse array of genes associated with both innate and adaptive immunity, including many cytokines, chemokines, adhesion proteins and stress response genes (Ghosh & Karin, 2002). It has been shown that the p65 sub-unit of NF-κB interacts with the PXR dimerisation partner RXRα, and disrupts binding to promoters (Gu *et al*., 2006). This interaction may account for the inhibition in drug metabolism by inflammation (Morgan, 1997). However, it is now clear that the interaction of NF-κB with the PXR has a reciprocal inhibitory effect on NF-κB activity (Zhou *et al*., 2006). This has been clearly documented in the gut, which has increased NF-κB target gene expression in PXR-null mice (Zhou *et al*., 2006). This may be of relevance to humans since PXR polymorphisms have been linked to increased inflammation of the bowel in man (Dring *et al*., 2006). A study has shown that PCN (a rodent-specific activator of the PXR) inhibits the expression of several NF-κB-dependent inflammatory genes in a mouse model of inflammatory bowel disease, an effect lost in $PXR^{-/-}$ mice (Shah *et al*., 2007).

It has also been revealed that PXR has a non-canonical function that results in an inhibition in the progression of liver fibrosis (Marek et al., 2005; Wallace *et al.,* 2008; Wright, 2006). Human fibrogenic myofibroblasts

express significant levels of PXR mRNA and protein. Treatment with PXR activators inhibits their trans-differentiation from hepatic stellate cells to fibrogenic myofibroblasts; inhibits myofibroblast expression of the major pro-fibrogenic cytokine TGFβ and markedly slows myofibroblast proliferation *in vitro* (Haughton *et al.,* 2006). Using mice with a disrupted PXR gene, it was also shown that pregnenolone 16α carbonitrile (PCN), a potent activator of rodent PXR, inhibited fibrosis *in vivo* in a PXR-dependent manner (Marek *et al.,* 2005). These observations may be of significant clinical value because some chronic liver disease patients are treated with PXR activators for extended periods, most notably patients with primary biliary cirrhosis (PBC). Resultant cholestasis over many years leads to chronic liver damage, fibrosis and cirrhosis. There is no treatment for PBC but many patients are often treated with the PXR activator rifampin (Khurana & Singh, 2006; Schuetz *et al.,* 2001). Therefore, it is imperative that PXR be studied in this work so that other avenues linking it to other regulators of inflammation, such as JNK or even mTOR, can be discovered. This will open up new strategies for PXR-based drug design to counter chronic and/or acute inflammation (Wallace *et al.,* 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 HepG2 culturing

3.1.1 Thawing of cells

HepG2, a human hepatocarcinoma cell line was obtained courtesy of Dr. Tan Mei Lan from Universiti Sains Malaysia. The cells were thawed at 37°C, and transferred to a sterile 15 mL centrifuge tube. Approximately 5 mL of Minimum Essential Medium (MEM) (Gibco, Invitrogen, USA), supplemented with 1% Pennicilin-Streptomycin (Gibco, Invitrogen, USA), 1% sodium pyruvate (Gibco, Invitrogen, USA), and 1% non-essential amino acids (Gibco, Invitrogen, USA) were added and the cells resuspended thoroughly. The cells were then centrifuged at 3 000 rpm using a microcentrifuge (Sorvall, Thermo Scientific, USA) for 5 mins. The supernatant were removed and the cell pellet resuspended in 4 mL MEM supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, Invitrogen, USA), which was subsequently transferred into a 25 cm^2 T-flask (TPP, Switzerland). The cells were incubated at 37^oC in a humid atmosphere of air containing 5% (v/v) CO_2 in a BD115 CO_2 incubator (Binder, USA). The medium was changed every 3 days until approximately 90% confluency were achieved.

3.1.2 Maintenance of cells

After 3 days, the spent medium was removed and the adherent cells washed with 4 mL of Phosphate Buffered Saline (PBS) (Amresco, USA) solution twice. About 4 mL of fresh MEM supplemented with 10 % FBS were added to the 25 cm^2 T-flask. The medium was changed every 3 days until approximately 90% confluency were achieved. For 75 cm^2 T-flasks, 15 mL of MEM supplemented with 10% FBS were used for cell maintenance and 4 mL of PBS were used for rinsing and aspirating the cells.

3.1.3 Subculturing of cells

The cells were rinsed with PBS as previously described, and 4 mL (for 75 cm^2 T-flasks) or 2 mL (for 25 cm^2) of TrypLE Express Stable (Invitrogen, USA) were added. The cells were then incubated at 37°C for 15 min in a 37°C, 5% CO_2 incubator, and subsequently the trypsin solution containing the cells was transferred in a sterile 15 mL centrifuge tube. Approximately 6 mL (for 75 cm^2 T-flasks) or 3 mL (for 25 cm^2) of MEM was then added to the centrifuge tube, and the cells were spun down at 3 000 rpm in a Sorvall Micro 17 microcentrifuge (Thermo Scientific, USA) for 5 min. After centrifugation, the supernatant was removed, and 4 mL of fresh MEM were added. The cells were thoroughly resuspended in 4 mL of fresh MEM supplemented with 10% FBS. The resuspended cells were then split into four 25 cm^2 T-flasks (1 mL into each). Each T-flask was topped up to 4 mL of fresh MEM supplemented with 10% FBS.

3.2 Treatment of cells

3.2.1 Dose and time response

When the cells reached approximately 50 - 60% confluency, they were washed twice with PBS. For dose response studies, 0, 5, 10, 20, and 50 ng/mL of TNF-α (Calbiochem, USA) together with MEM supplemented with 0.5% of FBS were added into respective flasks, and incubated for 24 h. For time course studies, 20 ng/mL of TNF-α in MEM supplemented with 0.5% of FBS were added into their respective flasks and incubated for 0, 2, 4, 8, 16 and 24 h. Total RNA and total protein were then extracted and quantified according to Section 3.4 and Section 3.5.

3.2.2 Inhibition of upstream kinases

To study the effects of inhibiting upstream kinases of other pathways on the activation of JNK, five sets of inhibitors were used. When the cells reached 50 – 60% confluency, the cells were washed twice with PBS, and the treated cells were dosed with 10 μM of PD98059, an MEK1 inhibitor (Calbiochem, USA) (Moon *et al.,* 2007); 10 μM of AG490, a JAK2 inhibitor (Calbiochem, USA) (Tsuchiya *et al.,* 2010); 1 μM of Herbimycin, a Hsp90 inhibitor (Calbiochem, USA) (Kasai & Kikuchi, 2010); 1 μM of SB203580, a p38 inhibitor (Calbiochem, USA) (Muniyappa & Das, 2008); and 100 nM of rapamycin, an mTOR inhibitor (Calbiochem, USA) (Sarbassov *et al*., 2006) in MEM supplemented with 0.5% FBS. The cells were then incubated at 37°C for 4 h, before they were washed twice with PBS, and all the cells were treated

with 20 ng/mL of TNF-α in MEM supplemented with 0.5% FBS. In addition to the five inhibited samples, two separate samples were treated without or in the presence of TNF-α as the unstimulated and stimulated control, respectively. The cells were incubated for 4 h in 37°C before the total RNA and cytoplasmic protein were extracted as described in Section 3.4 and Section 3.5. To ensure that the cells did not suffer any adverse effects which may be caused by the high concentrations of the inhibitors, they were monitored visually under an inverted microscope for any signs of apoptosis after 2 h of inhibitor pre-treatment.

3.3 Total RNA extraction and quantification

3.3.1 RNA extraction

Total RNA from the cells was extracted using the RNeasy Mini Kit (Qiagen, Germany). For this, 600 µL of the Buffer RLT was transferred to the T-flask and the cells were collected using a cell scraper (TPP, Switzerland). Subsequently the cell solution was transferred into a sterile 1.5 mL microcentrifuge tube (Axygen, USA). The cell solution was resuspended by vortexing before being lysed by passing the cells through a sterile 20 gauge (0.9 mm) needle for 5 times. When the cells were fully lysed, 600 µL of 70% ethanol were added to the tube, and the solution was mixed well by pipetting. The lysate was then transferred to the provided 2 mL spin column and centrifuged for 13 300 rpm in a microcentrifuge for 15 s. The flow through was discarded and the collection tube reused. Subsequently 700 µL of RW1

solution was added to the column, and centrifuged at 13 300 rpm in a microcentrifuge for 15 s. The flow through was discarded, and 500 µL of RPE was added to the column. The column was centrifuged at 13 000 rpm in a microcentrifuge for 15 s. The flow through was discarded, and 500 µL of RPE solution was again added to the column. The column was centrifuged in a microcentrifuge at 13 300 rpm for 2 mins. Using a new collection tube, the column was dried by centrifugation in a microcentrifuge at 13 300 rpm for 1 min. The column was then transferred to a new sterile 1.5 mL microcentrifuge tube. Subsequently, 30 µL of RNase-free water was added to the column, and the RNA was eluted by centrifugation in a microcentrifuge at 13 300 rpm for 1 min. Then, 6 µL of the eluted RNA was aliquoted into five separate 0.2 mL microcentrifuge tubes each as working solutions.

3.3.2 RNA quantification

For quantifying the concentration of RNA, 1 µL of RNA was used to determine its concentration and purity using a NanoDrop spectrophotometer (Thermo Fisher, USA). RNA formaldehyde agarose electrophoresis was performed to determine the integrity of RNA. Approximately 1.2 g of SeaKem LE agarose (Cambrex, USA) was added to 10 mL of 10X MOPS buffer (Amresco, USA) and 90 mL of DEPC-treated water to prepare 1.2% of formaldehyde agarose gel. The solution was then heated in a microwave to dissolve the agarose. About 1.8 mL of 37% formaldehyde (Merck, Germany) was then added when the solution was cooled down to approximately 60°C. The gel solution was cast in an electrophoresis tank and a 10-well comb

inserted into the gel. After the gel has solidified, the comb was removed and the tank was filled with 1X MOPS buffer. Then, 5 µL of RNA with 2 µL of RNA loading dye was loaded into a well. Electrophoresis was performed at 30 V for 30 min. The gel was then stained with ethidium bromide for 30 min, washed with water for 5 min twice, and visualised using the Fluorchem FC2 imager (Alpha Innotech, USA).

3.4 Protein extraction and quantification

3.4.1 Total protein extraction

Total protein was isolated from HepG2 cells after treatment using TRI-Reagent LS (Molecular Research, USA), according to the manufacturer's instructions. Briefly, cells were rinsed twice with PBS and scraped using a cell scraper (TPP, Switzerland). The detached cells were then homogenised by repeated pipetting in 0.75 mL Tri-Reagent LS. The homogenate was then transferred to a 1.5 mL microcentrifuge tube and incubated at room temperature for 5 min to allow complete dissociation of the nucleoprotein complex. Subsequently, 0.2 mL of chloroform was added and the mixture shaken vigorously for 15 s. The mixture was left at room temperature for 15 min and centrifuged at 13 300 rpm in a microcentrifuge for 15 min at 4^{o}C. Following centrifugation, the mixture was separated into a lower-pink phenol-chloroform (organic) phase, interphase and the colourless upper aqueous phase which contains protein, DNA and RNA, respectively. The aqueous phase was removed. Then, 0.225 mL of 100% ethanol was added and the samples mixed

by inversion, to precipitate DNA from the interphase and organic phase. Next, the samples were stored at room temperature for 2 - 3 min and DNA was sedimented by centrifugation at 3 500 rpm in a microcentrifuge for 5 min at 4^{o}C. The phenol-ethanol supernatant was removed for protein isolation. One volume of the phenol-ethanol supernatant was aliquoted into a microcentrifuge tube. The proteins were precipitated by adding 3 volumes of acetone. The samples were mixed by inversion for 10 - 15 s to obtain a homogeneous solution. Then, the samples were stored for 10 min at room temperature and the protein precipitate sedimented at 13 300 rpm in a microcentrifuge for 10 min at 4^{o}C. The phenol-ethanol supernatant was removed and the protein pellet was dispersed in 0.5 mL of 0.3 M guanidine hydrochloride in 95% ethanol + 2% glycerol (v/v). The pellet was dispersed using a pipet tip. After dispersing the pellet, another 0.5 ml aliquot of the guanidine hydrochloride/ethanol/glycerol wash solution was added to the sample and stored for 10 min at room temperature. The protein was sedimented at 11 000 rpm in a microcentrifuge for 5 min. The wash solution was removed and two more washes were performed in 1 ml each of the guanidine/ethanol/glycerol wash solution. The pellet was dispersed by vortexing after each wash to efficiently remove residual phenol. The final wash was performed in 1 ml of ethanol containing 2.5% glycerol (v/v). At the end of the 10 min ethanol wash, the protein was sedimented at 11 000 rpm in a microcentrifuge for 5 min. The alcohol was removed, the tube inverted and the pellet was air dried for 7 - 10 min at room temperature. After air-drying the protein pellet, 1% sodium dodecyl sulphate (SDS) solvent was added to the protein pellet. The pellet was gently dispersed and solubilized for 15 - 20 min by pipetting. The solubilised proteins were

stored at -20°C, if they were not required for immediate downstream processing.

3.4.2 Cytoplasmic and nuclear protein extraction

The stimulated cells were extracted using the Pierce NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher, USA) according to manufacturer's instruction. The cells were washed twice with ice-cold PBS, and subsequently 600 µL of PBS was added to the flasks. A cell scraper was used to scrape the cells. The cells were then collected into a sterile 1.5 mL microcentrifuge tube and resuspended by pipetting and vortexing. Following this, the cells were pelleted by centrifugation at 3 500 rpm in a microcentrifuge for 3 mins. The supernatant was subsequently removed and 100 µL of ice-cold CER I, together with 1 µL of Protease Inhibitor Cocktail II (100X) (Calbiochem, USA) were added. The tubes were vortexed at the highest speed for 15 s and immediately incubated on ice for 10 min. Then, 5.5 µL of CER II was added, vortexed at the highest speed of 13 300 rpm in a microcentrifuge for 5 s and incubated on ice for 1 min. The tubes were then vortexed for 5 min and centrifuged at 13 300 rpm in a microcentrifuge for 5 min. The supernatant (cytoplasmic proteins) were then transferred to cold tubes and stored at -80°C. For nuclear proteins, the pellets were subsequently resuspended with 50 µL of ice-cold NER together with 0.5 µL of protease inhibitor. The tubes were vortexed at the highest speed for 15 s and immediately incubated on ice for 10 min. The above process was repeated for four times. Then, the tubes were centrifuged at 13 300 rpm in a microcentrifuge for 10 min. The supernatant

(nuclear proteins) were then transferred to ice-cold tubes for immediate downstream processing.

3.4.3 Protein quantification

To quantify the amount of protein extracted, the DC Protein Assay (Bio-Rad, USA) was performed. Seven tubes (each containing 20 µL) consisting of 0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 µg/µL BSA were prepared using 10% SDS as solvent from stock BSA (Bio-Rad, USA) and used for standard curve construction. The protein samples (both cytoplasmic and nuclear) from Section 3.6.1 were diluted five-fold in 10% SDS. Approximately 4 µL of protein samples were diluted in 16 µL of 10% SDS. Five µL from all samples (each sample in triplicate) were then transferred into wells in a Nunc 96-well plate (Thermo Fisher, USA). Subsequently, 25 µL of Reagent A were added to all wells, followed by 200 µL of Reagent B. The plate was lightly shaken, and incubated in darkness at room temperature for 15 min. The plate was then read using an Infinite M200 optical plate reader (Tecan, Austria) at 750 nm. The average reading of each condition was obtained, and its concentration determined from the standard curve.

3.5 Polymerase Chain Reaction (PCR)

3.5.1 Primer design

The sequences for *JNK1* and *JNK2* genes were acquired from GenBank (http://www.ncbi.nlm.nih.gov/nuccore). The accession numbers for *JNK1* sub-isoforms are NM 002750.2, NM 139049.1, NM 139046.1 and NM 139047.1, while the accession numbers for *JNK2* sub-isoforms are NM 139068.2, NM 0002752.4, NM 139069.2 and NM 139070.2. As there are four sub-isoforms for each of the two genes, the primers were designed in conserved regions to amplify all four sub-isoforms of each gene. Two sets of primers were designed, *JNK1* and *JNK2* respectively, using Primer 3' Output (http://frodo.wi.mit.edu/). Homodimers, heterodimers and hairpins were analysed using the Premier Biosoft software available online (http://www.premierbiosoft.com/). The sequences of primers used for this study are as listed in Table 3.1. For the *LXR-α* and *PXR* primers, the sequences are as listed on Table 3.1.

Table 3.1: Nucleotide sequences of the primers used for RT-PCR and real-time RT-PCR and their expected product sizes.

Gene	Primer	Sequence	T_m / (°C)	Expected product size / bp
JNK1	JNK 1F	5'-CGTCTTCGTTCGCACTGTTG-3'	62.12	
	JNK2 R	5'-GCCTGTCGCACGTGATTGA-3'	59.87	175
JNK2	JNK2F	5'-GCCTGTCGCACGTGATTGA-3'	60.06	
	JNK2R	5'-GAACTCTGCTGATGGTGTTCC-3'	59.72	225
LXR-α	LXR-αF	5'-CGGGCTTCCACTACAATGTT-3'	57.63	
	LXR-αR	5'-TCAGGCGGATCTGTTCTTCT-3'	57.07	213
PXR	PXRF	5'-CAAATCTGCCGTGTATGTGG-3'	57.04	
	PXRR	5'-TCCCTGTCCGTTCACTTTTC-3'	57.3	289
β-actin	β-actinF	5'-CGTACCACTGGCATCGTGAT-3'	48.70	
	β-actinR	5'-CCATCTCTTGCTCGAAGTTC-3'	59.72	280

3.5.2 First-strand cDNA synthesis

The first step in the two-step reverse transcription is to subject the RNA to deoxyribonuclease (DNase) treatment, using the Mouse Moloney Murine Leukemia Virus (MMLV) 2-step Reverse Transcriptase kit (Promega, USA). The RNA was diluted to 50 μg/μL in RNase-free water. Then, 2 μL of the diluted RNA was pipetted into a 0.2 mL microcentrifuge cube (Axygen, USA), together with 1 μL of RQ1 buffer, 2 μL of RNase-free RQ1 DNase, and 5 μL of ribonuclease (RNase)-free water. The tube was placed in a 37°C water bath for 30 min. The DNase reaction was inhibited by adding 1 μL of RQ1 DNase. The tube was subsequently incubated at 37°C for 10 min. After DNase treatment, 11 μL of RNA and 1 μL random primers (250 ng/μL) were mixed together in an RNase-free 0.2 mL microcentrifuge tube. The tube was then incubated at 70°C for 10 min, and immediately placed on ice for 5 min. Subsequently, 5 μL MMLV 5X buffer, 1 μL dNTP (10mM), 0.4 μL RNasin ribonuclease inhibitor (40 U/μL), 1 μL MMLV-RT and 17.6 μL RNase-free water was added to the tube. The tube was further incubated at 37°C for 1 h. After that, it was incubated at 70°C for 10 min, and immediately placed on ice for another 2 min.

3.5.3 Gradient PCR

The first-strand cDNA synthesised in Section 3.6.1 was then used for gradient PCR to optimise the annealing temperature for the real-time PCR analysis. The kit used in this procedure was the GoTaq DNA Polymerase kit (Promega, USA). To amplify a target gene, 5 µL of 5X GoTaq PCR buffer, 2 µL of 25 mM $MgCl_2$, 1 µl of cDNA, 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM) and 1 µL of dNTP (400 µM), 0.2 µL of GoTaq polymerase (5 U/µL) and 13.8 µL of sterile water were added in a sterile 0.2 mL microcentrifuge tube. The PCR was conducted on a Mastercycler gradient thermocycler (Eppendorf, Germany) using the protocol described in Table 3.2. The PCR products were then visualised as described in Section 3.6.3.

3.5.4 PCR product visualisation

Agarose gel electrophoresis was carried out using 2% agarose gel in 0.5X TBE buffer (44.5 mM Tris-borate, 44 mM boric acid, 1 mM EDTA). Approximately 2.0 g of agarose was added to 10 mL of 0.5X TBE buffer to prepare 2% of agarose gel. Then, 2 µL of DNA with 1 µL of 6X DNA loading dye [0.25% bromophenol blue, 30% (v/v) glycerol] was loaded into a well. A 100 bp marker (Vivantis, Malaysia) was loaded as well. Electrophoresis was performed at 70 V for 1 h. The gel was then stained with ethidium bromide for 30 min, rinsed with water for 5 min twice, and visualised under UV in the Fluorchem FC2 imager (Alpha Innotech, USA).

Table 3.2: RT-PCR cycle used for amplifying human *JNK* cDNA

Step	Temperature	Time	Cycle
Initial denaturation	94°C	3 min	1
Denaturation	94°C	45 s	35
Annealing	59°C, 61°C, 63°C, 65°C, 67°C	1 min	
Extension	72°C	1 min	
Final extension	72°C	5 min	1

3.5.5 Gel extraction

Gel extraction was carried out using QIAquick Gel Extraction kit (Qiagen, Germany) following the manufacturer's instruction. Approximately 40 µL of the product was loaded into the well with 8 µL of 6X DNA loading buffer. Electrophoresis was performed at 70 V for 1 h. The band of interest was excised from the agarose gel previously stained with ethidium bromide using a sterile scalpel, and cut into fine pieces. One volume of the gel was then transferred into a clean 1.5 mL microcentrifuge tube. Then, 3 volumes of Buffer QG were added into the tube. The tube was incubated at 50°C for 10 min and vortexed every 2 min until the gel has completely dissolved, before adding 1 volume of isopropanol into the tube. Following this, 2 volumes of the sample was transferred to a QIAquick spin column and centrifuged at 13 300 rpm in a microcentrifuge for 1 min. The flow-through was discarded and the spin column was placed back in the same collection tube. The remaining 600 µL of the sample of transferred into the column, and spun for 13 300 rpm in a microcentrifuge for 1 min. Afterwards, the flow through was discarded and 500 µL of Buffer QG was added to the column. The column was centrifuged at 13 300 rpm in a microcentrifuge for 1 min. The flow-through was discarded and 750 µL of Buffer PE was added to the column. To wash, the column was centrifuged at 13 300 rpm in a microcentrifuge for 1 min. The flow-through was discarded and the column was centrifuged at 13 300 rpm in a microcentrifuge for 1 min to dry the column. The column was then transferred onto a sterile 1.5 mL microcentrifuge tube. To elute the DNA, 30 µL of sterile water was added directly to the QIAquick membrane and allowed to stand for 1

min, and the column was centrifuged at 13 300 rpm in a microcentrifuge for 1 min. The purified PCR product was visualised as described in Section 3.5.3. Then, 10 µL of the purified products was sent for sequencing (1st Base, Malaysia) to confirm the target sequence. The results were analysed for specificity using NCBI's BLASTN (http://blast.ncbi.nlm.nih.gov/).

3.5.6 One-step real-time RT-PCR

Quantifast SYBR Green RT-PCR (Qiagen, Germany) was used for performing one-step real-time RT-PCR. For the one-step reverse transcription process and subsequent quantitative real-time PCR, 12.5 µL Fast SYBR Green buffer, 2.5 µL of forward primer (10 µM), 2.5 µL of reverse primer (10 µM), 2 µL of RNA template (50 ng/µL) and 5.25 µL nuclease-free water were used per reaction. Amplification for β-actin was carried out to serve as the reference gene. All reactions were carried out in triplicates. The real-time PCR was performed on a MyIQ thermal cycler with an optical unit (Bio-Rad, USA). The one-step real-time PCR and melt curve protocols used are as listed in Table 3.3. The real-time PCR data was then analysed using the iQ5 software (Biorad). The PCR products were then visualised as described in Section 3.6.3.

Table 3.3: Real-time RT-PCR cycle and melting curve cycle

Step	Temperature	Time	Cycle
Reverse transcription	50°C	10 min	1
Initial denaturation	94°C	5 min	1
Denaturation	94°C	10 s	35
Annealing	*63°C, **58°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	5 min	1
Melting curve	95°C	1 min	1
	55°C	1 min	1
	60°C-95°C (set point temperature increased by 0.5°C	10 s	71

*Annealing temperature for *JNK1* and *JNK2*

**Annealing temperature for *LXR-α* and *PXR*

3.6 Western Blot

3.6.1 Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

Approximately 10 mL of resolving gel and 5 mL of stacking gel was prepared for this procedure as in Table 3.4. Ten percent ammonium persulphate and TEMED were added prior to polymerisation. Specifically, the resolving gel was added in between the glass plates to 75% of the plate height and layered with butanol. When the resolving gel has solidified (approximately 30 mins), the butanol was poured off and the gel was rinsed with sterile deionised water. Subsequently, the stacking gel was added above the resolving gel. A 10-well comb was inserted in between the plates. After the stacking gel has solidified for another 30 min, the comb was removed, and the wells rinsed with 1X SDS-PAGE running buffer.

The glass plates with the gel were then assembled in a SDS-PAGE tank, and the tank was filled with 1X SDS-PAGE running buffer to the required volume. Approximately 50 μg of all samples was added into sterile 0.2 mL microcentrifuge tubes together with 7 μL of 5X SDS loading buffer with 5% β-mecarptoethanol (Merck, Germany). The samples were heated at 99°C for 5 min and immediately cooled on ice afterwards. Each gel was loaded with a prestained, 7 – 175 kDa protein marker (New England Biolabs, USA). Electrophoresis was then carried out at 100 V for 90 min.

Table 3.4: Components of resolving gel and stacking gel for SDS-PAGE

	10% resolving gel	5% stacking gel
40% Bis-acrylamide (37.5:1) (Amresco, USA)	2.5 mL	0.625 mL
Resolving gel buffer (1.5 M Tris, 0.4 % SDS, pH 8.8)	2.5 mL	-
Stacking gel buffer (0.5 M Tris, 0.4 % SDS, pH 6.8)	-	1.25 mL
Sterile deionised water	4.89 mL	3.07 mL
10% ammonium persulphate (Amresco, USA)	100 µL	50 µL
TEMED (Bio Basic, Canada)	10 µL	5 µL

3.6.2 Membrane transfer

After the electrophoresis has completed, the gels were removed and the stacking gels were cut away. The gels were then immersed in Towbin transfer buffer (25 mM Tris, 192 mM glycine, and 20 % methanol in sterile deionised water) for 15 min. Meanwhile, Immobilon-P PVDF membranes (Millipore, USA) were cut to the gels' dimensions and soaked in methanol. Subsequently, the membranes were immersed in sterile deionised water for 2 min, before being transferred to the Towbin transfer buffer for 5 min. The filter papers (Bio-Rad, USA) were also cut to fit the gels' dimensions, and soaked in the Towbin transfer buffer for approximately 5 min. To assemble the Mini Trans-Blot (Bio-Rad, USA) transfer apparatus, a foam pad was placed on the anode side followed by a piece of filter paper. The foam pads were also soaked in Towbin transfer buffer prior to use. The polyacrylamide gel, which has been equilibrated in the transfer buffer, was laid on top of the filter paper followed by the PVDF membrane. Afterwards, a piece of filter paper was laid on top of the PVDF membrane followed by another foam pad. A glass rod was used to remove any air bubbles trapped. Lastly, the sandwich was assembled by clipping both the cathode side and anode side together tightly. The sandwiches were then assembled in the transfer tank and the tank was filled with ice-cold Towbin transfer buffer. The tank was then placed in a 4°C fridge. A stirrer was placed at the bottom of the tank to facilitate heat transfer. The high-intensity electrophoretic transfer was performed at 100 V for 30 min. When the transfer was completed, the membranes were immersed in methanol for 15 s and air-

dried on a filter paper (Whatman, USA) for 15 min. Each membrane was then placed between two filter papers and stored at -80°C.

3.6.3 Immunoblotting

Immunoblotting was performed using the SnapID Protein Detection System (Millipore, USA). The membrane was first left at room temperature for 10 min upon removal from -80°C. Then, the membrane was immersed in methanol for 15 s and subsequently soaked in sterile deionised water for 2 min. The membrane was processed according the the manufacturer's instructions.

Briefly, the membrane was placed in a blot holder. Then, 10 mL of blocking buffer (1% FBS in Tris Buffered Saline [TBS], supplemented with 0.1% Tween-20) was added onto the blot holder. Using a vacuum pump, the blocking buffer was then removed. Subsequently, 1 μL of rabbit primary antibody was added to 1 mL blocking buffer, amounting to a 1:1 000 dilution, and this solution was added to the blot holder. After 10 min of incubation, the solution was removed by vacuum pump. The membrane was then washed thrice with 10 mL wash buffer (0.1% Tween-20 in TBS) each time. After the third wash, 0.1 μL of goat anti-rabbit, HRP-linked secondary antibody (Cell Signaling Technology, USA) was added to 1 mL of fresh blocking buffer, amounting to a 1:10 000 dilution. The washed membrane was then immersed in the blocking buffer with the secondary antibody and incubated for 10 min. The membrane was then washed thrice with wash buffer as described previously.

Meanwhile, 1 mL of luminol reagent and 1 mL of peroxide reagent from the Immobilon Western HRP Chemiluminescent Substrate kit (Millipore, USA) was added into a sterile 15 mL centrifuge tube and equilibrated to room temperature for 15 min. After the final wash, the membrane was immersed in the chemiluminescent reagent and incubated in darkness for 5 min. The chemiluminescent signal was detected using the Fluorchem FC2 imager. To detect for other proteins, the membrane would have to be stripped prior to detection, as described in Section 3.7.4.

Antibodies used in this studied were all purchased from Cell Signaling Technology, USA: anti-β-actin #4967, anti-SAPK/JNK #9252, anti-phospho-SAPK/JNK (Thr183/Tyr185) #9251, anti-SEK1/MKK4 #9152, anti-phospho-SEK1/MKK4 (Ser257/Thr261) #9156, anti-MKK7 #4172, anti-phospho-MKK7 (Ser271/Thr275) #4171, anti-mTOR #2972, anti-phospho-mTOR (Ser2448) #2971, anti-Akt #9272, anti-phospho-Akt (Ser473) #9271, anti-phospho-PDK1 (Ser241) #3061, and anti-rabbit IgG HRP-linked antibody #7074. All antibodies used were raised from rabbits with the exception of antibody #7074, which was raised from goats.

3.6.4 Membrane stripping

For detection of other proteins, the membrane must first be stripped of any bound antibodies. The membrane was immersed in 10 mL of stripping solution (0.4 M glycine, 0.2 % SDS, in sterile deionised water, pH 2.2) and agitated for 30 min. After that, the membrane was washed twice with wash buffer for 10 min each time. Subsequently, the membrane was immersed in methanol for 15 s, air-dried for 15 min, and stored in -80°C in between two filter papers (Whatman, USA), as described in Section 3.7.3.

3.7 Statistical analysis

All statistical analysis of mRNA and protein expression was performed using the SPSS Statistics Standard software, version 18 (IBM, USA). A one-way ANOVA was used for all analysis using the LSD algorithm at a 95% confidence interval.

CHAPTER 4

RESULTS

4.1 Isolation of total cellular RNA

The HepG2 cells were allowed to proliferate until 75% confluency and total cellular RNA was isolated using RNeasy (Qiagen) as described in Section 3.3.1. The integrity of the total cellular RNA extracted was assessed via gel electrophoresis using 1% (w/v) denaturing agarose-formaldehyde gel (Section 3.3.2) and viewed using an UV transilluminator. Two distinct bands on the gel indicate intact, high quality 28S and 18S rRNA. As shown in Figure 4.1, the 28S band was approximately twice the intensity of the 18S band. This 2:1 ratio indicates that the total cellular RNA extracted was of high quality and integrity. Analysis of the concentration and purity of the samples were measured by reading the absorbance at 260 nm and 280 nm using a spectrophotometer (NanoDrop, Thermo Fisher Scientific) and the A_{260}/A_{280} ratio of the isolated RNA samples indicate the purity of the RNA isolated. The final preparation of total RNA used in this study had an A_{260}/A_{280} ratio of 1.8 – 2.0. Sambrook *et al.* (1989) have shown that an A_{260}/A_{280} ratio of 1.8 – 2.0 is considered an acceptable indicator of good RNA quality. Therefore, the A_{260}/A_{280} ratio obtained in this study indicate that the total RNA isolated from HepG2 cells was free of DNA and protein contamination, and was also of high quality.

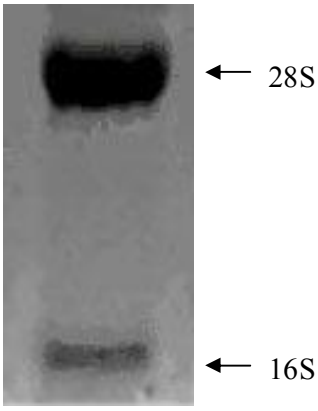

Figure 4.1: Formaldehyde agarose gel electrophoresis of total RNA extracted using RNeasy (Qiagen).
Total RNA was successfully extracted from HepG2 cells. The intensity of the 28S band (top band) was 2-fold higher than the 16S band (lower band), indicating high quality total RNA. The $A_{260/280}$ reading was 1.98.

4.2 Optimisation of conventional RT-PCR for untreated HepG2 cells

The primer sequences used in the RT-PCR reaction for *JNK1* and *JNK2* amplification are as shown in Table 3.1. The primers were designed specifically on different exons which span the introns, to avoid any co-amplification of contaminating genomic DNA.

The PCR reaction for *JNK1* and *JNK2* was optimised using cDNA generated from total RNA isolated from HepG2 cells as template and at different annealing temperatures as described in Section 3.5. After the PCR reaction, 2 μL of PCR products were subjected to 1% (w/v) agarose gel electrophoresis for further analysis. The gel was stained with ethidium bromide and viewed under an UV transilluminator (Alpha Innotech).

Figure 4.2 shows the results of the optimisation process. The DNA fragments which corresponded to the expected size of approximately 175 bp for *JNK1* and 225 bp for *JNK2* was successfully amplified. This indicated that the primers and the PCR reaction were specific. For the amplification of *JNK1* (Figure 4.2a) and *JNK2* (Figure 4.2b) primers, 63.0^{o}C was chosen as the optimum annealing temperature as it produced the highest intensity of the amplified products without primer dimers.

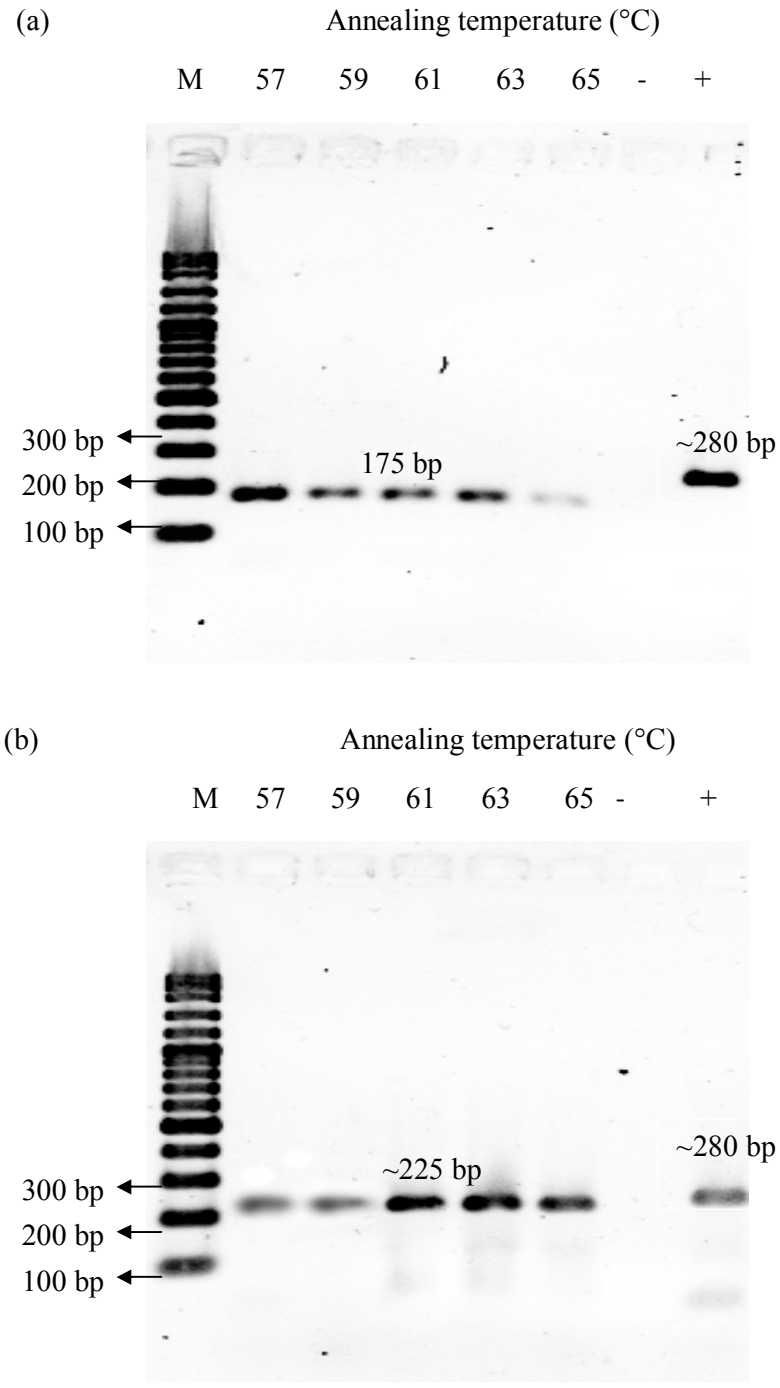

Figure 4.2: Gradient PCR of (a) *JNK1* and (b) *JNK2* primers using different annealing temperatures.
The optimum annealing temperature was 63°C as shown by the successful amplification of both *JNK1* and *JNK2*, without any primer dimers. Marker used was a 100 bp DNA ladder. '+' and '-' lanes were used as positive control (*β-actin*) and negative control, respectively. The results are representatives of three independent experiments.

4.3 Sequence confirmation

The PCR products were then purified using the QIAquick Gel Extraction Kit (Qiagen) as described in Section 3.5.5. To verify the specificity of the PCR primers in amplifying the desired products, a sequencing process was carried out. Here, the PCR products were sent 1st Base for the sequencing process together with the forward primers. The sequencing results were then compared with the sequences in the GenBank databases using nucleotide BLAST (BLASTN) as described in Section 3.5.5. The sequence alignments of *JNK1* and *JNK2* are shown in Appendix A(i) and A(ii), respectively. Using the BLASTN algorithm from NCBI, the product amplified using the primers Jnk1F and Jnk1R matches the accession numbers NM 002750, NM 139049, NM 139047 and NM 139046 which corresponds with *JNK1α1*, *JNK1α2*, *JNK1β1*, *JNK1β2* respectively with an E-value of $1e^{-62}$ and 97% similarity. For the primer pair JNK2F and JNK2R, the amplified product matches the accession numbers NM 139068, NM 002752, NM 139069 and NM 139047, which corresponds with *JNK2α1*, *JNK2α2, JNK2β1*, and *JNK2β2*, respectively. The α spliced isoforms has an E-value of $2e^{-91}$ and 98% similarity while the β spliced isoforms has an E-value of $5e^{-48}$ and 85% similarity. A lower E-value represents a more significant score. However, in the context of this study, the primers were specific in amplifying *JNK1* and *JNK2*, as shown by the discrete single bands in Figure 4.2.

4.4 Real-time RT-PCR

4.4.1 The effects of TNF-α on *JNK1* and *JNK2* expression

Based on Section 3.2.1, HepG2 cells were incubated with different concentrations of TNF-α. Total RNA extraction and real-time RT-PCR were performed as described in Section 3.3.1 and Section 3.5.6. The expression of *JNK1* and *JNK2* was normalised with *β-actin*, respectively. To ensure reproducibility, each independent experiment was performed three times.

As shown in Figure 4.3, the expression of *JNK1* and *JNK2* increases significantly at 20 ng/mL when the cells were treated with increasing concentrations of TNF-α (0 – 50 ng/mL). For *JNK1,* there was no change in normalised expression when stimulated with 5 ng/mL TNF-α, as compared to a ~1.50-fold increase in normalised expression when stimulated with 10 ng/mL TNF-α. However, the increase was not statistically significant. *JNK2* follows a similar trend where its expression was increased ~1.5- and ~2.0-fold when stimulated with 5 and 10 ng/mL TNF-α, respectively. Similar with *JNK1,* this increase was again not statistically significant.

However, *JNK1* and *JNK2* expression was increased significantly at 20 ng/mL to ~2.5- and ~2.25-fold respectively, relative to untreated cells. At 50 ng/mL of TNF-α stimulation, there was a further increase (~2.75- and ~2.5-fold for *JNK1* and *JNK2*, respectively) which was significant. The overall data

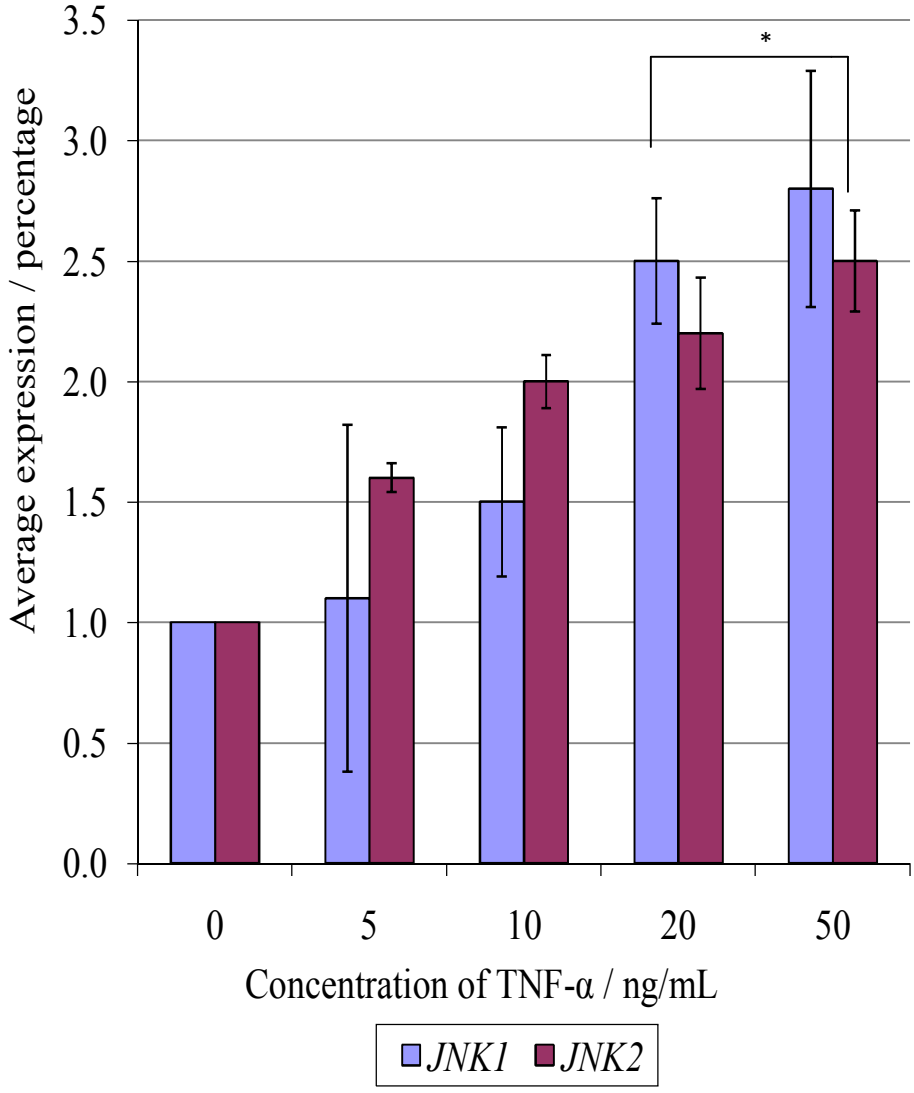

Figure 4.3: Average expression of *JNK1* and *JNK2* when stimulated at different dosages of TNF-α for 24 h.
After real-time PCR analysis, *JNK1* and *JNK2* expression increased significantly when subjected to 20 ng/mL of TNF-α. Expression of *JNK1* and *JNK2* increased ~2.5- and ~2.25-fold, respectively. Time 0 h indicates unstimulated control. The results are representatives of three independent experiments. * $p<0.05$ vs. control.

showed the upregulatory effects in both JNK mRNA levels when HepG2 were stimulated with increasing concentrations of TNF-α.

4.4.2 Time-course study on *JNK1* and *JNK2* expression

HepG2 cells were incubated for different durations (0 – 24 h) in the presence of 20 ng/mL of TNF-α as described in Section 3.2.1. Based on Section 3.3.1 and Section 3.5.6, total RNA extraction and real-time RT-PCR was performed. *β-actin* was used as the reference gene to normalise the expression of *JNK1* and *JNK2*. Each independent experiment was performed using samples in triplicate to ensure reproducibility.

Based on Figure 4.4, *JNK1* expression increased after 2 h to ~1.5-fold, but it was not statistically significant. *JNK2* was also induced ~1.5-fold but it was also not significant. The expression of *JNK1* and *JNK2* increases significantly at 4 h onwards, to ~3- and ~2.25-fold respectively, relative to untreated cells. Subsequently, there was a slight decrease in *JNK1* expression, from ~2.75-fold at 8 h and 16 h, to ~2.5-fold at 24 h, respectively. However, the reduction in expression was not significant. On the other hand, *JNK2* expression was consistent, remaining at ~2.25-fold from 4 h onwards. These results indicated that the increase in both *JNK* expressions would only be significant after 4 h of TNF-α treatment.

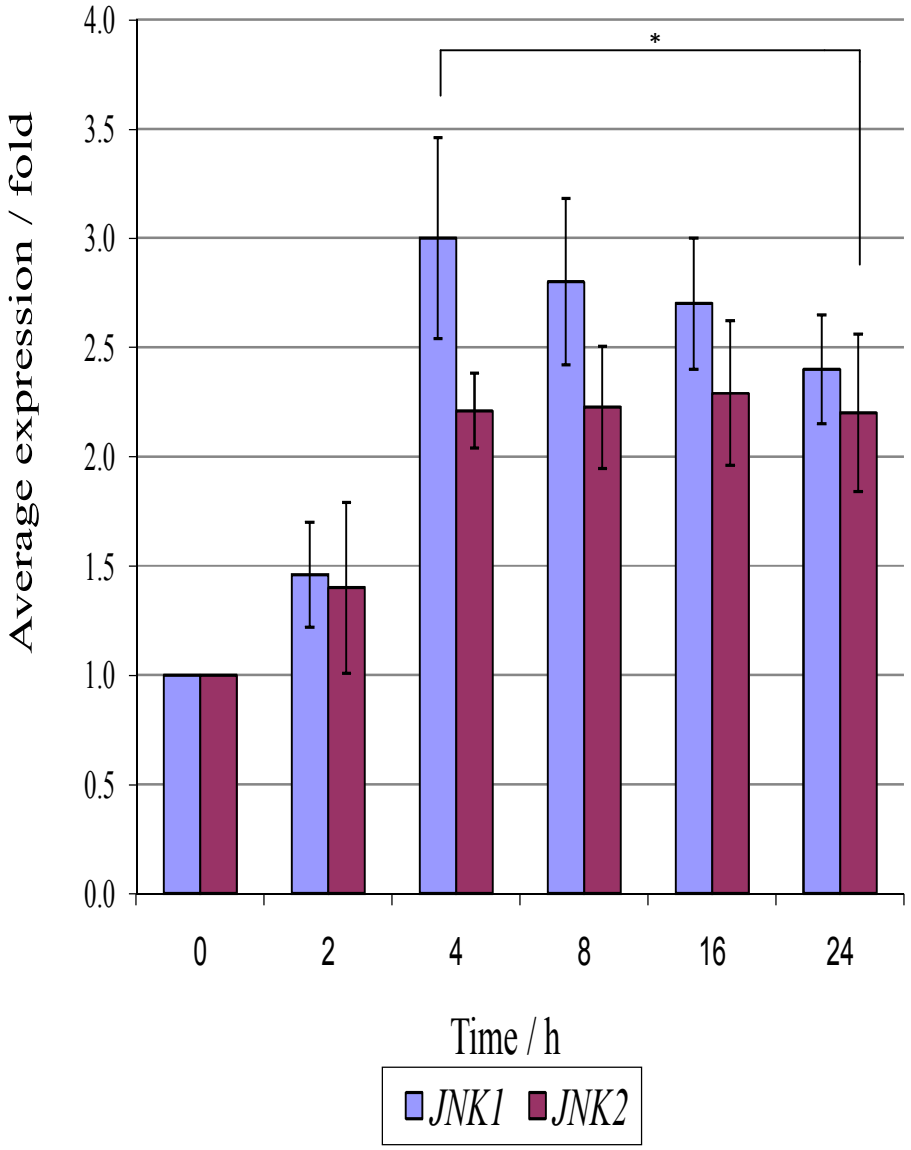

Figure 4.4: ***JNK* expression as normalised to *β-actin* after stimulation with 20 ng/mL of TNF-α for different durations.**
The expression of *JNK1* and *JNK2* as normalised to *β-actin* increased significantly (~3.0- and ~2.25-fold respectively) after 4 h of stimulation with 20 ng/mL of TNF-α, suggesting that *JNK* expression is regulated by TNF-α stimulation. Time 0 h indicates unstimulated control. The results are representatives of three independent experiments. * $p<0.05$ vs. control.

4.5 Western Blot

4.5.1 Effects of TNF-α on JNK1 and JNK2 expression

As described in Section 3.2.1, HepG2 cells were incubated for 24 h in the presence of different concentrations of TNF-α. Extraction of total protein and Western blotting were performed according to Section 3.4.1 and Section 3.6. The expression values were quantified using the FluorChem FC2 application from Alpha Innotech. Expression of JNK1 and JNK2 were then normalised with β-actin protein expression. To ensure reproducibility, each experiment was conducted three times.

As shown in Figure 4.5, the expression of JNK1 and JNK2 increased significantly when the cells were treated with increasing concentrations of TNF-α (0 – 50 ng/mL). At 5 and 10 ng/mL of TNF-α stimulation, JNK1 expression was ~1.16- and ~1.51-fold respectively, relative to untreated cells. As for JNK2, its expression was ~0.71- and ~1.86-fold respectively, relative to untreated cells. However, these figures were not statistically significant. JNK1 and JNK2 expression was increased significantly at 20 ng/mL to ~2.13- and ~2.85-fold respectively, relative to untreated cells. At 50 ng/mL, their expression was further increased significantly to ~2.32- and ~2.97-fold, respectively. The overall data (Figure 4.5) showed the upregulatory effects in JNK levels, consistent with the mRNA profile (Figure 4.4) when HepG2 were stimulated with increasing concentrations of TNF-α.

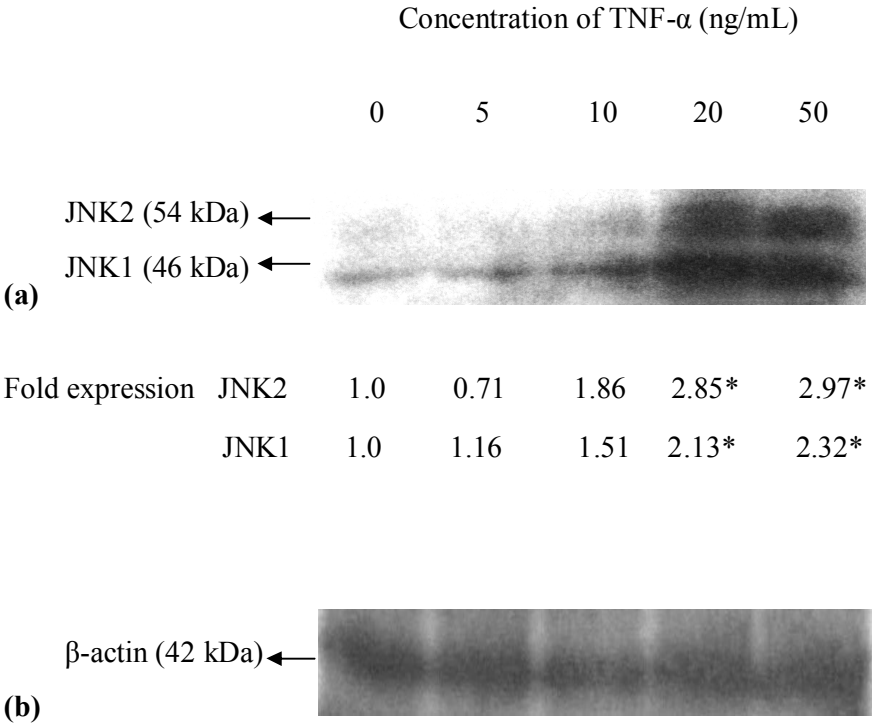

Figure 4.5: Expression of (a) p54 JNK2, top band; p46 JNK1, bottom band, when stimulated at different dosages of TNF- α for 24 h.

With 20 ng/mL of TNF- α stimulation, JNK1 and JNK2 were significantly expressed ~2.13- and ~2.85-fold, respectively. Time 0 h indicates unstimulated control. Expression values were normalised to time 0 for respective proteins. (b) Expression of β-actin as loading control. The results are representatives of three independent experiments. * $p<0.05$ vs. control.

4.5.2 Time-course study on JNK1 and JNK2 expression

HepG2 cells were incubated with 20 ng/mL of TNF-α for 0 - 24 h as described in Section 3.2.1. Based on Section 3.4.1 and Section 3.6, total protein was extracted and Western blotting was performed. The expression values were quantified as described earlier. To ensure reproducibility, each experiment was repeated three times.

As shown in Figure 4.6, the expression of JNK1 and JNK2 increased significantly after 4 h and 16 h respectively, following TNF-α treatment. Expression of JNK1 increased ~1.20-fold after 2 h of TNF-α stimulation. JNK1 was significantly induced after 4 h to ~1.32-fold. After 8 h, its expression was unchanged. It increased to ~1.38- and ~1.52-fold after 16 h and 24 h, respectively, although it was statistically insignifcant. After 2 h, JNK2 expression was induced ~1.18-fold, before increasing to ~1.28-fold at 4 h. At 8 h, its expression was increased to 1.40-fold. JNK2 expression was only increased significantly to ~1.78-fold after 16 h, relative to untreated cells (Figure 4.6). It further increased to ~2.0-fold after 24 h.

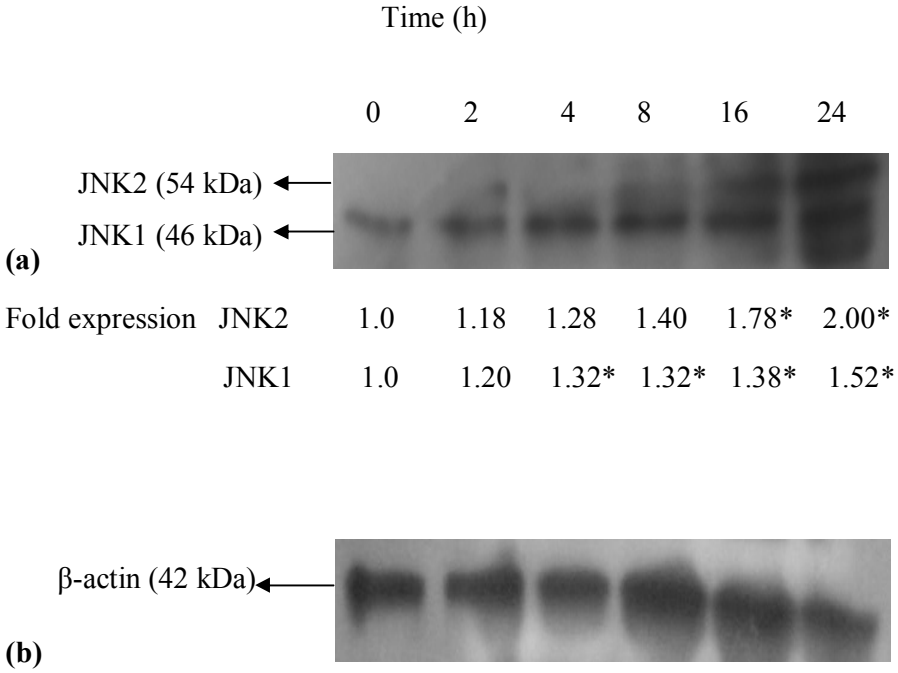

Figure 4.6: Expression of (a) p54 JNK2, top band; p46 JNK1, bottom band with 20 ng/mL TNF-α stimulation and (b) expression of β-actin as loading control for different durations.
With 20 ng/mL of TNF-α stimulation, JNK1 expression showed an increase of ~1.32-fold after 4 h, whereas JNK2 was observed to be significantly expressed ~1.78-fold only after 16 h of treatment. Time 0 h indicates unstimulated control. Expression values were normalised to time 0 for respective proteins. The results are representatives of three independent experiments. * $p<0.05$ vs. control.

These results indicate that TNF-α only exerts its effect on JNK1 and JNK2 expression after 4 h and 16 h, respectively, while the expression pattern with JNK1 is similar to its mRNA expression (Figure 4.4). It seems that JNK2 may be post-transcriptionally regulated as the protein expression was only significantly induced 12 h (Figure 4.6) after a significant induction in its mRNA levels was detected (Figure 4.4).

4.6 Elucidation of the role of mTOR-signalling on TNF-α-mediated *JNK* expression

4.6.1 Inhibition study with a panel of inhibitors

This study was done to examine the effects that pre-treatment with different kinase inhibitors have on *JNK* expression. Kinases which not are traditionally upstream of JNK, but are regulators of the Akt/mTOR pathway, were selected to show that inhibition of these kinases can affect *JNK* expression. These include the JAK, Hsp90 and mTOR itself. MEK1 was chosen to test if inhibition of an upstream parallel kinase would affect *JNK* expression. To check if a parallel kinase would affect *JNK* expression, the p38 kinase was chosen as a target for inhibition.

Briefly, HepG2 cells were incubated for with the different inhibitors for 2 h before stimulation with 20 ng/mL of TNF-α for 4 h as described in Section 3.2.2. Total RNA extraction and real-time RT-PCR was performed as described

in Section 3.3.1 and Section 3.5.6. Each independent experiment was performed using samples in triplicate to ensure reproducibility.

In cells without any inhibitor pre-treatment, TNF-α stimulation significantly induced both *JNK1* and *JNK2* to ~2.25-fold each (Figure 4.7). These results are in accordance with those obtained in Section 4.4.1. Pre-treatment using mTOR, p38, Hsp90 and JAK inhibitors almost abrogated *JNK1* and *JNK2* expression to pre-TNF-α stimulation levels. Specifically, mTOR, JAK, p38, and Hsp90 pre-treatment after TNF-α stimulation resulted in reduced *JNK1* expression (~1.25-, ~1.30-, ~1.37- and ~1.40-fold, respectively). These reductions in expression were significant when compared to TNF-α stimulation without any inhibitor pre-treatment (~2.25-fold). Similarly, *JNK2* expression was also significantly suppressed after pre-treatment with the MEK1, mTOR, JAK, p38, and Hsp90 inhibitors to ~1.13-, ~1.00-, ~1.25-, ~0.98- and ~1.28-fold, respectively.

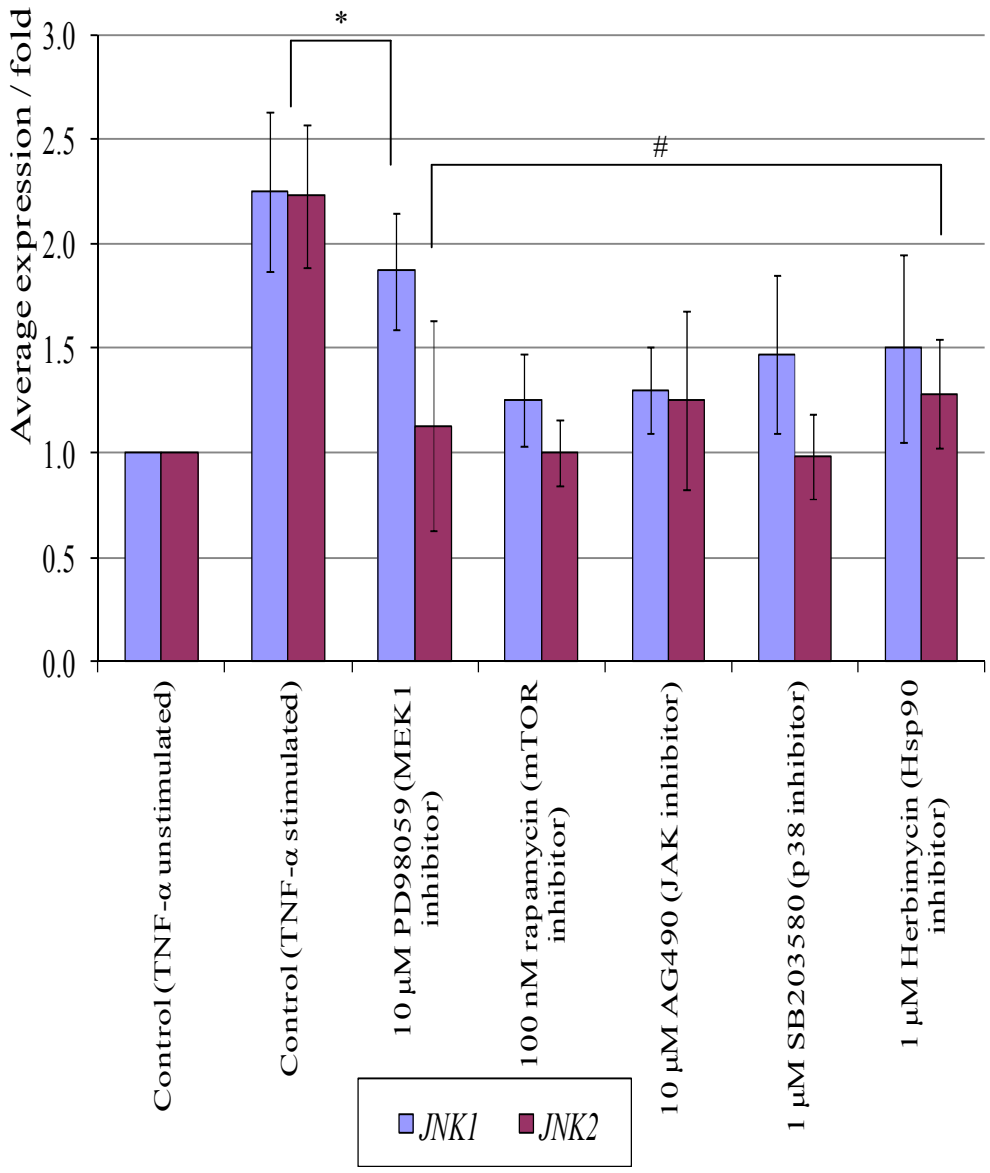

Figure 4.7: **Average expression of *JNK1* and *JNK2* when inhibited with different inhibitors prior to 20 ng/mL TNF-α stimulation.** Treatment by PD980589, rapamycin, AG490 and Herbimycin significantly reduced expression of both *JNK1* and *JNK2*. In contrast, there was a significant induction (~2.25-fold) in *JNK* in the control cells treated with TNF-α. The results are representatives of three independent experiments. * significant increase at $p<0.05$ vs. unstimulated control**.** # significant decrease at $p<0.05$ vs. stimulated control.

4.6.2 Optimisation of rapamycin concentration

As Sarbassov *et al*., (2006) has concluded that different concentrations of rapamycin affect JNK1 activation differentially, this experiment was conducted to determine the optimum rapamycin dosage which can reduce *JNK1* and *JNK2* expression as well. To determine the rapamycin concentration needed to inhibit *JNK* expression, HepG2 cells were incubated with different concentrations of rapamycin (0, 10, 20, 50, 100 ng/mL) for 2 h before being stimulated with 20 ng/mL of TNF-α for 4 h as described in Section 3.2.2. Based on Section 3.3.1 and Section 3.5.6, total RNA extraction and real-time RT-PCR was performed. Each independent experiment was repeated three times to ensure reproducibility.

Without rapamycin pre-treatment, TNF-α treatment significantly induced *JNK1* and *JNK2* to approximately ~2.5- and ~2.1-fold (Figure 4.8). Specifically, there was significant increases in *JNK1* expression (~2.31-, ~2.09- and ~1.79-fold) at dosages of 10, 20, 50 ng/mL of rapamycin pre-treatment, respectively. For *JNK2* expression, rapamycin pre-treatment at dosages of 10, 20, 50 ng/mL resulted in significant increases to ~2.06-, ~1.83- and ~1.76-fold, respectively. As observed in Figure 4.8, pre-treatment with 100 ng/mL of rapamycin significantly reduced *JNK1* and *JNK2* expression to pre-TNF-α treatment levels. Therefore, 100 ng/mL of rapamycin was subsequently used in all mTOR inhibition experiments in this study.

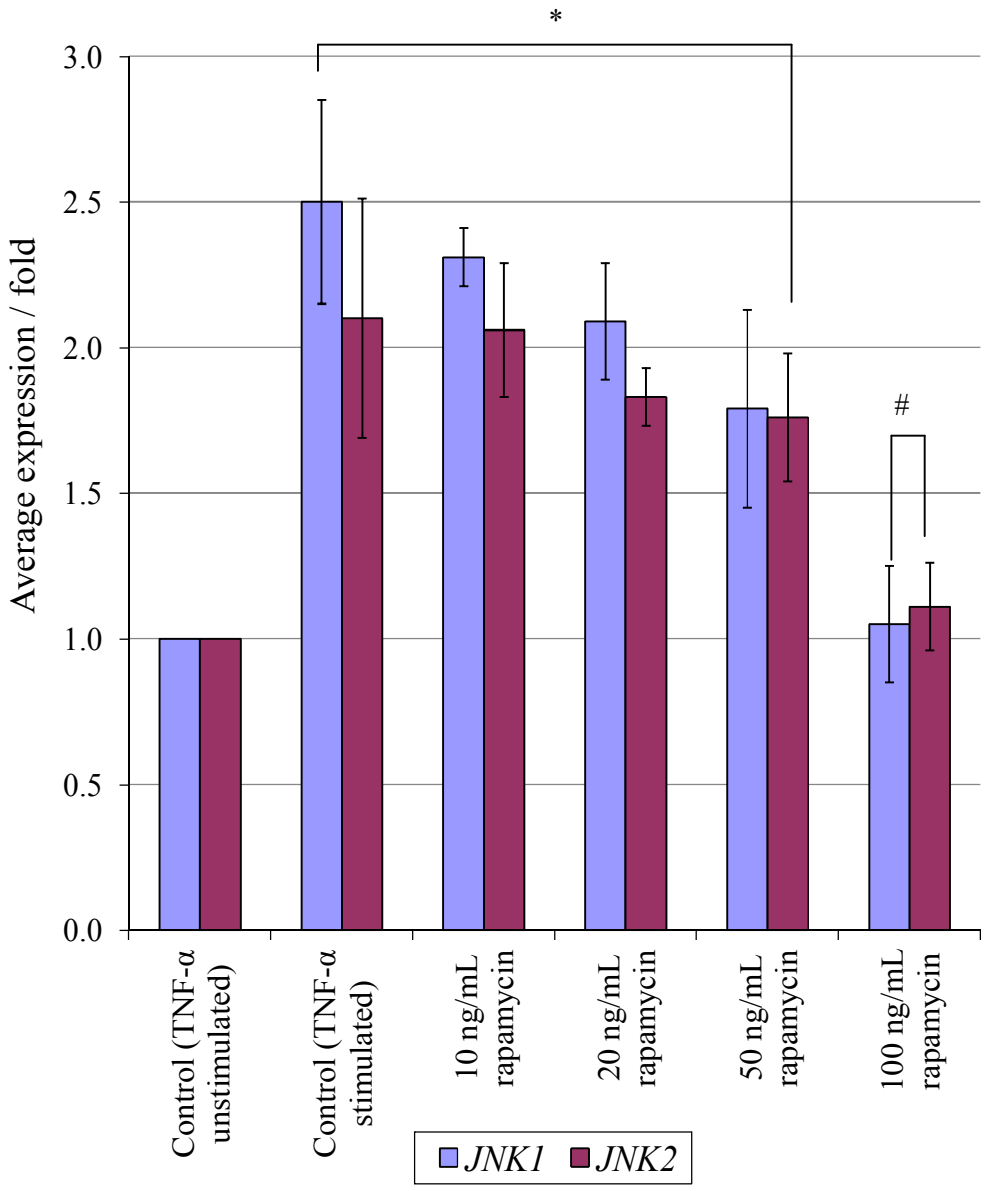

Figure 4.8: **Average expression of *JNK1* and *JNK2* when inhibited with different concentrations of rapamycin prior to 20 ng/mL TNF-α stimulation.**
Inhibition with 100 ng/mL of rapamycin significantly reduced expression of *JNK1* and *JNK2*. In contrast, *JNK1* and *JNK2* were significantly induced ~2.5- and ~2.1-fold, respectively, in control cells treated with TNF-α. The results are representatives of three independent experiments. * significant increase at $p<0.05$ vs. unstimulated control. # significant decrease at $p<0.05$ vs. stimulated control.

4.6.3 Determination of upstream JNK regulators

To determine the upstream events that lead to JNK activation, the activation status of different upstream kinases was investigated. Specifically of interest are the two pathways, the MAPKs and the Akt/mTOR kinases. As shown in Section 4.6.1, inhibition of the mTOR, p38, Hsp90 and JAK does indeed affect the expression of *JNK1* and *JNK2*. Briefly, HepG2 cells were incubated with 100 ng/mL of the mTOR inhibitor, rapamycin, before stimulation with 20 ng/mL of TNF-α for 2 h as described in Section 3.2.2. Extraction of cytoplasmic protein and Western blotting were performed according to Section 3.4.2 and Section 3.6. To check the expression and activation of the kinases, the Western blots were performed as described in Section 3.7. The membrane was stripped in between subsequent blots as described in Section 3.6.4. β-actin was used as loading control. Each experiment was conducted in triplicate to ensure reproducibility.

4.6.3.1 JNK1 and its upstream kinase, MKK7, are differentially phosphorylated under rapamycin treatment

The JNK signalling cascade comprises of MKK4/7, JNK1/2 and c-Jun (Bogoyevitch & Kobe, 2006). In all samples, JNK1 expression was constant 2 h after TNF-α stimulation (Figure 4.9a), regardless of rapamycin pre-treatment. JNK1 phosphorylation was significantly increased ~1.77-fold after 2 h of TNF-α stimulation in rapamycin-untreated samples as shown in Figure 4.9b. In rapamycin-treated samples, phosphorylation of JNK1 was abrogated and was not restored after 2 h of TNF-α stimulation (Figure 4.9b). However, there was no evidence of JNK2 expression in these two hours. As JNK2 was only expressed almost 16 h after TNF-α stimulation, as shown in Figure 4.6, these results were consistent with those obtained in the earlier time-course expression experiments.

Based on Figures 4.9c, the expression levels of c-Jun, the transcription factor, remained constant after 2 h regardless of rapamycin pre-treatment. There was no increase in c-Jun expression in rapamycin-treated cells compared to rapamycin-untreated cells. However, in rapamycin-untreated samples, c-Jun was phosphorylated at serine 63 once the cells were stimulated with TNF-α, up to ~2.29-fold (Figure 4.9d). This is in accordance with the classical model of c-Jun activation by JNK (Morton *et al.*, 2003). In rapamycin-treated cells, pre-

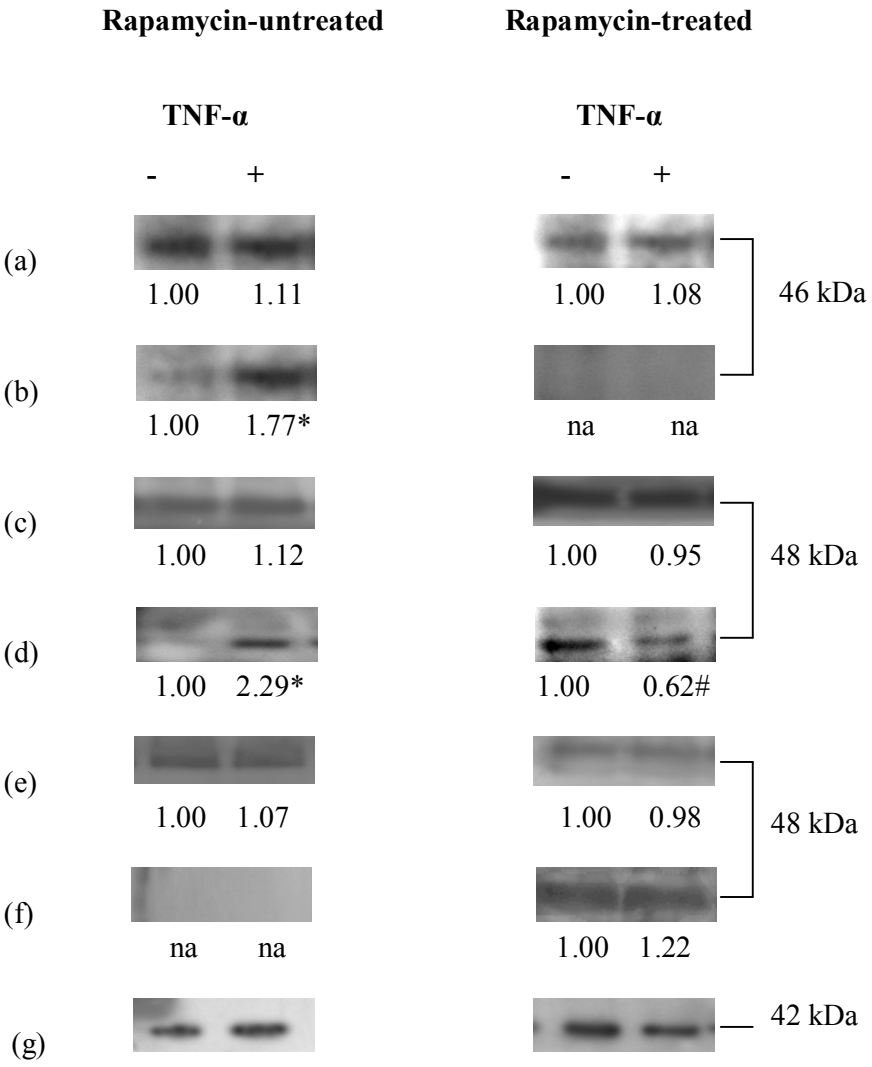

Figure 4.9: Western blots of (a) JNK1, (b) p-JNK1, (c) c-Jun, (d) pc-Jun (Ser 63), (e) MKK7, (f) p-MKK7, (g) β-actin.

TNF-α stimulation phosphorylates JNK1. Treatment with rapamycin abrogated JNK1 phosphorylation. Rapamycin and TNF-α both activate c-Jun singly, but treatment with TNF-α reduces c-Jun activation after rapamycin treatment. p-MKK7 was detected only in rapamycin-treated samples. β-actin was used as loading control. This is a representation of three separate experiments. Band densities were recorded beneath each blot and were normalised to their respective loading controls (phosphoproteins were normalized to total proteins and total proteins were normalized to β-actin). * significant increase at $p<0.05$ vs control. # significant decrease at $p<0.05$ vs control.

treatment with rapamycin appears to be sufficient to elicit a stress response via c-Jun phosphorylation (Figure 4.9d), which indicates that it is independent of JNK1 activation, as JNK1 activation has been abrograted earlier (Figure 4.9b).

However, subsequent stimulation with TNF-α gradually significantly reduces the c-Jun phosphorylation to ~0.62-fold (Figure 4.9d). Total MKK7 was expressed in both rapamycin-treated cells and rapamycin-untreated cells (Figure 4.9e). MKK7 was also phosphorylated in response to rapamycin pre-treatment (Figure 4.9f). Subsequent TNF-α stimulation did not significantly increase its phosphorylation.

4.6.3.2 Akt and mTOR phosphorylation are affected by rapamycin treatment

The Akt/mTOR pathway was investigated next for their response to TNF-α and subsequent JNK signalling. The activation of Akt and mTOR was therefore examined. For mTORC2 to activate Akt, Akt should be phosphorylated at serine 473 to exert its kinase activity (Sarbassov *et al.*, 2005b). The expression of Akt was detected both in rapamycin-untreated and –treated samples (Figure 4.10a). Based on Figure 4.10b, Akt was phosphorylated on its serine 473 residue upon rapamycin treatment, and after 2 h its phosphorylated state was significantly increased to ~1.48-fold. However, there was no phosphorylation of Akt at serine 473 in rapamycin-untreated samples.

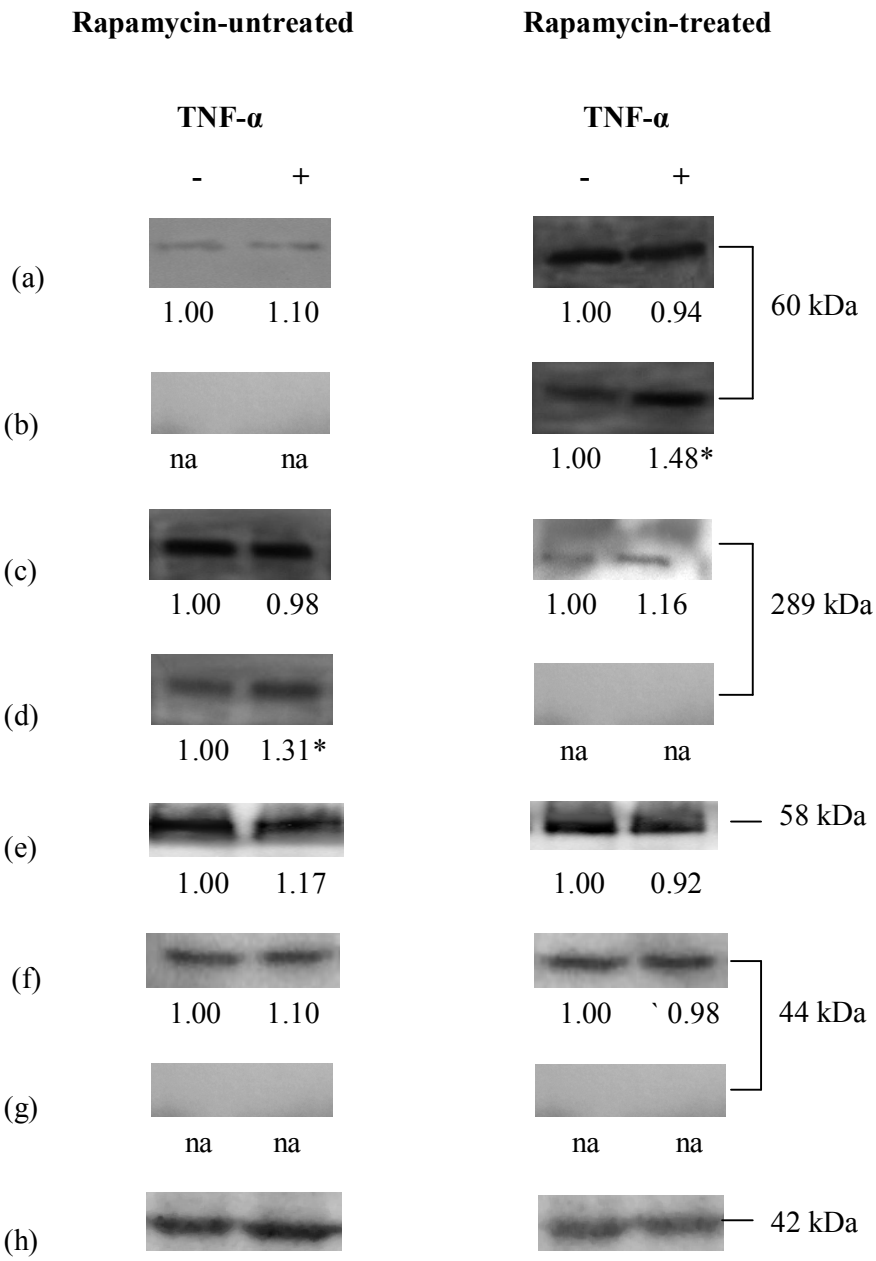

Figure 4.10: Western blots of (a) Akt, (b) p-Akt (Ser 473), (c) mTOR, (d) p-mTOR (Ser 2448), (e) p-PDK1, (f) MKK4, (g) p-MKK4, (h) β-actin.

Rapamycin treatment induces Akt phosphorylation at serine 473. However, treatment with rapamycin abrogated mTOR phosphorylation at serine 2448. PDK1 was phosphorylated under all conditions. β-actin was used as loading control. This is a representation of three separate experiments. Band densities were recorded beneath each blot and were normalised to their respective loading controls (phosphoproteins were normalized to total proteins and total proteins were normalized to β-actin). * significant increase at $p<0.05$ vs control.

According to Figure 4.10c, mTOR was expressed in both rapamycin-untreated and –treated samples. mTOR was constitutively phosphorylated at serine 2448, and stimulation by TNF-α increases its phosphorylation significantly to ~1.31-fold in rapamycin-untreated samples (Figure 4.10d). There was no phosphorylation of mTOR at serine 2448 in rapamycin-treated samples.

Based on Figure 4.10e, PDK1 was still phosphorylated when the cells were treated with rapamycin, verifying that PDK1 is constitutively active in this experiment and is not affected by rapamycin/TNF-α treatment. PDK1 activates Akt at threonine 308 (Brazil *et al*., 2004; Altomare & Testa, 2005; Woodgett, 2005), and this suggests that Akt phosphorylation at threonine 308 is not affected by rapamycin. Total MKK4 was detected in both rapamycin-treated and rapamycin-untreated samples (Figure 4.10f). However, no phosphorylation of MKK4 was observed (Figure 4.10g), suggesting that MKK4 was not involved in the TNF-α-JNK1 signalling cascade in HepG2 cells

4.7 Rapamycin affects the mRNA expression of *LXR-α* and *PXR*

As LXR-α and PXR were implicated in anti-inflammatory responses (Castrillo & Tontonoz, 2004), their mRNA expression in response to TNF-α and rapamycin treatment were studied. One set of HepG2 cells were incubated with 100 ng/mL of rapamycin for 2 h prior to stimulation with 20 ng/mL of TNF-α for 2 h; one control set stimulated with rapamycin for 2 h only; one

control set stimulated with TNF-α for 4 h only and another control set without any stimulation. Total RNA extraction and real-time RT-PCR was performed as described in Section 3.3.1 and Section 3.5.6. Each independent experiment was performed using samples in triplicate to ensure reproducibility.

The results in Figure 4.9d have shown that rapamycin and TNF-α activates the transcription factor c-Jun. It would be of interest if rapamycin and TNF-α can modulate the expression of the transcription factors *LXR-α* and *PXR* as well, as they have been implicated in playing a role in anti-inflammatory response. Based on Figure 4.11, TNF-α-mediated *LXR-α* and *PXR* expression was increased by ~17- and ~2-fold respectively, in cells pre-treated with rapamycin. Without rapamycin pre-treatment, TNF-α stimulation appears to repress *LXR-α* and *PXR* expression by almost ~2-fold. Rapamycin treatment alone causes a reduction in expression in *LXR-α* and *PXR* to ~0.5- and ~0.25-fold respectively .

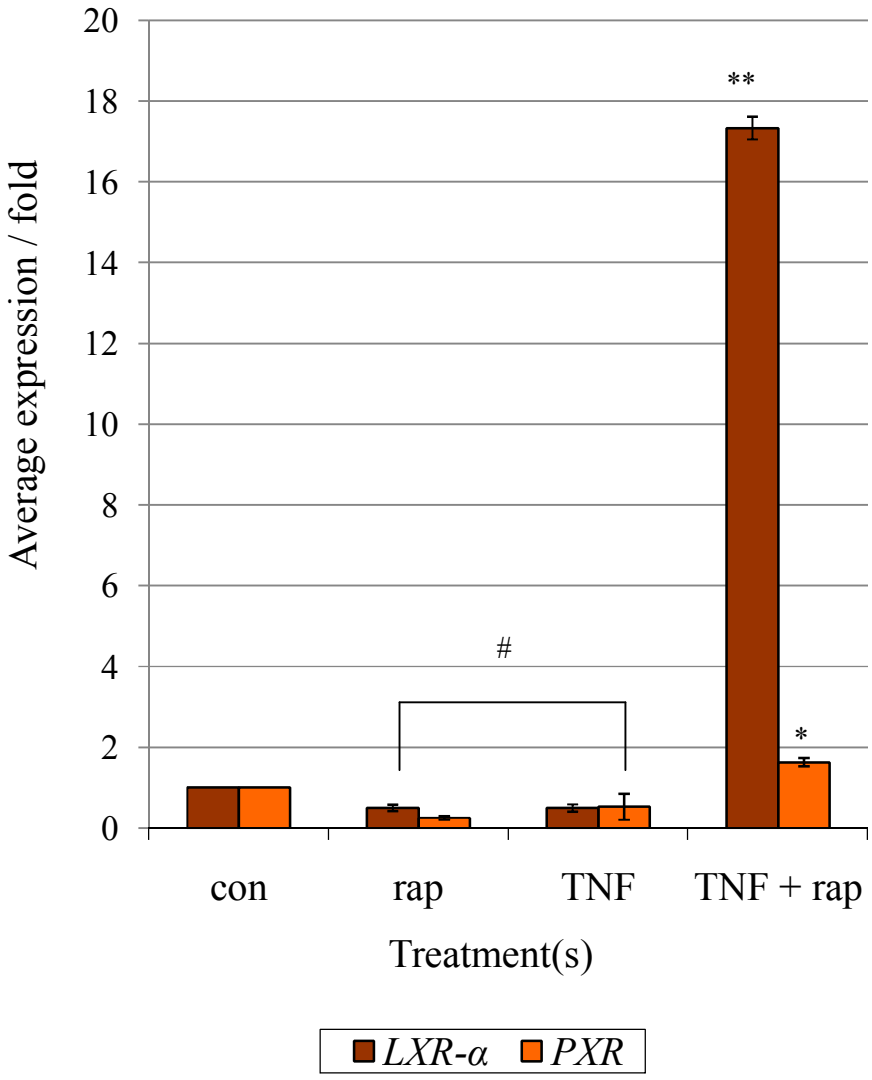

Figure 4.11: Average expression of *LXR-α* and *PXR* as normalised to β-actin in response to rapamycin, TNF-α, and rapamycin + TNF-α.
Treatment of TNF-α alone resulted in a significant decrease for *LXR-α* and *PXR* expression. Rapamycin treatment by itself causes a significant reduction in both *LXR-α* and *PXR* expression. However, pre-treatment with rapamycin followed by TNF-α stimulation caused a significant increase in induction for both *LXR-α* and *PXR* expression. The results are representatives of three independent experiments. # significant decrease at $p<0.05$ vs. control. * significant increase at $p<0.05$ vs. control. ** significant increase at $p<0.01$ vs. control.

CHAPTER 5

DISCUSSION

5.1 Primers specificity

As *JNK1* and *JNK2* isoforms mentioned in Section 2.1 are very similar structurally, primers were successfully designed to amplify all the sub-isoforms for each member (97% similarity for *JNK1*, 98% similarity for *JNK2α* isoforms). *JNK3* was excluded in this study as previous literature has shown that it was predominantly expressed only in human brain and testes cells (Yang *et al.,* 1997).

It was previously viewed that the 46 kDa molecule represents JNK1 and the 54 kDa molecule represents JNK2, respectively. However, it is now recognised that due to alternative splicing of the pre-mRNAs of JNKs, the 46 and 54 kDa variants represent two sub-isoforms of both JNK1 and JNK2. Both JNK1 and JNK2 are represented by two sub-isoforms, α and β, depending on the mutually exclusive use of exon 6α or exon 6β. An additional alternative splicing of the JNK1 and JNK2 pre-mRNAs can occur at the site between the last intron and exon. Both α1 and β1 transcripts of the JNK1 and JNK2 mRNAs use the conserved splicing site at this position, yielding 384 and 382 amino acid polypeptides with a molecular weight about 46 kDa. Alternative splicing at this site by using the cryptic splicing site within the last exon results in deletion of the 5 nucleotides at the 5′-terminus of the last exon and changes

of the reading frame, causing an extension of the COOH-terminal region, to generate α2 and β2 sub-isoforms of the JNK1 and JNK2 with a molecular weight of 54 kDa (Figure 2.2). The transcript of JNK1 α2/β2 yields a polypeptide of 427 amino acids and the transcript of JNK2 α2/β2 yields a polypeptide of 424 amino acids (Gupta *et al.,* 1996). The primers used in this study were designed to successfully amplify all eight of these sub-isoforms (*JNK1α1/β1/α2/β2* and *JNK2α1/β1/α2/β2*) as shown in Appendix C.

However, the expression levels of the JNK isoforms appear to be unequal. In several *JNK1* and *JNK2* knockout studies, the predominant forms of the JNK1 are the α1 or β1 isoforms and the major forms of JNK2 are the α2 or β2 isoforms (Liu *et al.,* 2004; Sabapathy *et al.,* 2004). The unequal expression of the different isoforms for a given JNK kinase is possibly determined by some unique characteristics of exons and the splicing sites. In the case of the α and β isoforms of the JNK1, the 6α exon may be much favourable for constitutive splicing because of the presence of 2 exonic splicing enhancer elements that were not found in the 6β exon (Chen *et al*., 2009). This might account for the predominance of JNK1α1 being expressed in the majority of tissues or cells.

The presence of the U2-U12-type hybrid intron 6 that prefers the usage of the α exon and the exon located down stream of the 6β exon in JNK2 genome might explain the predominance of JNK2α2 isoform (Chang *et al.,* 2007). Thus, these features of the JNK1 and JNK2 pre-mRNAs may explain why the 46 kDa JNK1α1 isoform and the 54 kDa JNK2α2 isoform are dominantly expressed in most cells or tissues. Subsequent JNK blotting experiments in this study were therefore only limited to the 46 kDa JNK1α1 isoform and the 54 kDa JNK2α2 isoform.

5.2 JNK mRNA and protein expression are TNF-α dose dependent

Based on the results obtained in Section 4.4.1, both JNK1 and JNK2 mRNA and protein expression increased significantly when stimulated with 20 ng/mL of TNF-α. Other reports have used varying dosages of TNF-α, from 10 to 100 ng/mL of TNF-α in insulinoma, fibroblast, osteoblast, HeLa, endothelial, cholangiocytes, and myoblast cells (Son *et al*., 2008; Ventura *et al*., 2004; Hishagimoto *et al*., 2006; Ahmed-Choudhury *et al*., 2005, Kim *et al*., 2005; Zhou *et al*., 2007; Mukai *et al*., 2007). In this study, 50 ng/mL of TNF-α was shown to increase JNK mRNA significantly but it was not significantly higher than stimulation with 20 ng/mL of TNF-α.

Furthermore, TNF-α is reported to cause a cytotoxic or necrotic effect within the cells (Hill *et al*., 1995). In HepG2 cells, 35 pmol/L of TNF-α was sufficient to kill 50% of the cells compared to 5 pmol/L of TNF-α for LM

fibroblasts, in a study of TNF-α cytotoxicity bioassays by Sidhu & Bollon (1993). Cross *et al*. (2008) have demonstrated that the effects of TNF-α are very much dose-dependent in neutrophils. Higher concentrations was shown to induce myeloid leukemia cell differentiation protein (Mcl-1) degradation, and lower dosages of TNF-α triggerred human fetal liver member of B-cell lymphoma/leukemia-2 (Bfl-1) expression. At concentrations of TNF-α of 1 ng/mL or less, the major effect is anti-apoptotic and at concentrations of TNF-α of 10 ng/mL will result in enhanced neutrophil apoptosis. These dose-dependent effects will have profound implications on how TNF-α may regulate neutrophil function *in vivo* and hence, the way anti-TNF-α therapy may affect immune function.

Similarly, van den Berg *et al*. (2001) also concluded that TNF-α prevents apoptosis over a wide concentration range in neutrophils as well. This effect is dependent on protein synthesis and is similar to the effects of treatment with other pro-inflammatory cytokines such as interferon-γ and granulocyte macrophage colony stimulating factor (GM-CSF). Furthermore, a dominant pro-apoptotic effect becomes apparent at higher concentrations of TNF-α (10–100 ng/mL). This process was mediated by the production of reactive oxygen species and dependent on the activation of CD11b/CD18. This was illustrated by the absence of the pro-apoptotic effect of TNF-α in neutrophils of patients with congenital defects in the expression or function of CD11b/CD18 molecules (i.e., LAD-1 and LAD-1/variant) or defects in the production of toxic oxygen metabolites (i.e., CGD).

Specifically, HepG2 cells are known to be resistant to TNF-α-induced apoptosis (Hill *et al.*, 1995). TNF-α was shown to be lethal to HepG2 only by combining TNF-α with cycloheximide or actinomycin D (Bai & Cederbaum, 2000; Leist *et al*., 1994). Cavin *et al*. (2004) have also shown 20 ng/mL of TNF-α would not cause apoptosis in HepG2 cells. Most conclusively, Plumpe *et al*. (2000) has shown that HepG2 cells were resistant to up to 200 ng/mL of TNF-α. As the cells were incubated for 24 h prior to total RNA extraction, 20 ng/mL was the best compromise to induce stress (and hence, JNK expression) in the cells and to prevent the cells from premature death due to apoptotic and/or necrotic signals induced by TNF-α.

5.3 Rapamycin blocks *JNK* expression

To deduce whether JNK signalling is affected by mTOR (and its upstream kinases), a panel of inhibitors was tested on HepG2, together with TNF-α stimulation. From initial results (Section 4.6.1), the Akt/mTOR cascade could affect the JNK pathway. AG490, a JAK2 inhibitor and herbamycin, an Hsp90 inhibitor, were also tested to see whether a kinase upstream of Akt/mTOR would affect JNK expression. SB203580, a p38 inhibitor; and PD98059, a MEK1 inhibitor, were tested to see if inhibition of parallel pathways of JNK would result in a compensatory response in JNK expression and activation.

Based on Figure 4.7, rapamycin, the mTOR inhibitor, significantly reduced the expression levels of both *JNK* isoforms in TNF-α stimulated cells to basal levels in TNF-α-unstimulated cells. AG490, the JAK2 inhibitor, and herbamycin, the Hsp90 inhibitor, also achieved similar results with rapamycin. As JAK2 lies upstream of Akt/mTOR pathway, inhibiting JAK2 would reduce mTOR activity in theory. A possible interplay between the JAK/STAT3 and AP-1/JNK signalling pathways has been suggested by Catterall *et al.* (2001). Ahmed-Choudhury *et al.* (2006) also hypothesised that a sustained rise in STAT3/JAK and JNK/ERK activity in the absence of persistent NF-*κ*B activation in CD40-induced cholangiocytes allows both STAT3 and the JNK/ERK pathways to act unopposed to promote apoptosis.

Reports from Delgoffe *et al.* (2009) and Ohji *et al.* (2006) have shown that Hsp90 interacts with raptor, a component in the mTORC1 complex. Therefore, inhibiting Hsp90 using herbamycin would result in reduced mTORC1 activity, and from Figure 4.7, *JNK* expression was reduced, suggesting that mTOR does indeed cross-talk into *JNK* transcription and/or activation. This was shown in Figure 4.7 where Hsp90 inhibition resulted in the reduction of *JNK1* and *JNK2* to basal levels after stimulation with TNF-α.

Unexpectedly, PD98059 significantly reduced *JNK2* expression only, and not *JNK1*. As MEK1 is the upstream kinase for ERK1/2 whose activity is antagonistic to JNK (Black *et al.*, 2002; David *et al.*, 2002; Shen *et al.*, 2003),

its inhibition should result in a decrease in JNK activity and/or transcription as the cells try to compensate for the lack of ERK's growth signals. An increase in expression of a suitable kinase that could drive growth and differentiation would be expected in this case, which is beyond the scope of this study. It may be an oncogenic event present in cancer cells, where inhibition of growth signalling did not reduce the inflammatory responses, as shown by the persistently induced *JNK1*. In this manner, JNK1 would act as a pro-survival molecule.

However, inhibition of p38 by SB203580 also reduces *JNK* expression significantly. JNK and p38 are more related activity-wise (inflammatory and tumourgenic signalling), and it is possible that p38 also plays a role in regulating the transcription of *JNK* via the transcription factors ATF-2, ELK-1 and MEF-2 (Herlaar & Brown, 1999; Ichijo, 1999). Therefore, by virtue of its JNK inhibitory ability and its mechanism of action at the utmost downstream end of the Akt/mTOR cascade, rapamycin, the inhibitor of mTOR, was chosen as the potential JNK inhibitor in this study.

A few studies have disputed the concentration of rapamycin needed to inhibit mTORC signalling. According to Kim *et al*. (2002), low concentrations of rapamycin (10 – 30 ng/mL) are sufficient to inhibit the kinase activity of mTORC1, but Sarbassov *et al*. (2006) confirmed that exposure to high concentrations (100 ng/mL) of rapamycin would inhibit mTORC2 activity as

well. In this study, the optimal concentration of rapamycin for *JNK1* and *JNK2* inhibition was studied. This was done by inhibiting mTOR using various concentrations of rapamycin. Figure 4.8 shows that 100 ng/mL of rapamycin significantly repressed *JNK1* and *JNK2* expression. However, Hornberger *et al*. (2007) has shown that rapamycin treatment failed to abrogate JNK2 phosphorylation due to mechanical stress. In human β-cells, rapamycin did not affect JNK2 phosphorylation, albeit under low glucose concentrations (Frankael *et al*., 2008).

From Figure 4.8, 100 ng/mL of rapamycin significantly repressed *JNK1* and *JNK2* levels, even when subjected to TNF-α stimulation. This suggests that mTOR may exert transcriptional control over *JNK1*, possibly via the mTORC1 complex, as the mTORC1 was significantly inhibited at 100 ng/mL (Sarbassov *et al*., 2006). Based on Figure 4.8, dosages lower than 100 ng/mL of rapamycin failed to inhibit *JNK1* and *JNK2* induction. This observation suggests that rapamycin at high concentrations (100 ng/mL) can have an inhibitory effect on *JNK* by repressing its transcription when stimulated by TNF-α, possibly via mTORC1.

5.4 JNK1 activation is abrogated by rapamycin pre-treatment

Based on Figure 4.9a, JNK1 was expressed and activated before TNF-α stimulation, but not JNK2. This is consistent with reports stating that the JNK1 isoform is preferentially expressed and activated over the JNK2 isoform, even

at basal state (Liu *et al*., 2004; Sabapathy *et al*., 2004). Elevated JNK1 activity was identified in more than 50% of HCC samples relative to non-cancerous liver tissues (Chang *et al*., 2009, Hui *et al*., 2008). Interestingly, the activation was specific to JNK1 and not to JNK2. Hepatocytes deficient in the NF-*κ*B activator IKKβ show reduced JNK phosphatase activity, which leads to sustained JNK1 activation and increased liver carcinogenesis (Kamata *et al*., 2005). Studies have revealed that JNK1, but not JNK2, is activated by various extracellular stimuli, such as TNF-α, UV, IL-1 and TPA, and is required for TNF-α-induced apoptosis (Liu *et al.,* 2004). In this study, TNF-α induces JNK1 activation up to ~1.77-fold (Figure 4.9b). Furthermore, JNK2 may interfere with JNK1 activity via its own "futile" phosphorylation (Liu *et al*., 2004). Liu *et al*. (2004) have also demonstrated that only JNK1, but not JNK2, was activated by TNF-α and is required for TNF-α induced apoptosis in the absence of NF-*κ*B activation. This could mean that JNK2 activation by TNF-α was negligible, and JNK2 appeared to interfere with JNK1 activation by TNF-α. Consistently, *JNK2* $^{-/-}$ fibroblasts were even more sensitive to TNF-α induced apoptosis (Liu *et al*., 2004).

In the context of human cells, persistent activation of the JNKs (either achieved through the over-expression of upstream activators or following exposure to appropriate external stimuli) have now been frequently associated with apoptotic cell death in a variety of cells, including HeLa cells (Widmann *et al.*, 1997), endothelial cells (Zhu *et al.*, 2001), rat mesangial cells (Zanke *et al.*, 1996), and the potentiation of apoptotic events in L929 fibrosarcoma cells

(Widmann *et al.*, 1997). Degenerating neurons in Alzheimer's disease have been recently shown to have increased activation of JNKs accompanied by their intracellular relocalisation (Zhu *et al.*, 2001). Here, it seems that JNK1 is basally activated, suggesting a prosurvival role for JNK1 in HepG2 cells.

However, the activation of JNK1 is abrogated by treating the cells with rapamycin (Figure 4.9b). As the Akt/mTOR pathway is implicated in serum withdrawal and nutrient sensing, this is the first evidence to link JNK expression and activation to the Akt/mTOR pathway in this study. Moreover, abnormal activation of the Akt/mTOR signalling pathway has been identified in HCC (Faivre *et al.,* 2006). A recent study showed that p-mTOR was expressed in 41% of HCC tumour tissue but not in adjacent cirrhotic liver tissue (Sieghart *et al.*, 2007) suggesting that mTOR might be a specific therapeutic target for HCC.

5.5 Rapamycin activates c-Jun without prior JNK1 activation

c-Jun was the the canonical substrate of JNK and its phosphorylation status was usually used to determine JNK activity (Bogoyovitch & Kobe, 2006). Based on Figure 4.9c, expression of total c-Jun was constant under all conditions. Activation of the transcription factor c-Jun at serine 63 was induced up to ~2.29-fold when stimulated with TNF-α in rapamycin-untreated cells (Figure 4.9d). However, as Figure 4.9d has shown, c-Jun activation proceeds

without the need for prior JNK activation in rapamycin-treated cells. Further TNF-α treatment resulted in a decrease in phosphorylation, to ~0.62-fold.

As c-Jun is the canonical substrate for JNK, it is expected that there would be no c-Jun phosphorylation without JNK phosphorylation (Bogoyevitch & Kobe, 2006). Paradoxically, despite the activation of JNK1 in HCC, no evidence currently links it to c-Jun activation in these tumours (Chang *et al*., 2009; Hui *et al*., 2008). There could be other MAPKs which can compensate for the loss of JNK activity in c-Jun phosphorylation, for example the ERK (Leppa *et al*., 1998). Recently, ERK8 has been shown to phosphorylate c-Jun directly in HCT15 colorectal cells (Xu *et al.,* 2010). In fact, ERK and JNK both phosphorylate JunD, a c-Jun related protein (Li *et al*., 2002). Another example is the exposure of sympathetic neurons in culture to the DNA-damaging agent etoposide resulted in phosphorylation and the subsequent upregulation of total c-Jun levels (Dunn *et al.,* 2002). In that study, the inhibition of JNK activity could not block either the c-Jun phosphorylation or upregulation or the subsequent death of these neurons (Dunn *et al.,* 2002).

Other studies have shown that c-Jun phosphorylation was increased without corresponding JNK activation. c-Jun was demonstrated to be activated by the constitutive photomorphogenic homolog (COP9) signalosome (Naumann *et al*., 2002), myocyte-specific enhancer factor 2C (MEF2C) (Coso *et al*., 1997), and calcium (Cruzalegui *et al*., 1999, Zanger *et al*., 2001). c-Jun

protein and transcriptional activity were upregulated in Hodgkin's lymphoma cells without a concomitant increase in c-Jun phosphorylation by JNK (Mathas *et al*., 2002).

It would appear that c-Jun would play a different role under rapamycin treatment. Although originally thought as the canonical substrate by JNK (hence the name), there are now more studies showing that ERK also activates c-Jun as described above. From this study, rapamycin treatment alone could induce a JNK-independent stress response (c-Jun activation), most likely via the ERK pathway. However, through yet unknown mechanisms, TNF-α could seemingly reverse the alternative c-Jun activation, reducing it over time. Possibly, HepG2 cells have adapted to cope with different types of stress, and rapamycin, already a c-Jun activator (Figure 4.9d), reverses the role of TNF-α in a negative feedback manner.

5.6 Possible mechanisms of rapamycin action in TNF-α signalling

5.6.1 MKK7 competes with Akt as a scaffold substrate

As shown in Figure 4.9e, MKK7 is consistently expressed in all conditions. However, MKK7 is observed to be activated in rapamycin-treated cells before TNF-α stimulation (Figure 4.9f). More importantly, in rapamycin-treated samples there is no activation of JNK1, whereas in rapamycin-untreated cells JNK1 activation was increased upon TNF-α stimulation (Figure 4.9b).

Similarly, MKK4 was also detected in rapamycin-treated samples but not its phosphorylated form (Figure 4.9g).

As with the case of mTOR and Akt as discussed in the following sections, MKK7 could be involved in a scaffolding complex, which was first described by Whitmarsh *et al.* (1998). Scaffold proteins for the JNK group of MAPK have also been identified. These include the JNK interacting protein (JIP) group of putative scaffolds, which includes the JIP1, JIP2 and JIP3 proteins. The JIP1 and JIP2 proteins are closely related proteins that bind to JNK, MKK7, and mixed-lineage protein kinases (Whitmarsh *et al*., 1998; Yasuda *et al*., 1999). The JIP3 protein is structurally unrelated to the JIP1 and JIP2 proteins. However JIP3, like JIP1 and JIP2, binds to JNK, MKK7, and members of the mixed-lineage group of MAPKKK (Kelkar *et al*., 2000). An alternatively spliced variant of JIP3 has also been reported (JSAP) which also appears to function as a scaffold for the assembly of a cytokine receptor module scaffold protein for the JNK signalling pathway (Ito *et al*., 1997).

This observation was also expressed by Song & Yong (2005), who hypothesised that JIP1 assembles Akt, MKK7 and JNK2; while JIP3 assembles MKK4, MKK7 and JNK1. The JSAP and JNK leucine zipper protein (JLP) was reported to interact with the module JNK, MKK4, and MEKK1 and p38 (Lee *et al*., 2002). As JNK1 and MKK7 would be complexed together in a signalling scaffold in JIP1, MKK7 would not need to be phosphorylated to

exert its kinase activity (Nihalani *et al*., 2003). As shown in Figure 4.9f, consistent with the scaffolding hypothesis, MKK7 was not observed to be phosphorylated in rapamycin-untreated samples. An inactive scaffold may cause MKK7 to be independently activated, and this was shown in Figure 4f where rapamycin pre-treatment activated MKK7. In fact, MKK7 was shown to be preferentially activated by TNF-α and cellular stresses (Moriguchi *et al*., 1997). Based on Figure 4.9d, this correlates with the results obtained in this experiment, where c-Jun was activated with corresponding MKK7 activation. Furthermore, as JNK1 has been implicated in priming the JIP1 scaffold in a positive feedback manner, the lack of activated JNK in rapamycin-treated cells will interfere with JIP1 priming (Nihalani *et al*., 2003), thus inhibiting the binding of MKK7 to the signalling complex. p-JNK1 would be detected as it would be freed from the complex to translocate into the nucleus to act on transcription factors (Cavigelli *et al*., 1995).

Therefore, it is plausible that for JNK activation to occur in response to TNF-α activation in HepG2 cells, JNK1 and MKK7 should be preferentially located within their respective scaffolds. As rapamycin appears to inhibit MKK4/7 scaffolding, it is postulated here that rapamycin inhibits JNK1 signalling at the MKK4/7 scaffold level.

5.6.2 Akt as a JNK1 inhibitor

Akt activation has been found to suppress the induction of JNK and p38 MAP kinase signalling pathways in response to various stresses (Berra *et al.*, 1998; Cerezo *et al.*, 1998; Okubo *et al.*, 1998). Some studies have linked Akt activation via serine 473 phosphorylation by mTOR, in a complexed form known as mTORC2 (Sarbassov *et al.*, 2005a; Hresko & Mueckler, 2005). However, previous literature has implicated mTOR as an Akt substrate (Nave *et al.*, 1999). Threonine 308 phosphorylation in Akt by PDK1 is also required for Akt to be fully activated (Alessi *et al.*, 1996).

Akt has been shown to inhibit JNK1 activity by binding to JIP1 (Kim *et al.*, 2002). JIP1 assembles the Mixed Lineage Kinase 3 (MLK3), MKK7 and JNK1 in a complex which can be disrupted by Akt binding. In this study, Akt expression in rapamycin-untreated samples is stable (Figure 4.10a), and pre-treatment with rapamycin activates Akt (Figure 4.10b). Akt phosphorylation was increased at serine 473 ~1.48-fold after TNF-α stimulation (Figure 4.10b). The increase of serine 473 phosphorylation is due to the action of TNF-α (Tsou *et al.*, 2010). Conversely, JNK1 is also deactivated (Figure 4.9b). In contrast, there was no detection of p-Akt in rapamycin-untreated samples (Figure 4.10b), strengthening the case that only phosphorylated Akt inhibit JNK1 signalling by binding to JIP1 and disrupting the formation of the MKK7-JNK1 scaffold. This contradicts observations from Kim *et al.* (2002), in which co-expression of wild-type or kinase-dead Akt reduced formation of a JIP1-mediated ternary complex between MLK3 and JNK1. However, the same authors Kim *et al.*

(2003) conceded that JIP1 phosphorylates Akt at serine 473. Other researchers have also established indirect links to Akt-JIP1 binding (Hao *et al*., 2008; Jaeschke *et al*., 2004; Morel *et al*., 2010). Taken together, these experiments demonstrate that p-Akt binding to JIP1 inhibits its ability to assemble active JNK signalling complexes. Presumably, rapamycin treatment will positively affect the binding affinity of Akt towards JIP1 by activating it at its serine 473 residue.

5.6.3 mTOR forms the active complex mTORC1 when stimulated withTNF-α

Anti-tumoural activity of rapamycin and its analogs have been reported in several breast cell line studies (Yu *et al*., 2001). Rapamycin, and likely its analogs (CCI779, RAD001, AP23573), are cell-type-dependent inhibitors of mTORC2 function as well as universal inhibitors of the mTORC1 pathway (Sarbassov *et al*., 2006). In the presence of rapamycin under serum deprived conditions, there was a decline in the cell proliferation of HepG2 cells (Varma & Kandelwal, 2007).

Earlier reports have shown that Akt phosphorylation at serine 473 is regulated by mTORC2, a complex consisting of mTOR, rictor, and mLST8 (Hresko & Mueckler, 2005; Sarbassov *et al*., 2005b). Based on Figure 4.10b, phosphorylation of Akt at the serine 473 residue suggests the presence of mTORC2 activity. This suggests that mTOR complexes into an active form

(mTORC2), and is in agreement with the hypothesis that mTOR complexes into mTORC1/2 as a more active signaling molecule (Sarbassov *et al.*, 2005b). In Figure 4.10c, total mTOR was detected in all samples but its activated form was only detected in rapamycin-untreated samples (Figure 4.10d). Stimulation with TNF-α increases its phosphorylation ~1.31-fold, which correlates with a report by Tsou *et al*. (2010). In rapamycin-untreated cells, mTOR may form the mTORC1, which is supported by the observation of mTOR phosphorylation at serine 2448 (Figure 4.10d). The mTORC1 would be assembled by the p70 S6 kinase (Chiang & Abraham, 2005; Holz & Blenis, 2005). Therefore, phosphorylation of mTOR at serine 2448 could be considered a marker for p70 S6 kinase activity (Chiang & Abraham; Holz & Blenis, 2005). Once the cells were pre-treated with rapamycin, the assembly of mTORC1 may be disrupted by rapamycin. This could encourage mTOR to form another active complex (mTORC2), as suggested by the phosphorylation of Akt at its serine 473 residue (Figure 4.10b). Furthermore, mTORC2 was observed to be resistant to low dosages of rapamycin treatment, while mTORC1 was not (Foster & Toschi, 2009; Sarbassov *et al*., 2006).

It might seem that mTORC1, located downstream of Akt, would be the upstream activator of JNK, instead of mTORC2. This is due to the observation that 100 ng/mL of rapamycin treatment abrogates JNK1 activation (Figure 4.9b), but not at lower concentrations. This correlates with the study conducted by Sarbossov *et al*. (2006), in which only rapamycin treatment higher than 100 ng/mL prohibits mTORC2 assembly. It is possible that JNK1 activation in

the HepG2 cell line is dependent on mTORC1 complexing and rapamycin inhibits JNK expression via the mTORC1 complex. It is therefore postulated that rapamycin inhibits mTORC1 as shown by the deactivation of JNK1 in rapamycin-treated cells (Figure 4.9b) and the decrease of mTOR phosphorylation at serine 2448 in rapamycin-treated cells (Figure 4.10d). It is hypothesised here that TNF-α signals to c-Jun via p70-mTORC1-JNK1 in rapamycin-untreated HepG2 cells. Consistent with this hypothesis, p70 S6 kinase was already shown to signal to JNK in bone cells (Takai *et al*., 2007). Conversely, with rapamycin treatment, it is suggested that TNF-α signals to c-Jun via mTORC2-Akt.

As shown in Figure 4.10e, PDK1 activation was consistent and not affected by rapamycin pre-treatment. This suggests that PDK1 is basally activated regardless of rapamycin treatment in this study. This agrees with earlier reports which concluded that PDK1 is constitutively active in mammalian cells (Alessi *et al*., 1997, Currie *et al*., 1999, Stephens *et al*., 1998). Interestingly, PDK1 has recently been shown to be a substrate of JNK (Frödin *et al*., 2002). Activation of JNK would activate ribosomal S6 kinase-2 (RSK2) and subsequently PDK1. The results gleaned from this study confirm that PDK1 is not affected by rapamycin treatment. This further reinforces the notion Akt is fully activated with rapamycin pre-treatment, as PDK1 activation is a marker of Akt phosphorylation at the threonine 308 residue (Sarbassov *et al*., 2005b); and conversely, Akt is phosphorylated at its serine 473 residue (Figure 4.10b).

A graphical representation of the mTOR complexing hypothesis as explained in this section is presented in Figure 5.1. Figure 5.2 shows the hypothetised location of mTORC1 in the TNF-α - JNK1 signalling pathway in this study. The interactions between JNK1, MKK7 and Akt are also graphically elaborated in Figure 5.2.

5.7 Effect of rapamycin on lipid transcription factors

Previously, it has been shown that TNF-α decreases the expression of *LXR-α* (Kim *et al*., 2007). To determine whether *PXR* expression will exhibit a similar pattern, HepG2 cells were treated with varying dosages of TNF-α.

From Figure 4.11, 20 ng/mL of TNF-α significantly reduced both *LXR-α* and *PXR* expression to ~0.5- and ~0.4-fold respectively. The reduction in *LXR-α* expression agrees with a recent study using Hep3B cells by Kim *et al*. (2007). Similar observations were made in HK2 (human proximal tubular) cells, 3T3-L1 cells and rabbit adipocytes, when TNF-α treatment significantly reduced *LXR-α* expression (Lu *et al*., 2006; Wang *et al*., 2005; Zhao & Dong, 2007).

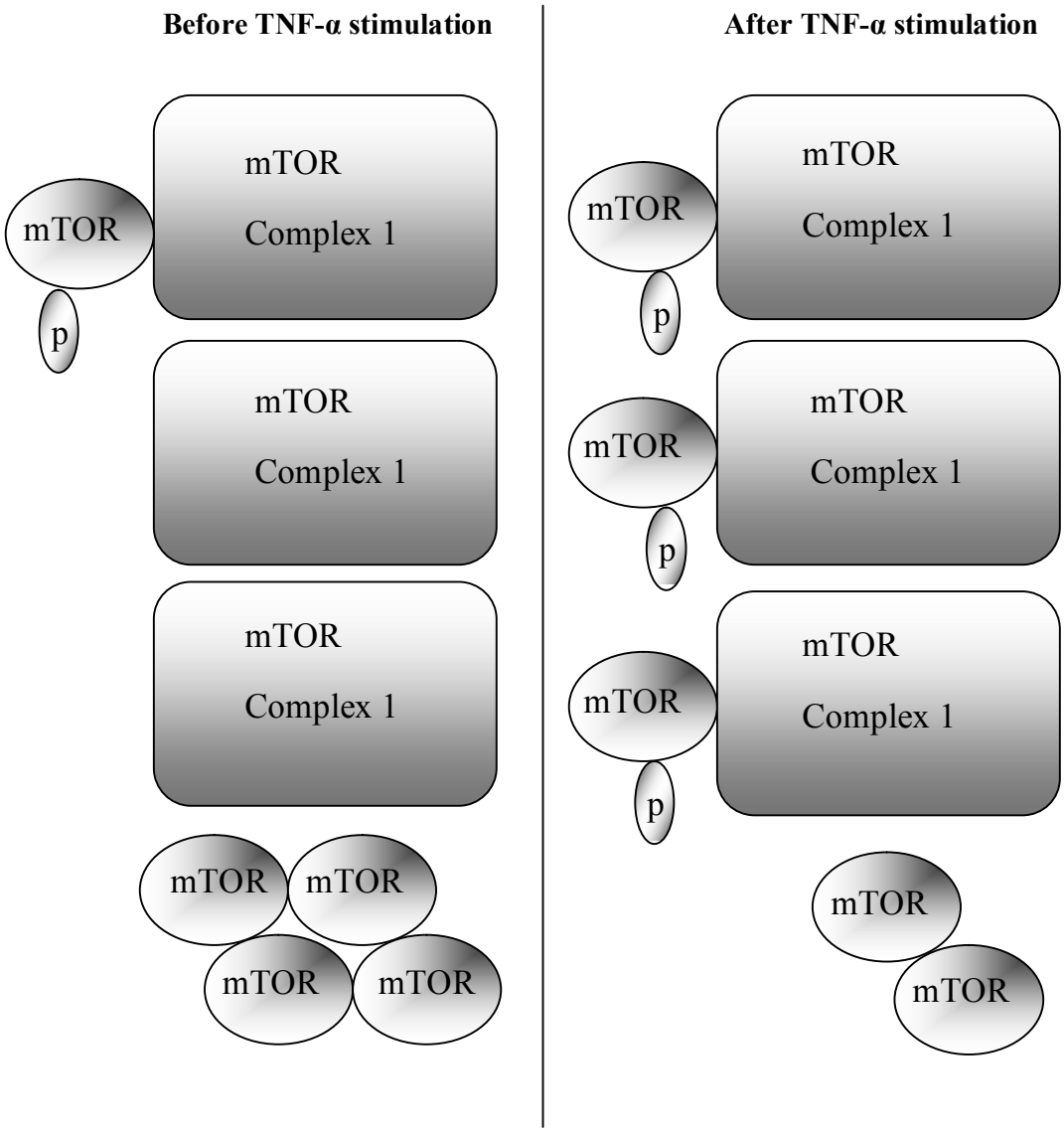

Figure 5.1: Hypothesis of mTOR complexing in HepG2 cells before (left panel) and after (right panel) TNF-α stimulation.
Before TNF-α stimulation, free mTOR molecules and mTOR Complex 1 are postulated to be in equilibrium. Presence of mTOR Complex 1 is detected by the phosphorylation of mTOR at its serine 2448 residue. After TNF-α stimulation, the free mTOR molecules are speculated to form more of the mTOR Complex 1, thus increasing the intensity of mTOR phosphorylation at serine 2448 in Western blots. Rapamycin completely blocks the formation of mTOR Complex 1, leading to formation of mTOR Complex 2. Therefore, phosphorylation of mTOR at serine 2448 is no longer possible.

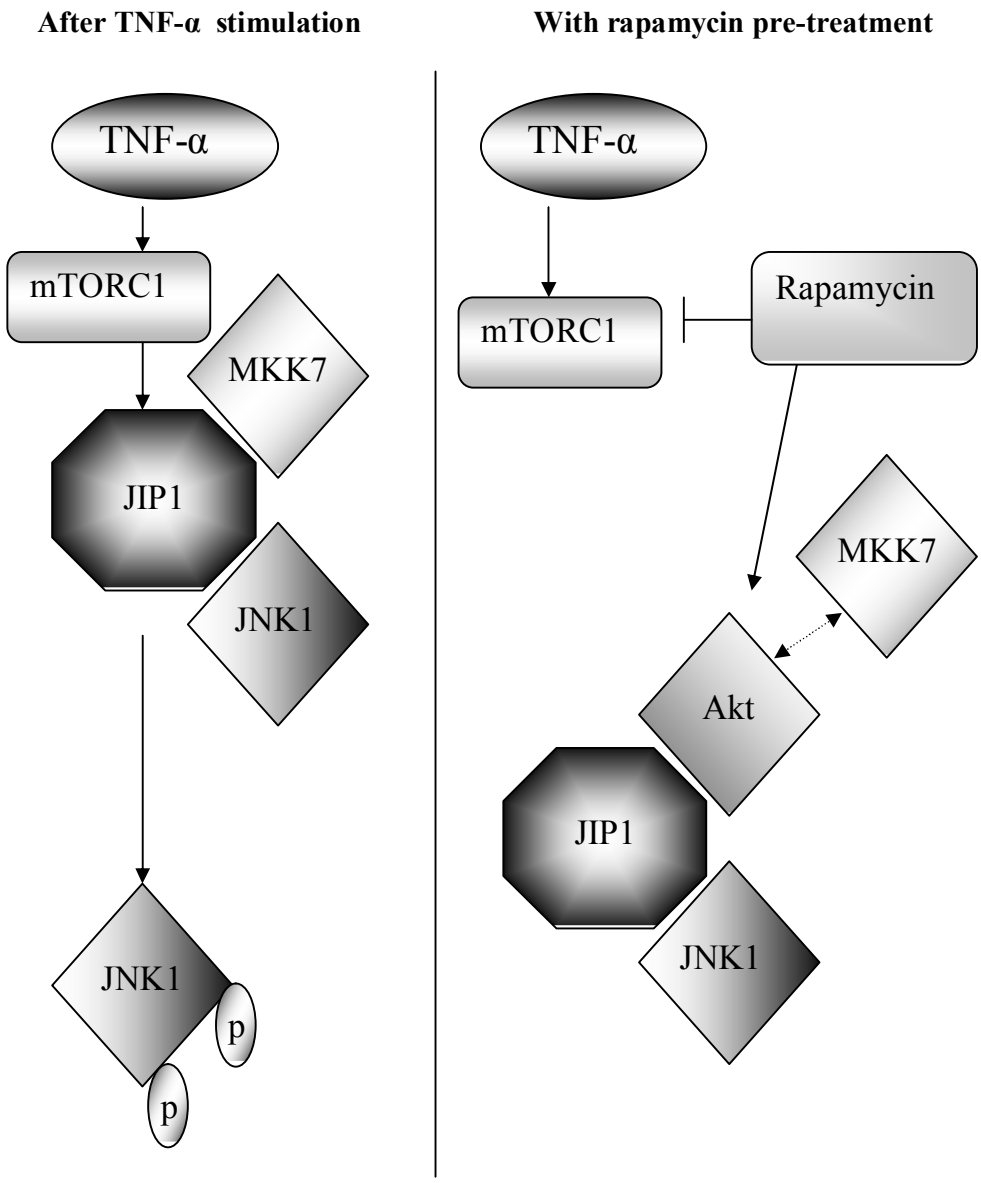

Figure 5.2: Graphical representation of hypothesised interactions among mTORC1, JNK1, MKK7, Akt and the JNK scaffold, JIP1 after TNF-α stimulation and rapamycin pre-treatment preceding TNF-α stimulation.
Upon TNF-α stimulation, mTORC1 is activated and the JNK1 signalling mechanism consisting of JIP, MKK7 and JNK1 are postulated to be assembled. This will cause successful phosphorylation of JNK1 and its subsequent release into the nucleus to act on its substrates. Rapamycin pre-treatment preceding TNF-α stimulation inhibits mTORC1 and also causes Akt to displace MKK7, thus preventing JNK1 phosphorylation and its subsequent release.

Indeed, RXR-α, which heterdimerises with LXR-α, was reduced upon stimulation with TNF-α as well (Kim *et al*., 2007). As for PXR, it has been reported that stimulation with the pro-inflammatory cytokines IL-6, IL-1 and LPS activates NF-*κ*B, a pro-inflammatory transcription factor, which in turn inhibit *PXR* expression (Assernat *et al*., 2006). Reciprocally, once the expression of NF-*κ*B was repressed, *PXR* expression was increased (Zhou *et al*., 2006). These findings conclusively show that the lipid metabolism and inflammatory pathways would eventually converge at some point.

The characteristic changes in lipid metabolism that occur during acute inflammation include hypertriglyceridemia, decreased hepatic fatty acid oxidation and ketogenesis, inhibition of bile acid synthesis, and decrease in serum HDL levels (Khovidhunkit *et al*., 2004). As both *LXR-α* and *PXR* expression are downregulated by TNF-α, they could be closely related to each other physiologically. It would be logical if their expression patterns mirror each other when exposed to certain external stimuli, such as ultraviolet radiation or reactive oxygen species, and in this particular study, TNF-α.

The repression of *LXR-α* and *PXR* when exposed to TNF-α would be mediated by kinases involved in signal transduction. As TNF-α is often implicated in JNK1 signalling (Bogoyevitch & Kobe, 2006), it is possible that JNK1 would be one of the important intermediates in this signalling cascade. Based on Figure 4.3, *JNK1* expression increased ~2.5-fold upon stimulation

with 20 ng/mL of TNF-α. This implies that the induction of JNK1 would repress *LXR-α* and *PXR* expression. However, as JNK1 is the most downstream of the MAPKs, it is probable that there may be still other upstream kinases which repression may induce an increase in *LXR-α* and *PXR* levels.

Interestingly, Kim *et al*. (2007) have also shown that the mRNA expression of the transcription factor PPAR-α, which primary function in mammals is to reduce lipid accumulation in cells was also repressed under TNF-α treatment. The downregulation of PPAR-α and its target genes have been implicated in the development of steatosis in most hepatocarcinoma cells (HCC) (Parent *et al*., 2007). Sustained mTOR activity may contribute to steatosis by impairing lipid homeostasis (Parent *et al*., 2007). Laplante and Sabatini (2010) also have shown that mTORC1 is involved in lipogenesis deregulation. Interestingly, both deregulation of JNK and mTOR has also been shown to contribute to HCC (Huang *et al*., 2010; Trieber, 2009; Wagner & Nebreda, 2009). As TNF-α impaired the induction of PPAR-α as well as *LXR-α* and *PXR*, it is therefore conceivable that mTOR signals downstream to JNK1 and subsequently LXR-α and PXR.

To see if mTOR inhibition affects the induction of *LXR-α* and *PXR*, the HepG2 cells were treated with rapamycin, an mTOR inhibitor, before the individual experiments were analysed. In Figure 4.11, TNF-α treatment significantly reduced the expression of *LXR-α* and *PXR* to ~0.5-fold. However,

pre-treatment with rapamycin, followed by TNF-α stimulation, resulted in a ~17- and ~2.0-fold increase in *LXR-α* and *PXR* expression respectively (Figure 4.11). Treatment with rapamycin only also resulted in a ~2.0-fold decrease in *LXR-α* and *PXR* expression. This confirms that the increase in *LXR-α* and *PXR* expression is not solely attributed to rapamycin treatment.

From these observations, it could be deduced that mTOR acts as an inhibitor in anti-inflammatory responses involving LXR-α and PXR. When the cells were stimulated with TNF-α alone, mTOR – possibly via JNK1 – inhibits the anti-inflammatory action of LXR-α and PXR, as shown in the decrease in the mRNA expression of both genes and the corresponding increase in *JNK1* (Figure 4.4). Removing the inhibitor – mTOR in this instance – will lead to upregulation of *LXR-α* and *PXR* expression when challenged with TNF-α.

The observations here suggest that rapamycin augments the anti-inflammatory response when stimulated with TNF-α by inducing the transcription of *LXR-α* and *PXR*. This hypothesis is backed by considerable evidence that has emerged indicating that, in addition to inducing genes involved in reverse cholesterol transport, LXRs reciprocally repress a set of inflammatory genes after bacterial, LPS, TNF-α, or IL-1β stimulation (Joseph *et al*., 2003). Examples of such genes include those involved in generation of bioactive molecules such as iNOS and COX2, IL-6 and IL-1β, the chemokines monocyte chemoattractant protein-1 (MCP-1) and MCP-3, and MMP9 (Joseph

et al., 2003; Castrillo *et al*., 2003a). Recently, PXR has been shown to have a significant effect on ablating the inflammatory response mediated by exogenous toxins and to have an important role in modulating inflammatory diseases of the bowel (Langmann *et al*., 2004; Shah *et al*., 2007; Zhou *et al*., 2006).

5.8 Future studies

As there were many drugs (inhibitors, cytokines) used in this study, there were some caveats that should be made known. The multiple targets involved present a complex network that may easily cross-talk with each other, or possibly negate each other's role in a negative feedback mechanism. Furthermore, potential toxic effects of the inhibitors and cytokines have not been studied explicitly in this experimental model. They may or may not affect downstream processes and signals, and due to the complexity of cellular signalling, events may be misinterpreted**.** Therefore, the arguments put forth here may be valid specifically for HepG2 cells only, under the exact experimental conditions in this study.

To improve upon this study, *in vivo* studies using *JNK* or *mTOR* knockout mice and siRNA-mediated knockdown on HepG2 cells could be done in the future. These experiments will futher our understanding of mTOR-JNK signalling at the physiological and transcriptional level, respectively.

mTOR complexing can be studied by co-immunoprecipitation experiments, using the other components in mTORC, namely Raptor, Rictor, etc. With rapamycin treatment, the mechanism in which rapamycin inhibits the assembly of mTORC can be investigated and its sequence of assembly can be elucidated. On the other hand, JNK scaffolding can be further examined. The identities of the MAPKKKs which accompanies JNK1/2 and MKK4/7 remains very much in doubt. Through co-immunoprecipitation, the specific kinases and their respective specific scaffolds which assemble them can be identified.

It is also imperative to investigate the effects of other transcription factors in the presence of upregulated *LXR-α* and *PXR*. As they have been implicated in anti-inflammatory reactions, and their upregulation correlates with reduced JNK1 expression in this study, there could be other mechanisms in which they can regulate the anti-inflammatory reaction. The transcriptional control and changes of mRNA stability of *LXR-α* and *PXR* can also be studied. For example, actinomycin-D treatment may be used to see if the LXR-α and PXR mRNA transcripts are stable when subjected to different treatments. The nuclear receptor activity can be measured with a functional promoter reporter assay by transient transfection of a luciferase reporter gene. If there is indeed transcriptional control on *LXR-α* and *PXR*, promoter analysis experiments such as identification of upstream *cis* elements can be done. Identification of interacting transcription factors (such as PPAR-α, RXR) can be carried out by electrophoretic mobility shift assay (EMSA), DNase I protection (footprinting) assay, methylation interference and ultraviolet crosslinking.

CHAPTER 6

CONCLUSION

In this study, JNK1 and JNK2 mRNA and protein were shown to be induced by TNF-α to almost ~2-fold. A panel of inhibitors were tested to see if there were novel pathways involved in TNF-α-JNK1 signalling. Rapamycin treatment was demonstrated to reduce TNF-α-induced *JNK1* expression, and thus linking mTOR in the TNF-α-JNK1 pathway.

A series of Western blots that were performed showed that TNF-α stimulation alone resulted in mTOR phosphorylation at its serine 2448 residue, a marker for mTORC1. However, both MKK7 and MKK4 were not activated. Consequently, JNK1 and its canonical substrate, c-Jun, was shown to be activated. Activation of c-Jun was observed at its serine 63. Rapamycin pre-treatment, however, abrogated the above-mentioned phosphorylations, with the exception of c-Jun. In contrast, Akt was phosphorylated at its serine 473 residue instead, indicating the formation of mTORC2 in the presence of rapamycin. MKK7 was also observed to be activated, but not MKK4 and JNK1. This suggests that the activated Akt molecule may impede MKK7 and JNK1 interaction, resulting in the inhibition of JNK1 activation. This is the first reported instance of JNK1 deactivation with corresponding Akt phosphorylation at its serine 473 residue.

This study also showed that treatment with TNF-α or rapamycin alone repressed the expression of the nuclear hormone receptors, *LXR-α* and *PXR*. However, rapamycin pre-treatment followed by TNF-α stimulation induced *LXR-α* and *PXR* expression, presumably to counter the effects of inflammation. This induction corresponded with the decrease in *JNK1*, as observed in this study. This suggests that TNF-α downregulation of the transcription factors are mediated through JNK1 stimulation. Further studies could be necessary to investigate the exact mechanism involved in the downregulation of *LXR-α* and *PXR*.

As a conclusion, this study has shown that TNF-α signalling to JNK1 is mediated by the mTOR/Akt signalling pathway, proving the hypothesis of this study.

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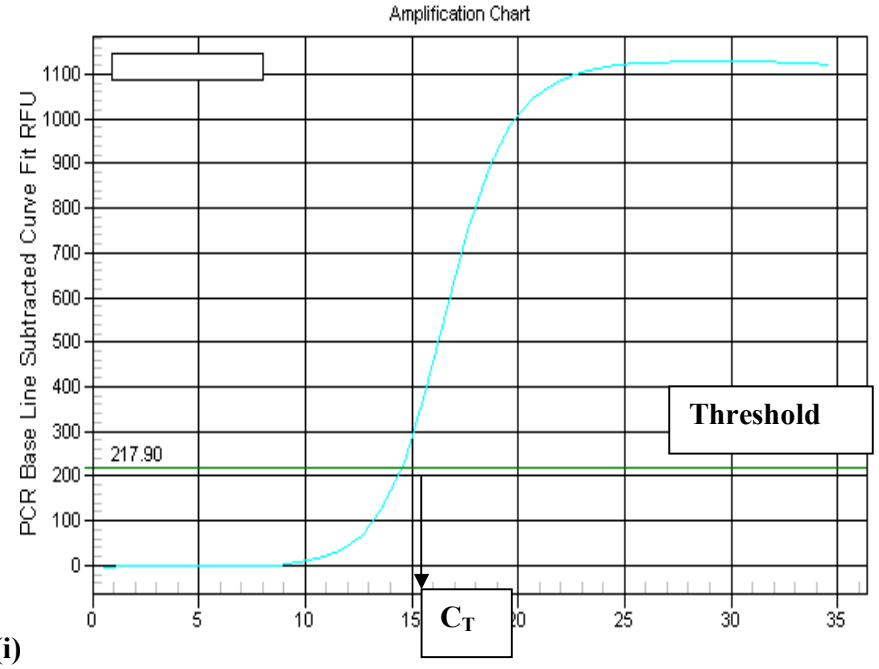
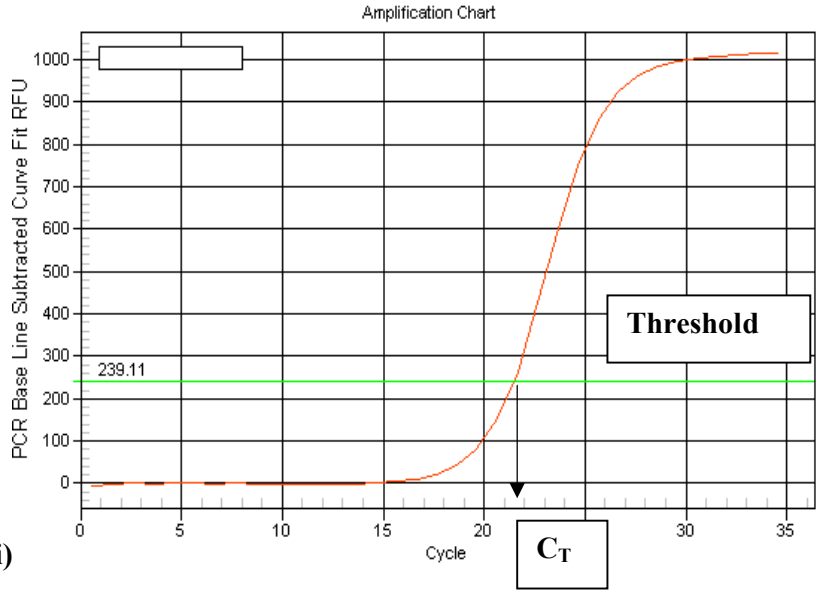
Appendix A

Graphical representation of the logarithmic scale in real-time RT-PCR amplification for (i) *β-actin*, (ii) *JNK1* and (iii) *JNK2*. The results were representatives of three independent experiments. The cycle number is shown along the X-axis and the arbitrary fluorescence units (which are fold increase over background fluorescence are shown on the Y-axis. The amplification plots have shown increase in fluorescence from the three genes. Based on the C_T values, *β-actin* is more highly expressed than both *JNK1* and *JNK2*.

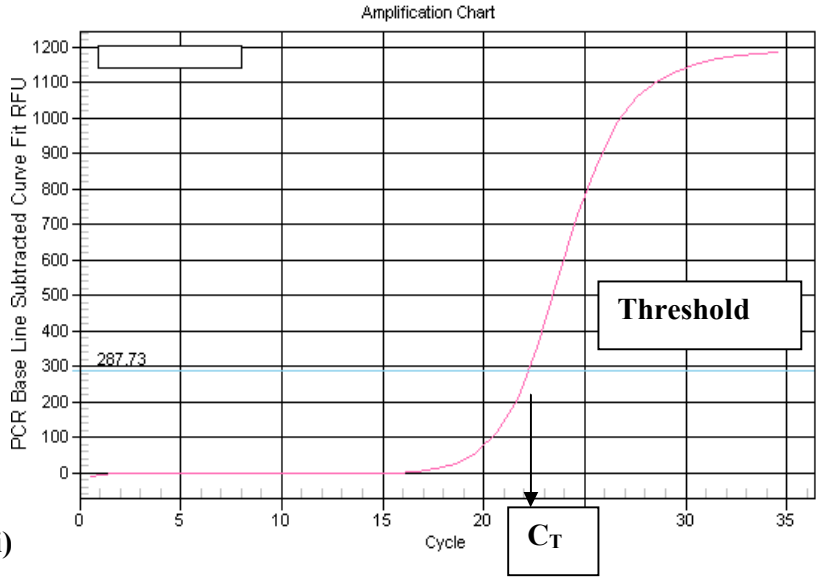


(i)

(ii)


Amplification Chart
PCR Base Line Subtracted Curve Fit RFU
1000
900
800
700
600
500
400
300
239.11
200
100
0
0
5
10
15
20
30
35
Cycle
Threshold
C_T

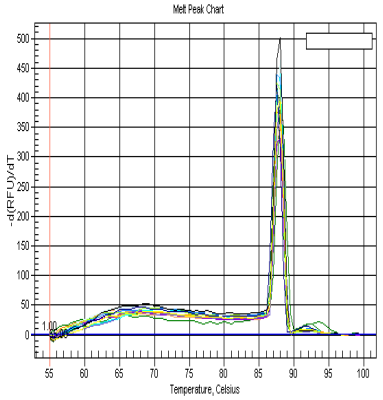
(iii)


Amplification Chart
PCR Base Line Subtracted Curve Fit RFU
1200
1100
1000
900
800
700
600
500
400
287.73
300
200
100
0
0
5
10
15
20
30
35
Cycle
Threshold
C_T

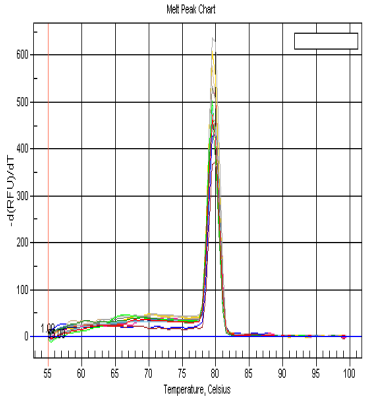
Appendix B

Melt peaks for (i) *β-actin*, (ii) *JNK1* and (iii) *JNK2*. The results were representatives of three independent experiments. The temperature is raised by 0.5°C and the change in the fluorescence is measured. At the melting point, the two strands of DNA will separate and the fluorescence will rapidly decrease. The software plots the rate of change of the relative fluorescence units (RFU) with time (T) [(-d(RFU)/dT] on the Y-axis vs. the temperature on the X-axis, and the peak is the melting temperature (T_m) of the respective PCR products.

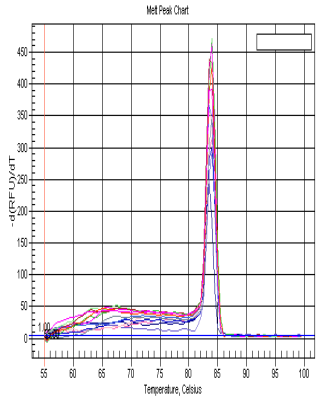
(i)



(ii)



(iii)



Appendix C

Comparison between the sequence of the RT-PCR products and the published (i-iv) *JNK1* (previously known as MAPK8) and (v-viii) *JNK2* (previously known as MAPK9) sequences from Genbank. *JNK1* was successfully amplifed using the primer set JNK1F and JNK1R without cross-amplifying *JNK2*. *JNK2* was succesfully amplifed using the primer set JNK2F and JNK2R without cross-amplifying *JNK1*. Identical nucleotides are indicated by vertical lines. The sequence of the RT-PCR product is shown on the upper line. Nucleotides underlined denote the region where the reverse primer anneals to during RT-PCR.

(i)

>ref|NM_002750.2| Homo sapiens mitogen-activated protein kinase 8 (MAPK8), transcript variant JNK1-α1, mRNA Length=1417

GENE ID: 5599 MAPK8 | mitogen-activated protein kinase 8 [Homo sapiens]
(Over 100 PubMed links)

Score = 244 bits (132), Expect = 1e-62
Identities = 142/146 (97%), Gaps = 3/146 (2%)
Strand=Plus/Plus

```
Query 15  GTGTTGAGA-TGGAGA-TCTACATTCACAGTCCTG-AACGATATCAGAATTTAAAACCTA  71
          |||| |||| |||||| |||||||||||||||||| |||||||||||||||||||||||||
Sbjct 52  GTGTAGAGATTGGAGATTCTACATTCACAGTCCTGAAACGATATCAGAATTTAAAACCTA  111

Query 72  TAGGCTCAGGAGCTCAAGGAATAGTATGCGCAGCTTATGATGCCATTCTTGAAAGAAATG  131
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 112  AGGCTCAGGAGCTCAAGGAATAGTATGCGCAGCTTATGATGCCATTCTTGAAAGAAATG  171

Query  132 TTGCAATCAAGAAGCTAAGCCGACCA  157
           |||||||||||||||||||||||||
Sbjct  172 TTGCAATCAAGAAGCTAAGCCGACCA  197
```

(ii)

>ref|NM_139049.1| Homo sapiens mitogen-activated protein kinase 8 (MAPK8), transcript variant JNK1-α2, mRNA Length=1412

GENE ID: 5599 MAPK8 | mitogen-activated protein kinase 8 [Homo sapiens]
(Over 100 PubMed links)

Score = 244 bits (132), Expect = 1e-62
Identities = 142/146 (97%), Gaps = 3/146 (2%)
Strand=Plus/Plus

```
Query 15  GTGTTGAGA-TGGAGA-TCTACATTCACAGTCCTG-AACGATATCAGAATTTAAAACCTA  71
          |||| |||| |||||| |||||||||||||||||| |||||||||||||||||||||||||
Sbjct 52  GTGTAGAGATTGGAGATTCTACATTCACAGTCCTGAAACGATATCAGAATTTAAAACCTA  111

Query 72  TAGGCTCAGGAGCTCAAGGAATAGTATGCGCAGCTTATGATGCCATTCTTGAAAGAAATG  131
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Sbjct 112 TAGGCTCAGGAGCTCAAGGAATAGTATGCGCAGCTTATGATGCCATTCTTGAAAGAAATG  171

Query 132 TTGCAATCAAGAAGCTAAGCCGACCA  157
          |||||||||||||||||||||||||
Sbjct 172 TTGCAATCAAGAAGCTAAGCCGACCA  197
```

(iii)

>ref|NM_139046.1| Homo sapiens mitogen-activated protein kinase 8 (MAPK8), transcript variant JNK1-β1, mRNA Length=1417

GENE ID: 5599 MAPK8 | mitogen-activated protein kinase 8 [Homo sapiens] (Over 100 PubMed links)

Score = 244 bits (132), Expect = 1e-62
Identities = 142/146 (97%), Gaps = 3/146 (2%)
Strand=Plus/Plus

```
Query 15  GTGTTGAGA-TGGAGA-TCTACATTCACAGTCCTG-AACGATATCAGAATTTAAAACCTA  71
          |||| |||| |||||| |||||||||||||||||| |||||||||||||||||||||||||
Sbjct 52  GTGTAGAGATTGGAGATTCTACATTCACAGTCCTGAAACGATATCAGAATTTAAAACCTA  111

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Sbjct 112 TAGGCTCAGGAGCTCAAGGAATAGTATGCGCAGCTTATGATGCCATTCTTGAAAGAAATG  171

Query 132 TTGCAATCAAGAAGCTAAGCCGACCA  157
          |||||||||||||||||||||||||
Sbjct 172 TTGCAATCAAGAAGCTAAGCCGACCA  197
```

(iv)

>ref|NM_139047.1| Homo sapiens mitogen-activated protein kinase 8 (MAPK8), transcript variant JNK1-β2, mRNA Length=1412

GENE ID: 5599 MAPK8 | mitogen-activated protein kinase 8 [Homo sapiens] (Over 100 PubMed links)

Score = 244 bits (132), Expect = 1e-62
Identities = 142/146 (97%), Gaps = 3/146 (2%)
Strand=Plus/Plus

```
Query 15  GTGTTGAGA-TGGAGA-TCTACATTCACAGTCCTG-AACGATATCAGAATTTAAAACCTA  71
          |||| |||| |||||| |||||||||||||||||| |||||||||||||||||||||||
Sbjct 52  GTGTAGAGATTGGAGATTCTACATTCACAGTCCTGAAACGATATCAGAATTTAAAACCTA  111

Quer  72  TAGGCTCAGGAGCTCAAGGAATAGTATGCGCAGCTTATGATGCCATTCTTGAAAGAAATG  131
          ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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(v)

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Score = 340 bits (184), Expect = 2e-91
Identities = 191/194 (98%), Gaps = 2/194 (1%)
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(vi)

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Score = 340 bits (184), Expect = 2e-91
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Strand=Plus/Plus

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(vii)

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(viii)

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Score = 196 bits (106), Expect = 5e-48
Identities = 166/195 (85%), Gaps = 4/195 (2%)
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Sbjct 1002 CATCAGCAGAGTTCA  1016
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Rapamycin pre-treatment abrogates Tumour Necrosis Factor-α down-regulatory effects on LXR-α and PXR mRNA expression via inhibition of c-Jun N-terminal kinase 1 activation in HepG2 cells

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Abstract The Liver X Receptor (LXR) and Pregnane X Receptor (PXR) are members of the nuclear receptor superfamily. Previously, they have been classified as important regulators of lipid homeostasis. However, recent studies have shown that they may be implicated in anti-inflammatory responses as well. This study shows that Tumour Necrosis Factor-α (TNF-α) treatment reduces both LXR-α and PXR mRNA expression. However, pre-treatment with rapamycin, an mTOR inhibitor, followed by TNF-α stimulation, significantly induces LXR-α and PXR mRNA expression to ~17- and ~2-fold, respectively. This suggests that mTORC1, a multi-molecular complex of which mTOR is a member, may act as a negative regulator that inhibits the induction of LXR-α and PXRas anti-inflammatory genes. It is also shown here that inhibition of JNK1 via the mTOR/Akt pathway coincides with the up-regulation of LXR-α and PXR mRNA, after TNF-α treatment. Together, these observations suggest that JNK1 possibly act downstream of mTORC1 as an LXR-α and PXR inhibitor. From the results gleaned in this study, rapamycin (and its analogues) may be used to reduce acute inflammation by promoting the induction of LXR-α and PXR as anti-inflammatory genes.

Keywords: Akt, c-Jun, homeostasis, inflammation, MKK7, transcription factor

INTRODUCTION

Several members of the nuclear receptor family, including LXR and PXR have emerged as important regulators of inflammatory signalling (Castrillo and Tontonoz, 2004). Previously, LXRs were only classified as cholesterol sensors that regulate the expression of genes involved in lipid metabolism in response to specific oxysterol ligands (Repa and Mangelsdorf, 2000). Conversely, the canonical function of the PXR is to sense elevations in xenobiotics and endobiotics and to orchestrate a response that promotes xenobiotic/endobiotic metabolism and excretion (Kliewer, 2003). Numerous studies have established that LXRs regulate gene expression linked to cholesterol metabolism in a tissue-specific manner. For example, LXR activation in rodent liver up-regulates Cyp7a1, a member of the cytochrome P450 family that is critical for bile acid synthesis (Peet et al. 1998). In the intestine, LXR controls the reabsorption of cholesterol via the expression of ABCG5 and ABCG8 (Yu et al. 2002). In peripheral cells such as macrophages, LXRs regulates the expression of a panel of genes involved in reverse cholesterol transport. In response to macrophage cholesterol overload, LXRs induce expression of the cholesterol efflux transporters ABCA1 and ABCG1, the apolipoproteins apoE and apoCs, and the phospholipid transfer protein, PLTP (Repa and Mangelsdorf, 2000; Zelcer and Tontonoz, 2006). PXR, on the other hand, regulates the expression of several key enzymes controlling the bile acid synthesis

pathway, lipid metabolism and glucose homeostasis (Eloranta and Kullak-Ublick, 2005; Handschin and Meyer, 2005; Moreau et al. 2008). The roles of LXR and PXR as intermediates in inflammatory signalling has also been described (Castrillo et al. 2003; Joseph et al. 2003; Langmann et al. 2004; Shah et al. 2006; Zhou et al. 2006). Since LXR and PXR are involved in both metabolic and inflammatory pathways, this makes them potentially attractive targets for the modulation of inflammation responses, especially towards TNF-α.

TNF-α down-regulates specific targets which include the nuclear receptors LXR and probably PXR (Kim et al. 2007). Upon TNF-α stimulation, the c-Jun N-terminal kinases (JNK) will be activated via its upstream kinase MKK7 (Bogoyevitch and Kobe, 2006). Subsequently, the activated JNK would translocate into the nucleus to activate its cognate transcription factor, c-Jun (Bogoyevitch and Kobe, 2006). There have been reports linking the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway to JNK activation (Wajant and Scheurich, 2001). However there was no direct attempt at studying the mechanisms of the mTOR/Akt/JNK interplay. There are still doubts as to whether rapamycin will inhibit JNK directly or indirectly via its many upstream kinases. This study will attempt to demonstrate that LXR-α and PXR mRNA repression by TNF-α via the JNK cascade is abrogated by mTOR inhibition. Insights obtained from this study may enable restoration of normal lipid homeostasis in acutely inflamed human cells in the future.

MATERIALS AND METHODS

Cell line and culture

The human hepatocarcinoma cell line, HepG2 cells, were obtained from ATCC (Manassas, VA, USA) and grown in Minimum Essential Medium with Earle salts (Invitrogen; Carlsbad, CA, USA) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and penicillin/streptomycin (100 units ml^{-1}) in a 5% CO_2 incubator maintained at 37ºC.

Cell treatment

For expression studies, cells were stimulated with 20 ng/mL of TNF-α (Millipore Corporation; Billerica, MA, USA). For rapamycin inhibition studies, 10 nM of rapamycin (Sigma-Aldrich; St. Louis, MO, USA) was added 2 hrs prior to TNF-α stimulation for 2 hrs.

Real-time RT-PCR

Expressions of human *Lxr-α, Pxr, Jnk1* and *β-actin* mRNA transcripts were detected by real-time RT-PCR. Briefly, total RNA from HepG2 cells was isolated using the RNeasy Mini kit (QIAGEN; Dusseldorf, Germany) and a real-time one-step RT-PCR performed using Quantifast SYBR Green (QIAGEN; Dusseldorf, Germany) on a MyIQ iCycler (Bio-Rad; Berkeley, CA, USA) using the protocol: reverse transcription at 10 min at 50ºC, initial denaturation for 5 min at 94ºC, 34 cycles of 10 sec at 94ºC, 30 sec at 58ºC (*Lxr-α*, *Pxr* and *β-actin*) or 63ºC (*Jnk1*), and 30 sec at 72ºC, followed by a 10 min final extension step at 72ºC. Melt curve analysis was performed as follows: 1 min at 55ºC , followed by 10 sec at 55ºC with an incremental 0.5ºC for 90 cycles. Primer sequences used to amplify the human *Lxr-α* and *Pxr* were as follows: 5'-TCAGGCGGATCTGTTCTTCT-3' (*Lxr-α* reverse), 5'-CGGGCTTCCACTACAATGTT-3' (*Lxr-α* forward), resulting in a 213-bp RT-PCR product, and 5'-TCCCTGTCCGTTCACTTTTC-3' (*Pxr* reverse), 5'-CAAATCTGCCGTGTATGTGG-3' (*Pxr* forward), resulting in a 289-bp RT-PCR product. Primer sequences used to amplify the human *Jnk1* were as follows: 5'-GCCTGTCGCACGTGATTGA-3' (*Jnk1* reverse) and 5'-CGTCTTCGTTCGCACTGTTG-3' (*Jnk1* forward), resulting in a 175-bp RT-PCR product. *β-actin* primers were used as an internal control. The primer sequences for human *β-actin* were as follows: 5'-CGTACCACTGGCATCGTGAT-3' (forward), 5'-CCATCTCTTGCTCGAAGTTC-3' (reverse), which yielded a 280-bp RT-PCR product.

Western blot analysis

Cells were harvested, washed in phosphate-buffered saline (PBS) and centrifuged. Cytoplasmic and nuclear proteins were extracted using the Nuclear and Cytoplasmic Extraction kit (Pierce, Thermo Fisher Scientific; Waltham, MA, USA). The cell lysates were subsequently resolved using SDS-PAGE,

transferred onto Immobilon-P membranes (Millipore Corporation; Billerica, MA, USA), and immunoblotted with anti-JNK/SAPK #9252, anti-phospho-SAPK/JNK (Thr183/Tyr185) #9251, anti-c-Jun #9165, anti pc-Jun (Ser 73) #9164, anti-MKK7 #4172, anti-phospho-MKK7 (Ser271/Thr275) #4171, anti-mTOR #2972, anti-phospho-mTOR (Ser2448) #2971, anti-Akt #9272, anti-phospho-Akt (Ser473) #9271, anti-rabbit IgG HRP-linked antibody (#7074). Anti-β-actin (#4967) was utilised as an internal control. The nuclear fractions were probed against c-Jun and pc-Jun only, while the cytoplasmic fractions were probed against the other antibodies. All antibodies were purchased from Cell Signaling Technology; Beverly, MA, USA. Immunoreactive bands were visualised and their densities analysed using the Fluorchem FC2 system (Alpha Innotech; San Leandro, CA, USA).

Statistical analysis

A one-way ANOVA was used to compare mean values for independent variables. Significant differences at 95% confidence ($p < 0.05$) and 99% confidence ($p < 0.01$) are depicted with an asterisk (*) and two asterisks (**) on each graph, respectively. Each experiment was repeated three times.

RESULTS AND DISCUSSION

Previously, it has been shown that TNF-α decreases the expression of LXR-α mRNA (Kim et al. 2007). To determine whether PXR mRNA expression will exhibit a similar pattern, HepG2 cells were treated with varying dosages of TNF-α. From Figure 1, 20 ng/mL of TNF-α significantly reduces both LXR-α and PXR mRNA expression to ~0.5- and ~0.4-fold respectively. The reduction in LXR-α mRNA expression agrees with a recent study using Hep3B cells by Kim et al. (2007). Similar observations were made in HK2 (human proximal tubular) cells, 3T3-L1 cells and rabbit adipocytes, when TNF-α treatment significantly reduced LXR-α mRNA expression (Wang et al. 2005; Lu et al. 2006; Zhao and Dong, 2007). Indeed, RXR-α, which heterodimerise with LXR-α, was reduced upon stimulation with TNF-α as well (Kim et al. 2007). As for PXR, it has been reported that stimulation with the pro-inflammatory cytokines IL-6, IL-1 and LPS activates NF-*κ*B, a pro-inflammatory transcription factor, which in turn inhibit PXR mRNA expression (Assenat et al. 2006). Reciprocally, once the expression of NF-*κ*B was repressed, PXR mRNA expression was increased (Zhou et al. 2006). These findings conclusively show that the lipid metabolism and inflammatory pathways would eventually converge at some point. The characteristic changes in lipid metabolism that occur during acute inflammation include hypertriglyceridemia, decreased hepatic fatty acid oxidation and ketogenesis, inhibition of bile acid synthesis, and decrease in serum HDL levels (Khovidhunkit et al. 2004). As both LXR-α and PXR mRNA expression were down-regulated by TNF-α, they could be closely related to each other physiologically. It would be logical if their expression patterns mirror each other when exposed to certain external stimuli, such as ultraviolet radiation or reactive oxygen species, and in this particular study, TNF-α.

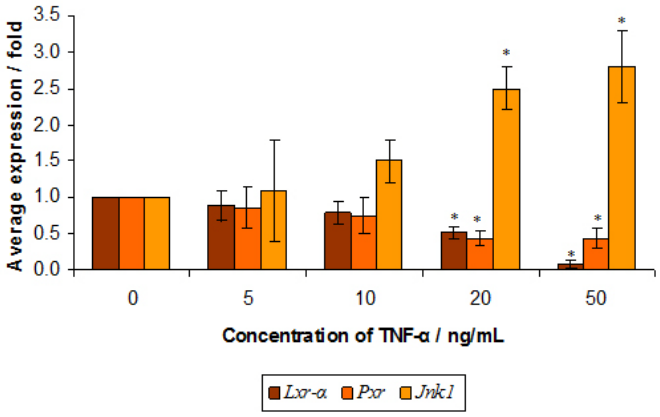

Fig. 1 Average mRNA expression of LXR-α, PXR and JNK1 as normalised to β-actin when treated with 0, 5, 10, 20 and 50 ng/mL of TNF-α for 24 hrs. * significant increase at $p < 0.05$ vs. control. Treatment of 20 ng/mL of TNF-α alone resulted in a significant decrease in mRNA expression for LXR-αand PXR mRNA expression. However, JNK1 mRNA was significantly increased.

The repression of LXR-α and PXR mRNA when exposed to TNF-α would be mediated by kinases involved in signal transduction. As TNF-α was often implicated in JNK1 signalling (Bogoyevitch and Kobe, 2006), it is possible that JNK1 would be one of the important intermediates in this signalling cascade. Based on Figure 1, JNK1 mRNA expression increased ~2.5-fold upon stimulation with 20 ng/mL of TNF-α. This increase in mRNA expression coincides with the decrease of LXR-α and PXR mRNA expression (Figure 1). Unfortunately, there is not enough substantive evidence in this study to suggest a concrete link between the two. However, knockdown of JNK1 has been shown to up-regulate PGC-1β (Yang et al. 2007), as a key activator of hepatic lipogenesis and lipoprotein secretion through activation of LXR-α (Wolfrum and Stoffel, 2006). As LXR-α auto-regulates its own transcription (Laffitte et al. 2001; Li et al. 2002, Ulven et al. 2004), it is conceivable that JNK1 may act to repress *Lxr-α* transcription through LXR-α inhibition. Kim et al. (2009) has proposed a contradictory argument, in which they hypothesised that JNK1 increases LXR-α activity, although this may be due to different cell types and experimental models. In any case, these reports suggest that there could be an interplay between JNK1 and LXR-α. Similar to our findings, Wang et al. (2010) has shown that activation of JNK1 coincides with reduced *Pxr* expression. As JNK1 is the most downstream of the mitogen activated protein kinases (MAPK) which is involved in inflammation (Wagner and Nebreda, 2009), it is probable that there may be still other upstream kinases which induction may cause a decrease in LXR-α and PXR mRNA levels. One viable option would be the mTOR. Sustained mTOR activity may contribute to steatosis by impairing lipid homeostasis (Parent et al. 2007) and also causes lipogenesis deregulation (Laplante and Sabatini, 2010).

To see if mTOR inhibition affects the induction of LXR-α and PXR mRNA, the HepG2 cells were treated with rapamycin, a mTOR inhibitor, before the individual experiments were analysed. In Figure 2, TNF-α treatment significantly reduced the expression of LXR-α and PXR mRNA to ~0.5-fold. This is in accordance with earlier experiments in this study (Figure 1). Rapamycin treatment alone causes a reduction in expression in LXR-α and PXR mRNA to ~0.5- and ~0.25-fold respectively (Figure 2). This shows that mTOR inhibition would not drive the expression of LXR-α and PXR mRNA by itself. However, pre-treatment with rapamycin, followed by TNF-α stimulation, resulted in a ~17- and ~2.0-fold increase in LXR-α and PXR mRNA expression respectively (Figure 2). From these observations, it could be deduced that mTOR acts as an inhibitor in TNF-α-mediated anti-inflammatory responses involving LXR-α and PXR. When the cells were stimulated with TNF-α alone, mTOR –possibly via JNK1– inhibits the anti-inflammatory action of LXR-α and PXR, as shown in the decrease in the mRNA expression of both genes and the corresponding increase in JNK1 mRNA expression (Figure 1). Removing the inhibitor –mTOR in this instance– will lead to up-regulation of LXR-α and PXR mRNA expression only when challenged with TNF-α. The observations here suggest that rapamycin augments the anti-inflammatory response only when stimulated with TNF-α by inducing the transcription of LXR-α and PXR mRNA. This hypothesis is backed by considerable evidence that has emerged indicating that, in addition to inducing genes involved in reverse cholesterol transport, LXRs reciprocally repress a set of inflammatory genes after bacterial, LPS, TNF-α, or IL-1β stimulation (Joseph et al. 2003). Examples of such genes include those involved in generation of bioactive molecules such as iNOS and COX2, IL-6 and IL-1β, the chemokines monocyte chemoattractant protein-1 (MCP-1) and MCP-3, and MMP9 (Castrillo et al. 2003; Joseph et al. 2003). PXR has been shown to have a significant effect on ablating the inflammatory response mediated by exogenous toxins and to have an important role in modulating inflammatory diseases of the bowel (Langmann et al. 2004; Shah et al. 2006; Zhou et al. 2006).

Subsequently, the role of JNK1 in regulating LXR-α and PXR mRNA expression was further examined. Recently, LXR-α, mTOR and JNK1 have been implicated in hepatic steatosis (Parent et al. 2007; Sabio et al. 2009; Kim et al. 2010). This provides a solid basis to further investigate their interplay. JNK1 has also been further identified as important regulators in hepatic steatosis and insulin resistance (Schattenberg et al. 2006; Sabio et al. 2009; Singh et al. 2009). Based on Figure 3a, JNK1 was constitutively expressed. JNK1 was activated after TNF-α stimulation (up to ~1.77-fold) in rapamycin-untreated cells. However, rapamycin treatment abrogated JNK1 activation in HepG2 cells (Figure 3b). Subsequent stimulation with TNF-α failed to re-establish JNK1 activation (Figure 3b). Apparently, it would seem here that rapamycin pre-treatment (and subsequent TNF-α stimulation) would up-regulate LXR-α and PXR mRNA expression (Figure 2), but abrogates JNK1 activation (Figure 3b).

To ensure that the abrogation of JNK expression is valid, the expression and activation of c-Jun, the canonical substrate of JNK, was studied. Figure 3c shows that total c-Jun expression remained constant regardless of any stimulation/treatment. From Figure 3d, TNF-α stimulation alone is shown to activate c-Jun in rapamycin-untreated samples, up to ~2.29-fold. However, rapamycin treatment alone

was also sufficient to induce c-Jun activation, as seen in rapamycin-treated samples (Figure 3d). Here, it would seem that c-Jun activation does not require prior JNK1 activation, as activated JNK1 was not detected under rapamycin treatment (Figure 3b). This suggests that there may be other kinases which may replace JNK1's role in activating c-Jun. Indeed, ERK8 has been shown to phosphorylate c-Jun directly in HCT15 colorectal cells (Xu et al. 2010). In neurons, the inhibition of JNK activity could not block either the c-Jun activation or up-regulation or their subsequent death (Dunn et al. 2002). In COP9 signalosome-directed c-Jun activation/stabilisation (Neumann et al. 1999) and calcium-regulated activation of c-Jun (Zanger et al. 2001), c-Jun activation was increased without corresponding JNK activation. The reduction in c-Jun activation (~0.62-fold) after rapamycin pre-treatment and subsequent TNF-α stimulation (Figure 3d) correlates with the increase in LXR-α and PXR mRNA expression (Figure 2). It is postulated here that rapamycin removes the molecular inhibitor that will prohibit LXR-α and PXR to counteract inflammation in cells (as induced here with TNF-α).

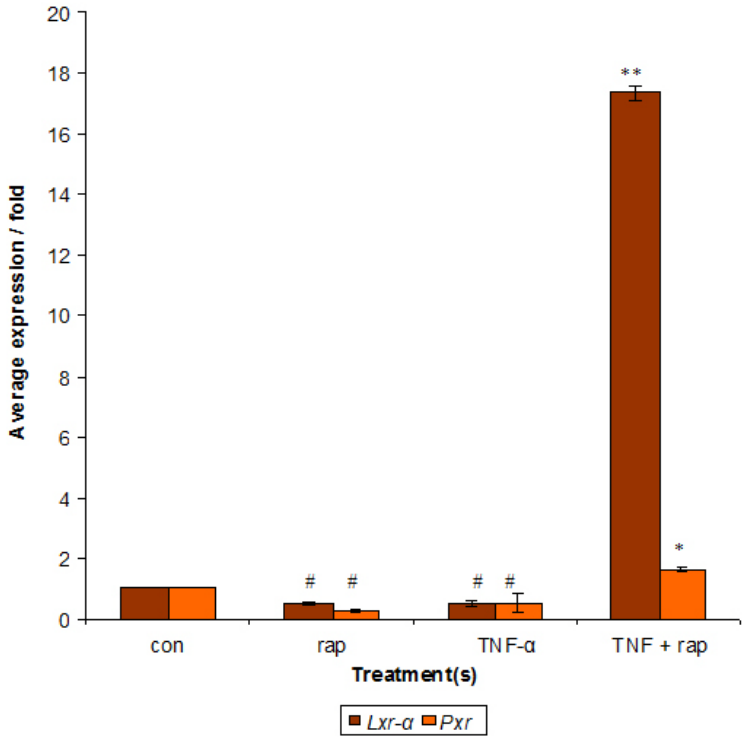

Fig. 2 Average mRNA expression of LXR-α and PXR as normalised to β-actin; untreated control (denoted by con bars), when treated with 100 ng/mL rapamycin only for 2 hrs (denoted by TNF-α bars) when stimulated with 20 ng/mL TNF-α only for 2 hrs (denoted by TNF-α bars) and when pre-treated with 100 ng/mL rapamycin for 2 hrs, subsequently followed by 20 ng/mL TNF-α for 2 hrs (denoted by TNF + rap bars). # significant decrease at $p < 0.05$ vs. control. * significant increase at $p < 0.05$ vs. control. ** significant increase at $p < 0.01$ vs. control. Treatment of TNF-α alone resulted in a significant decrease for LXR-α and PXRmRNA expression. Rapamycin treatment by itself causes a significant reduction in both *Lxr-α* and *Pxr*. However, pre-treatment with rapamycin followed by TNF-α stimulation caused a significant increase in induction for both LXR-α and PXR mRNA.

Next, the interaction between JNK1 and mTOR/Akt was studied. Previous experiments have shown that rapamycin, an mTOR inhibitor, successfully abrogates JNK1 activation as well as reduces LXR-α and PXR mRNA expression (Figure 2 and Figure 3b). Therefore, Western blots were performed using antibodies of components in the JNK1 cascade and the mTOR/Akt pathway, to look for any direct link that may herald cross-signalling between the two pathways. Based on Figure 4a, total MKK7, the specific JNK activator (Bogoyevitch and Kobe, 2006; Haeusgen et al. 2010), was detected in all samples. However, its activated form was only detected in rapamycin-treated samples (Figure 4b). As with the case of mTOR and Akt as discussed in the following sections, JNK1 and MKK7 could both be involved in a scaffolding complex, which was first described in yeast by Whitmarsh et al. (1998) and

further elucidated by Whitmarsh (2006). Scaffold proteins for the JNKs have been identified. These include the JNK interacting protein (JIP) group of putative scaffolds, which includes the JIP1, JIP2 and JIP3 proteins. The JIP1 and JIP2 proteins are closely related proteins that bind to JNK, MKK7, and mixed-lineage protein kinases (Whitmarsh et al. 1998; Yasuda et al. 1999). JIP1 and JIP2 binds to JNK, MKK7, and members of the mixed-lineage group of MAPKKK (Kelkar et al. 2000; Weston and Davis, 2002). Indeed, JIP1-deficient mice (Jaeschke and Davis, 2007) exhibit defects in JNK activation and insulin resistance. This observation was also expressed by Song and Yong (2005), who hypothesised that JIP1 assembles Akt, MKK7 and JNK1. As JNK1 and MKK7 would be complexed together in a signalling scaffold in JIP1, MKK7 would not need to be phosphorylated to exert its kinase activity (Nihalani et al. 2003). As shown in Figure 4b, consistent with the scaffolding hypothesis, MKK7 was not observed to be phosphorylated in rapamycin-untreated samples. An inactive scaffold may cause MKK7 to be independently activated, and this was shown in Figure 4b where rapamycin pre-treatment activated MKK7. In fact, MKK7 was shown to preferentially activated by TNF-α and cellular stresses (Moriguchi et al. 1997). Based on Figure 3d, this correlates with the results obtained in this experiment, where c-Jun was activated with corresponding MKK7 activation. p-JNK1 would be detected as it would be freed from the complex to translocate into the nucleus to act on transcription factors (Cavigelli et al. 1995).

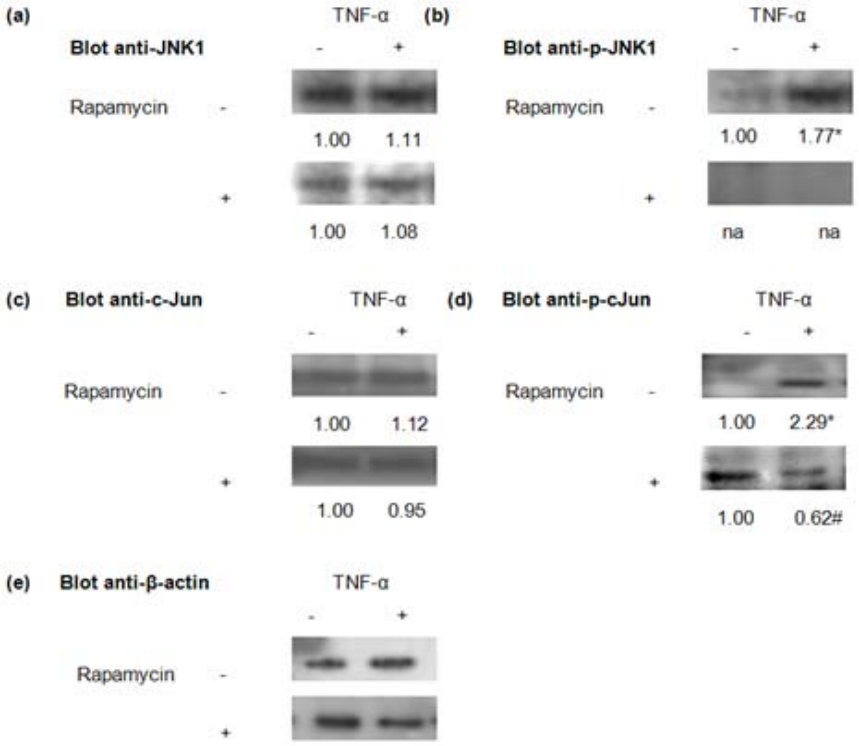

Fig. 3 Western blots of (a) JNK1, (b) p-JNK1, (c) c-Jun, (d) pc-Jun, (e) β-actin. This is a representation of three separate experiments. Rapamycin '-' indicates no pre-treatment and '+' indicates 2 hrs of rapamycin pre-treatment. TNF-α '-' signifies unstimulated samples and '+' signifies 2 hrs of TNF-α stimulation. * significant increase at $p < 0.05$ vs control. # significant decrease at $p < 0.05$ vs control. Treatment with rapamycin abrogated JNK1 activation. Rapamycin and TNF-α both activate c-Jun singly, but treatment with TNF-α reduces c-Jun activation after rapamycin treatment. β-actin was used as loading control. Densities for bands were recorded beneath each blot and were normalised to their respective loading controls.

Akt has been shown to inhibit JNK1 activity by binding to JIP1 (Kim et al. 2002). JIP1 assembles the Mixed Lineage Kinase 3 (MLK3), MKK7 and JNK1 in a complex which can be disrupted by Akt binding. In this study, Akt expression in rapamycin-untreated samples is stable (Figure 4c), and pre-treatment with rapamycin activates Akt (Figure 4d). Akt phosphorylation was increased at serine 473 ~1.48-fold after TNF-α stimulation (Figure 4d). The increase of serine 473 phosphorylation is due to the action of TNF-α (Tsou et al. 2010). Conversely, JNK1 is also deactivated (Figure 3b). In contrast, there was no

detection of p-Akt in rapamycin-untreated samples (Figure 4d), strengthening the case that only phosphorylated Akt inhibit JNK1 signalling by binding to JIP1 and disrupting the formation of the MKK7-JNK1 scaffold. This contradicts observations from Kim et al. (2002), in which co-expression of wild-type or kinase-dead Akt reduced formation of a JIP1-mediated ternary complex between MLK3 and JNK1. However, the same authors Kim et al. (2002) conceded that JIP1 phosphorylates Akt at serine 473. Other researchers have also established indirect links to Akt-JIP1 binding (Jaeschke et al. 2004; Hao et al. 2008; Morel et al. 2010). Taken together, these experiments demonstrate that p-Akt binding to JIP1 inhibits JIP1's ability to assemble active JNK signalling complexes. Presumably, rapamycin treatment will positively affect the binding affinity of Akt towards JIP1 by activating it at its serine 473 residue.

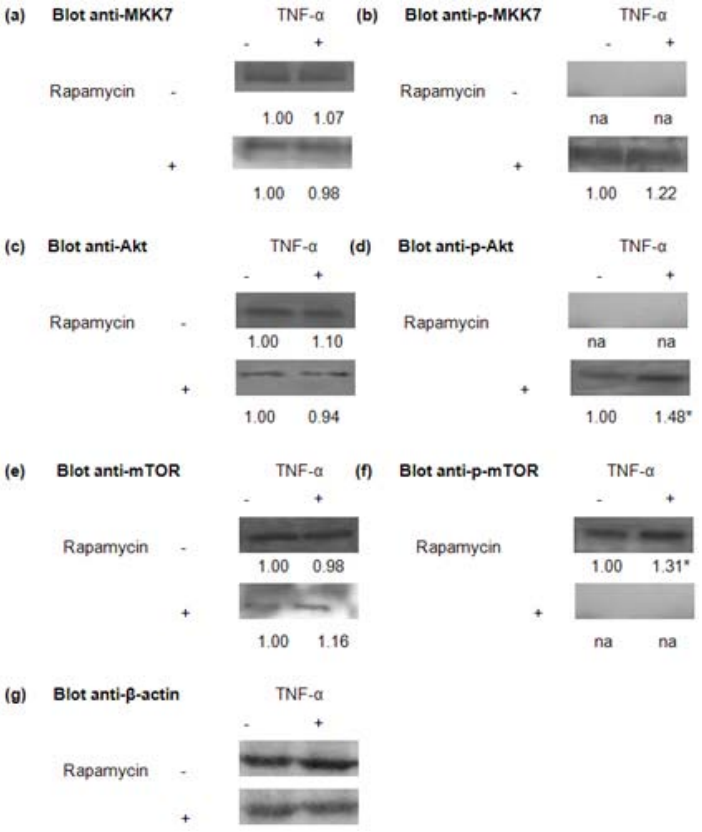

Fig. 4 Western blots of (a) MKK7, (b p-MKK7, (c) Akt, (d) p-Akt (S473), (e) mTOR, (f) p-mTOR (S2448) and (g) β-actin. Rapamycin '-' indicates no pre-treatment and '+' indicates 2 hrs of rapamycin pre-treatment. TNF-α '-' signifies unstimulated samples and '+' signifies 2 hrs of TNF-α stimulation. This is a representation of three separate experiments. * significant increase at $p < 0.05$ vs control.Pre-treatment with rapamycin activated MKK7 and Akt after TNF-α stimulation. However, rapamycin pre-treatment prevented mTOR phosphorylation at serine 2448. β-actin was used as loading control. Densities for bands were recorded beneath each blot and were normalised to their respective loading controls.

Rapamycin, and likely its analogs (CCI779, RAD001, AP23573), are cell-type-dependent inhibitors of mTORC2 function as well as universal inhibitors of the mTORC1 pathway (Sarbassov et al. 2006). Based on Figure 4d, phosphorylation of Akt at the serine 473 residue is considered a marker of mTORC2 activity (Hresko and Mueckler, 2005; Sarbassov et al. 2005; Jacinto et al. 2006). This suggests that mTOR complexes into an active form (mTORC2), and is in agreement with the hypothesis that mTOR complexes into mTORC1/2 as a more active signaling molecule (Sarbassov et al. 2005). In Figure 4e, total mTOR was detected in all samples but its activated form was only detected in rapamycin-untreated samples (Figure 4f). Stimulation with TNF-α increases its phosphorylation ~1.31-fold, which correlates with a report by Tsou et al. (2010). In rapamycin-untreated cells, mTOR may form the mTORC1, which is supported by the observation of mTOR

phosphorylation at serine 2448 (Figure 4f). The mTORC1 would be assembled by the p70 S6 kinase (Chiang and Abraham, 2005; Holz and Blenis, 2005). Therefore, phosphorylation of mTOR at serine 2448 could be considered a marker for p70 S6 kinase activity (Chiang and Abraham, 2005; Holz and Blenis, 2005). Once the cells were pre-treated with rapamycin, the assembly of mTORC1 may be disrupted by rapamycin. This could encourage mTOR to form another active complex (mTORC2), as suggested by the phosphorylation of Akt at its serine 473 residue (Figure 4f). Furthermore, mTORC2 was observed to be resistant to low dosages of rapamycin treatment, while mTORC1 was not (Sarbassov et al. 2006; Foster and Toschi, 2009). It is postulated here that TNF-α signals to c-Jun via p70-mTORC1-JNK1 in rapamycin-untreated HepG2 cells. Consistent with this hypothesis, p70 S6 was already shown to signal to JNK in bone cells (Takai et al. 2007). Conversely, with rapamycin treatment, it is postulated that TNF-α signals to c-Jun via mTORC2-Akt.

As conclusion, LXR-α and PXR mRNA are up-regulated upon TNF-α stimulation, only if rapamycin was administered beforehand. Although there was a lack of concrete evidence that JNK1 down-regulates LXR-α and PXR mRNA specifically, it was shown here in this study that LXR-α and PXR mRNA up-regulation (Figure 2) coincides with abrogation of JNK1 activation (Figure 3b), after TNF-α administration. Pre-treatment with rapamycin prior to TNF-α stimulation may release any inhibitory control that represses LXR-α and PXR from countering the effects of acute inflammation. Also, the formation of mTORC2 may also play a role in galvanising the response of LXR-α and PXR mRNA transcription. Based on results from this study, rapamycin (and its analogues) may be used to reduce acute inflammation by promoting the anti-inflammatory properties of LXR-α and PXR.

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