
# The effect of Mitogen-Activated Protein Kinase Phosphatase-2 (MKP-2) overexpression in prostate cancer cell function and its clinical prostate cancer progression

A thesis presented by

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# Abstract

MAP kinase signals have been reported as a crucial cancer chemopreventive and chemotherapeutic target due to their involvement in tumour cell growth, proliferation, apoptosis and survival. They consist principally of extracellular signal-regulated kinase (ERK), c-jun $NH_2$-terminal kinase (JNK) and p38 MAP kinase, which has been correlated with a more malignant phenotype in several tumour models and *in vivo.*

A key regulatory off switch for the MAP kinases is the dual specificity phosphatase, DUSP-4, also known as MAP kinase phosphatase-2 (MKP-2). This phosphatase is a type one DUSP, located in the nucleus, induced in cells in response to a number of extracellular stimuli and selective for ERK and JNK *in vitro*. This study was designed to examine the role of MKP-2 in the prostate cancer cell lines LNCaP Androgen sensitive (AS) and Androgen Insensitive (AI) in proliferation and apoptosis *in vitro* and cancer development *in vivo*.

MKP-2 was found to be expressed endogenously in both (AS) and (AI) cells. Adv.MKP-2 was then used as a tool to study the effect of MKP-2 overexpression in LNCaP (AS) and (AI). Immunofluorescent staining revealed strictly nuclear expression of the MKP-2 adenovirus, with more than 90% of the cells infected. In LNCaP (AS), ERK phosphorylation in response to EGF was transient whilst in LNCaP (AI) cells it was sustained. In contrast, JNK phosphorylation was sustained in both LNCaP (AS) and (AI) in response to Ultraviolet (UV) light C (60 $j/m^2$). Infection of both LNCaP (AS) and (AI) cells with Adv.MKP-2 significantly inhibited the phosphorylation of ERK and JNK. The kinetics of cell cycle progression was established. Stimulation with FCS over 48 h in LNCaP (AS) and 24 h in LNCaP (AI) caused a marked increase in cells both in S phase and $G_2$/M phase. Following infection with Adv.MKP-2 progression inhibited cell numbers in both S phase and $G_2$/M phase where they were reduced by over 50%. This correlated with a reduction in the expression of both cyclin B and D1. In contrast, Adv.MKP-2 did not modify apoptosis in both LNCaP cell lines stimulated by several agents including; UV-C, Doxorubicin and X-ray, These results indicate the potential for MKP-2 to influence cancer cell function proliferation but not apoptosis.

To specify the affects are mediated via dephosphorylation of ERK and/or JNK, we used a form of MAP kinase which has a triple substitution of arginine within positions R74, R75 and R76 to alanine and is unable to bind to ERK. Using this mutation further specifies the role of ERK or, in particular, JNK in regulating cellular proliferation, Adv.MKP-2-NLS1 caused inhibition of either EGF or UV-C ERK and JNK phosphorylation respectively in both LNCaP cells, whilst Adv.MKP-2-CI was not effective, suggesting that Adv.MKP-2-NLS1 can still bind and deactivate ERK. However, both NLS1 and CI MKP-2 adenoviruses inhibited cell cycle progression. The effect of either WT-MKP-2 or CI-MKP-2 on histone H3 phosphorylation was also assessed and both were found to cause substantial inhibition, suggesting the potential of a phosphatase-independent function for MKP-2 in the regulation of cell cycle progression. Finally, work using human prostate tissue confirmed that MKP-2 is expressed in invasive prostate tumours and localised in the nucleus, and staining intensity was positively correlated with PSA below 10 ng/ml levels and in early grade of Gleason score. These findings clearly suggest the potential for MKP-2 to be used as a possible gene tool in prostate cancer, which requires not only the inhibition of LNCaP cells proliferation *in vitro,* but PSA below 10 ng/ml levels and in early stage of Gleason score survival *in vivo*.

# Publications

**Poster presentation**

**Alnasser,** S & Plevin, R (14/02/2012). Role of MAP kinase phosphatase-2 (MKP-2) in prostate cancer postgraduate research day. Strathyclyde University, Glasgow, UK.

**Alnasser**, S & Plevin, R (22/06/2012). Role of MAP kinase phosphatase-2 (MKP-2) in prostate cancer Cell Biology, Cardiovascular and Neuroscience Postgraduate Research Day, Strathyclyde University, Glasgow,UK.

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**Papers**

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# Abbreviations

| | |
|---|---|
| **°C** | Degrees centigrade |
| **7–AAD** | 7-Amino-Actinomycin |
| **Ab** | Antibody |
| **Abs** | Absorbance |
| **ADP** | Adenosine monophosphate |
| **Adv.** | Adenovirus |
| **Ang II** | Angiotensin II |
| **ANOVA** | Analysis of variance |
| **AP-1** | Activating protein-1 |
| **APS** | Ammonium persulphate |
| **ASK** | Apoptosis signal regulating kinase |
| **ATP** | Adenosine triphosphate |
| **Bcl-2** | B cell lymphoma |
| **BH** | Bcl-2 homology domain |
| **BID** | Bcl-2 interacting-Bid |
| **BSA** | Bovine serum albumin |
| **CAD** | Caspase activated DNase |
| **CAR** | Coxackievirus-adenovirus receptor |
| **CARD** | Caspase associated receptor death domain |
| **Caspase** | Cysteine dependent aspartate specific protease |
| **cDNA** | Complementary DNA |
| **c-FLIP** | Cellular FLICE inhibitory protein |
| **CHX** | Cycloheximide |
| **c-IAP-1** | Cellular inhibitor of apoptosis |
| **CI** | Catalytic Inactive |
| **COX** | Cyclooxygenase |
| **CRM-1** | Chromosome region maintenence-1 |

| | |
|---|---|
| **DD** | Death domain |
| **DED** | Death effector domain |
| **DIABLO** | Direct IAP-binding protein with low Pi |
| **DISC** | Death inducing signalling complex |
| **DNIKKβ** | Dominant negative inhibitory kappa B kinaseβ |
| **DR** | Death receptor |
| **DTT** | Dithiothreitol |
| **DUSP** | Dual-specificity phosphatase |
| **E.coli** | Escherichia coli |
| **EB** | ERK Binding |
| **ECL** | Enhanced chemiluminescence |
| **EDHF** | Endothelium-derived hyperpolarising factor |
| **EDRF** | Endothelium-derived relaxing factor |
| **EDTA** | Ethylene diamine tetraacetic acid |
| **EGTA** | Ethylene glycol-bis (β-aminoethyl ether) N,N,N,N' -tetra acetic acid |
| **ERK** | Extracellular-regulated kinase |
| **ET-1** | Endothelin-1 |
| **FADD** | Fas-associated death domain |
| **FBS** | Foetal bovine serum |
| **FCS** | Foetal calf serum |
| **FGF** | Fibroblast growth factor |
| **GFP** | Green fluorescent protein |
| **Gm** | Grams |
| **GnRH** | Gonadotropin-releasing hormone |
| **GPCR** | G-protein coupled receptor |
| **GTP** | guanosine triphosphate |
| **H** | Hour (s) |
| **H2AX** | Histone variant A2 |
| **$H_2O_2$** | Hydrogen peroxide |
| **HEK** | Human embryonic kidney |
| **HEPES** | N-[12hydroxyethyl] piperazine-N'-[2-ethanesulphonic acid] |

| | |
|---|---|
| **HRP** | Horseradish peroxidise |
| **HSF** | Heat shock factor |
| **HSP** | Heat shock protein |
| **HUVECs** | Human umbilical vein endothelial cells |
| **IAP** | Inhibitor of apoptosis protein |
| **ICAD** | Inhibitor of caspase activated DNase |
| **ICAM-1** | Intracellular adhesion molecule-1 |
| **IKK** | Inhibitory kappa-B kinase |
| **IL-1β** | Interleukin-1 β |
| **INF-γ** | Interferon-gamma |
| **IP** | Inositol phosphates |
| **$IP_3$** | Inositol 1,4,5,-triphosphate |
| **ITR'S** | Inverted terminal repeats |
| **IκB** | Inhibitory kappa-B |
| **JIP** | JNK interacting protein |
| **JNK** | c-Jun N-terminal kinase |
| **kDa** | kilo-Dalton |
| **LPS** | Lipopolysaccharide |
| **MAPK** | Mitogen-activated protein kinase |
| **MAPKKK** | Mitogen-activated protein kinase kinase kinase |
| **MEEK** | MEK kinase |
| **MEF** | Mouse embryonic fibroblast |
| **MEK** | MAP kinase kinase |
| **MEKK** | MAP kinase kinase kinase |
| **Mg** | Milligrams |
| **Min** | Minute (s) |
| **MKB** | MAP kinase binding domain |
| **MKP** | Mitogen-activated protein kinase phosphatase |
| **mM** | Millimolar |
| **MMP** | Matrix metalloproteinase |
| **MOI** | Multiplicity of infection |

| | |
|---|---|
| **mRNA** | Messenger ribonucleic acid |
| **MSK** | Mitogen and stress kinases |
| **NEMO** | NF-κB essential modulator |
| **NES** | Nuclear export signal |
| **NF-κB** | Nuclear factor kappa-B |
| **NIK** | Nuclear factor kappa-B inducing kinase |
| **NLK** | Nemo-like kinase |
| **NLS** | Nuclear localisation sequence |
| **NO** | Nitric oxide |
| **NOS** | Nitric oxide synthase |
| **oxLDL** | Oxidized low density lipoprotein |
| **PARP-1** | Poly (ADP-ribose) polymerase enzyme |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase chain reaction |
| **$PGE_2$** | Prostaglandin E-2 |
| **$PGI_2$** | Prostacyclin |
| **PI3K** | Phosphatidyl inositol-3 kinase |
| **PIKK** | Phosphatidylinositol-3 kinase-related kinases |
| **PKC** | Protein kinase C |
| **$PLA_2$** | Phospholipase A-2 |
| **PMSF** | Phenylmethylsulfonylfluoride |
| **PS** | Phosphatidyl-serine |
| **ROS** | Reactive oxygen species |
| **RSK** | Ribosomal S6 kinase |
| **RTK** | Receptor tyrosine kinase |
| **SAPK** | Stress-activated protein kinase |
| **SDS** | Sodium dodecyl sulphate |
| **siRNA** | Small interfering RNA |
| **SMAC** | Second mitochondria derived activator of caspases |
| **SODD** | Silencer of death domain |
| **STAT** | Signal transducer and activator of transcription |

| | |
|---|---|
| **t-BID** | Truncated –Bcl-2 interacting protein |
| **TEMED** | N,N,N',N'-tetramethylenediamine |
| **TF** | Tissue factor |
| **TGFR** | Transforming growth factor receptor |
| **THD** | TNF homology domain |
| **THR** | Threonine |
| **TNFR-1-2** | TNF-receptor 1 & 2 |
| **TNF-α** | Tumour necrosis factor α |
| **TRAF2** | TNF receptor associated factor 2 |
| **TRAILR1-2** | TNF-related apoptosis inducing ligand receptor 1 &2 |
| **$TXA_2$** | Thromboxane A-2 |
| **TYR** | Tyrosine |
| **UV** | Ultraviolet |
| **VCAM-1** | Vascular cell adhesion molecule-1 |
| **VEGF** | Vascular endothelial growth factor |
| **VHR** | VH-1-related phosphatase |
| **XIAP** | x-linked mammalian inhibitor of apoptosis protein |
| **µg** | Micrograms |
| **µl** | Micro litre |
| **µM** | Micro molar |

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**CHAPTER 1**



**CHAPTER 1**

# 1. GENERAL INTRODUCTION

## 1.1 INTRODUCTION

### 1.1.1 Cancer progression and development

Cancer is now one of the most common conditions in the world, prevalent in the developed world, but increasing in incidence in other developing nations such as South America and Eastern Europe. In 2008, GLOBOCAN estimated there were about 12.7 million cancer cases and 7.6 million cancer deaths; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world (Jemal et al., 2011). Cancer is one of the most common conditions globally, second only to cardiovascular disease in prevalence.

There are many aspects of cancer which require classification and are often used interchangeably. Cancer is defined as a condition of the cell in which normal, finely regulated growth is dysregulated. Carcinogenesis or oncogenesis is the process which turns normal cells into cancer cells (Croce, 2008). It is characterised by a number of sequential changes at the genetic and cellular level that eventually re-programme a cell to undergo disorderly cell division. This results in the formation of a malignant mass of cells displaying abherrant growth characteristics. The cause of carcinogenesis is mutation in the genetic material of normal cells (Bertram, 2000). However, several mutations have to occur within certain categories of genes relevant to the growth process before a normal cell will turn into a cancer cell (Aminetzach et al., 2005).

Cancer is primarily a disease of the regulation of tissue growth. In order for a normal cell to convert to a cancer cell, genes that organise cell growth and differentiation must change or be deleted or amplifid. These changes can occur at multiple levels, from the loss of entire chromosomes to a mutation affecting a single DNA nucleotide (Paux et al., 2008). There are two large groups of genes that are affected by these changes. Firstly, there are genes which play a role in mediating the growth

process. They may be genes which are expressed at inappropriately high levels, for example the HERs (Human Epidermal Growth Factor Receptors), or alterations in genes that give the resultant protein new characteristics. In either case the consequence may be marginal or central to the growth process. Genes with the potential to cause cancer are defined as oncogenic and include Ras and Src (Malumbres and Barbacid, 2003, Chan and Chen, 2012).

In both cases, the expression of these genes enhances the proliferative phenotype of the malignant cells. There is also a set of genes known collectively as tumour suppressor genes, for example p53, that prevent cell division, survival, or other protective properties of cancer cells (Pearson, 2006) and often, disabled tumour suppressor genes lead to cancer-promoting genetic changes. Usually, changes must be made in many genes of either type to transform a normal cell into a cancer cell (Pennisi, 2007).

Additional changes in metabolic pathways are implicated in cancer (Wu and Zhao, 2013). For example, alteration of the glycolytic and pentose phosphatase pathways have been demonstrated (Marin-Valencia et al., 2012). Moreover, metabolism generates oxygen radicals, which contribute to oncogenic mutations (King et al., 2006). Altered metabolism is a key feature within the hypoxic cancer environment in relation to cell growth and survival. Excessive caloric intake is correlated with an increased risk of cancers, while caloric restriction is protective, perhaps through clearance of mitochondria or mitophagy, thereby reducing oxidative stress (Dang, 2012, Daye and Wellen, 2012).

In addition, cancer can take on a phenotype which compromises immune surveillance. i.e. antigen expression upon the cell surface is altered in such a way to avoid detection by the immune system. Alteration of the expression of antigens on the cell surface and increased expression of proinflammatory mediators may influence the survival of cancer patients and both provide mechanisms for tumour invasion (Zijlmans et al., 2007, Dinarello, 2006). In addition, many types of cancers cells can promote the expression of immunosuppresive factors such IL-6, IL-10 and VEGF (Sumimoto et al., 2006, Boni et al., 2010).

Several studies have indicated that angiogenic activators play an important part in the growth and spread of tumours. On immunohistochemical examination, the VEGF family and their receptors were found to be expressed in about half of the human cancers investigated, also, these factors were found to effect the prognosis of adenocarcinomas developed in the uterine cervix, endometrium, ovary and stomach (Nishida et al., 2006). Their actions are mediated through the development of new blood vessels.

A key additional aspect in relation to cancer progression is the concept of metastasis. Cancers eventually are fatal due to their unrestricted spread to distal sites and the establishment of multiple tumours. Essential for this process is the development of blood vessels, through angiogenesis, the migration of tumour cells including entry and survival in the circulation, peritoneal space or lymphatics, arrival in a distant target organ, mainly (but not always) followed by extravasation into the surrounding tissue, survival in the foreign microenvironment, proliferation, and further induction of angiogenesis, all the while evading apoptotic death or immunological response (Hunter, 2004, Coghlin and Murray, 2010).

Neve et al, (2006) summarised the functional characteristics of malignant tumour cells as the following: acquisition of self-sufficiency in grow the signals, loss of sensitivity to anti-growth signals resulting in uncontrolled growth, loss in the ability for programmed cell death (apoptosis), allowing growth despite genetic errors and external anti-growth signals; loss of capacity for senescence, leading to potentially limitless replication; continued acquisition of blood vessels allowing the tumour to grow beyond the limits of passive nutrient diffusion and the ability to invade neighboring tissue, and possible the development of metastases at distant sites. Finally, it should be noted that there is a general loss of the ability to repair genetic errors, which in turn leads to an increased rate of genetic mutation (genetic instability), (Neve et al., 2006), thus accelerating all the other functional changes outlined above.

Whilst these pathological changes are classical in cancer, other types of malignancy do not require these changes to be defined as cancerous. For example, in leukemia, malignancies of blood cells, as the cells circulate in the blood stream, it does not require tissue invasion. The multiple facets of cancer outlined above do not necessarily represent individual mutations; for example, disabling a

single gene coding for protein p53, can be sufficient to cause genetic instability, evasion of cells and blood vessel growth (Matlashewski et al., 1984;Tokino and Nakamura, 2000). Not all cancer cells divide; alternatively, a subset of cells in the tumour, called cancer stem cells, may replicate themselves and generate differentiated cells. Many features of this generalised process are apparent in prostate cancer, the subject of this thesis. However, each cancer has a number of distinguishing features which make it unique and require specialised understanding.

### 1.1.2 prostate cancer progression and development

Prostate cancer is the most commonly diagnosed non-cutaneous cancer in men worldwide. It is particularly prevalent in advanced countries such as, the United States and Western European countries and is the second leading cause of death caused by cancer in males, after lung cancer (Jemal et al., 2010).

The rate of tumour growth varies from very slow to moderately rapid; however, there are cases of aggressive prostate cancers. The cancer cells may metastasise from the prostate to other parts of the body, particularly the bones (90%), lung (46%), liver (25%), pleura (21%), and adrenals (13%). Prostate cancer may cause pain, difficulty in urinating, and problems during sexual intercourse, or erectile dysfunction (Bubendorf et al., 2000). About 5-10% of patients present with metastatic disease at diagnosis, and an additional 25% with localised or locally advanced disease will progress to metastasise during the course of their disease (Suarez et al., 2013).

There are several types of cells in the prostate; immunohistochemistry using 152 commercially available antibodies to examine cluster designation (CD) antigens have been used to characterise the luminal secretory epithelium, basal epithelial cells, the fibromuscular stroma, nerve sheath and other cell types (Liu and True, 2002, Tokar et al., 2005). However, all prostate cancers including adenocarcinoma, small cell carcinoma and rarer prostate cancer types start in the gland cells.

The NCCN guidelines use four factors in determining the recommended treatment: the stage and grade of the cancer, the patient's prostate-specific antigen (PSA) level and the estimated baseline life expectancy of the patient. Moreover, treatment for localised prostate cancer is recommended for

higher-risk patients. Overall risk can be estimated using an index of cancer stage and grade, PSA level, and comorbidity-adjusted life expectancy (Mohan et al., 2010). The primary goal of treatment is to target the men most likely to need intervention in order to prevent prostate cancer death and disability while minimising intervention-related complications. Common treatments include surgery to remove the prostate gland (radical prostatectomy), external beam radiotherapy (EBRT), interstitial radiotherapy (brachytherapy), freezing the prostate (cryotherapy), androgen deprivation therapy (ADT) and watchful waiting (active surveillance) (Mohan et al., 2010). All treatments have risks of complications, although frequency and severity may vary.

The growth of prostate epithelial cells needs physiological levels of androgen, both to stimulate proliferation and inhibit apoptotic death (Heinlein and Chang, 2004), so initial therapy for metastatic disease consists of androgenic suppression. However, this is only a palliative treatment with an effective duration of between 12 and 24 months (Chodak et al., 2002, Miyakita, 2005). After this stage, the tumour becomes castration-resistant and will progress even at undetectable levels of testosterone.

The sensitivity of prostate cancer to androgen means that it is viewed as a hormone-sensitive cancer regulated by activation of the androgen receptor (AR). A functional AR is required for prostate progression and normal prostate function (Roy et al., 1999). Androgen action can be reflected to function through an axis connecting the testicular synthesis of testosterone, its transport to target tissues and the conversion by 5α-reductase to the more active metabolite 5α-dihydrotestosterone (DHT) (Figure1.1). Testosterone and DHT utilise their biological effects through binding to the AR and inducing AR-mediated transcriptional activity. The androgen-induced transcriptional activation of AR is modulated by the interaction of AR with co-regulators and by phosphorylation of AR and AR co-regulators by growth factors (Heinlein and Chang, 2002a, Buchanan et al., 2001, Cunha et al., 1987). Unsurprisingly, AR activation is able to promote prostate carcinogenesis (Feldman and Feldman, 2001).

At an early stage in cancer progression, deprival of testosterone induces apoptosis and decreases cell survival, indicating that signalling through AR remains important for disease progression

(Heinlein and Chang, 2002b). Unfortunately, through time the cancer cell becomes more resistant to apoptosis upon androgen-deprivation therapy and androgen independence develops. A number of studies have shown that androgen-independent prostate cancer still depends on AR signalling but it is stimulated by very low levels of circulating androgen (Chen et al., 2008), suggesting a degree of hypersensitisation. This sensitivity is associated with a series of mutations within the AR that allows activation by low androgen levels or by other steroids. Different molecular drivers of sensitisation to androgens include increased stability of AR, AR overexpression and an upregulation in tyrosine kinase signalling (Chen et al., 2008, Feldman and Feldman, 2001, Navarro et al., 2002).

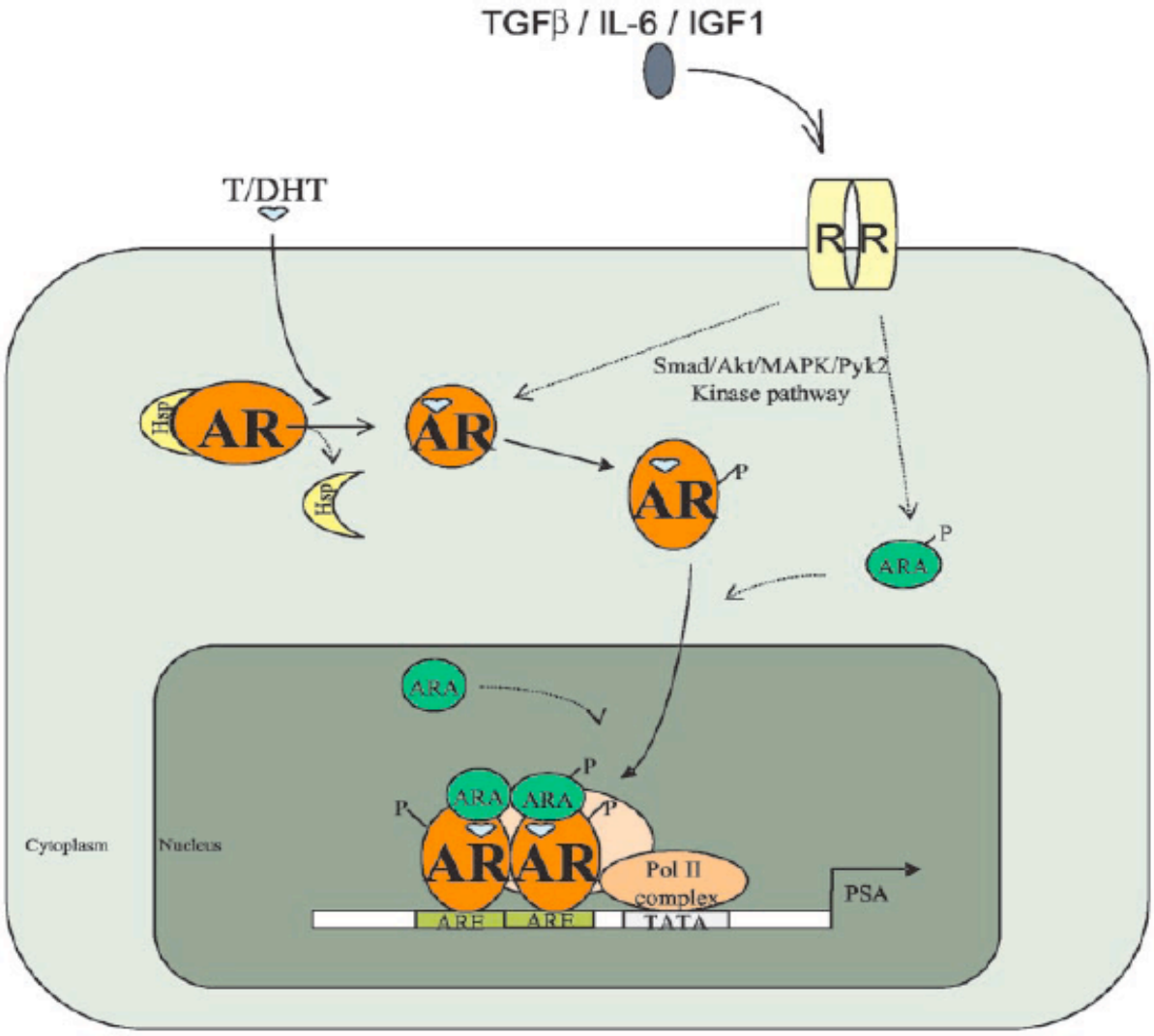


**Figure 1.1: Androgen-AR action in the prostate.** Testosterone (T) and DHT bind to AR and promote the association of AR essential coregulators (ARAs). AR then translocates to the nucleus and binds to ARAs in the promoter regions of target genes to induce cell proliferation and apoptosis. Other signal transduction pathways, such as those involving TGF, IL-6, and IGF-I, can also enhance AR activity via phosphorylation of AR and/or ARAs. Hsp, Heat shock protein; R, membrane receptor; P, protein phosphorylation (derived from Heinlein and Chang, 2004).

## 1.2 SIGNALLING PATHWAYS INVOLVED IN CANCER PROGRESSION

In order for the cell to function it must respond to changes from extracellular stimuli which mediate their actions via receptors. Receptor activation in turn stimulates a series of cascades which involve a number of signalling pathways to allow the cell to respond either immediatedly with, for example, contraction, or in the longer term via the transcription of new genes and the resultant proteins. These pathways can involve the immediate generation of a second messenger such as cAMP or a series of protein/protein interactions which eventually generates a series of active protein molecules often in the form of kinases. These kinases are key molecules involved in the regulation of proliferation and have the potential to be disrupted in cancer.

There are multiple pathways which have been implicated in prostate cancer (Jung et al., 2008). These include; the developmental signalling cascades such as HEDGEHOG, signalling via the Androgen Receptor (AR), Src, PI3K/AKT, NFκB, JAK/STAT and others. Several excellent reviews cover these and other pathways which are not directly examined in this thesis (Sheng et al., 2004, da Silva et al., 2013, Jain et al., 2012). However, a key signalling pathway frequently dysregulated in cancer and of potential importance in prostate cancer is the Ras/Raf/MEK/ERK (MAP kinase) pathway.

## 1.3 MITOGEN-ACTIVATED PROTEIN KINASES (MAP KINASES)

MAP Kinase signalling has been reported to be a crucial cancer chemotherapeutic target due to its involvement in tumour cell growth, proliferation, apoptosis and survival (Khattar and Kumar, 2010). In the following sections a detailed assessment of MAP kinase activation and function will be presented followed by an appraisal of its dysregulation in cancers.

### 1.1.2 MAP kinase signalling pathways

The MAP kinase signalling pathway is organised in a three-tiered kinase module where a MAP kinase is activated by phosphorylation of a MAP2 kinase which in turn is activated by a MAP3 Kinases (Chang and Karin, 2001). The three best-characterised MAP kinase pathways involve the

extracellular signal-regulated kinase (ERK), p38 MAP kinase and c-Jun $NH_2$ terminal kinase (JNK)/stress-activated protein kinase (SAPK) pathways (Chang and Karin, 2001, Boutros et al., 2008).

Over the last 25 years the MAP kinases have been closely studied in the context of cancer development and progression (Dhillon et al., 2007). MAP kinases can control both survival and cell death, dependent on cell type and context, through regulation of a number of different cytosolic and nuclear substrates. While ERK primarily increases survival and is involved in encouraging growth, differentiation and development, both p38 MAP kinase and JNK are thought to mediate apoptosis and inflammation but are also implicated in differentiation and development (Chang and Karin, 2001, Wada and Penninger, 2004). Upon activation, these cytosolic proteins translocate to the nucleus and control the activity of numerous proteins, including transcription factors (Chang and Karin, 2001)(see Figure 1.2A). As the MAP kinases play important roles in controlling different cellular processes, the regulation of their activity is of great importance. In the following sections, after a brief outline of MAP kinase signalling, current knowledge on the possible impact of these signalling pathways in cancers is presented.

## 1.4 MAP KINASE FAMILY

Within mammalian cell systems, three major MAP kinase cascades have been identified and grouped into subfamilies on the basis of sequence similarity, sensitivity to activation and mechanisms of upstream regulation by diverse MEK families. These include; the extracellular signal-regulated protein kinases (ERKs 1-4 and ERK5, also called big MAP kinase 1 (BMK1), ERK7/8), the c-Jun amino-terminal protein kinases known as stress-activated protein kinase (JNK 1/2/3 /SAPK α, β, γ) and isoforms of p38-MAP kinase (p38- α/ β/ γ/ δ) (Gupta et al., 1995, Gupta et al., 1996).

All of these MAP kinases have a Thr-X-Tyr (TXY) motif within their activation loop which is essential for function. Whereas Thr and Tyr are threonine and tyrosine, X is glutamate, proline or glycine, respectively, in ERK, JNK or p38 MAP kinase (Zhang and Dong, Walker and Lockyer, 2004). Activation as part of a signalling cascade may be achieved by triggering membrane receptors such as transforming growth factor β receptors (TGFR), G-protein-coupled receptors (GPCRs),

receptor tyrosine kinases (RTKs) and some cytokine receptors. Activation results in the recruitment of defined adaptor/scaffold proteins, which in turn initiates the cascade (Walker and Lockyer, 2004).

MAP kinases are activated by a wide range of external factors, and are frequently a focal point for activation of multiple subgroups of extracellular stimuli, for example growth factors. Nevertheless, there is strict control and a specific pattern of activation depending on the stimuli. For example, the two growth factors EGF and PDGF are recognised to stimulate short and prolonged activation of ERK in different cell types and as a result modify proliferation in different ways (Robin et al., 2004).

Other critical interactions also play a role, MAP kinases bind upstream and downstream target proteins through surface interactions that are achieved through the docking motifs outside the catalytic domain (Biondi and Nebreda, 2003, Bardwell et al., 2003). These interactions finely tune the output of each MAP kinase cascade. Furthermore, binding of MAP kinase signalling complexes to scaffold proteins determines the location and duration of MAP kinase activation, therefore regulating specific signalling outcomes (Kolch, 2005, Geest and Coffer, 2009) (See figure 1.2B).

Lastly, the controlled dephosphorylation of MAP kinases by phosphoprotein phosphatases also plays an essential role in determining the magnitude and duration of kinase activation and thereby the outcome of MAP kinase signalling (Raman et al., 2007, Caunt et al., 2008). Phosphoprotein phosphatases involved in the regulation of MAP kinase signalling can be categorised into three groups based on preference for dephosphorylating phosphotyrosine, phosphoserine/-threonine, or both, which can dephosphorylate both forms of residues. Dual specific phosphatases (DUSPs), which selectively inactivate MAP kinases are known as MKPs (Geest and Coffer, 2009, Caunt et al., 2008), have a variety of subcellular locations and can modulate a single class of MAP kinase or can regulate more than one MAP kinase pathway (Geest and Coffer, 2009). Their role in the regulation of MAP kinase function is highlighted in section 1.8.1.

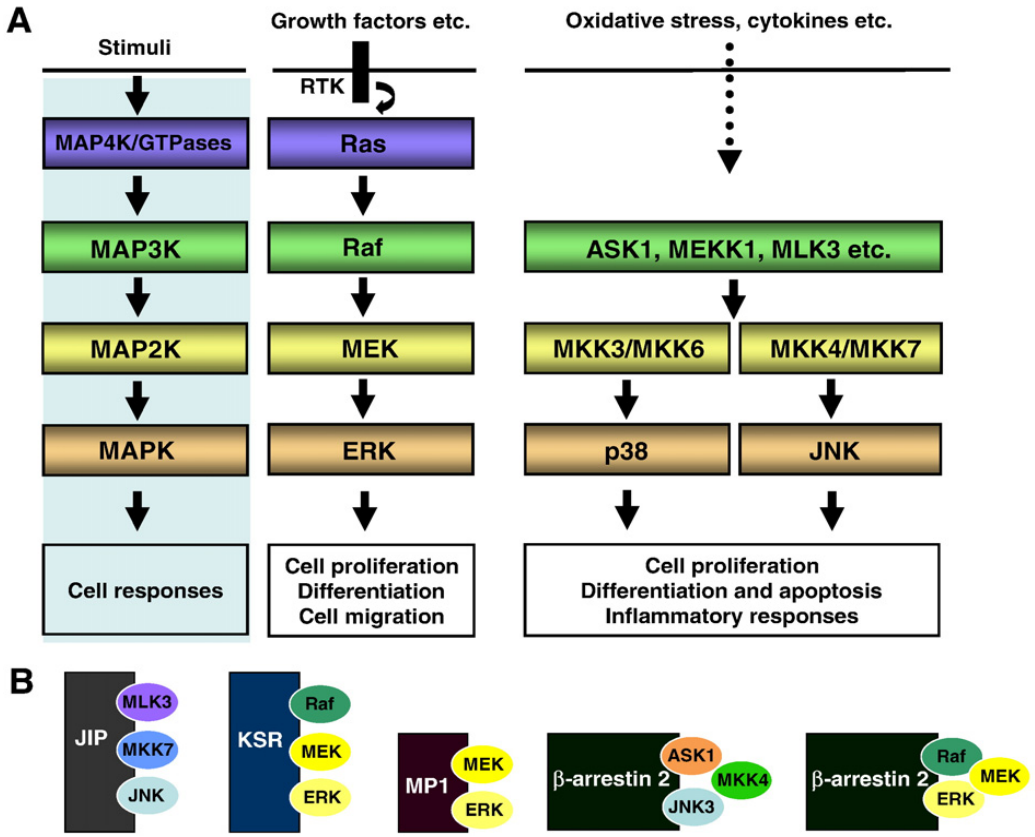


**Figure1.2: Mitogen-activated protein kinase (MAPK) signalling pathways.** A. MAP Kinase signalling pathways mediate intracellular signalling initiated by extracellular or intracellular stimuli. B. Scaffold proteins that facilitate activation of MAP Kinase signalling pathways include JIP for the JNK signalling pathway, KSR and MP1 for the ERK signalling pathway, and β-arrestin 2 for the ERK and JNK pathways (derived from Kim and Choi, 2010).

### 1.1.3 Extracellular signal-regulated kinase (ERK) family

The first mammalian MAP kinase isoforms discovered were ERK1 and ERK2 and, to date, at least six isoforms have been identified (ERK1-6). The main ERK1/2 isoforms share 83% amino acid identity and are therefore often regarded as functionally redundant (Boutros et al., 2008). As outlined above, ERKs are activated by a variation of extracellular agents, which include growth factors, neurotransmitters, hormones and chromosomal translocations such as BCR-ABL (Zhang and Liu, 2002, Rubinfeld and Seger, McCubrey et al., 2007). These extracellular factors, which can act through tyrosine kinase receptors, G protein-coupled receptors, ion channels (Fink et al., 2010, Uetz and Stagljar, 2006, Bell-Horner et al., Ma and Sansom, 2001) and other mechanisms, initiate a variety of intracellular signalling events that result in the activation of the ERK cascade (Geest and Coffer, 2009).

The coupling of events initiated at the plasma membrane to ERK itself requires adaptor proteins, which are themselves linked to guanine exchange factors (GEFs) of small GTP-binding proteins. Upon stimulation, the adaptor protein–GEF complex is translocated to the plasma membrane, where it activates the small GTP-binding protein Ras. This protein then further transmits the signal to the MAP3 kinase (MKKK) level of the cascade involving Raf-1, B-Raf, A-Raf and TPL2. The accumulation of active GTP-bound Ras, recruits Raf-1 to the plasma membrane where it is activated by a mechanism that is not yet fully understood (Geest and Coffer, 2009). Appropriate regulation of both Ras and Raf is crucial for the proper maintenance of cell proliferation, as activating mutations in these genes leads to oncogenesis (Roux and Blenis, 2004), Ras is mutated in 30% of all human cancers and B-Raf is mutated in 60% of malignant melanomas (Chong et al., 2003).

In some conditions, PKC and MLK3 can activate Raf-1 or B-Raf, respectively (Geest and Coffer, 2009). Afterward, the signal is transmitted down the cascade through MEK1 and MEK2 (MEKs). In this cascade of events, the MEKs are phosphorylated and activated by Rafs and other MAP3 kinases through serine phosphorylation at the typical Ser-Xaa-Ala-Xaa-Ser/Thr motif in their activation loop (Ser 218, 222 in human MEK1) (Alessi et al., 1994).

The activated MEKs are dual-specificity kinases that demonstrate a unique selectivity toward ERKs at the MAP kinase level (Seger et al., 1992). Two ERK genes are known (ERK1/2), and those encode two main proteins p42 and p44 (Butterfield et al., 1999), although there is the potential of spliced forms such as the rodent ERK1b (Yung et al., 2000), and the primate ERK1c (Aebersold et al., 2004). Activation of the ERKs is achieved by phosphorylation of both Tyr and Thr residues within the Thr-Glu-Tyr motif in their activation loop (Thr183, Tyr185 in human ERK2) and this appears to be mediated exclusively by MEKs.

The ERKs are ubiquitous Ser/Thr kinases that phosphorylate multiple substrates either in the cytosol e.g., $PLA_2$ (Lin et al., 1993), or the nucleus e.g., Elk1 (Janknecht et al., 1993). Most of the substrates of the ERKs are regulatory proteins themselves and they include one or more MAP kinase activated protein kinases (MAPKAPKs) (Roux and Blenis, 2004). The MAPKAPK tier includes the ribosomal S6 kinase (RSK), one of the first discovered substrates for ERK (Cargnello and Roux, 2011), the MAPK/SAPK-activated kinase (MSK) (Rubinfeld and Seger, 2005), the MAPK signal-interacting kinases 1 and 2 (MNK1, 2) (Fukunaga and Hunter, 1997), and MAP Kinase APK3/5 (Janknecht et al., 1993). Lastly, protein kinases such as GSK3 (Waskiewicz et al., 1997) and LKB1 have been identified as immediate substrates for MAPKAPKs, completing a plausible six-tiered MAP kinase cascade.

### 1.4.1 c-Jun N-Terminal Kinases /Stress-activated Protein Kinases (JNKs/SAPKs)

The c-Jun N-terminal kinases (JNKs), were first described in the early 1990s by Kyriakis and Avruch, originally categorised as stress-activated members of the mitogen-activated protein kinase MAPK family (SAPKs) (Bogoyevitch et al., 2010). To date, three distinct genes, JNK1, JNK2, and JNK3, encoding 10 kinase isoforms, have now been identified in mammals (Geest and Coffer, 2009). Like ERK1/2, JNKs/SAPKs are activated by the same dual phosphorylation ($Thr^{183}$,$Tyr^{185}$) within the activation loop T-P-Y (Kyriakis and Avruch, 2001).

JNK/SAPKs are activated by many various stimuli including both physical and chemical stress, as well as, UV radiation, inhibitors of translation, epidermal growth factor (EGF), hydrogen peroxide, cyclohexamide, serum deprivation, proinflamatory cytokines and heat shock (Derijard et al., 1994, Strniskova et al., 2002). Early studies showed that acute JNK activation and c-Jun phosphorylation promoted cell growth (Ma et al., 2012), while sustained JNK and c-Jun activation following stress-induced cell apoptosis, indicating that the kinetics of JNK may be important in defining cell function (Adler et al., 1999).

JNK1 and JNK2 are expressed in most cell types, while the JNK3 isoform is restricted primarily to the brain, heart and testis (Davis, 2000). Originally, three genes encoding MAP Kinase-related kinases were cloned from a rat brain cDNA library and these kinases turned out to have all the properties of the so-called SAPK 1α, β, γ (Kyriakis et al., 1994). The genes for SAPK 1α and SAPK β encode kinases of around 55 kDa, similar in size to JNK 2, whereas SAPK γ and JNK 1 have relative molecular masses of around 45 kDa. Of the two major isoforms, JNK2 exhibits 83% identity and similar regulation to JNK1.

JNK1 mediates a number of its functions via the regulation of key transcription factors such as c-Jun (Bogoyevitch and Kobe, 2006). Despite their close similarity, the two JNKs 1 and 2 differ greatly in their ability to interact with c-Jun. JNK2 binds c-Jun 25 times more avidly than JNK1 and as a result, has a lower $K_m$ toward c-Jun than JNK1. The structural basis for this difference has been investigated and traced to a small strand-like region close to the catalytic pocket of the enzyme (Kyriakis et al., 1994, Derijard et al., 1994, Kallunki et al., 1994). However, it is still unclear if a similar selectivity of interaction occurs for other substrate proteins, which includes the transcription factors ATF2, p53, DPC4, and NFAT4 (Cho and Choi, 2002). A critical additional function is in the regulation of AP-1 transcriptional activity, JNK appears to be essential for AP-1 activation caused by stress and some cytokines, but is not required for AP-1 activation in response to other stimuli (Spohn et al., 2010).

Through effects upon these transcription factor pathways, JNK has been linked with a number of

key cellular responses. As outlined above, JNK is implicated in cellular apoptosis (Dhanasekaran and Reddy, 2008, Kennedy and Davis, 2003), but also has the potential to regulate proliferation and the cell cycle. JNK1 has been implicated in the induction of apoptosis of immature germ cells when they detach from Sertoli cells (Li et al., 2009).

JNKs are also involved in cytokine production and other aspects of the inflammatory response and can be strongly activated in multiple cell types by lipopolysaccharide (LPS) or inflammatory cytokines such as TNF-α and IL-1 (Davis, 2000). It is significant that in Drosophila the JNK pathway is strongly activated in response to LPS (Sluss et al., 1996), suggesting that the JNK pathway is evolutionarily conserved in essential immune responses. Moreover, in fibroblasts, JNK can also be activated by inflammatory cytokines and double-stranded viral RNA (Chu et al., 1999). Using $JNK2^{-/-}$ fibroblasts, Chu et al. (1999) also determined that JNK is required for production of multiple cytokines for example type I interferon and IL-6 (Chu et al., 1999).

As with ERK1/2, JNK/SAPKs are regulated by a three tiered activation cascade. Phosphorylation is regulated by MAP2Ks, for SEK1/MKK4/JNKK1 and MKK7/JNKK2, and further upstream the MAP3 Kinases (such as MEKK1, ASK1, and TAK1) (Davis, 2000). The immediate upstream activating kinases have been characterised and include MEK4 (Kyriakis and Avruch, 2001). Several studies have shown that MEK4 specifically phosphorylates the (Tyr) residue, while MEK7 specifically phosphorylates the (Thr) residue (Hong and Privalsky, 2000). Moreover, MEK4 phosphorylates both JNK and p38 MAP kinase *in vitro,* whereas, MEK7 is specifically involved in the activation of JNK (Davis, 2000). Further upstream both Rho family GTPases and other kinases including members of the mixed-lineage protein kinase group and MEKK group of MAP3 Kinase (Davis, 2000) regulate inputs into MEK4 and 7. Signal transduction pathways that activate Rho proteins include tyrosine kinases; thus, Rho proteins may mediate the activation of JNK caused by some growth factors (Davis, 2000).

Activation of JNK by cytokine receptors also appears to be mediated by the TRAF group of adaptor proteins (Liu et al., 1996). Activation of the TNF receptor leads to binding of TRAF2, which is

required for JNK activation (Yeh et al., 1997). This adaptor protein has been reported to bind MEKK1 (Baud et al., 1999) and ASK1 (Nishitoh et al., 1998). It has been confirmed that the effect of TRAF2 on ASK1 requires the prior dissociation of ASK1 from the inhibitor thioredoxin (Liu et al., 2000) and is likely to involve regulated ASK1 dimerisation mediated by reactive oxygen species (Liu et al., 2000). The IL-1 receptor recruits another TRAF, TRAF6, which is essential for JNK activation (Korchnak et al., 2009). Mediators of TRAF6 have been reported, TRAF6 has been reported to bind MEKK1 (Yoshida et al., 2008), via the adaptor protein ECSIT (Tournier et al., 2001).

### 1.4.2 p38 MAP kinase family

The p38 MAP kinase family comprises p38α (MAPK14, SAPK2a, CSBP), p38β (MAPK11, SAPK2b), p38γ (MAP Kinase 12, ERK6, SAPK3) and p38δ (MAP Kinase 12, SAPK4) (Krens et al., 2006). Both p38α and p38β are broadly expressed isoforms that are involved in regulation of a number of diverse and often contradictory events including cell proliferation, differentiation, and development. In the latter, p38α knockout mice were found to be lethal because of defects in placental angiogenesis (Mudgett et al., 2000, Adams et al., 2000). Mice lacking p38β, p38γ or p38δ survive normally and do not demonstrate any obvious phenotypes (Ruiz-Bonilla et al., 2008). Moreover, the p38γ and p38δ double knockout mice were found to be viable and fertile and had no obvious health problems (Mudgett et al., 2000).

The p38 MAP kinase module is again a three tiered cascade, consisting of numerous MAP3 kinases, including MEKKs (1 to 4), MLK2 and -3, DLK, ASK1, Tpl2 (also termed Cot), and Tak1 and the MAP kinase kinase, MEK3 and MEK6 (also termed MKK3 and MKK6, respectively) (Chadee et al., 2002). MEK3 and MEK6 show a high degree of specificity for p38, as they do not activate ERK1/2 or JNK, however under some experimental conditions MEK4 possesses some MAP kinase kinase activity toward p38 MAP kinase, suggesting that MEK4 could represent a site of integration for the p38 and JNK pathways (Schoorlemmer and Goldfarb, 2002, Ma and Sansom, 2001). While MEK6 activates all p38 MAP kinase isoforms, MEK3 is more selective for p38α and p38β isoform MAP kinase (Roux and Blenis, 2004). The specificity in p38 MAP kinase activation is thought to

result from the creation of functional complexes between MEK3/6 and different p38 MAP kinase isoforms and the selective recognition of the activation loop (Mudgett et al., 2000). Activation of the p38 MAP kinase isoforms results from the MEK3/6-catalysed phosphorylation of the conserved T-G-Y motif in their activation loop.

In many mammalian cells, p38 MAP kinase isoforms are strongly activated by inflammatory cytokines and environmental stresses but not appreciably by mitogenic stimuli. This suggests a predominant role in inflammation and immune function. This is mediated through the activation of a broad range of relevant transcription factors such as ATF-1/2, C/EBP, STAT1, and STAT3 and other intermediate protein kinases such as p90 RSK, MAP kinase AP2 and -3, as well as a variety of other substrates including HSPs (Geest and Coffer, 2009, Crittenden and Filipov, 2008, Li et al., 2007).

Despite a major effect on immune function, it has become apparent through many studies that p38-MAP kinase has the potential to regulate cell growth in different cell types. In yeast, overexpression of mammalian p38-MAP kinase leads to a significant slowing of proliferation (Crittenden and Filipov, 2008). Yeast with a mutation of Spc-1, the yeast homologue of p38-MAP kinase, show a delay in progression to the $G_2$ phase of the cell cycle (Samejima et al., 1997), whilst microinjection of p38-MAP Kinase into NIH-3T3 cells results in $G_1$ arrest (Samejima et al., 1997, Lavoie et al., 1996). The activation of p38-MAP kinase also has a negative effect on cyclin D1 expression (McKay et al., 2000). All these studies suggest a link between p38-MAP kinase activity and control of cell cycle progression and perhaps other aspects of proliferation.

## 1.5 THE MITOGEN ACTIVATED PROTEIN KINASE (MAPK) SIGNALLING PATHWAY IN CANCER

Given the importance of the MAP kinases in the regulation of proliferation, it is perhaps not surprising that perturbations in the MAP kinase pathway has been a feature of cancer development and progression. Historically this has been most marked in the study of the ERK pathway. Early

studies investigating hyperproliferation mediated by viral and cellular oncogenes elucidated the link to intermediates of the MAP kinase, in particular Ras and Raf (Varmus, 1984). Indeed mutations are required to initiate ERK activation while Rap1 sustains this signal through B-Raf (Garcia et al., 2001).

### 1.5.1 ERK pathway in cancer

The ERK pathway has been implicated in cell growth and is dysregulated in one-third of all human cancers (Schubbert et al., 2007). Thus, a large number of cellular studies as well as, preclinical and clinical observations, have underpinned the acceptance of a role for ERK in cancer. Cellular studies have by and large recapitulated the clinical observations linking mutations in Ras and RAF proteins to cancer. Cells expressing mutated Ras give rise to in enhanced proliferation and cellular transformation. Finally, mouse models encoding such mutations have again supported a key role for ERK in tumour progression.

Mutations of K-Ras occur often in many human cancers including those of the lung and colon (Schubbert et al., 2007). Certainly, K-Ras mutations have been noticed in ~50% of colon cancers, in which mutations of N-Ras are rare (Malumbres and Barbacid, 2003). Transgenic mice expressing an activated form (G12D) of K-Ras manifest MEK-dependent hyperproliferation of colonic epithelial cells, while expression of the corresponding activated form (G12D) of N-Ras did not have such an effect. This suggests that K-Ras (G12D), but not N-Ras (G12D), activates the MEK-ERK signalling pathway and thereby promotes dysregulate the proliferation of colonic epithelial cells (Haigis et al., 2008, Calcagno et al., 2008).

The finding of mutated K-Ras in both early and late stages of CRC indicates that, as in pancreatic cancer, K-Ras mutations may be on early event in tumour progression (McLellan et al., 1993, Yuen et al., 2002, Nash et al., 2010). This correlates with findings in mice and in patient tissues. The study of animal models (Singh et al., 2010), indicates that K-Ras mutations may have a relevant role in non-small cell lung cancer (NSCLC) development. For example, in a mouse model which develops somatic human K-Ras mutations, by means of intrachromosomal *in vivo* recombination of

the mutant allele, the animals established lung carcinomas like human NSCLC and developed a series of morphological alterations like those defined in staging of the disease (Singh et al., 2010).

Mutations in the B-Raf gene involved with ~ 66% of malignant melanomas (Halilovic and Solit, 2008). The substitution of glutamate for valine at amino acid 600 (V600E) of B-Raf is the most common of these mutations and results in constitutive activation of B-Raf and the ERK signalling pathway (Wan et al., 2004). Mutations of B-Raf that do not affect its kinase activity, can increase MEK-ERK signalling as a result of the formation of a heterodimer between the mutant B-Raf and Raf-1 (Rushworth et al., 2006). Mutations of the Raf-1 gene are also found in acute myeloid leukemia (Steinmetz et al., 2004). One recent study, showed that B-Raf mutation detects a subset of papillary thyroid carcinoma (PTC) with increased risk of recurrence (Fernandez et al., 2013).

There is substantial evidence validating the importance of Raf in cancer (Shields et al., 2000) and this has prompted the development of Raf inhibitors, for example, Sorafenib. This drug demonstrated for the first time the potential to prolong survival in patients with advanced hepatocellular carcinoma (HCC) (Chaparro et al., 2008). Additionally, Treatment of metastatic melanoma with another Raf inhibitor PLX4032, in patients with tumours that transmit the V600E B-Raf mutation resulted in complete or partial tumour regression in the majority of patients (Flaherty et al., 2010).

Further downstream the ERK signalling pathway plays a role in several stages of cell attachment and spreading, key aspects of tumour development. The phosphorylation by ERK of proteins such as focal adhesion kinase, calpain, myosin light chain kinase, and paxillin (Huang et al., 2004) promotes cancer cell migration. The ERK pathway also stimulates the expression of matrix metalloproteinases and thereby promotes the degradation of extracellular matrix proteins and consequent tumour invasion (Chakraborti et al., 2003). Activated MEK1/2 has also been shown to up-regulate the expression of matrix metalloproteinase and to protect cancer cells from detachment-induced apoptosis or anoikis (Voisin et al., 2008). In addition to enhanced proliferation, the oncogenic potential of the ERK pathway is linked to its ability to promote cell survival. In MCF-7

cells nitric oxide is able to mediate apoptosis in part by inhibiting ERK activation (Feng et al., 2013).

Moreover, ERK1/2 signalling regulates the activities and levels of Bcl-2 family proteins such as anti-apoptotic protein MCL-1 and the pro-apoptotic protein BIM thereby promoting the survival of cancer cells (Balmanno and Cook, 2009). ERK mediates phosphorylation of the transcription factor FOXO3A, which results in proteasome-dependent degradation (Ley et al., 2003), and consequent down-regulation of FOXO3A-dependent transcription of the BIM gene (Satyanarayana and Kaldis, 2009). Moreover, ERK phosphorylates MCL-1 on threonine-163 within the PEST domain, resulting in stabilization of MCL-1 and promoting the survival of tumour cells. MCL-1 expression is increased in several types of cancer and is related with poor prognosis and resistance to anticancer drugs (Warr and Shore, 2008).

The dysregulation of the MAP kinase pathway is not limited to Raf and B-Raf mutations. Moreover, mutations in the epidermal growth factor receptor (EGFR), which activates the ERK pathway, often happen in lung and colorectal cancers (Hynes and MacDonald, 2009, Nagahara et al., 2005). Irregular activation of EGFR has been demonstrated in ~80% of cases of non-small cell lung cancer (Dy and Adjei, 2009). The most common mutation of EGFR is an in-frame deletion in the tyrosine kinase domain, which results in activation of downstream signalling such as that mediated by Raf-MEK-ERK and also PI3K-Akt pathways (McCubrey et al., 2007).

One recent study showed that hypermethylation within the promoter of Wnt inhibitory factor 1 (Wif1) is associated with an unfavorable prognosis in adenocarcinoma (AC) patients with EGFR mutation. Moreover, both *in vitro* and *in vivo* studies demonstrated that mTOR inhibitors prevent the growth of EGFR mutant lung cancer cells, even in the presence of HGF, additionally they indicated that mTOR inhibitors suppress angiogenesis by reducing VEGF production and endothelial proliferation motivated with various pro-angiogenic factors (Ishikawa et al., 2013).

Several other studies have shown that, paradoxically, in some instances NSCLC patients with

EGFR mutations have a more favorable prognosis than do patients with wild-type EGFR (Riely et al., 2006, Yoshida et al., 2007, Kosaka et al., 2009). Erlotinib and Gefitinib, both of which are inhibitors of the tyrosine kinase activity of EGFR are used to block the proliferation of non-small cell lung cancer cells (Zhu et al., 2008). As indicated above, components of signalling pathways activated by EGFR have also received excessive attention as potential targets for the development of new therapeutic drugs for lung cancer (Zhu et al., 2008, Wong et al., 2010).

### 1.5.2 JNK Pathway in Cancer

Perturbations in the JNK pathway are not readily linked to cancer as are those in the ERK pathway. No oncogenic mutations in upstream regulators or the JNKs themselves are implicated. Nevertheless, although under normal conditions Ras is implicated in the regulation of ERK activation, it also has a role in regulating the JNK pathways, linking JNK to cancer development (Wagner and Nebreda, 2009). JNK activity and phosphorylation of c-Jun has been showed to play a critical role in Ras-induced tumorigenesis, and Ras and c-Jun assist in cellular transformation (Kennedy and Davis, 2003). Ha-Ras expression promotes the phosphorylation of c-Jun on the same sites as JNK and c-Jun-deficient fibroblasts are resistant to Ras-induced transformation (Derijard et al., 1994, Johnson et al., 1996).

One essential function of c-Jun appears to be the transcriptional repression of the p53 gene, a key tumour suppressor (Schreiber et al., 1999, Eferl et al., 2003). In contrast to these outcomes, studies using MEFs from JNK1/2 knockout mice have indicated that JNK is not needed for Ras-induced transformation and tumorigenesis *in vivo*. Instead JNK may have a tumour-suppressive function that is connected to its ability to promote apoptosis (Kennedy and Davis, 2003). Despite this, JNK inhibitors have been considered for cancer therapy because of their ability to delay DNA repair in response to genotoxic drugs (Vasilevskaya and O'Dwyer, 2003). However, as these inhibitors have been shown to prevent apoptosis in several types cells (Chen, 2012, Heasley and Han, 2006, Durbin et al., 2009), their usefulness is unclear.

Dysregulation of JNK signalling may be linked to other pathways including the activation of nuclear factor kappa B (NF-κB) signalling, which can lead to the suppression of apoptosis (Bubici

et al., 2004). In cancer, JNK and NF-κB signalling often play opposing roles, with JNK activation being tumour suppressive whereas activation of NF-κB can prevent stress-induced apoptosis (Franzoso et al., 2003, Kennedy and Davis, 2003). In response to tumour necrosis factor alpha (TNF-α), the anti-apoptotic effect of NF-κB has been appeared to be mediated by the induction of genes that can suppress JNK activity (Javelaud and Besancon, 2001, Tang et al., 2002). This may have relevance to oncogenes such as Ras, which are potent inducers of sustained JNK activation; simultaneous activation of NF-κB may be required to repress JNK-induced apoptosis during tumorigenesis (Bubici et al., 2004). Thus, inhibition of NF-κB activity may be a suitable avenue to promote apoptosis in such cells via a JNK-dependent mechanism.

### 1.5.3 p38 MAP kinase pathway in Cancer

Studies examining the role of p38 MAP kinase in the regulation of cancer development and progressions are not as extensive for ERK nor JNK, however, some evidence has accumulated. Analysis of the phenotype of mice with mutation disrupted in either the MEK3 and MEK6 genes or the p38α gene has led to the suggestion that p38 MAP kinase can function as a tumour suppressor. The transforming potential of oncogenes is increased in fibroblasts from these animals as well as their tumorigenic potential when injected into nude mice (Bulavin and Fornace, 2004, Timofeev et al., 2005). Suppression of p38 MAP kinase function also plays a critical role in Ras-induced transformation (Timofeev et al., 2005). The tumour suppressive effects of p38 MAP kinase appear to be mediated in several different ways. p38 MAP kinase is involved in both the activation of p53 and in p53-induced apoptosis and acts as negative regulator of cell cycle progression (Bulavin and Fornace, 2004, Bradham and McClay, 2006). In addition, p38 MAP kinase is also activated by oncogenic stresses and plays a role in Ras-induced senescence in mouse embryo fibroblasts (Bulavin et al., 2003). Such findings suggest a decrease in p38 MAP kinase activity plays an important role in cancer. In support of this notion, p38 MAP kinase activity has been shown to be reduced in hepatocellular carcinomas in comparison to adjacent normal tissue, with tumour size inversely related to p38 kinase activity (Iyoda et al., 2003). Many chemotherapeutic agents require p38 MAP kinase activity for the induction of apoptosis (Olson and Hallahan, 2004, Bradham and McClay, 2006). Again there is the paradox that despite its involvement in apoptosis, inhibition of

p38 MAP kinase activity has been reported to enhance apoptosis in response to DNA-damaging agents such as doxorubicin and cisplatinin as well as the microtubule-disrupting agents taxol, vicristine and vinblastine (Deacon et al., 2003, Lee et al., 2006).

## 1.6 SIGNALLING PATHWAY OF MAPKS IN PROSTATE CANCER

MAP kinases have been extensively studied in different cancers including prostate cancer. The reader is referred to a number of excellent reviews (Dhanasekaran and Reddy, 2008, Papatsoris et al., 2007). Below the salient issues for each pathway will be highlighted, again combining clinical correlates, mouse models and cell studies.

### 1.6.1 ERK pathway in prostate cancer

A key consideration in assessing the involvement of ERK pathway in prostate cancer is the presence of extracellular mediators in the cancerous tissue. EGF and TGF-β are growth factors that can stimulate tumour progression partly by means of the ERK pathway (Thakur et al., 2009, Wilson et al., 2009). Moreover, several studies indicate that these factors are overexpressed in prostate cancer in comparison with normal tissue (de Miguel et al., 2000, Royuela et al., 1998, Leav et al., 1998, Putz et al., 1999). Moreover, stimulation of the Raf-1-MEK-ERK pathway is a feature of solid tumours such as prostate cancer (Rodriguez-Berriguete et al., 2010) even in the absence of mutation, suggesting growth factor induced activation of the pathway is important. Other extra activators may be involved; ERK phosphorylation is notably increased by TNF-α in a concentration-dependent manner in LNCaP cells, also, several studies indicate that TNF-α plays a role in the clinical conditions (Jiang et al., 2008, Chao et al., 2004). Furthermore, IL-6 is also implicated in prostate cancer via activation of the ERK pathway (McCubrey et al., 2006, Rodriguez-Berriguete et al., 2010). LNCaP cells, which produce IL-6, show an increase in proliferation, at least in part, due to ERK activation (Karkera et al., 2011).

Increased expression and activation of ERK pathway components is also a feature of prostate cancer. Raf has been revealed to be related to advanced prostate cancer, hormonal independence, metastasis,

and a poor prognosis (Rodriguez-Berriguete et al., 2010). Moreover, prostate cancer cell lines isolated from patients with advanced cancer such as LNCaP, PC3 and DU145 cells express high levels of active Raf kinase (McCubrey et al., 2007, McCubrey et al., 2006).

Additionally, high levels of ERK are observed in recurrent tumours when androgen ablation becomes refractory suggesting that an increase in ERK signalling may be involved in the progression of prostate cancer to androgen-independence. Supporting these findings is the observation that ERK activation in prostate epithelial cells significantly increases from benign prostatic hyperplasia (BPH) to prostate cancer (Royuela et al., 2002). This observation is contradictory to the finding that ERK activation is reduced in more advanced tumours from TRAMP (transgenic adenocarcinoma of the mouse prostate) mice (Greenberg et al., 1995, Uzgare et al., 2003).

Nevertheless, despite some contradictory data, numerous compounds that inhibit ERK signalling have been demonstrated to inhibit proliferation and increase apoptosis in various prostate cancer cell lines and in patients. One such compound is the biflavonoid morelloflavone, which is known to have anti-oxidative, anti-inflammatory, and anti-angiogenic properties in other settings (Mojzis et al., 2008). Using the MEK/ERK inhibitor U0126, activation of ERK was demonstrated to be necessary to sustain DNA synthesis in LNCaP, DU145 and PC3 prostate cancer cells and to have a role in regulating androgen induced gene transcription (Koreckij et al., 2009).

### 1.6.2 JNK pathway in prostate cancer

The precise role of JNK in prostate cancer development appears to be complex, with both proliferative and apoptotic roles reported. Different agents that activate apoptosis in prostate cancer cells *in vitro* increase JNK activity, which is required for cell death (Altuwaijri et al., 2003, Engedal et al., 2002, Lorenzo and Saatcioglu, 2008). Consistent with the role of JNK in increased apoptosis in prostate cancer cells, androgens have been shown to inhibit apoptosis with a concomitant significant drop in JNK phosphorylation (Lorenzo and Saatcioglu, 2008). In these experiments, blocking transcription by Actinomycin D, inhibiting AR signalling by the anti-androgen bicalutamide or AR

knockdown all effectively blocked the inhibition of JNK activation by androgen treatment (Lorenzo and Saatcioglu, 2008). These approaches indicated that the decrease in JNK activation by androgens requires AR-dependent transcriptional activation. Notably, an increase in phosphatase activity was observed in the presence of androgens when JNK phosphorylation was decreased (Lorenzo and Saatcioglu, 2008).

In contrast with these results, a more recent study indicated that JNK was upregulated in a prostate cancer mouse model (Vivanco et al., 2007). It has been shown that there was an increase in prostate tumours in PTEN null mice, which was associated with increased JNK activation. Also the authors demonstrated that JNK knockdown inhibited the growth of these cells (Vivanco et al., 2007). Moreover, parallel to JNK activation, AKT was individually activated (Vivanco et al., 2007). Consistent with these observations, significant association of c-Jun and c-Fos expression, direct downstream targets of JNK and components of the AP-1 complex, was observed in advanced prostate cancer in mice that are compound heterozygotes for null alleles of Pten and Nkx3.1 (Ouyang et al., 2008). In this model, c-Fos and c-Jun expression increased with increasing tumour grade and was related with ERK and JNK activation, which correlated with expression patterns in human tissues (Ouyang et al., 2008, Mehraein-Ghomi et al., 2008).

To date, there are only a few studies which assess JNK activity / expression activity in human tumour samples, however, either the activity or expression levels of one of the downstream targets of JNK, c-Jun, has been determined and shown to increase (Vivanco et al., 2007, Ouyang et al., 2008). Moreover, one recent *in vivo* study showed that inhibiting JNK reduced the survival of stem-like glioblastoma cells, indicating that JNK may be a clinically relevant therapeutic target for cancer stem cells (Matsuda et al., 2012). Woodfield and colleagues also demonstrated that whilst inhibition of JNK signalling in mutant tissues partially decreases proliferation, inhibition of JAK/STAT signalling rescues other aspects of the neoplastic phenotype (Woodfield et al., 2013), suggesting the potential of co-inhibition of JNK with another pathway as a novel strategy. How these observations link to prostate cancer is at present unclear.

### 1.6.3 p38 MAP kinase pathway in prostate cancer

Of the three major MAP kinases, p38 is the least characterised in relation to prostate cancer. However, studies to date indicate that is present in the basal and epithelial cells of the normal prostate where its expression significantly increases from BPH to prostate cancer and is associated with increased cell proliferation (Maroni et al., 2004, Ricote et al., 2006, Royuela et al., 2002).

## 1.7 THE MITOGEN ACTIVATED PROTEIN KINASE PHOSPHATES (MKPS)

The termination of kinase signalling by the action of phosphatases has been studied far less than that of the kinases themselves. Classes of protein phosphatases include the protein tyrosine phosphatases (PTP), serine/threonine phosphatases and the dual specific phosphateses (DUSPs) (Keyse, 2000). The first PTP was identified as early as 1989 by Walsh and his group, then cloned by Guan et al., 1991, almost ten years after the first protein tyrosine kinase (PTK) was discovered (Czernilofsky et al., 1980). Since then a large number of phosphatases have been identified with the potential to inactivate MAP kinase signalling by targeting either the tyrosine residue itself for example HePTP (Saxena et al., 1999), or the threonine residue via the action of PP2A (Alessi et al., 1995). The discovery of the MAP kinase phosphatases revealed a class of DUSPs capable of dephosphorylating both the Thr and Tyr residues (Mumby and Walter, 1993  Tonks, 2006, Sun et al., 1993, Alessi et al., 1995, Hanada et al., 1998).

## 1.8 OVERVIEW OF THE MKP FAMILY

In mammalian cell systems, to date, eleven members of the dual-specificity protein phosphatases (DUSP or VHR-like cysteine-dependent protein phosphatases) have been identified (Alonso et al., 2004). Such a group includes ten active enzymes and one protein, DUSP24/MK-STYX, that has many features of an MKP but does not have the active site cysteine that is necessary for catalysis (Wishart and Dixon, 1998). There is also a series of small DUSPs which have catalytic activity but lack some of the regions which make up the mammalian full length DUSPs (Dickinson and Keyse, 2006, Patterson et al., 2009).

All MKPs share a common structure (Fig 1.3), which includes a C-terminal catalytic domain that shows considerable sequence similarity to the prototypic dual-specificity protein phosphatase VH-1 of vaccinia virus and an N-terminal domain containing two regions resembling the catalytic domain of the Cdc25 phosphatase (Keyse and Ginsburg, 1993, Kondoh and Nishida, 2007, Keyse, 2008, Soulsby and Bennett, 2009, Patterson et al., 2009). An MKP domain situated towards the N-terminal domain also contains a cluster of basic amino-acid residues, which play an important role in MAP Kinase substrate recognition and binding (Camps et al., 2000, Owens and Keyse, 2007, Rohan et al., 1993, Kondoh and Nishida, 2007) (Fig 1.4A). It has been shown that several but not all MKPs are catalytically activated by substrate binding and specificity to its MAP Kinase binding (MKB) domain (Keyse and Ginsburg, 1993, Christie et al., 2005). Binding of phosphorylated MAP Kinase to the MKB domain alters the alignment of the DUSP domain. This conformational change, along with the interaction of the catalytic domain with the MAP Kinase, greatly enhances the catalytic activity of a number of MKPs, for example, MKP-1 by as much as 100-fold (Fig 1.4B) (Farooq et al., 2001, Stewart et al., 1999, Kondoh and Nishida, 2007).

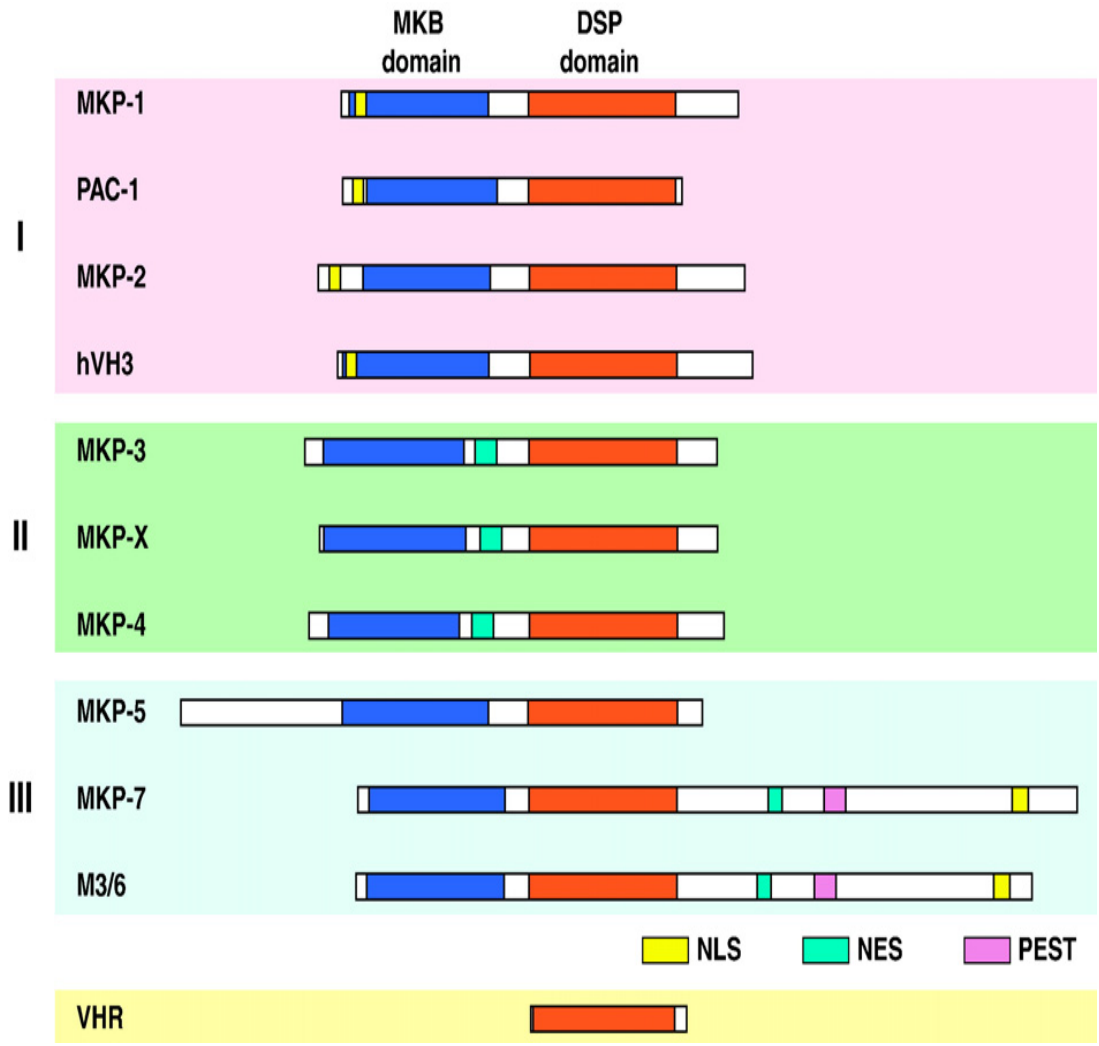


**Figure 1.3: Classification and domain structure of the MKP family.** Domain structures of the three subgroups of MKPs are shown. VHR is an atypical "MKP". In addition to the MAP Kinase binding (MKB) domain and dual-specificity phosphatase (DUSP) domain, nuclear localisation signal (NLS), nuclear export signal (NES), and PEST sequences are indicated (derived fromKondoh and Nishida, 2007).

MKPs are highly specific for MAP Kinases over other kinase substrates, but vary in the substrate specificity among the MAP Kinase family members. Furthermore, subcellular localisation is also a factor, and not all MKPs reside in the same sub-cellular location. Tissue distribution and induction by extracellular stimuli are two additional criteria on which to extend the classification (Cuadrado and Nebreda, 2010). Indeed, based on the sequence similarity, protein structure, substrate specificity, subcellular localisation and regulation by extracellular factors the MKP family can be divided into three groups (Table 1.1) (Stewart et al., 1999, Farooq et al., 2001).

Type I MKPs consist of a group of MKPs which localise in the nuclear compartment and are induced by many of the stimuli that simultaneously activate MAP Kinases. Therefore, it has been suggested that these MKPs play an important role in the feedback control of MAP Kinase signalling in the nucleus. Type I MKPs include the prototypic MKP-1, MKP-2, PAC1, and hVH3. MKPs in this group consist of 300 to 400 amino acid residues, and unsurprisingly contain a nuclear localisation signal (NLS) sequence in their N-terminus (Wu et al., 2005a).

The second group of MKPs, the Type II MKPs, are exemplified by the presence of a nuclear export signal (NES) and are localised in the cytoplasm (Patterson et al., 2009). Type II MKPs include MKP-3, MKP-X and MKP-4. Type II MKPs are also called the Pyst subfamily of dual-specificity phosphatases. MKP-3, MKP-X and MKP-4 are thus also called Pyst-1, 2 and 3, respectively. MKPs in this group show restricted tissue distribution and limited selectivity. For example, MKP-3 is selective for ERK over the other MAP Kinases (Groom et al., 1996, Muda et al., 1996a, Muda et al., 1996b).

The third group of Type III MKPs consist of MKP-5, MKP-7 and M3/6. Again they have a unique selectivity profile preferring JNK and p38 MAP kinase as substrates, but not ERK1/2. They contain both NLS and NES and have the capacity to reside in both nuclear and cytosolic compartments. They also have an extended region, either in the N- terminus (MKP-5) or in the C-terminus (MKP-7 and M3/6) in addition to the MKB and DUSP domains (Matsuguchi et al., 2001), called the PEST

domain, a sequence rich in proline (P), glutamic acid (E), serine (S), and threonine (T). The PEST domain may play a role in stability and is present in key metabolic enzymes, transcription factors, protein kinases, protein phosphatases and cyclins (Rogers et al., 1986).

Although not addressed extensively in this thesis there are the atypical “MKPs” that consist of only a catalytic domain with no recognised targeting or docking sequence. These phosphatases consist of the DUSP domain alone and a small regulating domain (approximately 200 amino acids). Nevertheless, some of these phosphatases are able to dephosphorylate MAP Kinases (Alonso et al., 2001, Vasudevan et al., 2005, Farooq and Zhou, 2004) and do so with some selectivity (Patterson et al., 2009).

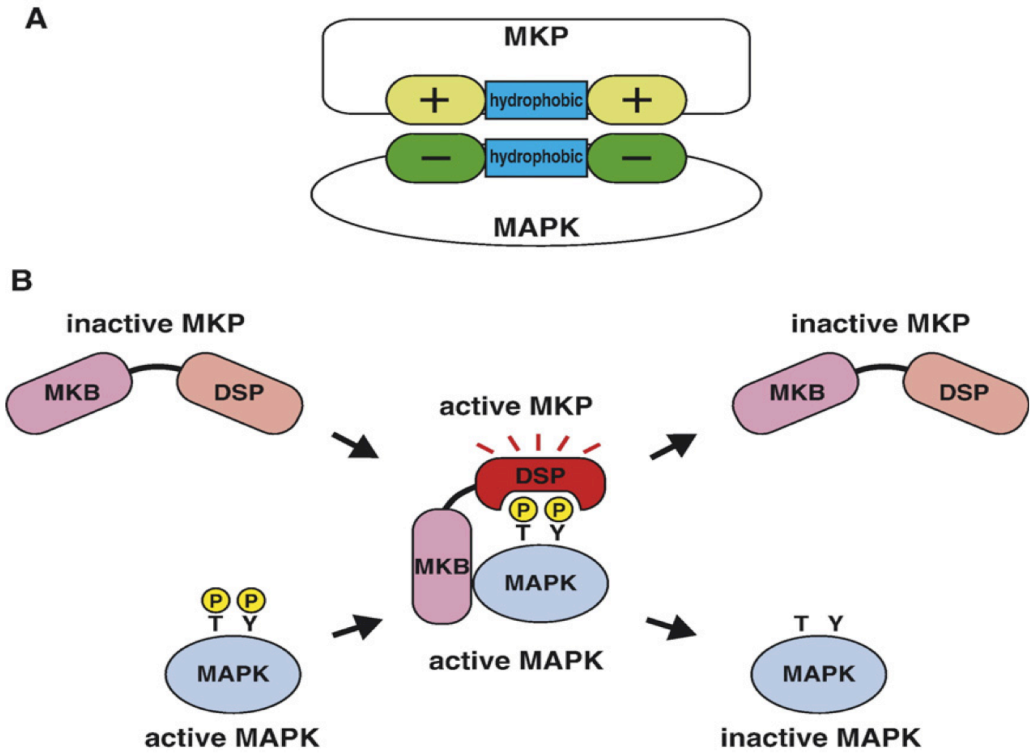


**Figure 1.4: Interaction of MKPs with MAP Kinases.** (A) The docking interaction between MAP Kinases and MKPs. The docking surface in the MKB domain of MKPs, which can be divided into three modules, binds to the corresponding sites in MAP Kinases. (B) Activation of MKPs by MAP Kinases. The dual-specificity phosphatase (DSP) domain in MKPs is inactive without its substrate. Binding of activated MAP Kinases to the MKB domain induces conformational changes in the DUSP domain, which causes the increase of its catalytic activity (derived from Kondoh and Nishida, 2007).

**Table 1.1: The MAP kinase phosphatases**

| Gene | MKP | Trivial Names | Subcellular Localisation | Substrate Specificity | Tissue distribution | References |
|---|---|---|---|---|---|---|
| DUSP-1 | MKP-1 | CL-100, hVH1, erp, 3CH134 | Nuclear | p38~JNK~ERK | Heart, skeletal muscle, pancrease, placenta, liver, testes, stomach, lung and brain. | (Farooq and Zhou, 2004; Keyse and Emslie, 1992; Noguchi et al., 19 |
| DUSP2 | PAC-1 | N.D | Nuclear | ERK~p38 | lymphoid cells (spleen and thymus) | (Gerondakis et al., 1994; Rohan et al., 1993; Ward et al., 1994). |
| DUSP4 | MKP-2 | hVH-2, Typ1, Sty8 | Nuclear | JNK~ERK1> p38 | Placenta, skeletal muscle, spleen, Kidney and brain, heart, liver, testis, and pancreas. | (Chu et al., 1996; Guan et al., 1995; Misra-Press et al., 1995). |
| DUSP5 | N.D | hVH3, B23 | Nuclear | ERK | Placenta, liver, heart, lung,brain,Kidney, pancreas,skeletal muscle | (Ishibashi et al., 1994; Martell et al., 1994). |
| DUSP6 | MKP-3 | rVH-6, Pyst1 | Cytoplasmic | ERK | Placenta,skeletal muscle, spleen, Kidney, and brain, heart, liver, testis, and pancreas. | (Groom et al., 1996; Muda et al., 1996a; Muda et al., 1996b). |
| DUSP7 | MKP-X | Pyst2, B59 | Cytoplasmic | ERK | Heart,brain, placenta, lung, skeletal muscle, kidney and pancreas. | (Smith et al., 1997, Shin et al., 1997) |
| DUSP9 | MKP4 | Pyst3 | Cytoplasmic | ERK > p38 | Placenta,kidney, embryonic liver | (Muda et al., 1997, Smith et al., 1999) |
| DUSP10 | MKP-5 | N.D | Cytoplasmic/ Nuclear | JNK~p38 | Heart, lung, skeletal muscle,liver,and kidney> brain, spleen and testis. | (Tanoue et al., 1999, Theodosiou et al., 1999) |
| DUSP16 | MKP-7 | MKP-M | Cytoplasmic/ Nuclear | JNK~p38 | N.D | (Masuda et al., 2001) |
| DUSP14 | MKP-6 | MKP-L | N.D | ERK~JNK | Human trachea, placenta, liver, heart and thyroid | (Marti et al., 2001) |
| N.D | MKP-8 | N.D | Cytoplasmic/ Nuclear? | p38 | Brain, heart, skeletal muscle | (Sanchez-Prieto et al., 2000; Vasudevan et al., 2005). |

- **N.D: not determined**

### 1.8.1 Type 1 Nuclear MKPs

#### 1.8.1.1 MAP Kinase Phosphatase-1 (MKP-1)

MKP-1, the first MKP discovered, was identified as an immediate early gene that is induced rapidly after exposure to growth factors, heat shock and oxidative stress (Charles et al., 1992, Keyse and Emslie, 1992, Valledor et al., 2000). MKP-1 expression is high in liver, placenta, lung and pancreas, with lower levels detected in heart and skeletal muscle (Martell et al., 1995). Since its discovery a large body of evidence has supported an important role in cellular regulation via dephosphorylation of MAP kinases. MKP-1 is involved in a negative feedback loop of MAP Kinase signalling, in a variety of different cell systems, studies have shown that MKP-1 expression is mediated predominantly by activation of ERK (Volmat et al., 2001, Franklin and Kraft, 1997). Expression levels appear to be modulated at multiple stages including stabilisation of the protein itself (Li et al., 2003).

Many extracellular factors up-regulate MKP-1 mRNA levels and protein in different cell types, for example, serum in mouse and rat fibroblasts (Bokemeyer et al., 1996), glucagon in rat hepatocytes (Schliess et al., 2000) and following dexamethasone-treatment in human breast epithelial cell lines, MCF-7 and MDA-MB-231 (Wu et al., 2004, Wu et al., 2005b). TGF-β signalling and retinoid acid signalling also upregulate MKP-1 expression (Mikami et al., 2006, Short et al., 2006), as do several hormones, such as endocannabinoids, glucocorticoids and parathyroid hormone (Aghaloo et al., 2006, Eljaschewitsch et al., 2006). Furthermore, DNA-damaging agents such as hydrogen peroxide also induce MKP-1 (Keyse and Emslie, 1992), possibly through activation of the tumour suppressor p53 (Li et al., 2003). Taken together these studies indicate the ubiquitous nature of MKP-1 induction.

There are a large number of studies to link MKP-1 to the development of different cancers. The gene locus of MKP-1 is on human chromosome 5q34, an area of chromosome 5 while correlates to malignancies (Emslie et al., 1994). This region is erased in leukaemias and translocation is linked to lymphoma. There is also strong causal evidence linking MKP-1 expression to cancer through clinical trial data, cellular outcomes and preclinical *in vivo* mice studies (see review below).

In addition to a role in cancer, MKP-1 has notable functions in other cell systems. An extensive amount of evidence has indicated a role for MKP-1 as a negative regulator of innate immunity, Chi et al. (2006), showed that stimulation of TLR-4 strongly induced MKP-1 mediated by both the TIR domain-containing adaptor-inducing IFN-β (TRIF) and myeloid differentiation factor 88 (MyD88) (Chi et al., 2006). Moreover, Hammer et al. (2006) demonstrated using a global microarray analysis of approximately 14,000 mouse genes, that following LPS stimulation 608 genes were upregulated in spleen cells derived from MKP-$1^{-/-}$ mice compared to wild-type mice. These included IL-10 and IL-6, in addition to some chemokines (Hammer et al., 2006).

Consistent with these findings another study showed that the half-lives of IL-6, IL-10 and TNF-α mRNAs were decreased by overexpression of MKP-1 in LPS-stimulated rat macrophages (Yu et al., 2011). These cellular studies were linked to *in vivo* effects, Zhang and co-workers demonstrated that MKP-$1^{-/-}$ mice were deficient in anti-influenza immunity but were unaltered in a model of autoimmune encephalomyelitis (Zhang et al., 2009).

Several studies have also linked MKP-1 expression and function to the effects of the glucocorticoids, which are able to strongly induce the protein and inhibit subsequent MAP kinase signalling (Chen et al., 2002, Kassel et al., 2001, Lasa et al., 2002, Abraham et al., 2006). For example in macrophages, overexpression of MKP-1 induced by dexamethasone was able to block stem cell factor induction through a reduction in p38 MAP kinase activity (Jeong et al., 2003). Another study showed that in comparison to wild-type mice, MKP-$1^{-/-}$ mice displayed enhanced mast cell degranulation and increased susceptibility to anaphylaxis. Glucocorticoids inhibited these effects to a lesser extent in MKP-$1^{-/-}$ mice (Maier et al., 2007). Finally, Zhang et al, (2009) also studied the kinetics of MAP kinase signalling in response to Ionomycin and PMA and established that JNK and ERK1/2 phosphorylation was increased in $CD4^{+}$ T-cells derived from MKP-$1^{-/-}$ mice compared with wild-type mice (Zhang et al., 2009). This suggests the potential for MKP-1 to also play a role in adaptive immune responses.

The MAP kinase signalling pathway has been implicated in other tissue systems, so it is of no surprise to find that MKP-1 is also implicated. For example, Reddy et al. (2004) showed that in

response to an atherogenic diet, the expression of MKP-1 mRNA was increased in atherosclerotic lesions of ApoE $^{-/-}$ mice (Reddy et al., 2004). Other studies have demonstrated that MKP-1$^{+/+}$ mice display a considerably higher degree of aortic atherosclerotic lesion formation compared with mice MKP-1$^{-/-}$ (Shen et al., 2010). MKP-1 has also been implicated in neurodegenerative disease (Taylor et al., 2013, Jeanneteau et al., 2010, Carrasco and Bravo, 1993, Oliva et al., 2006), diabetes (Weng et al., 2007), and arthritis (Toh et al., 2004).

#### 1.8.1.2 MAP kinase Phosphatase-2 (MKP-2)

MAP kinase phosphatase-2 (MKP-2) is a class I DUSP (known as DUSP4 and also hVH-2), which is an inducible nuclear protein of 394 amino acids (M.W 43 kDa) (Chen et al., 1996, Misra-Press et al., 1995, Lawan et al., 2012). MKP-2 was identified concurrently in rat and human and found to be induced by growth factors, oncogenes (Fu et al., 2000), phorbol 12-myristate 13-acetate (PMA), hormones, oxidative stress, and UV light, as well as LPS (Brondello et al., 1997, Chu et al., 1996, Jeffrey et al., 2006, Misra-Press et al., 1995).

MKP-2 was found to have a different tissue distribution from MKP-1, which indicated the potential of different physiological function for each phosphatase. Guan and Butch (1995), observed that MKP-2 was expressed in most rat tissues including spleen, testes, brain, heart and lung (Guan and Butch, 1995). Moreover, Chu et al, (1996) revealed that MKP-2 was expressed in most human tissues, with the highest amounts in prostate, testes, stomach and pancreas. Similarly, Misra-Press et al. (1995) discovered additional expression in human placenta and pancreas but undetectable amounts in the lung (Misra-Press et al., 1995). In some of these studies, expression was shown to be mediated by ERK1/2 (Brondello et al., 1997, Ryser et al., 2004).

Early biochemical studies indicated that MKP-2 was also located in the nucleus due to the presence of an NLS within the N-terminus defining it as a Type 1 DUSP. However, an additional study has shown the presence of an additional bipartite sequence further towards the C- terminus (Sloss et al., 2005). Sloss et al, (2005) also revealed that disruption of both putative localisation sequences was necessary for relocation of MKP-2 to the cytosol and that expression of either NLS alone was

sufficient to translocate the protein to the nucleus. Disruption of both sequences prevented the inactivation of JNK activity due to differential compartmentalisation of the phosphatase (Sloss et al., 2005).

MKP-2 has been found to dephosphorylate ERK and JNK *in vitro* (Chu et al., 1996, Brondello et al., 1997), whilst being ineffective against p38 MAP kinase despite binding strongly to this kinase (Chen et al., 2001). Activation of the ERK1/2 pathway induced MKP-2 expression (Brondello et al., 1997), but more recent studies also indicated that regulation of protein stability was a significant feature (Tresini et al., 2007), and may be physiologically relevant. For example, cellular senescence increased MKP-2 protein by blocking its degradation (Torres et al., 2003). Furthermore, MKP2 was also found to be induced by oxidative stress in a p53-dependent manner (Shen et al., 2006), suggesting that pathways in addition to ERK can regulate induction.

Studies performed *in vitro* confirm that the interaction of MKP-2 with and phosphorylation by ERK1/2 and JNK enhances its catalytic activity (Chen et al., 2001). However, the substrate specificity of MKP-2 *in vivo* is still unclear. Several studies suggest a predominant role in ERK dephosphorylation (Cagnol and Rivard, 2013, Jeong et al., 2013). Although it has been reported that MKP-2 dephosphorylates JNK, but not ERK1/2 in UV-C or cisplatin-treated cells (Cadalbert et al., 2005), in these cells, while JNK translocates to the nucleus, ERK1/2 remains within the cytoplasm. Therefore, MKP-2 may dephosphorylate nuclear JNK, but cannot access cytoplasmic ERK1/2 and this may direct its specificity in a cellular context.

As with MKP-1, MKP-2 has also been implicated in the development and progression of cancer (see below). However, MKP-2 is also implicated in other pathophysiological conditions, in particular inflammation and immune function which have been studied through the recent availability of DUSP-4 knockout mouse models. Cornell et al, (2010) showed that in MKP-2 knockout mice there was reduced mortality in response to LPS or cecal ligation and puncture which correlated with reduced levels of TNF-α and other cytokines (Cornell et al., 2010).

Furthermore, in another DUSP-4 deletion mouse model it was found that infection by the parasite Leishmania Mexicana (L Mexicana) was increased (Al-Mutairi et al., 2010b). Moreover, MKP-$2^{-/-}$ bone marrow-derived macrophages were found to have diminished ability to control the growth of the parasite, an effect linked to the regulation of iNOS and arginase activity (Al-Mutairi et al., 2010b). Other studies have also linked MKP-2 to T-cell responses (Huang et al., 2012), with impaired function linked to ageing (Yu et al., 2012). Schroeder et al, (2013) showed that whilst, $T_H1/T_H2$ responses were both altered in DUSP-4 $^{-/-}$ mice, this did not result in any significant change in disease phenotype (Schroeder et al., 2013).

Further studies utilising DUSP-4 deletion models to determine the physiological function and pathological roles of MKP-2 have not been forthcoming and studies are limited to cell systems. For example, overexpression of MKP-2 has been shown to limited cellular apoptosis due to inhibition of JNK (Cadalbert et al., 2005), whilst adenoviral MKP-2 has been used to prevent apoptosis in human endothelial cells (Al-Mutairi et al., 2010a). In other cell types, MKP-2 has been described as a mediator of oxidative damage-induced apoptosis through inhibition of ERK activation (Wang et al., 2007b). A recent study has also revealed the potential for MKP-2 to play a role in cell cycle progression, fibroblasts from DUSP-$4^{-/-}$ mice were more prone to arrest in $G_2$/M-phase than wild type counter parts (Lawan et al., 2011).

#### 1.8.1.3 PAC-1

PAC-1 was initially identified as a mitogen-inducible gene in human T-cells (Rohan et al., 1993). It is specifically induced in B cells and T cells upon activation (Grumont et al., 1996). Activation of the ERK1/2 pathway brings about PAC-1 expression (Rohan et al., 1993), whilst PAC-1 specifically dephosphorylates p38 MAP kinase and ERK1/2 *in vitro* (Ward et al., 1994, Chu et al., 1996). Significantly, while the interaction of the PAC-1 MKB domain with ERK1/2 increases PAC-1 catalytic activity, no interaction with p38 MAP kinase is observed (Zhang et al., 2005). Interestingly, pharmacological inhibition of JNK activity can reverse the reduced phosphorylation of ERK1/2 in $PAC1^{-/-}$ cells (Jeffrey et al., 2006), suggesting that loss of PAC-1 may decrease ERK1/2 activity by up-regulating JNK activity; therefore, it is likely that there is a cross-talk

between ERK1/2 and JNK. The manner in which PAC-1 targets JNK in the immune system has been examined further (Jeffrey et al., 2006, Pulido and Hooft van Huijsduijnen, 2008).

#### 1.8.1.4 hVH3

hVH3 is induced by heat shock and growth factors (Kwak and Dixon, 1995). In peripheral blood T lymphocytes, IL-2, IL-7 and IL-15 induce hVH3, whilst IL-2-induced ERK1/2 activation is inhibited by hVH3 (Kovanen et al., 2003), suggesting a negative feedback role for hVH3 in IL-2 signalling. Moreover, anti-CD3 stimulation of thymocytes conspicuously induces hVH3 (Tanzola and Kersh, 2006, Bokemeyer et al., 1997). Therefore, hVH3 may play an important role in T cells. hVH3 interacts with and dephosphorylates ERK1/2, but not JNK or p38 MAP kinase. It is also possible that hVH3 is a direct transcriptional target of the tumour suppressor p53 (Ueda et al., 2003), but further studies are required. Overexpression of DUSP5 has also been shown to suppress the growth of several types of human cancer cells (Wu, 2004, Ueda et al., 2003). Recent studies have shown that hVH3 can regulate subcellular localisation of ERK1/2 (Mandl et al., 2005, Kucharska et al., 2009).

### 1.8.2 Type II MKPs

#### 1.8.2.1 MAP Kinase Phosphatase-3 (MKP-3)

Controlling mechanisms for MKP-3 expression seem to be cell type-dependent, as although MKP-3 appears to be constitutively expressed in some cell types, it is induced by certain growth factors, such as basic fibroblast growth factors and nerve growth factors (Groom et al., 1996, Camps et al., 1998, Muda et al., 1996a). In contrast to the type I DUSPs, MKP-3 is relatively specific for ERK1/2 over the stress-related MAP Kinases (Groom et al., 1996, Dowd et al., 1998, Slack et al., 2001). Indeed to date, MKP-3 is the only MKP that has been shown to dephosphorylate ERK5. ERK1/2 binding to the MKB domain of MKP-3 increases its phosphatase activity (Muda et al., 1998, Muda et al., 1996b). MKP-3 is also phosphorylated by ERK1/2 and this facilitates proteasomal degradation of MKP-3 (Marchetti et al., 2005, Breitschopf et al., 2000).

Whilst MKP-3 contains a NES and is cytosolic, one key study has shown that MKP-3 shuttles between the cytoplasm and the nucleus and is capable of anchoring ERK1/2 in the cytoplasm, suggesting that MKP-3 may play a role in determining cytoplasmic localisation of ERK1/2 (Karlsson et al., 2004).

#### 1.8.2.2 MAP Kinase Phosphatase-X (MKP-X) and MAP Kinase Phosphatase-4 (MKP-4)

MKP-X has been shown able to form a complex with ERK1/2 and specifically targets ERK1/2 protein as its substrate *in vitro* (Groom et al., 1996). Furthermore, MKP-X has also been found to bind to and dephosphorylate JNK again, *in vitro* (Orlev et al., 2004, Levy-Nissenbaum et al., 2003b). Both protein and MKP-X mRNA were discovered to be expressed in bone marrow, peripheral leukocytes from acute myeloid leukaemia and several leukaemia cells (Levy-Nissenbaum et al., 2003a, Levy-Nissenbaum et al., 2003c). However, the biological function of MKP-X remains unclear.

Similarly, MKP-4 specifically blocks activation of ERK1/2 once expressed in cells (Levy-Nissenbaum et al., 2003b), and dephosphorylates p38 and ERK1/2 *in vitro* (Muda et al., 1997). MKP-4 shows an extremely restricted tissue distribution; it is expressed only in placenta, embryonic liver, kidney, migrating muscles and insulin-response tissues (Levy-Nissenbaum et al., 2003b, Muda et al., 1997). MKP-4 has been identified as a candidate gene involved in the negative regulation of insulin signalling (Xu et al., 2003).

### 1.8.3 Type III MKPs

#### 1.8.3.1 MAP Kinase Phosphatase-5 (MKP-5)

MKP-5 binds to and dephosphorylates p38 MAP kinase and JNK, but not ERK1/2 (Theodosiou et al., 1999, Tanoue et al., 1999). MKP-5 is expressed in heart, liver, lung, kidney and skeletal muscle. MKP-5 expression in cultured cells is increased by stress stimuli, including ATM-dependent double-strand DNA breaks (Masuda et al., 2000, Jeong et al., 2006). MKP-5 function within the

immune system has also been demonstrated (Zhang et al., 2004), and MKP-5 deficient mice show multiple defects in immune responsiveness to infection (Qian et al., 2009).

#### 1.8.3.2 MAP Kinase Phosphatase-7 (MKP-7)

MKP-7, the major phosphatase in the MKP type III family has a unique long C-terminal region that contains both NES and NLS, by which MKP-7 shuttles between the nucleus and the cytoplasm (Masuda et al., 2001, Tanoue et al., 2001). MKP-7 binds to and dephosphorylates p38 MAP kinase and JNK, but not ERK1/2 (Masuda et al., 2001, Tanoue et al., 2001). MKP-7 expression is induced by JNK activation (Han et al., 2002), and by LPS stimulation in macrophages (Matsuguchi et al., 2001). It has been shown that MKP-7 does bind to ERK1/2 through its C-terminal region, which is phosphorylated by ERK1/2 in response to several extracellular stimuli (Katagiri et al., 2005). This phosphorylation of MKP-7 by ERK1/2 suppresses the proteasomal degradation of MKP-7, enhancing its effect on JNK and p38 signalling (Masuda et al., 2001).

#### 1.8.3.3 M3/6

M3/6 (also called hVH-5) shows specificity towards the inactivation of JNK and p38 MAP kinase (Martell et al., 1995, Kawakami et al., 2003). M3/6 has both NLS and NES sequences within its C-terminal region that are very similar to that of MKP-7, and also shuttles between the cytoplasm and the nucleus. Like MKP-7, M3/6 binds to JIP-1 (Willoughby et al., 2003). M3/6 is expressed predominantly in the adult heart, brain, and skeletal muscle (Willoughby et al., 2003).

## 1.9 MKPS IN CANCER DEVELOPMENT AND PROGRESSION

Whilst studied in less depth than the associated MAP kinases, there is increasing evidence that perturbations in DUSP function play a significant role in the development of cancer. In general terms, studies are conducted along three major lines. Firstly, there is the process of establishing a clinical correlation between the tissue expression of the relevant DUSP and expression activity of the relevant MAP kinase substrate and the progression of the disease.

This may also be supported by genetic analysis of the chromosomal localisation relative to the deletion or mutation implicated in the cancer. Secondly, there are cellular studies which seek to make a correlation between DUSP expression and function and potential cellular transformation to a tumour phenotype. Thirdly, there are preclinical *in vivo* studies, using both mouse xenograft models assessing human tumour formation or specific mouse models where one or usually several mutations/deletions provide a model of a specific cancer type, for example PTEN/TRAMP. Overall, there has been a significant increase in MKPs studies because of their emerging role in controlling cancer cell growth and death (Keyse, 2008).

### 1.9.1 MKP-1 and cancer

In the context of cancer, the study of MKP-1 is the most advanced and worthy of description. Though MKP-1 knockout mice are developmentally normal (Dorfman et al., 1996), a number of studies have indicated that MKP-1 expression is altered in many cancer types including breast, lung, prostate, ovarian, pancreatic, liver, and gastric adenocarcinoma (Table 1.2). Furthermore, clinical studies have indicated that MKP-1 expression is correlated with cancer progression and may be useful as a prognostic marker. For instance, in human breast cancer, MKP-1 was found to be increased five-fold in malignant samples compared to non-malignant samples (Wang et al., 2003).

MKP-1 expression is also significantly higher in breast carcinomas even when poorly differentiated or in late stages of the disease (Loda et al., 1996). In human lung cancer, expression of MKP-1 is significantly higher in non-small cell lung cancer cells (NSCLC) than in small cell lung cancer cells (Vicent et al., 2004). Moreover, high levels of MKP-1 expression independently predict improved survival (Vicent et al., 2004). In pancreatic cancer, MKP-1 mRNA and protein levels are elevated, and down-regulation of MKP-1 expression results in decreased anchorage-dependent and -independent growth of tumours in a nude mouse tumour model and pancreatic cancer cells (Liao et al., 2003).

In human hepatocellular carcinoma, MKP-1 expression was decreased in tumour cells as compared

to normal hepatocytes, and reduced MKP-1 expression was found to be extensively correlated with serum alpha-fetoprotein levels and tumour size (Tsujita et al., 2005). In gastric adenocarcinoma, MKP-1 overexpression was related to the growth of human gastric adenocarcinoma (Bang et al., 1998). In addition, a study of 164 human epithelial tumours of different tissue origin by *in situ* hybridisation and immunohistochemistry showed that MKP-1 was overexpressed in the early phases of prostate, colon, and bladder carcinogenesis, with progressive loss of expression with elevated histological grade and in metastases (Loda et al., 1996).

MKP-1 has also been assessed in relation to cancer therapy and resistance. One recent study showed that overexpression of MKP-1, examined by immunohistochemistry, was associated with resistance to cetuximab-based chemotherapy in patients with metastatic colorectal cancer (mCRC), and indicates a role for MKP-1 as a negative predictive biomarker of response to cetuximab, mainly in KRAS wild-type patients (Montagut et al., 2010). Moreover, one recent study demonstrated that chemotherapy in combination with knockdown of MKP-1 was significantly more effective than chemotherapy alone (Park et al., 2013). Overall, these studies strongly suggest that MKP-1 plays a critical role in cancer development, can be a useful marker for predicting the survival of cancer patients and may be significant in synthetically-induced lethality and drug resistance (Wu, 2007).

Clinical observations have been underpinned by a large number of cellular studies. In tumour cells the chemotherapeutic drug cisplatin was found to induce apoptosis, but the trans-isomer of the same drug, transplatin, was ineffective. This was discovered to be due to the ability of transplatin to induce MKP-1 mRNA production, resulting in the inactivation of JNK (Wang et al., 2008, Ramesh et al., 2007, Wang et al., 2003). In addition, overexpression of MKP-1 increased breast cancer cell (MCF-7) resistance to $H_2O_2$-induced death by JNK activation (Wang et al., 2008, Wu and Bennett, 2005), and inhibition of MKP-1 expression also potentiated apoptosis (mediated by TNF-α) through JNK activation in rat mesangial cells (Guo et al., 1998). In the same cell type, another group showed that $H_2O_2$ was able to induce MKP-1, which in turn inhibited JNK activation and apoptosis (Xu et al., 2004).

### 1.9.2 MKP-2 and cancer

In comparison to MKP-1 there are far fewer studies assessing the role of MKP-2 in cancer and in particular prostate cancer, nevertheless evidence is accumulating. Genetic studies have shown that overexpression of MKP-2 correlates with hepatomas (Yokoyama et al., 1997), pancreatic tumours (Yip-Schneider et al., 2001), familial modulatory thyroid carcinoma (FMTC), multiple endocrine neoplasia (MEN), and papillary thyroid carcinoma (Hasegawa et al., 2008). In these studies, the mRNA levels of MKP-2 were found to be substantially increased, signifying a role for MKP-2 as a positive regulator of cancer proliferation. In chemical hepatocarcinogenesis in rats, MKP-2 was unnoticeable in normal liver but was strongly expressed in haematomas (Yokoyama et al., 1997).

Moreover, in human breast cancer, MKP-2 was increased 3-fold in malignant as compared to non-malignant samples (Wang et al., 2003). MKP-2 was also found to be down regulated in serous carcinomas as compared with serous borderline tumours in ovarian cancers (Levy-Nissenbaum et al., 2003c). Furthermore, Sieben et al, (2005) provided further indirect evidence to support a role for MKP-2 in the development of ovarian cancers (Sieben et al., 2005). Similar observations were made for oesophagogastric rib metastasis and pancreatic tumours (Barry et al., 2001). Indeed, in liver carcinoma the expression of MKP-2 was also dramatically increased by the homeobox gene of the HoxA10 family, which was associated with acute myeloid leukaemia (Wang et al., 2007a). Hasegawa et al, (2008) also showed that inhibition of MKP-2 attenuated the *in vitro* and *in vivo* proliferation of MKK-f cells, which was established from a mammary tumour developed in a RET-MEN2A transgenic mouse. This was mediated by the suppression of cyclin B1 expression, leading to dysregulation of the cell cycle (Hasegawa et al., 2008).

Whilst these studies demonstrate a positive correlation with cancer development, several other observations implicate DUSP-4 as a negative regulator of cancer. Several other screening studies have suggested that MKP-2 may be a tumour suppressor/cancer susceptibility gene (Cloos et al., 2006), linked to gene locus 1q8, that is frequently mutated in breast and prostate cancers (Armes et al., 2004). This is supported by the study of Venter et al. (2005) who showed that high rates of LOH

were observed at markers adjacent to or within MKP-2 in breast cancer cell lines, supporting their status as candidate tumor suppressor genes (TSGs) (Venter et al., 2005).

Consistent with these data is a recent genomic screen which relates DUSP4 loss with EGFR-mutant tumours in lung cancer within the 8p locus (Chitale et al., 2009). Furthermore, overexpression of MKP-2 (DUSP4) in glioblastoma cell lines led to a significant reduction of cellular proliferation and focus formation (Waha et al., 2010). Overall, it seems that MKP-2 can have both positive and negative roles in cancer development; however, definitive causal data is generally lacking.

**Table 1.2: Members of the MKP family that have been implicated in cancer development.**

| Phosphatase | Specificity | Subcellular location | Altered in | References |
|---|---|---|---|---|
| **MKP-1 (DUSP-1)** | ERK, JNK, p38 | nuclear | Bladder,breast, colon,lung, ovarian, prostate glioblastoma leukemia. | (Wang et al., 2003, Loda et al., 1996, Rauhala et al., 2005, Li et al., 2003, Furukawa et al., 2005, Xu et al., 2004, Bang et al., 1998, Yu H et al., 2012, Montagut et al.,2010, Masiero M et al., 2011, park et al., 2013) |
| **MKP-2 (DUSP-4)** | ERK, JNK, p38 | nuclear | Breast,liver, ovarian, pancreatic, colorectal, glioma, intestinal | (Wang et al., 2003, Keyse SM et al., 2008, Yokoyama et al., 1997, Benedikt et al., 2013, Waha A et al., 2010, Cagnol and Rivard, 2013, Rottenberg and Jonkers, 2012) |
| **MKP-3 (DUSP-6)** | ERK | cytoplasmic | Breast,ovarian, pancreatic | (Furukawa et al., 2003, Chen et al., 2007) |
| **MKP-4 (DUSP-9)** | ERK, p38 | cytoplasmic | Skin | (Christie GR et al., 2005) |
| **MKP-7 (DUSP-16)** | JNK, p38 | nuclear& cytoplasmic | Leukemia | (Hoornaert I et al., 2003, Zaidi SK et al., 2009) |
| **PAC-1 (DUSP-2)** | ERK, p38 | nuclear | Ovarian | (Givant-Horwitz et al., 2004) |
| **MKP-X (DUSP7)** | ERK | cytoplasmic | Leukemia | (Levy-Nissenbaum et al., 2003c, Levy-Nissenbaum et al., 2003a) |

### 1.9.3 MKP-3 and cancer

Several studies have shown that MKP-3 potential may have as a tumour suppressor gene (table 1.2). In pancreatic cancer, MKP-3 was slightly upregulated in dysplastic *in situ* carcinoma cells and primary pancreatic cancer, but down-regulated in invasive carcinoma, particularly in the poorly differentiated type (Furukawa et al., 2003). MKP-3 was not expressed in invasive carcinoma cells as compared to pancreatic intraepithelial neoplasia. In pancreatic cancer, involving intraductal papillary-mucinous neoplasms, deficient MKP-3 expression was detected in a relatively small fraction of intraductal carcinomas and intraductal adenoma/borderlines (Furukawa et al., 2005).

The advanced stage of the intraductal adenoma/borderline lesions correlated with loss of MKP-3 harboured mutations of KRAS2 (Furukawa et al., 2005). Loss of MKP-3 expression was associated with progression from pancreatic intraepithelial neoplasia to the invasive ductal carcinoma, although it was possibly associated with the initiation of intra-ductal papillary-mucinous neoplasms with mutated KRAS2 (Furukawa et al., 2005). In nude mice infected with a MKP-3 inducible cell line that was transmuted by Ha-ras, it was found that the treatment of the mice with the tetracycline equivalent doxycycline resulted in a considerable delay in tumour emergence and development as compared to the untreated control group, indicating a role of MKP-3 in tumour suppression (Marchetti et al., 2004).

### 1.9.4 Other members of the MKP family and cancer

In addition to MKP-1, MKP-2 and MKP-3, several other MKP family members have been implicated in cancer development including PAC1 and MKP-X (Table 1.2). A number of reviews summarise their involvement (Keyse, 2008, Wang et al., 2008, Wu, 2007, Marchetti et al., 2004).

## 1.10 ROLE OF MKPS IN PROSTATE CARCINOGENESIS

Whilst MKPs have been studied more extensively in other cancers, a body of evidence is slowly developing implicating their role in prostate cancer. Unsurprisingly, MKP-1 has been the most

extensively investigated, but the results have been equivocal. There has been far fewer studies conducted on other MKPs.

### 1.10.1 Role of MKP-1 in prostate cancer

Initially, Leav and co-workers detected the expression of ERK and MKP-1/DUSP1 following androgen stimulation in the dorsolateral prostate of Noble rats (Leav et al., 1996). In the same study, ERK and MKP-1/DUSP1 were shown to be highly expressed in dysplastic lesions, but MKP-1/DUSP1 mRNA and protein concentrations rapidly decreased in response to a combination of testosterone and estradiol-17β, which were identified as inducing dysplasia in the dorsolateral prostate (Leav et al., 1996). This indicated a link between androgens, MKP-1 and the potential of prostate dysfunction.

This work also linked MKP-1 to the potential of prostate cancer. MKP-1/DUSP1 mRNA was also found to be overexpressed in the initial phases of prostate cancer, followed by a loss of expression with metastases and higher histological grade (Leav et al., 1996). A later study found that DUSP1 mRNA expression was lower in hormone-refractory prostate carcinomas compared to benign prostate hyperplasia (BPH) or untreated prostate carcinomas. Moreover, normal prostate, BPH, and high-grade prostate intraepithelial neoplasia (PIN) expressed high levels of DUSP1 (Rauhala et al., 2005). Prostate cancer samples from patients not treated with androgen ablation had an elevated MKP-1/DUSP1 expression in the pre-invasive stage of prostate cancer, which decreased significantly with greater histological grade and advanced disease (Vaarala et al., 2012). In contrast, Rauhala et al, (2005) also observed lower levels of MKP-1/DUSP1 mRNA in hormone-refractory prostate cancer equal to levels observed in patients with BPH (Rauhala et al., 2005).

Much of this work has sought to make a correlation between the status of MKP-1 expression and the relative activities of the MAP kinases. Studies on prostatic intraepithelial neoplasia (PIN) lesions, showed an inverse association between MKP-1/DUSP1 and JNK, but not between MKP-1/DUSP1 and ERK1 (Magi-Galluzzi et al., 1998, Loda et al., 1996, Magi-Galluzzi et al., 1997).

The preferred substrates of MKP-1/DUSP1 are JNK and p38 MAP kinase, while ERK is known to diminish its degradation, thus one hypothesis derived from this work is that in prostate cancer, ERK increases the expression of MKP-1/DUSP1, thereby reducing JNK activity and inhibiting apoptosis. Indeed, the amount of cell death was found to be significantly increased in PIN lesions expressing low levels of MKP-1/DUSP1, demonstrating its protective role against cancer development. From patients treated with androgen ablation, MKP-1/DUSP1 and the anti-apoptotic protein Bcl-2, were found to be expressed at low levels while JNK was upregulated. Specifically, the expression levels of Bcl-2 and MKP-1/DUSP1 were only preserved in subpopulations of cells that did not undergo apoptosis (Magi-Galluzzi et al., 1997).

A number of cellular studies have linked these clinical observations to effects upon MAP kinase signalling and apoptosis. Expression of MKP-1/DUSP1 mRNA is upregulated in response to androgen treatment of LNCaP cells (Lorenzo and Saatcioglu, 2008). Srikanth et al, (1999) demonstrated that in DU145 cells, an androgen-independent prostate cancer cell line, ectopic expression of MKP-1/DUSP1 decreased FasL-induced activation of caspase-3 and caspase-1 and therefore prevented the cells from undergoing apoptosis. A similar study also indicated that activation of JNK by means of ectopic expression of ASK1, or constitutively active MEKK1, was inhibited in response to the expression of MKP-1/DUSP1 (Srikanth et al., 1999).

### 1.10.2 Other MKPs in prostate cancer

Whilst the evidence supporting the involvement of the MKPs in prostate cancer is small at this point, it is increasing. Table 1.3 gives an overview of the empirical response of DUSPs to different treatments in various prostate cancer cell lines. Much of the work has been restricted to cell lines and clinical correlates with little suitable and specific deletions models developed.

Most notably, the potential for MKP-5/DUSP10 to play a role in prostate cancer has been shown. MKP-5/DUSP10 overexpression reduced invasion of cultured prostate cancer cells and increased

dephosphorylation of p38 MAP kinase (Chen et al., 2004). Moreover, the expression of MKP-5/DUSP10 was increased in primary cultures of prostatic epithelial cells by 1,25-dihydroxyvitamin-D3 (1,25D) (Rauhala et al., 2005), which is known to inhibit prostate cancer cell growth and to have anti-inflammatory effects on different prostate cancer cell lines and xenografts (Krishnan et al., 2007). Furthermore, upregulation of MKP-5/DUSP10 by 1,25D treatment correlated with reduced interleukin 6 (IL-6) production and decreased p38 MAP kinase phosphorylation in prostatic epithelial cells derived from normal prostate tissues and localised adenocarcinomas (Nonn et al., 2006) (Table 1.3). Nevertheless, these limited studies highlight the need for much better understanding of DUSPs within the prostate cancer environment.

### 1.10.3 Role of MKP-2 in prostate Cancer

To date, no direct evidence either *in vivo* or *in vitro* has provided a causal link between MKP-2 and prostate cancer, but some indirect evidence has been accumulating. MKP-2 has been mapped to the 8p11-p12 gene locus, which is lost in several prostatic neoplasms (Emmert-Buck et al., 1995, Smith et al., 1997), and allelic loss in the short arm of chromosome 8 a frequent event in prostate cancer (Abate-Shen and Shen, 2000). Recently a novel variant of human MKP-2, MKP-2-S lacking the MAP kinase-binding site has been identified in a number of prostate cell lines (Cadalbert et al., 2010). However, it is unclear if this is relevant to prostate cancer development and a number of further studies need to be conducted.

**Table 1.3: Overview of DUSP expression in response to various treatment and their out come in different prostate cancer cell lines.**

| MKPs | Substrate specify | Cell type | Treatment | Effect | Refrences |
|---|---|---|---|---|---|
| **MKP-1/DUSP1** | p38-JNK-ERK | LNCaP<br>DU145 | R1881<br>Ectopic expression of MKP-1 | Upregulation No FasL-induced activation of caspase-1 and caspase 3 and no apoptosis<br>Upregulation | (Amoldussen et al; 2008, Vaarala et al; 2012, Srikanth et al; 1999, Rauhala et al; 2005, Magi-galluzzi et al; 1997, Loda et al; 1996, Lorenzo and Saactioglu et al; 2008, Leav et al; 1996) |
| **MKP-2/DUSP2** | ERK-JNK>p38 | LNCaP | R1881 | Upregulation | (Amoldussen et al; 2008, Emmert-Buck et al; 1995, Smith et al; 1997, Abateshen and shen et al; 200, Cadabert et al; 2010) |
| **MKP-3/DUSP6** | ERK>JNK-p38 | | | | |
| **MKP-4/DUSP9** | ERK>p38>JNK | | | | (Amoldussen et al; 2008) |
| **MKP-5/DUSP10** | P38-JNK>ERK | LNCaP/PC3/DU145<br>Primary prostate cells | 1,25D<br>Curcumin<br>Resveratrol | No change<br>Increase expression<br>Increase expression and inhibition of proinflamatory signalling | (Amoldussen et al; 2008, Rauhala et al; 2005, Krishnan et al; 2007, Nonn et al; 2006) |
| **MKPX/DUSP7** | ERK>JNK-p38 | LNCaP | R1881 | Upregulation | (Amoldussen et al; 2008) |
| **VH3/DUSP5** | ERK | | | | |
| **DUSP8** | P38-JNK>ERK | | | | |
| **MKP-6/DUSP14** | ERK-JNK>ERK | LNCaP | R1881 | Upregulation | (Amoldussen et al; 2008) |
| **MKP22/DSP2** | P38-JNK>ERK | | | | |
| **Atypical DUSPs VHR/ DUSP3** | ERK-JNK-p38 | LNCaP | R1881<br>Ectopic expression of VHR and treatment with TPA or TG | Upregulation<br>Decreased TPA or TG induced apoptosis | (Amoldussen et al; 2008) |

R1881 : is a methyltrienolone a synthetic androgen agonist.

## 1.11 AIM OF THE PROJECT

The MAP kinase phosphatases are a family of DUSPs which have the potential to play a significant role in the regulation of prostate cancer development. One of these, MKP-2, has not been studied to any great extent to date. Therefore, the aim of the work presented in this thesis was to understand better the role MKP-2 may play in prostate cancer cell function in the context of cancer development.

The first part of the study utilised over-expression of MKP-2 as an experimental tool to study the underlying effect of extrinsic and intrinsic MKP-2 on cell proliferation and apoptosis.

In the second part, clinical tissue from a cohort of prostate cancer patients was used to examine the relationship between MKP-2 expression and disease progression.

# 2. MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 General Reagents

All materials and reagents used were of commercial grade possible and were obtained from Sigma Aldrich Chemical Company Ltd. (Poole, Dorset, UK) unless otherwise stated.

**GE Healthcare Ltd (Buckinghamshire, UK), Amersham™ Hybond™**

ECL nitrocellulose membrane

**Roche diagnostics GmbH.**

Dithiothreitol (DTT)

**Bio-Rad Laboratories (Hertfordshire, UK).**

pre-stained SDS-Page molecular weight markers.

**Santa Cruz Biotechnology Inc. (CA, USA).**

Recombinant human TNF-α

**Cell Signalling Technology, Inc. (New England Biolabs, UK)**

Recombinant human EGF

**Boehringer Mannheim (East Sussex, UK).**

Bovine serum albumin (BSA, Fraction V)

**Corning Costar (Buckinghamshire, UK)**.

Nitrocellulose membranes

**Corning B.V (Buckinghamshire, UK).**

All tissue culture flasks, plates, dishes and graduated pipettes

**GIBCO BRL (Paisley. U.K).**

Foetal calf serum (FCS), L-glutamine, Geneticin (G418), Medium 199 with Earls salts (M199), Minimal Essential Medium (10 x), Non-essential amino acids, Opti-MEM® I Reduced Serum media, Penicillin/Streptomycin, Sodium Bicarbonate.

### 2.1.2 Reagents for Apoptosis.

Cisplatin (CALBIOCHEM)®

Doxorubicin hydrochloride (SIGMA), D1515-10MG

Hydrogen Peroxide (H2O2) (Sigma-Aldrich, Poole, UK)

X-ray (XRAD-225C) (PRECISION X-RAY INC®)

### 2.1.3 Adenoviruses.

Adv.MKP-2
Adv.MKP-2-NLS1
Adv.MKP-2-CI

### 2.1.4 Microscopy

**Merck-Calbiochem (Nottingham, UK)**

Mowiol

MG-132 (Carbobenzoxy-L-leucyl-L-leucyl-L-leucinal).

**Corning B.V. (Netherlands)**

Tissue culture plastic ware

**FlukaChemie GmbH (Germany)**

p-coumaric acid

**GE Healthcare (Kent, UK)**

Whatman™ 17 CHR Chromatography Paper

Amersham™ Hybond-ECL Nitrocellulose Membrane

**Gibco®-Invitrogen Life Technologies Ltd (Renfrewshire, UK)**

Versin (0.2% EDTA/PBS)

**Molecular Devices Corp. (Downingtown, PA, USA)**

MetaMorph Imaging Series software (version 7.0 or 7.6.4)

**Nikon Instruments (New York, USA)**

Nikon TE-300 Epifluorescence microscope

Nikon Eclipse TE2000-E inverted Epifluorescence microscope

40 x or 100 x oil-immersion Plan Fluor Objective Lens, NA=1.3

**VWR International Ltd (Leicestershire, UK)**

No. 0, (0.09-0.13 mm thick), circular glass 13 or 22 mm diameter coverslips.
0.8-1.0mm thick glass microscopy slides

**StratechSientific Ltd (Newmarket Suffolk, UK)**

DAPI (4,6-Diamidino-2-phenylindole, dihydrochloride) (17514-ABD)

### 2.1.5 Antibodies

**Santa Cruz Biotechnology Inc. (CA, USA).**

Mouse monoclonal anti-p-ERK (E-4)

Mouse monoclonal anti-T. ERK

Rabbit polyclonal T-p65

Rabbit polyclonal anti-MKP-2 (S-18)

Rabbit polyclonal anti-p38 (N-20)

Rabbit polyclonal T-p38

Rabbit monoclonal anti Cyclin D1

Mouse monoclonal anti Cyclin B1

**Invitrogen (Paisley, UK).**

Rabbit polyclonal anti-p-p38 (44684-G)

**Cell Signalling Technology, Inc. (New England Biolabs, UK)**

Rabbit polyclonal anti-p-JNK1 & 2 (44- 682G)

Mouse polyclonal anti-p-JNK1

Rabbit polyclonal anti-p-JNK 2

Rabbit polyclonal anti-p-ERK1 & 2

Rabbit polyclonal Phospho-cdc-2

**Secondary antibodies from Amersham®**
HRP-conjugated goat anti-rabbit IgG
HRP-conjugated anti-mouse IgG

## 2.2 CELL CULTURE

All cell culture work was performed in a class II cell culture hood under aseptic conditions. All the cells were grown in 75cm$^2$ flasks, unless otherwise stated.

### 2.2.1 PC3

Cryopreserved human prostate carcinoma epithelial cells, derived from a bone metastatic (PC3 cells) were purchased from American Type Culture Collection (ATCC). PC3 cells were cultured in RPMI 1640 containing 10% FCS, penicillin (25 units/ml), and streptomycin (25 μg/ml). These cells were maintained at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air. Medium was changed every 2-3 days thereafter until cells became confluent.

### 2.2.2 LNCaP Androgen Sensitive (AS) and Androgen Insensitive (AI)

Cryopreserved human prostate carcinoma, epithelial cells, derived from metastatic site, left supraclavicular lymph node (LNCaP)(AS) and subcutaneous co-injection of 2 non-tumorigenic human cell lines-LNCaP (AS), a prostate cancer cell line, and MS, a bone stromal cell line-into intact adult male mice resulted in formation of carcinomas that secreted prostate-specific antigen (PSA). In castrated hosts, upon cellular interaction with bone fibroblasts, observed the progression of these tumors from an androgen-dependent (AD) to an androgen-independent state (AI). Both were purchased from American Type Culture Collection (ATCC). LNCaP was cultured in RPMI 1640 containing 10% FCS, penicillin (25 units/ml), and streptomycin (25 μg/ml). These cells were maintained at 37°C in a humidified atmosphere of 5% CO2 and 95% air. Medium was changed every 2-3 days thereafter until cells became confluent.

### 2.2.3 Human Embryonic Kidney (HEK293) cells

Low passage human embryonic kidney (HEK) 293 cells were maintained in Minimal Essential Media (MEM) with Eagle's salts (GIBCO®, Invitrogen Ltd) supplemented with 10% (v/v) foetal calf serum (FCS), penicillin (250 units/ml), streptomycin (100 μg/ml), L-glutamine (27 mg/ml), 1x (v/v) non-essential amino acids and 0.375% (v/v) and sodium bicarbonate (GIBCO®, Invitrogen

Ltd). Cells were than incubated at 37°C in a humidified atmosphere with 5% $CO_2$ with media replaced every second day. At 90% confluence, the HEK 293 cells were passaged using 1x sodium sodium citrate (1x SSC) solution (8.78g NaCl and 4.41g sodium acetate (concentration molar)) dissolved in 100 ml of distilled water, and then pH was adjusted to 7.4. Cells were used for experimentation between passages 30 and 40.

### 2.2.4 Subculturing PC3 and LNCaP by trypsinisation

Cells were subcultured upon reaching approximately 90% confluency. The medium was removed and the cells washed twice with sterile 0.5% (w/v) trypsin, in 0.2% (w/v) EDTA, in phosphate buffered saline (PBS). The trypsin was then removed and the flask placed in an incubator at 37°C, 5% CO2 and 95% air, for 2-5 mins until the cells began to detach and change shape. The flask was then gently tapped to detach the cells completely from the surface and the cells were then washed in EBM-2 media and then diluted three times with additional EBM-2.

## 2.3 POLYMERASE CHAIN REACTION (PCR)

### 2.3.1 Total RNA extraction

RNA extraction was carried out using the GenElute Mammalian Total RNA isolation Kit (Sigma-Aldrich, UK). For cell lines grown in T-25 cell culture flasks, the growth medium was aspirated and the cell monolayer (no greater than 70% confluent) was washed with sterile PBS, pre-warmed to 37°C. The RNA extraction was carried out as per manufacturer's protocol in the kit manual. 500µl of the lysis solution was pipetted onto each flask and the cells lysed *in situ* by repeated rocking of the flask. Complete cell disruption and genomic DNA fragmentation was carried out by passing the lysate through the filtration columns provided with kit. The isolation procedure included the optional on-column DNase I digestion protocol to reduce the risk of genomic DNA carryover. Total RNA was eluted from the spin column into an RNase-free microcentrifuge tube by pipetting 50µl of the elution buffer directly onto the membrane and centrifuging for 1 min at full speed.

Eluted total RNA was quantified by Nanodrop 2000c spectrophotometer (Thermo Scientific, UK) and stored at -80°C. Integrity of the extracted RNA was assessed by the Experion Automated Electrophoresis System (Bio-Rad, UK) using the Std-Sens Total RNA Analysis kit. The RNA Quality Indicator (RQI) of the samples assayed had a value of >9.9, which indicated that they were of high quality.

### 2.3.2 First-strand cDNA synthesis.

A reverse transcriptase reaction was carried out with RNA samples to synthesise single–stranded cDNA templates for use in polymerase chain reactions (PCR). The first step in cDNA synthesis was carried out using 5µg of total RNA and Superscript III reverse transcriptase (Invitrogen, UK). The reaction was primed using 500ng of oligo (dT)18 primer. This reaction mixture was heated to 65°C for 5 min and then chilled on ice for 5 min. The total volume of the reverse transcriptase reaction was 20µl and included (final concentrations): 1x First Strand Buffer, 0.05mM DTT, and 0.5mM dNTP mix (0.5mM of dATP, dGTP, dCTP, and dTTP) and 200U of Superscript III reverse transcriptase. The reaction mixture was incubated at 42°C for 60 min and then terminated by heating at 70°C for 15 min. These samples were marked "RT+". A control to check for genomic DNA carryover in the RNA sample was set up in parallel. This reaction contained all of the aforementioned first strand synthesis components and total RNA but replaced the Superscript III reverse transcriptase with $H_2O$. This was designated as the "RT-" sample.

### 2.3.3 PCR primers

The oligonucleotide primers were synthesised by SIGMA®. Upon arrival they were resuspended to the appropriate stock concentration with ultra-pure water and stored at -20°C. For PCR applications, the primers were diluted to 10pmol/µl as a working concentration.

### 2.3.4 Polymerase chain reaction of MKP-2.

The long primers were designed to amplify a 264 bp and the short primers were designed to amplify a 202 bp fragment of MKP-2, from both LNCaP AS and AI cDNA. The long sense primer of MKP-2 (MKP-2-L), sequence was: 5'-CCGGGTTCTCTTCTCTTCC-3' and the antisense primer, MKP-2-L, had the sequence, 5'-GTGTTACAGCGCACGTTGAC-3'.The short sense primer of MKP-2 (MKP-2-S), sequence was: 5'-GACCGTCACAACTTCCTTGG-3' and the antisense primer, MKP-2-S, had the sequence, 5'-CTTCGTTAGCCAGGAACTGC-3'. GoTaq® Hot Start Polymerase Master Mix (Promega, UK) was used for the PCR. The conditions for a 50µl reaction were as follows: 1x GoTaq® Hot Start Polymerase Master Mix, 1µM sense primer, 1µM antisense primer,

and 1µl of cDNA. The reactions were carried out in 200ul thin-walled PCR tubes on a Primus 96 Plus thermal cycler (MWG Eurofins, Germany) under the following cycling conditions: Initial denaturation and activation of the hot-start polymerase of 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 54°C for 30 secs, 72°C for 1 min with a final extension period for 7 min at 72°C. Typically, three reaction tubes were set up per replicate study. The first tube would be a $H_2O$ control where the cDNA was replaced with $H_2O$. This would act as a false positive control as any amplicons generated from this would be derived from aberrant contamination of the reaction components or introduced during the setup. The second tube would be the "RT'' control to check for genomic DNA carryover in the RNA isolation. The final tube of each set would be the "RT+". Assuming the targeted gene of interest is expressed by the cells, only the "RT+" PCR tube should generate a PCR product of the expected size.

### 2.3.5 DNA agarose gel electrophoresis

The PCR products were electrophoresed for sizing and qualitative analysis using a horizontal submarine mini-gel apparatus (Bioscience Services, UK) and electrophoresis power supply (Kodak UK). 15 µl of the PCR reaction was added to 2 ul of gel loading buffer (Bioline, UK) and electrophoresed for 1 hour at 50-60 volts in a 1x TAE (89mM Tris-base, 2mM Na2-EDTA, 89mM Boric Acid, pH8.3) buffered 2% w/v agarose gel (Bioline, UK) with 1x TAE running buffer. 2ul of a 10mg/ml solution of ethidium bromide (Sigma, UK) was added to the agarose before pouring to allow visualisation of the electrophoresed DNA fragments on a 312 nm UV transilluminator (Syngene, UK). The size marker used was appropriate to the size of the amplicon and in this instance was Hyperladder II (Bioline, UK).

## 2.4 WESTERN-BLOT ANALYSIS

### 2.4.1 Preparation of Whole Cell Extracts

Cells were immediately washed twice with ice cold PBS before 200µl of pre-heated laemmli's sample buffer (63 mM Tris-HCl (pH6.8), 2 mM $Na4P_2O_7$, 5 mM EDTA, 10% (v/v) glycerol, 2% (w/v) SDS, 50 mM DTT, 0.007% (w/v) bromophenol blue) was added. The cells were then scraped and pushed through a 21-gauge needle to lyse the cells. The cells were then transferred to

Eppendorf tubes and boiled for 4 mins, to allow the proteins in the samples to be denature, before storing at -20 °C until use.

### 2.4.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gel kit apparatus was first cleaned in 70% ethanol before assembly, then distilled water was added to check the glass plates were flush and not leaking. Resolving gels were prepared containing an appropriate amount (7.5% (w/v), 9% (w/v), 10% (w/v), 11% (w/v) of acrylamide: (N, N'-methylenebis-acrylamide (30:0.8), 0.375 M Tris (pH 8.8), 0.1% (w/v) SDS and 10% (w/v) ammonium persulfate (APS)) and TEMED. The solution was poured between two glass plates assembled in a vertical slab configuration according to the manufacturer's (Bio-Rad) instruction and overlaid with 200 µl 0.1% (w/v) SDS. Following gel polymerisation, the layer of 0.1% SDS (w/v) was removed and a stacking gel containing (10% (v/v) acrylamide: N-methylenebis-acrylamide (30:0.8) in 125 mM Tris (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED) was poured directly on top of the resolving gel, and a teflon comb was immediately inserted into the stacking gel solution. After polymerisation was complete, the comb was removed and the polyacrylamide gel was assembled in a Bio-Rad Mini-PROTEAN IITM electrophoresis tank, with both reservoirs filled with electrophoresis buffer (25 mM Tris, 129 mM glycine, 0.1% (w/v) SDS). Aliquots of samples (20-30 µg/ml) were then loaded into the wells using a microsyringe. A prestained SDS-PAGE molecular weight marker of known molecular weights was run concurrently in order to identify the polypeptide of interest. Samples were electrophoresed at a constant voltage of 120 V, and then there was a wait until the bromophenol dye had reached the bottom of the gel.

### 2.4.3 Electrophoretic Transfer of Proteins to Nitrocellulose Membrane

The proteins separated by SDS-PAGE were transferred to nitrocellulose membrane by electrophoretic blotting following a standard protocol (Towbin et al., 1979). The gel was pressed firmly against a nitrocellulose membrane and assembled in a transfer cassette sandwiched between two pieces of Whatman 3 MM paper and two sponge pads. The cassette was immersed in transfer buffer (25 M Tris, 19 mM glycine, 20% (v/v) methanol) in a Bio-Rad Mini Trans-Blot TM tank and a constant current of 300 mA was applied for 2 h, whilst the tank was cooled by inclusion of an ice

reservoir. The presence of SDS in the resolving gel conferred a negative charge on the proteins and so the cassette was oriented with the nitrocellulose towards the anode.

### 2.4.4 Immunological detection of Protein

Following transfer of the proteins from the gel to the nitrocellulose membrane, the membrane was removed and blocked by incubation in 2% (w/v) BSA in NaTT buffer (150 mM NaCl, 20 mM Tris (pH 7.4), 0.2% (v/v) Tween-20) for 2 hrs on a platform shaker. The blocking buffer was then removed and membrane was incubated overnight with antibody specific to the target protein appropriately diluted in NaTT buffer containing 0.2% (w/v) BSA. On the following day, the membrane was washed in NaTT buffer every 15 mins for 90 mins with gentle agitation. The membrane was than incubated for a further 2 hrs at room temperature with secondary horseradish peroxidase-conjugated IgG directed against the first immunoglobulin diluted to 1:10000 in NaTT buffer containing 0.2% (w/v) BSA. After six washes in NaTT, as described previously, immunoreactive protein bands were detected by incubation with enhanced chemiluminescene (ECL) reagent for 2 min with agitation. The membranes were blotted onto a paper towel to remove any excess liquid. The blots were then mounted onto an exposure cassette and covered with cling film, then exposed to X-ray film (Kodak Ls X-OMAT) for the required time under darkroom conditions and developed using X-OMAT machine (KODAK M35-M X-OMAT processor).

### 2.4.5 Nitrocellulose membrane stripping and reprobing

Nitrocellulose membranes processed by Western blotting were reprobed for the subsequent detection of other cellulose bound proteins. This involved stripping the membrane of any previous antibody using stripping buffer (0.05 M Tis-HCl, 2% SDS, and 0.1 M of β-meracptoethanol). The membrane was incubated in 15 ml of stripping buffer for 60 min at 70$^{o}$C in an incubator/shaker (Stuart Science Equipment). The stripping buffer was discarded in a fume hood sink and the membrane washed three times with NATT buffer at 15-min intervals to remove residual stripping buffer. After the final NATT wash, membranes were then incubated overnight with primary antibody prepared in 0.2% BSA (w/v) in NATT buffer, then the same steps described in the

Immunological Detection of Protein section were followed. At this stage, the blots were ready for the immunological detection protocol.

## 2.5 INDIRECT IMMUNOFLUORESCENT MICROSCOPY

Cells were grown to approximately 70% confluence in 24-well plates on circular glass cover slips No.0. (0.9-0.13 mm thick) (13 mm diameter). Medium was replaced with serum-free media, and the next day cells were stimulated with an appropriate treatment. Then cover slips were washed with ice-cold PBS twice, prior to fixation with 500µl ice-cold methanol for 10 min. Cover slips were washed twice with ice cold PBS, then were incubated with 500µl 0.25% triton x100 in BPS for 10 min at room temperature. After permealisation of cells, three times 5-min wash with ice-cold PBS was applied, following which, the nonspecific binding was blocked by adding 500µl of blocking buffer (1% BSA (w/v) diluted in 0.25% triton/PBS) for 30 min at room temperature. Cover slips were then incubated with primary antibody, about 25µl/coverslip of rabbit polyclonal MKP-2 (1:50 in blocking buffer) kept in a humidified chamber for overnight at 4ºC. Cover slips were then washed three times each for 5 min with ice cold PBS, followed by the addition of 25µl/coverslip secondary antibody Fluorescein (FITC) conjugated Affini Pure donkey anti-Rabbit (1:100) incubated for 1 h in the dark. This was followed by three times 5-min fixed washes with ice cold PBS, then room temperature 500µl/well of 100ng/ml DAPI (4',6-diamidino-2-phenylindole) nuclear staining (1:2000 in PBS) was added in dark to cover slips for 5 min. Next they were twice washed with ice-cold PBS, and cover slips were mounted onto glass microscope slides embedded in 15µL Mowioland left to dry in cold room or at 4°C conditions overnight in the dark for visualisation by Nikon TE300-Ep-1 upright epifluorescence microscope (Nikon, Kingston upon Thames, UK). Cells were imaged at x100 or x40 magnification with an oil-immersion Plan Fluor objective lens. Images were collected using a cool digital Cool Snap-HQ CCD camera (Roper Scientific, Photometrics, and Tucson, AZ). MetaMorph Imaging Series 7.0 (Molecular Devices Corp., Downington, PA, USA) was used for control of image acquisition, processing and modification of all image data. The background average statistical correction editing function in MetaMorph was used to produce background corrected images. This was achieved by determining the average background level of fluorescence from regions of interest drawn adjacent to cells expressing fluorescence.

## 2.6 PREPARATION OF RECOMBINANT ADENOVIRUSES

### 2.6.1 Crude Adenoviral Lysates

Crude lysates of wild-type mitogen-activated protein kinase phosphatase-2 (Adv. MKP-2), and β-galactosidase (Lac*Z*) viruses were generated in HEK 293 cells by infecting a 75 $cm^2$ flask with 0.75 µl of original stock virus. Flasks were incubated at 37ºC, 5% $CO_2$, 95% air for 5-7 days until the cytopathic effect had occurred and the cells had started to detach from the flask. Cells were then removed from the flask and subjected to centrifugation (1500 rpm for 5 min). The supernatant was removed and pellet was washed twice with PBS and centrifuged at 1500 rpm for 5 mins. The collected pellet was then resuspended in HE buffer (10 mM HEPES pH 7.5, 1mM EDTA), frozen in liquid nitrogen and thawed in a 37ºC water bath. This was repeated a further two times. After the third cycle, cells were centrifuged at 1500 rpm for 5 min to pellet the debris and the supernatant, which constituted the crude adenoviral lysates, was collected in sterile tube and stored at -80ºC until required.

### 2.6.2 Generation and Purification of High-Titre Stocks of Adenovirus.

The high-titre stocks of adenovirus were generated by large scale amplification of the crude adenoviral lysates described in the section above (Crude Adenoviral Lysates). Twenty T175 flasks of HEK 293 cells were grown to 60-70% confluency. The media was then changed to 2% FCS media (19 ml) before adding 1ml of diluted virus media (100 µl crude adenoviral lysates added to 20 ml of HEK 293 medium). These flasks were incubated at 37ºC, 5% $CO_2$ until the cytopathic effect had observed and cells had detached from the flask. The cells were then removed together with medium from the flasks and pelleted by centrifugation in a 50 ml centrifuge tube at 1500 rpm for 5 min. The supernatant was collected in a sterile centrifuge tube (around 100 ml of total supernatants) and stored at 4ºC until use. Cells were resuspended in 20 ml of supernatant, frozen in liquid nitrogen and thawed in a 37ºC water bath' this was repeated a further two times. Subsequently, cells were resuspended by vortexing after each thaw. Following the third cycle, cells were pelleted by centrifugation at 1500 rpm for 5 min. Supernatant was collected in a sterile tube while the pellet was discarded. A BD Adeno-X virus purification kit from Clontech Laboratories was used for the adenovirus purification. The supernatant was subjected to filtering and then

incubation with benzonase (25 units/µl) (50 mM Tris-HCl pH 8.0, 20 mM NaCl, 2 mM $MgCl_2$, 50% glycerol) at 37ºC for exactly 30 min. Meanwhile, 1x dilution buffer and 1x wash buffer were prepared by diluting the provided 5x buffers with sterile $H_2O$, according to the manufacturer's instructions. The benzonase-treated filtrate was mixed with an equal volume of 1x dilution buffer and passed through BD Adeno-X syringe-Filter followed by washing with 1x washing buffer. Adenovirus was later eluted from the mega filter using the elution buffer and the elutate collected in sterile Eppendorf tubes and aliquots were then stored at -80ºC until titration.

### 2.6.3 Titration of Adenovirus by End-Point Dilution

Titration of the end point dilution method was used to quantify virus production (Nicklin and Baker, 1999). Serial dilutions of the adenovirus were infected in a 96- well plate of HEK 293 cells that reached approximately 70-80% confluency. The plate was incubated at 37ºC, 5% $CO_2$ overnight, after which the medium containing the adenovirus was replaced with fresh medium. The plate was then incubated for 5-7 days at 37º C, 5% $CO_2$ until the cytopathic effect was observed. Wells containing plaques were counted and the titre of adenovirus stock in terms of plaque-forming units (pfu) was calculated as in the example shown below in Figure 2.2.

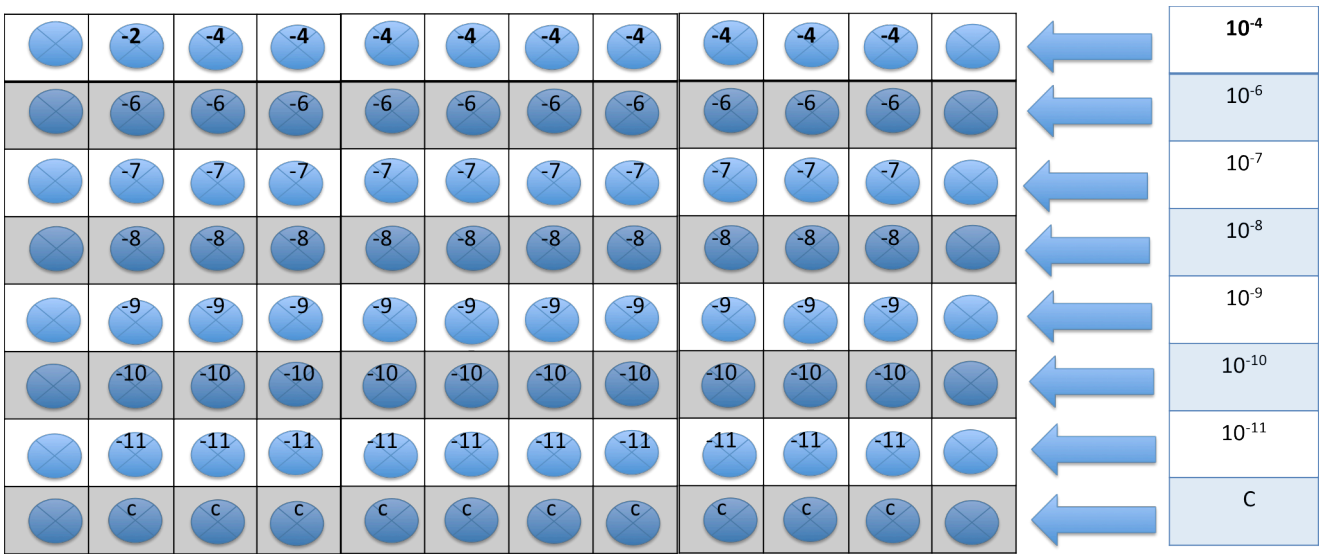


**Figure 2.1 : Layout of 96-well plate used for titration of adenovirus.**
The numbers represent adenoviral dilutions. Cells were sub-cultured into all wells and adenovirus was added in appropriate dilutions to the middle 10 wells in each row at the appropriate dilutions.

**Number of wells containing plaques:**

$10^{-4}$= 10/10= 100%

$10^{-6}$= 10/10 = 100%

$10^{-7}$= 10/10 = 100%

$10^{-8}$= 7/10 = 70%

$10^{-9}$= 5/10 = 50%

$10^{-10}$= 0

$10^{-11}$= 0

**The proportionate distance =**

$$\frac{\%\text{ wells positive above 50\%-50}}{\%\text{ wells positive above 50\% - \%wells positive below 50\%}}$$

$$= \frac{70-50}{50-20} = 0.67$$

**Log $ID_{50}$ = dilution factor at % wells positive above 50% + (proportionate x -1)**

= -8 + (0.67x -1)

= -8.67

$ID_{50}$ = $10^{-8.67}$

**TCID (Tissue culture infectivity dose) $_{50}$/100 µl =** $\frac{1}{10^{-8.67}} = 10^{8.67}$

**$TCID_{50}$/1 ml** = $10^{8.67}$ x dilution factor

= $10^{8.67}$ x10

= $10^{9.67}$

= 4.68 x$10^{9}$

**1 TCID50 = 0.7 plaque forming units (9 pfu), therefore final titre of adenovirus is**

4.68 x $10^9$ x 0.7= 2.21 x $10^9$ pfu/ml

= 3.27 x $10^6$ pfu/ µl

**Figure 2.2: Calculations for titration of adenovirus.**

### 2.6.4 Infecting PC3, LNCaP (AS) and (AI) with Adenovirus

To establish the appropriate volume of adenovirus to add to cells in order to give an appropriate multiplicity of infection (MOI), cells were grown to approximately 50-60% confluency in either 6- or 12-well plates. The cell number was determined using a haemocytometer. Before infection, the medium was changed to serum-free (0%) then an appropriate MOI of adenovirus (50-600 pfu/cell) was added to the cells and incubated for 24 h. Then cells were serum-starved with 0% FCS media for a further 48 h before stimulation. Later, cells were exposed to vehicle control, TNF-α, $H_2O_2$, UV-C, EGF or serum deprivation for the indicated times.

## 2.7 PROLIFERATION ASSAY

Confluent PC3, LNCAP (AS) and (AI) were trypsenised using trypsin-EDTA, seeded on coverslips in 24-well plates (5,000 cells/well) in 10% FCS-(F12 + Waymouth's medium) and allowed to attach for 24 h. Cell were starved with 0.1% serum containing media for 24 h and then stimulated further for 24, 48, 72 h with 10% FCS. Cultures were washed with PBS, and stained with Hematoxylin. The number of cells was determined by counting the number of cells in 10 random fields per coverslip.

### 2.7.1 Proliferation by Cell Counting using Hematoxylin Staining

Confluent LNCaP (AS) and (AI) cells were detached with trypsin-EDTA, seeded on coverslips into 24-well plates (5,000 cells/well) in 20% FCS-DMEM, and allowed to attach for 24 h. Cells were starved with serum-free media for 24 h and then stimulated for either 24, 48, 72 h with 10% FCS. Cultures were washed twice with cold PBS, fixed in 70% methanol for 30 min at room temperature and then washed (2x5 min) with cold PBS, and stained with 1 ml Hematoxylin for 20 min at room temperature. Excess Hematoxylin was removed by rinsing with distilled water until the coverslip became transparent and a drop of mounting medium (Mowial) was added on to the each coverslip. The number of cells was determined by counting the number of cells in 10 random fields per coverslip using MF-830 Microscope connected to Motic Images Plus 2.0 software.

## 2.8 CELL CYCLE ANALYSIS

Cell cycle profiles were analysed by staining intracellular DNA with propidium iodide. LNCaP (AS) and LNCaP (AI) were grown to confluency in 6-well plate, serum-starved for 48 h, and then released into growth media for another 24 h for LNCaP (AI) and for 48 h for LNCaP (AS). LNCaP cells were trypsinised and washed with PBS and prepared at $1x10^6$ in Eppendorf tubes. Cells were fixed in ice-cold 70% ethanol (dropwise while vortexing to ensure proper fixation of cells and prevent clumping) at 4° C overnight. Cells were washed with PBS and centrifuged at 2000 rpm for 10 min, then RNase A (50ug/ml) was added and they were incubated at 37° C for 1 h to ensure only DNA staining. Samples were stained with propidium iodide (PI) at 50 ug/ml. The cell cycle parameters from 10,000-gated events were read in the FACS scan flow cytometer using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). The data was analysed using FACS Diva (Becton Dickinson, Oxford, UK).

## 2.9 APOPTOSIS ASSAY BY FLOW CYTOMETRY

LNCaP (AS) and (AI) were grown on 6-well plates and were infected for 40 h then stimulated with X-ray, Doxorubicin or UV-C for a further 24 h prior to analysis. Supernatant was collected and cells were trypsinised and then pelleted at 1000 rpm for 5 min. The pellet was then resuspended in 500 **µ**l of 1x annexin binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl and 2.5 mM CaCl2). Phycoerthyrin-Annexin V and 7-AAD were added to the cells according to the manufacturer's instructions and the samples were analysed in the FACS scan flow cytometer using FACS Diva software (FACS scan, Becton Dickinson, Oxford, UK). The data was analysed using FACS Diva (Becton Dickinson, Oxford, UK) and RCS Express (De Novo Software, Canada) software. A total of 10,000 events were measured per sample. Gating was determined using PE-Annexin V FL-2 and 7–AAD FL-3 standards attached to beads (Becton Dickinson, Oxford, UK) and preliminary experiments conducted using paraformaldehyde and serum deprivation to define apoptotic and necrotic populations as outlined by the manufacturer's instructions.

## 2.10 hMKP2-FLAG in pcDNA3.1(+)

### 2.10.1 NLS1-hMKP2-FLAG in pcDNA3.1(+)

The wild-type human MKP2 (dual specificity phosphatase 4 (DUSP4), transcript variant 1. NM_001394.6) had under went site-directed mutagenesis to change the NLS1 motif from $^{73}$VRRRAKG$^{81}$ to $^{73}$VAAAAKG$^{79}$. This NLS1-hMKP2 has a C-terminal FLAG epitope and is inserted in the mammalian expression vector pcDNA3.1(+) (Invitrogen, Paisley, UK). Ampicillin is the selection antibiotic with bacterial culture while Neomycin should be used with mammalian cell lines

### 2.10.2 CI-hMKP2-FLAG in pcDNA3.1(+)

The wild-type human MKP2 (dual specificity phosphatase 4 (DUSP4), transcript variant 1. NM_001394.6) underwent site-directed mutagenesis at Cysteine$^{280}$ to give a Serine$^{280}$ and create a constitutively inactive hMKP2. This CI-hMKP2 had a C-terminal FLAG epitope and was inserted in the mammalian expression vector pcDNA3.1 (+) (Invitrogen, Paisley, UK). Antibiotic selection: Ampicillin (bacteria), Neomycin (cell lines).

### 2.10.3 WT-MKP2-FLAG in pEGFP-N1

The insert was wild-type human MKP2 (dual specificity phosphatase 4 (DUSP4), transcript variant 1. NM_001394.6) with a C-terminal FLAG epitope. It was inserted, in frame, into the GFP-mammalian expression vector, pEGFP-N1 (Clontech, CA, USA) to give the wt-MKP2-FLAG a C-terminal GFP fusion. Antibiotic selection: Kanamycin (bacteria), Neomycin (cell lines).

## 2.11 IMMUNOHISTOCHEMISTRY (IHC)

Immunohistochemistry is a method that allows for the detection of a cellular protein or other antigen within cells and tissues using an antibody specific for the desired antigen. The simplest immunohistochemical methods attach the marker directly to the primary antibody. In general, this direct immunohistochemical method does not have very high sensitivity. An alternative, more sensitive method is indirect immunohistochemistry. This involves using a second or “secondary”

antibody, labelled with either a visible marker (fluorochrome) or an enzyme that binds to the primary antibody bound to the antigen. This indirect approach generates an amplified signal. The method for this study was the Envision system (DAKO) that involves dextran polymer technology. Dako Envision detection reagent consists of a dextran backbone to which a large number of peroxidase (HRP) molecules and secondary antibody molecules have been coupled. There are several types of cells in the prostate; immunohistochemistry using 152 commercially available antibodies examining cluster designation (CD) antigens have been used to characterise the luminal secretory epithelium, basal epithelial cells, the fibromuscular stroma, nerve sheath and other cell types (Liu and True, 2002; Tokar et al., 2005). The secondary antibody coupled to the dextran backbone has been raised in goats. It reacts equally well with rabbit and mouse immunoglobulins. Following incubation with the Envision, the tissue is incubated with a substrate solution that consists of diaminobenzidine (DAB) chromagen and hydrogen peroxide. The HRP molecules on the Envision interact with the substrate solution to produce a crisp brown end product at the site of the target antigen/protein, which can be viewed using a light microscope.

**Immunohistochemistry involves the following steps**

### 2.11.1 Tissue preparation

All IHC was performed on 5μm, archival formalin-fixed, paraffin-embedded prostate tumour sections. Sections were dewaxed in xylene (2x4 minutes) and rehydrated through graded alcohol (100 % (2x2mins), 90 % (1x2mins), 70% ( 1x2 mins)) washes.

### 2.11.2 Antigen Retrieval

After formalin fixation and paraffin embedding of tissues, many antibodies react only weakly or not at all with their antigen. This is due to the fact that solvents, heat and fixatives can mask the antigen site. Formation of methylene bridges during fixation, which cross link proteins, mask antigenic sites therefore, it was necessary to include an antigen retrieval step, to break the protein cross-links and expose the antigenic binding site, in order to optimise immunohistochemical staining. Two different heat mediated methods of antigen retrieval were used for the antigens studied. The first involved incubating the tissue sections under pressure in 1L of TE buffer (1mM EDTA (Sigma), 5mM Tris (VWR), pH 8.0) or 1L Antigen Unmasking Solution (pH 6, 1:100 (Vector)) for five minutes. The

alternative method incubated tissue sections for twenty minutes at 96 ÆC in a water bath in 100ml Antigen Unmasking Solution (pH 6, 1:100 (Vector)), or Tris EDTA buffer (10mM EDTA (Sigma), 0.25mM Tris (VWR)) pH 9. All antigen retrieval steps were followed by a twenty-minute cool-down period.

### 2.11.3 Reduction of background staining: - Blocking steps

Peroxidase reacts with diaminobenzidine; therefore the presence of endogenous peroxidase activity in tissues is a common problem in IHC as it is a cause of background staining. Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide ($H_2O_2$) (VWR) for 10 mins, followed by a wash in water. A further cause of background staining is the formation of hydrophobic bonds between immunoglobulins and tissue proteins that results in the primary and secondary antibodies binding non-specifically to the tissue section rather than just the target antigen. To reduce this non-specific binding, tissue sections were incubated in 5% normal horse serum (Vector Laboratories) in TBS buffer (0.1MTris/HCl, 1.5M NaCl, pH 7.4) for twenty minutes.

### 2.11.4 Incubation with Primary Antibody

Antibody dilutions, incubation times and temperature were established for each protein investigated. All antibodies were diluted to the desired concentration in antibody diluent (DAKO). For each, a dilution series was performed, investigating various antibody titrations, incubation times and temperatures, to establish the optimal conditions in which to achieve the highest quality of staining, i.e. strongest specific antigen staining with the lowest non-specific background. It was crucial to have both a positive and negative control each time IHC was performed. The positive control confirmed that the chosen IHC method was working while the negative control checked the specificity of the antibody involved. Prostate tissues previously shown to have strong expression of the desired antigen were incubated with the appropriate antibody (MKP-2) and used as positive controls. The same tissues were used for negative controls by treating them with a negative isotype matched control reagent (DAKO).

### 2.11.5 Incubation with Secondary Antibody

Following incubation with antibody or negative control, the slides were thoroughly washed in TBS buffer twice for five minutes. The Envision detection method was used for all antigens. The slides were incubated with Envision for 30 min then washed twice for 5 min in TBS.

### 2.11.6 Detection & Visualisation

The chromagen used for staining the tissue sections was 3,3'-diaminobenzidine (DAB) (5ml distilled water ($dH_2O$), 2 drops of buffer solution, 4 drops of DABstock solution, and 2 drops of hydrogen peroxidase solution -Vector Laboratories). Slides were incubated with DAB for 5 to 10 min to allow brown staining to develop and were then washed in running water for 10 min.

### 2.11.7 Counterstaining

Tissue sections were counterstained with haematoxylin and Scott's Tap Water Substitute (S.T.W.S.). Slides were immersed in the haematoxylin for approximately thirty seconds, until a red colour was produced in the tissue section. Following this, slides were then submerged in S.T.W.S. for another thirty seconds, to produce a blue colour, in contrast to the brown positive staining of the antigen. The last steps involved dehydrating the tissues through a series of alcohol washes: (70% (1x1min), 90% (1x1min), 100 % (2x1min)) and xylene (2x1min), and then mounting the slides onto coverslips using DPX mountant (VWR) (Dibutyl Phtalate containing Xylene). Coverslips using DPX mountant (VWR) (Dibutyl Phtalate containing Xylene). 2.11.7. Histoscore Staining was scored blind by two independent observers (this author and Dr. Joanne Edwards) using a semi-quantitative weighted histoscore method also known as the Hscore system. Histoscores were calculated using the following formula: 0x % negative tumour cells + 1 x the % of cells staining weakly positive + 2x the %of cells staining moderately positive + 3 x the % of cells staining strongly positive.

The histoscore ranged from a minimum of zero to a maximum of 300. Results were considered discordant if the histoscores differed by more than 50. Both observers evaluated these cases. In addition, both intra-(variation in individual scoring) and inter-(variation between two observers) class correlation coefficients were calculated Agreement was considered excellent if the ICCC

value was > 0.7, anICCC of 1 indicates identical score. The mean of the two observers' histoscores was used for analysis. Changes in protein expression staining between MKP-2 expression and survival cases were defined as an increase or decrease outwith the 95% confidence interval for the difference in inter-observer variation (i.e. the mean difference between the histoscore that each observer assigns for protein expression plus or minus 2 standard deviations).

### 2.11.8 Statistical Analysis for Immunohistochemistry Studies.

All statistical analysis was performed using the SPSS version 15 for Windows. Basic descriptive statistics were performed to calculate the frequencies, mean, median and inter-quartile ranges for the histoscore for each protein investigated. These values were then used to establish appropriate cut-off points to define tumours as either low or high expressers of the desired protein. Wilcoxon Signed-Rank Tests were used to compare protein expression between and survival prostrate cancer patients. Correlations between protein expression levels in various sub-times to death from diagnosis and associations between the expressions of MKP-2 were calculated using the Spearman's Rank Correlation Test.

Kaplan-Meier life table analysis and Cox's multiple regression (multivariate survival analysis) were performed to estimate differences in prostate cancer related survival, in terms of time to biochemical relapse, time to death from relapse, disease=specific death and overall survival. Multivariate analysis combined the biological marker of interest with tumour grade at diagnosis, tumour grade at relapse (Gleason score), PSA at diagnosis, PSA at relapse, presence of metastasis at diagnosis, presence of metastasis at relapse and age to establish if it was independent of these known prognostic markers in influencing patient outcome. For survival analysis, patients were split into groups of those whose tumours expressed high levels of protein (above the median) and those who expressed low levels of protein (below the median). To establish the relative risk of a patient relapsing or dying as a result of either high or low levels of a particular protein in their tumour, hazard ratios (HR) were calculated using Cox regression analysis. A value of $p < 0.05$ was considered statistically significant.

## 2.12 SCANNING DENSITOMETRY

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

## 2.13 DATA ANALYSIS

All data shown were plotted as mean ± s.e.m and were representative of at least three separate experiments. The statistical significance of differences between mean values from control and treated groups were determined by either one-tailed Student's Unpaired t-test or a two-tailed one-way analysis of variance (ANOVA) with Dunnet's post-test ($P<0.05$ was considered significant).

# 3. EFFECT OF MAP KINASE PHOSPHATASE-2 (MKP-2) OVEREXPRESSION IN PROSTATE CANCER CELL FUNCTION

## 3.1 INTRODUCTION

As outlined in Chapter 1, prostate cancer is the second leading cause of death caused by cancer in males in the United States and Europe (Jemal et al., 2008). Both endogenous factors such as genetic mutations, hormones, immune conditions and environmental and lifestyle factors such as smoking, diet, radiation, and infectious agents have been shown to play a role in prostate cancer development and eventual metastasis (Fan et al., 2008). A variety of stimuli such as growth factors or UV-C and Hydrogen peroxide ($H_2O_2$) can mediate proliferative or pro-apoptotic protein expression (Wadgaonkar et al., 2004, Wang et al., 1999, Williams et al., 2006, Boutros et al., 2008, Geest and Coffer, 2009). These events are mediated by a variety of receptors and intrinsic signal transduction pathways. Among the signalling pathways involved in prostate cancer proliferation and apoptosis is the MAP kinase cascade, including ERK, JNK and p38 MAP kinases (discussed in section 1.4).

The MAP kinase family has been reported to play a major role in the control of many critical cellular processes including proliferation, apoptosis, and differentiation. They exert their effects on the cell by the phosphorylation of a multitude of cellular proteins including; transcription factors, cytoskeletal proteins and ion channels. Intermediates of the ERK pathways are dysregulated in one-third of all human cancers (Schubbert et al., 2007), whilst JNK appears to have both proliferative and apoptotic roles in prostate cancer (Altuwaijri et al., 2003, Engedal et al., 2002, Arnoldussen et al., 2008). These kinases are negatively regulated via dephosphorylation by specific MAP kinase phosphatases, MKPs. To date, eleven members of this family have been identified and their substrate specificities, subcellular distribution and regulation by extracellular stimuli defined (Keyse, 2008, Kondoh and Nishida, 2007, Soulsby and Bennett, 2009).

One of the earliest MKPs to be identified, MKP-2, is expressed in a wide range of tissues as an early gene induced of in response to diverse stimuli and localised within the nucleus (Chu et al., 1996, Misra-Press et al., 1995, Robinson et al., 2001). Originally, MKP-2 was found to display selectivity for ERK and JNK over p38 MAP kinase *in vitro*.

This chapter had two aims. The first was to estimate the level of endogenous MKP-2 in both LNCaP (AS) and (AI) as these cells represent different stages in prostate cancer development. This analysis included the assessment of both MKP-2-L and MKP-2-S transcripts. The second aim was to utilise an adenoviral version of MKP-2 (Adv.MKP-2) to overexpress MKP-2 in each cell type and assess the effects on ERK and JNK signalling and to determine the effects on cell cycle progression. This approach would provide an initial insight into the potential role of MKP-2 in prostate cancer cells.

## 3.2 CHARACTERISATION OF BOTH ENDOGENOUS EXPRESSION AND OVEREXPRESSION OF MKP-2 IN LNCaP (AS) AND (AI) CELLS

In order to assess the potential role of MKP-2 in both LNCaP (AS) and (AI) cells, an adenoviral construct of mitogen-activated protein kinase phosphatase-2 (MKP-2) was utilised. However, prior to this the levels of endogenous MKP-2 in both cell types was investigated. This includes assessment of both MKP-2-L and MKP-2-S transcripts.

### 3.2.1 Endogenous expression of MKP-2-L and MKP-2-S in both LNCaP (AS) and (AI) cells

The presence of both the full length and the short form of MKP-2 was investigated as shown in Figure 3.1. Panel A shows that there was substantial resting levels of full length MKP-2 in LNCaP (AI), with lesser amounts in LNCaP (AS). Much lower levels of MKP-2-S was observed in both cell types although again there was higher expression in LNCaP (AI). Stimulation with EGF for 2 h did not result in any observed increase in expression.

An extended time course using EGF (20 ng/ml) for 2, 4, and 24 h is shown in panel B. The figure shows that resting mRNA levels of MKP-2 from untreated cells were high in both LNCaP (AS) and (AI) lines. Again treatment with EGF for the times indicated did not change levels of MKP-2 expression in in either LNCaP (AI) and (AS).

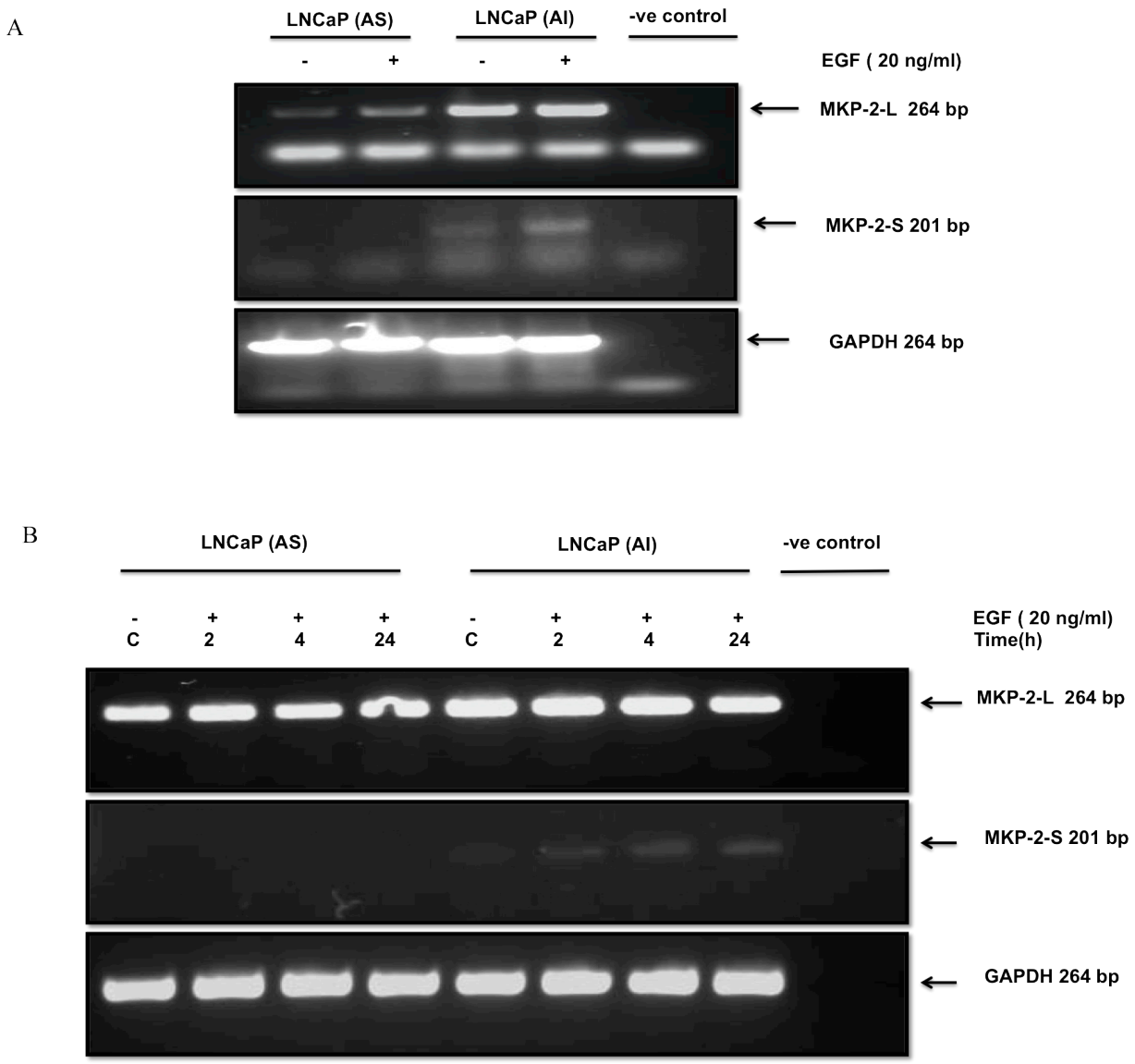


**Figure 3.1: Endogenous expression of MKP-2-L and MKP-2-S in both LNCaP (AS) and (AI) cells.** LNCaP cells were stimulated with EGF (20 ng/ml) for 2 h (panel A) and for 2, 4 and 24 h (panel B). Total RNA was prepared as outlined in (Section 2.3) and subjected to RT-PCR analysis with the following primers; MKP-2-L 264 bp and MKP-2-S 201 bp. Samples were separated on a 1.5% agarose gel. Each gel is representative of 3-4 experiments.

## 3.3 CHARACTERISATION OF OVEREXPRESSION OF MKP-2 IN PC3 CELLS

In order to investigate the potential for overexpression of MKP-2 to regulate MAP kinase activation in prostate cancer cell lines, initially PC3 and both LNCaP cell lines, were infected with MKP-2 adenovirus (Adv.MKP-2), to assess susceptibility of prostate cancer cells to adenoviral uptake over different times and at different concentrations of serum.

### 3.3.1 Time course of overexpression of adenoviral MKP-2 in PC3 cells

To evaluate the time course of infection with Adv.MKP-2, cells were grown to approximately 70% confluency in 12 well plates and infected with 500 pfu/cell of Adv.MKP-2 in serum free media for 0 – 72 h, and then assayed for MKP-2 expression by Western blotting. The results presented in figure 3.2, show that MKP-2 expression was evident after 24 h and reached maximum levels by 72 h. Fold expression was approximately 2.5 fold of background. However, infection with lacZ control virus had no effect on MKP-2 expression.

### 3.3.2 Concentration dependent overexpression of adenoviral MKP-2 in PC3 cells

To further evaluate the concentration dependent infection of MKP-2, cells were grown to approximately 70% confluency in 12 well plates and infected with increasing concentrations of adenovirus (100-500 pfu/cell) for 24, 48 and 72 h. The data presented in figure 3.3, demonstrated that at all time points, uptake of virus and expression of MKP-2 was evident. Highest expression was obtained at later times and at higher concentrations, between 300 and 500 pfu/cell at 48 and 72 h. Infection with lacZ control adenovirus virus had no effect on MKP-2 expression at any time points.

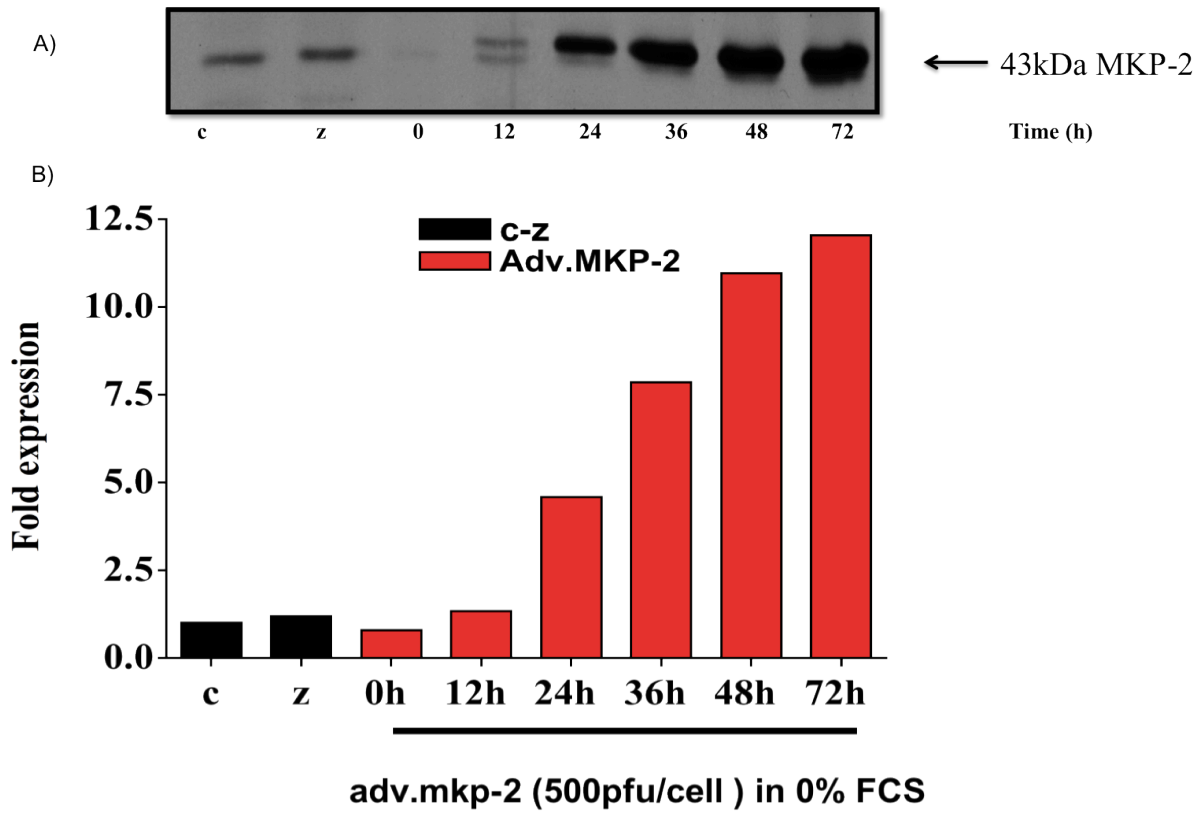


**Figure 3.2: Time course of infection Adv.MKP-2 (500 pfu/cell) in PC3 cell lines.** Cells were infected with (500 pfu/cell) of Adv.MKP-2 or Lacz (Z) in serum free media (0%) for the times indicated. Samples were assayed for a) cellular expression of MKP-2 (43 kDa), assessed by Western blotting as outlined in section 2.4, b) fold expression was quantified by scanning densitometry; n=1.

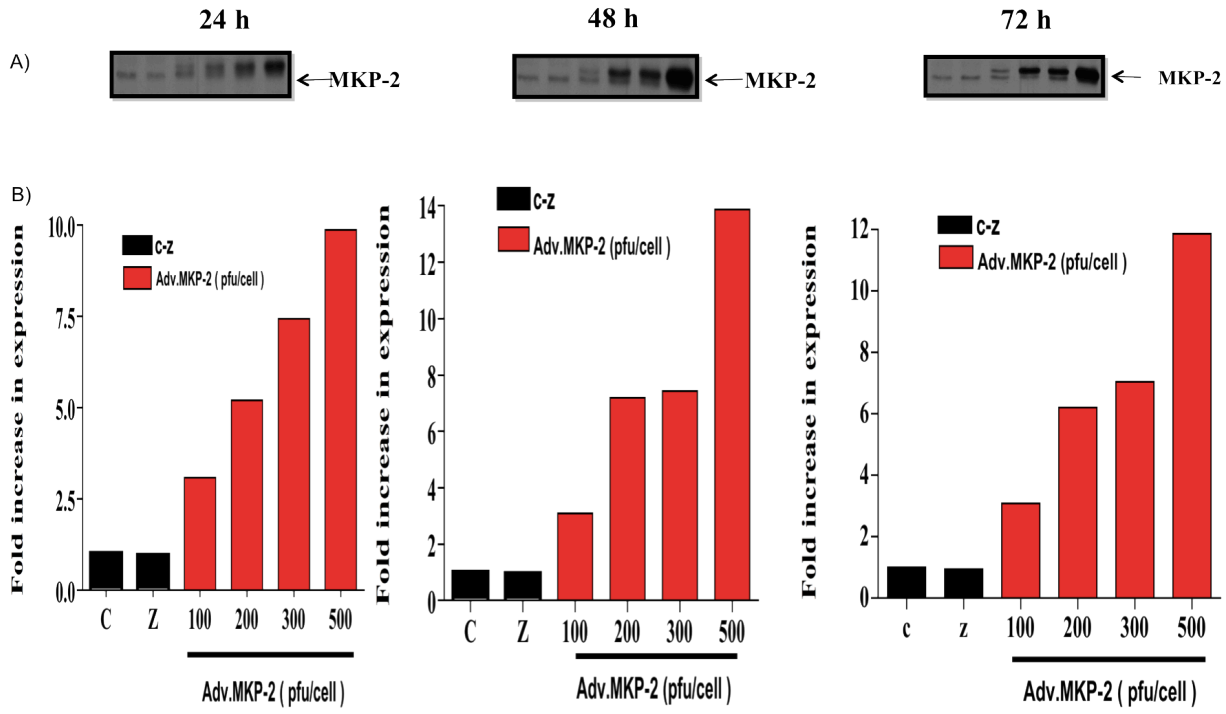


**Figure 3.3: Concentration-dependent prior to infection of PC3 cells with Adv.MKP-2.** Cells were rendered quiescent for 24 h with different concentrations (pfu/cell) of Adv.MKP-2 in serum free media for 24, 48 and 72 h. Samples were assayed for a) overexpression of MKP-2 (43 kDa) as outlined in section 2.6.4. Blots were quantified for b) fold expression by scanning densitometry; n=1.

## 3.4 CHARACTERISATION OF OVEREXPRESSION OF MKP-2 IN LNCaP (AS) CELLS

As overexpression of MKP-2 adenovirus has been demonstrated in PC3 cells, the overexpression of MKP-2 mediated by adenoviral infection in both LNCaP (AS) and (AI) were also examined.

### 3.4.1 concentration dependent overexpression of Adv. MKP-2 in LNCaP (AS) cells

Cells were infected with increasing concentrations of Adv.MKP-2 (100-500 pfu /cell) for 24, 48 and 72 h and assayed for expression of MKP-2 by Western blotting. The data is presented in figure 3.4. The figure demonstrates that expression was observed as early as 24 h and was maintained for 72 h. Expression was largely concentration dependant particularly at 24 h reaching a maximum between 300 and 500 pfu/cell. Expression was less concentration dependant over 48 h and 72 h as expression was at a maximum at all concentrations. Infection with lacZ control virus had no effect on MKP-2 expression.

### 3.4.2 Time concentration course overexpression of adenovirus MKP-2 in LNCaP (AI) cells

To evaluate the time course of infection with Adv.MKP-2, LNCaP (AI) cells were grown to approximately 70% confluency in 12 well plates and infected with 100, 200, 300 and 500 pfu /cell of Adv.MKP-2 in 10% FCS media for 0 – 72 h, and then assayed for MKP-2 expression by Western blotting. The results presented in figure 3.5, show that after a delay of 24 h, MKP-2 expression was evident after 48 h and reached maximum levels by 72 h. However, infection with lacZ control virus had no effect on MKP-2 expression.

### 3.4.3 Sub-cellular localisation of MKP-2 in LNCaP (AI) cells

To confirm the nuclear localisation of MKP-2 in LNCaP (AI), cells were infected with LacZ (200 pfu/cell) or increasing concentrations of Adv.MKP-2 for 40 h and then investigated using fluorescence microscopy (section 2.2.5), as shown in Figure 3.6. The Figure shows that no specific staining of MKP-2 was observed in cells infected with LacZ alone (data not shown), however at

increasing pfu enhanced cellular staining of MKP-2 was observed. At a pfu of 200, greater than 95% of the cells were infected and staining was strictly located to the nucleus. This was confirmed by nuclear staining with DAPI.

### 3.4.4 Sub-cellular localisation of MKP-2 in LNCaP (AS) cells

To confirm the nuclear localisation of MKP-2 in LNCaP (AS), cells were infected with LacZ (200 pfu/cell) or increasing concentrations of Adv.MKP-2 for 40 h and then investigated using fluorescence microscopy (section 2.2.5), as shown in Figure 3.7. The Figure shows no staining of MKP-2 was observed in cells infected with LacZ alone (data not shown), however at increasing pfu enhanced cellular staining of MKP-2 was observed. At a pfu of 200, greater than 95% of the cells were infected and staining was predominantly located to the nucleus. This was confirmed by nuclear staining with DAPI.

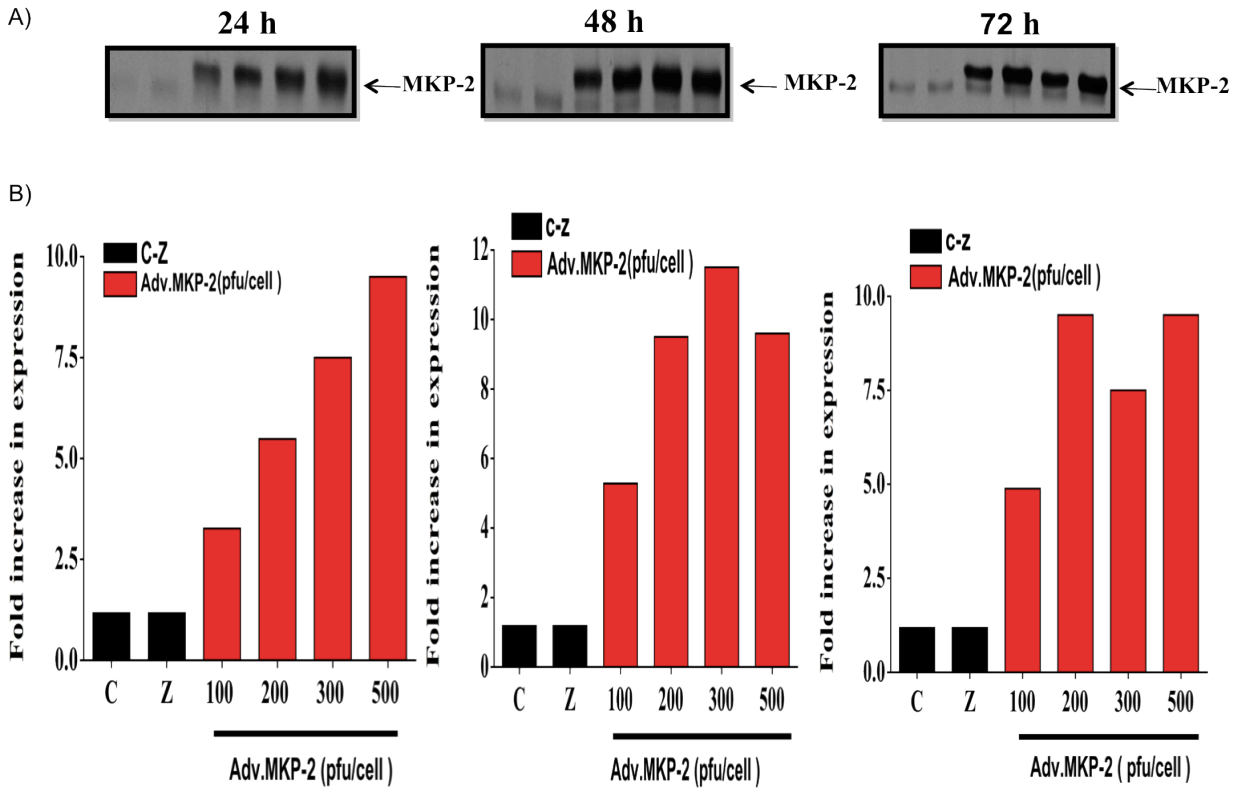


**Figure 3.4: Time and concentration dependent expression of Adv.MKP-2 in LNCaP (AS) cell lines.** Cells were infected with increasing concentrations of adenovirus MKP-2 (100-500 pfu/cell) in 10% FCS for 24, 48 and 72 h. Samples were assayed for a) cellular expression of MKP-2 (43 kDa) as outlined in section 2.6.4. Blots were quantified for b) fold expression by scanning densitometry; n=1.

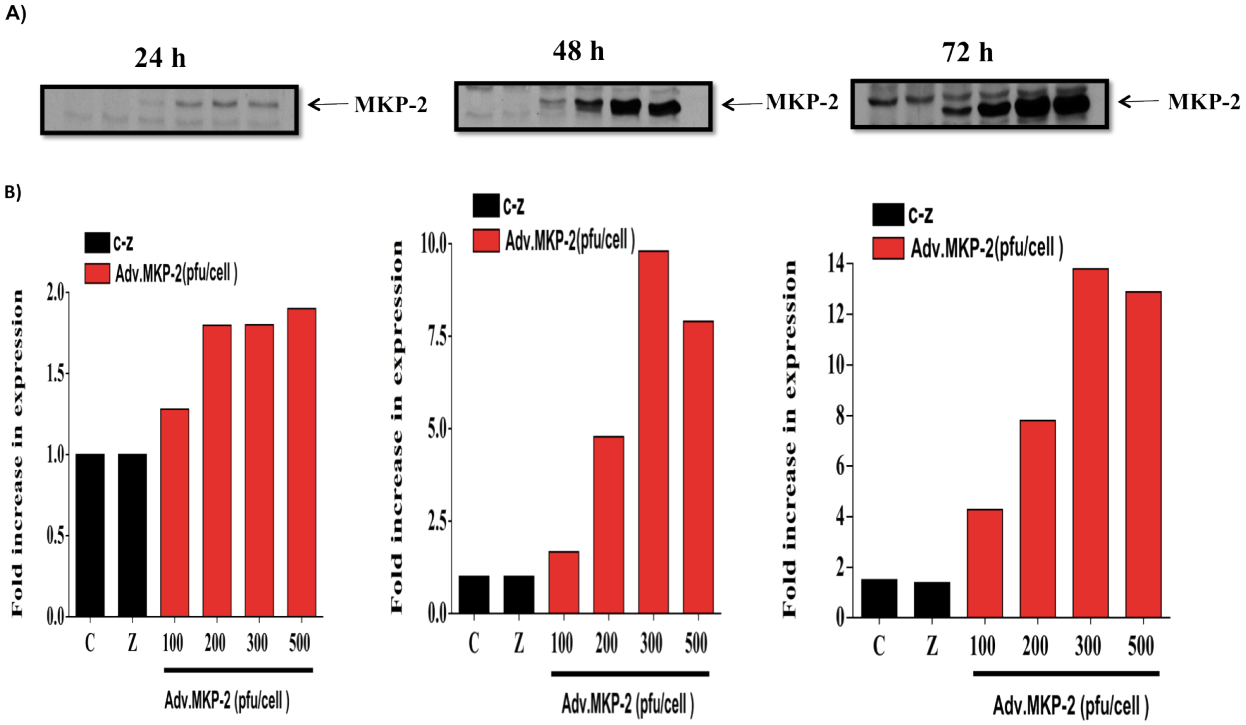


**Figure 3.5: Time and concentration dependent expression of Adv.MKP-2 in LNCaP (AI) cell lines.** Cells were infected with increasing concentrations of Adv.MKP-2 (100-500 pfu/cell) in 10% FCS for 24, 48 and 72 h. Samples were assayed for a) cellular expression of MKP-2 (43 kDa) by Western blotting as outlined in section 2.4. Blots were quantified for b) fold expression by scanning densitometry; n=1.

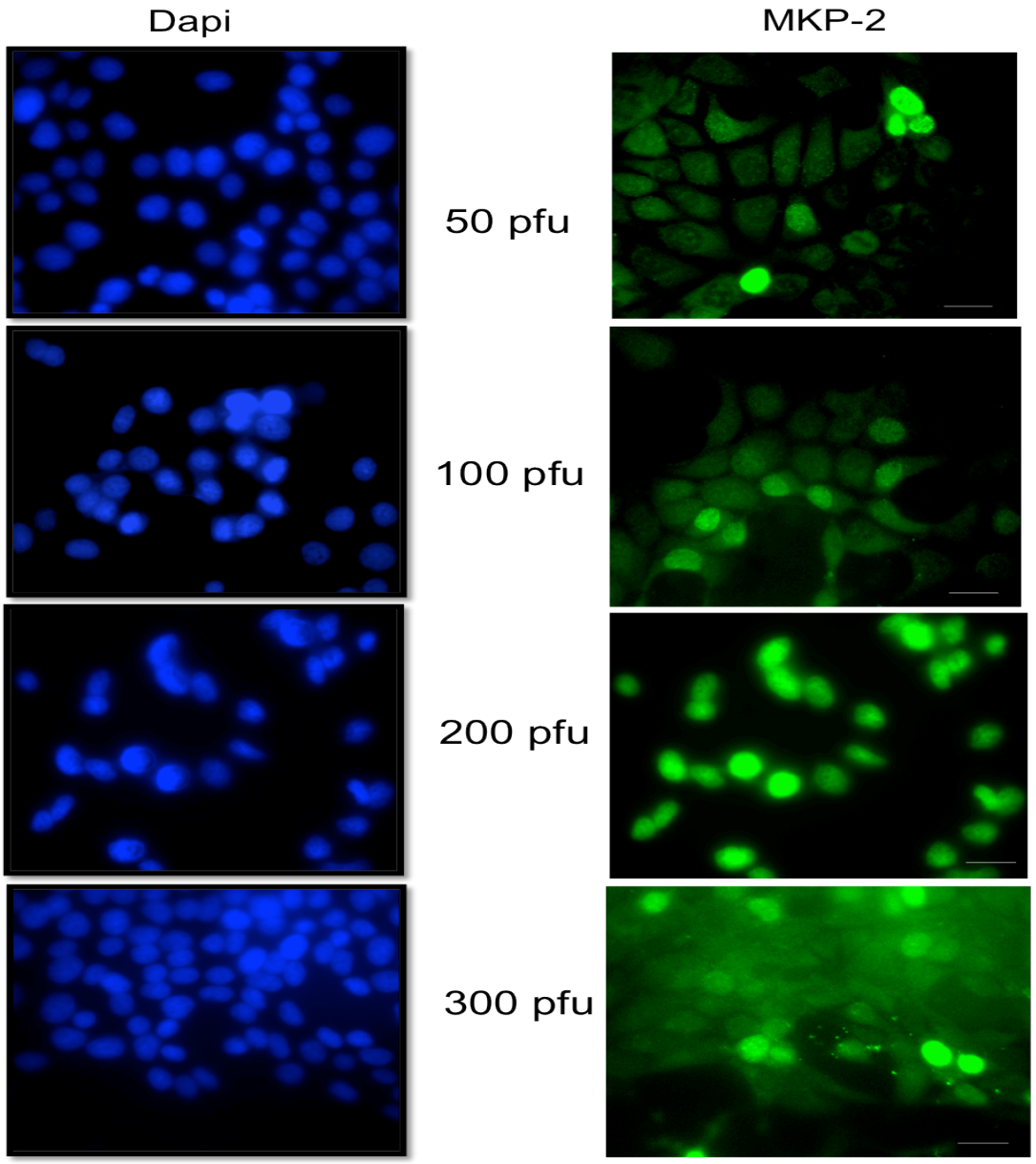


**Figure 3.6: Sub-cellular localisation of MKP-2 in LNCaP (AI) cell lines.** Cells were infected with increasing pfu/cell of Adv.MKP-2 for 40 h, then cells were fixed and stained for MKP-2 (1:100). Nuclei were visualised by DAPI (blue) staining and MKP-2 sublocalisation was visualized using FITC (green) staining using magnification of x40 as outlined in section 2.5. The results are representative of at least 4 independent experiments.

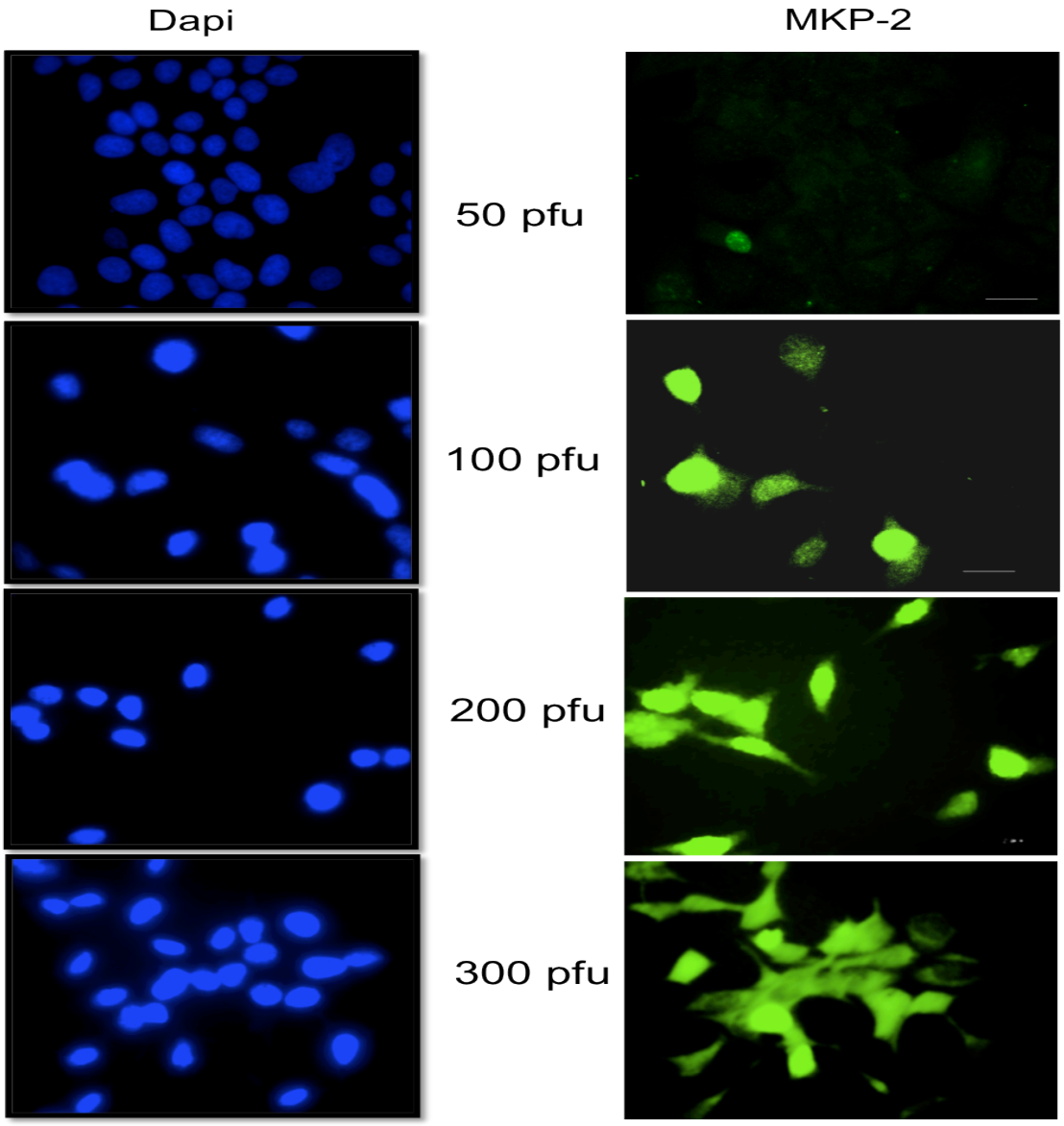


**Figure 3.7: Sub-cellular localisation of MKP-2 in LNCaP (AS) cell ines.** Cells were infected with increasing pfu/cell of Adv.MKP-2 for 40 h, then cells were fixed and stained for MKP-2 (1:100). Nuclei were visualised by DAPI (blue) staining and MKP-2 sublocalisation was visualized using FITC (green) staining using magnification of x40 as outlined in Section 2.5. The results are representative of at least 4 independent experiments.

## 3.5 CHARACTERISATION OF EGF AND TNF-α STIMULATED MAP KINASE SIGNALLING PATHWAYS IN LNCaP (AS) and LNCaP (AI) CELLS.

Initially we sought to identify ligands which would be able to stimulate both ERK and JNK MAP kinases as they are known targets of MKP-2. We initially used serum and EGF for ERK and also principally UV-C for JNK signalling.

### 3.5.1 Effect of quiescing of EGF and FCS-mediated activation of ERK in serum-starved in LNCaP (AS) cells

To evaluate how long a period serum starvation was required to reduce basal ERK activation in response to two type of growth stimuli, LNCaP (AS) cells were grown to approximately 80-90 % confluency in 6 well plates, quiesced for 24, 48, and 72 h, then stimulated with either FCS 10% or EGF (20 ng/ml) for 5 min. The data presented in Figure 3.8, in LNCaP (AS) demonstrated that increased ERK phosphorylation was observed after stimulation with EGF, but in response to FCS there was little stimulation at all three times point.

### 3.5.2 EGF-mediated ERK phosphorylation in LNCaP (AS) cells

Further characterisation of the growth factor, EGF is shown in Fig 3.9. The figure shows a strong increase in ERK phosphorylation in response to EGF, which reached a maximum between 5 and 15 min (fold stim. at 5 and 15 min respectively = 2.305 ± 0.194 and 1.644 ± 0.109, n=3). This response was transient, rapidly decreasing and returning to basal values by 30 min. Total ERK were unchanged, indicating equal protein loading. No further increases in ERK phosphorylation was observed between 30 and 480 min the longest time point examined.

### 3.5.3 EGF-mediated ERK phosphorylation in LNCaP (AI) cells

The effect of EGF was then compared in LNCaP (AI) cells. Figure 3.10, shows ERK phosphorylation (panel A) stimulated by EGF (20 ng/ml) over a 480 min period. In contrast to

LNCaP (AS) cells, ERK activation in LNCaP (AI) cells was stronger both in terms of magnitude and kinetics. The signal again reached a peak between 5 and 15 min, approximately 4-5 fold of basal values (fold stim. at 5 and 15 min respectively = 4.531 ± 0.093 and 3.781 ± 0.028, n=3). Phosphorylation levels declined slowly returning to basal values by 240 min, however, values at 120 min were still 2.5 fold greater than control. Total ERK values were unchanged, indicating equal protein loading.

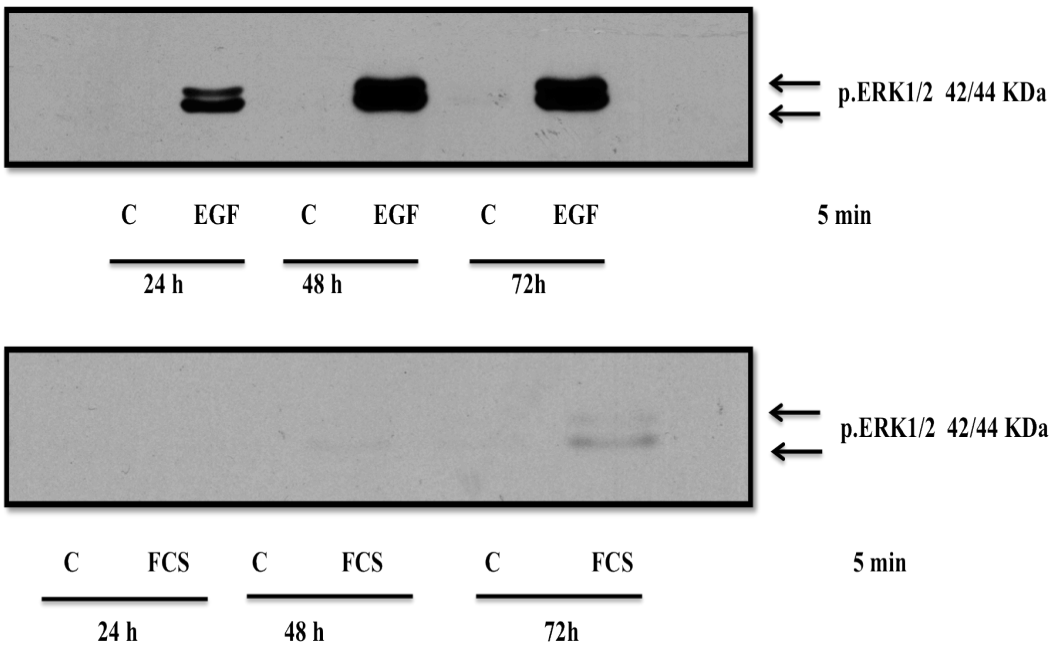


**Figure 3.8: Effect of quiescing on EGF and FCS-mediated ERK phosphorylation in LNCaP (AS) cell lines.** Cells were quiescent for 24, 48 and 72 h, then stimulated with either FCS 10% or EGF (20 ng/ml) for 5 min. Whole cell lysates were prepared, separated by SDS.PAGE as outlined in section 2.4, and then assessed for p-ERK1/2 (42/44 kDa).

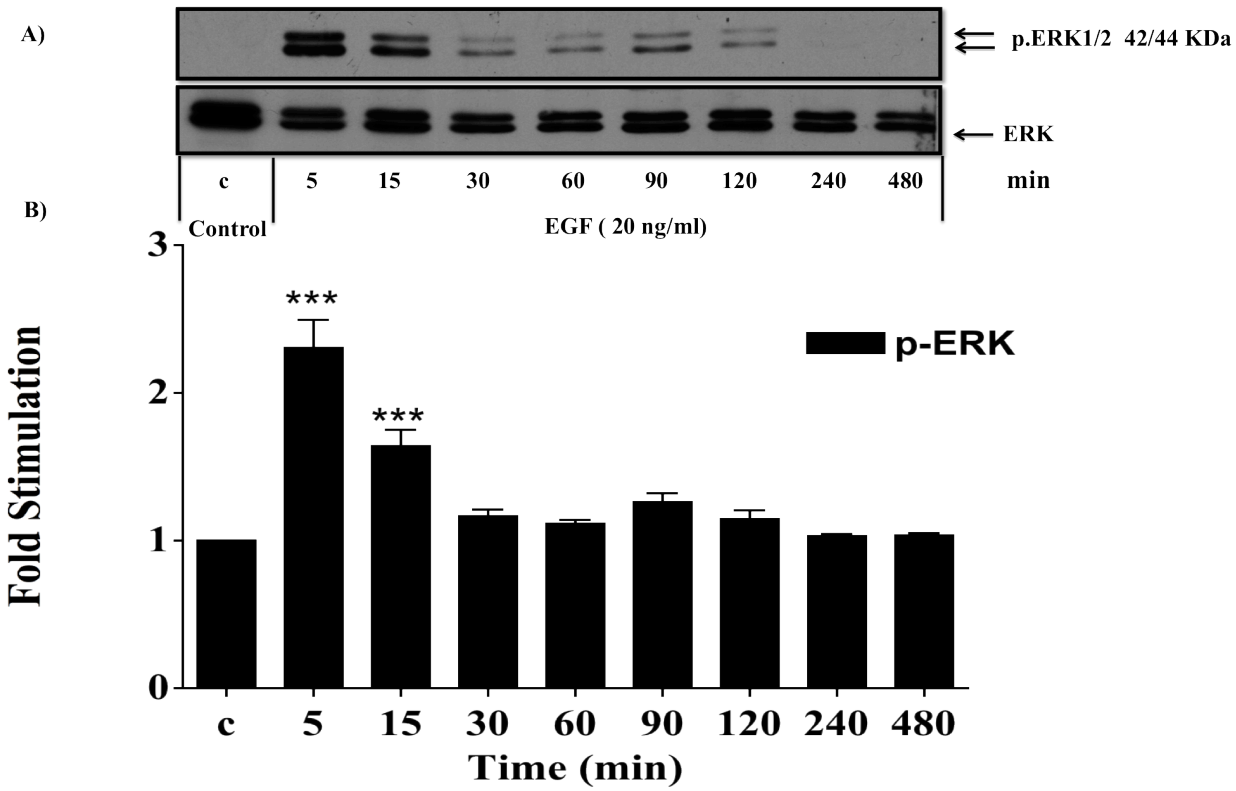


**Figure 3.9: Time course of EGF-mediated ERK phosphorylation in LNCaP (AS) cell lines.** Cells were stimulated with EGF (20 ng/ml) as indicated. Whole cell lysates were prepared, separated by SDS.PAGE, and then assessed for a) p-ERK1/2 (42/44 kDa) and ERK, as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m, ***P<0.001 at 5 and 15 min compared with control group. The results are representative of 3 independent experiments.

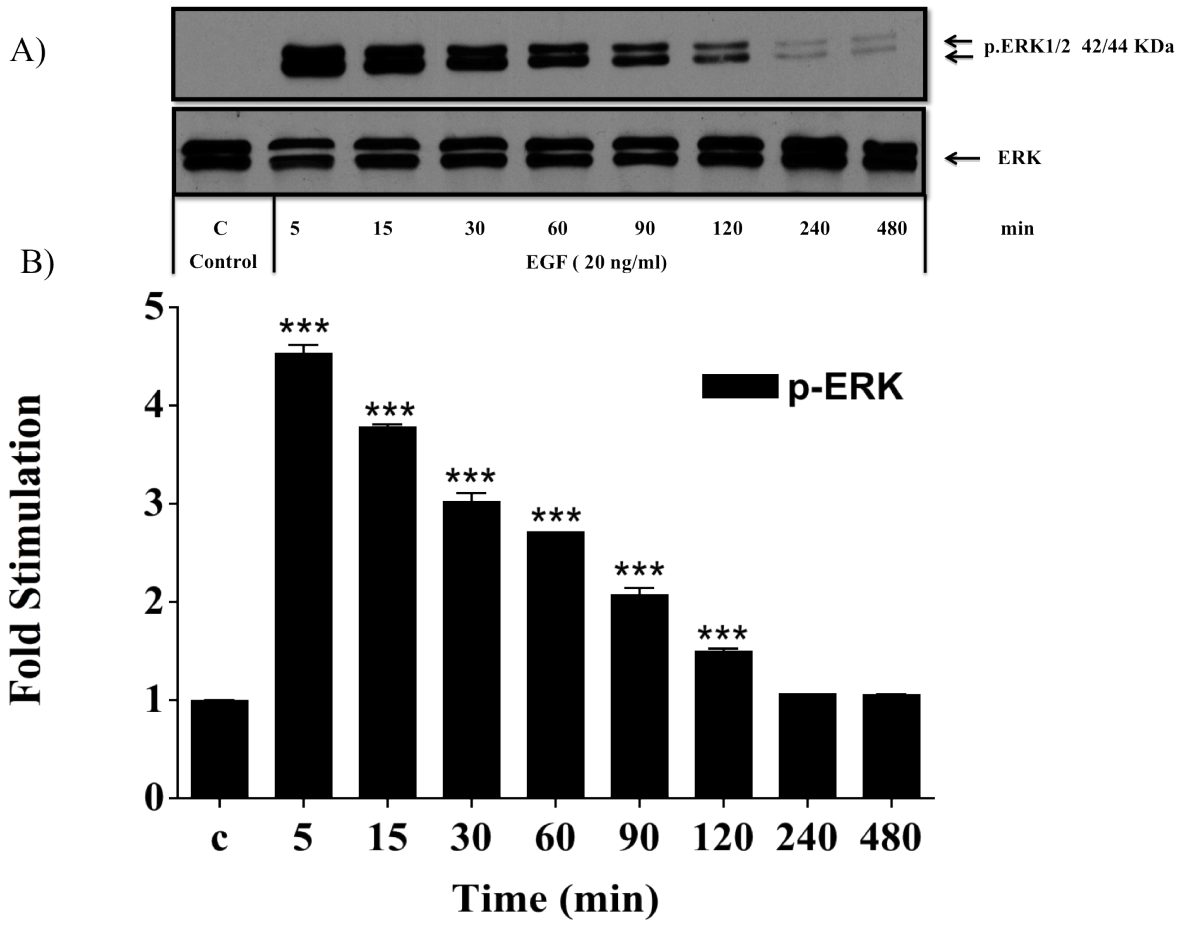


**Figure 3.10: Time course of EGF-mediated ERK phosphorylation in LNCaP (AI) cell lines.** Cells were stimulated with EGF (20 ng/ml) as indicated. Whole cell lysates were prepared, separated by SDS.PAGE and, then assessed for a) p-ERK1/2 (42/44 kDa) and ERK, as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m, ***P<0.001 from 5 until 120 min compared with control group. The results are representative of 3 independent experiments.

## 3.6 CHARACTERISATION OF UV-C STIMULATED MAP KINASE SIGNALLING PATHWAYS IN LNCaP (AS) AND LNCaP (AI) CELLS.

Having evaluated the time course of activation of ERK1/2 in LNCaP (AS) and (AI), particularly with respect to EGF, I sought to characterise further stimuli which would give a strong JNK signal. Two were examined, UV-C and TNF-α.

### 3.6.1 TNF-α mediated phosphorylation of JNK in LNCaP (AS) cells

The effect of TNF-α on the stress activated protein kinases such as JNK, was also examined in quiesced, LNCaP (AS) cells over a period of 90 min (figure 3.11). In contrast to ERK phosphorylation, JNK phosphorylation was minimal and slow in onset starting after 15 min and increasing up until 90 min, to approximately 1.5 fold of basal values. Total JNK levels were unchanged, indicating equal protein loading (panel A).

### 3.6.2 UV-C-mediated JNK phosphorylation in LNCaP (AS) cells

In order to find a more efficacious stimulant of JNK relevant to cancer, ultraviolet (UV) light C was used. This has been shown to be a good activator of JNK phosphorylation in a number of cell types (Dunkern et al., 2001, Tomicic et al., 2005). Figure 3.12 shows JNK phosphorylation (panel A) stimulated by UV-C (60 $J/m^2$) over a 480 min period. The figure shows a strong increase in JNK phosphorylation, which was apparent as early as 15 min and reached a maximum after 30 min (fold stim. at 30 min = 2.08 ± 0.07, n=3). Phosphorylation levels declined slowly returning towards basal values by 480 min, however, values at 120 min were still 1.8 fold greater than control. Total JNK levels were unchanged, indicating equal protein loading.

### 3.6.3 UV-C-mediated JNK phosphorylation in LNCaP (AI) cells

The effect of UV-C was then assessed in LNCaP (AI) cells. Figure 3.13 showed a strong increase in JNK phosphorylation in response to UV-C which reached a maximum between 30 and 60 min (fold stim. at 30 and 60 min respectively = 2.23 ± 0.16 and 2.30 ± 0.14, n=3). Similar to LNCaP (AS) cells, JNK phosphorylation levels also declined slowly returning to basal values by 240 min. JNK were unchanged, indicating equal protein loading.

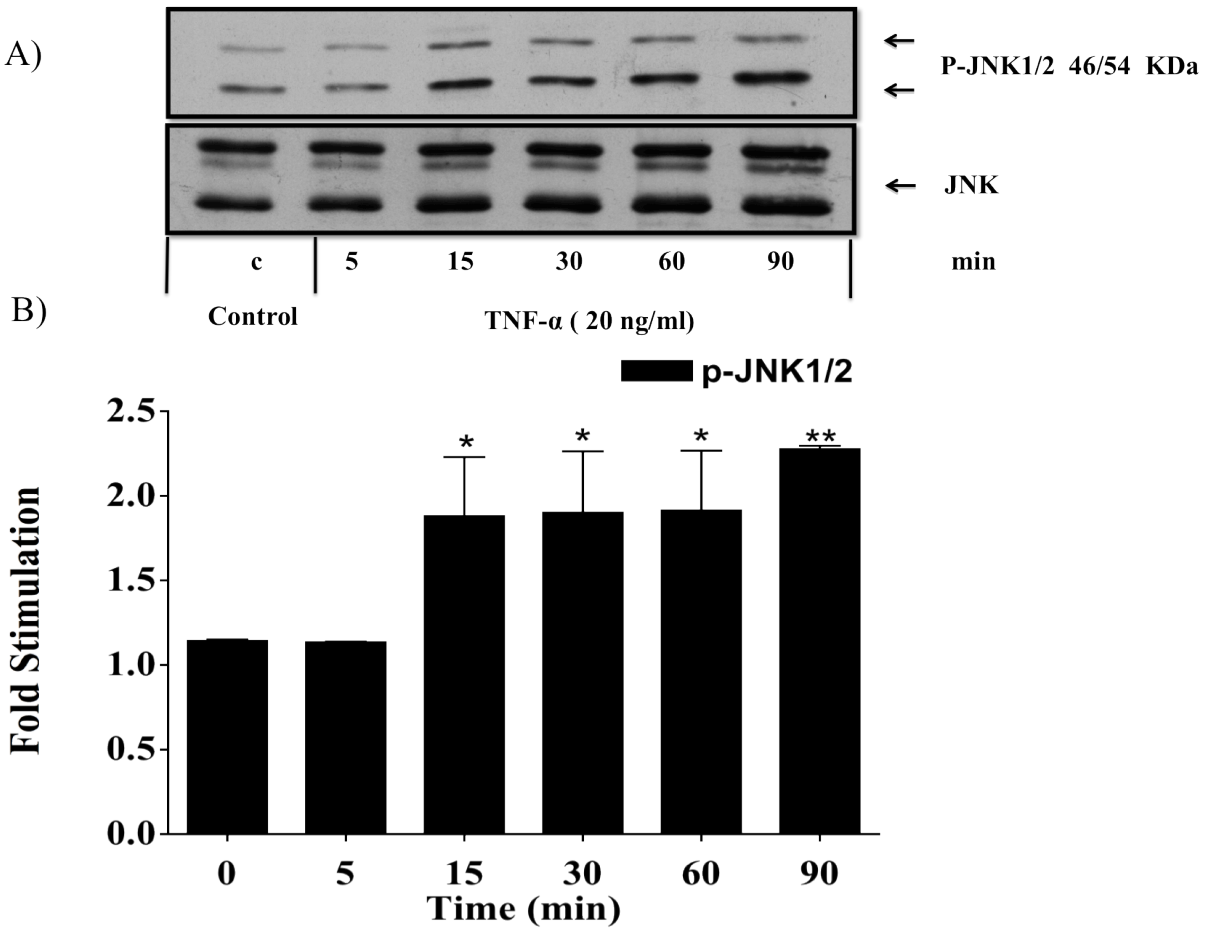


**Figure 3.11: Time course of TNF-α-mediated JNK phosphorylation in LNCaP (AS) cell lines.** Cells were stimulated with TNF-α (20 ng/ml) for the indicated times. Whole cell lysates were prepared, separated by SDS.PAGE, and then assessed for a) p-JNK (54/46kDa) and JNK, as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m.*P<0.05 at 15, 30 and 60 min and **P<0.01 at 90 min compared with control group. The results are representative of 3 independent experiments.

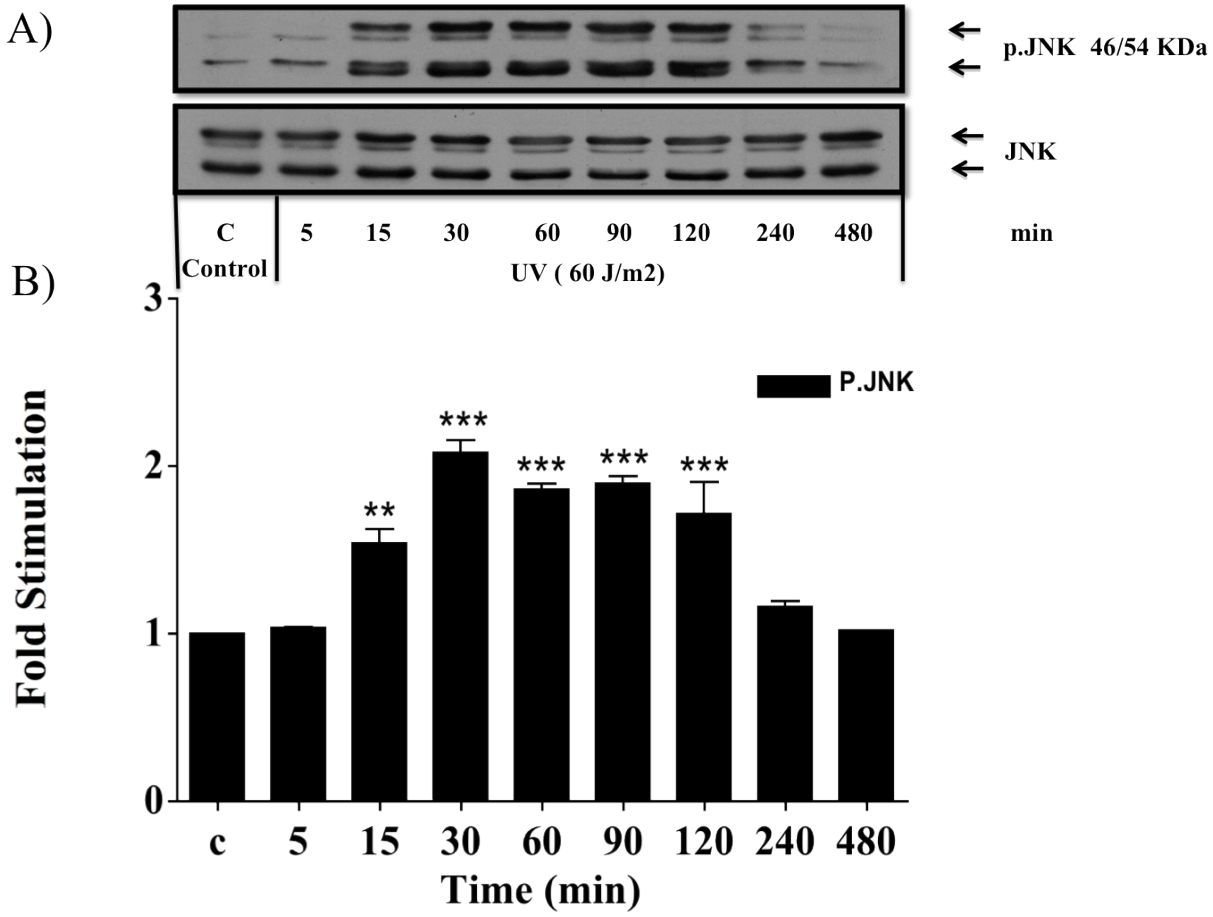


**Figure 3.12: Time course of UV-C-mediated JNK phosphorylation in LNCaP (AS) cell lines.** Cells were stimulated with UV-C (60 J/m$^2$) for the times indicated. Whole cell lysates were prepared, separated by SDS.PAGE, and then assessed for a) p-JNK (46/54 kDa) and JNK, as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. **P<0.01 at 5 min, ***P<0.001 at 15 to 120 min compared with control group. The results are representative of 3 independent experiments.

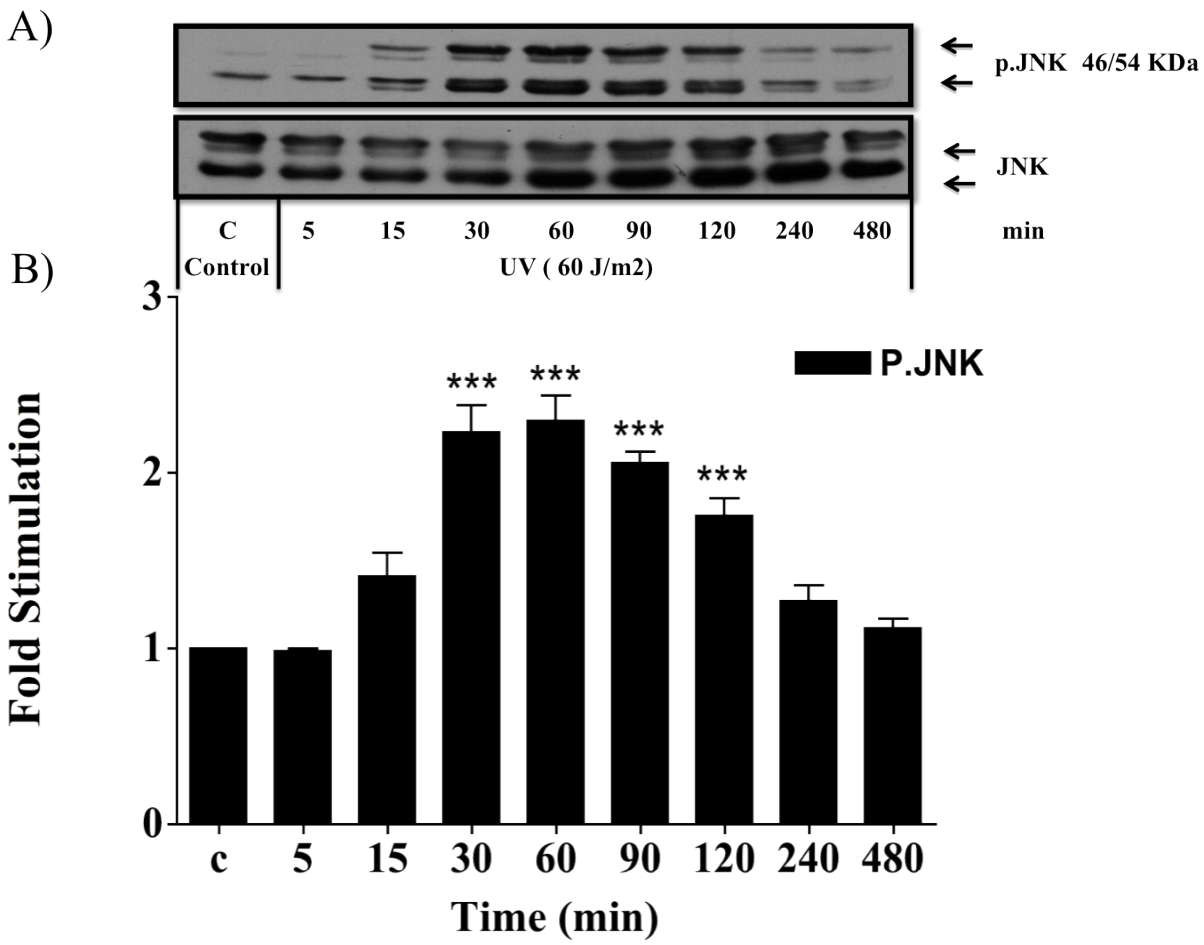


**Figure 3.13: Time course of UV-C-mediated JNK phosphorylation in LNCaP (AI) cell lines.** Cells were stimulated with UVC (60 J/m$^2$) for the times indicated. Whole cell lysates were prepared, separated by SDS.PAGE, and then assessed for a) p-JNK (46/54 kDa) and JNK, as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. ***P<0.001 at 30 to 120 min compared with control group. The results are representative of 3 independent experiments.

## 3.7 CHARACTERISATION OF THE EFFECT OF ADV.MKP-2 ON EGF MEDIATED ERK PHOSPHORYLATION IN LNCaP (AS) AND (AI) CELLS

Overexpression of Adv.MKP-2 in LNCaP (AS) and (AI), gave rise to a protein that was the correct size and nuclear located. The expectation is that the protein will be functional and able to dephosphorylate and inactivate ERK. However, a lack of ERK dephosphorylation is observed in cells either constitutively expressing MKP-2 (Robinson et al., 2001), or as demonstrated previously in the laboratory, following adenoviral infection (Al-Mutairi et al., 2010a). Other studies have clearly demonstrated that MKP-2 is expressed exclusively within the nucleus due to the presence of two nuclear localisation sequences (Chen et al., 2001, Sloss et al., 2005).

### 3.7.1 The effect of Adv.MKP-2 on EGF mediated ERK phosphorylation in LNCaP (AS) cells

Having established two agents which were able to generate ERK and JNK signals, the effect of Adv.MKP-2 on either signal was examined. Figure 3.14 shows the effect of Adv.MKP-2 (200 pfu/cell) upon ERK signalling in response to EGF. Stimulation with EGF induced a significant increase in ERK phosphorylation at all time points tested, although the size of the response was greater at 30 and 90 min compared with 240 min (EGF fold stim. at 30, 90 and 240 min = 2.21 ± 0.08, 2.59 ± 0.03 and 1.29 ± 0.12 respectively, n=3). Whilst EGF induced phosphorylation was slightly but not significantly decreased by infecting cells with LacZ (data not shown) in contrast, infection with Adv.MKP-2 (200 pfu/cell) significantly inhibited the phosphorylation of ERK by approximately 40% at 30 min and greater than 90% at 90 min and roughly 20% at 240 min (EGF + Adv.MKP-2 fold stim. at 30, 90 and 240 min = 1.57 ± 0.26, 1.08 ± 0.03 and 1.00 ± 0.02 respectively, **P< 0.01 and ***p<0.001 at 30 and 90 min respectively, n=3).

### 3.7.2 The effect of Adv.MKP-2 on EGF mediated ERK phosphorylation in LNCaP (AI) cells

Figure 3.15 shows the effect of Adv.MKP-2 (200 pfu/cell) upon ERK signalling in response to EGF in LNCaP (AI) cells. Stimulation with EGF induced a significant increase in ERK phosphorylation

at 30, 90 and 240 min (EGF fold stim. at 30, 90 and 240 min = 3.26 ± 0.08, 2.46 ± 0.33 and 1.42 ± 0.34 respectively, n=3). Phosphorylation was slightly but not significantly decreased by infecting cells with LacZ (data not shown) however, infection with Adv.MKP-2 (200 pfu/cell) significantly inhibited the phosphorylation of ERK. This was roughly 75% at 30 min, with approximately 60% inhibition at 90 min and 15% inhibition at 240 min (EGF + Adv.MKP-2 fold stim. at 30, 90 and 240 min = 1.53 ± 0.08, 1.11 ± 0.04 and 1.08 ± 0.07 respectively, ***P< 0.01 at 30 and 90 min, n=3).

### 3.7.3 The effect of Adv.MKP-2 on UV-C induced JNK phosphorylation in LNCaP (AS) cells

The effect of infection on UV-C stimulated JNK phosphorylation was also examined. In Figure 3.16, LNCaP (AS) were infected with Adv.MKP-2 (200 pfu/cell) for 40 h and, then exposed to UVC (60 $J/m^2$) for 30, 90 and 240 min. Under these conditions UVC alone caused a significant increase in JNK phosphorylation (UV-C fold stim. at 30, 90 and 240 min=1.69 ± 0.30, 1.28 ± 0.20 and 1.13 ± 0.15 respectively, n=3), which was not decreased by infecting cells with LacZ. However, infection with Adv.MKP-2 significantly inhibited the phosphorylation of JNK. This was noticeable at 30 min by approximately more than 75% and roughly 40% at 90 min and 20% inhibition at 240 min (UV-C + Adv.MKP-2 fold stim. at 30, 90 and 240 min = 1.18 ± 0.20, 1.03 ± 0.07 and 0.99 ± 0.01 respectively, *P< 0.05 at 30 min, n=3).

### 3.7.4 The effect of Adv.MKP-2 on UV-C induced JNK phosphorylation in LNCaP (AI) cells

Figure 3.17 shows that UV-C stimulation resulted in the usual substantial increase in JNK phosphorylation (UV-C fold stim. at 30, 90 and 240 min = 2.38 ± 0.27, 1.62 ± 0.26 and 1.35 ± 0.18 respectively, n=3). However, infection with Adv.MKP-2 significantly inhibited the phosphorylation of JNK and this was observed at all times points and greater than 40% (UV-C + Adv.MKP-2 fold stim. at 30, 90 and 240 min = 1.72 ± 0.08, 1.09 ± 0.05 and 0.93 ± 0.03 respectively, ***P< 0.001 and **p<0.01 at 90 and 240 min respectively, n=3).

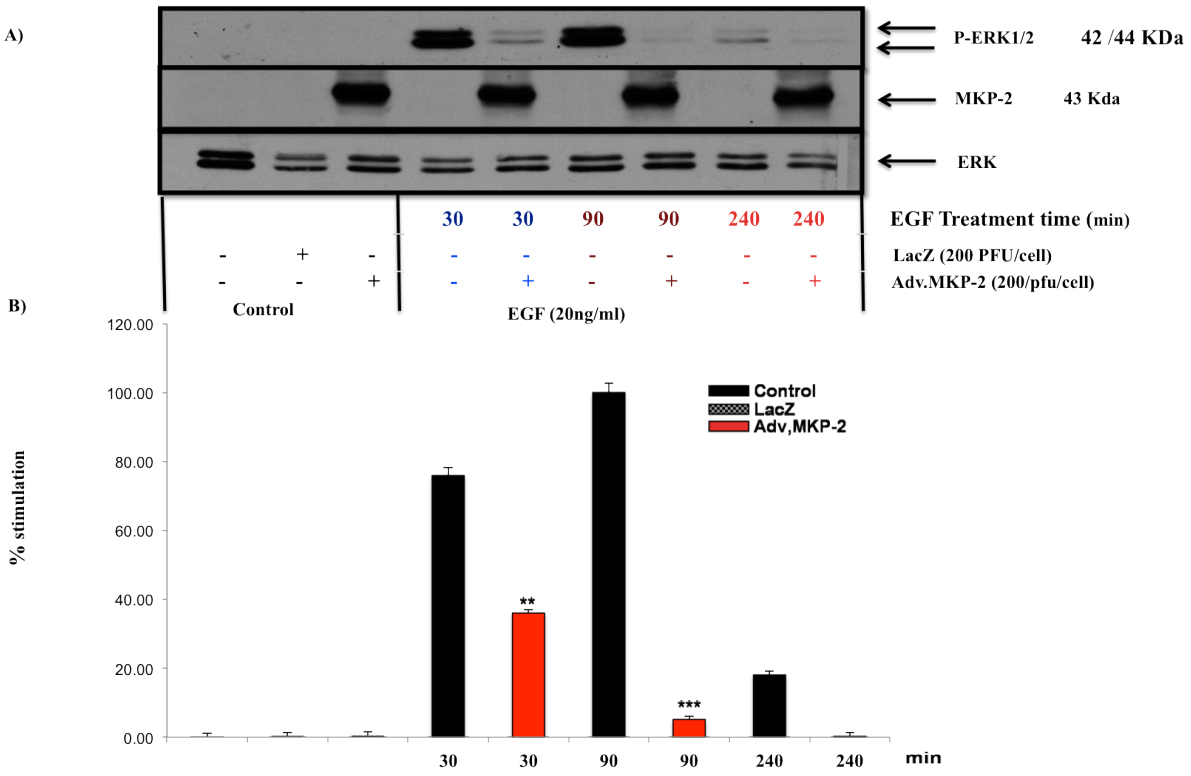


**Figure 3.14: The effect of Adv.MKP-2 on ERK phosphorylation mediated by EGF in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with EGF (20 ng/ml) for 30, 90 and 240 min. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p-ERK1/2 (42/44 kDa), MKP-2 (43 kDa) and ERK as outlined in Section 2.4. Blots were quantified for b) % stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, **P<0.01 in 30 min, ***P<0.001 at 90 min compared with EGF stimulated control. The results are representative of 3 independent experiments.

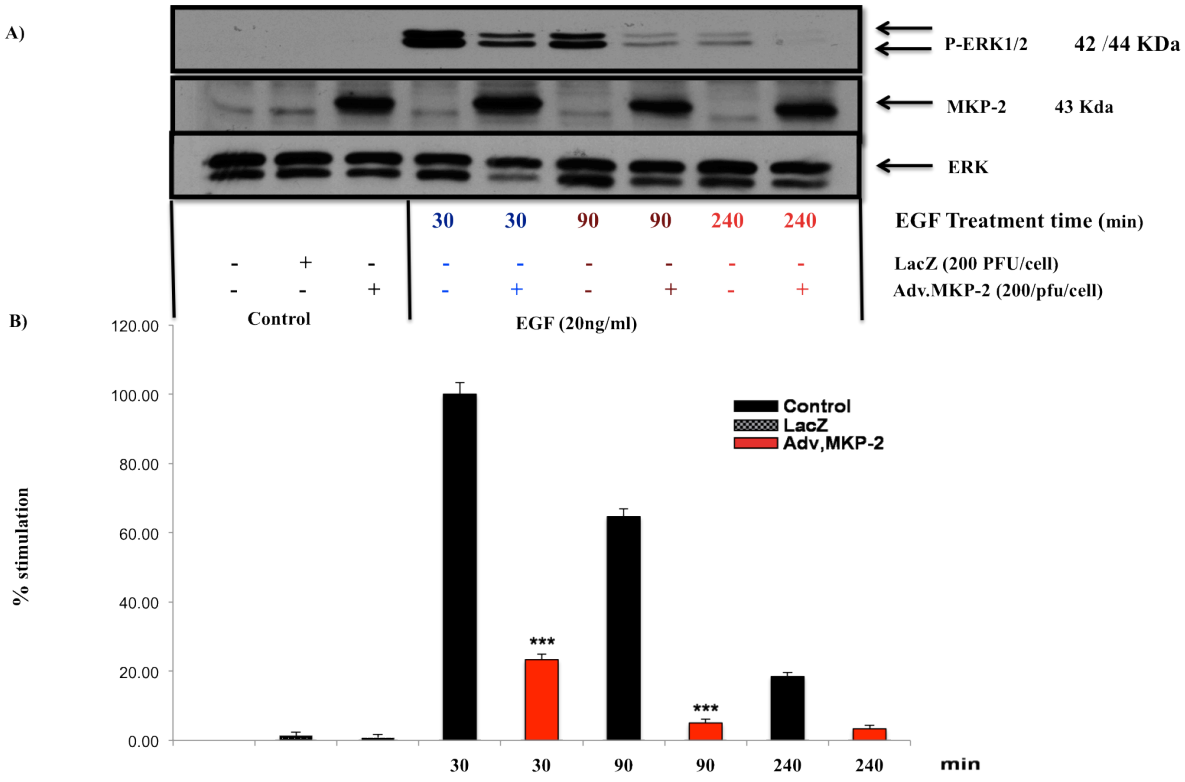


**Figure 3.15: The effect of Adv.MKP-2 on ERK phosphorylation mediated by EGF in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with EGF (20 ng/ml) for the times indicated. Whole cell lysates were prepared, separated by SDS-PAGE, and then assessed for a) p-ERK1/2 (42/44 kDa), MKP-2 (43 kDa) and ERK as outlined in Section 2.4. Blots were quantified for b) % stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, ***P<0.001 in 30 and 90 min compared with EGF stimulated control. The results are representative of 3 independent experiments.

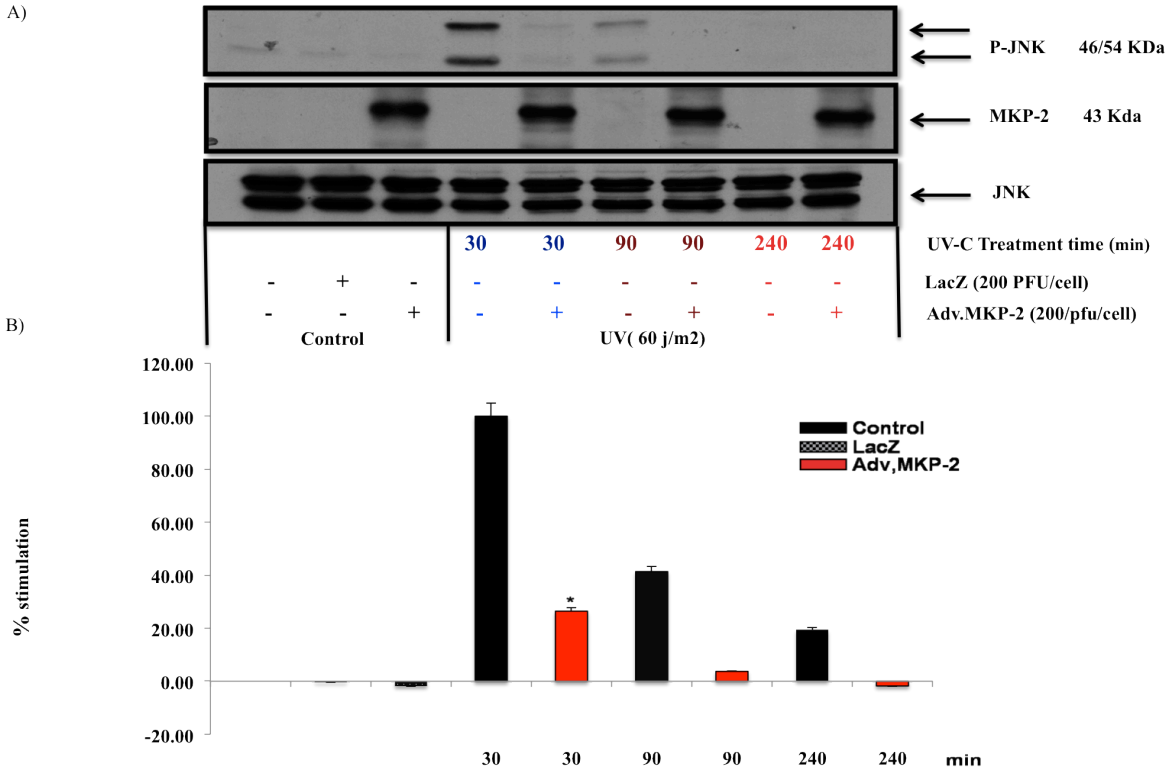


**Figure 3.16: The effect of Adv.MKP-2 on JNK phosphorylation mediated by UV-C in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with UV (60 J/m$^2$) for the times indicated (min). Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p-JNK (46/54 kDa), MKP-2 (43 kDa) and JNK as outlined in Section 2.4. Blots were quantified for b) % stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. *P<0.05 at 30 min compared with UV-C stimulated control. The results are representative of 3 independent experiments.

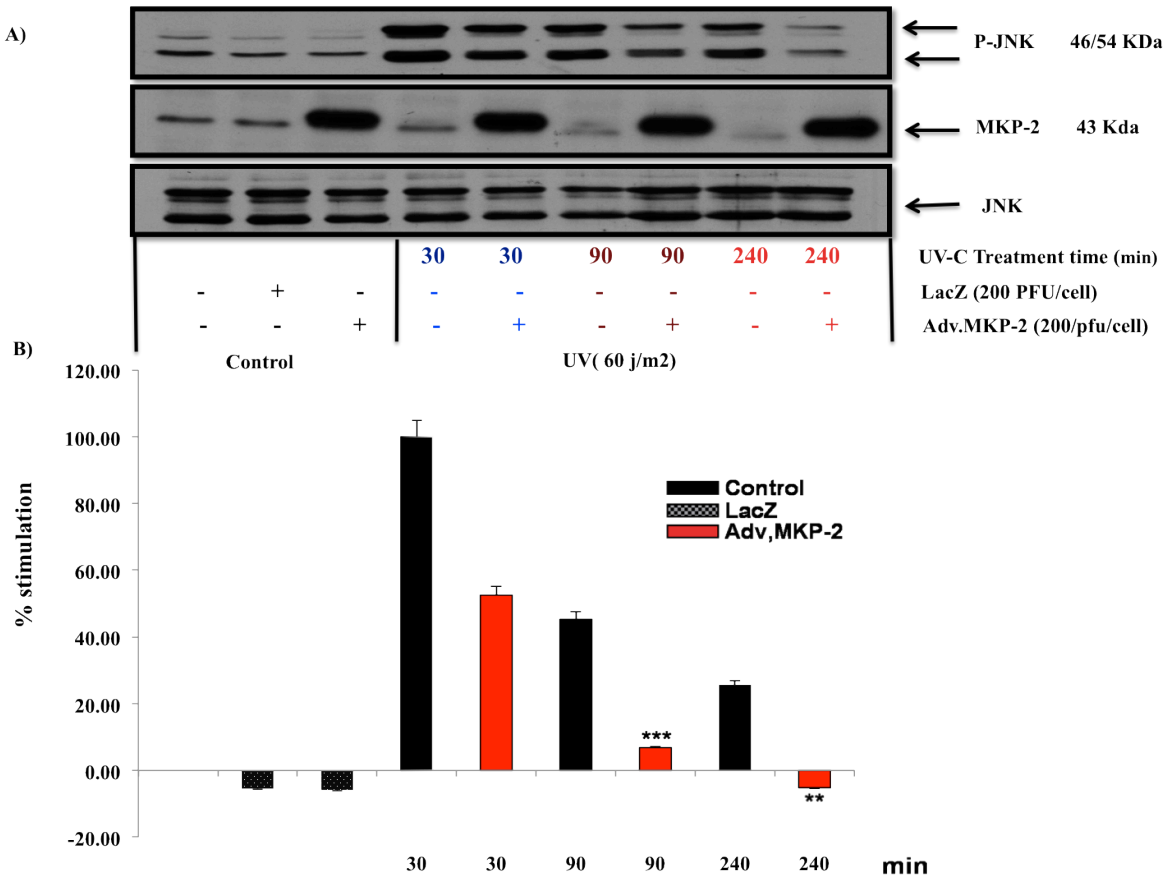


**Figure 3.17: The effect of Adv.MKP-2 on JNK phosphorylation mediated by UV-C in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with UV (60 J/m$^2$) for the time indicated as outlined in Section 2.6.4. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p-JNK (46/54 kDa), MKP-2 (43 kDa) and JNK as outlined in section 2.4. Blots were quantified for b) % stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. ***P<0.001 at 90 min, **P<0.01 at 240 min compared with UV-C stimulated control. The results are representative of 3 independent experiments.

## 3.8 THE EFFECT OF ADV.MKP-2 ON PROLIFERATION IN LNCaP (AS) AND (AI) cells

### 3.8.1 Overexpression of Adv.MKP-2 leads to a decrease in LNCaP (AS) proliferation: cell counting by haematoxylin staining

Having established from previous experiments that Adv.MKP-2 significantly inhibited ERK and JNK signalling in LNCaP (AS), further experiments were conducted to determine whether overexpression of Adv.MKP-2 could inhibit cell proliferation itself. For this purpose, LNCaP (AS) were infected with Adv.MKP-2 (200 pfu/cell) stimulated with FCS over 72 h and cellular proliferation assessed by cell haematoxylin staining. FCS stimulated a time-dependent increase in cell number which was over two fold by 24 h increasing to 3 fold at 72 h (10% FCS fold stim. at 24, 48 and 72 h = 214.0 ± 8.35, 267.0 ± 16.7, 375.3 ± 16.1 respectively, n=3). Following infection with Adv.MKP-2, cellular proliferation was reduced at all time points assessed. Moreover, inhibition of proliferation increased with increasing time, at 72 h proliferation was inhibited to a greater extent than at 48 h and 48 h more than at 24 h. Whilst infection with lacZ virus was without effect, cellular proliferation was significantly reduced at 24, 48 and 72 h (10% FCS + Adv.MKP-2 fold stim. at 24, 48 and 72 h = 76.3 ± 6.90, 96.6 ± 8.70 and 122.0 ± 24.60 respectively, n=3). Adv.MKP-2 also reduced basal cell proliferation to some extent which failed to return to basal levels throughout the time course. These results demonstrated that Adv.MKP-2 overexpression successfully inhibited the proliferation of these cells (see figure 3.18 panel A) and suggested that exogenous MKP-2 is responsible for the decrease in cell growth observed in these cells. It also suggests that MKP-2 could play a significant role in proliferation in these cells.

### 3.8.2 Overexpression of Adv.MKP-2 leads to a decrease in LNCaP (AI) proliferation: cell counting by haematoxylin staining

The effect of Adv.MKP-2 on LNCaP (AI) proliferation was then examined. Again FCS stimulated an increase in proliferation, however the increase in cell number for all time points was less than for AS cells, reaching approximately 2.8 fold over 72 h. (10% FCS fold stim. at 24, 48 and 72 h = 204.0± 8.41, 215.0 ± 17.9, 277.0 ± 14.4 respectively, n=3). Similar to LNCaP (AS), cellular proliferation was reduced in presence of Adv.MKP-2 at all time points assessed. Furthermore,

proliferation was reduced to a greater extent with increasing time, at 24 h there was less inhibition than at 48 h and 72 h, Adv.MPK-2 essentially reduced proliferation to resting levels. Whereas infection with lacZ virus was without effect, the reduction with Adv.MKP-2 was marked (10% FCS + Adv.MKP-2 fold stim. at 24, 48 and 72 h = 153.3 ± 11.90, 137.3 ± 2.02 and 119.0 ± 3.60 respectively, n=3). These results demonstrated that Adv.MKP-2 overexpression successfully inhibited the proliferative of these cells (see figure 3.19 panel A).

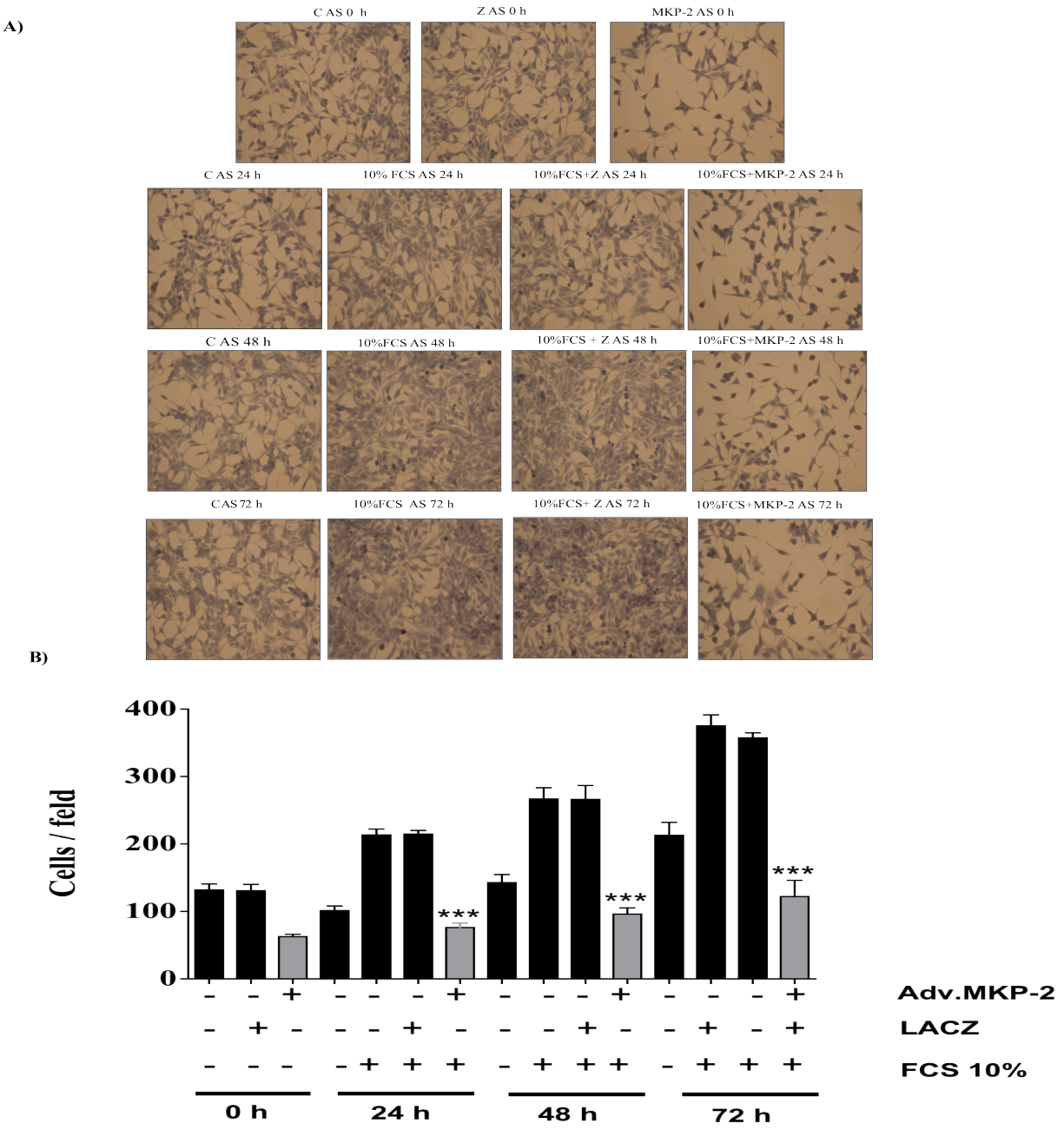


**Figure 3.18: The effect of Adv.MKP-2 on proliferation in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for either 24, 48, 72 h. Cultures were washed with PBS, and stained with haematoxylin as shown in panel A, (picture of CFs taken by Motic Images plus 2.0 software). The number of cells was determined from 10 random fields per each coverslip (see section 2.7.1). In panel B, Quantified data was analysed using two way ANOVA. Post hoc test was by Bonferroni's multiple comparison tests. Statistical significance is shown in relation to FCS stimulated control, at 24, 48 and 72 h, ***P<0.001. The results are from experiments performed in triplicate from 3 separate experiments (n=3).

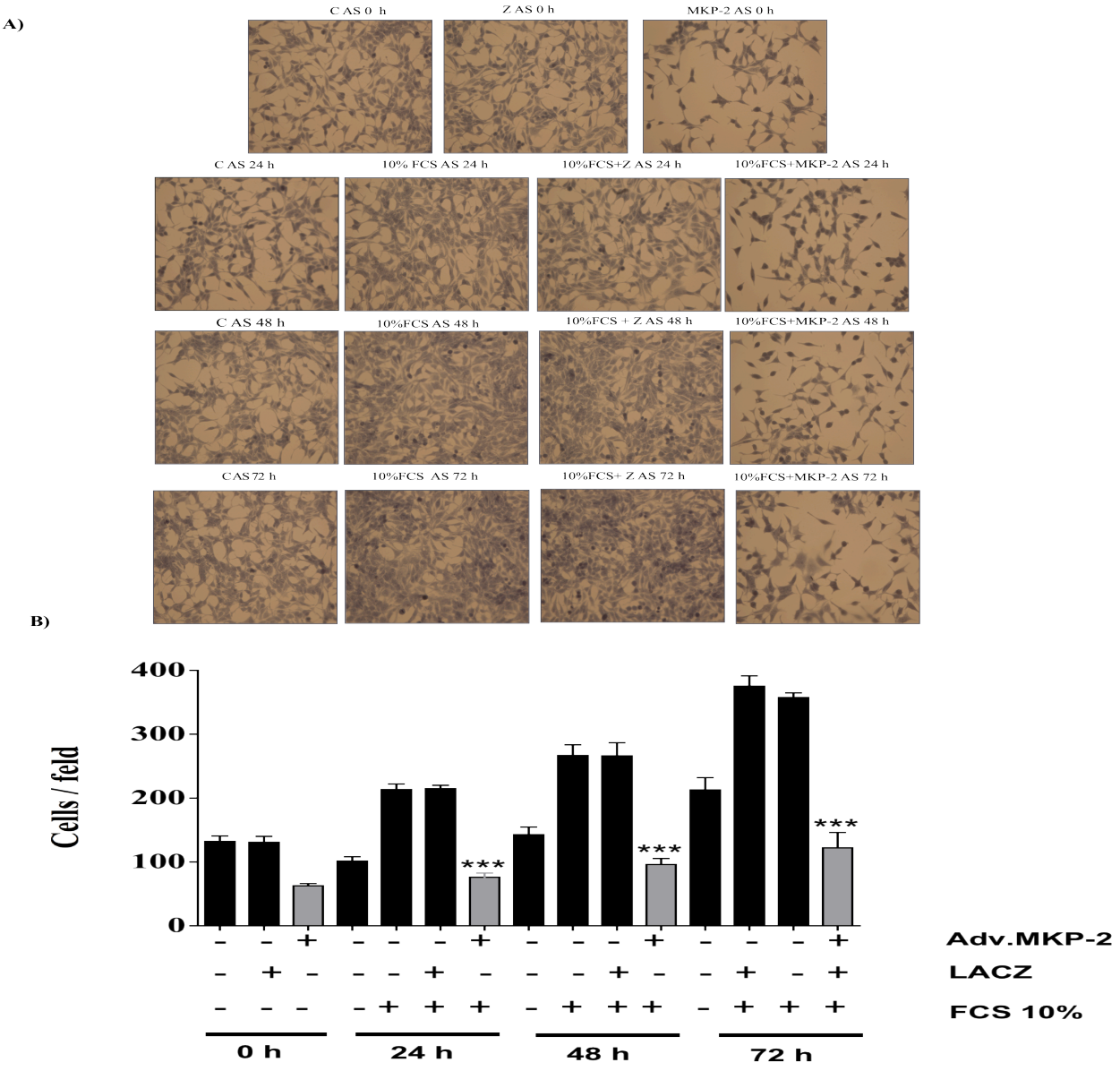


**Figure 3.19: The effect of Adv.MKP-2 on proliferation in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for either 24, 48, 72 h. cultures were washed with PBS, and stained with haematoxylin as shown in panel A, (picture of CFs taken by Motic Images plus 2.0 software). The number of cells was determined from 10 random fields per each coverslip (see section 2.7.1). In panel B, Quantified data was analysed using two way ANOVA. Post hoc test was by Bonferroni's multiple comparison tests. Statistical significance is shown in relation to FCS stimulated control, at 48 and 72 h, **P<0.01 and ***P<0.001 respectively. The results are from experiments performed in triplicate from 3 separate experiments (n=3).

## 3.9 THE EFFECT OF ADV.MKP-2 ON CELL CYCLE PROGRESSION IN LNCaP (AS) AND (AI) CELLS

Having established that Adv.MKP-2 overexpression is able to negatively regulate proliferation of both LNCaP (AS) and (AI) cells, the effect on individual stages of the cell cycle were examined. This was assessed using FACS analysis and these findings correlated with effects upon cell cycle protein expression and phosphorylation.

### 3.9.1 FCS mediated cell cycle progression in LNCaP (AS) cells

In order to assess the effect of Adv.MKP-2 on cell cycle progression, the growth factor FCS was used and cells stained using propidium iodide. Figure 3.20, shows cell cycle progression (panel A) stimulated by FCS (10 and 20%) for 24, 48 and 72 h in LNCaP (AS). Firstly, cells in the absence of stimulation where examined. It was found that the % of cells in S phase was (5.2, 5.2 and 3.6), whilst, into $G_2$/M phase was (3.0, 1.0 and 0.0) at 24, 48 and 72 h respectively. The figure shows that following stimulation for 24 h there was a minor increase of cells in S phase and, a corresponding decrease of cells in $G_1$. However, at 48 h there was a more marked entry of cells into S phase which increased from a control value of 5.2 ± 1.1 sem, to a stimulated value of 13.7 ± 3.1 sem. A similar increase was observed at both 48 and 72 h coupled with a corresponding decrease of cells in $G_1$ (approximately 25%).

### 3.9.2 FCS mediated cell cycle progression in LNCaP (AI) cells

The effect of FCS was then compared in LNCaP (AI) cells, figure 3.21 again shows cell cycle progression stimulated by FCS (10 and 20%) for 24, 48 and 72 h. In contrast to LNCaP (AS) cells, cell cycle progression in LNCaP (AI) was more rapid with an increase in cells entering S phase after 24 h, of 8.6 ± 5.1 sem, in Control vs 20.8 ± 4.1 sem, following serum stimulation. In addition, cells in S phase decreased slightly after 48 h and were further reduced by 72 h. Again these values corresponded to decrease cell numbers in phase $G_1$.

### 3.9.3 The effect of Adv.MKP-2 on cell cycle progression in LNCaP (AS) cells

Next the effect of Adv.MKP-2 on cell cycle progression was examined using cells stimulated with 10% FCS for 48 h. Figure 3.22 shows that FCS alone as expected, caused an increase in cells progressing into both S phase and $G_2$/M. Whilst LacZ was without effect, 10% FCS + Adv.MKP-2 significantly reduced cell numbers in both phases. Overall, a 50 to 60% reduction was observed for S phase and approximately 50% for $G_2$/M phase (10.5 and 18.1 vs 7.3 and 13.3 respectively, n=3). In contrast, LacZ was without significant effect.

### 3.9.4 The effect of Adv.MKP-2 on cell cycle progression in LNCaP (AI) cells

Figure 3.23 shows also the effect of Adv.MKP-2 (200 pfu/cell) upon cell cycle progression in response to FCS, in LNCaP (AI). Stimulation with FCS over 24 h caused a marked increase in cells both in S phase and $G_2$/M phase. Following infection with Adv.MKP-2 progression was inhibited, cells in both S phase and $G_2$/M phase where reduced by approximately 40% (20.9 and 23.4 vs 12.4 and 14.9 respectively, n=3). In contrast, LacZ without significant effect.

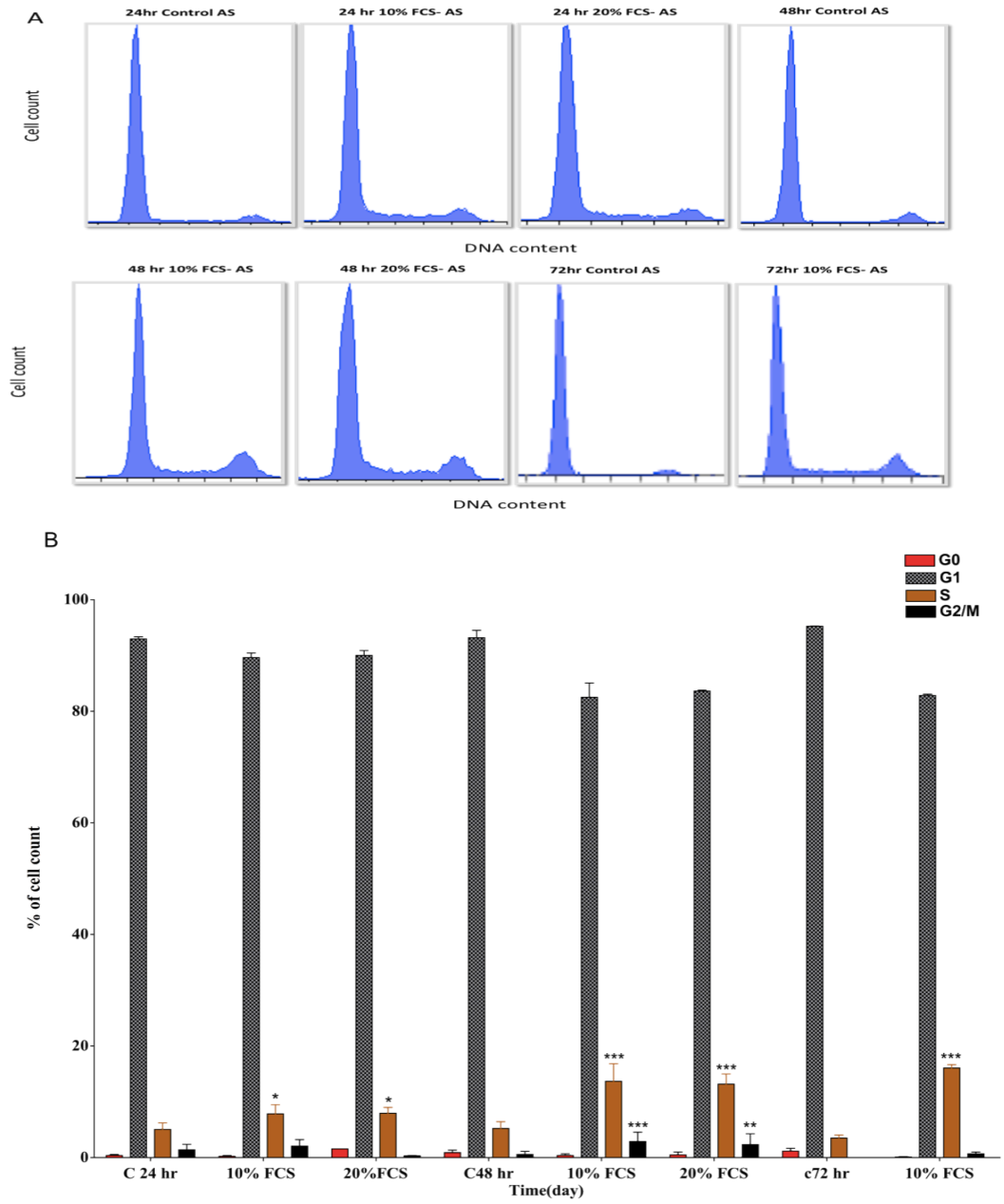


**Figure 3.20: Time course of FCS on cell cycle progression in LNCaP (AS) cell lines.** Cells stimulated with 10% and 20% FCS for the times indicated, then assessed by flow cytometery using propidium iodide (PI) for determination of total DNA content as outlined in Section 2.8. In (panel A), data are representative of actual analysis of cell cycle distribution for LNCaP (AS) cells performed in triplicate with similar results. In panel B, the bar chart quantifies the distribution of cells in the different phases of the cell cycle following FCS treatment, from three replicate experiments. Each value represents the mean ± s.e.m. ***P<0.001 with 10% FCS in both S and $G_2$/M phase at 48 h, compared with 48h control. The results are representative of 3 independent experiments.

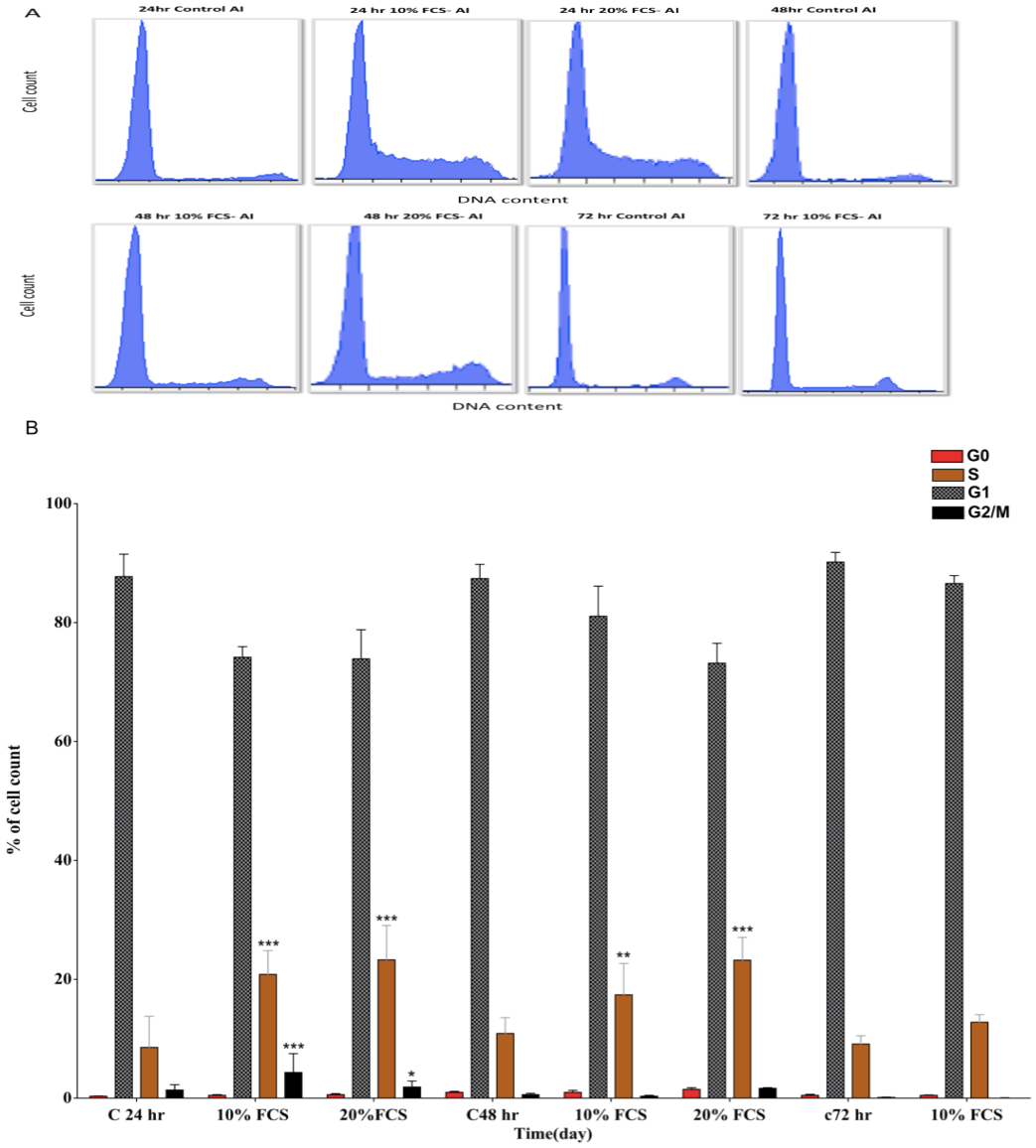


**Figure 3.21: Time course of FCS on cell cycle progression in LNCaP (AI) cell lines.** Cells stimulated with 10% and 20% FCS for the times indicated, then assessed by flow cytometry using propidium iodide (PI) for determination of total DNA content as outlined in Section 2.8. In panel A, data are representative of actual analysis of cell cycle distribution for LNCaP (AI) cells performed in triplicate with similar results. In panel B, the bar chart quantifies the distribution of cells in the different phases of the cell cycle following FCS treatment. Each value represents the mean ± s.e.m. **P<0.001 with 10% FCS. The results are representative of 3 independent experiments.

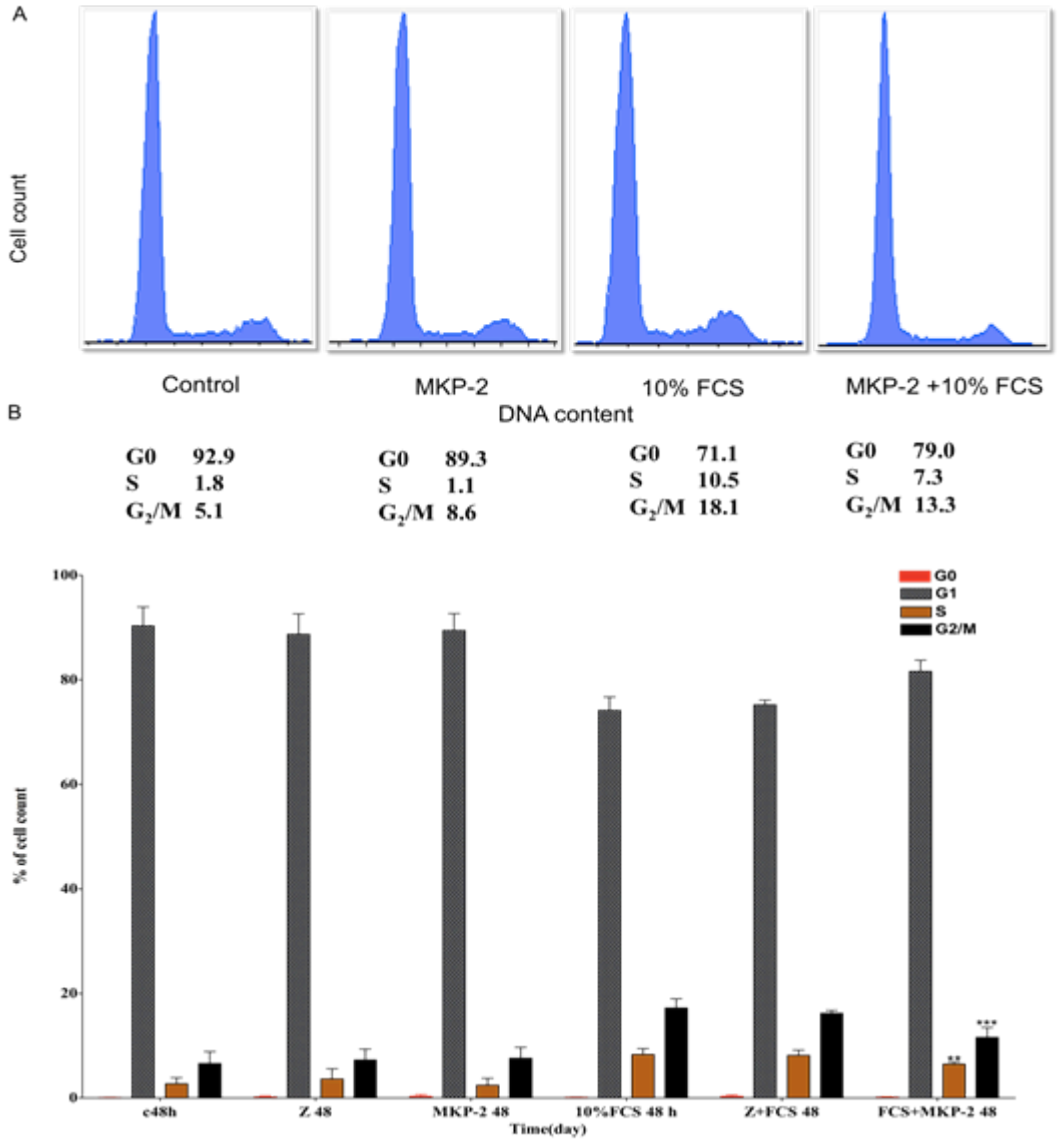


**Figure 3.22: The effect of MKP-2 on cell cycle progression in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior stimulation with 10 % FCS for 48 h, then assessed by flow cytometry using propidium iodide (PI) for determination of total DNA content as outlined in Section 2.8. In Panel A, a representative FACS plot is shown for 10 % FCS and distribution for LNCaP (AS) cells. Panel B, shows a bar chart of distribution of cells in the different phases of the cell cycle following FCS treatment, each value represents the mean ± s.e.m. Statistical analysis was by two-way ANOVA with a post hoc Dunnet's test, **P<0.01 and ***P<0.001, compared with 10% FCS stimulated control. The results are representative of 3 independent experiments.

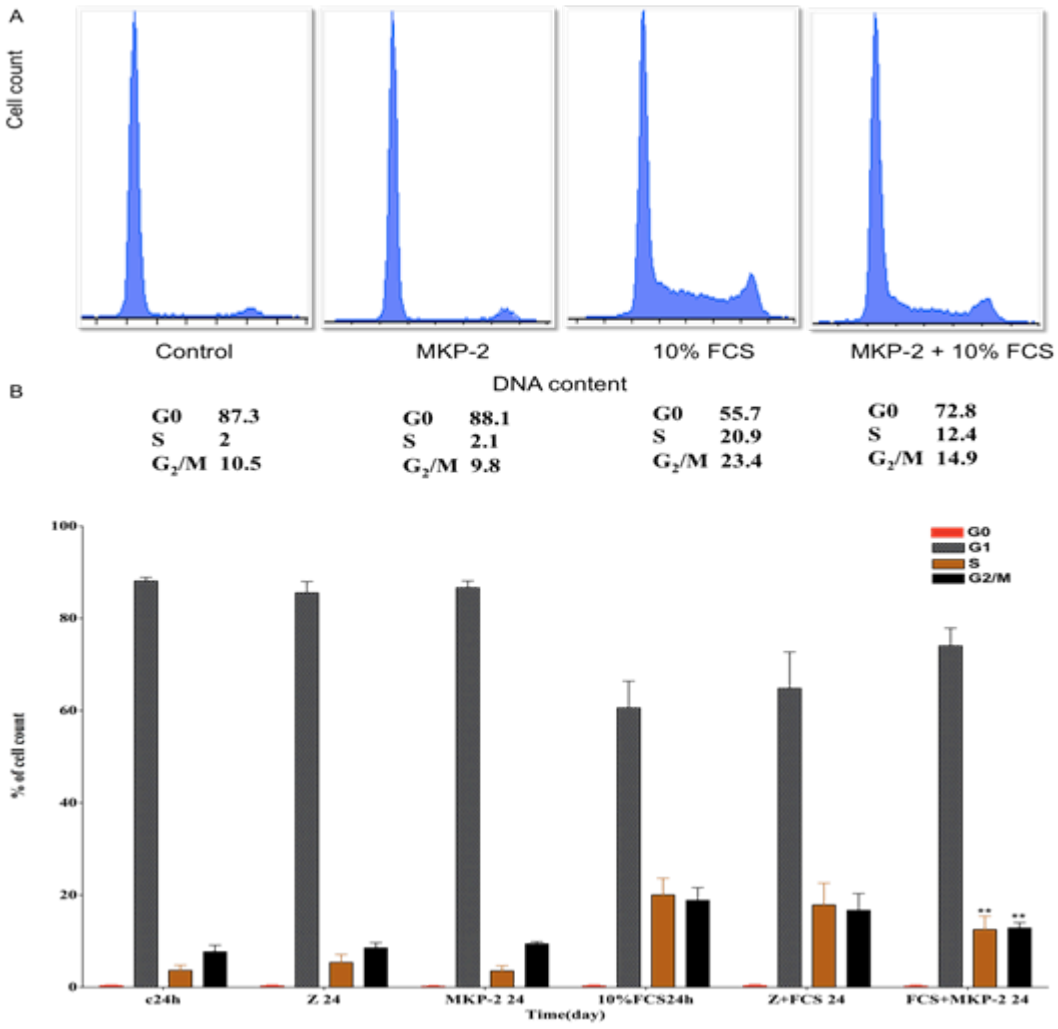


**Figure 3.23: The effect of Adv.MKP-2 on cell cycle progression in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior stimulation with 10 % FCS for 24 h, then assessed by flow cytometry using propidium iodide (PI) for determination of total DNA content as outlined in Section 2.8. In Panel A, a representative FACS plot is shown for 10 % FCS and distribution for LNCaP (AI) cells. In Panel B, the bar chart quantities distribution of cells in the different phases of the cell cycle following FCS treatment, each value represents the mean ± s.e.m. Statistical analysis was by two-way ANOVA, **P<0.01 with Adv.MKP-2 in both S and $G_2$/M phase at 24 h, compared with 10% FCS stimulated control. The results are representative of 3 independent experiments.

## 3.10 THE EFFECT OF ADV.MKP-2 ON CELL CYCLE PROTEIN REGULATOR IN LNCaP (AS) AND (AI) CELLS

### 3.10.1 The effect of Adv.MKP-2 on FCS mediated p-cdc2 phosphorylation in LNCaP (AS) cells

In order to pin-point further the effect of Adv.MKP-2 on the cell cycle a number of relevant cell cycle proteins were examined. Figure 3.24, shows the effect of Adv.MKP-2 (200 pfu/cell) upon cdc-2 phosphorylation a mailer for $G_2$/M phase. Stimulation with FCS induced a significant increase in cdc2 phosphorylation at 24 h and, this phosphorylation increased to a greater extend at 48 h (10% FCS fold stim. at 24 and 48 h = 2.83 ± 0.12 and 3.10 ± 0.24, respectively, n=3). Whilst LacZ was without effect, in contrast, infection with Adv.MKP-2 significantly inhibited the phosphorylation of cdc-2 at 24 h by approximately 50 % and greater than 60% at 48 h. (10% FCS + Adv.MKP-2 fold stim. at 24 and 48 h = 2.13 ± 0.06 and 2.27 ± 0.07, respectively, n=3, *P<0.05 and **P<0.01 respectively).

### 3.10.2 The effect of Adv.MKP-2 on FCS mediated p-cdc2 phosphorylation in LNCaP (AI) cells

Similar to LNCaP (AS), LNCaP (AI) was investigated to show the effect of Adv.MKP-2 (200 pfu/cell) upon cdc-2 phosphorylation (figure 3.25). Stimulation with FCS induced a significant increase in cdc-2 phosphorylation at 24 h and, this phosphorylation increased more at 48 h, (10% FCS fold stim. at 24 and 48 h = 3.10 ± 0.11 and 3.20 ± 0.12, respectively, n=3). Whilst LacZ without effect, in contrast, infection with Adv.MKP-2 significantly inhibited the phosphorylation of cdc-2 at 24 h by greater than 50 % and by approximately 40% at 48 h (10% FCS + Adv.MKP-2, fold stim. at 24 and 48 h = 2.30 ± 0.12 and 2.70 ± 0.14, respectively, n=3, ***p<0.001 and *p<0.05 respectively).

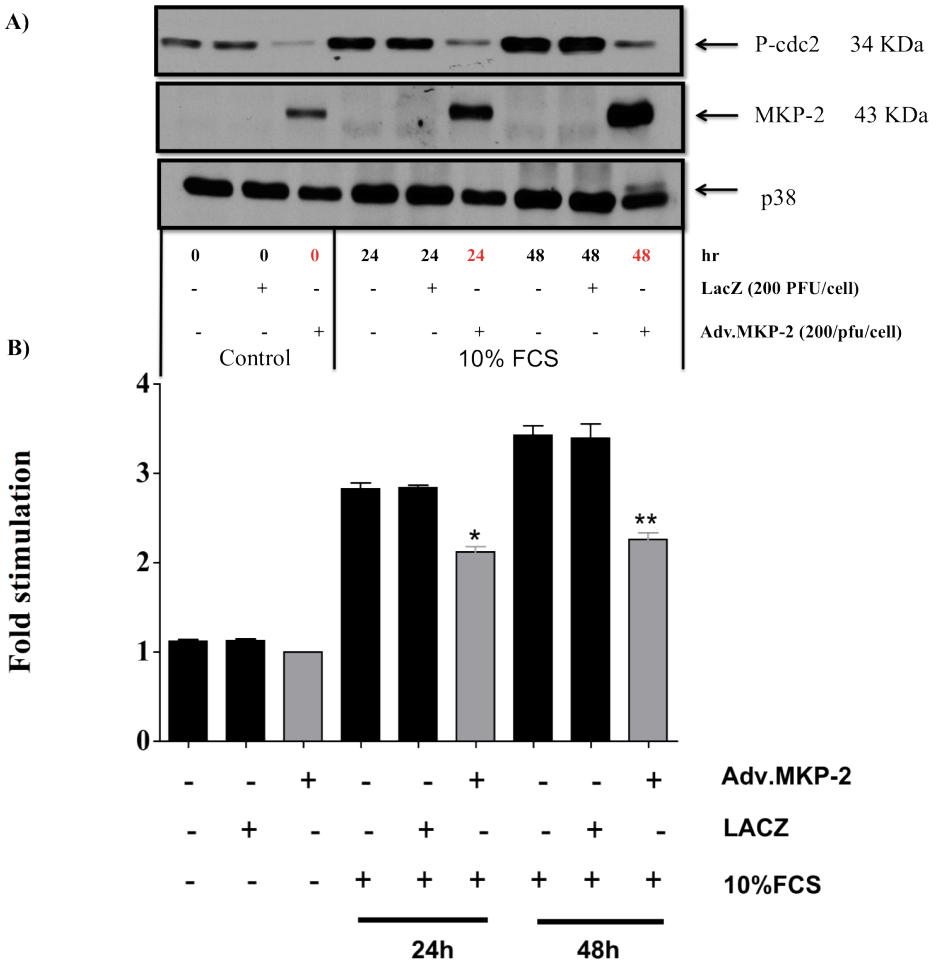


**Figure 3.24: The effect of Adv.MKP-2 on FCS stimulated cdc-2 phosphorylation in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p-cdc-2 (34 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, *P<0.05 and **p<0.01 at 24 and 48 h respectively, compared with FCS stimulated control. The results are representative of 3 independent experiments.

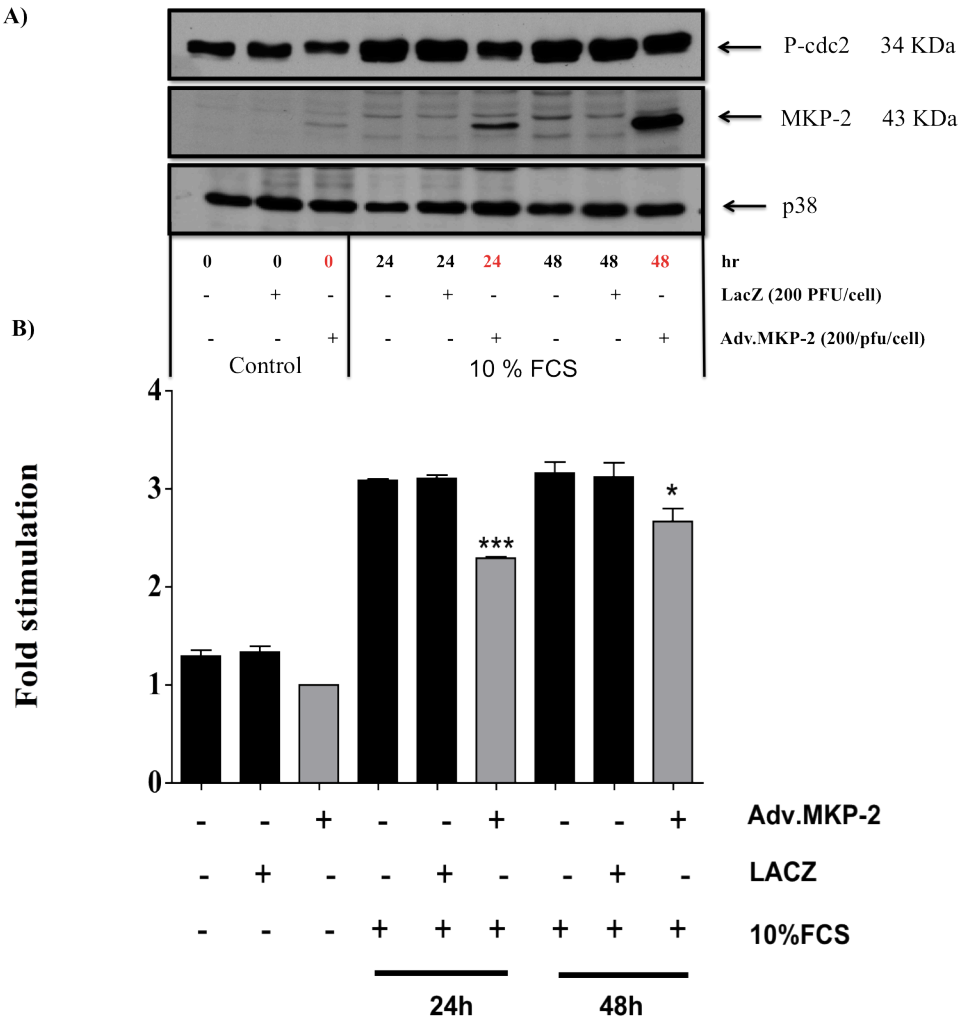


**Figure 3.25: The effect of Adv.MKP-2 on FCS stimulated cdc-2 phosphorylation in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p-cdc-2 (34 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, ***<0.001, *P<0.05 at 24 and 48 h respectively, compared with FCS stimulated control. The results are representative of 3 independent experiments.

### 3.10.3 The effect of Adv.MKP-2 on FCS mediated cyclin B1 expression in LNCaP (AS) cells

Figure 3.26, shows the effect of Adv.MKP-2 upon cyclin B1 expression as a further measure of $G_2$/M-phase. Stimulation with FCS induced a significant increase in cyclin B1 expression but only at 48 h (10% FCS fold stim. at 24 and 48 h = 1.32 ± 0.02 and 2.60 ± 0.10, respectively, n=3). Whilst LacZ was without effect, in contrast, infection with Adv.MKP-2 significantly inhibited the phosphorylation of cyclin B1 at 24 h by approximately 30 % and around 80% at 48 h. (10% FCS + Adv.MKP-2 fold, stim. at 24 and 48 h = 1.24 ± 0.02 and 1.30 ± 0.04, respectively, n=3, ***P< 0.001 at 48 h).

### 3.10.4 The effect of Adv.MKP-2 on FCS mediated cyclin B1 expression in LNCaP (AI) cells

Similarly, the effect of Adv.MKP-2 (200 pfu/cell) upon cyclin B1 was assessed in LNCaP (AI) cells (Figure 3.27). The figure shows that stimulation with FCS induced a significant increase of cyclin B1 phosphorylation at 24 h and this phosphorylation increased further at 48 h, (10% FCS fold stim. at 24 and 48 h = 2.70 ± 0.11 and 2.82 ± 0.12, respectively, n=3). Whilst LacZ was without effect, infection with Adv.MKP-2 significantly inhibited the phosphorylation of cyclin B1 at both 24 and 48 h greater than 80%. (10% FCS + Adv.MKP-2 fold, stim. at 24 and 48 h = 1.20 ± 0.01 and 1.40 ± 0.08, respectively, n=3, ***p<0.001 at 24 and 48 h). This confirms the potential of an effect upon $G_2$/M-phase of the cell cycle.

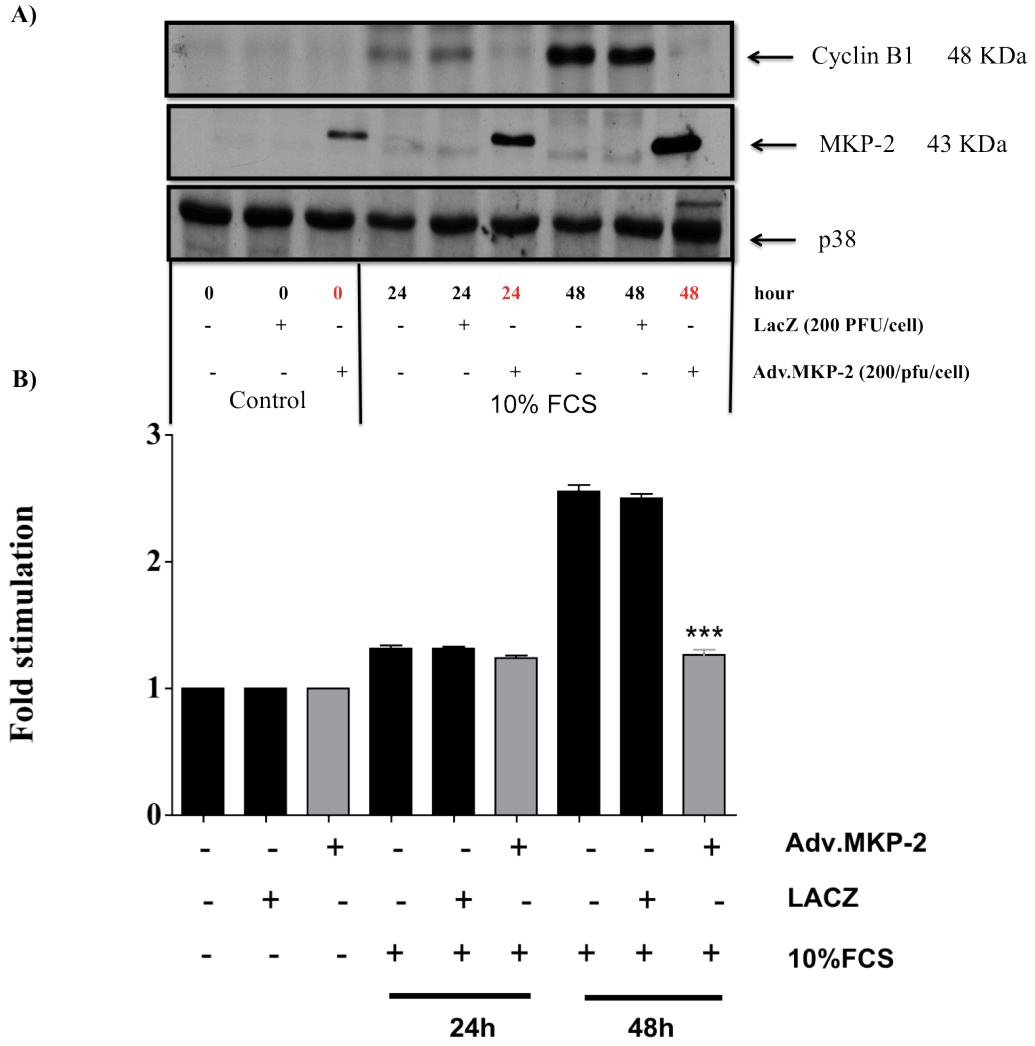


**Figure 3.26: The effect of Adv.MKP-2 on FCS stimulated cyclin B1 expression in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) cyclin B1 (48 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, ***P<0.001, compared with FCS stimulated control. The results are representative of 3 independent experiments.

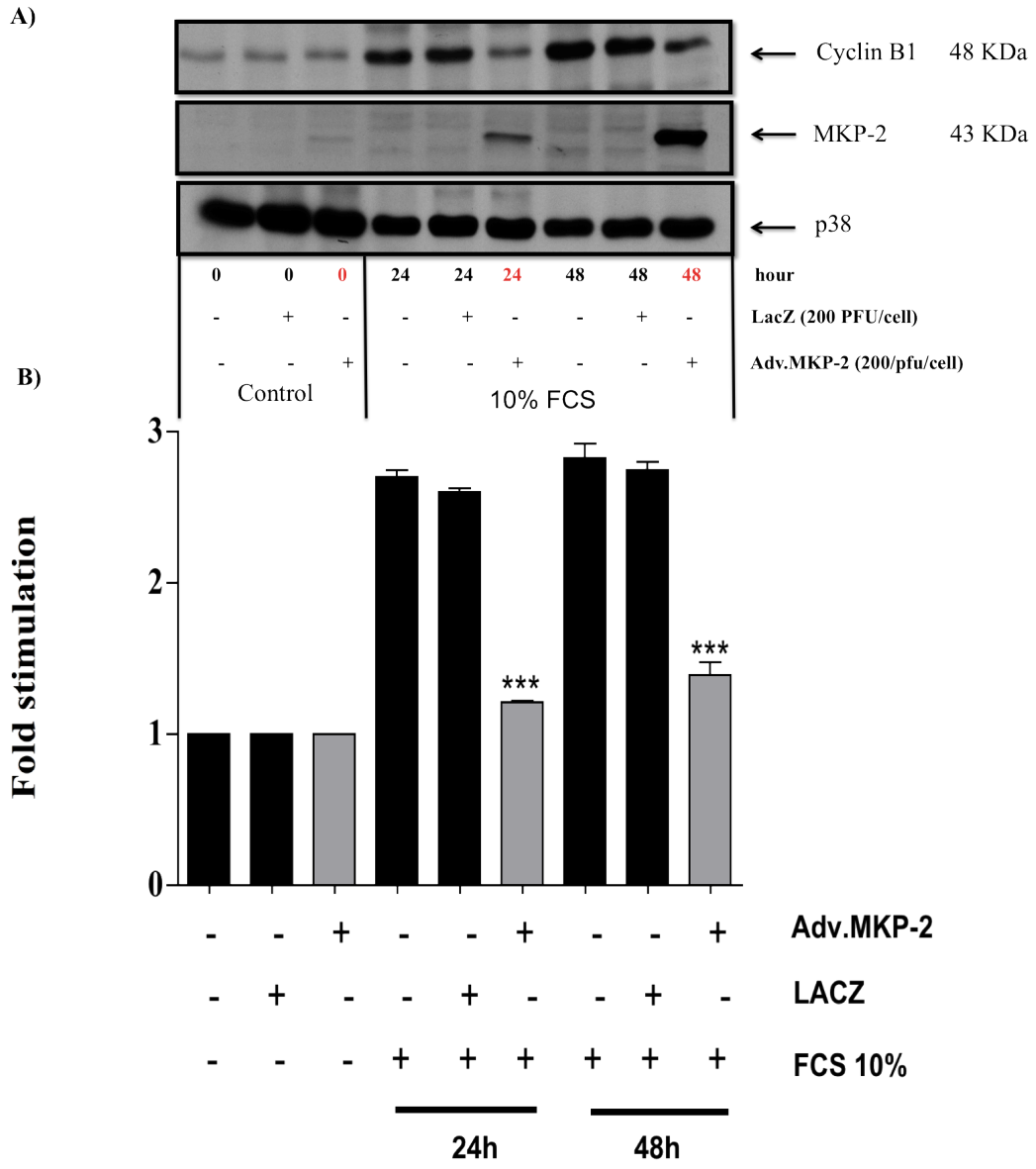


**Figure 3.27: The effect of Adv.MKP-2 on FCS stimulated cyclin B1 expression in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) cyclin B1 (48 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, ***P<0.01 at 24 and 48 h, compared with FCS stimulated control. The results are representative of 3 independent experiments.

### 3.10.5 The effect of Adv.MKP-2 on FCS mediated cyclin D1 phosphorylation in LNCaP (AS) cells

Figure 3.28, shows the effect of Adv.MKP-2 (200 pfu/cell) upon cyclin D1 signalling in response to a shorter period of FCS stimulation at 4 and 8 h. Stimulation with FCS induced significant increase of cyclin D1 phosphorylation at both 4 and 8 h (10% FCS fold stim, FCS at 4 and 8 h = 2.45 ± 0.11 and 2.64 ± 0.12, respectively, n=3). Whilst LacZ was without effect, infection with Adv.MKP-2 significant inhibited the phosphorylation of cyclin D1 at 4 h only, at 8 h there was inhibition but it was not significant. The inhibition was slightly more at 4 h, 55%, whilst at 8 h it was a 30%. (10% FCS + Adv.MKP-2 fold stim. at 4 and 8 h = 1.23 ± 0.02 and 1.38 ± 0.04, respectively, n=3, *$p<0.05$ at 4 h).

### 3.10.6 The effect of Adv.MKP-2 on FCS mediated cyclin D1 phosphorylation in LNCaP (AI) cells

The effect of Adv.MKP-2 (200 pfu/cell) upon cyclin D1 signalling in response to FCS at 4 and 8 h is shown in figure 3.29. The Figure shows that stimulation with FCS induced a significant increase of cyclin D1 phosphorylation at 4 h and this phosphorylation slightly increased at 8 h (10% FCS fold stim. at 4 and 8 h = 2.40 ± 0.11 and 2.62 ± 0.12, respectively, n=3). Although LacZ was without effect, infection with Adv.MKP-2 did not additionally inhibit the phosphorylation of cyclin D1 either at 4 or 8 h. (10% FCS + Adv.MKP-2, fold stim. at 4 and 8 h = 2.46 ± 0.10 and 2.68 ± 0.12, respectively, n=3).

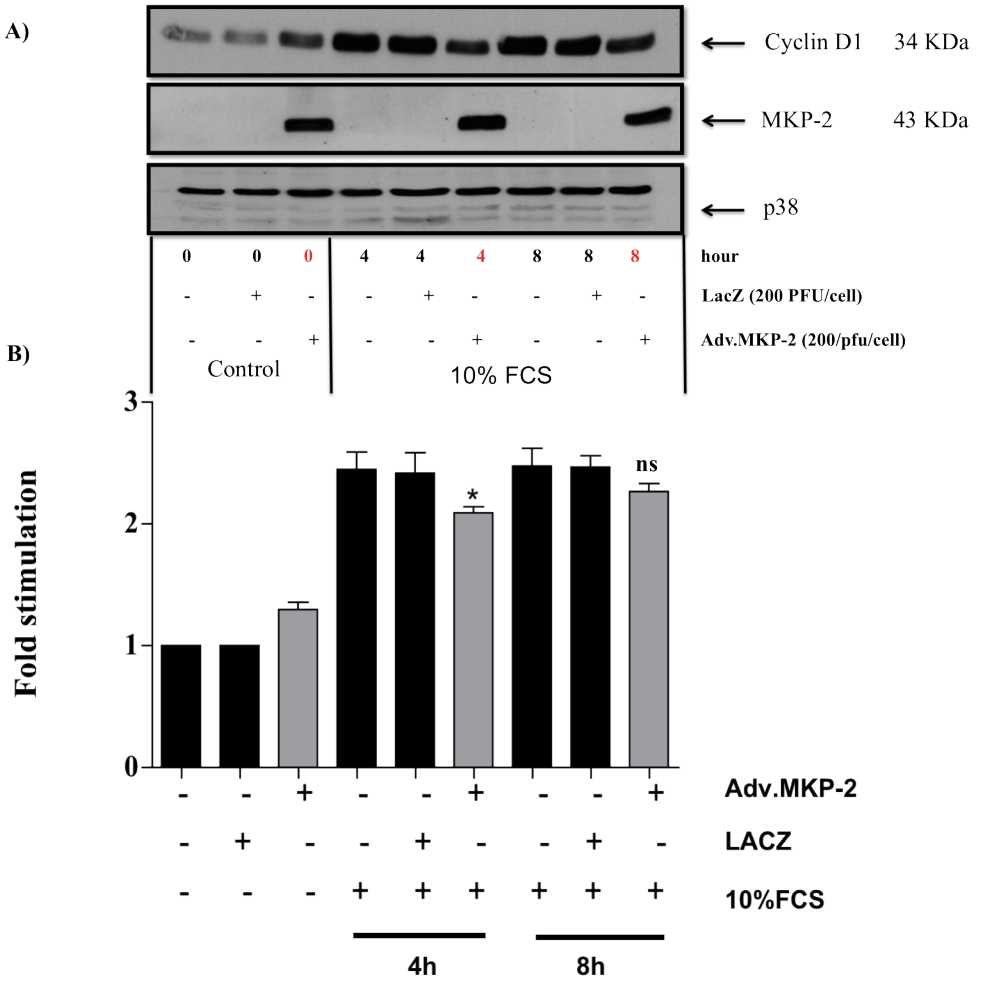


**Figure 3.28: The effect of Adv.MKP-2 on FCS stimulated cyclin D1 expression in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) cyclin D1 (34 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, *p >0.05 at 4 h, compared with FCS stimulated control. The results are representative of 3 independent experiments.

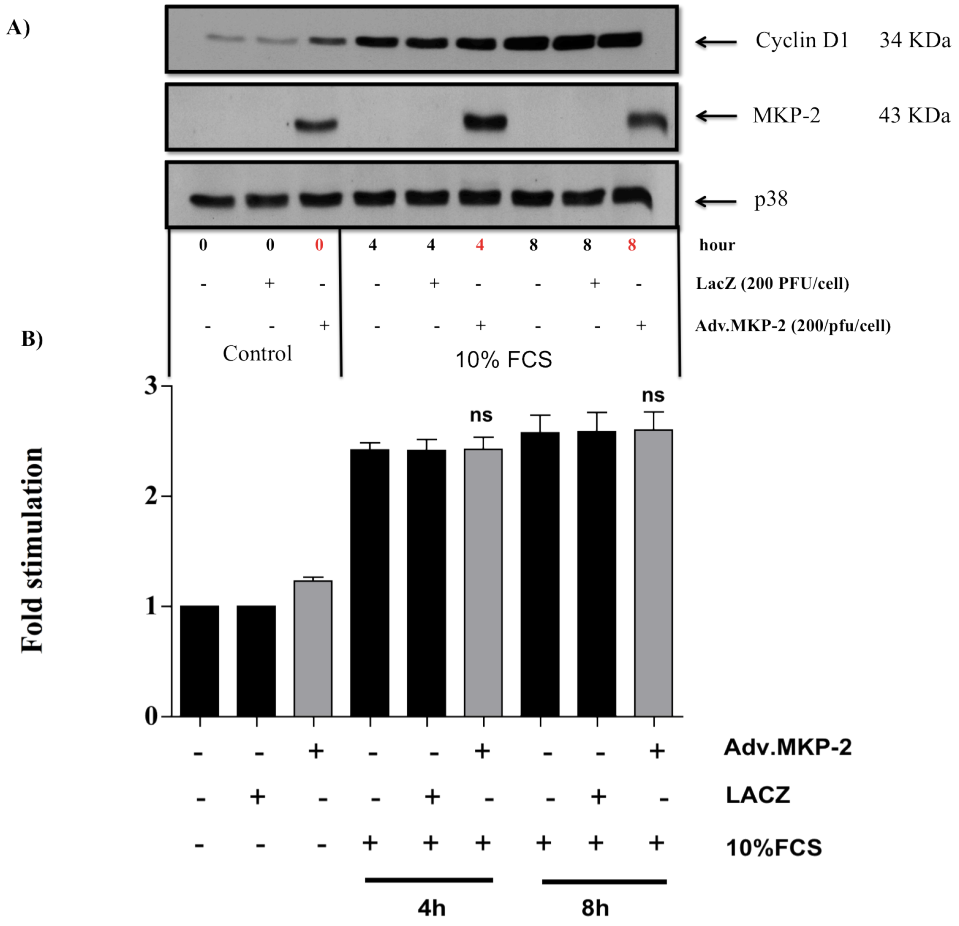


**Figure 3.29: The effect of Adv.MKP-2 on FCS stimulated cyclin D1 expression in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2 for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) cyclin D1 (34 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. The results are representative of 3 independent experiments.

## 3.11 THE EFFECT OF ADV.MKP-2 ON APOPTOSIS IN LNCaP (AS) AND (AI) CELLS

### 3.11.1 UV-C-mediated apoptosis in LNCaP (AS) cells

Having established in section 3.9, that Adv.MKP-2 was able to inhibit cell cycle progression, the potential for Adv.MKP-2 to modify LNCaP (AS) cell apoptosis using flow cytometry analysis was assessed. Several studies have demonstrated that Ultraviolet (UV) light C is a model agent to induce apoptosis in several cell lines (Dunkern et al., 2001, Tomicic et al., 2005, Zhao et al., 2012) and, this was initially characterised. Samples were analysed for Annexin V-phycoerythin and 7-Amino-Actinomycin-D (7-AAD) staining. Figure 3.30, shows a strong increase in apoptosis in response to UVC exposure (60 J/m2), which was apparent in early and late apoptosis, (% of apoptosis at 60 J/m2 = 52.7, compared with normal control = 25.2, n=1).

### 3.11.2 UV-C-mediated apoptosis in LNCaP (AI) cells

LNCaP (AI) cells were also examined. The data in figure 3.31 shows, a strong increase in apoptosis in response to both 60 and 90 ($J/m^2$) which was apparent in early and late apoptosis (% cell death at 60 and 90 $J/m^2$= 71.4 and 82.0, respectively, compared with control = 41.1, n=1).

### 3.11.3 Adv.MKP-2 infection does not modify UV-C mediated apoptosis in LNCaP (AS) cells

The effect of Adv.MKP-2 on UV-C mediated apoptosis was also examined in both cell lines. In figure 3.32, LNCaP (AS) cells were infected with Adv.MKP-2 (200 pfu/cell) for 40 h and then exposed to UVC (60 $J/m^2$). Samples were then analyzed for Annexin V-phycoerythin and 7-Amino-Actinomycin-D (7-AAD) staining. Stimulation with UV-C alone caused a significant increase in apoptosis (% cell death, control= 27.2 ± 10.5, UV-C= 50.2 ± 5.30, n=3), which was not reversed by infecting cells with Adv.MKP-2 + UV-C (% cell death at 60 J/m2 = 43.0 ± 8.50, n=3).

### 3.11.4 Adv.MKP-2 infection does not modify UV-C mediated apoptosis in LNCaP (AI) cells

Figure 3.33 shows, that UV-C induced an increase in apoptosis, in LNCaP (AI) cells were infected with Adv.MKP-2 (200 pfu/cell) for 40 h and, then exposed to UV-C (60 J/$m^2$). Stimulation with UV-C alone caused a significant increase in apoptosis (% cell death, control= 25.6 ± 2.70, LacZ= 25.3 ± 2.90, Adv.MKP-2= 34.4 ± 2.80, UVC= 60.4 ± 7.10, n=3). However again, Adv.MKP-2 did not reverse apoptosis induced by UV-C (% cell death at 60 J/m2 +Adv.MKP-2 = 51.3 ± 4.90, n.s, n=3).

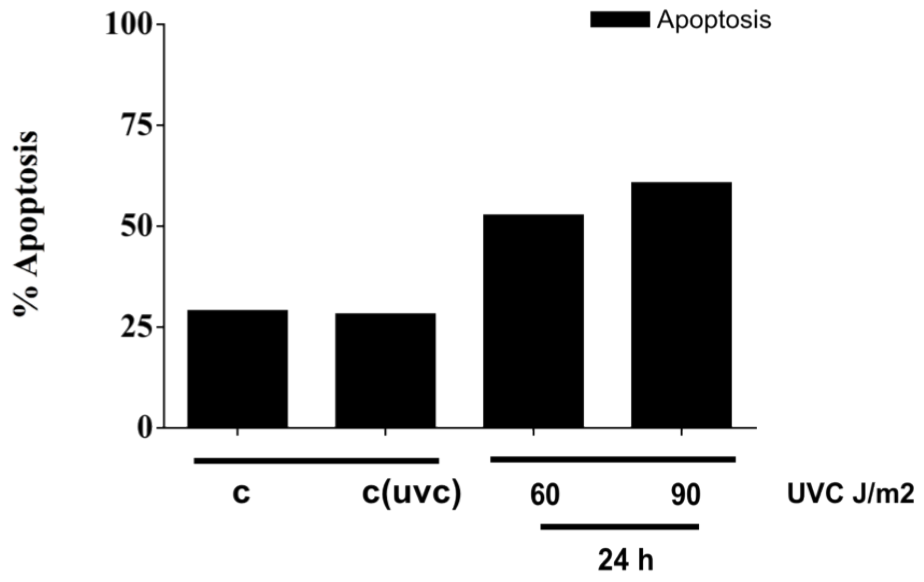


**Figure 3.30: UV-C-mediated apoptosis in LNCaP (AS) cell lines.** Cells were starved for 40 h prior to stimulation with UV-C (60 J/m$^2$) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9, n=1.

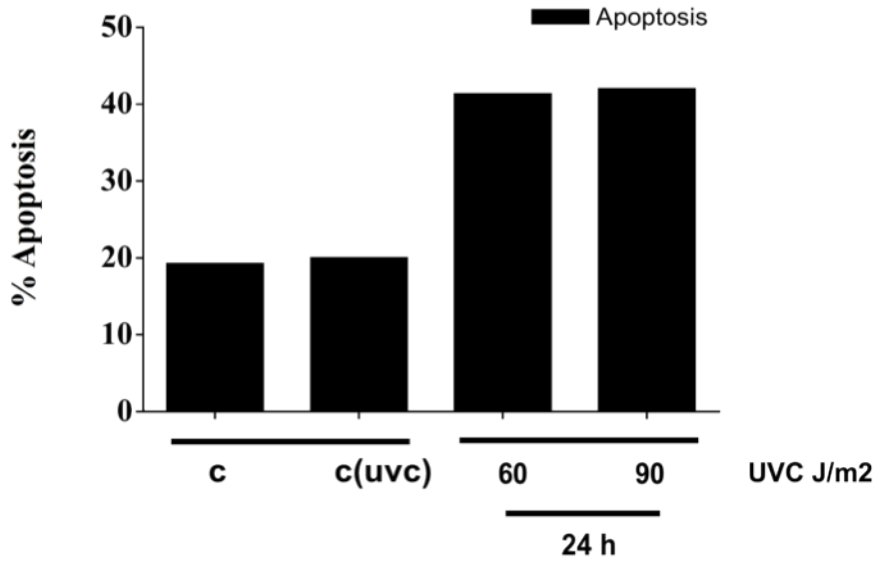


**Figure 3.31: UV-C-mediated apoptosis in LNCaP (AI) cell lines.** Cells were starved for 40 h prior to stimulation with UV-C (60 J/m$^2$) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9, n=1.

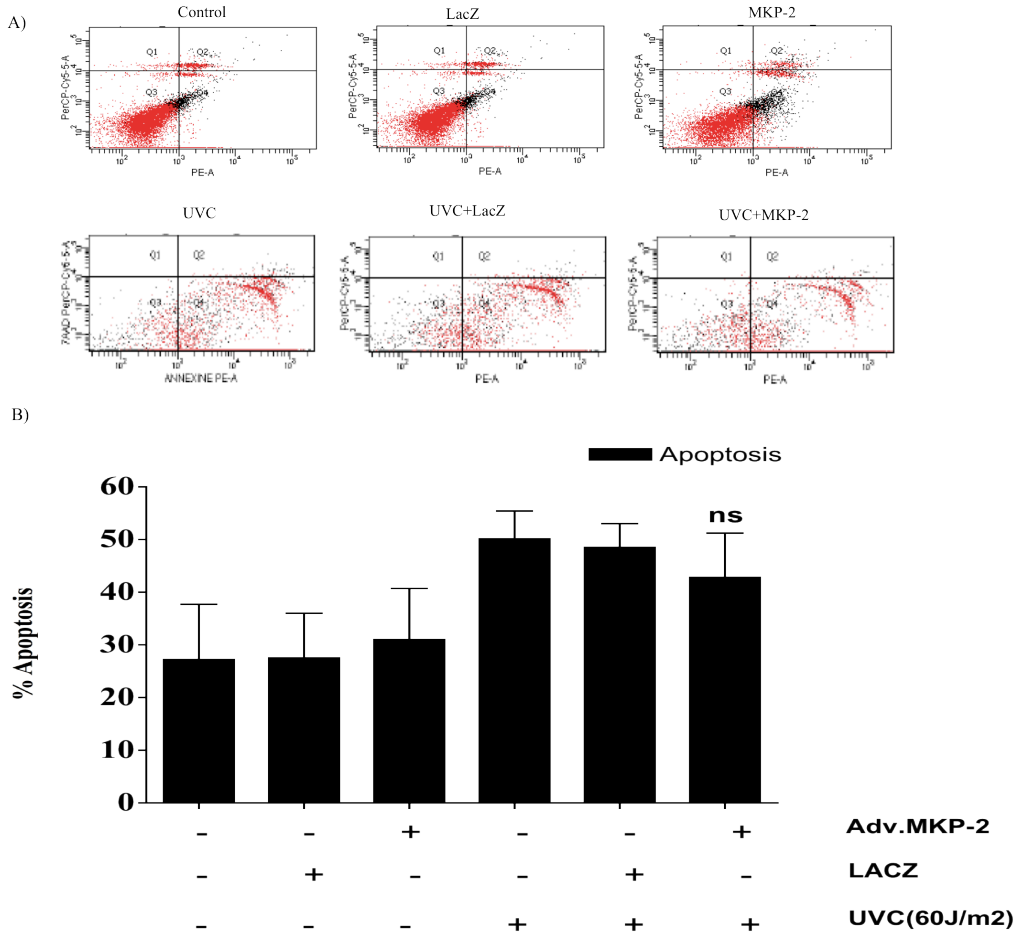


**Figure 3.32: Adv.MKP-2 infection does not modify UV-C mediated apoptosis in LNCaP (AS) cell lines.** Cells were infected with (200 pfu/cell) Adv.MKP-2 for 40 h prior to stimulation with UV-C (60 J/m$^2$) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9 (Panels A and B). Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. Each value represents the mean ± s.e.m of at least 3 experiments. n.s ($p>0.05$).

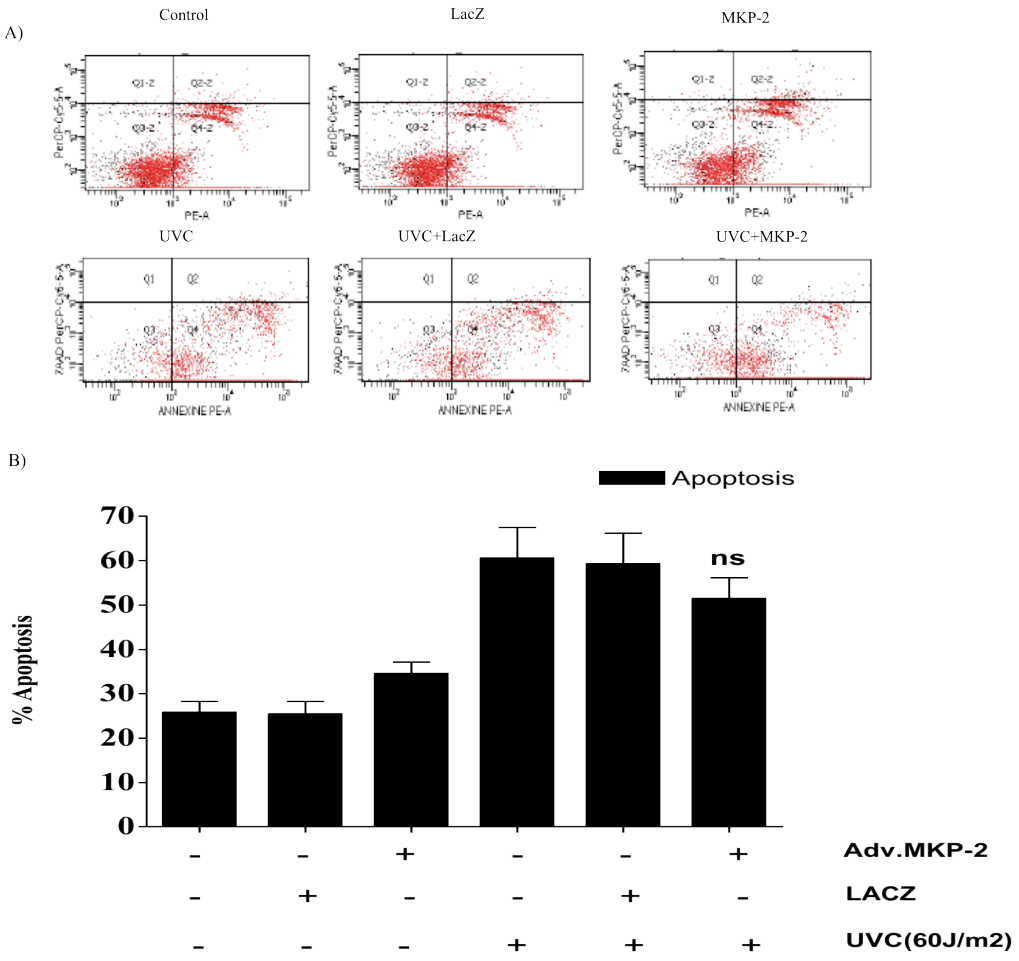


**Figure 3.33: Adv.MKP-2 infection does not modify UV-C mediated apoptosis in LNCaP (AI) cell lines.** Cells were infected with (200 pfu/cell) Adv.MKP-2 for 40 h prior to stimulation with UV-C (60 J/m$^2$) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9. (Panels A and B). Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. Each value represents the mean ± s.e.m of at least 3 experiments. n.s ($p>0.05$).

### 3.11.5 Adv.MKP-2 infection does not modify doxorubicin mediated apoptosis in LNCaP (AS) cells

In order to determine if the lack of effect of MKP-2 was due to the activator employed the effect of Adv.MKP-2 on apoptosis induced by doxorubicin was examined. LNCaP (AS) cells were infected with Adv.MKP-2 (200 pfu/cell) for 40 h prior to stimulation with doxorubicin (500 and 1000 nM) for a further 24 h. In figure 3.34, cells stimulated alone with doxorubicin showed a significant increase in apoptosis (% cell death; control = 26.8 ± 8.68, doxorubicin (500 and 1000 nM) = 59.1 ± 16.3, 62.5 ± 12.3, respectively. However, infecting cells with Adv.MKP-2 (200 pfu/cell) prior to stimulation with doxorubicin resulted in no significant change in apoptosis (% cell death; doxorubicin 500 and 1000 nM + Adv.MKP-2 = 70.5 ± 6.71, 71.7 ± 15.6 respectively, n.s).

### 3.11.6 Adv.MKP-2 infection does not modify doxorubicin mediated apoptosis in LNCaP (AI) cells

In figure 3.35, LNCaP (AI) cells were examined. Cells stimulated with doxorubicin showed a significant increase in apoptosis (% cell death; control= 22.5 ± 0.81, Adv.MKP-2 = 20.3 ± 1.244, doxorubicin (500 and 1000 nM) = 71.6 ± 0.64, 68.6 ± 10.4 respectively, n=3). However, infecting cells with Adv.MKP-2 (200 pfu/cell) prior to stimulation with doxorubicin 500 or 1000 nM resulted in no significant alteration in apoptosis (% cell death; doxorubicin 500 and 1000 nM + MKP-2 = 75.2 ± 1.02, 72.4 ± 9.42 respectively, n.s, n=3).

### 3.11.7 Adv.MKP-2 infection does not alter X-ray mediated apoptosis in LNCaP (AS) cells

As another alternative X-rays were utilised to further determine if Adv.MKP-2 could modify apoptosis. Initially the most suitable dose of X-rays for achieving apoptosis was determimined, a dose of (10 Gry) gave a suitable response and this compared favourably to previous studies (He et al., 2011). So to determine if Adv.MKP-2 was able to regulate LNCaP (AS) cells were again infected with Adv.MKP-2 (200 pfu/cell) for 40 h and then exposed to (10 Gry) for a further 24 h. Under these conditions figure 3.36 shows that x-ray alone caused a significant increase in apoptosis (% cell death; control= 26.8 ± 8.70, Adv.MKP-2 = 21.7 ± 10.8, X-ray = 55.8 ± 24.7, n=3). This

response was not modified infecting cells with Adv.MKP-2 (% cell death; X-ray + Adv.MKP-2 = 61.8 ± 11.8, n.s, n=3).

### 3.11.8 Adv.MKP-2 infection does not alter X-ray mediated apoptosis in LNCaP (AI) cells

Figure 3.37 shows, also the effect of Adv.MKP-2 (200 pfu/cell) upon apoptosis in response to X-ray in LNCaP (AI) cells. Stimulation with X-ray (10 Gry) alone caused a significant increase in apoptosis (% cell death; control= 17.9 ± 2.90, Adv.MKP-2= 19.1 ± 5.80, X-ray = 31.9 ± 9.67, n=3), which was not altered by infecting cells with Adv.MKP-2 (% of cell death; X-ray = 36.4 ± 4.00,n.s, n=3).

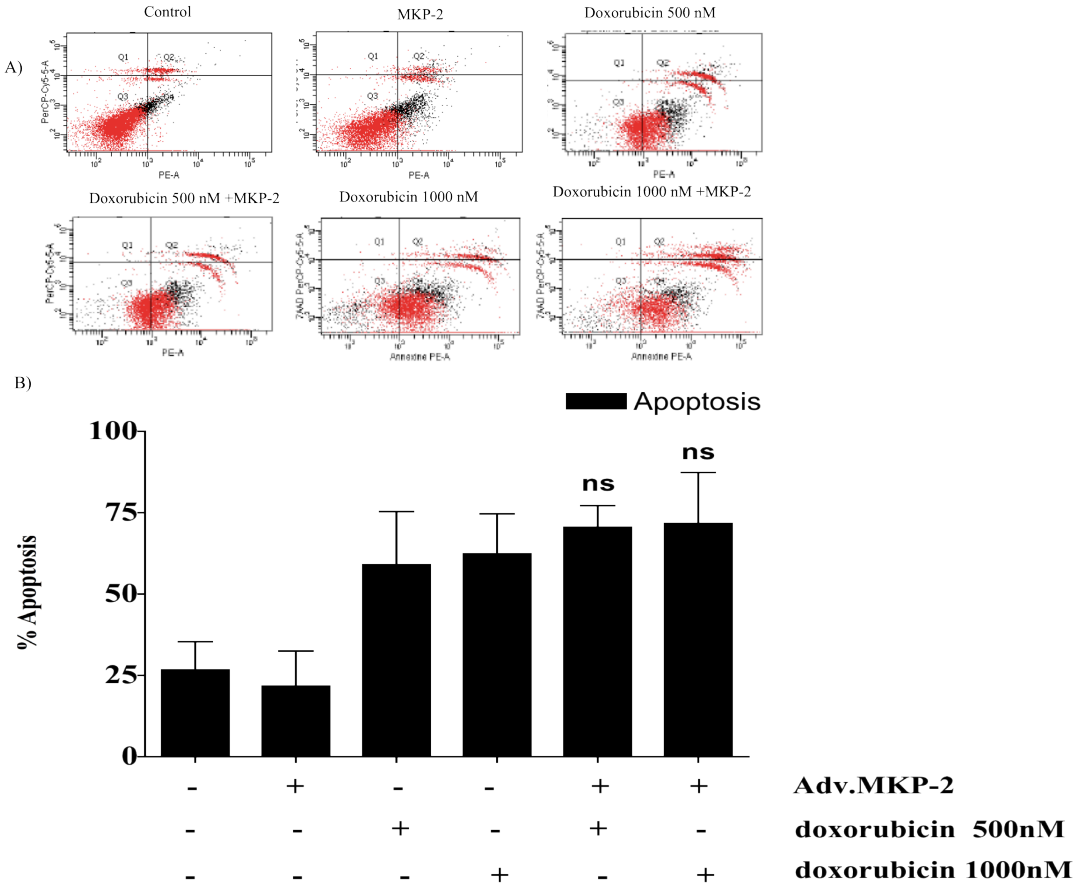


**Figure 3.34: Adv.MKP-2 infection does not alter doxorubicin-mediated apoptosis in LNCaP (AS) cell lines.** Cells were infected with (200 pfu/cell) Adv.MKP-2 for 40 h prior to stimulation with doxorubicin (500 and 1000 nM) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9 (Panels A and B). Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. Each value represents the mean ± s.e.m of at least 3 experiments. n.s ($p>0.05$).

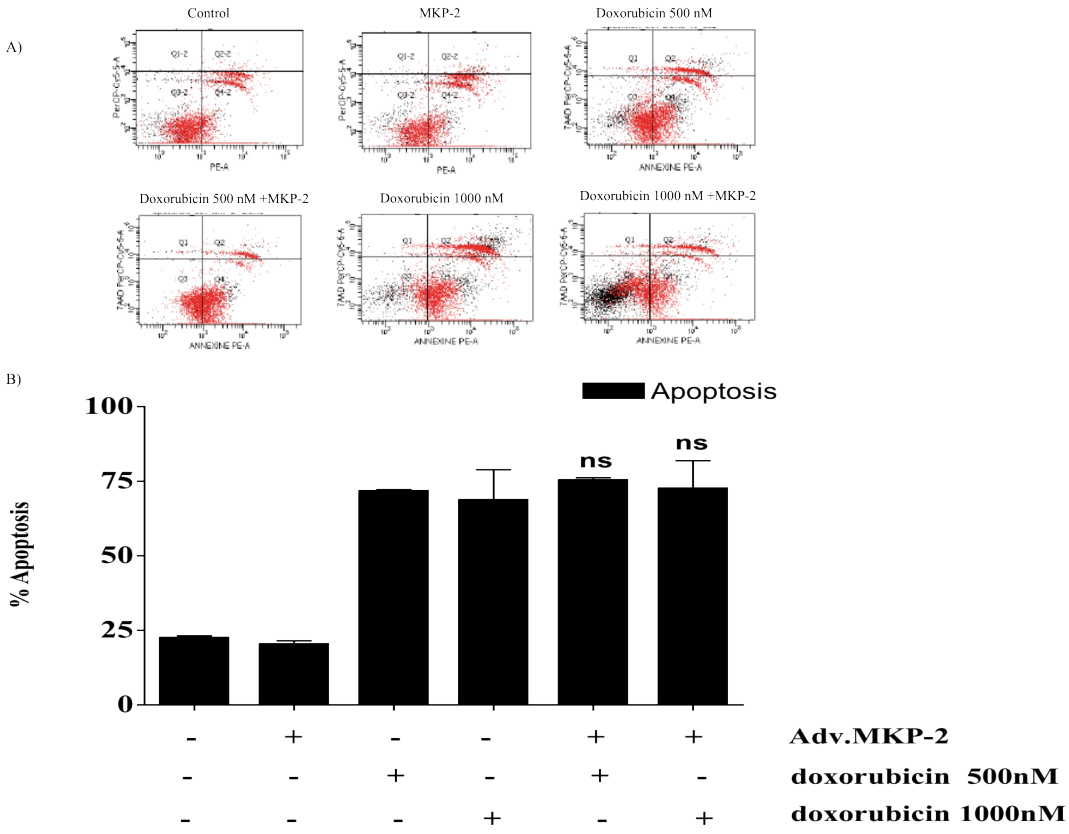


**Figure 3.35: Adv.MKP-2 infection does not alter doxorubicin-mediated apoptosis in LNCaP (AI) cell lines.** Cells were infected with (200 pfu/cell) Adv.MKP-2 for 40 h prior to stimulation with doxorubicin (500 and 1000 nM) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9. (Panels A and B). Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. Each value represents the mean ± s.e.m of at least 3 experiments. n.s ($p>0.05$).

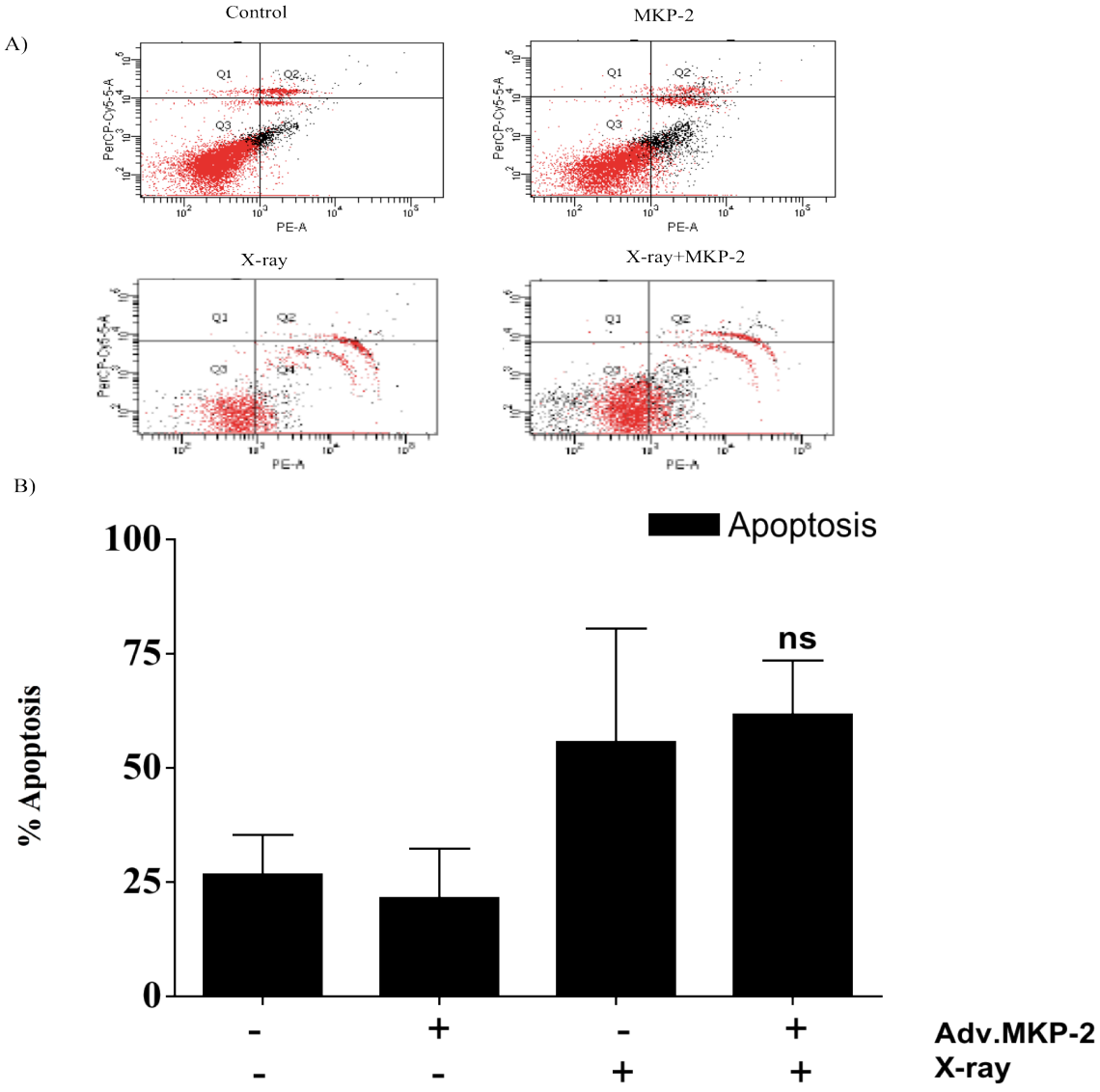


**Figure 3.36: Adv.MKP-2 infection does not modify X-ray mediated apoptosis in LNCaP (AS) cell lines.** Cells were infected with (200 pfu/cell) Adv.MKP-2 for 40 h prior to stimulation with X-ray (10 Gry) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9 (Panels A and B). Each value represents the mean ± s.e.m of at least 3 experiments. n.s ($p>0.05$).

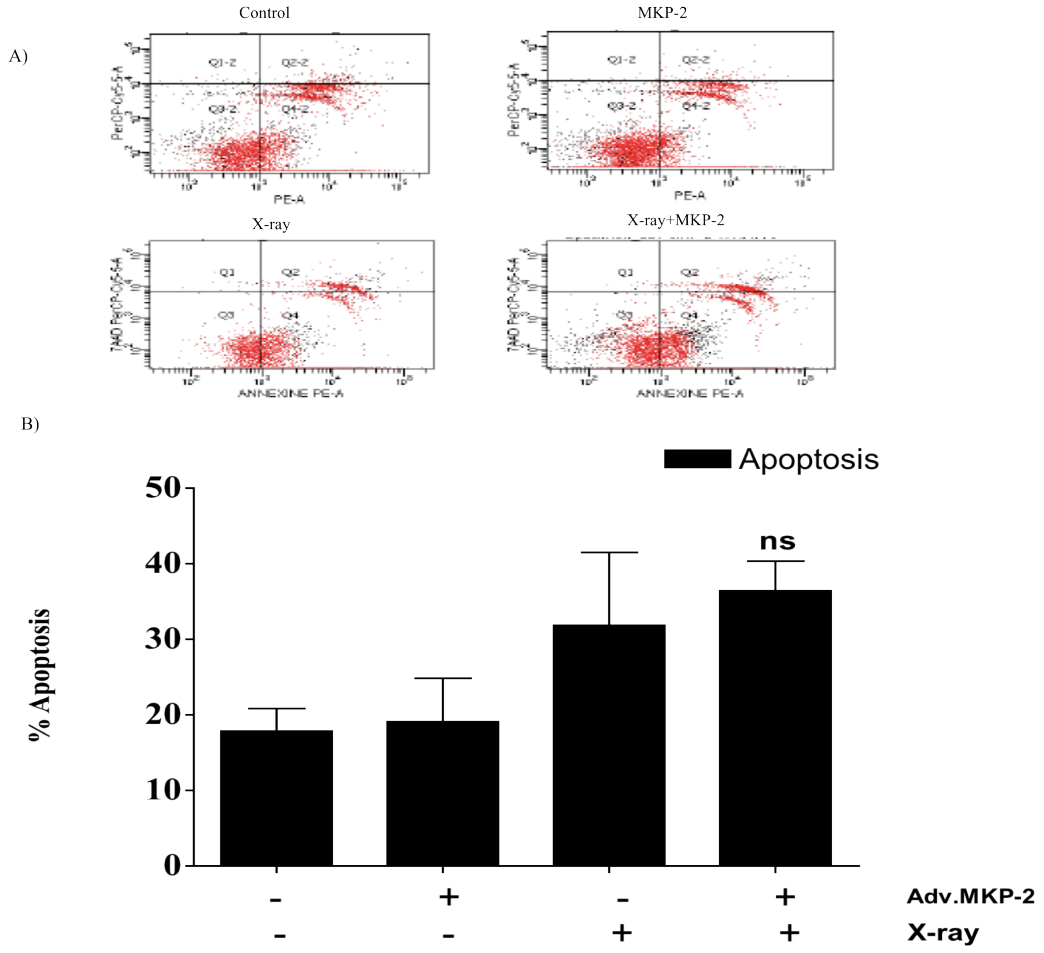


**Figure 3.37: Adv.MKP-2 infection does not modify X-ray mediated apoptosis in LNCaP (AI) cell lines.** Cells were infected with (200 pfu/cell) Adv.MKP-2 for 40 h prior to stimulation with X-ray (10 Gry) for a further 24 h. Samples were assessed for Annexin V and 7-AAD staining as outlined in section 2.9 (Panels A and B). Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. Each value represents the mean ± s.e.m of at least 3 experiments. n.s ($p>0.05$).

## 3.12 CHARACTERISATION OF DOXORUBICIN AND X-RAY ON JNK PHOSPHORYLATION IN LNCaP (AS) AND LNCaP (AI) CELLS.

Having established in section 3.8, that Adv.MKP-2 was unable to alter apoptosis in response to doxorubicin and X-ray exposure, the potential for doxorubicin and X-ray to mediate JNK phosphorylation in LNCaP (AS) and (AI) cells was further assessed.

### 3.12.1 Lack of effect of Doxorubicin and X-ray exposure on JNK phosphorylation in both LNCaP (AS) and (AI) cells

In order to find an effect stimulant of JNK relevant to cancer together with apoptosis, doxorubicin (1000 nM) was used. Figure 3.38, shows JNK phosphorylation (panel A) stimulated by doxorubicin (1000 nM) over a 480 min period. The figure shows no activation of JNK phosphorylation. Total JNK values were unchanged, indicating equal protein loading.

Similarly to doxorubicin, LNCaP (AS) and (AI) cells were stimulated using X-ray (10 Gry). Figure 3.38, shows JNK phosphorylation (panel B) stimulated by X-ray (10 Gry) over a 480 min period. The figure shows no activation of JNK phosphorylation. Total JNK values were unchanged, indicating equal protein loading.

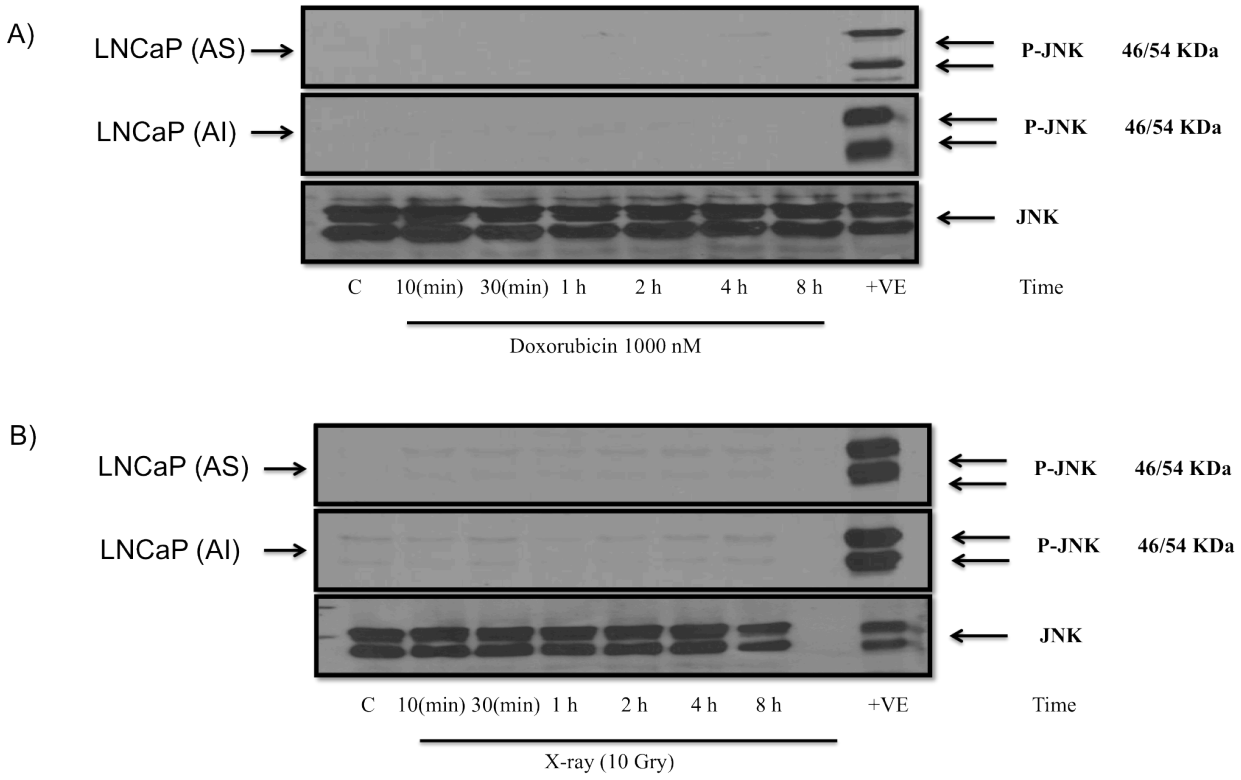


**Figure 3.38: Lack effect of Doxorubicin and X-ray on JNK phosphorylation in both LNCaP (AS) and (AI) cell lines.** Cells were stimulated with doxorubicin (1000 nM) for the indicated times and, then assessed for p-JNK (54/46kDa) and JNK (panel A). In panel B shows, cells were stimulated with X-ray (10 Gry) for the indicated times and then assessed for p-JNK (54/46kDa) and JNK. Whole cell lysates were prepared, separated by SDS PAGE as outlined in section 2.4. The results are representative of 2 independent experiments.

## 3.13 CHARACTERISATION OF CI-MKP2 AND NLS1-MKP2 ON EGF MEDIATED ERK PHOSPHORYLATION IN LNCaP (AS) AND LNCaP (AI) CELLS

The studies so far indicate the potential for overexpression of MKP-2 to regulate both ERK and JNK function and to regulate cell cycle progression but not apoptosis. It is still unclear if these affects are mediated via dephosphorylation of ERK and/or JNK. To test this hypothesis we used a form of MKP-2 which has a triple substitution of arginine within positions R74, R75 and R76 to alanine and is unable to bind to ERK (Chen et al., 2001, Sloss et al., 2005). Utilising this mutant will help us further specify the role of ERK or in particular JNK in regulating cellular proliferation. A catalytically inactive version of MKP-2 was also used as a further control because of the potential for MKP-2 to function as an anchoring protein for ERK in some studies (Caunt et al., 2008), which may in turn regulate the ERK pathway.

### 3.13.1 Sub-cellular localisation of both MKP-2-NLS1 and MKP-2-CI in LNCaP (AI) cells

To confirm the nuclear localisation of both MKP-2-NLS1 and MKP-2-CI in LNCaP (AI), cells were infected with either MKP-2-NLS1 or CI for 40 h and, then investigated using fluorescence microscopy as shown in figure 3.39. No staining of MKP-2 was observed in cells infected with LacZ alone (not shown), however MKP-2-NLS1 and CI gave enhanced cellular staining. At a pfu of 200 greater than 90% of the cells were infected and staining was strictly located to the nucleus. This was confirmed by nuclear staining with DAPI. A similar result was observed for LNCaP (AS) cells (not shown).

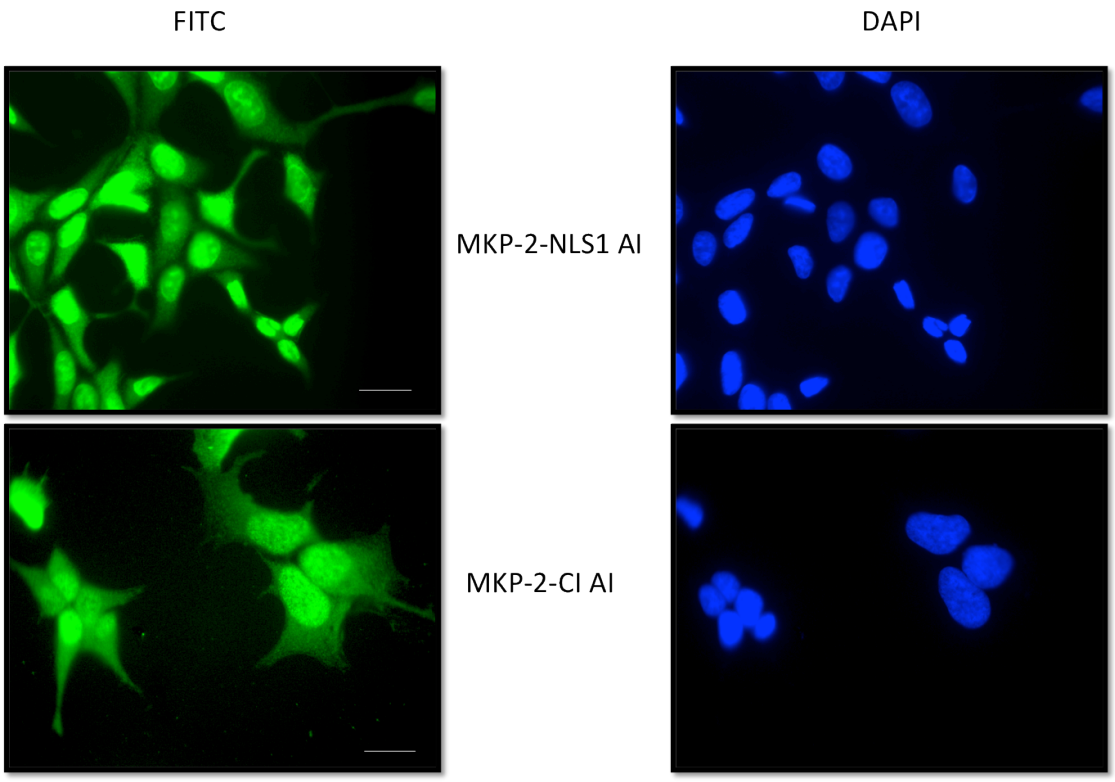


**Figure 3.39: Sub-cellular localisation of Adv.MKP-2-NLS1 and CI in LNCaP (AI) cell lines.** Cells were infected with 200 pfu/cell of Adv.MKP-2 for 40 h, then cells were fixed and stained for MKP-2 (1:100). Nuclei were visualised by DAPI (blue) staining and MKP-2 sub-localisation was visualized using FITC (green) staining using magnification of x40 as outlined in section 2.5. The results are representative of at least 4 independent experiments.

### 3.13.2 The effect of MKP2-CI and MKP2-WT on EGF mediated ERK phosphorylation in LNCaP (AS) cells:

To assess comparatively the levels of expression between MKP2-CI and MKP2-WT, LNCaP (AS) cells were infected either with MKP2-CI (50, 100 and 200 pfu/cell) or MKP2 construct (200, 300 and 500 pfu/cell) and quiesced for 40 h in serum free media, then stimulated with EGF (20ng/ml) for 30 min as shown in figure 3.40. Stimulation with EGF induced a significant increase in ERK phosphorylation at time points tested (EGF= 7.4), which was not decreased by infecting cells with LacZ. However, whilst infection with Adv.MKP-2-WT significantly inhibited the phosphorylation of ERK at all pfu tested (EGF+Adv.MKP-2= 3.0, 2.3 and 1.7 for 200, 300 and 500 pfu/cell, respectively), in the presence of Adv.MKP-2-CI there was no change (EGF+Adv.MKP-2-CI= 7.4, 8.0 and 8.0 for 50, 100 and 200 pfu/cell, respectively).

### 3.13.3 The effect of MKP2-NLS1 and MKP2-WT on EGF mediated ERK phosphorylation in LNCaP (AS) cells:

Expression levels between MKP2-NLS1 and MKP2-WT in LNCaP (AS) was also assessed (see figure 3.41), using the same conditions as outlined above. Stimulation with EGF induced a significant increase in ERK phosphorylation at time point tested (EGF= 6.1, 30 min), which again was not decreased by infecting cells with LacZ. However, infection with either MKP2-NLS1 or MKP2-WT significantly inhibited the phosphorylation of ERK in all concentrations of virus tested ((EGF+Adv.MKP-2-NLS1= 2.7, 2.4 and 1.9 for 50, 100 and 200 pfu/cell, respectively) and (EGF+Adv.MKP-2= 1.8, 1.5 and 1.1 for 200, 300 and 500 pfu/cell, respectively).

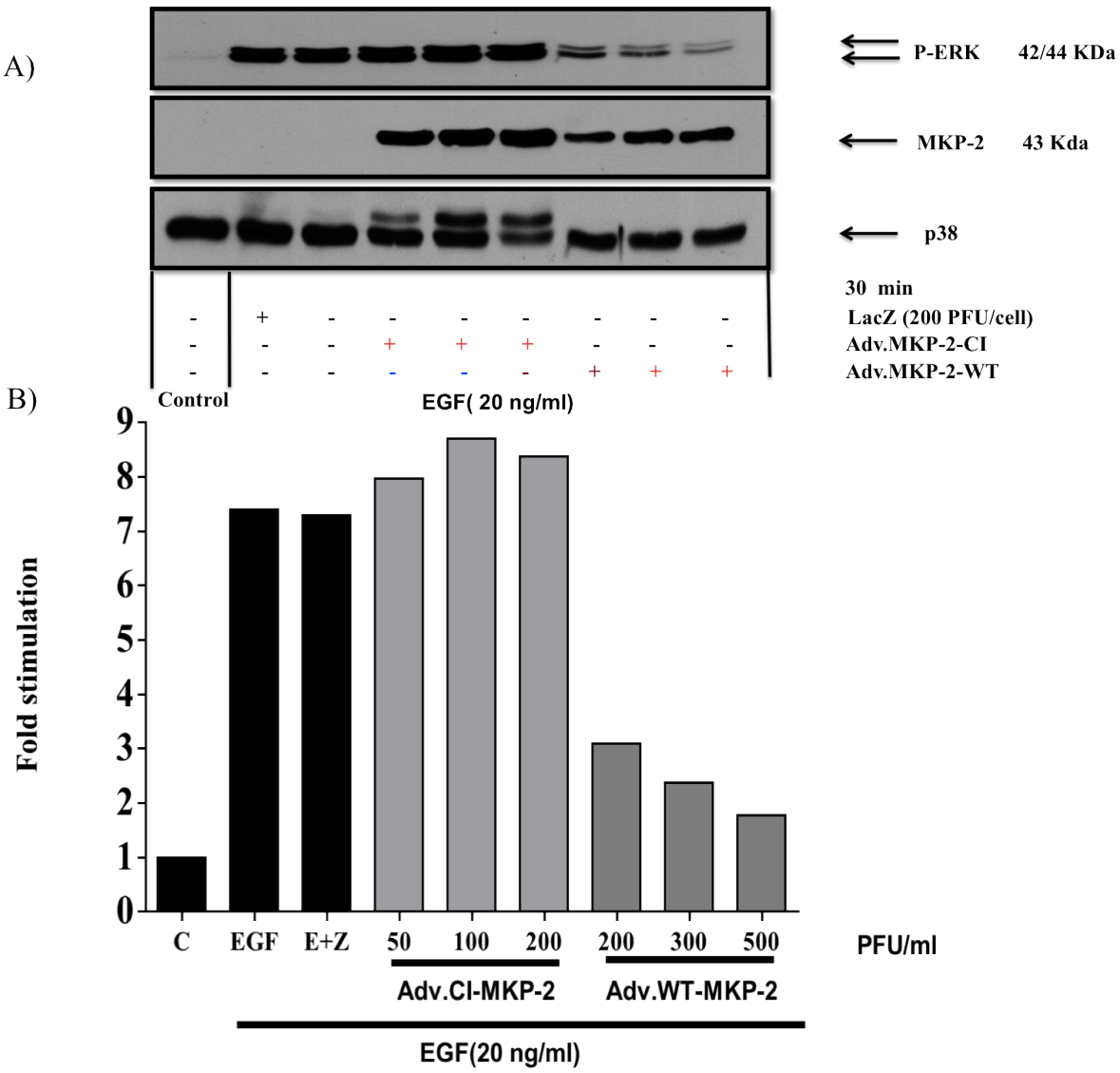


**Figure 3.40: The effect of MKP-2-CI and MKP-2-WT upon ERK phosphorylation stimulated by EGF in LNCaP (AS) cell lines.** Cells were infected with both MKP-2-CI and MKP-2-WT for 40 h prior to stimulation with EGF (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS.PAGE, and then assessed for a p-ERK1/2 (42/44 kDa), MKP-2-WT and MKP-2-CI (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; n=1

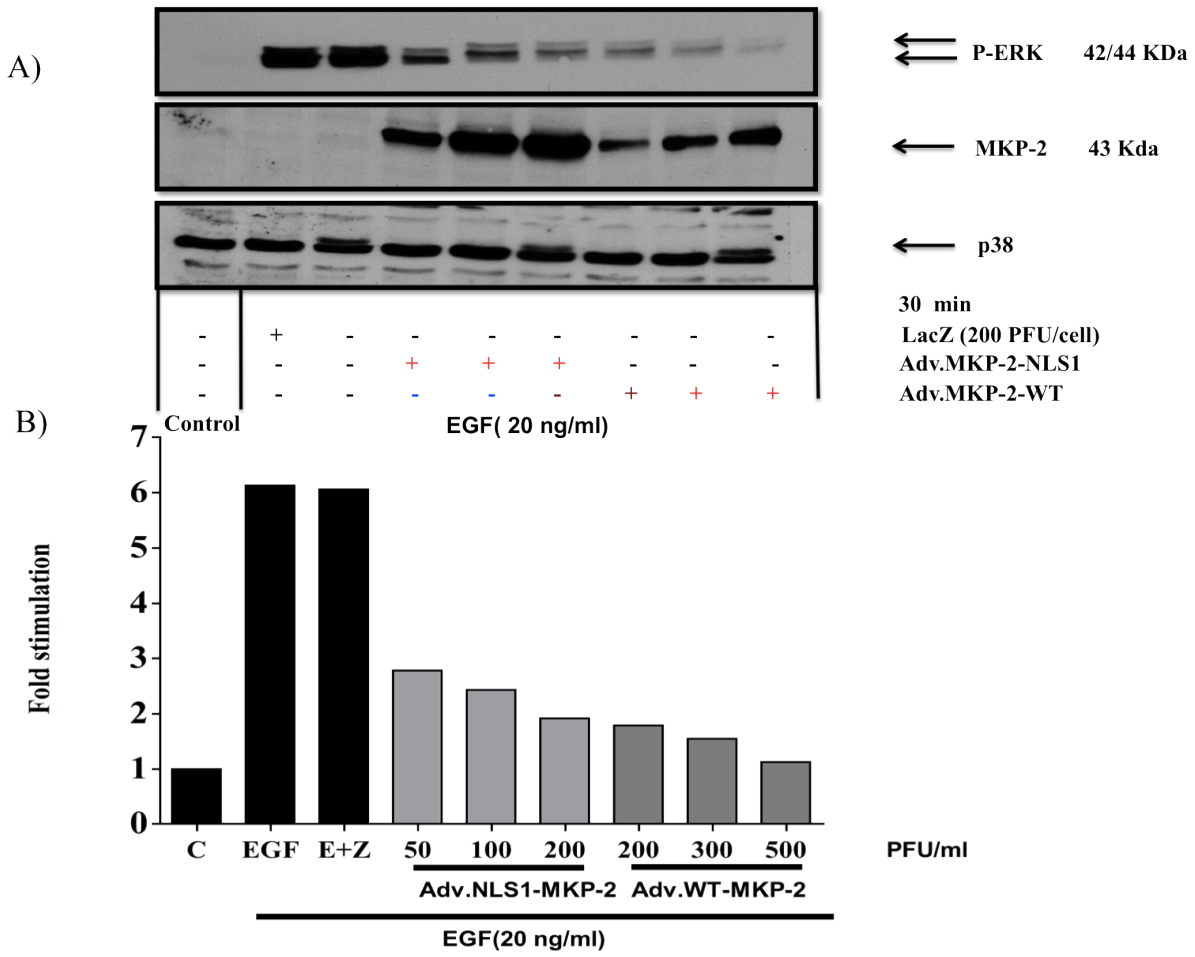


**Figure 3.41: The effect of MKP-2-WT and MKP-2-NLS1 upon ERK phosphorylation stimulated by EGF in LNCaP (AS) cell lines.** Cells were infected with both MKP-2-NLS1 and MKP-2-WT for 40 h prior to stimulation with EGF (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS.PAGE, and then assessed for a p-ERK1/2 (42/44 kDa), MKP-2-NLS1 and MKP-2-WT (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; n=1.

## 3.14 CHARACTERISATION OF THE EFFECT OF ADV.MKP-2-NLS1 AND MKP-2-CI IN LNCaP (AS) AND (AI) CELLS

Surprisingly Adv.MKP-2 had a significant inhibitory effect on ERK signalling. Nevertheless, this construct and MKP-2-CI was further examined at the level of JNK phosphorylation and cell cycle progression.

### 3.14.1 The effect of Adv.MKP-2 NLS1 and MKP-2-CI on UV-C induced JNK phosphorylation in LNCaP (AS) cells

To assess the effect of both Adv.MKP-2-NLS1 and CI in LNCaP (AS), cells were infected with either Adv.MKP-2-NLS1 or CI (200 pfu/cell) and quiesced for 40 h in serum free media then cells were incubated with UV-C (60 j/$m^2$) for 30 min (figure 3.42). Stimulation with UV-C induced a significant increase in JNK phosphorylation at the time point examined, (UV-C fold stim. at 30 min= 2.86 ± 0.15 n=3), which was not decreased by infecting cells with LacZ. However, infection with Adv.MKP-2-NLS1 inhibited the phosphorylation of JNK. This effect was significant and approximately 50% (UV-C + Adv.MKP-2-NLS1, fold stim. at 30 min= 1.43 ± 0.06, **P<0.01, n=3). Infection with Adv.MKP-2-CI did not alter the JNK response to UVC (UVC + Adv.MKP-2-CI, fold stim. at 30 min= 2.80 ± 0.18, n=3).

### 3.14.2 The effect of Adv.MKP-2-NLS1 and CI on JNK phosphorylation in LNCaP (AI) cells

In figure 3.43, shows that stimulation with UV-C induced a significant increase in JNK phosphorylation at 30 min (UV-C fold stim. at 30 min= 2.62 ± 0.10, n=3), which was not decreased by infecting cells with LacZ. However, infection with Adv.MKP-2-NLS1 significantly inhibited the phosphorylation of JNK. This was significant and approximately 50% (UV-C + Adv.MKP-2-NLS1, fold stim. at 30 min= 1.30 ± 0.060, **P<0.01, n=3), whilst infection with Adv.MKP-2-CI did not affect the JNK response (Adv.MKP-2-CI, fold stim. at 30 min= 2.61 ± 0.12 n=3).

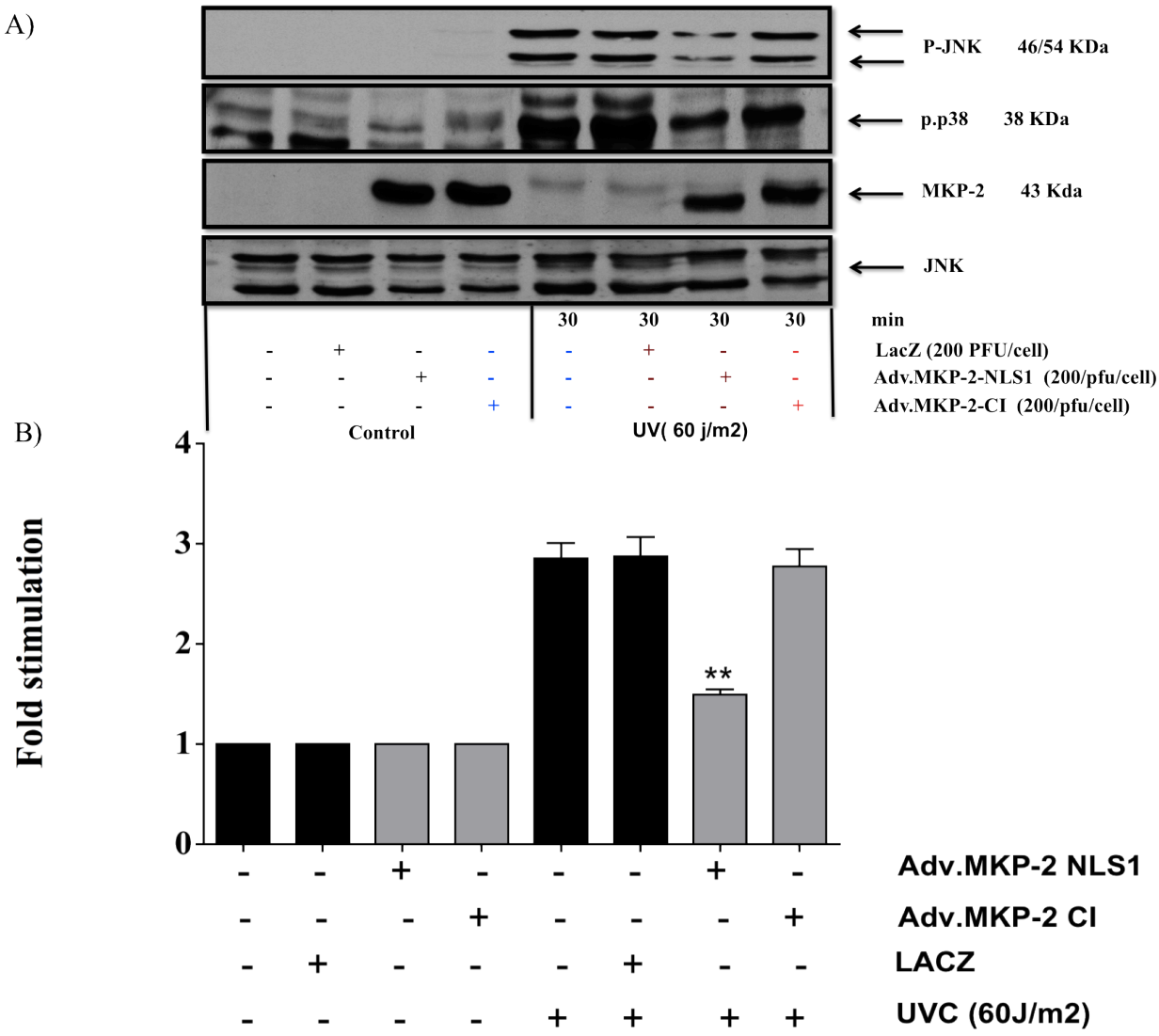


**Figure 3.42: The effect of MKP-2-NLS1 and MKP-2-CI upon JNK phosphorylation stimulated by UV-C in LNCaP (AS) cell lines.** Cells were infected with both MKP-2-NLS1 and MKP-2-CI for 40 h prior to stimulation with UV-C (60 j/m$^2$) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE, and, then assessed for a) p-JNK (46/54 kDa), MKP-2-NLS1 and MKP-2-CI (43 kDa) and p.p38 and JNK as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA, post hoc test by Dunnett's test **P< 0.01. The results are representative of 3 independent experiments.

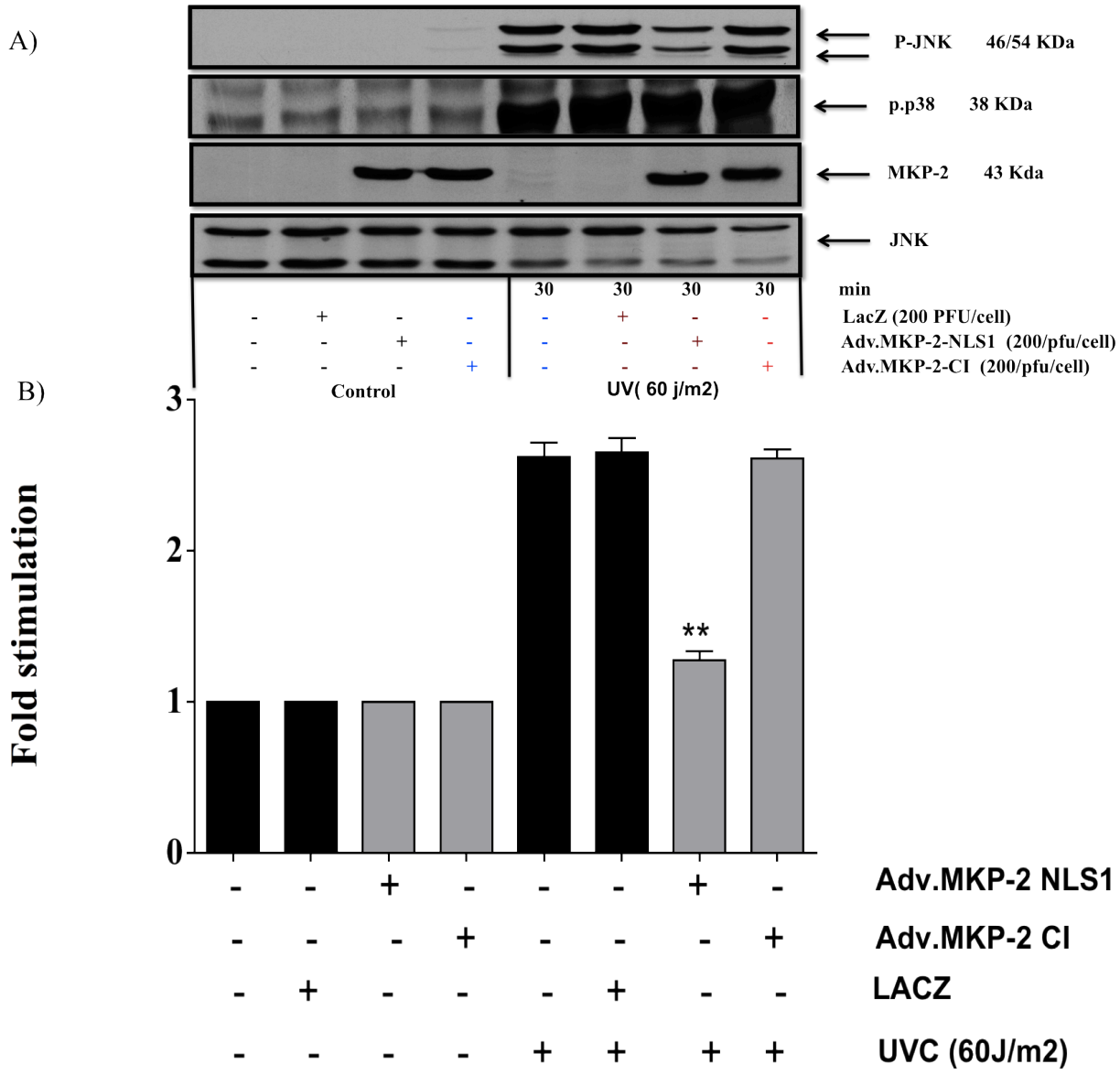


**Figure 3.43: The effect of MKP-2-NLS1 and MKP-2-CI upon JNK phosphorylation stimulated by UV-C in LNCaP (AI) cell lines.** Cells were infected with both MKP-2-NLS1 and MKP-2-CI for 40 h prior to stimulation with UV-C (60 j/m$^2$) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE, and, then assessed for a) p-JNK (46/54 kDa), MKP-2-NLS1 and MKP-2-CI (43 kDa) and p.p38 and JNK as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA, post hoc test by Dunnett's test **P<0. The results are representative of 3 independent experiments.

### 3.14.3 The effect of Adv.MKP-2-NLS1 and Adv.MKP-2-CI on EGF induced p-ERK in LNCaP (AS) cells

To specify the role of ERK in regulating cellular proliferation, LNCaP (AS) were infected with either Adv.MKP-2-NLS1 or CI (200 pfu/cell) and then stimulated with EGF (20 ng/ml) for 30 min (see figure 3.44). Stimulation with EGF induced a significant increase in ERK phosphorylation at the time point tested (EGF fold stim. at 30 min= 2.77 ± 0.21, n=3), which was not decreased by infecting cells with LacZ. However, infection with Adv.MKP-2-NLS1 significantly inhibited the phosphorylation of ERK. This was by as much as approximately 40% (EGF + Adv.MKP-2-NLS1, fold stim. at 30 min= 1.62 ± 0.11 n=3, **P<0.01). Infection with Adv.MKP-2-CI was without effect (EGF + Adv.MKP-2-CI, fold stim. at 30 min= 2.65 ± 0.19, n=3).

### 3.14.4 The effect of Adv.MKP-2 NLS1 and Adv.MKP-2-CI on p-ERK in LNCaP (AI) cells

Once the effect of Adv.MKP-2-NLS1 and CI on ERK phosphorylation in LNCaP (AS) was assessed, the effect in LNCaP (AI) cells was then investigated in figure 3.45, cells were infected with either Adv.MKP-2-NLS1 or CI (200 pfu/cell) and, then stimulated with EGF (20 ng/ml) for 30 min. Stimulation with EGF induced a significant increase in ERK phosphorylation at time points tested (EGF fold stim. at 30 min= 3.10 ± 0.11, n=3), which was not decreased by infecting cells with LacZ. However, infection with Adv.MKP-2-NLS1 significantly inhibited the phosphorylation of ERK. This was by as much as 70% (EGF + Adv.MKP-2-NLS1, fold stim. at 30 min= 1.36 ± 0.16, n=3, **P<0.01). Infection with Adv.MKP-2-CI was without effect (EGF + Adv.MKP-2-CI, fold stim. at 30, min= 3.22 ± 0.24, n=3).

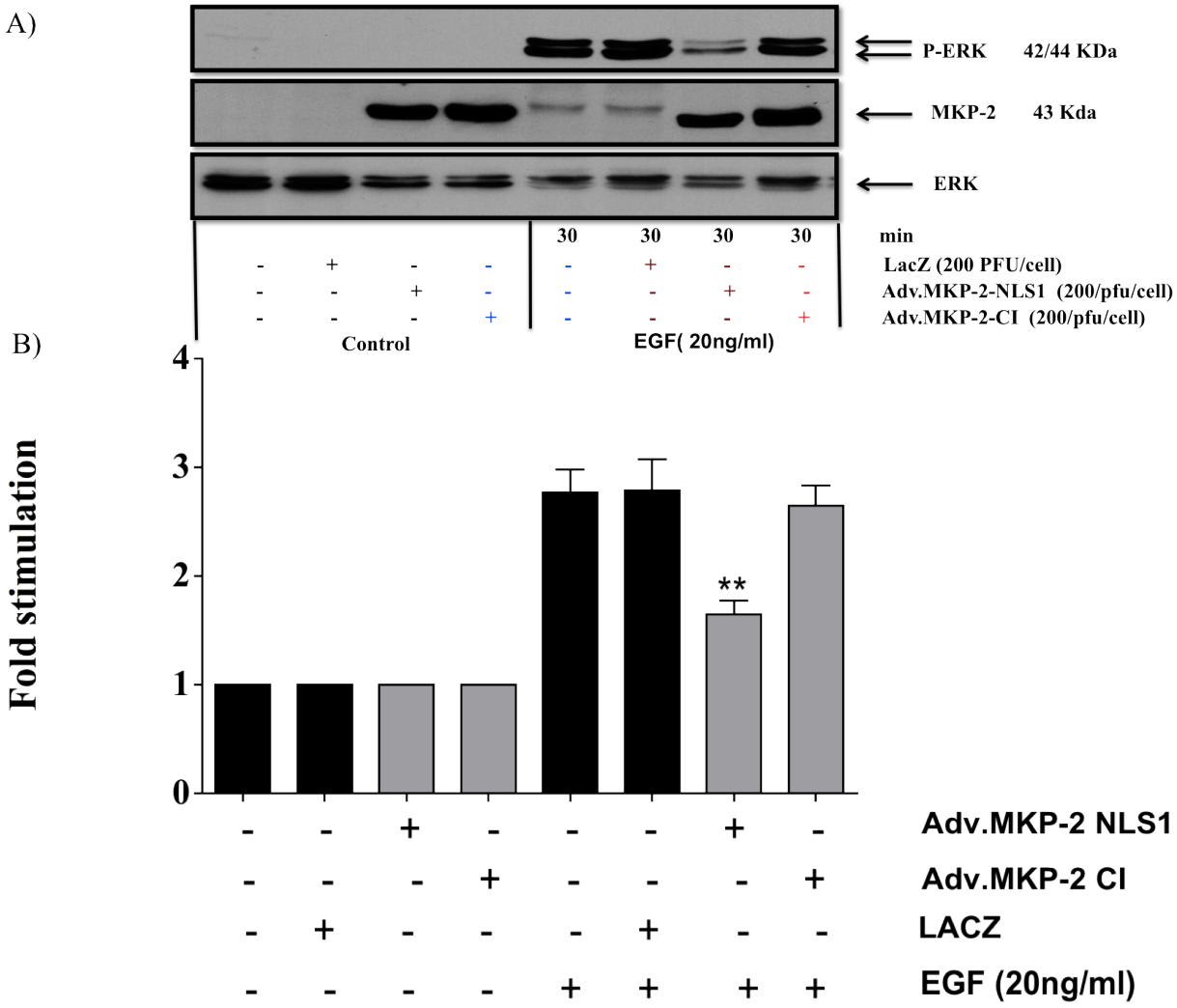


**Figure 3.44: The effect of MKP-2-NLS1 and CI upon ERK phosphorylation stimulated by EGF in LNCaP (AS) cell lines.** Cells were infected with both NLS1-MKP-2 and MKP-2-CI for 40 h prior to stimulation with EGF (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE, and, then assessed for a p-ERK1/2 (42/44 kDa), MKP-2-NLS1 and MKP-2-CI (43 kDa) and ERK as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, **P<0.01. The results are representative of 3 independent experiments.

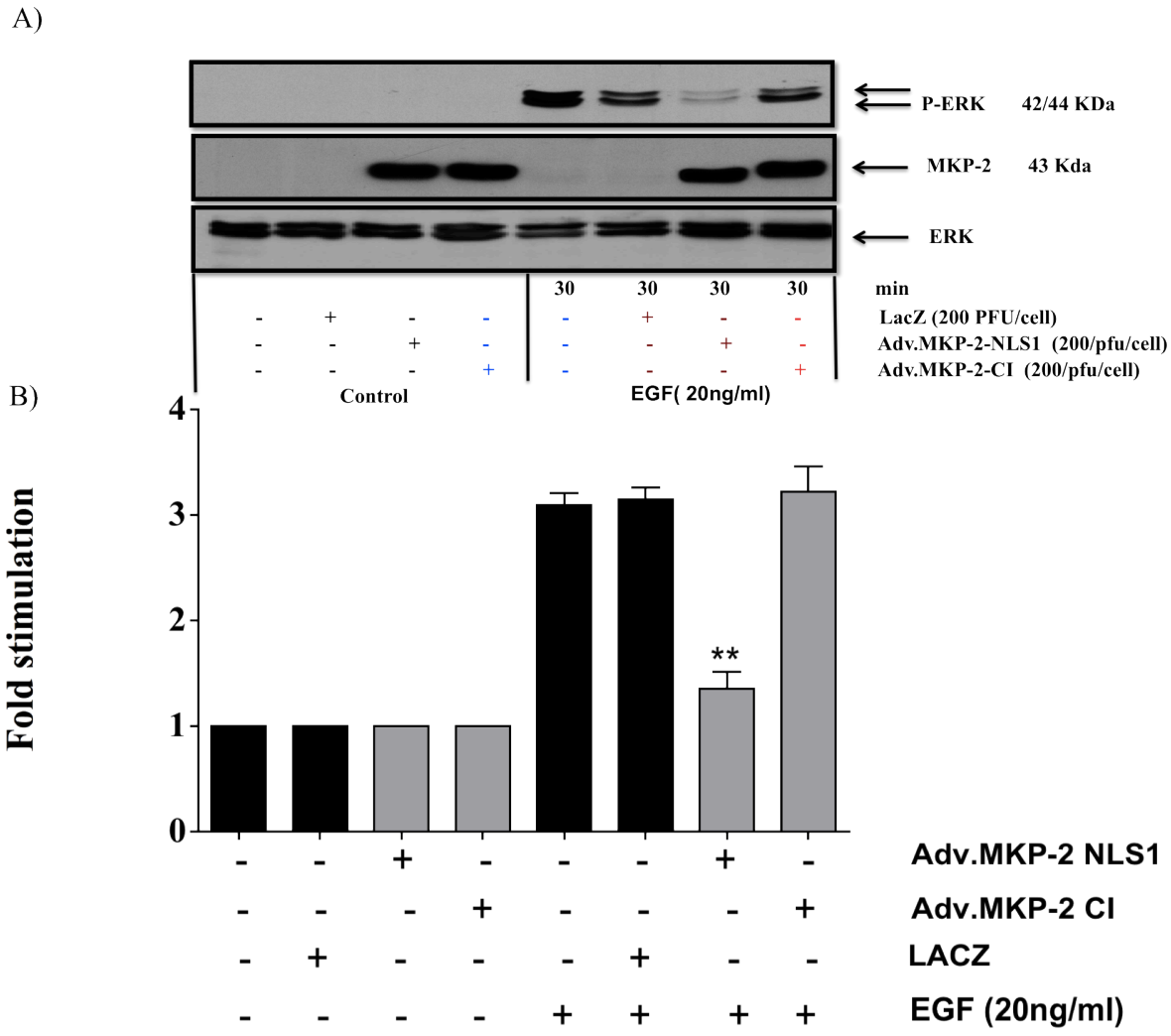


**Figure 3.45: The effect of MKP-2-NLS1 and CI upon ERK phosphorylation stimulated by EGF in LNCaP (AI) cell lines.** Cells were infected with both MKP-2-NLS1 and CI for 40 h prior to stimulation with EGF (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a p-ERK1/2 (42/44 kDa), NLS1-MKP-2 and CI-MKP-2 (43 kDa) and ERK as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test, **P<0.01. The results are representative of 3 independent experiments.

### 3.14.5 The effect of MKP-2-NLS1 and MKP-2-CI on cell cycle progression in LNCaP (AS) cells

Having established that Adv.MKP-2 NLS1, but not MKP-2-CI can strongly inhibit the ERK or JNK signalling and MKP-2-CI did not change ERK or JNK in response to either EGF or UVC, the effect MKP-2-NLS1 and MKP-2-CI on cell cycle progression was examined using 10% FCS for 48 h. Figure 3.46 shows, that FCS alone caused an increase in cells progressing into both S and $G_2$/M phase. Whilst Lacz was without effect, both MKP-2-NLS1 and MKP-2-CI significantly reduced cell numbers in both phases. Overall, a 90 to 95% reduction was observed for S phase entry (FCS= 9.60 ±1.70 s.e.m vs FCS + NLS1 and CI-MKP-2 = 1.60 ± 0.23 and 1.13 ± 0.20 s.e.m respectively, ***P<0.001, n=3) and approximately 50 to 60% for $G_2$/M phase (FCS=12.1 ± 0.93 s.e.m vs FCS + NLS1 and CI-MKP-2 = 4.80 ± 0.29 s.e.m and 3.40 ± 0.29 s.e.m respectively, ***P<0.001 for MKP-2-NLS1 and **P<0.01for MKP-2-CI, n=3).

### 3.14.6 The effect of MKP-2-NLS1 and MKP-2-CI on cell cycle progression in LNCaP (AI) cells

Figure 3.47, shows also the effect of MKP-2-NLS1 and MKP-2-CI (200 pfu/cell) on cell cycle progression in response to FCS, in LNCaP (AI). Stimulation with FCS over 24 h caused a marked increase in cells both in S phase and $G_2$/M phase entry, whilst Lacz was without effect. Following infection with either MKP-2-NLS1 or MKP-2-CI progression was significantly inhibited, cells entering S phase were reduced by more than 95% (FCS=17.2 ± 1.80 s.e.m vs FCS + NLS1 and CI-MKP-2 = 3.70 ± 0.40 s.e.m and 4.20 ± 0.17 s.e.m respectively, ± s.e.m, ***P<0.001, n=3) and into $G_2$/M phase where reduced by approximately 55% (FCS= 9.70 ± 2.30 s.e.m vs FCS + NLS1 and CI-MKP-2 = 5.30 ± 1.20 s.e.m and 6.60 ± 1.20 s.e.m respectively, ***P<0.001, n=3). In contrast Lacz was without significant effect.

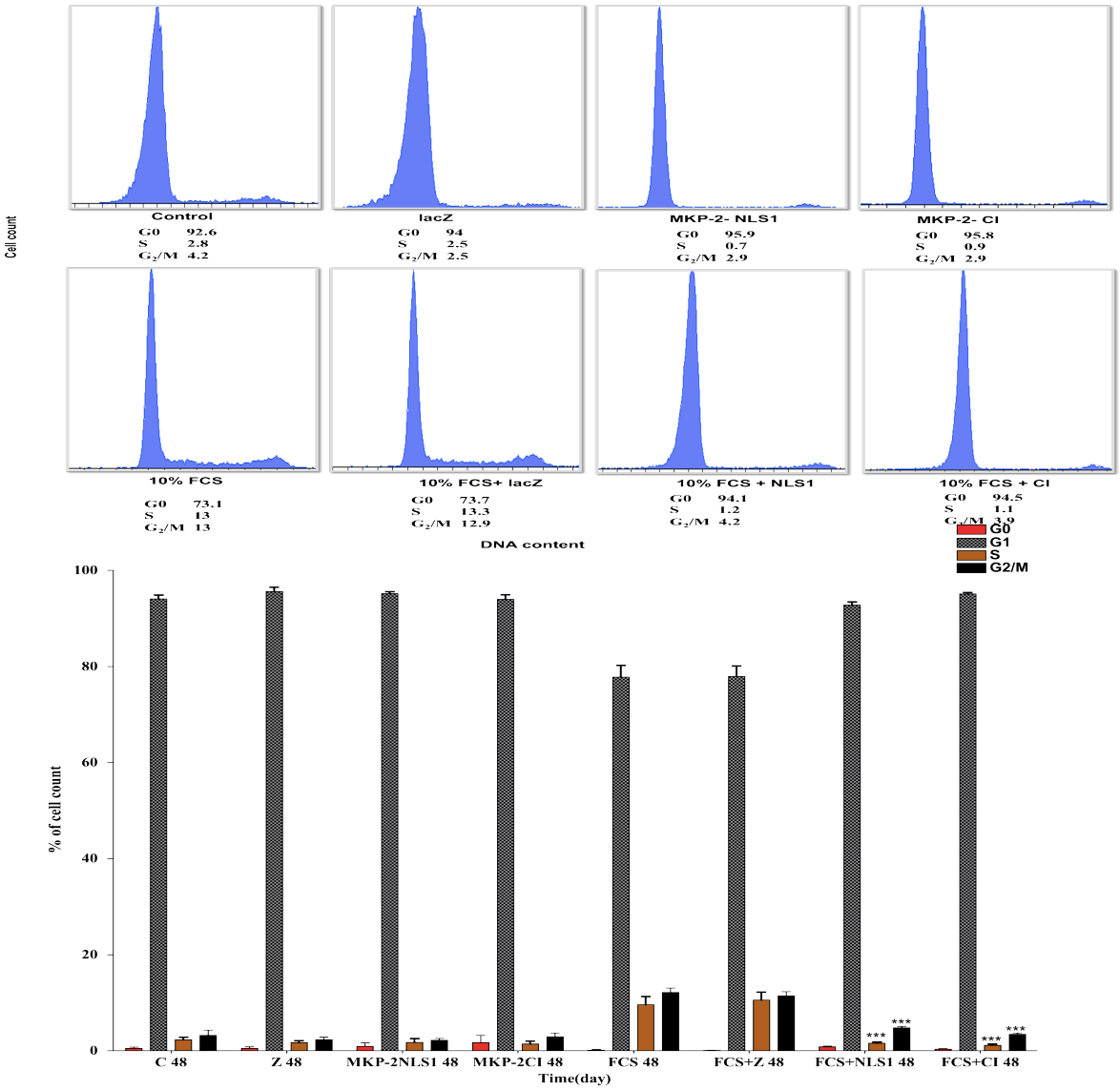


**Figure 3.46: The effect of MKP-2-NLS1 and MKP-2- CI on cell cycle progression in LNCaP (AS) cell lines.** Cells were infected with both Adv.MKP-2-NLS1 and MKP-2-CI for 40 h prior to stimulation with 10 % FCS for 48 h, then assessed by flow cytometery using propidium iodide (PI) for determination of total DNA content as outlined in section 2.8. (In Panel A), a representative FACS plot is shown for 10 % FCS and distribution for LNCaP (AS) cells (Panel B) shows, a bar chart of distribution of cells within in the different phases of the cell cycle following FCS treatment, each value represents the mean ± s.e.m. Statistical analysis was by two-way ANOVA. ***P<0.001, compared with 10% FCS stimulated control. The results are representative of 3 independent experiments.

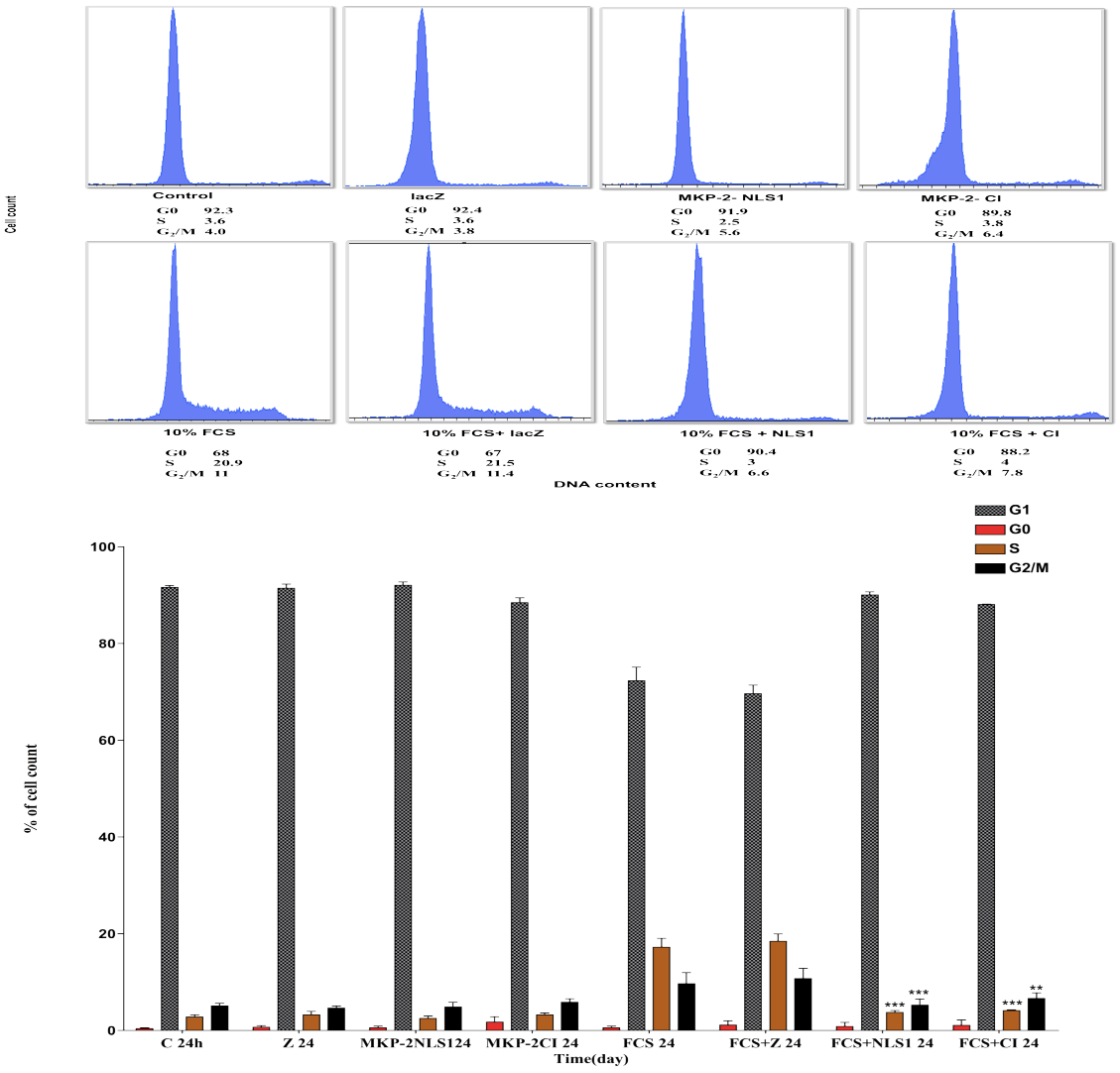


**Figure 3.47: The effect of MKP-2-NLS1 and MKP-2- CI on cell cycle progression in LNCaP (AI) cell lines.** Cells were infected with both Adv.MKP-2-NLS1 and MKP-2-CI for 40 h prior to stimulation with 10 % FCS for 24 h, then assessed by flow cytometery using propidium iodide (PI) for determination of total DNA content as outlined in section 2.8. (In Panel A), a representative FACS plot is shown for 10 % FCS and distribution for LNCaP (AI) cells (Panel B) shows, a bar chart of distribution of cells within the different phases of the cell cycle following FCS treatment, each value represents the mean ± s.e.m. Statistical analysis was by two-way ANOVA. ***P<0.001, compared with 10% FCS stimulated control. The results are representative of 3 independent experiments.

### 3.14.7 The effect of Adv. MKP-2-CI on FCS mediated cyclin D1 expression in LNCaP (AS) cells

In order to distinguish further the effect of Adv.MKP-2-CI on the cell cycle, cyclin D1 expression was examined. Figure 3.48 shows, the effect of Adv.MKP-2-CI (200 pfu/cell) upon cyclin D1 signalling in response to FCS. Stimulation with FCS induced a significant increase in cyclin D1 expression at 4 h, and this increased to a greater extent at 8 h (10% FCS fold stim. at 4 and 8 h = 5.94 ± 0.50 and 6.34 ± 0.80, respectively, n=3). Whilst LacZ was without effect, in contrast infection with Adv.MKP-2-CI significantly inhibited the phosphorylation of cyclin D1 at 4 h by approximately 70 % and greater than 80% at 8 h (10% FCS + Adv.MKP-2-CI fold stim. at 4 and 8 h = 2.03 ± 0.20 and 2.10 ± 0.20, respectively, **P<0.01 and ***p< 0.001 at 4 and 8 h respectively, n=3).

### 3.14.8 The effect of Adv. MKP-2-CI on FCS mediated cyclin D1 expression in LNCaP (AI) cells

Similarity to LNCaP (AS), the effect of Adv.MKP-2-CI on cell cycle regulatory proteins in LNCaP (AI) were examined. Figure 3.49 shows, the effect of Adv.MKP-2- CI (200 pfu/cell) upon cyclin D1 signalling in response to FCS. Stimulation with FCS induced a significant increase in cyclin D1 expression at 4 h and, this increased to a greater extent at 8 h (fold stim. at 4 and 8 h = 5.90 ± 1.00 and 6.80 ± 0.70, respectively, n=3). Whilst LacZ was without effect, infection with Adv.MKP-2-CI significantly inhibited the phosphorylation of cyclin D1 at both 4 and 8 h by approximately 55 %. (Adv.MKP-2-CI fold stim. at 4 and 8 h = 2.23 ± 0.41 and 3.63 ± 0.42, respectively, n=3, *p< 0.05 at both 4 and 8 h, n=3).

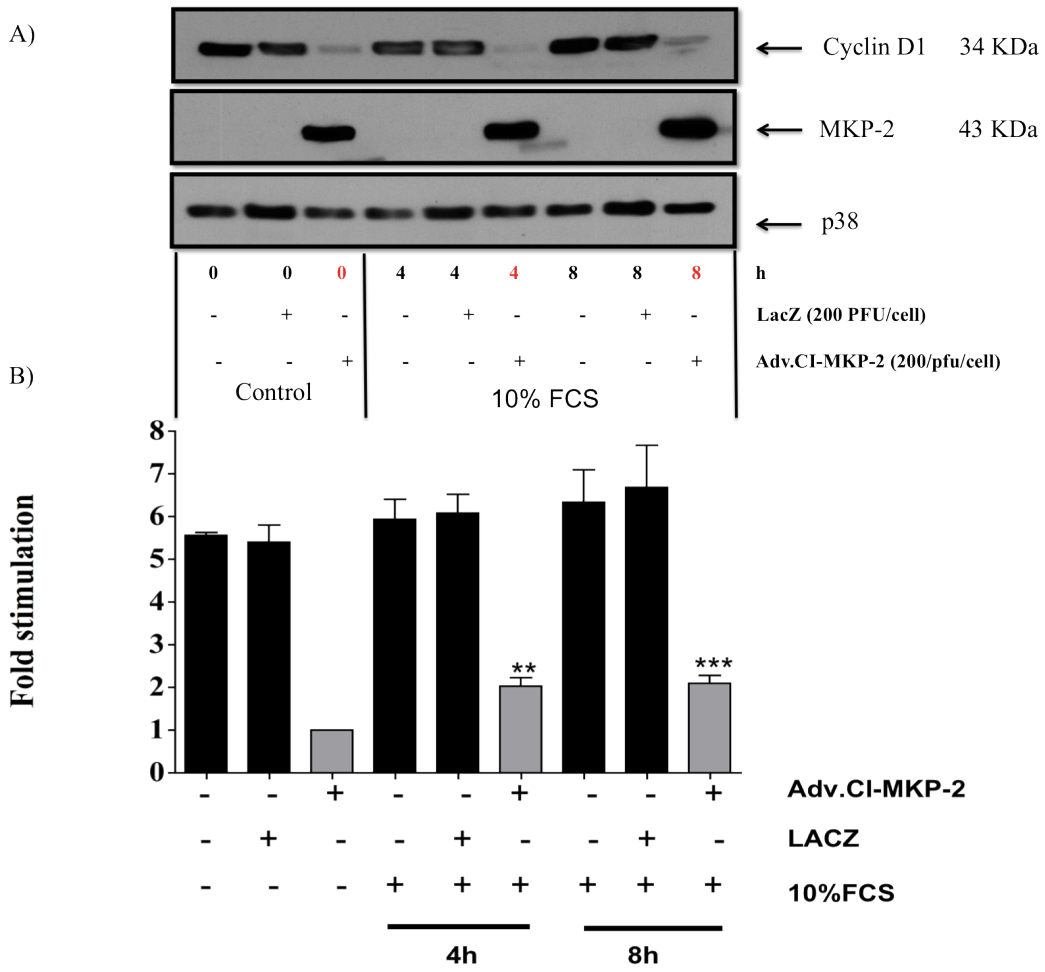


**Figure 3.48: The effect of Adv.MKP-2-CI on FCS stimulated cyclin D1 expression in LNCaP (AS) cell lines.** Cells were infected with Adv.MKP-2-CI for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) cyclin D1 (34 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. **P<0.01 and ***P<0.001 at 4 and 8 h respectively, compared with FCS stimulated control. The results are representative of 3 independent experiments.

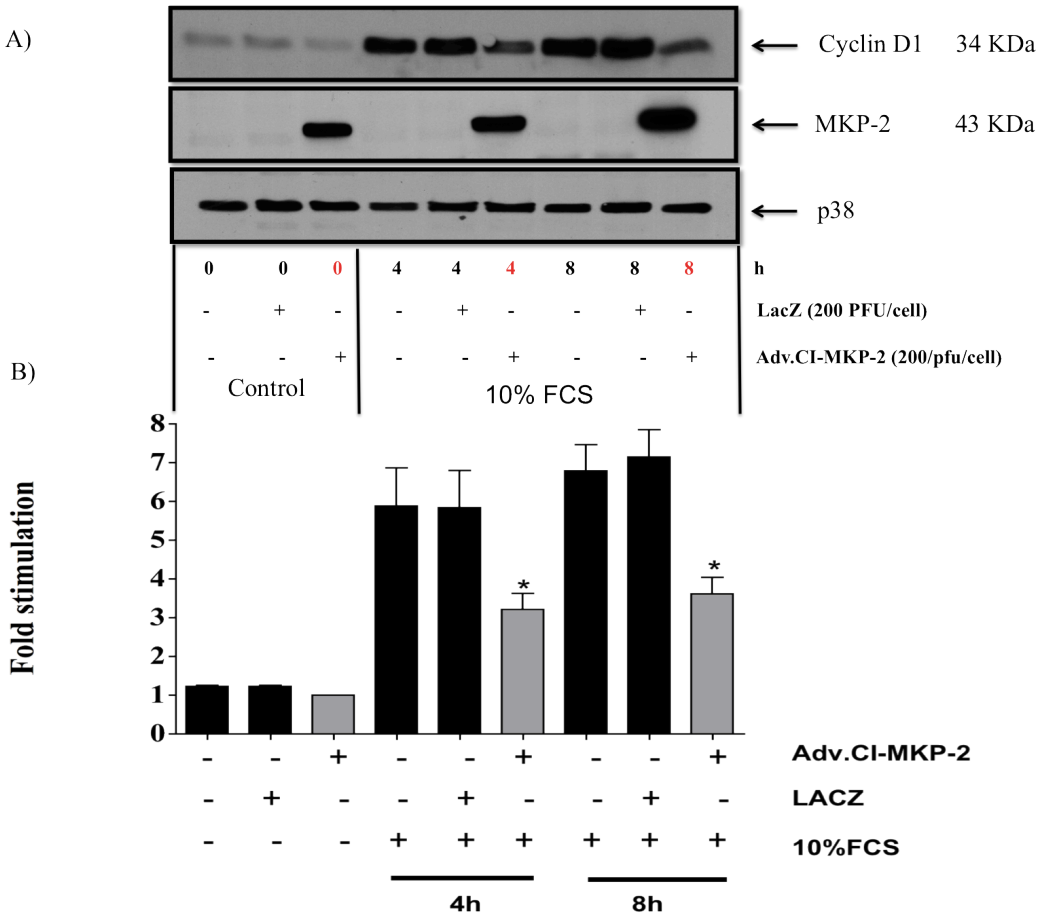


**Figure 3.49: The effect of Adv.MKP-2-CI on FCS stimulated cyclin D1 expression in LNCaP (AI) cell lines.** Cells were infected with Adv.MKP-2-CI for 40 h prior to stimulation with 10 % FCS for the times indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) cyclin D1 (34 kDa), MKP-2 (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. *P< 0.05 at 4 and 8 h, compared with FCS stimulated control. The results are representative of 3 independent experiments.

## 3.15 CHARACTRISATION OF THE EFFECT OF ADV.MKP-2-WT AND CI MEDIATED HISTONE H3 PHOSPHORYLATION IN LNCaP (AS) AND (AI) CELLS

Again surprisingly MKP-2-CI was able to inhibit entry into S-phase despite having little effect on ERK and JNK phosphorylation. This suggests that MKP-2 may be to function as scaffold protein without the need for phosphatase activity. A recent paper has indicated the potential for MKP-2 to regulate histone H3 phosphorylation independently of catalytic activity (Jeong et al., 2013). Therefore, in the experiments below this was examined in LNCaP (AS) and (AI).

### 3.15.1 The effect of MKP-2-WT and MKP-2-CI ON histone H3 phosphorylation in LNCaP (AS) cells

To determine if MKP-2 can regulate histone H3 in LNCaP (AS) (Figure 3.50), cells were infected with either Adv.MKP-2-CI (50, 100 and 200 pfu/cell) or Adv.MKP-2-WT (200, 300 and 500 pfu/cell), then stimulated with EGF (20 ng/ml) for 30 min. Stimulation with EGF did not induce a significant increase in histone H3 phosphorylation at the time points examined (EGF fold stim = 1.44 ± 0.21, n=3), and was not decreased by infecting cells with LacZ. However, infection with increasing pfu of Adv.MKP-2-CI or Adv.MKP-2-WT significantly inhibited basal levels of the phosphorylation of histone H3. CI-MKP-2 (200 pfu/cell) inhibited phosphorylation by approximately 95% (EGF + Adv.MKP-2-CI ( 200 pfu/cell), fold stim = 0.17 ± 0.01, ***$p<0.001$, n=3), whilst MKP-2-WT (500 pfu/cell) inhibited by approximately 76% (EGF + Adv.MKP-2-WT, fold stim = 0.24 ± 0.04, ***$p<0.001$, n=3)).

### 3.15.2 The effect of WT-MKP-2-WT and CI of P-histone H3 in LNCaP (AI) cells

The effect of CI-MLP-2 and WT-MKP-2 on histone H3 phosphorylation in LNCaP (AI) was also examined. As shown in figure 3.51, stimulation with EGF (20 ng/ml) induced a significant increase in histone H3 phosphorylation at the time point tested (EGF fold stim. = 1.72 ± 0.22, n=3), which was not decreased by infecting cells with LacZ. However, infection with increasing pfu of Adv.MKP-2-NLS1 or Adv.MKP-2-WT significantly inhibited the phosphorylation of histone H3. MKP-2-WT or CI (200 and 500 pfu) inhibited phosphorylation by approximately 75%, ((EGF + Adv.MKP-2-WT fold stim with 500 pfu = 0.231 ± 0.03, ***$P<0.001$, n=3), EGF + Adv.MKP-2-CI fold stim with 200 pfu = 0.248 ± 0.01, ***$P<0.001$, n=3)).

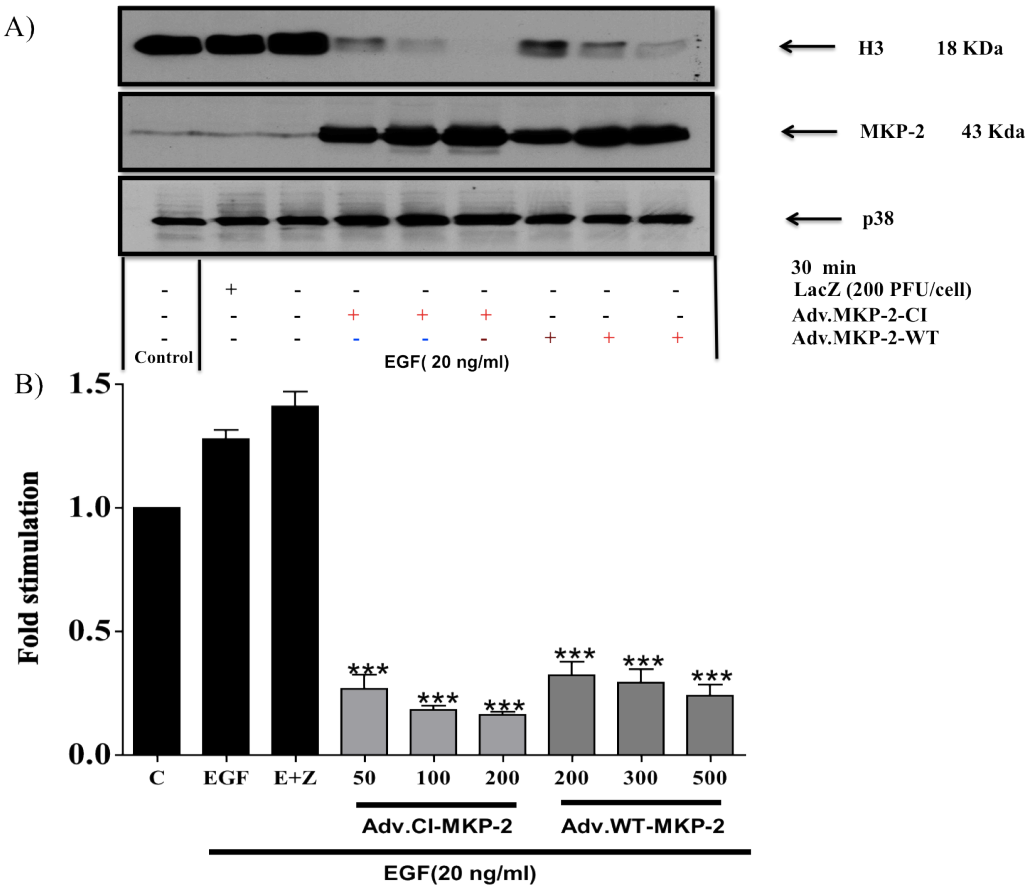


**Figure 3.50: The effect of Adv.MKP-2-CI and Adv.MKP-2-WT upon Histone H3 phosphorylation stimulated by EGF in LNCaP (AS) cell lines.** Cells were infected with both Adv.MKP-2-CI and Adv.MKP-2-WT for 40 hr prior to stimulation with EGF (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p.histone H3 (18 kDa), MKP-2-CI and MKP-2-WT (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA with post hoc Dunnett's test. ***P<0.001 for 50, 100 and 200 pfu for Adv.MKP-2-CI, and, ***P<0.001 for 200, 300 and 500 pfu for Adv.MKP-2-WT, compared with EGF stimulated control. The results are representative of 3 independent experiments.

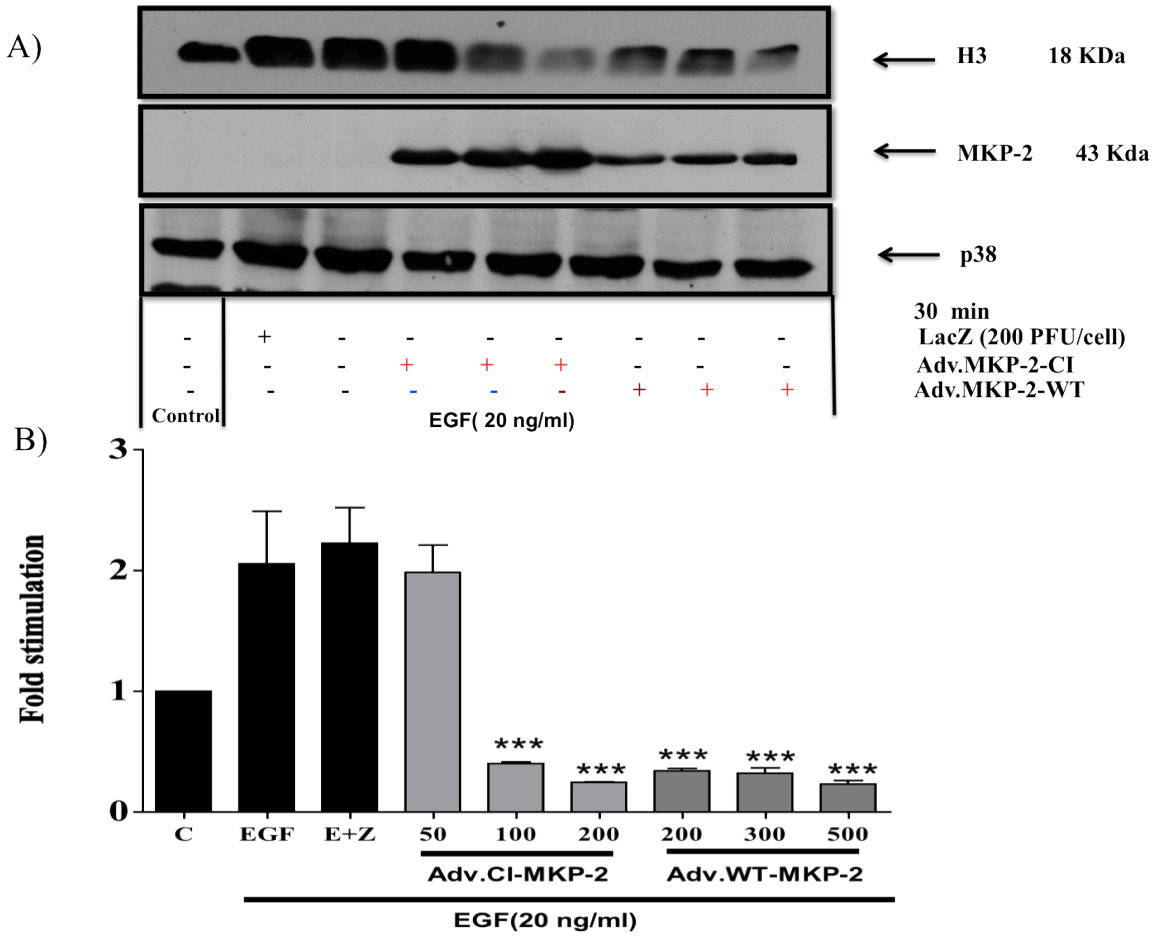


**Figure 3.51: The effect of Adv.MKP-2-CI and Adv.MKP-2-WT upon Histone H3 phosphorylation stimulated by EGF in LNCaP (AI) cell lines.** Cells were infected with both Adv.MKP-2-CI and Adv.MKP-2-WT for 40 h prior to stimulation with EGF (20 ng/ml) for the time indicated. Whole cell lysates were prepared, separated by SDS PAGE, and then assessed for a) p.Histone H3 (18 kDa), MKP-2-CI and MKP-2-WT (43 kDa) and p38 as outlined in section 2.4. Blots were quantified for b) fold stimulation by scanning densitometry; each value represents the mean ± s.e.m. Statistical analysis was by one-way ANOVA, post hoc test by Dunnett's test. ***P<0.001 for 100 and 200 pfu for Adv.MKP-2-CI, ***P<0.001 for 200, 300 and 500 pfu for Adv.MKP-2-WT, compared with EGF stimulated control. The results are representative of 3 independent.

## 3.16 Discussion

In this chapter, adenoviral-mediated overexpression of MKP-2 was used as an experimental pharmacological tool to reverse JNK and ERK-mediated responses in both LNCaP (AS) and (AI) cells, and thus gain some insight into its potential function in prostate cancer cells. It also indirectly shed light on its potential use in clinical conditions involving proliferation and apoptosis. This involved a number of approaches. First of all, the endogenous expression of MKP-2 was examined using PCR and Western blotting. Adv.MKP-2 infection was then tested against the activation of the MAP kinases ERK and JNK, and the resultant effect upon cell cycle progression and apoptosis examined. Two other MKP-2 adenoviruses were also used and they revealed some important information as to the role of MKP-2 in cancer cells.

Initially, the levels of endogenous expression in LNCaP (AS) and (AI) were examined using RT-PCR. It was found that both cell types expressed MKP-2 at moderate to high levels, although these were not quantified. This finding was similar to a recent study in our lab which demonstrated endogenous expression of MKP-2 in different human cell lines including PC3 (Cadalbert et al., 2010). This also included analysis of a truncated form of MKP-2 designated as MKP-2-S. Endogenous MKP-2-S transcripts and proteins were found in PC3 prostate and MDA-MB-231 breast cancer cells, and also in human prostate biopsies (Cadalbert et al., 2010). In contrast, relatively low levels seemed to be expressed in LNCaP cells.

However, it was found that stimulation with a series of agents were unable to enhance the expression of MKP-2. These agents included EGF (Figure 3.1), FCS and a number of other agents (not shown). Previous studies have shown that MKP-2 induction is regulated by ERK activation (Brondello et al., 1997, Tresini et al., 2007, Gomez et al., 2013); however, stimulation with EGF which strongly activated ERK did not increase expression. This suggests that in cancer cells of these types expression is either at a maximum under basal conditions or is regulated by another mechanism. Previous studies have shown that MKP-2 can be induced by hydrogen peroxide (Jeong et al., 2013) or by a p53=dependent mechanism (Shen et al., 2006).

We also found that expression of endogenous human MKP-2 by Western blotting was inconsistent in these cell lines. In the majority of blots it was found to be absent, but in some blots it was apparent. However, again, under activating conditions there was no further induction. This might be due to the stability of the protein. For example, cellular senescence increases MKP-2 protein by blocking its degradation (Torres et al., 2003) and, if LNCaP cells do not readily senesce then this may be relevant for MKP-2 expression. Other studies have indicated that MKP-2 can be induced but the majority of cell types involved are usually of rodent origin where induction seems to be consistently apparent (Ramesh et al., 2008). However, in human cells when PCR demonstrates expression, Western blotting outputs are not always shown or are inconsistent. An early study demonstrated that MKP-2 induction is regulated by ERK activation (Brondello et al., 1997), whilst more recently LH/hCG and 8-bromo-cAMP (8Br-cAMP) were found to control MKP-2 expression tightly; they both significantly increased MKP-2 mRNA levels (3-fold) (Gomez et al., 2013). This again suggests the potential of pathways other than ERK to regulate expression.

It has previously been demonstrated that cell lines express few tissue-specific (2%) or tumour-specific (5%) genes compared to tissue tumours (Sandberg and Ernberg, 2005) and this may be relevant for MKP-2 expression. Indeed, several recent studies have shown convincing detection of MKP-2 in tissue tumours; for example, one recent study showed high expression of MKP-2 in colorectal cancer tissue (Saigusa et al., 2013). Another study demonstrated frequent overexpression of MKP-2 in high frequent microsatellite unstable colorectal cancer (Groschl et al., 2013). Moreover, comparing between hepatocarcinogenesis and hepatoma tissue, no expression of MKP-2 could be detected in normal tissue, but was present in three out of five primary hepatomas studied and MKP-2 mRNA levels were also raised in ascites hepatoma cell lines related to normal liver (Yokoyama et al., 1997). These studies suggest a potential difference between tissues and cell lines.

In both LNCaP cell lines, EGF stimulated a very strong activation of ERK but a much smaller stimulation of either JNK or p38 MAP kinase. Furthermore, experiments examining EGF-induced ERK activation in LNCaP (AS) demonstrated that ERK phosphorylation was transient, occurring between 5 and 15 min. However, in LNCaP (AI) cells ERK phosphorylation was stronger, both in terms of magnitude and prolongation, and the activation was sustained until 2 h. The ERK pathway is often related to oncogenesis and the magnitude and kinetics of ERK activity appears to influence

the survival of carcinoma cells. In general terms, sustained nuclear ERK activation is associated with movement into $G_1$ and through S-phase (Shaul and Seger, 2007, Zhang et al., 1995, Keel and Davis, 1999, Mut et al., 2012). The activation of the ERK pathway is well recognised as being linked to proliferation in the context of cancer. For example, Verma et al, (2004) showed that activation of ERK by selenomethionine enhanced cell growth and this activation was evident within 60 min and lasted for up to 24 h (Verma et al., 2004). Moreover, Shin-Kang and co-workers showed that Tocotrienols caused inhibition of ERK phosphorylation for 6 h and led to a reduction in pancreatic cancer cell proliferation (Shin-Kang et al., 2011).

Nevertheless, activated ERKs can translocate to the nucleus, where they phosphorylate and regulate various transcription factors, such as Ets family transcription factors (e.g., Elk-1), eventually leading to changes in gene expression (Zuber et al., 2000, Schulze et al., 2004). One study showed that after EGF stimulation, ERK1/2 and Elk-1 were phosphorylated within 15 min and were immediately translocated into the nucleus (Mut et al., 2012). However, there are a number of contradictory studies showing that sustained activation of ERK can cause cell-cycle arrest (Sewing et al., 1997, Woods et al., 1997, Roovers and Assoian, 2000). Moreover, scaffold proteins could play an important role in the regulation of the magnitude of ERK activity. KSR (kinase suppressor of Ras) is a scaffold protein that prolongs the kinetics of ERK activity by directly binding to ERK itself (Morrison, 2001, Therrien et al., 1996).

A number of agents were also found to stimulate JNK in LNCaP cell lines, in particular UVC, which gave a strong signal which lasting for up to 2h in both cells types. However, the precise role of JNK in prostate cancer progression is unclear, as both proliferative and apoptotic roles have been proposed. Different agents that stimulate apoptosis in prostate cancer cells *in vitro* enhance JNK activity, the phorbol ester 12-O-tetradecanoyl-13-phorbol-acetate, UV irradiation, and (-)-Gossypol and Gonadotropin-releasing hormone (Altuwaijri et al., 2003, Lorenzo and Saatcioglu, 2008, Huang et al., 2006, Zhang and Roberson, 2006). The pro-apoptotic effect of JNK is shown in preclinical studies in mice; an increase in JNK activity is observed in CWR22 human prostate cancer xenografts following castration and leads to reversion of the tumour size progression through increased apoptosis (Arnoldussen et al., 2008). In contrast with these results, a recent study indicates that JNK is up-regulated in a prostate cancer model in mice (Vivanco et al., 2007).

In order to assess the potential effect of MKP-2 in cancer cell function in LNCaP (AS) and (AI) in relation to proliferation, an adenoviral construct of mitogen-activated protein kinase phosphatase-2 (MKP-2) was utilised (Al-Mutairi et al., 2010b). Increasing concentrations of the virus substantially reduced ERK activation in response to EGF. Indeed, recent studies have confirmed that MKP-2 plays an important, predominant role in ERK dephosphorylation (Cagnol and Rivard, 2013, Jeong et al., 2013, Ramesh et al., 2008, Balko et al., 2012). A recent study also demonstrated that DUSP4 overexpression inhibited the phosphorylation of ERK1/2 and reduced the viability of MDA-231 and BT-549 cells but had little effect on MDA-436 cells (Balko et al., 2012).

Several studies have shown that following stimulation, ERKs translocate to the nucleus within minutes (Lenormand et al., 1993, Caunt et al., 2008, Cagnol and Rivard, 2013, Balko et al., 2012). The fact that Adv.MKP-2 was able almost completely to abolish ERK phosphorylation suggested that ERK translocated to the nucleus and the results in this chapter demonstrate that Adv-MKP-2 was expressed only in the nucleus. This finding is consistent with the selectivity of MKP-2 *in vitro* and its nuclear location.

Overexpression of other MKPs using transfection or adenoviral-mediated infection has also revealed the ability to reduce ERK signalling. A recent study showed that overexpression of MKP-1 suppressed T47D cell proliferation in part, due to decrease of levels of phospho-ERK1/2 (Chen et al., 2011). Furthermore, MKP-1 dephosphorylated pERK1/2 and this led to the arrest of growth of osteoblasts of the MC-4 cells (Datta et al., 2010). Additionally, MKP-1 expression was reduced by dexamethasone induced ERK1/2 dephosphorylation, (Nicoletti-Carvalho et al., 2010), whilst time and concentration-dependent overexpression of MKP-1 correlated with dephosphorylation of ERK1/2 in BBI-treated MCF7 cells (Chen et al., 2005). Other MKPs such as PAC-1 also specifically dephosphorylated ERK1/2 in vitro (Ward et al., 1994, Chu et al., 1996), whilst MKP-4 blocked activation of ERK1/2 once expressed in cells (Levy-Nissenbaum et al., 2003b).

It is however, unclear if such overexpression studies give true insight into the specificity of the MKPs *in vivo*. One single study has demonstrated that induction of MKP-2 in an *in vivo* mouse model, as well as in cell lines, led to inhibition of ERK activity stimulated in response to gonadotropic hormones (Zhang and Roberson, 2006). However, other studies have shown that

overexpression of MKP-2 did not decrease basal ERK activity, and did not affect TPA-stimulated ERK activity in 308 cells (Warmka et al., 2004, Robinson et al., 2001). In addition, in EAhy926 cells, overexpression of MKP-2 had little effect on PMA-stimulated ERK phosphorylation despite its translocation to the nucleus (Robinson et al., 2001). These data suggest that ERK may not be the preferred substrate for MKP-2 in all circumstances, but again it is unclear what *in vivo* outcomes reflect these differences.

The same approach was used to determine if JNK was a substrate for MKP-2 in LNCaP cells lines. Results in this chapter indicated substantial inhibition of JNK signalling particularly in LNCaP (AS) cells, with less inhibition evident in (AI). Nevertheless, these studies were in agreement with previous work demonstrating inhibition of JNK signalling following MKP-2 overexpression. An early study showed that stable overexpression of MKP-2, resulted in inhibition of JNK activation in EAhy 926 endothelial cells (Robinson et al., 2001), and more recently, in UV-C or cisplatin-treated cells (Cadalbert et al., 2005). Wang et al. (2007a) showed that MKP-2 overexpression resulted in dephosphorylation of JNK in U937 cells (Wang et al., 2007a). Moreover, another study demonstrated that suppressing MKP-2 by siRNA in HEK 293 cells, led to prolonged JNK activation in response to $H_2O_2$ treatment (Teng et al., 2007). Again, these studies also imply that JNK was translocated to the nucleus following UVC activation as demonstrated by Charruyer et al., 2005 , or following cisplatin (Cadalbert et al., 2005) or TNF-α (Robinson et al., 2001).

Other types of MKPs also inactivate JNK; for example, MKP-X has been found to bind to and dephosphorylate JNK, again, *in vitro* (Orlev et al., 2004, Levy-Nissenbaum et al., 2003b). Furthermore, one class of MKP, MKP-5, which has both nuclear and cytosolic compartmentalisation, binds to and dephosphorylates p38 MAP kinase and JNK, but not ERK1/2 (Theodosiou et al., 1999, Tanoue et al., 1999). In addition, MKP-7 was found to bind to and dephosphorylates JNK and p38 MAP kinase (Masuda et al., 2001, Tanoue et al., 2001), whilst another study showed that overexpression of MKP-4 in 3T3-L1 cells inhibited JNK phosphorylation (Emanuelli et al., 2008).

Having established that overexpression of MKP-2 could reduce phosphorylation of the cognate MAP kinases, ERK and JNK, the subsequent effect upon proliferation was examined. There was a

marked decrease in proliferation following overexpression of MKP-2 in both LNCaP (AS) and (AI). This finding correlated with a decrease in cells within $G_2$/M phase. This was more marked in LNCaP (AI) than (AS) (Figure 3.23), but it should be noted that the proliferative characteristics of the cell were different in any event. Moreover, analysis of some of the cell cycle regulatory proteins were consistent with $G_2$/M phase arrest (Figure 3.24); for example, the expression patterns of cyclin B1 and p-cdc-2 were reduced following overexpression of Adv. MKP-2.

In the normal cell cycle, the transition from $G_2$ phase to mitotic phase is triggered by the accumulation of cyclin B1, which in turn mediates the phosphorylation a cdc-2 as part of the cyclin B1/cdc2 complex (Morgan, 1995, Baldin and Ducommun, 1995). In general, cells with a blocked cyclin B1/cdc2 activity would be arrested in the $G_2$ phase, while cells with an increased cyclin B1/cdc2 activity tended to enter and progress through mitosis (Wang et al., 2000). Early work has indicated that high cyclin B1 expression levels are found in prostate cancer (Mashal et al., 1996), and comparing between two prostate cancer cell lines, showed that 2-methoxyestradiol (2-ME) or docetaxelall caused apoptosis in both LNCaP and PC3 cells by inhibition of cyclin B1 (Gomez et al., 2007). Additionally, *in vivo*, AR showed a negative correlation with cyclin B1 expression in stroma of human prostate cancer samples (Li et al., 2012).

Previous studies performed in our laboratory have shown a potential role for MKP-2 in proliferation in mice, but do not correlate well with the studies conducted in this thesis. MKP-2 deficient MEFs display reduced proliferation and accumulate in $G_2$/M phase. This finding has not been replicated in any other DUSP KO models, and is the first to denote a role for an MKP at this stage in the cycle. However, analysis of some of the cell cycle controlling proteins were not consistent with $G_2$/M phase arrest, when the expression of patterns of cyclin B1 and p-cdc-2 were increased in MKP-$2^{-/-}$ MEFs when they might be expected to be reduced (Lawan et al., 2011). The same phenomenon was reported following siRNA mediated knock down of MKP-2 protein expression, which also resulted in attenuated proliferation of MKK-f cells *in vitro* and *in vivo* but was correlated with inhibition of cyclin B1 expression and cdc-2 phosphorylation (Hasegawa et al., 2008). More studies are required to examine this phenomenon.

However, looking at the effect of MKP-2 it is apparent that MKP-2 also affected $G_1$/S phase

transition and this may have indirect effect on $G_2$/M-phase cell number. This was due to the inhibition of cyclin D1 expression. The finding agrees with studies which demonstrate a role for ERK in the regulation of cyclin D1 expression and other early events in $G_1$ cycle progression; for example, ERK also regulates degradation of p21 by the 20S proteasome (Coleman et al., 2003). However, a major event in these processes is the regulation of cell-cycle entry and progression. ERKs serve in such a task by different mechanisms (for review see (Chambard et al., 2007). For example, ERKs play a major role in the transit from $G_0/G_1$ to S phase, being required for the transcriptional stimulation of D-type cyclins (Lavoie et al., 1996, Cheng et al., 1998, Sherr and McCormick, 2002).

The results with MKP-2 compare well with those assessing the prototypic DUSP, MKP-1. Constitutive expression of MKP-1 blocks $G_1$-specific gene expression (Brondello et al., 1995). In line with this result, cyclin D1 expression was reduced in a U28 clonal cell line induced to express MKP-1 (Manzano et al., 2002). Overexpression of MKP1 prevented human cancer cells from entering into the cell cycle (Li et al., 2003), whilst it was reported that in rat arterial smooth muscle cells overexpressing MKP-1, growth was arrested in the $G_1$ phase and entry into the S phase was blocked (Lai et al., 1996). Datta et al, (2010) showed that PTH and PTHrP increased MKP-1 expression and leading to growth arrest and a down-regulation of cyclin D1 in osteoblasts (Datta et al., 2010), *in vitro* as well as *in vivo* (Mahalingam et al., 2011). In addition, it has been demonstrated that the ERK inhibition by MKP-1 is sufficient to block DNA synthesis and cell cycle entry activated by oncogenic Ras (Sun et al., 1994).

A number of studies also implicate the ERK MAP kinase pathway in later stages of the cell cycle, (Chau and Shibuya, 1999, Liu et al., 2004, Dumesic et al., 2009); however, the input of DUSPs in this stage of the cycle is largely determined. Liu et al. (2007) demonstrated that MKP-4 reconstitution resulted in $G_2$/M associated cell death and microtubule disruption (Liu et al., 2007). Using RNA interference (RNAi), Rahmoni et al. (2006) showed that cells lacking VHR arrest at the $G_2$-M transition stage of the cell cycle and demonstrated the initial signs of senescence. Studies assessing other DUSPs have been limited and warrant further investigation.

Similarly to ERK function, it should also be noted that MKP-2 may be effective by disrupting JNK

activity in the cell cycle. For example, Gutierrez et al. (2010) showed that JNK activation is limited to $G_2$ and early M-phase, and this activation directly phosphorylates Cdc25C during cell cycle entry and the $G_2$/M DNA damage checkpoint (Gutierrez et al., 2010b). Moreover, activation of endogenous JNK occurs during $G_2$- and early M-phase (Gutierrez et al., 2010b, Lee and Song, 2008, Oktay et al., 2008). Another study showed that JNK is activated during $G_2$ and the beginning of mitosis (Gutierrez et al., 2010a). Similarly, JNK has been implicated in cell-cycle checkpoint activation in response to growth inhibitory stimuli (Tchou et al., 1999, Grosch et al., 2003). All these observations indicate that MKP-2 regulation of JNK is significant in the $G_2$/M phase but is likely obscured by effect in the $G_1$-S phase.

This chapter also assessed the potential for Adv.MKP-2 to regulate apoptosis in LNCaP (AS) and (AI) cells using flow cytometry analysis. This principally involved using UV-C, which has been shown to stimulate JNK activation, phosphorylation of its downstream target c-Jun, and cause sustained JNK activities in both cell lines. Indeed, a role for JNK in apoptosis mediated by UVC has been identified (Behrens et al., 1999, Wisdom et al., 1999). Due to MKP-2 having an effect both on ERK and JNK there may be some opposing effects. For example, ERK has been involved in survival and this may protect against apoptosis.

Furthermore, MKP-2 did not modify the apoptotic effect of two other agents, doxorubicin and X-ray (He et al., 2011, Tsakalozou et al., 2012). Significantly, these agents did not stimulate JNK activity in these cells either, which demonstrates that apoptosis could be induced in this cell line in the absence of JNK activation (Chathoth et al., 2009). This observation is similar to studies showing that JNK activation plays an important role in the mitochondrial apoptotic pathway induced by altered agent. However, numerous studies contradict this position. For example, Chang et al. (2013) showed that activation of JNK, through 2-methoxyestradiol (2-ME), induced JNK and mitochondria-dependent apoptosis in prostate cancer cells (Chang et al., 2013). Numerous studies have demonstrated that activated JNK is translocated to the mitochondria, where it initiates a decrease in membrane potential and subsequent release of cytochrome c (cyt c) into the cytosol, leading to caspase activation (Chauhan et al., 2003, Gao et al., 2005, Davoodpour and Landstrom, 2005). Additionally, a recent study demonstrated static load-induced chondrocyte apoptosis by increased JNK activation (Kong et al., 2013).

As shown in section 3.8, Adv.MKP-2 was unable to modify the apoptotic response to stimulation with doxorubicin, X-ray and UV-C. Similarly to these findings, overexpression of MKP-1 was associated with the suppression of JNK activation in breast cancer cells, which protecting them from apoptosis after treatment with doxorubicin, mechlorethamine and paclitaxel (Small et al., 2007).

Indeed, these findings contrast with other work conducted in our laboratory. In HUVECs, MKP-2 essentially abolished the phosphorylation of γ-H2AX and PARP-1 and reduced apoptosis in response to stimulation with TNF-α in combination with Adv.DNIKKβ (Al-Mutairi et al., 2010b), whilst another recent study showed that MKP-2 overexpression resulted in an inhibition of apoptosis in U937 myeloid leukaemia cells through the dephosphorylation of JNK (Wang et al., 2007a). Furthermore, conditional expression of MKP-2 was found to protect against genotoxic stress-mediated apoptosis in HEK293 cells (Cadalbert et al., 2005). Moreover, another recent study demonstrated that suppressing MKP-2 by siRNA in HEK 293 cells led to prolonged JNK activation in response to $H_2O_2$ treatment and apoptosis (Teng et al., 2007).

Several studies link DUSP expression to JNK inhibition and apoptosis in other cell types. One recent study demonstrated that inhibition of MKP-1 sensitised head and neck cancer to ϒ-radiation-induced apoptosis (Guan et al., 2012). In addition, Takeuchi et al. (2009) demonstrated that ectopic expression of MKP-1 induced inhibition of JNK activation and suppressed AG1478-induced apoptosis (Takeuchi et al., 2009). A previous study showed that VHR (DUSP3) has a direct role in the inhibition of JNK-dependent apoptosis in LNCaP cells (Arnoldussen et al., 2008). MKP-1 has also been implicated in anti-apoptotic effects through the suppression of caspase-3-mediated apoptosis in MEFs (Wu et al., 2005a). Furthermore, apoptosis induced by cisplatin was significant in MKP-$1^{-/-}$MEFs, while such changes were minimal in MKP-$1^{+/+}$ MEFs (Wang et al., 2006). Interestingly, the proteasome inhibitor MG132 increased MKP-1 in H4IIE rat hepatoma cells in a hyperosmotic environment and also activated caspase-3 (Lornejad-Schafer et al., 2005). Further, Lorenzo and Saatciglu (2008) demonstrated androgens inhibit apoptosis with an associated significant drop in JNK phosphorylation. (Lorenzo and Saatcioglu, 2008).

Whilst expression of WT-MKP-2 reduced either EGF or UV-C stimulated ERK and JNK activity respectively, it was unclear which component made the key contribution to either cell cycle arrest. It was also unclear if the co-inhibition of the ERK and JNK pathways was obscuring any potential effects on apoptosis through specific inhibition of each kinase separately. To test this hypothesis, we used a form of MAP kinase which has a triple arginine substitution within positions R74, R75 and R76 to alanine. This is the region within the KIM domain, a sequence which mediates the binding of the MAP kinases and in particular ERK (Chen et al., 2001, Sloss et al., 2005). Using this mutation could further specify the role of ERK or in particular, JNK, in regulating cellular proliferation. A catalytically inactive version of MKP-2 was also used as a further control, because of the potential for MKP-2 to function as an anchoring protein for ERK in some studies (Caunt et al., 2008). The R74-76 mutation also encompasses the NLS and is thus designated as NLS-MKP-2 (Sloss et al., 2005). This domain plays a role but is not essential for nuclear targeting of MKP-2 (Sloss et al., 2005), and indeed immunoflurescent staining demonstrated nuclear localisation of both constructs.

Thus, to determine if the NLS1 mutants were still able to function as specific phosphatases within the cell, their ability to inactivate both ERK and JNK was assessed. Unexpectedly, expression of Adv.MKP-2-NLS1 strongly inhibited EGF-stimulated ERK phosphorylation in both LNCaP (AS) and (AI), this observation suggests that the concomitant mutation of R74, R75 and R76 to alanine (NLS-1) did not abrogate ERK binding. As indicated above, Chen et al. (2001) revealed the ERK binding site as a cluster of three arginine residues R74-76 in the $NH_2$-terminal domain of MKP-2 (Chen et al., 2001).

Moreover, Sloss et al. (2005) demonstrated that the ability of both WT and CI-MKP-2 to co-precipitate ERK was lost following mutagenesis of this region (Sloss et al., 2005). However, these studies were carried out using transfection and it may be there is a law of mass action due to the viral-mediated expression of the protein, rather than an all or none effect; some binding and inactivation of ERK can be possible if there is a substantial amount of protein in the cell. Nevertheless, it should be noted that this domain is certainly implicated in the binding of other MKPs to ERK. For example, Slack et al. (2001) demonstrated that MKP-1 is able to interact selectively with ERK (Slack et al., 2001).

Moreover, Mandl et al, (2005) showed that the expression of DUSP5 in mammalian cells produces both nuclear translocation and sequestration of inactive ERK2 and the interaction is dependent on both the KIM within DUSP5 and the preserved CD site of ERK2 (Mandl et al., 2005).

On the other hand, expression of Adv.MKP-2- NLS-1 reduced UV-C-stimulated JNK activity in both LNCaP (AS) and (AI) cells, and this suggests that the nuclear located NLS-1-MKP-2 is still active towards JNK. This indicates that the KIM domain is not involved in either the interaction between MKP-2 and JNK or the catalytic mechanism. Similarly to these finding, Chen et al. (2001) found that through site-directed mutagenesis the KIM domain was able to reduce JNK activity in the cell but only to a minor extent (Chen et al., 2001). In addition, previous work in our lab showed that Adv.MKP-2-NLS1 inhibited JNK phosphorylation stimulated by both sorbitol or UV-C (Sloss et al., 2005). Moreover, Slack et al. (2001) showed that the basic motif on MKP-1, which is highly homologous to that of MKP-2, does not participate in the interaction between JNK1 and MKP-1 (Slack et al., 2001). Interestingly, mutation of an LXL motif (DSPs an amino acid motif, XILPXLXL, located approximately 80 amino acids from the active site in the cytosolic MAP kinase phosphatase, hVH5/M3/6, blocks JNK-mediated phosphorylation of hVH5 and reduces the phosphatase activity of hVH5 to JNK (Johnson et al., 2000), suggesting this as a potential site of JNK binding.

Secondly, the effect of Adv.MKP-2-CI either on EGF or UV-C stimulated ERK or JNK phosphorylation respectively, was examined and incorporated into the study as a further control. As expected, Adv.MKP-2-CI- had no phosphatase activity and did not reduce either ERK or JNK phosphorylation. Supporting to these findings, Cadalbert et al, (2005) showed that phosphorylation of cellular ERK and p38 MAP kinase activation following UV-C exposure appeared unchanged in the presence of CI-MKP-2 (Cadalbert et al., 2005), whilst Sloss et al, (2005) showed that WT-MKP-2 reduced JNK kinase activity in cells whereas CI-MKP-2 was without effect (Sloss et al., 2005).

However, to our surprise we found that CI-MKP-2 was equally as effective as WT-MKP-2 in reducing progression through both the S and $G_2$-M phases of the cell cycle. This surprising result

does not accord with a recognised role for MKP-2. There are a number of possibilities for these results, implicating the potential roles that MKPs may have in regulating cell function. The first potential is that, whilst MKP-2 was unable to dephosphorylate ERK, it may be able to sequester either ERK or JNK in the nucleus. This potentially has a number of repercussions for the cellular activation of pathways (Caunt et al., 2008).

The second possibility is found in a recent paper which suggests that MKP-2 has the potential to interact with additional substrates. One in particular is VRK-1, a serine threonine kinase which plays a key role in the regulation of proliferation. VRK-1 can phosphorylate several transcription factors, such as p53, ATF2, and c-jun (Sevilla et al., 2004a, Sevilla et al., 2004b, Vega et al., 2004), to regulate different stages of the cell cycle. However, a major role for VRK-1 within $G_2$/M phase is the phosphorylation of histone H3 and condensation (Zeitlin et al., 2001). A recent study has shown that MKP-2 can interact directly with VRK-1 to reduce histone H3 phosphorylation (Jeong et al., 2013), an effect which is not dependent on the phosphatase activity of MKP-2. In the final part of this study, the effect of MKP-2 was replicated in both LNCaP (AS) and (AI) cell. Both WT and CI-MKP-2 were able to reduce histone H3 phosphorylation substantially, supporting the data in a recent paper (Jeong et al., 2013) (see Figure 3.50).

This data suggests the potential of MKP-2 to function not only as a phosphatase but also as a scaffolding protein. Several recent studies have highlighted the potential importance of scaffolding proteins in a number of signalling pathways. Several kinases such as IKKα and Aurora B have been shown to have this property (Rosner, 2007). It is interesting that MKP-1 transiently dephosphorylates H3 on Ser 10 after stimulation of endothelial cells by VEGF or thrombin (Kinney et al., 2009), suggesting the potential of targets for a DUSP other than a MAP kinase. One possible interpretation is that Aurora B has an interaction with Raf/MEK/ERK signalling, moreover, Rosner et al, (2007) showed that Raf Kinase Inhibitory Protein regulates the mitotic spindle assembly checkpoint by controlling Aurora B Kinase activity, and the mechanism involves Raf/MEK/ERK signalling (Rosner, 2007).

Taken together, these results represent a new development in the field of DUSP function in cell signalling, as the only known substrates for MKP-2 and the other members of this family of

phosphatases are MAP kinase family members ERK, JNK or p38 MAP kinase. In the context of prostate cancer cell function, future studies could therefore focus on defining the role of MKP-2 in association with VRK-1 in LNCaP (AS) and (AI), and if related to histone H3 regulation during the M phase.

# 4. ROLE OF MITOGEN-ACTIVATED PROTEIN KINASE PHOSPHATASE-2 (MKP-2) IN CLINICAL PROSTATE CANCER PROGRESSION

## 4.1 INTRODUCTION

In the previous chapter, the potential of Adv.MKP-2 to inhibit both ERK and JNK signalling in LNCaP cell lines and decrease proliferation *in vitro* was highlighted. In addition, MKP-2 can regulate and inhibit histone H3 phosphorylation in both LNCaP (AS) and (AI). Based on these results, it was decided to investigate the expression levels of MKP-2 in clinical prostate cancer tissue further, to determine if a correlation with the cellular studies could be established. In the present study, MKP-2 expression in prostate cancer tissue was examined, and specifically linked to time to death from diagnosis.

The number of cohort studies assessing MKP-2 in human specmens cancer is low, but is increasing. A number of early papers demonstrated increased DUSP-4 expression in bone marrow leucocytes from small cohorts of patients with myelogenous leukaemia (Levy-Nissenbaum et al., 2003a, Levy-Nissenbaum et al., 2003c). Another, more recent, study has linked DUSP-4 to regulation of the HOXA gene in myeloid cells (Wang et al., 2007), and taken together, these suggest a role for MKP-2 in the development of these types of tumour.

Overexpression of DUSP-4 has also been revealed in tumours which have been profiled using Affimtetrix array approaches. In human melanoma, expression is increased 5.7-fold during the development from intermediate to thickness cutaneous melanoma tumours, which is related to progression and metastatic potential (Riker et al., 2008). The potential for MKP-2 to have metastatic effects is further exemplified in geneomic studies, and one study has suggested that MKP-2 expressed a gene that was found to be involved in signal transduction of receptor activity and cell growth of protein and DNA binding (Cloos et al., 2006). Expression is also enhanced in

ER-positive breast tumours and linked to predicting metastatic spread (Yu et al., 2007). Further studies have shown that overexpression of MKP-2 correlates with hepatomas (Yokoyama et al., 1997), pancreatic tumours (Yip-Schneider et al., 2001), familial modulatory thyroid carcinoma (FMTC), multiple endocrine neoplasia (MEN), and papillary thyroid carcinoma (Hasegawa et al., 2008).

In the case of ovarian tumours however, down regulation of MKP-2 is linked to increased activation of ERK and the progression of borderline tumours; this implies that MKP-2 can function as a negative regulator of cancer (Sieben et al., 2005). Consistent with this hypothesis, one recent study demonstrates a striking link between EGFR and KRAS mutations and reduced expression of DUSP-4 (Chitale et al., 2009), whilst other research demonstrated that a CpC region in the DUSP-4 gene is hypermethylated both in glioma cell lines and from tissue samples (Waha et al., 2010). MKP-2 is mapped into chromosome locus 1q8 that is frequently mutated or deleted in both breast and prostate cancers (Armes et al., 2004), and the work of Venter et al. (2005) further showed CHG alterations in multiple breast cancer cell lines within this locus. (Venter et al., 2005).

Thus, overall there is the potential for MKP-2 to be both a positive and negative regulator of cancer; however, to date no studies have link prostate tumour protein expression to clinico-pathological factor such as survival, PSA or other markers. The aim of this chapter was to conduct staining of a prostate cancer tissue array, to assess MKP-2 expression and to correlate this with disease progression.

## 4.2 CHARACTERISATION OF THE EFFECT OF MKP-2 IN CLINICAL PROSTATE CANCER PROGRESSION BY IMMUNOHISTOCHEMISTRY

Although it has been confirmed that the MKP-2 antibody is specific, numerous Western blotting studies show a single band of the predicted molecular weight, it is essential to confirm that the process of fixing the tissue with formalin and embedding in paraffin does not alter its antigenicity for immunocytochemical staining, cell pellets from LNCaP (AI) which were either untreated or infected with Adv.MKP-2-CI were utilised. The results in Figure 4.1 show that there was no staining in cell pellets in the absence of antibody (Panel A). However, there was specific staining for endogenous expression, as shown in panel B, whilst in panel C, there was a major increase in staining following overexpression of MKP-2-CI in LNCaP (AI) cells. This suggests that the procedure can be used to stain for MKP-2 in the prostate.

As no previous study had assessed the expression of MKP-2 in human prostate, it was essential to optimise staining conditions prior to assessing and clinical cohort. Initially, staining of MKP-2 was assessed using either of two buffering solutions (EDTA or citrate) with one of two blocking agents (NHS or Casein) and at different concentrations of MKP-2 antibody (1:100, 500 and 1000). The results in Figure 4.2 shows that EDTA with NHS and Casein were poor (panels B and F, respectively). The results in Figure 4.3 show that citrate with NHS was poor (panel B); however panel F showed the best staining results employed citrate with casein, with 1:100 MKP-2 concentration.

Immunohistochemistry for MKP-2 was employed to interrogate a cohort of 90 prostate cancer patients. Analysis was based on 84 patients and staining intensity assessed using the weighted histoscore technique (Kirkegaard et al., 2006). To confirm the observed scoring for MKP-2 staining, 10% rescoring from the total of both nucleus and cytoplasm was performed by Dr Joanne Edwards. The results in Figure 4.4 show there was no bias between observations relating to nuclear staining. An intra-class correlation coefficient, (ICC) of 0.96, was within the accepted limits. Similarly, for cytoplasmic staining there was no bias in in assessment (Figure 4.5), an ICC of 0.99 was obtained.

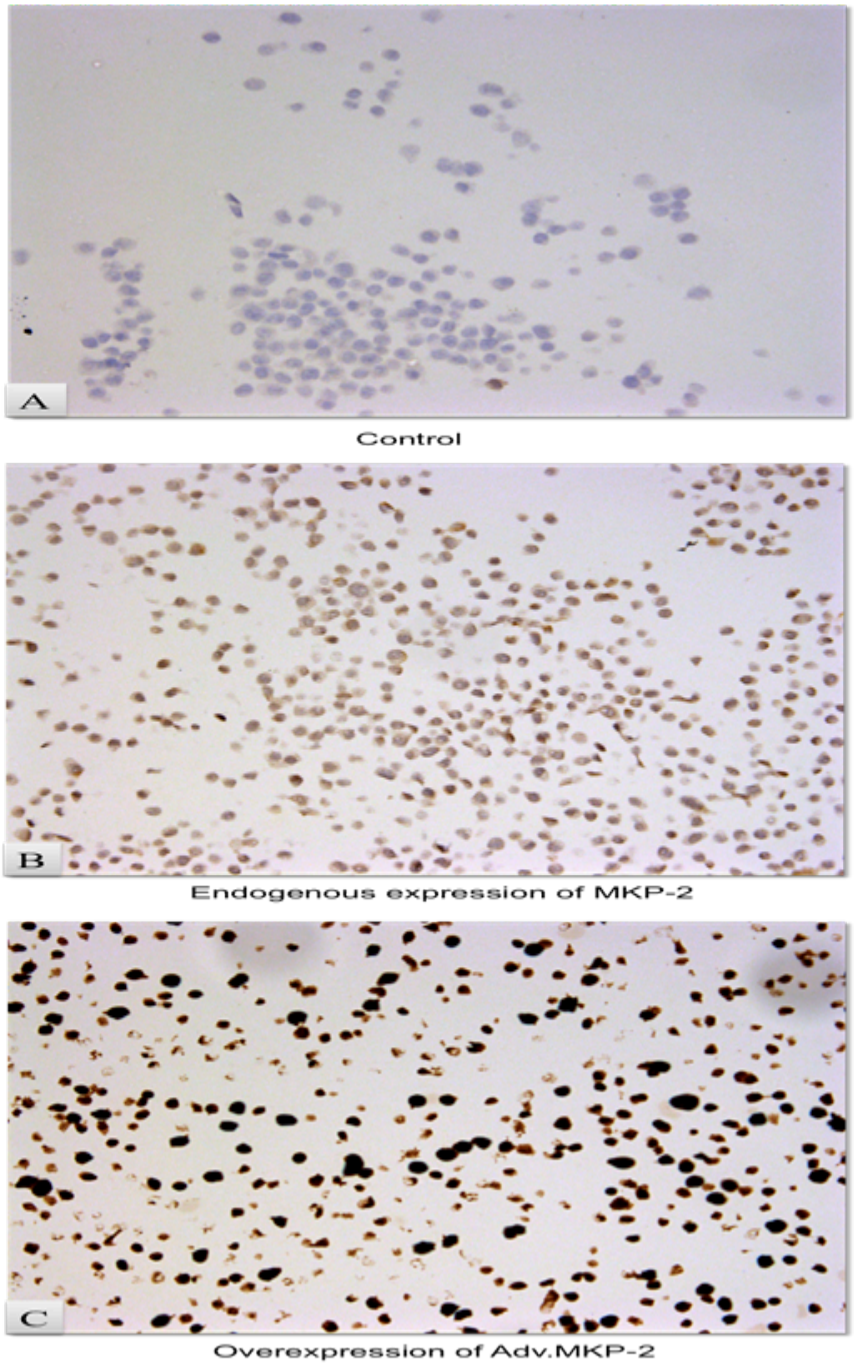


**Figure 4.1: Immunocytochemistry staining for Adv.MKP-2-CI in LNCaP (AI) cell lines.** Cells were either untreated or infected with Adv.MKP-2-CI (200 pfu/cell) for 40 h then got pelts and fixed the tissue with formalin and embedded it in paraffin then stained for MKP-2 as outlined in section 2.11. In panel A, LNCaP (AI) cells are absence of antibody. No virus staining of MKP-2 (panel B), overexpression of Adv.MKP-2-CI (panel C). The results are representative of at least 2 independent experiments.

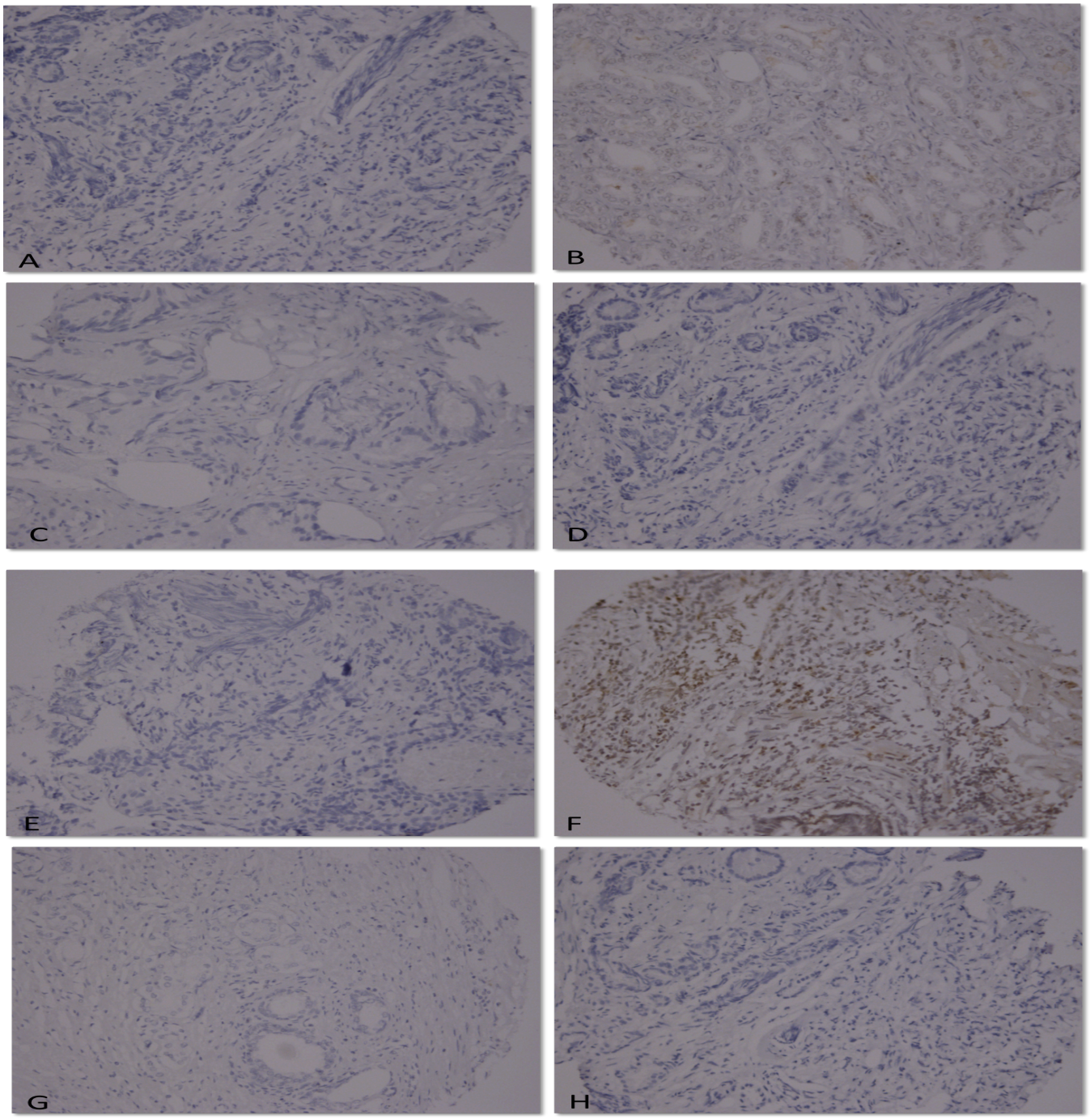


**Figure 4.2: Optimisation of MKP-2 immunohistochemistry staining.** Human Prostate cancer tissues were stained in either of concentrations of MKP-2 or buffer solution in (A, B, C, and D), used EDTA buffer with NHS (A) negative -MKP-2. (B) 1/100 MKP-2, (C) 1/500 MKP-2 and (D) 1/1000, While (E.F.G and H) used EDTA with Casein as outlined in section 2.11. The results are representative of at least 3 independent experiments.

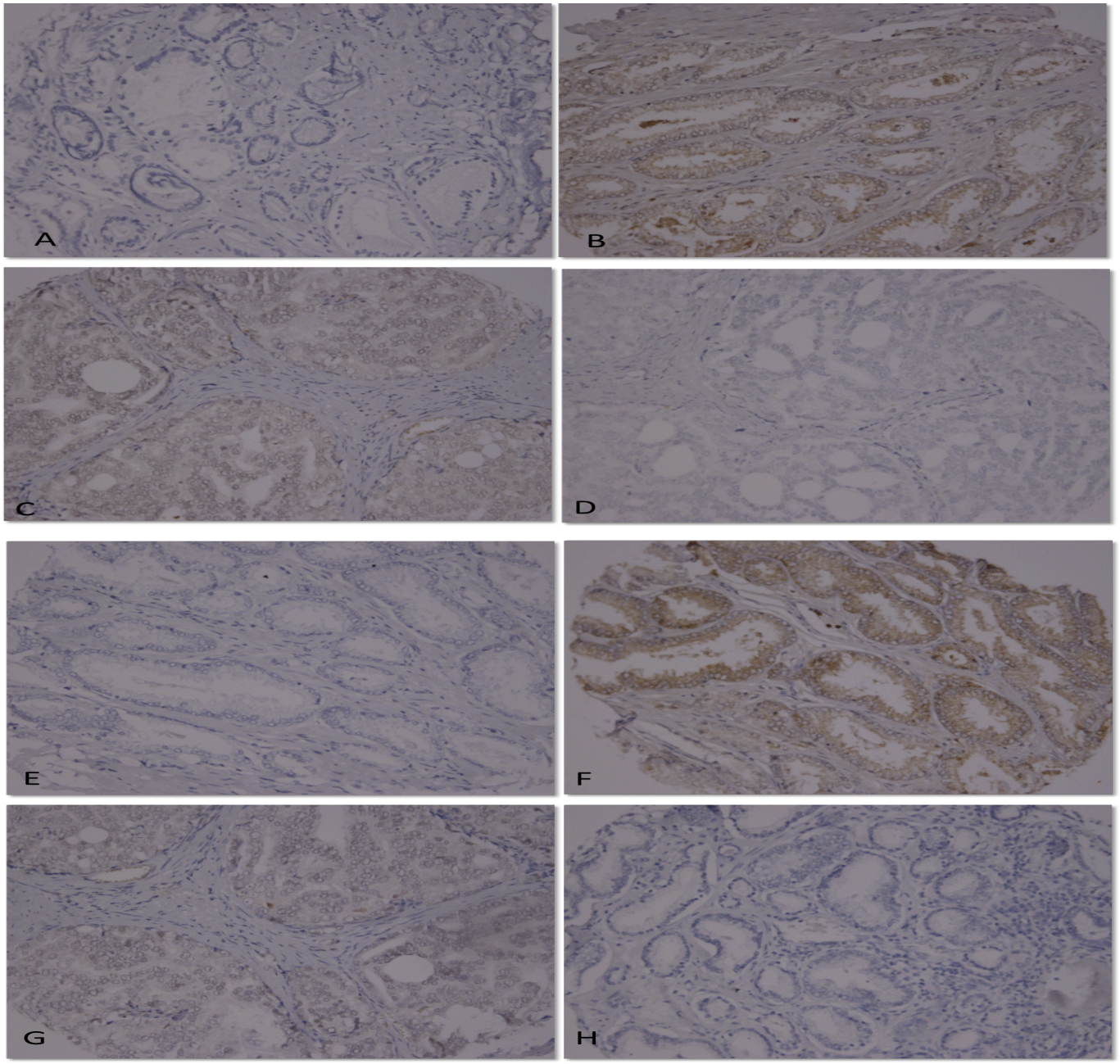


**Figure 4.3:Optimisation of MKP-2 immunohistochemistry staining.** Human Prostate cancer tissues were stained in either of concentrations of MKP-2 or buffer solution in (A, B, C, and D), used citrate buffer with NHS (A) negative -MKP-2. (B) 1/100 MKP-2, (C) 1/500 MKP-2 and (D) 1/1000, While (E.F.G and H) used citrate with Casein as outlined in section 2.11. The results are representative of at least 3 independent experiments.

### 4.2.1 Patient cohort

A cohort of 90 prostate cancer patients was utalised for this study. Patient characteristics are shown in Table 4.1. Twenty-three patients had metastases to local lymph nodes (3), bone (13) and at both sites (7). Forty-seven patients had biochemical relapse (median time to biochemical relapse 2.7 years, interquartile range 1.5–3.8). Twenty four patients were alive at the time of analysis, median follow-up was 11.7 years (interquartile range 9.9–14.0). Forty-six died of their disease (median time to death 4 years, interquartile range 1.9–7.2) and twenty deaths were attributed to intercurrent disease (median time to death 4.1 yr, interquartile range 0.9–5.5).

Table 4.2 shows associations with clinical parameters (grouped data) and outcome measures using Kaplan–Meier methods. When presence of metastases at diagnosis, age at diagnosis, Gleason score at diagnosis and PSA at diagnosis were analysed for this patient cohort only PSA at diagnosis ($p= 0.22$) and Gleason score at diagnosis ($p= 0.11$) were associated with shorter time to death from diagnosis.

Table 4.1: **cohort characteristics** (derived from Willder, et al. 2013).

| Clinical parameter | Percentage of patients (%) |
|---|---|
| **Age (years)** | |
| <70 | 37.8 |
| ⩾70 | 62.2 |
| **Gleason** | |
| <7 | 31.2 |
| =7 | 32.5 |
| >7 | 36.4 |
| **PSA at diagnosis** | |
| $<10\,ng\,ml^{-1}$ | 27.5 |
| $10–20\,ng\,ml^{-1}$ | 20.3 |
| $>20\,ng\,ml^{-1}$ | 52.2 |
| **Lymphovascular invasion** | |
| Absence | 93.3 |
| Presence | 6.7 |
| **Recurrence PSA** | |
| $<10\,ng\,ml^{-1}$ | 77.6 |
| $10–20\,ng\,ml^{-1}$ | 2.0 |
| $>20\,ng\,ml^{-1}$ | 20.4 |
| Abbreviation: PSA=prostate specific antigen. | |

Table 4.2: **Relationship between clinical parameters and clinical outcome measures** (derived from Willder, et al. 2013).

| | Time to biochemical relapse | Survival from biochemical relapse | Disease-specific survival |
|---|---|---|---|
| **Age (<70 vs >70 years)** | 0.260 | 0.385 | 0.020 |
| **Gleason (<7 vs .7 vs >7)** | 0.013 | 0.754 | 0.008 |
| **Diagnosis PSA (<10 vs 10–20 vs >20 ng ml$^{-1}$)** | 0.002 | 0.078 | 0.001 |
| **Recurrence PSA (<10 vs 10–20 vs >20 ng ml$^{-1}$)** | | 0.001 | 0.001 |
| **Lymphovascular invasion (presence vs absence)** | 0.001 | 0.612 | 0.114 |
| **Presence of metastases (presence vs absence)** | 0.001 | 0.008 | 0.001 |
| **Ki67 (<pmedian vs >median)** | 0.730 | 0.279 | 0.033 |

Abbreviation: PSA. prostate specific antigen.
The clinical variables were grouped and analysed by Kaplan–Meier methods with reference to clinical outcome measures as shown. Patients were considered to have biochemical relapse dependent on treatment; radical prostatectomy serum PSA 40.2 ng ml$^{-1}$, radical radiotherapy serum PSA of 2.0 ngml$^{-1}$ above the post-treatment nadir level, hormone treatment 2–3 consecutive rises in serum PSA levels above the nadir obtained at intervals of > 42 weeks (Roach et al, 2006; Cookson et al, 2007). Numbers in bold denote significant associations with P-value <0.05.

### 4.2.2 Localisation of MKP-2 expression in prostate cancer tissue

To identify the localisation of MKP-2 in prostate tumour and association with pathoclinico-characteristics immunohistochemistry was performed on the tissue micro array of 90 patients. Successful staining was performed for a total of 84 tumours on the array. A loss of 6 patients was due to missing cores, or no tumour present on the core, therefore MKP-2 expression could not be assessed. In figure 4.6 shows a histogram of the expression of MKP-2 in cellular membrane, however, only two patients were observed to express MKP-2 at this localization and only eight were observed to expression MKP-2 in the cytoplasm (figure 4.7). Due to the lack of staining in the membrane and low level of cytoplasm expression observed throughout, it was not deemed appropriate to apply these results to further statistical tests.

Figure 4.8 shows a histogram of the expression of MKP-2 in the nucleus; 100% of patient expressed MKP-2 in the nucleus, median expression was 140 histoscore units and interquartile range was 82-188. Patients were classified as having high expression levels if nuclear expression was above the median (>140 histoscore units (n=39)) and low expression levels if nuclear expression was below the median (<140 histoscore units (n=45)). Moreover, Figure 4.9 shows section staining of MKP-2. As mentioned above, the best staining results employed citrate with casein with 1:100 MKP-2 concentration, thus, this concentration was used through out panel A represents, is negative control and panel B is low expression MKP-2-positive in nucleus, whilst, panel C and D shows as high expression MKP-2 positive in the nucleus.

### 4.2.3 The effect of MKP-2 expression in the PSA and Gleason score to survival time

When nuclear MKP-2 levels were associated with patient outcome measures, no association was observed between time to relapse of disease specific survival. As prostate-specific antigen (PSA) has been used extensively as a serum biomarker to screen for prostate cancer and follow therapeutic responses, and the Gleason score is a pathological grading system used to help evaluate the prognosis of men with prostate cancer, it was important to examine the link between MKP-2 expression in PSA and Gleason score, and thus, to determine if MKP-2 expression was prognostic in different patient subgroups (ie those with low or high Gleason score, or those with low or high PSA at diagnosis) using Wilcoxon signed Rank tests to compare between protein expression and

survival in prostate cancer patients. Figure 4.10 shows cancer specific survival versus overall survival in relationship to Gleason score for prostate cancer tumours expressing in nucleus a low level of MKP-2 versus high levels of MKP-2 and compared using the log rank test. For patients whose tumours had a Gleason score of greater than 7, high levels of MKP-2 in the nucleus were observed to have shorter disease specific survival than those patients whose tumours expressed a low level of MKP-2 in the nucleus (Figure 4.10 A), while patients whose tumour had a Gleason score of below or equal than 7, high levels of MKP-2 in the nucleus were observed to have longer disease specific survival than those patient tumour expressed low levels of MKP-2, although this did not reach significance (P= 0. 29, figure 4.10 B).

In addition, Figure 4.11 shows relationship of MKP-2 with cancer specific survival in prostate cancer patients with PSA at diagnosis below 10 ng/ml (Figure 4.11A) or above 20 ng/ml (Figure 4.11B), expressing in nucleus low level of MKP-2 versus high levels of MKP-2.Those patients with high levels of nucleus MKP-2 expression in PSA at diagnosis below 10 ng/ml were observed to have longer median cancer specific survival compared to those with low MKP-2 expression, although this did not reach significance (P= 0.11).

The role of MKP-2 in prostate cancer therefore needs to be confirmed in a larger patient cohort, interesting trends were noted however, due to patient numbers and subcohort analysis performed significance was not reached. It is estimated that a cohort of 300 patients with a similar percentage of events would be required for a sufficient statistical power to validate appropriate biomarkers to level II evidence for either residual risk or associations with cancer specific survival.

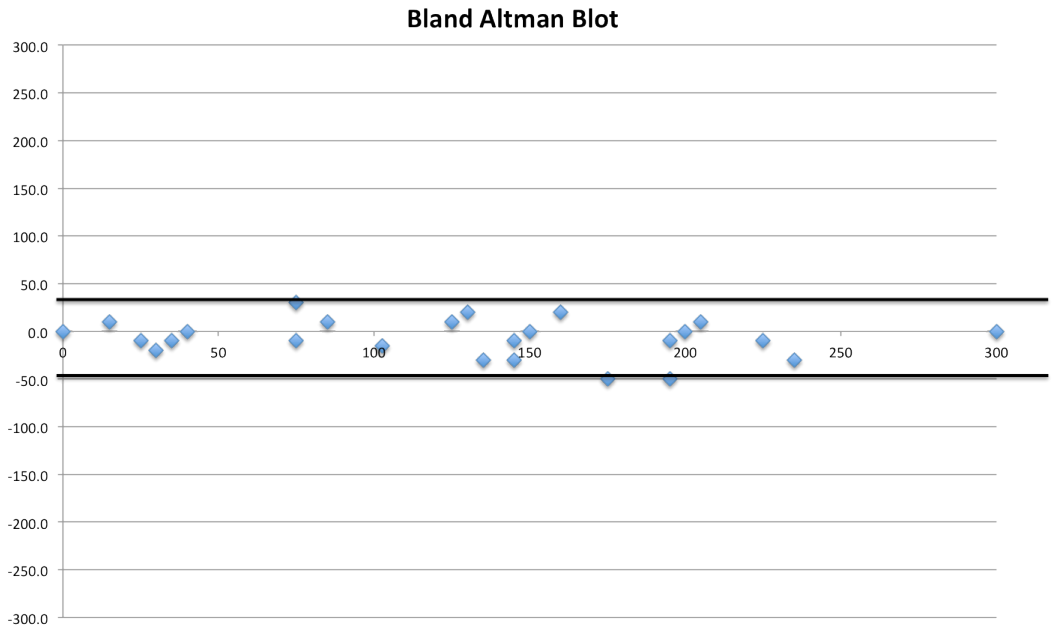


**Figure 4.4 A semi quantitative weighted histoscore to confirm the observed scoring of MKP-2 staining in nucleus (as the Hscore system).** The histoscore ranged from a minimum of zero to a maximum of 300. Results were considered discordant if the histoscores differed by more than 50 as outlined in section 2.11.7. There was no bias between observation and nuclear Intra-class correlation coefficient, (ICC = 0.96).

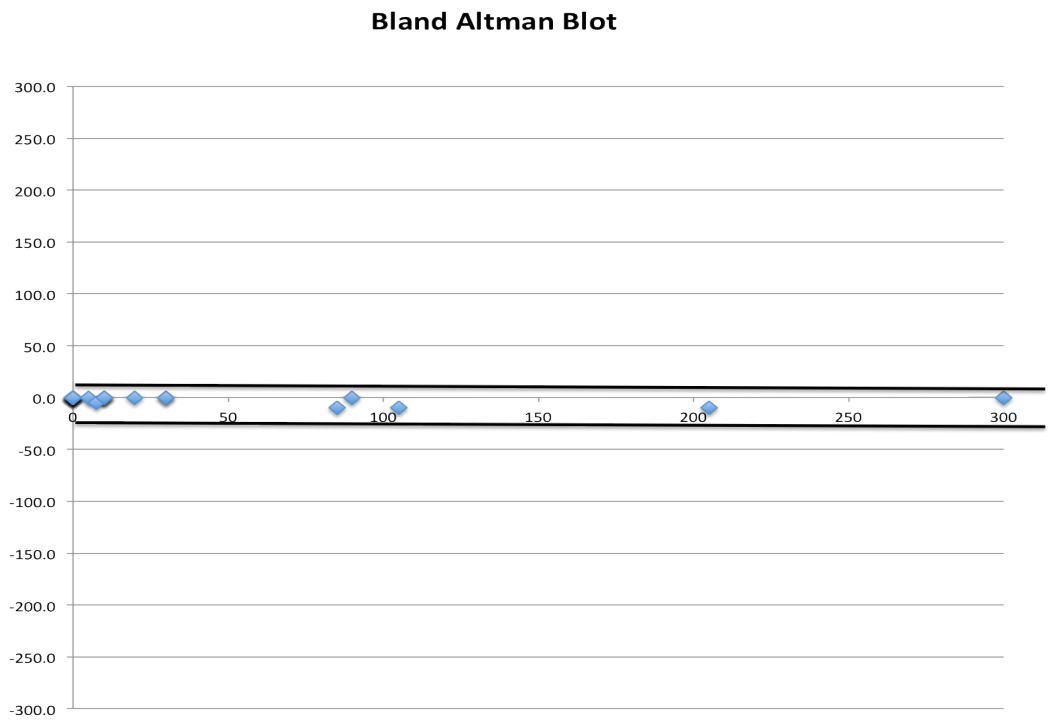


**Figure 4.5: A semi quantitative weighted histoscore to confirm the observed scoring of MKP-2 staining in cytoplasmic (as the Hscore system).** The histoscore ranged from a minimum of zero to a maximum of 300. Results were considered discordant if the histoscores differed by more than 50 as outlined in section 2.11.7. There was no bias between observation and cytoplasmic Intra-class correlation coefficient, (ICC = 0.99).

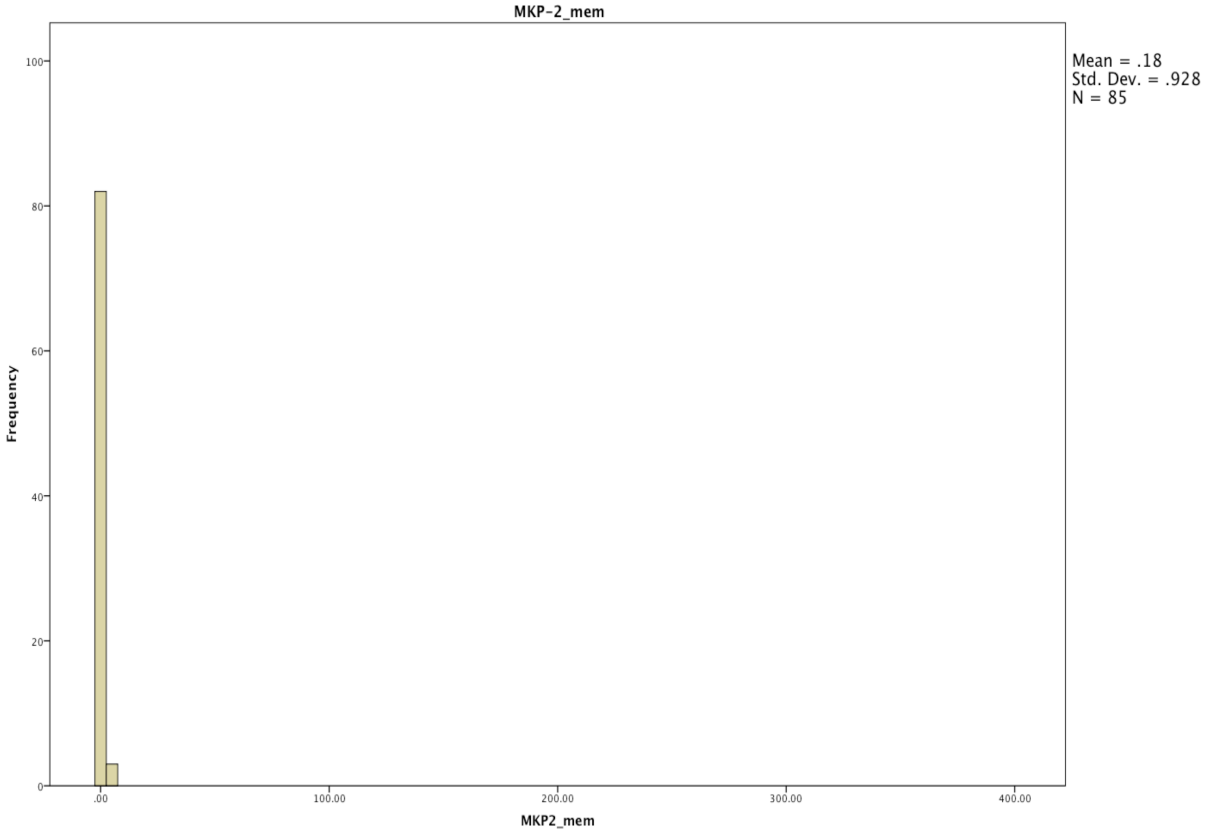


**Figure 4.6: A histogram of expression of MKP-2 in cellular membrane.** Section staining for MKP-2 was performed in cellular membrane, using the SPSS version 15 for Windows. Basic descriptive statistics were performed to calculate the frequencies, mean, median and inter-quartile ranges for the histoscore for each protein investigated as outlined in section 2.11.7. There was no staining observed in membrane, mean = 0.18. (n=84).

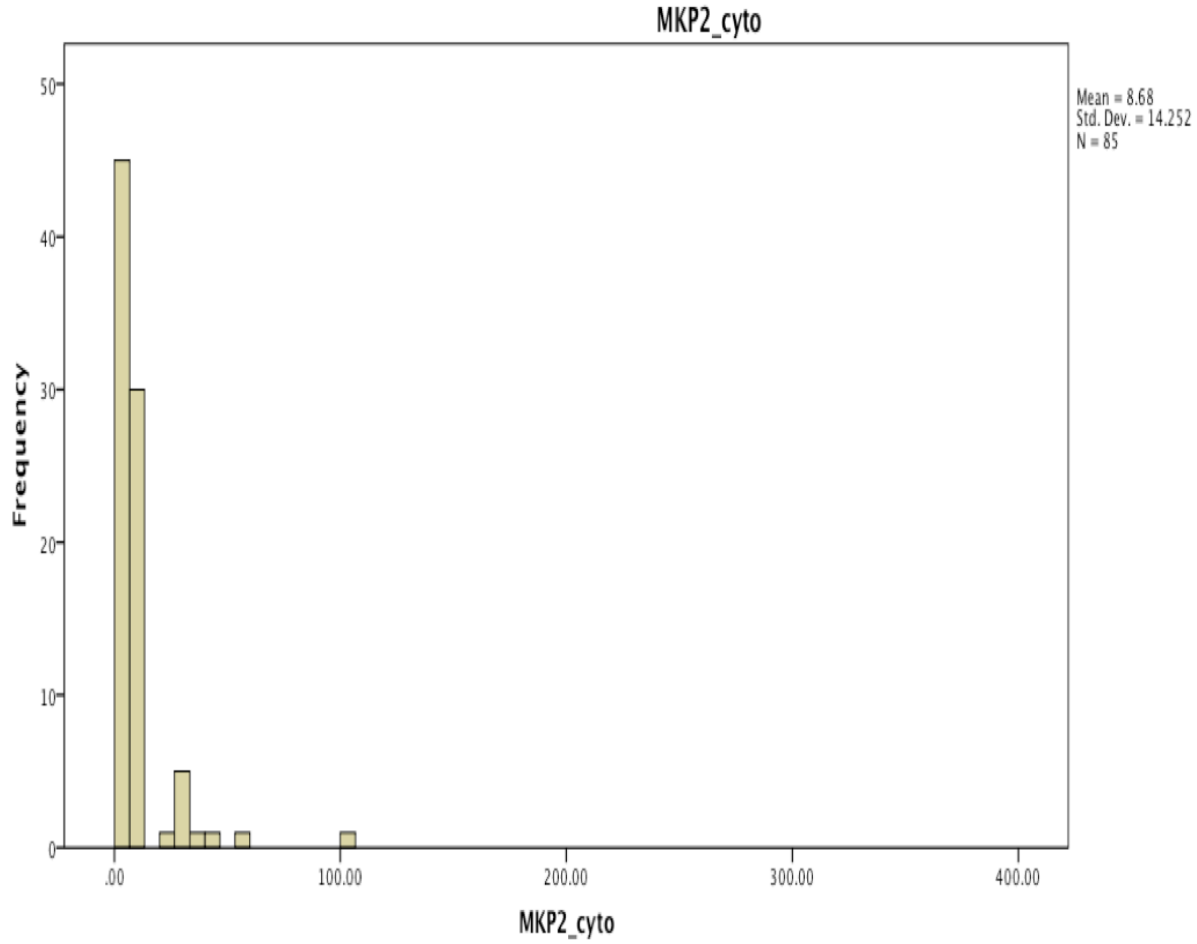


**Figure 4.7: A histogram of expression of MKP-2 in cytoplasm.** Section staining for MKP-2 was performed in cytoplasm, using the SPSS version 15 for Windows. Basic descriptive statistics were performed to calculate the frequencies, mean, median and inter-quartile ranges for the histoscore for each protein investigated as outlined in section 2.11.7. There was low expression in cytoplasm, mean = 8.68. (n=84).

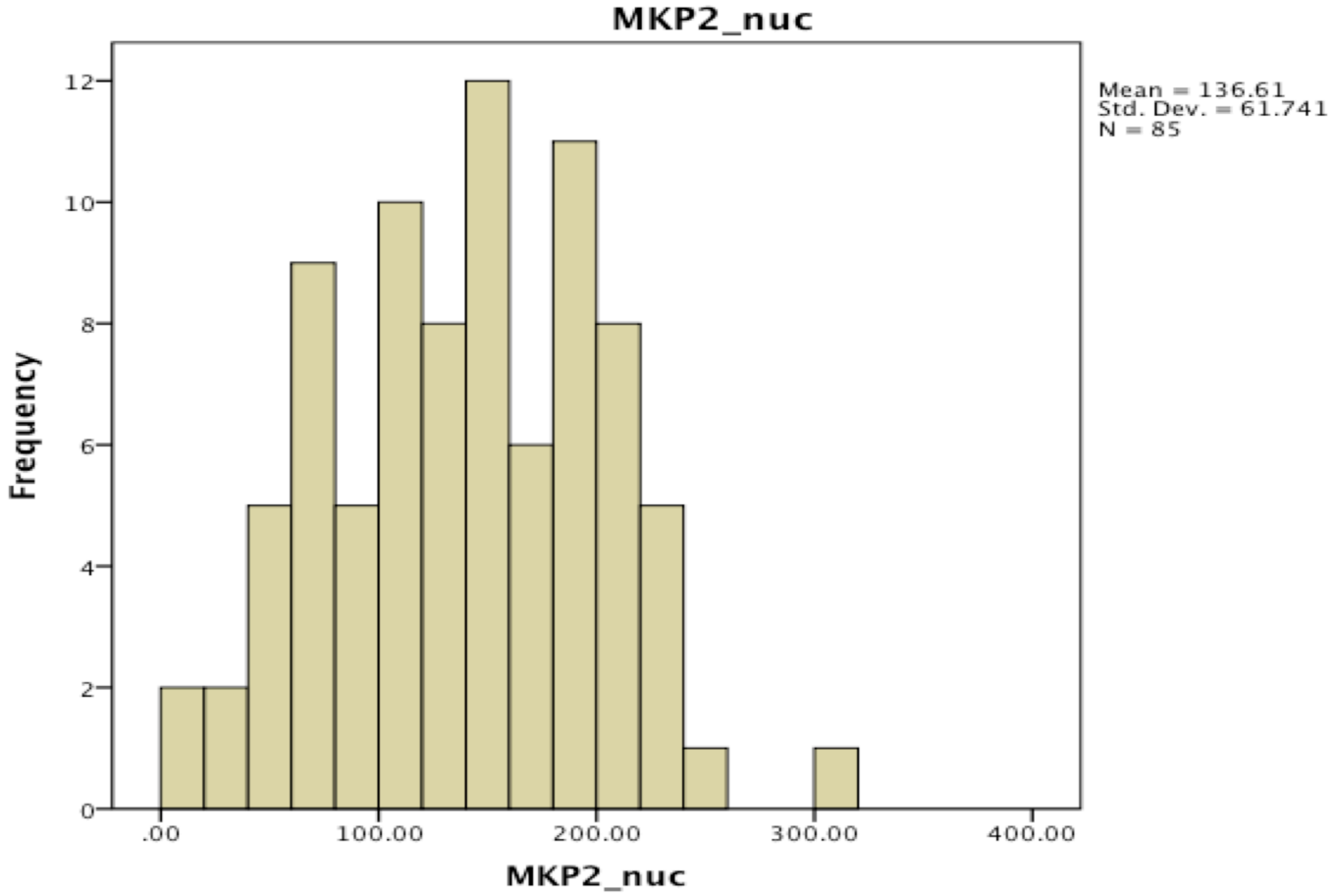


**Figure 4.8: A histogram of expression of MKP-2 in nucleus.** Section staining for MKP-2 was performed in nucleus, using the SPSS version 15 for Windows. Basic descriptive statistics were performed to calculate the frequencies, mean, median and inter-quartile ranges for the histoscore for each protein investigated as outlined in section 2.11.7. The expression was high in nucleus, mean = 136.6. (n=84).

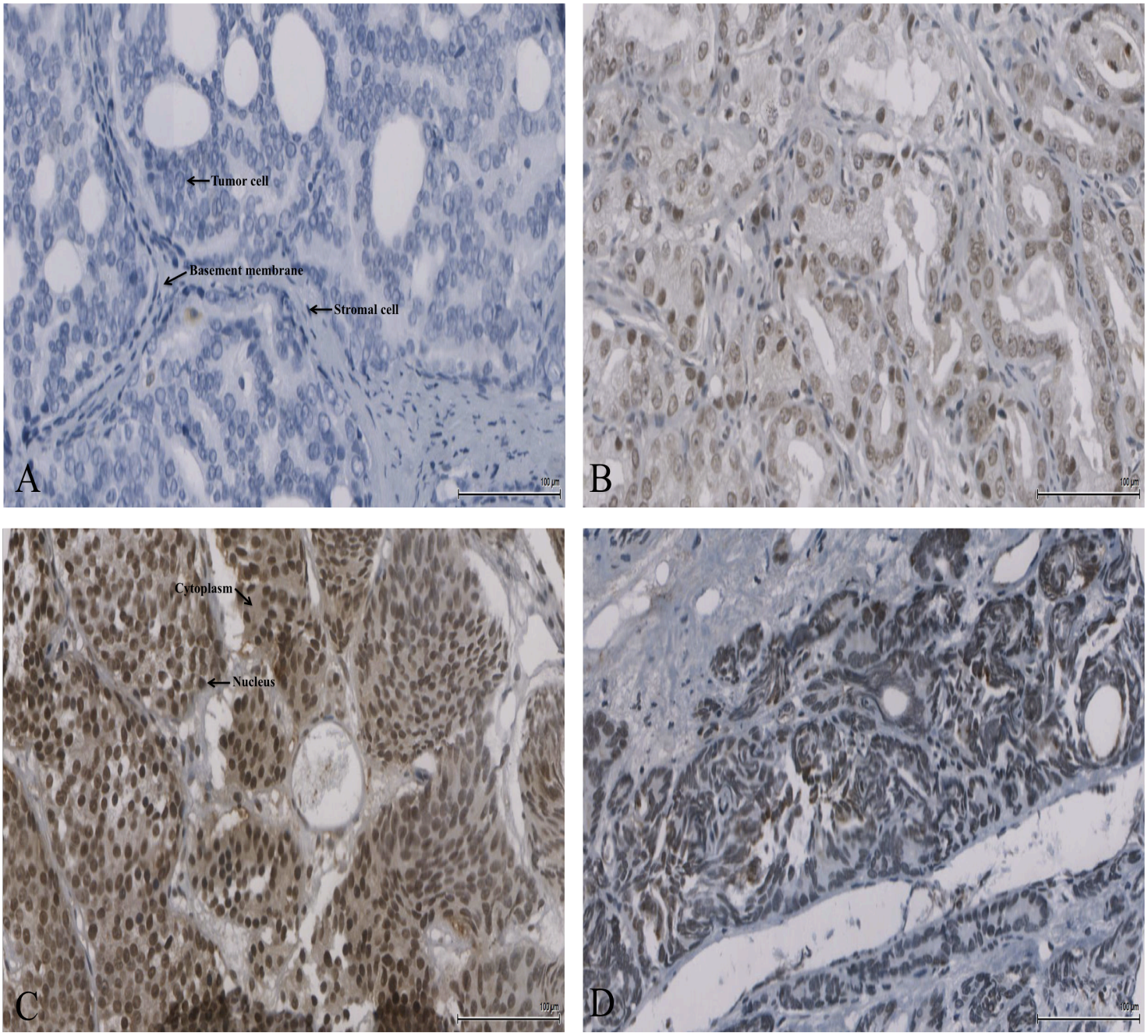


**Figure 4.9: MKP-2 immunohistochemistry in prostate cancer**. Section of cell types as indicated were fixed and stained for MKP-2 (1:100) as outlined in the method. In panel A, negative control, panel B, low expression MKP-2-positive (mean = 8.68 from 0-300), panel C and D, high expression MKP-2-positive (mean = 136.6 from 0-300). The results are representative of at least 3 slides a total from 84 patients.

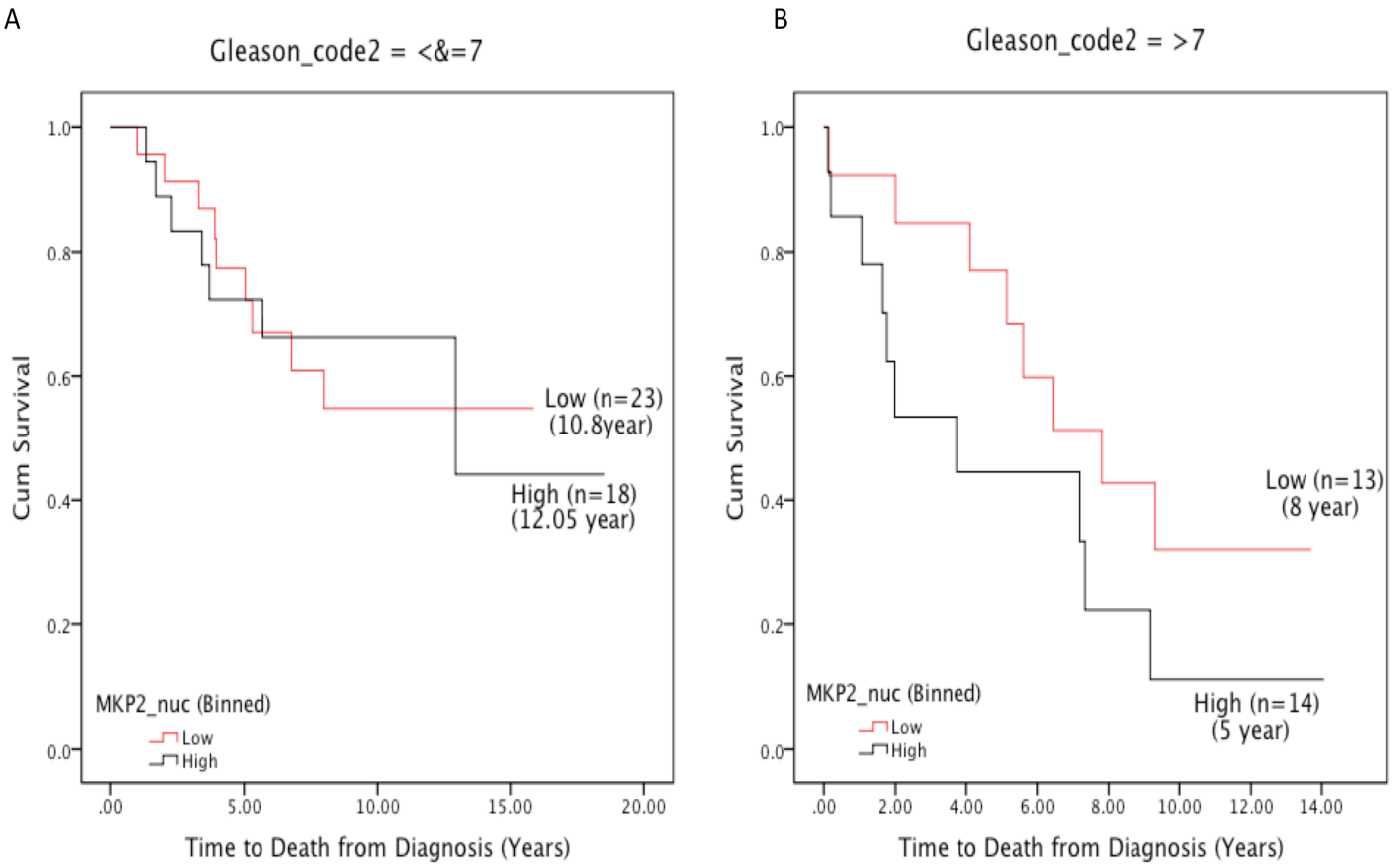


**Figure 4.10: The association between MKP-2 expression and survival prostate cancer patients to Gleason score.** To compare between protein expression and survival prostate cancer patients, Wilcoxon signed Rank tests were used as outlined in section 2.11.7. In panel A, Gleason score = < and = 7, panel B, Gleason score = >, and both representative association between prostate cancer specific and overall survival with Gleason score. The results are representative of 3 slides a total from 84 patients.

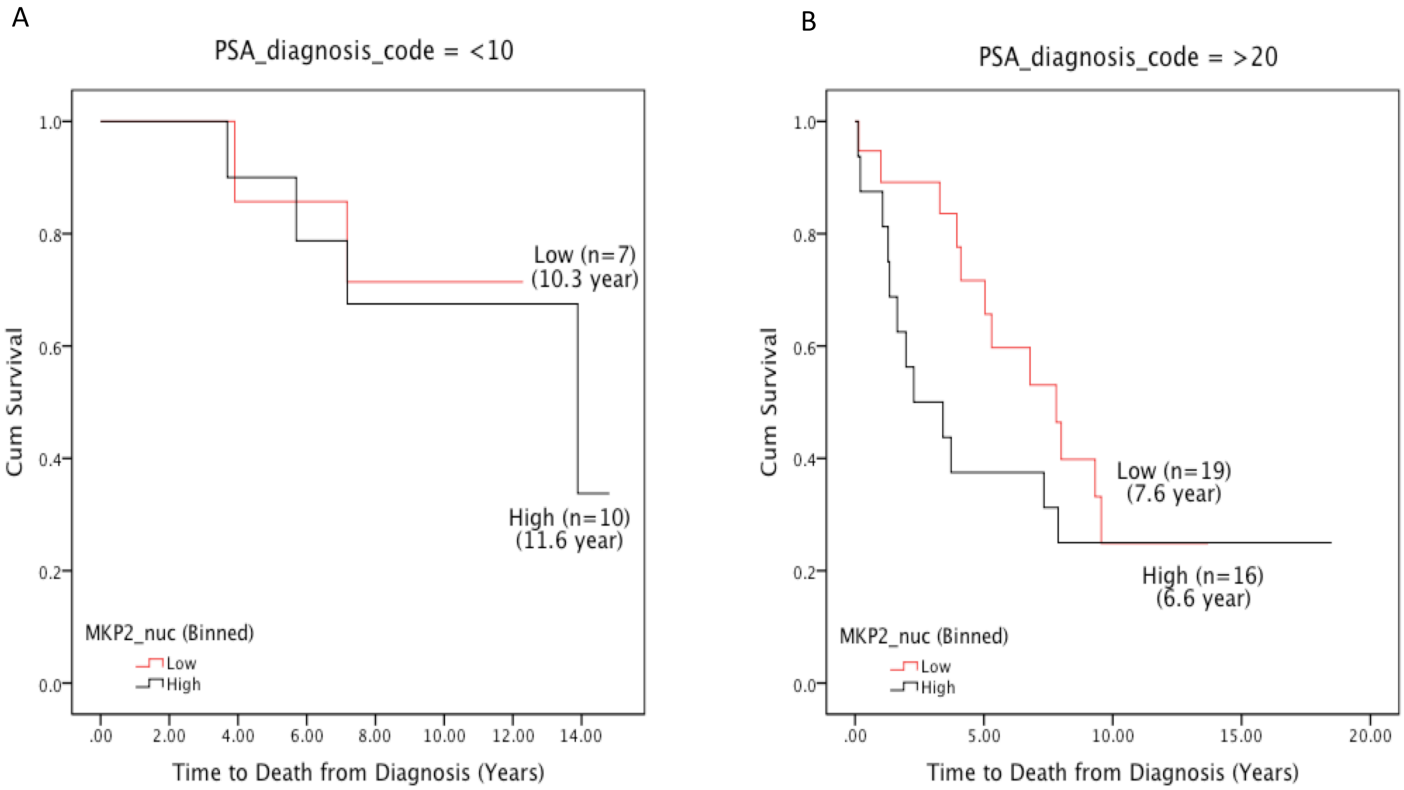


**Figure 4.11: The association between MKP-2 expression and survival prostate cancer patients to PSA.** To compare protein expression between and survival prostate cancer patients, Wilcoxon signed Rank tests were used as outlined in section 2.11.7. In panel A, PSA = < 10, panel B, PSA = > 20 and both representative association between prostate cancer specific and overall survival with PSA. The results are representative of 3 slides a total from 84 patients.

## 4.3 DISCUSSION

As discussed in the previous chapter, the cellular function of MKP-2 is not well described, particularly in the context of prostate cancer. There are very few data which have shown any cellular function of MKP-2 in prostate cancer cell lines and in Chapter 3, it was demonstrated that high levels of MKP-2 were associated with inhibition of proliferation. In this chapter, the potential function of MKP-2 was correlated with the expression in human prostate cancer tissues.

The initial objective was to confirm antibody suitable for IHC establish optimum conditions for MKP-2 staining and determine whether expression predominated within the nucleus, cytosol or plasma and subcellular membranes. A standard immunocytochemistry protocol was used based on using MKP-2 infected LNCaP (AI) cells. Endogenous levels were low as expected from the cellular studies. Using this protocol, MKP-2 was identified as being predominantly within the nucleus of prostate cancer cells, within each section although theidentify of the cell type was not established. This would be consistent with the fact that MKP-2 is -located in the nucleus, as confirmed using adenoviral infection in Chapter 3.

Our group has recently identified a splice variant of NV-MKP-2 which does not have an NLS (Cadalbert et al., 2010). Whilst we have show that in cancer cell lines it is retained in the nucleus, in other papers (Chen et al., 2001), removal of the R74A/R75A/R76A motif results in cytosolic localisation. It is therefore possible that cytosolic expression of MKP-2 in tissue sections may have reflected the presence of the new variant. However, this does not seem to be the case and supports the results in Chapter 3 showing very little expression of NV-MKP-2 in prostate cell lines. One study, in contrast to these findings, showed that DUSP4 expression in the cytoplasm of the colorectal cancer cells (Saigusa et al., 2013).

On examining 84 prostate tumours, a number of correlates were established, including metastases at diagnosis, age, Gleason score and plasma PSA level. These are diagnostic markers currently employed clinico-. It was demonstrated that high levels of MKP-2 expression in the nucleus were shown to have a higher cumulative effect than tumours which expressed a low level of MKP-2 in the nucleus, with PSA at diagnosis below 10 ng/ml, although this did not reach significance (P= 0.

29, figure 4.9). In addition, cancer specific survival versus overall survival in relationship to Gleason score for prostate cancer tumours expressing in nucleus a low level of MKP-2 versus high levels of MKP-2 and compared using the log rank test. For patients whose tumours had a Gleason score of greater than 7, high levels of MKP-2 in the nucleus were observed to have shorter disease specific survival than those patients whose tumours expressed a low levels of MKP-2 in the nucleus, while, patients whose tumour had a Gleason score of below or equal than 7, high levels of MKP-2 in the nucleus were observed to have longer disease specific survival than those patient tumour expressed low levels of MKP-2, although this did not reach significance (P= 0. 29, figure 4.10). Whilst these studies are difficult to assess due to the lack of statistical significance, this suggest that high expression of MKP-2 in early-grade prostate tissue biopsies, correlated with a longer time to survival in patients, this could suggest a beneficial role for mkp-2 in early-stage disease as a tumour suppressor. However, high expression of MKP-2 in late-grade prostate biopsies demonesraed poor survival, suggesting that MKP-2 may promote tumour progression and need to confirm in a larger independent cohort.

The significance of DUSP-4 in cancer has been examined in more depth in recent studies. Compared to control samples, CRC patients with either microsatellite instability (MSH-I) or stable tumours had significantly higher DUSP-4 levels (Groschl et al., 2012). In equivalent cell lines, MKP-2 overexpression was associated with proliferation indicating a positive role in cancer progression (Groschl et al., 2012). However, another more recent paper examined a cohort of 212 patients undergoing surgery for CRC (Saigusa et al., 2013). This study, whilst showing initially higher DUSP4 staining in such patients relative to matching controls, found a clear association of decreased DUSP-4 expression with advanced T classification, lymphatic invasion, advanced stage and liver and lung metastasis. Consistent with the results in this chapter, the study also demonstrated increased DUSP-4 expression with better prognosis. Indeed, another study examining resistance to chemotherapy in breast cancer cohorts revealed an association between DUSP-4 loss and poor patient outcome (Balko et al., 2012). Taken together, these data again suggest the potential for MKP-2 to function as a tumour suppressor gene.

These recent results are by an large consistent with previous studies which demonstrate loss in DUSP-4 is linked EGFR mutant tumours in lung adenocarcinomas cancers (Chitale et al., 2009),

whilst epigenetic downregulation of MKP-2 is associated with prolonged survival of patients with primary glioblastomas (Waha et al., 2010). However, in a later study, DUSP-4 deletion is actually associated with a better overall survival, making overall conclusions in this study difficult. Nevertheless, the majority of studies seem to link DUSP-4 to an anti-tumour function in cancers such as colon, neuroblastoma and breast, but for other cancers, the evidence is less clear. Furthermore, whilst in at least some cancers MKP-2 is linked to changes in ERK levels or associated with Ras or EGFR mutations this is not the case in all instances. A correlation between MKP-2 expression and ERK phosphorylation is a feature of very few of the existing studies. There are virtually no data associated with JNK levels nor changes in VRK1 and these parameters could be further examined.

There have been similar types of clinical study examining other MKPs, in particular MKP-1. For example, Rojo et al. (2009) showed that MKP-1 was expressed at low levels in normal breast and at high levels *in situ* carcinoma, and MKP-1 was overexpressed in approximately 50% of infiltrating breast carcinomas (Rojo et al., 2009). In another study, MKP-1 mRNA and ERK1/2 protein expression were assessed in 164 human epithelial tumours of diverse tissue origin by immunohistochemistry, and that MKP-1 was overexpressed in the early phases of prostate, colon, and bladder carcinogenesis, with progressive loss of expression with higher histological grade and in metastases (Loda et al., 1996). Moreover, immunohistochemistry in NSCLC resection specimens showed high levels of CL100/MKP-1 and an association was identified between high CL100/ MKP-1 expression levels and better survival in NSCLC, even for early-stage patients (Vicent et al., 2004). In addition, in clinical specimens, previous data had demonstrated an overexpression of the CL100/MKP-1 protein in tumours such as breast, colon, and prostate (Loda et al., 1996). Moreover, in immunohistochemistry results, both MAP kinase and MKP-1 were strongly expressed in severely dysplastic lesions (Leav et al., 1996).

In contrast to these results, using immunohistochemistry assay, Wang et al. (2011) indicated that the expression of MKP-1 was gradually reduced as tissue type moved from normal lung tissues to increasingly undistinguishable carcinoma, and it was negatively related with tumour differentiation (Wang et al., 2011). Moreover, in 2002, Denkert et al. showed that expression of MKP-1 was related to a significant reduction in ovarian carcinomas and LMP tumours compared to benign cysts

and normal surface epithelium (Denkert et al., 2002). Taken together, there is the potential for MKP-1 to be a positive and negative expression in cancer.

There are also studies looking at other MKPs. Wu et al. (2011) showed that reduced MKP-4 expression was related with poor prognosis in clear renal cells carcinoma. Liu et al. (2007) showed that DUSP-9 is downregulated in initial keratinocytes, but is absent in their malignant derivatives (Liu et al., 2007). One recent study indicates that MKP-4 status in tumours may be a useful clinical tool in estimating prognosis in patients with hepatocellular carcinoma (Liu et al., 2013).

In conclusion, MKP-2 is expressed in the invasive prostate tumours and localised as high expression more than 48% in the nucleus, but very low in cytoplasm and was not observed in cellular membrane. Analysis of 84 prostate tumours was examined and showed that high levels of MKP-2 expression in the nucleus to have disease specific survival more than those patients whose tumours expressed a low level of MKP-2 in the nucleus with PSA below 10 ng/ml at diagnosis. In addition, those patients with high levels of nuclear MKP-2 expression in Gleason score of below or equal than 7 at diagnosis also were observed to have longer median cancer-specific survival compared to those with low MKP-2 expression, while whose tumours had a Gleason score of greater than 7, high levels of MKP-2 in the nucleus were observed to have shorter disease specific survival than those patients whose tumours expressed a low level of MKP-2 in the nucleus. A future plan may also focus on ERK and JNK expression in prostate tumours and linked it to MKP-2 expression and correlate these expressions to other factors, such as survival.

# 5. GENERAL DISCUSSION

## 5.1 GENERAL DISCUSSION

The role of MAP kinase phosphatase-2 in cell function is still unclear. Whilst identified in 1995, it was not extensively examined and viewed primarily as a surrogate to the ubiquitous MKP-1 (Misra-Press et al., 1995). Attention was also directed to defining the roles of MKPs, which were distinct from MKP-1 such as MKP-3 and 7, which in turn allowed the classification of the family to be extended (Keyse, 1998). During this period evidence accumulated slowly which implicated the DUSP-4 gene as important in cancer and this trend has developed.

At the start of this thesis, the role of MKP-2 in prostate cancer was largely unknown. The study demonstrated for the first time that MKP-2 was endogenously expressed in both LNCaP (AS) and (AI) cells, although it was hard to detect at the protein level. As other studies imply that it is a gene which is normally induced in response to agonist stimulation, and linked to the negative feedback of MAP kinase, the relatively high levels may be associated with cancer development. This is consistent with a number of studies implying a role for MKP-2 in cancer (Cadalbert et al., 2010, Groschl et al., 2013, Hasegawa et al., 2008, Wang et al., 2003, Sieben et al., 2005, Wang et al., 2007, Armes et al., 2004, Chitale et al., 2009, Waha et al., 2010), but it remains unclear if it has a tumour-promoting role or an anti-tumour effect.

The demonstration that overexpression of MKP-2 reduced proliferation suggests the potential of anti-tumour role. This has been suggested in a number of recent studies with an effect mediated by inhibition of ERK as the primary mechanism (Cagnol and Rivard, 2013, Jeong et al., 2013, Ramesh et al., 2008, Balko et al., 2012). In this current study, overexpression did indeed inhibit ERK but also inhibited JNK, and strongly inhibited proliferation. Most of the current literature suggests that it is the regulation of ERK which is linked to this anti-proliferative effect, particularly in the context

of cancer progression (Chitale et al., 2009). A recent publication has also indicated that high levels of MKP-2 are associated with increased chemotherapy-induced apoptosis (Balko et al., 2012), showing the potential of a key role for MKP-2 in defining the usefulness of ERK pathway inhibitors as cancer therapeutic agents. This may be a highly significant area of future MKP-2 research, and as such, correlation of MKP-2 expression with ERK activation linked to defined prognostic markers is required for a more complete study of prostate cancer.

It should be noted that the ability of MKP-2 to regulate JNK may be a feature of MKP-2 in relation to cancer, although studies supporting this hypothesis are not numerous. Wang et al. (2007a) showed that MKP-2 overexpression resulted in an inhibition of apoptosis in U937 myeloid leukaemia cells by dephosphorylating JNK (Wang et al., 2007a), suggesting the potential of JNK modulation to be a feature of MKP-2 within blood- borne cancers.

In this thesis, MKP-2 was found not to influence apoptosis generated by a number of different agents such as UVC, X-ray and doxyrubicin. As some of these agents gave a strong JNK signal, this may suggest that within prostate cancer, regulation of JNK function does not contribute to cell survival or modify the effect of potential cancer interventions such as X-ray or doxyrubicin. Indeed, whilst these two latter agents caused LNCaP cells death, this was not associated with an increase in JNK signalling. Nevertheless, evidence indicates that JNK can directly regulate proliferation as well as apoptosis, thus potentially influencing tumour development. Therefore, the interaction between these two parts of the MAP kinase cascade may be of importance. One potential approach to examine this in detail which was considered, was the pharmacological inhibition of ERK or JNK. For example, the ERK inhibitors PD98059 and U0126, on docetaxel and vinorelbine induced cell growth suppression in LNCaP (AI), and regulation of caspase-mediated apoptosis in prostate cancer cells (Zelivianski et al., 2003, Hu et al., 2005). Moreover, several studies have shown that inhibition of JNK induces apoptotic, decreased DNA fragmentation and affects proliferation by arresting cells in the $G_2$/M in prostate cancer cells (Parra, 2012, An et al., 2007, Lorenzo and Saatcioglu, 2008, Ennis et al., 2005, Satomi, 2012), with these developments, it should be possible to identify ERK and/or JNK inhibitor have specific function on proliferation and cell cycle progression.

Nevertheless, there are questions regarding the selectivity of "specific" inhibitors, particularly the JNK inhibitor SP600125. Secondly the inhibitors target MAP kinases in all cellular compartments whilst MKP-2 directs inhibition within the nucleus. Therefore, in this thesis, the relative contribution of ERK and JNK inhibition to the overall effects of MKP-2 overexpression was examined by utilising a specific mutant of MKP-2, one which lacked the triple arginine 74-76. In particular, this would allow the JNK-dependent actions to be interrogated and circumvent the marked effect of MKP-2 overexpression on early $G_1$ progression due to inhibition of ERK. However, this mutation was not effective in that it still reduced ERK phosphorylation, suggesting that it was still able to bind to ERK, and promote dephosphorylation. This effect has recently been replicated in other cells such as endothelial cells (S.Roy, personal communication).

These findings may indicate the potential of sites other than Arg 74-76 to regulate binding of ERK, although the current literature does not support this contention. Rather, Chen et al. (2001) showed that ERK binding site was a cluster of arginine residues in the $NH_2$-terminal domain of MKP-2 (Chen et al., 2001). Moreover, Sloss et al. (2005) demonstrated that the ability of WT and CI-MKP-2 to co-precipitate ERK was lost following mutation of this region (Sloss et al., 2005). In addition, Mandl et al. (2005) showed that the expression of DUSP5 in mammalian cells produces both nuclear translocation and sequestration of inactive ERK2 and the interaction was dependent on both the KIM within DUSP5 and the preserved CD site of ERK2, (Mandl et al., 2005). The only difference between the current study and these previous approaches is the use of adenovirus. This may allow very high levels of MKP-2 into the cell and a law of mass action effect may be sufficient for binding to ERK to occur.

Using other constructs did, however, shed light on the potential of a novel phenomenon: a role for MKP-2 as a scaffold protein. Overexpression of the catalytically inactive MKP-2, whilst having no effect on either JNK or ERK, was able to inhibit proliferation. Indeed, in accordance with a recent paper, it was found that CI-MKP-2 was able to inhibit histone H3 phosphorylation (Jeong et al., 2013). This was demonstrated to be due to the ability of MKP-2 to bind to the cell cycle regulatory protein VRK1 and modify its ability to phosphorylate histone in position at Thr-3 and Ser-10 (Baek, 2011). Whilst follow-up experiments are required to demonstrate a direct interaction between MKP-

2 and VRK1, the results in this study using LNCaP cell lines further suggest that this potentially novel phenomenon may be widespread. Previous work has indicated the potential for MKP-1 to interact with other non MAP kinase substrates such as STAT1 and Histone H3, (Kinney et al., 2009). However, phosphorylation was involved in these targets and subsequent studies did not support these findings (Cuant 2012). Indeed, it is hard to equate these findings with the recognised mechanism of activation of MKP-1, phosphorylation by a MAP kinase and subsequent conformational changes to facilitate exposure of the active site (Marchetti et al., 2005). In contrast the proposed binding of MKP-2 to VRK1, it clearly does not require such a modification as no phosphatase activity is required. It remains to be demonstrated whether other MKPs can bind to VRK1 or if the interaction is specific for MKP-2. This may represent an exciting new area of research when assessing the function of MKP-2, and may reflect studies, from our laboratory, using DUSP-4 deletion mice which show marked effects of MKP-2 on cellular function in the absence of any large changes in kinase activation (Al-Mutairi et al., 2010, Lawan et al., 2011).

Other approaches could have been utilised to address some of the above issues. In particular, shRNA approaches could have bee used to knock down DUSP-4 and assess the effects on ERK and JNK and also cell cycle progression. Other studies have used siRNA to assess the function of MKP-2, however, in our hands knock down is only 50% maximum and unrevealing. Our laboratory is in the process of generating lentivial shRNA DUSP-4, and this may be used in future projects to assess function. This approach may also allow the re-introduction of different MKP-2 constructs including the NLS-1 mutant with in a null back ground further to dissect the role of MKP-2 in cellular function.

One of the main challenges in the effective management of prostate cancer is the identification of genes, such as DUSP-4, capable of predicting disease progression *in vivo*. In Chapter 4, the main aim of the study was to investigate the role of MKP-2 in the development and progression of prostate cancer and to determine if it influenced patient outcome. A key function of MKP-2, is dephosphorylation of MAP kinase. In the current study, increased levels of MKP-2 expression were significantly higher in nuclear prostate cancer tissue than in cytoplasmic or cellular membrane. This potentially argues a role for MKP-2-mediated mechanism in proliferation, which involves targeting the other proteins that were highly expressed in the nucleus and subsequent to phosphorylation

within the nucleus. Several studies reported the localised expression of MKP-2 in the nucleus (Chen et al., 2001, Cadalbert et al., 2010, Saigusa et al., 2013).

The relationship between MKP-2 expression and disease specific survival was tested. To the best of our knowledge, this is the first report to show the relationship between MKP-2 expression and distant metastases in clinical samples of prostate cancer tissues. Immunohistochemistry indicated that high levels of nuclear MKP-2 protein expression in prostate cancer tumours have greater cum survival than in those patients whose tumours expressed a low level of MKP-2 in the nucleus with PSA at diagnosis below 10 ng/ml (figure 4.11 A).

One possibility which remained to be examined was the potential for down-regulation of MKP-2 to regulate the expression of proliferation genes, mitosis genes and MAP kinase proteins, which remain important genes for proliferation. Moreover, PSA remains important as biomarker for screening in prostate cancer, but still there are some negative outcomes from its use, such as the test not always providing an accurate result; besides cancer, there are other conditions that can raise PSA levels, including an enlarged prostate (benign prostatic hyperplasia, or BPH), an inflamed or infected prostate (prostatitis); and PSA levels normally increasing with age. Finally, level of PSA may be affected by some drugs. For all of these reasons, it would be important for new biomarkers in order to be able to distinguish these conditions from aggressive prostate cancer. Over the last 25 years, no new blood test, genetic test or medical x-ray, has been able to replace PSA. Therefore, from the correlation between MKP-2 expression and PSA, MKP-2 may be able to replace PSA as a biomarker for the diagnosis of prostate cancer, in order to improve patient management, and to differentiate cancer from benign diseases of the prostate.

This thesis also showed that patients whose tumour had a Gleason score of below or equal than 7, high levels of MKP-2 in the nucleus were observed to have longer disease specific survival than those patient tumour expressed low levels of MKP-2 (Figure 4.10 A), in addition patients whose tumours had a Gleason score of greater than 7, high levels of MKP-2 in the nucleus were observed to have shorter disease specific survival than those patients whose tumours expressed a low level of MKP-2 in the nucleus (Figure 4.10 B). As the Gleason score is one of the strongest predictors of prostate cancer mortality in men with localised disease (Pound et al., 1999, Kryvenko et al., 2013).

This suggests that MKP-2 could play an important role in Gleason score and tumor stage in prostate cancer. No other data established a link between MKP-2 expression and Gleason score in prostate cancer, but one other study showed that, MKP-1 expression was increased in preinvasive stages of prostate cancer and was downregulated in higher stages and during androgen ablation (Magi-Galluzzi et al., 1997), This again indicates the prognostic value of MKPs in diagnostic evalution.

A few studies have demonestrated theshowed the phosphorylation of ERK in prostate cancer, but not JNK, for example, phosphorylation of ERK correlates with Gleason score and tumor stage in prostate cancer (Gioeli et al., 1999). So, this field needs more work to measure levels phosphorylation of ERK and JNK in prostate cancer tissue and correlate this with the expression with MKP-2. Moreover, as VRK-1 may play a hitherto unrecognised role in prostate cancer in relation to MKP-2, the function and role of VRK-1 needs to be established both in cell lines and correlated with clinical data. Whilst the potential targetting of MKP-2 therapeutically may be several years away the findings in this thesis brings further forward the potential to develop a pharmacological approach to regulating MKP-2 function.

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